Crystallization of threonyl-tRNA synthetase from *Thermus thermophilus* and preliminary crystallographic data

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Abstract Threonyl-tRNA synthetase from *Thermus thermophilus* (ttTRS) has been overproduced in *Escherichia coli*, purified and crystallized in solutions containing ammonium sulfate and glycerol. The crystals grew in the orthorhombic space group $C222_1$ with unit cell dimensions $a = 119.5$ Å, $b = 120.0$ Å, $c = 317.5$ Å. The asymmetric unit is constituted of two monomers and the crystals contain 66% solvent. This paper reports the first crystals of ttTRS and preliminary crystallographic results since the presumed crystals of ttTRS described in a previous paper [1] were crystals of aspartyl-tRNA synthetase [2].

Key words: Aminoacyl-tRNA synthetase; *Thermus thermophilus*; Crystallization; tRNA aminoacylation

1. Introduction

The aminoacyl-tRNA synthetases (aaRS) constitute a family of enzymes that catalyze the attachment of amino acids onto their cognate tRNA molecules, prior to participation of the aminoacylated tRNA in the protein synthesis. The aaRS can be divided into two classes on the basis of differences in active site topology and substrate recognition [3,4].

Threonyl-tRNA synthetase (TRS) from *Thermus thermophilus*, a homodimer of 151 kDa, belongs to class 2 (Cura et al., in prep.). We focused our interest on this aaRS because of its structural and functional particularities. TRS constitutes with the aaRS specific for serine (SRS), proline (PRS), histidine (HRS) and glycine (GRS) a subgroup of closely related enzymes (subclass 2a) according to strong sequence homologies in the active site [5,6]. In addition, X-ray structures of three members of this subclass have been determined: SRS from *Escherichia coli* [7], GRS from *T. thermophilus* [8] and HRS from *E. coli* [9], confirming the existence of a modular organization of these enzymes already observed for other aaRS [5]. However, TRS differs from the other subclass members by its larger size due to a particularly substantial N-terminal module comprising 45% of the whole protein weight which is conserved through evolution [10]. The function of this TRS-specific extension is still unknown. Furthermore, in *E. coli*, the expression of TRS is negatively autoregulated in vivo at the translational level [11]. It has been shown that the 5′ end of TRS mRNA adopts a tRNA-like structure that is recognized by the enzyme [12,13]. The molecular structure of TRS should give new insights into the function of the N-terminal domain and the interactions between the enzyme and its mRNA.

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The choice of *T. thermophilus* is the consequence of unsuccessful crystallization attempts with *E. coli* TRS: thermostable proteins appear to crystallize more easily than their mesophilic homologs. Furthermore, *T. thermophilus* is a thermophilic eubacterium close to *E. coli*. In particular, it has been shown that the extent of identity between homologous proteins and nucleic acids of the two species are generally high and that cross-reactions between aaRS and tRNA occur [14,15]. An *E. coli* strain overproducing the thermostable TRS has been constructed. This paper describes the crystallization of ttTRS isolated from the *E. coli* strain and the preliminary crystallographic data. Crystals of ttTRS have been reported previously [1], but it was demonstrated later [2] that the authors were dealing with aspartyl-tRNA synthetase.

2. Materials and methods

2.1. Materials and chemicals

Fractogel EMD trimethylaminoethyl (TMAE) 650M and Fractogel TSK butyl 650S were from Merck and Protein-Pack 300SW from Waters. Ultrafiltration YM10 Diaflo membranes were from Amicon. L-[^{14}C]threonine 8.6 GBq·mmol^{-1} was purchased from NEN. Total *E. coli* tRNA, restriction enzymes, T4 DNA polymerase and T4 DNA ligase were purchased from Boehringer. *E. coli* strain BL21(DE3)pLysE overproducing ttTRS was grown in LB medium at 37°C.

2.2. Protein purification

80 g of cells of the overexpressing strain BL21(DE3)pLysE were resuspended in 300 ml of a buffer containing 100 mM Tris-Cl, pH 7.6, 5 mM $MgCl_2$, 30 mM KCl, 4 mM 2-mercaptoethanol, 0.1 mM phenylmethylsulfonyl fluoride (PMSF) and were disrupted by freezing at −20°C followed by thawing. Cellular debris was pelleted by centrifugation at $3500 \times g$ for 15 min. Most of the thermolabile proteins from *E. coli* were flocculated by heating for 30 min at 70°C and removed by centrifugation at $3500 \times g$ for 20 min. The extract was then applied to a Fractogel EMD TMAE 650M column (20 cm^2 × 20 cm) equilibrated with 20 mM Tris-Cl buffer, pH 7.6, 5 mM $MgCl_2$, 0.5 mM dithiothreitol (DTE), 0.1 mM PMSF. The column was eluted with a linear gradient of 2.3 l from 0 to 200 mM NaCl (flow rate 2 ml·min^{-1}); TRS eluted at 130 mM NaCl. The active fractions were concentrated by ultrafiltration under nitrogen pressure in an Amicon concentration cell and precipitated in 2 M ammonium sulfate. The precipitate was dissolved in a small volume of 100 mM potassium phosphate buffer, pH 7.3, 4% glycerol, 0.1 mM DTE and loaded on a Fractogel TSK butyl 650S column (5 cm^2 × 25 cm) thermostated at 37°C and equilibrated with the same buffer containing additionally 1 M ammonium sulfate. The column was eluted with a reverse linear gradient of 1.5 l from 1 to 0 M ammonium sulfate. The fractions containing TRS (purity over 95% as estimated by polyacrylamide gel electrophoresis) were collected and concentrated up to 6 mg·ml^{-1} as described above.

2.3. Activity measurements

The enzyme was characterized by its tRNA aminoacylation capacity. The reaction mixture of a total volume of 50 μl contained 100 mM HEPES-Na, pH 7.5, 0.046 mM L-[^{14}C]threonine (130 cpm/pmol), 20 mM ATP, 30 mM KCl, 20 mM $MgCl_2$, 6 mg·ml^{-1} unfractionated tRNA from *E. coli* and an adequate amount of enzyme for initial rate

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determination. If necessary the enzyme was diluted in 20 mM Tris-Cl buffer, 30 mM KCl, 20 mM $MgCl_2$. The reaction was carried out at 37°C and the amount of [^{14}C]threonyl-tRNA formed at various incubation times was determined as described [15].

2.4. Analysis of purity of the protein

The purity of the protein was checked by 10% polyacrylamide gel electrophoresis (PAGE) in presence of sodium dodecylsulfate under the conditions described by Laemmli [16]. Gels were stained with Coomassie blue.

2.5. Crystallization

The sodium phosphate buffer in the enzyme preparation was exchanged with 20 mM Tris-Cl buffer, pH 7.5, containing 40 mM ammonium sulfate by successive microcentrifugations on an Amicon Centricon 30 microconcentrator. Crystallization trials were carried out in Linbro culture dishes (Flow Laboratories) using the vapor diffusion method [17]. Hanging drops were suspended from siliconized glass coverslips and sitting drops were placed upon siliconized glass microcupules (Perpetual Systems, Rockville, MD). The wells containing 500 μl of reservoir solution were sealed with paraffin oil and glass coverslips.

2.6. X-ray data collection

Data for the determination of crystallographic parameters were collected on a MarResearch image plate using a Rigaku rotating-anode X-ray source operating at 40 kV, 100 mA. Data for further analysis were recorded at 4°C on MarResearch 300-mm diameter image plates using two synchrotron radiation sources: the wiggler station W32 (λ = 0.91Å) of the Laboratoire pour l'Utilisation du Rayonnement Electromagnétique (LURE), Orsay, France [18], and the ID2 (High Brilliance) Beam Line 4 of the European Synchrotron Radiation Facility (ESRF) (λ = 0.95Å), Grenoble, France. Data were processed with the MARXDS (MarResearch, Hamburg, Germany [19]) data reduction software.

3. Results and discussion

The *thrS* gene-encoding TRS from *T. thermophilus* has been cloned and sequenced (Cura et al., in prep.). The gene was inserted into the overexpression vector pET3–1 [20]. Several clones obtained by transformation of the strain BL21(DE3)pLysE [20] were grown in Terrific Broth [21] at 37°C. The specific threonylation activity of unfractionated tRNA of the crude extract that has been heat treated for 10 min at 70°C in order to inactivate *E. coli* TRS was ~80 × higher than that of a crude extract from *T. thermophilus*. Overexpression of ttTRS was confirmed by analysis of the extract by SDS/PAGE which showed an accumulation of the protein.

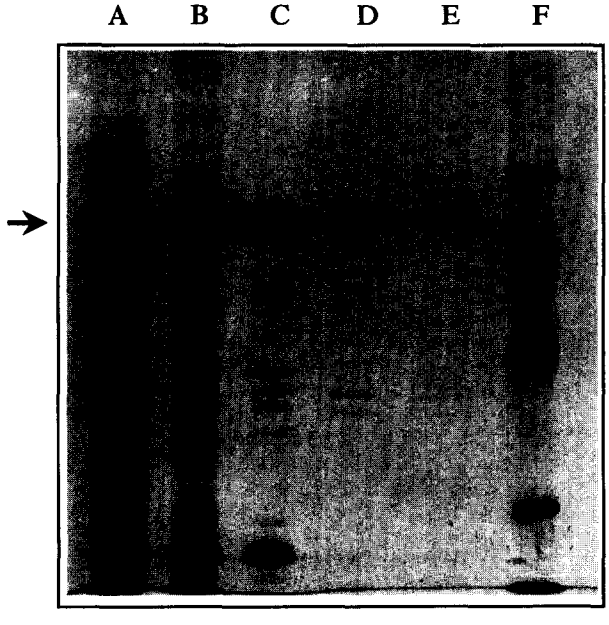

Fig. 1. Purification of ttTRS from overexpressing *E. coli* strain. Analysis was performed as described in section 2. Lane A: crude extract; lane B: extract after heat treatment; lane C: TMAE fraction: lane D: TSK butyl fraction; lane E: ttTRS from a washed and dissolved crystal: and lane F: molecular weight markers (94, 67, 43 and 30 kDa). The position of the ttTRS is indicated by an arrow.

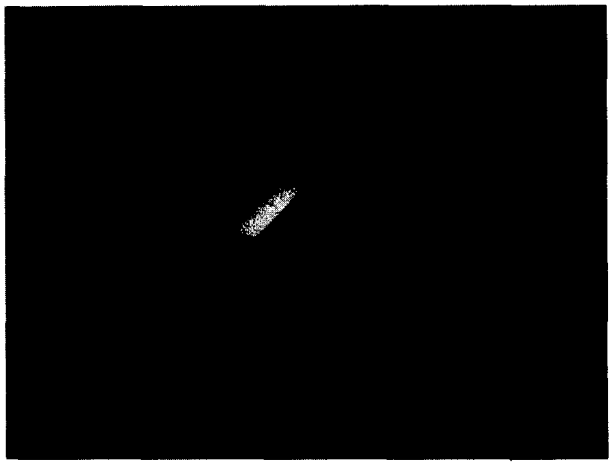

Fig. 2. Typical wedge-shaped ttTRS crystal suitable for X-ray analysis. The dimensions are 0.45 mm × 0.35 mm × 0.20 mm.

Taking advantage of the thermostability of the enzyme, the main purification step was the thermal denaturation and flocculation of most of the proteins from *E. coli*. The anion exchange chromatography removed all contaminating nucleic acids; finally, the hydrophobic interaction chromatography removed the last contaminating proteins, especially the thermostable T7 lysozyme coming from the expression system. We found that the presence of 4% glycerol (v/v) in the elution buffer prevented protein aggregates and, therefore, improved the purification. The specific activity of the enzyme fractions obtained after the different purification steps could not be quantified with enough accuracy to establish a table of purification, since *E. coli* $tRNA^{Thr}$ in the unfractionated tRNA we used in the test is poorly threonylated by ttTRS [15]. For this reason, the purification was followed by SDS/PAGE (Fig. 1). 200 mg of pure TRS, suitable for crystallization, were obtained from ~80 g of wet cells.

The molecular weight of the polypeptide chain of the overproduced TRS (75000) checked on SDS/PAGE is similar to that of the TRS purified from *T. thermophilus* [15]. By gel filtration on a Protein-Pack 300SW column under native conditions, TRS eluted as a globular protein of apparent molecular weight of 170 ± 10 kDa (data not shown). This result is in agreement with the dimeric structure of ttTRS and confirms the conservation of the oligomeric structure of this aaRS through evolution.

The first crystals of ttTRS were obtained with ammonium sulfate as the precipitant (concentration range: 1.3–1.9 M) in the presence of glycerol (concentration range: 0–18%) with protein concentration varying between 3 and 44 mg·ml^{-1}. The main effect of glycerol is to solubilize the protein and to avoid aggregates at high protein concentrations. The concentration of ammonium sulfate is increased in proportion with higher

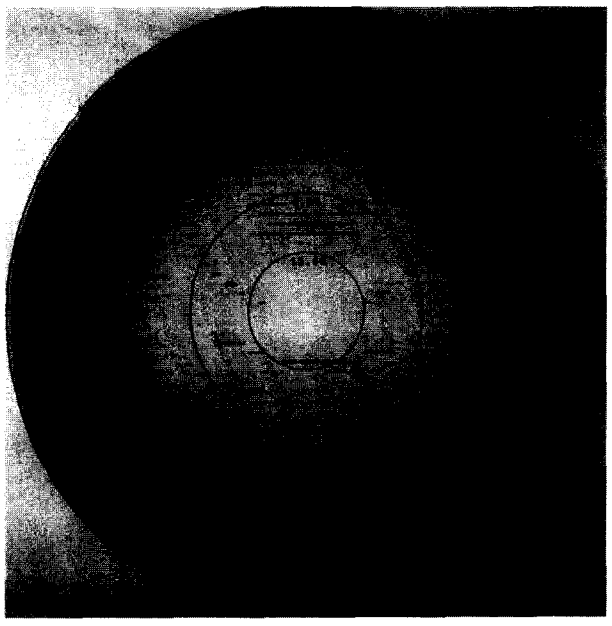

Fig. 3. A 1° oscillation photograph recorded on a MarResearch imaging plate (diameter 300 mm) at ESRF at 4°C. The crystal to imaging plate distance was 420 mm. Circles correspond to a resolution of 13.86, 6.94, 4.63, 3.48 and 2.79 Å.

glycerol concentration to keep the same nucleation effect. No crystals could be obtained when glycerol concentration exceeded 18%, even at high concentrations of ammonium sulfate.

Temperature has a dramatic effect on ttTRS solubility. The range between 4 and 37°C was explored. Since the protein solubility decreases with increasing temperature, we carried out the crystallization trials at 37°C. In these conditions, crystals grew faster and the number of crystals per drop decreased.

Crystals suitable for diffraction analysis were obtained in both hanging and sitting drops formed by mixing 5–15 μl of a protein solution (25–30 $mg \cdot ml^{-1}$) and an equal volume of a reservoir solution containing 1.4 M ammonium sulfate, 5% glycerol and 100 mM Tris-Cl, pH 7.5. Drops were equilibrated at 37°C against the reservoir solution. Crystals attained their maximum size within 1 or 2 weeks and then a secondary nucleation of hundreds of tiny crystals appeared. To prevent this phenomenon, the trials were transferred from 37 to 20°C once the crystals were formed. The crystals are wedge-shaped, most frequently having dimensions ~0.4 mm × 0.3 mm × 0.2 mm, the maximum observed being 1.0 mm × 0.7 mm × 0.3 mm (Fig. 2). Polypeptide chain integrity and activity of TRS in the crystals were checked by SDS/PAGE and aminoacylation tests to show that (1) crystals are composed of ttTRS (2) the properties of the enzyme are unaffected by the crystallization conditions. The ttTRS was also crystallized in the presence of the substrates for the formation of threonyl-adenylate (5 mM ATP, 5 mM threonine and 5 mM $MgCl_2$) in the conditions described for the free enzyme.

Crystals diffract to 2.7 Å at 4°C and belong to the orthorhombic space group $C222_1$ with unit cell parameters a = 119.5 Å, b = 120.0 Å, c = 317.5 Å (Fig. 3). The specific volume (V_M) is 3.70 $Å^3\ Da^{-1}$, assuming that two monomers are located in the asymmetric unit [22]. These values are in the range of those observed for other aaRS [2,23]. Identical parameters were observed for the crystals obtained in the presence of the substrates. The crystals are fragile and sensitive to manipulation or soaking. Considering the large c parameter, diffraction data were collected with a crystal to detector distance of 420 or 450 mm from crystals with low mosaicity to optimize spot separation. Initial native data sets indicated the existence of another crystal form for a minority of the crystals. They belong to the same space group, but the c parameter had shrunk to 313 Å, suggesting a fragility of the protein packing along this axis. However, these crystals do not diffract to a higher resolution than those from the major form. The two different crystals forms can grow in the same drop and are visually indistinguishable. The structure determination is in progress.

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