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Equilibrium Analysis of L-Phe-tRNA^{Phe} Complexes with L-Phenylalanyl Transfer Ribonucleic Acid Synthetase of *Escherichia coli* K 10†

Peter Bartmann, Till Hanke, Beate Hammer-Raber, and Eggehard Holler*

ABSTRACT: The interaction of tRNA^{Phe} and Phe-tRNA^{Phe} with L-Phe-tRNA synthetase has been investigated by equilibrium techniques. Dissociation constants were in the range of 1×10^{-7} M at pH 7.5. A 1:1 stoichiometry was demonstrated for tRNA^{Phe} by the equilibrium gel filtration method. Phe-tRNA^{Phe} was shown to be bound to the synthetase predominantly in a configuration wherein the L-phenylalanyl moiety is not in contact with the amino acid binding site. Using 2-*p*-toluidinylnaphthalene-6-sulfonate as a fluorescent reporter group, distinct steps of the synthetase-catalyzed reversal of the tRNA-aminoacylation could be followed as could the formation of the enzyme-bound adenylate and its breakdown to ATP and L-phenylalanine.

A mechanism of the catalytic action of aminoacyl-tRNA synthetases has been proposed recently for the L-isoleucine enzyme of *Escherichia coli* (Yarus and Berg, 1969; Eldred and Schimmel, 1972; Schimmel, 1973). Accordingly, the reaction can be separated into: (1) activation of the amino acid by formation of aminoacyl adenylate from ATP and amino acid; and (2) transfer of the aminoacyl moiety to cognate tRNA. Based on measurements under conditions of pH and temperature quite different from those required for optimal activity, it was concluded that overall reaction is controlled by the rate of dissociation of the aminoacyl-tRNA–enzyme complex. Evidence that this may not be valid under other conditions and for other enzymes emerged recently from fast kinetic measurements (Pingoud *et al.*, 1973) and from aminoacylation kinetics substituting ATP by thio analogs (E. Holler and W. Eckstein, unpublished observations). Even though it has been shown that aminoacyl-tRNA and amino acid can bind simultaneously to the enzyme (Yarus and Berg, 1969; Eldred and Schimmel, 1973; Hélène *et al.*, 1971; Charlier, 1972), the existence of only one type of aminoacyl-tRNA complex has been presumed in the quantitative treatment of the aminoacyl transfer step.

The aim of the present publication is to provide new evidence for the existence of two types of complexes and to present a sensitive fluorimetric method for the investigation of the transfer reaction by rapid kinetic techniques.

Materials and Methods

Preparation of Enzyme and tRNA. L-Phe-tRNA synthetase (specific activity 53,600 nmol mg^{-1} hr^{-1}) was prepared from *Escherichia coli* K IO in the presence of phenylmethanesulfon-

† From the Fachbereich Biologie, Universität Regensburg, 84 Regensburg, Federal Republic of Germany. *Received April 1, 1974.* This work was generously supported by a grant from the Deutsche Forschungsgemeinschaft and is part of the thesis of P. B. Dedicated to Professor Hermann Hartmann on the occasion of his sixtieth birthday.

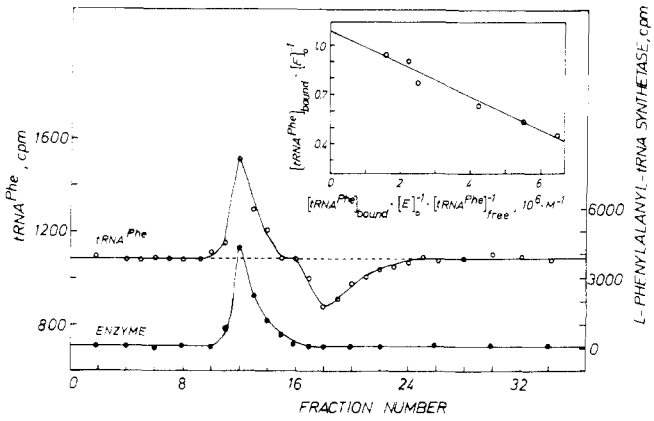

FIGURE 1: Equilibrium gel filtration. Elution profile for L-Phe-tRNA synthetase (●) and $tRNA^{Phe}$ (○) as measured by the standard aminoacylation assay; 100 μl of enzyme (6×10^{-7} M) and unfractionated tRNA (containing 6×10^{-7} M $tRNA^{Phe}$) were applied to the column. Results were evaluated according to Scatchard (1949) (inset). $tRNA^{Phe}$ concentration has been varied from 7×10^{-8} to 6×10^{-7} M.

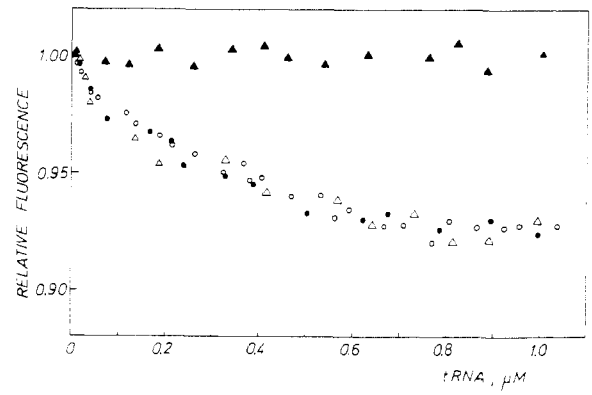

FIGURE 2: Titration of L-Phe-tRNA synthetase with (○) $tRNA^{Phe}$, (●) Phe-$tRNA^{Phe}$, (△) Phe-$tRNA^{Phe}$ in the presence of 1.5×10^{-4} M L-phenylalanine, and (▲) $tRNA^{Ile}$. The intrinsic tryptophan fluorescence was measured and corrected as described in the text.

yl fluoride as described by Hanke *et al.* (1974). $tRNA^{Ile}$ and $tRNA^{Phe}$ were prepared from unfractionated tRNA (Zubay, 1962) using chromatography on benzoylated DEAE-cellulose (Gillam *et al.*, 1967) and RPC-5 columns (Pearson *et al.*, 1971). $tRNA^{Ile}$ had an acceptance of 790 pmol and $tRNA^{Phe}$ of 1250 pmol per A_{260} unit (measured at pH 7 in H_2O). Also, $tRNA^{Phe}$ was purchased from Boehringer (Mannheim) and had an acceptance of 1090 pmol/A_{260} unit.

Preparation of [^{14}C]*Phe-tRNA*^{Phe}. $tRNA^{Phe}$, 4 μM, was aminoacylated in 5 ml of a mixture containing 100 mM Tris-HCl (pH 7.5), 10 mM KCl, 10 mM $MgCl_2$, 2 mM glutathione, 2 mM ATP, 20 μM L-[^{14}C]phenylalanine (10 Ci/mol), and 10 nM purified enzyme by incubating for 10 min at 28°. To this was added 0.55 ml of 1 M sodium acetate (pH 5.0), and the solution was applied to a column (0.7 cm × 2.5 cm) of DEAE-cellulose which had been preequilibrated at room temperature with a buffer containing 0.1 M sodium acetate (pH 5.0) and 0.2 M NaCl. After removal of enzyme and substrates by washing with the same buffer, Phe-$tRNA^{Phe}$ was eluted with a buffer containing 2.0 M NaCl, and precipitated by addition of 2 volumes of ethanol. After centrifugation, the precipitate was dissolved in 10 mM sodium acetate buffer (pH 5.0) containing 10^{-3} M $MgCl_2$ and stored at −20°. [^{14}C]Phe-$tRNA^{Phe}$ was found to be stable under these conditions for several months.

Chemicals. Uniformly labeled L- [^{14}C]phenylalanine with a specific radioactivity of 450 Ci/mol was obtained from Radiochemical Centre (Amersham). TNS[1] was purchased from Serva (Heidelberg), and all other chemicals (analytical grade) were from Merck (Darmstadt).

Measurement of Radioactivity. The filter paper disk method described by Mans and Novelli (1961) was used to determine the amount of aminoacylated tRNA. Radioactivity was counted in a Nuclear Chicago Isocap 300 scintillation spectrophotometer using toluene containing 0.5% 2,5-diphenyloxazole as scintillation liquid. Quenching corrections were made with reference to an internal standard.

Equilibrium Gel Filtration. The extent of complex formation between enzyme and $tRNA^{Phe}$ was measured according to Hummel and Dreyer (1962). Gel filtration on Sephadex G-100 (fine) was performed on 0.6 cm × 60 cm columns collecting fractions of 10 drops at a rate of 7 ml/hr. The elution buffer contained 20 mM Tris-HCl (pH 7.5), 0.2 mM EDTA, 5 mM $MgCl_2$, 0.1 M KCl, 0.3 mM dithiothreitol, and varying amounts of unfractionated tRNA. The enzyme which was applied to the column was dissolved in the same buffer. Activity was measured before and after each experiment. Recovery was 70–80% of total activity applied. Amounts of tRNA were determined according to the standard procedure described by Kosakowski and Böck (1970). The amount of $tRNA^{Phe}$ bound to the enzyme was determined from the average value of areas of the peak and trough in the elution profile. The amounts of bound tRNA were plotted as a function of free tRNA according to Scatchard (1949). Determination of the stoichiometry of complex formation took into account the recovery of the enzyme.

Fluorescence Titration Experiments. A Perkin-Elmer MPF-2A fluorescence spectrophotometer thermostated at 25 ± 0.5° and quartz cuvets from Hellma (Müllheim) were used for all experiments. When intrinsic tryptophan fluorescence was measured, excitation wavelength was 295 nm and emission wavelength 340 nm. Measurements were performed at pH 7.5 in sodium–potassium phosphate buffer, 0.025 M, including 0.5 mM EDTA, 8 mM $MgCl_2$, and 0.3 mM dithiothreitol. In each experiment a 0.1 μM solution of enzyme was titrated with purified $tRNA^{Phe}$ up to a final tRNA concentration of 1 μM. All other experiments have been performed in standard buffer, pH 7.5, consisting of 0.05 M Tris-HCl, 0.1 mM EDTA, 1 mM $MgCl_2$, and 0.3 mM dithiothreitol. In experiments with TNS as a reporter group, fluorescence was excited at 366 nm and emission was recorded at 445 nm. Concentration of the dye was 8 μM. The titrations were performed according to Kosakowski and Holler (1973). Deacylation of [^{14}C]Phe-$tRNA^{Phe}$ during the experiments was followed by pipetting 50-μl portions from the cuvet onto a filter paper and by counting the radioactivity as described by Mans and Novelli (1961). All corrections made in connection with the evaluation of the fluorescence results are discussed under Results. Dissociation constants were determined from plots according to Eadie (1942). Experiments were performed at least in triplicate.

Results

Equilibrium Gel Filtration. A redetermination of the stoichiometry and dissociation constant of the L-Phe-tRNA synthetase complex with $tRNA^{Phe}$ appeared to be necessary for two reasons. (1) Stoichiometry had to be based on a recently redetermined value for the molecular weight of 267,000 (Hanke *et al.*, 1974; Fayat *et al.*, 1974). For reasons of symmetry, the redetermined subunit structure $\alpha_2\beta_2$ would favor the

[1] Abbreviation used is: TNS, 2-*p*-toluidinylnaphthalene-6-sulfonate.

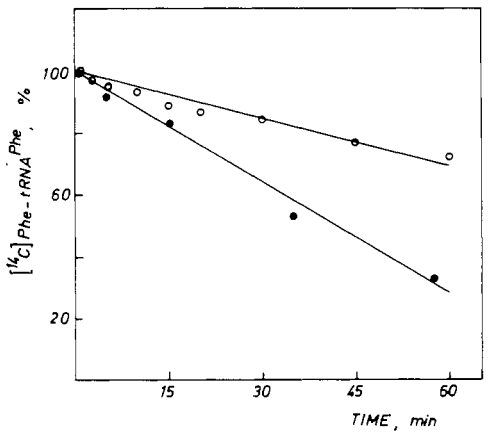

FIGURE 3: Deacylation of [^{14}C]Phe-tRNA^{Phe} in standard buffer pH 7.5, containing 0.05 M Tris-HCl, 0.1 mM EDTA, 0.3 mM dithiothreitol, and 1 mM $MgCl_2$ measured at room temperature. (○) 8×10^{-7} M [^{14}C]Phe-tRNA^{Phe}, (●) 8×10^{-7} M [^{14}C]Phe-tRNA^{Phe} and 4×10^{-7} M L-Phe-tRNA synthetase.

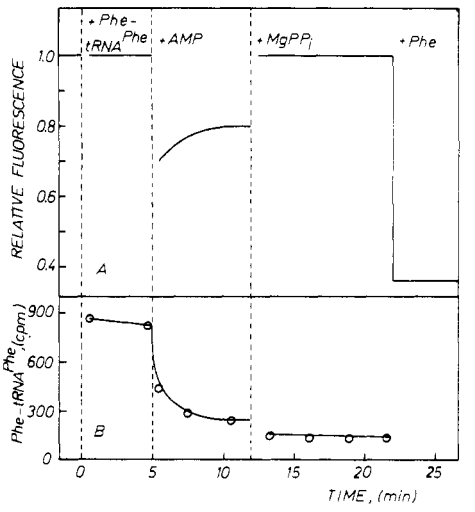

FIGURE 4: L-Phe-tRNA synthetase-catalyzed reversal of the aminoacylation reaction. (A) Relative fluorescence of TNS. (B) [^{14}C]Phe-tRNA^{Phe} determined by the filter paper assay. To a solution of L-Phe-tRNA synthetase (4×10^{-7} M), the following compounds have been added stepwise to give final concentrations of 1.4×10^{-6} M [^{14}C]Phe-tRNA^{Phe}, 1.5×10^{-3} M AMP, 7×10^{-5} M $MgPP_i$, and 4×10^{-4} M L-phenylalanine.

existence of two active sites. (2) Binding measurements had to refer to true equilibrium conditions like those reported by Hummel and Dreyer (1962).

Figure 1 inset shows the Scatchard plot obtained from gel filtration equilibrium measurements. The stoichiometry of the enzyme-tRNA^{Phe} complex is 1:1, taking into account the actual activity of enzyme, and the newly determined molecular weight. The dissociation constant was calculated from the slope to be 1.0×10^{-7} M.

Titration Experiments Using Intrinsic Tryptophan Fluorescence. Binding experiments with purified tRNA^{Phe} were performed by fluorimetric methods, which consumed less material than equilibrium gel filtration. The problem of correction for absorbance of light in the exciting beam was solved by using as a standard a solution of tryptophan having the same concentration as calculated for the enzymic tryptophan on the basis of the known amino acid content (Kosakowski and Böck, 1970). A correction curve, expressed as the fraction of quenching, was established as a function of tRNA^{Phe} concentration. Original fluorescence measurements of L-Phe-tRNA synthetase at varying concentrations of tRNA^{Phe} were first corrected for dilution and light scattering and finally by application of the fraction quenching curve. As a control, it was determined (Figure 2) that the corrected values of enzyme fluorescence remained constant when tRNA^{Ile} was used for titration. In the case of titration with Phe-tRNA^{Phe}, spontaneous and enzyme-catalyzed hydrolysis had to be considered (Schreier and Schimmel, 1972; Bonnet and Ebel, 1972). As may be seen from Figure 3, only 10% of the aminoacyl-tRNA was hydrolyzed during the first 10 min. Titrations were always finished within this period so that corrections were not necessary.

It is seen from Figure 2 that complex formation with tRNA^{Phe} followed a concentration dependence which is independent of the presence or absence of the aminoacyl moiety. For evaluation of the dissociation constant a Scatchard plot was applied. On the basis of the equilibrium gel filtration results, a 1:1 stoichiometry was assumed for the enzyme-tRNA complex, and thus the fraction of bound tRNA was calculated from the actually observed degree of fluorescence quenching divided by the degree of quenching for complete saturation of enzyme with tRNA.

The dissociation constants for tRNA^{Phe} and for Phe-tRNA^{Phe} were determined to be 1.2×10^{-7} and 1.1×10^{-7} M, respectively. Maximum quenching at saturation conditions was 7% for both tRNAs.

Simultaneous binding of L-phenylalanine to the enzyme-Phe-tRNA^{Phe} complex had no effect. This was demonstrated for saturating (1.5×10^{-4} M) concentration of amino acid (Figure 2).

Fluorescence Titrations Using TNS as a Reporter Group. The interaction of Phe-tRNA^{Phe} with the L-phenylalanine binding site was investigated *via* the fluorescence of TNS. The dye is known to bind specifically to the same site as does the amino acid (Holler and Kosakowski, 1973). Binding of L-phenylalanine displaces TNS and thus causes quenching of fluorescence. Other ligands like AMP or pyrophosphate do not alter the fluorescence.

Titration with Phe-tRNA^{Phe} was performed by stepwise addition of a total volume of 50 μl from a syringe to 700 μl of a solution containing the enzyme and TNS. A correction for the simultaneous dilution was made using the fluorescence decrease measured in a second titration with buffer instead of tRNA.

When Phe-tRNA^{Phe} was added to give a final, saturating concentration of 1.4 μM, fluorescence intensity remained unchanged (Figure 4A). Counting of a sample drawn from the cuvet indicated that essentially all radioactive amino acid was still present as aminoacyl-tRNA (Figure 4B). These results indicate that TNS is not displaced, when Phe-tRNA^{Phe} becomes bound to the enzyme. The addition of Phe-tRNA^{Phe} was followed by a titration with L-phenylalanine (Figure 5) in order to confirm the conclusion that the L-phenylalanine binding site had not been occupied by Phe-tRNA^{Phe}. The value determined for the dissociation constant of 4.0×10^{-5} M and the maximum quenching of approximately 64% at saturating concentration of amino acid compare well with the values of 3.0×10^{-5} M and 60%, respectively, which have been obtained for the formation of the binary complex (Holler *et al.*, 1973). Further experiments were designed to elucidate the involvement of the amino acid binding site. Subsequent to the addition of Phe-tRNA^{Phe} varying amounts of AMP were added to give final concentrations of 1.5–7.5 mM. Within the 30 sec needed for mixing, fluorescence dropped by approximately 30% and it then returned in a time-dependent reaction to a final level of 80% of the initial value (Figure 4A). Following the deacylation of Phe-tRNA^{Phe} (Figure 4B) by measuring radioactivity bound

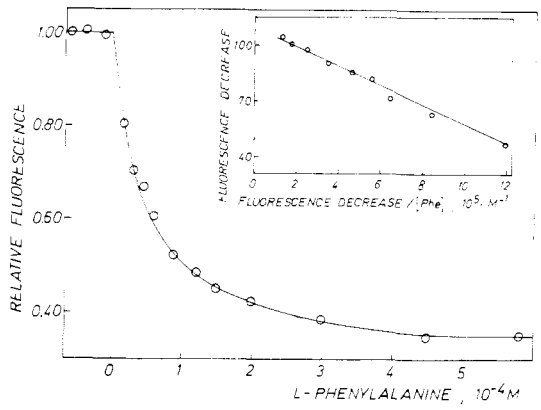

FIGURE 5: L-Phe-tRNA synthetase (2×10^{-7} M) was titrated with L-phenylalanine in the presence of saturating amounts of Phe-tRNA^{Phe} (1×10^{-6} M). Relative fluorescence of TNS was observed. In the inset, fluorescence quenching is plotted as a function of L-phenylalanine concentration according to Holler and Kosakowski (1973). Initial fluorescence was 164 (arbitrary) units.

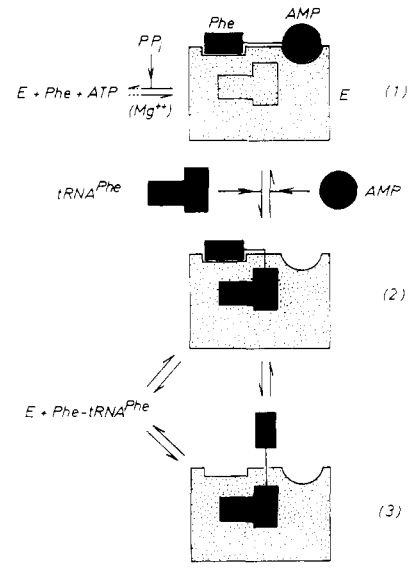

FIGURE 6: Formation of Phe-tRNA^{Phe} including results presented in this paper. Transfer of the L-phenylalanyl moiety from the adenylate (Phe-AMP) to tRNA^{Phe} is thought to result in an enzyme-Phe-tRNA^{Phe} complex which has the aminoacyl moiety in contact with its original binding site. This complex equilibrates to its thermodynamically more stable form in which the aminoacyl moiety is released from the amino acid binding site. In experiments at equilibrium conditions it is this complex being observed. Numbering refers to (1) L-Phe-tRNA synthetase-L-phenylalanyl adenylate complex; (2) the enzyme-L-Phe-tRNA^{Phe} complex with the L-phenylalanyl moiety in contact with the L-phenylalanine binding site; (3) the same complex, however, the L-phenylalanyl moiety released from this site.

to tRNA^{Phe}, it was found that within 30 sec after addition of AMP about one-third of the radioactivity had been liberated. This amount is equivalent to the amount of enzyme present, thus indicating that all Phe-tRNA^{Phe} which was complexed with the enzyme had immediately been deacylated. When the extent of AMP-induced fluorescence quenching (20%) was compared with the total quenching (64%) upon addition of saturating amounts of L-phenylalanine (Figure 4A, at the end of the experiment), it was seen that only a fraction of the enzyme must have been occupied by L-phenylalanine, probably as L-phenylalanyl adenylate (Holler and Calvin, 1972). The apparent discrepancy between this conclusion and the initial liberation of amino acid from tRNA will be dealt with under Discussion.

The final quenching after addition of AMP was completely eliminated when magnesium pyrophosphate was added. This would have been expected if quenching had been caused by formation of adenylate. The reappearance of the original level of fluorescence seems to be associated with a final drop of radioactivity.

Discussion

In previous investigations it has been reported that the rates of association and dissociation of aminoacyl-tRNA complexes with appropriate synthetases are significantly enhanced in the presence of the cognate amino acids or derivatives thereof (Yarus and Berg, 1969; Eldred and Schimmel, 1972; Hélène *et al.*, 1971; Charlier, 1972). Yarus and Berg (1969) have concluded from their results for the L-isoleucine system that the enzyme-Ile-tRNA^{Ile} complex may consist in a conformation in which the aminoacyl moiety does not bind to the amino acid specific site on the enzyme. Our present method appears to confirm these observations directly: Phe-tRNA^{Phe} does not displace the fluorescent reporter group, TNS, from the binding site under question. Additional evidence comes from titration experiments with Phe-tRNA^{Phe} in the presence of saturating concentrations of L-phenylalanine, and titration with L-phenylalanine in the presence of saturating concentrations of Phe-tRNA^{Phe}. In both cases, the values of the dissociation constants were found to be in agreement with the values measured for the formation of the binary complexes indicating that binding of amino acid and aminoacyl-tRNA are independent. While similar conclusion can be drawn from binding experiments with the enzymes specific for L-valine (Hélène *et al.*, 1971) or L-isoleucine (Yarus and Berg, 1969, Charlier, 1972), other phenomena like the rate enhancement mentioned above seem to indicate a configurationally mediated coupling of these sites. A known example of interdependence between the site binding the amino acid and the site binding tRNA is known from the investigations of L-arginyl-tRNA synthetase by Mehler and Mitra (1967). Our results are consistent with the reaction scheme shown in Figure 6. Here, the different complexes refer to the enzyme-L-phenylalanyl adenylate complex (1); the enzyme-Phe-tRNA^{Phe} complex (2), where the L-phenylalanyl moiety is in contact with the amino acid binding site; and the enzyme-Phe-tRNA^{Phe} complex (3) where the L-phenylalanyl moiety is not in contact with this site.

Our data are not sufficient to decide whether the Phe-tRNA^{Phe} can dissociate from both complexes or only from one of the two.

The reaction initiated by addition of AMP to the enzyme-Phe-tRNA^{Phe} mixture is very fast. Apparently, all aminoacyl-tRNA bound to the enzyme is deacylated at the moment when the nucleotide is added. Further release is probably controlled by displacement of tRNA^{Phe} by Phe-tRNA^{Phe} from the solution. The time scale compares readily with displacement kinetics reported previously for the L-isoleucine system (Yarus and Berg, 1969).

According to the commonly accepted scheme, the product of the reaction with AMP is expected to be enzyme-bound L-phenylalanyl adenylate. On the assumption that the adenylate displaces TNS competitively, it is surprising that only a fraction of the expected fluorescence decrease is observed. A possible explanation is that part of initially formed adenylate, or the enzyme-bound aminoacyl-tRNA itself, is hydrolyzed at high rate within mixing time. Appreciable hydrolysis has been observed during formation of L-isoleucyl-AMP (Iaccarino and Berg, 1969) and during acylation of tRNA with L-valyl-tRNA syn-

thetase (Bonnet and Ebel, 1972). The rate of hydrolysis must be comparable to the rate of formation of adenylate since attainment of a steady state is indicated by the time dependence of the fluorescence signal.

Addition of magnesium pyrophosphate causes decomposition of the adenylate into ATP and L-phenylalanine. Since the initial concentration of Phe-tRNA^{Phe} was more than 20-fold below the value of the dissociation constant for L-phenylalanine and more than 500-fold below that for magnesium–ATP, nearly all product formed upon addition of pyrophosphate would be expected to dissociate from the enzyme. This would explain why the level of fluorescence returns to the initial intensity. Decomposition is also reflected by the final drop in radioactivity assayed by the filter paper method. As the steady-state concentration of adenylate is rapidly being reduced, the amount of bound Phe-tRNA^{Phe} in equilibrium with bound adenylate has to be reduced simultaneously. Toward the end, the level of radioactivity decreases slowly, and is probably controlled by the slow dissociation of deacylated tRNA. The qualitative discussion of the reverse of the aminoacylation reaction catalyzed by L-phenylalanyl-tRNA synthetase is seen to be in agreement with most of the predictions made from previous models (Yarus and Berg, 1969; Eldred and Schimmel, 1972; Bonnet and Ebel, 1972). The ease of the fluorimetric method presented in this paper opens the possibility of detailed kinetic investigations in particular with the use of stopped-flow techniques. Such investigations are under way in our laboratory.

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