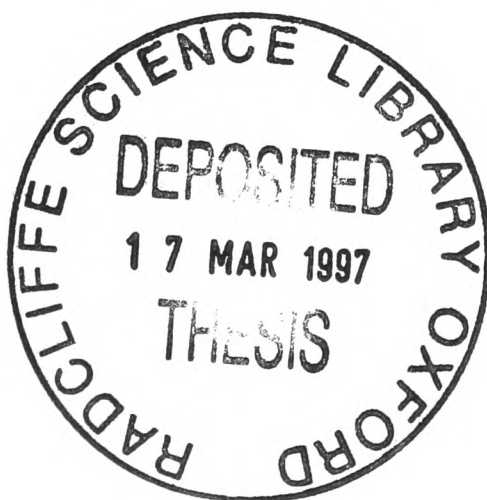

The Computer Simulation of Lipid Bilayers and Biological Membranes

by

Alan Jonathan Robinson

A thesis submitted for the degree of
Doctor of Philosophy in the
University of Oxford

Brasenose College, Oxford



Trinity Term 1996

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Computer simulations of lipid bilayers and biological membranes using molecular mechanics calculations have been undertaken in order to study these complex systems which are so vital in the control and functioning of many biological processes.

The preliminary research involved the development of a model that recreates experimentally observed properties. This is not a trivial task since structural data on lipids in the biologically relevant liquid crystalline phase are unavailable precisely because of their fluid nature. The starting configuration designed for simulation of lipids in the fluid phase contained four different lipid conformations. These reflected the most probable head group and glycerol moiety conformations plus gauche dihedrals were introduced into the hydrocarbon chains so that they resembled chains in the fluid phase and reduced the time required for equilibration molecular dynamics.

The bilayer model was then used to study cholesterol-lipid and peptide-lipid interactions. The cholesterol simulations illustrated how this molecule orders lipid chains by virtue of its rigid skeleton while the peptide simulations showed how cooperative the interactions between proteins and lipids are. Finally simulations of ion channels of gramicidin and melittin in membranes were accomplished and conclusions drawn on the nature and mechanism of the toxicity of melittin and of how water and ion translocation occurs along gramicidin channels.

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Thanks to Adam for playing me loads of records; train carriages will never be the same again.

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Chapter One

Introduction and Overview

1.1 Introduction

Biological membranes are complex fluid structures composed of lipid, protein and carbohydrate molecules all interacting to produce an extremely important cell organelle. Membranes have a variety of roles within cells, however their main function is as a highly selective barrier to the movement of molecules and ions between the interior and exterior of cells, as well as between regions within a cell. This selectivity is of vital importance for medicinal chemists and pharmacologists since many potential drugs will show no activity unless they can cross the cell membrane and enter the cytoplasm (Taylor and Kennewell, 1981).

This ability to function as a selectively permeable barrier is due to proteins within the membrane that act as molecular gates and pumps. Other proteins and glycoconjugates within the membrane act as receptors and are involved in the processes of intercellular communication and recognition; some are hormone receptors that induce changes in the physiological functioning of the cell when a hormone binds; others are photoreceptors in photosynthesis or photodectors for light detection (Stryer, 1981).

During the past three years the interest in the simulation of lipid bilayers has increased rapidly (for a review see Pastor, 1994). This is due largely to the increase in availability of high performance computers and has allowed the simulations of systems that can legitimately be called biological membranes.

The key areas of interest in membrane studies are the nature and behaviour of the lipid component and the modelling of small solutes (e.g. cholesterol), trans-membrane proteins (specifically ion channels) and glycoconjugates, e.g. glycolipids and glycoproteins within the

membrane. These areas are inherently suited to investigation by molecular modelling due to the difficulties in studying these systems experimentally because of the very nature of membranes which are fluids and hence make structural studies of them by conventional techniques far from straightforward. Indeed, so far it has not proved possible to determine the structure of an ion channel or other protein within a membrane at a high resolution (Branden and Tooze, 1991).

During the course of this thesis the aim has been to produce a realistic model of a lipid bilayer and then to investigate the behaviour of biologically relevant solutes within the bilayer, their effect on the lipid molecules and how they perturb the lipid bilayer. Of particular interest is the nature of protein-lipid interactions and their effect on protein structure since it is apparent that the geometry of proteins from any available crystal structure will differ from that in a hydrophobic lipid bilayer because of the radically differing environments around the protein. Paradoxically, a goal of the thesis was to produce a working model of a lipid bilayer in order to produce a simplified model that could mimic it since the simulation of full membranes is very expensive computationally. Furthermore, an understanding of biological membranes should aid in the drug design process, given that before any substance may enter a cell it must cross a cell membrane and so a membrane model could act as a quick filter to determine if a potential drug will show activity *in vivo* as compared to *in vitro*.

The complex fluid nature of bilayers makes the interpretation of molecular dynamic simulation data difficult but this has been overcome by the development of new techniques to analyse the data, and especially the use of computer visualisation techniques to rationalise otherwise complex phenomena.

1.2 Thesis Overview

Chapters one and two consider the general nature of the research, the properties of lipid molecules and bilayers and the parameters used for the molecular dynamic simulations. Chapter three introduces and discusses some of the programs and techniques that were developed and written to analyse molecular dynamic trajectories of the membrane systems.

Chapter four is about the first pure lipid bilayer simulations for comparison with the later work on much larger bilayers discussed in Chapter five. Chapter six describes the research on a system containing cholesterol and its effect on the properties of lipid molecules and the bilayer. The remaining chapters focus on lipid-peptide systems from the initial work on model single trans membrane helices in Chapter seven which led to the study of the toxin melittin (Chapter eight). Melittin is a single alpha helical peptide that interacts with membranes both as a surface adsorbant and as a trans membrane peptide. A speculative model is also proposed for the formation of a melittin pore structure. Finally in Chapter nine, a large scale simulation of gramicidin in a fully hydrated membrane is presented and the effect of the protein on the lipid chains and the surrounding water molecules analysed as well as the nature of water translocation through the pore.

The final chapter outlines the conclusions of this research and possible directions for future research.

Chapter Two

The Structure of Lipids and Bilayers

2.1 Introduction

It is over twenty years since J. S. Singer and G. Nicolson first proposed their fluid mosaic model for biological membranes (Singer and Nicolson, 1972). This model arose from a consideration of the constituent components isolated from membranes. The lipid molecules identified were all amphipathic in nature, the two main lipid components being the ionic phospholipids and cholesterol. In this work, only the phosphoglyceride class of phospholipids are considered, which have three main components, a glycerol backbone, two fatty acid chains, and a phosphorylated alcohol (see Figure 2.1). The polar head groups are hydrophilic and have an affinity for water, whilst the hydrocarbon tails are hydrophobic and have an aversion to aqueous environments. This chapter is a concise description of the properties of lipids (specifically dimyristoylphosphatidylcholine (DMPC)) and bilayers, for a more detailed treatment, see Cevc and Marsh, 1987.

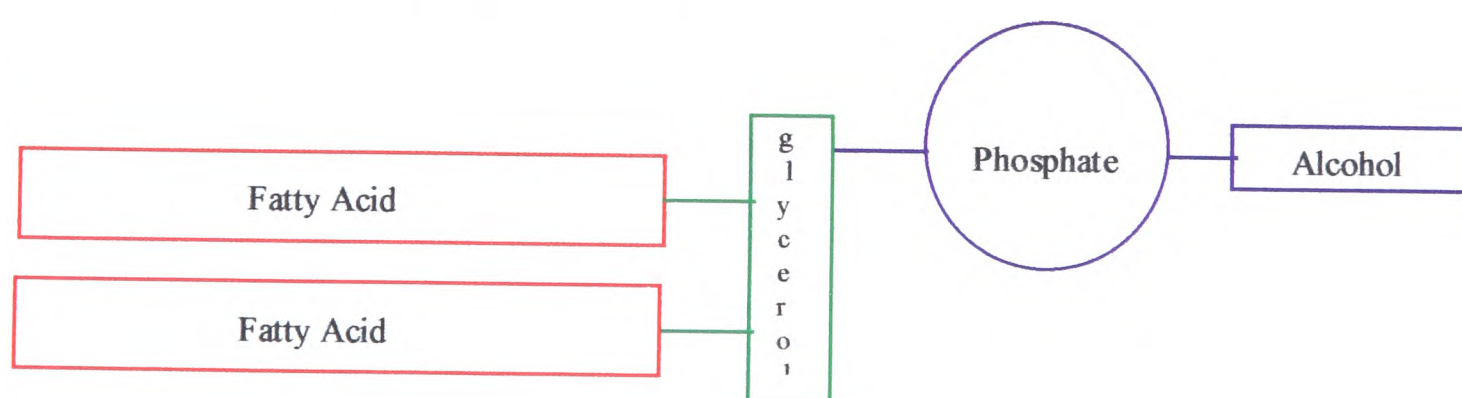


Figure 2.1 The three components of a phosphoglyceride.

Clearly in aqueous solutions these amphipathic molecules will try to arrange themselves so that the minimum hydrophobic surface area is exposed to water and for phospholipids, the

most obvious ways to achieve this is either by forming a micelle, or a bilayer arrangement (Fig 2.2).

In this bilayer structure the hydrophilic head groups interact with the water molecules and the hydrophobic tails interact with each other. It is these hydrophobic interactions that are the major driving force for the self-assembly of bilayers in aqueous solutions. Most membranes are between 60 and 100 Å thick, however they readily form bilayer sheets of macroscopic dimensions, e.g. 10^7 Å wide. The hydrophobic interior forms an effective barrier to the free diffusion of ions, water and other polar molecules. Lipid bilayers are cooperative structures held together by many noncovalent interactions including attractive van der Waals forces between hydrocarbon chains, and attractive electrostatic interactions (including hydrogen-bonding) between charged head groups and water (Cevc and Marsh, 1987).

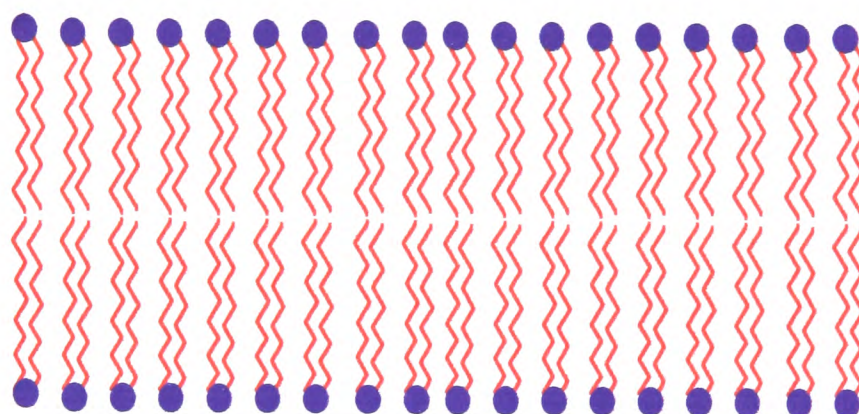


Figure 2.2 Schematic illustration of a bilayer of amphipathic molecules.

Singer and Nicolson proposed that the lipid molecules of a biological membrane formed a bilayer structure in which globular proteins floated. These proteins may be either integral and pass right through the membrane, or peripheral and only be associated with one side of the lipid bilayer assembly (Figure 2.3). Biological membranes also have the following characteristics:

1. They are fluid assemblies, lipids and proteins rapidly diffuse in lateral directions, unless there are specific interactions preventing them.

2. Flip-flop motions of lipids is very rarely seen, i.e. the movement of a lipid molecule from one side of the bilayer to the other.
3. Biological membranes are asymmetric, i.e. the two sides of a bilayer differ in their constitution.
4. Biological membranes contain a large amount of protein. The ratio of protein:lipid by weight can vary from 1:4 to 4:1.
5. There are many types of membrane proteins that have specific functions, e.g. ion channels and pores, enzymes, photoreceptors and receptors.

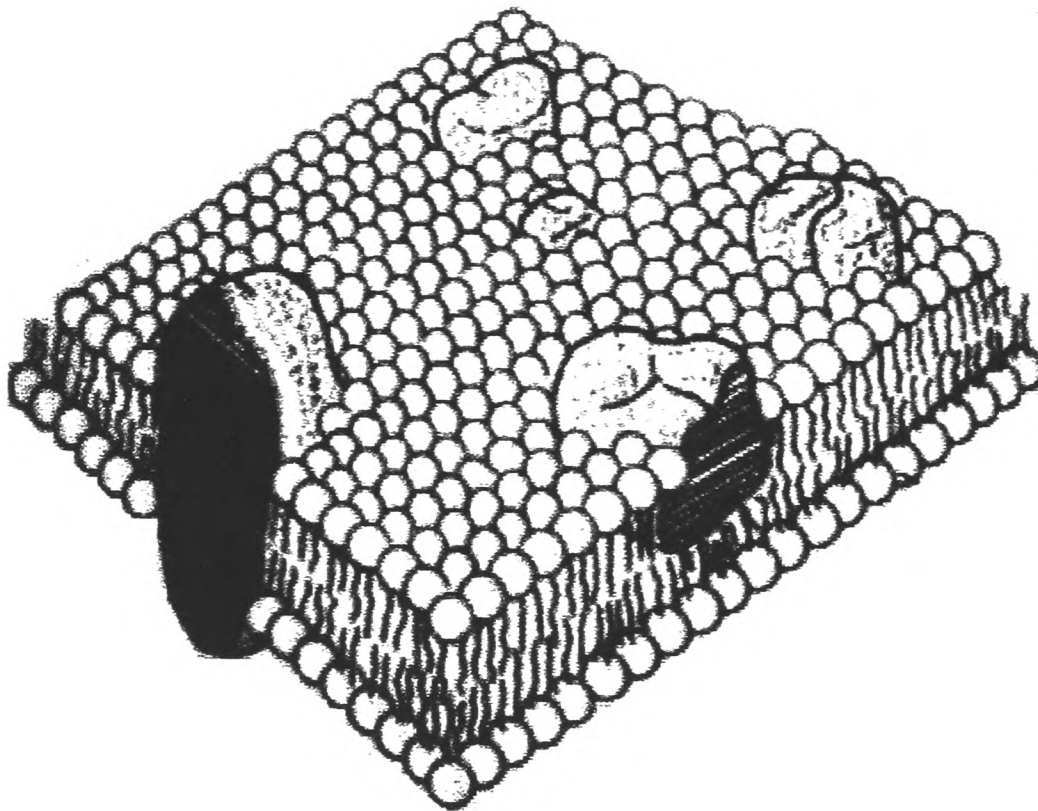


Figure 2.3 The fluid mosaic model of a biological membrane illustrating globular proteins floating in a bilayer of lipid molecules (from Taylor and Kennewell, 1981).

2.2 The Structure of Phospholipids

There are two main classes of phospholipids, the phosphoglycerides (Figure 2.4) and the sphingophospholipids. Phosphoglycerides have a phosphate and two fatty acids esterified to a glycerol. The fatty acid hydrocarbon chains usually have an even number of carbon units with the typical length being 16 or 18 carbons. If the hydrocarbon chain contains a double

bond, then this is almost exclusively *cis* as this leads to less disruption of the chain packing of hydrocarbon chains in the bilayer (see below).

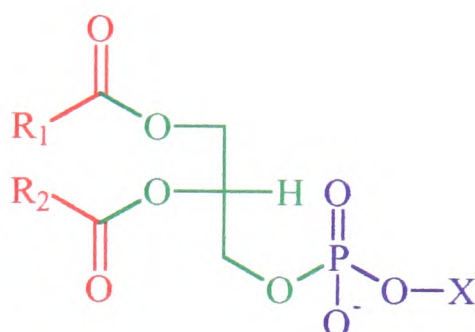


Figure 2.4 General structure of a phosphoglyceride. R_1 and R_2 are the hydrocarbon chains of the fatty acid. X is a group esterified to the phosphate that defines the phosphoglyceride. The colour scheme is the same as in Figure 2.1.

In biological membranes, the glycerol moiety of phospholipid molecules contains a chiral centre. Phosphoglycerides differ depending on what group (shown as X in Figure 2.4) is esterified to the phosphate, e.g. an organic base, an amino acid, or an alcohol.

In this thesis, the lipid studied was 1,2-dimyristoyl-sn-glycero-3-phosphatidylcholine (DMPC) and is shown in Figure 2.5. DMPC belongs to the phosphatidylcholine group of phosphoglycerides where a choline group is esterified onto the phosphate (i.e. X equals choline in Figure 2.4). Phosphatidylcholines are found in the membranes of most higher animals and although the head group of this class of phosphoglycerides is electronically neutral, it contains a large dipole because of the positive charge on the ammonium group and the negative charge on the phosphate group. This large dipole has a profound effect on the crystal structure of DMPC and probably on the fluid phase and its effect on lipid packing must be considered during model building.

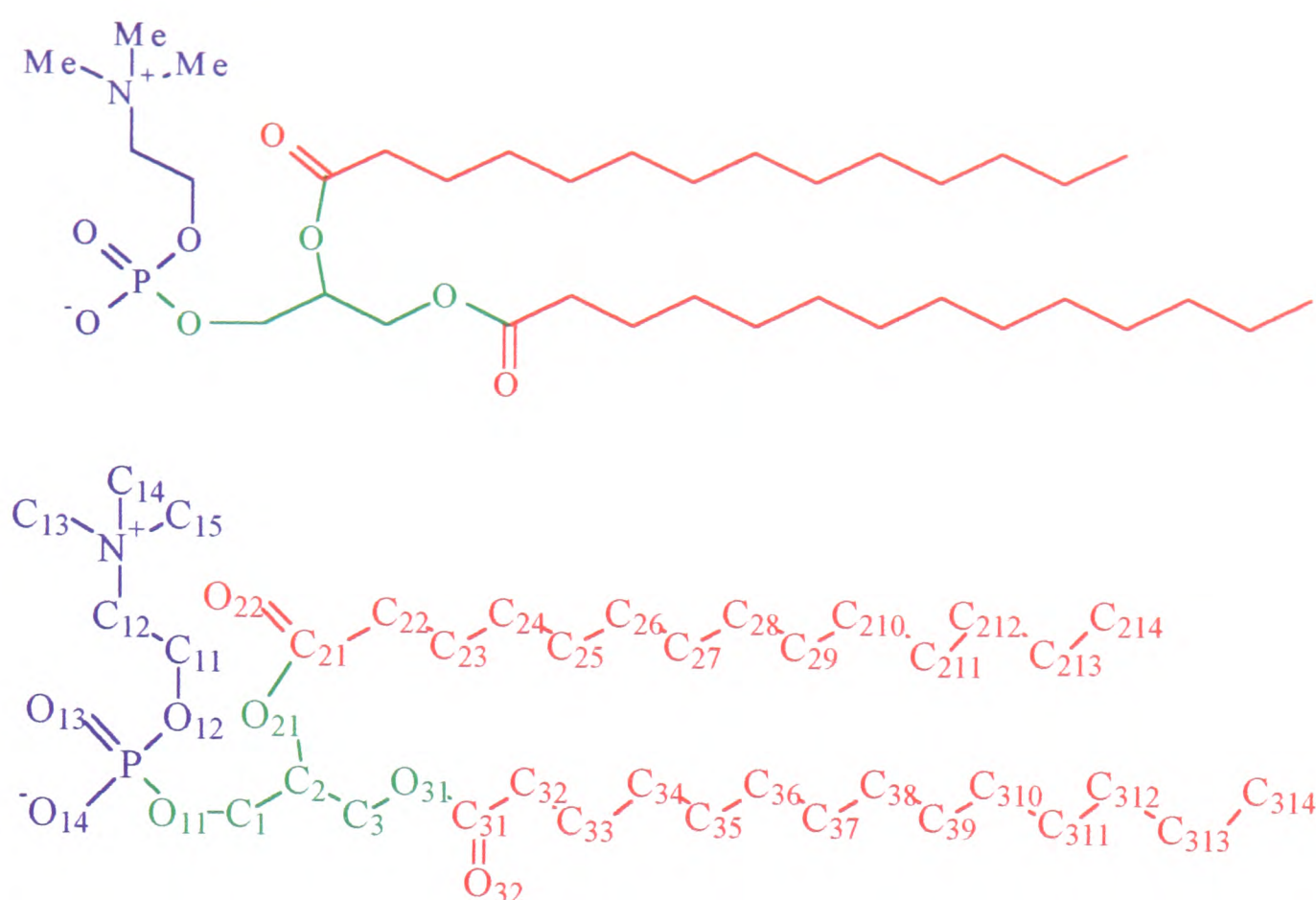


Figure 2.5 The structure of 1,2-dimyristoyl-sn-glycero-3-phosphatidylcholine (DMPC). The colour scheme is the same as in Figure 2.1. The second figure shows the atom naming scheme after Sundaralingham, 1972.

2.3 Crystal Structures of Phospholipid Bilayers

The crystal structures of some phospholipids have been determined (for a review, see Pascher et al, 1992) and may be used to make some predictions about the conformation of lipid molecules in bilayers which overcomes the difficulty of obtaining structural data on fluid lipid bilayers. However these predictions should be treated with caution since the environment of a lipid in a crystal structure is far removed from physiological conditions with the crystal structures containing very little water and being in a low temperature solid phase rather than the usual fluid phase of physiological conditions. The crystal structures are all characterised by configurations where the close packing of the lipid hydrocarbon chains (which contain no gauche conformations) appears to be the major concern and the head groups adopt conformations that allow this whilst also trying to minimise unfavourable electrostatic interactions between head groups.

In DMPC, the crystal structure consists of planes of bilayers stacked on top of each other (see Figure 2.6). The unit cell contains two different DMPC molecular conformations (A and B) which are displaced with respect to each other in the direction of the bilayer normal. This allows the bulky head groups to pack in a volume very similar to that needed for the hydrocarbon chains to be tightly packed. It must be emphasised that the crystal structure contains only two water molecules per DMPC lipid and the volume per lipid molecule is much smaller in this condensed crystal phase than a biological membrane under physiological conditions. However the following points can be noted and appear to have some validity for lipid bilayers in the fluid phase:

1. The head groups are oriented almost parallel to the bilayer surface with the P-N dipole making an angle of approximately 15° with the bilayer surface.
2. All the hydrocarbon chains are tilted with respect to the bilayer normal with an angle of approximately 20° .
3. The two hydrocarbon chains are not equivalent because of a bend in the Sn2 chain which ensures that the chains remain approximately parallel to each other and may pack together more efficiently.
4. The glycerol backbone is in an all trans conformation and the Sn1 chain continues on from it in the same direction. With the bend in one chain (see above) this also results in the chains not extending to the same depth, hence there is greater free volume in the bilayer than might be expected, and the carbonyl groups of each chain have different orientations and average depths within the bilayer.
5. The fundamental difference between the two conformations A and B is that the conformation of the head groups are approximate mirror images. Although the head groups are far more flexible in the fluid phase and adopt a range of conformations, these two particular conformations appear to be important.
6. The waters that are present in the crystal structure form a hydrogen-bonded network amongst the head groups that appears to stabilise the structure.

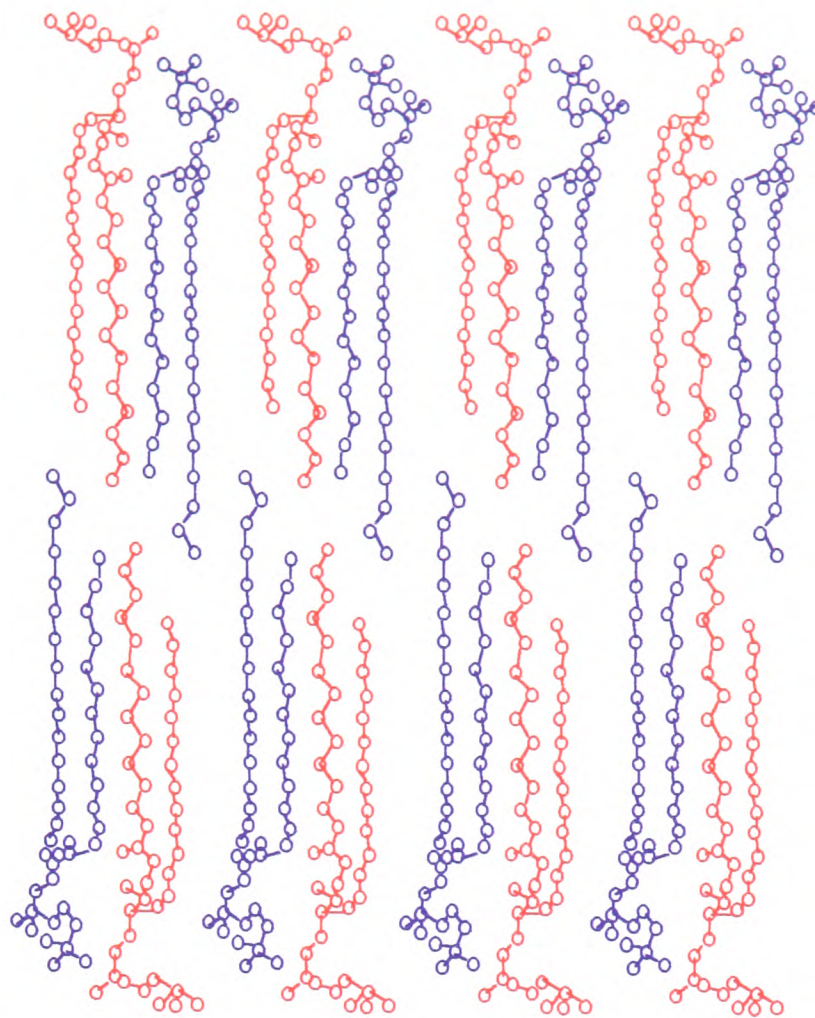


Figure 2.6 The crystal structure of 1,2-dimyristoyl-sn-glycero-3-phosphatidylcholine•2H₂O illustrating the lipid packing and two molecular conformations, A(blue) and B(red), present in the unit cell. (Pearson and Pascher, 1979).

2.4 The Structure and Dynamics of Fluid Phospholipid Bilayers

At 24°C, fully hydrated, pure crystals of DMPC undergo a phase transition from a gel phase (called the $P_{\beta'}$ phase) in which the lipid chains are cooperatively packed, to a fluid phase (called the L_{α} phase) characterised by hydrocarbon chains which are less well packed and possess gauche conformations along their length. The behaviour of lipids in the L_{α} phase is thought to be very similar to that in biological membranes. The lipid molecule can be divided into three main components, the head group, the glycerol moiety and the hydrocarbon chains. Structural information on these components is available from ^2H - and ^{31}P -NMR, ESR and X-ray and neutron diffraction experiments and all of this must be rationalised and included in any model of a bilayer.

2.4.1 The Hydrocarbon Chains

The hydrocarbon chains of fluid bilayers are not completely disordered and a high degree of cooperativity is maintained between chains which keeps them aligned with their neighbours in order to maintain favourable chain packing interactions. Dilatometric measurements show the change in volume of phospholipid bilayers when they melt is two-thirds that reported for normal hydrocarbons (Cevc and Marsh, 1987) which illustrates the greater degree of order of hydrocarbon chains in these bilayers compared to those in liquid paraffin. This desire to maintain favourable packing interactions puts constraints on the freedom of rotation about bonds in the hydrocarbon chains. The reproduction of the lipid chain dynamics and their rotational isomerism is vital in the validation of any computer model of a lipid bilayer.

The ability to undergo rotations about C-C bonds gives rise to the fluidity of bilayers. The introduction of gauche conformations upon chain melting is entropically favoured as it introduces rotational disorder, but the hydrocarbon chains are restricted to conformations which do not overly disrupt the ability of the chains to maintain favourable interactions, i.e. there is an enthalpic consideration. For example, a single gauche conformation near the top of a hydrocarbon chain (i.e. nearer the glycerol moiety) would introduce a tilt in the hydrocarbon chain that would lead to extensive disruption as neighbouring chains have to move in order to accommodate it (Figure 2.7b). Gauche conformations further down the chain should become less unfavourable as the chain length that must be accommodated for by the neighbouring chains gets shorter (Figure 2.7c).

The rotational isomeric model (Cevc and Marsh, 1987; Seelig and Seelig, 1974) proposes that gauche conformations can only occur as part of certain sequences, including kinks, so that the lipid chains remain oriented within 30° of the bilayer normal and the chain packing is not overly disturbed. Toward the termini of the lipid chains motion is less restricted and it is proposed that both the proportion of gauche dihedral angles and the rate of dihedral angle isomerisation should increase. For example, a gauche conformation is allowable at the top of a chain if it is part of an overall dihedral sequence such as a kink (Figure 2.7d) and introduces two gauche dihedral conformations into a chain without leading to a significant change in the direction of the chain, i.e. it causes minimal disruption in the packing of hydrocarbon chains around it, and corresponds to the dihedral sequence, gtg'. Other possible

sequences that lead to minimal disruption are jogs (gtttg'), double gauche sequences (tggtt and g'tgg) as well as single gauche conformations at the termini of chains.

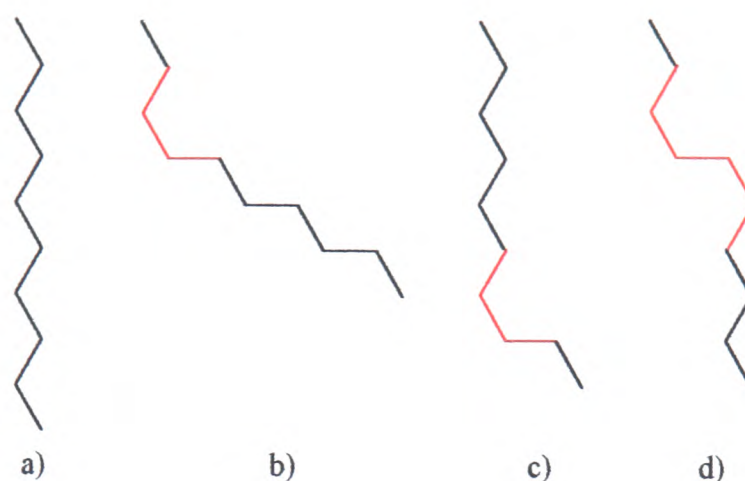


Figure 2.7 Four examples of hydrocarbon chain conformations. a) An all-trans conformation b) A chain with a single gauche conformation (highlighted in red) near the top of the chain c) A chain with a single gauche conformation (highlighted in red) near the bottom of the chain. d) A chain with a kink dihedral sequence (highlighted in red).

From FT-IR and ^2H -NMR experiments (Tuchtenhagen, Ziegler and Blume, 1994), the number of gauche conformations per chain in DMPC at 69°C has been estimated as 2.60 ± 0.37 excluding contributions from jogs and single gauche conformations and ~ 3.4 to 3.9 if they are included. The number of kink plus gtg, double gauche and chain termini single gauche sequences was determined to be 0.44 ± 0.07 , 0.67 ± 0.14 and 0.38 ± 0.05 , respectively. Interestingly, the FT-IR results also indicate that the total number of gauche conformations per chain depends on the chemical nature and charge on the head group. The values estimated previously from theoretical studies using a configurational partition function (Cevc and Marsh, 1987) estimated there to be 2.8 gauche conformations and 0.4 kinks per chain in a DMPC bilayer at 55°C .

The degree of motional disorder of methylene segments in hydrocarbon chains has conventionally been reported in terms of an order parameter (Seelig and Seelig, 1974; Davis, 1983). This may be reported as either a value for the whole chain, S_{CD} , or for individual methylene segments, S_n . Order parameters are widely reported as they may be evaluated directly in ^2H -NMR experiments on selectively deuterated lipids by recording quadrupolar splittings, or be calculated readily for lipid models using equation 1:

$$S_n = \frac{1}{2} \left(3 \langle \cos^2 \theta \rangle - 1 \right)$$

(1)

where θ is the angle that the C-D bond makes with the bilayer normal.

The quadrupolar splittings of signals in ^2H -NMR experiments is not the only way to determine order parameters experimentally and as Petersen and Chan (1977) discuss the value of order parameters is very much dependent on the method used to determine them. Electron spin resonance experiments suggest a continuous flexibility gradient along the hydrocarbon chain, whilst NMR experiments exhibit a plateau in the upper part of the chain which is taken to be a result of intermolecular interactions that disfavour gauche conformations here and hence cause an increase in molecular order (see Figure 2.8).

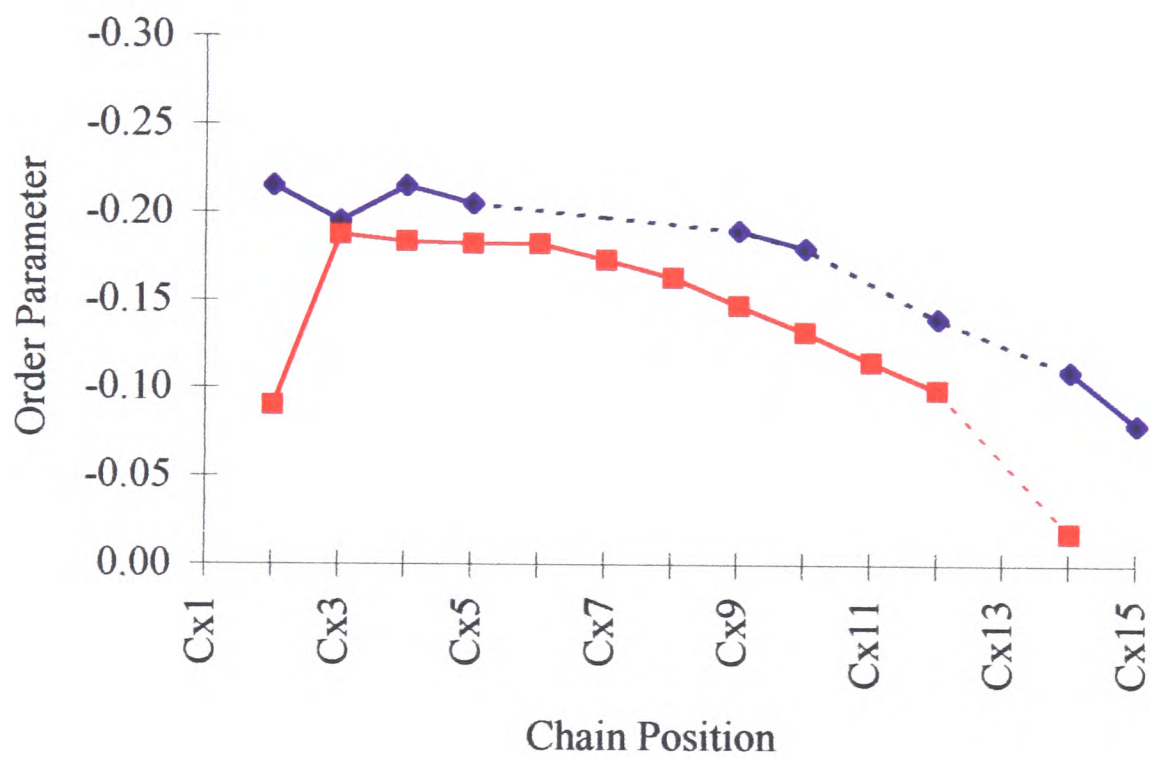


Figure 2.8 The experimental order parameter profiles from ^2H -NMR for the Sn2 chain ($x=2$) of DPPC at 50°C (blue line) by Seelig and Seelig, (1974) and the Sn2 chain ($x=3$) of DMPC at 60°C (red line) by Oldfield, et al. (1978).

N.B. To identify the methylene carbons in the two chains of phospholipids in graphs a general scheme is adopted of which Figure 2.8 is the first example. The fourteen carbon atoms of the DMPC Sn1 chain are identified as C3 1 to C3 14 and those of the Sn2 chain as C2 1 to C2 14 (see Figure 2.5 for naming scheme). Thus in graphs, the notation Cx 1 to Cx 14 is used so that both chains can be potted on a single graph where $x=3$ for the Sn1 chain and $x=2$ for the Sn2 chain.

Although the ESR experiments include the use of a probe that may perturb the system, the NMR experiments occur on a much longer time scale. Hence the two techniques may sample motions quite differently as the order parameter calculated from NMR includes contributions from both intramolecular motion (e.g. rotational isomerisation) and chain reorientation (e.g. rotation of the whole chain about its long axis, and ‘vibrations’ of the lipid within the cage formed by neighbouring molecules).

If the lipid reorients about an average director, i.e. the long axis sweeps out a cone, then NMR will sense the symmetry axis of this average chain orientation rather than the instantaneous chain orientation (Figure 2.9). Petersen and Chan (1977) propose that there is a tilt of the chains with respect to the bilayer normal because of motions in the glycerol and ester segments and that this tilt is locally cooperative covering all angles within a certain range and at a rate slow compared with that for dihedral isomerisations. NMR cannot distinguish between a normal distribution of chains about the director and a normal distribution about a permanently tilted orientation if the chains rotate about the director.

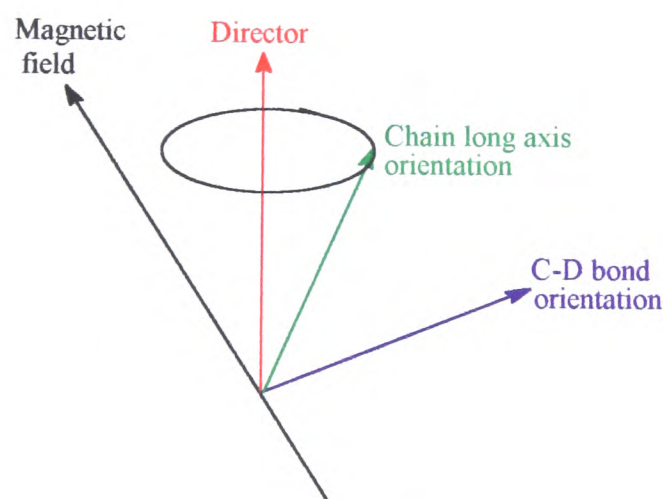


Figure 2.9 Representation of vector components that need to be considered in analysis of order parameters (after Petersen and Chan, 1977).

From a comparison of order parameters evaluated from ^2H - and ^1H -NMR, Petersen and Chan (1977) concluded that although the time-averaged orientation of a hydrocarbon chain is parallel to the bilayer normal, the most probable angle it makes with the normal is $27\text{--}34^\circ$ and its maximum deviation from the bilayer normal is no more than 50° . ESR experiments

(Gaffney and McConnell, 1974; Birrell and Griffith, 1976) showed that the long axis of a hydrocarbon chain was tilted by about 30° with respect to the bilayer normal. Oldfield, et al. (1978) used specifically deuterated cholesterol molecules as probes to determine the tilt of the hydrocarbon chains. Although the tilt of the cholesterol may not be identical to that of the lipid molecules, by extrapolating back to zero cholesterol concentration they concluded that at 60°C the most probable tilt angle of the lipid was around 25° from the bilayer normal. Strenk, et al. (1991) used a molecular frame-and-order matrix approach to determine that the most ordered axis of the DMPC molecule was tilted at an angle of $27 \pm 2^\circ$ from the bilayer normal. For the time-averaged quadrupolar interaction to be parallel to the bilayer normal requires that this most ordered axis samples most orientations about the bilayer normal within the time scale of the NMR experiment. This implies that either the lipid molecule sweeps out a cone in one fixed location, or that there is a rapid lateral diffusion with the molecule sampling a different orientation at each position in its lateral movement.

Thus although the time average of the system as detected by NMR proposes that the chains are oriented along the bilayer normal, analysis of the motions on shorter time scales indicates that the lipid chains are actually tilted.

2.4.2 The Head Group and Glycerol Moiety

Considerable effort has gone into attempting to deduce the conformation of the head groups in biological membranes yet the picture is still not clear. Experimental techniques to study the head group have included ^{31}P -, ^{14}N -, ^2H - and ^1H -NMR, neutron diffraction and infrared spectroscopy (see Büldt and Wohlgemuth, 1981). Because of the fluid nature of the L_α phase the NMR data are often difficult to interpret and involve comparing calculated deuterium quadrupolar splittings and phosphorous chemical shift anisotropies for models of head group structure with experimental values to evaluate possible head group dihedral angles. The head group of DMPC is described by five dihedrals, α_i (see Figure 2.10). The two limiting scenarios are completely free rotation in the head groups which was dismissed by the work of Seelig and colleagues (Seelig et al., 1977) and a completely rigid head group structure which

is consistent with experimental deuterium quadrupole splittings but not with the T1 deuterium relaxation times for head group hydrogens (Büldt and Wohlgemuth, 1981).

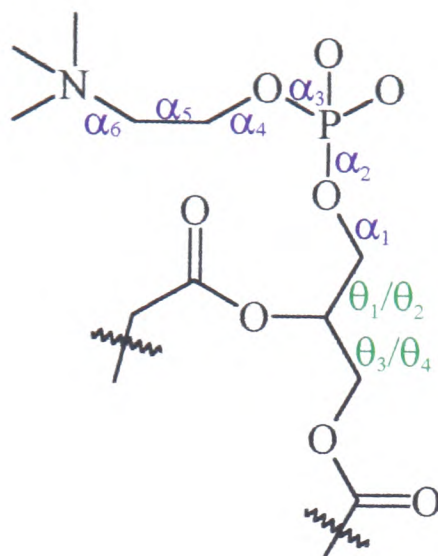


Figure 2.10 The dihedral naming scheme for the head group torsions of DMPC (after Sundaralingham, 1972).

Akutsu and Nagamori (Akutsu and Nagamori, 1991) deduced that the conformation of the polar head group of phosphatidyl choline in a liquid crystal bilayer to be close to one of those observed in the DMPC crystal structure. The current opinion from experimental data is that the two conformations observed in the crystal structures are also the most probable in the liquid crystalline state and interconvert via other conformations (Skarjune and Oldfield, 1980; Seelig et al., 1977; Gally et al., 1975). ^2H - and ^{31}P -NMR experiments on the L_α phase (Seelig et al., 1977; Seelig, 1978; Skarjune and Oldfield, 1979) are not consistent with models in which the polar head groups adopt a completely flexible or completely rigid head group structure. The work of Seelig and colleagues (Seelig et al., 1977; Seelig, 1978) propose two enantiomeric conformations, tggtg' for the dihedrals, α_1 to α_5 , which are in rapid dynamic equilibrium. Dufourc and colleagues (Dufourc et al., 1992) deduced from pulsed ^{31}P -NMR on DMPC bilayers that the θ_1 and α_1 torsions both show hindered rotation but that the α_2 dihedral angle exhibits free rotation. Using Raman spectroscopy to study the head group geometry and motional freedom of dipalmitoyl phosphatidylcholine (DPPC) in aqueous dispersions showed there to be considerable motional freedom about the C-O bonds in the phospholipid head group, i.e. α_1 and α_4 , and this is greater than in the P-O bonds, i.e. α_2 and α_3 (Bicknell-Brown et al., 1982).

Gally and co-workers (Gally et al., 1975) proposed the choline moiety to have a flexible temperature-dependent conformation and describe the choline group as having a bent structure which placed the ammonium and phosphate groups in close proximity, the stability of which increased with temperature. This result is consistent with Raman spectroscopy measurements (Akutsu, 1981) on the stretching vibration of C-N bonds which indicate they should be predominantly gauche.

Skarjune and Oldfield (1979) conclude that the head groups adopt a limited range of conformations after searching a limited range of conformational space of $\pm 40^\circ$ in 2° increments from the crystal structure dihedral angles. These conclusions are consistent with the range of energetically most stable conformations of glycerophosphatidylethanolamine calculated by Frischleder et al. (1981).

In the L_β crystal structure of DMPC, the dihedral θ_3 is trans. However, ^1H -NMR spin-coupling measurements on the L_α fluid phase by Hauser et al. (1988) indicate that three rotational isomers exist for θ_3 with dihedral torsions of trans, gauche⁺ and gauche⁻ existing in equilibrium with a ratio of 6.5:5:1. Furthermore, relaxation studies by Mayer et al. (1988) indicate rotational jumps between two conformers about the θ_3 torsion.

The large size of the phosphatidyl choline head group is not only responsible for the packing scheme observed in the crystal structure (Figure 2.6) and the tilt of the hydrocarbon chains in the P_β gel phase, but in the fluid phase makes it much easier for the glycerol moiety to undergo trans-gauche isomerizations and other tumbling motions that allow the lipid chains to move along the direction of the bilayer normal with respect to each other, i.e. the large head group increases the free volume available to the lipid hydrocarbon chains compared to other smaller head group structures such as phosphatidyl ethanolamine.

2.5 Summary

In this chapter, the basic structural and dynamic properties of phospholipid bilayers in both fluid and crystalline phases have been discussed. It is apparent that although the crystalline phase of DMPC is a useful source of molecular coordinates, there are important differences

between it and the structure and dynamics of DMPC molecules in the fluid L_α phase that must be accounted for and in the following chapters these experimental data will be used to construct models of bilayers and biological membranes.

Chapter Three

Analysing the Bilayer Simulations

3.1 Introduction

Biological membranes are fluids and hence their motions are intrinsically complicated. One of the main problems of molecular dynamic simulations is how to analyse this data and present it so that it may be understood and utilised by a general audience less familiar with computer modelling. To this end a series of programs have been developed and written and include programs that use novel computer visualisation techniques such as three-dimensional representation of otherwise complex data using the developing open standard Virtual Reality Modelling Language (VRML) and Silicon Graphics, Inc. Open Inventor modelling language. Furthermore VRML allows the distribution of the chemical data in a 3-D format to be made available on the Internet via the World Wide Web (Robinson, 1995; Robinson and Hardy, 1995). The emphasis has been on designing programs that produce results that can be compared directly with experimental data, or provide a general, atomic scale view of the fundamental structure, dynamics and properties of biological membranes which cannot be determined experimentally.

In this chapter, the main programs and the properties they calculate are considered and later chapters on different models will refer back here.

3.2 Calculation of Order Parameters

As outlined in Section 2.4.1, order parameters provide a measurement of the degree of motional disorder of atoms and are evaluated readily from the quadrupole splittings of NMR signals in oriented membranes. Order parameters may be related directly to the structure of the molecule using the equation 1:

$$S_n = \frac{1}{2} \left(3 \langle \cos^2 \theta \rangle - 1 \right) \quad (1)$$

where θ is the average angle that the C-D bond makes with the bilayer normal and S_n is the order parameter of the carbon atom. Thus to determine the order parameters of atoms in a computer model of a bilayer, it is only necessary to evaluate the average angle that the C-D bonds make with the z axis which has been defined as the bilayer normal. The only complication is that a united atom model is used for the hydrocarbon chains, so the C-D bonds are determined by assuming that bonds around the carbon are arranged tetrahedrally.

The average and standard deviation of the order parameters were calculated both as function of individual carbon atoms and as a function of the depth in the membrane.

3.3 Radial Distribution Functions

Radial, or pair, distribution functions are a common way of describing the structure of liquids (Allen and Tildesley, 1987) and of how a solvent packs around a solute, e.g. the size of successive coordination shells and the average number of solvent molecules in a coordination shell. It involves calculating the number of atoms per unit volume as a function of distance from a given atom. If a solute-solvent pair distribution function is being calculated, it is often reported relative to average solvent density. The minima in the pair distribution function are taken as the radii of successive coordination shells.

3.3.1 Regular Radial Distribution Functions

An example of a regular pair distribution function is given in Figure 3.1. The number of solvent molecules surrounding the solute at a distance, r , is found by counting the number of them in the interval $(r, r + \delta r)$ over all space, i.e. a spherical shell is considered. The pair distribution function (i.e. the number per unit volume) at r is this number divided by the volume of the shell.

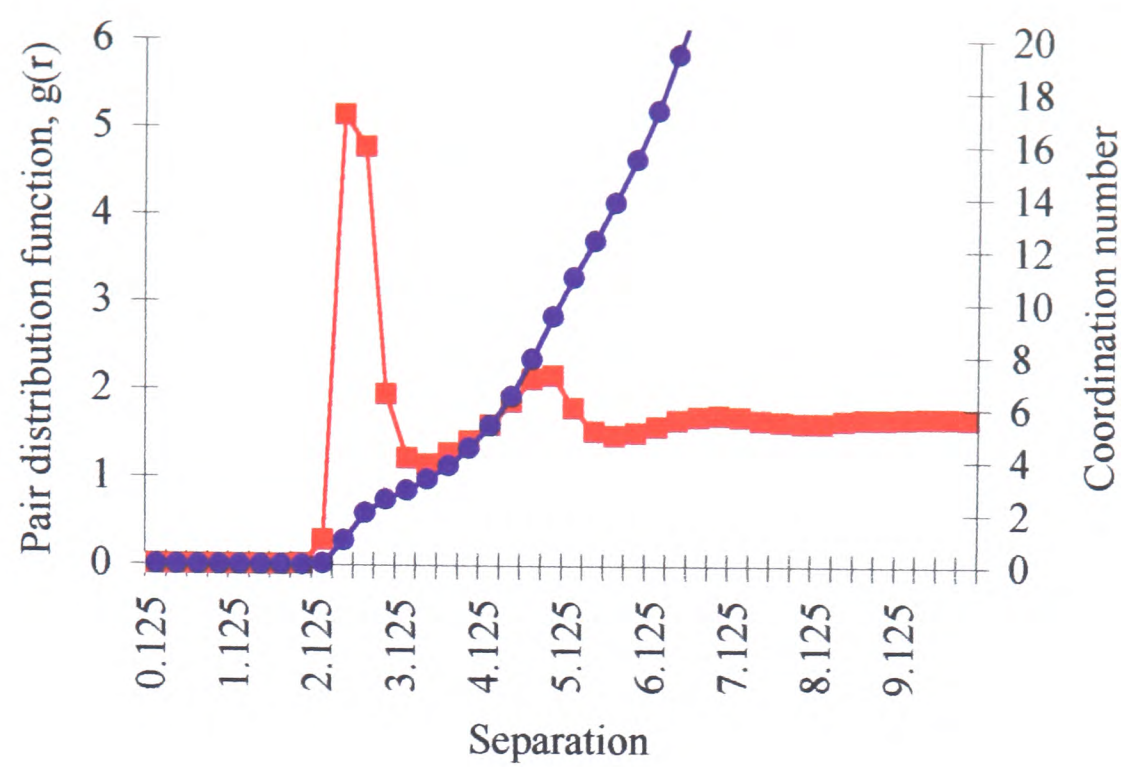


Figure 3.1 A pair distribution function (red) and the coordination number (blue) of an oxygen atom in the phosphate group of a lipid with the centre of geometry of water molecules as a function of distance.

This method of calculating pair distribution functions is correct so long as the distribution of solvent around the solute is fairly isotropic (Figure 3.2a). This is not the situation in lipid bilayers which have a very anisotropic solvent distribution because of their lamellar nature; the distribution of waters 9Å above the surface of the bilayer (point x in Figure 3.2b) will be very different to that 9Å away in the plane of the bilayer (point y in Figure 3.2b).

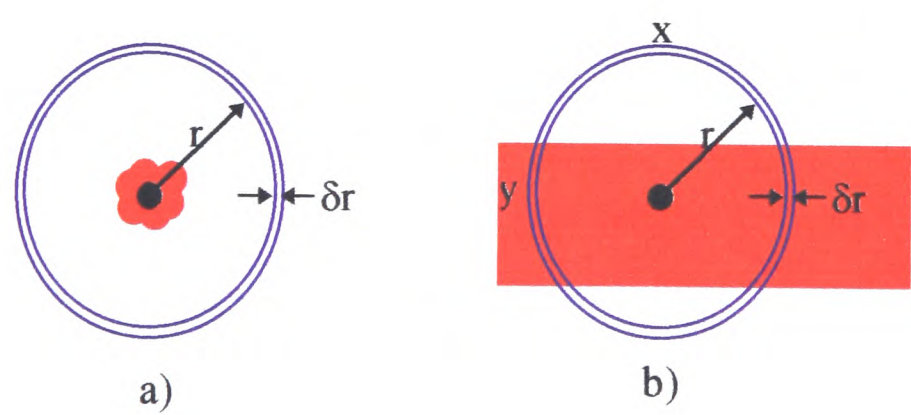


Figure 3.2 Comparison of water distribution at a distance r from the solute in a) an isotropic system and b) the anisotropic lipid bilayer system.

3.3.2 Two-Dimensional Pair Distribution Functions

The water density at point y in Figure 3.2b is far less than at point x , thus any structure in the pair distribution function at y would be overwhelmed by the contribution from bulk water at x . For this reason a new pair distribution function was devised; the two-dimensional pair distribution function. In this function solvent molecules that are displaced from the solute in the direction of the bilayer normal by more than a few angstrom's, i.e. those outside the first coordination sphere of the atom, are ignored. Rather than counting the number of solvent molecules in a spherical shell of thickness δr at a distance r from the solute, a cylindrical shell of thickness δh and radius r is considered (Figure 3.3).

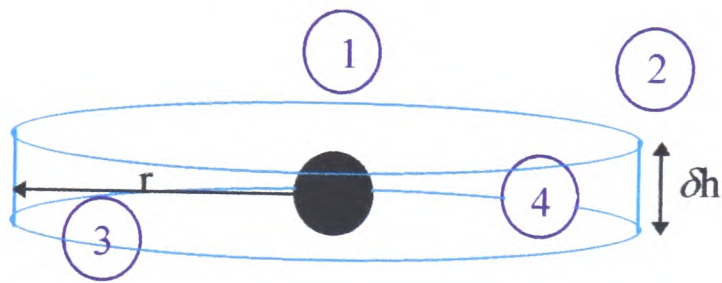


Figure 3.3 Calculating the two-dimensional pair distribution function. Solvent molecules 1 and 2 would not be considered; 3 and 4 would.

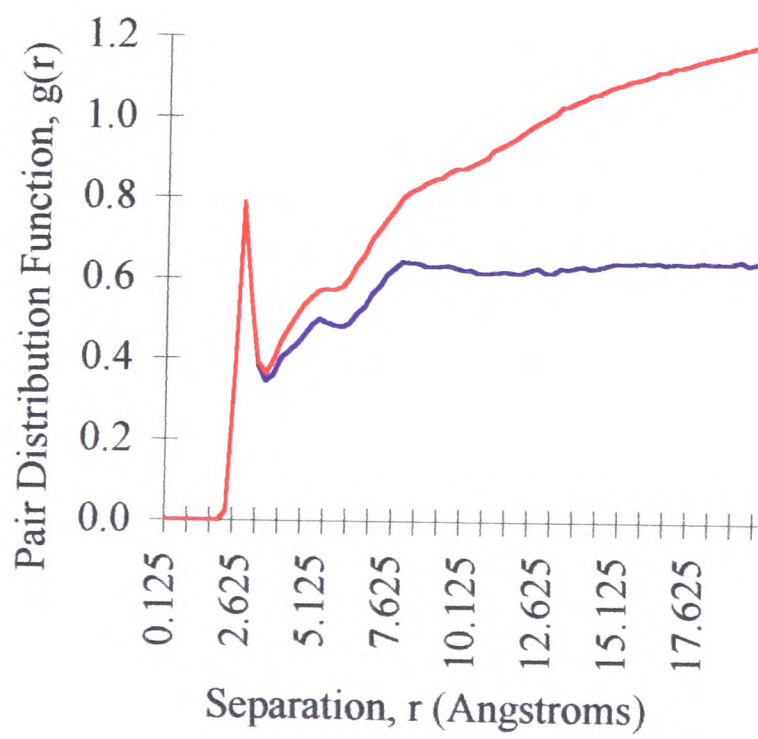


Figure 3.4 Comparison of two- (red) and three-dimensional (blue) pair distribution functions for the same solute atom with solvent.

Two- and three-dimensional plots for the same solute atom (a carbonyl oxygen) are illustrated in Figure 3.4. It is clear from this plot that the secondary structure around the carbonyl oxygen is better defined for the two-dimensional method.

3.4 Hydration Radii of Lipid Atoms

The size of the first coordination sphere around an atom is determined from the pair distribution functions of solute atoms with solvent by measuring the position of the first minimum in the pair distribution function (3.375Å in Figure 3.4). By integrating between zero separation and this hydration radius, the number of waters in the first hydration shell may be evaluated (~4 using Figure 3.4 or 3.1).

The hydration radii were calculated both as an average for an atom type over the course of the entire simulation and for each individual atom in the system over shorter periods of time (20-100 picoseconds). The latter calculations were done as part of an investigation into the permeability of membranes; the waters of the first coordination sphere of highly charged atoms are bound quite tightly (see Section 3.6) and will have an effect on the ability of solutes to cross from the aqueous phase to the hydrocarbon interior, since any solute must either push between the hydrated lipid head groups, or displace a water from the coordination sphere.

These hydration radii values were used to determine the topography of the surface of the membrane including the first coordination sphere waters, by building 3-D models of a time-averaged bilayer system with the radii of the lipid atoms set to the hydration radius (see Figure 3.5). The transparency of the water hydration spheres is dependent on the number of waters about the atom (see Section 3.5). Lipid atoms showing few waters within their first coordination sphere are more transparent, i.e. it is easier to move past these atoms than ones with greater numbers of waters bound. These models showed the lack of space between head groups when first coordination sphere waters are taken into consideration.

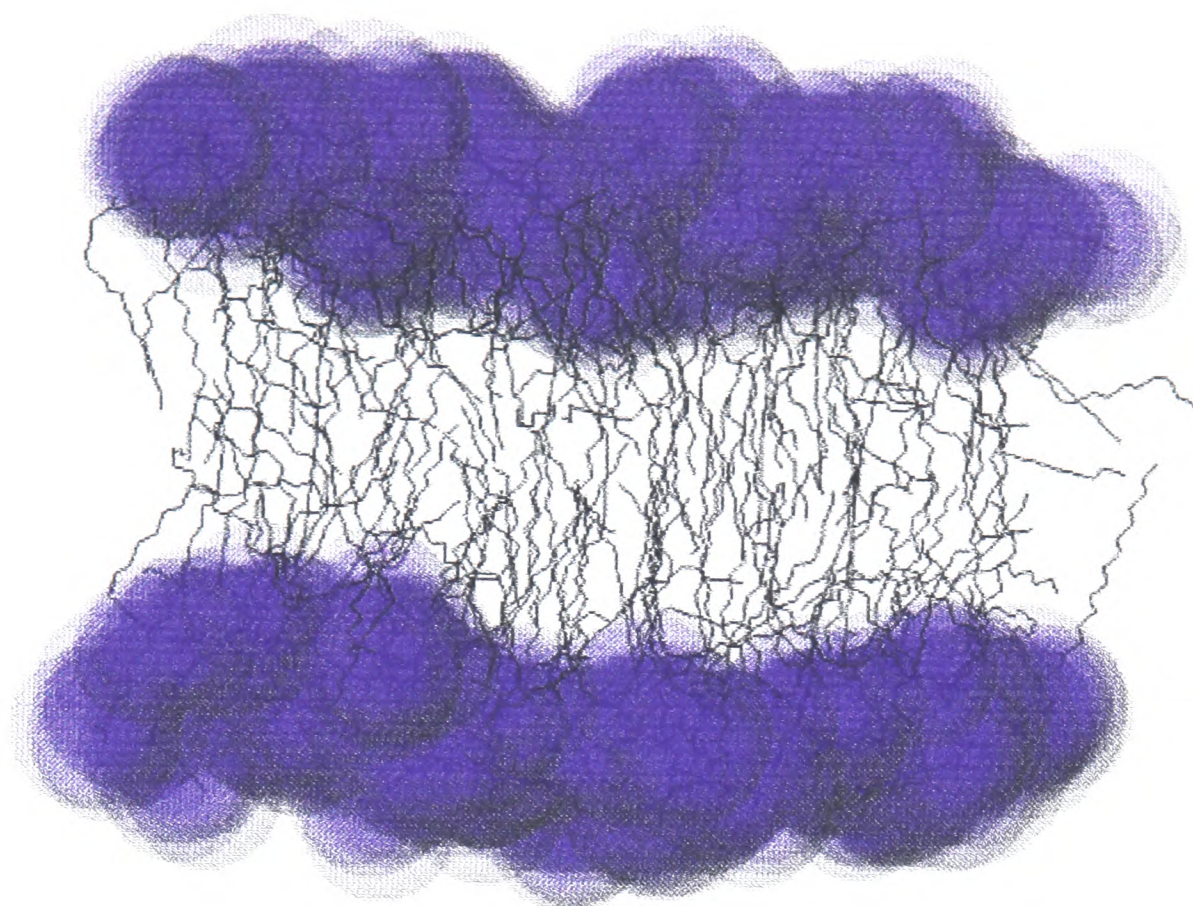


Figure 3.5 The hydration radii of the first coordination sphere of head group atoms of the lipids within a bilayer represented as transparent blue spheres. It is quite obvious that there is significant penetration of the water into the bilayer interior.

3.5 Water Coordination Numbers and Water Density about Lipid Atoms

There are few water molecules around lipid atoms situated deeper in the membrane which leads to pair distribution functions that are not well defined and may not even show minima. For this reason it was decided that ideal values of the hydration radii should be used for calculating the water coordination about these lipid atoms rather than values from their pair distribution function. Since the lipid atoms of the head groups appear well behaved, these were used to define the size of coordination spheres about carbon and oxygen atoms. Thus for carbon atoms, it is considered that any water within $4.6\text{\AA}$ of a lipid is in its first coordination shell and directly interacting with it, otherwise it is loosely, or not at all, associated with it. Similarly, for an oxygen atom the size of the first coordination shell is considered to be $3.3\text{\AA}$.

Thus for each atom of the lipid molecule, the pair distribution function with water was used to determine the number of waters interacting with it, i.e. the number of waters within a distance equivalent to being in the first coordination sphere of a fully solvated atom.

The coordination numbers calculated were used in 3-D models of bilayers to determine the transparency of hydration shells (see above). The lower the density of water around a lipid atom, the more transparent will be the hydration sphere. This gives a qualitative image of the hydration of the bilayer that can be used in discussions of solute permeability.

3.6 Residence Times of Water Molecules around Lipid Atoms

The mobility of water molecules in the head group region will have an important effect on the ease with which solutes may pass through this region. Since most waters in the core of the head group region are associated with a lipid atom, i.e. belong to a first coordination sphere, these water molecules must be either displaced, or moved aside for a solute molecule to pass through. To quantify how tightly first coordination water molecules are bound to lipid atoms, residence times for waters (defined as the average length of time that a water molecule persists within the first coordination sphere of a solute atom) were calculated for lipid atoms showing strong coordination. Atoms were deemed to have strong coordination if their pair distribution functions with water were sharp and well defined. The residence times are calculated in the manner described by Impey, Madden and McDonald (1983). In this method, a function $P_j(t, t_n; t^*)$ is used to describe the environment of the water molecule. It is equal to 1 if the water molecule j is in the first coordination sphere of the solute atom at both time t and $t+t_n$, without leaving it for longer than t^* in the intervening period, otherwise it is set equal to zero. P_j is used to define a new property, $n_i(t)$, which is equal to the number of water molecules persisting in the first coordination shell after a time, t , has elapsed for a solute atom i :

$$n_i(t) = \frac{1}{N_t} \sum_{n=1}^{N_t} \sum_j P_j(t_n, t; t^*) \quad (2)$$

NB, $n_i(t=0)$ is the normal coordination number of the solute atom. The factor t^* is introduced to account for water molecules that temporarily leave the first coordination sphere, but do not

become bulk water. The function $n_i(t)$ shows an exponential decay and thus an equation of the form $n_i(t) \sim n_i(0) \cdot \exp(-t/\tau_i^S)$ is fitted to the curve. The constant, τ_i^S , is the correlation time for this decay and is a simple definition of the residence time for water.

3.7 Bridging Water Molecules in the Lipid Bilayer

To investigate further the structure of water within the bilayer region, simple calculations were done to determine how many different first coordination spheres a water molecule belonged to at any one time, i.e. to determine whether water molecules form a hydrogen-bonded network that holds the head group region together. The presence of such bridging water molecules would also make it far more difficult for any solute molecule to pass through the head group region since it necessitates the displacement of a water molecule from a favourable location. To determine whether a water molecule was in a first coordination sphere of a lipid atom, the idealised hydration radii introduced in Section 3.5 were used to describe the size of the coordination shell. The number of coordination shells that water molecules belong to are reported as a function of depth through the lipid bilayer.

3.8 Time Correlation Functions

Correlation functions and correlation times are extremely useful functions to calculate for properties in molecular dynamics simulations since if a correlation is measured as a function of time, it describes the dynamic behaviour of a component of the system. The most common type of correlation function that is calculated is the autocorrelation function, $C_{AA}(t)$, which describes the correlation between a component A at time $t=0$ and a later time t . In stricter notation:

$$C_{AA}(\tau) = \langle A(\tau)A(0) \rangle = \frac{1}{\tau_{\max}} \sum_{\tau_0=1}^{\tau_{\max}} A(\tau_0)A(\tau_0 + \tau) \quad (3)$$

The FORTRAN77 subroutine used to calculate autocorrelation functions is taken from Allen and Tildesley (1987):

```

SUBROUTINE CORFUN(TRUN,TCOR,A,ACF,NORM)

INTEGER TRUN,TCOR
REAL A<TRUN>,ACF<0:TCOR>,NORM<0:TCOR>
INTEGER T,T0,TT0,TT0MAX
REAL A0

DO 100 T=0,TCOR
  ACF<T>=0.0
  NORM<T>=0.0
100 CONTINUE

DO 200 T0=1,TRUN
  A0=A<T0>
  TT0MAX=MIN<TRUN,T0+TCOR>
  DO 199 TT0=T0,TT0MAX
    T=TT0-T0
    ACF<T>=ACF<T>+A0*A<TT0>
    NORM<T>=NORM<T>+1.0
199 CONTINUE
200 CONTINUE

DO 300 T=0,TCOR
  ACF<T>=ACF<T>/NORM<T>
300 CONTINUE

RETURN
END

```

Extensive use is made of autocorrelation functions in the analysis of many dynamic properties of the bilayer system. The correlation time, t_A , of a property is calculated by fitting an equation of the form $C_{AA}(t) = C_{AA}(0) * \exp(-t/t_A)$ to a plot of $C_{AA}(t)$ against time.

3.9 Mobility of the Water Molecules

Mobility of the water molecules is measured in terms of its ability to undergo rotation and translation.

3.9.1 The Influence of Bilayer Structure on the Rotation of Water Molecules

The basic strategy in quantifying the freedom of waters to undergo rotation involved calculating the autocorrelation function of the water dipole both as a function of depth within the membrane and as a function of distance from the nearest lipid atom. The latter type of analysis will report how the rotational behaviour changes depending on the coordination sphere in which a water is located.

3.9.2 The Influence of Bilayer Structure on the Translation of Water Molecules

The translational mobility of water molecules is measured in terms of the diffusion coefficients and root mean square displacements and reported as a function of bilayer depth and as a function of distance from the nearest lipid atom. The diffusion coefficient, D , for diffusion in three dimensions is calculated using the Einstein relation:

$$2tD = \frac{1}{3} \left\langle \left| \mathbf{r}_i(t) - \mathbf{r}_i(0) \right|^2 \right\rangle \quad (4)$$

where $\mathbf{r}_i(t)$ is the position of the centre of geometry of the water molecule at time t .

Because of the anisotropic nature of the bilayer system, the root mean square displacement of water molecules was also calculated in directions parallel and perpendicular to the surface of the membrane and compared.

Exactly the same methodology is used to calculate the diffusion coefficients of lipid atoms.

3.10 Water Penetration into the Bilayer and Free Volume Calculations

Histograms were produced to show the extent to which water molecules penetrated into the lipid bilayer. The mechanism and prediction of diffusive behaviour of solutes within the lipid bilayer is central to understanding how drugs and other solutes may be designed to cross the biological membrane. It is believed that the barrier to non-electrolytes crossing lipid bilayers is the hydrophobic interior of the membrane composed of ordered hydrocarbon chains (Xiang, et al., 1992). The free-volume theory of Cohen and Turnbull (1959, 1970) describes

diffusion occurring only when the chains reorganise themselves so that enough available free volume comes together to form a cavity next to the solute that is large enough for it to move into.

Initial calculations on the amount of free volume within the bilayer were simplistic with a probe sphere the size of a TIP3P water molecule (Jorgensen et al., 1983) being systematically moved in 0.25Å steps in the x, y and z directions through the membrane. The TIP3P water molecule is essentially a sphere of radius 1.6Å centred about the oxygen because in the model the hydrogen is assigned a radius of 0Å. This technique provides no information about the number of cavities, their volume or their geometry, but does show the presence of cavities within the bilayer interior of sufficient size to contain a water molecule. Thus a plausible mechanism for diffusion involves the creation of cavities between hydrocarbon chains into which solute molecules can move, as well as a mechanism in which the presence of a solute molecule perturbs the surrounding chains causing a cavity to form.

A more sophisticated estimation of the free volume available to spherical solutes was carried out using a technique developed by Shah and co-workers from their work on polymers (Shah et al., 1989) and also used by Xiang (1993) on model lipid bilayers. At each sample point in the bilayer, the maximum sized sphere that can be placed there is evaluated and hence histograms for the size of permeant that can fit at any point determined. This analysis produces two distributions, N_i , which is the total number of spheres of any size located in a layer i of the bilayer and $N_{i,r}$, which is the number of spheres of radius r that can be placed in this layer i . The ratio of $N_{i,d}$ to N_i is the accumulated free volume fraction available in layer i (which is located at a depth z in the membrane) to a spherical solute of radius r :

$$f(V,z) = N_{i,r} / N_i \quad (5)$$

The total free volume fraction available as a function of depth in the bilayer is estimated by extrapolating $f(V,z)$ to $V=0$, i.e. $f(0,z)$.

3.11 Atomic and Electron Density Distribution Profiles

The distribution of atom types through the width of the bilayer is studied by averaging the positions of atoms along the z axis over the course of the simulation. For each frame of molecular dynamic simulation, the middle of the bilayer system, as defined by its centre of geometry, is translated to the origin so as to remove any drift of the system over the course of the simulation which would lead to a blurring of the results. These atomic density profiles are used to measure the thickness of different regions through the bilayer by evaluating the cumulative distributions for groups of atoms, rather than for individual atom types. The lipid molecule is divided into three components for such calculations; the atoms of the phosphatidyl choline head group, the glycerol moiety and the carbon atoms of the hydrocarbon chains. It is hoped that parameters including results from free space calculations to describe the barrier to diffusive behaviour and results from other calculations on the model bilayer can be associated with each of these regions to describe the behaviour of solutes within them in order to produce a simplistic bilayer model and circumvent the need for large molecular dynamic simulations.

For qualitative comparison with experimental results on membranes by x-ray diffraction (McIntosh, 1978; Lesslauer et al., 1972) electron density distributions are calculated using atom-centred values for the electron density for each atom. The number of electrons associated with each atom is consistent with the atom having neutral charge, the exceptions are the nitrogen and phosphorous which are assigned six and sixteen electrons respectively to reproduce the charge distribution within the head group.

3.12 The Tilt of the Hydrocarbon Chains

One of the main concerns during any molecular dynamic simulation is whether the system size is large enough that there are periodic boundary condition violations, i.e. molecules seeing their own image, or simulation artefacts. A possible artefact under periodic boundary conditions arises from cooperative motion between chains being propagated across the whole length of the box and hence amplified. This argument has been used to explain the observed cooperative tilt in the same direction of hydrocarbon chains in small bilayer systems (van der Ploeg and Berendsen, 1982). However it appears that although a gross overall tilt observed in

a model bilayer may well be an artefact, that individual chains show a tilt with respect to the bilayer normal is correct (cf. Chapter 4, Robinson et al., 1994). Thus for each system two descriptions of the hydrocarbon chain tilt with respect to the bilayer normal are calculated, the average individual tilt of a hydrocarbon chain and the average collective tilt over the whole system.

The tilts are evaluated using the method originally described by van der Ploeg and Berendsen (van der Ploeg and Berendsen, 1982 and 1983) in which the tilt angles are calculated by defining a unit vector, $\mathbf{S}_i$, that connects the mid-points of two bonds in a hydrocarbon chain. The average individual tilt of a hydrocarbon chain, θ_s , is the average of the angles that the vector $\mathbf{S}_i$ makes with the bilayer normal, $\mathbf{n}$:

$$\cos(\theta_s) = \frac{\mathbf{S}_i \cdot \mathbf{n}}{|\mathbf{S}_i| |\mathbf{n}|} \quad (6)$$

The average collective tilt of the hydrocarbon chains in the bilayer is evaluated by defining another vector, $\mathbf{T}$, which describes the average tilt vector of a layer. $\mathbf{T}$ is the average of the N individual $\mathbf{S}_i$ vectors:

$$\mathbf{T} = \frac{1}{N} \sum_{i=1}^N \mathbf{S}_i \quad (7)$$

The angle that $\mathbf{T}$ makes with the bilayer normal is the average collective tilt angle, θ_t . $\mathbf{T}$ is also used to measure the correlation between the chains by calculating the length of $\mathbf{T}$, i.e. $|\mathbf{T}|$. The closer $|\mathbf{T}|$ is to unity, the greater the correlation between chains. If the chains are fully aligned parallel to each other then $|\mathbf{T}|$ will be unity since $\mathbf{S}_i$ is defined as a unit vector.

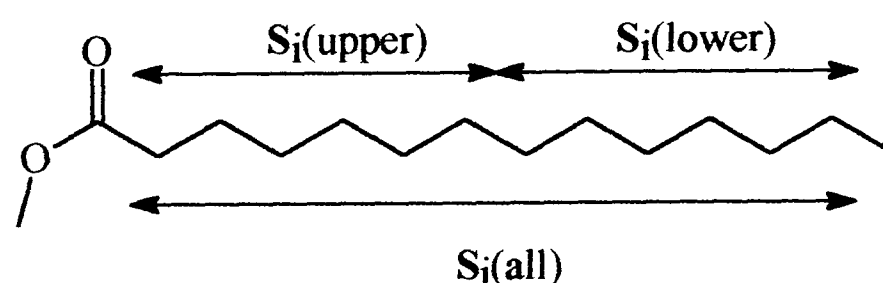


Figure 3.6 The three $\mathbf{S}_i$ vectors defined for each hydrocarbon chain used to calculate the tilt angles for different parts of the chain in the early simulations of lipid bilayers described in Chapter 4. Later simulations had the lipid chain divided into thirds rather than half, in an identical fashion.

Three different S_i vectors are defined for both the S_n1 and the S_n2 hydrocarbon chain (see Figure 3.6) and by comparing the individual and collective tilt angles values for different parts of the chains, ideas about the structure and packing of chains in these different regions may be inferred.

3.13 Dihedral Population and Pattern Sequence Recognition

Because of the large inter- and intramolecular interactions in the close-packed head group and hydrophobic core, it seems probable that free rotation about dihedrals is prohibited. In the hydrocarbon chains it has long been believed that gauche conformations may only appear as part of certain sequences (see Chapter 2) and there has been much speculation that the dihedral angles of the head groups show limited rotation. The limiting cases of completely flexible and completely rigid head groups have been discarded (see Chapter 4).

For each dihedral angle in a DMPC molecule, the average fraction of it as trans, gauche⁻ and gauche⁺ are calculated. From this the most probable phase for a dihedral angle can be determined. Since there is probably cooperativity between neighbouring dihedral angles, the probability of certain sequences occurring is investigated, including kinks and jogs.

In Figure 3.7 the numbering scheme for dihedral angles along the hydrocarbon chain is illustrated.

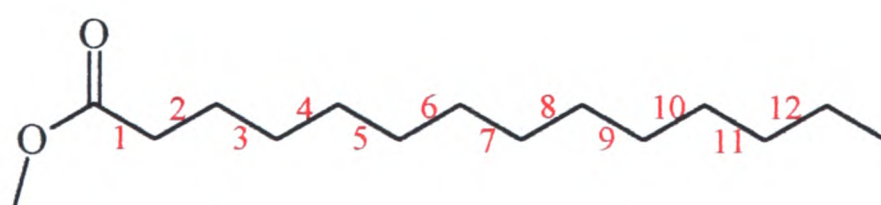


Figure 3.7 The numbering scheme for the twelve dihedral angles of the hydrocarbon chains.

3.14 Time Correlations of Hydrocarbon Chains

The autocorrelation function of S_i (see Section 3.12) can be evaluated and used to determine a correlation time that measures the length of time for which correlations persist:

$$C(\tau) = \langle \mathbf{S}_i(t + \tau) \cdot \mathbf{S}_i(t) \rangle \quad (8)$$

Long autocorrelation times are expected since the close packing of hydrocarbon chains leads to severe restriction of their motional freedom and this causes little phase space to be sampled during the course of a simulation.

The correlation times evaluated from $\mathbf{S}_i$ are not an ideal description of the extent of order in the bilayer because the correlation times calculated by fitting an exponential function to $C(t)$ can be longer than the simulation length. Of more interest is the extent of spatial and temporal correlations between chains and their neighbours. To investigate these, two new correlation functions were developed which use a vector $\Delta \mathbf{r}_i(t)$. $\Delta \mathbf{r}_i(t)$ is the difference between the individual tilt vector $\mathbf{S}_i$ at a time t and a vector describing the same lipid tilt vector, but averaged over the length of the simulation, i.e. $\langle \mathbf{S}_i(t) \rangle$. (See Figure 3.8).

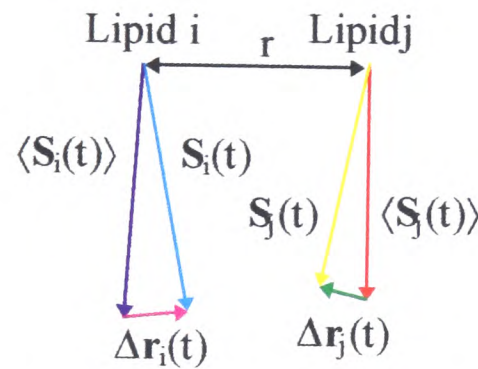


Figure 3.8 Schematic illustration showing the vectors defined and used for the calculation of a spatial correlation function of neighbouring lipid chains. The different terms are described in the text.

The equation to calculate the spatial correlation function has a simple form:

$$C(r)dr = \left\{ \frac{\langle \Delta \mathbf{r}_i(t) \cdot \Delta \mathbf{r}_j(t) \rangle}{|\Delta \mathbf{r}_i(t)| |\Delta \mathbf{r}_j(t)|} \right\} dr \quad (9)$$

To determine the temporal correlation between chains, the lipid neighbours are split into classes depending upon their distance from the lipid with which their correlation is being calculated. The size of these distance classes is determined from radial distribution functions

with each class being the distance from one minimum in the function to the next (cf. the calculation of sizes of successive solvation shells).

For each distance class, a correlation function is evaluated:

$$C_r(\tau) = \langle \Delta \mathbf{r}_i(t) \cdot \Delta \mathbf{r}_j(t + \tau) \rangle \quad (10)$$

N.B. For each analysis it must be decided whether or not the second chain of the DMPC is to be considered as a neighbour since the correlation between the two chains of a DMPC molecule will be much higher than for the chains of neighbouring lipids.

Finally, the autocorrelation function of $\Delta \mathbf{r}_i(t)$ is evaluated:

$$C(\tau) = \langle \Delta \mathbf{r}_i(t) \cdot \Delta \mathbf{r}_i(t + \tau) \rangle \quad (11)$$

The purpose of defining $\Delta \mathbf{r}_i(t)$ and its associated correlation functions is that it relates to the model proposed by Petersen and Chan (Petersen and Chan, 1977) in which NMR senses not the instantaneous orientation of the chain, $\mathbf{S}_i(t)$, but the time-average orientation of the chain, $\langle \mathbf{S}_i(t) \rangle$, resulting from the hydrocarbon chain sweeping out a cone (see Chapter 2).

3.15 Summary

A suite of programs has been written that allow the characterisation of structural and dynamic properties of lipid bilayers and their solutes in both a qualitative and quantitative manner.

Chapter Four

Simulations of Pure Lipid Bilayers

4.1 Introduction

Before a more complex and detailed model of a biological membrane containing proteins, sterols and other components can be attempted, a bilayer model must be developed which reproduces experimental observations on real lipid membranes.

During the course of this study several bilayer models have been tested which differ in the packing arrangement of the lipids, the starting conformations of the lipids and the number of lipids in the system. What is most startling has been the huge increase over the last three years in the size of the system that it is possible to investigate with molecular mechanics. This is because of the advances in computer architecture and programming, especially in the use and application of parallel and massively parallel machines. Our early simulations of explicit membrane models possessed forty or fewer lipid molecules and contained only seven or eight thousand atoms (Essex, 1992; Essex et al., 1994; Robinson et al., 1994, 1995). Now simulations of hydrated systems of over thirty-five thousand atoms which contain ion channels and greater than one hundred and sixty lipid molecules are routine.

When building a model of a membrane, the following points must be considered:

- The initial conformation of the lipid molecules in the starting structure
- How the lipid molecules are to be packed together in the starting structure
- The parameters to be used in the molecular mechanics calculations

The most obvious source of lipid conformation and bilayer structure would seem to be the crystal structure of DMPC (Pearson and Pascher, 1979). However as outlined in Section 2.3 the crystal structure of the L_β phase of DMPC is not an ideal structure to use for modelling

the packing of lipids in the fluid L_α phase because of the small degree of hydration and constraints of crystal packing in the solid phase.

Two models of pure bilayer models are presented in this thesis and exemplify the increasing complexity and integration of experimental observations in the building of initial starting structures as the membrane model evolved:

1. A forty lipid bilayer system with the lipid packing based on that of the crystal structure. Initial lipid conformations were adapted from the DMPC crystal structure, but with changes to make them consistent with data from NMR studies of the fluid phase. The hydrocarbon chains were initially in an all trans conformation and had to be melted during molecular dynamic simulations.
2. A seventy-two lipid system with a pseudo-random orientation of lipid molecules packed in a hexagonal arrangement. The conformations of the lipid head groups were based on details from both X-ray and NMR studies. Each hydrocarbon chain was built containing a kink and with the terminal dihedral angle being gauche, i.e. there are three gauche conformations per chain which is consistent with the experimental and theoretical studies as described in Chapter 3.

A seventy-two lipid system was also built using the same four lipid conformations of model 2, but with the packing of the lipids closer to that of the crystal structure and model 1. This structure was built using a specially written application that given the required head group surface area and number of lipids, automatically generates a monolayer structure that can subsequently be used to build a bilayer. Thus membrane models may be quickly and easily generated and this application was used in the production of large bilayers into which proteins were placed as described in later chapters. The structures produced are initially more ordered than in model 2 as the lipid sheets are built from repeating units of four lipids whose orientation with respect to each other is chosen so that the gross structure has a head group packing scheme close to that of the crystal structure. However, the lipids of model 2 are placed by hand so although there is local order to allow favourable interactions, there is no

overall order to the structure which is perhaps the more natural situation. Other groups have used a Monte Carlo approach to build membrane models as pioneered by Venable and co-workers (Venable et al., 1993), but it was felt that although the process used here is more time consuming, there is greater control of the initial lipid conformations and structure being generated and the model is just as valid.

The rest of this chapter describes the considerations that went into building the initial bilayer models and their subsequent analysis.

4.2 Conformations of the Lipid Molecules

A DMPC molecule can be divided into three components, the phosphatidyl choline head group, the glycerol moiety and the two fatty acid chains. Structural information on these components in the L_α phase is available from ^2H and ^3P NMR and X-ray and neutron diffraction experiments as discussed in Section 2.4. This information as well as the lipid crystal structure is used in building the starting lipid conformations.

In all the models constructed, the initial dihedral angle conformations of the head groups were taken from the ^2H and ^3P NMR experiments of Seelig et al. on DMPC in the L_α phase (Seelig et al., 1977; Seelig, 1978), i.e. the two enantiomeric conformations (t, g^+, g^+, t, g^-) and (t, g^-, g^-, t, g^+) for the dihedral angles ($\alpha_1, \alpha_2, \alpha_3, \alpha_4, \alpha_5$) (see Figure 2.10).

In the L_β crystal structure of DMPC the dihedral angle θ_3 is trans. However, ^1H spin-coupling measurements on the L_α phase indicate there are three rotamers for this dihedral angle with the ratio of trans:gauche⁺:gauche⁻ being 6.5:5.0:1.0 (Hauser et al., 1988) and relaxation studies by Hauser et al. show rotational jumps between two rotamers about the glycerol $\text{C}_2\text{-C}_3$ bond (Hauser et al., 1988). Furthermore, simulations of DMPC bilayers that contained only the trans rotamer in the initial lipid conformation had within 400 picoseconds of the start of the simulation about 15% of the lipid molecules having the gauche⁺ rotamer while the gauche⁻ rotamer was not observed to occur (Essex, 1992; Essex et al., 1994).

Thus for realistic simulations of the DMPC bilayer it is apparent that the gauche⁺ rotamer needs to be included in the starting structure. The source of the coordinates for this

alternative glycerol rotamer is DLPE.Me₂ which crystallises with the gauche⁺ conformation at θ_3 and differs from the phosphatidyl choline head group of DMPC by possessing one less methyl group on the N-methyl (i.e. DMPC could be described as DLPE.Me₃).

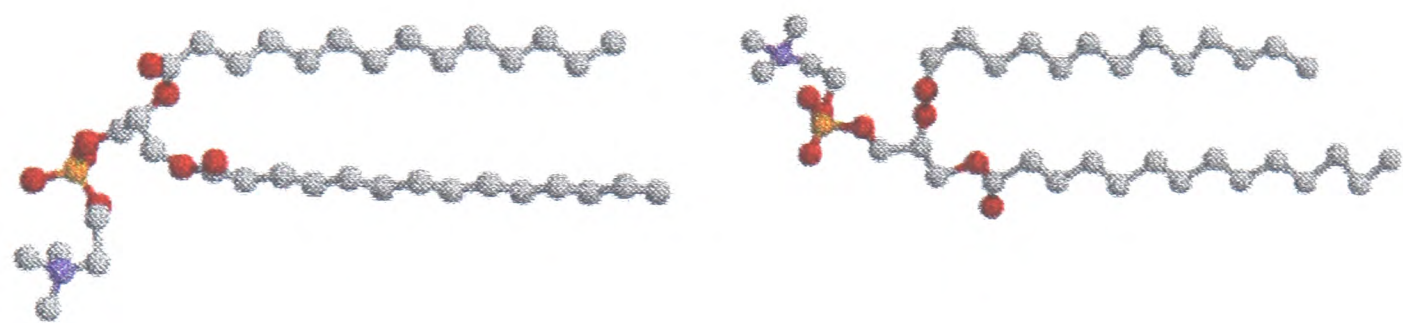


Figure 4.1 The two lipid conformations DMPC1 and DMPC2 used in the early bilayer models.

In model 1 described above, only two different lipid conformations, referred to as DMPC 1 and DMPC2, were used for which the dihedral angle values are listed in Table 4.1 and the structures shown in Figure 4.1.

Lipid	α_1	α_2	α_3	α_4	α_5	θ_1	θ_2	θ_3	θ_4
DMPC 1	-170°	60°	64°	145°	-81°	58°	177°	54°	-60°
DMPC 2	170°	-60°	-64°	-145°	81°	168°	-82°	166°	51°

Lipid	β_1	β_2	β_3	β_4	γ_1	γ_2	γ_3	γ_4
DMPC 1	148°	173°	-57°	176°	129°	-167°	166°	175°
DMPC 2	120°	179°	-134°	67°	102°	176°	-180°	180°

Table 4.1 The dihedral angles of the head groups and glycerol moieties of the two starting conformations used in model 1.

In this early model it was decided that the (t, g^+, g^+, t, g^-) head group conformation would occur with the trans θ_3 glycerol rotamer and the (t, g^-, g^-, t, g^+) head group conformation with the gauche⁺ θ_3 glycerol rotamer. In retrospect there is very little justification for this and later bilayer models contained four head group conformations by using all the combinations of the two glycerol rotamers with the two enantiomeric head group conformations (see Figure 4.2).

For the new conformations the value of the θ_1 dihedral angle was adjusted where necessary so that the P-N dipole remained approximately parallel to the surface of the bilayer in order to be consistent with ^{31}P NMR (Yeagle, 1977) and neutron diffraction studies (Büldt et al., 1978).

In model 1 the coordinates for the hydrocarbon chains are taken from the DMPC crystal structure and hence are in the all trans conformation. Once a bilayer model is constructed, molecular dynamic calculations have to be run to melt the hydrocarbon chains by introducing gauche conformations into them. In an attempt to reduce the time needed for equilibration later models had gauche conformations included in the initial lipid conformations so that each chain contained a kink and the terminal dihedral angle was gauche (see Figure 4.2).

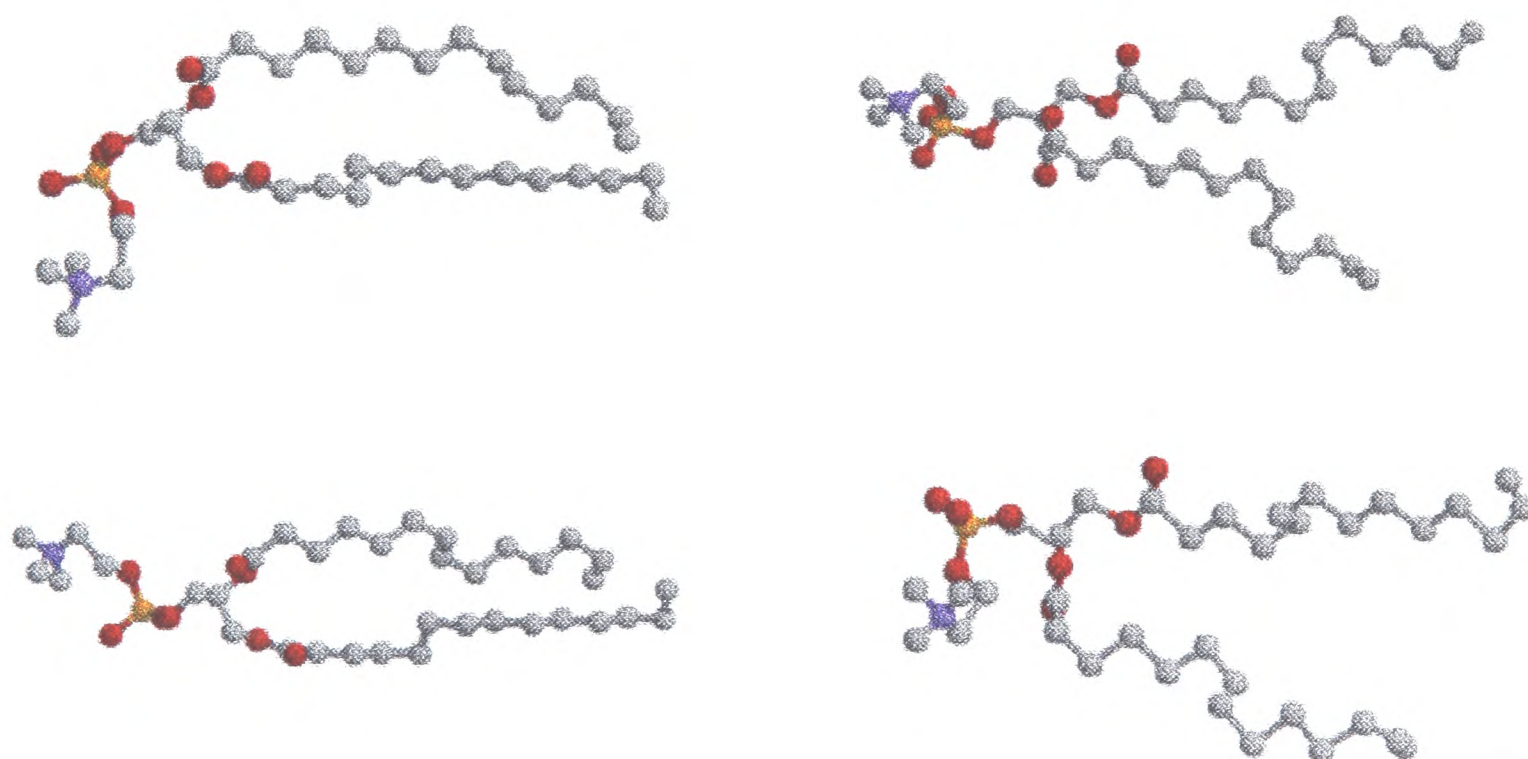


Figure 4.2 The lipid conformations used in later bilayer simulations that include the four possible conformations of the two enantiomeric head group conformations with the two glycerol moiety rotamers.

Since molecular dynamic simulations are done in the NVT ensemble it is essential that the dimensions of the model are identical to those of a system under normal atmospheric conditions. In the plane of the bilayer this equates to the head group surface area per lipid. Many values for this parameter have been published (see Hagle, 1983, and references therein) and the value recorded depends upon the hydration of the bilayer, the temperature and probably the experimental technique used to record it. A value of $66\text{\AA}^2$ is used in all pure bilayer models since this is consistent with most experimental recordings on the hydrated DMPC system at room temperature.

For the width of the membrane, results from data of X-ray scattering experiments are used which report the separation of phosphorous atoms across the membrane being $35\pm 1\text{\AA}$ (Lewis and Engelman, 1983) and are also consistent with measurements from ^2H NMR (Seelig and Seelig, 1974).

4.3 The Molecular Mechanics Program and Parameters

The molecular dynamics and energy refinement program AMBER was used in all simulations (Singh et al., 1988). The bond stretching, angle bending and dihedral angle energy terms are all contained in the standard AMBER force field except the parameters for the ester groups which are those obtained by Charifson and co-workers (Charifson et al., 1990) and have been used previously in DMPC simulations (Essex et al., 1994). The nonbonded and partial charges are based on the OPLS united atom parameter set of Jorgensen and co-workers (Jorgensen et al., 1983) and are completely consistent with the TIP3P water model that is used (Jorgensen et al., 1983) plus they have been optimised to reproduce solution phase behaviour. The additional bonus of using OPLS parameters for the lipid molecule is that once proteins are integrated into the membrane, they are consistent with the well respected and trusted OPLS nonbonded parameter set for proteins.

In line with current thinking, hydrogen bonding is treated as an electrostatic phenomenon rather than with the use of a 10-12 potential function since this reflects more closely the real situation.

When calculating the dihedral angle energy which includes the 1,4 nonbonded interactions, the van der Waals contribution is reduced by a factor of eight and the electrostatic part by a factor of two as recommended when using the AMBER united atom force field with OPLS parameters within the AMBER program (Jorgensen and Tirado-Reeves, 1988).

Lastly, two sets of improper torsions are defined which prevent the chiral centre at C2 from racemising and maintain the planarity of the ester groups.

4.4 Model 1: The First Attempt at Models of Pure Lipid Bilayers

[This work was published in The Biophysical Journal, Robinson et al., 1994. The description of results is not an in-depth analysis compared to subsequent work since later simulations are superior, however the work is included for comparison].

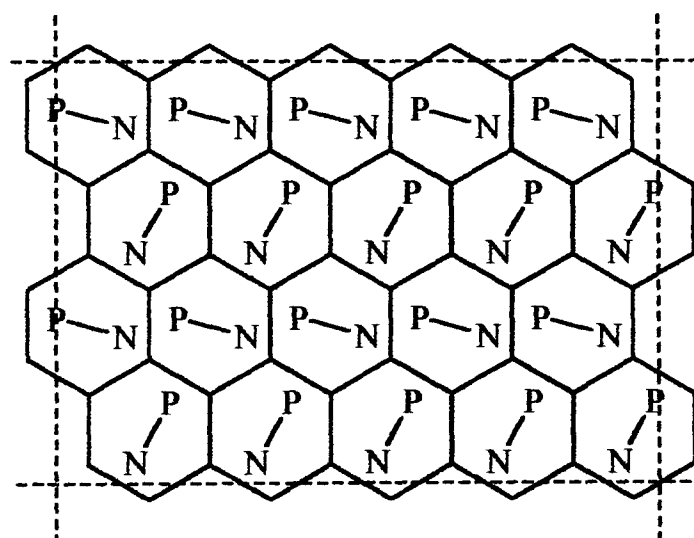


Figure 4.3 Schematic diagram of the surface of the membrane starting structure illustrating the hexagonal packing of the head groups and the relative orientation of the P-N dipoles. The dashed lines indicate the edges of the periodic box.

Using the two lipid conformations DMPC1 and DMPC2 described above for model 1, i.e. two enantiomeric head group conformations with different glycerol moiety rotamers and the hydrocarbon chains in an extended all trans conformation, a monolayer of twenty lipid molecules was designed. Rather than following previous work where an adaptation of the crystal structure which tries to account for the differences in volume between the phases was used (Damodaran and Merz, 1993; Essex, 1992; Essex et al., 1994); the lipids were equally

spaced in a hexagonal packing arrangement with the relative orientation of the molecules being similar to that observed in the crystal structure (see Figure 4.3).

The philosophy behind this arrangement is that within the crystal phase the structure adopted is governed by interactions between the lipid chains and their desire to be close packed. Conversely in the fluid liquid crystal phase it is the head groups which influence the structure.

The lipids of the monolayer were aligned with respect to the bilayer normal so that the phosphorous atoms were all co-planar with the surface of the membrane. This is in contrast to the situation in the crystal structure (see Figure 2.6) which differs because of the different packing considerations (see above). Two of these monolayers were placed together to form a bilayer structure such that the width of the membrane as measured by the separation of phosphorous atoms was consistent with the data of X-ray diffraction experiments (Lewis and Engelman, 1983) which necessitated having the chains interdigitated at the bilayer centre because of their extended conformation (see Figure 4.4).

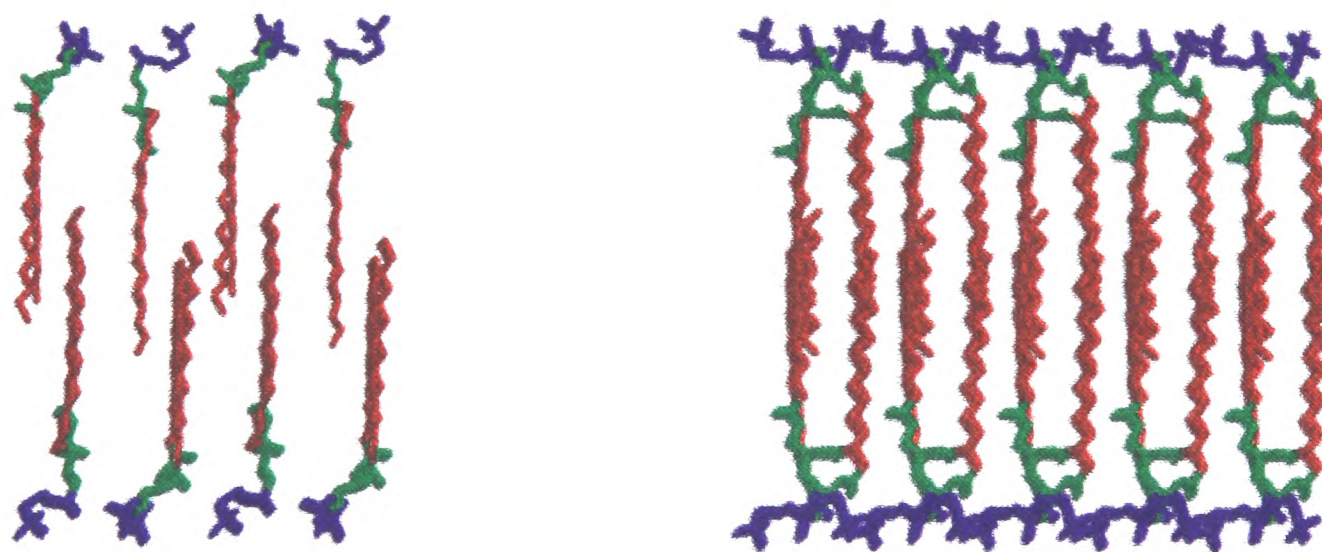


Figure 4.4 Two side views of the initial bilayer model, excluding waters, illustrating the interdigitation of the chains at the bilayer centre and the flat surface of the membrane. The head groups are coloured blue, the glycerol moiety and hydrophilic carbonyls groups are coloured green and the hydrocarbon chains are coloured red.

Next water molecules were added to the system and allowed to penetrate down to the carbonyl groups of the lipid ester linkages because the presence of water to this depth has been verified by Raman spectroscopy (Blechner et al., 1990).

A residue based cut-off of 9Å was applied for nonbonded interactions so that if an atom in a residue of a molecule was within 9Å of an atom in another residue, then the interactions between all the atoms in these two residues are calculated even if the separations of some are greater than the cut-off distance. The list of atom pairs which have nonbonded interactions was updated every 50 fs.

For the purposes of the molecular mechanics the lipid molecule was defined as a three residue molecule consisting of the head group, the Sn1 hydrocarbon chain with the glycerol moiety and the Sn2 chain. A potential problem with this method is that the head groups on one side of the bilayer do not interact with those of the other and hence in later simulations the lipid molecule was defined as a single residue to avoid the problems of split dipoles and charge separation. However, dividing the molecule into three residues dramatically reduces the size of the nonbonded pair list which leads consequently to shorter CPU times for the production of molecular dynamic trajectories. At the time of the study this approximation was justified because the available computer power necessitated the need to economise computer resources.

The initial model of the hydrated bilayer must undergo energy refinement to reduce unfavourable interactions, especially in the middle of the bilayer where interdigitation occurs and there are very large, positive nonbonded energies. The structure was energy refined in stages with first only the lipid groups being energy minimised followed by the waters alone before the whole system was energy refined. Next the hydrocarbon chains were “melted” from their crystal structure conformation by running 200 ps of molecular dynamic simulation on the lipids whilst keeping the positions of the water molecules constrained. The water molecules are held fixed because in the initial bilayer structure there are large gaps between lipid molecules into which water molecules tend to rush if they are allowed to move (see Figure 4.4). Once the hydrocarbon chains have melted the extent of interdigitation at the bilayer centre is reduced as is the free space between lipid molecules.

Before full molecular dynamic calculations, an energy refinement of all atoms was performed followed by 10 ps of molecular dynamic simulation on the water molecules to equilibrate their position around the head groups. All atoms were then energy refined and a preliminary molecular dynamic equilibration simulation run with a time step of 2 fs in case there were still any close contacts that had not been resolved by energy refinement calculations. As the main equilibration phase a molecular dynamic simulation with a time step of 5 fs was run for 200 ps which was followed by a subsequent 200 ps that was considered as the production run and analysed. Figure 4.5 shows lipid bilayer after 400 ps of molecular dynamics simulation on all atoms with the hydrocarbon chains showing an obvious tilt.

During molecular dynamic simulations, the system temperature was maintained at 323K using the Berendsen algorithm (Berendsen et al., 1984) with a relaxation time of 100 fs and the bond lengths were constrained using the SHAKE algorithm (Ryckaert et al., 1977).

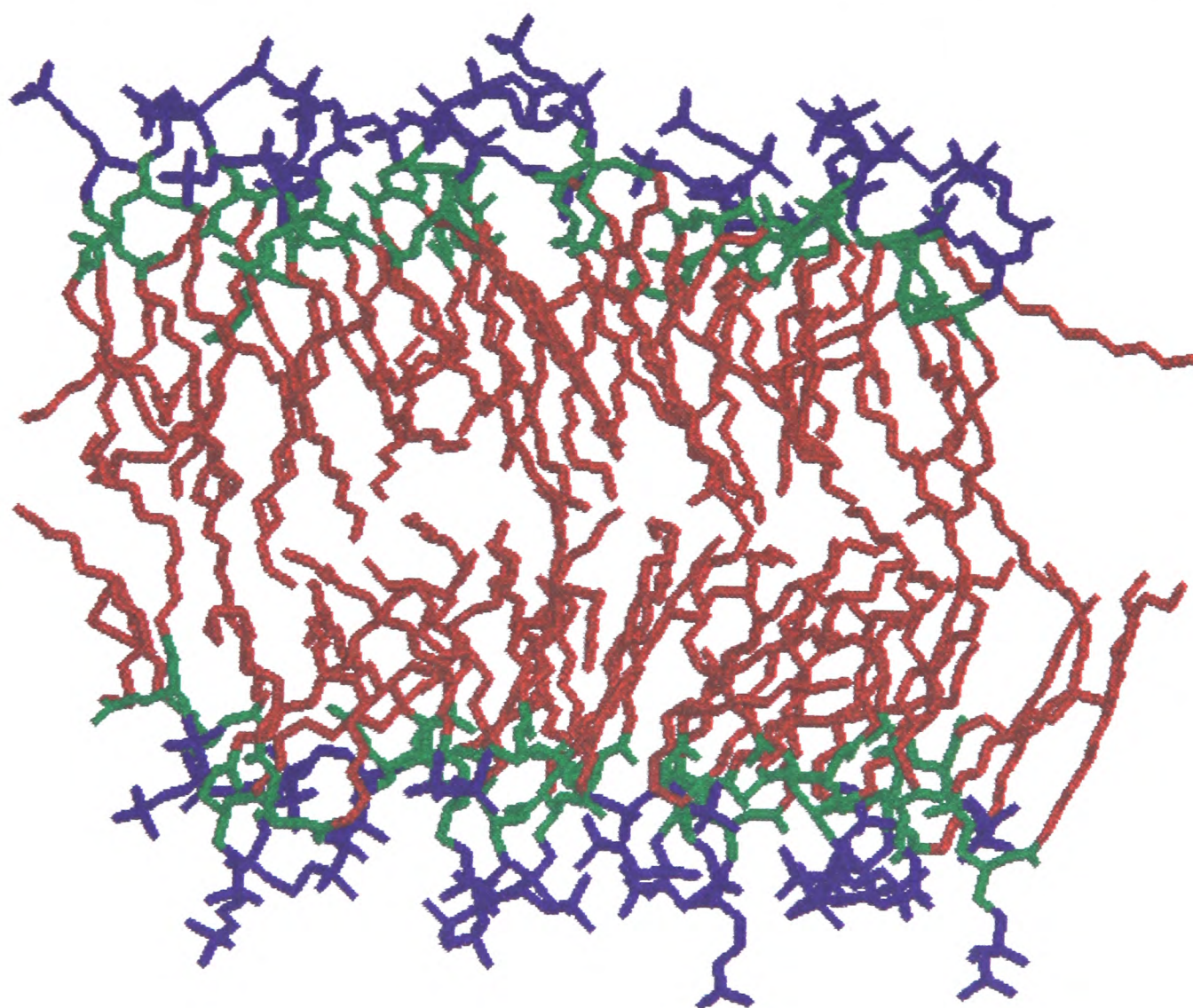


Figure 4.5 The lipid bilayer structure, excluding water molecules, after 400 ps of molecular dynamic simulation on all atoms. The head groups are coloured blue, the glycerol moiety and hydrophilic carbonyls groups are coloured green and the hydrophobic hydrocarbon chains are coloured red.

4.4.1 Dynamic Behaviour and Structure of the Lipid Head Groups

Previous simulations of phospholipid bilayers have not characterised fully the conformational behaviour of the head groups, but discussed the orientation of the P-N vector with the bilayer surface (Heller et al., 1989; Damodaran et al., 1992). Stouch (1993) notes that the head groups exhibit a number of conformations and that motion about some torsions is limited and Essex (1994) gives a description of the conformations observed.

The starting bilayer structure contained only two head group conformations but during the molecular dynamic simulations other conformations appeared rapidly and within 70 ps of the start of the equilibration phase there were already a large range present. Table 4.2 lists the populations of the head group dihedral angles α_1 to α_5 and Table 4.3 lists the populations of different head group structures as described by α_2 , α_3 and α_4 . The dihedral angles α_1 and α_5 are not used to classify the head group structure since α_1 is predominantly trans and α_5 is almost exclusively trans.

Dihedral Angle	trans	gauche	gauche ⁺	gauche ⁻
α_1	67%	33%	8%	25%
α_2	27%	73%	36%	37%
α_3	26%	74%	36%	37%
α_4	56%	44%	18%	26%
α_5	82%	18%	6%	12%

Table 4.2 The fractional distribution of each of the α_i head group dihedral angles from 200 ps of molecular dynamic simulation. The fraction of gauche bonds is further divided into the fraction of gauche⁺ and gauche⁻.

Table 4.3 also lists the percentage populations for the dihedral sequence (α_2 , α_3) which is responsible for the bend in the head group structure. For the dihedral angle sequence (α_2 , α_3) it is evident that there is not an even distribution of conformations with 43% possessing the (g, g) pattern and another 43% having at least one gauche dihedral angle. Ten percent have

the (g, g') conformation and only 4.8% have both α_2 and α_3 being trans dihedral angles. Thus there are a much greater range of head group conformations than proposed by Skarjune and Oldfield (1979), but the head groups are not completely conformationally free. Inter- and intramolecular interactions may be responsible for this with a preference for gauche dihedral angles to minimise the distance between opposite charged atoms in the zwitterionic head groups.

$(\alpha_2, \alpha_3, \alpha_4)$	Population	$(\alpha_2, \alpha_3, \alpha_4)$	Population	(α_2, α_3)	Population
(g^+, g^+, t)	13.0%	(g^-, g^-, t)	13.3%	(g^+, g^+)	21.7%
(g^+, g^+, g^+)	4.1%	(g^-, g^-, g^-)	4.8%	(g^-, g^-)	21.3%
(g^+, g^+, g^-)	4.6%	(g^-, g^-, g^+)	3.2%	(t, g^+)	10.5%
(g^+, t, t)	4.0%	(g^-, t, t)	4.8%	(t, g^-)	10.8%
(g^+, t, g^+)	4.1%	(g^-, t, g^-)	4.8%	(g^+, t)	10.0%
(g^+, t, g^-)	1.9%	(g^-, t, g^+)	2.0%	(g^-, t)	11.6%
(t, g^+, t)	8.1%	(t, g^-, t)	4.1%	(g^+, g^-)	4.8%
(t, g^+, g^+)	3.5%	(t, g^-, g^-)	3.9%	(g^-, g^+)	4.7%
(t, g^+, g^-)	2.1%	(t, g^-, g^+)	2.8%	(t, t)	4.8%
(t, t, g^+)	0.9%	(t, t, g^-)	2.6%		
(t, t, t)	1.3%				
(g^+, g^-, t)	3.3%	(g^-, g^+, t)	4.4%		
(g^+, g^-, g^+)	1.1%	(g^-, g^+, g^-)	0.1%		
(g^+, g^-, g^-)	0.3%	(g^-, g^+, g^+)	0.3%		

Table 4.3 The populations of the 27 head group structures defined by $(\alpha_2, \alpha_3, \alpha_4)$ and the nine by (α_2, α_3) from 200 ps of molecular dynamic simulation.

Analysis of the molecular dynamic trajectory shows the average dipole moment of the head groups as defined by a vector connecting the nitrogen and phosphorous atoms in the head group, makes an angle of approximately 5° with the bilayer surface, i.e. the phosphorous and

nitrogen atoms are almost co-planar. Furthermore the carbon atoms C11, C12 and the methyls attached to the nitrogen in the choline head group are also approximately equidistant from the bilayer centre. (The actual values are 17.9Å, 18.2Å and 18.5Å with a standard deviation of 3Å). These observations are consistent with neutron diffraction data (Büldt et al., 1978; Büldt et al., 1979), neutron diffraction data on selectively deuterated lipids (Seelig et al., 1977), X-ray diffraction experiments (Lesslauer et al., 1972) and ζ potential data (Makino et al., 1991). The latter data show the orientation of the head group to be dependent on temperature and ionic strength.

Table 4.4 lists the relative proportions for the three rotamers of the glycerol moiety, i.e. the dihedral angle about θ_3 . The dihedral angle is observed not to be rigid with the trans conformation being the more probable and the proportions are close to those reported experimentally (Hauser et al., 1988).

Rotamer, (θ_3, θ_4)	Population from NMR on DPPC*	Population from 200 ps molecular dynamic simulation
A (t, g ⁺)	52.0%	56.8%
B (g ⁺ , g ⁻)	40.0%	43.2%
C (g ⁻ , t)	7.5%	0.0%

* Hauser et al., 1988.

Table 4.4 The relative proportions of the three rotamers about the glycerol moiety as described by the θ_3/θ_4 dihedral angles.

4.4.2 Dihedral Angle Behaviour of the Hydrocarbon Lipid Chains

The fluidity in the hydrocarbon region arises from the rotations about the C-C bonds of the lipid chains. But dihedral angle rotation is not completely free because of the presence of neighbouring chains (see Section 2.4.1). The mean frequency of gauche dihedral angles during the molecular dynamic simulation is calculated as 2.6 for the Sn1 chain and 2.5 for

the Sn2 chain which compares favourably with the experimental values of 2.5 to 2.8 per chain (see Section 2.4.1).

The mean frequency of kinks is calculated as 0.4 per lipid chain over the course of the molecular dynamic simulation which compares very well with the value of 0.44 ± 0.07 proposed from FT-IR and ^2H -NMR experiments (Tuhtenhagen, Ziegler and Blume, 1994) for the number of kink (gtg) plus gtg conformations per chain. Theoretical calculations predict the number of kinks per myristoyl chain to be 0.5 at 55°C (Cevc and Marsh, 1987).

4.4.3 The Order Parameters of the Lipid Chains

The order parameter, S_{CD} , is a measure of the motional disorder of the methylene groups in a hydrocarbon chain (Seelig and Seelig, 1974; Davis, 1983) and is calculated from the molecular dynamic trajectory as described in Section 2.4.1. Contributions to the order parameter profile will include contributions from inter- and intramolecular motions, e.g. rotational isomerisation between trans and gauche conformations, the rate of trans-gauche isomerisation, and the reorientation of the lipid molecule as a whole (Mayer et al., 1988). The order parameter profiles calculated from 200 ps of molecular dynamic simulation are shown in Figure 4.6 with the standard deviation used for the error bars.

The shape of the calculated profiles is similar to that from NMR (Figure 2.8) but given the caveats about NMR derived order parameters (see Section 2.4.1), the close similarity in absolute magnitude between experimental and calculated order parameter profiles may be fortuitous. The turn in the Sn2 chain at C_{22} is retained during the simulation leading to the different behaviour of this methylene compared to its Sn1 chain counterpart because of the difference in orientation of the deuterons in the respective groups (Seelig and Browning, 1978). The plateau in the order parameter profiles for the upper part of the chains arises because the motional freedom of the methyls is restricted in this region compared to the lower part of the chains. The decrease in magnitude and the larger standard deviation of the order parameter at the termini of the chains is due to the greater torsional and motional freedom of these methylenes. The observed difference in magnitude of the order parameter

between the Sn 1 and Sn2 chains arises presumably from incomplete sampling of phase space in these relatively short simulations.

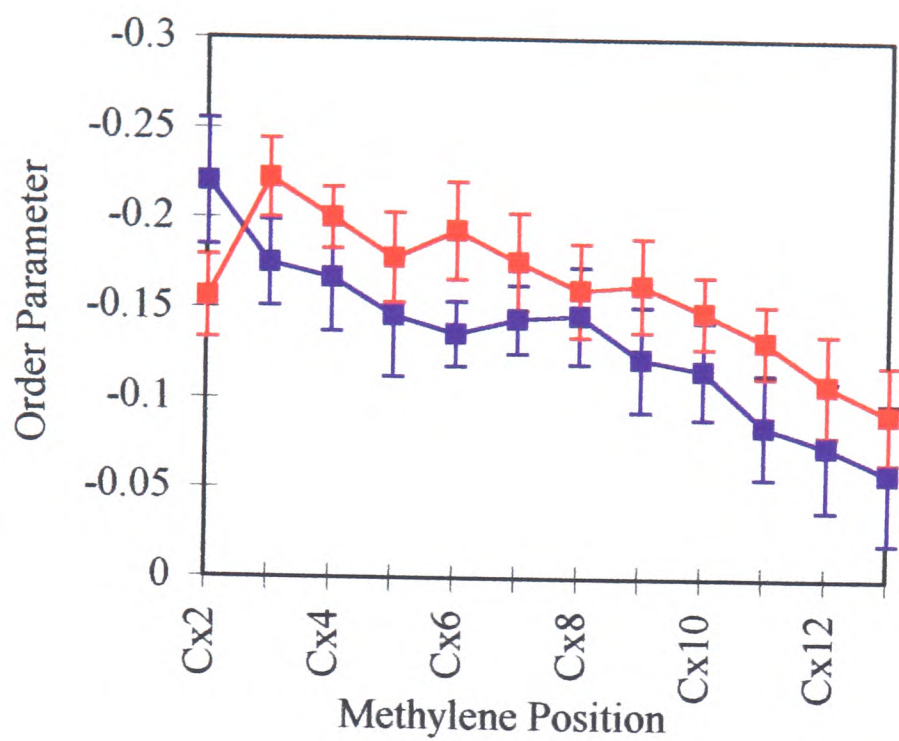


Figure 4.6 The calculated order parameter profile for the myristoyl chains of DMPC from 200 ps of molecular dynamic simulation. The Sn 1 chain is plotted in blue (x=3) and the Sn2 chain in red (x=2).

4.4.4 The Tilt of the Lipid Chains

In the following discussion, the definitions of the upper and lower parts of the hydrocarbon chains are as described in Section 3.12 and shown in Figure 3.6.

The tilt of a hydrocarbon chain was evaluated using the method of van der Ploeg and Berendsen (van der Ploeg and Berendsen, 1982; van der Ploeg and Berendsen, 1983) as described in Section 3.12.

From the distribution of individual chain tilt angles as defined by S_i and shown in Figure 4.7, the average angle that the upper part of a lipid chain makes with the bilayer normal is $35.4 \pm 14.4^\circ$ whilst the most probable angle is 28.8° . For the lower part of the lipid chains the average individual tilt angle is $44.9 \pm 19.5^\circ$ whilst the most probable tilt angle is 36.3° .

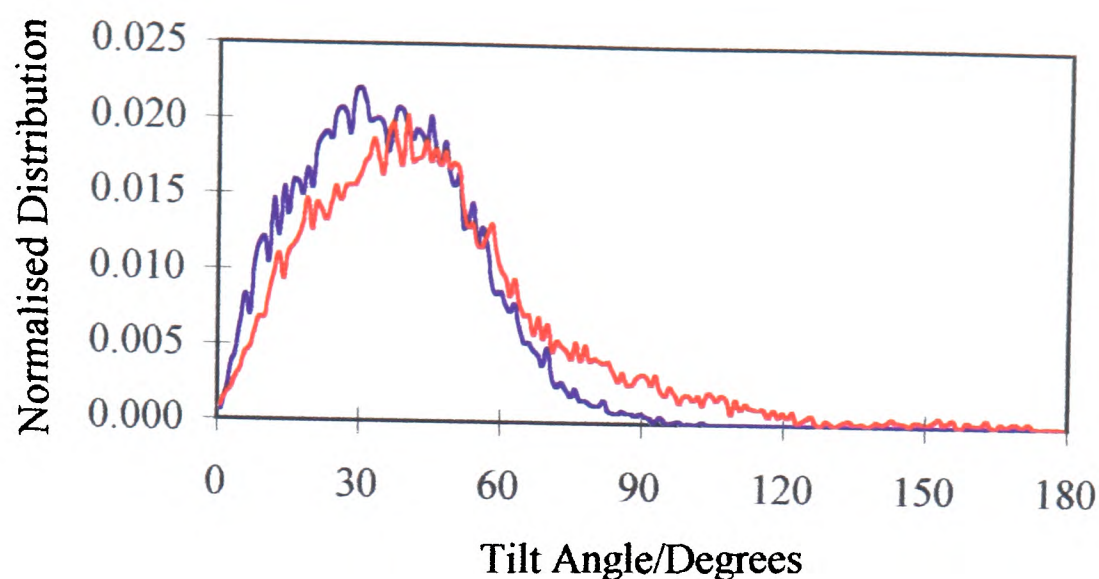


Figure 4.7 Normalised distribution of the individual chain tilt angles as defined by S_i . The distribution for the upper part of the chain is plotted as a blue line and the lower part of the chain in red.

A portion of the chains have tilt angles greater than 90° which corresponds to the vector used to define the direction of the lipid chain pointing up toward the glycerol moiety. For the upper part of the lipid chain the proportion of vectors showing this behaviour is less than 1%, but for the lower part of the lipid chain the situation is far more common with 7% of the chains directed toward the glycerol region, i.e. the lower part of the hydrocarbon chain has a much greater freedom in its orientation compared to the upper part of the chain.

The average collective tilt, T (see Section 3.12), in the upper part of the chains in one layer is $15.0 \pm 1.9^\circ$ and $13.7 \pm 4.0^\circ$ in the other layer. For the lower part of the chain it is $20.1 \pm 4.5^\circ$ and $11.8 \pm 4.3^\circ$ for the two layers of the bilayer. The non-zero value of the collective tilt shows that there is cooperation between the lipid chains, but since the magnitude of the collective tilt is less than that of the individual tilt angle, it is hoped that this is because not enough phase space has been sampled rather than periodic boundary violations leading to an exaggeration of the tilt.

Another possibility leading to the observed non-zero collective tilt is as a result of the ordered crystal-like starting structure in which the lipids are packed together and oriented in a very similar fashion (see Figure's 4.3 and 4.4). The simulations are not of sufficient length for lipid rotation about its long axis to occur thus there is no possibility that all phase space

has been sampled over the course of the simulation. To counter this, subsequent simulations had more disordered starting structures with lipids being placed in a variety of orientations whilst maintaining favourable electrostatic head group interactions.

The picture of the hydrophobic core that is starting to emerge is oriented lipid chains interacting with each other and forming microdomains in which the chains do show a collective tilt due to cooperative behaviour, but over a larger volume of the membrane and time-scale these average to give the expected zero collective tilt angle.

To investigate further whether the system size is large enough so that the collective tilt does not lead to (or is as a result of) periodic boundary violations and simulation artefacts, the correlation between the tilt of the hydrocarbon chains was evaluated from the length of $\mathbf{T}$, the collective tilt vector for the system (see Section 3.12). As the correlation between chains increases over the system, so $|\mathbf{T}|$ will tend toward unity (since it is the average of the unit vector, $\mathbf{S}_i$). For the upper part of the chain, $|\mathbf{T}|$ is 0.78 ± 0.02 and 0.82 ± 0.02 for the two layers whilst for the lower part of the chains it is 0.67 ± 0.03 and 0.70 ± 0.03 for the two layers. Not unexpectedly the correlation is greater in the upper part of the chain closer to the head group and corresponds to the plateau region on the order parameter profile.

4.4.5 Correlation Functions for Lipid Chain Behaviour

The autocorrelation function $C(t)$ of $\mathbf{S}_i$ is defined and discussed in Section 3.14 and measures the time over which correlations persist for the individual lipid chain vectors (see Figure 3.6). The correlation times are 1900 ps for the upper part of the chain and 1100 ps for the lower part whilst the components of the autocorrelation function in the x and y directions have correlation times of about 500 ps for both the upper and lower parts of the hydrocarbon chains. These times are in excess of the simulation length and must be treated as approximate, however long correlation times are not unexpected given the approximate parallel orientation and packing of the chains which will lead to large restrictions in their freedom of motion. From deuterium NMR, correlation times of 1000-100,000 ps are reported for overall reorientation of the lipid and correlation times of 1-500 ps for intramolecular motions (Mayer et al., 1988), i.e. correlation times of intramolecular motion occur on the

same time scale as molecular dynamic simulations, but intermolecular motions occur on a much longer time scale. These long correlation times stress the importance of a correct starting structure to avoid long equilibration times.

The correlation between motion of neighbouring chains was measured using the vector $\Delta \mathbf{r}_i(t)$ as described in Section 3.14 and the correlation function calculated every 20 ps to give the average and the standard deviation the average value which is plotted in Figure 4.8. The values need only be calculated every 20 ps since the properties of a system over a series of consecutive dynamic frames can be highly correlated (Allen and Tildesley, 1987).

From the values and standard deviation of $C(r)dr$ in Figure 4.8 it is apparent that the motion of the chains is correlated at separations of 4-6Å which corresponds to the nearest neighbour chains whilst at larger separation the motion is on average much less cooperative with the large standard deviation indicating that motion may vary between correlated and non-correlated.

4.4.6 The Dihedral Angle Distribution in the Lipid Chains

The fraction of gauche dihedral angles about a methylene segment has been estimated from NMR (Mendelsohn et al., 1989; Mendelsohn et al., 1991; Meier et al., 1986), although it should be emphasised that the experimental percentages are obtained by fitting NMR data of DPPC or DMPC to a model, and concluded that there are 3.6 to 4.2 gauche conformations per palmitoyl chain of DPPC. The values calculated for individual methylene groups are given in Table 4.5 with the experimentally determined values and the values as a function of dihedral angle listed in Table 4.6 and plotted in Figure 4.9 with the standard deviation. Values per methylene were calculated by averaging the proportions for dihedral angle on either side of the methylene position. From an analysis of wagging bands Senak and partners (Senak et al., 1991) proposed there to be 3.2 to 4.0 gauche conformations per palmitoyl chain of DPPC.

Methylene	Expt. (1991) [*]	Expt. (1989) [†]	Sn 1 (Sim.)	Sn2 (Sim.)
C _{x2}	-	-	41.3±5.1	53.4
C _{x3}	-	-	17.7±2.2	33.3
C _{x4}	21.0	20.7±4.2	26.8±2.4	29.5
C _{x6}	30.0	32.3±2.3	24.7±4.9	19.9
C _{x10}	20.0	19.7±0.8	26.2±4.0	20.2
C _{x12}	17.0	-	23.3±2.5	16.9
C _{x13}	40.0	-	23.5±5.1	15.5

^{*}Mendelsohn et al., 1991

[†]Mendelsohn et al., 1989

Table 4.5 The proportion of gauche dihedral angles as a function of methylene position from experiment and experiment (DPPC) and the molecular dynamic simulation (DMPC). [For the Sn 1 chain x=3 and for the Sn 1 chain x=2].

From Table 4.6, for the upper part of both the Sn 1 and Sn2 chains there is an alternating high-low variation in the proportion of trans dihedral angle while in the lower part of the chain the proportion of trans dihedral angles is approximately constant. This may be as a result of restricted motion in the upper part of the chain plus the glycerol moiety making alternate dihedrals more favourable to isomerisation. In the lower part of the chain isomerisation is less hindered and the dihedral angles are essentially equivalent.

The average number of any type of dihedral angle per chain will depend upon whether dihedral angle number one is included. Realistically it should not be included since it includes a carbonyl group and hence is very different in character from the rest of the hydrocarbon chain dihedral angles. Without its inclusion the proportion of trans dihedral angles in the lipid chains is 76.7% for the Sn 1 and 77.4% for the Sn2 which is equivalent to 8.4 trans or 2.6 gauche dihedral angles in an Sn 1 chain and similarly 8.5 and 2.5 for the Sn2 chain. With the inclusion of this dihedral then the number of gauche conformations per lipid chain rises to 3.3 for the Sn 1 and 3.2 for the Sn2.

Dihedral no.	Sn 1 average (%)	Sn 1 S.D. (%)	Sn2 average (%)	Sn2 S.D. (%)
1	28.8	5.2	31.3	5.5
2	88.8	2.6	61.9	4.1
3	75.1	3.6	71.6	8.5
4	71.3	2.6	69.5	4.6
5	78.8	2.3	84.9	2.6
6	71.1	5.0	75.4	2.0
7	78.7	2.6	86.1	2.1
8	77.0	5.3	75.3	3.5
9	71.1	4.2	82.3	4.5
10	76.6	4.1	77.8	2.7
11	77.0	4.2	81.9	5.0
12	76.5	2.9	84.3	4.7

Table 4.6 The mean and standard deviation (SD) for the proportion of trans dihedral angles as a function of dihedral number (See Figure 3.7 for the numbering scheme).

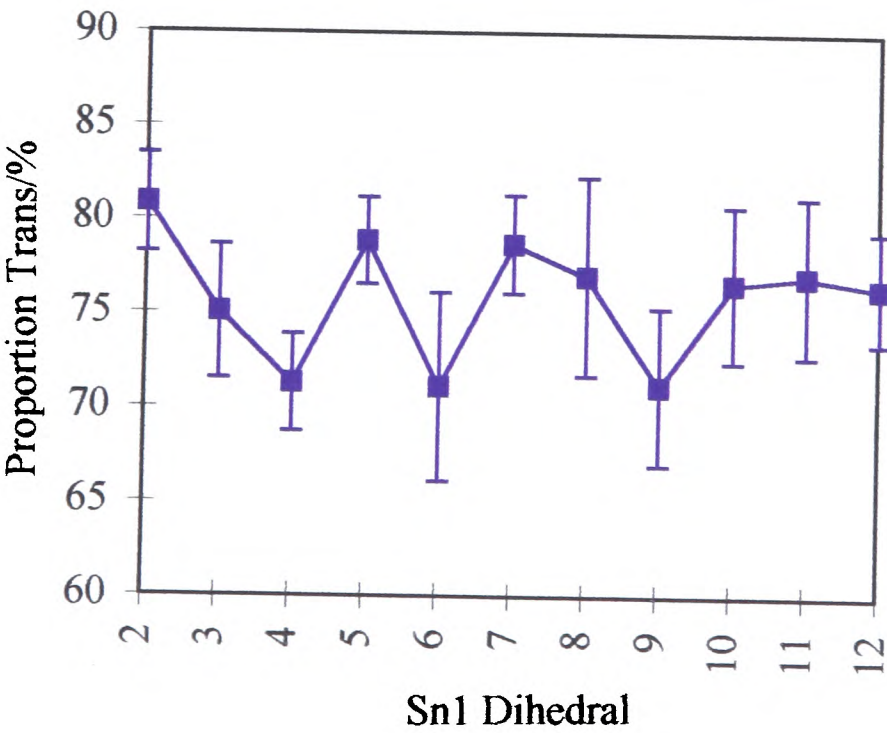


Figure 4.9 The proportion of trans dihedral angles as a function of dihedral position for the Sn 1 chain (See Figure 3.7 for numbering scheme).

Later work has integrated FT-IR and ^2H -NMR experiments (Tuchtenhagen, Ziegler and Blume, 1994) to study the dihedral angle distribution in hydrocarbon chains in lipid bilayers and concluded that there are 2.60 ± 0.37 gauche conformations per chain in DMPC at 69°C excluding contributions from single gauche conformations and jogs. For DPPC with the inclusion of single gauche conformations (1 per chain) and jogs (0.4 per chain) then the number of gauche conformations per chain is estimated to be ~ 3.4 to 3.9 per chain. DPPC differs from DMPC by having lipid chains that are two methylene segments longer so assuming that the probability of a dihedral angle being gauche is approximately independent of chain position then this converts to a figure of 2.9 to 3.3 gauche dihedral angles per myristoyl chain. This compares favourably with the simulation values, but it must be noted that these experiments were done at a temperature 20°C higher than the molecular dynamic simulation study. The results of Mendelsohn on DPPC (Mendelsohn et al., 1989; Mendelsohn et al., 1991) may also be extrapolated to give values of 3.1 to 3.6 gauche dihedral angles in a myristoyl chain.

Meier and co-workers report that the proportion of terminal dihedral angles being gauche is 35% (Meier et al., 1983). From the values of the simulation given in Table 4.5 it is obvious that the simulation does not reproduce the terminal dihedral angle population, yet it does reproduce behaviour such as the kink population and total average gauche population of the hydrocarbon chains. It was not clear if this was a failing of the force field or that the earlier experimental results were analysed incorrectly because of flaws in the model to which the data were fitted. Certainly where reported this is not the only hydrated bilayer model that has failed to reproduce the very large difference (up to 20%) in the probability between a gauche conformation occurring at the end of the chain compared to the rest of the length (Essex, 1992; Essex et al., 1994; Chiu et al., 1995; van der Ploeg and Berendsen, 1983) despite predicting correctly many other aspects of chain conformation behaviour so it is not just a simple problem with the parameter set used here.

In the next series of bilayer simulations the initial lipid molecule conformations had all the terminal dihedrals in the gauche conformation to investigate if it was an equilibration problem.

4.4.7 The Electron Density Profile across the Bilayer

The average electron density was evaluated over the 200 ps of molecular dynamic simulation using atom centred electron densities for the lipid and water molecules as outlined in Section 3.11 and is plotted in Figure 4.10. In agreement with experimental x-ray diffraction results the profile maybe divided into three regions; the head groups, the apolar region and the bilayer centre. The high electron density corresponds to the closely packed head groups containing the phosphorous atoms while the hydrocarbon shows a much lower density reflecting its more fluid-like nature. The dip at the centre corresponds to the bilayer interface where the greatest free volume is expected to occur.

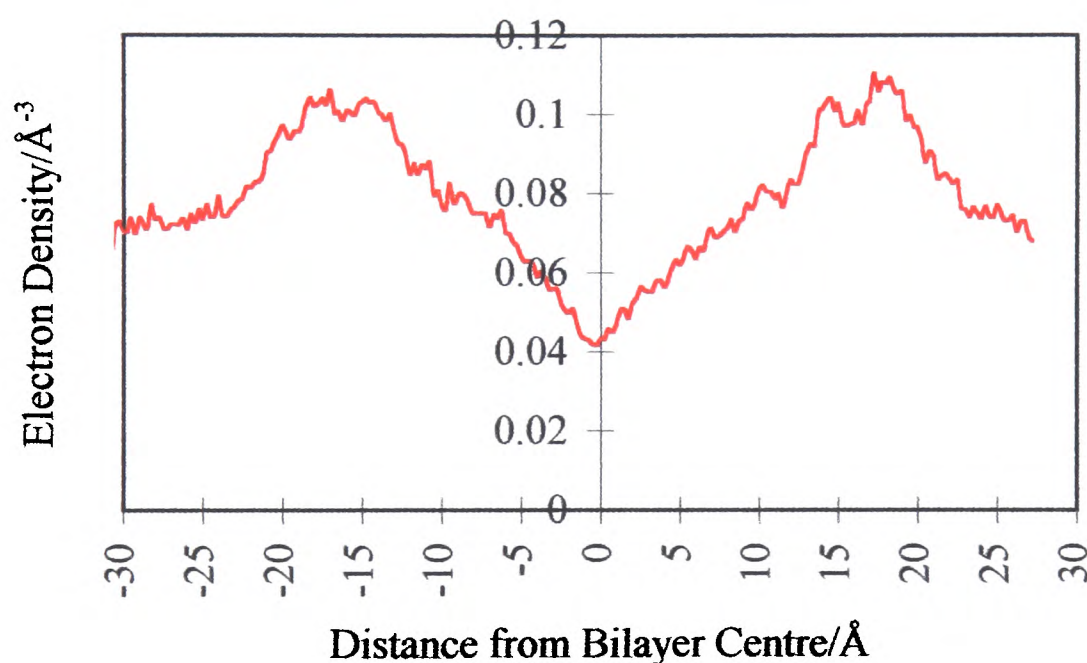


Figure 4.10 The average electron density distribution profile across the width of the bilayer evaluated using atom centred electron densities over the 200 ps of molecular dynamic simulation.

4.4.8 Summary of Results for the Small Scale Pure Lipid Bilayer Simulation

These simulations of a small, hydrated DMPC bilayer system were able to reproduce a number of experimental observations plus provide information on the possible structure and dynamics of lipid bilayers which is not accessible by conventional means. The behaviour of the fluid phase was reproduced and the head groups appear stable.

Although the head groups show a greater range of conformations than proposed from NMR experiments (Seelig et al., 1977; Seelig, 1978; Skarjune and Oldfield, 1979), they are not totally conformationally flexible. The inability to reproduce the gauche conformation at α_5 is probably due to a failure of the simulation technique rather than the model.

The upper and lower parts of the lipid chains show differences in their behaviour resulting from the upper part of the chain being motionally and conformationally restricted in order to maintain energetically favourable packing interactions.

The simulation reproduces the most probable tilt of a lipid with respect to the bilayer normal, but the simulation is not of sufficient length or the system of necessary size to sample enough phase space to reach the NMR time-average value of zero for the observed tilt.

The collective tilt arises because the motion and tilt of a lipid chain is correlated with those surrounding it leading to local regions, or microdomains, with a nonzero collective tilt. That individual lipids have a tilt is not unexpected given that each lipid comprises two hydrocarbon chains joined by the glycerol moiety and thus are necessarily quite close together here, but the ends are free and there is considerable free volume at the bilayer centre which the termini inhabit. So the termini of the chains of a lipid may be quite easily further apart than at the top which will usually lead to the chain being at an angle compared to the bilayer normal along which it was originally aligned in the starting structure (see Figure 3.4). Correlations of motion do not extend significantly beyond the nearest neighbour chains.

It was concluded (Robinson et al., 1994) that the system was of sufficient size to successfully model individual lipid chains and their intramolecular motions, but given the time needed for chains reorientation (Mayer et al., 1988; Meier et al., 1986) it is not surprising that the nonzero collective tilt of the layers could not be reproduced.

4.4.9 Conclusions about the Small Scale Bilayer Simulation

Although this model recreates many of the experimentally observed results and appears to behave correctly, the main problem is its limited size and short simulation time length. Furthermore the observed collective tilt angle may result from the crystal-like starting

structure with all the lipids oriented in either one of two directions. Thus subsequent models not only had a larger number of lipids and waters and were run for a greater length of time but also had a greater degree of disorder introduced into the starting structure.

In Chapter 5 a later pure lipid bilayer model is presented with better analysis of its properties and comparison with experimental data.

Chapter Five

Large Scale Simulations of Pure Lipid Bilayers

5.1 Introduction

Following the work in the previous chapter on pure lipid bilayers, better computer resources became available that allowed simulations of much larger bilayer systems.

The method used to build the starting membrane configuration was also reassessed in light of results from molecular dynamic simulations and a reconsideration of the experimental results on the behaviour and fluidity of lipid chains (e.g. Tuchtenhagen, Ziegler and Blume, 1994). The most dramatic change was an increasing departure from any resemblance to the crystal structure in the starting geometry both in terms of orientation of lipid molecules with respect to each other and the introduction of three gauche dihedral angles into each hydrocarbon chain in order that lipids in the starting structure resemble more closely those in the liquid phase (see Figure 4.2).

In this chapter a more in-depth analysis of the structure and dynamics of pure lipid bilayers will be presented and where appropriate compared with the results of Chapter 4. In addition the behaviour of water in and around the head group region will be considered and how it may effect the diffusion of permeants through the membrane. These are important topics since the nature and factors effecting solute permeation are still far from clear. (For a recent experimental study of water permeation, see Jansen and Blume, 1995).

5.2 Building the Starting Structure

As described in Chapter 4, one of the failures of the small scale bilayer model was the presence of a collective tilt which was probably a result of the short simulation time length,

the small size of the system and the highly oriented nature of the lipid chains in the starting structure leading to inadequate sampling of phase space (see Figure 4.4).

The structural problems were addressed in two ways; firstly although the lipid molecules are packed together in a hexagonal arrangement as before, they are not placed so that the relative orientation of all the head groups with respect to each other is so close to the crystal structure (as in Figure 4.3) but rather placed in a more random fashion, whilst maintaining favourable electrostatic interactions, in order that the initial packing of the lipid chains is not so regular (see Figure 4.4).

Secondly it was recognised that having the lipid chains in the extended all trans conformation not only required a significant computer CPU time to melt the bilayer, but might also introduce artefacts since the initial configuration is far removed from that of a liquid bilayer. Thus four different lipid conformations were used in the building of the starting structure (see Figure 4.2) which had three gauche conformations per lipid chain to be consistent with the figures proposed previously from experiments on lipid bilayers (see Section 4.4.6). In each chain two of the gauche dihedral angles were used to build a kink motif at some point along the length of the chain while the third gauche dihedral angle was introduced at the end of the hydrocarbon chain to investigate the belief that terminal dihedral angles in lipid hydrocarbon chains have a very high proportion of gauche dihedral angles (Meier et al., 1983; Tuchtenhagen, Ziegler and Blume, 1994). The previous simulations starting from 100% trans had not shown this large proportion of gauche dihedrals which may be an equilibration problem, so the simulation was started with 100% gauche instead and the final population compared with experimental and earlier simulation work.

The bilayer model was built in an analogous fashion to the previous forty lipid system with a monolayer of thirty-six lipid molecules being built by placing lipids using a template of hexagons which have a head group surface area of $66\text{\AA}^2$ (see Figure 4.3). However this time the lipids were placed by hand and careful consideration was given to their interaction with their neighbours especially since the lipid molecules are no longer as easy to pack together because of the introduction of the gauche torsions into the hydrocarbon chains. Two of these monolayers were put together to give a bilayer of width, as measured by the P-P distance across the membrane, of approximately $36\text{\AA}$.

The starting structure of this new system is very different from that of the previous smaller systems as can be seen in Figure 5.1 and looks more like the conventional text book picture of a fluid bilayer. There is still interdigitation between the two monolayers at the bilayer centre, but to a lesser extent than when the hydrocarbon chains are in an extended all trans conformation plus the large gaps between lipid molecules seen in Figure 4.4 are no longer present.

The system was then hydrated with TIP3P waters which were allowed to penetrate as far down into the bilayer as the carbonyl groups.

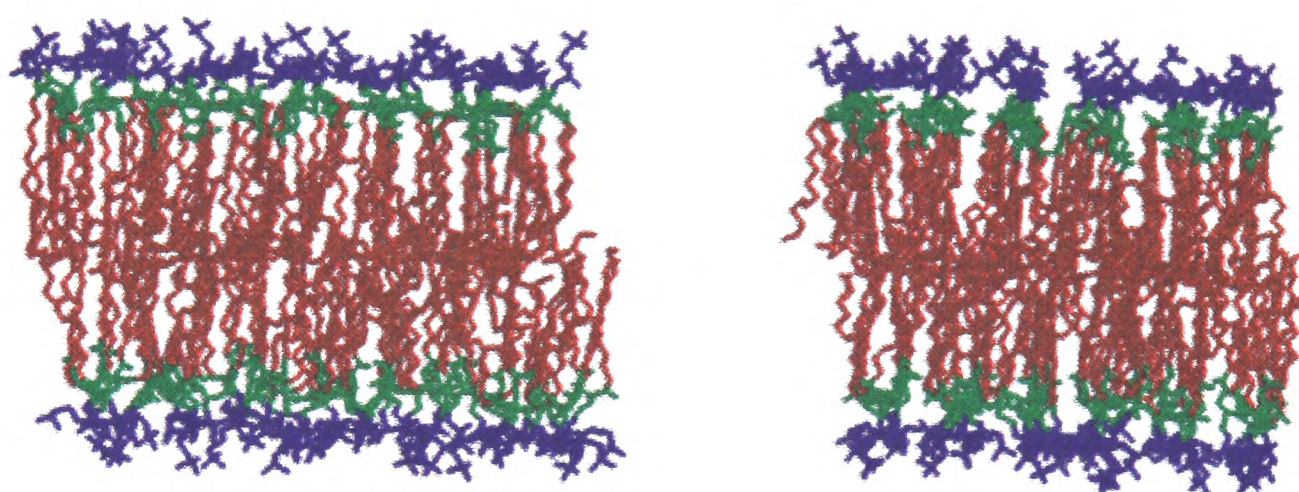


Figure 5.1 The starting structures of the large scale pure bilayer molecular dynamic simulation. The lipid head groups are shown in blue, the hydrocarbon chains in red and the glycerol moiety in green.

The molecular mechanics program AMBER was used for the simulations with the same parameters for all energy refinement and molecular dynamic simulation calculations as used previously, but this time version 4.0 was used. The major difference between AMBER 4.0 and 3.1 that was implemented in the simulations was the separate coupling of the temperature scaling of the lipid and water molecules which reduces the possibility of the solvent and solute of the system being at radically different temperatures, i.e. the “cold solute/hot solvent” problem. The lipid molecule is now defined as a single residue, unlike previously where it was defined as three (see Section 4.4) which implies that many lipid head groups will now see lipid head groups on the opposite side of the membrane which is highly desirable given the large charges in the head groups and the long range of electrostatic forces.

Before molecular dynamic simulations can be run on the system it needs to be energy refined to minimise unfavourable contacts generated during model building. This was done in stages with first just the waters being minimised followed by refinement of the lipid molecules. The main problem with the starting structure is the bilayer centre where the initial interdigitation and steric overlap of atoms in the chains causes extremely large van der Waals energies, but these are quickly resolved by energy minimisation. A short 10ps molecular dynamic simulation was done on the system with only the water molecules allowed to move in order to equilibrate their position at the bilayer interface before molecular dynamic calculations were initiated with all the atoms of the system free to move. The first 2.5ps used a time step of 1fs without the use of SHAKE in case there were still any close contacts that had not been resolved by energy minimisation, after which the system was run for over 700ps at 328K with a time step of 2fs and SHAKE implemented. The positions of the atoms were recorded every picosecond for subsequent analysis.

5.2.1 The Initial Starting Configurations of Other Research Groups

The scheme described here is in contrast to the methods used by other groups, many of which have been biased toward the crystal structure. In their DMPC model, Damodaran and Merz (1993) constructed the bilayer from the crystal structure by increasing the separation of the constituent lipid molecules such that the head group surface area of a single lipid was $\sim 68\text{\AA}^2$. Essex (1992) used a similar method but also reduced the extent of staggering between the two crystal structure conformations. Heller et al. (1993) built their monolayer from 25 units consisting of four 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphatidylcholine (POPC) molecules arranged by hand and gradually equilibrated using a combination of energy refinement and molecular dynamics. The POPC molecule was built using a modelling program and had all the dihedrals, save the unsaturated bond, in the trans conformation. Stouch (1993) describes his starting structure as two monolayers of 18 DMPC molecules in a periodic box with lateral dimensions of $34.5 \times 34.5\text{\AA}$ giving a head group surface area of $66\text{\AA}^2/\text{lipid}$.

Huang et al., (1994) used lipid molecules with chains in an extended all trans conformation and a highly ordered starting structure and with water molecules not penetrating past the phosphate groups. There was no interdigitation of the chains at the bilayer centre since they

ran constant pressure simulations at 1 atmosphere and proposed that the system would correct itself. However they did not account for the surface tension of the membrane that is of the order of - 100 atmospheres in the plane of the membrane (Israelachvili et al., 1980).

Shinoda et al., (1995) simulated their bilayer in the Nose-Parrinello-Rahman NPT ensemble again with the initial conformation of the lipid chains being in an extended all trans conformation and with all the lipid molecules being oriented regularly with respect to each other. They attempted to speed up equilibration by introducing degrees of freedom to the box angles as well as the lengths.

Venable et al. (1993) used the elegant method of producing 72 different lipid conformations from a Monte Carlo simulation with a Majacela field and placing these onto a two dimensional hexagonal lattice to avoid long equilibration times.

Finally Chiu et al. (1995) started from the crystal structure of DMPC but incorporated surface tension into their molecular dynamics program and the bilayer was observed to melt to a head group surface area per lipid of only $60\text{\AA}^2$ but with the lipid chains still showing fluidity.

5.3 Analysis of the Bilayer Simulation

The equilibration period of the simulation needed to be determined and from an analysis of the time dependence of various properties (e.g. the total energy of the system as shown in Figure 5.2), it was decided that the first 100ps would be considered as the equilibration phase. This is gratifyingly short given the time needed to melt and equilibrate the lipid chains of previous model starting structures.

Energy is a poor way to determine if a structure is at equilibrium since it is fairly insensitive to finer structural details, so structural properties were also considered and the variation in dihedral angle populations shown in Figure 5.3.

The plots of Figure 5.3 show that within 100ps the chain region appears to have reached an equilibrium with the average proportion of trans dihedral angles for the terminal dihedral angle and the average proportion of trans dihedral angles for the whole of a lipid chain having levelled out. It is recognised that dihedral angle populations are one of the slowest

components of the system to equilibrate, normally taking of the order of 100 to 200ps because of the infrequency of dihedral phase changes occurring (Huang et al., 1994; Stouch, 1993). The length of time between changes is also increased because of the chain packing interactions in the hydrocarbon region making isomerisation more difficult than might be expected.

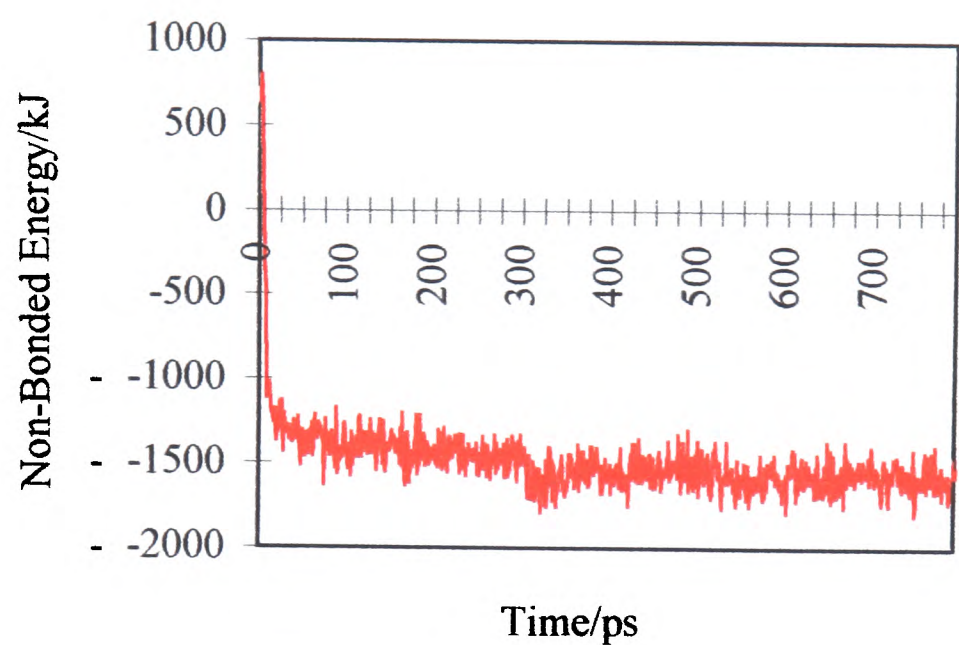


Figure 5.2 The value of the total non-bonded energy of the system as a function of time. The slight drop at 300ps is as a result of changing from all bonds being constrained with SHAKE to only bonds involving hydrogen being constrained.

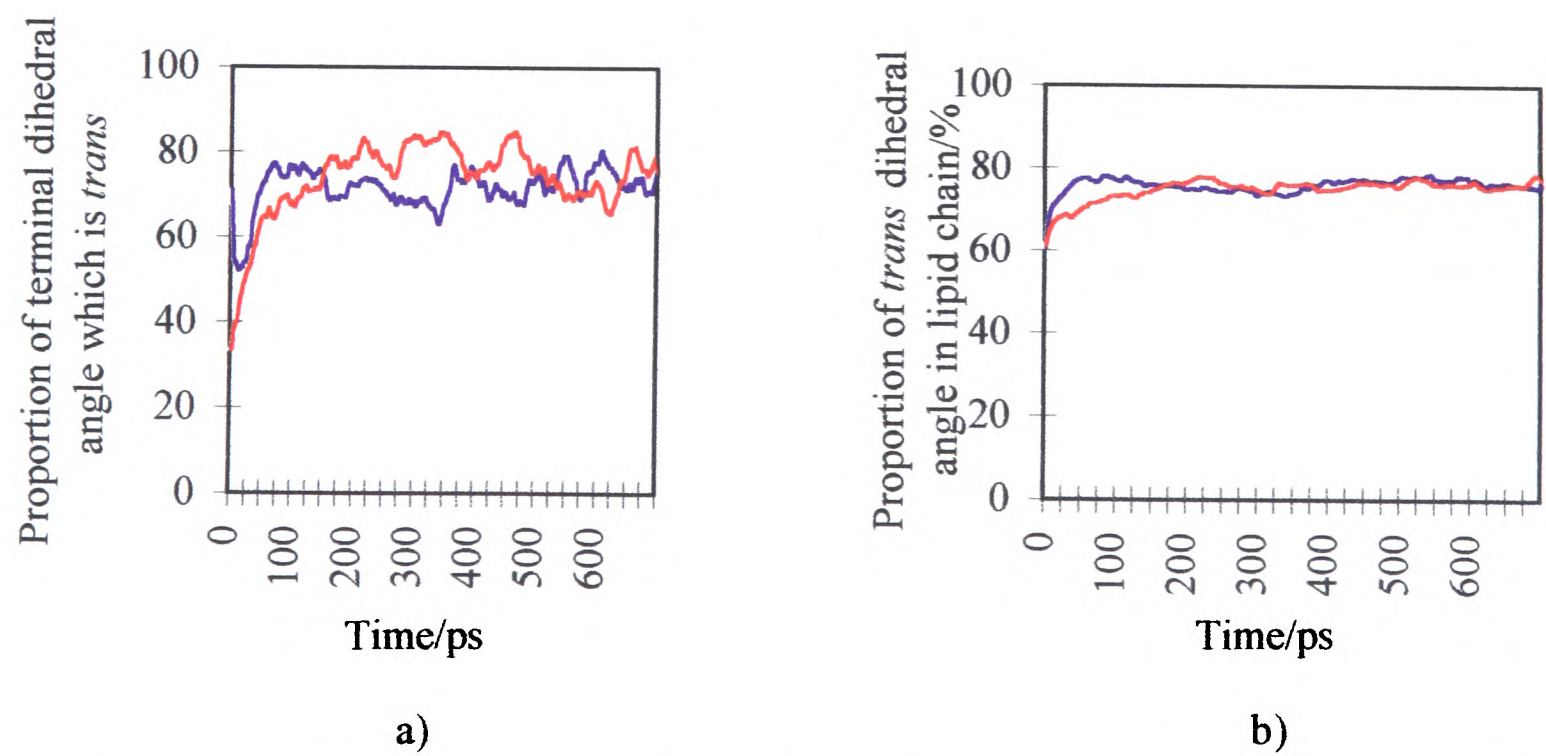


Figure 5.3 The variation in the proportion of trans dihedral angles for a) The terminal dihedral angle of the lipid chains b) the lipid chain as a whole over the course of the simulation. The Sn 1 chain is in blue and the Sn2 chain in red.

The equilibration of the system may also be investigated using the order parameters of the methylene chain segments and this is shown in Figure 5.4 for three different methylene segments along with the average value for the whole of the chain. This analysis is more interesting since the order parameters includes contributions from both the internal structural (i.e. the conformational state around the methylene) and the gross structure, i.e. the tilt and orientation of a methylene unit with respect to the bilayer normal.

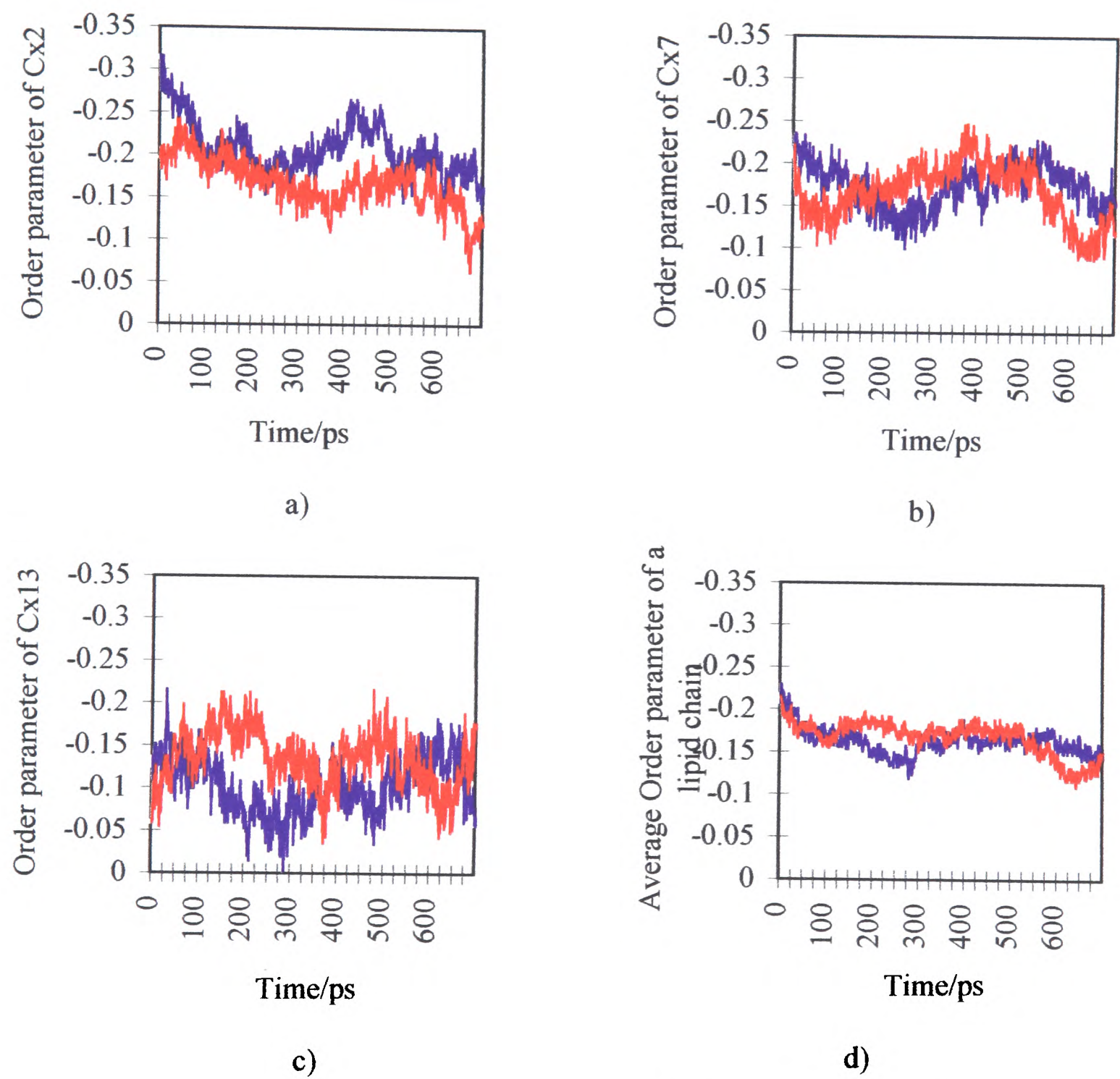


Figure 5.4 The variation in the order parameters of hydrogens in the methylene segments during the course of the molecular dynamic simulation for a) Cx2 b) Cx7 c) Cx 13 and d) Average over the whole chain. The Sn 1 chain is shown in blue and the Sn2 chain in red.

From the plot of simulation time versus average order parameter for the lipid chain it appears that at least 100ps is needed for the chains to equilibrate. For the values of individual methylenes the situation is more difficult, but the equilibration period appears to be of the order of 100 to 200ps. It was decided that the first 100ps should be considered as the equilibration phase and the subsequent 600ps be analysed. There are probably two stages to equilibration; firstly the reduction of large nonbonded energies, reorganisation of the head group/water region and isomerisation at the ends of the lipid chains which can occur quite rapidly, followed by a slower equilibration phase in which dihedral angles in the main part of lipid chains undergo isomerisation which requires a rearrangement of the surrounding lipid chains.

The analysis of the bilayer system is divided into three main sections:

1. Analysis of the lipid molecules
2. Analysis of the water molecules
3. Analysis of the interaction between the water molecules and the lipid bilayer

As in Chapter 4, the analysis of the bilayer will be split into the results for the bilayer as a whole and then the behaviour of the three separate components of the lipid molecules will be discussed, i.e. the head group, the glycerol moiety and the hydrocarbon chains. Finally the dynamics of water will be considered. The analysis will consider both static and dynamic properties of the system and where relevant comparisons will be drawn with experimental results and other simulations.

5.4 Behaviour of the Hydrocarbon Region of the Lipid Bilayer during the Molecular Dynamic Simulation

One of the goals of this work has been to use molecular dynamic simulations as a source of structural and dynamic information on lipid bilayer systems that is not available by conventional experimental techniques in order to develop simpler and less computationally

demanding models of bilayers to be used in further research on the interaction of membranes with molecules. Example bilayer models have already been developed and used to look at the integration of proteins within membranes (Milik and Skolnick, 1993), but these are simple and normally only involve a hydrophathy scale across the membrane to determine the interaction of a protein with the membrane. One of the aims of this work is to determine information that could be used to introduce fine structure into the model.

5.4.1 Electron Density Profiles and Atom Distributions across the Bilayer

The simplest method to describe the gross structure of the bilayer is as an atomic distribution profile across the width of the bilayer (see Section 3.11). This provides information on the distribution of individual atoms and groups of atoms as a function of position within the bilayer. In Figure 5.5 the distribution of all the atoms in the system averaged over 600ps is shown plus the separate components due to the atoms of water and of lipid molecules. The distribution for the lipid molecules must be corrected for the united atom model used in the simulation else the recorded density will be too small and this was done by assuming that the hydrogens lie in the same plane as the carbon, this is an approximation but was felt to be justified.

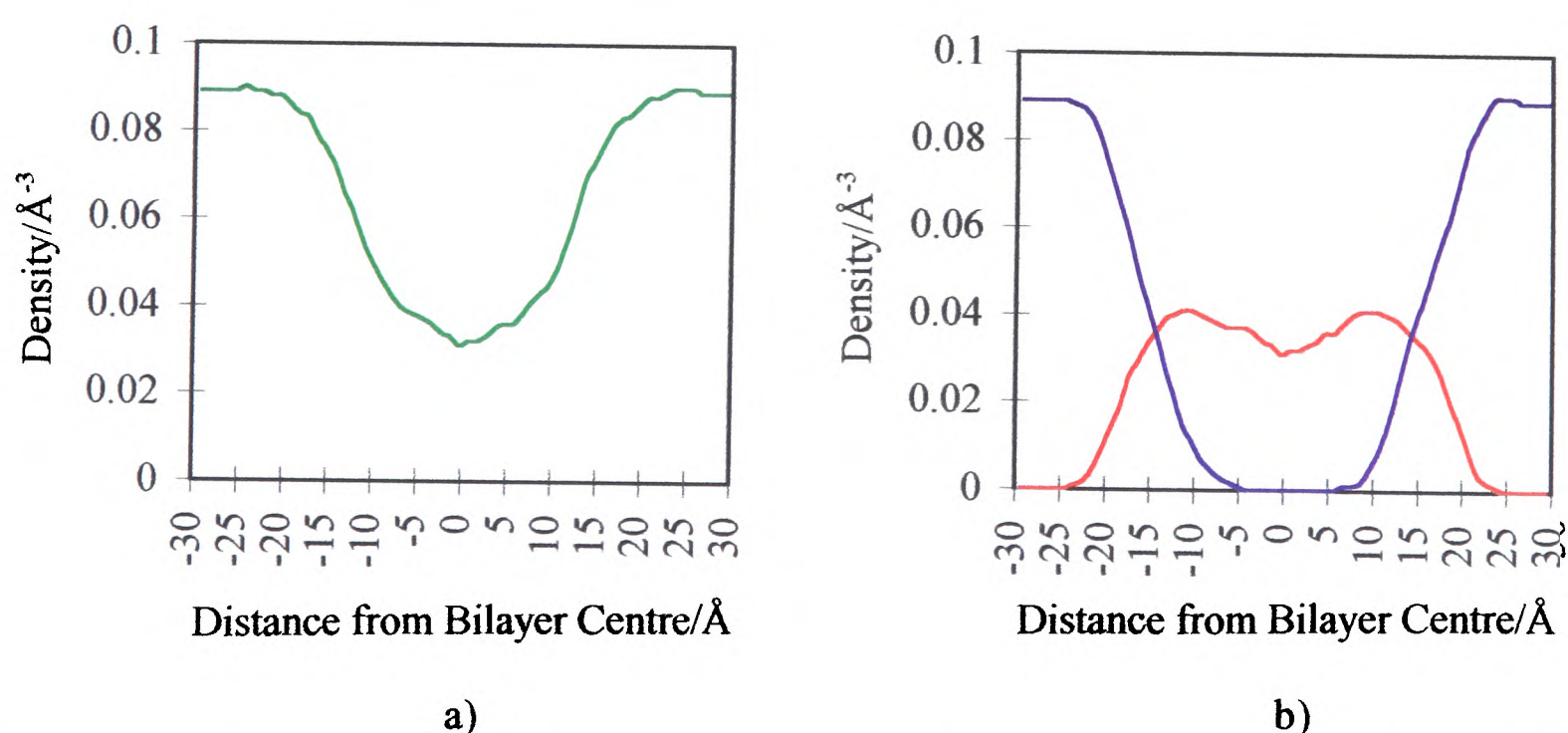


Figure 5.5 The distribution profiles across the united atom system for a) all atoms in the system b) the water molecules (blue) and the lipid molecules (red).

What is clear from Figure 5.5b is that water molecules penetrate quite deeply into the membrane, there is not a sharp membrane-water interface and the membrane surface is remarkable rough which is the consistent conclusion drawn by other researchers in the field using a variety of force fields and techniques.

In Figure 5.6 the atom distribution is plotted across the system with the inclusion of hydrogen atoms in the evaluation of the density. The profile now has a radically different form, but the characteristic dip at the centre of the bilayer is not only retained but appears more pronounced. The regions of highest density correspond to the bilayer interface where both water and head groups are together and in the hydrocarbon core approximately 5Å from the centre of the bilayer the origin of which may be determined by a more detailed analysis of the distribution of lipid atoms in the bilayer (see below).

Although it is tempting to equate qualitatively the density of a medium with its fluidity and hence the mobility of permeants within it, the aqueous phase and hydrocarbon core are not comparable because of the different packing and interaction energies between components. However it can be reasoned that the mobility of small solutes would be greater at the bilayer centre than in the rest of the hydrocarbon region because of its lower density.

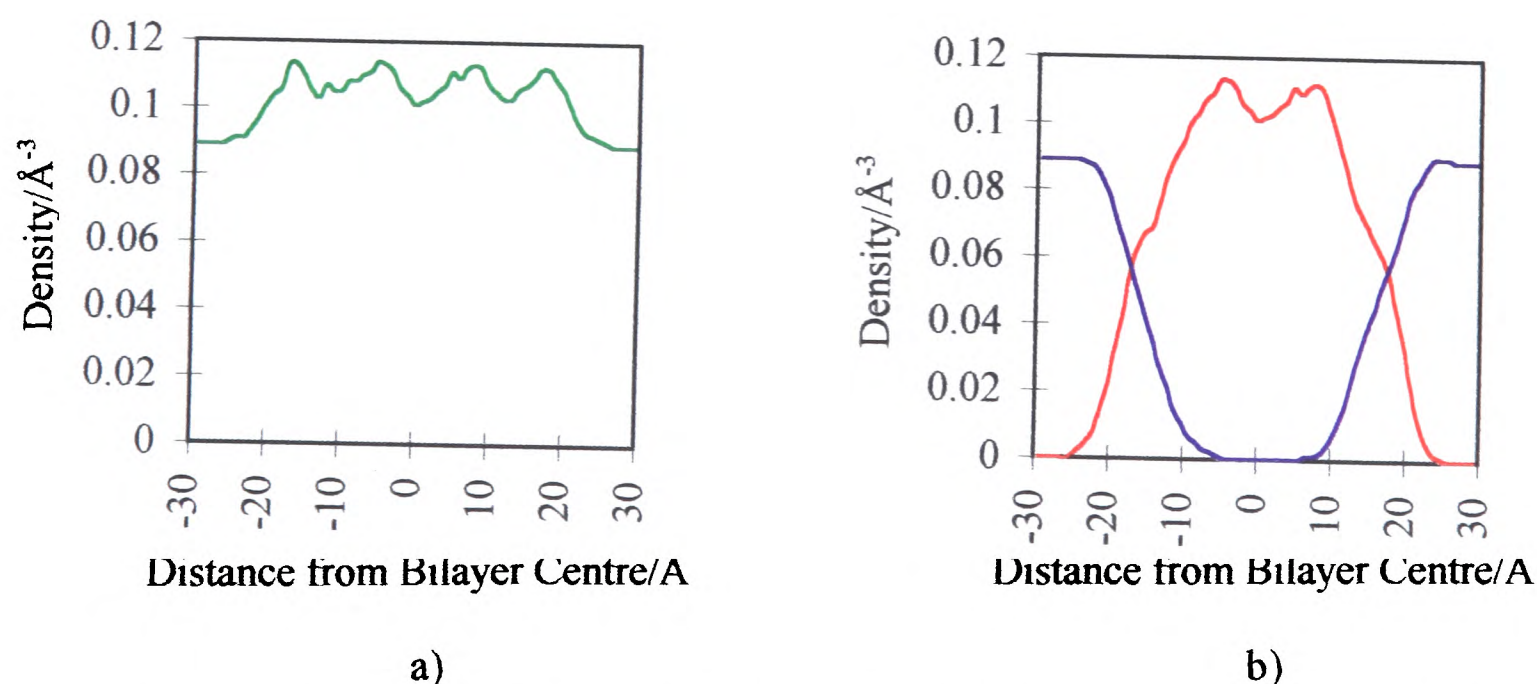


Figure 5.6 The distribution profiles across the system with hydrogen's included for a) all atoms in the system b) the water molecules (blue) and the lipid molecules (red).

The next analysis studied the distribution of the lipid components (i.e. the highly charged head groups showing strong hydrogen bonding, the glycerol moieties showing weaker hydrogen bonding and the apolar hydrocarbon chains) across the bilayer in an attempt to characterise different zones within the bilayer in terms of hydrogen bonding and electrostatic properties and the results for this are presented in Figure 5.7. A similar kind of analysis was done by Marrink and Berendsen, (1994) to define their “Four-Region” model of a lipid bilayer used in their description of the permeation process which divided the bilayer into regions of low head group density at the edge of the membrane-water interface, high head group density corresponding to the region in which the bulk of the head groups reside, high lipid chain density and low lipid chain density at the bilayer centre.

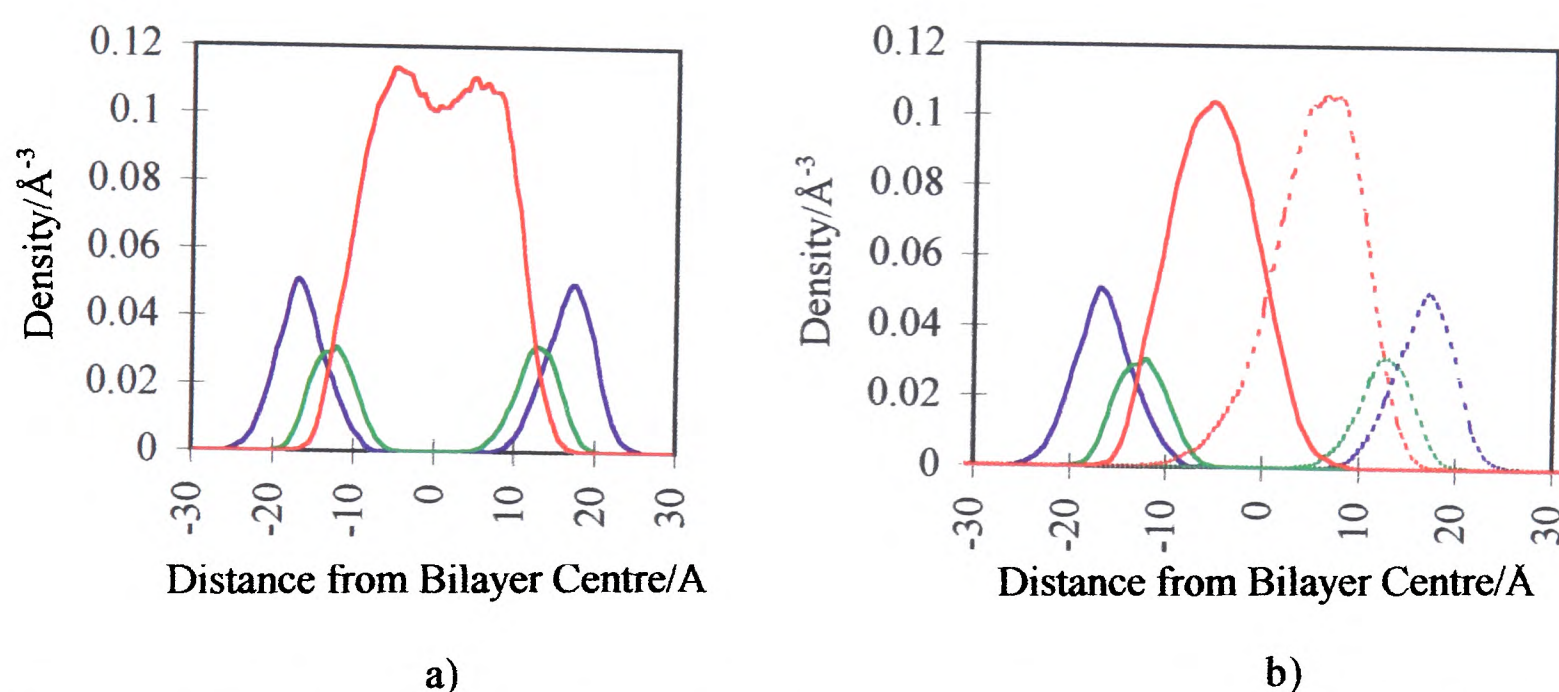


Figure 5.7 The atomic density distribution of the atoms comprising the lipid head groups (blue), the glycerol moiety (green) and the hydrocarbon chains (red) as a function of distance from the plane of the bilayer centre with a) The hydrocarbon region treated as a single entity b) The hydrocarbon chains of each layer considered separately. (An all atom approximation is used).

From Figure 5.7 the average width and distance from the bilayer of the three different components of the lipid bilayer can be evaluated and these results are reported in Table 5.1 with the width of a zone calculated from the points at which the density of the component has fallen to 10% of its maximum value. The results show that there is extensive interpenetration of these zones which reflects the fluid nature of the membrane and that the head group region is at least 10Å thick

Zone		Layer 1			Layer 2		
		Min.	Max.	Width	Min.	Max.	Width
Head Group Zone		-10Å	-23Å	13Å	10Å	23Å	10Å
Glycerol	moiety	-7Å	-18Å	11Å	7Å	18Å	11Å
Zone							
Lipid Chain Zone		+4Å	-14Å	18Å	-5Å	14Å	19Å

Table 5.1 The minimum and maximum distance from the membrane centre and the width of the zones defined by the three components of a lipid molecule.

Figure 5.7b shows that the hydrocarbon chains have a gaussian type distribution with the maximum at about 6Å from the bilayer centre and that the atoms of a lipid chain can extend right into the other side of the bilayer. The cumulative density for the hydrocarbon chains in each layer shows a dip at the bilayer centre (Figure 5.7) and this feature was analysed by calculating an atomic density profile across the profile for the Sn 1 and Sn2 chains separately (Figure 5.8).

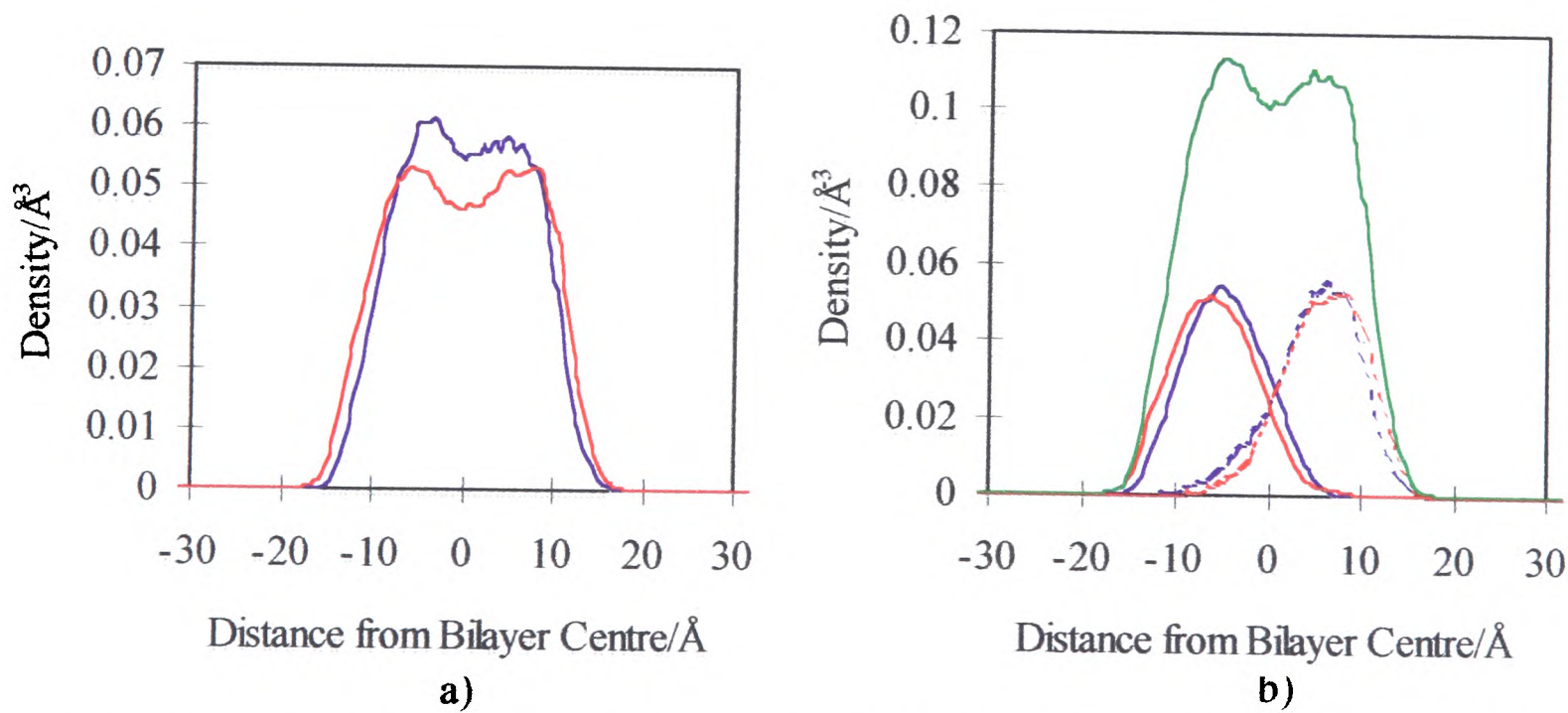


Figure 5.8 The atomic density profiles for the hydrocarbon lipid chains using an all atom approximation. a) The Sn 1 chain (blue) and the Sn2 chain (red) of both layers. b) The Sn 1 chain (blue) and the Sn2 chain (red) for each layer and the distribution for all the hydrocarbon atoms (green).

From Figure 5.8b, the Sn1 chain penetrates more deeply into the bilayer interior than the Sn2 chain and there is a greater probability of finding an Sn2 closer to the water/head group interface. The presence of the glycerol moiety which effectively extends the Sn1 chain (see Figure 2.5) is the reason for this and for other differences in behaviour e.g. the difference in the value of the order parameter for the first methylene segment of the hydrocarbon chains (see Figure 2.8).

Since a dip in density is still observed when the Sn1 chains are considered on their own (Figure 5.8a) then the lower density at the bilayer centre is not just because of the differing extents of penetration of the Sn1 and Sn2 chains. Rather it is because the lipid chains are fluid and there is a probability that there will be a gauche conformation somewhere along its length that will change its direction and hence reduce its penetration into the membrane. There may also be an entropic contribution since the reduced density allows greater rotational isomerism and motional freedom of the chain ends without also causing a penalty from unfavourable van der Waals interactions (Wilson and Pohorille, 1994)).

To define further the structure of lipid molecules and the effect of the glycerol moiety on the lipid chains, the average separation along the bilayer normal between pairs of atoms in lipid molecules was evaluated. (The atom pairs are listed in Table 5.3 and the distributions shown in Figure 5.9). A positive value for $C_x - C_y$ indicates that on average C_x is closer to the bilayer centre than C_y , i.e. it is further from the lipid/water interface.

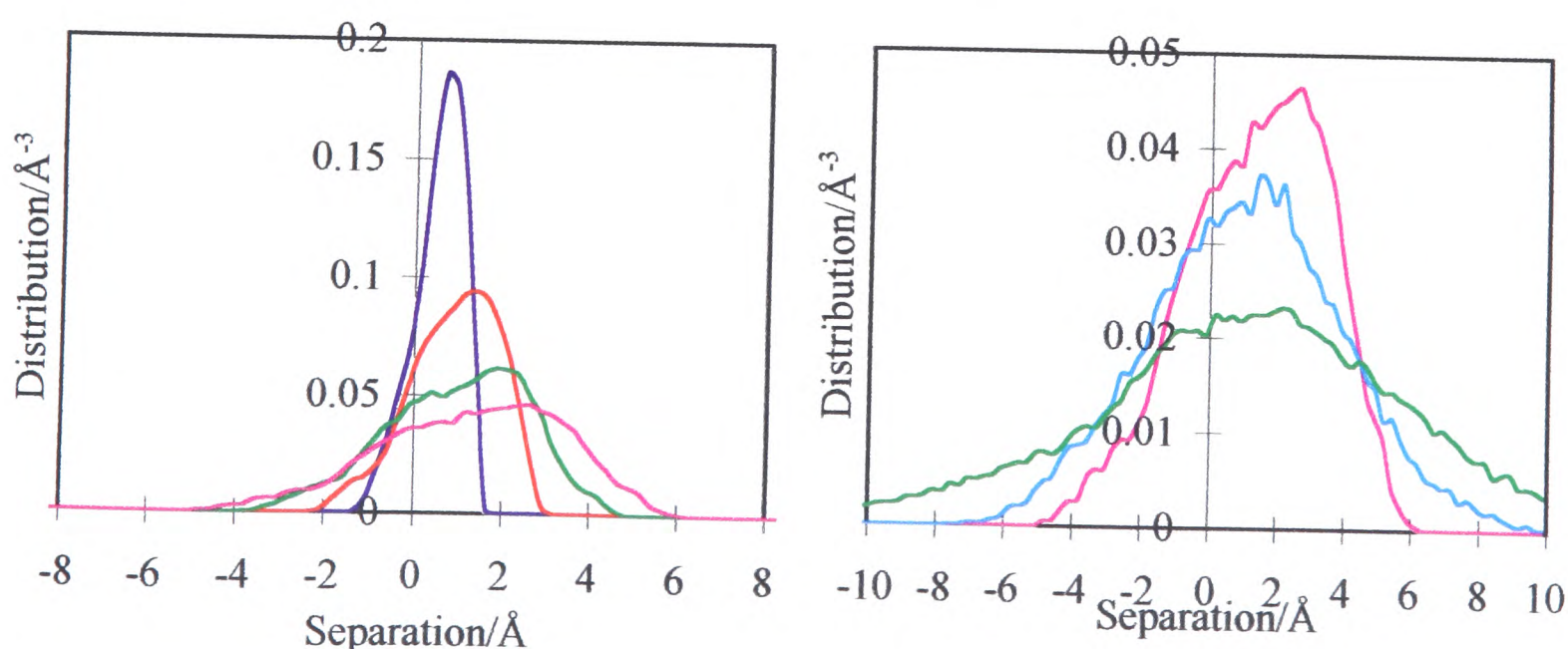


Figure 5.9 Normalised distribution profiles for the separation along the bilayer normal between pairs of atoms as described in Table 5.3.

Atom Pair	Average Separation	Standard Deviation	Colour in Figure 5.9
C ₃ - C ₂	0.52	0.44	Blue
O ₃₁ - O ₂₁	0.90	0.81	Red
C ₃₁ - C ₂₁	1.00	1.30	Green
C ₃₂ - C ₂₂	1.33	1.68	Magenta
C ₃₈ - C ₂₈	1.08	2.26	Cyan
C ₃₁₄ - C ₂₁₄	0.99	3.28	Olive

Table 5.3 Table identifying atom pairs used in calculating the average separation between them (Å) in lipid molecules and the colour scheme used in the plot of Figure 5.9. (See Figure 2.5 for atom numbering scheme).

The results show clearly that for the first four atom pairs listed in Table 5.3, the atoms of the Sn1 chain of a lipid molecule penetrate deeper into the lipid bilayer than the equivalent atoms of the Sn2 chain by on average 1Å and this arises from the glycerol moiety which introduces the C₃ atom into the Sn1 chain. (The distance along the bilayer normal between C₂ and O₃₁ is 1.52Å which includes the extra C₃ atom, but only 0.62Å between C₂ and O₂₁).

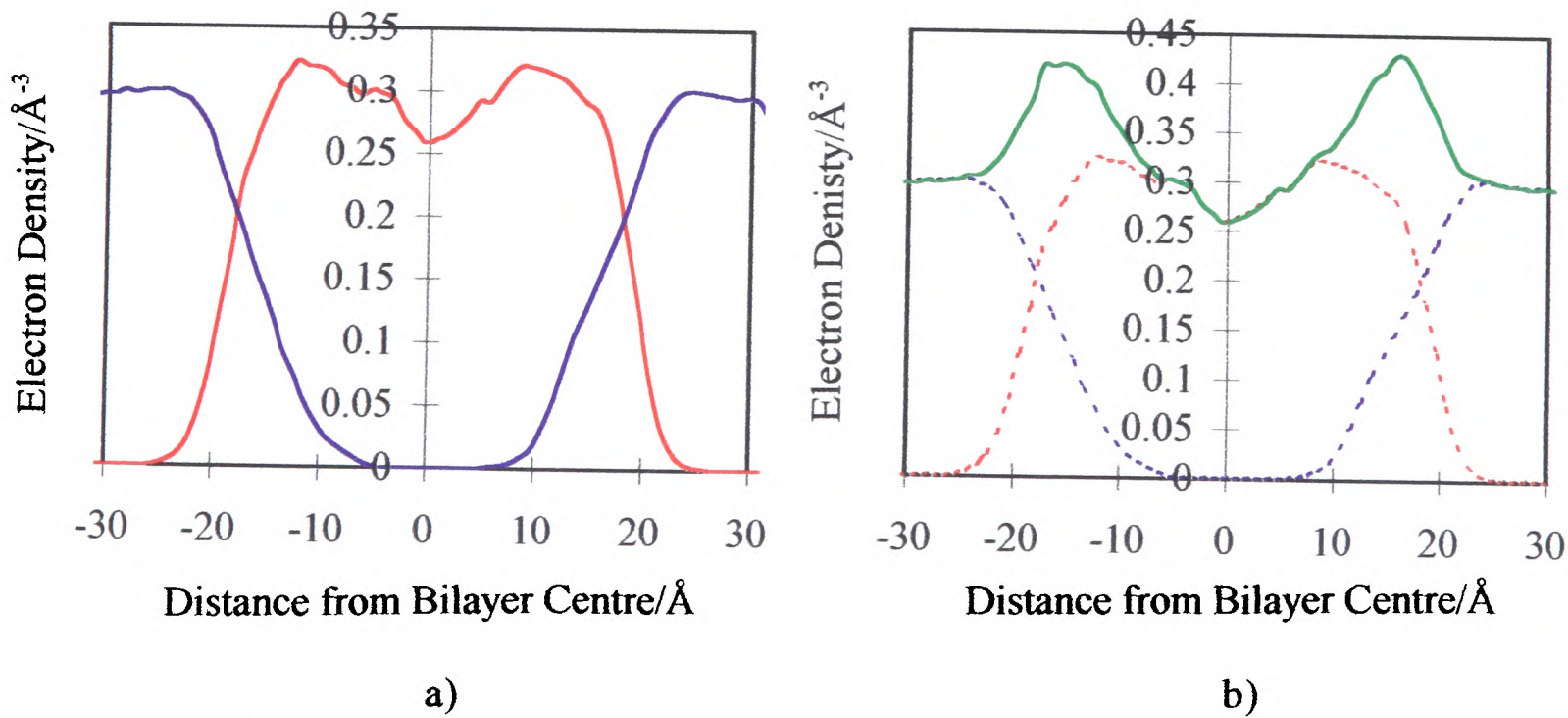


Figure 5.10 The calculated electron density profiles across the lipid bilayer. a) The lipid molecules (red) and water molecules (blue). b) The electron density profile for all atoms in the system (green) plus the lipid (red) and water (blue) components.

The electron density distributions were evaluated using the procedure described in Section 3.11 and are shown in Figure 5.10 for different components of the system and can be compared with the profiles calculated from x-ray and neutron diffraction experiments.

In common with the experimentally determined electron density profiles (McIntosh, 1978; McIntosh, 1990; Lesslauer et al., 1972), the highest electron density is seen in the electron rich head group region at $\pm 15\text{\AA}$ from the bilayer centre which contains a substantial amount of water. Shoulders are seen in the density at $\pm 5\text{\AA}$ and arise from the presence of the carbonyl groups at this depth.

What is apparent from these analyses is that the bilayer interior cannot be treated as a simple uniform hydrocarbon region as some models use for solubility and permeation calculations and is a consideration that is being developed by some other workers (Marrink and Berendsen, 1994).

5.4.2 The Order Parameters of the Methylene Segments

As described in detail previously (Section 2.4.1), order parameters calculated from molecular dynamic simulations are not directly comparable with those from NMR but it is still a useful parameter to measure the different motional behaviour of methylene groups along the length of the hydrocarbon chain. The average order parameters were calculated over the period 100-700ps and are shown in Figure 5.11. The variation in values of the order parameters during the simulation were plotted in Figure 5.4 and were used as a means to determine the time needed for equilibration of the system.

The calculated order parameters show the same features as the experimentally determined profiles (Figure 2.8) with the very different behaviour of C_{22} versus C_{32} , the approximate plateau between C_{x3} and C_{x9} (which was not observed for the previous small scale simulations in Section 4.4.3) before the fall in value toward the termini of the chains. That the value of the order parameter at the termini of the chains is not as small as observed experimentally is because the simulation is not long enough relative to the time scale nmr samples to explore fully this mobile region of the chains.

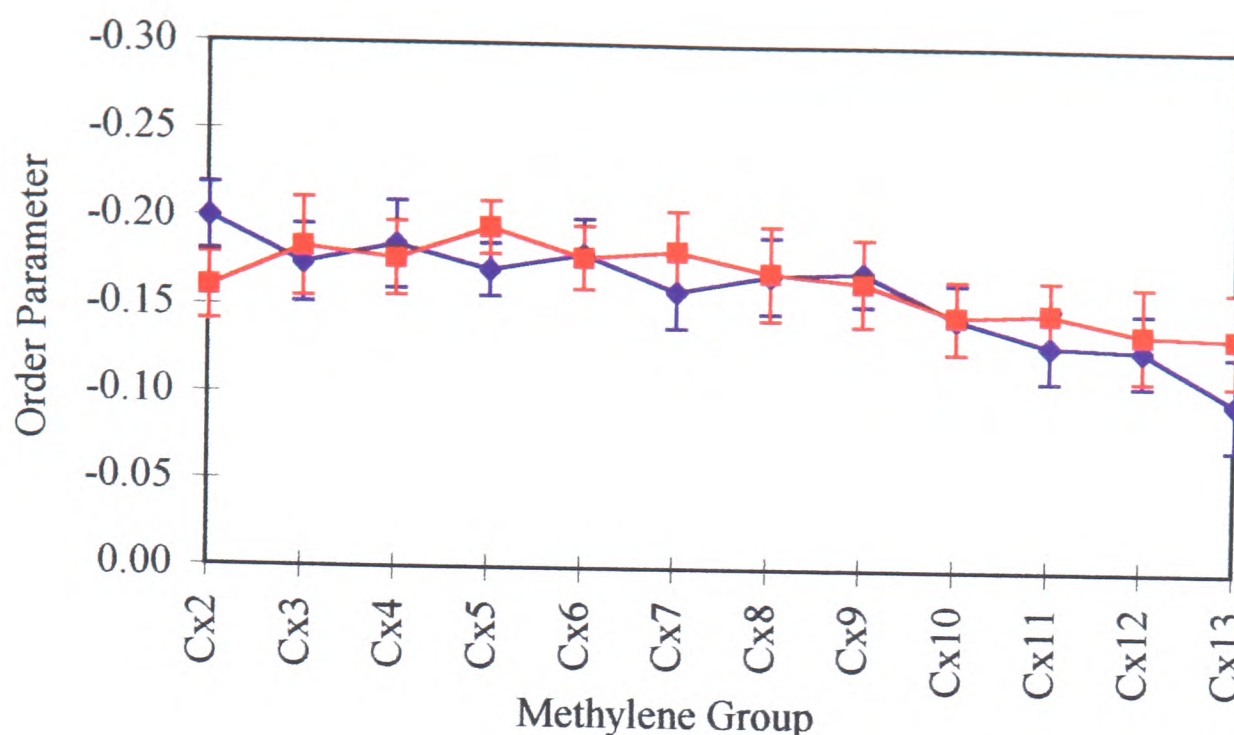


Figure 5.11 The order parameters for the methylenes of the Sn1 chain (blue and $x=3$) and the Sn2 chain (red and $x=2$).

The better reproduction of the shape of the profile has its origin in the better starting configuration of the system. Compared to the more ordered earlier system, it is not as easy for the chains to form a cooperative tilt because they are not all aligned in the same manner and thus the motion in the top part of the chains will be reduced leading to the observed plateau.

5.4.3 The Conformational Behaviour of the Lipid Chains

As described previously in Section 2.4.1, the fluidity of the hydrocarbon region arises from rotations about the carbon-carbon bonds. But because of interactions between chains, rotation is restricted to some extent with gauche dihedrals proposed to occur preferentially as part of certain patterns such as kinks and jogs.

The 600ps of molecular dynamic simulation was used to determine the probabilities of these patterns and other conformational properties. In all the discussions, the dihedral angle numbering scheme as illustrated in Figure 3.7 is used and in Figure 5.12 the probabilities of a dihedral angle being trans are plotted as a function of dihedral position.

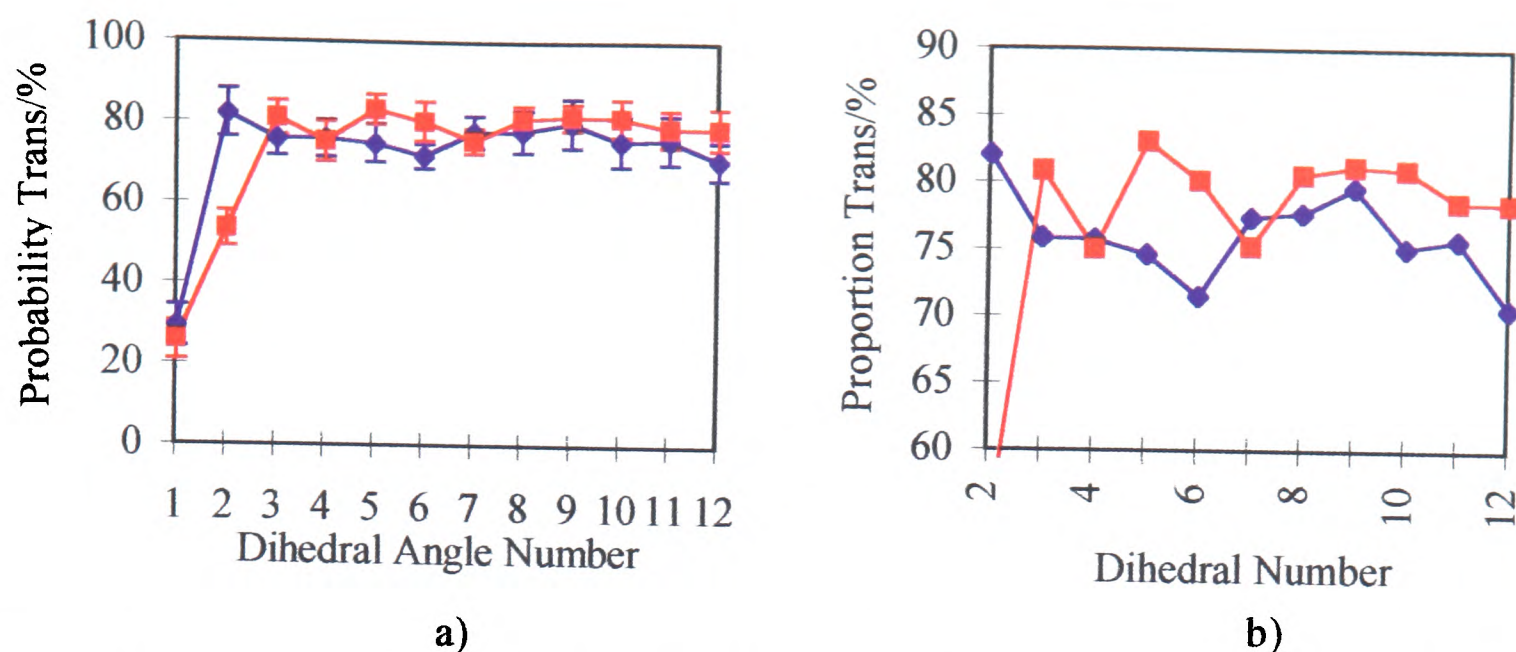


Figure 5.12 The probability(%) of a dihedral angle being trans is plotted for each dihedral angle in the hydrocarbon chains. (blue=Sn1 chain, red=Sn2 chain). a) All dihedral angles with the standard deviation included as error bars b) Dihedral angles 2 to 12 on a larger scale.

The Sn1 and Sn2 chains differ only at the second dihedral and this occurs because in the Sn2 chain that dihedral is partly responsible for the turn in the chain that directs it into the bilayer (see Figure 2.5) and hence can quite easily accommodate a gauche conformation whereas the Sn1 chain is already parallel to the bilayer normal at this point and will prefer to be trans.

The probability of a dihedral angle being trans is approximately 72% for both the Sn1 and Sn2 chains and compared to the early bilayer simulations (Section 4.4.6 and Figure 4.9) the odd-even oscillation in the values has disappeared showing better sampling in this larger system over a longer simulation length.

A decrease in the trans dihedral angle population is proposed to occur toward the termini of chains because of reduced constraints from neighbouring chains (see Section 2.4.1). Compared to the starting configuration in Chapter 4, the initial conformation of the present system had all the lipids with the terminal dihedral in the gauche conformation thus the population of trans dihedral angles observed has evolved over the course of the simulation. While the Sn1 chain does show a decrease in the probability of the terminal dihedral angles being trans compared to its immediate neighbours, the Sn2 chain shows a slight decrease and the results are far closer to the experimental situation than the simulation of Chapter 4 (cf. Figure 4.9), the results are still not ideal. The variation observed in the upper half of the chain

arises probably from inadequate sampling of torsional phase space because of the slow rate of isomerisation in this part of the chains (see Section 5.4.4).

Since the conformational state of a dihedral angle will effect the orientation of the chain and hence how it will interact with the surrounding hydrocarbon chains, it would seem that the conformational state of one dihedral will have an effect on the phase adopted by neighbouring dihedrals. To this end, the probability of a range of dihedral angle patterns and sequences was investigated, including the kink and jog sequences. In Table 5.4 the probabilities of different pairs of dihedral angles occurring adjacent to each other are listed as calculated from the molecular dynamic simulation. Using the average probability of a trans and gauche occurring within the hydrocarbon chain as calculated from the molecular dynamic simulation, i.e. 76% and 24% respectively, the statistical probabilities of these sequences were calculated and compared with the measured simulation values to determine if some sequences are more, or less, favoured than might be expected on a purely statistical basis.

Dihedral Pair	Sn 1	Sn2	Statistical
trans/trans	56%	57%	58%
trans/gauche	41%	41%	36%
gauche/gauche	4%	3%	3%
gauche/gauche'	0%	0%	3%

Table 5.4 The measured and calculated probabilities for different pairs of dihedral angles occurring adjacent to each other.

From Table 5.4 it is seen that for the simulation, the probability of two gauche conformations of opposite phase being adjacent is zero but from the statistical model which assumes that the dihedrals are independent of each other it is 3%. This arises because a gauche/gauche' sequence will have steric repulsion between the atoms and hence is disallowed. The other difference lies with the trans/gauche pair which is more probable than might be expected because of it making up for the missing contribution from the gauche/gauche' sequence. The

number of double gauche sequences per myristoyl chain is 0.44 which can be extrapolated to 0.52 for a palmitoyl chain (assuming that double gauche probability is independent of chain length) and compares not unfavourably with the experimental value for DPPC of 0.40 (Casal and McElheney, 1990; Mendelsohn and Senak, 1993).

The above procedure was repeated for patterns of three dihedral angles and the results are shown in Table 5.5. However any conclusions on differences between populations must be treated with caution as the differences are small, probably susceptible to noise and have contributions because of other sterically disallowed combinations which makes analysis difficult, i.e. intramolecular interactions have an impact on the probability of a particular dihedral sequence.

Dihedral Pattern	Sn 1	Sn2	Statistical
trans-trans-trans	42%	44%	44%
trans-trans-gauche	27%	28%	28%
trans-gauche-trans	17%	16%	14%
trans-gauche-gauche	6%	6%	4%
gauche-trans-gauche '	4%	4%	3%
gauche-trans-gauche	3%	3%	2%
trans-gauche-gauche '	0%	0%	4%
gauche-gauche-gauche	1%	0%	0%
gauche-gauche-gauche '	0%	0%	0%
gauche-gauche 'gauche	0%	0%	0%

Table 5.5 The probabilities from the simulation and from model calculations for sequences of triple dihedral angle sequences.

What is most apparent from Table 5.5 is that although the probability of a kink occurring is 33% larger than expected, the probability for the similar sequence gauche-trans-gauche is

50% more likely than expected when compared to a model in which all dihedrals act independently. This is contrary to the model in which it has always been assumed that kinks are much more strongly favoured than other combinations of two gauche and one trans dihedral angle because of interactions with neighbouring chains. It illustrates that motion of the hydrocarbon chains in the bilayer interior is actually relatively unhindered and the region is quite fluid despite the apparent ordering of the chains which must therefore behave in a fairly cooperative manner.

By extrapolation, the results of Table 5.5 can be used to give the approximate value per palmitoyl chain of 0.91 for the gtg and gtg ' sequences together (the simulation value is 0.77 per myristoyl chain) which is close to the experimental value for DPPC of 1.00 (Mendelsohn and Senak, 1993). Furthermore the simulation value of 0.44 kinks per myristoyl chain compares well with the theoretical value of 0.5/myristoyl chain calculated by Cevc and Marsh (1987) from the configurational partition function and the result of 0.5/myristoyl chain from interpolating the results of Schindler and Seelig (1987) on DPPC.

Number of trans dihedrals in run	Sn 1	Sn2	Calculated
1	76%	76%	76%
2	56%	57%	58%
3	42%	44%	44%
4	32%	33%	33%
5	25%	26%	25%
6	19%	20%	19%
7	15%	16%	15%
8	11%	13%	11%
9	9%	10%	8%
10	7%	8%	6%
11	5%	6%	5%

Table 5.6 The observed and calculated probabilities for different lengths of runs of trans dihedral angles.

This fluidity is also illustrated when the observed and calculated probabilities are compared for runs of trans dihedral angles in the hydrocarbon chains as shown in Table 5.6

From these calculations it is apparent that although kinks are slightly more favoured than expected, it is not clear if this is as a result of intermolecular chain packing interactions or the exclusion of other dihedral sequences because of intramolecular interactions. It does appear that dihedral isomerisation is relatively unrestricted but the chains remain oriented and the bilayer retains its integrity because of their attachment to the head group. Similar conclusions on the fluidity of bilayer chains were drawn by Venable et al (1993) based on the close similarities of both the diffusion coefficients and the average conformations of lipid chains in DPPC compared with hexadecane during molecular dynamic simulations.

5.4.4 Lifetime of Dihedral States in the Hydrocarbon Chains

The average length that a dihedral angle remained in the trans conformation was calculated for each torsion of the hydrocarbon chain and these are listed in Table 5.7. From experiments on bilayers it is reckoned that rate of interconversion is around once per 100ps in the main body of the chain but as fast as once every 10ps in the tails of the chains (Seelig and Seelig, 1980).

The life times calculated are of the right order of magnitude as the experimental results and from the times listed in Table 5.7 it appears that the average lifetime of a trans dihedral angle at the termini of the chains is shorter than for elsewhere in the chain reflecting the greater ease with which it should be able to undergo isomerisation because of the smaller impact it will have on surrounding chains, but because the standard deviations are very large for both chains this conclusion is questionable. However the difference between the lifetimes of other torsions along the chain is minimal apart from the second dihedral of the Sn2 chain which has a shorter life than expected and is probably due to the turn in the Sn2 chain near this dihedral angle causing it to undergo isomerisation more readily.

Dihedral Angle	Average lifetime for Sn 1 chain/ps	Standard Deviation	Average lifetime for Sn2 chain/ps	Standard Deviation
2	160	153	107	100
3	127	103	128	116
4	151	140	151	134
5	137	111	161	135
6	154	130	151	133
7	150	116	168	135
8	169	138	156	113
9	158	125	161	129
10	141	113	162	120
11	143	107	152	119
12	110	83	125	93

Table 5.7 The average and standard deviation of the lifetime of a trans dihedral angle in the hydrocarbon chain.

5.4.5 The Tilt of the Hydrocarbon Chains

As discussed previously in Section 2.4.1, experimental and simulation work points to individual lipid chains possessing a tilt with respect to the bilayer normal which averages to zero over the whole system and long time lengths. The observance of a tilt in the smaller bilayer model was taken as a sign that the simulation was neither long enough nor the system large enough.

Three vectors were used to describe different parts of the lipid chain which correspond to the upper section, the lower section and the whole of the chain as defined in Figure 3.7 and the distributions of the tilt angles that these vectors make with the bilayer normal are plotted in Figure 5.13.

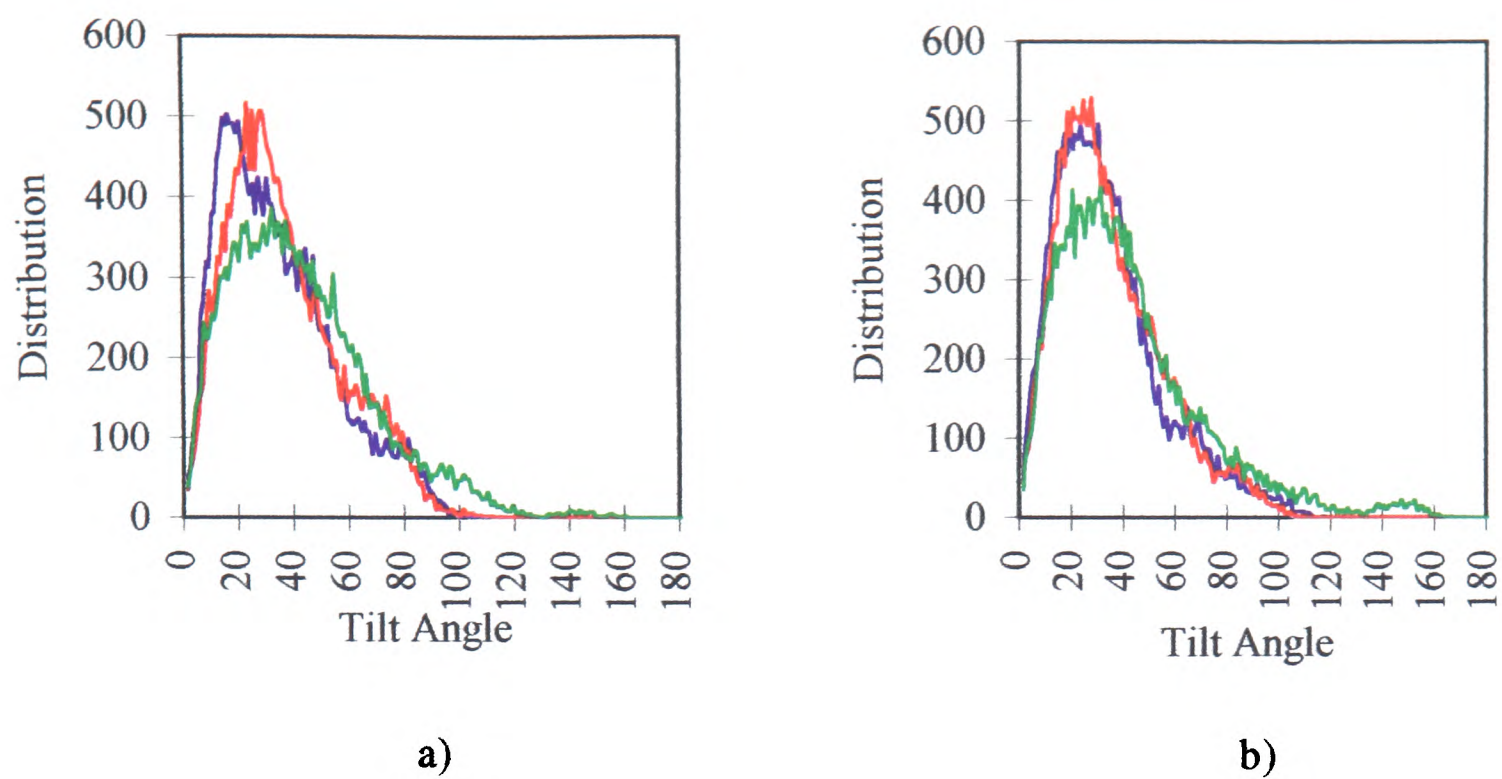


Figure 5.13 The distribution of the average tilt of the lipid chains with respect to the bilayer normal of the upper section (red), the lower section (green) and the whole of the chain (blue). a) Sn 1 chain b) Sn2 chain.

The vector defining the lower section of the chain shows a larger distribution than the other vectors illustrating the greater freedom of this section. A small portion of the chains show tilt angles greater than 90° which corresponds to the chain pointing toward the lipid/water interface rather than the bilayer centre and measurements have indicated that such behaviour occurs and this will be returned to. The most probable angle that a vector makes with the bilayer is in the range 20-30° which is entirely consistent with experimental observations (see Section 2.4.1) and the averages and standard deviations of the angle that each vector makes with the bilayer normal are listed in Table 5.8. The standard deviation is calculated from the difference between the angle for the whole system average over all time with the angle for individual lipids at each time frame.

As expected the lower part of the hydrocarbon chains shows both a larger average tilt and greater standard deviation than the upper part of the chain illustrating the increasing motional freedom toward the termini of the chains

Chain Vector	Average Tilt / Layer 1	Standard Deviation	Average Tilt / Layer 2	Standard Deviation
Sn 1/Whole chain	35°	17°	38°	19°
Sn 1/Upper part	37°	16°	38°	17°
Sn 1/Lower part	42°	20°	47°	25°
Sn2/Whole chain	34°	16°	36°	15°
Sn2/Upper part	35°	16°	36°	14°
Sn 1/Lower part	42°	21°	46°	24°

Table 5.8 The average tilt of lipid chains with respect to the bilayer normal and their standard deviation for both sides of the bilayer.

Chain Vector	Average Tilt / Layer 1	Standard Deviation	Average Tilt / Layer 2	Standard Deviation
Sn 1/Whole chain	8°	34°	18°	35°
Sn 1/Upper part	9°	36°	15°	35°
Sn 1/Lower part	7°	42°	21°	45°
Sn2/Whole chain	9°	33°	15°	34°
Sn2/Upper part	6°	35°	15°	33°
Sn 1/Lower part	13°	41°	15°	44°

Table 5.9 The collective tilt of the system for each of the chain vectors used to describe the lipid molecules over the course of the simulation. The large standard deviation reflects the large range of individual lipid chain tilts that occur in the system but that average to a value close to zero over the course of the simulation.

The collective tilt of the bilayer system is calculated using the method described in Section 3.12 and should be equal to zero to be consistent with experimental observations. The average value and standard deviation for each vector are listed in Table 5.9. The standard deviation is calculated from the difference between the collective tilt of the whole system

over all time with the tilt of individual lipids at each time frame and the large values show that a large amount of phase space has been sampled.

For the collective tilt there is less difference between the average for the upper and lower parts of the chain compared to the average tilt of individual lipids and this reflects the large range of situations that have been sampled over the course of the simulation.

Thus it appears that in terms of the collective system tilt and individual lipid chain tilt, the system size and simulation length is nearer the scale required to provide a working model of the bilayer, however the larger collective tilt in layer 2 of the bilayer is a potential problem. Compared to the earlier, smaller simulation one of the key elements that contributed to the greater confidence in the simulation of the hydrocarbon region was the greater degree of disorder introduced within the starting structure.

The simulation of Shinoda et al. (1995) is one of the few simulations of lipid bilayers that has not shown a collective lipid tilt but still shows local order. This system differs from all the others by not using a rectangular cell for the simulation and is a concept that should be examined more closely.

5.4.6 The Spatial Correlation between the Lipid Chains

The simplest method to describe the correlation between the lipid chains is using the length of the collective tilt vector, $\mathbf{T}$, which is the sum of the unit length chain vectors that describe the direction of individual lipid chains (see Section 3.12). If all lipid chains are perfectly aligned then the length of $\mathbf{T}$ will be 1.0 whilst in a totally random system it will be zero. The variation of the length of $\mathbf{T}$ with time was evaluated for both the Sn1 and Sn2 chains however there was little difference and no clear trend regarding differences between the upper and lower sections of the hydrocarbon chains and the average length of $\mathbf{T}$ was approximately 0.8 which is the same as the that of the earlier simulation (Section 4.4.4). It is clear that a finer method of measuring the correlation between lipid chains is required.

What we wish to know is how far the correlation between chains extends. To this end a spatial correlation function was devised in which the correlation between chains is calculated

as the average angle between chains as a function of their separation. The separation of the two vectors describing the chains is defined as the distance between their mid-points and the correlation between them is found from the result of their dot product and the distributions were normalised in exactly the same fashion as for the 2-d pair distribution functions described in Section 3.3.2. Correlation functions were calculated for each layer using vectors that represented the upper, middle and lower thirds of the lipid hydrocarbon chains in an analogous fashion to the two vectors defined previously that corresponded to the upper and lower halves of the lipid chains (see Figure 3.6) and Figure 5.14 shows the results for one of the layers in the system.

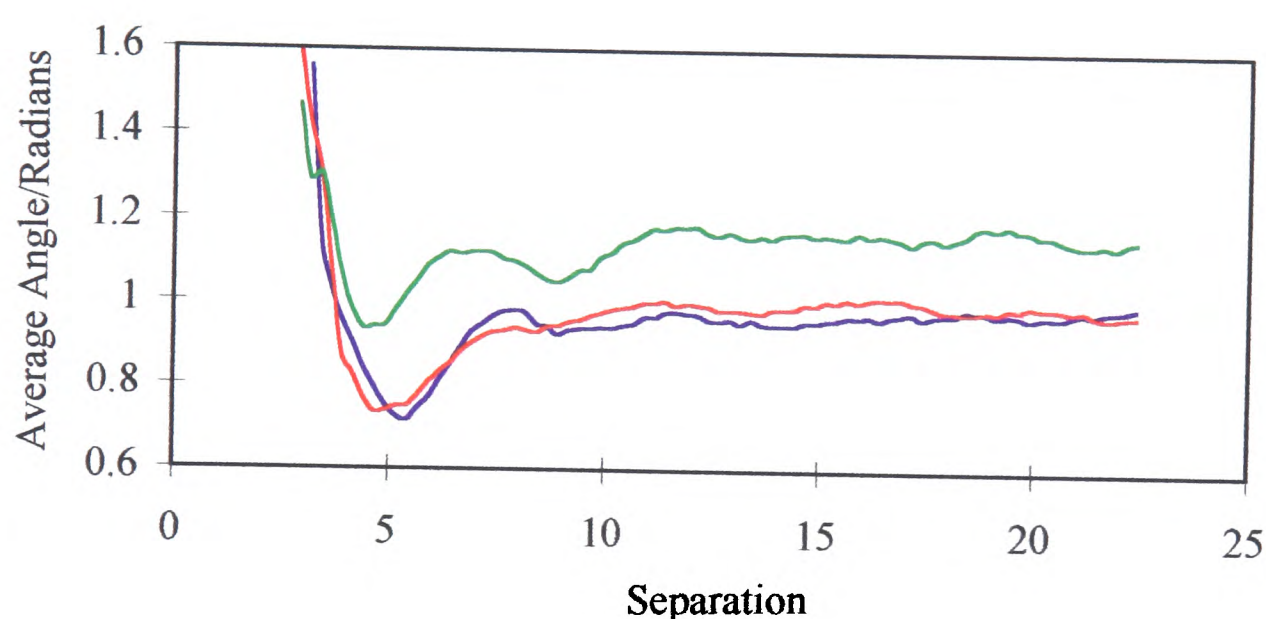


Figure 5.14 The correlation between chains as a function of distance as measured by the average angle between them for the upper (blue), middle (red) and lower third (green) parts of the lipid chains. N.B. Troughs imply separations at which the chains are more strongly aligned with one another.

Points that can be made from Figure 5.14 include:

1. The upper and middle portions show very similar behaviour.
2. The lower third section of the chains show a much greater average angle between chains than the other two sections.
3. The difference between peaks and troughs is quite large for the end third of the lipid chains.
4. The alignment of lipid chains extends over two shells (12 to 14Å).

More information about the nature of packing and interaction between hydrocarbon chains can be deduced by examining the standard deviation of the correlation functions (Figure 5.15) and the 2-d radial distribution of the chain vectors (Figure 5.16).

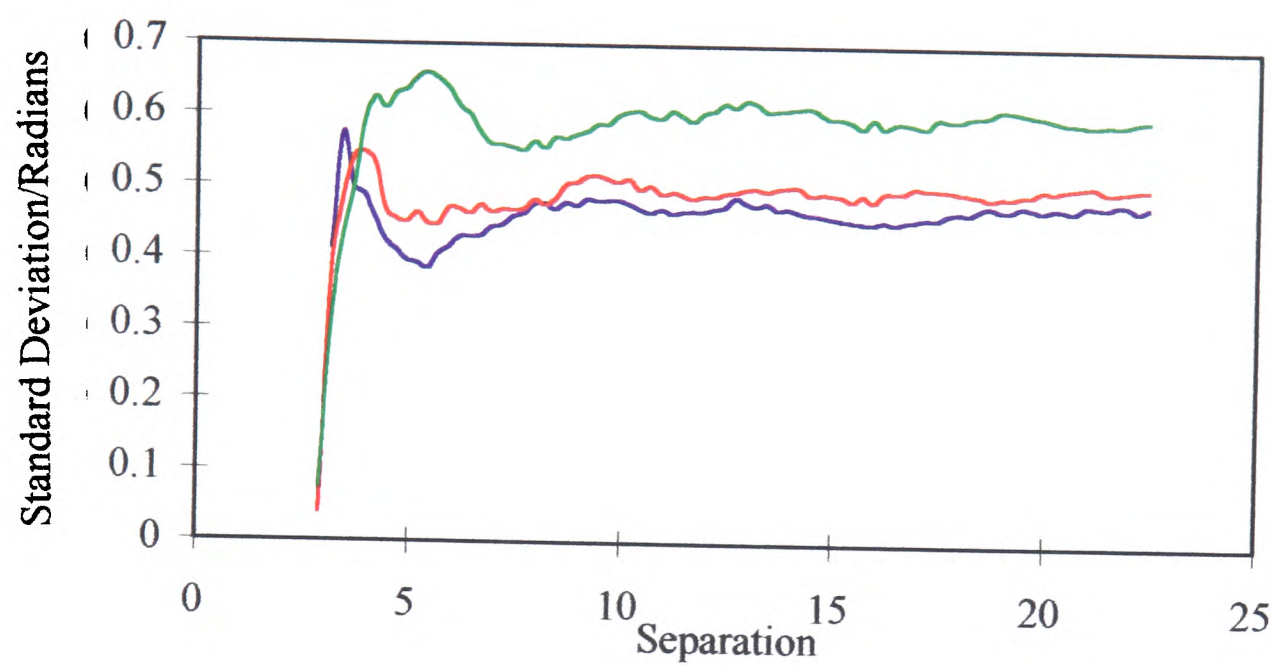


Figure 5.15 The standard deviation of the correlation function plotted in Figure 5.14. [Colour scheme as in Figure 5.14].

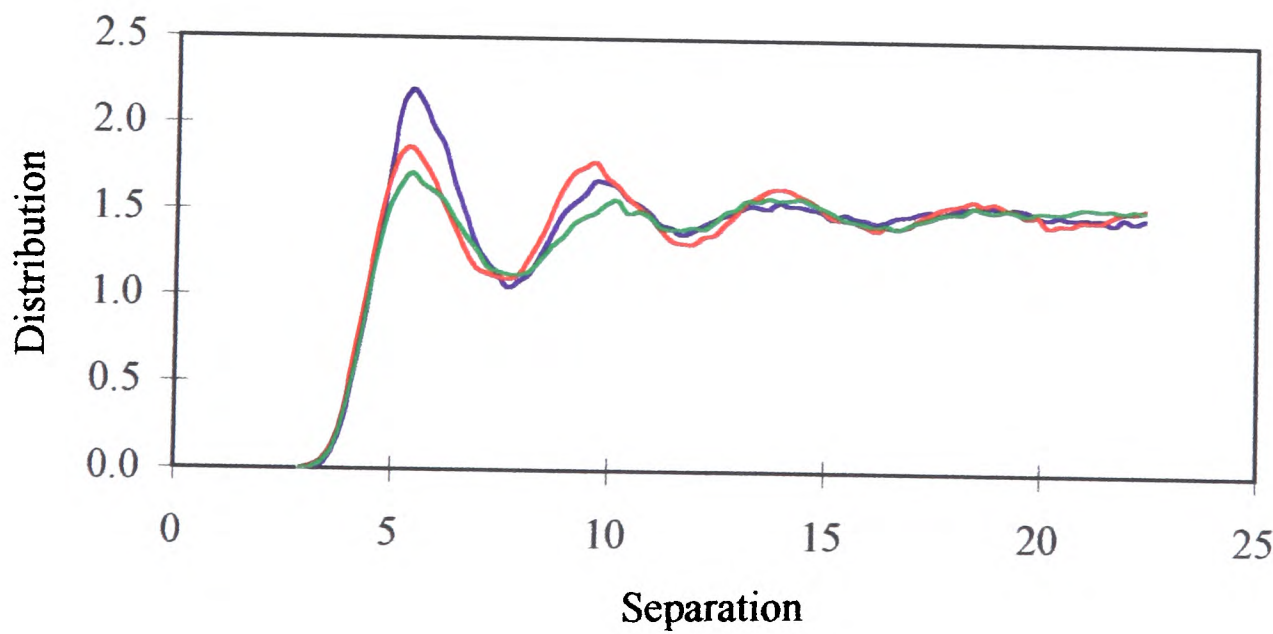


Figure 5.16 The 2-d radial distribution function for the midpoints of the vectors defining each portion of the lipid chain in one of the layers of the membrane. [Colour scheme as in Figure 5.14].

Conclusions about the degree of interaction and correlation between chains are reinforced by consideration of the standard deviation of the correlation function shown in Figure 5.15. The upper (and to a lesser extent the middle parts) of the chains behave as might be expected with the dips in the value of the standard deviation coinciding with the troughs in the correlation function and reflecting separations at which the chains are interacting more strongly. Furthermore the standard deviation is larger for the terminal third of the chains reflecting their greater freedom to tilt and move with respect to their neighbours without disturbing packing interactions as severely as higher in the chains.

The initially puzzling aspects of Figures 5.14 and 5.15 are the large difference in peak to trough height in Figure 5.14 and the peak in the standard deviation (Figure 5.15) where there would be expected to be a minimum. Thus although the large value of both the correlation function and the standard deviation suggest that packing and interaction should be less correlated between neighbours, in fact this appears not to be the case because of the obvious maxima and minima in the correlation function. That this part of the chain behaves differently from the middle and upper parts is less surprising when it is remembered that the lower part of the chains is not only interacting with neighbours in its own layer, but also with those of the other side of the bilayer and this is the extra factor that contributes to the ordering of this part of the chain and explains the otherwise anomalous behaviour.

From Figure 5.16 it is apparent that the packing of the chains extends over approximately four shells even though the value for the average tilt between tilts has levelled off after two (Figure 5.14). Thus although the tilt of the chains is no longer correlated after $11\text{\AA}$, the packing effect of the chains extends over $20\text{\AA}$. This is very close to half of the smallest box length ($22.5\text{\AA}$) which implies that some chains may indirectly sense the presence of the same chain twice. It also reflects the obvious highly ordered nature of the bilayer interior in terms of chain packing, though not in terms of direct interaction.

In conclusion, the correlation between the tilt of the chains extends for only $10\text{-}12\text{\AA}$, but the packing effect of chains is more extensive and lasts for up to $20\text{\AA}$ which is close to half the value of the minimum box length. Thus this simulation cell is probably close to the minimum size that a membrane simulation may be.

5.4.7 Diffusive Behaviour of the Lipid Molecules

The diffusive behaviour of selected atoms of the were determined by calculating the mean squared displacements as a function of time and then applying the Einstein relation approach to find the diffusion coefficient exactly as described in 'Computer Simulation of Liquids' (Allen and Tildesley, 1987) and outlined in Section 3.9.2. In all cases the contribution to diffusion from drift of the system as a whole was removed.

Experimental values for diffusion coefficients have been reported for DPPC molecules as a whole in membranes with 12% water (Konig et al., 1992) in terms of a short-time diffusion coefficient in the plane of the membrane and also perpendicular to it and these values were $0.02 \text{ Å}^2/\text{ps}$ and $0.0015 \text{ Å}^2/\text{ps}$ respectively. Fluorescence experiments on DMPC reported that the lateral diffusion of the molecule in the plane of the membrane at 45°C was between 0.010 and $0.012 \text{ Å}^2/\text{ps}$ (Vaz, Clegg and Hallmann, 1985).

The aim of the analysis was to determine how the mobility of the constituent atoms of a lipid molecule varied and if those nearer the centre of the bilayer showed greater diffusion, i.e. mobility. The diffusion coefficients were calculated both for specific atoms and as a function of depth in the membrane over the time scales of 5 to 30ps and 200 to 450ps where the latter range is the period in which the Einstein relation is expected to hold for regular liquids. The results for the 5 to 30ps range are illustrated in Figures 5.17 and 5.18 in units of $\text{Å}^2 \text{ ps}^{-1}$. The results for the 200 to 450ps analysis had the same shape as the shorter time scale analysis but are around twice as small which reflects the inability of the lipid molecules to undergo free diffusion, i.e. the motion of lipid molecules in the simulation is restricted to rattling motions within the cage formed by surrounding lipids and cannot swap positions, or hop, with neighbours as required for regular diffusion.

The most striking features of Figure 5.17 are the very similar behaviour of the Sn1 and Sn2 chains, the plateau in diffusion coefficients for the first nine carbon atoms of the chains and the steep increase in the value for last five atoms resulting in the terminal methyl group demonstrating a diffusion coefficient nearly twice that of carbons in the plateau region. These results clarify some features about the nature of motion of the chains since if the chain moved as a simple pendulum then it would be expected that the diffusion coefficient would increase steadily along the length of the chain which is clearly not the case. This hypothesis

was tested by evaluating the diffusion coefficients for diffusion in the plane of the bilayer (xy-direction) and also perpendicular to it (z-direction) and the results for each lipid chain are shown in Figure 5.18.

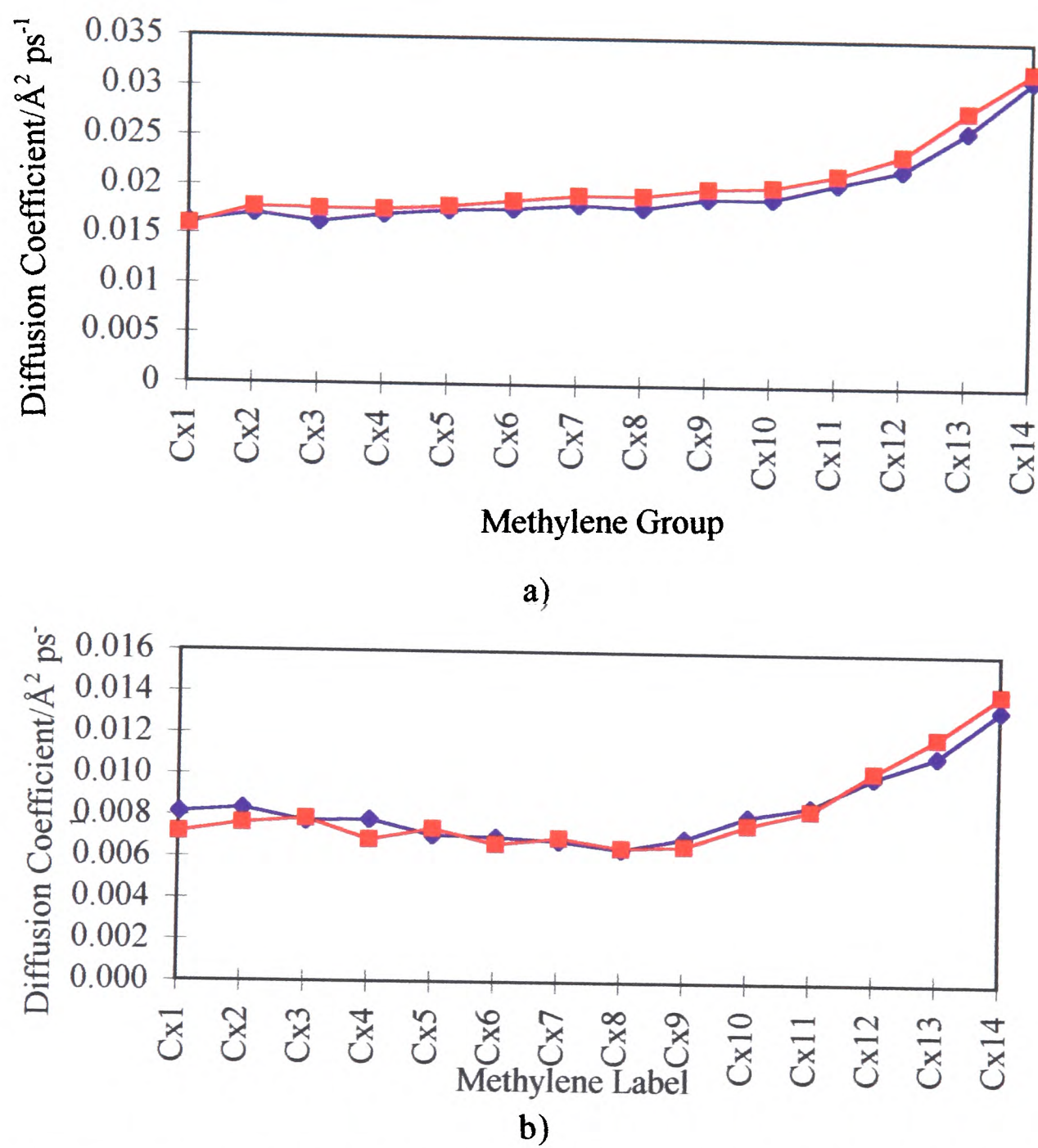
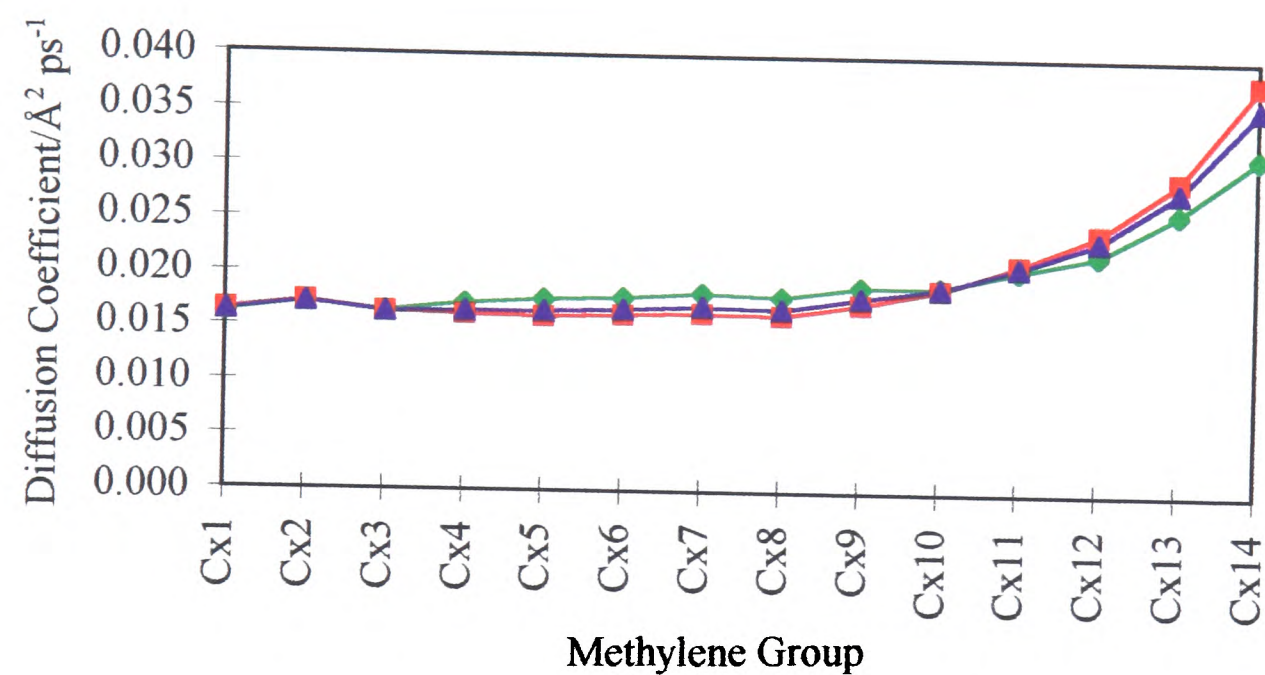


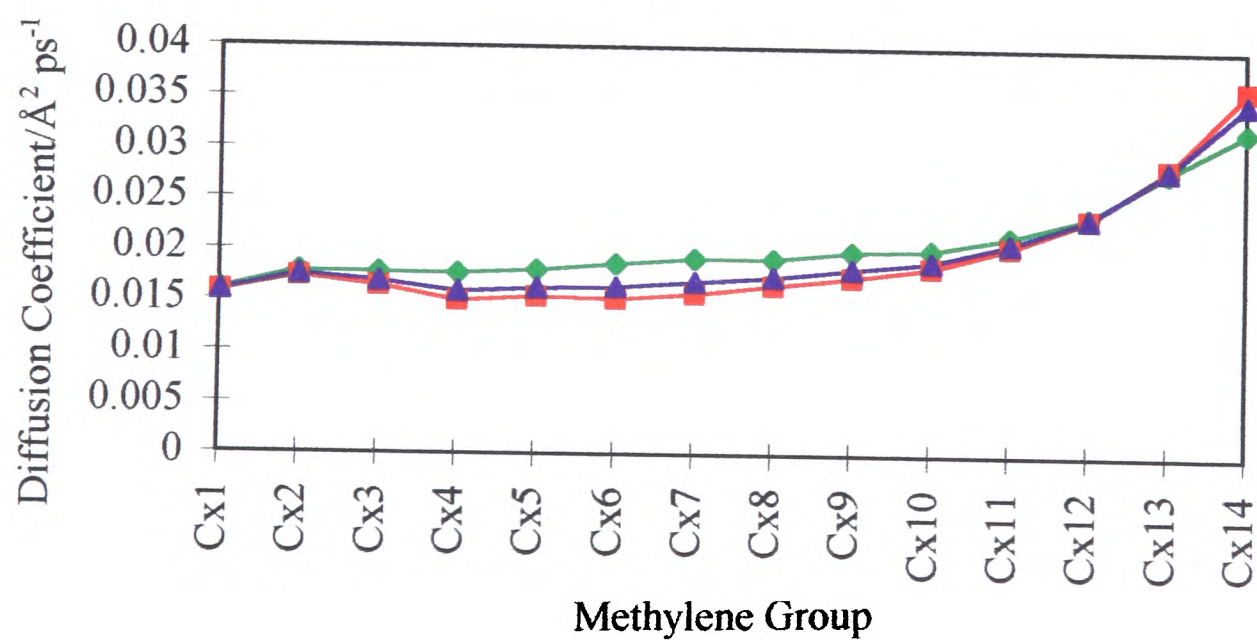
Figure 5.17 The Diffusion coefficients of carbon atoms along the hydrocarbon chains evaluated using the mean squared deviation and the Einstein relation in the time range a) 5 to 30ps and b) 200 to 450ps. The Sn1 chain is in blue and the Sn2 chain in red.

The value for the top part of the lipid chains should be closer to the value describing diffusion of the lipid molecule as a whole than the groups at the end and the agreement with the value reported by Konig (1992) is excellent.

The results of each chain are the same and show that the first nine methylenes of the lipid chains show identical diffusive behaviour while the terminal five show an increasing degree of freedom. which is consistent with a model in which motion is restricted in those chains nearer the head group than those at the end because of the different constraints of chain packing in different parts of the bilayer.



a)



b)

Figure 5.18 The diffusion coefficients for carbon atoms along the lipid chains for a) The Sn1 chain b) The Sn2 chain evaluated from the mean squared deviation in the scale 5 to 30ps. [D_{xyz} is in blue, D_{xy} in red and D_z in green].

So from Figure 5.18 it is apparent that the larger diffusion coefficient toward the termini of the chains is due to these atoms being able to diffuse more freely both in the plane of and perpendicular to the membrane surface which supports the notion that these atoms have greater motional freedom and that there is a mobility gradient along the length of the chain.

As a further point, the shape of the curves (i.e. plateau and then steep curve) is identical to the shape of order parameter curves observed experimentally which is known to be dependent on the motional behaviour of the chains. The calculated order parameters of Figure 5.4.2 did show a plateau but not the dramatic drop-off and this suggests that the simulation is of sufficient length to sample correctly the motional behaviour or “rattling” of the chains within the cage formed by surrounding chains plus dihedral isomerisation, but not the contribution from long time-scale “sweeping-out” motions and lipid reorientation about its long axis as discussed in Section 2.4.1. This may cause the lack of a drop-off because this important contribution to the motional freedom of the end groups of the chains is not included.

The mobility of chains within a bilayer must have an impact on and correlation with the ability of solutes within the membrane to diffuse past them. Since it has already been shown that there is a mobility gradient along the lipid chain it appears probable that solutes within the vicinity of the termini of lipid chains should have faster diffusion than those nearer to the lipid/water interface and Marrink and Berendsen (1994) have shown that the diffusion rate of water is fastest in the membrane interior where the lipid density is low. To investigate this phenomena further, the regular diffusion constants of methylene groups were evaluated as a function of distance from the bilayer centre as well as its components along the bilayer normal (D_z) and in the plane of the bilayer (D_{xy}) and all the results are plotted in Figure 5.19. The diffusion constants are only plotted up to 14Å from the bilayer centre as this was considered the limit of the hydrophobic region and the few chains that extend beyond this point are entering into the head group region.

The main observation that is that methylene groups in the centre of the bilayer diffuse faster than those nearer to the lipid/water interface. Furthermore this increase in diffusion is due to methylenes in the bilayer centre being able to undergo diffusion in the xy plane far more readily than they can nearer the surface (the red line of Figure 5.19). This behaviour can be

rationalised in terms of methylenes in the region 7 to 14Å having their motion in the xy plane restricted because of chain packing constraints while those in the bilayer centre are much more free to move around.

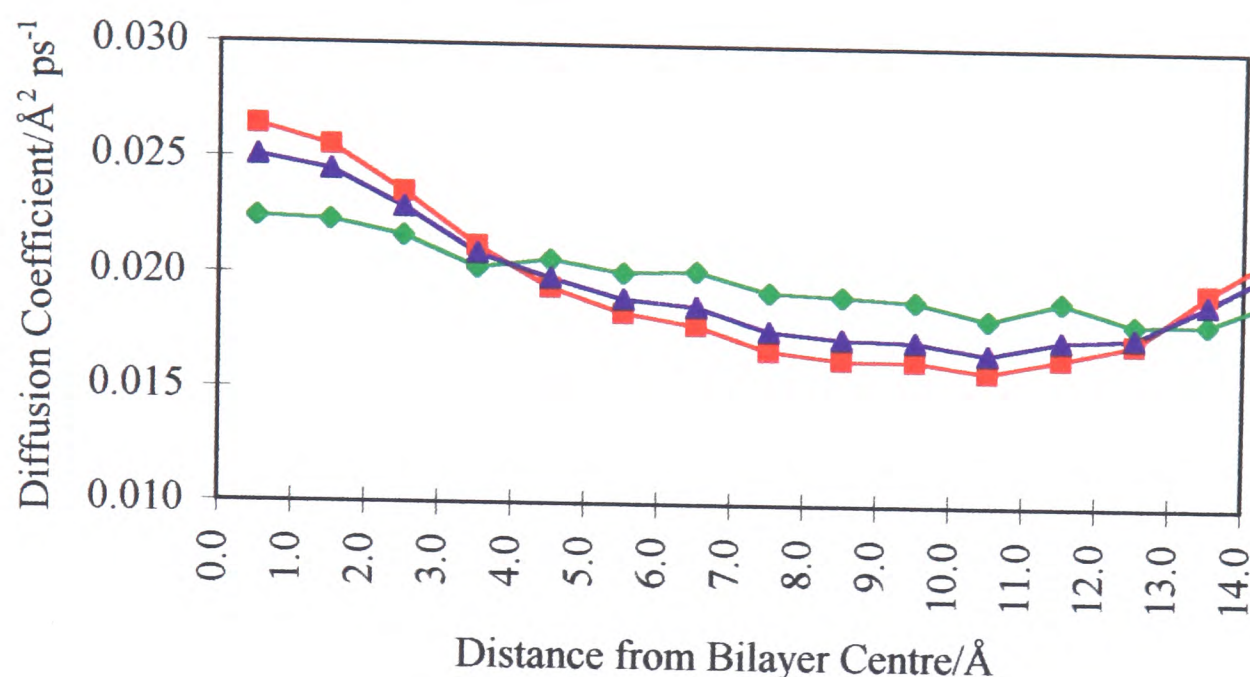


Figure 5.19 The diffusion coefficients of carbon atoms as a function of distance from the bilayer centre evaluated from mean squared deviations over the time scale of 5 to 30ps: D (blue), D_{xy} (red) and D_z (green).

5.4.8 Comparison of Simulation with Conclusions from NOE Experiments

NOE experiments on lipid bilayers are showing clearly that the hydrocarbon chains in lipid bilayers have much greater freedom than considered previously with evidence that up to 10% of terminal methyl groups are close enough to the N-methyl of a phosphatidyl choline head group to cause an NOE (Spooner, 1995), i.e. this provides evidence that either the terminal methyl of chains are penetrating right through to the other side of the bilayer, or that the chains are free enough to double back and interact with the head groups on their own side of the bilayer. The latter behaviour has already been identified clearly when calculating the tilt of chains with respect to the bilayer normal (see Section 5.4.5) and also consolidates the view in which chain motion is extensive yet averages to a situation in which the chain appears to be oriented along the bilayer normal.

Thus for each lipid the closest approach of the terminal methyls groups to an N-methyl group was calculated and used to construct the graphs shown in Figure 5.20. The first graph is the distribution of lipids that have a terminal methyl at the given distance from the closest N-methyl and the second graph is the cumulative distribution, i.e. the percentage of lipids that have a terminal methyl within the given distance from an N-methyl.

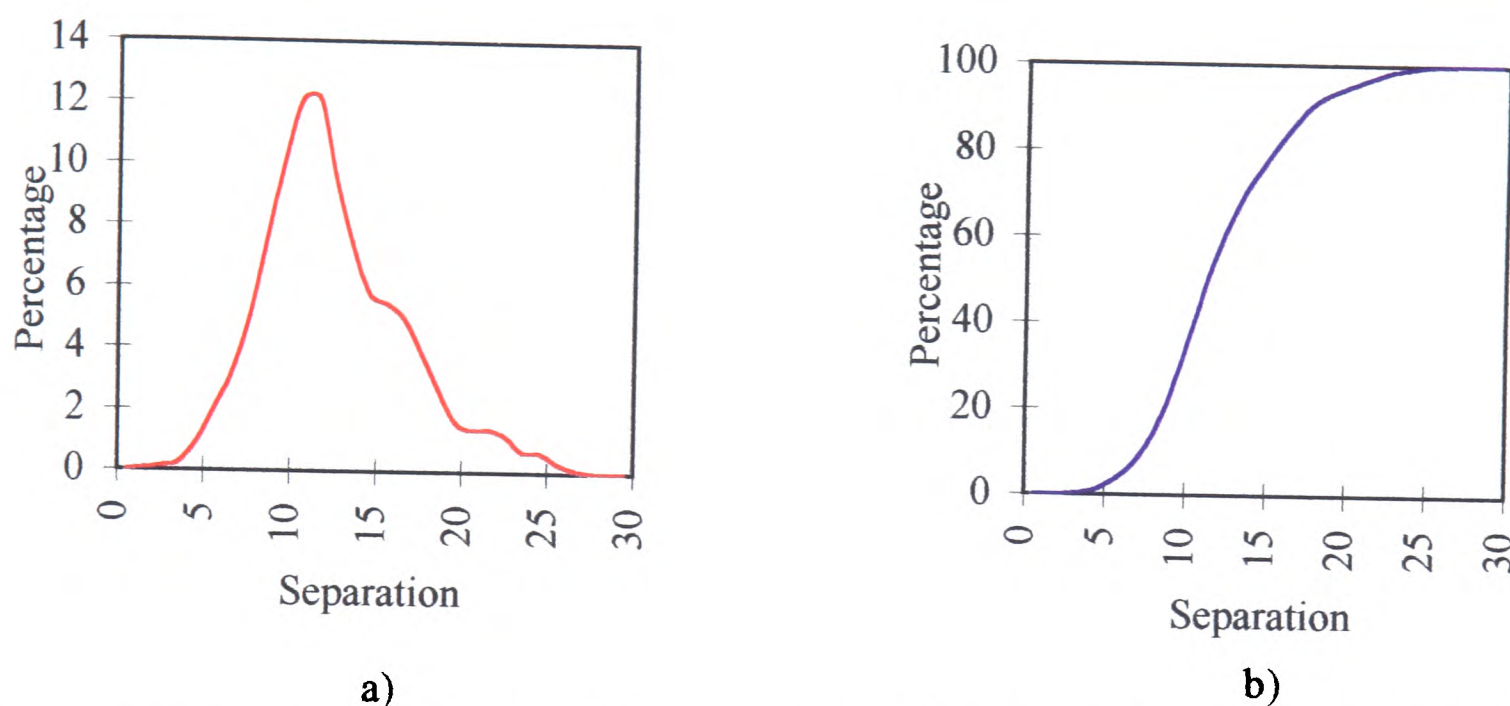


Figure 5.20 The distributions for a) the percentage of lipids that have a terminal methyl at the given distance from the closest N-methyl b) the percentage of lipids that have a terminal methyl within the given distance from an N-methyl.

The results illustrate that a portion of the lipids do have terminal methyl groups close to N-methyl groups however the results need to be corrected since the distances given are between carbon atoms, not hydrogens, because of the united atom approach used in the simulation. Allowing for this gives approximately 3% of the lipids having a N-methyl close enough to a terminal methyl group for an NOE to be expected (i.e. within $4.5\text{\AA}$). This is obviously smaller than the reported value and may arise from either poor sampling of lipid orientational distributions in the membrane, or the difficulty of determining the fraction from the NOE experiments.

5.4.9 Calculation of the Free-Volume within Membranes

The free-volume theory proposed by Cohen and Turnbull (Cohen and Turnbull, 1959; Turnbull and Cohen, 1970) concludes that “translational diffusion of a permeant occurs when statistical redistribution of free volume opens up a void of critical size in the immediate vicinity of the permeant” and so free-volume calculations are important in understanding the possible mechanisms and determining the ability for diffusion to occur within bilayers. Free-volume distributions were calculated from the molecular dynamics simulation as a function of distance from the bilayer centre using the basic theory as outlined by Xiang (Xiang, 1993). A free-volume distribution analysis involves calculating the probability that a spherical permeant of a given size may be placed in the membrane without colliding with any bilayer atoms. As stated, this probability was calculated as a function of depth within the membrane and the procedure involved placing a fine 2-d grid of points $0.5\text{\AA}$ apart in the plane of the membrane at a range of depths in the membrane and then for each point calculating the distance between it and the surface of the nearest atom (provided the point is not placed inside an atom). Permeant radius sizes were considered to increase from $0.0\text{\AA}$ in increments of $0.1\text{\AA}$. If a permeant of maximum size d can be placed at a point then it follows that all smaller sized permeants may also be placed there and thus a distribution was built up of the number of permeants of a given volume that can be placed at a depth d and this was normalised by dividing by the total number of points that were sampled at that depth, i.e. the number of points in the grid, to give a function, $f(V,d)$ where V is the volume of the permeant and d is the depth in the bilayer. The differential of $f(V,d)$ with respect to $-V$ is the probability distribution for cavity sizes available to spherical permeants. By extrapolating $f(V,d)$ to $V=0$ the total free-volume fraction available at a depth d may be estimated.

The character of cavities within the membrane interior may also be examined by recording the distribution of angles that a vector connecting the centre of a permeant with its nearest atom neighbour makes with the bilayer normal, but only if the permeant and atom are touching else it is ignored. Distributions are recorded for each possible size of permeant and the distributions are normalised by dividing by the total number of permeants of a volume V that can be placed at that depth. These distributions of contact angles are then compared with the theoretical distribution expected for a uniform distribution of atoms around a permeant and it is shown that the shape of the cavity must be ellipsoid since the probability of the

permeant touching an atom in the same plane of the membrane as it, is greater than it touching one above or below it. This behaviour is exactly as expected given the packing and general orientation of chains along the bilayer normal.

The average free-volume distributions were evaluated by analysing every 25 ps over the 600ps simulation with the depth in the membrane measured from the bilayer centre at 0Å up to 15Å from the centre in 1Å intervals and plots of the probability density, $f(r, z)$ are given in Figure 5.21 for $z=0$, $z=5$, $z=10$ and $z=15$ whilst in Figure 5.22 a plot of $f(r=0, z)$ versus distance from the bilayer centre is shown where r is the radius of the permeant. A graph of the distribution, $-df(V,d)/dV$ which is the probability for finding a cavity size that can accommodate a spherical permeant of radius r (i.e. volume $V = 4\pi r^3/3$) is shown in Figure 5.23.

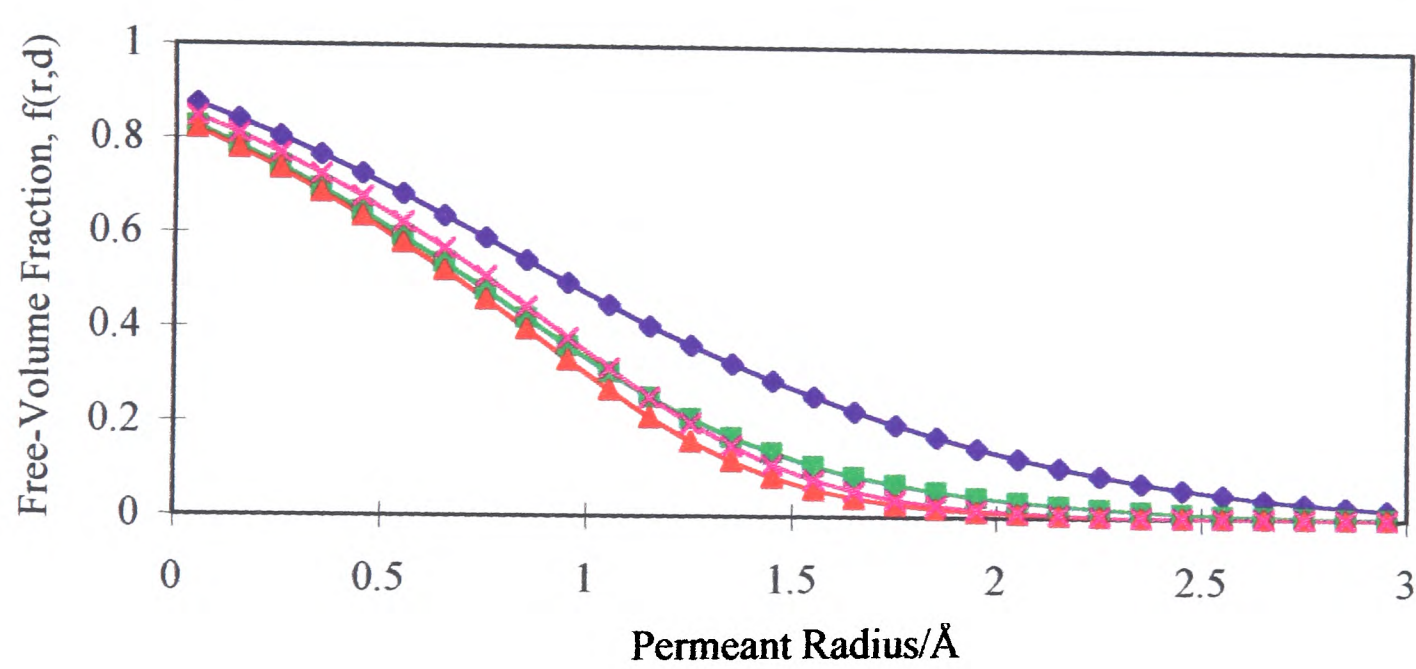


Figure 5.21 The free-volume fraction, $f(r,d)$, at four different distances from the bilayer centre for permeants with a radius of 0.05Å to 3.0Å. The distances are 0Å (magenta line), 5Å (red line), 10Å (green line) and 15Å (blue line) from the bilayer centre.

The first conclusion that may be drawn from Figure 5.21 is how rapidly the free-volume fraction falls of with increasing size of permeant and secondly that there is a far greater difference in the free-volume available at 15Å from the bilayer centre compared to the other three. This is not unsurprising given that at 15Å from the bilayer centre the water density is about 0.013Å^{-3} and corresponds to the head group region whilst 0Å, 5Å and 10Å all

correspond to the hydrocarbon region and the water density is at least an order of magnitude lower. Thirdly there is a greater free-volume fraction at the bilayer centre than there is at 5Å from the bilayer centre which is consistent with the lower lipid atom density observed at this point (see Section 5.4.1).

Figure 5.22 plots the total free-volume fraction available as a function of distance from the bilayer centre which was calculated by extrapolating plots of $f(r,d)$ back to $r = 0\text{\AA}$, i.e. zero permeant size and reinforces the points made about Figure 5.21 with a larger total free-volume fraction being observed at the bilayer centre than in other parts of the hydrocarbon region.

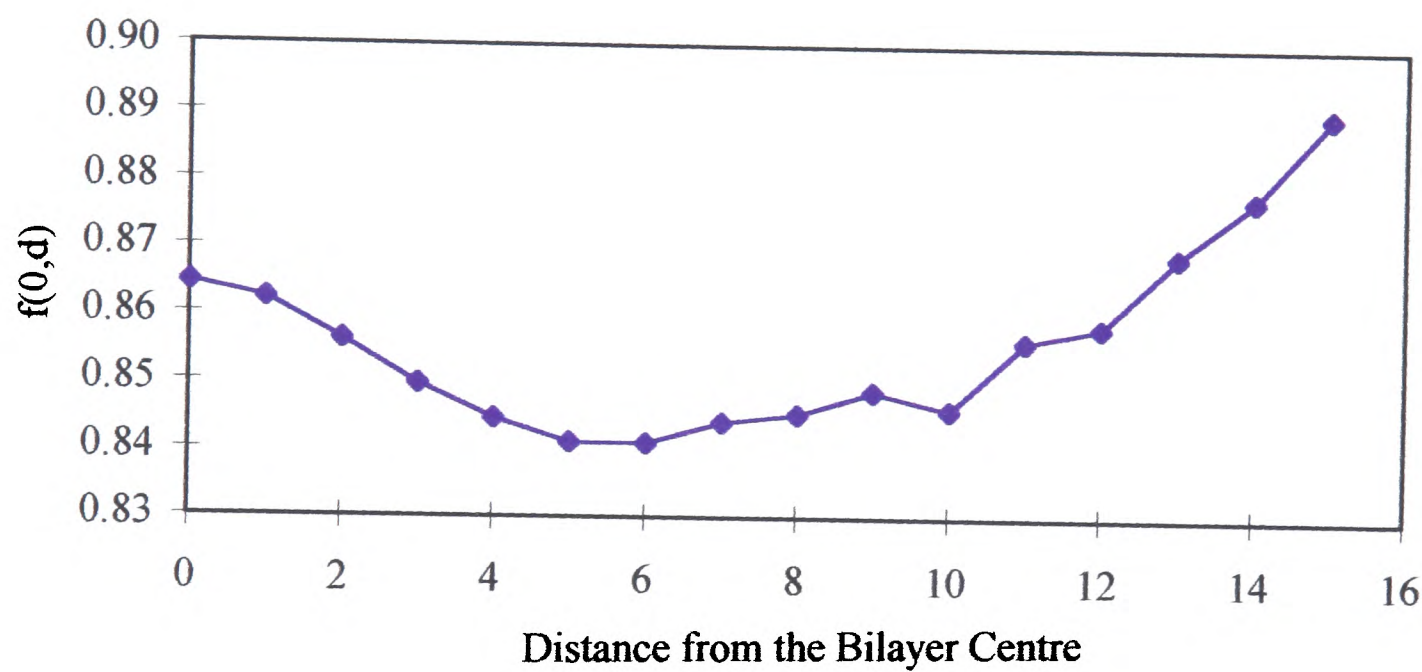


Figure 5.22 The total free-volume fraction available in the bilayer as a function of depth evaluated by extrapolating the function $f(r,d)$ back to $r = 0\text{\AA}$.

By evaluating the gradient of a plot of the free-volume fraction versus volume of the permeant, the probability of finding a cavity that can hold a permeant of a given volume may be found and this is plotted in Figure 5.23 on a logarithmic scale. The graph shows that the probability falls off extremely rapidly as the permeant size increases which is not surprising, as is the fact that the head group region (15Å from the bilayer centre) has a much higher probability of accommodating a large spherical permeant since the density of lipid atoms is relatively low here and water density (which is not included in calculating the free-volume) is high. The interesting result is that the probability of being able to accommodate a permeant of a given size is larger for the centre of the bilayer than for 7Å from the bilayer centre which

will be reflected in the ease of diffusion of permeants through the membrane at these different depths according to the free-volume theory of diffusion by Cohen and Turnbull (Cohen and Turnbull, 1959; Turnbull and Cohen, 1970).

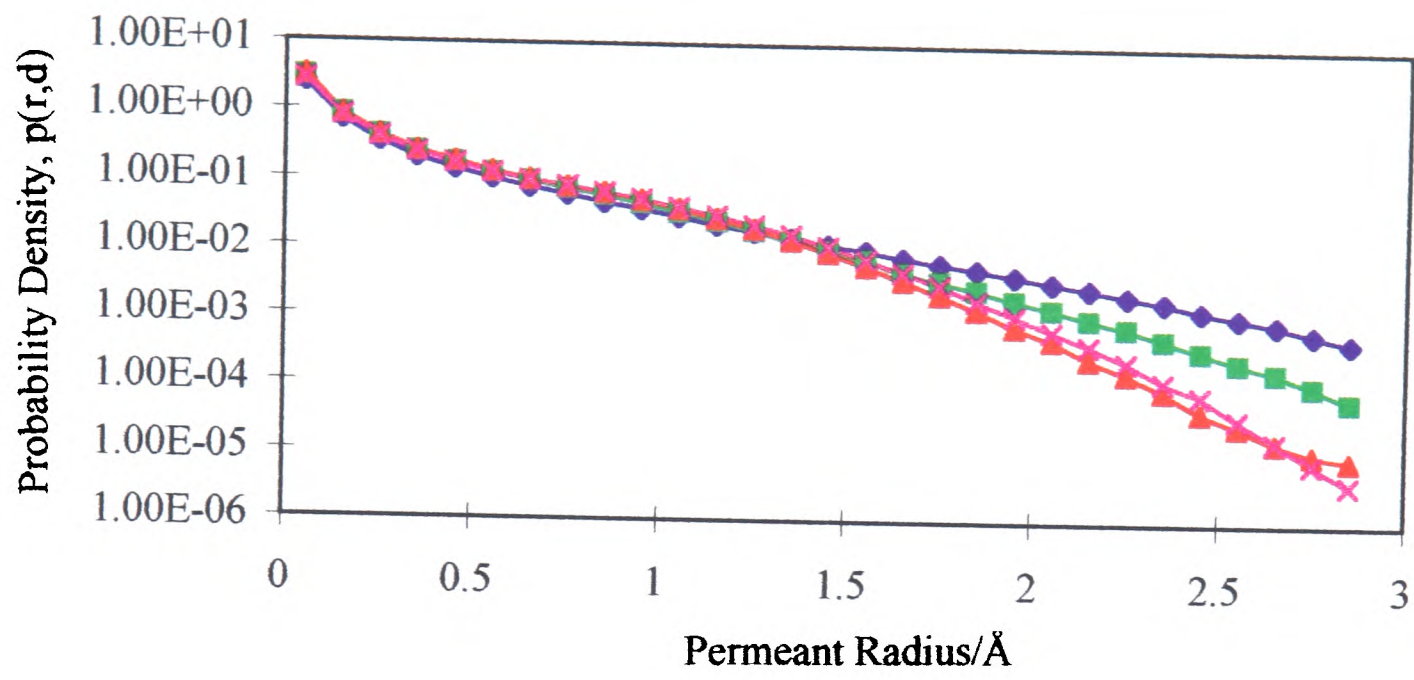


Figure 5.23 The probability density $p(r,d)$ of finding a cavity that can fit a permeant of given radius evaluated from $-df(V,d)/dV$, at four different distances from the membrane centre. [The colour scheme is as in Figure 5.2 1].

One possible permeant in a membrane is a water molecule, so as a function of depth in the membrane the probability of a water molecule being able to find a cavity was evaluated and the results presented in Figure 5.24. The plot shows that on moving from the head group to the hydrocarbon region (15Å to 10Å) there is rapid decline in the probability of water finding a cavity and this is also reflected in the density distributions calculated in Section 5.7.1. There is a small probability that water can find a cavity at the bilayer centre which is about 5% of it finding a cavity at 10Å from the centre.

Given that the calculated probabilities of finding a water cavity at 5Å and 10Å from the bilayer centre are 0.0002 13 and 0.000024 respectively and that the observed water density at 10Å from the bilayer centre is 0.0028 molecule/Å⁻³. Then the expected water density at 5Å is 0.00042 molecule/Å⁻³, however the observed value is only 0.00017 molecule/Å⁻³ and this reflects the energy penalty of moving a water into the hydrocarbon region. What it does

illustrate is that there is useful free-volume available in the bilayer interior which solutes may reside in and provide a pathway for diffusion through the membrane.

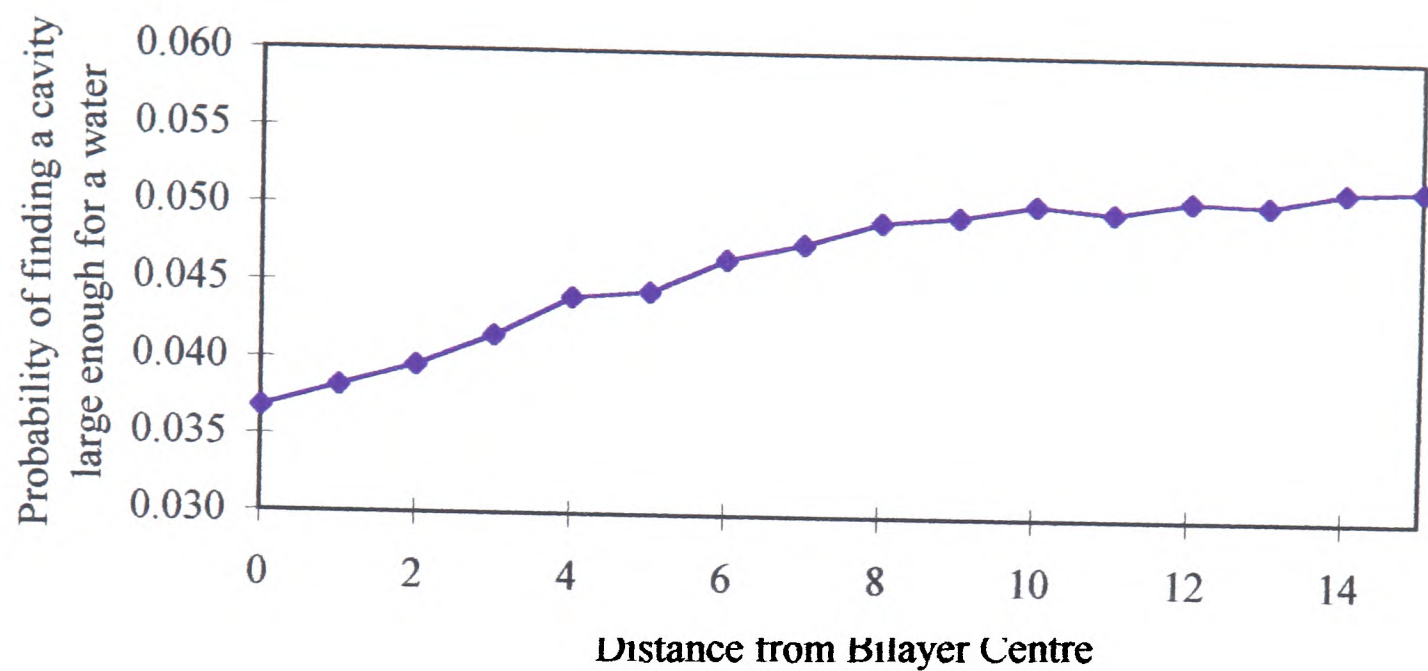


Figure 5.24 The probability of finding a cavity that can hold a spherical TIP3P water molecule (radius 1.8Å) as a function of distance from the bilayer centre evaluated from the calculated probability distribution, $p(r,d)$.

Most free-volume within the membrane interior is not useful in terms of diffusion since it is due to the space between neighbouring chains and the cavities are too small for normal sized permeants even if they are quite elongated. This type of free volume is known as interstitial free-volume and is reflected in Figures 5.21 and 5.23 as the very steep decline as the permeant size increases.

As a final exercise, the cavity shape at different depths in the membrane and for different sized permeants was investigated. Because of the orientation of chains it would be expected that most cavities sensed by permeants would be elliptical in shape and this is observed to be the case. The ideal distribution of contact angles against which the experimental values are compared are calculated from the equation $P(\theta) = 0.5(\cos \theta_1 - \cos \theta_2)$ which is the result from determining the surface area of a sector of a sphere defined by the two angles θ_1 and θ_2 and dividing by the total surface area of the sphere (see Xiang, 1993). The probability distribution for the contact angle is equal to the proportion of the surface area of a sphere that is available

to form this contact angle. In a qualitative manner, there is a greater probability that a point will hit the equator of a sphere than one of its poles because of the much greater difference in surface area at these locations.

The distributions are presented in Figure 2.25 for the bilayer centre and 5Å and 10Å from it and for two permeant sizes; 0.05Å radius and 0.9Å radius. The latter is the radius of a TIP3P water molecule which is considered spherical.

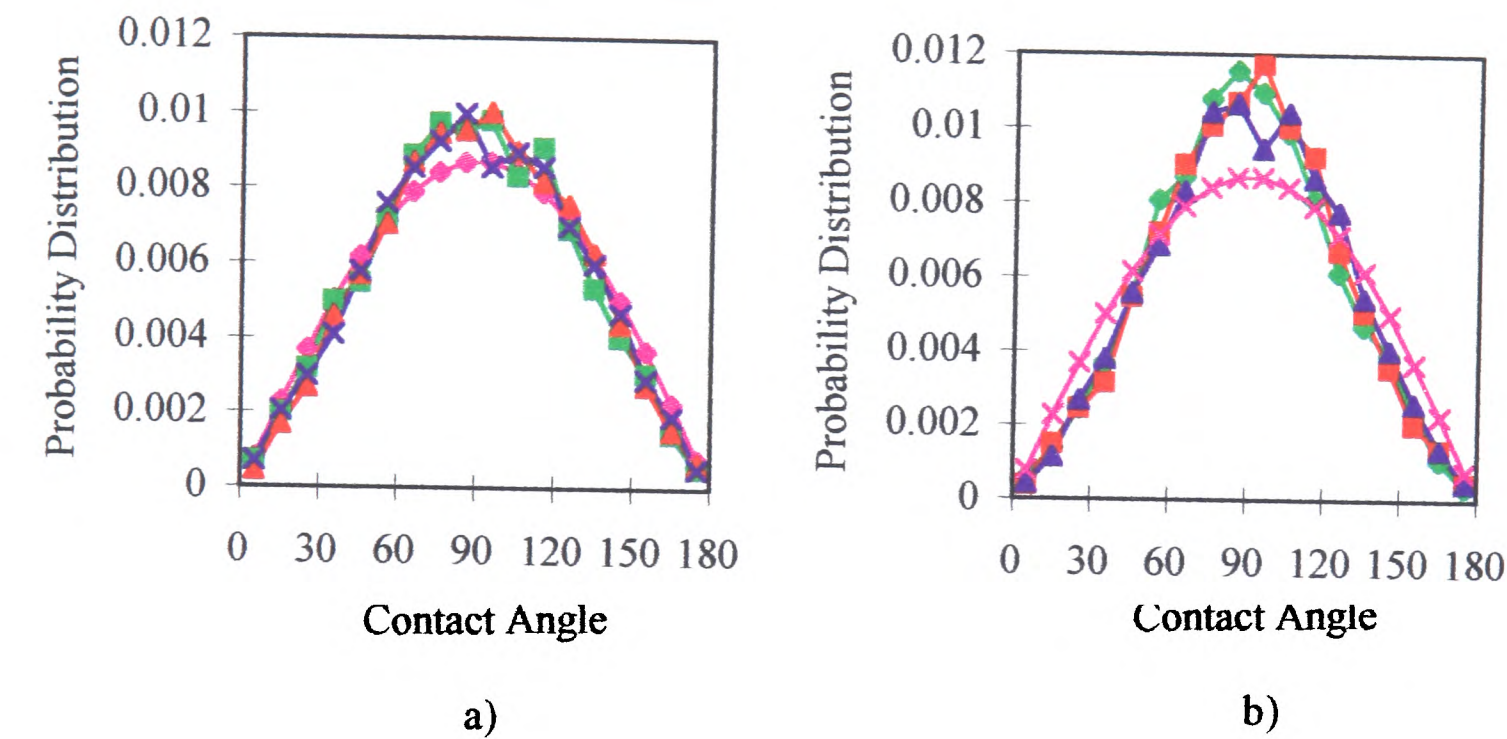


Figure 5.25 Two plots of the probability distribution for the contact angle with two different probe sizes at three different depths within the bilayer. a) Probe radius is 0.05Å b) Probe radius is 1.8Å. The three depths (as measured by distance from the bilayer centre) are 0Å (blue line), 5Å (red line) and 10Å (green line). The idealised distribution is shown in magenta.

The distribution in Figure 5.25a for the very small probe are very close to those of the ideal distribution and show that the cavities appear approximately spherical to the permeant because the cavities are so much larger than it.

The results shown in Figure 5.25b for the water sized probe show that compared to the ideal distribution, the probability is greater for contact angles corresponding to the permeant hitting lipid atoms around it in approximately the same xy plane, but less than expected for contacts with atoms above and below it along the z axis. (N.B. The bilayer normal is oriented

along the z axis, which is also the average direction that lipid chains are directed along). The variation of the profiles with depth shows that at 10Å from the bilayer centre the cavities are more elliptical in shape than those in the bilayer centre. This is consistent with a model in which the cavities at 10Å from the bilayer centre are due to the interstitial volume between packed, similarly oriented chains and are hence very elongated, whilst at the bilayer centre the chains are disordered and cavities are more spherical in shape.

Thus for a permeant the size of water, the free-volume cavities within the hydrophobic region appear to be elliptical in character with the long axis oriented along the bilayer normal.

5.6 The Behaviour of the Head Group Region during the Molecular Dynamics Simulation

The head group region is the first part of the membrane that a molecule will sense upon approaching it and is the first barrier to diffusion across the bilayer. It has already been shown that not only is the width of the head group really quite wide with some head groups transiently coming right out from the surface of the membrane, but that the extent of water penetration into it is extensive showing a strong interaction with the head groups and a moderate interaction with the carbonyl groups of the glycerol moiety (Figure 5.7 and Table 5.1 in Section 5.4.1). These observations are supported by neutron and x-ray diffraction studies that show the distribution of head group atoms along the bilayer normal to be up to 15Å (Wiener and White, 1992) and NMR studies which show a large number of waters to be associated with phosphatidylcholine head groups (Borle and Seelig, 1983). The protrusion of head groups from out of the bilayer surface has also been predicted from theoretical calculations (Israelachvili and Wennerstrom, 1992).

Furthermore the highly charged nature of the head groups will have a strong polarising effect on waters above the surface and this extends quite far from the surface (Alper, Bassolino and Stouch, 1993). Certainly the model that emerges from computer simulations of bilayers does not have the very ordered hydrocarbon chains and flat, well defined head group region depicted in text books (Stryer, 1981).

Water is an important component of the head group region and the behaviour of this water will differ from that of bulk water. Furthermore, the interaction between head groups and

water will have an important bearing on solute permeation of the membrane and this will be discussed in Section 5.7.8. It is even possible that the disorder and roughness possible in the bilayer surface introduces defects into the membrane structure that promotes unassisted ion transport and explains the high permeability of membranes to water and protons as the hydrocarbon region becomes more accessible and a high number of solutes are temporarily exposed to it (Deamer and Bramhall, 1986; Deamer and Nichols, 1989). Certainly the presence and properties of defects in the surface of the membrane is an important topic that will need to be addressed for biological membrane simulations, though it has been studied for much simpler systems (Wilson and Pohorille, 1994).

5.6.1 Effect of the Head Groups on Solute Approach

For charged molecules, the electrostatic nature of the surface of the membrane will obviously have an important bearing on how, where or even if potential permeants will approach because the electrostatic interaction has the longest range and is often the largest component of the interaction between molecules (Alper, Bassolino and Stouch, 1993. Alper, Bassolino-Klimas and Stouch, 1993). For many bilayers it is observed that the permeability to anions is greater than cations and this is taken as evidence of the surface having a net positive potential. This might be expected to be also true of DMPC bilayers since the end group of the DMPC head group is the positively charged choline and it might be that this extends further out from the bilayer than the phosphate of the head group. Experiments on the phosphatidyl choline head group show that generally the head group is oriented at about 20° to the membrane surface in the liquid crystalline phase (Akutsu and Nagamori, 1991; Seelig et al., 1977; Seelig et al, 1987; Wiener and White, 1992), which implies the phosphate groups are also relatively exposed at the surface.

To evaluate how the surface of the membrane appears to a charged solute a technique was devised in which 2-d layers of grids of points were placed parallel to the membrane surface at a series of distances from it. Each point was assigned a positive unit charge and its electrostatic interaction with all the layers of the bilayer calculated for a single frame of the molecular dynamic simulation and used to produce a map of the electrostatic interaction

energy of probe points over the surface at a given distance above it. The reason for these analyses was threefold:

- 1) To determine whether the surface of the membrane presents a uniform electrostatic surface, or if the surface is “patterned” with regions where a positively charged solute would be preferentially attracted plus regions that a negatively charged solute would favour.
- 2) To determine how far these electrostatic interactions extended and in the “patterned” surface case, at what point differences between regions became too blurred for the probe to distinguish between them.
- 3) To determine how, or if, the interaction energy maps changed over the course of the simulation.

In practical terms, the grid points were 0.5Å apart and given a point unit charge while the atoms were assigned the same nucleus-centred point charge used in the simulation. To produce the colour maps the intensity of the colour at a point is directly proportional to the value of the energy with negative interaction energies being shown in blue and positive ones in red. Furthermore, if an atom was found to be closer to a probe point than the van der Waals radius of the atom, then the contribution to the energy for that point from the atom was not included since it is unrealistic. This showed up on the maps as holes and identified regions where the surface lipid atoms were extending into the analysis region. Maps were calculated at 25Å, 30Å and 35Å from the centre of the bilayer since from Figure 5.7 it appears that few atoms of the lipid bilayer extend beyond this distance and solutes at 25Å could still be considered as within the aqueous phase and hence fairly free to diffuse over the surface of the membrane and choose where they wish to bind. Nine calculated maps are shown in Figures 5.26, 5.27 and 5.28 for different distances from the membrane at three different points in simulation.

Using the energy maps from the 400ps frame as an example, it can be seen that at 25Å from the bilayer centre the surface of the membrane is clearly divided into areas that favour either positive or negative solutes and that as the distance from the membrane increases, these differences tend to disappear or favour the approach of negatively charged species.

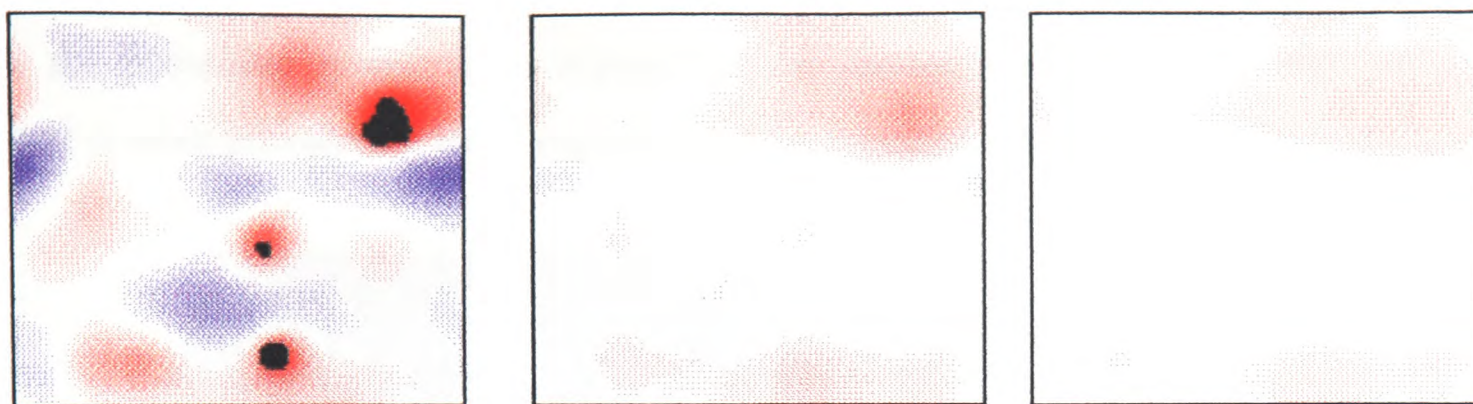


Figure 5.26 The electronic interaction energy maps of the membrane surface for the first step of the molecular dynamic simulation at 25, 30 and 35Å from the bilayer centre.

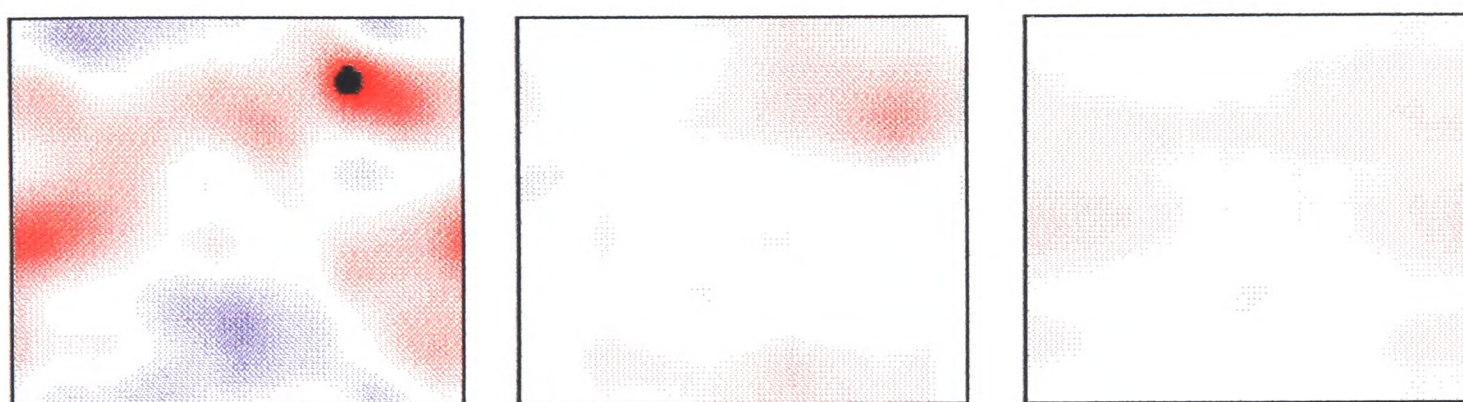


Figure 5.27 The electronic interaction energy maps of the membrane surface after 100 ps of molecular dynamic simulation on the bilayer at 25, 30 and 35Å from the bilayer centre.

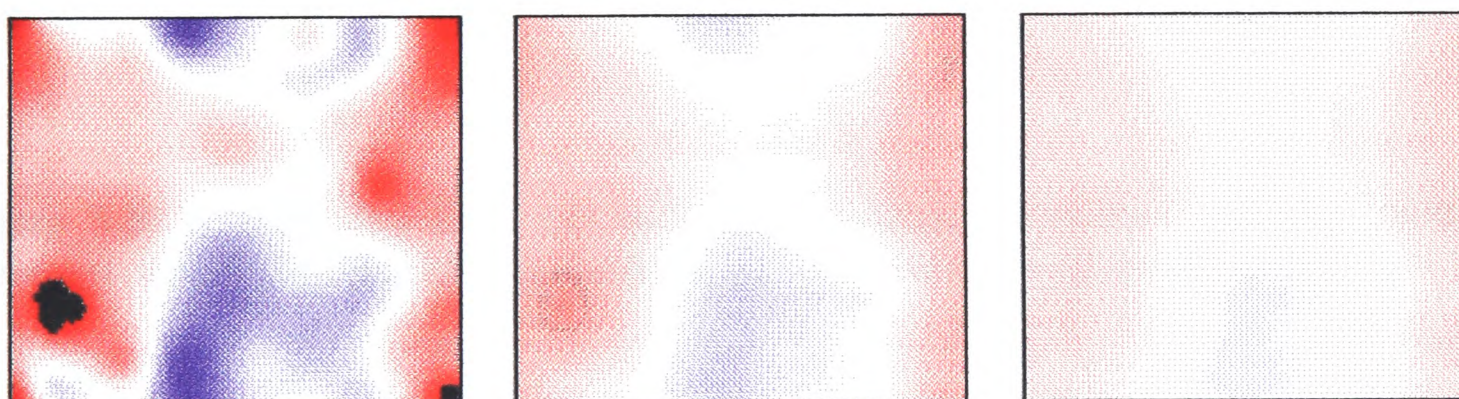


Figure 5.28 The electronic interaction energy maps of the membrane surface after 400 ps of molecular dynamic simulation on the bilayer at 25, 30 and 35Å from the bilayer centre.

These maps were compared with the atomic structure of the membrane in order to investigate how the electronic distributions arise and an example picture in Figure 5.30 illustrates how the 2-d maps were made semi-transparent and texture-mapped onto a plane at the relevant distance from the membrane model so that how lipid molecules contributed to the map could be determined. The most obvious conclusions are that regions favouring positive solutes must

have the phosphate groups more exposed at the surface whilst the N-methyl end of the choline is more prevalent at those regions where negatively charged solutes are preferred.

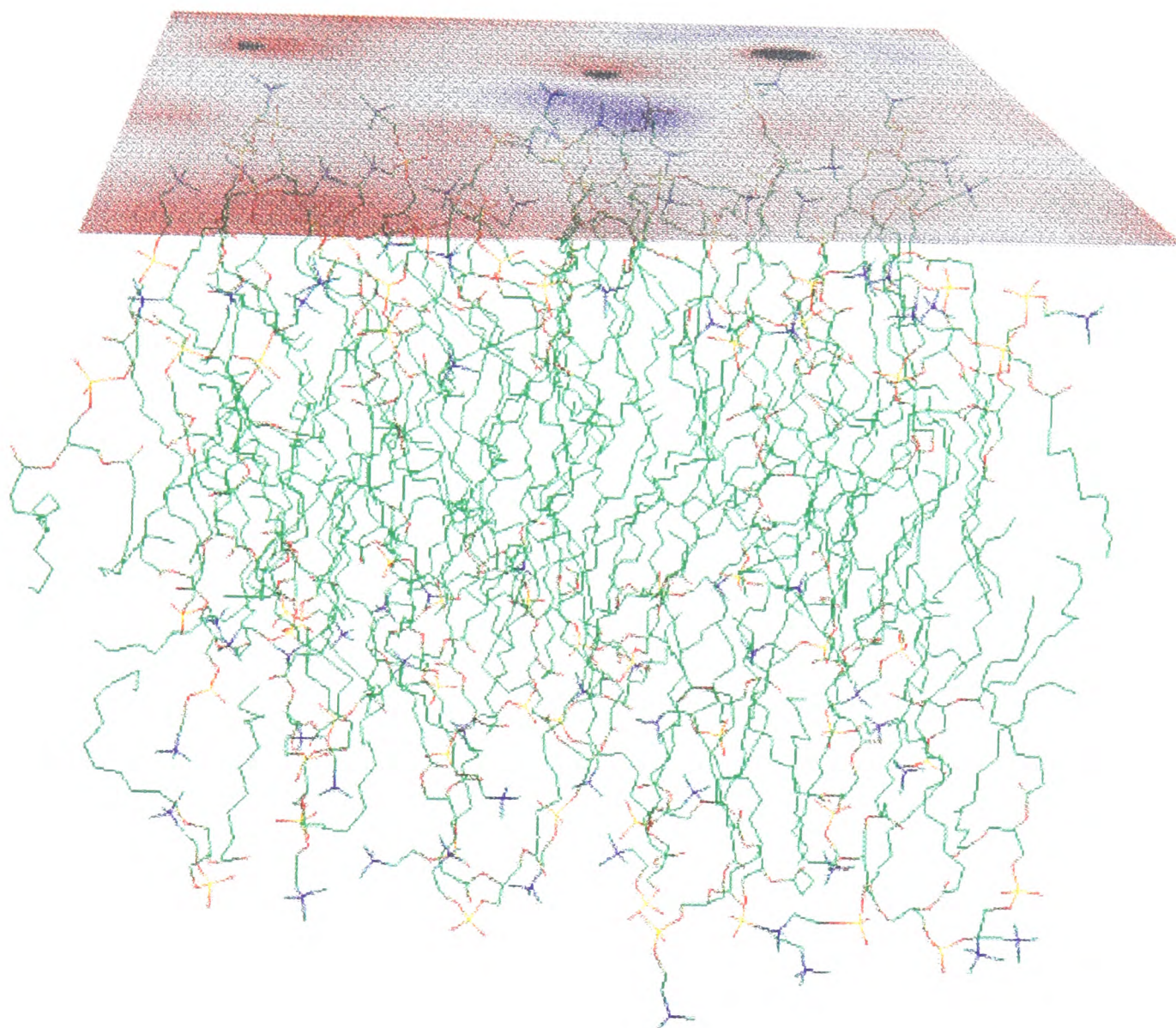


Figure 5.30 A snap shot from the molecular dynamic simulation with the electronic interaction energy map texture-mapped onto a plane above the membrane surface.

This model is consistent with the observations both by experiment and simulation that the phosphate and N-methyl parts of the choline head group tend to lie in the same plane so that both are exposed at the surface of the membrane. Furthermore, the patches in the maps arise from groupings of lipid molecules, not from individual molecules. The variation in the electrostatic interaction energy in the plane above the membrane surface illustrates how inhomogeneous it appears to a molecule diffusing over its surface.

To investigate how the electrostatic properties of the surface of the membrane change over time, electronic interaction maps were calculated every 25 ps at a distance of 25Å from the

bilayer centre and are displayed in Figure 5.31. From these figures it would appear that the usual lifetime of a charged region is of the order of 100ps.

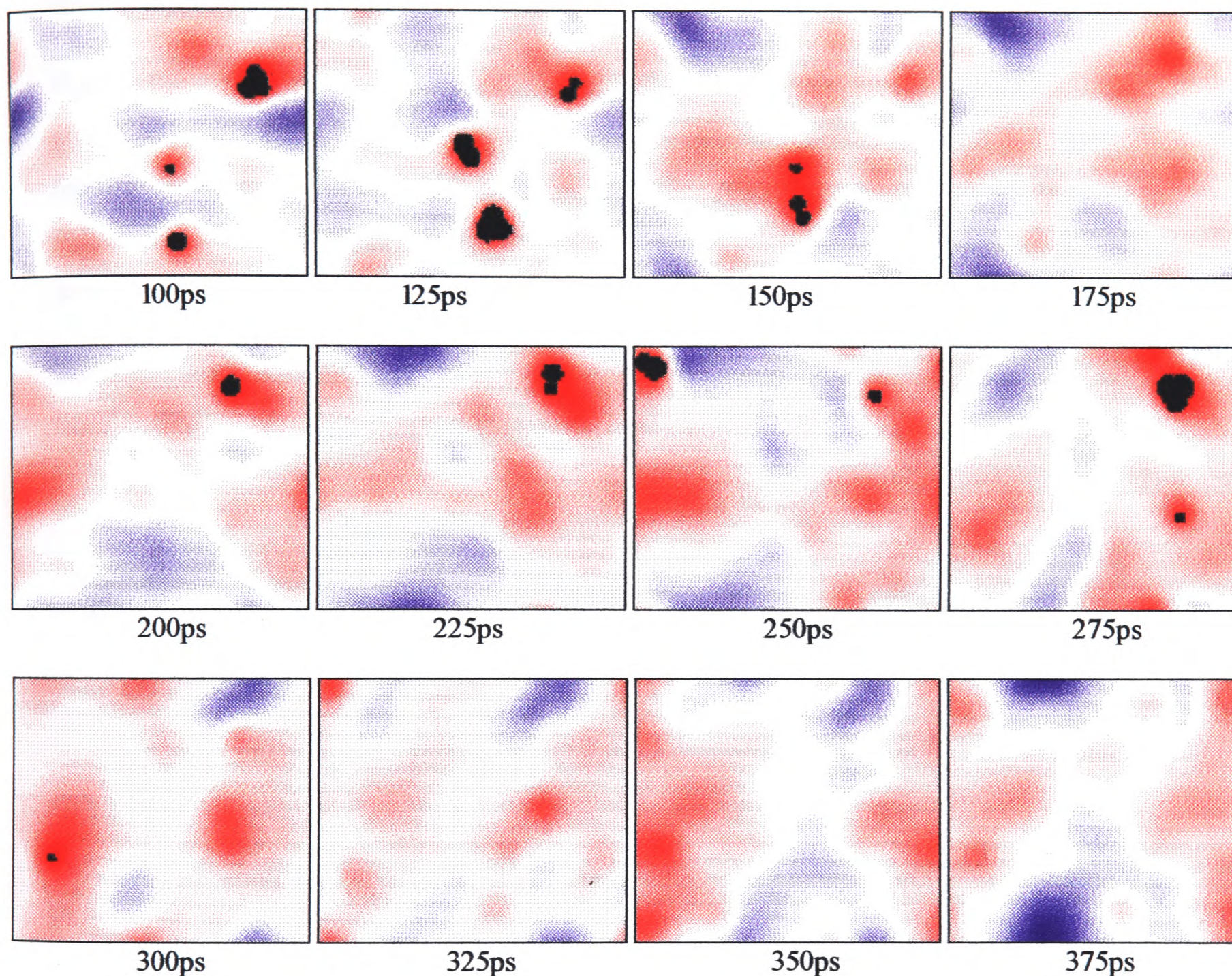


Figure 5.31 Snap shots of the electron density maps of the surface of the membrane taken every 25ps. Blue areas favour negatively charged solutes and red areas favour positively charged. The intensity of the colour is proportional to the strength of the interaction. Black areas signify regions in which the probe points have unacceptable van der Waals contacts with lipid atoms.

5.6.2 The Dihedral Angle Distribution in the Head Groups

An analysis was done of the average distribution of dihedral angles in the head group and in common with the previous analysis on the smaller system, it is observed that the most

probable angles for each dihedral angle are the same as the angles observed in the crystal structure (Pearson and Pascher, 1979) and proposed to occur in the fluid phase in early nmr experiments (Seelig, 1978). This is with the exception of the α_5 torsion which is predominantly trans rather than the gauche value reported experimentally (Akutsu, 1981).

Huang et al. (1994) also reported the failure to reproduce the gauche character of α_5 for the phosphatidylcholine (PC) head group, but did observe it occurring both in layers and single lipid simulations of phosphatidylethanolamine (PE). They ascribed this difference to unfavourable van der Waals contacts in the PC head group with its large methyl groups around the nitrogen compared to the much smaller hydrogens in PE.

The distributions were also calculated for the θ_1 and θ_3 angles of the glycerol moiety and are listed in Table 5.10 and illustrate the restricted rotation about this torsion.

Torsion Angle	Percentage Trans		Percentage Gauche ⁺		Percentage Gauche ⁻	
	Layer 1	Layer 2	Layer 1	Layer 2	Layer 1	Layer 2
α_1	79±5	75±6	11±4	9±4	11±3	16±6
α_2	18±5	18±5	42±3	39±4	41±5	43±5
α_3	20±4	22±6	34±4	32±4	46±5	46±6
α_4	58±6	59±5	23±5	21±5	19±4	20±8
α_5	78±7	80±6	11±4	9±4	11±4	11±7
θ_1	53±2	57±3	15±2	16±2	32±1	28±1
θ_3	62±2	64±3	38±2	37±3	0.0	0.0

Table 5.10 The distribution of dihedral angles in the head group and glycerol moiety torsions. The dihedral angle naming scheme is as illustrated in Figure 2.10.

The large range of head group angle exhibited will contribute to the roughness of the membrane surface which, as mentioned at the start of Section 5.6, may be an important

phenomena for creating transient defects and holes in the membrane surface that allow the rapid diffusion of water and other small molecules and ions.

5.7 The Interaction of Water with the Bilayer

The topics dealt with in this section involve the interaction of water with the membrane including:

- 1) The extent of penetration of water molecules into the bilayer
- 2) The hydration of lipid atoms
- 3) The variation on physical properties of water molecules as a function of distance from the lipid membrane
- 4) The strength of the association between water and lipid molecules and what effect this may have on the ability of solutes to penetrate the membrane.

Water permeation of bilayers is a subject vital to the understanding of general transport of small molecules into cells. Marrink and Berendsen (1994) have considered the problem of water transport through a membrane using a combination of free energy calculations and diffusion rate profiles. They found diffusion rate to be highest in the bilayer centre where the lipid density is lowest and concluded that the rate limiting step is the permeation through the dense part of the lipid tails where resistance is highest. From their simulations they proposed a new “four region” model which seems to be more realistic than the normal, simple “two-phase” solubility-diffusion model.

The normal model used to describe solubility and permeation in membranes is the homogeneous solubility-diffusion model in which permeation is considered to be a three stage process-

- 1) The molecule moves out of the aqueous phase and into the membrane
- 2) The permeant has to diffuse through the membrane interior

3) The permeant has to move back into the aqueous phase at the other side

Problems with this model include it treating the bilayer interior as a homogeneous phase, i.e. as resembling a liquid alkane which has been shown already is not the case.

A second diffusion model describes the diffusion of smaller permeants with a model in which the rate of diffusion is dependent on the free volume within the membrane and the mechanism involves a permeant hopping from one vacancy to the next as they occur (Walter and Gutknecht, 1986).

Diffusion has also been described as an inhomogeneous process with the head group region being non-Stokesian and the apolar part being a Stokesian fluid (Garrick et al. 1993).

Another model and one that can be directly studied by molecular mechanics simulations is that water diffusion occurs via defects in which a strand of water molecules extend into the bilayer interior with their presence in the hydrophobic interior stabilised by hydrogen bonds to each other.

5.7.1 The Extent of Water Penetration into the Lipid Bilayer

In Figure 5.7 the extent of water penetration into the bilayer is shown to be quite extensive with waters penetrating right into the hydrocarbon region although such behaviour is not expected on enthalpic grounds because of the loss in hydrogen bonding energy. As described in Section 4.4 water has been detected at the carbonyl groups in the bilayer from Raman experiments (Belchner et al., 1990) and radial distribution function of the two carbonyls are shown in Figure 5.32. By integrating these functions the average number of waters bound to each carbonyl was determined to be 0.63 for the Sn1 chain and 0.75 for the Sn2 chain in the first coordination sphere as defined by the first minimum in the radial distribution function. The higher value for the Sn2 chain no doubt reflects the closer proximity of this oxygen to the hydrophilic water/lipid interface as described in Section 5.4.1. Stouch (1993) reported that in their simulations of DMPC the carbonyl oxygen atoms are hydrogen bonded to waters for more than 50% of the simulation.

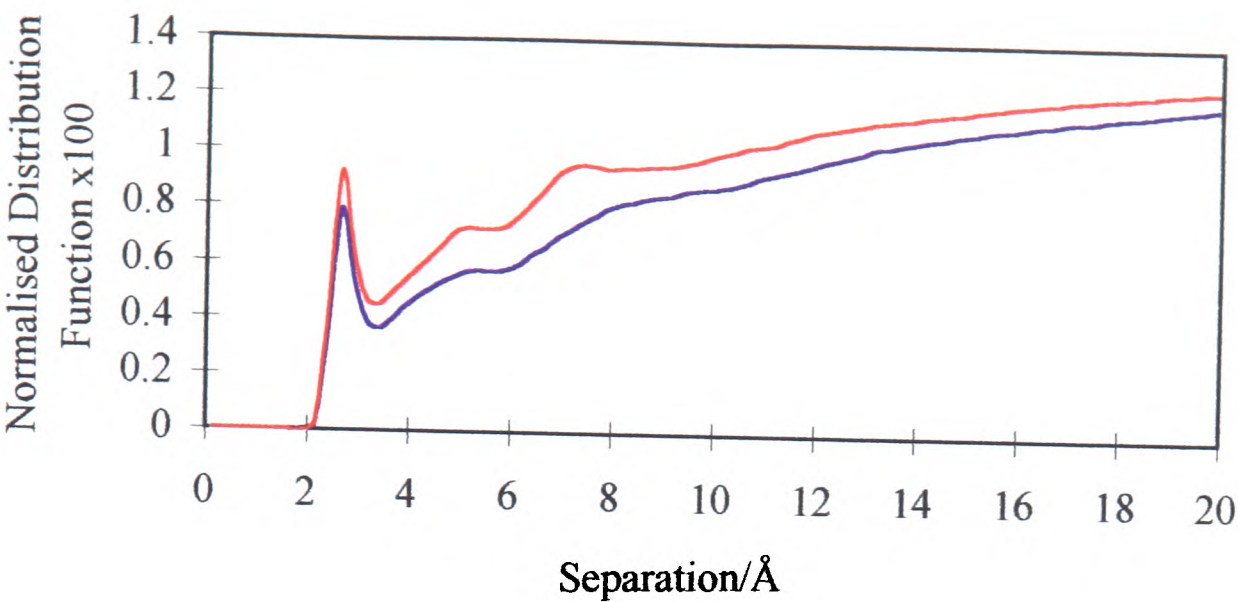


Figure 5.32 The radial distribution functions for the oxygen atoms of the Sn 1 (blue) and Sn2 (red) chain carbonyl groups with water.

Average radial distribution functions were calculated for all the atoms in a lipid molecule and the atoms that showed extensive hydration (i.e. those in the lipid head group) were used to define an idealised hydration radii of the first solvation spheres for a particular atom type. Those waters in the first solvation sphere will be more tightly associated with a lipid atom than those in the second and further coordination spheres and this idea was used to develop some hypotheses and models regarding the movement of solutes through the membrane, in particular the concept that these water molecules should be considered as part of the bilayer system rather than the solvent. In Figure 5.33a a lipid molecule is shown with the size of the first coordination sphere represented by a transparent blue sphere of the appropriate size which demonstrates how much larger the head group is than might be expected from a consideration of the lipid atoms alone.

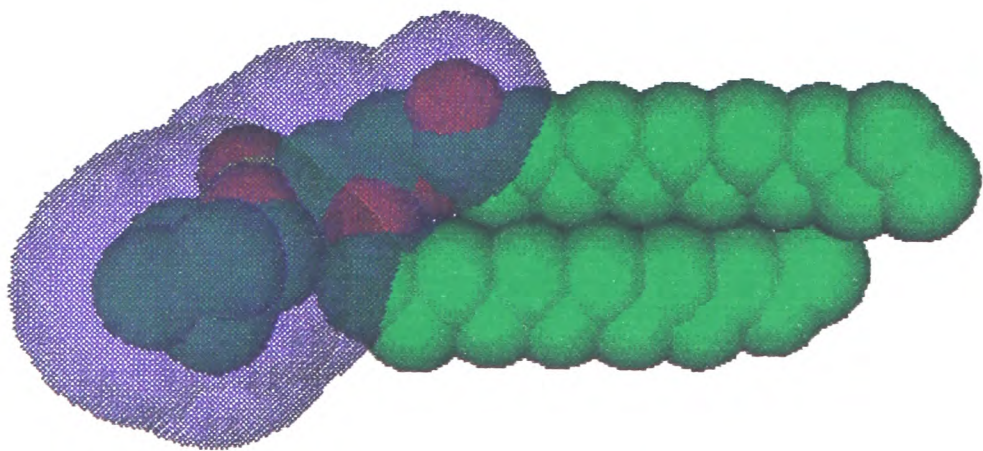


Figure 5.33a The first coordination spheres of atoms in the lipid head group represented as opaque spheres showing the extensive hydration.

It should be noted that simulation work done by Damodaran and Merz (1993) illustrates that there is a formation of a clathrate shell around the charged, but hydrophobic, choline group rather than a tight hydrogen bond network analogous to the one observed in DLPE.



Figure 5.33b A lipid molecule from the simulation with its first coordination sphere waters around it. Some of the water molecules are labelled according to the lipid atoms they are coordinated to. [A = N-methyl, B = phosphate and C = carbonyl oxygen].

By considering Figure 5.33a it is apparent that waters in the first coordination sphere of one lipid may also be within the first coordination sphere of a neighbouring lipid which would

differentiate the behaviour of that water even further from that of bulk water plus have a bearing on the movement of solutes through that region.

Figure 5.33b shows an actual lipid from the molecular dynamics simulation with the first coordination sphere waters shown around it. Those waters labelled A are coordinated to the N-methyl group, those labelled B are around the phosphate group and the water labelled C is hydrogen bonded to a carbonyl oxygen.

Figure 5.34 shows the results from a series of calculations in which the number of first coordination spheres water belonged to was determined as a function of depth in the membrane and reported as a percentage of the total number of waters at that depth in the membrane.

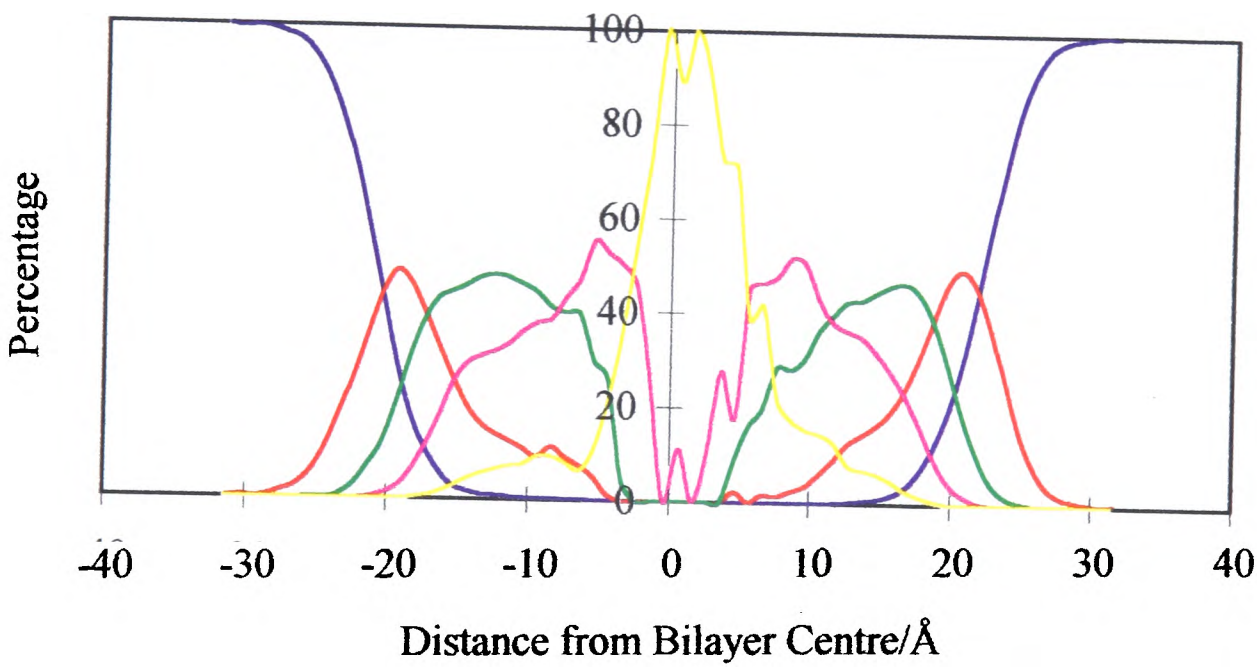


Figure 5.34 The variation in the number of first coordination spheres water molecules resides in as a function of depth in the membrane. [Blue=0, Red=1, Green=2, Magenta=3, Yellow=4 or more].

From Figure 5.34 it can be concluded that the deeper a water is in the membrane the greater the number of lipids with which it is in contact will be and in the head group region about half of the waters are bound to two lipids with the rest being associated with either one or three lipids. Only a small proportion of lipids are not in a first coordination sphere at all and this implies that virtually all the water in the head group region is directly associated with at

least one lipid molecule and these lipid molecules may form bridges between neighbouring lipid molecules. Such bridging waters can be clearly identified in the crystal structures of many lipids including DMPC and DLPE and in Figure 5.34b a snap shot from the molecular dynamic simulation is shown which illustrates a bridging water molecule between two phosphate groups. These bridging waters will serve to hold the head groups together and make it more difficult for solutes to pass through than might otherwise be expected.

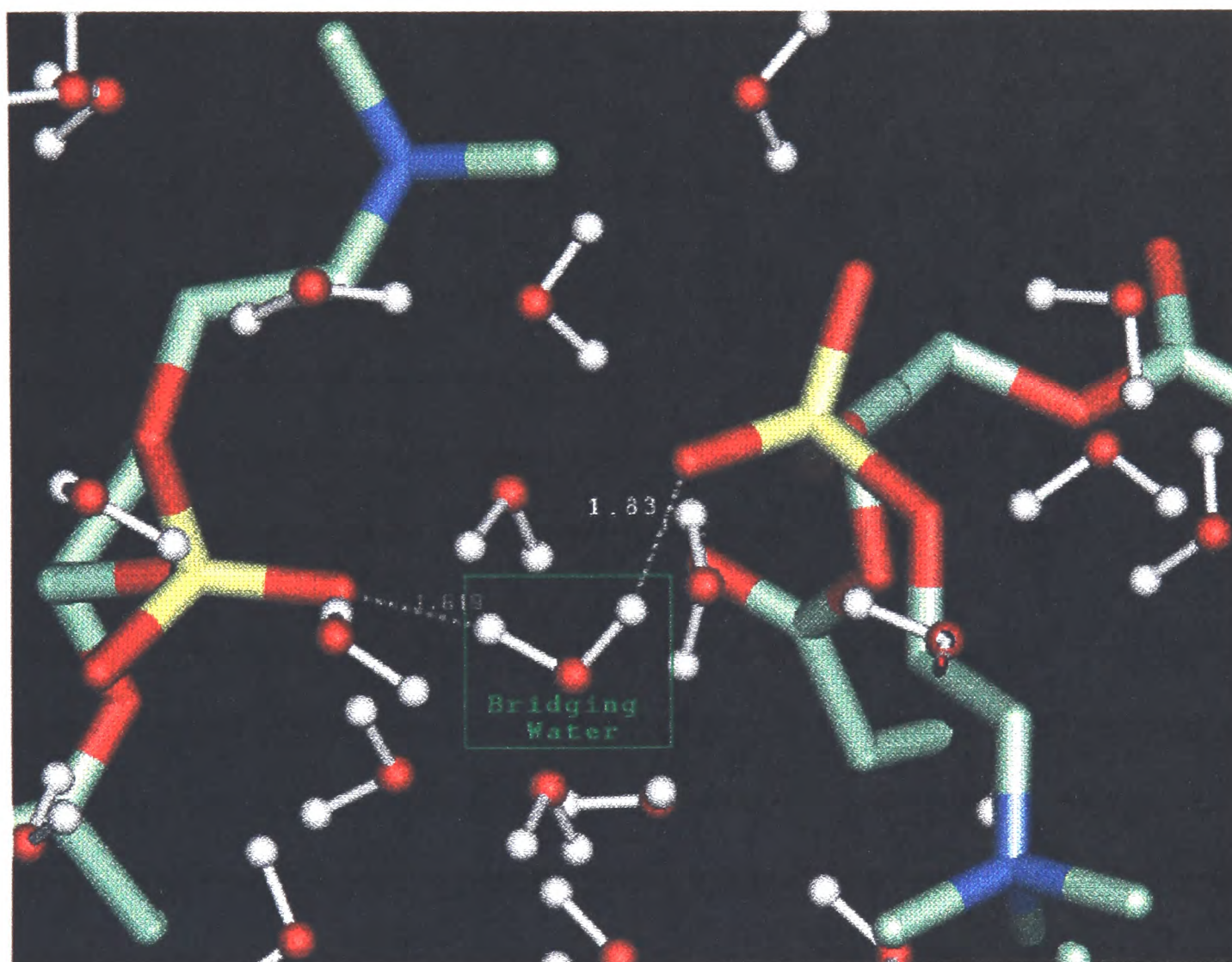


Figure 5.34b Two neighbouring lipids from the molecular dynamic simulation which are linked by a water molecule hydrogen bonded to each of their phosphate groups.

That virtually all the waters in the head group region are associated with at least one lipid has implications for the movement of solutes (including water) through it. For a solute to pass through the region it must either push past hydrated head groups, i.e. between the coordination shells of lipids, or exchange places with waters in these shells. A third mechanism would involve the formation of transient holes in the head group structure as lipid head groups move apart and are filled by water and other solutes which may then diffuse

further into the membrane (especially if they favour hydrophobic environments). Whether solute molecules will exchange positions with water molecules will depend upon how tightly bound these waters are to the lipid and this was investigated by calculating the average time that a water molecule spent in the first coordination sphere of an atom. This time is called the residence time and was calculated using the method described by Impey et al. (1983).

On a couple of occasions over the course of the simulation water molecules were observed to diffuse as far as the bilayer centre and as discussed above a number of cavities of sufficient size to accommodate water molecules do exist naturally at the bilayer centre and throughout the bilayer.

5.7.2 The Residence Times of Water Molecules around Lipid Atoms

To calculate the residence times of water around an atom a program was written that used the approach described by Impey et al., (1983) and determined if a water molecule was present in the first coordination sphere of an atom at a certain time, whether it would still be there a given time later without having left in the interim period. This distribution of the number of waters that remained still bound after a given length of time was averaged over all water molecules and all lipid atoms of the same type to give a function that decayed approximately exponentially.

Thus if $n_i(t=0)$ is the number of water molecules in the first coordination sphere at an initial time frame and $n_i(t)$ is the number of these same waters that remain in the shell after a time t , then $n_i(t) \sim n_i(t=0) \cdot \exp(-t/\tau_i)$ where τ_i is a correlation time which measures the persistence of waters in the first coordination sphere of a lipid atom i . (N.B. The number of water molecules around an atom at the initial time point is just its coordination number). By evaluating the gradient of a plot of $\ln(n_i(t))$ versus t , τ_i may be evaluated from the gradient of the line. The parameter τ_i was evaluated for the hydrated head group and glycerol atoms of the lipid and the results are listed in Table 5.11. N.B. The residence time of a water with another water is of the order of 2–4 ps thus the waters around a lipid atom are more tightly associated than they are to each other in the bulk solvent.

Extra notes on 5.7.3:

The literature value for the self-diffusion constant of TIP3P water at 298K is $0.24\text{\AA}^2/\text{ps}$ (Jorgensen et al., 1979). The values calculated here are rather larger than this and may arise from:

- a) The higher temperature at which the simulation was carried out (328K). At 323K the self-diffusion constant of water was reported to be around $0.5\text{\AA}^2/\text{ps}$ (Cuddeback, Koeller and Drickamer. 1953. J. Chem. Phys. 21: 589).
- b) The slightly lower water density of the system than is normal at 1 atmosphere because the simulation was carried out under constant volume conditions.

Future models will need to address this problem.

Atom Name	Residence Time/ps	Atom Name	Residence Time/ps	Atom Name	Residence Time/ps
O ₁₁	9.4	C ₁₃	8.1	O ₃₁	4.9
O ₁₂	5.7	C ₁₄	7.9	O ₃₂	8.7
O ₁₃	12.1	C ₁₅	8.0	O ₂₁	6.6
O ₁₄	12.1	C ₁	15.4	O ₂₂	8.9
C ₁₁	10.1	C ₂	11.9		
C ₁₂	8.6	C ₃	13.3		

Table 5.11 The residence times for selected lipid atoms (See Figure 2.5 for naming scheme).

The large residence times of waters around the hydrophobic choline group are consistent with the observed behaviour of water in clathrate structures where their reduced translational and rotational ability impedes the exchange of water between shells compared to polar solutes. The very large value for C₁ may be due to waters in this region of the membrane being involved in bridging between phosphates and generally trapped in the membrane. Further conclusions that may be drawn from these results will be discussed after other physical properties of the water molecules have been considered.

5.7.3 The Diffusive Behaviour of the Water Molecules

Marrink and Berendsen (1994) calculated free energy calculations and diffusion rate profiles of water and showed that both depend strongly on position within the membrane and that the diffusion rate is highest in the middle of the membrane where the lipid density is low. All recent analyses on simulated bilayer models have concluded that water is more restricted and less translationally mobile than bulk water.

To investigate the behaviour of the water molecules in the present system, the mean squared deviations of water molecules were calculated as a function of distance from the bilayer centre and from these a diffusion coefficient was calculated using the Einstein relation. In

Figure 5.35 the average mean squared deviation of the water molecules in one picosecond are shown as a function of distance from the bilayer centre.

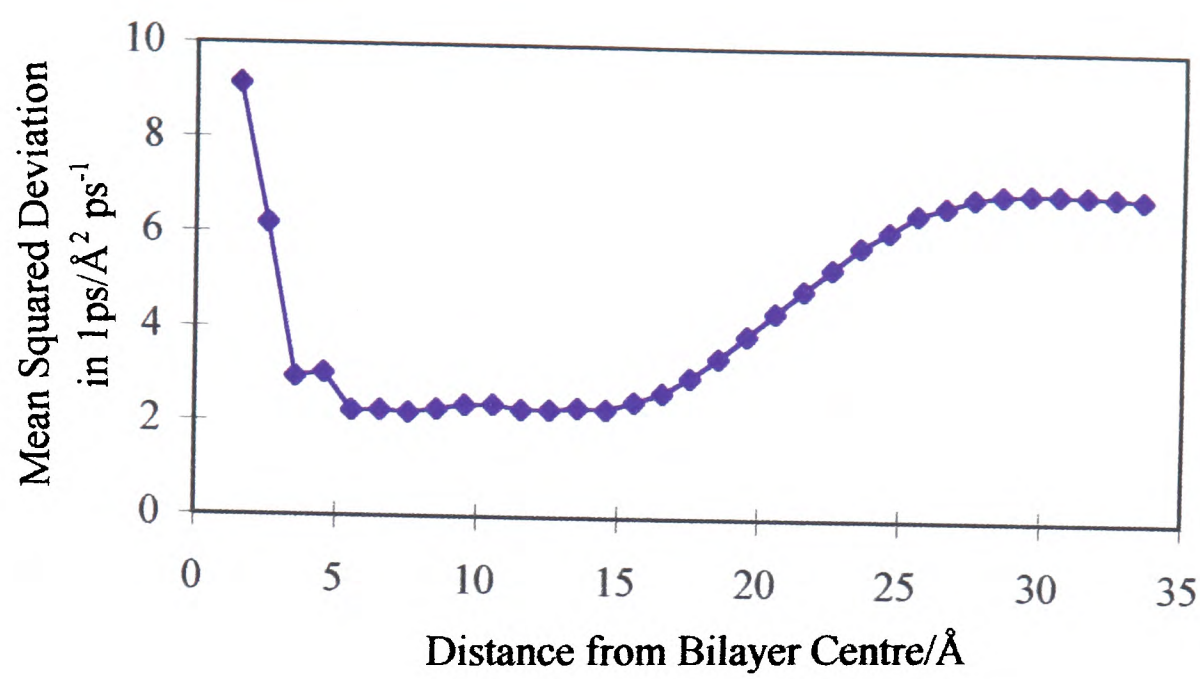


Figure 5.35 The average mean squared deviation of water molecules in one picosecond as a function of distance from the bilayer centre.

From Figure 5.35 it is interesting to note that within the hydrophobic core of the bilayer the mean-squared displacement of water molecules is approximately constant except at the bilayer centre where motion is observed to be quite unrestricted. The diffusion of water within the hydrophobic core is far less than that of free water (greater than 25Å from the bilayer centre) and traversing the head group region the mobility of water increases as the number of hydrogen bonds it forms to other groups increases which will limit its translational and rotational freedom. When the individual x, y and z components of the mean squared deviation in one picosecond were calculated for the water molecules as a function of depth within the membrane they were all observed to be approximately equal, i.e. on the one picosecond time scale the membrane appears isotropic to the water molecules which is consistent with fluorescent experiments on the diffusion of oxygen through membranes (Fishkoff and Vanderkooi, 1975).

Diffusion coefficients for the water molecules were calculated from the mean squared deviations using the Einstein relation over the time intervals 5-25ps and the results are shown in Figure 5.36.

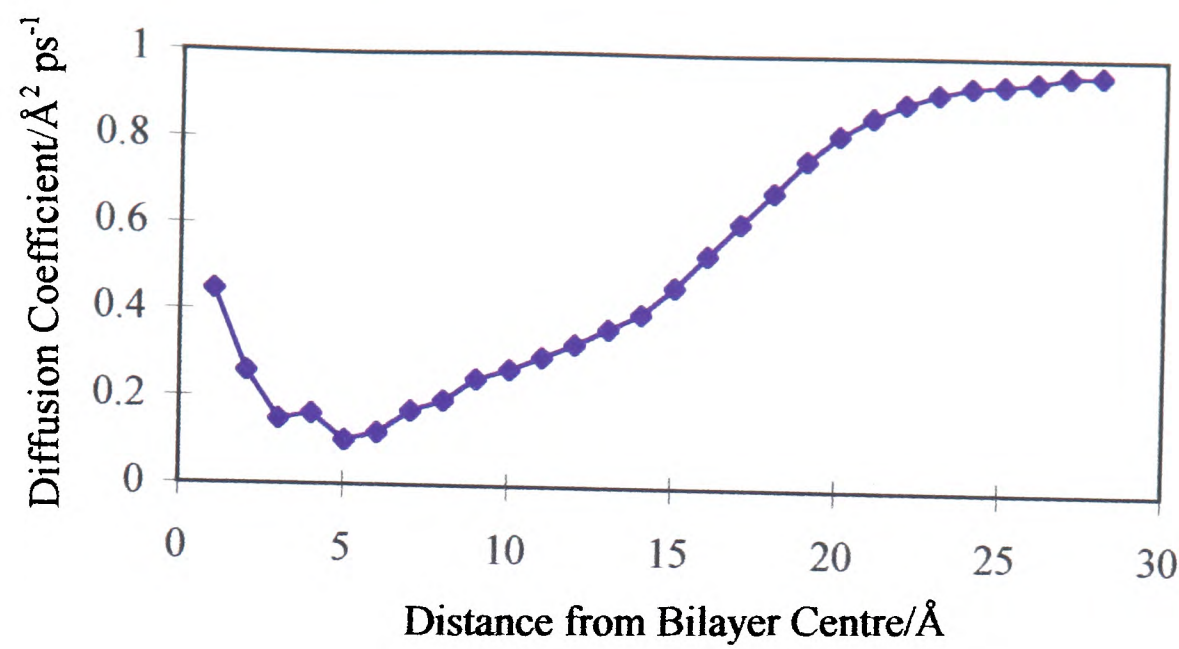


Figure 5.36 The diffusion coefficients of the water molecules as a function of their distance from the bilayer centre.

Marrink and Berendsen (1994) calculated the diffusion rate profile in the membrane interior from a force correlation method because there were few water molecules in this region during their shorter simulation. Their profile is similar in shape to Figure 5.36 but water diffusion at the bilayer centre is close to the expected value for bulk water whereas the rate in Figure 5.36 is about half of that of water in the bulk phase.

In common with the conclusions from the graph in Figure 5.35, the diffusion coefficients of water are quite large at the bilayer centre where it is known there is free volume available because of the lower lipid chain density and then increase as the water/lipid interface is approached. Between 5 and 15Å from the bilayer centre the line increases at a slower rate than between 15 and 20Å and this may reflect the different environments of the hydrocarbon region in the former case compared to the head group region of the latter case. That the diffusion of water in the bilayer is slower than in bulk water is probably related to their motion being restricted by the slow moving lipids (see Figure 5.19) which have diffusion coefficients of only 0.02Å²/ps. Within the head group region diffusion will be slowed down due to interaction with the charged atoms there, however the greater diffusion there than in the bilayer interior is probably due to the greater number of waters and fewer lipid atoms, i.e. the environment around a water in the hydrophilic head group region more closely resembles bulk water than does the hydrophobic core.

The diffusion coefficients were also calculated along the direction of the x, y and z axes as a function of distance from the bilayer centre and the results are shown in Figure 5.37.

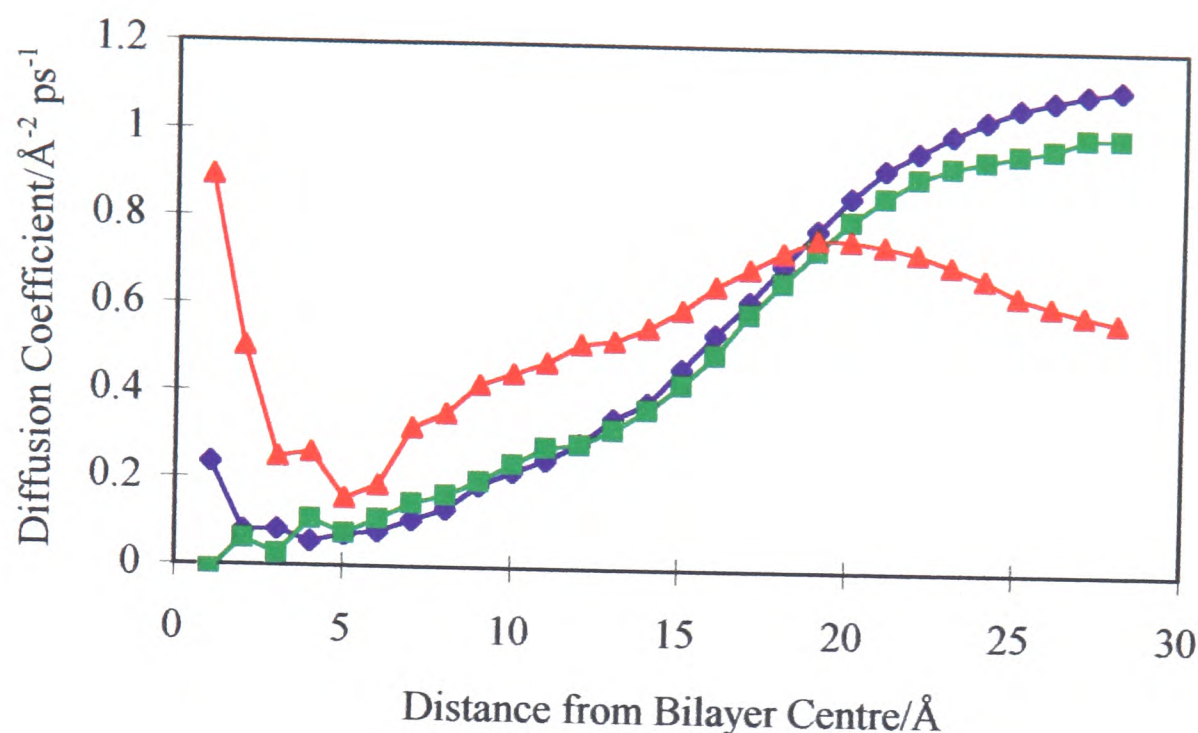


Figure 5.37 The diffusion coefficients calculated from the mean squared deviation along each of the three axes, x (blue), y (green) and z (red).

The diffusion coefficients of water in the x and y directions are approximately equal exactly as expected, but diffusion in the z direction shows interesting behaviour with it being larger than diffusion in the xy plane within the hydrophobic region. This may reflect the elongated shape of the cavities within this region as shown from the free-volume calculations in Section 5.4.9. Since the diffusion coefficients of water within the membrane in the xy plane are larger than those of the lipid (see Figure 5.19), it appears that water can diffuse in the plane of the membrane by moving between chains although at a slower rate than lateral diffusion along the z-axis. Once outside the head group region (13-20Å from the bilayer centre) then diffusion along the z direction is smaller than in the xy plane and is probably as a result of the strong electrostatic interactions preventing the water molecules from diffusing freely away from the bilayer surface but allowing relatively free diffusion along it. The phenomenon may be exacerbated by periodic boundary violations which mean the waters are effectively located between two membrane surfaces and Alper et al. (1993) have proposed that the influence of a bilayer surface extends 30-35Å into the solvating water phase and requires a

nonbonded cut-off of the order of 30Å to model correctly the water dynamics. Unfortunately such a cut-off is prohibitively large for most simulations of interesting systems.

5.7.4 The Rotational Behaviour of Water

To understand further the physical behaviour of water molecules in a membrane environment, the autocorrelation time for rotation of water molecules was calculated by defining a unit vector along the dipole moment of water and then fitting a simple exponential equation of the form $C(t) = C(0) \exp(-t/\tau_{rot})$, where τ_{rot} is the rotational correlation time and the correlation between two water molecules after a time t , was the result of the dot product of the unit vectors defined for the water with the results averaged over all water molecules. The rotational correlation times are shown as a function of distance from the bilayer centre in Figure 5.38.

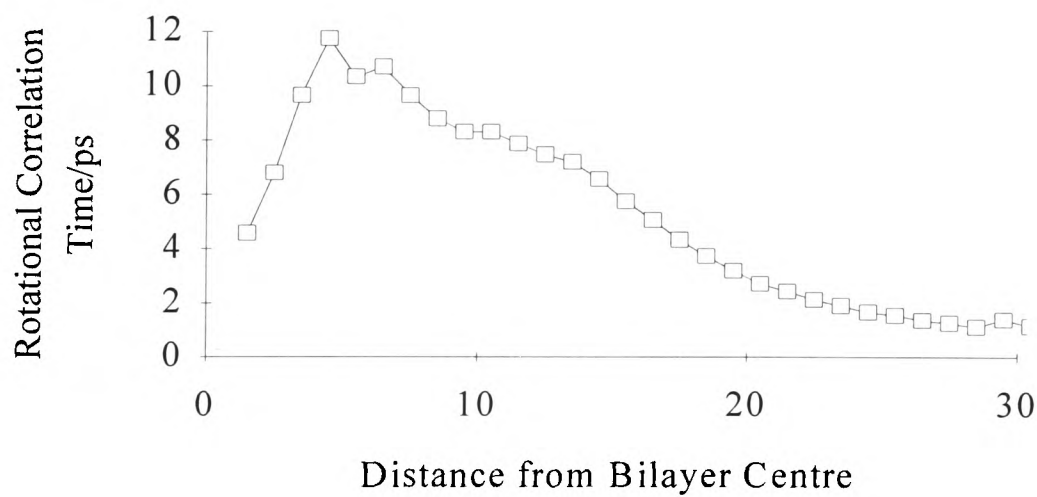


Figure 5.38 The calculated rotational correlation times as a function of distance from the bilayer interface.

As the surface of the membrane is approached from the bilayer centre then the rotational correlation time of the water molecules is seen to decrease from quite restrictive motion toward the value expected for bulk TIP3P water of 3ps (Jorgenssen et al., 1979). That the rotational correlation time of bulk water in the simulation is

lower (less than 2ps) probably reflects the lower water density in the system as was observed for the self diffusion constants in Section 5.7.3. The restrictive motion in the bilayer interior of the water molecules almost certainly arises from the ordered lipid chains that surround them.

5.7.5 Coordination of Water within the Bilayer

The translational and rotational properties of the water molecules will depend to a large extent upon the type and number of atoms that they are associated with. To evaluate the local environment of a water molecule within the bilayer a series of calculations were performed in which pair distribution functions were determined as a function of distance from the bilayer centre between firstly water molecules only and between secondly water molecules and both water and lipid atoms. From the pair distribution function for bulk water it was decided that any atom within 3.6Å would be considered to be associated with a water molecule and hence from the pair distribution functions the number of neighbours around a water molecule at a particular depth in the membrane was determined.

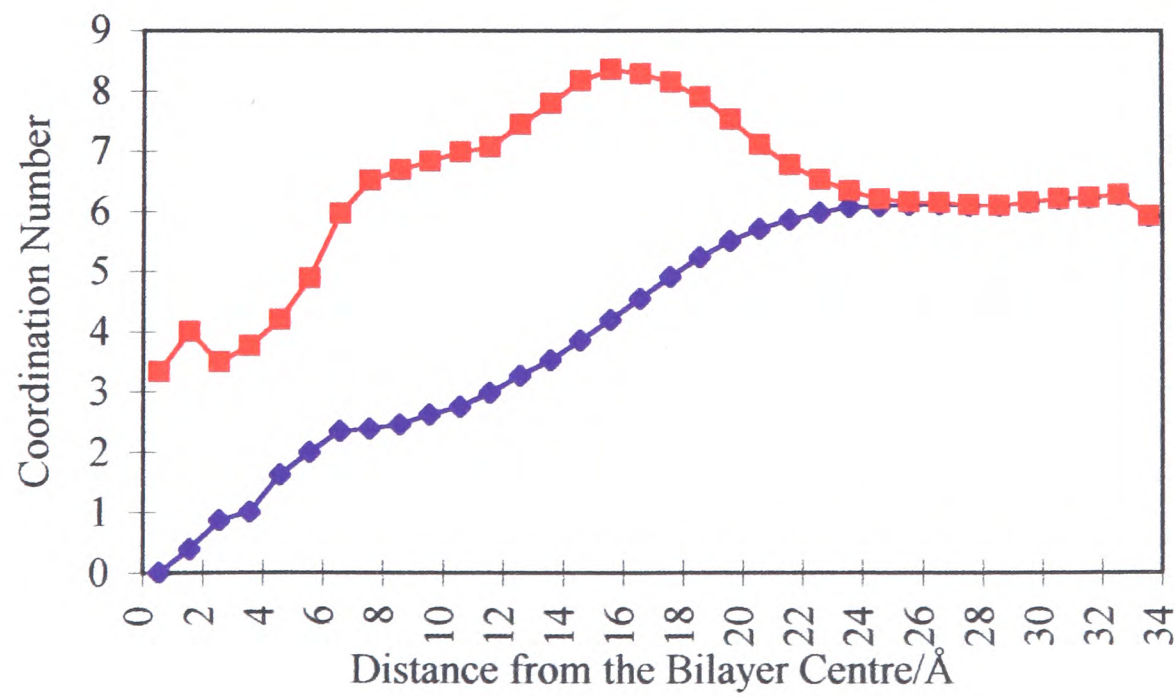


Figure 5.49 The coordination numbers of water molecules as a function of distance from the bilayer for a) water with water (blue line) b) water with both water and lipid (red line). An atom is considered to be coordinated if it is within 3.6Å.

The results of these calculations which show the number of neighbours around a water molecule are shown in Figure 5.49 and two important points may be drawn from the figure above:

- 1) It is apparent that very few waters occur singly except at the very centre of the bilayer and the number of waters around a water molecule increases as the bilayer surface is approached.
- 2) The number of neighbours a water molecule has in the head group region is higher than in bulk water.

The first observation is consistent with the defect model of water permeation in which a strand of water molecules enter the hydrophobic environment (see introduction to Section 5.7) and has also been identified by Marrink and Berendsen (1994) and supports other work in which it was shown that the propensity for water to form hydrogen bonds increased as the density of water decreased (Marrink, Berkowitz and Berendsen, 1993).

This second observation implies that (provided the actual interaction between neighbours is as strong as between water with itself and given that the head groups are fairly stationary) the water molecules within the head group region are essentially trapped in a matrix and this explains the observation that water in the head group region shows such a long rotational correlation time. Essentially the water in the head group region is an important structural component of the membrane and must be included in any model of a bilayer system. Since the water density is lower in the head region, the water molecules have to form hydrogen bonds to suitable lipid atoms rather than to each other.

5.7.6 The Orientation of Water Molecules

To determine the effect of the membrane on the average orientation of water molecules around it, the average cosine of the angle that the dipole vector of the water molecule made with the bilayer normal was calculated and is shown in Figure 5.50. The values in the range 0 to 5Å from the membrane centre must be treated with caution since the statistics will be very poor because few water molecules are found in this region. Water orientation profiles were also calculated by Chui et al. (1995) and showed similar behaviour except that it appeared

they had not enough water above the membrane to reach bulk conditions (i.e. the cosine of the angle did not show a smooth decay to zero at large distance from the membrane) and behaviour in the interior of the membrane was less well characterised. The water orientational profile calculation by Marrink et al. (1993) above DPPC again showed the orientation of water such that the hydrogens pointed toward the membrane and that this is to compensate for the dipole moment of the choline head groups but waters within the bilayer were not considered.

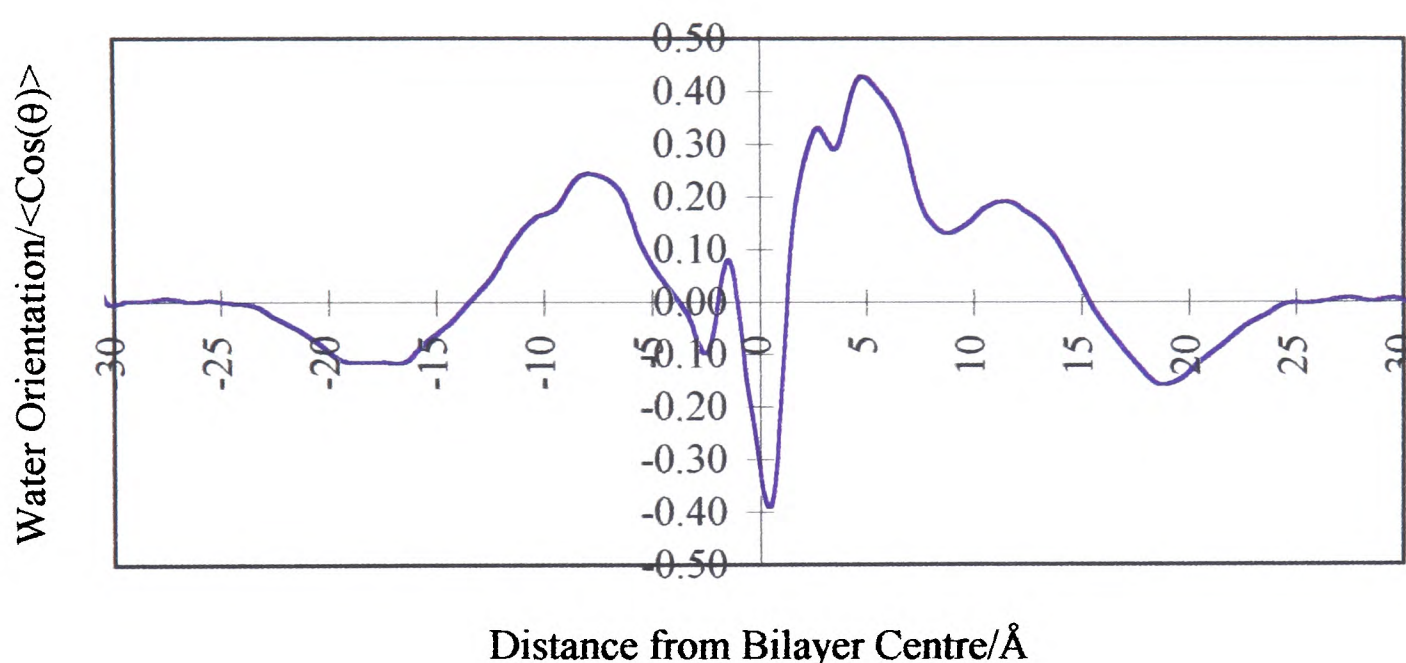


Figure 5.50 The average cosine of the angle that the water dipole vector makes with the bilayer normal as a function of distance from the membrane across the bilayer system.

It is quite obvious from Figure 5.50 that the membrane has an ordering effect on the water molecules and that the ordering on the inside of the membrane is of the opposite direction to the exterior. Above the surface of the membrane the waters show a preference to orient themselves so that their dipole is pointing toward the surface (i.e. the hydrogens are closer than the oxygen to the surface) while inside the membrane the reverse is true.

It is useful to compare this profile with the density distribution of groups in Figure 5.7 and Table 5.1 and draw some of the following conclusions. The orientation of the water above the membrane surface is consistent with the dipole moment of the head group (as defined by the nitrogen and phosphorous atoms) pointing away from the surface and the greater ability of the oxygens of the phosphate group to undergo hydrogen bonding by virtue of their larger

charge compared to the methyl groups at the positively charged choline end. The change in orientation of the water molecules at around 15Å from the bilayer centre is at about the same distance as the maxima in the head group distribution, so the reversal of the direction of the water dipole vector is from the phosphate groups now being on average closer to the surface than the water. A second contributing effect arises from the carbonyl groups of the lipid which will serve to orient the waters in the manner observed, i.e. the dipole vector of waters in the dipole centre is directed toward the surface. Gawrisch et al., (1992) have shown that the experimentally determined dipole moment of membranes arises from the orientation of waters in and above the surface.

As a final point, the ordering of water above a membrane surface has been proposed as the origin of the short ranged repulsive force between bilayer surfaces (Marcelja and Radic, 1976) commonly called the “hydration force” (Cevc and Marsh, 1987) and certainly if two DMPC bilayer surfaces were brought together it would result in a disruption of the preferred water orientation above the membrane surface and hence be energetically unfavourable. Berkowitz and Raghaven (1991) performed simulations on the gel phase of DLPE and observed that the orientational polarisation of the water molecules showed an oscillatory behaviour and hypothesised that the hydration force measured experimentally was the work required to break the network of hydrogen bonds in the water. Damodaran and Merz (1993) also give a good description and model for the origin of the hydration force based on simulations of DMPC and DLPE based bilayers.

5.7.7 The Variation in Charge Density across the Bilayer

When the charge density was calculated across the system using the OPLS charges then it became quite obvious that the lipid and water charge densities approximately mirrored each other leading to cancellation of charge across the system (Figure 5.51). Exactly the same behaviour was noted by Marrink et al. (1993). Thus it would seem that the water molecules are oriented because of the charged lipid atoms around them.

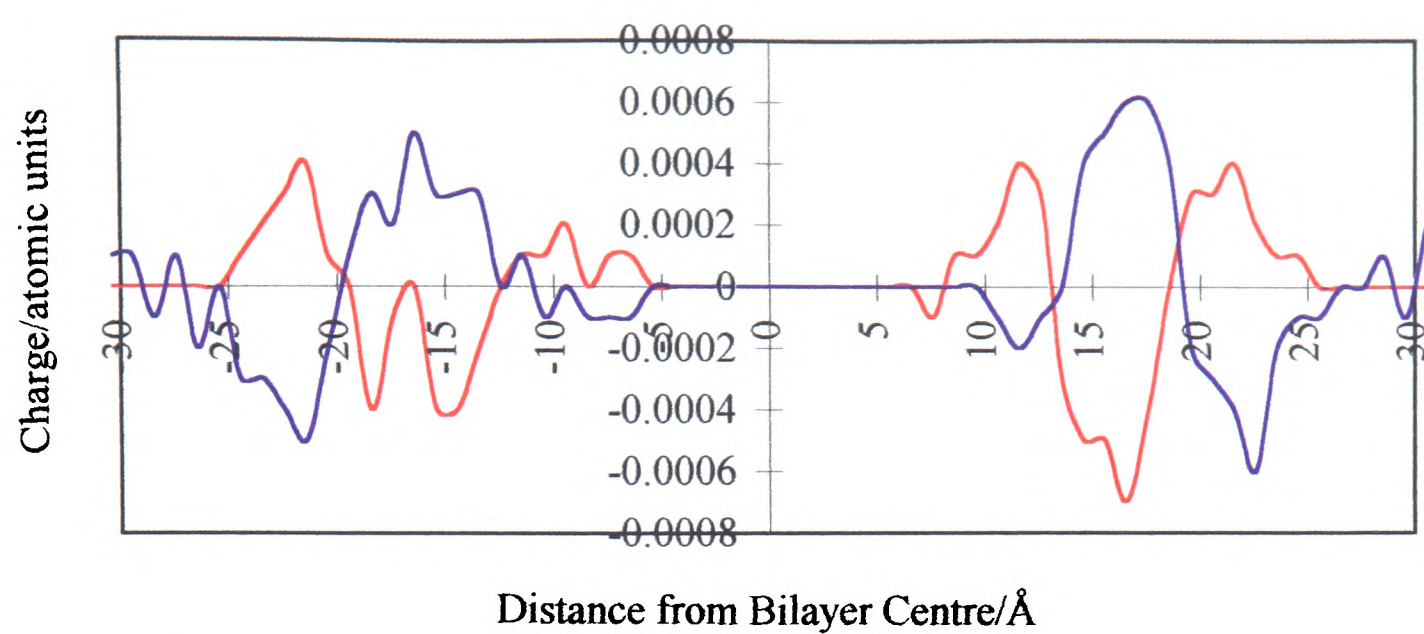


Figure 5.51 The charge density distribution across the system of the lipid atoms (red) and water atoms (blue).

The profile in Figure 5.51 can be used to describe the electronic environment of a permeant as it moves through the bilayer. Firstly it encounters a positively charged region that must correspond to the greater density of choline groups in this region followed by a swing to a negatively charged region as it encounters the phosphate groups. This behaviour is quite expected given that the P-N dipole is directed away from the bilayer (but not perpendicular to it). After this the electron density once again becomes positive. Why this is so is less clear, but may be due to the orientation of the carbonyl groups toward the bilayer surface and the corresponding dipole of the C=O bond directing itself to compensate for the dipole of the head groups. However it is not significant enough to effect the water orientation profile shown in Figure 5.50.

Comparing Figures 5.50 and 5.51 it would seem that ordering of the water occurs because of the phosphate groups (the negatively charged region of Figure 5.51) and the carbonyl region at the edge of the apolar region. It would appear that the cholines have little effect on the polarisation of the water which is consistent with them having a clathrate water structure (Damodaran and Merz, 1993).

5.7.8 Conclusions about Water and Solute Transport within a Bilayer

Marrink and Berendsen (1994) identified three types of water in their membrane: water molecules in a hydration cell which were either strongly or loosely bound and interstitial water trapped between the bilayers but not in a hydration cell and summarised their hypothesis of permeation process of water in membranes as follows:

“It is essentially determined by the free energy barrier that results from the breakage of interwater hydrogen bonds and the loss of electrostatic interaction. The real permeation process starts from the edges of strands of water molecules that already penetrate the membrane quite deeply. After having gained enough energy from random collisions to escape from these strands, the permeation is then limited by diffusion across the highly viscous part of the membrane. The permeation across the middle part is relatively easy.”

The scenario that emerges from the analysis presented here is that the water molecules around the head groups must be considered in any model of diffusion through the bilayer. Many waters within the head group region are associated with more than one lipid molecule and serve as bridging waters that hold head groups together. Such a matrix of waters and head groups will serve to make the entry of permeants into the bilayer more difficult since then they must either exchange places with associated waters, or the bilayer surface must become disrupted through the formation of defects that allow essentially unbound waters and solutes close to the apolar region. Given the rough nature of the membrane surface, it would appear that the latter situation is the easiest route for permeant entry. To an approaching permeant the bilayer surface will appear as a slightly positively charged environment since although the phosphate and choline groups are in similar planes and there is much charge cancellation, the positively charged cholines do extend further out from the membrane surface. Once the solute is closer to the membrane then the surface appears quite inhomogeneous with positive and negative regions exposed and it will be able to diffuse over the membrane surface until it finds a suitable binding site or defect into which it can enter.

Once inside the apolar region then the permeant can make its way across the membrane interface. Its progress across the bilayer will be aided by the orientation of the chains along the bilayer normal and the elliptical shape of the free volume within the interior. Movement

across the apolar region is slow, but the simulations do not include an electric field or concentration gradient that may accelerate permeant diffusion across the boundary and the effect of such contributions is a suitable topic for further study which has so far not been considered. The concentration gradient could be modelled by an extra pressure being exerted on one side of the system to mimic the osmotic pressure and the effect of this on water permeation and bilayer structure monitored.

5.8 Conclusions about the Large Scale Pure Bilayer Simulations

In this chapter, the development of a membrane model has been described and its behaviour during molecular dynamics simulation have been analysed extensively and shown to be consistent with experimental measurements on bilayers. Furthermore, since the model provides information at the atomic level about the motion of the system, the nature and dynamics of chain packing and the behaviour of the water around the head groups can be characterised and ideas proposed on the mechanism of passive solute and water permeation of the membrane.

The full atomic model is a good starting point for the future development of a force field that could mimic the physical properties of the bilayer for large scale biomolecular simulations such as trans membrane proteins. However in the subsequent chapters, explicit models of membranes are used since it appears that the direct interaction between lipid molecules and proteins may be important in the functioning of such proteins. Thus a suitable model may involve a force field that models the physical properties bilayer with the explicit inclusion of an annular ring of lipid molecules around the membrane solute, the dynamics of which are restricted so that they conform to normal behaviour as measured from previous molecular dynamic simulations of explicit membrane models.

Chapter Six

Simulations of Cholesterol in Lipid Bilayers

6.1 Introduction

[The work in this chapter appeared in *The Biophysical Journal* (Robinson et al., 1995)]

There have been fewer molecular dynamics simulations of bilayers with solutes incorporated than of pure bilayers, but this situation is changing rapidly with simulations including both smaller molecules and more ambitious protein structures. (Edholm and Johansson, 1987; Edholm and Nyberg, 1992; Bassolino-Klimas et al., 1993, Huang and Loew, 1995; Huang, Bertaccini and Loew, 1995; Woolf and Roux, 1996).

Cholesterol is an important solute to study because it is believed to be essential for the correct functioning of biological membranes and its physical effect on lipid bilayers has been studied extensively. It is also an uncomplicated molecule composed of a rigid ring system, a flexible hydrocarbon chain tail and a β -hydroxyl group. Its interaction and effect on lipid molecules should arise predominantly from physical non-covalent interactions and it appears not to complex strongly with phospholipids since ^{13}C experiments show the rate of cholesterol rotation to be faster than phospholipid rotation (Yeagle, 1980).

Cholesterol has a large and important effect on biological membranes that leads to a reduced permeability of the membrane, an increase in molecular order, condensation of the lipid molecules and a small increase in membrane thickness (for a review see Yeagle, (1985) or Bloom et al., (1991)). This is believed to arise from the rigidity of the steroid ring system causing trans-gauche isomerisations in surrounding lipid chains to become less probable (Stockton and Smith, 1976; Oldfield et al., 1978) and an increase in the fraction of trans dihedrals in lipid

chains around the rigid ring system which may lead to a small increase in bilayer width (McIntosh, 1978). However since the average number of trans dihedrals in a phospholipid does not increase (Pink et al., 1981; Bush et al., 1980), it follows that the lower part of the lipid hydrocarbon chain must then have an increased number of gauche conformations. Thus the torsional and translational motion of a lipid is reduced and consequently there is an increase in lipid molecular order (Lindblom, Johansson and Arvidson, 1981; Cornell and Keniry, 1983).

Cholesterol appears to cause little change in properties at the centre of the bilayer (Oldfield et al., 1978; Oldfield, Chapman and Derbyshire, 1971) and it has been proposed that the tail of cholesterol is much more conformationally flexible than its ring system. It is also proposed that the tail of cholesterol shows less motional freedom than the hydrocarbon chains around it (Dufourc et al., 1984; Opella et al., 1976; Kroon et al., 1975).

Monte Carlo simulation techniques have been used to calculate the effect of cholesterol on molecular order parameter profiles and showed a reduction in the ability of lipid chains adjacent to cholesterol to undergo torsional isomerisation (Scott and Kalaskar, 1989; Scott, 1991; Scott and McCullough, 1993). Edholm and Nyberg (1992) conducted a molecular dynamics simulation of cholesterol molecules in a model membrane which included neither head groups nor water molecules but provided important insights into the effect of cholesterol on lipid chain dynamics and conformation. They concluded that the effect of a cholesterol molecule extended for a radius of 1.25nm, leading to greater order as measured by molecular order parameters as well as increases in the fraction of trans bonds. These two effects were more pronounced in the upper and middle parts of the chain than at the terminus. An increase in bilayer thickness around the cholesterol was also reported.

In this chapter, the results of an analysis of 400ps of molecular dynamics from a computer simulation of four cholesterol molecules in a bilayer of thirty-six DMPC lipid molecule are described as is how cholesterol effects the lipid head groups and hydrocarbon chains and the behaviour of the cholesterol itself. This system is equivalent to a cholesterol concentration of about 11% cholesterol per mole lipid.

6.2 Initial System Configuration and Molecular Dynamics Protocol

The simulation package AMBER 3.1 (Singh et al., 1988) was used with nonbonded parameters based on the OPLS parameters of Jorgensen and co-workers (1988). The other parameters are from the standard AMBER force field apart from those parameters for the lipid ester groups taken from the work of Charifson and co-workers (1990).

The system was run under the same conditions as earlier successful bilayer simulations (see Chapter 4 and Robinson et al., (1994)) with a residue based cut-off of 9Å and the nonbonded pair list updated every 50fs. In the dihedral energy term, the van der Waals contribution was divided by eight and the electrostatic energy by two as recommended when using AMBER united atoms with OPLS parameters (Jorgensen et al., 1988). The system temperature was kept at 323K using the Berendsen algorithm (Berendsen et al., 1984) with a relaxation time of 100fs. To maximise the time step the SHAKE algorithm was used (Ryckaert et al., 1977).

The lipid molecules have the same conformations as the earlier small scale pure bilayer simulations (see Chapter 4) and coordinates for the cholesterol molecule were taken from the crystal structure (Pascher and Sundell, 1982) with parameters from the standard AMBER force field.

Model building was done with the aid of the QUANTA program (Molecular Simulations Inc, 1992) and involved constructing a bilayer from two monolayers as described in Chapter 4 and then replacing two lipid molecules in each layer with two cholesterol molecules. Cholesterol causes a condensation of lipid layers (Reiber, 1978) and the head group surface area of a lipid at the cholesterol concentration modelled should be approximately $62\text{\AA}^2$ (McIntosh, 1978) rather than the value of $66\text{\AA}^2$ observed for a pure lipid bilayer. Thus a pure lipid monolayer of twenty lipids was built such that when two lipid molecules were replaced by two cholesterol molecules, it had a total surface area equivalent to eighteen lipids with surface area of $62\text{\AA}^2$ plus two cholesterol molecules with a surface area of $38\text{\AA}^2$. The cholesterols were placed in the lipid lattice so that under periodic boundary conditions they were in equivalent sites and Figure 6.1 is a schematic view of the surface of the monolayer illustrating the positioning of the two cholesterols and Figure 6.2 shows the unhydrated starting structure. This bilayer was then

hydrated with TIP3P waters (Jorgensen et al., 1983) that penetrated down to the carbonyl oxygens of the lipids.

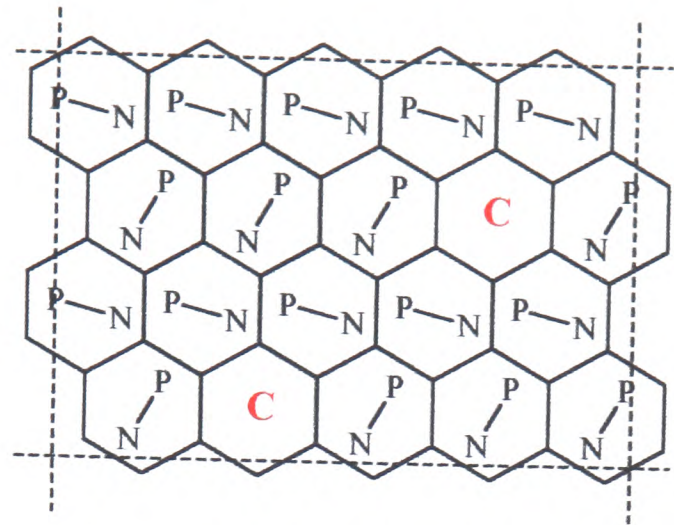


Figure 6.1 Schematic diagram of the surface of the monolayer showing the placing of the cholesterol molecules (C) with respect to the hexagonally packed lipids. Dashed lines mark the edges of the periodic box.

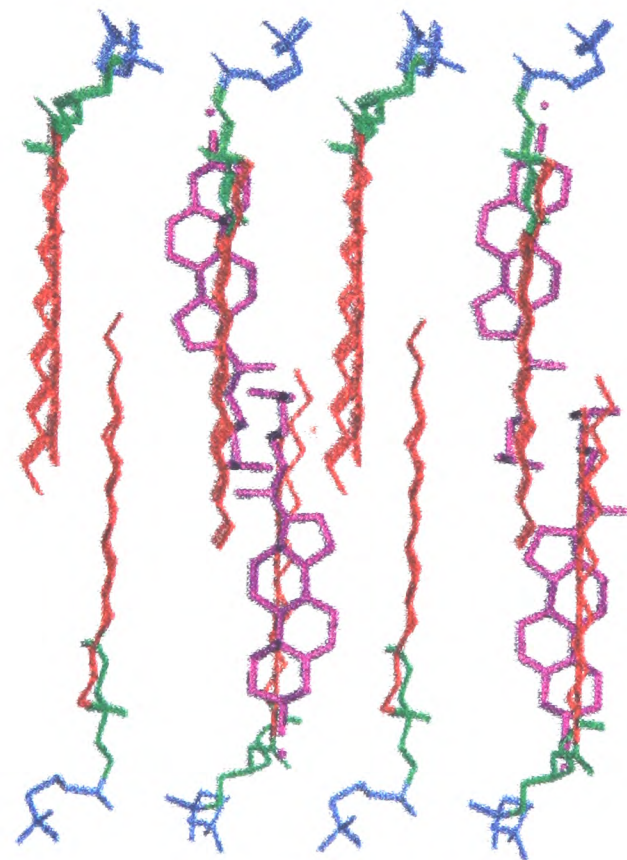


Figure 6.2 Side view of the unhydrated lipid bilayer structure with cholesterols in purple. Hydrocarbon chains are shown in red, glycerol in green and the head groups in blue.

Within the bilayer starting structure the closest contact between cholesterol atoms in opposite halves of the bilayer was 13Å while within a layer their closest contact was 23Å.

Although all four cholesterol molecules have their long axis parallel to the bilayer normal, in the starting configuration they were rotated about this axis to give different orientations of the cholesterol with respect to the surrounding lipids and to introduce some variation between sites. All cholesterol molecules were placed so that their hydroxyl group was in the same plane and oriented toward a carbonyl oxygen of lipid ester groups as suggested by ^{13}C nmr (Huang, 1976) and shown by X-ray and neutron diffraction (Franks, 1976; Worcester and Franks, 1976). Evidence that the cholesterol is this deep in the membrane comes from ^{31}P nmr that shows cholesterol is not present in the head group region (Yeagle et al., 1975).

Because of the ordered nature of the starting structure, great care was taken in the equilibration stage. Energy refinement and 20ps of molecular dynamics was done on the waters to equilibrate their positions around the lipid. Then the lipid molecules were energy minimised before both the waters and lipids together were energy refined followed by a further 20ps of molecular dynamics simulation on the water molecules alone. Finally, molecular dynamics on the hydrocarbon chains were run to introduce disorder into the chains and effectively melt the region. The chains in the present system showed as much disorder after 70ps as did those of the pure lipid bilayer after nearly 200ps of molecular dynamics (Robinson et al., 1994), i.e. the crystal-like starting structure broke down more quickly in the presence of cholesterol despite a reduction in volume per lipid. With the chains melted, 150ps of molecular dynamics were run with the waters, lipid molecules and hydrocarbon tail of the cholesterol free to move, but with the position of the cholesterol molecules held fixed and all bond angles and torsions of the cholesterol ring system held rigid. In the next 100ps the cholesterol molecules were free to diffuse but their ring systems were still held rigid by constraints. Finally all atoms were allowed to move freely with an equilibration sequence of 100ps before the production run of 400ps on which analysis was done. In conclusion, equilibration involved 350ps of molecular dynamics simulation on the lipid molecules before the production run of 400ps. A snapshot from the simulation, without waters, 200ps into the production run is shown in Figure 6.3.

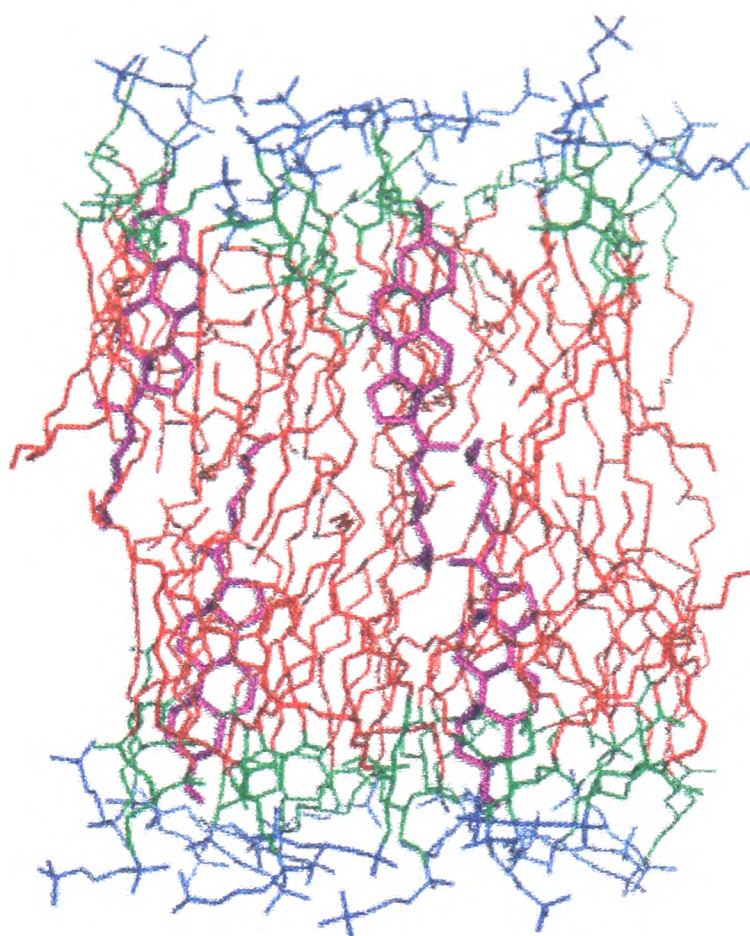


Figure 6.3 Side view of the unhydrated lipid bilayer structure 200ps into the production simulation. The cholesterol molecules are shown in purple, the hydrocarbon chains in red, the glycerol moieties in green and the head groups in blue.

6.3 Results of the Molecular Dynamics Simulation

Analysis of the behaviour of the cholesterol and lipid molecules during this final 400ps was accomplished using a program originally written by Essex (1992). Lipids were divided into those that had extensive contact with a cholesterol and those with little contact. Lipids with extensive contact with cholesterol were those that were within 5Å of a cholesterol for greater than 80% of the simulation time, whereas a lipid was classed as having little contact if it spent less than 30% of the production simulation in contact with a cholesterol. Cholesterol behaviour was determined in terms of the tilt of the long axis of cholesterol with respect to the bilayer normal, the conformations of the flexible hydrocarbon chain and the behaviour of the β -hydroxyl group.

6.4.1. Behaviour of the Cholesterol During the Simulation

From experiments by Dufourc et al. (1984) and Taylor, Akiyama and Smith (1981) the long axis of cholesterol is on average oriented perpendicular to the bilayer surface. However experiments by Oldfield et al. (1978) indicate that the most probable tilt of a cholesterol molecule is about 16° and that the cholesterol sweeps out a cone leading to the observed time-averaged tilt of 0° . The angle between the bilayer normal and a vector connecting the hydroxyl hydrogen with the final methylene of the tail of the cholesterols was evaluated from the simulation and the distribution is shown in Figure 6.4. Over the production simulation the cholesterol molecules show a tilt with the most probable tilt angle being 14° and 90% possessing a tilt of less than 24° . This is very close to the value reported by Oldfield and co-workers and as shown later, is less than the tilt measured in the surrounding lipid chains during the simulation.

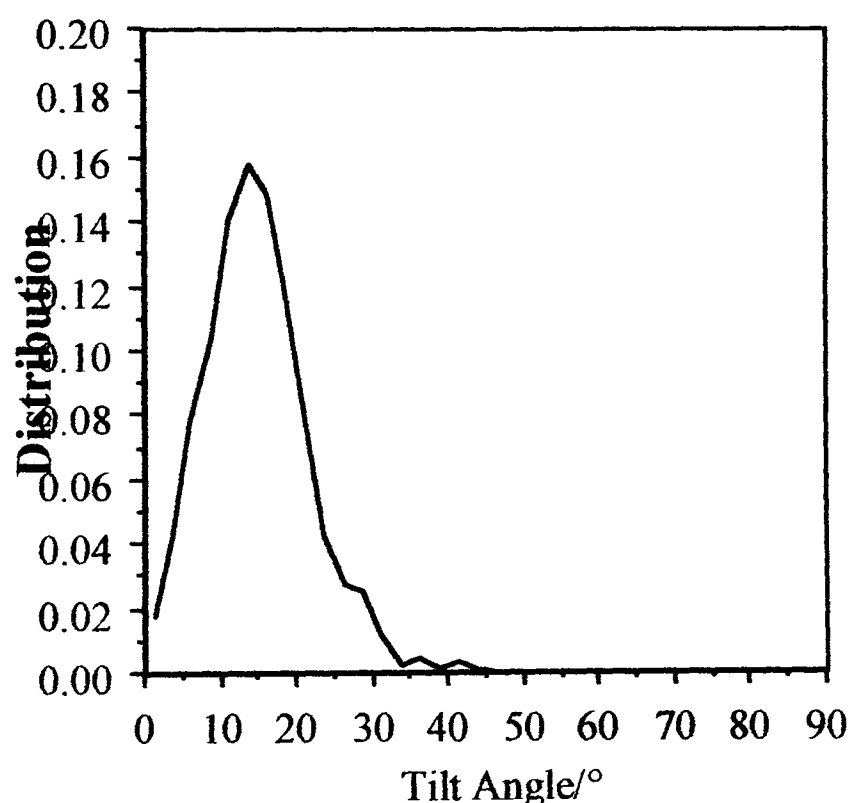


Figure 6.4 The tilt angle distribution of the cholesterol molecule with respect to the bilayer normal.

The conformation of the cholesterol chain is defined by five dihedral torsions, τ_1 to τ_5 , as illustrated in Figure 6.5 and in the crystal structure of cholesterol the tail is seen to exhibit six conformations (Duax, Griffin and Rohrer, 1980).

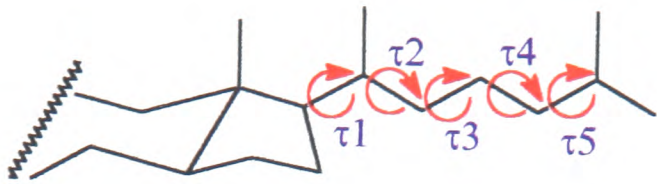


Figure 6.5 Diagram of the flexible hydrocarbon chain of cholesterol defining the dihedrals τ_1 to τ_5 .

An analysis of the populations of cholesterol tail conformations during the 400ps molecular dynamics production simulation showed main tail conformations as defined by (τ_3, τ_4) with 72% possessing the (trans, trans) conformation, 23% being (trans, gauche⁺) and 3% exhibiting the (gauche⁺, trans) pattern. The τ_3 and τ_4 dihedrals were used to define the conformation of the tail since τ_1 and τ_2 are both at least 99% trans. These conformations are some of those observed in the crystal structure where the (trans, trans) conformation is observed to occur in 72% of the cholesterol molecules and both (trans, gauche⁺) and (gauche⁺, trans) in 8% of the population.

6.4.2 The Behaviour of the Lipid Molecules

The behaviour of the lipid can be described by a variety of parameters such as the tilt of the hydrocarbon chains defined as the average angle a vector connecting two atoms makes with the bilayer normal (see van der Ploeg and Berendsen, (1983)), the population of kink defects, the number of trans bonds in lipid chains or the distribution of trans bonds as a function of chain position. Where appropriate these properties were considered for the Sn1 and Sn2 chains separately since the two chains are not equivalent due to their different attachments to the glycerol backbone. Three vectors were used to define each hydrocarbon chain corresponding to the upper half (nearer the head group), the lower half (nearer the bilayer centre) and the whole of the chain.

It is believed that the diffusion of small molecules through a membrane is aided by gauche conformations that form kinks in the surrounding chains providing a cage in which the solute

resides (Yeagle, 1987). Since cholesterol is believed to reduce the probability of kink formation by suppressing gauche formation in lipid chains adjacent to the rigid steroid ring, its presence should lead to a decrease in permeability so the kink population in chains as well as the distribution of trans bonds as a function of chain position and the total trans dihedral population for those lipid chains adjacent to cholesterol compared to those further away for the 400ps of molecular dynamics production simulation was characterised. From the subsequent work to this on large scale pure lipid bilayers that was presented in Chapter 5, the reduction in kink population may be a secondary effect of cholesterol reducing the number of gauche conformations in the upper part of the chains and hence reducing the formation of defect structures that allow permeation and the amount of free-volume available for solute diffusion within the membrane.

To determine the effect of cholesterol on lipid chain kink population, the Sn1 and Sn2 chains were considered separately and divided into two halves. For lipids neighbouring cholesterol, the upper half will be in contact with the main rigid steroid ring whilst the lower part will mainly be in contact with the more flexible cholesterol tail. Table 1 lists the results for 1-200ps and 201-400ps.

It is observed that in the current model, the cholesterol has a larger effect on the Sn2 chains than the Sn1 chains. Those Sn2 chains distant from a cholesterol molecule show a larger population of kinks in the upper half of the chain than do those lipid chains in extensive contact with cholesterol molecules. For the lower half of the chains it is observed there is little difference between the two sets for the occurrence of kinks. Thus for the Sn2 chain it appears that during the course of the simulation the cholesterol causes a decrease in the population of kinks within the upper half of a lipid chain where a solute would enter the membrane and hence acts as a barrier to diffusion through the membrane. In the lower half of the lipid chain, the flexible tail of cholesterol has little effect on kink population.

Chain	Cholesterol associated with chain.	Simulation Time / ps	Population of kinks in whole chain.	Population of kinks in upper half of chain.	Population of kinks in lower half of chain.
Sn1	Yes	1-200	2.63%	1.89%	3.36%
Sn1	No	1-200	2.69%	1.76%	3.63%
Sn1	Yes	201-400	3.01%	2.57%	3.46%
Sn1	No	201-400	2.75%	2.58%	2.89%
Sn2	Yes	1-200	1.98%	1.44%	2.52%
Sn2	No	1-200	2.50%	3.01%	1.99%
Sn2	Yes	201-400	3.22%	2.28%	4.14%
Sn2	No	201-400	3.76%	3.25%	4.27%

Table 6.1 The fractional population of kinks in the Sn1 and Sn2 chains. Comparison of those chains closely associated with a cholesterol with those that are not.

With regard to the total population of trans dihedral angles in Sn1 and Sn2 chains, the difference between chains next to cholesterol and those further away is about 2-3%, and the fractional population is around 78% for the Sn1 chains and 77% for the Sn2 chains over the 400ps of the production simulation. This absence of a difference is the same result as reported by Pink, Green and Chapman, (1981) and Bush, Adams and Levin, (1980).

To investigate further the effect of cholesterol on the number of dihedral torsions in the Sn1 and Sn2 chains, the lipid chains were divided in two and the average fraction of trans torsions per dihedral for these two regions compared. The results for 1-200ps and 201-400ps are given in Table 6.2. The simulation results show the upper half of Sn1 chains associated with cholesterol have a much larger fraction of the dihedrals being trans than those that are not. Thus the rigid steroid ring favours dihedrals to be trans in lipid chains in contact with it. The lower half of the lipid chain which is in contact with the flexible cholesterol tail has a lower fraction of trans torsions. The Sn2 chain does not show this behaviour clearly.

Chain	Cholesterol associated with chain.	Simulation Time / ps	Fraction of trans torsions in upper half of chain.	Fraction of trans torsions in lower half of chain.
Sn1	Yes	1-200	82.4%	76.6%
Sn1	No	1-200	75.6%	79.1%
Sn1	Yes	201-400	82.8%	80.2%
Sn1	No	201-400	76.4%	77.7%
Sn2	Yes	1-200	76.8%	76.6%
Sn2	No	1-200	76.0%	78.4%
Sn2	Yes	201-400	78.8%	81.1%
Sn2	No	201-400	80.2%	71.6%

Table 6.2 The fractional population of trans torsions in the Sn1 and Sn2 chains. Comparison of those chains closely associated with a cholesterol with those that are not.

The effect of this reduction in the population of trans dihedral angles will be that chains can pack more efficiently which leads to a reduction in free-volume suitable for solute diffusion and it is probably not so easy for water molecules to permeate into the membrane because of the probable lack of defect structures.

The tilt of the lipid chains during the simulation was measured by calculating the angle a vector connecting two ends of a chain makes with the bilayer normal. Figure 6.6 are the plots for the Sn2 chain over 400ps and shows that chains not in contact with cholesterol have a wider distribution of tilt angles, i.e. the cholesterol may limit the extent a lipid chain may tilt. This may arise from the promotion of trans dihedrals in the upper part of the lipid chains next to cholesterol as single gauche dihedrals in a lipid chain cause large deflections from the bilayer normal (Cevc and Marsh, (1987)). In comparison with Figure 6.4, we see that the lipid chains show a larger tilt than the cholesterol. The non-zero tilt of the lipid chains is consistent with experimental evidence as out-lined previously (see Chapter 3 and Robinson et al., 1994).

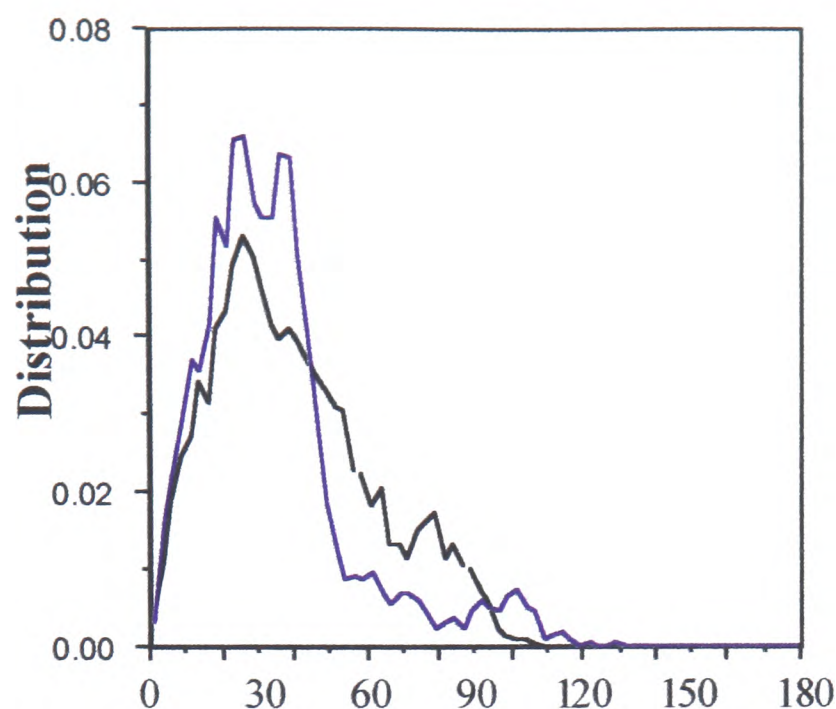


Figure 6.6 Comparison of the tilt angle distributions of the lipid Sn2 chains for those lipids associated with a cholesterol (blue line) with those that are not (black line).

Chain order parameters measure the extent of motional order of atoms in the hydrocarbon chains and may be calculated from molecular dynamics simulations as well as measured by ^2H nmr (Seelig and Seelig, 1974; Davis, 1983). Contributions to deuterium order parameters include inter- and intramolecular motions; isomerisation between trans and gauche isomers; the rate of trans-gauche isomerisation and the re-orientation of the lipid molecule as a whole (Mayer et al., 1988). An increase in molecular order is observed in experiments on DMPC bilayers to which cholesterol has been added (Stockton and Smith, 1976; Oldfield, Chapman and Derbyshire, 1971; Oldfield et al., 1978; Cornell and Keniry, 1983; Dufourc et al., 1984; Vist and Davis, 1990) and is interpreted as those lipid chains associated with the cholesterol having less freedom of motion and less ability to undergo trans-gauche isomerisations. As discussed previously (Robinson et al., 1994), the comparison of order parameter profiles from nmr which average over long time scales with those from the presently much shorter molecular dynamics simulations is not ideal.

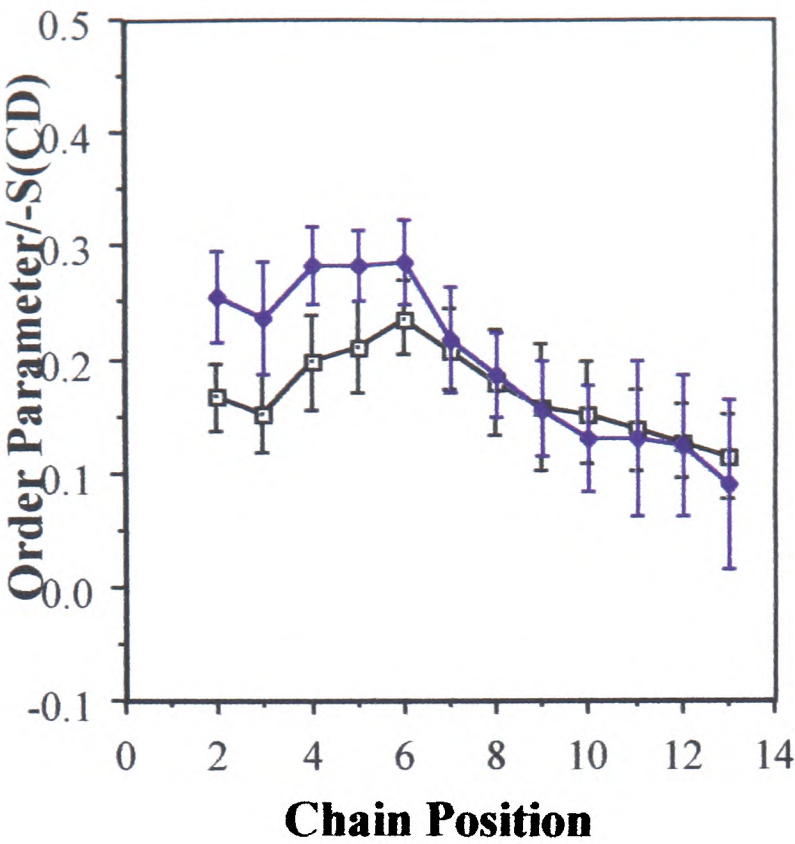


Figure 6.7 The deuterium order parameters of the Sn1 lipid chains for those lipids in contact with a cholesterol (blue line) compared with those that are not (black line).

In Figures 6.7 and 6.8 the deuterium chain order parameters calculated from 400ps of molecular dynamics are presented as a function of chain position for the Sn1 and Sn2 chains respectively with error bars being the standard deviation of the data. Whilst both chains show those chains nearer the cholesterol to have a larger order parameter, the Sn1 and Sn2 chains behave differently. For the Sn1 chain, the upper part of the lipid chain in contact with the rigid steroid ring is seen to have the greater motional order whilst for the Sn2 chain it is the lower half of the chain that has the greater order parameter. However, it should be noted that for the Sn2 chain the standard deviation is large for atoms in the upper part of the chain, thus it may be that the chains are close but not always interacting fully with the cholesterol molecule. The different behaviour of the Sn1 and Sn2 chains during the course of the simulation may also arise from the inequivalence of these two chains that leads to them being at different relative depths and the lower half of an Sn2 chain adjacent to a cholesterol molecule having restricted motion arising from the cholesterol tail not being able to pack well in the bilayer (Yeagle, 1985).

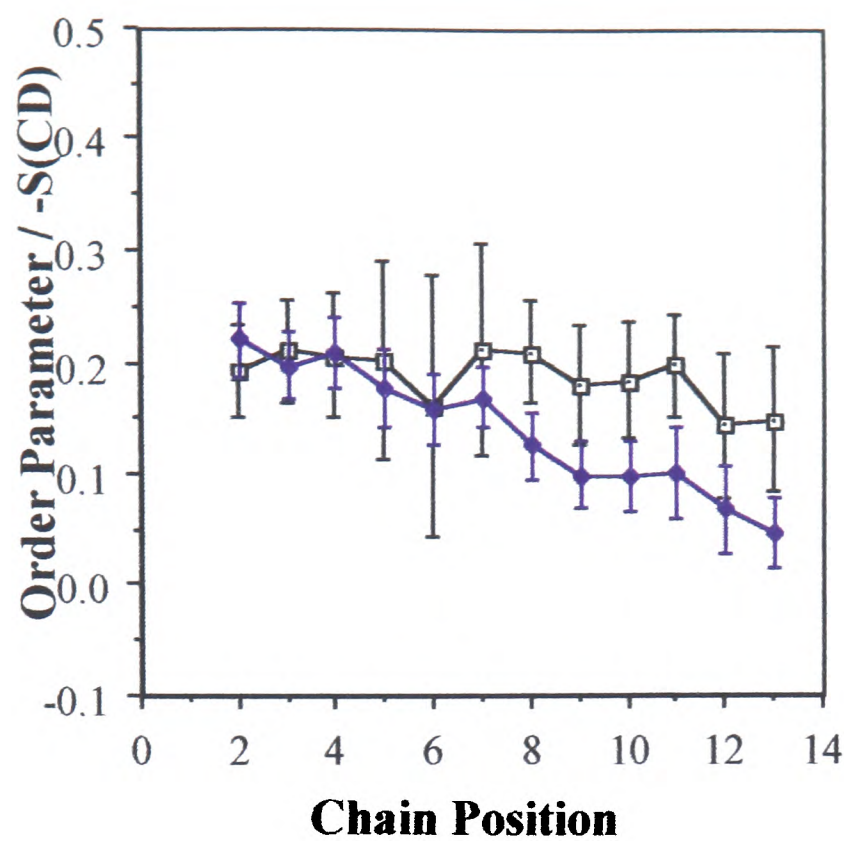


Figure 6.8 The deuterium order parameters of the Sn2 lipid chains for those lipids in contact with a cholesterol (black line) compared with those that are not (blue line).

The interaction of the β -hydroxyl group of cholesterol with the ester groups of lipids was discussed in the method section. Radial distribution functions were calculated to determine the interactions during the molecular dynamics production simulations of the β -hydroxyl group of cholesterol with the water molecules and with the lipid molecules. On average, a cholesterol hydroxyl was hydrogen-bonded to either 4 or 1.5 waters. The former corresponds to a hydrated cholesterol hydroxyl group and the latter to a cholesterol that has close contacts with a lipid molecule. In addition, radial distribution functions were calculated for the interaction of the cholesterol β -hydroxyl with phosphate and carbonyl groups and showed that hydrogen bonding does occur between the cholesterol and the oxygens of the phosphate in the head group and the glycerol carbonyls of the lipid. Thus the cholesterol hydroxyl can reside in two positions; deep in the membrane adjacent to the lipid carbonyls or nearer to the surface and interacting with the head groups or waters.

6.5 Conclusions on Cholesterol Simulations

The main physical effects of cholesterol have been successfully reproduced using molecular dynamics calculations on a fully hydrated, united atom model of a bilayer. These include an increase in motional order of those lipid atoms adjacent to the cholesterol as measured by the order parameter, an increase in the proportion of trans torsions in those dihedrals of the hydrocarbon chain next to the rigid steroid ring system and a decrease in the population of kinks in the upper part of lipid chains in contact with a cholesterol molecule. The cholesterol was also shown to have an effect on the tilt of hydrocarbon chains with respect to the bilayer normal with those chains adjacent to cholesterol not able to tilt as far as others. The increase in fraction of trans dihedrals and decrease in population of kinks in the upper part of the lipid chain will have an important effect on the ability of solutes to diffuse through the membrane and supports previous ideas on how cholesterol reduces the permeability of membranes.

The cholesterol is shown to be able to hydrogen bond to the oxygens of the phosphate in the head group, to carbonyl oxygens and to water molecules. This illustrates that cholesterol is relatively mobile within its site in the lipid bilayer and not associated strongly with the lipids.

In conclusion, the reproduction of observed experimental results provides the confidence to extend our model to other more complex systems and phenomena and the subsequent chapters consider the simulation of peptides and proteins in lipid bilayers.

Chapter Seven

Simulations of Lipid Bilayers Containing Simple Trans-Membrane Proteins

7.1 Introduction

Undoubtedly the most important components of biological membranes are the proteins which are responsible for and control the movement of both materials and information between the cell and its environment. It is very difficult to determine the structure and dynamics of proteins within membranes because of their fluid nature. No crystal structure of a protein within a membrane has been resolved to high resolution.

Preliminary work is being carried out by other groups on the nature of peptide-lipid interactions using molecular mechanics calculations (Edholm and Johansson, 1987; Woolf and Roux, 1994; Huang and Loew, 1995; Woolf and Roux, 1996) but the field is still extremely new and its development is linked intimately to advances in computer technology such as parallel and massively parallel computers plus the introduction of efficient parallelised versions of molecular mechanics programs. Attempts at modelling protein-lipid interactions have also been attempted using Monte Carlo techniques (Sperotto and Mouristen, 1991; Gersappe, Li and Blaza, 1993; Zhao and Reichert, 1994; Milik and Skolnick, 1993) usually by describing the bilayer environment with a hydropathy scale or simply as a hydrophobic region.

As a first exercise in exploring lipid-protein systems it was decided to start with the simplest systems possible, single alpha helices of alanines and alanines with glycine. Side chains introduce an extra order of complexity because of their interactions with the lipid chains and these will be treated in the next chapter where the toxin melittin is discussed. The models of this chapter are hypothetical because no experimental data could be found that related to these actual systems although experiments have been done on the model peptide Lys-Lys-

Gly-Leu_n-Lys-Lys-Ala-Amide (n=16 or 24) which have shown it to span the bilayer in an alpha helical conformation (Huschilt, Millman and Davis, 1989). The validity in simulating these peptides is because of the insights such simple systems can provide on the structure and dynamics of peptides in membranes that are not obtainable from conventional experiments. That the results and conclusions are relevant can be supported by the previous validation of the lipid bilayer model in Chapter 5 and the simplicity of the peptide coupled with the use of the trusted OPLS and AMBER parameter sets to describe it.

The peptides were given the alpha helical secondary structure since it is reported that polypeptides cannot integrate themselves into bilayers unless the C=O and NH groups are all hydrogen bonded and this condition can be satisfied intramolecularly with the alpha helix conformation (Engelman and Harvey, 1981; Jacobs and White, 1989).

The aim of the study was to investigate the effect of the peptide on the structure and dynamics of the lipid molecules around it and how being in a hydrophobic membrane environment altered the behaviour of the peptide compared to an aqueous environment. Thus simulations were run for peptides in both an aqueous environment and integrated into a membrane.

The two peptides studied were Ala₂₅ and Ala₁₁-Gly-Ala-Ala-Gly-Ala₁₁ which were chosen because the former is the simplest stable peptide that will span the bilayer membrane while the latter contains two glycines in a Gly-Ala-Ala-Gly pattern. The Gly-X-X-Gly pattern had been observed to occur in the centre portion of some trans-membrane proteins (e.g. glycophorin A) and since glycines introduce extra flexibility into alpha helices it was decided to test a hypothesis that such a sequence could act as a hinge at the centre of the bilayer where the two sides of the bilayer meet and may be preferred to a rigid structure in order to compensate for any differences in motion and tilt behaviour between each side of the bilayer.

7.2 Building the Starting Structures

The starting structures were constructed by taking a lipid bilayer structure as described and built in Chapter 5, removing a lipid molecule from each half of the bilayer and then inserting a peptide helix through the hole produced. The other lipids were then rearranged around the

peptide by hand to remove any close contacts and views of the starting structure are shown in Figures 7.1 and 7.2. For the Ala₁₁-Gly-Ala-Ala-Gly-Ala₁₁ system exactly the same modified lipid bilayer starting structure was used.

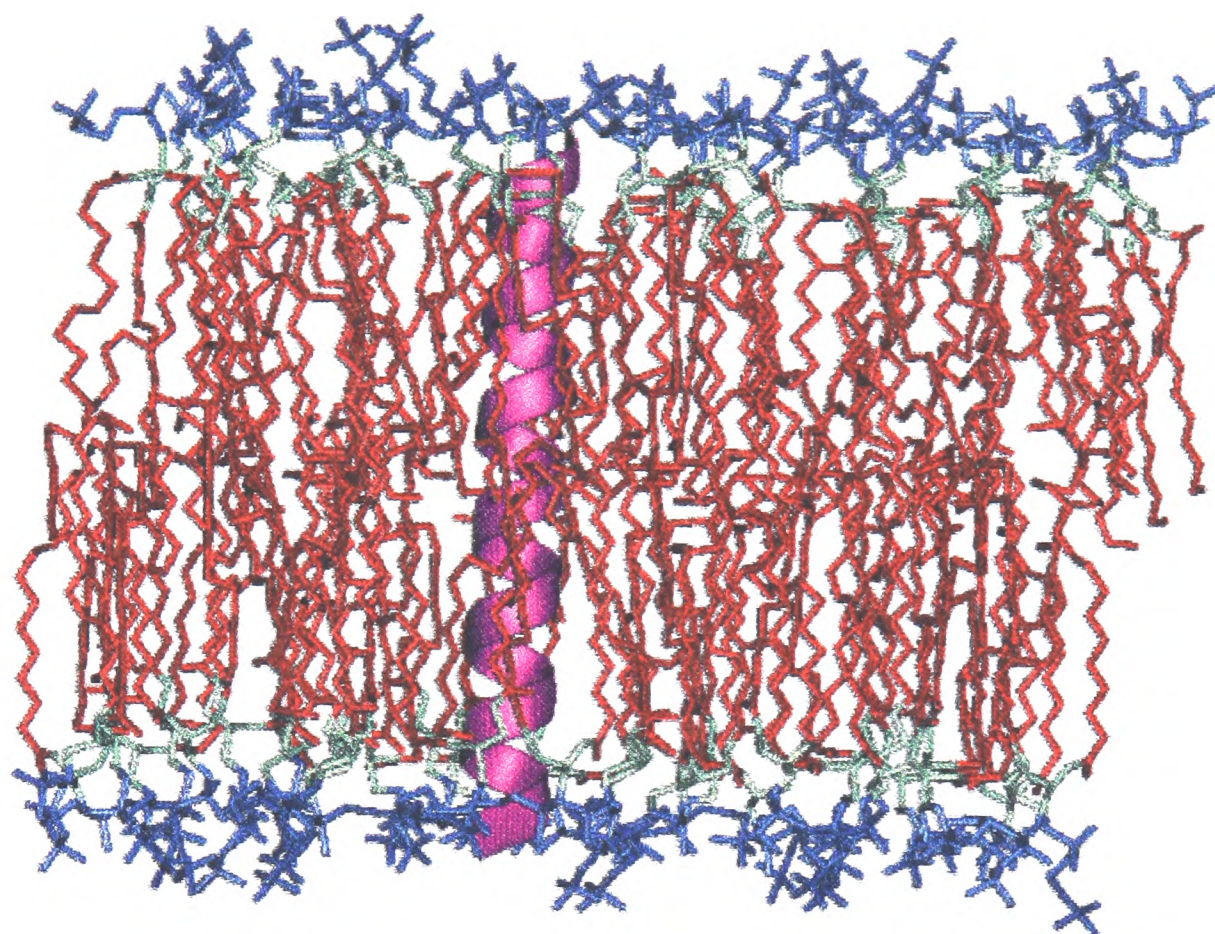


Figure 7.1 Starting structure of the membrane system with a Ala₂₅ inserted through it viewed from the side. The peptide is shown as a ribbon trace to emphasise its helical nature. Note that the peptide just spans the membrane width. The head groups are in blue, the hydrocarbon chains are in red and the glycerol moieties in green.

Each system contains 70 lipid molecules (35 per side) and before any molecular mechanics calculations were done, they were hydrated using the same procedure as described for the pure lipid bilayer in Chapter 5.

7.3 Molecular Mechanics Parameters and Simulation Protocol

The nonbonded parameters used for the peptide atoms were the widely used united atom OPLS parameter set of Jorgensen (Jorgensen and Tirado-Rives, 1988) in order to maintain consistency with the nonbonded parameter set used for the lipid molecules. AMBER 4.1 was

used for the set of simulations described herein with the same conditions as applied previously for the pure lipid bilayer simulations, i.e. NVT periodic boundary conditions at 328K with a residue based nonbonded cut-off of 8Å and the non-bonded pair list up-dated every 30fs with a time step of 2fs during the main body of the simulation.

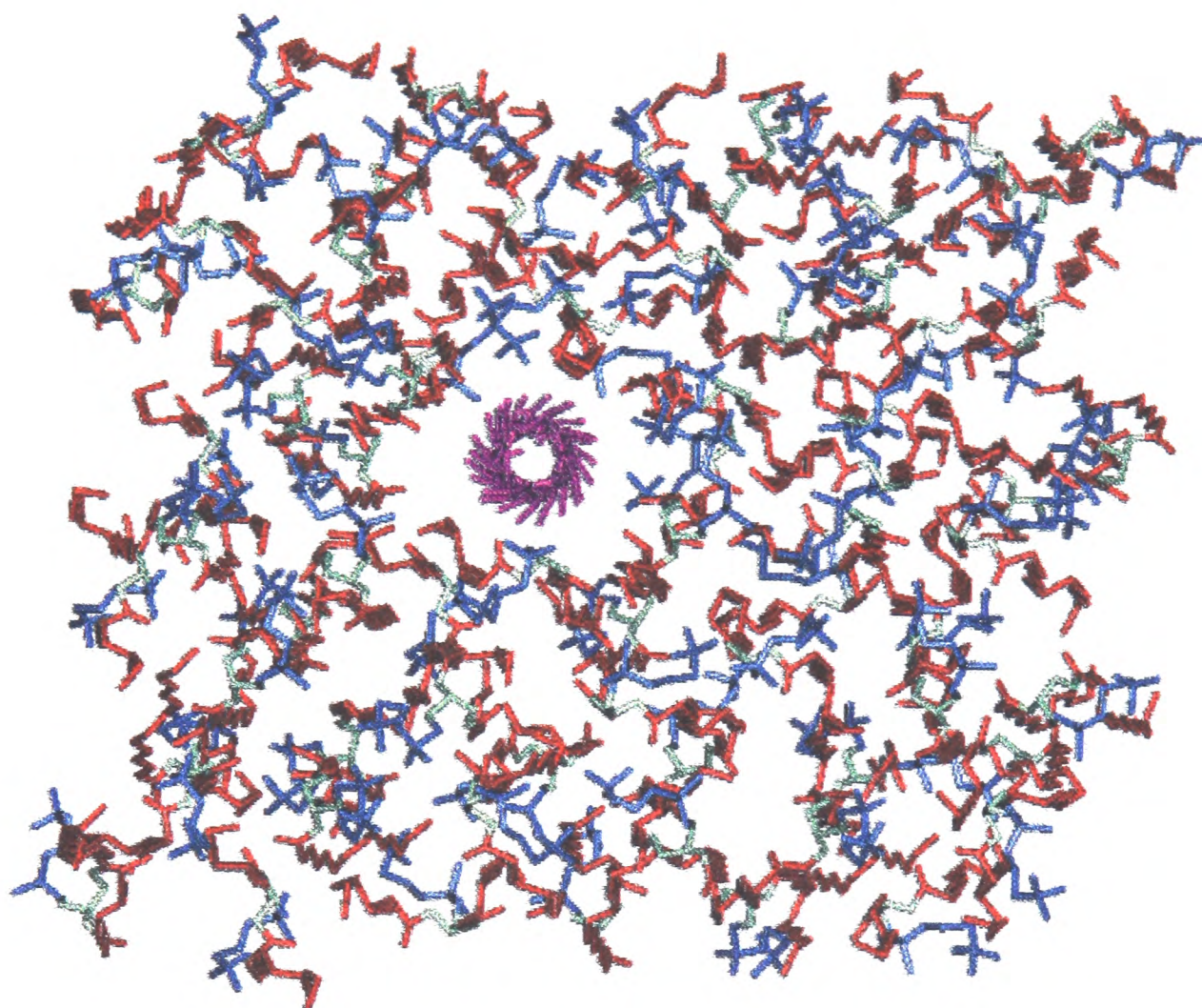


Figure 7.2 Starting structure of the membrane system with a Ala₂₅ (purple) inserted through it viewed from the top. The lipid head groups are in blue, the hydrocarbon chains are in red and the glycerol moieties in green.

To prepare the system for molecular mechanics calculations, the positions of the atoms in the system were energy refined. Firstly the lipid atoms were done because during the building of the bilayer and insertion of the peptide chain into it, many unfavourable nonbonded contacts were introduced. Following this, energy refinement on the whole system was carried out to relax the positions of the waters around the lipid head groups.

The first part of the molecular mechanics simulation consisted of two consecutive short runs of 5ps and 10ps using time steps of 0.5fs and 1fs respectively without SHAKE implemented

in order to relax further the atoms in the system. The main simulation runs continued from the output of these runs with a time step of 2fs and each proceeded for a further 600ps.

For simulations of the peptide in water, a box of TIP3P water was placed around a single Ala₂₅ peptide. The whole system was energy minimised before a short simulation of 10ps with only the positions of the water atoms allowed to move before the full molecular dynamic simulation was run for 500ps. The behaviour of the peptide in this aqueous environment was then compared with the behaviour of the peptide in the bilayer to ascertain the effect of the lipid molecules on the dynamics of the peptide.

7.4 Analysing the Results of the Molecular Mechanic Simulations

As outlined previously, the analyses of the system can be divided into-

- 1) The effect of the lipid environment on the behaviour of a peptide compared to an aqueous environment
- 2) The effect of the peptide on the physical properties of the lipid molecules
- 3) The differences in behaviour between Ala₂₅ and Ala₁₁-Gly-Ala-Ala-Gly-Ala₁₁ in order to determine if a hinge mechanism operates

The properties of the lipid molecules were analysed as a function of distance from the peptide using many of the programs described in Chapter 3 and used in Chapter 5 while to analyse the behaviour of the peptide different structural properties were calculated.

In all cases the normal to the bilayer surface was defined as the z axis and diffusion of the system as a whole was removed. It was decided that the first 100ps of the molecular mechanics simulation would be considered as the equilibration phase and the subsequent 500ps were analysed to investigate the properties of the system.

7.5 Analysis of the Behaviour of the Lipid Molecules in the Ala₂₅ Simulation

The basic procedure in analysing the effect of the inserted protein upon the lipid molecules was to analyse the properties of the lipid molecules as a function of their distance from the helical axis of the peptide. In Figure 7.3 the two-dimensional pair distribution functions are shown for the centre of the helix with the midpoint of the vector that defines the upper third of the hydrocarbon chains as defined in Section 5.4.6.

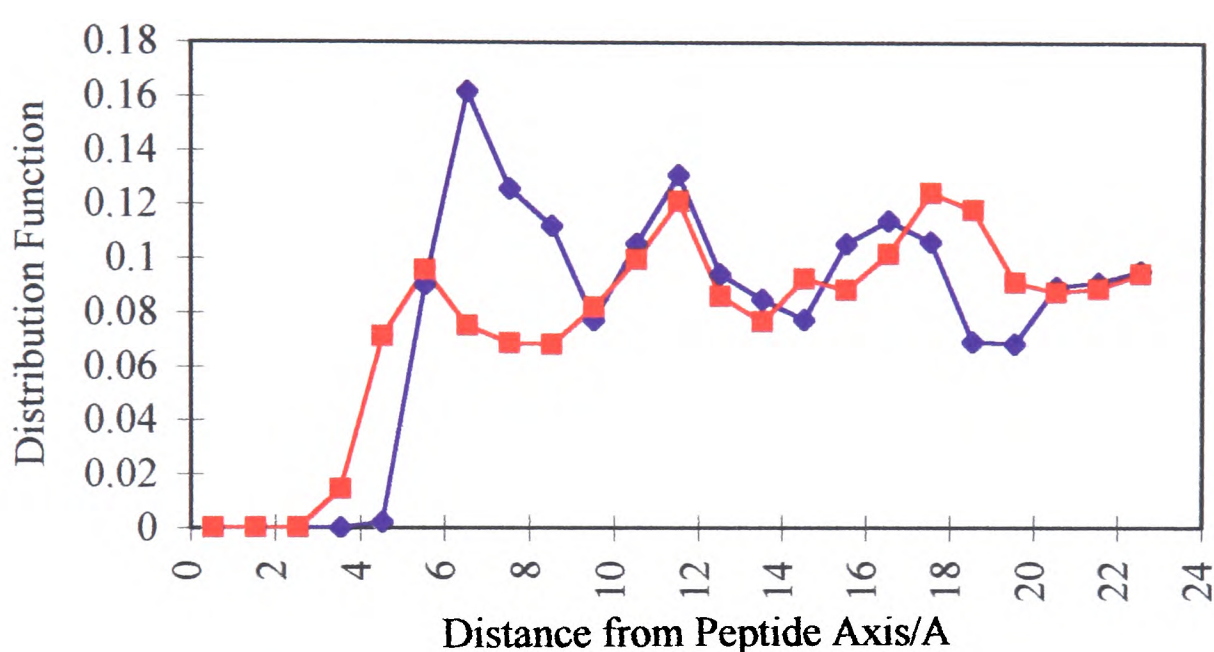


Figure 7.3 The normalised distribution function of the lipid chains with the centre of the peptide chain for layer 1 (blue) and layer 2 (red).

Upon the basis of Figure 7.3 it was decided that those lipids within 10Å of the central axis of the peptide could be classed as being associated with it and forming a first solvation shell. Other classes were defined such that the second shell was between 10 and 15Å, the third was from 15 to 20Å, etc.

From the figure it is evident that the two layers behave differently with one layer showing obvious packing around the peptide while the other layer does not have such a well defined first shell. The reasons for this become clear when the membrane system is displayed (see Figure 7.4) and it is apparent that the peptide has become tilted with respect to the bilayer normal in one layer (now defined as layer 2) while in the other layer (now defined as layer 1) the peptide has remained oriented along the bilayer normal.

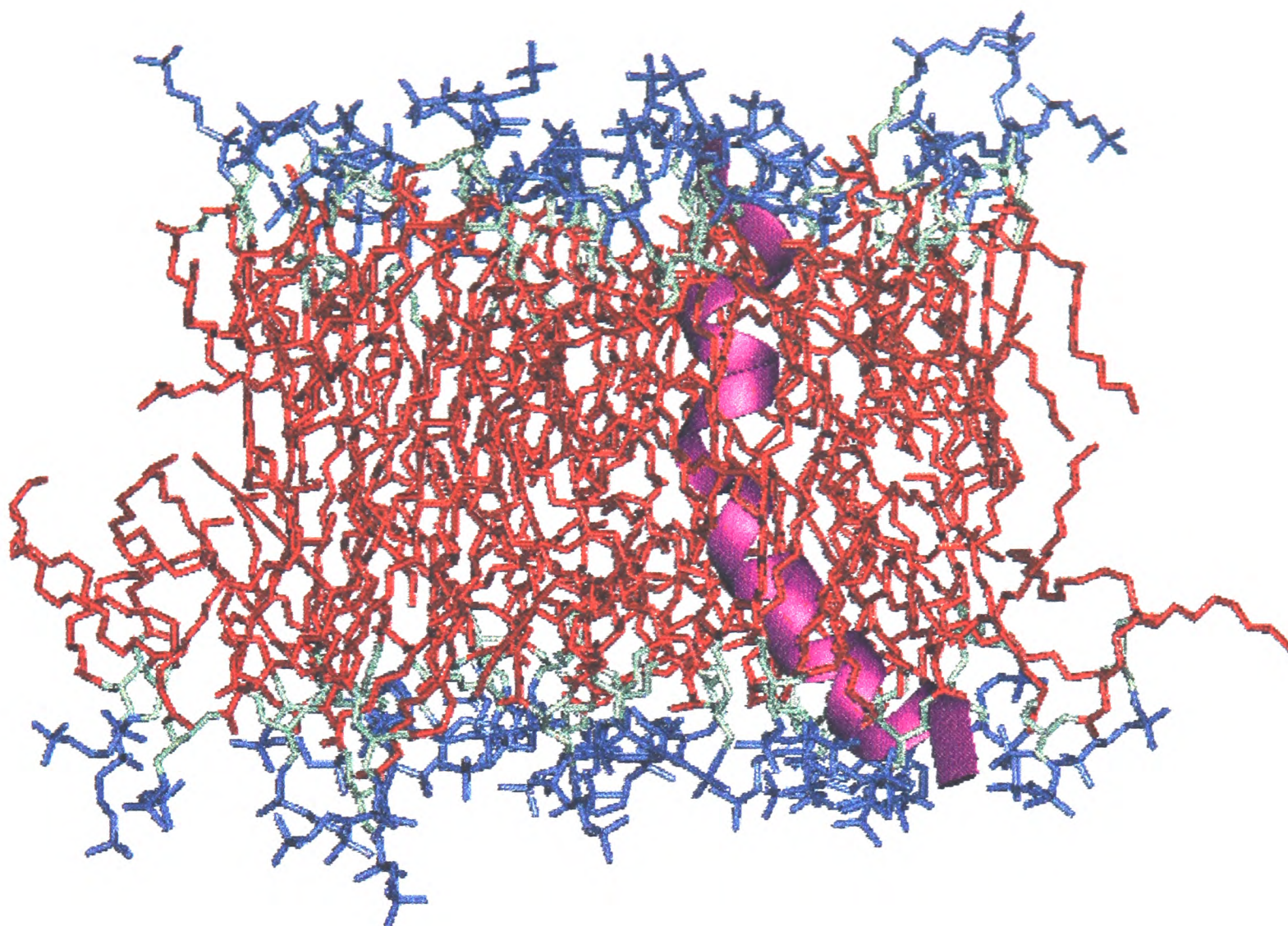


Figure 7.4 A side view of the membrane system showing the tilt of the peptide chain in one half of the bilayer (layer 2) while it remains parallel to the bilayer normal in the other half (layer 1).

This differing behaviour of the peptide in each layer is extremely interesting and has repercussions on the properties and behaviour of the lipid molecules. However the behaviour is not unexpected and represents the two possible extremes that could occur in the system:

- 1) That the peptide remains rigid and oriented along the bilayer and the lipid molecules must adjust their behaviour to compensate for its presence (the most notable example being in the ability of the lipid chains to tilt)
- 2) That the lipids and peptide behave cooperatively and that the natural inclination of the lipid chains to tilt is adopted by the peptide in order to maintain favourable packing between the chains.

This latter behaviour is contradictory to the behaviour of cholesterol in the lipid system (see Chapter 6) and may arise because the cholesterol molecules have a far more rigid structure.

7.5.1 The Tilt of the Peptide Chain in the Bilayer

Two helical axes were defined for the peptide which describe the general orientation in each half of the bilayer (see Figure 7.5) and in Figure 7.6 the results of calculating the angle that these axes make with respect to the bilayer normal are shown.

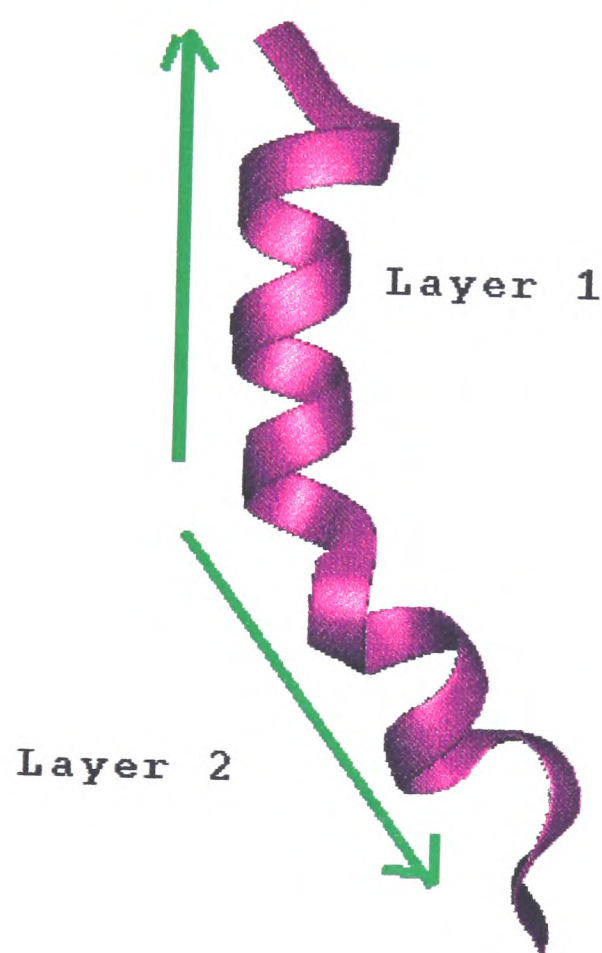


Figure 7.5 The two axes defining the direction of the helical axis of the peptide in each layer of the membrane.

It is evident from Figure 7.6 that the peptide segment in layer 2 possesses a large tilt with respect to the bilayer normal while the portion of the peptide in layer 1 remains oriented almost parallel to the bilayer normal and the most obvious question is what is the degree of cooperativity between the peptide and neighbouring lipid chains in terms of their tilting. The tilt that the two vectors make with each other is on average about 40° .

7.5.2 The Effect of Peptide Insertion upon the Tilt of the Lipid Chains

For each half of the bilayer the angle that vectors defining the parts of the lipid chains made with the bilayer normal were calculated and reported as a function of distance from the central axis of the peptide molecule. The Sn1 and Sn2 chains were considered together and the division of the chains was as in Section 5.4.6, i.e. each hydrocarbon chain was split into thirds with the orientation of each portion described by a vector connecting its ends. These vectors defining the chains were labelled top, middle and bottom with the top part of the hydrocarbon chain corresponding to that part connected to the glycerol moiety.

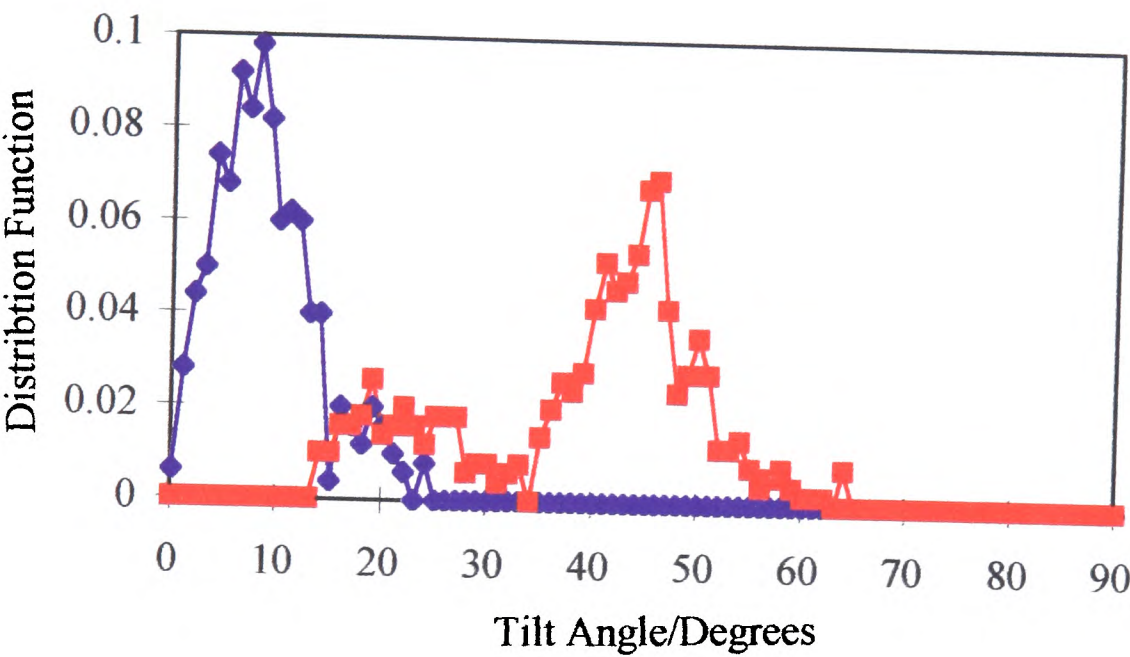


Figure 7.6 The distribution of tilt angles that the peptide makes with the bilayer normal in each half of the bilayer using the peptide axes defined in Figure 7.5. The distribution for layer 1 is shown in blue and that of layer 2 in red.

In general it was observed in layer 1 (where the peptide remains oriented along the bilayer normal) that those lipids closer to the peptide showed a narrower distribution and smaller values of the tilt angles compared to those more remote (see Figure 7.7). The effect of the peptide chain on reducing the tilt angle is quite expected because of the lipid chains packing around it and the peptide chain physically preventing chains tilting toward it because of its immobility. Moving away from the peptide then the hydrocarbon chains adopt a tilt expected for a lipid not under the influence of a membrane solute.

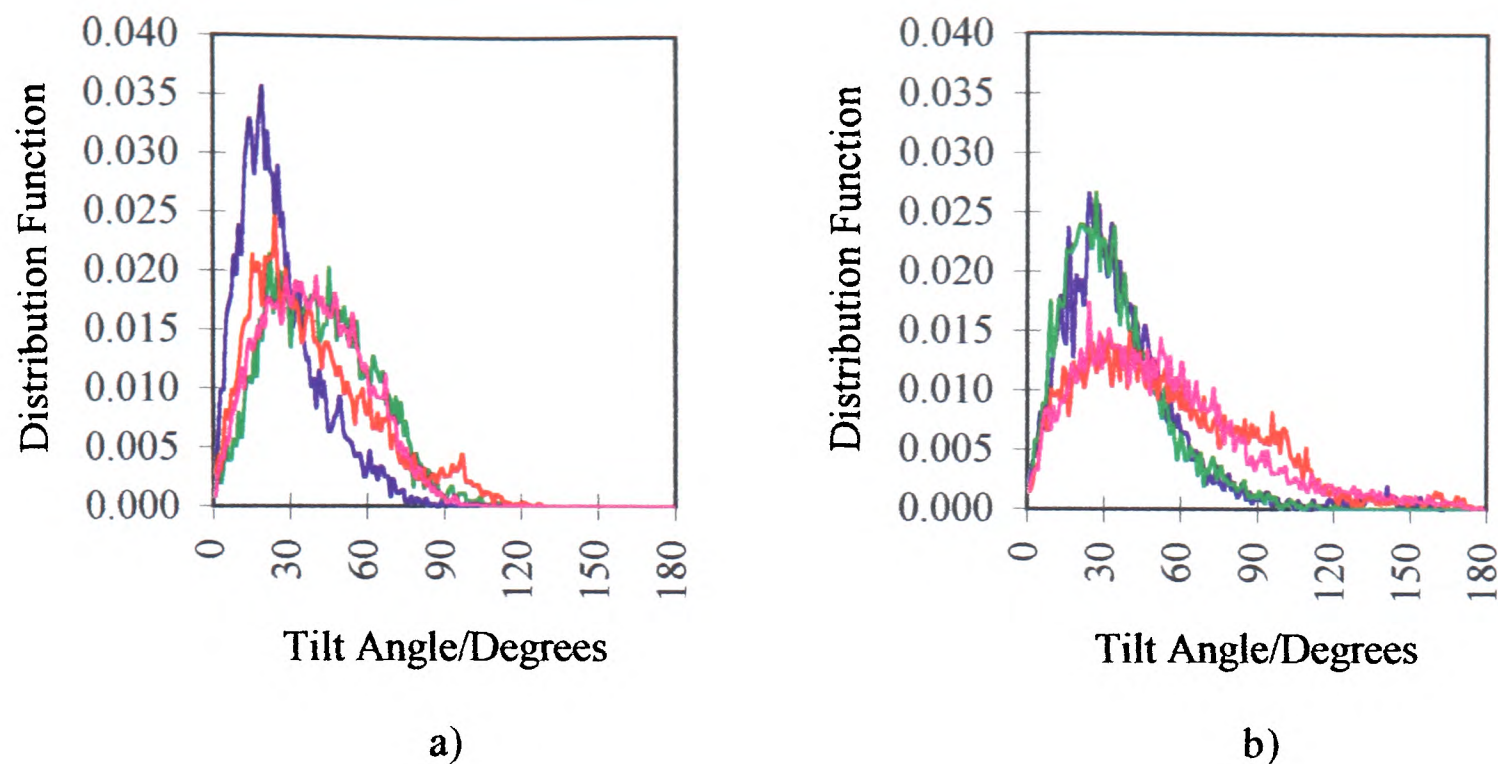


Figure 7.7 The variation in the tilt angle distributions as the distance from the peptide increases in layer 1 for a) the top part of the lipid chain b) the bottom part of the lipid chain. The distances from the peptide central axis are 5-10Å (blue), 10-15Å (green), 15-20Å (red) and 20-25Å (magenta).

In Figure 7.8 the tilt angle distributions for the three sections used to define the lipid chains are shown for lipids which are 5-10Å from the peptide central axis, i.e. neighbouring it, and for lipids which are 15-20Å distant from the peptide.

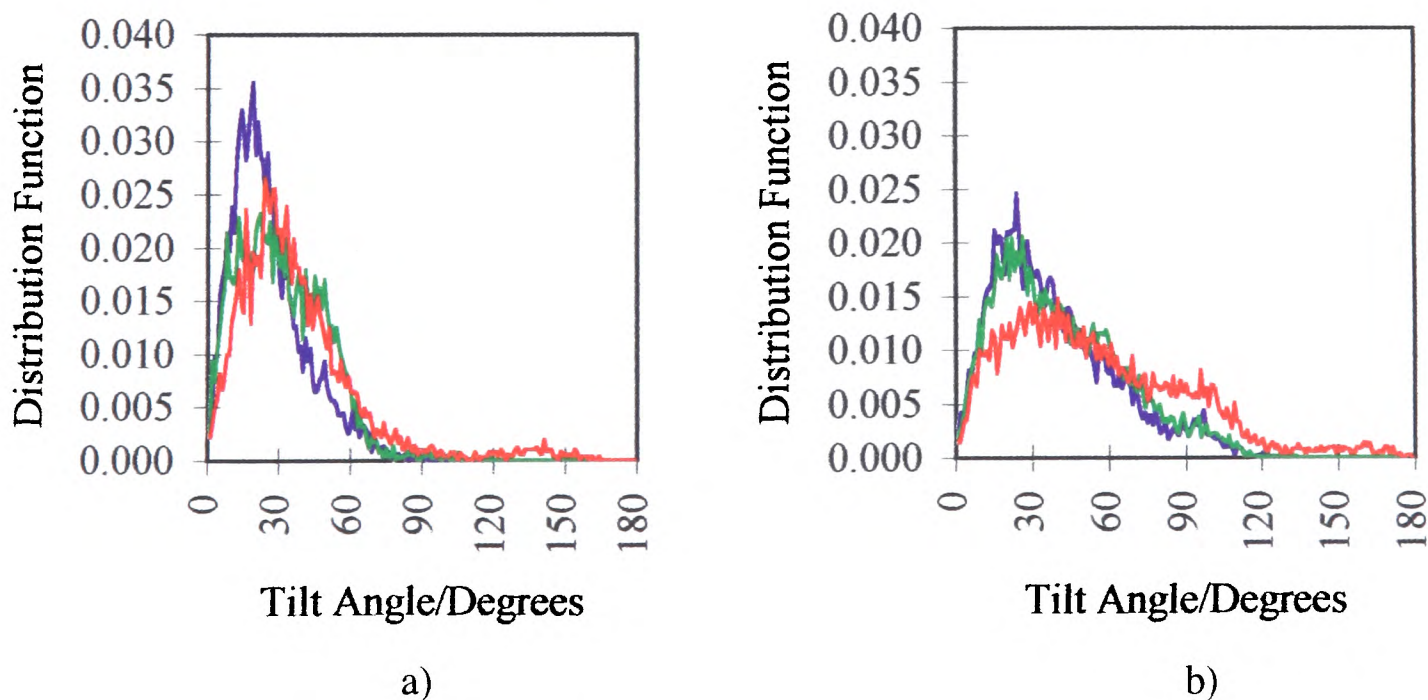


Figure 7.8 The comparative tilt angle distributions for the top (blue), middle (green) and bottom (red) sections of the lipid chains in layer 1 for a) 5-10Å from the peptide central axis b) 15-20Å from the peptide central axis.

From the figure it is quite apparent that the peptide reduces the tilt angle in all parts of the lipid chain. Most noticeably the bottom part of lipid chains in the immediate vicinity of the peptide show a very small range of tilt angles compared to lipid chains in the bulk of the bilayer illustrating that the peptide has quite a large ordering effect on the bilayer.

For layer 2 the behaviour is different and more interesting with the peptide having developed a bend at the interface between the hydrocarbon phase of the two layers but to a large degree still retaining its alpha helical structure in order to conserve hydrogen bonding (see Section 7.6.1). This has the effect of reducing the difference in tilt angles between lipids packed around the peptide and those further away (c.f. layer 1) because the tilt of the peptide is very similar to that of lipid chains, i.e. the peptide is behaving cooperatively with the lipids.

Figure 7.9 reports the tilt angle distributions of the top and bottom sections of the lipid chain at different distances from the peptide and illustrates how there is very little difference between them (c.f. Figure 7.7) while in Figure 7.10 the tilt angle distributions of the top, middle and bottom sections of the lipid chain at two different distances are presented.

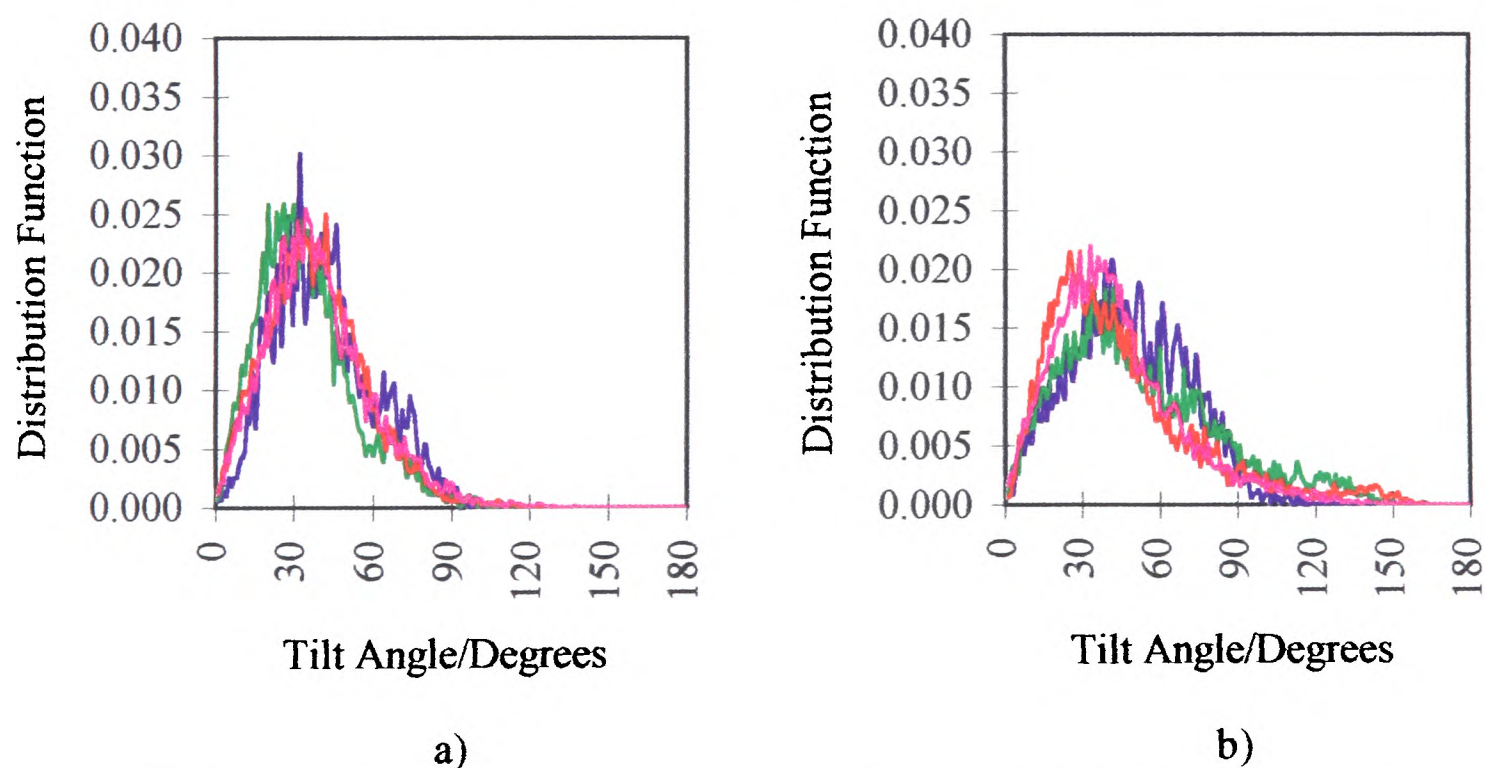


Figure 7.9 The variation in the tilt angle distributions as the distance from the peptide increases in layer 2 for a) the top part of the lipid chain b) the bottom part of the lipid chain. The distances from the peptide central axis are 5-10 Å (blue), 10-15 Å (green), 15-20 Å (red) and 20-25 Å (magenta).

Compared to Figure 7.8, the graphs in Figure 7.10 illustrate how little the presence of the peptide perturbs the tilt behaviour of the lipids which is evidence that the peptide has tilted

cooperatively with the lipid chains. The only noticeable difference between Figure 7.10a and 7.10b is that the bottom section of the lipid chains in close proximity to the peptide show a smaller proportion of lipid chain with very large tilt angles. To further ratify the extent of correlation between lipid and peptide tilt, the angle that the peptide axis make with neighbouring lipid chains was evaluated and this is discussed in Section 7.5.3

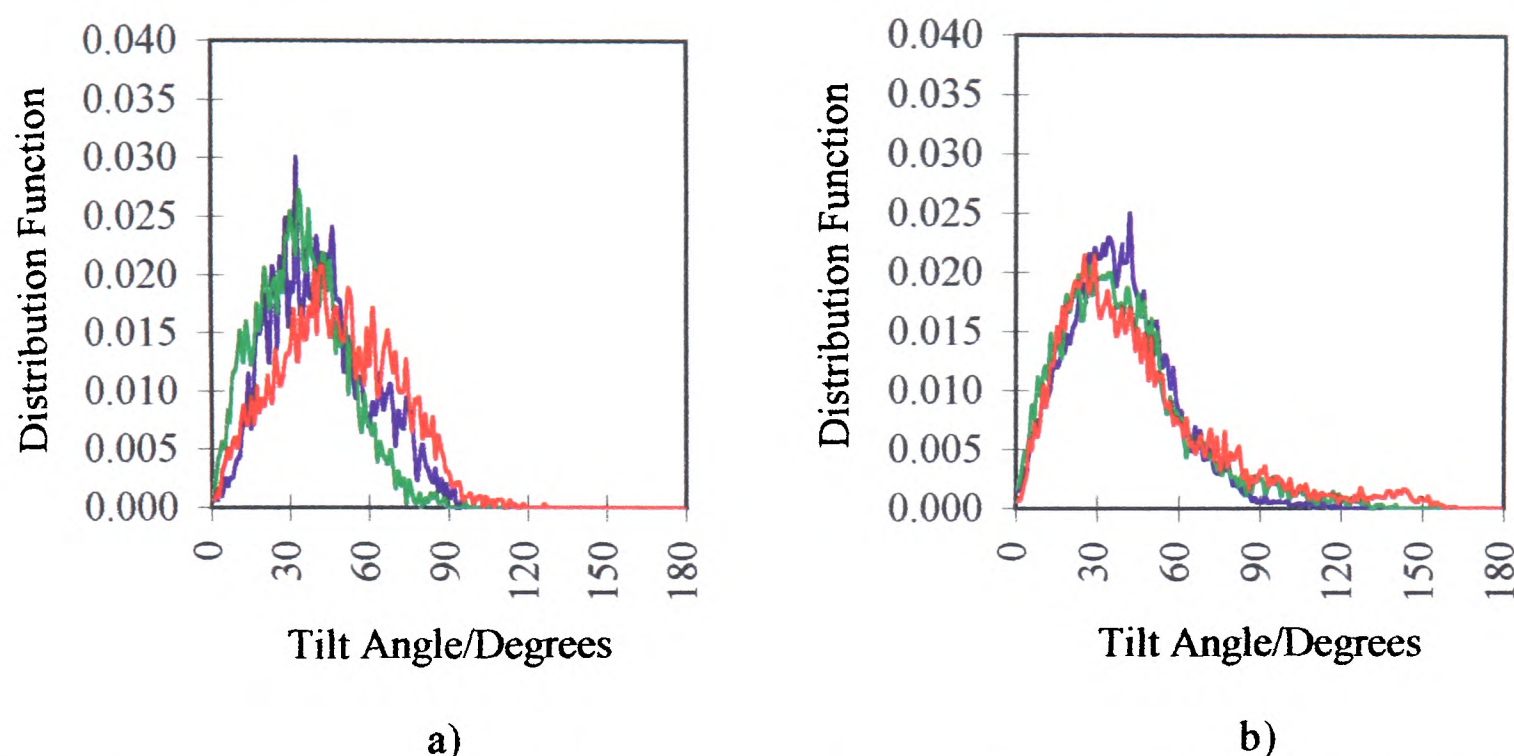


Figure 7.10 The comparative tilt angle distributions for the top (blue), middle (green) and bottom (red) sections of the lipid chains in layer 1 for a) 5-10Å from the peptide central axis b) 15-20Å from the peptide central axis.

7.5.3 The Correlation between Lipid Chain and Peptide Tilt in the Membrane

As an exercise in determining the extent of correlation between the tilt of the peptide and the lipid chains around it, the distributions of the angles between the two was calculated as a function of distance from the peptide in the region 5-10Å from the peptide axis for each layer and are shown in Figure 7.11. These graphs show that in layer 1 the lipid chains are oriented far more closely with the trans-membrane peptide than in layer 2 where there is a large range of angles which emphasises how the lipid chains are less well packed around the tilted peptide chain.

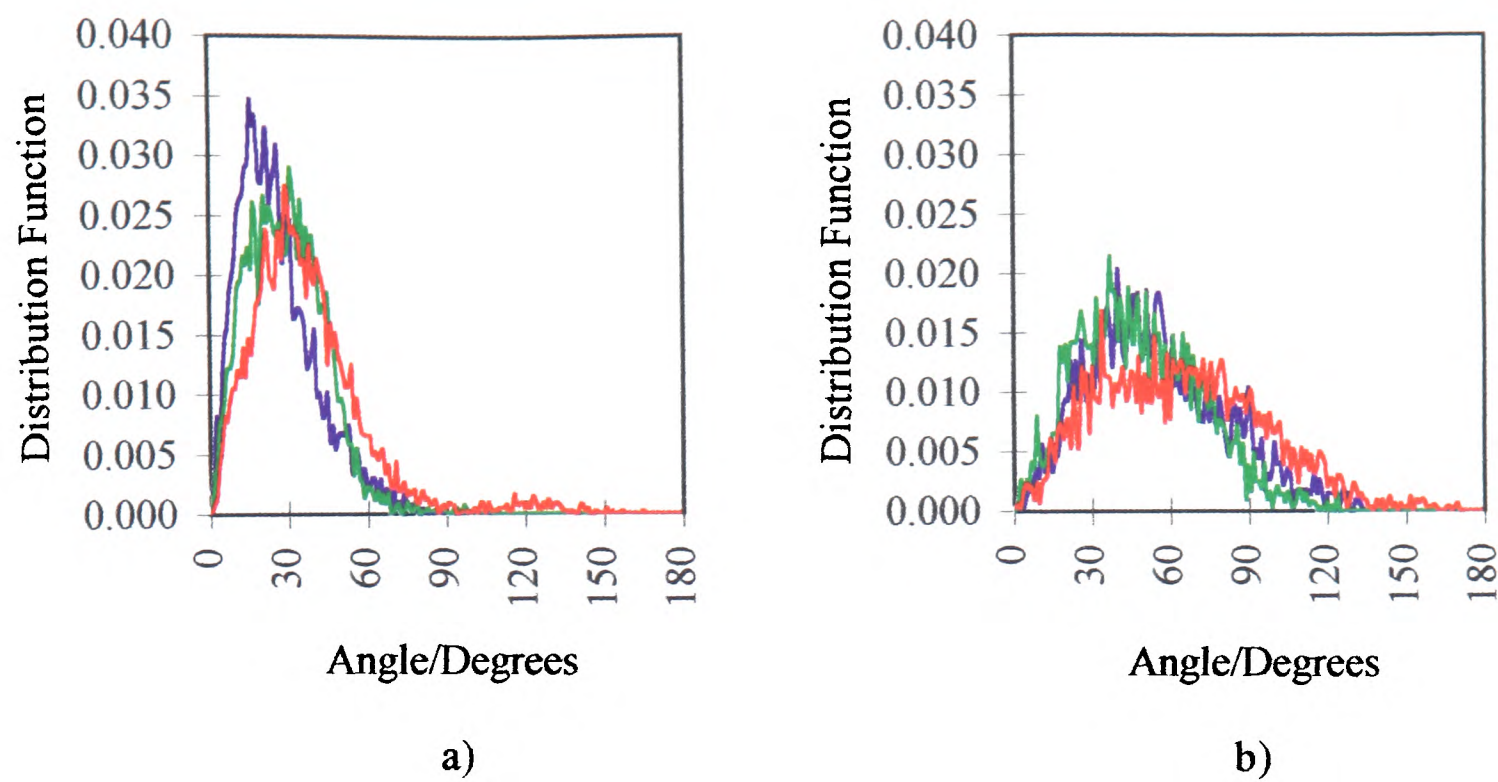


Figure 7.11 The distribution of the angles between the peptide axis and the top (blue), middle (green) and bottom (red) sections of the lipids in the region 5-10Å from the peptide chain in a) layer 1 b) layer 2.

The variation in the correlation between the top section of the lipid chain with the peptide as a function of distance from it for layer 1 and 2 are shown in Figure 7.12.

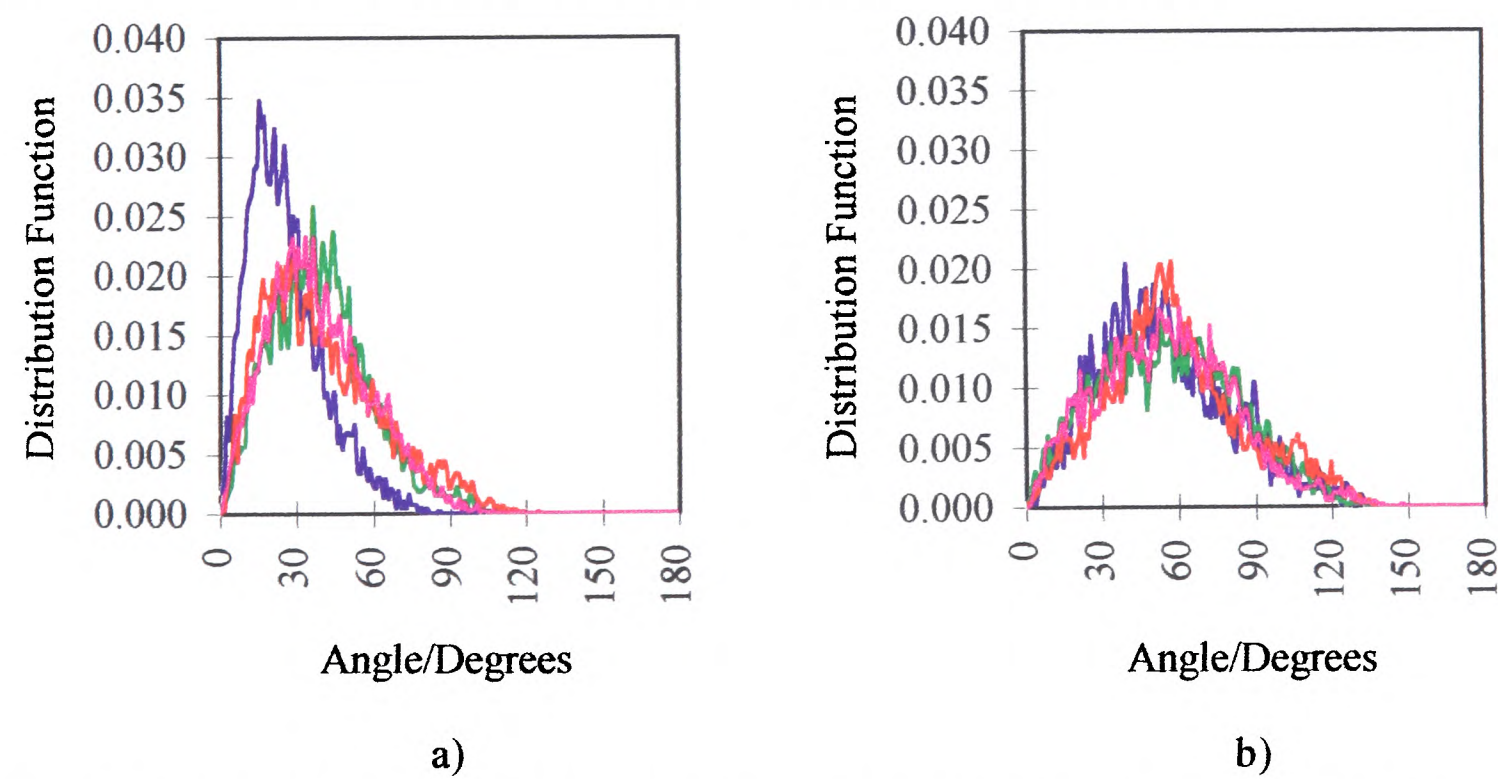


Figure 7.12 The difference in tilt angle between the top part of the lipid chains and the peptide for a) layer 1 and b) layer 2 over the four different regions of 5-10Å (blue), 10-15Å (green), 15-20Å (red) and 20-25Å (magenta) separation from the peptide axis.

From Figure 7.12 it is absolutely clear that in layer one the lipid chains in the 5-10Å region from the peptide are packing around it while in layer 2 there is little difference between any of the regions showing how the peptide has little influence on the structure of the layer in its tilted configuration.

7.5.4 The Order Parameter Profile of the Lipid Molecules

The order parameter profiles of the lipid chains were calculated for each layer as a function of the distance of the methylene unit from the peptide molecule to determine the how the motional behaviour of the lipid hydrocarbon chains is effected by the presence of the peptide. The Sn1 and Sn2 chains are not distinguished to improve the statistics in the calculation of the order parameters.

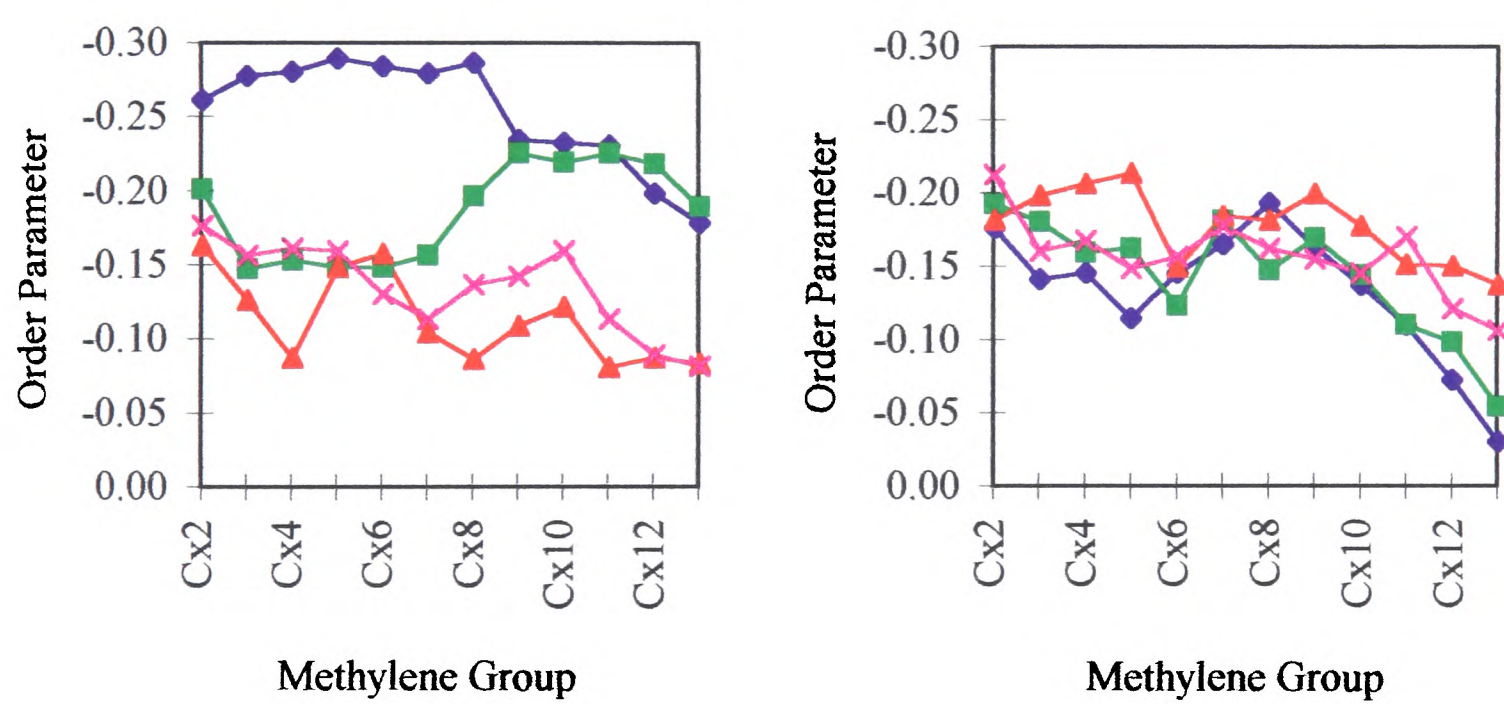


Figure 7.13 The order parameter profile of the methylene groups of hydrocarbon chains as a function of distance from the peptide chain for a) layer 1 and b) layer 2. Values for methylenes 5- 10Å from the peptide are in blue, those 10-15Å in green, 15-20Å are in red and finally those 20-25Å from the peptide are in magenta.

The plots of Figure 7.13 demonstrate quite clearly that in layer 1 the peptide chain is having an ordering effect on those lipid chains in close proximity to it and this is also felt by those chains in the next shell. Conversely in layer 2 the peptide appears to have very little effect on

the order parameters of the methylene groups reflecting its cooperative behaviour with the lipid chains with which it is in contact.

Fattal and Ben-Shaul (1993) used theoretical models and techniques to investigate the effect of hydrophobic mismatch between lipids and embedded proteins on the behaviour of lipid chains and the bilayer width. Their calculations showed that if the width of the hydrophobic section of the protein were wider than the normal bilayer width then it caused an increase in the order of lipid chains surrounding it. Conversely, if the hydrophobic length of the protein was less than the bilayer width then there was a decrease in the order of the lipid chains around the peptide compared to lipid molecules within the bulk.

Lipid bilayer polypeptide interactions were studied by Edholm and Johansson (1987) using a model membrane consisting of an array of hydrocarbon chains containing either polyglycine or glycophorin A. For the polyglycine there was a definite increase in order parameters of lipids around the peptide chain while for the glycophorin there was a slight increase. They concluded that the later behaviour was due to the bulky side chains present in the glycophorin A preventing the close packing of the lipid chains around the helix.

Certainly there is little consensus on the effect of peptide chains on the ordering of lipid chains around them and it is probably very dependant upon the nature of the side chains. For the peptide simulated here, the lack of bulky side chains allows for quite efficient packing of the lipid chains around it leading to an increase in their order when it is perturbing the bilayer environment as in layer 1, but little effect when it is behaving cooperatively with the lipid chains as in layer 2.

7.5.5 The Effect of the Peptide on Torsion Angle Distribution in the Lipid Dihedrals

To determine the effect of the peptide on the torsion angles of dihedrals along the length of the hydrocarbon chains, the average proportion of torsion angles with the trans dihedral angle was calculated as a function of distance from the peptide central axis. The results showed that there was no distinguishable difference between the dihedral populations of lipids chains that were next to the peptide compared with those further away for either layer 1 or layer 2.

Thus the difference in order parameters established previously is due to the influence of the peptide chain on the orientation of the chain, not on the dihedral population along the chain.

In the simple simulation of glycophorin A in a model bilayer reported by Edholm and Johansson (1987) the same behaviour was reported with little difference between average number of gauche bonds in lipid chains neighbouring the peptide and those in the bulk. But for the simulation of polyglycine it was observed that the average fraction of gauche bonds was approximately 5% lower for the lipid chains around the peptide but reached the normal bulk value at a distance of 20Å from it.

7.5.6 The Mobility of the Lipid Chains

The mobility of the lipid chains within the bilayer was characterised by determining the diffusion constants for carbon atoms of the methylene chains at different ranges of distance from the peptide. The diffusion constants were calculated using the mean squared displacements of the methylene carbons over the time differences of 100-350ps using the Einstein relation as described previously in Section 5.4.7 and the results are illustrated for the two layers in Figures 7.14 and 7.15. By using the long time scale it may be possible to determine if the lipid chains tend to stick to the peptide and hence have their mobility reduced compared to lipids in the bulk of the membrane. (N.B. The results have been averaged over both the Sn1 and Sn2 chains to improve the statistics for each methylene group).

Unfortunately the results are unlikely to be very accurate, especially for the region around the peptide because of the few lipids found within this region and so must be treated qualitatively. What is apparent is that for layer 1 the middle part of the chain is less mobile than for other regions while the bottom part of the chain is more mobile. For layer 2 the behaviour of methylenes next to the peptide are slightly anomalous, but the rest of the methylenes show the expected behaviour (see Section 5.4.7).

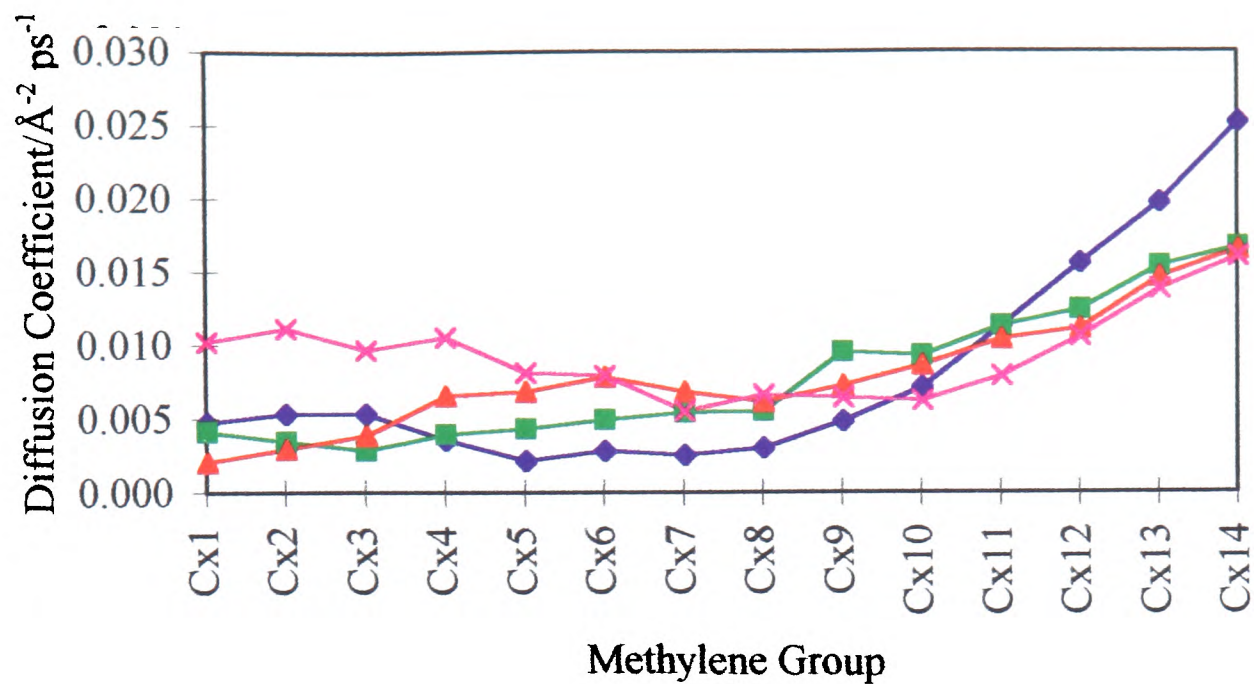


Figure 7.14 The diffusion coefficients for the lipid chain atoms of layer 1 at different distances from the peptide chain evaluated from the mean squared displacements using the Einstein relation. (Colour scheme is as in Figure 7.13).

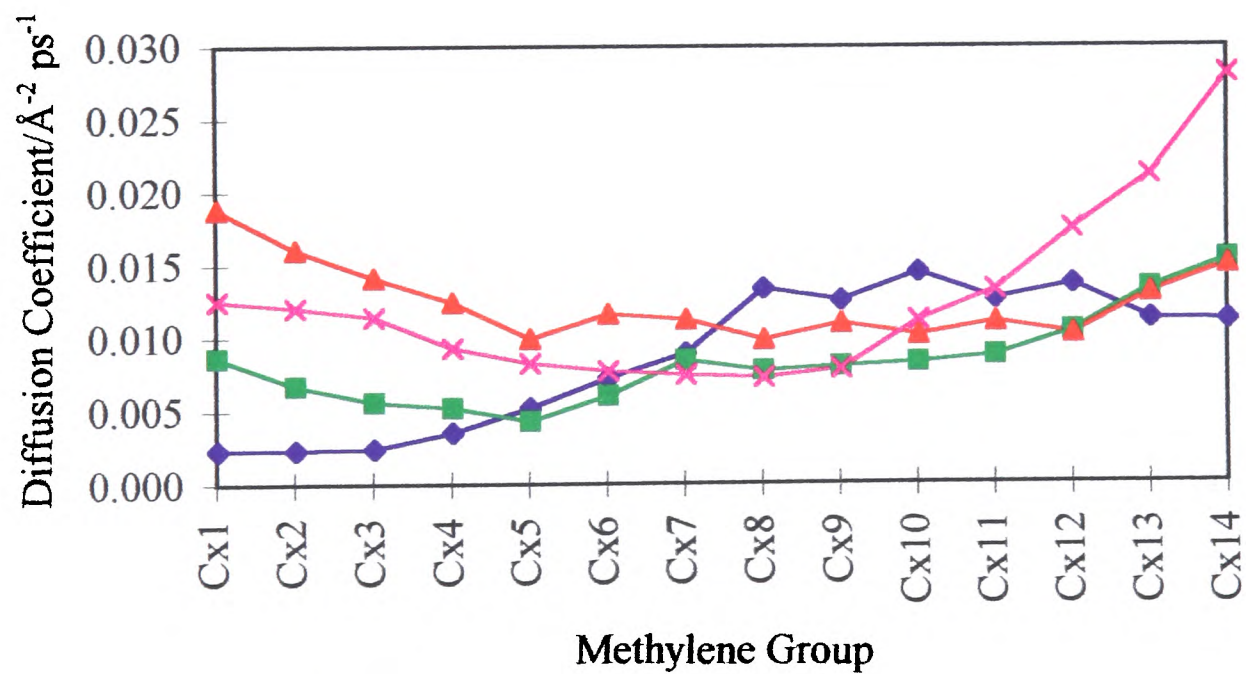


Figure 7.15 The diffusion coefficients for the lipid chain atoms of layer 2 at different distances from the peptide chain evaluated from the mean squared displacements using the Einstein relation. (Colour scheme is as in Figure 7.13).

What is also useful is the determination of the diffusion coefficients to describe mobility of the methylene groups around the peptide over a shorter time scale of 10-100ps, the results of which are given in Figure 7.16 for both of the layers. The values show that generally there is little difference between the lipid chains as their separation increases from the inserted

peptide in layer 2 which is consistent with the other analyses of this layer. For layer 1 the situation is less clear, but this may be due to poor sampling.

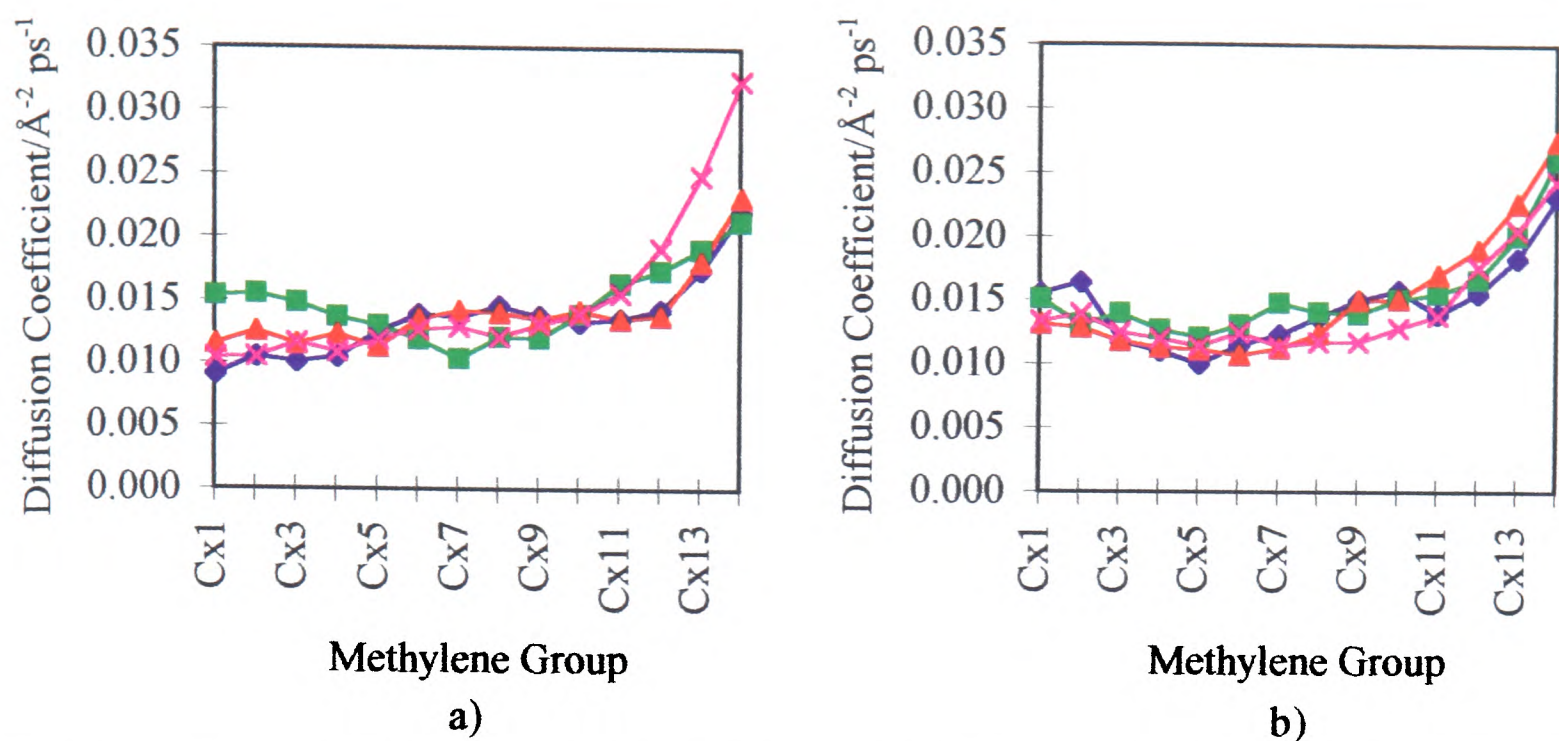


Figure 7.16 Short time-scale diffusion coefficients of a) layer 1 and b) layer 2 evaluated using the Einstein relation from the mean squared deviations of methylene groups over the 10-100ps time scale. [The colour scheme is the same as that in Figure 7.13].

7.5.7 Water Penetration into the Bilayer

Since the head group and its concomitant water structure (see Section 5.7) is broken up by the presence of the peptide, it was decided to observe if the peptide allowed water to enter the membrane more easily by comparing the extent of penetration of water into the membrane around the peptide with the rest of the bilayer. Unfortunately the analysis suffers from the low number of waters that can be sampled in the vicinity of the peptide, but the results are presented in Figure 7.16. For layer 1, two observations can be made. 1) The water density is slightly higher 8-15Å from the bilayer centre around the peptide compared to the rest of the bilayer which may reflect the less hydrophobic environment around the peptide compared to the bulk hydrocarbon phase. 2) Around the head group region, 15-20Å from the bilayer centre then the water density is observed to be slightly less than for the rest of the bilayer which could be due to the reduced hydrophilicity of the peptide in contrast to the highly charged amphipathic head group of the lipids.

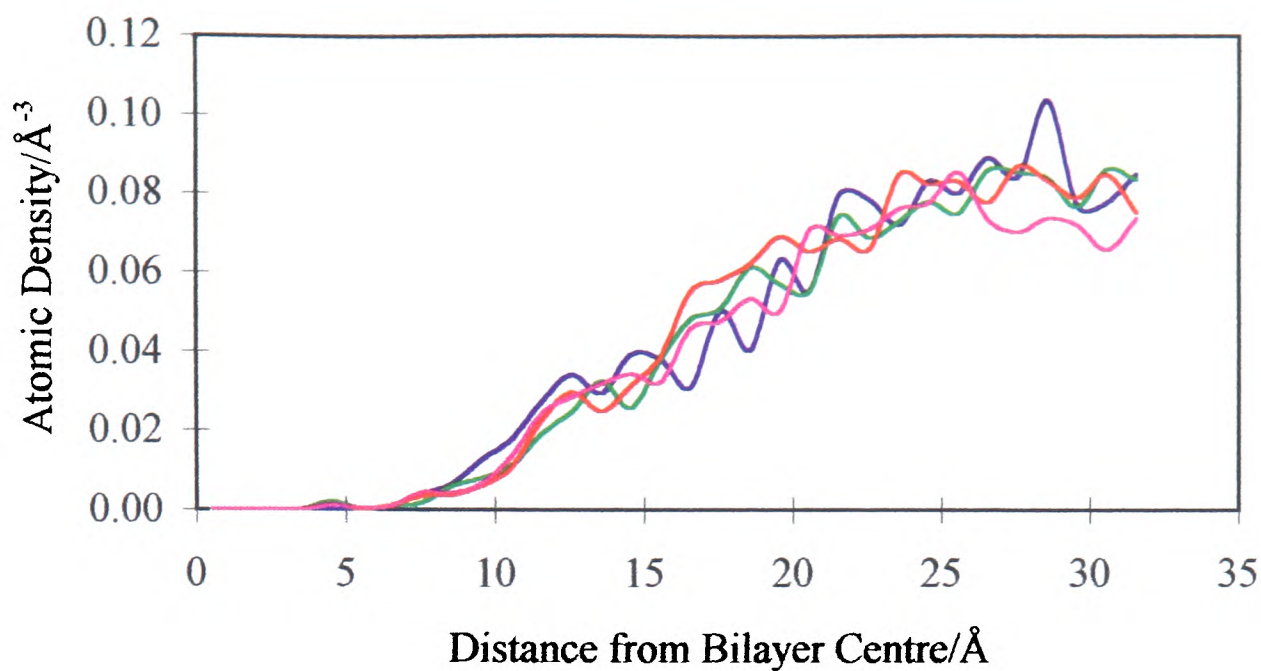


Figure 7.16 The extent of water penetration into layer 1 as a function of distance from the peptide central axis. [Colour scheme is the same as Figure 7.13].

The water penetration into layer 2 was also analysed and the density functions plotted in Figure 7.17 and it is observed that the peptide has a similar effect on water penetration into the lipid bilayer as it did in layer 1, i.e. lower water density around the head group region (19-23 $\text{\AA}$ from the bilayer centre) and higher in the hydrophobic core (6-10 $\text{\AA}$ from the bilayer centre).

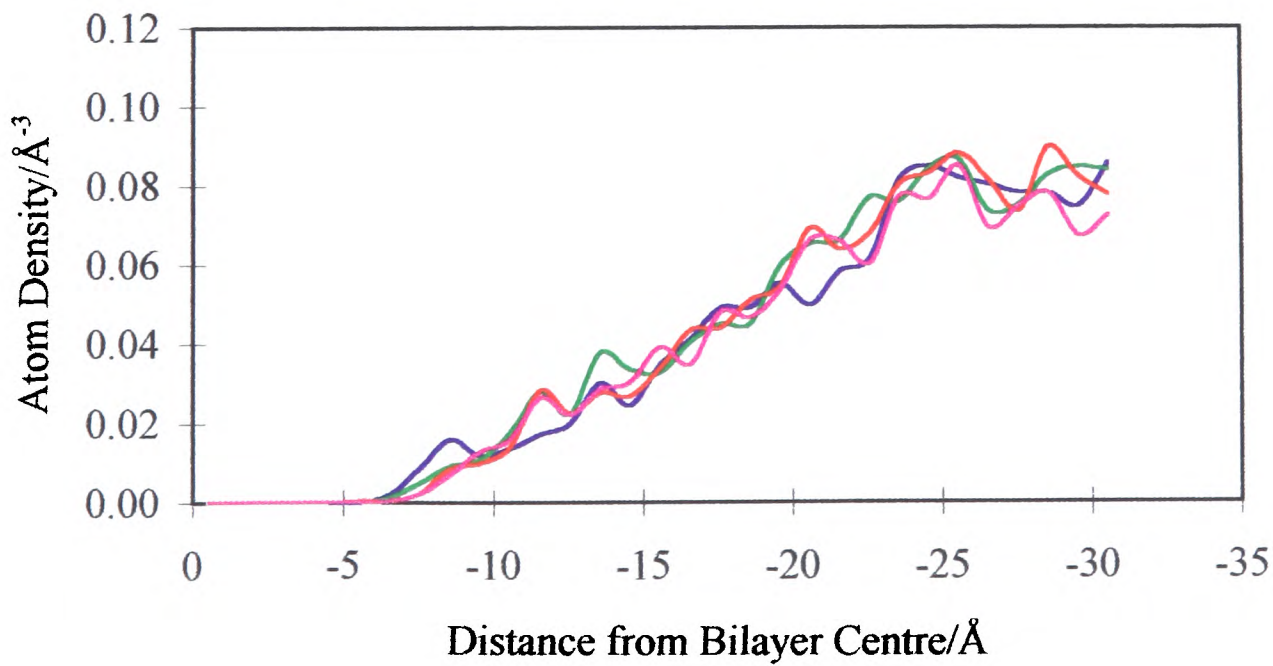


Figure 7.17 The extent of water penetration into the bilayer as a function of distance from the peptide central axis. [Colour scheme is the same as in Figure 7.13].

7.5.8 The Effect of the Peptide on Atom Density Profiles and Membrane Width

It has already been shown that the presence of a peptide causes water molecules to penetrate further into the membrane interior than would otherwise be expected, but what of its effect on the bilayer width? This aspect of the influence of the peptide on the lipid molecules was studied by examining the atom densities across the membrane as a function of the distance from the peptide central axis and the relevant plots are shown in Figure's 7.18 and 7.19 for layer 1 and layer 2 respectively. N.B. As in the previous calculations of atom density profiles a correction has been made to the model to allow for the united atom nature of the model.

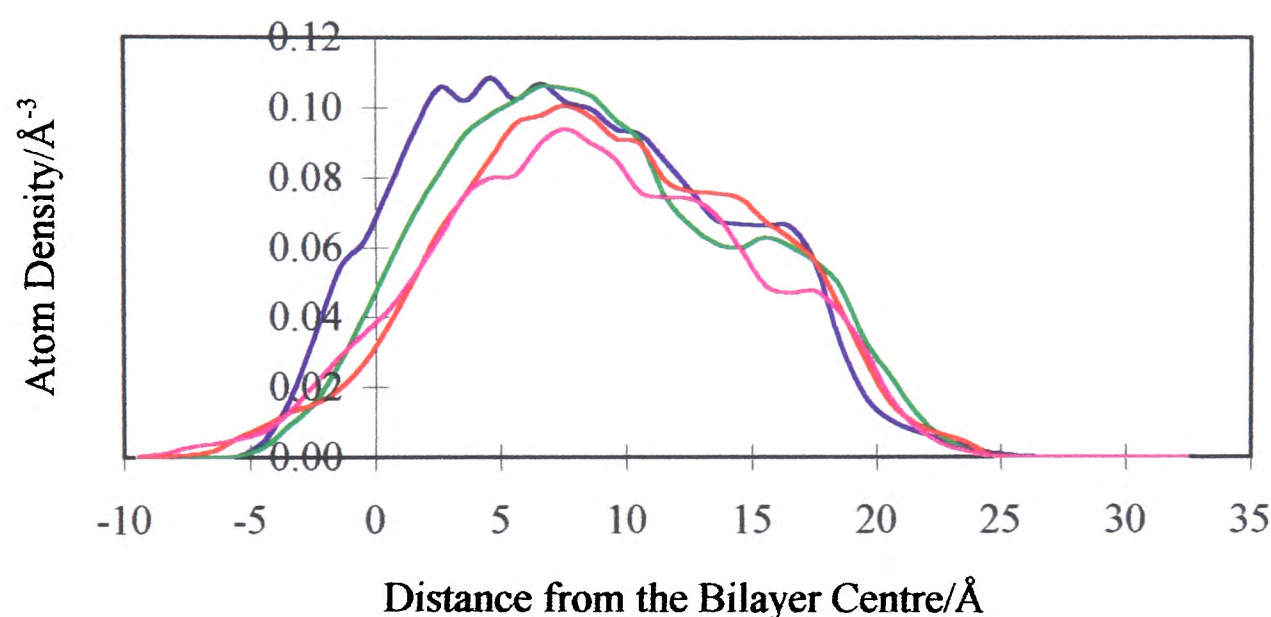


Figure 7.18 The atom density distributions across the bilayer measured from the bilayer centre for layer 1 of the membrane system. [Colour scheme is the same as in Figure 7.13].

For layer 1 the most dominating aspect of the plot is the much higher density of the lipid chains at the bilayer centre around the peptide which presumably arises from the lipid chains associated with the peptide having a much lower tilt angle and thus effectively penetrating further into the membrane interior than would be expected if they had a substantial tilt which would divert them from there.

For layer 2 a more complicated behaviour is observed with the lipids around the peptide (the blue line in Figure 7.18) not penetrating as far into the membrane plus having a low density in the head group/glycerol region of 11-18 Å from the bilayer centre, however the lipids do extend further into the solvent at the head group/water interface. The above observations all

indicate a more loosely packed arrangement of the lipids around the peptide compared to lipid molecules in the bulk or in layer 1.

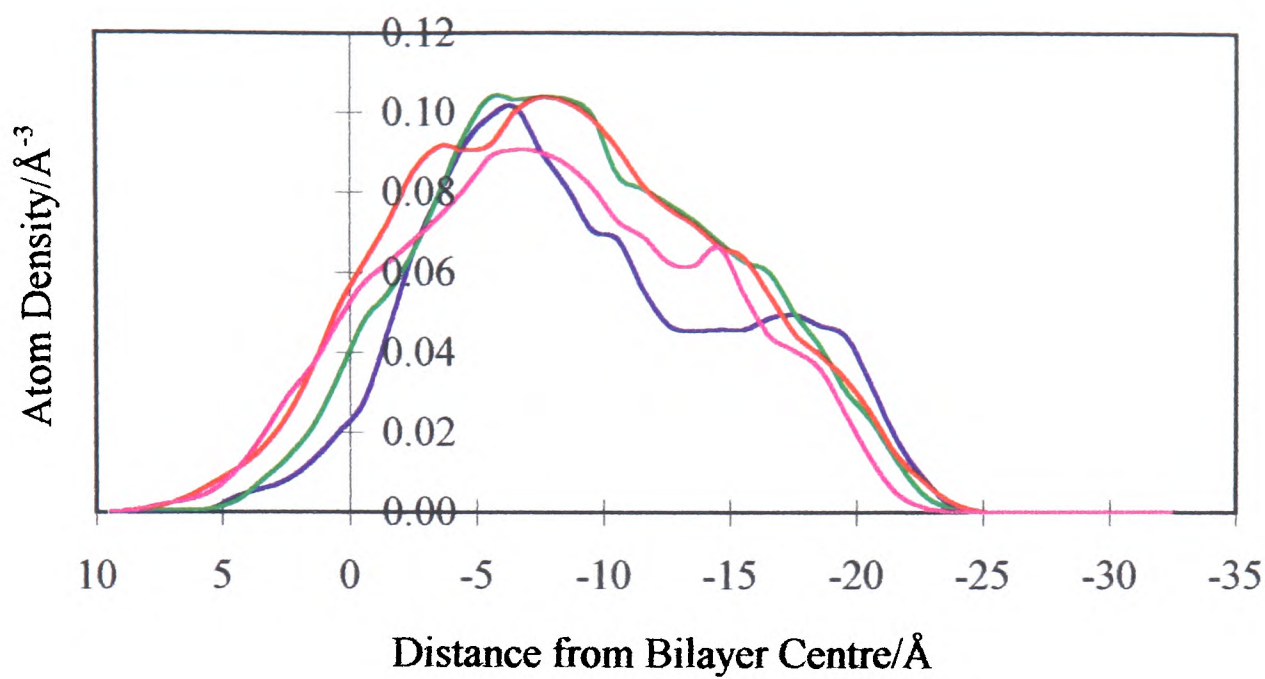


Figure 7.18 The atom density distributions across layer 2 with the bilayer centre being the origin. [Colour scheme is as in Figure 7.13].

7.6 Behaviour of the Peptide in the Bilayer

The behaviour of the Ala₂₅ peptide was analysed in order to contrast it with the behaviour of Ala₁₁-Gly-Ala-Ala-Gly-Ala₁₀, and to determine what effect a bilayer environment has on it compared to an aqueous one.

7.6.1 Hydrogen Bonding in the Peptide

The hydrogen bond lengths between the carbonyl oxygens and amide hydrogens which retain the alpha helical structure were calculated and are listed in Table 7.1. N.B. The hydrogen bonds are listed starting from the N-terminus.

The values show that some of the atom pairs are no longer hydrogen bonded, however an examination of the structure shows that these atoms have formed hydrogen bonds with other atoms instead and this helps maintain the alpha helical structure. Since there are no hydrogen

bond acceptor or donor groups in the lipid interior bar the few water molecules, it is natural that intra-molecular hydrogen bonding would be attempted with whatever atoms are available and is consistent with the observations of Engelman and Stetz, (1981) and Jacobs and White (1989) on peptides maintaining hydrogen bonding when within membranes.

Amino Acid	Average/Å	Standard Deviation/Å	Amino Acid	Average/Å	Standard Deviation/Å
1	5.9	0.6	12	2.0	0.2
2	4.3	0.8	13	2.1	0.2
3	4.0	0.4	14	2.1	0.2
4	1.9	0.2	15	2.0	0.2
5	4.3	0.7	16	2.0	0.2
6	2.1	0.3	17	2.5	0.4
7	7.1	0.5	18	2.5	0.6
8	5.4	1.4	19	2.0	0.3
9	4.6	0.6	20	3.6	0.9
10	5.9	0.6	21	6.5	1.2
11	2.3	0.4			

Table 7.1 The averages and standard deviations of the lengths of hydrogen bonds which maintain the alpha helical structure starting from the N terminus.

The values in Table 7.1 indicate a situation in which hydrogen bonding is very well conserved in the section of the peptide in layer 2 (bonds number 11-21), but in layer 1 it is quite poor in places. This might seem contrary to the expected situation, but can be rationalised as the section of the peptide in layer 1 being under greater stress than in layer 2 which has an impact on its structure. This stress in layer 1 arises from the peptide being aligned parallel with the bilayer normal while the preferred orientation of the lipid chains

around it is to be tilted which will lead to conflict. The large values for the 9th and 10th peptide hydrogen bonds arise from the breakdown of the structure to form the bend.

It is also seen that the ammonium and amine group of the end amino acid in layer one forms hydrogen bonds with an oxygen atom of a phosphate group at the bilayer/water interface (see Figure 7.19). Such hydrogen bonds could serve to anchor peptides in the bilayer. The presence of water at the bilayer/water interface is also undoubtedly one of the reasons contributing to the breakdown of the alpha helical structure at either end of the peptide chain.

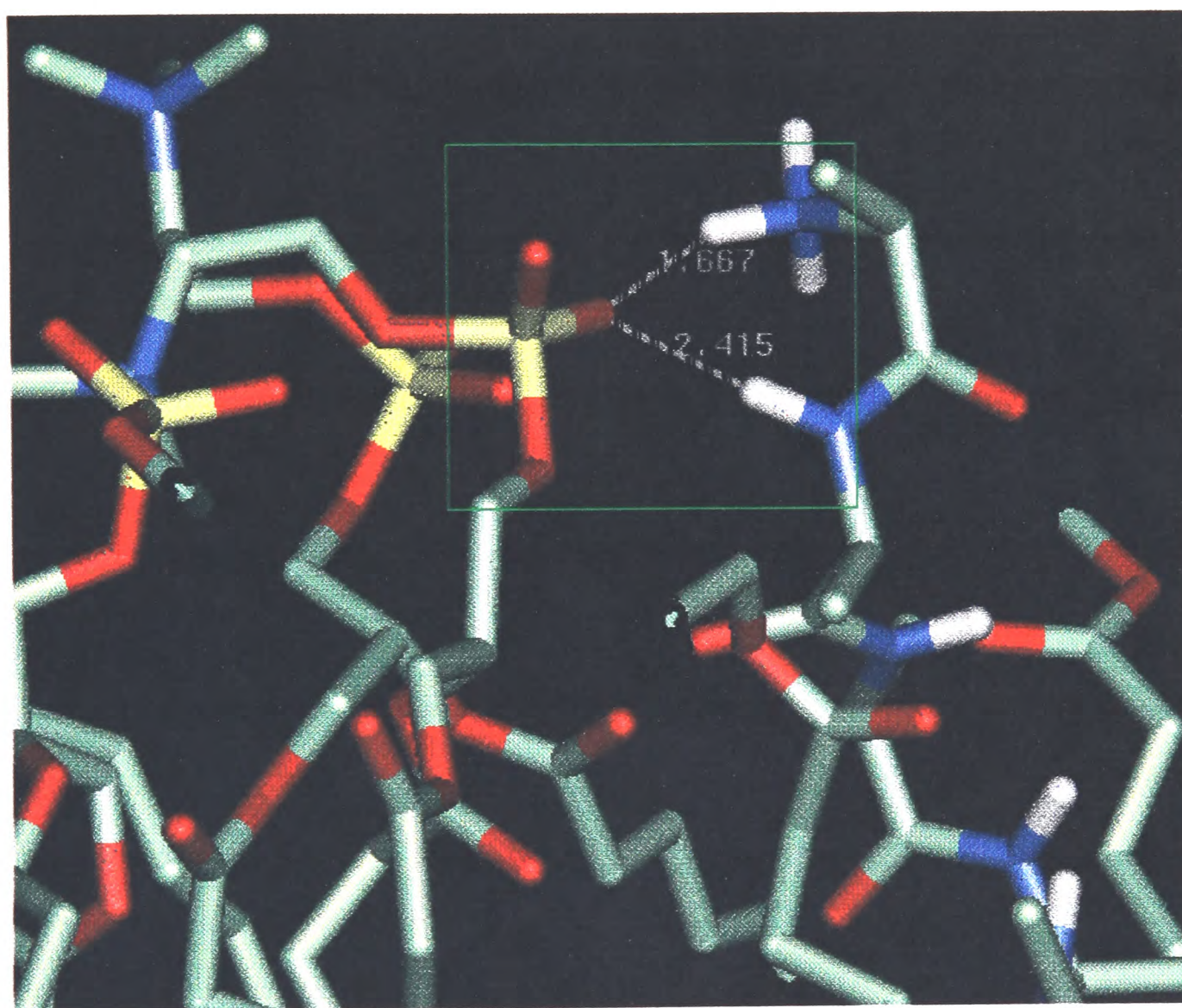


Figure 7.19 An example of hydrogen bonding between the peptide ammonium hydrogens (white) and the oxygens of the lipid phosphate group, high-lighted by a green box.

For this system there appears to be no evidence of hydrogen bonding between the peptide and the carbonyl groups of the glycerol moiety.

7.6.2 The Variation in Phi and Psi Angles of the Peptide within the Bilayer

The average values of the phi and psi angles were calculated over the course of the simulation along the length of the peptide chain and the results are presented in Figure 7.20.

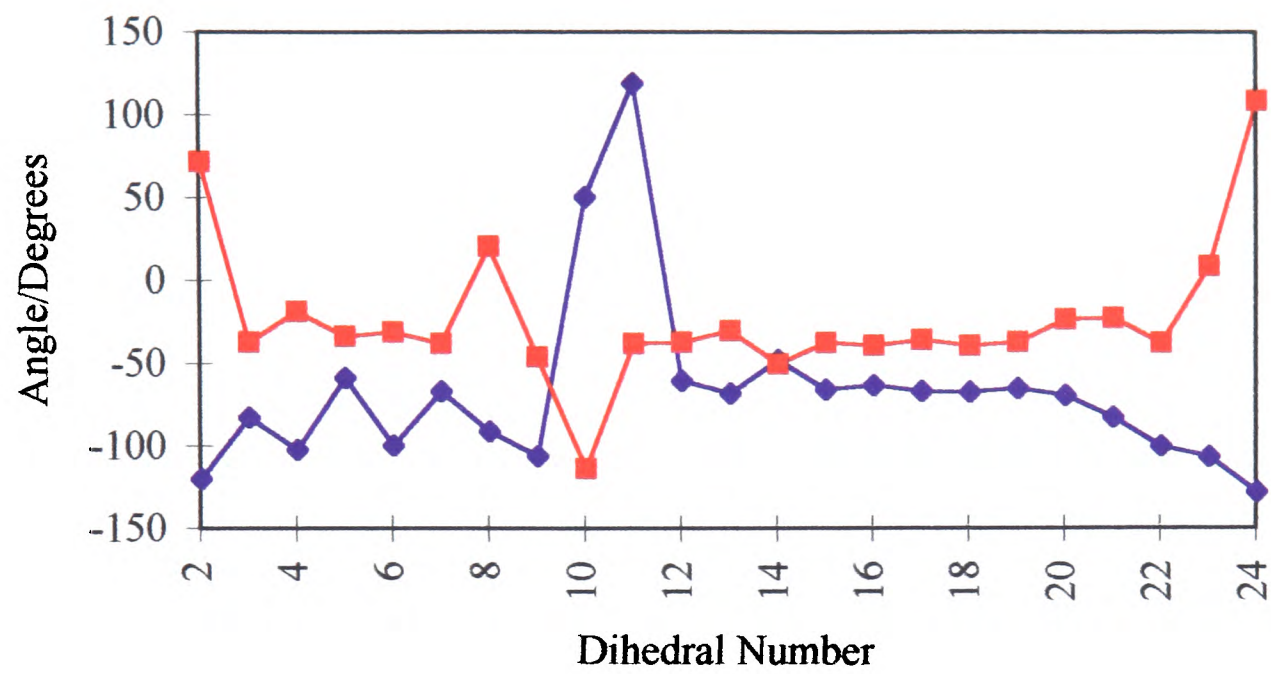


Figure 7.20 The variation in the value of the phi (blue) and psi (red) angles along the length of the peptide chain. Dihedrals 1-11 occur in layer 1 and dihedrals 12-25 are present in layer 2.

The divergence from the allowed phi and psi angle pairs as characterised by Ramachandran plots for alpha helices occur at the termini of the chain which is expected and also in the region of the peptide chain which occurs at the interface of the two monolayers, i.e. at the bilayer centre where it is observed that the chain bends. This breakdown of the structure and consequent loss of interaction energy lends weight to the hypothesis that a hinge may be present in trans-membrane peptides to allow easy bending of the peptide chain at the bilayer centre. Sections containing glycines are ideal candidates as they allow much greater freedom in the allowed phi and psi angle pairs in peptide chains.

7.7 Simulations of Ala₁₁-Gly-Ala-Ala-Gly-Ala₁₀ Peptides in Bilayers

The peptide Ala₁₁-Gly-Ala-Ala-Gly-Ala₁₀ system was chosen to study the likelihood that a Gly-X-X-Gly motif present in the centre of a trans membrane peptide could behave as a

simple hinge and allow the chain to bend easily without disrupting the rest of the alpha helical structure. A hinge would allow the peptide to accommodate the different motions and chain tilt distributions that may be occurring in either half of the hydrocarbon region of a bilayer.

The simulations were run in an identical fashion to the Ala₂₅ simulations including starting from the same bilayer starting structure but mutating the appropriate alanine residues of Ala₂₅ to glycine.

7.7.1 The Tilt of the Peptide within the Bilayer Relative to the Bilayer Normal and the Lipid Chains

For each layer the tilt that the axis of the peptide chain makes with the bilayer (see Figure 7.5) was calculated over the length of the simulation and the normalised histogram of the results is shown in Figure 7.21

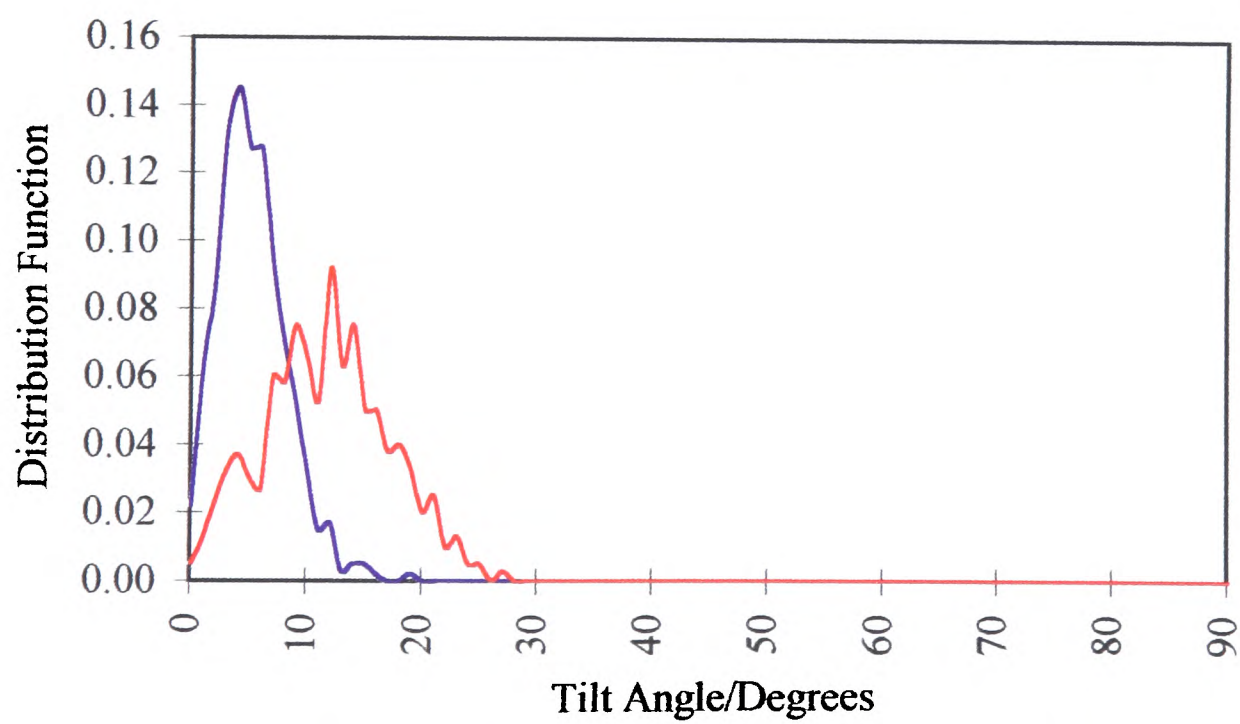


Figure 7.21 The tilt of the peptide chain with respect to the bilayer normal for layer 1 (blue) and layer 2 (red).

Although the section of the peptide in layer 2 does have a broader distribution than the part in layer 1, the magnitude of the tilt is certainly not as large as observed in the Ala₂₅ system (see

Section 7.5.1 and Figure 7.6). On examining snap shots of the dynamics simulation (see Figure 7.22), a slight bend in the peptide chain is sometimes observable, but this is certainly not as large as that seen in the simulation of Ala₂₅ and is transient.

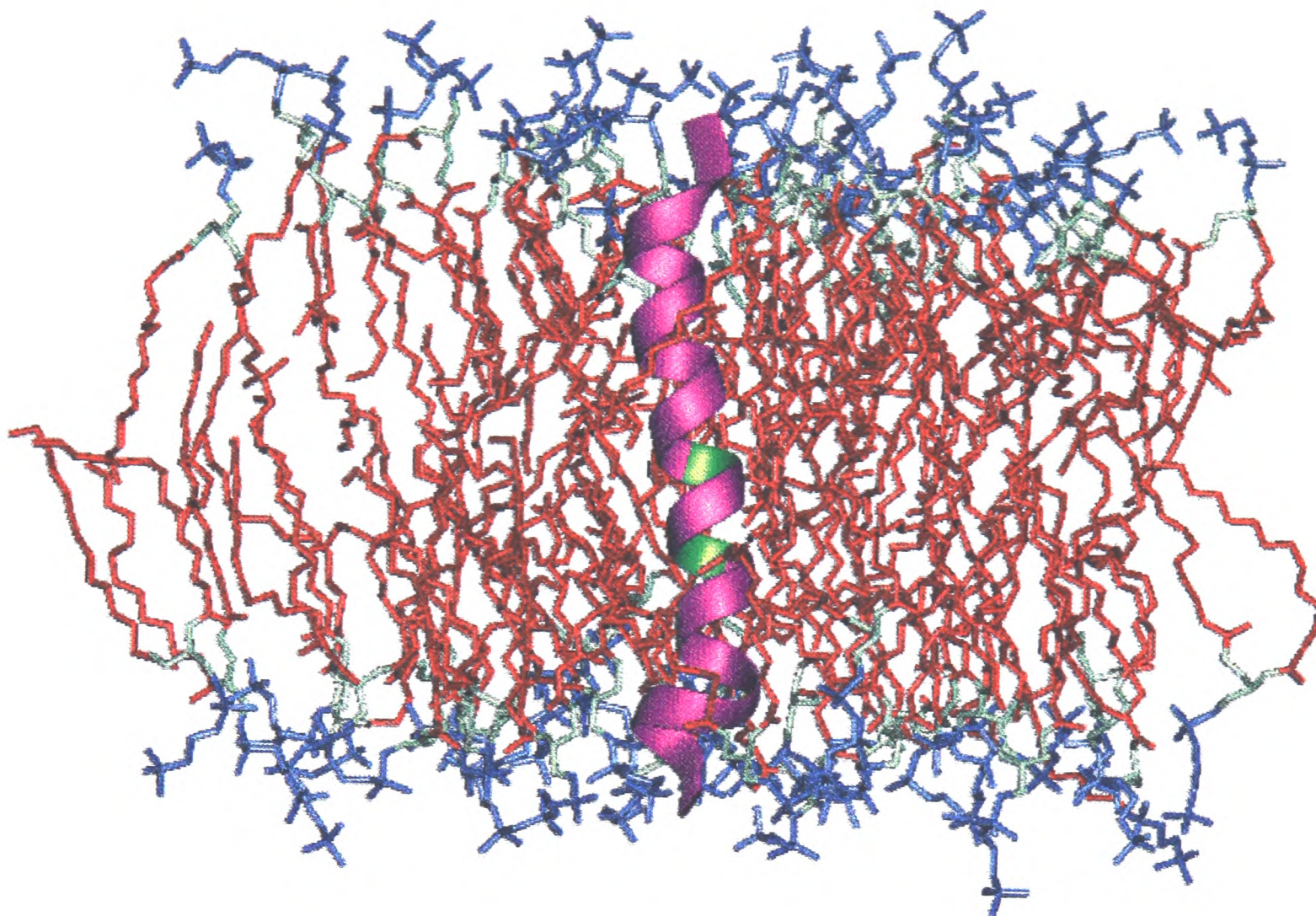


Figure 7.22 The structure of the bilayer at 400ps into the simulation. A slight tilt in the peptide chain is observable. The two glycine residues are coloured green and the rest of the peptide in purple.

The distribution of the angle between the peptide and lipid chains was also evaluated and showed very similar behaviour to layer 1 of the Ala₂₅ system with those lipids around the peptide illustrating a narrower range of possible tilt angles than those lipids further away.

7.7.2 Hydrogen Bonding within the Peptide

The average and standard deviation of the hydrogen bond lengths between carbonyl oxygens and the hydrogens of the nitrogen were calculated in order to characterise the stability of the peptide within in the bilayer and are listed in Table 7.2. As discussed previously,

intramolecular hydrogen bonding should be maintained within the bilayer interior as there are no other groups available with which it is possible to form interactions.

Amino Acid	Average/Å	Standard Deviation/Å	Amino Acid	Average/Å	Standard Deviation/Å
1	4.5	0.9	12 [*]	2.2	0.2
2	2.3	0.4	13	2.2	0.2
3	2.6	0.4	14	2.4	0.4
4	2.5	0.4	15 [*]	2.3	0.3
5	2.3	0.3	16	2.3	0.3
6	2.2	0.2	17	2.6	0.4
7	2.3	0.3	18	2.5	0.4
8 [*]	2.3	0.2	19	2.6	0.8
9	2.1	0.2	20	4.7	0.7
10	2.2	0.2	21	6.8	0.9
11 [*]	2.3	0.3			

Table 7.2 The average and standard deviation of the hydrogen bond lengths in the peptide over the course of the simulation. The first hydrogen bond is between the amino acids 1 and 5, the second between residues 2 and 6, etc. Amino acids marked with an asterisk are either a glycine (12 and 15), or forming a hydrogen bond with one (8 and 11).

Compared to the Ala₂₅ system it is apparent that this peptide is far more stable within the bilayer environment with all intramolecular hydrogen bonds well conserved and only at the termini of the amino acids does the hydrogen bond structure begin to break down between the amino acids. The hydrogen bonds that involve glycine have slightly larger average lengths and standard deviations, but the observed differences are very small (less than 0.1Å).

7.7.3 The Psi and Phi Angles of the Peptide within the Bilayer

To investigate further the structure of the peptide, the psi and phi angles along the length of the chain were calculated and the averages are presented in Figure 7.23 with the standard deviations in Figure 7.24. Although the average values are all similar in the main part of the peptide, the standard deviation of the psi angles shows two maxima which correspond to the two glycines and reflects their greater freedom of motion within an alpha helical structure.

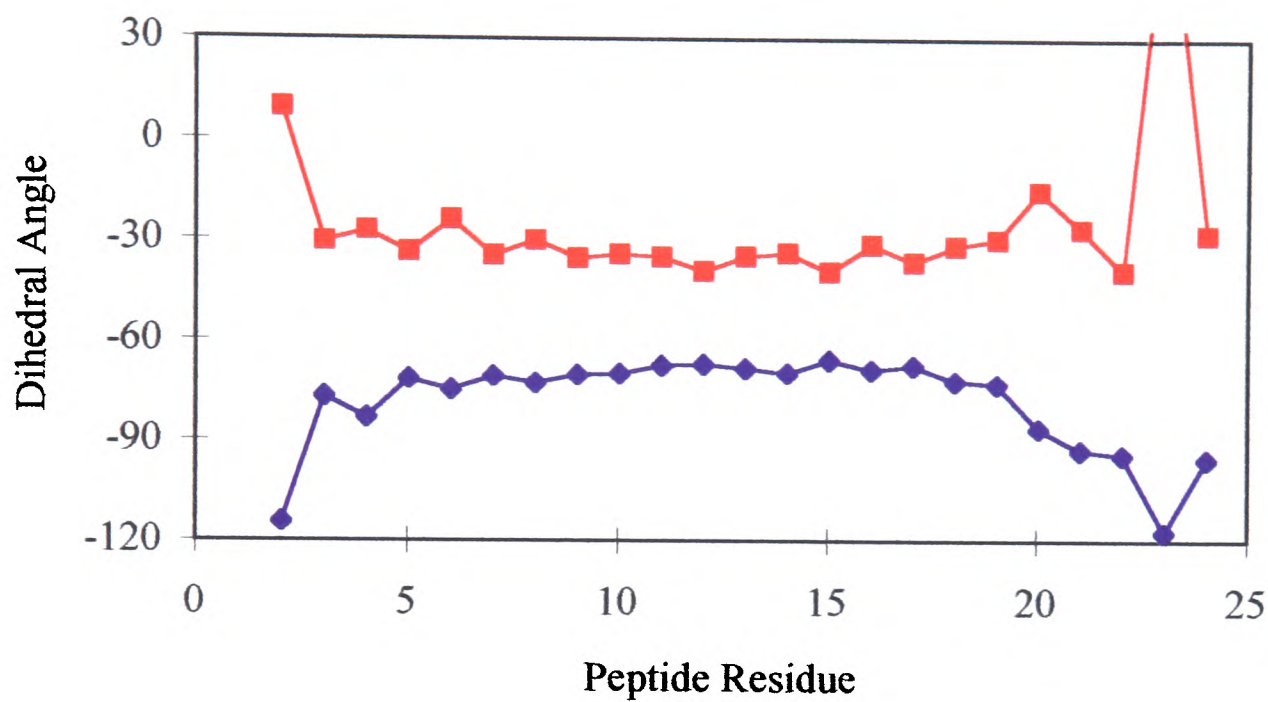


Figure 7.23 The psi (blue) and phi (red) angles of the peptide. Residues 12 and 15 are the glycine amino acids.

The phi angles of the two glycine residues (12 and 15) do show slightly different average values than the other alanines, plus the standard deviations for the psi angles are larger (see Figure 7.24) which all corresponds to the greater flexibility of the glycine residues.

It would appear that the glycine residues located in the middle of the peptide and placed in the centre of the bilayer do have a greater degree of flexibility than the surrounding alanines, however the differences are small.

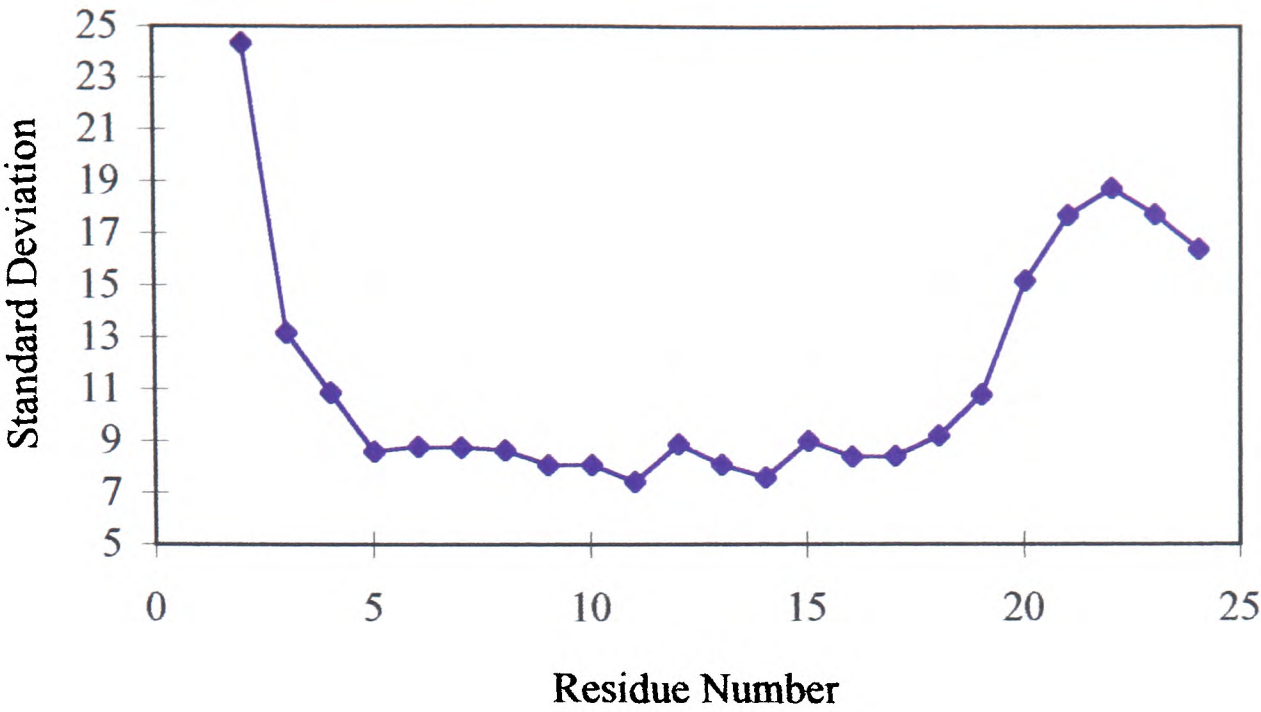


Figure 7.24 The standard deviation of the phi angles in the peptide. Residues numbers 12 and 15 correspond to the glycine amino acids and show slightly larger standard deviations than the alanine residues around them.

7.7.4 Conclusions on the Ala₁₁-Gly-Ala-Ala-Gly-Ala₁₀ Simulation

~~Unfortunately,~~ during the simulation of this peptide in a bilayer environment there was no clear evidence that the glycine behaved as a hinge structure although it could be construed that since the helix retained its alpha helical character, the presence of the glycines may have contributed to its stability. The glycines could provide weak links in the peptide chain that may flex and hence allow the peptide to bend within the bilayer environment without the energetically unfavourable breaking of hydrogen bonds and the deviation of psi and phi angles from preferred ranges of conformation. This could not occur as easily in the Ala₂₅ helix and may have contributed to the formation of the bend in the latter at the bilayer centre from which it did not recover.

7.8 Simulation of the Peptide Ala₂₅ in an Aqueous Environment

To study the effect that being in the bilayer environment may have on the structure of the peptide, a 500ps simulation of Ala₂₅ was run in a box of TIP3P water molecules under constant volume conditions and the structure and dynamics of the peptide chain compared with the results from the membrane simulation.

In terms of its general structure over the course of the simulation, the peptide retains a high degree of alpha helical character except at the ends where it started to unravel. For this reason the aqueous phase simulations were compared with the simulations of the glycine containing peptide as it was closer in structure to the aqueous phase peptide.

7.8.1 The Hydrogen Bonding and Psi and Phi Angle Behaviour

In Figure 7.25 and 7.26 the average and standard deviations of the hydrogen bond lengths with the peptide are compared for the membrane and aqueous simulations.

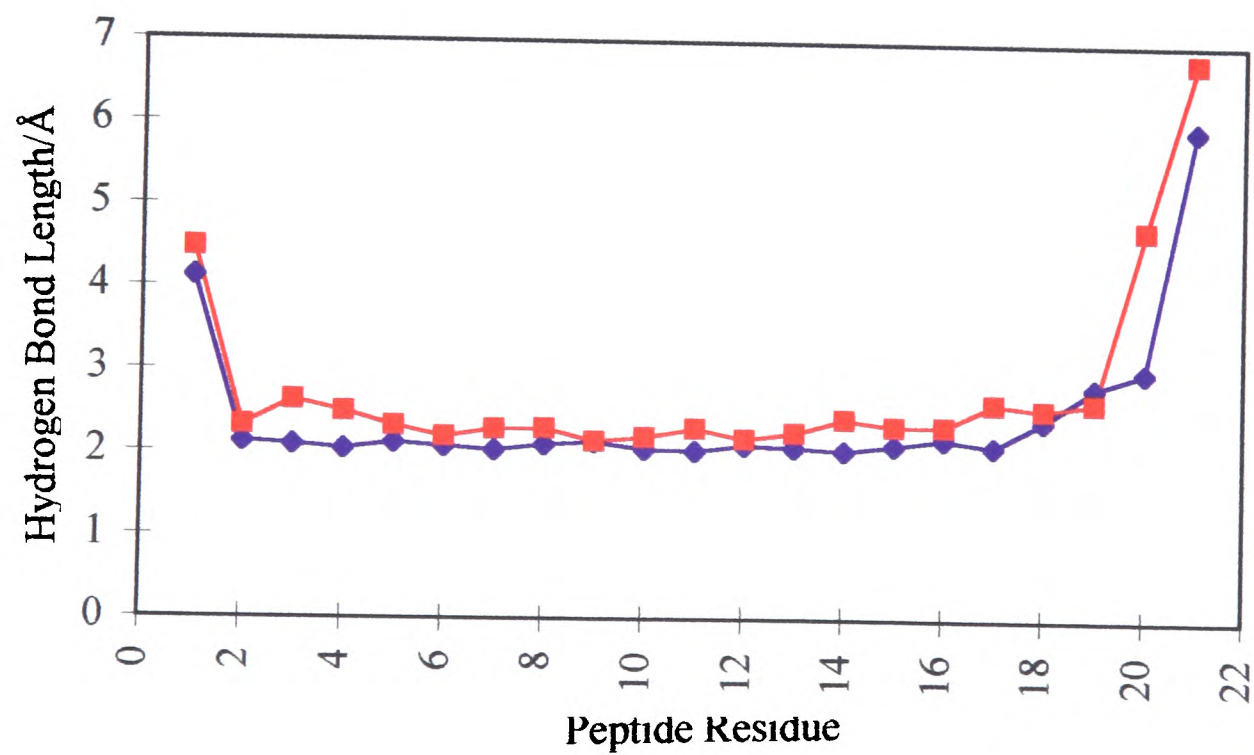


Figure 7.25 The lengths of the hydrogen bonds between residues in the peptide for the aqueous simulation (blue) and the membrane simulation (red).

From the two plots it can be concluded that the peptide is actually more flexible in the membrane than in the aqueous phase because not only are the hydrogen bonds generally

shorter in the aqueous phase simulations, but the standard deviations of the length are also smaller. This is consistent with a model in which the peptide within the membrane has not only greater free volume to move around in, but since the lipid chains are not held together by strong intermolecular forces as there are present in water (i.e. a hydrogen bonded network) which constrain the peptide, then the effective mobility of the peptide chain is greater since it is easier to move the chains apart, i.e. the peptide is not trapped within a strong solvent cage.

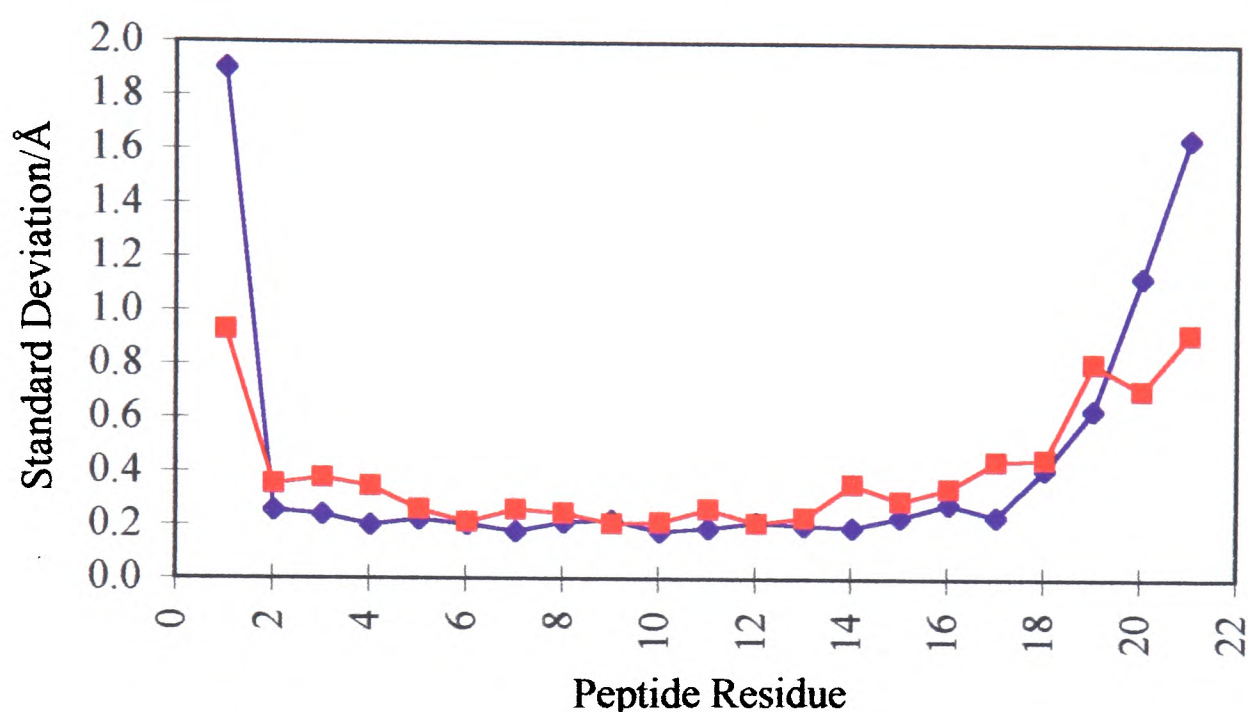


Figure 7.26 The standard deviations in the lengths of the hydrogen bonds in the alpha helix over the length of the simulation for the aqueous simulation (blue line) and the membrane simulation (red line).

7.8.2 Conclusions about Aqueous versus Membrane Peptide Simulations

As outlined in the previous section, if the alpha helical structure is retained, then the trans-membrane peptide has greater flexibility than when in the aqueous phase because of the free volume between chains in the bilayer plus the absence of strong intermolecular forces between hydrocarbon chains that hold the peptide rigid. i.e. The bilayer centre has a lower viscosity than water on the local scale allowing greater movement of the peptide.

However these same chains can also lead to behaviour such as the bend in the peptide chain of the Ala₂₅ simulation because of the extremely anisotropic environment of the lipid chains

(compared to water), perhaps forcing it to bend so it can be accommodated within the normal bilayer chain packing scheme in which the chains are tilted.

7.9 Conclusions of Chapter Seven

The trans-membrane proteins studied here are extremely simple because there are no large side chain groups but they illustrate some interesting features regarding the effect and interaction of peptides with lipids.

The simulation of Ala₂₅ is the most interesting because it represents a case in which for one half of the bilayer the lipid chains accommodate the protein and reduce their tilt angle while in the other half it is the peptide that changes its orientation so that it does not disrupt the preferred chain packing.

That two types of behaviour are observed during the simulations is quite natural given that both the peptide and the lipid molecules are relatively flexible but rigidity is imparted to the peptide because of its hydrogen bonding while the close packing of the lipid chains reduces their expected high flexibility. Whether the lipid or the peptide wins will depend upon a multitude of factors, however it seems perfectly reasonable that on normal time scales both situations will be encountered and interconvert.

Finally, the results of the analyses indicate that the peptides do have enough of a perturbing influence that the existence of an annular ring of lipids around peptides whose physical properties could be differentiated from lipids within the bulk is feasible.

In the next chapter a more complicated trans-membrane protein is considered, the bee venom melittin which contains side chains that will show interactions with the hydrocarbon chains and have a more complex behaviour than the peptides considered in this chapter.

Chapter Eight

Simulations of the Bee Venom Melittin with Lipid Bilayers

8.1 Introduction

Having studied the extremely simple Ala₂₅ and Ala₁₁-Gly-Ala-Ala-Gly-Ala₁₀ systems, the next logical stage of the research was to study a more complex peptide that is known to interact with lipid membranes. A toxic peptide is an ideal candidate to study because by its very nature it must perturb the structure and function of membranes. The peptide melittin which is the main component of the venom of the European honey bee was chosen and is a small peptide of 26 residues. Yet despite extensive experimental studies, its behaviour and interaction with lipid membranes is still not understood fully and it is thus a challenging and interesting system to study.

The reason for this uncertainty is because melittin actually has several modes of behaviour and which is observed depends upon experimental conditions and the experimental technique used to observe the system. What is known is that melittin causes cells to undergo hemolysis, lysis and fusion plus voltage-dependent ion-conductance which suggests that melittin has a trans membrane conformation and forms a voltage-gated channel or pore structure within the membrane. However there is also substantial evidence that melittin adsorbs strongly onto cell membrane surfaces and hence disrupts them. (For a review see Dempsey, 1990).

The powerful hemolytic activity of melittin could result from three possible modes of action:

- 1) The perturbation of bilayer lipid organisation due to the presence of the amphipathic helix in the head group region
- 2) The formation of ion permeable channels and voltage-gated pores

3) The peptide causes micellization of membranes leading to holes in the cell

It has been shown that the hemolytic activity of melittin is not related directly to its surface activity (Habermann, 1972) and experiments with synthetic analogues of melittin which retained its amphipathic character, but not its sequence, showed that neither a defined sequence nor a central proline are required for hemolytic activity (DeGrado et al, 1982). There is no direct evidence that voltage-gated channel formation is responsible for hemolytic activity

Since the exact nature of the interaction of melittin with membranes is unclear, a number of simulation studies were done which aimed to replicate possible stages in the mechanism of the interaction of melittin with DMPC bilayers. The order of events was-

1. Free melittin in the aqueous phase
2. Melittin adsorbed onto the surface of a membrane
3. A single melittin spanning the lipid bilayer
4. A group of aggregated melittin molecules forming a hypothetical pore structure

Step 1 with the melittin in the aqueous phase aimed to study its behaviour during its journey to the cell membrane and step 2 represents the first associated structure that the melittin and bilayer probably adopt with the melittin interacting with the lipid head groups. Stage 3 with a melittin spanning the bilayer is the stage that occurs after the melittin has moved from its surface adsorbed state. Finally in stage 4 a group of melittin molecules have come together to form a pore structure. This sequence of events are shown pictorially in Figure 8.1.

For each of these simulations the behaviour of the melittin was studied as well as its effect on the components of the lipid membrane with which it was interacting.

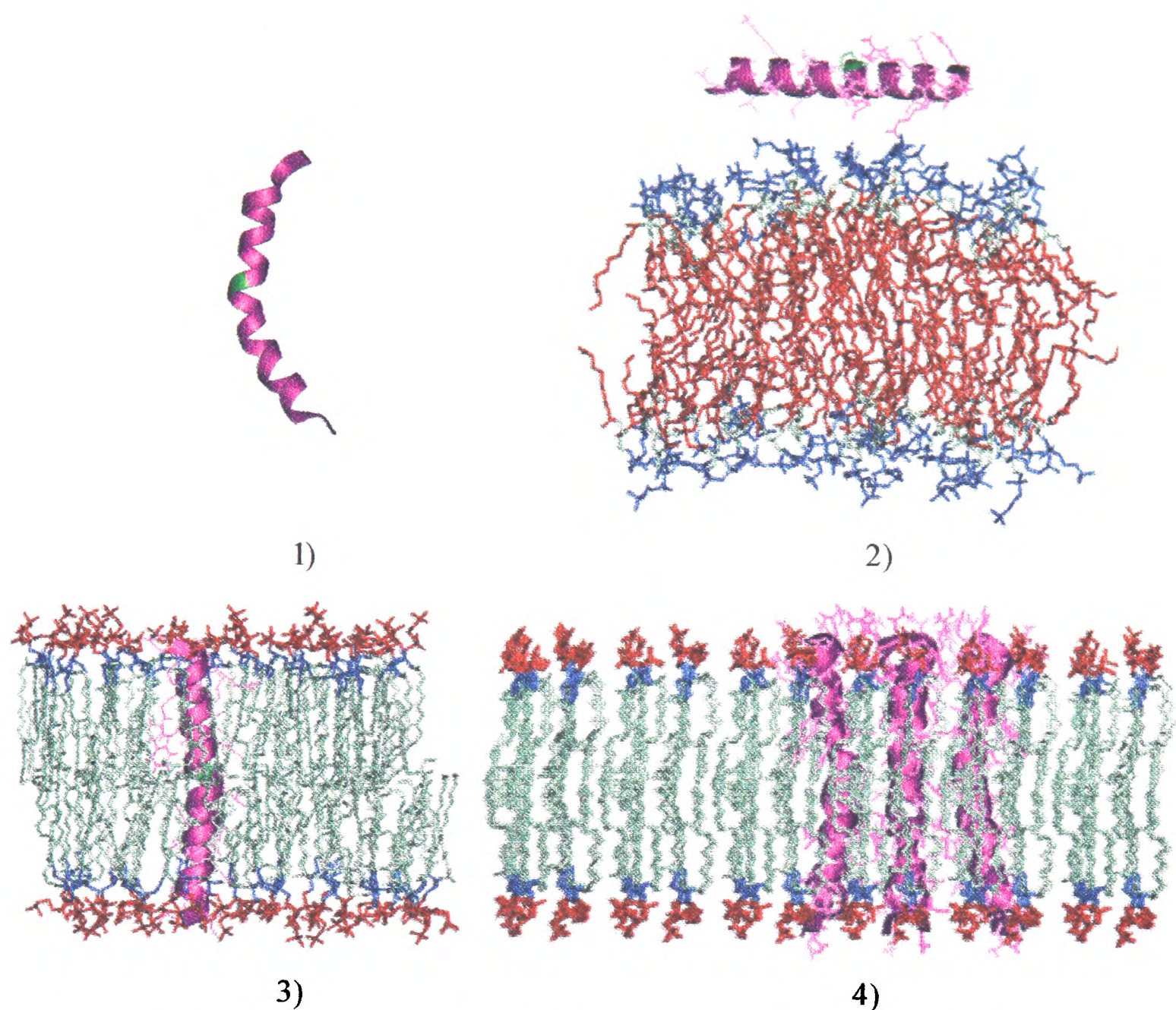
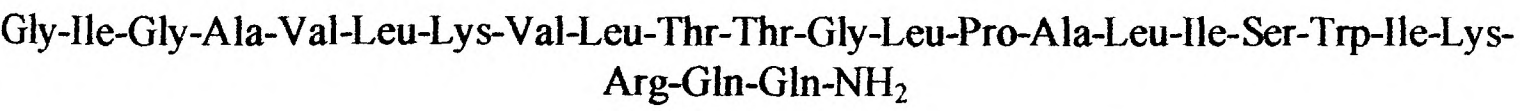


Figure 8.1 A possible sequence of events to describe the interaction of melittin with lipid bilayers leading to the possible formation of a pore structure.

8.1.1 The Molecular Structure of Melittin

The amino acid sequence of melittin is-



Although it is essentially a hydrophobic peptide, it has an overall charge of +6 from the four charged groups of the sequence Lys-Arg-Lys-Arg at the C-terminus which have a very basic nature, plus the N-terminal amino group and a single lysine at the other end of the peptide.

There are no acidic groups in the melittin molecule and if the melittin molecule is built as an alpha helix (see Figure 8.5) then an interesting structural property becomes apparent, the polar residues are all essentially located on one side of the helix and all the non-polar on the other, i.e. the peptide has amphipathic character and this subject will be returned to during the discussion of how melittin may bind to the membrane and go on to form a pore structure. Meanwhile Figure 8.2 shows a wheel diagram in which melittin in an alpha helical conformation is viewed down its long axis and the hydrophobicities of the groups are plotted on it showing their position with respect to each other.

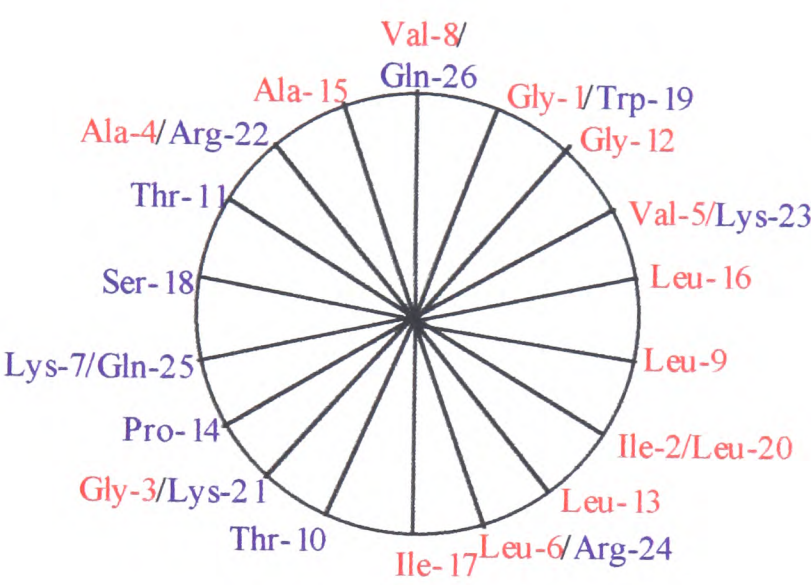


Figure 8.2 A wheel diagram of melittin showing the distribution of amino acids around the alpha helix. Hydrophobic residues are labelled in red and hydrophilic in blue.

Figure 8.2 shows that with the exception of the last few residues of the C-terminus which are all hydrophilic, over half of the peptide is hydrophobic around its circumference. Figures 8.3 and 8.4 plots the hydrophobicities of the amino acid residues along the length of the peptide on opposite sides, i.e. the first plot refers to the hydrophilic side and the second to the hydrophobic side. These series of plots emphasise the amphipathic nature of melittin which must be an important criterion in facilitating its mode of action.

Melittin has a proline as one of its central residues which in the alpha helical structure confers a tilt in the chain. Since previous simulations have shown that a trans membrane peptide will bend about the bilayer centre (see Chapter 7), this proline may well be an

important indication that melittin does have a trans membrane state and a bend is necessary to stabilise the structure in the bilayer environment.

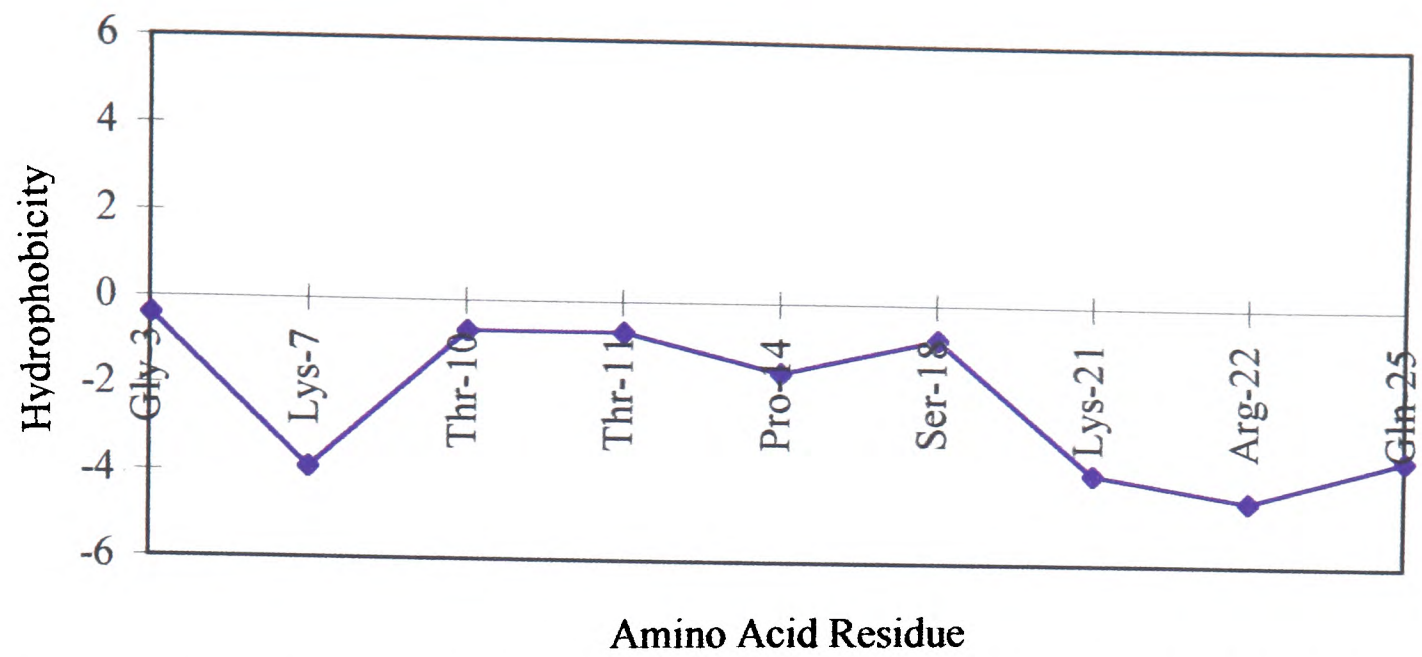


Figure 8.3 The hydrophobicities along the hydrophilic side of the melittin alpha helix.

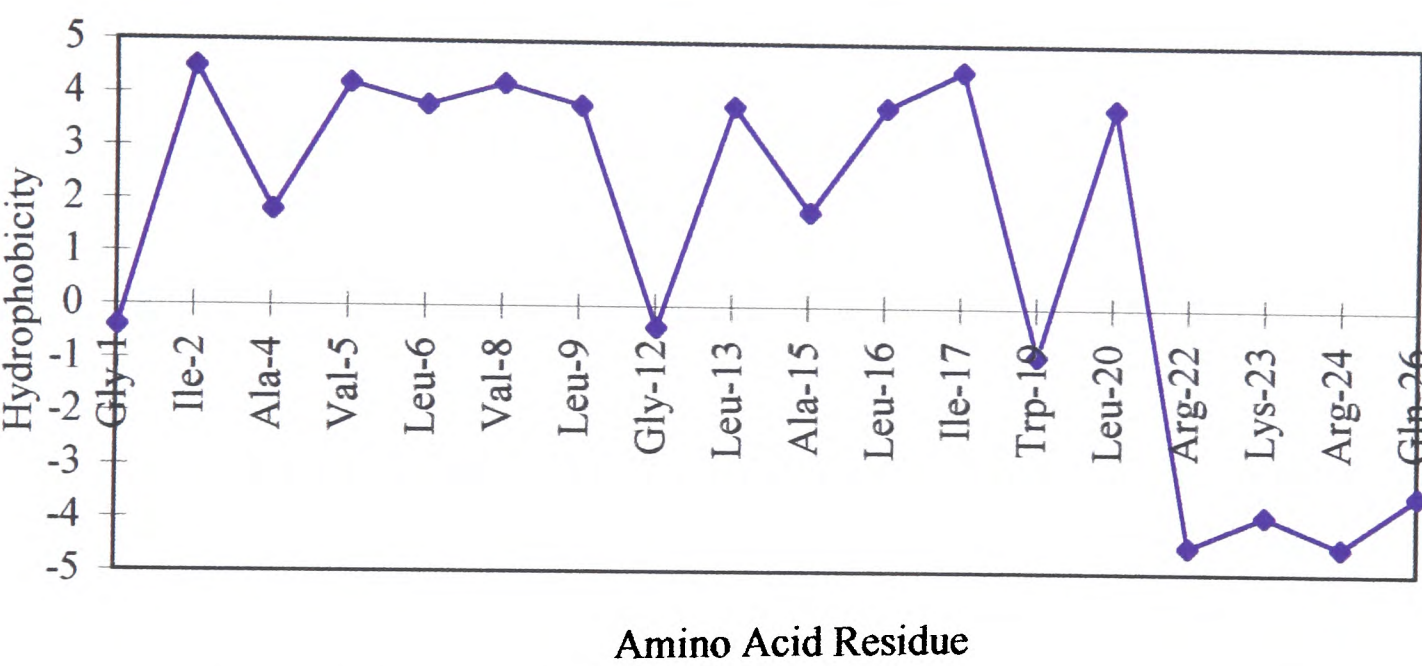


Figure 8.4 The hydrophobicities along the hydrophobic side of the melittin alpha helix.

NOE measurements of melittin in methanol show it to be monomeric and in the alpha helical conformation except around the proline residue emphasising the relaxation possible in alpha helical structure around this residue and is a situation which could also occur in a bilayer environment (Dempsey, 1988). Amide exchange studies show the proline residue increases flexibility at the centre of the bilayer which was concluded to be further evidence of a hinge structure that may be important to some of the behaviour of melittin (Pastore et al., 1989).

Amide exchange of melittin in methanol (Dempsey, 1988; Pastore et al., 1989) showed that the behaviour of the melittin can be characterised as two helical segments connected by a flexible segment containing the proline residue. Furthermore, the residues in the C-terminus half are observed to have more rapid exchange with solvent deuterons than the N-terminus half which indicates that the hydrogen bonds are stronger in the latter and thus the helix is more rigid.

8.2 The Binding of Melittin to Lipid Bilayers

There is still great debate about whether melittin binds to the surface of bilayers, is a trans membrane protein, or shows both types of behaviour. There is quite clear evidence that membrane associated melittin is alpha helical in conformation, e.g. from spectroscopic measurements (Drake and Hider, 1979; Vogel, 1987).

Electrophoretic mobilities analysed with a Gouy-Chapman double layer theory show that melittin is strongly adsorbed onto membrane surfaces (Ohki et al., 1994). X-ray and neutron diffraction studies show that melittin has more than one binding location in the membrane, suggested as corresponding to a surface binding mode and a more deeply buried mode (Bradshaw, Dempsey and Watts, 1994). Using fluorescence, CD and FTIR experiments to compare the alpha helical character of melittin in monolayers and bilayers it was hypothesised that melittin adsorbed onto the monolayer surfaces had less helical character than when it is inserted into bilayer membranes (Sui et al., 1994).

Micelle bound melittin is alpha helical with a loss of NOE contacts in the central region corresponding to residues 11-12 (Inagaki et al., 1989) which is consistent with their being additional flexibility in this region. These experiments also conclude that it is the non-polar side of the helix which is facing into the micelle interior since deuteron amide exchange with the solvent is slower for residues on the hydrophobic side.

Probably the best evidence of a trans bilayer configuration comes from solid state NMR experiments in which 10 analogues of melittin were synthesised which had ^{13}C enrichment of different peptide carbonyls along the chain (Smith et al., 1994). The NMR detected the

position of these carbon isotopes in oriented membranes and showed that the peptide was well aligned in bilayers and oriented about the bilayer normal.

Experiments to determine the order parameters of lipids in bilayers containing melittin have shown that in the gel phase the methylenes at the bilayer centre are more ordered while in the liquid phase the lipid chains are disordered (Dufourc, Bonmalm and Dufourcq, 1989).

It does appear that whether the peptide is aligned parallel or perpendicular to the membrane surface depends upon the degree of hydration of the bilayer. At low hydration experiments detect a trans bilayer orientation while at higher hydration (which is closer to the natural state of biomembranes) the bilayer is oriented with the surface. For example CD experiments on the gel phase showed alpha helical segments of melittin to be oriented approximately perpendicular to the oriented bilayers (Vogel and Jahnig, 1986; Vogel, 1987). Neutron diffraction experiments at higher hydration detected a surface parallel orientation (Dempsey, 1990). The binding site of melittin was probed indirectly through experiments in which amino groups were chemically modified to determine their solvent accessibility and suggested that the melittin was bound at the surface (Stanislowski and Ruterjans, 1987).

An important factor missing in many of these experiments which undoubtedly effects the orientation of the melittin is a trans membrane potential. The ability of melittin to form voltage-gated ion channels is voltage dependent, implying that the melittin undergoes a voltage dependent reorientation in the membrane in order to attain a trans bilayer configuration (Kempf et al., 1981). Measurements by Hanke et al., (1983) show the N-terminal section of melittin moves across a membrane under the influence of a trans membrane potential.

Certainly the interaction of melittin with bilayers is complex and the binding mode is dependent on bilayer composition, phase, hydration and the presence of a trans membrane potential.

The binding of melittin to membranes has also been simulated using a bilayer modelled with a hydrophathy scale (Milik and Skolnick, 1993). Their Monte Carlo simulation showed that the melittin had a random conformation outside the bilayer but when it adsorbed onto the

surface it gained a slightly ordered helical structure. After a while the N-terminus moved into the bilayer interior and eventually found the other side of the bilayer and became oriented perpendicular to the bilayer surface. In effect the melittin behaves as two helical fragments which are connected by a flexible fragment containing the Pro-14.

Evidence for the existence of melittin aggregating to form pore structures will be summarised in Section 8.12.

8.3 Simulations of Melittin in Water

Melittin is surprising soluble in water despite its high number of hydrophobic groups but this solubility is necessary for it to travel from its point of entry to the target site of the cell membrane. The consensus on the behaviour of melittin in water is that its structure and aggregation state are highly dependant upon conditions, but essentially that it exists either on its own with no discernible secondary structure (Talbot et al, 1979; Dawson et al, 1979; Knoppel, Eisenberg and Wickner, 1979) or in a tetramer of alpha helical coils that places hydrophobic residues together and hence shields them from the aqueous environment (Terwilliger and Eisenberg, 1982). However when melittin is in the presence of a bilayer surface then it preferentially adopts the alpha helical conformation (Drake and Hider, 1979; Vogel, 1987; Ohki et al., 1994).

The simulations of melittin in water focused only on the behaviour of a single molecule in water and the initial structure of melittin was built in a modelling program and assigned an alpha helical secondary structure. The OPLS nonbonded parameter set was used for melittin to maintain consistency with the TIP3P water model and the subsequent simulations of melittin with bilayers.

All energy calculations were done using the AMBER 4.1 molecular mechanics program in the NVT ensemble at 328K. Equilibration of the system involved a minimisation of the water molecules followed by a 50ps molecular mechanics simulation to relax and equilibrate the water molecules around the peptide before the positions of all atoms within the system were energy refined. The molecular mechanics simulation of the hydrated system was 470ps long

with the first 70ps being considered as the equilibration phase. Figure 8.5 shows some snapshots of the melittin molecule during the simulation.

Over the course of the simulation a pronounced bend appears in the melittin structure about the proline residue, however the peptide retains its alpha helical structure. A bend of 60° around Pro-14 is seen in the crystal structure of the tetrameric state (Terwilliger and Eisenberg, 1982) and a smaller bend of 20° was observed to occur in a short molecular dynamic simulation of melittin in methanol (Bazzo et al, 1988).

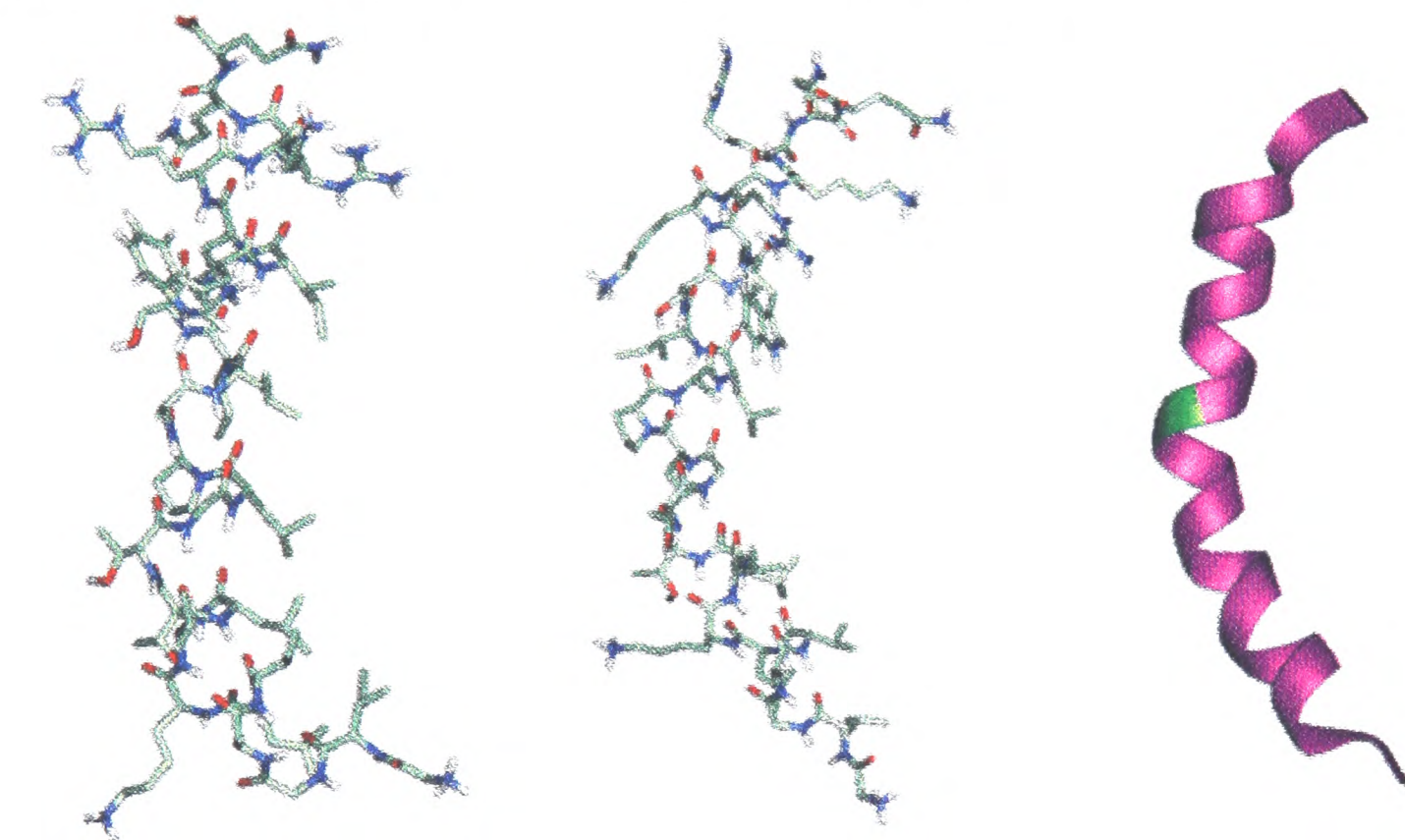


Figure 8.5 Snapshots of the melittin molecule during the simulation after a) 70ps b) 400ps c) 400ps using a helix representation with the proline coloured green.

8.3.1 Hydrogen Bonding in Melittin

The average and standard deviations of the lengths of the hydrogen bonds that hold the peptide in the alpha helix conformation were calculated over the 400ps simulation and are presented in Figure 8.6 and 8.7 respectively.

Over the course of the simulation, hydrogen bonding is maintained between the residues that hold the peptide in the alpha helical conformation, i.e. there is no breakdown of the alpha helical structure, except at the termini of the chains.

The residues Gly-1, Gly-3, Ala-4, Ile-20, Lys-21 and Arg-22 show long hydrogen bond lengths yet only residue Lys-21 has a large standard deviation which implies that some of the other residues must be locked in place by other intramolecular hydrogen bonded interactions.

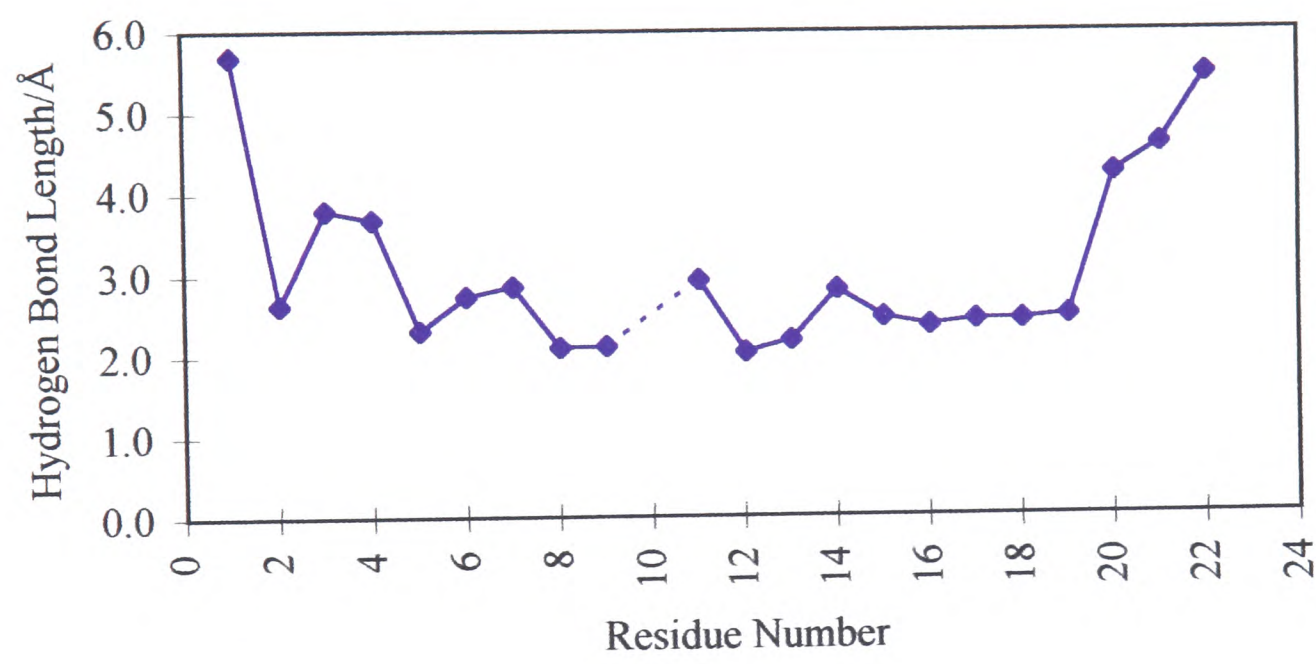


Figure 8.6 The hydrogen bond lengths between residues. The value for the 10th residue, threonine, is missing because a hydrogen bond cannot be formed with the proline.

An examination of an example structure from the melittin simulation shows that some of these residues do indeed form other intramolecular hydrogen bonds, e.g. the carbonyl oxygen of Ile-2 is hydrogen bonded to the amine of Val-5 and similarly there are hydrogen bonds between the main chain atoms of Gly-3 and Leu-6, Arg-22 and Arg-24 and finally between Ile-20 and Lys-23. Some of the side chains also form hydrogen bonds with other groups within the structure, e.g. the hydroxyl hydrogen of Ser-18 and the carbonyl of the Pro-14, and between the hydroxyl hydrogen of Thr-11 and the carbonyl of Lys-7.

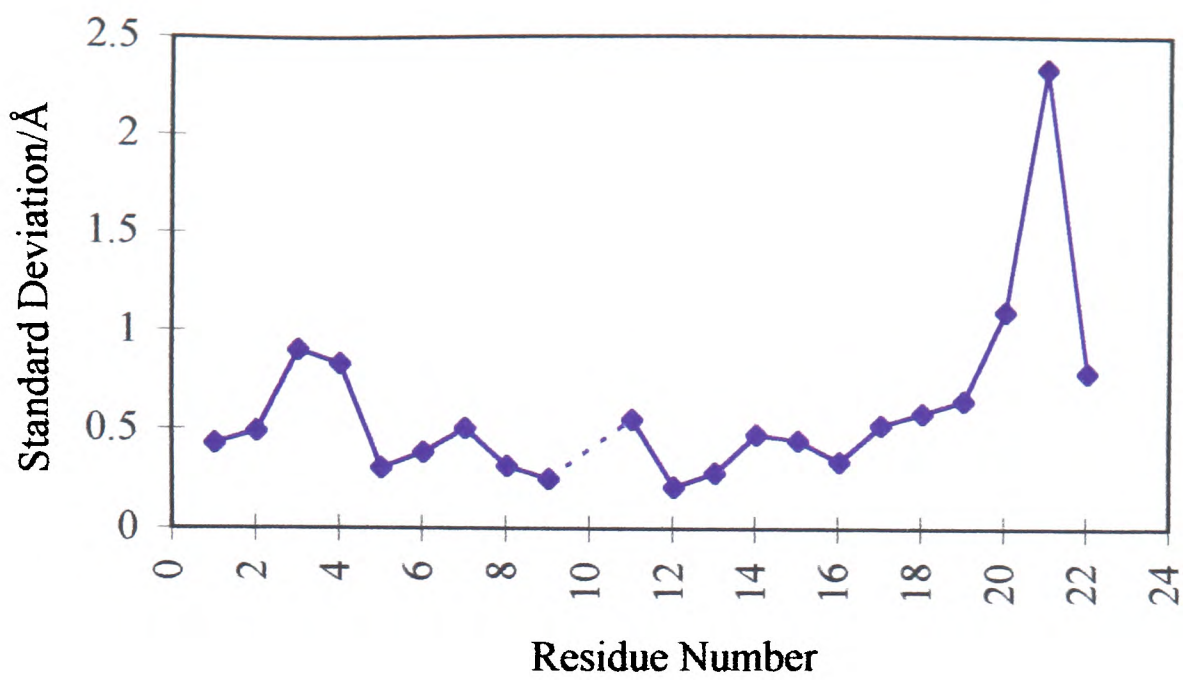


Figure 8.7 The standard deviations in the lengths of the hydrogen bonds between residues.

8.3.2 The Behaviour of the Psi and Phi Angles of Melittin during the Simulation

Figure 8.8 shows the average and standard deviation of the psi angles calculated for the melittin over the course of the simulation and Figure 8.9 shows the phi angles.

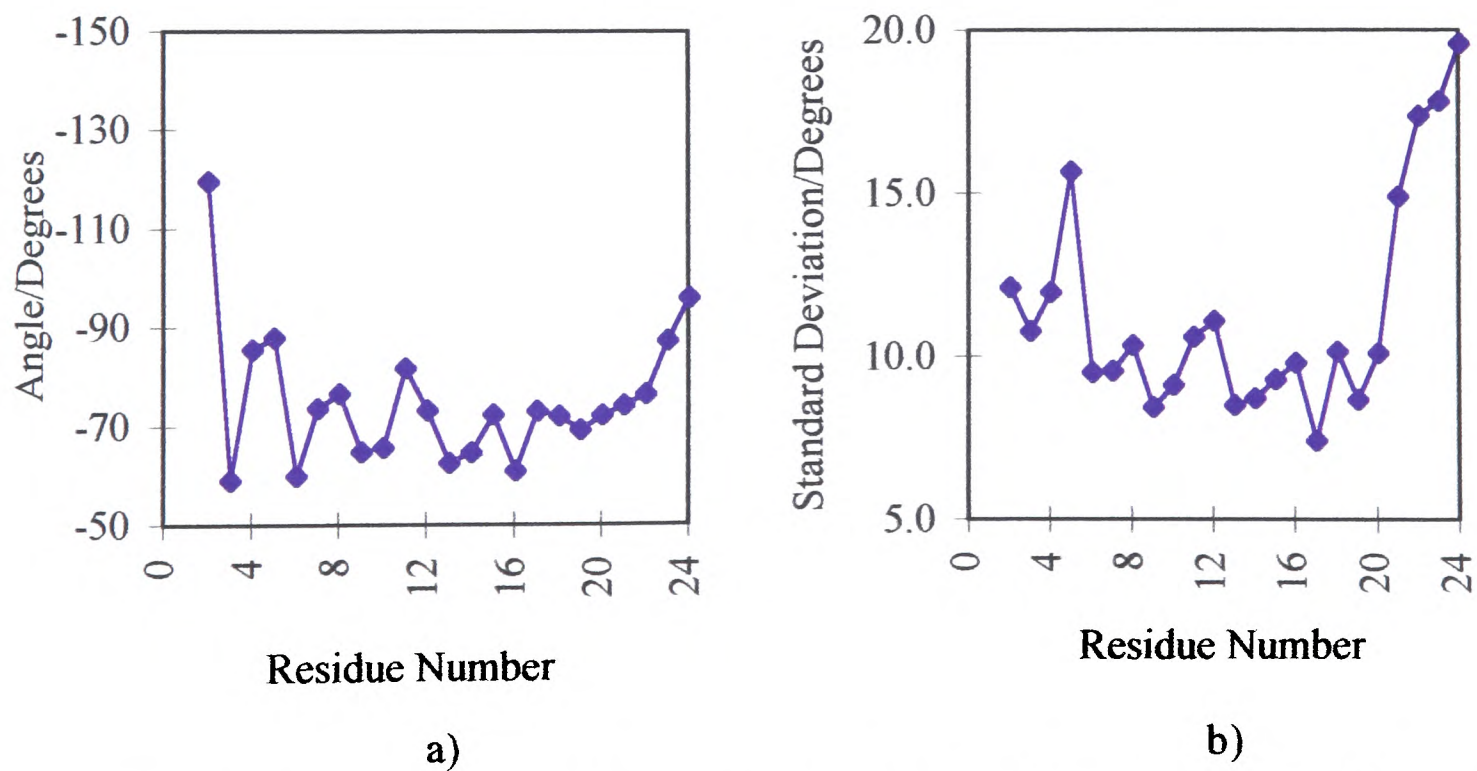


Figure 8.8 The a) average and b) the standard deviation for each residue of the melittin molecule over the course of the simulation in the aqueous phase.

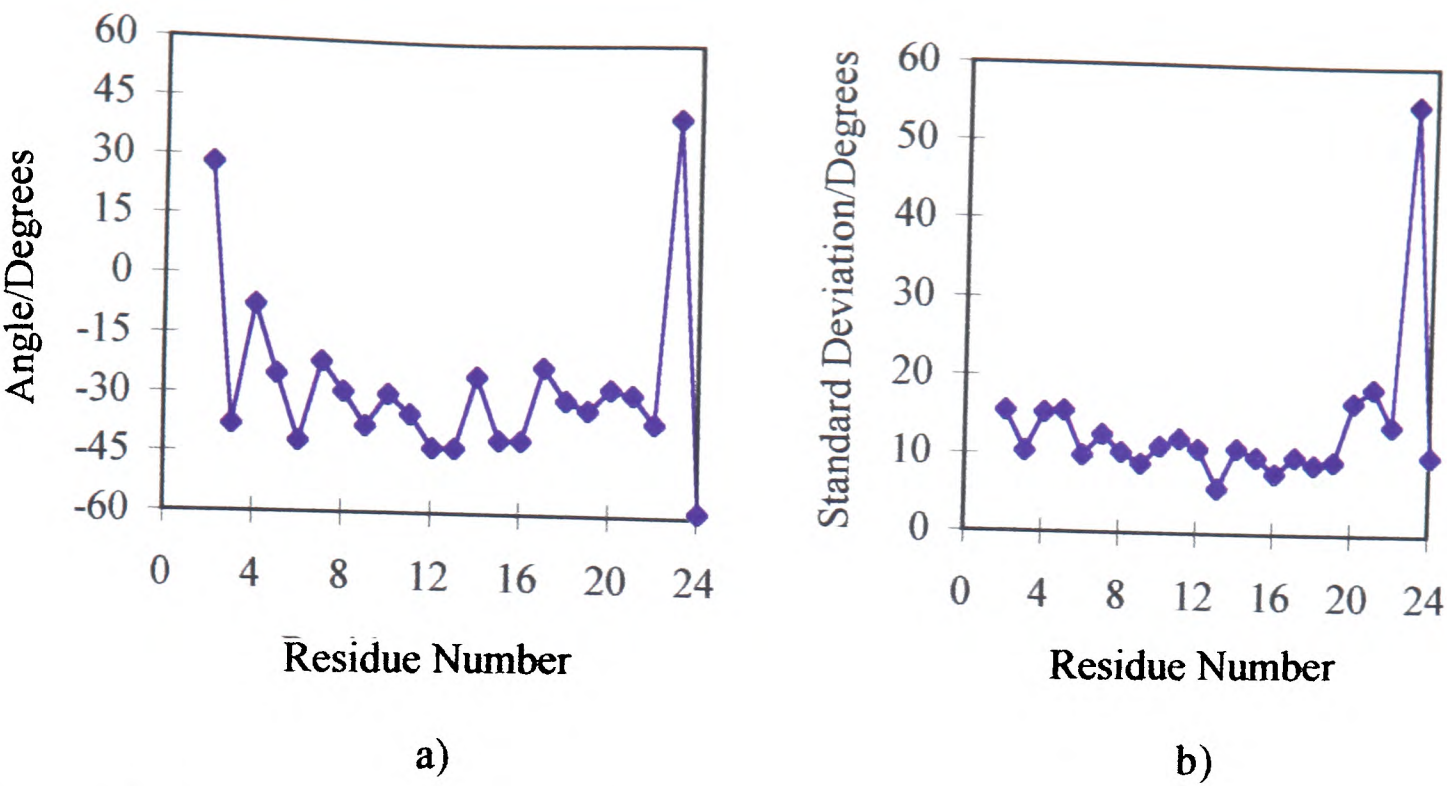


Figure 8.9 The a) average and b) standard deviation of the phi angles over the course of the simulation for the melittin molecule.

Figures 8.8 and 8.9 provide further evidence that during simulations of melittin in the aqueous phase it retains much of its alpha helical character except at the termini of the chains where it starts to unwind.

8.4 Conclusions on the Simulation of Melittin in Water

From the short simulation studied here, it would appear that the melittin molecule in the alpha helical conformation is stable under the simulation conditions. This is in conflict with experimental evidence on the behaviour of melittin in water and may simply reflect the melittin molecule being stuck in a potential energy minimum because of the strong intramolecular hydrogen bonding from which it cannot escape during the course of the simulation.

8.5 Simulations of Melittin Adsorbed onto the Surfaces of Membranes

There is plenty of evidence that melittin molecules may become adsorbed onto lipid bilayer surfaces (e.g. Ohki et al., 1994) and if insertion of the melittin molecule through the bilayer is to occur, then this is probably a preliminary stage (see Dempsey, 1990). It is also well recognised that when peptides with little or no secondary structure in free solution (see Section 8.4) bind to lipid bilayers then secondary structure is introduced (Kaiser and Kezdy, 1983). This occurs because when a peptide associates with an interface the probability of secondary structure formation increases dramatically as motion of the peptide is reduced and lowers the entropic barrier to protein folding occurring (Wattenbarger et al., 1990; Chan et al., 1991).

All of the studies on melittin binding to membranes indicate that the peptide has an alpha helical conformation plus studies of melittin in methanol show that it adopts an alpha helical conformation with its ^1H -NMR spectra very close to that observed for melittin associated with phosphatidylcholine micelles. For this reason the initial conformations of melittin built for the simulations had an alpha helical secondary structure.

Since the melittin molecule is amphipathic it was apparent that there were two opposite models for the interaction of melittin with the bilayer surface. The first has the hydrophobic side approaching the bilayer surface and the second has the charged side approaching the head groups. In the first model it is very simple for the melittin molecule once it is on the surface of the bilayer to lose solvating water and move further into the membrane so that the hydrophobic side is completely shielded from water. In the second model, once the melittin is within the head group region it is necessary for it to rotate through 180° for the hydrophobic portion which is facing into the aqueous phase to become shielded. These two mechanisms are shown schematically in Figure 8.10.

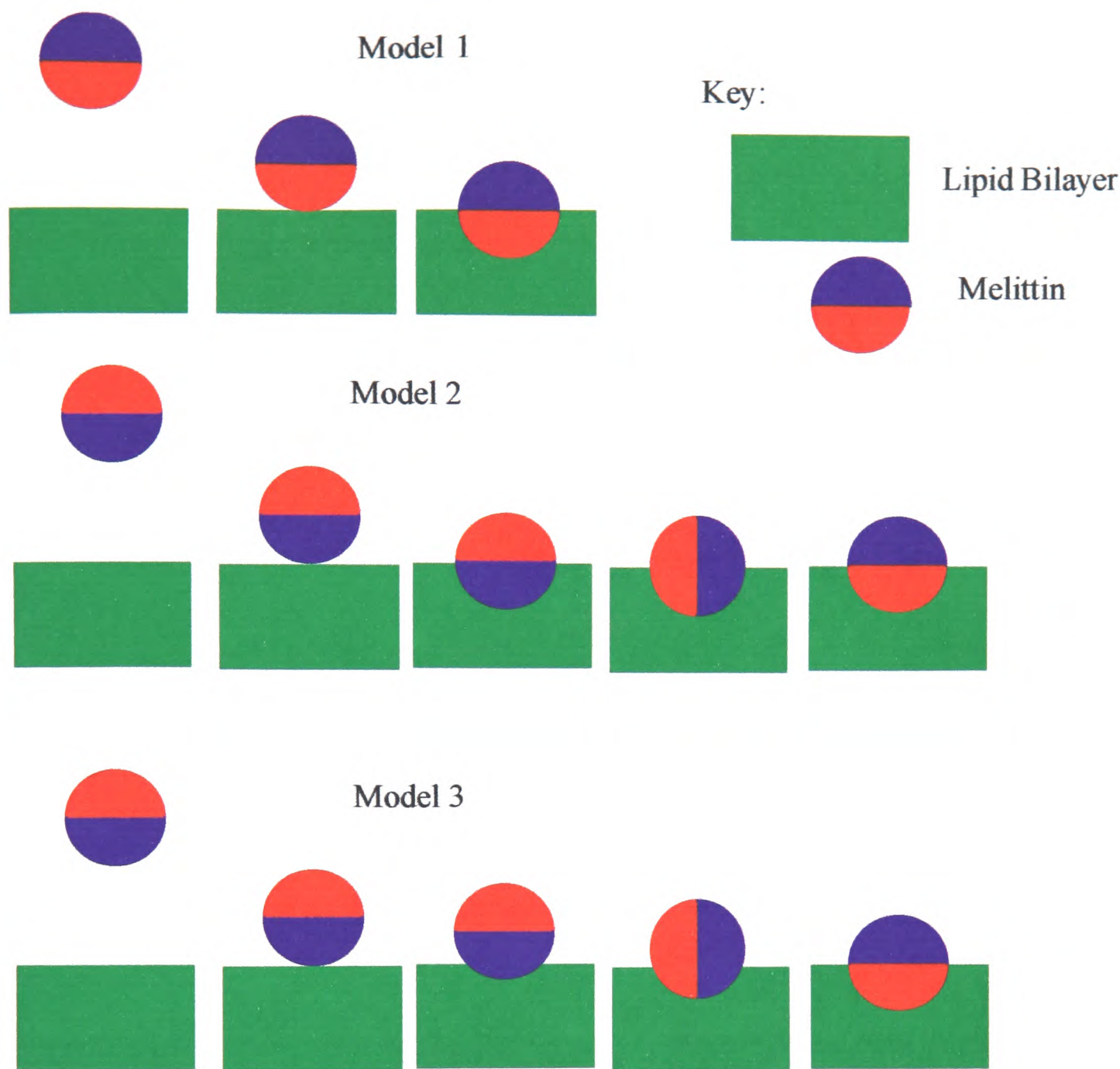


Figure 8.10 Three possible mechanisms for the adsorption of melittin (viewed along its long axes) into the head group region of bilayers. The hydrophobic half of the peptide is shown in red and the hydrophilic in blue. Model 1 has the hydrophobic side of the melittin approaching the bilayer and model 2 has the charged, hydrophilic side which then necessitates the melittin turning in the membrane. Model 3 is a more refined version of model 2.

A third plausible mechanism involves the melittin molecule approaching the bilayer with its charged side and becoming associated with the surface, then rather than integrating itself deeper into the membrane and then rotating, it rolls into the membrane, i.e. the rotation occurs as the melittin becomes imbedded deeper into the membrane (see Figure 8.10).

All these models presuppose that the hydrophobic side of the melittin will wish to become embedded within the hydrophobic core of the bilayer as it has a very low water concentration.

8.5.1 Simulation of Adsorbed Melittin- Model 1

In this model the melittin was placed on the surface of a pre-equilibrated bilayer structure and the system hydrated with TIP3P water molecules (see Figure 8.11).

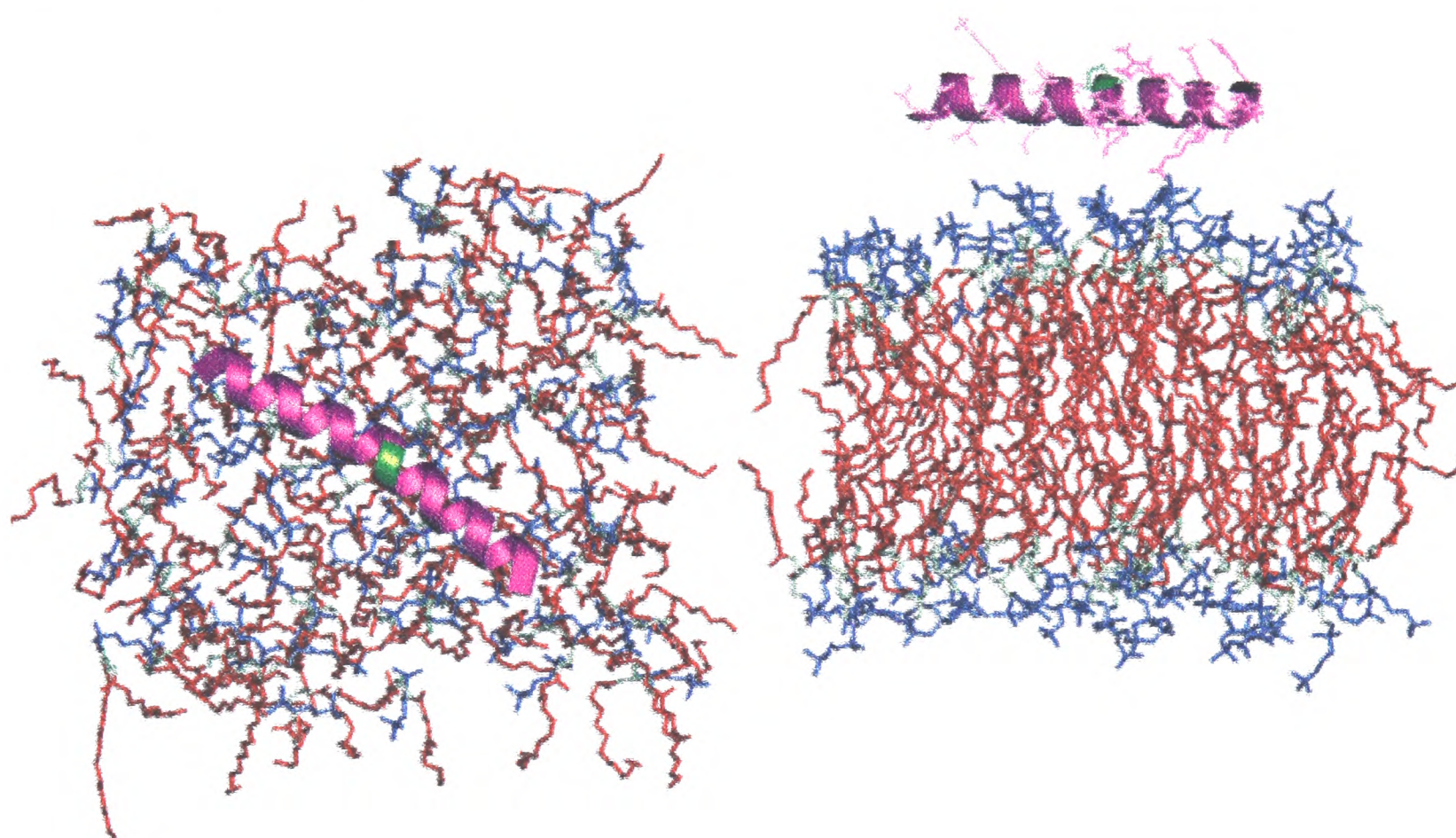


Figure 8.11 Views from the top and side of the starting structure of model 1 with melittin above the surface of a bilayer. The view from the side includes the side groups and in both diagrams the proline group is highlighted in green.

The simulation was run for 200ps to observe how the structure of the peptide and bilayer would adapt to each other and snap shots from the simulation at 0, 50, 100 and 150ps are shown in Figure 8.12.

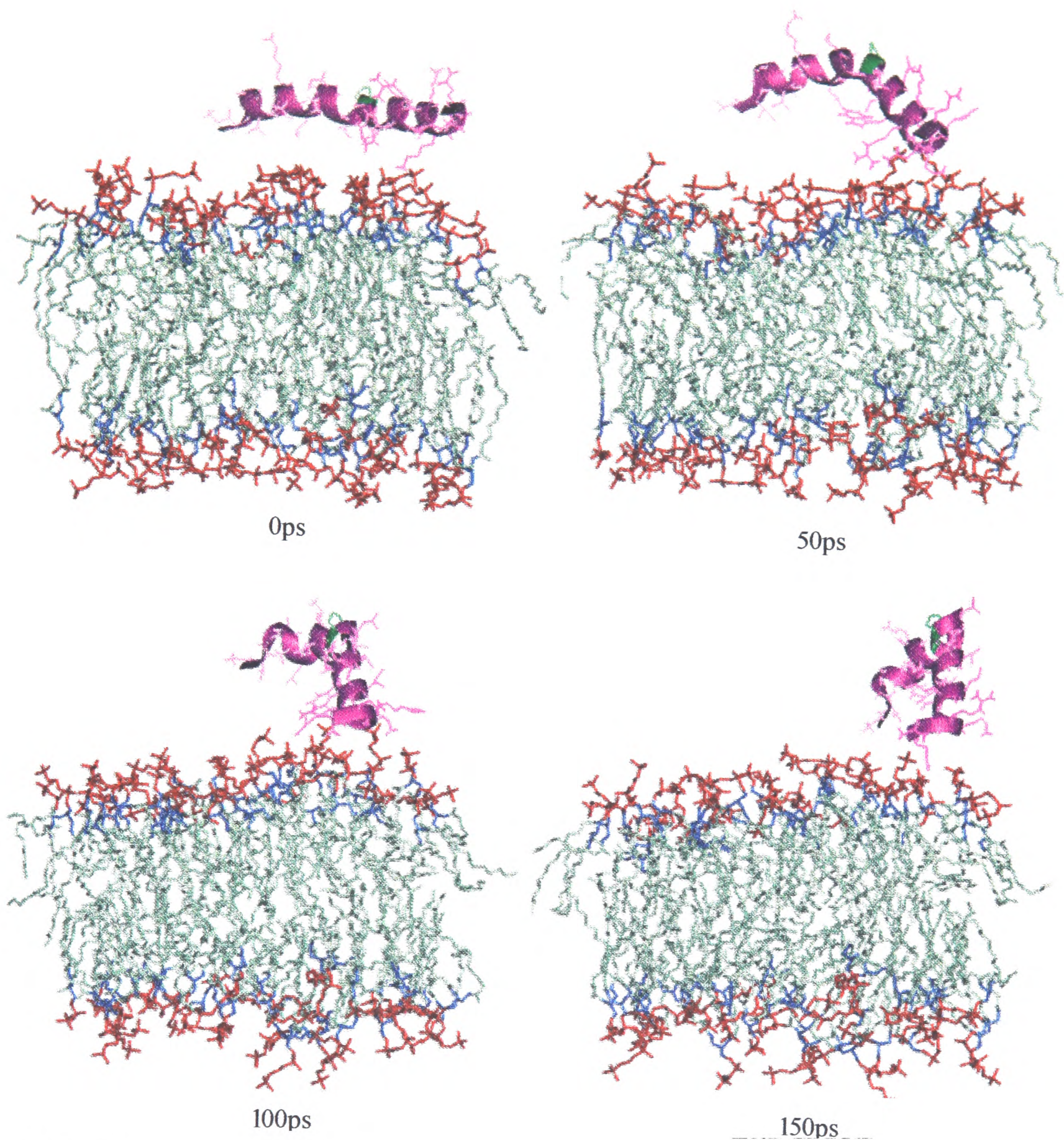


Figure 8
every 50p

Very rapidly in the simulation a bend occurred at the Gly-12 group and the very highly charged and basic C-terminus of the melittin moved into the surface of the membrane. The alpha helical structure of the peptide was preserved along the chain except at the bend. The

bend was so large that the C-terminus of the melittin was aligned almost perpendicular to the bilayer surface (see Figure 8.13) and allowed all the charged residues to interact with the head groups.

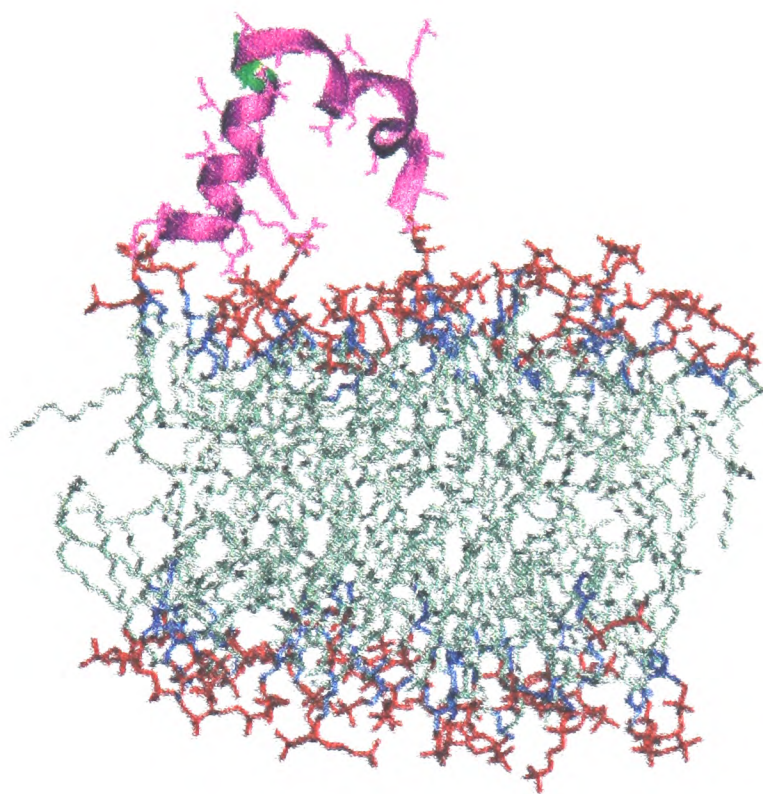


Figure 8.13 A view of the melittin on the surface of the membrane illustrating the almost perpendicular alignment of the peptide with the bilayer surface.

From the trajectory of the simulation it can be speculated that the actual mechanism of melittin insertion into the membrane is by the highly charged end pushing its way into the membrane followed by the rest of the peptide, rather than it simply lying on the surface and slipping in. The effect of the melittin on the head groups is that in the region where it is pointing at the surface the head groups have oriented themselves so that phosphate groups are able to hydrogen bond with the lysines and arginines while the positively charged N-methyl ends of the head group move out of the way. Thus it would appear that the lysine and arginine groups around the C-terminus of the melittin act as “grappling hooks” in order to secure themselves to the membrane surface before insertion occurs. Experiments have already shown that phosphate-arginine binding is extremely favourable in the melittin system (Drake and Hider, 1979). The bend at the Gly-12 residue is also consistent with the NOE experiments of Inagaki et al., (1989) which showed a loss of NOE at residues 11-12. More about the binding of the melittin to the head groups will be discussed in Section 8.5.2.

Unfortunately there is little point in continuing the simulations since the system is at constant volume and the lipids of the bilayer will not be able to move in order to accommodate the peptide.

8.5.1 The Dynamics of the Melittin during the Simulation

The dynamic behaviour of the melittin on the surface of the bilayer was characterised by calculating the average and standard deviation of the length of hydrogen bonds within the alpha helical structure and by following the psi and phi angles along the peptide backbone.

The results for the hydrogen bond calculations are shown in Figure 8.14 and quite clearly show that the alpha helical hydrogen bonding pattern is maintained for residues 12 to 21 which is the section of the peptide pointing into the membrane. The greatest deviations are shown by Leu-9 and Thr-11 which are supposed to be hydrogen bonded to Leu-13 and Ala-15 but become disrupted because of the bend at Gly-12.

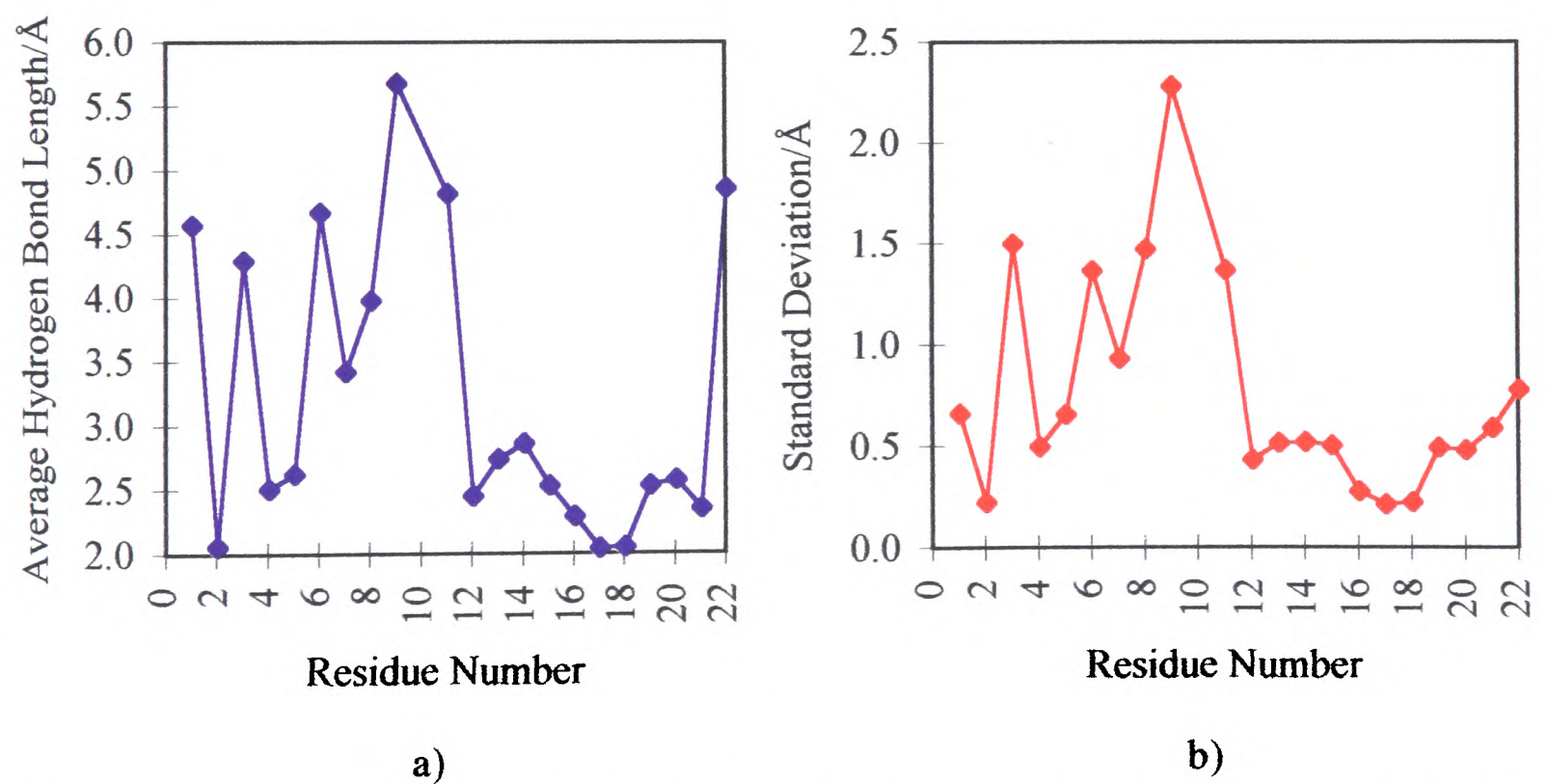


Figure 8.14 The a) average and b) standard deviation of the lengths of hydrogen bonds between residues that maintain the alpha helical structure in peptides.

In a similar fashion the psi and phi angles were calculated and are presented in Figure 8.15 and show that the largest deviation in the expected psi angle occurs at Gly-12 where it is seen from the simulation that the turn occurs. That a glycine should show such a large deviation from the expected psi angle in a alpha helix is not unexpected given their known greater flexibility and during the course of the simulation the angle changes from the normal value of around -50° to +120°.

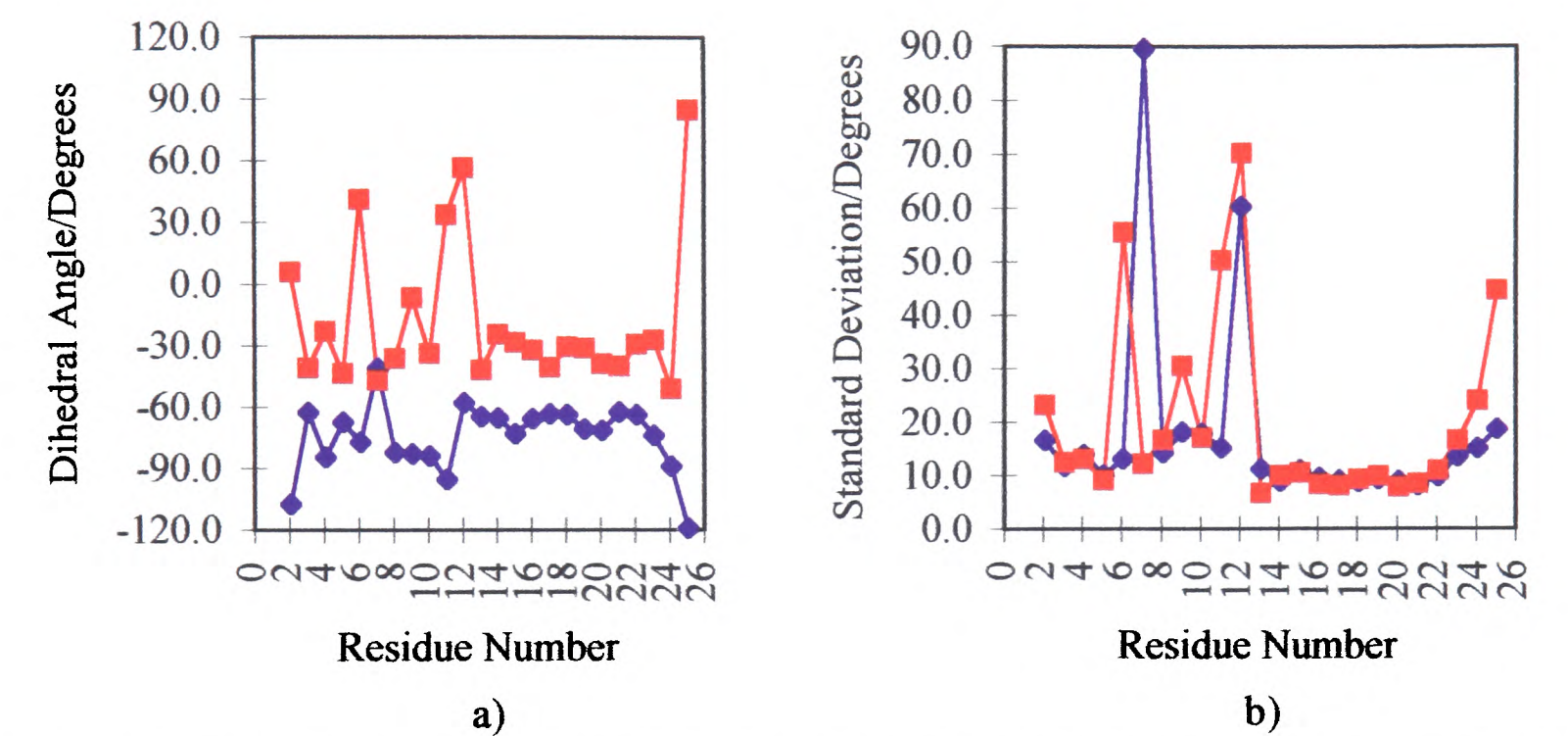


Figure 8.15 The a) average and b) standard deviation of the psi (red) and phi (blue) angles of the melittin molecule over the course of the simulation.

A large standard deviation is observed for the phi angle of Gly-12 which also reflects its greater flexibility.

8.5.2 The Interaction between Melittin and the Head Groups

Figure 8.16 is a surface view of the membrane and the final five residues of the melittin which are interacting with the head groups. It should be emphasised that because of the tight turn about the Gly-12 residue, these 5 residues are all in the same plane above the bilayer surface. Furthermore, it can be seen that the end of the melittin is pointing into a hole between the lipid head groups (drawn in red) and the lipid head groups are oriented so that

opposite charged groups are in close proximity. As mentioned previously, the arginines of melittin do have an affinity for phosphate groups (Drake and Hider, 1979).

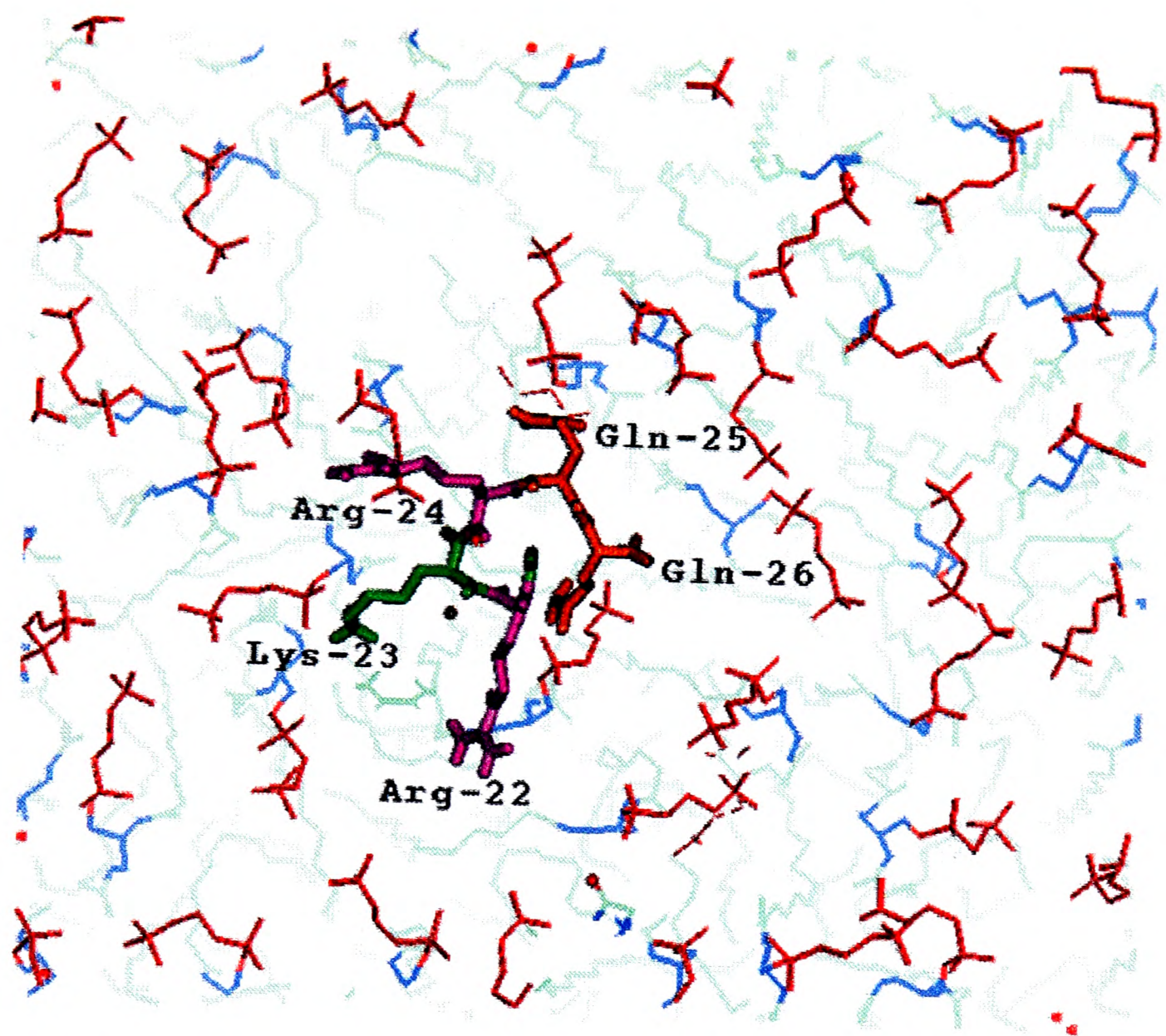


Figure 8.16 The surface of the bilayer with the lipid head groups drawn in red and the last 5 residues of the melittin C-terminus as labelled in the diagram. Glutamines are shown in orange, arginine in purple and lysine in green.

It appears from Figure 8.16 that Arg-24 and Gln-25 have both pulled lipids toward themselves by interacting with the phosphate groups, hence leaving a large hole in the membrane just above them in the picture. This will obviously be an ideal point for free water to enter into the membrane and hence lead possibly to lysis of the cell. However calculations on the extent of water penetration into the bilayer showed no difference between the side with melittin bound and the other side.

Experiments where the C-terminus of the melittin has been modified have shown that it is very important in the binding of melittin to membrane surfaces since removing the residues Lys-21 to Gln-26 reduced the activity of melittin by two-thirds but did not stop pore formation since ion migration was still observed to occur. In addition, work by Werkmeister et al., (1993) where one of the two Lys-Arg sequences was cleaved from the C-terminus reduced cytolysis by 8-fold and they concluded that arginine residues are very important since blocking them leads to an inhibition of cytolysis. They also concluded that a basic sequence at the C-terminus was not in itself sufficient for binding to the cell surface and at least one arginine is needed for binding to mammalian membranes. They hypothesised that part of the melittin molecule is involved in cell recognition, a proposal that is also proposed by Portlock, Clague and Cherry (1990).

This experimental work can be seen to have direct relevance to the manner in which the simulation has evolved in which the C-terminus has moved into the head group region and there are large contacts between the arginine and lysine residues with the phosphates of the head groups.

8.6 Simulation of Adsorbed Melittin- Model 2

The second model for the adsorption of melittin onto the surface of a bilayer is perhaps a little more natural, since it has the charged side of the melittin approaching the charged bilayer surface of the membrane but in fact this turned out not to be the case. The simulation protocol was identical to that used previously for model 1 of melittin adsorption and a view of the starting structure of the simulation is shown in Figure 8.17.

The simulation of the system lasted 300ps and six snapshots from the simulation taken every 50ps are shown in Figure 8.18 to illustrate the changing structure of the melittin in the presence of the bilayer.

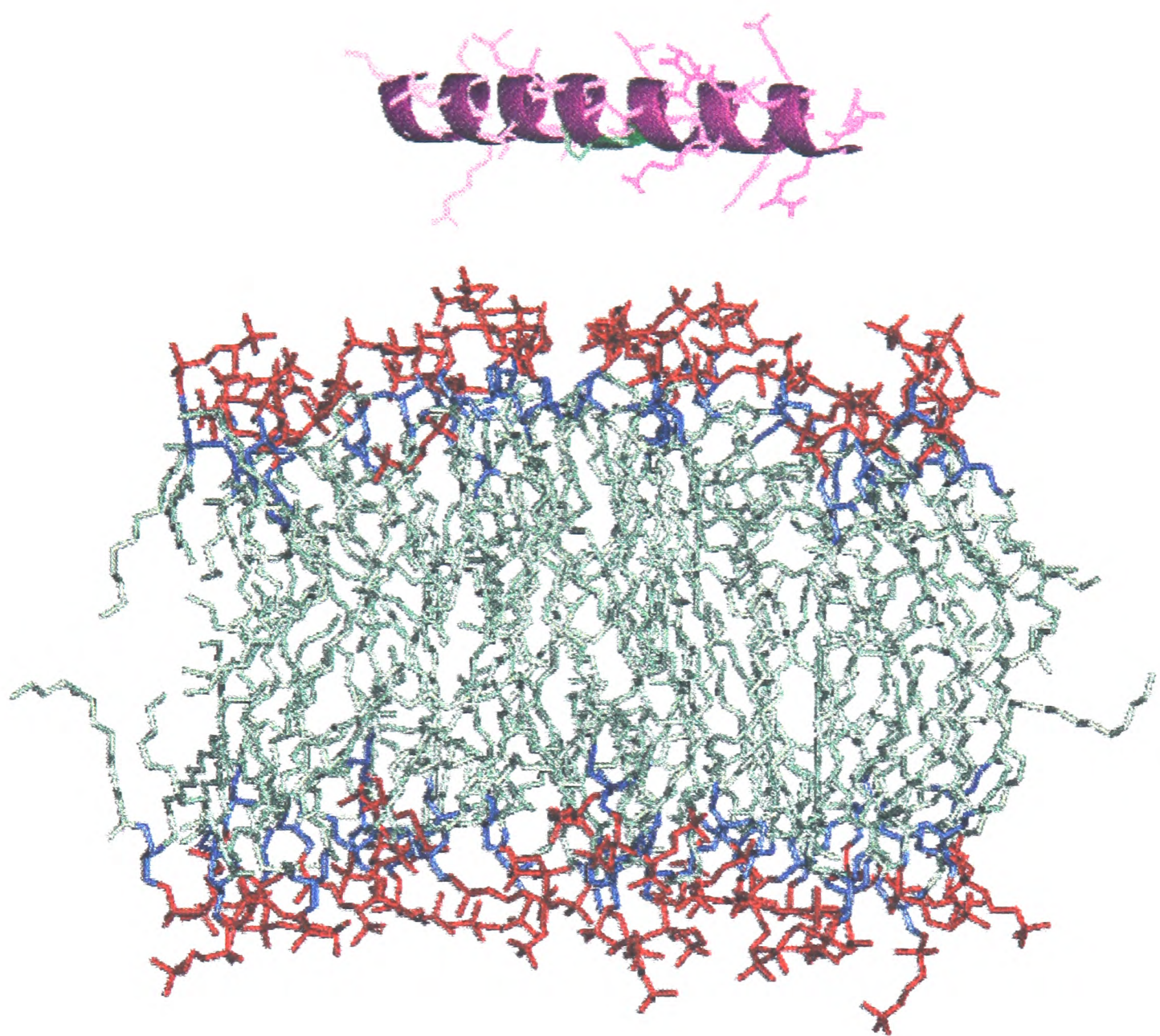
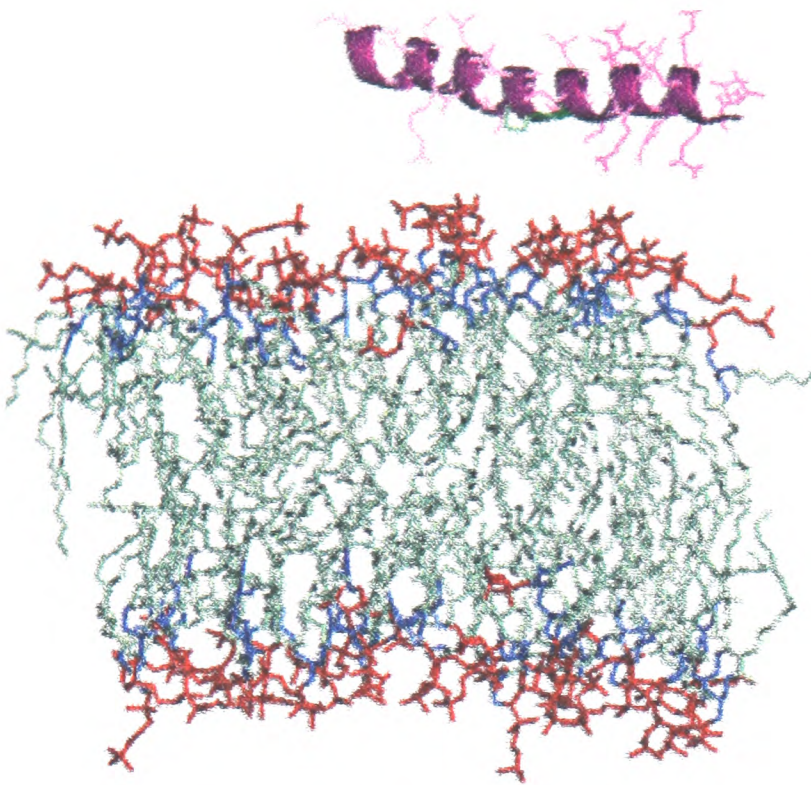
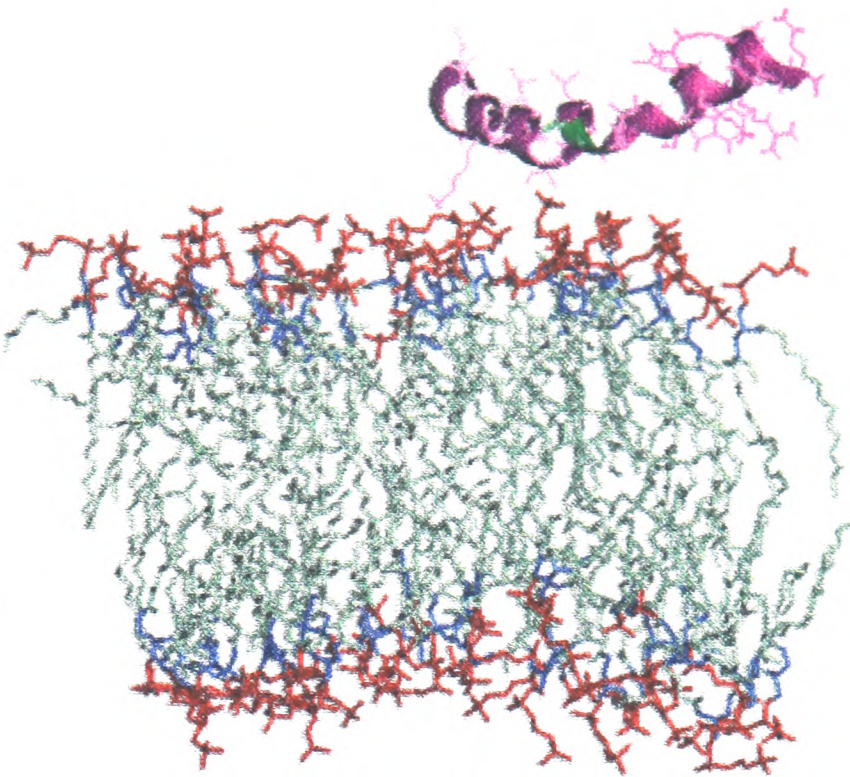


Figure 8.17 The starting structure of the second model of melittin adsorbed onto a bilayer surface.

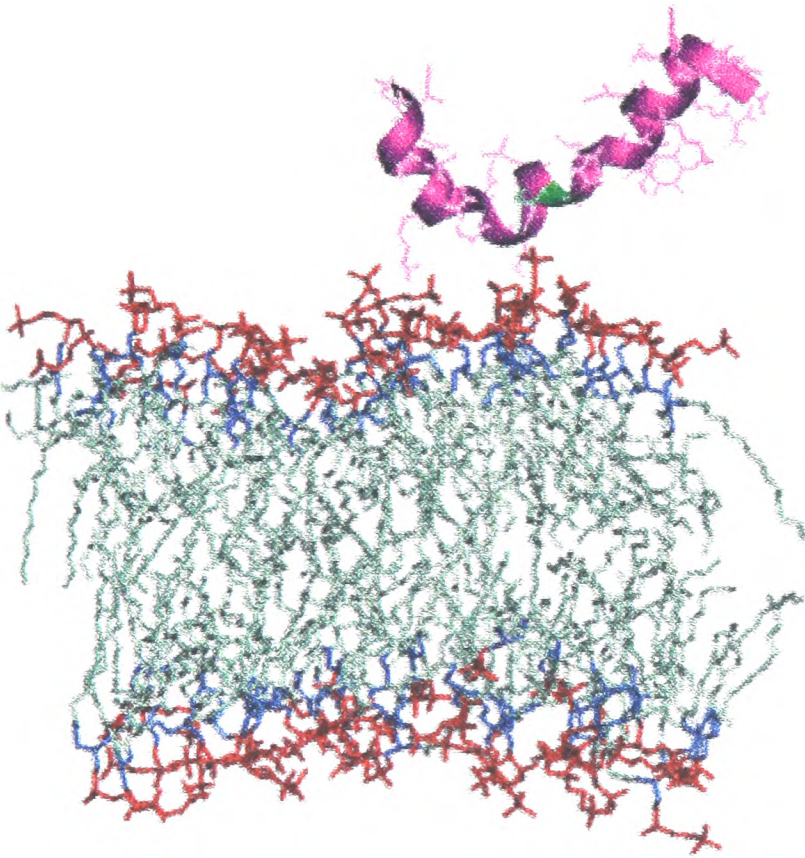
Figure 8.18 shows that once again in the presence of the lipid bilayer, a bend is forming in the melittin chain, however this time the effect of it is to turn the peptide away from the bilayer so that it is less associated than it was at the beginning. There is still a degree of interaction between the peptide and the bilayer surface and it can be seen in the final snapshot of Figure 8.18 that some of the lipid head groups are protruding from the bilayer in order to interact with the peptide.



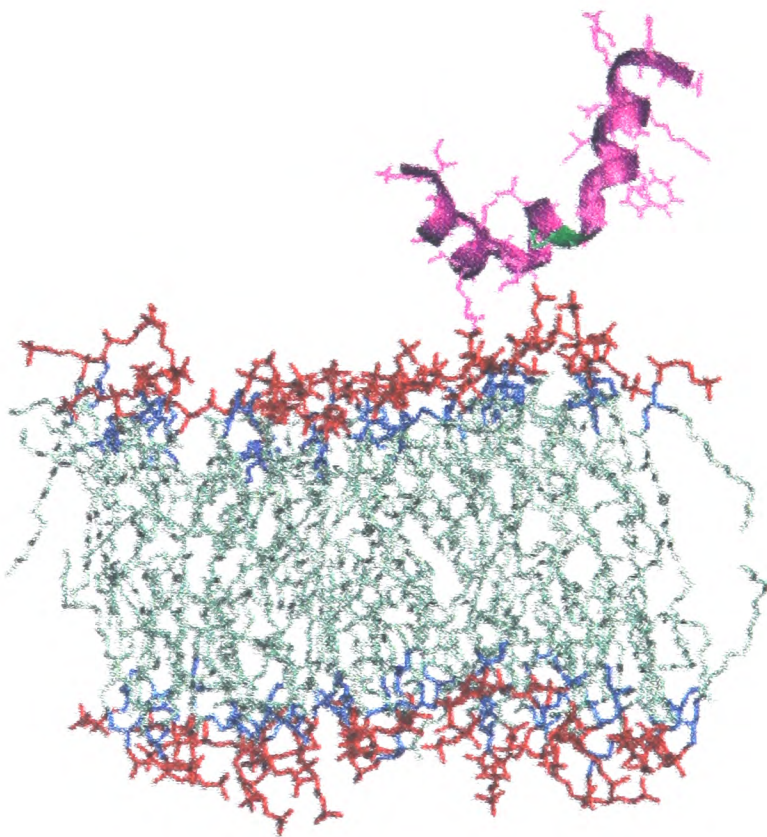
0ps



50ps



100ps



150ps

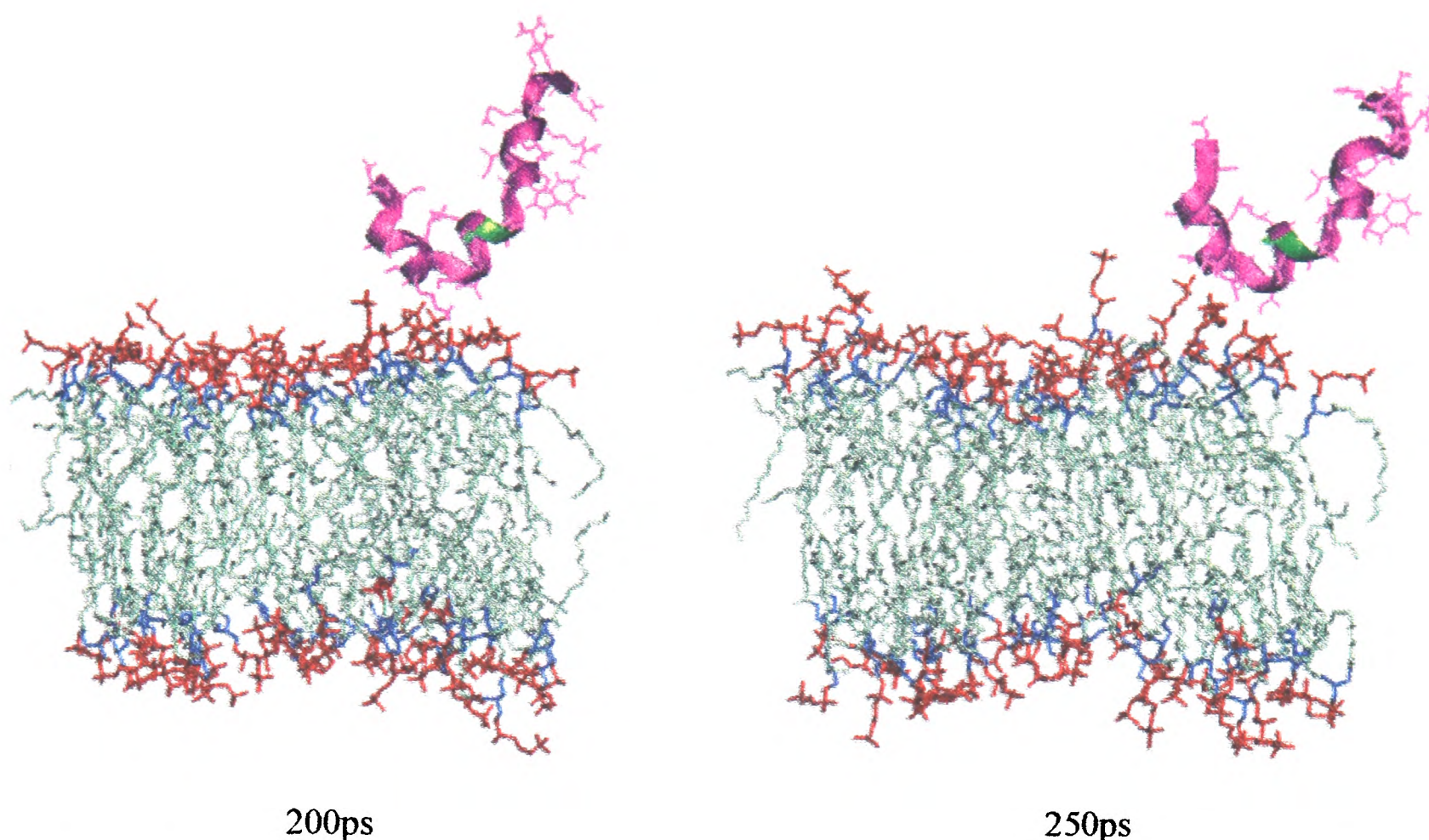


Figure 8.18 Six snap shots from the molecular dynamics simulation of model 2 of melittin adsorbed onto the surface of a bilayer taken every 50ps from the start of the simulation. The proline residue is shown in green.

8.6.1 The Dynamics of the Melittin during the Simulation

Exactly as in the simulation of model 1 the hydrogen bond lengths and psi and phi angles of the melittin were recorded over the duration of the simulation to determine how and where the bend in the melittin occurred.

The average lengths and standard deviations of the hydrogen bond lengths along the length of melittin are plotted in Figure 8.19 and demonstrate that the melittin chain is quite flexible.

Compared to the first model of melittin on a bilayer surface then hydrogen bonding is not particularly well maintained over the course of the simulation.

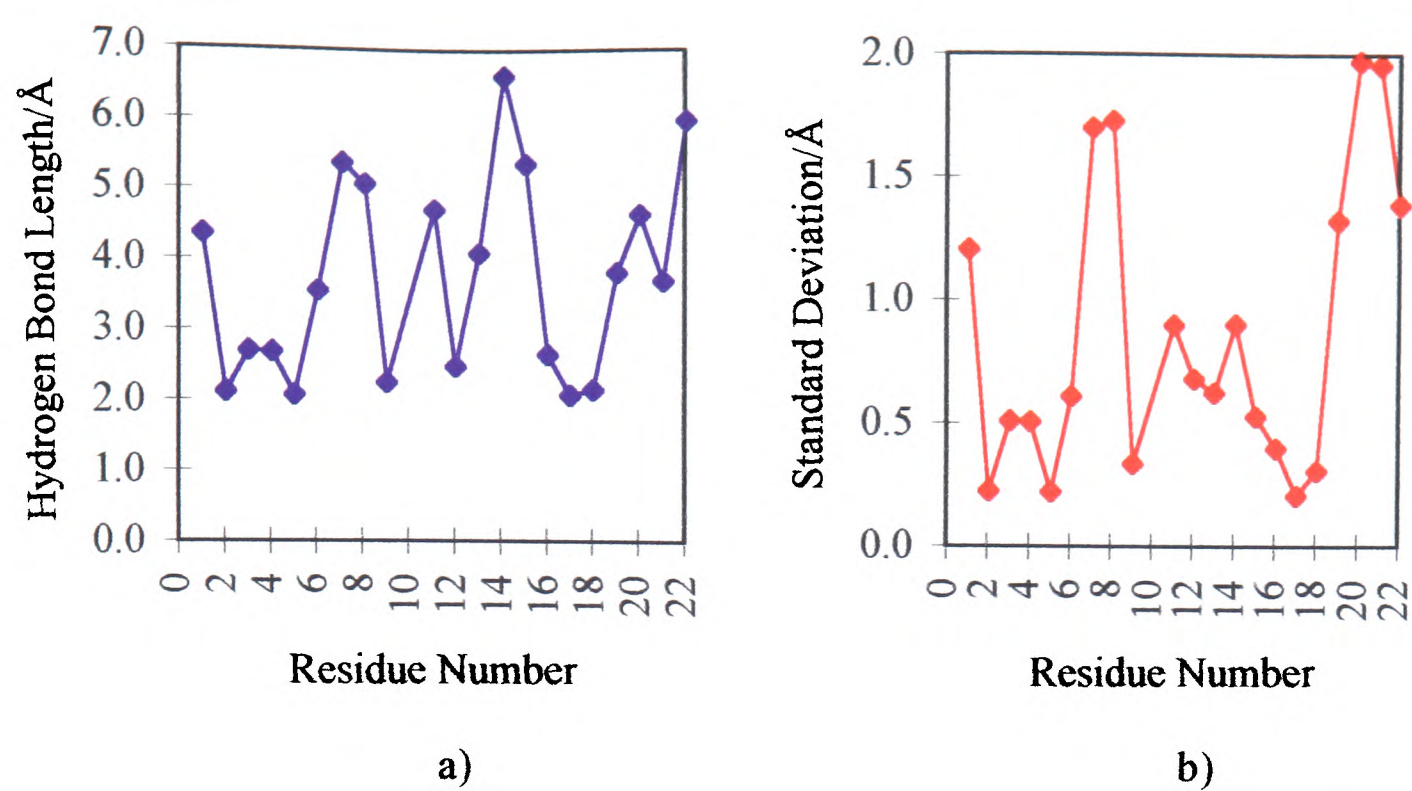


Figure 8.19 The a) average and b) standard deviations of the lengths of the hydrogen bond lengths holding the alpha helix together over the course of the simulation.

When the plots of the variation in the phi and psi angles are examined (see Figure 8.20) then the same conclusion may be drawn. The large standard deviation in the phi angle of Gly-12 is no surprise given the previous behaviour of this central glycine.

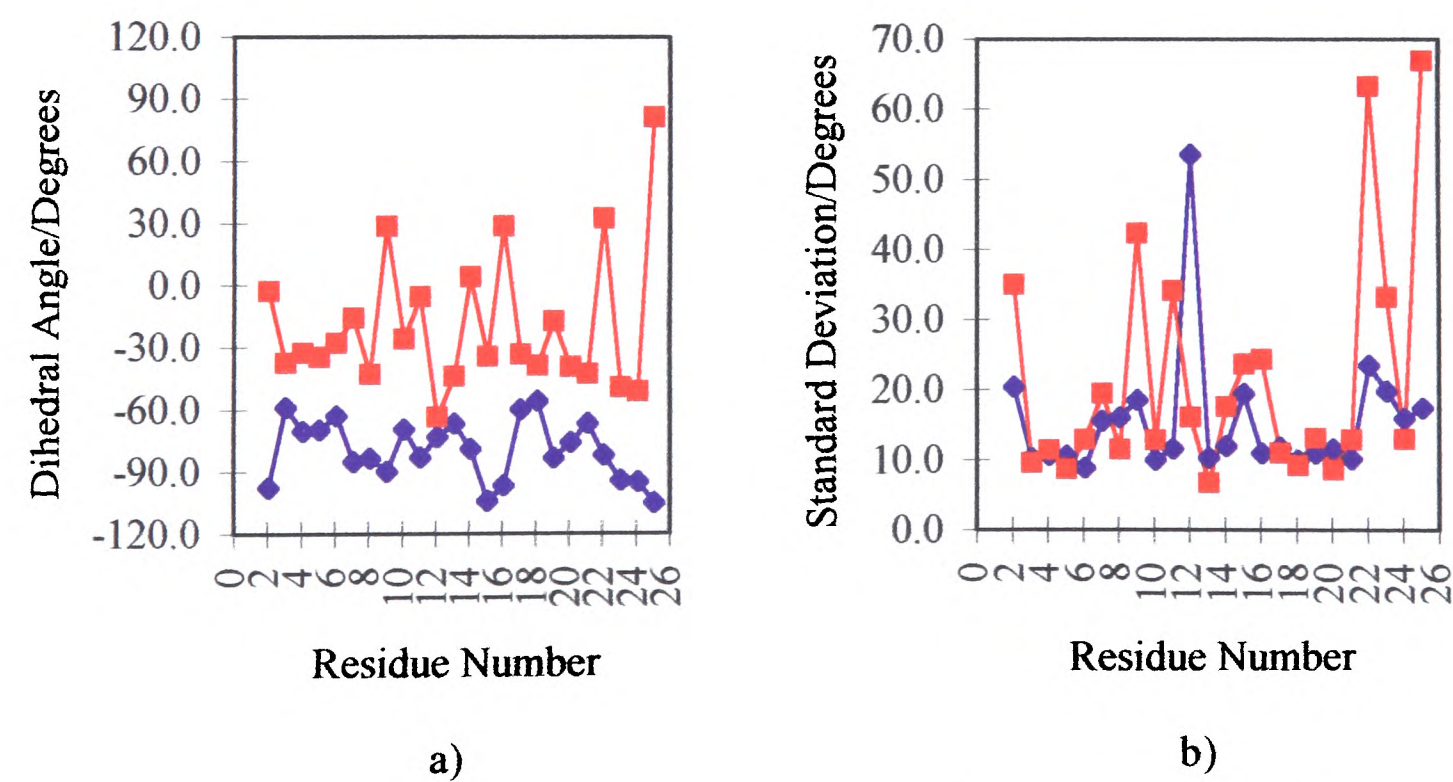


Figure 8.20 The a) average and b) standard deviation of the psi (red) and phi (blue) angles of the melittin molecule over the course of the simulation.

8.6.2 Conclusions about the Behaviour of Model 2

From the pictures of the structure over the course of the simulation and the results presented in Section 8.6.1 it is quite apparent that in the melittin has not become associated with the membrane even though it would be expected that since the charged surfaces of the melittin and the bilayer are facing each other, some kind of attractive force may have developed. The main cause of this failure to associate appears to be the overwhelming desire of the melittin to form a bend about the Gly-12 position that effectively turns the highly charged C-terminus groups away from the bilayer surface.

8.7 Conclusions on the Simulations of Single Molecules of Melittin in Aqueous Environments

It is interesting to note that in the simulation of melittin in water there was very little sign of any kind of bend forming in the chain, yet when the melittin molecule is under the influence of the charged bilayer surface it acquires a bend about the Gly-12 residue very rapidly. This supports other work that proposes bilayer environments have a profound effect on the conformational state of proteins in close proximity to them compared to aqueous solution (Kaser and Kedsey, 1983; Wattenberg et al., 1990; Chan et al., 1991).

Comparing the behaviour of melittin in the two surface adsorbed models, it appears that melittin is more likely to become further integrated into the melittin layer if it approaches with the apolar side than the polar side because of the formation of the bend in the molecule. Furthermore a possible mechanism for the entry of the melittin into the bilayer and origin of toxicity can be proposed based on the C-terminus of the melittin behaving like a “grappling hook” and anchoring itself to the phosphate groups of the bilayer surface (see Figure 8.16) and then opening up holes in the head group region through which water may flow into the hydrocarbon region. Once the melittin is anchored to the bilayer surface then it could start to integrate itself more fully into the membrane.

Assuming that the melittin does integrate itself into the membrane then the next stage of the research was to model both single melittin molecules in a bilayer, and a more speculative melittin model in which single molecules aggregate to form a channel.

8.8 Simulations of Melittin within a Bilayer

Compared to the earlier simulations of peptides in membranes (see Chapter 7) a new problem arises with the integration of melittin and other peptides into a bilayer, namely that the presence of often large side chains must be accounted for. Melittin includes some very large and bulky side chains, e.g. tryptophan, that will have a profound impact on the ease with which melittin may be placed within the membrane. The melittin starting structure used in the bilayer structure was taken from the previous simulation of it in water with the peptide exhibiting a small bend and in Figure 8.21a the starting conformation of the melittin is illustrated alongside the initial structure of the membrane system.

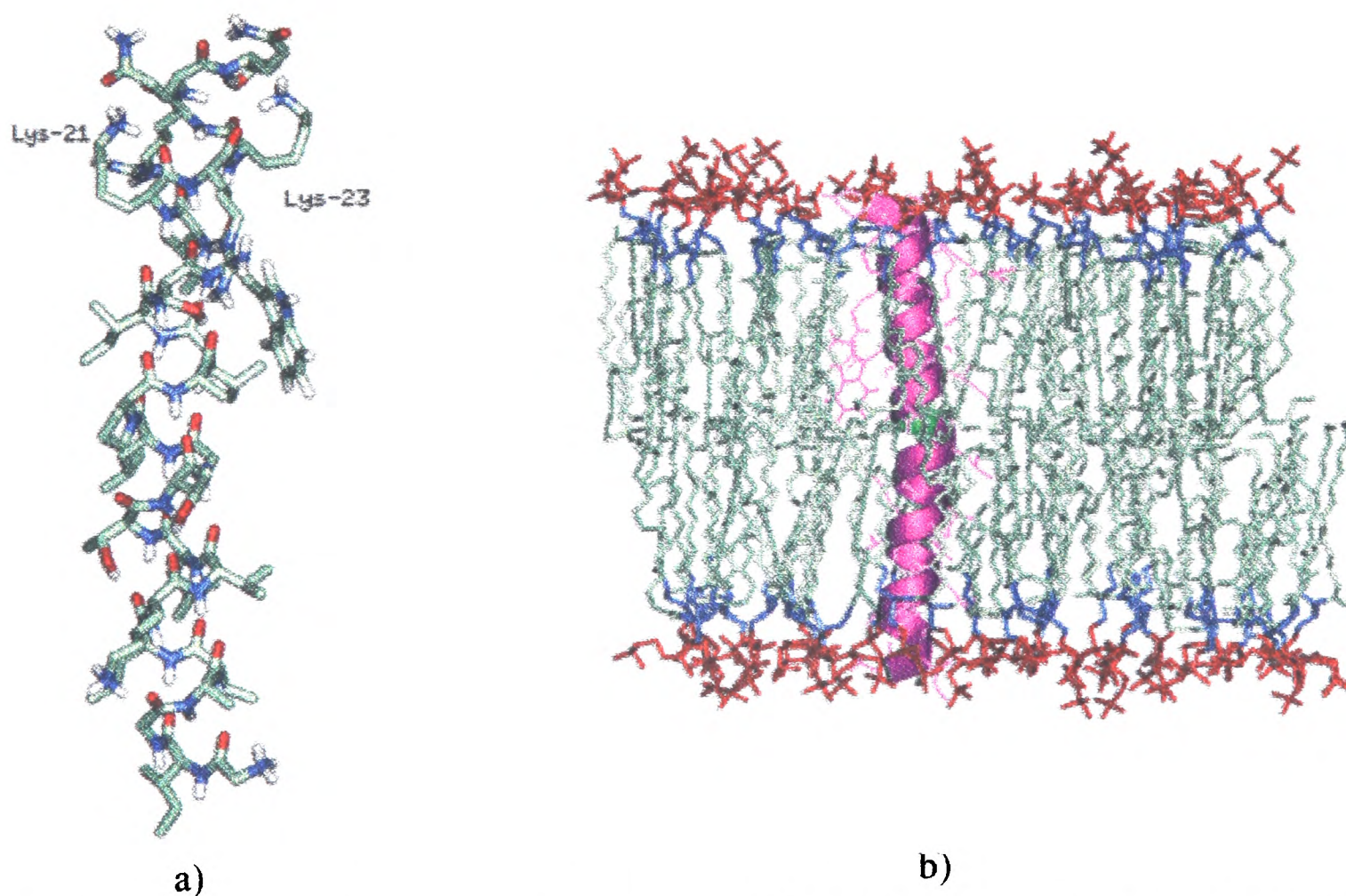


Figure 8.21 The starting conformation of the melittin molecule and its placement in the lipid bilayer. In the first figure the Lys-21 and Lys-23 residues are labelled and illustrate how they have been oriented to point away from the bilayer interior once inside in the membrane. The proline residue of melittin at the bilayer centre is coloured green in the second figure.

By considering the van der Waals volume of the melittin molecule compared to that of the lipid molecules and their packing density, it seemed appropriate that a single molecule of melittin would replace 3 lipid molecules in each half of the bilayer. Thus a previously minimised bilayer was taken and three lipid molecules removed from each side to form a channel through the membrane into which the melittin molecule was placed so that the polar residues at each end of the melittin were in the region of the charged head groups. This arrangement is far from ideal since the lipids have been removed randomly and thus with the aid of stereo viewing software and glasses the lipid molecules around the lipids were better packed and then individual lipid chains were examined and individual torsion angles adjusted to remove the more obvious bad contacts between the lipid and peptide. Following this, some of the torsions of the side chains of the melittin were adjusted to improve their interaction with the surroundings, e.g. the side chains of some lysines and arginines were modified so that they were directed toward the water rather than into the bilayer interior (see Figure 8.21a).

In Figure 8.21b it can be seen how the residues of the melittin extend much further into the plane of the bilayer (especially the threonine) than did the methyl residues of alanine in the previous model of a peptide in a membrane (see Chapter 7).

The whole system was then fully hydrated with TIP3P water molecules which were allowed to penetrate to the carbonyl groups of the lipid molecules.

The simulation of the system lasted 700ps with the first 100ps being considered the equilibration phase for the lipid molecules to relax around the peptide. In contrast to previous simulations of melittin in membranes (Robinson, unpublished results) the melittin was not constrained during molecular dynamic simulation since it was felt this would lead to artefacts in the behaviour of the lipids around the melittin, especially given the observations in the last chapter about the close interplay between the lipids and peptides. In Figure 8.22 some snapshots are shown of the melittin within the bilayer during the simulation to illustrate its behaviour.

It must be noted that the N-terminus of the peptide is in layer 1 while the C-terminus is in layer 2.

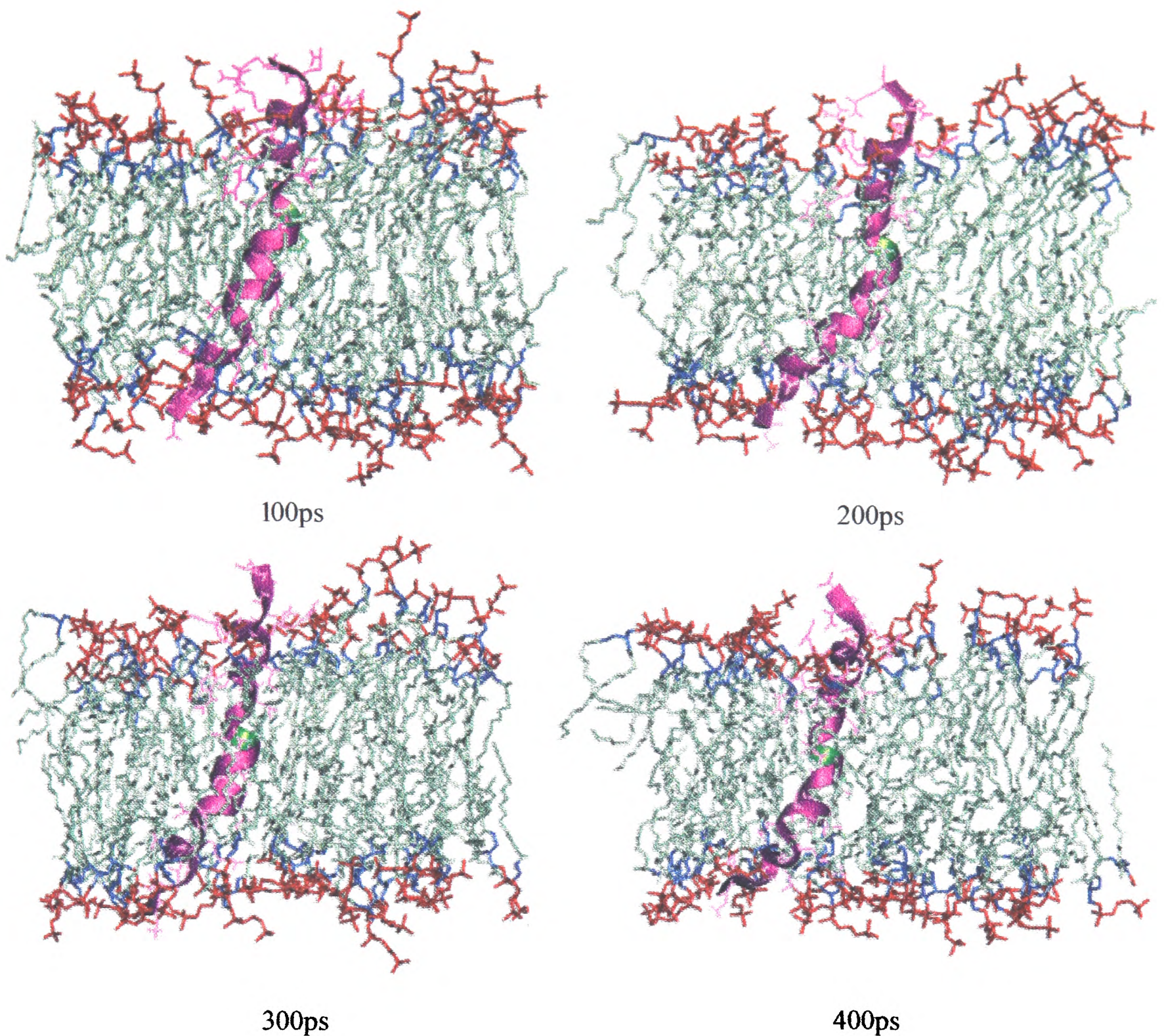


Figure 8.22 A series of snap shots from the melittin in a membrane simulation to illustrate the behaviour of the melittin at 100, 200, 300 and 400ps. The proline of melittin is coloured green.

In analysing the behaviour of the system, the following points were considered:

- 1) The behaviour of the melittin in the bilayer as a whole
- 2) The behaviour of the melittin side chains, especially their interaction with lipids
- 3) The effect of the melittin on the dynamics of lipids in the bilayer

8.9 The Behaviour of Melittin within the Membrane

Examining first the series of snap shots from the molecular dynamics trajectory some points may be made immediately:

- 1) The melittin has developed a bend at the centre of the bilayer
- 2) The charged residues at either end of the melittin have moved right into the head group/water interface
- 3) The tryptophan ring has changed its orientation from being toward the centre of the bilayer (Figure 8.2 1) to being in the vicinity of the glycerol moieties
- 4) The bilayer has appeared to have become thinner

These points will be discussed below.

8.9.1 The Bending of the Melittin

In Figure 8.23, the distribution of the angles between the two sections of the peptide in each half of the bilayer are plotted and clearly show that the a definite tilt is present.

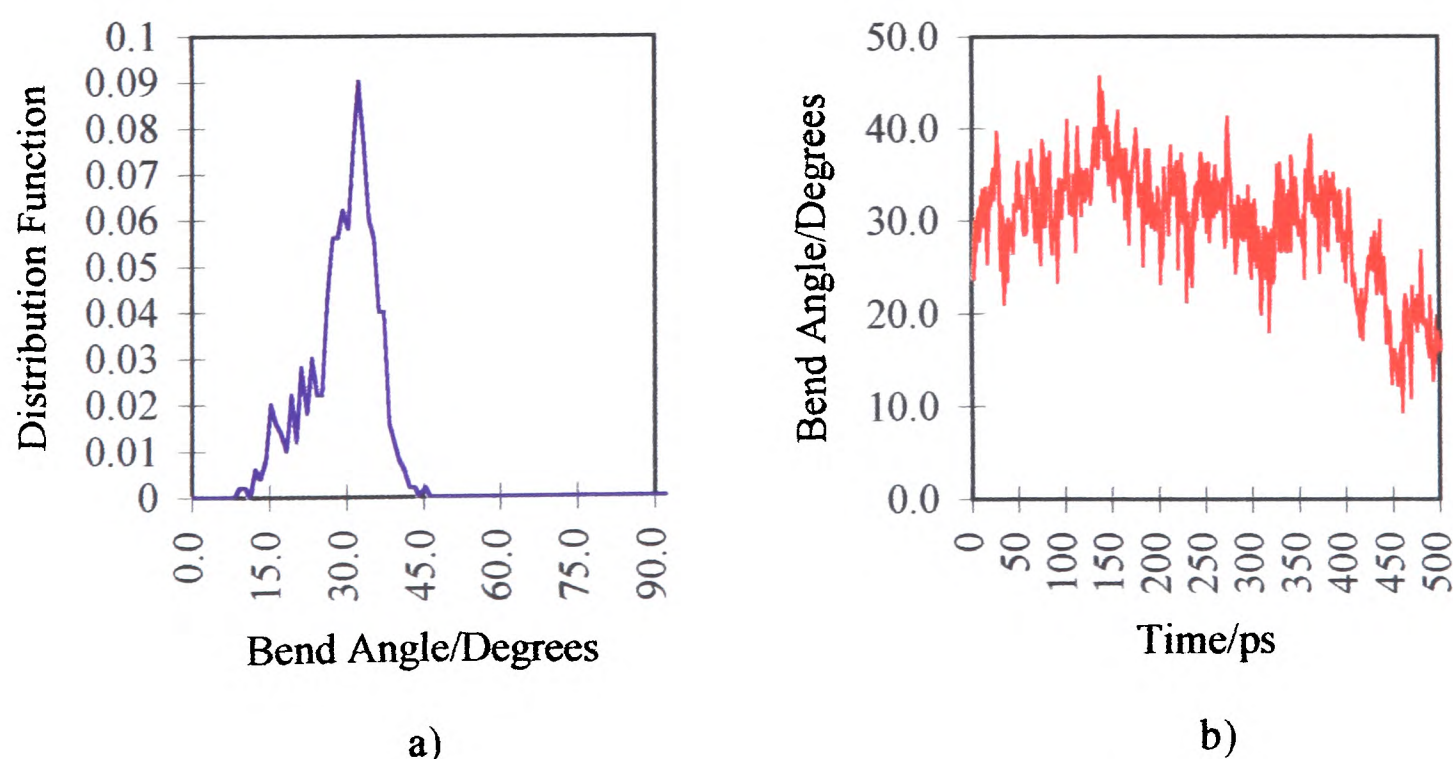


Figure 8.23 a) The distribution of bend angles in the melittin (blue) and b) the change in the tilt angle over the course of the simulation (red).

This tilt occurs within 100ps of the start of the simulation with the melittin section in layer 2, i.e. residue 14 to 26, being tilted with respect to the bilayer normal. The average bend in the peptide upon moving from one half of the bilayer to the other is around 30° although it can be seen that in the later stages of the simulation this bend angle tends toward 20° .

An important consideration is where and how the bend occurs in the melittin chain. From the snap shots from the simulation it is apparent that the bend occurs at the centre of the bilayer between the Pro-14 and Gly-12 residues which both reside in the middle of the chain and their presence is known to introduce conformational flexibility into peptide chains (Pastore et al., 1989) which once again introduces the concept of a hinge structure being present in trans bilayer peptides to accommodate for the different motions that could be occurring in each half of the bilayer. The nature of the bend was investigated further by calculating the average and standard deviation of the hydrogen bond lengths between residues over the course of the simulation as well as the psi and phi angles along the backbone.

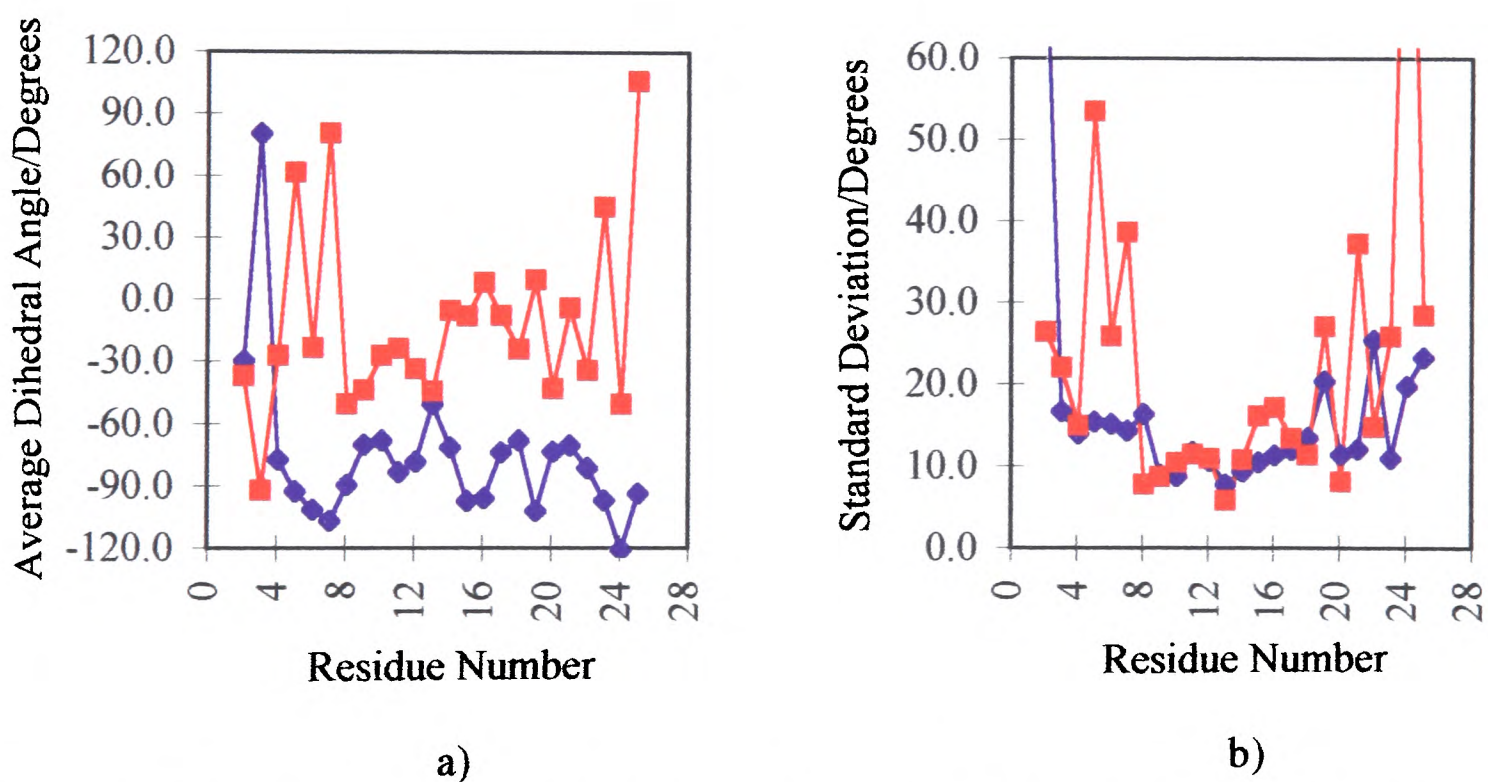


Figure 8.24 The a) average and b) standard deviations of the psi (red) and phi (blue) angles along the length of the melittin. (NB The residues 1 to 13 occur in layer 2 and 14 to 26 in layer 1).

The plots of Figures 8.24 and 8.25 illustrate that although the alpha helical structure is approximately conserved, the chain is fairly flexible and it is not possible to easily determine

a definite residue at which the bend occurs. By examining individual structures from the dynamic simulation it was concluded that the turn occurred primarily about the residues Leu-13, Pro-14 and Ala-15.

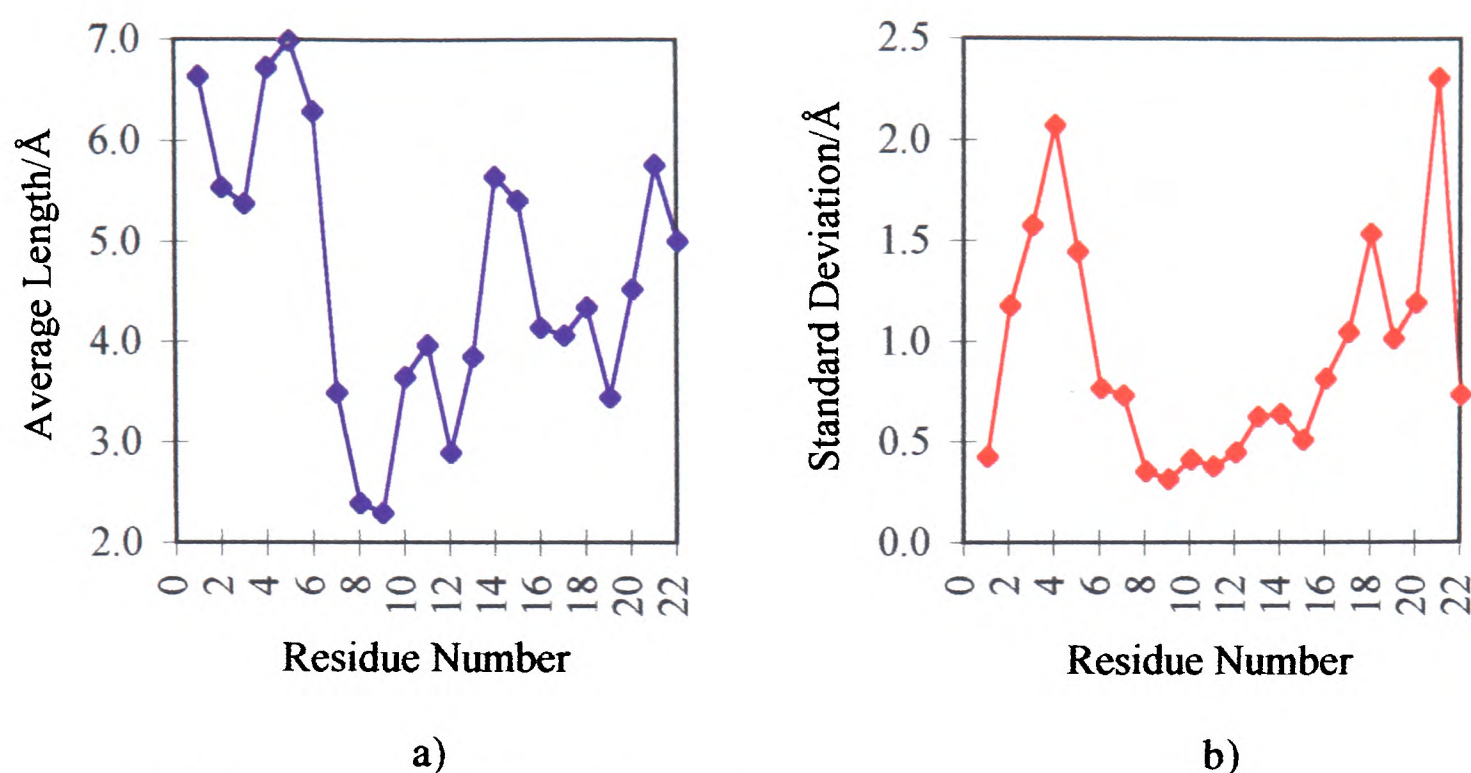


Figure 8.25 The a) average) and b) standard deviation of the length of hydrogen bonds connecting residues in the alpha helical structure.

8.9.2 The Interaction of the Melittin with the Head Groups

In Figure 8.26 a snap shot from the molecular dynamic simulation after 300ps is displayed to demonstrate the interaction of the C-terminus of melittin with the surrounding head groups. The head groups are coloured so that the negatively charged phosphate is blue and the positively charged N-methyls are red. It can be seen clearly that the majority of head groups have oriented themselves so that the phosphates are directed toward the basic lysine, arginine and glutamine residues and that this end of the melittin is securely anchored in the membrane by this ring of positively charged residues.

These observations on the importance of the arginine and lysine of the C-terminus binding to the phosphate head groups is consistent with the results of experiments in which these groups were cleaved off (see Section 8.5.2), but it was also observed that pore formation still

occurred when this was done which implies they are not essential for stabilising the melittin in a trans bilayer configuration.

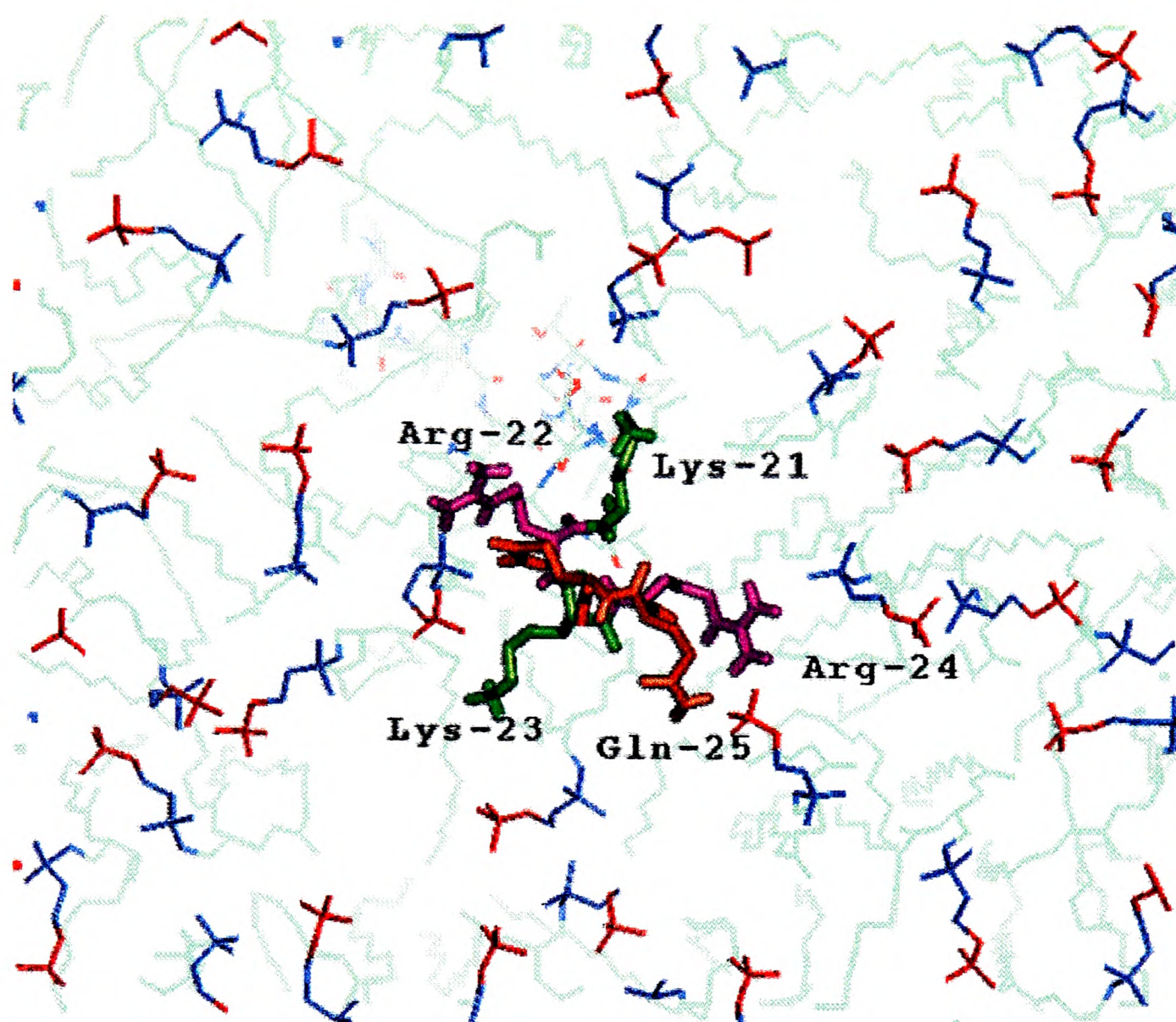


Figure 8.26 The surface of the bilayer and the last five residues of the melittin molecule to illustrate how the melittin binds securely to the head group region. The phosphate groups of the lipid are blue, the N-methyls groups are red while the lysine, arginine and glutamine residues of the melittin are coloured green, purple and orange respectively.

In Figure 8.27 the outer surface of the membrane is shown with the N-terminus penetrating through into the head group region. This end of the melittin only has two charged residues, Lys-7 and the end residue thus it would be expected that its interaction with the head group region should be less than the other end of melittin which has implications for the penetration of the melittin into the bilayer (see below).

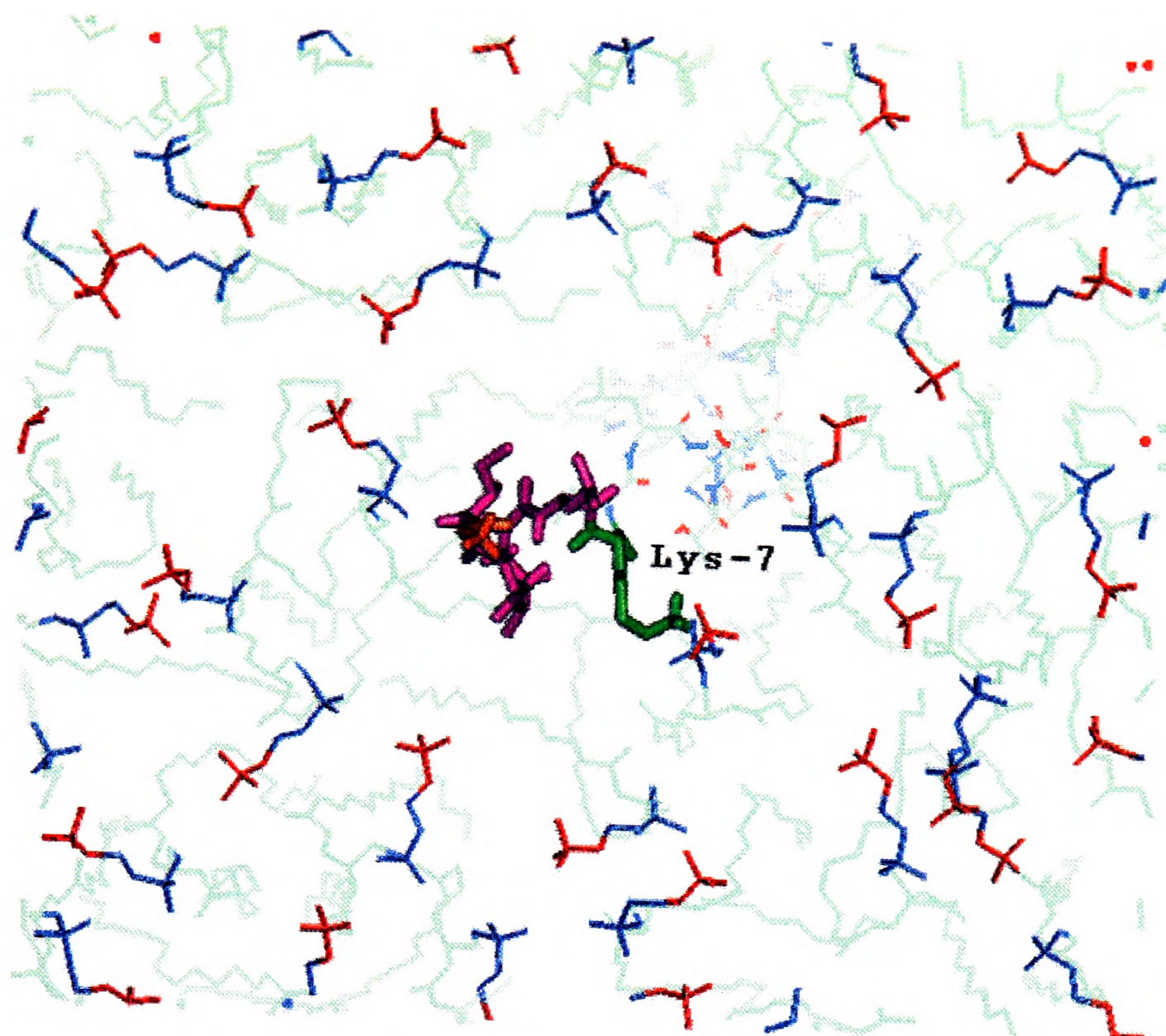


Figure 8.27 The surface of the bilayer and the last five residues of the N-terminus of the melittin molecule to illustrate how the melittin binds to the head group region. The phosphate groups of the lipid are blue, the N-methyls groups are blue, the charged Lys-7 is coloured green, the nonpolar residues 2 to 6 are coloured purple and the terminal glycine is orange.

Figure 8.27 clearly demonstrates that the interaction of the N-terminus of melittin with the head group region is restricted to the Lys-7 associating with a phosphate group and there is little or no reorientation of the head groups around the peptide. Compared to the C-terminus, the N-terminus is fairly apolar, which should make it far easier for this end of the peptide to move from the extracellular side of the membrane to the intracellular surface which has implications for the formation of a pore structure in the membrane and experiments by Hanke et al., (1983) have shown that under the influence of a trans membrane potential it is the N-terminus that moves across the bilayer (c.f. the Monte Carlo simulations of Milik and Skolnick, (1993) described in Section 8.2).

8.9.3 The Interaction of the Melittin with the Lipid Molecules

To determine the extent of interaction between the charged atoms of the melittin with those of the lipid molecules and assess whether hydrogen bonding occurred between the two, pair distribution functions were calculated. The main question addressed was whether the hydroxyl hydrogens of threonine and serine in melittin were able to form hydrogen bonds (i.e. were within $2.25\text{\AA}$) of the carbonyl groups in the fatty acid chains of the lipid molecules.

Both the threonine residues (Thr-10 and Thr-11) and the serine (Ser-18) spend only about 1% of the simulation actually hydrogen bonded to a carbonyl group of the lipid, however the polar hydrogen of the tryptophan (Trp-19) spends approximately 6% of the duration of the simulation hydrogen bonded to a lipid carbonyl (see Figure 8.27). During the course of the simulation (see Figure 8.22) the tryptophan residue is seen to reside close to the lipid-water interface where it is able to associate with lipid carbonyl oxygens. Research by Chattopadhyay and Rukmini (1993) showed that the tryptophan is located in a motionally restricted region of the membrane which offers considerable resistance to solvent reorientation by measuring the red edge excitation shift in fluorescence emission spectroscopy. This data are consistent with the tryptophan exhibiting hydrogen bonding to lipid carbonyls and the motionally restricted interfacial water. It is also in agreement with previous fluorescence quenching and nmr experiments which indicate that the tryptophan is localised to a shallow position near the membrane interface.

The charged ammonia of Lys-7 is calculated to spend 17% of the simulation hydrogen bonded to oxygens of the phosphate group, however it is always within at least $4.75\text{\AA}$ of such an oxygen. Similarly the residue Lys-21 (which is also approximately the same depth in the membrane on the other side) is hydrogen bonded 20% of the time and always within $4.0\text{\AA}$ of a potential hydrogen bond accepting oxygen. The residue Lys-23 is only hydrogen bonded 7% of the time and always within $5.5\text{\AA}$ of a phosphate oxygen.

Arg-22 shows the largest propensity to form hydrogen bonds with the phosphate oxygens spending at least 88% of the simulation in such a state and always being within $2.5\text{\AA}$ of a phosphate oxygen (see Figure 8.26). Arg-24 shows a weaker association at 32% and but is always within $5.75\text{\AA}$ of a phosphate oxygen.

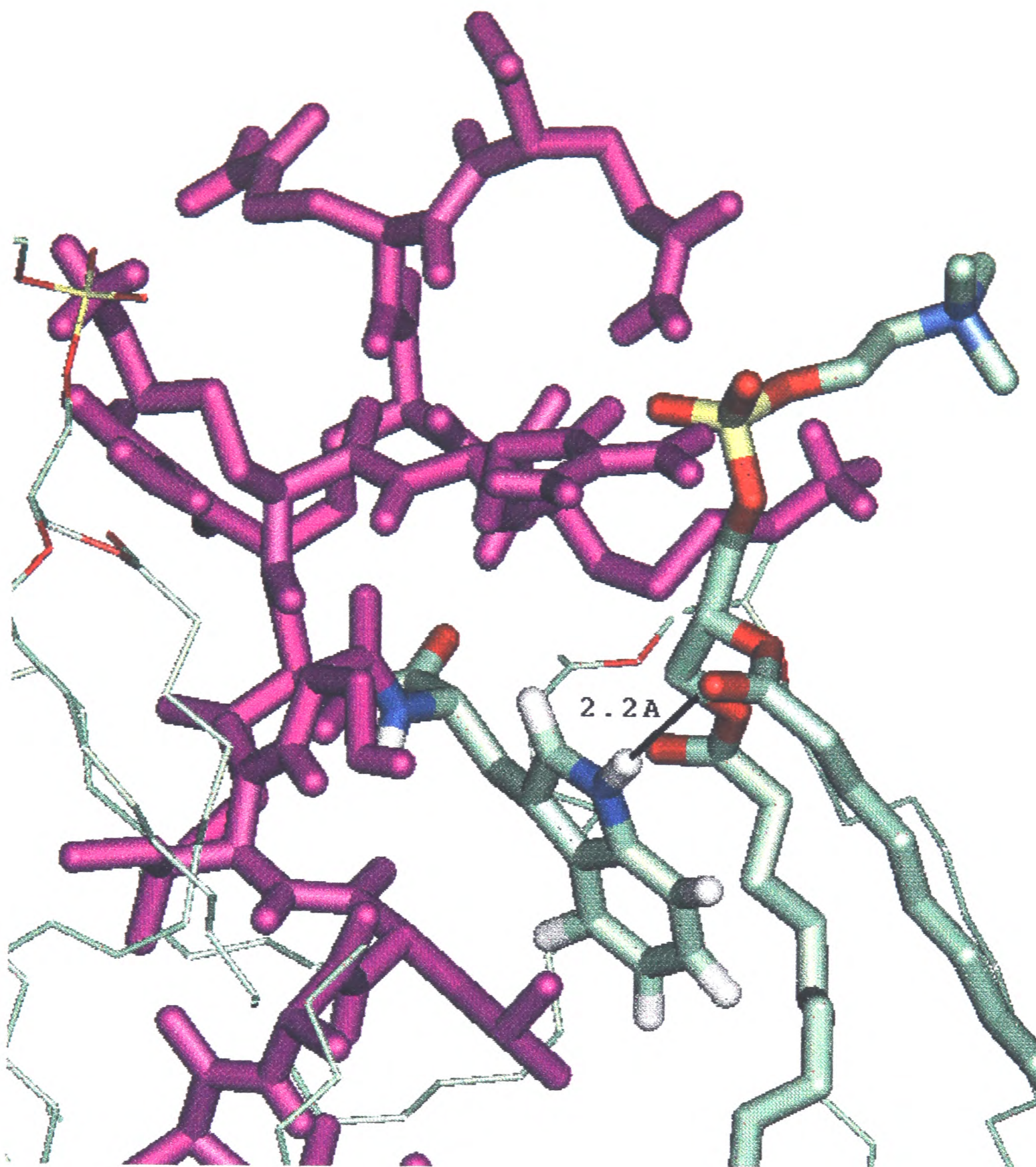


Figure 8.27 Snap shot from the simulation showing a hydrogen bond between the Trp-19 residue and a lipid carbonyl group which is 2.2Å in length. The hydrogen bond is shown as a black line and the peptide is shown in purple apart from the Trp-19 residue.

8.10 The Effect of Melittin on the Lipid Molecules

Compared to the previous peptide simulations in Chapter 7 it would be expected that melittin would show different behaviour because of the presence along it of various bulky side chains,

e.g. the tryptophan aromatic ring. To measure the extent of the effect of melittin on the lipid chains analyses of the dynamics and structure of the chains were performed.

8.10.1 The Effect of Melittin on the Lipid Order Parameters

As in the previous peptide simulations (see Chapter 7) the order parameters of methylene groups of the Sn1 and Sn2 chains together were calculated as a function of distance in bands of width 5Å from an axis describing the position and orientation of the melittin in each half of the lipid bilayer and the results are given in Figure 8.28.

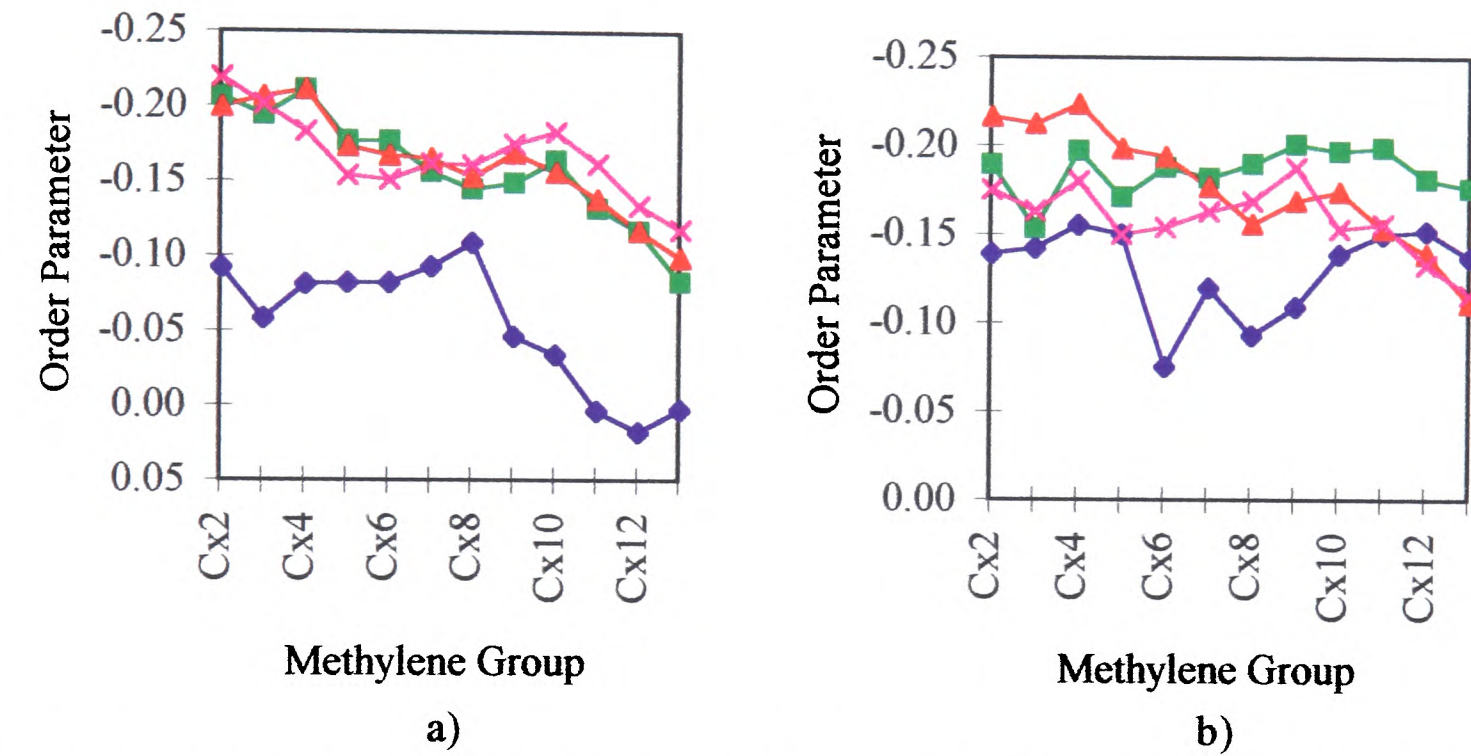


Figure 8.28 The calculated order parameters for the methylene groups of the lipid chains as a function of distance from the an axis describing the melittin for a) layer 1 containing the C-terminus of melittin and b) layer 2 containing the N-terminus of the melittin chain which is also tilted. (5-10Å is blue, 10-15Å is green, 15-20Å is red and 20-25Å is magenta).

For both layers, but especially for layer one that contains the C-terminus of the melittin and the large tryptophan residue the order parameters are much smaller for the methylene groups adjacent to the melittin than those further away. Thus the melittin molecule promotes disorder in the chains around it. This is in contrast to the simulations of Ala₂₅ in bilayers which had the order being increased, probably because the lipid chains could quite pack easily around the rigid peptide chain but is consistent with the order parameters measured

experimentally (Dufourc, Bonmalin and Dufourcq, 1989). Increasing disorder in the chains will probably make it easier for other solutes to pass through this region as disordered chains will not pack as efficiently and thus there is greater useful free volume into which solutes may move.

8.10.2 Effect of Melittin on the Tilt of the Lipid Chains

The effect of the presence of the melittin on the tilt of vectors describing the lipid chains, conveniently described as the top, middle and bottom lipid chain vectors, was measured and showed that in both layers the melittin did not have a large impact on the tilt of the lipid chains except at the bilayer centre where it significantly broadened the range of tilt angles observed for those lipids near to the peptide.

8.10.3 the Effect of Melittin on Bilayer Thickness

Bilayer thickness was defined using atom density profiles across the bilayer as a function of distance from the melittin molecule using only the fatty acid chains of the lipids and the results (seen in Figure 8.29) indicate that the bilayer in the vicinity of the melittin is thinner than the rest of the bilayer.

This thinner bilayer and greater disorder in the chains will serve to make the bilayer less restrictive to the diffusion of solutes across it. Furthermore studies on the extent of water penetration into the bilayer showed that although there is no significant difference between the amount of water found deeper in the bilayer around the melittin compared to the bulk, there is a substantially greater amount of water penetrating deeper into the head group/glycerol region.

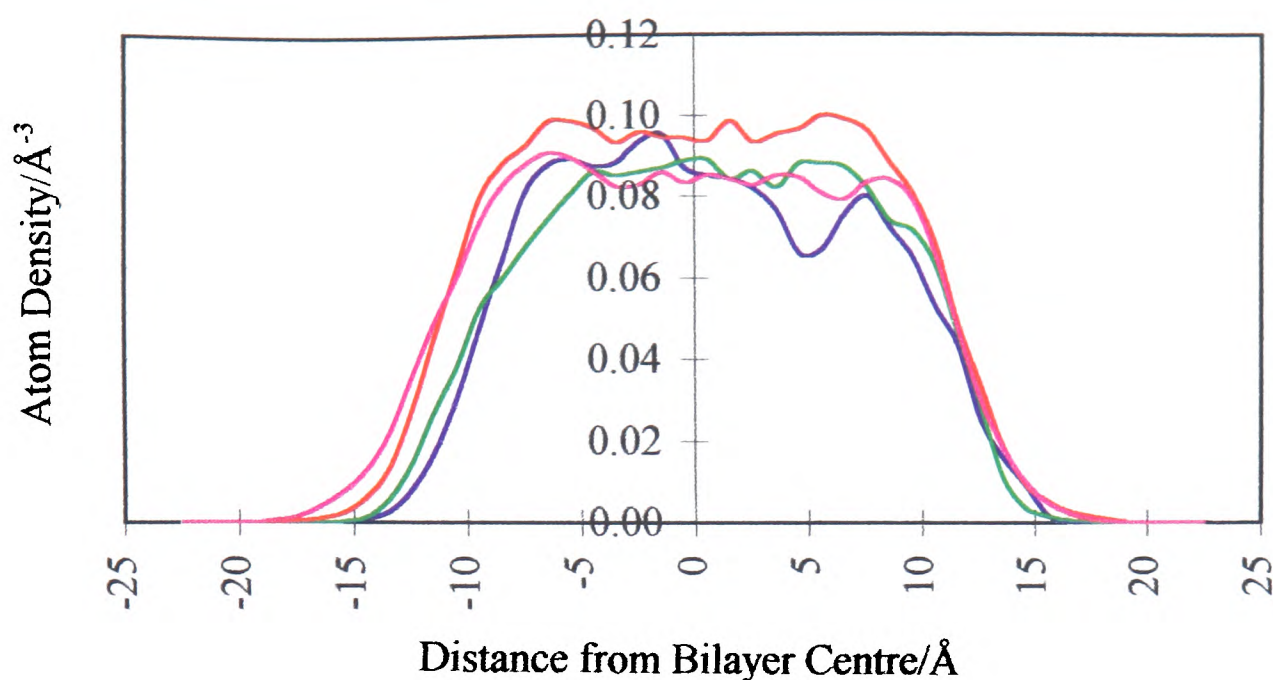


Figure 8.29 The thickness of the bilayer as measured using the atom density profile across the bilayer for the fatty acyl chains of the lipid molecules. (The colour scheme is the same as in Figure 8.28).

8.11 Conclusions on the Melittin Bilayer System

The ability of melittin to bend may be an important part of its function both in the membrane and during its interaction with and possible entry into the membrane. The four charged residues at the C-terminal end of the melittin show a large favourable interaction with the oxygen atoms of the phosphate head groups of the lipids, while the other end of the chain has a single lysine that interacts with the phosphate oxygens. Within the membrane the peptide is observed to bend at the bilayer centre between the glycine and proline residues. The effect of the melittin on the membrane is to cause a slight reduction on the thickness of the bilayer and increase disorder in those lipid chains immediately surrounding it.

8.12 A Hypothetical Model for a Melittin Pore in a Membrane

There is a general belief that it is feasible that melittin molecules within a bilayer may aggregate to form a pore structure which behaves as a voltage-gated ion channel but there is

no conclusive structural evidence on which to base the structure. When melittin binds to membranes it is observed that there is a voltage dependent increase in conductance that is consistent with the formation of channels allowing ion migration in response to a reorientation of melittin in the membrane (Kempf et al., 1981). This reorientation and aggregation of the melittin molecules may only occur in the presence of a trans membrane potential to provide a driving force for the N-terminus to cross the bilayer (Hanke et al., 1983). Since the melittin pores have discrete levels of conductance it implies that this pore is an actual channel formed by melittin molecules rather than ion migration occurring as a result of reorganisation of the lipids by melittin (Hanke et al., 1983).

To build a melittin pore structure involves a consideration of the mechanism by which the melittin molecule may enter the membrane to determine their orientation within the membrane, the distribution in the melittin of hydrophobic and hydrophilic groups to understand how they will interact with each other if molecules pack together and how many melittin molecules need to pack together in order to shield hydrophilic groups from the hydrophobic bilayer interior yet still leave a pore hole large enough for solutes to travel through.

From the previous study of a melittin molecule in a bilayer it is patently obvious that one end of the melittin is far more strongly bound to the head group region than the other by virtue of having more charged groups (i.e. two lysine residues and two arginine residues, plus two polar glutamines) while the other end has a single lysine residue to anchor it with the rest of the residues being extremely hydrophobic. It is therefore not unreasonable that it would be the less polar end of the peptide that would traverse the hydrophobic region of the bilayer leading to all the melittin molecules being oriented the same way in the membrane and this is supported by experimental evidence (Hanke et al., 1983). Such a hypothesis leads to a large reduction in the number of pore models that are possible and need be considered.

The second stage of building the model was to decide how many melittin molecules would form the pore. There is no conclusive evidence about how many melittin molecules may aggregate to form a pore, but the consensus appears to be that it is variable (Schwarz, Zong and Popescu, 1992) and probably depends on composition of the lipid bilayer. Tetrameric

models have been proposed by Vogel and Jahnig, (1986) and Tosteson and Tosteson, (1981) while Talbot, Faucon and Dufourcq, (1987) proposed that pores would be formed by between three and ten melittin molecules.

Decisions on the number of melittin molecules to use for the simulations were made using simple geometric considerations of the hydrophilic surface of the melittin combined with measurements of the pore size for different packing arrangements. From Figure 8.2 it can be seen that one side of the helix is predominantly hydrophilic and within the hydrophobic environment the helices should pack together to mask this portion from the lipid chains. Since this section covers an angle of $\sim 125^\circ$ this implies that at least 5 or 6 melittin molecules probably aggregate.

When five or six melittin chains are packed together the resultant pores both have an inside diameter that is of sufficient size to accommodate water molecules. The final model that was used had six melittin molecules arranged in a hexagonal pattern about $10\text{\AA}$ apart and three different views of the pore are shown in Figure 8.30. The actual pore was built by creating an idealised pore (see Figure 8.30a) and placing the melittin molecules inside the cylinders that represented the idealised peptide position.

Problems that had to be resolved in constructing the pore models included

- 1) How to orient the chains given that they contain a bend in the middle.
- 2) How to allow for the lipids being conical in shape because of the bulky side chains at one end but not the other.
- 3) How to arrange side chains that may have steric clashes with side chains of neighbouring melittins.
- 4) How to arrange the hydrophilic side chains that occur inside the pore.

The six melittin molecules were packed together to form the pore with any groups that showed steric clashes being manipulated. The resulting structure had the tyrosine rings on the

outside with the other hydrophobic groups while the polar groups in the chain were on the inside, e.g. the serine and two threonines, and is shown in Figure 8.30.

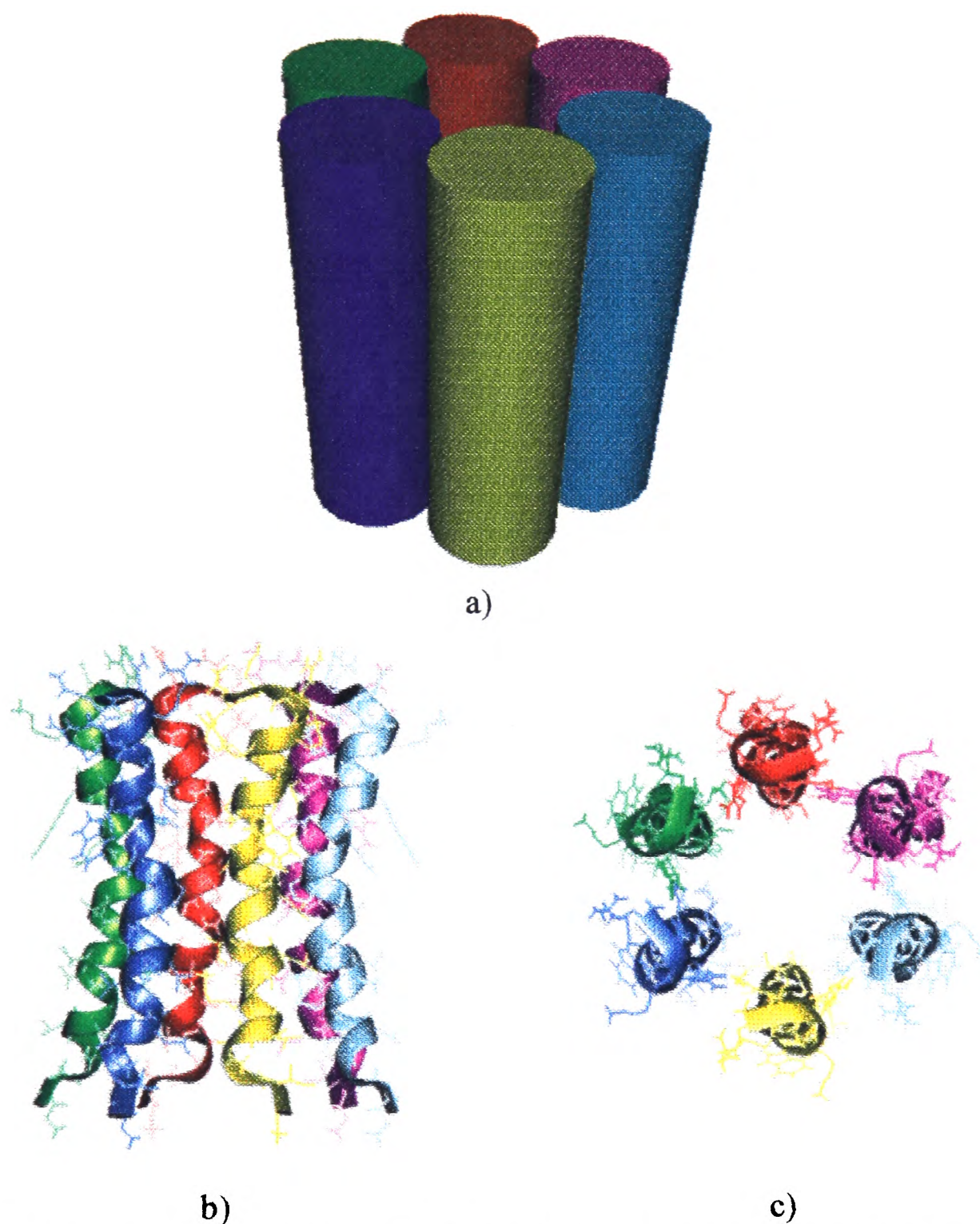


Figure 8.30 Different representations and views of the melittin pore a) An idealised structure that was used as a template to build the model b) The structure highlighting the alpha helical chain of the peptides viewed from the side c) The packing of the helices viewed from the top.

The bilayer that the melittin molecule was to be solvated in was built using a specially written program that creates bilayers of any size using the same criterion as for previous hand built bilayers. The program builds a membrane consisting of the four different lipid

conformations described previously (see Section 4.1) arranged in a hexagonal packing array with the relative orientation of the head groups based on that observed in the crystal structure of DMPC. The melittin pore was inserted into the bilayer and lipid molecules with which it had obvious collisions were removed while others around it were rearranged to resemble a close packing of the lipid chains around the protein giving a bilayer containing a total of 198 lipid molecules.

In the final stage of constructing the membrane model, water molecules were placed within the pore formed by the melittin molecules using a modified version of the hydration option of AMBER and the final structure is shown in Figure 8.31.

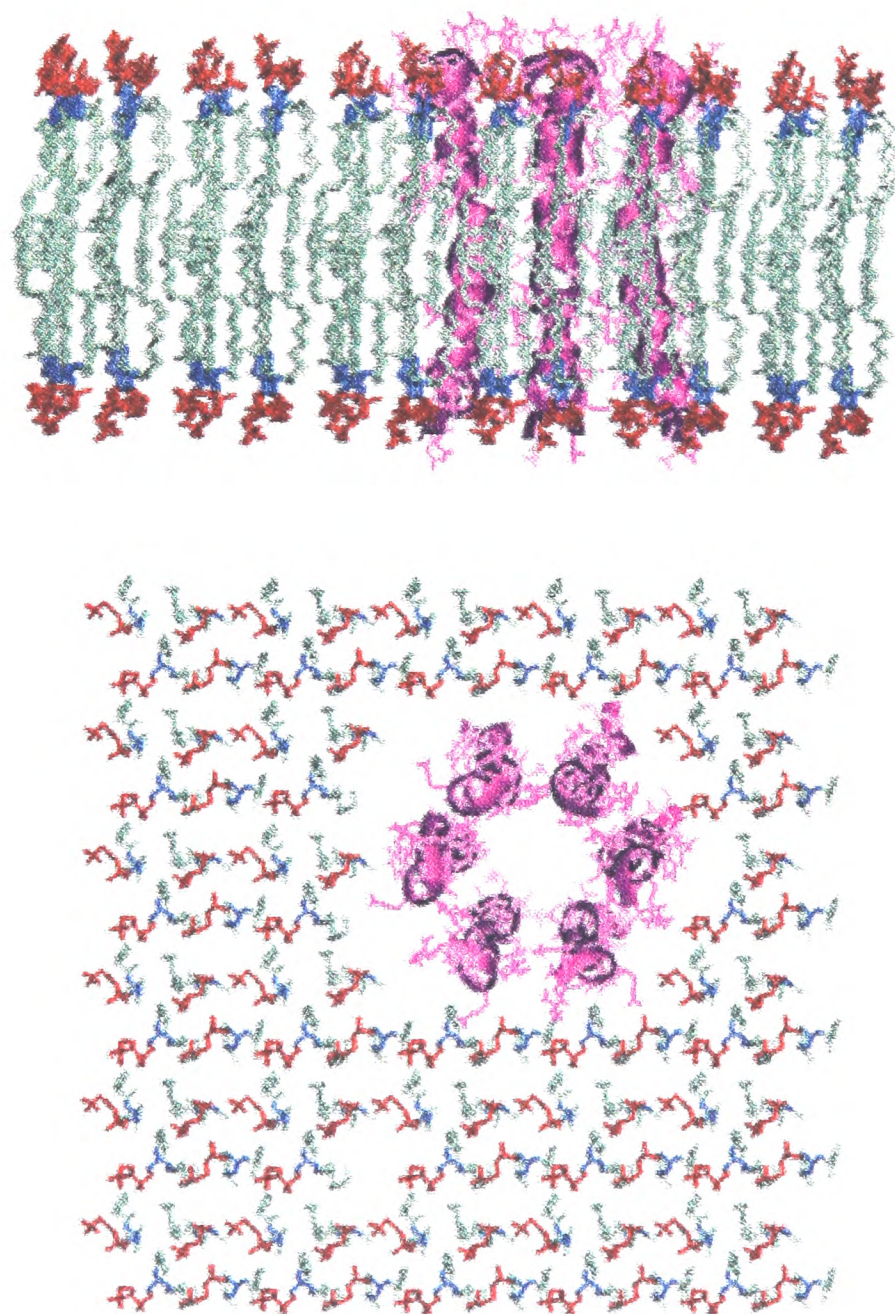


Figure 8.31 The hypothetical model of a melittin pore in a bilayer of DMPC molecules before molecular mechanics calculations have been performed viewed from the side and the top.

The bilayer model was hydrated with TIP3P water molecules to give a system containing nearly 36,000 atoms which required it to be run using parallelised versions of the AMBER4.1 program.

The equilibration of the system involved a series of three energy minimisations on firstly the lipid molecules, then on the water and lipid molecules together and finally on all molecules in the system.

This energy refined structure was taken and using AMBER4.1 with the same parameters as used in the previous simulations of melittin in bilayers, 100ps of equilibrating molecular dynamic simulation were run with the first 50ps having only the lipid molecules able to move and the second 50ps with both the lipid and water molecules able to move. The objective of this part of the simulation was to relax and equilibrate the lipid and water molecules around the pore without disturbing the integrity of the pore. To compensate for a system that may have too low a density, a short constant pressure simulation was run to slightly shrink the system and remove any large empty volume that might be present.

Molecular mechanics simulations were run on the system for a further 550ps but within approximately 100ps the pore had started to lose its regular hexagonal packing structure with helices moving apart and lipid chains moving between them (see Figures 8.32 and 8.33), but in the interval 100 to 550ps the structure of the pore changed very little.

At first sight, one possibility why this may have occurred is because the peptide chains in the starting structure were not packed together tightly enough or that the lipid density around the peptide chains around the pore was not high enough again leading to the melittin molecules moving apart despite the very careful equilibration procedure. However with deliberation and an examination of the pore structure it is clear that:

- 1) A pore structure does still exist since there is a clear hole in the membrane in which water molecules are present but lipid chains do not penetrate
- 2) At the N-terminal end of the melittin there is only one polar group so there is actually very little incentive for the melittin chains to remain in a close packed array and they must be relatively free to move

3) At the C-terminal end of the melittin there are four residues with positive charges in the same vicinity which must lead to unfavourable contacts

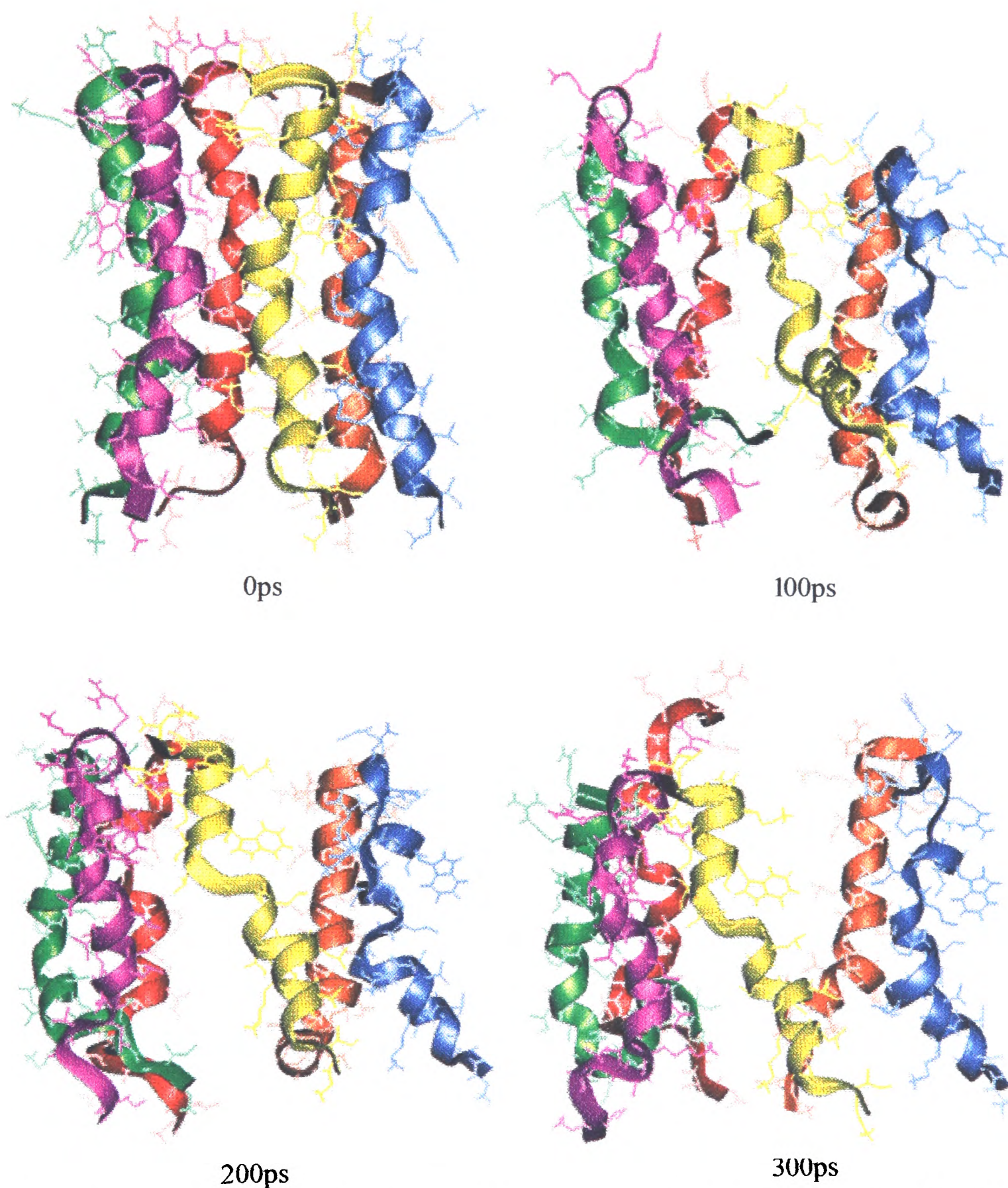


Figure 8.32 Four snapshots taken every 100ps from the molecular mechanics simulation showing the change in the packing of the six melittin helices when viewed from the side.

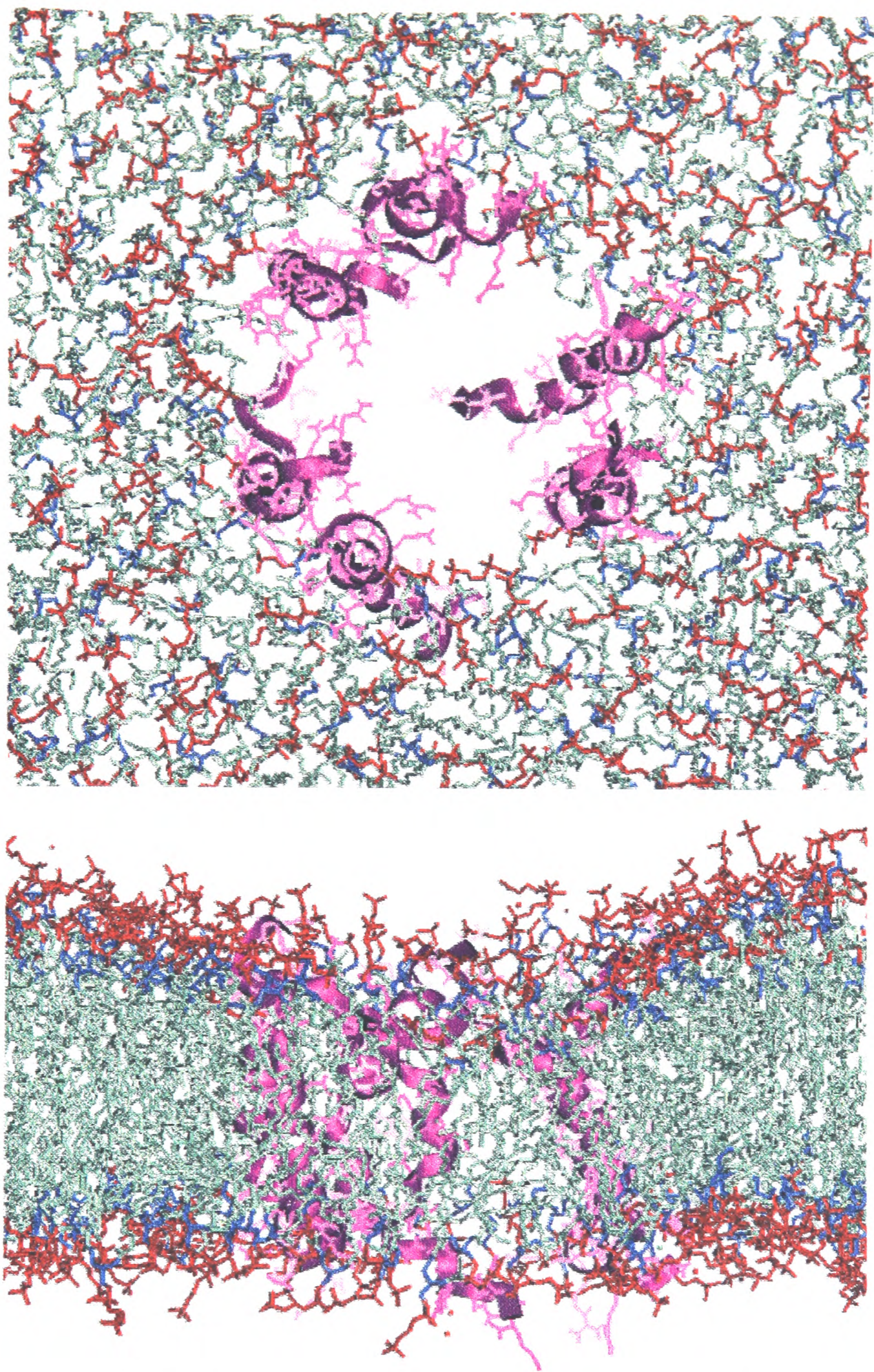


Figure 8.33 The simulation of the melittin pore in the membrane observed from the top at 400ps showing that although the structure of the pore is not as regular as at the start (see Figure 8.32a), a hole still exists in the membrane.

Certainly it is too early in the research to use this result as an indication that pore formation definitely does or does not occur in the membrane for melittin and the system will require further simulation and more study. However the model does support a hypothesis in which the melittin molecules do form a flexible, less well-defined pore structure which is not unexpected given the lack of large, favourable intermolecular interactions to bind them together and so remain in the tightly packed arrangement of the starting structure. However the presence of relatively hydrophilic residues occurring together on one side of the molecule does provide a driving force for them to aggregate.

An interesting observation of the system is that the six melittins could be considered to have formed three dimer structures (see Figure 8.33) so some close association between the melittins occurs. The separation of melittin molecules could be as a result of the melittin molecules needing to distance themselves at the C-terminal end because of the short distance between like-charged residues and even needing phospholipid head groups to come between them to mediate their interactions. It has been suggested that the role of the proline residues in melittin is to allow the peptide to bend so that the charged groups may become separated and if Pro-14 is replaced by Ala-14 then the ability of melittin to form a voltage-gated channel is reduced (Dempsey, 1990).

The final noticeable effect that the melittin pore has on the lipid bilayer is that there is a definite thinning of the membrane around the pore on the side out of which the N-terminal end of the melittin molecules protrude. This may reflect the Lys-7 group pulling the head groups down to interact with it.

8.13 The Behaviour of the Melittin Molecules of the Pore within the Membrane

Generally the alpha helical structure of the peptides appears to be maintained during the simulation except at the termini of the chains where it is interacting with waters and the charged head groups.

Pair distribution functions were calculated for a selection of the polar hydrogens of the melittin molecules with oxygen atoms of the phospholipids to characterise some features of

the interaction between the pore and bilayer. It was observed that the residues Lys-7, Thr-10, Thr-11 and Ser-18 showed very little interaction with oxygen atoms of the lipid molecules which is consistent with their position within the interior of the pore. Conversely the Trp-19 residue shows hydrogen bonding to the carbonyl oxygens of the fatty acid chains and are found quite near the surface which is consistent with experimental observations (Chattopadhyay and Rumini, 1993).

Comparing Lys-21 which is placed on the inside of the pore with Lys-23 which is on the outside then Lys-21 is observed to be hydrogen bonded to phosphate oxygen's for only 2% of the time while Lys-23 is hydrogen bonded 8%.

Another interesting characteristic is that the Arg-22 residue shows extensive hydrogen bonding to phosphate oxygens while for Arg-24 it is virtually non-existent. This suggests that the role of Arg-22 is to bond the melittin to the bilayer while the Arg-22 is mostly in aqueous phase and is the origin of the selectivity of the pore for anions which is observed experimentally since they form a ring of positive charge around the entrance (Tosteson and Tosteson, 1981).

8.14 Conclusions on the Simulations of Hypothetical Melittin Pores in Membranes

It must be emphasised that it is still not clear if melittin molecules do form an actual pore structure within bilayers let alone any direct structural evidence on which to base a model. This simulation is an exercise in trying to determine the validity of a possible model and test the membrane bilayer model and parameters that have been developed.

What can be concluded is that although the pore structure is not stable in the regular hexagonal arrangement, it does appear to relax to a less well-defined, but stable, pore-like structure. This probably occurs because although the intermolecular interactions between melittin molecules are not sufficient to hold the pore together tightly, the hydrophilic segment of the molecule provides a driving force for them to form a loose association and produce a hole in the membrane through which water and other small solutes may pass which is in line with some of the toxic effects that melittin is observed to cause.

Chapter Nine

Simulations of the Ion Channel Gramicidin in Lipid Bilayers

9.1 Introduction

The melittin pore studied in the previous chapter was a hypothetical model while the subject of this chapter is the ion channel gramicidin A which has been studied extensively as a model ion channel. Ion channels are important since they provide an energetically favourable pathway for the movement of ions across the apolar bilayer.

Gramicidin A is a peptide showing antibiotic activity and is produced by the aerobic soil bacterium *Bacillus brevis* (Hotchkiss and Dubos, 1940). It consists of 15 alternating L and D amino acids with a formyl group at the N-terminus and an ethanolamine group at the C-terminus (Sarges and Witkop, 1965) which because of the alternating D and L residues is able to form β -type helices in a lipid environment. These β -type helices are characterised by the side chains pointing outwards while the carbonyl groups point alternately upwards and downwards which gives a helix with a hydrophilic pore of diameter $\sim 4\text{\AA}$ running through it. In membranes these gramicidin peptides adopt the $\beta^{6.3}$ conformation (Weinstein et al., 1980) and dimerise head-to-head by hydrogen bonding between the N-H and C=O groups of the two units (Veatch and Stryer, 1977; Cifu et al., 1992). The structure of this channel has now been fully characterised and solved in membranes using 2-D NMR (Arseniev et al., 1985). This structure is used in the current simulations (see Figure 9.1).

Although the natural function of gramicidin in *Bacillus brevis* is probably not as an ion channel but as a cofactor in RNA synthesis (Fisher and Blumenthal, 1982), it behaves as a monovalent cation selective, single filing pore when integrated into membranes. Gramicidin A has been studied extensively because while exhibiting behaviour very similar to that of more complex biological ion channels, it is very simple structurally and this makes it an ideal

model channel for both experimental and simulation work. (For a review of gramicidin, see Killian, (1992)).

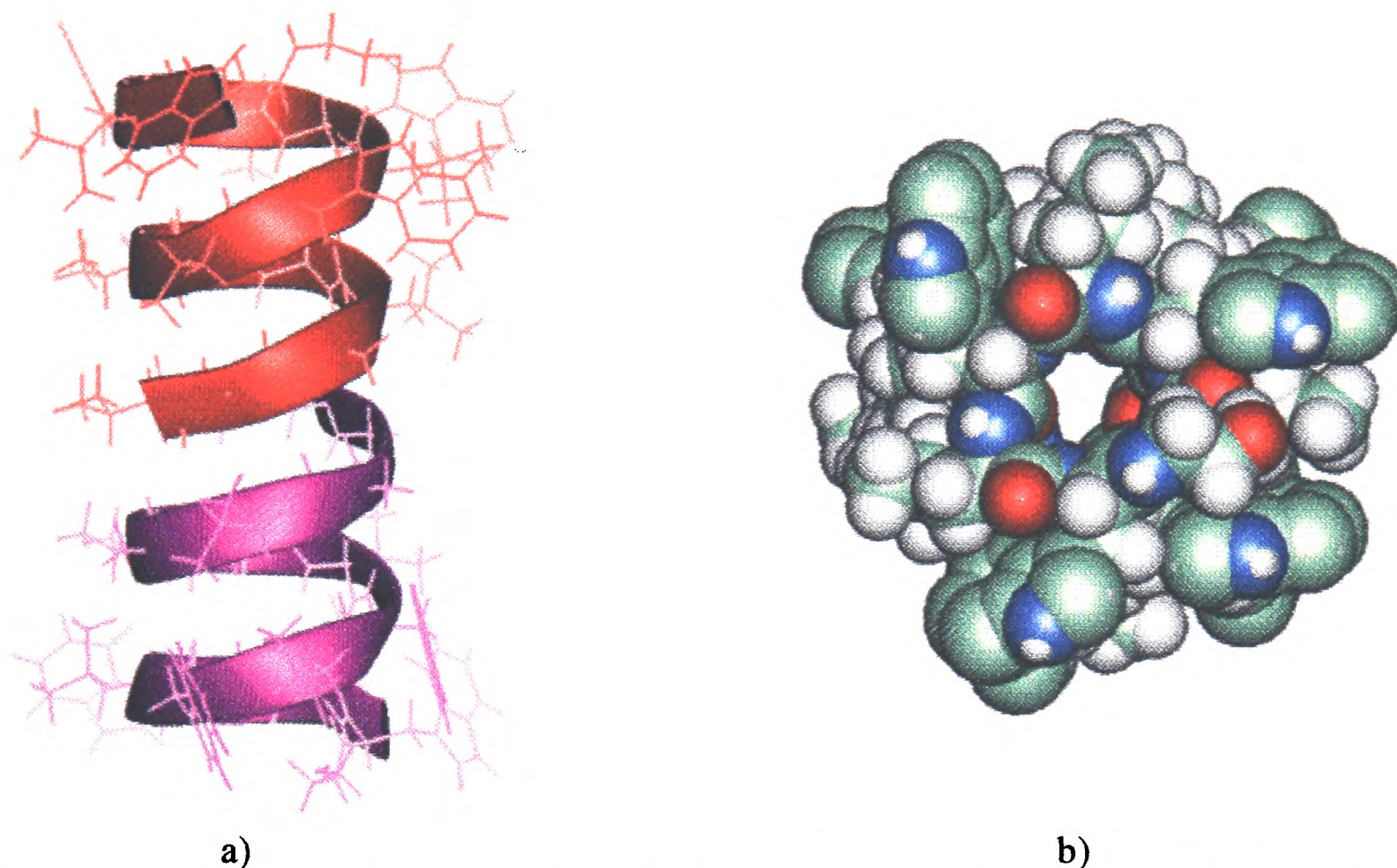


Figure 9.1 Two views of the protein gramicidin from a) the side to illustrate the two subunits in the structure and b) above with the gramicidin as a space filling model to show the hole that exists down the centre of the protein.

There have been previous theoretical studies of gramicidin in model membranes to investigate ion transport, (Chiu et al., 1989; Jordan, 1987; Mackey, Berens and Wilson, 1983; Roux and Karplus, 1991a; Roux and Karplus, 1991b; Roux and Karplus, 1993; Roux, Prod'homme and Karplus, 1995) and one simulation of gramicidin A in an explicit bilayer environment (Woolf and Roux, 1994; Woolf and Roux, 1996) although this only contained 16 lipid molecules in contrast to the 166 simulated here.

The simulations of Mackay, Berens and Wilson (1984) considered a water-filled gramicidin channel and ran short molecular dynamic simulations of 5ps to determine the behaviour of water molecules and ions within the pore. They observed that the water molecules were oriented with respect to each other in order to allow hydrogen bonding to their neighbours and the carbonyls of the channel walls and that their motions were highly correlated.

The early simulations of Roux and Karplus (Roux and Karplus, 1991a; Roux and Karplus, 1991b) used periodic poly(L,D)-alanine β -helix structures to model the interior of gramicidin channels. Water molecules and either Na^+ or K^+ were placed in the channel and the potential of mean force of the ions calculated as a function of distance along the channel (Roux and Karplus, 1991a). These calculations were performed because it is believed that the free energy profile along the channel is the main factor responsible for the ion selectivity of the gramicidin channel and an understanding of both the rate of ion passage through the channel and the origin of selectivity necessitates a knowledge of both dynamic and thermodynamic contributions. It is observed that the water molecules are all aligned in the channel and show hydrogen bonding both to their neighbours and the carbonyls of the channel walls. The free energy profile of Na^+ indicates a sequence of well-defined binding sites along the channel with a binding site characterised by the ability of an ion to interact with four surrounding carbonyl oxygens plus two neighbouring water molecules. The ion hops between these binding sites through transition states in which it loses contact with two of the carbonyl oxygens, but becomes associated with another two at the new binding site. In the absence of any ions the water molecules are observed to have a tendency to occupy specific locations in the pore. The selectivity arises because different sized ions have an effect on the ability of their water neighbours to hydrogen bond to them and reside in favourable positions in the pore.

Mobilities of water, Na^+ and K^+ inside the model gramicidin channels were evaluated (Roux and Karplus, 1991b) and showed there to be almost free diffusion of water while motion of the Na^+ was controlled by local interactions and collisions with neighbouring carbonyls and the two nearest waters. The activation free energy barrier to water movement is small enough ($\sim 0.2 \text{ kcal/mol}$ (Roux and Karplus, 1991a)) that water transport is observed to proceed as a continuous diffusive process.

In reports of simulations of water transport in gramicidin dimers (Roux and Karplus, 1991b), the translational mobility and rotation of water molecules along the channel was observed to vary significantly. The water molecules around the entrance of the channel were observed to show much slower diffusion and orientational relaxation because of the orientation of the carbonyls in the β -helical structure at these locations.

In more detailed molecular dynamic simulations to calculate the free energy profile of Na^+ , the gramicidin A channel was modelled explicitly with caps of water molecules at either end of the pore and the membrane described using a collection of neutral Lennard-Jones particles (Roux and Karplus, 1993). The simulations were used to estimate the free energy profile of Na^+ in three regions corresponding to the transition of the ion from bulk solvation to single file, the single file at the mouth of the pore and at the junction of the two monomer units at the bilayer centre. Binding sites were found at the extremities of the channel and the principle energy barrier was at the entrance of the channel in the single file region.

The molecular dynamics simulations of Woolf and Roux (1996) studied details of lipid-protein interactions using a fully hydrated DMPC bilayer. The initial structure of gramicidin A surrounded by 16 lipid molecules was built using a Monte Carlo type procedure in which lipid molecules with random conformations were placed around the peptide and then the number of bad contacts between peptide and lipid was evaluated and if the number was too high then the structure was rejected. In this manner 300 systems were investigated of which seven were used for molecular mechanics simulations. During simulations it was observed that the gramicidin A caused an ordering of the chains around it but the effect was not uniform because of the irregular shape of the protein. The most energetically favourable interactions between the lipid and protein were observed to arise from van der Waals and electrostatic interactions of approximately equal magnitude.

The work of this chapter was two-fold; first to consider how a trans membrane protein should be placed into a membrane environment with regard to lipid packing, and secondly to assess the effect of the bilayer and the gramicidin on each other. Of particular interest is how the waters behave in and around the pore plus the interaction of the phenanthroline rings with the lipid molecules and the interstitial water since this is the first system large enough to examine these behaviours properly.

The two models that were built and simulated differed in respect to the initial packing of the lipid molecules around them. In the first model the lipid molecules were arranged randomly around the gramicidin while in the second an appreciation of the structure of the gramicidin was taken into account and the lipid molecules placed around the channel in a more ordered fashion.

9.2 Building Models of Gramicidin in a Membrane

Gramicidin is composed of two identical alpha helical peptides that come together to form a pore structure that traverses the lipid bilayer and is selective for single valent cations. Two views of the structure taken from the NMR structure (Arseniev et al., 1985) are shown in Figure 9.1 to illustrate the two sub-units and the pore through the centre of the structure.

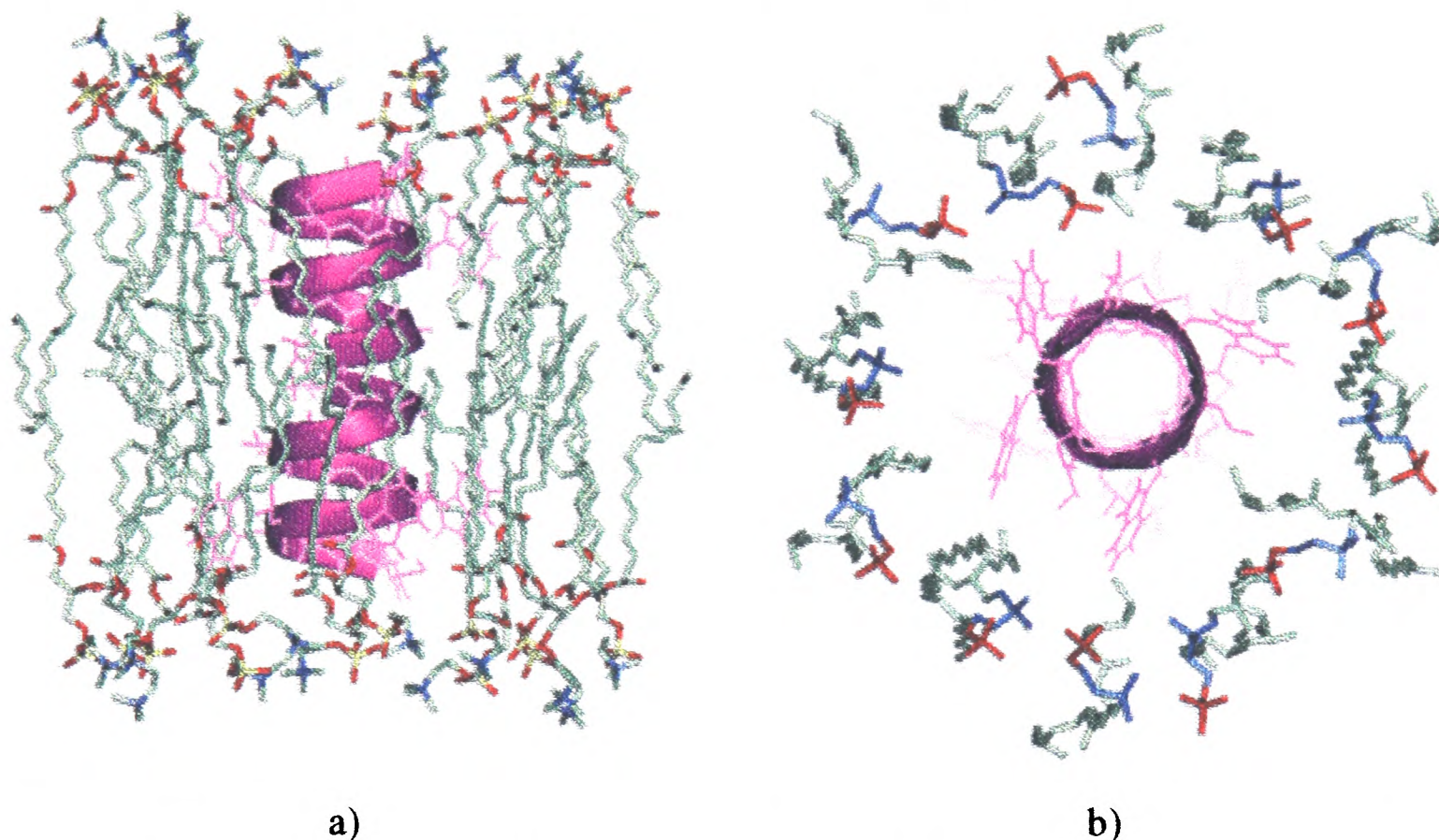


Figure 9.2 The gramicidin protein with a ring of twelve lipids around each sub-unit of it. In the first part of the figure it can be seen that the gramicidin only just spans the hydrophobic region while in the second figure the negative phosphate groups have been coloured blue and the positive N-methyls have been coloured blue to show the relative arrangement of the head groups (NB Only the lipids on one side have been shown).

At either end of the gramicidin channel there are four tryptophan residues that form a ring around the channel and these large and bulky groups must have a particular function. A tryptophan residue is also present in melittin and as was shown in the previous chapter it was quite capable of forming hydrogen bonds with the carbonyl groups in the fatty acyl chains of surrounding lipids. It was this observation that led to the two different membrane models that were constructed. In the first model the lipids were simply placed around the gramicidin in a fashion which maintained close packing of the chains to the protein and favourable interactions between the lipid head groups (see Figure 9.2) while in the second model the lipids were oriented so that the carbonyl groups of the fatty acyl chains were directed towards

the polar hydrogen of the tryptophan residues which led to a very different orientation of the head groups with respect to each other (see Figure 9.3).

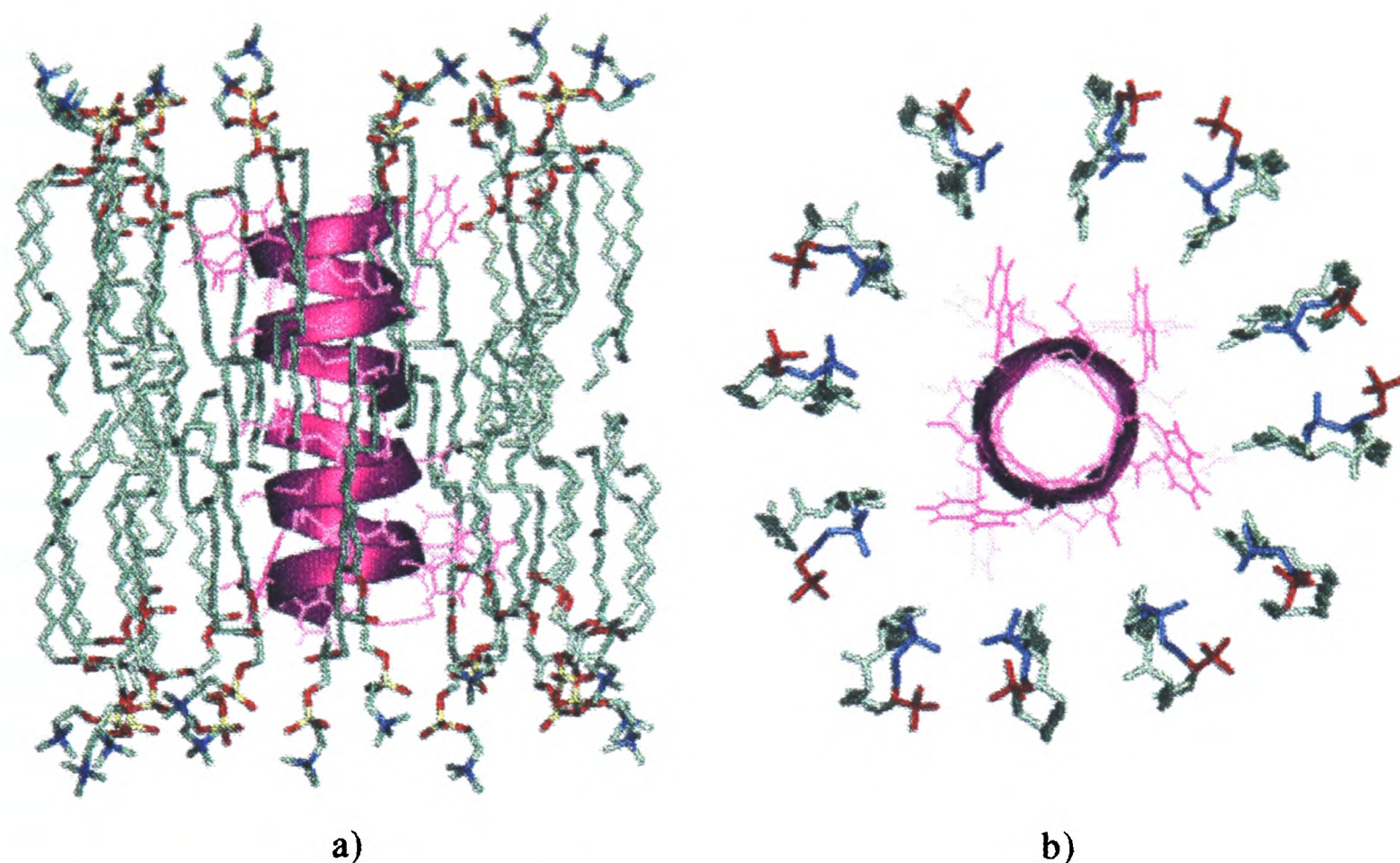


Figure 9.3 The second model of stage one in which the lipid molecules are oriented so that the carbonyl groups face into the gramicidin. (Colour scheme is the same as in Figure 9.2).

The purpose of these variations was to determine how stable each geometry was and to decide how important the starting lipid structure is on the dynamics of the protein. Furthermore, given the results from previous simulations, the probability of a molecule reorienting along its long axis is very small on the time scale of present simulations. Thus it is immensely important to make sure that the starting configuration of the system is as close as possible to the optimum.

The model building was divided into two main stages for both systems, in the first stage a ring of twelve annular lipids was placed around each sub-unit of the gramicidin and carefully monitored energy minimisations and molecular dynamic simulations were carried out to equilibrate the positions of these lipids around the gramicidin as seen in Figures 9.2 and 9.3. In the second stage, the gramicidin with its annular lipids was placed into a lipid bilayer and the positions of lipid molecules adjusted so that there were no close contacts or large

vacancies in the bilayer after which a series of energy refinement calculations were done followed by the main molecular mechanics simulation of the system.

During the energy refinements and molecular dynamic simulations of stage one, a layer of water was added to each side of the bilayer so that the head groups were not undergoing simulation without explicit water present. These waters mediate interactions between the head groups which would otherwise have free and unhindered motion.

In Figure 9.2b it can be seen that the lipids have been oriented so that the head groups all have favourable electrostatic interactions. In Figure 9.3b it is apparent that having all the carbonyl groups directed toward the gramicidin results in the phosphate groups (coloured blue) all forming a ring of negative charge around the pore.

During the equilibration of the second model in which the head groups of the same charge were quite close (see Figure 9.3b), the lipid head groups were observed to reorient themselves to maximise favourable electrostatic interactions.

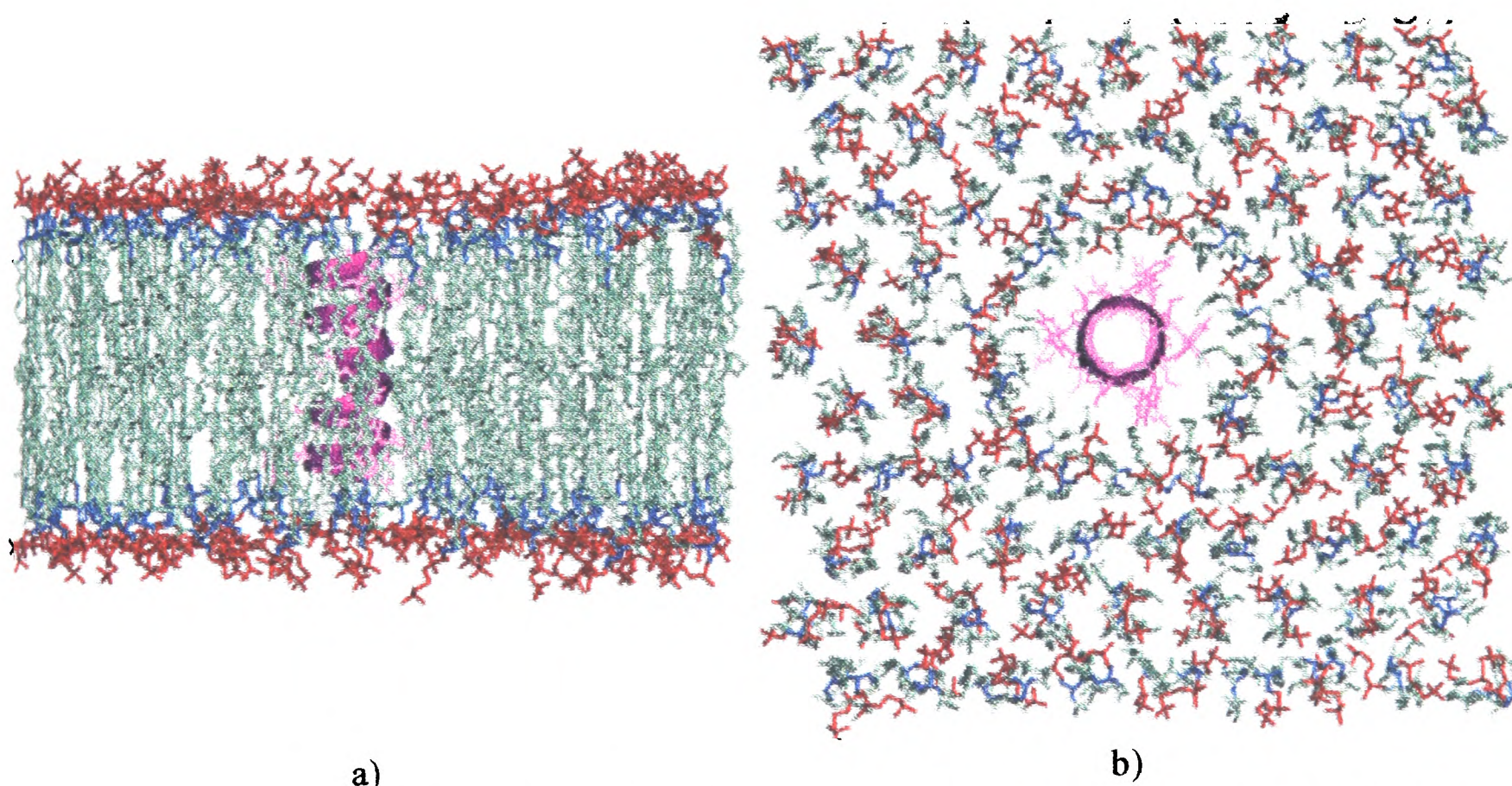


Figure 9.4 One of the starting configurations of the gramicidin membrane models. In the second part of the figure the annular ring of lipids may just be differentiated from the rest of the lipids.

In Figures 9.2 and 9.3 it can be seen that the gramicidin channel is about the width of the hydrophobic region in DMPC and places the tryptophan residues adjacent to the carbonyl groups. Of the two models, it is more probable that the first one in which the lipids are placed to maximise head group interactions is closer to the situation in real membranes and a picture of the starting configuration of gramicidin in a membrane is shown in Figure 9.4.

In the simulation by Woolf and Roux (1996) the membrane structure simulated was essentially analogous to the starting conformation shown in Figure 9.2, but rather than solvate the structure in a bilayer environment, hexagonal periodic boundary conditions were used so that the lipid to protein ratio is 8:1 and 45% by weight of water.

9.3 The Simulations of Gramicidin in Lipid Bilayers

Both models were simulated under NVT conditions using OPLS parameters in AMBER4.1 in exactly the same fashion as previous simulations for over 600ps with the first 100ps being considered as an equilibration phase.

For both models, the β -type helical structure of the gramicidin remains completely intact for the whole of the simulation, including the end sections which for other systems have shown a tendency to lose their integrity (see Chapters 7 and 8). However these peptides all had the termini of their chains exposed to the aqueous phase while for gramicidin both ends are buried in the hydrophobic core. This result shows that the gramicidin molecule is completely stable within the bilayer environment and it is the nature of the interaction between the lipid molecules, gramicidin side chains and water molecules that needs to be investigated.

9.3.1 The Order Parameters of the Lipid Chains

The deuterium order parameters of methylene segments of the Sn1 and Sn2 chains were calculated as a function of distance from the central axis of the gramicidin in 5Å bands. Since very few lipid chains come closer than 10Å to the gramicidin, the order parameters shown in Figure 9.5 are for the ranges 10-15Å, 15-20Å, 20-25Å and 25-30Å from the central axis.

The effect of gramicidin on lipid chain order is a complex phenomenon. At lipid to peptide ratios of greater than 15:1 then the quadrupolar splittings are seen to increase linearly with gramicidin concentration up to about 30% greater than the value for pure bilayers but then for the ratios 15:1 to 4:1 the splittings are observed to decrease slowly (Rice and Oldfield, 1979). ESR studies using the nitroxide free radical spin label detect motion on the 10^{-8} second time scale and indicate the presence of a motionally restricted annulus of lipid around the peptides in bilayers (Jost et al., 1973; Hesketh et al., 1976). Generally it is observed that there is an increase in chain segmental order parameters from spin label experiments (Chapman et al., 1977; Cornell et al., 1978). Furthermore Raman spectroscopy indicates a reduction in the number of gauche isomers in chains around peptides which is normally taken as an indication of an increase in chain order (Chapman et al., 1977). On the longer ^2H -NMR time-scale peptides are observed to cause a disordering of the hydrocarbon chains at least in the region of the terminal methyls (Oldfield et al., 1978).

In the simulation of Woolf and Roux (1996) the order parameters of those lipids around the gramicidin were seen to be larger which corresponds to the peptide ordering the lipid chains. However the effect was not uniform and this was hypothesised to be due to the irregular surface of the peptide because of the side chains of the amino acids that protrude out into the bilayer.

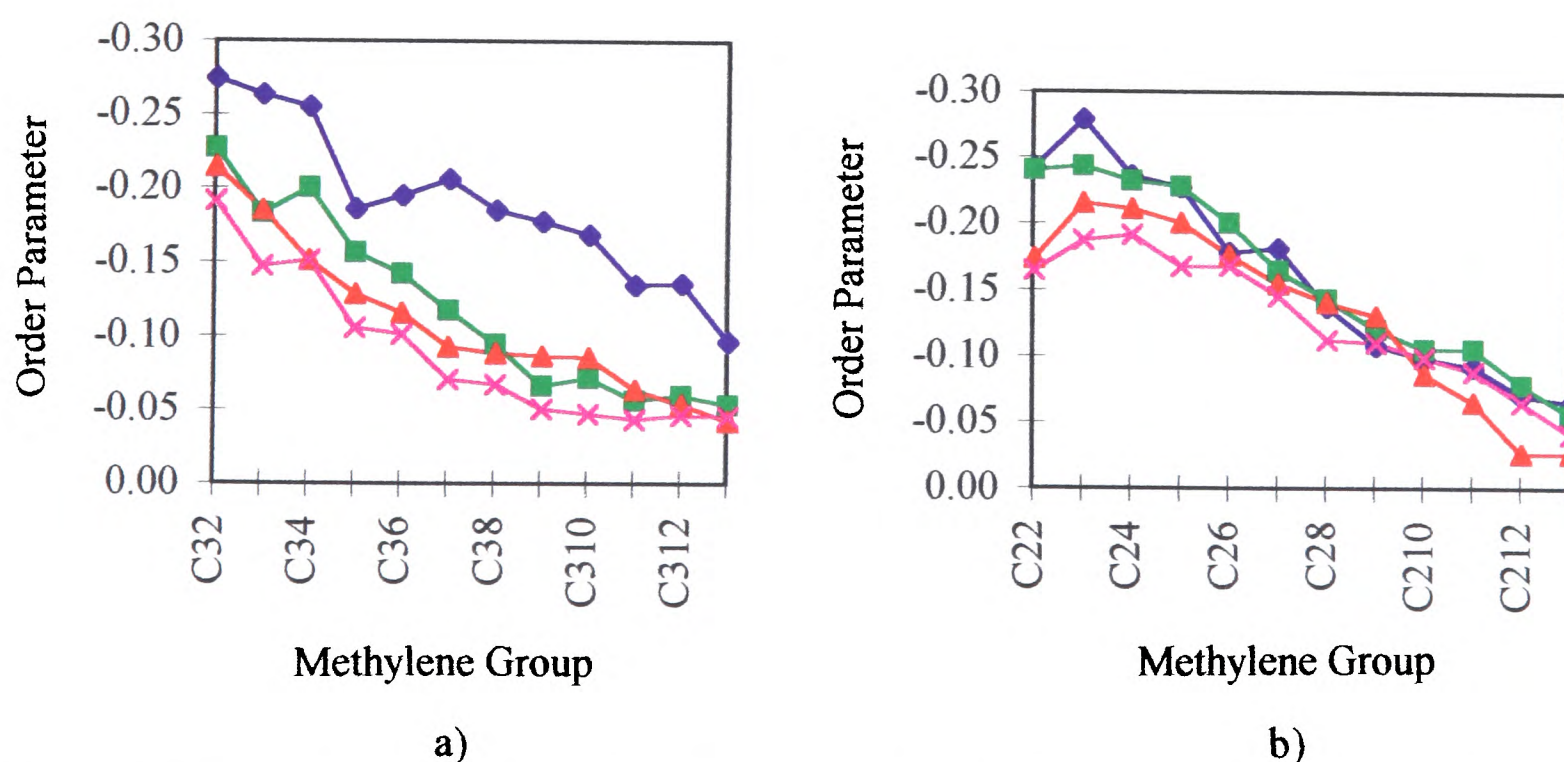


Figure 9.5 The calculated deuterium order parameters of the methylene segments for the a) Sn1 and b) Sn2 lipid chains as a function of the methylene group from the central axis of the gramicidin. The ranges 10-15Å, 15-20Å, 20-25Å and 25-30Å are shown in red, green, blue and magenta respectively.

For the Sn 1 chains in particular it is seen that the closer the lipid chains are to the protein the greater the ordering effect it has on the chains. The origin of the ordering effect on lipids has already been discussed in Chapter 7 but arises essentially because the gramicidin is a large immovable object and hence it reduces the motional and torsional freedom of chains directly in contact with it plus the lipids interact with its surface and are attracted to the surface as has been hypothesised previously to explain the increase in the molecular order parameter of DMPC in the presence of gramicidin (Cornell and Keniry, 1981).

9.3.2 The Tilt of the Hydrocarbon Chains around the Gramicidin

In the same manner as previously, the orientation of the lipid chains was considered to be described by three vectors which correspond to the top, middle and end of the fatty acyl chains. The distributions of the tilt angle that these vectors make with the bilayer normal were calculated as a function of distance from the central axis of the gramicidin. Not unexpectedly it was observed that those chains closer to the protein had not only a smaller range of tilt angles but that the probability of a chain possessing a smaller tilt angle was increased.

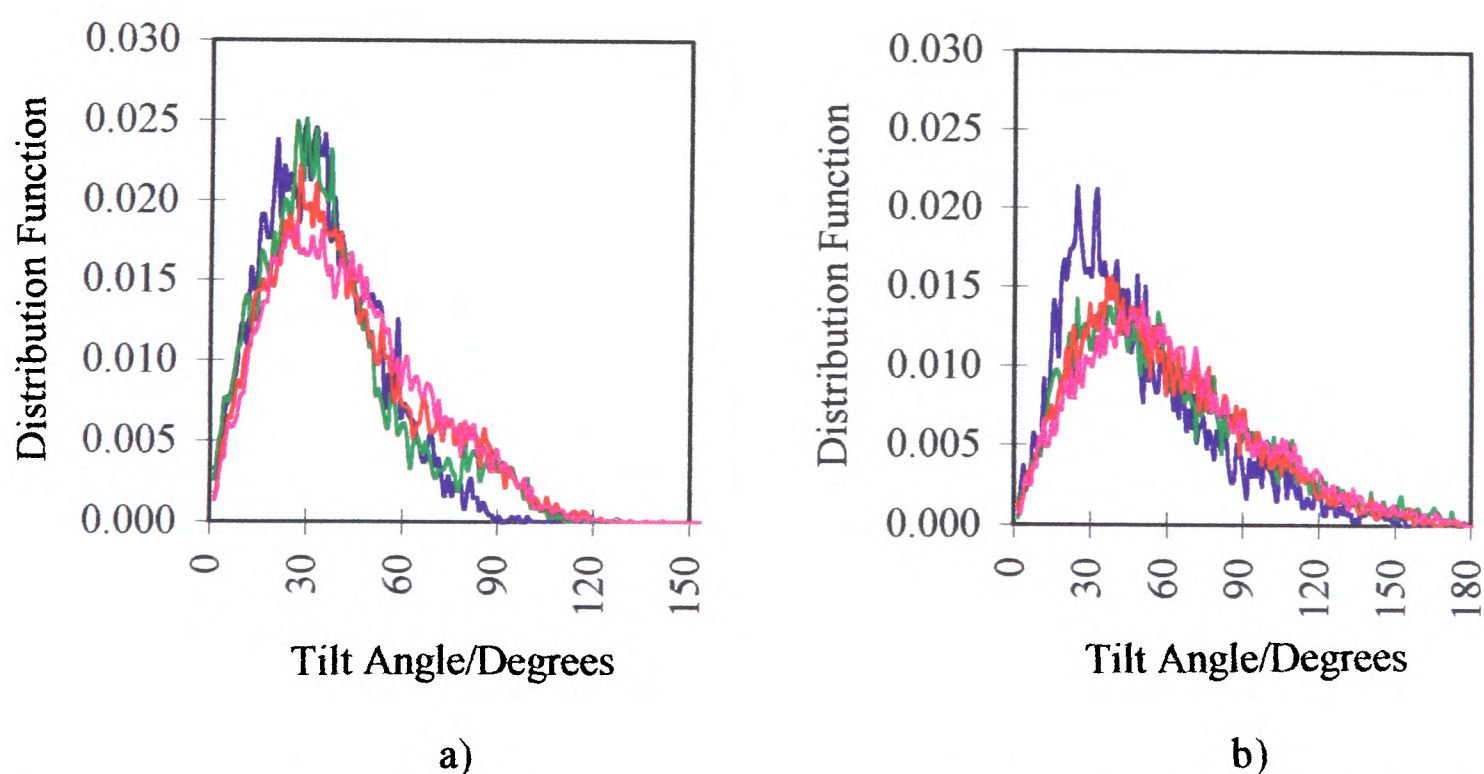


Figure 9.6 The distribution of tilt angles that vectors describing the orientation of a) the top of the lipid chain and b) the end of the lipid chain make with the bilayer normal as a function of distance from the central axis of the gramicidin. [Colour scheme is as in Figure 9.5].

Two of these distributions are shown in Figure 9.6 for the top and bottom of the Sn1 chains of the lipid molecules in bands of 5Å width starting from 10Å from the centre of the gramicidin. These results are in agreement with those from proton enhanced ^{13}C NMR which indicate that gramicidin reduces the range of angles that the long axis of DMPC molecules can experience and hence causes a change in the molecular order parameter (Cornell and Keniry, 1983). Thus the gramicidin has the effect of reducing the natural tilt of lipid chains around them as has also observed for smaller trans membrane peptides (see Chapters 7 and 8).

9.3.3 The Distribution and Shape of the Gramicidin in the Bilayer

The atomic density of the gramicidin across the bilayer was calculated as a function of distance from its central axis to determine the average shape of the gramicidin within the bilayer and the extent of penetration of the gramicidin side chains into the bilayer laterally, i.e. parallel to the bilayer surface. The atomic density profiles were calculated using the methodology of the 2-d radial distribution functions described in Chapter 3 in bands of width 1Å starting from the central axis of the gramicidin across the width of the bilayer in 1Å strips and three are shown in Figure 9.7.

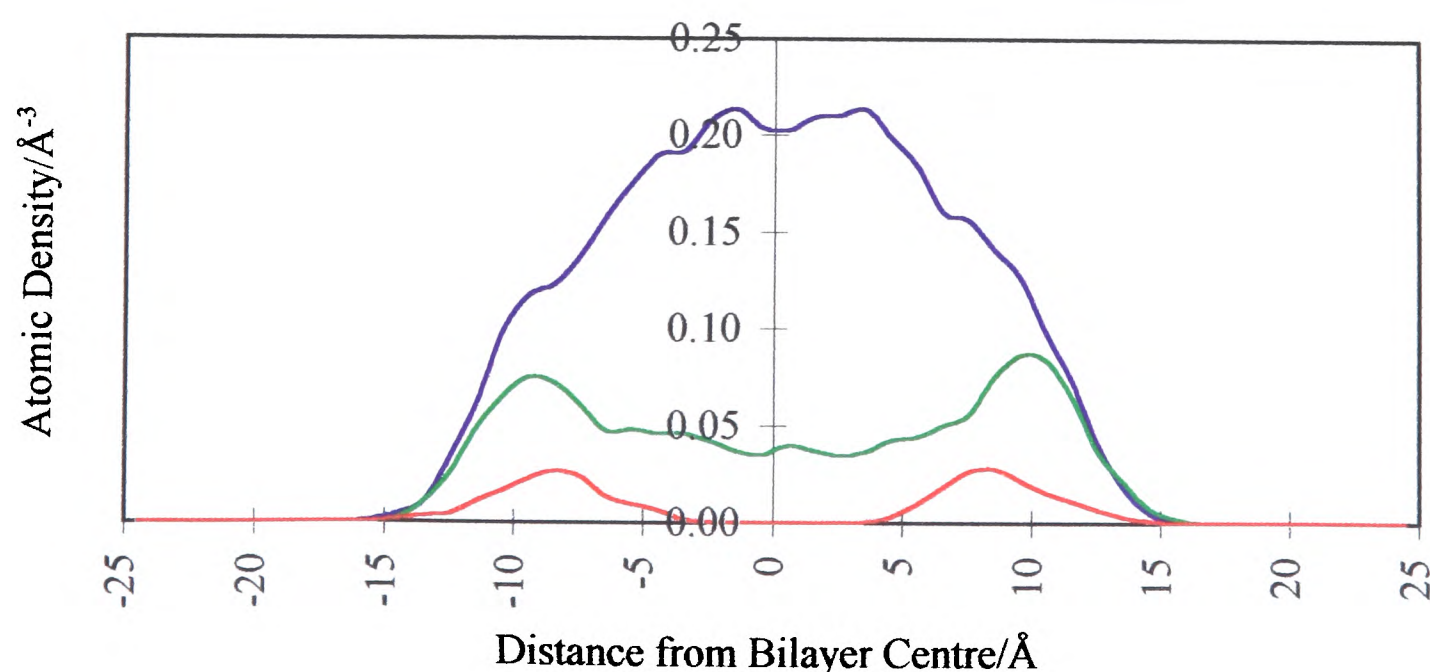


Figure 9.7 Selected atomic density profiles of gramicidin across the bilayer as a function of distance from the bilayer centre for distances of 3-4Å (blue), 4-5Å (green) and 9-10Å (red) from the central axis of the gramicidin.

Examining Figure 9.7, the very large density profile between 3 and 4Å from the central axis of gramicidin corresponds to the atoms that make up the straight walls of the channel and hence the high density is as a result of the large number of atoms present at this distance. Between 4 and 5Å from the central axis it can be seen that the density is highest at the ends and lower in the middle which is due to the large tryptophan residues extending into the bilayer at either end of the channel compared to the smaller residues in the bilayer centre. Finally between 9 and 10Å from the central axis it can be seen quite clearly that the only gramicidin atoms penetrating this far are those of the phenanthroline rings with the density dropping to zero at the bilayer centre. These profiles illustrate that the gramicidin is far from cylindrical in shape with the diameter of the end of the channel extending up to 22Å because of the bulky phenanthroline residues, but falling to around 14Å at the bilayer centre.

The profiles also show that the length of the gramicidin is around 25Å which is much less than the usual width of a membrane of around 40Å and reflects how the channel is embedded in the hydrocarbon region of the bilayer and will have a group of waters above it that are effectively penetrating into the head group region but are quite free moving (see Section 5.7) because they are not associated with lipid head groups. Having examined the system it is not unreasonable that one structural role for the tryptophan residues is to prevent the close approach of any lipid head groups to the entrance of the pore that would hinder solutes entering it. This is discussed in the next section.

9.3.4 The Packing of the Lipid Molecules around the Gramicidin

Atomic density profiles were calculated at a series of distances from the central axis of the gramicidin pore in order to determine how the lipids packed around the pore and a selection of these are shown in Figure 9.8 illustrating that the head groups do not extend into the region above the entrance to the pore (0-2Å from its centre) but between 5 and 10Å from the centre of the pore the head groups are wrapping over the tryptophan residues. Furthermore it is concluded that the lipids bend around the bottom of the tryptophan residues so that the lipid chains are also packed around the thin central part of the pore (see above).

If the regions of very low atomic density for lipid molecules between 5 and 10Å from the bilayer centre are compared with the density profiles in Figure 9.7 then it can be seen that these dips coincide with the high densities from the typtophan residues extending into the bilayer. In addition, the peak in the lipid density from 0 to 5Å from the bilayer centre for lipid chains between 9 and 10Å from the gramicidin central axis is because the gramicidin is thinner at its centre than at its ends and the lipid chains wrap around the tryptophan residues and into this region to interact with the apolar peptide residues. Moving further away from the gramicidin pore then the density profiles become closer to that expected for a normal bilayer (McIntosh, 1990).

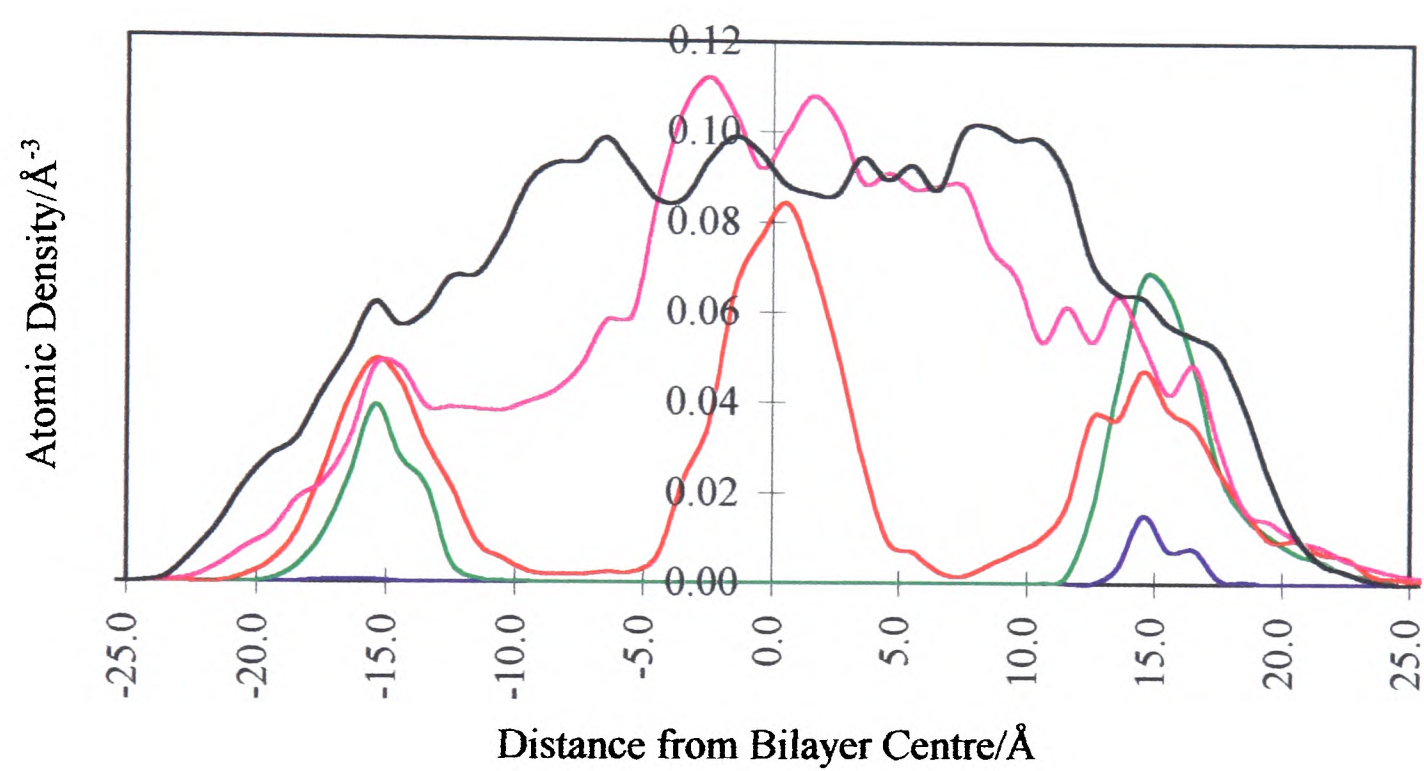


Figure 9.8 The atomic density profiles of the lipid molecules across the bilayer as a function of distance from the central axis of the gramicidin: 2-3Å (blue), 5-6Å (green), 7-8Å (red), 9-10Å (magenta) and 14-15Å (black).

Thus from these density plots it is evident that the lipid molecules are associated fully with the gramicidin in order to maximise their interactions with it to the extent of wrapping around the phenanthroline residues. The profiles also indicate that the bilayer width is thinner around the pore than in the bulk of the bilayer. The lack of head groups around the pore entrance also means that there will be a greater amount of water at this depth in the

membrane than would otherwise be expected and it is quite free moving because it will not be associating directly with charged head groups (see Section 9.3.5 and 9.3.6).

9.3.5 Water Penetration into the Bilayer

Results from fluorescence emission life-time measurements show that gramicidin increases the number of water molecules that are found in the hydrocarbon region of POPC layers (Ho, 1992) so the penetration of water molecules into the bilayer was calculated as a function of distance from the central axis of gramicidin for the simulation.

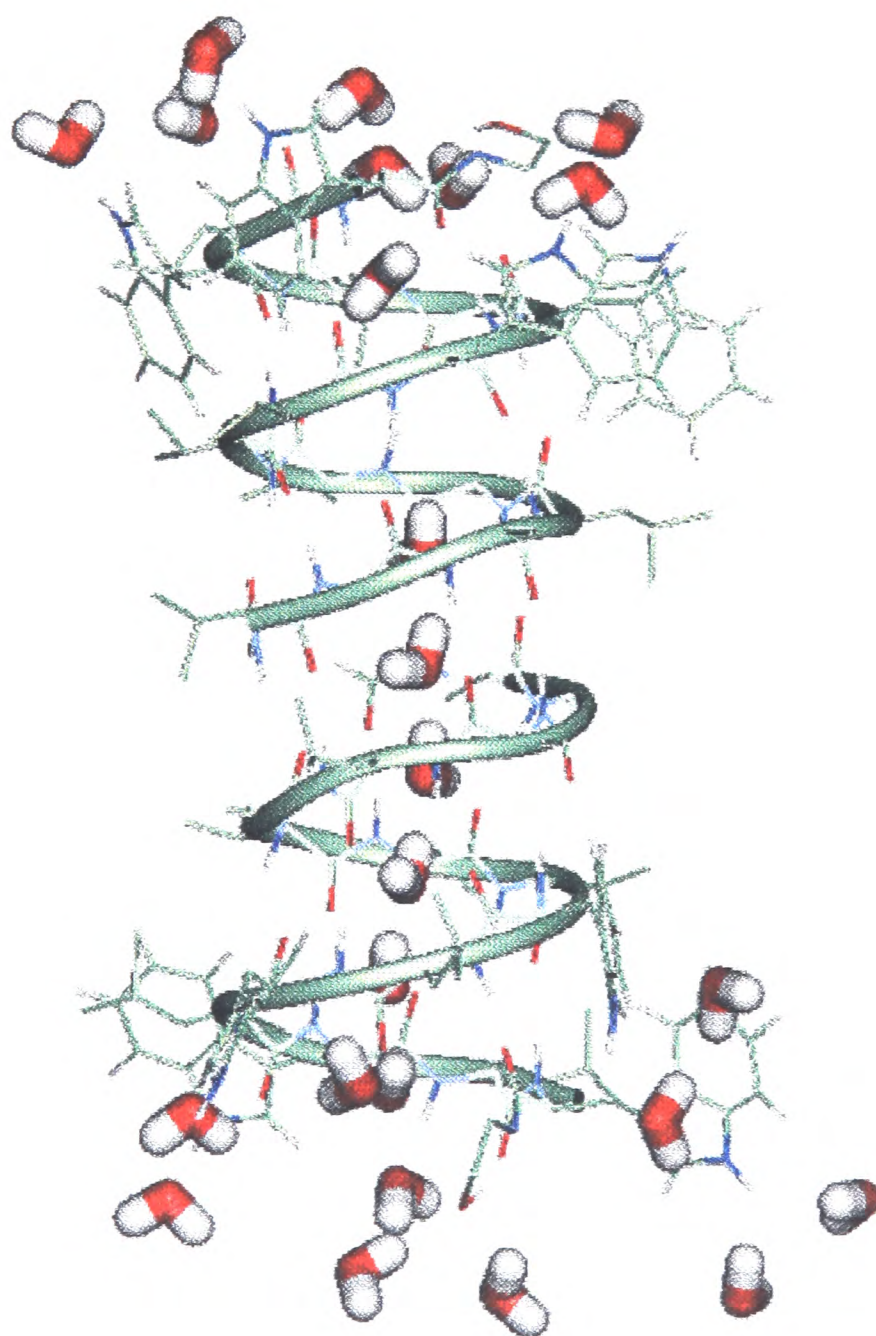


Figure 9.9 A snap shot from the molecular dynamic simulation at 500ps showing the thread of hydrogen bonded waters extending through the pore plus the water molecules hydrogen bonded to the residues (particularly the tryptophans) above and around the sides of the pore.

The two main observations are that there is a thread of highly oriented, hydrogen bonded waters extending through the pore (see Figure 9.9) and that the water density above the pore is greater than at the same depth in the rest of the bilayer (see Figure 9.10) which reflects the lack of lipid head groups in this region (see above).

The waters inside the pore were not present at the start of the simulation which was at first considered to be a problem with the model. However within 100ps of the start of the simulation water molecules had diffused into it.

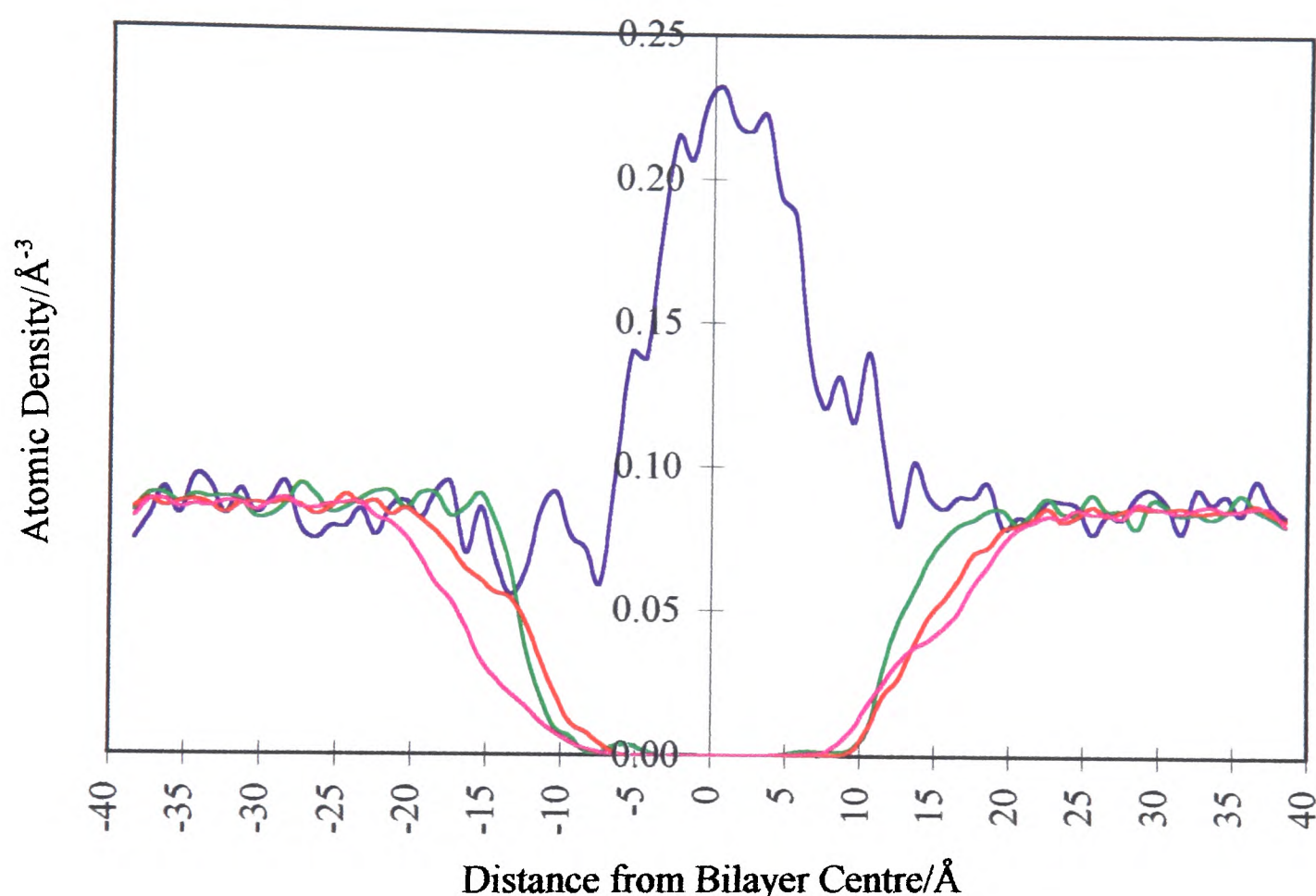


Figure 9.10 Four atomic density profiles of water across the bilayer as a function of distance from the bilayer centre for distances of 0-1Å (blue), 2-3Å (green), 9-10Å (red) and 19-20Å (magenta) from the central axis of gramicidin.

From osmotic and diffusional permeability measurements, five to six water molecules are expected to be present in the pore (Rosenberg and Finkelstein, 1978a) while electrokinetic measurements indicate that there are probably seven to nine water molecules present (Rosenberg and Finkelstein, 1978b; Levitt, 1984). During the course of the simulations

between six and ten water molecules are observed to be located within the pore of the gramicidin.

The hydrogen bonded orientation of waters within a gramicidin pore has also been observed in other simulations (Chiu et al., 1989; Mackey, Berens and Wilson, 1983; Roux and Karplus, 1983; Roux and Karplus, 1995). The water penetration into the bilayer was evaluated by calculating a series of atomic density profiles of water across the through the bilayer using bands of both 1Å and 5Å in width from the central axis of gramicidin (c.f. 2-d radial distribution functions described in Chapter 3) and a selection of them are shown in Figure 9.10.

The water density profile calculations show that water density at a depth considered normally as being in the head group region is far greater above the pore. This is because the gramicidin is only the width of the hydrocarbon region and the head groups do not extend into the region above the entrance to the pore because of the bulky tryptophan groups around the ends of the pore (see Figure 9.10).

9.3.6 The Mobility of Waters around the Pore Entrance

Since the waters around the pore are not directly associated with the highly charged lipid head group atoms they should have a much greater mobility than other waters in the head group region whose motion is restricted because of their role as bridging links between lipids. The mobility of waters at different depths in the membrane and as a function of distance from the pore in bands of width 4Å was determined by calculating their rotational correlation times. The static behaviour of water within the pores during the simulations was not considered appropriate for the calculation of diffusion coefficients from mean squared displacements although this was done by Roux and Karplus (1991) to yield values of 0.77 and 0.084 Å²/ps.

The correlation function was evaluated by calculating the correlation between the dipole of the water molecule at a time t and a later time $t + \Delta t$ over the course of the simulation. The

correlation was measured as the cosine of the angle between the two vectors describing the dipole.

As a first result, the average correlation between the dipoles after 1ps (i.e. the cosine of the angle) is shown in Figure 9.11 as a function of distance from the bilayer centre for four different distance classes from the centre of the pore: 0-4Å, 4-8Å, 8-12Å and 12-16Å.

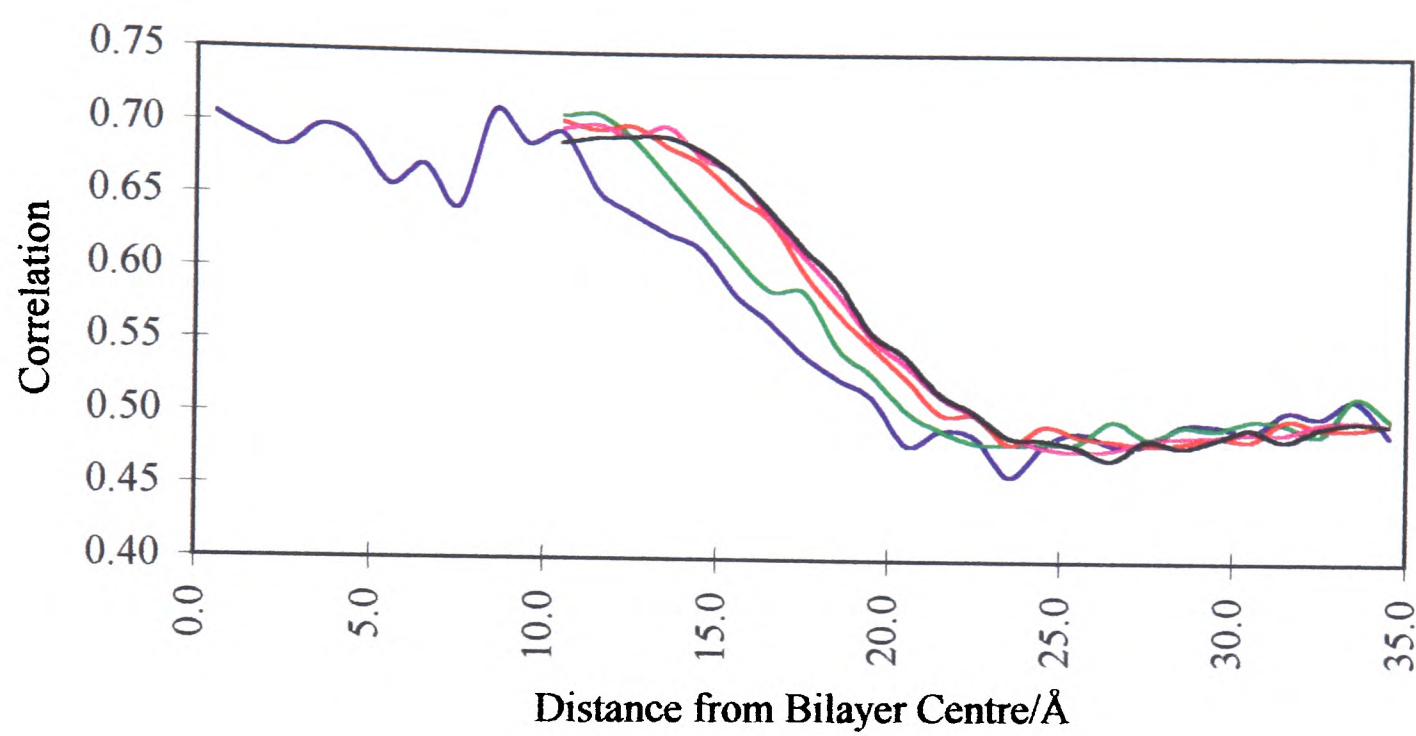


Figure 9.11 The correlation between the dipoles of water after 1ps as a function of distance from the bilayer centre for four different distance classes from the centre of the pore: 0-4Å (blue), 4-8Å (green), 8-12Å (red), 12-16Å (magenta) and 16-20Å (black).

The results of Figure 9.11 show quite clearly that those waters directly above the pore (0-4Å from the pore centre) are far more mobile than those at a similar distance from the bilayer centre but much further away from the pore and reflect the lack of highly charged atoms in the immediate vicinity of these former waters. The water molecules at a distance of 4-8Å from the pore centre are not as mobile as those directly above the pore, but freer than those at the same depth in the rest of the membrane. This is most probably because although they are associated with phospholipid head groups, they are not completely surrounded by them unlike the water molecules in the main bulk of the bilayer. The correlation for 0.0 to 10.0Å from the bilayer centre is only shown for the distance class 0 to 4Å from the pore centre

because very few water molecules are found at these depths anywhere else in the membrane except inside the pore (see Figure 9.10).

In Figure 9.12 the rotation correlation times are shown for the four different distance classes.

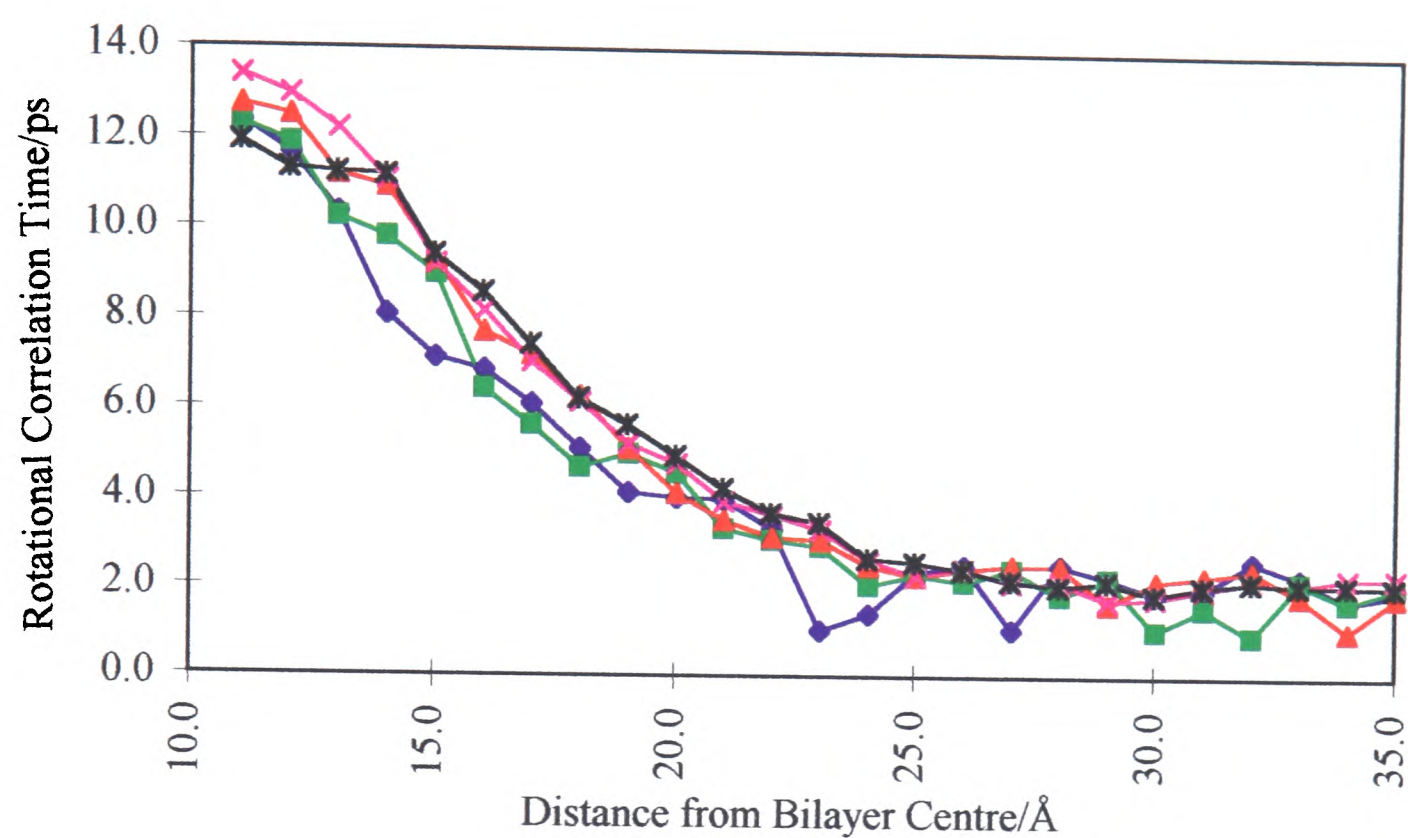


Figure 9.12 The rotation correlation times as a function of distance from the bilayer centre for the distance classes: 0-4Å (blue), 4-8Å (green), 8-12Å (red), 12-16Å (magenta) and 16-20Å (black).

The results shown in Figure 9.12 are completely consistent with those in Figure 9.11 and reflect the greater freedom of waters above the pore compared to those at the same depth in the membrane further away because the water molecules in the latter case are associated with highly charged phospholipid head groups which restrict their motion. The tryptophan molecules could be playing a steric role in preventing the lipid head groups coming to close to the pore and thus restricting the motion of water molecules around the entrance to the pore.

The greater freedom of the waters above the pore means it is far easier for a potential solute to swap places with a water and then be able to move into the pore and calculations on the free energy profile of Na⁺ moving into the pore (Roux and Karplus, 1993) showed no sharp

energy barrier because solvation by the carbonyls is equivalent to solvation by water molecules.

9.3.7 Hydrogen Bonding Interactions of the Tryptophan Residues

The tryptophan residues of gramicidin have an important role in stabilising the β -helical structure of the pore within bilayers and a critical function in the ability of gramicidin to function as an ion channel (Becker et al., 1991). Experiments in which tryptophans were substituted by phenylalanines lead to a drop in the conductance of the channel and a proposal that each tryptophan residue independently effects the conductance of the channel because of interactions between the dipole of the tryptophan and the approaching ion (Becker et al., 1991).

Results from hydrogen exchange (Takeuchi, Nemoto and Harada, 1990), IR and Raman spectroscopy (Bouchard and Auger, 1993) and fluorescence emission spectroscopy (Mukherjee and Chattopadhyay, 1994) show that the tryptophans are motionally restricted because of hydrogen bonds with either neighbouring interstitial waters or lipid carbonyls. However proton enhanced ^{13}C NMR of DMPC layers containing gramicidin gave results that were consistent with the peptide not causing significant conformational changes in the carbonyl region of the bilayer but having a major effect on the molecular order parameters by reducing the range of angles through which the long axis of DMPC moves (Cornell and Keniry, 1983).

In the simulations by Woolf and Roux (1996), although tryptophans were seen to hydrogen bond with carbonyls, phosphates and waters, the association with lipids tended to be short lived.

In the present simulation, pair distribution functions were calculated for the polar hydrogen of the eight tryptophan residues with oxygen atoms of the phospholipid and of the water molecules to determine the extent to which these residues are hydrogen bonded and the results are presented in Table 9.1.

	Phosphate O ₁₃ /O ₁₄	Phosphate O ₁₁ /O ₁₂	Carbonyl O ₂₂ /O ₃₂	Water
Trp- 10	42%	10%	0%	80%
Trp- 12	30%	0%	1%	100%
Trp- 14	71%	1%	0%	0%
Trp- 16	90%	28%	0%	80%
Trp-27	0%	0%	0%	100%
Trp-29	27%	21%	0%	100%
Trp-31	7%	4%	0%	0%
Trp-33	0%	0%	19%	100%

Table 9.1 The percentage of time that the polar hydrogen of tryptophan spends hydrogen bonded (i.e. within 2.1Å) of different types of oxygen atoms during the simulation.

Because of the symmetry of the gramicidin pore, residues Trp- 10, Trp- 12, Trp- 14 and Trp- 16 are equivalent to Trp-27, Trp-29, Trp-31 and Trp-33 respectively. From the values it can be concluded that, apart from Trp-14/Trp-31, all the residues are hydrogen bonded to water molecules that have penetrated into the bilayer. Hydrogen bonding is also observed to occur with the oxygen atoms of the phosphate group, but little hydrogen bonding is seen with the carbonyl oxygens. This is less than the extent of interaction expected from other experimental observations (see above) and may be as a result of the starting structure configuration. The lipid carbonyl oxygens and tryptophan polar hydrogen do interact, it is just that their approach is not close enough to be considered a conventional hydrogen bond, but given the low density of polar atoms at this depth in the membrane the interaction is probably more important than would be considered in a conventional aqueous environment.

Possible structural roles of the tryptophans are to hydrogen bond to the lipid groups and hence anchor the pore in the membrane, and to act as a steric block to head groups blocking the entrance to the pore. This is in addition to their contribution to conductance by virtue of their dipole moments (Becker et al., 1991).

In Figure 9.13 and 9.14 some structures are shown illustrating the interaction of the lipid and water molecules with the gramicidin pore (NB Only molecules which have atoms within 3.0Å of gramicidin atoms are included).

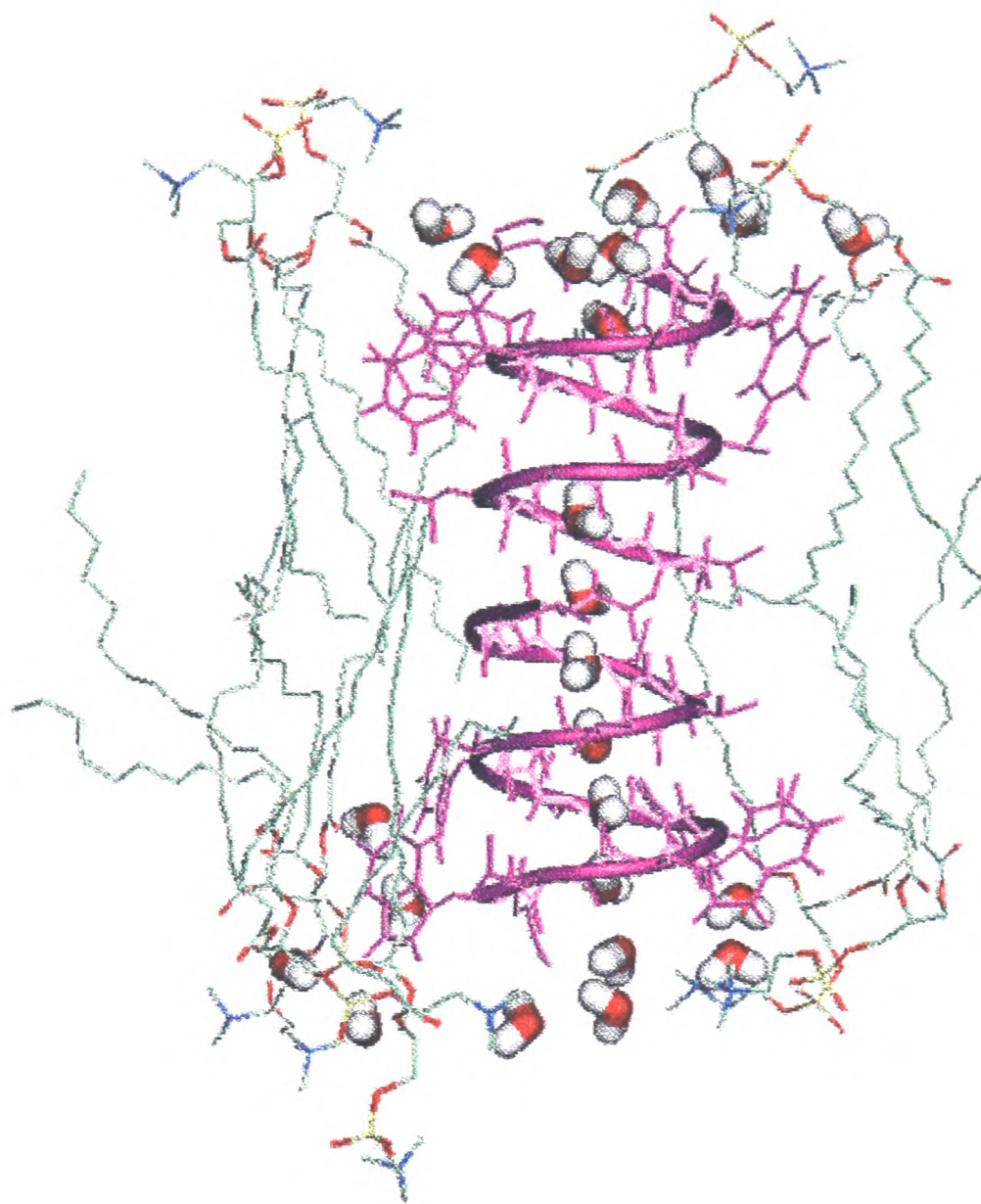


Figure 9.13 Snapshot from the molecular dynamic simulation at 500ps illustrating the packing of the chains around the gramicidin.

The structure in Figure 9.13 illustrates how the lipid head groups do not encroach into the region of space above the entrance to the pore and also how the lipids fold themselves around the tryptophan group so that they keep in contact with the gramicidin molecule (see Section 9.3.4). Figure 9.14 illustrates one of the occurrences of a hydrogen bond occurring between a carbonyl oxygen of a lipid and the polar hydrogen of a tryptophan.

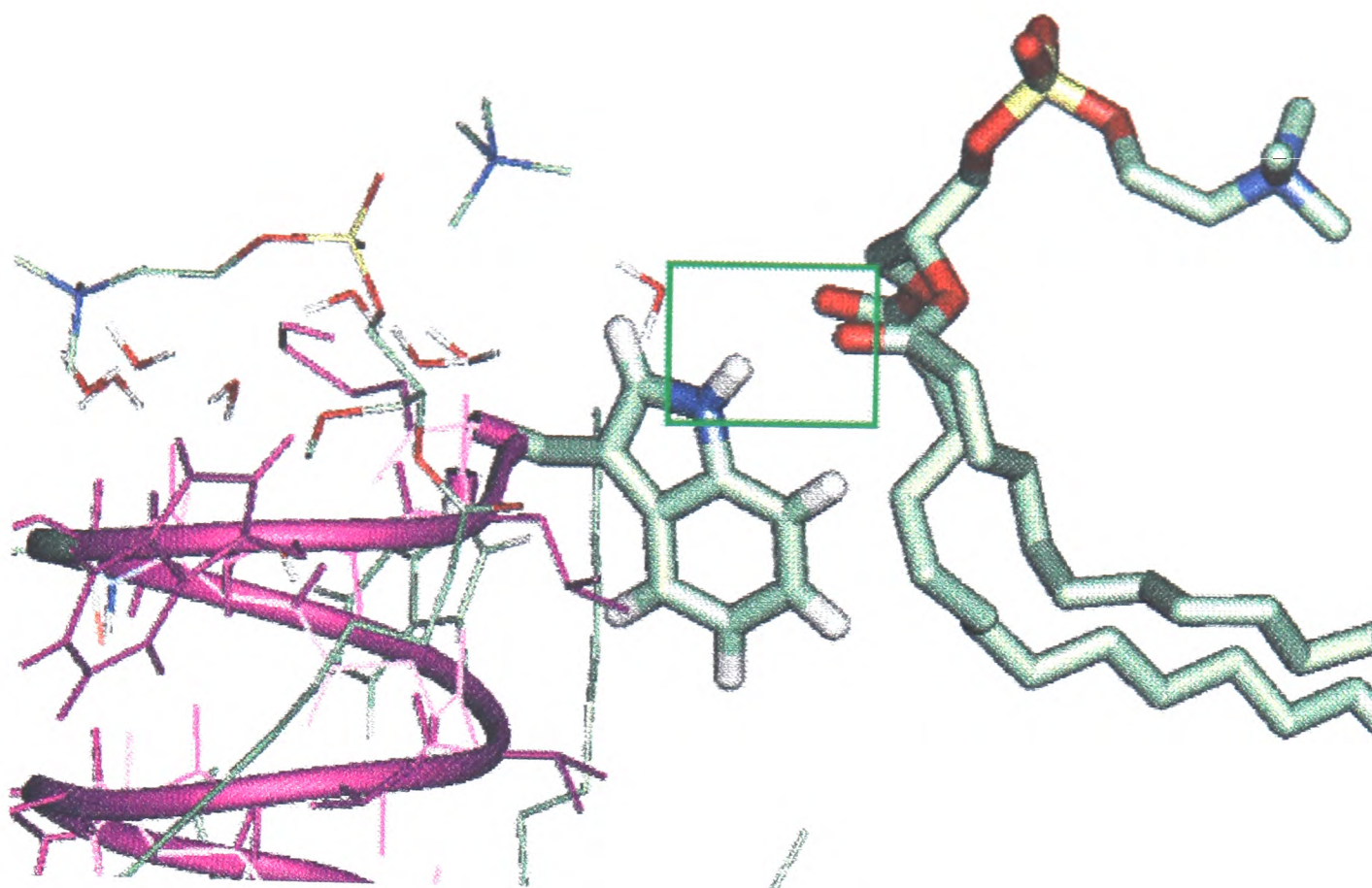


Figure 9.14 Snapshot from the molecular dynamic simulation at 500ps illustrating a hydrogen bond between the polar hydrogen of a tryptophan residue and a lipid carbonyl oxygen (inside the green box).

9.3.8 Electrostatic Surface Properties of the Bilayer

In the same manner as before (see Section 5.6.1), the electrostatic interaction energy of a particle over the surface of the membrane with the lipid molecules and peptide was calculated. The probe atom was given a charge of +1 and a radius of $0.8\text{\AA}$ (which are the approximate values for a sodium ion) and its interaction with the bilayer in a plane above the surface calculated using a fine grid of points $0.5\text{\AA}$ apart. This plane was $25\text{\AA}$ from the bilayer centre which places it above all but a couple of the head group atoms. It was reasoned that particles in this plane will still have relatively free diffusion over the surface of the membrane and be able to choose where they wish to bind.

In Figure 9.15 the surface of the membrane is shown with the electrostatic map shown superimposed on top as a semi-transparent texture map. Areas of red indicate unfavourable interactions between the probe and the surface while area of blue correspond to favourable

interactions. The black patches are points at which the probe atom is intersecting with the van der Waals radius of a lipid atom and hence having a bad steric contact.

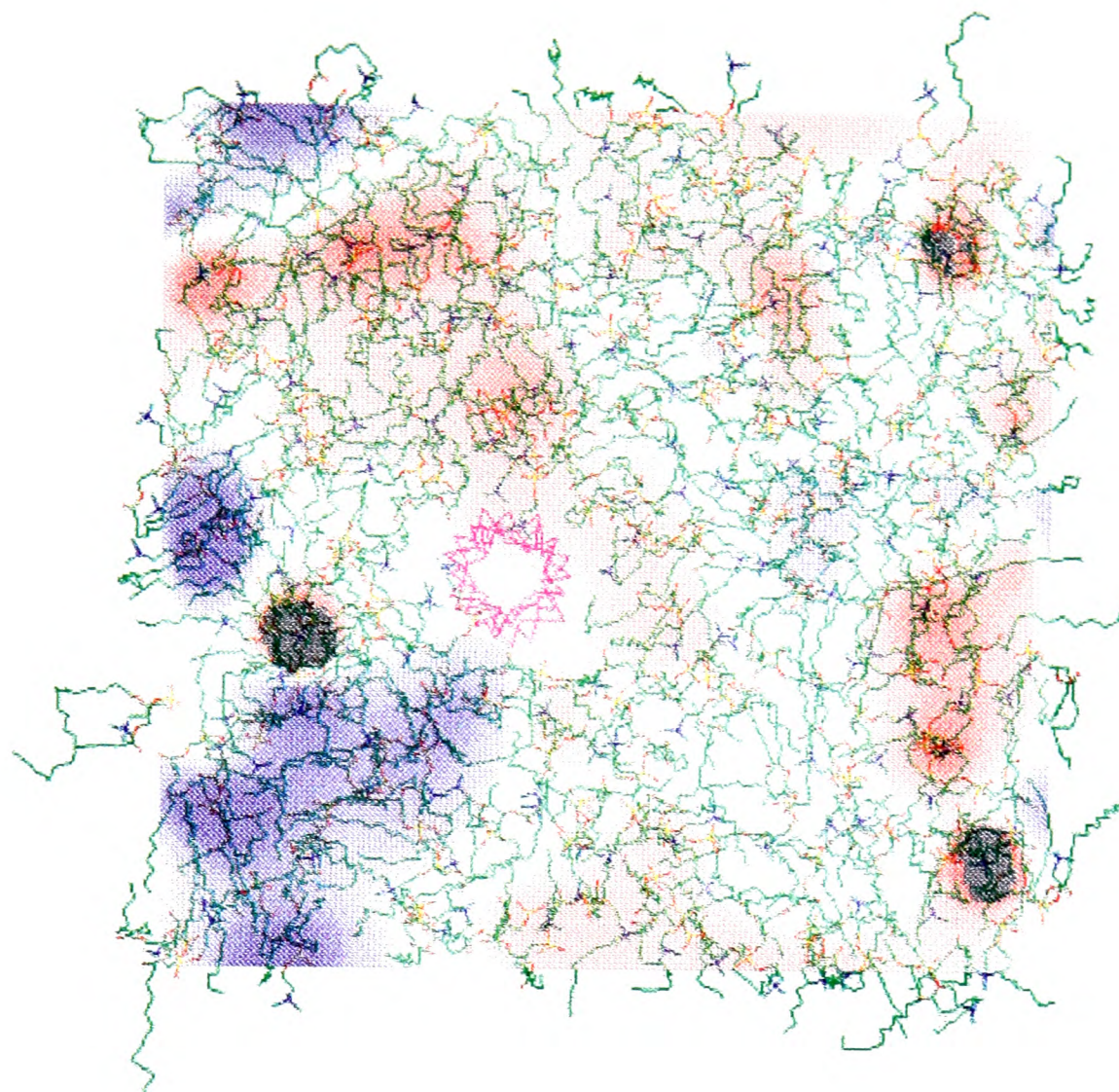


Figure 9.15 The surface view of the lipid bilayer at 500ps with the electrostatic interaction map texture mapped over it and made semi-transparent. (See text for details of energy map colouring). The gamicidin backbone is shown in purple.

From Figure 9.15 it is seen that the gamicidin has no effect on the electrostatic interaction energy map compared to the surrounding lipid molecules which arises because it is not as close to the plane as the surrounding lipid head groups. Furthermore the head groups of the surrounding lipid molecules are not oriented in such a way so as to attract cations to the pore although there is no reason why they should unless the tryptophans have some ordering effect that has not arisen on the time scale of the current simulation.

Figure 9.16 is a side view of the system to show the position of the plane in which the energy calculations are done above the membrane surface.

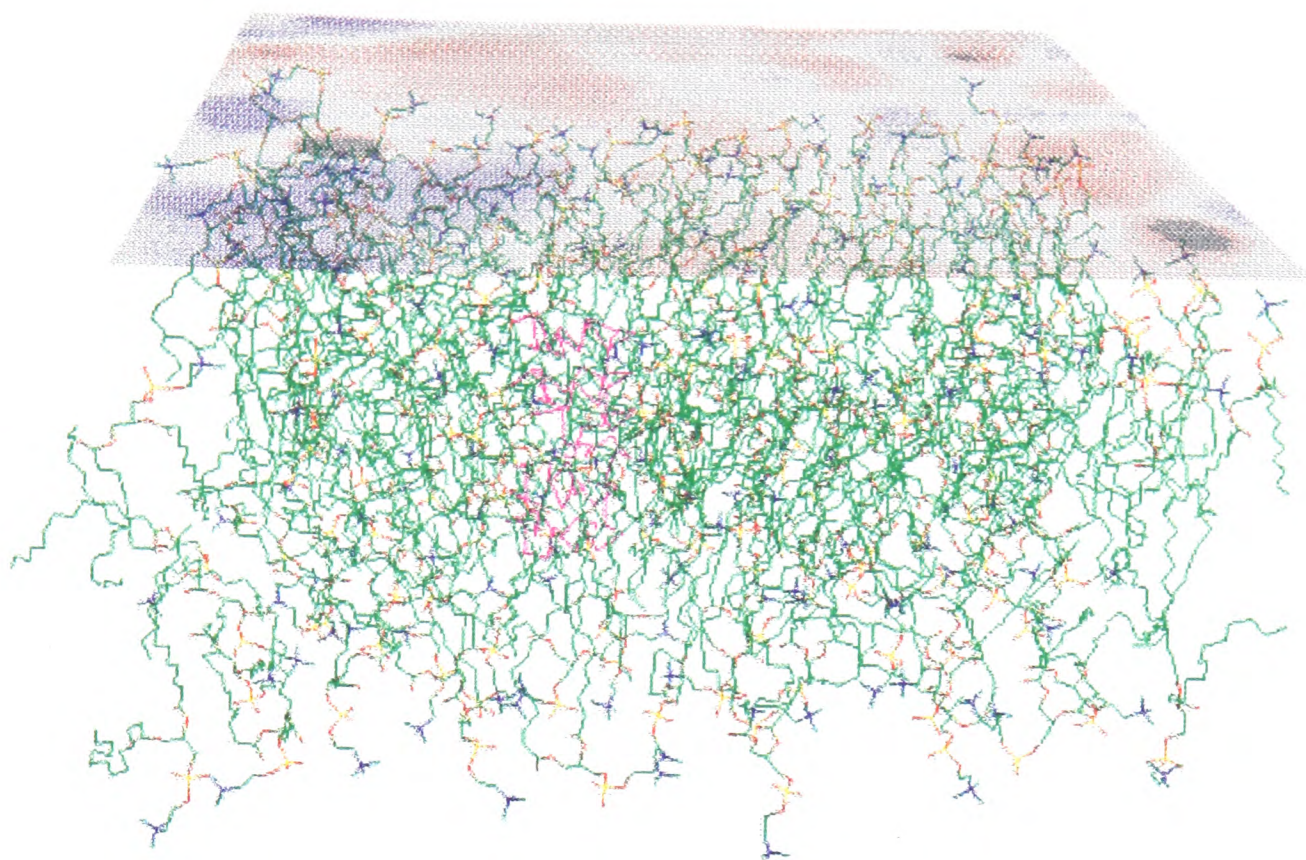


Figure 9.16 A side view of the bilayer with the electrostatic interaction map placed at the appropriate distance above it.

9.3.9 The Behaviour of Water Molecules in and Around the Pore

It was decided that at the start of the simulation no water would be placed within the gramicidin pore in order to investigate how and if water would enter it during the course of the simulation which is in contrast to the simulation of Woolf and Roux (1996) in which ten waters were inserted into the pore. It was observed that within 50ps of the start of the molecular dynamic simulation that water molecules had started to enter the pore and during the next 50ps the pore filled with water. From following an animation of the simulation the following points were noted:

- 1) The pore is not always filled completely with water. There may be gaps between water molecules
- 2) The pore waters are oriented with respect to each other so as to allow good hydrogen bonds with neighbouring water molecules

3) The pore waters are also oriented so they may form hydrogen bonds with carbonyl groups on the inside walls of the pore

4) At the entrance to the pore the carbonyl oxygen atoms and amide hydrogen atoms form a crown of hydrogen bond acceptors and donors that can interact and orient surrounding water molecules

It must be emphasised that no driving force has been applied to make the water molecules move through the pore. They are undergoing passive diffusion only.

The first process that was investigated was the mechanism of how initial waters entered the pore. The first molecule approaches the mouth of the pore and becomes hydrogen bonded to a carbonyl and then moves so that its other hydrogen is hydrogen bonded to a carbonyl on the other side of the pore (see Figure 9.17a). It is now sitting right in the centre of the entrance when another water molecule approaches it from above and hydrogen bonds to it (see Figure 9.17b). The first water makes a few abortive attempts to enter deeper into the pore but keeps returning to the mouth until one time it moves into the pore and its partner follows it (see Figure 9.17c) leading to the situation where the first water is hydrogen bonded both to a carbonyl on the side of the pore and to its water partner which has now moved into the mouth of the pore and is also hydrogen bonded to one of the carbonyls forming the 'crown' of hydrogen bonding atoms around the entrance to the pore (see above). Gradually these two waters move further into the pore and meet water molecules coming from the other side of the gramicidin (see Figure 9.17d).

Thus it appears that for a water molecule to move from the mouth of the pore into the pore interior, it must also pull a water molecule to which it is hydrogen bonded into the mouth of the pore to fill the position it has just vacated. If its partner does not follow it then it usually returns to the position whence it came.

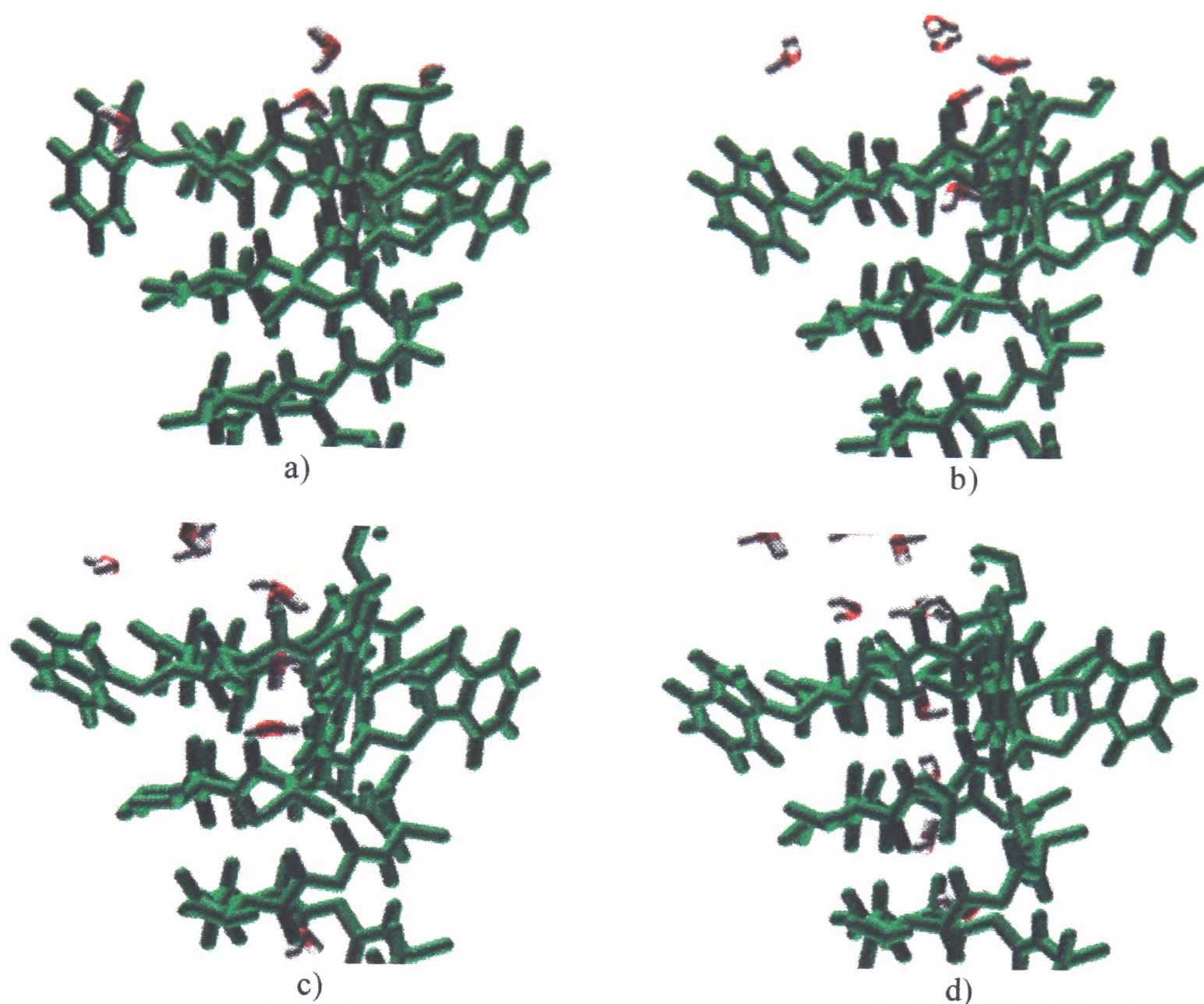


Figure 9.17 Four snap shots from the molecular dynamic simulation at 20, 41, 60 and 70ps respectively that illustrate how water enters into the pore. a) A water sits at the mouth of the pore. b) The water is still around the mouth of a pore but is also hydrogen bonded to a water above it. c) The water has moved into the pore followed by the other water to which it is hydrogen bonded (NB A water can be seen at the bottom of the frame that has come from the other side of the membrane. d) A thread of oriented water molecules has formed in the pore.

To study the motion of waters in the pore a program was written that extracted the coordinates of those waters that entered into the pore. In Figure 9.18 the z coordinate for some of these water molecules that show an extensive interaction with the pore are plotted as a function of time (The z axis is the bilayer normal and the direction along which the pore is oriented).

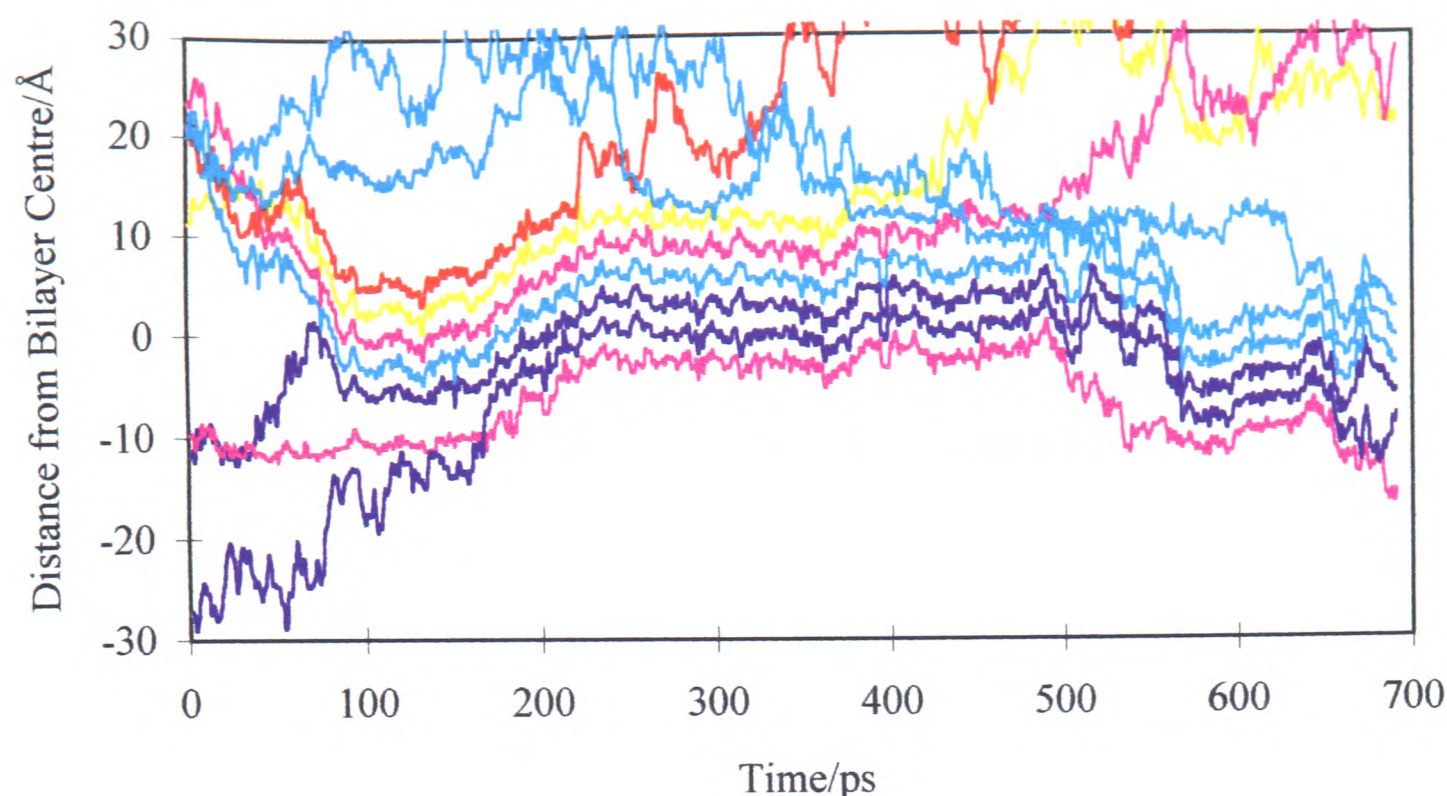


Figure 9.18 The z coordinate for some water molecules that show an extensive interaction with the pore. The pore ranges from -10 to $+10$ Å along the bilayer normal.

With the pore extending from -10 Å to $+10$ Å it can be seen that the motion of the waters in that range is very highly correlated with their neighbours probably because of the strong hydrogen bonding between them which maintains their separation. It can also be seen from the figure that no water actually passes from one side of the membrane to the other although a single water molecule (the cyan line in Figure 9.18) does move from being free water on one side of the membrane to being at the mouth of the pore on the other side. It does not actually leave the pore on the other side.

Highly correlated water dynamics were also seen in the simulations of water in models of the gramicidin channel by Roux and Karplus (1991) with the waters exhibiting rapid oscillations on the 0.8 ps time-scale within a well 1.5 Å in length and momentum was observed to be transmitted rapidly to distant neighbours due to the hydrogen bonded chain network.

In Figure 9.19 the region 0 to 100 ps of Figure 9.18 is expanded to show the entry of water into the membrane.

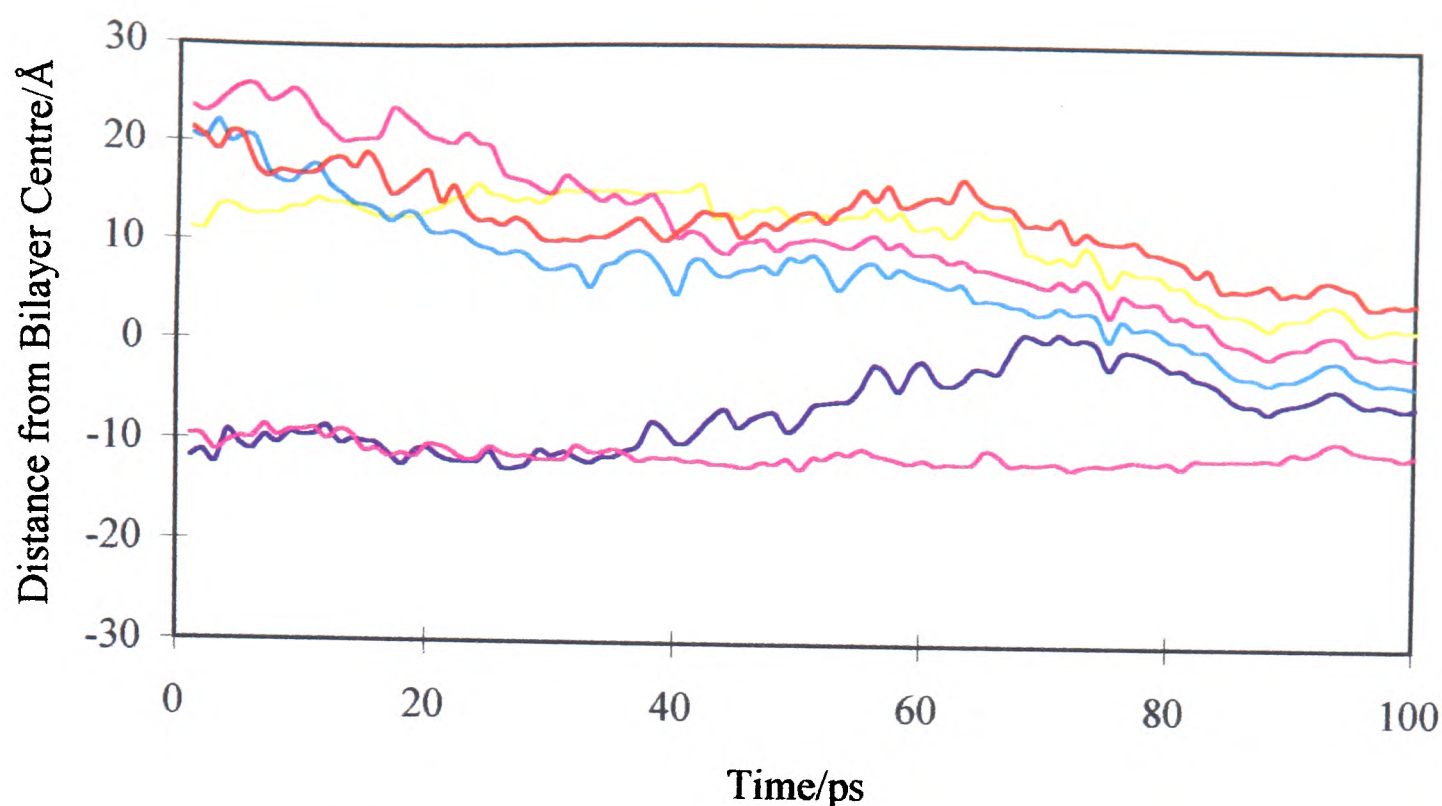


Figure 9.19 The z coordinate of some waters in and around the membrane during the first 100ps of the simulation.

Comparing Figure 9.19 to the four diagrams in Figure 9.17, the cyan line corresponds to the first water to enter the pore and the dark blue line is the water that meets it from the other side. The abortive attempts of the water to enter deeper into the membrane can be seen at 35, 41 and 53 ps as troughs in the cyan line. Only when the water to which it hydrogen bonds moves as well does it start successfully to move deeper in the membrane (after 60ps in Figure 9.19) and such behaviour is also shown by the other water molecules.

The position of a water molecule is recorded every picosecond so on this time scale it can be said that the movement of waters within the pore is highly correlated to the extent of being instantaneous along the channel length; i.e. as one moves, the others instantly follow suit along the length of the pore. At 393ps in Figure 9.18 a “blip” can be seen in the position of the water molecules through the length of the pore. This is caused by a free water moving into the mouth of the pore to fill a void which has the knock-on effect of pulling a water molecule that is quite deep in the pore back towards the mouth of the pore so that it can hydrogen bond to this newly arrived water. As this water moves it pulls the rest of the waters in the pore along with it to the extent that a water above the pore mouth at the other end of the channel is pulled into the mouth of the pore (N.B. This particular water is not shown in Figure 9.17).

Thus it has been shown that hydrogen bonding is vital in the diffusion (and hence probably general translocation) of water through the pore. The simulation shows a mechanism in which a water molecule approaches the entrance of the pore and becomes hydrogen bonded to the water sitting in the entrance of the pore. As a water exits out of the mouth of the pore this is transmitted along the length of the pore by the hydrogen bonds between the thread of waters to the other side of the membrane and a water at the entrance of the pore moves into the pore and pulls a previously free water to which it is bonded into the mouth of the pore in a concerted action. This whole process is aided by the crown of hydrogen bonding atoms at the mouth of the gramicidin which serve to orient water molecules in the mouth so that free water may become associated easily with water in the pore. It appears that the pore usually contains five to six water molecules at any one time plus a couple associated in the entrances at either end.

Three different types of water-pore interaction behaviour can be characterised during the simulation, the first type are waters that are within the pore for nearly the whole duration of the simulation. The second are those that may enter into the pore but not for a long length of time and do not penetrate very deeply. The third are those water molecules that diffuse into the vicinity of the pore, sit above the mouth and may just move into it, but exit rapidly. The difference between the second and third type of waters is that in the former a shift in the positions of water molecules occurs in the pore which leads to the water being pulled in, but this is not a common occurrence. Usually nothing happens and the water molecule is only temporarily associated with the water in the mouth of the pore. Trajectories over 600ps are shown in Figure 9.20 for examples of these three different types of water.

These different behaviours can be compared qualitatively with the free energy profiles of Na^+ in gramicidin (Roux and Karplus, 1993) that show that the energy barrier to entry to the pore is not the change in the solvation of the ion moving from bulk water to single file, but upon moving into the first part of the entrance of the channel. Furthermore, models based on ^{13}C NMR and low resolution x-ray structures suggest that an ion binding site lies close to the mouth of the pore and this is the rate limiting barrier to trans membrane diffusion.

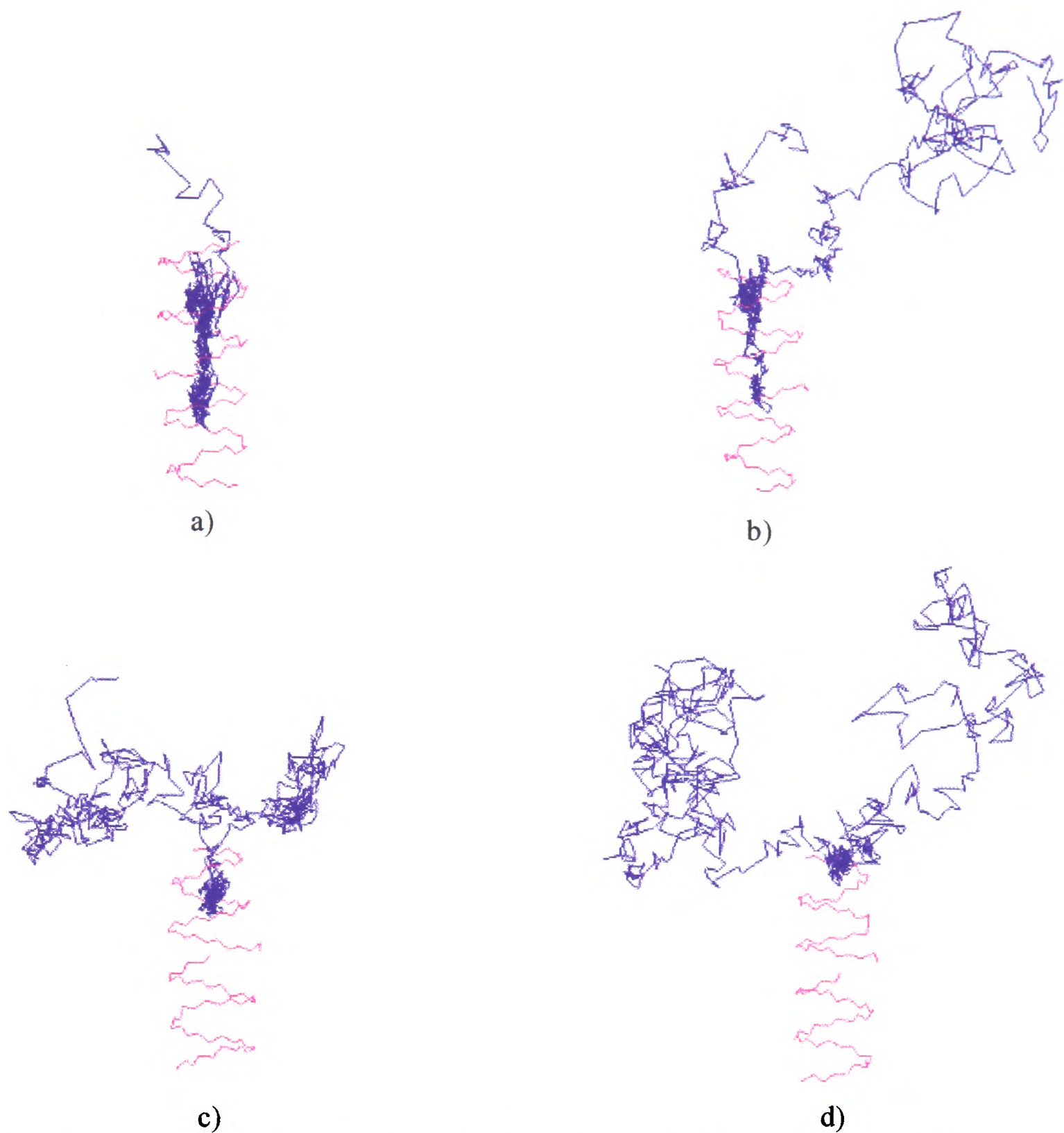


Figure 9.20 Trajectories of four water molecules (blue) that illustrate the three different types of behaviour by water molecules a) type 1 b) type 2 c) type 2 and d) type 3. Only the back bone of the gramicidin is shown (magenta).

9.4 Conclusions on the Gramicidin Simulation

The simulation of gramicidin in a lipid bilayer environment has been a success with the gramicidin molecule itself being completely stable and the lipid molecules around it orienting themselves so as to enhance their interactions with it and negate any free volume.

The gramicidin itself exhibits a thread of highly oriented, hydrogen bonded waters through it that were not present at the start of the simulation and it is speculated that the tryptophan residues may have a role in preventing the lipid head groups from encroaching over the entrance to the pore and blocking it. Since the pore is only as wide as the hydrophobic region this leads to a pocket of waters penetrating into the membrane head group region above the pore. These waters have a greater freedom of motion than other waters at a similar depth in the membrane because they are not associated with lipid head groups which restrict their motion. This greater motion means that it is much easier for solute molecules to swap positions with these waters and then diffuse into the pore. The tryptophan residues may also hold the head groups back in order to maintain the motional freedom of these waters as well as encouraging ion transport because of their dipole moment (Becker et al., 1991).

The polar hydrogen of the tryptophan residues is able to form hydrogen bonds with water molecules and with the oxygen atoms of the lipid phosphate groups and the carbonyl groups in the fatty acyl chain which may serve to anchor it in the membrane and stabilise the β -helix structure.

The gramicidin does not contribute to the electrostatic surface that approaching permeants will interact with and the head groups do not appear to orient themselves around the pore so as to promote the movement of cations toward the pore entrance. In this sense it appears that an ion finding the pore is essentially a random process.

In Section 9.3.9 the behaviour of water in and around the pore was analysed and demonstrated a model in which water motion was highly correlated because of hydrogen bonding between water molecules. A mechanism for the translocation of water through the membrane was also proposed based on the simulation results in which water molecules become oriented and hydrogen bonded to other waters within the pore and are then pulled into the pore as waters exit at the other side. The waters within the pore and at its mouth are able to hydrogen bond with atoms of the gramicidin.

The success of the simulation reflects the care that went into building the starting configuration and the simulation procedure as well as the parameters used. These have already been ratified by the success of previous simulations and the next stage in these

simulations would be the inclusion of an ion and a trans membrane potential in order to model fully the movement of ions across the bilayer region.

Chapter Ten

Conclusions on the Computer Simulations of Lipid Bilayers and Biological Membranes

10.1 Introduction

During the period of this research the complexity and size of simulations of lipid bilayers and biological membranes have increased incredibly. This work covers the evolution of membrane simulations from simple, pure lipid bilayer simulations with only 40 lipid molecules to the simulation of biological membranes containing ion channels, hundreds of lipid molecules and thousands of waters.

As well as increasing the size and complexity of the system, the techniques used in building and simulating the membranes have also undergone a revolution with the design of programs that can produce lipid bilayers of any size containing lipid molecules arranged in a pseudo random orientation to avoid the long equilibration times when starting from crystal structure-like starting configurations and ensure that the initial system is as close as feasibly possible to the fluid phase.

With the increasing availability of high performance parallel computers running efficient molecular mechanic programs and using trusted parameter sets, membrane simulations are entering a renaissance period during which our understanding of the behaviour of proteins in membranes and their interactions with lipid environments should show rapid acceleration.

10.2 Direction of the Research

The work of this thesis can be divided into three components:

- 1) The design of lipid bilayer starting configurations that are stable during molecular mechanics simulations and reproduce experimentally observed parameters of the biologically important liquid crystalline fluid phase
- 2) Systems containing peptides, proteins and other solutes may be constructed with these liquid crystalline lipid bilayer models in order to simulate the behaviour of solutes within a hydrophobic bilayer environment, their interaction with the lipid molecules and how this interaction may influence their functionality
- 3) The production of programs and techniques to probe these systems, explore fully their dynamics and then visualise the often complex results in a manner that is understandable by a general audience.

10.3 Summary of the Research

In Chapter 4 the initial lipid bilayer simulations were presented and analysed including a discussion of the important considerations involved in building models of bilayers. These centre around removing any resemblance of the starting configuration to the crystal structure which may bias the simulation and introduce artefacts into the model that cannot be corrected during the course of the simulation. This care in building the starting configuration is required because the rotation of the lipid molecules about the long axis takes of the order of many nanoseconds rather the picosecond time scale of molecular mechanics simulations. The final pure lipid bilayer model presented in Chapter 5 contained four different lipid molecule conformations that reflected the different combinations of the most probable head group and glycerol moiety conformations with gauche dihedrals introduced into the hydrocarbon chains to introduce the disorder observed experimentally and remove the need for lengthy molecular dynamic simulations to equilibrate and melt them.

Chapter 6 described a simulation of cholesterol in a lipid bilayer which illustrated the ordering effect of cholesterol on lipids because of its rigid ring system and was also the first system in which the introduction of solutes into lipid bilayers was attempted.

In chapters seven to nine inclusive, the extremely important topic of the interactions between lipids and peptides was considered starting from simple, model trans membrane peptides of polyalanine introduced in Chapter 8 in which it was shown that the peptide and surrounding lipid molecules behaved in a cooperative manner with either the lipid molecules arranging themselves around a peptide oriented along the bilayer normal or the peptide becoming tilted with respect to the bilayer normal in order to match the natural state of the surrounding lipids. The possible role of hinge structures in trans membrane peptides that stabilise their structure was also examined using a Gly-Ala-Ala-Gly motif and it was observed that such structures may play an important role since the peptide containing the hinge showed less disruption in its hydrogen bonding and psi and phi dihedral angles than did the polyalanine peptide.

In chapter 8 the interaction and possible mode of functioning of the bee venom melittin was considered in terms of surface adsorbed structures, single trans membrane helices and the aggregation of trans membrane helices to form a hypothetical pore structure. In line with experimental evidence the interaction between the highly charged C-terminus of the melittin was seen to be very important in the surface adsorbed state and suggested plausible mechanisms both for the entry of the melittin deeper into membranes and for how melittin in a surface adsorbed state may cause lysis and cytolysis by severely disrupting the head group structure. It was observed how the Pro-15 and Gly-12 residues may be extremely important in controlling the binding of the melittin to the cell and stabilising the melittin within the membrane by forming a bend in the peptide which may act as a hinge structure for single trans membrane configurations and also allow greater separation of like charged species when these trans membrane helices aggregate to form a pore. The pore structure was built from an analysis of the often contradictory experimental evidence, geometrical considerations of the structure of the melittin peptide and mechanisms for its possible mode of entry into the cell since there is no experimental structural data on the pore other than between three and ten melittin peptides may aggregate to form it. The resultant pore structure is shown to be moderately stable within the bilayer and retain enough integrity to function as an effective pore for water and anions by virtue of charged groups around the entrance to the pore. Further work needs to be done on the system and in light of the current work a new and very promising model has been devised that contains five melittin molecules that will be

equilibrated in a radical new model membrane environment before inclusion in an explicit membrane model.

Finally in Chapter 9, attention turned to the ion channel gramicidin for which the structure in a bilayer model has been solved by 2-D NMR and the first large scale explicit simulation of gramicidin in a membrane was tackled. This involved a consideration of how to place the irregularly shaped polypeptide into the bilayer without introducing bias and artefacts and this was accomplished using a two stage equilibration procedure.

The analysis of the simulation involved a consideration of the dynamics of waters in and around the pore and the interaction of the tryptophan rings both with interstitial waters and the lipid molecules and how the presence of the tryptophan rings may serve a structural function in stopping the phospholipids from blocking the pore and retain a plug of freely diffusing waters above the entrances of the pore which would allow easier access of permeants to the mouth of the pore. In addition a possible mechanism for the entry, translocation and exit of water molecules from the pore was suggested based on an observation of their behaviour during the simulation.

10.4 Future Directions for Research

Paradoxically one of the current outcomes of this research is to use the results to build structurally simpler models of bilayers. This has arisen from a consideration of the peptide simulations and that of gramicidin in particular which has shown that the effect of peptides and proteins on lipids is far more significant than vice versa. In addition the most problematic and worrying stage in the simulation of polypeptides within bilayers is the equilibration stage in which the protein is first inserted into the membrane with lipid molecules surrounding it. There is no experimental evidence to guide how these lipid molecules may be initially placed and an inappropriate conformation could have an unnoticed but disastrous impact on the protein structure. In light of these observations and conclusions it seemed appropriate that a more homogeneous and fluid model of the bilayer is required for the initial equilibration and dynamics of the protein which will recreate the physical environment of a lipid bilayer, but

without the direct, harsh lipid-peptide interactions that may perturb the protein, i.e. the model membrane will have a softer impact on the solute.

The model conceived involves replacing discrete lipid molecules with moieties that will recreate the physical nature of different parts of the bilayer. For example the lipid phosphatidylcholine head group region will be replaced by a collection of “diatomic” species that model the zwitterionic nature of the head group while the lipid hydrocarbon chains will be modelled by a collection of uncharged methyl-sized spheres at the same density as measured for the explicit membrane and will reproduce the hydrophobic environment of the region. Because the moieties are all very mobile they should sample far more phase space and equilibrate around the inserted solute quite quickly and in the head group region favourable interactions should be found far more quickly than with explicit lipid molecules. It should be emphasised that this model is intended to be used for far larger membrane solutes than currently simulated, e.g. the size of bacteriorhodopsin and upwards where the structure of the peptide in the bilayer is liable to be quite rigid. The membrane model will be used primarily for the equilibration phase and when more detailed simulations are required, a program will be devised that recognises groups of the simple moieties that can be connected together by virtue of being within an appropriate bonding distance and “morphed” to form explicit lipid molecules.

Other simulations should focus on the inclusion of other physical properties that influence the behaviour of membranes and their solutes, e.g. concentration gradients that cause osmotic pressures and the inclusion of trans membrane potentials during the simulation of ion channels.

In conclusion, the simulation of lipid bilayers and their solutes has been remarkably successful given the initial concerns several years ago that the simulations of lipid bilayers let alone solutes within them were far too large and complex to be attempted. The only negative note that must be considered is that many of the force fields used today are getting old and simulations being done are probably starting to stretch them to their limits. The time will soon come for the consideration of more powerful and accurate force fields to simulate even larger and more dynamically interesting systems.

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