Pumping ions

Membrane proteins present a particular challenge to structural biologists. Good three or two-dimensional crystals are the most important prerequisite for any direct insight into their atomic structure and molecular mechanisms, yet membrane proteins are notoriously difficult to crystallize. One of the few exceptions is bacteriorhodopsin (BR) which forms near-perfect two-dimensional crystals already in the cell membrane of a salt-loving bacterium where it pumps protons out of the cell, using sunlight as a source of energy. These 2D crystals are ideally suited for structure analysis by electron microscopy and image analysis. Accordingly BR was the first membrane protein for which a three-dimensional structure was determined originally at 7 Å (ref. 1). Throughout the past 20 years, BR has fueled the development of new and better electron crystallographic techniques at ever higher resolution. Today, it is one of the most-investigated and best-understood of all membrane proteins. An atomic model based on a 3.5 Å 3D map was published in 1990[2], followed by the refined structure[3] and now by a 3 Å map[4].

The ground-breaking work by Henderson and colleagues[2] had shown the structure of the seven trans-membrane helices very clearly and resulted in a detailed model of proton pumping. However, the loops connecting the helices on the membrane surface were not resolved and were thought to be disordered. In the paper now published by Kimura *et al.*[4], the loops are almost as clearly seen as the trans-membrane regions. This is due to two technical advances. For one, Kimura *et al.* prepared very flat specimens of the two-dimensional BR crystals which enabled them to collect data at higher tilt angles than before. They also used an electron microscope capable of recording higher resolution detail, by a combination of low specimen temperature (close to 4K), high voltage (300 kV) and the extra benefit of a highly coherent electron beam produced by a field emission gun. Together these factors account for the greater completeness of the data and thus for the better definition of their map.

Both membrane surfaces show the strategic location of charged residues around the entrance and exit of the proton channel. It is likely that these side chains help to conduct protons to the channel on the cytoplasmic side and disperse them on the exoplasmic side (Fig. 1). The authors draw attention to a potential feature of their map which may enable them to distinguish negatively charged residues from uncharged ones: since electrons are charged particles, they are sensitive to the charge distribution in the crystals. Atoms carrying negative charges have negative electron scattering factors at low resolution. Therefore negatively charged aspartates and glutamates should in principle show up less well than other side chains. This effect had already been noted in the 3.4 Å map of the plant light harvesting complex[5] which

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Fig. 1 A ribbon diagram of the 3 Å structure of bacteriorhodopsin, determined by electron microscopy of two-dimensional crystals[4]. Charged residues on the inner (top) and outer surfaces and in the proton channel are circled. The likely pathway of protons being pumped through the membrane (light blue) is indicated by green arrows. The retinal bound in the centre of the α-helical bundle is purple, and the newly visualized β-sheet structure on the extracellular side is light green. This figure was kindly provided by Y. Kimura (ref. 4).

was determined by the same method. However, some of the asparates and glutamates on the BR surface which have well-defined densities in the 3 Å map and are tentatively presented as uncharged by Kimura *et al.*, seem to be exposed to the aqueous medium and are unlikely to be protonated at neutral pH. It is therefore not yet clear whether electron crystallography can actually tell the charge of an amino acid side chain, or whether the effect is partly obscured by noise.

Bacteriorhodopsin, probably the most simple and one of the most ancient ion pumps in living cells, continues to fascinate structural biologists and to inspire new technological developments. Very recently, tiny but well-ordered three-dimensional crystals of BR were grown in a cubic lipid phase[6]. These crystals were found to diffract X-rays to 2 Å in the microfocus beamline at the European Synchrotron Radiation Facility (ESRF) and have yielded a structure of BR at 2.5 Å resolution which has just been published[7]. The X-ray structure was solved by molecular replacement, using the atomic coordinates determined by electron crystallography[3] as a search model. It is therefore not surprising that the two structures are very similar overall, although the X-ray map contains small regions of density in the proton-pumping channel which have been interpreted as water molecules[7]. It is surprising, however, that some of the loops which show up clearly in the 3 Å structure reported by Kimura *et al.*[4] are less well defined or completely absent in the X-ray map[7]. Since it is unlikely that these loops are more highly ordered in two-dimensional crystals, this observation suggests that a 3 Å map with good phases, determined directly by electron crystallography, contains as much, or more structural detail than a 2.5 Å X-ray map.

Electron crystallography of two-dimensional crystals is now an established method. Although it does not yet produce structures at the same alarming rate as X-ray crystallography of soluble proteins, it has a firm place in the arsenal of structural biologists when it comes to tackling macromolecules that do not form well-ordered 3D crystals easily, in particular filamentous and membrane proteins. In the near future we will see several more exciting and unique structures determined by this powerful technique.

Werner Kühlbrandt

Max-Planck-Institut für Biophysik, Heinrich-Hoffmann-Str. 7, D-60528 Frankfurt / Main, Germany.

Correspondence should be addressed to W.K. *e-mail: kuehlbrandt@biophys.mpg.de*

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