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Crystallization and preliminary X-ray analysis of the apo form of *Escherichia coli* tryptophanase

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Tryptophanase from *Escherichia coli* is a pyridoxal phosphate-dependent homotetrametic enzyme with a subunit weight of 52 kDa. It has been crystallized in the apo form by the hanging-drop vapour-diffusion method using polyethylene glycol 400 as a precipitant and magnesium chloride as an additive. The crystals belong to the orthorhombic space group *F*222, with unit-cell parameters $a = 118.4$, $b = 120.1$, $c = 171.2$ Å. A 97.8% complete data set to 1.9 Å resolution was collected at a rotating-anode source from a single frozen crystal. Packing-density considerations agree with a monomer in the asymmetric unit with a solvent content of 55%. Tryptophanase mutants W330F and Y74F were crystallized under the same conditions and the crystals diffracted to a resolution limit of 1.9 Å. Data sets of wild-type crystals soaked with L-tryptophan or pyridoxal phosphate were collected, as well as of Y74F mutant soaked with both.

1. Introduction

Tryptophanase (tryptophan indole-lyase; EC 4.1.99.1; Trpase) is a widely distributed bacterial pyridoxal phosphate-dependent enzyme that catalyzes α,β-elimination and β-replacement of L-tryptophan and a variety of other β-substituted L-amino acids (Snell, 1975). It consists of four identical subunits. Each monomer binds one molecule of pyridoxal phosphate (PLP), which forms an aldimine bond with a lysine residue. The enzyme requires monovalent cations (K^+, NH_4^+, Tl^+) for its activity (Suelter & Snell, 1977). The subunit molecular weight of Trpase from *Escherichia coli* is 52 kDa. PLP is bound to Lys270. The crystal structure of Trpase from *Proteus vulgaris*, which shares 40% identity with the *E. coli* enzyme, has been reported (Isupov *et al.*, 1998). However, most kinetic and mechanistic studies have been performed on the Trpase from *E. coli* and there is a need for a high-resolution crystal structure of the enzyme. Although biochemical studies of Trpase have been carried out for many years, X-ray structural investigations have been unsuccessful owing to difficulties in obtaining well diffracting crystals. The first crystals of *E. coli* Trpase were obtained as early as 1965 (Newton *et al.*, 1965). The first crystals of the *E. coli* enzyme suitable for X-ray research were reported in 1991 (Kawata *et al.*, 1991) and improved in 1994 (Dementieva *et al.*, 1994), but the structure never followed. Our research was aimed at obtaining high-quality crystals of *E. coli* Trpase. In addition to the wild-type enzyme, we have chosen two mutants as targets for crystallization: W330F and Y74F. The first exhibits a significantly different behaviour with respect to cold inactivation (Erez *et al.*, 1998, 2002), while the second is catalytically inactive and might be suitable for observing both the cofactor and the substrate in the same crystal structure.

2. Materials and methods

2.1. Protein expression and purification

The *E. coli* wild-type (WT) Trpase was overexpressed in *E. coli* SVS 370 cells containing the *tnaA* gene on plasmids using a previously described procedure (Phillips & Gollnick, 1989). Single colonies of cells grown on Luria broth (ampicillin agar plate), were brought into 5 or 10 ml of medium containing 1%(*w*/*v*) casein enzymatic hydrolysate, 0.2% yeast extract (Difco), 0.2% KH_2PO_4, 0.002% pyridoxine–HCl and 0.01% ampicillin (10–20 µl at 50 µg µl^{-1}) adjusted to pH 7.3–7.4 with NaOH.

The cells were grown at 310 K overnight with gentle agitation (200 rev min^{-1}). The following day a portion of the culture was diluted (1:100) into fresh warmed medium and growth was allowed to continue until the cells reached an optical density ($OD_{600\,nm}$) of approximately 0.4–0.6.

Induction was carried out with IPTG (at a final concentration of 1 m*M*). After induction, the cell suspension was incubated for an additional 3 h. The cells were then collected by centrifugation for 30 min at 10 000*g* and stored at 193 K. After thawing, the cells were resus-

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pended in 0.1 m*M* potassium phosphate pH 7.0 (4.2 ml per gram of cells), 2 m*M* EDTA, 5 m*M* 2-mercaptoethanol, 0.1 m*M* PLP. The yellow suspension was cooled on ice and sonicated for 5 min in 1 min increments with a Heat Systems-Ultrasonic Inc. sonicator. The cell debris was removed by centrifugation for 40 min at 25 000*g*. The supernatant was adjusted to pH 6 with 1 *M* acetic acid and diluted with distilled water for protamine treatment (Morino & Snell, 1970). 1 ml of 2%(*w*/*v*) protamine sulfate was added dropwise to each 7.5 ml of supernatant at room temperature and the resultant cloudy solution was centrifuged for 40 min at 25 000*g*. The clear yellow supernatant was then brought to 40% saturation with $(NH_4)_2SO_4$ by the addition of solid $(NH_4)_2SO_4$ at 277 K and the pH was adjusted to 7.0. The resultant precipitate was removed by centrifugation for 40 min at 25 000*g*. After precipitation with $(NH_4)_2SO_4$, the supernatant was loaded onto a Sepharose CL-4B column previously equilibrated with buffer containing 40% saturated $(NH_4)_2SO_4$, 0.1 *M* potassium phosphate (KPB) pH 7.0, 2 m*M* EDTA and 0.1 m*M* PLP. The column was then washed with 5–6 volumes of this buffer. The enzyme was eluted with 0.1 *M* KPB, 20% saturated $(NH_4)_2SO_4$, 2 m*M* EDTA and 0.1 m*M* PLP.

The eluate was dialysed against 0.06 *M* KPB pH 7.7 containing 2 m*M* EDTA, 0.1 m*M* PLP, 5 m*M* 2-mercaptoethanol and loaded onto a DEAE-Sepharose A-50 column (Honda & Tokushige, 1986). The column was washed with 0.1 *M* KPB and the protein was eluted at 0.4 *M* KPB.

Additional purification was achieved by size-exclusion chromatography on Sephacryl S-300-HR. The apo enzyme was prepared by overnight dialysis of the holo enzyme in the cold against 0.2 *M* sodium phosphate–ethylenediamine pH 8.0, 2 m*M* EDTA and 5 m*M* 2-mercaptoethanol. The degree of conversion was monitored by measuring the specific activity of Trpase, as described by Suelter *et al.* (1976).

2.2. Crystallization and X-ray analysis

Initial screening for crystallization conditions was performed using the commercially available Crystal Screen and Crystal Screen II from Hampton Research, utilizing the hanging-drop vapour-diffusion technique. Drops were prepared on siliconized cover slips and contained 2 µl protein solution at a concentration of 30 mg ml^{-1} and 2 µl reservoir solution at 293 K. The droplets were equilibrated against a reservoir volume of 0.5 ml. Single crystals were obtained using condition No. 23, along with amorphous precipitate. Further optimization included the addition of 5 m*M* 2-mercaptoethanol to the reservoir solution, changing the volume of the protein solution in the drop to 3 µl and raising the protein concentration to 50 mg ml^{-1}. The crystals appeared after approximately one week and rapidly achieved a maximum size of 0.5 mm (Fig. 1). Soaking was performed in the mother liquor lacking 2-mercaptoethanol and containing 50 m*M* L-tryptophan, 3–6 m*M* PLP or both (in the case of the Y74F mutant crystals). Crystals soaked with PLP acquired an intense yellow colour, indicating that the substrate was bound.

Diffraction data were collected using a Rigaku RU3H rotating-anode generator (Cu $K\alpha$ radiation) and MAR345 image-plate detector at 100 K. Before freezing, crystals were transferred to Paratone oil (Hampton Research) and the excess liquid removed. Flash-freezing was performed in liquid nitrogen.

Table 1
Summary of data-collection and processing statistics.

Data in parentheses are for the highest resolution shell. The space group is *F*222.

Crystal	Unit-cell parameters (Å)			Resolution (Å)	R_{sym} (%)	Completeness (%)	Unique reflections	$I/\sigma(I)$
	a	*b*	*c*					
WT	118.4	120.3	171.3	1.9	6.1 (28.9)	97.8 (99.3)	47614	24 (4.2)
Y74F	118.7	120.2	171.7	1.9	5.4 (40.1)	97.6 (97.8)	47181	19 (2.9)
W330F	118.4	119.8	171.4	1.9	5.5 (40.2)	95.9 (99.8)	45788	28 (2.7)
WT + 3 m*M* PLP	118.7	119.5	172.0	1.9	3.6 (43.0)	99.6 (98.1)	48490	31 (2.9)
WT + 6 m*M* PLP	118.8	118.8	171.9	2.9	4.7 (34.8)	98.9 (97.0)	13575	30 (3.4)
WT + 50 m*M* Trp	119.0	120.7	172.6	2.0	5.0 (55.1)	98.9 (91.9)	41544	28 (2.3)
Y74F + 5 m*M* PLP and 50 m*M* Trp	118.6	120.1	172.1	1.8	4.3 (35.6)	98.0 (100)	56883	46 (4.6)

3. Results

Seven data sets were collected from single crystals of apo WT and mutant Trpase both with and without soaking (Fig. 2). Data-collection statistics are summarized in Table 1. All data sets were indexed in space group *F*222 with similar unit-cell parameters. Data processing was performed using the *HKL* package (Otwinowski & Minor, 1997). Calculation of the Matthews coefficient (Matthews, 1968) yielded a value of 2.9 Å^3 Da^{-1}, assuming the presence of one monomer in the asymmetric unit, which corresponds to a solvent content of 55%. The phase problem will be solved by molecular replacement using the structure of

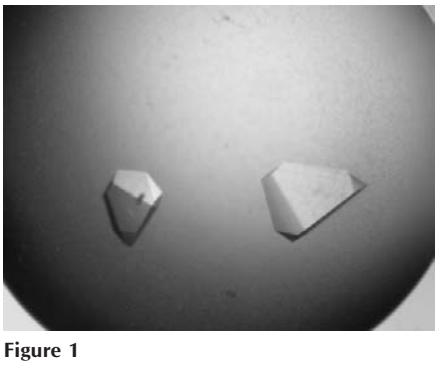

Figure 1
Crystals of apo WT Trpase from *E. coli*. The longest crystal dimension is 0.5 mm.

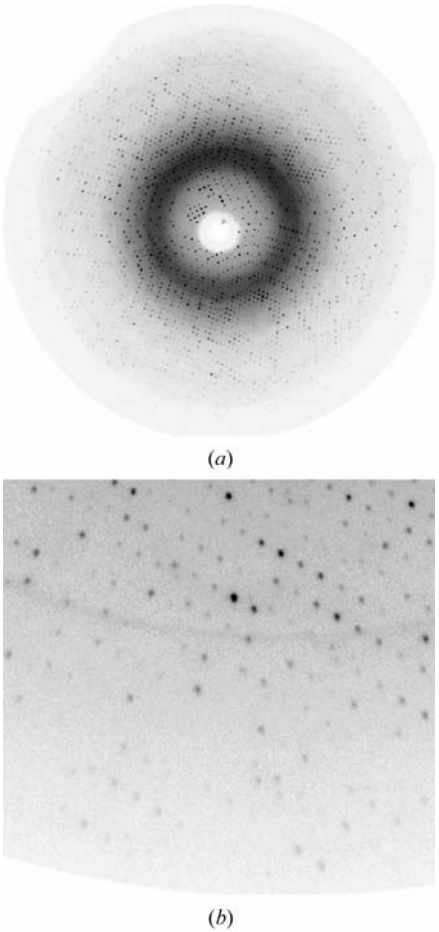

Figure 2
(*a*) X-ray diffraction pattern of apo WT Trpase from *E. coli*. The oscillation range per frame was 1° and the exposure time was 300 s. (*b*) The edge of the image corresponds to 1.9 Å resolution.

Trpase from *P. vulgaris* (PDB code 1ax4) as a search model. Knowledge of the three-dimensional structure of *E. coli* Trpase will provide a structural basis for previous studies on this enzyme and will serve as a platform for structure-based design of inhibitors. The latter might be of practical importance as Trpase has been shown to be involved in biofilm formation (Di Martino *et al.*, 2002)

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