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Expression, purification and preliminary X-ray characterization of *N*-acetyl-γ-glutamyl-phosphate reductase from *Thermus thermophilus* HB8

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N-Acetyl-γ-glutamyl-phosphate reductase (AGPR) catalyses the NADPH-dependent reduction of *N*-acetyl-γ-glutamyl phosphate to give the *N*-acetylglutamic semialdehyde. A recombinant form of AGPR from *Thermus thermophilus* HB8 has been crystallized by the hanging-drop vapour-diffusion technique using PEG 4000 as a precipitating agent. The crystals grew as colourless prisms, with unit-cell parameters $a = b = 90.9$, $c = 139.5$ Å, $\alpha = \beta = 90$, $\gamma = 120°$. The crystals belong to the hexagonal space group $P6_222$ or $P6_422$ and are most likely to contain a monomer in the asymmetric unit, with a V_M value of 2.19 Å^3 Da^{-1}. The crystals diffract to a resolution of 2.2 Å at beamline BL44B2 of SPring-8.

1. Introduction

Arginine is synthesized from glutamate in an eight-step biosynthesis (Davis, 1986; Cunin *et al.*, 1986). *N*-Acetyl-γ-glutamyl-phosphate reductase (AGPR; *N*-acetylglutamate-γ-semialdehyde dehydrogenase) catalyses the third step of the arginine biosynthesis, in which *N*-acetyl-γ-glutamyl phosphate, the product of the second step catalysed by acetylglutamate kinase, undergoes reductive dephosphorylation to give *N*-acetylglutamic semialdehyde, which is converted to ornithine by acetylornithine aminotransferase and acetylornithine deacetylase. AGPR reversibly catalyses the NADPH-dependent reduction of *N*-acetyl-γ-glutamyl phosphate. The reaction catalysed by AGPR is presumed to be similar to those catalysed by glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and aspartate-β-semialdehyde dehydrogenase (ASADH); GAPDH and ASADH perform oxidative phosphorylation of glyceraldehyde-3-phosphate and reductive dephosphorylation of β-aspartyl phosphate, respectively (Fersht, 1999; Biellmann *et al.*, 1980). The overall subunit structure of ASADH from *Escherichia coli* was shown to be similar to that of GAPDH by X-ray structure determination, with the catalytic nucleophile (cysteine) being conserved, despite the sequence identity between the two enzymes being only 15% (Hadfield *et al.*, 1999; Skarzynski *et al.*, 1987).

AGPR from *Thermus thermophilus* HB8 (tAGPR), which has been cloned and overexpressed in *E. coli*, has 348 residues per subunit and a molecular weight of 37 956 Da. A homology search using *FASTA* (Pearson & Lipman, 1988) and *CLUSTALW* (Thompson *et al.*, 1994) indicated that tAGPR has significant sequence identity to ASADHs from many species, with identities of 25% (the highest identity) and 13% for ASADH from *Methanococcus jannaschii* and *E. coli*, respectively. Furthermore, the sequence of tAGPR is 16% identical to that of the GAPDHs from *E. coli* and *Bacillus stearothermophilus*. Thus, tAGPR is likely to have an overall fold that is similar to, but to have a detailed structure that significantly differs from, those of the ASADH and GAPDH enzymes. The consensus sequence PGC*xx*T (*x* is any amino acid) is observed in most of the AGPRs whose sequences have been deposited in the SWISS-PROT data bank. The Cys residue of this sequence, which is conserved in the ASADH and GAPDH enzymes, is assumed to be the catalytic nucleophile of tAGPR (Ludovice *et al.*, 1992).

Structure determination of tAGPR should help to clarify the role of the active-site residues and the mechanism of NADPH and substrate recognition, as the three-dimensional structure of AGPR is not available. Moreover, a comparison of the structure of tAGPR with those of the GAPDH and ASADH enzymes will provide some insight into the catalytic mechanism of the reductive dephosphorylation or the oxidative phosphorylation described above and the evolutionary relationship of the NADPH-dependent dehydrogenase. In this communication, we report the crystallization and preliminary X-ray diffraction studies of tAGPR.

2. Experimental

2.1. Expression and purification

Using *T. thermophilus* HB8 genomic DNA and a set of primers (5′-ATATCATATGGTA-AGGGTGGGGATCCTGGGGGCCTCGG-3′ and 5′-ATATAGATCTTTATTACGGCCA-TAACCCCTCCTTGGGAAGC-3′) as a

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template, the tAGPR gene was amplified by PCR and cloned into pT7Blue (Novagen). After confirmation of the nucleotide sequence, the tAGPR gene was ligated into the expression vector pET-11a (Novagen) at the *Nde*I/*Bam*HI sites. The resulting expression plasmid was used to transform *E. coli* strain BL21(DE3) (Novagen). The transformant was cultured in Luria broth containing 50 µg ml^{-1} ampicillin and 0.1% glucose at 310 K until the density of the culture reached 4×10^8 cells ml^{-1}. The cells were incubated for a further 4 h in the presence of 1 m*M* IPTG and then harvested by centrifugation.

The cells were suspended in 20 m*M* Tris–HCl, 5 m*M* 2-mercaptoethanol, 50 m*M* NaCl pH 8.0 and then disrupted by sonication. The cell lysate was incubated at 343 K for 10 min and then ultracentrifuged (200 000*g*) for 60 min at 277 K. Solid ammonium sulfate was added to the resulting supernatant to a final concentration of 1.5 *M* and the solution was applied onto a Resource ISO column (Amersham Biosciences) equilibrated with 50 m*M* sodium phosphate pH 7.0 containing 1.5 *M* ammonium sulfate. The enzyme was eluted with a linear gradient of 1.5–0 *M* ammonium sulfate in 50 m*M* sodium phosphate pH 7.0. Fractions containing tAGPR were desalted and applied onto a Resource Q column (Amersham Biosciences) equilibrated with 20 m*M* Tris–HCl pH 8.0. tAGPR was eluted with a linear gradient of 0–0.5 *M* NaCl in 20 m*M* Tris–HCl pH 8.0. Fractions containing tAGPR were desalted and applied onto a CHT2-I column (Bio-Rad) equilibrated with 10 m*M* sodium phosphate pH 7.0. tAGPR was eluted with a linear gradient of 10–250 m*M* sodium phosphate pH 7.0. Fractions containing tAGPR were concentrated and loaded onto a HiLoad 16/60Superdex 75 pg column (Amersham Bioscience) equilibrated with 20 m*M* Tris–HCl and 150 m*M* NaCl pH 8.0. The peak fractions were concentrated and stored at 277 K. The recombinant enzyme was assayed at 298 K by a previously reported procedure (Ludovice *et al.*, 1992). The enzyme has a specific activity of 0.848 units mg^{-1}, which is comparable to that of the enzyme from *Neurospora crassa* (0.953 units mg^{-1}; Wandinger-Ness *et al.*, 1986).

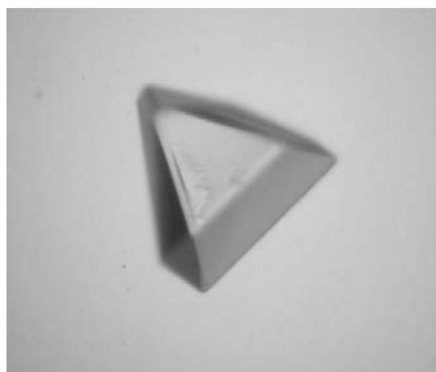

Figure 1
A hexagonal crystal of *N*-acetyl-γ-glutamyl-phosphate reductase from *T. thermophilus* HB8.

2.2. Crystallization

Transparent colourless crystals of tAGPR were grown by the hanging-drop vapour-diffusion method at 293 K (Fig. 1). Initial trials for the crystallization conditions were performed using sparse-matrix (Jancarik & Kim, 1991) kits from Hampton Research (Crystal Screens I and II). Minute crystals appeared from solution No. 6 of Crystal Screen kit I [30%(*w*/*v*) PEG 4000, 0.1 *M* Tris–HCl pH 8.5, 0.2 *M* $MgCl_2$] and the crystallization conditions were optimized with different PEG and Tris–HCl concentrations. A droplet of 6 µl protein solution [2.0 mg ml^{-1} protein, 4.5%(*w*/*v*) PEG 4000, 100 m*M* $MgCl_2$, 50 m*M* Tris–HCl pH 8.5] was equilibrated against 400 µl of reservoir solution [9.0%(*w*/*v*) PEG 4000, 200 m*M* $MgCl_2$, 100 m*M* Tris–HCl pH 8.5] to produce crystals of tAGPR.

2.3. Data collection

For preliminary characterization, crystals were mounted in glass capillaries with a small amount of mother liquor and intensity data for unit-cell parameter and space-group determination were collected on an R-AXIS IV++ image-plate detector with Cu *K*α radiation from a Rigaku rotating-anode generator operated at 40 kV and 100 mA. Data collection from the native crystals was performed at 100 K using a wavelength of 1.00 Å from the Synchrotron Radiation Source at the SPring-8 BL44B2 and a MAR CCD165 detector system (Hyogo, Japan). Prior to flash-freezing, the crystals were soaked for a few seconds in a solution containing 8 µl reservoir solution and 2 µl PEG 400. The crystals were then picked up using a 0.5 mm fiber loop (Hampton Research) and flash-frozen in a liquid-nitrogen stream at 100 K. The data were processed using *MOSFLM* (Leslie, 1992) and *SCALA* from the *CCP*4 suite (Collaborative Computational Project, Number 4, 1994).

3. Results and discussion

Crystals of tAGPR appeared within a week of incubation and grew to maximum dimensions of $0.1 \times 0.1 \times 0.13$ mm (Fig. 1). From the diffraction data collected using the R-AXIS IV++, the space group of the crystal was determined to be hexagonal $P6_222$/$P6_422$, with unit-cell parameters $a = b = 90.9$, $c = 139.5$ Å (Table 1). Evaluation of the crystal-packing parameter showed that the lattice can accommodate one molecule per asymmetric unit (V_M = 2.19 Å^3 Da^{-1}), with an estimated solvent content of approximately 34% in the unit cell. This is within the range of values for typical protein crystals (Matthews, 1968). A native data set with 17 958 unique reflections has been collected, giving a data-set completeness of 99.8% at 19.8–2.20 Å and an R_{merge} of 7.4% (Table 1). These data indicated that the crystals were of good quality for X-ray structural analysis. The crystals showed no significant decay upon exposure. SeMet-substituted tAGPR was crystallized under the same conditions as native tAGPR. Crystals of the SeMet-substituted enzyme diffracted to 2.0 Å resolution using synchrotron radiation at the BL44B2 beamline, SPring-8, Hyogo, Japan. The X-ray structure determination of tAGPR based on MAD (SeMet) methods is currently under way.

Table 1
Crystal data and intensity data.

Values in parentheses refer to the last resolution shell.

Space group	$P6_222$ or $P6_422$
Unit-cell parameters (Å)	a = 90.9, b = 90.9, c = 139.5
Temperature (K)	100
Wavelength (Å)	1.00
Resolution range (Å)	19.8–2.2 (2.28–2.20)
No. of reflections	287874
No. of unique reflections	17958
Completeness (%)	99.8 (99.8)
R_{merge}† (%)	7.4 (24.9)
Mean $I/\sigma(I)$	30.2 (7.3)

† $R_{merge} = \sum_{hkl}\sum_i |I_{hkl,i} - \langle I_{hkl}\rangle| / \sum_{hkl}\sum_i I_{hkl,i}$, where I is the observed intensity and $\langle I\rangle$ is the average intensity for multiple measurements.

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