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Expression, purification, crystallization and preliminary crystallographic analysis of phosphoserine aminotransferase from *Bacillus alcalophilus*

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Phosphoserine aminotransferase (PSAT; EC 2.6.1.52) from *Bacillus alcalophilus*, an obligatory alkalophile with optimum growth at pH 10.6, was overexpressed in *Escherichia coli*, purified and crystallized under two different conditions using the hanging-drop vapour-diffusion method. Crystals were obtained using trisodium citrate dihydrate or PEG 400 as a precipitating agent. Crystals grown in the presence of trisodium citrate belong to the orthorhombic space group $C222_1$, with unit-cell parameters $a = 105.6$, $b = 136.6$, $c = 152.0$ Å, and those grown in the presence of PEG 400 belong to the orthorhombic space group $P2_12_12$, with unit-cell parameters $a = 143.7$, $b = 84.3$, $c = 67.4$ Å. Complete data sets were collected to 1.7 and 1.6 Å resolution, respectively, at 100 K using synchrotron radiation. Analysis of the structure of *B. alcalophilus* PSAT may reveal structural features that contribute to enzyme adaptability at high pH values.

1. Introduction

Extremophilic microorganisms have attracted considerable interest in recent years as a unique source of extremophilic enzymes for industry (Schiraldi & De Rosa, 2002; Demirjian *et al.*, 2001). Moreover, studies on proteins from extremophiles can provide useful information about modification of proteins in order to preserve their functional state under biotechnologically relevant conditions (Jaenicke, 2000). The value of such information is constantly rising owing to the increasing industrial and biomedical application of proteins. These data are also required for the engineering of ultrastable proteins (Hough & Danson, 1999). Along with temperature, pressure and salinity, pH is one of the most important factors affecting protein stability and function. However, despite numerous studies focused on proteins from thermophiles, psychrophiles, barophiles and halophiles, studies on proteins from alkalophiles and acidophiles have been scarce (Jaenicke & Böhm, 1998).

Phosphoserine aminotransferase (PSAT; EC 2.6.1.52), a vitamin B_6-dependent enzyme, is a member of subgroup IV of the aminotransferases. It catalyses the reversible conversion of 3-phosphohydroxypyruvate to L-phosphoserine, which is subsequently hydrolysed to L-serine by L-phosphoserine phosphatase (EC 3.1.3.3; Walsh & Sallach, 1966). These reactions are the final steps of the 'phosphorylated pathway' of L-serine biosynthesis in many organisms, ranging from bacterial cells to higher animals (Hester *et al.*, 1999).

Bacillus alcalophilus (Vedder, 1934) is an obligate alkalophile with optimum growth at pH 10.6. However, the internal cytoplasmic pH remains only slightly alkaline (pH 9.2), even when the environmental pH is 11.0 (Horikoshi, 1999). Nevertheless, enzymatic studies of PSAT from *B. alcalophilus* showed optimum enzymatic activity at around pH 10, which is essentially high for a cytoplasmic enzyme (data to be published). The crystal structure of *Escherichia coli* PSAT has been reported to 2.3 Å (Hester *et al.*, 1999). The coordinates of PSAT from the facultative alkalophile *B. circulans* ssp. *alkalophilus* have been deposited in the PDB (PDB code 1bt4; 2.3 Å resolution), but the structure has not yet been reported. Structural comparison of similar enzymes with different pH optima and stability may elucidate the nature of the structural features that make proteins stable and active under extreme environmental conditions. Furthermore, understanding the principles and mechanisms of stability at high pH in proteins may be useful for protein engineering and practical applications in industry, biotechnology and medicine.

Here, we report the high-level expression, purification, crystallization and preliminary crystallographic analysis of PSAT from *B. alcalophilus*, the third member of the phosphoserine aminotransferase family to be crystallized.

2. Methods and results

2.1. Expression and purification

The pBALC-PSAT plasmid encoding PSAT from *B. alcalophilus* (GenBank accession code AF204962) was generously provided by Natalia Battchikova, Turku University, Finland (N. Battchikova, M. Koivulehto & T. Korpela, work in preparation). *E. coli* strain BL21 (DE3) was used for protein expression. The yield of soluble recombinant protein was very low when standard expression conditions including induction with 1 m*M* isopropyl-β-D-thiogalactopyranoside (IPTG) and a growth temperature of 310 K were used. Despite the high expression level, PSAT was almost completely incorporated into insoluble inclusion bodies (Fig. 1). Screening of IPTG concentrations and different post-induction temperatures was used for optimization. Finally, a sufficient amount of soluble protein for structural studies (Fig. 1) was obtained after expression using the following conditions. Cells were grown in LB medium containing 100 µg ml^{-1} ampicillin at 310 K until OD_{600} reached 0.7. The culture was induced with 10 µ*M* IPTG for 12 h at 295 K and harvested by centrifugation at 5000*g* for 15 min at 277 K. The precipitated cells were resuspended in 100 m*M* Tris–HCl pH 8.0 containing 100 µ*M* pyridoxal-5′-phosphate (PLP), 1 m*M* DTT, 1 m*M* PMSF, 5 m*M* EDTA and homogenized by sonication. The lysate was centrifuged at 20 000*g* for 20 min at 277 K. Ammonium sulfate was added to the supernatant to 70% saturation. The mixture was incubated for 1 h on an ice bath with agitation, centrifuged at 20 000*g* for 20 min at 277 K and the pellet was discarded. The supernatant was dialysed extensively against 20 m*M* bis-tris pH 6.5 to remove ammonium sulfate. Protein solution was filtered through a 0.22 µm filter and loaded onto a DEAE CL-6B column (1.5 × 12 cm, Econo-Pac, BioRad) pre-equilibrated with 20 m*M* bis-tris pH 6.5. After extensive washing, proteins were eluted with a linear gradient of 0.15–0.5 *M* NaCl. Fractions containing PSAT were pooled, dialyzed against 20 m*M* Tris–HCl pH 8.5 and loaded onto an AX 300 HPLC anion-exchange column (25 × 0.46 cm, Eprogen, USA). After washing with 0.1 *M* NaCl in 20 m*M* Tris–HCl pH 8.5, protein was eluted with 0.35 *M* NaCl in 20 m*M* Tris–HCl pH 8.5. Fractions containing >0.5 mg ml^{-1} PSAT were pooled and concentrated using Amicon Centriprep (YM-30) filters. Sephacryl S-200 size-exclusion chromatography was performed with 50 m*M* Tris–HCl pH 8.0 containing 0.15 *M* NaCl (column dimensions 100 × 1.5 cm, BioRad). A single peak corresponding to 80–82 kDa apparent molecular weight was obtained after gel filtration, in good agreement with the theoretical molecular weight of the dimeric PSAT (80.41 kDa) calculated from the amino-acid sequence.

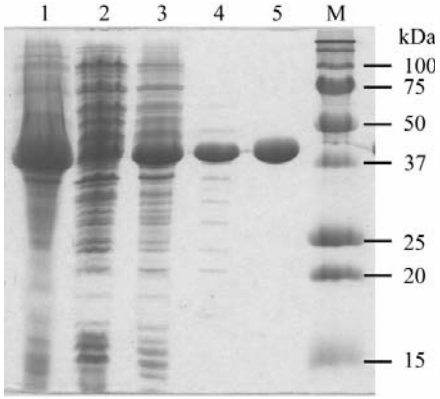

Figure 1
Expression under different conditions and purification of PSAT. Proteins were separated using 12% SDS–PAGE and stained with Coomassie Blue R-250. Lane 1, insoluble inclusion bodies (310 K, 1 m*M* IPTG); lane 2, soluble cell fraction (310 K, 1 m*M* IPTG); lane 3, soluble cell fraction (295 K, 10 µ*M* IPTG); lane 4, supernatant after addition of ammonium sulfate; lane 5, purified PSAT used for crystallization; M, molecular-weight markers.

The specific activity of the purified enzyme measured at pH 8.2 according to Hirsch & Greenberg (1967) was 25–30 U mg^{-1}. Typically, the activity assay was carried out using 0.5–1 µg of PSAT in 1 ml of reaction mixture containing 0.5 m*M* 3-phosphohydroxypyruvate, 8 m*M* glutamate, 32 m*M* ammonium acetate, 0.2 m*M* NADH, 20 µ*M* pyridoxal-5′-phosphate, 12 U glutamate dehydrogenase (Roche) in 50 m*M* Tris–HCl pH 8.2. Reaction was started by the addition of 3-phosphohydroxypyruvate and the decrease in NADH absorption at 340 nm was measured at 298 K.

Protein concentration was determined from the absorption of a 0.1%(*w*/*v*) solution in a 1 cm path-length cuvette at 280 nm. The absorption value of 0.729 was calculated from the amino-acid sequence according to the method of Gill & von Hippel (1989).

2.2. Crystallization

PSAT from *B. alcalophilus* was crystallized by the hanging-drop vapour-diffusion method using Crystal Screen HR2-910 (Hampton Research) and Linbro 24-well cell-culture plates. The drops consisted of 2 µl protein solution (10–15 mg ml^{-1} in 10 m*M* Tris–HCl pH 8.0) and 2 µl reservoir solution. The drops were equilibrated against 700 µl reservoir solution at 289 K. Very thin needle-like crystals were obtained from three different conditions. However, crystals of reasonable size for crystallo-

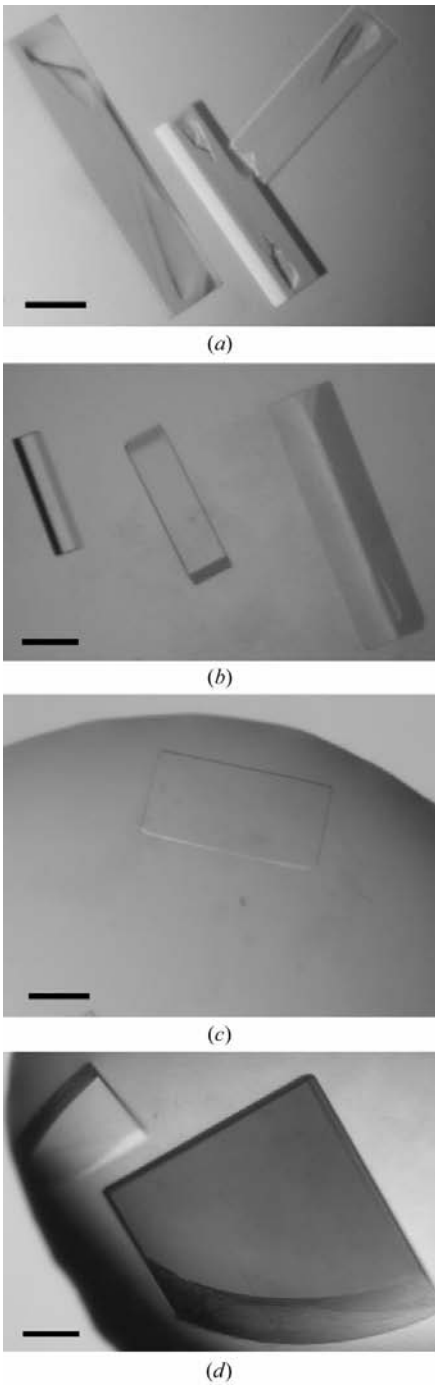

Figure 2
Crystals of phosphoserine aminotransferase obtained from different conditions: (*a*) condition 38 of Crystal Screen HR2-910 (Hampton Research); (*b*) 1.4 *M* trisodium citrate dihydrate, 0.1 *M* Tris–HCl pH 8.5; (*c*) condition 23 of Crystal Screen HR2-910 (Hampton Research), protein concentration 10 mg ml^{-1}; (*d*) condition 23 of Crystal Screen HR2-910 (Hampton Research), protein concentration 30 mg ml^{-1}. The bar represents 0.2 mm.

graphic studies (Fig. 2*a*) were obtained after 1 d from condition 38 (1.4 *M* trisodium citrate dihydrate, 0.1 *M* HEPES pH 7.5). These crystals were further improved (Fig. 2*b*) by using 0.1 *M* Tris–HCl pH 8.5 instead of HEPES pH 7.5. In addition, plate-like crystals were obtained after initial crystallization trials in condition 23 [0.2 *M* magnesium chloride hexahydrate, 30%(*v*/*v*) PEG 400, 0.1 *M* HEPES pH 7.5]. These crystals (Fig. 2*c*) were only 10–15 µm thick even after two months and diffracted very poorly. However, well diffracting crystals that were up to 150 µm thick were obtained from condition 23 using 30–35 mg ml^{-1} protein solution (Fig. 2*d*).

2.3. Data collection and preliminary X-ray analysis

Complete data sets for both crystal forms were collected from single crystals at the EMBL X11 beamline at the DORIS storage ring, DESY, Hamburg. Crystals were flash-cooled to 100 K in a nitrogen-gas cold stream using an Oxford Cryosystems Cryostream. A crystal from condition 38 (form I) was transferred into reservoir solution containing 15% glycerol as cryoprotectant for ∼5 s before cooling. No additional cryoprotectant was needed for the crystal from condition 23 (form II) owing to the high concentration [30%(*v*/*v*)] of PEG 400 in the mother liquor. Diffraction data were collected on a MAR CCD detector with a diameter of 165 mm. The exposure time was 10–15 s per image (dose mode) and the oscillation range was 0.4°. 250 images were collected in total at a wavelength of 0.811 Å from the form I crystal. 200 images were collected at a wavelength of 0.811 Å from one part of crystal form II and an additional 100 images were collected from another part of the crystal after a small translation. Data were processed with *DENZO* and *SCALEPACK* (Otwinowski & Minor, 1997). The final statistics for the data collection are summarized in Table 1. Complete data sets to 1.7 and 1.6 Å resolution were collected from crystal forms I and II, respectively. Form I crystals belong to the orthorhombic space group $C222_1$, with unit-cell parameters a = 105.6, b = 136.6, c = 152.0 Å. Assuming a dimer in the asymmetric unit, the Matthews coefficient (Matthews, 1968) is 3.451 Å^3 Da^{-1}, which corresponds to a solvent content of ∼64%. Form II crystals belong to the orthorhombic space group $P2_12_12$, with unit-cell parameters a = 143.7, b = 84.3, c = 67.4 Å. Assuming a dimer in the asymmetric unit for crystal form II, the Matthews coefficient is 2.568 Å^3 Da^{-1}, which corresponds to a solvent content of ∼50%. The presence of a dimer in the crystals is in agreement with the results obtained by gel filtration and the well known feature of aminotransferases to be functional only in the dimeric form.

Table 1
Data-collection statistics.

Values in parentheses correspond to the highest resolution shell.

Crystal	Form I	Form II
Space group	$C222_1$	$P2_12_12$
Unit-cell parameters (Å)	a = 105.6, b = 136.6, c = 152.0	a = 143.7, b = 84.3, c = 67.4
No. of crystals	1	1
No. of observations	2738511	2316323
No. of unique reflections	121000	106389
Resolution range (Å)	20.0–1.7 (1.75–1.70)	20.0–1.6 (1.65–1.60)
Completeness (%)	99.7 (99.7)	96.4 (96.4)
$R_{\rm merge}$	3.5 (19.4)	5.3 (9.8)
$I/\sigma(I)$	14.5 (3.7)	17.6 (10.3)

3. Discussion

PSAT from *B. alcalophilus* was expressed in *E. coli* cells. However, a very poor yield of soluble protein was initially obtained under standard expression conditions. In order to slow the expression rate, both the IPTG concentration and the temperature were decreased. The highest expression level of soluble protein was achieved using 10 µ*M* IPTG and a post-induction temperature of 295 K. Such optimization along with an increased post-induction time allowed expression of >50 mg of PSAT per litre of bacterial culture.

Salting out with ammonium sulfate was used as the first purification step. The protein showed a relatively high stability against high concentrations of ammonium sulfate and did not precipitate even in the presence of up to 70% ammonium sulfate saturation. However, most of the *E. coli* proteins were precipitated under these conditions and PSAT with more than 90% purity was obtained after this step. Further purification using anion-exchange chromatography and gel filtration increased the purity to >95% as judged by SDS–PAGE. The final yield of purified PSAT was >30 mg per litre of bacterial culture.

PSAT was successfully crystallized and complete high-resolution data sets were collected from two crystal forms. The structure will be determined by molecular replacement (Navaza, 1994) using *E. coli* PSAT as a search model (sequence identity ∼41%). Structure determination will provide insights into protein-structure adaptability and function at high pH values. Moreover, the best current resolution for collected data sets (1.6 Å; Table 1) is essentially higher than that of the reported *E. coli* PSAT structure (2.3 Å; Hester *et al.*, 1999). The high-resolution structure of *B. alcalophilus* PSAT may reveal unresolved mechanistic features of the PSAT family. In addition, detailed comparison of the two crystal forms will provide a better understanding of the flexibility of residues involved in crystal-packing contacts and their potential role in the adaptability of PSAT to high pH values.

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