Three-dimensional Crystals of an Integral Membrane Protein: An Initial X-Ray Analysis

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ABSTRACT Matrix protein, a pore-forming transmembrane protein spanning the outer membrane of *Escherichia coli*, has been obtained in a variety of three-dimensional crystal forms amenable to both electron microscope and x-ray analyses. Successful association into large crystals depended on the use of α-octyl glucoside, a detergent with relatively low affinity for the protein. Electron micrographs of thin-sectioned crystals show a high degree of order. Preliminary crystallographic data suggest that the crystals, which exhibit diffraction to 3.8 Å, have a cubic space group.

Matrix protein is an abundant and well-characterized polypeptide (9) that forms gated pores (12) across the bacterial outer membrane. Previous investigations of its structure (13) relied on its availability as two-dimensional hexagonal arrays, obtained by extraction of cell envelopes by dodecyl sulfate (9). Such preparations exhibited triplet indentations around local threefold axes at a resolution of ~25 Å (13). Because the protein contains pores (8), we tentatively interpreted these indentations to correspond to the orifices of the channels. Conductance measurements in planar lipid bilayers demonstrated that the minimal pore diameter is on the order of 10 Å. The channels are water-filled and can assume either of two states: open or closed (12). Recently, we have succeeded in solubilizing the large aggregates (13), which previously resisted all attempts at dissociation without concomitant denaturation (10), to trimers (M_r 110,000), using mild, nonionic detergents (11). The resulting monodisperse solution allowed attempts to crystallize this membrane protein. Our rationale for doing so was as follows: (*a*) In view of our functional investigations resolving single channels (12), high-resolution structural data could allow relating structure and function of this pore protein on a molecular level. This appears particularly attractive because the channels exist in two states that are likely to be related by conformational changes. (*b*) With respect to secondary structure, matrix protein, unlike bacteriorhodopsin with its seven α-helices spanning the membrane (3), appears to satisfy the requirement of neutralizing its polar peptide bonds within the membrane by way of hydrogen-bonding them in antiparallel β-pleated sheets (9, 10). The significance of this type of secondary structure in membrane proteins cannot be evaluated without a structural resolution exceeding 4 Å. Furthermore, the possible role of β-structure as regards both formation and conformational changes of pores has been discussed recently (4). (*c*) The polarity of matrix protein is very high (1, 9), and its sequence does not exhibit large nonpolar fragments (1). Because the protein apparently hardly protrudes into the aqueous environment (11), the question arises whether most of the charged residues are indeed exposed in the hydrophilic channels or whether ion-pairing in the protein's interior could occur. This question, of course, also requires high structural resolution.

MATERIALS AND METHODS

Matrix protein trimers ($A^{0.1\%}_{278\,nm}$ = 1.41; cf. reference 9) were extracted (11) with 3% β-OG[1] (Calbiochem-Behring Corp., American Hoechst Corp., La Jolla, Calif.) in standard buffer (20 mM sodium phosphate, 3 mM NaN_3, pH 7.0). Further purification by gel filtration[2] yielded the protein with a purity of >95%, as judged from dodecyl sulfate gel electrophoresis. Gas chromatographic analyses (11) indicated residual amounts of lipopolysaccharides.

Strategy

Stacking of two-dimensional arrays to obtain three-dimensional crystals appeared to promise little because of the limited size and long-range disorder of matrix protein fragments (13). Also, interactions in the third dimension were unlikely to approach the strength of those in the two-dimensional lattices. Crystallization from monodisperse, solubilized protein therefore appeared preferable but required, in addition to solubilization, a high micellar concentration and small detergent micelles to promote interactions of protein trimers. As conventional detergents such as Triton X-100 did not meet these criteria (2), we initially used β-OG. Because of the resulting two-phase system (cf. below), we proceeded to synthesize and characterize its optical isomer.[2]

Crystallization

IN β-OG: The fusion of a 10-μl drop containing 10 mg/ml protein (in standard buffer, 1% [wt/vol] β-OG and 0.1 M NaCl) with a 10-μl drop containing PEG 4000 (25% [wt/vol] in 0.1 M NaCl and standard buffer) caused spontaneous formation of a new phase. After incubation of a drop under a coverslip for 15–30 min, light microscope observation revealed numerous microcrystals in droplets within the liquid phase. Vapor diffusion experiments according to published procedures (6) gave similar crystal forms. Drops (50 μl of 5 mg/ml protein) and 5% (wt/vol) PEG 4000 in standard buffer with β-OG were placed into depression slides and allowed to equilibrate with a solution (40 ml) containing either PEG

[1] Abbreviations used: α(or β)-OG, *n*-octyl-α(or β)-D-glucopyranosides. PEG, polyethylene glycol.

[2] The details of the procedures mentioned, and the synthesis of α-OG, are submitted for publication.

(50%) or phosphate (0.4–0.5 M). Within 1 wk, a second, heavier liquid phase appeared, in which crystals were soon observed. In selected cases, glutaraldehyde fixation was performed by exposing microcrystals to 5 ml of a 2.5% (vol/vol) solution of the fixative in the vapor diffusion chamber. Crystallization by equilibrium dialysis (15) was performed under conditions analogous to those described in detail below, except that 18% PEG in the outside compartment was needed to attain the required protein concentrations. However, such conditions caused formation of a two-phase system, analysis of which showed a volume ratio of 6:1 between upper and lower phases. The latter contained 100% of the protein and 85% of β-OG. If PEG was present in the compartment containing protein, its distribution of 5% in the lower phase and 95% in the upper phase suggested that it is not necessary for crystallization. The small volume of the lower phase, its high viscosity, and the growth of the crystals in clusters made mounting single crystals for x-ray analysis extremely difficult. A further complication was that any contact of crystals with the upper phase produced irreversible disordering.

IN α-OG: Crystals were grown by means of equilibrium dialysis (15) in glass tubes (50 mm × 3 mm inner diameter) that were sealed at one end with a dialysis membrane. About 200 μl of a protein solution (20 mg/ml) in standard buffer, containing 1% α-OG but no PEG, was inserted into the glass tubes, which were then placed into a vial containing standard buffer with 12% PEG 6000 and 1% α-OG. The protein, prepared with β-OG, had been dialyzed previously against α-OG. The quantitative removal of β-OG was monitored with ^{14}C-labeled detergent. Because of the high Krafft point[2] of α-OG (42°C), crystallizations were carried out between 37° and 45°C. After 36–48 h, the reservoir solution was exchanged for one supplemented with 1 M NaCl. Several hours later, a shower of microcrystalline material appeared. After 4 d, bipyramidal crystals grew along the walls of the tube. Full bipyramids were rare, and large crystals were often interpenetrated by smaller ones. The best results were obtained at 37°C, but the potential for supersaturation of α-OG at this temperature[2] necessitates delicate handling of such solutions.

RESULTS AND DISCUSSION

Initial crystallization attempts with β-OG promptly yielded microcrystals with high reproducibility. They were birefringent and assumed a uniform shape with distinct edges and vertices. Three crystal habits could be observed, which, in the order of decreasing populations, were (*a*) rhombohedral plates, (*b*) hexagonal plates, and (*c*) hexagonally shaped prisms. The longest dimension of the crystals was limited to <50 μm, apparently because the condensation of the heavier phase in small droplets. In Fig. 1 *a*, an obliquely sectioned crystal with a nonorthogonal lattice is shown. Its basic repeats are ℓ_1 = 122 Å and ℓ_2 = 112 Å (ℓ, length), with an interaxial angle α of 67°. Very few dislocations or holes appear in the plane of sectioning. Glutaraldehyde (2.5%) caused the crystals to turn bright yellow within 12 h. The resulting insolubility in standard buffer containing 1% β-OG indicates that the crystals indeed consist of protein, and appropriate controls confirmed that no other component in the buffer used reacts with glutaraldehyde. Although the majority of microcrystals were large (20–50 μm body diagonals), the formation of small droplets produced extremely thin platelets in some preparations. These, as well as embedded crystals, appear promising for high-resolution studies of the various microcrystalline forms according to procedures described previously (3).

In an effort to obtain large crystals suitable for x-ray analyses, crystals were obtained by equilibrium dialysis. Though the use of β-OG yielded crystals with body diagonals up to 0.5 mm, their quality was unsatisfactory, particularly because the presence of this detergent caused the formation of an unfavorable two-phase system (see Materials and Methods). The experiments did show, however, that a high protein concentration (180–200 mg/ml), rather than the presence of PEG, was responsible for crystal growth. Subsequently, α-OG was substituted for β-OG, and PEG was used as a concentrating agent only in the outer compartments of Zeppezauer tubes. These conditions provided a single-phase system that gave either large crystal clusters or well-formed, though incomplete, bipyramids. A crystal cluster was washed carefully with crystallization buffer, and subsequently solubilized in SDS. Gel electrophoresis in this detergent yielded a single band with the properties characteristic for matrix protein (9). A fragment of a cluster is shown in Figure 1 *b*; diameters of up to 1 mm were observed. Bipyramids were smaller (their longest dimensions extending to 0.2 mm), but they exhibited sharp edges and vertices. They were colorless and not birefringent. A double crystal was isolated and mounted in a glass capillary. A still photograph (Fig. 2) exhibits Bragg diffractions extending to spacings of 3.8 Å. This high-order diffraction, together with the absence of optical activity, strongly suggests that the crystals have cubic symmetry (14). The reciprocal space distances of the observed lattice (Fig. 2 *a* and *b*), because of the orientation of one of the crystals, are indeed consistent with a cubic space group. Within experimental error, *b* would be the face diagonal of a cube, and *a*, the length of its side. We therefore conclude, tentatively, that the most probable lattice constants[3] are a = b = c ≃ 154 Å, with $\alpha = \beta = \gamma = 90°$. These values yield a unit cell volume (~3.94×10^6 Å^3) that, in conjunction with the size of the matrix protein trimer (M_r 110,000), limits the number of possible space groups. Thus, either P23 or $P2_13$ would satisfy the above constraints, if a trimer were in the asymmetric unit. This also would yield a reasonable value (2.94 Å^3/dalton) for the volume-to-mass ratio (5). This analysis is internally consistent, because computer modeling of a cubic lattice with the above cell constants and the observed angles of misalignment predicts the appearance of both the +*l* lattice and the −*l* zone at the positions where they appear in Fig. 2 (computer pattern not shown). The definitive assignment of the space group

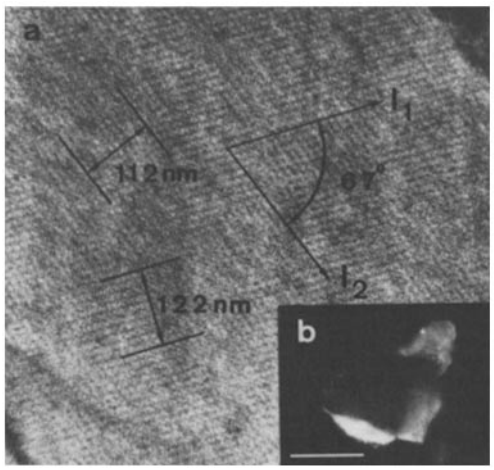

FIGURE 1 (*a*) Oblique thin section through a rhombohedral microcrystal of matrix protein obtained by vapor diffusion in β-OG. The dark portions correspond to the embedding plastic stained by OsO_4. The distances shown represent 10 lattice units. Analysis of this photograph by optical diffraction reveals maxima to a resolution of ~50 Å. Microcrystals were collected and dehydrated, using a graded series of ethanol. After embedding in Epon 812, the samples were sectioned on an LKB 2128 Ultratome (LKB-Produktor AB, Bromma, Sweden), mounted on gold 400-mesh grids, stained with OsO_4 vapor, and observed in a Philips EM301 operating at 80 kV. (*b*) A fragment of a crystal obtained in a Zeppezauer tube (15) in α-OG. This crystal, even though appearing disordered, exhibited Bragg diffraction to 4.9 Å. Bar, 0.5 mm.

[3] Such lattice constants, together with the results obtained by electron microscopy, further support our conclusion that these crystals are derived from protein.

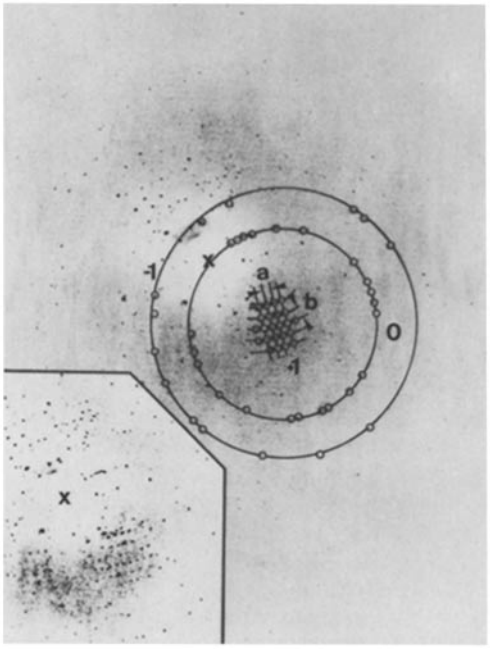

FIGURE 2 Diffraction pattern from a still photograph obtained from a double crystal grown in the presence of 1% α-OG. A distinct zero-order layer (*0*) and a first layer (*−1*) are marked on the photograph. The *+1* layer appears to cut Ewald's sphere tangentially, exhibiting several lattice lines. Discrete diffraction spots are clearly visible out to spacings of 4.2 Å, with the limit currently at 3.8 Å. They are not shown here because of the considerable falling off of the intensities. The diffraction by the second crystal probably produces the speckled background. An original diffraction pattern is shown in the *inset*. The 1-h exposure was taken at 20°C on a Philips generator (PW 1130; 44 kV, 32 mA) with unfiltered Cu K_α radiation from a sealed-off tube, using an Enraf-Nonius precession camera set at 75 mm. *X* indicates the origin.

remains to be established. Furthermore, we do not yet know whether this crystal form is related by space group or packing arrangement to the microcrystals discussed above. Our present efforts concentrate on assuring a steady supply of crystals suitable for high-resolution x-ray analysis. A complementary electron microscopic examination of the rhombic microcrystalline forms, obtained through vapor diffusion, has been started as well.

After completion of the studies described above, we learned that three-dimensional crystals have been obtained also with bacteriorhodopsin, using β-OG (7). The resolution attained in that case (currently 8 Å) does not exceed the one obtained from two-dimensional arrays previously (3), but it does provide a further example of an integral membrane protein that, given proper conditions, can be induced to form crystals amenable to high resolution analyses. It will be interesting also to determine the influence of the stereochemistry of the detergents used.

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