Detergent-free membrane protein crystallization

Peter Nollert[a,1], Antoine Royant[b,c], Eva Pebay-Peyroula[c,d], Ehud M. Landau[a,*]

[a] *Department of Molecular Microbiology, Biozentrum, University of Basel, Klingelbergstrasse 70, CH-4056 Basel, Switzerland*
[b] *ESRF, 6 rue Jules Horowitz, P.O. Box 220, F-38043 Grenoble Cedex 1, France*
[c] *Université Joseph Fourier, Grenoble, France*
[d] *Institut de Biologie Structurale CEA/CNRS, 41 rue Jules Horowitz, F-38027 Grenoble Cedex 1, France*

Received 29 June 1999; received in revised form 22 July 1999

Abstract A comprehensive understanding of structure-function relationships of proteins requires their structures to be elucidated to high resolution. With most membrane proteins this has not been accomplished so far, mainly because of their notoriously poor crystallizability. Here we present a completely detergent-free procedure for the incorporation of a native purple membrane into a monoolein-based lipidic cubic phase, and subsequent crystallization of three-dimensional bacteriorhodopsin crystals therein. These crystals exhibit comparable X-ray diffraction quality and mosaicity, and identical crystal habit and space group to those of bacteriorhodopsin crystals that are grown from detergent-solubilized protein in cubic phase.

© 1999 Federation of European Biochemical Societies.

Key words: Bacteriorhodopsin; Crystallization; Lipidic cubic phase; Membrane protein; Purple membrane

1. Introduction

Membrane proteins participate in all the interactions between the cell and its surroundings, performing such vital activities as solute transport, charge separation, conversion of energy and signal transduction. It is predicted that over a third of the approximately 6000 proteins of *Saccharomyces cerevisiae* are integral membrane proteins [1], and it is generally accepted that knowledge of their structures at high resolution is a prerequisite for the comprehensive understanding of their functions. However, fewer than 20 structures of membrane proteins have been elucidated at atomic resolution to date, in contradistinction to soluble proteins, of which thousands of high resolution structures are available. This discrepancy is due to three major difficulties: firstly, purification of fully functional membrane proteins from low-abundant natural sources [2]; secondly, overproduction of sufficient amounts in order to provide samples for NMR, electron crystallography, X-ray or neutron diffraction [3]; and thirdly, the notoriously weak tendency of membrane proteins to crystallize [4]. Because the most widely used methodology in structural biology, X-ray crystallography, requires well-diffracting crystals, the latter presents the most important obstacle in the field. These difficulties are all rooted in the special surface properties of transmembrane proteins, which possess both a hydrophobic core embedded in the non-polar bilayer region, and two polar surfaces that are solvated by water molecules and ions.

The fluid mosaic model [5] has been a successful representation of biological membranes since its introduction in the early 1970s, explaining their peculiar architecture and the extreme stability of integral membrane proteins therein. This notwithstanding, the conventional approach to functional and structural studies of membrane proteins by biochemical and biophysical methods, including crystallization, generally includes the replacement of the native lipid environment by synthetic detergents [6], which are not components of biological membranes. This solubilization procedure yields detergent/protein co-micelles and renders membrane proteins water-soluble, which may thus be treated analogously to soluble proteins [7]. Indeed, the first reported 3D crystallizations of membrane proteins – those of bacteriorhodopsin [8] and porin [9] – were obtained from aqueous detergent solutions. Recently, perhaps due to the rather limited success of this conventional approach, two novel concepts were introduced, both yielding crystals which diffract to high resolution. In the first, an Fv fragment of a conformation-specific monoclonal antibody, which binds to a discontinuous epitope at the periplasmic side of bacterial cytochrome *c* oxidase, was used in the co-crystallization with the enzyme [10]. The second concept [11,12] introduced bicontinuous lipidic cubic phases as a matrices for the crystallization of bacteriorhodopsin, a seven α-helical photon-driven proton pump residing in the purple membrane of *Halobacterium salinarum* [13,14]. This matrix, consisting of lipid, protein and aqueous solution in appropriate proportions, forms a transparent, non-birefringent and highly viscous material [12,15]. Bacteriorhodopsin molecules, once incorporated into this lipidic array, diffuse, nucleate and eventually yield well-ordered three-dimensional crystals [16]. Both concepts, however, still rely on the initial treatment of the native membrane with detergents, a procedure that requires the screening of many different detergent types and that frequently conflicts with maintaining protein stability for the period needed for crystallization [6].

We have thus set out to test the feasibility of obtaining well-ordered crystals excluding the use of detergents. Purple membrane, consisting of bacteriorhodopsin and a unique class of lipids [17], can be readily isolated from *H. salinarum* without the use of detergent [18]. We reasoned that by direct incorporation of the purple membrane into the lipidic matrix, we might be able to omit the solubilization in detergent micelles altogether, a procedure always associated with crystallization of membrane proteins, but often also with their denaturation [6].

*Corresponding author. Fax: (41) (61) 267-2118.
E-mail: landau@ubaclu.unibas.ch

[1] Present address: Department of Biochemistry and Biophysics, University of California, San Francisco, CA 94143-0448, USA.

0014-5793/99/$20.00 © 1999 Federation of European Biochemical Societies. All rights reserved.
PII: S0014-5793(99)01014-5

2. Materials and methods

Purple membranes isolated from *H. salinarum* strain S9 were a gift from Dr. Georg Büldt, Jülich, Germany, and were used without further treatment. Cubic phases were prepared by mixing monoolein (1-monooleoyl-*rac*-glycerol from Sigma or Nu-Check-Prep) with an aqueous suspension of the purple membrane to yield a 60% (w/w) monoolein lipidic matrix, analogous to the procedure described earlier [11,12]. The final concentration in the cubic phase was 3.5 mg bacteriorhodopsin/ml. Crystallization was induced by the addition of solid Na/K-phosphate to the preformed cubic phase to yield a final con-

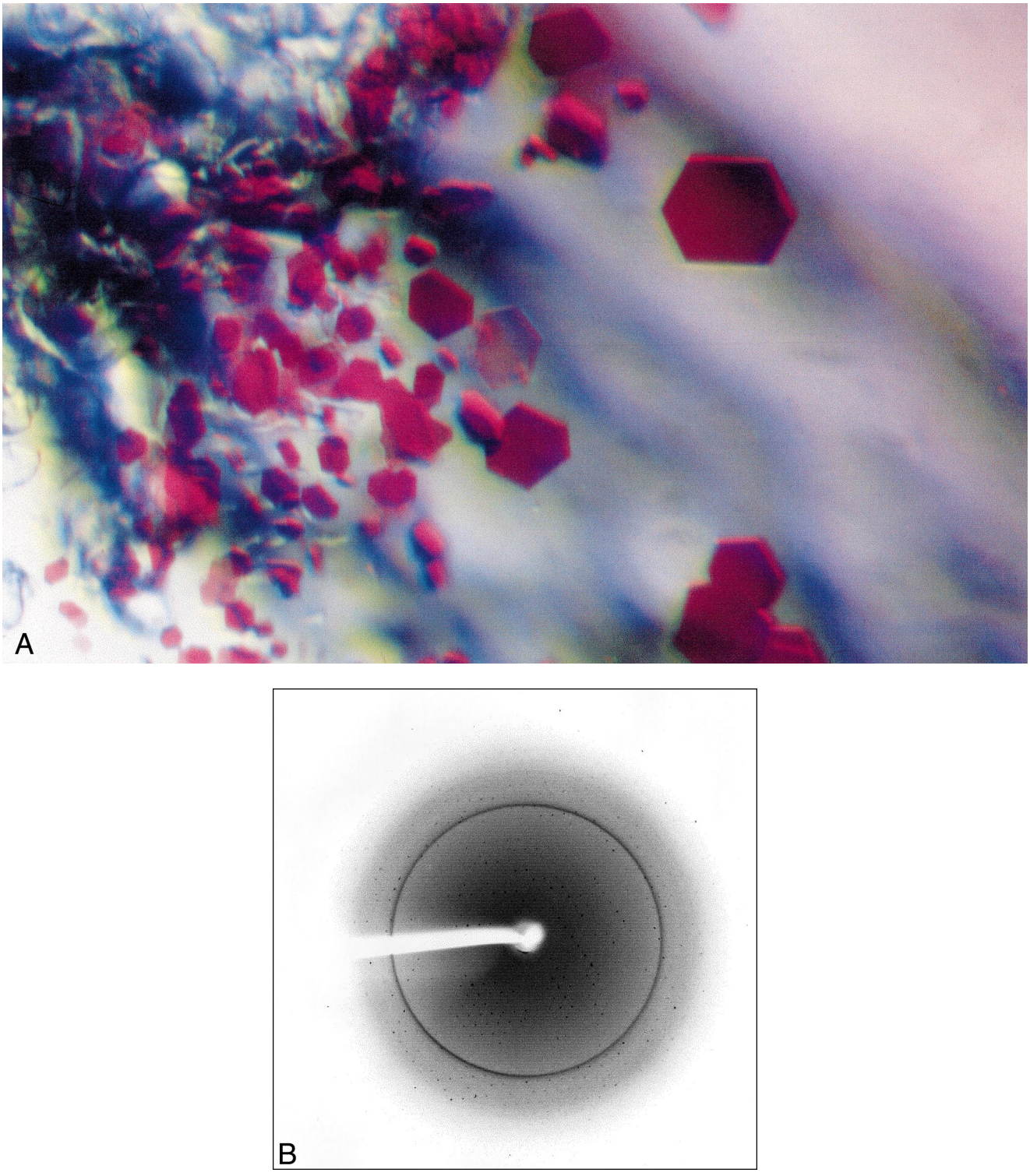

Fig. 1. A: Photograph of hexagonal micro-crystals of bacteriorhodopsin grown from purple membrane patches of *H. salinarum* in a 60% (w/w) monoolein lipidic matrix. B: Diffraction image of a micro-crystal of bacteriorhodopsin obtained on beam line ID14-EH3 at the ESRF, Grenoble, France. Diffraction spots range up to 2.8 Å. The marked ring at 4.5 Å stems from the interchain distance in the lipidic matrix in which the crystal is embedded.

centration of ca. 0.2–0.3 mg Na/K-phosphate/mg cubic phase, followed by a short centrifugation in a desk-top centrifuge at 20°C. Thereafter, the crystallization setups were stored in the dark at 20°C and 12°C, respectively. Crystals of bacteriorhodopsin were flash frozen in liquid nitrogen and maintained at 100 K during data collection. Diffraction experiments were performed on beam line ID14-EH3 at the ESRF [19], Grenoble, France. The bacteriorhodopsin crystal was rotated over 60° in steps of 1°. Data were integrated to 3.5 Å using the program MOSFLM (version 6.00) by A.G.W. Leslie, with new auto-indexing using Fourier analysis [20]. Further scaling was carried out with the CCP4 suite [21].

3. Results and discussion

The three-dimensional crystallization of bacteriorhodopsin in the absence of detergent was carried out in the simplest possible way: detergent-free isolated purple patches were mixed with 1-monooleoyl-*rac*-glycerol, forming a transparent, highly viscous and non-birefringent purple gel. Analogous to our earlier experiments with bacteriorhodopsin [11,12], these membranous materials were perfectly stable. The formation of hexagonal micro-crystals (Fig. 1A) was induced by the addition of solid Na/K-phosphate. Crystals grew slowly (1–6 months at 20°C or 12°C) to sizes of up to 50 μm in the largest dimension. Diffraction to 2.8 Å resolution is shown in Fig. 1B, and data were integrated to 3.5 Å (Table 1). Under similar experimental conditions, i.e. similar crystal size and beam intensity, the diffraction quality and mosaicity were comparable, and the crystal habit and space group were identical to those of bacteriorhodopsin crystals reported earlier [11,16], which were grown in the presence of the detergent *n*-octyl-β-D-glucopyranoside.

A crystallization mechanism involving annealing of entire purple patches inside the lipid crystalline material does not seem reasonable due to the large size of the patches and the geometrical mismatch between the planar patches and the curved membrane of the cubic phase. We suggest therefore a cubic phase-induced disaggregation of purple patches to smaller units, possibly bacteriorhodopsin monomers, followed by lateral diffusion in the three-dimensionally curved membrane and subsequent salt-triggered three-dimensional crystallization. We have previously shown that the detergent-solubilized integral membrane protein bacteriorhodopsin can indeed be crystallized in a membranous cubic phase [11]. Here we demonstrate that this membrane protein can be crystallized directly from its native membrane, omitting any potentially denaturing detergent-dependent procedures.

Previously two attempts to obtain three-dimensional crystals of bacteriorhodopsin starting from purple membrane were reported, one utilizing stacking of the purple membrane [14] and the other employing successive fusion of preformed vesicular bacteriorhodopsin assemblies [22]. The latter requires the use of a detergent, octylthioglucoside, and results in crystals that differ in space group (*P*622) and protein packing from the ones described here. Although mechanistic similarities with our cubic phase crystallization were inferred, there are clear differences. The purple membrane is exposed to amphiphiles with different spontaneous curvatures. In the cubic phase approach more than half of the crystallization volume is occupied by the lipid, forming a dense membranous network, thus dramatically limiting the space available for diffusion and association of large preformed aggregates such as crystalline purple membrane sheets. Furthermore, the crystals produced by the vesicle fusion approach contain detergent; the protein arrangement in these crystals differs from that of the purple membrane, while the crystals described herein consist of protein and purple membrane lipids that closely resemble the packing in the purple membrane [23].

We anticipate this crystallization method to be applicable likewise to other proteins that are naturally enriched in a biological membrane. For other membranes, novel separation procedures need to be established in order to achieve the high initial purity required for crystallization. In conclusion, we have demonstrated, contrary to the accepted dogma, that detergents are not needed for the three-dimensional crystallization of integral membrane proteins.

Table 1
Crystallographic data

Resolution range (Å)	29–3.5
R_{sym} (%)	9.1
$< I/\sigma >$	7.1
number of reflections:	
total	10 178
unique	1 888
completeness (%)	65
redundancy	5.4

The unit cell is $a = b = 60.64$ Å, $c = 108.44$ Å, $\alpha = \beta = 90°$, $\gamma = 120°$, the space group is $P6_3$.

Acknowledgements: We are grateful to Dr. J.P. Rosenbusch for insightful discussions and continuous support, to G. Rummel and A. Hardmeyer for expert technical assistance and to Drs. H. Belrhali and W. Burmeister for their help at the ESRF. We thank Dr. Georg Büldt for the generous gift of purple membrane. These studies were supported by Swiss National Science Foundation's SPP Biotechnology Grants 5002-46092 and 5002-55179, by EU-Biomed Grant PL 950990 and by EU-Biotech Grant PL 970415.

References

[1] Walker, J. and Saraste, M. (1996) Curr. Opin. Struct. Biol. 6, 457–459.
[2] Kühlbrandt, W. (1992) Curr. Opin. Struct. Biol. 2, 503–504.
[3] Schertler, G.F.X. (1992) Curr. Opin. Struct. Biol. 2, 534–544.
[4] Landau, E.M. (1998) in: Biomembrane Structures (Haris, P.I. and Chapman, D., Eds.), pp. 23–38, IOS Press, Amsterdam.
[5] Singer, S.J. and Nicolson, G.L. (1972) Science 175, 720–731.
[6] Rosenbusch, J.P. (1990) J. Struct. Biol. 104, 134–138.
[7] Helenius, A. and Simons, K. (1975) Biochim. Biophys. Acta 415, 29–79.
[8] Michel, H. and Oesterhelt, D. (1980) Proc. Natl. Acad. Sci. USA 77, 1283–1285.
[9] Garavito, R.M. and Rosenbusch, J.P. (1980) J. Cell Biol. 86, 327–329.
[10] Ostermeier, C., Iwata, S., Ludwig, B. and Michel, H. (1995) Nature Struct. Biol. 2, 842–846.
[11] Landau, E.M. and Rosenbusch, J.P. (1996) Proc. Natl. Acad. Sci. USA 93, 14532–14535.
[12] Rummel, G., Hardmeyer, A., Widmer, C., Chiu, M.L., Nollert, P., Locher, K.P., Pedruzzi, I., Landau, E.M. and Rosenbusch, J.P. (1998) J. Struct. Biol. 121, 82–91.
[13] Oesterhelt, D. and Stoeckenius, W. (1973) Proc. Natl. Acad. Sci. USA 70, 2853–2857.
[14] Henderson, R. and Unwin, P.N.T. (1975) Nature 257, 28–32.
[15] Luzzati, V., Delacroix, H., Gulik, A., Gulik-Krzywicki, T., Mariani, P. and Vargas, R. (1997) Curr. Top. Membr. 44, 3–25.
[16] Pebay-Peyroula, E., Rummel, G., Rosenbusch, J.P. and Landau, E.M. (1997) Science 277, 1676–1681.
[17] Kates, M. (1992) Biochem. Soc. Symp. 58, 51–72.
[18] Oesterhelt, D. and Stoeckenius, W. (1974) Methods Enzymol. 31, 667–678.

[19] Burmeister, W.P., Bougeois, D., Kahn, R., Belrhali, H., Mitchell, E.P., McSweeney, S.M. and Wakatsuki, S. (1998) SPIE 3448, 188–196.
[20] Steller, I., Bolotovsky, R. and Rossmann, M.G. (1997) J. Appl. Crystallogr. 30, 1036–1040.
[21] Collaborative Computational Project Number 4 (1994) Acta. Crystallogr. D 50, 760–763.
[22] Takeda, K., Sato, H., Hino, T., Kono, M., Fukuda, K., Sakurai, I., Okada, T. and Kouyama, T. (1998) J. Mol. Biol. 283, 463–474.
[23] Belrhali, H., Nollert, P., Royant, A., Menzel, C., Rosenbusch, J.P., Landau, E.M. and Pebay-Peyroula, E. (1999) Structure 7, 909–917.