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Kinetics of Acyl Transfer Ribonucleic Acid Complexes of *Escherichia coli* Phenylalanyl-tRNA Synthetase. A Conformational Change Is Rate Limiting in Catalysis†

Mireille Baltzinger‡ and Eggehard Holler*

ABSTRACT: Kinetics of complex formation between phenylalanyl-tRNA synthetase and Phe-tRNA^{Phe} have been measured by the stopped-flow technique. Either the protein intrinsic fluorescence or the fluorescence of the added indicator 6-(*p*-toluidinyl)naphthalene-2-sulfonate was observed. Identical results were obtained with each method. Acyl-tRNAs with variable structures of the acyl and tRNA moieties were examined. Kinetics were measured as a function of pH and at different ionic strengths. Kinetic constants were compared with those of enzymatic phenylalanylation of tRNA^{Phe}. The results are as follows. (1) Phe-tRNA^{Phe} binds to phenylalanyl-tRNA synthetase in two, mutually exclusive types of complexes, one at the tRNA-specific binding site of the enzyme and the other in a region which involves the Phe-specific binding site of the enzyme [Holler, E. (1980) *Biochemistry 19*, 1397–1402]. The Phe site directed association includes a conformational change of the complex that is rate limiting. (2) The conformational change and catalytic tRNA aminoacylation follow similar values of rate constants irrespectively of pH and ionic strength. It is concluded that aminoacylation is limited by the kinetics of a conformational change of the nascent enzyme·Phe-tRNA^{Phe} complex. (3) The nature of Phe site directed binding was probed by variation of the structure of Phe-tRNA^{Phe}. Both the acyl and the tRNA moieties are recognized by the enzyme. Of the acyl moiety, only the phenyl ring but not the amino group is essential for binding. The amino group can be acetylated or replaced by a hydroxyl group. Protonation of the amino group results in loss of Phe site directed binding. It gives a pK_a = 6.9, which is close to that for protonation of a phenylalanine ester. (4) Rate constants were only slightly affected by addition of 200 mM NaCl at pH 7.5, indicating that the contribution by electrostatic forces was probably minimal. Mg^{2+} ions were essential for Phe site directed binding. Complexation of enzyme, Phe-tRNA^{Phe}, and Mg^{2+} either was random or was at preequilibrium with the conformational change. (5) Binding of Phe-tRNA^{Phe} at the tRNA-specific site of the enzyme was studied in the presence of in situ synthesized phenylalanyl adenylate. The reaction was bimolecular with rate constants of 50 μM^{-1} s^{-1} and 15 s^{-1} for association and dissociation, respectively.

The knowledge of the rate-limiting reaction of tRNA aminoacylation is one of the goals directed toward an understanding of the catalytic mechanism. A search for this reaction can be traced back to work by Yarus & Berg (1969) and subsequently to that by Schimmel and co-workers (Schimmel, 1973). Using classical radioactive techniques, they found evidence that dissociation of the enzyme·aminoacyl-tRNA complex was rate limiting. Employment of rapid sampling techniques revealed later that the aminoacyl transfer from AMP to tRNA was the slowest reaction (Pingoud et al., 1973; Bartmann et al., 1975a; Fersht & Kaethner, 1976; Fasiolo & Fersht, 1978).

We have reported in a preliminary note that a complex between phenylalanyl-tRNA synthetase and Phe-tRNA^{Phe} [1] exhibits kinetics which are reminiscent of those for tRNA aminoacylation (Holler, 1976). Equilibrium experiments have revealed that the reaction between the enzyme and Phe-tRNA^{Phe} is complex and involves at least two binding sites (Güntner & Holler, 1979). The kinetics, which show similar properties as in the case of tRNA aminoacylation, were found to belong to a type of complex that involved the Phe-specific binding site of the enzyme (Holler, 1976, 1980; Holler et al., 1981a). In the present paper, we attribute these kinetics to a conformational change of this particular enzyme·Phe-tRNA^{Phe} complex. We confirm and extend previous results concerning the structural requirements of an acyl-tRNA and the effects of salt and pH of the reaction mixture.

Materials and Methods

Chemicals. L-[^{14}C]Phenylalanine was a product of Amersham Buchler. TNS was obtained from Serva (Heidelberg). ATP, tRNA^{Phe} from *Escherichia coli* and from brewers yeast

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[1] Abbreviations: EDTA, (ethylenedinitrilo)tetraacetic acid; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol; TNS, 6-(*p*-toluidinyl)-naphthalene-2-sulfonate; Phe-tRNA^{Phe}, L-phenylalanyl ester of tRNA^{Phe}; Phe-tRNA$^{Phe}_{HNO_2}$, L-phenylalanyl ester of tRNA$^{Phe}_{HNO_2}$; Phe(OH)-tRNA$^{Phe}_{HNO_2}$, DL-β-phenyllactyl ester of tRNA$^{Phe}_{HNO_2}$; *N*-Ac-Phe-tRNA$^{Phe}_{Ac}$, *N*-acetylphenylalanyl ester of tRNA$^{Phe}_{Ac}$; Phe-tRNA^{Phe}2′NH, phenylalanyl ester of tRNA^{Phe}2′NH$_2$; Phe-tRNA^{Phe}3′NH, phenylalanyl ester of tRNA^{Phe}3′NH$_2$; Ile-tRNA^{Phe}, isoleucyl ester of tRNA^{Phe}; Phe-tRNA$^{Val}_1$, phenylalanyl ester of tRNA$^{Val}_1$; Phe-tRNA$^{Phe}_{hy}$, phenylalanyl ester of tRNA$^{Phe}_{hy}$; tRNA$^{Phe}_{HNO_2}$, tRNA^{Phe} after treatment with nitrous acid; tRNA$^{Phe}_{Ac}$, tRNA^{Phe} after acetylation of base X; tRNA$^{Phe}_{Ac}$, tRNA^{Phe} cross-linked between positions 8 and 13; tRNA^{Phe}2′NH$_2$, tRNA^{Phe} with the 2′-OH of the terminal adenosine substituted by NH$_2$; tRNA^{Phe}3′NH$_2$, tRNA^{Phe} with the 3′-OH of the terminal adenosine substituted by NH$_2$.

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(Phe acceptance 1.4 nmol/A_{260} unit), and $tRNA_1^{Val}$ (Val acceptance 1.13 nmol/A_{260} unit) were purchased from Boehringer (Mannheim). All other chemicals were analytical grade and obtained from Merck (Darmstadt). L-Phenylalanine methyl ester was homogeneous as analyzed (Kosakowski & Holler, 1973).

Enzymes. L-Phenylalanyl-tRNA synthetase (EC 6.1.1.20) from *E. coli* K10 was prepared as described by Hanke et al. (1974). The specific activity was 53 000 nmol mg^{-1} h^{-1} and 1.9 active sites per enzyme (M_r 270 000). L-Phenylalanyl-tRNA synthetase from Baker's yeast (EC 6.1.1.20) was a generous gift from P. Remy and F. Fasiolo (Strasbourg). Inorganic pyrophosphatase (EC 3.6.1.1) from yeast was purchased from Boehringer (Mannheim) in the form of a 1 mg/mL (200 units/mg) suspension in 3.2 M ammonium sulfate.

Modifications of tRNA. $tRNA^{Phe}_{HNO_2}$ [and Phe(OH)-$tRNA^{Phe}_{HNO_2}$] was synthesized by incubation with nitrous acid as has been described (Güntner & Holler, 1979). $tRNA^{Phe}_{h\nu}$ was prepared by UV irradiation as in Favre et al. (1979) and was the same material as described (Holler et al., 1981a). Phe-$tRNA^{Phe}$2′NH and Phe-$tRNA^{Phe}$3′NH were a gift of M. Sprinzl (Bayreuth). N-Acetylation of [^{14}C]Phe-$tRNA^{Phe}$ (*E. coli*) with *N*-hydroxysuccinimide ester of acetic acid was performed according to Bartmann et al. (1974a). The yield was 100% of the theoretical as determined by the method of Schoffield & Zamecnick (1968). It was verified that the amino group of base X had been acetylated, employing the reaction with fluorescamine (Sprinzl & Faulhammer, 1978).

Aminoacylation of tRNA. Phe-$tRNA^{Phe}$ was prepared as described by Bartmann et al. (1974b). Phe-$tRNA^{Phe}_{h\nu}$ and Ile-$tRNA^{Phe}$ were identical with the material used by Güntner & Holler (1979). Phenylalanylation of $tRNA^{Val}$ (*E. coli*) was performed as described (Roe et al., 1973).

Buffers. Routine buffer was 50 mM Tris-HCl, pH 7.5 (25 °C), 10 mM $MgCl_2$, 0.1 mM EDTA, and 0.2 mM dithioerythritol unless noted otherwise. Ionic strength of the above buffer was maintained at all pH values by addition of NaCl or by reduction of buffer concentrations, respectively. As in the case of Ile-tRNA synthetase (Holler et al., 1980), specific buffer effects were not observed for Tris-HCl, glycine–NaOH, and potassium phosphate.

Kinetics of Aminoacylation of tRNA. Unless noted otherwise, phenylalanylation of $tRNA^{Phe}$ was followed by the method of Kosakowski & Böck (1970).

Stopped-Flow Measurements. Kinetics were observed via the fluorescence of added TNS as has been described in detail (Bartmann et al., 1975a; Holler, 1980). We shall refer to this as the "TNS method". In some experiments, formation of complexes was followed via protein intrinsic fluorescence (285-nm excitation wavelength and a Corning 0-54 cutoff filter with 90% transmission at 340 nm in the emission light path). Rate constants (k_{obsd}) were calculated from first-order plots.

Results

Kinetics of Enzyme·Acyl-tRNA Complexes. Phenylalanyl-tRNA synthetase complexes of acyl-tRNAs were observed by their ability to decrease the fluorescence intensity of TNS (Güntner & Holler, 1979; Holler, 1980). When TNS occupies the Phe-specific binding site of the enzyme, it becomes highly fluorescent. Its displacement, by competition with acyl-tRNA, is concomitant with the extinction of the fluorescence (Güntner & Holler, 1979). In all cases, the extinction followed a single exponential time dependence. Rate constants were measured as a function of [acyl-tRNA] $\gg$ $[E]_0$ (total enzyme concentration).

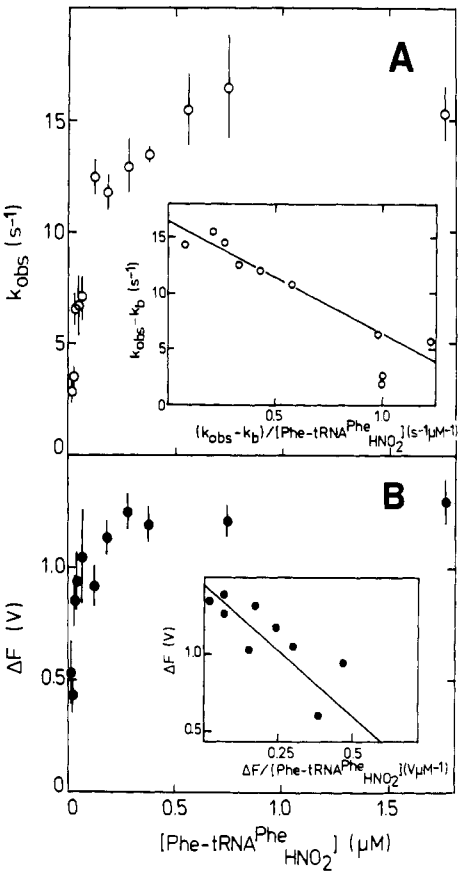


FIGURE 1: Kinetics of enzyme·Phe-$tRNA^{Phe}_{HNO_2}$ complex formation by stopped-flow techniques. Solutions, 16–170 nM phenylalanyl-tRNA synthetase and respectively 0.03–3.5 μM Phe-$tRNA^{Phe}_{HNO_2}$, were mixed in a 1:1 (v/v) ratio in the stopped-flow apparatus. Both solutions contained 50 mM Tris-HCl (pH 7.5, 25 °C), 10 mM $MgCl_2$, 0.1 mM EDTA, 0.2 mM dithioerythritol, and 5 μM TNS. (A) First-order rate constants are plotted as a function of ligand concentration. A linear extrapolation for $[Phe\text{-}tRNA^{Phe}_{HNO_2}] \lesssim 0.1$ μM (not shown) was used to determine an approximate value of k_b. Data were replotted as in the inset of panel A. They were expected to follow a linear relationship [see Holler (1980)]. Values of k_b were varied until the best fit was obtained. From the linear interpolation of the inset, $k_f K_1$(obsd) and K_1(obsd) were determined from the intercept and slope, respectively. Values of k_b, $k_f K_1$(obsd), and K_1(obsd) are summarized in Table I. (B) The reaction amplitude, in terms of a decrease in fluorescence intensity, is given as a function of ligand concentration. The dependence was brought into a linear form according to Eadie (1942) as shown in the inset. ΔF_{max} and K_{II}(obsd) were determined from the intercept and slope, respectively. Values are listed in Table I.

A typical dependence is seen in Figure 1 for Phe-$tRNA^{Phe}_{HNO_2}$. The reaction monitored by the TNS method could be described by the mechanism represented in eq 1 and 2 (Holler, 1980). Complex $(E\cdot acyl\text{-}tRNA)_1$ involves the

$$E + acyl\text{-}tRNA \underset{K_1}{\rightleftharpoons} (E\cdot acyl\text{-}tRNA)_1 \quad (1)$$

$$E + acyl\text{-}tRNA \underset{k_b}{\overset{k_f}{\rightleftharpoons}} (E\cdot acyl\text{-}tRNA)_2 \quad (2)$$

tRNA-specific binding site ("tRNA site directed binding") and complex $(E\cdot acyl\text{-}tRNA)_2$ part or all of the Phe-specific binding site of the enzyme ("Phe site directed binding"). The second mode of binding (eq 2) has been explored in detail (Holler, 1980). It has been found to involve a collisional complex followed by a conformational change. Equation 2 is then substituted by eq 3.

$$E + acyl\text{-}tRNA \underset{K_1}{\rightleftharpoons} (E\cdot acyl\text{-}tRNA)_1 \underset{k_b^{II}}{\overset{k_f^{II}}{\rightleftharpoons}} (E\cdot acyl\text{-}tRNA)_{II} \quad (3)$$

Table I: Kinetics of Phenylalanyl-tRNA Synthetase·Acyl-tRNA Complexes[a]

acyl-tRNA	k_b (s^{-1})	$k_f K_1$(obsd) (s^{-1})	k_f (μM^{-1} s^{-1})	K_1(obsd) (μM)	K_{II}(obsd) (μM)	$\Delta F_{max}/\Delta F_{Phe\to\infty}$	K_{II}[b] (μM)
Phe-tRNA^{Phe} [c]	2–3	13	32	0.4	0.09[d]	0.75[d]	0.1
N-Ac-Phe-tRNA$^{Phe}_{Ac}$	3	15	19	0.8	0.09	0.9	0.2
Phe(OH)-tRNA$^{Phe}_{HNO_2}$	2 ± 1	65	81	0.8	0.03	1.0	0.02
Phe-tRNA$^{Phe}_{HNO_2}$	1 ± 0.5	16	160	0.1	0.017	0.9	0.006
Phe-tRNA^{Phe}2′NH	4	nd	nd	nd	nd	$\lesssim$0.08	$\gtrsim$4.6[e]
Phe-tRNA^{Phe}3′NH	3	11	39	0.4	0.28	0.55	0.08
Phe-tRNA$^{Phe}_{h\nu}$ [f]	2.9	0.45	1.3	0.35	0.3	0.13	2.3
Ile-tRNA^{Phe} [g]	5 ± 2	0.3	5	0.06	0.06	0.06	1.0

[a] 50 mM Tris-HCl buffer (pH 7.6, 25 °C), 10 mM $MgCl_2$, 0.1 mM EDTA, 0.2 mM dithioerythritol, and 5 μM TNS. Standard deviations were at maximum 30% unless stated otherwise. [b] Calculated as k_b/k_f according to eq 6. [c] Holler (1976, 1980). [d] Günter & Holler (1979). [e] Calculated from eq 4 and 5 and $\Delta F_{max}/\Delta F_{Phe\to\infty} \leq 0.08$ by assumption of K_1(obsd) = 0.4 μM. [f] Holler et al. (1981a). [g] Analysis was carried out as described (Holler et al., 1981a).

If formation of complex (E·acyl-tRNA)$_{II}$ is the only reaction with a decrease in fluorescence intensity, the following relations hold:

$$k_{obsd} = k_b + k_f K_1(obsd)\frac{[acyl\text{-}tRNA]}{K_1(obsd) + [acyl\text{-}tRNA]}$$

$$k_f = \frac{k_f^{II}}{K_1} \qquad k_b = k_b^{II} \qquad K_1(obsd) = \frac{1}{1/K_1 + 1/K_1} \tag{4}$$

$$K_1 = \frac{[E][acyl\text{-}tRNA]}{[(E\cdot acyl\text{-}tRNA)_I]} \qquad K_{II} = \frac{[E][acyl\text{-}tRNA]}{[(E\cdot acyl\text{-}tRNA)_{II}]}$$

$$K_1 = \frac{[E][acyl\text{-}tRNA]}{[(E\cdot acyl\text{-}tRNA)_I]}$$

Dissociation constants K_1, K_1, and K_{II} and rate constants k_f, k_b, k_f^{II}, and k_b^{II} are defined by the above equations (eq 1–4).

Reaction amplitudes (ΔF) were measured as a function of acyl-tRNA concentration in terms of the decrease in fluorescence intensity. With ΔF_{max} and $\Delta F_{Phe\to\infty}$ the maximum intensity decrease for saturating concentrations of acyl-tRNA and L-phenylalanine (in place of acyl-tRNA), respectively, values of apparent dissociation constants K_{II}(obsd) could be determined.

$$\Delta F = \Delta F_{max}\frac{[acyl\text{-}tRNA]}{K_{II}(obsd) + [acyl\text{-}tRNA]}$$

$$\frac{\Delta F_{max}}{\Delta F_{Phe\to\infty}} = \frac{1/K_{II}}{1/K_1 + 1/K_I + 1/K_{II}} \tag{5}$$

$$K_{II}(obsd) = \frac{1}{1/K_1 + 1/K_I + 1/K_{II}}$$

K_1(obsd), K_{II}(obsd), $k_f K_1$(obsd), and k_b were "operational" constants and were obtained from dependencies such as in Figure 1A,B by methods described previously (Holler, 1976; Güntner & Holler, 1979; Holler et al., 1981a). The dissociation constant K_{II} of the complex (E·acyl-tRNA)$_{II}$ was calculated according to eq 6. Constants k_f^{II}, K_I, and K_1 could

$$K_{II} = K_{II}(obsd)\frac{\Delta F_{Phe\to\infty}}{\Delta F_{max}} = \frac{K_1(obsd)K_{II}(obsd)}{K_1(obsd) - K_{II}(obsd)} = \frac{k_b}{k_f} \tag{6}$$

not be determined because of insufficient experimental data. Operational constants have been measured for a variety of acyl-tRNAs and are summarized in Table I.

It may be objected that the kinetics contain errors due to the presence of TNS. With regard to the dissociation constant of the enzyme·TNS complex (20 μM; Holler & Kosakowski, 1973) and the employed concentrations of TNS (5 μM), 20% of the enzyme is calculated to be in the complex. Competition effects are considered to be negligible under this condition in regard to other methodical errors (Bartmann et al., 1975a). Furthermore, kinetics of enzyme–TNS interactions were fast (k_{obsd} = 200–250 s^{-1}; Pimmer & Holler, 1979) in comparison with the slower kinetics observed here. Errors derived from the contribution of the TNS kinetics were calculated to be at the worst 10–20% the value of the measured rate constants. Nevertheless, we have followed the reaction between enzyme and Phe-tRNA^{Phe} in the absence of TNS by measurement of the protein intrinsic fluorescence. because of small reaction amplitudes, these measurements were tedious in comparison with measurements by the TNS method. Complex formation was indicated as a decrease in fluorescence intensity. Kinetics followed the same operational kinetic constants as in the case of the TNS method. Uncharged tRNA^{Phe} could also provoke a decrease in intensity (Farelly et al., 1971; Bartmann et al., 1975b); however, this reaction was too fast to be measured by the stopped-flow technique.

As an advantage over the TNS method, protein intrinsic fluorescence offered the possibility to observe the association of Phe-tRNA^{Phe} to the tRNA-specific binding site of the enzyme. Phe site directed binding was excluded by the presence of in situ synthesized phenylalanyl adenylate. Enzyme (0.05–0.2 μM) was preincubated with 5 mM Phe and 0.1 mM ATP. Since 10 mM Mg^{2+} was present, this solution must contain a mixture of enzyme with either Phe or adenylate occupying the Phe-specific binding site (Pimmer & Holler, 1979). Rapid mixing with a solution containing Phe-tRNA^{Phe} (0.6–4.8 μM) and the smaller substrates at concentrations identical with those in the preincubation mixture was followed by an intensity decrease. Reaction amplitides were of the order of 6% or less of the emitted light which was not corrected for stray light. First-order rate constants, as a function of acyl-tRNA concentration, were linear (not shown). Rate constants of association and dissociation were determined from the slope and intercept, respectively, of the linear dependence according to $k_{obsd} = k_{-1} + k_1$[Phe-tRNA^{Phe}] (Bernasconi, 1976) to be k_1 = 50 ± 10 μM^{-1} s^{-1} and k_{-1} = 15 ± 5 s^{-1}, respectively. The value of the dissociation constant k_{-1}/k_1 = 0.3 μM compares favorably with the value K_1(obsd) = 0.4 μM obtained from measurements with the TNS method (Table I).

Variation of the Structure of Acyl-tRNA. The structure of Phe-tRNA^{Phe} was varied in order to probe for the topography of the binding sites, in particular the site involving the phenylalanine binding pocket. Parameters responded by factors as high as 10 [in the case of K_1(obsd)] or 100 (in the case of k_f) except for k_b, which seemed to be constant (3 ± 2 s^{-1}) for all acyl-tRNAs examined. Substrate structure was

varied by modification of either the acyl moiety or the tRNA moiety.

Within the acyl moiety, the noncognate side chain of L-isoleucine was shown to bind poorly in the Phe site directed mode (K_{II} = 1.0 μM for Ile-tRNA^{Phe} in comparison to K_{II} = 0.1 μM for Phe-tRNA^{Phe}, Table I). Replacement of the phenylalanine amino group by a hydroxyl moiety in the case of Phe(OH)-tRNA$^{Phe}_{HNO_2}$, or acetylation in the case of *N*-Ac-Phe-tRNA$^{Phe}_{Ac}$, improved the proportion bound in the Phe site directed mode (comparison of values for $\Delta F_{max}/\Delta F_{Phe\to\infty}$). The effect of different points of attachment involving the ester bond (at either the 2′ or the 3′ carbon of the terminal adenosine of tRNA^{Phe}) has been examined via introduction of the imino group instead of the ester oxygen because of stability against hydrolysis (Sprinzl & Cramer, 1975). As a result, the kinetic parameters of Phe-tRNA^{Phe}3′NH were almost identical with those of Phe. In contrast, Phe-tRNA^{Phe}2′NH exhibited marginal binding to the Phe-specific site ($\Delta F_{max}/\Delta F_{Phe\to\infty}$ = 0.08). Inhibition studies with *N*-Ac-Phe-tRNA$^{Phe}_{Ac}$ indicated that Phe-tRNA^{Phe}2′NH attached to the tRNA-specific site of the enzyme.

On the side of the tRNA moiety, binding to the Phe-specific site was no longer observed after replacement of homologous tRNA^{Phe} by yeast tRNA^{Phe} (5 μM, K_{diss} = 8 μM; Holler et al., 1981b) or by noncognate tRNA^{Val} (7 μM). Kinetics were too fast to be measured in the case of phenylalanine methyl ester (5 mM, K_{diss} = 0.75 mM; Kosakowski & Holler, 1973). UV light induced cross-linking between bases in positions 8 and 13 of tRNA^{Phe} (Favre et al., 1979; Holler et al., 1981a) was disadvantageous for Phe site directed binding (Table I). In contrast, treatment of tRNA^{Phe} with nitrous acid (Güntner & Holler, 1979) intensified the association via this binding mode. It is evident from these results that Phe site directed binding involves recognition of both the phenylalanine and the tRNA^{Phe} moiety.

Effect of pH. The observation of an increased proportion of association via the Phe-specific binding site of the enzyme in cases where the phenylalanine amino group had been removed or acetylated suggested the ionization of the amino group to affect binding of Phe-tRNA^{Phe}. The pH dependence of complex formation should give an answer to this question.

pH profiles of k_f and k_b (rate constants in eq 2) were measured in the range pH 6.5–9 (Figure 3A). Dissociation constants K_{II} were calculated as k_b/k_f (see eq 6). Values of k_f varied only in the range 6.5–7.5 while values of k_b and K_{II} followed further changes in the range above pH 8 (Figure 3A). This latter aspect will not be considered further. Here the profiles in the neutral pH range coincide with the pH profile of the reaction amplitude (Figure 2). Because $k_b = k_b^{II}$ is characteristic for the conformational change of Phe site directed binding, the pH profile indicates that protons associate at the level of the E·Phe-tRNA^{Phe} complex and inhibit any rearrangement. Following the association of protons, the rate constant $k_f^{II} = k_f K_I$ would apply in a similar manner if K_I is pH independent.

If the pH dependence of the kinetics is related to ionization of the phenylalanine amino group, kinetics, in the case of Phe(OH)-tRNA$^{Phe}_{HNO_2}$ and *N*-Ac-Phe-tRNA$^{Phe}_{Ac}$, should be independent of pH. Parameters for *N*-Ac-Phe-tRNA$^{Phe}_{Ac}$ have been measured at pH 6.0 to be k_f = 58 ± 9 μM^{-1} s^{-1}, k_b = 5 ± 2 s^{-1}, K_I(obsd) = 0.8 ± 0.2 μM, K_{II} = 0.07 ± 0.01 μM, and $\Delta F_{max}/\Delta F_{Phe\to\infty}$ = 0.7 ± 0.1, which are in acceptable agreement with parameters at pH 7.5 in Table I.

The magnitude of the reaction amplitude, in terms of the relative intensity decrease, $\Delta F_{max}/\Delta F_{Phe\to\infty}$, was found to increase sharply between pH 6.5 and 7.5 in the case of Phe-tRNA^{Phe}, but to stay almost constant in the case of Phe-(OH)-tRNA$^{Phe}_{NHO_2}$ and *N*-Ac-Phe-tRNA$^{Phe}_{Ac}$ (Figure 2). In the case of Phe(OH)-tRNA$^{Phe}_{HNO_2}$, it was verified that the pH dependence was not a result of modification of tRNA^{Phe} by showing that in the case of Phe-tRNA$^{Phe}_{HNO_2}$ kinetics were again pH dependent (Figure 2). This finding indicated again that the ionization of the phenylalanine amino group counteracted binding to the Phe-specific site of the enzyme. With regard to binding at the tRNA-specific site, we have previously found that association of enzyme and tRNA^{Phe} became tighter as the pH was lowered from pH 5 to pH 6 (Bartmann et al., 1975b).

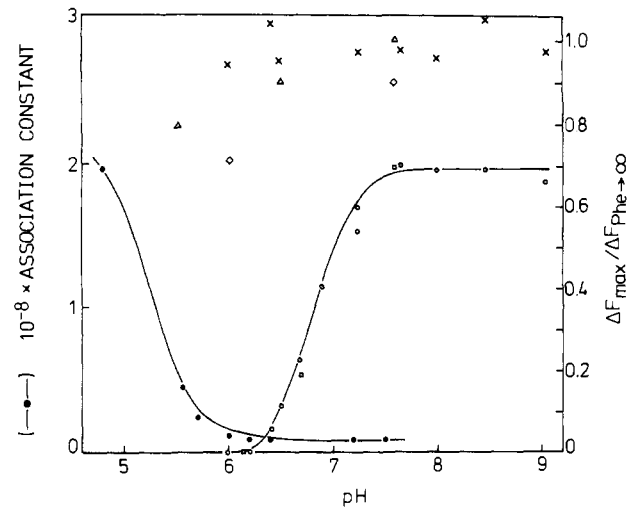


FIGURE 2: Stability of complexes as a function of pH. tRNA^{Phe} (●) dissociation constants have been published (Bartmann et al., 1975b) for enzyme·tRNA^{Phe} complexes as measured via protein intrinsic fluorescence. The drawn curve has been computed on the basis of the formalism presented elsewhere (Holler et al., 1980). It was assumed that ionization involves two protons and a shift of a pK_a = 5.3 (free enzyme and/or tRNA) to pK_a = 6.0 in the enzyme·tRNA^{Phe} complex. The maximum value of the association constant is assumed to be 2.1 × 10^8 M^{-1} and the minimum value 0.077 × 10^8 M^{-1}. Acyl-tRNA: the various symbols refer to (○) Phe-tRNA^{Phe}, (□) Phe-tRNA$^{Phe}_{HNO_2}$, (△) Phe(OH)-tRNA$^{Phe}_{HNO_2}$, and (◇) *N*-Ac-Phe-tRNA$^{Phe}_{Ac}$. Amplitudes were determined by extrapolation from concentration dependences to saturation by acyl-tRNA. In the case of Phe-tRNA^{Phe}, some of the amplitudes have been measured at an assumed "saturating" concentration of 1.2 μM. Amplitudes for Phe (5 mM) in arbitrary units are also indicated (×). They were used as values for $\Delta F_{Phe\to\infty}$. The drawn curve was computed on the basis of two protons involved in ionization of free acyl-tRNA and/or enzyme following a pK_a = 6.9. Values of $\Delta F_{max}/\Delta F_{Phe}$ were 0.75 at maximum and 0 at minimum.

The reaction amplitude is a complex parameter according to eq 5. If we intend an analysis of the pH dependence, we will assume that tRNA site directed binding is pH independent in this range and that the amount of complex (E·Phe-tRNA^{Phe})$_I$ is negligible in comparison with complex (E·Phe-tRNA^{Phe})$_{II}$ (see eq 3). The pH dependence will be described on the basis of the following equation:

$$\begin{bmatrix} \text{Phe-tRNA}^{Phe} \\ + \\ \text{E} \end{bmatrix} H^+_n \underset{nH^+}{\rightleftharpoons} \begin{matrix} \text{Phe-tRNA}^{Phe} \\ + \\ \text{E} \end{matrix} \rightleftharpoons (\text{E·Phe-tRNA}^{Phe})_{II} \quad (7)$$

it is assumed that both enzyme and Phe-tRNA^{Phe} must be in the less ionized form in order to associate at the Phe-specific binding site. The data in Figure 2 were treated according to Holler et al. (1980) by plotting log ($\Delta F_{max}/\Delta F_{Phe\to\infty}$) vs. pH. A minimum number of two protons were determined and an apparent ionization constant of pK_a = 6.9.

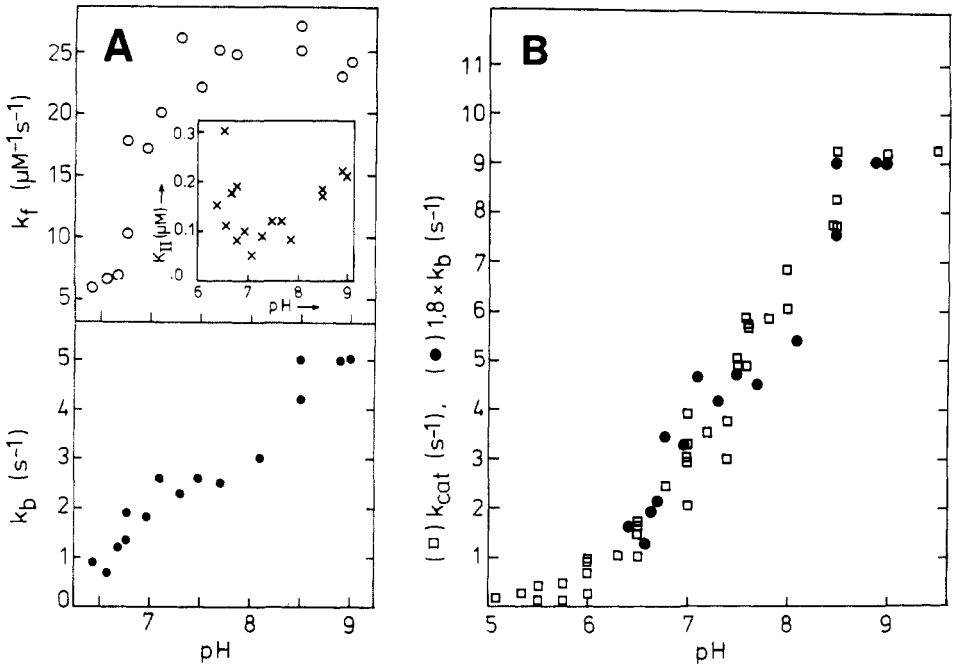


FIGURE 3: Formation of enzyme·Phe-tRNA^{Phe} complex. pH dependence of kinetic constants. (A) Kinetic constants of enzyme·Phe-tRNA^{Phe} complex as measured by stopped-flow techniques in the presence of 5 μM TNS. First-order rate constants, k_{obsd}, were measured at low concentrations of ligand, 40 nM $\leq$ [Phe-tRNA^{Phe}] $\leq$ 200 nM (20 nM enzyme). Values of k_{obsd} were approximated for each pH by a line which gave the best fit. Values of k_f were calculated from the slope of the line and k_b values from the intercept of these plots (not shown). Values of K_{II} shown in the inset were calculated according to $K_{II} = k_b/k_f$. (B) Catalytic rate constants of tRNA^{Phe} phenylalanylation (□) compared with the rate constants k_b (●) from panel A. These values of k_b had been multiplied by a factor of 1.8 as discussed in the text. Buffers were potassium phosphate (pH 5.5–7.0), Tris-HCl (pH 7.0–8.5), and glycine–NaOH (pH 8.5–9.5). Ionic strength was that of 0.1 M NaCl (pH 7.5, 25 °C) adjusted by variation of buffer concentration and/or addition of NaCl. Other conditions were 10 mM $MgCl_2$, 0.2 mM reduced glutathione, and 4 mM ATP. [^{14}C]Phe and tRNA^{Phe}, respectively, were varied at concentrations allowing for extrapolation to enzyme saturation [see also Holler et al. (1980)].

Effect of Mg^{2+}. Mg^{2+} ions have been reported to affect properties of phenylalanyl-tRNA synthetase (Hanke et al., 1975; Pimmer & Holler, 1979; Favre et al., 1979; Holler et al., 1981a,b). The extent of decrease in fluorescence intensity of TNS due to complex formation of enzyme and Phe-tRNA^{Phe} has been found to increase between 1 and 10 mM Mg^{2+} (Bartmann et al., 1975a; Holler, 1976).

In the experiments above, Mg^{2+} had been present in each of the reactant solutions prior to the reaction, and solutions of enzyme had been mixed with solutions of Phe-tRNA^{Phe}. We examined now, by changing the order of mixing, whether this affected the observed kinetics. Preincubation of only enzyme or Phe-tRNA^{Phe} with $MgCl_2$ had no effect. Rapid mixing of a combined solution of enzyme (40 nM) and Phe-tRNA^{Phe} (0.1–3 μM) in the presence of 0.5 mM EDTA with a solution of 20 mM $MgCl_2$ was also without effect; the kinetic parameters were $k_f = 40 \pm 8$ μM^{-1} s^{-1}, $k_b = 4 \pm 2$ s^{-1}, K_I(obsd) = 0.4 $\pm$ 0.1 μM, and $K_{II} = 0.1 \pm 0.05$ μM. The invariance indicated that the sequence of addition of enzyme, Phe-tRNA^{Phe}, and Mg^{2+} was random or, if ordered, at rapid preequilibrium with the conformational change.

We envisaged that the observed pH dependence could be a reflection of a pH-dependent association between enzyme and Mg^{2+}. We examined binding of Mg^{2+} at pH 6.0 by means of a titration experiment similar to that described by Pimmer & Holler (1979) at pH 7.5. The titration curve was biphasic, following dissociation constants of 0.38 and 6.0 mM (10 mM sodium phosphate buffer). The agreement with the results of the pH 7.5 experiments was acceptable (0.16 and 4.1 mM in 50 mM Tris-HCl) and indicated that the Mg^{2+} dependence could not be responsible for the pH dependence.

Effect of Ionic Strength. Interactions between aminoacyl-tRNA synthetases and tRNAs are typically salt sensitive (Pingoud et al., 1973; Krauss et al., 1976). Values of binding constants are reported to decrease as much as 100-fold for an increase of ionic strength from 10 to 200 mM. By addition of 200 mM NaCl (in the presence of 50 mM Tris-HCl buffer, pH 7.5) and comparison of the results with the parameters of Table I, we intended to probe whether Phe site directed binding is similarly salt sensitive. Kinetic parameters were $k_f = 45 \pm 2$ μM^{-1} s^{-1}, $k_b = 5 \pm 2$ s^{-1}, K_I(obsd) = 0.6 $\pm$ 0.1 μM, and $K_{II} = 0.1$ μM. Since the values were similar to those for Phe-tRNA^{Phe} in Table I (in the absence of added NaCl), we concluded that Phe site directed association was not typical for the binding of a tRNA. One possibility is the absence of (significant) electrostatic interactions.

Comparison of Rate Constants k_b of the Phe Site Directed Complex and k_{cat} of tRNA^{Phe} Phenylalanylation. A similarity between the rate constants k_{cat} of tRNA phenylalanylation and k_b of dissociation of the Phe site directed complex has been reported (Holler, 1976). These values were found to follow a unique dependence of Mg^{2+} concentration. We extend this finding to the dependence on pH and ionic strength, respectively.

The pH profile of the catalytic rate constant, k_{cat}, is shown in Figure 3B together with the pH profile of k_b, the rate constant of the conformational change that is limiting for dissociation. Values increase over a broad range from pH 6 to pH 9.5, where k_{cat} is at maximum 9 $\pm$ 0.5 s^{-1}. Values of both rate constants follow the same pH profile within experimental error. The absolute values of k_{cat} seem to be higher by a factor of 1.8 with respect to values of k_b. The meaning of this factor is not clear. It could be due partly to different ionic strengths (50 mM Tris-HCl, pH 7.5, for k_b and 100 mM NaCl for k_{cat}) and to the presence of substrates at catalytic conditions. In general, a factor of 2 seems to be an acceptable discrepancy among results found under such different conditions.

We examined the dependence of the catalytic rate constant on ionic strength. Conditions at 25 °C were 26 pM enzyme, 6 μM $tRNA^{Phe}$, 96 μM [^{14}C]Phe (10 μCi/μmol), 2 mM ATP, 1 mM reduced glutathione, 10 mM $MgCl_2$, 50 mM Tris-HCl (pH 7.5), and 0, 50, 100, and 200 mM NaCl. Values of k_{cat} were 3.64, 4.56, 4.36, and 4.03 s^{-1}, respectively. At 200 mM NaCl, the concentration of $tRNA^{Phe}$ seemed not to be saturating; with 12 μM $tRNA^{Phe}$, k_{cat} was 5.15 s^{-1}. These values are in agreement with those measured for k_b of the conformational change.

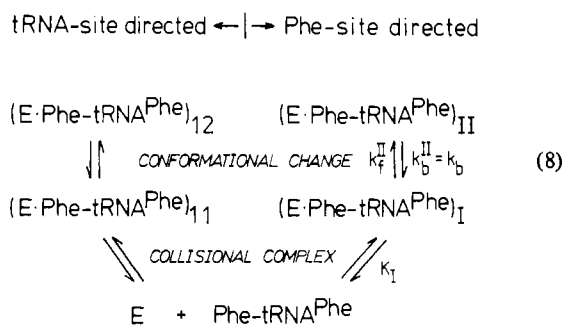
Discussion

Rearrangement of the Enzyme·Phe-tRNA^{Phe} Complex Is Rate Limiting. In an effort to understand the thermodynamics of an aminoacyl-tRNA synthetase, we found two competing sites for the binding of Phe-$tRNA^{Phe}$ to phenylalanyl-tRNA synthetase of *E. coli* (Güntner & Holler, 1979). The result seemed reasonable, considering that severe inhibition by the product of a synthesis of tightly bound substrates must be avoided. The competing binding modes were tRNA and Phe site directed, respectively. Phe site directed binding was established by displacement of radioactively labeled Phe and by other competition experiments (Güntner & Holler, 1979). In our present investigation, we followed Phe site directed binding of a series of structurally modified Phe-$tRNA^{Phe}$ substrates via the displacement of the fluorescent indicator TNS from the phenylalanine-specific binding site of the enzyme.

Kinetics of occupation of the two sites by the various acyl-tRNAs were consistent with a scheme for parallel association with the enzyme at the tRNA- and Phe-specific binding sites, respectively (eq 1 and 2). Prevention of binding to the Phe-specific site in the presence of in situ synthesized phenylalanyl adenylate still allowed association of Phe-$tRNA^{Phe}$ at the tRNA-specific site. Hindrance of access to the tRNA-specific site in the presence of saturating amounts of $tRNA^{Phe}$ did not abolish binding of Phe-$tRNA^{Phe}$ to the Phe-specific site (Holler, 1980).

An inspection of the kinetic parameters in Table I and of those in Holler (1980) revealed that eq 1 and 2 were oversimplified. Phe site directed binding most likely included a conformational change that determined the observed kinetics. tRNA site directed binding might as well involve a conformational change though at a considerable rate. Conformational changes as a result of tRNA binding have been demonstrated for several aminoacyl-tRNA synthetases [see Holler et al. (1981b) and references cited therein]. In the case of phenylalanyl-tRNA synthetase of *E. coli*, this has been interpreted as a "contraction" of the enzyme molecule (Holler et al., 1981b). If such a contraction occcurs on binding of substrate tRNA, it is reasonable to expect a "decontraction" as product aminoacyl-tRNA dissociates from the enzyme. Indeed, we have observed a similar contraction in the case of the Phe-$tRNA^{Phe}$-like derivative Phe(OH)-$tRNA^{Phe}_{NHO_2}$ (N. Ford and E. Holler, unpublished results), which binds exclusively to the Phe-specific site of the enzyme (Table I). We think that the oversimplified eq 1 and 2 are appropriately replaced by eq 8. Here, we have assumed that tRNA site directed binding involves another conformational change, which explains why the value of the rate constant of association, 50 μM^{-1} s^{-1}, is almost 1 order of magnitude below the expected value of a diffusion-controlled reaction (Krauss et al., 1973).

Conformational Change Could Be Rate Limiting in tRNA Aminoacylation. The mixed anhydrate bond of an aminoacyl adenylate is highly reactive according to its group transfer potential (Holler & Calvin, 1972). One wonders then why



(8)

synthesis of the aminoacyl-tRNA ester bond should be rate determining in the catalytic reaction (Pingoud et al., 1973; Bartmann et al., 1975a; Fersht & Kaethner, 1976; Fasiolo & Fersht, 1978). It seems likely, as we have evidence now, that a conformational change is rate determining. We have argued that association and dissociation of tRNA and, respectively, aminoacyl-tRNA with an aminoacyl-tRNA synthetase involve conformational changes of considerable magnitude (Holler et al., 1981b). It is conceivable, as we find now, that these rates are similar to or identical with turnover rates. A similar conclusion has appeared recently from kinetic work about the association of tRNA with yeast phenylalanyl-tRNA synthetase (Krauss et al., 1979).

Rate constants of catalytic tRNA phenylalanylation, k_{cat}, and of the conformational change of enzyme·Phe-$tRNA^{Phe}$, k_b, are almost of identical magnitude irrespectively of pH and ionic strength. Furthermore, the rate constant k_f of the conformational change in the reverse direction and the catalytic rate constant for AMP-dependent deacylation of Phe-$tRNA^{Phe}$ have identical values (Baltzinger & Holler, 1982). From these findings, we conclude that the rate-limiting reaction is one and the same for both phenomena, the catalysis and the conformational change.

Although the similarity of the values is striking, we have doubts that the ultimate Phe site directed complex, (E·Phe-$tRNA^{Phe}$)$_{II}$ of eq 8, is on the catalytic pathway. One reason is that this complex does not accumulate during tRNA aminoacylation (Baltzinger & Holler, 1982). The other reason is as follows. It has been shown that the primary point of phenylalanylation is the 2′ carbon of the terminal adenosine of $tRNA^{Phe}$ (Sprinzl & Cramer, 1975). Under the assumption that Phe-$tRNA^{Phe}$2′NH mimics this primary product (Fraser & Rich, 1973), we would expect it to bind in the Phe site directed mode. Phe-$tRNA^{Phe}$3′NH does not have the correct geometry and is expected to be disqualified for this type of binding. The results in Table I indicate the opposite situation, almost no binding of Phe-$tRNA^{Phe}$2′NH while binding of Phe-$tRNA^{Phe}$3′NH almost equals that of Phe-$tRNA^{Phe}$. We conclude that among the rapidly established 2′–3′ isomers, it is the 3′ form that is preferred. If the complex of Phe-$tRNA^{Phe}$, as we measure it, is not on the catalytic pathway, the similarity of rate constants suggests that nascent Phe-$tRNA^{Phe}$ has an at present, unknown configuration, which requires the presence of the 2′-hydroxyl group in the substrate $tRNA^{Phe}$, and which allows similar interactions as does Phe-$tRNA^{Phe}$3′NH. An example of such an intermediate case is proposed in the following paper (Baltzinger & Holler, 1982).

Nature of the Phe Site Directed Complex. Results of structure variation of both the aminoacyl and the tRNA moiety reveal considerable sensitivity of the rate constant k_f, but almost none of k_b (Table I). The mechanistical difference between these rate constants is that k_b refers to the conformational change, while k_f is more complex and reflects both k_f^{II} of the conformational change and K_I of the collisional

complex (eq 4). If it is agreed that the conformational change is independent of structure variation, recognition of acyl-tRNA must occur at the level of the collisional complex $(E{\cdot}Phe\text{-}tRNA^{Phe})_I$.

This complex contains sites recognizing the phenylalanine and the $tRNA^{Phe}$ moiety, respectively. Since $Phe(OH)\text{-}tRNA^{Phe}_{HNO_2}$, *N*-Ac-Phe-$tRNA^{Phe}_{Ac}$, and $Phe\text{-}tRNA^{Phe}$ are recognized almost equally well, we assume that the binding site of the Phe moiety is not the same as that for phenylalanine or that it is not fully assembled. N-Acetylated amino acids are not accepted by aminoacyl-tRNA synthetases, probably for sterical reasons, and deaminated L-phenylalanine binds considerably less tightly to phenylalanyl-tRNA synthetase than the intact amino acid (Güntner & Holler, 1979). Although the binding site may not be fully assembled, it distinguishes between side chains of phenylalanine and isoleucine and probably between the D and L configurations (Güntner & Holler, 1979).

Recognition of noncognate tRNA in the case of $Phe\text{-}tRNA^{Val}_I$, of heterologous tRNA in the case of yeast $Phe\text{-}tRNA^{Phe}$, and of modified $tRNA^{Phe}$ in the case of $Phe\text{-}tRNA^{Phe}_{HNO_2}$ and $Phe\text{-}tRNA^{Phe}_{h\nu}$ (Table I) requires a subsite for tRNA binding adjacent to the site for Phe. Another possibility of recognition is that association with the phenylalanine binding site is only possible for a defined conformer of acyl-tRNA. The concentration of conformer depends on the degree of modification. Such an explanation has been forwarded recently (Holler et al., 1981a).

The pH dependence of rate constants k_b and k_f (Figure 3) and of the reaction amplitude (Figure 2) reflects ionization of groups which, if ionized, prevent the conformational change. At least two protons are involved which follow an apparent ionization constant of $pK_a = 6.9$. One of the ionizing groups is the phenylalanine amino group; the other one could be a histidine side chain of the enzyme. The reasons in favor of the amino group are the pH independence observed for binding of $Phe(OH)\text{-}tRNA^{Phe}_{HNO_2}$ and *N*-Ac-Phe-$tRNA^{Phe}_{Ac}$ and the fact that the amino group of phenylalanine esters ionizes with a pK_a of 6.64–7.2 (Perrin, 1965; Schuber & Pinck, 1974). That histidine side chains (pK_a = 6.7–7.1; Tanford & Hauenstein, 1956) are essential for catalysis by phenylalanyl-tRNA synthetase has been shown by titration with group-specific reagents (Hennecke & Böck, 1974). Whether the detrimental effect of ionization is caused by juxtaposition of the positive charges, by formation of a salt bridge between the phenylalanine ammonium group and the tRNA moiety (or the protein), or by some degree of hydrophobicity of the binding site remains to be established. This last possibility is supported by the observed insensitivity against an increased ionic strength.

It should be noted that the pH ranges for Phe site directed binding of $Phe\text{-}tRNA^{Phe}$ and for tRNA site directed binding of $tRNA^{Phe}$ are not the same (Figure 2). Techniques like the nitrocellulose filter assay of Yarus & Berg (1970) must fail to detect the Phe site directed complex if they use slightly acidic pH.

Mg^{2+} ions in the millimolar range are essential for Phe site directed complex formation (Holler, 1976). Mg^{2+} in this concentration range is also known to promote an enzyme form with a more extended shape (Holler et al., 1981b). It is concluded that one of the effects of Mg^{2+} is to provide the extended shape that can accommodate $Phe\text{-}tRNA^{Phe}$ in the Phe site directed binding mode.

Phenylalanyl-tRNA synthetase has been shown to contain two active sites which interact anticooperatively (Bartmann et al., 1975a,b; Pimmer & Holler, 1979). It is not possible to consider Phe site directed and tRNA site directed binding both to occur at tRNA-specific sites, which belong to different active sites of a particular enzyme molecule. It has been shown that $tRNA^{Phe}$ and $Phe\text{-}tRNA^{Phe}$ form complexes in a competitive fashion (Güntner & Holler, 1979; Holler, 1980), while $tRNA^{Phe}$ interacts anticooperatively with the two active sites (Bartmann et al., 1975b). Another argument against such a consideration is that $Phe(OH)\text{-}tRNA^{Phe}_{HNO_2}$ has been found to displace [^{14}C]Phe in the presence of saturating concentrations of $tRNA^{Phe}$ (Güntner & Holler, 1979). It cannot be excluded that Phe site directed and tRNA site directed binding involve different active centers of one and the same enzyme molecule. It is of interest whether the observed conformational change would be part of the mechanism of anticooperativity and half-of-the-sites reactivity.

Conclusion. We have presented evidence for a conformational change to be rate determining for tRNA aminoacylation. The conformation of nascent $Phe\text{-}tRNA^{Phe}$ in the complex, which would then undergo the conformational change, may have a geometry at the phenylalanine-tRNA junction, which is presently unknown. In the following paper (Baltzinger & Holler, 1982), we discuss a tetrahedral intermediate as one possibility. We have characterized the complex with respect to structural requirements of the aminoacyl and the tRNA moieties. The outcome of all results is that it does not require the tRNA-specific binding site of the enzyme. The tRNA moiety of $Phe\text{-}tRNA^{Phe}$ is bound and recognized by a different site. The Phe moiety is accommodated by an apparently incompletely assembled binding pocket for L-phenylalanine. Our findings implicate that, in the course of a catalytic cycle, substrate $tRNA^{Phe}$ and product $Phe\text{-}tRNA^{Phe}$ come and go via different sites. We suspect that the switch from one site to the other is part of the conformational change.

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Catalytic Mechanism of Phenylalanyl-tRNA Synthetase of *Escherichia coli* K10. Conformational Change and $tRNA^{Phe}$ Phenylalanylation Are Concerted†

Mireille Baltzinger‡ and Eggehard Holler*

ABSTRACT: Catalytic phenylalanylation of $tRNA^{Phe}$ and the reverse reaction, AMP-dependent deacylation of Phe-$tRNA^{Phe}$, have been measured by steady-state and pre-steady-state techniques, including rapid sampling and fluorescence stopped-flow methods. (1) Stoichiometry of adenylate synthesis under steady-state phenylalanylation of $tRNA^{Phe}$ indicates half-of-the-sites reactivity. (2) Identity of values of rate constants under pre-steady- and steady-state conditions demonstrates that the rate-limiting steps in catalysis are bond making for phenylalanylation and bond breaking for AMP-dependent deacylation, respectively. (3) Values of catalytic rate constants are the same as those for the conformational change of the Phe site directed enzyme·Phe-$tRNA^{Phe}$ complex [Baltzinger, M., & Holler, E. (1982) *Biochemistry* (preceding paper in this issue)]. (4) A model is developed that accounts for the observed concert of chemical and geometrical reactions as well as for experimental evidence that nascent Phe-$tRNA^{Phe}$ may not be the same as in solution. In this model, nascent Phe-$tRNA^{Phe}$ is thought to be the tetrahedral intermediate that is formed by nucleophilic attack of the adenylate by the tRNA. It awaits the conformational change in order to break down into Phe-$tRNA^{Phe}$ and AMP. The model can serve as a unifying basis for an interpretation of discrimination against noncognate amino acids and tRNAs and also gives an explanation why severe product inhibition is not observed [Güntner, C., & Holler, E. (1979) *Biochemistry 18*, 2028–2038].

The catalysis of tRNA aminoacylation by aminoacyl-tRNA synthetases involves, in a minimum mechanism, an activation of the amino acid by synthesis of an adenylate and a subsequent transfer of the activated amino acid to the tRNA. We have reported on the mechanism of aminoacyl adenylate synthesis (Holler & Calvin, 1972; Pimmer & Holler, 1979). The present paper deals with the mechanism of phenylalanylation of $tRNA^{Phe}$, catalyzed by *Escherichia coli* phenylalanyl-tRNA synthetase. The particular mechanism is considered to be representative for at least a group of aminoacyl-tRNA synthetases. The reaction under study is interesting with respect to the question of other (covalent) intermediates (Thiebe, 1975; Kovaleva et al., 1978; Remy & Ebel, 1976) and with regard to rejection of misactivated noncognate amino acids (Fersht, 1977). Problems of thermodynamics and kinetics of product release have also been connected with this

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