

The simulation approach to bacterial outer membrane proteins (Review)

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Summary

The outer membrane of Gram-negative bacteria serves as a protective barrier against the external environment but is rendered selectively permeable to nutrients and waste by proteins called porins. Other outer membrane proteins (OMPs) provide the membrane with a variety of other functions including active transport, catalysis, pathogenesis and signal transduction. A relatively small number of crystal or NMR structures of these proteins are known, and it is therefore essential that the maximum possible information be extracted. In this respect, computational techniques enable extrapolation from time- and space-averaged static structures to dynamic, physiological events. Electrostatics approaches have been used to investigate the structures of porins. The stochastic simulation of ion trajectories through these channels has been possible with Brownian dynamics, which treats the membrane and solvent approximately, enabling the prediction of conduction properties. Molecular dynamics has also been applied, enabling fully atomistic descriptions of 'virtual outer membranes'. This has provided atomic resolution descriptions of solute permeation through porins. It has also yielded insights into the dynamics of gating in active transporters and ion channels, as well as providing clues to catalytic mechanisms in outer membrane enzymes. Additionally, simulations are beginning to reveal the common features of interactions between membrane proteins and lipids, with biological implications for OMP folding, stability and mechanism. Future prospects include the simulation of longer, larger and more complex outer membrane systems, with more accurate descriptions of inter-atomic forces.

Keywords: Outer membrane protein, porin, lipid-protein interactions, Brownian dynamics simulation, molecular dynamics simulation.

Introduction

Outer membrane proteins (OMPs) are found in many prokaryotes and in certain organelles of eukaryotic cells. Computational methods have been used to predict that ca. 2–3% of the genes in Gram-negative bacteria encode integral OMPs, and a significant proportion of these are expressed ubiquitously (e.g. ca. 0.5% of the genome, equivalent to 20 OMPs, are abundant in *E. coli*; Molloy *et al.* 2000, Wimley 2003). To date, only about 20 unique OMP crystal or NMR structures have been solved, all from bacterial sources (see http://blanco.biomol.uci.edu/Membrane_Proteins_xtal.html). In contrast to the α -helical nature of all other known (inner) membrane protein structures, the

basic architecture of the bacterial OMP is the β -barrel domain. These are closed barrels, consisting of transmembrane anti-parallel β -strands that are strongly tilted with respect to the barrel axis; each strand is connected by short turns on the inner side of the membrane and by long, mobile loops on the extracellular side. Matching the bilayer environment, the outer surface of the barrel is strongly hydrophobic, whilst at the membrane-solvent interface, amphipathic aromatic groups (i.e. Trp, Tyr) are generally observed (Schulz 2000). OMPs also exist in mitochondria, chloroplasts and peroxisomes; many of these are probably β -barrels (e.g. the mitochondrial voltage-dependent anion channel), but it is now suggested that integral $\beta\beta$ -helical proteins may also exist in these outer membranes (Wimley 2003). No simulation work has been performed on these OMPs because of a lack of structural data, and they are therefore not further discussed in this review.

OMPs are found in the outer membranes (OMs) of Gram-negative bacteria, as well as in the cell envelopes of certain Gram-positive bacteria and possibly even archaea (Nikaido 2003). The OM itself is highly asymmetric. Its inner leaflet, which faces the periplasmic space, is composed of phospholipid, similar in composition to the inner membrane. By contrast, the outer leaflet contains large lipopolysaccharide (LPS) molecules, which contain multiple saturated fatty acid tails and heterogeneous, charged polysaccharides that are cross-linked by divalent cations. The structure of LPS varies greatly between species and is even modified within a single cell in response to changes in environmental conditions to protect, for example, against host defence proteins. The OM protects the cell against toxic agents: the combination of a highly charged sugar region and tightly ordered, gel-like hydrocarbon chains results in low permeability. Nevertheless, to aid in the exchange of nutrients and waste, the membrane is rendered selectively permeable to solutes smaller than ca. 600 Da by pore-forming OMPs called porins. Thus, the sieve-like OM sharply contrasts with the tightly coupled, energy-transducing inner membrane. Along with these classical, 'non-specific' porins and the similar but solute-specific porins, a number of other OMPs with varying functions exist, many of whose structures are known (Figure 1). These non-porin OMPs range in function from enzymes and active transporters, to structural linkers and proteins involved in defensive or pathogenic recognition events (Koebnik *et al.* 2000, Koronakis *et al.* 2000, Vandeputte-Rutten *et al.* 2001a, Prince *et al.* 2002, Chimento *et al.* 2003). Reflecting this diversity in function, the structures of OMPs vary in the number and stagger of the β -strands, the shape and contents of the transmembrane pore, the structure of the extracellular loops, and their oligomerization state (Schulz 2000).

Because of the lack of structural data available for membrane proteins, it is important that we derive the maximum possible information from the structures available

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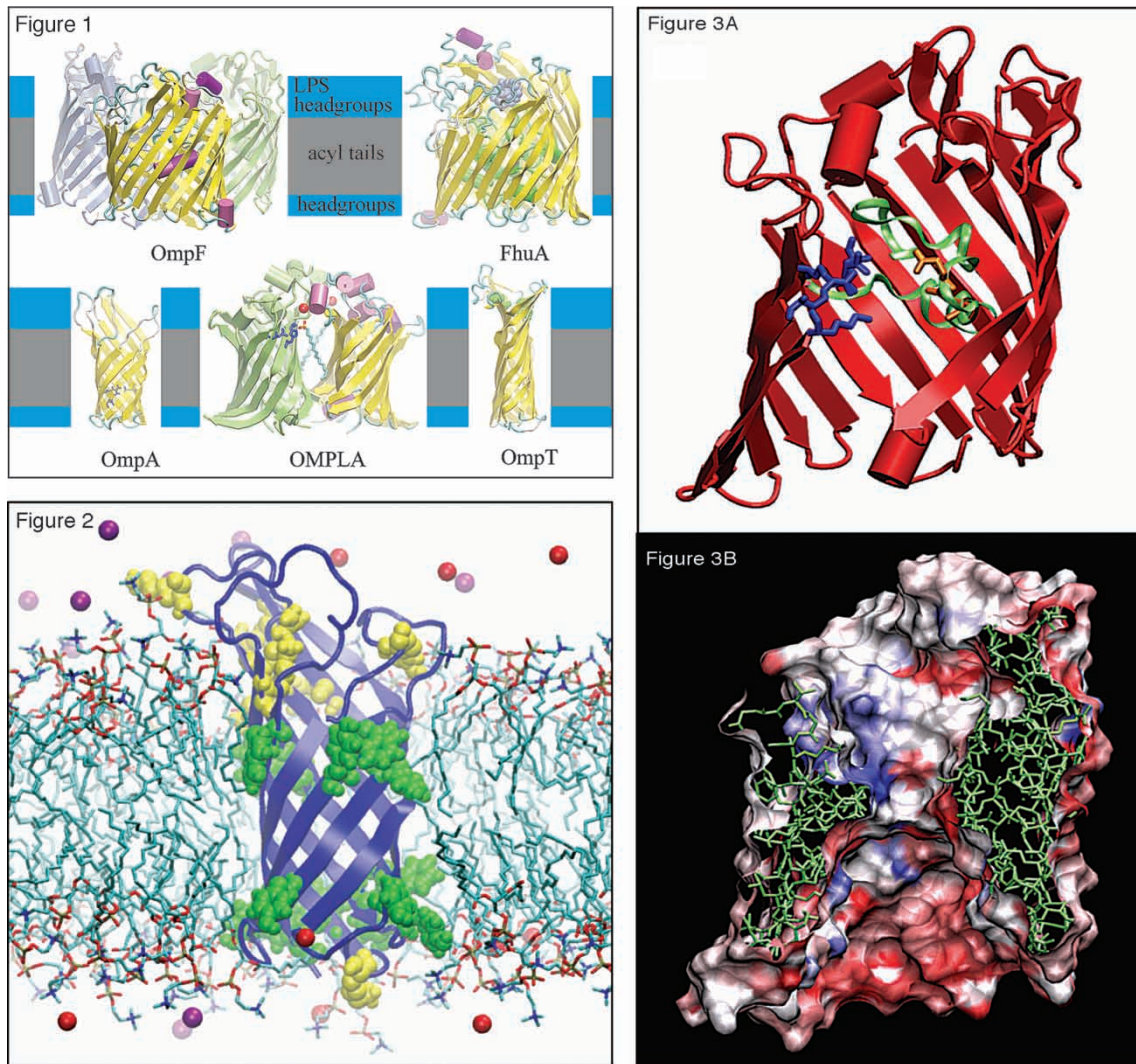


Figure 1. Structures of OMPs from representative structural classes for which MD simulations have been performed (see text for details). The outer membrane is indicated schematically, with the extracellular and periplasmic sides at the top and bottom, respectively. Except where stated, the proteins are displayed in cartoons format, coloured by secondary structure. OmpF (pdb code 2OMF), a member of the homotrimeric, 16 β -stranded general porin family. One of the three monomers is coloured by secondary structure. FhuA (pdb code 1FCP), a member of the 22 β -stranded TonB-dependent receptor family involved in ferrichrome-iron transport. The ca. 150 residue, amino-terminal, internal cork domain is shown in green cartoon format. The ferrichrome-iron ligand is shown in ice-blue space-filling format. OmpA (pdb code 1BXW), a small, eight-stranded β -barrel implicated in structural and ion channel functions. Some of the β -strands are rendered transparent, revealing in blue bonds format the salt bridge residues implicated in gating. OMPLA (pdb code 1FW3), a 12- β -stranded outer membrane phospholipase which becomes active in its homodimeric, calcium-bound form (represented here). One of the two monomers is coloured by secondary structure. The other is coloured in green, with its catalytic triad residues displayed in blue bonds format. The 'catalytic site' calcium ions are shown as red spheres, and the active site-bound substrate analogues, located at the monomer/monomer interface, are shown in bonds format, coloured by atom type. OmpT (pdb code 1I78), a 10- β -stranded outer membrane protease which belongs to the 'omptin' family. Its Asp/His catalytic dyad is located on the extracellular side of the extended β -barrel, displayed in green space-filling format. The figure was made with vmd (Humphrey *et al.* 1996).

Figure 2. Typical MD simulation set-up for a membrane protein embedded in a bilayer (Bond *et al.* 2002). The protein, OmpA, is shown in blue cartoons format. Aromatic residues at the lipid-water interface are implicated in anchoring proteins in the outer membrane and are shown in green space-filling format. Lys and Arg residues are shown in yellow space-filling format, and are able to 'snorkel' up from the bilayer region into the solvent. The membrane is composed of dymyristoylphosphatidylcholine (DMPC) molecules, which are represented in bonds format, coloured by atom type. Sodium and chloride ions in the bulk solvent are displayed as red and purple spheres; the ca. 5000 water molecules are omitted for clarity. The figure was made with vmd (Humphrey *et al.* 1996).

to us. Since the first crystal structure of a porin was obtained, a multitude of computational methods has been applied to further our understanding of OMPs, at various levels of molecular detail. Indeed, simulations of most of the known structural classes of OMPs have now been performed (Figure 1). We will begin this review by describing how computational methods have been used to analyse and predict ion conductance properties of porins, before discussing how more ambitious simulations have been integrated with structural data to gain insight into solute, lipid and protein dynamics, providing information on the functional implications of the conformational dynamics of this important class of membrane proteins.

The computational methods discussed differ in their level of granularity—i.e. the level of detail used to describe the membrane proteins and their environment. Coarse-grained methods based on Poisson-Boltzmann theory focus primarily on electrostatic interactions. The protein, solvent and membrane are treated as continuum dielectric regions. A static analysis of such a system description, treating only the charges on the protein explicitly, enables qualitative visualization and quantitative calculation of the electrostatic protein surface. Explicit description of the stochastic trajectories of individual ions, whose pathways are affected by the mean force of the respective implicit regions of the system and charges on the protein, is possible with Brownian dynamics simulations. More fine-grained methods employ the molecular dynamics simulation technique. This enables a fully atomistic representation of the entire system, including protein, membrane, water and ions, and results in a trajectory of all atoms undergoing thermal fluctuations at equilibrium (Figure 2). Modifications of the algorithms also enable the simulation of non-equilibrium processes. These more fine-grained approaches are desirable from the point of view that they should provide the highest level of accuracy and do not neglect solvation effects and potentially important correlations between atoms. Moreover, the complete conformational mobility offered theoretically enables the observation of biologically important protein and lipid conformational changes. By contrast, the coarse-grained techniques are less computationally demanding than molecular dynamics owing to the reduced number of particles and the simpler description of forces on these particles.

Electrostatics and ion conduction in porins

The crystal structures of several bacterial porins provided clues as to mechanisms of solute permeation (Schirmer 1998, Koebnik *et al.* 2000). Computational studies enable one to test and refine the proposed mechanisms. Classical

porins are homotrimers, each monomer consisting of a 16-stranded β -barrel (Figure 1). The long, mobile loops face the extracellular medium, except for loop L2, which folds over the top of the barrel and makes stabilizing interactions with an adjacent monomer, and the exceptionally long loop L3, which folds into the barrel and forms the minimum cross-section of the transmembrane pore (7 by 11 Å in OmpF) at the 'eyelet' or constriction zone, across which there is a segregation of basic and acidic residues (Figure 3). Based on examination of the structures, it was suggested that the variable size and electric field across the eyelet of each porin formed the primary determinant of selectivity. Computational studies have been used to analyse the electrostatics of interactions of ions with porins, thereby confirming and extending this hypothesis.

An early qualitative approach enabled the visualization of the surface electrostatic potential for the first OMP whose crystal structure was obtained, namely the porin from *Rhodobacter capsulatus*, allowing a relation of the potential to its selectivity (Weiss *et al.* 1991). Subsequently, a more quantitative approach has enabled the inclusion in the calculation of the protein, solvent, and membrane as continuum regions of defined permittivity. The electrostatic potential is calculated by solving (numerically) the Poisson-Boltzmann equation, given the charge density (on the protein) and dielectric constant of different regions (Davis and McCammon 1990). This method was used to investigate two homologous porins from *E. coli*: the cation-selective OmpF and the anion selective PhoE (Karshikoff *et al.* 1994). An advantage of this technique is that it enables the calculation of pK_A values of ionizable groups. Thus, in both porins, some of the basic residues at the constriction zone displayed unusual titration behaviour that may stabilize the strong electrostatic field inside channel and could also explain experimentally observed pH-dependent changes in effective channel size. Moreover, the electrostatic potential for the two channels revealed a screw-like transverse electric field. The electrostatic field, strongest at the constriction zone, would facilitate the dehydration of polar solutes and may aid in the transport of molecules with strong dipole moments. Additionally, the selectivities of the two porins were predicted to be due primarily to differences in electrostatic potential at the extracellular mouths, similar to the proposal from earlier analyses of *R. capsulatus* porin (Weiss *et al.* 1991). Further continuum electrostatic potential calculations were subsequently performed on other porins, revealing that the biologically relevant difference in selectivity at low ionic strength between OmpK36 and the homologous OmpF is due to electrostatic features (Dutzler *et al.* 1999), and that the characteristic potential and titration behaviour for the extremely narrow charge constriction in classical porins from

Figure 3. The features of the channel within OmpF porin. (a) The protein is shown in transparent red cartoons format. Some of the β -strands are omitted, revealing the pore. Loop L3 (green ribbons format) folds back into the barrel lumen, and contains two acidic residues at the eyelet (red bonds format). On the barrel wall opposite, four basic residues (blue bonds format) complete the charge constellation in the constriction zone, which determines the major conductance properties of the porin. (b) The inner molecular surface is shown with a similar orientation to that in (a), coloured by electrostatic potential on a scale from red (negative) through white to blue (positive). Residues within the cut-away molecular surface are shown in green bonds format. Note the segregation of positive and negative charge on opposite sides of the channel, which produces a particularly strong transverse electrostatic field at the constriction zone; this is thought to be important for dehydration of polar solutes, and exclusion of hydrophobic molecules. The electrostatic potential was calculated by solving the Poisson-Boltzmann equation using Grasp (Nicholls *et al.* 1991) with default parameters, and the membrane was not considered. The figures were made with vmd (Humphrey *et al.* 1996).

the α -, β -, and γ -proteobacteria, represented by Omp32, explain their selectivity and unusual electrophysiological channel properties (Zachariae *et al.* 2002).

An extension of the continuum representation of electrostatics enables explicit simulation of ion transport through channels. With the Brownian dynamics (BD) method, ion trajectories are generated by numerically integrating stochastic equations of motion over discrete timesteps, using a potential function, which is a sum of the interaction of an ion with the protein charges and the dielectric boundaries (Chung and Kuyucak 2002). This method is suitable for large open channels and results in statistically significant simulations of ion flux. One such study simulated thousands of independent ion trajectories through various porins (Schirmer and Phale 1999). Qualitatively, anions and cations were observed to follow different paths, according to the respective charge segregation at the constriction of each porin. Quantitatively, the authors were able to predict accurately the relative conductance and ion selectivity of each porin and also their dependence on ionic strength (related to ionic screening effects). An extension of this study successfully predicted permeability characteristics for a number of OmpF mutants (Phale *et al.* 2001). Taken together, these two studies seem to justify the assumptions made for the purpose of deriving conductance properties from large, open-channel structures. Nevertheless, certain difficulties remain, mainly as a consequence of the description of the steady-state ion conduction process *via* an equilibrium electrostatics theory. An attempt to solve these problems was made by combining the BD method with a Grand Canonical Monte Carlo (GCMC) algorithm, which allows Boltzmann-weighted creation and destruction of particles, enabling the simulation of a fluctuating non-equilibrium state (Im *et al.* 2000). The method (GCMC/BD), which also includes a microscopic representation of the transmembrane potential, allowed a qualitative prediction of the cation specificity of OmpF, and also reproduced the experimentally observed asymmetric current–voltage relationship, with different ion fluxes being seen at opposite applied transmembrane potentials. Further refinement of the potential function governing the multi-ion trajectories should lead to even more accurate prediction of macroscopic properties under non-equilibrium conditions. In particular, the inclusion within the GCMC/BD method of a general reaction field, arising from implicit salt in the exterior bulk solvent and polarization at the system dielectric boundaries, resulted in greatly improved predictions of cation selectivity and channel conductance for OmpF (Im and Roux 2001, 2002a).

Simulations applied to solute behaviour in porins

Although BD methods enable the simulation of statistically meaningful ion trajectories with timescales comparable to real permeation processes, there is a neglect of microscopic detail and in particular of protein mobility, as BD methods (generally) treat the protein as a static object. Such approximations inherent in this approach may have important consequences for an accurate and complete understanding of solute transport. Although more computationally demand-

ing, molecular dynamics (MD) simulations circumvent some of these limitations. In particular, it is possible to represent the protein, lipid, and solvent in atomic detail and with unrestrained conformational mobility. In MD simulations, interactions between explicit atoms are described by an empirical potential function, alongside numerical integration of the classical equations of motion over discrete timesteps (Karplus and McCammon 2002b). In a typical simulation using current methods, a complete OMP structure is docked into a bilayer (see, e.g., Faraldo-Gómez *et al.* 2002) consisting of a few hundred phospholipids (an approximation to the complex OM lipid composition), before placing this system into a box of salt solution of dimensions ~ 10 nm³. The result is a virtual patch of OM, containing between $\sim 10^4$ and $\sim 10^5$ atoms (Figure 2). Subsequent MD simulations result in a ‘trajectory’ of the system (i.e. the atomic coordinates of the system vs. time) extending over a time-scale of tens of nanoseconds, from which structural and dynamical quantities can be calculated. A snapshot from a representative simulation illustrates the complexity of such a system (Figure 3; Bond *et al.* 2002). In addition to OMPs, similar MD simulations have been extended to a wide variety of membrane proteins including ion channels, water-transport proteins, proton pumps and ABC transporters, as well as to pure lipid bilayers and detergent micelles (Merz and Roux 1996, Tieleman *et al.* 1997, Tobias *et al.* 1997, Forrest and Sansom 2000, Domene *et al.* 2003a).

Several MD studies have investigated equilibrium solute behaviour in OmpF. An early 1-ns MD simulation of this porin (Tieleman and Berendsen 1998), fully solvated at a low salt concentration (~ 0.1 M) and embedded in a simple bilayer, enabled a detailed analysis of water and ions within the wide aqueous channel. The mobility of water molecules in the pore was reduced in comparison with bulk solvent, a result which has been consistently observed in simulations of other membrane channels, and which has consequences for the estimation of macroscopic properties from simple geometric (Smart *et al.* 1996) and continuum electrostatics models (e.g. Im and Roux 2002c). Moreover, the large variation in water diffusion and ordering across the pore is consistent with previous electrostatics analysis. A more extended simulation study of OmpF in a bilayer has since been performed, with the system bathed in a solution of 1M KCl (equivalent to > 200 of each ion; Im and Roux 2002c). Because of the limited timescales presently accessible to the MD method, it was important to generate a starting ion configuration as close as possible to equilibrium. Therefore, the initial positions of ions were determined according to the electrostatic potential calculated from the Poisson equation, using a Metropolis Monte Carlo simulation. The resulting 5-ns trajectory resulted in a statistically meaningful average ion density along the pore. A screw-like separation of cations and ions along the channel was found, consistent with the strong electric field, and more potassium ions were located in the pore in accord with the observed cation selectivity of OmpF. These are conclusions that have also been derived from BD simulations, as discussed above. Indeed, a recent study compared ion behaviour in the channel of OmpF using MD and BD, along with Poisson-Nernst-Planck (PNP) electrodiffusion theory, which considers the solvent and

ions as a continuum (Im and Roux 2002a). All three methods appeared to reproduce the equilibrium ion distribution with similar accuracy, including the screw-like pathway for anions and cations. Nevertheless, certain features of the ion conduction mechanism are inaccessible from the more approximate methods, owing especially to the continuum dielectric description of water in the BD and PNP approaches. For example, an interesting insight into OmpF permeation was gained from MD, when it was observed that the total solvation number of ions was preserved along the length of the pore, because water molecules and protein residues contributed in complement to maintain a constant solvation shell (Im and Roux 2002b). This feature is important for the high-throughput of ion channels (Roux *et al.* 2000). Moreover, the cation selectivity was potentially explained by the fact that, whilst isolated potassium ions could permeate the channel, chloride could only pass the constriction zone when paired with potassium.

A number of atomistic simulations have been performed with the aim of observing directional (i.e. non-equilibrium) ion or solute transport in porin channels. Several of these studies have attempted to correlate macroscopic conduction properties with microscopic ion–protein interactions. For example, an early simulation of OmpF was carried out, with a constant force across the pore applied to a sodium ion as an approximation to a transmembrane potential (Suenaga *et al.* 1998). This was used to identify Asp113 on loop L3 at the constriction zone as important in binding cations during the permeation process, particularly at low salt concentration. As an extension of this study, single-channel conductance measurements for different alkali metal ions through OmpF were combined with free energy calculations based on the original ion-bound Asp113 configuration (Danelon *et al.* 2003). It was concluded that the ion binding affinity to the central aspartate residue increased with atomic radius, and that this was correlated with greater conduction rates because of increased local ion concentrations within the pore. However, an explicit bilayer was omitted and so the outer surface of the protein was fixed. This lack of detail, combined with the short simulation times and consequent lack of statistically significant sampling, means that extensions to these studies are probably required for confirmation of the results. Furthermore, in a recent MD study of the strongly anion selective porin Omp32 from *Delftia acidovorans* (Zachariae *et al.* 2003), no explicit bilayer was included and, consequently, a major part of the β -barrel had to be constrained. However, multiple simulations were performed under different conditions with a combined length of ca. 50 ns, and these included a reconstruction of the potential of mean force (PMF) for anion translocation around the constriction zone, constructed from multiple umbrella sampling simulations. Chloride ion transport was observed to move in a stepwise manner along a ‘basic ladder’ in the channel. The constriction zone at the centre consists of three arginine and no compensating acidic residues, in contrast with other porins. The transfer mechanism from one residue to the next, both at equilibrium and with an electric field applied, proceeded via thermal fluctuations to overcome small energy barriers and was aided by motion of the flexible basic side-chains. The PMF energy minimum was observed at the

constriction zone, where chloride ions remained bound to Arg75 and Arg38 for prolonged periods of time, consistent with a previous continuum electrostatics study which showed this region to be an electrostatic potential well (Zachariae *et al.* 2002). At this site, anions interacted not only with arginines but also with polar groups from other residues; indeed, the protein solvated the ions in a complimentary manner with water molecules to maintain constantly a full hydration shell throughout channel conduction, as previously observed in the MD simulations of OmpF (Im and Roux 2002b). Moreover, the strong binding of chloride at the constriction prevented the entry of further anions into the channel; this could explain the curious observation that the conductance of Omp32 decreases with increasing salt concentration, in contrast with many other porins. The study of Omp32 highlights the strengths of the MD technique: a detailed description of the ion conduction mechanism was possible, which could not be achieved via BD simulations because of the exceptionally small constriction size and the evident requirement for side-chain flexibility in the mechanism (Zachariae *et al.* 2003).

Simulations have also been carried out to analyse the transport of non-ionic solutes through OmpF (Robertson and Tieleman 2002). Non-equilibrium MD was recently performed, by attaching a ‘virtual spring’ to the dipolar molecules alanine and methylglucose and pulling the spring along the pore axis. In contrast with the previous MD and BD ion trajectories described above, the solutes did not follow screw-like pathways during transport; the rate of transport may have been too rapid to observe this phenomenon. Nevertheless, the molecules aligned with the transverse electric field at the eyelet and desolvation as the pore narrowed resulted in compensatory hydrogen bonds being made with the protein wall, in a similar manner to the MD ion trajectories.

Computational studies have also been applied to maltose transport by the solute-specific porin maltoporin. The protein is similar in structure to classical porins, except that its constriction zone is composed of three loops rather than one, and the pore contains residues that confer solute specificity, namely a helical line of aromatic residues (the ‘greasy slide’) and an opposing group of polar residues (the ‘ionic track’), which facilitate sugar transport (Koebnik *et al.* 2000). The conjugate peak refinement method explores possible reaction pathways and transition states (Dutzler *et al.* 2002), and revealed a fast relay of hydrogen bonds between sugar residues and ‘ionic track’ side-chains along the protein channel wall, helping to explain both the specificity and rate of sugar transport.

Simulations applied to conformational change: channel gating

Whilst it is possible to capture some of the fundamental elements of ion transport in wide aqueous channels using coarse-grained methods such as BD (Im and Roux 2002a), certain functions of OMPs involve protein conformational changes more amenable to investigation via full atomistic MD simulations. In particular, the permeability of the bacterial

OM may be regulated by a number of gating mechanisms specific to each OMP channel or transporter. The first such mechanism to be investigated using simulations was the voltage gating of porins in planar lipid bilayers, whereby membrane potentials of ca. 100 mV or more induce closure of the channels (Koebnik *et al.* 2000). Although the biological significance of this process has been questioned (on account of the low electrical potential across the OM), it is feasible that the presence of highly charged LPS or other local environmental conditions may modulate the channel closure characteristics, or that the gating serves as a 'fail-safe' upon mis-incorporation of porins into the inner membrane (Nikaido 2003). Initially, it was generally assumed that loop L3 played a key role in gating, owing to its constrictive nature and the high electrostatic potential at the centre of the pore (Figure 3). The lack of a porin crystal structure in the closed state lead early on to a number of MD studies to investigate the conformational flexibility of the loop, particularly at the eyelet (Björkstén *et al.* 1994, Soares *et al.* 1995, Watanabe *et al.* 1997). Although the results suggested that the loop could move significantly and hence diminish the pore size, these simulations were all performed in the absence of ions, solvent or bilayer, and, consequently, it was necessary for large regions of the protein to be constrained, leading to some reservations concerning the physical realism of the results (Watanabe *et al.* 1997). Subsequently, the more detailed MD studies described (Tieleman and Berendsen 1998, Im and Roux 2002b) have shown that, at least under equilibrium conditions, the crystal structure conformation of loop L3 of bilayer-embedded OmpF is very stable in both low- and high-molarity salt solutions. This result has been supported by several cross-linking studies, which show that covalent tethering L3 to the barrel wall does not prevent voltage gating (e.g. Phale *et al.* 1997). Along with the observation of voltage gating activity in large, aqueous β -barrels lacking any sort of constriction (Bainbridge *et al.* 1998), these results call into question the role of L3 in voltage gating of OmpF and related porins.

Simulation studies have also been applied to investigate gating in OMP transporters without large, aqueous channels, such as FhuA. This is a large, monomeric, 22-stranded β -barrel with long, extracellular-facing loops, as well as a novel amino-terminal, globular 'cork' domain of ca. 150 amino acids, which occludes the β -barrel (Ferguson *et al.* 1998, Locher *et al.* 1998; Figure 1). FhuA is a member of the homologous TonB-dependent receptor family of proteins, which tightly bind iron-chelating siderophores or vitamin B₁₂; the crystal structures of a number of these OMPs with ligands bound and unbound are known, including FepA (Buchanan *et al.* 1999), FecA (Ferguson *et al.* 2002), and BtuB (Chimento *et al.* 2003). They are all 'scavenger' transporters, which couple the import of ligand against a concentration gradient to the proton motive force across the inner membrane, via interaction with the periplasm-spanning TonB and with the assistance of the inner membrane proteins ExbB and ExbD (Faraldo-Gómez and Sansom 2003). A number of allosteric conformational changes are likely to be involved in this multi-step mechanism, and this has been partially unravelled with the aid of 10-ns simula-

tions of membrane-embedded FhuA in the ligand-free and bound states (Faraldo-Gómez *et al.* 2003).

The first step involves conformational changes in some of the loops of the cork domain induced by ferrichrome binding, which are allosterically propagated to regions that interact with TonB and thereby signal that ligand is loaded (Faraldo-Gómez and Sansom 2003, Chimento *et al.* 2003). Along with loops from the β -barrel, the cork loops form a binding pocket involving multiple protein–ligand interactions, and some long-lived hydrogen bonds with iron-coordinating groups are persistently maintained during the MD simulations (Faraldo-Gómez *et al.* 2003). The next stage of transport may be initiated by movement of the extracellular loops, as indicated by the change in conformation of loop L8 so as to close the binding pocket during the simulation of the siderophore-bound state (Faraldo-Gómez *et al.* 2003). This suggests an 'air-lock' gating hypothesis, whereby ligand-binding induces a closure of the extracellular 'hatch', enabling directional transport upon the formation of some kind of channel. This mechanism is supported by *in vivo* cross-linking studies (Scott *et al.* 2002), and by the FecA crystal structure (Ferguson *et al.* 2002), which revealed closing of the extracellular hatch in the ligand-bound state via conformational changes in loops L8 and also L7. Finally, the electrochemical energy of the inner membrane is somehow transduced via TonB into further conformational changes, causing the transport of ligand from the extracellular hatch to the periplasm; this may proceed by the formation of a channel within the β -barrel, or by partial or complete removal of the plug (Faraldo-Gómez and Sansom 2003). In this context, a potential channel-forming, water-filled region has been identified at part of the interface between the cork domain and barrel wall in FhuA (Ferguson *et al.* 1998). Simulation analysis suggests that this cavity remains too small to accommodate the siderophore, due largely to the maintenance of multiple hydrogen bonds between plug and barrel, and is actually smaller towards the periplasmic end in the ligand-bound state (Faraldo-Gómez *et al.* 2003). This therefore suggests that the plug domain would need to undergo a significant motion or change in conformation to allow passage of siderophore. Interestingly, the interface between the barrel and plug domains was observed to be extensively solvated during simulation. Moreover, the associated water molecules were longer-lived and less permeable for the ligand-bound FhuA on a 10-ns timescale. These tightly bound waters may thus reduce the activation energy of dissociation of the plug-barrel interface, by providing alternative hydrogen-bonding donors and acceptors.

Thus, the interplay between static structural information and simulations of protein and water dynamics are leading to the elucidation of the mechanisms of gating in active transport. Simulations have also been applied to the gating process in an atypical ion channel, OmpA from *E. coli*. This small, monomeric protein is composed of an eight-stranded amino-terminal β -barrel domain (OmpA^{NT}), located in the OM, and a globular carboxy-terminal domain of unknown structure, which lies in the periplasm (Figure 1). OmpA or similar proteins are expressed at high levels in almost all Gram-negative bacteria, and maintain the structural integrity of the cell envelope by linking the OM to the periplasm, as

well as serving recognition roles for bacterial conjugation and pathogenesis (Koebnik *et al.* 2000). In contrast with porins, the crystal structures of OmpA^{NT} reveal that the extracellular loops all point away from the β -barrel, whilst the barrel interior itself does not contain a continuous channel, but rather several aqueous cavities interrupted by charged and polar sidechains (Pautsch and Schulz 1998, 2000). However, many independent studies have shown that OmpA^{NT} forms small ion channels (conductance ca. 60 pS in 1M KCl) in planar lipid bilayers (e.g. Arora *et al.* 2000). Additionally, the complete protein is able to form higher conductance ion channels (conductance ca. 300 pS in 1M KCl). It has been suggested that formation of the higher conductance channels may involve conversion to a porin-like conformer with additional β -strands contributed by the C-terminal domain (Nikaido 2003). Irrespective of the mechanism of formation of the higher conductance pores, it would seem that some kind of conformational change is necessary to gate between the closed OmpA^{NT} crystal structure and a more mobile form that would allow transient 60 pS pore formation in this domain. The physiological importance of this phenomenon is unclear because of the high permeability already conferred to the OM by porins. Nevertheless, for some bacterial cells, OmpA homologues are the major OM channels, as is the case for OprF in *Pseudomonas aeruginosa*; the consequent lower permeability of the cell envelope confers resistance to a wide range of antibiotics (Nikaido 2003).

To explore the possible gating mechanisms of OmpA^{NT}, multi-nanosecond MD simulations of this protein were performed in membrane mimetic environments (Bond *et al.* 2002). The side-chains making up the aqueous cavities within the β -barrel were observed to be quite mobile in response to thermal fluctuations, leading to significant water diffusion. In fact, only one region was observed to be prohibitive to complete water permeation events during simulation, at an Arg-Glu salt bridge within the centre of the pore. Using molecular modelling, the Arg side-chain was rotamerized, coordinating it with a nearby alternative, unpaired Glu side-chain. This was hypothesized to be a potential gating mechanism that would lead to an open state; accordingly, simulation of this putative open-state conformation led to complete water permeation events on the nanosecond timescale, and its empirically estimated conductance was in good agreement with experimental data. This kind of electrostatic-switch gating mechanism is not without precedent, as illustrated by pore formation in annexins (Benz and Hofmann 1997) and chloride channels (Dutzler *et al.* 2003). Furthermore, the solution NMR structure of OmpA^{NT} in detergent micelles has provided additional information on the protein dynamics; in particular, a gradient of flexibility was identified along the axis of the β -barrel, which the authors speculate may have a role in pore formation (Arora *et al.* 2001). Comparative simulations of OmpA^{NT} in a detergent micelle and a lipid bilayer resulted in the observation of a similar gradient of flexibility (Bond and Sansom 2003); however, this gradient was more prominent in the more mobile micelle environment. The increased mobility led to spontaneous conformational changes in the salt bridge region originally identified by molecular modelling as a potential gate (Bond *et al.* 2002), resulting in water

permeation through the pore. Further NMR relaxation experiments seem to support the possibility that pore formation may occur via conformational dynamics of side-chains in the region of the proposed gate (Tamm *et al.* 2003).

Simulations applied to conformational change: OMP catalysis

MD simulations have also been used to aid the investigation of catalytic mechanisms of OM enzymes. OMPLA is an outer membrane phospholipase that degrades phospholipids only in perturbed cell membranes, and it is implicated in toxin release. It consists of a 12-stranded β -barrel, the interior of which contains water-filled cavities formed by a hydrogen-bonding network; the formation of a channel is prevented by the loops, turns, and termini that fold over the centre of the barrel (Figure 1). A number of structural (Snijder *et al.* 1999, 2001) and biochemical (Snijder and Dijkstra 2000) investigations of OMPLA's enzyme activity have revealed that it is regulated by calcium-dependent, reversible dimerization. The enzyme is completely inactive in the monomeric form, but active in the dimeric state and when two calcium ions are bound per monomer, one at the 'catalytic site' (where it aids catalysis by stabilizing negatively charged intermediates) and one at a lower affinity site between loops L3 and L4. The β -barrel of OMPLA consists of a convex and a flat side, and the dimerization interface occurs at the flat side, consisting primarily of hydrophobic interactions along with a few critical hydrogen bonds. The active site of each monomer consists of a serine hydrolase catalytic triad, located on the outside of each barrel near to the dimerization interface and on the outer membrane leaflet side.

Surprisingly, the crystal structures revealed very little difference between the monomeric and dimeric forms of OMPLA. A series of 5-ns MD simulations of different forms of bilayer-embedded OMPLA have been used to compare the dynamics of the monomer and dimer, to look for clues that may explain the process of conversion between the respective inactive and active catalytic states, and also to investigate the role of calcium and water in the catalytic mechanism (Baaden *et al.* 2003). Of particular interest was the fact that dimerization and substrate-analogue binding progressively decreased dynamic fluctuations in the structure of OMPLA, especially around the calcium-binding sites. In particular, the presence of calcium at the active site seemed to stabilize the arrangement of the catalytic triad, by positioning water molecules that form an intricate hydrogen bonding network with the triad sidechains; this observation is also suggestive of a role of one of the long-lived water molecules in the catalytic mechanism. By contrast, simulation of the calcium-free OMPLA monomer revealed no stable water-mediated hydrogen bonding network, resulting in multiple conformational states of the active site residues such that a configuration likely to be productive in catalysis was unstable and present for only about a third of the trajectory. Finally, whilst the substrate-binding clefts remained stable during simulation of the OMPLA dimer when covalently bound to acyl tail-containing substrate analogues at each active site, simulations lacking the inhibitor actually resulted in the collapse of

these clefts, thus reducing the likelihood of phospholipid entry. This may explain the experimental observation that dimeric OMPLA exists in equilibrium between active and inactive conformations; bilayer perturbation is presumably required to open the blocked state observed during simulation, thus preventing unregulated lipolysis of the cell membrane.

Lipid–protein interactions

MD simulations also offer the prospect of analysis of the interactions between membrane proteins and the surrounding lipid molecules. This is of some importance, as only limited data on lipid–protein interactions may be obtained by examination of interactions in those crystal structures where some lipid molecules are present (Fyfe *et al.* 2001, Lee 2003). Early simulations provided a preliminary glimpse at lipid–protein interactions (Tieleman *et al.* 1999), but more detailed analysis was hampered by the relatively short simulation times (~ 1 ns) relative to the known multi-nanosecond dynamics of lipid exchange in bilayers. As simulations of membrane proteins out to ~ 20 ns and beyond are entirely feasible, it is possible to obtain a more reliable picture of lipid–protein interactions.

Particular attention has been paid to the interactions between amphipathic aromatic (i.e. tryptophan and tyrosine) side-chain ‘belts’ on the surface of the protein and interfacial region of the bilayer (Yau *et al.* 1998). These aromatic belts are thought to anchor the protein within the mobile and flexible membrane. OmpA possesses a clear aromatic belt at each end of the molecule: five such residues in the upper (extracellular) and six in the lower (periplasmic) respective belts. There is also an effective third aromatic belt provided by four Tyr residues of the extracellular loops, above the transmembrane β -barrel region. Note that Trp and Tyr residues are able to form hydrogen bonds with interfacial water molecules and polar head groups of lipids (see Figure 4(a)), whilst also forming hydrophobic interactions with the bilayer core. Density profile analysis of multi-nanosecond MD simulations of OmpA in a DMPC bilayer reveal that the aromatic belts clearly overlap with the locations of the lipid headgroups (Domene *et al.* 2003b). The mobility of the extracellular loops leads to conformational changes resulting in a narrower, more ‘uniform’ aromatic belt that matches the bilayer interface. Moreover, all the tyrosine rings become more perpendicular to the membrane plane, optimizing interfacial interactions; this may result in tighter lipid–protein packing. In the bacterial outer membrane, the extracellular loops would be expected to form multiple interactions with the rather more complex lipopolysaccharide (LPS) headgroups of the outer leaflet of this membrane. This is important because the resultant tight linkage between OmpA and the membrane is essential for maintaining bacterial cell integrity.

In addition to amphipathic aromatic residues, it is proposed that basic side-chains (i.e. lysine and arginine) also play an important role in lipid–protein interactions (Killian and von Heijne 2000; see Figure 4(b)). The outer membrane protease OmpT provides an example of the characterization of

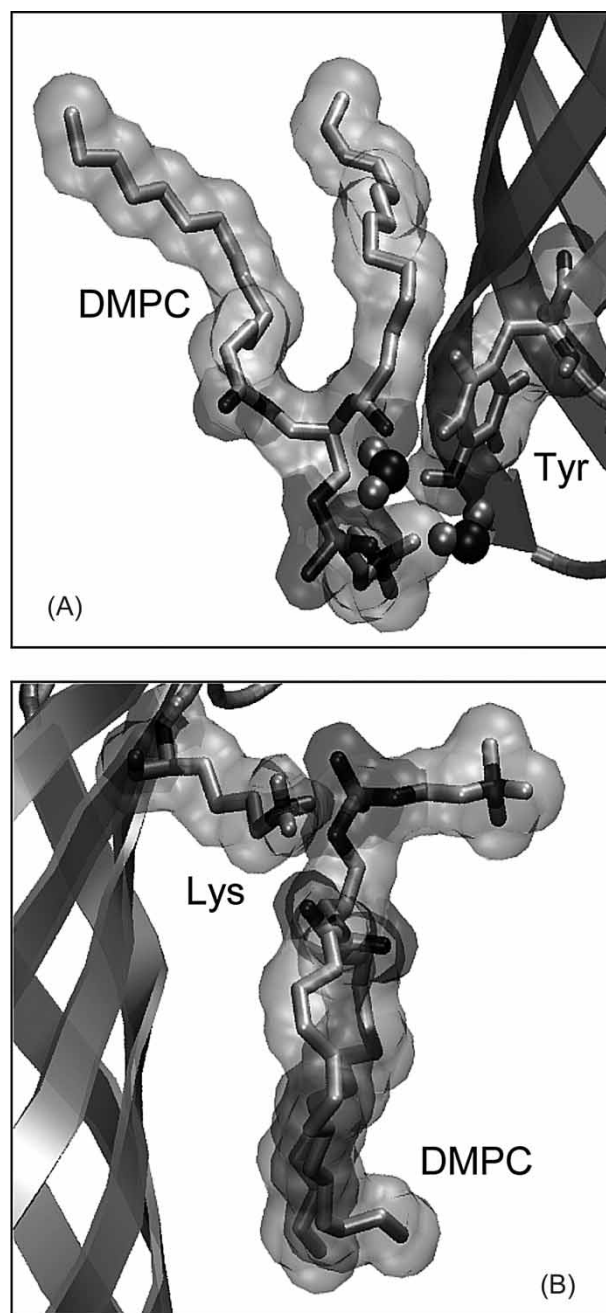


Figure 4. Protein–lipid interactions observed during simulations of OmpA in a DMPC bilayer. The protein is shown in cartoons format. Selected side-chains and lipids are shown in light grey bonds format, with nitrogen and oxygen atoms coloured black. Their molecular surfaces (probe radius of 1Å) are also shown in transparent grey format. (a) A tyrosine residue in the lower aromatic belt of OmpA is interacting with a lipid molecule. Its hydroxyl group is hydrogen bonded with the DMPC glycerol backbone and headgroup via bridging water molecules (shown in space-filling format), whilst the aromatic ring is making van der Waal's contacts with the acyl lipid chain. (b) The amine group of a lysine residue on the outside of OmpA above the extracellular membrane surface is shown forming an ionic bond with a DMPC phosphate group.

such interactions via simulation. On the basis of comparing the crystal structures of FhuA with bound LPS and of OmpT it

was suggested (Vandeputte-Rutten *et al.* 2001b) that a LPS binding site for OmpT consisting of a cluster of predominantly basic side-chains. In the FhuA structure, the primary contacts from the protein are via a lysine and arginine cluster to the diphosphate moiety of the Lipid A portion of LPS.

Analysis of multiple 10-ns simulations of OmpT in a DMPC bilayer (M. Baaden and M. S. P. Sansom, unpublished data) have focused on the principal contacts between the phosphate of DMPC and the OmpT side-chains of the proposed LPS binding site. The results of this analysis revealed long-lasting hydrogen bonds from the DMPC molecule to all of the residues in the proposed LPS binding site. A more detailed analysis of the lipid-protein interactions in the simulations suggested that the interactions are quite dynamic, with the phosphate of a DMPC molecule being passed back and forth between the side-chains of Arg138, Arg175 and Lys226. It is conceivable that the diphosphate of Lipid A may form more stable interactions, possibly with more than one basic side-chain simultaneously, thus supporting the proposed preferential binding of LPS at this site.

Reflections and future directions

Coarse-grained methods, used for the analysis of electrostatics interactions between protein surfaces and ions, have proven to be useful in describing some of the structural determinants of macroscopic properties such as selectivity and conductance in the wide aqueous channels found in porins. However, with the advances in structural biology that have led to the achievement of a multitude of crystal (Koebnik *et al.* 2000, Wimley 2003), and more recently NMR (Arora and Tamm 2001, Tamm *et al.* 2003, Fernández and Wüthrich 2003), structures of membrane proteins, it has emerged that OMPs are not always static, hollow barrels and hence require more advanced computational simulations to relate their mobility and interactions with their environment to their biological function. Most such biomolecular simulations have used classical MD methods, which have improved considerably since their first application to proteins over 25 years ago (Karplus and McCammon 2002a). Advances in algorithms, forcefields and computing power have experienced a rapid evolution, from preliminary explorations of the (*in vacuo*) conformational flexibility of porins, to the investigation of solute behaviour in confined pores, and presently to the role of protein, water and lipid dynamics in active transport, gating and catalysis.

One of the main limitations to the application of MD to biological systems is the relatively high computational power required. This restraint manifests itself in two (overlapping) ways. First, accepting that the current time regime for MD simulations is of the order of tens of nanoseconds, larger-scale motions of protein chains are insufficiently sampled during simulations. Indeed, a comparative simulation study of a number of membrane proteins shows that for all but the most simple structures, 10 ns is insufficient to sample fully their respective conformational sub-spaces, especially for the more mobile, extra-membrane domains (Faraldo-Gómez *et al.*, unpublished data). On the other hand, recent simulations of OmpA in the crystal unit cell environment

(P. J. Bond and M. S. P. Sansom, unpublished data) suggest that extending simulations from by an order of magnitude (i.e. from a ~ 10 -ns to a ~ 100 -ns timescale) yields similar trends in the sampling of protein conformational dynamics for different structural domains. This lends some confidence to extrapolating to even longer timescales. Validation may be aided by recent advances in NMR relaxation techniques to enable detailed comparison between experiment and MD of motions over different time regimes (Tamm 2003).

Second, many biological processes of interest occur on a timescale as yet only indirectly accessible by simulation. Examples of such phenomena include ligand binding to proteins and large-scale domain conformational changes (on a ~ 1 - μ s to ~ 1 -ms timescale), and protein folding (~ 1 ms and above). Nevertheless, preliminary studies are beginning to shed light on these and related aspects of the long timescale conformational dynamics of proteins (Simmerling *et al.* 2002, Chowdhury *et al.* 2003). It is expected that, with continued improvements in methodology and computing power, increases in both simulation length and system complexity will be attained, enabling direct observation of these more complex phenomena *in silico*. This will also result in the ability to carry out high-throughput simulations with the aim of drawing common features of the dynamics of OMPs, and eventually the simulation of large-scale 'virtual outer membranes' will become possible (Arinaminpathy *et al.* 2003). Additionally, advances in simulation timescales will enable further comparison and integration with structural and folding experiments. This should also have a direct impact on the accuracy of forcefield parameters.

Beyond these sampling issues, certain improvements in the simulation methodology for OMPs will be necessary. For example, most simulations of OMPs to date have employed relatively simple phospholipid bilayers, yet bacterial OMPs contain LPS in their outer leaflet. LPS is thought to have effects on the folding and insertion of membrane proteins (Nikaido 2003) and may also influence the conformational dynamics of OMPs within the membrane. In this context, the first co-crystallized structure of LPS bound to a membrane protein was obtained (Ferguson *et al.* 2000), whilst preliminary simulations of LPS bilayers have been performed (Lins and Straatsma 2001, Schroll and Straatsma 2002). Along with refinements in biological detail, standard molecular mechanics forcefields should also see improvements in the near future. For example, application of methods that include some treatment of electronic polarizability of atoms (Halgren and Damm 2001) may result in a more accurate description of ions and their interaction with OMPs during transport, and of the influence of the polar membrane interface on OMP structure and dynamics.

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