Yeast Phenylalanyl-tRNA Synthetase: Symmetric Behavior of the Enzyme during Activation of Phenylalanine As Shown by a Rapid Kinetic Investigation†

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ABSTRACT: The adenylation of phenylalanine catalyzed by phenylalanyl-tRNA synthetase was investigated in the absence of tRNA, by rapid kinetic measurements using 6-(*p*-toluidinyl)naphthalene-2-sulfonate (TNS) as a nonspecific fluorescent reporter group. It is shown that each protomer of the enzyme is able to catalyze independently the adenylation of phenylalanine by ATP, as well as the reversion by pyrophosphate, at least in the absence of tRNA. The kinetic rate constants of synthesis and pyrophosphorolysis are respectively found equal to 100 ± 20 s^{-1} and 150 ± 50 s^{-1}. The symmetric behavior of the enzyme is consistent with a symmetric binding of 2 mol of phenylalanine to the enzyme as shown by equilibrium dialysis experiments. The affinity of phenylalanyladenylate for the enzyme could be characterized by an equilibrium constant of 0.2 × 10^9 M^{-1}.

Phenylalanyl-tRNA synthetase has been shown to belong to the tetrameric class of aminoacyl-tRNA synthetases, consisting of nonidentical subunits ($\alpha_2\beta_2$). Functionally the enzyme could be characterized as a dimer by the presence of two active sites for phenylalanyladenylate and $tRNA^{Phe}$ (Fasiolo et al., 1974, 1977). Fluorescence equilibrium measurements (Lefèvre et al., 1980) as well as steady-state kinetic studies (Fasiolo et al., 1974, 1977, 1981) revealed negative interactions between various couples of ligands leading to an asymmetric behavior of the enzyme.

In the present work, we were first interested in the activation of phenylalanine by ATP, the first step of the reaction catalyzed by phenylalanyl-tRNA synthetase. For better insight into the mode of interaction between the two active sites, a pre-steady-state kinetic analysis of the reaction was undertaken, in the absence of tRNA.

Experimental Procedures

Materials

Native phenylalanyl-tRNA synthetase (EC 6.1.1.20) was purified to homogeneity from bakers' yeast by using the standard procedure described previously (Fasiolo & Ebel, 1974). The specific activity was 3200–3600 nmol min^{-1} mg^{-1} with 2 mol of active sites/mol of enzyme (Fasiolo et al., 1977). Inorganic pyrophosphatase (EC 3.6.1.1) (specific activity 500–600 units mg^{-1}), 6-(p-toluidinyl)naphthalene-2-sulfonate (TNS),[1] and ATP were purchased from Sigma (St. Louis, MO). Uniformly labeled L-[^{14}C]phenylalanine (400–500 Ci/mol) was obtained from the Commissariat à l'Energie Atomique (France). All other reagents were of the highest purity grade from Merck (Darmstadt, Germany). Dialysis bags were obtained from Union Carbide Corp. (Chicago, IL).

Methods

Unless otherwise stated all the experiments were done in a standard buffer containing 50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 0.1 mM EDTA, and 0.2–0.5 mM dithioerythritol.

Equilibrium dialysis was performed in eight Lucite cells designed according to Furlong et al. (1972). Each compartment was filled with 50–60 μL of the appropriate solution in the standard buffer containing 10% glycerol. One chamber of each cell was filled with phenylalanyl-tRNA synthetase (20 μM) and the other with [^{14}C]phenylalanine (8–1000 μM, 10 Ci/mol). The dialysis was run for 4–15 h at 4 °C, and samples of 40 μL or duplicates of 20 μL were withdrawn from each chamber and counted in 5 mL of Bray's solution.

Nonequilibrium Dialysis. The method of Colowick & Womack (1969) has been previously used to measure the stoichiometry of enzyme·aminoacyladenylate complex or the binding of amino acids or aminoacyl-tRNA to aminoacyl-tRNA synthetases (Kosakowski & Böck, 1971; Hyafil et al., 1976; Güntner & Holler, 1979). Here this technique was applied to the determination of the kinetic rate constant for the dissociation of the phenylalanyladenylate·enzyme complex.

The upper chamber (V = 0.45 mL) contained in the standard buffer 3.5 μM enzyme which was previously incubated 5 min at 37 °C in the presence of 6.6 μM [^{3}H]-phenylalanine (40 Ci/mmol), 13 mM ATP/$MgCl_2$, and 7 units/mL inorganic pyrophosphatase to saturate the enzyme with labeled phenylalanyladenylate. The lower chamber of 200 μL was flushed at a constant rate (4 mL/min) with the standard buffer.

Stirring of the upper and lower chambers was respectively ensured by a rotating glass stick and a magnet.

The measurement of the dialysis rate was carried out at room temperature. So that the diffusion rate of aminoacyl-adenylate through the membrane could be increased, the latter was made more porous by $ZnCl_2$ treatment under the following conditions: dialysis bags previously moistened were immersed in a solution of $ZnCl_2$ (320 g of $ZnCl_2$ + 180 mL of H_2O) for 6 min, then rinsed 3 times in 1 mM HCl, and finally extensively washed in water. Dissociation of labeled phenylalanyladenylate was followed after addition of 150 μL of 4 mM chemically synthesized phenylalanyladenylate (Berg, 1958) (chase experiment). Fractions of 1 mL were collected at the exit of the lower chamber. Aliquots (0.4 mL) were diluted in 5 mL of Beckman Ready Solv M.P. solution for

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[1] Abbreviations: TNS, 6-(*p*-toluidinyl)naphthalene-2-sulfonate; EDTA, (ethylenedinitrilo)tetraacetic acid; Phe, phenylalanine; Phe~AMP, phenylalanyladenylate; PP_i, inorganic pyrophosphate; DTE, dithioerythritol; Tris, tris(hydroxymethyl)aminomethane.

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liquid scintillation counting. The radioactivity was determined in an Intertechnique scintillation counter (Model SL 30).

Fluorescence Measurements with 6-(p-Toluidinyl)-naphthalene-2-sulfonate (TNS) as Indicator. (*a*) *Corrected emission spectra* were recorded by using a Fica 55 spectrofluorometer. The excitation and the emission monochromators were operated with bandwidths of 5 and 7.5 nm. The excitation wavelength was 330 nm.

(*b*) *Fluorescence Titration.* The association of phenylalanyl-tRNA synthetase and ligand was monitored by the fluorescence quenching of enzyme-bound TNS in a Jobin-Yvon 3C fluorometer with an excitation wavelength of 330 or 360 nm and an emission wavelength of 430–435 nm (bandwidths 10 nm). Aliquots (1–2 μL) of ligand solution (typically 10^{-3}–10^{-4} M) were added from a Hamilton syringe to 0.4–1 mL of enzyme·TNS solutions. The concentrations of TNS usually used were in the range 10–20 μM. Dissociation constants were determined from Scatchard-type plots (1949) taking into account the stoichiometry of the E·L complex determined by equilibrium dialysis. The concentration of ligand bound to the enzyme could be calculated as [L bound] = [active sites]$_0(\Delta F/\Delta F_{max})$. The symbol ΔF refers to the observed fluorescence quenching resulting from the addition of ligand and ΔF_{max}, the maximum decrease of fluorescence at saturating concentrations of ligand.

Rapid Kinetic Measurements. Stopped-flow fluorescence measurements were done on a Durrum-Gibson D-110 instrument equipped with the Durrum fluorescence accessory. Excitation at 366 nm of the enzyme-bound TNS was ensured by a 100-W high-pressure Hg lamp (HBO 100W/2, Osram, Germany). An UG11 Schott filter was set at the entrance of the monochromator. Fluorescence was observed at 90 °C through a low-pass Kodak–Wratten filter type 2B (L9101). Reaction was initiated at 22–25 °C. Both solutions contained TNS at a concentration of 20 μM. All concentrations given in the text correspond to the final values after mixing. The dead time of the spectrometer was 4–5 ms. Fluorescence intensities were stored in a transient analyzer (Datalab or Biomation with dual-time base) and transferred into a Computer Automation LSI 2/20. One thousand to two thousand fluorescence values were stored per experiment. Determination of the first-order rate constant was performed by the computer with the help of nonlinear iterative regression procedures.

Results

(*1*) *Effect of Ligand Binding on the Fluorescence of Enzyme-Bound TNS.* In the presence of yeast phenylalanyl-tRNA synthetase, TNS exhibits a strong enhancement of fluorescence similarly to what has been described for the *Escherichia coli* isoleucyl-tRNA synthetase (Holler et al., 1971) and phenylalanyl-tRNA synthetase (Holler & Kosakowski, 1973). The addition of phenylalanine or ATP to a mixture containing enzyme and TNS leads to a decrease of the fluorescence intensity of the enzyme-bound TNS. A 25% quenching is observed in the presence of 1 mM ATP (Figure 1). Under conditions where the synthesis of phenylalanyl-adenylate occurs (in the presence of both ATP and phenylalanine) a 70% quenching of the fluorescence is observed. These changes allowed us to analyze the reactions of phenylalanyladenylate formation and of its reversion by pyrophosphate, as well as the interaction of phenylalanyl-tRNA synthetase with phenylalanine and phenylalanyladenylate.

(*2*) *Binding of Phenylalanine to Phenylalanyl-tRNA Synthetase.* The equilibrium binding of phenylalanine was monitored by measurements of enzyme·TNS fluorescence or equilibrium dialysis. The dissociation constant (K_{Phe}) of the

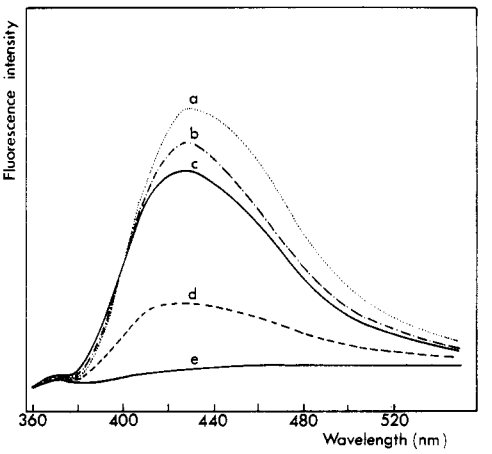


FIGURE 1: Corrected emission spectra of phenylalanyl-tRNA synthetase·TNS complex (a), in the presence of 1 mM ATP/$MgCl_2$ (b), 1 mM Phe (c), and 1 mM phenylalanine plus 1 mM ATP/$MgCl_2$ (d). Fluorescence of free TNS (e). Mixtures were buffered with 50 mM Tris-HCl (pH 7.5) and 6 mM $MgCl_2$. Enzyme was 0.2 μM and TNS 20 μM. Excitation was at 330 nm ($\Delta\lambda_{exc}$ = 5 nm, $\Delta\lambda_{em}$ = 7.5 nm). The small maximum at 370 nm corresponds to the Raman diffusion of water.

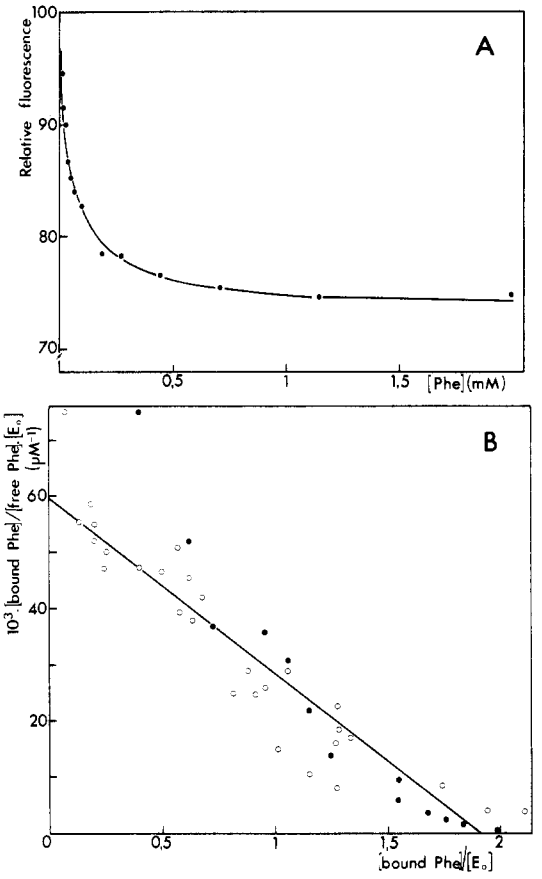


FIGURE 2: Binding of phenylalanine to phenylalanyl-tRNA synthetase and to phenylalanyl-tRNA synthetase·TNS complex. The data were obtained in 50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 0.1 mM EDTA, and 0.2 mM DTE. (Panel A) Fluorescence titration. (Panel B) Data plotted according to Scatchard (1949). Equilibrium dialysis in the presence of 10–20 μM phenylalanyl-tRNA synthetase and variable amounts (5–1000 μM) of [^{14}C]phenylalanine (○). Fluorescence titration in the presence of 10 μM phenylalanyl-tRNA synthetase and 10 μM TNS (●). The concentration of bound phenylalanine was calculated from [Phe bound] = [active sites]$(\Delta F/\Delta F_{max})$.

enzyme·Phe complex was found equal to 32 μM as is illustrated by the Scatchard plot in Figure 2B. The stoichiometry of

Table I: Kinetic Parameters and Equilibrium Constants of the Synthesis and Pyrophosphorolysis of Phenylalanyladenylate Catalyzed by Phenylalanyl-tRNA Synthetase

parameters	analysis at equilibrium	stopped-flow analysis
K_{Phe} (μM)	32 ± 3[a] (n = 2) 36[b] (n = 1) 30 ± 10[c]	58 ± 8
K_{ATP} (mM)	1.1[c] 0.8[c]	0.68 ± 0.05
$K_{PP_i}^{Phe\sim AMP}$ (mM)	0.08[c]	0.25 ± 0.15
$K_{Phe\sim AMP}$ (nM)	4.4	
k_f (s^{-1})		100 ± 10[d] 80 ± 10[e]
k_b (s^{-1})		150 ± 50
k_f/k_b		0.6

[a] Equilibrium dialysis, this work; two binding sites observed. [b] Fasiolo et al. (1977): equilibrium dialysis, one binding site observed. [c] Fasiolo et al. (1977, 1981), Michaelis constant in the ATP-PP_i exchange reaction; kinetics were monophasic. [d] This work, Figure 5A. [e] This work, Figure 5B.

phenylalanine binding was checked by radioactive measurements taken in parallel (Table I and Figure 2B). The results clearly showed two binding sites per mol of phenylalanyl-tRNA synthetase which exhibit the same affinity for phenylalanine, in contrast to earlier results (Fasiolo et al., 1977); this point is taken up under Discussion.

The dissociation constant determined by fluorescence titration in the presence of TNS (Figure 2A) is in excellent agreement with the value obtained from the equilibrium dialysis experiment. This allows us to rule out any interference between the binding of TNS and phenylalanine to the enzyme.

(*3*) *Interaction of Phenylalanyladenylate with Phenylalanyl-tRNA Synthetase.* (*a*) *Fluorescence Measurements.* The stoichiometry of phenylalanyladenylate bound to the pseudo-dimeric phenylalanyl-tRNA synthetase has been studied earlier by different radioactive methods (Fasiolo et al., 1977). The results clearly indicated the binding of two phenylalanyladenylate molecules per mol of enzyme. The experiments performed in this study deal with the optical equivalence of the two active sites when adenylation is followed in the presence of TNS. It is indeed necessary to know whether the fluorescence changes observed during phenylalanyladenylate synthesis reflect the functioning of both sites. Figure 3 illustrates the magnitude of fluorescence quenching as a function of phenylalanine concentration which reacts with an ATP-enzyme complex in the presence of pyrophosphatase. The stoichiometry of synthesized phenylalanyladenylate was close to 2 (Figure 3A; n = 1.7). If the titration is performed with phenylalanyl-tRNA synthetase which was previously incubated with an equimolecular amount of phenylalanine and an excess of ATP in order to saturate the enzyme with 1 mol of phenylalanyladenylate, only one adenylate is titrated (Figure 3, panel B). In this case, the observed fluorescence quenching of enzyme-bound TNS is nearly half that observed when titration is started with "free" enzyme·ATP complex. The final magnitude of fluorescence of the phenylalanyladenylate·enzyme·TNS complex is 25–30% that of the enzyme·TNS complex. These results indicate that the fluorescence change is rigorously proportional to the fraction of occupied sites. Titration of the Phe·enzyme complex with ATP yielded erratic data probably because of ATP hydrolysis.

(*b*) *Equilibrium Constant for Phenylalanyladenylate Binding.* The strength of the enzyme–phenylalanyladenylate interaction was determined by measurement of the association and dissociation rate constants of the phenylalanyladenylate·enzyme complex. The association, as followed by

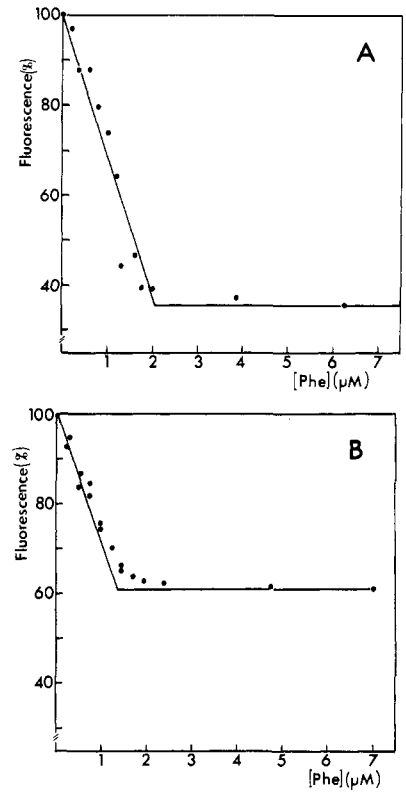


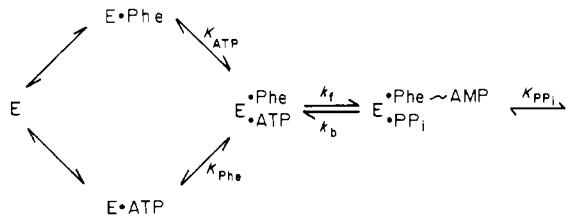
FIGURE 3: Fluorescence titration of phenylalanyl-tRNA synthetase under conditions of phenylalanyladenylate formation. (Panel A) Enzyme (1.2 μM) titrated by phenylalanine in the presence of 3.5 mM ATP/$MgCl_2$. (Panel B) Enzyme (1.3 μM) incubated with 1.2 μM phenylalanine and 0.48 mM ATP/$MgCl_2$ prior to titration with phenylalanine. Experiments were carried out in standard buffer containing 10–20 μM TNS and 12 units/mL inorganic pyrophosphatase. The stoichiometries are obtained by calculating the Phe concentrations which correspond to the intersections of the final plateau with the initial slope of the curve.

fluorescence stopped-flow analysis, is characterized by a rate of 1.6×10^6 M^{-1} s^{-1} (Lin et al., 1983). But this technique was not useful to evaluate the dissociation rate constant because of its low value. We used therefore the technique of non-equilibrium dialysis described by Colowick & Womack (1969). Dissociation of [^{3}H]Phe~AMP bound to the enzyme was followed in a chase experiment by mixing a large excess (1 mM) of unlabeled chemically synthesized phenylalanyladenylate with the radioactive complex. As shown in Figure 4, [^{3}H]Phe~AMP diffused through the membrane with a rate constant of 7.2×10^{-3} s^{-1}. This rate of diffusion is limited by the dissociation of the complex as indicated by a control experiment where dissociation of the complex was achieved prior to dialysis. In this case we observed a much higher rate for the diffusion of [^{3}H]Phe~AMP through the membrane.

The equilibrium of phenylalanyladenylate·enzyme complex is thus characterized by a dissociation constant of 4.4×10^{-9} M.

(*4*) *Kinetic Analysis of the Enzyme-Catalyzed Phenylalanyladenylate Synthesis and Its Reversion by Pyrophosphate.* In the previous section, it has been shown that TNS was a good reporter group to monitor the formation of phenylalanyladenylate catalyzed by phenylalanyl-tRNA synthetase. Stopped-flow analysis of the reaction was therefore undertaken to determine the kinetic constants of the activation reaction. The procedure was similar to that reported previously (Holler & Calvin, 1972; Hyafil et al., 1976; Pimmer & Holler, 1979; Mazat et al., 1982).

Results were analyzed according to the scheme



E•Phe~AMP + PP$_i$

The time dependencies of phenylalanyladenylate formation and of enzyme-bound TNS fluorescence changes followed the same rate constant which was expressed by

$$k_{obsd} = k_f\left(\frac{[Phe]_0}{K_{Phe} + [Phe]_0}\right)\left(\frac{[ATP]_0}{K_{ATP} + [ATP]_0}\right) + k_b\left(\frac{[PP_i]_0}{K_{PP_i} + [PP_i]_0}\right) \quad (1)$$

Expression 1, which has been written for monomeric enzymes, can be used for oligomeric enzymes, provided that the active sites are independent and equivalent. Furthermore, the following conditions have to be fulfilled:

(i) Synthesis and pyrophosphorolysis of the adenylate are the rate-limiting steps of the overall scheme. A consequence is that all the association–dissociation reactions can be considered in preequilibrium. (ii) The binding of phenylalanine and ATP is independent. Therefore

$$K_{Phe} = \frac{[E][Phe]}{[E\cdot Phe]} \qquad K_{ATP} = \frac{[E][ATP]}{[E\cdot ATP]}$$

(iii) The dissociation constant for PP$_i$ is defined with respect to the enzyme·adenylate species:

$$K_{PP_i} = \frac{[E\cdot Phe\sim AMP][PP_i]}{\left[\begin{matrix}\cdot Phe\sim AMP \\ E\cdot PP_i\end{matrix}\right]}$$

(iv) The rate of dissociation of aminoacyladenylate·enzyme complex is low enough to assume that all synthesized adenylate remains bound to the enzyme in the time scale of the experiment. (v) Competition between PP$_i$ and ATP is neglected. (vi) Substrate concentrations remain constant during the reaction

$$[E]_0 \ll [Phe]_0, [ATP]_0, [PP_i]_0$$

Evaluation of the kinetic and equilibrium parameters according to the reaction scheme described above was done by studying the concentrations dependencies of k_{obsd}.

The interference of the back-reactions was usually eliminated by choosing appropriate experimental conditions: (i) in the case of synthesis reaction, either by working in the presence of pyrophosphatase or by choosing an enzyme concentration low enough so that the concentration of the PP$_i$ produced during the experiment remains very low with respect to its dissociation constant; (ii) in the case of the pyrophosphorolysis reaction, the rate of adenylate formation was usually made negligible by keeping the enzyme concentration low enough. When the rate of synthesis could not be neglected, the measured k_{obsd} were corrected for the synthesis.

The results are illustrated in Figures 5 and 6. The values of the kinetics and equilibrium constants are summarized in Table I.

(*a*) *Phenylalanyladenylate Synthesis.* The synthesis of phenylalanyladenylate was visualized as a decrease in fluorescence intensity of TNS bound to the enzyme. The amplitude of the reaction indicated the occupation of both sites by phenylalanyladenylate. Indeed at low concentration of ATP or phenylalanine, where the reaction within the dead time of the stopped flow was negligible, a 60–70% quenching of fluorescence was observed (after subtraction of the blank signal), a value which corresponds to the saturation of the two active sites by phenylalanyladenylate (see above).

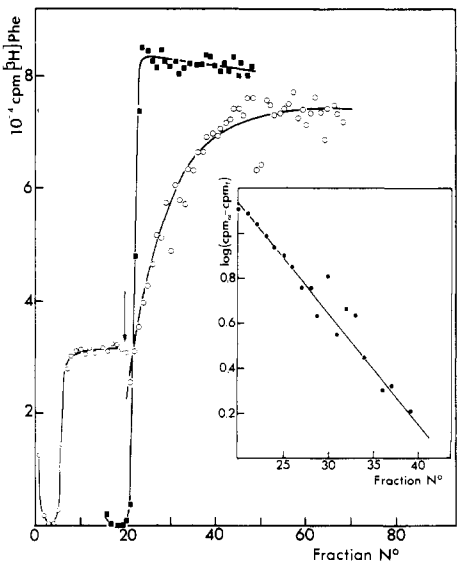


FIGURE 4: Nonequilibrium dialysis. Phenylalanyl-tRNA synthetase (3.5 μM) was incubated for 2 min at 37 °C prior to dialysis, in 450 μL of standard buffer containing 6.6 μM [^{3}H]phenylalanine (40 Ci/mmol), 13 mM ATP/$MgCl_2$, and 11 units/mL inorganic pyrophosphatase. Dialysis was performed at 22 °C. After radioactivity in the effluent reached a steady state ($\simeq$30 000 cpm/aliquot) 150 μL of chemically synthesized unlabeled phenylalanyladenylate was added as indicated by the arrow, to give a final concentration of 1.1 mM (chase). Fractions of 1 mL were collected at the exit of the lower chamber (10 fractions/158.5 s), and radioactivity was measured on a 400-μL aliquot of each fraction. A control was done with [^{3}H]-Phe~AMP·enzyme complex synthesized in the same conditions but mixed with unlabeled phenylalanyladenylate 12 min before dialysis (■). (Insert) First-order plot gives a rate constant of $k = 7.2 \times 10^{-3}$ s^{-1} for phenylalanyladenylate dissociation.

In all the concentration ranges investigated (3 mM ATP/$MgCl_2$ and 0.005–0.5 mM phenylalanine, Figure 5, panel A; or 0.5 mM phenylalanine and 0.01–4 mM ATP/$MgCl_2$, Figure 5, panel B) the decrease of fluorescence intensity followed single exponential curves. As indicated in Figure 5, panel A, the presence of pyrophosphatase had no effect on the kinetics. Results were the same whether phenylalanyl-tRNA synthetase was preincubated with either phenylalanine or ATP/$MgCl_2$ or directly mixed with both reactants. The results indicate that under our conditions both sites of phenylalanyl-tRNA synthetase catalyze the synthesis of phenylalanyladenylate simultaneously and independently.

(*b*) *Phenylalanyladenylate Pyrophosphorolysis.* Pyrophosphorolysis was followed by an increase of the fluorescence intensity of TNS bound to the enzyme·phenylalanyladenylate complex. The dependence of the rate constants as a function of PP$_i$ concentration is illustrated in Figure 6. In all cases single exponential curves were obtained. Data were similar whether one site or both sites were occupied by phenylalanyladenylate. In the case where pyrophosphorolysis was followed at one site, the amplitude of the reaction characterized by an increase of the fluorescence intensity was the half of that observed when both sites were working. Kinetic and equilibrium parameters of the reaction are summarized in Table I.

Discussion

We have shown that TNS serves well as an accurate reporter group in the activation of phenylalanine catalyzed by yeast

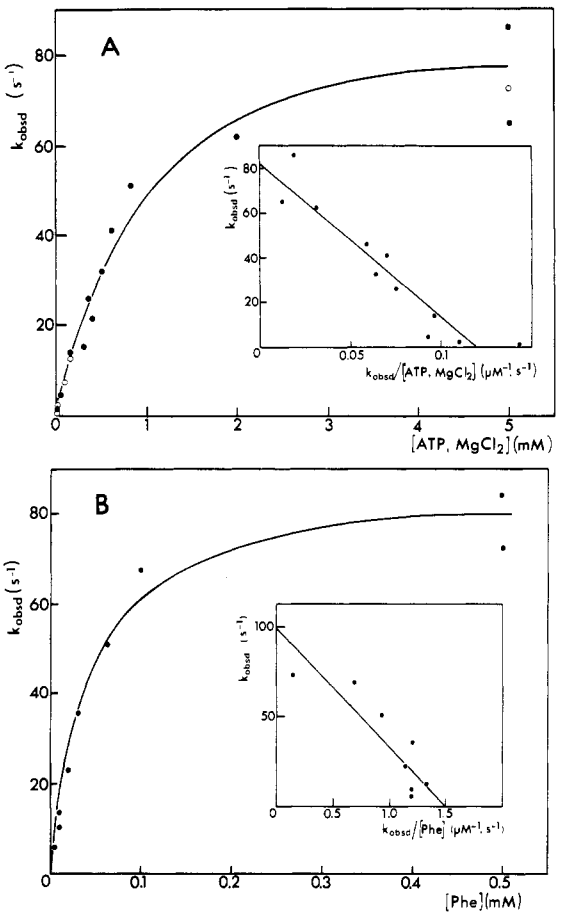


FIGURE 5: Rate of synthesis of phenylalanyladenylate as a function of substrate concentrations. The values of the rate constants (k_{obsd}) were determined by stopped-flow measurements from the time-dependent decrease in fluorescence intensity of enzyme-bound TNS, which was fitted with a single exponential. Measurements were made in the standard buffer (22–25 °C) containing 0.2 μM phenylalanyl-tRNA synthetase, 20 μM TNS, and 0.5 mM phenylalanine (panel A) or 3 mM ATP/$MgCl_2$ (panel B) without (●) or with (○) 10 units/mL inorganic pyrophosphatase. Data were plotted according to Eadie (1942) in the figure insert.

phenylalanyl-tRNA synthetase. Although the fluorescence emission of the bound dye is affected by the presence of substrates, it has been shown by Dibbelt (1981) that TNS had no effect on the Michaelis constant for phenylalanine and ATP when the aminoacylation of tRNA was followed. This is an indication that the catalytic site is not directly involved in the binding of TNS to phenylalanyl-tRNA synthetase. The fluorophore was therefore used as a nonspecific reporter group. In such fluorescence studies the choice of TNS and protein concentrations is extremely important for the determination of dissociation constants (McClure & Edelmann, 1967). With the conditions used in this work ([E] $\lesssim$ [TNS] $\ll K_{TNS}$ = 0.4 mM; Dibbelt, 1981) no interference was observed between TNS and substrate binding.

Binding of Phenylalanyladenylate. The fluorescence quenching of the enzyme-bound TNS in the presence of ATP and phenylalanine corresponds to the synthesis of enzyme·phenylalanyladenylate complex. Indeed, in the absence of pyrophosphate (addition of pyrophosphatase to the reaction mixture), maximal quenching of fluorescence is observed even at very low concentrations of the substrates, which, when the affinities for ATP and amino acid are taken into account, would only lead to a very low saturation fraction. The sum of the fluorescence quenchings observed respectively in the presence of saturating concentrations of phenylalanine and

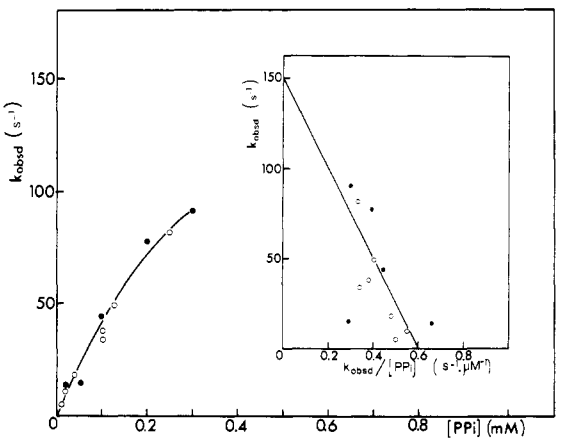


FIGURE 6: Stopped-flow analysis of the pyrophosphorolysis of phenylalanyladenylate. Phenylalanyladenylate·enzyme complex was prepared in one syringe by preincubation of 0.4 μM phenylalanyl-tRNA synthetase, 100 μM ATP/$MgCl_2$, 50 μM phenylalanine, and 5 units/mL inorganic pyrophosphatase in the first set of experiments (●). In the second set of experiments, pyrophosphorolysis was followed at one active site only, by preincubation of 0.4 μM enzyme, 0.4 μM phenylalanine, 20 μM ATP/$MgCl_2$, and 10 units/mL pyrophosphatase (○). The other syringe contained various amounts of PP_i in the presence of Phe and ATP at the same concentration as in the first syringe. Standard buffer contained 20 μM TNS in both syringes. Values of k_{obsd} (●) have been corrected for the resynthesis of phenylalanyladenylate (k = 6 s^{-1}).

ATP ($\Delta F \lesssim 60\%$) is a little bit less than the quenching due to Phe~AMP synthesis (ΔF = 70–75%), which could mean that some rearrangement of the phenylalanyladenylate·enzyme complex occurs, which is responsible for the observed difference. Titration of phenylalanyl-tRNA synthetase by phenylalanine in the presence of nearly saturating concentrations of ATP, which gives an equivalence of 2 mol of amino acid added per mol of enzyme, also demonstrates the "optical equivalence" of both sites (at a macroscopic level), since for a saturation fraction of 0.5, the decrease of fluorescence is half the maximal quenching. However, at the present time, we cannot conclude that the affinities of both sites for Phe~AMP are really identical, since the enzyme concentration in the titration experiment is much higher than the dissociation constant of the complex. If they are not, the observed rate constant for phenylalanyladenylate dissociation, which has been determined by nonequilibrium dialysis, will be an apparent rate constant combining the microscopic dissociation constants on both sites. Indeed the time constant observed for the appearance of dialyzable radioactivity in the nonequilibrium dialysis experiment reflects the dissociation of the enzyme·Phe~AMP complex, since (i) prior to the addition of an excess of unlabeled Phe~AMP, the concentration of the enzyme·Phe~AMP complex remains constant due to the large excess of ATP (this is shown by the constancy of the plateau value for the radioactivity, reflecting a constant level of the free labeled species) and (ii) after the chase experiment, the free adenylate is in large excess (>300-fold) with respect to the enzyme, which allows us to neglect the hydrolysis on the enzyme template; as a control the spontaneous rate of hydrolysis has been measured and found to lie in the range of 10^{-3} s^{-1} (result not shown), which is much lower than the rate constant observed for the dissociation of the enzyme·adenylate complex under the same conditions (7×10^{-3} s^{-1}).

Binding of Phenylalanine. In earlier studies (Fasiolo et al., 1977), it had been reported that phenylalanyl-tRNA synthetase bound only 1 mol of phenylalanine/mol of $\alpha_2\beta_2$ enzyme, in the absence of other ligands. However, the enzyme was observed to synthesize 2 mol of phenylalanyladenylate in the

Table II: Comparison between the Experimental Kinetic Parameters of the ATP-PP_i Exchange Reaction and Calculated Values on the Basis of the Stopped-Flow Analysis

varying substrate	fixed substrates (mM)	k_{cat}[a] (s^{-1}) exptl	k_{cat} calcd[b] step a	k_{cat} calcd[b] step b	K_m (mM) exptl	K_m calcd[b] step a	K_m calcd[b] step b
Phe	ATP (2),[c] PP_i (2)	28	18	48	ND	0.035	0.026
	ATP (1),[d] PP_i (0.2)	23	27	33	0.05	0.026	0.022
	ATP (4),[d] PP_i (2)	28	27	52	0.03	0.032	0.025
ATP	Phe (0.03),[d] PP_i (0.02)	31	27		1.0	0.9	
	Phe (2),[d] PP_i (2)	23	57		0.8	4.5	

[a] k_{cat} = moles of substrate per mole of site (25 °C). [b] Calculated values of k_{cat} and K_m according to eq 2 and 3 assuming $k_f = 100\ s^{-1}$, $k_b = 150\ s^{-1}$, $K_{ATP} = 0.7$ mM, $K_{PP_i} = 0.2$ mM, and $K_{Phe} = 0.04$ mM; step a takes into account a competitive inhibition of pyrophosphate toward ATP and step b excludes any competition between ATP and pyrophosphate. [c] Lin et al. (1982). [d] Fasiolo et al. (1981).

presence of inorganic pyrophosphatase.

The data of the fast kinetic analysis performed in this study were difficult to reconcile with an asymmetric binding of phenylalanine. Therefore, the binding of phenylalanine to the enzyme was reinvestigated. Contrary to the findings of Fasiolo et al. (1977), it was unambiguously observed that phenylalanyl-tRNA synthetase did bind 2 mol of phenylalanine per $\alpha_2\beta_2$ oligomer, with identical microscopic dissociation constants equal to that previously reported (see Table I). The reason for the failure to detect two binding sites in the early studies is not yet understood. One possibility would be a difference in the enzyme batches used (resulting for instance from proteolysis), but it must be mentioned that the purification scheme of phenylalanyl-tRNA synthetase was identical in early and recent studies. However, in the presence of tRNA, Fasiolo et al. (1974) observed the existence of two binding sites for phenylalanine, with microscopic dissociation constants differing by a factor of 10 ($K_1 = 4\ \mu M$; $K_2 = 40\ \mu M$). These results were confirmed in our investigations (data not shown): the binding of tRNA to phenylalanyl-tRNA synthetase results in an asymmetric binding of 2 mol of Phe per $\alpha_2\beta_2$ oligomer with dissociation constants equal to those previously reported by Fasiolo et al. (1974).

Kinetics of the Phenylalanyladenylate Synthesis. Analysis of the kinetics of the reaction has shown that the enzyme catalyzed phenylalanyladenylate synthesis and the reversion by PP_i according to a scheme which involved independent active sites, as indicated by single exponential curves. The results agree with an identical behavior of each protomer of phenylalanyl-tRNA synthetase. This conclusion is supported by the comparison between the kinetic rate constants of synthesis (k_f) and pyrophosphorolysis (k_b) with the catalytic rate constant (k_{cat}) of the ATP-PP_i exchange reaction for each protomer. Indeed k_{cat}, which is defined by $v = k_{cat}$[E·Phe~AMP·PP_i] is related to k_f and k_b by (Cole & Schimmel, 1970; Mulivor & Rappaport, 1973)

$$k_{cat}^{-1} = (k_f S_{ATP})^{-1} + (k_b S_{PP_i})^{-1} \quad (2)$$

at infinite phenylalanine concentration and

$$k_{cat}^{-1} = (k_f S_{Phe})^{-1} + (k_b S_{PP_i})^{-1} \quad (3)$$

at infinite ATP concentration. S_{ATP}, S_{Phe}, and S_{PP_i} are defined as follows:

$$S_{ATP} = \frac{[ATP]_0}{K_{ATP}(1 + [PP_i]_0/K_{PP_i}) + [ATP]_0}$$

$$S_{Phe} = \frac{[Phe]_0}{K_{Phe} + [Phe]_0}$$

$$S_{PP_i} = \frac{[PP_i]_0}{K_{PP_i} + [PP_i]_0}$$

The expressions of the K_m values for Phe and ATP in the exchange reaction are the following:

$$K_m(Phe) = K_{Phe}\left(1 + \frac{k_f S_{ATP}}{k_b S_{PP_i}}\right)^{-1}$$

$$K_m(ATP) = K_{ATP}\left(1 + \frac{[PP_i]_0}{K_{PP_i}}\right)\left(1 + \frac{k_f S_{Phe}}{k_b S_{PP_i}}\right)^{-1}$$

In Table II, calculated values for k_{cat} (on the basis of eq 2 and 3 at various substrates concentrations) were compared with experimental values. The comparison supports the conclusion that each protomer of phenylalanyl-tRNA synthetase is able to catalyze independently the adenylation of phenylalanine by ATP. However, it may be noted that the competition between ATP and PP_i occurs to a lower extent than expected assuming a dissociation constant of 200 μM for pyrophosphate. This can be explained if we assume that PP_i binds preferentially to the enzyme·phenylalanyladenylate complex rather than to the free enzyme.

In conclusion, our results agree with a symmetric behavior of phenylalanyl-tRNA synthetase catalysis of adenylation of phenylalanine by ATP, at least in the absence of $tRNA^{Phe}$. Despite the existence of two equivalent binding sites for $tRNA^{Phe}$, it was shown earlier that tRNA markedly affected the enzyme characteristics, inducing an asymmetric behavior of the protein which could be correlated to (i) an asymmetric binding of phenylalanine (Fasiolo et al., 1974) and (ii) a conformational change of the enzyme, in the presence of ATP, which was complete upon binding of a single tRNA molecule (Lefèvre et al., 1980). This study must therefore be extended to the analysis of the reaction in the presence of $tRNA^{Phe}$.

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Registry No. L-Phenylalanyladenylate, 35874-27-2; L-phenylalanine, 63-91-2; pyrophosphate, 14000-31-8; ATP, 56-65-5; phenylalanyl-tRNA synthetase, 9055-66-7.

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Fast Kinetic Study of Yeast Phenylalanyl-tRNA Synthetase: An Efficient Discrimination between Tyrosine and Phenylalanine at the Level of the Aminoacyladenylate·Enzyme Complex†

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ABSTRACT: The discrimination by yeast phenylalanyl-tRNA synthetase between phenylalanine and tyrosine has been studied with highly purified tyrosine. It is shown that commercial grade tyrosine contains enough contaminating phenylalanine (≃0.5% molar ratio) to perturb significantly the study of tyrosine misactivation. Highly purified tyrosine (9-fold recrystallized, containing less than 5×10^{-5} mol/mol contaminating phenylalanine) has been used. It is shown that tyrosine is indeed misactivated by phenylalanyl-tRNA synthetase, as shown by ATP–PP_i exchange reaction. The maximal velocity of ATP–PP_i exchange in the presence of tyrosine is, however, less than 20% of that observed in the presence of phenylalanine. Titration experiments of phenylalanyl-tRNA synthetase by tyrosine in the presence of ATP or by chemically synthesized tyrosyladenylate, in the presence of 6-(*p*-toluidinyl)naphthalene-2-sulfonate (TNS) as a fluorescent reporter probe, reveal that phenylalanyl-tRNA synthetase has a rather poor affinity for tyrosyladenylate [$K_{Tyr\sim AMP} \simeq (1–2) \times 10^{-6}$ M]. This is confirmed by stopped-flow measurement of the kinetic association (1×10^6 M^{-1} s^{-1}) and dissociation (≃2 s^{-1}) constants. The forward rate constant for tyrosyladenylate was found ($k_f^{Tyr\sim AMP} = 10 \pm 2$ s^{-1}) much lower than that observed for the cognate amino acid phenylalanine ($k_f^{Phe\sim AMP} = 100 \pm 20$ s^{-1}). In contrast, the pyrophosphorolysis rate constant ($k_b^{Tyr\sim AMP} = 200 \pm 60$ s^{-1}) was found to be at least equal to or even higher than that observed for phenylalanine ($k_b^{Phe\sim AMP} = 150 \pm 50$ s^{-1}). The discrimination between tyrosine and phenylalanine by phenylalanyl-tRNA synthetase thus appears to correspond to the model proposed by J. J. Hopfield [Hopfield, J. J. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 4135–4139].

The selection of amino acids during protein biosynthesis has to be extremely precise. In the absence of an absolute recognition of a given amino acid by the cognate aminoacyl-tRNA synthetase, several mechanisms have been suggested, which would increase the overall accuracy of the tRNA aminoacylation. These mechanisms act either at the level of the aminoacyladenylate·enzyme complex prior to the transfer of the amino acid residue to the tRNA (Hopfield, 1974) or at the level of the enzyme-bound aminoacyl-tRNA (Fersht & Kaethner, 1976). In the particular case of yeast phenylalanyl-tRNA synthetase (EC 6.1.1.20) it has been suggested by Igloi et al. (1978) that the correction mechanism ensuring the distinction between phenylalanine and tyrosine might be of the second type. These authors also showed that the misactivation of tyrosine by yeast phenylalanyl-tRNA synthetase was very efficient, since the maximal velocity of the ATP–PP_i exchange in the presence of tyrosine was 53% of that observed for the cognate amino acid. On the other hand, we found that the complex between phenylalanyl-tRNA synthetase and tyrosyladenylate obtained by incubation of the enzyme with an excess ATP and labeled tyrosine could not be isolated by Sephadex filtration, under conditions where the cognate complex was almost quantitatively recovered. But when phenylalanyl-tRNA synthetase was incubated with labeled ATP and commercial grade tyrosine, an aminoacyladenylate·enzyme complex could be isolated, the properties of which were reminiscent of enzyme-bound phenylalanyladenylate rather than tyrosyladenylate. As already underlined by Fersht et al. (1980), the purity of the noncognate amino acid under investigation is a very critical factor, since the cognate substrate

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