Acta Crystallographica Section D
Biological Crystallography
ISSN 0907-4449

Crystallization and preliminary X-ray diffraction studies of the lipopolysaccharide core biosynthetic enzyme ADP-L-glycero-D-mannoheptose 6-epimerase from *Escherichia coli* K-12†

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Received 1 June 1998
Accepted 9 November 1998

† A preliminary report of this work was presented at the American Society for Biochemistry and Molecular Biology Meeting, San Francisco, California, August 24–29, 1997.

ADP-L-glycero-D-mannoheptose 6-epimerase is a 240 kDa NAD-dependent nucleotide diphosphosugar epimerase from *Escherichia coli* K12 which catalyzes the interconversion of ADP-D-glycero-D-mannoheptose and ADP-L-glycero-D-mannoheptose. ADP-L-glycero-D-mannoheptose is a required intermediate for lipopolysaccharide inner-core and outer-membrane biosynthesis in several genera of pathogenic and non-pathogenic Gram-negative bacteria. ADP-L-glycero-D-mannoheptose 6-epimerase was overexpressed in *E. coli* and purified to apparent homogeneity by chromatographic methods. Three crystal forms of the epimerase were obtained by a hanging-drop vapor-diffusion method. A native data set for crystal form III was collected in-house on a Rigaku R-AXIS-IIC image plate at 3.0 Å resolution. The form III crystals belong to the monoclinic space group $P2_1$. The unit-cell parameters are a = 98.94, b = 110.53, c = 180.68 Å and β = 90.94°. Our recent results show that these crystals diffract to 2.0 Å resolution at the Cornell High Energy Synchrotron Source. The crystal probably contains six 40 kDa monomers per asymmetric unit, with a corresponding volume per protein mass (V_m) of 4.11 Å^3 Da^{-1} and a solvent fraction of 70%.

1. Introduction

The outer membrane, unique to Gram-negative bacteria, exhibits low permeability to hydrophobic agents, rendering the bacteria refractory to antibiotic therapy (Coleman & Leive, 1979). Lipopolysaccharide (LPS) is an integral component of the outer membrane. L-Glycero-D-mannoheptose is a seven-carbon sugar typical of the core region of the lipopolysaccharide of enteric and nonenteric Gram-negative bacteria. LPS synthesis requires the enzyme ADP-L-glycero-D-mannoheptose 6-epimerase (Coleman, 1983). The ADP-L-glycero-D-mannoheptose 6-epimerase (henceforth referred to as epimerase) catalyzes the interconversion of ADP-D-glycero-D-mannoheptose and ADP-L-glycero-D-mannoheptose (Coleman, 1983; Coleman *et al.*, 1992), which is the last step in the ADP-L-glycero-D-mannoheptose biosynthetic pathway. ADP-L-glycero-D-mannoheptose is the precursor of L-glycero-D-mannoheptose. The epimerase is encoded by the *rfaD* gene (Pegues *et al.*, 1990). RfaD mutant strains exhibit the classic heptoseless phenotype which includes mucoidal colonies, reduced growth rates and viability, a truncated lipopolysaccharide structure and loss of the intrinsic barrier function of the outer membrane (Coleman & Leive, 1979; Coleman, 1983; Coleman *et al.*, 1992; Pegues *et al.*, 1990). The native epimerase from *Escherichia coli* is composed of six identical subunits and has a molecular mass of 240 kDa. The N-terminus of the primary structure of each epimerase subunit contains the fingerprint sequence Gly*X*Gly*XX*Gly, which is common to proteins that bind NAD or FAD. One molecule of nicotinamide adenine dinucleotide (NAD^+) is bound to each subunit (Pegues *et al.*, 1990; Ding *et al.*, 1994). ADP-L-glycero-D-mannoheptose 6-epimerase has properties which suggest that it belongs to a group of epimerases involving NAD^+-dependent redox catalysis (Glaser, 1972). UDP-galactose 4-epimerase, the group prototype, catalyzes the NAD^+-dependent interconversion of UDP-galactose and UDP-glucose. UDP-galactose 4-epimerase has been studied extensively both catalytically and structurally (Thoden *et al.*, 1997). There is an absence of significant amino-acid sequence homology between UDP-galactose 4-epimerase and ADP-L-glycero-D-mannoheptose 6-epimerase; however, the NAD-binding sites of the two enzymes share certain key invariant residues (Wierenga *et al.*, 1986; Ding *et al.*, 1994; Thoden *et al.*, 1997).

In this article, we report the crystallization and preliminary X-ray diffraction studies of the first LPS core biosynthetic enzyme, ADP-L-glycero-D-mannoheptose 6-epimerase. Crystals of the epimerase were obtained in the presence of NAD^+, spermidine, PEG 400 and the epimerase catalytic inhibitor ADP-glucose.

2. Materials and methods

2.1. Protein purification

ADP-L-glycero-D-mannoheptose 6-epimerase, overproduced in *E. coli* strain CL627, was purified to apparent homogeneity employing a two-step purification protocol (Ding *et al.*, 1994). To obtain epimerase of suitable quality for crystallization experiments, the epimerase (Ding *et al.*, 1994) was subjected to an additional ion-exchange chromatographic step employing the Pharmacia Biotech Smart System. Epimerase (2 mg) obtained by the two-step purification protocol was applied to a MonoQ anion-exchange column (HR 5/5, 1 ml bed volume) pre-equilibrated with TEM buffer (10 m*M* Tris, 10 m*M* EDTA, 0.1 m*M* β-mercaptoethanol, pH 8.0) containing 0.4 *M* KCl. The bound proteins (Fig. 1) were eluted with 19 ml of a 0.4–1.0 *M* KCl linear gradient in TEM buffer. A major epimerase peak which eluted at 0.8 *M* KCl was collected, desalted against water and concentrated with an Amicon Centricon-10 microconcentrator.

2.2. Crystal growth and analysis

For crystallization, protein concentrations between 2.5 and 4 mg ml^{-1} were used. Crystals were grown from hanging drops, *via* the vapor-diffusion technique, at room temperature (Wlodawer & Hodgson, 1975). The drops were prepared by mixing equal volumes of protein and reservoir solutions (final drop volumes of 10 or 20 µl). The drops were routinely equilibrated against 1 ml of reservoir solution. Employing the general crystal-growth protocol outlined above, three crystal conditions were established (see Fig. 2). For crystallization condition 1, epimerase samples (5 and 10 µl of 2.5 mg ml^{-1} epimerase) were equilibrated against a reservoir solution containing 15%(*w*/*v*) PEG 8000, 50 m*M* potassium acetate pH 6.9, 2 m*M* spermine, 5% glycerol, 1 m*M* dithiothreitol, 3 m*M* NaN_3 and 3.75 m*M* NAD (see Fig. 2*a*). For condition 2, 5–10 µl of 3.0 mg ml^{-1} epimerase was equilibrated against a reservoir solution containing 13%(*w*/*v*) PEG 8000, 0.1 *M* sodium cacodylate pH 6.8, 0.15 *M* magnesium acetate, 3 m*M* NaN_3 and 1 m*M* ADP-glucose. Crystallization conditions 1 and 2 resulted in two crystal forms (see Figs. 2*a* and 2*b*) that diffracted poorly. For the third condition, 5–10 µl of 4 mg ml^{-1} epimerase was equilibrated against 2 *M* ammonium sulfate, 2% PEG 400, 0.1 *M* *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic sodium (HEPES, sodium salt) buffer pH 7.5, 1 m*M* ADP-glucose, 1 m*M* NaN_3 and 20 m*M* spermidine. Crystals (designated form III, see Fig. 2*c*) were observed within 3–5 d.

The form III crystals were sensitive to X-ray radiation and only survived for a few hours in the beam at room temperature. For cryo-crystallography, the crystals grown at room temperature were transferred for less than 1 min to a solution containing 25–30%(*v*/*v*) glycerol as the cryoprotectant. This was followed by flash freezing in a 95 K nitrogen-gas stream. The stability of the crystals during soaking was monitored microscopically.

A native 3.0 Å data set was collected on an R-AXIS IIc imaging-plate system with Cu $K\alpha$ X-rays generated from a Rigaku RU200 rotating-anode generator operated at 50 kV and 100 mA. Data reduction and scaling were performed using *DENZO* and *SCALEPACK*, respectively (Otwinowski & Minor, 1997). Subsequently, X-ray diffraction data were collected using an ADSC Quantum 4 CCD detector on station F1 at the Cornell High Energy Synchrotron Source (CHESS). The crystals diffracted to beyond 2.0 Å resolution. A complete native data set was obtained. These data were processed using the programs *MOSFLM* and *SCALA* from the *CCP*4 program suite (Collaborative Computational Project, Number 4, 1994).

3. Results and discussion

X-ray diffraction quality crystals of epimerase were prepared in the presence of spermidine and the epimerase catalytic

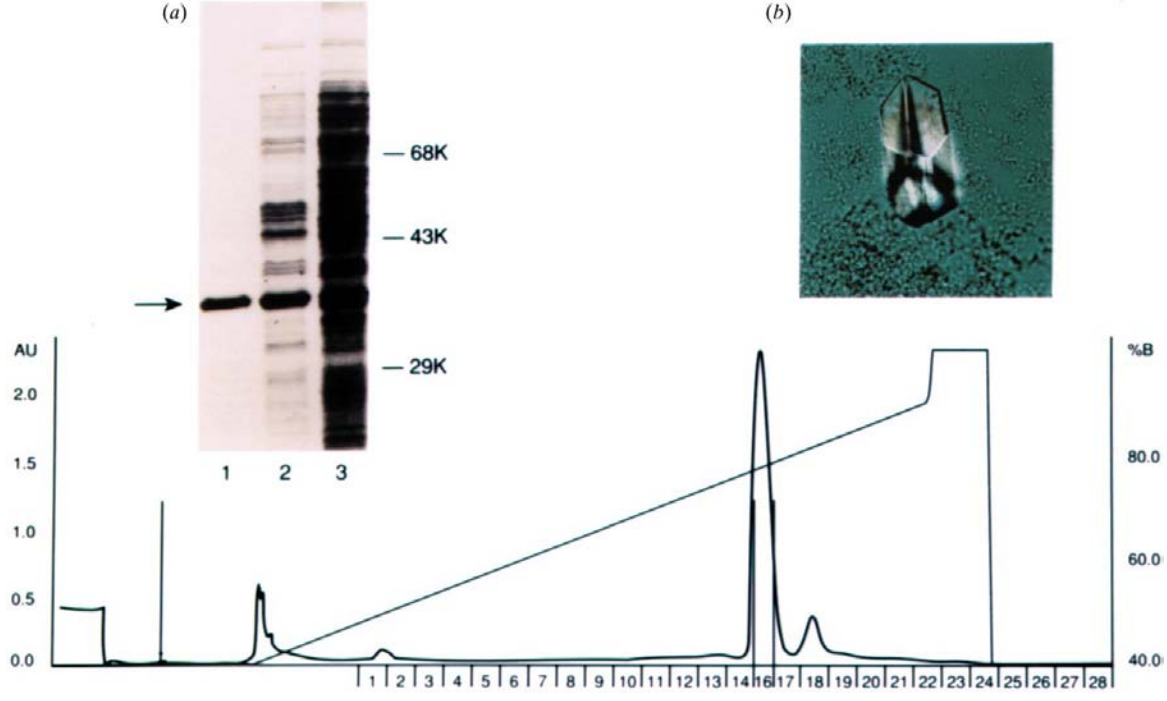
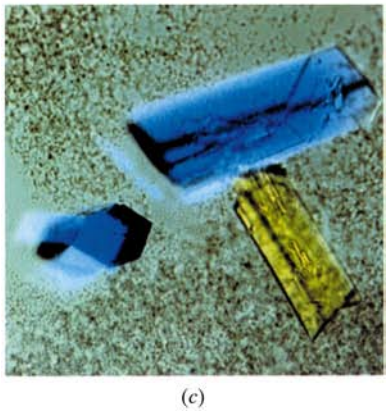


Figure 1
Chromatographic profiles of ADP-L-glycero-D-mannoheptose 6-epimerase on a MonoQ ion-exchange column. A pure preparation of epimerase was applied to the column. Protein elution was monitored by absorbance at 280 nm. Inset (*a*): SDS–PAGE analysis of protein fractions from previous purification steps. Lanes 1, 2 and 3 are protein fractions from a blue Sepharose CL-6B column, 0.6 *M* KCl fraction from hydrophobic chromatography and crude extract, respectively. Inset (*b*): a crystal of epimerase obtained with protein from the major protein peak resolved by MonoQ chromatography.

inhibitor ADP-glucose. Crystals appeared within 3–5 d and grew to dimensions of 0.6 × 0.25 × 0.1 mm (see condition 3, §2.2). These crystals diffracted beyond 3.0 Å on a laboratory X-ray source and beyond 2.0 Å at the synchrotron radiation source (Fig. 3). Crystals obtained under the same conditions in the absence of spermidine were more difficult to flash-freeze, less stable in the X-ray beam and showed higher mosaicity in their diffraction. These findings were consistent with earlier observations that additives such as spermidine affect protein–protein and protein–solvent interactions, sometimes improving growth and the quality of macromolecular crystals (Dock-Bregeon & Moras, 1992). A 3.0 Å native data set was collected on a single crystal using the Rigaku R-AXIS IIC imaging-plate system as described in §2.2. 198243 reflections were collected of which 66236 were independent, representing 84.7% of the unique data between 50.0 and 3.0 Å. The $R_{\rm merge}$ is 9.6%. The data collected at CHESS extended the resolution to 2.0 Å. The number of unique reflections was increased to 239829 from a total of 573756 diffraction measurements. This data set had a low $R_{\rm merge}$ (6.6%) and high completeness (92.4%). The crystals belong to the monoclinic space group $P2_1$. For the two datasets the unit-cell parameters and data statistics are given in Table 1. Assuming an hexameric enzyme of molecular mass 240 kDa in the asymmetric unit, a crystal-packing parameter V_M (Matthews, 1968) of 4.11 Å^3 Da^{-1} and a solvent fraction of 70% were calculated.

(*a*)

(*b*)



(*c*)

Figure 2
Crystals of ADP-L-glycero-D-mannoheptose 6-epimerase. (*a*) Crystals obtained (average dimensions 0.15 × 0.15 × 0.1 mm) in the presence of PEG 8000, NAD^+ and spermine (condition 1). (*b*) Crystals obtained (average dimensions 0.3 × 0.3 × 0.07 mm) in PEG 8000, ADP-glucose and Mg^{2+} (condition 2). (*c*) Epimerase crystals (average dimensions 0.6 × 0.25 × 0.1 mm) grown in the presence of PEG 400, ammonium sulfate, ADP-glucose and spermidine (condition 3). Epimerase crystals shown in micrographs (*b*) and (*c*) were obtained from protein purified by an additional ion-exchange chromatographic step.

Table 1
Data-collection statistics.

X-ray source	Rigaku RU200, 50 kV, 100 mA	CHESS F1
Wavelength (Å)	1.54	0.919
Unit-cell parameters		
a (Å)	98.9	98.9
b (Å)	110.5	108.7
c (Å)	180.7	180.6
β (°)	90.94	90.96
Mosaicity (°)	0.7	0.4
Resolution† (Å)	50–3.0 (3.11–3.0)	9–2.0 (2.11–2.0)
Total No. of reflections	198243	573756
No. of unique reflections	66236	239829
Completeness† (%)	84.7 (60.1)	92 (78.1)
$R_{\rm merge}$†‡	0.096 (0.16)	0.066 (0.218)
$I/\sigma(I)$	10.3	11.7

† Numbers in parentheses indicate the statistics for the last resolution shell. ‡ $R_{\rm merge}$ is defined as $\sum|I_h - \langle I_h\rangle|/\sum I$.

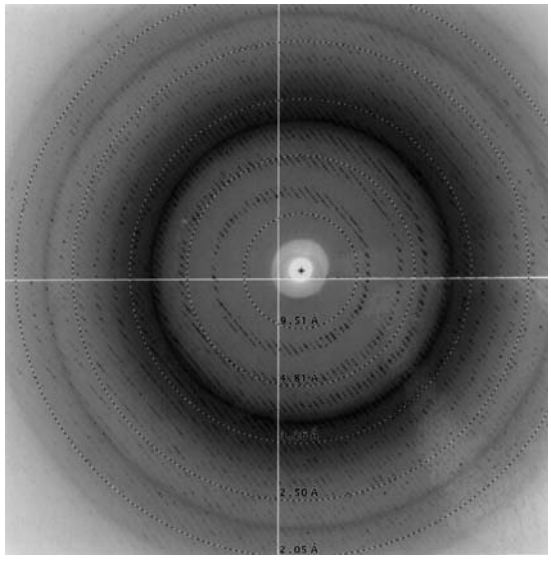


Figure 3
The X-ray diffraction pattern of epimerase collected on CHESS station F1 from a single crystal placed 199.5 mm from an ADSC Quantum 4 CCD detector. The exposure time was 25 s for each 0.75° oscillation. Resolution rings are indicated.

4. Conclusions

In conclusion, we have produced diffraction-quality crystals of ADP-L-glycero-D-mannoheptose 6-epimerase in the presence of spermidine and have collected a 2.0 Å resolution data set. Further structural determinations are in progress.

The determination of the high-resolution three-dimensional structure of the epimerase should provide insight into the catalytic mechanism of the interconversion of ADP-D-glycero-D-mannoheptose and ADP-L-glycero-D-mannoheptose.

We are indebted to Dr S. Ashraf Ahmed for sharing his knowledge of protein crystallization with us and to Dr Edith Wilson Miles for the critical reading of the first manuscript draft. The authors would like to acknowledge the provision of beam time at the Cornell High Energy Synchrotron Source, which is supported by the NSF under award DMR-9311772 and the Macromolecular Diffraction at CHESS (MacCHESS) facility supported by award RR-01646 from NIH.

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