Mechanism of tRNA–Aminoacyl-tRNA Synthetase Recognition: Influence of Aminoalkyladenylates†

Gerhard Krauss,*,‡ Stephen M. Coutts,§ Detlev Riesner,¶ and Guenter Maass

ABSTRACT: The influence of aminoacyl-5′-AMP on the recognition process between $tRNA^{Phe}$ (yeast) and phenylalanyl-tRNA synthetase (yeast) has been studied using an analogue of the aminoacyladenylate, phenylalaninyl-5′-AMP (Phe-ol-AMP). The complex formation between tRNA and synthetase in the presence of Phe-ol-AMP has been followed by fluorescence titrations and fast kinetic techniques. Previous investigations had shown that the mechanism of interaction between $tRNA^{Phe}$ and its cognate synthetase includes a recombination step between the two macromolecules that is almost controlled by diffusion and a fast conformational change of the complex (Krauss, G., Riesner, D., & Maass, G. (1976) *Eur. J. Biochem. 68,* 81). A two-step reaction scheme is also valid in the presence of Phe-ol-AMP. Rate constants and the distribution of fluorescence change and reaction enthalpy between the two reaction steps have been determined at different temperatures. At 5 °C the following rate constants are obtained: $k_{12} = 3 \times 10^7\ M^{-1}\ s^{-1}$, $k_{21} = 19\ s^{-1}$, $k_{23} = 10\ s^{-1}$, $k_{32} = 70\ s^{-1}$. The recombination step, however, in the presence of the inhibitor is no longer diffusion controlled. The conformational change is slowed down by more than an order of magnitude and thus comes close to being the rate-limiting step of the overall aminoacylation process. The 3′-terminal adenosine of $tRNA^{Phe}$ is involved in the rearrangement of the tRNA within the ternary complex since no slow conformational change is observed when $tRNA^{Phe}$ lacking the 3′-terminal adenosine is used as a substrate. It is concluded that the observed reactions are related to the process of aminoacylation by providing optimal orientation of the reactants for the transfer of the activated amino acids to the 3′-terminal ribose of the tRNA.

The recognition of tRNA molecules by their cognate aminoacyl-tRNA synthetases has been observed by various techniques (for reviews, see Söll & Schimmel, 1974; Kisselev & Favorova, 1974). One approach has been to apply physical techniques to study the mechanism of complex formation. It has been suggested earlier that the interaction between tRNAs and their cognate aminoacyl-tRNA synthetases comprised more than one reaction step (Yarus & Berg, 1969; Eigen, 1964). By employing fast kinetic methods it was later shown for three enzyme systems that the specific interaction between tRNAs and their cognate enzymes involved a recombination step followed by a fast conformational change (Riesner et al., 1976; Krauss et al., 1976). The recombination step is nearly controlled by diffusion and the conformational change of the complex occurs in less than 1 ms.

A comparison of the binding mechanism of cognate, noncognate, and modified tRNAs in the phenylalanine specific system from yeast allowed more insight into the biological meaning of the two-step binding process. The specificity of interaction between the tRNAs and phenylalanyl-tRNA synthetase (PRS) is expressed in both the recombination step and the conformational change, since the latter is not observed in nonspecific binding of $tRNA^{Tyr}$ to PRS (Krauss et al., 1976). Furthermore, it was shown (Krauss et al., 1977) that the integrity of the 3′-terminal CCA end is a prerequisite for the occurrence of the conformational change. The conformational change was interpreted as a rearrangement of both the tRNA and the synthetase, providing proper alignment of the 3′-terminal adenosine with the active site of the enzyme.

For studies of individual binding equilibria, e.g., the binding of tRNA and the amino acid to the enzyme, fast kinetic techniques such as stopped-flow or T-jump are particularly well suited. If one wants to look at more complicated reactions, e.g., catalysis, then steady-state analyses or quenched flow techniques (Fersht & Jakes, 1975) are the methods of choice. However, different approaches have to be employed if one wants to obtain information about conformational changes which directly precede or follow the catalytic steps. For example, if a suitable competitive inhibitor may be found, the reaction steps near the catalytic conversion of substrate to product can be studied by fast kinetic techniques. Suitable specific and competitive inhibitors of aminoacyl-tRNA synthetase have been described in the literature (Cassio et al., 1967), and one, phenylalaninyl-5′-AMP (Phe-ol-AMP), has been employed in the present study to investigate the conformational changes in or proximal to the tRNA binding site of PRS having its amino acid and ATP binding sites occupied. Aminoalkyladenylate inhibitors are structurally very similar to proposed intermediates in the activation of amino acids by ATP and it was therefore our hope that additional intermediates on the pathway of aminoacylation of $tRNA^{Phe}$ could be

† From the Institut für Klinische Biochemie und Physiologische Chemie, Abteilung Biophysikalische Chemie, Medizinische Hochschule Hannover, Karl-Wiechert-Allee 9, D-3000 Hannover 61, West Germany, and the Department of Biochemical Sciences, Princeton University, Princeton, New Jersey 08540. *Received October 13, 1977.* This work was supported by grants from the Deutsche Forschungsgemeinschaft (Schwerpunktprogramm Struktur-Funktion von Biopolymeren), the Fonds der Chemischen Industrie, and National Institutes of Health Grant GM 07654 to J. R. Fresco.

‡ Address correspondence to this author at: Institut für Klinische Biochemie und Physiologische Chemie, Abteilung Biophysikalische Chemie, Medizinische Hochschule Hannover, Karl-Wiechert-Allee 9, D-3000 Hannover 61, West Germany.

§ Present address: USV Pharmaceutical Corporation, Tuckahoe, New York 10707.

¶ Present address: Institut für Organische Chemie und Biochemie, Technische Hochschule Darmstadt, D-6100 Darmstadt, West Germany.

[1] Abbreviations used: DTE, dithioerythritol; EDTA, ethylenediaminetetraacetic acid; PRS, phenylalanyl-tRNA synthetase; T-jump, temperature jump; Phe-ol-AMP, phenylalaninyl-5′-AMP; $tRNA_{CC}^{Phe}$, $tRNA^{Phe}$ lacking the 3′-terminal adenosine.

0006-2960/78/0417-2443$01.00/0

detected by occupation of the amino acid activating site of PRS with Phe-ol-AMP. This approach showed additional promise in light of experiments with $tRNA_{CC}^{Phe}$, which indicated that the CCA end of the tRNA adapted itself to the active site of the enzyme (Krauss et al., 1977). We report here studies of complex formation between $tRNA^{Phe}$ and PRS in the presence of Phe-ol-AMP and compare the results with data obtained in the absence of the competitive inhibitor.

Experimental Section

Materials

Phenylalanyl-tRNA synthetase from commercial baker's yeast was purified and characterized as outlined previously (Krauss et al., 1976). The preparation of pure $tRNA^{Phe}$ from crude baker's yeast tRNA was according to Litt (1968). $tRNA_{CC}^{Phe}$ was prepared according to Sprinzl & Cramer (1975) and characterized as given in Krauss et al. (1977). All reagents and solvents were of the highest purity available from commercial sources.

L-Phenylalaninol was a product of Fluka AG (Buchs, Switzerland). Triisopropylbenzenesulfonyl chloride was obtained from Aldrich Chemicals (Milwaukee, Wis.) and was recrystallized from dry petroleum ether (bp 30–60 °C). P-L Biochemicals (Milwaukee, Wis.) furnished 5′-adenosine monophosphate. Pyridine was distilled first over ninhydrin and then over barium oxide and was stored under nitrogen, over Linde molecular sieve, type 4a.

Methods

The Synthesis of Phenylalaninyl-5′-AMP. N-tert-Butyloxycarbonyl-L-phenylalaninol was synthesized according to Sandrin & Boissonnas (1966).

N^6,$O^{2'}$,$O^{3'}$-Triacetyl-5′-AMP was synthesized by modification of the method of Rammler & Khorana (1962). The acid form of 5′-AMP (2.0 g, 6 mmol) was dissolved in a minimum of 50% pyridine and passed through a 3 × 2 cm column of Dowex 50-X4 (pyridinium form). The column was washed with water and the combined effluents were evaporated to dryness at 30 °C. The residue was rendered anhydrous by coevaporation with dry pyridine (10 mL) three times at 30 °C. The dry residue was suspended in 100 mL of anhydrous pyridine and treated with 40 mL of acetic anhydride. After stirring for 48 h in the dark at room temperature, the pale yellow solution was stripped in vacuo at 30 °C to a thick oil, to which 100 mL of 50% pyridine was added. After 2 h at room temperature, the water–pyridine solution was evaporated in vacuo at 30 °C, and the resulting viscous oil was rendered anhydrous by coevaporation with dry pyridine (three times). The dry residue was dissolved in 4 mL of anhydrous pyridine and dropped into 360 mL of rapidly stirred ether (under nitrogen). The precipitate was collected by centrifugation, washed three times with ether, and dried in vacuo over P_2O_5. The product was stored over desiccant at −18 °C and was isolated with a yield of 92% (3.55 g). Thin-layer chromatography on cellulose sheets (ethanol/1 M ammonium acetate; 5/2) revealed a major UV absorbing spot with an R_f of 0.40, corresponding to triacetyl-5′-AMP, and a minor spot with an R_f of 0.77, corresponding to an acetyl adduct of pyridine (Rammler & Khorana, 1962). Phenylalaninyl-5′-AMP was synthesized by a modification of the method of Sandrin & Boissonnas (1966). Dry N^6,$O^{2'}$,$O^{3'}$-triacetyl-5′-AMP (117 mg, 0.19 mmol) was placed in a flask with a threefold excess of dry *N-tert*-butyloxycarbonyl-L-phenylalaninol (145 mg, 0.6 mmol) and dissolved in 2 mL of anhydrous pyridine. The pyridine was removed in vacuo at 30 °C and the procedure repeated twice more. The dry residue was finally dissolved in 1.0 mL of anhydrous pyridine, and triisopropylbenzenesulfonyl chloride (95 mg, 0.31 mmol) was added. The flask was fitted with a drying tube, the contents mixed, and then left at room temperature in the dark for 24 h. The solution was then concentrated to a gum in vacuo at 30 °C and dried under a stream of nitrogen. The residue was triturated twice with petroleum ether (boiling point range 30–60 °C), and once with diethyl ether. The acetyl groups were deblocked with ammonium hydroxide, the N^{α}-*tert*-butyloxycarbonylphenylalaninyl-5′-AMP was purified by ether precipitation from methanol, and the amino blocking group was removed by trifluoroacetic acid as described by Sandrin & Boissonnas (1966). The final product of Phe-ol-AMP, after purification over a column of silica gel, was obtained in 82% yield (76 mg, 0.16 mmol), and gave a single spot (both UV and ninhydrin positive) upon thin-layer chromatography on silica gel (acetone/water; 8/2), with an R_f of 0.48. The UV spectrum (λ_{max}, 260 nm; λ_{min}, 228 nm) was identical with that reported by Sandrin & Boissonnas (1966).

ATP-pyrophosphate exchange kinetics catalyzed by crude yeast aminoacyl-tRNA synthetase preparations, measured according to the method of Simlot & Pfaender (1973) at constant and saturating levels of ATP while varying the phenylalanine concentration, yielded an inhibition constant of 5×10^{-7} M. A value of 2.5×10^{-6} M was obtained for the same compound by Cassio et al. (1967), using an *E. coli* synthetase preparation. Experiments were performed in 0.03 M potassium phosphate (pH 7.2), 0.5 mM EDTA, 10 mM $MgCl_2$, 0.5 mM dithioerythritol, and 0.2 M KCl if not otherwise stated.

Titrations. Binding constants for complex formation between $tRNA^{Phe}$ and the PRS·Phe-ol-AMP complex were obtained from fluorescence titrations using the Y-base fluorescence of $tRNA^{Phe}$ as indicator. Evaluation of the titration data was performed as outlined in Krauss et al. (1977). The stoichiometry of the tRNA–synthetase complexes was determined from the heights of the boundaries in sedimentation velocity runs in an analytical ultracentrifuge as described earlier (Krauss et al., 1975).

Fast Kinetic Measurements. The Y-base fluorescence of $tRNA^{Phe}$ was monitored in T-jump and stopped-flow experiments. The T-jump measurements were carried out and evaluated as described by Coutts et al. (1975) and Riesner et al. (1976). The magnitudes of the temperature rise were between 3 and 5 °C. The dependence upon concentration of the measured relaxation times was analyzed in terms of a two-step scheme:

$$E + tRNA \underset{k_{21}}{\overset{k_{12}}{\rightleftharpoons}} (E \cdot tRNA)^{+} \underset{k_{32}}{\overset{k_{23}}{\rightleftharpoons}} (E \cdot tRNA) \quad (1)$$

where

$$K_I = \frac{k_{12}}{k_{21}}, K_{II} = \frac{k_{23}}{k_{32}}$$

and the binding constant from equilibrium measurements is expressed as:

$$K_{assoc} = K_I(1 + K_{II}) \quad (2)$$

Reaction 1 requires the presence of two relaxation times, the concentration dependence of which is given by Eigen & de Mayer (1963):

$$\frac{1}{\tau_{1,2}} = -\frac{(\alpha_{11} + \alpha_{22})}{2} \times \left[1 \mp \sqrt{1 - \frac{4(\alpha_{11}\alpha_{22} - \alpha_{12}\alpha_{21})}{(\alpha_{11} + \alpha_{22})^2}}\right] \quad (3)$$

where $\alpha_{11} = -k_{12}(C_{tRNA} + C_E)$, $\alpha_{12} = k_{21}$, $\alpha_{21} = k_{12}(C_{tRNA} + C_E) - k_{32}$, $\alpha_{22} = -(k_{21} + k_{23} + k_{32})$, and where C_E and C_{tRNA} are the concentrations of free binding sites on the enzyme and free tRNA, respectively. The rate constants were evaluated by their dependence upon concentration according to eq 3, as outlined in detail by Riesner et al. (1976). Rapid mixing experiments were carried out in a stopped-flow spectrofluorimeter of high sensitivity as described by Pingoud et al. (1975). Ternary complex experiments were carried out by mixing the binary complex of PRS and Phe-ol-AMP with $tRNA^{Phe}$. Advantage was taken of the dependence upon ionic strength of the tRNA-synthetase interaction in dissociation experiments. A solution containing the ternary complex of $tRNA^{Phe}$·PRS·Phe-ol-AMP without added KCl was mixed with buffer containing 0.4 M KCl to yield a final concentration of 0.2 M KCl. Initial rates of aminoacylation were determined under conditions of Giégé et al. (1974).

Results

1. Equilibria

Binding of Phe-ol-AMP to the Synthetase. The influence of Phe-ol-AMP on the aminoacylation reaction was followed by measuring steady-state kinetics at various concentrations of the amino acid. Phe-ol-AMP behaves as a competitive inhibitor. The inhibition constant was determined to be 2×10^{-7} M, indicating a high affinity of Phe-ol-AMP to the active site of the enzyme. Measurement of the inhibition constant using ATP–pyrophosphate exchange yielded a similar value of 5×10^{-7} M. Under the ionic strength conditions used in the kinetic experiments, the inhibition constant of Phe-ol-AMP increases by a factor of 2 to 3. The inhibitory effect of Phe-ol-AMP is thus comparable to that of other aminoalkyladenylates tested with pure synthetase (Cassio et al., 1967; Blanquet et al., 1972).

It was not possible to derive binding parameters for the interaction between PRS and Phe-ol-AMP from fluorescence titrations since Phe-ol-AMP does not affect the tryptophan fluorescence of the enzyme. However, it was recently reported that two aminoacyladenylates are formed by PRS during catalysis (Fasiolo et al., 1977), so we have assumed that PRS also binds two molecules of Phe-ol-AMP. In the tRNA binding studies reported here the concentrations of Phe-ol-AMP were in the range between 100 and 500 μM, thus always providing a large excess of inhibitor over PRS. Saturation of both sites of the enzyme was ensured by the high concentration of Phe-ol-AMP employed, together with its high affinity constant.

Binding of $tRNA^{Phe}$ to the Complex between PRS and Phe-ol-AMP. Equilibrium data for the interaction between $tRNA^{Phe}$ and the complex between Phe-ol-AMP and PRS were determined from ultracentrifugation experiments and fluorescence titrations. The results are presented in Table I, together with data obtained in the absence of Phe-ol-AMP. The presence of Phe-ol-AMP does not change the stoichiometry of the tRNA–synthetase complex; the strength of binding, however, is increased by a factor of two to three. Since there was no experimental indication that the binding sites for $tRNA^{Phe}$ become nonequivalent upon addition of Phe-ol-AMP, the tRNA-binding sites were considered to be equivalent. Also under aminoacylation conditions the 2:1 stoichiometry of the tRNA–synthetase interaction is preserved as can be seen clearly in sedimentation velocity runs in the analytical ultracentrifuge of a solution containing 0.8 μM PRS, 3 μM $tRNA^{Phe}$, 5×10^{-4} M phenylalanine, 5×10^{-4} M ATP, and 2×10^{-4} M pyrophosphate. This result is in contrast to observations of Fasiolo et al. (1977) who report a 1:1 complex under similar conditions. We ascribe the discrepancy to the different methods used for the determination of the stoichiometry. The advantage of the ultracentrifugation technique as compared with the nitrocellulose filter technique used by Fasiolo et al. (1977) has been discussed by us in an earlier paper (Krauss et al., 1977).

TABLE I: Equilibrium Parameters for Complex Formation between $tRNA^{Phe}$ and Phenylalanyl-tRNA Synthetase in the Presence and Absence of Phe-ol-AMP.[a]

Conditions	Fluorescence change (%)	Binding constant[b] (M^{-1})	$tRNA^{bound}$/enzyme[c]
5×10^{-5} M Phe-ol-AMP, at 5°C	−25	$2 \pm 0.5 \times 10^6$	2
5×10^{-5} M Phe-ol-AMP, at 25 °C	−25	$1.5 \pm 0.5 \times 10^6$	2
No Phe-ol-AMP added, at 25 °C	−25	$8 \pm 2 \times 10^5$	2

[a] Determined in 0.03 M potassium phosphate, pH 7.2, 0.2 M KCl, 10 mM $MgCl_2$, 1 mM EDTA, 0.5 mM dithioerythritol. [b] Obtained from fluorescence titrations. [c] Data from sedimentation experiments in the analytical ultracentrifuge according to Krauss et al., 1975.

2. Kinetics

General Outline of the Experiments. We have shown previously (Riesner et al., 1976) that the interaction between tRNAs and their cognate aminoacyl-tRNA synthetases is characterized by a two-step reaction scheme. This mechanism was deduced from an investigation of the concentration dependence of the relaxation times in T-jump experiments. A leveling off of the relaxation times at high concentrations of the reactants was indicative of a conformational change following the recombination step. Other binding mechanisms compatible with the saturation behavior of the relaxation times were excluded by analyzing the amplitudes of the observed effects. In the present work we have applied a similar analysis. Additionally we have performed stopped-flow experiments in order to corroborate the T-jump data in such cases where relaxation effects were small or negligible. If possible, one of the reactants in the stopped-flow experiments was present in large excess so that the reaction time obeyed the same formalism as the relaxation time in the T-jump experiments. In all kinetic experiments, 0.2 M KCl was present; the reasons for selecting this ionic strength have been outlined earlier (Krauss et al., 1976). Due to the high excess of Phe-ol-AMP over synthetase in the T-jump experiments, a possible shift of the PRS–Phe-ol-AMP binding equilibrium upon the increase of the temperature could be excluded.

The kinetics of complex formation between $tRNA^{Phe}$ and PRS in the presence of Phe-ol-AMP reveal the existence of a fast and a slow process, which can be observed separately in either stopped-flow or T-jump experiments. The observation of two processes in the presence of Phe-ol-AMP implies that at least two steps are involved. Compared with the earlier experiments in the absence of Phe-ol-AMP (Krauss et al., 1976), the conformational change is slower by a factor of 20 to 50. The characteristic features of the processes will be dealt with separately below.

Fast Processes. Just one relaxation time is observed in temperature jumps of the ternary complex at 25 °C. This process is observable only in the presence of synthetase and Phe-ol-AMP and therefore is ascribed to the binding of tRNA

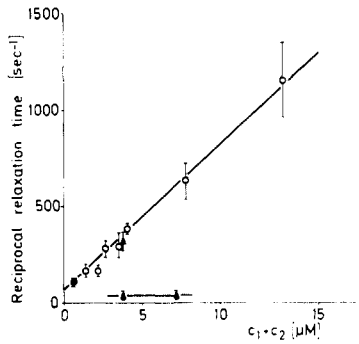

FIGURE 1: Binding of $tRNA^{Phe}$ to the complex between Phe-ol-AMP and phenylalanyl-tRNA synthetase at 25 °C, stopped-flow and T-jump data. Dependence of the reciprocal relaxation times on the sum of the concentration of free reactants. The error bars indicate the experimental error obtained for approximately five experiments with the same solution. (○) Reciprocal relaxation times measured in T-jump experiments. (▲) Reciprocal time constants obtained from stopped-flow association experiments under conditions of excess enzyme binding sites. At $C_1 + C_2 \approx 4$ μM, a fast (upper filled triangle) and a slow process (lower filled triangle) can be observed. At $C_1 + C_2 \approx 7$ μM, only the slow process is detected whereas the fast one is hidden in the dead time of the stopped-flow apparatus. The lower drawn line shows the concentration independent behavior of the slow process. (●) Reciprocal time constant from a stopped-flow dissociation experiment. C_1, concentration of free $tRNA^{Phe}$; C_2, concentration of free tRNA binding sites on the Phe-ol-AMP·PRS complex.

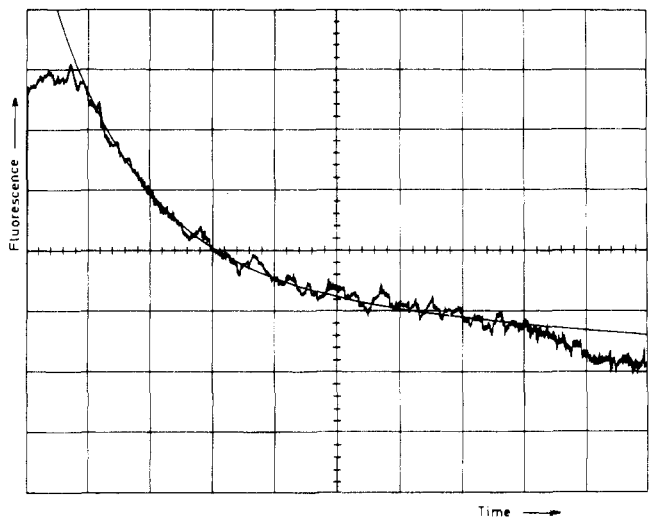

FIGURE 2: Oscilloscope trace of a stopped-flow association experiment with $tRNA^{Phe}$ and phenylalanyl-tRNA synthetase in the presence of Phe-ol-AMP at 5 °C. A fast fluorescence change is followed by a slow one. A simulated exponential with $1/\tau = 145\ s^{-1}$ is superimposed over the fast effect. Concentrations after mixing: 0.62 μM $tRNA^{Phe}$, 1.25 μM phenylalanyl-tRNA synthetase in 0.03 M potassium phosphate, 10 mM $MgCl_2$, 0.2 M KCl, 1 mM EDTA, 0.5 mM dithioerythritol, and 5×10^{-5} M Phe-ol-AMP. Oscilloscope settings: 100 mV/unit; time base of the first 8 units is 5 ms/unit; that of the last 2 units is 50 ms/unit, 100 μs rise time; total signal, 3.5 V.

to the complex between PRS and Phe-ol-AMP. The reciprocal of the relaxation time, $1/\tau$, is linearly dependent on the sum of the concentrations of free tRNA and enzyme (Figure 1). However, due to the small amplitudes, the concentration dependence of the $1/\tau$ values could not be followed up to the concentration range where saturation of the $1/\tau$ values was achieved in the absence of Phe-ol-AMP (Krauss et al., 1976). From the equilibrium measurements it was known that the binding of $tRNA^{Phe}$ to the complex between PRS and Phe-ol-AMP leads to a quenching of the Y-base fluorescence. The increase of fluorescence observed in the T-jump experiments indicates a dissociation of the tRNA from the ternary complex

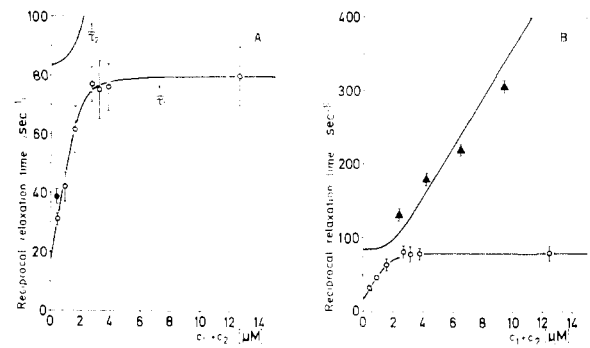

FIGURE 3: Binding of $tRNA^{Phe}$ to the complex between Phe-ol-AMP and phenylalanyl-tRNA synthetase at 5 °C. (A) T-jump data: dependence of the reciprocal relaxation times (○) on the sum of the free reactants. The drawn lines represent the theoretical curves of $1/\tau_1$ and $1/\tau_2$ as obtained for the rate constants in Table II. (●) Reciprocal time constant of a stopped-flow dissociation experiment. (B) Same data as in A, together with data from stopped-flow association experiments (▲) performed in the presence of excess enzyme binding sites. Other conditions as in Figure 1.

and a negative ΔH value of the observed binding process.

A fast process can be resolved in stopped-flow association experiments at 25 °C. It is accompanied by a quenching of the Y-base fluorescence and its time constants are in agreement with the T-jump data (Figure 1).

At 5 °C a fast process in the time range of 1 ms is observed in the T-jumps which is detectable in both the presence and the absence of the synthetase and Phe-ol-AMP. This effect originates from the binding of Mg^{2+} to the tRNA (unpublished results) and does not indicate an interaction between tRNA and synthetase; it is not observed in this time range at 25 °C. Due to this effect it was not possible to resolve another fast process equivalent to the fast effect observed at high temperatures. However, good resolution of the fast process is achieved in stopped-flow experiments at 5 °C (Figure 2). This process becomes faster with increasing concentrations of free reactants (Figure 3B), but is much slower than that observed at 25 °C.

Slow Processes. In stopped-flow association experiments at 25 °C a slow, concentration independent, process with a time constant of about 30 s^{-1} is observable and is associated with a small quenching of the Y-base fluorescence. Under the same conditions no slow effect could be detected in the T-jump experiments. In a stopped-flow dissociation experiment a time constant of about 110 s^{-1} is measured, which is in agreement with the T-jump data (Figure 1). At 5 °C the situation in the T-jump experiments changes remarkably. A slow process is observable (Figure 4) and its $1/\tau$ value increases with increasing concentrations, reaching saturation over a narrow concentration range at a $1/\tau$ value of 80 s^{-1} (Figure 3). In contrast to the measurements at 25 °C, the T-jump effects at 5 °C are associated with a quenching of the Y-base fluorescence. From this observation it follows that the reaction enthalpy of the observed process has a positive sign. The reaction enthalpy at 5 °C is calculated from the fluorescence amplitudes to be +8 ± 4 kcal/mol. In the stopped-flow association experiments at 5 °C (i.e., mixing $tRNA^{Phe}$ with the binary complex of PRS and Phe-ol-AMP), a slow process independent of concentration is also observed with a time constant of about 40 s^{-1} (Figure 2). It is clearly separated from the fast process.

The appearance of a slow process in the T-jump experiments at low temperatures only indicates that the process has a temperature dependent reaction enthalpy. To follow this point in more detail, the temperature dependence of the $1/\tau$ values

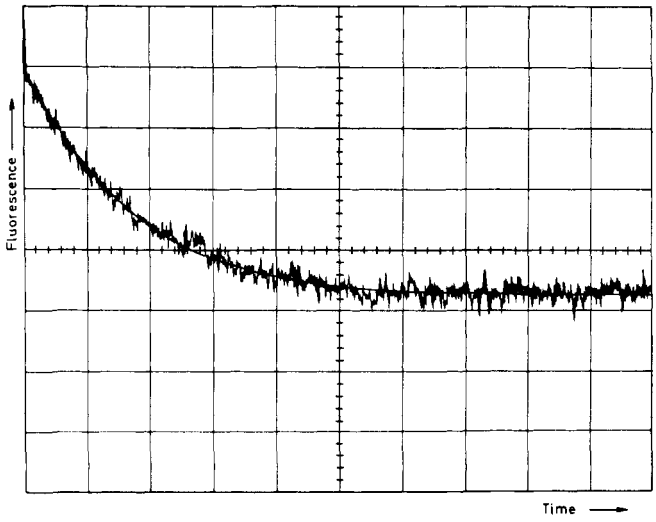

FIGURE 4: Oscilloscope trace of a temperature-jump experiment with $tRNA^{Phe}$ and phenylalanyl-tRNA synthetase in the presence of Phe-ol-AMP, 1.0 μM $tRNA^{Phe}$, 1.0 μM phenylalanyl-tRNA synthetase, and 50 μM Phe-ol-AMP in 0.03 M potassium phosphate (pH 7.2), 0.2 M KCl, 10 mM $MgCl_2$, 1 mM EDTA, 0.5 mM dithioerythritol, at 5 °C. Oscilloscope settings: 10 ms/unit, 10 mV/unit, rise time 50 μs; total signal, 4 V, 300 mV compensated. The simulated exponential with $1/\tau = 58\ s^{-1}$ is superimposed.

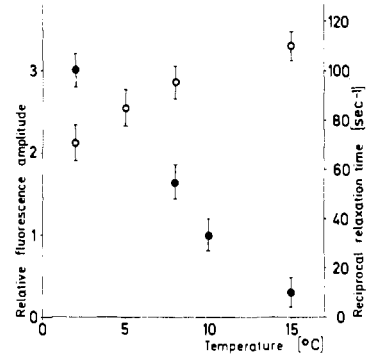

FIGURE 5: Binding of $tRNA^{Phe}$ to the complex between Phe-ol-AMP and phenylalanyl-tRNA synthetase; temperature dependence of amplitudes and relaxation times of the slow effect in T-jump experiments. (●) Amplitude; (○) slow relaxation time. The error bars indicate the experimental errors obtained for about five experiments with the same solution.

of the slow process and of its associated amplitudes was examined in the temperature range between 5 and 20 °C. As is shown in Figure 5 the amplitude of the slow relaxation time decreases with increasing temperature and vanishes at a temperature around 15 °C. Evidently the reaction enthalpy of the slow process increases with decreasing temperature and reaches a relatively high positive value at 5 °C. In the absence of Phe-ol-AMP a similar temperature dependence of the reaction enthalpy is not observed. Temperature dependent reaction enthalpies in reactions involving synthetases have been reported for the binding of isoleucine to isoleucyl-tRNA synthetase from *E. coli* (Hinz et al., 1976). The interpretation of the temperature dependence of reaction enthalpies, however, is difficult. The relaxation time of the slow process is only slightly dependent on the temperature indicating that the slow process at low temperature is not identical with the fast process at high temperatures.

Evaluation of the Rate Parameters. The combined T-jump and stopped-flow data show that the mechanism of the complex formation between $tRNA^{Phe}$ and PRS in the presence of Phe-ol-AMP includes at least two steps:

$$tRNA^{Phe} + (PRS\cdot Phe\text{-}ol\text{-}AMP) \underset{k_{21}}{\overset{k_{12}}{\rightleftharpoons}} (tRNA^{Phe}\cdot PRS\cdot Phe\text{-}ol\text{-}AMP)^{+} \underset{k_{32}}{\overset{k_{23}}{\rightleftharpoons}} (tRNA^{Phe}\cdot PRS\cdot Phe\text{-}ol\text{-}AMP)$$

where $^{+}$ indicates a ternary complex with a conformation different from the final complex. In this reaction scheme the fluorescence change and reaction enthalpy of the first step will be denoted as ΔF_{12} and ΔH_{12}, respectively, and those of the second step as ΔF_{23} and ΔH_{23}, respectively. The two-step reaction mechanism is valid both at high and low temperatures. However, some remarkable differences in the binding process show up when going from 5 to 25 °C. Therefore, the evaluation of the kinetic data at the different temperatures will be dealt with separately.

At 25 °C a fast, concentration dependent process was detected in the T-jump experiments. In the stopped-flow experiments, however, a slow, concentration independent process was observed in addition to the fast effect. Since the fast process is clearly separated from the slow process, the time constant can be evaluated using an approximation of eq 3 (see Materials section). If the recombination step is fast compared with the conformational change, the relaxation times are given by (Eigen & de Mayer, 1963):

$$\frac{1}{\tau_2} = k_{12}(C_E + C_{tRNA}) + k_{21} \quad (4)$$

and

$$\frac{1}{\tau_1} = k_{32} + \frac{k_{12'}}{k_{12'} + k_{21}} k_{23} \quad (5)$$

where $k_{12'} = k_{12}(C_E + C_{tRNA})$ and where C_E and C_{tRNA} are the concentrations of free binding sites on the enzyme and free tRNA, respectively.

According to eq 4 the values for k_{12} and k_{21} may be obtained from the slope and intercept of a plot of the fast relaxation process vs. the concentrations of free reactants (Figure 1). With the concentrations of reactants employed here, the ratio $k_{12'}/(k_{12'} + k_{21})$ is approximately 1; the slow process therefore provides $k_{32} + k_{23}$. The individual values for k_{23} and k_{32} can be calculated by using the equilibrium constant

$$K_{assoc} = (k_{12}/k_{21})(1 + k_{23}/k_{32}) \quad (6)$$

which is obtained from fluorescence titrations. The four rate constants are listed in Table II.

TABLE II: Rate Constants for the Binding of $tRNA^{Phe}$ to Phenylalanyl-tRNA Synthetase in the Presence of Phe-ol-AMP.

Temp	Rate constants	K_{assoc} (M^{-1})	Mean error factor[c]
5 °C[a]	$k_{12} = 3 \times 10^7\ M^{-1}\ s^{-1}$ $k_{23} = 10\ s^{-1}$ $k_{21} = 19\ s^{-1}$ $k_{32} = 70\ s^{-1}$	2×10^6	1.8
25 °C[b]	$k_{12} = 7.5 \times 10^7\ M^{-1}\ s^{-1}$ $k_{23} = 10\ s^{-1}$ $k_{21} = 70\ s^{-1}$ $k_{32} = 20\ s^{-1}$	1.6×10^6	1.6

[a] From the data in Figure 3. [b] From the data in Figure 1. [c] For determination of the mean error factors see Riesner et al., 1976.

Aminoacylation experiments performed under the same conditions as the T-jump measurements provide a rate constant for charging of 2 s^{-1} at 25 °C. This value comes close to the smallest rate constant (k_{23}) evaluated from the T-jump data.

The plot in Figure 1 also includes the result of a stopped-flow dissociation experiment, where the dissociation of the tRNA from the ternary complex in a solution containing 1 μM PRS, 2 μM $tRNA^{Phe}$, and 100 μM Phe-ol-AMP was followed. The reaction observed occurs with a time constant of 110 s^{-1}. This value is faster than the individual values of k_{21} and k_{32} because under these conditions both dissociation and association contribute to the reaction time. The rate constants obtained from the T-jump experiments are consistent with this interpretation.

From the combined T-jump and stopped-flow data at 25 °C, an estimate of the relative distribution of reaction enthalpy and fluorescence change during each of the two steps of the binding process can be obtained. As is indicated from the sign of the fluorescence change of the fast effect in the T-jumps, the reaction enthalpy ΔH_{12} of the first step is negative. Furthermore the recombination step includes most of the fluorescence quenching. With respect to the conformational change, the following combinations of reaction enthalpy ΔH_{23} and fluorescence changes ΔF_{23} are possible: (a) $\Delta F_{23} \neq 0$ and $\Delta H_{23} = 0$; (b) $\Delta F_{23} = 0$ and $\Delta H_{23} \neq 0$; (c) $\Delta F_{23} = 0$ and $\Delta H_{23} > 0$, $\Delta H_{12} < 0$; (d) $\Delta F_{23} = 0$ and $\Delta H_{23} = 0$. The small and slow fluorescence quenching in the stopped-flow association experiments at 25 °C implies a negative ΔF value of the conformational change. In order to arrive at this conclusion one has to remember that in our stopped-flow association experiments the enzyme was in excess over the tRNA and its absolute concentration was at least a factor of 4 higher than the reciprocal binding constant. Consequently, all tRNA present was complexed during the initial recombination and the subsequent conformational transition could not lead to an additional binding of tRNA. It follows that the possibilities b and d can be excluded. If case a were true a slow effect in the T-jump should have shown up because of the coupling of the two steps. Thus case c alone can explain the lack of the slow effect in the T-jump. In this case the ΔH of the slow effect is compensated by the ΔH of the fast effect. The experimental results therefore seem to correspond best to a reaction scheme where the reaction enthalpies of the two steps have opposite signs and are of approximate equal magnitude.

At 5 °C a slow relaxation process is observable in the T-jump experiments that saturates at higher concentrations. The concentration dependence of the $1/\tau$ values is evaluated according to a two-step reaction scheme. The rate constants as determined from the computer fitting procedure are depicted in Table II. Due to the relatively small uncertainty of the $1/\tau$ values measured at low temperature the four rate constants are obtained from a regression analysis independent of equilibrium data. The value for K_{assoc} evaluated from the kinetic data agrees very well with the value obtained from the fluorescence titrations. The knowledge of the four rate constants permits the calculation of the concentration dependence of the relaxation time of the fast process; the fast effect could not be measured in the T-jump experiments because of the interference with the Mg^{2+} binding to $tRNA^{Phe}$ (see above). The fast, concentration dependent process of the stopped-flow association experiments fits approximately the concentration dependence of the fast $1/\tau$ value predicted from the computer fit (Figure 3). The rate of the concentration independent process in the stopped-flow association experiments at 5 °C is lower than that corresponding to the $1/\tau$ values in the saturation region of the plot of the T-jump data (Figure 3). A simulation of the stopped-flow experiments in an analogue computer with the rate constants evaluated from the T-jump experiments yielded a time constant of 60 s^{-1} for the slow process. The difference between this value and the experimentally measured value of 30 s^{-1} is ascribed to the uncertainty in the evaluation of the rate constants from the T-jump data.

It is noteworthy that the equilibrium between the intermediate and the final complex in the reaction scheme in Table II is more on the side of the intermediate complex. The strong interdependence between the two processes is manifested in the rather sharp curvature in the plot in Figure 2, where the transition from a concentration dependent to a concentration independent behavior occurs over a small concentration range of free reactants.

Binding of $tRNA_{CC}^{Phe}$. Previously published experiments have shown that the terminal A of $tRNA^{Phe}$ is involved in a conformational change of the tRNA–synthetase complex (Krauss et al., 1977). To examine whether or not this holds also in the presence of Phe-ol-AMP, we have performed stopped-flow experiments by mixing $tRNA_{CC}^{Phe}$ with the complex between PRS and Phe-ol-AMP at a low temperature. In these experiments, no slow, concentration independent process could be observed. Evidently the terminal A is also involved in the conformational change of the reaction scheme in Table II.

Discussion

The results reported in this paper indicate that both the kinetic and equilibrium parameters of the binding of $tRNA^{Phe}$ to PRS are changed in the presence of the analogue of the aminoacyladenylate, phenylalaninyl-5′-AMP. The strength of the tRNA binding to the synthetase is increased in the presence of the Phe-ol-AMP by a factor of 2–3. It suggests that the Phe-ol-AMP bound to the enzyme provides by itself additional contact points for the tRNA or induces structural changes in the enzyme to provide stronger binding of the tRNA. With ATP or phenylalanine alone a similar effect is not observed (Krauss et al., 1973); evidently both phenylalanine and ATP need to be present in the active site to bring about the increase of the binding constant for the tRNA.

No experimental evidence could be found to indicate that Phe-ol-AMP changes the equivalence of the two tRNA binding sites. In contrast, Blanquet et al. (1973) have reported that for the methionine specific system from *E. coli*, the two tRNA binding sites are detectable only in the presence of methionyl-AMP. Evidently in this case the structural changes of the tRNA–synthetase complex induced by the analogue are much more pronounced and cannot be compared directly with the results reported in the present paper.

It is evident from stopped-flow and temperature-jump studies that the binding of $tRNA^{Phe}$ to PRS includes at least two steps. This has been found in the presence of Phe-ol-AMP (this work) as well as in the absence of the smaller substrates (Krauss et al., 1976). Both steps (the recombination and the subsequent conformational change), however, are remarkably slower when Phe-ol-AMP is present. It may be a question of semantics whether the steps observed in both systems are the same processes with different rates, or whether or not the fairly slow process in the presence of Phe-ol-AMP is an additional conformational transition. For the following reasons the second point of view seems to be more reasonable: first, in the presence of Phe-ol-AMP the recombination step is no longer controlled by diffusion; its rate constant is about an order of magnitude lower than the value that has been estimated for a diffusion-controlled binding of the tRNA (Krauss et al., 1973). In the

absence of Phe-ol-AMP, the observed recombination step could be considered to be basically identical with the formation of the encounter complex. The recombination step in the presence of Phe-ol-AMP, however, is too slow in order to be diffusion controlled. In addition to the formation of the encounter complex it has to include an additional process. This process cannot be observed separately and could correspond to the fast conformational change observed in the absence of Phe-ol-AMP.

The second result that distinguishes the present conformational change from that in the absence of Phe-ol-AMP relates to the temperature dependence of the reaction enthalpy. The reaction enthalpy of the conformational change is strongly dependent upon temperature in the presence of Phe-ol-AMP whereas a similar dependence could not be observed in experiments in the absence of Phe-ol-AMP.

The slow conformational change which appears only in the presence of Phe-ol-AMP indicates stricter requirements for the binding of the tRNA and may express additional specificity in the process of adaptation of the tRNA to the active site of the enzyme. Thus we propose that this conformational change is necessary to optimize the relative orientation of the reactants in order to facilitate the transfer of the activated amino acid to the tRNA. In this context, it should be mentioned that this conformational change is only a factor of 3–5 faster than the overall aminoacylation reaction.

The slow process found in the present work as a consequence of the presence of Phe-ol-AMP has to be well distinguished from reports of slow effects for tRNA–synthetase interactions in other systems measured in the absence of small substrates. Measurements of $tRNA^{Tyr}$ interaction with tyrosyl-tRNA synthetase from *E. coli* showed a slow effect only for the binding of the second $tRNA^{Tyr}$ (Riesner et al., 1976). A slow effect was also reported for the Ser-specific system from yeast (Rigler et al., 1976); this effect, however, is in contradiction to stopped-flow experiments in the same and related systems (Pingoud et al., 1973; Blanquet & Dessen, 1976).

In the present work with PRS we have found characteristic differences in reaction enthalpy and fluorescence quenching between the recombination step and conformational change. The recombination step comprises a large part of the total fluorescence quenching of the Y base, and the conformational change results in an additional small quenching. Evidently the conformational change leads to a limited rearrangement of the tRNA within the ternary complex. This arrangement is still large enough in order to be monitored by the fluorescence of the Y base.

The occurrence of the slow conformational change in the presence of Phe-ol-AMP is dependent on the integrity of the CCA end of $tRNA^{Phe}$. No slow conformational transition is observable in the presence of $tRNA_{CC}^{Phe}$. This observation is in accordance with models suggested for the structure of tRNA–synthetase complexes in which it was proposed that the anticodon and the acceptor region are involved in the recognition process (Krauss et al., 1976; Rich & Schimmel, 1977). The fast conformational change reported earlier also requires an intact CCA end of the tRNA (Krauss et al., 1977). It has been suggested above that the fast conformational change precedes the slow one. In this case the fast conformational change would be a prerequisite for the slow conformational change and the result with $tRNA_{CC}^{Phe}$ reported in the present paper would be easily comprehensible. In any case, it may be concluded that the 3′-terminal adenosine is also involved in the slow conformational change, which indicates that we are measuring physical effects which are connected with the process of aminoacylation, and which occur near the active site of the enzyme.

Acknowledgments

We thank Dr. F. Peters for supply of the computer programs and Dr. A. Pingoud for critically reading the manuscript. The expert technical assistance of Mrs. M. Möllering and Miss B. Ziegler is gratefully acknowledged.

References

Blanquet, S., & Dessen, Ph. (1976) *J. Mol. Biol. 103,* 765.
Blanquet, S., Fayat, G., Waller, J. P., & Iwatsubo, M. (1972) *Eur. J. Biochem. 24,* 461.
Blanquet, S., Iwatsubo, M., & Waller, J. P. (1973) *Eur. J. Biochem. 36,* 213.
Cassio, D., Lemoine, F., Waller, J. P., Sandrin, E., & Boissonnas, R. A. (1967) *Biochemistry 6,* 827.
Coutts, S. M., Riesner, D., Römer, R., Rabl, C. R., & Maass, G. (1975) *Biophys. Chem. 3,* 275.
Eigen, M. (1964) in 15th *Mosbach Kolloquium der Gesellschaft für Physiologische Chemie,* p 344, Springer-Verlag, Berlin.
Eigen, M., & de Mayer, L. (1963) in *Techniques of Organic Chemistry* (Weissberger, A., Ed.) Vol. VIII, part II, p 895, Wiley, New York, N.Y.
Fasiolo, F., Ebel, J. P., & Ladzunski, M. (1977) *Eur. J. Biochem. 73,* 7.
Fersht, A. R., & Jakes, R. (1975) *Biochemistry 14,* 3350.
Giégé, R., Kern, D., Ebel, J. P., Grosjean, H., Dettenan, S., & Chantrenne, H. (1974) *Eur. J. Biochem. 45,* 351.
Hinz, H. J., Weber, K., Flossdorf, J., & Kula, M. R. (1976) *Eur. J. Biochem. 71,* 437.
Kisselev, L. L., & Favorova, O. (1974) *Adv. Enzymol. 40,* 141.
Krauss, G., Römer, R., Riesner, D., & Maass, G. (1973) *FEBS Lett. 30,* 6.
Krauss, G., Pingoud, A., Boehme, D., Riesner, D., Peters, F., & Maass, G. (1975) *Eur. J. Biochem. 55,* 517.
Krauss, G., Riesner, D., & Maass, G. (1976) *Eur. J. Biochem. 68,* 81.
Krauss, G., Riesner, D., & Maass, G. (1977) *Nucleic Acids Res. 4,* 2253.
Litt, M. (1968) *Biochem. Biophys. Res. Commun. 32,* 507.
Maelicke, A., Engel, G., Staehelin, M., & Cramer, F. (1974) *Eur. J. Biochem. 42,* 311.
Pingoud, A., Riesner, D., Boehme, D., & Maass, G. (1973) *FEBS Lett. 30,* 1.
Pingoud, A., Boehme, D., Riesner, D., Kownatzki, R., & Maass, G. (1975) *Eur. J. Biochem. 56,* 617.
Rammler, D. H., & Khorana, H. G. (1962) *J. Am. Chem. Soc. 84,* 3112.
Rich, A., & Schimmel, P. R. (1977) *Nucleic Acids Res. 4,* 1649.
Riesner, D., Pingoud, A., Boehme, D., Peters, F., & Maass, G. (1976) *Eur. J. Biochem. 68,* 71.
Rigler, R., Pachmann, U., Hirsch, R., & Zachau, H. G. (1976) *Eur. J. Biochem. 65,* 307.
Sandrin, E., & Boissonnas, R. (1966) *Helv. Chim. Acta 49,* 76.
Simlot, M., & Pfaender, P. (1973) *FEBS Lett. 35,* 201.
Söll, D., & Schimmel, P. R. (1974) *Enzymes, 3rd Ed. 10,* 489.
Sprinzl, M., & Cramer, F. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72,* 3049.
Yarus, M., & Berg, P. (1969) *J. Mol. Biol. 42,* 171.