

Computational Studies of
Ligand-Water Mediated Interactions in Ionotropic
Glutamate Receptors

by

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Abstract

Careful treatment of water molecules in ligand-protein interactions is required in many cases if the correct binding pose is to be identified for molecular docking. Water can form complex bridging networks and can play a critical role in dictating the binding mode of ligands. A particularly striking example of this can be found in the ionotropic glutamate receptors (iGluRs), a family of ligand gated ion channels that are responsible for a majority of the fast synaptic neurotransmission in the central nervous system that are thought to be essential in memory and learning. Thus, pharmacological intervention at these neuronal receptors is a valuable therapeutic strategy. This thesis relies on various computational studies and X-ray crystallography to investigate the role of ligand-water mediated interactions in iGluRs bound to glutamate and α -amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid (AMPA).

Comparative molecular dynamics (MD) simulations of each subtype of iGluRs bound to glutamate revealed that crystal water positions were reproduced and that all but one water molecule, W5, in the binding site can be rearranged or replaced with water molecules from the bulk. Further density functional theory calculations (DFT) have been used to confirm the MD results and characterize the energetics of W5 and another water molecule implicated in influencing the dynamics of a proposed switch in these receptors. Additional comparative studies on the AMPA subtypes of iGluRs show that each step of the calculation must be considered carefully if the results are to be meaningful.

Crystal structures of two ligands, glutamate and AMPA revealed two distinct modes of binding when bound to an AMPA subtype of iGluRs, GluA2. The difference is related to the position of water molecules within the binding pocket. DFT calculations investigated the interaction energies and polarisation effects resulting in a prediction of the correct binding mode for glutamate. For AMPA alternative modes of binding have similar interaction energies as a result of a higher internal energy than glutamate.

A combined MD and X-ray crystallographic study investigated the binding of the ligand AMPA in the AMPA receptor subtypes. Analysis of the binding pocket show that AMPA is not preserved in the crystal bound mode and can instead adopt an alternative mode of binding. This involves a displacement of a key water molecule followed by AMPA adopting the pose seen by glutamate.

Thus, this thesis makes use of various studies to assess the energetics and dynamics of water molecules in iGluRs. The resulting data provides additional information on the importance of water molecules in mediating ligand interactions as well as identifying key water molecules that can be useful in the *de novo* design of new selective drugs against iGluRs.

To Mamoo

“I’m a doctor” now...

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List of Publications

1. R. Vijayan, **M. A. Sahai**, T. Czajkowski and P. C. Biggin (2010). A comparative analysis of the role of water in the binding pockets of ionotropic glutamate receptors *Phys. Chem. Chem. Phys.* 12, 14057-14066.
2. **M. A. Sahai** and P. C. Biggin (2011). Quantifying Water-Mediated Interactions in a Glutamate Receptor; A DFT Study. *J. Phys. Chem. B.* (under review).

Abbreviations

3-21G	A split valence basis set with 3 functions for the atomic core and 3 functions splitted in the form (2+1) functions for the valence shell
6-31G*	A split valence basis set with 6 functions for the atomic core and 4 functions splitted in the form of (3+1) functions for the valence shell while adding polarization functions to heavy atoms, using the 6-component type d functions
6-31G**	A split valence basis set with 6 functions for the atomic core and 4 functions splitted in the form of (3+1) functions for the valence shell while adding polarization functions to heavy atoms, using the 6-component type d functions in addition to p functions for hydrogen atoms
Ψ	Wavefunction
E	Energy
$\hat{H}$	Hamiltonian Operator
ACPA	(RS)-2-amino-3-(3-carboxy-5-methyl-4-isoxazolyl) propionic acid
ALS	Amyotrophic Lateral Sclerosis
AMBER	Assisted Model Building with Energy Refinement
AMPA	α -amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid
ATD	Amino-terminal Domain
B3LYP	Becke's three-parameter exchange functional with LYP correlation
B97D	Grimme's functional including dispersion
BD	Brownian Dynamics
BRH or Br-HIBO	(S)-2-amino-3-(4-bromo-3-hydroxy-isoxazol-5-yl) propionic acid
BSSE	Basis Set Superimposition Error
CHARMM	Chemistry at HARvard Molecular Mechanics
CNS	Central Nervous System
CPW399	2-amino-3-(1,3,5,6,7-pentahydro-2,4-dioxo-cyclopenta[e]pyrimidin-1-yl) propanoic acid
CypA	Cyclophilin A
DFT	Density Functional Theory
EGF	Epidermal Growth Factor
ETF	Exponential-type Functions
F-Will	5-fluoro-willardiine;
GAMESS	General Atomic and Molecular Electronic Structure System
Glu	Glutamate
GluA2	AMPA-subtype Ionotropic Glutamate Receptor 2
GGA	Hybrid Meta Gradient Generalized Approximation
Gyr	DNA Gyrase
GROMACS	GRONingen MACHine for Chemical Simulations
GTF	Gaussian-type Functions
GTO	Gaussian-type Orbitals
HF	Hartree Fock
HIV	Human Immunodeficiency Virus

iGluRs	Ionotropic Glutamate Receptors
I-Will	5-iodo-willardiine.
JAWS	Just Add Water Molecules
KA	Kainic Acid
LBD	Ligand Binding Domain
LINCS	Linear Constraint Solver
LYP	Lee-Yang-Parr correlation functional
MAPKs	Mitogen-activated protein kinases
MC	Monte Carlo
MD	Molecular Dynamics
MeTetAMPA	2-amino-3-[3-hydroxy-5-(2-methyl-2H-tetrazol-5-yl)isoxazol-4-yl]propionic acid
MM-GB/SA	Molecular Mechanics-Generalized Born Surface Area
M.O.	Molecular Orbital
MP2	Møller-Plesset second order perturbation method
MPW1B95	hybrid meta GGA (a combination of meta GGA with Hartree-Fock exchange)
NMDA	N-methyl-D-aspartic acid
NMR	Nuclear Magnetic Resonance
OPSL-AA	Optimised Potentials for Liquid Simulations All Atom
PCA	Principal Component Analysis
PDB	Protein Data Bank
PES	Potential Energy Surface
PME	Particle Mesh Ewald
POVRAY	The Persistence of Vision Raytracer
PxA	Pleomorphic Xanthoastrocytoma
QM	Quantum Mechanics
RESP	Restricted Electrostatic Potential
RHF	Restricted Hartree Fock method
RMSD	Root Mean Square Deviation
RMSF	Root Mean Square Fluctuation
SCF	Self Consistent Field
STO	Slater-type Orbitals
STO-3G	Three GTOs contracted to a single STO
TMD	Transmembrane Domain
TS	Transition State
VMD	Visual Molecular Dynamics
X-ray	X-ray Crystallography

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Chapter 1

Introduction

“I could have done it in a more complicated way”, said the Red Queen, immensely proud
Lewis Carroll, ca. 1870

1.1 Accounting for water molecules in drug design

Water constitutes the cellular environment where biomolecules interact. Specifically, it plays a crucial role in mediating interactions between ligands and macromolecular receptors. Water is also important for protein folding and stability, and is known to actively participate in many catalytic processes in the cell (Nelson et al., 2002). The most obvious contribution of water molecules within the binding pockets of receptor proteins is forming bridging contacts between the ligand and the protein (Lu et al., 2007), but they may also contribute indirectly by optimising the rotamers of receptor side chains within the pocket when the ligand is present. As such, its importance has been increasingly recognised through protein-protein and protein-ligand interaction studies with respect to rational drug design (Ladbury, 1996). However, despite the wealth of structural information, the precise role of water molecules in mediating receptor-ligand interactions remains unclear (Whitesides and Krishnamurthy, 2005).

In general, solvation effects are known to influence the overall binding of drugs, crucial for lead optimisation where a 100-fold difference in binding affinity is significant but corresponding to only ~ 3 kcal mol⁻¹ of the binding free energy (Mancera, 2007; de Beer et al., 2010; Wong and Lightstone, 2010). In a survey of Protein Data Bank (PDB) structures, ~ 4.6 ligand bound water molecules are found in each ligand-protein complex (Lu et al., 2007). However, formal analytical expressions based on statistical mechanics that exist to calculate binding free energies of these water molecules are extremely difficult to compute in practice (Gilson et al., 1997), the main reason being that it is difficult to understand the range of effects mediated by water.

Many methods have been used to identify areas of hydration in protein binding sites. They include those based on conservation across protein families such as in T4 lysozyme (Zhang and Matthews, 1994), aspartic proteinases (Prasad and Suguna, 2002), Rossmann dinucleotide-binding domains (Bottoms et al., 2002), serine proteases (Sreenivasan and Axelsen, 1992) and legume lectins (Sreenivasan and Axelsen, 1992). Other programs rely on calculating the physical interactions of Lennard-Jones, electrostatics and hydrogen bonding in GRID (Godford, 1985), 'Just Add Water Molecules' (JAWS) (Michel et al., 2009b), *Consolv* (Raymer et al., 1997), WaterScore (Garcia-Sosa et al., 2003) and Watermap (Young et al., 2007; Abel et al., 2008; Beuming et al., 2009). In particular Watermap is a protocol that uses the inhomogenous solvation theory (Lazaridis, 1998a; b) to calculate the free energy cost of transferring a water molecule from a protein hydration site to bulk solvent.

In the context of drug-design, once a lead compound(s) is identified and X-ray structures are available for optimisation, there are two issues to deal with: 1) dealing

with water in docking calculations and 2) deciding if displacing a water during lead optimisation will improve binding. Indeed, this has been the focus of recent computational studies (Barillari et al., 2007; Pan et al., 2007; Abel et al., 2008; Michel et al., 2009a; Yu and Rick, 2009).

In these calculations it is important to consider whether the representation used for the water molecules is adequate. Continuum solvent models representing a homogenous dielectric (Edinger et al., 1997; Ghosh et al., 1998; Bashford and Case, 2000; Tsui and Case, 2000) and quantum models can represent water molecules (Borgis and Staib, 1995; Lobaugh and Voth, 1997). However, even quantum mechanical treatments of water have difficulty reproducing all the properties of water accurately (Paesani and Staib, 1995; Guillot, 2002). Implicit solvent models have been developed as a tool for lead optimisation (Michel et al., 2006). However, there have been successful efforts to incorporate the effects of explicit water models such as SPC and SPC/E (Mark and Nilsson, 2001; Amira et al., 2004), TIP3P (Jorgensen et al., 1983; Sun and Kollman, 1995; Mark and Nilsson, 2001), TIP4P (Neumann, 1986; Horn et al., 2004) and TIP5P (Mahoney and Jorgensen, 2000) into docking programs such as Autodock (Osterberg et al., 2002), GOLD (Verdonk et al., 2003; Verdonk et al., 2005), SLIDE (Schnecke and Kuhn, 2000), FlexX (Rarey et al., 1999; Jacobsen, 2002), FITTED (Corbeil et al., 2007) and Glide (Friesner et al., 2004). A recent study assessed the inclusion of water molecules on ligand enrichment against 24 targets (Huang and Shoichet, 2008). It was found that for 12 of them enrichment was increased substantially but for the remainder there was little enrichment. Another study found that exploiting commonly observed waters in cross-docking could lead to

improvement in pose prediction most, but not all of the time (Thilagavathi and Mancera, 2010).

Molecular dynamics (MD) studies have focused on identifying and characterising the interactions between the protein, ligand and conserved water molecules (Arinaminpathy et al., 2006) and free energy calculations (Barillari et al., 2007; Knight et al., 2009) have been used to further elucidate the functions of these water molecules and probe their apparent stabilising role in the protein binding site. A measure of the free energy (Kollman, 1993; Chipot and Pearlman, 2002) is ultimately what is required in order to assess the stability of water molecules within binding sites. However, obtaining sufficient accuracy of these calculations, especially for charged systems (which encompasses most biological systems) (Mobley et al., 2006) is particularly challenging and an area of intense investigation (Deng and Roux, 2009).

One factor in these calculations is the underlying simplicity of the force-field which may or may not account for interactions at the necessary level required. Recently, the use of the molecular mechanics-generalized Born surface area (MM-GB/SA) (Srinivasan et al., 1998) either for re-scoring or as part of the docking scoring function has emerged as a practical compromise between the higher accuracy of a Poisson-Boltzmann solver and a fast, efficient method to calculate free energies. Even with these simplified models, the calculations are still time-consuming enough to be prohibitive at the level of virtual screening. Nevertheless they do form the underlying physical basis of many scoring functions employed in virtual high-throughput screening methods and consequently it is in our interest to improve them as much as possible.

1.2 Water molecules mediate receptor-ligand interactions

Many binding pockets have a complex network of water molecules and in some instances this has already been exploited to give increased drug potency. For example, in the case of HIV-1 protease, cyclic urea derivatives were designed to displace a conserved water molecule (Grzesiek et al., 1994; Lam et al., 1994; Hodge et al., 1996; Pan et al., 2007). Another example is the displacement of a water molecule with a cyano group at the p38 α MAPK binding site which yielded a 60-fold improvement in K_i (Liu et al., 2005; Michel et al., 2009a). Displacement of a water molecule by a cyano group in the active site of scytalone dehydratase also improved binding (Chen et al., 1998; Michel et al., 2009a). However, displacement by cyano groups can also worsen affinity. Wissner *et al.* (Wissner et al., 2000) made a similar attempt in their work to displace a structural water of EGF receptor kinase, resulting in a threefold decrease in binding affinity. Monte Carlo (Hastings, 1970) free energy perturbation calculations by Michel *et al.* (Michel et al., 2009a) on EGF receptor kinase displayed an overestimation of the relative binding energy of the modified ligand showing that careful analysis is necessary for accurate results. Another example where removal of a water molecule creates an unfavourable effect is in the case of inhibitors against CypA, where a methylhydroxyl group decreased binding of a (5-hydroxynorvaline)-2-cyclosporin inhibitor by eight- to nine-fold (Mikol et al., 1995). Additionally, Barillari *et al.* (Barillari et al., 2007) performed Monte Carlo free energy perturbation calculations coupled with replica exchange (Sugita and Okamoto, 2000; Nagasima et al., 2002; Woods et al., 2003) to calculate the binding energy of 54 water molecules in HIV-1 protease, neuraminidase, trypsin and PxA ligand-

protein complexes. Their data showed a statistically significant lower binding free energy for a water molecule known to be displaced by ligands versus those not displaced (-6.2 vs. -3.7 kcal mol⁻¹).

The net effect of displacing a water molecule to improve binding depends on the free energy of transferring the water molecule to bulk solvent and the new interactions of the modified ligand. Transferring structural water to bulk solvent is entropically favourable, and its enthalpy depends on the relative strength of protein contacts versus water contacts in the bulk (Wong and Lightstone, 2010). Dunitz (Dunitz, 1994) estimated the net free energy to be between 0 and 2 kcal mol⁻¹, but the contribution of individual water molecules is highly dependent on the protein environment (Raymer et al., 1997). Jia *et al.* (Jia et al., 2008) found that if individual water molecule are to contribute to the stability of a protein-ligand complex then it depends on the ligand in question. Free energy calculations yielded values ranging between -0.4 and -7.7 kcal mol⁻¹ for water stability in the binding site of GyrB for the binding of novobiocin (Yu and Rick, 2009).

Related to the problem of displaceable water molecules is the concept that water molecules can be considered as part of the drug rather than part of the protein. This can lead to the situation where similar compounds that might be expected to adopt the same binding pose on the basis of their chemical structure actually adopt different poses within the binding pocket. A good example of this can be found in structures of the ionotropic glutamate receptors, which are an important set of potential drug targets within the central nervous system.

The ionotropic glutamate receptors (iGluRs) are ion channel receptors activated by the carboxylate anion of glutamic acid, glutamate (Glu) (Figure 1.1), the major excitatory neurotransmitter in the vertebrate central nervous system (CNS) (Mayer, 2005b). The ion channel opens in response to the binding of Glu, causing the depolarisation of the post-synaptic terminal, while desensitisation limits the effective ion flow to a few milliseconds following binding of glutamate. Ionotropic glutamate receptors play key roles in various physiological processes, in particular synaptic plasticity, which is thought to be essential in memory and learning (Dingledine et al., 1999). Furthermore, they have been associated with abnormal neuronal activity leading to a wide range of neurodegenerative diseases such as Alzheimer's, Parkinson's, Huntington's chorea, amyotrophic lateral sclerosis (ALS), epilepsy, and ischemic stroke (Parsons et al., 1998; Dingledine et al., 1999; Barnes and Slevin, 2003; Hynd et al., 2004; Mayer and Armstrong, 2004). Thus, pharmacological intervention at these neuronal receptors is a valuable therapeutic strategy.

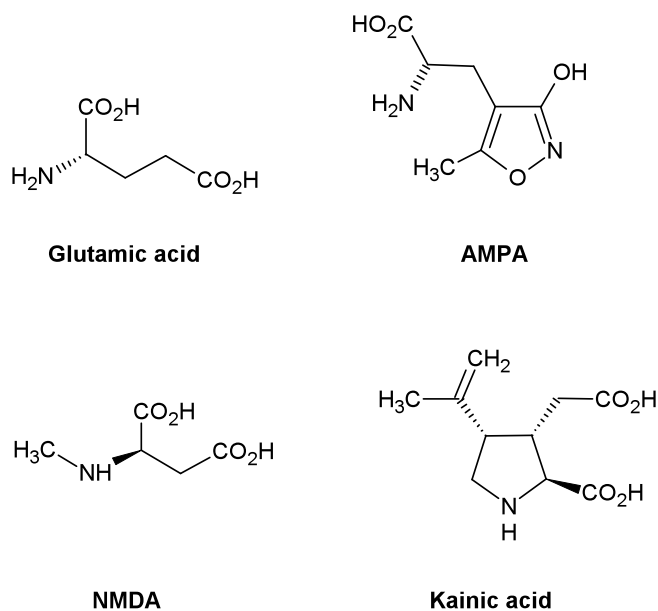


Figure 1.1 Structures of ligands used to classify ionotropic glutamate. All structures are represented in their unionized form.

1.3 Ionotropic glutamate receptors, subunits and assemblies

The ionotropic glutamate receptors (iGluRs) can be classified, both in terms of sequence similarity and pharmacology, into three major subclasses: α -amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid (AMPA), *N*-methyl-D-aspartic acid (NMDA) and kainic acid (KA) receptors (Collingridge et al., 2009). These pharmacologically active ligands are depicted in Figure 1.1. There is a fourth sub-family; the delta (δ) subunits which are believed to be incapable of forming functional receptors of their own (Lomeli et al., 1993; Naur et al., 2007; Hansen et al., 2009). Despite unique functional properties and varying degrees of sequence identity (between 20 and 70%), the major structural features

are conserved in all known iGluR subunits (McFeeters and Oswald, 2004; Mayer, 2006; Bowie, 2008).

iGluRs are integral membrane proteins that assemble as heteromeric or homomeric receptors from subunits within their respective families (Dingledine et al., 1999). Most evidence indicates that, similar to K⁺ channels, iGluRs are tetramers assembled as a dimer-of-dimers (McFeeters and Oswald, 2004). In the case of the AMPA receptors they are assembled from the GluA1-4 subunits (Mayer and Armstrong, 2004). Subunits GluK1- 5 are activated by kainate and form the KA receptors (Mayer, 2006), and NMDA preferring receptors are formed by combinations the GluN1-3 (Dingledine et al., 1999) subunits.

Biochemical studies have established that the iGluR subunits are modular proteins made up of an amino-terminal domain (ATD), a ligand binding domain (LBD) (D1 and D2), and a transmembrane domain (TMD) which is composed of three membrane spanning subdomains (TM1, TM2 and TM3), a cytoplasmic re-entrant loop (P) and a C-terminal intracellular region (Armstrong et al., 1998; Dingledine et al., 1999; Mayer, 2006) (Figure 1.2).

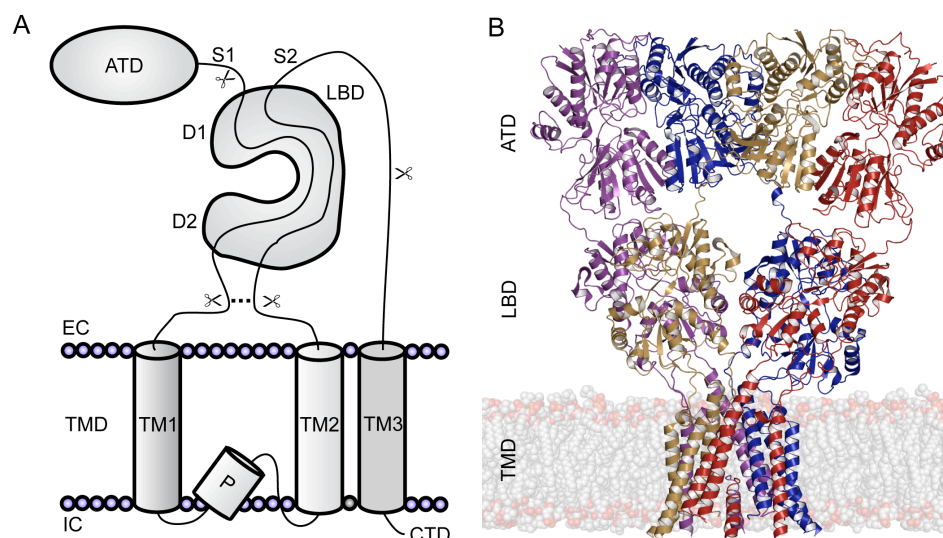


Figure 1.2 (A) Block diagram of a glutamate receptor monomer. The amino terminal domain (ATD) and the clamshell shaped ligand binding domain (LBD) reside in the extracellular (EC) region. The LBD is formed by two discontinuous segments (S1 and S2) that form the two halves of the clamshell D1 and D2. Crystal structures of the iGluR S1S2 construct is formed by cutting and splicing regions shown with a (X). The membrane bound region consists of three transmembrane helices (TM1-TM3) and a reentrant helix (P). (B) Cartoon of the homo-tetrameric GluA2 receptor. Adapted from (Vijayan et al., 2010).

Recently, the structure of a full-length tetrameric GluA2 construct was solved to a resolution of 3.6 Å (Sobolevsky et al., 2009). However, because working with the full-length protein is difficult, it has in the past been more common to perform structural work with the soluble domains; the ligand binding core domain (Mayer, 2006) and the N-terminal domain (Clayton et al., 2009; Jin et al., 2009; Kumar et al., 2009). As such, most GluA2 structural efforts have focused on the extracellular ligand binding domain since understanding iGluR/ligand interactions paves the way for potential therapeutic treatments.

1.4 Project objectives and aims

Currently there are over 80 crystal structures for the GluA2 ligand binding domain (Figure 1.2) (Sutcliffe et al., 1996; Armstrong et al., 1998; Armstrong and Gouaux, 2000; Mayer et al., 2001; Hogner et al., 2002; Kasper et al., 2002; Furukawa and Gouaux, 2003; Hogner et al., 2003; Lunn et al., 2003; Mayer, 2005a; Naur et al., 2005; Mayer, 2006; Mayer et al., 2006; Plested and Mayer, 2007; Ahmed et al., 2008; Gill et al., 2008; Kasper et al., 2008; Maltsev et al., 2008; Hansen et al., 2009; Sobolevsky et al., 2009). These X-ray structures provide a wealth of information about the interactions of agonists, antagonists, and allosteric modulators particularly with respect to the glutamate receptor subunit GluA2. The crystal structures not only reveal the manner in which compounds interact with GluA2, but also highlight the importance of water molecules in ligand-receptor binding. The goal of this thesis is to assess the role of water molecules within the binding pocket of GluA2. Considering the high degree of amino acid sequence identity between ligand binding domains of iGluR subunits in general and between the AMPA subunits GluA1-GluA4 in particular, much of this information may also be used for analysis in subunits other than GluA2, such as the newly crystallised GluA3 (Ahmed et al., 2008) and GluA4 (Gill et al., 2008; Kasper et al., 2008) structures.

Using various computational approaches, such as quantum mechanics and MD simulations, I will attempt to address the following questions: 1) *What dynamics in the LBD facilitate water displacement?* 2) *Which water molecules are important in mediating protein-ligand interactions and which are targeted for displacement?* 3) *Can simple*

ligand-system interaction energies alone predict the correct binding mode? 4) How accessible is the ligand binding domain to various conformations of ligands?

After briefly outlining the methods (**Chapter 2**) used in this thesis, the immediate goal is to compare the role of water in the binding pockets of various iGluR receptors (**Chapter 3**). Following that I will compare the simple interaction energies of water molecules with the agonists glutamate and AMPA in the GluA2 binding core (**Chapter 4**). Subsequently, I plan to investigate the water and protein dynamics within the AMPA subtype of glutamate receptors (**Chapter 5**). Finally, I will show how dynamic the ligand binding domain can be and the different binding modes available for one particular ligand, AMPA (**Chapter 6**). Ultimately, my work will provide information that will be extremely useful in the design of new subtype specific ligands that will maximise the interactions with conserved water molecules and will target those that can be displaced for iGluRs.

Chapter 2

Theory and Methods

“I do not like it, and I am sorry I ever had anything to do with it.”

Erwin Schrödinger

2.1 Introduction to computational chemistry

In computational chemistry we take the view that we are simulating the behaviour of real physical entities, albeit with the aid of intellectual models such as quantum mechanics (QM), molecular dynamics (MD), Monte Carlo (MC) and Brownian dynamics (BD) (Leach, 1996). Consequently, as these models improve they reflect more accurately the behaviour of atoms and molecules in the real world. The results of such methods essentially describes the potential energy surface (PES) by either using the laws of classical physics characterised by force fields or by solving the Schrödinger equation; equation (2.1)*:

$$\hat{H}\Psi = E\Psi \quad (2.1)$$

* This equation is a simplification of the time independent Schrödinger equation $\hat{H}_{\text{tot}}\Psi_{\text{tot}} = E_{\text{tot}}\Psi_{\text{tot}}$, equation (2.2), describing the energy of the total system and will be discussed in more detail in Section 2.2.

In this equation, Ψ is the wavefunction, E is the total energy of the system and $\hat{H}$ is the Hamiltonian operator.

The work described in this thesis employed both MD and QM methods extensively for simulating proteins and small molecular fragments. Hence, these methods are briefly reviewed in this chapter.

2.2 Quantum mechanical calculations

2.2.1 The Schrödinger Equation

Ab initio quantum mechanical theory, as its name implies, approaches problems “from the beginning” and are based on the Schrödinger equation. It assumes only the knowledge of fundamental universal constants such as Planck’s constant (h), the charge (e), and the rest-mass (m_0) of the electron as well as the atomic number (Z) of the component in a given molecule (Csizmadia, 1976).

Firstly, the time independent Schrödinger equation is defined according to equation (2.2) (Csizmadia and Daudel, 1981; Foresman and Frisch, 1996)

$$\hat{H}_{\text{tot}}\Psi_{\text{tot}}=E_{\text{tot}}\Psi_{\text{tot}} \quad (2.2)$$

This equation includes the total Hamiltonian ($\hat{H}_{\text{tot}}$), an operator, which describes the position of the atomic nuclei and each and every possible interaction between all the nuclei and all the electrons, which make up the molecule. In this sense the Hamiltonian

is the mathematical equivalent of the interactions within the chemical structure of a molecule. The total wave function (Ψ_{tot}) represents the simultaneous distribution of all the electrons in the field of all the atomic nuclei. Consequently, the wavefunction is the mathematical equivalent of the structure resulting from all the interactions prescribed by the Hamiltonian operator. Finally, the total energy of the system is specified by E_{tot} .

Due to the fact that the masses of the atomic nuclei are much greater than those of the electrons, Born and Oppenheimer suggested that the nuclear and electronic coordinates may be separated. Such partitioning, amounting to the following relationships given in equations (2.3) and (2.4), splits equation (2.2) into equations (2.5) and (2.6) (Csizmadia and Daudel, 1981)

$$\hat{H}_{\text{tot}} = \hat{H}_{\text{el}} + \hat{H}_{\text{nuc}} \quad (2.3)$$

$$\Psi_{\text{tot}} = \Psi_{\text{el}} \Psi_{\text{nuc}} \quad (2.4)$$

$$\hat{H}_{\text{el}} \Psi_{\text{el}} = E_{\text{el}} \Psi_{\text{el}} \quad (2.5)$$

$$\hat{H}_{\text{nuc}} \Psi_{\text{nuc}} = E_{\text{nuc}} \Psi_{\text{nuc}} \quad (2.6)$$

Equation (2.5) describes the “electronic motion” or more precisely the electronic distribution while equation (2.6) describes the “molecular motion” which is frequently referred to as “molecular vibrations”.

According to the Born-Oppenheimer approximation the nuclear kinetic energy will vanish and the nuclear-nuclear repulsion becomes a constant (Csizmadia and Daudel, 1981). This implies that the electronic Schrödinger equation (2.5) can be solved for stationary atomic nuclei where the geometry is regarded as a set of variable parameters.

Gradient geometry optimisations will vary these geometrical parameters to find the optimum structure. If vibrational characteristics are needed, then the nuclear Schrödinger equation (2.6) may be solved subsequently. This is illustrated in Figure 2.1.

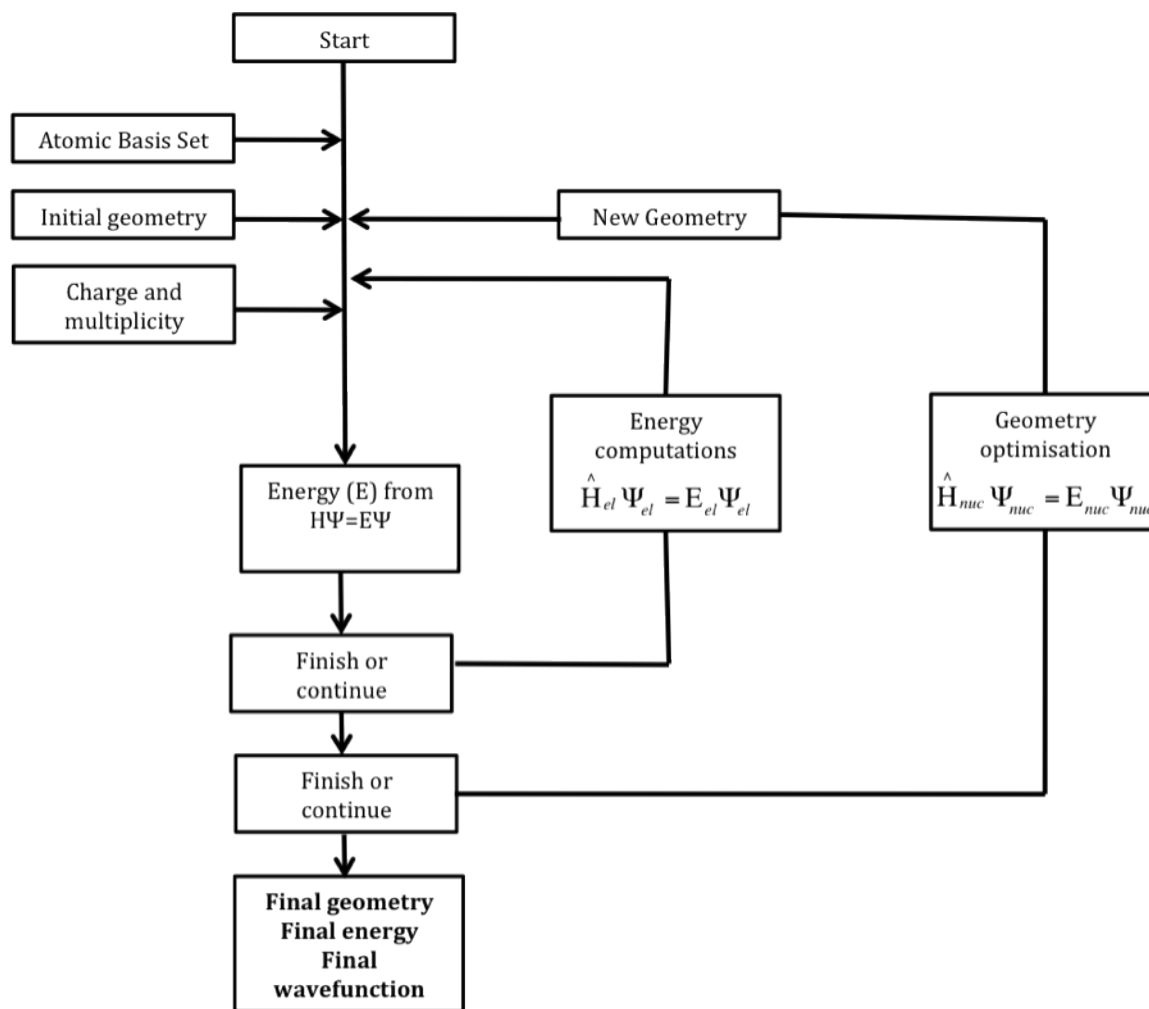


Figure 2.1 A schematic illustration of the iterative nature of *ab initio* molecular computations.

2.2.2 Molecular orbital theory

The basic premise of Molecular Orbital (M.O.) theory (frequently referred to as the Hartree-Fock method) is the allocation of electron pairs, one with an α spin and one with a β spin, to the available molecular orbitals. It is assumed that the total *many-electron* wave function, which describes the molecule, may be constructed from a set of *one-electron* wave functions (M.O.'s). Thus, the Schrodinger equation, which is a *many-electron* wave equation, is converted by the application of HF theory to many *one-electron* wave equations (HF-equations) (Ogretir and Csizmadia, 1991), Figure 2.2, within the variational principle[†].

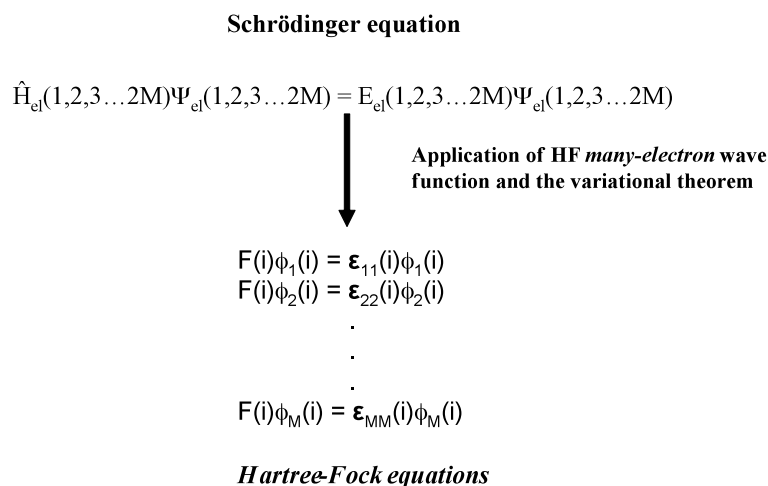


Figure 2.2 The application of HF Theory to the Schrödinger equation converts one many-electron wave equation to many one-electron wave equations. The variational principle is applied in this process.

[†]The variational principle says that, for the ground state of any antisymmetric normalised function of the electronic coordinates, which we will denote Ξ , the expectation value for the energy corresponding to Ξ will always be greater than the energy for the exact wavefunction:

$$E(\Xi) > E(\Psi); \quad \Xi \neq \Psi$$

In other words, the energy of the exact wavefunction serves as a lower bound to the energies calculated by any other normalised antisymmetric function. Thus, the problem becomes one of finding the set of coefficients that minimise the energy of the resultant wavefunction.

In the HF equations the Fock operator (F) describes all the interactions one electron experiences in the field of the rest of the electrons and the contributing atomic nuclei. The one-electron wavefunctions (ϕ_i) are the molecular orbitals (M.O.'s). Note that for the accommodation of $2M$ electrons, M molecular orbitals are required due to double occupancy.

The procedure that is involved in computing the HF molecular orbitals is implemented by Roothaan's algorithm and is frequently referred to as the Self Consistent Field (SCF) procedure. It is called SCF because this mathematical problem can only be solved iteratively and the name implies that the field around the electron created by all other electrons, in addition to all the atomic nuclei, is varied until no further change or until self consistency is achieved.

This thesis also reports calculations with Density Functional Theory (DFT), which computes electron correlation via general *functionals*[‡] of the electron density. DFT functionals partition the electronic energy into several components which are computed separately; the kinetic energy, the electron-nuclear interaction, the Coulomb repulsion, according to the Hamiltonian, and also into an exchange-correlation term accounting for the remainder of the electron-electron interaction (which is itself divided into separate exchange and correlation components in most actual DFT formulations).

Varieties of traditional functionals have been defined, generally distinguished by the way that they treat the exchange and correlation components. Local exchange and

[‡] A functional is defined in mathematics as a function of a function. In Density Functional Theory, energy is a functional that is a function of the electron density, which is in turn a function of molecular geometry according to equation (2.7).

$$E=f[\rho(\text{geometry})] \quad (2.7)$$

correlation functionals involve only the values of the electron spin densities while gradient-corrected functionals involve both the values of the electron spin densities and their gradients.

There are also several hybrid functionals, which define the exchange functional as a linear combination of Hartree-Fock, local, and gradient-corrected exchange terms; this exchange functional is then combined with a local and/or gradient-corrected correlation functional. This thesis makes use of Becke's three-parameter exchange functional with the Lee-Yang-Parr (LYP) correlation functional (Becke, 1993), and the hybrid meta gradient generalized approximation (GGA) (a combination of meta GGA with Hartree-Fock exchange) functional MPW1B95 (Zhao and Truhlar, 2004; 2005).

These so called DFT methods offer the prospect of tackling large systems in a broad context with reasonable effort and appears to be less basis set dependent than more advanced wave function methods can be. However, treatment of noncovalent interactions must be addressed on a functional and basis set level, significantly complicating general applicability. Conventional DFT techniques fail to treat dispersion effects completely. Recently, the DFT community has developed a variety of methods for the treatment of van der Waals (dispersion) interactions, including treatments with semiempirically dispersion corrected functionals. One such functional, the B97D Grimme's functional including dispersion has been employed in this thesis where interaction energies need to include dispersion interactions in the energy term (Grimme, 2006).

2.2.3 Basis set

A basis set or function is a mathematical representation of the molecular orbitals within a molecule (which in turn combine to approximate the total electronic wavefunction) used to perform the molecular computation. The basis set can be interpreted as restricting each electron to a particular region of space. Larger basis sets impose fewer constraints on electrons and more accurately approximate exact molecular orbitals.

A basis set is also a linear combination of atomic orbitals. For the atomic orbitals two types of analytic functions are used to represent them in molecular computations (Csizmadia, 1976; Csizmadia and Daudel, 1981; Ogretir and Csizmadia, 1991) (Figure 2.3).

- (1) Slater-type orbitals (STO) or Exponential type functions (ETF)
- (2) Gaussian-type orbitals (GTO) or Gaussian-type functions (GTF)

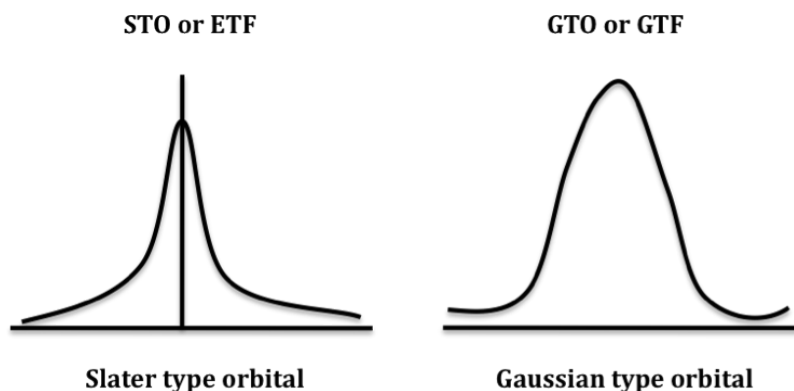


Figure 2.3 The schematic representation of Slater-type orbitals (STO) and Gaussian-type orbitals (GTO).

Slater type orbitals (STOs) are used as basis functions due to their similarity with the eigenfunctions of the hydrogen atom. However, from a computational point of view

the STOs have the severe shortcoming that most of the required integrals needed in the course of the SCF procedure must be calculated numerically which drastically decreases the speed of a computation. As a result, STOs are expanded in terms of Gaussian-type orbitals (GTOs) or, in other words, the GTO basis set is contracted to a set of STOs, as functional forms are needed. As an example, the contraction of a set of three Gaussian-type functions to a single Slater function results in the very popular (STO-3G) basis set; equation (2.8).

$$\eta_i^{STO} = d_{i1}\eta_1^{GTO} + d_{i2}\eta_2^{GTO} + d_{i3}\eta_3^{GTO} \quad (2.8)$$

This thesis makes use of the split valence basis set 6-31G** (Foresman and Frisch, 1996). The split valence basis set 6-31G** contains 6 functionals for the atomic core and 4 sets of functions (3 + 1) in the valence region which provides a more accurate representation of orbitals. The 6-31G* basis sets also adds polarisation functions to heavy atoms, using the 6-component type d functions in addition to p functions for hydrogen atoms (Foresman and Frisch, 1996).

2.2.4 Geometry optimisations

A potential energy surface is a mathematical relationship linking molecular structure and resultant energy. A minimum is the bottom of a valley on the potential energy surface (Foresman and Frisch, 1996). From such a point, motion in any direction leads to an increase in energy. A minimum can be a local minimum, meaning that it is the lowest point in some limited region of the potential surface, or it can be a global minimum, the lowest energy point anywhere on the potential surface. Minima occur at equilibrium

structures for the system, with different minima corresponding to different conformations.

Peaks and ridges correspond to maxima on the potential energy surface. A peak is a maximum in all directions. A low point along a ridge is a local minimum in one direction and a maximum in the other. A point which is a maximum in one direction and is a minimum in all other is called a saddle point. A saddle point corresponds to a transition structure connecting the two equilibrium structures. Geometry optimisations attempt to locate minima on the potential energy surface, thereby predicting equilibrium structures.

At all critical points, i.e. at both minima and saddle points, the first derivative of the energy, known as the gradient, is zero (Figure 2.4). Since the gradient is the negative of the forces, the forces are also zero at such a point. A point on the potential energy surface where the forces are zero is called a stationary point or critical point.

A geometry optimisation begins at the molecular structure specified as its input, and steps along the potential energy surface. It computes the energy and the gradient at that point, and on the basis of the gradient vector it then determines how far and in which direction to make the next step. The gradient indicates the direction along the surface in which the energy decreases most rapidly from the current point as well as the steepness of that slope.

The optimisation algorithm available in the Gaussian '03 (Frisch et al., 2004) and Gaussian '09 (Frisch et al., 2009) programs also computes the values of the second derivative of the energy with respect to the molecular coordinates, updating the matrix

force constants (k) (known as the Hessian). These force constants specify the curvature of the surface of that point. As long as the curvature is positive (within the two nearest inflection points of the potential energy function) the vibrational frequency associated with the reaction coordinate is real at the minimum energy point and in its vicinity (Figure 2.4). The vibrational frequency associated with the reaction coordinate at the transition state (TS) is imaginary.

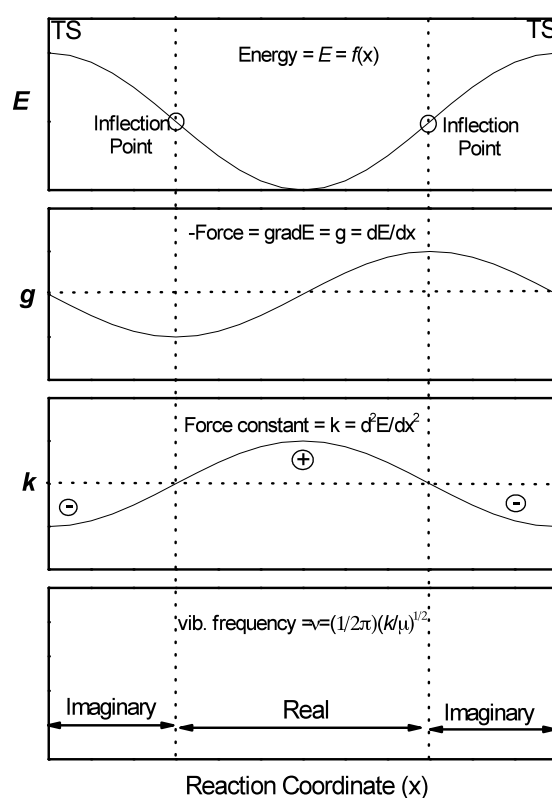


Figure 2.4 Mathematical characteristics of an idealised potential energy curve (top), its first derivative (g), its second derivative (k), and its real and imaginary domains (bottom) of the vibrational frequency associated with the reaction coordinate.

2.3 Molecular dynamics simulations

2.3.1 Potential functions

Molecular dynamics (MD) solves Newton's equations of motion for N interacting atoms of a system (Leach, 1996). The position of every atom is recorded with respect to time and the interactions between atoms are defined by a force-field (Leach, 1996). Running MD on proteins allows us to visualise and study what happens to a protein on a very small time-scale. With today's computational capabilities, we can run simulations up to hundreds of nanoseconds (Leach, 1996). This time-scale is still inadequate for observing some physiological phenomena such as channel gating but can still be used to observe different types of protein motions on different time-scales (Becker et al., 2001).

From the raw data, it is possible to extract information about the larger scale motions of the protein using various techniques such as principal component analysis (PCA) (Leach, 1996). Molecular dynamics provides a useful complement for experimental structural biology and is used here, along with various other methods, to study the structural variability and behaviour of proteins.

The principal approximation made in MD is the Born-Oppenheimer approximation as explained in detail in Section 2.2.1. This enables the electronic and nuclear terms to be separated and thus the potential energy of a molecule in its ground electronic state can be considered as a function of the nuclear coordinates only (Leach, 1996). This approximation enables us to simplify the description of the dynamics of atoms, and it also allows us to represent the interactions and repulsions among atoms as

functions depending on the nuclei positions only. In this assumption, the classical Newton's laws of motion are used.

Newton's equation of motion for a system of N interacting atoms (Leach, 1996):

$$m_i a_i = F_i \rightarrow m_i \frac{\partial^2 r_i}{\partial t^2} = F_i, i = 1 \dots N \quad (2.9)$$

This equation describes the motion of a particle of mass m_i along a coordinate r_i with force F_i . The force at time t , $F(t)$, is calculated for all pairs of particles of the system at position $r(t)$. From the force $F(t)$, the acceleration $a(t)$ is computed and by knowing the velocity $v(t)$ and acceleration $a(t)$, the new position $r(t+\Delta t)$ and the new velocity $v(t+\Delta t)$ are obtained. Δt is the time interval called the time step and the force is assumed to be constant during the time step. The *Verlet* (Verlet, 1967) and *leap-frog* (Hockney et al., 1974; Leach, 1996) algorithms are most commonly used to integrate the equations of motions for MD simulations. These assume the positions, velocities and accelerations can be approximated as Taylor series expansions. Positions are updated using the forces $F(t)$.

The force field describes the total potential energy of the system (Leach, 1996; Becker et al., 2001). The potential energy function of the system is broken down into the various energy types of the system i.e. bonded and non-bonded interactions according to equation (2.10). It also determines the parameters used for force-constants and atomic charges. These parameters are based on experimental data such as X-ray crystallography, NMR, infra-red spectroscopy and quantum mechanical calculations (Leach, 1996).

$$V(r) = V_{bonded}(r) + V_{non-bonded}(r) \quad (2.10)$$

The non-bonded interactions are those between atoms that are separated by at least three bonds (1,4 interaction) or atoms from different molecules.

$$V_{non-bonded}(r) = V_{electrostatics}(r) + V_{Van-der-Waals}(r) \quad (2.11)$$

Bonded interactions are those between atoms directly bound to each other in the same molecule.

$$V_{bonded}(r) = V_{bonds}(r) + V_{angles}(r) + V_{dihedrals}(r) + V_{impDihedrals}(r) \quad (2.12)$$

In equation (2.13), b_i is the bond length with $b_{i,0}$ being the reference value with $k_{i,b}$ as the force constant.

$$\sum V_{bonds} = \sum_{i=1}^{N_{bonds}} k_{i,b} (b_i - b_{i,0})^2 \quad (2.13)$$

In equation (2.14), θ_i is the bond angle value and θ_{i0} is the reference angle with $k_{i,\theta}$ as the force constant.

$$\sum V_{angles} = \sum_{i=1}^{N_{angles}} k_{i,\theta} (\theta_i - \theta_{i,0})^2 \quad (2.14)$$

The dihedral term, which represents the energy term for rotating about the central bond between four consecutively bonded atoms (Figure 2.5), has force constant k , n is the periodicity or multiplicity, and δ is the phase. Φ represents the angle between two planes.

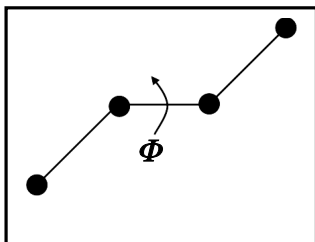


Figure 2.5 A schematic for a dihedral angle showing rotation about the central bond.

$$\sum V_{dihedrals} = \sum_{i=1}^{N_{dihedrals}} k_{i,\Phi} [1 + \cos(n_i \Phi_i - \delta_i)] \quad (2.15)$$

In order to keep the planar groups planar and to prevent molecules flipping over to their mirror images, improper dihedrals are used. Ψ represents the torsion angle between four atoms of a molecule.

$$\sum V_{impDihedrals} = \sum_{i=1}^{N_{impDihedrals}} k_{i,\Psi} (\Psi_i - \Psi_{i,0})^2 \quad (2.16)$$

For the non-bonded interactions, the electrostatic term is calculated using a Coulombic potential according to equation (2.17). q_i and q_j are the charges on the atoms i and j , r_{ij} is the separation distance on the atoms i and j , and ϵ_0 is the relative permittivity of free space.

$$\sum V_{electrostatics} = \sum_{pairs(ij)} \frac{q_i q_j}{4\pi\epsilon_0 r_{ij}} \quad (2.17)$$

Van-der-Waals interactions describe the dispersive and exchange-repulsive interactions between atoms and are modeled using the Lennard-Jones function according to equation (2.18). The collision diameter is σ (the energy is zero at this separation) and the well depth is ϵ .

$$\sum V_{Van-der-Waals} = \sum_{pairs(ij)} 4\epsilon_{ij} \left[\left(\frac{\sigma_{ij}}{r_{ij}} \right)^{12} - \left(\frac{\sigma_{ij}}{r_{ij}} \right)^6 \right] \quad (2.18)$$

2.3.2 Force-fields

There are various MD packages with different force-fields. Some of the more popular MD force-fields are AMBER (Weiner et al., 1984), CHARMM (Brooks et al., 1983), GROMOS (Hermans et al., 1984; van Gunsteren et al., 1996), and OPLS-AA (Jorgensen and Tirado-Rives, 1988; Tirado-Rives and Jorgensen, 1990; Jorgensen et al., 1996). Each can offer certain advantages depending on the system being simulated. Where MD studies have been carried out in this work, the OPLS-AA (Optimised Potentials for Liquid Simulations) force-field in the GROMACS MD package has been used. This includes every atom explicitly.

2.3.3 Periodic boundaries

In order to mimic a bulk fluid environment periodic boundary conditions are used (Leach, 1996). This enables a simulation to be carried out with a small number of particles with characteristics of a much larger and fluid environment. This also enables ‘macroscopic’ properties to be calculated from simulations using a relatively small number of particles and ensures that when a particle leaves the box during a simulation, it is replaced by an image particle that enters from the opposite side. Thus, the number of particles in the central box remains constant. Other shapes can also be used, however in the studies carried out in this work, the boxes used were cubic.

2.3.4 Long-range electrostatic interactions and PME

The calculation of non-bonded interactions (electrostatic and van der Waals) is the most time-consuming part of a MD simulation (Leach, 1996). In principle non-bonded interactions should be computed between every pair of particles in the system including their images. However, this is inefficient. For this reason, the calculation can be approximated using a cut-off. It is assumed that the largest contribution to the potential and forces comes from the near neighbours of the particle and a truncation can be made. The interactions between pairs of particles further than a spherical cut-off value are set to zero. For van der Waals interactions, the potential becomes significant after a distance of about 2.5 Å in the Lennard-Jones potential. This makes van der Waals interactions short range interactions.

On the other hand, electrostatic interactions are long-range interactions and their effects are felt at a considerably greater distance than the cut-off commonly used for short-range interactions (Leach, 1996). A variety of methods have been developed to calculate long-range forces. However the most commonly used is the Ewald summation method (Ewald, 1921). A variant of this method is employed for the simulations in this thesis. This method can be employed for calculating the full electrostatic energy of a unit cell. In the Ewald sum method, each point charge in the system is surrounded by a charge distribution of equal magnitude and opposite sign; this distribution is conveniently taken to be Gaussian and acts like an ionic atmosphere to screen the interactions between neighbouring charges. Now, the screened interactions are short-ranged and the total screened potential is calculated by summing over all the particles in the central box and all the images in real space. Then, another charge distribution of opposite sign is added

to the first added Gaussian distribution and this cancelling distribution is summed in reciprocal space.

A fast implementation of the Ewald summation is the Particle Mesh Ewald (PME) method (Sarman et al., 1998). In this method Fast Fourier Transformations are used to approximate the reciprocal space term of the Ewald sum. Here, charges are interpolated to grid points. This procedure greatly reduces the computational cost of the reciprocal space sum.

2.3.5 Temperature and pressure coupling

It is necessary to control the temperature and pressure of a system due to drift as a result of integration errors, force truncation, drift during equilibration, and heating due to frictional forces. Both the weak coupling scheme of Berendsen (Berendsen et al., 1984) or the extended ensemble of Nosé-Hoover (Nose, 1984; Hoover, 1985) and the newly introduced Velocity Rescaling Method is available for use in the GROMACS package. The Berendsen algorithm uses weak coupling to an external heat bath with first-order kinetics given temperature T_0 . This means that a temperature deviation decays exponentially with the time constant τ .

$$\frac{dT}{dt} = \frac{T_0 - T}{\tau_T} \quad (2.19)$$

This system is efficient for relaxing a system to the target temperature and thus is the method of choice during the equilibration phase of an MD simulation. However, once the system has reached equilibrium, it is important to use a correct thermodynamic

ensemble. The method introduced by Nosé and later modified by Hoover, introduces a thermal reservoir and a friction term in the equations of motion. The friction parameter or ‘heat bath’ variable is a dynamic quantity with its own equations of motion. This is the method of choice after the equilibration stage, although usually the differences are negligible (Holian et al., 1995).

The Berendsen algorithm (Berendsen et al., 1984) and the extended Parrinello-Rahman method (Parrinello and Rahman, 1981) are supported by GROMACS for pressure coupling. The coordinates and box vectors are rescaled at every step with a matrix μ . This matrix has the effect of a first-order kinetics relaxation of the pressure towards a reference pressure P_0 .

$$\frac{dP}{dt} = \frac{P_0 - P}{\tau_p} \quad (2.20)$$

The Berendsen scaling in GROMACS can be done isotropically, semi-isotropically or anisotropically.

An isotropic scale factor is equal for all three directions i.e. for bulk water, which is an isotropic system. Semi-isotropic pressure coupling is where the x/y directions are scaled independently from the z direction i.e. applicable for a lipid bilayer; and in anisotropic coupling, the scaling factor is calculated independently for each of the three axes (Leach, 1996).

In this thesis simulations were performed in an isobaric-isothermal ensemble maintained at 1 bar and 300 K by coupling to an external bath using the Nosé-Hoover algorithm.

2.3.6 Constraints

Constraints are requirements that bonds and angles are forced to meet throughout the simulation without affecting the other internal degrees of freedom. If molecules are allowed flexibility, it will result in conformational changes. The two most common methods for applying constraints in MD are the SHAKE (Ryckaert et al., 1977) and LINCS (Hess et al., 1997) algorithms.

LINCS is a more recent non-iterative constraint algorithm implemented in GROMACS, which is faster and more stable than SHAKE, which is iterative. In LINCS, bonds are reset to their correct lengths after an unconstrained period of the simulation. Constraints are used to increase the time-step of a simulation. The time-step is dependent on the high frequency motions (e.g. bond vibrations), which are of less interest than lower frequency motions of a protein; with constraint methods, the high frequency bond motions are ignored.

2.3.7 Energy minimisation

An important step in setting up a simulation is minimising the energy of the system. Energy minimisation removes atomic clashes and strained geometry that may cause simulation instability. A system which is energy minimised corresponds to a stable arrangement of atoms. We obtain a low-energy configuration of the system by finding the *nearest local minimum* of the potential energy landscape. Two of the more common energy minimisation algorithms are the steepest descent and conjugate gradient methods, both based on the first derivative of the potential energy function (Leach, 1996).

In the steepest descent method, a line-search is used to locate the minimum value in the function along a line through the multi-dimensional space and the current step is not dependent on the previous step. This method is good for relieving the highest energy features of an initial configuration and thus is useful when the system is far away from its energy minimum. The conjugate gradient method takes the previous step into account and refines its direction of movement down the gradient. This method performs better when the system is near the energy minimum (Leach, 1996). The steepest descent method is used for the energy minimisations carried out in this work as it is ideal for removing bad contacts in high energy configurations.

2.3.8 Making topologies

GROMACS contains topologies for common biological molecules. However, topologies have to be created when wanting to run simulations of non-protein molecules. Ligands such as AMPA, used in the simulations and discussed in **Chapters 4** and **6** did not have available topologies and thus they had to be generated. The process is discussed in detail in **Chapters 4** and **6**.

2.4 Visualisation and graphics

Simulation data and crystal structures were visualised in VMD (Humphrey et al., 1996), PyMOL (DeLano, 2004), and UCSF Chimera (Pettersen et al., 2004). Images were rendered using the Tachyon renderer (Stone, 1998), VMD, POVRAY, PyMOL and UCSF Chimera.

Chapter 3

A Comparative Analysis of the Role of Water in the Binding Pockets of Ionotropic Glutamate Receptors

'Everything should be made as simple as possible, but not simpler.'

Albert Einstein

This chapter was based on the article (Vijayan et al., 2010):

R. Vijayan, M. A. Sahai, T. Czajkowski and P. C. Biggin (2010). A comparative analysis of the role of water in the binding pockets of ionotropic glutamate receptors *Phys. Chem. Chem. Phys.* 12, 14057-14066

Abstract

This chapter compares multiple MD simulations of the ligand binding core of four glutamate-bound iGluR structures (GluA2, GluK1, GluK2, and GluN2A) and one apo structure (GluA2) using two different water models and oligomerisation states for GluA2 and GluK1. The ligand binding core of ionotropic glutamate receptors represents an example where the binding pocket has many crystallographically reported waters, but their precise role remains unclear. It is also uncertain to what extent the dynamic properties of these waters is conserved across the different receptor subtypes. Regardless of the water model, the MD simulations revealed that crystal water positions were reproduced and that all but one water molecule in the binding site can be rearranged or replaced with water molecules from the bulk that enter the binding site through transient water channels. Further density functional theory calculations demonstrate that whilst it is not needed in order to be able to predict the correct binding pose, it does contribute a large favourable interaction energy. It was also observed that one conserved water has a much stronger interaction with the protein in GluA2, GluK1 and GluK2 compared to the GluN2A receptor. The position of this water molecule has been implicated in influencing the dynamics of the proposed switch in the GluA2 and GluK1/2 receptors.

3.1 Introduction

Ultimately, for any given water, it is the influence of its presence or absence on the free energy of ligand binding that will provide the most predictive power in terms of ligand affinity. However, such calculations are computationally expensive and challenging and remain an area of intense investigation. Thus, for systems such as the ligand binding core of the ionotropic glutamate receptors where there are many water molecules present, it is difficult to know which water molecule one should focus the available computational power. However, it does provide an opportunity to exploit comparative dynamics (Pang et al., 2005) in order to ascertain which of those water molecules are likely to be important in terms of docking predictions, but also in broader functional terms.

Molecular dynamics (MD) studies have previously provided insights into the dynamics of the NMDA receptor (Kaye et al., 2006; Kaye et al., 2007) and the AMPA receptor (Mendieta et al., 2001; Arinaminpathy et al., 2002; Arinaminpathy et al., 2003; Mendieta et al., 2005; Pang et al., 2005; Speranskiy and Kurnikova, 2005; Arinaminpathy et al., 2006; Mamonova et al., 2008). As such the series of structures of the LBD from different families of glutamate receptor, all of which bind glutamate, present an opportunity to examine the similarities and differences in the behaviour of water molecules within the binding pocket using comparative molecular dynamics.

To compare and contrast the behaviour of the binding site across the iGluR family, MD simulations were performed on glutamate-bound structures: one each of

the NMDA (GluN2A) (Furukawa et al., 2005) and AMPA (GluA2) (Armstrong and Gouaux, 2000) receptors and two of the KA (GluK1 and GluK2) (Swanson et al., 1997; Mayer, 2005a; Mayer et al., 2006) receptors along with a ligand free structure of the AMPA (GluA2) receptor (Armstrong and Gouaux, 2000). Additional simulations were performed on the apo form of the GluA2 receptor to assess water accessibility in the absence of the ligand. Two different sets of calculations were performed on all five receptors using either the TIP4P (first set) or TIP3P (second set) water models, in order to assess if different water models have any effects on the dynamics of the protein and, more importantly, dynamics and behaviour of water molecules. In the second set of calculations, different oligomerisation states, specifically dimers for GluA2, GluA2 (apo) and GluN2A were studied to identify any differences in dynamics between the two subunits.

3.2 Methods

3.2.1 MD simulations

Five X-ray crystal structures, as listed in Table **3.1**, were used in this study. This included glutamate-bound structures from each of the iGluR family, namely GluA2 (AMPA; PDB ID: 1FTJ), GluA2 (apo) (AMPA, PDB ID: 1FT0), GluK1 (KA; PDB ID: 2F36), GluK2 (KA; PDB ID: 1S50) and GluN2A (NMDA; PDB ID: 1A5S). A dimer was simulated for GluA2, GluA2 (apo) and GluK1 while only a monomer was simulated for GluK2, GluN2A and GluA2 (apo). Water molecules within 4 Å of a

protein residue were retained in the initial structure while the rest were discarded. Missing side chain atoms in the GluA2, GluA2 (apo) and GluN2A structures were added using the WHATIF “Complete a structure” web interface (<http://swift.cmbi.ru.nl>). A missing threonine residue in the GluN2A structure was added using MODELLER version 8v1 (Sali and Blundell, 1993).

Table 3.1 iGluR structures used for MD simulations. Two 30 ns MD simulations were performed. Each simulation was repeated with different velocities to give a total of 20 simulations. In the first set of calculations the TIP4P water model was used while in the second set used the TIP3P water model.

Subtype	PDB ID	Resolution (Å)	Ligand	First Set (TIP4P) Oligomerisation State	Second Set (TIP3P) Oligomerisation State
GluA2	1FTJ	1.90	Glutamate	Monomer	Dimer
GluA2	1FTO	2.00	None	Monomer	Dimer
GluK1	2F36	2.11	Glutamate	Monomer	Dimer
GluK2	1S50	1.65	Glutamate	Monomer	Monomer
GluN2A	2A5S	1.70	Glutamate	Monomer	Monomer

MD simulations were performed using the GROMACS version 3.3.1 and 3.3.3 suite of tools (Lindahl et al., 2001). In the first set of calculations the OPLS-AA forcefield parameters (Jorgensen et al., 1996) were used to describe the protein, and the TIP4P water model (Kaminski et al., 2001) was chosen to solvate the monomers of all proteins in a $80 \text{ Å} \times 80 \text{ Å} \times 80 \text{ Å}$ box and subjected to periodic boundary conditions (Vijayan et al., 2010). In the second set of calculations this procedure was repeated but instead the TIP3P water model (Jorgensen et al., 1983) was chosen to solvate a $90 \text{ Å} \times 90 \text{ Å} \times 90 \text{ Å}$ box for the monomers and a $100 \text{ Å} \times 100 \text{ Å} \times 100 \text{ Å}$ box for the dimers, all of which were then subjected to periodic boundary conditions. Counterions were

added to neutralise the system and water molecules were randomly replaced with cations and anions to make a 150 mM solution. Simulations were performed in an isobaric-isothermal ensemble maintained at 1 bar and 300 K by coupling to an external bath using the Berendsen algorithm (Berendsen et al., 1984). Electrostatics were handled using the particle mesh Ewald method (Essman et al., 1995) with a real space cut-off of 10 Å. Protein bonds were constrained using LINCS (Hess et al., 1997) and water molecules using SETTLE (Miyamoto and Kollman, 1992). An integration timestep of 2 fs was used.

Simulations consisted of first minimising the energy of the system using the steepest descent method with a tolerance of $100 \text{ kJ mol}^{-1} \text{ nm}^{-1}$ ($2.39 \text{ kcal mol}^{-1} \text{ Å}^{-1}$). A short 200 ps MD run was then performed, where the heavy atoms of the protein were restrained using a harmonic potential with a force constant of $1000 \text{ kJ mol}^{-1} \text{ nm}^{-2}$ ($2.39 \text{ kcal mol}^{-1} \text{ Å}^{-2}$). Finally, 30 ns unrestrained production simulations were performed for each structure. The set of simulations described above were repeated but with different initial velocities.

3.2.2 Quantum mechanical calculations

Interaction energies in the crystal structures were calculated with Quantum Mechanical (QM) models of the binding site. Each model was assembled by considering the glutamate ligand, water molecules within 5 Å from the ligand and all amino acids within a distance of 10 Å from the ligand and water molecules. Table 3.2 gives a list of the interacting residues from the subunits that were chosen. The

numbering employed here is according to the mature sequence as employed by Armstrong and Gouaux (Armstrong and Gouaux, 2000) for ease of comparison. The side chain of each residue was explicitly considered and the peptide bond was replaced with a C α -H bond to reduce the size of the QM model system.

Single point density functional theoretical calculations were carried out with the Gaussian '03 (Frisch et al., 2004) or Gaussian '09 (Frisch et al., 2009) programs. The interaction energies for the conserved water molecule were calculated using the function counterpoise method of Boys and Bernardi (Boys and Bernardi, 1970). The 6-31G** basis set was used with the B3LYP (Lee et al., 1988; Becke, 1993) functional as a compromise between computational expedience and reliability of the values for BSSE. To investigate the influence of the dispersion term additional calculations using the Grimme (Grimme, 2006) functional (B97D) were performed.

Table 3.2 List of interacting residues selected for the QM model calculations from the four proteins GluA2, GluK1, GluK2 and GluN2A.

GluA2	GluK1	GluK2	GluN2A
Glu402	Glu426	Glu409	Glu394
Tyr405	Tyr429	Tyr412	Glu396
Tyr450	Tyr474	Tyr457	His466
Pro478	Pro501	Pro485	Ser492
Leu479	Leu502	Leu486	Leu493
Thr480	Thr503	Ala487	Thr494
Arg485	Arg508	Arg492	Arg499
Leu621	Leu641	Leu624	Arg618
Ile623	Ile643	Ile626	Thr631
Gly648	Gly668	Gly652	Gly664
Thr649	Ala669	Ala653	Thr665
Leu650	Val670	Val654	Val666
Asp651	Arg671	Glu655	Pro667
Ser652	Asp672	Asp656	Asn668
Gly653	Gly673	Gly657	Gly669
Ser654	Ser674	Ala658	Ser670
Thr655	Thr675	Thr659	Thr671
Lys656	Met676	Met660	Glu672
Thr667	Ser706	Asn690	Arg673
Phe668	Leu720	Phe704	Val694
Thr686	Leu721	Leu705	Ile709
Tyr702	Met722	Met706	Tyr710
Glu705	Glu723	Glu707	Asp712
Met708	Ser726	Thr710	Val715
Lys730	Lys747	Lys731	Tyr742
Tyr732	Tyr749	Tyr733	

3.3 Results

3.3.1 Comparison of X-ray crystal structures

In these calculations it is important to pay special attention to water molecules that were found to interact directly with the ligand in X-ray crystal structures. A structural alignment using the program STRAP (Gille and Frommel, 2001), was used to locate the water oxygens in the crystal structures that defined a series of potential water-binding sites, W1-W11 (Figure 3.1).

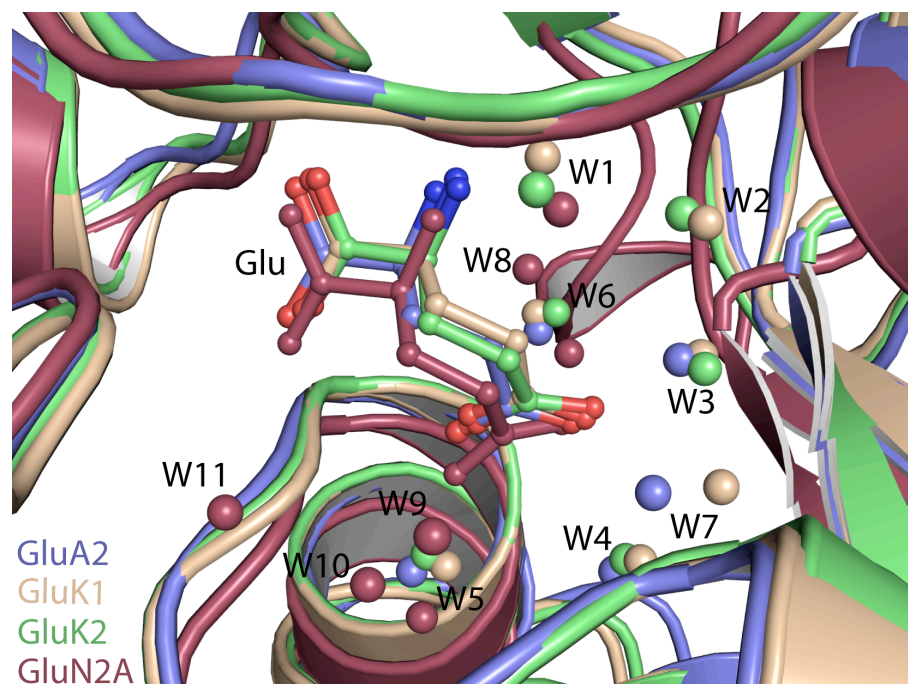


Figure 3.1 Structural alignment of GluA2 (teal), GluK1 (peach), GluK2 (green) and GluN2A (mauve) structures performed with STRAP (Gille and Frommel, 2001). This scheme was translated to the GluK2 structure with 6 bound water molecules, GluA2 with 5 bound water molecules and GluN2A with 7 bound water molecules. Additional identifiers W8-W11 were used for 4 water molecules that were specific to GluN2A.

The position of these water molecules with respect to the protein and ligand are used throughout this chapter.

3.3.2 Comparison of the structural behaviour of the proteins

Before focusing on the behaviour and characterisation of water molecules within the binding pocket, a comparison of the overall dynamics of the different receptors is necessary. An indication of the structural stability of a protein in a simulation is generally provided by its root mean square deviation (RMSD) with respect to a reference structure, in this instance the crystal structure, over the course of a

simulation. The RMSDs of protein C α atoms for the first set of calculations can be seen in Figure **3.2**. In all simulations, the RMSD quickly flattens suggesting a stable relaxation of the structure from the X-ray crystal state. The drift from the starting crystal structure is between 1.5 – 2.5 Å for all simulations. The RMSDs of protein C α atoms for the second set of calculations can be seen in Figure **3.3**. The drifts are comparable.

The root mean square fluctuation (RMSF) of C α atoms from all simulations gives an indication of regions in a protein that exhibit varying degrees of motion. The RMSFs for the first and second set of calculations are shown in Figure **3.4** and **3.5**, respectively. The increased fluctuations at the N and C-termini of these receptors could be attributed to unbonded protein segments that are free to fluctuate in solution.

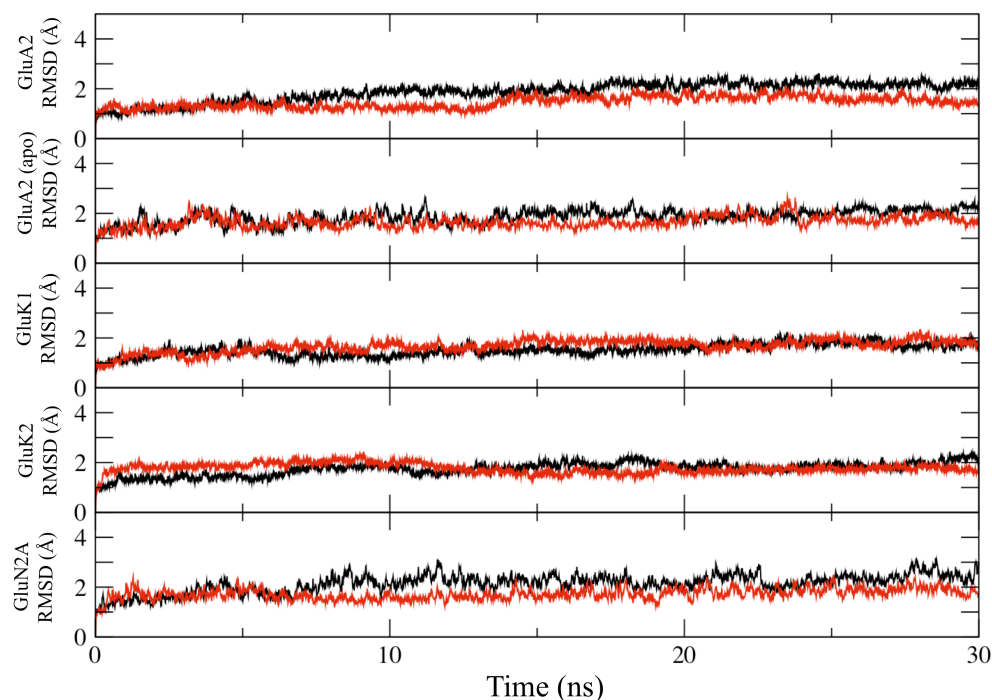


Figure 3.2 Root mean square deviation (RMSD) of all protein C α atoms calculated as a function of time (run 1; red, run 2; black) for the first set of calculations, which were all monomers. All RMSDs plateau off indicating relaxation to a stable state. The TIP4P water model was used in all simulations. Adapted from (Vijayan et al., 2010).

In Figure 3.4, scanning from left to right, the first two peaks encompass regions in flexible loop 1 and loop 2 that are known to exhibit significant motion. The region that follows contains a series of peaks corresponding to sub-domain D2 (Figure 1.2). In all structures studied here, D2 exhibits higher motion compared to D1, apart from loops 1 and 2, which is consistent with NMR relaxation dynamics data (McFeeters and Oswald, 2002) and the higher beta factor observed for residues in D2 in these structures and other ligand bound structures (Vijayan et al., 2010). Furthermore, in the apo GluA2 simulations, in the absence of a ligand that holds the clamshell shut, the fluctuations in D2 are consistently higher than the glutamate-bound simulations. The RMSFs for the second set of calculations follow the same

fluctuation trends for the monomers as can be seen in Figure 3.5. However, there is increased fluctuation at the dimerisation interface for GluA2, GluA2 (apo) and GluK1.

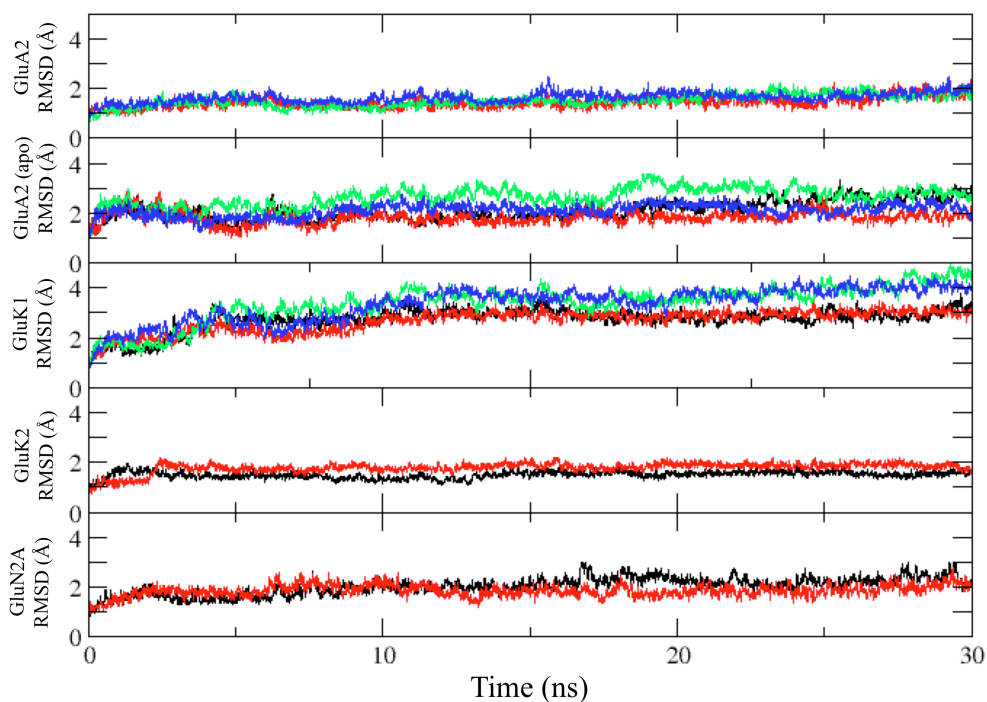


Figure 3.3 Root mean square deviation (RMSD) of all protein C α atoms calculated as a function of time for the second set of calculations. GluA2, GluA2 (apo) and GluK1 were dimer simulations. Chain A in the dimers and monomers are depicted as follows - run 1; red and run 2; black. Chain B in the dimer simulations are represented as follows – run 1; green and run 2; blue. All RMSDs plateau off indicating relaxation to a stable state. The TIP3P water model was used in all simulations.

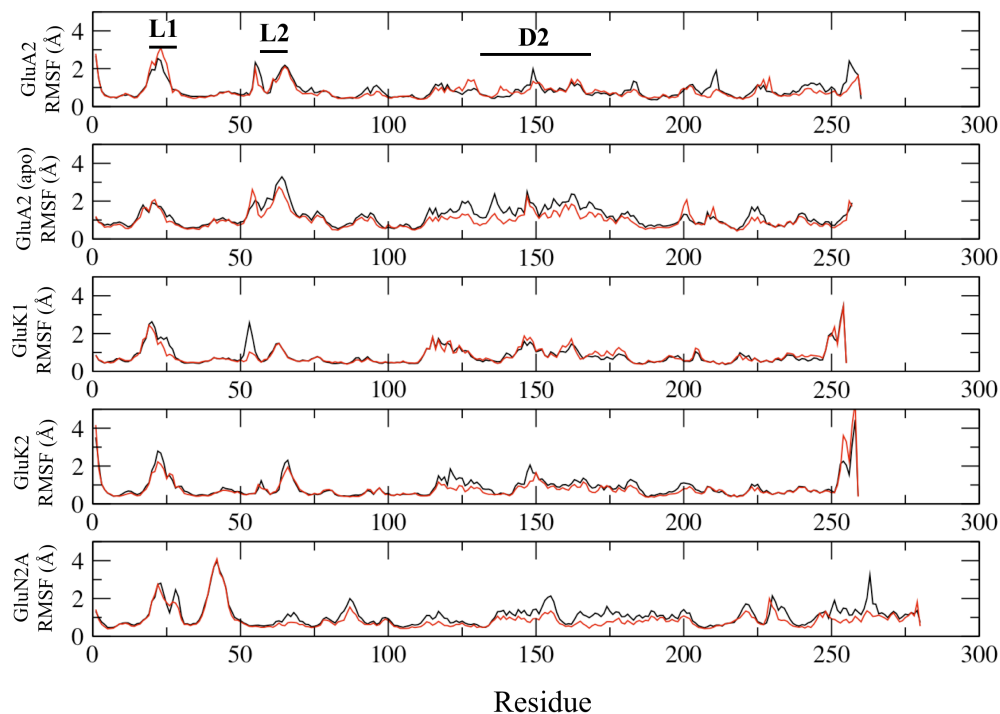


Figure 3.4 Root mean square fluctuation (RMSF) of protein C α atoms (run 1; red, run 2; black) for the first set of calculations. Regions of high fluctuation include loop 1 (L1) and loop 2 (L2) in domain D1 and residues in domain D2. Domain D2 of the apo GluA2 structure exhibits a higher motion with respect to the other structures due to the absence of a ligand that holds the clamshell shut. The hinge residues, located on either side of domain D2, mark a region of comparatively low fluctuation. The TIP4P water model was used in all simulations. Adapted from (Vijayan et al., 2010).

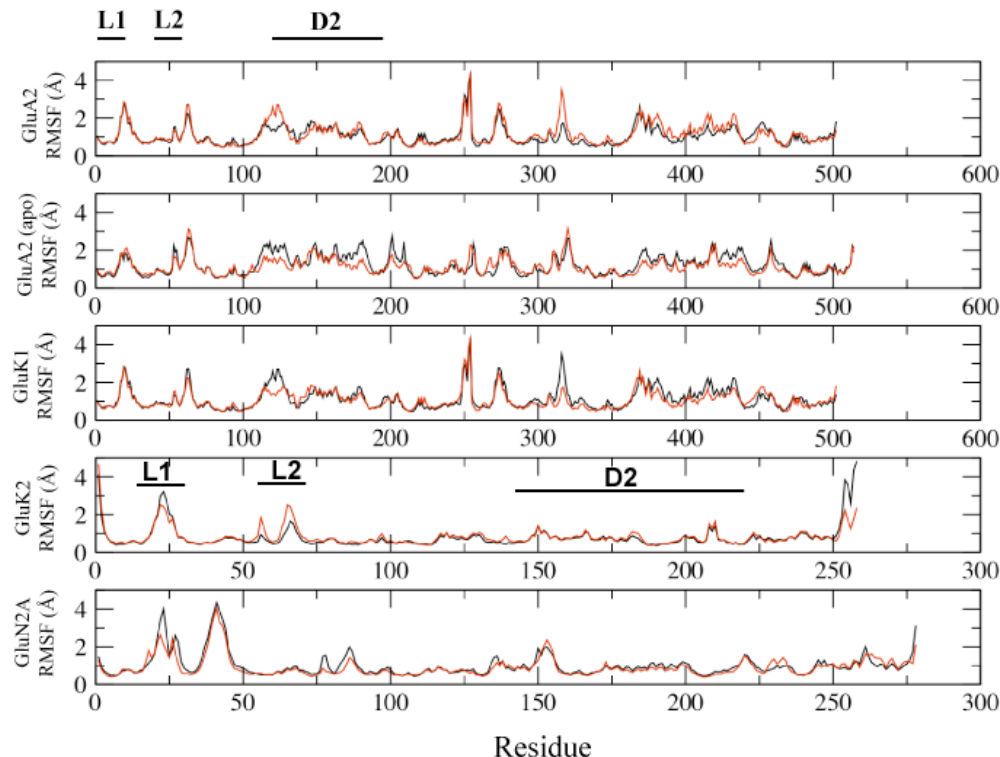


Figure 3.5 Root mean square fluctuation (RMSF) of protein C α atoms (run 1; red, run 2; black) for the second set of calculations. Regions of high fluctuation include loop 1 (L1) and loop 2 (L2) in domain D1 and residues in domain D2. The TIP3P water model was used in all simulations.

3.3.3 Interdomain motion

It is also of interest to study how the two sub-domains D1 and D2, when they assemble to form a clamshell structure that opens/closes to accommodate a range of ligands, behaves in the glutamate-bound and apo structures. The distance between the center of mass of the two sub-domains was computed for all simulations (set one Figure 3.6 and set two Figure 3.7). The majority of ligand bound structures appeared to show little variation in the interdomain distance. In contrast the apo structure in the first set of calculations exhibits closure in both runs indicated by a large drop in the D1-D2 distance at ~12 ns in the first simulation and at ~25 ns in the second

simulation. The speed of the transition is remarkably fast as has been observed previously with a different force-field (Arinaminpathy et al., 2006).

Since choice of water model and dimerisation does not appear to significantly affect the dynamics of these proteins, close inspection was made of the hinge region of GluA2 (apo) monomer in the first set of calculations. These simulations showed that a conformational change in a lysine (Lys730) in the hinge preceded this closing motion (Figure **3.8A,B**). Lys730 in the apo structure dissociated from Glu705 and formed a hydrogen bond with Asp728 in the hinge, which has been speculated to stabilise the hinge in the closed conformation (Armstrong and Gouaux, 2000). Further evidence was provided by the GluK2 run 1 simulation (where the opposite movement is observed as one would expect for an opening movement of the cleft). At ~12.5 ns the lysine at the equivalent position, Lys731, breaks its hydrogen bond with the hinge-based Asp729 and forms a hydrogen bond with Glu707. This was accompanied by a sharp increase in the D1-D2 distance (Figure **3.6**). It would seem as though the conformation of the lysine is acting as a switch which determines the preferred state of the cleft. The same sharp increase in the D1-D2 distance can be seen in the second set of calculations for GluK1: run 1. Thus this hypothesis was explored by rerunning the GluA2 (apo) simulations with a restraint ($1000 \text{ kJ mol}^{-1} \text{ nm}^{-2}$ on the carbon of the carboxylic acid of Glu and the nitrogen of Glu side chain) designed to maintain the hydrogen-bond between Glu705 and Lys730 (Figure **3.8C**). The cleft in those simulations did not undergo any distinct closing motion. Since the lysine, aspartate and glutamate residues are not conserved in GluN2A, the

stabilisation of the hinge in NMDA receptors might be accomplished through alternative pathways.

Given the speed of closure and the influence of this hinge along with similar observations from other research groups, it would appear that it is the biasing of this switch (presumably by the presence of ligands) which controls how fast the domains will close.

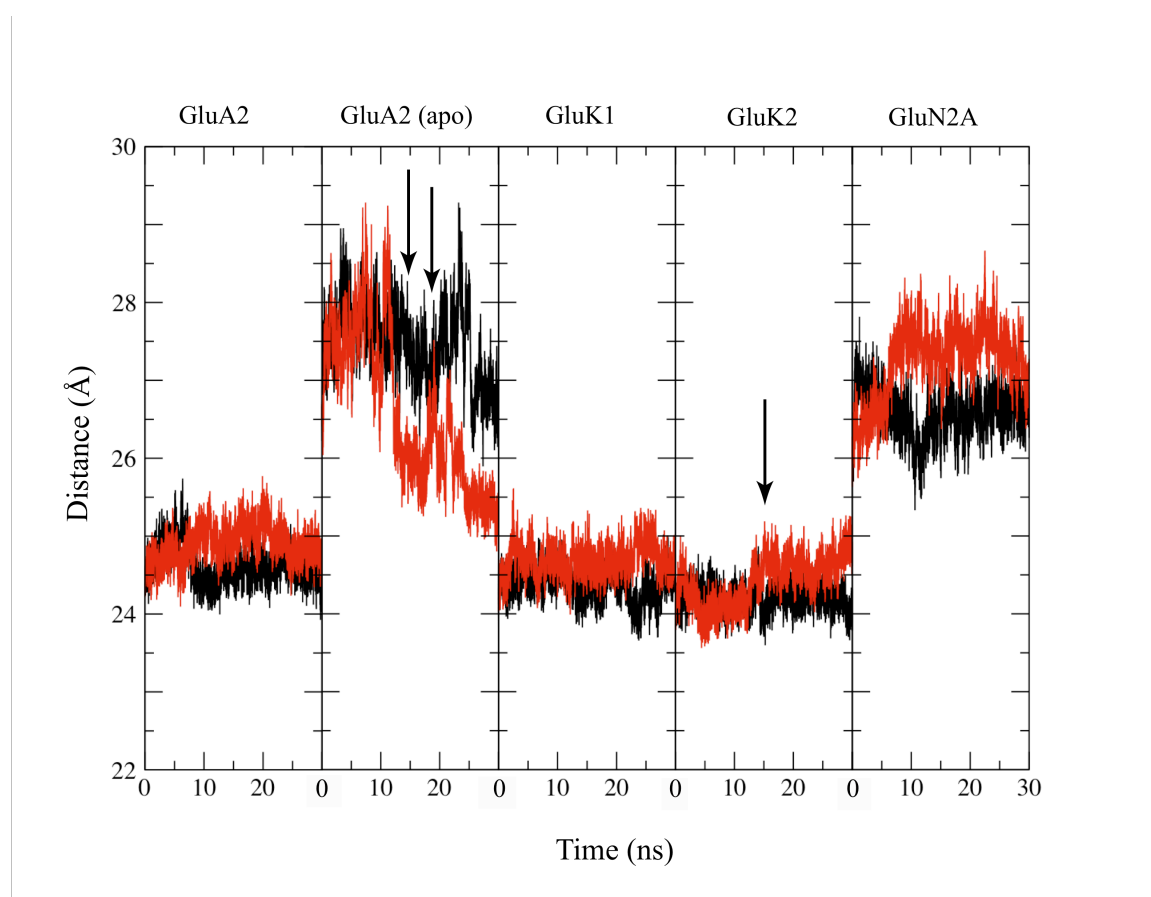


Figure 3.6 The distance between the center of mass of the C α atoms of the two sub-domains D1 and D2 for the first set of simulations (run 1; red, run 2; black), which were all. Arrows indicate a major change in D1-D2 distance and occurs around the time where a lysine residue in the hinge changes conformation. The TIP4P water model was used in all simulations. Adapted from (Vijayan et al., 2010).

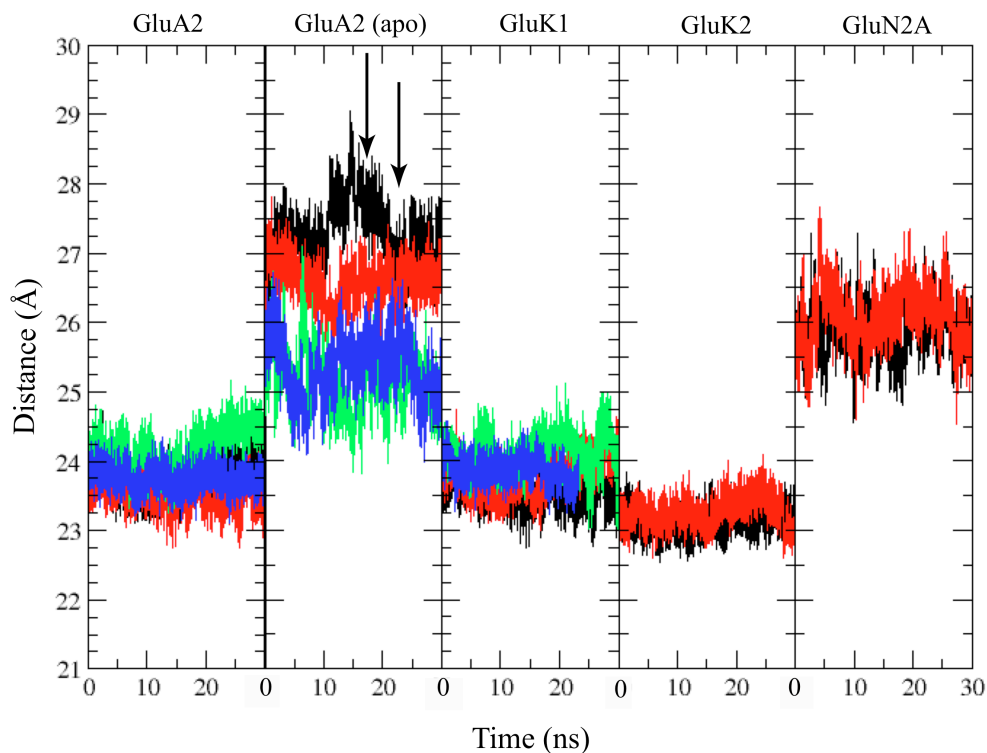


Figure 3.7 The distance between the center of mass of the C α atoms of the two sub-domains D1 and D2. GluA2, GluA2 (apo) and GluK1 were dimer simulations. Chain A in the dimers and monomers are depicted as follows - run 1; red and run 2; black. Chain B in the dimer simulations are represented as follows - run 1; green and run 2; blue. Arrows indicate a major change in D1-D2 distance and occurs around the time where a lysine residue in the hinge changes conformation. The TIP3P water model was used in all simulations.

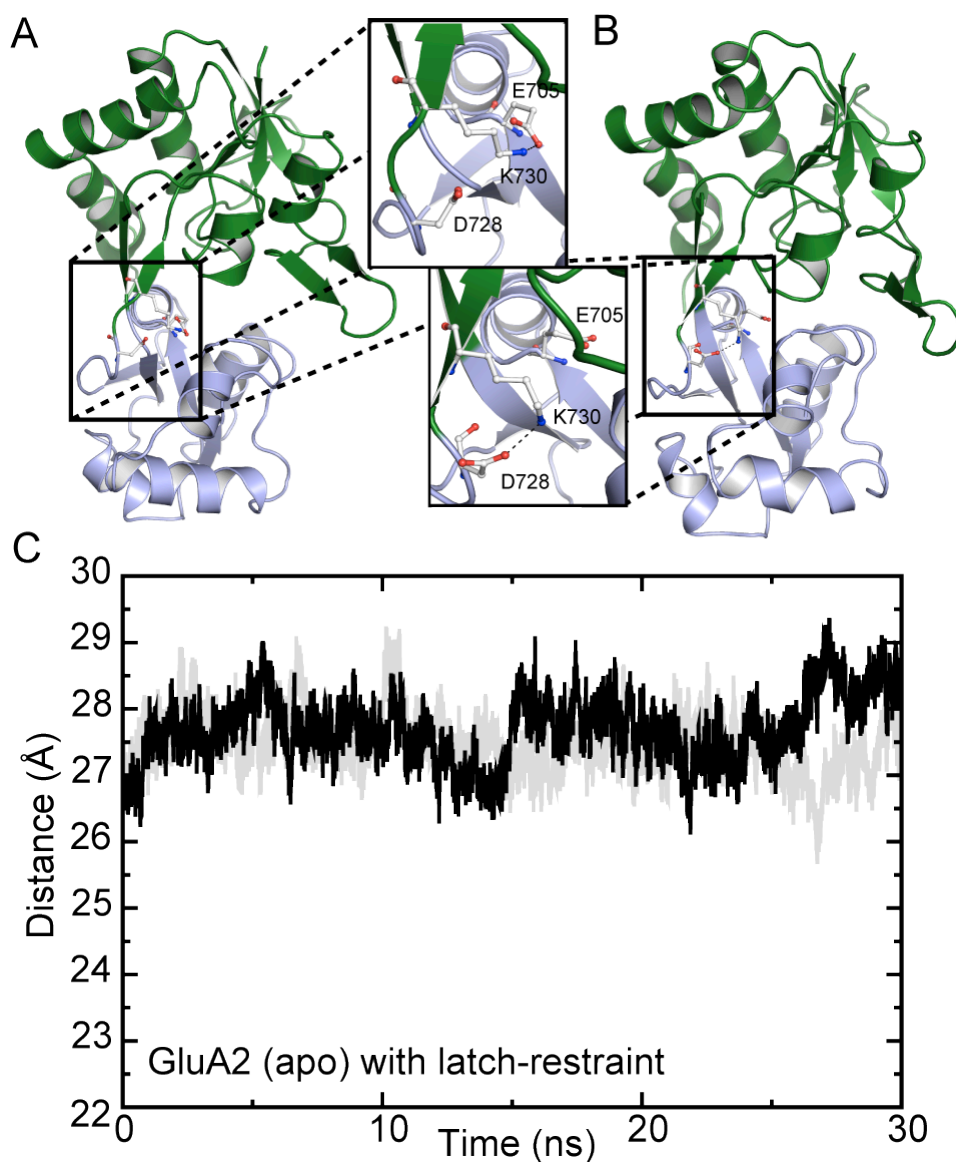


Figure 3.8 In the GluA2 apo structure, Lys730 in the hinge region breaks its hydrogen bond with Glu705 (A) and associates with Asp728 in the hinge region (B). This is accompanied by a reduction in D1-D2 distance, indicative of domain closure. (C) A test of the latch whereby the hydrogen bond between Lys730 and Glu705 is restrained prevents closure of the GluA2 apo system (compare with Figure 3.6). The TIP4P water model was used in these simulations. Taken from (Vijayan et al., 2010).

3.3.4 Ligand protein interactions

A summary of residues found to interact with the bound glutamate in each of the crystal structures is listed in Table 3.3. All residues listed in Table 3.3 were also found to interact with the bound glutamate throughout the course of MD simulations.

Table 3.3 Residues within 3.2 Å of the bound glutamate. Residue numbering is based on the mature sequence. These contacts were maintained throughout MD simulations.

GluA2	GluK1	GluK2	GluN2A
Tyr450	Tyr474	Tyr457	His 466
Pro478	Pro501	Pro485	Ser492
Thr480	Thr503	Ala487	Thr494
Arg485	Arg508	Arg492	Arg499
Ser654	Ser674	Ala658	Ser670
Thr655	Thr675	Thr659	Thr671
Glu705	Glu723	Glu707	Asp712
Tyr732	Tyr749	Tyr733	Tyr742

Table 3.4 Average number (and one standard deviation) of hydrogen bonds formed by glutamate with binding site residues and water molecules. Hydrogen bonds were calculated using a distance cut-off of 3.5 Å and acceptor-donor-hydrogen angle set to 30°.

Subtype	First Set		Second Set	
	Simulation 1	Simulation 2	Simulation 1	Simulation 2
GluA2	12.1 ± 1.0	12.7 ± 1.3	12.3 ± 1.1	12.5 ± 0.8
GluK1	12.2 ± 1.0	11.5 ± 0.9	12.5 ± 1.2	12.0 ± 1.0
GluK2	11.7 ± 1.3	11.1 ± 1.0	11.9 ± 1.0	12.0 ± 1.2
GluN2A	13.3 ± 1.0	12.9 ± 1.2	13.5 ± 1.2	13.0 ± 1.1

The ligand resides in a cavity forming a rich set of hydrogen bonds with both protein residues and water molecules (Figure 3.9). The average number of hydrogen bonds formed by the ligand with protein residues and water molecules is summarised in Table 3.4. In both sets of calculations, GluN2A appears to have the highest number of hydrogen bonds, partly due to an increased number of water molecules in the binding site, while GluA2, GluK1 and GluK2 have a similar number of hydrogen bonds.

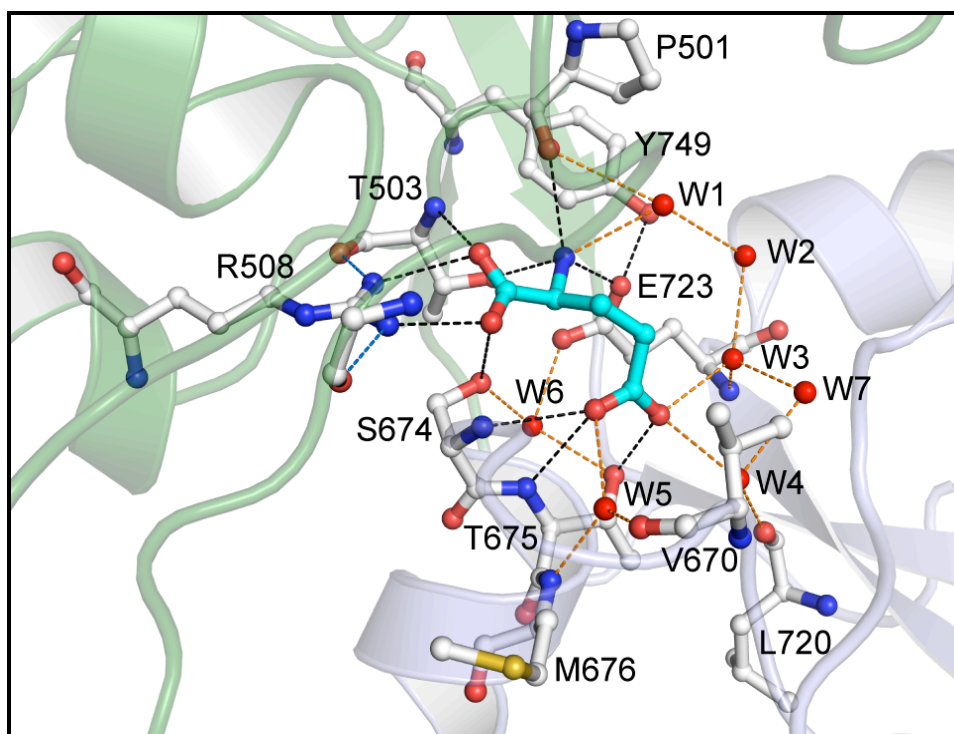


Figure 3.9 Cartoon detailing the complex hydrogen-bond network between the ligand, protein and water (oxygen atoms only displayed) for GluK2. Taken from (Vijayan et al., 2010).

As observed in the crystal structures, the ligand glutamate interacts with sub-domain D1 in an identical manner across the sub-types and the two sets of calculations. A conserved arginine (GluA2: Arg485; GluK1: Arg508; GluK2:

Arg492; GluN2A: Arg499) (Table 3.3) forms a bidentate salt bridge with the α -carboxyl group of the ligand. One of the ligand α -carboxyl oxygens formed a hydrogen bond with a backbone amide group of a residue in D1 (GluA2: Thr480; GluK1: Thr503; GluK2: Ala487; GluN2A: Thr494). The side chain hydroxyl group of the threonine was also found to make contact either with the α -carboxyl or the α -amino group (Figure 3.10A and B). The other α -carboxyl oxygen formed hydrogen bonds with the backbone amide group of a residue in domain D2 (GluA2: Ser654; GluK1: Ser674; GluK2: Ala658; GluN2A: Ser670). Furthermore, the side-chain hydroxyl group of the serine made contact with the same α -carboxyl group of the ligand. Owing partly to the dynamics of the D2 domain, the side-chain and main chain groups were found to be capable of interacting with either the α -carboxyl or the γ -carboxyl group of the ligand (Figure 3.10C and D).

The α -amino group forms a near tetrahedral hydrogen-bonded arrangement with contributions from domains D1 and D2. A backbone carbonyl oxygen (GluA2: Pro478; GluK1: Pro501; GluK2: Pro485; GluN2A: Ser492) and a side chain threonine (GluA2: Thr480; GluK1: Thr503; GluN2A: Thr494) hydroxyl group contributes two hydrogen bonds. However, as mentioned above, the threonine hydroxyl group was occasionally found to interact with the α -carboxyl group as well. A water molecule was found to take its place instead hydrogen bonding to the α -amino group. It is important to note that this threonine is not conserved in GluK2, but is replaced with an alanine, thereby eliminating a hydrogen bond. An additional water molecule, W1 (Figure 3.1), forms a hydrogen bond with the α -amino group, consistent with MD

observations. The third hydrogen bonding partner is an acidic residue from β strand 10 (GluA2: Glu705; GluK1: Glu723; GluK2: Glu707; GluN2A: Asp712).

In the GluN2A crystal structure, a water mediated hydrogen bond links the shorter aspartate residue to the amino group of the ligand. However, simulations showed that the water was quickly displaced and the aspartate formed a direct hydrogen bond with the ligand (Figure **3.10E** and **F**).

3.3.5 Water in the binding site

Unlike the α -carboxyl and α -amino interactions in the binding site that are mainly direct protein-protein hydrogen bonds, the γ -carboxyl interactions with the protein are largely water mediated. The backbone amide and side-chain hydroxyl group of a conserved threonine (GluA2: Thr655; GluK1: Thr675; GluK2: Thr659; GluN2A: Thr671) and a serine/alanine (GluA2: Ser654; GluK1: Ser674; GluK2: Ala658; GluN2A: Ser670) hydrogen bonds with the γ -carboxyl group, while 3 water molecules at sites W3, W4 and W5 in GluA2, GluK1 and GluK2 bridge the ligand to the protein. Water occupancy computed from MD trajectories in the second set of calculations showed distinct locations preferred by water molecules in the binding site in good agreement with crystallographic locations (Figure **3.11**). This observation is quite remarkable given all but one of these water molecules is able to exchange with the bulk through the formation of transient water channels. Furthermore waters are able to readily move between the sites W1 to W4. The timescale for water exchange is of the order of nanoseconds.

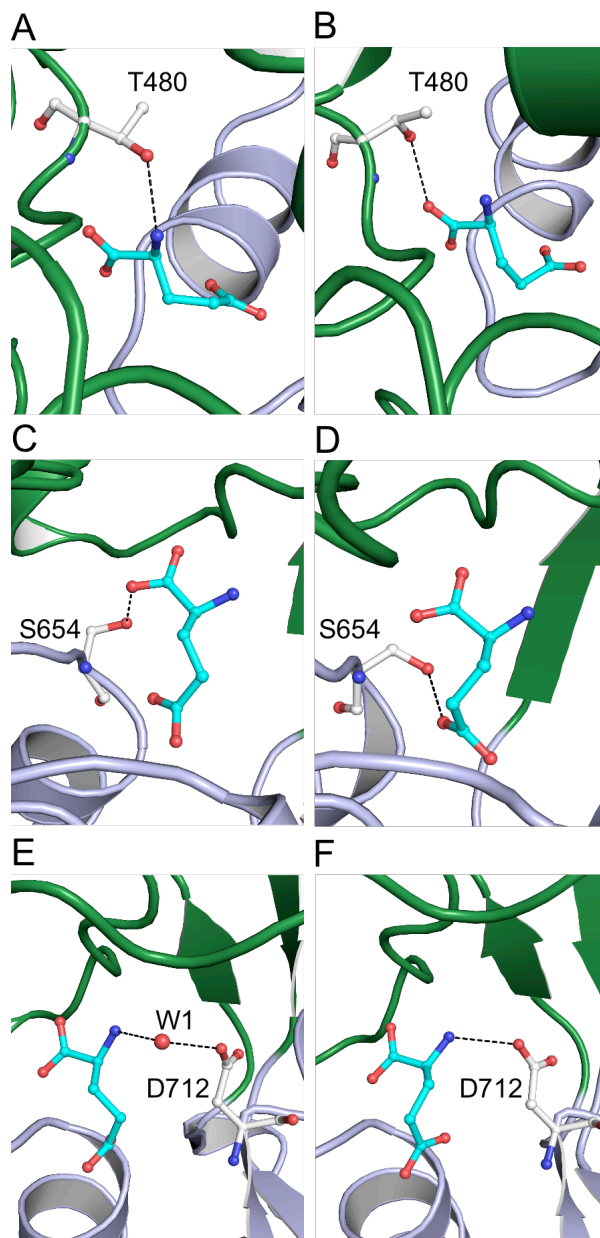
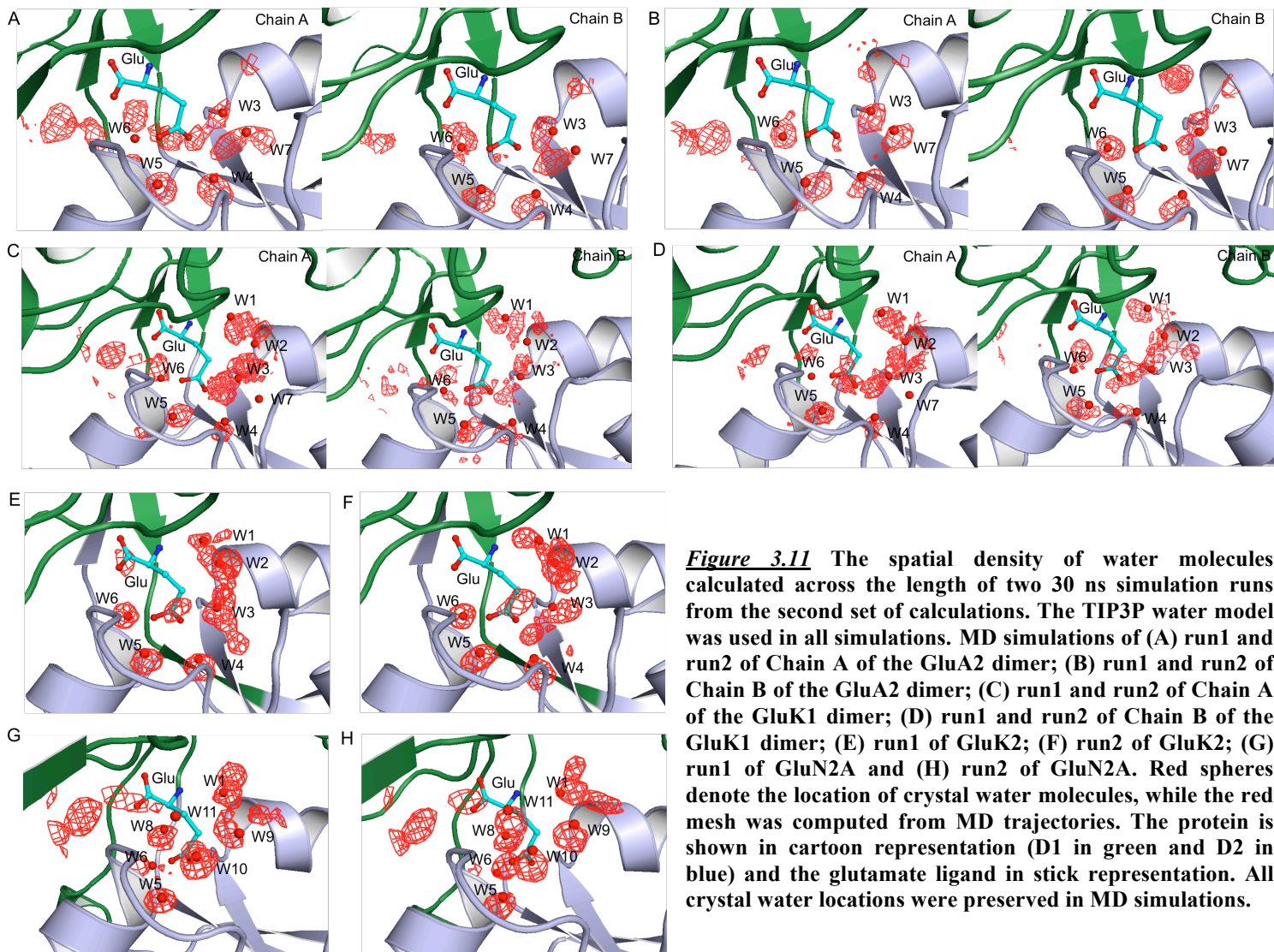


Figure 3.10 The ligand is shown in cyan ball-and-stick representation. (A) and (B): GluK2 lacks a threonine in domain D1 that hydrogen bonds with the α -amino group of the glutamate ligand, which is conserved in GluA2, GluK1 and GluN2As. Threonine (T480 in GluA2) is capable of forming hydrogen bonds with either the α -carboxyl group or the α -amino group. (C) and (D): Alternate hydrogen bonding of a serine (S654 from GluA2) that is conserved in GluA2, GluK1 and GluN2A. The serine is capable of hydrogen bonding to either the α -carboxyl group or the γ -carboxyl group of the glutamate ligand. (E) In the GluN2A crystal structure, unlike a conserved glutamate in GluA2, GluK1 and GluK2 the equivalent shorter Asp712 interacts with the ligand via an intermediate water molecule, W1 (red sphere). (F) MD simulations using the TIP4P water model show that this aspartate residue is able to directly interact with the α -amino group of the ligand.

One water molecule, in W5, was never replaced with water from the bulk in any of the glutamate-bound simulations. The water in W5 is hydrogen bonded to the γ -carboxyl group of the ligand, the backbone amide group of a residue in helix F (Lys656 in GluA2, Met676 in GluK1, Met660 in GluK2 and Glu672 in GluN2A), and the backbone carbonyl group of a residue in the coil between helix F and β -strand 9 (Leu650 in GluA2, Val670 in GluK1, Val654 in GluK2, and Val666 in GluN2A).

Since only the protein backbone is involved, neither of these two residues is consistently conserved across the iGluR family. Analysis of crystal structures and MD simulations showed that the W5 water resides in a cavity that is occluded by ligand binding (Figure **3.12A**). Simulations of the apo GluA2 monomer structure from the first set of calculations and apo structures with the modeled glutamate that lacked this water molecule showed that waters bind to the W5 site in the absence of a ligand (Figure **3.12B**). This is a consequence of the ligand binding, followed by cleft closure, making this location inaccessible to water molecules (Vijayan et al., 2010). Furthermore in the GluA2 apo simulations, once a water molecule enters from bulk, it is also never swapped with bulk (Figure **3.12B**). A survey of high-resolution iGluR LBD structures from the PDB showed that a water molecule at the W5 site is present in almost all (>80) ligand bound crystal structures (Table **3.5**). The only significant exception was the apo structure considered in this study.



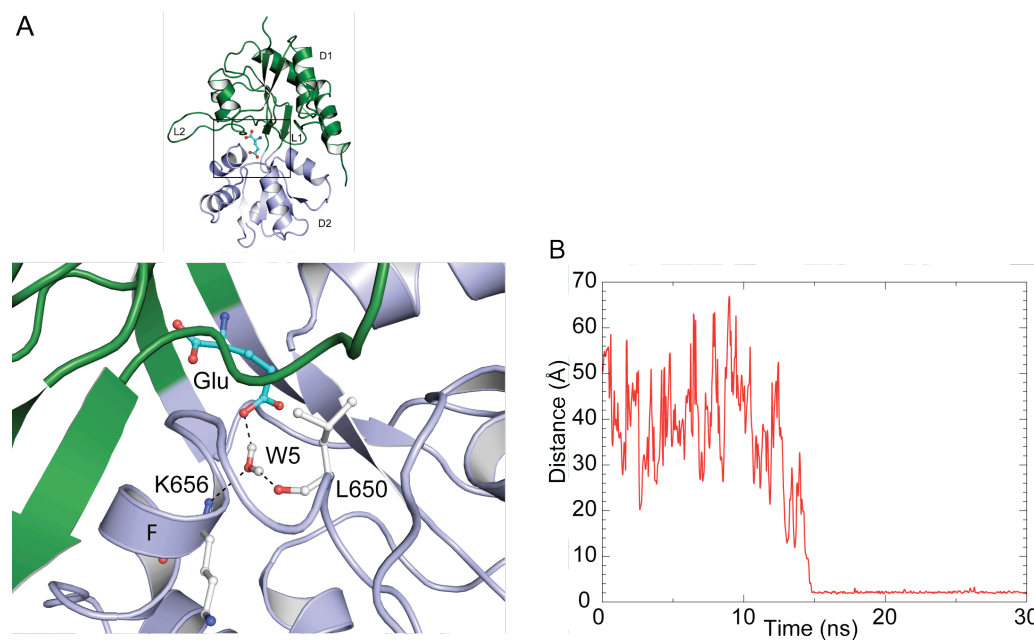


Figure 3.12 (A) In the GluA2 monomer simulation, water molecule W5 (shown in ball and stick representation) hydrogen bonds to the backbone of two protein residues and the ligand glutamate. Hydrogen atoms of amino acids have been omitted for clarity (B) Water molecule trajectory (Lys656-NH—Water-O distance) to the W5-binding pocket in one of the apo simulations. The TIP4P water model was used in these simulations. Taken from (Vijayan et al., 2010).

Table 3.5 iGluR structures that were checked for the conserved water molecule in the W5 site.

Receptor	Subtype	PDB ID
AMPA	GluA2	1FTJ, 1FTK, 1FTL, 1FTM, 1FTO [‡] , 1FW0, 1GR2, 1LB8, 1LB9 [‡] , 1LBB, 1LBC, 1M5B, 1M5C, 1M5D, 1M5E, 1M5F, 1MM6, 1MM7, 1MQD, 1MQG, 1MQH, 1MQI, 1MQJ, 1MS7, 1MXU, 1MXV, 1MXW, 1MXX, 1MXY, 1MXZ, 1MY0, 1MY1, 1MY2, 1MY3, 1MY4, 1N0T, 1NNK, 1NNP, 1P1N, 1P1O, 1P1Q, 1P1U, 1P1W, 1SYH, 1SYI, 1WVJ, 1XHY, 2AIX, 2AL4, 2AL5, 2ANJ, 2CMO [‡] , 2GFE, 2I3V, 2I3W, 2P2A, 2UXA, 3B6Q, 3B6T, 3B6W, 3B7D, 3BBR
Kainate	GluK1	1TXF, 1VSO, 1YCJ, 2F34, 2F35, 2F36, 2OJT [‡] , 2PBW, 3C31, 3C32, 3C33, 3C34, 3C35, 3C36
Kainate	GluK2	1S50, 1S7Y, 1S9T, 1SD3, 1TT1, 2I0B, 2I0C
NMDA	GluN2A	2A5S, 2A5T

[‡] Structures that do not have a water at W5. These are low-resolution structures. The notable exception is 1FTO, the GluA2 ligand-free structure.

Further characterisation of the interaction energies of water in the W5 site with the components of the binding pocket are shown in Table 3.6. It can be seen that the interaction energy is favourable and has significant contributions from both the ligand (glutamate) and the protein. Whilst the contribution from the ligand is approximately the same across all systems, the protein contribution in the GluN2A system is much more favourable. Closer examination revealed that in this system the water makes more hydrogen bonds with the protein.

Table 3.6 Full breakdown of interaction energies (A) and basis set superposition errors (B) of water at site W5 in each QM system in kcal mol⁻¹. Ws; all other waters (eg. for the W1-Ws cell, Ws means all other waters), L; ligand (glutamate), P; protein, LP; ligand and protein, WsL; waters and ligand, WsP; waters and protein, WsLP; waters and ligand and protein.

(A)

W5 Fragment	Ws	L	P	LP	WsL	WsP	WsLP
GluA2	0.84	-10.54	-6.10	-12.24	-5.84	-6.30	-9.53
GluK1	0.71	-11.38	-6.40	-14.28	-7.29	-6.39	-11.71
GluK2	0.88	-12.18	-7.26	-14.01	-8.45	-7.23	-12.22
GluN2A	1.86	-13.88	-18.66	-24.00	-10.06	-9.52	-30.80

(B)

W5 Fragment	Ws	L	P	LP	WsL	WsP	WsLP
GluA2	0.25	2.81	7.99	8.97	2.45	8.11	8.82
GluK1	0.24	2.74	8.72	9.58	2.39	8.83	9.44
GluK2	0.10	2.80	8.88	9.70	2.37	9.26	9.60
GluN2A	0.88	3.56	2.89	6.53	3.66	9.48	5.63

A water in the W6 site is the last water molecule that is conserved across all four systems, but exhibits sub-type dependent behaviour. In the GluA2, GluK1 and GluK2 simulations, waters in the W6 site exhibit long residence times and are swapped only once or twice during the period of the simulations. However for the GluN2 simulations the swapping rate is much higher. This is reflected in the interaction energies based on single point calculations of binding site residues taken from the crystal structures as shown in Table 3.7.

Table 3.7 Full breakdown of interaction energies (A) and basis set superimposition errors (B) of water at the W6 site in each QM system in kcal mol⁻¹. Ws; all other waters (eg. for the W1-Ws cell, Ws means all other waters, L; ligand (glutamate), P; protein, LP; ligand and protein, WsL; waters and ligand, WsP; waters and protein, WsLP; waters and ligand and protein.

(A)

W6 Fragment	Ws	L	P	LP	WsL	WsP	WsLP
GluA2	1.22	-2.86	-37.59	-37.39	-1.32	-35.16	-34.91
GluK1	0.94	-2.62	-37.00	-36.93	-1.20	-33.93	-35.12
GluK2	0.50	-1.55	-36.72	-35.64	-0.65	-32.52	-33.70
GluN2A	0.32	-3.70	-4.25	-19.83	-3.91	-6.82	-18.45

(B)

W6 Fragment	Ws	L	P	LP	WsL	WsP	WsLP
GluA2	0.00	0.48	10.89	10.59	0.41	10.62	10.48
GluK1	0.00	0.53	10.75	10.45	0.44	11.96	10.42
GluK2	0.00	0.51	10.28	9.19	0.45	11.28	10.47
GluN2A	0.00	2.44	7.25	8.60	2.37	10.84	10.45

Additional calculations with a dispersion correction (Grimme, 2006) are shown in Tables 3.8 and 3.9. These results have been expanded on from the previously published data to gain a clearer understanding (Vijayan et al., 2010) and show small differences across the four receptor subtypes, but the overall trends are maintained.

Table 3.8 Full breakdown of dispersion-corrected interaction energies (A) and basis set superposition errors (B) of water at the W5 site in each QM system in kcal mol⁻¹. Ws; all other waters (eg. for the W1-Ws cell, Ws means all other waters), L; ligand (glutamate), P; protein, LP; ligand and protein, WsL; waters and ligand, WsP; waters and protein.

(A)

W5 Fragment	Ws	L	P	LP	WsL	WsP	WsLP
GluA2	0.43	-10.27	-10.92	-17.01	-6.06	-11.48	-14.67
GluK1	-1.49	-11.25	-11.23	-19.38	-7.52	-13.02	-15.83
GluK2	0.59	-12.13	-11.86	-18.64	-8.62	-12.80	-17.41
GluN2A	1.11	-13.40	-17.18	-28.04	-10.31	-15.75	-25.39

(B)

W5 Fragment	Ws	L	P	LP	WsL	WsP	WsLP
GluA2	0.26	2.70	7.89	8.78	2.39	8.00	8.62
GluK1	0.24	2.65	8.65	9.43	2.33	8.78	8.98
GluK2	0.10	2.70	8.03	9.90	2.32	9.38	9.45
GluN2A	0.91	3.40	6.99	8.83	3.58	7.75	8.83

Table 3.9 Full breakdown of dispersion-corrected interaction energies (A) and basis set superposition errors (B) of water at the W6 site in each QM system in kcal mol⁻¹. Ws; all other waters (eg. for the W1-Ws cell, Ws means all other waters), L; ligand (glutamate), P; protein, LP; ligand and protein, WsL; waters and ligand, WsP; waters and protein.

(A)							
W6 Fragment	Ws	L	P	LP	WsL	WsP	WsLP
GluA2	1.09	-3.69	-40.90	-39.89	-2.24	-38.81	-37.88
GluK1	0.83	-3.49	-40.37	-39.70	-2.12	-36.92	-36.33
GluK2	0.42	-2.71	-39.84	-37.41	-1.75	-29.67	-17.28
GluN2A	0.27	-3.83	-11.05	-24.95	-4.03	-10.59	-24.48

(B)							
W6 Fragment	Ws	L	P	LP	WsL	WsP	WsLP
GluA2	0.00	0.51	10.64	10.35	0.43	10.39	10.23
GluK1	0.00	0.56	10.53	11.94	0.47	12.81	11.89
GluK2	0.00	0.58	10.59	9.05	0.49	20.31	8.20
GluN2A	0.00	2.73	6.82	8.76	2.45	6.79	8.59

3.4 Discussion

3.4.1 Binding modes are dynamic

Ligand-protein interactions are dynamic with a considerable contribution given entropically to the binding mode of ligands. Thus, computing the free energy of binding is likely to be extremely challenging for systems where there are considerable fluctuations in the binding mode. The situation is complicated by the fact that the iGluR receptors can exist in several conformational states prior to ligand binding. An experimental measurement of affinity will encompass the energetics of conformational rearrangement of the protein. In such cases it is prudent to gain a full understanding of the regions of the protein that are likely to contribute significantly to that term.

A series of equilibrium 30 ns simulations for a series of ionotropic glutamate receptors gave evidence for the behaviour of one set of interactions that appears to correlate with the overall motion of the lobes; the salt bridge behaviour of Lys730. In the open-cleft conformation Lys730 forms an interaction with Glu705, but in order for the cleft to undergo closure the Lys730 breaks the salt bridge and forms an alternative contact with Asp728. The reverse appears to coincide with cleft-opening. This is in agreement with recent work by Kurnikova and colleagues (Mamonova et al., 2008) who examined the role of Glu705 specifically and with crystallographic observations of the open and closed cleft (with ligand bound) forms of the protein.

3.4.2 Roles for specific water sites

In the glutamate-bound state Lys730 forms a salt-bridge to Asp728, and still interacts with Glu705 indirectly via a water molecule at the W6 site. The MD suggests that waters in the W6 site do not play a causative role in the behaviour of the switch although there were distinct changes in cleft separation. More investigation is needed to state conclusively the role of water at the W6 position.

Since waters in the W5 site are neither swapped nor displaced in simulations, they may be structurally significant. The location of W5 is such that it forms part of the “floor” of the binding pocket with which the γ -carboxy side chain of glutamate can interact. To understand what contribution a water in the W5 site makes to the interaction energy of the ligand with the system, a fragment-based QM approach, was used to calculate the interaction energy of the ligand with the (model) system in the presence and absence of a water at the W5 site (Table 3.10).

Table 3.10 Interaction energies in kcal mol⁻¹ of the the ligand, glutamate with the protein and waters. L; ligand (glutamate), W_{sall}P; all waters and protein; W_{sall-w5}P; all waters except W5 and protein.

Subunit	L-W _{sall} P	L-W _{sall-w5} P	W5 contribution
GluA2	-179.86	-177.55	-2.31
GluK1	-172.03	-167.63	-4.39
GluK2	-94.75	-89.33	-5.42
GluN2A	-106.68	-97.31	-9.37

The results show that a water in this position makes a favourable contribution to the interaction energy and that it makes its strongest contribution in the GluN2A structure (of the order of 9 kcal/mol). Taken together with the other DFT results and the MD simulations, this suggests that polarisation effects contribute to the overall affinity of the ligand in a subtype specific manner, but that is less crucial for dictating the presence or absence of a water at a specific position. **Chapter 4** discusses this aspect in more detail.

3.5 Conclusions

This chapter demonstrates the value of performing relatively simple calculations to provide a more complete characterisation of the binding pocket and the expected behaviour of the waters within it. Results from two sets of MD simulations and one representative set of QM calculations demonstrate that the binding site of ionotropic glutamate receptors are remarkably flexible, but despite this flexibility, the water distribution in the presence of glutamate is conserved across the iGluR family.

The latter is made even more notable by the observation that all but one of water binding sites are transiently accessible from bulk solution. One water binding site at W5 is however accessible in the apo (open-cleft) form of the protein and effectively becomes part of the protein prior to ligand binding. However, its presence is not a requirement to predict the correct binding mode and its role appears to be to enhance the affinity of glutamate for the receptor.

The simulations also identified another water at the W6 site, which behaves differently in the GluA2 and GluK1/2 systems compared to the GluN2A. This water may play a distinct role in modulating the transition of the cleft between its open and closed states. This transition has been suggested to be different in NMDA receptors (GluN2A) compared to non-NMDA receptors (GluA2, GluK1/2). While the glutamate-bound structure exhibited limited rigid body motion of the D1 and D2 domains, the ligand-free apo structure was found to display a closing motion centered on the hinge region of the LBD. This was associated with the switch in conformation of a lysine residue, which may stabilise the hinge in the closed conformation by the formation of a hydrogen bond with an aspartate located in the hinge. On the basis of this study **Chapter 4** will focus on more detailed calculations on specific water molecules with respect to different ligands.

Chapter 4

Quantifying Water-Mediated Interactions in a Glutamate Receptor

'There is no quantum world. There is only an abstract quantum mechanical description.'

Niels Bohr

This chapter was based on the article (Sahai and Biggin, 2011):

M. A. Sahai and P. C. Biggin (2011). Quantifying Water-Mediated Interactions in a Glutamate Receptor; A DFT Study. *J. Phys. Chem. B.* (under review).

Abstract

It is becoming increasingly clear that careful treatment of water molecules in ligand-protein interactions is required in many cases if the correct binding pose is to be identified in molecular docking. Water can form complex bridging networks and can play a critical role in dictating the binding mode of ligands. A particularly striking example of this can be found in the ionotropic glutamate receptors. Despite possessing similar chemical moieties, crystal structures of glutamate and α -amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid (AMPA) in complex with the ligand binding core of the GluA2 ionotropic glutamate receptor revealed, contrary to all expectation, two distinct modes of binding. The difference appears to be related to the position of water molecules within the binding pocket. However, it is unclear exactly what governs the preference for water molecules to occupy a particular site in any one binding mode. In this chapter density functional theory (DFT) calculations are used to investigate the interaction energies and polarisation effects of the various components of the binding pocket. The results show i) the energetics of a key water molecule are more favourable for the site found in the glutamate-bound mode compared to the alternative site observed in the AMPA-bound mode, ii) that polarisation effects are important for glutamate but less so for AMPA, iii) ligand-system interaction energies alone can predict the correct binding mode for glutamate, but for AMPA alternative modes of binding have similar interaction energies and iv) the internal energy is a significant factor for AMPA but not for glutamate.

4.1 Introduction

Prior to the crystal structures of iGluRs and on the basis of pharmacophoric models, it was thought that the two agonists glutamate and AMPA would adopt similar poses within the GluA2 binding pocket. However, crystal structures revealed that AMPA adopts a slightly different binding mode with a water molecule assuming a position that would normally be occupied by the γ -carboxyl group of glutamate (Figure 4.1) (Armstrong and Gouaux, 2000). It has also been reported that docking studies that did not take into account the crystallographic waters failed to obtain the correct poses (Greenwood and Liljefors, 2004). This unexpected difference in binding mode raises some interesting questions; i) What is the underlying cause for the difference? ii) Can the differences in the position of water molecules and binding pose be rationalised in terms of interaction energies? iii) does AMPA cause polarisation effects that glutamate does not? iv) Can the correct binding mode be predicted by energetic (enthalpic) considerations alone?

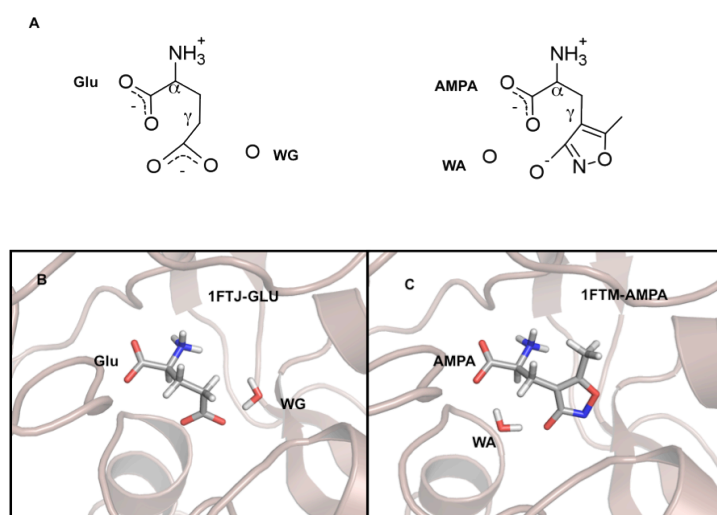


Figure 4.1 (A) Chemical structures of glutamate and AMPA. The 3-oxyanion oxygen and nitrogen from the ring of AMPA were thought to mimic the γ -carboxylate of the glutamate. Cartoons of (B) glutamate from the 1FTJ structure and (C) AMPA from the 1FTM structure bound to GluA2 along with the water molecules that act as surrogate ligands. WG occupies the site occupied by the ring nitrogen of AMPA. WA sits in the site occupied by left-most (in this view) carboxylate oxygen of glutamate.

In order to address these questions a quantum mechanical level treatment is used to accurately calculate interaction energies of both glutamate and AMPA in the two different binding modes. *Ab initio* calculations have been used previously in order to account for subtype specificity between GluA1 and GluA3 where the difference in the binding pocket was a phenylalanine instead of a tyrosine (Banke et al., 2001). Here density functional theory (DFT) is used to calculate the interaction energies of components of the GluA2 binding site with AMPA and glutamate. The calculations demonstrate that interaction energies alone are able to discriminate between “correct” binding mode for glutamate, but not for AMPA. The calculations show that the interaction energy of a water found in the glutamate-bound crystal structure (1FTJ) is more favourable than its counterpart at a different position in the AMPA-bound crystal (WA in Figure 4.1C). Polarisation effects were also found to be stronger in glutamate than in AMPA and that the difference in strain energy between alternative conformations of glutamate is very small, but is over 2 kcal mol⁻¹ for the alternative conformation of AMPA.

4.2 Methods

The model-systems have been assembled using the high-resolution crystallographic structures of the GluA2 ligand binding core bound with glutamate and AMPA available at the Protein Data Bank with PDB Code 1FTJ (resolution 1.9 Å) and 1FTM (resolution 1.7 Å), respectively. Since the crystal structures were composed of 3 protomers (chains

A, B and C), only one monomer (i.e. chain A) was selected as the starting geometry for a molecular mechanics energy minimisation. This choice was based on previous work done on GluA2 protomers (Arinaminpathy et al., 2002). In total, four model systems were prepared (Figure 4.2); two “wildtype” where the ligand (glutamate or AMPA) is in the pose observed crystallographically which are referred to as 1FTJ-Glu and 1FTM-AMPA and two where the ligand is in the alternative pose (Figure 4.2). For glutamate in the 1FTM structure this achieved by converting AMPA to glutamate (by matching equivalent torsion angles, deleting atoms and converting the isoxazole nitrogen to an oxygen). For AMPA in the 1FTJ structure, AMPA was fitted to the C, C α , C β , C γ and C δ atoms of glutamate such that the amide of AMPA fitted onto the side chain carboxylate of glutamate. Missing side chains of the protein were completed with the WHAT IF web interface at <http://swift.cmbi.kun.nl/whatif/>. All the water molecules reported in the crystal structure were included in the minimisation. The system was then solvated using a box of TIP3P (Jorgensen et al., 1983) water molecules. Counterions were added to neutralise the system and water molecules were randomly replaced with cations and anions to make a 150 mM solution. The system was then minimised using the steepest descent algorithm, until it converged with a force tolerance of 100 kJ mol⁻¹ nm⁻¹. GROMACS version 3.3.1 (Van der Spoel et al., 2005) was used in these calculations utilising the all atom OPLS-AA force field (Jorgensen et al., 1996; Kaminski et al., 2001). This energy minimised structure was then chosen as the representative structure to assemble the quantum mechanical (QM) model.

The QM models were assembled by considering the specific ligand of interest, water molecules within 5 Å from the ligand and all amino acids within a distance of 10 Å

from the ligand and water molecules. In the end 23 interacting residues from the GluA2 receptor were chosen that included Glu402, Tyr450, Pro478 - Thr480, Arg485, Gly648 - Lys656, Thr686, Tyr702 - Glu705, Met708, Lys730 and Tyr732 (Figure 4.3). The numbering employed here is according to the mature sequence as employed by Armstrong and Gouaux (2000) for ease of comparison (Armstrong and Gouaux, 2000). It is important to note that residues Arg485, Thr480 and Pro478 from domain 1 and Ser654, Thr655 and Glu705 from domain 2 directly interact with both ligands. The side chain of each residue was shortened to keep only those hydrogen-bonded interactions with the ligand or adjacent residues. Additionally, each peptide bond was replaced with a C α -H bond to reduce the size of the QM model system.

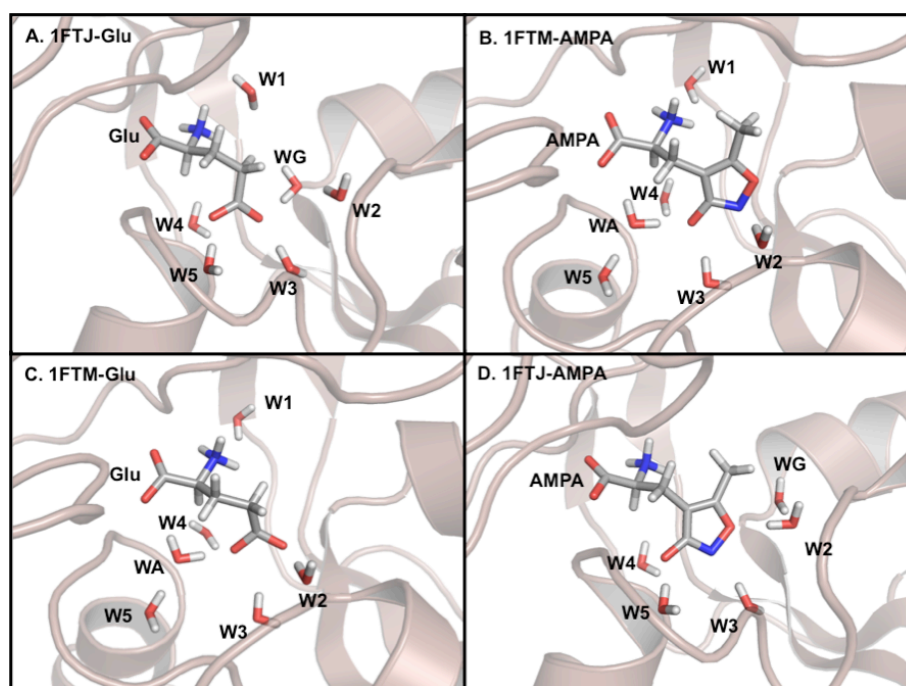


Figure 4.2 Cartoon illustrating the four model systems. (A) Glutamate in the 1FTJ structure. (B) AMPA in the 1FTM structure. (C) Glutamate in the 1FTM structure and (D) AMPA in the 1FTJ structure. All water molecules within 5Å of the ligand are shown explicitly.

The final QM models consisted of 357 atoms for 1FTJ-Glu and 1FTM-Glu, 358 atoms for 1FTJ-AMPA and 361 atoms for 1FTM-AMPA. As such each model has 23 residues, the ligand and 6 water molecules, except 1FTJ-AMPA, which only maintained 5 water molecules within 5 Å from the ligand after the energy minimisation step.

Each structure was initially optimised using the *ab initio* Restricted Hartree Fock (RHF) method (Roothan, 1951) with the 3-21G split valence basis set (Ditchfie et al., 1971; Hehre et al., 1972; Hariharan and Pople, 1973). To take into account the partially constraining effect of the protein environment on the active-site geometry, during the geometry optimisation procedure the positions of the alpha carbons were kept “frozen” and the bonded hydrogen atoms used to emulate the missing peptide bond. Also, for contiguous amino acidic residues the positions of atoms involved in the peptide bond were frozen.

The RHF/3-21G geometry provided structural parameters that were then used as the input in a subsequent theoretical refinement step with the inclusion of electron correlation effects to obtain more reliable energy. These single point density functional theory calculations were carried out with the Gaussian ‘03 program (Frisch et al., 2004). Both the B3LYP (Lee et al., 1988; Becke, 1993) and the MPW1B95 (Zhao and Truhlar, 2004; 2005) functionals were used.

Interaction energies were calculated for a series of fragments that form the major components of the binding pocket (waters, protein and ligands). The interaction energies for any two fragments can systematically be corrected for by the basis set superposition

error (BSSE) using the function counterpoise method of Boys and Bernardi (Boys and Bernardi, 1970). As such, the interaction energies can be described as in equation 4.1.

$$\Delta E_{\text{Interaction}} = \Delta E_{\text{Total}} - (\Delta E_{\text{Fragment 1}} + \Delta E_{\text{Fragment 2}}) \quad (4.1)$$

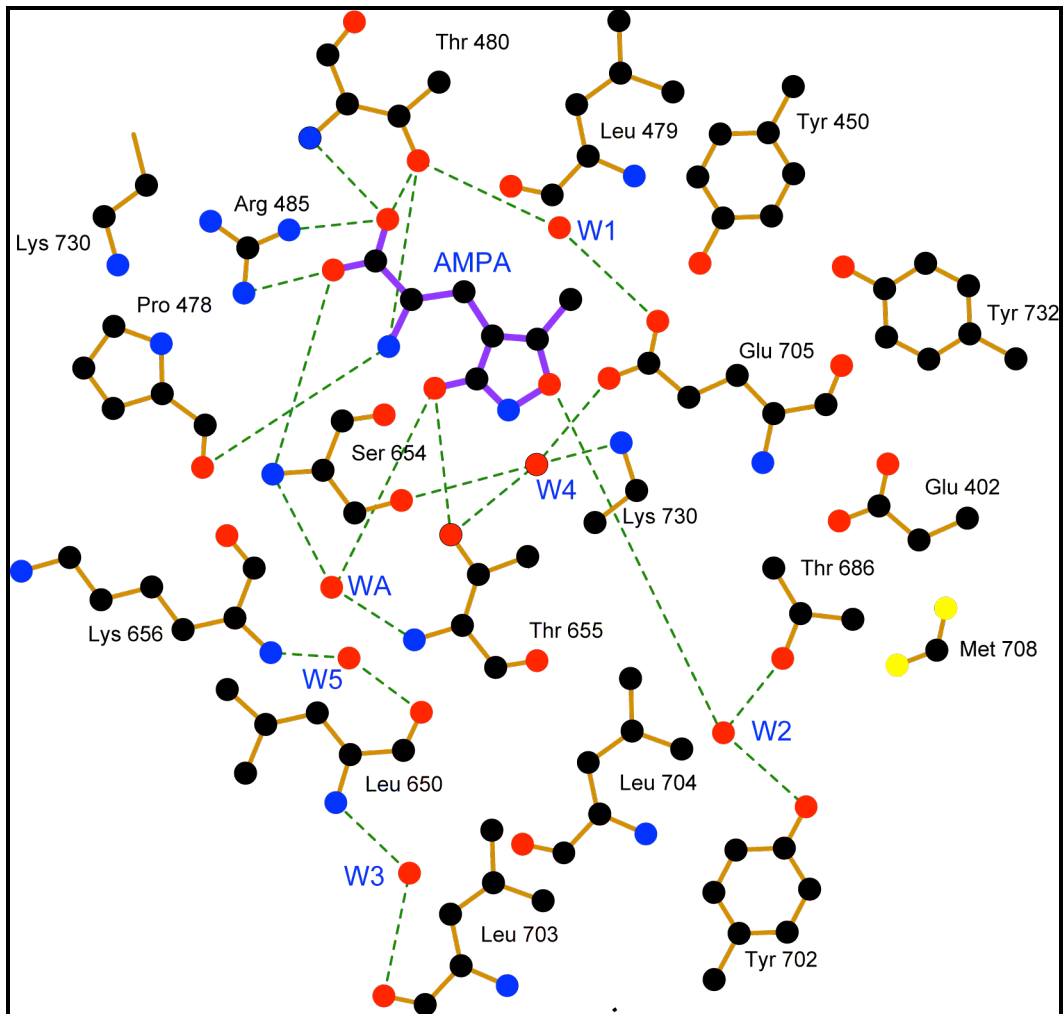


Figure 4.3 Ligplot diagram of the isolated QM region used in the density functional calculations for 1FTM-AMQ. This representation includes the 23 interacting residues from the GluA2 receptor (Glu402, Tyr450, Pro478 - Thr480, Arg485, Gly648 - Lys656, Thr686, Tyr702 - Glu705, Met708, Lys730 and Tyr732), six water molecules (WA, W1-W5) and the ligand. The ligand shown in this representation is AMPA and similarly for 1FTJ-AMQ. The ligand is glutamate in 1FTM-Glu and 1FTJ-Glu.

4.3 Results

4.3.1 Basis set choice

It is important to first assess the influence of different levels of theory on the interaction energies. As such, two water molecules (WG and W5) were selected from the 1FTJ-Glu system (see Figure 4.2A), and the interaction energies were calculated with the MPW1B95 and B3LYP methods (Table 4.1). The MPW1B95 (Zhao and Truhlar, 2004; 2005) meta-hybrid method was first used in conjunction with three different basis sets. These included the more mathematically complete 6-31G* and 6-31G** basis sets (Petersson et al., 1988; Petersson and Allaham, 1991) as well as the locally dense basis set (LDBS) (DiLabio and Wright, 1998) approach, where the model-system can be partitioned into two very different regions, which are assigned basis sets of different accuracy. Specifically, the atoms directly involved in the formation of hydrogen bonds (including the ligand and water molecules) were described by the 6-31G* basis set. The 3-21G (Ditchfie et al., 1971; Hehre et al., 1972; Hariharan and Pople, 1973) basis set was chosen for all remaining atoms. The basis set superimposition error (BSSE) was found to be the smallest at the MPW1B95/6-31G** level of theory with comparable interaction energy values for each water molecule (Table 4.1). The B3LYP/6-31G** level of theory was also investigated and in this context the BSSE is larger than the MPW1B95/6-31G** level of theory. However, although reportedly less accurate in its description of hydrogen-bond interactions than MPW1B95 (Zhao and Truhlar, 2004; 2005) it still offers a good compromise between computational expedience and reliability of the values for

BSSE. Thus the B3LYP/6-31G** level of theory was used for the series of calculations for all fragments as detailed in Table 4.2.

4.3.2 Ligand-protein interaction energies

Analysis of the ligand to protein+water interaction energies (L-WsP cells, Table 4.2) reveals that glutamate is much more favourable in its crystallographically observed mode compared to AMPA in its crystal structure (1FTM-AMPA). Comparing glutamate in alternative binding poses, the L-WsP interaction energy for 1FTJ-Glu ($-380.13 \text{ kcal mol}^{-1}$) is considerably more favourable than 1FTM-Glu ($-304.81 \text{ kcal mol}^{-1}$). However, the situation is not simply reversed for AMPA; in this instance the energies of interaction between AMPA and protein are similar in both modes of binding. The L-WsP interaction energy for 1FTM-AMPA ($-327.93 \text{ kcal mol}^{-1}$) is slightly less favourable than for 1FTJ-AMPA ($-329.53 \text{ kcal mol}^{-1}$). Thus glutamate clearly has a strong preference for a binding mode, but AMPA it would seem is energetically capable of adopting a ‘glutamate-binding’ pose.

Table 4.1 Interaction energies with counterpoise correction (ΔE_{CP} in kcal mol⁻¹ and Basis Set Superposition Error (BSSE) for 2 specific water molecules (WG and W5) in the binding site of 1FTJ-Glu system at four different levels of theory.

Water	MPWB1K/gen [†]		MPWB1K/6-31G*		MPWB1K/6-31G**		B3LYP/6-31G**	
	ΔE_{CP}	BSSE	ΔE_{CP}	BSSE	ΔE_{CP}	BSSE	ΔE_{CP}	BSSE
WG	-14.81	8.39	-15.71	7.70	-15.91	7.61	-12.29	9.26
W5	-12.76	7.72	-13.68	7.22	-13.89	7.16	-9.49	8.74

[†]gen refers to the locally dense basis set (LDBS) approach.

Table 4.2 Fragment interaction energies of waters and ligand in each QM system in kcal mol⁻¹. Fragments are defined as follows: WX; waters where X is 1-5 or A or G as described in Figure 4.2. Ws; all other waters (eg. for the W1-Ws cell for 1FTJ-Glu, Ws would mean W2, W3, W4, W5 and WG), L; ligand (AMPA or Glu), P; protein, LP; ligand and protein, WsL; waters and ligand, WsP; waters and protein, WsLP; waters and ligand and protein.

Fragment	Ws	L	P	LP	WsL	WsP	WsLP
1FTJ-GLU							
W1	0.09	1.98	-57.13	-13.18	2.10	12.26	-3.65
W2	-1.90	-3.22	3.56	-34.21	-7.04	-0.67	-29.05
W3	0.42	4.43	-61.78	-29.15	4.65	-52.58	-23.18
W4	0.11	5.27	-83.72	-22.52	5.34	-38.83	-21.79
W5	-0.12	-9.43	-52.17	-15.27	-8.62	5.14	-14.87
WG	-4.00	-13.28	-45.08	-3.10	-19.37	-15.19	-7.87
L	-	-	-	-	-	-380.13	-
1FTJ-AMQ							
W1	-	-	-	-	-	-	-
W2	-0.37	-3.79	-24.21	-26.85	-3.41	-30.48	-30.47
W3	-0.50	-9.42	-6.16	-12.72	-8.84	13.35	-18.01
W4	0.24	5.65	-47.94	-25.44	5.73	-12.32	-24.56
W5	0.50	-10.33	61.64	-16.48	-9.02	24.28	-14.87
WG	0.93	-5.80	-11.21	-13.35	-3.89	-81.47	-9.75
L	-	-	-	-	-	-329.53	-
1FTM-AMQ							
W1	0.41	-1.43	2.09	-4.69	0.03	-20.95	4.21
W2	0.27	-2.28	-0.27	-37.59	-1.36	-24.21	-37.21
W3	1.69	-13.68	-9.27	-18.05	-9.74	10.59	-14.65
W4	0.07	4.04	-49.34	-20.69	4.69	-26.70	-19.55
W5	-2.17	-3.56	-4.90	-5.92	-11.23	-12.12	-11.76
WA	0.30	-11.85	0.60	-5.58	-15.70	18.39	-6.26
L	-	-	-	-	-	-327.93	-
1FTM-GLU							
W1	-0.85	1.49	-10.07	-8.36	0.55	-10.95	-1.26
W2	0.77	-2.64	-20.55	-21.54	-1.15	-20.34	-20.40
W3	1.59	-10.60	-7.82	-13.62	-7.08	-30.77	-10.70
W4	-0.83	2.68	-53.96	-22.96	2.82	-51.65	-23.68
W5	-2.54	-3.00	-5.30	-5.60	-11.00	-7.11	-12.03
WA	-0.05	-11.54	2.11	-3.62	-16.21	4.13	-5.59
L	-	-	-	-	-	-304.81	-

Table 4.3 Basis Set Superimposition Error Energies for water and ligand fragments in each optimised QM system in kcal mol⁻¹. Fragments are defined as follows: WX; waters where X is 1-5 or A or G as described in Figure 4.2. Ws; all other waters (eg. for the W1-Ws cell for 1FTJ-Glu, Ws would mean W2, W3, W4, W5 and WG), L; ligand (AMPA or Glu), P; protein, LP; ligand and protein, WsL; waters and ligand, WsP; waters and protein, WsLP; waters and ligand and protein.

Fragment	Ws	L	P	LP	WsL	WsP	WsLP
1FTJ-GLU							
W1	0.00	0.61	6.47	6.53	0.60	6.48	15.47
W2	2.28	0.12	8.05	9.35	3.17	8.66	9.41
W3	0.73	0.14	8.86	9.16	0.86	9.06	8.92
W4	0.00	0.77	9.33	8.72	0.72	9.28	8.67
W5	0.08	1.97	8.06	8.12	1.81	7.88	8.11
WG	1.75	4.51	6.18	6.07	6.15	7.16	7.53
L	-	-	-	-	-	32.18	-
1FTJ-AMQ							
W1	-	-	-	-	-	-	-
W2	2.21	1.09	6.63	7.60	2.87	7.77	8.71
W3	0.65	1.49	6.58	7.63	2.03	8.10	7.95
W4	0.00	0.55	10.22	9.32	0.53	10.01	9.28
W5	0.03	2.24	7.77	8.31	2.06	7.69	8.17
WG	1.59	1.49	4.26	5.39	3.02	4.31	6.67
L	-	-	-	-	-	89.40	-
1FTM-AMQ							
W1	0.00	0.59	16.42	3.24	0.40	-15.17	10.76
W2	0.00	0.68	8.39	9.64	0.61	8.79	9.72
W3	0.12	3.09	7.32	7.94	2.57	12.24	7.62
W4	0.00	0.14	8.41	7.24	0.13	8.24	7.16
W5	1.57	0.14	7.17	7.27	2.49	7.84	8.01
WA	1.74	5.72	6.55	8.85	6.54	7.40	9.46
L	-	-	-	-	-	38.97	-
1FTM-GLU							
W1	0.01	0.35	5.98	5.91	0.33	8.90	13.85
W2	0.05	0.29	7.55	7.95	0.32	8.10	8.04
W3	0.20	2.78	7.32	8.13	2.37	7.39	7.95
W4	0.01	1.13	8.51	7.63	0.94	8.24	7.60
W5	1.57	0.09	7.71	7.69	2.51	11.86	8.57
WA	1.78	5.07	6.35	8.28	6.28	7.20	9.08
L	-	-	-	-	-	41.79	-

4.3.3 Water interaction energies

Given the central role of water molecules in these binding poses the interaction energies of the water molecules were investigated. Figures 4.4-4.7 describe the water interactions in the four models including the “pharmacophoric water”, which is the water that appears to act as a surrogate part of the ligand in either the glutamate (WG, see Figure 4.2A and 4.2D) or AMPA (WA, see Figure 4.2B and 4.2C) complexes. Before an in-depth analysis one might expect that the interaction energy of WG or WA would be more favourable in the poses adopted in the crystal. However, the interaction energy of the pharmacophoric water with the remainder of the system (WA-WsLP and WG-WsLP cells in Table 4.2) presents a different perspective of what is observed in the crystal structures. Regardless of the ligand bound WA appears to be iso-energetic with a $0.67 \text{ kcal mol}^{-1}$ difference between the glutamate-bound ($-5.59 \text{ kcal mol}^{-1}$) mode and the AMPA-bound ($-6.26 \text{ kcal mol}^{-1}$) mode. In the 1FTJ-based models, WG appears to also be iso-energetic albeit with a slightly greater preference of $1.88 \text{ kcal mol}^{-1}$ for the AMPA-bound ($-9.75 \text{ kcal mol}^{-1}$) mode than the glutamate-bound ($-7.87 \text{ kcal mol}^{-1}$) mode. One might expect these to be exactly isoenergetic, but the optimisations are done in the presence of ligands and thus there will be some small effects on the final positions of the protein atoms dictated by the ligand. In the 1FTJ-based models, WG forms hydrogen bonds (Figure 4.4A and 4.5A) with the backbone NH group of Glu705, the water W2 and an oxygen atom from the ligand (one of the carboxylate oxygens for Glu, and the ring oxygen for AMPA). In the 1FTM-based models, WA forms hydrogen bonds (Figure 4.6A and 4.7A) with the OH group of Ser 654, the backbone NH of Thr 655, W5 and two oxygen atoms from the ligand in each case.

The detailed breakdown of how this water interacts with the various components of the binding pocket (the WG or WA rows in Table 4.2) shows that the overall interaction energy (WG/WA-WsLP) does not arise from the contribution of the ligand solely but instead appears to be related to the whole environment of the system in which each ligand is bound. Table 4.2 also demonstrates that the fragments are clearly non-additive in nature. WG appears to have favourable interaction energies with all fragments. However, the interaction of WA with protein alone appears to be non-favourable.

W1 was found to have the least favourable interaction energies in all calculations, and indeed in none of the models does W1 make a hydrogen bond with the ligand. W2 is most energetically favourable in the 1FTM-AMPA system. W2 makes contacts with Tyr 702 and Thr 686 in all models. The pattern of interaction energies relates directly to the number and geometry of the hydrogen bonds formed. W3 makes hydrogen bonds with Leu 650 and Leu 730 in all four models (Figure 4.4-4.7D), and a third hydrogen bond to the ligand. The interaction energy (Table 4.2) is most favourable in the 1FTJ-Glu mode and is a result of an optimal arrangement of both ligand and protein.

W4 was found to maintain comparable interaction energies across the models, which could be associated with the fact that it forms a tetrahedral arrangement with mostly charged side chain of the residues; Glu 705, Lys 730, Ser 654 and Thr 655 in all four models except 1FTM-Glu (Figure 4.7E) which only makes three hydrogen bonds with Lys 730, Ser 654 and Thr 655. This water does not form any direct interaction with the ligands and occupies a well defined site apparently serving a structural role, presumably helping to define the shape of the binding pocket.

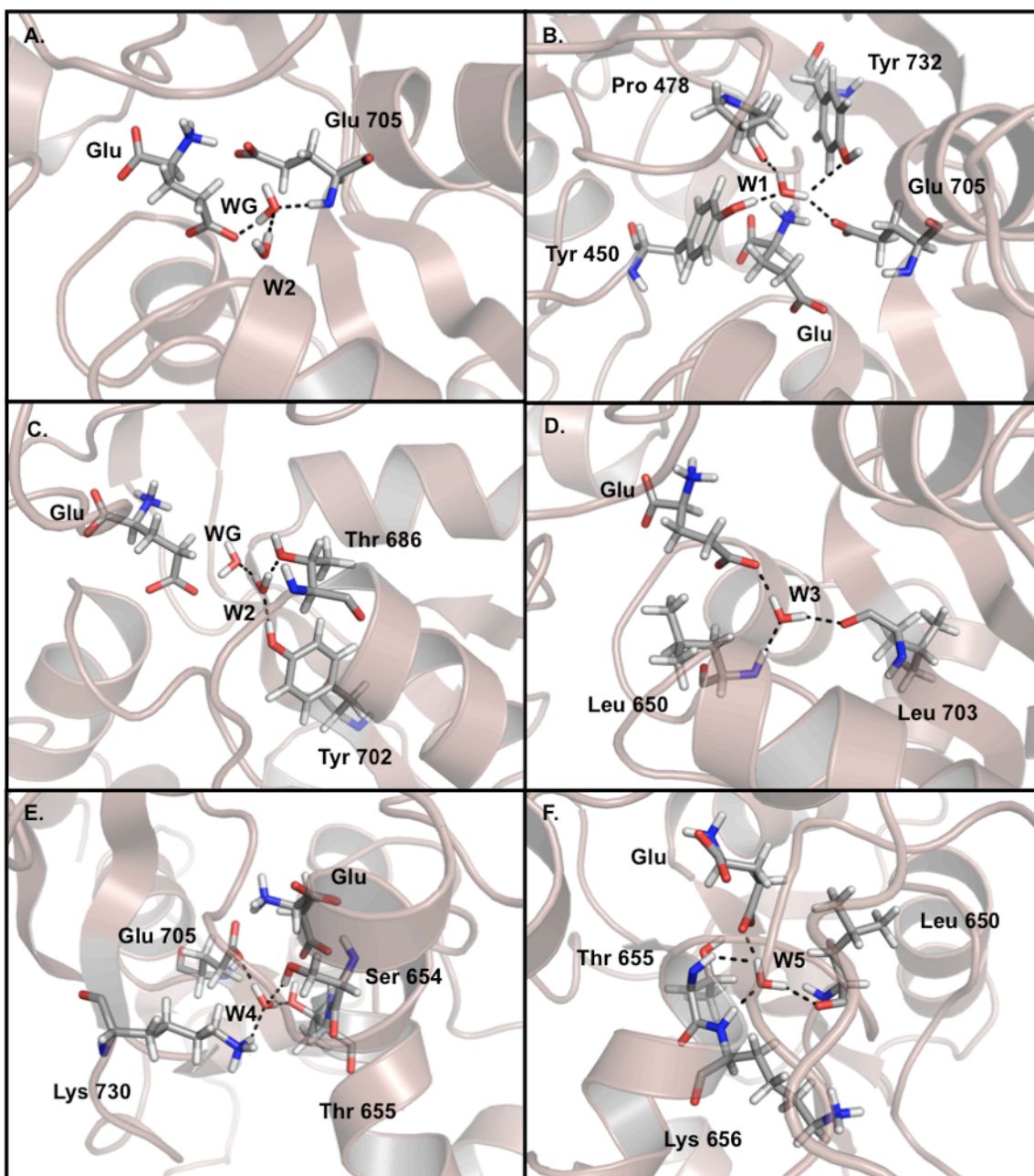


Figure 4.4 Hydrogen bond interactions for individual water molecules for the 1FTJ-Glu model system (A) WG, (B) W1, (C) W2, (D) W3, (E) W4 and (F) W5. All protein residues, ligand and water molecules that interact with WG and W1-W5 are labelled.

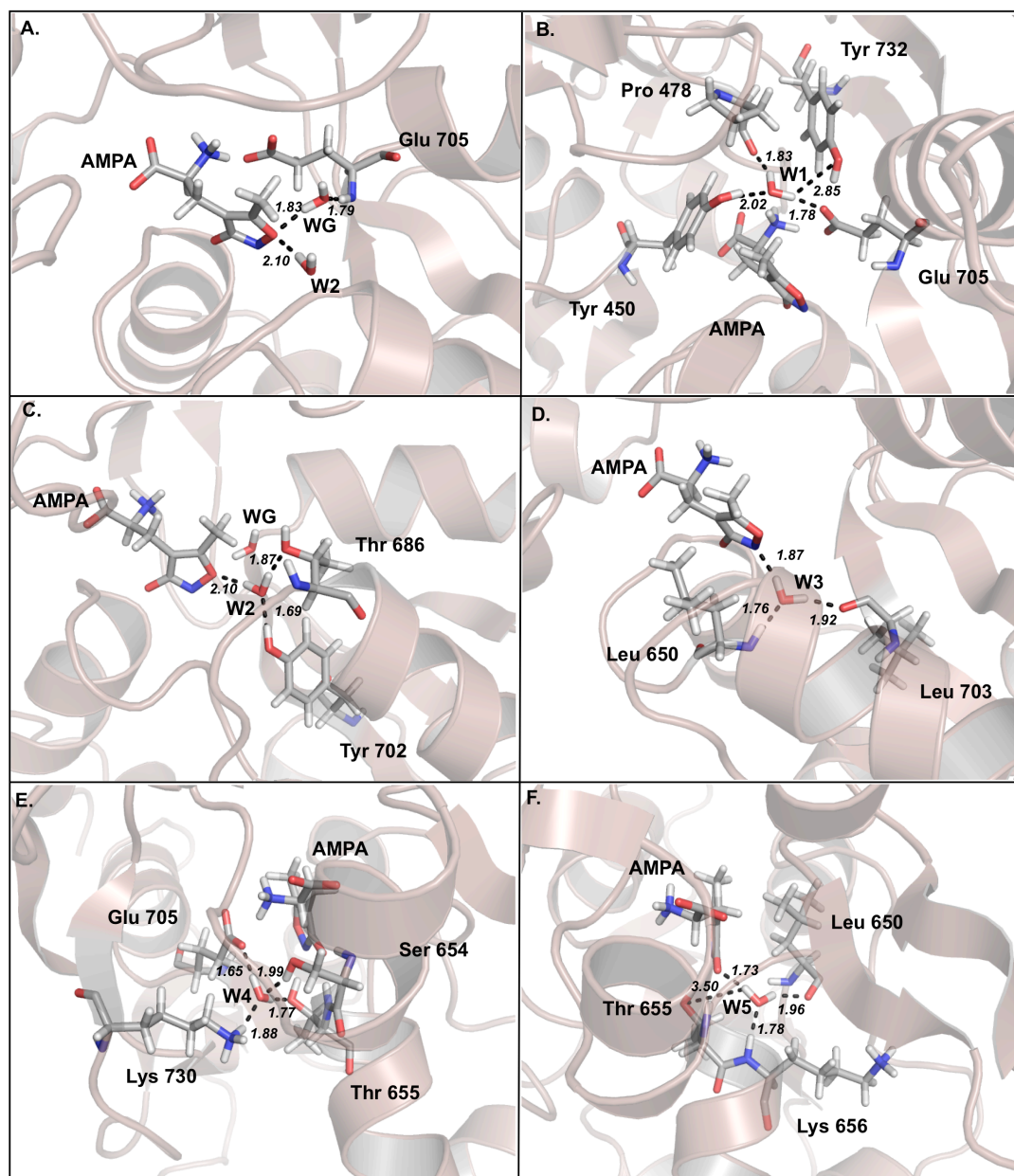


Figure 4.5 Hydrogen bond interactions for individual water molecules for the 1FTJ-AMPA model system (A) WG, (B) W1, (C) W2, (D) W3, (E) W4 and (F) W5. All protein residues, ligand and water molecules that interact with WG and W1-W5 are labelled.

W5 is another water molecule that occupies a well defined site and is also a well conserved water molecule, found in the majority of published high-resolution ligand-bound iGluR structures. In the 1FTJ derived models (Figures **4.4F** and **4.5F**) W5 makes four direct hydrogen bonds to the ligand, Leu 650, Thr 655 and Lys 655 and has an identical interaction energy of $-14.87 \text{ kcal mol}^{-1}$ regardless if glutamate or AMPA is bound. In the 1FTM derived models W5 can also be considered iso-energetic with a $0.27 \text{ kcal mol}^{-1}$ between the glutamate-bound ($-11.76 \text{ kcal mol}^{-1}$) mode and the AMPA-bound ($-12.03 \text{ kcal mol}^{-1}$) mode. It does not make any direct hydrogen bond with the ligand but instead makes a hydrogen bond with WA. W5 makes three further hydrogen bonds with Leu 650 and Lys 656 in 1FTM-AMPA (Figure **4.6F**) and with the backbone carbonyl of Leu 650, backbone NH of Lys 656 and backbone NH of Gly 653 in 1FTM-Glu (Figure **4.7F**).

4.3.4 Polarisation

A method commonly employed to calculate partial charges for compounds that are not already parameterised in modern force-fields is to use the restricted electrostatic potential (RESP) fitting method (Bayly et al., 1993). RESP calculations were performed on the ligands within the four models in order to assess to what extent polarisation from the environment in the different binding modes might be a factor. The results are shown for the four models in Figure **4.8**. The calculations show that there is reasonable agreement at the amino and carboxylic acid groups in all four systems (which is to be expected). The greatest variation is observed at the OE1 and OE2 atoms of Glu, which have an asymmetric charge distribution, presumably reflecting the local environment in each case

(WG or WA). In contrast there is much less variation observed at the OE2 (or OE1) atoms of AMPA in 1FTM or 1FTJ.

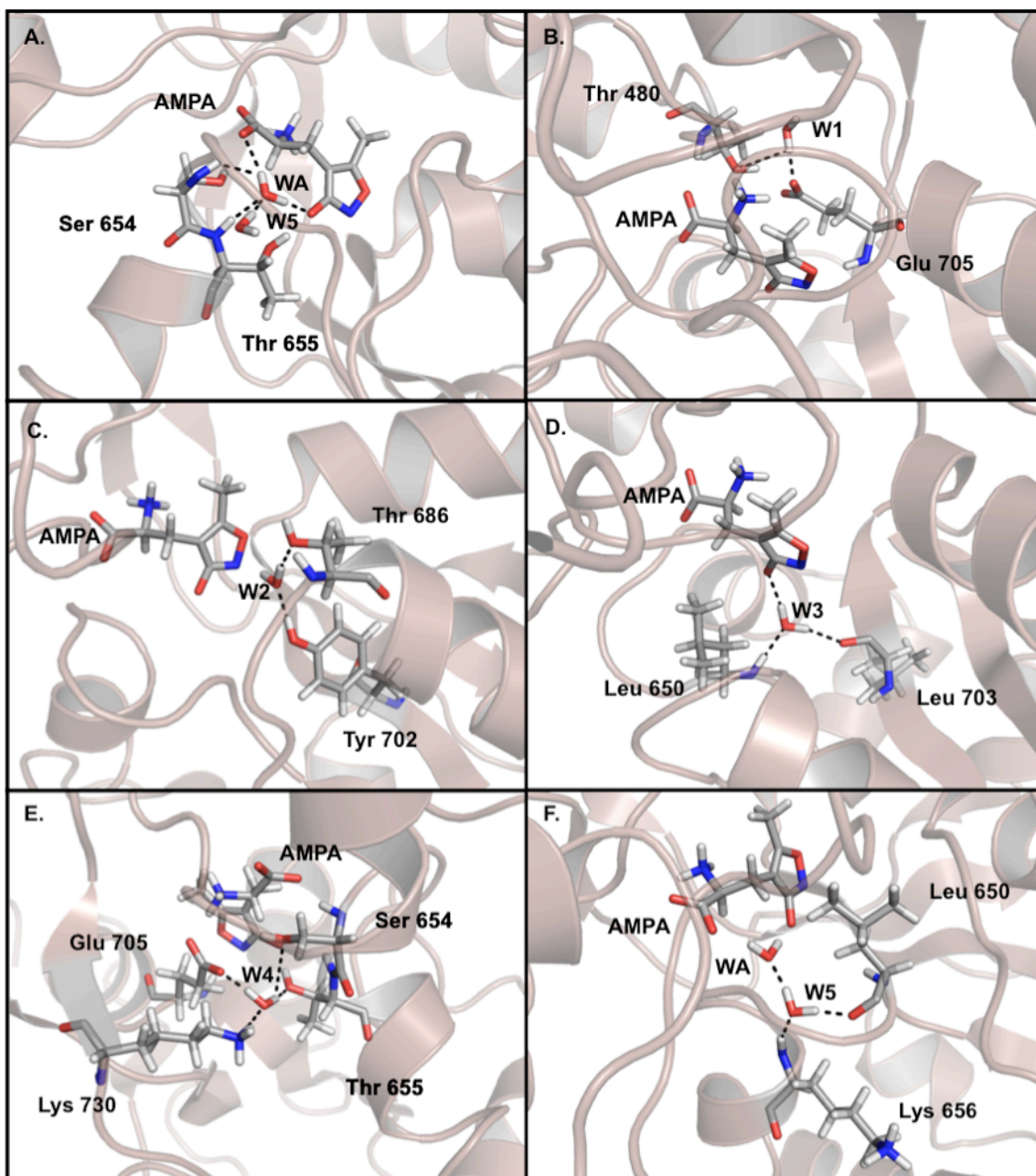


Figure 4.6 Hydrogen bond interactions for individual water molecules for the 1FTM-AMPA model system (A) WA, (B) W1, (C) W2, (D) W3, (E) W4 and (F) W5. All protein residues, ligand and water molecules that interact with WA and W1-W5 are labeled.

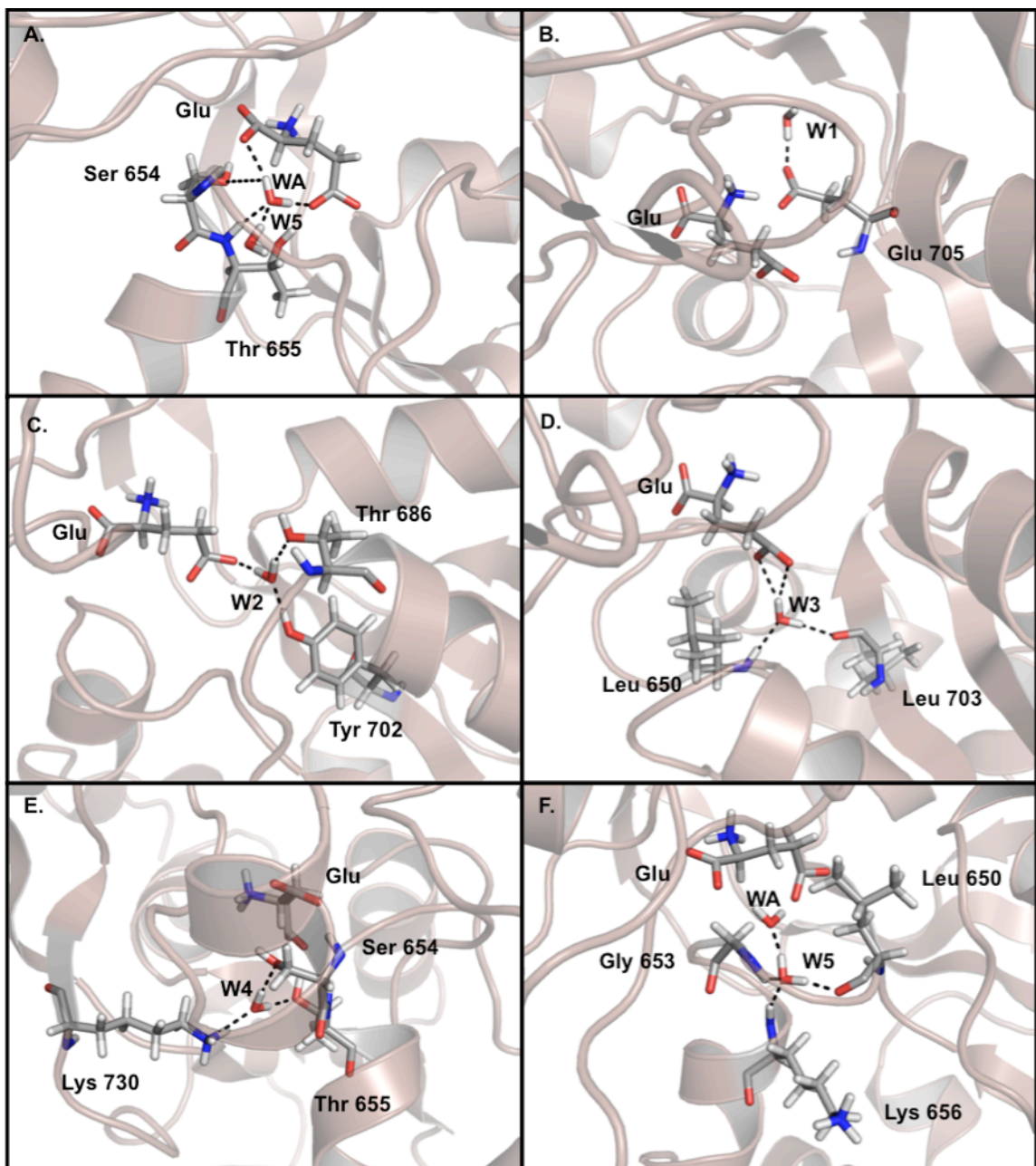


Figure 4.7 Hydrogen bond interactions for individual water molecules for the 1FTM-Glu model system (A) WA, (B) W1, (C) W2, (D) W3, (E) W4 and (F) W5. All protein residues, ligand and water molecules that interact with WA and W1-W5 are labelled.

4.3.5 Internal energies

The internal energies of the different fragments were also examined as isolated fragments in the gas phase. Differences between the internal gas phase energies for the water molecules were all under $0.25 \text{ kcal mol}^{-1}$. The results for the ligands suggest that gas phase internal energy of glutamate is raised by $13.35 \text{ kcal mol}^{-1}$ when placed in the AMPA-binding mode (1FTM-Glu system). Similarly, when AMPA is positioned in the glutamate binding mode, the internal energy of AMPA rises by $8.19 \text{ kcal mol}^{-1}$. When using the Polarizable Continuum Model (PCM) of solvent with a dielectric $\epsilon=10$ (Miertus et al., 1981), the internal energy for glutamate in the 1FTM derived system is only $0.15 \text{ kcal mol}^{-1}$ higher than the glutamate conformation observed crystallographically in 1FTJ. However, AMPA in 1FTJ is still $2.06 \text{ kcal mol}^{-1}$ more unfavourable compared to AMPA in 1FTM, suggesting that internal strain is a considerable factor in dictating the binding mode of AMPA, but is not a contributing factor to the glutamate mode of binding.

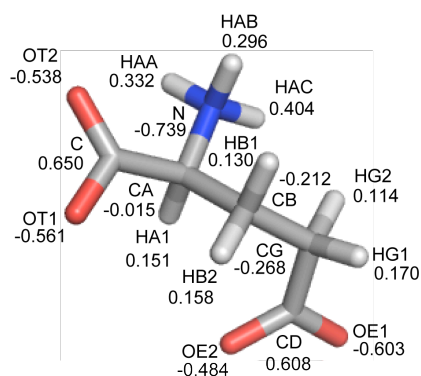
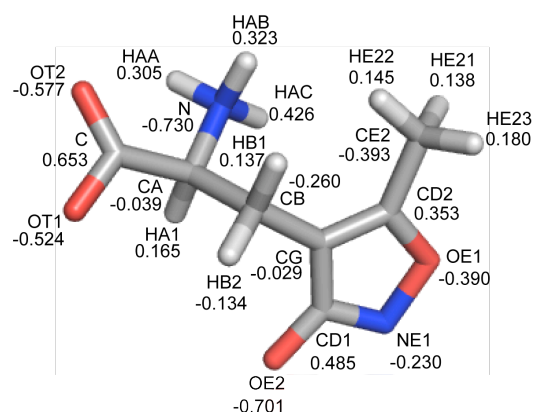
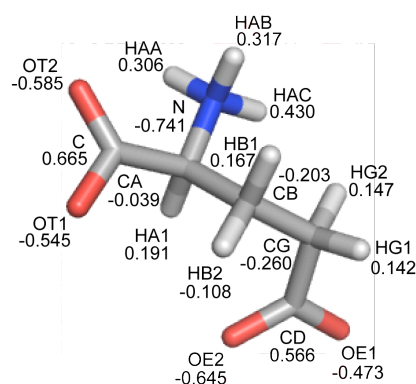
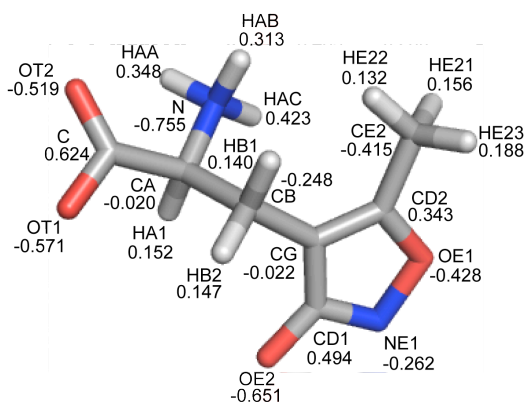
A. 1FTJ-Glu**B. 1FTM-AMPA****C. 1FTM-Glu****D. 1FTJ-AMPA**

Figure 4.8 Comparison of the resulting partial charge on atoms using the RESP (Bayly et al., 1993) method (A) Glutamate in 1FTJ structure. (B) AMPA in the 1FTM structure. (C) Glutamate in the 1FTM structure and (D) AMPA in the 1FTJ structure. The overall charges are similar except at the carboxyl and amino terminal ends where interactions with protein residues and waters take place.

4.4 Discussion

The crystallographically determined binding pose of AMPA was rather contrary to expectation. Prior to structural knowledge it was anticipated that the 3-oxyanion and ring nitrogen of AMPA would superimpose on the γ -carboxylate oxygens of glutamate. Instead a water molecule occupies the position of one of the carboxylate oxygen atoms (WA in Figure 4.2B). Similarly, the position of ring nitrogen in the GluA2-glutamate complex is also occupied by a water molecule (WG in Figure 4.2A). In this sense this water molecule can be considered as part of the ligand in each case. However it is not clear why this water molecule should preferentially occupy different sites with respect to these ligands and hence why the resulting binding pose for AMPA differs from glutamate. Previous docking studies have shown that consideration of this water molecule is essential to retrieve the correct binding pose for AMPA and that omission of this water molecule leads to a docking pose that overlays with the glutamate-GluA2 complex (Greenwood and Liljefors, 2004). In the context of drug-design, there has been a lot of work aimed at determining whether water molecules in a binding site can be displaced by modifications to existing ligands. Indeed (RS)-2-amino-3-(3-carboxy-5-methyl-4-isoxazolyl) propoionic acid (ACPA), an analogue of AMPA where the 3-oxyanion moiety is replaced by a carboxylic acid, interacts directly with the side-chain OH of Thr 655 (Hogner et al., 2002). ACPA has both a lower IC_{50} ($IC_{50} = 20$ nM compared to 79 nM for AMPA) (Madsen and Wong, 1992) and lower EC_{50} ($EC_{50} = 0.74$ μ M (Hogner et al., 2002) compared to 3.5 μ M for AMPA (Madsen et al., 1993). The improvement may be more modest than expected as ACPA loses an additional water

interaction that AMPA makes, the overlay is not quite perfect and there are small differences in the binding pose and the conformation of binding pocket residues.

In this chapter the aim was to examine to what extent the observed differences in binding orientation between AMPA and glutamate could be attributed to stabilisation effects of water molecules and how the water molecules interact with the protein and/or ligand. Because polarisation effects may be critical to this analysis *ab-initio* calculations were used to calculate the interaction energies between the various components of the binding site.

These calculations show that a simple interaction energy calculation between the ligand and the rest of the system is sufficient to be able to predict the correct mode of binding for glutamate, but not for AMPA. Clearly, entropic effects, which are neglected in these calculations, must play a more dominant role in the case of AMPA.

Table 4.2 shows that the “pharmacophoric water” (WG/WA) has no energetic preference for the ligand (glutamate or AMPA) it was crystallised with. The interaction energies in each case are essentially iso-energetic pointing to the possibility that the binding mode must be due to more than the presence of the ligand alone. One contributing factor is polarisation effects which do not appear to be pairwise additive for these systems. One notable example is the clearly defined W5 site which influences the binding of the ligand (Vijayan et al., 2010). Although the interaction energies are iso-energetic for W5-WsLP, the breakdown across each subtype is different in each case. The results here suggest that consideration of the binding pocket in the absence of ligand(s) may not always give the correct answer without taking into consideration the

significant effects from polarisation. This is a particularly important aspect in the context of drug design as there is increasing interest in being able to predict how readily water molecules in the active site are displaced (Michel et al., 2009a).

Docking programs often have an energetic term that accounts for the internal or strain energy of the ligand and indeed this can be a contributing factor to the binding pose. The most useful measure of this strain energy though is the comparison between binding modes and in solvent. These calculations showed that the differences in internal energy for the glutamate conformations using the PCM solvent model are very small ($0.15 \text{ kcal mol}^{-1}$). Previous calculations of glutamate in solution suggest that the energy difference between two minima for glutamate in solution may be as small as $0.1 \text{ kcal mol}^{-1}$ and that the conformation in the crystal structure is intermediate between these two minima (Odai et al., 2003). Thus internal strain is not likely to be a factor for dictating the pose that glutamate adopts. On the other hand, the results for AMPA suggest that internal strain could be a contributing factor. Recently, Butler *et al.* (Butler et al., 2009) demonstrated via the use of *ab-initio* calculations, in conjunction with a solvent model, that bioactive (as observed by X-ray crystallography) conformations of ligands are very close to conformations expected in solution, suggesting that the best docking poses would impose little conformational strain energy on the ligand (less than $0.5 \text{ kcal mol}^{-1}$ in two thirds of the cases examined). The strain for AMPA in the 1FTJ is considerably above this level (at $2.06 \text{ kcal mol}^{-1}$). Together, the results support the notion that scoring functions must incorporate terms other than interaction energies in an accurate fashion if they are to be useful (Tirado-Rives and Jorgensen, 2006).

The results from the RESP calculations suggest that polarisation effects can be quite significant in the case of glutamate but less so for AMPA. The charge assignments in the region of the molecule where the environment is the same in the four systems (the amino and carboxyl groups) are in good agreement with each other suggesting that the differences observed for the glutamate side chain group are genuine and system dependent. The RESP method is one of the standard methods for calculating charges for ligands in force fields for which parameters are not already available. These calculations are usually performed in the gas phase. The variation in these charges shown in Figure 4.8 demonstrates that this may potentially be a source of error if simple point charges are used to evaluate electrostatic contributions in molecular mechanics or docking approaches. Large, conformationally-dependent variation in charge assignment for glutamate has been observed before; Odai et al reported the variation in Mulliken charges for four distinct conformations of glutamate in solution (Odai et al., 2003). More systematic work is clearly required in this area to assess how significant these effects are in general.

4.5 Conclusion

This chapter has shown how various components of the binding site of GluA2 contrive to dictate the binding mode for glutamate and AMPA. Simple ligand-system interaction highlight that for glutamate there is very little internal strain energy for the different binding modes and that it could readily adopt any one of those conformations. The water, W5, which could be considered part of the ligand or part of the protein, appears to

be energetically favourable regardless of the ligand present (and indeed appears slightly more favourable when AMPA is bound). Although simple interaction energies of ligands and waters could partly account for the observed glutamate-binding mode, they do not appear to account for the crystallographically observed binding mode of AMPA. Although there is more internal strain energy of AMPA in the 1FTJ-AMPA system, the overall interaction energy of AMPA with the system is more favourable (than in 1FTM). Thus, there must be other factors, which control the pose of AMPA; most likely an entropic contribution, which is not considered here. Although interaction energies can go some way to providing insight into what factors are controlling binding, they do not provide a complete picture.

There are many reasons why compounds may adopt different binding modes within a receptor, ultimately dictated by the free energy of binding, but with many different contributions. The problem of computing that free energy is complex because often the difference in energy that dictates different binding poses is a very small difference between two very large numbers. Thus we must strive to be as accurate as possible if we are to make progress on this front. An accurate consideration of the role of waters within the binding pocket is likely to be critical. These results suggest that for establishing trends, treatment at the B3LYP/6-31G** level is sufficient, but that absolute values are in general much lower than that reported for MPWB95/6-31G** for the two test cases discussed in Section 4.3.1.

Finally, the results also indicate that polarisation effects could mean that initial partial charge assignments based on gas phase calculations may lead to problems. More work is required to assess those effects in detail. Nonetheless, there is much work in

progress in developing polarisable models for molecular mechanics (Lopes et al., 2007)
so there is good reason to be optimistic on that front.

Chapter 5

Water Dynamics within the AMPA subtype of Glutamate Receptors: Combined QM and MD calculations

'Newton, forgive me.'

Albert Einstein

Abstract

This chapter focuses on the ligand binding core of three glutamate-bound iGluR structures from the AMPA subtype of the glutamate receptors, GluA2, GluA3 and GluA4. The recently published crystal structures of GluA3 and GluA4 bound to glutamate make this a possibility since only the LBD of GluA2 was previously available. The goal of this study is to compare the water dynamics in these receptors and to also provide any structural insight into protein dynamics. Firstly, a quantum mechanical study is performed to assess the nature of the energetic contribution of individual water molecules in the binding sites. The method developed in Chapter 4 is used here. Secondly, multiple MD simulations are performed on each structure. The MD simulations reveal that crystal water positions were reproduced and are comparable to those results from Chapter 3. GluA2 and GluA3 are comparable in their water and protein dynamics. However, results from the GluA4 structure show a difference in dynamics between the two chains of the dimer. Density functional theory calculations support these results by providing a series of simple interaction energies that are remarkably different from one chain to the next. Other observations include a methionine (M708 in GluA3 and M709 in GluA4) side chain flipping into a new conformation and the preservation of the arrangement of the water molecules in the binding site between the subtypes, despite the fact that the position of GluA2 Y702 is instead substituted by a phenylalanine in GluA3 (F702) and GluA4 (F703). The results from this study can provide further information into subtype specificity to aid in ligand design.

5.1 Introduction

Previously, only crystal structures of the LBD of GluA2 complexed with various agonists and antagonists were available for computational studies. It was only recently that the crystal structures of the AMPA receptor subtypes GluA3 (Ahmed et al., 2008) and GluA4 (Gill et al., 2008; Kasper et al., 2008) were determined. This has allowed the possibility of comparative computational studies across the AMPA subtype of glutamate receptors.

An alignment of amino acids comprising the ligand binding cores of GluA2, GluA3 and GluA4 is shown in Figure 5.1. 38 amino acids differ among all three subunits. Most of these residues are primarily either facing solvent or taking part in the formation of hydrophobic cores within both domains D1 and D2 (Kasper et al., 2008). Studies have shown that AMPA receptor agonists with different efficacy induce distinct conformations of the ligand binding core of these receptors by inducing the D1-D2 domain closure (Armstrong and Gouaux, 2000; Hogner et al., 2002; Ahmed et al., 2008; Gill et al., 2008; Kasper et al., 2008). These AMPA receptor agonists can be classified into two groups: AMPA related analogs (e.g. (S)-Br-HIBO or BRH (Coquelle, 2000; Kasper et al., 2008) and willardiine-like compounds (e.g. (S)-2-amino-3-(1,3,5,6,7-pentahydro-2,4-dioxo-cyclopenta[e]pyrimidin-1-yl) propionic acid ((S)-CPW399) (Campiani et al., 2001) (Figure 5.2). These compounds show a certain degree of selectivity for GluA1/GluA2 over GluA3/GluA4 and vice versa respectively. This range of selectivity may be due to the presence of a tyrosine in GluA2 (Tyr702), which is a phenylalanine (F) in GluA3 (Phe702) and GluA4 (Phe703) (Y/F in Figure 5.1). However, computational studies to investigate these differences are limited since the crystal structures of these compounds are not available for GluA3 and GluA4. At present

these proteins have only been complexed with the agonists glutamate and AMPA and the partial agonist kainate for GluA4.

As a result, this chapter focuses on the glutamate-bound structures of these receptors providing the groundwork for comparative protein dynamics as well as investigating the water-mediated interactions present within the LBD. Firstly, a QM description of the interaction energies within each LBD is used to characterise any water-mediated interactions. Following that a standard MD study will attempt to compare the protein dynamics in these receptors and provide any additional information into water dynamics through density calculations.

The conservation scoring is performed by PRALINE.

The scoring scheme works from 0 for the least conserved alignment position, up to 10 for the most conserved alignment position.

The colour assignments are:

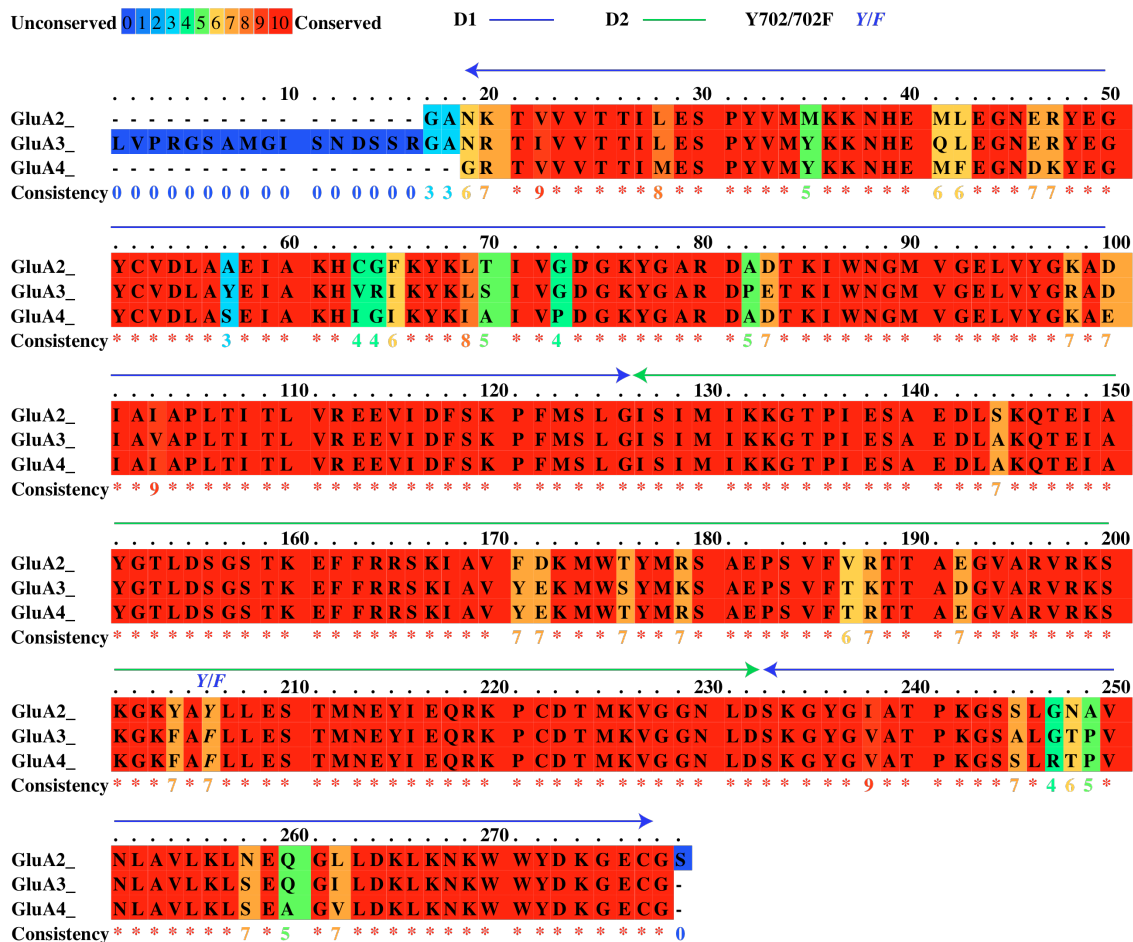


Figure 5.1 Multiple sequence alignment of the full length receptors of GluA2 (PDB ID: 1FTJ); GluA3 (PDB ID: 3DP4) and GluA4 (3FAS). Domain 1 is shown between blue lines while domain 2 is shown between green lines. The tyrosine residue at position 106 (Y/F) corresponds to Y702 in GluA2 or F702 in GluA3 and F703 in GluA4. The sequence alignment was performed using the PRALINE web interface (<http://www.ibi.vu.nl/programs/pralinewww/>).

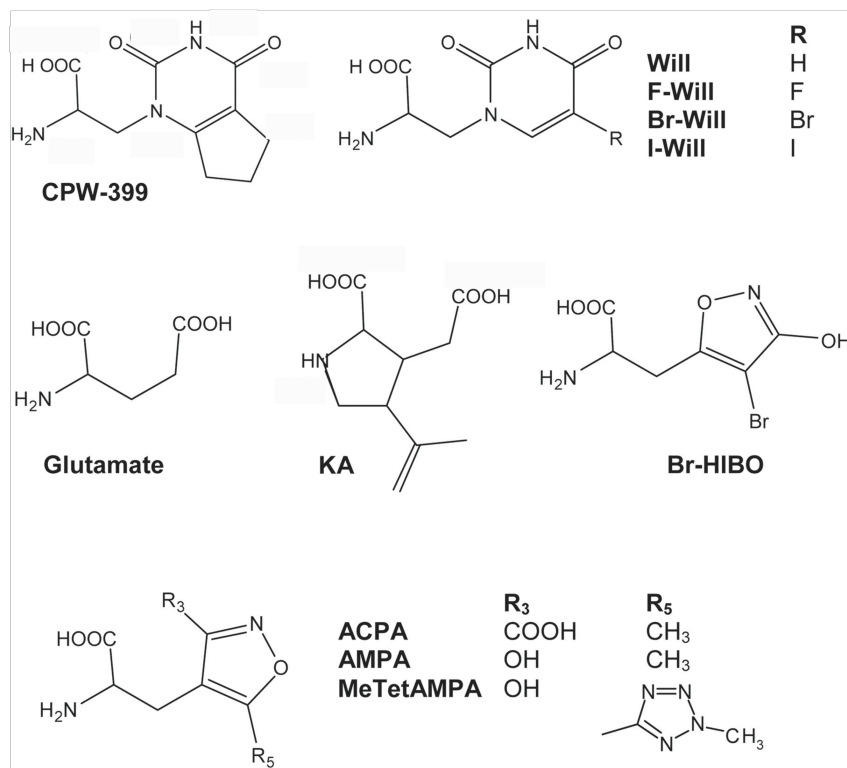


Figure 5.2 Chemical structures of AMPA receptor agonists. See abbreviations for compound names. Adapted from (Frandsen et al., 2005).

5.2 Methods

5.2.1 Calculations performed

Table 5.1 gives a brief outline of the calculations performed using the two computational methods (MD and QM) in this chapter for the three receptors GluA2 (Armstrong and Gouaux, 2000), GluA3 (Ahmed et al., 2008) and GluA4 (Kasper et al., 2008). For GluA2 bound to glutamate the MD results were taken from **Chapter 3**.

Table 5.1 iGluR structures bound to glutamate that were used in the calculations.

Subtype	PDB ID	Resolution (Å)	Ligand	Oligomerisation State
GluA2 [§]	1FTJ	1.90	Glutamate	Dimer
GluA3	3DLN	1.91	Glutamate	Monomer
GluA4	2FAS	1.40	Glutamate	Dimer

5.2.2 Quantum mechanical calculations

The model-systems were assembled using the high-resolution crystallographic structures of the GluA2, GluA3 and GluA4 ligand binding core bound with glutamate as listed in Table 5.1. The method was similar to the one in **Chapter 4** without the optimisation step prior to the single point calculation of the interaction energies. This offers a good compromise between computational expedience and reliability of the QM values and was

[§] The MD simulation results are those reported in **Chapter 3** for the second set of calculations.

tested prior to those optimised results reported in **Chapter 4** and used in **Chapter 3**. Only one monomer (chain A) for GluA2 and GluA3 was selected as the starting geometry for a molecular mechanics energy minimisation. This choice was based on previous work done on GluA2 protomers (Arinaminpathy et al., 2002). However, the GluA4 crystal structure was a dimer and as such both chains A and B were subjected to energy minimisations before continuing on with assembling the QM models. GluA4 was used as a test case in order to observe any differences between interaction energies in the two monomers (chain A and B). In total, four model systems were prepared for the monomers GluA2, GluA3 and the dimer GluA4, each resembling the model seen in Figure 4.2A. These structures were then prepared for energy minimisation using the protocol described in **Chapter 4**, after which were used to assemble the quantum mechanical (QM) models.

The QM models were assembled by considering the specific ligand of interest, water molecules within 5 Å from the ligand and all amino acids within a distance of 10 Å from the ligand and water molecules. In the end 26 interacting residues from each receptor was chosen. They are listed in Table 5.2. The numbering employed here is according to the mature sequence as employed by Armstrong and Gouaux (2000) for ease of comparison (Armstrong and Gouaux, 2000).

Table 5.2 List of interacting residues selected for the QM model calculations from the three proteins GluA2, GluA3, GluA4.

GluA2	GluA3	GluA4
Glu402	Glu402	Glu403
Tyr405	Tyr405	Tyr406/
Tyr450	Tyr450	Tyr451
Pro478	Pro478	Pro479
Leu479	Leu479	Leu480
Thr480	Thr480	Thr481
Arg485	Arg485	Arg486
Leu621	Leu621	Leu622
Ile623	Ile623	Ile624
Gly648	Gly648	Gly649
Thr649	Thr649	Thr650
Leu650	Leu650	Leu651
Asp651	Asp651	Asp652
Ser652	Ser652	Ser653
Gly653	Gly653	Gly654
Ser654	Ser654	Ser655
Thr655	Thr655	Thr656
Lys656	Lys656	Lys657
Thr667	Thr667	Thr668
Phe668	Phe668	Phe669
Thr686	Leu686	Thr687
Tyr702	Phe702	Phe703
Glu705	Glu705	Glu706
Met708	Met708	Met709
Lys730	Lys730	Lys731
Tyr732	Tyr732	Tyr733

The final QM models consisted of 443 atoms for GluA2, 439 atoms for GluA3 and the two dimers, such that each model has 26 residues and the ligand. Six water molecules remained within 5 Å from the ligand after the energy minimisation step for GluA2. However, only five and four water molecules remained in GluA3 and GluA4 respectively after the energy minimisation step.

Single point density functional theoretical calculations were carried out with the Gaussian '03 program (Frisch et al., 2004). The interaction energies for the conserved water molecule were calculated using the function counterpoise method of

Boys and Bernardi (Boys and Bernardi, 1970) as described in detail in **Chapter 4**. The 6-31G** basis set was used with the B3LYP (Lee et al., 1988; Becke, 1993) functional as a compromise between computational expedience and reliability of the values for BSSE.

5.2.3 MD simulations

Three X-ray crystal structures, as listed in Table **5.1**, were used in the MD simulations. This included glutamate-bound structures from the AMPA sub-group, namely GluA2 (PDB ID: 1FTJ), GluA3 (PDB ID: 3DLN) and GluA4 (PDB ID: 3FAS). A dimer was simulated for GluA2 and GluA4 while only a monomer was simulated for GluA3. All the water molecules reported in the crystal structure were included in the simulations. 2 x 30 ns unrestrained production simulations were performed for each structure following the protocol established in **Chapter 3**.

5.3 Results and Discussion

5.3.1 Results from the quantum mechanical calculations

Results from the QM calculations are reported in Tables **5.3** and **5.4** and the discussion for this study is discussed in Sections **5.3.1.1** and **5.3.1.2**.

5.3.1.1 Ligand-protein interaction energies

Analysis of the ligand to protein+water interaction energies (L-WsP cells, Table **5.3**) reveals that glutamate is favoured in the following order: GluA3 ($-188.78 \text{ kcal mol}^{-1}$) > GluA2 ($-179.86 \text{ kcal mol}^{-1}$) > GluA4 (Chain B: $-161.25 \text{ kcal mol}^{-1}$) > GluA4 (Chain A: ($-150.79 \text{ kcal mol}^{-1}$)). These results do not offer any definitive preference between the dimer pairs i.e. GluA1/GluA2 versus GluA3/GluA4. However, within the dimer pair of GluA3/GluA4, glutamate bound to GluA3 is found to be more favoured than GluA4 and that GluA4 chain B is more favoured than chain A.

Table 5.3 Fragment interaction energies of waters and ligand in each QM system in kcal mol⁻¹. Fragments are defined as follows: WX; waters where X is 1-5 or G as described in Figure 2. Ws; all other waters (eg. for the W1-Ws cell for 1FTJ-Glu, Ws would mean W2, W3, W4, W5 and WG), L; ligand (Glu), P; protein, LP; ligand and protein, WsL; waters and ligand, WsP; waters and protein, WsLP; waters and ligand and protein. The interaction energies for GluA2 were taken from Chapter 4 for ease of comparison with those of GluA3 and GluA4.

Fragment	Ws	L	P	LP	WsL	WsP	WsLP
GluA2 (Chain A)							
W1	0.24	3.65	-13.68	-9.16	3.15	-13.02	-0.94
W2	-3.27	-0.53	-20.28	-19.03	-4.73	-23.57	-22.58
W3	2.89	-12.23	-8.58	-18.09	-5.53	-6.56	-13.10
W4	1.22	-2.86	-37.59	-37.39	-1.32	-35.16	-34.91
W5	0.84	-10.54	-6.10	-12.24	-5.84	-6.30	-9.53
WG	-2.00	-12.80	-4.18	-12.56	-14.39	-4.62	-12.31
L	-	-	-	-	-	-179.86	-
GluA3 (Chain A)							
W1	-	-	-	-	-	-	-
W2	-3.19	-7.18	5.62	0.65	-12.25	2.92	-3.41
W3	0.28	-12.44	-7.05	-16.95	-10.25	-6.39	-14.65
W4	1.57	-3.36	-37.41	-38.75	-1.21	-34.86	-35.65
W5	0.52	-11.90	-6.16	-15.33	-7.53	-6.31	-12.19
WG	0.85	-13.22	-5.45	-14.75	-9.89	-4.35	-11.31
L	-	-	-	-	-	-188.78	-
GluA4 (Chain A)							
W1	-	-	-	-	-	-	-
W2	-	-	-	-	-	-	-
W3	1.55	-12.19	-13.34	-24.41	-5.77	-12.49	-0.69
W4	0.44	-2.19	-36.70	-36.13	-1.30	-35.32	-35.60
W5	0.52	-13.20	-7.46	-16.48	-9.38	-7.46	-0.84
WG	-2.45	0.78	-10.78	-6.44	2.85	-14.89	-1.04
L	-	-	-	-	-	-150.79	-
GluA4 (Chain B)							
W1	-	-	-	-	-	-	-
W2	-0.36	-13.94	3.43	-5.44	-7.72	1.51	-1.87
W3	-8.61	-13.47	-1.31	-10.62	-20.21	-4.90	-17.03
W4	-	-	-	-	-	-	-
W5	0.92	-12.35	-6.84	-15.10	-9.90	-6.39	-16.68
WG	-6.21	1.94	-14.21	-13.75	-3.82	-21.27	-17.30
L	-	-	-	-	-	-161.25	-

Table 5.4 Basis Set Superimposition Error Energies for water and ligand fragments in each optimised QM system in kcal mol⁻¹. Fragments are defined as follows: WX; waters where X is 1-5 or G as described in Figure 4.2. Ws; all other waters (eg. for the W1-Ws cell for 1FTJ-Glu, Ws would mean W2, W3, W4, W5 and WG), L; ligand (Glu), P; protein, LP; ligand and protein, WsL; waters and ligand, WsP; waters and protein, WsLP; waters and ligand and protein. The interaction energies for GluA2 were taken from Chapter 4 for ease of comparison with those of GluA3 and GluA4.

Fragment	Ws	L	P	LP	WsL	WsP	WsLP
GluA2 (Chain A)							
W1	0.03	3.44	7.29	9.36	3.40	7.33	17.68
W2	1.88	1.01	7.18	7.61	2.68	7.90	8.21
W3	0.62	2.84	7.35	8.42	2.39	7.68	8.24
W4	0.00	0.48	10.89	10.59	0.41	10.62	10.48
W5	0.25	2.81	7.99	8.97	2.45	8.11	8.82
WG	2.04	6.03	5.59	8.38	6.83	6.99	9.25
L	-	-	-	-	-	45.24	-
GluA3 (Chain A)							
W1				-			
W2	4.63	1.86	4.84	5.89	6.00	7.67	8.29
W3	3.32	2.82	6.79	7.85	4.96	9.23	9.58
W4	0.00	0.67	11.16	10.87	0.56	10.99	10.76
W5	0.15	2.76	8.10	9.21	2.30	8.21	8.96
WG	2.42	6.00	5.74	8.55	6.69	7.60	9.49
L	-	-	-	-	-	43.85	-
GluA4 (Chain A)							
W1				-			
W2				-			
W3	2.82	3.40	5.84	7.24	5.07	8.24	9.00
W4	0.01	0.52	10.67	10.61	0.45	10.48	10.46
W5	0.07	2.80	7.95	9.12	2.57	8.32	9.07
WG	1.84	2.89	6.78	7.98	3.75	7.84	8.32
L	-	-	-	-	-	43.95	-
GluA4 (Chain B)							
W1				-			
W2	3.59	5.34	3.86	6.88	6.75	6.44	8.17
W3	1.90	3.21	7.64	8.85	3.88	9.02	9.55
W4				-			
W5	0.12	3.17	8.57	9.63	2.53	8.66	9.38
WG	1.87	2.27	7.18	8.07	3.55	8.72	9.16
L	-	-	-	-	-	42.60	-

5.3.1.2 Water interaction energies

Following the central theme of this thesis, the key role of water molecules in these binding sites through interaction energies of the water molecules was investigated. The water interactions in these four models including the “pharmacophoric water”, which is the water that appears to act as a surrogate part of the ligand in either the glutamate (WG: Figure 4.2A and D) is represented in Figures 4.3 (for GluA2) and 5.3 (for GluA3 and GluA4).

Firstly, how the “pharmacophoric water” interacts with the various components of the binding pocket (WG-WsLP in Table 5.3) is reported (Figure 4.3A and 5.3A). WG is favoured in the following order: GluA4 (Chain B: $-17.30 \text{ kcal mol}^{-1}$) > GluA2 ($-12.31 \text{ kcal mol}^{-1}$) > GluA3 ($-11.31 \text{ kcal mol}^{-1}$) > GluA4 (Chain A: $-1.04 \text{ kcal mol}^{-1}$). Overall, the difference between GluA4 (chain B) to GluA3 and GluA2 is $\sim 5\text{-}6 \text{ kcal mol}^{-1}$. However, the difference between GluA4 (chain A) to GluA3 and GluA2 is $\sim 10\text{-}11 \text{ kcal mol}^{-1}$. It would be important to study the dynamics within this chain to understand these results. This is reported in Section 5.3.2.3. Table 5.3 also demonstrates that the fragments are clearly non-additive in nature.

W1 cannot be compared across this family of receptors as it is only present in GluA2 (Figure 4.3B and 5.3B). In the previous MD studies this position is prone to being occupied by bulk water (Chapter 3). In GluA2 W1 does not make a hydrogen bond with the ligand. W2 is not present in GluA4 (Chain B) but is most energetically favourable in GluA2. W2 makes contacts with Tyr 702/703 and Thr 688/689 in all models. The pattern of interaction energies relates directly to the number and geometry

of the hydrogen bonds formed (Figure **4.3C** and **5.3C**). W3 makes hydrogen bonds with Leu 650/651 and Leu 730/731 in all four models (Figure **4.3D** and **5.3D**), and a third hydrogen bond to the ligand. The interaction energy (Table **5.3**) is comparable across the receptors to $\sim 3\text{--}4$ kcal mol⁻¹ as a result of an optimal arrangement of both ligand and protein, except GluA4 (chain A at -0.69 kcal mol⁻¹).

W4 was found to maintain comparable interaction energies across the models except GluA4 (Chain B) where it was not present. This could be associated with the fact that it forms a tetrahedral arrangement with the charged side chains of the residues; Glu 705/706, Lys 730/731, Ser 654/655 and Thr 655/656 in all four models (Figure **4.3E** and **5.3E**). This water does not form any direct interaction with the ligands and occupies a well defined site apparently serving a structural role, presumably helping to define the shape of the binding pocket.

W5 has been previously reported as a well conserved water molecule (**Chapter 3**) that is found in the majority of published high-resolution ligand-bound iGluR structures. The computed interaction energy for W5 is favoured in the following order: GluA4 (Chain B: -16.68 kcal mol⁻¹) > GluA3 (-12.19 kcal mol⁻¹) > GluA2 (-9.53 kcal mol⁻¹) > GluA4 (Chain A: -0.84 kcal mol⁻¹). One immediate observation is the large difference between GluA4 (chain A) and GluA4 (chain B) at ~ 16 kcal mol⁻¹. Such a large difference would suggest less stability of this water position in chain A, either because it is sterically strained or perhaps because it makes different interactions to the ligand and protein when compared to chain B. From these interaction energies it is difficult to accurately explain this difference since W5 still makes four hydrogen bonds with the backbone carbonyl of Leu 650/651, backbone NH of Lys 656/657 and backbone carbonyl

of Thr 655/656 and the carboxylate group of glutamate (Figure 4.3F and 5.3F). The dynamics within GluA4 (chain A) shed some light on this discrepancy. This is reported in Section 5.3.2.3.

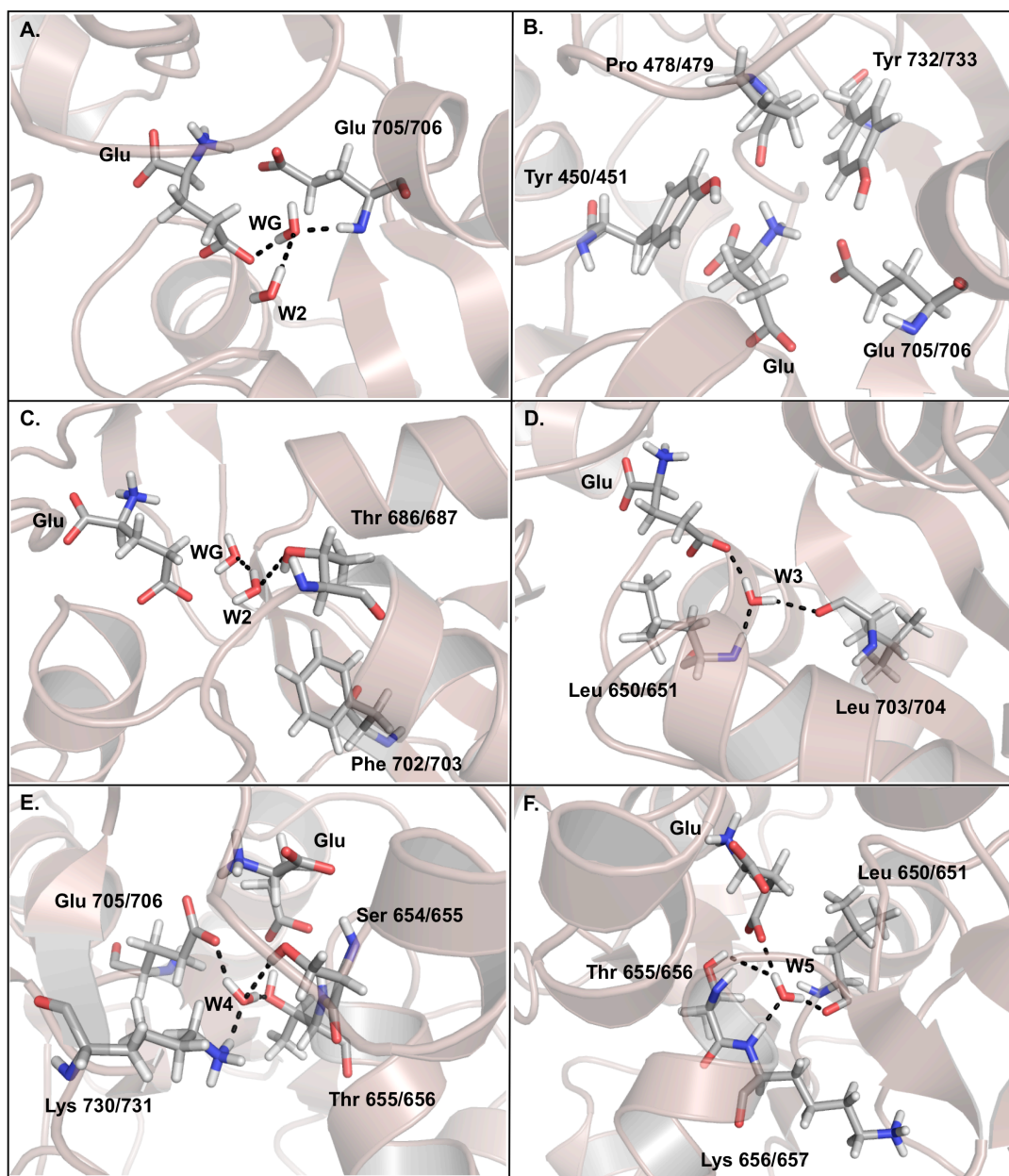


Figure 5.3 Hydrogen bond interactions for individual water molecules for the model systems of GluA3 and GluA4 (chain A and chain B) (A) WG, (B) W1, (C) W2, (D) W3, (E) W4 and (F) W5. W2 is not present in GluA4 (chain A) and W4 is not present in GluA4 (chain B). All protein residues, ligand and water molecules that interact with WG and W1-W5 are labelled.

5.3.2 Results from the MD simulations

5.3.2.1 Comparison of the structural behaviour of the proteins

The drift of each simulated structure from its initial crystallographic conformation provides information on the quality of the simulations. The drift was measured in terms of the root mean square deviation (RMSD) of the C α atoms from the initial structures as a function of time. For the two simulations in each receptor, GluA2, GluA3 and GluA4, after an initial rise the C α RMSD plateaus at $\sim$ 1-2.50 Å (Figure 5.4). This is indicative of a stable simulation.

The root mean square fluctuation (RMSF) of C α atoms from all simulations gives an indication of regions in a protein that exhibit varying degrees of motion. The RMSFs are shown in Figure 5.5. The increased fluctuations at the N and C-termini of these receptors could be attributed to unbounded protein segments that are free to fluctuate in solution. These results are also comparable to those reported earlier (Pang et al., 2005) and in **Chapter 3**.

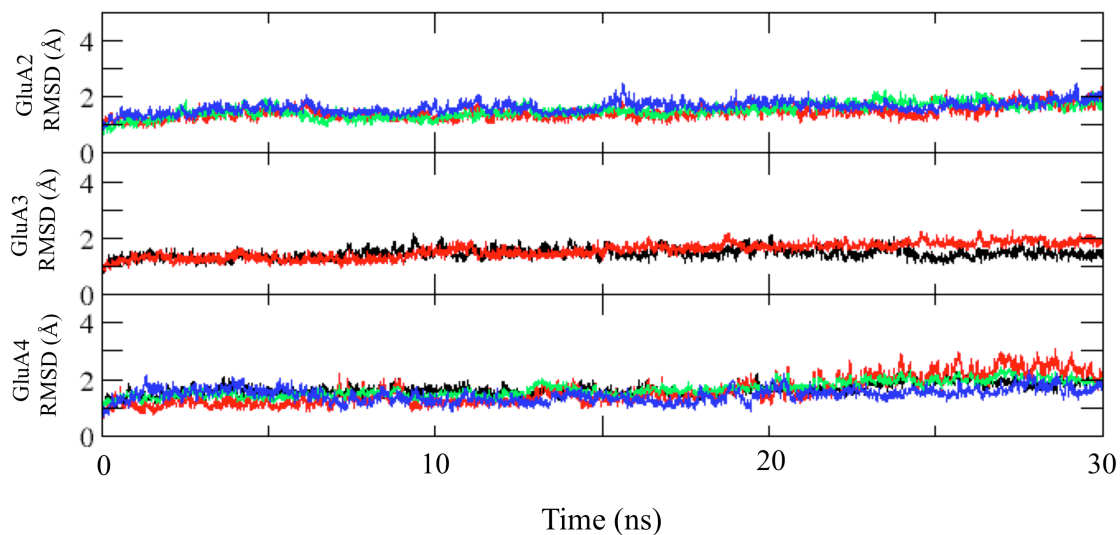


Figure 5.4 Root mean square deviation (RMSD) of all protein C α atoms calculated as a function of time for the AMPA subtype of receptors. GluA2 and GluA4 were dimer simulations. Chain A in the dimers and monomers are depicted as follows - run 1; red and run 2; black. Chain B in the dimer simulations are represented as follows - run 1; green and run 2; blue. All RMSDs plateau off indicating relaxation to a stable state.

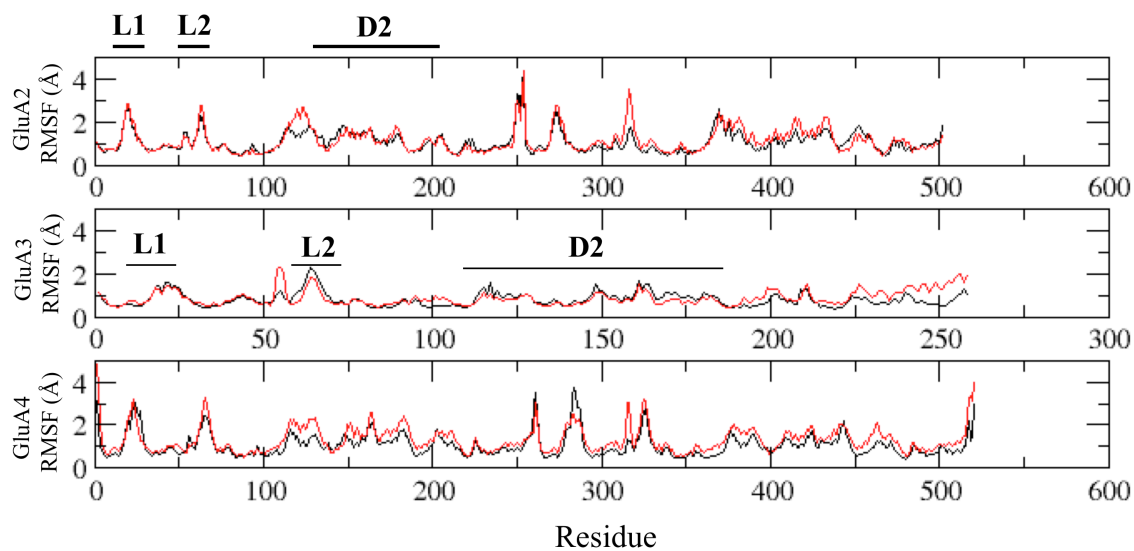


Figure 5.5 Root mean square fluctuation (RMSF) of protein C α atoms (run 1; red, run 2; black) for the AMPA subtype of receptors. Regions of high fluctuation include loop 1 (L1) and loop 2 (L2) in domain D1 and residues in domain D2.

5.3.2.2 Interdomain motion

To assess the extent of closure of the ligand binding domain, the distance between the centers of mass of D1 and D2 as a function of time in the simulations was calculated. The results from Figure 5.6 show that the GluA2 simulations displays an average distance of ~ 24 Å between D1 and D2. In the GluA3 and GluA4 simulations a sharp increase in the D1-D2 distance between 23.4-24.5 Å can be seen. A conformational change of a key lysine residue implicated in acting as a switch (**Chapter 3**) seems to be the cause.

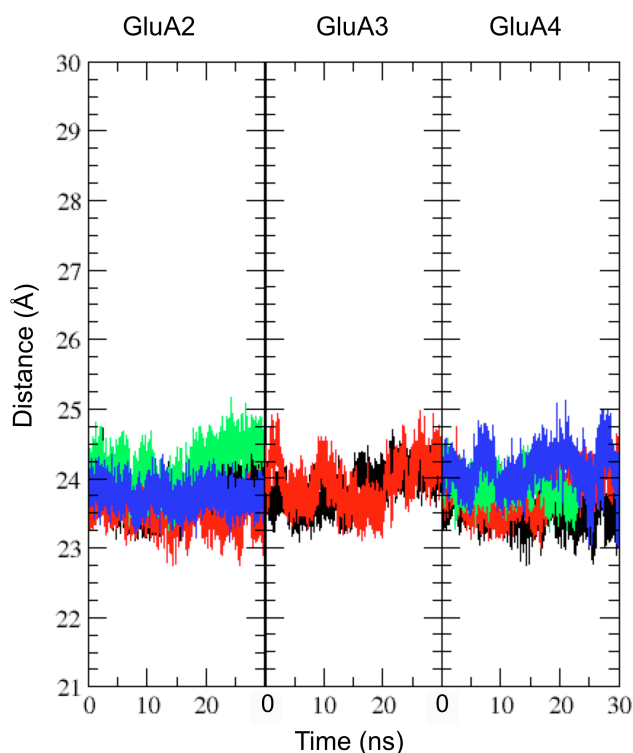


Figure 5.6 The distance between the center of mass of the C α atoms of the two sub-domains D1 and D2. GluA2 and GluA4 were dimer simulations. Chain A in the dimers and monomers are depicted as follows - run 1; red and run 2; black. Chain B in the dimer simulations are represented as follows - run 1; green and run 2; blue.

5.3.2.3 Hydrogen bonding and ligand stability

The binding site for each of these simulations is comprised of an intricate network of hydrogen bonds. These interactions involve direct contact with the ligand and key

residues of D1 (Arg485, Thr480, and Pro478) and D2 (Ser654, Thr655, and Glu705). The lengths and angles of these H-bonds are summarised in Table 5.5. All sets of calculations appear to have similar number of hydrogen bonds except GluA4 which has slightly less.

Table 5.5 Average number (and one standard deviation) of hydrogen bonds formed by glutamate with binding site residues and water molecules. Hydrogen bonds were calculated using a distance cut-off of 3.5 Å and acceptor-donor-hydrogen angle set to 30°.

Subtype	Simulation 1	Simulation 2
GluA2	12.3 ± 1.1	12.5 ± 0.8
GluA3	12.1 ± 1.1	11.9 ± 1.3
GluA4	11.3 ± 0.9	10.9 ± 1.1

The MD results thus far do not show remarkable differences between the subtypes as they have only looked at the protein chains as a whole, which is not surprising given that GluA2 and GluA3/GluA4 are ~90% identical (Ahmed et al., 2008). Any differences between these receptors should arise from within the agonist-binding site and those residues interacting with the ligand. These variations have been previously shown to activate these subtypes differentially (Wahl et al., 1996; Coquelle, 2000; Vogensen et al., 2007; Ahmed et al., 2008).

One such way of characterising the difference between the subtypes is to look at the binding site and any water mediated protein-ligand interactions by means of the protocol used in **Chapter 3**, to calculate the spatial density function for the waters in GluA3 and GluA4. This can then be compared to the X-ray crystal structures and those previous results for GluA2 (Figure 3.11A and B). Water occupancy computed from

MD trajectories showed distinct locations preferred by water molecules in the binding site in good agreement with crystallographic locations (i.e WG, W3 and W5) (Figure 5.7). This observation is comparable to those seen in **Chapter 3**. For GluA4 water sites W4 (chain A) and W2 (chain B) that were not occupied in the crystal structure have new density (labelled “new”) due to bulk waters being able to readily move into these sites. The timescale for water exchange is of the order of picoseconds. Overall, there appear to be consistent areas of “new” density in all simulations.

Figure 5.8 and Figure 5.9 display a close up representation of keys residues in the binding sites of GluA3 and GluA4, respectively. One immediate observation is the conformational change seen for the Met 708/709 side chain. In the X-ray structure it is straight and pointing into the binding site and then ends up pointing away at the end of the 30 ns simulations (Figure 5.8 and 5.9). More importantly, however, is the phenylalanine residue at position 702 and 703 in GluA3 and GluA4, respectively. When compared to GluA2, there is a tyrosine (Y702) at this position (Figure 5.1). The simulations show that regardless of the residue at this position the water positions are conserved, which is in direct agreement with previous mutational studies (Frandsen et al., 2005; Ahmed et al., 2008; Kasper et al., 2008). One other important observation that relates back to the QM results for GluA4 shows that the geometry of the glutamate ligand in chain A is highly strained and water occupancy around the ligand is not the same as in chain B. This could explain why the interaction energies for all the computed waters were highly unfavoured as the majority of waters after the simulation except WG, the “pharmacophoric water” and W4 and W5 do not occupy well defined sites (Figure 5.9).

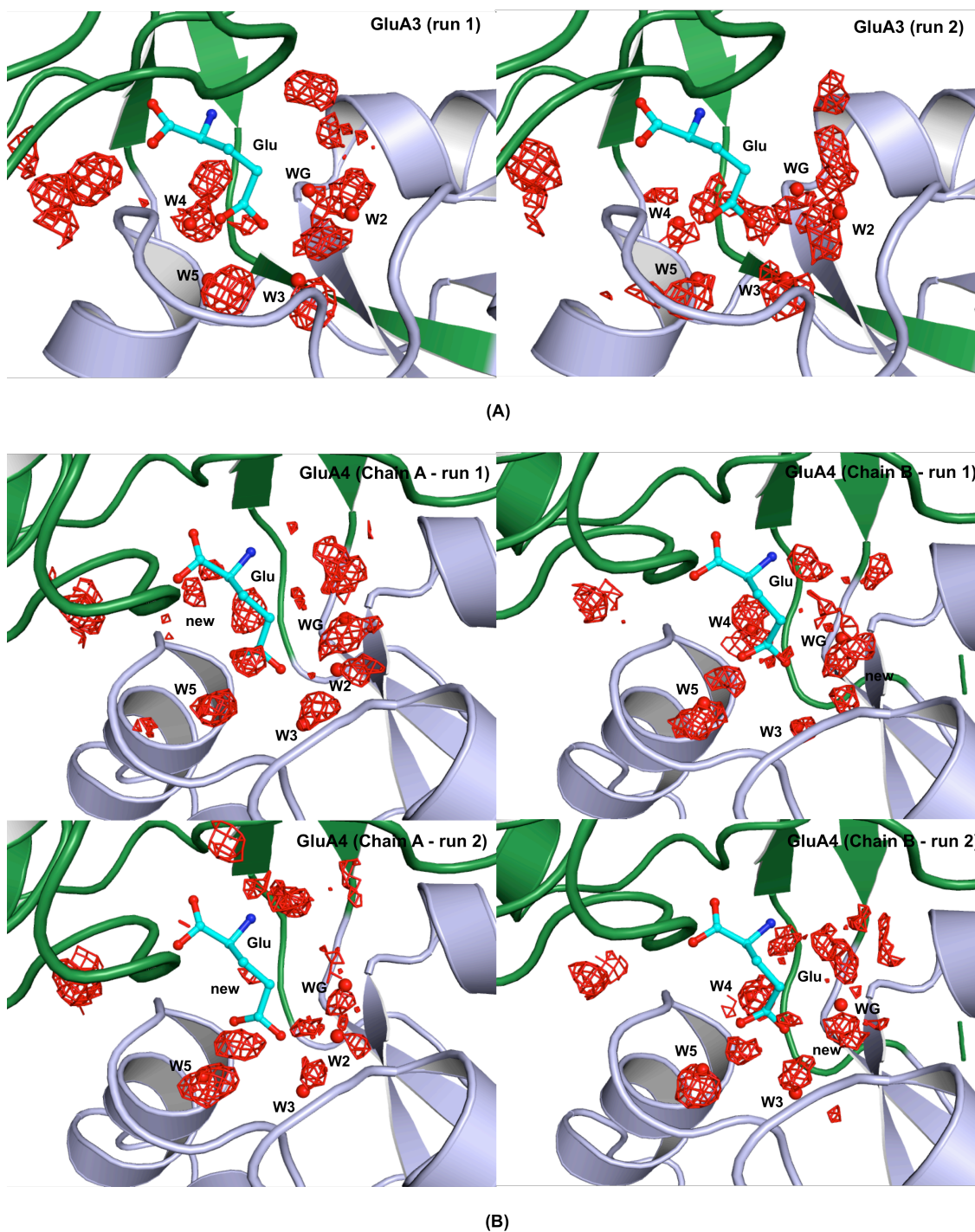
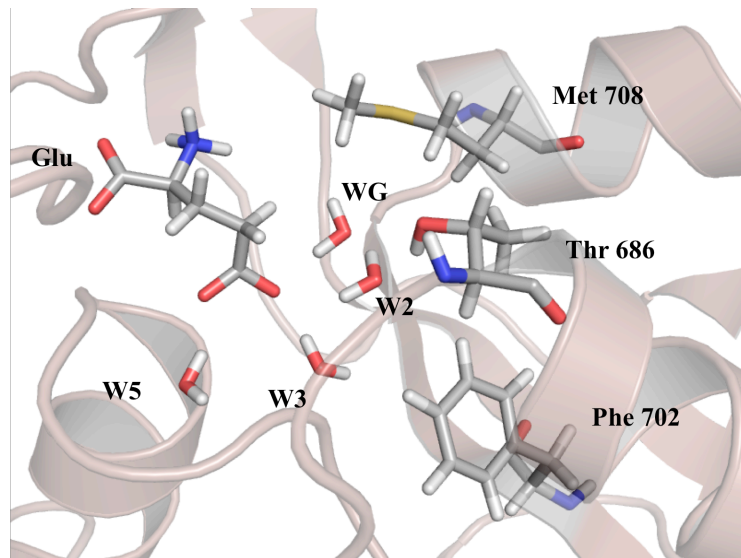
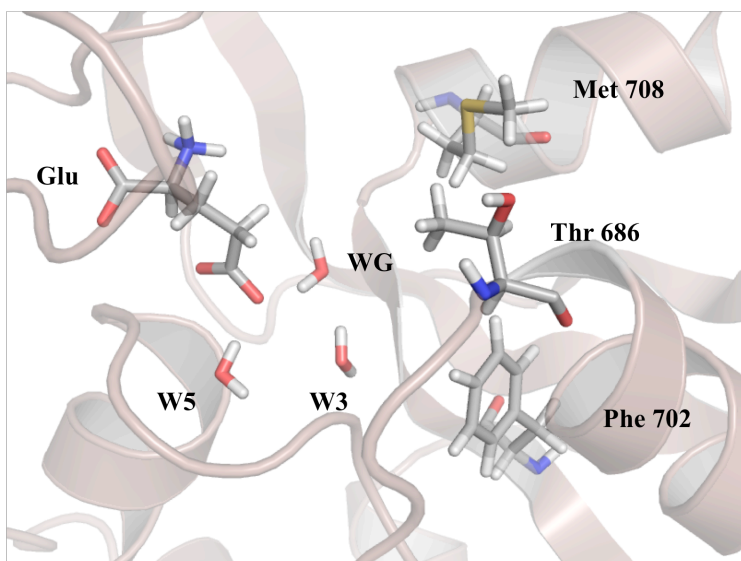


Figure 5.7 The spatial density of water molecules calculated across the length of two 30 ns simulation runs for the AMPA subtype of receptors. MD simulations of (A) run1 and run2 of the GluA3 monomer; (B) run1 and run2 of Chain A and Chain B of the GluA4 dimer. Red spheres denote the location of crystal water molecules, while the red mesh was computed from MD trajectories. The protein is shown in cartoon representation (D1 in green and D2 in blue) and the glutamate ligand in stick representation. All crystal water locations were preserved in MD simulations. New density is labelled as “new” and appears to be consistent in all simulations.



(A)



(B)

Figure 5.8 Arrangement of water molecules 5Å from Glu as well as key residues F702 and T686 in the (A) crystal structure of GluA3 and (B) after the 30 ns simulation.

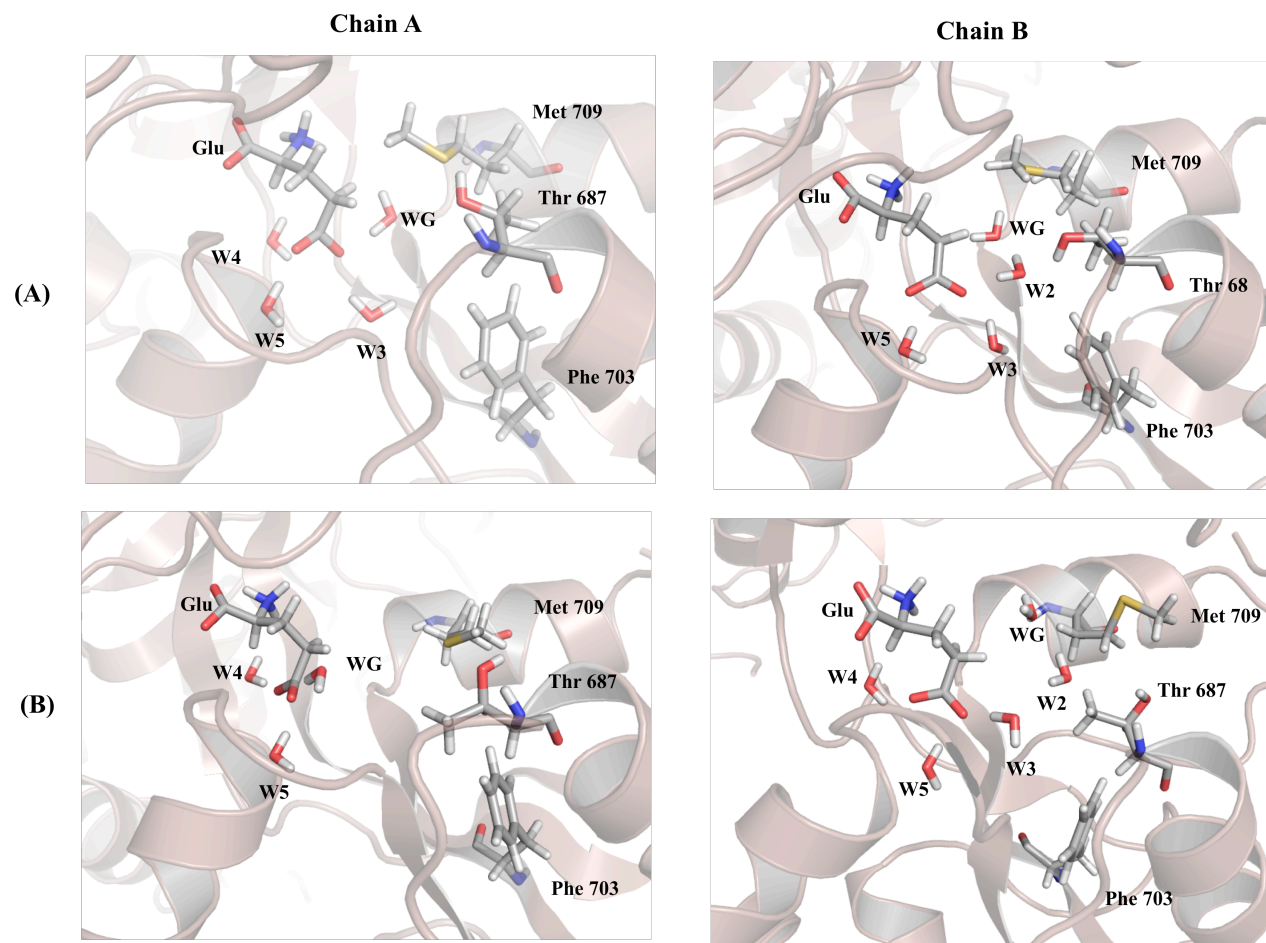


Figure 5.9 Arrangement of water molecules 5Å from Glu as well as key residues F702 and Thr686 in (A) the crystal structure of GluA4 and (B) after the 30 ns simulation for both Chain A and Chain B.

5.4 Conclusions

This chapter presents another example whereby performing simple QM calculations can provide a preliminary insight into the characteristics of the binding pocket and the expected behaviour of the waters within it, which can subsequently be supported by MD simulations. Not only do the results show that the binding site of the AMPA subtype of glutamate receptors are flexible, but also that despite this flexibility the water dynamics in the presence of glutamate is conserved across the iGluR family (when compared to the results from **Chapter 3**).

The QM results demonstrate that the receptors show comparable interaction energies except for chain A in GluA4. These results were further supported by the MD results which show a strained glutamate in the binding site and fewer hydrogen bonds since only 3 out of 5 crystal water molecules remain after the simulation. Obviously, not all chains display the same degree of dynamics and this could arise from the crystal lattice or the initial crystallographic refinement. Clearly, when performing comparative studies of different subtypes each step of the calculation, from the starting crystal structure to the selection of the protein chain, must be considered carefully if the results are to be meaningful.

This work paves the way for further comparative computational studies for AMPA subtypes once more crystal structures are available for other agonists such as CPW and Br-HIBO (BRH) for GluA3 and GluA4 (Figure **5.10**). One immediate question to ask is whether an addition or subtraction of the hydroxyl group of Y702 in GluA2 and a possible change in the corresponding water molecule can change affinity for different

ligands when compared to GluA3 and GluA4. Such information may facilitate the process of identifying subtype selective agonists through future free energy calculations.

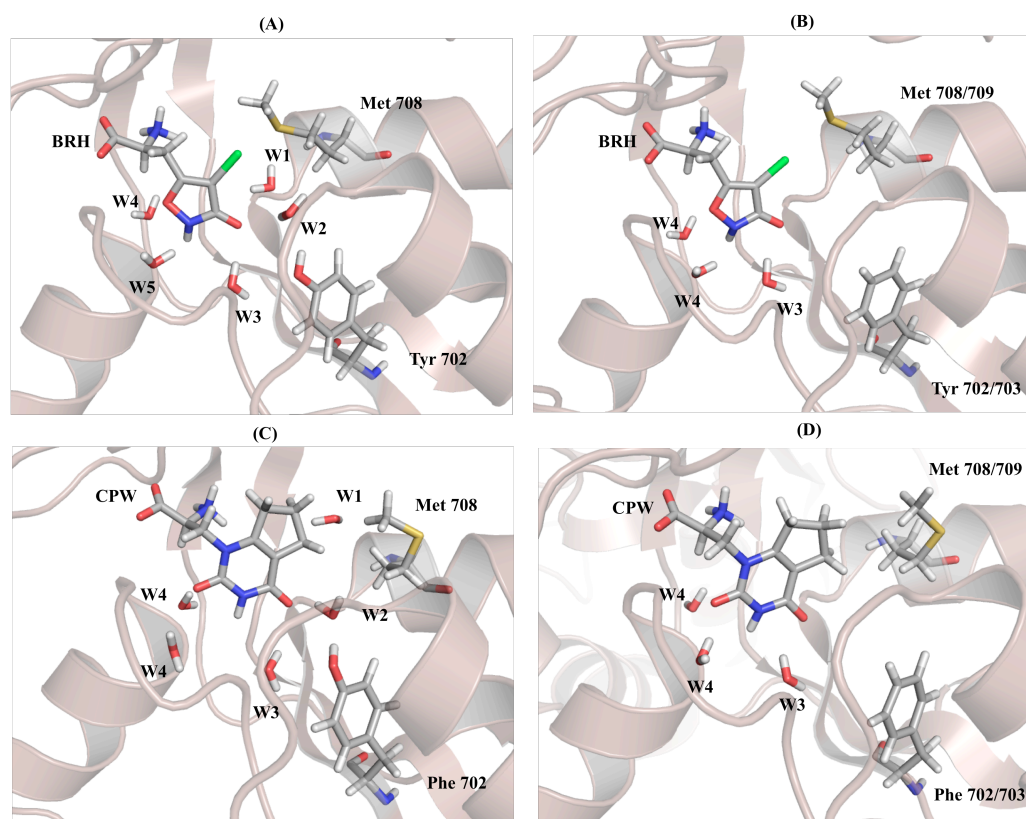


Figure 5.10 Starting structures for future free energy calculations to study the influence of Tyr at position 702 in GluA2 when it is replaced with a Phe at 702/703 in GluA3/GluA4 (A) BRH bound to GluA2 (B) BRH bound to GluA3/GluA4 (C) CPW bound to GluA2 (D) CPW bound to GluA3/GluA4.

Chapter 6

Binding Mode Promiscuity: Molecular Simulations of the Full Agonist AMPA with the Glutamate Receptor

‘Theory guides. Experiment decides.’

Anonymous

Abstract

The crystal structure of GluA2 bound to AMPA displays a different binding mode when compared to GluA2 bound to glutamate. Previously QM calculations were used to investigate this difference in binding modes (Chapter 4). In this chapter MD simulations are used to further study this phenomenon. Here, multiple MD simulations of the ligand binding core of the GluA2, GluA3 and GluA4 subunits of the glutamate receptor in complex with AMPA were performed. Analysis of the binding pocket shows that AMPA is not preserved in the crystal bound mode and can instead adopt an alternative mode of binding. This involves a displacement of a key water molecule previously described as the “pharmacophore” water molecule (WA: Chapter 4) followed by AMPA adopting the pose seen by glutamate when bound to the GluA2, GluA3 and GluA4 subunits. Other observations include a methionine (M708) side chain flipping into a new conformation. These changes result in an altered pattern of hydrogen bonding at the agonist binding site. Examination of water molecules surrounding the ligand reveals that mobility in distinct subsites will be an important consideration in the design of drugs against these receptors. Various program packages were used to investigate whether parameterisation and the use of different force-fields could explain the alternate binding pose. However, these methods and additional mutational studies (M708A) had no influence on AMPA remaining in the crystal binding pose. X-ray crystallisation was also performed to determine other conditions that could capture this alternate binding mode. Well-diffracting crystals were obtained from the previously reported condition and a new crystal structure was determined in complex with AMPA. However, AMPA was again captured in the original binding mode. These results were supported from similar simulations with the Br-HIBO (BRH) compound in complex with GluA2.

6.1 Introduction

Chapter 4 discussed how the crystal structures of glutamate and AMPA revealed two different binding modes when bound to GluA2. This was contrary to the expectation that the two agonists glutamate and AMPA would adopt similar poses within the GluA2 binding pocket (Figure 4.1A). The crystal structures revealed that in the AMPA structure a water molecule assumes a position that would normally be occupied by the γ -carboxyl group of glutamate (Figure 4.1B and C) (Armstrong and Gouaux, 2000) effectively acting like part of the ligand.

Previously quantum mechanical calculations (**Chapter 4**) were used to shed some light on this difference by calculating simple interaction energies. This study was able to quantify the water molecules in the binding site energetically. However, it was not able to definitively explain the preference for AMPA or glutamate for their respective binding modes. This chapter is an extension of that study and uses MD simulations of AMPA-bound to the crystal structure of GluA2 and the recently published GluA3 (Ahmed et al., 2008) and GluA4 (Gill et al., 2008; Kasper et al., 2008) structures. These simulations also present an opportunity to examine the similarities and differences in the behaviour of water molecules within the binding pocket, by using comparative molecular dynamics.

The calculations performed by varying ligand parameterisation, mutations and by using two different force-fields (GROMACS and AMBER) revealed that AMPA does not remain in the crystal bound mode. Instead it adopts the glutamate bound mode very early

on in the simulation (~200-500 ps) and remains in that position for the remainder of the 20 ns run. X-ray crystallisation was also used to investigate AMPA bound to GluA2 in an attempt to reproduce the simulation bound mode. Further simulations revealed that BRH bound to GluA2 also displays the same alternative binding mode when compared to the crystal structure.

6.2 Methods

6.2.1 Parameterising AMPA

Three methods of parameterising the agonist AMPA were used. In the first method AMPA was geometry-optimised quantum mechanically with Gaussian '03 program (Frisch et al., 2004) at the B3LYP/6-31G* level of theory and RESP charges were assigned using Antechamber (Pearlman et al., 1995). In the second method PRODRG (Schuettelkopf and van Aalten, 2004) was used to add hydrogens and GAMESS (Schmidt, 1993) at the RHF with Pople's 6-31G split valence basis set was used to assign RESP charges on a non-optimised structure of AMPA using Antechamber (Pearlman et al., 1995). In the third method atomic charges and masses for AMPA were assigned manually by matching atom types with those within the OPLS-AA (Jorgensen et al., 1996) force-field library. The first method was used to parameterise BRH.

6.2.2 MD simulations performed

Table 6.1 gives a brief outline of the calculations performed with the two MD program packages used in this chapter. Two 20 ns MD simulations were performed with GROMACS for GluA2 (Armstrong and Gouaux, 2000), GluA3 (Ahmed et al., 2008) and GluA4 (Kasper et al., 2008) complexed with AMPA and GluA2 complexed with (S)-2-amino-3-(4-bromo-3-hydroxy-isoxazol-5-yl) propionic acid (Br-HIBO or BRH) (Hogner et al., 2002). The methodology is detailed in Section 6.2.3. Only one 20 ns simulation was performed with AMBER for GluA2 complexed with AMPA and the methodology is detailed in Section 6.2.4. Each simulation was repeated with different velocities to give a total of 7 simulations.

Table 6.1 iGluR structures used for MD simulations bound to AMPA.

Subtype	PDB ID	Resolution (Å)	Ligand	Oligomerisation State
GROMACS simulations (2 x 20 ns)				
GluA2	1FTM	1.70	AMPA	Monomer
GluA2	1M5C	1.65	BRH	Monomer
GluA3	3DP4	2.11	AMPA	Monomer
GluA4	3FAT	1.90	AMPA	Monomer
AMBER simulation (1 x 20 ns)				
GluA2	1FTM	1.70	AMPA	Monomer

6.2.3 MD with the GROMACS program package

Four X-ray crystal structures, as listed in Table 6.1, were used in this study. This included AMPA-bound structures from the AMPA sub-group, namely GluA2 (PDB ID: 1FTM), GluA3 (PDB ID: 3DP4) and GluA4 (PDB ID: 3FAT) as well as the GluA2-BRH bound structure, PDB ID: 1M5C. Only one monomer (i.e. chain A) was

selected from each crystal structure as the starting geometry for each MD simulation. All the water molecules reported in the crystal structure were included in the simulations. 2 x 20 ns unrestrained production simulations were performed for each structure following the protocol established in **Chapter 3**.

6.2.4 MD with the AMBER program package

A simulation was performed with AMPA-bound to GluA2 (PDB ID: 1FTM) using the ff03 parameters of the AMBER force field (Pearlman et al., 1995) (Table 6.1). Only one monomer (i.e. chain A) was selected from each crystal structure as the starting geometry for each MD simulation. All the water molecules reported in the crystal structure were included in the simulations. Missing hydrogen atoms were added to the X-ray crystallographic structure using the LEaP (Pearlman et al., 1995) module available in the AMBER package. The van der Waals and electrostatic interactions were calculated with a cut-off of 12 Å. A full conjugate gradient minimisation was performed until the root mean square of the Cartesian elements of the gradient was less than 0.001 kcal mol⁻¹ Å⁻¹. Only residues within a distance of less than 10 Å from the ligand were allowed to move during the simulation, and a constraint of 10,000 kcal mol⁻¹ Å⁻² was imposed on all remaining atoms. The MD simulation heating was started from 0 to 300 K in 100 ps and then, a simulation of 20 ns was carried out at constant temperature (300K) with an integration step of 2 fs. The SHAKE (Ryckaert et al., 1977) algorithm was used to constrain the stretching of bonds involving hydrogen atoms.

6.3 Results and Discussion

6.3.1 Comparison of the structural behaviour of the proteins

The following results are from the simulations performed with GROMACS. The drift of each simulated structure from its initial crystallographic conformation provides information on the quality of the simulations. The drift was measured in terms of the root mean square deviation (RMSD) of the C α atoms from the initial structures as a function of time. For the two simulations in each receptor, GluA2, GluA3 and GluA4, after an initial rise the C α RMSD reaches a plateau at $\sim$ 1-2.50 Å (Figure 6.1). This is indicative of a stable simulation.

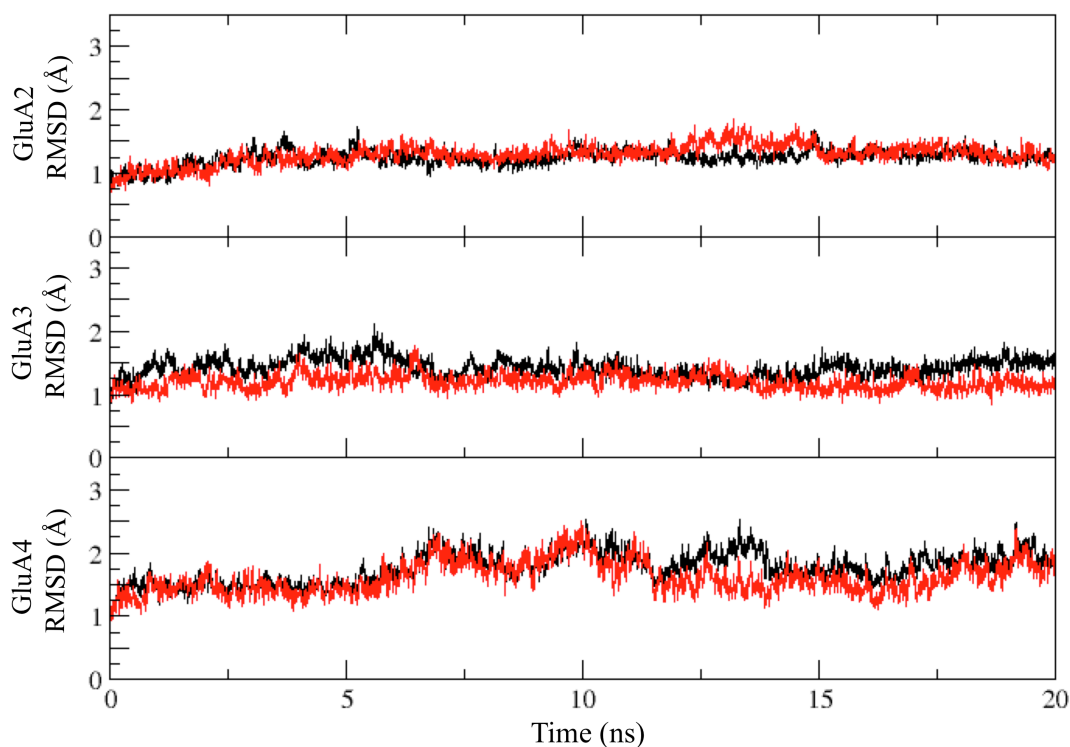


Figure 6.1 Root mean square deviation (RMSD) of all protein C α atoms calculated as a function of time (run 1; red, run 2; black). All RMSDs plateau off indicating relaxation to a stable state.

The root mean square fluctuation (RMSF) of C α atoms indicate regions in the protein that exhibit varying degrees of motion are shown in Figure 6.2. The increased fluctuations at the N and C-termini of these receptors could be attributed to unbounded protein segments that are free to fluctuate in solution. These results are also comparable to those reported earlier, where there are noticeable regions exhibiting fluctuations especially around loop 1 (residues 18-24), loop 2 (45-60) and domain 2; residues 110-140, 147-149 and 167-17 (Pang et al., 2005) and in **Chapter 3** and **5**.

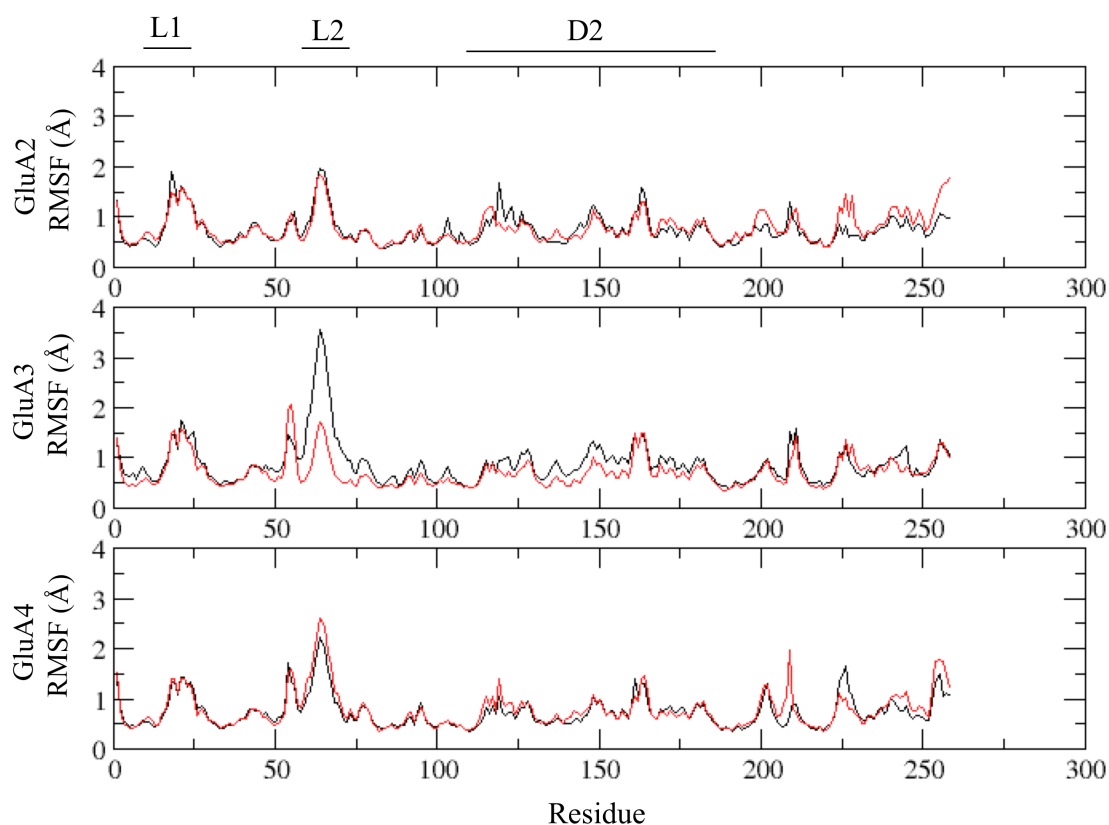


Figure 6.2 Root mean square fluctuation (RMSF) of protein C α atoms (run 1; red, run 2; black). Regions of high fluctuation include loop 1 (L1) and loop 2 (L2) in domain D1 and residues in domain D2. The hinge residues, located on either side of domain D2, mark a region of comparatively low fluctuation.

6.3.2 Interdomain motion

To assess the extent of closure of the ligand binding domain, the distance between the centers of mass of D1 and D2 as a function of time in the simulations was calculated. The results from Figure 6.3 show that the GluA2 simulations displays an average distance of ~ 24 Å between D1 and D2. In the GluA3 simulations the distance between D1 and D2 fluctuates between 23.4-24.5 Å while in the GluA4 simulations the distance between D1 and D2 fluctuates slightly lower between 23-24 Å.

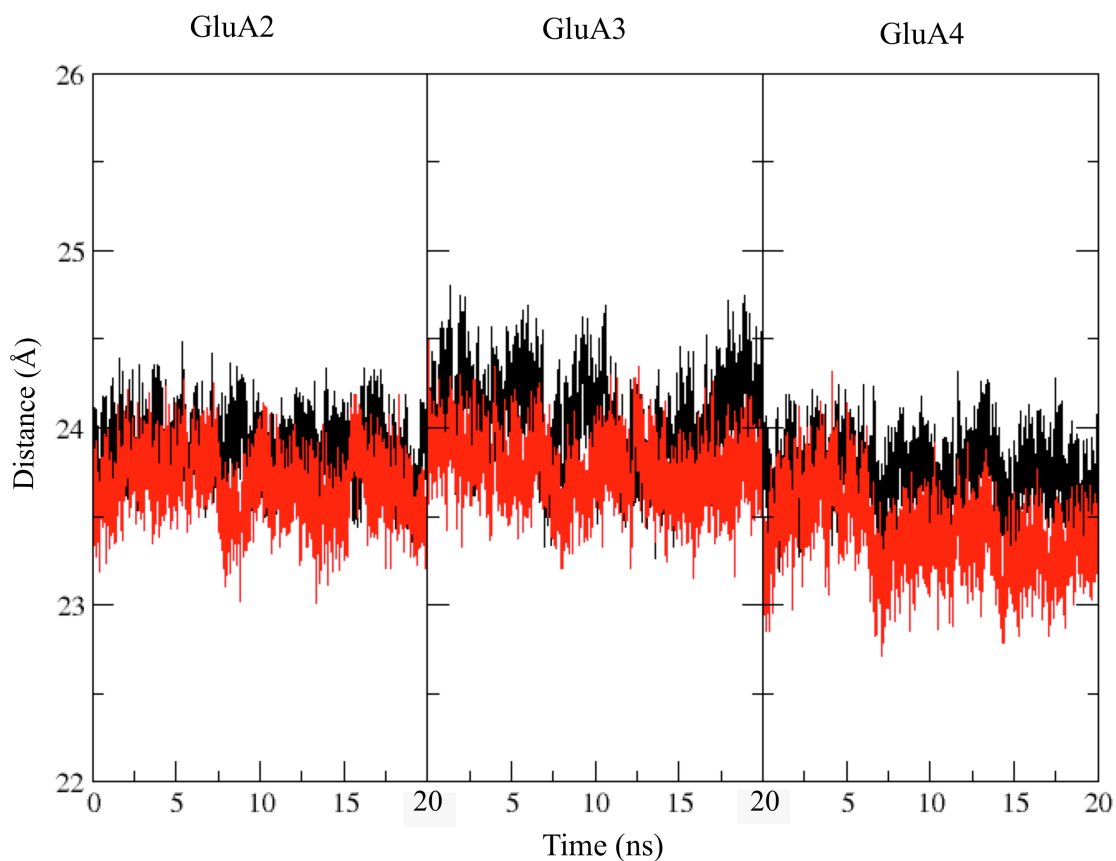


Figure 6.3 The distance between the center of mass of the C α atoms of the two sub-domains D1 and D2 (run 1; red, run 2; black).

6.3.3 Hydrogen bonding and ligand stability

The binding site for each of these simulations is comprised of an intricate network of hydrogen bonds. These interactions involve direct contact with the ligand and key residues of D1 (Arg485, Thr480, and Pro478) and D2 (Ser654, Thr655, and Glu705). The lengths and angles of these H-bonds are summarised in Table 6.2. All sets of calculations appear to have a similar number of hydrogen bonds.

Table 6.2 Average number (and one standard deviation) of hydrogen bonds formed by AMPA with binding site residues and water molecules. Hydrogen bonds were calculated using a distance cut-off of 3.5 Å and acceptor-donor-hydrogen angle set to 30°.

Subtype	Simulation 1	Simulation 2
GluA2	11.9 ± 1.0	11.7 ± 1.2
GluA3	12.0 ± 1.0	11.5 ± 0.9
GluA4	11.8 ± 1.1	12.0 ± 1.0

To gain a better understanding of the positions of water molecules in the active site, the calculated spatial density function for the waters in the two sets of simulations for GluA2, GluA3 and GluA4 were compared to the original crystal structures. It is expected that the calculated spatial density would overlay on the positions of water molecules as seen in crystal structure (Figure 4.2B), indicating that the MD treatment on the system was a good description and is comparable to what was found from the X-ray crystallography structures; GluA2 (PDB ID: 1FTM), GluA3 (PDB ID: 3DP4 and GluA4 (PDB ID: 3FAT). However, the overlay of the calculated spatial densities from the GluA2 simulations (represented as pink wireframes) on the PDB structure (1FTM) give some unexpected results (Figure 6.4). Although it appears that from the calculated

spatial density the majority of water molecules are preserved from the crystal structures, no density was found to represent the water molecule in the position of the pharmacophore water, WA, from the X-ray structure (Figure 4.1C). Additionally, it appears that during the simulation, bulk water swapped with W1, while WA migrated out of the binding core after ~200-500 ps with AMPA remaining in this alternative pose for the remainder of the 20 ns. Simulations repeated with the various parameterisations of AMPA did not yield the crystal binding mode either.

The most important observation from these spatial density calculations is that there appear to be three similar positions in each of the binding cores that are occupied by water molecules. These correspond to W2, W3 and W4 in both the 1FTM-Glu and 1FTJ-AMPA structures as in Figure 4.2B. New density (labelled as “new”) also appears indicating movement of bulk water into the binding site.

In the context of the current work this is important in identifying which water molecules are conserved during the simulations. However, the empty position where WA should reside in the GluA2 binding core raises some question about what motions have occurred during the MD simulation to cause it to move out of position.

A simple superimposition of run 1 of the simulated 1FTM-AMPA with the crystal structure shows that during the simulation AMPA shifted and adopted the position similar to that of glutamate. Figure 6.5 shows the extent of the movement of the AMPA molecule. However, when the torsional angles were tracked, the structure as such did not change drastically. There were no significant changes in χ_1 and χ_3 , with only a minor deviation in χ_2 . This is represented in Figure 6.6.

Further analysis show that D1 interactions are comparable to the crystal structure as seen in **Figure 6.7**. However, Met708 seems to have flipped resulting in a steric clash with the methyl group of AMPA, triggering it to move away during the simulation and reorient itself in the binding core. Further mutational studies were done to test whether the side chain extension of Met708 does actually influence the movement of WA. However, the M708A mutation does not maintain the original crystal binding mode and once again AMPA moves with WA being displaced (Figure **6.8**).

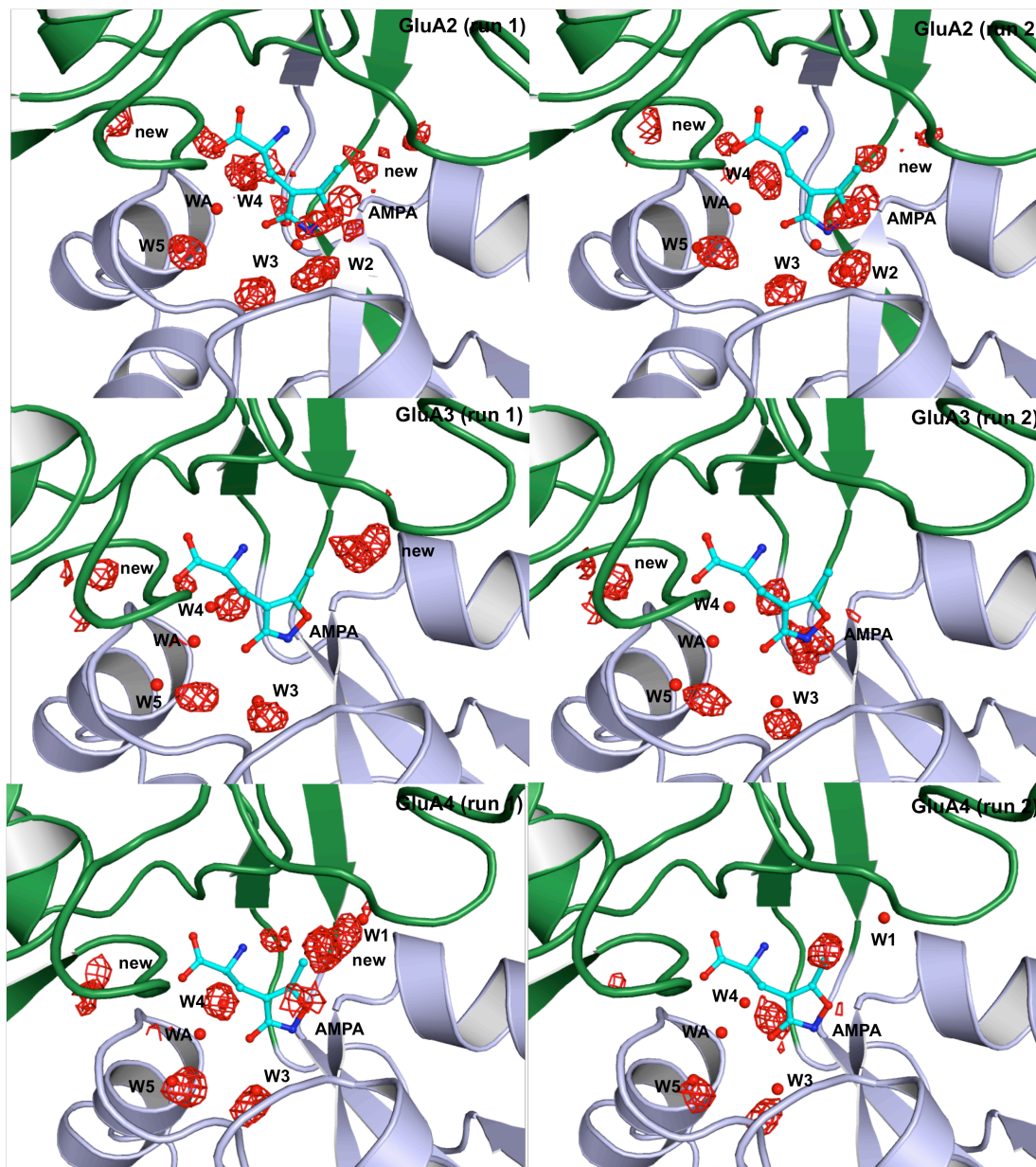
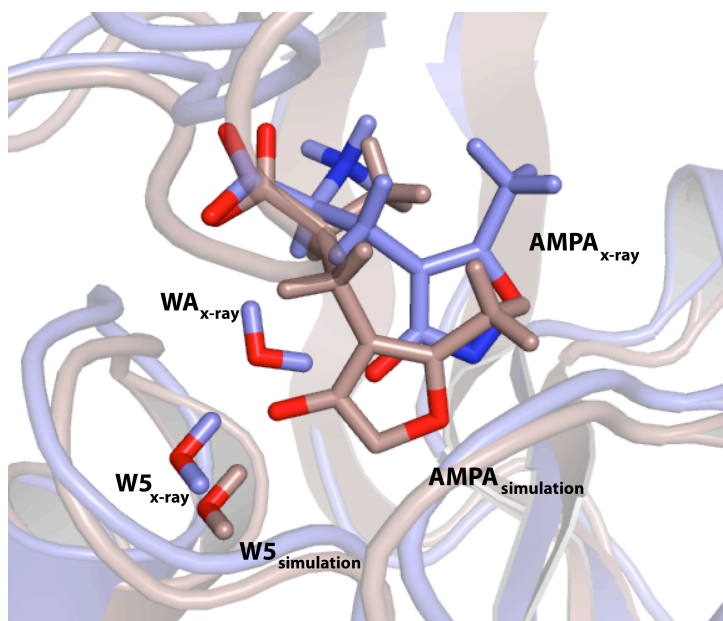
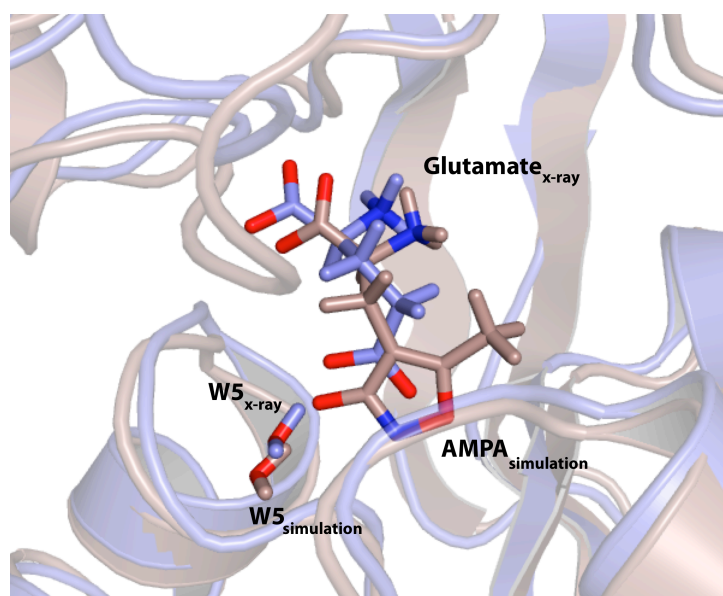


Figure 6.4 The spatial density of water molecules calculated across the length of two 20 ns simulation runs for GluA2, GluA3 and GluA4. Red spheres denote the location of crystal water molecules, while the red mesh was computed from MD trajectories. The protein is shown in cartoon representation (D1 in green and D2 in blue) and the AMPA ligand in stick representation. New density is labelled as “new”.



A



B

Figure 6.5 (A) Superimposition of the binding core of the AMPA-bound GluA2 simulation (salmon) with the crystal structure of AMPA-bound GluA2 structure (blue): 1FTM. (B) Superimposition of the binding core of the AMPA-bound GluA2 simulation (salmon) with the crystal structure of glutamate-bound GluA2 structure: 1FTJ (blue).

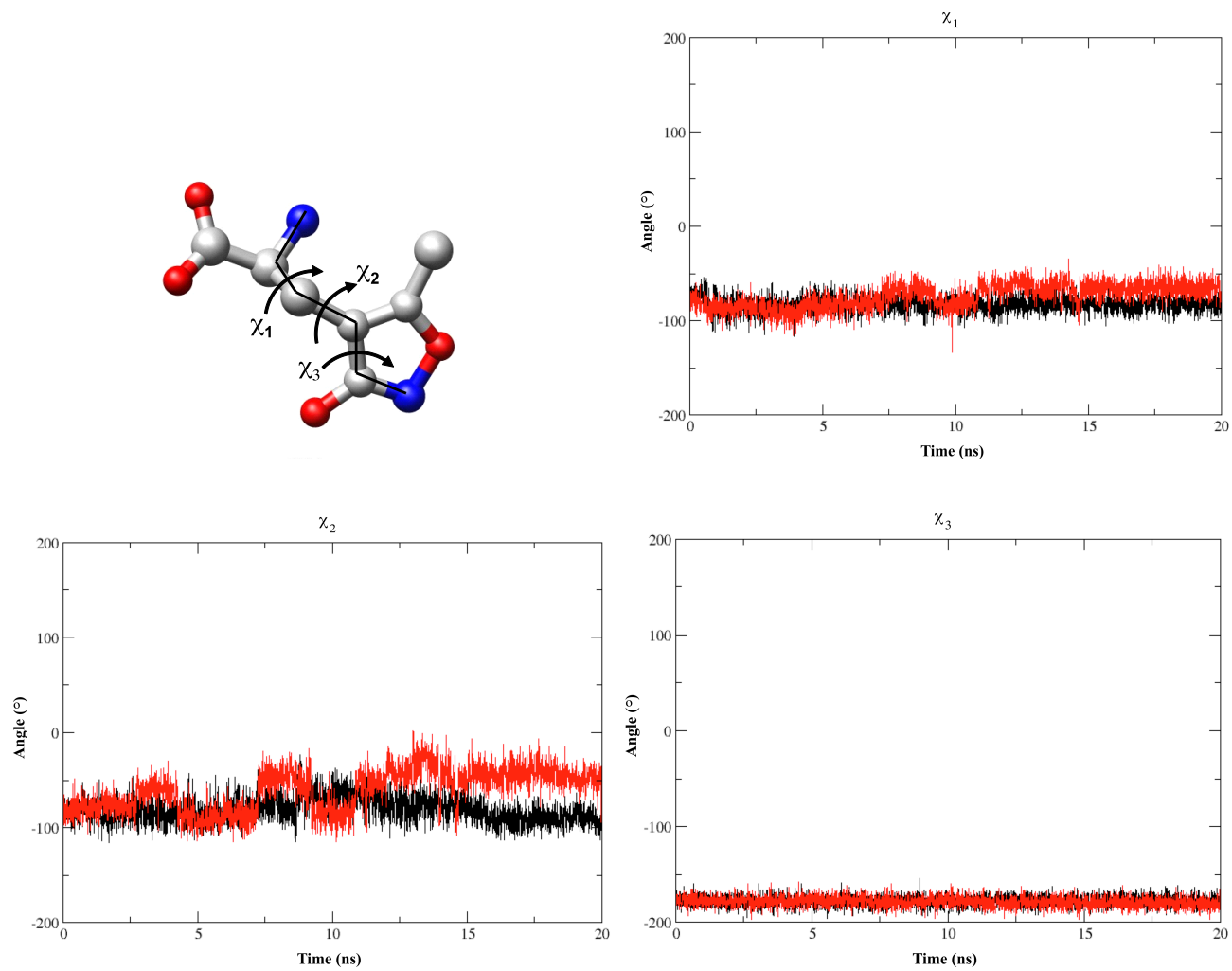
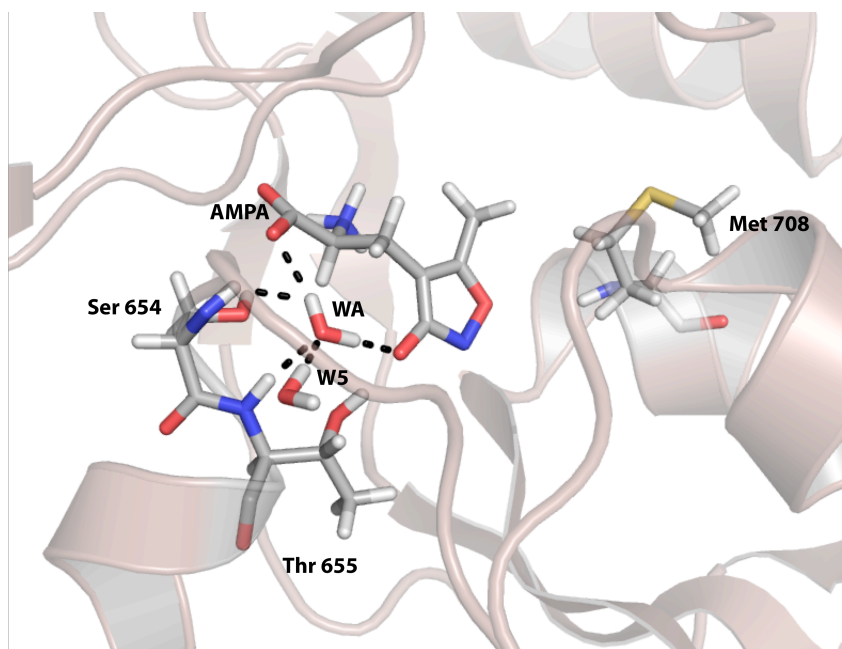
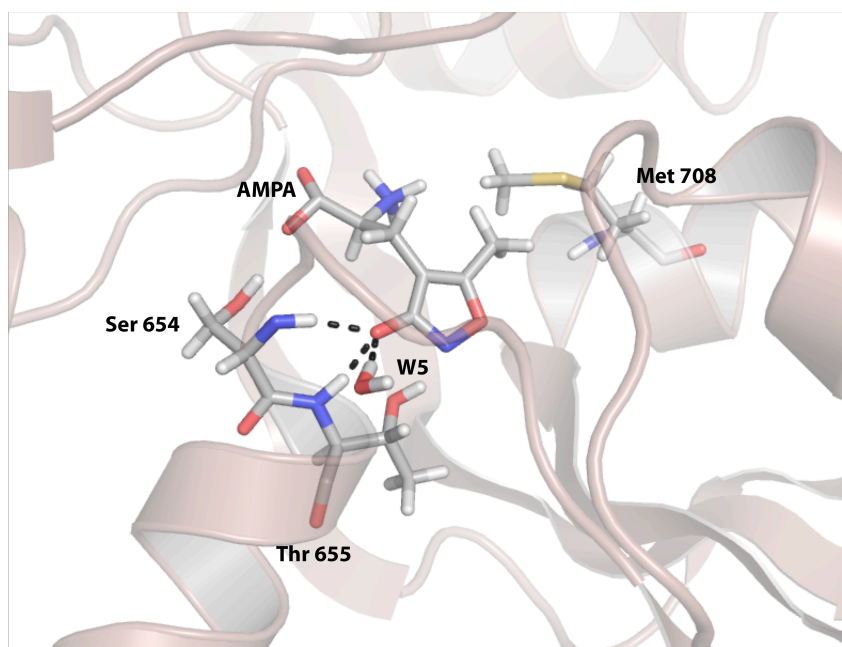


Figure 6.6 Torsional angle (χ_1 , χ_2 and χ_3) as a function of time for the GluA2.

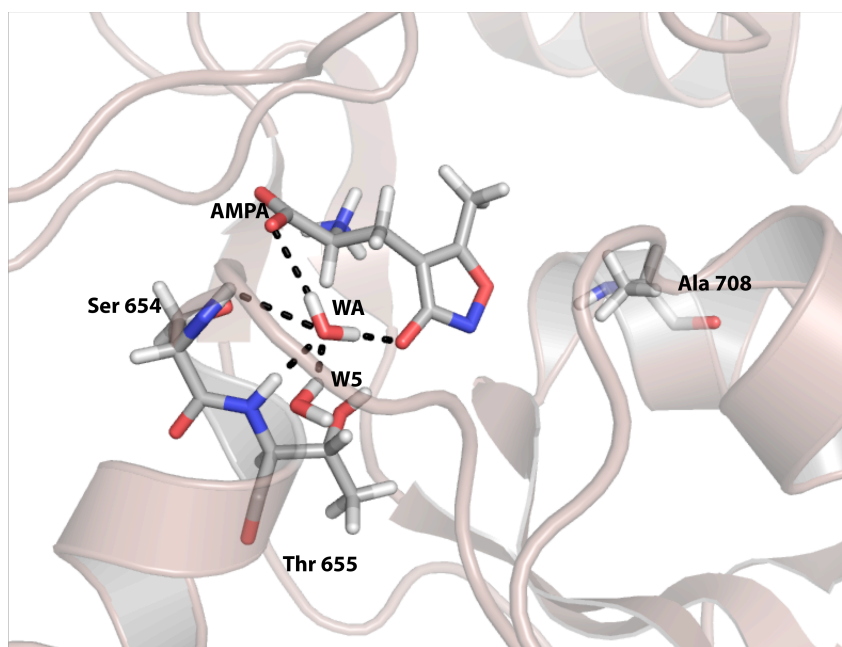


A

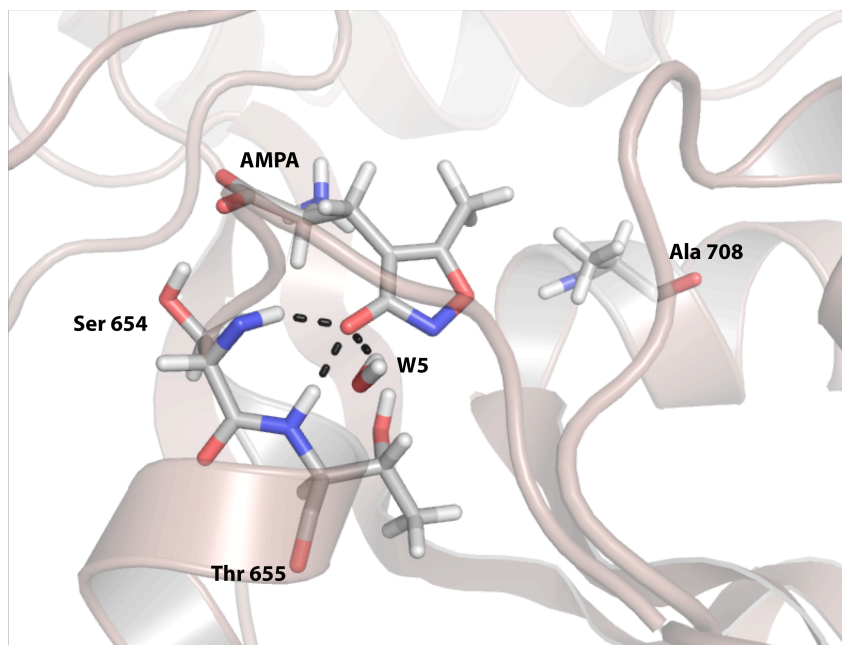


B

Figure 6.7 (A) Crystal structure arrangement of the GluA2 binding site bound to AMPA. WA is present and makes hydrogen bonds to protein W5 and ligand. M708 has a bent orientation of the side chain away from the binding site. (B) Structural arrangement after the simulation of the GluA2 binding site bound to AMPA. WA has been displaced and M708 has an extended orientation of the side chain pointing towards the binding site.



A



B

Figure 6.8 M708A mutational studies (A) Crystal structure arrangement of the GluA2 binding site bound to AMPA. WA is present and makes hydrogen bonds to protein W5 and ligand. (B) Structural arrangement after the simulation of the GluA2 binding site bound to AMPA. WA has been displaced. The mutation M708A does not appear to affect the movement of AMPA.

6.3.4 X-ray crystal structure of GluA2 complexed with AMPA at 1.90 Å

Further to the MD simulations, X-ray crystallography could offer another route to capturing the alternative binding mode for AMPA. This study strived to obtain a crystal in a different condition or crystal form. With the help of collaborators at the Division of Structural Biology (STRUBI)** at the University of Oxford, various solution parameters, precipitant concentrations, as well as pH were varied. However, the best crystals were obtained at 277K and in the condition previously published (Armstrong and Gouaux, 2000). Crystals such as that in Figure 6.9 were used for data collection.

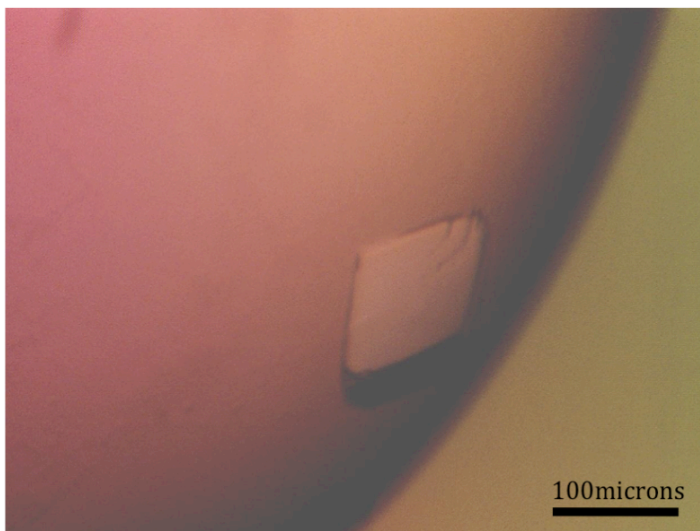


Figure 6.9 A crystal of GluA2 complexed with AMPA used for data collection.

** Miss Amber Clayton performed the molecular biology, including protein expression and purification. Dr A. Radu Aricescu performed the data collection and refinement.

Data processing and refinement included molecular replacement of the protein backbone on the 1FTM structure (Armstrong and Gouaux, 2000) resulting in a crystal structure determined to 1.90 Å resolution. However, AMPA was again captured in the original binding mode (Figure 6.10).

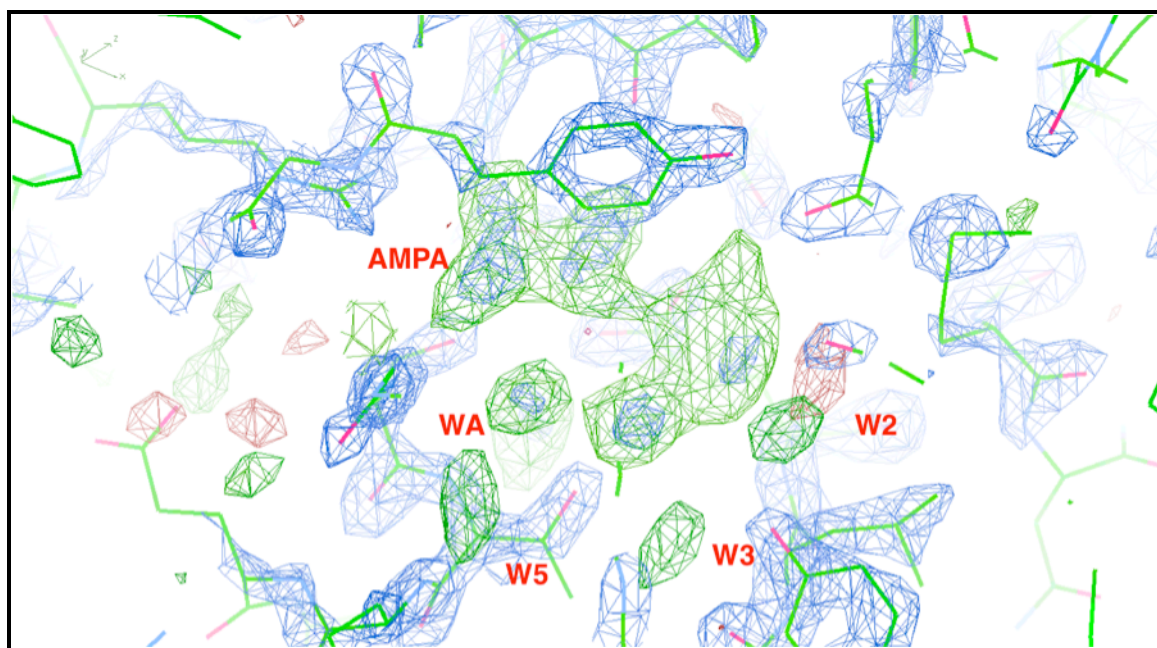


Figure 6.10 Superimposition of the calculated electron density on the protein backbone of 1FTM. The positions of AMPA and all waters including WA are clearly resolved.

6.3.5 Simulations of GluA2 with BRH

Two 20 ns MD simulations of GluA2 with BRH were performed. An analysis of the structural behaviour showed that these simulations were stable (Figure 6.11). However, a simple superimposition of run 1 of the simulated protein with the crystal structure shows that during the simulation BRH shifted and adopted the position similar to that of glutamate. Figure 6.12 shows the extent of the movement of the BRH molecule.

This similarity to AMPA is not surprising considering the structural resemblance of the two ligands. They both occupy the same position in the binding mode in the crystal structure but during the MD simulation they relax into the glutamate binding mode.

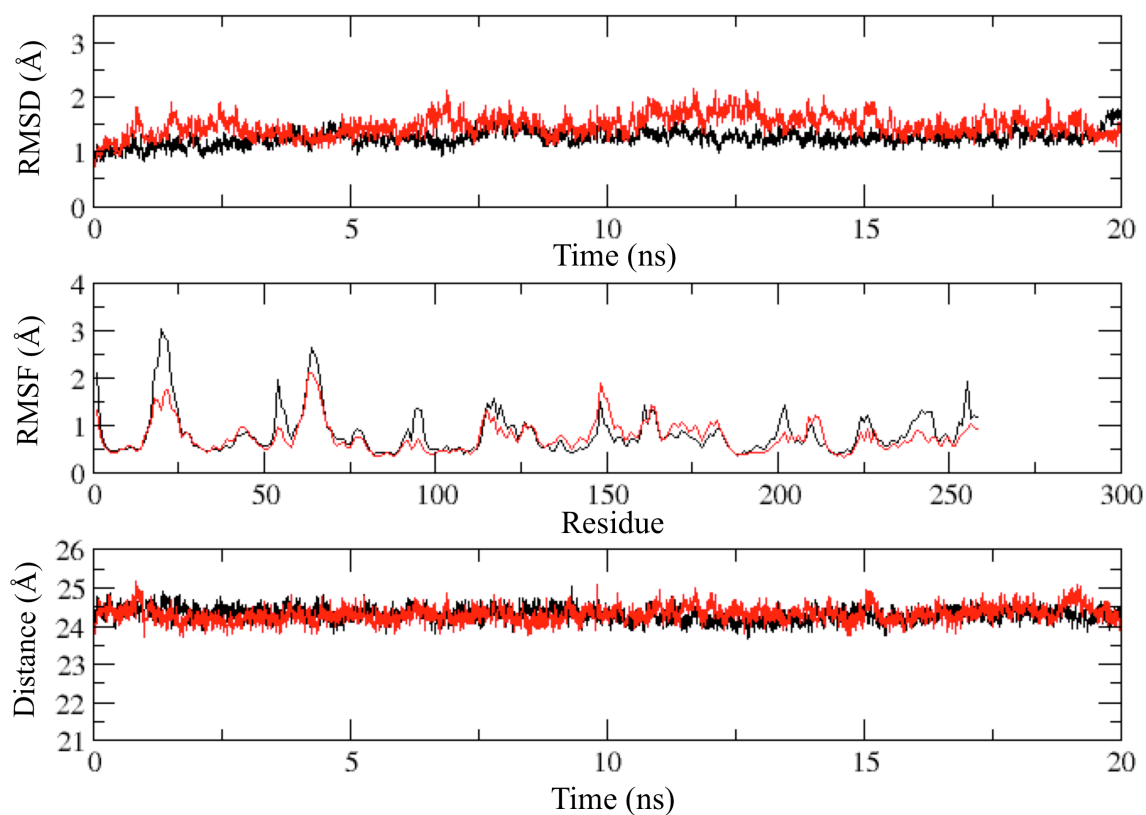


Figure 6.11 Root mean square deviation (RMSD) and root mean square fluctuation (RMSF) of the C α atoms and distance between the center of mass of the C α atoms of the two sub-domains D1 and D2 (run 1; red, run 2; black) for GluA2 complexed with BRH (run 1; red, run 2; black).

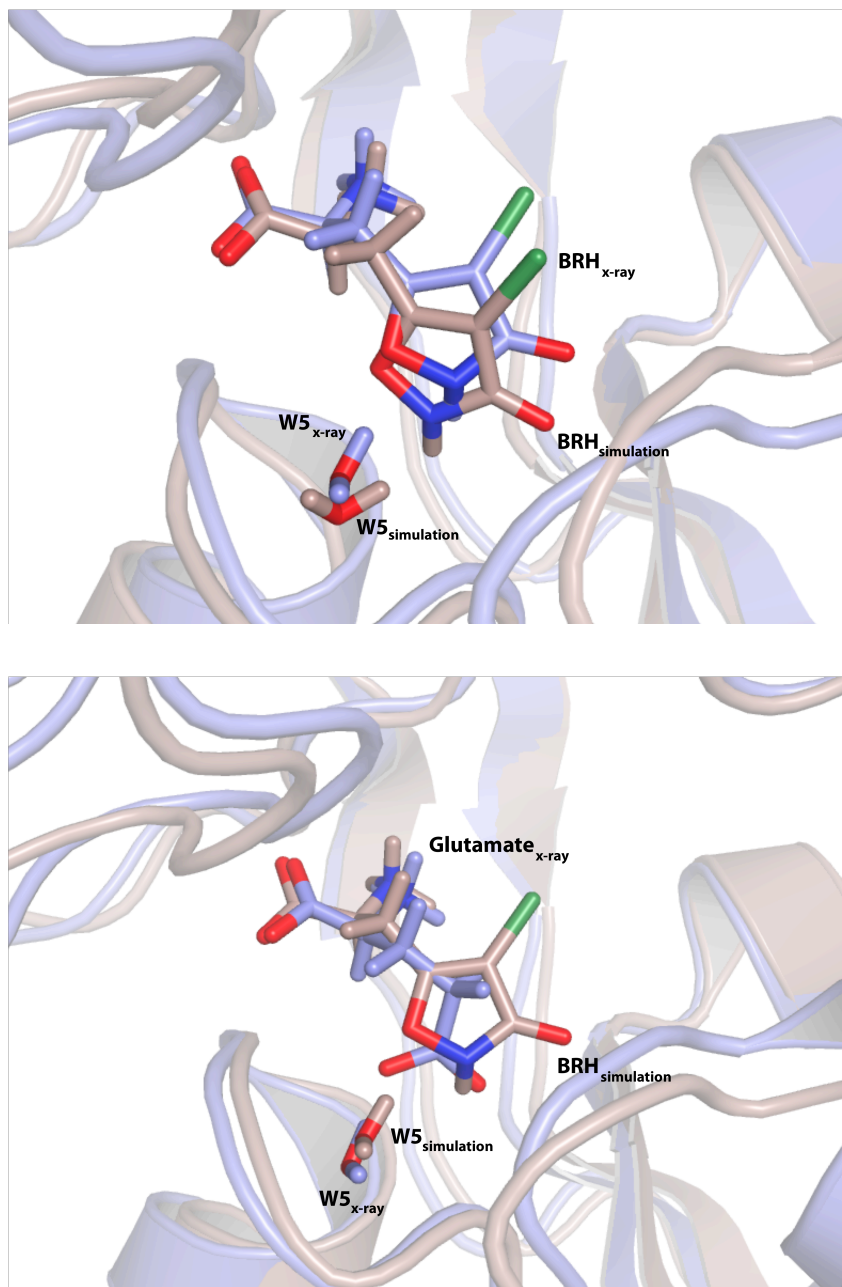


Figure 6.12 (A) Superimposition of the binding core of the BRH-bound GluA2 simulation (salmon) with the crystal structure of BRH-bound GluA2 structure (blue): 1M5C. (B) Superimposition of the binding core of the BRH-bound GluA2 simulation (salmon) with the crystal structure of glutamate-bound GluA2 structure: 1FTJ (blue).

6.4 Conclusion

This chapter has shown that MD simulations do not always reproduce what is seen in the crystal structure. In this particular case MD simulations of AMPA bound to GluA2, GluA3 and GluA4 present an alternative binding mode when compared to the crystal structure. The two force-fields, GROMACS and AMBER and three methods of parameterising the ligand were used to investigate the reason for this disparity and also to see if the method of preparing the system for the simulations is a contributing factor. However, they revealed the same alternative binding mode. Thus, there must be other factors which control the pose of AMPA. Further analysis of the binding site revealed that this alternative pose involves a displacement of a key water molecule previously described as the “pharmacophore” water molecule (**Chapter 4**). Other observations include a methionine (M708) side chain flipping into a new conformation. Mutating this methionine into alanine did not offer any further explanation. These changes result in an altered pattern of hydrogen bonding at the agonist-binding site.

The goal of the X-ray crystallography was to capture the alternative binding mode in a different precipitant condition and different crystal form. However, the only viable crystals that could be used for data collection came from the condition previously published (Armstrong and Gouaux, 2000). Therefore, it is not surprising that the same binding mode was discovered in this new crystal. Further, optimisation of different lead conditions where crystals were formed but not large enough for data collection could present an opportunity for crystallographers to find this alternative binding mode.

Despite the fact that this chapter looked at different parameterisations, different MD force-fields, a mutant, a rigorous QM energy study (**Chapter 4**) and a new crystal structure the reason why AMPA adopts a different binding mode during the MD simulations is still not known. Obviously, there are many reasons why compounds can adopt different binding modes within a receptor and one such contributing factor is temperature. The crystals were all grown at 277K while the simulations were performed at 300K, so temperature effects could be explored further.

Preliminary MD simulations have been performed to investigate whether temperature effects could possibly play a part in allowing AMPA to change its binding mode. Simulations performed at a biologically relevant 300K versus the 277K at which the crystals were grown were compared. Previously, AMPA changed binding mode while displacing WA at ~200-500 ps of the total 20 ns computed at 300K. However, in half of the 10ns simulations (6 were performed) AMPA remained in the crystal binding mode for the entire duration. It is interesting that the 277K simulations either moved AMPA into the alternative binding mode within a few hundred picoseconds or stayed in the crystallographic mode for the entirety of the simulation. This would suggest a sensitivity to the initial velocities of the water molecules. If this were true, one would expect that WA will be more likely to move into the bulk if it has a high initial velocity. Additionally, the higher the temperature of the simulation, the more likely WA is to have a higher starting velocity. Work is currently being carried out by Mr. Gregory Ross (SBCB Unit) to help validate this model by observing WA's kinetic energy in a series of simulations and noting the time it takes to be displaced (if at all). Hopefully from this

work a better understanding of AMPA's and WA's dynamics in the binding pocket of iGluRs will emerge.

Finally, **Chapter 4** has also indicated that polarisation effects could mean that initial partial charge assignments of the ligand, based on gas phase calculations, may lead to problems in the MD results. As such, this can be another area to investigate.

The results from these simulations show that careful consideration of the simulation protocol is important in cases where water dynamics are important. Additionally this becomes extremely important when exploiting positions of water molecules in the binding site for the design of new drugs against these receptors.

Chapter 7

Summary

'Briefly summarised, what I did can be described as simply an act of desperation.'

Max Planck

7.1 Lessons from iGluR structures

The first publication in 1998 of the crystal structure of a soluble construct of the LBD of the AMPA-type receptor GluA2 (Armstrong et al., 1998) stimulated extensive studies on GluA2, as well as on other iGluR subunits. More than 80 crystal structures have been reported on the LBD of GluA2 alone. The structures of wildtype and mutant GluA2 proteins, combined with functional data, have led to models of receptor activation and desensitisation by agonists, inhibition by antagonists and block of desensitisation by positive allosteric modulators, as well as provided some understanding of subunit selectivity and the role of binding site water molecules. Every new crystal structure of an iGluR offers the opportunity, through structural and computational studies, to extend this knowledge in aid of *de novo* design of new selective drugs.

The goal of this thesis is to add to this growing field through computational methods and X-ray crystallography by focusing on a single aspect of these studies: the importance of water molecules in the binding site that mediate receptor ligand interactions. The insights from this thesis can be summarized here:

7.1.1 Comparative analysis of the role of water in the binding pockets of iGluRs

In **Chapters 3** and **5** relatively simple QM calculations and standard MD simulations have provided a more complete characterisation of the binding pocket and the expected behaviour of the waters within it.

In **Chapter 3**, a comparison was made between representative structures from the receptor subtypes AMPA, NMDA and KA bound to glutamate, i.e. GluA2, GluK1, GluK2 and GluN2A and one apo structure (GluA2-apo). Here results from two sets of MD simulations and one representative set of QM calculations demonstrate that the binding site of ionotropic glutamate receptors is remarkably flexible, but despite this flexibility, the water dynamics in the presence of glutamate is conserved across the iGluR family. One water molecule (W5) has been found to be conserved across iGluRs while another water at the W6 site plays a distinct role in modulating the transition of the cleft between its open and closed states. This transition has been suggested to be different in NMDA receptors (GluN2A) compared to non-NMDA receptors (GluA2, GluK1/2). This was associated with the switch in conformation of a lysine residue, which may stabilise the hinge in the closed conformation by the formation of a hydrogen bond with an aspartate located in the hinge.

Chapter 5 compares structures from within the AMPA receptor subtype, i.e. GluA2, GluA3 and GluA4 bound to glutamate. The overall findings from this study show that the structures from the AMPA receptor subtype have comparable protein dynamics with a flexible LBD. Additionally, the water dynamics in the presence of glutamate is conserved. However, insights from the QM interaction results show that not all chains display the same degree of dynamics when comparing chain A and chain B from the GluA4 dimer. It is suggested that all steps in comparative studies should be considered carefully if results are to be meaningful.

7.1.2 Quantifying water-mediated interactions in a GluA2 bound to glutamate and AMPA

Chapter 4 discussed how simple ligand-system QM interaction energies could highlight important distinctions to explain the binding modes of two agonists of GluA2, glutamate and AMPA. The presence of the conserved water, W5 (from **Chapter 3**), appears to be energetically favourable regardless of the ligand present. Simple interaction energies of ligands and waters can partly account for the observed glutamate-binding mode, however, they can not account for the crystallographically observed binding mode of AMPA. Thus, there must be other factors that control the pose of AMPA, most likely an entropic contribution, which cannot be accounted for from QM calculations.

7.1.3 Binding Mode Promiscuity of AMPA bound to the AMPA Receptor subtypes

Chapter 6 used MD simulations and X-ray crystallography to investigate the binding mode of the agonist AMPA bound to GluA2, Glu3 and GluA4. MD simulations found an alternative binding mode from AMPA when compared to the crystal structure. Further investigations through the use of the two force-fields, GROMACS and AMBER and three methods of parameterising the ligand revealed the same alternative binding mode. The study then turned to X-ray crystallography in the hopes of capturing this alternative binding mode in a different precipitant condition and different crystal form. However, the only viable crystals that could be used for data collection came from the condition previously published (Armstrong and Gouaux, 2000) and did not capture the pose.

7.2 Future Directions

The results from this thesis have provided preliminary insights into water-mediated interactions in iGluRs. Further computational studies may emerge from within these chapters and could offer complementary information to the data available in this field. They include:

- Application of standard techniques such as thermodynamic integration and free-energy perturbation to calculate the free-energy of binding for individual water molecules within the binding site of GluA2 subunits. This can be further subdivided:
 - a) *Application to the GluA2 subunit with AMPA and Glu*: The propensity for displacement of water molecules will be calculated specifically in the 1FTJ-Glu

and 1FTM-AMPA complexes. Obviously, this would only be possible once the binding mode of AMPA in the crystal structure can be maintained, through investigation of temperature and polarisation effects.

- b) *Application to the GluA2 subunit and other agonists*: Based on (a), ligand-water-receptor interaction predictions can be made for GluA2 in complex with different agonists such as kainate and quisqualate.
- c) *Application to AMPA subtype residue mutations*: (S)-Br-HIBO (BRH) and (S)-CPW399 (CPW) are examples of compounds that show selectivity in GluA2 receptors. The expression and crystallisation of a mutant of the LBD of GluA2 where Tyr702 (as found in GluA1 and GluA2) was converted into a Phe (as found in GluA3 and GluA4) led to structures of this mutant GluA2 as well as of wildtype GluA2 in complex with BRH and CPW (Frandsen et al., 2005). These structures provided a likely explanation to the molecular mechanism behind the GluA1/GluA2 selectivity and led to the identification of key residues for ligand recognition. It is the absence of a water molecule in the mutant LBD that disrupts the hydrogen binding architecture and subsequently allows the binding of the agonist to Tyr702 in wildtype GluA2. **Chapter 5** has already provided some data for the wildtype forms of GluA2, GluA3 and GluA4 bound to glutamate and can be extended through free energy calculations to calculate the binding energies of the ligand due to this point mutation.
- d) *Application of the methodology to other iGluR subtypes*; GluK1, GluK2 and GluN2A, where W5 has been seen to be conserved. The binding free energy of glutamate in these various iGluR subtypes can be calculated to answer the

following questions: 1) *How strongly is Glu bound in these receptors?* 2) *What defines selectivity?*

7.3 Water, water everywhere...

A fitting expression of the central theme of this thesis comes from an excerpt from the poem “*The Rime of the Ancient Mariner*” written by the English poet Samuel Coleridge, in 1797–98:

*“Water, water, everywhere,
And all the boards did shrink;
Water, water, everywhere,
Nor any drop to drink.”*

It is safe to say that water molecules play a crucial role in mediating the interaction between a ligand and any macromolecular receptor including iGluRs (Barillari et al., 2007). This thesis focused on studying the dynamics and energetics of water molecules to identify and quantify those that are important in mediating ligand-receptor interactions. It is true that the concepts and methods in this thesis can be transferred to other ligand gated ion channels such as the nicotinic acetylcholine receptors, or nAChRs. As long as at least one crystallographic structure of a target protein complexed with an inhibitor is available and water molecules are present in the binding site, then the binding free energy

of those water molecules can be calculated. This information can then be used to generate new ligands that maximize the interactions with conserved water molecules and target water molecules that can be displaced.

Ultimately the results herein and an understanding of the nature and role of each water molecule in the active site of a protein could greatly increase the efficiency of rational drug design approaches. In general if the propensity of a water molecule to be displaced can be determined, then synthetic efforts may be most profitably applied to the design of specific ligands with the displacement of this water molecule in mind.

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