MEMBRANE PROTEINS:
STRUCTURES, INTERACTIONS AND MODELS

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MEMBRANE PROTEINS: STRUCTURES, INTERACTIONS AND MODELS

PROCEEDINGS OF THE TWENTY-FIFTH JERUSALEM SYMPOSIUM ON QUANTUM CHEMISTRY AND BIOCHEMISTRY HELD IN JERUSALEM, ISRAEL, MAY 18–21, 1992

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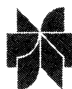

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PREFACE

The 25th Jerusalem Symposium represents a most significant highlight in the development and history of these meetings. Living within the decimal system we have celebrated with much pleasure the 10th and the 20th Jerusalem Symposia. With this one we experience a feeling of particular satisfaction because 25 years is different from, is more than, two decades and a half. It is a quarter of a century. It seems thus as if we have changed the dimension of our endeavour. In no way do we loose the sense of modesty with respect to the significance of these meetings. For the organizers, however, they do represent a continuity of efforts which we feel happy to have been able to carry out.

At this occasion it seems useful to say a few words about the origin of the Jerusalem Symposia and to recall the name of a colleague who played an essential role in their creation and has been a most efficient and devoted co-organizer of the seven first of them. This was Professor Ernst Bergmann, one of the most distinguished founders of Israeli Science and a world famous physico-organic chemist.

These Symposia were conceived by Ernst Bergmann and one of us (B.P.) as a means to commemorate the tremendous historical event of the reunification of Jerusalem which occurred as a result of the events of June 1967 known as the six-days war. This was the first time, since a long time, that this Holy City became a free City where everybody could come, walk, pray before the shrine he loves and respects. The idea of International Symposia on fundamental modern scientific subjects, bringing together to this place scientists from all parts of the world, appeared as the most appropriate way for scientists to celebrate this memorable happening.

The dreaming scientists were lucky to have a common, good and very generous friend who made it possible for them to transform the dream into reality. This was the Baron Edmond de Rothschild who, when informed about the project, was immediately enthusiastic. He liked the idea and, we are happy to say, trusted its authors to be able to make it a succes. His help was total and decisive. Continuing the glorious tradition of generosity of his family he gave them the means to do the work. These Symposia are as much his creation as that of the scientists involved.

The first Jerusalem Symposium took place in October 1968 and since then the Symposia continued on a yearly basis, uninterrupted by whatever events occurred, war (the Yom Kippur war in 1973) or death, Professor Bergman's death on April the 6th 1975 at the eve of the 8th Jerusalem Symposium.

However great and irreplaceable a loss his disappearance represented, the flame was kept alight and the movement continued.

We dare think that, on their modest scale, the Jerusalem Symposia have been a significant event in a number of their aspects. They contributed, we believe, in a very positive way to scientific exchanges and contacts and, we hope, also to the progress of science. Altogether over a thousand of distinguished scientists participated in these meetings, some of them in several, and the contacts produced at these occasions generated a number of interesting and mutually profitable collaborations.

It is our great pleasure to acknowledge that from the beginning, the Israel Scientific authorities and in particular the Israel Academy of Sciences and Humanities and the Hebrew University of Jerusalem became intimately and indefatigably associated with these Symposia. The prestige that their distinguished patronage added to our undertaking helped greatly to increase its international renown. Moreover, holding the Symposia in the beautiful setting of the Israel Academy offered an incomparably pleasant atmosphere of calm and serenity for the lectures and discussions.

Many persons have contributed to the success of these Symposia. Enumerating the names of all of them would just be too long. But we would like to mention specifically the name of Mrs. Avigail Hyam, Secretary General of the Israel Academy for many years who has taken care with remarkable skill and admirable devotion of all the material aspects of the local organization of these Symposia, from the first one up to this one and who, we hope, will continue to do so for the future ones. We address her our deep thanks.

The organization of the historical 25th Jerusalem Symposium was entrusted to Mrs Pullman both as a testimony of our gratitude for her constant and vigilant help in the preparation and holding of a number of these meetings, her efficient and succesful organization of some of them and because of her mastership of the subject chosen for this 25th gathering. We owe her particular thanks for having conceived, organized and directed this symbolically particularly important Symposium.

Joshua JORTNER and Bernard PULLMAN.

ELECTRON CRYSTALLOGRAPHY OF MEMBRANE PROTEINS

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ABSTRACT. The technical capabilities of electron diffraction and high resolution electron microscopy have recently been advanced to the point that three-dimensional density maps can be obtained at sufficiently high resolution to allow an interpretation in terms of the structure at atomic resolution. The high scattering intensity of electrons compared to x-rays results in the fact that two-dimensional crystals, one molecule thick, are ideal specimens for electron crystallography. In the event that large, well ordered, three-dimensional crystals cannot be obtained, which are required for x-ray diffraction, electron diffraction now represents a practical alternative for high resolution structure analysis. The conditions needed to obtain two-dimensional crystals are, in general, very different from those which can produce three-dimensional crystals. As a result, attempts to obtain two-dimensional crystals complement the more traditional approach, which would be to obtain crystals for x-ray crystallography.

I. Introduction

The use of electron diffraction and high resolution electron microscopy to determine the structure of biological macromolecules at atomic resolution is a very recent development. The initial idea of using electrons for the crystallographic structure analysis of thin samples of ordered biological macromolecules is more than 20 years old (DeRosier and Klug, 1968; Hoppe et al., 1968). The approach has become a well established, routine method for structure determination at low resolution (Amos et al., 1982; Glaeser, 1985), using either negatively stained samples or even the more sophisticated, frozen-hydrated type of sample (Dubochet et al., 1988). Although advances in methodology were made very early, which even made it possible to obtain the structure of bacteriorhodopsin at 0.7nm resolution (Henderson and Unwin, 1975), the technological capability of obtaining an interpretable density map at much higher resolution finally was realized only in the past few years (Henderson et al., 1990). The solutions to previous instrumental and methodological limitations which have been developed in advancing the work on bacteriorhodopsin are now also leading to rapid progress on crystals of other membrane proteins (Walian and Jap, 1990; Jap et al., 1991; Kuhlbrandt and Wang, 1991). From this recent work it is now clear that electron crystallography has become sufficiently well developed as a method of high resolution structure analysis that it can be regarded as a very good alternative to x-ray crystallography.

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There are two reasons why the development of high resolution electron crystallography may prove to be especially important for membrane-protein structural biology. The first reason has to do with the considerable difficulty that still exists in obtaining the large, well ordered crystals of detergent solubilized membrane proteins that are needed for x-ray diffraction work. As will be discussed further below, the conditions that produce thin, sheet-like crystals of membrane proteins are completely different from those which are needed to produce large, three-dimensional crystals. Electron crystallography therefore provides a second chance to get suitable crystals, in those cases where the effort to obtain crystals for x-ray diffraction seems to be unsuccessful. It should be stated, however, that there is still too little experience, as yet, to know whether crystallization of membrane proteins as thin sheets will prove to be generally more successful than has so far been the case for three-dimensional crystals. The second reason why electron crystallography will be important has to do with the fact that membrane proteins can be crystallized within the plane of a lipid bilayer. Not only does this fact allow for structural studies in a more native environment, it also makes it much easier to design experiments in which there is direct access to the membrane protein for functional studies. The work of Unwin and co workers on conformational changes in gap junctions (Unwin and Ennis, 1984) and the acetylcholine receptor (Unwin et al., 1988), although at low resolution, represent good examples of how straightforward it can be to relate structure and function when working with thin, sheet-like crystals.

2. Theoretical and Experimental Aspects.

The formal mathematical basis of electron crystallography is, in most respects, identical to that of x-ray crystallography. Since electrons are scattered by the (shielded) coulomb potential of the atomic nuclei, however, the density maps obtained in electron crystallography are maps of the coulomb potential, while those obtained in x-ray crystallography are maps of the electron density.

A practical consequence of the difference in the physical mechanism of scattering is that electrons are scattered more than 100,000 times more strongly than x-rays are. The advantage of this strong interaction is that good, single-crystal electron diffraction patterns can be obtained from samples that are as small as 2 μm in diameter, and only one molecule thick. The physical characteristics of electron scattering therefore make electron crystallography ideally suited for membrane proteins which have been crystallized within the plane of a lipid bilayer. It is equally important to recognize, however, that the strong scattering of electrons imposes a strong limitation on how thick the sample can become, before multiple scattering effects begin to invalidate the single-scattering (i.e. kinematic scattering) approximation upon which the crystallographic structure analysis must be based. Experimental measurements (Glaeser and Ceska, 1989) as well as theoretical simulations (Ho et al., 1988) suggest that multiple scattering effects may begin to be significant at a protein crystal thickness greater than 20nm or so.

Perhaps the most significant difference between electrons and x-rays, however, comes from the fact that electrons can be focused to produce a high resolution image, whereas x-rays cannot. Under suitable conditions of sample thickness, accelerating voltage, and resolution (Glaeser, 1982), the image intensity of the electron microscope image is proportional to the projection of the coulomb potential in the sample. The calculated Fourier transform of the image intensity therefore retains the crystallographic phase information that is needed to ultimately calculate the three-dimensional density

map. While it is true that the calculated Fourier transform also provides reasonable estimates of the amplitude of the crystallographic structure factor, much more accurate values of the diffraction amplitude can be obtained from the square root of the electron diffraction intensities, just as is done for x-ray diffraction studies.

Data collection in high resolution electron crystallography consists of two separate steps, the collection of diffraction patterns and the collection of images. The data in a single diffraction pattern or in a single image corresponds to data on a single "central section" in Fourier space, i.e. on a plane in Fourier space which passes through the origin and which is inclined at an angle that is determined by the tilt angle of the specimen. As is illustrated in Figure 1, both types of data are collected for as wide a

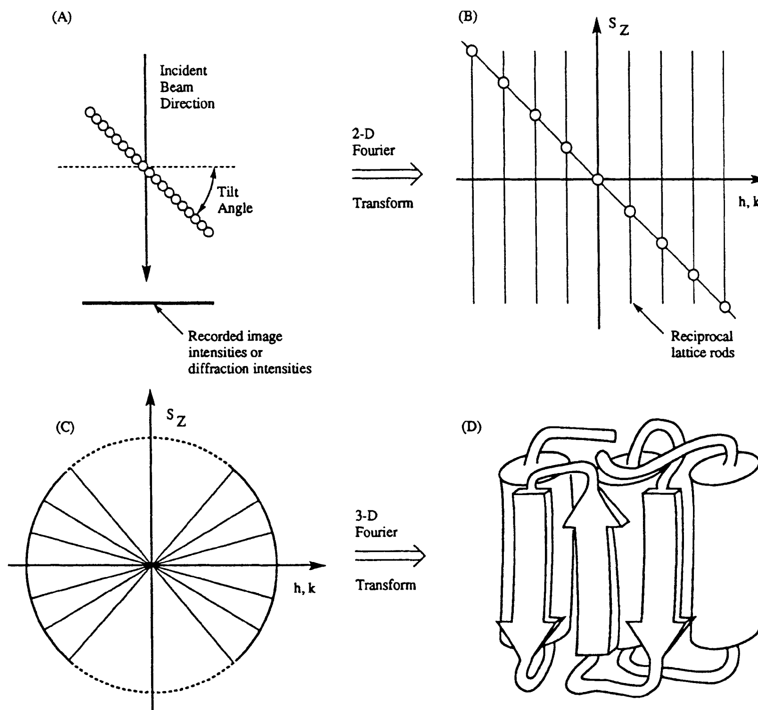

Figure 1. Schematic diagram to illustrate the main steps in three-dimensional electron crystallography at high resolution. (A) Electron diffraction patterns and electron microscope images are recorded separately for specimens tilted over a wide range of angles relative to the incident beam. (B) The data on any one image or diffraction pattern corresponds to the points in reciprocal space where a plane (central section) intersects the reciprocal lattice rods; while the Fourier transform of a two-dimensional crystal is continuous along these reciprocal lattice rods, it is, of course, discretely sampled in the other two dimensions. (C) Phases, obtained from the calculated Fourier transform of the image, are combined with diffraction amplitudes, obtained as the square root of the diffraction intensities. Data from many different tilt angles are merged to give a three-dimensional set of complex structure factors. (D) An inverse, three-dimensional Fourier transform is used to calculate a three-dimensional density map in real space. This density map must then be "interpreted" by fitting the known protein sequence to the observed densities, as in x-ray crystallography.

range of specimen tilt angles as is practical. The amplitudes from the diffraction patterns are then combined with the phases from the images to produce a three-dimensional data set of crystallographic structure factors. A three-dimensional inverse Fourier transform then produces a three-dimensional density map. If the map is obtained at sufficiently high resolution, it can be interpreted by fitting the known amino acid sequence to the map, giving rise to an atomic resolution model of the molecular structure of the specimen.

As a general rule, the effects of radiation damage are a far more important consideration in electron crystallography than they are in x-ray crystallography. The reason for this difference is due mostly to the fact that the crystals which are used in electron diffraction experiments are so small. As a matter of fact, the minimum crystal size is determined by the limitations of radiation damage rather than by the strength of electron scattering or by any optical or instrumental limitations. In learning to cope with radiation damage, one must first recognize that the total electron exposure should be kept below about 500 electrons/nm^2 at a specimen temperature below -100 C. The low specimen temperature makes it possible to use an electron exposure that is about 5 to 7 times higher than the maximum exposure that can be used at room temperature (Hayward and Glaeser, 1979), but even at low temperature the electron diffraction pattern is almost completely destroyed at exposures that are significantly above 500 electrons/nm^2. A more subtle effect of radiation damage is to cause small, apparently random movements of the specimen, presumably due to mechanical stresses resulting from radiolytic processes. While these movements have no effect on the electron diffraction pattern, they are the principal factor that make it difficult to get images, and hence phases, at a resolution above 0.7nm (Henderson and Glaeser, 1985). It has therefore been a significant development in high resolution electron crystallography to discover that the beam-induced movement effect can be practically eliminated by recording images in which only a very small area of the specimen is illuminated at any one time (Downing, 1991).

Two different methods can be used to preserve a native, hydrated state for specimens, within the vacuum of the electron microscope. Of these, perhaps the more intuitive approach is to freeze a very thin, aqueous film, surrounding the specimen, and to examine the specimen on a cold stage at a temperature so low that the ice does not even sublime (Taylor and Glaeser, 1974). With the introduction of techniques to freeze the water in the vitreous state (Dubochet et al., 1988), the "frozen-hydrated" specimen technique has become a very popular one. The other alternative is to embed the specimen in a thin film of an appropriate material which can maintain a hydrated state in vacuum, even at room temperature. Glucose was the first such material that was identified as being effective for this purpose (Unwin and Henderson, 1975), but several other materials have been found to be even more effective in specific cases, and no doubt many others will be identified in the future.

A few other practical considerations can be mentioned here briefly, although the basic references will have to be consulted for more complete details. Commercially available cold stages have generally not been as stable as their room-temperature counterparts (Downing and Chiu, 1990). While this causes no problems for electron diffraction work, there currently are very few installations where the cold stage is sufficiently stable to make it possible to obtain images at 0.35nm or better, especially at high tilt angles (Henderson et al., 1990). Imperfect specimen flatness can also be a practical factor that limits how easy it is to collect a high resolution data set at high tilt angles (Glaeser, 1991). While improvements in specimen flatness will certainly be a welcome technological advance in the years to come, the current difficulties in this

respect are not so severe that they should in any way discourage the use of the electron crystallographic approach. Finally, electron crystallography imposes formidable demands on the speed of available computational facilities and on the size of available disk memory. Both factors have become so inexpensive in the past few years, however, that the cost of setting up the needed computing facilities has become a small part of the total equipment costs.

In summary, the niche that can be occupied by electron crystallography in the world of structural molecular biology is completely distinct from that which has been served by previously existing structural techniques. The uniqueness of this niche originates from the very high scattering power of electrons, which in turn makes it possible to carry out single-crystal diffraction studies with sheet-like specimens that are only one molecule thick. Integral membrane proteins which have been crystallized within the plane of the lipid bilayer, and peripheral membrane proteins which have been crystallized on the surface of either a lipid monolayer or a lipid bilayer are likely to represent the largest single class of structural problems which stand to benefit from the development of electron crystallography.

3. How to Prepare Crystals of Integral Membrane Proteins That Are Suitable for High Resolution Electron Crystallography.

There currently are three different methods that are known by which it is possible to obtain crystals that can be used for high resolution structural studies. The first of these can be referred to as "negative purification," i.e. the enrichment of a membrane fraction by the removal of other, unwanted proteins and lipids. The second method can be called "purification and reconstitution," and it uses standard methods for the purification of integral membrane proteins in detergent. The third method is to simply crystallize the protein in detergent-solubilized form. Although intended to produce three-dimensional crystals that are suitable for x-ray diffraction studies, this third method sometimes produces thin, sheet like crystals that are ideal for electron diffraction. For a much more extensive discussion of the crystallization of membrane proteins, the reader should consult the recent review by Kuhlbrandt (1992).

If an integral membrane protein already forms crystalline arrays in the native cell membrane, as is the case for bacteriorhodopsin and for gap junctions, then a negative purification approach is the natural route to take. As shown by the work with bacteriorhodopsin (Baldwin and Henderson, 1984), such native crystalline arrays can be further annealed and fused by the use of detergents at a concentration below that which will solubilize the crystalline array. Generalization of this approach will no doubt benefit from further studies which would show us how detergents can affect the rim energy (Fromherz et al., 1986) of a membrane patch, or even clean the rim of adsorbed contaminants. It would also be useful to learn more about how detergents can increase the fluidity and diffusion of membrane proteins within crystalline arrays, at least in areas near to the rim. If the initial crystalline order is rather poor, selective removal of "excess" lipids with an appropriate detergent may possibly improve the crystalline order, as has been demonstrated by Sikerwar et al. (1991) for gap junctions.

It is not necessary for the membrane protein of interest to be in a pre-existing crystalline array for the negative purification approach to work, however. Cytochrome oxidase (Fuller et al., 1979) and the outer membrane porin of E. coli (Steven et al., 1977) are two examples where detergents were used to extract excess lipid from membrane fractions that had been enriched for the protein of interest, with the subsequent formation of crystalline arrays. The use of phospholipase to remove excess

lipid has also been demonstrated to be an effective way to bring about the formation of crystalline arrays (Mannella, 1984). Another approach that works is to add an inhibitor of protein function to membranes that are highly enriched for the protein of interest. Vanadate, for example, induces the crystallization of both the Na^+/K^+ ATPase (Skriver et al., 1981; Mohraz et al., 1987) and the Ca^{++} ATPase (Taylor et al., 1984), in their respective, enriched membrane fractions. It is likely that the effect of inhibitors in this case is to lock the protein into a unique conformation, and so to favor a unique class of protein-protein contacts.

The formation of 2-dimensional (2-D) crystals by reconstitution of detergent solubilized, purified protein should prove to be a much more generally applicable approach. The basic approach is an extremely simple one, assuming that the detergent solubilized, purified protein is already at hand. All that needs to be done is to mix the detergent-solubilized protein with detergent-solubilized lipid at a high protein-to-lipid ratio, and remove the detergent, usually by dialysis (Leonard et al., 1987). As little as 20 μg of protein (i.e. 20 microliters at 1 mg/ml) can be used for each crystallization trial, yielding enough material to make 10 or more electron microscope grids. When the approach works, as is the case for bacterial porins (Jap, 1988; Sass et al., 1989), the results can be extremely good. Complications in such a simple-appearing method can include the effort needed to find precisely the right protein-lipid ratio, selection of the right lipid type (which may depend upon both the polar head group and on the acyl chain composition), and selection of the right detergent; one does not want the critical micelle concentration for mixed micelle components to be such that the lipids separate from the detergent before the protein does, or vice versa, resulting in lipid vesicles and precipitated protein.

Although it is natural that the published literature does not show it, the number of cases where purification and reconstitution has failed to produce well-ordered crystals greatly exceeds the successes. The most disappointing type of failure is the one in which the protein and lipid reconstitute into large membrane sheets with a mass density like that of purple membrane, but the protein is not crystalline; this was the outcome of Kong's attempt to get 2-D crystals of halorhodopsin (Kong, 1989). Other modes of failure that have been encountered by different groups, for various proteins, include both the formation of particulate, lipoprotein-like complexes rather than the desired 2-D sheets, at the high protein-lipid ratios that are desired for crystallization, and the formation of 2-D crystals which are not well ordered at high resolution. Too little work has as yet gone into the effort to crystallize membrane proteins by purification and reconstitution, however, to conclude that success will be the exception rather than the rule; as more is learned about what makes the 2-D crystallization of some proteins succeed, it is likely that the same biochemical techniques and know-how will be applicable to systems which previously had failed.

The fact that sheet-like crystals are sometimes formed by detergent-solubilized membrane proteins under conditions that were meant to produce 3-D crystals (Kuhlbrandt, 1987; Stokes and Green, 1990) is, without question, unexpected. When this does happen, however, the result is just as useful as if crystals suitable for x-ray diffraction had been obtained. The electron crystallographic work on the photosynthetic light-harvesting complex reported in this volume by Kuhlbrandt is a good example of how a high resolution density map can be obtained from such crystals, when no structural progress could be made by other methods.

Before concluding, it should also be mentioned that tube-like, or cylindrical "crystals", (i.e. ordered assemblies) are sometimes obtained by both the "negative purification" approach and by the "purification and reconstitution" approach. Examples

include the nicotinic acetylcholine receptor (Toyoshima and Unwin, 1990) and the Ca^{++} ATPase (Taylor et al.,1984). Helical (cylindrical) samples have one significant advantage in that a full 180 degree range of tilt angles is represented in a single image, without any need to actually tilt the specimen. The disadvantage of such specimens is that there are relatively few unit cells present in such images, which in turn limits the attainable signal-to-noise ratio that can be achieved in the subsequent data processing. It is impossible to obtain high resolution "single crystal" diffraction patterns from such samples, although there is no limitation, in principle, to getting useful information from the high resolution fiber diffraction patterns that could be obtained from oriented "rafts" of such cylindrical specimens. The difficulty of averaging data from a very large number of images of such specimens, and the lack of single-crystal electron diffraction intensities means that progress in getting interpretable density maps at atomic resolution from cylindrical specimens will be very much more difficult than it is for 2-D crystalline sheets.

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HIGH-RESOLUTION ELECTRON MICROSCOPY OF MEMBRANE PROTEINS

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ABSTRACT. The determination of membrane protein structures at high-resolution requires crystallographic methods. Progress with x-ray crystallography of three-dimensional (3D) crystals has been less rapid than was hoped 10 years ago. However, electron microscopy and electron diffraction of two-dimensional (2D) crystals now offers a viable alternative. The 3D structures of three membrane proteins, namely bacteriorhodopsin (Henderson et al, 1990), PhoE porin (Jap et al, 1991) and plant light-harvesting complex (Kühlbrandt & Wang, 1991), have to date been determined by this method at resolutions ranging from 3.5 to 6 Å.

1. Two-dimensional crystallization of membrane proteins

Methods for growing 2D crystals of membrane proteins have been summarized in a recent review (Kühlbrandt, 1992a). Some proteins, such as bacteriorhodopsin, already occur as 2D crystals *in vivo*. Others can be induced to form crystalline arrays within isolated membranes. The most promising approach is to start with the isolated, purified protein in detergent solution, as for 3D crystallization. The solubilized protein is mixed with lipid, also in detergent solution, and the detergent is then gradually removed by dialysis or absorption. Frequently, 2D crystals result.

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