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Crystallization and preliminary X-ray analysis of the periplasmic receptor (PotF) of the putrescine transport system in *Escherichia coli*

DMITRY G. VASSYLYEV,[a]*† TATSUKI KASHIWAGI,[a]‡ HIDEYUKI TOMITORI,[b] KEIKO KASHIWAGI,[b] KAZUEI IGARASHI[b] and KOSUKE MORIKAWA[a] at [a]*Biomolecular Engineering Research Institute, 6-2-3 Furuedai, Suita, Osaka 565, Japan, and* [b]*Faculty of Pharmaceutical Sciences, Chiba University, 1-33 Yayoi-choi, Inage-ku, Chiba 263, Japan.*
E-mail: dmitry@crl.mei.co.jp

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Abstract

The primary receptor (PotF) of the putrescine transport system in *E. coli* has been crystallized by the hanging-drop vapor-diffusion technique. The crystals belong to the space group $P2_12_12$ with unit-cell dimensions a = 269.4, b = 82.33 and c = 93.74 Å. The crystals diffract beyond 2.2 Å with a rotating-anode X-ray source. A complete data set from the native crystals has been collected and processed at 2.3 Å resolution. Two heavy-atom derivatives have been prepared from the same Pt compound at 293 and 277 K. The difference Patterson maps revealed completely different major heavy-atom sites between these two derivatives.

1. Introduction

Polyamines (putrescine, spermidine and spermine) are the small aliphatic cations that are involved in crucial cellular processes, such as the biosynthesis of proteins and nucleic acids (Tabor & Tabor, 1984; Pegg, 1988). In cells, the polyamine content is regulated by polyamine biosynthesis and degradation, and by the uptake/excretion activities of polyamine transport systems, which transfer the polyamines through the plasma membrane. Two polyamine uptake systems with different substrate specificities have been characterized in *E. coli* (Kashiwagi *et al.*, 1990; Kashiwagi, Miyamoto, Nukui, Kobayashi & Igarashi, 1993; Pistocchi *et al.*, 1993). The putrescine uptake system consists of the PotF, PotG, PotH, and PotI proteins, while the PotA, PotB, PotC, and PotD proteins are involved in both spermidine and putrescine transport, with a strong preference toward spermidine. In these transport systems, the PotF (PotD) protein is the primary receptor of putrescine (spermidine/putrescine) in the periplasmic space. PotH/PotI (PotB/PotC) are trans-membrane proteins, which form a channel for putrescine (spermidine/putrescine) in the membrane. PotG (PotA) is an ATP binding protein, which presumably provides the energy for putrescine (spermidine/putrescine) transport through the membrane. Recently, the membrane protein encoded by the *potE* gene in *E. coli* has been shown to possess both uptake and excretion activities for putrescine (Kashiwagi, Shibuya, Tomitori, Kuraishi & Igarashi, 1997). The crystal structure of the PotD protein in complex with spermidine has been solved by our group (Sugiyama, Matsuo *et al.*, 1996; Sugiyama, Vassylyev *et al.*, 1996). The atomic model revealed that the overall topology and the fold are similar to those of other periplasmic binding proteins, with the highest three-dimensional homology to the maltodextrin-binding protein (Sharff, Rodseth, Spurlino & Quiocho, 1992; Spurlino, Lu & Quiocho, 1991). PotD exhibits 35% sequence homology with PotF. However, PotD preferentially binds spermidine, while PotF can recognize only putrescine and has no affinity to the other polyamines. We initiated a crystallographic analysis of PotF to determine the mechanism of PotF substrate binding at the atomic level, which accounts for the strong specificity of PotF for putrescine. We now report the crystallization and preliminary X-ray analysis of PotF.

2. Materials and methods

2.1. *Crystallization procedures*

PotF consists of 370 amino acids (M_r = 41 000), including a 26-amino-acid signal peptide. It was expressed and purified as described previously (Pistocchi *et al.*, 1993). The hanging-drop vapor-diffusion technique was used for the crystallization. The extensive screening of crystallization conditions using protein concentration of 20 mg ml^{-1} and different precipitants at various concentrations and pH values has been carried out to obtain the PotF crystals. No crystals appeared under most of these conditions. In particular, the crystallization procedure, which was used successfully for the crystallization of PotD, the closest homologue of PotF (Sugiyama, Matsushima *et al.*, 1996), did not lead to the formation of any PotF crystals. Small microcrystals occasionally appeared within two or three weeks under the following conditions. The protein solution was concentrated to 20 mg ml^{-1} in a 20 m*M* Tris–HCl buffer (pH 7.0). Drops (5 µl) containing 2.5 µl of protein solution, 1.25 µl of H_2O, and 1.25 µl of a reservoir solution [2.5 *M* ammonium sulfate, 6% glycerol, and 200 m*M* cacodylate (pH 5.0)] were equilibrated with 1 ml of reservoir solution. The crystallization drops, which were preliminary equilibrated with 1 ml of reservoir solution for 5 d and did not provide any crystals, were used to grow the microcrystals through microseeding followed by macroseeding techniques. At first, the microcrystals were transferred to a crystal-free drop for micro-seeding. A few small macrocrystals (0.2 × 0.1 × 0.05 mm) appeared in the drop within two weeks. Each of these small crystals was washed in a three protein-free stock solutions with ammonium sulfate concentrations of 2.5, 2.75 and 3 *M*, respectively, and then was transferred to a new crystallization drop. Crystals of size 0.4 × 0.2 × 0.1 mm could be grown using macroseeding in a two-week period. However, this procedure required a large amount of protein material and produced only a few good crystals in a two-month period. In addition, the crystals were extremely sensitive to X-rays. Therefore, attempts were undertaken to improve the crystallization procedure. The new crystallization

† Present address: International Institute for Advanced Research, 3-4 Hikaridai, Seika, Soraku, Kyoto, 619-02, Japan.
‡ Present address: Central Research Laboratories, Ajinomoto Co., Inc., 1-1, Suzuki-cho, Kawasaki-ku, Kawasaki, 210, Japan.

conditions provided up to ten large crystals (average size 0.6 × 0.4 × 0.3 mm) in one 5 µl drop at 285 K within 5 d. The protein solution was concentrated to 40 mg ml^{-1}. Drops containing 2.5 µl of protein solution, 1.25 µl of H_2O, and 1.25 µl of a reservoir solution [2.0 *M* ammonium sulfate, 6% glycerol, and 200 m*M* cacodylate (pH 5.0)] were equilibrated with 1 ml of reservoir solution. The new crystals were much more stable to X-ray irradiation than the previous ones, although the crystal form was the same. Furthermore, they diffracted beyond 2.2 Å with a rotating anode X-ray source.

2.2. *X-ray analysis of the native crystals*

Data from the largest native PotF crystal (1.0 × 0.5 × 0.3 mm) have been collected at room temperature using a Rigaku FR-C rotating-anode generator, operated at 50 kV and 60 mA (0.1 × 1 mm electron-beam focus on the copper anode) and equipped with a RAXIS-IIC imaging-plate detector, at the International Institute for Advanced Research (IIAR, Kyoto, Japan). After focusing the X-ray beam by a system of mirrors (Charles Supper, USA) the beam size was about 0.1 × 0.1 mm, which allowed us to translate the crystal six times during data collection to avoid radiation damage. 240 frames for a full data set have been collected from one crystal. The oscillation range, the exposure time and the crystal-to-detector distance were fixed at 0.4°, 12 min and 120 mm, respectively, for each frame during the data collection. The diffraction data were processed by the programs *DENZO* and *SCALEPACK* (Otwinowski, 1993; Minor, 1993).

2.3. *Heavy-atom derivatives*

The native PotF crystals were soaked for 24 h at room temperature in a solution containing 2.3 *M* ammonium sulfate, 6% glycerol, 200 m*M* cacodylate and 4 m*M* K_2PtCl_4 to obtain the heavy-atom derivative. To reduce the possibility of obtaining non-isomorphic heavy-atom derivative crystals, another heavy-atom compound preparation was carried out in parallel; the concentration of K_2PtCl_4 was decreased to 2 m*M* and the soaking time was extended to 5 d at 277 K. Complete three-dimensional data sets were collected at room temperature for the crystals from both preparations using the rotating-anode X-ray source at IIAR.

3. Results and discussion

The PotF crystals belong to the space group $P2_12_12$, with unit-cell dimensions a = 269.4, b = 82.33 and c = 93.74 Å. A complete data set at 2.3 Å resolution has been collected and processed for the crystals of the native protein (Table 1). According to the molecular weight and unit-cell parameters, the asymmetric unit may contain from three to six molecules, with putative solvent contents ranging from 70 to 40%. The self-rotation function was calculated with the program *AMoRe* (Navaza, 1994) to examine the non-crystallographic rotational symmetry between the PotF monomers in the asymmetric unit. No prominent peaks corresponding to three-, four-, five- or sixfold rotation axes were found. Although a number of peaks in the rotation function indicate possible twofold symmetry, the signal-to-noise ratio for these peaks was too small to assign the non-crystallographic symmetry (NCS) between the PotF monomers. The cross-rotation function (the PotD coordinates were used as a search model) also showed no clear solution. Thus, we initiated a search of heavy-atom derivatives to solve the PotF structure.

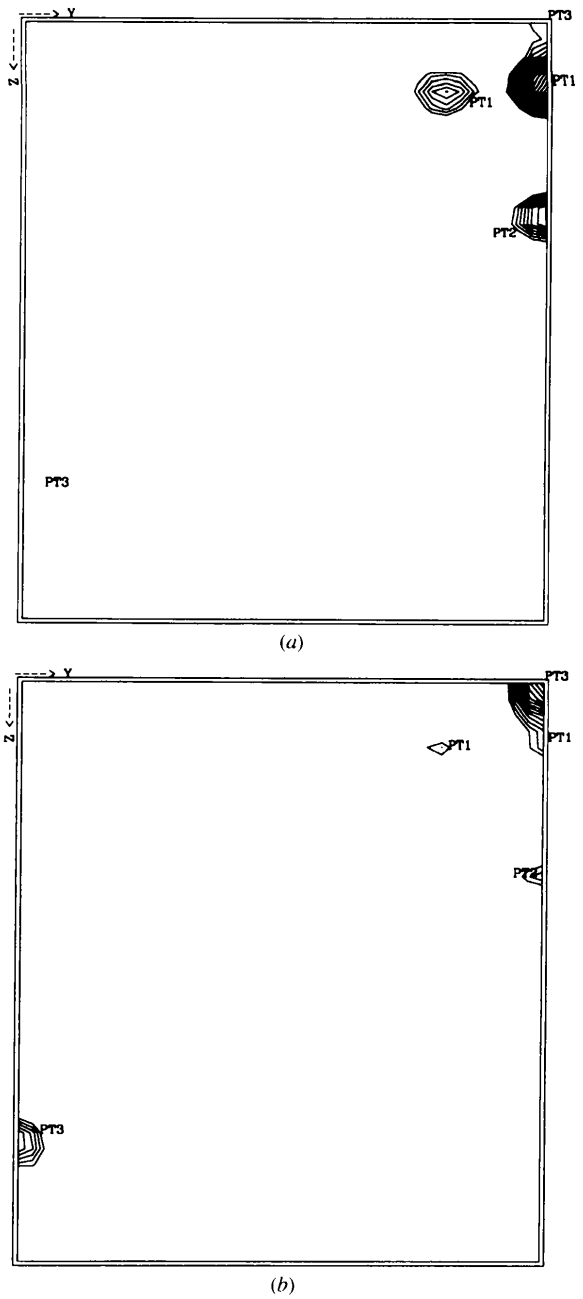

Fig. 1. Section ($x = \frac{1}{2}$, $y = 0 - \frac{1}{2}$, $z = 0 - \frac{1}{2}$) of the difference Patterson maps calculated with the data in the resolution range of 15–4 Å for the two K_2PtCl_4 heavy-atom derivative preparations. The contours are drawn starting from a density level equal to 4σ of the density, with 1σ increments in both maps. (*a*) The soaking time was 24 h at room temperature. The Patterson map shows two major heavy-atom sites (PT1), (PT2). (*b*) The soaking time was 5 d at 277 K. There is only one major heavy-atom site (PT3), and its position is different from the major sites in Fig. 1(*a*).

Table 1. *Data collection and isomorphous statistics for the native and the heavy-atom derivative crystals of PotF*

Native				Heavy-atom derivatives				
Resolution (Å)	R_{merge} $I > 0.5\sigma(I)$	Completeness $I > 0.5\sigma(I)$ (%)	$I > 2\sigma(I)$ (%)	Resolution (Å)	K_2PtCl_4 (293 K) R_{merge}†	R_{iso}‡	K_2PtCl_4 (277 K) R_{merge}	R_{iso}
50–4.60	0.041	98.6	97.0	6.00	0.046	0.183	0.036	0.127
3.65	0.054	97.8	95.6	4.76	0.073	0.163	0.053	0.115
3.19	0.074	97.0	92.0	4.16	0.075	0.148	0.053	0.112
2.90	0.102	95.7	85.7	3.78	0.108	0.165	0.070	0.119
2.69	0.147	94.4	77.0	3.51	0.148	0.181	0.094	0.133
2.53	0.185	92.3	67.0	3.30	0.199	0.210	0.115	0.144
2.40	0.219	90.7	59.0	3.14	0.252	0.247	0.147	0.157
2.30	0.260	85.3	49.0	3.00	0.300	0.260	0.192	0.165
Overall	0.069	94.0	79.0	—	0.090	0.180	0.067	0.126

† $R_{merge} = \sum_h \sum_i |I_{hi} - \langle I_h \rangle| / \sum_h \sum_i I_{hi}$; I_{hi}, $\langle I_h \rangle$, intensity of ith observation and mean intensity for the reflection with $h = \{h,k,l\}$, respectively. ‡ $R_{iso} = \sum |F_{nat} - F_{der}|_{nat}$; F_{nat}, F_{der}, structure factors of the native and the heavy-atom derivative, respectively.

Diffraction data at 3 Å resolution were collected for the two K_2PtCl_4 heavy-atom derivative preparations (Table 1). The heavy-atom positions were determined for these two Pt derivatives from the respective difference Patterson maps. The major heavy-atom sites appeared to be completely different between these two derivatives (Fig. 1). The heavy-atom positions were refined and the phases were calculated by use of the *MLPHARE* program (Otwinowski, 1991), and the initial electron-density map was calculated after phase improvement by solvent flattening and histogram matching, using the *DM* program in *CCP*4 (Collaborative Computational Project, Number 4, 1994). Long fragments of α-helices and β-strands are observed in this electron-density map, and the presence of four molecules in the asymmetric unit was confirmed. However, the electron density is too poor to trace the entire polypeptide chain, and it is too difficult to recognize the precise NCS operators to improve the phases with NCS averaging. The screening of other heavy-atom derivatives is now in progress.

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