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 \textit{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 \(\alpha\)-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 \(\AA\), have a cubic space group.

Matrix protein is an abundant and well-characterized polypeptide \cite{9} that forms gated pores \cite{12} across the bacterial outer membrane. Previous investigations of its structure \cite{13} relied on its availability as two-dimensional hexagonal arrays, obtained by extraction of cell envelopes by dodecyl sulfate \cite{9}. Such preparations exhibited triplet indentations around local threefold axes at a resolution of \(\sim 25 \AA\) \cite{13}. Because the protein contains pores \cite{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 \(\AA\). The channels are water-filled and can assume either of two states: open or closed \cite{12}. Recently, we have succeeded in solubilizing the large aggregates \cite{13}, which previously resisted all attempts at dissociation without concomitant denaturation \cite{10}, to trimers \((M, 110,000)\), using mild, nonionic detergents \cite{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 \cite{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 \(\alpha\)-helices spanning the membrane \cite{3}, appears to satisfy the requirement of neutralizing its polar peptide bonds within the membrane by way of hydrogen-bonding them in antiparallel \(\beta\)-pleated sheets \cite{9, 10}. The significance of this type of secondary structure in membrane proteins cannot be evaluated without a structural resolution exceeding 4 \(\AA\). Furthermore, the possible role of \(\beta\)-structure as regards both formation and conformational changes of pores has been discussed recently \cite{4}. (c) The polarity of matrix protein is very high \cite{1, 9}, and its sequence does not exhibit large nonpolar fragments \cite{1}. Because the protein apparently hardly protrudes into the aqueous environment \cite{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 \((M, 110,000)\) \cite{11} were extracted \cite{11} with 3\% \(\beta\)-OG \cite{9} (Calbiochem-Behring Corp., American Hoechst Corp., La Jolla, Calif.) in standard buffer \((20 \text{ mM sodium phosphate, 3 mM NaCl, pH 7.0})\). Further purification by gel filtration \cite{10} yielded the protein with a purity of \(>95\%\), as judged from dodecyl sulfate gel electrophoresis. Gas chromatographic analyses \cite{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 \cite{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 \cite{2}, we initially used \(\beta\)-OG. Because of the resulting two-phase system \cite{cf. below}, we proceeded to synthesize and characterize its optical isomer \cite{7}.

Crystallization

1. **IN \(\beta\)-OG:** The fusion of a 10-\(\mu\)l drop containing 10 mg/ml protein in standard buffer, 1\% \([\text{wt/vol}]\) \(\beta\)-OG and 0.1 M NaCl with a 10-\(\mu\)l drop containing PEG 4000 (25\% \([\text{wt/vol}]\) 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 \cite{6} gave similar crystal forms. Drops (50 \(\mu\)l of 5 mg/ml protein) and 5\% \([\text{wt/vol}]\) PEG 4000 in standard buffer with \(\beta\)-OG were placed into depression slides and allowed to equilibrate with a solution \((40 \text{ ml})\) containing either PEG

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\(1\) Abbreviations used: \(\alpha\) (or \(\beta\))-OG, \(\alpha\)-octyl-\(\alpha\) (or \(\beta\))-D-glucopyranosides. PEG, polyethylene glycol.

\(2\) The details of the procedures mentioned, and the synthesis of \(\alpha\)-OG, are submitted for publication.
only in the outer compartments of Zeppezauer tubes. These
succeeded for β-OG, and PEG was used as a concentrating agent
for large crystal clusters or well-formed, though incomplete, bipy-
ramids. A crystal cluster was washed carefully with crystalliza-
tion buffer, and subsequently solubilized in SDS. Gel electro-
phoresis in this detergent yielded a single band with the prop-
erties characteristic for matrix protein (9). A fragment of a
cluster is shown in Fig. 1b; 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. 2a 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 constants2 are a = b
= c = 154 Å, with α = β = γ = 90°. These values yield a unit
cell volume (∼3.94 × 10⁶ Å³) that, in conjunction with the size of
the matrix protein trimer (Mt, 110,000), limits the number of
possible space groups. Thus, either P23 or P2₃,3 would satisfy
the above constraints, if a trimer were in the asymmetric unit.
This also would yield a reasonable value (2.94 Å³/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 +1 lattice and the −1 zone
at the positions they appear in Fig. 2 (computer pattern not
shown). The definitive assignment of the space group

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