A feast of membrane protein structures in Madrid

Workshop: Pumps, channels and transporters: structure and function

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Introduction

Determining the atomic-resolution structure of integral membrane proteins is an adventure for those with a strong stomach, but the results from the last 3 years formed a veritable feast of structures at the recent meeting in Madrid (February 12–14, 2001). Structure is an essential prerequisite to understanding how membrane proteins function and, in addition, how their functions can be modified by small molecules. This is of paramount importance in the pharmaceutical industry, which produces many drugs that bind to membrane proteins (e.g. Prozac and Imigran), and recognizes the potential of many recently identified G protein-coupled receptors, ion channels and transporters as targets for future drugs. This conference discussed many issues that are critically important for membrane protein structure determination, namely increasing the expression levels of complex mammalian membrane proteins and making high-quality two-dimensional (2D) or three-dimensional (3D) crystals; some of these issues were covered by a recent Workshop discussed in a previous report (Thomas, 2001). The major part of the Juan March meeting described how recent structural information on channels, pumps and transporters has led to detailed models of how ions and molecules cross a membrane, and is the subject of this report.

The aquaporin family: molecular discrimination of water and glycerol by channels

The aquaglyceroporin family currently comprises more than 200 members, found in eubacteria, plants and animals, which transport a variety of substrates including water, by AQP1, and glycerol, by GlpF. The atomic resolution model of AQP1 at 3.8 Å resolution (A. Engel, Basel, Switzerland) was determined by electron cryo-microscopy (EM) of 2D crystals (Murata et al., 2000) and the 2.2 Å resolution structure of GlpF (R. Stroud, San Francisco, CA) was determined by X-ray diffraction of 3D crystals (Fu et al., 2000). The structures (Figure 1) are remarkably similar to each other (1.53 Å r.m.s.d. of the Cα backbone) considering they are only 26% identical in sequence and they transport different molecules. Both structures suggested plausible mechanisms for solute translocation and preferences. AQP1 allows the passage of water molecules through the 30 Å-long channel, but it does not transport H+. Reorientation of water molecules at a constriction in the middle of the channel due to the formation of H-bonds to two conserved Asn side chains breaks the H-bonded water chain, effectively preventing H+ conductance because the ‘H+ wire’ is broken. The high-resolution structure of GlpF, determined from crystals grown in the presence of glycerol, provided beautiful evidence for the mechanism of transport. Three molecules of glycerol were clearly visible aligned along the amphipathic channel in GlpF. The hydroxyl groups on the glycerol molecules were all H-bonded to amino acid residues along a polar strip inside the channel, the methylene groups on the glycerol oriented towards the hydrophobic face. Specificity for glycerol over other larger alcohols was proposed to be due to the precise spatial orientation of H-bonds. The inability of GlpF to...
transport water was suggested to be due to the channel being more hydrophobic and narrower than the channel in AQP1; this would necessitate extensive breakage of intra-water H-bonds that could not be substituted by H-bonds to the channel walls, an extremely unfavourable process energetically. Stroud’s group is currently refining the structure of GlpF without any glycerol in the channel. Intriguingly, preliminary results suggest that there appear to be no major conformational changes of the Cα backbone (compare to the Ca2+-ATPase and bacteriorhodopsin structures discussed below). This is reminiscent of the bacterial maltoporin which also showed no gross conformational changes in the presence or absence of substrate (Schirmer et al., 1995). This may be a common feature of transporters that work by facilitative diffusion.

P-type ATPases: coupling ATP hydrolysis to ion efflux

The P-type ATPases are members of a large family of ion-transporters that transport ions by direct hydrolysis of ATP, the phosphorylated intermediate in the transport cycle giving the family its name; the family archetype is the Ca2+-ATPase from muscle sarcoplasmic reticulum. The 2.6 Å resolution structure of the Ca2+-ATPase (C. Toyoshima, Tokyo, Japan) in the calcium-bound (E1) conformation showed 10 transmembrane α-helices (Toyoshima et al., 2000), as predicted from the 8 Å structure determined from tubular crystals by EM (Zhang et al., 1998), linked to three large intracellular domains (Figure 2). Two calcium ions were efficiently co-ordinated by oxygen atoms from amino acid backbone carbonyl groups and carboxylate side chains in two unwound proline-rich regions in the middle of transmembrane helices 4 and 6. One puzzling feature of this structure is that the ATP binding site in the N domain is 25 Å away from Asp351, a residue that is in the P domain and is auto-phosphorylated during the reaction cycle. The reason was apparent after fitting the atomic structure to the 8 Å resolution EM data for the Ca2+-ATPase in the decavanadate-inhibited state, which is thought to resemble the autophosphorylated E2 conformation (C. Toyoshima; D. Stokes, New York). A comparison of the two structures showed dramatic conformational changes; the N domain rotates 20° towards the P domain and the A domain rotates through 90° to place the conserved TGE5 amino acid sequence in juxtaposition to the P domain. These domain motions were predicted to move transmembrane helices 1–6 to allow the efflux of two Ca2+ ions into the sarcoplasmic reticulum. C. Toyoshima has recently managed to collect diffraction data to 3.3 Å resolution from 3D crystals of the Ca2+-ATPase stabilized in a Ca2+ free state, a form of the protein that putatively resembles the autophosphorylated E2 conformation. This structure may resolve some of the issues surrounding the domain movements during ATP hydrolysis and, hopefully, will show how these changes affect the Ca2+ binding sites and lead to vectorial Ca2+ movement.

Biological aspects of Ca2+-ATPase regulation are under investigation using EM (D. Stokes). Phospholamban is a single transmembrane helix protein that inhibits the Ca2+-ATPase in cardiac muscle. 2D crystals of a Ca2+-ATPase-phospholamban complex showed that one molecule of phospholamban induces the formation of Ca2+-ATPase dimers; dimerization could inhibit ion transport by preventing the large conformational changes in the reaction cycle. In addition, the binding site of the toxin thapsigargin has been localized to the luminal loops of the Ca2+-ATPase, presumably locking them in a fixed conformation and thus preventing the movements necessary for ion translocation.

The structure of the Ca2+-ATPase is also having an impact on the studies of other P-type ATPases. The 8 Å resolution structure of the H+-ATPase from the fungus Neurospora crassa has been modelled (W. Kuhlbrandt, Frankfurt, Germany) using the Ca2+-ATPase structure (Stokes et al., 1999). Intriguingly, a 9 Å projection map of the Arabidopsis thalaliana H+-ATPase AHA2 (J. Dietrich, Frankfurt, Germany, and W. Kuhlbrandt), which was expressed and purified in yeast (M. Palmgren, Copenhagen, Denmark) looks rather different from that of the N. crassa H+-ATPase, although the basis for this difference may have to await a 3D reconstruction.

The F1F0 ATPase: rotary catalysis and the number of c-subunits

The Nobel prize-winning structure of the soluble F1 portion of the F1F0 ATPase (J.E. Walker, Cambridge, UK) has now been...
extended to include the structures of various inhibited states (inhibition using efrapeptin, aurovertin, DCCD or AlF3–), including one structure in which the central stalk region was in an ordered state (in the case of inhibition by DCCD; Gibbons et al., 2000). Despite these advances, the fundamental question regarding how proton movement through the Fo domain causes the rotation of the central stalk, leading to ATP synthesis, remains unanswered, as there is at present no complete structure of an F1Fo–complex. An electron density map of a subcomplex of the yeast enzyme at 3.9 Å resolution revealed 10 c-subunits forming the ring structure of the Fo domain, in contact with the foot of the central rotary stalk (Stock et al., 1999). In the intact enzyme, it is likely that the c-ring and central stalk rotate as an ensemble.

The number of c-subunits in the Fo domain is of critical importance, because it defines the number of protons transported per molecule of ATP synthesized. From kinetic experiments and calculations, this number was predicted to be three to four. Since the Fo domain contains three catalytic binding sites for ATP and it was commonly believed that the number of protons transported per ATP should be unitary, a number of nine or 12 c-subunits per Fo domain was expected. It was, therefore, a real surprise that the yeast Fo domain contained 10 c-subunits, implying that 10 protons are required to synthesize three molecules of ATP. The surprises continued with the analysis of Fo domains from chloroplasts and the bacterium Ilyobacter tartaricus (H. Stahlberg, Basel, Switzerland). Unlike the Escherichia coli Fo domain c-ring, that from chloroplasts and Ilyobacter could be solubilized, purified and reconstituted in an intact ring structure. The Ilyobacter c-subunit ring formed 2D crystals that were analysed by atomic force microscopy (AFM) and cryo-EM, the latter yielding a 6.9 Å resolution projection structure; there were 11 subunit c-molecules per ring (Stahlberg et al., 2001). The chloroplast c-subunit ring formed only badly ordered crystals, and were therefore analysed by AFM; these had 14 subunit c-molecules per ring (Seelert et al., 2000). In these experiments both subunit c-rings were also shown to contain additional density in the centre of the rings, and this has been attributed to a lipid plug. An added bonus from the AFM images was that they also showed a number of broken rings which had the same diameter as the intact rings; the implication of this observation is that the number of subunits per ring is dictated by the sequence of the subunit c (D.J. Muller, unpublished observations). Whether the number of subunits per ring is regulated in
Bacteriorhodopsin: an archael light-driven proton pump

Two approaches to the study of bacteriorhodopsin (bR) were highlighted at the meeting. H. Luecke, Irvine, CA, described the 1.55 Å resolution structure of bR in the ground state, the high resolution allowing the unambiguous assignment of bound water molecules which are important in the proton translocation pathway (Luecke et al., 1999b). Comparison of the high-resolution structure of bR with the recently determined 1.8 Å resolution structure of the archaeabacterial Cl\textsuperscript{−} pump, halorhodopsin (hR) (Kolbe et al., 2000) showed that the structures are extremely similar; this has prompted the controversial suggestion (H. Luecke) that, like hR, bR transports an anion (OH\textsuperscript{−}) rather than a proton, during part of the translocation process. Recent work in Luecke’s laboratory has also resulted in high resolution structures of bR mutants that allow the trapping of photocycle intermediates without significant contamination with other intermediates (Luecke et al., 1999a). The structures of the E204Q and D96N mutants (early and late M intermediates) clearly show isomerization of the retinal chromophore, and movements of W182 and the adjacent water molecule. In contrast any movement of the extracellular ends of helices F and G were poorly defined; these movements are present in trapped photocycle intermediates of wild-type bR analysed by electron diffraction (Subramaniam et al., 1999). In addition (R. Henderson, Cambridge, UK), electron diffraction of a triple mutant (D96G/F171C/F219L) that is locked in the ‘open’ conformation revealed a 3.5 Å and 2.0 Å displacement of the ends of helices F and G compared to the ground state (Subramaniam and Henderson, 2000).

Techniques: the problem of overexpressing membrane proteins

The deluge of genome sequences, with ~25% of the genes encoding integral membrane proteins has meant that it has never been easier to identify and clone homologues of your favourite membrane protein. The next step is to overexpress and purify the membrane protein. Although this has been spectacularly successful for some eubacterial transporters, the overexpression of many mammalian transporters and receptors has yielded <0.1 mg of functional protein per litre of culture (Grisshammer and Tate, 1995). The reasons for this are not understood for most membrane proteins. An example of a problematic membrane protein is the serotonin transporter (SERT) which has now been expressed in bacteria, yeast, insect cells and mammalian cells, functional expression only occurring in insect and mammalian cells (C. Tate, Cambridge, UK). This is probably for three reasons: (i) N-linked glycosylation improves the folding and stability of the transporter; (ii) the molecular chaperone calnexin is involved in the folding of SERT; (iii) SERT requires cholesterol to maintain its structure. The combination of these factors effectively precludes functional expression in bacteria or yeast. The poor expression in mammalian and insect cells could be due to the synthetic apparatus of the cell being overwhelmed by the production of a complex protein that requires multiple factors to fold, or merely to the toxicity of the transporter.

The ionotropic glutamate receptors: molecular details of agonism and antagonism

Ionotropic glutamate receptors (GluRs) contain three transmembrane regions that are proposed to form tetrameric assemblies in a manner similar to the bacterial KcsA potassium channel whose structure has been solved. Unlike the potassium channel, GluRs contain large extracellular domains that bind agonists and antagonists. The difficulty with working on the intact channel, combined with homologies between specific GluR sequences (a region of the N terminal domain termed the S1 domain, and a large loop between transmembrane regions 2 and 3, termed the S2 domain) and those of bacterial periplasmic binding proteins, prompted the development of a bacterial expression system in which these external domains are fused into a single functional polypeptide (S1S2) using a linker. N. Armstrong, NY, reported the crystal structures of S1S2 in the unliganded state, and complexed either with the agonists glutamate, quisqualate or AMPA, with the partial agonist kainate, or with the antagonist DNQX (Armstrong and Gouaux, 2000). The S1S2 protein comprised two domains separated by a cleft in which all the agonists and antagonists bound. The full agonists caused a 20° rotation between the domains that virtually closed the cleft, whereas the partial agonist kainate led to only a 12° rotation and partial closure. In contrast, when the antagonist DNQX bound to the cleft, there was minimal domain movement. Another important feature of the structures is the 5 Å increase in the separation between the transmembrane attachment points in the dimer when the ligand is bound compared to the unliganded state. Because the linker is situated where the transmembrane domains would be, this finding suggests that domain closure could directly affect the movement of transmembrane domains. Further insights into this movement may come from S1S2 crystals that contain more of the external domain and from reconstructions of the intact channel using EM and single particle imaging (D. Madden, NY).
this procedure has been improved recently by using fluorinated lipids to form the monolayer, making it far more resistant to the addition of detergents in the chamber which can cause solubilization and dispersal of normal lipids (Lebeau et al., 2001).

The future

The future for determining the structure of integral membrane proteins is looking bright. Established 3D crystallization protocols are already allowing the analysis of intermediates for H+ translocation in bR and this success will inevitably be extended to other channels and transporters. Medium resolution EM structures are now revealing novel architectures for many membrane proteins; presentations at this meeting included NhaA, the Na+/H+ antiporter (3D structure at 7 Å resolution; Williams, 2000), the multidrug transporter EmrE (7 Å resolution projection structure; Tate et al., 2001), with a 3D reconstruction in progress (C. Tate), and the bacterial translocon SecYEG, whose 3D reconstruction is also in progress (C. Tate), and the bacterial translocon SecYEG, whose 3D reconstruction is also in progress (C. Tate), and the bacterial translocon SecYEG, whose 3D reconstruction is also in progress (C. Tate). In addition, the projection structure of a CIC-type chloride channel was recently published (Mindell et al., 2001). The road from sequence to structure for membrane proteins is not, however, smooth. The ease of overexpression of many bacterial transporters is making them prime candidates for structure determination, but the most medically and pharmaceutically important membrane proteins are human, and they are much more difficult to express in large quantities. This problem needs urgent attention. Our ability to crystallize membrane proteins would also benefit from a greater understanding of the influence of lipids on the structure of membrane proteins; this information might be useful in determining ways to improve detergents and stabilizing agents that could maintain the solubilized membrane protein in an active conformation. The optimism and enthusiasm in the membrane protein field for structure determination will ensure further developments where problems exist, and a steady stream of medium- and high-resolution structures in the future.

References


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