

Influence of Modified tRNA^{Tyr} on the Activation of Tyrosine Catalyzed by Tyrosyl-tRNA Synthetase from *Saccharomyces cerevisiae*

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Yeast tyrosyl-tRNA synthetase (TyrRS, EC 6.1.1.1) is a homodimeric enzyme capable of binding only one molecule of its macromolecular substrate, tRNA^{Tyr}. The reactive intermediate tyrosyl adenylate is formed from tyrosine and ATP in the first reaction step, which can be conveniently assayed by pyrophosphate exchange. In order to determine the number of active sites per homodimer, the kinetics of pyrophosphate exchange was measured in the presence of the tRNA^{Tyr} analogue unable to accept the amino acid. The analogue was found to form the expected equimolar complex with dimeric enzyme. It was a competitive inhibitor of pyrophosphate exchange with respect to ATP and non-competitive with respect to tyrosine. Inhibition cannot exceed 50%, suggesting the simplest model in which yeast TyrRS is a symmetrical dimer, possessing two identical active sites, both capable of catalyzing the formation of tyrosyl adenylate.

Key words: modified tRNA^{Tyr}, pyrophosphate exchange, *Saccharomyces cerevisiae*, tyrosyl-tRNA synthetase, tRNA^{Tyr}.

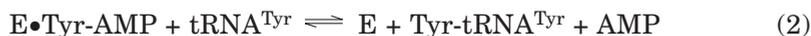
INTRODUCTION

Aminoacyl-tRNA synthetases (aaRS, EC 6.1.1) catalyze the covalent attachment of amino acids to the 3'-ends of their cognate transfer RNAs. The

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fidelity of translation of the genetic code relies on the correct aminoacylation of tRNAs by their cognate aaRS, since errors in the aminoacylation reaction result in the attachment of an incorrect (noncognate) amino acid to tRNA and ultimately in the incorrect amino acid insertion into the growing polypeptide chain. There are 20 aaRS types in the cell, one for each amino acid and for each tRNA isoacceptor family. Only exceptionally, some organisms carry more than one gene for a given aaRS or, on the other hand, lack the genes for some synthetases.¹

Tyrosyl-tRNA synthetase (TyrRS, EC 6.1.1.1) catalyzes the aminoacylation of tRNA^{Tyr} in a two-step reaction. Tyrosine is first activated with ATP to form tyrosyl adenylate (Tyr-AMP) and pyrophosphate (Eq. 1), and then Tyr-AMP is attacked by the 3'-terminal ribose of the tRNA^{Tyr} to form tyrosyl-tRNA and AMP (Eq. 2).



Amino acid activation can be assayed by steady-state kinetics of pyrophosphate exchange, whereby isotopically labeled pyrophosphate distributes between the bulk pyrophosphate in solution and the β , γ phosphates of the ATP in solution.²

Homodimeric TyrRS from *Bacillus stearothermophilus* is one of the best-studied enzymes of this group. Although there are two active sites per dimer, this TyrRS displays half-of-the-sites activity by binding tightly only one tyrosine and forming only one tyrosyl adenylate per two active sites.³ A single molecule of tRNA^{Tyr} binds across both subunits of the dimer, with its 3'-terminus positioned in the active site of the N-terminal domain in one subunit and its anticodon loop bound to the C-terminal domain of the other subunit.⁴ TyrRS from yeast *Saccharomyces cerevisiae* is also a homodimer.⁵ The monomer consists of 394 amino acids, as determined from the primary structure of the yeast TyrRS gene.⁶ Despite the lack of strong overall sequence similarity between the yeast TyrRS and the corresponding eubacterial enzymes, there is a striking conservation of residues thought to be important for recognition of their common amino acid substrate, tyrosine. On the other hand, bacterial and eukaryotic TyrRSs do not catalyze the aminoacylation of heterologous RNA^{Tyr} substrates, suggesting that tRNA^{Tyr} recognition differs between bacteria and eukaryotes.⁷ This is not surprising, since tRNA^{Tyr} is unique among tRNAs due to the phylogenetic variability in the length of its variable arm. In bacteria it belongs to type II tRNAs, which contain an extended variable arm, whereas in eukaryotes it is a typical type I tRNA, with a short variable arm.

Since the yeast TyrRS also binds one tRNA^{Tyr} per dimer,^{8,9} we wanted to determine the number of active sites capable of exchanging pyrophosphate in the presence of a tRNA^{Tyr} analogue. In this paper we describe the inhibition of pyrophosphate exchange by periodate oxidized tRNA^{Tyr}, unable to accept the amino acid. Non-modified tRNA^{Tyr} cannot be used in such studies because transfer of tyrosine to tRNA^{Tyr} would occur immediately after the formation of the Tyr-AMP intermediate. The results suggest that binding of one modified tRNA^{Tyr} molecule to dimeric enzyme blocks the exchange reaction on one subunit only.

EXPERIMENTAL

Purification of tRNA^{Tyr}

The tRNA^{Tyr} was prepared from bulk brewer's yeast tRNA, enriched in tyrosine isoacceptor, kindly supplied by Professor G. Dirheimer of Strasbourg, by repeated chromatography on benzoylated DEAE-cellulose according to the method of Maxwell *et al.*¹⁰ Pure tRNA^{Tyr} accepted at least 1.4 nmol tyrosine/A_{260 nm} unit, determined in an aminoacylation assay as described elsewhere.⁸ In order to confirm the purity level, tRNA^{Tyr} was treated with NH₄⁺-loaded ion exchange beads and subjected to analysis by the matrix-assisted laser desorption/ionization time-of-flight mass spectrometry.¹¹ A mass resolution (full width of peak at half height) m/dm = 220 was achieved in the linear mode of operation.

Oxidation of tRNA^{Tyr}

Purified tRNA^{Tyr} (3.8 nmol) was treated with 0.4 μmol sodium periodate in sodium acetate (50 mmol dm⁻³) pH = 5.0 (final volume, 500 μl). The reaction was performed in the dark at room temperature. After one hour, the reaction was stopped by addition of 4 μmol ethylene glycol. Oxidized tRNA^{Tyr} (tRNA^{Tyr_{ox}}) was precipitated (0.3 mol dm⁻³ sodium acetate, pH = 5.2 and three volumes of ethanol) overnight at -20 °C. The precipitate was resuspended in Tris/HCl (20 mmol dm⁻³) pH = 7.0, magnesium acetate (1 mmol dm⁻³). In the aminoacylation assay,⁸ tRNA^{Tyr_{ox}} accepted 20 pmol tyrosine/A_{260 nm} unit. Oxidized tRNA^{Tyr} was subjected to electrophoresis on 10% acrylamide/bisacrylamide (19 : 1) gel containing 8 mol dm⁻³ urea in electrophoresis buffer (Tris, 90 mmol dm⁻³; boric acid, 90 mmol dm⁻³; Na₂EDTA, 2 mmol dm⁻³; pH = 8.0). The samples were denatured for 2 minutes at 65 °C in urea (4 mol dm⁻³), 1% bromophenol blue, and 1% xylene cyanol FF, Na₂EDTA (5 mmol dm⁻³) prior to loading the gel. After electrophoresis, the gel was stained with silver.¹²

Gel-retardation Assay

The complexes between tyrosyl-tRNA synthetase and both tRNA^{Tyr} and tRNA^{Tyr_{ox}} were prepared according to Rubelj *et al.*⁸ The amount of tyrosyl-tRNA synthetase was 5 pmol and both tRNA^{Tyr} and tRNA^{Tyr_{ox}} varied in a range from 1 to 25 pmol. The preformed complexes were subjected to electrophoresis on 6% acrylamide/bisacrylamide (40 : 1) gel containing 5% glycerol in electrophoresis buffer

(Tris, 50 mmol dm⁻³; boric acid, 25 mmol dm⁻³; Na₂EDTA, 1 mmol dm⁻³; pH = 8.0). Electrophoresis took place at 4 °C, 210 V, 18 mA, and lasted 4 hours. The gel was stained with silver.¹³

Pyrophosphate Exchange

The extent of adenylate formation catalyzed by TyrRS was assessed by the pyrophosphate exchange reaction.¹⁴ Standard reaction mixtures contained HEPES/KOH (100 mmol dm⁻³) pH = 7.2–7.5; MgCl₂ (10 mmol dm⁻³); ³²P-PP_i (4–7 cpm/pmol, (0.5 mmol dm⁻³); KF (10 mmol dm⁻³); final volume 100 μl. The temperature was kept at 30 °C. When the *K_M* for ATP* was determined, the concentration of ATP varied from 0.2 to 8.5 mmol dm⁻³, and tyrosine was kept at a constant concentration (1 mmol dm⁻³). For determination of *K_M* for tyrosine, tyrosine concentration was in the range from 0.004 to 0.2 mmol dm⁻³ and ATP was kept at a constant concentration (2 mmol dm⁻³). For determination of *K_M* for tyrosine and ATP in the presence of tRNA^{Tyr_{ox}}, the concentration of tRNA^{Tyr_{ox}} was 92 nmol dm⁻³. In all reaction mixtures, the concentration of TyrRS was 50 nmol dm⁻³. The reactions were performed in triplicates for each concentration of substrates.

When the ability of the tRNA^{Tyr_{ox}} to inhibit the pyrophosphate exchange reaction was studied, the tRNA^{Tyr_{ox}} concentration in the standard reaction mixture varied from 0 to 1500 nmol dm⁻³. The concentrations of ATP and tyrosine were 2 mmol dm⁻³ and 1 mmol dm⁻³, respectively. The concentration of TyrRS was 50 nmol dm⁻³.

RESULTS AND DISCUSSION

The Ability of Oxidized tRNA^{Tyr} to Form a Complex with TyrRS

Oxidized tRNA^{Tyr} possesses 2'- and 3'-aldehyde groups on the terminal ribose, and has no significant ability to accept tyrosine (see Experimental). The oxidation procedure did not cause any cleavage of tRNA^{Tyr}, as confirmed by denaturing polyacrylamide gel electrophoresis (data not shown). In order to determine the influence of oxidation of the terminal ribose on the ability of tRNA^{Tyr} to make a complex with TyrRS, the gel retardation assay of the preformed complexes between TyrRS and both tRNA^{Tyr} and tRNA^{Tyr_{ox}} was performed. Mixtures containing molar ratios tRNA^{Tyr} / TyrRS and tRNA^{Tyr_{ox}} / TyrRS from 0.2 to 5 were prepared and subjected to polyacrylamide gel electrophoresis (Figure 1). Both non-modified and oxidized tRNA^{Tyr} have the same electrophoretic properties (lanes a and f) and form complexes (lanes b–e and g–j) of equal mobility, previously shown to have the stoichiometry of one tRNA^{Tyr} per dimeric enzyme.⁸ Furthermore, the amount of tRNA remaining unbound at all input ratios (lanes b–e and g–j) appears to be equal with both tRNAs, suggesting the same affinity of the enzyme for

* ATP was always present as ATP-MgCl₂ complex prepared by mixing 1 : 1 molar amounts of ATP and MgCl₂.

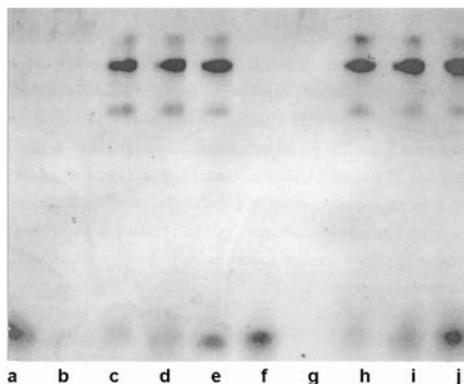


Figure 1. Polyacrylamide gel electrophoresis of preformed complexes between yeast TyrRS and oxidized and non-oxidized tRNA^{Tyr}. a) 25 pmol tRNA^{Tyr}; b) 1 pmol tRNA^{Tyr}; c) 5 pmol tRNA^{Tyr}; d) 10 pmol tRNA^{Tyr}; e) 25 pmol tRNA^{Tyr}; f) 25 pmol tRNA^{Tyr_{ox}}; g) 1 pmol tRNA^{Tyr_{ox}}; h) 5 pmol tRNA^{Tyr_{ox}}; i) 10 pmol tRNA^{Tyr_{ox}}; j) 25 pmol tRNA^{Tyr_{ox}}. Lanes b–e and g–j contained 5 pmol TyrRS. Gel was stained with silver.

tRNA^{Tyr} regardless of the structural change on the 3'-terminal ribose. Hence, the periodate-modified tRNA^{Tyr} can be successfully used as the analogue of the third substrate in studying the kinetics of the reaction between the first two substrates.

Determination of the Kinetic Parameters in the Activation Reaction

The first step of aminoacylation reaction is the activation of amino acid *via* formation of aminoacyl adenylate. The extent of tyrosyl adenylate formation catalyzed by TyrRS was assessed in the presence and absence of tRNA^{Tyr_{ox}}. The kinetic parameters were evaluated from the Wolf-Hanes plots and are summarized in Table I. Oxidized tRNA^{Tyr} showed inhibition of the competitive type when ATP was used as variable substrate and of the non-competitive type when tyrosine was used as variable substrate. In a related study it has been observed that tRNA^{Tyr}, modified to remove 3'-terminal adenosine which accepts tyrosine, had no significant effect on the rate of pyrophosphate exchange reaction catalyzed by TyrRS from *B. stearothermophilus*.¹⁵ In contrast, our results undoubtedly show that modified tRNA^{Tyr} possessing terminal adenosine with diol groups oxidized to aldehyde is an inhibitor of pyrophosphate exchange catalyzed by the yeast enzyme, indicating that the 3'-terminal adenosine of modified tRNA^{Tyr} competes for the same active site with ATP. The non-competitive inhibition pattern with respect to tyrosine could be explained by the structural changes of the tyrosine

TABLE I

Kinetic parameters for yeast TyrRS in the pyrophosphate exchange reaction

	Substrate	
	Tyrosine	ATP
$K_M / \text{mmol dm}^{-3}$	0.03	0.4
$K_M / \text{mmol dm}^{-3}$ with $\text{tRNA}^{\text{Tyr}_{\text{ox}}}$, 92 nmol dm^{-3}	0.03	1.3
$k_{\text{cat}} / \text{s}^{-1}$	14.4	17.3
$k_{\text{cat}} / \text{s}^{-1}$ with $\text{tRNA}^{\text{Tyr}_{\text{ox}}}$, 92 nmol dm^{-3}	9.0	16.9
$K_I / \mu\text{mol dm}^{-3}$ for $\text{tRNA}^{\text{Tyr}_{\text{ox}}}$	0.15	0.041
	non-competitive	competitive

^a All kinetic parameters were evaluated from the Wolf-Hanes plots.

binding site induced by $\text{tRNA}^{\text{Tyr}_{\text{ox}}}$ binding. Since the inhibition constant for ATP is by an order of magnitude higher than the inhibition constant for tyrosine, it seems that the binding of ATP to the TyrRS is more inhibited by $\text{tRNA}^{\text{Tyr}_{\text{ox}}}$ than the binding of tyrosine.

For most aaRS, the activation of amino acid substrate by ATP to form an enzyme-bound aminoacyl adenylate intermediate (step I) precedes the tRNA binding and the transfer of amino acid to the 3'-end of the tRNA (step II). This raises the possibility of the formation of adenylate intermediate influencing the tRNA binding and aminoacylation.^{16,17,18} The exceptions are arginyl-, glutaminyl- and glutamyl-tRNA synthetases, which are not able to activate amino acid without the presence of cognate tRNA. Crystallographic¹⁹ and biochemical²⁰ data showed that in the absence of tRNA^{Gln} the active site of glutaminyl-tRNA synthetase from *Escherichia coli* is not completely formed. In some other cases, tRNA binding may enhance the accuracy of amino acid selection. For example, seryl-tRNA synthetase from *S. cerevisiae* exhibits more than an order of magnitude higher affinity for serine in the reaction performed in the presence of tRNA than in the activation step.²¹ It was concluded that SerRS displays tRNA-dependent amino acid recognition. In the aminoacylation reaction catalyzed by TyrRS from yeast, Freist and Sternbach²² and Plohl and Kučan⁵ reported the K_M for ATP of 0.4 mmol dm^{-3} . The K_M values for tyrosine were $0.012 \text{ mmol dm}^{-3}$ and $0.008 \text{ mmol dm}^{-3}$, respectively. In this work, we have determined K_M values for ATP and tyrosine (0.4 mmol dm^{-3} and $0.03 \text{ mmol dm}^{-3}$, respectively) in the pyrophosphate exchange reaction. The kinetic parameters evaluated in the

aminoacylation reaction and pyrophosphate exchange reaction do not differ significantly: K_M values for ATP are the same and K_M value for tyrosine is 2.5 times higher in the pyrophosphate exchange reaction than in the aminoacylation reaction. It seems that binding of tRNA^{Tyr} has no major influence on the affinity of the TyrRS for ATP and tyrosine. This is consistent with the random bi-uni uni-bi ping-pong mechanism previously proposed for TyrRS from yeast,²² in which the binding of tRNA^{Tyr} to the TyrRS occurs after formation of tyrosyl adenylate and dissociation of pyrophosphate. In such a sequence of substrate binding, tRNA^{Tyr} could not change the affinity of the enzyme for ATP and tyrosine.

Number of Active Sites for Tyrosine Activation

Dimeric TyrRS (α_2) from yeast forms a noncovalent complex with one molecule of tRNA^{Tyr} when the macromolecules are present in equimolar concentrations.^{8,9} The same (α_2)•tRNA^{Tyr} noncovalent complex is formed in the molar excess of tRNA^{Tyr}. As demonstrated by the gel retardation assay, TyrRS does not distinguish between oxidized and non-oxidized tRNA^{Tyr} in the formation of noncovalent (α_2)•tRNA complexes (Figure 1). Furthermore, tRNA^{Tyr_{ox}} competes with the formation of tyrosyl adenylate at the 3'-end tRNA^{Tyr} binding site. To examine whether TyrRS possesses two identical active sites for tyrosine activation, the influence of tRNA^{Tyr_{ox}} (concentration: 0–1500 nmol dm⁻³) on the velocity of the pyrophosphate exchange reaction was examined. The other substrates were held at a constant concentration (ATP, 2 mmol dm⁻³ and tyrosine, 1 mmol dm⁻³). The dependence of the velocity of the pyrophosphate exchange reaction on the tRNA^{Tyr_{ox}} concentration is shown in Figure 2. The velocity decreases by half when the concentration of the tRNA^{Tyr_{ox}} reaches the value of about 900 nmol dm⁻³. Further raising of the tRNA^{Tyr_{ox}} concentration has no influence on the velocity of the pyrophosphate exchange reaction.

The fact that one half of TyrRS cannot be inhibited by tRNA^{Tyr_{ox}} supports the conclusion that yeast TyrRS possesses two active sites for tyrosine activation and does not display half-of the sites activity. In Figure 3, we present the model in which TyrRS has two active sites for pyrophosphate exchange, and only one molecule of tRNA^{Tyr_{ox}} can be bound to the enzyme dimer. There is no cooperativity between the two active sites. If the bound 3'-end of tRNA^{Tyr_{ox}} prevents formation of tyrosyl adenylate at one active site, the other exchanges pyrophosphate with normal activity. In such a case, the overall activity in the reaction mixture containing a molar excess of tRNA^{Tyr_{ox}} is decreased to 50% (Figure 3, Model 1).

Contrary to that, our results are not consistent with the model in which TyrRS is a symmetrical dimer that exhibits substrate-induced asymmetry

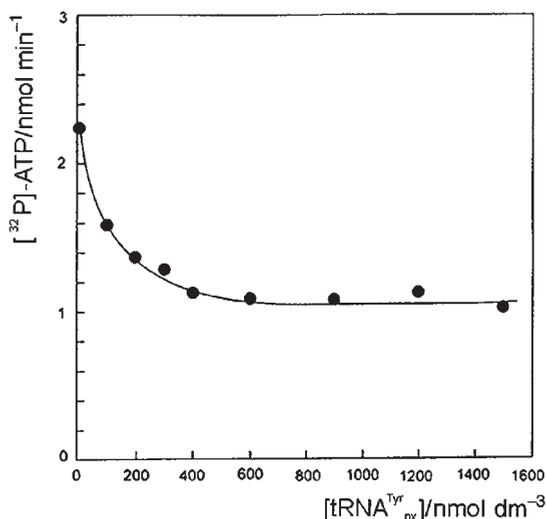


Figure 2. Dependence of the velocity of the pyrophosphate exchange reaction on the concentration of $\text{tRNA}^{\text{Tyr}}_{\text{ox}}$.

in each cycle, displaying half-of-the-sites activity. If half-of-the-sites activity is induced by binding of ATP or tyrosine or by the formation of tyrosyl adenylate, and in every catalytic cycle the active site is randomly chosen, the binding of the $\text{tRNA}^{\text{Tyr}}_{\text{ox}}$ to one site would prevent binding of ATP and tyrosine and hence the tyrosyl adenylate formation at that site, but would still allow the formation of tyrosyl adenylate on the other site of the enzyme. Thus, approximately one site will work per dimer molecule of the enzyme and the activity would not significantly decrease (at least not to 50%). On the other hand, one could imagine a model in which binding of the tRNA^{Tyr} to the enzyme induces half-of-the-sites activity. It is reasonable to assume that tRNA^{Tyr} would inactivate the active site that does not bind the 3'-CCA end of the tRNA^{Tyr} . In such a case, $\text{tRNA}^{\text{Tyr}}_{\text{ox}}$ would completely inactivate the pyrophosphate exchange reaction.

Symmetry of the Tyrosyl-tRNA Synthetase Homodimer

In the above discussion, all models assume that unligated TyrRS is a symmetrical dimer possessing two identical active sites. The half-of-the-sites activity models assume that binding of each substrate (ATP, tyrosine, tRNA) or the formation of tyrosyl adenylate at one active site induce inactivation of the other site, and hence induce asymmetry of TyrRS. These models also assume that in every catalytic step the active site and the tRNA

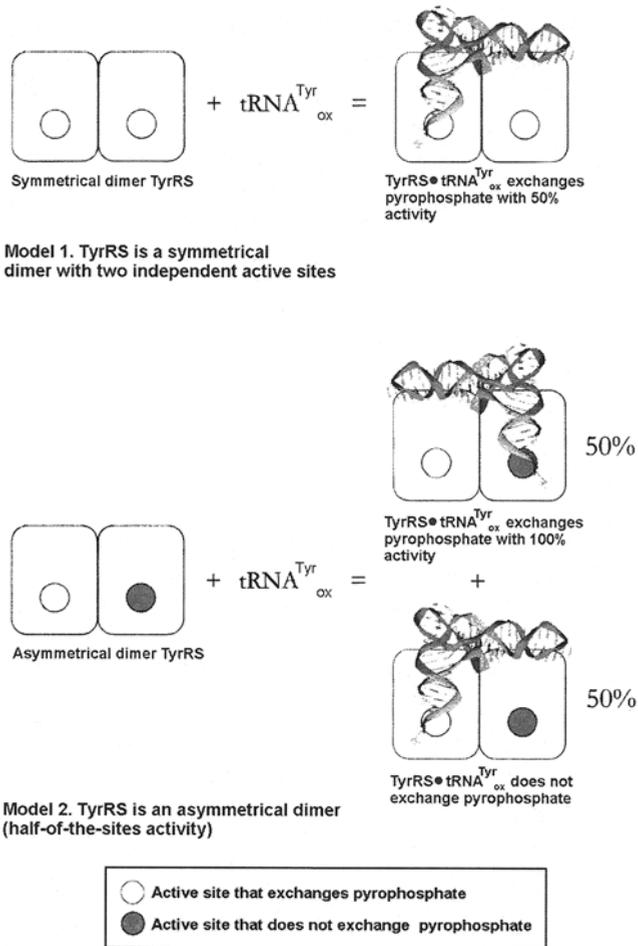


Figure 3. Proposed models for yeast TyrRS exchanging pyrophosphate with 50% activity in the presence of a high molar excess of tRNA^{Tyr}_{ox}.

binding site are chosen randomly. The TyrRS from *B. stearotherophilus* crystallizes as a symmetrical dimer with each subunit having a complete active site.^{23,24} The results of the solution studies appear to contradict those of X-ray crystallography, since the enzyme-substrate complexes are asymmetrical in solution because the enzyme exhibits half-of-the-sites activity.³ Ward and Fersht¹⁵ have presented evidence that unligated TyrRS is also asymmetrical in solution. Each dimer is active at only one site, and the same active site is used for each catalytic cycle. The used site is randomly distributed between the subunits and there is no evidence that either catalysis or binding of substrate is required to induce asymmetry. It is not known

whether TyrRS from yeast is asymmetrical in solution. If we suppose that Ward and Fersht's model for *B. stearothermophilus* TyrRS is applicable to the yeast counterpart, then the 50% decrease of the activity in the pyrophosphate exchange reaction at the molar excess of tRNA^{Tyr_{ox}} is explained by Model 2 (Figure 3). The model assumes that the asymmetrical dimer of the TyrRS could bind tRNA^{Tyr_{ox}} in two orientations with the same affinity. The first orientation is with the 3'-CCA end of the tRNA^{Tyr_{ox}} bound to the catalytically active site, and these enzyme•tRNA complexes possess no pyrophosphate exchange activity. In the second orientation, the 3'-CCA end of the tRNA^{Tyr_{ox}} binds to the catalytically inactive site and these enzyme•tRNA complexes exchange pyrophosphate with normal activity. Since only half of the enzyme molecules are in catalytically active complexes, the overall activity is decreased to 50%. However, it does not seem plausible that tRNA^{Tyr} would not sense the inherent asymmetry of the TyrRS, and bind in both orientations with the same affinity.

In summary, we conclude that tRNA^{Tyr_{ox}} prevents pyrophosphate exchange at the site where the 3'-CCA end of the tRNA is bound, most probably by sterical competition with the binding of ATP. Binding of tRNA^{Tyr_{ox}} also impairs the binding of tyrosine to the active site. The results presented in this paper support the simple model in which the yeast TyrRS is a symmetrical dimer possessing two identical active sites, both capable of catalyzing the formation of tyrosyl adenylate (Figure 3, Model 1). Our results do not support the idea that half-of-the-sites activity is conserved in yeast TyrRS.

ABBREVIATIONS AND CODES

PP_i – pyrophosphate, AMP – adenylate, TyrRS – tyrosyl-tRNA synthetase, tRNA^{Tyr_{ox}} – oxidized tRNA^{Tyr}, DEAE-cellulose – diethylaminoethyl-cellulose.

Non-covalent bonds are represented by • and covalent bonds by -.

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SAŽETAK

Utjecaj modificirane tRNA^{Tyr} na aktivaciju tirozina kataliziranu tirozil-tRNA sintetazom iz kvasca *Saccharomyces cerevisiae*

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Tirozil-tRNA sintetaza iz kvasca (EC 6.1.1.1) homodimerni je enzim, koji može vezati samo jednu molekulu svog makromolekulnog supstrata tRNA^{Tyr}. U prvom koraku reakcije, iz tirozina i ATP nastaje reaktivni međuprodukt tirozil-adenilat; reakcija se može pratiti izmjenom pirofosfata. Da bi se odredio broj aktivnih mjesta po molekuli homodimera, izmjena pirofosfata mjerena je u prisutnosti analoga tRNA^{Tyr}, koji se nije mogao aminoacilirati. Nađeno je da analog tvori očekivani ekvimolarni kompleks s dimernim enzimom i pokazao se kompetitivnim inhibitorom izmjene pirofosfata s obzirom na ATP, a nekompetitivnim s obzirom na tirozin. Inhibicija nije prelazila 50%, što navodi na najjednostavniji model u kojemu je TyrRS simetričan dimer s dva aktivna mjesta za sintezu tirozil-adenilata.