The Crystallization of Outer Membrane Proteins from *Escherichia coli*

**STUDIES ON lamB AND ompA GENE PRODUCTS**

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R. Michael Garavito†, Ursula Hinz, and Jean-Marc Neuhaus‡

From the Biozentrum der Universität Basel, Abteilung Mikrobiologie, Klingelbergstrasse 70, CH-4056 Basel, Switzerland

The outer membrane protein LamB from *Escherichia coli* has been crystallized from detergent-containing solutions. Several different crystal habits can be obtained under the same ionic and precipitant conditions by altering the detergent head group composition of the protein-detergent mixed micelle or by adding polar organic compounds. Two crystal forms have been partially characterized as P1 and C2221, the former diffracting to beyond 4 Å resolution and the latter to 6 Å. The detergents used were β-octyl glucoside, octyl tetraoxyethylene, and octyl polyoxyethylene (polydisperse) either alone or as mixtures. In some experiments, the addition of small nonionic amphiphiles having n-butyl alkyl tails significantly influenced crystallization. The experiments suggest that the detergent region of the mixed micelle plays a critical role in crystal formation. Using the methods developed here for LamB and also for matrix porin (Garavito, R. M., Jenkins, J. A., Jansonius, J. N., Karlsson, R., and Yamato, I., 1983; Garavito and Jenkins, 1983) an additional protein from the outer membrane, OmpA, has been obtained as a microcrystalline preparation.

The outer membrane of Gram-negative bacteria is a highly differentiated structure involved in the passage of nutrients, genetic material, and information from the outside to the bacterium (Lugtenberg and Van Alphen, 1983; Osborn and Wu, 1980). Recently, three integral membrane proteins from the outer membrane of *Escherichia coli* have been isolated and purified to homogeneity: matrix porin (OmpF; Rosenbusch, 1981; Garavito et al., 1983), LamB (Garavito et al., 1983), and conjugin (OmpA; Hinz, 1983). Our work on these proteins has given great promise for the detailed analysis of membrane structure.

LamB, the λ phage receptor, is involved in passive diffusion of maltose and maltodextrins across the outer membrane (Szmelcman and Hofnung, 1975; Luckey and Nikaido, 1980). LamB has also been implicated in forming a receptor complex with the maltose-binding protein MalE (Bavoil and Nikaido, 1981; Ferenci and Boos, 1980; Neuhaus et al., 1983). Neuhaus (1982a, 1982b) has shown that LamB exists as a stable trimer when solubilized by detergents. The lamB gene sequence (Clement and Hofnung, 1981) revealed that the trimer would have a molecular weight of 142,000. The regions of the amino acid sequence involved in phage absorption and monovalent antibody binding have been recently determined (Clement et al., 1983; Gabay et al., 1983). Less is known about the ompA gene product. OmpA forms part of the receptor for F pilus-mediated conjugation (Havekes and Hoekstra, 1976; Schweizer and Henning, 1977). It is apparently monomeric (M0 ~ 35,000) when isolated using detergents (Hinz, 1983), based on the gene sequence (Chen et al., 1980).

The development of methods to grow crystals of integral membrane proteins suitable for x-ray crystallography holds great promise for the detailed analysis of membrane structure (Michel, 1982a; Garavito et al., 1983). The published crystallization procedures for membrane proteins do not differ markedly from the more "classical" methods of producing crystals of soluble proteins (see McPherson, 1982). The method developed in Basel uses primarily a PEG3/NaCl/detergent system (Garavito et al., 1983; Garavito and Jenkins, 1983) while Michel (1982a, 1982b, 1983) uses an ammonium sulfate/detergent combination. In both systems success depends on the proper choice of detergent(s). The most successful generally have 1) been nonionic (or zwitterionic), 2) been monodisperse, 3) moderate to high critical micellar concentrations, and 4) relatively small micellar sizes. Michel has also noted that the addition of small organic compounds can favorably affect the crystallization of membrane proteins (Michel, 1982b, 1983). We discuss the crystallization behavior of LamB in the presence of detergents with different nonionic head groups or small organic compounds, as well as the implications of this work for crystallization of membrane proteins in general. Finally, we report the preparation of microcrystalline material from OmpA.

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† To whom correspondence should be addressed.

‡ Present address, Max-Planck-Institut für Biologie, D-7400 Tübingen, West Germany.

† Nomenclature of Osborn and Wu (1980).

2 J. M. Neuhaus, U. Hinz, J. P. Rosenbusch, and I. Yamato, manuscript in preparation.

3 The abbreviations used are: PEG, polyethylene glycol; β-OG, β-octyl glucoside; C6E4, octyl tetraoxyethylene; C6POE, octyl polyoxyethylene (polydisperse, average oxyethylene units in head group is 5); EG, ethylene glycol; ethyl ether; EGI, ethylene glycol isopropyl ether; EGB, ethylene glycol butyl ether; HTO, 1,2,3-heptane-triol.

4254
**Experimental Procedures**

*Materials*—All chemicals were reagent grade or purified before use. Polyethylene glycol 4000, for gas chromatography, was purchased from Merck. The detergents used were β-octyl glucoside (Sigma), octyl tetraoxetylenylene (Bachem) and octyl polyoxyethylene (synthesized according to Rosenbusch et al., 1982). The Cellosolve compounds ethylene glycol isoproyl ether and ethylene glycol butyl ether were obtained from Fluka and ethylene glycol ethyl ether from Merck. Glycolate butyl ester (technical grade), purchased from Fluka, was vacuum distilled before use. The compound 1,2,3-heptane-triol was a kind gift from Dr. H. Michel (Munich).

*Protein Preparation*—LamB was prepared by detergent extraction of *E. coli* W3110 outer membranes and subsequent chromatography according to the procedure of Neuhaus (1982a, 1982b). The protein was stored in 0.1 M NaCl, 20 mM sodium phosphate, 3 mM NaN3, 1 mM dithiorethiol, and 1% C8-POE at pH 7.0 and protein concentration, 20 mg/ml. OmpA was isolated and purified according to Hinz, 1983. This protein was stored in the above buffer except that 1% β-OG replaced C8-POE and dithiothreitol. LamB and OmpA were judged free of protein and lipid contaminants, particularly murein lipoprotein and bacterial glycolipids.

*Crystallization Procedures*—The drop test (Garavito and Rosenbusch, 1980) was used as a quick method to determine the crystallization potential of the protein. A drop (10–20 μl) of protein and detergent solution was laid on top of another drop of buffer containing a precipitant, either PEG or ammonium sulfate. The drops were incubated in a closed chamber with the humidity controlled by a reservoir solution. If needed, the drops could be concentrated by vapor diffusion.

Standard vapor diffusion was done using glass depression slides, small glass cups, or 150-μl plastic microtest wells (Limbro). These were enclosed in a plastic box with at least 20-fold volume excess of reservoir buffer over the sample volume. Microdialysis was done using 50-μl dialysis cells from Cambridge Repetition Engineers. Unless otherwise specified, buffer for crystallization was 20 mM sodium phosphate, 0.1 M NaCl, and 3 mM NaN3, pH adjusted to 7.0. Most of the polar organic compounds used in the crystallization experiments were volatile and thus were added to the vapor diffusion reservoir at the desired final concentration as well as to the protein solution.

*X-ray Diffraction*—Crystals were mounted in glass capillaries along with a small amount of the nondetergent-containing reservoir buffer to control the humidity but prevent crystal wetting. X-ray photographs were taken on Enraf-Nonius precession cameras using GX-13 or GX-20 rotating anodes (Elliot-Macconi) with 300 μm focus cups and graphite monochromators and operating at 40 kV and 60 mA. Diffraction images were recorded using Kodak No-Screen or CEA Reflex 25 (Ceaverken AB) x-ray film.

**Results**

LamB—The drop test procedure readily gave microcrystals if PEG 4000 was used (see Fig. 1 of Garavito et al., 1982). After overlaying a drop of 10–15 mg/ml of LamB and either 1% β-OG or C8-POE on a drop of 18% PEG 4000 in buffer, microcrystals appeared in 2–3 days with incubation against 18% PEG 4000. The predominant crystal habits were thin flat rhombic or square plates. Well shaped small crystals, either long thin plates (Fig. 1a) or hexagonal columns (Fig. 1b) also occasionally grew. A similar assortment of microcrystals could be obtained using polyvinylpyrrolidone 10,000 (Fluka) as a precipitant. Experiments with ammonium sulfate yielded no discernible crystalline material. Dialysis experiments with 20 mg/ml of LamB against 0.7% (w/v) β-OG, ammonium sulfate (up to 3.1 M), and occasionally 5% (v/v) ethanol or Cellosolve solvents also resulted in neither microcrystal formation nor precipitation.

The positive results with the PEG experiments led us to explore vapor diffusion with this compound. Large crystals (Fig. 2a) were obtained from 5 mg/ml of LamB in buffer with 3.6% (w/v) PEG 4000, 0.25% (w/v) C8-POE, and 0.25% (w/v) β-OG when equilibrated with the reservoir (25.2% (w/v) PEG 4000, 0.7 M NaCl, 0.14 M sodium phosphate, and 3 mM NaCl, 0.14 M sodium phosphate, and 0.1 M sodium phosphate) and the presence of 5% (v/v) EGB causes thin hexagonal plates to grow without significant phase separation of the mother liquor. The arrowhead indicates an edge-on view of one crystal. The bars represent 0.1 mm.

**Fig. 1.** LamB microcrystals produced by the drop test. A drop of LamB solution containing 1% β-OG in buffer (see text) was placed on a drop of buffered 18% (w/v) PEG 4000. Spontaneous phase separation occurred and, after a few days, microcrystals appeared: a) long, thin plates or b) hexagonal columns. The bars in the photograph represents 40 μm.

**Fig. 2.** LamB crystallization by vapor diffusion in C8-POE/β-OG detergent mixture. a, large irregular bipyramides were grown at 25.2% (w/v) PEG 4000, 0.7 M NaCl, and 0.14 M sodium phosphate. b, lower vapor diffusion conditions (18% PEG 4000, 0.5 M NaCl, and 0.1 M sodium phosphate) and the presence of 5% (v/v) EGB causes thin hexagonal plates to grow without significant phase separation of the mother liquor. The arrowhead indicates an edge-on view of one crystal. The bars represent 0.1 mm.
Phase separation of the protein/detergent solution preceded crystal formation similar to that seen for matrix porin (Garavito et al., 1983). Addition of EGE did increase the size of the crystals but did not improve their quality. The colorless crystals had an irregular bipyramidal shape and some optical activity. They diffracted x-rays to about 8 Å resolution and were presumed to have the space group P1 (cell constants: $a = 114$ Å, $b = 166$ Å, $c = 208$ Å, $\alpha = 97.2^\circ$, $\beta = 74.9^\circ$, $\gamma = 90.5^\circ$). P1 crystals grown using polyvinylpyrrolidone 10,000 instead of PEG 4,000 revealed ordered diffraction to 4 Å with the synchrotron x-ray source at European Molecular Biology Laboratory Outstation, Hamburg (courtesy of Dr. H. D. Bartunik).

In vapor diffusion experiments where the conditions were lowered to 18% (w/v) PEG 4000, 0.5 M NaCl, and 0.1 M sodium phosphate, no crystals formed. Furthermore, the addition of 5% (v/v) EGE, EGI, ethanol, or 1-propanol did not have an effect. Upon the addition of 5% (v/v) EGB or 3% (v/v) 1-butanol, well formed but thin (5-7 µm) hexagonal plates grew within 2 weeks without significant phase separation (Fig. 2b). The crystals diffract x-rays to 7 Å despite their thinness. They are assumed to be hexagonal judging from their shape and optical activity. The addition of HTO (at 1% (w/v) initial concentration) resulted in the growth of small poorly formed chunky crystals.

If 0.3% (w/v) C$_6$E$_4$ was used instead of the β-OG/C$_6$-POE mixture, large chunky prisms grew at the lower vapor diffusion conditions (Fig. 3a). Significant phase separation of the mother liquor was not observed. Using the detergent mixture 0.3% (w/v) C$_6$E$_4$ and 0.2% (w/v) β-OG at the start of vapor diffusion improves the quality of the crystals (Fig. 3b) though they are thinner (40 µm). These crystals are birefringent and diffract x-rays well to 6 Å, again despite their thinness. The observed diffraction patterns of the principal zones show mm symmetry, and the c* axis exhibits systematic extinctions following the $l = 2n + 1$ rule. The hk0 zone displays $h + k = 2n + 1$ extinctions to a resolution of about 12 Å, the edge of our precession photographs. Thus, we have tentatively assigned the space group to be C2221 ($a = 119$ Å, $b = 218$ Å, $c = 203$ Å).

Vapor diffusion experiments, with an initial C$_6$E$_4$ concentration of 0.3%, against a reservoir buffer containing 5% (v/v) EGB yielded another crystal habit (Fig. 3c). These large well formed tetragonal columns showed little optical activity. They grew within 2 weeks and without noticeable phase separation. Glycolate butyl ester produced similar though less well formed crystals. X-ray data are not yet available for these crystals though it is known that they are not well ordered. HTO was the least effective of the amphiphilic compounds with a butyl alkyl tail; small bipyramidal crystals formed along with a flocculent precipitate.

OmpA—The drop test using 6–7 mg/ml of protein in buffer containing 1% β-OG and 18% (w/v) PEG 4000 yielded microcrystals if equilibrated with more than 22% (w/v) PEG 4000 in the reservoir. Replacing PEG with ammonium sulfate gave no positive results. Hence, vapor diffusion experiments were begun using PEG. OmpA (3–4 mg/ml) in buffer with 1% β-OG and 3.6% (w/v) PEG 4000 gave rise to crystals within 3 months after equilibration with 25% (w/v) PEG 4000, 0.7 M

Fig. 3. Effects of the addition of β-OG or the small amphiphiles EGB and glycolate butyl ester. Vapor diffusion experiments with LamB in 0.3% (w/v) C$_6$E$_4$ (see text) yielded the large chunky prisms seen in a. Crystals of improved quality, b, appeared when 0.2% (w/v) β-OG was added to the initial mixture. Another crystal habit grew, as shown in c, when 5% (v/v) EGB (or glycolate butyl ester) was added instead. The bars represent 0.2 mm.

Fig. 4. Microcrystalline preparation of OmpA. A lozenge-shaped microcrystal of OmpA grown from a PEG/NaCl/β-OG system. The bar represents 50 µm.
NaCl, and 0.14 M sodium phosphate. The lozenge-shaped crystals (Fig. 4) were well formed, though few in number and small (60 × 20 × 10 μm). No diffraction data are yet available. The addition of amphiphilic compounds resulted only in precipitation or gelation. It is obvious that we have not found the optimal conditions for the crystallization of OmpA. However, larger scale purification of the protein should allow us to search a wider range of variables.

**DISCUSSION**

We have shown that the outer membrane protein LamB forms several different crystal habits. The surprising result in this study is the dramatic effect on crystallization that occurs upon changing from a monodisperse detergent (C_{12}E_{6}) to a chemically similar though polydisperse detergent (C_{8}-POE) with a slightly larger head group size. The critical micellar concentrations for these two detergents are almost identical (7 mM), though they do differ in micelle aggregation number and cloud point behavior. The study of matrix porin crystallization (Garavito et al., 1983) suggests that the protein-detergent **mixed micelle** is the species which crystallizes and that alteration of the micelle surface by exchanging or mixing detergents can affect crystal formation. Which specific features of the detergent layer control crystallization are not known, though it is clear that head group structure and hydration affect such micellar characteristics as aggregation number, micellar size, and cloud point (Kreshcek, 1975; Rosen, 1978; Wannerström and Lindman, 1979).

The addition of polar organic compounds also perturb the detergent head group region of the micelle (Kreshcek, 1975; Rosen, 1978). Thus, it is not surprising that the crystallization of LamB (this study), bacteriorhodopsin (Michel, 1982b), and the bacterial photosynthetic reaction center (Michel, 1982a) is influenced by the presence of small amphiphiles. The most effective amphiphiles, ITO (see Michel, 1983), EGB, and glycolate butyl ester, are noneonic and contain an n-butyl moiety. In our PEG system, ITO does not seem to have the same efficacy as the other amphiphiles. However, it should be noted that the insolubility of EGB and glycolate butyl ester in solutions containing ammonium sulfate will limit their use in the crystallization procedure of Michel.

The PEG/NaCl system that we use often produces a phase separation of the detergent solution (Garavito et al., 1983), though the formation of a two-phase system is not obligatory for the crystallization of LamB. Interestingly, the crystallization of bacteriorhodopsin (Michel, 1982b) and matrix porin (Garavito et al., 1983) also occurs near or at conditions where detergent phase separation is observed. The extent of this demixing or deconsolution varies with detergent composition^4^ or the presence of small amphiphiles (Michel, 1983). Several groups (Corti and Degiorgio, 1981; Triolo et al., 1982; Zulauf and Rosenbusch, 1983) have shown that phase separation of detergent solutions at higher temperatures or solute concentrations is the end result of extensive micelle attraction and subsequent aggregation. Given the correlation between detergent phase separation and crystallization, a certain degree of micelle clustering might play a role in crystal formation, possibly by encouraging the close approach of protein-containing detergent micelles, thus enhancing the production of crystal nuclei. The ideal situation might be where protein-protein interactions and micelle aggregation are balanced such that protein-detergent micelles predominate in these crystals.

Our work with another E. coli outer membrane protein, OmpA, gives us the impression that many other membrane proteins could be crystallized. However, the preparation of large single crystals suitable for high resolution X-ray diffraction remains the major hurdle. Small crystal size and a significant variability of crystal yield from experiment to experiment are the primary problems. What should be noted about these and other experiments on membrane proteins (Michel, 1982a; Michel, 1982b; Garavito et al., 1983) is that a new set of variables arises from the role of the detergent in crystal formation. More extensive knowledge of the physical chemistry of protein-detergent micelles is necessary for the better understanding of membrane protein crystallization (Zulauf, 1983). However, at the present time procedures are available to crystallize integral membrane proteins.

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**Note Added in Proof**—Crystals of LamB have both recently been produced by D. Tserngouli and A. Tucker at the European Molecular Biology Laboratory Outstation, Heidelberg, using essentially identical methods as ours.

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