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Overexpression, purification and crystallization of tyrosyl-tRNA synthetase from the hyperthermophilic archaeon *Aeropyrum pernix* K1

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Hyperthermophilic archaeal tyrosyl-tRNA synthetase from *Aeropyrum pernix* K1 was cloned and overexpressed in *Escherichia coli*. The expressed protein was purified by Cibacron Blue affinity chromatography following heat treatment at 363 K. Crystals suitable for X-ray diffraction studies were obtained under optimized crystallization conditions in the presence of 1.5 *M* ammonium sulfate using the hanging-drop vapour-diffusion method. The crystals belonged to the tetragonal space group $P4_32_12$, with unit-cell parameters $a = b = 66.1$, $c = 196.2$ Å, and diffracted to beyond 2.15 Å resolution at 100 K.

1. Introduction

In protein biosynthesis, maintaining the accurate amino-acid sequence that is important for correct protein structure and function relies on the accurate enzymatic activities of aminoacyl-tRNA synthetases (ARSs). These enzymes catalyze a two-step amino-acylation reaction. An amino acid selected by a cognate ARS is activated by the formation of its aminoacyl-adenylate and the aminoacyl moiety from this adenylate is subsequently transferred to a conserved adenosine residue of the 3′-terminal CCA of the cognate tRNA. The ARS accurately recognizes its cognate amino acid and tRNA and discriminates non-cognate amino acids and tRNAs. The fidelity of this crucial reaction is ensured by the specificity of ARS to the amino acid and its base-specific or tertiary structural interactions with the tRNA (Giegé *et al.*, 1998; Asahara *et al.*, 1993).

Tyrosyl-tRNA synthetase (TyrRS) is one of the most researched ARSs and there have been extensive advances in studies of its functional aspects. TyrRS recognizes particular bases and structures of tyrosine tRNA that vary across kingdoms. On the basis of the length of the variable arm, tRNAs are classified into either class I or class II. The class that tyrosine tRNAs belong to differs according to their origin, while the class of other tRNAs is maintained across the three primary domains, namely, bacteria, archaea and eukarya. Bacterial tyrosine tRNA belongs to the class II tRNAs, which are characterized by a long variable arm, while archaeal and eukaryotic tyrosine tRNAs belong to the class I tRNAs, which have short variable arms. On one hand, bacterial TyrRS recognizes the idiosyncratic variable arm by using its flexible C-terminal domain (Yaremchuk *et al.*, 2002); on the other hand, archaeal and eukaryotic TyrRSs recognize the unique C1–G72 base pairs that are not present in other tRNAs (Kobayashi *et al.*, 2003; Fechter *et al.*, 2000, 2001). Thus, TyrRS provides interesting insights into evolutionary differences in molecular recognition; therefore, understanding its structural information in detail is crucial.

TyrRS from the hyperthermophilic archaeon *Aeropyrum pernix* K1 is an 81.6 kDa homodimeric protein comprising two 364 amino-acid residue polypeptides. *A. pernix* TyrRS is strikingly thermostable. It recognizes the C1–G72 base pair of its cognate class I tyrosine tRNA and specifically attaches a tyrosine residue to it (unpublished data). Since a large number of archaeal species survive in extreme habitats resembling those of ancient times, the hyperthermophilic archaeal *A. pernix* TyrRS will be a good model for studying the molecular-recognition mechanism that has been adapted by TyrRS in two considerably distinct tRNA structures. In this study, the cloning,

purification, crystallization and preliminary X-ray diffraction analysis of *A. pernix* TyrRS are described.

2. Experimental procedures

2.1. Cloning and expression of *A. pernix* TyrRS

The gene encoding *A. pernix* TyrRS was isolated from genomic DNA (Kawarabayasi *et al.*, 1999) using PCR amplification. For PCR, a forward primer (5′-CAT G**CC ATG G**TC CGC GTG GAT GTT GAG GAG-3′) with an *Nco*I restriction-enzyme cleavage site (bold) and a reverse primer (5′-CGC **GGA TCC** GCA TGC *CTA* TCT CGT AAC CTT ACC TTC TAT-3′) with a *Bam*HI cleavage site (bold) and a termination codon (italicized) were employed. PCR products were inserted into pGEM-T Easy vector (Promega, Madison, WI, USA). DNA fragments digested with *Nco*I–*Bam*HI were subcloned into corresponding sites of the expression vector pET-28a(+) (Novagen, Madison, WI, USA). None of the histidine tags are present on the expressed *A. pernix* TyrRS. The expression vectors that encode *A. pernix* TyrRS were transformed into *Escherichia coli* BL21-CodonPlus (DE3)-RIL strain (Stratagene, La Jolla, CA, USA). The transformants were cultured in 10 l Luria–Bertani (LB) medium containing 20 µg ml^{-1} kanamycin at 310 K to an optical density of 0.6 at 600 nm. Protein expression was allowed to proceed for 4 h after induction by the addition of 1 m*M* isopropylthio-β-D-galactoside (IPTG). Cells were harvested by centrifugation (6000*g* for 20 min).

2.2. Purification

The harvested cells were suspended in 80 ml lysis buffer (10 m*M* Tris–HCl pH 8.0, 10 m*M* KCl, 10 m*M* $MgCl_2$, 0.1 m*M* phenylmethylsulfonyl fluoride, 5 m*M* β-mercaptoethanol), disrupted by sonication, centrifuged (8000*g* for 30 min) and the supernatant obtained was heated at 363 K for 1 h to simplify the purification step. Proteins from *A. pernix* K1 are remarkably thermostable; therefore, cloned *A. pernix* TyrRS could be easily separated from the heat-denatured host-cell proteins by centrifugation (8000*g* for 30 min) after the heat treatment. The clarified supernatant was treated with 6 *M* urea and *A. pernix* TyrRS was refolded twice in 5 l buffer *A* (10 m*M* Tris–HCl pH 8.0, 10 m*M* KCl, 10 m*M* $MgCl_2$, 5 m*M* β-mercaptoethanol) to eliminate the effects of misfolding. The dialyzed supernatant was subjected to a Cibacron Blue 3GA affinity column (Sigma, St Louis, MO, USA; 2.5 × 7.0 cm; Li *et al.*, 1999) equilibrated with buffer *A*. After washing the column with 300 ml buffer *A* containing 300 m*M* NaCl, *A. pernix* TyrRS was eluted with 100 ml buffer *A* containing 1.5 *M* NaCl. Fractions were combined, dialyzed against buffer *A* and concentrated to 10 mg ml^{-1} using a Centriprep YM-10 filter (Millipore, Bedford, MA, USA).

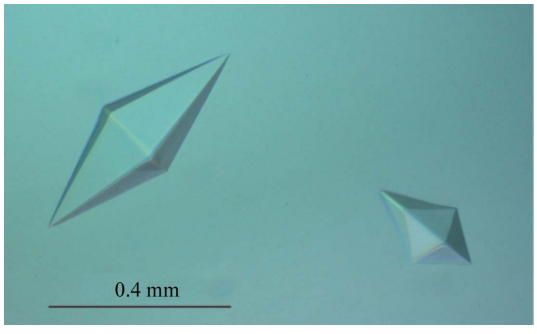

Figure 1
Typical crystals of *A. pernix* TyrRS grown using the hanging-drop vapour-diffusion method.

2.3. Crystallization and data collection

Initial crystallization conditions were assessed by the hanging-drop vapour-diffusion method using different concentrations of precipitants; namely, ammonium sulfate, polyethylene glycol 6000, 1,6-hexanediol and 2-propanol. A protein solution concentrated to 100 mg ml^{-1} was used for initial screening. Bipyramid-shaped crystals appeared in the presence of 1.5 *M* ammonium sulfate under different pH conditions (pH 6.0–8.5) at 293 K. Finally, 1 µl protein solution (10 mg ml^{-1}) and 1 µl reservoir solution consisting of 1.5 *M* ammonium sulfate and 100 m*M* Tris–HCl pH 8.0 were mixed and droplets were equilibrated against 400 µl reservoir solution (on 24-well plates; Asahi Techno Glass Co., Tokyo, Japan) at 293 K. Crystals suitable for X-ray analysis were obtained within 3 d (Fig. 1).

X-ray diffraction experiments were conducted using an R-AXIS VII imaging-plate X-ray detector with a Cu $K\alpha$ X-ray diffractometer (Rigaku, Japan; λ = 1.5418 Å). After soaking the *A. pernix* crystals in a cryoprotectant solution (42% xylitol in reservoir solution), they were mounted in a nylon loop (Hampton Research, Aliso Viejo, CA, USA). Subsequently, these crystals were flash-frozen in a nitrogen stream at 100 K. Diffraction data were collected in 0.5° oscillation steps over a range of 100°. The collected data sets were processed using the programs *DENZO* and *SCALEPACK* from the *HKL*2000 package (Otwinowski & Minor, 1997).

2.4. Aminoacylation assay

The aminoacylation activity was assayed as outlined by Sherman *et al.* (1992). Aminoacylation reactions were performed at 338 K in a reaction mixture consisiting of 100 m*M* HEPES–NaOH pH 8.0, 10 m*M* KCl, 10 m*M* $MgCl_2$, 2 m*M* ATP, 6.1 µ*M* (^{14}C)-tyrosine (410 mCi mmol^{-1}) and 8 mg ml^{-1} total tRNA from *A. pernix* K1 cells. After pre-incubation for 3 min, the reactions were initiated by adding *A. pernix* TyrRS (1.0 µg ml^{-1}) to the reaction mixture. The reactions were stopped at constant time intervals by spotting an 8 µl aliquot obtained from 40 µl of the reaction mixture onto Whatman 3MM filter papers (Whatman, Maidstone, Kent, UK) that had been soaked in 5% trichloroacetic acid. After the filter papers had been washed three times (10 min each) with cold 5% trichloroacetic acid, they were dried completely. The radioactivities of the filter papers were measured using a liquid-scintillation counter.

3. Results and discussion

A. pernix TyrRS was cloned and overexpressed in *E. coli*. The expressed protein was purified by Cibacron Blue affinity chromatography following heat treatment at 363 K. The nucleic acids that were tightly bound to *A. pernix* TyrRS could not be removed using ion-exchange column chromatography (Saldanha *et al.*, 1995). The high affinity of *A. pernix* TyrRS to Cibacron Blue 3GA, which can bind enzymes requiring adenylic cofactors such as NAD^+, NADH and ATP, enabled single-step column chromatography purification. This purification method was extremely rapid and facile compared with the conventional methods, which use several chromatographic steps, and the purified protein was remarkably soluble. The purity of *A. pernix* TyrRS was detected using denaturing gel electrophoresis and its enzyme activity was determined using an aminoacylation assay. The specific activity of purified *A. pernix* TyrRS was 487 U mg^{-1} (1 U of aminoacyl-tRNA synthetase activity was defined as the amount of the enzyme that catalyzes the incorporation of

Table 1
Data-collection statistics for *A. pernix* TyrRS.

Values in parentheses refer to the highest resolution shell.

X-ray source	Cu $K\alpha$
Wavelength (Å)	1.5418
Space group	$P4_32_12$
Unit-cell parameters (Å)	$a = b = 66.1$, $c = 196.2$
Resolution (Å)	50.0–2.15 (2.23–2.15)
R_{merge} (%)	6.5 (33.2)
Completeness (%)	99.7 (99.9)
Average $I/\sigma(I)$	55.7 (11.9)
Average redundancy	7.4 (7.3)
Unique reflections	24590 (2392)
Observed reflections	898560

1 nmol amino acid into aminoacyl-tRNA in 10 min), which was considerably higher than that of human TyrRS (Jia *et al.*, 2003).

Crystals of *A. pernix* TyrRS suitable for X-ray diffraction studies were obtained under optimized crystallization conditions in the presence of 1.5 *M* ammonium sulfate using the hanging-drop vapour-diffusion method. The crystals diffracted X-rays to beyond 2.15 Å resolution and belonged to the tetragonal space group $P4_12_12$ or $P4_32_12$, with unit-cell parameters $a = b = 66.1$, $c = 196.2$ Å. The native data set contained a total of 898 560 reflections, which reduced to 24 590 unique reflections with 99.7% completeness. The data-collection statistics for *A. pernix* TyrRS are summarized in Table 1. Matthews coefficient calculations suggested that the V_M value of the crystal was 2.6 Å^3 Da^{-1} (Matthews, 1968), assuming the presence of one *A. pernix* TyrRS molecule per asymmetric unit; this V_M value corresponded to a solvent content of 52.9%.

A sequence-homology search conducted using *BLAST* (Altschul *et al.*, 1997) for *A. pernix* TyrRS showed 42% homology to *Methanococcus jannaschii* TyrRS (PDB code 1j1u; Kobayashi *et al.*, 2003). The molecular-replacement method using the program *MOLREP* (Vagin & Teplyakov, 1997) from the *CCP*4 package (Collaborative Computational Project, Number 4, 1994) was employed for structural analysis of *A. pernix* TyrRS using *M. jannaschii* TyrRS as the starting model. After rotation, translation and fitting calculations in the resolution range 42.2–3.0 Å, a solution was found when space group $P4_32_12$ was adopted. The crystal contained one molecule per asymmetric unit; therefore, the dimerization axis of *A. pernix* TyrRS appeared to coincide with the crystallographic axis. Model building and structure determination are currently in progress.

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