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Crystallization and preliminary X-ray crystallographic analysis of *Escherichia coli* RbsD, a component of the ribose-transport system with unknown biochemical function

Min-Sung Kim,[a] Hyangee Oh,[b] Chankyu Park[b] and Byung-Ha Oh[a]*

[a]National Creative Research Initiative Center for Biomolecular Recognition, Department of Life Science and Division of Molecular and Life Science, Pohang University of Science and Technology, Pohang, Kyungbuk 790-784, South Korea, and [b]National Creative Research Initiative Center for Behavioral Genetics, Department of Biological Sciences, Korea Advanced Institute of Science and Technology, Yusong-Ku, Taejon 305-710, South Korea

Correspondence e-mail: bhoh@postech.ac.kr

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The *Escherichia coli* high-affinity ribose-transport system consists of six proteins encoded by the *rbs* operon (*rbsD*, *rbsA*, *rbsC*, *rbsB*, *rbsK* and *rbsR*). Of the six components, RbsD is the only one whose function is unknown. In order to gain insights into the function of RbsD by structural analysis, we overexpressed and crystallized the protein as a first step toward this goal. RbsD was overexpressed in *E. coli* and crystallized using the hanging-drop vapour-diffusion method at 296 K. The crystals belong to the monoclinic space group *C*2, with unit-cell parameters $a = 285.9$, $b = 92.3$, $c = 93.3$ Å, $\beta = 105.0^\circ$. The unit cell is likely to contain 64 molecules of RbsD, with a crystal volume per protein mass (V_M) of 2.43 Å^3 Da^{-1} and a solvent content of about 49.3% by volume. An equilibrium centrifugation analysis demonstrated that RbsD (MW = 15 292 Da) exists as an octamer in solution, suggesting that the asymmetric unit contains two octameric assemblies of RbsD. A native data set to 2.7 Å resolution was obtained from a flash-cooled crystal.

1. Introduction

D-Ribose is a pentose sugar that is an energy source as well as a component of nucleic acids. In *E. coli*, D-ribose is transported into the cell by several mechanisms. Firstly, high-affinity transport of D-ribose is achieved by one of the ATP-binding cassette transport systems (Ames, 1986; Iida *et al.*, 1984). Secondly, D-ribose can be transported at low affinity by the D-xylose transport system (Song & Park, 1998) and the D-allose transport system (Kim *et al.*, 1997). At least six proteins are involved in high-affinity D-ribose transport. These proteins are encoded by the *rbs* operon composed of *rbsD*, *rbsA*, *rbsC*, *rbsB*, *rbsK* and *rbsR* (Bell *et al.*, 1986; Iida *et al.*, 1984). In the periplasmic space, ribose-binding protein (RBP), encoded by *rbsB*, binds D-ribose which crosses the outer membrane by simple diffusion from the outside (Willis & Furlong, 1974). RBP can then interact with the membrane-bound permease (RbsC) that transports ribose across the inner membrane (Furlong, 1982). In the cytoplasmic space, ATP-binding protein (RbsA) assists permease function by coupling the energy from ATP to drive the transport of the sugar molecule (Buckel, 1986). The transported D-ribose is phosphorylated into D-ribose-5-phosphate by ribokinase (RbsK) before it can be used for the synthesis of nucleotides, tryptophan and histidine or before entering the pentose phosphate pathway (Anderson & Cooper, 1969). The last component, RbsR, is the repressor that binds to the transcriptional start site of the *rbs* operon. The affinity of RbsR for the *rbs* operator is reduced by addition of ribose *in vitro*, consistent with ribose being the transcriptional inducer of the operon (Mauzy & Hermodson, 1992). The *rbsD* gene is at the beginning of the *rbs* operon and the intercistronic spacings between *rbsD* and *rbsA* and between *rbsA* and *rbsC* are only seven and four base pairs without counting the translation terminator codons. In addition, the ribosome-binding site of *rbsA* or *rbsC* overlaps with the last part of the preceding gene (Bell *et al.*, 1986). It has been suggested that such close spacing enables translational coupling of adjacent genes (Anderson & Cooper, 1970). In the sequence analysis, RbsD shows no predicted transmembrane domain and exhibits sequence similarity to RbsD homologues in other organisms and to FucU, which is a component of the fucose operon. The biochemical functions of RbsD and FucU are not known.

Recently, it was shown that several mutations in PtsG, the glucose-specific transporter of the phosphoenolpyruvate:carbohydrate phosphotransferase system, allowed transport of D-ribose and D-ribose-dependent growth of an *E. coli* strain defective in both the high-affinity and the low-affinity ribose transporters (Oh *et al.*, 1999). Further analyses of the mutants revealed that maximal level of enhanced growth and transport was observed when both the *rbsK* and *rbsD* genes were

expressed. The results indicate that the transported ribose is phosphorylated not by PtsG but by ribokinase and that RbsD somehow plays a critical role in the PtsG-mediated ribose transport. The requirement for *rbsD* was also demonstrated in the low-affinity transporters. The role of RbsD is likely to be independent of specific types of transporters and involved in a step after membrane transport, such as acceleration of the ribose metabolism (Oh *et al.*, 1999).

Structural information from RbsD could lead to the identification of the biochemical function of RbsD and its homologues. The elucidation of their functions will provide a complete picture of the active transport of sugar molecules. In this report, we present data demonstrating that RbsD forms an octameric assembly in solution and describe preliminary X-ray crystallographic analyses of RbsD.

2. Protein purification

The *rbsD* gene was amplified using the polymerase chain reaction (PCR) technique with forward (5′-TACATATGATGAAAA-AAGGCACC-3′) and reverse (5′-GCTC-GAGTCAGAACGTCACGCCAGCAC-3′) primers. The PCR products were purified, digested with *Xho*I and *Nde*I and then ligated into the pET21b vector (Novagen). The resulting vector was introduced into *E. coli* BL21 (DE3) strain. The expression of the recombinant RbsD protein was induced by 1 m*M* isopropyl-D-thiogalactopyranoside at an optical density of 0.3–0.4 at 310 K for 8 h. Bacterial lysates were prepared by sonication in buffer *A* (pH 7.3) consisting of 50 m*M* Tris–HCl, 100 m*M* NaCl and 1 m*M* EDTA. After centrifugation at 15 000 rev min^{-1} for 1 h, the supernatant was brought to 30% saturation with ammonium sulfate, stirred for 3 h and centrifuged at 6000 rev min^{-1} for 30 min at 277 K. The resulting precipitant was resuspended in 30 ml buffer *A* and dialyzed against the same buffer. The dialyzed solution was loaded onto a Q Sepharose fast-flow column (Amersham Pharmacia Biotech) and eluted with a linear NaCl gradient from 0.1 to 1 *M* in buffer *A*. The eluted solution was concentrated and loaded onto a Hiload 26/60 Superdex 200 size-exclusion column (Amersham Pharmacia Biotech) equilibrated with buffer *B* (pH 7.3) consisting of 20 m*M* Tris–HCl, 100 m*M* NaCl and 1 m*M* EDTA. Finally, using a vacuum-dialysis membrane (Schleicher & Schuell), the protein solution was concentrated to 10 mg ml^{-1} against buffer *B*.

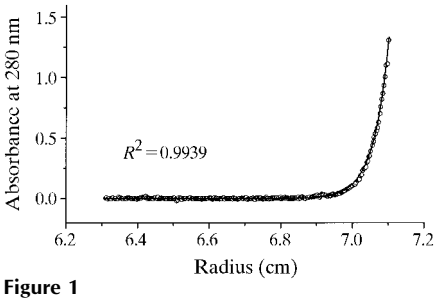

Figure 1
Equilibrium centrifugation of RbsD. An equilibrium was attained in 40 h. The circles indicate the experimental data and the solid line indicates the calculated curve for octameric species. R^2, the coefficient of determination, indicates an excellent fit to an ideal octameric species model. R^2 is defined as $\sum(\hat{y}_i - \langle y\rangle)^2 / \sum(y_i - \langle y\rangle)^2$, where y_i, $\hat{y}_i$ and $\langle y\rangle$ are the observed, fitted and average values of y, respectively.

3. Equilibrium centrifugation analysis

Since RbsD was eluted from the size-exclusion column as if it was a multimeric protein, we investigated the oligomeric state of RbsD in solution by equilibrium centrifugation. Sedimentation equilibrium measurement was performed at 293 K on a Beckman Optima XL-A analytical ultracentrifuge, using a four-hole rotor with a standard double-sector cell at a rotor speed of 10 000 rev min^{-1}. The protein sample contained 0.2 mg ml^{-1} RbsD in buffer *B*. The values of the two variables, absorbances at 280 nm *versus* radial positions, were obtained (Fig. 1). The partial specific volume of RbsD was calculated to be 0.7402 cm^3 g^{-1} from the amino-acid sequence of the protein according to the description in the literature (Zamyatnin, 1984). The apparent molecular weight of RbsD was calculated by fitting the data set to a single-species model using the software *BMPD-Nonlinear regression* provided by the manufacturer. The analysis demonstrated an average molecular mass of 127 000 ± 1600 Da, suggesting that RbsD associates into an octamer in solution, given the calculated octameric molecular mass of RbsD.

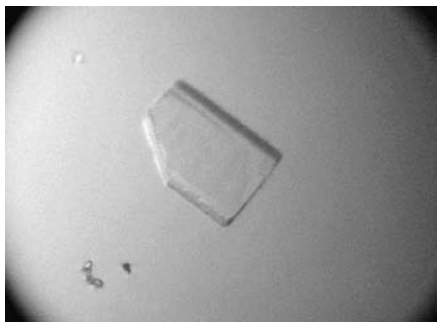

Figure 2
Crystal of RbsD; typical dimensions are 0.3 × 0.2 × 0.05 mm.

Table 1
Crystal information and data-collection statistics.

Values in parentheses refer to the highest resolution shell, 2.7–2.8 Å.

Source	6B1, PLS
Wavelength (Å)	1.0000
Space group	*C*2
Unit-cell parameters (Å,°)	a = 285.9, b = 92.3, c = 93.3, β = 105.0
Resolution range (Å)	20–2.7
Completeness (>1σ, %)	92.9 (83.1)
R_{sym}† (%)	8.1 (13.5)
$I/\sigma(I)$	15.26 (2.8)

† $R_{sym} = \sum|I_{obs} - I_{avg}|/I_{obs}$.

4. Crystallization and X-ray crystallographic study

In the early stages of this study, we used RbsD with a His_6 tag at the C-terminus for crystallization trials. Although crystals were obtained from this sample, they suffered from very high mosaicity. The C-terminal segment is hydrophobic and may be involved in octamer formation. We solved this problem by re-cloning, purifying and crystallizing RbsD without a tag. 2 µl droplets of the final purified protein solution were mixed with an equal volume of a precipitant solution containing 0.4 *M* NaCl, 14% polyethylene glycol 8000 and 0.1 *M* 2-(*N*-cyclohexylamino)ethanesulfonic acid pH 9.2. The mixture was then equilibrated on a cover slip against 1 ml of the precipitant solution as a reservoir solution in a 24-well tissue-culture plate at 296 K. Crystals of RbsD appeared in the droplets in less than 3 d and reached maximum dimensions in a week (Fig. 2). Diffraction data were collected from a flash-cooled crystal using synchrotron radiation from beamline 6B1 at Pohang Light Source, Korea. Since the crystals were sensitive to radiation damage, they were cooled at 100 K using a cryo-cooling system (Oxford Cryosystems) for full data collection. Before data collection, the crystal was soaked briefly in a cryoprotectant solution, which was the precipitant solution containing 25% glycerol. Diffraction data (Table 1) were obtained and processed using the programs *DENZO* and *SCALEPACK* (Otwinowski, 1993). We calculated the crystal volume per molecular weight (V_M) to be 2.43 Å^3 Da^{-1} with a solvent content of 49.3% by volume (Matthews, 1968), assuming one unit cell to

contain 64 protomers. This corresponds to two octameric assemblies per asymmetric unit. A search for suitable heavy-atom derivative crystals is in progress.

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