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Crystallization *in cubo*: general applicability to membrane proteins

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Obtaining well ordered crystals of membrane proteins is the single most serious stumbling block in the pursuit of their high-resolution structures. The applicability of lipidic cubic phase-mediated crystallization is demonstrated on a diverse set of bacterial membrane proteins: two photosynthetic reaction centres, a light-harvesting complex and two retinal proteins, halorhodopsin and bacteriorhodopsin. Despite marked differences in molecular dimensions, subunit composition and membrane origin, one single lipid, monoolein, is sufficient to form a crystallization matrix for all the aforementioned systems. Therefore, the lipidic cubic phase approach is proposed as a general method for crystallizing membrane proteins.

We dedicate this paper to Dr Vittorio Luzzati on the occasion of his 75th birthday.

1. Introduction

A comprehensive understanding of fundamental membrane-associated biological processes such as energy conversion, transport and signal transduction cannot be attained unless the high-resolution structures of the membrane proteins involved are known. The success of X-ray crystallography, the most widely used method to determine protein structures, critically depends on the availability of well ordered three-dimensional crystals. Whereas well over 4000 structures of soluble proteins have been solved to resolutions better than 2.5 Å, the number of high-resolution structures of membrane proteins is still limited to fewer than 20. Conventionally, methods for solubilization, purification and subsequent crystallization of membrane proteins have employed detergents. These form belts covering the hydrophobic membrane-spanning domains, rendering the proteins hydrophilic. Crystallization of such protein/detergent mixed micelles is thought to proceed similarly to that of soluble proteins and resulted in the first crystals of membrane proteins (Michel & Oesterhelt, 1980; Garavito & Rosenbusch, 1980). However, this approach could not be applied successfully to a large number of membrane proteins. In developing an alternative concept, we reasoned that membrane proteins might crystallize more readily in a lipidic environment, provided that they can be incorporated into an appropriate lipidic matrix, retain their native properties and diffuse in three dimensions. We demonstrated the feasibility of this idea by crystallizing bacteriorhodopsin (bR) in bicontinuous lipidic cubic phases (Landau & Rosenbusch, 1996). This enabled the elucidation of the X-ray structure of the protein at high resolutions from microcrystals grown in a monoolein-based cubic phase (Pebay-Peyroula *et al.*, 1997; Luecke *et al.*, 1998, 1999*a*; Belrhali *et al.*, 1999). Bacteriorhodopsin in such crystals retains biological activity since upon photoexcitation it undergoes a photocycle which is indistinguishable from that observed in the native purple membrane (Heberle *et al.*, 1998). Indeed, crystals grown in lipidic cubic phases recently yielded the first high-resolution structures of two intermediate states in the photocycle of bR (Edman *et al.*, 1999; Luecke *et al.*, 1999*b*). The cubic phase crystallization methodology also proved effective for small molecules and soluble proteins (Landau *et al.*, 1997; Rummel *et al.*, 1998).

Bacteriorhodopsin is unusual among membrane proteins since most of its mass is membrane-embedded, existing as highly ordered two-dimensional crystalline arrays in purple membranes (Blaurock & Stoeckenius, 1971; Henderson & Unwin, 1975). Clearly, in order to establish the general applicability of lipidic mesophase crystallization, the method must be successfully applied to a variety of membrane proteins with different characteristics. Here, we demonstrate the feasibility of the concept of lipidic cubic phase-mediated crystallization by extending it to two photosynthetic reaction centres from *Rhodopseudomonas viridis* (RCvir) and *Rhodobacter sphaeroides* (RCsph), the light-harvesting complex 2 from *Rhodopseudomonas acidophila* (LH2) and to halorhodopsin from *Halobacterium salinarum* (hR).

2. Materials and methods

2.1. Crystallization

Prior to incorporation into cubic phases, RCsph and RCvir were solubilized in dodecyl-dimethylamineoxide. All other proteins were solubilized in *n*-octyl-*β*-D-glucopyranoside. The solubilization detergent affects the phase behavior of monoglycerides and a comprehensive understanding of these effects requires complete phase diagrams to be established. The two detergents used in this study, however, behave in a similar way. bR and hR were purified as described by Landau & Rosenbusch (1996) and Duschel *et al.* (1988). RCvir, RCsph and LH2, provided by Drs G. Fritzsch, F. Reiss-Husson and T. Howard, respectively, were used without further purification. Crystallization of all systems was carried out at 298 K in the dark using 60%(*w*/*w*) monoolein (from Sigma or Nu-Check-Prep) cubic phases with 3–9 mg ml^{-1} protein as described previously for bR (Landau & Rosenbusch, 1996; Rummel *et al.*, 1998). RCsph, RCvir and LH2 were first reconstituted into the lipidic matrix, which resulted in stable homogenously coloured transparent and non-birefringent cubic phases, and crystallized upon the addition of precipitants. The final overall concentrations for crystallization were as follows. RCvir: 750 m*M* sodium acetate, 750 m*M* HEPES pH 7.5, 37.5 m*M* cadmium sulfate, 2.6%(*w*/*v*) 1,2,3-heptanetriol, 0.1 mg µl^{-1} sodium/potassium phosphate pH 7.5. RCsph: 17%(*v*/*v*) jeffamine M600, 750 m*M* HEPES pH 7.5, 0.05 mg µl^{-1} ammonium sulfate. LH2: 150 m*M* magnesium chloride, 75 m*M* Tris pH 8.5 and 2.5%(*w*/*v*) 1,6-hexanediol. These conditions differed from those employed in crystallization from detergent solutions (Fritzsch, 1998; Arnoux *et al.*, 1989; Prince *et al.*, 1997). The initially viscous material partially liquefied following the appearance of crystals. Bacteriorhodopsin and hR were crystallized as described for bR (Landau & Rosenbusch, 1996; Rummel *et al.*, 1998).

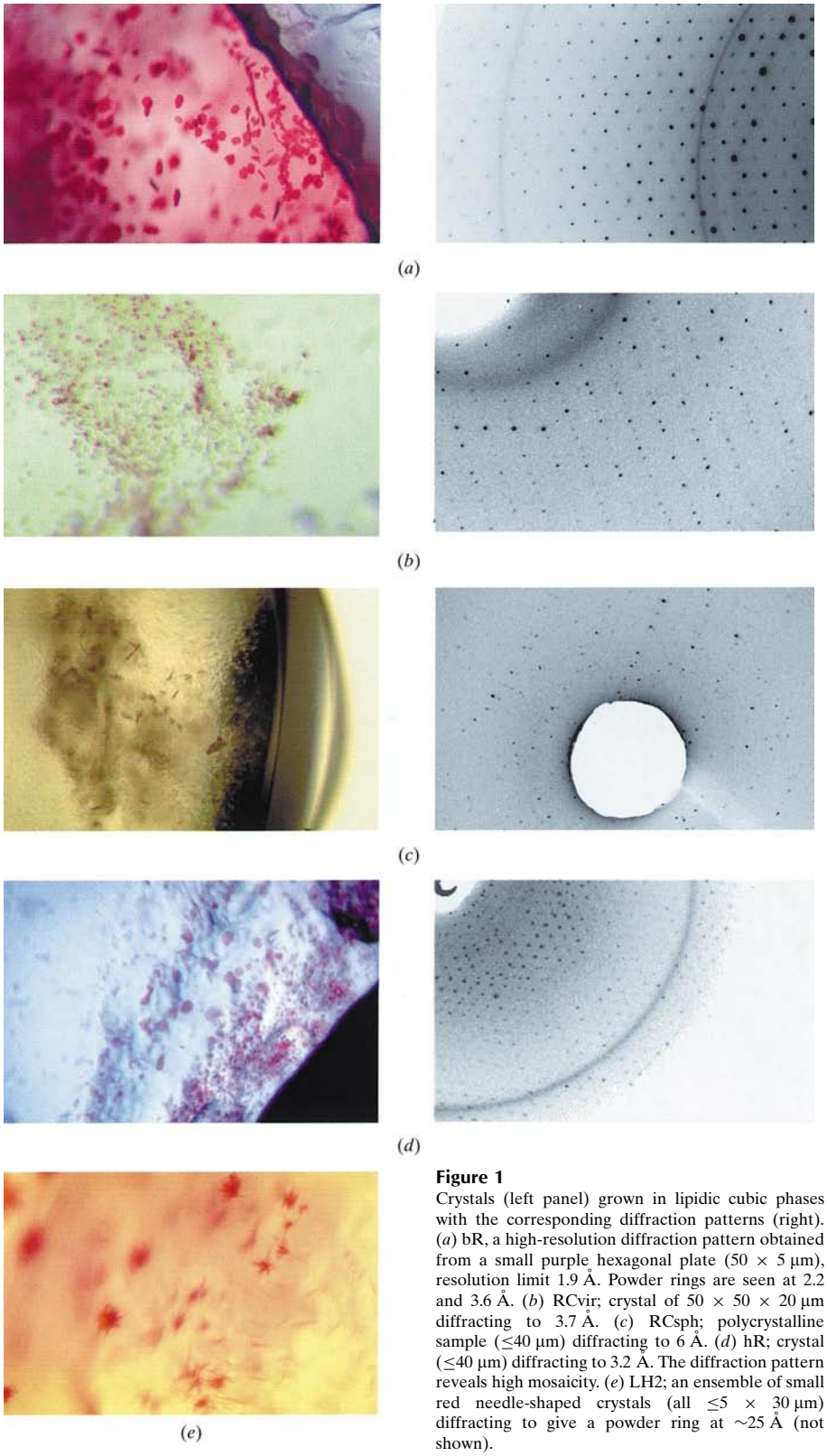

Figure 1
Crystals (left panel) grown in lipidic cubic phases with the corresponding diffraction patterns (right). (*a*) bR, a high-resolution diffraction pattern obtained from a small purple hexagonal plate (50 × 5 µm), resolution limit 1.9 Å. Powder rings are seen at 2.2 and 3.6 Å. (*b*) RCvir; crystal of 50 × 50 × 20 µm diffracting to 3.7 Å. (*c*) RCsph; polycrystalline sample (≤40 µm) diffracting to 6 Å. (*d*) hR; crystal (≤40 µm) diffracting to 3.2 Å. The diffraction pattern reveals high mosaicity. (*e*) LH2; an ensemble of small red needle-shaped crystals (all ≤5 × 30 µm) diffracting to give a powder ring at ∼25 Å (not shown).

2.2. Diffraction and data analysis

X-ray diffraction experiments on flash-cooled (100 K) crystals of bR, hR and RCsph were performed on the microfocus beamline ID13 (Engström *et al.*, 1995). Diffraction of crystals of RCvir was conducted on beamline ID14-EH3 (Burmeister *et al.*, 1999). Owing to the small sizes of LH2 crystals, powder diffraction of an ensemble of needles was collected on ID13.

3. Results and discussion

The four chromophore-containing membrane proteins and bR were incorporated into monoolein-based cubic phases by mixing monoolein (1-monooleoyl-*racemo*-glycerol) with the respective detergent/membrane protein solutions to form stable homogeneous materials which, upon addition of precipitants, yielded coloured crystals (Fig. 1). These were small, ranging from a few micrometres to 80 µm, and exhibited the

Table 1
Diversity of membrane proteins crystallized *in cubo*.

Protein	Organism	Mass of complex† (Da)	Number of peptide chains in complex‡	Mass of subunits (Da)	PDB code
RCvir, photosynthetic reaction centre	*Rsp. viridis*	132197	4	*C*, 37390; *H*, 28484; *L*, 30433; *M*, 35890	1prc
RCsph, photosynthetic reaction centre	*Rb. sphaeroides*	88580	3	*H*, 25646; *L*, 29760; *M*, 33174	1pss
LH2, light-harvesting complex 2	*Rsp. acidophila*	90369	18	*A*, 5505; *B*, 4536	1kzu
hR, halorhodopsin	*H. salinarum*	30256	1§	30256	
bR,bacteriorhodopsin	*H. salinarum*	80355	3, 1§	26785	1qhj

† Peptide only. ‡ In crystal. § In detergent solution.

following habits: hexagonal plates for bR and hR, needles for LH2, rectangular plates for RCvir and both rectangular and hexagonal plates for RCsph. The five proteins differ widely (Table 1) in subunit composition, size and relative proportions of the hydrophobic and hydrophilic surface areas (Fig. 2). Remarkably, these differences did not restrict the applicability of the crystallization method. The conditions of lipidic cubic phase-based crystallization applied were identical for all systems used with respect to the matrix-forming lipid [monoolein at an initial concentration of 60%(*w*/*w*)], but differed in the type of additives and detergents used. Moreover, the precipitants used here were different from those used to obtain crystals from membrane protein–detergent mixed micelles. A bicontinuous lipidic cubic phase appears to be essential for crystal formation, because bR does not crystallize under the same conditions in a micellar cubic phase (Landau & Rosenbusch, 1996). Moreover, bR in the detergent-solubilized state does not crystallize at the pH and phosphate concentration used *in cubo*.

The resulting crystals diffracted X-rays with the characteristics and the resolutions described in the legend to Fig. 1. The best diffraction, besides bR, was obtained with RCvir crystals and hR crystals, which diffracted to 3.7 and 3.2 Å, respectively. The LH2 needles are protein crystals: their colour corresponds to the protein in solution and originates from bound chromophores. Moreover, the needles exhibit typical properties of crystalline materials, such as having facets and being birefringent when observed with crossed polarizers. Owing to the very small size of the needles, diffraction from single crystals was not possible. However, as expected from an ensemble of protein crystals of that size, a spotty diffraction ring was obtained at 25 Å resolution.

It is remarkable that membrane proteins with diverse membrane-spanning domains and with hydrophilic domain sizes larger than the aqueous channel diameter of the monoolein cubic phase (Briggs *et al.*, 1996) can be incorporated into the lipidic matrix (Loewen *et al.*, 1999) where they form crystals. This indicates the prevalence of the previously underestimated plasticity and dynamics of the supramolecular assembly of monoglycerides (Clerc *et al.*, 1997). Lateral diffusion of large proteins in the curved membrane relies critically on this plasticity. Very recently, we demonstrated that even purple-membrane patches could be incorporated into a monoolein-based cubic phase, yielding bR crystals without any detergent treatment (Nollert *et al.*, 1999). Considering the properties of membrane proteins and the adaptability of lipidic mesophases, we anticipate more membrane proteins to be crystallized in lipidic mesophases, amongst them some which are notoriously unstable in detergent solution. Hence, such '*in cubo*' crystallization expands the repertoire of crystallization methods and as such can help to elucidate novel structure–function relationships in other membrane protein systems.

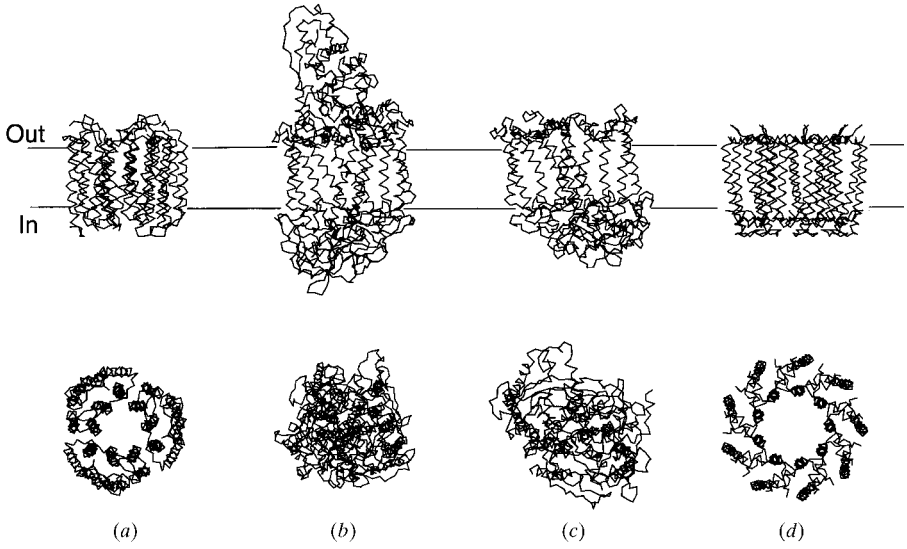

Figure 2
Comparison of the crystal structures of (*a*) bR (Pebay-Peyroula *et al.*, 1997), (*b*) RCvir (Deisenhofer *et al.*, 1995), (*c*) RCsph (Stowell *et al.*, 1997) and (*d*) LH2 (Prince *et al.*, 1997). The structures are viewed parallel (top) and perpendicular (bottom) to the membrane. These proteins vary in their membrane integral and extramembranous domain sizes, as well as in their oligomeric states and subunit compositions: bR is a homotrimer, RCvir and RCsph consist of four and three different subunits, respectively, and LH2 is a nonamer. The membrane boundaries are indicated by solid lines in the upper panel. The figure shows C^{α} backbones of the corresponding structures (prepared with *MOLSCRIPT*; Kraulis, 1991).

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