Modelling the packing of transmembrane helices: application to aquaporin-1

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Integral membrane proteins constitute at least 20% of most genomes [1] and yet few of their structures are known to high resolution. It is evident that the majority of membrane proteins are made up of bundles of transmembrane (TM) α-helices. This paper is concerned with how molecular modelling techniques, guided by experimental data, may be used to generate candidate structures for the TM domains of α-helical membrane proteins. Thus, molecular modelling may be used to integrate available data relating to the structure of a membrane protein. Such data may arise from sequence analysis, from experimental studies of membrane protein topology, and from low resolution structural studies [e.g. from electron microscopy (EM) and/or spectroscopy]. Restrained molecular dynamics (MD) simulations may be used to generate models compatible with such data. These models may be used as the starting point for further simulation studies, and/or to aid the design of further experiments. This approach is illustrated via its application to human aquaporin-1 (Aqp1).

The aquaporins (Aqp) are a family of membrane proteins which enable passive transport of water, but not of ions, across cell membranes [2,3]. Ten Aqp species have been described in mammalian cells (Aqp0−Aqp9). Of these, Aqp3, 8 and 9 are able to transport urea and/or glycerol in addition to water. Prediction studies and experimental topology data [4,5] suggest that the Aqp monomer contains six TM helices with their N- and C-termini on the intracellular face of the membrane (Figure 1). This is supported by FTIR spectroscopic studies, which indicate a high α-helical content, with the helices approximately perpendicular to the bilayer plane [6]. This predicted topology has been confirmed by medium (6–7 Å)-resolution EM images of two dimensional crystals of Aqp [7–9]. These images reveal six rods of density which are of the dimensions expected for TM α-helices.

The proposed topology is illustrated in Figure 1. There is 20–40% sequence identity between the members of the Aqp family, indicating that they share a common fold. A conserved Asn-Pro-Ala (i.e. NPA) sequence motif occurring in two loops within the Aqp monomer (between helices H2 and H3, and between H5 and H6) is a ‘fingerprint’ motif for Aqps. A cysteine residue (Cys-189 in Aqp1) immediately before the second NPA motif is responsible for the inhibition of water transport by externally applied HgCl₂. Simultaneous mutation of this residue to serine, and of the equivalent residue just before the first NPA motif ( Ala-73) to cysteine, also yields HgCl₂ sensitivity. On this basis the ‘hourglass’ model has been proposed [4] in which the two NPA-containing loops fold back into the membrane and line a narrow aqueous channel through the centre of the protein.

To model the packing of TM helices in Aqp1 we have used a multi-stage approach, which is analogous to the two-stage model of...
membrane protein folding. In this model [10] the TM helices are proposed to form first, and then to pack together within the membrane to give the intact protein. In our modelling procedure, we first predict the location of the TM helices within the membrane protein sequence, and their orientation (N<sub>IN</sub>C<sub>OUT</sub> versus N<sub>OUT</sub>C<sub>IN</sub>) relative to the bilayer plane. Subsequently, restrained MD simulations are used to pack the helices together into a model of a TM helix bundle. The resulting model(s) may then be 'refined' by extended MD simulations in the presence of a lipid bilayer.

The small number of high resolution structures known for membrane proteins seriously impedes development of empirical potentials for the packing of TM helices, making ab initio prediction of TM helix packing almost impossible. However, restraints upon possible modes of TM helix packing within a given membrane protein may be obtained from: (i) analysis of multiply aligned sequences; (ii) experimental protein chemistry and mutagenesis data; (iii) low resolution (9–6 Å) EM structures. A number of computational techniques may be used to model packing of TM helices subject to such restraints.

Methods for the prediction of TM helices from sequences were developed a number of years ago. On average TM helix prediction methods are quite reliable, achieving approximately 85–90% accuracy [11]. However, there are difficulties when using such predictions as the starting point for molecular modelling. The main problem is one of exact definition of the extent of TM α-helices. Although when applied to a membrane protein the different methods generally agree in the number of TM helices predicted, and in the location within the sequence of the hydrophobic cores of those helices, they may differ considerably as to the exact start and end residues for each helix. We have predicted TM helices for human Aqp1 using a number of methods: (i) MEMSAT [12]; (ii) TMAP [13,14]; (iii) PHDhtm [11]; (iv) PHDtopology [15]; (v) TopPred2 [16,17]; (vi) DAS [18]. The number of predicted TM helices varied between five (PHDhtm) and seven (MEMSAT and TopPred2). However, the consensus is six TM helices, in agreement with the EM images. The major discrepancies between the different predictions cluster around the first NPA motif, which may correspond to this region forming a loop which folds back into the membrane. Furthermore, although PHDtopology, DAS and TMAP all predict six TM helices, they disagree with respect to the start/end positions of the helices. In particular, PHDtopology predicts rather shorter TM helices than the other methods. For subsequent modelling we employed a consensus definition of the TM helices based on a combination of the TMAP and MEMSAT predictions.
Further analysis of the sequences of predicted TM helices may be used to place restraints on their possible orientations within a six helix bundle. Analysis of multiply aligned sequences of bacterial photosynthetic reaction centres, for which a three dimensional structure was known, suggested that residues within the interior of the helix bundle were more highly conserved than those on the exterior (lipid-facing) surfaces of the helices [19]. This produces a periodicity in conservation/variability of aligned residues along the length of each predicted TM helix. Such periodicities may be detected via Fourier analysis using e.g. PERSCAN [20]. Periodicity analysis of multiply aligned sequences of TM helices across a family of membrane proteins can thus provide restraints on the orientation adopted by a predicted TM helix within a helix bundle. PERSCAN analysis of the six Aqp helices provided particularly clear-cut results, providing restraints on helix orientations within AqpI models.

EM images of Aqp helix bundles provide further restraints on helix packing. The EM images of Cheng et al. [8] were used to provide ‘target’ restraints for the TM helices. These images provided approximate coordinates for six rods (labelled A to F in Figure 2A) of density, sectioned 7 Å above and below the mid-plane of the membrane. The aim of the model-building protocol is to automatically generate models in which the helices match these target restraints, and are oriented such that their sequence-conserved faces are towards the centre of the bundle.

AqpI TM helix bundles were modelled using restrained MD with a simulated annealing protocol (SA/MD [21–23]). The starting point for SA/MD is a Cx template, which provides an initial model of the TM helix bundle as a set of idealized helices containing only Cx atoms. The starting positions and orientations of these idealized helices embody the initial assumptions and the restraints upon the models. From the EM images of AqpI it is evident that the six helices lie at the corners of an irregular hexagon [8]. Thus, in the Cx template six idealized helices were positioned on a regular hexagon with an interaxial separation of 9.4 Å between adjacent helices (Figure 2B). The helices were
oriented such that their residue-conserved faces were towards the centre of the bundle. In all Cx templates the N- and C-termini were intracellular. Furthermore, in accordance with the analysis of, for example, Bowie [24] and with the EM images, Cx templates had the helices placed at the apices of the hexagon in either a clockwise or anticlockwise fashion, i.e. sequence adjacent helices were spatially adjacent. Thus, 12 Cx templates were possible, and each of these was used as a starting model for generation of an ensemble of 25 structures by using SA/MD.

Three classes of restraint were used: (i) intra-helix distance restraints, to maintain a-helicity within each TM segment; (ii) inter-helix distance restraints, to maintain helix orientations defined by sequence periodicity analysis; (iii) 'target' restraints on helices, to maintain their positions and orientations seen in the EM images. All three classes of restraint were implemented by adding terms to the Xplor [25] potential energy function used in the MD simulations.

For modelling TM helix bundles we employed a SA/MD protocol based on methods used for NMR structure determination [26] and for prediction of helix packing within the GCN4 leucine zipper helix dimer [27,28]. In SA/MD the temperature of the simulation is used to control sampling of conformations. By starting at a high temperature and then progressively decreasing the temperature to 300 K, large changes in conformation are permitted at the start of the simulation, whilst towards the end of the simulation only much smaller changes occur. The starting point of Stage 1 of SA/MD is the Cx template discussed above. The other backbone and sidechain atoms are superimposed on the Cx atoms of the corresponding residue. These atoms 'grow' from the Cx atoms, the positions of which remain fixed throughout Stage 1. Annealing starts at 1000 K, after which the system is cooled from 1000 K to 300 K, in steps of 10 K and 0.5 ps. Electrostatic interactions are not included during Stage 1. Five structures were generated from each Cx template, corresponding to multiple runs of the process with different random number seeds. Structures from Stage 1 were each subjected to, for example five MD runs (Stage 2), resulting in an ensemble of $5 \times 5 = 25$ final structures from a single Cx template. During Stage 2 the various restraints are introduced at this point, whilst the Cx positional constraints are removed. Also during Stage 2 electrostatic interactions are introduced into the potential energy function. At the end of Stage 2, a 5 ps burst of constant temperature in vacuo MD is performed, followed by 1000 steps of energy minimization.

Application of this procedure to the 12 Cx templates [which differed in the order in which the helices, H1–H6, were assigned to the density-derived target restraints, A–F; see Figure 2(B) for Model 7] resulted in 12 ensembles of models, each ensemble containing 25 structures for a six-TM helix bundle. At the outset, it was hoped that it would be possible to rank these 12 models in terms of how well they fitted the experimental data and/or in terms of the strength of their helix–helix interactions. However, this was not possible. Each of the 12 models fitted the helix orientation and target restraints equally well, and the helix–helix interaction energies were approximately the same for all of the models. Thus, all 12 models had to be taken onto the next stage of the modelling procedure. Of course, these 12 models could in principle be used to design experiments to distinguish between them, based on predicted patterns of cross-linking between spatially adjacent helices for example.

In order to attempt discrimination between the alternative models, some of the inter-helix loops, including those containing the two NPA-motifs, were added. This was also important because mutagenesis data indicate that the NPA-containing inter-helix loops play an important functional role. Examination of the predicted topology for Aqp1 (Figure 1) reveals that the L1 (between H1 and H2) and L4 (between H4 and H5) loops are relatively short; it is therefore feasible to model these. Furthermore, it has been suggested [4,5] that the L2 and L5 loops containing the NPA motifs fold back into the bilayer (the 'hourglass' model) and contribute to the water-permeation pathway. Density in the EM images in the approximate centre of the helix bundle has been identified with these NPA loops [7,8]. So, the suggested locations of the NPA loops were included as approximate restraints. The L3 loop (between H3 and H4) is long and therefore it has not been incorporated into the Aqp1 model.

For the short L1 and L4 loops the only restraint applied was connectivity to the two adjacent helix termini to which they are attached. For the re-entrant NPA loops an approximate 'target' restraint was used to bias the models towards the suggested locations of these loops in the EM
images. No secondary structure restraints were applied to either class of loop. The Cα coordinates of one selected structure from each ensemble of models (i.e. one structure each for Models 1–12) was used as a new Cα template. Cα template coordinates for the L1 and L4 loops were calculated by taking points scattered about a vector linking the Cα of the C-terminus of H1 (or H4) and the Cα of the N-terminus of H2 (or H5). For the NPA loops a similar procedure was used. Points around a V shape projecting into the centre of the helix bundle (with the apex of the V in the position indicated by the EM images) were used. The new Cα template was then input to a further run of the SA/MD procedure. During Stage 2 the NPA-containing loops were restrained to somewhat approximate 'target' coordinates derived from the published EM images [8]. The L1 and L4 loops were not restrained.

An example of a model derived by this approach (Model 7) is shown in Figure 3(A). It can be seen that the two NPA loops are folded back into the centre of the six helix bundle in a quasi-symmetrical fashion, as suggested by the original 'hourglass' model [4]. This considerably reduces the cross-sectional area of the pore within the six-helix bundle. Both NPA loops adopt a turn conformation in their central region; the L1 and L4 loops lie on the surface of the molecule; the shorter L4 loop adopts a turn conformation, whilst the longer L1 loop has a more irregular conformation. Note that loops were only added to eight of the 12 models (Models 1, 3, 4, 6, 7, 8, 9 and 10). Examination of the other models (Models 2, 5, 11 and 12) revealed that the NPA loops could not be added to them in a manner compatible with the restraints. Thus, it was possible to exclude some models on the basis of this consideration, resulting in a total of $25 \times 8 = 200$ possible structures for the Aqp1 TM helix bundle.

Model 7 (Figure 3B) was used to test a procedure for further refinement, as it appeared to have a pore-like region running through the centre of the molecule, and its second NPA-motif (loop L5) adopted a conformation such that its mercury-sensitive Cys residue was exposed at the extracellular mouth of the pore. However, it must be stressed that Model 7 was not unambiguously 'better' than the seven other loop-containing models (i.e. Models 1, 3, 4, 6, 8, 9 and 10). Furthermore, the position of the first NPA loop (L2) did not correspond very well with the density in the EM image, suggesting that the restraints applied to the NPA loops needed to be improved.

Model 7 was 'refined' by embedding it in a phospholipid palmitoyl-oleoyl-phosphatidylcholine (POPC) bilayer, solvating the protein/bilayer and running a 1.3 ns MD simulation, without any restraints. The rationale behind this was to test whether the mode of packing of the helices within the model was retained during unrestrained simulations in a lipid bilayer environment. Such bilayer MD simulations have been used both with experimental membrane protein structures (e.g. OmpF porin [29]) and with models of ion channels formed by symmetrical $\alpha$-helix bundles (M. S. P. Sansom, D. P. Tieleman and H. J. C. Berendsen, unpublished results). The stability of a protein model in such a simulation may be measured via the Cα atom root mean square deviation between the initial ($t = 0$) structure and the structure at the end of the simulation. For Model 7 of Aqp1 this was approximately 1.8 Å. Fluctuations in the structure over the course of the simulation were greater for the loops than for the helices. This suggests that the packing of helices within this model is stable. Of course, a low root mean square deviation (RMSD) does not prove that a structure is correct. Rather, a low RMSD in such a simulation is a necessary but not a sufficient condition for a correct model. However, the results from this simulation suggest that running bilayer MD simulations for all eight possible models may provide a way in which to distinguish between them. The bilayer MD simulation for Model 7 was also analysed in terms of the extent to which a water-filled pore was formed. Although water molecules entered one mouth of the 'pore' they did not fully penetrate it. This suggests that the pore is not correctly configured, and provides a further argument for re-examination of the restraints applied to the NPA loops.

Overall, we have demonstrated how restrained MD simulations may be used to build plausible models of Aqp1 in a quasi-objective fashion. These models may be refined by extended MD simulations in the presence of a lipid bilayer. The most immediate task for the future is to improve the models of the loops by constructing restraints which encapsulate the EM data more accurately. This will then generate a number of possible models, each of which will need to be refined using bilayer MD simula-
Figure 3

Model 7 of Aqp1, including inter-helix loops

(A) Cα traces of five structures from the 25 in the Model 7 ensemble. The view is the same as in Figure 2(A) with the extracellular face of the molecule towards the viewer. (B) A single Model 7 structure (in white and 'ribbons' format) embedded in a POPC bilayer (light grey) with water molecules (dark grey) on either side of the membrane. The grey spheres correspond to the carbonyl oxygens of the fatty acyl chains, and so define the limits of the hydrophobic core of the bilayer.
tions. The refined models may then be compared with the available experimental data. For example, it should be possible to use models to predict approximate single pore water permeabilities (using an approach similar to that used to predict an approximate ionic conductance from a channel model [30]), which may then be compared with experimental estimates.

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