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FOR THE RECORD

Purification, characterization, and crystallization of *Escherichia coli* ribokinase

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Abstract: Ribokinase phosphorylates ribose to form ribose-5-phosphate in the presence of ATP and magnesium. The phosphorylated sugar can enter the pentose phosphate pathway or be used for the synthesis of nucleotides, histidine, and tryptophan. Ribokinase belongs to the PfkB family of carbohydrate kinases, for which no three-dimensional structure is currently known. We describe an improved purification protocol for *Escherichia coli* ribokinase and give evidence from light-scattering and gel filtration studies that the protein forms a dimer in solution. Several types of crystals are also described that have been obtained of apo ribokinase, ribokinase in the presence of ATP, and in a ternary complex with an ATP-analogue and ribose. The latter crystals give the best X-ray diffraction. A complete data set has been collected at the synchrotron source in Hamburg, to 2.6 Å resolution using a frozen crystal. The crystals belong to space group $P6_122$ or $P6_522$ with cell parameters $a = b = 95$ Å and $c = 155$ Å.

Keywords: crystallization; purification; ribokinase; X-ray diffraction

D-ribose is one of the most abundant and important sugars in biology. It is a component of RNA, DNA, ATP, and many cofactors, as well as a useful source of energy. Before entering the metabolic pathways, ribose must first be phosphorylated. The resulting D-ribose-5-phosphate can then be used either for synthesis of nucleotides, histidine, and tryptophan, or as a component of the pentose phosphate pathway. The phosphorylation of ribose is carried out by ribokinase, which requires ATP and magnesium. A single type of ribokinase has been found in bacteria, plants, and animals. Sequence comparisons have shown that it belongs to the PfkB family of carbohydrate kinases (Wu et al., 1991), also called the ribokinase family (Bork et al., 1993). This family includes ribokinase, ketohexokinase, adenosine kinase, fructokinase, 2-dehydro-3-deoxygluconokinase, 1-phosphofructokinase, and the minor 6-phosphofructokinase (PfkB). No three-dimensional structure of a protein in this family has so far been determined.

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In the present paper, we describe an improved purification protocol for the *Escherichia coli* (*E. coli*) ribokinase and present data showing that this enzyme forms a dimer in solution. We also discuss the crystallization of the protein and describe preliminary X-ray data collection from a number of crystal forms.

Results and discussion: *Purification:* The full-length *E. coli* ribokinase, 309 amino acids, has been over-expressed in the cytoplasm of an *E. coli* strain with a non-functional rbs operon, and purified to near homogeneity. A typical 4-L culture gives 20 g of wet cells. After cell lysis and ammonium sulfate precipitations, ribokinase constitutes 20% of the total amount of protein, as estimated from gels and ribokinase activity assays. A DEAE column improves purity to about 50% and a RedA dye-affinity column results in a preparation that is approximately 95% pure. As a final step, a MonoQ column is used. This column gives four incompletely resolved peaks with ribokinase activity (hereafter referred to as peaks I–IV).

After pooling corresponding peaks from four consecutive runs, peaks I–IV contain 10 mg, 10 mg, 3 mg, and 2 mg of ribokinase, respectively. This is a significant improvement of yield over the previously described method of purification (Hope et al., 1986). Although these species presumably differ in surface charge, the differences between them are not yet completely characterized. Peak I is more homogenous than peak II, as judged by IEF, SDS, and native gels. In the final peak fractions, 30 U corresponds to 1 mg of ribokinase; peak I and peak II have equal activity within the limits of experimental error.

Evidence for dimer formation: Both gel filtration and dynamic light-scattering studies show that peak I ribokinase is found primarily as a dimer, while in peak II a mixture of dimer and monomer is observed.

In the gel filtration experiment, peak I ribokinase gave a single peak at 7.5 mL elution and peak II split up into one large peak at 7.5 mL and a smaller peak at 9 mL. These values correspond well to those expected for a dimer and a monomer, respectively. Bovine serum albumin (67 kDa) and glucose/galactose-binding protein (33 kDa), which were used as standards in the calibration of the column, gave peaks at 7.5 mL and 8.6 mL, respectively. An estimate of the relative area of the two peaks obtained from the

peak II run, suggests that approximately 80% is a dimer and 20% elutes at the expected monomer position.

The dynamic light-scattering studies showed that peak I is monodisperse with an estimated molecular weight of 62 kDa, which also, within error limits, corresponds to a dimer. Peak II has polydisperse behavior, suggesting a mixture of species.

Crystallization: We have obtained a number of different types of ribokinase crystals, as summarized in Table 1.

Under the crystallization conditions described for ribokinase in the presence of ATP, two different types of crystals are obtained in the same drop. The first are well-formed box-shaped tetragonal crystals with one large cell parameter (c = 343 Å), which complicates data collection. The other crystals are mis-shapen and do not diffract well enough to determine the space group. These latter crystals are also observed under similar conditions but in the absence of ATP, and are obtained with both peaks I and II. Both of these mis-shapen crystal types can be used to seed apo ribokinase drops where PEG 2000 monomethyl ether replaces the PEG 8000.

The apo ribokinase drops that are streak-seeded one to two days after setup give two new higher quality crystal forms within one to two additional days. Each of these forms could be obtained with both peaks I and II. Those belonging to space group P1 diffract to 2.8 Å resolution on a rotating anode X-ray source. These crystals cannot be obtained reproducibly, although they are more easily obtained with fresh protein. The other crystals belong to space group P321, $P3_121$, or $P3_221$ and diffract to 3.7 Å without freezing. We have tried a number of cryoprotectant solutions but have not yet succeeded in freezing these crystals without a drastic increase in their mosaicity that results in streaked spots.

Ribokinase/ribose/AMP-PNP crystals using ammonium phosphate as the precipitant appear after a few days. These hexagonal crystals belong to either space group $P6_122$ or $P6_522$ and diffract, when frozen, to 2.9 Å resolution on a rotating anode X-ray source and to 2.6 Å at the synchrotron beam line X11 of the EMBL Outstation at DESY, Hamburg. Hexagonal crystals grown with ammonium sulfate as the precipitant take at least six weeks to grow, but otherwise have the same appearance and behavior as those grown with ammonium phosphate. Crystals obtained from peak I were larger than those obtained with peaks I and II combined.

Estimation of the number of monomers in the asymmetric unit according to Matthews (1968) is consistent with the presence of dimers in the various crystals (Table 1). For the P1 crystals, there seem to be four monomers/asymmetric unit, which may correspond to two dimers. The other crystal forms, where we know the space group, have only one monomer in the asymmetric unit, but in each there is a crystallographic twofold symmetry axis, and therefore, dimer formation is possible.

Data collection: The crystals grown in ammonium phosphate are particularly suitable for structure solution. They have a small unit cell, diffract to a reasonable resolution, and are easy to crystallize and to freeze. We have obtained a data set extending to 2.6 Å resolution at the EMBL Outstation, DESY, Hamburg with R_{merge} 6.9% (23.4%), $I/sigI$ 20.3 (7.1), and completeness 96.9% (98.2%), where numbers in parentheses correspond to the highest resolution bin (2.64–2.6 Å). These crystals are obtained reproducibly and will be used to screen for heavy atom derivatives.

More effort is required to find suitable cryo conditions before a good data set from the apo crystals can be obtained. The results with the crystals of the ternary complex suggest that a fuller characterization of the aggregation behavior of the protein will be an important factor in improving crystal quality. A possible correlation between the dimer/monomer equilibrium and the ligands bound by the enzyme is now being investigated. The fact that inclusion of different ligands affects the crystal form may indicate that conformational changes are taking place.

Materials and methods: *Culture growth:* The plasmid and strain used for expression of *E. coli* ribokinase were gifts from Mark Hermodson, Purdue University. The ribokinase gene has been cloned behind a trp promoter in the plasmid pJGK10 (Hope et al., 1986). The strain MRi240 (*Drbs, recA*^{56}, *nal*^{R}, *srl*$^{+}$), carrying the plasmid, was grown in Vogel-Bonner minimal medium containing 0.05% casamino acids, 0.4% glycerol, and 50 mg/L ampicillin at 37 °C, as described by Hope et al. (1986). At A_{600} 0.5–0.6 the promoter was induced by adding 25 mg/L β-indole acrylic acid. The cells were harvested after 16 h of induction by centrifugation (6,000 × *g*, 15 min).

Table 1. *The different crystal forms*

Ribokinase form	Size of crystal (mm)	Space group	Cell parameters						Highest resolution (Å)	mol./a.u.[a]	V_M[a]
			a (Å)	b (Å)	c (Å)	α (°)	β (°)	γ (°)			
apo	0.4 × 0.2 × 0.1	P1	64	66	94	71	73	62	2.8	4	2.5
apo	0.2 × 0.2 × 0.05	$P3_x21$	62	62	127	90	90	120	3.7	1	2.1
ATP	0.2 × 0.2 × 0.2	Tetragonal	62	62	343	90	90	90	3.6	2 or 4[b]	2.5
Ribose/AMP-PNP (Am.sulf.)	0.7 × 0.1 × 0.1	$P6_x22$	96	96	156	90	90	120	2.7[c]	1	3.1
Ribose/AMP-PNP (Am.phos)	0.7 × 0.1 × 0.1	$P6_x22$	95	95	155	90	90	120	2.6[c]	1	3.1

[a]Estimated number of molecules per asymmetric unit based on common values for V_M (Matthews, 1968). V_M = cell volume (Å^3)/molecular weight in cell (Da).
[b]Depending on whether the space group is $P4_x2_x2$ or $P4_x$.
[c]Under cryo conditions at beam line X11 of EMBL Outstation at DESY, Hamburg.

Purification: The purification involved substantial modifications of that described by Hope et al. (1986). Unless otherwise stated, purification was carried out at 4 °C. Cell pellets were resuspended in 2 vol of 10 mM Tris-HCl, pH 7.8, 10 mM EDTA, and incubated for 30 min with 2 mg lysozyme/g cells at 0 °C. An aliquot of 5 μL/mL Pefabloc SC (Pentapharm AG, Basel, Switzerland) was added before a 3-min sonication. After centrifugation (26,500 × *g*, 25 min) streptomycin sulfate (Sigma Chemical Co., St. Louis, MO) was added to a concentration of 1% followed by another centrifugation.

The 30–70% pellet from ammonium sulfate precipitations was dissolved in the smallest possible volume of 25 mM sodium phosphate pH 6.8 and dialyzed against the same buffer. The sample was then applied to a 2 × 11.5 cm DEAE Sepharose (Pharmacia, Uppsala, Sweden) column equilibrated with 25 mM sodium phosphate, pH 6.8. A linear salt gradient of 0–0.4 M NaCl in the same buffer was then applied; ribokinase eluted between 175 and 230 mM NaCl. The pooled fractions were dialyzed against 20 mM bis-Tris, pH 6.2, 20 mM $MgCl_2$ and then applied to a 20 mL Affinity DyeMatrex™ Gel RedA column (Amicon, Beverly, MA). The sample was loaded onto the column at a flow rate of 0.3 mL/min, the flow was then stopped for 15 min to allow binding. The protein was eluted by washing with the same buffer, but without Mg^{2+}, at a flow rate of 0.5 mL/min. The pooled ribokinase-containing fractions were concentrated to a volume of about 8 mL and dialyzed against 20 mM Tris-HCl, pH 7.8.

The final purification step was done at room temperature with a MonoQ HR 5/5 column (Pharmacia). In each run, between 3 and 6 mg protein were loaded onto the column in a 2 mL volume, at a flow rate of 1 mL/min, followed by washing with 10 mL buffer. A gradient in three steps was then used to elute the protein; 0–15% in 2 mL, 15–45% in 26 mL, and finally 45–100% in 10 mL. The elution buffer was 20 mM Tris-HCl, pH 7.8 containing 0.7 M NaCl. Ribokinase eluted in four partially resolved peaks between 210 and 315 mM NaCl.

Assays: A coupled assay consisting of three reaction steps was used to measure ribokinase activity as described by Hope et al., 1986. In our hands, this assay was not very reproducible, presumably because it involves coupled reactions in three steps, and is influenced by the composition of sample and buffer. We, therefore, used the kinetics as a guide throughout the purification and not as an absolute measurement of how much enzyme was obtained. Total protein concentration was measured with the BioRad protein assay using bovine serum albumin as a standard. To check the purity, Pharmacia's PhastSystem was used. The SDS gels used contained 12.5% polyacrylamide and the native gels 20%. pIs were determined on a PhastGel IEF 3-9, with Pharmacia's Low pI Kit for calibration.

Dynamic light-scattering studies: The dynamic light-scattering machine used was a Model DP-801 instrument from Protein Solutions (Charlottesville, Virginia). The experiments were performed at room temperature with 1 mg/mL of protein in 20 mM Tris-HCl, pH 7.8, and 0.21 M NaCl.

Gel filtration studies: The column used was Novarose PrePac SE-100/17 (Pharmacia) at a flow rate of 0.5 mL/min, with 20 mM Tris-HCl, pH 7.8 containing 0.2 M NaCl as the buffer. As standards, 0.5 mg/mL bovine serum albumin, 67 kDa, and 0.3 mg/mL glucose/galactose-binding protein, 33 kDa, were used. The ribokinase samples had a concentration of 1 mg/mL. The sample volume per run was 0.2 mL and the study was performed at room temperature.

Crystallization: All crystals were grown at room temperature using the hanging drop vapor diffusion technique (McPherson, 1989). Four different starting conditions, with crystals of varying quality, were found using the Hampton Crystal Screening kit I (Hampton Research).

Ribokinase with or without ATP: A 2 μL sample of 11 mg/mL of ribokinase in 20 mM Tris-HCl, pH 7.8, 0.2 M NaCl, and 20 mM ATP, where needed, was mixed with an equal volume of 0.1 M Na acetate, pH 4.8, 0.2 M NH_4 acetate, and 18–23% PEG 8000, and equilibrated against the latter solution.

Apo ribokinase: A 2 μL sample of 11 mg/mL of ribokinase in 20 mM Tris-HCl, pH 7.8, and 0.2 M NaCl was mixed with an equal volume of 0.1 M Na acetate, pH 4.8, 0.2 M NH_4 acetate, and 22–33% PEG 2000 mono-methyl ether, and equilibrated against the latter solution. After 1–2 days of incubation the drops were streak-seeded either using ribokinase/ATP crystals or apo ribokinase crystals.

Ribokinase with ribose and the ATP analogue, AMP-PNP: A 2 μL sample of 11 mg/mL of ribokinase in 20 mM Tris-HCl, pH 7.8, 0.2 M NaCl, 1 mM ribose, 20 mM Li AMP-PNP (5′-adenylylimido-diphosphate; Sigma Chemical Co.) and 20 mM $MgCl_2$ was mixed with either an equal volume of 2.1–2.4 M $NH_4H_2PO_4$ and 0.1 M Tris-HCl, pH 8.4, or an equal volume of 2.0 M $(NH_4)_2SO_4$. The 4 μL drop was then equilibrated against the ammonium phosphate or the ammonium sulfate solution. These crystals could be frozen in a liquid nitrogen stream after a 2–5 min soak in an equivalent mother liquor containing 20% glycerol.

Data collection: Data were collected either on a Rigaku R-AXIS imaging plate mounted on a rotating anode X-ray source or on a MAR imaging plate at the EMBL beam line X11 at DESY, Hamburg. The images were integrated and the data scaled using the HKL suite of programs (Otwinowski, 1993).

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