Published in final edited form as:

Curr Opin Struct Biol. 2012 August ; 22(4): 520–528. doi:10.1016/j.sbi.2012.04.003.

Membrane protein structure determination by electron crystallography

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Abstract

During the past year, electron crystallography of membrane proteins has provided structural insights into the mechanism of several different transporters and into their interactions with lipid molecules within the bilayer. From a technical perspective there have been important advances in high-throughput screening of crystallization trials and in automated imaging of membrane crystals with the electron microscope. There have also been key developments in software, and in molecular replacement and phase extension methods designed to facilitate the process of structure determination.

I. Introduction

The methods of electron crystallography were developed to solve the structure of bacteriorhodopsin in 1975 [1], which represented the first 3D structure of an integral membrane protein. This method has the distinct advantage of using a lipid bilayer as the medium for crystallization, unlike X-ray crystallography, which generally studies membrane proteins solubilized in a detergent micelle. Specifically, the more natural membrane environment is likely to favor a native conformation and potentially to allow conformational changes in response to ligands or binding partners. Because two-dimensional crystals of membrane proteins are microscopic, electron cryo-microscopy (cryo-EM) combined with image processing is the usual route to solving their 3D structure. The success of this approach is evident in the large numbers of membrane protein structures that have been solved at medium and high-resolution (for a table of all structures to date, see ref. [2]). Here we review recent structures that elucidate interactions between membrane proteins and lipid as well as conformational changes that are relevant to their function. In addition, we review technical developments that promise to facilitate the screening of larger numbers of crystallization conditions, and to expedite data analysis and structure determination once suitable crystals have been obtained.

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Protein/lipid interactions

The anisotropic nature of the lipid bilayer has a strong influence over the structure and function of membrane proteins [3,4]. In particular, the bilayer has three distinct zones: 1) a hydrophobic core, which is composed of lipid acyl chains, 2) hydrophilic layers on either side of the core occupied by charged lipid head groups, and 3) aqueous regions with unique dielectric properties at the periphery. This heterogeneous environment places distinct physical and chemical constraints on the structure of membrane proteins. Furthermore, a large variety of lipids are present in lipid membranes, which differ in length and saturation of their acyl chains as well as in the charge and size of their head groups. The specific lipid composition varies from organism to organism and from organelle to organelle and influences the design and behavior of resident membrane proteins. In order to understand the corresponding principles, it is important to study the structural and chemical interactions between membrane proteins and their surrounding lipids.

Structures of membrane proteins determined by both X-ray and electron crystallography often reveal a population of tightly bound lipids [5]. These lipids are generally bound by hydrophobic, van der Waals forces between the lipid acyl chains and the transmembrane surface of the protein, as well as by ionic coupling between the lipid head groups and the hydrophilic protein surface found at the boundary of the membrane. However, the majority of lipids in a biological membrane are not bound in any specific way. Instead, these so called annular lipids form a shell around the protein and engage in transient and relatively nonspecific interactions with the protein [6]. Such annular lipids are typically not seen in X-ray crystal structures, either because they are removed during purification or because they are not involved in any lattice interactions and are therefore free to diffuse around the micelle surrounding the transmembrane region of the protein.

In contrast, an intact lipid bilayer is an integral part of the two-dimensional crystals used for electron crystallography, and lipid molecules within the plane of the bilayer often mediate crystal contacts. For this reason, electron crystallographic structures of bacteriorhodopsin (bR) and aquaporin provide a more complete picture of the protein-lipid interactions. In the structure of bR at 3.5 Å resolution [7], 30 lipids were associated with the trimer. Because these crystals were derived from native membranes, the constituent lipids came from the original bacterial membrane. Almost a decade later, the structure of aquaporin-0 (AQP0) from eye lens at 1.9 Å resolution revealed a belt of nine well-defined lipid molecules (Fig. 1) at the perimeter of each protein monomer, which a total of 20 associated with the tetramer [8,9]. In this case, AQP0 was fully delipidated during purification and then reconstituted in a bilayer composed of synthetic dimyristoyl phosphatidylcholine (DMPC). Both bR and AQP0 structures showed that the lipid acyl chains tend to occupy grooves in the protein surface, where they make hydrophobic interactions with apolar side chains as well as with atoms in the polypeptide backbone. The AQP0 structure also revealed lipids outside the shell of annular lipids, which lack direct interactions with the protein and thus constitute the bulk of the bilayer. Predictably, these bulk lipids are more mobile and therefore have higher temperature factors and less well defined structures relative to the annular lipids. It is nevertheless remarkable that the bilayer as a whole was sufficiently well ordered to reveal essentially all of its constituent lipid molecules.

In order to investigate whether the structures of polar head group and acyl chains affect either membrane protein conformation or crystal packing, AQP0 was recently crystallized with a completely different set of lipids. Specifically, *E. coli* polar lipids (EPL) were used, which substitute the phosphatidylcholine headgroup of DMPC with a mixture of phosphatidylethanolamine (~67%) and phosphatidylglycerol (~23%) and substitute the saturated 14-carbon acyl chains of DMPC with a mixture of longer, partially unsaturated

acyl chains (16:0, 17:0, and 18:1 being the dominant species). Nevertheless, the 2.5 Å structure revealed that the conformation of AQP0 does not appear to change (Fig. 1) and that the distance between the phosphate groups in DMPC and EPL bilayers is almost identical [10]. The head groups of EPL interacted differently with AQP0 than those of DMPC, but the acyl chains in both bilayers occupied similar positions at the periphery of the membrane helices. This result suggested that AQP0 was the primary determinant of membrane structure and that the acyl chains of the annular lipids were simply filling grooves in the protein surface. Somewhat surprisingly, lipid head groups had a negligible effect both on protein conformation and on the ability of annular lipids to adapt to the hydrophobic surfaces of the transmembrane domain. This result may reflect the particular physiological role of AQP0 in the eye lens, where it is responsible for forming planar intercellular junctions between fiber cells and thus maintaining the transparency of this tissue.

In other systems, lipid composition does seem to have notable effects on protein structure. Like AQP0, crystals of the Cu transporter CopA were produced by reconstitution into exogenous lipids. Unlike AQP0, a radically different crystal form resulted from changing the lipid from DOPC to a mixture of DMPC and DOPE (4:1 weight ratio), even though the crystallization conditions otherwise remained the same [11]. Although the resolution was too low to evaluate the lipid interactions at an atomic scale, it was clear that the membrane domain tilted by 30° in DMPC, which is consistent with the 25% decrease in thickness of the hydrophobic core of DMPC membranes relative to DOPC [12]. This tilt greatly altered the geometry of the CopA dimer composing the unit cell and induced an inverted curvature in the corresponding tubular crystals. More importantly, there was evidence of shear between transmembrane helices, which was postulated to pull on one of the cytoplasmic domains and lead to a physiologically relevant conformational change. Similarly, coupling between bilayer thickness, in this case mediated by lateral tension, and the conformation of the transmembrane helices is thought to play a role in gating the mechanosensitive channel as documented by EPR [13]. Lipid composition of the endoplasmic reticulum is also a determinant of membrane protein topology during biosynthesis. Specifically, the presence of phosphatidylethanolamine appears to regulate the charge density on the membrane surface and thus enforce the positive-inside rule [4]. But membrane proteins exhibit a wide range of sensitivity to their lipid environment. In the case of channelrhodopsin-2 (ChR2), a recent electron crystallographic study showed that the dimer interface was unaffected by switching lipids from DMPC to EPL. Indeed, the stability of this interface appears to be a particularly extreme case, because the ChR2 dimer is stable enough to survive even in SDS [14]. It is therefore not surprising that this ChR2 dimer is thought to represent the functional unit with ions being conducted through the dimer interface. Thus, the take-home message seems to be that in some cases the interplay between the membrane structure and protein conformation are part of the design and mechanism of membrane proteins, whereas in other cases the membrane simply plays the role of a passive solvent.

Mechanism of transporters

For proteins that are responsive to the physical properties of the bilayer, it stands to reason that the membrane environment of two-dimensional crystals will favor their native conformation. Furthermore, physiologically relevant conformational changes may be more readily accommodated by these crystals [15]. Although it is common to trap proteins in different conformational states by including relevant ligands during crystallization, concomitant changes in crystal packing have the potential to confound interpretation of the structural changes. It is simpler and more straightforward to compare the structures before and after adding the ligand to pre-existing crystals. In this way, electron crystallography has been used to study conformational changes by either applying physiologically relevant stimuli or adding ligands to preformed membrane crystals of nicotinic acetylcholine receptor

[16], bR [17], rhodopsin [18], and EmrE [19,20]. In the case of bR and rhodopsin [21], the corresponding conformational changes could not be tolerated by the 3D crystals used for X-ray crystallography [22].

In a more recent example, membrane crystals of the Na^+/H^+ antiporter from *E. coli* (NhaA) have been used to study the transport cycle. An initial electron crystallographic map of NhaA at 7 Å [23] and the ensuing atomic structure by X-ray crystallography [24] were both obtained at pH 4, i.e., where the transporter is inactive (Fig. 2e). To obtain mechanistic insight into transport, the membrane crystals were soaked in buffers at higher pH and with Na^+ and Li^+ ions [25], an approach that has not been possible with the 3D crystals. Above neutral pH NhaA becomes activated and a conformational change involving the ordering of the N-terminus was observed in the membrane crystals. When Na^+ was then added at the higher pH, an additional conformational change was ascribed to movement of one of the transmembrane helices (Fig. 2f), leading to a model for activation and transport of the ions.

The membrane crystals of CopA offer another example of conformational changes that are relevant to function. In particular, the coupling between membrane helices, which bind ions and mediate their transport, and cytoplasmic domains, which bind ATP and harness the energy of hydrolysis, is evident in comparisons of different structures. The first report showed changes in the cytoplasmic domains consistent with addition of a phosphate analogue [26]. Unpublished comparison of the more recent structure from electron crystallography [11] with the even more recent structure from X-ray crystallography [27] not only confirms the influence of phosphate analogues on the juxtaposition of cytoplasmic domains, but also illustrates how bilayer-induced shear of the membrane helices pulls on one of the cytoplasmic domains and pushes the pump towards the conformation that binds Cu^+ (Fig. 2a–d).

Finally, insights into the mechanism of membrane protein biogenesis have recently been provided by electron crystallography. Membrane crystals of the bacterial translocon SecYEG have been produced together with a peptide mimic of the signal sequence. The 7 Å structure shows that the membrane environment preserved the back-to-back arrangement of SecYEG dimers and that only one of the two channels was occupied by the signal sequence [28]. Conformational changes associated with the signal sequence suggest a mechanism for initiating the transport of the signal sequence and opening of the channel. The structure also helps explain how only one member of the functional SecYEG dimer is active.

High-throughput screening of crystallization trials

Over the last two decades, structure determination by X-ray crystallography has been greatly facilitated by developments in hardware and software and by a strong emphasis on automation. Sophisticated robotics are now routinely used for setup and evaluation of crystallization trials; synchrotron beam lines are fully automated for screening and data collection and robust software facilitates structure determination. In contrast, methods for electron crystallography are predominantly manual and structure determination can take several years, even after optimal crystals are obtained. To improve this situation, a number of laboratories have been developing strategies to automate crystallization and data collection as well as streamlining software for structure determination. These developments promise both to increase the breadth of parameters that can be surveyed during crystallization trials and to accelerate the rate at which electron crystallographers solve their structures.

Aside from a few special cases, in which crystallization occurs *in situ* within the native cellular membrane, membrane crystals are typically grown by reconstitution of purified, detergent-solubilized membrane proteins into lipid bilayers (see Box 1). Crystallization

requires screening of key parameters: i.e. type of phospholipid, lipid-to-protein ratio (LPR), pH, temperature, type of detergent, divalent cations, ionic strength, ligands, inhibitors, and amphiphiles. Using manual screening methods, these parameters can only be surveyed in a relatively limited fashion, potentially missing truly optimal conditions, or in some cases failing even to obtain crystals. To increase throughput, two independent approaches are under development for crystallization screening on a 96 well basis. The first approach relies on dialysis blocks with wells holding 5–50 μ l of protein sample, each associated with independent buffer reservoirs with 0.5–1.0 ml of dialysis buffer [29,30]. Using a commercial liquid-handling robot to refresh reservoir buffers frequently, detergent removal over a period of 4–14 days has been demonstrated. The second approach relies on cyclodextrins to bind detergent in a stoichiometric complex, thus gradually removing it from the mixed micelles of protein, lipid and detergent [31]. Molar ratios of 1–2 (cyclodextrin:detergent) have been shown effective for complexing a range of non-ionic detergents and a custom liquid-handling robot has been built for systematic addition of nanoliter volumes of cyclodextrin stock solutions to 10–50 μ l wells of protein [32]. Both approaches have been effective in producing membrane crystals and are being used to screen a broad array of parameters affecting the process. Liquid-handling robots are also being employed to prepare negatively stained grids, using magnetic platforms to hold down Ni grids during the pipetting steps required for the staining process [30,33,34].

Box 1

Pipeline for electron crystallography

Structure determination by electron crystallography begins with vesicles derived from a biological membrane, which could be either from natural sources or from a heterologous expression system. This biological membrane has a heterogeneous population of membrane proteins embedded in a lipid bilayer (a). Detergent is used to solubilize this membrane (b), thus placing each of the proteins in a mixed micelle of lipid and detergent. A small population of detergent molecules remain unassociated with micelles and the concentration of these individual detergent molecules corresponds to the critical micelle concentration (cmc), which is characteristic of each detergent species. Generally speaking, detergents with a long acyl chain will have a low cmc and detergents with a short acyl chain will have a high cmc. Like in X-ray crystallography, column chromatography is used to purify the protein of interest, producing a homogeneous population of proteins still solubilized in detergent micelles (c). These micelles may still contain some endogenous lipids, or in some cases lipid is added during purification to improve protein stability. Unlike X-ray crystallography, extra lipid is added to the preparation and dialysis is then used to remove the detergent, thus reconstituting the purified membrane protein back into a lipid bilayer (d). The rate of detergent removal is significantly influenced by the cmc of the detergent, since micelles cannot move across the dialysis membrane and equilibration only involves the population of individual detergent molecules. Thus, short-chain detergents are removed much more quickly than long-chain detergents. Typically, a large number of conditions are tested and the resulting samples must be evaluated by electron microscopy, which has motivated several groups to develop robotic systems for imaging the samples (e). With luck and persistence, two-dimensional crystals are formed, which consist of proteins organized in a regular array within the plane of the membrane (f). These crystals are then prepared for cryo-EM (g), in which both images and electron diffraction are recorded (h). Amplitudes from the diffraction patterns are combined with phases from the images and after merging data together from a wide variety of tilt angles, a three-dimensional structure is generated (i). Molecular images used for this figure were created by David S. Goodsell at the RCSB

PDB and they included the following entries from the database: 2ZXE, 2OAU, 2BG9, 2RH1, 1KYO, 1NLQ, 1IVO, 1M17, 2JWA.

These 96-well crystallizations generate large numbers of samples that must be evaluated by electron microscopy. Screening of these samples represents a huge bottleneck in the pipeline, given the logistics of inserting samples into the electron microscope followed by imaging multiple locations at several different magnifications. To increase the speed and efficiency of this process, four different systems have evolved for automated insertion and imaging of negatively stained samples. The first system is based on an articulated 5-axis robotic arm that uses forceps to pick up individual EM grids, to load them into the standard specimen holder, and then to manipulate the holder through the airlock of a Tecnai F20 electron microscope [34]. A variant of this system divided the sample insertion robot into two coordinated parts: a SCARA robot to pick up EM grids with a vacuum probe and to load them into the sample holder, and a Cartesian robot to place this holder into a JEOL 1230 electron microscope [35]. In both cases, specimen insertion and imaging is controlled by Leginon [36], a program which goes on to acquire a series of representative images from each sample and to place them in a database for later evaluation. An advantage of this approach is that modifications to the microscope are not required. In contrast, two other systems employ carousels carrying 96–100 grids, which are mounted within the vacuum of the microscope, thus expediting sample exchange. The Gatling gun inspired the first of these designs, where 100 grids are loaded into cartridges that are spirally mounted onto a cylindrical drum. This design was implemented on a Tecnai T12 microscope using DigitalMicrograph scripts to orchestrate the process and to acquire images [37]. The second design was based on the so-called auto-loader built by FEI for their Titan line of microscopes. Placing the 12-grid cassettes onto an 8-position carousel extended the capacity to 96 samples and a Tecnai T12 microscope was customized to accept the assembly [33]. Custom software was developed both to control sample insertion and to collect images, which included a sophisticated algorithm to identify 2D crystals based on their shape and to evaluate their order based on diffraction patterns [38].

All these automated imaging systems have the potential to generate thousands of images that need to be scored for crystallization and archived. Currently an experienced electron crystallographer carries out the time consuming process of scoring. Approaches for the automated evaluation of crystallization trials are under development and we expect that they will be available in the near future, thus directing the crystallographer to the most promising samples. There are also efforts underway to facilitate the archiving of data associated with

the crystallization trials. Very recently the laboratory information management system (LIMS) called Sesame [39] has been updated to track protein targets through the two-dimensional crystallization pipeline, including uploading of images from the Legion database and recording of crystallization scores [35].

A newly developed optical microscopy holds promise as a more rapid alternative to electron microscopy in screening two-dimensional crystallization trials. The technique is referred to as Second Order Nonlinear Optics of Chiral Crystals (SONICC) and it relies on frequency doubling of light that occurs with high efficiency in chiral crystals. The method benefits from a complete absence of background signal from aggregated material or from non-chiral crystals from buffer components such as salt. Unlike UV microscopy, which is much less sensitive, SONICC is compatible with plastics used for microtiter plates that are employed both for dialysis and for the cyclodextrin methods of two-dimensional crystallization. The current technology has been licensed to Formulatrix and has been shown effective for imaging small 3D protein crystals in solution [40] and in lipidic cubic phase [41], and has even been shown to detect 2D crystals of bacteriorhodopsin [42]. Although these preliminary results are exciting, further developments are necessary to optimize the design in order to routinely apply it to smaller, more poorly ordered crystals that typically result from a two-dimensional crystallization screen.

Software for structure determination

Improvements in data processing software are critical to the advancement of electron crystallography. The groundwork was laid at the Medical Research Council (MRC) in the 1970's and 1980's in a successful effort to solve the atomic resolution structure of bacteriorhodopsin [43]. Over the last 5–10 years, a number of developments have sought to enhance and extend this software, including 2dx, XDP, IPLT and EMIP. The 2dx software package [44] (<http://www.2dx.unibas.ch>) provides a graphical user interface to the original MRC programs and streamlines certain steps with an eye toward automating the process, which has potential to accelerate structure determination considerably. 2dx also includes some novel features for finding defocus and for using maximum likelihood to correct in-plane lattice defects (so-called unbending). Similarly, the XDP software provides a user interface to the MRC programs for processing electron diffraction patterns [45]. In contrast, IPLT is a new development for processing both images and electron diffraction (<http://www.iplt.org>). This program takes advantage of a modern, object oriented programming architecture and incorporates new strategies for correcting lattice distortions and untangling electron diffraction patterns from overlapping crystals [46,47]. Processing of electron diffraction with IPLT is currently fully functional and modules for processing of images are still under development. Indeed, IPLT is designed to be extensible and appears to offer a good platform for incorporating new algorithms on an ongoing basis. Finally, the EMIP user interface has been developed for Fourier-Bessel reconstruction of crystals with helical symmetry [48] (<http://cryoem.nysbc.org/EmIP.html>). EMIP provides a front-end to a complex series of programs and scripts, thus guiding less experienced users through the process. A real-space alternative for helical crystals has also been implemented in SPARX [49], which may be a more effective reconstruction approach for helical crystals that are not as straight. Both alternatives for helical reconstruction require knowledge of the helical symmetry, which requires expertise and experience in interpreting the corresponding diffraction patterns.

Images are the conventional source of phase information in electron crystallography, but there have been significant developments in using either molecular replacement or phase extension as an alternative. Although image phases are generally of high quality, the ability to acquire these phases beyond 6Å resolution remains a technical challenge due to sample

drift, charging and optical properties of the electron microscope, all of which do not effect electron diffraction. Molecular replacement, which is a common procedure in X-ray crystallography, was only recently used in electron crystallography to solve the 1.9 Å structure of AQP0 [8]. As in X-ray crystallography, molecular replacement method relies on the availability of a closely related structure. Phase extension offers a more general method, which shows great promise for electron crystallography [50]. An approach tailored for electron crystallography starts by combining low-resolution phases from images with amplitudes from electron diffraction to produce an initial, low-resolution 3D map (e.g., at 6–7 Å resolution). This map is used to place poly-alanine helical fragments to produce a starting model that is used to extend the phases. After combining experimental and model phases to produce a new, higher-resolution map, density modification is used to improve the map and thus to allow a more accurate model to be built. The efficacy of this approach was demonstrated on three membrane proteins, whose phases quickly increased from 6 Å to atomic resolution and, in the process, revealed density for ligands and lipids that were never included in the model. This phase extension procedure represents an exciting alternative to high-resolution imaging and has potential to greatly accelerate structure determination for well ordered membrane protein crystals.

Conclusions

These various developments illustrate that despite a period of inactivity, electron crystallography is enjoying a Renaissance, with developments occurring at all stages of the pipeline. As the improved methods come into common use, we can look forward to routinely evaluating the structure of membrane proteins within their native bilayer environment. Given perennial questions regarding the effects of detergent and of a crystalline environment on the conformation and intermolecular interactions of proteins, we believe that electron crystallography will have a valuable and ongoing role to play in elucidating the structure and function of membrane proteins.

Acknowledgments

The authors thank Dr. Andreas Engel for providing the diffraction pattern and molecular structure used in Box 1. The authors gratefully acknowledge support from the National Institutes of Health (R01 GM095747 and U54 GM094598).

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Highlights

Two-dimensional crystals provide a native lipid environment for membrane proteins

Lipid-protein interactions have been studied by electron crystallography

Conformational changes associated with transport mechanism have been studied

Methods for high-throughput crystallization screens are under development

Phase extension may facilitate structure determination at atomic resolution

Figure 1.

Interactions between aquaporin and its annular lipids. The two structures compared in this figure resulted from electron crystallographic analysis of two-dimensional crystals produced in DMPC (a) and *E. coli* polar lipids (b). In each case, seven lipids are shown distributed around the periphery of AQP0. The remarkable observation was that bilayer thickness and AQP0 conformation was not affected by this rather substantial change in lipid composition. Furthermore, individual aliphatic chains can be seen occupying the same grooves on the surface of AQP0. The take-home message seems to be that, at least in the case of AQP0, the lipid adapts itself to the surface of this protein. PDB codes for the structure are 2B6O in (a) and 3M9I in (b).

Figure 2.

Conformational changes in CopA and NhaA evaluated by electron crystallography. (a–b) The map of CopA determined by helical reconstruction of membrane crystals is shown in grey and cytoplasmic domains were fitted with a homology model (PDB code 3J08). This atomic model fits the map densities extremely well, illustrating that at 9 Å resolution, elements of secondary structure are visible in the experimental map. (c–d) The X-ray structure for CopA (PDB code 3RFU) fits the EM map very poorly, reflecting a conformational change in the molecule. The central phosphorylation (P) domain has been aligned in panels (a–d), but comparison of (b) with (c) shows that all the other domains have shifted. This conformational change is partially attributable to the phosphate analogue included in the X-ray crystallization buffer, but also reflects the thin DMPC bilayer used for the two-dimensional crystals, which results in a 30° tilt in the membrane domain. (e) X-ray structure for the NhaA dimer (PDB code 1ZCD) fitted to the 3D map determined by electron crystallography. (f) Projection map summarizing difference densities for NhaA induced by high pH (blue) and by binding of Na⁺ or Li⁺ substrate ions (red). The helices of NhaA are also shown in grey. The authors conclude that pH-dependent activation of NhaA results from ordering of its N-terminus and that substrate binding causes movement of the periplasmic end of helix IV.