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Crystallization and preliminary crystallographic analysis of *Thermus thermophilus* leucyl-tRNA synthetase and its complexes with leucine and a non-hydrolysable leucyl-adenylate analogue

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Leucyl-tRNA synthetase from *Thermus thermophilus* (LeuRSTT) is the first LeuRS to be crystallized. Two crystal forms of the native enzyme have been obtained using the hanging-drop vapour-diffusion method with ammonium sulfate as a precipitant. Crystals of the first form belong to space group *I*422 and have unit-cell parameters $a = b = 312.4$, $c = 100.4$ Å. They diffract anisotropically to 3.5 Å resolution in the *c*-axis direction and to only 6 Å resolution in the perpendicular direction. Crystals of the second form, which can be obtained native or with leucine or a leucyl-adenylate analogue bound, belong to space group $C222_1$ and have unit-cell parameters $a = 102.4$, $b = 154.1$, $c = 174.3$ Å. They diffract to 1.9 Å resolution and contain one monomer in the asymmetric unit. Selenomethionated LeuRSTT has been produced and crystals of the second form suitable for MAD analysis have been grown.

1. Introduction

Aminoacyl-tRNA synthetases (aaRSs) are a family of enzymes which catalyze the specific attachment of an amino acid to the 3′-end of its cognate tRNA. The high specificity of these enzymes for both the amino acid and tRNA are critical to the fidelity of protein synthesis. A full understanding of the exquisite selectivity of aaRSs requires knowledge of the three-dimensional structure of the enzymes and their complexes with different substrates (Cusack, 1995; Arnez & Moras, 1997). Leucyl-tRNA synthetase (LeuRS) is a large (100 kDa) monomeric class Ia synthetase closely related to isoleucyl-tRNA synthetase (IleRS) and valyl-tRNA synthetase (ValRS). Sequence and structural homologies suggest that these three enzymes evolved from a common ancestral enzyme, an early divergence from the other class I aminoacyl-tRNA synthetases (Brown & Doolittle, 1995). One of the common functional features of IleRS, ValRS and LeuRS is the presence of a distinct editing activity by which misactivated or mischarged non-cognate amino acids, chemically similar to the cognate amino acid, are hydrolysed (Jakubowski & Goldman, 1992). This editing activity is essential for the fidelity of protein synthesis. Biochemical and crystallographic results suggest that the hydrolytic activity of IleRS is associated with an independently folded domain inserted into the Rossmann-fold catalytic domain (Schmidt & Schimmel, 1995; Lin *et al.*, 1996; Nureki *et al.*, 1998). In IleRS, the synthetic and putative editing active sites are separated by about 25 Å (Nureki *et al.*, 1998; Silvian *et al.*, 1999) and the mechanism by which the misactivated or mischarged amino acid translocates between the two active sites remains obscure. On the other hand, LeuRS is distinct from ValRS and IleRS in recognizing cognate tRNAs with a long variable arm, which it does in an unusual manner (Asahara *et al.*, 1993). It was shown that the tertiary structure unique to $tRNA^{Leu}$ in addition to a base-specific interaction with the discriminator base A73 is crucial for the recognition by LeuRS. Neither the anticodon nucleotides nor the variable arm of $tRNA^{Leu}$ (with the exception of an archaeal system; Soma *et al.*, 1999) are used as recognition determinants of $tRNA^{Leu}$ (Asahara *et al.*, 1993). In addition to its aminoacylation activity, the yeast mitochondrial LeuRS (mtLeuRS) is involved in mRNA splicing of the genes which encode mitochondrial cytochrome *b* and a subunit of cytochrome oxidase (Herbert *et al.*, 1988). Recently, the ability of human mitochondrial and *Mycobacterium tuberculosis* LeuRS to complement a yeast null strain lacking the endogenous mtLeuRS was shown (Martinis *et al.*, 1999).

Leucyl-tRNA synthetase is one of the very few synthetases whose crystal structure is not yet known. In an attempt to understand the structural basis for specific recognition of $tRNA^{Leu}$ and leucine by LeuRS and to provide insight into the mechanisms of aminoacylation, editing and splicing, we have undertaken structural studies of LeuRS from the hypothermophilic bacteria *T. thermophilus*. Here,

we report the crystallization and preliminary crystallographic studies of this enzyme and its complexes with leucine and a non-hydrolysable sulfamoyl analogue of leucyl-adenylate.

2. Methods and results

2.1. Purification of the native and selenomethionine-enriched *T. thermophilus* leucyl-tRNA synthetase

LeuRSTT was cloned, sequenced and overexpressed in *Escherichia coli* strain BL21(DE3)pLysS as described by Tukalo *et al.* (2000). The cells carrying the recombinant plasmid were grown at 310 K in 4 l Luria–Bertani medium containing 50 μg ml^{-1} kanamycin and 34 μg ml^{-1} chloramphenicol until the OD_{600} reached 0.6–0.8. Overexpression was induced by addition of 1 m*M* IPTG; after 4 h of induction at 310 K, the cells were harvested by centrifugation (15 min, 6000*g* at 277 K). The cell pellet was resuspended in ice-cold lysis buffer containing 100 m*M* Tris–HCl pH 8.0, 5 m*M* EDTA, 30 m*M* 2-mercaptoethanol, 5 m*M* phenylmethylsulfonyl fluoride (PMSF), Complete (a protease-inhibitor cocktail, one tablet per 25 ml extraction buffer), 4% glycerol and 20 m*M* lysozyme and homogenized by sonication. After 30 min incubation at room temperature with DNAase (25 mg ml^{-1}) and 1 m*M* $MgCl_2$, the crude lysate was clarified by centifugation for 30 min at 10 000*g* and incubated at 343 K for 40 min. Most of the host *E. coli* proteins were denatured and precipitated by heating and were removed by centrifugation for 30 min at 20 000*g*. The supernatant was dialysed against 20 m*M* Tris–HCl buffer pH 7.9 containing 5 m*M* $MgCl_2$, 0.1 m*M* PMSF, 2 m*M* DTT, 1 m*M* NaN_3 (buffer *A*) and absorbed on a DEAE-Sepharose column (2.5 × 55 cm) equilibrated with buffer *A*. The leucyl-tRNA synthetase was eluted with a 2 × 0.8 l linear gradient of 0.03–0.3 *M* sodium chloride in buffer *A*. The fractions containing LeuRS activity were pooled, dialyzed in buffer *A* and chromatographed on a heparin Sepharose CL-6B column (1 × 40 cm). A 1.0 l linear 0–0.25 *M* KCl gradient in buffer *A* was used to elute the LeuRS. All enzyme-purification steps were carried out at 277 K. The final yield was about 20 mg of pure LeuRS from 1 l of cells. The electrophoretic mobility and aminoacylation activity of the purified recombinant enzyme were identical to those of the LeuRS purified directly from *T. thermophilus* cells. Purified LeuRSTT was concentrated to about 20 mg ml^{-1} for crystallization experiments, using a Centriprep-30 concentrator (Amicon).

For purification of selenomethionine-labelled LeuRS, the transformed *E. coli* BL21(DE3)pLysS cells were grown in minimum medium which was supplemented 30 min before induction with selenomethionine and sufficient amounts of other amino acids known to inhibit methionine biosynthesis (Van Duyne *et al.*, 1993). The purification of selenomethionine-enriched LeuRS was identical to that of unlabelled LeuRS and yielded about 8 mg of pure enzyme from 1 l of cells.

2.2. Crystallization and X-ray analysis

Crystallization experiments were performed at different constant temperatures using the hanging-drop vapour-diffusion method. Two crystal forms of LeuRS were obtained using ammonium sulfate as a precipitant. Plentiful extremely thin needle-like crystals of LeuRSTT were obtained from 44% ammonium sulfate solution at 279 K after 2 d and were not of suitable dimensions for diffraction experiments. An attempt to enlarge these crystals by microseeding or by the variation of protein or precipitant concentration was unsuccessful. One of the known approaches to control the crystal nucleation and grow larger crystals is to increase the temperature during crystallization (Blow *et al.*, 1994), since proteins are often more soluble at higher temperature. Initial incubation of the protein solution under conditions which promote nucleation followed by an increase in the incubation temperature can in some cases produce rapid crystal growth from previously formed nuclei and prevent new nucleation. Because many small crystals appeared after 2 d of equilibration against 44% ammonium sulfate solution at 279 K, trials were therefore carried out in which equivalent drops were equilibriated for various times at 279 K (2–24 h) and then transferred to 285 K for different times and then to 295 K. The best results were obtained under the following conditions. 10 μl drops containing 3–4 mg ml^{-1} LeuRS in 40 m*M* Tris–HCl pH 7.5–8.3, 2 m*M* DTT, 5 m*M* $MgCl_2$, 1 m*M* NaN_3 and 8% ammonium sulfate were equilibrated for 10 h at 279 K against 800 μl reservoir solution containing 44% ammonium sulfate in 100 m*M* Tris–HCl pH 7.5–8.3 and the drops were then transferred to 285 K for 2 d. Usually, after 2 d at 285 K only a few crystals with approximate dimensions 20 × 20 × 50 μm were visible in the drops. To prevent new nucleation and provide rapid crystal growth, the drops with crystals were carefully transferred to 295 K. After 2 d of incubation at 295 K, the crystals reached their maximum size of approximately 1300 × 500 × 500 μm. For diffraction measurements, the crystals were stabilized for one week at room temperature against 50% saturated ammonium sulfate and then transferred to cryoprotectant (52% saturated ammonium sulfate, 100 m*M* Tris–HCl pH 7.5–8.3, 2 m*M* DTT, 5 m*M* $MgCl_2$ and 30% glycerol) for 1–2 h. Diffraction data on crystals frozen to 100 K were collected on various beamlines at ESRF, Grenoble. These crystals belong to the space group $I422$ or $I4_122$, with unit-cell parameters of $a = b = 312.4$, $c = 100.4$ Å at 100 K. They are anisotropic, diffracting to 3.5 Å resolution in the *c*-axis direction and only to 6 Å resolution in the perpendicular direction, and thus did not permit determination of the structure of the enzyme.

A higher quality crystal form of the native enzyme and its complexes with leucine and leucyl-adenylate analogue was obtained at room temperature by crystallization from precipitate. 10 μl drops containing 15–20 mg ml^{-1} of native or selenomethionated enzyme in 50 m*M* bis–tris propane buffer pH 6.0–6.5, 2 m*M* DTT, 20 m*M* $MgCl_2$, 1 m*M* NaN_3 and 18–20% ammonium sulfate were equilibrated at room temperature against 38% ammoniun sulfate solution in 100 m*M* bis-tris propane buffer pH 6.0–6.5. A few clusters of extremely thin plate-like crystals grew in each drop after about two weeks from the precipitate, with individual crystals reaching maximum dimensions of about 500 × 300 × 5 μm. Single crystals were grown to the maximum size of 1800 × 900 × 60 μm by microseeding or macroseeding. The seed stock was produced by crushing one previously obtained cluster of crystals in 30% ammonium sulfate solution. Drops with a protein concentration of 10 mg ml^{-1} in 50 m*M* bis-tris propane buffer pH 6.0–6.5, 2 m*M* DTT, 20 m*M* $MgCl_2$, 1 m*M* NaN_3 and 15% saturated ammonium sulfate were equilibrated for 2 d against 30% ammonium sulfate solution at room temperature before the seeds were added. These crystals belong to space group $C222_1$, with unit-cell parameters $a = 102.4$, $b = 154.1$, $c = 174.3$ Å at 100 K, and diffract to about 1.9 Å resolution. The protein used for crystallization as well as that from washed and solubilized crystals was verified to be full-length LeuRSTT by SDS–PAGE. To obtain structural information on substrate specificity and enzyme mechanism, LeuRSTT has also been co-crystallized with leucine and a leucyl-

adenylate analogue 5′-*O*-[*N*-(leucyl)-sulfamoyl]adenosine. This compound was synthesized by a method analogous to that described for the alanyl compound (Ueda *et al.*, 1991), except that 2′,3′-*O*-isopropylidene-5-*O*′-sulfamoyladenosine was reacted with *tert*-butoxycarbonyl-L-leucine-*N*-hydroxysuccinimide ester. The crystals of LeuRSTT complexed with either leucine or the sulfamoyl analogue of leucyl-adenylate were obtained under identical crystallization conditions. They belong to the same space group as found for the ligand-free enzyme ($C222_1$), but with slight differences in unit-cell parameters. Similar crystals grown with selenomethionated LeuRSTT should permit the determination of the structure by the MAD method. There are 23 methionines in the enzyme, which is of molecular weight 101 kDa.

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