Yeast Phenylalanyl-tRNA Synthetase: Modulation of Catalytic Properties by Variation of Affinities for Phenylalanine in the Presence of $tRNA^{Phe}$ and Aminoacyl-$tRNA^{Phe}$

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ABSTRACT: The binding of phenylalanine to yeast phenylalanyl-tRNA synthetase, in the presence of $tRNA^{Phe}$ or aminoacyl-$tRNA^{Phe}$, has been investigated by fluorescence titrations in the presence of 6-(*p*-toluidinyl)naphthalene-2-sulfonate (TNS) and by the kinetics of the ATP–PP_i exchange reaction. Phe-$tRNA^{Phe}$ and *N*-acetyl-Phe-$tRNA^{Phe}$ were found to behave as competitive inhibitors in the binding of phenylalanine to its specific binding site, as shown by an increase of the K_m for phenylalanine in the ATP–PP_i exchange reaction. The decrease of affinity for phenylalanine was confirmed by TNS fluorometric titrations in the presence of the nonhydrolyzable derivative *N*-acetyl-Phe-$tRNA^{Phe}$. The degree of competition between phenylalanine and Phe-$tRNA^{Phe}$ was dependent upon the integrity of the 3′-terminal adenosine of $tRNA^{Phe}$. Indeed, Phe-$tRNA^{Phe}_{ox-red}$ affected to a lesser degree the binding of phenylalanine. On the other hand, $tRNA^{Phe}$ was shown to be a noncompetitive inhibitor with respect to phenylalanine, reducing the V_{max} of the isotopic exchange by a factor of 3, whereas the K_m(Phe) value remained unchanged. By stopped-flow fluorescence measurements, it was shown that the rates of synthesis and pyrophosphorolysis of enzyme-bound Phe~AMP were not altered in the presence of $tRNA^{Phe}$. The decrease in V_{max} for the ATP–PP_i exchange reaction could be correlated with a partial dissociation of Phe~AMP from the enzyme, promoted by the binding of native $tRNA^{Phe}$. This dissociation of Phe~AMP from the enzyme–$tRNA^{Phe}$ complex found expression in an incomplete transfer of the phenylalanine moiety from the two adenylates bound to the enzyme. An alternative explanation could be that upon fixation of the tRNA one of the two enzyme-bound Phe~AMP becomes unreactive. Such behaviors may account for half-of-the-sites reactivity and biphasic dependencies during steady-state aminoacylation of the $tRNA^{Phe}$.

Phenylalanyl-tRNA synthetase from baker's yeast is a "pseudodimeric" enzyme, each protomer consisting of two different types of subunit (Fasiolo et al., 1974; Fasiolo & Ebel, 1974). We have shown earlier that activation of phenylalanine by ATP, in the absence of $tRNA^{Phe}$, is catalyzed simultaneously at the same rate by the two protomers (Baltzinger et al., 1983). However, many studies from our laboratory as well as from other laboratories reported an asymmetric behavior of the enzyme (Berther et al., 1974; Fasiolo et al., 1974, 1977; Lefèvre et al., 1980). It is well established that such behavior is triggered by the binding of native $tRNA^{Phe}$ and appears by the existence of nonequivalent binding sites for phenylalanine as well as nonlinear steady-state kinetics for the aminoacylation reaction of $tRNA^{Phe}$.

In the present report we confirm that phenylalanyl-tRNA synthetase, when complexed with native $tRNA^{Phe}$, behaves like an asymmetric enzyme and exhibits half-of-the-sites reactivity during the ATP–PP_i exchange reaction. On the contrary, Phe-$tRNA^{Phe}$ is unable to induce such behavior. However, we show that the latter inhibits the activation step by competition for the phenylalanine-specific binding site.

Materials and Methods

Phenylalanyl-tRNA synthetase (EC 6.1.1.20) was purified from baker's yeast as described earlier (Fasiolo & Ebel, 1974). The specific activity was 3200–3600 $nmol \cdot min^{-1} \cdot mg^{-1}$ with 2 mol of active sites/mol of enzyme under the conditions previously described (Fasiolo et al., 1970). ATP, 6-(*p*-toluidinyl)naphthalene-2-sulfonate and yeast inorganic pyrophosphatase (EC 3.6.1.1) of specific activity 500–600 $units \cdot mg^{-1}$ were purchased from Sigma. $tRNA^{Phe}$ (1720 $pmol/A_{260}$) and $tRNA^{Tyr}$ (800–1000 $pmol/A_{260}$) were purified from unfractionated brewer's yeast tRNA (Boehringer) by countercurrent distribution (Dirheimer & Ebel, 1967) and BD-cellulose chromatography (Ehrlich et al., 1980). Uniformly labeled L-[^{14}C]phenylalanine (400–500 Ci/mol) was obtained from the Commissariat à l'Energie Atomique (Saclay, France); sodium [^{32}P]pyrophosphate (3.5 Ci/mmol) was a product of New England Nuclear (Dreieich, West Germany). All other reagents were of the highest purity grade from Merck (Darmstadt, West Germany).

Aminoacylation of $tRNA^{Phe}$. [^{14}C]Phe-$tRNA^{Phe}$ (5–10 mg) was prepared as described previously (Baltzinger et al., 1979) and freed from the synthetase and small ligands of the aminoacylation mixture by chromatography on DEAE-cellulose (column 1–2 mL) equilibrated with 100 mM sodium acetate, pH 4.5, containing 200 mM NaCl. The elution of [^{14}C]-Phe-$tRNA^{Phe}$ was performed with 2 M NaCl in the same buffer and followed by an ethanolic precipitation.

N-Acetylation of [^{14}C]Phe-$tRNA^{Phe}$. The reaction was carried out by using the *N*-hydroxysuccinimide ester of acetic acid according to Bartmann et al. (1974). The yield of acetylation was 100% of the theoretical one as determined by the method of Schoffield & Zamecnick (1968).

Preparation of Phe-$tRNA^{Phe}_{ox-red}$. Native $tRNA^{Phe}$ was oxidized and reduced according to the method described by Cramer et al. (1968). The aminoacylation of $tRNA^{Phe}_{ox-red}$ was carried out under the same conditions as described above for native $tRNA^{Phe}$ except that the incubation time in the presence of phenylalanyl-tRNA synthetase was prolonged 3–4 times. The yield of aminoacylation was 96%. Controls of spontaneous and enzymatic (in the presence of 2 μM synthetase) hydrolysis

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of Phe-tRNA$^{Phe}_{ox-red}$ revealed a much greater stability than that of native Phe-tRNA^{Phe} ($t_{1/2}$ > 180 min for the spontaneous as well as the enzymatic deacylation of Phe-tRNA$^{Phe}_{ox-red}$ measured in 50 mM Tris-HCl, pH 7.8, and 10 mM $MgCl_2$).

ATP–PP$_i$ Exchange Reaction. The pyrophosphate exchange assay was monitored as described by Fasiolo et al. (1981) with the following modifications. ATP was 5 mM, $MgCl_2$ was 15 mM, [^{32}P]pyrophosphate (pH 7.8) was 2 mM (specific activity 500–2000 cpm/nmol), and phenylalanine was varied from 10 μM to 5 mM in a 100 mM Tris-HCl buffer, pH 7.8. Final concentrations of tRNA^{Phe} or *N*-acetyl-Phe-tRNA^{Phe} in the reaction mixture are specified in the legends to figures. The reaction was initiated at 37 °C by the addition of 5.8 nM phenylalanyl-tRNA synthetase in a final volume of 100 μL. Initial rates were measured after incubation times shorter than 10 min. In order to avoid the aggregation of the charcoal, resulting from the precipitation of tRNA present in the reaction mixture, the reaction was stopped by the addition of 100 μL of 15% perchloric acid containing 0.4 M sodium pyrophosphate. After 10 min on ice, 100 μL of a 4% activated charcoal suspension was added and the samples were vortexed, filtered through glass fiber disks (Whatman GFC), and washed 5 times with 4-mL portions of water.

Stoichiometry of Phenylalanine Transferring Sites. Stoichiometry measurements were performed as described in Baltzinger & Holler (1982). Enzyme–[^{14}C]Phe~AMP was synthesized in situ by the reaction of 4–40 μM [^{14}C]phenylalanine (450 mCi/mmol), 5 mM ATP, and 2 μM phenylalanyl-tRNA synthetase in 100 mM Hepes, pH 7.5, containing 15 mM $MgCl_2$ and 10 units/mL pyrophosphatase, for 5–10 min at room temperature. An aliquot of 35 μL was spotted onto a parafilm sheet that was applied on ice and rapidly mixed with 15 μL of a solution of tRNA^{Phe} containing a large excess of unlabeled phenylalanine. The concentrations after mixing were respectively 30–80 μM tRNA^{Phe} and 20 mM phenylalanine. The amounts of [^{14}C]Phe~AMP and [^{14}C]Phe-tRNA^{Phe} were determined by thin-layer chromatography on cellulose plastic sheets according to Jakubowski et al. (1977), using 1–2 μL of the reaction mixture. Similarly, the amount of [^{14}C]Phe-tRNA^{Phe} was monitored on the remaining mixture by TCA precipitation on paper disks (Whatman 3MM).

Fluorescence Measurements. (*1*) *Fluorescence titrations* of the phenylalanine binding sites were performed in the presence of TNS on a Jobin Yvon (JY 3C) fluorometer as previously described (Baltzinger et al., 1983). In order to avoid any interference between the fluorescence of TNS and that of the Y base of tRNA^{Phe}, the excitation wavelength was set at 366 nm. Emission was observed at 435 nm. It was verified by direct competition experiments with respect to phenylalanine that TNS did not alter the binding of phenylalanine. Indeed, the K_m values for phenylalanine in the aminoacylation reaction, determined in the presence of [TNS] ≤ 100 μM, were found equal to 28 ± 2 μM and 5 ± 1 μM, values that are very close to those described previously by Fasiolo et al. (1974).

(*2*) *Stopped-Flow Experiments.* Kinetics of adenylation in the presence of native tRNA^{Phe} were observed via the fluorescence of added TNS in a Durrum-Gibson stopped-flow apparatus under the conditions previously described (Baltzinger et al., 1983).

(*3*) *Standard Buffer.* Unless otherwise stated all the fluorescence experiments were done in a standard buffer containing 50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 10^{-4} M EDTA, and 20 μM TNS.

RESULTS

Binding of Phenylalanine to the Enzyme in the Presence of Phe-tRNA^{Phe}. (*a*) *Fluorescence Measurements in the Presence of TNS.* The enzyme–TNS complex was titrated with phenylalanine in the presence of *N*-acetyl-Phe-tRNA^{Phe} or Phe-tRNA$^{Phe}_{ox-red}$, which are known to be resistant against hydrolysis. In both cases, the fluorescence intensity of enzyme-bound TNS was observed to decrease in a monophasic manner, covering the concentration range of 0.5 μM–5 mM phenylalanine. The values of the dissociation constants for phenylalanine were calculated from the slope of the linearized plot as described under Figure 1. From Table I, the following observations emerged: (1) In the presence of *N*-acetyl-Phe-tRNA^{Phe}, the strength of the enzyme–phenylalanine interaction was greatly diminished [by a factor of 9 (or 50) when compared to the binding in the absence (or presence) of native tRNA^{Phe}]. (2) tRNA$^{Phe}_{ox-red}$ did not improve markedly the binding of phenylalanine as did native tRNA^{Phe}; however, in the presence of Phe-tRNA$^{Phe}_{ox-red}$, the interaction of phenylalanine with its binding site was decreased by a factor of 4. (3) In the presence of tRNA^{Phe}, the Eadie plot for Phe binding derived from the fluorometric titration remained monophasic, contrary to what was observed by equilibrium dialysis (Fasiolo et al., 1974). Nevertheless, the result still indicates a stronger binding of phenylalanine [K_{Phe}(app) = 10 μM], as compared to the binding in the absence of tRNA^{Phe} [K_{Phe}(app) = 62 μM]. (4) The amplitudes of fluorescence quenching at saturating amounts of phenylalanine were found to be variable,

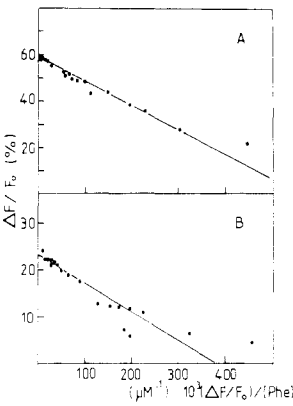


FIGURE 1: Fluorometric titration of enzyme–TNS–tRNA^{Phe} complex (A) and enzyme–TNS complex (B) with phenylalanine. The reaction mixture contained 0.5 μM phenylalanyl-tRNA synthetase with 2.3 μM tRNA^{Phe} (A) or without tRNA^{Phe} (B). The fluorescence quenching is calculated with respect to the initial fluorescence intensity (in the absence of phenylalanine) and is plotted as a function of phenylalanine concentration, according to the method of Eadie (1942). The values of dissociation constants (K_{Phe}) and maximum fluorescence quenching (ΔF_{max}) are given in Table I.

Table I: Fluorometric Study of the Binding of Phenylalanine to Phenylalanyl-tRNA Synthetase in the Absence or Presence of Various tRNA^{Phe} Derivatives[a]

enzyme complexes with	K_{Phe} (μM)[b]	ΔF_{max} (%)[b]
control without tRNA	62	26
tRNA^{Phe} (2.3 μM)	10	57
N-acetyl-Phe-tRNA^{Phe}		
12.5 μM	570	25
5 μM	530	30
tRNA$^{Phe}_{ox-red}$ (3.1 μM)	36	38
Phe-tRNA$^{Phe}_{ox-red}$ (2.2 μM)	120	16

[a] Experimental conditions are 50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 0.1 mM EDTA, 20 μM TNS, and 0.2–0.5 μM phenylalanyl-tRNA synthetase. Concentrations of tRNA^{Phe} present in the mixture are given. [b] The evaluation of the parameters (dissociation constant, K_{Phe}, and maximum fluorescence quenching, ΔF_{max}) is described in the legend of Figure 1. Standard experimental deviations are in the order of 10–20%.

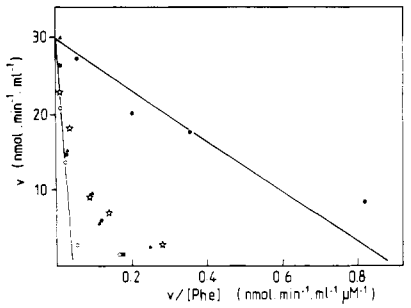


FIGURE 2: Rate of ATP–PP_i isotopic exchange as a function of phenylalanine concentration and inhibition by Phe-$tRNA^{Phe}$ or *N*-acetyl-Phe-$tRNA^{Phe}$. The reaction mixture contained 100 mM Tris-HCl, pH 7.8, 5 mM ATP, 15 mM $MgCl_2$, 2 mM [^{32}P]PP_i (388 cpm/mmol), 5.9 nM phenylalanyl-tRNA synthetase, and 5–5000 μM phenylalanine. Radioactivity incorporated in ATP was measured after 6 min at 37 °C either in the absence (●) or in the presence of 0.32 μM (☆), 1.3 μM (■), or 5 μM (○) *N*-acetyl-Phe-$tRNA^{Phe}$ or 5 μM (▲) Phe-$tRNA^{Phe}$ (actually, in the latter case, the rate of exchange was monitored 7 min after the addition of 5 μM $tRNA^{Phe}$; it was controlled such that the maximum extent of tRNA aminoacylation was already reached after 3 min of reaction). The data were plotted according to Eadie representation (1942).

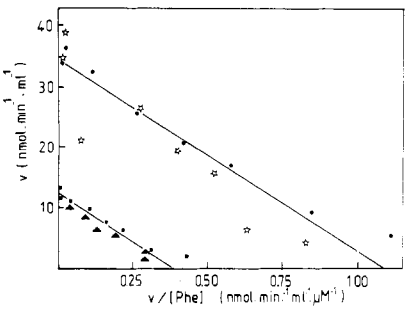


FIGURE 3: Rate of ATP–PP_i isotopic exchange as a function of phenylalanine concentration and inhibition by $tRNA^{Phe}$. Experimental conditions were the same as in Figure 2 except that much larger concentrations of $tRNA^{Phe}$ were used and that the radioactivity incorporated in ATP was measured after 3 min at 37 °C, to reduce the percentage of Phe-$tRNA^{Phe}$ formed during the incubation (it was controlled such that Phe-$tRNA^{Phe}$ never exceeded 4–5% of the input tRNA). The rates of exchange were measured in the presence of 84 μM $tRNA^{Phe}$ (■) or 56 μM $tRNA^{Phe}$ plus 0.5 mM AMP (▲). Control experiments were carried out in the presence of 2 mg/mL noncognate $tRNA^{Tyr}$, either in the absence (●) or in the presence (☆) of 0.5 mM AMP.

depending on the tRNA complexed to the enzyme [see, for example, $tRNA^{Phe}$ and $tRNA^{Phe}_{ox-red}$ (Table I)]. As TNS is a nonspecific reporter group (Holler & Calvin, 1972; Dibbelt, 1981; Baltzinger et al., 1983), this probably reveals different conformations of the complexed enzyme, although no significant changes could be observed for the slight TNS fluorescence increase induced by the binding of $tRNAs^{Phe}$ (native or modified, data not shown). (5) The maximum quenching of TNS fluorescence, at saturating concentration of phenylalanine is 2-fold lower, in the presence of both *N*-acetyl-Phe-$tRNA^{Phe}$ and Phe $tRNA^{Phe}_{ox-red}$ compared to the fluorescence intensity measured in the presence of the deacylated tRNAs.

(*b*) *Competition between Phenylalanine and Phe-$tRNA^{Phe}$ in the ATP–PP_i Exchange Reaction.* The ATP–PP_i exchange reaction, catalyzed by phenylalanyl-tRNA synthetase, is characterized by a Michaelis–Menten constant of 32 μM for phenylalanine and a catalytic rate constant of 84 s^{-1} (Fasiolo et al., 1981; Baltzinger et al., 1983; this study, Figure 2). As can be seen on Figure 2, Phe-$tRNA^{Phe}$, like *N*-acetyl-Phe-$tRNA^{Phe}$, inhibits the exchange reaction in a competitive manner with respect to phenylalanine. The data indicate a significant increase of the K_m for phenylalanine, in the presence of *N*-acetyl-Phe-$tRNA^{Phe}$ concentrations above 1 μM. At lower concentrations of *N*-acetyl-Phe-$tRNA^{Phe}$, the Eadie plots are no longer linear (Figure 2). The value of the Michaelis constant [K_m(Phe)] for the low concentration range of variable substrate is the same as the one measured in the absence of $tRNA^{Phe}$. Such behavior can be explained by assuming that only a fraction of phenylalanyl-tRNA synthetase is saturated with *N*-acetyl-Phe-$tRNA^{Phe}$ and therefore two distinct populations of enzyme with different reaction parameters compete for phenylalanine. It is therefore difficult to analyze the dependence of the apparent K_m for phenylalanine at varying inhibitor concentrations.

Phe-$tRNA^{Phe}_{ox-red}$ was found less effective as an inhibitor even at a concentration of 5 μM (data not shown). This result supports the data obtained by fluorometric titration (see above).

The competitive inhibition between phenylalanine and *N*-acetyl-Phe-$tRNA^{Phe}$ in the ATP–PP_i exchange reaction as well as in the fluorometric titration indicates that the phenylalanine binding site is no longer available when the enzyme is saturated with Phe-$tRNA^{Phe}$.

In order to verify that the observed inhibition is a consequence of $tRNA^{Phe}$ aminoacylation, we looked for the effect of native $tRNA^{Phe}$ on the ATP–PP_i exchange reaction. Owing to the relatively high rate of the aminoacylation reaction at the present enzyme concentration, a very large excess of $tRNA^{Phe}$ was used, so that the amount of Phe-$tRNA^{Phe}$ formed during the time necessary to measure the rate of exchange did not exceed 5% of the input tRNA. The results are illustrated on Figure 3. In that case a noncompetitive behavior is observed, the K_m for phenylalanine being constant, while the V_{max} of the reaction is reduced by a factor of 3.

The specificity of the inhibitory effect of native $tRNA^{Phe}$ was checked by use of the noncognate $tRNA^{Tyr}$. The rate of isotopic exchange was not modified by the addition in the assay of 2 mg/mL $tRNA^{Tyr}$. Furthermore, it was verified that the inhibition of isotopic exchange produced by $tRNA^{Phe}$ is not a consequence of AMP production during aminoacylation. Indeed, the addition of 0.5 mM AMP in both cases (presence or absence of tRNA) did not modify the rate of exchange under our test conditions.

As inhibition by native $tRNA^{Phe}$ proceeds with no change of the K_m for phenylalanine, this behavior suggests that tRNA binding does not alter significantly the ternary phenylalanine–ATP–enzyme complex. This result seems in contradiction with the binding experiments described by Fasiolo et al. (1974), which showed an increase in the affinity of the enzyme–$tRNA^{Phe}$ complex for phenylalanine at least at one of the two binding sites. One must admit that ATP abolishes the positive effect of tRNA on the binding of phenylalanine. This point will be dealt with under Discussion.

Inhibition of the isotopic exchange by native $tRNA^{Phe}$ can result either from a decrease in the affinity for PP_i or from an alteration of the kinetic constants k_f (for the adenylate synthesis) and/or k_b (for the reverse pyrophosphorolysis). Fluorescence stopped-flow experiments were performed in order to determine the way in which occupation of the tRNA binding site controls the activation reaction.

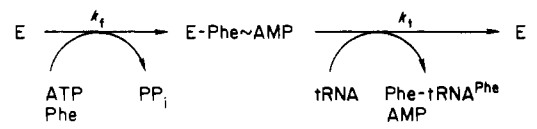
Kinetic Analysis of the Enzyme-Catalyzed Phenylalanyl-adenylate Synthesis and Pyrophosphorolysis in the Presence of $tRNA^{Phe}$. Stopped-flow analysis of the reaction was undertaken to determine the kinetic constants of the activation reaction in the presence of native $tRNA^{Phe}$. The procedure was similar to that reported previously (Holler & Calvin, 1972; Hyafil et al., 1976; Pimmer & Holler, 1979; Mazat et al.,

Table II: Kinetic Parameters of the Activation Step of Phenylalanine in the Absence or Presence of $tRNA^{Phe}$

	parameters	$-tRNA^{Phe}$ [a]	$+tRNA^{Phe}$
synthesis of Phe~AMP	K_{Phe} (μM)	58 ± 8	60 ± 20
	k_f (s^{-1})	100 ± 10	120 ± 30
pyrophosphorolysis	K_{PP_i} (μM)	250 ± 150	600 ± 200
	k_b (s^{-1})	150 ± 50	130 ± 60
ATP–PP_i exchange reaction	K_m(Phe) (μM)	33 ± 3	32 ± 3
	k_{cat} (s^{-1})[b]	85 ± 5	36 ± 2

[a] From Baltzinger et al. (1983). [b] Moles of substrate per mole of enzyme (37 °C).

1982; Baltzinger et al., 1983). The equations describing the synthesis of Phe~AMP were derived according to the reaction scheme



where E = phenylalanyl-tRNA synthetase; Phe~AMP = phenylalanyladenylate, k_f = the kinetic rate constant of adenylation, and k_t = the kinetic rate constant of tRNA acylation; reverse reactions have been neglected because PP_i was hydrolyzed by added inorganic pyrophosphatase and AMP concentrations were assumed to be small with respect to K_{AMP}.

The saturation of phenylalanyl-tRNA synthetase by Phe~AMP was monitored by the time-dependent decrease in TNS fluorescence according to the equation

$$\frac{d(\Delta F)}{dt} \sim \frac{d[E\text{–}Phe\sim AMP]}{dt} = k_f[E]S_{ATP}S_{Phe} - k_t[E\text{–}Phe\sim AMP]S_{tRNA} \quad (1)$$

where S_{ATP}, S_{Phe}, and S_{tRNA} are the saturation fractions by ATP, Phe, and tRNA, respectively. From eq 1 the observed first-order rate constant for phenylalanine activation was derived:

$$k_{obsd} = k_f \frac{[Phe]_0}{K_{Phe} + [Phe]_0} \frac{[ATP]_0}{K_{ATP} + [ATP]_0} + k_t \frac{[tRNA]_0}{K_{tRNA} + [tRNA]_0} \quad (2)$$

Expressions 1 and 2 have been written, according to Baltzinger et al. (1983) and Baltzinger & Holler (1982), by assuming that (i) all the association–dissociation steps are rapid and in preequilibrium with respect to the synthesis of both the adenylate and transfer reactions, (ii) all substrate binding reactions are independent and characterized by equilibrium dissociation constants K_{Phe}, K_{ATP}, and K_{tRNA}, and (iii) coupling between sites (Fasiolo et al., 1974, 1977) was neglected. As to other assumptions, refer to Baltzinger et al. (1983) and Baltzinger & Holler (1982).

As expected from eq 2, the hyperbolic dependence of k_{obsd} as a function of phenylalanine concentration was found at fixed ATP and $tRNA^{Phe}$ concentrations. From the data illustrated in Figure 4A, a rate constant of 120 ± 30 s^{-1} was determined for the synthesis of phenylalanyladenylate in the presence of native $tRNA^{Phe}$ with a K_m for phenylalanine of 60 ± 20 μM. These parameters are quite similar to those determined in the absence of native $tRNA^{Phe}$ (Baltzinger et al., 1983; this paper, Table II). The transfer rate constant (k_t = 8 s^{-1}) that could be derived by extrapolation at zero concentration of phenylalanine (Figure 4A, eq 2) was found to be in agreement with

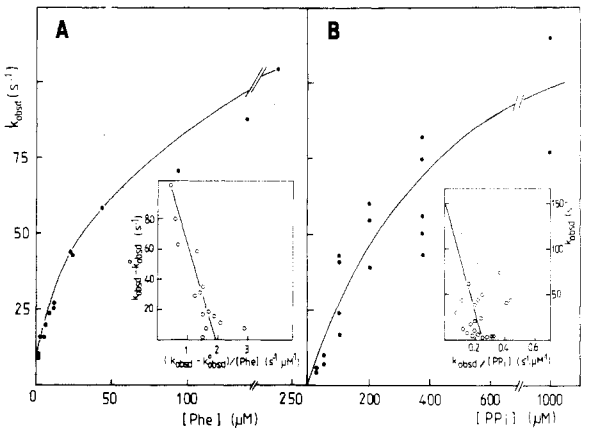


FIGURE 4: (A) Rate of synthesis of phenylalanyladenylate in the presence of native $tRNA^{Phe}$ as a function of phenylalanine concentration. The values of the rate constants k_{obsd} (see eq 1 and 2) were determined by stopped-flow measurements, monitoring the fluorescence quenching of enzyme-bound TNS. As shown by eq 2, the extrapolated ordinate at zero phenylalanine concentration is equal to k_t. Experimental data were fitted with a single exponential decay. The measurements were done in the standard buffer containing 0.2 μM phenylalanyl-tRNA synthetase, 0.7 μM $tRNA^{Phe}$, 3 mM ATP, 3 mM $MgCl_2$, and 5–10 units/mL inorganic pyrophosphatase. (B) Rate of pyrophosphorolysis of phenylalanyladenylate in the presence of native $tRNA^{Phe}$ as a function of pyrophosphate concentration. The enzyme–adenylate complex was prepared in situ in one syringe of the stopped-flow apparatus and mixed with various amounts of PP_i in the presence of a fixed concentration of $tRNA^{Phe}$. Initial concentrations after rapid mixing were 0.2 μM phenylalanyl-tRNA synthetase, 0.4 μM phenylalanine, 20 μM ATP, 5 units/mL inorganic pyrophosphatase, and 1.2 μM $tRNA^{Phe}$ in the standard buffer. In the inserts, data are plotted according to the Eadie representation (1942).

the previous values determined by quenched-flow experiments under similar conditions (25 °C) by Fasiolo & Fersht (1978).

Since $tRNA^{Phe}$ does not affect the rate constant of Phe~AMP synthesis, the decrease of velocity observed in the ATP–PP_i exchange reaction could reflect a much slower rate for the pyrophosphorolysis step or a marked decrease in the affinity for pyrophosphate. We therefore analyzed the increase of TNS fluorescence in the reverse reaction as described previously (Baltzinger et al., 1983). First-order kinetics were observed for the increase of TNS fluorescence. The rate constants exhibited a concentration dependence with respect to PP_i corresponding to a $K_m(PP_i)$ = 600 ± 200 μM and a $k_{pyrophosphorolysis}$ = 150 ± 50 s^{-1} as computed from an Eadie plot of the experimental data (Figure 4B).

The kinetic parameters of the activation step in the absence or presence of native $tRNA^{Phe}$ are summarized in Table II. $tRNA^{Phe}$ increases the value of $K_m(PP_i)$ without affecting the kinetic rate constant for the reverse pyrophosphorolysis. As already mentioned above, no modification was observed for the kinetic parameters of the direct reaction (synthesis of Phe~AMP). Taking into account the PP_i concentration (2 mM) used in the isotopic exchange reaction, the observed decrease in affinity for pyrophosphate could not account for the 3-fold decrease of the V_{max} of this reaction, when measured in the presence of native $tRNA^{Phe}$. Owing to the rather high dead time of the stopped-flow apparatus (5 ms), we could not analyze the amplitudes of both reactions and therefore no information could be gained as to the number of active sites actually activating phenylalanine in the presence of $tRNA^{Phe}$. A complete inhibition of phenylalanine activation at one active site could explain the reduction of V_{max} of the ATP–PP_i exchange reaction. [As already discussed by Fasiolo et al. (1981), the depletion of the adenylate–enzyme concentration due to the transfer reaction can only account for a decrease

Table III: Stoichiometries of Phenylalanyladenylate–Enzyme Complexes and Yield of Transfer to $tRNA^{Phe}$

[Phe] in the incubation mixture (μM)	mol of Phe~AMP formed/mol of enzyme in the absence of tRNA	mol of Phe-$tRNA^{Phe}$ formed/mol of enzyme[a] estimated by		residual Phe~AMP[a] after transfer	yield of transfer (%)	
		TCA precipitation	TLC		TCA precipitation	TLC
24	1.7	1.0	1.3	0.4	59	76
48	1.6	1.1	1.3	0.6	69	81
4.5[b]	1.2	1.0	1.2	0.2	83	100
4.5[c]	1.0	0.85	1.1	0.2	85	110

[a] The yield of transfer was measured under single turnover conditions upon addition of $tRNA^{Phe}$ (75 μM final concentration) solution containing a large excess of unlabeled phenylalanine (20 mM) to preformed enzyme–adenylate complex (2 μM enzyme). [b] Adenylate mixture preincubated for 9 min at room temperature. [c] Adenylate mixture preincubated for 9 min at room temperature plus 9 min at 0 °C.

of the exchange rate lower than or equal to 20%.]

Stoichiometry of Phenylalanine Transferring Sites. In the absence of PP_i and $tRNA^{Phe}$, phenylalanyl-tRNA synthetase synthesizes 2 mol of the Phe~AMP, which remains tightly bound to the enzyme template (Fasiolo et al., 1977; Baltzinger et al., 1983). The fate of this (2:1) enzyme–Phe~AMP complex after mixing with native $tRNA^{Phe}$ was followed under conditions of single-turnover experiments (chase experiment in the presence of an excess of unlabeled phenylalanine as described under Materials and Methods). The results are summarized in Table III. Only 58–68% of the phenylalanine engaged in the adenylate–enzyme complex was found tranferred to tRNA when the TCA-precipitable radioactivity was measured. The ratio of Phe-$tRNA^{Phe}$ formed to the input enzyme never exceeded 1 under these conditions. This incomplete transfer of phenylalanine when more than one active site per enzyme molecule is occupied by phenylalanyladenylate cannot be explained by a partial hydrolysis of the latter upon binding of tRNA, as previously observed for the noncognate tyrosyladenylate (Lin et al., 1984) and *Escherichia coli* phenylalanyladenylate system (Baltzinger & Holler, 1982). Indeed, the missing fraction of radioactive phenylalanine could always be recovered under the form of phenylalanyladenylate by thin-layer chromatography.

Surprisingly, the yield of transfer was much higher (85–100%) when only one or less than one active site per enzyme molecule was initially filled with adenylate. However, it must be stressed that higher yields of transfer were measured when Phe-$tRNA^{Phe}$ was estimated by thin-layer chromatography rather than TCA precipitation. But this could arise from higher blank values, because both Phe-$tRNA^{Phe}$ and phenylalanyl-tRNA synthetase remain at the origin of the chromatogram in the solvent system used. We conclude from the above observations that the binding of $tRNA^{Phe}$ either promotes the dissociation of one of the enzyme-bound adenylates, while the other one is consummed in transferring the phenylalanine moiety to the tRNA, or blocks the reactivity of one Phe~AMP bound to the enzyme. These two explanations would be perfectly consistent with (i) the observed reduction in the V_{max} of the ATP–PP_i exchange reaction (which is therefore efficiently catalyzed at only one of the active sites) and (ii) a biphasic behavior during steady-state aminoacylation of $tRNA^{Phe}$. The simultaneous occurrence of half-of-the-sites reactivity in the ATP–PP_i exchange reaction and biphasic Lineweaver and Burk plots in tRNA aminoacylation will be further discussed later.

Discussion

Nonequivalence of Phe-$tRNA^{Phe}$ and $tRNA^{Phe}$ on the Enzyme Template. The competitive inhibition between Phe-$tRNA^{Phe}$ (or *N*-acetyl-Phe-$tRNA^{Phe}$) and phenylalanine in the ATP–PP_i exchange reaction suggests that phenylalanine either free or linked to the tRNA does bind to the same receptor site of the enzyme. Similar observations have already been reported as well as in the same yeast system (Thiebe, 1982) as in other systems like the *E. coli* phenylalanyl-tRNA synthetase (Holler, 1976; Güntner & Holler, 1979) and methionyl-tRNA synthetase (Jacques & Blanquet, 1976). However, if the decrease in the affinity for phenylalanine observed in the presence of Phe-$tRNA^{Phe}$ would result from a direct competition at the phenylalanine binding subsite, one would expect that Phe-$tRNA^{Phe}$ binding promotes an efficient quenching of TNS fluorescence as does phenylalanine binding. On the contrary, Phe-$tRNA^{Phe}$ slightly enhances the fluorescence of enzyme-bound TNS (10–15%) as does nonacylated $tRNA^{Phe}$ (data not shown). The observed competition would therefore most likely result from allosteric conformational changes, rather than from a direct competition. The allosteric mechanism is further supported by the following observations: (i) *N*-Acetyl-Phe-$tRNA^{Phe}$ strongly interferes with the binding of phenylalanine, although it is known that α-NH_2 modifications of amino acids hinder the fixation to their stereospecific binding sites (Santi & Danenberg, 1971; Holler et al., 1975). (ii) The maximum theoretical ratio of bound phenylalanine per mole of enzyme should be 4 (two phenylalanyl-tRNA and two phenylalanine residues). Indeed, Sephadex chromatography of an enzyme–tRNA complex in the presence of constant phenylalanine and ATP concentration shows that if the stoichiometry rarely reaches 4, it is usely higher than 3 (data not shown). (iii) Free and tRNA-linked phenylalanine behave differently in photoaffinity labeling experiments (Baltzinger et al., 1979, and references cited therein).

Whereas acetylation of the α-NH_2 group of the tRNA-linked phenylalanine does not hinder the competition between the aminoacyl moiety and the free phenylalanine for the enzyme binding sites, on the contrary, modification of the 3′-terminal adenosine of $tRNA^{Phe}$ by oxidation–reduction, which leads to the opening of the ribose ring, also significantly reduces the inhibitory effect of $tRNA^{Phe}_{ox-red}$ on phenylalanine binding. Similar results were already reported for the *E. coli* phenylalanyl system (Baltzinger & Holler, 1982). It should be emphasized that the critical role of the 3′-terminal adenosine of $tRNA^{Phe}$ has already been revealed in the ultraviolet cross-linking experiments (Baltzinger et al., 1979; Renaud et al., 1981) as well as in the study of $tRNA^{Phe}$-induced discrimination between tyrosine and phenylalanine by phenylalanyl-tRNA synthetase (Lin et al., 1984).

While Phe-$tRNA^{Phe}$ decreases the affinity of phenylalanyl-tRNA synthetase for phenylalanine, $tRNA^{Phe}$ has the opposite effect (Fasiolo et al., 1974; Baltzinger et al., 1983; this paper, Table I). Once more, this effect appears to result from a conformational change of the protein triggered by the 3′-terminal adenosine of $tRNA^{Phe}$. Indeed, $tRNA^{Phe}_{ox-red}$ only poorly affects the binding of phenylalanine to phenylalanyl-tRNA synthetase as compared to native $tRNA^{Phe}$. However, it remains to be understood why monophasic titration curves

are observed when the binding of phenylalanine to phenylalanyl-tRNA synthetase in the presence of $tRNA^{Phe}$ is monitored by the fluorescence quenching of TNS, contrary to the biphasic binding shown by equilibrium dialysis. However, it should be stressed that the unique dissociation constant measured by fluorescence titration (10 μM) is intermediate between the two values determined previously by equilibrium dialysis (Fasiolo et al., 1974). This situation is somewhat reminiscent of that observed for the binding of $tRNA^{Phe}$ to phenylalanyl-tRNA synthetase in the presence of ATP. Although two molecules of tRNA are bound per mole of enzyme as seen in the fluorescence of the wybutine residue, the fluorescence quenching of the enzyme tryptophan residues only reports the binding of a single molecule of tRNA (Lefèvre et al., 1980). These results suggest that, in the presence of native $tRNA^{Phe}$ and/or small ligands, the enzyme may behave in an asymmetric manner, leading to an anticooperative binding of phenylalanine.

The noncompetitive inhibition of the ATP–PP_i exchange activity measured in the presence of native uncharged $tRNA^{Phe}$ once more indicates that $tRNA^{Phe}$ and Phe-$tRNA^{Phe}$ behave in a completely different way when binding to the enzyme. As we could show that the direct and reverse rate constants of the activation reaction were not significantly altered in the presence of $tRNA^{Phe}$, one must conclude that "the fraction of active protomers" per enzyme molecule is reduced. The 2–3-fold decrease in the maximal velocity of the exchange reaction would therefore be compatible with a half-of-the-sites reactivity together with a slight decrease of the steady-state level of the aminoacyladenylate–enzyme complex resulting from the transfer reaction.

Symmetric or Asymmetric Behavior of Phenylalanyl-tRNA Synthetase during Catalysis. In the absence of $tRNA^{Phe}$ or in the presence of Phe-$tRNA^{Phe}$, the enzyme appears to behave symmetrically in the amino acid activation reaction, the two protomers being active in the isotopic exchange. Indeed, the maximal velocity of the exchange reaction, measured at saturating concentrations of substrates, is quite compatible with the theoretical value, calculated on the basis of the direct and reverse rate constants of the activation reaction, determined by stopped-flow measurements (Baltzinger et al., 1983; this paper, Table II).

In the presence of uncharged native $tRNA^{Phe}$, an asymmetric behavior of the enzyme is revealed as well in the binding of phenylalanine under noncatalytic conditions as in the Michaelis constant for phenylalanine during steady-state aminoacylation of $tRNA^{Phe}$ (Fasiolo et al., 1974). However, it must be kept in mind that the similarity between the equilibrium dissociation constants measured for phenylalanine by equilibrium dialysis in the presence of tRNA [6 and 30 μM; Fasiolo et al. (1974) confirmed by our results, 4 and 40 μM] and the K_m values in the aminoacylation reaction (3–9 and 28–33 μM; Fasiolo et al., 1977; Berther et al., 1974) is only casual. Indeed, the equilibrium dissociation constants for phenylalanine and the Michaelis constants in the ATP–PP_i exchange reaction and in the tRNA aminoacylation reaction are respectively related by eq 3 and 4:

$$K_m(\text{Phe}) = K_{\text{Phe}}[k_b/(k_f + k_b)] \quad (3)$$

$$K_m(\text{Phe}) = K_{\text{Phe}}[k_t/(k_f + k_t)] \quad (4)$$

where k_f and k_b are respectively the rate constants for the synthesis and pyrophosphorolysis of phenylalanyladenylate and k_t is the transfer rate constant.

According to the experimental values shown in Table II (k_f = 120 s^{-1}; k_b = 150 s^{-1}) and Figure 4A (k_t = 6–8 s^{-1}), the ratio $K_m(\text{Phe})/K_{\text{Phe}}$ would be respectively 0.5 in the activation reaction and 0.05 in the aminoacylation reaction. The K_m(Phe) values reported above for tRNA aminoacylation (3–9 and 28–33 μM) should therefore correspond to equilibrium dissociation constants of 60–180 and 560–660 μM for phenylalanine under catalytic conditions, values much higher than those measured by equilibrium dialysis of phenylalanine in the presence of tRNA (4 and 40 μM; Fasiolo et al., 1974). Once more, ATP appears to antagonize phenylalanine binding. The calculated dissociation constant of 60–180 μM under catalytic conditions is in very good agreement with the 33 μM Michaelis constant measured in the ATP–PP_i exchange reaction (eq 3). However, the 600 μM dissociation constant should correspond to a second Michaelis constant of 300 μM in the activation reaction. It is not yet understood why this second value has not been observed, although the concentration of the variable substrate (phenylalanine) has been varied up to 5 mM. A likely explanation would be that high concentrations of pyrophosphate (used in the exchange reaction but absent in tRNA aminoacylation) still amplify the asymmetric behavior of the enzyme, possibly switching off completely one catalytic center per enzyme molecule (for example, by competition with ATP and/or $tRNA^{Phe}$).

Conclusion

The data presented above described a symmetric behavior of yeast phenylalanyl-tRNA synthetase with two active protomers in the amino acid activation by ATP, in the absence of $tRNA^{Phe}$ as well as in the presence of Phe-$tRNA^{Phe}$. Nonacylated $tRNA^{Phe}$ induces an asymmetric behavior of phenylalanyl-tRNA synthetase, which is expressed by a reduced affinity of one of the two enzyme protomers for phenylalanyladenylate. The data are consistent with two nonequivalent binding sites for phenylalanine in the presence of $tRNA^{Phe}$. Our results show that phenylalanyl-tRNA synthetase differentiates between binding of $tRNA^{Phe}$ and Phe-$tRNA^{Phe}$ and regulates its catalytic activity at least in vitro by varying its fraction of saturation by phenylalanine when complexed with free or esterified $tRNA^{Phe}$.

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Direct Photoaffinity Labeling of Tubulin with Guanosine 5′-Triphosphate†

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ABSTRACT: Irradiation of tubulin in the presence of [^{3}H]GTP or [^{3}H]GDP at 254 nm led to the covalent incorporation of nucleotide into the protein. The specific nature of the labeling was shown in the following manner: with tubulin depleted of exchangeable nucleotide, the amount of labeling increased to a plateau value as the [^{3}H]GTP concentration was increased, with saturation being reached at a ratio of approximately 1.5; the same amount of labeling was obtained with GTP/tubulin ratios of 1 and 100; [^{3}H]GMP was not incorporated into the dimer, nor did GMP inhibit the incorporation of [^{3}H]GTP; [^{3}H]ATP was not incorporated; [^{3}H]GTP incorporation did not occur into denatured tubulin or into serum albumin. When [α-^{32}P]GTP was used in the irradiation experiments, sodium dodecyl sulfate–polyacrylamide gel electrophoresis of the carboxymethylated protein demonstrated that the incorporated label was associated with the β subunit. The radiation treatment did cause changes in the tubulin molecule resulting in a decrease in assembly competence and in sulfhydryl groups, but these effects were minimized when a large excess of GTP was present during irradiation. Labeling of tubulin in the assembled state was much less than that observed in the free state.

The principal protein component of microtubules (MTs),[1] tubulin, is a dimer of M_r 100 000. The dimer, which consists of nonidentical monomers, α and β, contains 2 mol of guanine nucleotide, one at a readily exchangeable site and the other at a nonexchangeable site (Weisenberg et al., 1968). Upon polymerization of tubulin to form MTs, the exchangeable nucleotide is hydrolyzed to GDP (Kobayashi, 1975; Weisenberg et al., 1976), although under some conditions hydrolysis is not coincident with assembly (Carlier & Pantaloni, 1981). In addition, assembly can occur in the presence of nonhydrolyzable GTP analogues (Weisenberg et al., 1976; Arai & Kaziro, 1976). At the present time the role GTP plays in the polymerization process is unclear.

To localize and characterize the exchangeable GTP site, use has been made of photoaffinity analogues of GTP, such as 8-azido-GTP (Geahlen & Haley, 1979) and 3′-(*p*-azidobenzoyl)-GTP (Maccioni & Seeds, 1983) as well as the periodate oxidation product of GTP 2-[(guanylformyl)methoxy]-3,3,3-triphosphopropanal (Maccioni & Seeds, 1983; Kirsch & Yarbrough, 1981). Geahlen & Haley (1979) concluded that the 8-azido-GTP binds to the β subunit, although significant nonspecific binding to the α subunit also occurred. More recently, Haley et al. (1983) reported that the α subunit was labeled exclusively by this photoaffinity analogue. Maccioni & Seeds (1983) found that their analogues bound equally well to the α and β subunits but the binding was

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[1] Abbreviations: Mes, 2-(*N*-morpholino)ethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; Pipes, 1,4-piperazinediethanesulfonic acid; MTs, microtubules; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); DTT, dithiothreitol; Me_2SO, dimethyl sulfoxide; PED buffer, 100 mM Pipes, 1 mM EGTA, and 1 mM DTT, pH 6.9; PEMD buffer, PED buffer containing 1 mM $MgSO_4$; PEI, poly(ethylenimine).

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