Conformational Transitions of a tRNA–Aminoacyl-tRNA Synthetase Complex Induced by tRNAs Bearing Different Modifications in the 3′ Terminus†

Gerhard Krauss,* Friedrich von der Haar,‡ and Guenter Maass

ABSTRACT: The influence of modifications of the 3′-terminal adenosine of $tRNA^{Phe}$ (yeast) on the complex formation between this tRNA and phenylalanyl-tRNA synthetase (yeast) has been investigated by using fluorescence titrations and fast kinetic techniques. Subtle changes in the 3′ terminus are reflected by distinct alterations in the two-step recognition process which had been demonstrated earlier for the native substrate $tRNA^{Phe}_{CCA}$ [Krauss, G., Riesner, D., & Maass, G. (1977) *Nucleic Acids Res. 4*, 2253–2262]. Binding experiments with $tRNA^{Phe}_{CC}$, $tRNA^{Phe}_{CCA\text{-}ox\text{-}red}$, $tRNA^{Phe}_{CC2'dA}$, $tRNA^{Phe}_{CC3'dA}$, $tRNA^{Phe}_{CC\text{-}formycin}$, and $tRNA^{Phe}_{CC\text{-}formycin\text{-}ox\text{-}red}$ confirm that the 3′-terminal adenosine participates in a conformational change of the tRNA–synthetase complex. This is valid in both the absence and presence of phenylalaninyl-5′-AMP, the alkyl analogue of the aminoacyladenylate. As compared to $tRNA^{Phe}_{CCA}$, a slower conformational change is observed with the competitive inhibitor $tRNA^{Phe}_{CC\text{-}formycin\text{-}ox\text{-}red}$. The reaction enthalpy and/or the quench of the Y-base fluorescence that accompany the conformational change are altered upon binding of $tRNA^{Phe}_{C2'dA}$, $tRNA^{Phe}_{CC3'dA}$, and $tRNA^{Phe}_{CC\text{-}formycin}$. It is evident that the final adaptation between tRNA and its synthetase in the complex is determined by the chemical nature of the 3′-terminal nucleotide. This is of vital importance for the specificity of the aminoacylation process.

Overall protein biosynthesis proceeds with an error rate of less than 1 in 3000 (Loftfield et al., 1963). Aminoacylation of tRNA, being a prerequisite, must thus occur with a comparable or even lower error rate. The thermodynamic data on the tRNA–synthetase interaction (Lam & Schimmel, 1975; Krauss et al., 1976) that are at present available do not provide a clue for understanding the high specificity of the aminoacylation process in vivo. Alternatively, a dynamic recognition process between tRNAs and aminoacyl-tRNA synthetases has been postulated (Ebel et al., 1973). Experimental evidence for this concept, based mainly on steady-state investigations on the aminoacylation of modified tRNAs, begins to accumulate slowly (von der Haar & Gaertner, 1975; Krauss et al., 1977; von der Haar & Cramer, 1978). To establish the occurrence of a dynamically controlled recognition process more firmly, it is necessary to perform physicochemical measurements on the binding mechanism of the modified tRNAs.

By use of fast kinetic techniques it could be shown for the phenylalanine-specific system from yeast that the recognition process includes a recombination step and a subsequent conformational change of the complex (Krauss et al., 1976), the latter step being dependent on the presence of the 3′-terminal adenosine of $tRNA^{Phe}$ (Krauss et al., 1977). Furthermore, it was found that the presence of phenylalaninyl-5′-AMP in the active site of the enzyme introduces profound changes in the tRNA-binding mechanism (Krauss et al., 1978). A similar conclusion was drawn from inhibition studies with $tRNA^{Phe}$ modified at the 3′-terminal acceptor moiety (von der Haar & Gaertner, 1975). In the present paper we therefore studied the mechanism of complex formation between phenylalanyl-tRNA synthetase (yeast) and $tRNA^{Phe}$ species that carry different modifications at the 3′-terminal adenosine. Our data show that the final structure of the tRNA–synthetase complex both in the presence and in the absence of phenylalaninyl-5′-AMP is strongly determined by the chemical nature of the 3′-terminal purine nucleotide.

Experimental Section

Materials

Phenylalanyl-tRNA synthetase (PRS)[1] from yeast was isolated and characterized as given in Krauss et al. (1976). The preparation of $tRNA^{Phe}_{CC}$ from the unfractionated tRNA (yeast) was according to Schneider et al. (1972). In the presence of tRNA nucleotidyltransferase, ATP, phenylalanyl-tRNA synthetase, and phenylalanine, 1 A_{260} unit of $tRNA^{Phe}_{CC}$ accepted 1.5 nmol of phenylalanine. The 3′-terminal adenosine as well as its analogues formycin, 3′-deoxyadenosine, and 2′-deoxyadenosine was incorporated as described (Sprinzl et al., 1978). Oxidation and reduction of the 3′-terminal ribose of $tRNA^{Phe}_{CCA}$ and $tRNA^{Phe}_{CCF}$ were performed as outlined earlier (von der Haar et al., 1971). The amount of incorporation of modified nucleotides at the 3′ end was determined by nucleoside analysis according to Sprinzl et al. (1978). With the exception of $tRNA^{Phe}_{CC3'dA}$, the extent

† From the Institut für Klinische Biochemie und Physiologische Chemie, Abteilung Biophysikalische Chemie, Medizinische Hochschule Hannover, Karl-Wiechert-Allee 9, D-3000 Hannover 61, Federal Republic of Germany, and the Max-Planck-Institut für Experimentelle Medizin, Abteilung Chemie, D-3400 Göttingen, Federal Republic of Germany. *Received March 28, 1979.* This work was supported by grants from the Deutsche Forschungsgemeinschaft (Schwerpunktprogramm Struktur-Funktion von Biopolymeren) and the Fonds der Chemischen Industrie.

* Correspondence should be addressed to this author at the Institut für Klinische Biochemie und Physiologische Chemie, Abteilung Biophysikalische Chemie, Medizinische Hochschule Hannover, D-3000 Hannover 61, Federal Republic of Germany.

‡ Present address: Max-Planck-Institut für Experimentelle Medizin, Abteilung Chemie, D-3400 Göttingen, Federal Republic of Germany.

[1] EDTA, ethylenediaminetetraacetic acid; PRS, phenylalanyl-tRNA synthetase; Phe-ol-AMP, phenylalaninyl-5′-AMP; T-jump, temperature jump; $tRNA^{Phe}_{CCN}$, $tRNA^{Phe}$ carrying the nucleotide N at the 3′ terminus; $tRNA^{Phe}_{CC}$, tRNA lacking the 3′-terminal AMP; $tRNA_{CCF}$, $tRNA_{CC2'dA}$, and $tRNA_{CC3'dA}$, tRNA with 3′-terminal formycin (9-deaza-8-aza-adenosine), 2′-deoxyadenosine, and 3′-deoxyadenosine, respectively, instead of AMP; $tRNA_{CCF\text{-}ox\text{-}red}$ and $tRNA_{CCA\text{-}ox\text{-}red}$, tRNA with the 3′-terminal cis diol group oxidized by periodate to a dialdehyde moiety and subsequently reduced by borohydride to two CH_2OH groups.

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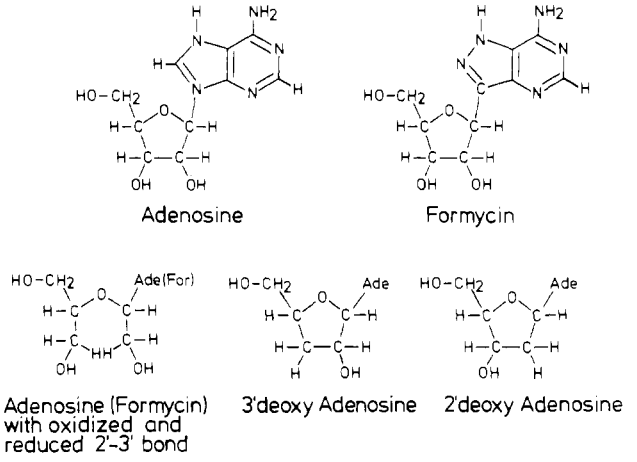

FIGURE 1: Structures of nucleotides at the 3′ terminus of $tRNA^{Phe}_{CCN}$.

of incorporation was more than 90%. For those modified tRNAs that could serve as substrates for aminoacylation, the nucleoside analysis was confirmed by aminoacylation. 3′-dA was incorporated to 78 ± 3% according to nucleoside analysis whereas aminoacylation pointed to an incorporation of about 90%. Phenylalaninyl-5′-AMP (Phe-ol-AMP) was synthesized and characterized as described previously (Krauss et al., 1978). All reagents and solvents were of the highest purity available from commercial sources.

Methods

Determination of Equilibrium Parameters. The stoichiometry of the tRNA–synthetase complexes was obtained from sedimentation velocity determinations carried out with an analytical ultracentrifuge as outlined earlier (Krauss et al., 1975). Binding constants for complex formation between $tRNA^{Phe}$ and PRS or the PRS·Phe-ol-AMP binary complex were derived from fluorescence titrations, utilizing the Y base of $tRNA^{Phe}$ as an indicator of complex formation. The Y-base fluorescence was excited at 313 nm and observed at 460 nm. Titration data were evaluated as described earlier (Krauss et al., 1973).

Fast Kinetic Measurements. In all kinetic experiments the fluorescence of the Y base of $tRNA^{Phe}$ was monitored. T-jump experiments were performed as described by Coutts et al. (1975) and Riesner et al. (1976), where the magnitudes of the temperature jumps were between 3 and 5 °C. The dependence upon concentration of the measured relaxation times was analyzed in terms of a two-step reaction scheme as outlined in Riesner et al. (1976) and Krauss et al. (1978). Evaluation of the rate constants were performed as described in detail by Riesner et al. (1976). For rapid mixing experiments, a stopped-flow spectrofluorometer as described by Pingoud et al. (1975) was used. Association experiments were carried out by mixing $tRNA^{Phe}$ with PRS or PRS·Phe-ol-AMP. All 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.

Results and Discussion

The structures of the 3′-terminal nucleotides of the modified tRNAs used are shown in Figure 1. Whereas $tRNA_{CC2'dA}$ and $tRNA_{CCF\text{-}ox\text{-}red}$ are competitive inhibitors of the aminoacylation reaction, all other modified tRNAs can serve as substrates for aminoacylation (von der Haar & Gaertner, 1975).

Table I: Equilibrium Parameters for Complex Formation between $tRNA^{Phe}_{CCN}$ and Phenylalanyl-tRNA Synthetase[a]

tRNA species	fluorescence change (%)	binding constant[b] (M^{-1})	$tRNA^{bound}$/enzyme[c]
$tRNA^{Phe}_{CCA}$	−(25 ± 2)	8×10^5	2
$tRNA^{Phe}_{CC2'dA}$	−25	9×10^5	2
$tRNA^{Phe}_{CC3'dA}$	−25	9.8×10^5	2
$tRNA^{Phe}_{CCA\text{-}ox\text{-}red}$	−25	8.5×10^5	2
$tRNA^{Phe}_{CCF}$	−18	6.7×10^5	2
$tRNA^{Phe}_{CCF\text{-}ox\text{-}red}$	−35	8.7×10^5	2
$tRNA^{Phe}_{CC}$[e]	−25	2.7×10^5	2

[a] Determined in 0.03 M potassium phosphate, pH 7.2, 0.2 M KCl, 10 mM $MgCl_2$, 1 mM EDTA, and 0.5 mM dithioerythritol, at 5 °C. [b] Obtained from fluorescence titration. [c] Data from sedimentation experiments in the analytical ultracentrifuge according to Krauss et al. (1975). [d] From Krauss et al. (1975). [e] From Krauss et al. (1977).

Binding of $tRNA^{Phe}_{CCN}$ to Phenylalanyl-tRNA Synthetase. (*1*) *Equilibria.* The stoichiometry and binding constants for complex formation between $tRNA^{Phe}_{CCN}$ and PRS are given in Table I, together with the data for the binding of $tRNA^{Phe}_{CC}$ and $tRNA^{Phe}_{CCA}$. The data show that, within the limits of error, the strength of complex formation is not influenced by the modifications of the 3′-terminal nucleotide. The data also demonstrate that two binding sites on the synthetase are occupied with the same affinity by the modified tRNAs.

Differences with respect to the binding behavior of unmodified $tRNA^{Phe}$ are seen only in the amount of quenching of the fluorescence of the Y base. A smaller fluorescence quench is observed upon binding of $tRNA^{Phe}_{CCF}$ to PRS, whereas binding of $tRNA^{Phe}_{CCF\text{-}ox\text{-}red}$ is accompanied by an increase in the fluorescence quenching as compared to binding of $tRNA^{Phe}_{CCA}$. An interpretation of the latter finding requires an examination of the relative contributions of the formycin and the Y-base residues to the emission spectrum of $tRNA^{Phe}_{CCF\text{-}ox\text{-}red}$ when excited at 313 nm. Oxidation of the terminal ribose cleaves the ribofuranose ring, resulting in a reduced stacking of the formycin residue in $tRNA^{Phe}_{CCF\text{-}ox\text{-}red}$. As a consequence, the quantum yield of the formycin fluorescence is strongly increased (Maelicke et al., 1974). The main contribution to the emission spectrum of $tRNA^{Phe}_{CCF\text{-}ox\text{-}red}$ in the wavelength region between 390 and 480 nm probably arises from the fluorescence of formycin, although it is not possible to observe the Y-base and the formycin fluorescence separately. The large quenching of the formycin fluorescence observed upon binding of $tRNA^{Phe}_{CCF\text{-}ox\text{-}red}$ to PRS is presumably due to an increased degree of stacking of the formycin residue in the tRNA–synthetase complex. This stacking may include interactions with aromatic residues of the protein or interactions with the adjacent nucleotides of the tRNA. The reduced quenching of the Y-base fluorescence in the case of $tRNA_{CCF}$ is difficult to explain. One possible explanation is that there is a slightly altered topography of the Y base in the complex, which does not express itself in the strength of binding.

(*2*) *Kinetics.* The kinetics of the binding of $tRNA^{Phe}_{CCN}$ to PRS were studied by investigating the concentration dependence of the relaxation times in temperature-jump experiments. The binding mechanism for $tRNA^{Phe}_{CCA}$ and $tRNA^{Phe}_{CC}$ was derived earlier by utilizing data from similar experiments, whereby a leveling off of the reciprocal relaxation times at high concentrations of the reactants indicated a two-step mechanism for the binding between $tRNA^{Phe}_{CCA}$ and the enzyme (Krauss et al., 1976). On the other hand, in the case of $tRNA^{Phe}_{CC}$, the linear dependence of the reciprocal

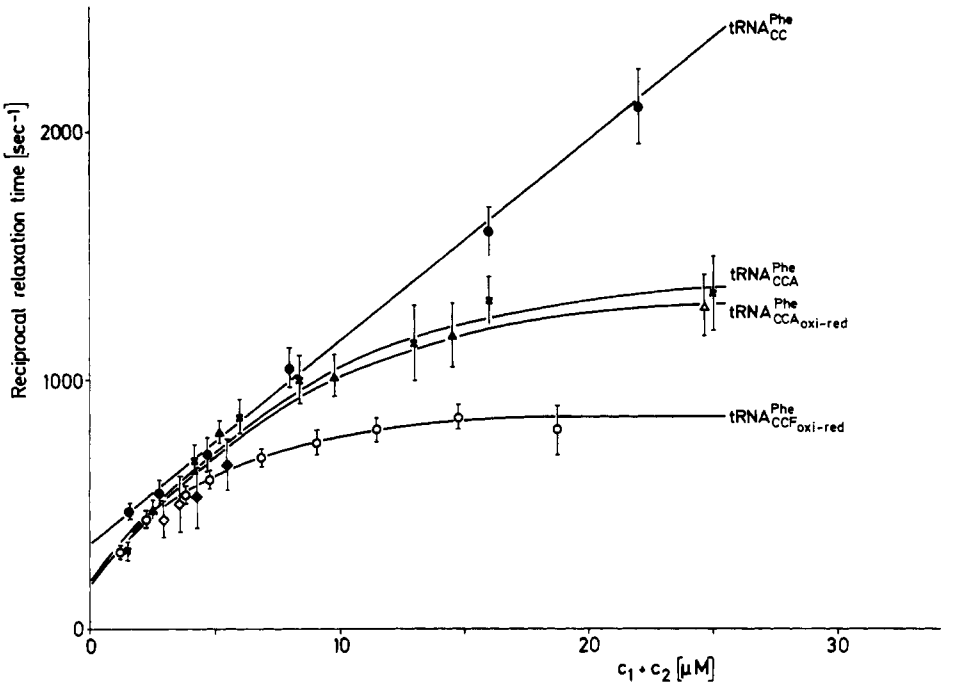

FIGURE 2: Binding of $tRNA^{Phe}_{CCN}$ to phenylalanyl-tRNA synthetase at 20 °C; temperature-jump data. Dependence of the reciprocal relaxation times on the sum of the concentrations of free reactants. (●) $tRNA^{Phe}_{CC}$; (×) $tRNA^{Phe}_{CCA}$ [from Krauss et al. (1977)]; (○) $tRNA^{Phe}_{CCF\text{-}ox\text{-}red}$; (△) $tRNA^{Phe}_{CCA\text{-}ox\text{-}red}$; (◇) $tRNA^{Phe}_{CC2'dA}$; (◆) $tRNA^{Phe}_{CC3'dA}$. The drawn lines represent the theoretical curves of $1/\tau_1$ as obtained for the rate constants in Table II. A fit to the data for $tRNA^{Phe}_{CC2'dA}$ and $tRNA^{Phe}_{CC3'dA}$ has not been performed. For sake of clarity not all measured points have been included.

Table II: Rate Constants for the Binding of $tRNA^{Phe}_{CCN}$ to Phenylalanyl-tRNA Synthetase[a]

tRNA species	k_{12} (M^{-1} s^{-1})	k_{21} (s^{-1})	k_{23} (s^{-1})	k_{32} (s^{-1})	mean error factor[c]
$tRNA^{Phe}_{CCA}$[b]	1.7×10^8	700	1160	500	2.0
$tRNA^{Phe}_{CCA\text{-}ox\text{-}red}$	1.5×10^8	600	1020	470	2.5
$tRNA^{Phe}_{CCF\text{-}ox\text{-}red}$	2×10^8	660	615	340	1.8
$tRNA^{Phe}_{CC}$[b]	0.85×10^8	320			1.3

[a] From the data in Figure 2. [b] From Krauss et al. (1977). [c] For determination of the mean error factors see Riesner et al. (1976).

relaxation times on the concentrations pointed to a one-step binding process (Krauss et al., 1977).

The observation of a second step is based on fluorescence measurements throughout all kinetic experiments. For several modified tRNAs a change in the rate, fluorescence quantum yield, or reaction enthalpy of the second step can be detected. On the basis of the present data, however, it is not possible to decide whether the experimental differences observed point to a new conformational change or whether one is dealing with the same conformational change but with slightly altered physicochemical parameters. The results obtained with $tRNA^{Phe}_{CCN}$ are summarized in Figure 2 and Table II, together with the earlier data on the binding of $tRNA^{Phe}_{CC}$ and $tRNA^{Phe}_{CCA}$ (Krauss et al., 1977).

The behavior of the individual $tRNA^{Phe}_{CCN}$ is as follows.

$tRNA^{Phe}_{CCA\text{-}ox\text{-}red}$. A two-step mechanism has to be postulated for the binding of $tRNA^{Phe}_{CCA\text{-}ox\text{-}red}$ to PRS. The rate constants are very similar to those determined for the binding of unmodified $tRNA^{Phe}$. In addition, the amplitudes of the observed relaxation effects are also comparable to those observed with unmodified $tRNA^{Phe}$.

$tRNA^{Phe}_{CCF\text{-}ox\text{-}red}$. The kinetic data for this tRNA species can also be analyzed by a two-step process. In comparison to that of unmodified $tRNA^{Phe}$, the rate of the conformational change is slower, as indicated by the lower saturation level of the relaxation times. The recombination step, however, is unaffected. $tRNA^{Phe}_{CCF\text{-}ox\text{-}red}$ is nonchargeable (von der Haar & Gaertner, 1975) and is a competitive inhibitor of the aminoacylation process. This behavior becomes understandable in the light of the present results, since the final positioning of the tRNA on the enzyme is evidently altered as a consequence of the presence of the modified formycin nucleotide at the 3′ terminus.

$tRNA^{Phe}_{CC2'dA}$, $tRNA^{Phe}_{CC3'dA}$, and $tRNA^{Phe}_{CCF}$. The kinetic data are not adequate to reveal two steps for these tRNAs. The amplitudes of the relaxation effects are too small or, in the case of $tRNA^{Phe}_{CCF}$, no relaxation effects can be observed at all. The data for $tRNA^{Phe}_{CC2'dA}$ and $tRNA^{Phe}_{CC3'dA}$ are included in Figure 2. The changes in the relaxation effects are difficult to explain. One possible explanation could be a reduced reaction enthalpy, ΔH, of complex formation. The reaction enthalpy for complex formation between $tRNA^{Phe}_{CCA}$ and PRS was determined to be -3.5 ± 1 kcal/mol (Krauss et al., 1976). It is conceivable that a further reduction of this rather small value could occur without affecting the binding constant, by compensating the reduction in ΔH by an increase in entropy.

The kinetic experiments on the binding of the modified tRNAs show that the interaction of the synthetase with the tRNA is influenced by the nature of the 3′-terminal nucleotide in a rather complex way. In spite of this, several conclusions concerning the structural requirements at the CCA end can be drawn. Evidently, both hydroxyl groups of the 3′-terminal ribose are necessary for the conformational change to occur correctly. If one of the OH groups of the terminal ribose is missing, changes in the reaction enthalpy of complex formation become evident. It is not necessary, however, that the terminal ribose ring is intact. The latter conclusion is derived from the observation that $tRNA^{Phe}_{CCA\text{-}ox\text{-}red}$ behaves very similarly to $tRNA^{Phe}_{CCA}$ in the kinetic experiments. In this context it should be mentioned that earlier work of von der Haar et al.

Table III: Equilibrium Parameters for Complex Formation between $tRNA^{Phe}_{CCN}$ and Phenylalanyl-tRNA Synthetase in the Presence of Phe-ol-AMP[a]

tRNA species	fluorescence change (%)	binding constant[b] (M^{-1})	$tRNA^{bound}$/enzyme[c]
$tRNA^{Phe}_{CCA}$[d]	(25 ± 2)	(1.6 ± 0.3) × 10^6	2
$tRNA^{Phe}_{CC2'dA}$	25	1.3 × 10^6	2
$tRNA^{Phe}_{CC3'dA}$	25	1.5 × 10^6	2
$tRNA^{Phe}_{CCA\text{-}ox\text{-}red}$	25	1.2 × 10^6	2
$tRNA^{Phe}_{CCF}$	23	1.0 × 10^6	2
$tRNA^{Phe}_{CCF\text{-}ox\text{-}red}$	48	6.0 × 10^6	2
$tRNA^{Phe}_{CC}$	28	1.8 × 10^6	2

[a] Determined in 0.03 M potassium phosphate, pH 7.2, 0.2 M KCl, 10 mM $MgCl_2$, 1 mM EDTA, and 0.5 mM dithioerythritol, at 5 °C. [b] Obtained from fluorescence titration. [c] Data from sedimentation experiments in the analytical ultracentrifuge according to Krauss et al. (1975). [d] From Krauss et al. (1975).

(1971) suggests that the terminal adenosine in $tRNA^{Phe}_{CCA\text{-}ox\text{-}red}$ adopts a similar configuration as in $tRNA^{Phe}_{CCA}$. The distinct behavior of $tRNA^{Phe}_{CCF}$ and $tRNA^{Phe}_{CCF\text{-}ox\text{-}red}$ is attributed to a slightly different orientation of the OH groups of the ribose compared to those of the intact $tRNA^{Phe}$. Prusiner et al. (1973) have shown that the puckering of the sugar in the formycin nucleotide is different from that in, e.g., an adenosine nucleotide. Another explanation for the altered binding of $tRNA_{CCF}$ and $tRNA_{CCF\text{-}ox\text{-}red}$ is offered by a possible formation of hydrogen bonds between the –NH–N= in the five-membered ring of formycin (see Figure 1) and the amino acid side chains of the protein.

Binding of $tRNA^{Phe}_{CCN}$ to PRS in the Presence of Phenylalaninyl-5'-AMP. Previously we have shown that the presence of Phe-ol-AMP in the active site of PRS leads to remarkable changes in the kinetic parameters of the binding of tRNA to the enzyme (Krauss et al., 1978). Now we have elaborated these investigations by performing binding experiments with $tRNA^{Phe}_{CCN}$ in the presence of Phe-ol-AMP. In all experiments Phe-ol-AMP was present in 50–100-fold molar excess over PRS in order to guarantee saturation of both active sites on the enzyme [cf. Krauss et al. (1978)]. All binding experiments were carried out at 5 °C, since our previous investigations had shown that the best signals in the temperature-jump experiments, carried out in the presence of Phe-ol-AMP, were obtained in this temperature range (Krauss et al., 1976).

(*1*) *Equilibria.* Binding constants, fluorescence quenching, and stoichiometry of complex formation in the presence of Phe-ol-AMP are delineated in Table III. These data were obtained from fluorescence titrations and ultracentrifugation experiments. With the exception of $tRNA^{Phe}_{CCF\text{-}ox\text{-}red}$, the modified tRNAs bind within the limits of error with the same affinity to PRS·Phe-ol-AMP as does $tRNA^{Phe}_{CCA}$. As compared to the situation in the absence of Phe-ol-AMP (Table I), the binding constants are about a factor of 2 higher for all modified tRNAs. The sole exception, $tRNA^{Phe}_{CCF\text{-}ox\text{-}red}$, has a four- to fivefold greater affinity for PRS·Phe-ol-AMP than has unmodified $tRNA^{Phe}$. This is in agreement with the K_I value determined in steady-state kinetics (von der Haar & Gaertner, 1975). The large fluorescence quenching of about –50% observed upon binding of this tRNA is mainly due to the quenching of the fluorescence of the formycin residue. The quenching of the formycin fluorescence in the PRS·$tRNA_{CCF\text{-}ox\text{-}red}$ complex is enhanced in the presence of Phe-ol-AMP. The analogue may provide additional stacking interactions for the formycin residue in the active site of the enzyme, thus resulting in a stronger quenching of the formycin fluorescence.

Table IV: Rate Constants for the Binding of $tRNA^{Phe}_{CCN}$ to Phenylalanyl-tRNA Synthetase in the Presence of Phe-ol-AMP[a]

tRNA species	k_{12} (M^{-1} s^{-1})	k_{21} (s^{-1})	k_{23} (s^{-1})	k_{32} (s^{-1})	mean error factor[b]
$tRNA^{Phe}_{CCA}$	3 × 10^7	19	10	70	1.8
$tRNA^{Phe}_{CC}$	5 × 10^7	26	3	76	2.2
$tRNA^{Phe}_{CCA\text{-}ox\text{-}red}$	3.5 × 10^7	37	5	115	1.8
$tRNA^{Phe}_{CC2'dA}$	2.6 × 10^7	13	3	71	2.0
$tRNA^{Phe}_{CC3'dA}$	3.6 × 10^7	19	4	103	2.0
$tRNA^{Phe}_{CCF}$	2.7 × 10^7	29	4	77	2.0
$tRNA^{Phe}_{CCF\text{-}ox\text{-}red}$	1.5 × 10^7	2.8	3	24	1.7

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

(*2*) *Kinetics.* In the temperature-jump experiments a saturation of the relaxation times with increasing concentration is observed for all $tRNA^{Phe}_{CCN}$ species investigated. Therefore, evaluation of the data was performed according to a two-step mechanism:

$$\text{tRNA} + \text{PRS·Phe-ol-AMP} \rightleftharpoons (\text{tRNA·PRS·Phe-ol-AMP})^* \rightleftharpoons \text{tRNA·PRS·Phe-ol-AMP} \quad (1)$$

The plot of the reciprocal relaxation times vs. the sum of the concentrations of free reactants is depicted in Figure 3. The rate constants evaluated from these experiments are given in Table IV. The data show that the basic mechanism of binding, as determined for $tRNA^{Phe}_{CCA}$ in the presence of Phe-ol-AMP (Krauss et al., 1978), is retained with the modified tRNAs: the recombination step cannot be considered to be diffusion-controlled, and the rate of the conformational change is more than 1 order of magnitude slower than that of the process observed in the experiments in the absence of Phe-ol-AMP. However, the slow conformational change, observable only in the presence of Phe-ol-AMP, is very sensitive to the chemical nature of the 3'-terminal nucleotide of $tRNA^{Phe}$. In the binding experiments with $tRNA^{Phe}_{CCA\text{-}ox\text{-}red}$ and $tRNA^{Phe}_{CC3'dA}$, a slightly faster conformational change is detected, whereas the recombination step is not affected. In the complex formation between PRS·Phe-ol-AMP and $tRNA^{Phe}_{CCF\text{-}ox\text{-}red}$, both the conformational change and the recombination step are slowed down.

The values of the rate constants for binding of $tRNA^{Phe}_{CCF}$ and $tRNA^{Phe}_{CC2'dA}$ are nearly identical with the values measured in corresponding experiments with $tRNA^{Phe}_{CCA}$. However, the amplitudes of the temperature-jump experiments are smaller by a factor of 3–4 for these two tRNAs. In stopped-flow experiments on the association of $tRNA^{Phe}_{CC2'dA}$ and $tRNA^{Phe}_{CCF}$ with PRS·Phe-ol-AMP, the conformational change cannot be observed as a separate process. However, the failure in detecting the slow process in the stopped-flow experiments does not necessarily mean that the slow process does not occur. A simulation of the binding process using an analogue computer shows that, if an unfavorable distribution of the fluorescence quenching occurs between the two steps of the binding mechanism, the slow process may prove to be hardly detectable. Thus, it may be a reduction of the fluorescence change of the slow process that prevents the detection of the process in the stopped-flow experiments. This reduction of the fluorescence change and/or a reduction of the reaction enthalpy of this process may provide the explanation for the smaller relaxation amplitudes observed in

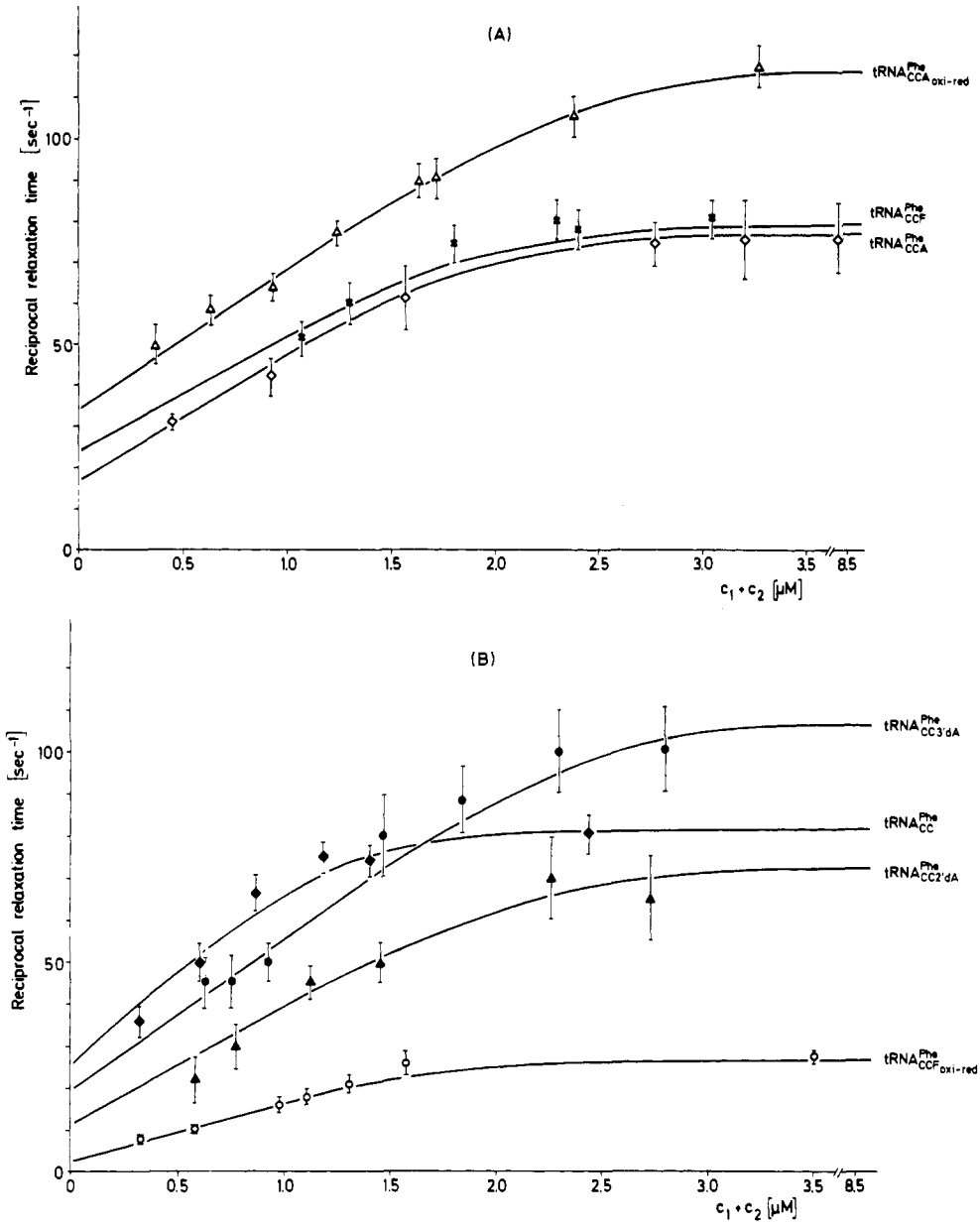

FIGURE 3: Binding of $tRNA^{Phe}_{CCN}$ to the complex between Phe-ol-AMP and phenylalanyl-tRNA synthetase at 5 °C; temperature-jump data. Dependence of the reciprocal relaxation times on the sum of the concentrations of free reactants. (A) (△) $tRNA^{Phe}_{CCA\text{-}ox\text{-}red}$; (×) $tRNA^{Phe}_{CCF}$; (◇) $tRNA^{Phe}_{CCA}$. (B) (●) $tRNA^{Phe}_{CC3'dA}$; (◆) $tRNA^{Phe}_{CC}$; (▲) $tRNA^{Phe}_{CC2'dA}$; (○) $tRNA^{Phe}_{CCF\text{-}ox\text{-}red}$. The drawn lines represent the theoretical curves of $1/\tau_1$ as obtained for the rate constants in Table IV.

the T-jump experiments upon binding of $tRNA^{Phe}_{CC2'dA}$ and $tRNA^{Phe}_{CCF}$ to PRS·Phe-ol-AMP.

An interesting result that is not immediately evident from the data in Figure 3 and Table IV shows up in T-jump studies on the binding of $tRNA^{Phe}_{CC}$. The amplitudes of the relaxation effects have signs opposite to those in corresponding experiments with the other modified or unmodified tRNAs. The change in the sign of the amplitude can be attributed to a change in the sign of ΔH or the fluorescence change of the process under investigation. As reported in our previous paper (Krauss et al., 1978), no slow conformational change was observable in stopped-flow experiments upon binding of $tRNA^{Phe}_{CC}$ to PRS·Phe-ol-AMP. On the basis of this observation, we concluded that the mechanism for binding $tRNA^{Phe}_{CC}$ did not include a slow rearrangement of the ternary complex. Our present temperature-jump data would argue against that conclusion, since we have shown here that a two-step mechanism is also valid for the binding of $tRNA^{Phe}_{CC}$ to PRS·Phe-ol-AMP. However, as indicated by such effects as the change in the sign of the relaxation, the intermediate complex and/or the final complex of the reaction scheme (eq 1) are not identical with those detected in the binding mechanism of $tRNA^{Phe}_{CCA}$ and the other modified tRNAs.

The mechanism of complex formation between the various modified tRNAs and PRS·Phe-ol-AMP includes at least two steps. This is valid also for the binding of $tRNA^{Phe}_{CC}$. Evidently, the second step in the reaction scheme (eq 1) does not depend on the presence of the 3′-terminal adenosine. The conformational change observed in the absence of Phe-ol-AMP, however, requires the presence of the 3′-terminal adenosine (Krauss et al., 1977). This confirms the conclusion derived earlier (Krauss et al., 1978) that the two conformational changes are distinct processes. The appearance of the slow conformational change must be correlated to other parts of the $tRNA^{Phe}$ molecule beyond the CCA end.

In the experiments in the presence of Phe-ol-AMP, we observe an influence of the structure of the 3′-terminal nucleotide on the fluorescence change that accompanies the

second step. In the earlier paper (Krauss et al., 1978) we had concluded that the second step leads to a limited rearrangement of the tRNA in the ternary complex. This conclusion can now be extended. We can ascribe the second step to a rather large conformational change that extends from the CCA end to the region of the Y base of the $tRNA^{Phe}$. Otherwise, the presence of modifications at the CCA end could not be monitored by the Y base. The profound rearrangement is most probably due to a change in the conformation of the protein. This interpretation is in accordance with the strongly temperature dependent reaction enthalpy of the second step. Similar temperature-dependent reaction enthalpies are not observed in structural transitions of $tRNA^{Phe}$ (Hinz et al., 1977). It may be that the PRS·Phe-ol-AMP complex contains flexible regions that are very sensitive to modifications at the CCA end.

The present data show that the chemical nature of the 3′-terminal adenosine in $tRNA^{Phe}$ plays a critical role in determining the final orientation of the tRNA in the complex with the cognate synthetase. The binding behavior of the modified tRNAs, as indicated by the kinetic constants and the distribution of fluorescence changes and/or reaction enthalpy on the two steps, differs from that of the intact $tRNA^{Phe}$. This finding is in accordance with studies on the steady-state kinetics of the modified tRNAs in this and other synthetase systems. It could be shown, for instance, that $tRNA^{Phe}_{CC2'dA}$ and $tRNA^{Phe}_{CCF\text{-}ox\text{-}red}$, although both are competitive inhibitors of the aminoacylation reaction, differ with respect to the influence on the AMP–PP_i exchange (von der Haar & Gaertner, 1975). It was also found that the replacement of the 3′-terminal adenosine by the formycin moiety led to changes in the AMP-independent hydrolysis of aminoacyl-tRNA (von der Haar & Cramer, 1976). Furthermore, in various systems, including the Phe system (yeast), the velocity of AMP formation is decreased in the presence of $tRNA^{Phe}_{CCF}$ as compared to $tRNA^{Phe}_{CCA}$ (von der Haar & Cramer, 1976). With respect to the terminal 2′- and 3′-OH groups of tRNAs, it follows from these studies that the two OH groups have distinct functions in the aminoacylation process. The nonaccepting 3′-OH group of $tRNA^{Phe}$ could be shown to participate in AMP formation and AMP-independent hydrolysis of Phe-$tRNA^{Phe}$. Such a nonaccepting OH group is also involved in proofreading functions (von der Haar & Cramer, 1976). In the Ile system from yeast, the 3′-OH group has a triggering function for the aminoacylation of the cognate tRNA (von der Haar & Cramer, 1978). These steady-state data reveal a strong sensitivity of the amino acid activation as well as the aminoacylation toward the structure of the 3′-terminal adenosine. The physicochemical investigations presented here suggest similar conclusions. Differences in the binding behavior are observed, e.g., upon replacing adenosine by formycin or upon removing the 2′- or 3′-OH group.

A direct correlation between the present binding data and the steady-state data is only partially possible. The finding that the strength of binding $tRNA^{Phe}$ to PRS is not influenced by the modification at the CCA end is in accordance with reports on the K_M values for the different tRNAs ($tRNA^{Phe}_{CC3'dA}$, $tRNA^{Phe}_{CCF}$, and $tRNA^{Phe}_{CCA\text{-}ox\text{-}red}$). The fivefold higher affinity of $tRNA^{Phe}_{CCF\text{-}ox\text{-}red}$ to PRS·Phe-ol-AMP, compared to that of $tRNA^{Phe}_{CCA}$, is reflected in a lower K_I value (stronger inhibition) observed for this tRNA (von der Haar & Gaertner, 1975). However, this observation seems not to follow a more general pattern since the association constant for binding $tRNA_{CC2'dA}$ to PRS·Phe-ol-AMP does not differ from that for $tRNA^{Phe}_{CCA}$, although a lower K_I has also been determined for this tRNA (von der Haar & Gaertner, 1975). The relationship between the data obtained in the absence and that obtained in the presence of Phe-ol-AMP is not yet fully understood. In comparing these data, we find that the differences in the binding mechanism of the various $tRNA^{Phe}_{CCN}$ species seem to be less prominent in the presence than in the absence of Phe-ol-AMP. In the presence of Phe-ol-AMP a two-step mechanism could be verified for all $tRNA^{Phe}_{CCN}$ species, whereas in the absence of Phe-ol-AMP a two-step mechanism cannot be proved for at least one tRNA species, $tRNA^{Phe}_{CC}$. In our opinion the set of data determined in the absence of Phe-ol-AMP is better suited for a comparison with steady-state results. In the Phe system a formation of an aminoacyladenylate in both active sites is observable only in the presence of pyrophosphatase (Fasiolo et al., 1977). Therefore, the situation we have simulated in the experiments with Phe-ol-AMP does not fully correspond to the situation that is found in steady-state experiments. The binding of the $tRNA^{Phe}_{CCN}$ species to PRS·Phe-ol-AMP reflects rather the binding to a transition-state analogue than to a real intermediate of the aminoacylation reaction.

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Physical Properties of Collagen–Sodium Dodecyl Sulfate Complexes†

J. William Freytag,§ Milton E. Noelken, and Billy G. Hudson*

ABSTRACT: Sodium dodecyl sulfate (NaDodSO$_4$)–polyacrylamide gel electrophoresis and gel filtration chromatography of protein–NaDodSO$_4$ complexes are frequently used to characterize collagen-like polypeptide components in mixtures obtained from extracts of basement membranes. However, electrophoresis yields anomalously high apparent molecular weights for collagenous polypeptides when typical globular proteins are used as molecular weight standards, and the use of gel filtration chromatography for this purpose was suspect because Nozaki et al. [Nozaki, Y., Schechter, N. M., Reynolds, J. A., & Tanford, C. (1976) *Biochemistry 15*, 3884–3890] found that asymmetric particles, including NaDodSO$_4$–protein complexes, coeluted with native globular proteins of lower Stokes radius, when Sepharose 4B was used. To understand these effects and to improve the characterization of collagenous polypeptides, we investigated the secondary structure of NaDodSO$_4$–collagen complexes with the use of circular dichroism, measured the NaDodSO$_4$ content, studied the dependence of electrophoretic mobility on gel concentration, and extended work on gel filtration by use of a more porous gel, Sepharose CL-4B. We found that the anomalous behavior of collagen chains on NaDodSO$_4$–polyacrylamide gel electrophoresis is due in large part to treatment of data and that the method can be used to determine rather accurate values for the number of residues per polypeptide chain. Our gel filtration results indicated that reliable molecular weights can be obtained when Sepharose CL-4B is used. These methods can be applied equally well to collagenous and noncollagenous polypeptides.

Polyacrylamide gel electrophoresis in the presence of the anionic detergent NaDodSO$_4$[1] has proved to be an excellent method for estimating the molecular weights of proteins. It is especially useful for analysis of mixtures, such as those obtained by extraction of membranes, before the components can be separated and characterized by methods which have a stronger theoretical foundation, such as equilibrium sedimentation. However, collagen and related collagenous proteins chains, which are widely distributed in nature and are important components of many tissues, were found to behave anomalously on NaDodSO$_4$–polyacrylamide gel electrophoresis. Furthmayr & Timpl (1971), for example, found that the α_1-collagen chain, molecular weight 96 000, migrated at a slightly lower rate than the human serum albumin dimer, molecular weight 132 000, thus behaving as if it had a molecular weight 40% higher than the true value. A similar effect was noted for the α_2-collagen chain, and, in addition, the two collagen chains could be easily resolved from each other, although they presumably have almost identical molecular weights. Aggregation was an unlikely cause for these effects since a good linear relationship, in the molecular range 5000–96 000, was found between log molecular weight and electrophoretic mobility of α_1-collagen and four fragments obtained from it by cyanogen bromide cleavage. This would occur only if all the five species were either unaggregated or, rather unlikely, partially aggregated to a degree that varied in a unique, monotonic manner with molecular weight. Furthermore, a closely lying parallel line was obtained for the α_2-collagen chain and several of its cyanogen bromide fragments. Furthmayr & Timpl (1971) suggested that there was a fundamental conformation difference between NaDodSO$_4$ complexes of typical proteins and those of collagenous proteins, which have unusual amino acid compositions and sequences, and that this was responsible for the atypical behavior of the latter on NaDodSO$_4$–polyacrylamide gel electrophoresis. The subtlety of the conformation effect was indicated by the difference between the log molecular weight–mobility relationship of the α_1-collagen chain and its cyanogen bromide fragments and that of the α_2-collagen chain and its fragments. While this seemed to be a reasonable explanation, it was incomplete in the sense that other factors such as binding of NaDodSO$_4$ were not considered, and there were no independent conformation studies.

A second method commonly used to estimate molecular weights is gel filtration chromatography in the presence of NaDodSO$_4$. This has been used several times to characterize collagenous components of basement membranes [e.g., Grant et al. (1973) and Clark & Kefalides (1979)]. However, Nozaki et al. (1976) have shown that a fundamental assumption underlying this method, i.e., elution volume is determined solely by the Stokes radius, is invalid. NaDodSO$_4$–protein complexes and other asymmetric particles were shown to coelute from Sepharose 4B columns with native

† From the Department of Biochemistry, University of Kansas Medical Center, Kansas City, Kansas 66103, and the Department of Biochemistry, Oklahoma State University, Stillwater, Oklahoma 74074. *Received March 9, 1979.* This work was supported by National Institutes of Health Grant AM 18381.

§ Taken in part from the Ph.D. thesis of J.W.F., Department of Biochemistry, University of Kansas Medical Center, Kansas City, KS, 1978. Present address: Department of Biochemistry, Duke University Medical Center, Durham, NC 27710.

[1] Abbreviations used: NaDodSO$_4$, sodium dodecyl sulfate; BSA, bovine serum albumin; EDTA, ethylenediaminetetraacetic acid (tetrasodium salt); CD, circular dichroism; *N*, number of residues in a polypeptide chain.

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