SHORT COMMUNICATIONS

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Crystallization and preliminary crystallographic analysis of galactose-1-phosphate uridylyltransferase from *Escherichia coli*.

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Abstract

Galactose-1-phosphate uridylyltransferase catalyzes the formation of UDP-galactose during normal cellular metabolism, making it an essential enzyme in all cells. The enzyme from *Escherichia coli* has been crystallized at pH 5.9 in the presence of phenyl-UDP (P^1-5′-uridyl-P^2-phenyl diphosphate), a substrate analog, using PEG 10 000 in combination with Li_2SO_4 and NaCl. Crystals belong to space group $P2_12_12$ with unit-cell dimensions a = 58.6, b = 217.6 and c = 69.6 Å. There is one dimer or two subunits in the asymmetric unit. Crystals are relatively insensitive to X-ray radiation and diffract beyond 2.5 Å resolution. A low-resolution native data set has been recorded.

Introduction

Galactose-1-phosphate uridylyltransferase (E.C. 2.7.7.12) catalyzes the interconversion of UDP-glucose (UDP = uridine-5′-diphosphate) and galactose-1-phosphate with UDP-galactose and glucose-1-phosphate. Attenuation or deficiency of enzyme activity in humans results in the potentially lethal disease galactosemia (Kalckar, 1960).

Kinetic analyses have demonstrated that the enzyme follows a double-displacement reaction characterized by a covalent uridylyl-histidine-enzyme intermediate (Wong & Frey, 1974*a*,*b*; Field, Reznikoff & Frey, 1989; Kim, Ruzicka & Frey, 1990) shown in Fig. 1. This enzyme is unique among nucleotidyltransferases that use phosphates as acceptor groups because it does not utilize nucleoside di- or triphosphates as nucleotidyl donor substrates (Wong & Frey, 1974*a*). Galactose-1-phosphate uridylyltransferase from *E. coli* is a 79 288 Da dimer of identical subunits (Saito, Ozutsumi & Kurahashi, 1967; Field, 1988). Work by Ruzicka (1993) has demonstrated the presence of 1.0 g-atom of zinc and substoichiometric amounts of iron associated with each monomer. Divalent metals appear to be required for activity and might function to polarize the α-phosphoryl group of the nucleotidyl donor thereby facilitating uridylyl transfer.

In order to improve understanding of the molecular basis of nucleotidyl transfer, and how enzyme dysfunction culminates in galactosemia, a structural study of galactose-1-phosphate uridylyltransferase from *E. coli* was initiated. This paper describes the details of protein purification, crystallization, and the preliminary crystallographic analysis of enzyme crystals grown in the presence of phenyl-UDP (P^1-5′-uridyl-P^2-phenyl diphosphate).

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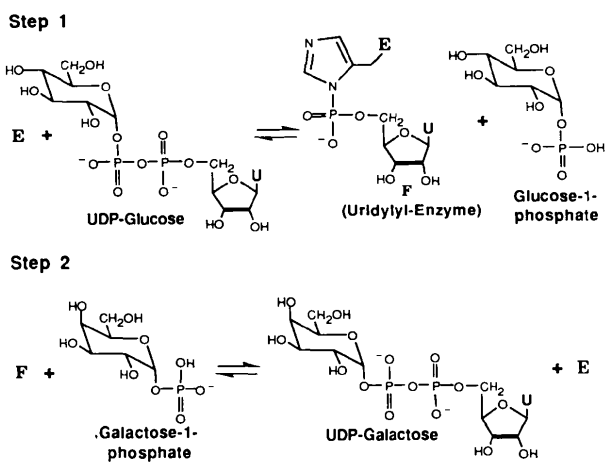

Fig. 1. Schematic diagram of the double-displacement mechanism of galactose-1-phosphate uridylyltransferase as it occurs on the solvated enzyme. The free enzyme is labeled *E*. The covalent uridylyl-enzyme intermediate is labeled *F*. The two half reactions of catalysis are labeled Step 1 and Step 2, respectively. The enzymatic nucleophile is histidine, as described in the text.

Materials and methods

Materials

Chloramphenicol was obtained from Boehringer-Mannheim (Indianapolis, IN, USA). Succinic acid, PMSF, NaN_3, bicinate, Li_2SO_4 and Cibacron Blue 3G-A were obtained from Sigma (St Louis, MO, USA). *N*-2-Hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES), ethylenediamine tetraacetic acid (EDTA) and *N*-α-tosyl-L-lysine chloromethyl ketone hydrochloride (TLCK) were from Research Organics (Cleveland, OH, USA). NaCl and NaOH were from Mallinkrodt (Paris, KY, USA). Polyethylene glycol (PEG) 10 000 was from Aldrich (Milwaukee, WI, USA) and 2-mercaptoethanol was from Eastman Kodak (Rochester, NY, USA). High-resolution Sephacryl S-100 and fast-flow Q-Sepharose were from Pharmacia (Uppsala, Sweden). Phenyl-UDP was the generous gift of Dr James Burke (Enzyme Institute). All other materials were from the references noted.

Overexpression

Overexpression of galactose-1-phosphate uridylyltransferase was achieved with a modified version of the BL21(DE3), pLysS, pT7E2-containing system constructed by Swanson & Frey (1993). Removal of *galE* and replacement with *galT* from *E. coli*, generated BL21(DE3), pLysS, pTZ18ROT (Wedekind, 1993) which produces three to four times more enzyme than previously reported (Field *et al.*, 1989).

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Cell storage and growth

Procedures were carried out according to Field *et al.* (1989). Changes included the addition of 68 mg ml^{-1} chloramphenicol during all growths and the elimination of isopropylthio-β-D-galactoside (IPTG). 0.5 l growths were inoculated with agar plugs cut from SOB Petri plates. The plugs contained colonies that originated from frozen cell stocks. Liquid growths were harvested at an absorption level, A_{600}, of 1.6.

Protein purification

Enzyme preparations proceeded by modifying the procedure of Arabshahi, Brody, Smallwood, Tsai & Frey (1986). A standard buffer (SB), consisting of 10 m*M* HEPES pH 7.0, and 10 m*M* 2-mercaptoethanol was used for all preparative techniques, except where noted. 20 g of cells were thawed in 100 ml of SB containing 0.5 m*M* TLCK, 1 m*M* phenylmethylsulfonyl fluoride (PMSF) and 1 m*M* EDTA. The pH was adjusted to 8.0 with 1 *M* NaOH which activated the endogenous lysozyme. Cellular debris and nucleic acids were pelleted after sonication. The supernatant was diluted to 20 mg ml^{-1} with SB containing 50 m*M* NaCl and 1 m*M* PMSF and loaded onto a 4.5 × 45 cm column of Q-Sepharose equilibrated with SB and 1 m*M* PMSF. Protein was eluted with a (2 × 2.0 l) linear gradient from 50 to 600 m*M* NaCl. Fractions were pooled based upon specific activity measurements (Wong & Frey, 1974*a*), concentrated, and applied to a 3.2 × 95 cm column of high-resolution Sephacryl S-100 buffered with SB containing 50 m*M* NaCl. Then the protein was applied to a 5 × 16 cm column of Cibacron Blue 3G-A equilibrated with 5 m*M* sodium bicinate containing 10 m*M* 2-mercaptoethanol. Protein was eluted with a linear gradient (2 × 1.3 l) of 0 to 750 m*M* NaCl. The quality of protein from the final column was assessed by specific activity measurements and by polyacrylamide gel electrophoresis (PAGE) (Laemli, 1970) which served as the final criterion for the pooling of fractions. Protein concentrations were estimated from A_{280} values and the extinction coefficient of 7.24 × 10^4 M^{-1} cm^{-1} (Ruzicka, 1993). The resultant protein was pressure concentrated, dialyzed overnight against SB containing 100 m*M* NaCl and frozen as pellets in liquid nitrogen. The protein was stored at 136 K.

Crystal screening procedures

These were carried out in hanging-drop vapor-diffusion experiments (McPherson, 1982). Initial precipitation points were identified by use of the sparse matrix sampling procedure of Jancarik & Kim (1991). All experiments were carried out at 293 and 277 K. Promising precipitates prompted the use of PEG, 2-methyl-3,4-pentadiol (MPD) and the salts of sulfate and phosphate for additional empirical screening. The pH was screened over a range from 4.0 to 9.0. PEG yielded the most promising crystals in the presence of substrates and nucleotides, which included UDP-glucose, UDP-galactose, TDP-glucose, UDP and TDP (thymidine-5′-diphosphate). Attempts to improve the crystal morphology led to the use of the following cations and anions in combination with PEG 8000 and 10 000: Na^+, K^+, Li^+, NH_4^+, $N(CH_3)_4^+$, $N(CH_3CH_2)_4^+$, Mg^{2+}, Ca^{2+}, Ba^{2+}, Ni^{2+}, Zn^{2+}, Cd^{2+}, Cl^-, Br^-, SO_4^{2-}, HPO_4^{2-} and $CH_3CO_2^-$. Additives such as β-octylglucoside, PEG 400, dioxane and dimethyl sulfoxide (DMSO) were also examined. The best diffraction-quality crystals were grown in the presence of phenyl-UDP from reservoirs of PEG 10 000, Li_2SO_4 and NaCl buffered at pH 5.9. Phenyl-UDP was added to the enzyme, then microfuged prior to the addition of precipitant. The precipitant was layered upon the protein to give a 15 µl droplet.

X-ray diffraction experiments

These were performed by mounting crystals in thin-walled quartz capillary tubes (Rayment, 1985). Crystal slippage during data collection was prevented by coating the crystal with a thin film of Formvar (Rayment, Johnson & Suck, 1977). Precession photographs were recorded for 20 h at 277 K using Ni-filtered Cu $K\alpha$ radiation from a Rigaku RU 200 rotating-anode X-ray generator with a 200 µm focal spot, operated at 50 kV and 50 mA with a crystal-to-film distance of 10 cm. X-ray data were collected at a crystal-to-detector distance of 24 cm.

Results and discussion

Galactose-1-phosphate uridylyltransferase crystals were grown from >14.5% (*w*/*w*) PEG 10 000, 400 m*M* Li_2SO_4, 250 m*M*

Fig. 2. Typical crystals of galactose-1-phosphate uridylyltransferase grown in the presence of phenyl-UDP, a substrate analog, and photographed under polarized light. The dimensions were 0.91 × 0.23 × 0.11 mm. Growth occurred at 277 K from PEG 10 000 as a precipitant.

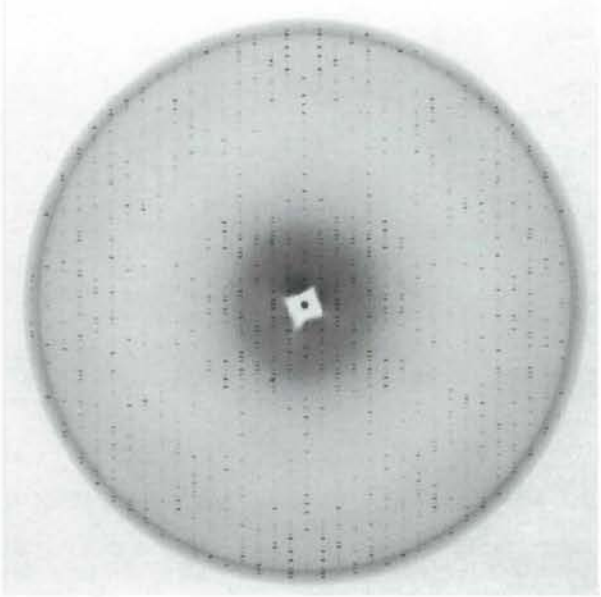

Fig. 3. A 13° precession photograph of the *hk*0 zone.

Table 1. *Native data statistics*

Resolution (Å)	Overall	∞–7.59	7.59–5.37	5.37–4.39	4.39–3.80	3.80–3.40
R_{merge} (%)*	4.0	2.7	4.1	3.4	3.8	5.2
No. of independent reflections	11672	1089	2129	2715	3001	2738
Theoretical	12897	1268	2142	2723	3186	3578

* $R_{merge} = \Sigma|I_{hi} - I_h|/\Sigma I_{hi} \times 100$.

NaCl, 4 m*M* phenyl-UDP and 1 m*M* NaN_3, buffered at pH 5.9 with 100 m*M* sodium succinate. The initial protein concentration was between 17.5 and 23 mg ml^{-1}. Single crystals appeared at 277 K in 5–6 d. Crystals grow as rods reaching typical dimensions of 1.0 × 0.2 × 0.1 mm in 3 weeks (Fig. 2). The space group was determined to be $P2_12_12$ based upon the *mm* symmetry of the *hk*0 and 0*kl* zones and their respective upper levels together with systematic absences of the form (2*n* +1) for the *h*00 and 0*k*0 reflections, with cell dimensions *a* = 58.6, *b* = 217.6 and *c* = 69.6 Å (Fig. 3). The Matthews coefficient or V_m for one dimer or two subunit equivalents per asymmetric unit corresponds to 2.89 $Å^3$ Da^{-1}, which falls within the normal range (1.68–3.53 $Å^3$ Da^{-1}) exhibited by globular proteins (Matthews, 1968). Still setting photographs indicate strong diffraction to 2.6 Å resolution. The crystals are resistant to radiation damage and diffract strongly for >40 h in the X-ray beam. A typical 13° precession photograph with a 3.4 Å resolution limit shows little intensity fall off (Fig. 3). Native data to 3.4 Å resolution were collected on a Siemens X-1000 area detector and processed with the data-reduction software *XDS* (Kabsch, 1988*a*,*b*). A total of 23 916 reflections were recorded from two crystals which reduced to 11 672 of 13 080 possible independent reflections with an R_{merge} of 4.0%, where $R_{merge} = \Sigma|I_{hi} - I_h|/\Sigma I_{hi} \times 100$. Table 1 shows R_{merge} as a function of resolution.

Presently, a single uranyl derivative has been obtained. Difference Patterson maps and the origin-removed Patterson correlation method of Rossmann (1960) and Terwilliger & Eisenberg (1983), have revealed a single major binding site for this heavy atom. A search for additional heavy-atom derivatives is in progress. Upon completion of this study it should prove possible, in conjunction with previous chemical and mechanistic studies, to establish a structural model for nucleotidyl transfer.

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