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Crucial role of pyrophosphate in the aminoacylation of *E. coli* $tRNA^{Phe}$ by yeast phenylalanyl-tRNA synthetase

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Rapid inactivation of the yeast phenylalanyl-tRNA synthetase in the course of aminoacylation of the heterologous *E. coli* $tRNA^{Phe}$ is observed. This inactivation occurs due to the formation of the tight complex of the enzyme with the pyrophosphate formed during the aminoacylation reaction. This complex is shown to be the normal intermediate of the reaction. Possible inactivation mechanism and correlation between structural differences of yeast and *E. coli* $tRNAs^{Phe}$ with the changes in the enzymatic mechanism of aminoacylation are discussed.

Phenylalanyl-tRNA synthetase; Pyrophosphate; tRNA; Enzyme inactivation

1. INTRODUCTION

Aminoacyl-tRNA synthetases are the enzymes that catalyze the specific aminoacylation of tRNAs by cognate amino acids. The first stage of the aminoacylation reaction, activation of amino acid, involves the formation of aminoacyladenylate and pyrophosphate (PP_i) from ATP and amino acid. The second stage of the reaction is the transfer of aminoacyl from aminoacyladenylate to tRNA [1]. Pyrophosphate, formed at the activation stage is known to be a potent inhibitor of aminoacylation [2–5], especially in heterologous systems. Cleavage of pyrophosphate by inorganic pyrophosphatase can tremendously increase the activity of aminoacyl-tRNA synthetase [2–4]. Covalent modification of several aminoacyl-tRNA synthetases by pyrophosphate also was shown [6]. Here we present evidence that pyrophosphate plays a key role in discrimination of *E. coli* $tRNA^{Phe}$ by yeast phenylalanyl-tRNA synthetase, by forming a stable complex with the enzyme.

2. MATERIALS AND METHODS

We used [^{3}H]phenylalanine (Amersham, 25 Ci/mmol), [γ-^{32}P]ATP (Obninsk, Russia, 5,000 Ci/mmol), $tRNA^{Phe}$ *E. coli* (Boehringer), yeast $tRNA^{Phe}$ and inorganic pyrophosphatase (670 U/mg, Sigma). *E. coli* phenylalanyl-tRNA synthetase was a kind gift of Prof. O.I. Lavrik (Novosibirsk Institute of Bioorganic Chemistry, Russia). Yeast phenylalanyl-tRNA synthetase was purified to homogeneity by ammonium sulfate precipitation, DEAE-Toyopearl 650M, HA-Ultrogel and MonoQ chromatography.

Enzyme activity was determined at 37°C. Aminoacylation was performed in 50 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 25 mM KCl, 1 mM dithiothreitol (DTT), 3 mM ATP, 5 μM [^{3}H]Phe and 1 μM $tRNA^{Phe}$. Reaction was initiated by addition of the enzyme to the final concentration of 4 nM unless otherwise specified. Reaction was stopped by 5% trichloracetic acid (TCA), the tRNA precipitate was collected on GF/C filters and radioactivity was counted in toluene scintillator. One unit of enzyme activity corresponds to formation 1 nmol aminoacyl-tRNA per 1 min.

Preincubation of yeast phenylalanyl-tRNA synthetase was performed in the aminoacylation buffer at 37°C for 15 min. Concentrations of ATP and Phe were as in the aminoacylation test.

Inorganic pyrophosphatase was added to assay mixtures up to the final concentration of 20 U/ml.

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3. RESULTS

The kinetics of aminoacylation of $tRNA^{Phe}$ by yeast and *E. coli* enzymes are presented in Fig. 1. As can be clearly seen, initial rate as well as plateau level of aminoacylation of *E. coli* $tRNA^{Phe}$ by the yeast enzyme is about 2 orders of magnitude lower than in the homologous system. This phenomenon cannot be explained by inactivity of *E. coli* tRNA because it is fully aminoacylated by homologous enzyme. Both the rate of aminoacylation and plateau level in the heterologous system are significantly increased after addition of inorganic pyrophosphatase to the reaction mixture (Fig. 1). In contrast pyrophosphatase has no effect on aminoacylation of yeast $tRNA^{Phe}$ by yeast phenylalanyl-tRNA synthetase (data not shown). Addition of a new aliquot of yeast phenylalanyl-tRNA synthetase after the reaction with *E. coli* $tRNA^{Phe}$ reaches a plateau, leads to the appearance of an identical kinetic curve (Fig. 2). This behavior is an exclusive feature of enzymes which undergo self-inactivation in the course of reaction (see [7] for references). Addition of *E. coli* phenylalanyl-tRNA synthetase to this reaction mixture results in complete aminoacylation of tRNA. The level of aminoacylation of *E. coli* $tRNA^{Phe}$ by the yeast enzyme is

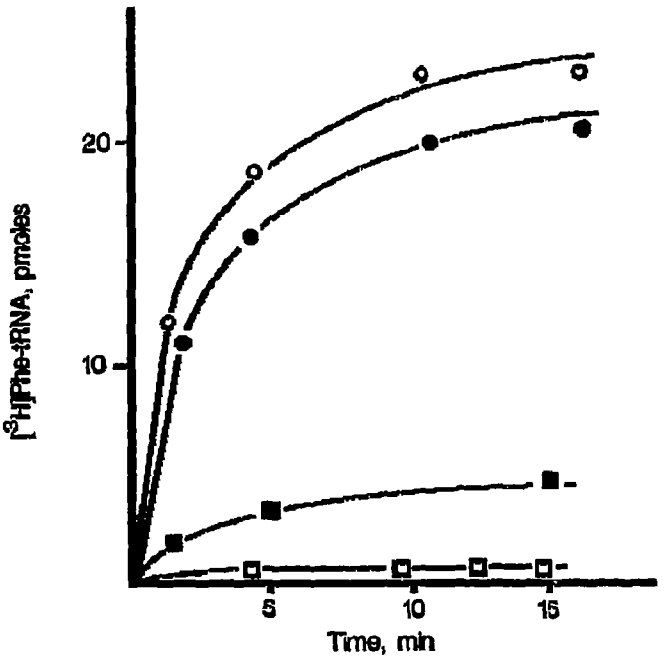

Fig. 1. Aminoacylation of $tRNA^{Phe}$ in different systems. Yeast (●) and *E. coli* (□) $tRNA^{Phe}$ with yeast phenylalanyl-tRNA synthetase. *E. coli* $tRNA^{Phe}$ with the *E. coli* enzyme (○), *E. coli* $tRNA^{Phe}$ and the yeast enzyme in the presence of inorganic pyrophosphatase (■).

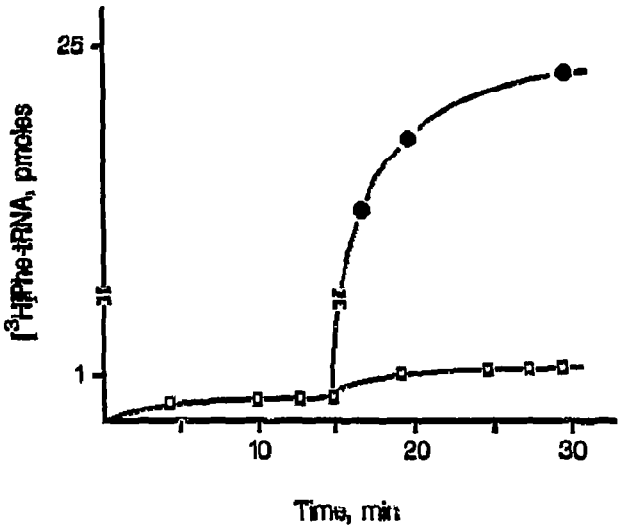

Fig. 2. Aminoacylation of *E. coli* $tRNA^{Phe}$ by the yeast enzyme. After 15 min of reaction, an additional aliquot of enzyme (□) or 25 pmols of yeast $tRNA^{Phe}$ (●) was added to the mixture.

proportional to the amount of the enzyme added (Fig. 3). One molecule of enzyme seems to be able to perform a single aminoacylation reaction in these conditions. Preincubation of yeast phenylalanyl-tRNA synthetase in a reaction mixture containing only ATP and Phe results in complete inactivation of the enzyme with respect to the aminoacylation of *E. coli* $tRNA^{Phe}$, while it has no effect on activity towards yeast tRNA (Fig. 4). Addition of inorganic pyrophosphatase to the preincubation mixture prevents this inactivation (Fig. 4). Preincubation with ATP or Phe alone does not influence enzyme activity. Catalytic constants of tRNA aminoacylation by yeast phenylalanyl-tRNA synthetase are summarized in Table I. As is clearly seen from these data, aminoacylation of *E. coli* $tRNA^{Phe}$ is characterized by higher K_m and significantly lower V_{max} values. Addition of pyrophosphatase increases the V_{max} of the reaction, while having only a moderate effect on the K_m value.

4. DISCUSSION

The potent inhibitory effect of pyrophosphate on heterologous aminoacylation, as well as the activation effect of inorganic pyrophosphatase are well known [2–5]. To explain the unexpectedly low K_i value of pyrophosphate (10^{-6} M) it was suggested that pyrophosphate competed with tRNA for aminoacyladenylate bound to the enzyme [2]. However, this explanation is not consistent with our results: the concentration of pyrophosphate formed in our experiments is less than 10^{-8} M, which is significantly lower than the K_i value. Surprisingly, it appeared that inactivation of yeast phenylalanyl-tRNA synthetase occurred in the course of the reaction with *E. coli* $tRNA^{Phe}$. This inactivation is pyrophosphate-dependent, since it is prevented by inorganic pyrophosphatase. Inactivation occurs during the first catalytic act, because only one *E. coli* Phe-$tRNA^{Phe}$ molecule can be formed when the yeast enzyme is added to the mixture of all three substrates. This inactivation can be explained by formation of a tight, probably covalent complex of the enzyme with pyrophosphate. Covalent complexes of tryptophanyl-tRNA synthetase with pyrophosphate are already known [8,9]. Pyrophosphate derivatives of this enzyme were formed from ATP and amino acids, or by incubation with PP_i.

Table I

Kinetic constants of the yeast and *E. coli* $tRNA^{Phe}$ aminoacylation by yeast phenylalanyl-tRNA synthetase

	$tRNA^{Phe}$ yeast	$tRNA^{Phe}$ *E. coli*	$tRNA^{Phe}$ *E. coli* + pyrophosphatase
K_m, μM	0.17	3.2	1.8
V_{max}, pmols/min	3.86	0.0085	0.43
V_{max}/K_m, relative units	1.0	$1.1 \cdot 10^{-4}$	$1.0 \cdot 10^{-2}$

Pyrophosphorylation of other aminoacyl-tRNA synthetases, including yeast phenylalanyl-tRNA synthetase, also was shown [10]. However, the role of these derivatives in enzyme functioning was unclear.

In our experiments, preincubation of phenylalanyl-tRNA synthetase with ATP and Phe in the absence of pyrophosphatase leads to the conversion of the enzyme into the form which is inactive in aminoacylation of *E. coli* $tRNA^{Phe}$ but is completely active with yeast tRNA. The presence of pyrophosphatase in the preincubation mixture prevents this inactivation. This means that the pyrophosphate-containing form of the enzyme exists as a normal intermediate of the aminoacylation reaction. The pyrophosphate-containing enzyme formed by preincubation with Phe and ATP is unable to aminoacylate heterologous *E. coli* tRNA. While yeast $tRNA^{Phe}$ is able to dissociate enzyme-bound PP_i and thus allow continued aminoacylation, *E. coli* tRNA is not.

Briefly, our results could be summarized as follows: phenylalanyl-tRNA synthetase is pyrophosphorylated in the course of aminoacyladenylate formation. This intermediate is comparatively stable, and PP_i dissociates from it slowly. Dissociation is facilitated by the binding of homologous tRNA or by the cleavage of PP_i by pyrophosphatase. The step of pyrophosphate dissociation is a limiting stage in the aminoacylation of *E. coli* $tRNA^{Phe}$ by the yeast enzyme. This scheme is consistent with the effect of pyrophosphatase on the catalytic constants of *E. coli* $tRNA^{Phe}$ aminoacylation: having practically no effect on the affinity of phenylalanyl-tRNA synthetase to tRNA (K_m), it increases tremendously V_{max} by changing the limiting step, which seems to be the dissociation of PP_i.

What is the structural basis for the differences in aminoacylation of *E. coli* and yeast $tRNA^{Phe}$? As it was shown, recognition of $tRNA^{Phe}$ by yeast phenylalanyl-tRNA synthetase is mainly determined by nucleotides at positions 20, 34, 35, 36 and 73 [11,12]. The only difference between yeast and *E. coli* $tRNAs^{Phe}$ in these positions is substitution of G_{20} by U_{20}, which determines the poor aminoacylation of *E. coli* tRNA by yeast enzyme [12]. If U_{20} was replaced by G_{20} in the transcript of *E. coli* $tRNA^{Phe}$ gene obtained in vitro by T7 RNA-polymerase, it became identical in its kinetic parameters to the transcript of the yeast $tRNA^{Phe}$ gene [12]. So, the observed differences in the mechanism of yeast and *E. coli* $tRNA^{Phe}$ aminoacylation by the yeast enzyme could be clearly correlated with this G_{20}–U_{20} substitution.

Generally it is considered that the substitutions of nucleotides in the recognition points of tRNAs lead to their inactivation due to the dramatic decrease of the k_{cat} of aminoacylation [13]. In the case of alanine minihelices, substitution of the nucleotide at position 73 resulted in the blockage of the transfer of aminoacyl moiety from aminoacyladenylate to acceptor RNA [14]. Our results demonstrate the possibility that tRNA inactivation may occur mainly due to the change of enzymatic mechanism of reaction caused by the substitution of the tRNA nucleotide, important for the recognition.

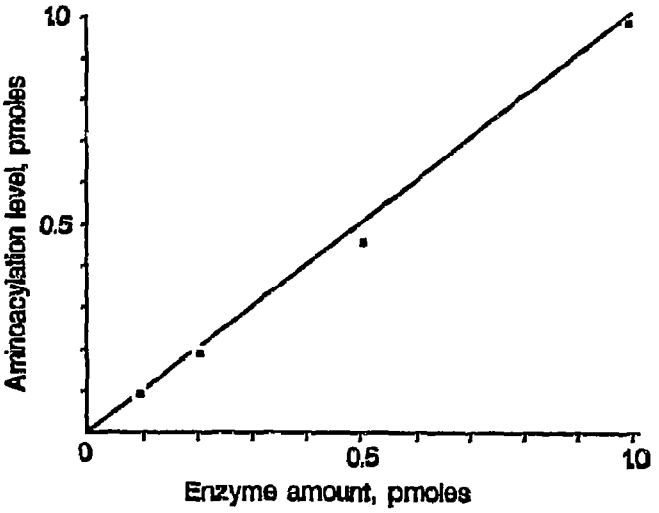

Fig. 3. Effect of the yeast phenylalanyl-tRNA synthetase concentration in the reaction mixture on the limiting level of *E. coli* $tRNA^{Phe}$ aminoacylation.

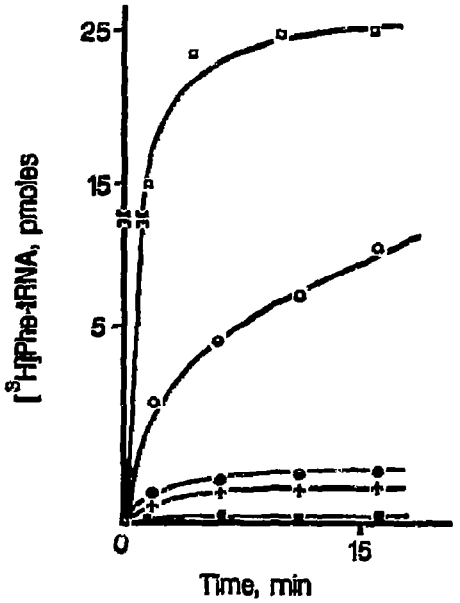

Fig. 4. Effect of preincubation on the aminoacylation of yeast and *E. coli* $tRNAs^{Phe}$ by yeast phenylalanyl-tRNA synthetase. Reaction was initiated by addition of the corresponding tRNA. Activity of phenylalanyl-tRNA synthetase with *E. coli* $tRNA^{Phe}$: no preincubation (+); preincubation with ATP and Phe in the presence (○) or in the absence (■) of inorganic pyrophosphatase; preincubation with Phe only (●). Activity of phenylalanyl-tRNA synthetase with yeast $tRNA^{Phe}$: preincubation with ATP and Phe (□).

In addition to the differences in the nucleotide sequence, the tRNAs used in this study differ also in base modification. There is some evidence [15], although not confirmed [16], that modifications of G_{10} in *E. coli* $tRNA^{Phe}$ can significantly change its substrate proper-

ties for yeast phenylalanyl-tRNA synthetase. Further studies using transcripts of corresponding genes will allow us to establish more clearly the structural differences in tRNAs which lead to the changes in mechanism of the reaction.

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