

Single-Molecule Chemistry Studies with Engineered α -Hemolysin Pores

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Für meine Eltern

Abstract

Single-Molecule Chemistry Studies with Engineered α -Hemolysin Pores

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Engineered protein nanopores can be used to investigate a wide range of dynamic processes in real time and at the single-molecule level, for example covalent bond making and breaking or the interaction of ligands with their cognate binding sites. The detection of such processes is accomplished by monitoring the current carried by ions through the pore in an applied potential, which is modulated as molecules of interest interact with engineered binding sites within the pore. In contrast to ensemble measurements, where the behaviour of individual molecules is obscured by averaging, single-channel recordings can identify short-lived intermediates and rare reaction pathways, thereby adding to our understanding of fundamental processes in chemistry and biology. The goal of my thesis work was to engineer α -hemolysin (α HL) pores to gain insight into such processes.

Chapter 1 provides an overview of common techniques used to study single-molecule processes, in particular single channel recordings. General techniques to engineer ion channels and pores are presented, followed by examples of how the α HL pore has been engineered to monitor dynamic processes at the single-molecule level.

Chapter 2 describes how α HL pores can be chemically modified with a tridentate "half-chelator" ligand. Single channel recordings show that this modified pore

can be used to determine rates of chelation and the stability of divalent metal ion complexes. The modified pore can also be used as a stochastic sensor for the detection of different divalent metal ions in solution.

Chapter 3 investigates the chelate-cooperativity between two half-chelator ligands installed in close proximity in the α HL pore, as they form a full complex with a single Zn^{2+} ion. The single channel recordings reveal a two step process, in which the Zn^{2+} ion must first bind to one of the two half-chelators, before the second one completes the complex. The rate constants for all the major steps of the process are determined and the extent of cooperativity between the half-chelators is quantified.

Chapter 4 demonstrates that genetically encoded subunit dimers of α HL can be used to control the subunit arrangement in the heptameric pore. Although techniques exist to prepare heteroheptameric pores, pores containing more than one type of modified subunit are not commonly used because it is impossible to distinguish between the permutations of the pore. By using subunit dimers, heptamers in which two defined subunits are adjacent to each other can be formed, which increases the range of structures that can be obtained from engineered protein nanopores.

Chapter 5 explores the possibility of following the nuclease activity of a metal complex in the α HL at the single-molecule level. The Rh(III) complex $[\text{Rh}(\text{bpy})_2\text{phzi}]^{2+}$ binds strongly to CC mismatches in dsDNA, and on activation with UV light promotes the cleavage of one of the two strands. To follow this reaction by single channel recording, a piece of dsDNA with the bound Rh-complex was immobilised in the α HL pore and the single current changes under UV irradiation were monitored. The preliminary data indicate that the rate of the photocleavage reaction can be measured.

Declaration

The work described in this thesis was carried out between September 2007 and October 2011, in the laboratory of Prof. Hagan Bayley at the Chemistry Research Laboratory of the University of Oxford. All the work described within this thesis is entirely my own, unless stated otherwise in the text. With the exception of chapter 4, this work has not been submitted previously for any other degree at the University of Oxford or any other university. The work in chapter 4 was developed from a Chemistry Part II project carried out by myself at the University of Oxford. The initial results were published in the thesis "Engineering of a Transmembrane Pore: Changing the Stoichiometry and the Arrangement of an α -Hemolysin Pore by Concatenation of Subunits", which was submitted for the degree of Master of Chemistry in June 2006. A detailed list of experiments that contributed to the Part II thesis or the DPhil thesis is appended to chapter 4. Publications stemming from this work are detailed prior to the document contents.

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Abbreviations

AFM	Atomic force microscopy
Bis-Tris	2-[bis(2-hydroxyethyl)imino]-2-(hydroxymethyl)-1-3-propanediol
bpy	2, 2'-bipyridyl
chrysi	5,6-chrysenequinone diimine
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
DPhPC	1,2-diphytanoylphosphatidyl choline
dppz	dipyrido[3,2-a:2',3'-c]phenazine
dsDNA	Double-stranded DNA
DTT	Dithiothreitol
<i>E. coli</i>	Escherichia coli
EDTA	Ethylenediaminetetraacetic acid
IDA	Iminodiacetic acid
IVTT	In vitro transcription and translation
K _d	Dissociation constant
K _f	Formation constant
LB	Luria-Bertani
Luk	Leukocidin protein
MIDA	Methyl-iminodiacetic acid
MOPS	3-(4-morpholinyl)-1-propanesulfonic acid
MTS	Methanethiosulfonate
NTA	Nitrilotriacetic acid
OPSS	Orthi-pyridyl disulfide
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
PDF	Probability density function
phen	1, 10-phenanthroline
phzi	benzo[a]-phenazin-5,6-quinone diimine
PIDA	Propyliminodiacetic acid
rRCM	Rabbit red blood cell membrane
RT	Room temperature (23°C)
s.d.	Standard deviation
SDS	Sodium dodecyl sulphate

ss	Single-stranded
TIRF	Total internal reflection fluorescence
Tris	2-amino-2-(hydroxymethyl)-1-2-propanediol
UV	Ultraviolet
wt	Wild-type
α HL	α -Hemolysin
β CD	β -cyclodextrin
β PFT	β -barrel pore forming toxin

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Chapter 1

General introduction

1.1 Single-molecule studies in biology and chemistry

Single-molecule techniques have emerged as powerful tools to study biological and chemical processes at the molecular level. Many biological processes such enzyme catalysis [1, 2], the movement of a molecular motor along its track [3, 4], the folding of proteins [5] and the opening and closing of ion channels [6, 7] can be visualised as a sequence of molecular events (transitions between the distinct kinetic states of the system) that are stochastic in nature. The same applies to chemical reactions (transformations involving only small molecules), however, only few studies have investigated chemical reactions at the single-molecule level. Therefore many concepts presented in this section will deal with biological processes, but I will provide chemical examples where possible.

In classical ensemble experiments the properties of individual molecules are obscured by population averaging and one can only obtain simplified kinetic parameters to describe the system (e.g. k_{cat} for enzymes [8, 9], the mean displacement of an actin filament on immobilised myosin filaments [10], k_{app} for a chemical reaction). Single-molecule techniques can identify rare or transient intermediates and heterogeneity in the distribution of molecular properties [11, 12]. Moreover, many single-molecule techniques have excellent spatial and temporal resolution, making them suitable for

the study of dynamic processes in real time [13, 14]. Single-molecule processes can be studied using a variety of techniques. The choice of technique depends on the variable to be observed. The examples presented below do not constitute a comprehensive review, rather they are meant to illustrate the power, but also the limitations of the different approaches. Commonly used single-molecule methods are fluorescence, force measurements and electrophysiological techniques.

1.1.1 Single-molecule fluorescence studies

Fluorescence methods have been used very extensively in single-molecule studies and they are particularly useful to study particle-particle interactions and enzyme turnover. When a fluorophore absorbs a photon, it reaches an excited electronic state. Through collisions with other molecules in solution, the molecule can relax to the lowest vibrational state of the electronically excited state, however, the surrounding molecules cannot accept the large amount of energy required for the fluorophore to relax to the electronic ground state. Instead the excited molecule spontaneously emits a photon of longer wavelength (Figure 1.1). This process is termed fluorescence [15]. Although it is possible to image intrinsically fluorescent

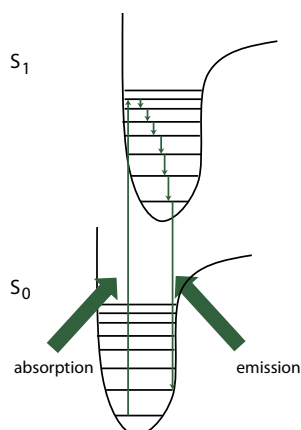


Figure 1.1: Steps leading to fluorescence: A fluorophore absorbs a photon and makes a transition to an excited electronic state. The fluorophore undergoes radiationless decay to the lowest vibrational state of the excited electronic state by interaction with surrounding molecules. A radiative transition then occurs to the electronic ground state. Figure adapted from [15].

particles, the majority of particles require labelling with an extrinsic fluorophore [16]. Often the fluorophore is linked to the particle in such a way that any perturbation of the particle (e.g. bond cleavage by an enzyme [16], a conformational change [17] or an interaction with another particle [14]) leads to a change in the fluorescence properties, thereby dynamic processes can be monitored. For single-molecule experiments it is important that the fluorescence of only one particle is collected at any one time. To achieve this, the concentrations of the reagents is kept low, such that only one fluorophore is present per detection limited volume [14]. Another way to achieve this is to drastically narrow the field of excitation. This can be done by using an evanescent wave to excite the fluorophores (as in total internal reflection fluorescence, TIRF, microscopy) that can only permeate a distance of 100-200 nm (Figure 1.2A). Wang *et al.* used TIRF microscopy to determine the single-molecule binding kinetics of the bacteriophage λ -repressor CI and its target DNA (Figure 1.2) [18]. In their experiments, green fluorescently-labelled DNA with transcription factor binding sites was immobilised on a coverslip and incubated with red fluorescently-labelled transcription factor. Both molecules were visualised by TIRF microscopy. Co-localised red and green spots revealed the binding of a transcription factor to a piece of DNA.

A second method to limit the field of excitation is to bring a laser beam to a near diffraction limited spot by focusing it through a high numerical aperture objective on a microscope (confocal microscopy). Confocal microscopy was used to monitor the formation and dissociation of individual metal chelator complexes [20]. In this experiment the chelator was tethered to a glass surface via a piece of DNA. A complementary piece of DNA with a fluorescent label was hybridised to the chelator strand. In the absence of divalent metal ions the label fluoresced, however, when divalent metal ions were bound to the chelator the fluorescence was quenched by electron transfer [21] (Figure 1.3A).

Eid *et al.* also limited the field of excitation in their experiment by confining the fluorophore to a zeptoliter (10^{-21} l) hole in the metal coating of a glass slide [22].

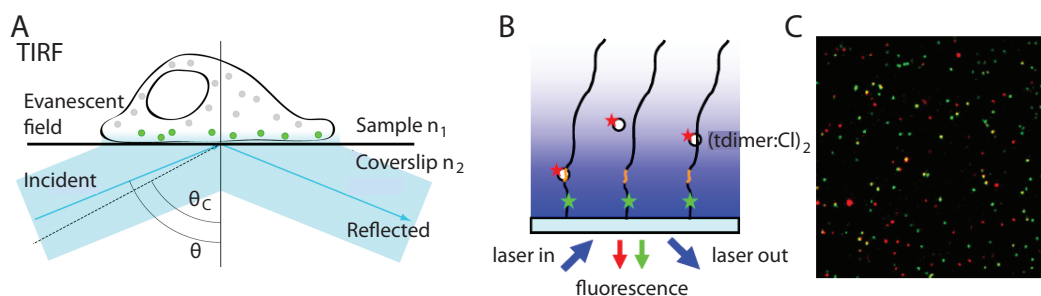


Figure 1.2: Total internal reflection fluorescence microscopy: **A** The excitation beam is directed at the sample at an angle θ that is larger than the critical angle θ_c , leading to total internal reflection of the beam. An evanescent wave is generated on the opposite side of the interface. The evanescent field has a thickness of 100-200 nm, therefore only fluorophores on the surface are excited. This panel was adapted from [19]. **B** Green fluorescently-labelled DNA molecules with transcription factor binding sites are immobilised on a coverslip and incubated with red fluorescently-labelled transcription factor. Both molecules are visualised by TIRF microscopy. **C** Superposition of red and green fluorescence images. Co-localised red and green spots indicate transcription factor binding to the DNA. Panels B, C were adapted from [18].

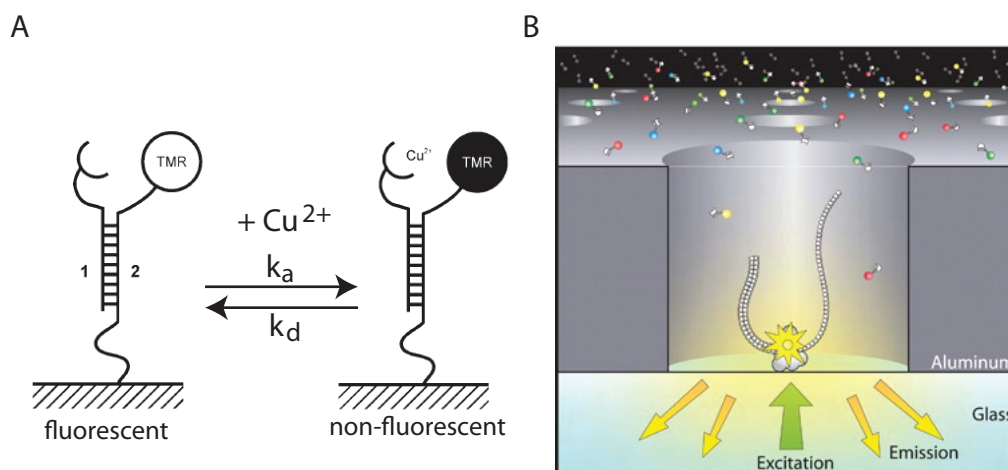


Figure 1.3: Single-molecule fluorescence studies: **A** Real-time detection of metal ion chelation: A chelator is tethered to a glass surface via a piece of DNA. A complementary piece of DNA with a fluorescent label is hybridised to the chelator strand. In the absence of divalent metal ions the label fluoresces, however, when divalent metal ions are bound to the chelator the fluorescence is quenched. Panel adapted from [20]. **B** Real-time DNA sequencing from single polymerase molecules: A single-molecule of DNA template-bound $\phi 29$ polymerase is immobilised at the bottom of a zeptoliter sized hole and illuminated by laser light from below. The dimension of the hole does not permit the propagation of light into the bulk solution. The incorporation of fluorescently-labelled phospholinked dNTPs can be monitored in real time and at the single-molecule level. Panel adapted from [22].

Since the size of the hole is smaller than the wavelength of light, the excitation light cannot propagate into the bulk of the solution and only the molecules inside the

hole are visualised. It has been shown that the observation volume accessible to this technique is 10 zeptoliters (four orders of magnitude smaller than the diffraction limit of light), which means that it is possible to work with fluorophores at concentrations of up to 200 μM and still observe only one fluorescent molecule per detection volume [23]. With this method the DNA synthesis by a single $\phi 29$ polymerase could be visualised: the polymerase was immobilised with a template DNA strand at the bottom of a zeptoliter hole and nucleotides that had been conjugated to fluorescent labels via a penta-phosphate linker were added to the bulk solution. Each type of modified nucleotide (A, T C and G) carried a different label. When a modified nucleotide entered the active site of the immobilised enzyme to base-pair with the template strand, a fluorescent pulse was recorded. As the phosphodiester bond with the emerging strand was formed, the fluorescent by-product was released and diffused out of hole. This terminated the fluorescence signal. For each nucleotide that was added to the emerging strand, a fluorescent signal was recorded, allowing the template DNA to be sequenced in real time (Figure 1.3B).

Although fluorescence methods are very powerful, they suffer from some drawbacks. Following prolonged excitation, fluorophores can break down (photobleaching) rendering them useless for further investigation. For single-molecule experiments the concentration of fluorescent particles is usually in the nanomolar range. However, many equilibrium binding processes in biology have K_d values in the micromolar range, therefore they cannot be studied at the reagent concentrations compatible with single-molecule fluorescence experiments [14].

1.1.2 Single-molecule force measurements

Molecular scale force drives many biological processes, for example cellular motility and DNA replication and segregation [24]. Conversely, the elimination or reduction of motion through the binding of ligands to their receptors or the folding of biopolymers involves the formation of bonds to overcome thermal and other forces [24]. Force spectroscopy can be used to probe such forces at the single-molecule

level.

Optical tweezers are a type of optical trap for particles, which is created by focusing a laser beam to a diffraction limited spot through a high numerical aperture microscope objective. The narrowest point of the focused beam contains a very strong electric field gradient, which attracts dielectric particles towards the focus and immobilises them. For small displacements, the force required to remove a particle from its equilibrium position is linearly proportional to the displacement. In a seminal paper Svoboda and co-workers demonstrated that the steps taken by individual kinesin molecules on immobilised microtubules could be monitored if the kinesins were attached to an optically trapped silica bead and the displacement of the bead was mapped (Figure 1.4) [25].

Atomic force microscopy is normally used to map large areas of a surface. In these experiments a microscopic probe on a cantilever is brought into close contact with the specimen surface. As the probe is scanned across the surface of the probe, the varying forces between the tip and the surface lead to a deflection of the cantilever, from which the topography of the surface can be constructed. However, AFM can also be used to measure the stability of protein folds. To do so, the AFM tip is brought into direct contact with proteins immobilised on a sample surface. A fraction of a protein will adsorb onto the tip. The sample surface is then retracted vertically and the force required to move the tip is measured as a function of distance (Figure 1.4). The force-induced unfolding of proteins can then be monitored at the single-molecule level. This technique was used to monitor the reversible unfolding of several immunoglobulin domains of a titin molecule [26]. Although powerful, force methods are naturally limited to mechanochemical problems. Interestingly AFM has also been used to probe the mechanical strengths of individual Si-C and S-Au bonds [27]. In this experiment an amylose chain was attached either to a silicon oxide surface or to a gold surface. The AFM tip was used to stretch the chain and the force that was required to dissociate the chain from the surface was measured. This is a rare example of the strength of an individual chemical bond being measured

at the single-molecule level.

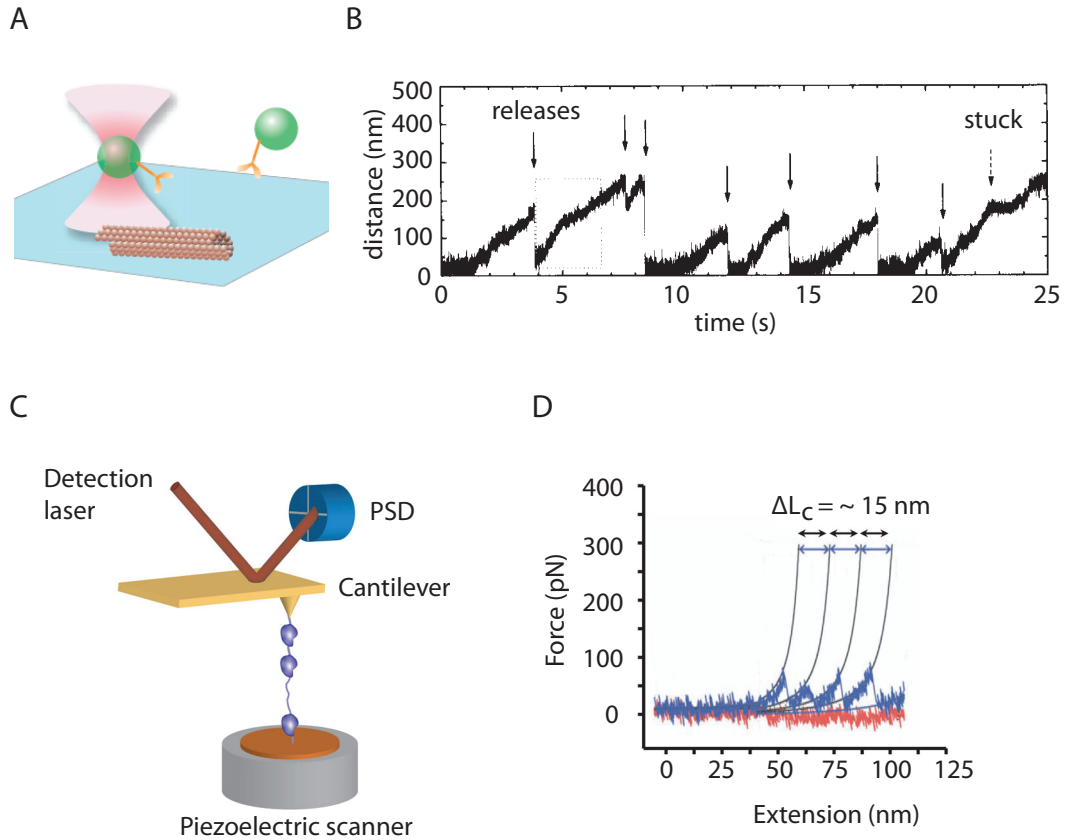


Figure 1.4: Single-molecule force measurements: **A** A silica bead is sparsely coated with kinesin molecules and captured in an optical trap. The trapped bead is brought to a microtubule immobilised on a glass coverslip and a kinesin molecule attaches to the microtubule. Panel adapted from [24]. **B** The displacement generated by the individual kinesin molecule as it walks along the microtubule is determined from the displacement of the bead in the optical trap. Multiple cycles of movement, release (indicated by arrows) and reattachment can be seen. Panel adapted from [25]. **C** Illustration of an atomic force microscope. A cantilever with a sharp tip is held above a piezoelectric scanning stage. A protein molecule is attached to the sample surface and also the AFM tip. As the piezoelectric stage is retracted, the molecule experiences a force. This force can be quantified by measuring the deflection of the cantilever as a function of distance. **D** A molecular force probe measurement shows the force-induced unfolding of four folded domains of a hypothetical protein. Panels C and D are adapted from [24].

1.1.3 Electrical measurements through individual ion channels and pores

Ion channels and pores are transmembrane proteins that allow the passage of certain substances across a cell membrane down their electrochemical gradients. Although

a pore is the focus of this thesis, the electrophysiological techniques used for its characterisation were developed initially for the study of ion channels. For this reason this section will provide examples from both classes of protein. Ion channels allow the selective passage of dehydrated ions (eg. K^+ , Na^+) from one side of a bilayer to the other through a narrow channel [7, 28]. They are not constitutively open, rather they activate in response to stimuli such as changes in the membrane potential or binding of a ligand. They are involved in a range of processes, for example the transmission of fast impulses in the nervous system, the release of insulin in pancreatic cells and most types of muscle contraction [28].

In contrast to ion channels, biological pores have a larger diameter and they allow the passage of fully hydrated ions and molecules of low molecular weight with little selectivity [29]. Many pores are of bacterial origin, for example the outer membrane of *E. coli* is densely covered with OmpF and maltoporin pores [30]. However, some bacteria also release pore-forming toxins as a form of attack [29, 31]. Pore forming toxins, especially α -hemolysin, have been investigated in great detail, and have proven to be excellent protein engineering targets, as will be discussed later.

Since both channels and pores are involved in the flux of ions across cell membranes, their function can be assessed by measuring the ionic current through the protein under an applied potential. The voltage clamp technique was developed in the late 1940s by Cole [28] and it can be used to measure the combined current through a large number of ion channels (Figure 1.5A). However, it was the development of the “patch-clamp” technique by Sakmann and Neher in the late 1970s [34] (Figure 1.5B) that allowed the electrophysiological characterisation of individual ion channels in cells for the first time. Patch clamping involves attaching a glass pipette that contains a microelectrode against a patch of cell membrane. By applying a slight negative pressure, a GigaOhm ($G\Omega$) seal forms. When a potential difference is applied, the current through an individual ion channel contained in the patch of membrane can be measured. Sakman and Neher received the 1991 Nobel Prize in Physiology and Medicine in recognition of their work. Patch clamping

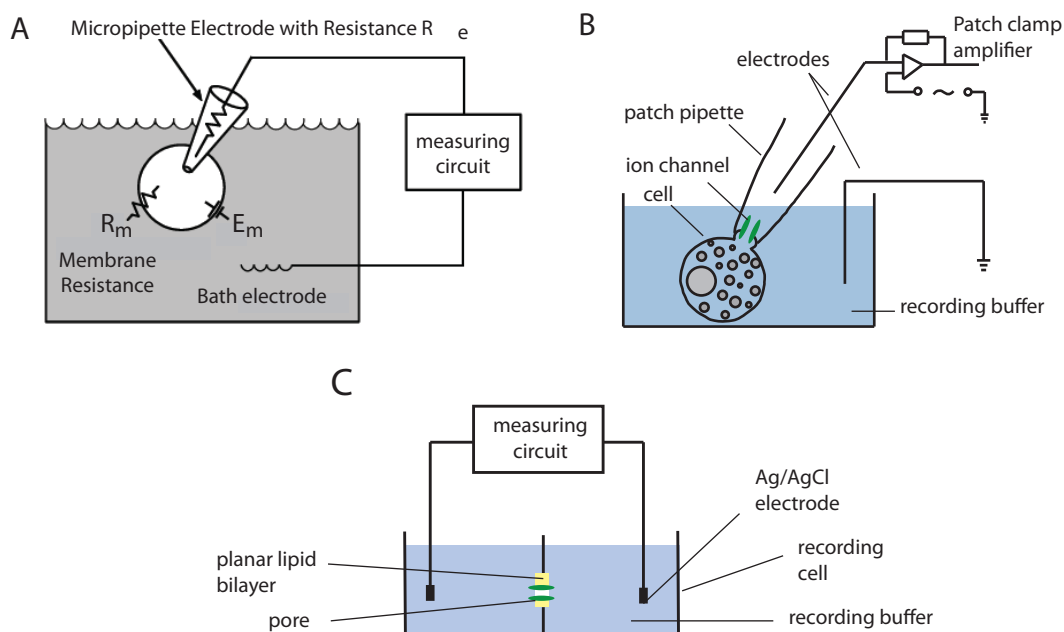


Figure 1.5: *Electrophysiological techniques. A Voltage Clamp technique: A potential difference is applied between an intracellular and a bath electrode. The resulting current through a large number of ion channels in the cell membrane is measured. Panel adapted from [32]. B Patch clamp technique: A glass pipette containing a microelectrode is attached to a patch of cell membrane. A slight negative pressure is applied and a $G\Omega$ seal is formed. The single channel current through ion channels contained in the patch can be measured under an applied potential. Panel adapted from [33]. C Single channel recording from pores reconstituted into a planar lipid bilayer. The recording cell consists of two buffer compartments that are separated by a Teflon sheet. The Teflon has a small $100\text{--}120\text{ }\mu\text{m}$ aperture, over which the planar lipid bilayer is formed. Individual pores are inserted into the bilayer by adding a solution of protein in detergent to the buffer. The single channel current under an applied potential is measured through a set of Ag/AgCl electrodes.*

allows the study of ion channels and pores in their native environment, however, the complexity of biological systems can create difficulties. The activity of many channels can be modulated, for example by phosphorylation through protein kinases [28] or by interaction of the protein with particular lipids [35]. Furthermore the ion channels of intracellular organelles are not readily available to patch pipettes. Such complications can be overcome by studying individual ion channels in artificial bilayers of defined composition.

Artificial planar lipid bilayers (Figure 1.5C) can be formed by “painting” lipids dissolved in an organic solvent onto an aperture that separates two aqueous compartments [36]. Alternatively, the planar bilayer can be formed by folding

together two lipid monolayers, each present at an air-water interface, over an aperture [37]. The experiments described in this thesis were carried out using folded planar lipid bilayers. A typical setup for single channel recordings consists of a recording chamber with two buffer compartments (1 ml each) that are separated by a 25 μm thick piece of Teflon film. The Teflon film contains a 100-120 μm aperture over which the bilayer is formed. To do so, the aperture is first pre-treated on both sides with a small amount of a hydrophobic oil such as hexadecane or squalene. This creates an oil annulus around the aperture. Next, the recording solution is added to the recording chamber and a drop of phospholipid in volatile solvent (e.g. pentane) is applied to the surface of the recording solution. As the solvent evaporates, a monolayer of phospholipids forms on the surface. Sequential lowering of the buffer solution levels folds the two monolayers together over the aperture to form a vertical lipid bilayer. Channel proteins or pores can be inserted into the bilayer by adding detergent solubilised protein to the recording buffer, or by fusing protein-containing vesicles with the bilayer [36]. To carry out single channel recordings, a very dilute solution of the detergent-solubilised protein is added to the recording buffer, such as to minimise the chance of multiple protein insertion events. The solution is then stirred mechanically until a single channel inserts into the lipid bilayer. Channel or pore insertion is evident as a jump of the current from zero to that of the open channel or pore under an applied potential. When the recording buffer is no longer stirred, the probability of additional protein insertion events is decreased, but not eliminated. The process of bilayer formation, channel insertion and electrical recording is described in more detail in the Materials and Methods section of this thesis.

Early single channel recordings focused on the biophysical characterisation of native ion channels, for example to understand factors such as the effect of the applied voltage or ligand concentration on the activity of the channel. However, with the advances of molecular biology and chemistry, it has become possible to probe the structural role of individual amino acids or to engineer proteins with completely

novel properties. The next section provides an overview of the commonly used techniques to engineer channels and pores and will lead to example of how such proteins can be used to investigate dynamic processes at the single-molecule level.

1.2 Engineered channels and pores

1.2.1 Genetic engineering

At the simplest level systematic site-directed mutagenesis can help identify the functional role of individual amino acids in a protein. For example, it was found that the E71A mutation suppresses the inactivation of KcsA potassium channels [38]. It was therefore inferred that this amino acid plays a role in the gating mechanism of the protein. Site-directed mutagenesis can also be used to engineer proteins with enhanced properties. Channelrhodopsins are a family of proteins that function as light-activated ion channels. They have emerged as popular tools to study neuronal circuits, because channel activation and therefore neuronal excitability can be brought about by light and does not require impalement of the neuron with electrodes. By systematically mutating the channelrhodopsins, some variants were found that showed enhanced light sensitivity or improved kinetics of activation and it is hoped that properties such as the conductivity, the ion selectivity and the spectral response of the channel can also soon be addressed [39, 40].

Mutations are not limited to single amino acids. The role of protein domains can be explained if they are transferred between related proteins. When the voltage sensing domain of a eukaryotic potassium channel (Kv2.1) was replaced with the paddle motif from an archaeobacterial potassium channel (KvAP), the chimera channels still showed voltage dependent gating, confirming that the paddle motif is in fact a modular voltage sensor [41]. Chimera channels can also be used to enhance expression levels of some proteins. For example, it was found that the eukaryotic inward rectifier Kir3.1 would not express in *E. coli* and it was therefore difficult to obtain sufficient amounts of the protein for crystallisation trials. However, when the

extracellular domain of a bacterial KirBac channel was fused to the transmembrane domain of the Kir3.1 channel, the protein did express well and sufficient protein was produced to grow crystals for the structure determination by X-ray crystallography [42].

Finally, many ion channels can comprise different types of subunits in varying numbers. For example, one type of nicotinic acetylcholine receptor (nAChR) can comprise α and β subunits, and the possible channel stoichiometries are $\alpha_2\beta_3$ and $\alpha_3\beta_2$. By creating genetic fusions between α and β subunits, Zhou *et al.* created nAChRs of defined subunit composition which were characterised by single channel recording [43] (Figure 1.6A). Similarly Jayasinghe *et al.* created concatemers of the LukF and LukS subunits of the leukocidin pore, which were used to prove an alternating subunit arrangement of the leukocidin pore [44] (Figure 1.6B).

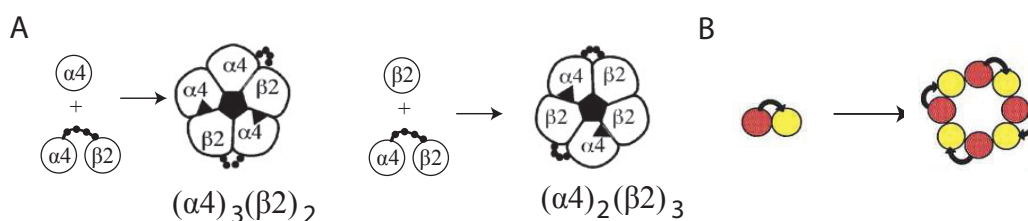


Figure 1.6: Subunit concatemers give rise to channels of pre-determined subunit arrangement. **A** The combination of wild-type α subunits with dimers comprising one α and one β subunit of the acetylcholine receptor gives rise to $\alpha_3\beta_2$ channels only. Similarly, the combination of wild-type β subunits with dimers comprising one α and one β subunit gives rise to $\alpha_2\beta_3$ channels only. Panel adapted from [43]. **B** Subunit dimers of LukF and LukS give rise to octameric leukocidin pores with an alternating arrangement of subunits. Panel adapted from [44]

1.2.2 Covalent chemical modification

The modifications described so far are limited to the twenty naturally occurring amino acids, however many more modifications can be introduced by chemically modifying amino acid side chains. Although several side chains can be made to react readily (e.g. cysteine, lysine, arginine [45]), the relatively high abundance of some amino acids in proteins (e.g. lysine, $\sim 7\%$ of the yeast proteome [46]) make selective modification of only one residue difficult. The low natural abundance of

cysteines ($\sim 1\%$ of the yeast proteome [46]) and the high nucleophilicity of the thiol group as thiolate make cysteine residues an ideal target for the selective chemical modification of proteins. Cysteines react specifically with a variety of reagents that can be incorporated into larger probes, for example, they can be alkylated with iodoacetamide reagents, they can undergo Michael additions with maleimides, and they can form disulfide bonds with methanethiosulfonate (MTS) or ortho-pyridyl disulfide (OPSS) reagents (Figure 1.7) [45, 47].

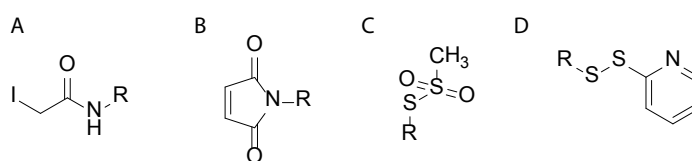


Figure 1.7: Cysteine modification reagents: **A** iodoacetamides **B** maleimides **C** Methanethiosulfonates (MTS) **D** ortho-pyridyl disulfides (OPSS)

Mindell and co-workers [48] used a combination of site-directed mutagenesis and chemical modification to map residues that form the channel entrance of the diphtheria toxin. In their experiments the residues surrounding the channel entrance were mutated to cysteine. The protein was then subjected to analysis by single channel recording in planar lipid bilayers. When an MTS reagent was added to the recording buffer, a sharp decrease in the single channel current was observed for some mutants only. The authors inferred that only the residues accessible to the reagent can be located at the channel entrance. Remarkably, the authors also observed formation of the covalent bond between the cysteine residue and the MTS reagent at the single-molecule level (Figure 1.8).

Covalent chemical modification at cysteine can also be used to engineer switches into ion channels channels. Volgraf *et al.* [49] covalently attached a glutamate agonist to the ligand binding site of an ionotropic glutamate receptor. The reagent used for the reaction consisted of a glutamate headgroup, a maleimide for reaction with the protein and an azobenzene photoswitch (Figure 1.9). When the protein was illuminated at 380 nm, the azobenzene group isomerised from trans to cis, allowing the ligand to bind to the protein and thereby activate the channel. The

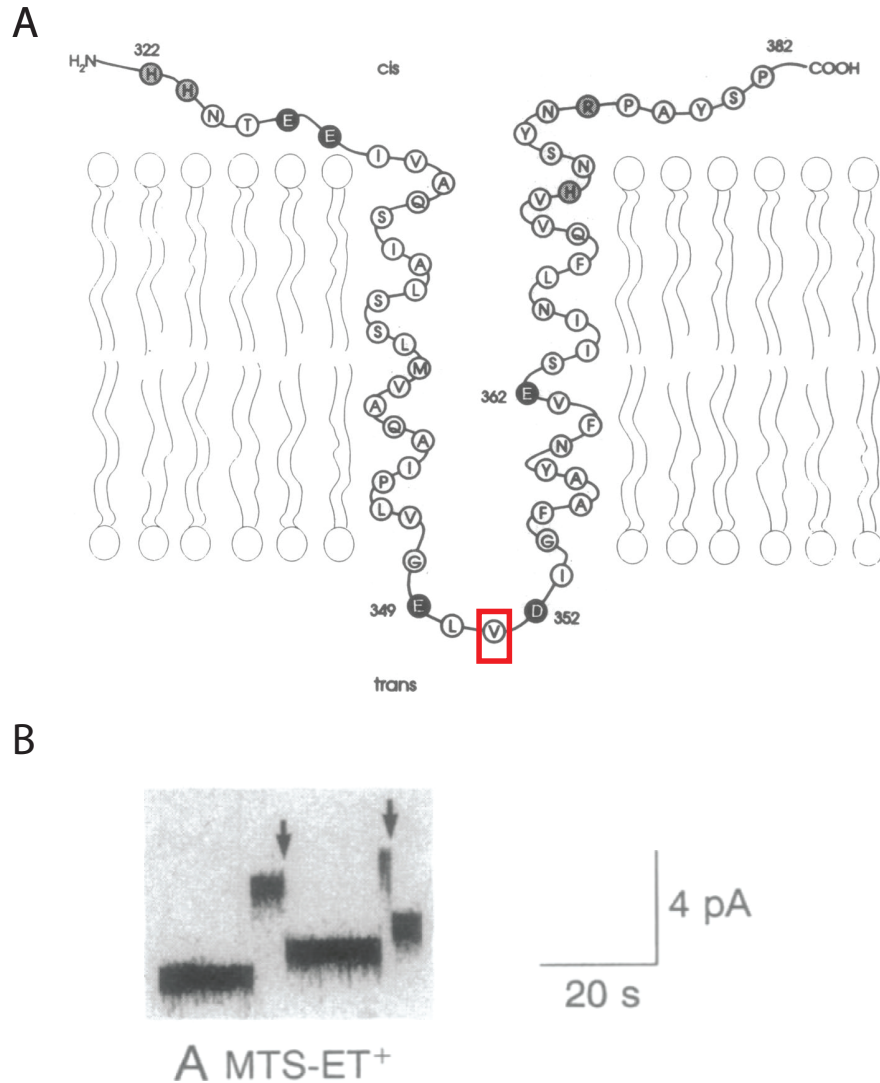


Figure 1.8: Reactions of V351 diphtheria toxin channels with MTS derivatives. **A** Proposed transmembrane topography of the diphtheria toxin channel forming domain. Residue V351 is boxed. **B** Single channel recording of a diphtheria toxin channel. A channel opens, as can be seen by an increase in the conductance. After a further 6 s the reaction of V351C with the MTS reagent occurs (indicated by the arrow), resulting in a drop of the conductance of the channel by a factor of 3. A second channel then opens, and V351C of this channel also reacts with the MTS reagent approximately 2 s after the channel opened. Figure adapted from [48].

isomerisation could be reversed by illuminating the protein at 500 nm. This switch permits optical control of the function of the ion channel.

The covalent modification at cysteine residues is also commonly used to incorporate structural probes into channels, for example pairs of fluorophores for fluorescence resonance energy transfer (FRET) measurements [50] or spin labels for electron

paramagnetic resonance spectroscopy [51].

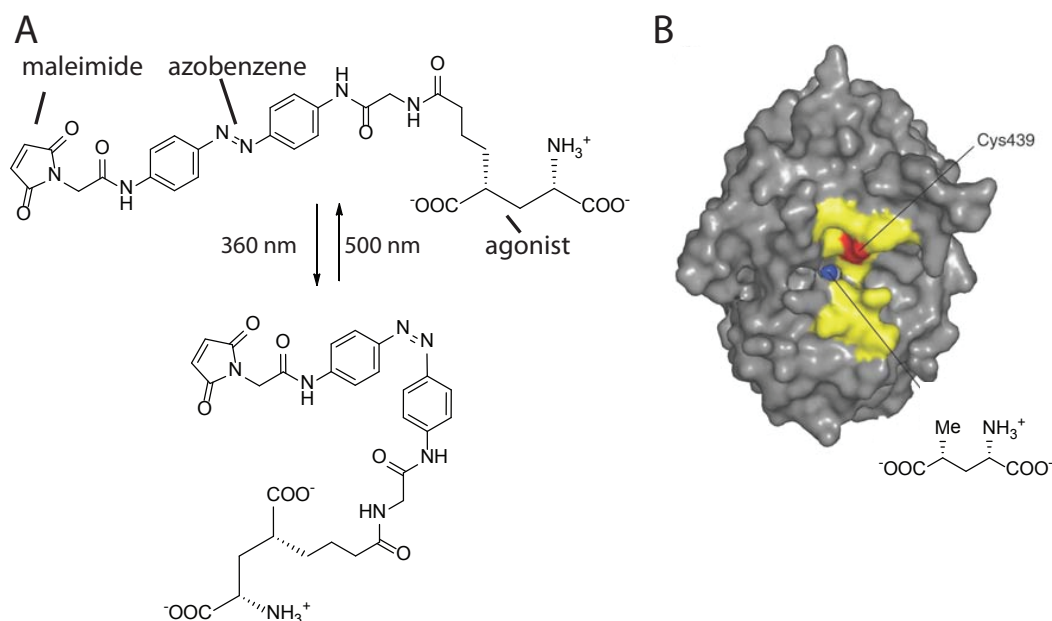


Figure 1.9: Covalent modification at cysteine to introduce an optical switch into a glutamate receptor. **A** Structure of the glutamate ligand used for the chemical modification of the protein. **B** View into the ligand binding site (blue) of the *iGluR6* glutamate receptor. Residue 439 (yellow) was mutated to cysteine and used to covalently attach the glutamate agonist to the protein. Figure adapted from [49].

1.2.3 Un-natural amino acids and semi-synthetic proteins

Although the chemical modification of cysteine residues is very versatile, there are some limitations. As indicated previously, it is not usually possible to modify more than one reagent-accessible cysteine residue selectively and one has to ensure that the reaction has gone to completion. Moreover, most modifications will be relatively large, as the molecule of interest has to be coupled to a reactive group in order to be incorporated into the protein. This can be a problem if the effect of small structural differences on the function of a channel is to be probed. One way to avoid such problems is to use genetically encoded un-natural amino acids. For this technique an amber stop codon (TAG) is incorporated at the position coding for the residue of interest. A suppressor tRNA that recognises the stop codon is then loaded with the un-natural amino acid and added to the translation system

or injected into cells (e.g. *Xenopus laevis* oocytes) that will express the protein. Using a series of proline analogs, this method was used to show that the cis-trans isomerisation of a proline residue of the 5-HT₃ receptor acts as a switch that opens the channel [52]. Similarly the binding mode of acetylcholine to the α subunit of the acetylcholine receptor was revealed in detail. It was thought that binding was mediated by the cation- π interaction between the choline group and a tryptophan residue at the ligand binding site. When the hydrogens on the aromatic ring of the tryptophan residue were substituted with electron-withdrawing fluorines, the interaction between the ligand and the protein was weakened, as would be expected for a cation- π interaction [53].

Although this powerful technique has been used to incorporate more than 70 unnatural amino acids into proteins [54], low protein yields are common because tRNA release factors compete with the amber suppressor tRNA at the amber codon. Chemically synthesised proteins avoid some of the problems described above completely. Solid-phase peptide synthesis can be used to synthesise peptides of up to 50 amino acids in length and almost any type of unnatural amino acid can be incorporated into the peptide. However, as most proteins are longer than 50 amino acids, it is usually necessary to combine fragments in order to obtain full length proteins. Native chemical ligation, which involves the ligation of two synthetic fragments, has been used for the total synthesis of functional HIV-1 Vpu channels [55] and also of functional MscL channels [56]. It is also possible couple the synthetic fragment with a much larger recombinant fragment. This technique is termed expressed protein ligation and has been used to prepare functional KcsA [57] and NaK [58] channels and also OmpF pores [59]. Synthetic methods are useful for incorporating unnatural amino acids with azide side chains into proteins. The azide side chain reacts selectively and rapidly with alkynes in the presence of Cu²⁺ ions (click chemistry) and provides a unique handle for introducing functionality into proteins. Click chemistry has been used to modify gramicidin A, a 15 residue peptide that forms channels in lipid bilayers [60]. The technique could also become more

useful for the modification of ion channels. One major drawback of the synthetic approach is the limited size and low solubility of the synthetic fragment [61], as well as the fact that the protein must be refolded properly before its function can be assessed [62].

1.2.4 Directed evolution techniques

Directed evolution techniques have been used very extensively to optimise the function and characteristics of cytosolic proteins and they have even been used to develop proteins with new functions [63]. However, they have barely been used for the evolution of ion channel or pore function, mainly because there are no high-throughput screening techniques to assess the function of such proteins. Although recent years have seen lots of progress with the development of the automated patch-clamp method, the state of the art machines (e.g. the IonWorks Barracuda machine, Molecular Devices) can at present only process 384-well plates which is insufficient to screen the huge diversity that can be encoded in a cDNA library. Furthermore, automated patch clamping does not work well on cells that express the protein of interest only transiently [64].

1.3 Engineered α -hemolysin pores

The α -hemolysin (α HL) pore has been engineered very extensively for a variety of applications, for example for the controlled permeabilization of cells [65, 66], stochastic sensing [67], nucleic acid detection and sequencing [68, 69], the examination of single-molecule chemistry [70] and the construction of prototissues based on droplets connected by bilayers [71, 72]. One of the reasons why α HL is so suitable for protein engineering purposes is that the protein structure is exceptionally stable. The protein can tolerate extensive mutagenesis (both in the β -barrel and in the cap domain) and chemical modification [73], and it is stable in SDS at up

to 65°C [74]. Furthermore, the protein has very well-defined electrophysiological properties. The pore does not gate at potentials between ± 100 mV and it shows very little ion selectivity [73]. Before providing examples of engineering α HL pores for the study of dynamic processes at the single-molecule level, an overview of the protein is presented.

1.3.1 Overview of the α -hemolysin pore: Structure and assembly

α -Hemolysin (α HL) is a 293 residue water soluble polypeptide that is secreted by *Staphylococcus aureus*. The 32 kDa monomer can bind to susceptible cell membranes, on which it oligomerises to form a heptameric transmembrane pore. α HL belongs to the structural family of small β -barrel pore-forming toxins (β PFTs). Other members of this family include γ -hemolysin, leukocidin and the Pantone-Valentine leukocidins from *S. aureus* [75], hemolysin II [76] and cytotoxin K [77] from *B. cereus*, cytolysin from *V. cholerae* and aerolysin from *A. hydrophila* [78]. β PFTs are a potent and ubiquitous class of virulence factors. Once assembled, these proteins destroy target cells by permeabilization of the cell membrane to ions, water, and molecules of low molecular weight, which results in lysis for some cell types [79]. α HL assembles on many different types of membrane, for example rabbit and human erythrocytes [80], human platelets and monocytes [80], as well as supported [81] and planar lipid bilayers [82]. The susceptibility of different cell types or substrates to attack by α HL varies by several orders of magnitude [80]. Recent evidence has suggested that α HL binding to the cell membrane is facilitated by metalloprotease 10 (ADAM 10) receptors [83], but in the absence of these receptors, the protein can also bind to clusters of phosphatidylcholine lipid headgroups instead [84].

1.3.2 Structure of α HL

The structure of the α HL oligomer in detergent has been solved by X-ray crystallography to 1.9 Å resolution [85]. The crystal structure shows that the oligomer is a heptamer. An AFM study suggested that α HL can form hexamers

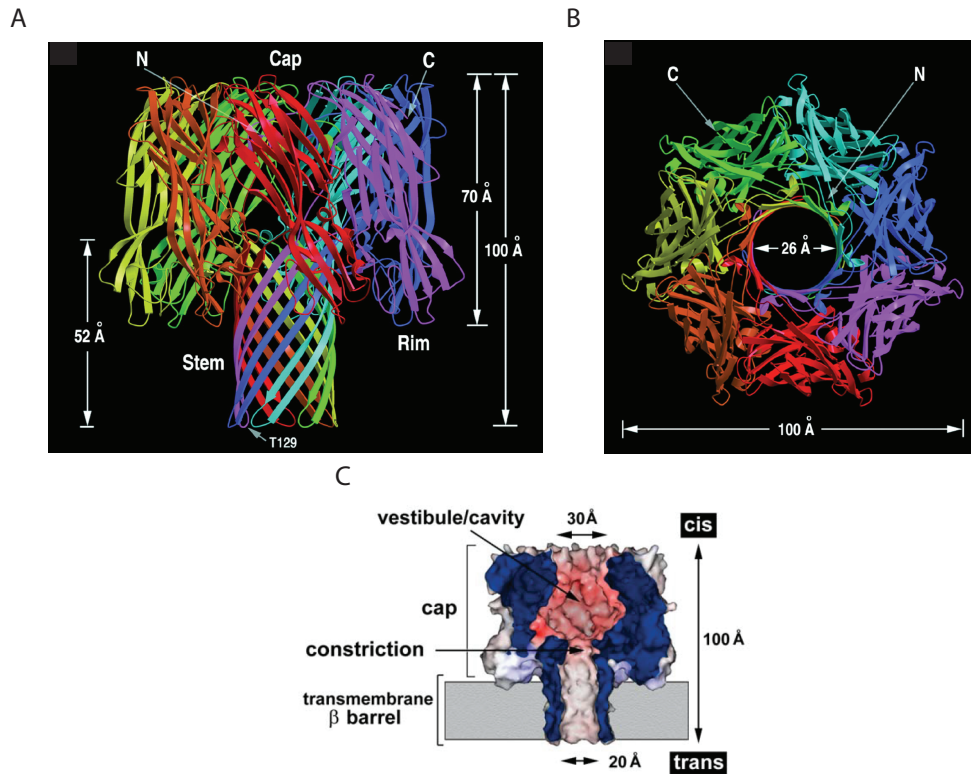


Figure 1.10: *Crystal structure of the α HL heptamer. **A + B** Side and top view of the heptamer. Each subunit is shown in a different colour. **C** Cross-section through the heptamer showing the dimensions of the aqueous channel that spans the protein.*

under certain conditions [86]. Therefore the possibility exists that a small fraction of pores are hexamers. Chapter 4 will investigate if the α HL oligomer can be forced to adopt stoichiometries other than a heptamer (e.g. a hexamer or an octamer).

The crystal structure shows that α HL forms a mushroom-shaped oligomer that measures approximately 100 Å in height by up to 100 Å in diameter. The oligomer has three main domains: the stem, cap, and rim domains. The stem domain comprises 14 β strands, of which 2 are contributed by each monomer. Together they form a righthand β -barrel with a height of 52 Å and a diameter of 26 Å. The cap domain is composed of seven β -sandwiches and the amino latches of each protomer. The amino latch (Ala1-Val20) of one subunit makes extensive contacts with the inner β -sheet of the next clockwise subunit. The amino latch used to be considered essential for the formation of lytic pores [87]; however, more recent experiments have shown that up to 17 residues can be deleted from the N-terminus while retaining

the ability to form pores [88]. This made the generation of genetically encoded subunit dimers of α HL feasible, in which the N-terminus of one subunit is linked to the C-terminus of the next clockwise subunit. This will be discussed in chapter 4. The rim domains protrude from the underside of the heptamer. They are involved in only few subunit interactions and are in either close or direct contact with the lipid bilayer. A solvent-filled channel runs along the seven-fold axis of the protein from the top of the cap domain through a constriction at Met113 to the β -barrel. The width of this channel varies. At the narrowest part of the protein (the constriction at M113) it is 14 Å wide, whereas at the widest part in the cap domain it is 46 Å wide.

1.3.3 The assembly pathway of α HL

The crystal structure of the α HL heptamer represents the end point of the assembly of the protein. No crystal structure is available for the monomer of α HL. However, the crystal structures of monomeric LukF and LukS, which form the bicomponent octameric leukocidin pore and which share approximately 20-30% sequence homology with α HL, have been solved (Figure 1.11B, [89, 90]). The Luk structures serve as prototypes for the starting point in the assembly of α HL. The fold of the Luk monomers resembles that of the α HL protomers in the heptameric pore, with the exception of the amino latch and stem domains, which are folded against the core of the protein in the monomer, but which protrude away from the core of the protein in the oligomer. The formation of the oligomer therefore involves the re-arrangement of these domains. More recently, the crystal structure of the monomer [91] and heptamer [92] of the *Vibrio cholerae* cytolysin (VCC, a small β PFT) were determined. The cytolytic domain of the VCC monomer has a low extent of sequence homology with both α HL (16.5%) and LukF (12.9%), but the VCC monomer fold bears a strong resemblance to the LukF monomer and the oligomer fold bears a strong resemblance to the α HL heptamer (Figure 1.11C). These structures show that the monomers and oligomers of β PFTs share similar

folds and re-inforce the concept that the β PFTs share a common assembly pathway.

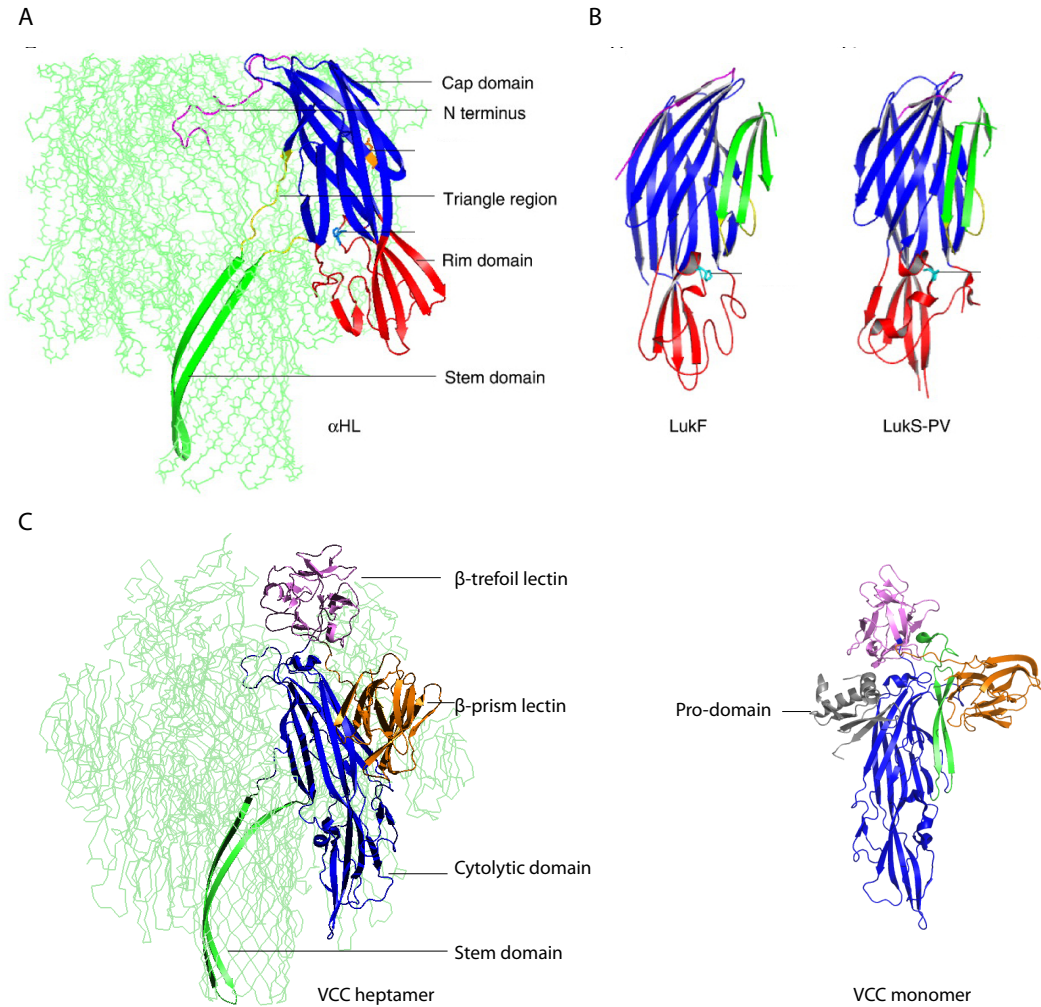


Figure 1.11: Crystal structures of monomers and oligomers of β PFTs. **A** The α HL heptamer (PDB code 7AHL). **B** The LukF and LukS monomers (PDB codes 1LKF and 1T5R). **C** The heptamer and monomer of the *V. cholerae* cytotoxin (PDB codes 3O44 and 1XEZ). Panels A and B were adapted from [88]. Panel C was generated from the PDB files in PyMOL.

The current model for the assembly of the α HL oligomer comprises four distinct stages (Figure 1.12): first, water-soluble monomers (1) bind to the membrane. The membrane-bound monomers (2) then form a prepore (3) by lateral diffusion and through intersubunit interactions. The prepore is then converted into the transmembrane pore (4), which involves the insertion of the stem domains into the lipid bilayer and the N-terminus latching onto the next subunit [88, 93]. The prepore

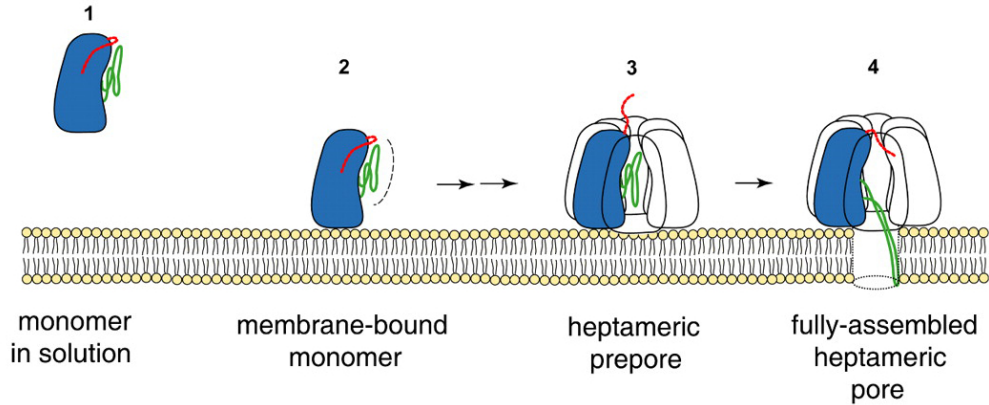


Figure 1.12: The assembly pathway of α HL oligomers. The assembly proceeds via four distinct stages: Water soluble monomers (1) bind to the membrane. Membrane-bound monomers (2) form a prepore (3). The prepore is converted to a transmembrane pore (4). Figure adapted from [88].

consists of a ring of seven subunits, which are tightly associated with the membrane and which do not scramble when the prepore is solubilised in mild detergent [94]. At the prepore stage, the stem domain of a subunit remains folded against the β -sandwich folds of the cap domain, and the N-terminus has not yet latched onto the next subunit [88]. A kinetically competent prepore intermediate was demonstrated by using an α HL mutant with a genetically engineered switch [95]. The switch consisted of a pentahistidine motif in the hinge region between the cap domain and the stem domain. In the presence of Zn^{2+} the protein assembly was arrested at the prepore stage, however, after treatment with EDTA the lytic transmembrane pore was formed.

The assembly of the lytic transmembrane pore from membrane-bound monomers of α HL is a very rapid process, making a detailed characterisation of this pathway difficult. Thompson et al. followed the assembly of fluorescently-labelled monomers of α HL on a lipid bilayer by TIRF microscopy [96], and found that the assembly proceeded within 5 ms, the time resolution of the experiment. No lower oligomeric complexes were detected in these experiments and it was suggested that the assembly is diffusion limited.

1.3.4 Assembly of mixed oligomers and their separation

For many applications it is desirable to modify only one of the seven subunits of the α HL pore. To achieve this, the modified subunit carries also a genetically encoded C-terminal oligoaspartate (D₈) extension. When the modified subunits are combined with wild-type subunits on rabbit red blood cell membranes, mixed oligomers assemble. These can be separated by SDS-polyacrylamide gel electrophoresis, as the D₈ tail alters the electrophoretic mobility of the oligomers (Figure 1.13). With

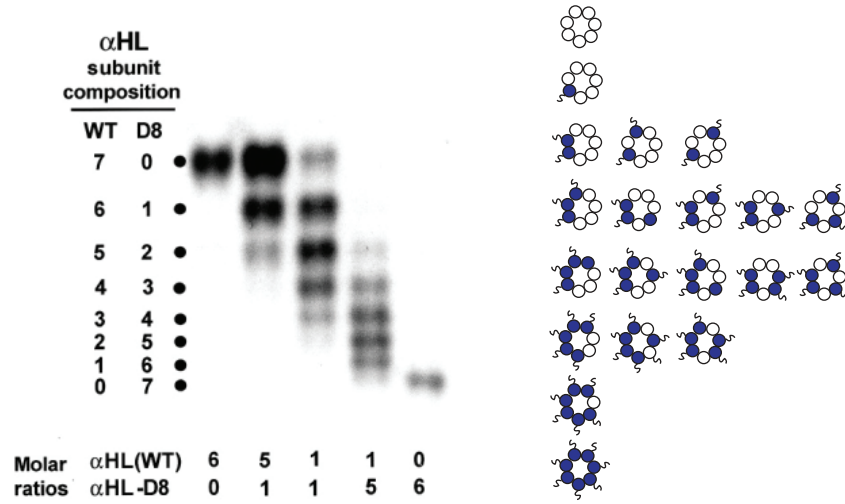


Figure 1.13: The separation of heteroheptameric pore by SDS-PAGE. The heteroheptamers are formed by assembling mixtures of monomeric wt- α HL subunits and α HL-D8 subunits on rabbit red blood cell membranes. Figure adapted from [97].

this technique all eight possible combinations (i.e. wt₇, wt₆(mod-D8)₁, wt₅(mod-D8)₂, ..., (mod-D8)₇) can be separated. However, pores that contain more than one modified subunit exist in different permutations that cannot be separated by this method. A technique to overcome this problem will be presented in chapter 4, where it will be shown that it is possible to produce pores with two adjacent modified subunits.

1.3.5 Engineered α -hemolysin pores to study dynamic processes at the single-molecule level

Electrical measurements can be used to monitor the interaction of analytes with the α HL pore at the single-molecule level. Under an applied potential, a current flows through the pore that is carried by the ions in the recording buffer. In the absence of an analyte, this single channel current is very stable and does not show any deflections. An analyte is then added to the recording buffer. Each time it binds to an engineered site in the pore, the single channel current is modulated (Figure 1.14). The amplitude and duration of the binding events can give information about

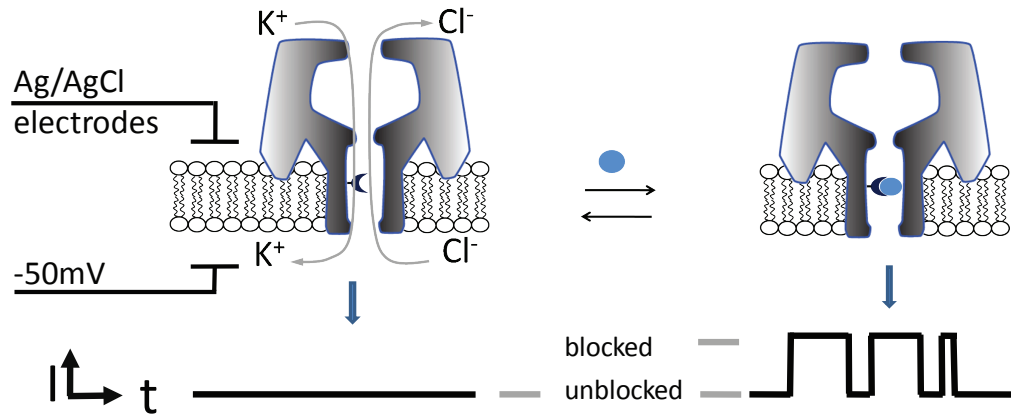


Figure 1.14: Single channel recording to study dynamic processes: A single α HL pore is inserted into a planar lipid bilayer. Under an applied potential a current flows through the pore that is carried by the ions of the recording buffer. Each time an analyte binds to an engineered site in the pore, the single channel current is modulated. The amplitude and dwell time of the block give information regarding the identity of the analyte, whereas the frequency of events is related to the concentration of the analyte.

the identity of the analyte, whereas the frequency of the events is related to the concentration of the analyte. By analysing a large number of events that are fit to exponential distributions, the mean dwell time of the analyte in the pore (τ_{off}) and the mean inter-event interval (τ_{on}) can be determined. The τ values are related to the rate constants of the interaction through $k_{\text{off}} = 1/\tau_{\text{off}}$ and $k_{\text{on}} [\text{A}] = 1/\tau_{\text{on}}$, where $[\text{A}]$ is the concentration of the analyte. A detailed derivation of the rate constants is given in the appendix, chapter 8.3. Furthermore from the law of mass action we

can define a formation constant $K_f = k_{on}/k_{off}$.

Much of the early work in the Bayley group focused on the stochastic detection of analytes in solution. To this end, specific analyte-binding sites had to be engineered into the protein and this was mostly achieved by using either genetic and/or chemical modification techniques. For example, Braha *et al.* used site-directed mutagenesis to introduce a cluster of four histidines into the β -barrel of α HL, to create a divalent metal ion binding site [98]. In single-channel recordings, this protein could be used for the simultaneous stochastic detection of two or more divalent metal ions in solution, as the binding of each type of metal ion resulted in binding events with distinct amplitude and dwell time. By performing a kinetic analysis, the identity and concentration of each metal ion could be determined.

If the analyte is too large to enter the pore, the binding site can also lie on the periphery of the protein. Cheley *et al.* incorporated a kinase inhibitor peptide sequence into one of the loops that connect the β -strands of the transmembrane barrel of α HL (Figure 1.15A) [99]. This protein was used for the stochastic detection of the C subunit of protein kinase A. Furthermore, this system could be used to study how the synergistic binder ATP affects the binding of the kinase to the inhibitor peptide at the single-molecule level.

Howorka *et al.* chemically modified the pore to create binding sites. In their experiments, mutants of α HL that carried a cysteine on the cap domain were reacted with a disaccharide ligand to which an OPSS group had been added (Figure 1.15B) [100]. These “glycopores” were used for the stochastic detection of the lectin from *Bauhinia purpurea* (BP). By using pores that carried multiple disaccharide ligands the authors also investigated the multivalent nature of the BP-disaccharide interaction, although the results of this study did not show any substeps of the multivalent interaction.

A third method to engineer specific binding sites into the α HL pore, is to use a non-covalent adapter that transiently lodges in the lumen of the pore. β -cyclodextrin has a residence time of up to tens of seconds inside the pore and it can form host-

guest complexes with small organic molecules. These host-guest interactions can be seen as an additional block of the current on top of the blocked level generated by the β -cyclodextrin molecule alone (Figure 1.15 C). Gu *et al.* used α HL pores equipped with β -cyclodextrin adapters for the simultaneous stochastic detection of three different adamantane derivatives [101].

The application of α HL pores in the study of dynamic processes is not limited to stochastic sensing. The pore can also be used as a nanoreactor to follow chemical reactions at the single-molecule level. To do so, a reactive residue must be created inside the pore on which the reaction can take place. Using the nanoreactor approach, Shin *et al.* studied the kinetics of the reversible bond formation between a cysteine residue and an organoarsenic (III) compound at the single-molecule level (Figure 1.16A) [102], which had not been observed by any other technique. Since the bilayer separates two compartments that can hold two different reagents, the nanoreactor can also function as a reaction vessel to monitor the turnover of otherwise irreversible reactions. In this setup, one reagent is introduced from the *trans* side of the bilayer and reacts at a defined site within the pore. A second reagent has access to the reaction from the *cis* side of the bilayer and cleaves the bond formed by the *trans* reagent (Figure 1.16B, [103]). This setup was tested by introducing Ellman's reagent (DTNB, 5,5'-dithiobis-(2-nitrobenzoic acid)) to the *trans* side of a bilayer containing an α HL pore with a cysteine at position 117. DTNB reacted with the cysteine to form a mixed disulfide, which would normally be an irreversible reaction. However, dithiothreitol was present in the *cis* compartment and could diffuse into the pore to cleave the disulfide. This re-generated the free thiol and the cycle could start over. Using the nanoreactor approach Luchian *et al.* could detect each intermediate of the cycle and obtained kinetic parameters to describe the system (Figure 1.16B) [103].

Finally Lu *et al.* used the nanoreactor approach to observe a primary kinetic isotope effect [104]. In their experiments they recorded the irreversible reaction between a cysteine on the protein and a quinone reagent to give a substituted hydroquinone.

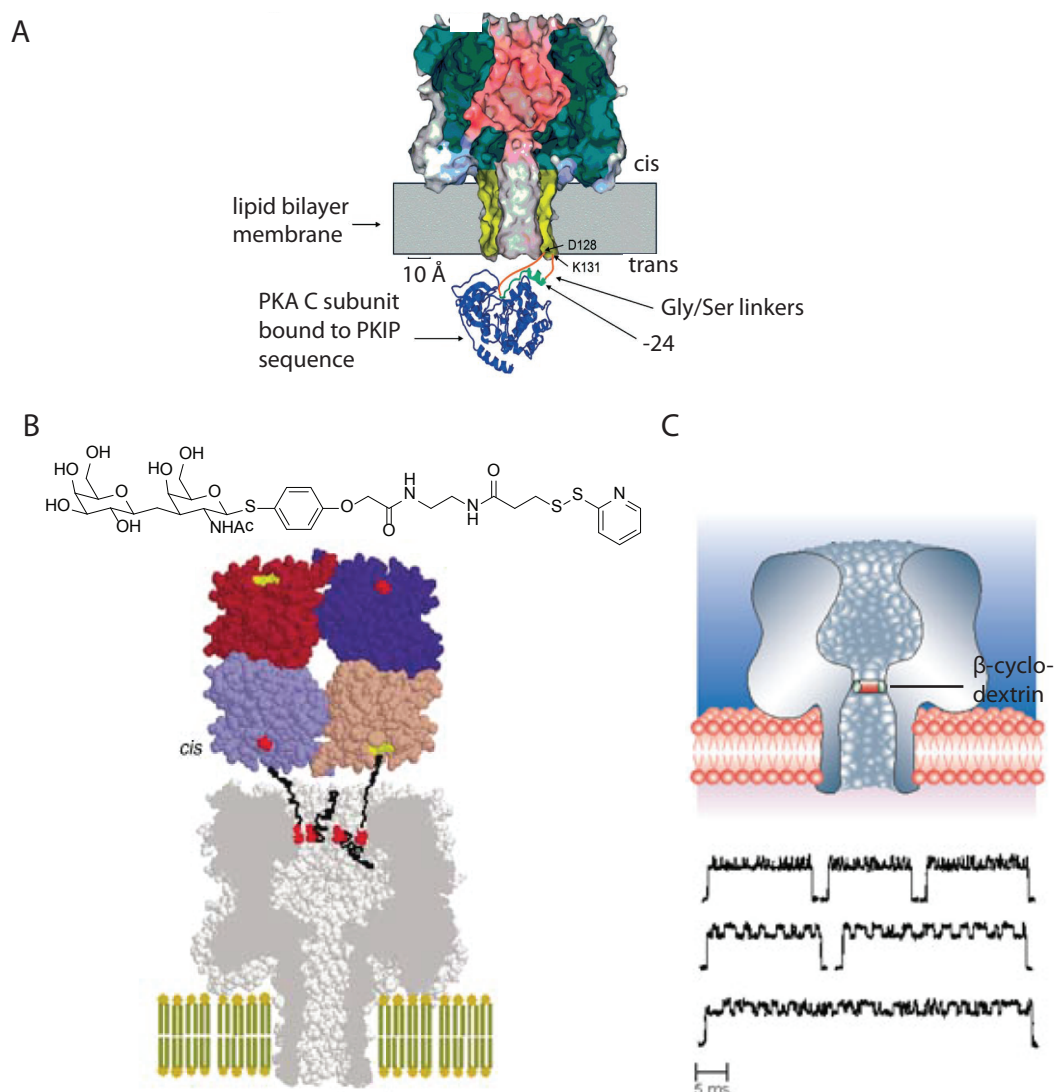


Figure 1.15: Detection of a variety of analytes by stochastic sensing. **A** Model of an α HL pore carrying the PKIP inhibitor peptide. The C subunit of protein kinase A is shown docked to the peptide. Panel adapted from [99]. **B** Top: Structure of the dissacharide ligand used for the chemical modification of the protein. Bottom: Model of the glycopore with a bound BP lectin. Panel adapted from [100]. **C** Detection of organic molecules using a non-covalent adapter. A β -cyclodextrin molecule is lodged in the lumen of the pore, where it can still form host-guest complexes with small organic molecules. In this example binding of adamantane molecules can be seen as the current deflections of the β -cyclodextrin-blocked level. Panel adapted from [67].

This reaction proceeds via a reductive 1,4-Michael addition mechanism. The authors could detect a primary hydrogen-deuterium isotope effect during the prototropic rearrangement of the initial adduct (Figure 1.16C). Since individual reaction intermediates could be visualised, the isotope effect could be measured regardless of

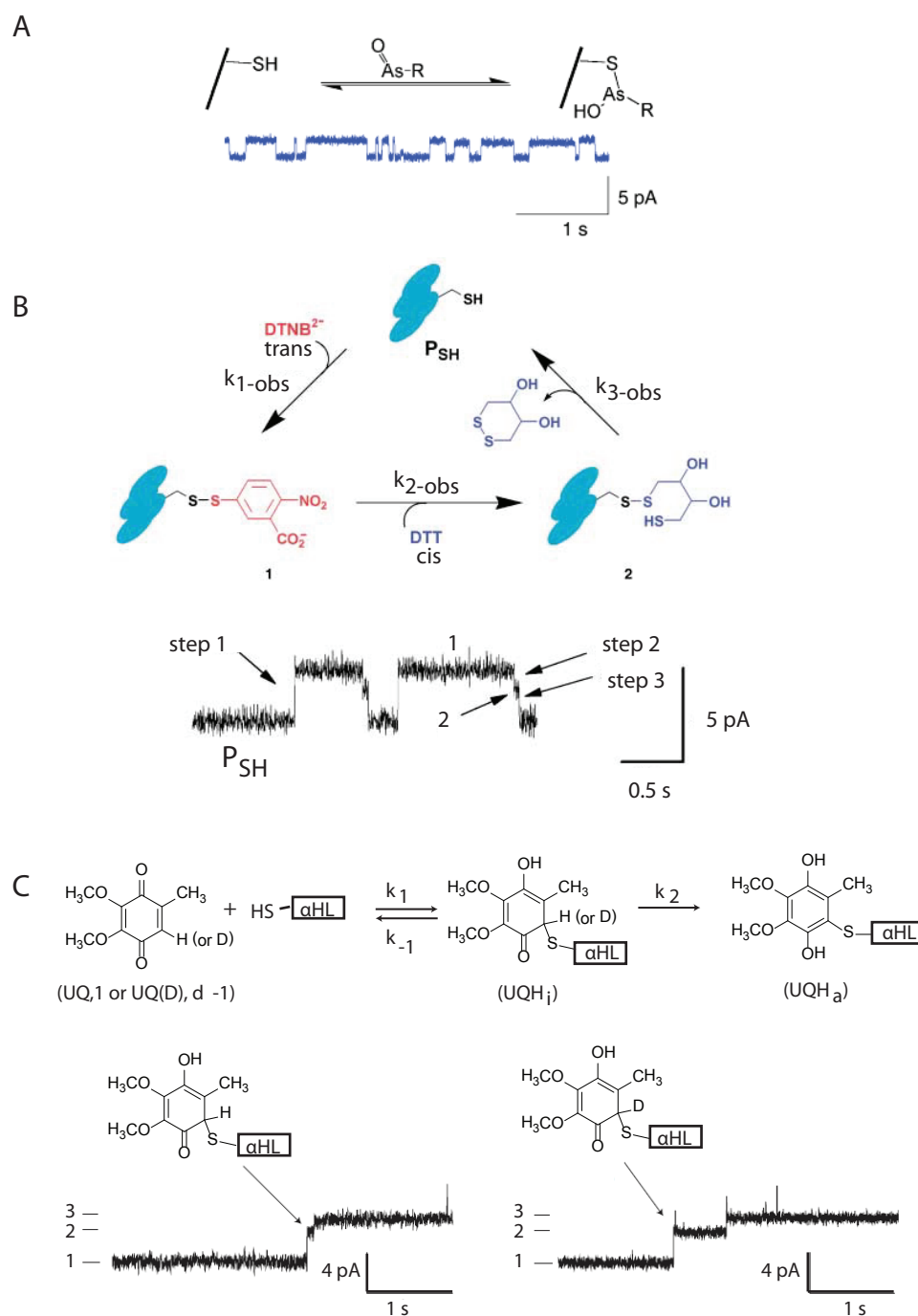


Figure 1.16: The nanoreactor approach to study chemical reactions at the single-molecule level. **A** The reversible bond formation between a cysteine on the protein and an organoarsenic (III) reagent monitored by single channel recording. Panel adapted from [102]. **B** Kinetics of a three-step chemical reaction with spatially separated reactants. Panel adapted from [103]. **C** Observation of a hydrogen-deuterium primary kinetic isotope effect. Panel adapted from [104].

whether the step involved is rate limiting. This would not be possible in ensemble measurements.

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Chapter 2

Single molecule kinetics of divalent metal ion chelation using an engineered α -hemolysin pore.

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Author contributions: AFH, SHS and HB designed the experiments; SHS synthesised the half-chelator ligand; AFH prepared the protein, carried out the single channel recordings and performed the data analysis; AFH and HB wrote the manuscript.

2.1 Chapter overview

This chapter demonstrates how a divalent metal ion chelation site can be created in the α HL pore by site-directed mutagenesis and chemical modification. It will be shown that single channel recording of this engineered pore with different divalent

metal ions can be used to determine the rates of chelation, as well as the formation constants of the resulting complexes. Furthermore, it will be shown that this pore can be used for the simultaneous stochastic detection of divalent metal ions in solution.

2.2 Introduction

Divalent metal ion chelators play an important role in numerous areas, including basic biology and medicine, as illustrated by the three examples below:

Ca^{2+} acts as an important signalling molecule in biological systems and it is involved processes such as the transmission of fast impulses in the nervous system and in muscle contraction [1]. The free Ca^{2+} concentration inside a cell is tightly buffered to about 100 nM [2, 3]. However, when Ca^{2+} channels in the cell membrane or intracellular Ca^{2+} stores open, the Ca^{2+} concentration inside the cell can rise locally to 100 μM over a timecourse of a few hundred microseconds [4]. Considering the importance of Ca^{2+} in biological systems, it is crucial that the Ca^{2+} dynamics in a cell can be measured with high temporal and spatial resolution. One approach to study the role of such Ca^{2+} transients is the use of fluorescent Ca^{2+} indicators, which consist of a divalent metal ion chelator coupled to a fluorophore. When Ca^{2+} binds to the indicator, the spectral properties of the fluorophore change, permitting the local changes in Ca^{2+} concentration to be imaged. A good Ca^{2+} indicator must bind Ca^{2+} preferentially over other divalent metal ions (e.g. Mg^{2+}) and with high affinity [5]. In order to follow the timecourse of fast Ca^{2+} oscillations, the chelator must bind Ca^{2+} rapidly, too [4, 6].

A second example illustrating the importance of chelators are oral iron chelators that are given to patients with iron overload disease. Iron is essential for many biological processes, such as oxygen transport, energy generation and DNA synthesis [7]. However, it is also a redox active metal and if allowed to build up inside a cell it can lead to oxidative stress and ultimately cell death [8]. Some people suffer from

iron overload diseases, which prevent their bodies from excreting excess iron [9]. Such diseases can cause fatal damage to the tissues and organs of the patient. Iron overload diseases can be managed by giving the patient oral iron chelators, which must bind iron strongly and selectively to work efficiently [9, 10].

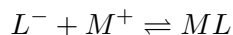
A third example illustrating the role of chelators are Positron emission tomography (PET) probes. PET is a method to visualise the location of tumours in the body. Prior to the PET scan, a radiotracer (a substance containing a radioisotope) is injected into the patient's bloodstream for distribution across the body. Tumours often have a higher metabolism than other tissues, so radiotracers (e.g. 2-deoxy-2-[^{18}F]fluoro-D-glucose) tend to accumulate there [11]. Once the radiotracer has been distributed, the positron emission is recorded by a scanner. Recently efforts have been made to develop more specific PET probes that consist of a biologically active molecule (e.g. a peptide, protein or antibody) targeting a specific cell type or tissue, which is coupled to a chelator with a bound radiometal (e.g. ^{68}Ga) [12]. In order for these PET probes to function properly, the chelator should have a high affinity for the radiometal and the rate of dissociation of the radiometal from the chelator should be slow.

These three examples illustrate that a thorough understanding of the kinetics and thermodynamics of metal ion chelation is required for the design of many medicinal and biological probes.

2.2.1 Techniques to measure the stability of divalent metal ion complexes and their rates of formation and dissociation

The stability of chelator-divalent metal ion complexes is most commonly determined by the potentiometric titration of the chelator with a divalent metal ion. In order for the metal ion (M^{+}) to bind, the chelator (which is often referred to as a ligand,

L) must deprotonate, so there are two equilibria that need to be considered:



Therefore by following the H^+ ion concentration (i.e. the pH) during a titration of a mixture of M and LH with acid or base, and knowing the acid dissociation constant of HL, the stability constant for the formation of ML can be determined [13]. The stability constants of a very large number of metal complexes with organic and inorganic ligands have been determined by this method [14]. Other methods to measure the stability of divalent metal ion-chelator complexes include UV-Vis spectroscopy [15], isothermal titration calorimetry [16] and NMR spectroscopy [17].

There are only few papers that have addressed the kinetics of complex formation. One way to measure the rate of complex formation is by NMR line shape analysis [18–20]. For example, in the presence of a divalent metal ion, the methylene protons of methyl-iminodiacetic acid (MIDA) give rise to two NMR peaks with characteristic resonance frequencies, one for the free MIDA ligand (ν_A) and one for the metal-bound MIDA ligand (ν_B , Figure 2.1). The free and metal-bound ligand are in equilibrium, with a rate of exchange of k . In the limit of slow exchange, that is when k smaller than the difference in resonance frequency ($|\nu_A - \nu_B| = \delta\nu$) between the two peaks, two distinct resonance peaks are observed. In the limit of slow exchange, the line width of each peak at 1/2 height ($\Delta\nu$) is related to the rate of exchange through $\Delta\nu = k/\pi$. If the temperature is increased, the rate of exchange will also increase and the two resonance peaks first broaden, then start to shift towards one another, then broaden further until they merge into a single wide resonance peak. At the point of coalescence, it is found that $k = \pi\delta\nu/\sqrt{2}$. If the temperature of the experiment is increased further still, k will exceed $\delta\nu$ and the single mean resonance peak will begin to sharpen. In the limit of fast exchange, $\Delta\nu = \pi(\delta\nu)^2/2k$ [21]. Therefore

the rate of exchange can be calculated from the line width of the NMR peaks under the conditions of slow, intermediate and fast exchange. The fastest rates that can be measured by this method are in the order of 100-1000 s⁻¹ [22]. For example, the rate of dissociation of MIDA from the Zn.(MIDA)₂ complex was found to be 51 s⁻¹ at 308 K and pH 8.0 [18]. To a lesser extent the rate of complex formation has also been measured by other ensemble techniques such UV-Vis spectroscopy [23].

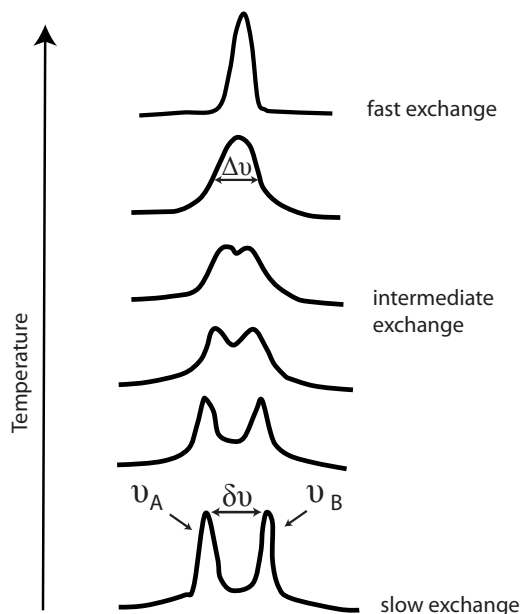


Figure 2.1: Illustration of NMR resonances for the exchange reaction $MIDA + Zn^{2+} \rightleftharpoons Zn.MIDA$ at different temperatures. Figure adapted from [21].

Despite the importance of divalent metal ion chelators in biology and medicine, our understanding of the thermodynamic basis of chelation [15, 16] and the kinetics of metal-ion binding and dissociation is far from complete, as there are complications to the ensemble approaches above. For example, a chelator such as EDTA can promote the exchange of a metal ion from a preformed M.EDTA complex; metal complexes can undergo protonation and proton transfer can be rate determining in metal ion exchange; exchange rates can vary depending on the presence of monovalent cations, e.g. Na⁺ versus K⁺. These factors have lead to the postulation of complex mechanisms for metal chelation with numerous rate constants that must often be reconciled with a relative paucity of data. Single-molecule methods should

be capable of shedding light on complex multi-step processes, including chelation. For example, by using a protein nanoreactor, Braha et al. were able to monitor the association and dissociation of individual divalent metal ions with clusters of imidazole ligands [24, 25]. Recently, single molecule fluorescence was used to examine the formation and dissociation of a Cu^{2+} complex [26]. However, in neither case were the steps in complex formation resolved.

This chapter will show how the rates of divalent metal ion chelation can be measured in real time and at the single molecule level by using an engineered αHL pore, thereby providing kinetic and thermodynamic data that would be difficult to obtain by other methods. Secondly it will be demonstrated that the αHL pore can be used as a stochastic sensor for the detection of divalent metal ions, either individually or in a mixture. Chapter 3 will deal with the more complex kinetics of the interaction of two chelation sites.

2.3 Results

2.3.1 Design of the chelation site

In order to investigate the kinetics of divalent metal ion chelation with the αHL pore, a chelation site had to be created inside the pore. For this purpose a “half-chelator” ligand was designed (Figure 7.2), which comprises the N-propyliminodiacetic acid (PIDA) group and an iodoacetamide group for covalent attachment to the protein via mutant cysteine residues. It was hoped that one half-chelator would provide a single divalent metal binding site, whereas two half-chelators in close proximity could come together to form a complex with a single divalent metal ion in a similar fashion to EDTA. The latter is the focus of the next chapter. The half-chelator ligand was first synthesised by Seong-Ho Shin (University of Oxford) as described in the Materials and Methods section, although I prepared subsequent batches of this compound. Residue 117 was chosen for modification with the half-chelator ligand, because this position had been used previously to observe the covalent bond

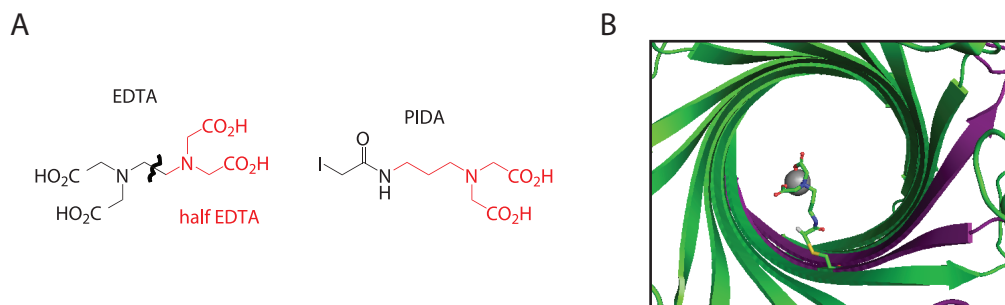


Figure 2.2: **A** Structures of EDTA and the half-chelator ligand. **B** Molecular model of an α -hemolysin pore generated in PyMOL. The view inside the β -barrel depicts a PIDA ligand with a bound divalent metal ion, which are attached to one of the seven subunits of the protein. The modified subunit is coloured purple, the wild-type subunits are green.

formation between an organoarsenic(III) compound at a cysteine residue at the single molecule level [27], and also the cis-trans isomerisation of an azobenzene molecule covalently attached to a cysteine residue [28]. Figure 7.2 shows a molecular model of a single half-chelator at position 117 on one of the subunits in the α HL pore.

2.3.2 Protein preparation

The monomeric polypeptide α HL(117C)-D8 was prepared by in vitro transcription and translation (IVTT) of the gene α HL(117C)-D8 (a gift from Seong-Ho Shin, University of Oxford). The protein was radiolabelled with [35 S]-methionine to permit visualisation of the protein band on SDS-PAGE gels by autoradiography at a later stage. IVTT provides a convenient method to synthesise up to 5 ng/ μ l of protein in 1 h, which is a sufficiently high yield for the experiments described in this thesis. By comparison, the expression and purification of proteins from recombinant sources typically takes several days. Following the IVTT, the protein was passed through a gel filtration column to remove molecules of low molecular weight that could interfere with the chemical modification reaction. The eluted protein was diluted with Tris.HCl buffer (pH 8.0) containing 0.5 mM DTT to ensure that the cysteine residues were reduced and deprotonated. The protein was reacted with the half-chelator ligand (20 mM final concentration) for 1 h at RT and in the dark, as the iodoacetamide group is light sensitive. The protein was then passed through a

gel filtration column to remove excess reagent. The extent of chemical modification was assessed by gel shift experiments. Chemical modification of α HL often produces an upward shift of the protein band on SDS-PAGE gels compared to the unreacted protein and to the wild-type protein, but this was not so with the half-chelator ligand (Figure 2.3), probably because the reagent was small compared to the size of the protein. The negative charge of the PIDA group could also counter the effect of the increased size on the mobility of the modified protein in the gel. To confirm

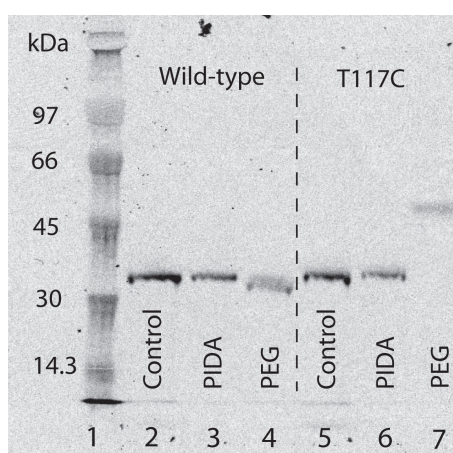


Figure 2.3: 12% SDS-PAGE gel of monomers of α HL, showing the gel shifts caused by chemical modification of a cysteine residue. Lane 1: 14 C-protein molecular weight markers. Lane 2: wild-type α HL after reaction with water. Lane 3: wild-type α HL after reaction with the half-chelator reagent. Lane 4: wild-type α HL after reaction with mPEG-OPSS-5000. Lane 5: T117C-D8 after reaction with water. Lane 6: T117C-D8 after reaction with the half-chelator reagent. Lane 7: T117C-D8 after reaction with mPEG-OPSS-5000.

that the cysteines were fully reactive, a control reaction with polyethylene glycol 5000 orthopyridyl disulfide (mPEG-OPSS-5000, a cysteine-specific labelling reagent that results in a large gel shift of modified α HL [28]) was performed under the same reaction conditions. Reaction of T117C-D8 with mPEG-OPSS-5000 resulted in an upward shift of the band on SDS-PAGE gels (Figure 2.3), with no band corresponding to unreacted protein remaining. The wild-type protein (which does not contain any cysteine residues) did not produce a gel shift. These results confirm that the reaction between the polypeptide T117C-D8 and mPEG-OPSS-5000 is specific and goes to completion. Since iodoacetamide reagents are known to be excellent reaction partners with cysteine residues [29], it was assumed that the

half-chelator reagent had also reacted fully with the protein, but was too small to produce a gel shift. Next, heteroheptamers of α HL were prepared by assembling mixtures of wild-type and chemically modified subunits on rabbit red blood cell membranes [28]. A total of eight different heptamers can be made when wild-type and chemically modified subunits are combined [e.g. (WT)₇, (WT)₆(mod-D8)₁, (WT)₅(mod-D8)₂, ..., (mod-D8)₇], however, only the (WT)₆(mod-D8)₁ oligomer was of interest for this project. The different oligomers were separated on a 5% SDS-PAGE gel by electrophoresis. This is possible because the chemically modified subunit carries an oligoaspartate (D₈) extension, which specifies the electrophoretic mobility of oligomers containing different numbers of wild-type and chemically modified subunits in the gel (Figure 2.4) [30]. It should be noted that the proteins

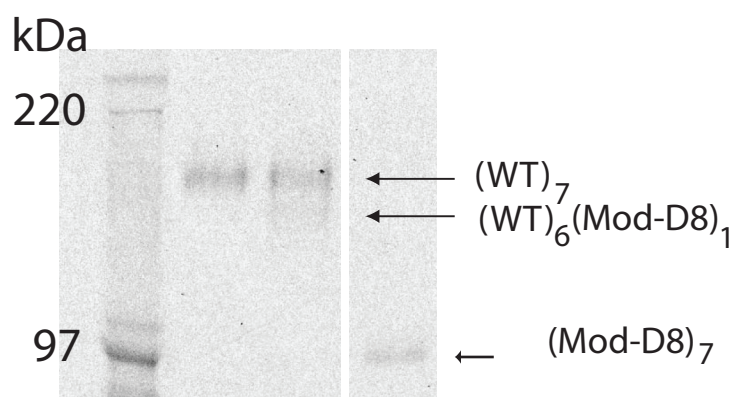


Figure 2.4: 5% SDS-PAGE gel showing oligomers of α HL. Left lane: Heptamers comprising wild-type subunits only. Middle lane: Heptamers formed from wild-type and 117C-PIDA-D8 subunits. Right lane: Heptamers formed from 117C-PIDA-D8 subunits only. Mod, engineered subunit; (WT)₆(Mod-D8)₁, the heteroheptamer containing six wild-type and one engineered subunit. The oligomers had been formed by allowing wt and/or modified monomers of α HL to assemble on rabbit red blood cell membranes. The membranes containing the assembled oligomers were then solubilised in Laemmli sample buffer and loaded onto the gel.

were subjected to electrophoresis under non-denaturing conditions, therefore the molecular weight of the protein does not match that of the marker. In the experiment shown in Figure 2.4, three oligomerisation reactions were set up, which contained wild-type and chemically modified proteins in the ratios 7:0, 6:1 and 5:2 only. This increases the likelihood of producing (WT)₆(mod-D8)₁ oligomers, and indeed only

2 of the possible 8 oligomer bands are seen. Following visualisation of the protein bands on the dried gel by autoradiography, the band containing the (WT)₆(mod-D8)₁ oligomer was excised from the gel and rehydrated in buffer. The gel fragment was then crushed and filtered through a cellulose acetate column to obtain a solution of protein in SDS that was used for single-channel recordings. The (WT)₆(mod-D8)₁ oligomer will be referred to as the PPIDA pore from here on.

2.3.3 Single channel recordings of the PPIDA pore

The PPIDA pore was characterised by single-channel recording in planar lipid bilayers. The single channel recordings described in this chapter were all carried out under an applied potential of -50 mV and in buffer containing 2 M KCl, 10 mM 3-morpholinopropane-1-sulfonic acid (MOPS), pH 7.0. Under these conditions the PPIDA pore carried a single-channel current of -73.8 ± 2.5 pA, whereas the wild-type pore carried a single channel current of -79.1 ± 1.8 pA. This provided the first indication that the PPIDA pore had been modified. Next, the interaction of the PPIDA pore with different divalent metal ions was investigated.

2.3.3.1 The interaction of the PPIDA pore with Zn²⁺

The addition of Zn²⁺ to the trans recording chamber resulted in the fluctuation of the single channel current between two discrete levels separated by $\Delta I_{\text{Zn}} = 1.6 \pm 0.1$ pA, where ΔI is the current difference between the pore partially blocked by Zn²⁺ and that of the unoccupied pore (Figure 2.5A). The frequency of Zn²⁺-binding events increased as the Zn²⁺ concentration was increased and no binding events were seen in buffer containing 1 mM EDTA or with WT₇ pores (data not shown). A kinetic analysis was carried out in order to determine the rate of chelation of Zn²⁺ by the PPIDA pore. For this analysis it was assumed that the half-chelator and Zn²⁺ reversibly form a binary complex (Figure 2.5B) and that the Zn²⁺ “concentration” inside the pore equals that in solution [31]. The rates of transitions between the

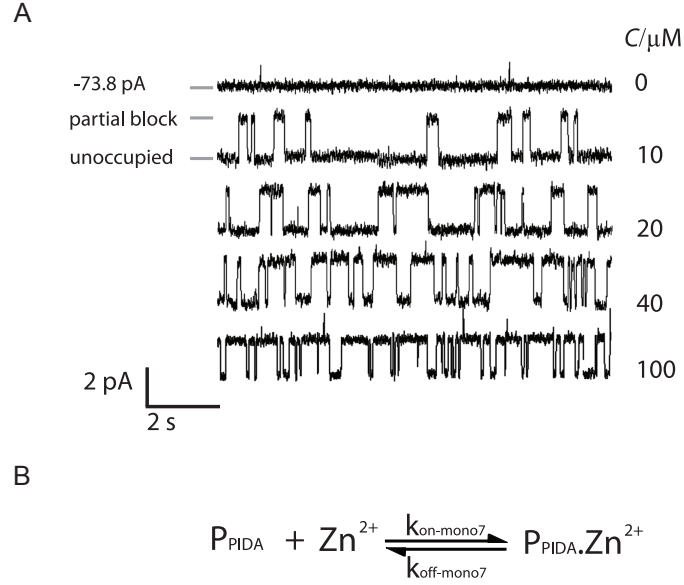


Figure 2.5: The interaction of the PPIDA pore with Zn^{2+} . **A** Single-channel recordings at -50 mV with a buffer containing 2.0 M KCl and 10 mM MOPS ($\text{pH } 7.0$) in both chambers, and 0 to $100 \text{ } \mu\text{M ZnCl}_2$ in the trans chamber. The two current levels of the PPIDA pore correspond to the unoccupied pore and to the partial block caused by Zn^{2+} binding to the half-chelator in the pore. **B** Proposed kinetic scheme describing Zn^{2+} binding to the PPIDA pore.

unblocked pore and the pore partially blocked by Zn^{2+} were then modelled on a Markov chain [32], which requires the following assumptions:

1. The pore exists in either one of two distinct states, the unblocked pore or the pore partially blocked by Zn^{2+} . The two states are observable through the current level of the unblocked pore and the partially blocked pore, respectively.
2. Transition between the two states have a fixed probability of occurrence and depend only on the current state, i.e. the system has no memory of previous transitions.

The dwell times (τ) of each state of a Markov process follow single exponential distributions, with a mean dwell time of $\bar{\tau}$ [32]. For the PPIDA pore, the mean dwell time of the unblocked pore level is $\bar{\tau}_{\text{on}}$ and the mean dwell time of the partially blocked pore level is $\bar{\tau}_{\text{off}}$. For the bimolecular reaction between the PPIDA pore and

Zn^{2+} (Figure 2.5B), it can be shown that $\bar{\tau}_{\text{on}}$ is related to k_{on} through

$$1/\bar{\tau}_{\text{on-Zn}} = k_{\text{on-Zn}} \cdot [\text{Zn}^{2+}]$$

A detailed derivation of this equation is given in the Appendix (chapter 8.3). Similarly, the dissociation of Zn^{2+} from the PPIDA pore is a unimolecular reaction, for which it can be shown that

$$1/\bar{\tau}_{\text{off-Zn}} = k_{\text{off-Zn}}$$

The determination of the rate constants therefore involves two steps: First $\bar{\tau}_{\text{on}}$ and $\bar{\tau}_{\text{off}}$ for each Zn^{2+} concentration are derived from dwell time histograms. Second, the on- and off-rates are determined graphically from a plot of $1/\bar{\tau}$ against the Zn^{2+} concentration. This process is described in detail below.

In order to construct dwell time histograms for the unblocked and partially blocked pore, a table of dwell times in each state had to be generated. This was done automatically, by processing the single channel recording traces with the “Single Channel Search” algorithm of the Clampfit 10.2 software (Molecular Devices). In this algorithm, a current level is assigned to each observed level of the single channel current. In the example shown in Figure 2.6, the current corresponding to the unblocked pore was assigned level 0 and the current corresponding to the partially-blocked pore was assigned level 1. The search algorithm then “idealised” the single channel recording, that is, it translated the recording trace to a series of events that were categorised as belonging to either level 0 or level 1. The amplitude and dwell time of each event was recorded in a table.

Data sets comprising 300 or more dwells were then used to construct dwell time histograms, which were fitted to single exponential functions [33, 34]. In the single channel recording field, dwell time histograms are frequently plotted over the logarithm of dwell time, across equally spaced bins [35]. This permits dwell times that span several orders of magnitude to be represented in a single plot.

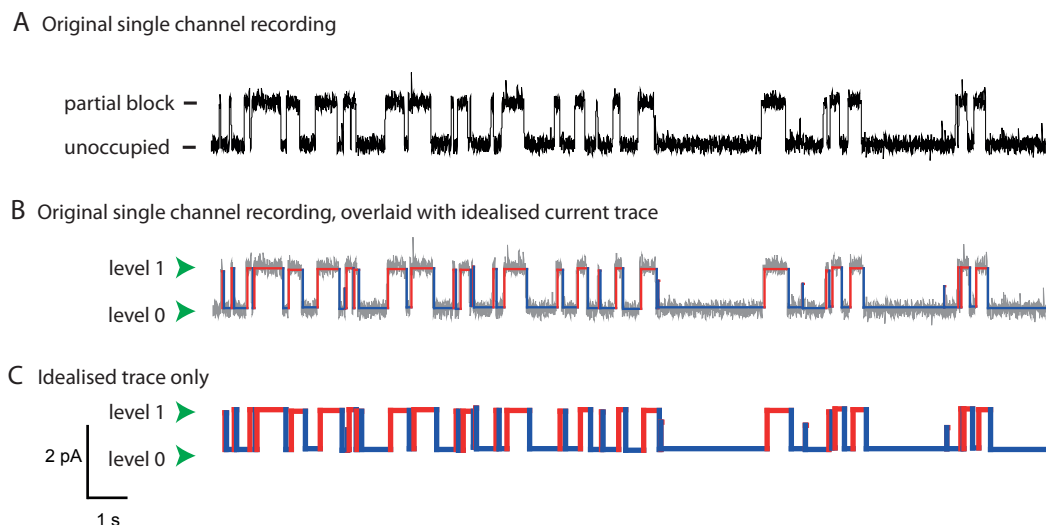


Figure 2.6: *Generation of an idealised current trace from a single channel recording. **A** Original single channel recording showing Zn^{2+} binding events with the PPIDA pore. **B** Original single channel recording, overlaid with the idealised current trace generated by the single channel search algorithm. **C** Idealised current trace only.*

Furthermore, as several states could be associated with one conductance class, logarithmic dwell time plots make it easier to obtain a visual impression of the number of exponential terms in a dwell time distribution. This is because each state gives rise to a single exponential term, which appears as a peaked function in the histogram [35, 36]. In keeping with this convention of display, the dwell time histograms for the PPIDA pore were also plotted across the logarithm of dwell time and fitted with single log exponential functions to determine $\bar{\tau}$ (Figure 2.7A).

The reciprocal of $\bar{\tau}$ was then plotted against the Zn^{2+} concentration to determine the rate constants for the complex formation (Figure 2.7B). The forward and reverse rate constants ($k_{\text{on-Zn}} = 1.5 \pm 0.2 \times 10^5 \text{ M}^{-1}\text{s}^{-1}$ and $k_{\text{off-Zn}} = 4.0 \pm 0.2 \text{ s}^{-1}$) derived from the $\bar{\tau}$ values yield a formation constant of $K_{\text{f-Zn}} = 3.8 \pm 0.5 \times 10^4 \text{ M}^{-1}$ at 24°C ($n=4$ independent experiments).

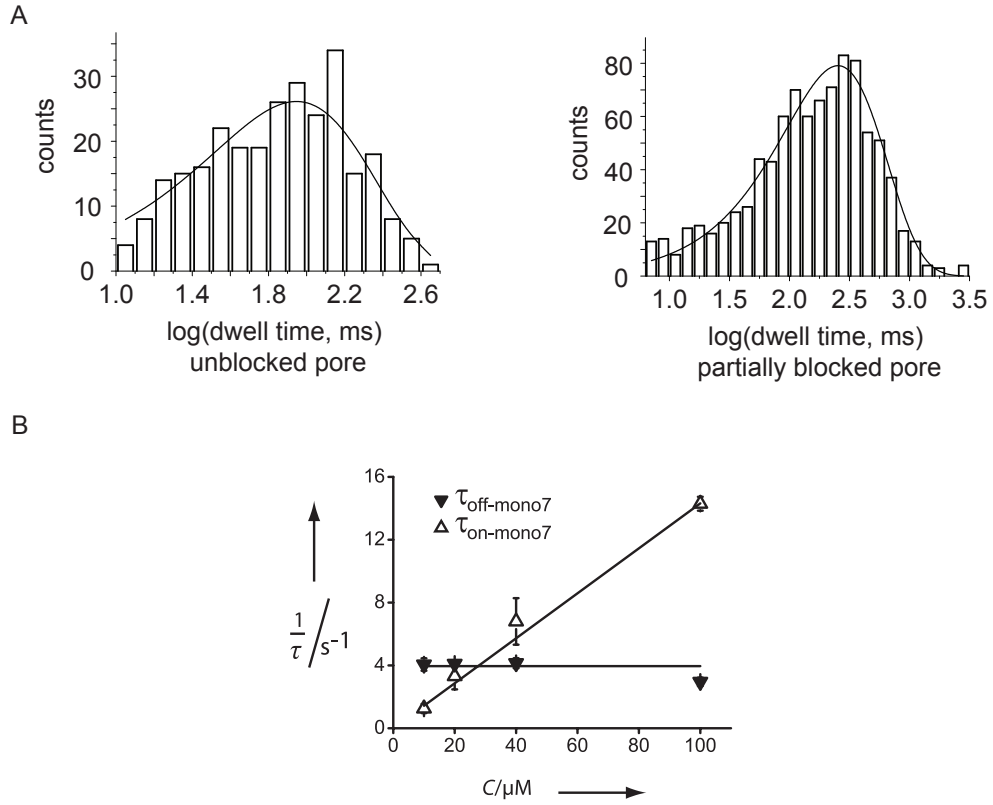


Figure 2.7: Kinetic analysis of Zn^{2+} binding events with the PPIDA pore. **A** Representative $\log(dwel\ time)$ histograms of the unblocked and blocked pore at $100\ \mu M\ Zn^{2+}$. The histograms are fitted with log-exponential functions. **B** Reciprocals of the mean inter-event intervals (τ_{on-Zn} ; $\blacktriangledown$) and the dwell times of Zn^{2+} (τ_{off-Zn} ; $\triangle$) versus the Zn^{2+} concentration. The values of τ_{on-Zn} and τ_{off-Zn} were determined by fitting dwell-time histograms for each Zn^{2+} concentration to single exponential functions. For a simple bimolecular interaction: $k_{off} = 1/\tau_{off}$ and $k_{on} = 1/\tau_{on}$. $[Zn^{2+}]$.

2.3.3.2 The interaction of the PPIDA pore with other divalent metal ions

Since Zn^{2+} gave a very characteristic current signature, it was investigated if the PPIDA pore could also be used for the stochastic detection of other divalent metal ions.

The addition of Cd^{2+} to the trans recording chamber resulted in a fluctuation of the ionic current between two discrete levels separated by ΔI_{Cd} of 2.6 ± 0.2 pA (Figure 2.8A). By performing the same kinetic analysis for the Cd^{2+} binding events (Figure 2.8B) that had been used for the Zn^{2+} binding events, it was found that $\tau_{\text{off-Cd}} = 20.7 \pm 1.9$ ms, which yields $k_{\text{off-Cd}} = 47 \pm 4$ s⁻¹. A value of $k_{\text{on-Cd}} = (5.6 \pm 0.7) \times 10^4$ M⁻¹s⁻¹ was derived from a plot of the reciprocal of the mean inter-event interval ($\tau_{\text{on-Cd}}$) against the Cd^{2+} concentration (Figure 2.8C). The forward and reverse rate constants for Cd^{2+} binding yield a formation constant of $K_{\text{f-Cd}} = (1.2 \pm 0.2) \times 10^3$ M⁻¹.

The addition of Mn^{2+} to the trans recording chamber also resulted in a fluctuation of the single channel current between two discrete levels separated by $\Delta I_{\text{Mn}} = 3.2 \pm 0.2$ pA. (Figure 2.9A). A kinetic analysis of the binding events (Figure 2.9B) revealed that $\tau_{\text{off-Mn}} = 3.7 \pm 1.2$ ms, yielding $k_{\text{off-Mn}} = 290 \pm 20$ s⁻¹. A plot of the reciprocal of the interevent interval ($\tau_{\text{on-Mn}}$) against the Mn^{2+} concentration (Figure 2.9C) was used to derive $k_{\text{on-Mn}} = (1.6 \pm 0.2) \times 10^4$ M⁻¹s⁻¹. The forward and reverse rate constants for Mn^{2+} binding yield a formation constant of $K_{\text{f-Mn}} = (5.5 \pm 0.8) \times 10^1$ M⁻¹.

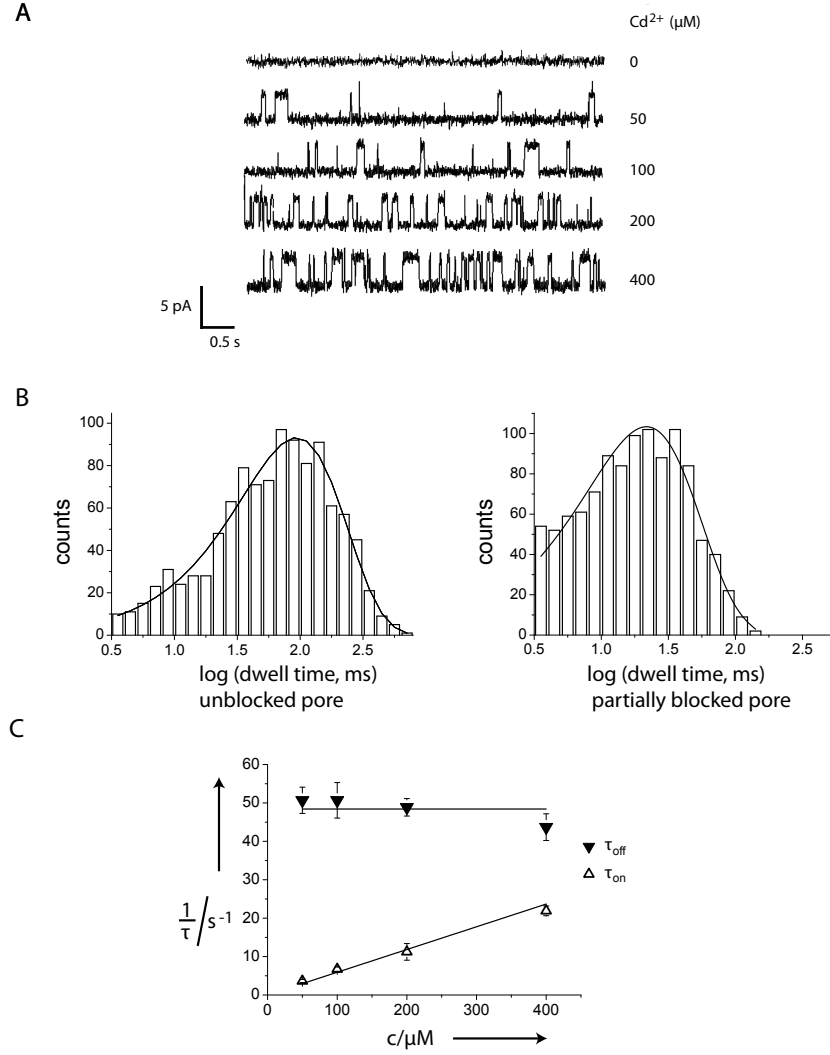


Figure 2.8: Cd^{2+} binding to the PPIDA pore. **A** Single-channel recordings at -50 mV with a buffer containing 2.0 M KCl and 10 mM MOPS (pH 7.0) in both chambers, and 0 to 400 μM CdCl_2 in the trans chamber. **B** Representative log(dwel time) histograms of the unblocked and blocked pore at 200 μM Cd^{2+} . The histograms are fitted with log-exponential functions. **C** Reciprocals of the mean inter-event intervals ($\tau_{\text{on-Cd}}$; ▼) and the dwell times of Cd^{2+} ($\tau_{\text{off-Cd}}$; △) versus the Cd^{2+} concentration.

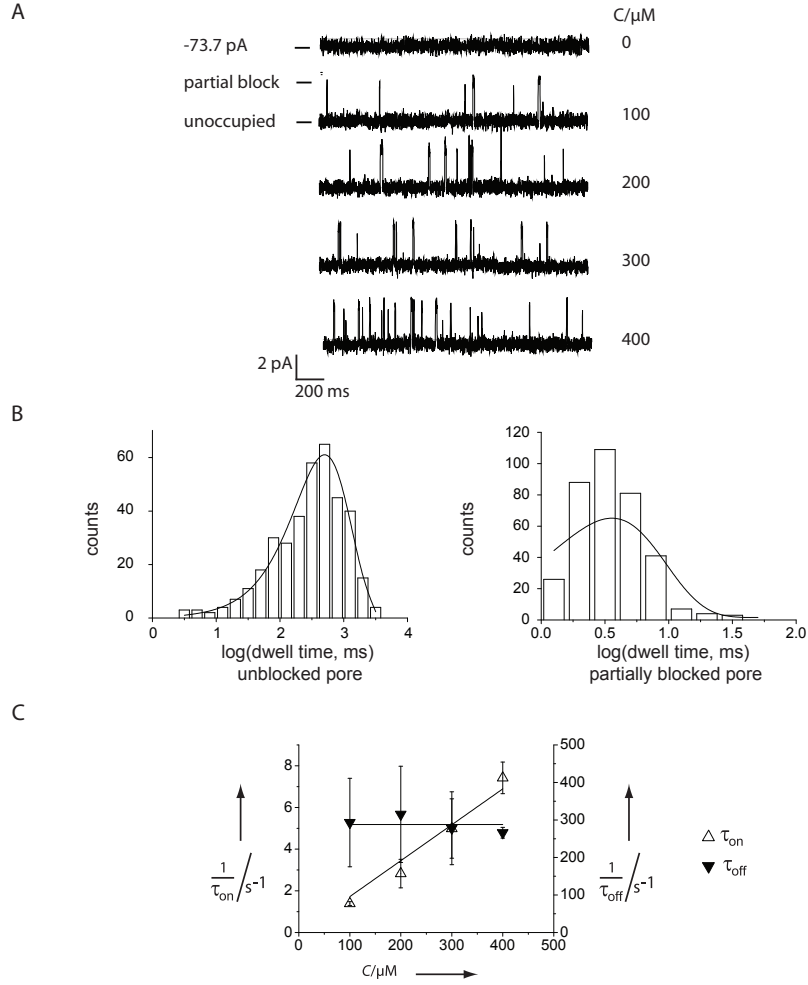


Figure 2.9: Mn^{2+} binding to the PPIDA pore. **A** Single-channel recordings at -50 mV with a buffer containing 2.0 M KCl and 10 mM MOPS (pH 7.0) in both chambers, and 0 to 400 μM MnCl_2 in the trans chamber. **B** Representative log(dwel time) histograms of the unblocked and blocked pore at 200 μM Mn^{2+} . The histograms are fitted with log-exponential functions. **C** Reciprocals of the mean inter-event intervals ($\tau_{\text{on-Mn}}$; ▼) and the dwell times of Mn^{2+} ($\tau_{\text{off-Mn}}$; Δ) versus the Mn^{2+} concentration.

2.3.3.3 The simultaneous detection of Zn^{2+} and Cd^{2+} by the PPIDA pore

Knowing that the PPIDA pore can detect different divalent metal ions independently, the next step was to determine if two divalent metal ions could also be detected simultaneously in a mixture.

The addition of $10\ \mu\text{M}\ \text{Zn}^{2+}$ and $50\ \mu\text{M}\ \text{Cd}^{2+}$ to the trans recording chamber resulted in two types of binding events with the PPIDA pore. From the current level of the unblocked pore (level 1) smaller amplitudes blockades to level 2 ($\Delta I = 1.7 \pm 0.2\ \text{pA}$) and larger amplitude blockades to level 3 ($\Delta I = 2.8 \pm 0.2\ \text{pA}$) were observed (Figure 2.10). The amplitude of the blockades mirror those observed for Zn^{2+} and Cd^{2+} individually. The frequency of events increased as the concentration of either analyte was increased.

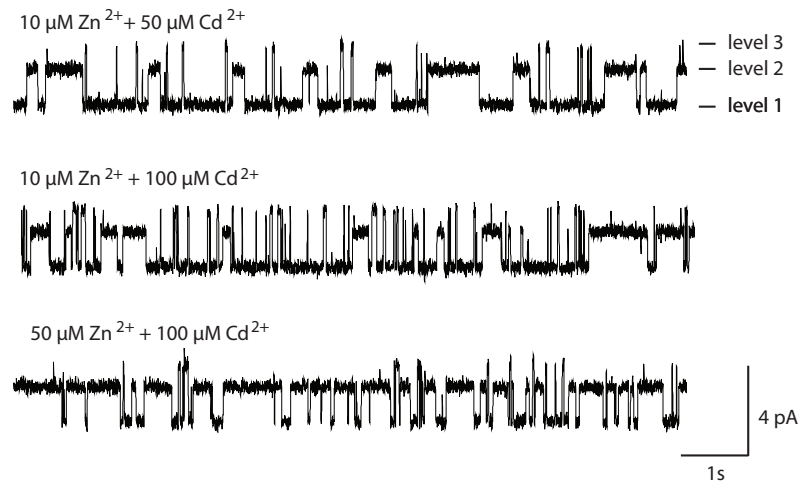


Figure 2.10: The simultaneous detection of Zn^{2+} and Cd^{2+} in solution. **A** Single channel recording of the PPIDA pore at $-50\ \text{mV}$ in $2\ \text{M}\ \text{KCl}$, $10\ \text{mM}\ \text{MOPS}$ ($\text{pH}\ 7.0$). Zn^{2+} and Cd^{2+} were added to the trans recording chamber. For each set of concentrations $n=3$.

To analyse the kinetics of the two types of block, a list of dwell times in each of the three states was generated by using the “Single Channel Search” algorithm of the Clampfit 10.2 software, as described previously. The dwell times of Zn^{2+} or Cd^{2+} in the pore ($\bar{\tau}_{\text{off}}$) can be estimated readily from the dwell time histograms of the blocked levels, by fitting the histograms with single exponential functions. By using

$k_{\text{off}} = 1/\bar{\tau}_{\text{off}}$, the value of the rate constant can then be derived. The determination of k_{on} , however, is more difficult. Two methods exist to determine k_{on} for Zn^{2+} or Cd^{2+} from the idealised trace. In addition to the general assumptions made for a kinetic Markov analysis (distinct number of states, fixed probabilities for the transitions between states that depend only on the current state of the system) both types of analysis will also assume that

1. The two metal ions interact with the pore independently (i.e. when Zn^{2+} is bound, then Cd^{2+} cannot bind simultaneously and vice versa).
2. The dwell time of one type of metal ion in the pore is unaffected by the presence of the second type.

Figure 2.11A describes the kinetic model that will form the basis of both types of analysis.

The first method of analysis described here has been used by Braha et al. [24] for the simultaneous detection of metal ions using a genetically engineered αHL pore and involves determining the fractional occupancy for each divalent metal ion in the pore. For this purpose an edited trace of events for each divalent metal ion is constructed, in which the periods of occupancy by the other divalent metal ion are deleted (Figure 2.11B). Using the edited trace, $\bar{\tau}_{\text{on}}$ is then determined from a dwell time histogram. Again, k_{on} is determined graphically from a plot of $1/\bar{\tau}_{\text{on}}$ against $[\text{Zn}^{2+}]$. The process is then repeated for the other divalent metal ion.

If there are more than two states under investigation, this traditional method of determining the rates of transitions by fitting dwell time histograms with exponential functions has several drawbacks: first, it is extremely laborious, as it involves manually editing the recording traces. Second, for a system of N states, a maximal number of $2(N-1)$ kinetic parameters can be resolved. This is generally inadequate for more complex kinetic models, especially if they involve loops. Furthermore, the histogram fitting method makes no use of the correlation between adjacent levels.

A second method to determine the rates of transitions between different states, which

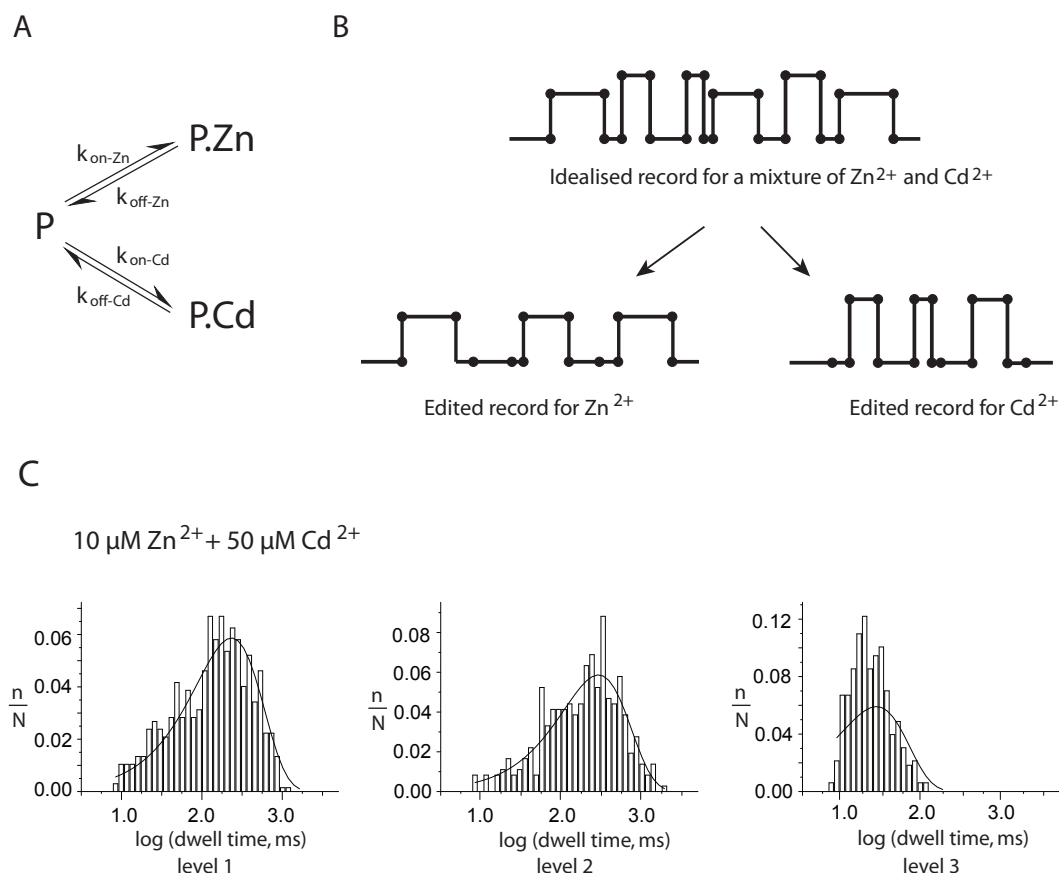


Figure 2.11: Determination of rate constants for mixtures of analytes. **A** Kinetic scheme describing the binding of Zn^{2+} and Cd^{2+} to the PPIDA pore. **B** Method of editing traces to determine the fractional occupancy of Zn^{2+} and Cd^{2+} in the pore as described by Braha et al. [24]. **C** Log(dwel time) histograms and probability density functions for levels 1, 2, and 3 constructed in QuB. In this experiment the analytes were $10 \mu\text{M Zn}^{2+}$ and $50 \mu\text{M Cd}^{2+}$.

overcomes the limitations of histogram fitting, is the maximum likelihood approach. In maximum likelihood fitting, a kinetic model (e.g. Figure 2.11A) is fitted directly to observed dwell time sequences such that the probability of the observed dwell time sequences is maximised [37]. For the kinetic analysis of Zn^{2+} - and Cd^{2+} -binding by the PPIDA pore, this was done by using the “Maximum Interval Likelihood” (MIL) algorithm of the QuB software package. The program input consisted of the idealised dwell time sequences that had been generated by the Clampfit single channel search algorithm, as well as the kinetic model with initial kinetic parameters (the default setting was $k=1$ for all rate constants). The MIL algorithm works by calculating

the gradients of the likelihood function, and then maximising the likelihood using a fast variable metric optimiser [38]. The MIL output therefore includes the MIL rate constants, a log likelihood (LL) score of the rate constants and the “gradient” (the derivative of the LL with respect to each model parameter). In addition, the program derives a probability density function (PDF) and time constants (τ) computed from the final model parameters for each conductance class. The PDF is displayed as an overlay to the dwell time histogram of the original data (Figure 2.11C).

The quality of the MIL rate constant estimates and the validity of the kinetic scheme is judged by

1. The quality of the fit of the predicted PDF to the dwell time histograms: If the PDFs and histograms do not match, then the kinetic scheme on which the MIL rate constants are based must be revised. Assuming that each conductance class corresponds to only one state, this involves changing the connectivity between the states.
2. The log likelihood (LL) score of the model: A higher LL indicates a better fit.
3. The gradient of the likelihood function: Good fits have a gradient near 0, indicating a local maximum on the likelihood surface.

The MIL rate constants determined in QuB are apparent rate constants for a given concentration of either Zn^{2+} or Cd^{2+} . To calculate k_{on} , the apparent on-rate in a recording was normalised to the concentration of the analyte and the average value was calculated from nine independent experiments (three for each concentration set). k_{off} , which is independent of the concentration, was calculated by averaging the apparent off rates. The QuB analysis of these experiments gave $k_{\text{on-Zn}} = (2.1 \pm 0.4) \times 10^5 \text{ M}^{-1}\text{s}^{-1}$, $k_{\text{off-Zn}} = 4.7 \pm 0.6 \text{ s}^{-1}$, $k_{\text{on-Cd}} = (3.9 \pm 1.0) \times 10^4 \text{ M}^{-1}\text{s}^{-1}$ and $k_{\text{off-Cd}} = 39 \pm 4 \text{ s}^{-1}$.

2.3.4 Discussion

A comparison of the amplitude of the block (ΔI), k_{on} , k_{off} and K_f for Zn^{2+} , Cd^{2+} and Mn^{2+} binding to the PPIDA pore is made in Table 2.1.

	Zn^{2+}	Cd^{2+}	Mn^{2+}
ΔI (pA)	1.6 ± 0.1	2.6 ± 0.2	3.2 ± 0.2
effective ionic radius CN=6 ($\text{\AA}$)	-	-	0.83 (high spin)
effective ionic radius CN=4 ($\text{\AA}$)	0.60	0.78	-
τ_{off} (ms)	251 ± 13	20.7 ± 1.9	3.7 ± 1.2
k_{off} (s^{-1})	4.0 ± 0.2	47 ± 4	290 ± 20
k_{on} ($\text{M}^{-1}\text{s}^{-1}$)	$(1.5 \pm 0.2) \times 10^5$	$(5.6 \pm 0.7) \times 10^4$	$(1.6 \pm 0.2) \times 10^4$
K_f (M^{-1})	$(3.8 \pm 0.5) \times 10^4$	$(1.2 \pm 0.2) \times 10^3$	$(5.5 \pm 0.8) \times 10^1$
$\log(K_f)$ with MIDA	7.63	6.75	5.39

Table 2.1: Comparison of Zn^{2+} , Cd^{2+} and Mn^{2+} binding to the PPIDA pore. The data for the effective ionic radii were taken from [39]. CN = coordination number. The data for $\log(K_f)$ with MIDA were taken from [14]. MIDA = Methyl-imminodiacetic acid.

Each divalent metal ion gives rise to binding events with distinct fixed current amplitudes (ΔI), when analysed either individually or in a mixture. It was attempted to relate the amplitude of the block to the size of the divalent metal ion. However, this was not straightforward. The effective radii of many metal ions have been determined from the crystal structures of the metal-oxygen or metal-halide complexes [39]. However, these data show that the effective ionic radius of a metal depends on the complex environment: the ligand coordination number, and the electronic spin and oxidation state of the metal are known to affect the ionic radius. To estimate the ionic radii of the metals on the half-chelator several assumptions had to be made: first, it was assumed that the oxidation state of the metals is stable, and second that the Mn^{2+} ion (d^5) on the half-chelator is high spin (the Mn^{2+} complexes with the chelating ligands en, EDTA and oxalate are known to be high spin too [40]). For Zn^{2+} and Cd^{2+} (d^{10}) there are no spin effects to take into account. Third, the ligand coordination number of the complexes had to be

estimated. The half-chelator ligand is tridentate, so there are vacant coordination sites on the metals. These are likely to be occupied by water or chloride ligands, which typically have very fast exchange rates [41] and would not be observed at the time resolution of the experiments described here (~ 0.2 ms). Mn^{2+} complexes typically have a ligand coordination number of 6, because the octahedral geometry of the complex affords the highest crystal field stabilisation energy of the d-orbital electrons. As the both Zn^{2+} and Cd^{2+} have the full d^{10} electronic configuration, the complexes of these metal ions can gain no crystal field stabilisation energy by adopting a particular geometry. Therefore, the ligand coordination number of these complexes is determined by the steric requirements of the ligands and 4-coordinate tetrahedral complexes are typically favoured, although other coordination geometries are known [42]. Using these assumptions, the size of the divalent metal ions was estimated by reference to the literature data [39]. It was found that the size of the metal ion is related to the amplitude of the partial block of the ionic current, that is, a small ion results in small block, whereas a large ion results in a large block (Table 2.1). Further experiments would be required to determine if this trend would be followed by other divalent metal ions.

The dwell time (τ_{off}) of Zn^{2+} in the PPIDA pore is an order of magnitude longer than that of Cd^{2+} , which is in turn an order of magnitude longer than that of Mn^{2+} . This is reflected directly in the values of k_{off} for the complexes. The values for k_{on} differ less between the different metal ions than the values of k_{off} : k_{on} for Zn^{2+} is three-fold faster than for Cd^{2+} , which is in turn four-fold faster than k_{on} for Mn^{2+} . The experiments involving the simultaneous detection of Zn^{2+} and Cd^{2+} show that the presence of one divalent metal ion does not perturb the binding of the other. Indeed, as summarised in Table 2.2, ΔI , k_{on} and k_{off} for a given metal are the same within experimental error in both types of experiment. Interestingly, since the stability constant of the complexes is given through the ratio of k_{on} to k_{off} , these experiments show that k_{off} dominates the magnitude of the formation constant, K_f .

	Zn ²⁺	Cd ²⁺
$\Delta I_{\text{independent}}$ (pA)	1.6 ± 0.1	2.6 ± 0.2
$\Delta I_{\text{simultaneous}}$ (pA)	1.7 ± 0.2	2.8 ± 0.2
k_{on} (independent, M ⁻¹ s ⁻¹)	$(1.5 \pm 0.2) \times 10^5$	$(5.6 \pm 0.7) \times 10^4$
k_{on} (simultaneous, M ⁻¹ s ⁻¹)	$(2.1 \pm 0.4) \times 10^5$	$(3.9 \pm 1.0) \times 10^4$
k_{off} (independent, s ⁻¹)	4.0 ± 0.2	47 ± 4
k_{off} (simultaneous, s ⁻¹)	4.7 ± 0.6	39 ± 4

Table 2.2: Comparison of Zn²⁺ and Cd²⁺ binding by the PPIDA pore, either independently or simultaneously. The measurements were obtained in buffer containing 2M KCl, 10 mM MOPS, pH 7.0 and under an applied potential of -50 mV. The rates for the simultaneous experiments were calculated from 9 independent experiments (3 for each set of concentrations). The rates for the independent experiments are from table 2.1.

It is not straightforward to make a detailed comparison of the rates determined in these experiments to those reported by other researchers using different techniques, because only few rate constants have been measured directly. A value of 50 s⁻¹ was determined by NMR line-shape analysis for Zn(MIDA)₂²⁻ → Zn.MIDA + MIDA²⁻ (MIDA = methyl-imminodiacetic acid) [18], which compares reasonably with the k_{off} value of 4 s⁻¹ determined here. However, the authors of the NMR study also found that the exchange rate was independent of pH over the range 6.0 to 9.0, which is surprising considering that the chelator must re-protonate in the process of metal dissociation. A more detailed analysis of how the pH of the recording solution affects k_{on} , k_{off} and K_f for Zn²⁺ binding by the PPIDA pore will be given in chapter 4. In the context of this chapter it is important to note that the formation of the half-chelator.Zn²⁺ complex is only possible when the half-chelator is in the deprotonated state [5, 16, 43], the probability of which is determined by the pH of the recording buffer and the pK_a of the half-chelator. Therefore the values of the rate constants and the formation constant are expected to depend on the pH. It is possible that the pore itself also affects the magnitude of the rate constants, as the half-chelator is surrounded by other amino acid side chains inside the β -barrel of the protein, which may affect its interaction with Zn²⁺. In particular, there is a lysine residue at position 147 on the same subunit, whose α -carbon is separated from the α -carbon of residue 117 by 14 Å. This distance leaves the possibility of

an electrostatic interaction between the half-chelator and the lysine residue, which could result in a lower k_{on} value. Further, the trans opening of the β -barrel contains 14 aspartate residues (D127 and D128 on each of the seven subunits) that may interact with Zn^{2+} . The wt αHL pore did not show any interaction with Zn^{2+} in single channel recordings filtered with a 5 kHz low-pass Bessel filter, therefore any interaction between the aspartate residues and Zn^{2+} must be extremely transient and is unlikely to affect k_{on} . To investigate if K147 and D127/D128 do indeed affect k_{on} , the experiments described in this chapter could be repeated with a mutant αHL pore, in which the charged residues at positions 127, 128 and 147 are replaced with uncharged amino acids (e.g. alanines).

The effect of the pore structure on the rate of complex formation can also be estimated by comparing k_{on} to the rate of the diffusion-limited reaction between Zn^{2+} and the half-chelator in solution. The following derivation was adapted from Alberty et al. [45].

If the half-chelator and the Zn^{2+} ion were diffusing freely in bulk solution, then the rate of diffusion of each reaction partner would be given by Fick's first law through

$$dq = 4\pi r^2 D \left(\frac{\partial c}{\partial r} \right) dt \quad (2.1)$$

where dq is the quantity diffusing through a spherical surface of radius r in time dt , c is the concentration and D is the diffusion coefficient. D depends on the viscosity of the solution, the size and shape of the diffusing molecule and its interaction with the solvent. The differential equation for spherically symmetric diffusion with the diffusion coefficient independent of the concentration is given through

$$\frac{\partial(rc)}{\partial t} = D \frac{\partial^2(rc)}{\partial r^2} \quad (2.2)$$

Smoluchowski showed that the quantity q that flows into a fixed spherical sink of radius R in time t , from an infinite volume of initially homogenous solution of concentration c_0 , may be obtained by integration of the equation above and is given

by

$$q = 4\pi DRc_0[t + \frac{2R\sqrt{t}}{\sqrt{\pi D}}] \quad (2.3)$$

After a transient phase of diffusion, the term $t^{1/2}$ becomes negligible, and the rate of flow into the spherical sink becomes constant. In order to allow for Brownian motion of the particles, the sum of the diffusion coefficients, D_{12} (in $\text{cm}^2 \text{s}^{-1}$) = $D_1 + D_2$, of the two particles is used. In applying the Smoluchowski equation to a reaction, R is replaced by the sum R_{12} (in cm) of the radii of the reactants, which is also called the reaction cross section. Therefore the second order rate constant k (in $\text{M}^{-1}\text{s}^{-1}$) for the reaction between two unlike molecules is expressed as

$$k = \frac{4\pi N}{1000} R_{12} D_{12} \quad (2.4)$$

Using this equation it could be shown that the rate of reaction between a fumerate ion and a fumerase enzyme approaches the diffusion limit of $1.8 \times 10^9 \text{ M}^{-1}\text{s}^{-1}$ at 298 K [45]. To estimate the diffusion limited rate between a Zn^{2+} ion and a half-chelator in solution, D_1 , D_2 and R_{12} must be estimated. The diffusion coefficient for Zn^{2+} in water at 298 K is $7 \times 10^{-6} \text{ cm}^2 \text{s}^{-1}$ [46]. The diffusion coefficient of the half-chelator in water is not known, however, the diffusion coefficient for the amino acid leucine is also $7 \times 10^{-6} \text{ cm}^2 \text{s}^{-1}$ [47] and will be used as an approximation. Again, the reaction cross-section is unknown, but the value of $5 \times 10^{-8} \text{ cm}$ utilised in the fumerase study is a reasonable estimate. These parameters predict a diffusion-limited rate of $5 \times 10^8 \text{ M}^{-1}\text{s}^{-1}$ at 298 K, which exceeds the value of k_{on} measured by single channel recording by three orders of magnitude. However, the estimate assumes that the half-chelator is fully deprotonated. Since the pK_{a} of the half-chelator is approximately 9.5 and k_{on} was measured at pH 7.0, it is not surprising that k_{on} is lower than the calculated diffusion-limited rate. Further, in the experiments described in this chapter, the divalent metal ions are binding against a voltage gradient, which can also alter rates [31]. Additional experiments could be carried out to determine in how far the pH and the applied voltage affect the rate of complex formation and whether this alone

can account for the lower values of k_{on} .

The relative stabilities of the complexes determined here mirror the stability of the MIDA complexes reported in the literature ($\text{Zn}^{2+} > \text{Cd}^{2+} > \text{Mn}^{2+}$), but the actual numbers are smaller. As indicated by Toone et al. [16], it is difficult to find data that can be compared fairly, because the buffer ions and the pH of the reaction can have a large impact on the stability of metal-chelator complexes and few experiments are carried out under comparable conditions.

Overall these experiments have shown that the complex formation between an engineered chelator and divalent metal ions can be monitored in real time and at the single molecule level, thereby establishing a new means to obtain kinetic and thermodynamic data for the formation of metal ion complexes. It was shown that the PPIDA pore can be used as a stochastic sensor to detect the presence and amount of different divalent metal ions, both individually and simultaneously in a mixture. In principle this approach could be extended to the detection of other divalent metal ions. The single-molecule approach presented here avoids many of the complexities encountered in ensemble experiments and others can be ruled out. For example, there is no excess chelator able to promote divalent cation exchange, and no evidence was seen for the additional states that might indicate exchange mediated by slow proton transfer [20, 48]. These experiments therefore enhance our ability to obtain information about the chelation of divalent metal ions.

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Chapter 3

Single molecule kinetics of two-step divalent cation chelation

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3.1 Chapter Overview

This chapter investigates the binding kinetics and equilibria of an α HL pore containing two half-chelator ligands with Zn^{2+} ions. It will be shown that a single Zn^{2+} ion can be bound simultaneously by two half-chelators in close proximity, in a fashion similar to EDTA. Remarkably a two step process is visualised at pH 4.0, in which the Zn^{2+} ion must first bind to either one of the two half-chelators, before

the second one completes the complex. The rate constants for the eight major steps of this process are determined. As the formation of the full complex includes an intramolecular step, the extent of chelate cooperativity between the two half-chelators is also assessed.

This chapter begins by providing an overview of the different types of cooperativity, how cooperativity can be quantified and how it relates to the concept of multivalency. These concepts will then be applied to the analysis of the kinetics and equilibria of P(PIDA)₂ pore (the pore containing two half-chelators) with Zn²⁺.

3.2 Introduction to cooperativity

Cooperativity is a key mechanism that regulates the behaviour of complex molecular processes in biology and chemistry, for example protein folding [1, 2], enzyme kinetics [3] and molecular recognition [4]. Two types of cooperativity have to be distinguished: allosteric and chelate cooperativity [5–7]. While allosteric cooperativity has been widely recognised in biological systems, the assessment of chelate cooperativity (which is the focus of this chapter) has largely been unsatisfactory. Indeed, many authors struggle to quantify cooperativity adequately [5, 6]. Recently chelate cooperativity has received renewed attention in the field of supramolecular chemistry [4, 8, 9] and the findings should further our understanding of this topic in other areas. A definition of the different types of cooperativity and how they can be measured in terms of the binding equilibria involved is given below. An explanation of how multivalency relates to cooperativity will also be given.

3.2.0.1 Allosteric Cooperativity

Allosteric cooperativity describes a process whereby binding of a ligand to a protein causes a conformational change of the protein, which facilitates the binding of subsequent ligands. The classic example illustrating this process is

the interaction of oxygen with haemoglobin. Once the first molecule of oxygen is bound by haemoglobin, the binding of subsequent molecules of oxygen is facilitated [10]. Allosteric cooperativity is also seen in some enzymes, e.g aspartate transcarbamoylase, in which binding of the substrate at one of the catalytic sites facilitates substrate binding at the other sites [11]. Allosteric cooperativity is easy to quantify if the microscopic association constants for the formation of the mono-liganded protein (K_1) and the doubly-liganded protein from the mono-liganded protein (K_2) are known. If K_2 is larger than K_1 , then there is positive cooperativity between the two binding sites. If K_1 is larger than K_2 , then there is negative cooperativity between the two sites. If $K_1 = K_2$, then there is no interaction between the two sites. Some authors define a thermodynamic interaction parameter α [5, 6] that describes the level of cooperativity between the two sites:

$$\alpha = \frac{K_2}{K_1} \quad (3.1)$$

When $\alpha = 1$, the two sites are independent; when $\alpha > 1$ there is positive cooperativity between the two sites and when $\alpha < 1$ there is negative cooperativity between the two sites. It should be noted that α is a dimensionless parameter. Allosteric cooperativity is frequently associated with conformational changes of a protein [11]. The mechanism of allostery is still under debate. The “induced fit” model developed by Koshland, Nemethy and Filmer [12] proposes that ligand binding effects a conformational change of the protein. In contrast to this, the “conformation selection” model by Monod, Wyman and Changeux [13] proposes that a population of proteins exists in several distinct states that are in equilibrium with each other. Ligands bind preferentially to some states over others, thereby shifting the equilibrium between the states. Recent evidence suggests that induced fit and conformation selection models may co-exist and the dominance of one pathway over the other depends on the experimental conditions [14]. In chemical systems allosteric cooperativity has also been associated with the electronic polarisation of

a receptor [15] or long-range electrostatic interactions between ligands [16].

3.2.0.2 Chelate Cooperativity

Chelate cooperativity describes the enhanced thermodynamic and kinetic stability of multivalent interactions compared to the corresponding monovalent interactions [17]. The classic example illustrating this process is divalent metal ion chelation by EDTA (Figure 3.1). The formation of fully chelated M.EDTA is a two-step

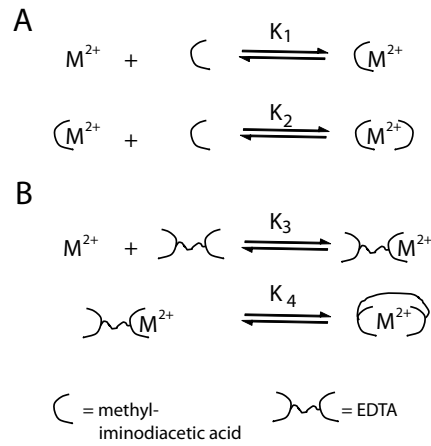


Figure 3.1: Steps involved in divalent metal ion chelation by **A)** MIDA and **B)** EDTA.

process, in which the two methyl-iminodiacetic acid (MIDA) chelation groups have to subsequently bind to the metal. The overall formation constants for fully chelated M^{2+} by MIDA and EDTA are given through

$$K_f(\text{M.MIDA}_2) = K_1 \times K_2 \quad (3.2)$$

$$K_f(\text{M.EDTA}) = K_3 \times K_4 \quad (3.3)$$

Experimentally it has been found that $K_f(\text{M.EDTA})$ exceeds $K_f(\text{M.MIDA}_2)$ by one to two orders of magnitude, depending on the divalent metal ion under investigation [18]. The origin of this effect has been primarily attributed to the fact that formation of fully chelated EDTA includes an intramolecular step, whereas formation of M.MIDA_2 includes only intermolecular steps. To quantify such an effect

microscopically, K_1 - K_4 in Figure 3.1 must be known (footnote ¹). In the case of allosteric cooperativity, one could simply define a parameter α , which is the ratio of the two association constants, K_4 and K_2 . This is not possible in the present case. Since the association constants have different units, the Gibbs free energy changes associated with the two equilibria are not additive [5, 19]. One possible solution to quantify the extent of cooperativity between the two chelation groups is to define the effective molarity, M_{eff} [20, 21], of the second MIDA group on EDTA. M_{eff} is given through

$$M_{\text{eff}} = \frac{K_f^{\text{intra}}}{K_f^{\text{inter}}} = \frac{K_4}{K_2} \quad (3.4)$$

It represents the amount of free EDTA (or MIDA) that would have to be present in solution to achieve the same extent of chelation as observed in the intramolecular case. From this definition of M_{eff} it also follows that if $[\text{EDTA}] > M_{\text{eff}}$, then the intermolecular reaction to form $\text{M}(\text{EDTA})_2$ would dominate over the intramolecular reaction to form $\text{M}(\text{EDTA})$ (Figure 3.2). From the formation constants of

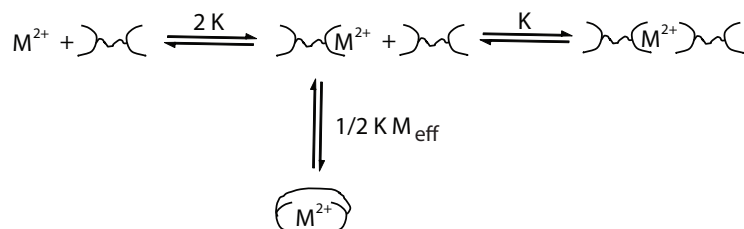


Figure 3.2: The relationship between K_{inter} , K_{intra} and M_{eff}

$\text{Ni}(\text{MIDA})_2$ and $\text{Ni}(\text{EDTA})$ [18], M_{eff} for EDTA is estimated to be 14 M (footnote ²). Therefore the intermolecular reaction would not be expected to contribute to the equilibria under regular experimental conditions. However if M_{eff} was in the mM range (and this is frequently the case in synthetic systems [5]) then the intramolecular reaction would dominate at low ligand concentrations and the

¹For the Ni complexes of MIDA and EDTA [18]: $\log(K_{\text{Ni,MIDA}}) = 8.67$, $\log(K_{\text{Ni,MIDA}_2}) = 15.85$, $\log(K_{\text{Ni,EDTA}}) = 18.52$. Therefore $\log(K_1) = 8.67$, $\log(K_2) = 7.18$, $\log(K_3) \approx \log(K_1) = 8.67$, $\log(K_4) = 9.85$

² $\log(K_2) = 7.18$, $\log(K_4) = 9.85$, therefore $K_2 = 1.31 \times 10^3 \text{ M}^{-1}$, $K_4 = 1.90 \times 10^4$ and $M_{\text{eff}} = K_4/K_2 = 14 \text{ M}$

intermolecular reaction would dominate at high ligand concentrations [5]. Since the contributions of inter- and intramolecular reactions to equilibria vary with the concentration of the ligand, the two types of reaction can only be compared under specific experimental conditions, but not in general thermodynamic terms.

In practical terms, the magnitude of M_{eff} depends on the length and flexibility of the linker between the two chelation groups and reflects the proximity of the two reactive groups (i.e. the second MIDA ligand and the divalent metal ion) and whether they have the correct orientation for complex formation [22, 23].

Chelate cooperativity isn't limited to the formation of inorganic complexes. Many biological processes are driven by this type of cooperativity, for example protein folding [1, 2]. When a small part of a denatured protein folds, a network of stabilising interactions (hydrophobic interactions, H-bonds, salt bridges, van der Waals forces etc.) is established that then promotes the folding of the remainder of the structure. Similarly when part of the folded protein structure is denatured by heat or chemicals, not only are the interactions within that part of the structure lost, but also the long range interactions to other parts of the protein, causing the whole structure to unfold rapidly. This is evident in the sharp transitions between the folded and unfolded state of the protein in response to denaturants (Figure 3.3). Cooperativity ensures that proteins are either fully folded or unfolded, but not partly folded (which could lead to the interference with other cellular processes) [24].

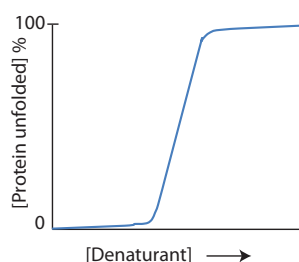


Figure 3.3: *Protein folding and unfolding are cooperative processes*

3.2.0.3 Multivalency vs. chelate cooperativity

The apparent strength of interaction between two particles can be stronger if the interaction is mediated by many ligand-receptor interactions than if it is mediated by a single interaction. However, it is a common misconception that the strength of multivalent interactions stems from cooperativity, in fact cooperativity in multivalent systems is probably extremely scarce [9]. Although positive cooperativity would enhance the interaction between particles, multivalent ligand-receptor interactions can exhibit exceptional strength in the absence of cooperativity. For example, Kitov et al. [25] created a pentavalent glycoprotein inhibitor (STARFISH) for the pentameric Shiga-like toxin. STARFISH displays a 10-million-fold increase in inhibition of the toxin compared to the monovalent ligand [$IC_{50}(\text{penta})=0.24 \text{ nM}$; $IC_{50}(\text{mono})=2.1 \text{ mM}$]. If the data from this study are analysed in detail, one can calculate $K_{\text{mono}} \approx [IC_{50}(\text{mono})]^{-1} = 4.8 \times 10^2 \text{ M}^{-1}$; $K_{\text{penta}} \approx [IC_{50}(\text{penta})]^{-1} = 4.2 \times 10^9 \text{ M}^{-1}$. Formation of the fully liganded Shiga-like toxin with independent ligands is given through $K_5 = (K_{\text{mono}})^5 = 2.5 \times 10^{13} \text{ M}^{-5}$, which exceeds K_{penta} by four orders of magnitude. The effective molarity of a monovalent ligand in the STARFISH inhibitor can be estimated by using

$$M_{eff} = \sqrt[5]{\frac{K_{penta}}{K_5}} = 175 \text{ mM} \quad (3.5)$$

In order to assess cooperativity in multivalent systems (i) the exact number of binding partners in the experiment must be known, (ii) binding data for the monovalent and multivalent interactions must be available and (iii) microscopic association constants for substeps in the formation of the final complex must be known in order to calculate the exact effective molarities for each step. The last point can present considerable challenges in ensemble measurements, as many intermediates are too short lived to be detected. Single channel recording, which has a temporal resolution of up to $25 \mu\text{s}$, could identify short-lived intermediates and

therefore promises to be an excellent tool for the investigation of cooperativity in multivalent systems. This chapter will show how single channel recording of a pore containing two half-chelator ligands can be used to identify both the partially and fully chelated Zn^{2+} complex, the rates and equilibria leading to their formation and finally how these data can be used to calculate the effective molarity of the second half-chelator ligand.

3.3 Results

3.3.1 The design of two chelation sites in the α HL pore

In order to assess the level of cooperativity between two half-chelator ligands, an α HL pore containing two-half chelators in the β -barrel of the protein had to be created. The half-chelators had to be positioned in such a way that they could both participate in the complex formation with a single divalent metal ion. To identify suitable sites for the covalent modification of the protein, molecular models of the α HL pore (PDB code 7AHL) were created in PyMOL (Figure 3.4). The experiments of the previous chapter had established that position 117 is a good site for the installation of a single half-chelator. Therefore it was decided that one of the two half-chelators would also be installed at residue 117. The crystal structure of [bis(iminodiaceto-N,O,O)Ni(II)] [26] served as the starting point for the molecular models. First the remainder of the two half-chelator ligands (the PIDA molecule minus the iodine atom) was built onto the Ni(II) complex by using the PyMOL Build tool. The PyMOL Mutagenesis Wizard tool was used to mutate residue 117 on one subunit from threonine to cysteine, and the sulfur atom of the cysteine residue was then fused to the α -carbon of one of the two PIDA molecules on the Ni(II) complex. This positioned the second ligand favourably for connection with residue 143 on the same subunit, which was therefore chosen as the second site for mutagenesis and subsequent chemical modification. This pore is called P(PIDA)₂. It was also attempted to install the second PIDA ligand at position 115 rather than 143 on

the same subunit, as model building had suggested that a second half-chelator at this position would also form a full complex with the first half-chelator at position 117. However, single channel recordings with this pore did not indicate that this full complex was formed, possibly due to steric hindrance. The investigation of the pore doubly modified at position 115 and 117 was therefore not continued.

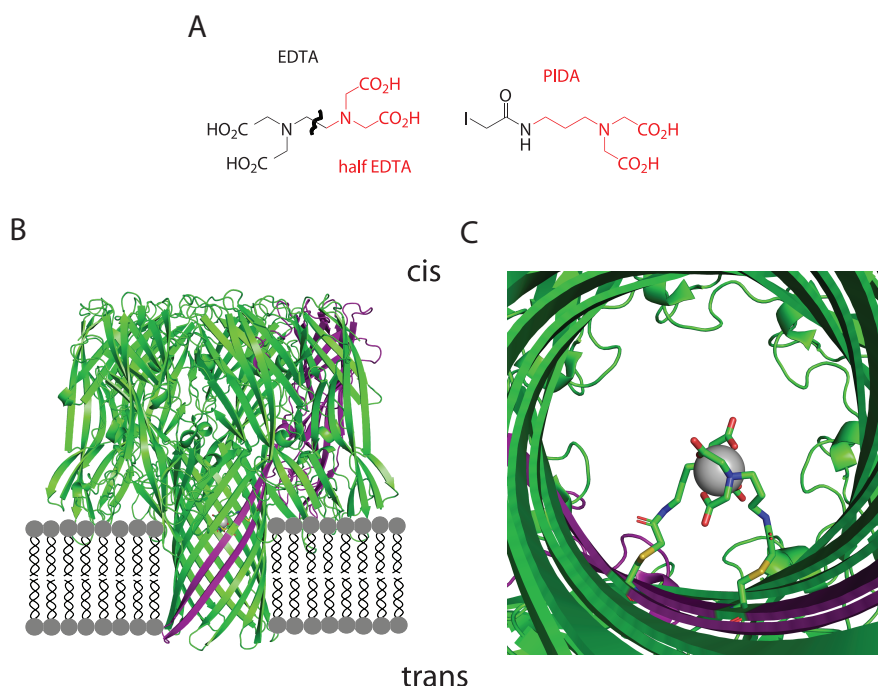


Figure 3.4: Molecular model of the $P(\text{PIDA})_2$ pore. **A)** The chemical structure of EDTA and PIDA. **B)** The $P(\text{PIDA})_2$ pore comprises six wild-type subunits (green) and one modified subunit (purple). **C)** View inside the β -barrel of the modified $P(\text{PIDA})_2$ pore. Two molecules of PIDA are connected to the protein backbone at positions 117 and 143 and form a complex with a divalent metal ion (grey sphere).

3.3.2 Preparation of the $P(\text{PIDA})_2$ pore

The monomeric polypeptide $\alpha\text{HL}(117\text{C}/143\text{C})\text{-D8}$ was prepared by in vitro transcription and translation (IVTT) of the gene $\alpha\text{HL}(117\text{C}/143\text{C})\text{-D8}$ (a gift from Seong-Ho Shin, University of Oxford). The monomeric protein was chemically modified with the half-chelator ligands using the method described in chapter 2.3.2. In order to assess the extent of chemical modification of the monomer, gel shift experiments were performed with the proteins wt αHL , $\alpha\text{HL}(117\text{C})\text{-D8}$,

and α HL(T117C/143C)-D8 using either water or PIDA or mPEG-OPSS-5000 as the reagent (Figure 3.5). As expected, the reaction with water did not result in a gel

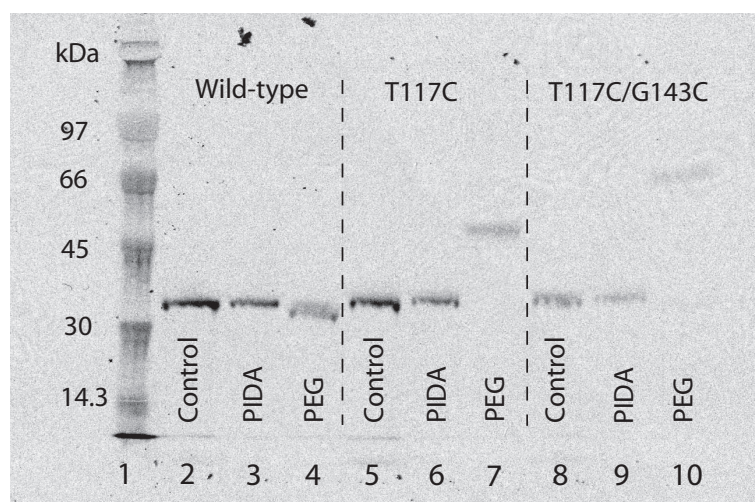


Figure 3.5: 12% SDS-PAGE gel of monomers of α HL, showing the gel shifts caused by chemical modification of one or two cysteine residues. Lane 1: 14 C-protein molecular weight markers. Lane 2: wt α HL (water control). Lane 3: wt α HL after reaction with the half-chelator reagent. Lane 4: wt α HL after reaction with mPEG-OPSS-5000. Lane 5: α HL(T117C)-D8 (water control). Lane 6: α HL(T117C)-D8 after reaction with the half-chelator reagent. Lane 7: α HL(T117C)-D8 after reaction with mPEG-OPSS-5000. Lane 8: α HL(T117C/143C)-D8 (water control). Lane 9: α HL(T117C/143C)-D8 after reaction with the half-chelator reagent. Lane 10: α HL(T117C/143C)-D8 after reaction with mPEG-OPSS-5000. Lanes 1-7 of this gel are the same as the gel shown in chapter 2.3.

shift for any of the three proteins. The reaction with PIDA also did not result in a detectable gel shift for any of the proteins, although this was not surprising for α HL(T117C/143C)-D8 considering that α HL(T117C)-D8 had not resulted in gel shift either (the single channel recordings in chapter 2 showed that this protein was modified). However, the reaction with mPEG-OPSS-5000 confirmed that both cysteines in α HL(T117C/143C)-D8 were fully reactive. The protein band for mPEG-OPSS-5000-modified α HL(T117C/143C)-D8 had a slower electrophoretic mobility than the band for mPEG-OPSS-5000-modified α HL(T117C)-D8, indicating that both cysteines had reacted.

The chemically modified monomers were then mixed with wt α HL subunits and assembled on rabbit red blood cell membranes. The different oligomers were then separated in a 5% SDS-PAGE gel by electrophoresis (Figure 3.6) and the band

corresponding to the $(WT)_6(mod-D8)_1$ oligomer (i.e. the $P(PIDA)_2$ pore) was excised from the gel, reconstituted in buffer and was used for the single channel recordings.

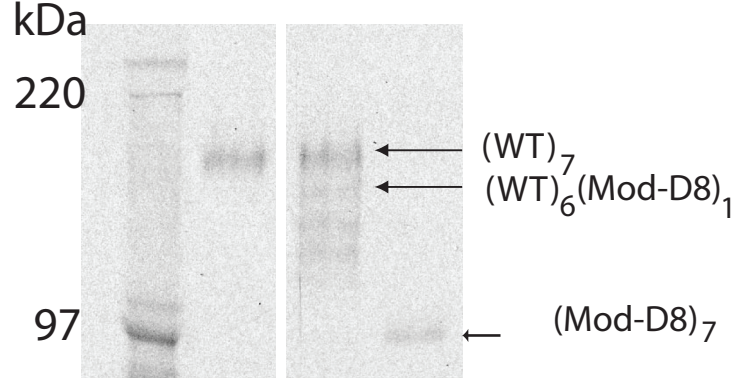


Figure 3.6: 5% SDS-PAGE gel showing oligomers of αHL . Left lane: Heptamers comprising wild-type subunits only. Middle lane: Heptamers formed from wild-type and 117C/143C- $PIDA_2$ -D8 subunits. Right lane: Heptamers formed from 117C/143C- $PIDA_2$ -D8 subunits only. Mod, engineered subunit; $(WT)_6(Mod-D8)_1$, the heteroheptamer containing six wild-type and one engineered subunit.

3.3.3 Zn^{2+} binding by the $P(PIDA)_2$ pore

The Zn^{2+} binding experiments described in the previous chapter were repeated with the $P(PIDA)_2$ pore. $P(PIDA)_2$ had a single-channel current of -67.0 ± 2.8 pA at -50 mV in 2 M KCl, 10 mM MOPS, pH 7.0 (Table 3.1), which is less than the single channel current of the $PPIDA$ pore under the same recording conditions. This indicates that

Protein	pH	Open Pore Current (pA)	Magnitude of partial block (ΔI , pA)	
WT_7	7.0	-79.1 ± 1.8	-	
$PPIDA$	7.0	-73.8 ± 2.5	1.6 ± 0.1	
$P(PIDA)_2$	7.0	-67.0 ± 2.8	3.7 ± 0.3	
$PPIDA$	4.0	-75.7 ± 1.0	1.60 ± 0.003	
$P(PIDA)_2$	4.0	-56.4 ± 2.0	Level A	1.0 ± 0.04
			Level B	2.5 ± 0.2
			Level C	3.7 ± 0.1

Table 3.1: Single channel currents of unoccupied αHL pores and after partial blockade by Zn^{2+} . All values were calculated from at least three independent experiments and are quoted as the mean $\pm$ s.d. The measurements were recorded at -50 mV in buffer containing 2.0 M KCl on both sides of the bilayer. At pH 4.0, three distinct blocked states (levels A to C) were observed after the addition of Zn^{2+} to the $P(PIDA)_2$ pore. The magnitude of the block is quoted relative to the unoccupied pore current.

the P(PIDA)₂ pore had been doubly modified. Upon the addition of 10 μM Zn^{2+} to the trans recording chamber, very long binding events ($\Delta I = 3.7 \pm 0.3$ pA) were observed that lasted from tens of seconds to several minutes (Figure 3.7A). These events were interpreted to represent complex formation between both half-chelators and a single Zn^{2+} ion. Because the binding events were so long, it was difficult to perform a detailed kinetic analysis. Furthermore, no intermediate states leading to fully liganded Zn^{2+} could be observed. To overcome these obstacles, the pH of the recording buffer was lowered from 7.0 to 4.0. The formation of the half-chelator. Zn^{2+} complex and the fully chelated Zn^{2+} complex is only possible when the half-chelators are in the deprotonated state [27–29], the probability of which is determined by the pH of the recording buffer (the pK_a values of *N*-methyliniminodiacetic acid, MIDA, are 2.5 and 9.5) [30]. A detailed explanation of how the pH of the recording buffer affects the rates is given in the appendix, chapter 8.3.3. It was hoped that lowering the pH of the recording buffer would (i) slow down k_{on} sufficiently to permit the visualisation of partially chelated Zn^{2+} and/or (ii) limit the lifetime of the complex to permit the analysis of a larger number of events.

At pH 4.0, various Zn^{2+} binding events were observed with the P(PIDA)₂ pore (Figure 3.7B). Rare short binding events resulted in two distinct partially blocked current levels: level A ($\Delta I = 1.9 \pm 0.04$ pA) and level B ($\Delta I = 2.5 \pm 0.2$ pA). By comparison, Zn^{2+} binding events in the PPIDA pore, at pH 4.0, had a single ΔI value of 1.6 ± 0.03 pA (Figure 3.7C). The short binding events in the P(PIDA)₂ pore were interpreted to correspond to the two possible complexes between a Zn^{2+} ion and one or the other of the half-chelators at positions 117 and 143. The two different ΔI values for the block must result from the slightly different environments of the half-chelators inside the β barrel, a phenomenon that is also seen with cysteine-arsenic(III) adducts [31]. With the P(PIDA)₂ pore, at pH 4.0, long Zn^{2+} binding events were also seen, which comprised three different current levels. The long events always began as a step from the unoccupied pore level to either level A or level B (i.e. complex formation by one half-chelator), followed by a step to a new current level,

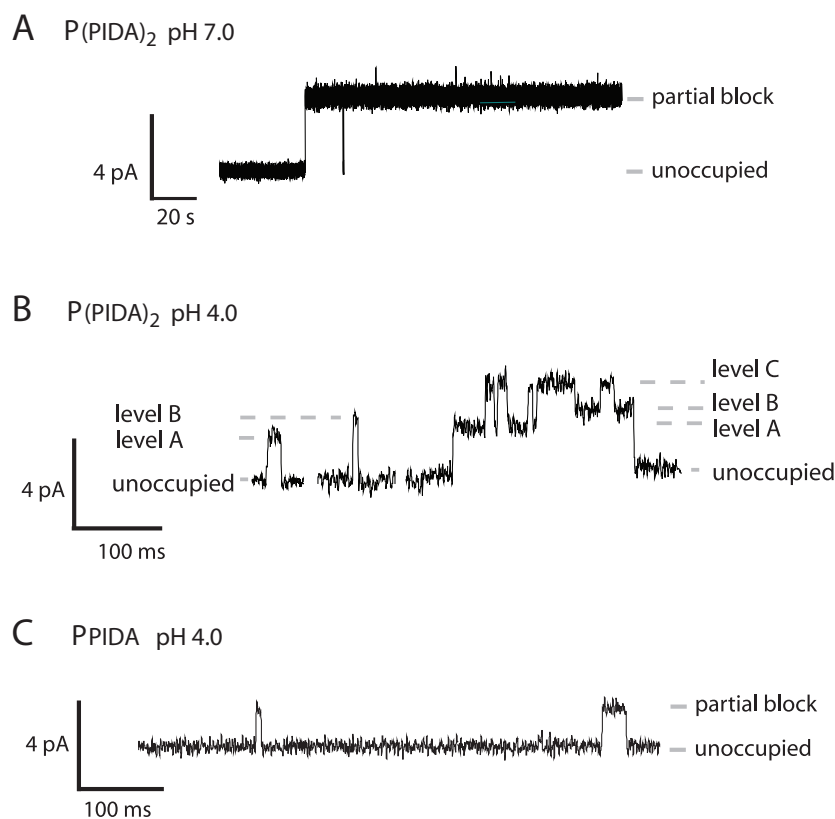


Figure 3.7: Binding of Zn^{2+} to the P(PIDA)_2 and PPIDApores. **A)** Single-channel recording of the P(PIDA)_2 pore at -50 mV with a buffer containing 2.0 M KCl and 10 mM MOPS (pH 7.0) in both chambers, and 10 μM Zn^{2+} in the trans chamber. The Zn^{2+} binding events were very long, lasting from tens of seconds to several minutes. **B)** Complex formation between the P(PIDA)_2 pore and Zn^{2+} at pH 4.0 proceeds through intermediate steps. Single-channel recording at -50 mV with a buffer containing 2.0 M KCl and 10 mM potassium acetate (pH 4.0) in both chambers, and 1 mM ZnCl_2 in the trans chamber. The four different current levels correspond to the unoccupied pore; a single Zn^{2+} ion bound to one or the other of the half-chelators (levels A and B); the Zn^{2+} ion bound to both chelators (level C). Sub-steps during the association and dissociation of each tridentate half-chelator were not observed. **C)** Single-channel recording of the PPIDApore at -50 mV with a buffer containing 2.0 M KCl and 10 mM potassium acetate (pH 4.0) in both chambers, and 400 μM Zn^{2+} in the trans chamber.

C. As supported by subsequent findings, level C represents the full chelator. Zn^{2+} complex formed by the participation of both half-chelators. From level C, current steps to level A or B were observed, but steps directly to the level of the unoccupied pore were never seen. Therefore, the association of Zn^{2+} to form the full chelator complex is a process with two major steps, featuring an intermediate state (A or B) in which the metal ion is complexed by only one half-chelator (Figure 3.8A).

As with the PPIDA pore, sub-steps involving the dissociation or association of one or two of the three coordinating arms of a half-chelator are too fast to detect. The reaction rates in the binding scheme for Zn^{2+} by the P(PIDA)_2 pore (Figure 3.8A) were determined by maximum likelihood fitting of dwell time sequences, using the same method that is described in detail in chapter 2. Briefly, a single channel search was conducted with the Clampfit software, which generated a table of dwells in each of the four states (unoccupied, A, B, C). The MIL algorithm of the QuB software was then used to derive the value of the rate constants (Table 3.2), by fitting the kinetic model directly to the dwell time records. The quality of the rate constant

	Level A	Level B	Level C (via A)	Level C (via B)
k_{on}	$k_{\text{on-A4}}$ $2490 \pm 370 \text{ M}^{-1}\text{s}^{-1}$	$k_{\text{on-B4}}$ $2340 \pm 60 \text{ M}^{-1}\text{s}^{-1}$	$k_{\text{on-AC4}}$ $51 \pm 5 \text{ s}^{-1}$	$k_{\text{on-BC4}}$ $56 \pm 7 \text{ s}^{-1}$
k_{off}	$k_{\text{off-A4}}$ $27 \pm 2 \text{ s}^{-1}$	$k_{\text{off-B4}}$ $23 \pm 3 \text{ s}^{-1}$	$k_{\text{off-AC4}}$ $32 \pm 4 \text{ s}^{-1}$	$k_{\text{off-BC4}}$ $35 \pm 5 \text{ s}^{-1}$
K_{f}	$K_{\text{f-A4}}$ $92 \pm 15 \text{ M}^{-1}$	$K_{\text{f-B4}}$ $102 \pm 13 \text{ M}^{-1}$	$K_{\text{f(C via A)4}}$ $147 \pm 26 \text{ M}^{-1}$	$K_{\text{f(C via B)4}}$ $163 \pm 37 \text{ M}^{-1}$

Table 3.2: Kinetic constants for the formation of complexes between the P(PIDA)_2 pore and Zn^{2+} at pH 4.0. The kinetic constants were obtained by using the QuB software package. Formation constants (K_{f}) were calculated by using $K_{\text{f}} = k_{\text{on}}/k_{\text{off}}$.

estimates was assessed by comparing the probability density functions generated by QuB to the dwell time histograms of the four levels (Figure 3.8B) and they were found to be in close agreement. Furthermore, plots of the reaction rates $v_{\text{A-on}}$ and $v_{\text{B-on}}$ at pH 4.0, (s^{-1} , from QuB), versus the Zn^{2+} concentration are of the form $v = k[\text{Zn}^{2+}]$, where k is $k_{\text{on-A4}}$ or $k_{\text{on-B4}}$ ($\text{M}^{-1}\text{s}^{-1}$), which confirms that the formation of both half-chelator. Zn^{2+} complexes involves a bimolecular reaction (Figure 3.8C). By contrast, the rates of formation of C from A, and B, are independent of the concentration of Zn^{2+} (Figure 3.8C), confirming that C contains a single Zn^{2+} ion, i.e. the two half-chelators are not simultaneously occupied at pH 4.0, each by one Zn^{2+} ion ("transparent" state, Figure 3.8A). If the kinetic scheme is correct, then at equilibrium the product of the rate constants for a clockwise movement around the diamond in Figure 3.8A must equal the product for the anticlockwise movement

[32]. It was found that $k_{\text{on-A4}}k_{\text{on-AC4}}k_{\text{off-BC4}}k_{\text{off-B4}} = 96 \pm 25 \times 10^6 \text{ M}^{-1}\text{s}^{-4}$ and $k_{\text{on-B4}}k_{\text{on-BC4}}k_{\text{off-AC4}}k_{\text{off-A4}} = 113 \pm 22 \times 10^6 \text{ M}^{-1}\text{s}^{-4}$, which are within experimental error the same. The rate constants in Table 3.1 yield the formation constants $K_{\text{f-A4}} = 92 \pm 15 \text{ M}^{-1}$ and $K_{\text{f-B4}} = 102 \pm 13 \text{ M}^{-1}$ for the half-chelator(A). Zn^{2+} and half-chelator(B). Zn^{2+} complexes respectively, and $K_{\text{f(C via A)4}} = 147 \pm 26 \text{ M}^{-1}$ and $K_{\text{f(C via B)4}} = 163 \pm 37 \text{ M}^{-1}$ for the formation of the full chelator. Zn^{2+} complex. The latter values should be and are the same ($K_{\text{f-C4}}$) within experimental error.

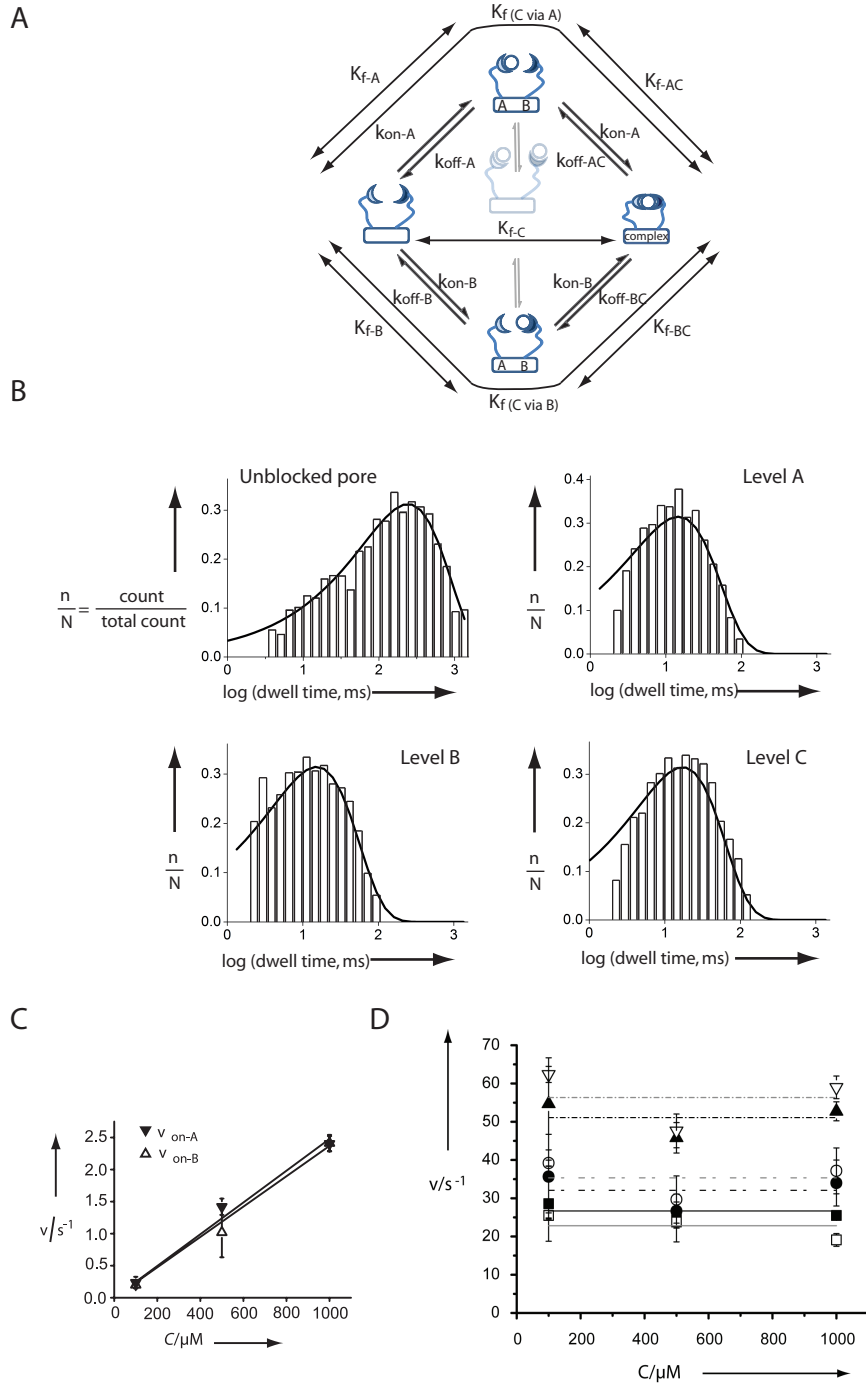


Figure 3.8: Binding kinetics of Zn²⁺ to the P(PIDA)₂ pore. **A)** Proposed kinetic scheme describing the formation of the complex at pH 4.0. **B)** Dwell time histograms and probability density functions describing the lifetimes of the four states of the P(PIDA)₂ pore at pH 4.0 with 1 mM Zn²⁺ present in the recording buffer. **C)** The Zn²⁺ concentration-dependence of the reaction rates for chelation sites A (v_{on-A}, ▼) and B (v_{on-B}, △). **D)** Unimolecular reaction rates for the P(PIDA)₂ pore complexed with Zn²⁺ at pH 4.0, plotted against the Zn²⁺ concentration in the recording buffer. Values are presented as the mean value ± standard deviation (n = 3).

3.3.4 Determination of the effective molarity of the second half-chelator and estimation of the overall affinity constant at pH 7.0

As explained in the introduction, the level of cooperativity between the two half-chelators is best measured in terms of the effective molarity (M_{eff}) of the second half-chelator. The present experimental system is particularly suited to this task, as the microscopic association constant for each step leading to fully chelated Zn^{2+} are known. M_{eff} is the concentration of PIDA that theoretically would be present in solution to obtain the same extent of full chelation of Zn^{2+} as observed in the intramolecular case. (Figure 3.9) M_{eff} also represents the amount of free PIDA

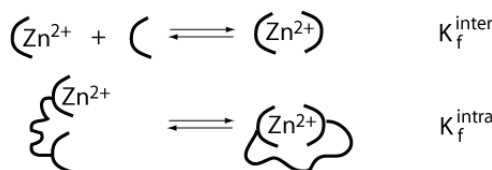


Figure 3.9: $M_{\text{eff}} = K_f^{\text{intra}} / K_f^{\text{inter}}$. K_f^{inter} has the units of M^{-1} , while K_f^{intra} is dimensionless

that would have to be added to the experiment to favour the formation of a new intermolecular complex between half-chelator A or B, Zn^{2+} and a free PIDA molecule (over the intramolecular complex formed by half-chelators A and B and Zn^{2+}), although this particular experiment was not carried out. In the calculations below (Table 3.3), it is assumed that $K_f^{\text{intra}} = K_{\text{f-AC4}}$ and that $K_f^{\text{inter}} = K_{\text{f-A4}}$, giving $M_{\text{eff}} = 17$ mM. The use of $K_{\text{f-B4}}$ and $K_{\text{f-BC4}}$ gives a similar result. These calculations are likely to overestimate K_f^{inter} , as successive formation constants for Zn^{2+} .amino acid complexes tend to decrease [18]. The value of 17 mM therefore represents a minimum estimate of M_{eff} . M_{eff} values of the same order of magnitude were obtained for the intramolecular binding of a ligand covalently attached to the surface of a protein [21] and for the bivalent binding of porphyrins to N-methylimidazole-functionalised

	Level A	Level C (via A)
k_{on}	$k_{\text{on-mono7}}$ $1.5 \pm 0.2 \times 10^5 \text{ M}^{-1}\text{s}^{-1}$	$k_{\text{on-AC7}}$ 2550 s^{-1}
k_{off}	$k_{\text{off-mono7}}$ $4.0 \pm 0.2 \text{ s}^{-1}$	$k_{\text{off-AC7}}$ 4.0 s^{-1}
K_f	$k_{f\text{-mono7}}$ $3.8 \pm 0.5 \times 10^4 \text{ M}^{-1}$	$K_{f\text{-C7}}$ $2.5 \times 10^7 \text{ M}^{-1}$

Table 3.3: Kinetic constants for the formation of complexes between the PPIDA pore and Zn^{2+} and estimated kinetic constants for the $P(\text{PIDA})_2$ pore at pH 7.0. The kinetic constants for PPIDA were obtained as described in the text. $k_{\text{on-AC7}}$ and $K_{f\text{-C7}}$ for $P(\text{PIDA})_2$ were estimated by using $M_{\text{eff}} = 17 \text{ mM}$ and the values for $k_{\text{on-mono7}}$ and $K_{f\text{-mono7}}$ for PPIDA. Because ΔI for Zn^{2+} block of the PPIDA pore is similar to ΔI for level A in the $P(\text{PIDA})_2$ pore, it is likely, but not certain, that level A represents Zn^{2+} binding to the half-chelator at position 117. The estimates for $P(\text{PIDA})_2$ tacitly assume that the k_{off} values for the two steps in chelation by $P(\text{PIDA})_2$ are the same at a given pH, which is not quite true (e.g. $k_{\text{off-A}}$ and $k_{\text{off-AC}}$, Table 3.2)

gold nanoparticles [33]. A single value of M_{eff} should be applicable at all pH values. Therefore, with M_{eff} in hand, Zn^{2+} binding at pH 7.0 can be reconsidered. In chapter 3 the experiments with PPIDA (the pore containing a single half-chelator) had given $k_{\text{on-mono7}} = 1.5 \times 10^5 \text{ M}^{-1}\text{s}^{-1}$, $k_{\text{off-mono7}} = 4.0 \text{ s}^{-1}$ and $K_{f\text{-mono7}} = 3.8 \times 10^4 \text{ M}^{-1}$. $k_{\text{on-mono7}}$ is 60 times higher than $k_{\text{on-A4}}$, which is not surprising as the deprotonated half-chelator ($\text{pK}_a \sim 9.5$) is expected to bind more rapidly than the protonated form. $k_{\text{off-mono7}}$ is 6.8 times lower than $k_{\text{off-A4}}$. The origin of this reduction in rate is less obvious, although the half-chelator must become reprotonated during one of the (invisible) sub-steps involved in dissociation. By using $M_{\text{eff}} = K_f^{\text{intra}} / K_f^{\text{inter}}$ and putting $K_f^{\text{inter}} = K_{f\text{-mono7}}$, it is found that $K_f^{\text{intra}} = 646$ ($= K_{f\text{-AC7}}$, or $K_{f\text{-BC7}}$), which is far greater than the value of 1.6 for $K_{f\text{-AC4}}$ (at pH 4.0). The overall affinity constant at pH 7.0 is then

$$K_{f\text{-C7}} = K_{f\text{-mono7}} \times K_f^{\text{intra}} = 2.5 \times 10^7 \text{ M}^{-1}$$

an increase of more than five log units compared to the value at pH 4.0. The partitioning of the intermediate A (or B) between the fully chelated form C and the form with double Zn^{2+} occupancy (Figure 3.8A) will be in the ratio $M_{\text{eff}}/[\text{Zn}^{2+}]$ to

1. Therefore double occupancy will only be observed at very high $[\text{Zn}^{2+}]$, and indeed no evidence for this species was found. The value of $K_{\text{f-mono7}}$ and $K_{\text{f-AC7}}$ are lower than the values for binding a first and a second MIDA molecule in solution [30], but the literature values are based on fully deprotonated MIDA ($\text{pK}_a = 9.5$) and the value for the second MIDA is in units of M^{-1} . These estimates of the rate constants make sense in the light of additional observations at pH 7.0. First, only a single step upon Zn^{2+} binding is seen (Figure 3.7A). Because $k_{\text{on-AC7}} \sim 2550 \text{ s}^{-1}$ ($M_{\text{eff}} \cdot k_{\text{on-mono7}}$), the mean life-time of the intermediate with one half-chelator occupied is $\tau_{\text{on-AC7}} = 1/k_{\text{on-AC7}} \sim 390 \text{ }\mu\text{s}$, which would be difficult to observe under the present recording conditions. Second, the overall dwell time of Zn^{2+} at the binding site in P(PIDA)_2 in all states (A, B and C) is given by $\tau_{\text{ABC}} \sim (1/k_{\text{off-A}})(1 + k_{\text{on-AC}}/2k_{\text{off-AC}})$ [34]. A statistical factor of 2 is applied because there are two pathways for dissociation of the fully chelated Zn^{2+} (complex C). At pH 7.0, this equation predicts $\tau_{\text{ABC}} = 80 \text{ s}$. Knowing this, additional measurements were performed at $\text{pH} = 7.0$ and it was found that $\tau_{\text{ABC}} = 193 \text{ s}$ (95% confidence interval 130, 316 s, $n = 20$ events), which is in reasonable agreement given the assumptions made in the calculation (Table 3.3).

As noted in the previous chapter, it is difficult to compare the rate constants determined in these experiments with work reported in the literature, because few rate constants have been measured directly in ensemble measurements. Recall that a value of 50 s^{-1} was determined by NMR line-shape analysis for $\text{Zn(MIDA)}_2^{2-} \rightarrow \text{Zn.MIDA} + \text{MIDA}^{2-}$ [30]. This exchange rate was independent of pH over the range 6.0 to 9.0, but no explanation was given by the authors for this observation. By comparison, the experiments described in this chapter at pH 4.0 revealed a rate constant of $\sim 35 \text{ s}^{-1}$ for the dissociation of the second PIDA ligand from the full complex (Table 3.2). It was also found that the dissociation rate for the first PIDA lacked a strong dependence on pH: pH = 4.0, $k = \sim 25 \text{ s}^{-1}$; pH 7.0, $k = 4 \text{ s}^{-1}$ (Table 3.2, Table 3.3). The affinities of P(PIDA)_2 for Zn^{2+} are lower than those in the literature for EDTA ($\log K_{\text{f}} = 16.5$) [18], although as Toone and colleagues have indicated [27]

it is difficult to find data that can be compared given the effects of buffer ions and the need for rigorous control over pH during titration experiments. As discussed in chapter 3, the pore structure and the applied potential could also affect k_{on} . The most significant factor contributing to the difference in affinity between EDTA and the P(PIDA)_2 pore for Zn^{2+} is likely to be structural. It has been shown that small structural changes can dramatically alter the affinity of chelators. For example, the affinity of PDTA, with one additional methylene between the two nitrogens, for Ca^{2+} is 10^4 times lower than that of EDTA [27]. Given the additional conformational freedom of the two PIDA groups in P(PIDA)_2 , compared with the two arms of EDTA, which must in binding Zn^{2+} produce unfavorable entropic contributions as well as decreased relief from unfavorable electrostatic interactions [27], it is hardly surprising that the affinity of P(PIDA)_2 is respectable, but not exceptionally strong.

3.4 Summary

This chapter described Zn^{2+} binding by the P(PIDA)_2 pore, the pore containing two half-chelators. At pH 7.0 very long lasting binding events were observed, which made it impossible to perform a detailed kinetic analysis or to determine the stability of the complex. By lowering the pH of the recording buffer to 4.0, a two-step process could be visualised, in which the Zn^{2+} ion must first bind to one of the two half-chelators before the second one completes the complex. All of the major rate and formation constants involved in this process were determined. As the formation of the full complex by participation of both half-chelators includes a second intramolecular step, the extent of cooperativity between the two half-chelators was determined by calculating the effective molarity of the second half-chelator. This was found to be 17 mM. With this value at hand, k_{on} for the second intramolecular step for the complex formation at pH 7.0 (which was too fast to detect by single channel recording) was estimated from the monovalent interaction. Similarly, the overall dwell time of the Zn^{2+} ion in the P(PIDA)_2 pore was estimated, as was the overall

formation constant for the complex at pH 7.0. The estimates are in agreement with the limited experimental data available at pH 7.0.

Overall, these data highlight the wealth of additional chemical information available from the single molecule approach compared to ensemble measurements. The utility of the effective molarity of the second half-chelator as a means to compare the inter- and intramolecular reactions involved in the complex formation was demonstrated.

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Chapter 4

Dimers of α -hemolysin expand the protein engineering toolbox for protein nanopores

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The work described in this chapter was developed from a Chemistry Part II project carried out by AFH at the University of Oxford. The initial results were published in the thesis "Engineering of a Transmembrane Pore: Changing the Stoichiometry and the Arrangement of an α -Hemolysin Pore by Concatenation of Subunits", which was submitted for the degree of Master of Chemistry. A detailed list of experiments that contributed to the Part II thesis is appended to this chapter.

4.1 Chapter Overview

This chapter explores the use of subunit dimers of α HL as a new means to engineer α -HL pores. The work was motivated by two specific goals: The first goal was to investigate whether subunit dimers of α HL would assemble to give oligomers with new stoichiometries. Since it is known that many β -barrel pore forming toxins can adopt variable pore stoichiometries, it was hoped that by oligomerising subunit dimers it would be possible to obtain α HL pores comprising six or eight subunits, however, this could not be achieved. Subunit dimers of α HL always oligomerised to give functional heptamers, implying that one or more subunits were excluded from the central pore. The results of this chapter show that the α HL pore resists all structures except those that possess seven subunits immediately surrounding the central axis.

The second goal of this work was to increase the scope for genetic and chemical modifications of the pore. By using existing techniques it is possible to prepare heteroheptameric pores. However, pores containing more than one type of modified subunit are not commonly used, because it is impossible to distinguish between the permutations of the pore. By using subunit dimers, heptamers in which two modified subunits are adjacent to each other can be formed, thereby increasing the range of structures that can be obtained from engineered protein nanopores.

4.2 Introduction

4.2.1 The stoichiometries of β -barrel pore forming toxins

α HL belongs to the large structural class of β -barrel pore forming toxins (β PFTs). The β PFTs can be further divided into the family of cholesterol-dependent cytolysins (CDCs), the family of small β PFTs (which includes α HL) and the family of AB toxins [1]. The β PFTs share some basic structural motifs. They are all multimeric proteins comprising several subunits and each subunit contributes either one or two β -hairpins to a cylindrical transmembrane β -barrel [2]. But although they are

structurally related, different members of the β PFTs class of proteins form oligomers of different stoichiometries.

The CDCs form large pores comprising 30 to 50 protein subunits arranged around a central axis [3], with each subunit contributing two β -hairpins to the transmembrane barrel. Even for a given protein, the number of subunits per oligomer is variable. For example the pneumolysin oligomer from *Streptococcus pneumoniae* comprises 38-44 subunits [4]; the perfringolysin O oligomer from *Clostridium perfringens* comprises 35-50 subunits [5].

Oligomers from the family of small β PFTs typically comprise seven or eight protein subunits, with each subunit contributing one β -hairpin to the transmembrane barrel of the oligomer [2]. A high resolution crystal structure shows that α HL is a heptamer [6], although hexamers may also exist under certain conditions [7]. By contrast, the structurally-related leukocidin pore is an octamer comprising each of two classes of subunits (LukF and LukS), both of which have a low extent of sequence identity with α HL [8, 9].

Interestingly, the protective antigen of anthrax toxin, an AB toxin which is structurally similar to the α HL pore but lacks sequence similarity, exists in both heptameric and octameric forms [10, 11]. These results suggest that it might be possible to manipulate the subunit stoichiometry of the α HL pore, and this was attempted in this study.

4.2.2 The permutation of subunits in multimeric pores and channels

Many ion channels and pores comprise different types of subunit. For such proteins the number of copies of each type of subunit and the relative orientation of the subunits in the oligomer is important and can have a profound effect on the function of the protein. For example, the LukF and LukS subunits of the leukocidin pore are not shuffled randomly, rather each oligomer contains four subunits of each type, which alternate around a central axis [8]. Many ligand-gated ion channels

(e.g. nicotinic acetylcholine receptors, GABA_A receptors) contain different types of subunit. The precise architecture of the channel determines the affinity for agonists, antagonists and modulators and consequently the function of the channel [12].

Although wt α HL is a homoheptamer, engineered pores have been prepared that contain mixtures of wt and modified subunits [8]. Heteroheptameric pores are prepared by introducing a modification (e.g. a mutation) to a subunit containing a C-terminal oligoaspartate (D₈) extension, and co-oligomerising the modified subunit with wt subunits on rabbit red blood cell membranes (rRBCMs). The D₈ extension specifies the electrophoretic mobility of the oligomer in SDS-PAGE gels, allowing the separation of pores that contain different numbers of each type of subunit [13] (Figure 4.1).

However, in the case of heteromers, the permutation of the subunits around the central axis of the pore has not been controlled. For example, where two of the seven subunits have been altered, there are three possible permutations (Figure 4.1). A second goal of the present work was to demonstrate such control.

4.2.3 The use of concatenated subunits to study heteromeric pores and ion channels

One means to control subunit stoichiometry and permutation is to use concatenated subunits produced by genetic engineering, which has most often been used with ion channels [14]. The concatenated subunits are encoded in a tandem gene, which translates into fused subunits that are covalently linked between the N- and the C-terminus. The position of each subunit in the concatemer is pre-determined, allowing the effect of the number and type of each subunit on the function of the oligomer to be studied. This approach has been widely utilised, for example the structure and function of K⁺ channels [15], GABA_A receptors [16], nicotinic acetylcholine receptors [17] and large conductance mechanosensitive channels [17] have been investigated in this way. This method was also used to show the

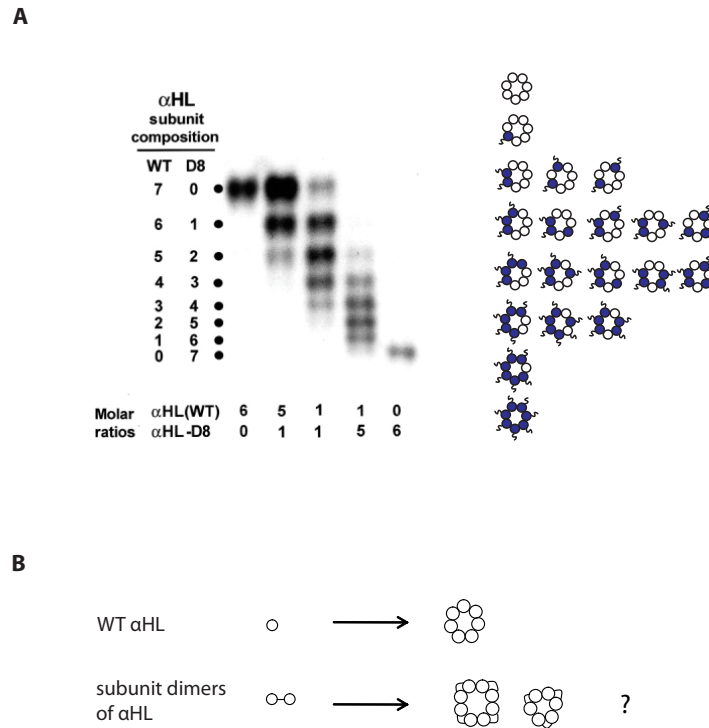


Figure 4.1: Separation of heteroheptamers generated from α HL and α HL-D8 and possible stoichiometries of pores containing subunit α HL. **A**, heteroheptamers generated from the monomeric subunits α HL and/or α HL-D8 (figure adapted from [13]). **B**, wt α HL forms heptameric pores with seven subunits arranged around a central axis (2_01_77)¹. Subunit dimers of α HL could adopt novel stoichiometries, such as an octamer comprising four subunit dimers (2_41_08) or a hexamer comprising three subunit dimers (2_31_06).

¹ The following nomenclature for α HL is used: 2_m1_nX , where m indicates the number of subunit dimers in the pore; n indicates the number of subunit monomers in the pore; X , when known, indicates the number of subunits immediately surrounding the central axis, and hence lining the lumen.

alternating LukS/LukF subunit arrangement of the leukocidin pore [8]. Because the N- and C- termini of the subunits in the α HL pore lie on the same side of the lipid bilayer, the creation of concatemers of α HL seemed feasible. Subunit dimers of α HL were prepared with two goals in mind. The first goal was to investigate whether subunit dimers of α HL would assemble to give oligomers with new stoichiometries (Figure 4.1). The second goal was to increase the scope for genetic and chemical modifications of the pore. By using subunit dimers, it was aimed to form heptamers in which two modified subunits are adjacent to each other.

4.3 Results and discussion

4.3.1 Design and expression of subunit dimers of α HL

The termini of the individual subunits in a concatemer are usually linked by short peptide sequences. Such linkers are kept as short as possible to prevent the formation of secondary structure in the linker and to prevent the formation of undesired oligomeric structures. Undesired oligomers include those with interspersed subunits (Figure 4.2 A, structure 2) and those with concatenated subunits that are incorporated into separate oligomers (structure 3). As yet, no interspersed subunits have been recognised in channels or pores formed from concatenated subunits, but a subunit dimer can offer one subunit to each of two acetylcholine receptors if the linker between the subunits is sufficiently long [17]. However, if the linker between the two subunits is short, then one subunit is excluded from the central pentameric pore of a single receptor. The same principle could apply to concatemers of α HL; for example, a very short linker might force the exclusion of subunits from the central pore (Figure 4.2 A, structure 4). In a PyMOL model, the crystal structure of the α HL heptamer (Figure 4.2 B) was used to estimate the length of a desirable linker between two adjacent subunits. The distance from the C terminus (Figure 4.2 B, blue) of a specified subunit (red) to the N terminus (green) of the next anticlockwise subunit (yellow), via Phe-42 (cyan) and Thr-12 (purple) on the protein surface, is 37 Å. A suitable linker should be longer than this to cover the uneven protein surface and to prevent strain of the linker. This model for the linker assumes that the N terminus of one subunit latches tightly onto a second, as seen in the crystal structure of the wt heptamer. However, work in the Bayley laboratory has revealed that the N terminus of α HL is not critical for the formation of oligomers, and up to 17 residues can be deleted while retaining the ability to form pores [18]. Furthermore, the related *Vibrio cholerae* cytotoxin has no such N-terminal domain [19]. Therefore the possibility that the N terminus of the second subunit (yellow, Figure 4.2 A) of an α HL subunit dimer is flexible had to be considered, which would shorten the required

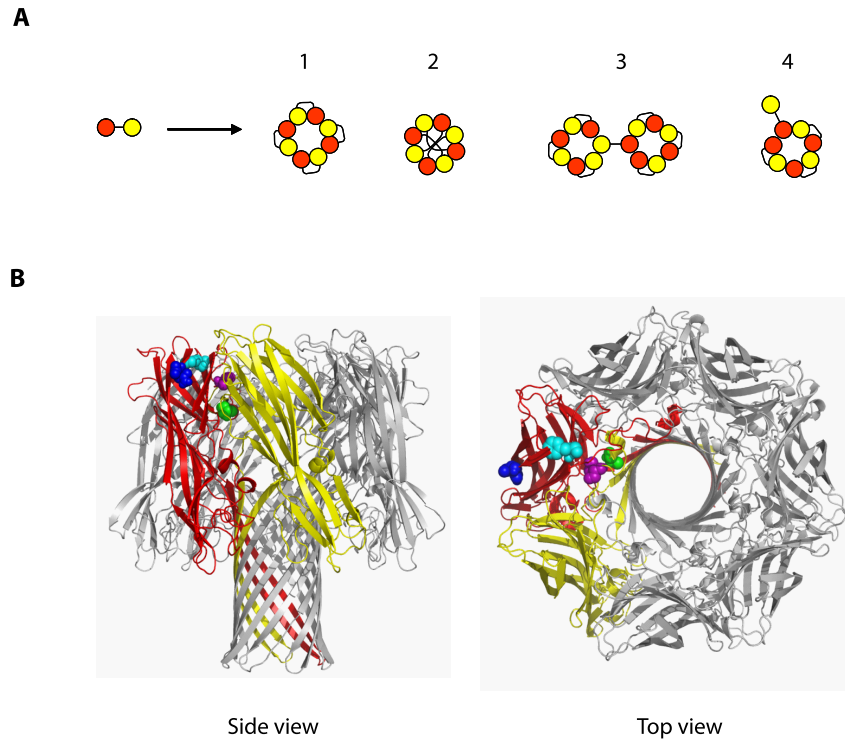


Figure 4.2: Structural considerations for the design of subunit dimers of α HL. **A**, possible arrangements of subunit dimers of α HL within oligomers. (1) An octamer comprising four subunit dimers arranged to form the central pore (2_41_08). (2) The subunits from the subunit dimers are interspersed, in nonadjacent positions in the oligomer. (3) A subunit dimer spans two oligomers, both of which are 2_41_07 oligomers. (4) A subunit is excluded from the central pore of the oligomer (2_41_07). **B**, the structure of the α HL heptamer (PDB code 7AHL) was used to determine the length of the linker required to connect the C terminus of the first subunit (red) in a subunit dimer to the N terminus of the second subunit (yellow). In a PyMOL model, the distance across the protein surface from the C terminus (blue) via Phe-42 (cyan) and Thr-12 (purple) (all on the red first subunit) to the N terminus (green) on the yellow second subunit was estimated to be 37 Å. This represents the minimum length of the linker if the N terminus of the second (yellow) subunit is not displaced.

length of the linker. For this reason, the optimal length of the linker was explored experimentally. A tandem gene encoding a subunit dimer in a pT7 expression vector (Figure 4.3 A) was prepared by PCR followed by homologous recombination in *E. coli*. The two subunit genes were separated by a sequence encoding a serine/glycine linker. Such linkers have been employed to link subunits of the leukocidin pore [8] and are thought to be conformationally flexible [20]. Four different linkers from 5 to 15 amino acids in length (Figure 4.3 B) were designed. In PyMOL models

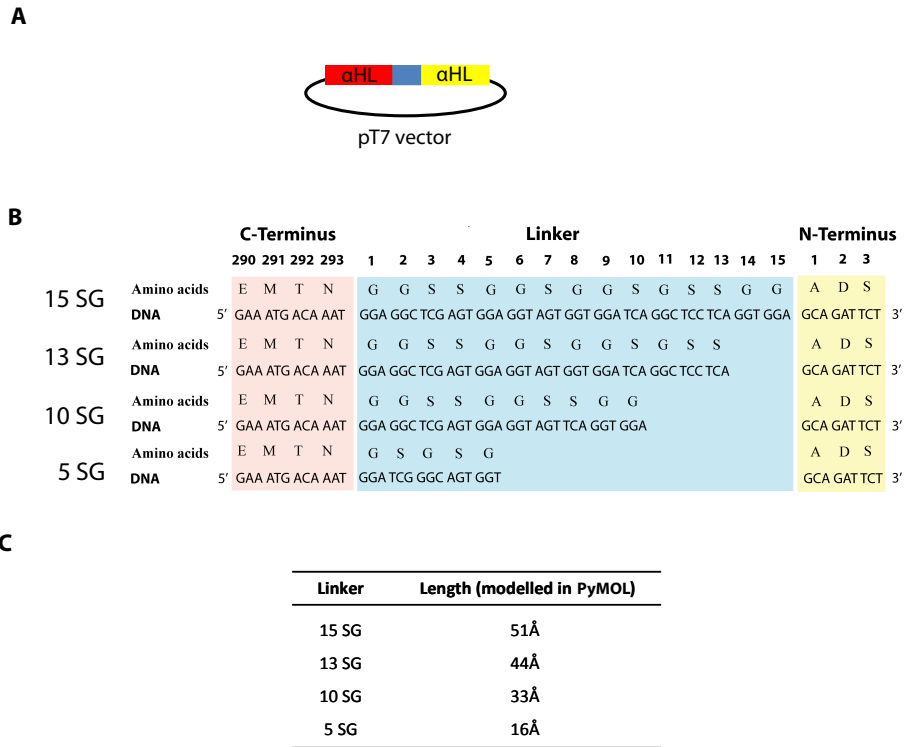


Figure 4.3: Design of the serine-glycine peptide linkers. **A**, schematic of the subunit dimer gene in a pT7 vector. The 3' end of one full-length gene of α HL (red) was connected to the 5' end of a second full-length gene (yellow) through a peptide linker (blue). **B**, DNA and amino acid sequences of linkers containing from 5 to 15 serines and glycines. **C**, the lengths of the linkers in B were estimated from PyMOL models of the corresponding peptide sequences by measuring the through-space distance between the C α atoms at the termini of the elongated peptides.

of the elongated sequences, the lengths between the terminal C α atoms are 16 to 51 Å (Figure 4.3 C). Subunit dimers of α HL were prepared by coupled in vitro transcription and translation. SDS-PAGE analysis of the radiolabelled proteins (Figure 4.4 A) revealed a polypeptide that migrated slightly faster than the 69-kDa marker, consistent with a dimer of 66 kDa. The expression levels of all the dimer constructs were two to five times lower than the wt monomer, as seen in the diminished intensities of the bands (Figure 4.4 A). Upon incubation with rRBCMs, all of the dimers formed oligomers, which appeared as two closely spaced diffuse bands when nondenatured samples were run in SDS-polyacrylamide gels (Figure

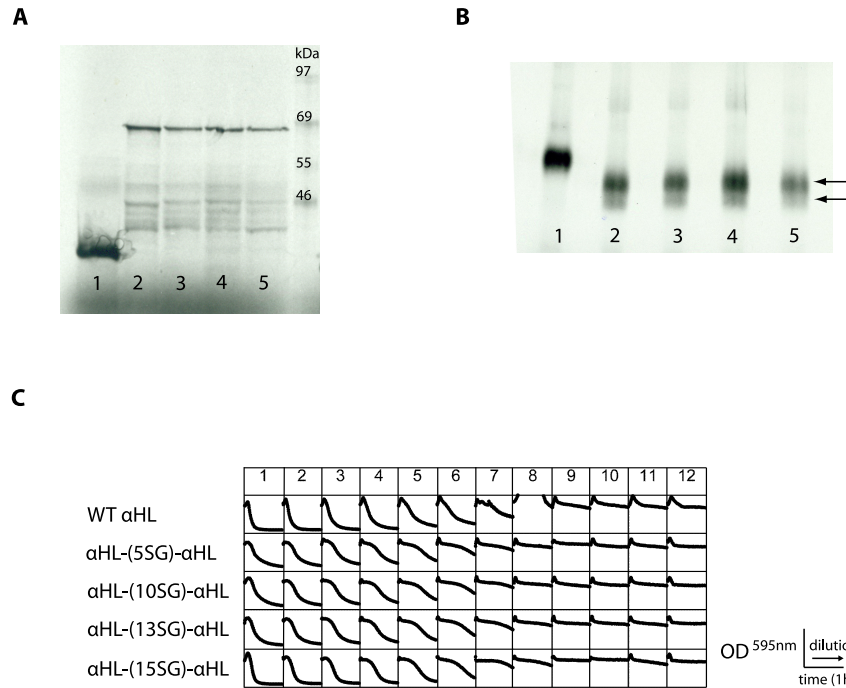


Figure 4.4: Expression and haemolytic activity of subunit dimers of α HL. **A**, 10% Bis-Tris polyacrylamide gel showing monomers and dimers of α HL synthesized by coupled IVTT. Lane 1, wt α HL monomer; lane 2, α HL-(5SG)- α HL subunit dimer; lane 3, α HL-(10SG)- α HL dimer; lane 4, α HL-(13SG)- α HL dimer; lane 5, α HL-(15SG)- α HL dimer. **B**, 5% Tris.HCl SDS-polyacrylamide gel showing oligomers formed by monomers and dimers of α HL on rRBCMs. The proteins in lanes 15 are the same as those shown in A. The two closely spaced diffuse bands of oligomers formed by the subunit dimer preparations are indicated by arrows. **C**, haemolytic activity assay with rabbit erythrocytes. Monomers and dimers of α HL were synthesized by IVTT and serially 2-fold diluted across the row of a microtiter plate. Pore formation was initiated by adding washed rabbit erythrocytes, and the rate of haemolysis was monitored by observing the decrease in light scattering at 595 nm over 1 h.

4.4 B). The oligomers have faster electrophoretic mobilities than the wt heptamer, suggesting that they may have a different subunit stoichiometry or arrangement. A haemolytic activity assay (Figure 4.4 C) was performed to measure the ability of subunit dimers to form lytic pores. For this assay, an equal amount of rabbit red blood cell membranes is added to the wells of a 96 well plate. To each row, a serial dilution of α HL subunit monomers or dimers is added. As the subunits assemble into transmembrane pores, the red blood cell membranes undergo lysis. This is monitored as a decrease in light scattering at 595 nm. The haemolytic activity

assay showed that all subunit dimers assembled to form lytic pores. Monomeric α HL has an HC_{50} value (the concentration required for 50% haemolysis) of ~ 25 ng/ml at 20 °C [9]. By comparison, the subunit dimers of α HL have HC_{50} values of ~ 50 -100 ng/ml. This activity may in part arise from truncated species that act as monomers, as discussed below.

4.3.2 Does the linker-internal N terminus sequence of the subunit dimer bind tightly within the cap domain?

Because all the dimers formed oligomers with equal efficiency, the linker-internal N terminus sequence between the two subunits presumably does not latch tightly onto the first of the two subunits. To further probe the conformation of the linker-internal N terminus sequence, limited proteolysis experiments were carried out. Proteinase K is a serine protease with broad specificity, which cleaves the N terminus of α HL after Ile-7 and Ile-14 when these sites are accessible [21]. In the wt heptamer, the N termini of the seven subunits are buried deeply in the cap domain of the protein and are therefore resistant to proteolytic cleavage [18, 21]. However, truncation mutants of α HL lacking part of the N terminus (but not the cleavage sites) are sensitive to protease cleavage, presumably because the shortened N termini are no longer occluded within the cap domain [18]. Limited proteolysis of oligomers made from subunit dimers should therefore provide insight into the conformation of the linker-internal N terminus sequence. Proteinase K treatment rendered gel-purified α HL oligomers made from subunit dimers (Figure 4.4 B) more electrophoretically mobile in SDS gels (Figure 4.5), but this was not the case for the wt heptamer (Figure 4.5). The proteolysed oligomers made from subunit dimers were also heated in SDS sample buffer at 90 °C to disrupt the intersubunit interactions. Electrophoresis revealed the formation of a protein band with a slightly faster mobility than the wt monomer, implying that cleavage had taken place in the linker-internal N terminus sequence to yield truncated monomers. At lower concentrations of proteinase K, the same experiment yielded several protein bands that correspond to incomplete

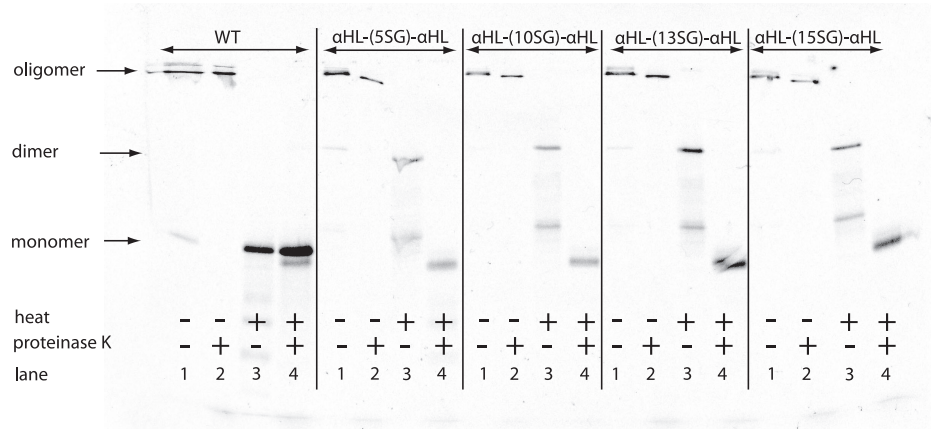


Figure 4.5: Limited proteolysis assay to probe the conformation of the N termini and linkers in oligomers formed from subunit dimers of α HL. SDS-PAGE-purified oligomers of α HL in 20 mM Tris.HCl buffer, pH 8.0, were split into four equal samples and subjected to different treatments. Lane 1, no treatment; lane 2, proteinase K (0.5 mg/ml final) for 5 min, sample not heated; lane 3, no proteinase K, sample heated to 95 °C for 10 min in SDS-containing sample buffer; lane 4, proteinase K (0.5 mg/ml final) for 5 min and heated to 95 °C for 10 min in SDS-containing sample buffer.

digestion (Figure 4.6). Because even the shortest linker between the subunit dimers in oligomers is accessible to proteinase K, it was concluded that the linker-internal N terminus sequences of all four types of subunit dimer do not bind tightly within the cap domain. Therefore, a shorter linker would be preferable to a longer linker, which could lead to the undesired oligomeric structures mentioned earlier (Figure 4.2). However, it was decided to proceed with a 10SG linker rather than a 5SG linker, as it offers more possibilities for the introduction of unique restriction sites on the DNA that encodes it. This will ultimately be important for preparing extended concatemers of α HL (subunit trimers, tetramers, etc.), as it will allow mutant subunit genes to be cut and pasted into pre-made concatemer genes.

4.3.3 Stoichiometries of oligomers made from α HL subunit dimers

The limited proteolysis experiments described above included a control condition, in which the oligomers were heated but not treated with proteinase K. Only dimers of α HL were expected to be recovered; however, a mixture of dimers (major component) and monomers (minor component) was found (Figure 4.5). Closer

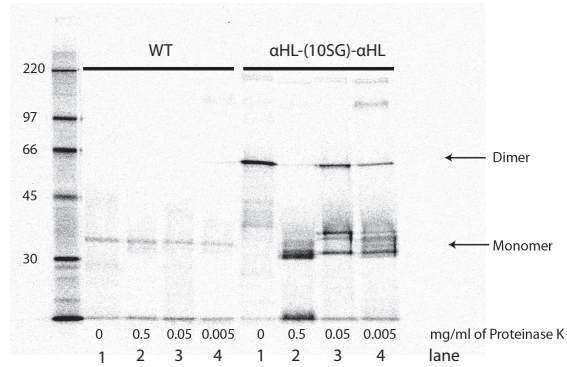
A**B**

Figure 4.6: *Limited proteolysis assay to probe the conformation of the N-terminus and linker of oligomers comprising dimers of α HL.* **A**, SDS-PAGE purified oligomers of α HL (wt₇ and oligomers made from α HL-(10SG)- α HL subunit dimers) in 20 mM Tris.HCl buffer (pH 8.0) were split into 4 equal samples and subjected to different treatments. (Lane 1) water control; (Lane 2) Proteinase K (0.5 mg/ml final); (Lane 3) Proteinase K (0.05 mg/ml final); (Lane 4) Proteinase K (0.005 mg/ml final). After incubation at RT for 10 min, 10 mM PMSF was added, which inhibits proteinase K. All samples were heated in SDS sample buffer at 90 °C for 10 min prior to electrophoresis. **B**, Putative proteinase K cleavage sites.

inspection of the IVTT products (Figure 4.4 A) revealed weak bands that migrate more slowly than the monomer but faster than the dimer. It was suspected that these protein bands represent truncated dimers, generated by either incomplete transcription of the subunit dimer gene or incomplete translation of the mRNA. Mixtures of full-length concatenated protein subunits and truncated protein subunits have been found in oligomers built from concatemers of the P2X receptor and the K_v-1.2/1.1 potassium channel, but their precise origin remains obscure [22, 23]. In the present experiments, the ratio of truncated dimer to full-length dimer is greater in the heated oligomers than in the original IVTT reaction, suggesting that truncated dimers (which would be structurally similar to monomers) are

preferentially incorporated into oligomers. Because subunit dimers cannot form a heptameric central pore, without the exclusion of one or more subunits from the ring, this result provided the first hint that α HL resists all but a heptameric stoichiometry. For example, such a stoichiometry could be achieved by combining three subunit dimers with one truncated dimer (effectively a monomer). At this point, however, it was not clear why the oligomers made from the subunit dimers showed a different electrophoretic mobility compared with the wt heptamer, especially if they actually share the same stoichiometry with respect to the number of subunits surrounding the central pore. However, it is known that oligomers made from certain mutants of α HL can run anomalously fast. For example, the heptamer made from α HL-D₈, a mutant that carries an oligoaspartate extension at the C terminus [9], has similar mobility to denatured phosphorylase B (97 kDa) in a 5% Laemmli gel, although its actual molecular mass is 240 kDa. To avoid producing a mixture of truncated species and dimers by IVTT of the subunit dimer gene, a gene with a C-terminal His₆ affinity tag was used. Because only full-length protein carries the tag, Ni-NTA purification should give only subunit dimers of α HL. This was confirmed by SDS-PAGE (Figure 4.7 A). By comparison, the nonpurified dimer contained a truncated protein, in amounts that varied from batch to batch (compare Figure 4.4 A with 4.7 A). The purified α HL subunit dimer was allowed to oligomerise in the presence of rRBCMs. The band corresponding to the oligomer in a 5% polyacrylamide gel (Figure 4.7 B) was sharp compared with the band derived from the oligomer made from nonpurified dimers. Faint bands could be detected above and below the main band generated from purified subunit dimers, indicating the presence of other types of oligomer. The major band again had a faster mobility than the wt heptamer. When the major oligomer made from purified subunit dimers was gel-purified and heated, only dimers were released (Figure 4.7 C). Therefore, the truncated proteins seen in Figure 4.5 are not the result of proteolytic cleavage during or after oligomer assembly, rather they are IVTT products. It was tried to elucidate the stoichiometry of the oligomers made from purified His-tagged subunit dimers by gel-shift experiments, which had

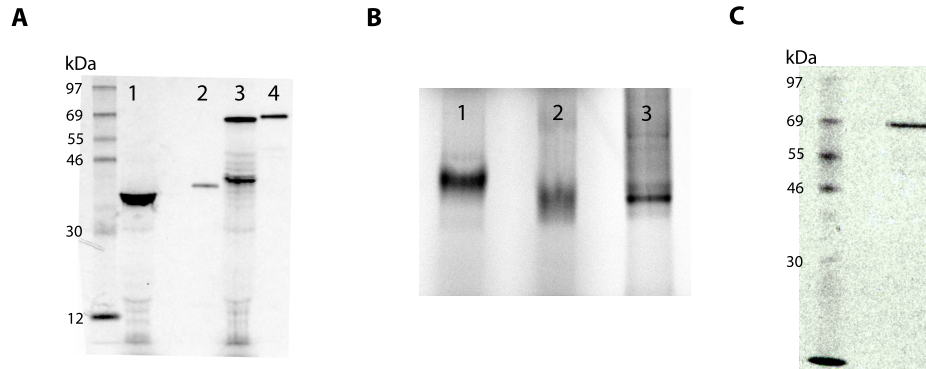


Figure 4.7: Oligomers made from His-tagged subunit dimers of α HL that had been purified by Ni-NTA affinity chromatography. **A**, monomers and dimers of α HL in a 12% Bis-Tris polyacrylamide gel. Lane 1, wt α HL monomer, no purification; lane 2, α HL-H6 monomer, purified with Ni-NTA magnetic beads; lane 3, α HL-(10SG)- α HL subunit dimer, no purification; lane 4, α HL-(10SG)- α HL-H6 subunit dimer, purified with Ni-NTA magnetic beads. **B**, oligomers of α HL in a 5% Tris.HCl SDS-polyacrylamide gel. Lane 1, oligomers from wt α HL monomer; lane 2, oligomers from α HL-(10SG)- α HL dimer, no purification; lane 3, oligomers from α HL-(10SG)- α HL-H6 subunit dimers, purified with Ni-NTA magnetic beads. **C**, oligomer of purified α HL-(10SG)- α HL-H6, cut from a 5% Tris.HCl SDS-polyacrylamide gel (B), heated to 95 °C for 10 min in SDS-containing sample buffer and re-run in a 12 % Bis-Tris polyacrylamide gel.

been used successfully to determine the heptameric stoichiometry of the α HL pore [24] and the octameric stoichiometry of the leukocidin pore [8, 9]. The most likely oligomers to be formed by subunit dimers of α HL are a hexamer or an octamer (Figure 4.8 A). α HL-(10SG)- α HL-H6 was co-oligomerised with α HL-(10SG)- α HL-D8H6 and a stepladder of four bands was expected to be seen for a hexamer, or five bands for an octamer, as the number of subunits bearing the D8H6 extension specifies the electrophoretic mobility of the oligomer. This method cannot resolve the different permutations for a given stoichiometry, so the protein band corresponding to an octamer that comprises two subunit dimers with an H6 tag and two subunit dimers with a D8H6 tag would contain two distinct proteins. In one permutation, the H6-tagged and D8H6-tagged subunit dimers would alternate around the central axis, in the other permutation the H6-tagged and D8H6-tagged subunit dimers would be adjacent to each other (Figure 4.8 A). However, the experiment did not result in a clear pattern (Figure 4.8 B). The lack of a clear band pattern for oligomers

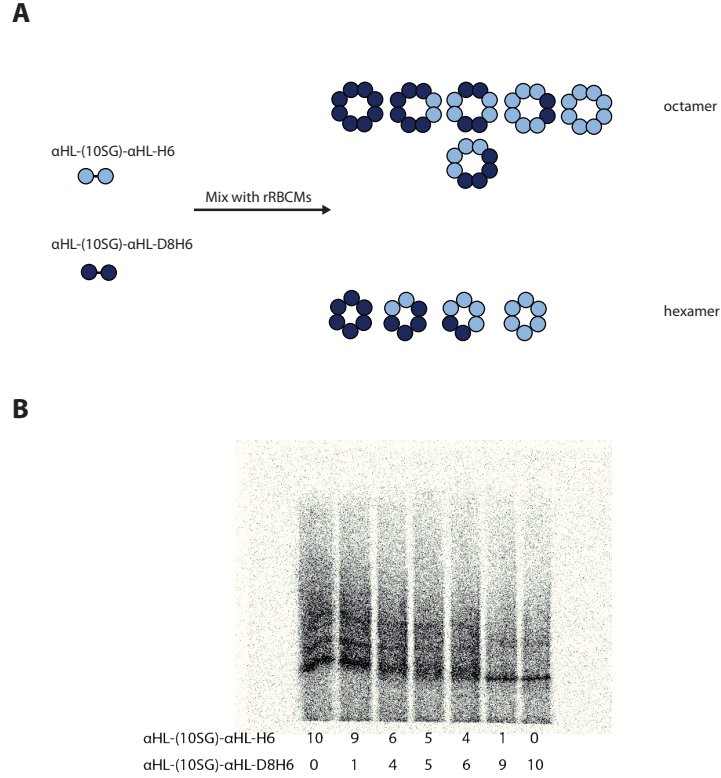


Figure 4.8: Analysis of oligomers containing subunit dimers of α HL. **A**, Possible combinations of subunit dimers in octameric and hexameric pores formed from α HL-(10SG)- α HL-H6 and α HL-(10SG)- α HL-D8H6. **B**, 5% polyacrylamide gel of oligomers formed on rRCMs from mixtures of purified α HL-(10SG)- α HL-H6 and α HL-(10SG)- α HL-D8H6. The ratios of the constituent proteins are given below the lanes.

made from α HL-(10SG)- α HL-H6 and α HL-(10SG)- α HL-D8H6 excludes a hexameric (2_31_6) or octameric (2_41_8) oligomer structure, leaving a heptamer with excluded subunits as a possible structure. It is hard to predict how many subunits would be excluded from the central pore, but because both α HL-(10SG)- α HL-H6 and α HL-(10SG)- α HL-D8H6 alone give rise to minor oligomer bands as well as one major band (Figure 4.4 B and Figure 4.8), it was suspected that several different arrangements can exist (e.g. 2_41_7 and 2_51_7).

4.3.4 Oligomers made from subunit dimers of α HL form functional heptamers with excluded subunits

To settle the question of pore stoichiometry conclusively, single channel recordings of oligomers made from subunit dimers were carried out. The unitary conductance of β -barrel pores is extremely sensitive to the pore stoichiometry and mutation. For example, the conductance of the octameric leukocidin pore is more than three times that of the heptameric α HL pore under the same recording conditions [25]. Furthermore, systematic mutation of only one residue (Met-113) in homoheptameric α HL pores produces pores with conductance values that differ by as much as 24% [26]. Protein from the major bands of oligomers was extracted from an SDS-polyacrylamide gel (Figure 4.4 B) and multiple pores were allowed to insert into planar lipid bilayers in 2.0 M KCl, 10 mM MOPS, pH 7.0, under an applied potential of -50 mV (Figure 4.9). By assuming that each current step corresponds to the insertion of a single pore, current amplitude histograms were constructed. wt α HL gave uniform quiet channels with a unitary conductance of 1.71 ± 0.26 nS (n=50, Figure 4.9 A). By comparison, the major oligomers made from either nonpurified subunit dimers or purified subunit dimers gave noisier channels with wide distributions of conductance values (Figure 4.9, B and C). The histograms indicate that a fraction of the pores made from subunit dimers share the same single channel conductance values as the wt heptamer, suggesting that they might also possess heptameric central pores. It was suspected that either the linkers between the subunit dimers or subunits excluded from the central pore might be responsible for the noise observed in the recordings and that they might also partially block the channel, resulting in lower conductance values. Therefore the oligomers made from purified subunit dimers were subjected to limited proteolysis with proteinase K, which had previously been shown to cleave the linkers and release any excluded subunits that might be attached to them. Following proteolysis, the oligomers made from purified subunit dimers gave quiet uniform channels with a mean unitary conductance similar to the wt heptamer, 1.59 ± 0.22 nS (n=161, Figure 4.9 D).

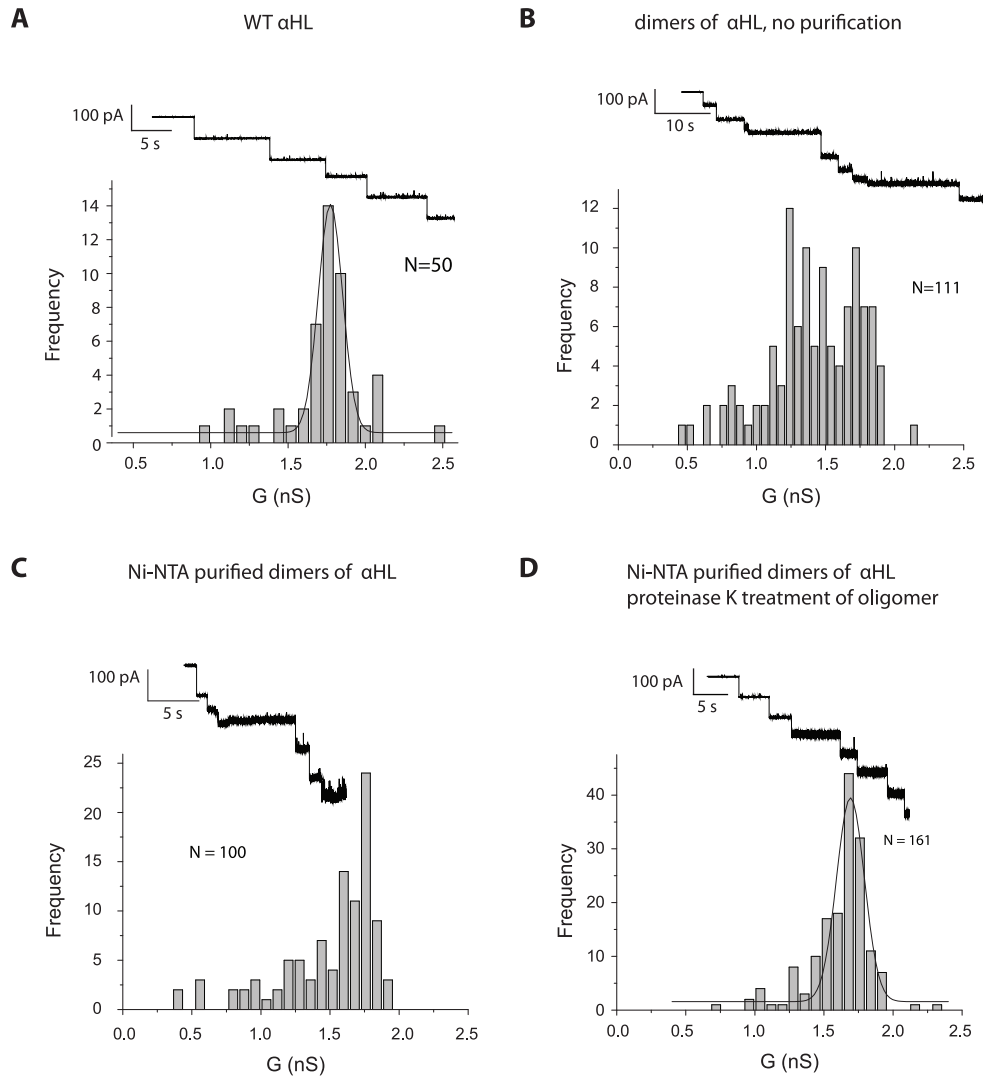


Figure 4.9: Representative pore insertion events and unitary conductance histograms. α HL oligomers were extracted from SDS-polyacrylamide gels, before insertion into planar lipid bilayers. The oligomers were made from **A**, wt α HL. **B**, subunit dimers of α HL. **C**, Ni-NTA-purified subunit dimers of α HL. **D**, Ni-NTA-purified subunit dimers of α HL, and the oligomers were treated with proteinase K before the recording was made. The current traces were recorded in 2.0 M KCl, 10 mM MOPS, pH 7.0, at an applied potential of -50 mV.

The unitary conductance values of these pores mirrored those of wt heptamers over potentials ranging from -100 to +100 mV (Figure 4.10 A). The binding of the molecular adapter β -cyclodextrin (β CD) to these pores was also investigated (Figure 4.10 B). The mean dwell time (τ_{off}) of β CD in the α HL pore can vary by up to 5 orders of magnitude between various mutants [26], so it is another sensitive indicator

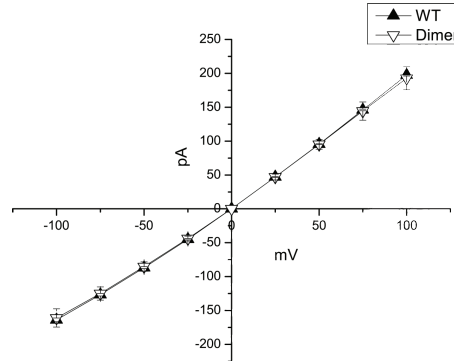
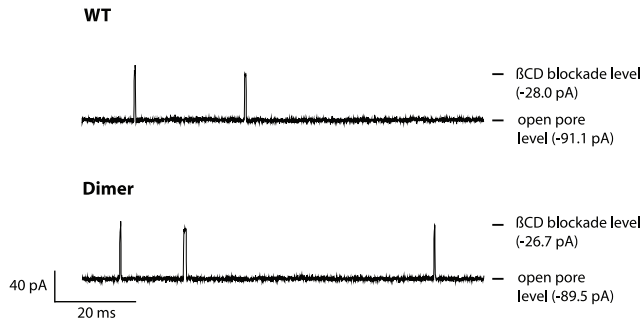
A**B**

Figure 4.10: Comparison of oligomers made from monomers of α HL and Ni-NTA-purified subunit dimers of α HL. Oligomers made from subunit dimers were treated with proteinase K. **A**, current-voltage (I - V) relationships obtained in 2.0 M KCl, 10 mM MOPS, pH 7.0. Mean values $\pm$ S.D. for each point were calculated from at least five different pores. **B**, single channel recording traces showing β -cyclodextrin binding to oligomers of α HL. The recordings were obtained in 2.0 M KCl, 10mM MOPS, pH 7.0, at -50 mV. β -Cyclodextrin (40 μ M) was added to the trans recording chamber. The mean residence time (τ_{off}) of β CD in the pore was 0.39 ± 0.03 ms ($n = 5$) for wt pores and 0.33 ± 0.03 ms ($n = 5$) for oligomers made from subunit dimers. The inter-event intervals (τ_{on}) were 84 ± 14 and 115 ± 14 ms, respectively.

of the pore architecture. The dwell times of β CD in wt pores and pores made from purified subunit dimers and then subjected to limited proteolysis were the same within experimental error (0.33 ± 0.03 and 0.39 ± 0.03 ms, respectively). It was therefore concluded that oligomers made from subunit dimers adopt a heptameric central pore architecture, with one or more excluded subunits.

4.3.5 A single subunit dimer can be incorporated into a heptameric α HL pore

Although it was not possible to change the stoichiometry of the α HL pore by the concatenation of subunits, a second aim was to incorporate a single modified dimer into a heptameric pore. Because the experiments of this chapter had established that oligomers made from subunit dimers readily incorporate monomeric subunits, the next step was to make mixed oligomers. The assembly on rRBCMs of different ratios of subunit dimers and monomers that carried a C-terminal D8H6 extension resulted in a stepladder of oligomeric bands upon SDS-PAGE analysis (Figure 4.11A, bands B – F). As expected from previous experiments with $(\alpha$ HL-D8)₇ [9], the $(\alpha$ HL-D8H6)₇ oligomer showed the fastest electrophoretic mobility. With increasing amounts of subunit dimer, the oligomer bands shifted upwards, suggesting that untagged subunit dimers are incorporated into the oligomers in various ratios. To analyse the composition of the mixed oligomers, they were separated by electrophoresis in a preparative 5% gel (Figure 4.11B) and the bands were extracted for further analysis. After heating the oligomers from bands 1 to 4 (Figure 4.11B) in SDS to disrupt the intersubunit interactions, the constituent polypeptides were separated in a second gel and the ratio of monomer to dimer was determined (Figure 4.11C). Band 2 (Figure 4.11, B and C) was expected to contain an oligomer that includes one subunit dimer and five monomers ($2_1 1_5 7$, Figure 4.11D), and the intensities of the bands after heating support this view (expected 29% dimer, 71% monomer; observed 31, 69%). Similarly, band 3 (Figure 4.11, B and C) includes two dimers and three monomers ($2_2 1_3 7$, Figure 4.11D; expected 57, 43%; observed 55, 45%). Band 4 (Figure 4.11, B and C) was initially thought to include three dimers and one monomer ($2_3 1_1 7$). However, after heating, it was found that this oligomer contains a higher percentage of monomers than expected (expected 86, 14%; observed 74, 26%). A second possibility is an oligomer with two monomers and three dimers, with one subunit excluded from the central pore ($2_3 1_2 7$, Figure 4.11D; expected 75, 25%).

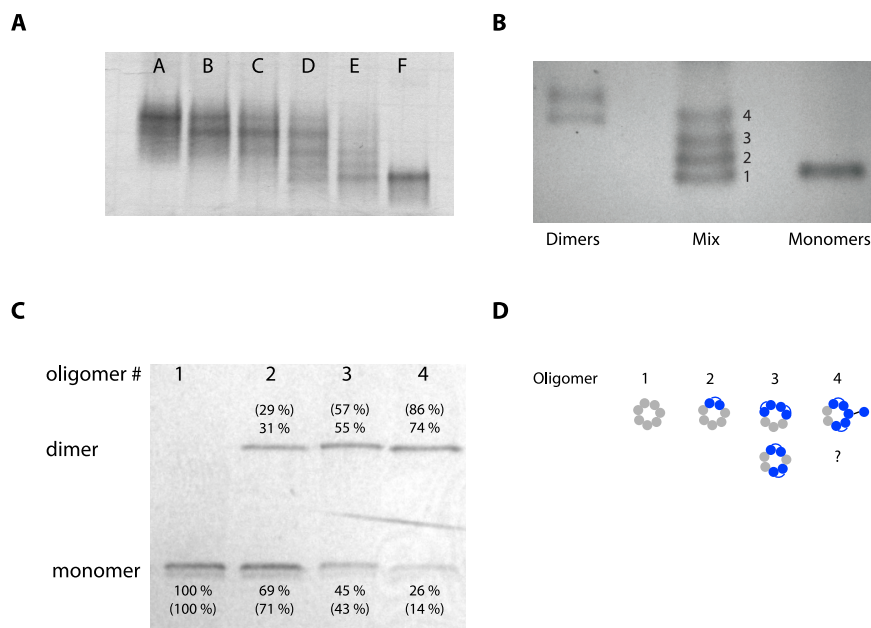


Figure 4.11: Composition of oligomers containing monomers and subunit dimers of α HL. **A**, mixtures of monomers and dimers of α HL were assembled on rRBCMs and subjected to electrophoresis in a 5% SDS-polyacrylamide gel. The monomers carried a D8H6 tail to allow separation of the oligomers. Lane A, dimer/monomer ratio 5:0; lane B, dimer/monomer 4:1; lane C, dimer/monomer 3:2; lane D, dimer/monomer 2:3; lane E, dimer/monomer 1:4; lane F, dimer/monomer 0:5. **B**, the experiment in A was repeated on a larger scale, and the samples with different ratios of monomer to dimer were combined ("Mix"). **C**, the oligomers in bands 1-4 from B were excised from the gel and heated to 95 °C for 10 min to disrupt the subunit interactions. The polypeptides were then subjected to electrophoresis in a 12% SDS-polyacrylamide gel to resolve the constituents, and the relative intensities of the bands were measured from an autoradiogram by using Quantity One software (Bio-Rad). The predicted intensities of the bands is given in parentheses. Lane 1, 2_01_77 ; lane 2, 2_11_57 ; lane 3, 2_21_37 ; lane 4, 2_31_17 . **D**, proposed stoichiometries of the oligomers 1-4 in B.

4.3.6 Introduction of functionality across two subunits of the α HL pore

To demonstrate the utility of a subunit dimer in a heptameric pore, the half-chelating ligand PIDA (Figure 4.12A) was introduced on each of the two subunits within a dimer of a 2_11_57 pore. The experiments in chapter 3 showed that two PIDA ligands on the same subunit can come together to form a complex with a single divalent metal ion [27]. This requires the ligands to be in close proximity, which can be achieved by placing them on two adjacent subunits in the heptamer but not on two

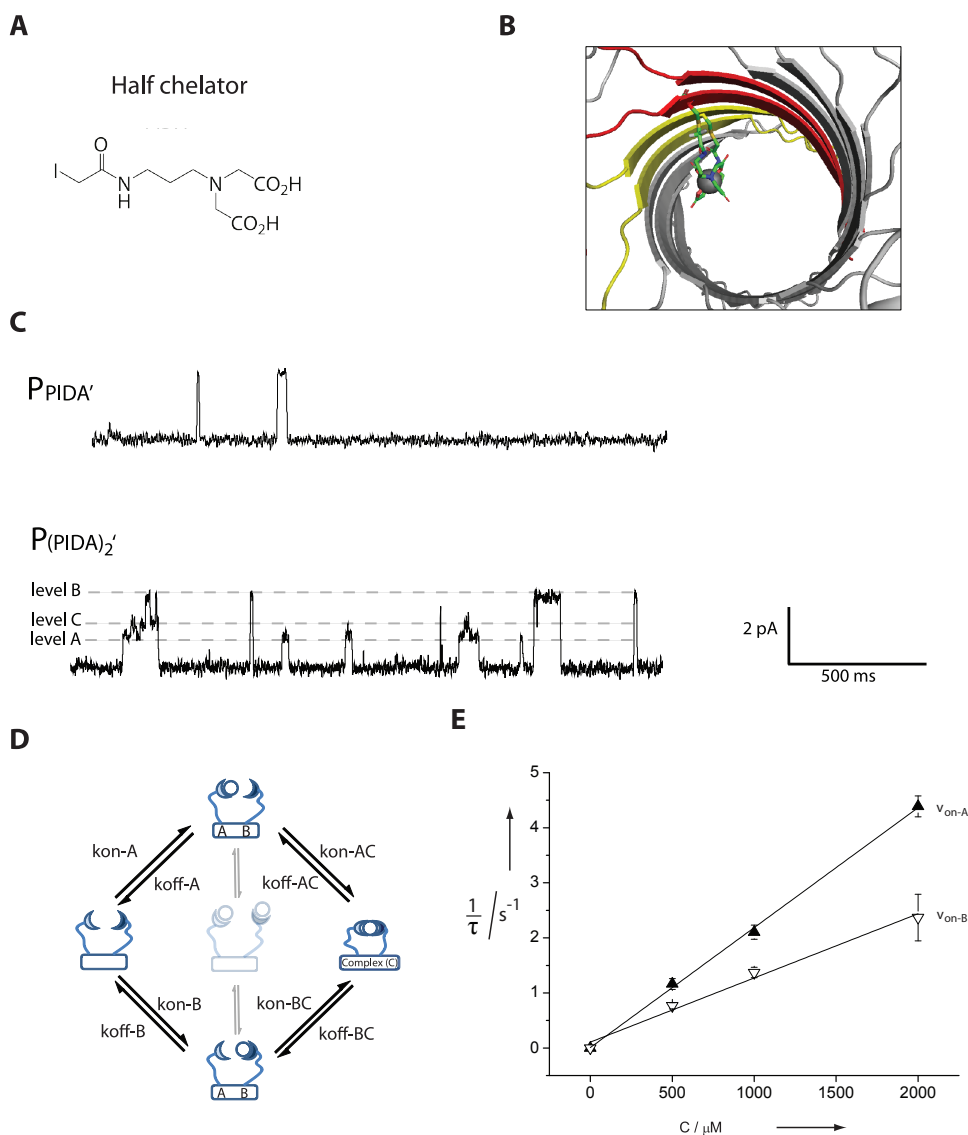


Figure 4.12: Half-chelators installed on two adjacent subunits in the α HL heptamer. **A**, structure of the half-chelator reagent containing the PIDA group. **B**, molecular model of the $P(PIDA)_2'$ pore. The two half-chelators are attached to cysteine residues at position 145 on the first subunit (red) and position 117 on the second subunit (yellow) of the subunit dimer. **C**, single channel recordings at -50 mV with a buffer containing 2.0 M KCl, 2 mM succinic acid, pH 4.0 , in both chambers and 500 μ M $ZnCl_2$ in the trans chamber. The $P(PIDA)'$ pore contains a single half-chelator at position 117, while the $P(PIDA)_2'$ pore contains two half-chelators, one at each at positions 145 and 117. The four current levels for the $P(PIDA)_2'$ pore correspond to the unoccupied pore, a single Zn^{2+} ion bound to one or the other of the half-chelators (levels A and B), and Zn^{2+} fully complexed with both half-chelators (level C). **D**, proposed kinetic scheme. **E**, The Zn^{2+} concentration dependence of the reaction rates for chelation site A (v_{onA}) and B (v_{onB}).

nonadjacent subunits. Two types of modified pore were prepared (containing one or two PIDA ligands), by adapting the method described in chapters 2 and 3. PPIDA' has a single PIDA ligand at position 117 on the second subunit of the subunit dimer and is similar to the original PPIDA pore, which has a single PIDA ligand at position 117 on one of the seven monomeric subunits [27]. The P(PIDA)₂' pore has one PIDA ligand at position 145 on the first subunit of the dimer (red) and a second PIDA ligand at position 117 on the second subunit of the dimer (yellow) (Figure 4.12B). The distance between the two PIDA ligands in the P(PIDA)₂' pore is similar to that in the original P(PIDA)₂ pore, which had one PIDA ligand at each of positions 117 and 143 on one of the seven monomeric subunits (the distances between the two sets of C α atoms are both 8.5 ± 0.1 Å in the wt heptamer structure, Protein Data Bank code 7AHL). The PPIDA' and P(PIDA)₂' pores were characterised by single channel recording in planar lipid bilayers, and their properties were compared with the PPIDA and P(PIDA)₂ pores from the original study. The PPIDA' pore carried a single channel current of -86.1 ± 1.6 pA at -50 mV in 2.0 M KCl, 2 mM succinic acid, pH 4.0, which is larger than the value of -75.7 ± 1.6 pA reported for the PPIDA pore under similar conditions. The recordings were performed at low pH because Zn²⁺ binds very tightly to P(PIDA)₂ at neutral pH values. The addition of Zn²⁺ to the trans side of the PPIDA' pore resulted in the fluctuation of the ionic current between two discrete levels separated by $\Delta I = 2.7 \pm 0.1$ pA, where ΔI is the current difference between the level partially blocked by Zn²⁺ and that of the unoccupied pore (Figure 4.12C, upper panel). A kinetic analysis of the binding events gave $k_{\text{on-mono4}} = 1300 \pm 400$ M⁻¹s⁻¹ and $k_{\text{off-mono4}} = 24 \pm 6$ s⁻¹ to yield an overall formation constant of $K_{\text{f-mono4}} = 52 \pm 19$ M⁻¹. The k_{on} value is about half that of the original PPIDA pore (Table 4.1), although the k_{off} values are nearly the same. The percentage block by Zn²⁺ relative to the unblocked pore is 3.1% for PPIDA' and 2.1% for PPIDA (Table 4.2). Therefore, in this regard, pores that include subunit dimers differ only subtly from similar pores made from monomers alone. The P(PIDA)₂' pore carried a single channel current of -67.1 ± 2.2 pA under

	PPIDA	PPIDA'
$k_{\text{on-mono4}}$	$k_{\text{on-A4}}$ $2500 \pm 400 \text{ M}^{-1}\text{s}^{-1}$	$k_{\text{on-B4}}$ $1300 \pm 400 \text{ M}^{-1}\text{s}^{-1}$
$k_{\text{off-mono4}}$	$k_{\text{off-A4}}$ $4 \pm 0.2 \text{ M}^{-1}\text{s}^{-1}$	$k_{\text{off-B4}}$ $24 \pm 6 \text{ M}^{-1}\text{s}^{-1}$
K_f	K_{f-A4} $85 \pm 16 \text{ M}^{-1}$	K_{f-B4} $52 \pm 19 \text{ M}^{-1}$

Table 4.1: *The association and dissociation rate constants for Zn^{2+} with the PPIDA' and PPIDA pores. The measurements were made in 2 M KCl, 2 mM succinic acid, pH 4.0 (PPIDA'), or 2 M KCl, 10mM potassium acetate, pH 4.0 (PPIDA), at an applied potential of -50 mV. The kinetic constants were obtained by using the Clampfit 10.2 software package. The formation constants (K_f) were calculated by using $K_f = k_{\text{on}}/k_{\text{off}}$.*

the conditions described above, which is again larger than the -56.4 ± 2.0 pA found for the P(PIDA)_2 pore. After the addition of Zn^{2+} to the trans recording chamber, both individual and complex current blockades were observed (Figure 4.12C, lower panel). The individual binding events to either level A ($\Delta I = 1.3$ pA) or level B ($\Delta I = 3.1$ pA) are consistent with Zn^{2+} binding to one or the other of two different sites. The complex blockades of the $\text{P(PIDA)}_2'$ pore included three different current levels and always began as a step from the unoccupied pore to either level A or level B, followed by a step to a new current level, C ($\Delta I = 2.0$ pA, Table 4.2). As supported by subsequent findings, level C represents a Zn^{2+} cation fully chelated by both PIDA ligands. Current steps to level A or B from level C were observed, but steps from C directly to the level of the unoccupied pore were never detected. The reaction rates for each step in the binding scheme for Zn^{2+} by the $\text{P(PIDA)}_2'$ pore (Figure 4.12D) were determined by fitting the mean lifetimes of the four conductance states (unoccupied, A, B, and C) to the scheme by using the QuB software package (Table 4.3). Plots of the rates $v_{\text{A-on}}$ and $v_{\text{B-on}}$ at pH 4.0 versus the Zn^{2+} concentration are of the form $v = k[\text{Zn}^{2+}]$, where k is $k_{\text{on-A4}}$ or $k_{\text{on-B4}}$ ($\text{M}^{-1}\text{s}^{-1}$), which confirms that both proposed half-chelator. Zn^{2+} complexes are formed in a bimolecular reaction (Figure 4.12E). As a further test of the kinetic scheme, note that at equilibrium the product of the rate constants for clockwise movement around the diamond (Figure

Protein	Open pore current (pA)	Magnitude of current block by Zn^{2+} (ΔI)		% current block by Zn^{2+}
PPIDA'	-86.1 ± 1.6	2.7 ± 0.1		3.1
PPIDA	-75.7 ± 1.0	1.6 ± 0.03		2.1
P(PIDA)₂'	-67.1 ± 2.2	Level A	1.3 ± 0.1	1.9
P(PIDA) ₂	-56.4 ± 2.0		1.9 ± 0.04	3.4
		Level B	3.1 ± 0.2	4.6
			2.5 ± 0.2	4.4
		Level C	2.0 ± 0.2	3.0
			3.7 ± 0.1	6.6

Table 4.2: Single channel currents through unoccupied αHL pores and after blockade by Zn^{2+} . The values were calculated from at least three independent experiments and are quoted as the mean $\pm$ S.D. The measurements were recorded at -50 mV in buffer containing 2.0 M KCl, 2 mM succinic acid, pH 4.0 (PPIDA' and P(PIDA)₂' in boldface), or 2.0 M KCl, 10 mM potassium acetate, pH 4.0 (PPIDA and P(PIDA)₂ in normal font). A single channel current of -100 pA corresponds to a unitary conductance of 2 nS. The magnitude of the block is quoted with respect to the unoccupied pore current.

4.12D) must equal the product for anticlockwise movement. It was found that $k_{\text{on-A4}}k_{\text{on-AC4}}k_{\text{off-BC4}}k_{\text{off-B4}} = 33 \pm 9 \times 10^6 \text{ M}^{-1}\text{s}^{-4}$ and $k_{\text{on-B4}}k_{\text{on-BC4}}k_{\text{off-AC4}}k_{\text{off-A4}} = 31 \pm 11 \times 10^6 \text{ M}^{-1}\text{s}^{-4}$, which are the same within experimental error. The properties of the P(PIDA)₂' pore are similar to those previously reported for the P(PIDA)₂ pore [27]; however, there are some differences. Although level C of the P(PIDA)₂' pore corresponds to the largest current block (ΔI), level C of the P(PIDA)₂' pore lies between level A and B. As noted previously, small changes within the lumen of the pore can have unpredictable effects on pore currents [26, 28], i.e. there is no reason to assume that fully complexed Zn^{2+} should always produce the largest current block. Neither is it possible to unambiguously assign level A or level B to one of the two locations of the PIDA ligand in either the P(PIDA)₂ or the P(PIDA)₂' pore, although, based on the observed sequence of transitions and the Zn^{2+} binding kinetics, the two levels must correspond to Zn^{2+} binding events. In contrast to the two sites in the P(PIDA)₂ pore, the sites (145 and 117) in the P(PIDA)₂' pore although similar are no longer almost kinetically equivalent. For example, for P(PIDA)₂', $k_{\text{on-A4}}$ is

	Level A	Level B	Level C (via A)	Level C (via B)
k_{on}	$k_{\text{on-A4}}$ 2200 $\pm$ 100 $\text{M}^{-1}\text{s}^{-1}$ 2500 $\pm$ 400 $\text{M}^{-1}\text{s}^{-1}$	$k_{\text{on-B4}}$ 1400 $\pm$ 200 $\text{M}^{-1}\text{s}^{-1}$ 2300 $\pm$ 100 $\text{M}^{-1}\text{s}^{-1}$	$k_{\text{on-AC4}}$ 29 $\pm$ 2 s^{-1} 51 $\pm$ 5 s^{-1}	$k_{\text{on-BC4}}$ 23 $\pm$ 2 s^{-1} 56 $\pm$ 7 s^{-1}
k_{off}	$k_{\text{off-A4}}$ 24 $\pm$ 2 s^{-1} 27 $\pm$ 2 s^{-1}	$k_{\text{off-B4}}$ 23 $\pm$ 2 s^{-1} 23 $\pm$ 3 s^{-1}	$k_{\text{off-AC4}}$ 42 $\pm$ 13 s^{-1} 32 $\pm$ 4 s^{-1}	$k_{\text{off-BC4}}$ 22 $\pm$ 5 s^{-1} 35 $\pm$ 5 s^{-1}
K_{f}	$K_{\text{f-A4}}$ 92 $\pm$ 11 M^{-1} 92 $\pm$ 15 M^{-1}	$K_{\text{f-B4}}$ 59 $\pm$ 9 M^{-1} 100 $\pm$ 10 M^{-1}	$K_{\text{f(C via A)4}}$ 65 $\pm$ 22 M^{-1} 150 $\pm$ 22 M^{-1}	$K_{\text{f(C via B)4}}$ 62 $\pm$ 19 M^{-1} 160 $\pm$ 40 M^{-1}

Table 4.3: *Kinetic constants for the formation of complexes between the $P(\text{PIDA})_2'$ and $P(\text{PIDA})_2$ pores and Zn^{2+} .* The measurements were recorded at -50 mV in buffer containing 2.0 M KCl, 2 mM succinic acid, pH 4.0 ($P(\text{PIDA})_2'$), or 2.0 M KCl, 10 mM potassium acetate, pH 4.0 ($P(\text{PIDA})_2$). The kinetic constants were obtained by using the QuB software package. The formation constants (K_{f}) were calculated by using $K_{\text{f}} = k_{\text{on}}/k_{\text{off}}$. $P(\text{PIDA})_2'$ is shown in boldface, and $P(\text{PIDA})_2$ is in normal font.

2200 $\pm$ 100 $\text{M}^{-1}\text{s}^{-1}$, although $k_{\text{on-B4}}$ is 1400 $\pm$ 200 $\text{M}^{-1}\text{s}^{-1}$. Note that both $k_{\text{on-A4}}$ and $k_{\text{on-B4}}$ for formation of the half-chelator complexes, as well as $k_{\text{on-AC4}}$ and $k_{\text{on-BC4}}$ for formation of the fully chelated complex, are lower for the $P(\text{PIDA})_2'$ pore by comparison with the corresponding rate constants of the $P(\text{PIDA})_2$ pore (Table 4.3). These differences could arise because different buffers were used for the two sets of experiments (succinic acid, pH 4.0, for $P(\text{PIDA})_2'$; potassium acetate, pH 4.0, for $P(\text{PIDA})_2$). Alternatively, there may be small structural changes in pores that contain subunit dimers, which affect the rate constants. In any event, the effects are small, and the fundamental behaviors of the $P(\text{PIDA})_2$ and the $P(\text{PIDA})_2'$ pores are similar.

4.4 General implications and future prospects

Although the utility of the concatemer approach has been carefully documented, the data presented in this chapter reinforce the need for rigorous analysis of translation products to ensure that only full-length concatemers are produced. Full-length

subunit dimers were obtained by Ni-NTA purification of a His₆-tagged protein. By placing the affinity tag at the C terminus of the protein, it could be ensured that only fully translated proteins would bind to the Ni-NTA column. This approach, however, cannot solve the problem of proteolytic cleavage of the concatemer after translation, which has been a problem in other studies [22]. Even though there are several examples of pore-forming proteins that can form oligomers with variable stoichiometries, these experiments have shown that α HL oligomers made from subunit dimers resist all but a heptameric pore stoichiometry. This implies that one or more subunits must be excluded from the central pore. Subunit exclusion is not uncommon for ion channels made from concatenated subunits, when there are more subunits to be accommodated in the oligomer than in the native protein [17, 29]. Based on these findings, a method to prepare α HL pores containing one subunit dimer and five monomers was developed, allowing mutations and/or chemical modifications to be introduced selectively on adjacent subunits of the α HL pore. The metal chelation experiments show that pores containing subunit dimers behave similarly to pores containing only monomeric subunits, showing that they assemble correctly. Subunit concatenation has therefore widened the scope for engineering the α HL pore and might be expanded to extended concatemers (trimers, tetramers, etc.). Ultimately, the approach could allow the experimenter to control the nature and position of every subunit in the pore. For practical reasons, it would be useful to introduce unique restriction sites into each linker at the DNA level, to allow the cutting and pasting of specific subunit genes into the construct.

4.5 Description of work included in AFH's Chemistry Part II thesis and work carried out for the D.Phil. thesis

Some experiments shown in this chapter are described at least in part in my Part II thesis. Because much of the work presented in the Part II thesis was preliminary,

the experiments were repeated and improved over the course of my D.Phil. studies and there is some overlap. I have indicated where this is the case.

The following experiments were carried out during my Part II studies:

- the design of the subunit dimers and oligopeptide linkers based on the crystal structure of the α HL heptamer
- preparation of the genes HL-(nSG)-HL (where n= 5, 10, 13, 15)
- IVTT of the genes HL-(nSG)-HL (cf. Figure 4.4A, although the gel shown came from an experiment carried out during my D.Phil. studies)
- oligomer formation of subunit dimers on rRBCMs (cf. Figure 4.4B, although the gel shown came from an experiment carried out during my D.Phil. studies)
- haemolytic activity assay of subunit dimers (Figure 4.4C)
- the limited proteolysis assay shown in Figure 4.6
- the limited proteolysis assay shown in Figure 4.5 was carried out during my D.Phil. studies, however the gel combines several smaller experiments described in the Part II thesis
- the preparation of mixed oligomers comprising the subunit dimers α HL-10SG- α HL and monomers α HL-D8H6 (Figure 4.11A), but **not** the analysis of the number of constituent subunits (Figure 4.11B and C)
- preliminary single-channel recording data

The following experiments were carried out during my DPhil studies:

- the analysis of the oligomers comprising the subunit dimers α HL-10SG- α HL and monomers α HL-D8H6, specifically the number of constituent subunits (Figure 4.11B and C)
- preparation of His-tagged subunit dimers, their purification by Ni-NTA affinity chromatography and oligomerisation on rRBCMs (Figure 4.7)

- the preparation of mixed oligomers comprising the Ni-NTA purified subunit dimers α HL-10SG- α HL-H6 and α HL-10SG- α HL-D8H6 (Figure 4.8)
- all single channel recording experiments (Figure 4.9, Figure 4.10)
- modification of the subunit dimers with the PIDA group, preparation of the PPIDA' and P(PIDA)₂' pores and all related single channel recordings (Figure 4.12)

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Chapter 5

A light-activated single-molecule nuclease (preliminary data)

5.1 Chapter overview

This chapter explores the possibility of following the nuclease activity of a metal complex in the α HL pore at the single molecule level. The complex chosen for these experiments was $[\text{Rh}(\text{bpy})_2\text{phzi}]^{3+}$, which binds strongly to CC mismatches in dsDNA, and which on activation by UV light causes DNA strand cleavage. To follow this process at the single molecule level, a piece of duplex DNA (with the bound Rh-complex) was immobilised in the α HL pore and the single current changes under UV irradiation were monitored. Preliminary data indicate that the photocleavage reaction does proceed inside the pore and that the rate of individual reactions can be monitored.

5.2 Introduction

Chapters 2 and 3 established that the α HL pore can be engineered to bind divalent metal ions. Divalent metal ion binding sites in proteins play a prominent role in enzyme catalysis [1, 2], so the question arose of whether a catalytic site could

be built into the α HL pore. The initial ideas for this project focused on (S)-1-[*p*-(bromoacetamido) benzyl] ethylenediaminetetraacetate (FeBABE, Figure 5.1) [3].

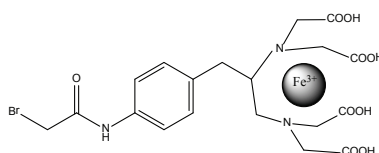


Figure 5.1: *The structure of the cleavage agent FeBABE*

FeBABE is a commercially available EDTA derivative [4], which can be used to map peptide or nucleic acid binding sites on a protein. For this purpose, the reagent is conjugated to mutant cysteine residues in the putative binding site of a protein. The derivatised protein is then mixed with the peptide or nucleic acid binding partner. When hydrogen peroxide is added, the Fe-EDTA complex generates diffusible hydroxy radicals as well as a Fe-EDTA-peroxo species [5]. The radicals are generated close to the peptide/nucleic acid backbone and react, resulting in cleavage of the binding partner. This reaction is unspecific and the cleavage position is determined primarily by the distance between the Fe-EDTA complex and the binding partner.

Based on the experiments described in Chapters 2 and 3, it should be facile to install such a cutting reagent in the α HL pore, either in the β barrel or in the cap domain. Further, a lot of work in the nanopore field has focused on immobilising ssDNA strands in the α HL pore [6, 7], with a view towards developing an electrical method to sequence DNA strands. Therefore, it should also be possible to bring a piece of DNA into close contact with a cutting reagent.

However, a major drawback of using FeBABE is that it must be activated by peroxide and that a reducing agent (e.g. ascorbic acid) is required to regenerate the Fe(III) complex [3]. There was a concern that peroxide could have unpredictable effects on the protein. Furthermore, the cleavage reaction is known to be unspecific, so if different parts of the ssDNA backbone were cleaved, then a

range of electrophysiological signals might be observed that would be hard to analyse systematically. For this reason, a range of other metal-based nucleases was considered to determine if they would be more suitable for these experiments.

The only known natural examples of metal-based nucleases that bind to DNA non-covalently are the bleomycins, a family of cytotoxic agents from *Streptomyces verticillus* [8, 9]. Bleomycins are glycopeptide antibiotics that bind to the minor groove of DNA. Most bleomycins have a coordinated Fe(II) ion, which forms a high valent Fe-oxo species with O₂. Activated bleomycin can abstract a 4'-hydrogen atom from the deoxyribose sugar of DNA, which ultimately leads to scission of the DNA backbone. Like FeBABE, the bleomycins also require a reductant to regenerate the complex, so they are not suitable for the experiments described in this chapter. However, the idea of using a nuclease that binds to DNA non-covalently was investigated further.

A further class of synthetic DNA-binding metallo-nucleases are the octahedral Rh(III) complexes bearing large aromatic ligands, e.g. [Rh(phen)₃]³⁺ (phen = 1, 10-phenanthroline) [10]. These complexes are coordinatively saturated and inert to ligand substitution. [Rh(phen)₃]³⁺ binds non-covalently to dsDNA, mainly through hydrophobic interactions in the minor groove and by partial intercalation of the phenanthroline ligand [11]. Rh(III) complexes of this type have useful photochemical properties. On activation with UV light, the complex undergoes a ligand-to-metal charge transfer that results in the formation of a ligand-based cation- π radical. This radical is positioned closely to the backbone of the bound DNA and abstracts a hydrogen atom from the deoxyribose sugar, which leads to cleavage of one of the two strands [12]. Such Rh(III) complexes would be of interest to this project, because the activation of the complex can be brought about by light rather than chemicals. To date, a range of Rh(III) complexes with different aromatic ligands have been synthesised, some of which bind to specific DNA regions (Figure 5.2) [10].

Besides groove binding and intercalation, Rh(III) complexes can also bind to DNA by

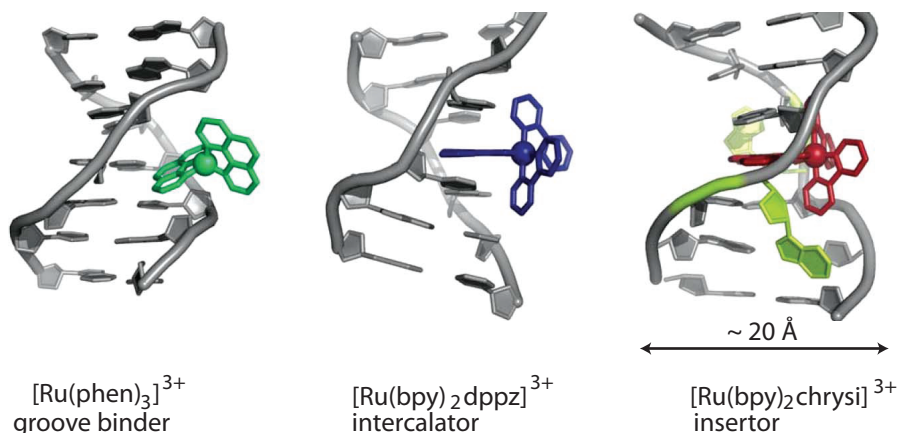


Figure 5.2: DNA binding modes of Rh(III) complexes. *phen* = 1, 10-phenanthroline, *bpy* = 2, 2'-bipyridyl, *dppz* = dipyrido[3,2-*a*:2',3'-*c*]phenazine, *chrysi* = 5,6-chrysenequinone diimine. Figure adapted from [10].

insertion. Intercalation and insertion both involve π -stacking of the ligand between the bases of the DNA. The difference between the two binding modes is that for intercalation the ligand is inserted between two existing base pairs. Insertion on the other hand involves the ejection of the mismatched base pair from the helix, followed by insertion of the ligand, which serves as a π -stacking replacement [10]. Metallo-insertors have sterically expansive ligands, e.g. 5,6- chrysene quinone diimine (*chrysi*) or benzo[*a*]-phenazin-5,6-quinone diimine (*phzi*), which are too large to intercalate into dsDNA, due to steric clash with the sugar rings of the DNA. Insertors do, however, bind to mismatch sites in the helix with high affinity. The more thermodynamically destabilising the mismatch is, the stronger is the interaction with the insertor. Two specific Rh(III) insertor complexes have been developed for DNA base mismatch detection: $[\text{Rh}(\text{bpy})_2\text{chrysi}]^{3+}$ and $[\text{Rh}(\text{bpy})_2\text{phzi}]^{3+}$. Of these two, the *phzi* complex has a slightly higher affinity ($K_f \approx 10^8 \text{ M}^{-1}$ [13]) for CC mismatch sites, so it will be the focus of this chapter.

No detailed information is available for the the quantum yield ¹ of the DNA cleavage reaction by the $[\text{Rh}(\text{bpy})_2\text{phzi}]^{3+}$ complex, nor has a detailed mechanism for the strand cleavage been determined. However, these data are available for the DNA photocleavage by the related $[\text{Rh}(\text{phen})_2\text{phi}]^{3+}$ complex (*phi* = 9,10-

¹The quantum yield of the cleavage reaction is defined as $\theta = \frac{\text{number of DNA strands cleaved}}{\text{number of photons absorbed}}$

phenanthrenequinone diimine). The cation- π radical of this complex abstracts a C3'-hydrogen from the backbone of the DNA, which after several reaction steps (Figure 5.3) leads to scission of the strand [12]. The quantum yield for the elimination of the base by-product is $\approx 10^{-4}$ [12]. It is likely that the photoactivation of the $[\text{Rh}(\text{bpy})_2\text{phzi}]^{3+}$ complex involves a similar degradation mechanism and that the reaction proceeds with a similar quantum yield.

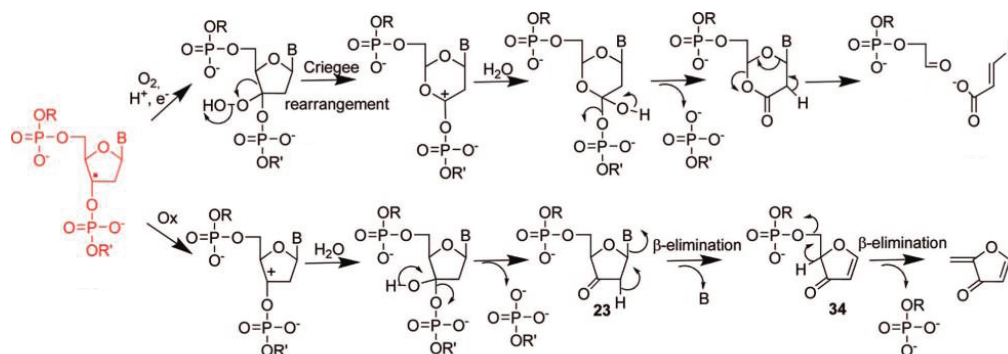


Figure 5.3: Mechanism of the DNA strand cleavage, following the abstraction of a 3'-hydrogen from a deoxyribose sugar. Figure adapted from [14]

5.2.1 Experimental design

To monitor the DNA photocleavage reaction by the Rh-complex inside the α HL pore, it was decided to immobilise a piece of dsDNA (containing a single CC mismatch site) in the pore. The strand containing the cleavage site would have a long polyA overhang, that allows the DNA to be driven into the pore electrophoretically. It is known that the ssDNA segment can enter the β -barrel of the protein, but that the dsDNA segment cannot [6]. Therefore, the dsDNA segment can be held in the pore vestibule under an applied potential (Figure 5.4), which would be evident as a partial block of the single channel current. Since the affinity of the Rh-complex for the CC mismatch site is high ($K_f \approx 10^8 \text{ M}^{-1}$), the Rh-complex can be introduced into the pore by pre-incubation of the duplex DNA with complex, which is considerably simpler than covalently modifying the pore with the complex. Furthermore, following the

photocleavage of a strand, the Rh-complex does not need to be regenerated, rather a new Rh-bound DNA duplex can be captured by the pore and the experiment can start over. As the Rh-complex is buried deeply in the dsDNA segment (the width of the Rh-bound dsDNA is approximately 20 Å, Figure 5.2), it should not affect the entry of the dsDNA segments into the pore vestibule (width = 30 Å).

Photoactivation of the Rh-bound DNA duplex would result in cleavage of the longer strand in the pore, and it was hoped that the reaction would cause a perturbation of the single channel current level associated with the immobilised strand. Such a signal could allow the reaction to be monitored in real time. Throughout the experiment the applied potential would exert a force on the longer of the two DNA strands. While the longer strand is fully hybridised to the shorter oligonucleotide, this force should be insufficient to "unzip" the duplex. However, following the cleavage of the double strand in the middle of the duplex, a much shorter section of the longer strand would be hybridised, and this could lead to unzipping of the longer strand [15]. The longer strand would probably translocate through the β -barrel. The shorter remaining duplex could either escape to the cis recording chamber under thermal motion [15] or also unzip and translocate, causing the single channel current to return to the level of the unblocked pore. The process of cleavage and translocation is illustrated in Figure 5.4B.

5.3 Results

5.3.1 Synthesis of the rhodium complex and bulk cleavage experiments

The complex $[\text{Rh}(\text{phzi})(\text{bpy})_2]\text{Cl}_3$ (Figure 5.5) was synthesised as a racemic mixture according to a published method [16]. It is known that the Δ -enantiomer of the complex has a much higher affinity for B DNA than the Λ -enantiomer, because the shape of the Δ Rh-complex is complementary to the shape of the B DNA helix. However, the separation of the two enantiomers is technically challenging, and a

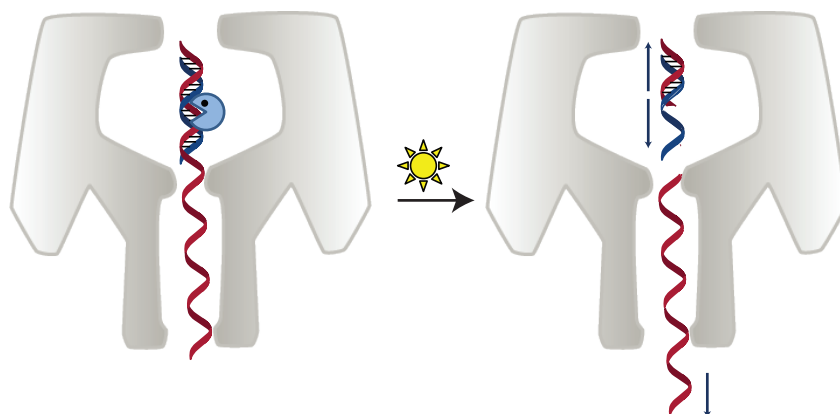


Figure 5.4: Photocleavage of the duplex region of an immobilised DNA strand in the α HL pore.

racemic mixture can be used instead [16]. A detailed description of the synthesis and characterisation of the Rh-complex is described in the Materials and Methods section.

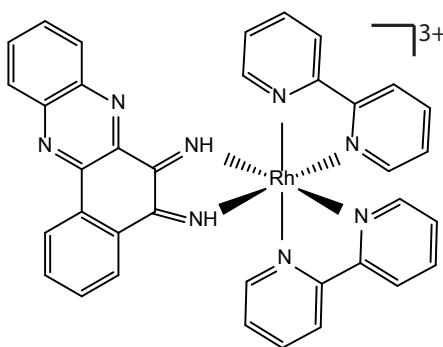


Figure 5.5: The structure of the metalloinsertor $[Rh(phzi)(bpy)_2]^{3+}$.

Next, dsDNA sequences were designed which (i) contain a CC mismatch to bind the Rh complex and (ii) have a long poly(A) overhang on one strand to thread the DNA through the β -barrel. The dsDNA segment was the same as the one used in a previous photocleavage experiment with the metalloinsertor Δ -Rh(bpy)₂(chrysi)³⁺ [17]. From experiments of the Bayley group, it is known that roughly 15 nucleotides of an immobilised oligonucleotide strand span the β -barrel of α -HL under an applied

potential [7]. For the initial experiments, a 5' extension of 18 adenine residues was therefore added to one of the strands. The structure of the duplex DNA is shown in Figure 5.6A. Although it is known that the photocleavage of the phosphate backbone adjacent to a CC mismatch occurs site-specifically [10], it was not clear on which of the two strands or on which side of the mismatch the scission would occur. This was therefore determined experimentally.

For these experiments, an equimolar amount of the two oligonucleotides in water (12-40 μM , $T_m = 60^\circ\text{C}$) were hybridised by heating to 90°C and then slowly cooling the solution to room temperature. The resulting duplex DNA had a concentration of 9-15 μM and was combined with two molar equivalents of the Rh complex in 100 mM NaCl, 20 mM NaP_i (pH 7.1), i.e. 1 molar equivalent of the Δ -enantiomer of the complex, assuming a 1:1 mixture of the two enantiomers. The DNA and the Rh-complex were incubated on the bench for 30 min in the dark. The fractional occupancy (θ) of the DNA mismatch site with the Rh-complex is given through

$$\theta = \frac{2[A] + K_d - \sqrt{(2[A] + K_d)^2 - 4[A]^2}}{2[A]} \quad (5.1)$$

where $[A] = [\text{DNA}] = [\text{Rhodium complex}]$. A detailed derivation of this expression is presented in the appendix, chapter 8.4. For $[A] = 10 \mu\text{M}$ and $K_d = 10^{-8} \text{ M}$, $\theta = 0.96$, i.e. the DNA should be almost saturated with the Rh-complex under the experimental conditions described here.

For the bulk photocleavage experiments, irradiation was carried out between 5 and 60 minutes, using a 300 W Xe arc lamp equipped with a 280-370 nm band-pass filter. The oligonucleotide fragments were then separated by electrophoresis in a 20% polyacrylamide/8M urea denaturing gel and visualised with "Stains All" (Figure 5.6B).

The two control conditions (lane 1, no Rh-complex + 5 min $h\nu$; lane 2, Rh-complex - $h\nu$) show only the two starting oligonucleotides (a 27-mer and a 45-mer), but no photocleavage products, confirming that cleavage does not occur in the absence

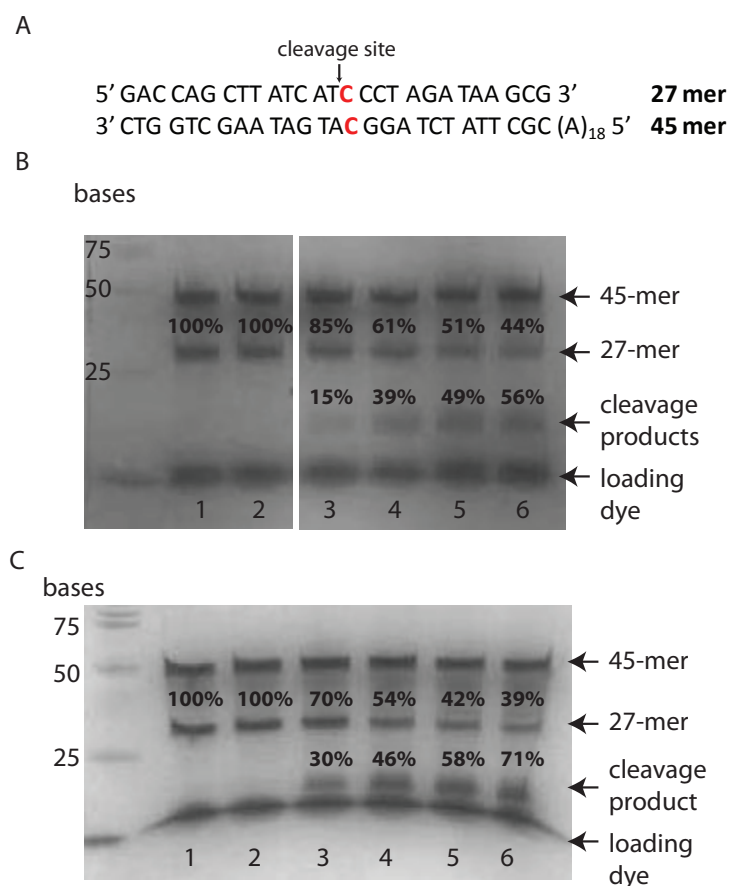


Figure 5.6: Photocleavage of a DNA duplex by $Rh(phzi)(bpy)_2^{3+}$. **A** Sequence of the DNA duplex and proposed cleavage site. **B** 20% polyacrylamide/8M urea gel showing photocleavage fragments after reaction in buffer containing 100 mM NaCl, 20 mM NaP_i (pH 7.1). Lane 1: DNA, no Rh-complex. 5 min $h\nu$; Lane 2: DNA, Rh-complex, no $h\nu$; Lane 3: DNA, Rh-complex, 5 min $h\nu$; Lane 4: DNA, Rh-complex, 15 min $h\nu$; Lane 5: DNA, Rh-complex, 30 min $h\nu$; Lane 6: DNA, Rh-complex, 60 min $h\nu$. The relative intensity of the bands is displayed above the band. **C** Photocleavage products after reaction in buffer containing 1M KCl, 10 mM MOPS (pH 7.0). Lanes 1-6 are the same as for panel B.

of the Rh-complex or irradiation. On irradiation of the Rh-bound DNA duplex, a cleavage band (<25 bases) is observed, which becomes more intense as the irradiation time is increased. The intensity of the 45-mer remains unchanged throughout the experiment, however, the intensity of the 27-mer decreases. The reaction does not go to completion and after 60 min roughly half of the 27-mer has been cleaved.

These experiments show that the photocleavage reaction occurs at a specific site on the 27-mer and it is likely that the cleavage site is the same as had been observed with the Δ -Rh(bpy)₂(chrysi)³⁺ complex [17]. If the site is indeed the same, then

the cleavage reaction should have revealed two bands, a 13-mer and a 14-mer. It is likely that the resolution of the gel (Figure 5.6B) was insufficient to resolve both bands.

It is known that the efficiency of the cleavage reaction depends on the power of the light source. For experiments described here, irradiation with a 1000 W Xe-Hg arc lamp for 30-60 min, or a solar simulator for 15 min is recommended to effect cleavage. For these preliminary experiments only a 300 W Xe arc lamp was available, so it is not surprising that the reaction did not go to completion.

As the cleavage experiments were to be carried in conjunction with single channel recording, the photocleavage reaction was also investigated in the high salt buffer required for the electrical recording, rather than the phosphate buffer recommended for this reaction [16]. This control experiments is required, as the electrostatic interaction between the Rh-complex and the DNA would be screened by a high concentration of salt ions, and therefore the interaction between the DNA and the Rh-complex could be weaker. The photocleavage reaction was repeated in buffer containing 1M KCl and 10 mM MOPS (pH 7.0). Figure 5.6C shows that the reaction generates cleavage products as readily in high salt conditions as it does in low salt conditions and should therefore be compatible with the single channel recordings.

The initial experimental design had the cleavage site on the longer of the two oligonucleotides, so that on photocleavage the long poly(A) overhang would translocate through the pore. Even if the photocleavage reaction itself does not generate a perturbation of the single channel current, the translocation of the poly(A) overhang would cause the current to return to the level of the unblocked pore, inferring that cleavage had taken place. As the previous experiments had established that the cleavage site was in fact on the shorter oligonucleotide, new oligonucleotides were used, in which the poly(A) extension was added to the cleavage strand (Figure 5.7A). The length of the overhang was also increased from 18 As to 30, as this length of overhang had been used successfully in combination with a

DNA stem-loop to immobilise a strand of DNA in the α HL pore under an applied potential [18]. Photocleavage experiments in bulk solution (Figure 5.7B) show that adding an extension of 30 As to the cleavage strand has no effect on the cleavage efficiency. In this case, both cleavage products (a 14-mer and a 43-mer) are resolved well on the gel.

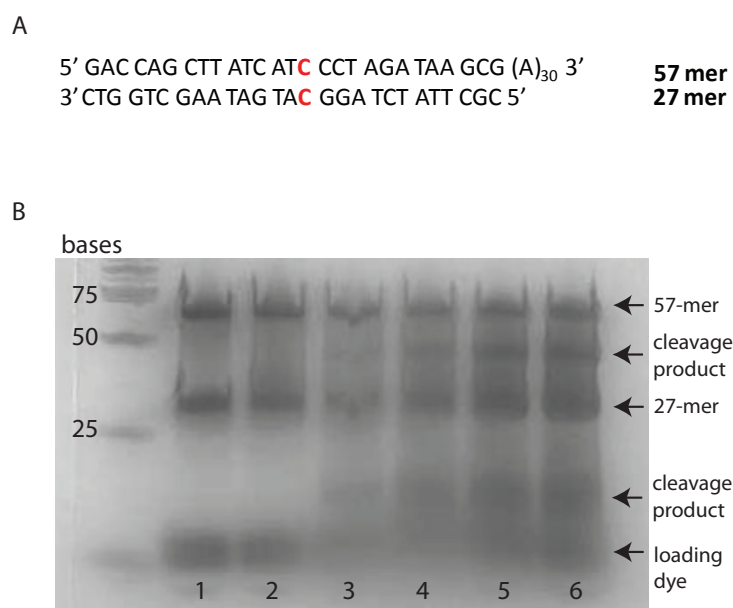


Figure 5.7: Photocleavage of a DNA duplex by $Rh(phzi)(bpy)_2^{3+}$. **A** Sequence of the DNA duplex and proposed cleavage site. **B** 20% polyacrylamide/8M urea gel showing photocleavage fragments after reaction in 100 mM NaCl, 20 mM NaP_i (pH 7.1). Lane 1: DNA, no Rh-complex. 5 min hv; Lane 2: DNA, Rh-complex, no hv; Lane 3: DNA, Rh-complex, 5 min hv; Lane 4: DNA, Rh-complex, 15 min hv; Lane 5: DNA, Rh-complex, 30 min hv; Lane 6: DNA, Rh-complex, 60 min hv.

5.3.2 Investigation of the DNA cleavage reaction by single channel recording

As it was clear that the photocleavage experiments would take tens of minutes to proceed, it had to be ensured that the DNA strands could be immobilised in the pore efficiently for this length of time. Furthermore, it was assumed that the Rh-complex would remain bound to the DNA when added to the recording chamber. As the Rh-bound DNA is added to the experiment, it is diluted by a factor of 100

by the recording buffer, to approximately 100 nM. According to equation 5.1, under equilibrium conditions the fraction of Rh-bound mismatch sites is only 0.7 when $[A]=10^{-7}$ M, so fewer strands would be expected to cleave. However, this would not be an issue if the rate of dissociation of the Rh-complex from the DNA was slow, and as the subsequent experiments show no evidence for dissociation was found.

Immobilising the DNA in the pore for tens of minutes turned out to be challenging and a large number of recording conditions had to be screened until a satisfactory result was obtained. The initial capture experiments using the pre-hybridised oligonucleotides (sequences shown in Figure 5.7A, approximately 100 nM final concentration) were performed in buffer containing 2 M KCl, 10 mM MOPS (pH 7.0) and under an applied potential of +120 mV. The recording chamber was equipped with a sapphire window on the side of one buffer compartment, to allow irradiation of the bilayer with UV light. An applied potential of +120 mV was chosen, because the DNA capture by the wt α HL pore is low at potentials of less than +120 mV [19, 20]. Under these recording conditions the pore had a single channel current of 234 pA. The DNA duplex was readily captured by the pore and two types of blockade were observed (Figure 5.8A).

Type A blockades were most common and had a mean single channel current of 13.7 pA (corresponding to a 94% block relative to the open channel current). These blocking events had a mean dwell time of 340 ms, which is surprisingly short for an immobilised strand. Type B blocking events were rarer and the single channel currents associated with this type of event were broadly distributed between 60 and 130 pA, corresponding to 49-69% blockades of the single channel current. Type B events had a similar dwell time of 470 ms.

Maglia et al. [19] also described several types of blocking events when a ss 92-mer was captured by the wt α HL pore under an applied potential of +120 mV and in buffer containing 1 M KCl, 25 mM Tris.HCl (pH 8.0). When the oligonucleotide translocated through the β -barrel, a 89% block of the single channel current was observed. When the oligonucleotide entered the vestibule of the pore, but did

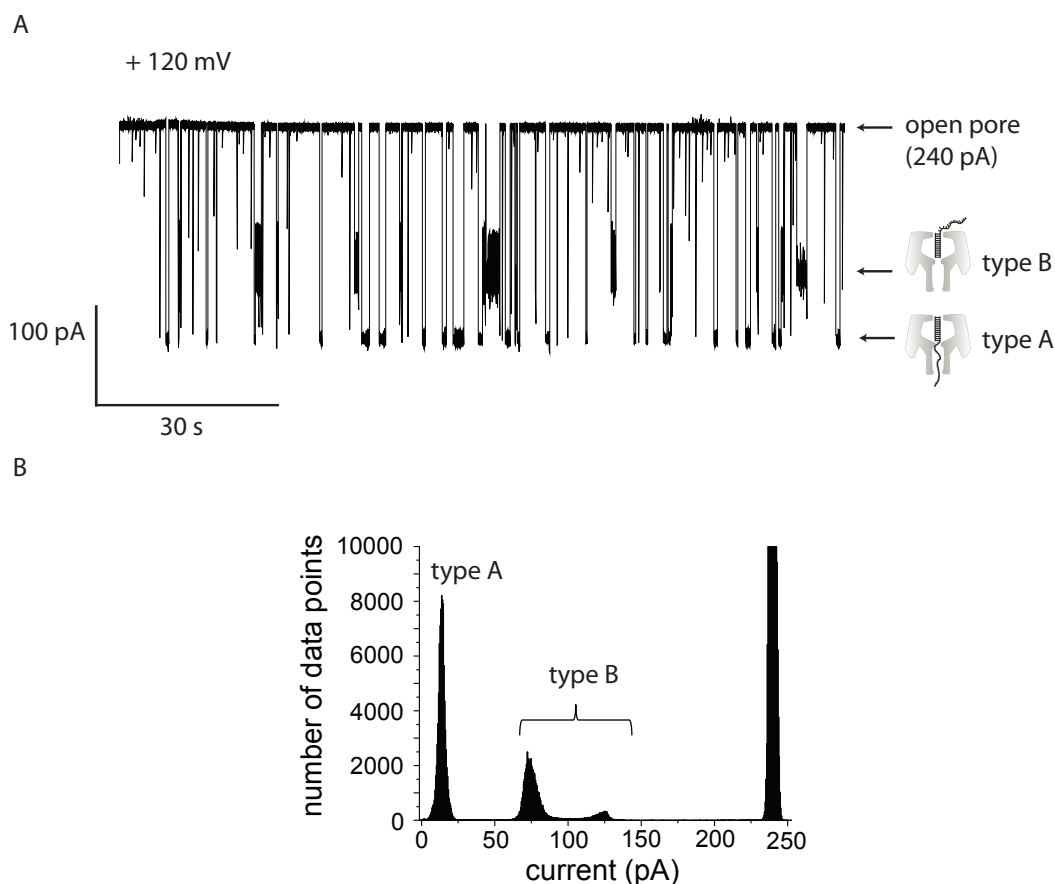


Figure 5.8: DNA capture by the wt α HL pore. **A** Single channel recording in 2M KCl, 10 mM MOPS (pH 7.0) at +120 mV, with 150 nM DNA duplex added to the cis chamber. **B** Amplitude histogram of the recording trace shown in panel A.

not enter the β -barrel to translocate, a broad distribution of blocking events was observed that had a mean value of 42%. Both types of blocking event observed by Maglia *et al.* were very brief, lasting less than 1 ms. Based on these observations, it is likely that the type A events correspond to a configuration in which the ssDNA segment has entered the β -barrel and the dsDNA segment is immobilised in the vestibule. Type B events likely correspond to the dsDNA segment having entered the vestibule first, with the ssDNA located outside of the cap domain. These two configurations are indicated in the cartoon in Figure 5.8A. The dwell times of the type A events indicate that DNA is temporarily immobilised in the pore, but then escapes. This could either be because the applied potential is too low to maintain the DNA in the pore against thermal agitation and repulsive forces [20], or because

the potential difference exerts such a strong force on the longer ssDNA segment that it dissociates from the duplex and translocates through the pore [15]. An additional step corresponding to the translocation of the shorter segment of ssDNA through the pore was not observed, but may have been missed due to insufficient temporal resolution of the experiment. Since the recordings had to proceed for tens of minutes, the size of the data files had to be kept as small as possible. This was done by applying a 5 kHz low-pass Bessel filter to the recording (rather than the 40 kHz filter used by Maglia et al.), which limited the temporal resolution of the experiment to 0.2 ms and may have been insufficient to resolve the translocation of short segments of ssDNA.

To test these two hypotheses, the capture experiments were repeated at a lower applied potential of + 80 mV (Figure 5.9). Under these recording conditions the pore carried a single channel current of 173.3 ± 5.6 pA ($n = 4$). The DNA blocking events were less frequent than at +120 mV, but a qualitative analysis showed that the type A blocking events had longer dwell times of tens of seconds.

It was not possible to perform a dwell time analysis, because only 50 blocking events above the 2 ms detection threshold were observed in a 30 min recording. The increased dwell time of the duplex DNA in the pore at lower applied potentials indicates that it is the stability of the duplex that limits the dwell time of the DNA in the pore at high potentials. Although a high positive potential helps to drive the DNA into the pore in the first place, it also creates a strong pull on the ssDNA segment that destabilises the duplex, and leads to the dissociation of the two strands. Knowing that the duplex would be more stable at lower applied potentials, an attempt was made to prolong the residence time of the DNA in the pore by lowering the voltage to +50 mV after the DNA had been captured at +80 mV. This worked remarkably well, and it was possible to hold the DNA duplex in the pore for at least 60 min (Figure 5.9, $n = 3$). The same was true when the duplex DNA was pre-incubated with two equivalents of the Rh-complex, and again the DNA could be held in the pore for at least 60 min ($n = 4$, data not shown). The residual current

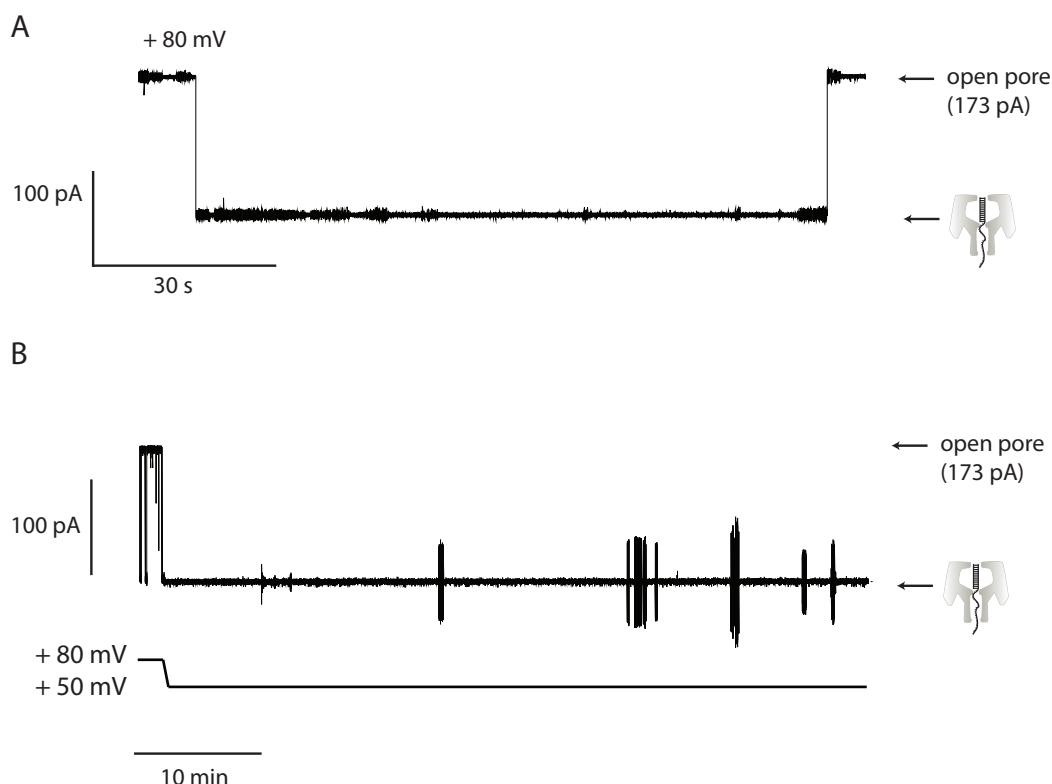


Figure 5.9: DNA capture by the wt α HL pore in buffer containing 2M KCl, 10 mM MOPS (pH 7.0), with 150 nM DNA duplex added to the cis recording chamber. **A** DNA capture at +80 mV **B** The DNA is initially captured by the pore at +80 mV and then held in the pore at +50 mV according to the voltage protocol shown.

associated with the DNA blockades (with or without the Rh-complex) at +50 mV was 7.4 ± 0.8 pA ($n=7$). The DNA capture events are fully reversible, and the DNA duplex can be expelled from the pore by briefly switching from a positive to a negative applied potential (Figure 5.10).

Following the expulsion of the duplex DNA, the unblocked pore at +50 mV has a lower single channel current of 99 ± 5.2 pA ($n = 4$).

Next, photocleavage experiments were carried out in the α HL pore. A piece of duplex DNA (pre-incubated with the Rh-complex) was briefly immobilised in the pore using the variable voltage protocol and then expelled by reversing the polarity of the applied potential (Figure 5.11A). This was done to establish the single channel current of the pore at both +80 mV and +50 mV. The DNA capture process was repeated, and a piece of duplex DNA (+Rh-complex) was immobilised in the pore.

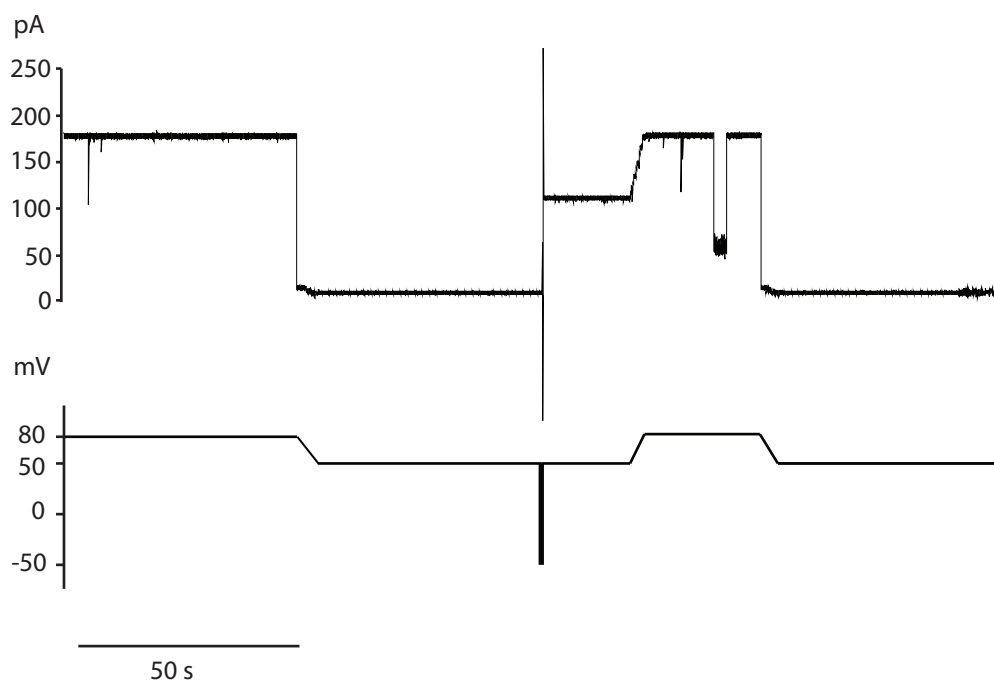


Figure 5.10: *Duplex DNA capture and release by the α HL pore using a voltage protocol. The initial DNA capture is performed at +80 mV, and once captured the DNA is held in the pore at +50 mV. When the potential is briefly reversed to from +50 mV to -50 mV, the duplex DNA is expelled from the pore. The pore does not capture DNA at +50 mV, so the potential is increased to +80 mV to repeat the cycle of capture and immobilisation*

20 min after the beginning of the recording, the bilayer containing the pore was irradiated continuously with UV light to effect cleavage of the duplex DNA. In experiment 1 (Figure 5.11B), the pore returned to the level of the unblocked pore 26.5 min after the irradiation had begun, implying that the DNA had been cleaved. Reversing the polarity of the potential at this point did not alter the single channel current of the pore, showing that the current jump had resulted from the removal of the DNA from the pore and not the insertion of a second pore into the bilayer. It was hoped that the cleavage event would result in a perturbation of the single channel current, however no perturbations were detected prior to the cleavage event (Figure 5.11C).

The experiment was repeated four times and the time elapsed between the beginning of the irradiation and the return to the open pore level was measured. Out of the five pores investigated, four pores showed events consistent with cleavage within 40

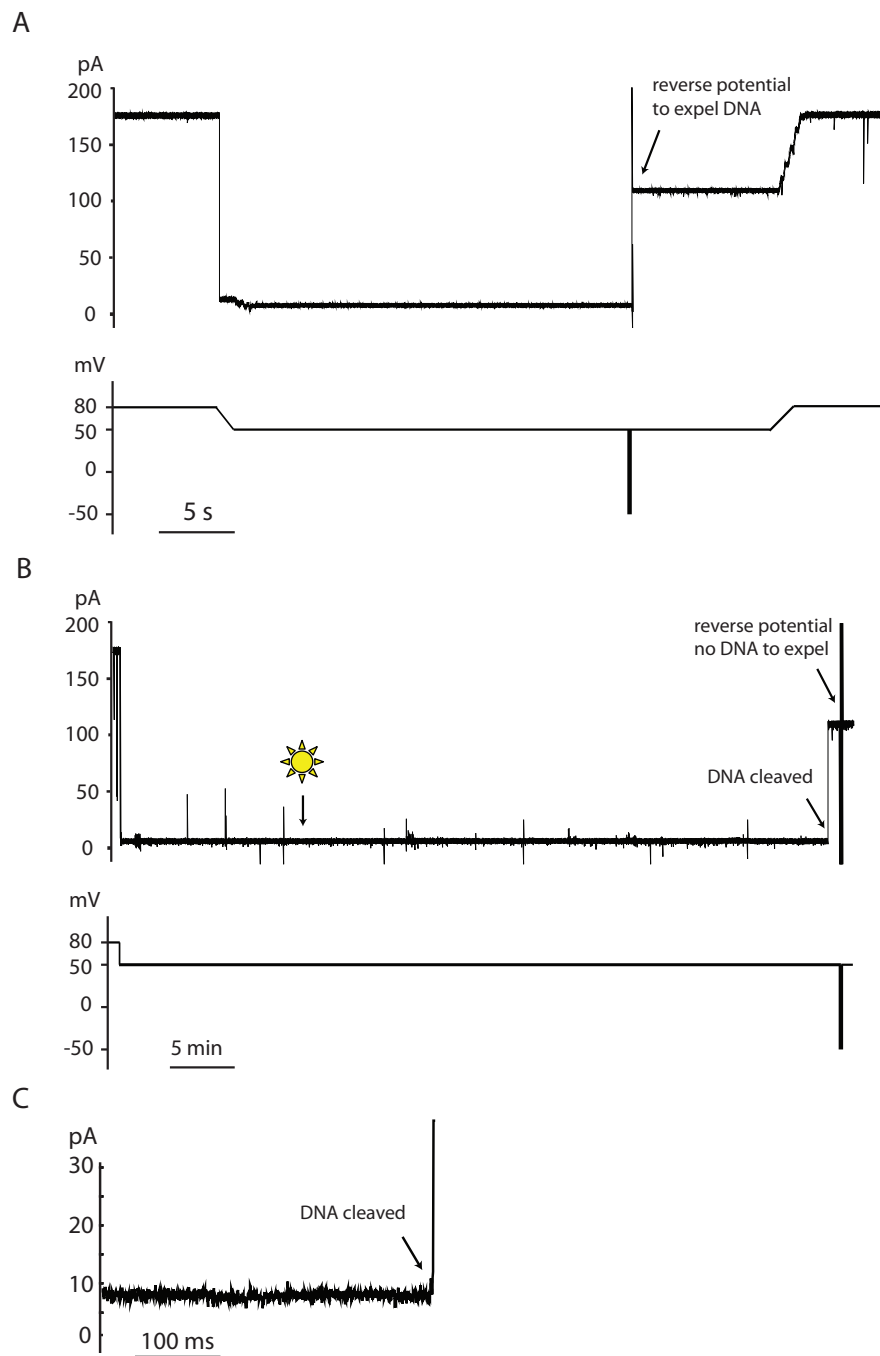


Figure 5.11: DNA photocleavage in the α HL pore. **A** A piece of duplex DNA (pre-incubated with the Rh-complex) is captured by the α HL pore using a variable voltage protocol and expelled by reversing the polarity of the voltage. **B** The same pore as shown in panel A. A new piece of DNA is immobilised in the pore. The beginning of the continuous UV irradiation is shown by the sun cartoon. **C** Magnification of the recording trace shown in panel B, immediately before the cleavage event.

min of irradiation. The mean time elapsed for the pores that did cleave was 14.0 ± 10.8 min ($n = 4$), however, the times were widely distributed (table 5.1).

The results of the single channel recording imply that the dsDNA segment was

pore number	time elapsed between irradiation onset and the return to the open pore level (min)
1	26.5
2	9.6
3	18.4
4	no change within 40 min
5	1.6

Table 5.1: *Time elapsed between irradiation of the Rh-DNA complex in the pore and the cleavage of the DNA*

cleaved by the Rh-complex on irradiation with UV light. The resulting shortened duplex ruptured under the applied potential, causing the DNA to translocate through the pore. The times elapsed between the onset of the irradiation and the return to the open pore level are consistent with the bulk experiments carried out previously (Figure 5.7).

5.4 Conclusions and future directions

The preliminary results presented in this chapter suggest that DNA photocleavage by a Rh-complex can be carried out in an α HL pore, and that the time taken for a single reaction to occur can be measured.

Further experiments are required to confirm the proposed DNA cleavage in the α HL pore: First, it needs to be established that the DNA remains stably in the pore when irradiated with UV light in the absence of the Rh-complex. Second, the stability of DNA duplex in the pore needs to be investigated more systematically. Specifically it needs to be shown that a 14 nucleotide dsDNA segment is insufficient to immobilise the DNA in the pore under an applied potential and will lead to rupture of the strands. Third, the cleavage event is implied from a jump of the single channel current from the level of the pore blocked by the oligonucleotide to the level of

the open pore. It is assumed that the long ssDNA fragment translocates through the pore, but the electrical signal cannot distinguish between this mechanism and a mechanism in which the oligonucleotide escapes to the cis-side (possibly without cleavage). Although the control experiments exclude the latter mechanism, it would be good to prove the former. This could be achieved by replacing the poly(A) overhang with a different type of biopolymer (Figure 5.12), that results in a lesser block of the single channel current. Both hexaethylene glycol phosphate oligomers [18] and abasic (1',2'-dideoxy) residue oligomers [21] result in a smaller block of the single channel current than regular oligonucleotides and could be used for this purpose. On translocation of the mixed polymer, two types of signal would be

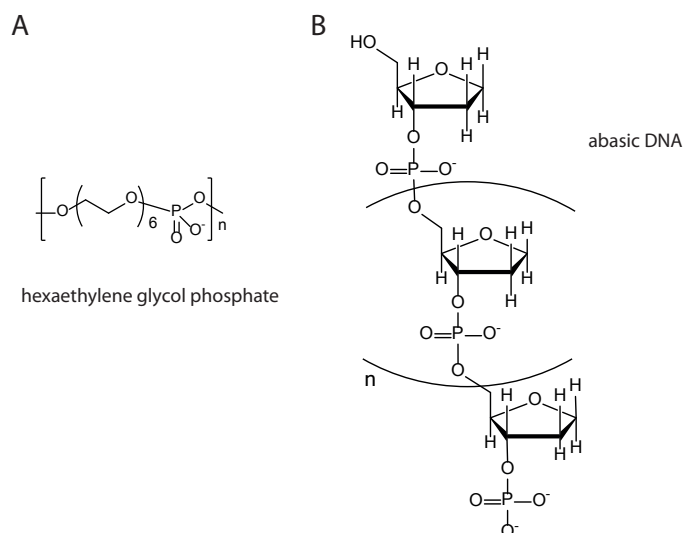


Figure 5.12: Structure of **A** poly (hexaethylene glycol phosphate) and **B** abasic DNA.

observed: Initially a smaller block corresponding to translocation of the biopolymer segment, followed by a larger block that corresponds to the translocation of the DNA segment through the β -barrel. Finally, the rate of reaction could be accelerated by using a more powerful light source, e.g. a 1000 W Xe arc lamp or a solar simulator, allowing a larger number of cleavage events to be analysed.

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Chapter 6

Perspectives

The work described in this thesis could be expanded to address more complex problems at the single molecule level. For example, it may be possible to develop the PPIDA pore described in chapter 2 into a more sophisticated tool. When a divalent metal ion binds to the single half-chelator in the PPIDA pore, three vacant coordination sites remain on the metal ion. As mentioned previously, these sites are usually occupied by water or chloride ligands, the exchange rates of which are too fast to detect by single channel recording. However, the vacant coordination sites on the metal ion can also be occupied by a secondary ligand. This can be a second half-chelator ligand, as was shown with the $P(PIDA)_2$ pore in chapter 3, but in principle it should be possible to add a secondary ligand to the recording buffer and to detect its interaction with the metal ion on the half-chelator in the pore. This idea and some potential applications are explored below.

Transition metal complexes play a prominent role in catalysis, for example they can act as a Lewis acid catalyst in Diels-Alder (DA) reactions. A DA reaction is a [4+2] cycloaddition between a substituted alkene and a conjugated diene, which proceeds through a single transition state [1]. When the diene is cyclic and the alkene is asymmetric, there are two possible ways in which addition can occur: the larger side of the alkene may be under the ring (endo addition), or over the ring (exo addition, Figure 6.1A). Lewis acids accelerate the rate of DA reactions, because

they act as an electron withdrawing group on the alkene, thereby making it more susceptible to attack by the conjugated diene [1].

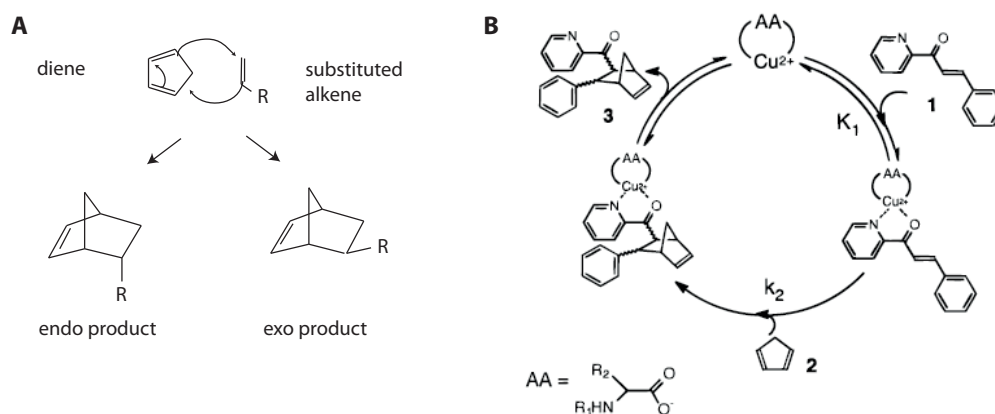


Figure 6.1: Mechanism of a Diels-Alder reaction. **A** A Diels-Alder reaction is a $[4+2]$ cycloaddition that proceeds through a single transition state. The endo and exo product of the reaction are shown. **B** Catalytic cycle of a Diels-Alder reaction catalysed by a Cu²⁺-amino acid chelate. Panel adapted from [2].

Otto et al. carried out a DA reaction catalysed by a Cu²⁺-amino acid chelate in water, which was found to proceed with a second order rate constant of $\sim 1 \text{ M}^{-1}\text{s}^{-1}$ [2] (Figure 6.1B). The reaction conditions suggest that it may be possible to follow the course of a DA reaction on a half-chelator in the α HL pore by single channel recording. By adding one reagent (as well as the Cu²⁺ ion) to one side of the recording chamber and the second reagent to the other side of the recording chamber, the reaction could only proceed when the reagents come into contact inside the pore. As well as being able to monitor this reaction in single time and at the single molecule level, it would be interesting to see if the asymmetric environment of the pore would drive the enantioselectivity of the reaction. For example, when a DA reaction was carried out on a Cu²⁺ complex that had been intercalated in a DNA helix, the chiral environment of the DNA helix strongly favoured the formation of the endo over the exo product (endo:exo = 98:2) [3]. The same may be true for DA reactions carried out in the α HL pore.

A second important type of ligand that is known to form complexes with divalent metal ions (especially Cu²⁺) are the amino acid neurotransmitters released in the

nervous system and at neuromuscular junctions (NMJs) [4]. The sympathetic NMJ is the point of communication between a neuron in the sympathetic division of the nervous system and a smooth muscle cell. Signals generated at the sympathetic NMJ drive smooth muscle contraction, which is involved in processes such as the contraction of blood vessels and dilation of the eye pupil [5]. When an action potential arrives at the nerve terminal of a sympathetic neuron, the depolarisation activates voltage-gated Ca^{2+} -channels, leading to the influx of Ca^{2+} from the extracellular fluid into the nerve terminal. The rise in Ca^{2+} concentration then triggers the fusion of intracellular vesicles containing noradrenaline (NA) with the cell membrane. Vesicular release of NA can also be triggered pharmacologically in some tissues by exposure to epibatidine [6]. NA is released into the extracellular space that separates the neuron and the smooth muscle cell, and binds to G-protein coupled receptors (GPCRs) embedded in the smooth muscle cell membrane. NA binding to the GPCR triggers a cellular signalling cascade that results in the release of Ca^{2+} from intracellular stores, which in turn leads to contraction of the smooth muscle cell (Figure 6.2A).

The characterisation of the NMJ continues to be of strong scientific interest, however, at present there is no established technique to study the amount of neurotransmitter released from vesicles directly. Instead, researchers infer release by measuring Ca^{2+} transients in neurons or smooth muscle cells by using fluorescent indicators [8]. It is also possible to estimate the number of vesicles that have fused with the neuronal membrane by taking capacitance measurement of the membrane [9]. A probe containing the PPIDA pore that is brought into the immediate vicinity of the NMJ could afford the detection of NA directly. Such a probe might consist of a patch-clamp pipette, over whose minute ($\sim 10\text{ }\mu\text{m}$) opening a bilayer is formed and into which an αHL pore is inserted. Such pipettes have been described by Heitz et al. [7] (Figure 6.2B) and furthermore, the design described by the authors uses polymerisable lipids that enhance the stability of the probe. The patch pipette would be filled with recording buffer containing Cu^{2+} to allow the formation of the

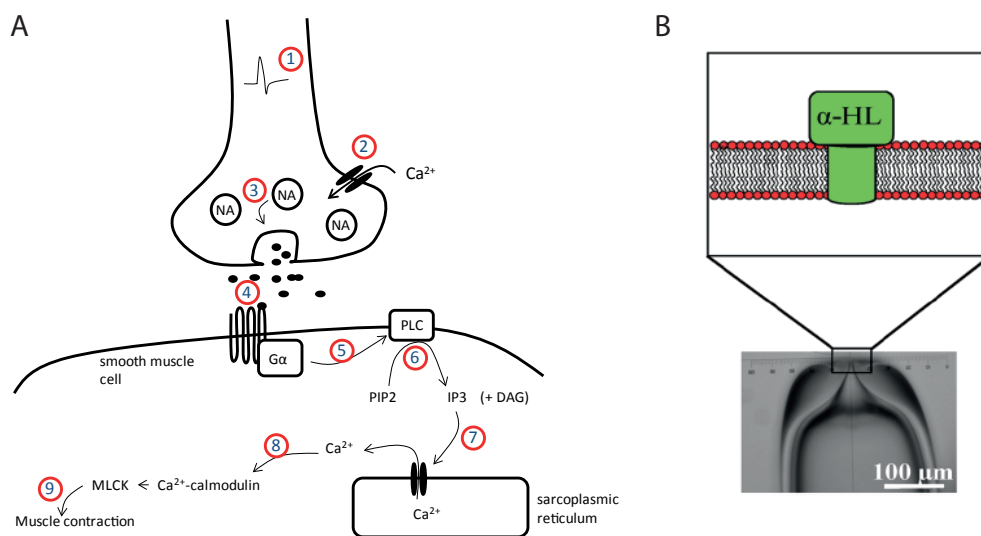


Figure 6.2: **A** Steps leading to smooth muscle contraction at the neuromuscular junction. 1. An action potential arrives at a nerve terminal. 2. Voltage-gated Ca^{2+} channels open, leading to the influx of Ca^{2+} from the extracellular fluid. 3. The increased Ca^{2+} concentration triggers the fusion of neurotransmitter vesicles with the membrane. 4. Neurotransmitters are released into the neuromuscular junction, diffuse and bind to G-protein coupled receptors (GPCRs) on the smooth muscle membrane. 5. Neurotransmitter binding to the GPCR activates the associated G-protein, which in turn activates the enzyme phospholipase C (PLC). 6. PLC catalyzes the conversion of phosphatidylinositol 4,5-bisphosphate (PIP2) to inositol 1,4,5-triphosphate (IP3) and diacylglycerol (DAG). 7. IP3 binds to IP3-activated Ca^{2+} -channels on the sarcoplasmic reticulum, leading to an increase in intracellular $[\text{Ca}^{2+}]$. 8. Ca^{2+} activates the Ca^{2+} /calmodulin kinase, which in turn activates myosin light chain kinase (MLCK). 9. MLCK phosphorylates the myosin light chain of the muscle fibre, allowing it to crosslink with actin filaments, which results in muscle contraction. **B** Schematic of an ion channel functionalised bilayer that is suspended across the opening of a micropipette [7].

half-chelator. Cu^{2+} complex. Continuous neurotransmitter release would be evoked by bath application of epibatine and the amount of NA released could be measured directly. This technique may overcome some of the limitations presented by other techniques.

A final further application of the work described in this thesis is the creation of a “molecular walker” in the pore. Synthetic molecular walkers have received much attention in recent years, and they have been developed with the aim of creating molecular machines that can transport nanoscale cargoes along a track. For example, Yin and colleagues constructed a DNA motor that moved along a DNA track [10]. The track contained three anchorages at which the walker, a six-nucleotide DNA

fragment, could be bound. At each step the walker was ligated to the next anchorage, then cut from the previous one by a restriction enzyme. Each cut destroyed the previous restriction site, and each ligation created a new site, in such a way that the walker only moved in one direction. In a second example, von Delius et al. created a two-legged 21-atom molecular walker that moved along a molecular track [11]. A hydrazide group on the walker acted as one foot and a sulfur-based group acted as the other. The molecular track consisted of alternating aldehyde and disulfide groups. By switching between acidic and alkaline conditions, the molecule took steps along the track, which could be followed by NMR spectroscopy.

The chelation experiments of chapters 3 and 4 describe the formation of a full complex between two half-chelator ligands and a single Zn^{2+} ion. In both sets of experiments, the distance between the two modified residues in the β -barrel is roughly 8.5 Å. This suggests that other pairs of residues separated by the same distance could also be functionalised with half-chelator ligands and that the formation of the full complex might be observed. This could be achieved by either modifying subunit monomers or dimers or higher order concatemers. Extending this idea further, it should be possible to build a molecular track of half-chelator ligands in the β -barrel of the α HL pore. A Zn^{2+} ion could "walk" along this track by alternately forming partial and fully chelated complexes with the ligands. This idea is illustrated for three ligands in Figure 6.3. Although a Zn^{2+} ion walking along a track of chelators cannot carry additional cargo, this system could still provide insights into the fundamental behaviour of the walker. The movement of the walker in the pore could be followed in real time and at the single molecule level by single channel recording, and the exact number of step before dissociation could be determined. A further advantage of this experimental system is that the Zn^{2+} ion should walk without the need to switch experimental conditions (eg. the addition of an enzyme or a change of pH). Finally if the chelators are positioned along the length of the protein, then it may be possible to bias the direction of the walk through the applied voltage (Figure 6.4).

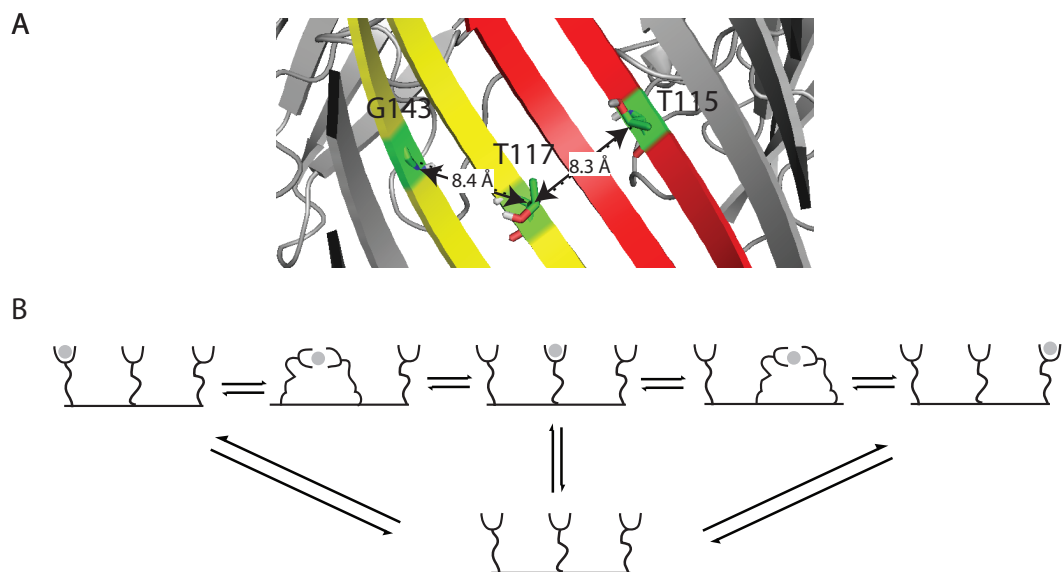


Figure 6.3: A molecular track of half-chelator ligands on a subunit dimer in the α HL pore. **A** Possible sites on a subunit dimer (red and yellow) for the the chemical modification. The C_{α} - C_{α} distances between the residues are approximately 8.5 Å. **B** Scheme illustrating the walk of a Zn^{2+} ion along a track consisting of 3 half-chelator ligands.

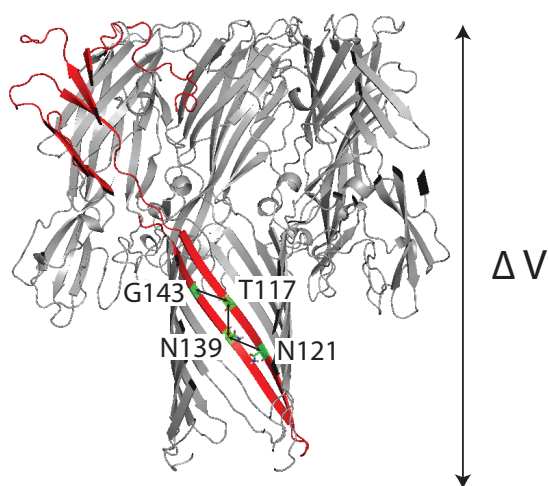


Figure 6.4: A molecular track of four half-chelator ligands on a single subunit in the α HL pore. Possible sites for the the chemical modification with the half-chelator ligand are shown. The C_{α} - C_{α} distances between the residues are all approximately 8.5 Å.

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Chapter 7

Materials and methods

7.1 Molecular biology

7.1.1 Mutant generation

7.1.1.1 Genes and plasmids

All α HL genes were assembled in the pT7-SC1 expression vector [1]. A detailed vector map is given in the appendix, chapter 8.1. The vector contains a T7 promoter and terminator sequence. The α HL gene is flanked by the NdeI and HindIII restriction sites and transcribed under control of the T7 promoter. The pT7-SC1 vector also contains a β -lactamase gene conferring ampicillin/carbenecillin resistance and the pBR322 origin of replication.

7.1.1.2 Site-directed mutagenesis by QuikChange PCR

Single mutations were introduced into the α HL gene by using the QuikChange II XL kit (Stratagene) according to the manufacturer's instructions, using the α HL(RL3) gene [2] in the pT7-SC1 expression vector as a template. The α HL(RL3) gene encodes the wt α HL polypeptide, but contains additional restriction sites.

7.1.1.3 PCR followed by homologous recombination

Dimers of α HL were made by PCR, followed by homologous recombination in *E. coli*. Briefly, two PCR reactions were set up with overlapping primers to generate the two halves of the dimer construct. Each reaction generated one of copy of the α HL gene, the serine glycine linker and half of the vector. The PCR reactions were carried out with the Expand Long Template PCR system (Roche). Each reaction contained DNA template (pT7-SC1 containing a single WT α HL gene, 30 ng), dNTPs (1.75 μ l, 10 mM), primer 1 (1 μ l, 100 μ M, either SC46 or SC47), primer 2 (1 μ l, 100 μ M, either HL_nSG/46 or HL_nSG/47, where n is the number of residues in the linker), reaction buffer 1 (5 μ l), DMSO (3 μ l) and polymerase (1 μ l) made up to a final volume of 50 μ l with water. Thermal cycling was carried out as follows: 1 cycle at 94°C (5 min); 18 cycles at 94°C (50 s), 60°C (50 s), 68°C (9 min); 1 cycle at 68°C (7 min).

7.1.2 DNA preparations

7.1.2.1 Preparation of XL10-Gold competent cells

A small amount of XL10-Gold cells was removed from a frozen aliquot of ultracompetent cells, by scratching a sterile inoculating loop across the surface of the cells. The cells were then streaked out onto LB agar plates containing chloramphenicol (1x) and grown at 37°C for 14 h. A single colony was picked and used to inoculate 100-200 ml of LB medium containing chloramphenicol (1x) and grown to an OD₆₀₀ of 0.5. The cells were then harvested by centrifugation (10 min, 2000 rpm, 4°C) and gently resuspended in 1/10 of their original volume in ice cold TSS buffer. The cells were then rapidly aliquoted into pre-chilled Eppendorf tubes standing in a stand on a mixture of dry ice and ethanol. The cells were stored at -80°C.

- Chloramphenicol (1000x): 34 mg/ml in ethanol
- TSS buffer: 10% (w/v) PEG-8000, 5% (v/v) DMSO, 25 mM MgCl₂ in LB

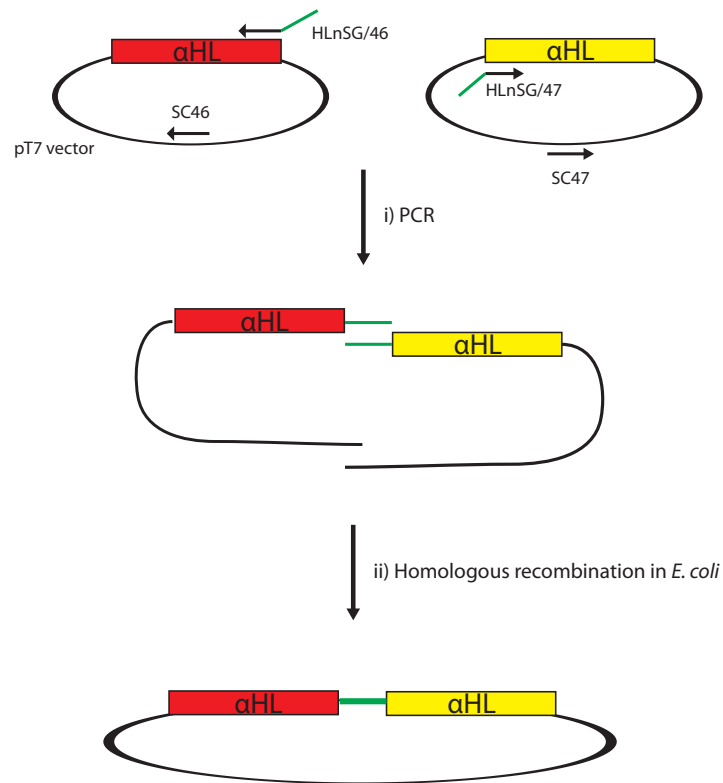


Figure 7.1: PCR and homologous recombination to generate dimers of α HL

medium

- LB medium: 1% (w/v) tryptone, 1% NaCl, 0.5% yeast extract
- LB Agar plates: LB medium with 1.5% (w/v) agar

7.1.2.2 Transformation of competent cells

An aliquot of competent cells was thawed on ice. 40 μ l of the cells were mixed with 0.5 μ l of plasmid DNA or up to 3 μ l of PCR reaction products in pre-chilled Eppendorf tubes. The cells were incubated on ice for 20 min, heat shocked at 42°C for 45 s and then chilled on ice for a further 2 min before being spread onto plates containing ampicillin (1x) or carbenicillin (1x). The cells were grown at 37°C overnight.

- Ampicillin (1000x): 50 mg/ml in H₂O
- Carbenicillin (1000x): 50 mg/ml in 1:1 (v/v) ethanol: H₂O
- LB Agar plates: 1% (w/v) tryptone, 1% NaCl, 0.5% Yeast Extract, 1.5% (w/v) agar

7.1.2.3 Small scale plasmid preparation (Mini Prep)

A single colony of freshly transformed XL10-Gold cells containing the plasmid of interest was used to inoculate 5 ml of LB medium supplemented with ampicillin (1x) or carbenicillin (1x) in a 15 ml plastic tube. The tubes were incubated at 37°C overnight with shaking at 200 rpm. The bacteria were then harvested by centrifugation (3800 rpm, 10 min, 4°C). The DNA was extracted from the bacterial pellet using a plasmid purification kit (Qiagen, Miniprep kit) according to the manufacturer's instructions.

- Ampicillin (1000x): 50 mg/ml in H₂O
- Carbenicillin (1000x): 50 mg/ml in 1:1 (v/v) ethanol: H₂O
- LB medium: 1% (w/v) tryptone, 1% NaCl, 0.5% Yeast Extract

7.1.2.4 Large scale plasmid preparation (Maxi Prep)

A single colony of freshly transformed XL10-Gold cells containing the plasmid of interest was used to inoculate 100 ml of LB medium supplemented with either ampicillin (1x) or carbenicillin (1x) in a 500 ml baffled flask. The culture was grown at 37°C overnight, with shaking at 200 rpm. The bacteria were harvested by centrifugation (3800 rpm, 20 min, 4°C). The DNA was extracted from the bacterial pellet using a plasmid purification kit (Qiagen, Maxiprep kit) according to the manufacturer's instructions. Following the final precipitation step, the DNA was redissolved in TE buffer to a final concentration of 400 ng/μl.

- Ampicillin (1000x): 50 mg/ml in H₂O

- Carbenicillin (1000x): 50 mg/ml in 1:1 (v/v) ethanol:H₂O
- LB medium: 1% (w/v) tryptone, 1% NaCl, 0.5% Yeast Extract
- TE buffer: 10 mM Tris.HCl, 1 mM EDTA, pH adjusted to 8.0

7.1.2.5 DNA quantification

The concentration of DNA samples was determined by measuring the absorbance of a 2 μ l sample at 260 nm on a Nanodrop spectrophotometer (Thermo Scientific).

7.1.3 DNA analysis

7.1.3.1 Agarose gel electrophoresis

Powdered agarose was combined with TAE buffer (1x) to give a 0.8% solution and boiled to dissolve the solids. After cooling to about 50°C, ethidium bromide was added to a final concentration of 0.5 μ g/ml. The gel was cast in an appropriate chamber (Bio-Rad). A 5 μ l sample of plasmid DNA or PCR product was mixed with 1 μ l nucleic acid sample buffer (6x, Bio-Rad) and loaded to the wells of the gel. Electrophoresis was carried out in TAE buffer at 100-120 V for 20-30 min. The DNA was visualised and imaged under UV illumination on a gel imaging system (Gel Doc, Bio-Rad).

- TAE buffer (50x): 40 mM Tris.HCl pH 8.0, 20 mM acetic acid, 1 mM EDTA

7.1.3.2 DNA photocleavage by [Rh(bpy)₂(phzi)]³⁺

Oligonucleotides were purchased from ATD Bio (University of Southampton). The synthesis scale was 0.2 μ M and the oligonucleotides had been purified by HPLC by ATD Bio. The oligonucleotides typically had concentrations between 12-40 μ M. For the photocleavage experiments, an equimolar amount of complementary (except for a single CC mismatch) oligonucleotides were mixed in an Eppendorf tube and heated to 90°C for 10 min on a heating block. The heating block was then switched off and the contents of the tube were allowed to slowly cool to RT, allowing the

oligonucleotides to anneal to give short segments dsDNA. Rh(bpy)₂(phzi) was made up as a twice concentrated solution in H₂O (e.g. if the concentration of the duplex DNA was 15 μ M, the Rh complex was made up to 30 μ M). This was necessary as the Rh complex is chiral, and only half of the racemic mixture was expected to be active. 10-20 μ l of the DNA duplex were mixed with an equal volume of the Rh complex solution and incubated at RT for 15 min. Irradiation (5-30 min) was carried out under a 300 W Xe arc lamp that was equipped with a condenser, an IR water filter (refrigerated by a circulating bath), a 280-370 nm coloured glass filter (Oriel #59154), a fibre optic focusing assembly and a fibre optic bundle. Control reactions that lacked either the Rh complex or the UV irradiation were also carried out. Following the irradiation, the oligonucleotide fragments were analysed on urea-polyacrylamide gels.

7.1.3.3 Polyacrylamide gel electrophoresis

20% polyacrylamide gels were prepared by mixing the gel ingredients in a 50 ml plastic tube and gently heating the tube in a hot water bath. The tube was vortexed from time to time until all the solids had dissolved. The gel was cast between glass plates (mini Protean system, Bio-Rad) and a comb was inserted. 10-20 μ l of oligonucleotide solution (10-40 μ M) were mixed with an equal volume of TBE-urea sample buffer (2x, Bio-Rad) and heated to 60°C for 10 min, before being loaded to the wells of the gel. Electrophoresis was carried out in TBE buffer (1x) at 200 V for 60 min. The gel was then stained with Stains All (Sigma Aldrich) solution for 10 min and destained in water overnight. Both the staining and the destaining step were performed in the dark.

- Urea-polyacrylamide gel: (for one 1.5 mM mini Protean gel) 4.8 g urea, 6.7 ml of 30% acrylamide/bis 37.5:1, 0.2 ml of 10% w/v ammonium persulfate, 1 ml TBE buffer (10x), 5 μ l TEMED
- TBE buffer (10x): 89 mM Tris, 89 mM boric acid, 2 mM EDTA, pH adjusted

to 8.4

- Stains All solution: 0.2% (w/v) Stains All in 1:1 formaldehyde:water

7.1.3.4 DNA restriction digests

0.5-2.0 μg of plasmid DNA were digested with 1-5 units of restriction endonuclease (NEB) for 1 h. The reaction volume was 20 μl and the reaction buffer and temperature were selected according to the manufacturer's recommendations. The restriction fragments were separated by agarose gel electrophoresis.

7.1.3.5 DNA sequencing

The sequences of all genes were verified by DNA sequencing at the Source BioScience LifeSciences facility (Department of Biochemistry, University of Oxford). The genes described in this thesis are carried in the pT7 expression vector, which contains a T7 promoter and terminator sequence. DNA sequencing was therefore carried out with the T7F and T7R primers, which anneal to these sequences.

7.1.4 Protein expression by coupled in-vitro transcription and translation

Protein expression by coupled in vitro transcription and translation (IVTT) was carried out by using an *E. coli* T7-S30 expression system for circular DNA (Promega). The amino acid mixture minus methionine and the amino acid mixture minus cysteine (both 1 mM) were mixed in equal volumes to give a working mixture of all twenty amino acids. The T7-S30 extract was supplemented with rifampicin (1 μl of 500 $\mu\text{g}/\text{ml}$ rifampicin per 150 μl) to inhibit transcription by the endogenous *E. coli* RNA polymerase. For a 25 μl reaction, premix solution (10 μl) was combined with the complete amino acid mixture (2.5 μl), plasmid DNA (4 μl , 400 ng/ μl), T7 S30 extract (7.5 μl) and [^{35}S]L-methionine (1 μl , MP Biomedicals, 1174 Ci/mmol, 10 mCi/ml). The reaction was incubated at 37°C for 60 to 90 min and then centrifuged

for 8 min at 4°C at 25,000 x g to pellet any insoluble proteins. The supernatant was removed and stored at -80°C.

7.1.5 Protein chemical modification

α HL polypeptides containing one or more mutant cysteine residues were prepared by IVTT. The monomeric protein also contained a C-terminal D8 extension, to allow for the separation of hetero-oligomers at a later stage. 25 μ l of the freshly prepared IVTT protein was subjected to gel filtration in 200 mM Tris.HCl, pH 8.0, on a Micro Bio-Spin 6 gel filtration column (Bio-Rad). The protein in the eluent was reduced for 10 min in 200 mM Tris, pH 8.0, containing 1 mM DTT (67.5 μ L total volume). 2,2'-(3-(2-Iodoacetamido)propylazanediyl)diacetic acid (half-chelator, 7.5 μ L, 200 mM stock in water) was added and the solution was incubated at room temperature for 1 h in the dark. In control experiments, the same volume of water (7.5 μ L) or 100 mM mPEG-OPSS-5000 (Nektar Transforming Therapeutics) was added to the IVTT protein instead of the half-chelator reagent. After the reaction, gel filtration was performed with Micro Bio-Spin 6 columns. A portion (2 μ L) of each eluate was analysed by SDS-PAGE. The remainder of the reaction mix was stored at -80°C.

7.1.6 Analysis of monomers of α HL by SDS-polyacrylamide electrophoresis

0.5 μ l of radiolabelled IVTT protein was made up with sample buffer (XT, Bio-Rad) to a final volume of 20 μ l and heated to 90°C for 10 min. The protein was then applied to the wells of a 10% or 12% Bis-Tris gel (Bio-Rad). Electrophoresis was carried out in MOPS running buffer (Bio-Rad) at 200 V for 60 min. The gel was washed with water for 15 min to remove any unincorporated ³⁵S-methionine, dried onto Whatman paper (3 MM Chr) and an autoradiograph or phosphorimage was obtained.

7.1.7 Haemolytic activity assays

Monomers or dimers of α HL (5 μ l, 100 to 400 ng/ μ l) were prepared by IVTT, diluted with MBSA (95 μ l) and then subjected to twofold serial dilutions across the 12-well row of a microtitre plate, such that the final volume in each well was 50 μ l. An equal volume of 1% washed rabbit erythrocytes in MBSA was quickly added to each well, starting with the most dilute sample. Haemolytic activity was recorded for 1h by monitoring the decrease in light scattering at 595 nm with a Bio-Rad microplate spectrophotometer, using the Microplate Manager 5.2 software.

- MBSA buffer: 10 mM MOPS pH 7.4, 150 mM NaCl, 1 mg/ml bovine serum albumin

7.1.8 Oligomer formation on rabbit red blood cell membranes

20 to 100 μ l of IVTT protein was mixed with 5 μ l of rabbit red blood cell membranes (rRBCMs, a gift from E. Mikhailova, $\sim$ 4 mg protein/ml) in MBSA buffer. For homo-oligomers, only one type of IVTT protein (eg wt α HL) was mixed with rRBCMs. For hetero-oligomers, different ratios of protein containing the mutation/modification and the C-terminal D8 extension were co-oligomerised with wt protein on rRBCMs. In both cases oligomer formation proceeded over 1 h at 37°C. The membranes containing the assembled oligomers were recovered by centrifugation at 25,000 x g for 8 min.

- MBSA buffer: 10 mM MOPS pH 7.4, 150 mM NaCl, 1 mg/ml bovine serum albumin

7.1.9 Separation of heteroheptamers of α HL by SDS-PAGE

The rRBCM pellet containing the assembled heptamers was solubilised in 30 μ L Laemmli sample buffer (1x) without heating and loaded to one lane of a 5% SDS-polyacrylamide gel. The gel was run in TGS running buffer at 50 V overnight. The D8, -(Asp)₈, tail allows the separation of heptamers with different combinations of

subunits [3]. The gel was dried under vacuum onto Whatman 3MM Chr paper and exposed to X-ray film for 3 h. The band that corresponded to the (WT)₆(Mutant-D8)₁ heteroheptamer was excised [4]. The gel was hydrated in 300 μ l TE buffer and the paper was removed. The gel was then crushed with a plastic pestle and the resulting suspension was incubated at room temperature for 30 min. The material was filtered through a 0.2 μ m cellulose acetate filter (Rainin) at 16,000 x g for 30 min. The filtrate was aliquoted and stored at -80°C.

- Laemmli sample buffer (2x): 125 mM Tris.HCl, pH 6.8, 4.6% SDS, 20% glycerol, 0.02% bromophenol blue
- 5% polyacrylamide gel: Running gel (35 ml): 8.75 ml of 1.5 M Tris.HCl pH 8.0, 5.8 ml of 30% acrylamide/bis 37.5:1, 19.8 ml of water, 105 μ l of 20% (w/v) SDS, 580 μ l of 10% (w/v) ammonium persulfate, 15.5 μ l TEMED
- TGS running buffer: 25 mM Tris.HCl, 192 mM glycine, 0.1% SDS
- TE buffer: 10 mM Tris.HCl, 1 mM EDTA, pH 8.0

7.1.10 Limited proteolysis assays

Proteinase K (Sigma Aldrich) was dissolved in water (5 mg/ml) and used immediately. Limited proteolysis was performed on gel-purified oligomers. The enzyme (2 μ l, or water for the control reactions) was mixed with the protein (18 μ l in 20 mM Tris.HCl, pH 8.0) and incubated at 21°C for 6 min. The reaction was quenched by the addition of phenylmethylsulfonyl fluoride (2 μ l, 100 mM in isopropanol). The oligomers were mixed with Laemmli sample buffer (1x final concentration) and a portion was heated to 95°C for 10 min before electrophoresis in a 10% Bis-Tris polyacrylamide gel (Bio-Rad) in MOPS running buffer (Bio-Rad).

7.2 Molecular Modelling

A model of the modified α HL pore was created from the X-ray crystal structure (PDB code 7AHL) in PyMol (Delano Scientific). The mutant T117C had been used previously to observe covalent bond forming reactions at the single molecule level [4], so we reasoned that this position would also be suitable for the observation of complex formation between the half-chelator and a divalent metal ion. Fusing one of the half-chelator ligands to residue 117 positioned the second ligand favorably for connection with residue 143 on the same subunit, which was therefore chosen as the second site for mutagenesis and subsequent chemical modification. The PyMol Mutagenesis Wizard was used to introduce cysteine residues at positions 117 and 143 on one of the seven subunits of the pore. The ligand/metal ion complex was generated from the crystal structure of (bis(iminodiaceto-N,O,O)Ni(II) [5], onto which the remainder of the ligand was built by using the PyMol Build tool. The ligand/metal ion complex was then fused onto the cysteine residues of the α HL heteroheptamer.

7.3 Chemistry

7.3.1 Synthesis of the half-chelator ligand

The chemical syntheses described in this subsection were initially carried out by Seong-Ho Shin, University of Oxford. AFH produced subsequent batches of the half-chelator.

7.3.1.1 Synthesis of *tert*-Butyl 2,2'-(3-azidopropylazanediyl)diacetate (2)

3-Azido-propylamine (**1**) [6] (710 mg, 7.09 mmol) and *tert*-butyl bromoacetate (2.4 ml, 16 mmol) were added to a suspension of sodium bicarbonate (2978 mg, 35.5 mmol) in acetonitrile (70 ml). The reaction was stirred for 27 h at room temperature. The solvent was evaporated and the residue was diluted with water (20 ml). The

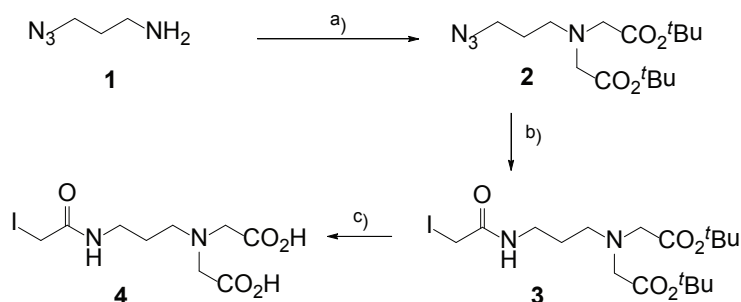


Figure 7.2: Synthesis of the half-chelator ligand. a) *tert*-butylbromoacetate, $\text{NaHCO}_3/\text{CH}_3\text{CN}$, RT, 27 h; b) PPh_3 , $\text{H}_2\text{O}/\text{CH}_3\text{CN}$, 50° C, 5 h, then iodoacetic acid *N*-hydroxysuccinimide ester, RT, 2 h; c) TFA, CH_2Cl_2 , 0° C, 20 h.

product was extracted from the aqueous layer with diethyl ether (2 x 30 ml). The organic extract was dried with anhydrous MgSO_4 and concentrated. Purification was carried out by silica gel flash chromatography, eluting with hexanes: diethyl ether (5:1). The product was obtained as colorless oil (1.41 g, 61%). $R_f = 0.2$ (hexanes: diethyl ether, 5:1), ^1H NMR (200 MHz, CDCl_3) δ 3.42 (br, 4H), 3.39 (m, 2H), 2.79 (m, 2H), 1.73 (m, 2H), 1.46 (br, 18H).

7.3.1.2 *tert*-Butyl 2,2'-(3-(2-iodoacetamido)propylazanediyl)diacetate (3)

Triphenylphosphine (452 mg, 1.72 mmol) and water (200 μL) were added to a solution of *tert*-butyl 2,2'-(3-azidopropylazanediyl)diacetate (515 mg, 1.57 mmol) in acetonitrile (10.5 ml). The solution was heated at 50° C for 5 h. The water was then removed by adding anhydrous MgSO_4 and filtering. Iodoacetic acid *N*-hydroxysuccinimide ester (422 mg, 1.50 mmol) was added to the filtrate, and the mixture was stirred for 2 h at room temperature. The solution was concentrated and purified by silica gel flash chromatography, eluting with hexanes: ethyl acetate (3:2) to yield a pale yellow oil (471 mg, 67%). $R_f = 0.45$ (hexanes: ethyl acetate, 1:1), ^1H NMR (200 MHz, CDCl_3) δ 8.41 (N-H, br, 1H), 3.75 (s, 2H), 3.44 (m, 2H), 3.38 (s, 4H), 2.77 (t, 2H), 1.62 (m, 2H), 1.47 (s, 18H).

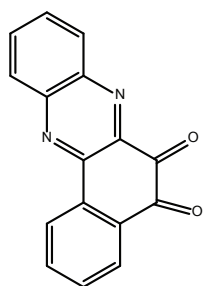
7.3.1.3 2,2'-(3-(2-Iodoacetamido)propylazanediyl)diacetic acid (half-chelator, 4)

tert-Butyl 2,2'-(3-(2-iodoacetamido)propylazanediyl)diacetate (307 mg, 0.65 mmol) was dissolved in methylene chloride (6.5 ml) and trifluoroacetic acid (223 μ L, 3.26 mmol) was added at 0°C. After 20 h at room temperature, the same amount of trifluoroacetic acid was added, again at 0°C. After 7 h, the solvent was removed and the product was used for protein modification without further purification. ^1H NMR (200 MHz, 100 mM Na phosphate, pD 7.8 in D_2O), δ 3.81 (s, 4H), 3.71 (s, 2H), 3.27 (m, 4H), 1.93 (m, 2H); HRMS (ESI): Calcd. for $\text{C}_9\text{H}_{14}\text{IN}_2\text{O}_5$ $[\text{M}-\text{H}]^-$ 356.9947, found 356.9951.

7.3.2 Synthesis of $[\text{Rh}(\text{phzi})_1(\text{bpy})_2]\text{Cl}_3$

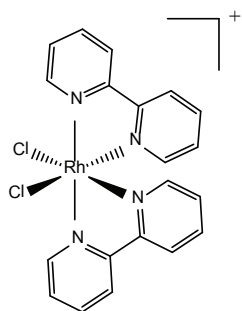
The DNA mismatch inserter $\text{Rh}(\text{phzi})_1(\text{bpy})_2^{3+}$ was synthesised according to a published protocol [7].

7.3.2.1 Synthesis of 3,4- benzo[a]phenanzine quinone



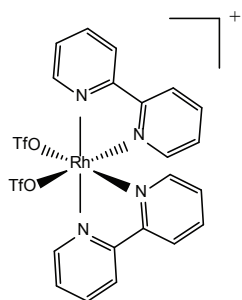
2.5g (20 mmol) 2,3-dichloro-1,4-naphthoquinone and 2.0 g (20 mmol) *o*-phenylene diamine were dissolved in 150 ml pyridine and boiled under reflux for 1 h. After cooling, filtration of the solution through a glass filter afforded a red-brown solid (70% yield). 4.51 g of this product were combined with 10 ml glacial acetic acid, 1 ml nitric acid (70%) and 3 ml ddH_2O and boiled under reflux for 1 h. After cooling, filtration afforded a yellow-orange solid, that was washed with 15 ml of EtOH and then 15 ml of diethyl ether. The product was dried under vacuum. Yield 42%. ^1H NMR (400 MHz, dichloromethane, D_2), δ 8.8 (d, 1H), 8.3-8.1 (m, 3H), 8.1-7.8 (m, 3H), 7.7 (t, 1H).

7.3.2.2 Synthesis of $[\text{Rh}(\text{bpy})_2\text{Cl}_2]\text{Cl}$



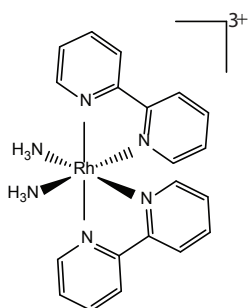
320 mg RhCl_3 (1.4 mmol) and 25 mg hydrazine monohydrochloride were dissolved in 6.25 ml ddH₂O. To this, a solution of 425 mg (2.3 mmol) 2,2'-bipyridyl in 10 ml ethanol was added. The reaction was frozen in liquid N₂ and was deoxygenised by the repeated application of vacuum, followed by backfilling with nitrogen gas. The reaction was then boiled under reflux for 30 min. The reaction was cooled and stored at 4°C overnight to promote crystallisation. The reaction was centrifuged (3800 rpm, 30 min, 4°C) to compact the very fine crystals and the solvent was decanted. The product was dried under vacuum. Yield 47%. ¹H NMR (400 MHz, in DMSO, D₆): δ 9.70 (d, 2H), 9.0 (d, 2H), 8.9 (d, 2H), 8.6 (t of d, 2H), 8.3 (t of d, 2H), 8.15 (m, 2H), 7.8 (d, 2H), 7.6 (m, 2H).

7.3.2.3 Synthesis of $[\text{Rh}(\text{bpy})_2(\text{OTf})_2]\text{OTf}$



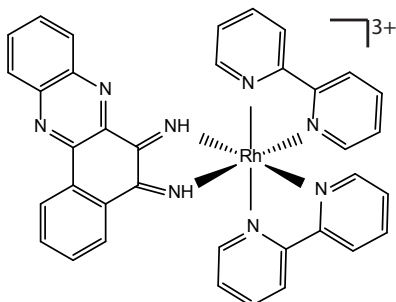
This reaction was carried under an Ar atmosphere, using dry glassware. 320 mg (0.64 mmol) $[\text{Rh}(\text{bpy})_2\text{Cl}_2]\text{Cl}$ was combined with 3.2 g (=1.86 ml) triflic acid and stirred at RT for 6 h. 300 ml of rapidly stirring diethyl ether were cooled in an acetone/dry ice bath. The reaction product was added dropwise to the diethyl ether and a yellow-white solid precipitated. The diethyl ether was allowed to warm to RT and the yellow product was removed by filtration through filter paper.

7.3.2.4 Synthesis of $[\text{Rh}(\text{bpy})_2(\text{NH}_3)_2][\text{OTf}]_3$



500 mg (0.6 mmol) of $[\text{Rh}(\text{bpy})_2(\text{OTf})_2]\text{OTf}$ were combined with 50 ml NH_4OH and boiled under reflux for 1 h. The solvent was then removed on a rotary evaporator and the product dried under vacuum. Yield 80%. ^1H NMR (400 MHz, in acetone, D_6): δ 9.42 (d, 2H), 9.04 (d, 2H), 8.88 (d, 2H), 8.72 (t, 2H), 8.39 (t, 2H), 8.22 (t, 2H), 7.95 (d, 2H), 7.68 (t, 2H), 5.06 (br, 6H).

7.3.2.5 Synthesis of $[\text{Rh}(\text{bpy})_2(\text{phzi})][\text{Cl}_3]$



100 mg (0.1 mmol) $[\text{Rh}(\text{bpy})_2(\text{NH}_3)_2][\text{OTf}]_3$ were combined with 35 mg (0.125 mmol) 3,4-benzo[*a*]phenazine quinone in 50 ml MeCN and stirred rapidly at RT. 2 ml of 0.4 M NaOH were added and the reaction was allowed to stir for 3 h at RT. The reaction was stopped by adding 2 ml of 0.4 M HCl. The solvent was removed by rotary evaporation.

7.3.2.6 Purification of $[\text{Rh}(\text{bpy})_2(\text{phzi})][\text{Cl}]_3$ by cation exchange chromatography

20 g of Sephadex SP-C25 resin were equilibrated with 0.05 M MgCl_2 (500 ml) used to fill a glass column. The column was then washed with H_2O (500 ml). $[\text{Rh}(\text{bpy})_2(\text{phzi})][\text{Cl}_3]$ was dissolved in a small amount of H_2O and applied to the top of the column, which was then washed with H_2O (500 ml). The complex

was eluted with MgCl_2 solution, which was applied in 500 ml batches of increasing concentration. The starting concentration of MgCl_2 was 0.01 M, which was increased by 0.01 M for each 500 ml batch. The green product started to elute at 0.08 M MgCl_2 and was collected in 100-250 ml fractions, which were analysed by UV-Vis spectroscopy. For all fractions λ_{max} (observed)= 305 nm, 314 nm, 343 nm. [λ_{max} (expected)= 304 nm, 314 nm, 343 nm [8]]. The total column eluent (approximately 1300 ml) was then concentrated on a reversed phase column (Waters, 2g, C18 Sep Pak) that had been equilibrated with MeOH. The column was then washed with H_2O to flush out the MgCl_2 . The product was eluted with approximately 12 ml of (1: 1: 0.001) H_2O : MeCN: TFA. Following elution, the solvents were removed by rotary evaporation and the product was dried under vacuum. Yield 20%. λ_{max} (observed) 305 nm, 314 nm, 343 nm. [λ_{max} (expected) 304 nm, 314 nm, 343 nm]. ESI-MS (m/z): $[\text{M}+2\text{H}^+]$ found 671 (expected 671).

7.4 Single channel recordings

The αHL pores described in this thesis were characterised by single channel recording in folded planar lipid bilayers. In these experiments a single αHL pore was reconstituted into an artificial lipid bilayer, and the single channel current under an applied voltage was measured.

7.4.1 Instrumentation overview

An overview of the experimental setup is given in Figure 7.3, top panel.

Electrical measurements were made with an Axopatch 200B patch clamp amplifier (Molecular Devices). The amplifier headstage was connected to the recording chamber by a set of Ag/AgCl electrodes within in a grounded Faraday cage to shield the set-up from electrical noise. The experiments described in this thesis were acquired in the voltage clamp mode, using resistive WHOLE-CELL ($\beta=1$) headstage settings. In voltage clamp mode, the headstage of the amplifier

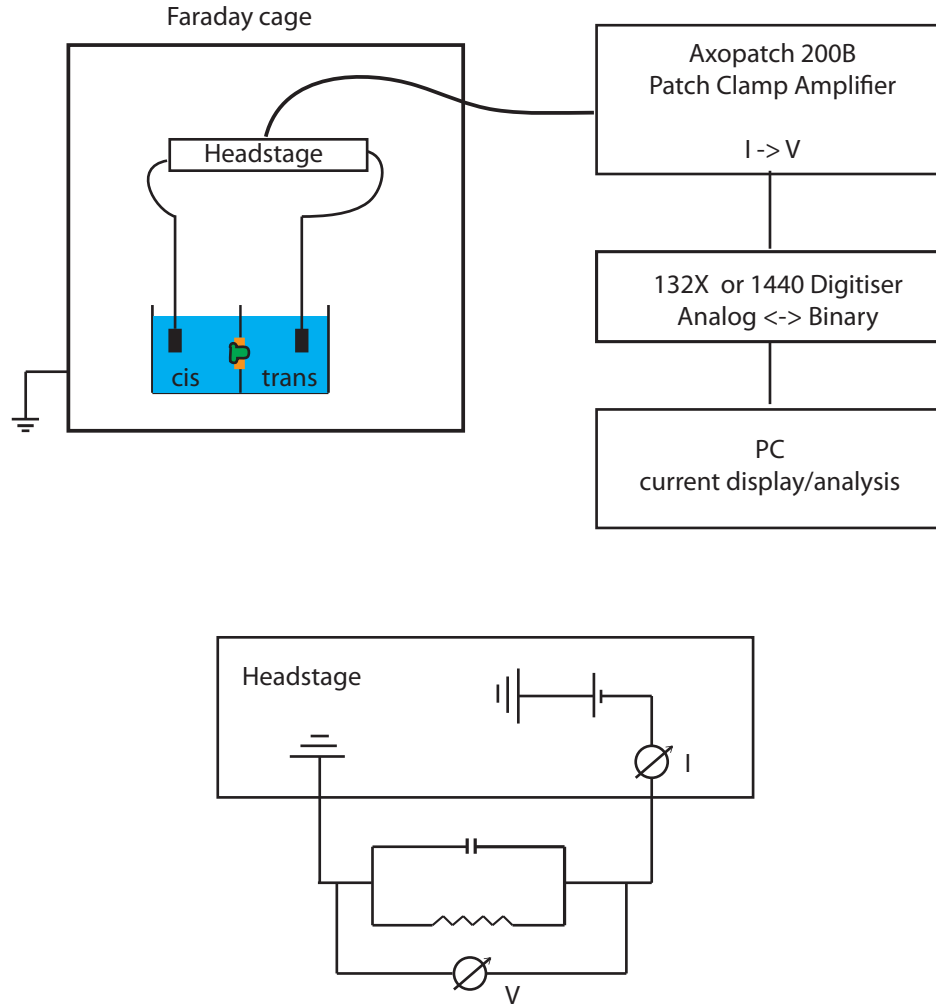


Figure 7.3: *Top panel: Overview of equipment required for single channel recording. Bottom panel: A simplified electrical recording circuit.*

applies a fixed potential difference between the electrodes and the single channel current through the nanopore is measured [9]. A simplified representation of the electrical measurement circuit is given in Figure 7.3, bottom panel.

The analog current signal is converted to an analog voltage signal by the amplifier headstage (a 1 pA signal generates a 1 mV output), and is then fed into a 16-bit digitiser (132x or 1440 series, Molecular Devices) for conversion to a binary signal. The digitiser is connected to a computer for display and analysis of the digitised recording traces.

7.4.2 Recording chamber

The electrical recordings were carried out in custom made vertical planar bilayer chambers, which had been machined from Delrin [10]. The chamber contained two buffer compartments (1 ml each) separated by a 25 μm thick piece of Teflon film (Goodfellow), which had been glued between the two buffer compartments using silicone glue (Sylgard 184, Dow Corning). The Teflon film contained a 100-120 μm aperture, over which a planar lipid bilayer was formed. The aperture was created

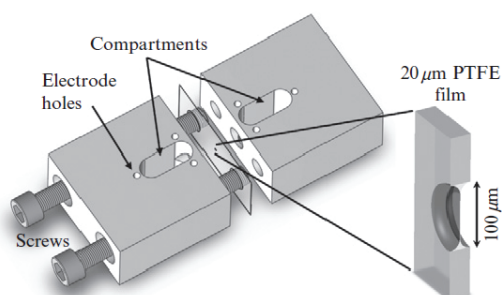


Figure 7.4: Model of a vertical planar bilayer chamber. Figure adapted from [10].

by making a small indentation on the Teflon film with a needle. The film was then ‘zapped’ with a custom built, high voltage spark generator [10]. The spark passed through the weakened material, which caused local melting and led to the formation of an aperture. The size of the aperture was measured under a light microscope with a graticule. The ‘zapping’ process was repeated until an aperture of approximately 100-120 μm had been created. For the electrical recordings under UV irradiation a modified recording chamber was used, which was fitted with a sapphire window on the side of one compartment to allow irradiation of the bilayer [11].

7.4.3 Electrodes

Electrical contact between the recording buffer and the amplifier headstage was made through Ag/AgCl electrodes. By convention, the working (active) electrode connects to the trans recording compartment, whereas the ground electrode connects to the

cis compartment. The electrodes were prepared by soldering a 1-2 cm piece of silver wire (1.5 mm, 99.99%, Sigma Aldrich) to one end of an electrical wire. The other end was equipped with a pin adaptor for connection with the amplifier headstage. The soldered connection was covered in heat shrink insulation to prevent contact with the recording buffer. The silver wire surface was then roughened by brushing with sand paper and immersed into 50% NaClO solution (Fisher Scientific) overnight. The AgCl-coated electrode was rinsed with plenty of distilled water. The electrodes were then set in agar bridges to prevent Ag^+ leaking into the recording buffer. For this purpose, a 200 μl pipette tip (Gilson) was filled with melted agarose/KCl solution, into which the electrode was inserted. A small rubber stopper acted as a seal, which was further secured by wrapping with parafilm. The tip of the pipette tip was cut off with a scalpel to leave a 1 mm opening.

- Agarose/KCl solution: 3% (w/v) low melting point agarose in 3 M KCl

7.4.4 Planar lipid bilayer formation

Planar lipid bilayers were formed according to a method published by Montal and Mueller [12]. The aperture was pretreated by applying one drop ($\sim 5 \mu\text{l}$) of hexadecane in pentane solution to both sides of the Teflon film and the pentane solvent was allowed to evaporate. This created an oil annulus around the rim of the aperture. The two compartments of the recording chamber were each filled with 1 ml of recording buffer and the electrodes were connected. Even under zero applied potential from the amplifier, a very small potential difference existed between the two compartments and a current could be measured. One drop of lipid solution in pentane ($\sim 5 \mu\text{l}$) was then applied directly to the surface of the recording buffer and the solvent was allowed to evaporate. A monolayer was formed across the aperture by lowering the level of the recording buffer in one compartment to below the level of the aperture with a pipette, and then raising it to above the level of aperture again. This process was repeated in the other compartment, thereby creating a bilayer. This led to a drop of the current to zero, indicating that an electrical seal

had formed between the two compartments.

The quality of the bilayer was then assessed by measuring its capacitance. The capacitance of a bilayer is given through $I = C \times dV/dt$. By applying a triangular wave function (which has a constant dV/dt component for each sweep, Figure 7.5) a properly formed bilayer will generate a square current output.

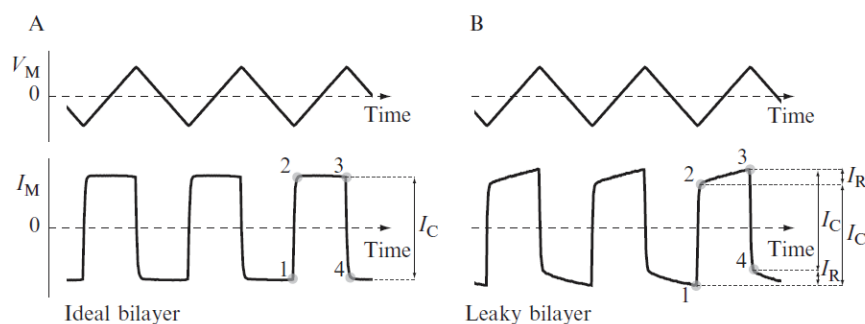


Figure 7.5: *Measurement of the bilayer capacitance. A triangular wave voltage applied across a capacitor produces a square-wave current output. (A) The bilayer capacitance can be determined by measuring the peak-to-peak magnitude of the square wave current output (I_C). (B) A leak in the bilayer is visualised by the triangular ohmic deviation (I_R) from the ideal square wave. Figure adapted from [10]*

A commercial analog waveform generator was used to feed the triangular wave into the patch-clamp amplifier and the waveform had been calibrated such that a square wave current of 100 pA corresponded to a bilayer capacitance of 100 pF. A $\sim 120 \mu\text{m}$ aperture normally gave rise to a bilayer with a capacitance of 100-110 pF. If the capacitance of the bilayer was smaller than this, protein nanopores would not readily insert. In this case the bilayer was broken by ‘zapping’ and reformed again. Similarly, a non-square wave current output indicated that the bilayer was leaky and it was therefore broken and reformed until it reached the required characteristics.

- hexadecane in pentane solution: 10% (vol:vol) solution of hexadecane in pentane (both from Sigma Aldrich)
- lipids in pentane solution: 10 mg/ml of 1,2-diphytanoyl-*sn*-glycero-3-phosphocholine (DPhPC, Avanti Polar lipids)

7.4.5 Nanopore insertion into planar lipid bilayers

Once the planar lipid bilayer had been formed, 0.2-0.5 μl of SDS-solubilised oligomers of αHL (~ 0.5 ng/ml) were added to the recording buffer in the cis chamber. The solution was stirred gently with a magnetic stirrer bar. Insertion of a pore resulted in a jump of the single channel current from zero to the value of the pore. The stirring was then stopped and the recording commenced.

7.4.6 Data acquisition

7.4.6.1 Patch clamp amplifier gain settings

The 132x/1440A digitiser (Molecular Devices) has a dynamic input range of ± 10 V, which divides the signal into 2^{16} (65 536) values (bins), providing a resolution of 0.305 mV/bin ($20/2^{16}$) [10]. The headstage of the amplifier converts a 1 pA signal to a 1 mV output, corresponding to a digitiser dynamic range of ± 10 nA and a resolution of 0.305 pA/bin. In many cases a higher resolution of the current is desirable. This is achieved by means of a second amplification of the voltage output by the patch clamp amplifier (=gain). For example a gain of x10 scales a 1 mV output (from a 1 pA signal) to 10 mV. This results in a *reduced* digitiser dynamic range (± 1 nA), however the resolution *increases* to 0.0305 pA/bin. To maximise the quantisation between current levels, it is therefore best to record at the highest possible gain setting. The single channel recordings described in this thesis were typically recorded at a gain of x20 (dynamic range ± 500 pA, 0.015 pA/bin).

7.4.6.2 Filtering

The single channel recording traces were filtered in order to remove high frequency electrical noise from the recording. Filtering improves the signal-to-noise ratio of the recording and also prevents aliasing of the signal. An amplifier in-built low pass Bessel filter (80 dB/decade) was used to remove high frequency components of the signal. Low pass filters work by rejecting frequencies above the corner frequency of

the filter but passing frequencies below it. Although many types of low pass filter exist (Bessel, Butterworth, Chebyshev...), Bessel filters are most suited to time-domain analysis, as they represent the electrical signal most faithfully [9]. It is important to select the correct corner frequency of the filter in order to represent the electrical signal accurately. If a trace is not filtered sufficiently, it may be impossible to distinguish between several closely spaced current levels, as the noise associated with each level will be larger than the separation between the levels (Figure 7.6A and B). Although increasing the level of low pass filtering makes the distinction between current levels clearer, overfiltering will lead to the disappearance of short lived intermediates and perturb the amplitude and dwell time of events [13], as illustrated in Figure 7.6C. In practical terms, the corner frequency of the low pass filter is usually set to greater than five times the inverse of the mean dwell time of the events to be sampled [13], that is 1 kHz for a 5 ms mean dwell time. The pre-set corner frequencies of the Bessel filter of the AxoPatch 200B amplifier are 1, 2, 5, 10, and 100 kHz. If additional filtering is required a digital Gaussian filter can be applied post-acquisition (Figure 7.6D). The response characteristic of a Bessel filter is well approximated by the Gaussian filter response, and the two actually become identical as the number of poles in the Bessel filter becomes large [14]. The cut-off frequency of the digital Gaussian filter can be set to any value desired.

7.4.6.3 Sampling rate

In addition to selecting the appropriate filter for single channel recording, attention must be paid to selecting the correct sampling rate. The Nyquist sampling theorem states that the sampling frequency should be faster than twice the highest-frequency component within the signal [15] to prevent aliasing of the signal. In the context of single channel recording this means sampling at at least twice the corner frequency of the Bessel filter, although sampling at 5-10 times that frequency gives much better results [15].

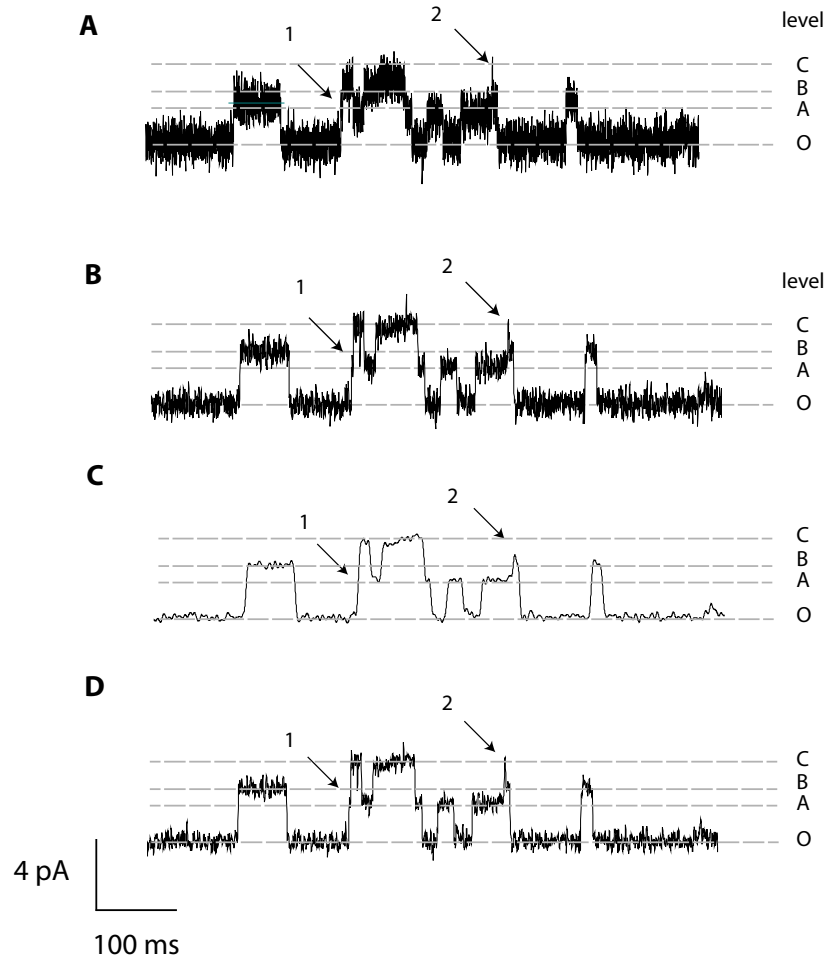


Figure 7.6: The effect of Bessel and Gaussian filters on three identical single channel recording traces acquired at a sampling rate of 10 kHz. **A**, 2 kHz Bessel filter (amplifier in-built, 80 dB/decade). This trace has not been filtered sufficiently to distinguish clearly between current levels A and B. **B**, 1 kHz Bessel filter (digital, applied post-acquisition, 160 dB/decade). This trace has been filtered appropriately. The events indicated by arrows 1 and 2 have distinct dwell times and amplitudes. However, there is some overlap between current levels A and B. **C**, 100 Hz Bessel filter (digital, applied post-acquisition, 160 dB/decade). This trace has been overfiltered. Although the separation between the current levels is very clear, the event indicated by arrow 1 has disappeared. The event indicated by arrow 2 has a much smaller amplitude (leading to the wrong current level assignment!) and longer dwell time than the original signal. **D**, 1 kHz Bessel filter (amplifier in-built, 80 dB/decade), followed by a digital 500 Hz Gaussian filter. Careful post-acquisition filtering with a Gaussian filter has improved the separation of the current level, without compromising the dwell times of the events.

7.4.7 Data Analysis

The filtered single channel recording traces were analysed using the Clampfit 10.2 software (Molecular Devices). A single channel search was performed to identify the unoccupied and partially blocked states of the pore. For the single channel search algorithm, the experimenter assigns a current threshold to each level of the pore, e.g. level 0 for the open pore, level 1 for the first blocked state etc. Every time the single channel current crosses the threshold the nature, amplitude and dwell time of the event is recorded in the results window. Together these data generate an idealised current trace (Figure 7.7). For a simple 2-level analysis, the single channel

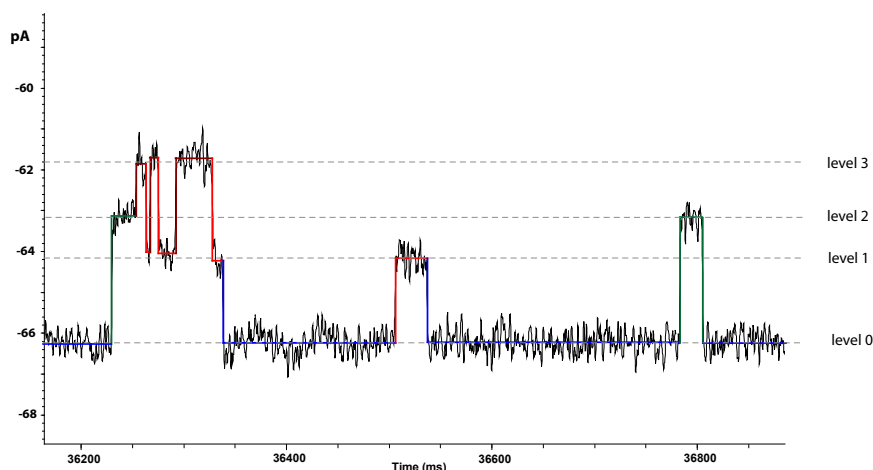


Figure 7.7: Typical single channel search events analysed in Clampfit 10.2. Levels 0-3 are set manually and the software automatically assigns data points to one of the four levels. The nature, amplitude and dwell time of the events is recorded leading to the generation of an idealised recording trace (overlaid in colour).

search results were then processed further in Clampfit 10.2.

For PPIDA, The dwell times (τ) of the states were determined by fitting dwell-time histograms to single exponential functions. The rate constants for the half-chelator. Zn^{2+} complex were calculated by using OriginPro 8 (Microcal Software): $1/\tau_{\text{on-mono7}} = k_{\text{on-mono7}}[\text{Zn}^{2+}]$; $1/\tau_{\text{off-mono7}} = k_{\text{off-mono7}}$. The rate constants for Zn^{2+} binding to the P(PIDA)_2 pore were calculated by analysis of idealized single channel recording traces, obtained in Clampfit, with the software QuB (State University of New York at Buffalo). Briefly, the program derives the lifetimes of all four states

(O, A, B, C) from the idealized trace. Next, the user defines a kinetic scheme to describe the observed states of the channel. The QuB software then applies a MIL algorithm to the dwell times and calculates rate constants that maximize the probability of the observed sequence of states. The MIL output also includes dwell time histograms for each state, the predicted probability density function (PDF) for the event durations, and the log likelihood for the model. The validity of the kinetic scheme can be evaluated by assessing the quality of the fits of the PDFs to the dwell time histograms.

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Chapter 8

Appendix

8.1 Gene and vector maps

8.1.1 Vector maps

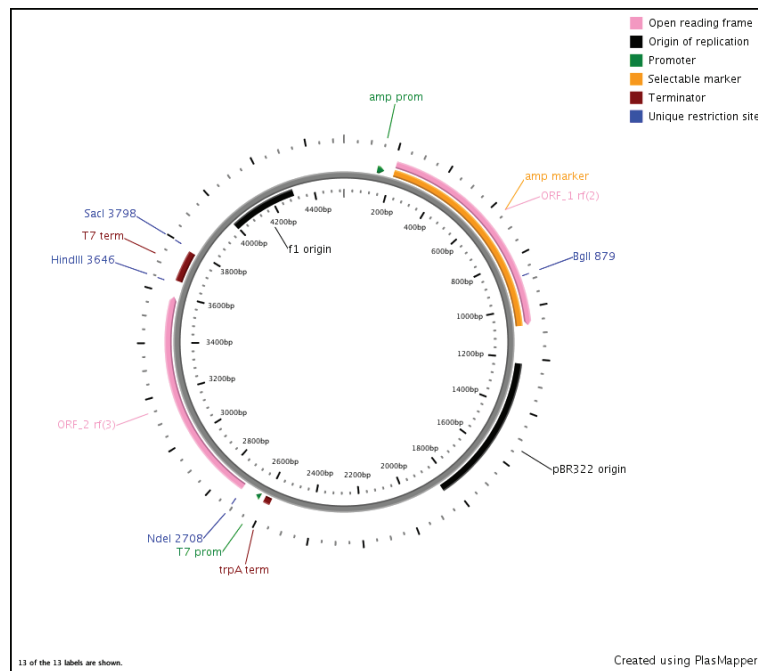


Figure 8.1: Map of the α HL gene in the pT7-SC1 vector(ref Cheley Protein Eng 1997) (generated by PlasMapper ref). The vector contains a T7 promotor and terminator sequence. The α HL gene is flanked by the NdeI and HindIII restriction sites. The vector also contains a β -lactamase gene conferring ampicillin/carbenecillin resistance and the pBR322 origin of replication.

8.1.2 Sequence of the wt α HL gene

The NdeI and HindIII restriction sites are underlined and the stop codon is in boldface.

CAT ATG GCA GAT TCT GAT ATT AAT ATT AAA ACC GGT ACT ACA
GAT ATT GGA AGC AAT ACT ACA GTA AAA ACA GGT GAT TTA GTC
ACT TAT GAT AAA GAA AAT GGC ATG CAC AAA AAA GTA TTT TAT
AGT TTT ATC GAT GAT AAA AAT CAC AAT AAA AAA CTG CTA GTT
ATT AGA ACA AAA GGT ACC ATT GCT GGT CAA TAT AGA GTT TAT
AGC GAA GAA GGT GCT AAC AAA AGT GGT TTA GCC TGG CCT TCA
GCC TTT AAG GTA CAG TTG CAA CTA CCT GAT AAT GAA GTA GCT CAA
ATA TCT GAT TAC TAT CCA AGA AAT TCG ATT GAT ACA AAA GAG TAT
ATG AGT ACT TTA ACT TAT GGA TTC AAC GGT AAT GTT ACT GGT
GAT GAT ACA GGA AAA ATT GGC GGC CTT ATT GGT GCA AATG TTT
CGAT TGGT CAT ACA CTG AAA TAT GTT CAA CCT GAT TTC AAA ACA
ATT TTA GAG AGC CCA ACT GAT AAA AAA GTA GGC TGG AAA GTG
ATA TTT AAC AAT ATG GTG AAT CAA AAT TGG GGA CCA TAC GAT
CGA GAT TCT TGG AAC CCG GTA TAT GGC AAT CAA CTT TTC ATG
AAA ACT AGA AAT GGT TCT ATG AAA GCA GCA GAT AAC TTC CTT
GAT CCT AAC AAA GCA AGT TCT CTA TTA TCT TCA GGG TTT TCA
CCA GAC TTC GCT ACA GTT ATT ACT ATG GAT AGA AAA GCA TCC
AAA CAA CAA ACA AAT ATA GAT GTA ATA TAC GAA CGA GTT CGT
GAT GAT TAC CAA TTG CAT TGG ACT TCA ACA AAT TGG AAA GGT
ACC AAT ACT AAA GAT AAA TGG ACA GAT CGT TCT TCA GAA AGA
TAT AAA ATC GAT TGG GAA AAA GAA GAA ATG ACA AAT **TAA** TGT
AAA TTA TTT GTA CAT GTA CAA ATA AAT ATA ATT TAT AAC TTT AGC
CGA AAG CTT

8.1.3 Sequence of the α HL(RL3) gene

The NdeI and HindIII restriction sites are underlined and the stop codon is in boldface.

CAT ATG GCA GAT TCT GAT ATT AAT ATT AAA ACC GGT ACT ACA
GAT ATT GGA AGC AAT ACT ACA GTA AAA ACA GGT GAT TTA GTC
ACT TAT GAT AAA GAA AAT GGC ATG CAC AAA AAA GTA TTT TAT
AGT TTT ATC GAT GAT AAA AAT CAC AAT AAA AAA CTG CTA GT T
ATT AGA ACA AAA GGT ACC ATT GCT GGT CAA TAT AGA GTT TAT AGC
GAA GAA GGT GCT AAC AAA AGT GGT TTA GCC TGG CCT TCA GCC
TTT AAG GTA CAG TTG CAA CTA CCT GAT AAT GAA GTA GCT CAA
ATA TCT GAT TAC TAT CCG CGG AAT TCG ATT GAT ACA AAA GAG TAT
ATG AGT ACG TTA ACG TAC GGA TTC AAC GGT AAT GTT ACT GGT
GAT GAT ACA GGA AAA ATT GGA GGC CTT ATT GGT GCA AAT GTT
TCG ATT GGT CAT ACA CTT AAG TAT GTT CAA CCT GAT TTC AAA
ACA ATT CTC GAG AGC CCA ACT GAT AAA AAA GTA GGC TGG AAA
GTG ATA TTT AAC AAT ATG GTG AAT CAA AAT TGG GGA CCA TAC
GAT CGA GAT TCT TGG AAC CCG GTA TAT GGC AAT CAA CTT TTC
ATG AAG ACT AGA AAT GGT TCT ATG AAA GCA GCA GAT AAC TTC
CTT GAT CCT AAC AAA GCA AGT TCC CTA TTA TCT TCA GGG TTT
TCA CCA GAC TTC GCT ACA GTT ATT ACT ATG GAT AGA AAA GCA
TCC AAA CAA CAA ACA AAT ATA GAT GTA ATA TAC GAA CGA GTT
CGT GAT GAT TAC CAA TTG CAT TGG ACT TCA ACA AAT TGG AAA
GGT ACC AAT ACT AAA GAT AAA TGG ACA GAT CGT TCT TCA GAA
AG A TAT AAA ATC GAT TGG GAA AAA GAA GAA ATG ACA AAT TGA
TAAG CTT

8.2 List of primers

Name	sequence
SC46	ATAAAGTTGCAGGACCACTTCTG
SC47	CAGAAGTGGTCCTGCAACTTTAT
HL5SG/46	GGATCGGGCAGTGGTGCAGATTCTGATAT
HL10SG/46	GGAGGCTCGAGTGGAGGTAGTTCAGGTGGAGCAGA TTCTGATAT
HL13SG/46	GGAGGCTCGAGTGGAGGTAGTGGTGGATCAGGCTC CTCAGCAGATTCTGATAT
HL15SG/46	GGAGGCTCGAGTGGAGGTAGTGGTGGATCAGGCTC CTCAGGTGGAGCAGATTCTGATAT
HL5SG/47	ACCACTGCCCCGATCCATTTGTCATTTCTTC
HL10SG/47	TCCACCTGAACTACCTCCACTCGAGCCTCCATTTG TCATTTCTTC
HL13SG/47	TGAGGAGCCTGATCCACCACTACCTCCACTCGAGC CTCCATTTGTCATTTCTTC
HL15SG/47	TCCACCTGAGGAGCCTGATCCACCACTACCTCCAC TCGAGCCTCCATTTGTCATTTCTTC
T7F	TAATACGACTCACTATAGGG
T7R	GCTAGTTATTGCTCAGCGG
T117C-FWD	GATACAAAAGAGTATAGTAGTACTTTATGTTATGGATTCAACGG
T117C-REV	CCGTTGAATCCATAACATAAAGTACTACTATACTCTTTTGTATC
T145C-FWD	GCAAATGTTTCGATTGGTCATTGTCTTAAGTATGTTCAACC
T145C-REV	GGTTGAACATACTTAAGACAATGACCAATCGAAACATTTGC
SHS129	GCAAATGTTTCGATTTGCCATACACTTAAC
SHS128	GTTAAGTGTATGGCAAATCGAAACATTTGC

Table 8.1: *List of primers*

8.3 Single Molecule Kinetics

8.3.1 Unimolecular reactions

Consider a simple reaction, for which a population of molecules in one state, A, spontaneously undergoes a change into a different state, B.



Processes such as radioactive decay or the dissociation of an agonist from a receptor are commonly described by this scheme. From the reaction scheme, a differential equation can be set up that describes the rate of transformation:

$$\frac{d[A]}{dt} = -k_A[A] \quad (8.2)$$

By integrating both sides of the equation it follows that

$$\int \frac{1}{[A]} \frac{d[A]}{dt} d[t] = -k_A \int dt \quad (8.3)$$

$$\ln[A] = -k_A t + c \quad (8.4)$$

By considering the boundary condition that when $t=0$, $A=A_0$, it is found that:

$$\ln[A] = -k_A t - \ln[A_0] \quad (8.5)$$

or

$$\frac{[A]}{[A_0]} = e^{-k_A t} \quad (8.6)$$

In this example the value of the rate constant can be determined experimentally from a plot of $\ln[A]$ against t .

Consider now a reversible process of the same type, but at the single molecule level. In order to do so, three assumptions must be made: All transition events (i) have a discrete probability of occurrence, (ii) are random and (iii) do not depend on any

previous state of the system [1].



Since a single molecule is under consideration, the rate constants of the reaction can no longer be determined from the change of the concentration of the molecule over the timecourse of the reaction. Instead, an exponential probability distribution must be derived, which describes the probability that individual events of residence in state A are greater than a variable of time, t :

$$P(t) = \text{Prob}(\tau > t) \quad (8.8)$$

An expression for this probability is given by Colquhoun [2]:

$$P(t) = e^{-k_{AB}t} \quad (8.9)$$

$P(t)$ is a cumulative distribution function, which expresses the summed probability of all events that last longer than time t . A second common form of this equation is the probability density function, $f(t)$, for which the area under the curve between any two times, t_1 and t_2 , is the probability of events that lasts longer than t_1 but less than t_2 . It is found by taking the derivative of $P(t)$:

$$P(\tau \leq t) = F(t) - 1 = P(t) - 1 = e^{-k_{AB}t} \quad (8.10)$$

$$\frac{dF(t)}{dt} = f(t) = k_{AB} e^{-k_{AB}t} \quad (8.11)$$

Three graphical representations of an exponential distribution are shown in Figure 8.2.

Two well known parameters used in describing probability density functions are the population mean, μ , and variance, σ^2 , which are defined as follows:

$$\mu = \int_0^{\infty} x f(x) dx \quad (8.12)$$

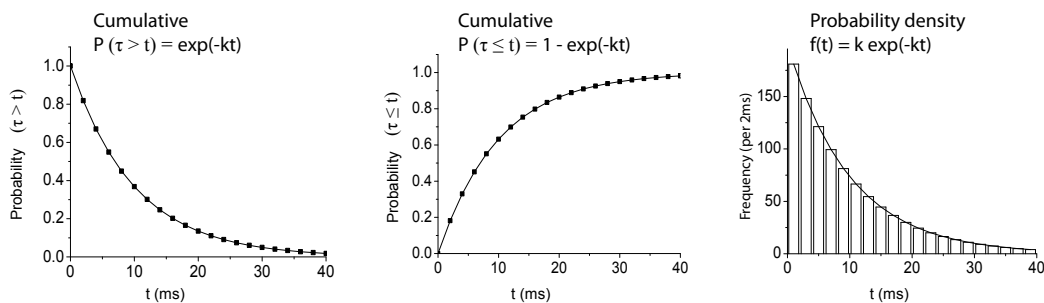


Figure 8.2: Three representations of dwell time probability functions (Figure adapted from [1]). In these examples the rate constant, k , equals 100 s^{-1} . The theoretical probability density function (p.d.f.) is shown for a bin width of 2 ms. and a total of 1000 events.

$$\sigma^2 = \int_0^\infty (x - \mu)^2 f(x) dx = \int_0^\infty x^2 f(x) dx - \mu^2 \quad (8.13)$$

If the above equations are applied to the probability density function mentioned earlier, a simple relationship is obtained:

$$\mu = \sigma = k^{-1} \quad (8.14)$$

It follows that for an exponential distribution, the mean dwell time equals the inverse of the rate constant, which also equals the standard deviation of the population. It is common practise to refer to the mean of this distribution as τ rather than μ .

8.3.2 Bimolecular reactions

Next consider a simple bimolecular reaction between a protein, P, and an analyte, A:



Again, a differential equation can be set up to describe the rate of formation of the product.

$$\frac{d[A]}{dt} = -k_{on}[P][A] \quad (8.16)$$

However, in the single molecule experiments under consideration the amount of protein is never depleted, so this reaction then becomes pseudo first order

$$\frac{d[A]}{dt} = -k'_{on}[A] \quad (8.17)$$

and therefore

$$\tau_{on} = \frac{1}{k'_{on}[A]} \quad (8.18)$$

The rate of dissociation of the analyte from the protein is a unimolecular reaction, as described in the previous section. Therefore

$$\tau_{off} = \frac{1}{k_{off}} \quad (8.19)$$

Under the conditions of dynamic equilibrium:

$$K_f = \frac{k_{on}}{k_{off}} \quad (8.20)$$

8.3.3 The pH dependence of the rate of chelation

To illustrate how the pH of the recording buffer affects the rates of complex formation and dissociation, consider a simplified protonation-deprotonation equilibrium of the half-chelator PIDA, which takes into account only the NH proton:



Only the deprotonated form of the half-chelator PIDA forms divalent metal ion complexes:



The acid dissociation constant for the equilibrium in (8.21) is

$$K_a = \frac{[PIDA][H^+]}{[PIDA.H]} \quad (8.23)$$

The total concentration of half-chelator in the absence of M is the sum of the concentrations of the protonated and deprotonated species:

$$[PIDA]_t = [PIDA] + [PIDA.H] \quad (8.24)$$

Combining (8.23) and (8.24) gives:

$$[PIDA] = \frac{K_a[PIDA]_t}{K_a + [H^+]} \quad (8.25)$$

For a macroscopic reaction the initial rate of formation of metal.half-chelator is:

$$v_{PIDA.M} = k_{PIDA.M}[PIDA][M] \quad (8.26)$$

Substituting (8.25) into (8.26) gives:

$$v_{PIDA.M} = \frac{k_{PIDA.M}K_a}{[K_a + [H^+]]}[PIDA]_t[M] \quad (8.27)$$

Therefore, we can define a new rate constant k_{on} :

$$k_{on} = \frac{k_{PIDA.M}K_a}{[K_a + [H^+]]} \quad (8.28)$$

Therefore the observed initial rate constant k_{on} for metal.half-chelator complex formation is a function of the pH and the pK_a of the half-chelator. Importantly, the rate, $v_{PIDA.M}$, is proportional to $[PIDA]_t$ and $[M]$ under all conditions. It can be shown that the macroscopic rate constant (k_{on}) is equal to $1/(\tau_{on}[M])$ in the single-molecule case. Therefore by lowering the pH of the recording solution, the rate of complex formation should slow down and permit the detection of the intermediate state in which Zn^{2+} is bound by one half-chelator only. Since the dissociation of the full complex must involve protonation of the half-chelator ligands, lowering of the pH should also reduce the lifetime of the complex, thereby facilitating a kinetic analysis.

8.4 The fractional occupancy of the DNA mismatch site with the Rh-complex

This derivation was adapted from [3]. Consider the following equilibrium:



The total amount of DNA or Rh-complex in the system is given through

$$[DNA] = [DNA]_{free} + [DNA.Rh] \quad (8.30)$$

$$[Rh] = [Rh]_{free} + [DNA.Rh] \quad (8.31)$$

The dissociation constant for the equilibrium is therefore

$$K_d = \frac{[DNA]_{free}[Rh]_{free}}{[DNA.Rh]} \quad (8.32)$$

As the Rh-complex binds to the DNA, the amount of free Rh-complex and free DNA decreases, so we cannot approximate the amount of free DNA as the amount added to the experiment, nor the amount of free Rh-complex as the amount added to the experiment. By substituting equations 8.30 and 8.31 into equation 8.32 we obtain

$$K_d = \frac{([DNA] - [DNA.Rh])([Rh] - [DNA.Rh])}{[DNA.Rh]} \quad (8.33)$$

If we multiply both sides of the equation by $[DNA.Rh]$ and subtract the term $K_d[DNA.Rh]$ from both sides of the equation, then we obtain

$$0 = ([DNA] - [DNA.Rh])([Rh] - [DNA.Rh]) - K_d[DNA.Rh] \quad (8.34)$$

which can be rearranged to

$$0 = [DNA.Rh]^2 - ([DNA] + [Rh] + K_d)[DNA.Rh] + [DNA][Rh] \quad (8.35)$$

Solving the quadratic equation for above for $[DNA.Rh]$, we find that

$$[DNA.Rh] = \frac{([DNA] + [Rh] + K_d) - \sqrt{([DNA] + [Rh] + K_d)^2 - 4[DNA][Rh]}}{2} \quad (8.36)$$

Since the amount of Rh-complex and DNA added to the experiment are equal, we can simplify equation 8.36

$$[DNA.Rh] = \frac{(2[A] + K_d) - \sqrt{(2[A] + K_d)^2 - 4[A]^2}}{2} \quad (8.37)$$

$$\text{where } [A] = [DNA] = [Rh] \quad (8.38)$$

Finally, the fractional occupancy (θ) of the DNA binding sites with the Rh-complex is given through

$$\theta = \frac{[DNA.Rh]}{[DNA]} \quad (8.39)$$

$$= \frac{(2[A] + K_d) - \sqrt{(2[A] + K_d)^2 - 4[A]^2}}{2[DNA]} \quad (8.40)$$

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