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L-Phenylalanine:tRNA Ligase of *Escherichia coli* K10. A Rapid Kinetic Investigation of the Catalytic Reaction†

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ABSTRACT: The kinetics of the amino acid activation and the transfer of the amino acid to tRNA have been investigated for L-phenylalanine:tRNA ligase of *Escherichia coli* K10 by stopped-flow and radioactive techniques. The rapid kinetics were followed by the observation of the displacement of the fluorescent dye, 6-*p*-toluidinylnaphthalene-2-sulfonate from the binding site of L-phenylalanine under conditions where a single active site of the enzyme was involved. The following results are of particular interest. (1) Equilibrium binding of L-phenylalanine and $tRNA^{Phe}$ indicates in each case two sites of interaction with an approximately tenfold difference of the binding affinity. (2) Experimental conditions of the kinetic investigation were chosen to favor reactions at the high affinity binding sites. Under those conditions, the rate constants have been evaluated at 1 m*M* magnesium to be in the range 12–25 sec^{-1} for the activation reaction and 42–77 sec^{-1} for the reverse, the variation of the values depending on those of the dissociation constants used for computation. The rate constant for the transfer reaction is 0.05 sec^{-1} and for the reverse 0.19 sec^{-1}. The forward reaction is rate limiting for the overall reaction at single turnover and steady-state conditions. (3) All rate constants depend on the concentration of magnesium. Evidence is provided that the transfer occurs via a productive enzyme-$tRNA^{Phe}$ complex which is in a magnesium-dependent equilibrium with an unproductive complex, high magnesium favoring the former. The position of the tRNA-CCA end in the productive complex is such, that the fluorescent dye can be displaced by Phe-$tRNA^{Phe}$. The thermodynamics of the overall reaction have been treated on the basis of the partial reactions. The free enthalpy of the completed reaction was calculated to be very close to zero. The significance of the adenylate intermediate is discussed with respect to the product inhibition expected on the basis of the tendency of $tRNA^{Phe}$ and L-phenylalanine to form tight complexes with the enzyme.

Previous investigations have provided considerable evidence that the aminoacylation of tRNA as catalyzed by L-amino acid:tRNA ligases proceeds via an activation of the amino acid and a subsequent transfer of this moiety to tRNA (Kisselev and Favorova, 1974, and references therein). Despite many efforts to establish the kinetics of these enzymes, none of them has been investigated beyond the time limits of the classical radioactive techniques as there are the ATP-[^{32}P]PP_i exchange and ^{14}C amino acid labeling, to provide a detailed knowledge of the overall kinetics at conditions which are optimal for catalysis. In the present work we have attempted to resolve the catalytic steps, in particular those of the transfer reaction by means of stopped-flow experiments. We have also made classical radioactive measurements under single turnover and steady-state conditions. However, we have not been following the kinetics of substrate binding. In connection with previous results we have drawn conclusions about the catalysis by L-phenylalanine:tRNA ligase (*Escherichia coli*) which may be also valid for amino acid:tRNA ligases in general.

Materials and Methods

L-Phenylalanine:tRNA ligase (specific activity 53,600 nmol mg^{-1} hr^{-1}) was prepared from *E. coli* K10 in the presence of phenylmethanesulfonyl fluoride as described by Hanke et al. (1974). Unfractionated tRNA was obtained according to Zubay (1962) from *E. coli* K10. Chromatography on benzoylated DEAE-cellulose (Gillam et al., 1967) and RPC-5 columns (Pearson et al., 1971) was applied to obtain $tRNA^{Phe}$ having an amino acid acceptance of 1250 pmol/A_{260} unit (measured at pH 7 in H_2O). Enzyme activity and tRNA charging capacity were determined as described by Kosakowski and Böck (1970). Protein concentrations were measured according to Lowry et al. (1961) and Waddell (1965).

[^{14}C]Phe-$tRNA^{Phe}$ was prepared as described previously (Bartmann et al., 1974). Uniformly labeled L-[^{14}C]phenylalanine with a specific radioactivity of 450 Ci/mol was ob-

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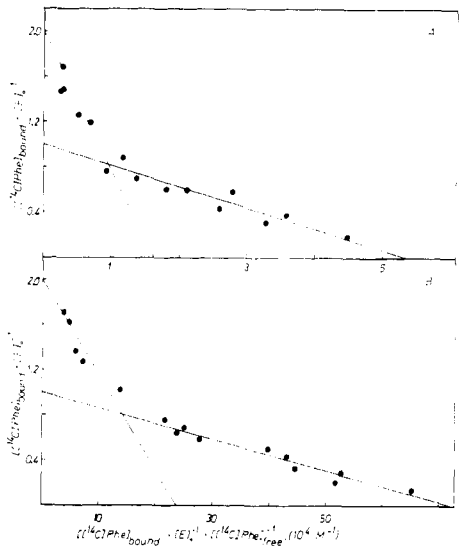

FIGURE 1: Equilibrium dialysis. (A) L-Phenylalanine:tRNA ligase was dialyzed against L-[^{14}C]phenylalanine at concentrations varying from 1 to 700 μM. (B) Dialysis of L-phenylalanine:tRNA ligase (4 μM) against L-[^{14}C]phenylalanine at concentrations varying from 0.5 to 50 μM in the presence of 5 mM adenosine in both dialysis cell compartments. Experiments were performed in buffer A as described under Materials and Methods.

tained from Radiochemical Centre (Amersham). TNS[1] was purchased from Serva (Heidelberg) and adenosine from Boehringer (Mannheim). L-Phenylalaninol was from Fluka (Buchs) and has been analyzed for homogeneity as described (Kosakowski and Holler, 1973). All other chemicals (analytical grade) were from Merck (Darmstadt).

Equilibrium dialysis was performed in lucite cells designed according to Englund et al. (1969). Each compartment had a volume of 200 μl and was filled with 100 μl of the appropriate solution. Visking dialysis membranes (Serva, Heidelberg) were used throughout. Experiments were performed in 0.05 M Tris-HCl (pH 7.5), 0.1 mM EDTA, 0.4 mM dithioerythritol, and 1 mM $MgCl_2$ (buffer A) or 10 mM $MgCl_2$ (buffer B). The dialysis was run for 5 hr at 22°. No unspecific binding of [^{14}C]Phe to protein was found using bovine serum albumin in a control experiment. Two 20-μl samples for each compartment were pipetted into 10 ml of dioxane containing 0.5% diphenyloxazole, and radioactivity was counted in a Nuclear Chicago Isocap 300 scintillation counter. Counting efficiency as determined by the use of an internal standard was 80%.

DEAE-Cellulose Filter Assay. The assay was performed at room temperature similar to the procedure described by Santi and Anderson (1974). The reaction mixture contained buffer A, 0.5 μM L-phenylalanine:tRNA ligase, and 0.5 μM [^{14}C]Phe-tRNA^{Phe} in a total volume of 300 μl and was incubated at 10°. Deacylation of the aminoacyl-tRNA was started by the addition of AMP in final concentrations varying from 30 μM to 6 mM. 20-μl samples were drawn at certain time intervals and pipetted onto DEAE-cellulose filter discs (Whatman DE 81, 23 mm) which had been presoaked in 0.1 M glycine-HCl buffer (pH 2.3). After 1 min the filter discs were washed with 10 ml of the glycine buffer and 3 ml of 70% ethanol. Radioactivity was counted after drying using a toluene cocktail including 0.5% diphenyloxazole with a counting efficiency of 65%. The procedure described was found to result in a low background (<100 cpm) and to retain [^{14}C]Phe-tRNA^{Phe} almost quantitatively (>95%). It was demonstrated in control experiments that no further deacylation of the aminoacyl-tRNA occurred after the sample had been pipetted on the filter discs presoaked with the glycine buffer.

Formation of the enzyme–aminoacyl adenylate complex was followed as described by Bartmann et al. (1975a) using detection on DEAE-cellulose filter discs. Instead of Tris-HCl buffer, a 0.05 M potassium phosphate buffer (pH 6) including 1 mM $MgCl_2$, 0.4 mM dithioerythritol, and 0.1 mM EDTA was applied.

Equilibrium Fluorescence Measurements. A Perkin-Elmer MPF-2A fluorescence spectrophotometer thermostated at 25 ± 0.5° and quartz cuvets from Hellma (Müllheim) were used for all experiments. These were performed exactly as described previously (Kosakowski and Holler, 1973; Bartmann et al., 1974).

Kinetic Measurements. Kinetics were followed by observing the time-dependent fluorescence change after rapid mixing of the reactants in a Durrum-Gibson stopped-flow spectrophotometer equipped with a Durrum 16400 fluorescence attachment. Excitation light was provided by a 75-W xenon lamp. After passing the Durrum monochromator and a UVD 25 filter (Hitachi Perkin-Elmer, max. transmittance at 320 nm with a band width of 160 nm) light of 320 nm entered the 20-mm reaction cell. Light intensity was observed at 90° to the incident beam through a cut-off filter (Corning 0-52, 75% transmittance at 370 nm). After preamplification by the photomultiplier the signal entered a Hewlett-Packard oscilloscope 181A equipped with a dual channel vertical amplifier (50 MHz) 1801A and a time base and delay generator 1822A. The traces were stored and photographed using a Dumont-Land Polaroid camera. The apparatus was thermostated using a Haake thermostat FK 10 at 10 or 25 ± 0.1°. The dead time was 1.6 msec. For each tracing, 0.2 ml from each drive syringe were mixed. The rate constant for the binding of TNS to the enzyme was found to be about 200 sec^{-1}. The slow kinetics of the dye did not allow observation of the kinetics for the formation of the enzyme–amino acid complex. Buffer A was used throughout except for experiments determining the magnesium dependence of the reaction. In these cases the appropriate amounts of $MgCl_2$ were added to buffer A. All solutions contained TNS in equal concentration (2.6 μM). Formation of the enzyme–L-phenylalanyl adenylate complex was measured via observation of the displacement of fluorescent dye. The reaction was started by rapid mixing of a solution containing 0.2 μM enzyme and 0.5 μM [^{14}C]Phe-tRNA^{Phe} with a solution containing varying amounts of AMP (20 μM–3 mM) unless otherwise stated. Each set of experiments was performed in less than 4 min. Deacylation of the aminoacyl-tRNA within this time was less than 5% of the total [^{14}C]Phe-tRNA^{Phe} (Bartmann et al., 1974).

Formation of the enzyme–aminoacyl adenylate complex was observed at low concentration of ligands as described by Holler and Calvin (1972). Experiments were performed by mixing 0.4 μM enzyme with 4 μM L-phenylalanine and 200 μM MgATP or 2 μM L-phenylalanine and 100 μM MgATP including inorganic pyrophosphate (sodium salt) at concentration varying from 0.5 to 30 μM. Formation of the enzyme–aminoacyl adenylate intermediate and the subsequent transfer of the aminoacyl moiety to tRNA^{Phe} was observed by mixing a solution containing 1 μM enzyme, 1 μM L-phenylalanine, and 2 μM tRNA^{Phe} with a solution

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

Table I: Stoichiometry and Dissociation Constants of Enzyme–Substrate Complexes at 25°.

Ligand	Experimental Condition	Dissociation Constant (μM)	Occupation of Site	K_{diss} (2)/ K_{diss} (1)
L-Phenylalanine	1 mM magnesium	19 (18); 27;[a] 30[b]	1	
		260 (280)	2	14
	0–10 mM magnesium + 0–2 μM $tRNA^{Phe}$	30–35[b]	Not determined	
	10 mM magnesium + 20 μM $tRNA^{Phe}$	170 (87)	1 + 2	
	1 mM magnesium + 5 mM adenosine	1.3 (1.2); 1.6[c]	1	
		15 (16)	2	12
MgATP	10mM magnesium + 5 mM L-phenylalaninol	1.2;[b] 1.9[a]	1[a]	
$tRNA^{Phe}$	1 mM magnesium	0.09[d]	1[d]	
		0.12[e]		
		1.2[d]	2[d]	10
	11 mM magnesium	0.2[f]	Not determined	

[a] Determined by nonequilibrium dialysis (Kosakowski and Böck, 1971; Kosakowski and Holler, 1973). [b] Fluorimetric titration in the presence of 2–8 μM TNS. [c] Determination as under *b* in the presence of 10 mM magnesium and 10 mM adenosine (Holler et al., 1975). [d] Equilibrium gel filtration (Bartmann et al., 1974, 1975b). [e] Fluorimetric titration observing emission at 340 nm (excitation 300 nm). Evaluation based on the assumption of a 1:1 stoichiometry (Bartmann et al., 1974, 1975b). [f] Determination according to the method used under *e*; values in parentheses were calculated for macroscopic dissociation constants (see Klotz and Hunston, 1971).

containing 4 mM MgATP.

Experimental errors are in the order of 20% for dissociation constants and up to 50% for rate constants. Factors of 2 should not be taken too seriously for kinetic results (cf. Holler and Calvin, 1972). The kinetic treatment is often considered as an approximation with respect to experimental conditions. However, it can be shown that methodical errors would not seriously affect the results.

Results

Stoichiometry and Dissociation Constants of Enzyme–Substrate Complexes. Molecular weight and subunit structure of L-phenylalanine:tRNA ligase have been recently redetermined to be 270,000 and $\alpha_2\beta_2$ instead of previously 180,000 and α_4, respectively (Hanke et al., 1974; Fayat et al., 1974). On the basis of this new molecular weight previous stoichiometry results of single sites per enzyme tetramer were now predicted two sites (Hanke et al., 1974). Reinvestigation then revealed two sites for L-phenylalanyl adenylate and $tRNA^{Phe}$ (Bartmann et al., 1975b). The present results from equilibrium dialysis experiments indicate also two binding sites for L-phenylalanine whether (a) in the absence of additional ligands (Figure 1A), (b) in the presence of the synergistic (Kosakowski and Holler, 1973) effector adenosine (Figure 1B), or (c) in the presence of magnesium and $tRNA^{Phe}$ at elevated concentrations with 10 μM enzyme, 10 mM magnesium, 20 μM $tRNA^{Phe}$, and L-[^{14}C]phenylalanine varying between 5 and 350 μM.

Except in the latter case (not shown), Scatchard plots were biphasic indicating two nonequivalent sites. Apparently they become indistinquishable (linear Scatchard plot with an intersection to indicate 2.2 ± 0.3 binding sites) as saturating concentrations of magnesium ($K_{Mg^{2+}}$ = 5 mM, Hanke et al., 1975) and $tRNA^{Phe}$ ($K_{tRNA(2)}$ = 1.2 μM, Table I) are approached. Identical sites are predicted on the basis of the $\alpha_2\beta_2$ subunit structure. The observed "asymmetry" suggests anticooperativity as has been proposed for binding of $tRNA^{Phe}$ (Bartmann et al., 1975b). The microscopic dissociation constants for binding of L-phenylalanine were evaluated from the slopes in Figure 1 according to Klotz and Hunston (1971). Their values differ more than tenfold. Whether the similar ratios $K_{diss(2)}/K_{diss(1)}$ for L-phenylalanine and $tRNA^{Phe}$ are indicative of a similar property for secondary binding remains to be established. In the case of yeast L-phenylalanine:tRNA ligase clearly different ratios have been reported (Fasiolo et al., 1974).

Table I contains results for the binding of MgATP and $tRNA^{Phe}$. Preliminary experiments for the nucleotide have been obtained in the presence of L-phenylalaninol, a synergistic effector, indicating only one site per tetramer (Kosakowski and Holler, 1973). However, it is to be expected that a second nonequivalent binding site can be detected at increased concentrations. The binding of ATP is a function of magnesium (Kosakowski and Holler, 1973). This is to be expected because of formation of MgATP (Martell and Schwarzenbach, 1956). Moreover, magnesium has been shown to affect the enzyme quaternary structure as well as the active site conformation (Hanke et al., 1975; Holler et al., 1975). The formation of enzyme complexes with either L-phenylalanine or $tRNA^{Phe}$ was not significantly dependent on the concentration of magnesium under the conditions applied (1–11 mM magnesium in excess of $tRNA^{Phe}$). The results (Table I) were compared with the values determined from fluorescence titration in the presence of 6-*p*-toluidinylnaphthalene-2-sulfonate. The values are similar in magnitude suggesting that the reporter group reflects binding of the amino acid to the high affinity site. We have previously reported that several molecules of the fluorescing compound interact with the enzyme, one of them exhibiting particular high affinity and enhanced fluorescence in the bound state (Holler and Kosakowski, 1973). Competition experiments with L-phenylalanine have demonstrated that dye and amino acid compete for the same site. Consequently, at low dye concentration the high affinity complex prevails which exhibits complete fluorescence quenching upon saturation with the amino acid. This is seen for 2 μM concentration of dye; at 8 μM concentration only 75% quenching of the fluorescence is observed. At these concentrations, titration curves in the form of Eadie (1942) plots are strictly linear, indicating together with the other observations that TNS functions as a genuine competitive reporter group at the high affinity site. This conclusion must be kept in

Table II: Kinetic Parameters of the L-Phenylalanine Activation Reaction at pH 7.5, 25°, and 1 mM Magnesium.

Parameters	Values
Slope ($10^5 M^{-1}$ sec^{-1})	1.4 ± 0.1[a,b]
Intercept (sec^{-1})	0.42 ± 0.04;[a] 0.12 ± 0.01[b]
K_{Phe} (μM)	10;[e] 19–35 (Table I)
K_{MgATP} (mM)	0.3;[c] 1.0;[d] 1.5[e]
K_{PP_i} (mM)	0.3;[c] 0.55[e]
k_f (sec^{-1})	12–22;[f] 32;[g] 13–25[h]
k_b sec^{-1})	42;[i] 77[j]
$k_f/(k_b$	0.3–0.5;[k] 0.4[l]
K_{eq} (μM)[m]	67;[a] 58;[b] 64[e]

[a] Determined from a linear plot of k_{obsd} vs. pyrophosphate concentration at $(Phe)_0$ = 2 μM and $(ATP)_0$ = 100 μM. The measurement is described in the text. [b] Determined as under *a* except that $(Phe)_0$ = 1 μM and $(ATP)_0$ = 50 μM. Errors are given as standard deviations. [c] From titration in the presence of TNS. See also Kosakowski and Holler (1973). [d] Michaelis–Menten constant from Santi et al. (1971a) in the presence of ca.17 mM free magnesium. [e] Mulivor and Rappaport (1973) in the presence of 3.3 mM free magnesium. [f] Calculated from Table I and *a* and *c*. [g] Calculated from *a* and *e*. [h] Calculated from Table I and *b* and *c*. [i] Calculated from *a* and *c*. [j] Calculated from *a* and *e*. [k] Calculated from *f* and *j*. [l] Calculated from *g* and *k*. [m] Calculated from the slope and intercept. Details see text.

mind for the evaluation of the kinetic experiments.

Amino Acid Activation Reaction. The formation of enzyme-bound L-phenylalanine adenylate was measured in the absence of tRNA by observation of the time-dependent fluorescence quenching due to displacement of TNS. At the low concentrations of L-phenylalanine applied, displacement following the formation of the enzyme–amino acid complex was negligible. The procedure was similar to that reported for the L-isoleucine system (Holler and Calvin, 1972). Rate constants k_{obsd} were evaluated from first-order plots of the time-dependent fluorescence decrease. They were measured as a function of the pyrophosphate concentration at two different, but constant concentrations of L-phenylalanine and ATP. The resulting values were linear functions of the pyrophosphate concentration in the range investigated (0–15 μM). The two sets of points could be approximated by a pair of parallel lines (results not shown). Slopes and intercepts with the k_{obsd} axis are given in Table II. The evaluation of the elementary rate constants of the amino acid activation reaction was based on the following equation (Holler and Calvin, 1972):

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

The rate constants k_f and k_b refer to the formation of the enzyme-adenylate-PP_i complex from the enzyme-L-phenylalanine-ATP (as MgATP) complex and its reverse reaction, respectively. The equation is a simplification and is limited to low concentrations of reactants, where initial concentrations are $(Phe)_0 \ll K_{Phe}$, $(ATP)_0 \ll K_{ATP}$, $(PP_i)_0 \ll K_{PP_i}$, and $(Phe)_0$, $(ATP)_0$, $(PP_i)_0 \gg (E)_0$, the total enzyme concentration. The dissociation constants K_{Phe}, K_{ATP}, and K_{PP_i} pertain to the formation of the ternary Michaelis–Menten complex and the ternary enzyme-product complex, respectively.

Equation 1 rests on the assumption of random preequilibrium for the formation of the ternary enzyme-substrate complex. This condition appears to be fulfilled on the basis of the results of steady-state kinetic analysis (Santi et al., 1971a; Mulivor and Rappaport, 1973). A second assumption is that the product L-phenylalanyl adenylate remains tightly bound, or at least does not dissociate from the enzyme to a measurable extent. The possibility of isolating the enzyme–adenylate complex from the substrates observed with this and other enzymes (Kisselev and Favorova, 1974; Bartmann et al., 1975b) is in accord with this requirement. Also, fluorescence quenching when evaluated as a first-order plot was usually linear against time over more than 90% of the reaction.

The values of the dissociation constants of eq 1 were assumed to be equal to the values of the dissociation constants for the corresponding binary complexes. This approximation appears to be justified when reference is made to the similarity of values obtained from equilibrium measurements and the kinetic analysis of the ATP-[^{32}P]PP_i exchange (Mulivor and Rappaport, 1973). The assumption also finds support from the investigations with various substrate analogs (Kosakowski and Holler, 1973; Holler and Kosakowski, 1973; Holler et al., 1975). The dissociation constants used and the parameters evaluated from the slopes and intercepts on the basis of eq 1 are listed in Table II. Also listed are values for the equilibrium constant which is defined by

$$K_{eq} = \frac{(E)(ATP)(Phe)}{(E\cdot Phe \sim AMP)(PP_i)} = \frac{\text{slope (Figure 3)}}{\text{intercept (Figure 3)}}(Phe)_0(ATP)_0 \quad (2)$$

(Holler and Calvin, 1972) and which can be determined without the knowledge of dissociation constants. The values in the table provide additional support for the validity of our basic assumptions: an almost identical set of parameters was determined for two different initial concentrations of substrates. Moreover, the values of K_{eq} are comparable with the value determined previously by a completely different method (Mulivor and Rappaport, 1973). It is of interest to compare k_b with k_{ap}, the rate constant of the ATP-[^{32}P]PP_i exchange reaction (the same notation is used as by Mulivor and Rappaport, 1973). For this purpose, the species X is interpreted as being identical with the ternary enzyme-product complex, E·Phe ~ AMP·PP_i. Then the rate constant is identified with $k_{ap} = k_b/K_{PP_i}$. The value of K_{PP_i} = 0.55 mM is taken from the same work. Since these authors have not given the concentration of the enzyme, their value for V_{Max} is used for computation under the reasonable assumption of $(E)_0$ = 5 nM which is based on our own results. With k_b = 77 sec^{-1} (Table II) we calculate k_{ap} = 1.4×10^5 M^{-1} sec^{-1} which compares with $k_{ap} \simeq 0.9 \times 10^5$ M^{-1} sec^{-1} of Mulivor's work. From the ATP-[^{32}P]PP_i exchange data of Santi et al. (1971a) we computed k_b = 81 sec^{-1} taking their value of k_{cat} = 18 μmol/(min mg). Again, this value compares favorably with our results.

The similarities considered above for the different investigations can, however, be only qualitative, because the rate of formation of enzyme–adenylate depends on the concentration of magnesium present. While the stopped-flow measurements were performed at 1 mM free magnesium, 3.3 mM were applied in Mulivor's experiments and even higher concentrations in Santi's work. An increase of the exchange rate has been discussed in detail by Santi et al. (1971a) who agree with us that magnesium is required in addition to the formation of MgATP and $MgPP_i$ (Cole and Schimmel, 1970).

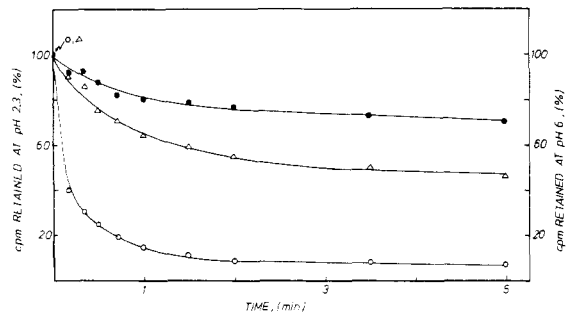

FIGURE 2: Deacylation kinetics of [^{14}C]Phe-tRNA^{Phe} in the presence of AMP at pH 7.5, 10°. The deacylation was followed applying the DEAE-cellulose filter assay as described under Materials and Methods. Deacylation in the presence of 0.3 mM AMP (○) and additional 0.6 mM L-phenylalanine (●). Simultaneous detection of aminoacylated tRNA^{Phe} and aminoacyl adenylate–enzyme complex in the presence of 0.3 mM AMP (△) as determined by the filter assay described by Bartmann et al. (1975a).

The Aminoacyl Transfer Reaction. We have recently suggested that the transfer of the L-phenylalanyl moiety can be investigated by observing the reporter group monitored formation of enzyme-bound adenylate starting from Phe-tRNA^{Phe} and AMP (Bartmann et al., 1974). Before turning to such experiments we looked for a direct measurement by means of radioactive labeling. Kinetic results of these attempts are given in Figure 2. Following the amount of radioactive [^{14}C]Phe-tRNA^{Phe} which is retained on DEAE filters at acidic conditions, biphasic kinetics were observed. An initial jump, too fast to be resolved by this technique, was followed by a measurable time dependence. At the acidic conditions applied, only tRNA is retained on the filter. When the conditions were adjusted to neutral pH, the initial jump disappeared while the slow release remained unchanged. It has been shown that under these conditions of the filter assay both tRNA and adenylate were retained almost quantitatively by the filter disc (Bartmann et al., 1975a). From the results it was concluded that the initial jump corresponded to the rapid transfer of the aminoacyl moiety from tRNA^{Phe} to AMP. The conclusion was confirmed by the inhibition experiment in the presence of saturating L-phenylalanine. Here, the amino acid specific binding site would be occupied preventing the formation of adenylate from Phe-tRNA^{Phe} and AMP. Accordingly, the initial jump was not seen in the presence of L-phenylalanine following the extent of the deacylation under the acidic conditions of the filter assay. Subsequent stopped-flow experiments monitoring the fluorescence of the enzyme-bound reporter group exhibited quenching kinetics which fitted the time elapsed between mixing and drawing of the first sample. The initial jump was evaluated as a function of AMP concentration at fixed concentrations of the other reactants. The double-reciprocal plot according to Eadie (1942) revealed a saturation phenomenon with a half-maximum concentration of 0.15 mM AMP (10°, 1 mM Mg^{2+}). All the radioactive experiments were performed at 10° since the release of the radioactive label was too fast at room temperature to be measured. This was found to be due to a highly increased instability of the enzyme-bound adenylate when AMP was present. The slow kinetic phase seen in Figure 4 presumably reflects hydrolysis of the adenylate. This reaction was not further investigated.

Stopped-flow measurements were undertaken to elucidate the rapid, unresolved part of the transfer reaction. As in the radioactive experiments we chose conditions where

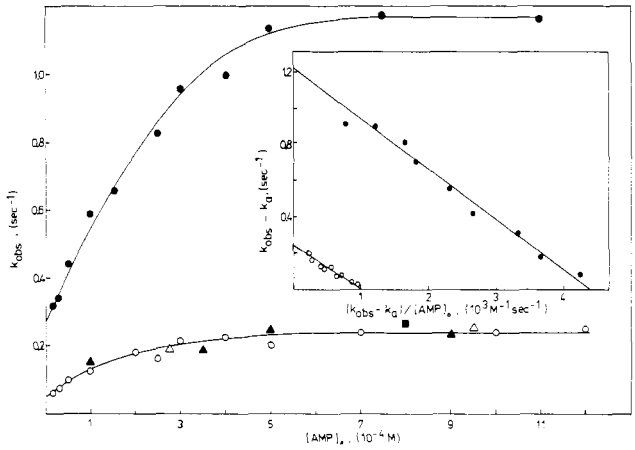

FIGURE 3: Deacylation of Phe-tRNA^{Phe} in the presence of AMP as determined by stopped-flow measurements. Rate constants k_{obsd} were determined from the slopes of first-order plots. Experiments were performed as described under Materials and Methods at 1 mM (○) and 1.4 mM (●) $MgCl_2$. Final concentrations of reactants were 0.1 μM enzyme and 0.25 μM Phe-tRNA^{Phe} (○, ●); 0.25 μM enzyme and 0.25 μM Phe-tRNA^{Phe} (▲); 0.5 μM enzyme and 0.1 μ Phe-tRNA^{Phe} (△); 0.1 μM enzyme and 1.0 μM Phe-tRNA^{Phe} (■). The plot in the inset refers to the linearization procedure described in the text and previously (Holler and Calvin, 1972). Temperature was 25°.

Phe-tRNA^{Phe} was in excess of enzyme, at a concentration $>K_{\text{Phe-tRNA}^{Phe}}$ (0.1 μM) almost saturating a single binding site but not interacting to a measurable extent with the second site of the enzyme. Recently, it has been established for other amino acid:tRNA ligases that the dissociation of the enzyme–tRNA complex could be a comparably rapid process leading to preequilibrium between the complex and the free Phe-tRNA^{Phe} (Pingoud et al., 1973; Krauss et al., 1973). With this in mind it was not expected that first-order plots of the time-dependent fluorescence quenching would be linear throughout the reaction. We have, however, observed linearity over more than 50% of the total signal. We have tested the reliability of the values for k_{obsd} determined from these plots by varying the concentrations of enzyme and Phe-tRNA^{Phe} as indicated in Figure 3. It is seen that the fluctuations are within experimental error.

Rate constants k_{obsd} were determined from the slopes of the initially linear first-order plots. They are given as a function of AMP concentration in Figure 3. The saturation type of the function and our knowledge of the products and enzyme–reactant complexes suggest an evaluation of the kinetic results on the basis of the following reaction scheme

$$\text{AMP} + \text{E·Phe-tRNA}^{Phe} \overset{K_{AMP}}{\rightleftharpoons} \text{AMP·E·Phe-tRNA}^{Phe} \quad (3)$$

$$\text{AMP·E·Phe-tRNA}^{Phe} \underset{k_a}{\overset{k_{da}}{\rightleftharpoons}} \text{Phe}\sim\text{AMP·E·tRNA}^{Phe} \quad (4)$$

where k_a, k_{da}, and K_{AMP} refer to the rate constants of tRNA acylation and deacylation, respectively, and to the dissociation constant of AMP from the ternary complex. The dissociation of the enzyme–tRNA complexes has not been taken into consideration since conditions were such as to approximately saturate the enzyme with the ribonucleic acid (cf. Table I and Bartmann et al., 1974). It can be shown that under conditions of preequilibrium for AMP binding (eq 3) the apparent first-order rate constant, k_{obsd}, follows

$$k_{obsd} = k_a + k_{da}\frac{(AMP)_0}{K_{AMP} + (AMP)_0}, \quad (5)$$

Table III: Kinetic Parameters of the L-Phenylalanyl Transfer Reaction at pH 7.5, 25°.[a]

Parameter	Magnesium 1.0 mM	Magnesium 1.4 mM
k_a (sec^{-1})	0.05	0.26
k_{da} (sec^{-1})	0.19	0.95
K_{AMP} (mM)	0.24	0.28

[a] Parameters were evaluated from Figure 3 as described in the text.

Table IV: The Degree of Transfer during Formation of the Enzyme–Adenylate from Phe-tRNA^{Phe} and AMP.

Reactant Concn: Enzyme (μM)	AMP (mM)	Phe-tRNA^{Phe} (μM)	% Transfer[a]
0.05	1.0	0.1	25[b]
0.1	1.6	0.25	55[b]
0.1	1.0	1.0	75[b]
0.5	0.65	0.1	70[c]

[a] Reference is made to complete transfer obtained under the assumption that equilibrium eq 4 is completely on the side of the enzyme–adenylate. [b] Determined from the observed fluorescence quenching at equilibrium with reference to quenching at saturating concentration of L-phenylalanine at 25°. [c] Determined from the initial jump observed during the release of radioactivity from charged tRNA^{Phe} at 10°. Reference is made to the total amount of radioactivity applied.

with $(AMP)_0 \gg (E)_0$. The equation can be rearranged so that a plot of $k_{obsd} - k_a$ against $(k_{obsd} - k_a)/(AMP)_0$ would give a straight line (cf. Holler and Calvin, 1972). An approximate value of k_a was obtained from the intercept, $(AMP)_0 \rightarrow 0$, of Figure 3 and was subsequently changed in small steps until a straight line was calculated giving the best fit to the linearized plot in the figure inset. The values for k_{da} and for K_{AMP} were taken from the intercept and slope, respectively, of the linear plot. The results are summarized in Table III.

Regarding the ratio of the rate constants k_{da}/k_a, it is found that the equilibrium of the transfer reaction (eq 4) is on the side of the aminoacyl adenylate. This is an interesting finding and will be considered in the Discussion section. Another interesting point is the magnesium dependence of the kinetics. Thus, a 40% increase leads to a fivefold enhancement of the rate constant without displaying an effect on the equilibrium constants K_{AMP} and k_{da}/k_a.

The overall quenching as measured for every stopped-flow experiment at equilibrium deserves some attention. Table IV contains relative values with reference to the quenching observed after mixing of the enzyme with saturating amounts of L-phenylalanine. At present concentrations of enzyme and Phe-tRNA^{Phe} used in the stopped-flow experiments and at saturating concentration of AMP, maximum quenching was close to 60%, in cases of higher Phe-tRNA^{Phe} up to 75%. In the case of the radioactive experiment where enzyme was fivefold higher than Phe-tRNA^{Phe} and $K_{Phe\text{-}tRNA^{Phe}}$, also 70% of the radioactive amino acid was transferred to AMP as concluded by the magnitude of the initial jump. The higher values can be reconciled with the predicted 80% quenching when regarding only equilibrium eq 4 and $k_{da}/k_a = 4$.

The dissociation constant K_{AMP} = 0.24–0.28 mM at 25° (Table III) and the half-maximum concentration of 0.15 mM at 10° for AMP determined from the radioactive initial jump may be compared by setting the expression $K_{AMP}k_a/k_{da}$ equal to the half-maximum concentration. This is appropriate if the transfer process is at equilibrium at the time of the radioactive measurement. By assuming similar values for K_{AMP}, k_a, and k_{da} at 10° as have been measured at 25°, a half-maximum concentration of 0.06–0.07 mM is estimated. The value is low compared with the actually measured one indicating the temperature dependence of the parameters involved. Agreement of the value of K_{AMP} was obtained with titration experiments in the presence of the fluorescent reporter group, where the competition with ATP was measured, since AMP by itself does not induce fluorescence quenching. However, comparison with the inhibition constant obtained in previous ATP–[^{32}P]PP$_i$ exchange work indicates a tenfold higher value for this method (Santi et al., 1971b). An obvious explanation cannot be given at present; however, it is not unlikely that the higher value reflects the higher concentration of magnesium and also the competition between AMP and PP$_i$ not taken into account in the exchange experiments.

The Overall Reaction. The results obtained from the analysis of the amino acid activation and the transfer reaction as they were investigated separately are not necessarily valid for the combined reactions. In this section we provide evidence that they are compatible with the results from the overall reaction obtained by single turnover and steady-state experiments.

The formation of the L-phenylalanyl adenylate as the intermediate is directly visualized in single turnover experiments using the fluorescence technique (Figure 4). The concentration of L-phenylalanine is such that its complex formation with the enzyme is negligible. Phe-tRNA^{Phe} as it is formed during the reaction does not displace the reporter group TNS as we have demonstrated previously (Bartmann et al., 1974). Thus the formation of the enzyme-bound adenylate can be seen as a transient decrease of the fluorescence intensity for TNS, if a rapid formation of the intermediate is followed by the slower transfer of the L-phenylalanyl moiety to tRNA^{Phe}. Both phases, the initial quenching and the subsequent dequenching, were analyzed as first-order plots over more than 60% of the particular total fluorescence signals. From the values of the apparent single exponential rate constants the values of k_f and k_a for the amino acid activation and the transfer to tRNA^{Phe}, respectively, were estimated. With the formalism presented earlier (Holler and Calvin, 1972) it can be shown that at conditions of saturating concentration of ATP and $(Phe)_0 \ll K_{Phe}$, in the absence of PP$_i$, the rate constant can be approximated by $k_{obsd} \simeq k_f(Phe)_0/K_{Phe}$. With the values k_{obsd} = 1.9 sec^{-1}, $(Phe)_0$ = 0.5 μM, and K_{Phe} = 19 μM the rate constant is calculated to be k_f = 72 sec^{-1} which is compatible with the values in Table II. For the transfer of the L-phenylalanyl moiety from AMP to tRNA^{Phe} the rate constant k_{obsd} = 0.04 sec^{-1} = k_a was measured in agreement with the value established in the separate transfer experiment (Table III). The results of the stopped-flow experiments are consistent with the transfer reaction being the rate-limiting step of the overall reaction. In that case, the value of the rate constant k_a is predicted to be the same as that determined for the radioactive charging assay at appropriate conditions. In the presence of 2 mM MgATP and 1 mM free magnesium but at conditions otherwise identical with those of the aminoacylation standard assay at 25°, a

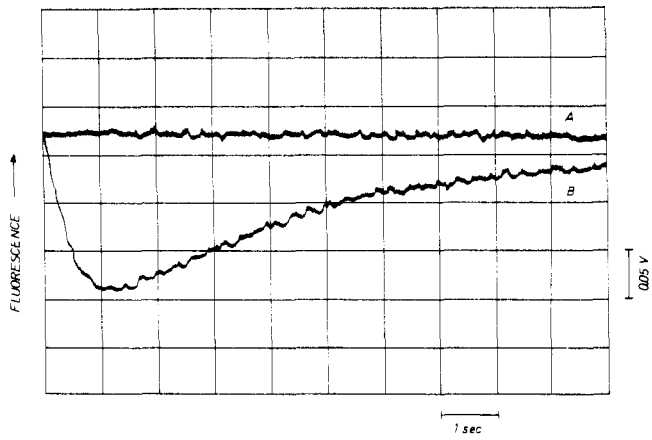

FIGURE 4: Oscilloscope display for the formation of the enzyme–aminoacyl adenylate complex and the subsequent transfer of the amionacyl moiety to $tRNA^{Phe}$ as observed after rapid mixing of 1 μM enzyme, 1 μM L-phenylalanine, and 2 μM $tRNA^{Phe}$ with 4 mM MgATP, each solution containing buffer A and 2.6 μM TNS. Formation of the aminoacyl adenylate intermediate is reflected by fluorescence quenching, while acylation of the $tRNA^{Phe}$ leads subsequently to a fluorescence increase. The horizontal trace indicates the level of fluorescence after equilibrium has been established. Conditions were 25° and pH 7.5, 1 mM magnesium.

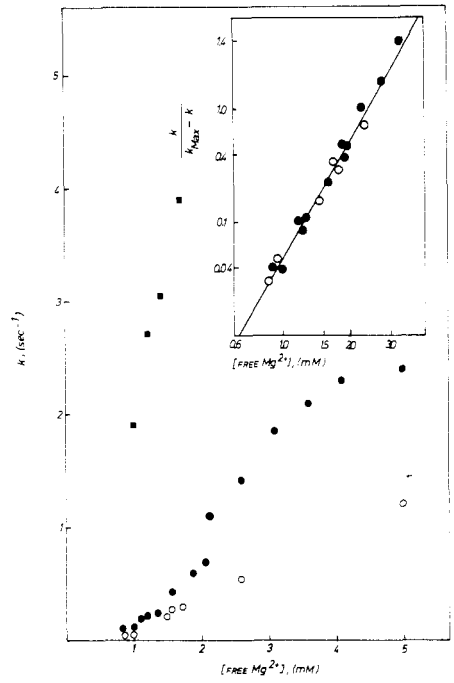

FIGURE 5: Cooperative effects of magnesium on the rate constants of the amino acid activation and transfer reaction at 25°. The rate constants were determined as function of the magnesium concentration. (■) $k = k_{obsd}$ determined from the rapid initial fluorescence decrease as depicted in Figure 4, and reflects the formation of the adenylate ($(PP_i)_0 = 0$, cf. eq 1 of the text). A Hill coefficient, n, of approximately 1.5 was determined from a double-logarithmic plot on the basis of the relation $k_{obsd} \simeq (k_{max}/K_{Mg^{2+}})\cdot[\text{Magnesium}]^n$, which is derived from $k_{obsd} = k_{max}[\text{magnesium}]^n/(K_{Mg^{2+}} + [\text{magnesium}]^n)$ for $[\text{magnesium}]^n \ll K_{Mg^{2+}}$, the apparent dissociation constant of magnesium binding. (●) k_{obsd} obtained for the aminoacylation under standard assay conditions. Since acylation of $tRNA^{Phe}$ is considered as the rate-limiting step in the catalysis, $k = k_{obsd}$ equals k_a. (○) $k = k_a$ obtained from the stopped-flow experiments following the formation of the adenylate from Phe-$tRNA^{Phe}$ and AMP (cf. eq 5). Measurements were performed as those for Figure 3 at a fixed concentration of $(AMP)_0$ = 1 mM. The rate constant determined from the first-order plots was to a good approximation equal to $k_1 + k_{da}$. With the assumption that magnesium affects k_a and k_{da} equally (cf. Table III) the value for k_a could be calculated. The results (except for the formation of the adenylate) are plotted according to Hill (1910) in the figure inset using k_{max} equal to 2.45 sec^{-1} (●) and equal to 1.2 sec^{-1} (○).

charging rate of 1270 nmol mg^{-1} hr^{-1} was measured at 25°. When this value is divided by the molar amount of the enzyme, the rate constant equals 0.1 sec^{-1} which has to be compared with $k_a = 0.05$ sec^{-1} (Table III). The agreement is acceptable considering that the assay concentrations of the substrates might have enabled some participation of the second active site.

The Effect of Magnesium on the Activation and Transfer Reaction. The rate constant (k_f) of the adenylate formation determined at 25° from experiments similar to that in Figure 4, the rate of tRNA charging in the overall reaction, and the rate constant k_a of the transfer reaction, derived on the basis of the assumption mentioned under Figure 5, are all strongly dependent on the concentration of free magnesium. A plot of the charging rate vs. the concentration of the free cation is sigmoidal (Figure 5), the Hill constant (Hill, 1910) from the double-logarithmic plot being approximately 3.5 (Figure 5, inset). The same is found for the rate constant of the transfer reaction but as the preliminary results in Figure 5 suggest, not for the rate constant of the adenylate formation. The similarity confirms our foregoing conclusion that the single turnover and the steady-state kinetic experiments exhibit the same rate-limiting elementary step. The high cooperativity which is exerted by a minimum of four magnesium ions must occur at the level of the enzyme–adenylate–$tRNA^{Phe}$ complex. Since the rate constant of the reverse reaction is enhanced by the same ratio (cf. Table III) this cooperativity exists equally at the level of the enzyme–AMP–Phe–$tRNA^{Phe}$ complex.

Charging of tRNA at 25° was conducted at varying concentrations of Tris-HCl in order to verify that the observed magnesium dependence was not an artifact caused by complexation between the buffer and the cation. Since rates were reproducible, any important buffer effect was ruled out.

The sites of interaction with magnesium leading to the enhanced rates of the transfer reactions may be located on both the $tRNA^{Phe}$ and the enzyme component of the complexes. Binding of magnesium to the enzyme has been demonstrated in the 0.1–10 mM range (Kosakowski et al., 1972; Hanke et al., 1975). This binding probably accounts for the increasing rate of the adenylate formation which exhibits a minimum Hill constant of 1.5 (Figure 5). With tRNA the cation is known to interact at several levels (Römer et al., 1970; Rialdi et al., 1972; Lynch and Schimmel, 1974). The level with the lowest affinity is characterized by binding in the 0.1–10 mM region and has been realized by enzymic and structural investigations (Riesner et al., 1973; Robison and Zimmerman, 1971). Whether this binding involves conformational changes within the $tRNA^{Phe}$ molecule essential for the charging reaction remains to be established. In conclusion, it is conceivable that a productive enzyme–$tRNA^{Phe}$ complex exists besides others which are unproductive or less productive, and which is stabilized by uptake of magnesium via protein *as well* as the tRNA.

The observed strong dependence of reaction rates is scarcely supported from results in literature except it seems to be in agreement with those reported by Santi et al. (1971a) when proper corrections are made for magnesium uptake by the substrates. Preliminary results, however, ob-

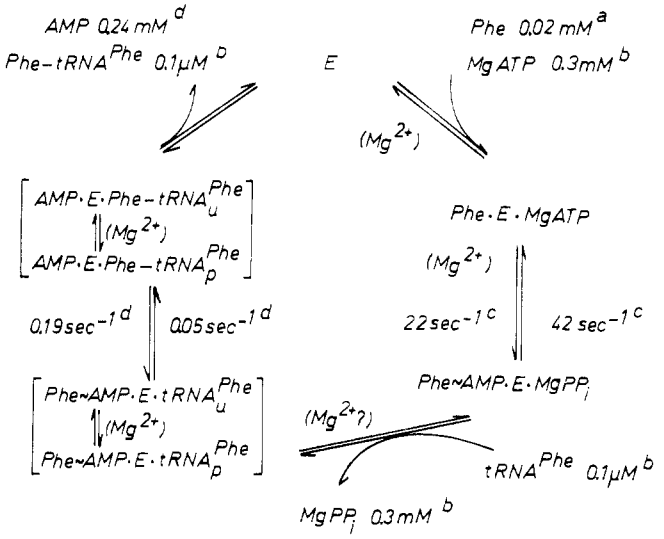

FIGURE 6: The reaction path for the formation of Phe-tRNA^{Phe} catalyzed by L-phenylalanine:tRNA ligase at 1 mM magnesium (pH 7.5) and 25°. The addition of the substrates and products to the enzyme occurs at random preequilibrium with the amino acid activation and amino acid transfer. Productive and unproductive enzyme-tRNA complexes are designed by the subscript p and u, respectively. Magnesium-dependent steps are indicated by (Mg^{2+}). Rate (sec^{-1}) and dissociation constants (M) have values as taken from (a) Table I, (b) Kosakowski and Holler (1973), (c) Table II, (d) Table III. For further details see text.

tained in our laboratory do reflect comparable magnesium dependencies for the binding of MgATP, L-phenylalaninol, and for the fluorescence quenching accompanying the formation of the enzyme–Phe-tRNA^{Phe} complex in the presence of TNS. These results seem to be in contrast to the magnesium insensitivity (in the range 1–11 mM Mg^{2+}) observed for the binding of L-phenylalanine and tRNA^{Phe} mentioned afore. This subject is of considerable interest in our laboratory and results will be published in detail elsewhere.

We have demonstrated with Phe-tRNA^{Phe} the existence of two complexes with the enzyme (Bartmann et al., 1974). Only one of these complexes is productive, namely the one in which the tRNA-linked L-phenylalanyl moiety protrudes into the binding site for the amino acid. At 1 mM magnesium it was shown that the equilibrium between these complexes is on the side of the unproductive complex in which the amino acid moiety cannot displace the fluorescent reporter group 6-*p*-toluidinylnaphthalene-2-sulfonate occupying the amino acid binding site. We presume that an increase in the concentration of magnesium then shifts this equilibrium toward the productive complex thereby enhancing the rate constants for the transfer reactions. The effect of magnesium binding to the enzyme–tRNA complex is such that the CCA arm of the tRNA moves into the active site assuming a final position where the L-phenylalanine moiety may be close to its binding site.

We have tested this prediction by repeating the binding experiment for Phe-tRNA^{Phe} to the enzyme in the presence of TNS at 11 mM magnesium. Contrary to the observation at 1 mM cation and in agreement with the above model we found an almost complete displacement of the fluorescent dye from the amino acid binding site. Titration experiments at high magnesium indicated the dissociation constant of 0.1 μM for the enzyme–Phe-tRNA^{Phe} complex as determined from the fluorescence quenching accompanying the displacement of the dye. This value compares with the similar value of 0.2 μM for uncharged tRNA^{Phe} which indicates that the amino acid moiety only protrudes into but does not bind to this site.

Discussion

Kinetics. Our present knowledge about the kinetics of tRNA^{Phe} charging in the presence of L-phenylalanine: tRNA ligase is summarized in Figure 6. The reaction scheme takes into account the findings by Santi et al. (1971a) that the overall reaction follows a ping-pong mechanism and also of Mulivor and Rappaport (1973) that binding of the substrates and products is of random order. The latter finding is consistent with our observation that the binding of the substrates and products is in rapid preequilibrium with both the formation of the adenylate and the transfer reaction. The binding of tRNA^{Phe} appears to occur at random with respect to the various stages of the reaction as indicated by the very similar values of the dissociation constants under a variety of conditions of other ligands present (Bartmann et al., 1974, 1975b).

The following points are of interest. (1) The rate-limiting step of the overall reaction is the transfer of the L-phenylalanyl moiety from AMP to tRNA^{Phe}. Because of the magnesium-dependent equilibrium between productive and unproductive enzyme–tRNA^{Phe} complexes, the transfer rate is increased at higher concentrations of the cation reaching a maximum value of approximately k_a = 2.4 sec^{-1} at saturation (cf. Figure 5). Even then the transfer step is still slower than the amino acid activation by a factor >20 because this step also depends on the cation concentration (Figure 5). At all concentrations of magnesium the data are consistent with the transfer being rate limiting and not the dissociation of the charged tRNA from the enzyme complex. This is concluded from the very similar values of rate constants under a variety of experimental conditions including single-turnover and steady-state measurements. The finding is in agreement with that of others (Pingoud et al., 1973; Evans and Nazario, 1974; Fasiolo and Ebel, 1974; Bartmann et al., 1974) for the L-phenylalanine, L-serine, and L-arginine specific enzymes. This finding is opposed by the conclusions obtained previously for the L-isoleucine and L-valine specific enzymes (Yarus and Berg, 1969; Hélène et al., 1971; Schimmel, 1973). Whether the opposing results reflect different intrinsic enzyme properties or the particular experimental conditions of pH and temperature, remains to be determined.

(2) During steady-state conditions, the enzyme bound adenylate is transiently accumulated. The accumulation predicts values for the Michaelis–Menten constants which are lower than those of the corresponding dissociation constants of ATP and L-phenylalanine. This idea has been treated in an earlier communication (Holler and Kosakowski, 1973). Since no other intermediate seems to be built up between the Michaelis–Menten complex of the amino acid activation reaction and the formation of the ternary enzyme–adenylate–pyrophosphate complex, the Michaelis–Menten constants of the ATP–[^{32}P]PP_i exchange reaction appear of similar magnitude as the dissociation constants (Holler et al., 1973, and references therein).

(3) The kinetics of the formation of the adenylate and those of the subsequent formation of Phe-tRNA^{Phe} are in line with the assumption that the intermediate is an obligatory step on the reaction path. However, it cannot be excluded that under the particular condition of a rapid preequilibrium between the intermediate and an unknown in-

Table V: Free Enthalpies (25°, 1 atm, pH 7.5) of the Various Reaction Steps along the Catalytic Path at Equilibrium between Substrates and Products at 1 m*M* Magnesium.

Reaction Path[a]	Parameter[a]	ΔG° (kcal)
E + Phe + MgATP ⇌ Phe•E•MgATP	K_{Phe}	−6.5
	K_{MgATP}	−4.8
Phe•E•MgATP ⇌ Phe ~ AMP•E•$MgPP_i$	k_f/k_b	+0.4
Phe ~ AMP•E•$MgPP_i$ + $tRNA^{Phe}$ ⇌	$K_{tRNAPhe}$	−9.7
Phe ~ AMP•E•$tRNA^{Phe}$ + $MgPP_i$	$K^{-1}_{PP_i}$	+4.9
Phe ~ AMP•E•$tRNA^{Phe}$ ⇌	k_a/k_{da}	+0.8
AMP•E•Phe-$tRNA^{Phe}$		
AMP•E•Phe-$tRNA^{Phe}$ ⇌	K^{-1}AMP	+5.0
E + AMP + Phe-$tRNA^{Phe}$	K^{-1}Phe-$tRNA^{Phe}$	+9.7
Phe + MgATP + $tRNA^{Phe}$ ⇌		−0.2
$MgPP_i$ + AMP + Phe-$tRNA^{Phe}$		

[a] Taken from Figure 9.

Table VI: Equilibrium Constants (k_f/k_b) of the Formation of an Enzyme–Adenylate–$MgPP_i$ Complex from the Enzyme–Amino Acid–MgATP Complex as Calculated from ATP–[^{32}P]PP_i Exchange Results.

Ligase Specific for	K_X[a] (m*M*)	K_{PP_i}[b] (m*M*)	k_f/k_b[c]
L-Methionine[d] (*E. coli*)	0.07	5	0.014
L-Tryptophan[e] (*E. coli*)	0.345	10	0.035
L-Tryptophan[f] (beef pancreas)	0.014	0.16	0.09
L-Phenylalanine[g] (*E. coli*)	0.24	0.55	0.44
L-Phenylalanine (*E. coli*)			0.3–0.5[h]
L-Isoleucine (*E. coli*)			0.045–0.2[i]

[a] The equilibrium constant is defined as K_X = (E•AA ~ AMP)-($MgPP_i$)/(X), where X has been identified by us with the ternary enzyme–amino acid–MgATP complex (AA = amino acid). [b] Dissociation constant K_{PP_i} = (E•AA ~ AMP)($MgPP_i$)/(E•AA ~ AMP•$MgPP_i$) assumed to be of identical value with (E)($MgPP_i$)/(E•$MgPP_i$). [c] k_f/k_b = (E•AA ~ AMP•PP_i)/(E•AA•MgATP) = K_X/K_{PP_i}. [d] Blanquet et al. (1974). [e] Penzer and Plumbridge (1974). [f] Knorre et al. (1974); Zinov'ev et al. (1974). In their terminology are: $K_Z^{(1)} = K_X$ and $K_Z^{(2)} = K_{PP_i}$. [g] Mulivor and Rappaport (1973). [h] Table II. [i] Holler and Calvin (1972), stopped-flow technique.

termediate X (not accumulated) which is *on* the path, the adenylate intermediate is a dead-end intermediate. The problem has been discussed in general by Loftfield (1972) and has not been solved in the similar case of acylenzyme intermediates observed with a variety of esterases.

(4) The approximately 20-fold increase of rate of transfer by the relatively small shift of the free magnesium concentration from 1 to 3 m*M* suggests a possibility of in vivo regulation of protein synthesis via the concentration of this cation. Another, but less effective regulatory possibility might be the simultaneous function of the two active sites at particular high concentrations of substrates (cf. Table I). The approximately twofold higher rates measured for the steady-state formation of Phe-$tRNA^{Phe}$ in comparison with the single turnover formation (cf. Figure 5), together with previous results of stoichiometry measurements indicating two molecules of adenylate bound (Bartmann et al., 1975b) are consistent with at least partially simultaneous functioning of the active sites at conditions of the standard aminoacylation assay. Recently, Fersht (1975) has suggested half-of-the-sites reactivity of amino acid:tRNA ligases with negative cooperativity. Our present results are consistent without such an assumption (including our thermodynamical considerations). Clearly, further investigations have to be conducted to answer this problem at an even more sophisticated level.

Thermodynamics. A single turnover of substrates is fully described by the rate and equilibrium constants depicted in Figure 6. The overall equilibrium constant between substrates and products is known to be approximately 1 (the free enthalpy of the reaction $\Delta G \simeq 0$) (Berg et al., 1961) thus allowing a crucial test of the validity of approximations and equilibrium constants of the various enzyme-ligand complexes used. The calculation has been performed for the condition of 1 m*M* magnesium and the results are given in Table V. It is seen that the prediction has been verified.

When the free enthalpy is depicted as a function of the reaction coordinate (not shown), it will be seen that a single minimum is obtained at the position of the quaternary complex between enzyme, adenylate, $MgPP_i$, and $tRNA^{Phe}$. The existence of such a complex cannot be excluded on an experimental basis. Because of its symmetry we find this free energy profile interesting.

As demonstrated in an earlier investigation (Holler and Calvin, 1972) we are surprised at the ease by which the adenylate and $MgPP_i$, while still bound to the enzyme, are formed from the enzyme bound amino acid and MgATP. As pointed out previously, this reaction is facilitated by several kilocalories in comparison to the reaction as it would proceed between free reactants in solution. If this shift of the equilibrium in favor of the adenylate is enzymically meaningful it should be also verified with other, if not all, amino acid:tRNA ligases. Several enzyme systems have been recently analyzed via their ATP–[^{32}P]PP_i exchange kinetics, and we have taken these cases to calculate the pertaining ratios k_f/k_b as indicated in Table VI. Accordingly, these values represent free reaction enthalpies of the order of 0–2.5 kcal, which compare favorably against approximately 5 kcal to be expected for the reaction in the free solute state (Holler and Calvin, 1972).

Product Inhibition. The question arises, why the energetically less stabile adenylate is formed as an intermediate? The answer becomes evident when the strength of binding is considered between the reaction product, Phe-$tRNA^{Phe}$, and the enzyme. When the dissociation constants are compared for the binding of the product Phe-$tRNA^{Phe}$ and the substrate $tRNA^{Phe}$ virtually no difference is seen (Bartmann et al., 1974). Yet, the aminoacyl moiety which is added as the result of the catalytic reaction has as such been favorably interacting with the enzyme at the level of a substrate. It is the free enthalpy of this interaction which is compensated by part of the chemical free enthalpy becoming available during the transfer of the aminoacyl moiety from AMP to $tRNA^{Phe}$. This part is in excess over the free enthalpy required for the formation of the amino acid-tRNA ester bond. If the free enthalpy of the L-phenylalanyl-enzyme interaction would not have been compensated, the dissociation constant of the enzyme-Phe-$tRNA^{Phe}$ complex would be less than of the order of 10^{-10} *M*, and the catalytic reaction would be completely inhibited after the first round by the tight binding of the product.

Then, another question arises how to store considerably more free enthalpy in the adenylate bond than is available from the splitting of MgATP into AMP and $MgPP_i$. We

have discussed this question previously (Holler and Calvin, 1972; Kosakowski and Holler, 1973; Holler et al., 1975), and came to the conclusion that for reasons of the considerable binding affinity of $MgPP_i$ the substrate MgATP must be bound to the enzyme under strain. A similar suggestion has been also put forward by Santi et al. (1971a). A second event which is presumed to contribute to the strain in the ternary enzyme–amino acid–MgATP complex is the reaction previously characterized as synergistic formation of enzyme–ligand complexes (Kosakowski and Holler, 1973; Holler et al., 1975). This reaction is described as a considerably better binding of a substrate in the presence of an analog of the second substrate, as for instance of MgATP in the presence of L-phenylalaninol. However, the cooperativity is not seen when one substrate binds in the presence of the other. This suggests that free enthalpy which is released in the former case is used to build up strain in the latter. The strain then must be relieved during the subsequent formation of the adenylate, thereby constituting the extra free enthalpy needed to explain the relatively favorable equilibrium constants k_f/k_b. Even though considerably more experimental details have to be established, the present example of amino acid:tRNA ligase catalysis has reached a state where the complete mechanism is becoming conceivable.

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