Fasiolo, F., & Ebel, J. P. (1974) *Eur. J. Biochem. 49*, 257–263.
Fasiolo, F., Remy, P., Pouyet, J., & Ebel, J. P. (1974) *Eur. J. Biochem. 50*, 227–236.
Fasiolo, F., Ebel, J. P., & Lazdunski, M. (1977) *Eur. J. Biochem. 73*, 7–15.
Fasiolo, F., Remy, P., & Holler, E. (1981) *Biochemistry 20*, 3851–3856.
Furlong, C. E., Morris, R. G., Krandrach, M., & Rosen, B. P. (1972) *Anal. Biochem. 47*, 514–526.
Güntner, C., & Holler, E. (1979) *Biochemistry 10*, 2028–2038.
Holler, E., & Calvin, M. (1972) *Biochemistry 11*, 3741–3752.
Holler, E., & Kosakowski, H. M. (1973) *Biochem. Biophys. Res. Commun. 54*, 1532–1539.
Holler, E., Bennett, E. L., & Calvin, M. (1971) *Biochem. Biophys. Res. Commun. 45*, 409–416.
Hyafil, F., Jacque, Y., Fayat, G., Fromant, M., Dessen, P., & Blanquet, S. (1976) *Biochemistry 15*, 3678–3685.
Kosakowski, H. M., & Böck, A. (1971) *Eur. J. Biochem. 24*, 190–200.
Lefèvre, J. F., Ehrlich, R., & Remy, P. (1980) *Eur. J. Biochem. 103*, 155–159.
Lin, S. X., Baltzinger, M., & Remy, P. (1983) *Biochemistry* (following paper in this issue).
Mazat, J. P., Merle, M., Graves, P. V., Merault, G., Gandar, J. C., & Labouesse, B. (1982) *Eur. J. Biochem. 128*, 389–398.
McClure, W. O., & Edelmann, G. M. (1967) *Biochemistry 6*, 559–572.
Mulivor, R., & Rappaport, H. P. (1973) *J. Mol. Biol. 76*, 123–134.
Pimmer, J., & Holler, E. (1979) *Biochemistry 18*, 3714–3723.
Scatchard, G. (1949) *Ann. N.Y. Acad. Sci. 51*, 660–665.

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

S. X. Lin, M. Baltzinger, and P. Remy*

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

† From the Laboratoire de Biochimie, Institut de Biologie Moleculaire et Cellulaire du CNRS, 67084 Strasbourg Cedex, France. *Received June 28, 1982.*

0006-2960/83/0422-0681$01.50/0

is far more reactive than its competitor. This contamination problem is particularly acute in the case of the tyrosine–phenylalanine couple, owing to their similarities. The above results suggested that contaminant phenylalanine was at least partly responsible for the ATP–PP_i exchange activity observed in the presence of tyrosine. This prompted us to reinvestigate the misactivation of tyrosine by phenylalanyl-tRNA synthetase in the presence of highly purified tyrosine. This paper will show that indeed phenylalanine was partly responsible for the effects observed in earlier reports. Nevertheless, highly purified tyrosine can be adenylated by yeast phenylalanyl-tRNA synthetase. The kinetic and equilibrium constants of the aminoacyladenylate formation, and of the reverse pyrophosphorolysis step, have been determined by stopped-flow measurements. The affinity of the noncognate tyrosyladenylate for phenylalanyl-tRNA synthetase and the kinetic binding and dissociation constants have been measured with chemically synthesized tyrosyladenylate. The values obtained are compared to those measured in the cognate system (Baltzinger et al., 1983). The discrimination mechanism is discussed.

Materials and Methods

L-Tyrosine and L-phenylalanine were bought from Merck (Darmstadt, FRG). 6-(*p*-Toluidinyl)naphthalene-2-sulfonate (TNS)[1] as well as phenylalanyl- and tyrosylhydroxamates and inorganic pyrophosphatase (EC 3.6.1.1) was purchased from Sigma (St. Louis, MO). Radioactive chemicals (^{14}C-labeled amino acids and ^{32}P-labeled pyrophosphate) were from the Commissariat à l'Energie Atomique and from New England Nuclear Center. Yeast phenylalanyl-tRNA synthetase was purified according to Fasiolo & Ebel (1974). All other chemicals were of the best available purity.

Purification of Commercial Tyrosine. The contamination of phenylalanine in commercial tyrosine was assayed by amino acid analysis on a Durrum D500 apparatus, using a large amount of tyrosine (50–100 nmol). The contaminating phenylalanine was detected as a shoulder on the tyrosine peak and was estimated to be in the range of 0.5% (mol/mol). Such a contamination being incompatible with a study of tyrosine misactivation, the commercial amino acid was purified by successive recrystallizations in water, according to the following procedure:

Three grams of commercial tyrosine was dissolved in about 800 mL of boiling bidistilled water. About 7.5–10 μCi of [^{14}C]phenylalanine (450 mCi/mmol) was added as a tracer, and the solution was filtered on a sintered glass while still hot. After the solution was cooled in ice, the crystals of tyrosine which formed were recovered by filtration on a sintered glass. The recrystallization step was carried out 4- or 9-fold. At each step, the absorbancy of the hot solution was measured (after a convenient dilution) at 274.8 nm ($\epsilon_M = 1405 \pm 7$), and the radioactivity was measured on an aliquot of the same solution. The decrease of the "specific radioactivity" (cpm/OD unit) of the solution allowed us to follow the improvement of purity of the tyrosine sample.

Chemical Synthesis of L-Phenylalanyl- and L-Tyrosyl-adenylates. The adenylates of phenylalanine and tyrosine were synthesized as described by Berg (1958) and stored at −20 °C. Immediately before use, samples were freed from contaminating AMP on a Dowex 1 × 8 column, and the aminoacyladenylate concentrations were determined by the hydroxamate method (Berg, 1958). Standards were prepared with the commercial hydroxamates.

ATP–PP_i Exchange Reaction. The activation of phenylalanine or tyrosine was followed by measuring the ATP–[^{32}P]PP_i exchange, under conditions similar to those described by Fasiolo et al. (1981). The incubation mixture contained 130 mM Tris-HCl buffer, pH 7.5, 10 mM $MgCl_2$, 2 mM ATP, and 2 mM [^{32}P]pyrophosphate (specific activity around 400 cpm/nmol). For the cognate system, the reaction was conducted in the presence of 2 mM phenylalanine; in the noncognate system, varying concentrations of recrystallized tyrosine were used, as indicated later on.

The reactions were initiated upon addition of phenylalanyl-tRNA synthetase (respectively 5 and 50 nM in the cognate and noncognate systems). After variable incubation times at 37 or 25 °C (1–10 min), 100-μL aliquots were pipetted out in a mixture containing 100 μL of 15% $HClO_4$ and 0.4 mM pyrophosphate and 300 μL of a 2% Norit suspension. The charcoal was then filtered onto Whatman GF/C glass-fiber disks, washed with 20 mL of water, and dried. The [^{32}P]ATP adsorbed on the Norit was then measured by scintillation counting in Omnifluor/toluene (4 g/L).

Separation of Aminoacyladenylates from Free Amino Acids on Cellulose Plates. The phenylalanyl- and tyrosyladenylates were synthesized enzymatically in a mixture containing 50 mM Tris-HCl buffer, pH 7.5, 3 mM ATP, 10 mM $MgCl_2$, 4 mM 2-mercaptoethanol, 40 units/mL inorganic pyrophosphatase, 4.25 μM phenylalanyl-tRNA synthetase, and 100 μM [^{14}C]phenylalanine (450 mCi/mmol) or [^{14}C]tyrosine (388 mCi/mmol). After variable incubation times at 37 °C (1–70 min) 12-μL aliquots were pipetted out and mixed with 5 μL of 0.6 M HCl to stop the reaction. Fractions of 1 μL were then applied to a thin-layer cellulose plate (polygram CEL 300), and the chromatogram was developed at 4 °C as described by Jakubowsky et al. (1977). The radioactive spots were localized by using a two-dimensional spark chamber (Beta-Camera LB 290 BE-Berthold, FRG), then cut out, and counted in an Omnifluor/toluene scintillator (4 g/L).

Fluorescence Measurements in the Presence of 6-(p-Toluidinyl)naphthalene-2-sulfonate. 6-(*p*-Toluidinyl)-naphthalene-2-sulfonate (TNS) was used as a nonspecific fluorescent reporter group. Fluorescence spectra were recorded on an absolute spectrofluorometer FICA 55; kinetics and titration experiments were carried out on a JY3C fluorometer from JOBIN-YVON. The excitation wavelength was usually 330 nm, and the emission wavelength was 435 nm. The bandwidths were equal at the excitation and emission (respectively 7.5 or 10 nm for FICA or JOBIN-YVON fluorometers).

The free TNS has a very faint fluorescence which is greatly enhanced upon binding to phenylalanyl-tRNA synthetase. Contrary to what was observed for the *Escherichia coli* enzyme by Holler & Kosakowski (1973), the binding of TNS is not specific for the phenylalanine acceptor site of the yeast enzyme, as shown by Dibbelt (1981). An important fluorescence quenching is observed upon aminoacyladenylate formation, as already reported in the case of isoleucyl-tRNA synthetase by Holler & Calvin (1972). The reporter group was used in conditions ensuring a minimal interference with the phenomena under study: the TNS concentration was usually from 5 to 30 μM and the enzyme concentration in the range 0.1–4 μM. Thus

$$[E]_0 \ll [TNS]_0 \ll K_{TNS}$$

where $[E]_0$ = input concentration of enzyme, $[TNS]_0$ = input

[1] Abbreviations: TNS, 6-(*p*-toluidinyl)naphthalene-2-sulfonate; AA, amino acid; Tyr∼AMP, tyrosyladenylate; Phe∼AMP, phenylalanyl-adenylate; PP_i, inorganic pyrophosphate; AMP, adenosine 5′-monophosphate; ATP, adenosine 5′-triphosphate; Tris, tris(hydroxymethyl)-aminomethane.

concentration of TNS, and K_{TNS} = dissociation constant of the TNS-enzyme complex [in the range of 400 μM according to Dibbelt (1981)].

The titration of phenylalanyl-tRNA synthetase by tyrosyladenylate was studied under two different experimental conditions:

(i) The first condition was with in situ formed aminoacyladenylate. The reaction mixture contained 50 mM Tris-HCl buffer, pH 7.5, 3 mM ATP, 10 mM $MgCl_2$, 0.1 mM dithiothreitol, 20 μM TNS, 20 units/mL inorganic pyrophosphatase, and 4.25 μM phenylalanyl-tRNA synthetase. Increasing concentrations of tyrosine were then added, and after a time interval sufficient to reach the equilibrium, the fluorescence level was recorded.

(ii) The second experimental condition was with chemically preformed tyrosyladenylate. A conventional titration was performed, the mixture containing 50 mM Tris-HCl buffer, pH 7.5, 10 mM $MgCl_2$, 10 μM TNS, 0.1 mM dithiothreitol, and 0.2–1 μM phenylalanyl-tRNA synthetase. After each addition of tyrosyladenylate, the fluorescence level was recorded as quickly as possible. The dissociation constant measured by using this technique was only approximative, because of the interfering cumulative hydrolysis of tyrosyladenylate in solution ($t_{1/2}$ = 9 min) which prevents an accurate determination of the ligand concentration. So that this interference could be avoided, the preliminary results were confirmed by using a fresh solution of enzyme and aminoacyladenylate for each concentration and measuring the level of fluorescence within a few seconds after the solutions were mixed.

Stopped-Flow Experiments. They were performed on a Durrum-Gibson apparatus equipped with a fluorescence detection and a high-pressure Hg lamp (HB0100 W/2, Osram, Germany) as the light source. The excitation of enzyme-bound TNS was performed with the Hg line at 366 nm and a UG11 Schott filter at the entrance of the monochromator. The fluorescence was detected at right angle, using a Kodak–Wratten filter type 2B. The signals were recorded on a transient analyzer (Biomation or Datalab with dual-time bases), transferred into a Computer Automation LSI 2/20, and analyzed by using a nonlinear least-squares regression. The superimposition of the experimental data and the calculated regression curve was then displayed on an oscilloscope. The dead time of the apparatus was about 4–5 ms. The experiments were usually carried out at 0.2 μM phenylalanyl-tRNA synthetase (unless otherwise stated), in 50 mM Tris-HCl, pH 7.5, 10 μM TNS, and 10 mM $MgCl_2$ (final concentrations). Under these conditions, the total signal in the presence of enzyme was usually adjusted to 8 V (the buffer alone brought a contribution of around 4 V to this signal). When the binding of chemically synthesized aminoacyladenylate to phenylalanyl-tRNA synthetase was studied, a 2-fold concentrated enzyme solution was mixed with aminoacyladenylate at variable concentrations in 0.5 mM HCl. This acidic solution of aminoacyladenylate was used to avoid as completely as possible the hydrolysis of the latter prior to mixing with the enzyme solution. The use of such an acidic solution did not alter the final pH of the reaction mixture and did not induce any change of the fluorescence of the enzyme·TNS complex in the time scale under investigation. When the in situ adenylation of tyrosine by phenylalanyl-tRNA synthetase was studied, the observed rates of reaction were slow enough to allow the study in the normal JOBIN JY3C fluorometer. The mixing of solutions was done by hand, using a rapid flush of the solution in the cuvette with the help of a syringe. The "dead time" under these conditions was usually less than 5 s.

Table I: Purification of Commercial Tyrosine

product	[^{14}C]Phe/Tyr[a] (cpm/μmol)	Phe/Tyr (mol/mol)
commercial tyrosine	999	5.6×10^{-3} [b]
4 × recrystallized tyrosine	20	1.2×10^{-4}
9 × recrystallized tyrosine	8.8	4.9×10^{-5} [c]

[a] [^{14}C]Phe was added to tyrosine prior to recrystallization, to monitor the purification (see Materials and Methods). [b] Determined by amino acid analysis on a Durrum D 500 analyzer. [c] This figure is an upper limit for the residual contamination, since it cannot be excluded that [^{14}C]Phe also contains contaminating labeled tyrosine, which would result in a constant residual specific activity for the purified tyrosine, whatever the number of recrystallization steps.

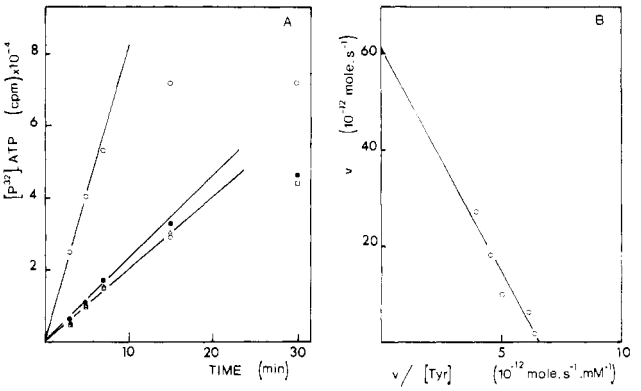


FIGURE 1: (A) Comparison of ATP–PP_i exchange in the presence of phenylalanyl-tRNA synthetase and commercial or recrystallized tyrosine. Initial concentrations were 2 mM ATP, 2 mM [^{32}P]PP_i (about 1200 cpm/nmol), 130 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, and 100 nM PheRS. (○) Commercial tyrosine (2 mM); (●) 4-fold recrystallized tyrosine (2 mM); (□) 9-fold recrystallized tyrosine (2 mM); (△) 9-fold recrystallized tyrosine (2 mM) plus phenylalanine (0.1 μM). (B) Tyrosine concentration dependence of the ATP–PP_i exchange in the presence of phenylalanyl-tRNA synthetase at 37 °C. Initial concentrations were 2 mM ATP, 2 mM [^{32}P]PP_i (about 400 cpm/nmol), 130 mM Tris-HCl buffer, pH 7.5, 10 mM $MgCl_2$, and 50 nM phenylalanyl-tRNA synthetase in the presence of a varying concentration of tyrosine.

Results and Discussion

Purification of Commercial Tyrosine. Previous results of Igloi et al. (1978) suggested a very efficient misactivation of tyrosine by yeast phenylalanyl-tRNA synthetase. The main difficulty in this type of study is a possible contamination of the noncognate amino acid under study by minute amounts of the cognate amino acid, especially in the case of tyrosine, since phenylalanine is closely related to the latter. Indeed, amino acid analyses, carried out on a Durrum 500 amino acid analyzer, using a large amount of tyrosine (up to 100 nmol), revealed a contamination by phenylalanine (detected as a shoulder on the tyrosine peak), in the range of 0.5% (mol/mol). The purification of the commercial tyrosine was carried out by successive recrystallizations in water, which appears to be the simplest and most efficient technique, due to the large difference between the solubilities of L-tyrosine and L-phenylalanine (respectively 0.196 and 20 g/L at 0 °C) as well as to the large difference in the temperature coefficients of tyrosine and phenylalanine solubilities [$\log s(\text{Tyr}) = -0.708 + 0.0146t$; $\log s(\text{Phe}) = 1.30 + 0.007t$]. The residual contamination of phenylalanine was estimated as indicated under Materials and Methods. Table I shows the maximum residual

Table II: Kinetic Constants in the ATP-PP_i Exchange Reaction

amino acid	K_M (mM)	V_{max} (%)	k_{cat} (s^{-1})[b] (25 °C)
Phe	0.030[a]	100	56
4 × recrystallized Tyr	8.2	19.2[c]	
9 × recrystallized Tyr	9.2	17.1[c]	9.6
Tyr[d]	1.5	53	

[a] From Fasiolo et al. (1977). [b] Moles of product per mole of enzyme. [c] Calculated assuming a mean value of 8.6 mM for K_M. [d] From Igloi et al. (1978).

contaminations of recrystallized tyrosine by phenylalanine. As can be seen on Figure 1A the contamination of tyrosine by phenylalanine greatly enhanced the ATP–PP_i exchange catalyzed by yeast phenylalanyl-tRNA synthetase, since the use of a 4-fold recrystallized tyrosine resulted in a 3.4-fold lower ATP–PP_i exchange rate. Even this 4-fold recrystallized tyrosine still contains enough contaminating phenylalanine to affect the ATP–PP_i exchange, since further recrystallization still depresses the ATP–PP_i exchange. The residual contamination in the 9-fold recrystallized tyrosine can be considered as negligible, since upon addition to the reaction mixture of phenylalanine in an amount equivalent to the residual contamination, almost no increase of the ATP–PP_i exchange rate can be detected. All the following experiments were therefore carried out by using the 9-fold recrystallized tyrosine.

Kinetic Constants for the ATP–PP_i Exchange Reaction. Figure 1B shows the Eadie (1942) plot of the data of ATP–PP_i exchange in the presence of 9-fold recrystallized tyrosine. The maximal velocity using tyrosine as substrate is close to 20% of that observed in the presence of phenylalanine, as shown in Table II, the K_m for tyrosine being around 8–9 mM. These values differ significantly from the values previously reported by Igloi et al. (1978), which were respectively 53% and 1.5 mM. This large difference may be attributed to a contamination of the tyrosine used by these authors by phenylalanine. Indeed, the latter being a much better substrate than tyrosine for phenylalanyl-tRNA synthetase, increasing concentrations of phenylalanine in tyrosine will result in apparent V_{max} and K_M closer to those observed for phenylalanine. It is important to stress that due to the large difference in the affinities for phenylalanine and tyrosine ($K_{Phe} \simeq 30$–40 μM; $K_{Tyr} \simeq 8$–9 mM), even a "faint" contamination of tyrosine by phenylalanine will bring an important contribution to the reaction.

Determination of the Dissociation Constant for the Tyrosyladenylate. Figure 2 shows the titration of the phenylalanyl-tRNA synthetase·ATP complex, in the presence of the fluorescent reporter TNS, either by phenylalanine or tyrosine. As can be seen, the titration by phenylalanine is much sharper than that by tyrosine, but the maximum quenching of fluorescence of the TNS reporter appears to be the same. Since it has been shown by Baltzinger et al. (1983) that the binding of phenylalanyladenylate occurred on both sites of phenylalanyl-tRNA synthetase and that the fluorescence quenching produced on both sites was the same, we will assume the situation to be identical for the tyrosyladenylate. Indeed, the maximum quenching of fluorescence observed is close to the one measured in the presence of phenylalanine. It must be kept in mind that the intercept of the asymptote and the tangent at the origin of the curve is equal to $n[E]_0 + K_D$ (where $[E]_0$ = input concentration of enzyme, n = number of binding sites, and K_D = dissociation constant of the complex). In the case of the phenylalanyladenylate, the dissociation constant has been found equal to 4×10^{-9} M (Baltzinger et al., 1983). Since the experiment has been performed at 0.2 μM phenylalanyl-tRNA synthetase, $n[E]_0 = 4 \times 10^{-7}$ M, and the abscissa essentially reflects the stoichiometry of the complex. The higher value of the abscissa in the case of the tyrosyladenylate most likely reflects a much higher dissociation constant of the complex with phenylalanyl-tRNA synthetase. This prompted us to make titration experiments with chemically synthesized tyrosyladenylate.

Figure 3 shows one titration experiment. The estimation of the dissociation constant from the curve gives a value close

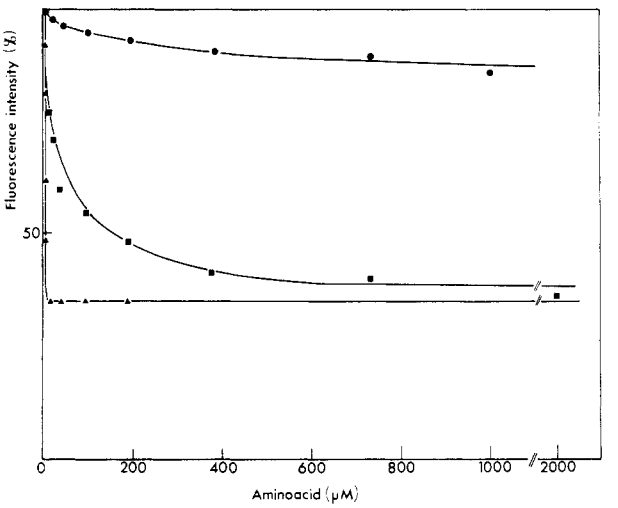


FIGURE 2: Titration of phenylalanyl-tRNA synthetase by tyrosine or phenylalanine in the presence of ATP, using TNS as a fluorescent reporter probe. Initial concentrations were 50 mM Tris-HCl buffer, pH 7.5, 3 mM ATP, 10 mM $MgCl_2$, 0.1 mM dithiothreitol, 40 μM TNS, and 4.25 μM phenylalanyl-tRNA synthetase. (●) Titration with tyrosine in the absence of inorganic pyrophosphatase; (■) titration with tyrosine in the presence of 20 units/mL inorganic pyrophosphatase [the percentage of fluorescence quenching at 110 μM Tyr was used for the estimation of $K_{Tyr\sim AMP}$ by TLC method (see text)]; (▲) titration with phenylalanine in the presence of 20 units/mL inorganic pyrophosphatase.

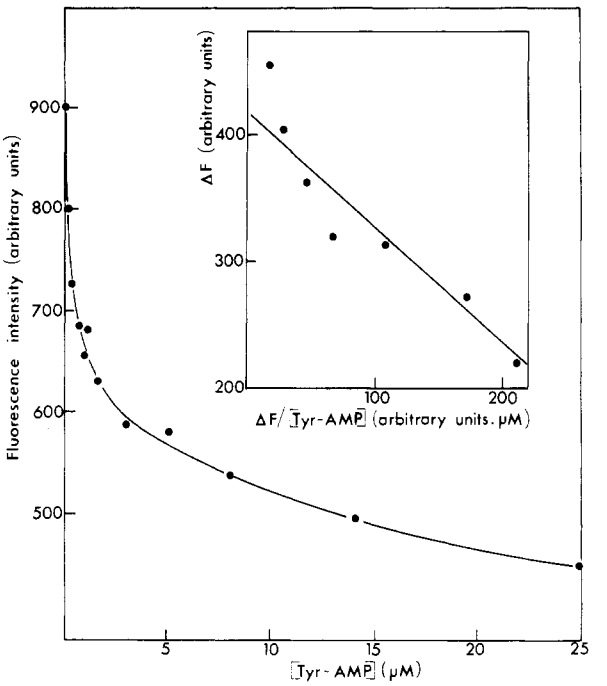


FIGURE 3: Titration of phenylalanyl-tRNA synthetase by chemically synthesized tyrosyladenylate. Initial concentrations were 50 mM Tris-HCl buffer, pH 7.5, 10 mM $MgCl_2$, 10 μM TNS, 0.1 mM dithiothreitol, and 0.2 μM phenylalanyl-tRNA synthetase. (Insert) Eadie (1942) plot of the data.

to 2 μM, as shown by the Eadie plot of the data.

Evidence for Unbound Tyrosyladenylate in the Adenylation Mixture and Estimation of the Dissociation Constant by Thin-Layer Chromatography. If the dissociation constant of the tyrosyladenylate·phenylalanyl-tRNA synthetase complex is in the range of 2 μM, it should be possible to characterize free aminoacyladenylate in the reaction mixture in the enzyme concentration range used (0.2–10 μM). This investigation was undertaken by thin-layer chromatography as described under Materials and Methods. The total amount of aminoacyladenylate formed in the presence of 4.25 μM phenylalanyl-tRNA synthetase and 110 μM tyrosine was found equal to 10.8 μM which gives a stoichiometry of 2.55 aminoacyladenylates synthesized per mol of enzyme. This figure is significantly higher than that obtained in the cognate system, since under the conditions used, the stoichiometry for phenylalanyladenylate rarely exceeds 1.6 or 1.7 mol/mol. The total concentration of tyrosyladenylate remained constant during a large period of time (at least from 3 to 10 min of incubation). As will be shown later, the association–dissociation equilibrium of tyrosyladenylate and enzyme is much faster than the hydrolysis reaction. One can therefore assume that the system is in true equilibrium:

$$\text{E} + \text{tyrosyladenylate} \rightleftharpoons \text{E·tyrosyladenylate}$$

In the above experiment, the fractional saturation of phenylalanyl-tRNA synthetase was estimated from a parallel experiment in the presence of the fluorescent reporter TNS, by measuring the ratio of the fluorescence quenching at 110 μM tyrosine to the maximum quenching of fluorescence. It was found to be 0.71. When the above stoichiometry of 2.55 aminoacyladenylates per mol of enzyme is taken into account, an apparent dissociation constant can be calculated for tyrosyladenylate, which is equal to

$$K^{app}_{Tyr\sim AMP} = \frac{([E]_0 - [\text{E·adenylate}])[\text{Tyr}\sim\text{AMP}]_{free}}{[\text{E·adenylate}]} = 1.95\ \mu\text{M}$$

The true dissociation constant will be somewhat lower, due to the competition by ATP toward free enzyme. The above result is therefore in good agreement with the dissociation constant measured in the titration experiments. This dissociation constant must be considered as exceptionally high, compared to the value measured for the cognate phenylalanyladenylate [4 nM according to Baltzinger et al. (1983)]. The consequence of this large difference will be discussed later, together with the differences observed at the level of the kinetic constants.

Stopped-Flow Study of the Binding of Tyrosyladenylate to Phenylalanyl-tRNA Synthetase. Phenylalanyl-tRNA synthetase was rapidly mixed in the stopped-flow spectrofluorometer with a solution of chemically synthesized tyrosyladenylate (or phenylalanyladenylate as a control). A large fluorescence quenching was observed. The kinetics were treated according to the general scheme

$$\text{E} + \text{Ad} \underset{k_d}{\overset{k_a}{\rightleftharpoons}} \text{E·Ad}$$

where E = phenylalanyl-tRNA synthetase, Ad = aminoacyladenylate, k_a = kinetic association constant, and k_d = kinetic dissociation constant. As shown by Czerlinsky (1966), when $[\text{Ad}]_0 \gg [\text{E}]_0$

$$k_{obsd} = k_d + k_a[\text{Ad}]_0$$

where k_{obsd} = observed kinetic constant for the fluorescence modification, $[\text{Ad}]_0$ = input concentration of aminoacyladenylate, and $[\text{E}]_0$ = input concentration of enzyme. Figure 4C and Table III show the data obtained by this technique. Both phenylalanyl- and tyrosyladenylates appear to bind to phenylalanyl-tRNA synthetase in a simple bimolecular process. The measured kinetic association constant is, however, too low ($=10^6$ M^{-1} s^{-1}) for a diffusion-controlled reaction (k_{assoc} in the range 10^8–10^9 M^{-1} s^{-1}). However, it must be pointed out that independent determinations of the kinetic association constants of methionyl- and methioninyladenylate for the *E. coli* methionyl-tRNA synthetase (Blanquet et al., 1972), using the

Table III: Comparison of the Association–Dissociation Equilibrium of Phenylalanyl- and Tyrosyladenylate to Phenylalanyl-tRNA Synthetase

kinetic and equilibrium constants	nature of the adenylate: phenylalanyl-adenylate	nature of the adenylate: tyrosyl-adenylate
k_a (M^{-1} s^{-1})	1.6×10^6	10^6
k_d (s^{-1})	$\simeq 7 \times 10^{-3}$ [a]	2–3
$K_D = k_d/k_a$ (M)	$\simeq 4 \times 10^{-9}$	$2\text{–}3 \times 10^{-6}$
K_D measured by titration experiments (M)		2×10^{-6}

[a] According to Baltzinger et al. (1983).

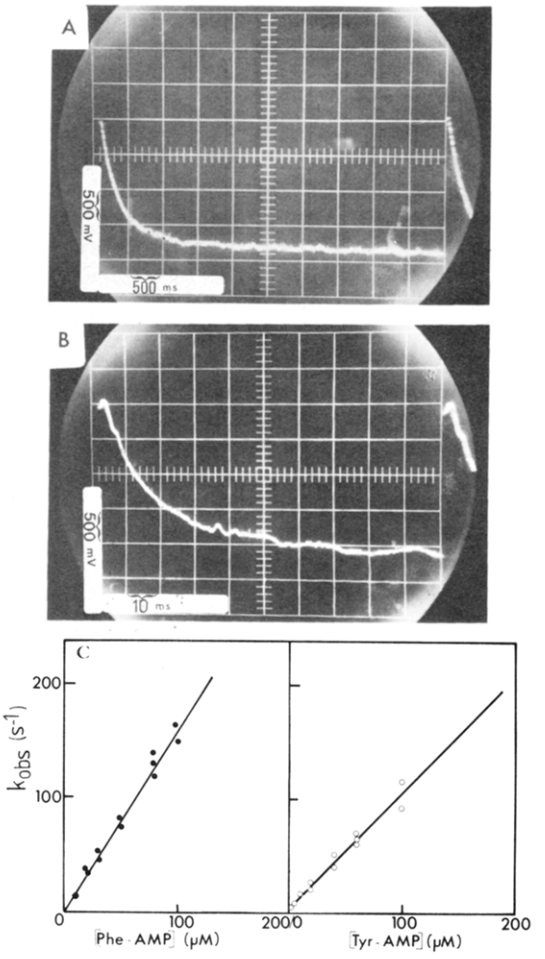
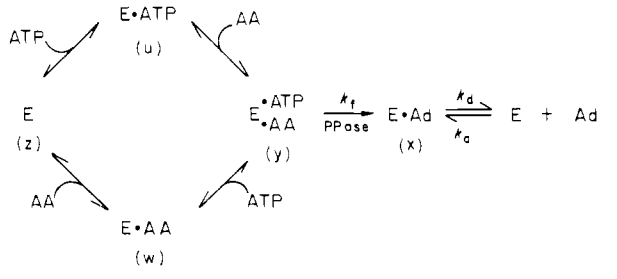


FIGURE 4: Stopped-flow investigation of the binding of aminoacyladenylates to phenylalanyl-tRNA synthetase. Initial concentrations after rapid mixing were 50 mM Tris-HCl buffer, pH 7.5, 10 μM TNS, 10 mM $MgCl_2$, 0.2 μM phenylalanyl-tRNA synthetase, and varying aminoacyladenylate concentration at 25 °C. (A) Oscilloscope display of the phenylalanyladenylate binding (phenylalanyladenylate 1 μM); sensitivity 500 mV/division; time scale 500 ms/division. (B) Oscilloscope display of the tyrosyladenylate binding (tyrosyladenylate 60 μM); sensitivity 500 mV/division; time scale 10 ms/division. (C) Dependence of the observed rate constants upon aminoacyladenylate concentration.

intrinsic fluorescence of the protein, yielded similar values, respectively 1.5×10^6 and 6×10^6 M^{-1} s^{-1}. In the latter case, the authors explained the low value of the k_{assoc} by an hydrolysis of the adenylate, leading to a large uncertainty in the adenylate concentrations. Such an explanation does not hold in the present case, since the adenylate was kept in 1 mM HCl, where it is rather stable and the effective concentration was measured just prior to the experiment, using the hydroxamate titration (Berg, 1958). Several explanations can be proposed for the low value of these association constants, keeping in mind that these constants are considered to be the product of three terms: the frequency of collisions, a steric factor to account for the fraction of molecules which are in the proper orientation, and an activation energy term to allow for the fraction of molecules that are sufficiently thermally activated to react. The apparent "sluggishness" of the complex formation could therefore arise from very restricting steric factors and/or from a strain imposed by the enzyme to the adenylate molecule, which would explain that only a small fraction of the adenylate molecule would have the "strained" configuration allowing a proper fit in the binding site. But whereas the extrapolation of k_{obsd} at zero concentration of aminoacyl-adenylate appears to be significantly different from zero in the case of tyrosyladenylate (2–3 s^{-1}), it cannot be distinguished from zero when phenylalanyladenylate is used. Using a modification of the technique of Collowick & Womack (1969), Baltzinger et al. (1983) could measure accurately the k_d for phenylalanyladenylate and found it to be 7×10^{-3} s^{-1}. The kinetic constants measured for the binding of tyrosyl-adenylate are in perfect agreement with the equilibrium dissociation constant measured by titration with chemically synthesized tyrosyladenylate.

Determination of the Rate Constant for the Formation of Tyrosyladenylate. When the kinetics of tyrosine adenylation by phenylalanyl-tRNA synthetase were studied, in the presence of TNS as a reporter group, very slow reactions were observed. For instance, in the presence of 120 μM tyrosine, the maximum extent of fluorescence quenching was only observed after 4 min of reaction. In the cognate system, for an appropriate phenylalanine concentration (corresponding to the same percentage of saturation by the amino acid, equal to 0.013), the observed rate constant would have been close to 1 s^{-1}, assuming a formation kinetic constant of 100 ± 20 s^{-1} (Baltzinger et al., 1983) which means that the maximum quenching of fluorescence would have been reached after a few seconds (4–5). It was thus of interest to study the reason for such a slow reaction in the case of tyrosine. Keeping in mind that the tyrosyladenylate has a rather poor affinity for the enzyme, let us consider the activation scheme



For the sake of simplicity, we will assume that both active sites of phenylalanyl-tRNA synthetase work independently one from another, and the enzyme will be treated as a monomer (with a 2-fold higher concentration). This is a reasonable assumption, since in the cognate system, Baltzinger et al. (1983) indeed observed that both sites were independent. In all the following treatment, we will assume that

$$[AA]_0, [ATP]_0 \gg [E]_0$$

where $[AA]_0$ = input concentration of amino acid, $[ATP]_0$ = input concentration of ATP, and $[E]_0$ = input concentration of enzyme (expressed in sites).

A further assumption will be that that the tyrosine concentration is kept low enough so that the rate of adenylation is low compared to the rate of dissociation. Therefore, we will assume that all binding reactions are fast with respect to the tyrosyladenylate formation. The following equation can be established (see Appendix):

$$\frac{1}{([E]_0 - x)^2} = 2Bt + \frac{1}{[E]_0^2} \tag{1}$$

$$B = \frac{S_{ATP} k_f [Tyr]}{K_{Tyr}[E]_0 \left([E]_0 + \frac{K_{ATP} + [ATP]}{K_{ATP}} K_{Ad} \right)}$$

with S_{ATP} being the saturation fraction of the enzyme by ATP. This equation will be essentially valid for the initial rates of reaction (see Appendix). Actually, it proved to be perfectly valid for $\Delta F \leq {}^1/_2 \Delta F_{max}$. In each experiment, x was measured as a function of time, by comparison of the fluorescence quenching at time t to the maximum quenching of fluorescence:

$$x = \frac{\Delta F_t}{\Delta F_{max}} [E]_0$$

As shown in Figure 5A, the plots of $([E]_0 - x)^{-2}$ as a function of time exhibit a good linearity. The ordinate intercepts of the straight lines obtained for different tyrosine concentrations are very close to 0.25 μM^{-2} which can be expected from eq 1. The experimental data therefore seem in good agreement with the reaction scheme described above. The slopes of the straight lines in Figure 5A are a function of the tyrosine concentration. A new plot of these slopes as a function of tyrosine concentration (Figure 5A) will afford a straight line, the slope of which is equal to

$$\frac{2 k_f S_{ATP}}{K_{Tyr}[E]_0 \left([E]_0 + \frac{K_{ATP} + [ATP]}{K_{ATP}} K_{Ad} \right)}$$

From a first set of experiments performed at tyrosine concentrations equal to 10, 15, 20, and 30 μM a value of $k_f^{Tyr \sim AMP} = 10 \pm 2$ s^{-1} was determined, which is considerably slower than that for the cognate system (100 ± 20 s^{-1}). Therefore, it was possible to enlarge the range of tyrosine concentration, still keeping satisfied the condition that equilibrium I remains fast compared to the adenylation. Figure 5B shows that the tyrosine concentration can be increased up to 120 μM without substantial deviation from the linearity in the plots of $([E]_0 - x)^{-2}$ vs. time. For higher concentrations, the plots are no longer linear. Furthermore, it should be emphasized that all the above measurements were carried out in less than 60 s, which allows us to neglect the hydrolysis of free aminoacyl-adenylate, since the half-life of tyrosyladenylate under the same experimental conditions was found to be 9 min at 25 °C.

The above results demonstrate important differences between the activation of the cognate (phenylalanine) vs. noncognate (tyrosine) amino acid. An important discrimination takes place at the level of the rate constant of the adenylation reaction, and a still more critical factor is the difference in the affinities for the cognate and noncognate aminoacyl-

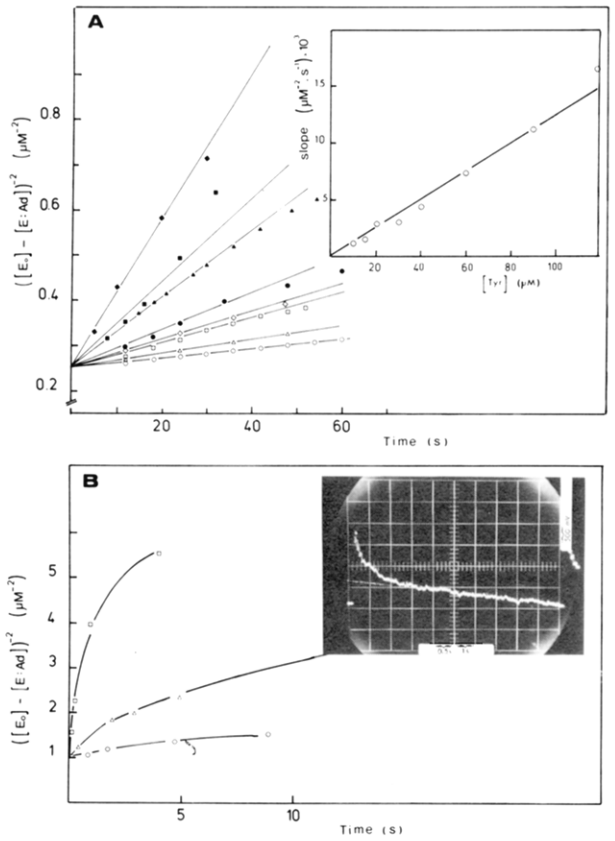


FIGURE 5: Kinetics of tyrosine activation in the presence of phenylalanyl-tRNA synthetase at varying concentrations of tyrosine. (A) Low tyrosine concentrations (kinetics followed on a conventional fluorometer). Initial concentrations were 1 μM phenylalanyl-tRNA synthetase, 3 mM ATP, 10 mM $MgCl_2$, 20 units/mL pyrophosphatase, 20 μM TNS, 0.1 mM dithiothreitol, and 50 mM Tris-HCl, pH 7.5. Tyrosine concentrations: (○) 10, (△) 15, (□) 20, (◊) 30, (●) 40, (▲) 60, (■) 90, and (♦) 120 μM. (Insert) Plot of the slopes of the linear graphs vs. tyrosine concentration, according to eq A14 of the Appendix. A K_{ATP} = 0.7 mM was used as reported by Fasiolo et al. (1977) and Baltzinger et al. (1983). (B) High tyrosine concentrations (kinetics followed on a stopped-flow apparatus). Initial concentrations as in (A), except 0.5 μM phenylalanyl-tRNA synthetase. Tyrosine concentrations: (○) 200, (△) 400, and (□) 1000 μM. (Insert) Oscilloscope display showing the interference of the dissociation of tyrosyladenylate from the enzyme, yielding a very slow process (1 mM tyrosine). Sensitivity 500 mV/division; time scale 0.5 s/division and 1 s/division by the Biomation.

adenylates. In the case of tyrosine activation, the dissociation of the enzyme·aminoacyladenylate complex perturbs significantly the kinetics of the enzyme·aminoacyladenylate accumulation: the formation of the enzyme·adenylate complex appears clearly biphasic, due to the rather slow dissociation reaction, compared to the fast adenylate formation (insert to Figure 5B). This is also clearly demonstrated by the fact that the time course of the adenylation, which is independent of the enzyme concentration in the cognate system, varies with the latter parameter in the noncognate system (Figure 6). Indeed the enzyme concentration markedly affects the saturation of phenylalanyl-tRNA synthetase by tyrosyladenylate under our experimental conditions, since the enzyme concentrations used throughout this study are close to or smaller than the dissociation constant.

Stopped-Flow Determination of the Pyrophosphorolysis Rate Constant. When the misactivation of tyrosine is studied in the absence of inorganic pyrophosphatase, using TNS as a reporter probe, the fluorescence quenching is quite small (Figure 2), suggesting that the pyrophosphate produced during the reaction is sufficient to displace the equilibrium. It was

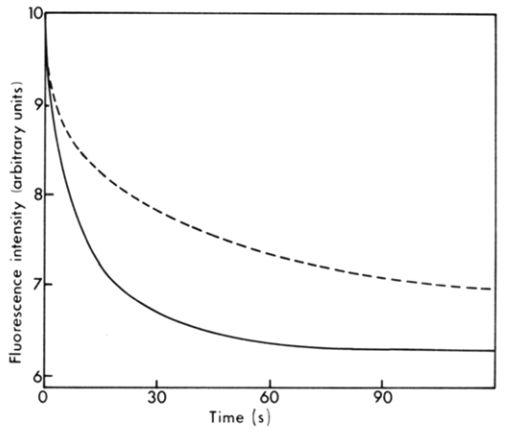
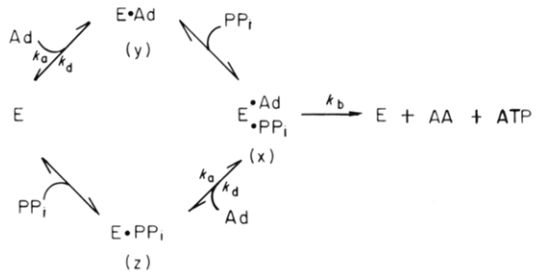


FIGURE 6: Influence of phenylalanyl-tRNA synthetase concentration upon tyrosine misactivation. Initial concentrations were 3 mM ATP, 80 μM tyrosine, 20 units/mL pyrophosphatase, 80 μM TNS, 10 mM $MgCl_2$, 50 mM Tris-HCl buffer, pH 7.5, and 0.1 mM dithiothreitol. Spectrofluorometric records at (—) 8 and (---) 1 μM phenylalanyl-tRNA synthetase.

therefore impossible to study the adenylation reaction in the presence of varying concentrations of pyrophosphate. Furthermore, we could not study the direct pyrophosphorolysis of preformed tyrosyladenylate·enzyme complex, because of the poor affinity of phenylalanyl-tRNA synthetase for the noncognate aminoacyladenylate. A roundabout procedure was used, consisting in the study of the binding of chemically synthesized tyrosyladenylate to the enzyme, in the presence of varying concentration of pyrophosphate. Phenylalanyl-tRNA synthetase in a 2-fold concentrated buffer containing $MgCl_2$ and TNS was rapidly mixed with a solution of tyrosyladenylate and pyrophosphate in 1 mM HCl. (The presence of 1 mM HCl did not perturb the pH of the enzyme solution and did not result in any modification of the fluorescence in the time scale under study.) The kinetic pathway is



Provided that $[E]_0 \ll [Ad]_0$, $[PP_i]_0$ where $[E]_0$ = input concentration of enzyme (expressed in sites), $[Ad]_0$ = input concentration of tyrosyladenylate, and $[PP_i]_0$ = input concentration of pyrophosphate, we can assume that the formation of the $[E^{Ad}_{\cdot PP_i}]$ complex from free enzyme, tyrosine, and ATP is negligible because of the very low concentration of the free ligands.

If ΔF is the fluorescence change

$$\frac{d\Delta F}{dt} \propto \frac{d(y + x)}{dt}$$

$$\frac{d(y + x)}{dt} = k_a[E][Ad] - k_d y - k_b x + k_a z[Ad] - k_d x \quad (2)$$

Writing $[E]_0 = [E] + x + y + z$ and assuming that the association–dissociation equilibrium with pyrophosphate in front of the pyrophosphorolysis step is fast, thus

$$y \simeq \frac{K_{pp}}{[PP_i]_0} x$$

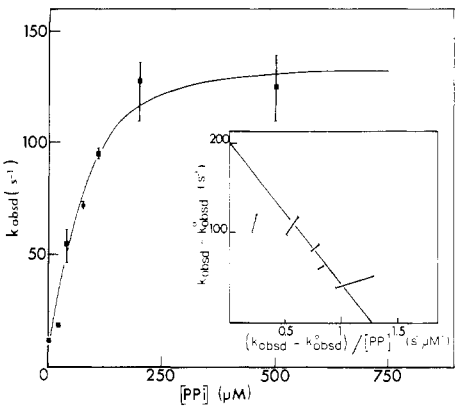


FIGURE 7: Binding of tyrosyladenylate to phenylalanyl-tRNA synthetase in the presence of varying concentration of pyrophosphate. Initial concentrations after rapid mixing were 0.4 μM phenylalanyl-tRNA synthetase, 10 μM tyrosyladenylate, 20 μM TNS, 10 mM $MgCl_2$, 0.1 mM dithiothreitol, and 50 mM Tris-HCl buffer, pH 7.5. Observed rate constant at varying pyrophosphate concentration. (Insert) Eadie (1942) plot of the data.

where K_{pp} is the equilibrium dissociation constant for pyrophosphate. Writing $m = x + y$

$$x = \frac{[PP_i]_0}{K_{pp} + [PP_i]_0} m \qquad y = \frac{K_{pp}}{K_{pp} + [PP_i]_0} m$$

Substituting in eq 2

$$\frac{dm}{dt} = k_a[E]_0[Ad]_0 - \left(k_d + k_a[Ad]_0 + k_b \frac{[PP_i]_0}{K_{pp} + [PP_i]_0} \right) m$$

Therefore

$$k_{obsd} = k_{obsd}^0 + k_b \frac{[PP_i]_0}{K_{pp} + [PP_i]_0}$$

with $k_{obsd}^0 = k_d + k_a[Ad]_0$, where k_{obsd} is the observed rate constant.

The experimental data are shown in Figure 7. The pyrophosphorolysis rate constant for the tyrosyladenylate can be estimated to $k_b^{Tyr\sim AMP} = 200 \pm 60\ s^{-1}$ and the equilibrium dissociation constant for pyrophosphate is $K_{PP} = 160 \pm 50$ μM. This last value is in agreement with the equilibrium dissociation constant for PP_i measured in the pyrophosphorolysis of Phe~AMP by Baltzinger et al. (1983) (≃200 μM). The pyrophosphorolysis rate constant for tyrosyladenylate is close to or even higher than that observed for phenylalanyladenylate (≃150 s^{-1}) (Baltzinger et al., 1983). Since the formation rate constant for tyrosyladenylate is much lower than that observed for the cognate aminoacyladenylate, it explains that the equilibrium in the noncognate system is much more sensitive to pyrophosphate concentration, as underlined at the beginning of this section.

Comparison between the Catalytic Rate Constant of the ATP–PP_i Exchange and the Adenylation and Pyrophosphorolysis Rate Constants. As shown by Baltzinger et al. (1983), the adenylation and pyrophosphorolysis rate constants are related to the catalytic rate constants of the ATP–PP_i exchange at varying tyrosine concentrations according to

$$k_{cat}^{-1} = k_f^{-1} \frac{K_{ATP} + [ATP]}{[ATP]} + k_b^{-1} \frac{K_{pp} + [PP_i]}{[PP_i]}$$

With the values of k_f and k_b determined above for the activation step of tyrosine, the calculated k_{cat} will vary from 2 to 7 s^{-1} depending upon the efficiency of the competition between pyrophosphate and ATP. The observed value of 4.8 s^{-1} (Table II) is therefore compatible with the calculated values.

Conclusion

The above results emphasize the critical importance of amino acid purity in studies devoted to misaminoacylation. Yeast phenylalanyl-tRNA synthetase, which had been earlier (Igloi et al., 1978) reported to misactivate efficiently tyrosine, does indeed form tyrosyladenylate but much less easily than described. Indeed, a strong discrimination between the cognate (phenylalanine) and noncognate (tyrosine) amino acids results from important differences at the level of the affinities for the amino acids (K_{Phe} = 40 μM; K_{Tyr} = 9 mM), the affinities for the aminoacyladenylates ($K_{Phe\sim AMP}$ = 4 nM; $K_{Tyr\sim AMP}$ = 1–2 μM), and the forward rate constants for the formation of aminoacyladenylates ($k_f^{Phe\sim AMP} = 100 \pm 20\ s^{-1}$; $k_f^{Tyr\sim AMP} = 10 \pm 2\ s^{-1}$).

This difference in the forward rate constants is still amplified by the similitude of the pyrophosphorolysis rate constants in both systems ($k_b^{Phe\sim AMP} \simeq 150\ s^{-1}$; $k_b^{Tyr\sim AMP} \simeq 200\ s^{-1}$), resulting in a much higher sensitivity of the tyrosyladenylate·enzyme complex to the simultaneously formed pyrophosphate. All the above factors contribute to reducing the level of the intermediate tyrosyladenylate·enzyme complex and thus ensure an efficient discrimination between phenylalanine and tyrosine. The differences in equilibrium and kinetic constants will lead to a discrimination factor around 2000 between phenylalanine and tyrosine. This factor is close to the maximal error rate in protein biosynthesis which has been estimated to lie around $^1/_{3000}$ (Loftfield & Vanderjagt, 1972). The estimated discrimination factor between phenylalanine and tyrosine may even be underestimated, since we did not take into account the low affinity of tyrosyladenylate for phenylalanyl-tRNA synthetase. Indeed, the rather fast dissociation of the noncognate aminoacyladenylate makes it a good candidate to obey the Hopfield's model (Hopfield, 1974). It is important to remind that this model requires an almost "irreversible" dissociation of the complex between the enzyme and the noncognate aminoacyladenylate. This "irreversibility" may have several origins: (i) a hydrolysis of the liberated adenylate (which in vivo might be promoted by nucleophilic substances); (ii) a competition by the free amino acid and ATP toward free enzyme (since these ligands are usually present at high concentrations with respect to their dissociation constants); (iii) a trapping by tyrosyl-tRNA synthetase of the tyrosyladenylate synthesized by the noncognate phenylalanyl-tRNA synthetase, taking into account the internal concentration in aminoacyl-tRNA synthetases and the respective equilibrium constants. The above reasons may make unnecessary an additional proofreading step at the level of the aminoacylated tRNA. However, further experiments are in progress to detect a transfer of the misactivated tyrosine to $tRNA^{Phe}$ possibly followed by a correction step.

Acknowledgments

We gratefully acknowledge the help of Drs. Zana, Sturm, and Lang in making available their stopped-flow apparatus and computation facilities. We thank Professor Boulanger for carrying out the amino acid analyses.

Appendix

According to the kinetic pathway for the formation of aminoacyladenylate in the noncognate system (see text), writing z = [E], u = [E·ATP], w = [E·AA], y = [E^{ATP}_{AA}], and x = [E·Ad] and assuming that all association–dissociation equi-

libria are fast in front of the irreversible aminoacyladenylate formation, we have

$$\frac{d(x + [Ad])}{dt} = k_f y \quad (A1)$$

$$\frac{z[Ad]}{x} = K_{Ad} \qquad \frac{z[ATP]}{u} = K_{ATP}$$
$$\frac{u[AA]}{y} = K_{AA}{}^{ATP} = K_{AA} = \frac{z[AA]}{w} \quad (A2)$$

$$[E]_0 = z + u + y + x + w \quad (A3)$$

where $[E]_0$ is the input enzyme concentration and K_{Ad}, K_{ATP}, and K_{AA} are the equilibrium dissociation constants of the complexes between phenylalanyl-tRNA synthetase and respectively aminoacyladenylate, ATP, and amino acid. Taking eq A2 into account, eq A3 can be written as

$$[E]_0 = \left(\frac{K_{ATP}}{[ATP]} + 1 + \frac{[AA]}{K_{AA}} + \frac{[AA]}{[ATP]}\frac{K_{ATP}}{K_{AA}}\right)u + x \quad (A4)$$

If $[AA] \ll K_{AA}$, eq A4 will be reduced to

$$[E]_0 = \frac{u}{S_{ATP}} + x \quad (A5)$$

where S_{ATP} is the saturation fraction by ATP. All reactants can now be expressed as a function of x:

$$u = S_{ATP}([E]_0 - x) \quad (A6)$$

$$y = \frac{[AA]}{K_{AA}} S_{ATP}([E]_0 - x) \quad (A7)$$

$$z = \frac{K_{ATP}}{K_{ATP} + [ATP]}([E]_0 - x) \quad (A8)$$

$$[Ad] = K_{Ad}\frac{K_{ATP} + [ATP]}{K_{ATP}}\frac{x}{[E]_0 - x} \quad (A9)$$

Thus

$$x + [Ad] = x + \frac{K_{Ad}(K_{ATP} + [ATP])}{K_{ATP}}\frac{x}{[E]_0 - x}$$

$$x + [Ad] = x\frac{\left([E]_0 + \frac{K_{ATP} + [ATP]}{K_{ATP}}K_{Ad}\right) - x}{[E]_0 - x} = x\frac{A - x}{[E]_0 - x} \quad (A10)$$

where $A = [E]_0 + [(K_{ATP} + [ATP])/K_{ATP}]K_{Ad}$. Under our experimental conditions, A is usually much larger than x: for instance, if $[E]_0$ is in the range 1–2 μM, x will be lower than 0.5–1 μM (since we restrict ourselves to the time scale where the adenylate-enzyme complex concentration is lower than half the input enzyme concentration, i.e., $\Delta F < {}^1/_2\Delta F_{max}$) whereas A will be in the range of 10 μM. Thus, eq A10 can be simplified as follows:

$$x + [Ad] \simeq A\frac{x}{[E]_0 - x} \quad (A11)$$

Then, according to eq A1 and A7

$$\frac{d\left(A\frac{x}{[E]_0 - x}\right)}{dt} = k_f\frac{[AA]}{K_{AA}}S_{ATP}([E]_0 - x) \quad (A12)$$

The integrated form of eq A12 is

$$\frac{1}{([E]_0 - x)^2} = 2\frac{S_{ATP}k_f[AA]}{K_{AA}[E]_0\left([E]_0 + \frac{K_{ATP} + [ATP]}{K_{ATP}}K_{Ad}\right)}t + \text{constant} \quad (A13)$$

Of course, for $t = 0$, $x = 0$, and thus the integration constant is equal to $1/[E]_0{}^2$; therefore

$$\frac{1}{([E]_0 - x)^2} = 2\frac{S_{ATP}k_f[AA]}{K_{AA}[E]_0\left([E]_0 + \frac{K_{ATP} + [ATP]}{K_{ATP}}K_{Ad}\right)}t + \frac{1}{[E]_0{}^2} \quad (A14)$$

Registry No. L-Tyrosyladenylate, 50466-77-8; L-phenylalanyl-adenylate, 35874-27-2; L-phenylalanine, 63-91-2; L-tyrosine, 60-18-4; phenylalanyl-tRNA synthetase, 9055-66-7.

References

Baltzinger, M., Lin, S. X., & Remy, P. (1983) *Biochemistry* (preceding paper in this issue).

Berg, P. (1958) *J. Biol. Chem. 233*, 608–611.

Blanquet, S., Fayat, G., Waller, J. P., & Iwatsubo, M. (1972) *Eur. J. Biochem. 24*, 461–469.

Collowick, S. P., & Womack, F. C. (1969) *J. Biol. Chem. 244*, 774–777.

Czerlinsky, G. H. (1966) *Chemical Relaxation: An Introduction to Theory and Application of Stepwise Perturbation*, Marcel Dekker, New York.

Dibbelt, L. (1981) Ph.D. Thesis, Munchen, FRG.

Eadie, G. S. (1942) *J. Biol. Chem. 146*, 85–93.

Fasiolo, F., & Ebel, J. P. (1974) *Eur. J. Biochem. 49*, 257–263.

Fasiolo, F., Ebel, J. P., & Lazdunski, M. (1977) *Eur. J. Biochem. 73*, 7–15.

Fasiolo, F., Remy, P., & Holler, E. (1981) *Biochemistry 20*, 3851–3856.

Fersht, A. R., & Kaethner, M. M. (1976) *Biochemistry 15*, 3342–3346.

Fersht, A. R., Shindler, J. S., & Tsui, W. C. (1980) *Biochemistry 19*, 5520–5524.

Holler, E., & Calvin, M. (1972) *Biochemistry 11*, 3741–3752.

Holler, E., & Kosakowski, H. M. (1973) *Biochem. Biophys. Res. Commun. 54*, 1532–1539.

Hopfield, J. J. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 4135–4139.

Igloi, G. L., von der Haar, F., & Cramer, F. (1978) *Biochemistry 17*, 3459–3468.

Jakubowsky, H. Z., Pastuzyn, A., & Loftfield, R. B. (1977) *Anal. Biochem. 82*, 29–37.

Loftfield, R. B., & Vanderjagt, M. A. (1972) *Biochem. J. 128*, 1353–1359.