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Crystallization and preliminary X-ray analysis of the primary receptor (PotD) of the polyamine transport system in *Escherichia coli*

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Abstract

The primary receptor (potD, $M_r = 39\,000$) of the polyamine transport system in *Escherichia coli* has been crystallized by the vapor-diffusion method. Two crystal forms were obtained in the presence of spermidine, and were examined by X-ray analysis. Form I crystals, which diffract to 2.5 Å resolution, belong to the space group $P2_1$, with unit-cell dimensions $a = 145.3$, $b = 69.1$, $c = 72.5$ Å and $\beta = 107.6°$. Four molecules are contained in an asymmetric unit. These form two dimers that are related to each other by a local translation of about half of the until cell along the a axis. The two protein molecules in each dimer are similarly related by a local dyad. Form II crystals diffract to 1.8 Å resolution and belong to the space group $I4_1$, with unit-cell dimensions $a = b = 130.3$ and $c = 38.7$ Å. They contain one molecule per asymmetric unit.

1. Introduction

Polyamines, such as putrescine, spermidine and spermine, are ubiquitous in all living organisms, and are implicated in a wide variety of biological reactions, including nucleic acid and protein syntheses (Tabor & Tabor, 1984; Pegg, 1988). It is a crucial in cell biology to understand the detailed mechanisms of polyamine biosynthesis and transport by which the cellular polyamine contents are controlled. Although the biosynthetic pathways for polyamines have been studied extensively, the transport mechanism remains obscure (Tabor & Tabor, 1984; Pegg, 1988). The polyamine transport gene (pPT104) in *Escherichia coli* has been cloned and characterized (Kashiwagi *et al.*, 1990). the proteins encoded by pPT104 constitute the spermidine-preferential uptake system, which belongs to a periplasmic transport system (Ames, 1986; Furlong, 1987). This spermidine-transport machinery consists of four protein subunits, potA, -B, -C and -D, the primary structures of which have been deducted from the nucleotide sequences of their clones (Furuchi, Kashiwagi, Kobayashi & Igarashi, 1991; Pistocchi *et al.*, 1993). The potA ($M_r = 43\,000$) protein, which is associated with the inner surface of the cytoplasmic membrane, is a strong candidate for an ATP-hydrolyzing energy-generating factor. In fact, the potA protein contains a consensus nucleotide-binding sequence, which is similar to the sequences of the α and β subunits of *E. coli* ATPase (Walker, Saraste, Runswick & Gay, 1982), the hisP protein (Higgins *et al.*, 1982) and the malK protein (Gilson, Nikaido & Hofnung, 1982). Both the potB ($M_r = 31$) and potC ($M_r = 29\,000$) proteins have six transmembrane-spanning segments linked by hydrophilic segments of variable length, and they probably form channels for spermidine and putrescine. The potD protein is a polyamine-binding protein present in the periplasmic space, and it regulates the polyamine content in cells. This protein binds both putrescine and spermidine, although it preferentially binds spermidine (Kashiwagi, Miyamoto, Nukui, Kobayashi & Igarashi, 1993). To obtain a more detailed insight into the molecular mechanism of the preferential spermidine transport, we have initiated a crystallographic study of the potD protein.

2. Crystallization and X-ray diffraction

The potD protein was purified according to the protocol described previously (Furuchi *et al.*, 1991). The purity was verified by sodium dodecyl sulfate polyacrylamide electrophoresis (SDS–PAGE). For crystallization, the protein was concentrated using a Centriprep-10 concentrator. In the presence of spermidine, crystals appeared in two different forms, which were designated as forms I and II. Form I was most frequently grown at 285 K by the sitting-drop mode of the vapor-diffusion method. A protein solution containing 5–10 mg protein ml^{-1}, 10 m*M* Bis–tris buffer (pH 7.0), and 20 m*M* spermidine trihydrochloride was mixed with an equal volume of a reservoir solution containing 30%(*w*/*v*) polyethyleneglycol 10 000 and 20 m*M* Bis–tris buffer (pH 7.0). The form I crystals were grown to an average size of $1.3 \times 0.6 \times 0.2$ mm within three weeks. The form II crystals were produced by combining the techniques of vapor diffusion and repeated seeding. The crystallization solutions were prepared by mixing an equal volume of the protein solution and the reservoir solution. The protein solutions contained 5–10 ml^{-1} mg protein and 20 m*M* spermidine trihydrochloride in 10 m*M* HEPES buffer (pH 7.0), while the reservoir solution contained 30%(*w*/*v*) polyethyleneglycol 4 000 and 40 m*M* HEPES buffer (pH 7.0). Small crystals appeared within a week. Repeated seeding was carried out to produce diffraction-quality crystals. Before seeding, the mixture had been equilibrated at room temperature with adequate reservoir solutions for at least 2 d, and the seeds were introduced into the mixture within a week. Thus, suitable crystals for X-ray diffraction were obtained, with dimensions of $0.6 \times 0.6 \times 0.4$ mm. Interestingly, small form II crystals sometimes appeared on the surface of the crystallization drops under the conditions that produce form I crystals, although these form II crystals did not grow to a large size. No crystals could grow in crystallization solutions lacking spermidine trihydrochloride. This result implies that the conformation of the potD protein might be different, depending upon the presence or absence of the substrate.

Diffraction studies were carried out using a precession camera (Enraf–Nonius & Huber). The diffraction patterns of the form I crystals exhibit systematic weakness of the $h = 2n + 1$ reflections at lower resolution. The form I crystal was found to

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belong to the space group $P2_1$, with unit-cell dimensions $a = 145.3$, $b = 69.1$, $c = 72.5$ Å and $\beta = 107.6°$. There are four molecules in an asymmetric unit. The packing density of the crystal, V_m, is calculated to be 2.24 Å^3 Da^{-1}, which is well within the range normally found for protein crystals (Matthews, 1968). This value corresponds to a solvent content of 45%. The form II crystal belongs to the space group $I4_1$, with unit-cell parameters $a = b = 130.3$ and $c = 38.7$ Å, and contains one molecule in an asymmetric unit. It has a similar solvent content to that of the form I crystal.

Intensity data were collected from each crystal form, using an automated oscillation camera system (DIP-320, MAC Science) with a cylindrical imaging-plate detector (Miyahara, Takahashi, Amemiya, Kamiya & Satow, 1986; Amemiya *et al.*, 1988), which is equipped on a Cu rotating-anode generator operated at 50 kV, 90 mA at 277 K. The intensities recorded on the area detector were evaluated by the program *WELMS* (Tanaka *et al.*, 1990), and were processed by the program *PROTEIN* (Steigemann, 1974). The completeness of reflections for the intensity data of crystal forms I and II [$I > 2\sigma(I)$] were 76.5% to 2.7 Å resolution and 90.0% for the data to 1.8 Å resolution, respectively. The $R_{\rm merge}$ values were 5.3% for the intensity data of crystal form I, and 5.6% for those of crystal form II. Furthermore, another 2.5 Å resolution data set has been collected from the form I crystal, using the macromolecular-oriented Weissenberg camera (Sakabe, 1991), installed at the beamline 6A2 of the Synchrotron Radiation Source in the National Laboratory for High Energy Physics, Tsukuba. The wavelength was set to 1.00 Å. The diffraction patterns were digitized by a BA-100 reader (Fuji film). The intensity data were evaluated using the program *WEIS* (Higashi, 1989), and were processed by the programs *COMBINE* and *SCALE* from the same program package. These programs were implemented on a FACOM VP2600 vector computer. The completeness and $R_{\rm merge}$ values of this data set were 78.0 and 6.0%, respectively. It should be noted that the diffraction pattern from the form I crystal systematically indicates weak intensities for the h odd indexed reflections (Fig. 1). On the other hand, no remarkable intensity difference could be observed between $h + k$ even and odd indexed reflections. These findings suggest that half of the four independent protein molecules were related to the other half by a local translation of about $\frac{1}{2}$ of the unit-cell length, along the a axis.

The self-rotation function was calculated for the resolution range from 10.0 to 3.5 Å, using the program *X-PLOR* (Brünger, 1990). One significant peak, which is 1.33 times larger than the second highest peak, was observed on the section $\kappa = 180°$, indicating the presence of non-crystallographic twofold symmetry. The position of the peak yields the polar angles $\psi = 90°$, $\varphi = 36°$, and $\kappa = 180°$ (Fig. 2). Thus, the non-crystallographic twofold axis is found to be approximately perpendicular to the crystallographic b axis. These results suggest that the protein molecules in the pair are related by the twofold axis, and that two pairs were packed with a local translation of about $\frac{1}{2}$ of the unit-cell length, along the a axis. The observation of a strong

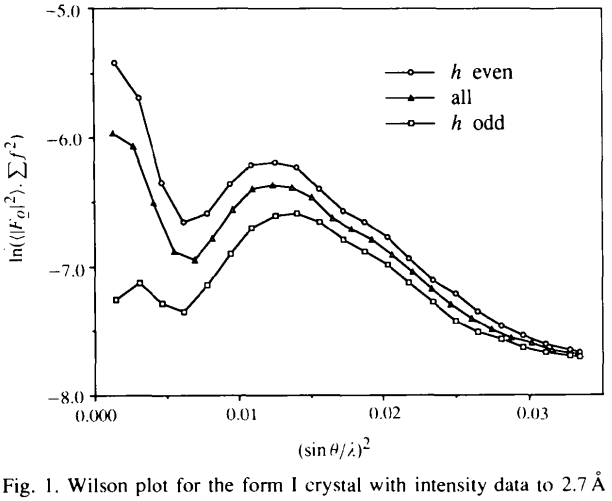

Fig. 1. Wilson plot for the form I crystal with intensity data to 2.7 Å resolution. The intensity data are classified into three groups in which the indexes of the reflections are $h = 2n$ and $h = 2n + 1$. The $\langle|F_o|^2\rangle$ values of the h odd reflections are notably weaker than those of the h even indexed reflections, and in particular, at low resolution. This fact implies that similar oriented protein molecules were packed in the unit cell with a local translation of about $\frac{1}{2}$ of the unit-cell length, along the a axis.

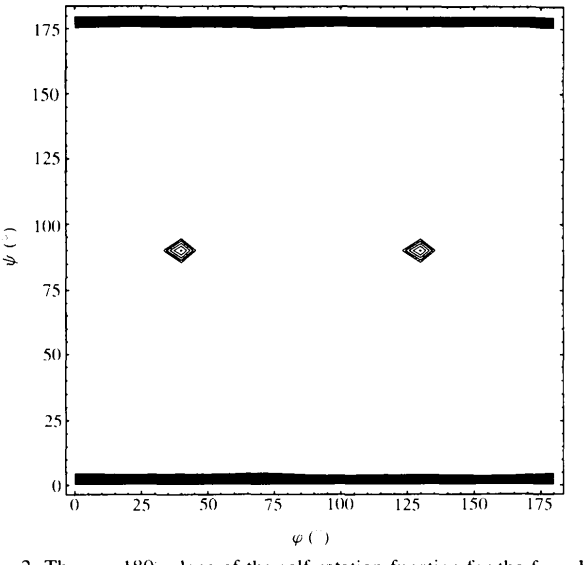

Fig. 2. The $\kappa = 180°$ plane of the self-rotation function for the form I crystal. A total of 16043 reflections between 10.0 and 3.5 Å resolution were included in the calculations. A single peak and its symmetry-equivalent peak were found at polar angles $\psi = 90°$, $\varphi = 36°$ and 126°, $\kappa = 180°$, respectively.

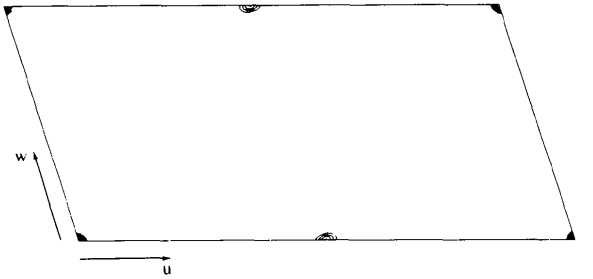

Fig. 3. Patterson map of the form I crystal at the section, $v = 0$. The map was calculated with all of the structure factors between 15.0 and 3.0 Å resolution. The origin peak is scaled to a value of 100, and the map has been contoured from 3 to 80 in steps of 6. The peak height at (0.5, 0, 0) is about 0.25 times that of the origin peak.

non-origin peak at approximately (0.5, 0, 0) in the Patterson synthesis is consistent with the above interpretation (Fig. 3).

Various heavy-atom compounds have been screened for both crystal forms. A few derivatives have yielded promising difference Patterson maps for the form I crystal.

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