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Phenylalanyl-tRNA synthetase from *Thermus thermophilus* HB8

Purification and properties of the crystallizing enzyme

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Phenylalanyl-tRNA synthetase from *Thermus thermophilus* HB8 was isolated, characterized and crystallized. The enzyme is a tetramer of $\alpha_2\beta_2$-type structure, its molecular mass being 264 kDa. Molecular masses of the enzyme subunits are 40 (α) and 92 (β) kDa. The optimal temperature conditions of the $tRNA^{Phe}$ aminoacylation, catalyzed by this enzyme, are close to 80°C. K_M values for $tRNA^{Phe}$ from *E.coli*, for $tRNA^{Phe}$ from *T. thermophilus* HB8, for phenylalanine and ATP, as well as their temperature dependencies were determined. The enzyme crystals were grown by the hanging drop technique at 4°C in the presence of ammonium sulfate.

Phenylalanyl-tRNA synthetase; $tRNA^{Phe}$; Thermodynamic parameter; Crystallization; (*Thermus thermophilus*)

1. INTRODUCTION

Phenylalanyl-tRNA synthetases (EC 6.1.1.20) of various origins belong to the group of aminoacyl-tRNA synthetases of a rare (for these enzymes) subunit structure of the $\alpha_2\beta_2$ type [1]. It is quite possible that the different oligomeric structure can be associated with peculiarities in the realization of the main function, tRNA aminoacylation, as well as with some additional functions of the enzyme. The study of the three-dimensional structure of aminoacyl-tRNA synthetases is an important step in the understanding of the reaction mechanism and the character of the enzyme–substrate interactions. Until recently crystals of aminoacyl-tRNA synthetases of the $\alpha_2\beta_2$ type were not available. We were the first to isolate and crystallize phenylalanyl-tRNA synthetase from the extreme thermophile *Thermus thermophilus* HB8 [2]. Of particular interest is the structural-functional analysis of the interaction of phenylalanyl-tRNA synthetase with $tRNA^{Phe}$ and other substrates, which can have some peculiarities in the case of the enzyme from extreme thermophile.

The present paper is devoted to the purification and characterization of the crystallizing phenylalanyl-tRNA synthetase from *T. thermophilus* HB8. Thermodynamic characteristics of the enzyme interaction with substrates of tRNA aminoacylation were investigated.

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Abbreviation: BSA, bovine serum albumin

2. MATERIALS AND METHODS

T. thermophilus HB8 was provided by 'Biolar' (Olaine, Latv. SSR). The total tRNA from *E. coli* and *T. thermophilus* HB8 was isolated as described in [3]. Protein concentrations were determined by ultraviolet absorption at 228.5 and 234 nm [4], with BSA used as a standard. Phenylalanyl-tRNA synthetase activity was determined in the reaction of tRNA aminoacylation, carried out in the following mixture: 0.1 M Tris-HCl, pH 8.0, 10^{-2} M $MgSO_4$, 5×10^{-3} M ATP, 10^{-5} M [^{14}C]phenylalanine, 4 mg/ml crude tRNA from *E. coli*, 0.2 mg/ml BSA, 10^{-2} M KCl and 800–0.5 μg/ml protein, depending on the purification step. The quantity of the obtained [^{14}C]phenylalanyl-tRNA was estimated from the value of radioactivity absorbed on paper filters FN-16 impregnated with 5% trichloroacetic acid. One unit of enzyme activity is defined

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by the quantity of enzyme, which catalyzes aminoacylation of 1 nmol $tRNA^{Phe}$ for 1 min at 55°C. K_M values were estimated by the graphic method of Eisenthall and Cornish-Bowden [5] within the following concentration ranges: ATP 3×10^{-5} to 1.4×10^{-4} M, phenylalanine 2.0×10^{-7} to 2.5×10^{-6} M, $tRNA^{Phe}$ from *E. coli* 0.96×10^{-7} to 9.6×10^{-7} M, $tRNA^{Phe}$ from *T. thermophilus* 0.6×10^{-7} M. The molecular mass of the enzyme was determined by gel filtration on a column (0.9 × 50 cm) of Sephacryl S-300. Molecular masses of the enzyme subunits were determined by electrophoresis in 5% polyacrylamide gel in the presence of SDS [6]. Protein crystallization was carried out by the hanging drop technique [7]. A solution of 30% saturated ammonium sulfate in 20 mM imidazole-HCl buffer (pH 7.8) was used as precipitant.

2.1. *Purification of phenylalanyl-tRNA synthetase from* T. thermophilus *HB8*

The initial extract was obtained by incubating the cells at 4°C for 1 h with lysozyme (1 g/kg cells) and DNase (2 mg/kg cells) in 0.02 M Tris–HCl, pH 8.5, containing 5×10^{-3} M $MgCl_2$. Nucleic acids were precipitated by 1% streptomycin sulfate. Phenylalanyl-tRNA synthetase was salted out by ammonium sulfate in the 30–40% saturation range. Then it was dialyzed and chromatographed on a DEAE-cellulose column (5.0 × 100 cm) with 8 l potassium phosphate buffer gradient from 0.05 to 0.25 M, with pH changing from 8.0 to 7.0. The fraction containing phenylalanyl-tRNA synthetase activity, was salted out by ammonium sulfate (45% saturation) and chromatographed on a column (2.5 × 70 cm) with polyvinyl sorbent Toyopearl HW-65. The enzyme was eluted with 2 l gradient of 30–10% saturation of ammonium sulfate in 0.02 M Tris–HCl, pH 8.0, containing 5×10^{-3} M $MgCl_2$ (buffer A). The active fraction was salted out, dialyzed and applied to a DEAE-Sephadex A-50 column (2.5 × 40 cm). 2 l linear 0–0.4 M KCl gradient in buffer A was used to elute the enzyme activity. The active fraction was salted out, dialyzed and chromatographed on a Heparin-Sepharose column (1 × 15) using 0.5 l of a 0–0.3 M KCl gradient in buffer A. The obtained preparation of phenylalanyl-tRNA synthetase was stored in buffer A with 50% glycerine at −20°C. All the steps of enzyme purification were carried out at 4°C, all buffers containing 1 mM NaN_3.

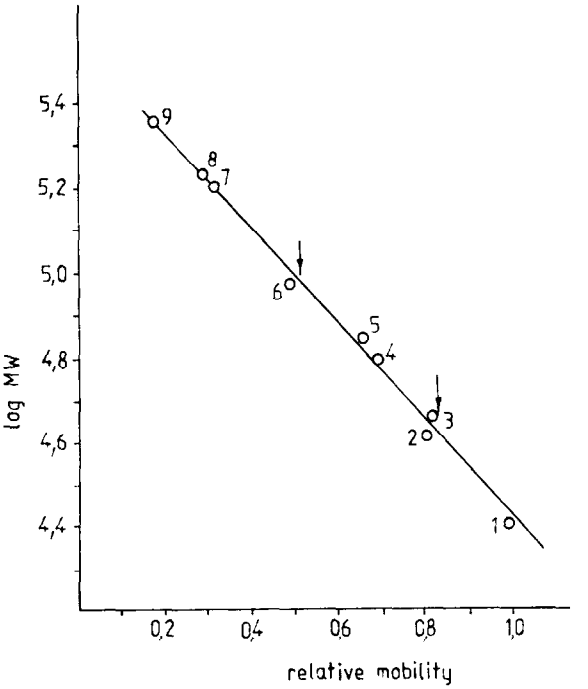

Fig.1. Determination of the molecular-mass of denatured phenylalanyl-tRNA synthetase from *T. thermophilus* with polyacrylamide gel electrophoresis in the presence of SDS. (1) Chymotrypsinogen (25 kDa); (2) α-subunit of RNA polymerase (40 kDa); (3) ovalbumin (45 kDa); (4) catalase (60 kDa); (5) BSA (67 kDa); (6) β-subunit of RNA polymerase (155 kDa); (7) β′-subunit of RNA polymerase (155 kDa); (8) myosin (220 kDa). Positions of α and β subunits of enzyme are marked (↓).

3. RESULTS AND DISCUSSION

Phenylalanyl-tRNA synthetase from *T. thermophilus* HB8 was purified to apparent

Table 1

Purification of phenylalanyl-tRNA synthetase from *T. thermophilus*

No.	Step	Protein (mg)	Activity (units)	Specific activity (units/mg)	Fold	Yield (%)
1	Crude extract	66450	8705	0.131	1	100
2	Streptomycin sulfate precipitation	53935	8738	0.162	1.24	100.4
3	Ammonium sulfate fractionation	30768	10707	0.348	2.66	123
4	DEAE-cellulose	4476	10719	2.395	18.3	123.1
5	TSK-gel HW-65	433	9333	21.54	164.4	107.2
6	DEAE-Sephadex	106.7	8544	80.1	611.5	98.1
7	Heparin-Sepharose	42.7	8448	198	1511.5	97

homogeneity. The enzyme purification data are summarized in table 1. The yield of the purified enzyme was 42.7 mg from 1 kg of the cells.

The molecular mass of the native enzyme was determined by gel filtration and proved to be equal to 245 kDa. The existence of two polypeptide chains with molecular masses of 40 kDa (α) and of 92 kDa (β) was found by denaturing the polyacrylamide gel electrophoresis technique. The results are given in fig.1. The data give evidence for an oligomeric structure of $\alpha_2\beta_2$-type enzyme, similar to most phenylalanyl-tRNA synthetases of different origins [1].

It was of great interest to evaluate the optimal temperature conditions for the tRNA aminoacylation reaction catalyzed by this enzyme. The data are presented in fig.2. The optimal temperature for aminoacylation of $tRNA^{Phe}$ from *E. coli* is shown to be equal to 70°C, while that of $tRNA^{Phe}$ from *T. thermophilus* is about 80°C. The specific activi-

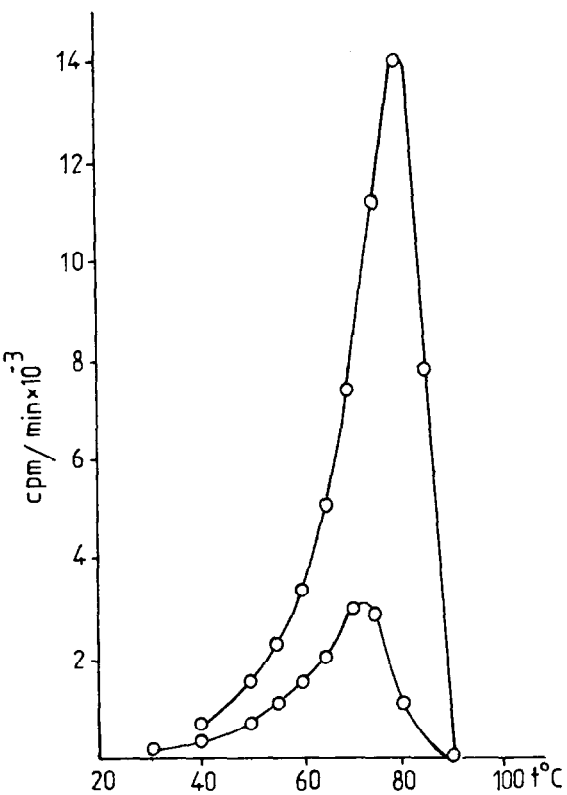

Fig.2. Dependence of tRNA aminoacylation rates catalyzed with phenylalanyl-tRNA synthetase from *T. thermophilus* on temperature. (1) *E. coli* $tRNA^{Phe}$; (2) *T. thermophilus* $tRNA^{Phe}$.

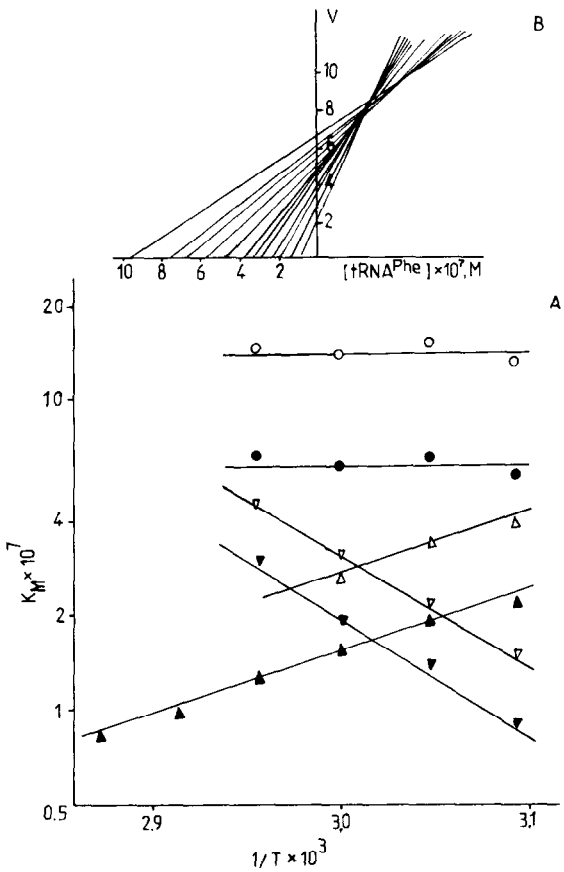

Fig.3. (A) Dependencies of $tRNA^{Phe}$ (*E. coli*) (▼, ▽) and $tRNA^{Phe}$ (*T. thermophilus*) (▲, △). K_M values in reaction of tRNA aminoacylation catalyzed with phenylalanyl-tRNA synthetase from *T. thermophilus* on temperature ($1/T$) (dependencies for two active sites of functional dimer); the same dependencies of K_M values of phenylalanine (●,○). (B) Eisenthall–Cornish-Bowden plots of the rates of *E. coli* tRNA aminoacylation catalyzed with *T. thermophilus* phenylalanyl-tRNA synthetase on tRNA concentration at 65°C.

ty of the enzyme under optimal conditions in the case of tRNA from *T. thermophilus* was equal to 2770 units/mg.

K_M values and their temperature dependencies were estimated for ATP, phenylalanine, $tRNA^{Phe}$ from *E. coli* and *T. thermophilus* in the aminoacylation reaction, catalyzed by thermophilic phenylalanyl-tRNA synthetase. Two K_M values were clearly seen for all the substrates in Eisenthall–Cornish-Bowden coordinates (data for tRNA are presented in fig.3), which are probably due to a functional dimeric structure of thermophilic phenylalanyl-tRNA synthetase. A

negative cooperativity exists in the binding of all substrates.

K_M values for ATP are observed to increase with temperature, indicating a decrease of ATP affinity to the enzyme, while for phenylalanine the affinity does not depend on temperature (see fig.3.).

Of considerable interest is the case of the opposite influence of temperature upon the affinity of specific (thermophilic) and non-thermophilic (from *E. coli*) tRNA to phenylalanyl-tRNA synthetase from *T. thermophilus* which is clearly seen in fig.3. The thermophilic tRNA affinity is seen to grow with temperature, while that for the tRNA from *E. coli* decreases. These data give evidence for principal differences in the character of protein-nucleic acid interactions in the case of thermophilic systems.

Gibbs' energy levels ($\Delta G°$) and the corresponding $\Delta S°$ and $\Delta H°$ values for the interaction of all substrates with thermophilic phenylalanyl-tRNA synthetase were determined. In the case of $tRNA^{Phe}$ from *E. coli*, these values are equal to -43.2 kJ/mol for the binding of one $tRNA^{Phe}$ molecule and to -41.9 kJ/mol for the binding of two $tRNA^{Phe}$ molecules. In the case of thermophilic $tRNA^{Phe}$, these values are equal to -41.3 kJ/mol and -40.7 kJ/mol. However, in the case of *E. coli* $tRNA^{Phe}$, $\Delta H°$ and $\Delta S°$ are negative values: -71.3 kJ/mol ($\Delta H°$), -85.7 J·mol^{-1}·K^{-1} ($\Delta S°$), and -71.3 kJ/mol ($\Delta H°$), -89.7 J·mol^{-1}·K^{-1} ($\Delta S°$) for the binding of one and two $tRNA^{Phe}$ molecules, respectively, while in the case of thermophilic $tRNA^{Phe}$, they are positive (37.5 kJ/mol ($\Delta H°$), 240.3 J·mol^{-1}·K^{-1} ($\Delta S°$), and 37.5 kJ/mol ($\Delta H°$), 238.4 J·mol^{-1}·K^{-1} ($\Delta S°$)). The difference in $\Delta G°$ values for tRNA interactions with two centers of thermophilic phenylalanyl-tRNA synthetase is due to entropy difference. A considerable contribution is made by the positive entropy into the $\Delta G°$ value in the case of the thermophilic system. It may be supposed that the increase in the efficiency of the tRNA-enzyme complex formation with temperature is provided by a considerable contribution of hydrophobic interactions [8]. Further analysis of the type of interactions in this system will be of certain interest.

Crystals of phenylalanyl-tRNA synthetase from

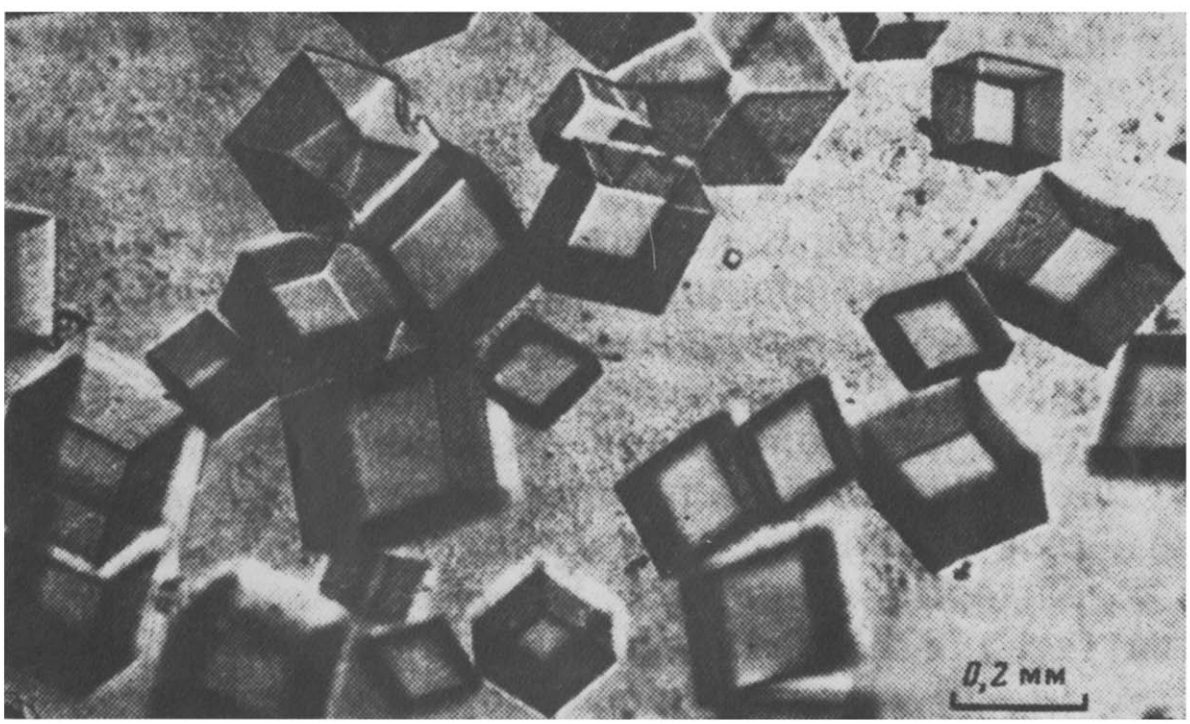

Fig.4. Microphotograph of crystals of phenylalanyl-tRNA synthetase from *T. thermophilus*.

T. thermophilus were grown at 4°C with ammonium sulfate used as precipitant. Each droplet of solution (10 μl) with protein concentration of 3–5 mg/ml in 20 mM imidazole-HCl buffer (pH 7.8), 1 mM $MgCl_2$, 1 mM NaN_3 and 15% saturated ammonium sulfate was equilibrated with 1 ml of 25–30% ammonium sulfate in the same buffer. Crystals appeared after 1–2 weeks and grew up to maximal dimensions in a few weeks. Efforts were made to crystallize phenylalanyl-tRNA synthetase from *E. coli* MRE-600, but to no avail. Crystals of phenylalanyl-tRNA synthetase from *T. thermophilus* have the form of a parallelepiped with 0.3 × 0.3 × 0.3 mm dimensions. Fig.4 presents a photograph of the crystals. The specific activity of the crystals was determined, and gel-electrophoresis was carried out in the presence of SDS. Perfect identity with the initial preparation of the enzyme has been shown. The crystals are suitable for X-ray structural analysis with the resolution of 3.5–4 Å, which is being developed at present [7].

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