Two-dimensional Electron Crystallography

Thomas Braun, *University of Basel, Basel, Switzerland*

Andreas Engel, *University of Basel, Basel, Switzerland*

Electron crystallography allows the structural analysis of two-dimensional (2D) protein crystals up to atomic resolution. Planar 2D crystalline arrays are reconstituted from lipids and membrane proteins, which are thus regularly arranged and in a close-to-native environment.

Advanced article

Article Contents

doi: 10.1038/npg.els.0003044

Introduction

Biological membranes fulfil two main functions: (1) to separate the cell from the surrounding environment and to subdivide the (eukaryotic) cell into compartments; (2) communication and interaction with the environment or different compartments. These seemingly contradictory functions are closely related to the structural and chemical composition of biological membranes. The first is a property of the membrane lipids that build up a hydrophobic barrier for water and water-soluble solvents. The second function is primarily mediated by proteins embedded in the lipid bilayer. They are responsible for transport of matter, energy conversion, cell adhesion and signal transduction.

Therefore, it is not surprising that around 30% of the sequenced genes appear to code for membrane-spanning proteins, of which only 60 crystal structures are available, compared to over 10 000 unique structures of soluble proteins. This lack of structural information is in huge contrast to the enormous medical interest. For instance, around 50% of all modern therapeutics are targeted to G protein-coupled receptors (GPCRs) (Howard *et al*., 2001).

Several techniques allow structural information of membrane proteins to be collected, but all of them suffer from the notorious problems related to handle membrane proteins. They are adapted to the lipid bilayer and tend to denature when extracted from this environment. Membrane proteins have a belt of hydrophobic residues in the transmembrane region. This region has to be stabilized by detergent molecules during preparation for structural analysis. Unfortunately, many detergents suitable for solubilization of membranes induce aggregation of fragile membrane proteins.

Nevertheless, 3D crystallization of membrane proteins has recently improved, leading to several new membrane-protein structures. The strength of X-ray crystallography is the established technology that allows data to be collected and structures to be solved to high resolution with enormous efficiency. However, this route to establish the atomic structure of a membrane protein is still risky as high-resolution 3D crystals are needed. Solution nuclear magnetic resonance (NMR) is the other established method for atomic structure determination. It does not require 3D crystals and allows the dynamics of a protein to be measured. Progress in assessing the structure of large complexes has been reported. Difficulties with the stability of solubilized membrane proteins kept at high concentrations, however, can be a problem.

As alternatives, techniques for the structural exploration of membrane proteins in their native environment in a lipid bilayer and under physiological buffer solution have been developed. The solid-state NMR could become a potential technique as demonstrated recently (Patzelt *et al*., 2002). Electron crystallography, for which the protein is reconstituted in 2D crystals in the presence of lipids is another promising technique. This approach restores the native environment of membrane proteins and thus their biological activity. Cryo-electron microscopy (cryo-EM) then allows the assessment of the static 3D structure of highly ordered 2D crystals at atomic resolution, yet even badly ordered crystals can give valuable structural information. Furthermore, the atomic force microscope (AFM) can provide subnanometer spatial resolution when operated in buffer solution (Engel and Müller, 2000). The outstanding signal-to-noise (S/N) ratio provided by the AFM makes the observation of single protein conformations and their dynamics possible. By combining such structural methods with biochemical experiments, spectroscopy, bioinformatics and molecular dynamics, a deep insight of the function of membrane proteins can be reached as demonstrated for aquaporins (Murata *et al*., 2000).

In a successful electron crystallography project, three main milestones have to be reached: (1) highly ordered 2D crystals with diameters of about 1–10 μm have to be produced; (2) high-resolution electron microscopic images have to be recorded; and (3) intensive image processing has to take place to obtain a 3D representation of the protein. The aim of this review is to discuss the technical aspects of

2D crystallography from crystallization to structural analysis at atomic resolution.

2D Crystallization of Membrane Proteins

2D crystals consisting of membrane proteins and lipids are obtained in three different ways: (1) by inducing regularly packed arrays of a highly abundant protein in its native membrane; (2) by reconstitution of the purified membrane protein into a lipid bilayer at high protein density; and (3) by the monolayer technique. In the case of (2) and (3), the purified and solubilized protein is mixed with solubilized lipids and the detergent is subsequently removed to reconstitute the protein in a membrane bilayer. After the completion of the crystallization process, the samples are negatively stained, and observed in the electron microscope (EM) for the crystallinity of the sample.

In general, membrane proteins are expressed in very low amounts in their native environment; thus, they have to be overexpressed recombinantly in suitable host cells. This is especially important for mammalian proteins and can be a major challenge on its own. Once obtained in sufficient amounts, the protein has to be separated from the host-cell proteins. Purification involves solubilization of the original membrane with detergent and subsequent separation steps. The pure protein, often complexed with residual lipids, is kept in solution by an appropriate amount of detergent. Bound lipids may contribute to the stability of the membrane protein and may be essential for successful 2D crystallization.

During the crystallization process, the interaction between the detergent–protein complexes and detergent–lipid is weakened, such that the hydrophobic surfaces of the proteins are exposed, leading to aggregation of protein and lipid. The detergent is either removed by dialysis, inactivated by binding to Biobeads or brought below the critical micelle concentration (CMC) by dilution. The chosen technique depends on the detergent(s) used and influences the rate in which the protein–lipid interaction is increased.

The critical moment in the life of a membrane protein during the crystallization process is the time point when the lipid molecules replace the detergent molecules to stabilize the protein. The absorption–desorption kinetics of the detergent molecule is not necessarily the same for the protein and the mixed micelles or the lipid molecules, respectively. The chance for the protein to interact with the appropriate lipids early enough increases when the crystallization cocktail can be incubated before detergent removal. If the interactions between the detergent–protein and detergent–lipid are not balanced suitably during the reconstitution, unspecific aggregation occurs and the protein is lost.

Many membrane proteins are destabilized upon solubilization, especially when short-chain (high CMC) detergents are used. Smaller but harsher detergents are an advantage for dialysis-driven 2D crystallization. Proteins can be effectively solubilized with low CMC detergents that replace the lipid and keep the hydrophobic surfaces of the protein shielded from water. Unfortunately, such detergents are not easily removed. Thus, the choice of detergent is critical: there is a fine balance between disruption of the membrane to solubilize a membrane protein and preserving its structural integrity. Furthermore, a suitable lipid mixture has to be found for a protein–detergent system to achieve reconstitution and crystallization of the protein.

With an excess of lipid over protein, the protein is mainly incorporated into lipid bilayers, similar to its native state. In an excess of protein over lipid, however, some of the protein aggregates, most likely in a denatured form. An important parameter is therefore the lipid:protein ratio (LPR), which should be low enough to promote crystal contacts between protein molecules, but not so low that the protein is lost to aggregation.

Despite an appropriate lipid and detergent combination, several proteins have been reported to depend on additives for crystallization: divalent ions seem to be especially important, among them Mg^{2+} having a special role, likely as a result of its interaction with the lipids. **See also**: Crystallization of proteins: two-dimensional

Data Acquisition

Two major problems in electron microscopy have to be overcome to reach high-resolution 3D structures. First, the strong interaction of electrons with matter leads to rapid sample destruction and second, the crystals have to be prepared such that the high-resolution structure is preserved within the vacuum of the electron optical system. Interestingly, the same answer was found for both problems by one technique – cryo-EM (Dubochet *et al.*, 1988). With this technique, crystals are adsorbed on to a thin carbon layer, surplus liquid is blotted away and the sample is layer of amorphous ice or in layer of carbohydrates substituting water, which conserves fine frozen quickly in liquid ethane. By this preparation method, the crystals are embedded in a thin structures of the 2D crystal (**Figure 1**). As a result of the low temperature of the specimen (at least liquid nitrogen temperature, 90 K), the sample is less susceptible to beam-induced damage, allowing images to be recorded at electron doses of approximately $500e^{-}/nm^{-2}$. If the sample is cooled down to liquid helium temperature (4.2 K), electron doses of $2000e^{-}/nm^{-2}$ are possible without intolerable sample damage (Fujiyoshi, 1998). **See also**: Electron cryomicroscopy

EM can record data in two ways. First, images of the crystal can be taken acquiring both amplitude and phase information. Second, electron diffraction patterns can be

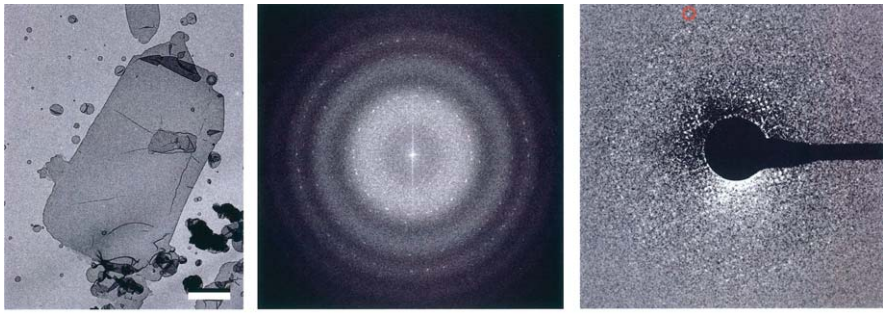

Figure 1 Electron microscopic images and electron diffraction of 2D crystals. (a) Overview image of a vitrified 2D crystal. Scale bar, 2 μm. (b) Power spectrum of a high-resolution image of a 2D crystal. The periodic arrangement of the protein in the 2D crystal leads to a periodic signal in the recorded image. Therefore, the $|modulus|^2$ of the Fourier transform, i.e. the power spectrum, shows discrete spots, in which the structural information of the protein is concentrated. Furthermore, noise (all information outside the spots) and the CTF (see text) is visible, (c) Electron diffraction of a highly ordered 2D crystal which is not affected by the CTF but does not carry phase information. The red circle indicates order 26,0 at 3.7 Å resolution.

taken to measure the amplitudes directly, similar to the diffraction experiments in X-ray crystallography.

In the imaging mode, the electrons scattered by the sample are focused in the image plane of the optical system, where scattered and unscattered electrons interfere to form an image. Only the elastically scattered (and coherent) electrons contribute to a high-resolution image, whereas the inelastically scattered electrons lead to sample damage and noise. The phase contrast is very weak when in focus, but can be greatly enhanced by moving out of focus because the phase difference between the scattered and unscattered electrons becomes $\pi(2n+1)/2$. The phase shift of the elastically single scattered electrons is proportional to the coulomb potential of individual atoms the electron passed by. Therefore, the ensemble of all single scattered electrons produce a projection of the coulomb potential, which is dominated by the atom's nuclei rather than the electron shells as in X-ray crystallography.

Figure 1b shows the power spectrum of a 2D crystal image. This spectrum reveals: (1) the discrete spots representing the crystal information (see image processing); (2) concentric rings (Thon rings) with gaps in between where no structural information is available; and (3) the envelope modulation decreasing the signal intensity at higher resolutions.

Thon rings and envelope function reflect the specific nature of the contrast transfer function (CTF) of EMs. The decrease in contrast is due to the partial incoherence of the electron beam. Modern EMs are equipped with field emission guns exhibiting a high degree of coherence so that the contrast decrease is acceptable even at high resolution. The Thon rings with alternating positive and negative contrast are the result of the phase shift between scattered and unscattered electrons being multiples of π. These two effects have to be corrected prior to any further image processing steps.

Electron diffraction is not affected by the CTF and the envelope function. The directly measured amplitudes can be combined with the phases from the images during image processing. Electron diffraction is not an absolute requirement for determining a structure, but it allows a fast judgement of the crystal quality, helps correcting the CTF and provides high-resolution information. Provided a related structure is available, such data can be used for molecular replacement approaches.

Image Processing

Images recorded by cryo-EM have an extremely low S/N ratio. Therefore, the structure of a 2D crystal can only be seen after image processing. In the power spectrum, the crystallinity of the sample is manifested in discrete spots containing the structural information of the periodically arranged protein. In contrast to X-ray crystallography, not only the amplitude of the diffraction pattern but also the corresponding phase can be measured, both of which are read from the calculated Fourier transform. A Fourier peak filtering step allows noise reduction (**Figure 2c**). After this simple image-processing step, some structural features can usually be recognized in the filtered image (**Figure 2d**, inset). Crystal defects (distortions, lattice defects and mosaicity) become visible. Since the image is available, lattice distortions can be corrected, which should only be done after CTF correction. For the former, the cross-correlation of the image with a small reference (**Figure 2e**) is calculated and the real position of the unit cells are detected (**Figure 2f**) (Saxton and Baumeister, 1982). The reference may be a small area of the original image housing a few unit cells or may be the average of other image(s). The unit-cell positions are compared to the theoretical lattice vectors and a field of shift vectors describing the crystal distortions can be calculated (**Figure 2g**). This information is used to interpolate the original image for unbending the crystal structure. By this real-space procedure, the diffraction spots are focused (cf. the insets in **Figure 2h**). This combination of

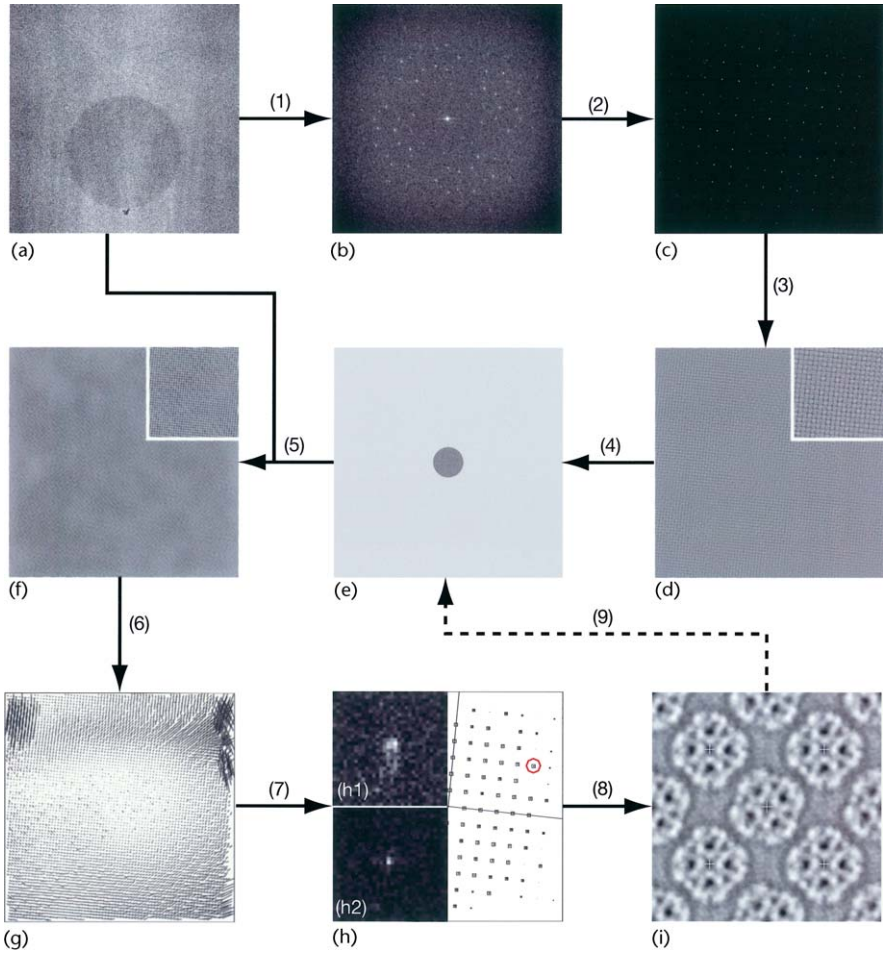

Figure 2 Fourier peak-filtering and unbending of 2D crystals. The raw image (a) is Fourier transformed (1) and the crystal lattice is indexed in the power spectrum (b) of the raw image. Note that in this case, two crystalline layers of the flattened crystalline vesicle can be separated as they are rotated with respect to each other. For the Fourier peak-filtering, the spots containing all the crystal information are cut out, and the amplitudes outside the mask-area (containing the other crystal layer and noise) is set to 0 (2) as illustrated in (c). The inverse Fourier transform (step (3), as shown in (d)) reveals the packing of the crystal (inset). To unbend the 2D crystal, a reference (e) is generated (4) and a cross-correlation (5) with the raw image is calculated. The cross-correlation (f) reveals the positions of the unit cells. These can be compared to the index of the crystal (ideal crystal) (6) to generate distortion vectors (g). This information is then used to interpolate the raw image to unbend the crystal, but this step is only executed after the correction of the CTF (7). As a result, the spots of the power spectrum are better focused (h). In inset (h1), peak 5,3 (indicated with a red circle) is depicted before unbending and in (h2), after unbending. Amplitudes and phases of the spots are read out and combined with the data of other crystals (8) to yield a final projection map. Such a result can be seen in the 3.7 Å map of GlpF (i) revealing the typical tetrameric structures of an aquaglyceroporin. This map can also be used to generate a synthetic reference for the unbending procedure (9).

crystallographic methods in Fourier space and image processing methods in real space allows the resolution to be improved by a factor of 2 (Henderson *et al*., 1990).

To get a 3D structure, the 2D crystal has to be tilted in the EM, thus 'side-views' of the protein are recorded (see **Figure 3**). These images are Fourier peak filtered and unbent in the same way as the untilted ones (**Figure 2**). The different images are combined in the Fourier space according to the central section theorem, shifting the phase centre of each projection to the origin of the Fourier space. The amplitude and phases of the crystals are concentrated on lattice lines in the z^* direction (see **Figure 3b**). Since there is no repetition in the z direction of the crystal, the lines are sampled continuously in the Fourier space and can be interpolated to obtain a regularly spaced 3D sampling (**Figure 3c**). Note that not all of the Fourier space can be sampled as a result of the maximal tilt angle (around 60°) of the sample, thus leaving a cone without data.

In theory, 0° and high tilt angle projections would give all the information to obtain a high-resolution 3D representation of the unit cell. Practical limitations necessitate the recording of images at low tilt angles. Bending of the carbon support film and the 2D crystals leads to crystal distortions. This has a larger affect at high tilt angles than around 0°

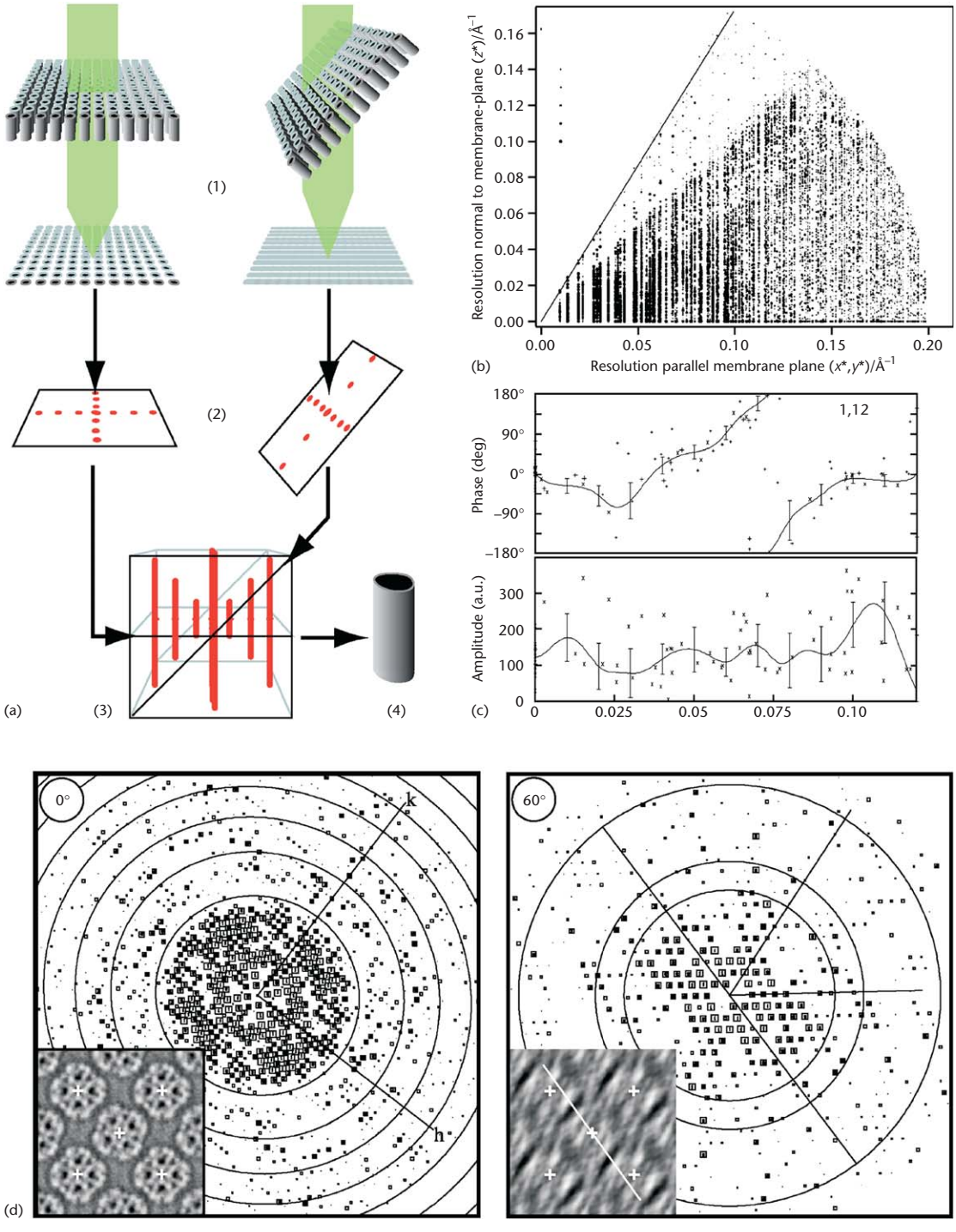

Figure 3 3D reconstruction of membrane proteins by 2D crystallography. (a) To obtain a 3D reconstruction from 2D crystals, projections are recorded at different tilt angles (1). The images are Fourier-filtered and processed (2), and the Fourier transforms are combined in the 3D Fourier space according to the central section theorem (3). The inverse Fourier transform reveals the unit cell structure (4). The discrete orders in the Fourier transform from the crystal are aligned in continuous lattice lines since the sample is not periodic in the *z* direction. The lattice lines are regularly interpolated to sample the 3D Fourier space on a cubic raster. Back-transformation of the combined data finally leads to the representation of the 3D unit cell (4). (b) Azimuthal projection of the sampling in z^* direction. The different tilt angles can be distinguished. In this case, a maximal nominal tilt angle of 60° was applied indicated with a black line revealing the missing cone. The lattice lines are visible; an example is given in (c). Amplitude and phase of lattice line 1,12 revealing a *z* resolution of $(1/7\,\text{Å})^{-1}$. The plotted curve indicates the interpolation of the lattice line. (d) Power spectra of an untilted (0°) and 60° tilted 2D crystal. The inset shows the Fourier-filtered projection map from an unbent image. Perpendicular to the tilt axis (line in the 60° panel), the resolution is reduced as a result of support nonflatness and charging.

tilts. The second problem concerns charging effects. Inelastically scattered electrons induce positive charges on the sample, which leads to the deflection of the electron beam at tilt angles $\neq 0^\circ$ and thus image shifts for tilted samples. All effects result in a decrease in the resolution perpendicular to the tilt axis as shown in **Figure 3d**. Finally, a continuous set of tilted projections greatly reduces the initial problem of merging the 2D data in 3D space.

Data Interpretation

The combination of highly ordered 2D crystals and electron microscopy can result in 3D maps of resolution better than 3 Å. Even low-resolution maps (resolution worse than 4 Å) can give useful biological information, especially when combined with other techniques. As an example, the calcium pump of the sarcoplasmatic reticulum at 7 Å resolution complemented the high-resolution X-ray structure with a different conformation suggesting large domain movements.

A combination of mass spectroscopy, AFM and electron microscopy of 2D crystals allowed the identification and structural characterization of a putative Ca-binding domain in the *C*-terminus of the AQP1 protein. The high S/N ratio of the AFM technique also allowed the conformational space of the surface loops of membrane proteins to be sampled as demonstrated for AqpZ, porin OmpF and bacteriorhodopsin (BR). **See also**: Water channels

Interpreting a cryo-EM potential map in terms of an atomic protein structure is still a challenging task, even when relatively high-resolution data of better than 4 Å are available. The first step is to determine the macromolecular fold and to trace the backbone through the map, which can be achieved by visual inspection. Structural clues can further be obtained through bio-informatics methods. Extensive sequence alignments and the analysis of correlated mutations led to valuable insights in the case of AQP1. In the case of α-helical proteins, automated procedures have been developed to determine both the location and direction of individual helices in the map. When used in conjunction with constraints from the sequence (e.g. the maximal length of a loop), macromolecular folds can be unambiguously derived. Once the fold has been determined, an initial model of the backbone structure can be generated. What follows are many rounds of manual model (re)building and refinement. The structures of BR and AQP1 have been solved by EM in this way and were later confirmed by X-ray crystallography. These successes demonstrate that even at a resolution of around 3.5 Å, a cryo-EM map contains sufficient structural information to uniquely define the atomic structure. The practical challenge is to find this optimal structure in the high-dimensional search space. Since an exhaustive search is not feasible, there is no straightforward general method to arrive at the correct (or best possible) solution at this level of resolution. The available tools are for a substantial part the same as used in X-ray crystallography, but if used inappropriately, the usually lower resolution may lead to inaccurate interpretations of the data, slow convergence of the refinement, or poor model quality. In contrast to X-ray crystallography, experimental phase information is available in the case of EM. This advantage is not as yet fully exploited and requires the development of novel automated techniques specifically targeted at model building and refinement of EM data (Gonen *et al*., 2004).

Conclusions

Microscopy techniques open important possibilities to assess the structure, dynamics and function of membrane proteins. Electron diffraction and microscopy allow the structure of proteins to be determined at atomic-scale resolution. Compared to X-ray crystallography, the microscopy methods are in many respects less advanced simply because only a small community push their development. Yet these methods offer many attractive advantages and will gain importance in the future.

References

Dubochet J, Adrian M, Chang JJ *et al*. (1988) Cryo-electron microscopy of vitrified specimens. *Quarterly Reviews of Biophysics* **21**: 129–228.

Engel A and Müller DJ (2000) Observing single biomolecules at work with the atomic force microscope. *Nature Structural Biology* **7**(9): 715–718.

Fujiyoshi Y (1998) The structural study of membrane proteins by electron crystallography. *Advanced Biophysics* **35**: 25–80.

Gonen T, Cheng Y, Kistler J and Walz T (2004) Aquaporin-0 membrane junctions form upon proteolytic cleavage. *Journal of Molecular Biology* **342**: 1337–1345.

Henderson R, Baldwin JM, Ceska T *et al*. (1990) Model for the structure of bacteriorhodopsin based on high-resolution electron cryo-microscopy. *Journal of Molecular Biology* **213**: 899–929.

Howard A, McAllister G, Feighner S *et al*. (2001) Orphan G-protein-coupled receptors and natural ligand discovery. *Trends in Pharmacological Sciences* **22**(3): 132–140.

Murata K, Mitsuoka K, Hirai T *et al*. (2000) Structural determinants of water permeation through Aquaporin-1. *Nature* **407**(6804): 599–605.

Patzelt H, Simon B, terLaak A *et al*. (2002) The structures of the active center in dark-adapted bacteriorhodopsin by solution-state NMR spectroscopy. *Proceedings of the National Academy of Sciences of the USA* **99**(15): 9765–9770.

Saxton WO and Baumeister W (1982) The correlation averaging of a regularly arranged bacterial cell envelope protein. *Journal of Microscopy* **2**(127): 127–138.

Further Reading

Crowther RA, Henderson R and Smith JM (1996) MRC image processing programs. *Journal of Structural Biology* **116**(1): 9–16.

Henderson R, Baldwin JM, Downing KH, Lepault J and Zemlin F (1986) Structure of purple membrane from *Halobacterium halobium*: recording,

measurement and evaluation of electron micrographs at 3.5 Å resolution. *Ultramicroscopy* **19**: 147–178.

Jap BK, Zulauf M, Scheybani T, *et al*. (1992) 2D crystallization: from art to science. *Ultramicroscopy* **46**(1–4): 45–84.

Stahlberg H, Fotiatis D, Scheuring S, *et al*. (2001) Two dimensional crystals: a powerful approach to assess structure, function and dynamics of membrane proteins. *FEBS Letters* **504**: 166–172.