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Crystallization and preliminary crystallographic studies of the anthranilate synthase partial complex from *Salmonella typhimurium*

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Anthranilate synthase catalyzes the first step in the biosynthesis of tryptophan from chorismate. The anthranilate synthase partial complex from *Salmonella typhimurium* has been crystallized in space group $P2_12_12$ with unit-cell dimensions a = 116.7, b = 101.2 and c = 66.8 Å.

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1. Introduction

In *S. typhimurium* the first two steps in the tryptophan biosynthetic pathway are catalyzed by the multifunctional allosteric enzyme, anthranilate synthase (ASI; E.C. 4.1.3.27)/ *N*-(5′-phosphoribosyl)anthranilate synthase (ASII; E.C. 2.4.2.18), encoded by the *trp*E (ASI) and *trp*(G)D (ASII) genes. ASI (TrpE) (520 amino acids) catalyzes the formation of anthranilate from chorismate and ammonia as an independent subunit and from chorismate and glutamine when complexed with ASII [Trp(G)D] (531 amino acids). ASII consists of a glutamine amidotransferase (TrpG) domain, which enables the complex to utilize glutamine as an ammonia source, and an *N*-(5′-phosphoribosyl)anthranilate synthase domain, which catalyzes the production of *N*-(5′-phosphoribosyl)anthranilate from anthranilate and 5-phosphoribosyl-1-pyrophosphate. The TrpG domain of ASII can be separated from the *N*-(5′-phosphoribosyl)anthranilate synthase domain by proteolytic cleavage or genetic mutation (Bauerle *et al.*, 1987). The independent TrpG domain assembles normally with the ASI subunit, forming a partial complex with full anthranilate synthase activity. The TrpG domain represents the N-terminal 193 amino acids of ASII in *S. typhimurium*. The partial complex [$(\mathrm{TrpE})_2(\mathrm{TrpG})_2$] is analogous to the complex that exists naturally in *S. marcescens* and numerous other bacteria (Crawford, 1989).

The anthranilate synthase complex is regulated by feedback inhibition by the binding of one molecule of L-tryptophan to a regulatory site within the ASI subunit (Caligiuri & Bauerle, 1991*a*,*b*). The enzyme requires a divalent metal cofactor for activity. The Mg^{2+} ion is probably used *in vivo* (Robinson & Levy, 1979). Kinetic, NMR and EPR studies of the Mn^{2+} bound complex indicate that the divalent cation interacts directly with chorismate (Summerfield *et al.*, 1988). The ASII amidotransferase activity functions *via* a catalytic triad and has no metal requirement (Zalkin & Smith, 1998).

The crystallization of anthranilate synthase was undertaken as the first step in the elucidation of its structure, mechanism of catalysis and mode of inhibition. Crystals were grown in the presence of L-tryptophan and in both the absence and presence of Mg^{2+}.

2. Materials and methods

Expression plasmid pSTC25 for the *S. typhimurium* partial complex was made by the engineering of tandem stop codons (TAG, TAA) at Leu194 and Glu195 of the *trp*(G)D gene cloned in the phagemid pSTS23 [*trp*E$^+$(G)D$^+$; Chatterji, 1995] by oligonucleotide site-directed mutagenesis (Kunkel, 1985). Anthranilate synthase was purified from cultures of *E. coli* strain CB694 [W3110 Δ*trp*E-A2 *tna2 bgl*R/F′ *pro*AB$^+$ *lac*I^q *lac*Z ΔM15 *Tn10*(*Tet*^{r})] carrying pSTS25. Cells were grown in a New Brunswick fermentor to an A_{550} of 2.3 in 10 l of M9 minimal media (Sambrook *et al.*, 1989) supplemented with 0.1% casamino acids and 25 µg ml^{-1} of tryptophan. The cells were collected by centrifugation and resuspended in buffer *A* [100 m*M* potassium phosphate buffer pH 7.0, 10% glycerol, 1.0 m*M* dithiothreitol (DTT) and 0.1 m*M* EDTA]. The cells were then sonicated and centrifuged at 17500*g* for 20 min and the pellet discarded. 0.23 g of ammonium sulfate per millilitre of supernatant was added and the precipitate was collected by centrifugation at 17500*g* for 20 min. The pellet was resuspended in buffer *A* and dialyzed against the same buffer with three changes over 24 h. The preparation was then applied to a 1.0 l Amicon Matrex Gel Orange A column and washed with four column volumes of buffer *A*. The partial complex was eluted from the column with buffer *A* containing 1.0 m*M* tryptophan. The preparation was then dialyzed against 50 m*M* 3-(*N*-morpholino)propanesulfonic acid (MOPS) pH 7.0 and 1.0 m*M* DTT

with three changes in 18 h. The protein purity was checked by SDS–PAGE and by ion-exchange chromatography. Pure protein ran as two distinct bands of the appropriate molecular weight by SDS–PAGE and appeared as a single peak when eluted from a Pharmacia mono-Q column in 20 m*M* Bis–Tris propane pH 6.5 with a gradient of 0–1.0 *M* KCl (data not shown).

Enzyme activity was measured fluorometrically by monitoring the production of anthranilate from chorismate with glutamine as the ammonia donor. The standard reaction mixture contained 0.5 m*M* chorismate, 20 m*M* glutamine, 100 m*M* potassium phosphate (pH 7.0) and 10 m*M* $MgCl_2$ in a volume of 1.0 ml. Anthranilate was excited at 308 nm and monitored at 388 nm at room temperature (293 K) using a Shimadzu spectrofluorometer. The specific activity of the partial complex was 1600 units mg^{-1}; a unit of activity is defined as production of anthranilate at a rate of 1.0 n*M* min^{-1}.

Polyethylene glycol (PEG) was purchased from Sigma Chemicals (St Louis, MO) and recrystallized with the following protocol: 40 g of PEG was dissolved in 480 ml of acetone and heated to 323 K. The PEG–acetone mixture was filtered and 920 ml of anhydrous ethyl ether added. PEG was allowed to recrystallize overnight at 253 K. The precipitate was collected on filter paper, washed with two volumes of cold anhydrous ethyl ether and dried.

The purified protein was crystallized by the hanging-drop method. 10 µl of 5–10 mg ml^{-1} of the partial complex and 10 µl of the well solution were mixed and placed on a cover slip. The cover slip was then inverted and placed over the solution in a well of a Linbro tissue-culture plate. Wells were sealed with mineral oil or silicon grease.

3. Results and discussion

Three sets of reservoir conditions produced crystals: (i) 9.5–10% PEG 8000, 100 m*M* MOPS pH 7.0, 245 µ*M* L-tryptophan, 5 m*M* DTT and 10 m*M* NaCl; (ii) 5.5% PEG 6000, 50 m*M* MOPS pH 7.0, 245 µ*M* L-tryptophan and 10 m*M* EDTA; (iii) 11% PEG 6000, 10 m*M* MOPS pH 7.0, 10 m*M* $MgCl_2$ and 245 µ*M* L-tryptophan. All three crystallization conditions produced crystals at 277 K over a period of 6–24 months.

Crystals were mounted in 0.7–1.0 mm diameter quartz or glass capillary tubes. Mounted crystals were characterized by precession photographs using Ni-filtered Cu $K\alpha$ radiation (λ = 1.54 Å). Crystals grown in the absence of Mg^{2+} diffracted for approximately 48 h at room temperature, while crystals grown in the presence of Mg^{2+} diffracted for approximately 96 h at room temperature. Both crystal types (+Trp, $-Mg^{2+}$ and +Trp, $+Mg^{2+}$) diffracted to a resolution of 2.8 Å on precession films. A native data set (+Trp, $-Mg^{2+}$) representing 100° in φ was measured on a MAR Research area detector at 293 K. Data were indexed with *DENZO* and symmetric reflections merged with *SCALEPACK* (Otwinowski, 1993). Merging statistics are given in Table 1. The crystal used for data collection was redissolved in water and analyzed by SDS–PAGE. There was no detectable change in the molecular weight of either subunit of the crystallized enzyme.

Table 1
SCALEPACK merging statistics for the anthranilate synthase data set (+Trp, $-Mg^{2+}$).

The linear *R* factor is calculated as the sum of the absolute value of the intensity minus the average intensity over the sum of the intensity, while the square *R* factor is calculated as the sum of the intensity minus the average intensity squared over the sum of the intensity squared. The percentage of the asymmetric unit measured and the number of lattice points measured are also given as a function of resolution range. 75% of measured reflections had a redundancy of 2 or greater.

Resolution limit (Å)	$\langle I\rangle$	$\langle I/\sigma(I)\rangle$	Linear *R* factor	Square *R* factor	Percentage of asymmetric unit measured	Lattice points
80.0–5.36	1260.3	13.65	0.084	0.093	78.1	2395
5.36–4.25	1319.5	11.58	0.111	0.107	84.3	2453
4.25–3.71	896.0	7.63	0.167	0.158	86.1	2463
3.71–3.37	631.4	4.84	0.222	0.199	80.8	2300
3.37–3.13	398.6	2.73	0.331	0.292	60.1	1709
3.13–2.95	247.4	1.66	0.462	0.444	28.7	816
2.95–2.80	167.9	1.27	0.749	0.451	8.1	228
All reflections	876.4	7.27	0.135	0.125	61.3	12364

The presence of glycerol inhibited crystallization and its addition disrupted the crystalline lattice. The removal of glycerol present in the purification buffers was important in stabilizing the tryptophan-bound form of the enzyme and promoting crystallization.

Both crystal forms are orthorhombic with unit-cell parameters a = 116.7, b = 101.2 and c = 66.8 Å. The 00l axis does not obey the systematic absence of $l = 2n + 1$, identifying it unambiguously as a twofold axis. Observed systematic absences of $h = 2n + 1$ on the h00 axis and $k = 2n + 1$ on the 0k0 axis make the most likely space-group assignment $P2_12_12$. Data-set merging statistics support this assignment.

Space group $P2_12_12$ contains four asymmetric units per unit cell. With two $(TrpE)_2(TrpG)_2$ tetramers per unit cell, the crystal contains approximately 49% solvent (calculated using a protein density of 1.3 g ml^{-1}) and has V_m = 2.53 Å^3 Da^{-1} (Matthews, 1968).

A 5 d day soak of crystals in CH_3HgCl produced significant changes in precession photographs. The increase in intensity of a diffraction spot along the twofold rotation axis was the main indication that mercury was bound to the crystal. Other precession film intensity changes support this observation.

The crystallographic twofold symmetry observed corresponds to a molecular twofold symmetry between (TrpE)(TrpG) pairs in the heterotetramer. We infer that both halves of the heterotetramer bind one molecule of tryptophan and the enzyme is fully inhibited. A more detailed analysis of the metal binding awaits the determinations of the structures of the apo and magnesium-bound crystal forms.

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