Journal of Bioenergetics and Biomembranes, Vol. 16, Nos. 5/6, 1984

SHORT REVIEW

Crystallization of Intrinsic Membrane Proteins

Takayuki Ozawa[1]

Received April 20, 1984; revised June 25, 1984

Abstract

After many years of discouraging failures, it is now possible to crystallize the intrinsic membrane proteins and to obtain structural information from diffraction studies on the crystals. The strategy for the crystallization consists of depletion of boundary phospholipids from the protein and complex formation with specific ligands.

Key Words: crystal; membrane proteins; diffraction; negative staining; freeze fracture; phospholipids; ligand.

Introduction

With the discovery of *Atmungsferment,* Warburg (1925) recognized the firm binding of this insoluble enzyme, a typical intrinsic membrane protein, to cell structure, and remarked that the *Atmungsferment* molecule was as inaccessible as the stars. Since then, many researchers have endeavored to obtain pure membrane proteins. As reviewed by Green (1962), Hatefi *et al.* (1962 a,b) fragmented the electron-transfer chain of the mitochondrial inner membrane into four complexes and reconstituted the chain from the complexes. However, the isolated complexes still contained some boundary phospholipids and contaminating polypeptides; e.g., the number of subunits and the specific heme and copper content of Complex IV, cytochrome *c* oxidase, have been reported in many papers but with wide variations. Extensive purification and crystallization of these intrinsic membrane proteins will solve the problems. Preparation of three-dimensional crystals of the proteins, however, has been difficult due to the proteins' bimodal surfaces (Green, 1972) and the asymmetric distribution of hydrophobic and hydrophilic areas, which favor the formation of membranous vesicles or sheets, as schematically shown in

[1]Department of Biomedical Chemistry, Faculty of Medicine, University of Nagoya, Nagoya 466, Japan.

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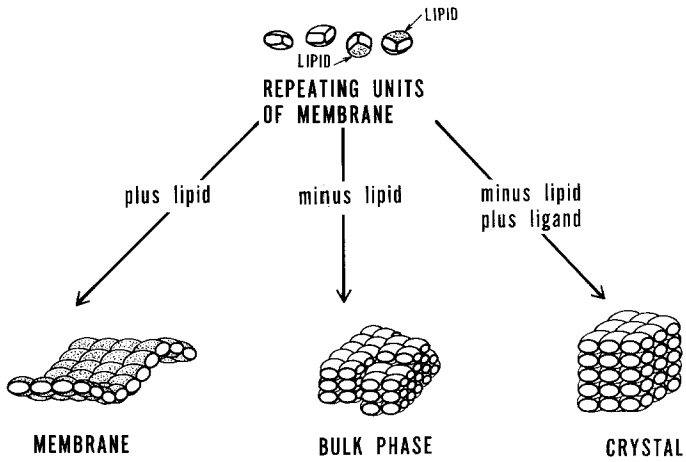


Fig. 1. Molecular array of the intrinsic membrane proteins under three different conditions. Principles of the molecular array of the proteins described in Green *et al.* (1967) and Ozawa *et al.* (1980a) are combined and schematically represented.

Fig. 1. Yonetani (1961) reported a crystalline preparation of cytochrome oxidase, but his results have not been generally accepted.

In the middle 1960's, Green and his group started to reconstruct a model biomembrane. Until that time, the Danielli–Davson model (1935) and the Robertson model (1964), which indicate that lipid bilayers are sandwiched between two layers of protein, were widely accepted since either model can explain the three layers of biomembrane observed by electron microscopy. On the other hand, biochemical evidence which contradicts these models had been accumulated. The first demonstration that an intrinsic membrane protein, cytochrome oxidase, can reform membranous vesicles was reported by McConnell *et al.* (1966). Green and Perdue (1966) and Green and Tzagoloff (1966) presented notions indicating that membrane proteins, but not lipid bilayer, plays a major role in the formation of membranes, giving a new aspect to biomembrane structure. An electron microscopist, Oda (1968), carefully checked the notions, and reported that cytochrome oxidase containing less phospholipids than the sample used by McConnell *et al.* (1966) formed two-dimensional crystalline arrays. In his preparation, "green membrane," prepared in the presence of deoxycholate, the arrays seem to be in the form of flat sheets rather than vesicles, and the symmetry is *pg*. More precise electron micrographic images were presented by Seki and Oda (1970) and Seki *et al.* 1970). Using a nonionic detergent, Wakabayashi *et al.* (1970) and Vanderkooi *et al.* (1972) reported another crystalline array of cytochrome oxidase sheets with characteristic *pgg* symmetry. Electron-diffraction studies on these

"two-dimensional crystals" of cytochrome oxidase have been carried out by Henderson *et al.* (1977) and Fuller *et al.* (1979, 1982), and a lopsided "Y" shape was proposed for the oxidase monomer, using a sheet with *pg* symmetry (Fuller *et al.*, 1979). Molecular arrays of three-dimensional crystals of the oxidase obtained in our laboratory (Ozawa *et al.*, 1982) showed *pg* symmetry. Weiss and his group also reconstructed a three-dimensional structure for cytochrome bc_1 complex by optical diffraction (Leonard *et al.*, 1981) or by neutron scattering (Perkins and Weiss, 1983) using two-dimensional sheets of the complex with *pg* symmetry. Three-dimensional crystals of the complex (Ozawa *et al.*, 1983) showed hexagonal symmetry. Therefore, no definite correlation of molecular array of the proteins seems to exist between two- and three-dimensional crystals.

By virtue of bimodal interaction of the membrane proteins, and phospholipids, these two-dimensional sheets could be easily prepared by simple dialysis of the protein–phospholipid complex. However, the resolution in diffraction studies on these sheets is limited to *ca.* 25 Å (Fuller *et al.*, 1979), and a high-resolution structural analysis has not been possible.

Ligand for Three-Dimensional Crystals

As mentioned above, observations that cytochrome oxidase, *viz.* the oxidase molecules with 35% or more phospholipids, forms membranous vesicles and the oxidase with *ca.* 20% phospholipids arrays in crystalline sheets, seem to us to suggest that three-dimensional crystal could be formed when boundary phospholipids are eliminated from the surface of the oxidase molecule. However, there was a difficulty to be overcome. As Green *et al.* (1967) cited during their study on reformation of membranes by electron-transfer complexes, the bimodal nature of these intrinsic membrane proteins, *viz.* hydrophilic on one side and hydrophobic on the other, leads the proteins either to form membranes with lipid or to precipitate without lipid (Fig. 1). In our laboratory, the bimodal nature of cytochrome oxidase was found to be masked by complex formation with a polar ligand, cytochrome *c*, during affinity chromatography using immobilized cytochrome *c* (Ozawa *et al.*, 1975). The oxidase–cytochrome *c* complex as a whole was found to be more polar and less bimodal than the oxidase molecule. By small angle x-ray scattering, conformational change in the oxidase molecule induced by complex formation with cytochrome *c* was clearly demonstrated (Sato *et al.*, 1983).

The first three-dimensional crystals of the intrinsic membrane protein were obtained as a 1:1 complex of cytochrome oxidase (M_r = 130,000) and cytochrome *c* (Ozawa *et al.*, 1980a). The oxidase was almost completely depleted of phospholipids by hydrophobic interaction chromatography

(Ozawa *et al.*, 1979), except for two moles of tightly bound cardiolipin. The oxidase was dispersed in an ionic detergent, cholate. Cytochrome *c* was added to complex with the oxidase, and crystals were obtained by removal of detergent by dialysis against buffer for 24 hr. The crystals contained both the oxidase and cytochrome *c* in a 1:1 ratio, but detergent and phospholipids, except the cardiolipin, were absent. Similarly, cytochrome bc_1 complex (M_r = 235,500) was crystallized as a form of a complex with cytochrome *c* (Ozawa *et al.*, 1980b). Henderson (1980) cited that complex formation of the proteins with a relatively polar ligand is a promising strategy for crystallization, referring to two other groups' reports on crystallization of membrane proteins using β-octylglucoside, the smallest commercially available detergent. In the case of bacteriorhodopsin (bR) (Michel and Oesterhelt, 1980) and porin from *E. Coli.* (Garavito and Rosenbusch, 1980), the proteins were crystallized without removal of the detergent, so the protein molecule in the crystals is in the form of a complex with the detergent.

Michel and Oesterhelt (1980) crystallized bR from purple membrane by salt precipitation after solubilization of the membrane with octylglucoside. The crystals were obtained by means of vapor diffusion of the bR solution against 2.5–2.8 M salt for a week. Two different crystal forms, one needle shaped and the other cube shaped, were obtained, depending on the nature of the salt used and the pH. The needle-shaped crystal was ordered to at least 8 Å resolution, judging from the outermost reflections of X-ray, and showed strong linear dichroism. The purple bR chromophore was oriented almost exactly along the needle axis. So, further studies on three-dimensional structure should be useful to determine the chromophore orientation.

Garavito and Rosenbusch (1980) solubilized the pore-forming protein spanning the outer membrane of *E. coli,* porin, in the presence of octylglucoside. Then, the protein was precipitated using polyethyleneglycol (PEG), resulting in microcrystal formation after 15–30 min, as observed under a light microscope. They obtained birefringent rhombic plates and prisms using β-octylglucoside and nonbirefringent but larger bipyramids using α-octylglucoside synthesized by themselves. The larger bipyramids were grown by means of equilibrium dialysis against buffer containing PEG and α-octylglucoside. They gave X-ray diffraction spots out to 3.8 Å resolution which was close to 3 Å that are required to identify the positions of the amino acid residues in a protein structure. The lattice constants reported were $a = b = c \cong$ 154 Å, with $\alpha = \beta = \gamma = 90°$ in the space group of either $P23$ or $P2_13$, suggesting a trimer of porin (M_r = 110,000) in the asymmetric unit.

Michel (1982) crystallized the photosynthetic reaction center from *Rhodopseudomonas viridis* after molecular sieve chromatography in the presence of the detergent, *N,N*-dodecyldimethylamine *N*-oxide (DDAO), which is similar in size and polarity to β-octylglucoside. Crystallization was

achieved by vapor diffusion of the solubilized reaction center against 2.4–3 M ammonium sulfate in the presence of small amphiphilic molecules, heptane-1,2,3-triol and triethylammonium phosphate. Rapid crystallization leads to small crystals, whereas upon slow crystallization large square columns are obtained. The large crystals diffract X-ray to 2.5 Å resolution. The space group of these tetragonal crystals is $P4_12_12$ or $P4_32_12$ with unit cell dimensions of 223 × 223 × 114 Å. By SDS-gel electrophoresis, the crystal contains four different protein subunits including a membrane-bound cytochrome. Judging from the resolution, the crystals will hopefully lead to revelation of the structure of the reaction center and the arrangement of pigments. Michel (1983) gave the rationale behind his usage of the small amphiphilic molecules. The molecules are thought to replace the detergent, the molecular size of which is too large to fit perfectly into the proteins' crystal lattice. The nonpolar part of the molecules bind to hydrophobic surfaces of the proteins, arraying their polar heads to the outside. Larger parts of the proteins' polar surfaces are not covered by the molecules. Thus, a relatively polar protein–ligand complex as a whole could be expected.

During the course of the studies on purification of cytochrome oxidase, it was found that one mole of cardiolipin per mole of heme *a, i.e.,* two moles of cardiolipin per mole of the oxidase, bound tightly to the oxidase (Ozawa *et al.,* 1980a). Differing from other phospholipids, the binding between cardiolipin and the oxidase is so tight that the cardiolipin could not be removed without destruction of the molecular structure of the oxidase. This oxidase–cardiolipin complex behaves in a much more polar manner than the original oxidase of bimodal nature. Removal of excess boundary lipids from the surface of the oxidase molecules etches in a relatively polar oxidase–cardiolipin surface, in which the cardiolipin confers a certain hydrophilicity on the oxidases by sticking its polar head over hydrophobic areas and penetrating its nonpolar tail into crevices between polypeptide subunits (Ozawa, 1982). Forced removal of cardiolipin by 10% cholate resulted in cleavage of the oxidase into two genetically different groups of polypeptide subunits (Tada *et al.,* 1981). Similarly, removal of tightly bound cardiolipin from cytochrome bc_1 complex resulted in preferential dissociation of iron sulfur protein from the complex (Shimomura and Ozawa, 1982).

In an attempt to crystallize these cytochromes with their tightly bound cardiolipin as a built-in ligand, the boundary phospholipids, excepting the tightly bound cardiolipin, were eliminated from cytochrome oxidase by hydrophobic interaction chromatography. The oxidase, further purified by affinity chromatography, was dispersed in the nonionic detergent Triton X-100 and dialyzed slowly against 10 mM buffer for 1 week. By this procedure, three-dimensional crystals of the oxidase–cardiolipin complex were obtained as shown in Fig. 2A, B (Ozawa *et al.,* 1982). Electron

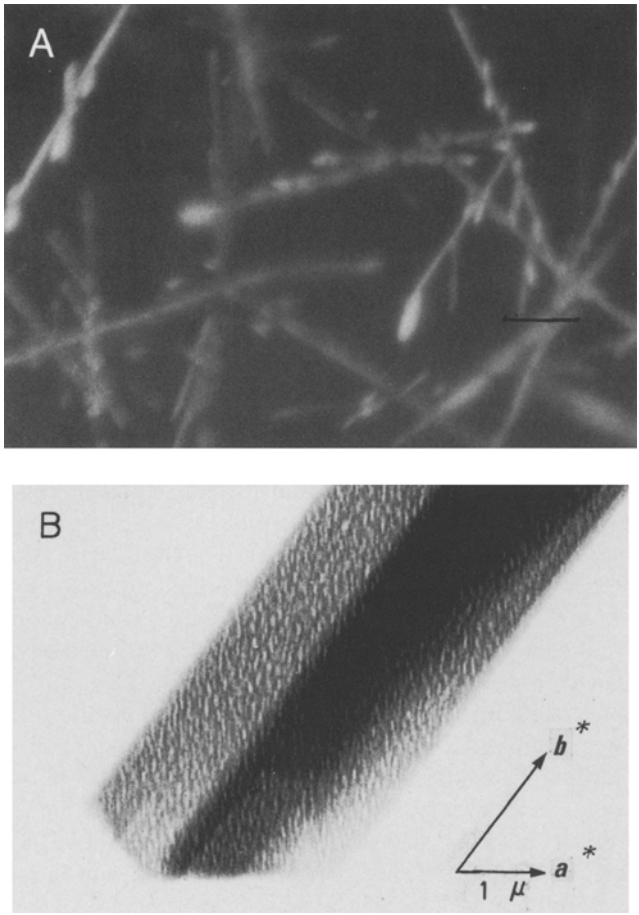
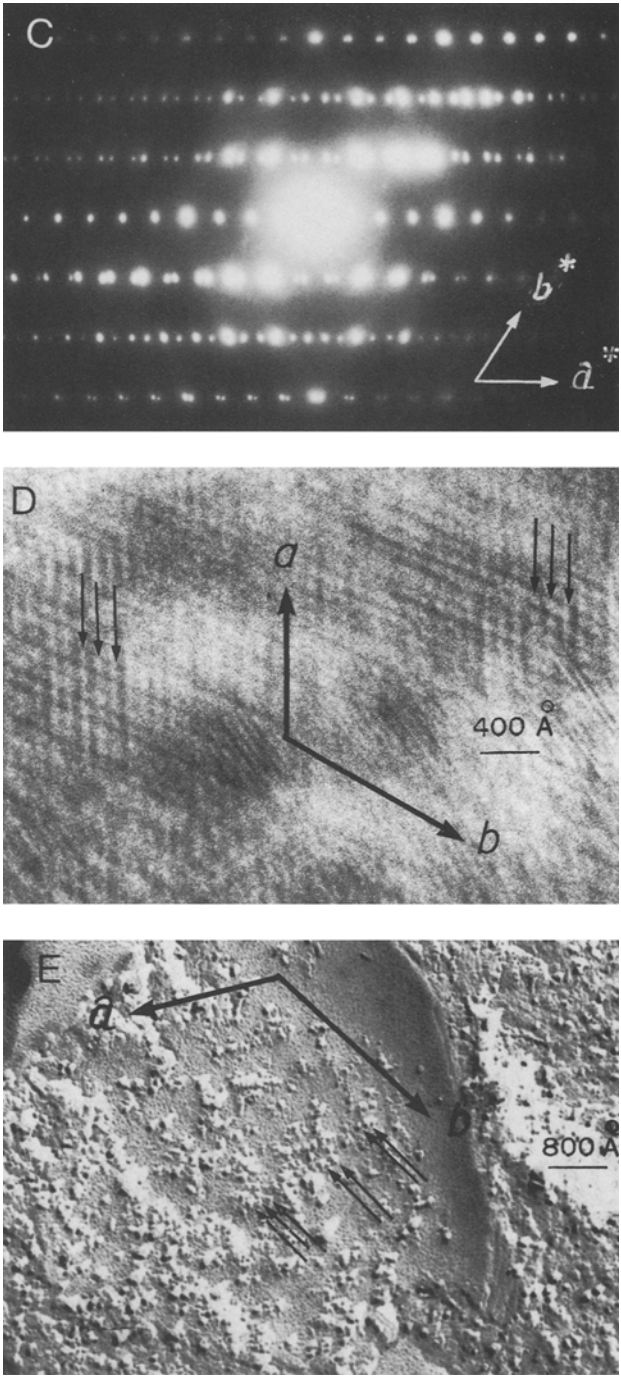


Fig. 2. Crystals of cytochrome oxidase, their electron diffraction patterns, negative-stained image, and freeze-fracture replica. (A) Crystals of the oxidase under polarized light. Bar = 100 μm. (B) Crystals of the oxidase were fixed with 1% glutaraldehyde overnight, then were negatively stained with 2% ammonium molybdate, pH 7.4. (C) An electron diffraction pattern from (h k 0) plane of an unstained single crystal with 1000-kV electrons was photographed under an illumination level of = 0.25 electrons/Å^2/sec. 1 cm: 0.32 Å^{-1}. (D) An image of (h k 0) plane of the crystal was taken as described in (B). Arrows indicate individual oxidase molecules. (E) Freeze-fracture replica of (h k 0) plane of the crystal. Arrows indicate individual oxidase.



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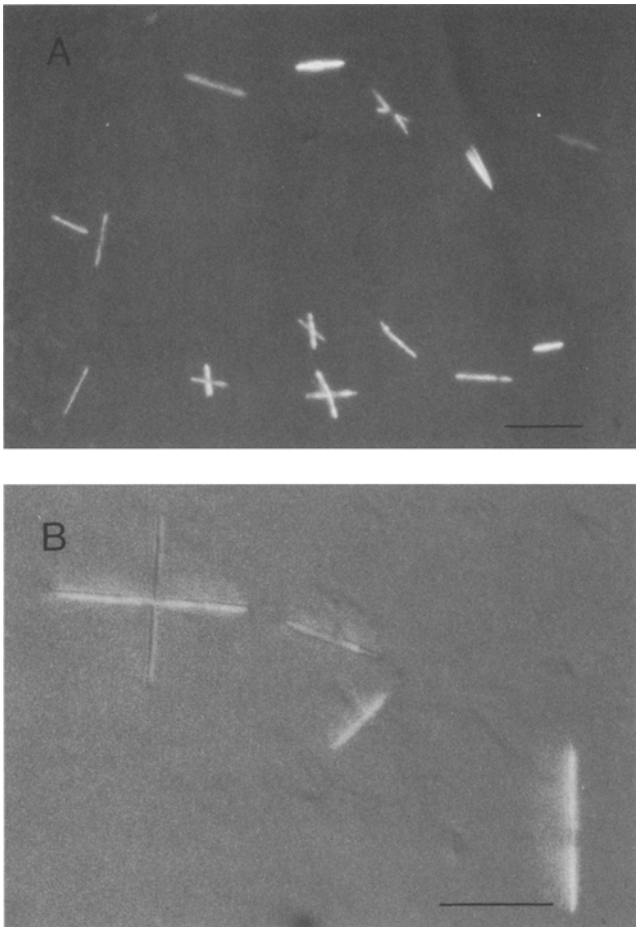

Fig. 3. Crystals of cytochrome bc_1 complex and cytochrome bc_1–cytochrome c complex. (A) Crystals of cytochrome bc_1 complex under polarized light. Bar = 50 μm. (B) Crystals of cytochrome bc_1–cytochrome c complex under polarized light. Bar = 20 μm. (C) Crystals of cytochrome bc_1 complex were fixed with 1% glutaraldehyde overnight. The electron diffraction pattern from (h k 0) plane of an unstained single crystal with 1000-kV electron was photographed under an illumination level of = 0.15 electron/Å^2/sec. 1 cm: 0.23 Å^{-1}. (D) Crystals of the bc_1 complex were fixed with 1% glutaraldehyde overnight, then were negatively stained with 2% ammonium molybdate, pH 7.4.

diffraction patterns (Fig. 2C) of the crystal using 1000-kV electron as well as images of negatively stained and freeze-fractured replica of crystal (Fig. 2D, E) demonstrated that the crystal showing *pg* symmetry is monoclinic in the space group of $P2$ with unit cell dimensions a = 92 Å, b = 84 Å, c = 103 Å, with $\alpha = \beta = 90°$, $\gamma = 126°$. The crystal diffracted electron to beyond 1.2 Å

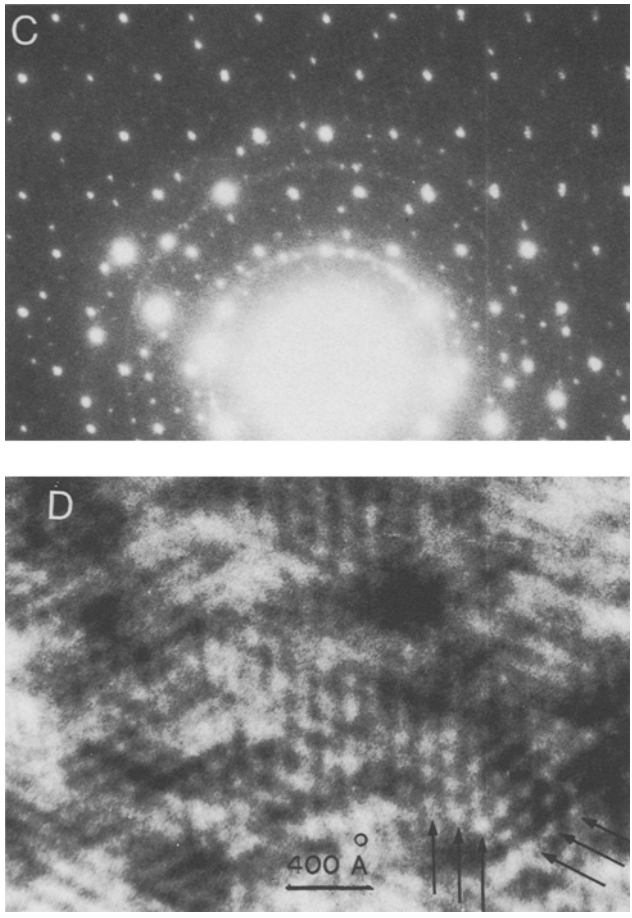

Fig. 3. Continued.

resolution. Chemical analyses showed seven polypeptide subunits in the crystals of cytochrome oxidase–cardiolipin complex but no detergent and other lipids. By a similar procedure (Ozawa *et al.*, 1983), cytochrome bc_1 complex containing nine polypeptide subunits and seven moles of tightly bound cardiolipin was crystallized (Fig. 3A) as well as its complex with cytochrome *c* (Fig. 3B). Electron diffraction patterns (Fig. 3C) and electron micrographs (Fig. 3D) of the crystals of cytochrome bc_1–cardiolipin complex demonstrate that the crystals are in hexagonal symmetry with unit cell dimensions $a = b = 113$ Å, $c = 132$ Å, with $\alpha = \beta = 90°$, $\gamma = 120°$. Bragg diffraction extends to spacings of 1.0 Å.

It can be concluded that the lower the phospholipid or detergent content in the membrane protein crystal, the better the resolution of diffraction. In the

case of "two-dimensional crystals" of cytochrome oxidase (Fuller *et al.*, 1979), the resolution is 25 Å in the presence of 20–30% (w/w) of phospholipids, whereas it is 1.2 Å in the case of three-dimensional crystals (Ozawa *et al.*, 1982) with two moles of tightly bound cardiolipin. Although the chemical compositions of the crystals of bR (Michel and Oesterhelt, 1980), porin (Garavito and Rosenbusch, 1980), and photoreactive center (Michel, 1982) are not described and estimation of the phospholipid or detergent content in these crystals is difficult, the resolution of diffraction, e.g., 8 Å in bR, 3.8 Å in porin, and 2.5 Å in photoreactive center, seems to be closely related to the phospholipid or detergent content in these crystals. Other factors which influence the resolution limit might be the mode of existence of these molecules and the "small amphiphilic molecule" or their interaction with protein molecules.

Strategy for Crystallization

Green (1972) proposed "the bimodal principle of membrane construction": all biological membranes conform to the same constructional principle, namely, the packing of bimodal proteins with bimodal phospholipids. Both polar and nonpolar interactions between two bimodal molecules stabilize membrane structure. The concept of bimodal protein (Fig. 1) is based on the fact that the presence of both detergent and salt, which interfere with hydrophobic and hydrophilic interactions, respectively, is obligatory to break membrane structure, e.g., in the case of "green–red separation" of mitochondrial inner membrane. Green *et al.* (1967) found that phospholipids are asymmetrically distributed among surfaces of each membrane protein. They demonstrated reformation of two-dimensional membrane structure by simple dialysis of each electron-transfer complex with phospholipids.

For the crystallization of the membrane proteins, the first obligatory step is removal of excess phospholipids from the proteins to dodge the bimodal principle. The next important strategy is binding of the membrane proteins with the ligand which would cover the hydrophobic surfaces of the proteins and confer some hydrophilicity on the bimodal proteins, resulting in a protein–ligand complex of unimodal nature as a whole, even if not completely unimodal (Fig. 4). Polar interactions among the unimodal complexes in three dimensions would lead to crystal formation.

When the specific polar ligand such as cytochrome *c* (Ozawa *et al.*, 1980a,b) is available, complex formation of the protein with the ligand results in an unimodal protein–ligand complex as a whole. In the case of flavoprotein (Yagi and Ozawa, 1963, 1964), complex formation between the enzyme and its substrate or product makes the complex crystallize more readily than the enzyme alone. Conformational changes in the oxidase molecule on complex

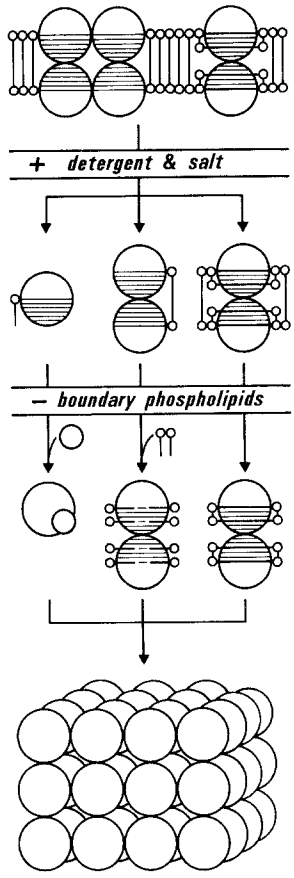

Fig. 4. Strategy for crystallization of membrane proteins. The scheme outlines the process of crystallization of the membrane proteins. As Green (1972) noted, membranes are composed of the bimodal proteins (spheres) and phospholipids (dumbbells). After removal of boundary lipids, complex formation of the proteins with the specific polar ligand (small spheres), amphiphilic detergent (half-dumbbells), or with cardiolipin (double-dumbbells) promotes formation of the unimodal protein–ligand complex and the three-dimensional crystal.

formation with cytochrome *c* are demonstrated by X-ray scattering (Sato *et al.*, 1982), and reflect the changes in the bimodal surfaces of the oxidase.

When there is no specific ligand available, "amphiphilic molecule" of small molecular size such as heptanetriol is a promising ligand (Michel, 1983) when one can control the molecule content in the crystal. The polar areas of the protein and the amphiphilic molecules interact as in a phospholipid micelle, while the molecules mask the hydrophobic areas of the protein.

Some of the boundary phospholipids, cardiolipin, in cytochrome are tightly bound to the protein, probably maintaining structural integrity of the molecule (Ozawa, 1982). They seem to act as a built-in ligand in the protein molecule. A relatively polar cytochrome–cardiolipin complex could be produced by removal of excess boundary lipids, and be crystallized.

This strategy for crystallization of the proteins is schematically presented in Fig. 4.

Retrospect and Perspective

Green *et al.* (1967) inspired us with the concept that (a) the bimodal membrane proteins form two-dimensional sheets with lipid and (b) that they form bulk precipitates without lipid, as shown in Fig. 1. The concept led to the demise of the lipid-bilayer model of membrane (Robertson, 1964) and opened the door to study of the "two-dimensional crystal" of the membrane proteins. Here it seems reasonable to add the third principle: (c) the unimodal protein–ligand complexes form three-dimensional crystalline arrays without lipid (Fig. 1).

Although the X-ray or electron diffraction studies mentioned here allow determination of the symmetry of the crystal and the dimensions of the unit cell, more precise information on the structure and chemical details of these membrane proteins will be obtained by further diffraction analyses.

The strategy shown in Fig. 4, viz. metamorphosis of the bimodal membrane proteins to the unimodal protein–ligand complexes, could be applicable for the crystallization of other membrane proteins such as receptors or pump proteins.

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