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New routes to membrane protein structures

Practical course: Current methods in membrane protein research

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Introduction

Membrane proteins are essential for cellular life: they contribute to the multitude of processes whereby cells interact with their environment, generate energy and maintain their cellular milieu and structure. Consequently, about a quarter of the genes in most genomes encode membrane proteins. Due to the nature of their environment in the hydrophobic lipid bilayer, membrane proteins are generally much more difficult to study than are soluble proteins, a problem which becomes apparent both when trying to obtain crystals and when attempting to determine high-resolution structures of these proteins.

In June 2000, a group of 21 young European scientists assembled at the European Molecular Biology Laboratory in Heidelberg, Germany, for a practical course entitled 'Current methods in membrane protein research'. Here, they learned the most recent methods available for studying membrane proteins. The course touched on many areas relating to the study of this diverse grouping of proteins. However this review will focus mainly on the subject that is the final aim of many of these methods: the determination of high-resolution structures of membrane proteins. To initiate these kinds of structural studies, one needs to have a regular supply of milligram quantities of purified membrane protein, preferable solubilized in a detergent in which the protein is still functionally active. Again, this is by no means a trivial task, but the methods that are being developed to facilitate this are beyond the focused theme of this report.

Packing your protein in 2D

Jean-Louis Rigaud and Gervaise Mosser (Paris, France) described the use of two-dimensional (2D) crystals of membrane proteins to study their structure using various microscopy techniques. With Bettina Böttcher (Heidelberg, Germany), they also demonstrated methods for obtaining 2D crystals from purified and reconstituted membrane proteins, and for the application of electron crystallography to study these crystals. In one interesting new methodology which was described, ternary micelles containing histidine-tagged membrane proteins, reconstitution lipids and detergent, are bound to a Ni_2^+ functionalized lipid layer formed at an air–water interface (Figure 1). Subsequent reconstitution induces the formation of a lipid bilayer around the oriented membrane proteins (Figure 1). Crystal formation can then potentially be induced by this method which has been used

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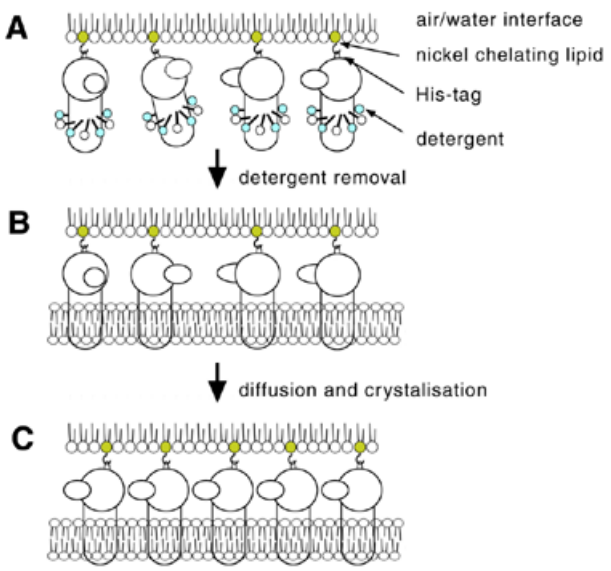

Fig. 1. Proposed mechanism for 2D crystallization of membrane proteins on a functionalized lipid layer. Adapted from Levy *et al.* (1999). (**A**) Binding of the membrane protein–lipid–detergent micelles to the lipid–detergent layer via the His-tag; (**B**) reconstitution of a bilayer around the affinity-bound membrane proteins by detergent removal; (**C**) diffusion of the lipid–protein complexes, leading to 2D crystallization. Lipid molecules are in yellow (modified) or white (unmodified), and detergent molecules are in blue.

successfully for the *Escherichia coli* porin, FhuA, and an F_oF_1–ATP synthase from *Bacillus* (Levy *et al.*, 1999). Levy and co-workers described progress with melibiose permease (MelB), a secondary transport protein from *E. coli*, with which they have produced tubular 2D crystals that diffract to 30 Å. Jean-Louis Rigaud also described the usefulness of reconstituted proteoliposomes to analyze the function of membrane proteins. As an example, the *E. coli* outer membrane porin FhuA was reconstituted into liposomes. This porin also acts as a receptor for bacteriophage T5 and, when this phage is added to the liposomes, direct binding to the liposome surface can be observed with the aid of electron microscopy. Strikingly, some of the bacteriophage appear to be 'empty', illustrating that they have injected their DNA into the liposome via FhuA (Lambert *et al.*, 2000).

Werner Kühlbrandt (Frankfurt, Germany) also described methods for the formation of 2D crystals, which his group studies using cryo-electron microscopy (cryo-EM). He stressed the advantages of using 2D crystals; only relatively small quantities of starting material are required for these studies (an important consideration for membrane proteins), and these kinds of crystals form relatively easily compared to those needed for 3D methods. Unfortunately, obtaining the large flat crystals that are required for cryo-EM is very difficult and labour-intensive. Also the highest resolution ever achieved by this method is much lower (2.8 versus ~1 Å), probably because of the less rigid structure of the 2D lattice compared to the 3D lattice. However, the method does work, as exemplified by the recent solution of the structure of the membrane transport protein NhaA (Williams, 2000). In this, the first structure of a secondary transport protein to be elucidated, 3D information was obtained from 2D crystals by recording a series of cryo-EM images with the crystals progressively tilted away from the horizontal. This method resolved the structure of the protein to 7 Å in the membrane plane, and 14 Å in the vertical plane, and Kühlbrandt described recent progress in his laboratory to refine this structure further, to <5 Å resolution.

Getting more from your 2D crystals—the power of AFM

Continuing the exploitation of 2D crystals, Henning Stahlberg and Simon Scheuring from Andreas Engel's group in Basel, Switzerland, described the group's work on applying the technique of atomic force microscopy (AFM) to the study of membrane proteins (for a recent review see Engel and Müller, 2000). This microscopy technique uses a tiny stylus on a cantilever that is dragged across the crystal surface, and the deflections recorded are used to map the surface topology. Scheuring made the analogy to a blind man scanning the environment with a stick to explore the road ahead. Deflections of his stick are recorded and assembled into an image in his brain. The technique does not provide resolution as high as that obtained using either electron or X-ray crystallography, but unlike these other methods, it allows the user to monitor conformational changes of the proteins *in situ*.

D. Müller in the group of A. Engel has used the AFM to map surface features of bacteriorhodopsin in 2D crystals and, by altering the force applied to the stylus of the AFM, was able to detect movements of flexible loop regions of the protein. Stahlberg described progress studying crystals of aquaporins and other members of the major intrinsic protein (MIP) family, for which the group has been using AFM techniques to complement EM techniques. Data from experiments with the AFM have allowed D. Fotiadis, also from the group of A. Engel, to determine the orientation of the proteins in the 2D crystals (Fotiadis *et al.*, 2000). By increasing the pressure on the AFM stylus when scanning the MIP 2D crystals, the protein could be scraped away from the crystals which revealed a lower layer of protein with a different structure. They assumed that this corresponds to the protein in an inverted orientation. They knew from *in vitro* studies that treatment of the protein with carboxypeptidase removed a 5 kDa fragment from the cytoplasmic side of the 30 kDa protein. Hence, they treated the 2D crystals with carboxypeptidase, examined which layer of the crystal had an altered surface topology and then assigned this to be the cytoplasmic side of the protein in the crystal.

As an example of another application of AFM technology, Scheuring described its use in monitoring a function-related conformational change of a membrane protein. It had been known for many years that the *E. coli* outer membrane porin OmpF could be opened and closed by varying either the membrane potential or the pH of whole *E. coli* cells. However, the 3D crystals that could be formed contained only porins which were in the 'open' form. Starting with 2D crystals of OmpF, D. Müller used the AFM to record the exterior surface of the protein while varying the pH. A noticeable change in the conformation of OmpF was observed as the pH was lowered, and these surface changes were modelled to overall pH-dependent changes in the protein structure. However, it is

important to remember that these structural changes were being mapped onto proteins with known structures that had been solved using traditional methods.

Going to the next dimension….

The highest resolution structures of membrane proteins have come from 3D crystals. However, these crystals are the most difficult to generate. Two new approaches to efficiently generating 3D crystals that have been used successfully to solve membrane protein structures were described and demonstrated. These could, in theory, be applied to many different membrane proteins.

Hartmut Michel and Carola Hunte (Frankfurt, Germany) described a method that was initially developed by Michel and colleagues to solve the structure of *Paracoccus denitrificans* cytochrome *c* oxidase. The idea is to isolate monoclonal antibodies that specifically bind to a protein of interest and to use these antibody fragment–protein complexes to generate crystals. This increases the hydrophilic surface of the solubilized membrane protein and encourages packing of the complex into a crystal lattice. Hunte has isolated a monoclonal antibody which binds with high-affinity to the yeast cytochrome bc_1 complex. Its Fv fragment was expressed in *E. coli* and mixed with purified bc_1 complex to form a stable co-complex for crystallization. Hunte presented her recently solved structure of the bc_1 complex to 2.3 Å resolution. Her data clearly illustrate that, in the crystal lattice, contacts between the membrane protein subunits were mediated by the Fv fragment (Figure 2) (Hunte *et al.*, 2000). Michel observed the same to be true in crystals of cytochrome *c* oxidase, suggesting that use of the antibody fragments is very important for crystal formation. Hunte also presented preliminary work with NhaA, the Na^+/H^+ transporter also being studied with 2D methods in Werner Kühlbrandt's laboratory. The group has isolated a specific monoclonal antibody against NhaA that will potentially make possible co-crystallization of NhaA. It will ultimately be interesting to compare structures of this secondary transporter that have been determined using the 2D- and 3D-based methods.

Ehud Landau (Galveston, TX) described the development of the lipidic-cubic phase as a matrix in which to produce well-ordered 3D crystals of membrane proteins, and detailed the progress his group has made using this method to obtain structures of bacteriorhodopsin in the ground and intermediate states (see Pebay-Peyoula *et al.*, 2000). The lipidic-cubic phase is a special 3D arrangement of lipids that form a continuous curved bilayer with a cubic structure, between which aqueous solutions can move (Figure 3). Membrane proteins can be incorporated into this bilayer and diffuse freely within it. One of the drawbacks of this approach is that, despite being a more natural environment for membrane proteins, conditions for crystal formation must be determined empirically for each protein (as is the case for other detergent/lipid-based methods). Landau described his successes in obtaining highly ordered crystals of bacteriorhodopsin using this approach. He also described somewhat less ordered crystals that were obtained for several other proteins: the photosynthetic reaction centers from *Rhodopseudomonas viridis* and *Rhodobacter sphaeroides*; the light harvesting complex 2 (LH2) from *Rhodopseudomonas acidophila*; and

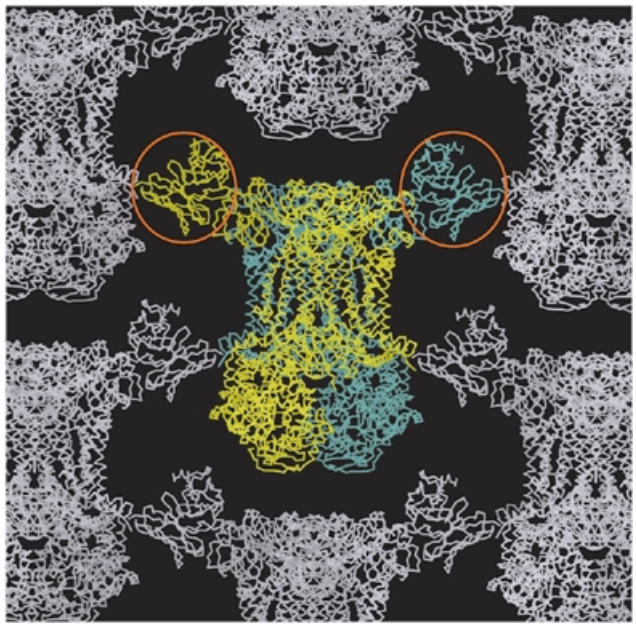

Fig. 2. Crystal contacts between the cytochrome bc_1 Fv fragment co-complex (coloured yellow and green, respectively, in the central complex) are mediated by the Fv fragment (encircled in red). Image provided by Dr Carola Hunte.

halorhodopsin (hR) from *Halobacterium salinarum*. Bacteriorhodopsin, a light-driven proton pump from *H. salinarum* has been particularly resistant to forming well-ordered 3D crystals, despite numerous attempts using conventional detergent-based techniques. The lipidic-cubic phase method has been very successful in providing 3D crystals of bacteriorhodopsin, although notably high-resolution structures have also come from pioneering electron crystallography studies performed by Richard Henderson and colleagues. Bacteriorhodopsin undergoes a photocycle in which light is used to catalyse proton translocation across the membrane. The combined studies of the groups of Landau, Henderson and others have recently provided a series of structures for various intermediates in this photocycle, three of which were recently published in *Nature* (Royant *et al.*, 2000; Sass *et al.*, 2000; Subramaniam and Henderson, 2000). These structures are particularly exciting, as movements of the membrane protein can be followed during the complete catalytic cycle at the atomic level.

NMR—a future in solving membrane protein structures?

An emerging method for studying large integral membrane protein structures is by nuclear magnetic resonance (NMR), using either isolated helical/loop regions or whole proteins. Marc le Maire (Gif-sur-Yvette, France) gave a presentation on the recently solved structure of the sarcoplasmic reticulum Ca^{2+}-ATPase (Toyoshima *et al.*, 2000). His group has worked on this protein for many years, and he presented an insightful comparison between the 2.6 Å resolution structure and the vast amount

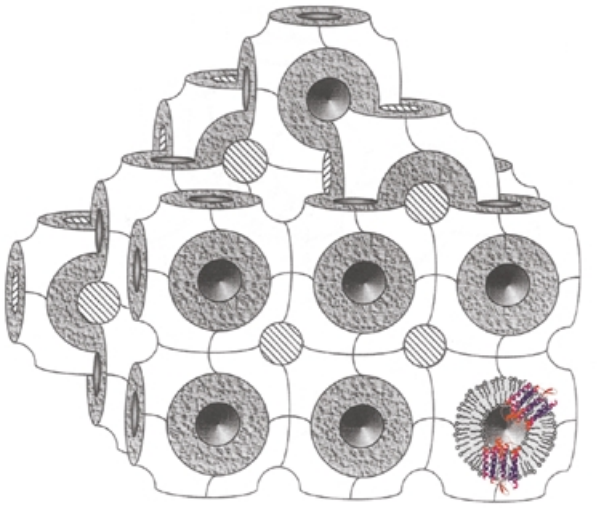

Fig. 3. Schematic model of a bicontinuous lipidic-cubic phase composed of monoolein, water and a membrane protein. The matrix consists of two compartments; a membrane system with an infinite three-dimensional periodic minimal surface is interpenetrated by a system of continuous aqueous channels (shown in black). The detailed section (bottom right) shows the curved lipid bilayer (with inserted membrane protein molecules) enveloping a water conduit. Image provided by Professor Ehud Landau.

of data in the literature from biochemical, molecular biological and structural studies on this important ATPase. One approach that the group had used to obtain structure/function information for this protein involved determining the NMR solution structures of synthetic peptides corresponding to different helices of this ATPase. Among these was a detergent-solubilized peptide corresponding to the M6 helix, which was known to contain residues important for binding of Ca^{2+} ions. Le Maire's structure indicated that the M6 helix was in fact 'split', with the Ca^{2+}-binding residues present in a flexible region in the middle of the polypeptide. This surprising observation was corroborated by the crystal structure solved by Toyoshima *et al.* (2000), illustrating the success of this method in studying isolated membrane helices. The work of Philip Yeagle (Storrs, CT) was also discussed at the meeting, as his group has done similar experiments with helices and, more recently, with loop regions for bacteriorhodopsin and rhodopsin. In most cases their NMR structures match exactly those determined by X-ray methods (Katragadda *et al.*, 2000), again illustrating the virtues of NMR as a tool in cases in which X-ray structures are more difficult to obtain.

Bruno Miroux (Meudon, France), who is collaborating with Michael Sattler and Matti Saraste (Heidelberg, Germany), described an ambitious project to determine the structure of a large integral membrane protein by NMR. These investigators are interested in mitochondrial uncoupling proteins (UCPs), a set of proteins which contain 6-transmembrane helices. Each UCP is made up of three repeats of a 2-transmembrane-helix domain of ~100 amino acids. Large quantities of the C-terminal repeat of this domain have been purified from *E. coli* inclusion bodies. The groups are now attempting to solve the structure of this domain solubilized in detergent micelles using multidimensional liquid-state NMR techniques. If the project is successful, it will mark the start of a gradual transition towards studying larger domains of membrane proteins by non-crystallographic techniques.

Outlook

After 10 days of intensive lectures and practicals, what were the take-home messages for the young membrane protein biologists? Certainly it was clear that the two greatest obstacles to obtaining membrane protein structures are getting the proteins to form well-ordered crystals and getting enough starting material. It is a sobering thought that most of the membrane proteins whose structures have been solved are stable proteins from abundant sources, some of which even naturally form 2D arrays, e.g. bacteriorhodopsin in the purple membrane of *H. salinarum*. Tina Iverson from Doug Rees's lab (Pasadena, CA) presented a fascinating talk about the structure of the fumarate reductase complex from *E. coli*, which is one of two bacterial respiratory complex II proteins for which structures have recently been solved (Iverson *et al.*, 2000). The solution of the structure of the *E. coli* fumarate reductase was particularly important, as the protein had been overexpressed and purified from *E. coli*. It is one of only a handful of available structures for membrane proteins that are not naturally abundant (Luna-Chavez *et al.*, 2000). Although this example and other structures like the mechanosensitive channel (MscL) and potassium channel (KscA) are promising for studying a broader spectrum of membrane proteins, they have all been of bacterial origin. Much work is now being dedicated to expressing eukaryotic membrane proteins in *E. coli* (for examples of recent progress in this area see Grisshammer and Tate, 1999).

Whereas the production of large quantities of a protein of interest is requisite for good structure results, it is no guarantee of success. In spite of the recent methodological advances mentioned in this report, there is no easy route to a membrane protein structure. While NMR methods are useful for looking at isolated helices or loop regions, 2D or 3D crystals are currently the only routes to obtaining high-resolution structures. Both 2D and 3D methods have their merits, although the 3D methods are likely to result in a higher resolution structure. The co-crystallization method expounded by Hunte is very attractive and has been successful in all cases tested so far, but realistically requires at least 3 years to complete. The greatest challenge lies in isolating an antibody that is suitable for this method; it must bind tightly to the membrane protein, allowing formation of a stable co-complex and, preferably, should hold it in a fixed conformation (this usually requires a discontinuous epitope). Hunte had prepared 35 monoclonal antibodies for the yeast cytochrome bc_1 complex and only one bound to the native protein with high affinity! However, improved ELISA-techniques allow the efficient selection of monoclonal antibodies which recognize native epitopes of membrane proteins, a method Hunte has introduced for NhaA, the Na^+/H^+ transporter (Padan *et al.*, 1998). Also, the alternative use of lipidic-cubic phases has been extremely successful in providing crystals of bacteriorhodopsin, although it remains to be seen whether other proteins of different structures can be crystallized in this environment.

In comparison to 3D crystals, 2D crystals are easier to produce, and can also give relatively high-resolution structures from EM techniques. Again, the methods involved are not necessarily rapid. For example, it took 3 years to obtain the data for NhaA that was used to generate the low resolution 3D model (Williams, 2000). Nevertheless, even this is probably still much quicker than the time it would have taken to determine a structure using 3D crystals and X-ray crystallography. The real advantage with 2D crystals, however, is that one can probably do more with them en-route to a high resolution structure (i.e. collect data for a projection map and study the surface topology using AFM), which makes it slightly safer when commencing a project solely aimed towards producing a structure. Ideally though, 2D and 3D methods should be used in combination. Hopefully the participants of the course will now be in a position to initiate these projects with many other classes of membrane protein, and will come up with a few tricks of their own on the way.

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