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THE STRUCTURAL STUDY OF MEMBRANE PROTEINS BY ELECTRON CRYSTALLOGRAPHY

YOSHINORI FUJIYOSHI

Department of Biophysics, Faculty of Science, Kyoto University, Sakyo-ku, Kyoto 606-8502, Japan

Over the last few years, structural biology has grown into one of the most important fields in modern biology. While molecular biology is continually revealing many new proteins indispensable for life on earth, only their atomic structure can help us to really understand how these proteins accomplish their important biological functions. The information which can be drawn from a structure analysis of a protein strongly depends on the resolution achieved in the study. The higher the resolution of the determined structure can give the more information on the relationship between structure and function of a biological macromolecule. Electron microscopy provides useful biological information over a wide range of resolutions and this is summarized in Table I. However, the ultimate goal of every structural study of a protein will always be to determine its atomic structure, and from X-ray crystallographic and electron microscopic studies we know that a resolution of about 3.0 Å is sufficient to interpret structural detail on the level of amino acid side chains.

The foundation for crystallography was laid in 1913 by W. H. Bragg and his son W. L. Bragg (*1*). Due to methodological innovations, especially the development of the isomorphous replacement technique pioneered by Perutz (*2*), dramatic progress in recombinant techniques in molecular biology, and the massive progression

V. SPECIMEN PREPARATION TECHNIQUES

Electron crystallography requires 2D crystals which are easily deformed by very weak mechanical forces. The deformation causes blurring and/or extinguishing of diffraction spots particularly in the case of highly tilted specimens. Both images and electron diffraction patterns from highly tilted specimens are essential for high-resolution structure analysis. This means that a specimen support which minimizes the deformation of the specimen is of primary importance for data collection in high-resolution electron crystallography.

1. New Molybdenum Grids

As stated earlier, an electron cryo-microscope is required for the observation of proteins at high-resolution to reduce beam-induced radiation damage and to overcome the problem of specimen dehydration in the vacuum of the electron microscope by embedding the specimen in amorphous ice. However, cooling of the specimen leads to cryo-crinkling of the carbon film which is caused by different shrinkage of the carbon film and the supporting electron microscope grid when the temperature is lowered. This presents a serious problem when specimens are cooled to low temperatures. Booy and

Fig. 16. Electron microscopic system as currently installed in the International Institute for Advanced Research (IIAR, Matsushita, Ltd.). The 3rd version of our electron cryo-microscope is equipped with a super-fluid helium stage, a cryo-transfer device, an FEG, a slow scan CCD camera, and a spot scan system.

Pawley found that grids made of titanium, molybdenum, or tungsten dramatically reduce the cryo-crinkling effect (*21*), and more recently the use of molybdenum grids has become standard in electron cryo-microscopy. However, the surfaces of commercially available molybdenum grids are not flat and smooth, and the rough surface of these grids can also induce the carbon film to crinkle as shown in Fig. 17a and b.

We designed various types of molybdenum grids specifically for high-resolution electron crystallography. All the newly designed grids were 3 mm in diameter and had a thickness of 20 μm. While one grid design had circular holes 50 μm in diameter, a different design featured circular holes 100 μm in diameter. Holes with hexagonal shape were also created to increase the ratio of observable area, and various arrangements of the holes were tested. All grids were carefully manufactured by photochemical etching to preserve a smooth surface especially around the hole edges. After the etching, the grids were washed with sulfuric acid and distilled water. Ultrasonication in chloroform was the final step in cleaning of the grids. All these grids were checked in a scanning electron microscope (a typical example is shown in Fig. 17c) and the carbon film supported by such grids displayed no crinkling as shown in Fig. 17d.

2. *Carbon Films by Spark-less Evaporation*

While high quality molybdenum grids are important for electron crystallography, the flatness of the carbon film itself is even more important. Only an atomically flat surface of the specimen support film can be expected to preserve the planarity of the 2D crystals to the highest degree. A high quality carbon film can also reinforce the 2D crystals which adhere to it and reduce beam induced movement. Butt *et al.* examined the flatness of carbon films by atomic force microscopy and developed a multiple evaporation technique to prepare very flat carbon films (*22*). Although in our laboratory this multiple evaporation technique sometimes yielded very flat carbon films, the results were not very reproducible.

In our procedure to produce flat carbon films, a pure carbon rod (a spectroscopic carbon electrode 3 mm in diameter with a purity of 99.9999%) is thinned to about 1.5 mm diameter and mounted in a JEE400 vacuum evaporator. The most important

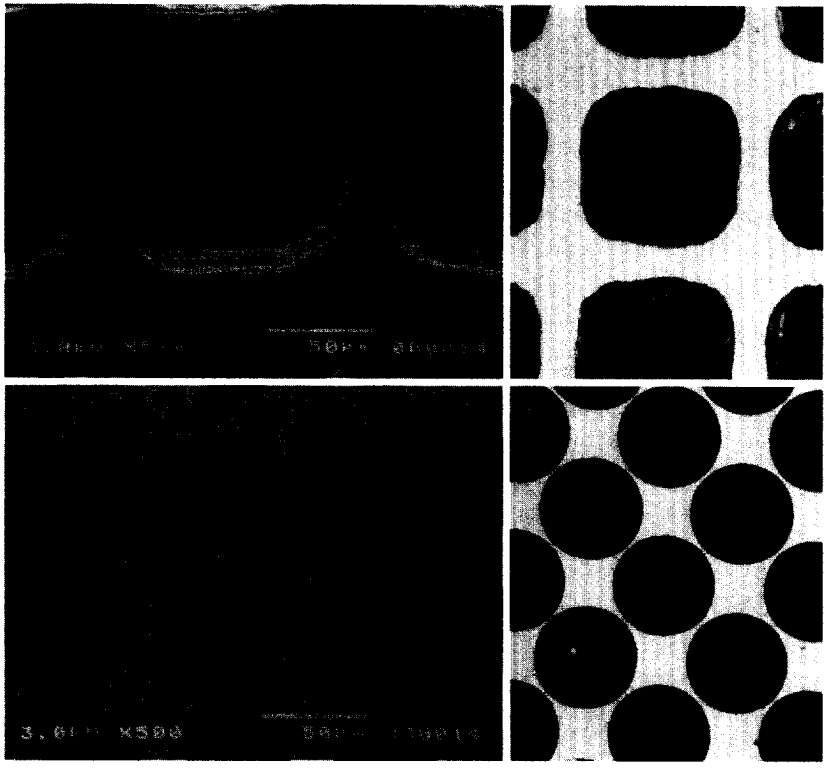

Fig. 17. Carbon film on molybdenum grids. (a) When imaged with a scanning electron microscope, the surface of a commercially available molybdenum grid appears quite rough. (b) The optical micrograph illustrates that a carbon film deposited on such a grid shows crinkles. (c) The surface of photochemically etched molybdenum grids is much smoother and (d) they induce no crinkling of the carbon film.

requirement for the preparation of flat carbon films is the spark-less evaporation of the carbon, and the most reliable technique to achieve this is a pre-evaporation of the carbon rod before preparation of the actual carbon film on mica. For this purpose, we thin an approximately 7 mm long stretch at one end of the carbon rod. While the freshly cleaved mica is covered, we evaporate carbon until the carbon stops sparkling during evaporation. Then, the cover over the mica is removed and the carbon deposited very slowly and carefully on the mica surface, avoiding any sparks. Spar-

edges of the holes of the EM grid. For highly tilted specimens, this grid must be used because at a tilt angle of 70°, the 50 μm holes are reduced to ellipses with a short axis of only 17 μm, which is relatively small to record high quality diffraction patterns. We therefore used molybdenum grids with a hole size of 100 μm which enabled us to record diffraction patterns of bR 2D crystals at a tilt angle of 70°. However, the flatness of the bR 2D crystals prepared on these grids was slightly inferior to the preparations on the grids with a hole size of 50 μm.

VI. STRUCTURE OF BACTERIORHODOPSIN

1. Proton Pump

bR is a membrane protein found in *H. salinarium* (*24*) with a bound retinal molecule which serves to absorb light energy. bR functions as a very efficient proton pump and absorption of a single photon by the retinal suffices to transport a proton from the inside of the cell to the outer medium. Many studies have been performed on bR and revealed the functional intermediates of the bR photocycle (*25*). These studies laid the basis for the proposed mechanism of the proton pumping by bR (*7*). However, details of the proton pumping mechanism by which a proton is actually transported from the inside to the outside are not known. It is not clear how protons are efficiently introduced into the channel of bR, how they are carried along the channel, or how the protons are released from the other side of the protein. To address these challenging questions, high-resolution electron crystallography has been recognized as one of the most promising tools.

Continuing on from their pioneering work, Unwin and Henderson (*6*), they were able to determine an electron crystallographic density map of bR at a resolution of 3.5 Å. Based on this 3D map, Henderson and his coworkers built an atomic model of the protein including the retinal molecule (*7*) and deposited the atomic co-ordinates in the Brookhaven Protein Data Bank (PDB). They thereafter improved their atomic model by crystallographic refinement with electron diffraction data corrected for diffuse scattering and additional phase information calculated from 30 new images of tilted specimens (*26*). The atomic coordinates of the resulting new atomic model of bR have also been registered in the PDB (2BRD).