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Leucyl-tRNA synthetase from *Thermus thermophilus*. Purification and some properties of the crystallizing enzyme

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Leucyl-tRNA synthetase from Thermus thermophilus (LeuRSTT) was purified to homogeneity using a five-step purification procedure. The enzyme was characterized and crystallized. Molecular mass determinations of the native and denatured proteins indicate monomeric structure of LeuRSTT with the molecular mass of about 101 kDa. The protein obtained is remarkably thermostable and retains 97 % of its initial aminoacylation activity after 1 hour of incubation at 88 °C. Crystals of LeuRSTT were obtained from ammonium sulfate solution by the vapour diffusion techniques. The crystals quality was improved by crystallization from the precipitate.

Introduction. Aminoacyl-tRNA synthetases (aaRSs) catalyze the specific esterification of an amino acid to the 3′-end of its cognate tRNA. Through a two-step reaction, aaRSs use first ATP to form the activated intermediate, aminoacyl-adenylate, and then transfer the amino acid to the 3′-end of their cognate tRNA. The reaction is highly specific, although in some cases, to ensure the fidelity of the genetic code translation, the aaRS relies on an editing activity to hydrolyze misactivated amino acids which are similar to the cognate amino acid [1, 2]. The family of twenty aaRSs are divided into two distinct classes of ten enzymes each on the basis of the primary and tertiary structures information [3, 4]. Also, all tRNA molecules can be classified into two types according to the length of the variable arm. Type I tRNAs (most of tRNA molecules) have a short variable arm comprising four or five nucleotides. Type II tRNAs ($tRNA^{Tyr}$ from eubacteria and organelles of lower eukaryotes, $tRNA^{Leu}$ and $tRNA^{Ser}$) have a long variable arm composed of more than ten nucleotides.

Leucyl-tRNA synthetase (LeuRS) is a monomeric class I enzyme which recognizes type II tRNA. It was shown by mutational studies that *Escherichia coli* LeuRS recognized $tRNA^{Leu}$ in a manner different from the standard recognition mode [5]. Only the discriminator base A73 is required for the base-specific recognition by LeuRS while the anticodon, the variable stem loop and the acceptor stem sequences are not essential for the specific recognition by LeuRS. To define the recognition mode and structural identity elements of $tRNA^{Leu}$ by LeuRS as well as to get detailed information on substrate specificity and enzyme mechanism it is necessary to determine the crystal structure of the enzyme alone and in the complex with substrates. So far crystal structures of sixteen from twenty different synthetases have now been determined together with a variety of substrate complexes [6, 8]. LeuRS is one of four aaRSs which crystal structure is not known yet. Here, we report the purification, some properties and crystallization of LeuRSTT.

Materials and Methods. *Materials.* DEAE Sepharose Fast Flow, Heparin Sepharose CL-6B were from «Farmacia LKB Biotechnology» (Sweden). Hydroxylapatite was from «Bio-Rad» (USA). TSK-Gel HW-65 from «Toyo Soda MFG» (Japan). Polypepton and yeast extract were from «Difco» (USA). Unfractionated tRNAs from *E. coli*, protease inhibitor cock-

tail tablets (completeTM) and phenylmethylsulfonyl fluoride were from «Boehringer Mannheim» (Germany). Protein markers for molecular mass determinations, HEPES and ATP were purchased from «Sigma Chemical Co.» (USA). ^{14}C-labelled leucine was from «Amersham, UK» (Great Britain). Ammonium sulfate, polyethylene glycol, MES and bis-Tris-propane were from «Fluka, Biochem.» (Switzerland).

Purification of T. thermophilus LeuRS and tRNA^{Leu}. The *T. thermophilus* (strain HB8 or HB27) cells were harvested by centrifugation, washed and disrupted by a French Press in 100 mM Tris-HCl buffer, containing 2 mM dithiothreitol, 0.1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, completeTM (1 tabl./25 ml extraction buffer), 5 mM $MgCl_2$ and 1 mM NaN_3. The extract was centrifuged for 2 hours at 105000 *g* and the supernatant was subjected to 30—65 % ammonium sulfate fractionation. The precipitate containing LeuRS activity was dissolved in 20 mM Tris-HCl buffer, pH 7.5 containing 5 mM $MgCl_2$, 0.1 mM phenylmethylsulfonyl fluoride, 2 mM DTT, 1 mM NaN_3 (buffer A), dialyzed against buffer A and absorbed on a DEAE-Sepharose column (5 × 55 cm) equilibrated with buffer A. The LeuRS was eluted with 2 × 2.5 L linear gradient of 0.03 M to 0.3 M sodium chloride in buffer A. The fractions containing LeuRS activity were salted out by ammonium sulfate (50 % saturation) and applied to a Toyopearl HW-65 column (3 × × 80 cm) equilibrated with 40 % of ammonium sulfate in buffer A. The proteins were eluted with 2 × 2 L gradient of 40—10 % saturation ammonium sulfate in buffer A. Fractions containing LeuRS activity were pooled, dialyzed and chromatographed on a hydroxylapatite column (3 × 40 cm) with 2 × 2 L potassium phosphate buffer linear gradient from 0.01 M to 0.25 M (pH 7.9). After dialysis in buffer A of the active fractions the enzyme was loaded 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 steps of the enzyme purification were carried out at 4 °C.

T. thermophilus tRNA^{Leu} was purified from bulk tRNA using BD-cellulose, anion-exchange DEAE 5PW (HPLC, «Beckman», USA) and C8 reverse-phase chromatography (I. Krikliviy, D. Rozhko and M. Tukalo, unpublished results).

Enzyme activity. The aminoacylation reaction mixture contained 50 mM Na-Hepes, pH 7.3, 15 mM $MgCl_2$, 30 mM KCl, 0.5 mg/ml bovine serum albumin (BSA), 7 mM ATP, 0.1 mM DTT, 0.096 mM L-[^{14}C]leucine («Amersham») diluted with non-labelled amino acid to 68420 cpm/nmol, 4 mg/ml unfractionated *E. coli* tRNA and 0.001—1 mg/ml of protein depending upon the purity of the enzyme. The reaction was conducted at 55 °C for 1 min and the samples were placed on GF/A filters. The filters were washed three times in 5 % trichloroacetic acid, ethanol, 50:50 (vol/vol.) ethanol/ether and the radioactivity was measured by liquid scintillation counting.

The K_m values for ATP, leucine and tRNA^{Leu} were determined at 65 °C in the standard mixture containing 10 μg/ml of LeuRSTT and varying concentration of ATP (10—300 μM), leucine (0.5—50 μM) or tRNA (0.05—2.5 μM) respectively (using ENZFITTER programme).

The molecular mass determination. The molecular mass of the enzyme was determined by gel filtration on a column (1.1 × 72 cm) of Sephadex G-200 and by PAGE under native and denaturing conditions. Under native conditions it was conducted in 5 % and 6 % polyacrylamide gels. Under denaturing conditions it was conducted in 12.5 % polyacrylamide gel in the presence of 0.1 % SDS as described by Laemmli [9].

Measurements of thermal stability of LeuRS. The heat stability of the aminoacylation activity of LeuRSTT was analyzed by incubating 100 μl of the enzyme solution (1.5 mg/ml) in 100 mM Na-Hepes, pH 7.3 containing 15 mM $MgCl_2$ with or without the small substrates at the indicated concentration. At various times, 10-μl aliquots were removed, diluted in cold enzyme dilution buffer containing 100 mM Na-Hepes, pH 7.3, 2 mM DTT, 10 % glycerol, 15 mM $MgCl_2$ and 5 mg/ml BSA. The remaining aminoacylation activity was determined by initial rate measurements as described above.

Crystallization procedure. Crystallization trials were conducted at different constant temperatures (6, 12, and 20 °C) using the hanging-drop vapor-diffusion method. The drops of 4—10 μl were allowed to equilibrate against 0.8 ml reservoir solution. Conditions such as the nature of the precipitant, the composition and pH of the buffer and the concentration of the protein were varied.

Results and Discussion. LeuRSTT was purified from both HB8 and HB27 strains to homogeneity using a five-step purification procedure (Table 1). The final yield was approximately 20 mg of the pure enzyme from 520 g cells with a specific activity of 2212 U/mg (1 U of the enzyme catalyzes the formation of 1 nmol leucyl-tRNA/min at 65 °C). Gel filtration gives M_r of 100 kDa. PAGE under native conditions reveals an apparent M_r of 102 kDa. SDS/PAGE shows a single band corresponding to a polypeptide chain of about 101 kDa. The kinetic constants of the aminoacylation reaction catalyzed by

Table 1
Purification of leucyl-tRNA synthetase from T. thermophilus HB8

Purification step	Total protein, mg	Specific activity, U/mg	Total activity, U	Purification, fold	Yield, %
Crude extract*	35230	0.72	25319	1	100
Ammonium sulfate fractionation	12364	1.93	23826	2.7	94
DEAE-Sepharose	1284	18.54	23800	25.7	94
Toyopearl HW-65	354	64.58	22859	89.7	90
Hydroxylapatite	42	534.71	22458	742.7	89
Heparin Sepharose	19.8	1117	22123	1551	87

*Obtained from 520 g cells.

Table 2
Kinetic constants of aminoacylation reaction catalyzed by LeuRS from T. thermophilus at 65 °C

K_m, M			k_{cat}, min^{-1}
ATP	Leucine	$tRNA^{Leu}$	
$5.5 \cdot 10^{-5}$	$9.1 \cdot 10^{-6}$	$3.7 \cdot 10^{-7}$	220

LeuRSTT for ATP, leucine and $tRNA^{Leu}$ are reported in Table 2. There is no significant difference in K_m and k_{cat} values of LeuRSTT for all three substrates compared with those of LeuRS from *E. coli* [10]. The optimal temperature for the tRNA aminoacylation reaction catalyzed by LeuRSTT is about 70 °C for $tRNA^{Leu}$ from *T. thermophilus* and about 65 °C for $tRNA^{Leu}$ from *E. coli* (Fig. 1). The thermal stability of the enzyme was investigated in the range from 50 to 90 °C. The enzyme is remarkably thermostable. It retains 100 % of its ability to aminoacylate tRNA after 1 h of incubation at 85 °C (data not shown) and about 97 % at 88 °C. ATP and leucine alone do not affect the thermal inactivation of the enzyme at 88 °C. Surprisingly, when ATP and leucine or ATP, leucine and inorganic pyrophosphate are present together significant inactivation of LeuRS occurs after 1 h at 88 °C (Fig. 2). In contrast, the protection of GlyRS from thermoinactivation by ATP, glycine or glycyladenylate was shown for *T. thermophilus* GlyRS [11], ATP or tyrosyladenylate for TyrRS, histidine or histidyladenylate for HisRS and prolyladenylate for ProRS from *T. thermophilus* (Yaremchuk et al., unpublished results). In all these cases the protection of the aaRS from thermal inactivation by substrates correlates with conformational changes of the enzyme promoted by substrates binding ([11], Cusack S., Yaremchuk A. and Tukalo M., unpublished results).

To study the mechanism of amino acid recognition and activation and the specific recognition of cognate tRNA by *T. thermophilus* LeuRS we tried to crystallize this enzyme alone and in complex with the substrates. Initial screening of crystallization conditions was conducted using a sparse matrix sampling reagents from Hampton Research (Crystal screen 1 and 2, Grid screens including ammonium sulfate,

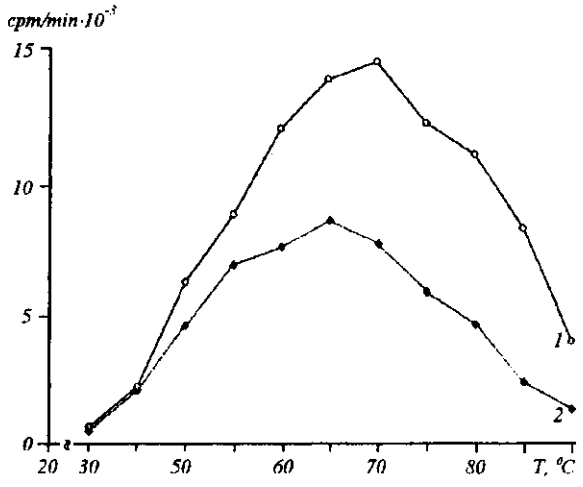

Fig. 1. Dependence of the rate of tRNA aminoacylation catalyzed by LeuRSTT on temperature: *1* — *T. thermophilus* $tRNA^{Leu}$; *2* — *E. coli* $tRNA^{Leu}$

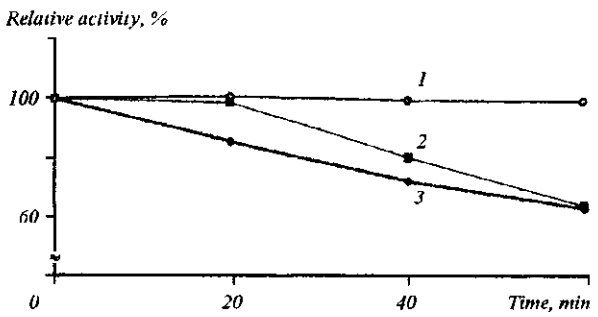

Fig. 2. Thermal stability of LeuRSTT in the absence and in the presence of small ligands. The enzyme (1.5 mg/ml) was incubated at 88 °C in 100 mM Na-Hepes, pH 7.3, and 15 mM $MgCl_2$ either in the absence (*1*) or in the presence (*2*) of 1 mM ATP and 2 mM leucine and in the presence (*3*) of 1 mM ATP, 2 mM leucine and 1 mM PP_i. The remaining activity was measured after 0—60 min incubation at 88 °C

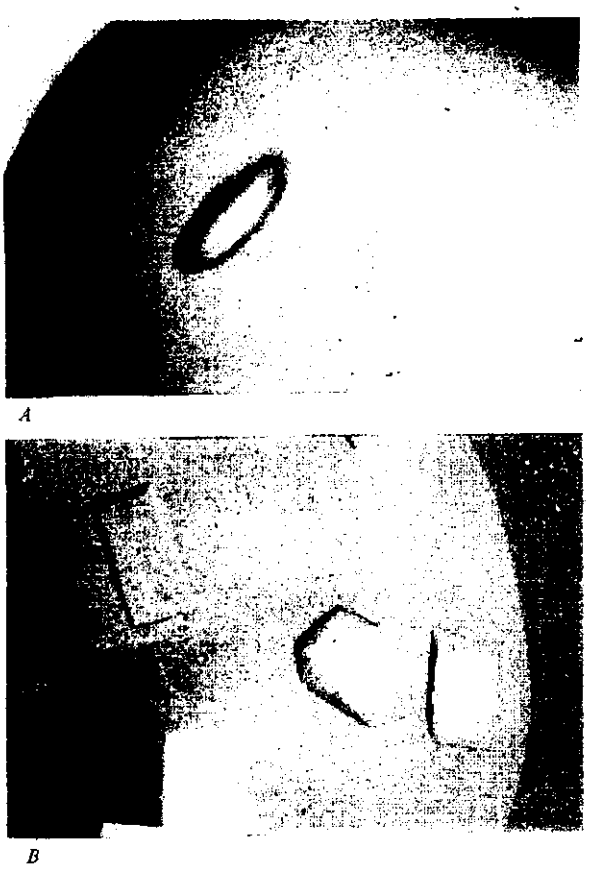

Fig. 3. Crystals of LeuRSTT: *a* — not well-shaped crystals and diffracting to 9 Å resolution; *b* — crystalls obtained by crystallization from precipitate and diffracting to 3.5 Å resolution

polyethylene glycol 6000, sodium chloride, 2-methyl-2,4-pentanediol and Crystal screen cryo formulation) without any success. Precipitants alone, such as ammonium sulfate, PEG, sodium formate, sodium citrate, sodium chloride and MPD at different concentrations over a broad range of pH and protein concentration did not yield crystals suitable for X-ray structure determination. Extremely thin needle-like crystals were obtained from 48 % ammonium sulfate solution at pH 7.5—8.3 at 6 °C and were not of suitable dimensions for diffraction experiments. Not well-shaped crystals (Fig. 3, *a*) and diffracting to 9 Å resolution were obtained at 20 °C from 38—40 % ammonium sulfate solution at pH 7.5 in the presence of 5 % glycerol after two—three weeks and are very poorly reproducible. SDS/PAGE shows degradation of the enzyme after one week at 20 °C and even crystals contain two polypeptide chains with molecular mass of about 100 and 96 kDa (data not shown). Better-quality crystals of LeuRSTT were obtained under the following conditions: to prevent protein degradation 10 μl drops containing 8—10 mg/ml LeuRS in 40 mM Tris-HCl, pH 7.5, 2 mM DTT, 10 mM $MgCl_2$, 1 mM NaN_3, and 15 % ammonium sulfate were equilibrated for 12—14 hours at 6 °C against 800 ml reservoir solution containing 54—58 % ammonium sulfate in 100 mM Tris-HCl, pH 7.5. After rapid precipitation of the macromolecules the drops were transferred to 12 °C and ammonium sulfate concentration was decreased to 40—42 %. Under these conditions well-shaped crystals appeared in two days and grew from precipitate rapidly over a 4—5 days period to a maximum of 0,8 mm in the longest dimension (Fig. 3, *b*). After stabilization for one week against 48 % saturated ammonium sulfate in 100 mM Tris-HCl, pH 7.5 crystals were mounted in quartz capillaries with a small amount of reservoir solution for X-ray diffraction measurements. These crystals diffract to 3.5 Å resolution. The protein from washed and solubilized crystals as well as the protein used for crystallization was verified as full-length LeuRSTT by SDS/PAGE and enzymatic ability to aminoacylate tRNA. X-ray diffraction analysis of these crystals is under way. Small crystals of LeuRSTT in complex

with $tRNA^{Leu}$ were obtained using ammonium sulfate as a precipitant but they were not of suitable dimensions for X-ray analysis. The gene for LeuRSTT has been recently cloned, sequenced end expressed in *E. coli* (Tukalo et al., unpublished results) and the work is under way to obtain high diffracting crystals of recombinant LeuRSTT and its complexes with substrates.

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Лейцил-тРНК синтетаза із *Thermus thermophilus*. Очищення і деякі властивості кристалів ферменту

Резюме

Лейцил-тРНК синтетазу із Thermus thermophilus (ЛейРСТТ) виділено в гомогенному стані з використанням п'яти стадій очищення. Фермент охарактеризовано та отримано його кристали. Визначено молекулярну масу нативного і денатурованого білка. Встановлено, що ЛейРСТТ являє собою мономер з молекулярною масою 101 кДа. Отриманому ферментові притаманна значна термостабільність і він зберігає 97 % аміноацилюючої активності після інкубації протягом 1 год при температурі 88 °С. Кристали ЛейРСТТ одержано методом дифузії парів із використанням як осаджувача розчину сульфату амонію.

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Лейцил-тРНК синтетаза из *Thermus thermophilus*. Очистка и некоторые свойства кристаллов фермента

Резюме

Лейцил-тРНК синтетаза из Thermus thermophilus (ЛейРСТТ) выделена в гомогенном состоянии с использованием пяти стадий очистки. Фермент охарактеризован и получены его кристаллы. Определена молекулярная масса нативного и денатурированного белка. Установлено, что ЛейРСТТ представляет собой мономер с молекулярной массой 101 кДа. Полученный фермент обладает значительной термостабильностью и сохраняет 97 % аминоацилирующей активности после инкубации в течение 1 ч при температуре 88 °С. Кристаллы ЛейРСТТ получены методом диффузии паров с использованием в качестве осадителя раствора сульфата аммония.

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