Structure of *Escherichia coli* tryptophanase purified from an alkaline-stressed bacterial culture

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Tryptophanase is a bacterial enzyme involved in the degradation of tryptophan to indole, pyruvate and ammonia, which are compounds that are essential for bacterial survival. Tryptophanase is often overexpressed in stressed cultures. Large amounts of endogenous tryptophanase were purified from *Escherichia coli* BL21 strain overexpressing another recombinant protein. Tryptophanase was crystallized in space group $P6_522$ in the apo form without pyridoxal 5'-phosphate bound in the active site.

1. Introduction

Tryptophanase (Tnase), also known as tryptophan indole-lyase (EC 4.1.99.1), is a widely distributed bacterial enzyme that catalyses the reaction in which L-tryptophan is degraded to indole, pyruvate and ammonia *via* α,β -elimination and β -replacement mechanisms (Snell, 1975). Tnase is pyridoxal 5'-phosphate (PLP)-dependent. Monovalent cations (K^+ or NH_4^+) are required for the binding of PLP to a lysine residue in the active site, leading to the functionally active form (Toraya *et al.*, 1976). Tnase can act in reverse to form L-tryptophan at high concentrations of pyruvate and ammonia (Watanabe & Snell, 1972). It appears to have a lack of substrate and stereochemical specificities because it degrades many other β -substituted L-amino acids (Watanabe & Snell, 1977) and also D-tryptophan in the presence of high concentrations of ammonium phosphate (Shimada *et al.*, 1996, 1997).

In many bacteria, the degradation products of tryptophan are biologically essential compounds that are necessary for survival (Yanofsky, 2007). In *Escherichia coli* and in several other species of pathogenic bacteria such as *Vibrio cholerae*, indole generated by the action of Tnase acts as a cell-to-cell signalling molecule in quorum sensing, biofilm formation and the expression of multidrug-exporter genes (Mueller *et al.*, 2009). Tnase has been isolated from several bacterial species such as *E. coli* (Newton & Snell, 1964, 1965), *E. aureus*, *Bacillus alvei*, *Aeromonas alkalescens*, *Shigella alkalescens*, *Proteus vulgaris*, *P. morganii* and *Symbiobacterium thermophilum* (Simard *et al.*, 1975). The enzymes from all of these species have a molecular weight of about 210 kDa. The most extensively studied Tnase is that from *E. coli*. Tnase functions as a tetramer of four identical 52.8 kDa subunits in *E. coli*. Each monomer binds one molecule of PLP, which forms an aldimine bond to the Lys270 residue in the active site. The dissociation of holo Tnase into monomers occurs only under denaturing conditions or at a pH above 8.7 (Metzler, Metzler *et al.*, 1991; Metzler, Viswanath *et al.*, 1991). However, apo

Table 1
Crystallization.

Method	Hanging-drop vapour diffusion
Plate type	VDX Plate, 24-well (Hampton Research)
Temperature (K)	291
Protein concentration (mg ml ⁻¹)	10–17
Buffer composition of protein solution	50 mM Tris–HCl pH 7.4, 300 mM NaCl
Composition of reservoir solution	25–30% PEG 4000, 30 mM ammonium sulfate, 100 mM Tris–HCl pH 9, 20 mM β -mercaptoethanol
Volume and ratio of drop	2 μ l, 1:1 ratio
Volume of reservoir (ml)	1

Tnase dissociates into dimers below 25°C and into monomers below 2°C (Gdalevsky *et al.*, 2004).

X-ray structural investigations have been actively pursued to add to the knowledge and understanding of the biochemical properties of *E. coli* Tnase, but these studies were prevented by difficulties in obtaining well diffracting crystals. The first crystals of *E. coli* Tnase were described as early as 1965 (Newton *et al.*, 1965) but only in 1991 were tetragonal crystals obtained, which belonged to space group $P4_12_12$ and were suitable for X-ray analysis (Kawata *et al.*, 1991). Since *P. vulgaris* and *E. coli* Tnase share 51% sequence identity, the biochemical properties of *E. coli* Tnase were deduced from the determination of the structure of its homologue from *P. vulgaris* (Dementieva *et al.*, 1994; Isupov *et al.*, 1998). *E. coli* Tnase was also crystallized in space group $P4_12_12$ in the apo form (Ku *et al.*, 2006). The structure of *E. coli* Tnase in its apo form shows a closed conformation. Other X-ray structures of recombinant wild-type and mutants of apo *E. coli* Tnase were also reported in another crystal form (space group $F222$) and revealed a wide-open conformation, reflecting the flexibility of the enzyme (Kogan *et al.*, 2004, 2009; Tsesin *et al.*, 2007). Here, we present the crystal structure of wild-type endogenous apo Tnase from *E. coli* refined at 2.8 Å resolution in space group $P6_522$, showing an open conformation.

2. Experimental procedures

2.1. Expression and purification

During the production of the recombinant form of human thymidylate synthase (hTS), native Tnase from *E. coli* Rosetta (DE3) pLysS strain was also overexpressed, probably owing to stress conditions from the alkaline pH value, as discussed below, and was co-purified. Cells transformed with pET-17xb expressing hTS were grown at 37°C in 2×YT medium (16 g tryptone, 10 g yeast extract, 5 g NaCl per litre of H₂O) supplemented with ampicillin at 50 μ g ml⁻¹ until an OD 600 nm of 0.6 was reached. Protein expression was then induced with 1 mM isopropyl β -D-1-thiogalactopyranoside (IPTG) and the cell culture was further incubated at 30°C overnight. The cells were harvested by centrifugation and stored at –80°C. The cells were resuspended in lysis buffer (buffer A; 20 mM potassium phosphate pH 7.8, 500 mM NaCl, 10 mM MgCl₂, 1 mM DTT, 0.1 mM EDTA). Cell lysis was completed by sonication and the cell extract was centrifuged

Table 2
Data-collection and refinement statistics.

Values in parentheses are for the highest resolution shell.

Wavelength (Å)	0.98
Resolution range (Å)	47.01–2.78 (2.88–2.78)
Space group	$P6_522$
Unit-cell parameters (Å, °)	$a = b = 158.24$, $c = 387.76$, $\gamma = 120$
Total reflections	3101724 (312087)
Unique reflections	72775 (7136)
Multiplicity	42.6 (43.7)
Completeness (%)	99.88 (99.99)
Mean $I/\sigma(I)$	25.72 (2.54)
Wilson B factor (Å ²)	56.3
R_{merge}	0.20 (2.26)
R_{meas}	0.21
Reflections used for R_{free} (%)	5
R_{work}	0.21 (0.30)
R_{free}	0.24 (0.35)
No. of non-H atoms	
Total	13098
Macromolecules	12841
Ligands	144
Water	113
No. of protein residues	1629
R.m.s.d., bonds (Å)	0.003
R.m.s.d., angles (°)	0.70
Ramachandran favoured (%)	97.0
Ramachandran allowed (%)	2.8
Ramachandran outliers (%)	0.2
Clashscore	1.52
Average B factor (Å ²)	
Overall	60.9
Macromolecules	60.5
Ligands	106.1
Solvent	47.6

at 20 000g for 30 min at 4°C. The clarified supernatant was precipitated successively with different ammonium sulfate solutions (0, 35 and 80%) on ice and then centrifuged at 20 000g for 30 min at 4°C. The pellet was resuspended in 5 ml buffer *B* (10 mM potassium phosphate pH 7.8, 1 mM DTT, 0.1 mM EDTA) and dialyzed in 5 l of the same buffer overnight at 4°C. Tnase was then purified in three steps by FPLC using 15Q Sepharose anion-exchange chromatography (GE Healthcare, $V_{\text{column}} = 30$ ml, gradient from 100% buffer *B* to 100% buffer *C*, where buffer *C* consists of 1 M potassium phosphate pH 7.8, 1 mM DTT, 0.1 mM EDTA). Fractions from the main peak (containing Tnase) were pooled and ammonium sulfate was added to a final concentration of 1 M prior to the second purification step using phenyl Sepharose Fast Flow chromatography ($V_{\text{column}} = 50$ ml, gradient from 100% buffer *D* to 100% buffer *E*, where buffer *D* consists of 20 mM potassium phosphate pH 7.8, 1 M ammonium sulfate, 1 mM DTT, 0.1 mM EDTA and buffer *E* consists of 20 mM potassium phosphate pH 7.8, 1 mM DTT, 0.1 mM EDTA). The last purification step corresponded to preparative gel filtration on Superdex 200 16/60 (GE Healthcare HiLoad, $V_{\text{column}} = 120$ ml) using an elution buffer consisting of 50 mM Tris–HCl pH 7.4, 300 mM NaCl. The protein was concentrated using Amicon Ultra-15 5K (Millipore) and was characterized by SDS–PAGE. A single but diffuse protein band was observed on SDS–PAGE (data not shown). After digestion with trypsin from SDS–PAGE gel slices, the resulting mixture was analyzed

by MS and MALDI-MS/MS. *E. coli* Tnase was unambiguously identified using the *Mascot* v.2.2 (Matrix Science) peptide mass-fingerprinting program (Perkins *et al.*, 1999).

2.2. Crystallization

The native protein was crystallized using the hanging-drop vapour-diffusion method with 25–30% PEG 4000, 30 mM ammonium sulfate, 20 mM β -mercaptoethanol, 100 mM Tris–HCl pH 9 (Table 1). Crystals were transferred into the mother liquor containing 10% ethylene glycol before flash-cooling in liquid nitrogen.

2.3. X-ray data collection and processing

Preliminary X-ray diffraction data were collected on the PROXIMA-1 beamline at SOLEIL, Saint Aubin, France and the final data were collected using an ADSC Quantum Q315r detector on the ID23-1 beamline at the ESRF, Grenoble, France and were processed using *XDS* (Kabsch, 2010). The crystals belonged to space group $P6_522$, with unit-cell parameters $a = b = 158.24$, $c = 387.76$ Å, $\gamma = 120^\circ$. The unit-cell parameters and data-collection statistics are reported in Table 2. Molecular replacement was performed with *Phaser* using the *E. coli* Tnase structure (PDB entry 2c44; Ku *et al.*, 2006) as a template. Further refinement was performed in *PHENIX* (Adams *et al.*, 2010). Manual building and structure adjustments were performed with *Coot* (Emsley *et al.*, 2010) assisted by programs from the *CCP4* suite (Winn *et al.*, 2011).

3. Results and discussion

3.1. Overall structure

The $P6_522$ crystal form contains four molecules in the asymmetric unit related by the dihedral D_2 symmetry, forming a tetramer of Tnase as in PDB entry 2c44 (Fig. 1). Pairwise superposition of the four chains gives an average r.m.s.d on C^α atoms of 0.514 Å. The same regions lack electron density in $2F_o - F_c$ and $F_o - F_c$ maps for each chain: amino-acid residues 136–147, 289–308 and 398–413 have no electron density. The last 17 amino acids at the C-terminus are also absent, except in chain A, where an extended C-terminal part can be traced unambiguously. The structure does not contain any PLP ligand linked to Lys270 and thus is in an apo form. A sulfate ion occupies the location of the phosphate group of PLP, as reported previously (PDB entry 2c44; Ku *et al.*, 2006). In PDB entry 2c44, tryptophanase was also purified from *E. coli* strain JM109 during the overexpression of a recombinant form of *Leishmania donovani* S-adenosylhomocysteine (SAH) hydrolase, and although the protein was expressed and crystallized under conditions similar to those for the apo form published by Ku and coworkers (PDB entry 2c44), the structure is closer to another apo form (PDB entry 2oqx; Tsesin *et al.*, 2007), with r.m.s.d.s of 1.556 and 0.681 Å, respectively (Fig. 2). The structure determined by Ku and coworkers (PDB entry 2c44) is a closed apo form (with a sulfate ion in the binding site), whereas Tsesin and coworkers (PDB entry 2oqx) obtained another apo form in an open conformation

with a HEPES molecule in the binding site. The differences between the closed and open apo forms have already been analysed by Tsesin *et al.* (2007). In the structure with PDB code 2oqx there is only one molecule in the asymmetric unit and the quaternary-structure organization is formed by molecules related by crystallographic symmetry. In our reported structure four molecules are present in the asymmetric unit forming the tetramer, and in the noncrystallographic symmetry tetramer the same regions in each molecule lack visible electron density. We have performed mass spectrometry, which showed that there was no proteolysis of the purified and crystallized protein. Hence, these regions, 136–147, 289–308 and 398–413, are highly flexible in the apo form. The regions present in PDB entry 2oqx that are not visible in our structure are engaged in crystal contacts in PDB entry 2oqx. Our structure has a greater solvent content (63% solvent) and loose crystal contacts between molecules. The missing regions do not contribute to crystal packing. Our structure is less biased by crystal packing than PDB entry 2oqx and is probably more flexible.

3.2. Extra tetramer contacts

There is very little variation from the 2c44 and 2oqx structures in the tetramer organization except in the N- and C-terminal parts, which form extra intermolecular contacts in the tetramer (Fig. 3). The N-terminal end of molecule A (residues 1–5) makes hydrophobic contacts with helix $\alpha 15$ of chain C. Phe4 of chain A is in close proximity to Phe434 of chain C. The same interaction between Phe4 and Phe434 is observed between chains B and D, chains C and A and chains D and B.

The C-terminus of chain A adopts a unique conformation. While in all other structures the C-terminus folds back on the

Figure 1
Structure of the tryptophanase tetramer in space group $P6_522$ (PDB entry 4up2). A tetramer is present in the asymmetric unit: molecule A is shown in green, molecule B in cyan, molecule C in magenta and molecule D in yellow.

small domain, the region 452–468 is more extended and forms a short sequence (459–464) that interacts with the region 81–95 of molecule *B* (Fig. 4), forming a hydrophobic pocket

between Phe464 of chain *A* and Tyr80 and Ile97 of chain *B*. The buried surface area calculated by *PISA* is 22 836 Å², which is larger than those for PDB entries 2c44 (21 450 Å²)

Figure 2

Comparison of various forms of apo tryptophanase. (*a*, *b*) Superimposition of a monomer of the apo form of tryptophanase in space group *P*₆₅₂₂ (green; PDB entry 4up2) on a monomer of apo tryptophanase in space group *F*₂₂₂ (purple; PDB entry 2oqx). The overall r.m.s.d. is 0.681 Å. PDB entry 4up2 is in green and PDB entry 2oqx is in magenta, with regions missing in PDB entry 4up2 coloured yellow (136–147), orange (289–308) and red (398–413). A molecule of HEPES present in the ligand pocket of PDB entry 2oqx is shown in ball-and-stick representation and indicates the location of the PLP-binding site. The view in (*b*) is rotated by 90°, with the PLP-binding site at the front and the N-terminus at the back of the figure. The C-termini of PDB entries 4up2 and 2oqx end in opposite directions. (*c*, *d*) Superimposition of the apo form (PDB entry 4up2; green) and PDB entry 2c44 (yellow). A sulfate ion is present in PDB entries 4up2 (pink) and 2c44 (orange) in the binding site for PLP. The overall r.m.s.d. is 1.72 Å owing to the displacement of the C-terminal domain. Although PDB entries 4up2 and 2c44 are both apo forms obtained by serendipity while overproducing a recombinant protein in *E. coli*, PDB entry 4up2 is in the open form, as is PDB entry 2oqx.

Figure 3
(a) Detail of the intermolecular contacts involving the N-terminus of molecule A (green) and helix $\alpha 15$ of molecule C. (b) Detail of the intermolecular contacts involving the C-terminus of molecule A (green) and helix $\alpha 3$ of molecule B. The $2F_o - F_c$ maps are at a 1.5σ contour level.

and 2oqx (20 680 Å²). These extra intermolecular contacts between the N- and C-terminal ends of the subunits may contribute to the tetramer stabilization.

3.3. Production of Tnase during alkaline stress

It has previously been reported that bacteria exposed to stress can overexpress proteins which can be purified and crystallized by serendipity instead of the expected target (Ku *et al.*, 2006; David *et al.*, 2002). For example, YodA, a metal-

binding protein, is overexpressed in conditions of metal stress in *E. coli* (David *et al.*, 2003). Tryptophanase is involved in indole production, which is a signal for cell survival (Lee & Lee, 2010). Chant & Summers (2007) illustrated the biological relevance of *E. coli* Tnase in the cell cycle and in the maintenance of multicopy plasmids. They reported that *E. coli* Tnase interacts directly with a short transcript (Rcd; Chant & Summers, 2007) and that this binding enhances the affinity of *E. coli* Tnase for its substrate, leading to the increased production of indole. The accumulation of indole then inhibits *E. coli* growth in a concentration-dependent manner.

Endogenous Tnase was thus purified and crystallized from an *E. coli* culture overexpressing another protein. We measured an alkaline pH of 8.3 in the *E. coli* culture at the end of the overnight induction at 30°C. Among the multiple effects of pH on gene expression in *E. coli*, alkaline induction of Tnase has been well established (Blankenhorn *et al.*, 1999; Stancik *et al.*, 2002). *E. coli* Tnase is overexpressed and becomes one of the most abundant proteins during alkaline stress. On deamination, *E. coli* Tnase produces pyruvic acid, which can be further catabolized anaerobically to acetic and formic acids. It is proposed that *E. coli* Tnase induction offers an effective mean of neutralizing alkaline stress by a reverse process producing acidic compounds. Therefore, the purification of Tnase from an *E. coli* culture overexpressing recombinant protein may be a common artifact.

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Figure 4
Comparison of the C-terminal region of chain A in space group $P6_522$ (green) and in space group $F222$ (purple; PDB entry 2oqx). The $2F_o - F_c$ map is at a 1.5σ contour level.

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