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# Phenylalanyl-tRNA Synthetase Induced Conformational Change of *Escherichia coli* tRNA$^{Phe}$†

A. Favre,* J. P. Ballini, and E. Holler

ABSTRACT: Binding of *Escherichia coli* tRNA$^{Phe}$ to phenylalanyl tRNA synthetase induces a broadening of the main positive dichroic band ($\lambda_{max}$ 265 nm) of the tRNA, resulting in a 5-nm red shift in the range of 260–280 nm. A second effect is to reduce the rate of photo-cross-linking between residues in positions 8 and 13 by a factor of 2.3. These effects are only detected with the cognate *active* synthetase but not with other proteins including the methionyl-tRNA synthetase. Formation of the tRNA enzyme complex is required since both effects are lowered by the addition of monovalent cations which are known to decrease the association constant of tRNAs for their synthetases or on addition of a competitor tRNA such as the tRNA$^{Phe}$ from yeast. Detailed analysis of the stoichiometry of "protection" against cross-linking revealed that only the strong and not the weak tRNA binding site on the enzyme participates in the tRNA–enzyme rearrangement. The cooperative binding of two magnesium ions to the tRNA–enzyme complex is required for the rearrangement to occur. In a very similar way, the catalytic phenylalanylation depends upon $Mg^{2+}$. It is likely that the rearrangement is involved in the mechanism of acylation of tRNA$^{Phe}$. The molecular nature of the rearrangement, as far as tRNA$^{Phe}$ is concerned, was deduced from the photochemical and spectroscopic properties of the tRNA, including the 4-thiouridine luminescence. We propose that the helical structure of tRNA$^{Phe}$ is slightly altered in the course of the rearrangement of the enzyme–tRNA complex. The protection effect against the light-induced cross-linking observed when the enzyme–tRNA$^{Phe}$ complex is formed is shown to be due to a decreased probability for the 8 and 13 residues to assume a highly reactive conformational state necessary for the 8–13 link formation.

Fast kinetics studies of different tRNA, aminoacyl-tRNA synthetase systems, such as the serine system from yeast, the tyrosine system from *E. coli* (Riesner et al., 1976), and the phenylalanine system from yeast (Krauss et al., 1976; Rigler et al., 1976), have shown that tRNA binding to its cognate ligase proceeds in two steps. The first one, the recombination step, is in first approximation diffusion controlled. The second step involves a rearrangement of the synthetase–tRNA complex and is assumed to be responsible for the recognition

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of the tRNA.

So far there is, however, little direct evidence for a conformational change of both the bound tRNA and the bound synthetase. Results of circular dichroism studies of the *E. coli* glutamate specific system imply that both the tRNA and the synthetase undergo conformational changes as the complex is formed (Willick & Kay, 1976). An earlier circular dichorism study of the *E. coli* tyrosine system (Otha et al., 1967) also suggested a change in the structure of the enzyme. Furthermore, the variation of the Y base fluorescence upon binding of yeast $tRNA^{Phe}$ to its cognate ligase could be viewed as an indication of a structural change in the anticodon region of the tRNA (Krauss et al., 1976). However, proton magnetic resonance studies of the *E. coli* glutamate specific system (Shulman et al., 1974), as well as the hydrogen exchange studies of the *E. coli* isoleucine specific system (Yarus, 1972), failed to detect any conformational change on the side of the tRNA.

Here, we have examined the phenylalanine-specific system from *E. coli* by two complementary approaches. Circular dichroism was applied to investigate the structural changes of both the nucleic acid and the protein part of the complex. The other approach provides evidence about the region of the $tRNA^{Phe}$ tertiary structure that is involved in the rearrangement. This method is based on the photo-cross-linking technique developed by Favre et al. (1969–1975). Both approaches led to the conclusion that the formation of the specific enzyme–tRNA complex involves a structural change of the $tRNA^{Phe}$.

## Materials and Methods

*Material.* Phenylalanyl-tRNA synthetase (EC 6.1.1.20) was prepared from *E. coli* K. 10 as described by Hanke et al. (1975). In the final stage of the preparation, the enzyme at a concentration between 30 and 37 $\mu$M was kept in 50% glycerol buffer (0.05 M Tris-HCl, pH 7.5, containing 0.1 mM EDTA[1] and 0.2 mM DTT). The specific activity was 50 000 nmol $mg^{-1}$ $h^{-1}$.

$tRNA^{Phe}$ from *E. coli* was purchased from Boehringer. Its content in 8–13 link was lower than 5% and its amino acid acceptance was 1000–1200 pmol/$A_{260}$ units (measured at pH 7 in $H_2O$). The concentration of $tRNA^{Phe}$ was measured by absorption at 260 nm by using an extinction coefficient of 570 000 L $mol^{-1}$ $cm^{-1}$ in a 10 mM $Mg^{2+}$, 50 mM Tris-HCl buffer at pH 7.

Uniformly labeled L-[$^{14}$C]phenylalanine (400–500 $\mu$Ci/$\mu$mol) was purchased from Radiochemical Center (Amersham), and ATP was from Boehringer (Mannheim). All other reagents were of the highest purity grade from Merck (Darmstadt).

*Methods.* Phenylalanyl-tRNA synthetase activity was determined as described by Kosakowski & Böck (1970). Glass fiber filters (Whatman, GF/C) were counted in a Packard Isocap 300 with 80% counting efficiency.

Intramolecular cross-linking of tRNA between 4-thiouridine in position 8 and cytidine in position 13 was achieved by the method of Favre et al. (1971). The tRNA solutions to be compared (400–800 $\mu$L) in 4 × 10 mm quartz cuvettes were placed on a cell holder thermostated at 17 °C. Four cuvettes were simultaneously irradiated, the short side of the cuvette facing the incident light. The light source was a HB0 200-W superpressure mercury lamp (Osram) mounted in a Cunow lantern. The far and medium ultraviolet light from the lamp, which could have a deleterious effect on the major pyrimidines of the tRNA, or on the added proteins, was cut off with a MTO J 324a filter. Under these conditions, the efficient spectral lines were at 336 and 366 nm. The entrance faces of the cuvettes were placed at a distance of 15 cm from the light source so that the incident fluence is quasiuniform.

In the course of a cross-linking experiment, the concentration of the 8–13 link was followed by the sodium borohydride reduction assay that converts the 8–13 link into a highly fluorescent product (Favre & Yaniv, 1971). In order to increase the sensitivity and accuracy of the method, the sample in the final stage of the reduction is brought to pH 4.5 with sodium acetate. As a result, the remaining $NaBH_4$ is destroyed, preventing the formation of bubbles and increasing the quantum yield of the emission by a factor of 2.

The studies were performed in a 0.05 M Tris-HCl buffer, pH 7.5 (see Results), and, since high Tris concentration (1 M) decreases the final yield of 8–13 link, possible artefacts were carefully examined and excluded. Also the mechanism of the Tris inhibition effect was delineated. Indeed, in the presence of 1 M Tris-HCl buffer, pH 7.5 at 25 °C, the final yield of 8–13 link formation in total *E. coli* tRNA is 50% of the control (cacodylate buffer). $Mg^{2+}$ antagonizes to some extent the Tris effect and, in the presence of 10 mM $Mg^{2+}$, the final yield is 80%. A ionic strength effect is excluded, the final yield being 100% in 1 M sodium phosphate or sodium cacodylate as well as in the presence of 1 M ammonium chloride or 0.5 M ammonium sulfate. Correlated with the quenching effect observed in Tris buffer is a blue shift of the 4-thiouridine absorption $\lambda_{max}$ which shifts from 337 ± 1 nm for the control to 335 nm in 1 M Tris plus 10 mM $Mg^{2+}$ and to 331 nm in 1 M Tris in absence of $Mg^{2+}$. These results strongly suggest that it is chelation and the subsequent tautomerization of 4-thiouridine by Tris (or contaminant molecules) which triggers a new photoreaction of this residue.

Dichroic spectra were performed with a Jouan Dichrograph II or a Jouan Dichrograph III.

Fluorescence measurements were done with either a Hitachi Perkin-Elmer 2 MPF or a Jouan "Bearn" spectrofluorometer. All measurements were performed at a controlled temperature of 17 ± 0.1 °C.

Absorption spectra were run at room temperature with a Cary 15 spectrophotometer.

The luminescence emission spectra of 4-thiouridine were obtained at 10 °C with a spectrofluorimeter built for the detection of very weak fluorescence (Vigny & Duquesnes, 1974). A right angle arrangement is used. The method employs photon counting which gives an improvement of the signal-to-noise ratio by increasing counting time.

The lifetime of 4-thiouridine emission was measured with a single-photon counting apparatus. Excitation was provided by a nanosecond Ortec lamp and the 337-nm $N_2$ line isolated with an interferential MTO filter ($\lambda$ 337.1 nm, $\Delta\lambda$ = 3 nm). Emission at wavelengths shorter than 490 nm was cut off with an MTO V J 49 filter.

## Photochemical Kinetics

In a pure tRNA species the photoreactions of 4-thiouridine are in general described by eq 1.

$$S^4U \begin{cases} \nearrow \text{8–13 link} \\ \searrow \text{other photoproducts} \end{cases} \quad (1)$$

If the entrance face of the cuvette is uniformly irradiated with a monochromatic beam of light and if solutes concen-

[1] Abbreviations used: CD, circular dichroism; EDTA, ethylenediaminetetraacetate; DTT, dithiothreitol; UV, ultraviolet.

trations are maintained uniform throughout the solution volume, then the rate constant $k_t$ for $S^4U$ photolysis can be simply derived from eq 9 of Ballini et al. (1976)

$$k_t \propto F\frac{1 - 10^{-D}}{D}\epsilon(\Phi + \Phi') \quad (2)$$

where $F$ is the light flux (einstein $s^{-1}$), $D$ is the total optical density at the irradiation wavelength, and $\epsilon$ is the extinction coefficient (L $mol^{-1}$ $cm^{-1}$). $\Phi$ and $\Phi'$ are respectively the quantum yields of 8–13 link formation and of the contaminant reactions. When $D$ is low, eq 2 can be further simplified and the rate constant $k$ (or $k'$) for the individual processes becomes proportional to $F\epsilon\Phi$ or $F\epsilon\Phi'$. Since $\Phi$ is independent of the irradiation wavelength $\lambda$ in the range 310–380 nm (Ballini et al., 1976), the expression of $k$ for a beam of polychromatic light is

$$k \propto \Phi\int_{\lambda_1}^{\lambda_2} F(\lambda)\epsilon(\lambda)d\lambda \quad (3)$$

In our experimental conditions, $k$ (and $k'$) is independent of time (absorbance never exceeds 0.06 unit in the range 320–380 nm). The concentration $c$ of the cross-linked tRNA at time $t$ is related to $c_0$, the initial concentration of 4-thiouridine by

$$c = c_0\frac{k}{k + k'}(1 - e^{-(k+k')t}) \quad (4)$$

## Results

*Kinetics of tRNA$^{Phe}$ Cross-Linking.* Irradiation with near-ultraviolet light (310–380 mm) of a number of *E. coli* tRNAs specifically triggers photoreaction(s) of 4-thiouridine. In all tRNA species examined so far, containing 4-thiouridine in position 8 and cytidine in position 13, this results in the formation of a covalent bridge between the two residues (Favre et al., 1969, 1975). This photoreaction is not only relevant to the native structure of tRNAs in solution but also to the crystalline structure of yeast tRNA$^{Phe}$. Indeed in the crystal, uridine-8 and cytidine-13 are found stacked on each other with orientations compatible with the formation of the covalent bridge (Kim et al., 1973).

The kinetics of cross-link formation can be easily followed by the fluorometric assay (Favre et al., 1971), allowing the determination of the rate constant $k$ and of the final yield. tRNA$^{Phe}$ is known to be quantitatively cross-linkable with first-order kinetics in a cacodylate buffer (Favre et al., 1975), and these findings were first extended to the 0.05 M Tris-HCl buffer, pH 7.5, containing 0.1 mM EDTA, 0.2 mM DTT, and 0–10 mM $MgCl_2$ used to study the interaction of tRNA$^{Phe}$ and its cognate ligase (Bartmann et al., 1975). However, at high concentrations (1 M), commercial Tris decreases the final yield (see Methods). Also the rate constant $k$ is not affected by the EDTA and DTT ingredients in the buffer.

Upon mixing tRNA$^{Phe}$ with an equimolar amount of L-phenylalanyl-tRNA synthetase, the kinetics of cross-linking are strikingly affected (Figure 1), the ratio of the rate constants for free, $k_f$, and bound, $k_b$, tRNA$^{Phe}$ being 2.3 ± 0.1. The final plateau is independent of the presence of the synthetase, which excludes the possibility of competing side reactions. Since excitation is to the red of tryptophan absorbance, the protein should not have been photodenatured, and, indeed, the enzyme activity remains stable during the course of an experiment (Figure 1). Protection of tRNA$^{Phe}$ against cross-linking has been reproducibly obtained with different concentrations and preparations of the tRNA and the enzyme (Table I).

According to eq 4 (Materials and Methods), the decreased value of the rate constant observed when the enzyme is present

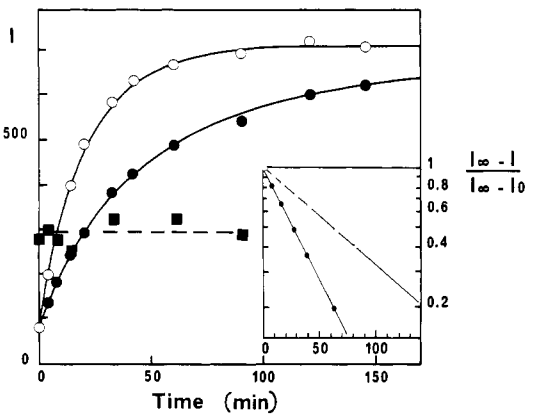


FIGURE 1: Kinetics of tRNA$^{Phe}$ (3.96 $\mu$M) cross-linking in the absence (—○—) and presence (—●—) of phenylalanyl-tRNA synthetase (3.53 $\mu$M). The two samples in a 0.05 M Tris-HCl buffer containing 0.1 mM EDTA, 0.2 mM DTT, and 10 mM $MgCl_2$ were simultaneously irradiated at 14 °C, and the amount of cross-link was determined by fluorometry as described under Methods. $I$ represents the fluorescence intensity (expressed in arbitrary units), $I_0$ and $I_\infty$ being respectively the values of $I$ at time zero and at infinite time, i.e., when the reaction has proceeded to completion. Notice the presence of 10% cross-linked tRNA$^{Phe}$ prior to irradiation. The dotted line (■- - -■) indicates that the enzyme activity determined by initial velocity measurements according to Kosakowski & Böck (1970) remains unaffected. The insert is a logarithmic representation indicating first order according to eq 4.

Table I: Rate and Extent of tRNA$^{Phe}$ Cross-Linking in the Presence and Absence of Phenylalanyl-tRNA Synthetase[a]

| | expt no. | | | | | |
|---|---|---|---|---|---|---|
| | I | II | III | IV | $V_a$ | $V_b$ |
| $T_t$ | 0.80 | 1.0 | 3.96 | 4.95 | | I.01 |
| $E_t$ | 0.90 | 1.1 | 3.53 | 5.00 | | 1.21 |
| extent (%) | 99 | 100 | 94 | 100 | 97 | 99 |
| $k_f/k_b$ | 2.1 | 2.2 | 2.7 | 2.6 | 2.25 | 2.30 |

[a] Experiments I-V were performed in standard buffer, the $MgCl_2$ concentration being between 9 and 11 mM. The a and b subscripts of experiment V refer to assays performed in the absence (a) and presence (b) of 0.5 mM ATP plus 0.5 mM phenylalanine. The tRNA$^{Phe}$ and enzyme total concentration $T_t$ and $E_t$ are expressed in $\mu$M units. The extent of cross-linking refers to the plateau value in the presence of enzyme relative to the plateau reached with the free tRNA.

may be due either to a decreased value of $\Phi$ and (or) $\epsilon$. As shown in Figure 7 (insert), the absorption spectrum of tRNA$^{Phe}$ in the presence or absence of enzyme shows little if any variation in the range 330–380 nm, thus indicating a decreased value of $\Phi$.

*Phenylalanyl-tRNA Synthetase Induced Change of the tRNA$^{Phe}$ Dichroic Spectra.* The dichroic spectra of tRNA$^{Phe}$ in the range of the wavelengths 225–320 nm is shown in Figure 2. The overall shape and positions of the positive and negative peaks as well as their magnitudes are in agreement with the results reported by others (Willick & Kay, 1971; Blum et al., 1972). The spectrum of an equimolar amount of phenylalanyl-tRNA synthetase shows a weak contribution in the range from 260 to 320 nm and a large negative trough in the region of the amide bond absorbance (Figure 2a). Formation of the synthetase–tRNA$^{Phe}$ complex causes an increased ellipticity of the main positive band of the tRNA, centered at 263 nm. This increase is accompanied by a broadening of the band and by a 5-nm red shift of the maximum. The spectrum of the complex is clearly distinct from the sum of the spectra obtained for the free synthetase and free tRNA (Figure 2b). Since the contribution by tRNA$^{Phe}$ is by far dominant in the

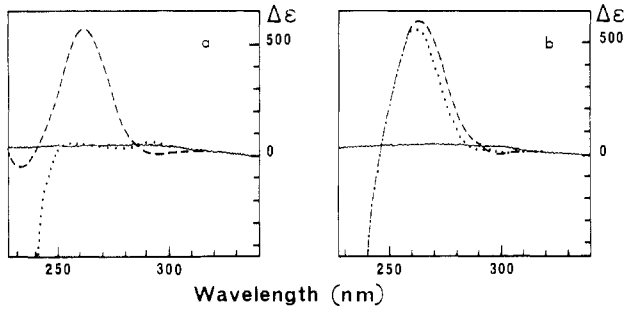


FIGURE 2: Dichroic spectra of free and bound $tRNA^{Phe}$. All experiments were performed at 22 °C in standard Tris buffer (pH 7.5). Figure 2a shows the dichroic spectra of free $tRNA^{Phe}$ (1.03 μM) (---) and of the free phenylalanyl-tRNA ligase (1.10 μM) (····). Figure 2b shows that the spectra of the complex between $tRNA^{Phe}$ (1.02 μM) and the ligase (1.09 μM) (---) are distinct from the sum of the spectra of both components (····).

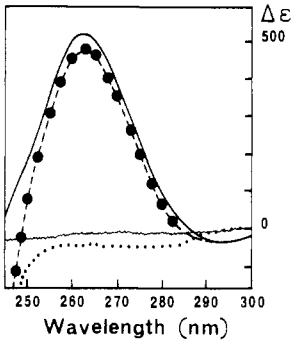


FIGURE 3: Dichroic spectra of $tRNA^{Phe}$ in the presence of a noncognate ligase. The spectra of $tRNA^{Phe}$, 0.8 μM (—), of the *E. coli* methionyl-tRNA synthetase, 0.92 μM (····), and of the mixture (---) were recorded in the conditions of Figure 2. The points (●) represent the spectra of the mixture calculated as the sum of the contributions of the two components.

range 260–290 nm, it can be inferred that it is the $tRNA^{Phe}$ dichroic spectrum that is different in the presence or absence of the cognate ligase. On the contrary, no change was detected for the contribution of the synthetase that dominates in the region between 225 and 250 nm.

*Demonstration of Specificity.* The effects described above are attributed to the specific interaction of $tRNA^{Phe}$ and its cognate ligase as judged from the following results.

(i) Other proteins either unrelated to the phenylalanyl-tRNA synthetase such as bovine serum albumin (data not shown) or functionally related to it such as the *E. coli* methionyl-tRNA synthetase are clearly unable to alter either the dichroic spectra (Figure 3) or the rate of cross-linking (Figure 4).

(ii) None of the effects noted earlier can be detected with the heat-denatured phenylalanyl-tRNA synthetase (Figure 4), indicating the requirement of the functional structure of the enzyme.

(iii) Both effects are abolished when the binding affinity for the formation of the specific complex between $tRNA^{Phe}$ and its cognate ligase is decreased, for example, by the addition of monovalent cations (Krauss et al., 1975, 1976; Blanquet et al., 1973; Pingoud et al., 1973). Increasing the $Na^+$ concentration up to 0.2 M does not affect the cross-linking rate of free $tRNA^{Phe}$ (Figure 5a), although it abolishes the protection effect by the ligase. The behavior of the $K^+$ cations (Figure 5b) is more complex since it significantly decreases the rate of cross-linking of free $tRNA^{Phe}$. However, when the latter effect is taken into account, both $K^+$ and $Na^+$ act similarly. The simplest interpretation of the data is that, as

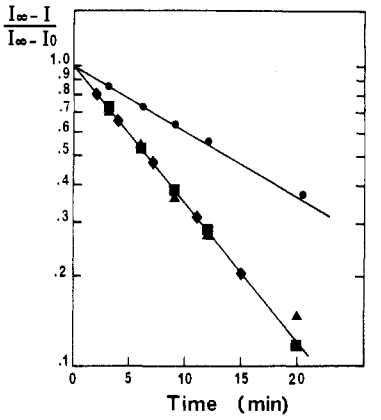


FIGURE 4: Kinetics of $tRNA^{Phe}$ cross-linking in the presence of different proteins. Four cuvettes containing $tRNA^{Phe}$, 0.8 μM, alone (■), or in the presence of heat-denatured phenylalanyl-tRNA synthetases, 0.9 μM (▲) (heated for 5 min at 100 °C), methionyl-tRNA synthetase, 0.9 μM (■), or native phenylalanyl-tRNA synthetase, 0.9 μM (●), were simultaneously irradiated as described in Figure 1. Again $I$ represents the fluorescence intensity of reduced aliquots (see Methods), $I_0$ and $I_\infty$ referring to time zero and to the plateau level of photoreaction.

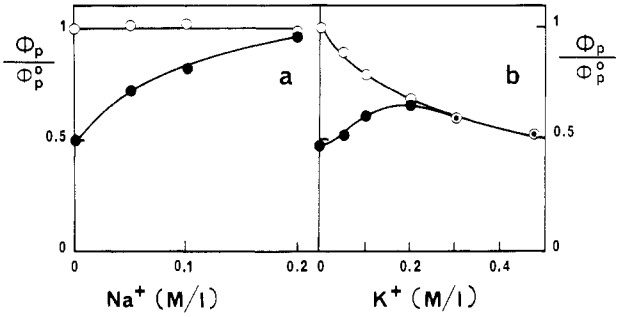


FIGURE 5: Effect of the $Na^+$ and $K^+$ cations on the kinetics of $tRNA^{Phe}$ (0.89 μM) cross-linking in the absence (○) or presence (●) of phenylalanyl-tRNA synthetase (0.94 μM). The experiments were conducted as described under Methods and under the conditions of Figure 1. In every case, the kinetics of cross-linking were of first order. The photochemical rate constants and, according to eq 3, the relative values of the photochemical quantum yields, $\Phi_P/\Phi_P^0$, were calculated from the slopes of the straight lines obtained as shown in Figure 1 (insert) or in Figure 4. In this experiment, $\Phi_P$ and $\Phi_P^0$ are the quantum yields measured in the absence and in the presence of added salt, respectively.

Table II: Effect of Yeast $tRNA^{Phe}$ on the Protection of *E. coli* $tRNA^{Phe}$ by Its Cognate Ligase[a]

| $tRNA^{Phe}$ yeast concn (μM) | 0 | 5.2 | 26 | 104 |
|---|---|---|---|---|
| rel rate constant | 0.54 | 0.57 | 0.62 | 0.69 |

[a] $tRNA^{Phe}$ from *E. coli* at a final concentration of 0.84 μM was cross-linked under the conditions of Figure 1 in the presence of the cognate ligase (0.89 μM) and of various amounts of yeast $tRNA^{Phe}$. The rate constant was determined according to Figure 1 and is given relative to that of free $tRNA^{Phe}$.

expected, the dissociation constant $K_D$ increases with ionic strength and that, at a concentration of monovalent cation of 0.2 M, the complex is almost fully dissociated. This interpretation is supported by the circular dichroism data (not shown). The spectra of $tRNA^{Phe}$ look slightly affected by the addition of KCl at a final concentration of 0.2 M but become insensitive to the addition of an equimolar amount of the cognate ligase.

(iv) A competitor tRNA able to displace *E. coli* $tRNA^{Phe}$ from its binding site on the enzyme should increase the rate of cross-linking. Indeed, this is observed when yeast $tRNA^{Phe}$

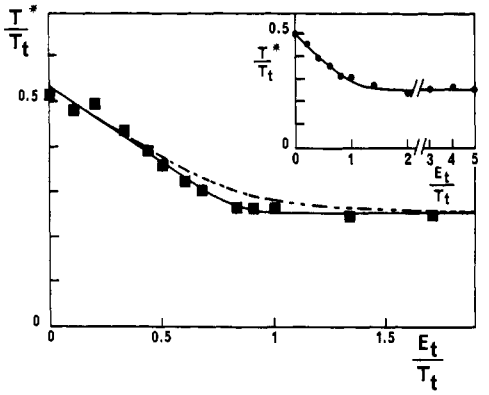

FIGURE 6: Stoichiometry of the protection effect. The amount of cross-linked tRNA$^{Phe}$, $T^*$, is determined after irradiation in conditions where half of the tRNA$^{Phe}$ is cross-linked in the absence of enzyme. The amount of cross-linked tRNA$^{Phe}$ is then determined as a function of the total enzyme concentration $E_t$, the total tRNA input being $T_t$ = 5 $\mu$M. The experimental data are represented by (■). The theoretical curve (---) was computed according to the single site model according to eq 8–10 of the Appendix, assuming a dissociation constant $K_D$ of 0.1 $\mu$M. The insert shows the experimental results obtained with $T_t$ = 1 $\mu$M.

is used as a competitor (Table II). Assuming that the two tRNAs do compete for the same site on the enzyme allows us to calculate the ratio of their respective binding constants. Thus, binding of *E. coli* tRNA$^{Phe}$ is found to be 150–200 times stronger than that of yeast tRNA$^{Phe}$. This is in agreement with the Michaelis–Menten constant of 8 $\mu$M for the yeast tRNA$^{Phe}$ as opposed to the constant (0.1 $\mu$M) of *E. coli* tRNA$^{Phe}$ for the phenylalanylation by the *E. coli* enzyme.

(v) Finally, the protection effect obeys a defined stoichiometry. Phenylalanyl-tRNA synthetase can bind a total of two tRNA$^{Phe}$ molecules. The association is anticooperative and proceeds with dissociation constants of 0.1 and 1 $\mu$M, respectively (Bartmann et al., 1975). The stoichiometry of protection was determined by measuring the fraction $T^*/T_t$ of tRNA$^{Phe}$ containing the 8–13 link as a function of the total enzyme concentration $E_t$, in conditions where free tRNA$^{Phe}$ is half cross-linked. Figure 6 shows the data obtained when $T_t$, the total tRNA concentration, is fixed at either 1 or 5 $\mu$M. It is clear that the "strong" binding site is implied in the protection effect. Attribution of an eventual role to the weak site is more difficult and requires quantitative evaluation of $T^*/T_t$. This is detailed in the Appendix and rules out a contribution of the weak site.

*Role of Mg$^{2+}$.* All observations above are strikingly dependent on the Mg$^{2+}$ concentration (in the range of 1–10 mM), although this does not affect the dissociation constant of the tRNA$^{Phe}$ strong binding site of its cognate ligase (Bartmann et al., 1975). The dichroic spectra of free or enzyme-bound tRNA$^{Phe}$ were, therefore, examined in the range of wavelengths 210–400 nm at low (1 mM) and high (10 mM) concentrations of the divalent cation. In addition to the CD signal shown in Figure 2a, the spectrum of free tRNA$^{Phe}$ presents a negative peak, $\lambda_{max}$ 290 nm, generally assigned to the nucleic bases $n\pi^*$ transitions in the geometry of RNA (Willick & Kay, 1971), followed by a weak positive peak ($\lambda_{max}$ 340 nm) which is assigned to 4-thiouridine. This peak has been observed in a number of *E. coli* tRNAs containing this residue (Saneyoshi et al., 1972; Willick & Kay, 1971). The apparent conflict between the CD spectra of tRNA$^{fMet}$ reported by the two groups is probably due to a high level of 8–13 link in the sample showing a negative band at 340 nm. A negative band is constantly observed in the fully cross-linked tRNAs (A. Favre, personal observation).

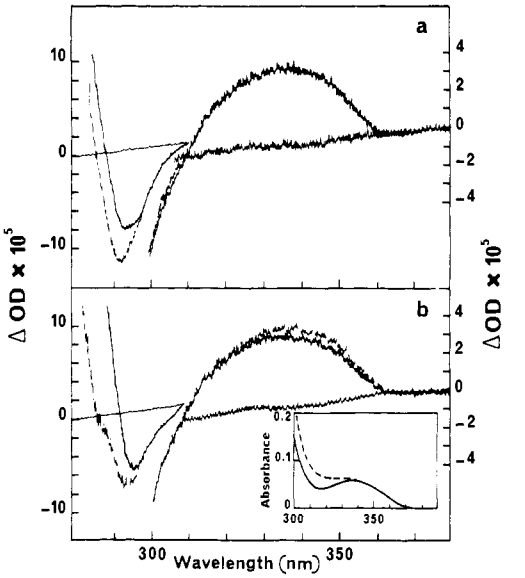

FIGURE 7: Dichroic spectra of tRNA$^{Phe}$ in the range 280–380 nm. The spectra were obtained in standard pH 7.5 buffer at 22 °C in the presence of either 0.6 mM (---) or 10 mM Mg$^{2+}$ (—). (a) tRNA$^{Phe}$ (4.68 $\mu$M) alone; (b) tRNA$^{Phe}$ and phenylalanyl-tRNA synthetase at concentrations of 4.1 $\mu$M and 4.65 $\mu$M at low Mg$^{2+}$ and 3.8 $\mu$M and 4.3 $\mu$M at high Mg$^{2+}$, respectively. The insert shows the absorption spectra of tRNA$^{Phe}$ (4.6 $\mu$M) alone (—) or in the presence of the cognate synthetase, 5 $\mu$M (---).

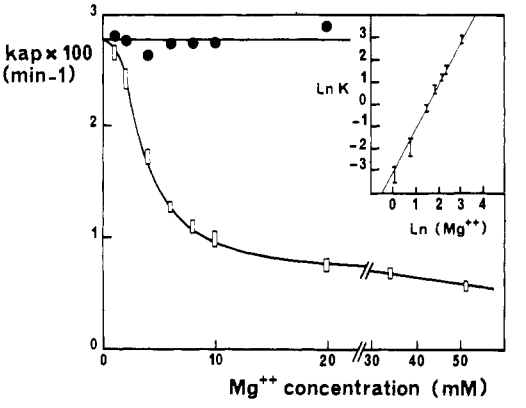

FIGURE 8: Mg$^{2+}$ dependence of the cross-linking rate. The rate constant $k_{ap}$ was determined according to Figure 1 and plotted as a function of the Mg$^{2+}$ concentration. Free tRNA$^{Phe}$ (●); enzyme-bound tRNA$^{Phe}$ (□). Total concentrations of enzyme and tRNA$^{Phe}$ were 1.05 $\mu$M and 1.01 $\mu$M, respectively. The insert shows a plot of $K$ as a function of the Mg$^{2+}$ concentration which, according to the logarithmic form of eq 6, yields values of $x_{ap}$ and $n$.

Increasing the Mg$^{2+}$ concentration hardly affects the CD spectra of free tRNA$^{Phe}$ in the range 210–280 mm (data not shown) nor in the near-ultraviolet region between 320 and 400 nm (Figure 7). The 290-nm peak shows a greater sensitivity as previously noticed by Willick & Kay (1971). Addition of a stoichiometric amount of phenylalanyl-tRNA synthetase at low Mg$^{2+}$ results in simple signal additivity in the whole range of wavelengths. Increasing the amount of Mg$^{2+}$ triggers the red-shift effect previously described in the range 260–285 nm and slightly affects the 340-nm positive band (Figure 7).

More quantitative data can be obtained from the effect of the divalent cation on the rate of cross-linking. Although the kinetics remain unaffected in free tRNA$^{Phe}$ when the Mg$^{2+}$ concentration is raised, the initial rate strikingly decreased in the presence of the cognate synthetase. The kinetics remain of the apparent first order, allowing the determination of $k_{ap}$ (Figure 8). Since the same transition point (approximately 3.5 mM) is obtained by both the photochemical kinetics and

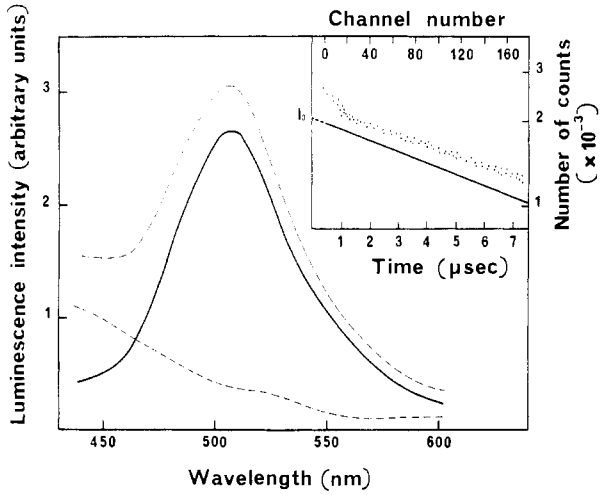


FIGURE 9: Luminescence spectra of 4-thiouridine in *E. coli* $tRNA^{Phe}$. The emission spectra of $tRNA^{Phe}$ (5 $\mu$M) for 335-nm excitation light was determined as described under Methods. The contribution of 4-thiouridine (—) was obtained by subtracting from the observed spectrum (---) the background emission observed after total quenching of the $tRNA^{Phe}$ luminescence with 2 M KBr (---). The insert shows a lifetime determination. The continuous line (—) is computed by subtracting the background emission from the experimental signal (···). The dotted line represents extrapolation of the 510-emission decay to time zero (the time of excitation).

the CD technique, it is likely that *enzyme-bound* $tRNA^{Phe}$ can exist under two forms. The relaxed form R is indistinguishable from free $tRNA^{Phe}$ and is converted into a modified form Q at high $Mg^{2+}$ according to

$$R + nMg^{2+} \rightleftharpoons Q \tag{5}$$

with

$$x_{ap} = \frac{Q}{R}\frac{1}{(Mg^{2+})^n} \tag{6}$$

In our experimental conditions, that is, total $tRNA^{Phe}$ concentration close to 1 $\mu$M and an excess of the enzyme and taking into account the magnitude of the dissociation constant, $K_D$ = 0.1 $\mu$M (Bartmann et al., 1975), almost all $tRNA^{Phe}$ molecules are bound to the enzyme. The rate $dT^*/dt$ of cross-link formation is therefore

$$\frac{dT^*}{dt} = k_f(R) + k_s(Q) \tag{7}$$

and by integration

$$T^* = T_t(1 - e^{-k_{ap}t}) \tag{8}$$

with

$$k_{ap} = \frac{k_f + k_s(Q/R)}{1 + (Q/R)} \tag{9}$$

The values of $k_{ap}$ and of the $Q/R$ ratio can be easily determined (eq 8 and 9) from the experimental data as a function of the $Mg^{2+}$ concentration. A double-log plot of the $Q/R$ ratio, derived from eq 6, then allows the determination of $n$ = 2 and of the apparent dissociation constant ($x_{ap}$) for $Mg^{2+}$ of 4.1 ± 0.6 mM (Figure 8).

*4-Thiouridine Luminescence.* An insight into the processes that led to the formation of the 8–13 link is provided by a study of the 4-thiouridine luminescence. On excitation by light of 335 nm, in aqueous solution at 300 K, 4-thiouridine emits a weak luminescence ($\lambda_{em}$ 540 nm, decay time $\tau$ = 200 ns for the free nucleoside, and $\lambda_{em}$ 515 nm, $\tau$ = 5 $\mu$s in native tRNA

Table III: Determination of the Quantum Yields, $\Phi_P$, $\Phi_L$, and the Lifetime of Emission, $\tau$, of 4-Thiouridine in Free and Bound $tRNA^{Phe}$[a]

| | $Mg^{2+}$ concn (mM) | $\Phi_P \times 10^3$ | $\Phi_L \times 10^3$ | $\tau$ ($\mu$s) |
|---|---|---|---|---|
| free $tRNA^{Phe}$ | 1 | 5.0 | 1.1 | 7.5 |
| | 10 | 5.0 | 1.0 | 6.7 |
| bound $tRNA^{Phe}$ | 1 | 5.0 | 1.2 | 8.3 |
| | | | $1.5_a$ | |
| | 10 | 2.2 | $1.3_b$ | 8.8 |
| | | | $1.5_c$ | |

[a] All data were obtained in the standard buffer at 12 °C with $tRNA^{Phe}$ and its cognate ligase at a concentration of 5 $\mu$M each. The subscripts a–c refer to the different ways of determination of $\Phi_L$ as described under Results. In method c, $\Phi_L$ is derived from the product $I_0\tau$, $I_0$ being the number of photons that are obtained by extrapolation to time zero of the monoexponential decay curve of Figure 9.

(Figure 9); Favre et al., 1969; Favre, 1974; Shalitin & Feitelson, 1973, 1976). The latter authors have proposed that the emitter state E* is a triplet state. Quenching experiments by halide anions have indicated that a long-lived excited species is a common intermediate in both the 8–13 link formation and the radiative deactivation, the two processes being quenched with the same efficiency (Favre, 1974). Furthermore, the emitter state is the one which is sensitive to quenching. This deduction results from the measured lifetime of 200 ns in aqueous solution (Shalitin & Feitelson, 1973) which approaches closely the value of 170 ns obtained from quenching experiments (Favre, 1974).

A simple scheme taking into account these findings is shown

$$\begin{array}{l} \text{photoproducts} \\ \overset{h\nu}{S^4U \longrightarrow} S_1^* \rightarrow\rightarrow E^* \overset{\alpha_P \nearrow \quad \alpha_D}{\alpha_L \searrow} \begin{array}{l} \longrightarrow S^4U \\ S^4U + h\nu' \end{array} \end{array} \tag{10}$$

with $\alpha_P$, $\alpha_D$, and $\alpha_L$ being respectively the rate constants for photoproduct formation, radiative deactivation (luminescence), and nonradiative deactivation. $\alpha_D$ includes the intrinsic unimolecular decay constants and the pseudo-unimolecular term(s) due to external quencher(s). If $\Phi^*$ is the quantum yield for interconversion between the first excited singlet state $S_1^*$ and the emitter state E*, then the expressions for the quantum yields are

$$\Phi_P = \Phi^*\alpha_P\tau \tag{11}$$

$$\Phi_L = \Phi^*\alpha_L\tau \tag{12}$$

the lifetime being

$$\tau = \frac{1}{\alpha_D + \alpha_L + \alpha_P} \tag{13}$$

We confronted certain difficulties in determining $\Phi_L$ when $tRNA^{Phe}$ is complexed to the cognate enzyme. This is due to a contaminant emission accompanying the enzyme preparation. Three approaches were used. In the first one, the emission spectrum was determined by subtracting the contribution of the enzyme measured independently. $\Phi_L$ was also determined directly from the emission spectrum of the tRNA–enzyme mixture, the 510-nm emission being abolished by cross-linking the tRNA (Favre et al., 1971). A third estimation was derived from the monoexponential decay curves obtained after a short excitation flash (insert of Figure 9).

As shown in Table III, both $\Phi_L$ and $\tau$ increase when the enzyme is added to the $tRNA^{Phe}$ irrespective of the $Mg^{2+}$

concentration. At 300 K, $\Phi^*$ is not accurately known but is certainly much larger than $\Phi_L$ or $\Phi_P$. Internal conversion processes are generally assumed to be slightly temperature dependent and, at 77 K, $\Phi_L$ approaches 15% (Favre, 1974; Shalitin & Feitelson, 1976). Therefore, in our conditions, $\alpha_D$ is much larger than $\alpha_L$ or $\alpha_P$ and the lifetime becomes $\tau \simeq \alpha_D^{-1}$ (eq 13). The lifetime data of Table II, therefore, indicate that $\alpha_D$ *decreases* upon the tRNA–synthetase binding. The simplest interpretation of this effect is that the enzyme protects state E* against solvent or oxygen quenching. These data also rule out the possibility of energy transfer from E* to an eventual acceptor on the enzyme or of an impurity accompanying the enzyme preparation. The important point, however, is the remarkable constancy of the ratio $\Phi_L/\tau$, $\Phi_L/\tau \simeq 150\ s^{-1}$, irrespective of the $Mg^{2+}$ concentration and of the presence or absence of the enzyme. Hence, the product $\Phi^*\alpha_L$ is constant (refer to eq 12 and 13). This finding suggests that both $\Phi^*$ and $\alpha_L$ remain practically unchanged upon binding of tRNA$^{Phe}$ to its synthetase. This interpretation agrees with the spectroscopic observations shown in Figure 7. The low value of $\Phi_P$ observed in the presence of synthetase at 10 mM $Mg^{2+}$ is, therefore, due to a decreased efficiency of conversion of state E* into 8–13 link.

## Discussion

Classical enzymes such as lysozyme, carboxypeptidase, or chymotrypsin are known to respond to the binding of their substrates by conformational rearrangements (Blackburn, 1976; Imoto et al., 1972). A rearrangement has been suggested for the association of L-isoleucine and isoleucyl-tRNA synthetase and for complexes of other aminoacyl-tRNA synthetases with their cognate tRNAs (Holler & Calvin, 1972; Riesner et al., 1976; Krauss et al., 1976). The present findings of a change in the circular dichroic spectrum and in the rate of the light-induced intramolecular cross-linking suggest a conformational rearrangement of the substrate tRNA$^{Phe}$ when associating with phenylalanyl-tRNA synthetase. Specific interaction between tRNA$^{Phe}$ and its cognate synthetase is required for the following reasons. (a) Denatured synthetase and a noncognate aminoacyl-tRNA synthetase such as methionyl-tRNA synthetase or any other protein have no effect. (b) The magnitude of the effects depends on the concentration of the enzyme and reflects the value of the dissociation constant of the enzyme–tRNA$^{Phe}$ complex. (c) The protection is diminished by competition with tRNA$^{Phe}$ (yeast). (d) Increasing ionic strength destabilizes the complex and in parallel diminishes the protection effect. (e) Magnesium ions in the mM range are required as do other catalytical properties of the synthetase (Bartmann et al., 1975).

The next question concerns the molecular nature of the rearrangement. The red shift of the tRNA$^{Phe}$ dichroic spectrum is reminiscent of the effect reported by Willick & Kay (1976) for the Glu-specific system. Red shifts of the 260–260-nm positive dichroic band of tRNAs are known to occur in conditions that alter the RNA conformation (Wolfe et al., 1968; Prinz et al., 1974). In contrast to what is observed in the model system, the 260–280-nm CD signal of the bound tRNA$^{Glu}$ or tRNA$^{Phe}$ increases slightly (Figure 2). By analogy it is suggested that the complex formation triggers a minor change in the helical structure of the tRNAs. A nuclear magnetic resonance study of the tRNA$^{Glu}$ system indicated that, despite this change, all hydrogen bonds present in the free tRNA$^{Glu}$ remained intact in the complex (Shulman et al., 1974).

Formation of the tRNA–enzyme complex leaves the absorption spectrum of the 4-thiouridine in position 8 unchanged and hardly affects its dichroic spectrum (Figure 7), thus excluding the possibility of a large permanent perturbation in the vicinity of that base. This is also confirmed from the 4-thiouridine luminescence data. In contrast to the large variation of the quantum yield $\Phi_L$ and the lifetime $\tau$ observed with free 4-thiouridine in various solvents (unpublished results), both $\Phi_L$ and $\tau$ increase only slightly upon complex formation (Table III). Therefore, the possibility of a large reorientation of residue 8 relative to its neighbors must be excluded. Brun et al. (1975) have suggested that the intercalation of aromatic amino acid side chains between bases may be involved in tRNA binding, a possibility that seems less likely in view of the results of Seeman et al. (1976). In any case, intercalation in the vicinity of the 4-thiouridine has to be excluded. On the other hand, the putative participation of a nucleophilic group of the enzyme facilitating the hydrogen exchange at C-5 of certain pyrimidines (Shoemaker & Schimmel, 1977) is a possibility compatible with our results.

The photochemical quantum yield (Table I) decreases strikingly upon complex formation. This effect was discussed under Results in the light of the luminescence data and shown to be due to a decreased efficiency of conversion of the excited 4-thiouridine (state E*) into the photoproduct. This step involves the formation of a thietane intermediate (Bergstrom & Leonard, 1972; Fourrey et al., 1974) which is an immediate precursor of the photoproduct. Formation of the thietane should critically depend upon the relative positions and orientations of residues 8 and 13. Correspondingly several explanations exist as to why $\Phi_P$ is diminished. (a) The enzyme would induce a conformational change around residues 8 and 13. (b) tRNA$^{Phe}$ would exist on a variety of conformers in fast equilibrium and the enzyme would bind more efficiently the less reactive of them. In other words, some kind of tRNA$^{Phe}$ transconformation would be prevented in the complex. (c) Another case should be considered where the occurrence of the "reactive" conformer of tRNA$^{Phe}$ is not an intrinsic property of the tRNA, but rather is due to an external supply of energy. This would occur if part of the excess electronic energy of excited 4-thiouridine contributes to increase the mobility of this residue, for example, by relaxing its H-bond system. In the tRNA–enzyme complex, the tRNA structure around residue 8 would be maintained rigidly, decreasing the efficiency of thietane formation. Taking note of 4-thiouridine spectroscopic data of Figure 7, the simple hypothesis a is made unlikely. On the other hand, it is not yet possible to determine which of the explanations (b or c) is valid.

The tRNA$^{Phe}$ conformational rearrangement in this complex is triggered by the cooperative binding of two $Mg^{2+}$ ions with $K_D$ = 4.1 mM. It is of interest that the tRNA$^{Phe}$ dichroic spectrum is sensitive to the binding of three $Mg^{2+}$ with $K_D$ = 3.2 mM in the presence of 0.2 M $K^+$ (Willick & Kay, 1971). As far as the synthetase is concerned, a structural and catalytical role has been assigned to this cation (Hanke et al., 1975; Bartmann et al., 1975). Despite these effects, the dissociation constant of the tRNA$^{Phe}$–enzyme complex is independent of $Mg^{2+}$ (Bartmann et al., 1975). This could be explained as a compensation between the free-energy contributions due to the $Mg^{2+}$-dependent rearrangement of the complex and to the macromolecular association.

The subtle conformational distortion of bound tRNA$^{Phe}$ described above appears to be involved in the rate-limiting step of the acylation reaction. This is indicated by the dependence of the CD and photochemical measurements upon $Mg^{2+}$ (Figure 8) which closely parallels the activation of the en-

zymatic phenylalanylation of $tRNA^{Phe}$ by this cation (Bartmann et al., 1975; Holler et al., 1976). The analysis above demonstrates that the rearranged form of $tRNA^{Phe}$ is metastable; that is, $tRNA^{Phe}$ recovers its original conformation when released from the enzyme. Is the metastable conformation locked into a stable form as a result of the amino acylation? Relevant to this question is the report of Potts et al. (1977) of a conformational change of $tRNA^{Phe}$ from yeast which does occur at high $Mg^{2+}$ after amino acylation. Preliminary experiments with the *E. coli* system indicate, however, a close similarity between the free uncharged or acylated, $tRNA^{Phe}$ molecules as judged by the cross-link assay.

Phenylalanyl-tRNA synthetase can accommodate a total of two $tRNA^{Phe}$ molecules. The association follows negative cooperativity (Bartmann et al., 1975). The binding of the first $tRNA^{Phe}$ molecule triggers the delicate molecular rearrangement of the tRNA. If this rearrangement is a prerequisite for catalysis, then half of the sites reactivity is predicted.

Acknowledgments

We are indebted to Drs. J. P. Waller and S. Blanquet for a kind gift of pure methionyl-tRNA synthetase from *E. coli*.

## Appendix: Relationship between $T^*/T_t$, the Fraction of $tRNA^{Phe}$ Cross-Linked at Time $t$ of the Photoreaction, and the Enzyme Concentration $E_t$

A quantitative interpretation of the stoichiometry data (Figure 6) requires knowledge of the dependence of $T^*/T_t$ ($T_t$ is the total $tRNA^{Phe}$ concentration) upon $E_t$. Since the binding affinity of the cross-linked $tRNA^{Phe}$ molecules for the cognate synthetase is lowered by a factor of at least 100 (E. Holler and A. Favre, manuscript in preparation), the total concentration in the tRNA–enzyme complex continuously decreases during irradiation.

(*a*) *A Single Binding Site on the Enzyme.* Neglecting the concentration of complex formed between enzyme and cross-linked tRNA, T*E, the basic set of equations governing both the equilibrium between free and bound $tRNA^{Phe}$ and the photochemical behavior is

$$TE = K(T)(E) \tag{A1}$$

$$T_t = T + TE + T^* \tag{A2}$$

$$E_t = E + TE \tag{A3}$$

$$dT^*/dt = k_f(T) + k_b(TE) \tag{A4}$$

At any time of the photoreaction, $T^*$ is related to the concentration of free tRNA $T$ by

$$T^* = T_t - T\left[1 + \frac{KE_t}{1 + KT}\right] \tag{A5}$$

which after differentiation yields

$$-\frac{dT^*}{dt} = \frac{dT}{dt}\left[1 + \frac{KE_t}{(1 + KT)^2}\right] \tag{A6}$$

Combining eq A6 and A4 and rearranging

$$dT\frac{(1 + KT)^2 + KE_t}{K(T)(1 + KT)(\alpha + T)} = -k_f t \tag{A7}$$

with

$$K\alpha - 1 = \frac{k_b}{k_f}KE_t \tag{A8}$$

Integration of eq A7 yields the desired relationship

$$a \ln \frac{T}{T_0} + b \ln \frac{\alpha + T}{\alpha + T_0} - c \ln \frac{K^{-1} + T}{K^{-1} + T_0} = -k_f t \tag{A9}$$

with

$$c = \frac{k_f}{k_b} \qquad a = \frac{1 + KE_t}{c + KE_t}c \qquad b = \frac{c^2 + KE_t}{c + KE_t}$$

$T_0$ is the concentration of free $tRNA^{Phe}$ at time zero of the photoreaction. $T_0$ is a root; $T_0 \leq T_t$ of eq A10 obtained by combining eq A1–A3:

$$KT_0^2 - T_0[K(E_t - T_t) + 1] - T_t = 0 \tag{A10}$$

Given numerical values of $K$, $T_t$, $k_b$, $k_f$, and $t$, one can successively solve eq A10, A9, and A5 for a given value of $E_t$. It is of interest to compare the dependence of $T^*$ upon $E_t$ with the dependence of $V_0$, the initial rate of the photoreaction, upon $E_t$

$$\frac{V_0}{(V_0)_{max}} = \frac{k_b T_t + (k_f - k_b)T_0}{k_f T_t} \tag{A11}$$

where $(V_0)_{max}$ is the value of $V_0$ in the absence of enzyme.

(*b*) *Case of Two Binding Sites on the Enzyme.* Phenylalanyl-tRNA synthetase can accommodate a total of two $tRNA^{Phe}$ molecules and the association follows negative cooperativity (Bartmann et al., 1975). This can be accounted for by a simple sequential model (insert of Figure 10), where only the free enzyme $E_0^0$ is assumed to be in a symmetric form. Structural asymmetry of the enzyme is induced by binding the first $tRNA^{Phe}$ molecule (association constant $K_1$) to yield the $E_0^1$ form. $E_0^1$ then weakly binds a second tRNA molecule (association constant $K_2$) to yield $E_1^1$. The concentration $T_0$ of free $tRNA^{Phe}$ at time 0 of the photoreaction is related to $T_t$, $E_t$, $K_1$, and $K_2$ by eq A12; $0 \leq T_0 \leq T_t$:

$$2K_1K_2T_0^3 + 2K_1T_0^2[1 + K_2(2E_t - T_t)] + T[1 + 2K_1(E_t - T_t)] - T_t = 0 \tag{A12}$$

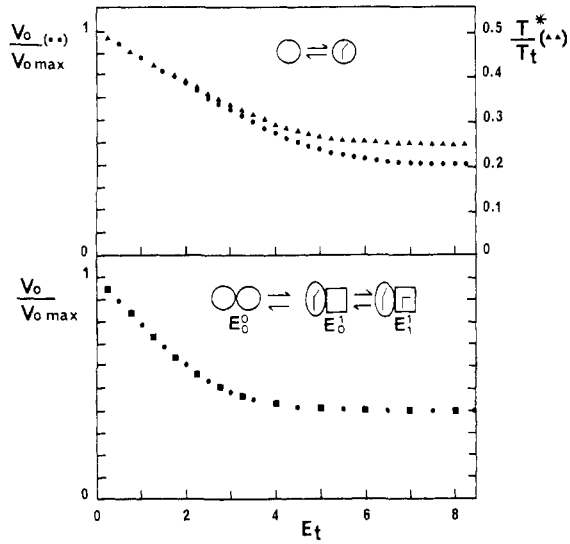


FIGURE 10: Dependence of $T^*/T_t$ and $v_0/(v_0)_{max}$ upon $E_t$, the total enzyme concentration. The upper panel represents the values of $v_0/(v_0)_{max}$ (●) and $T^*/T_t$ (▲) calculated in the case of the single binding site model. The lower panel shows the dependence of $v_0/(v_0)_{max}$ in the case of the anticooperative model assuming either exclusive protection by the "strong" binding site (○) or equal protection by both the sites (●■ ●■). The parameters used for the calculations were $K = 10\ \mu M^{-1}$ (Bartmann et al., 1975), $T_t = 5\ \mu M$, $k_b/k_f = 0.385$ (Table I), and $k_f t = 0.693$ (50% cross-linking of free $tRNA^{Phe}$ at time $t$).

The initial rate of the photoreaction $v_0$ can be easily derived by assuming either protection at the "strong" site on the enzyme (eq A13) or equal protection at both sites (eq A14)

$$\frac{V_0}{(V_0)_{max}} = \frac{T_0 + E_1^1}{T_t} + \frac{k_b}{k_f}\frac{E_0^1 + E_1^1}{T_t} \quad \text{(A13)}$$

$$\frac{V_0}{(V_0)_{max}} = \frac{T_0}{T_t} + \frac{k_b}{k_f}\frac{E_0^1 + 2E_1^1}{T_t} \quad \text{(A14)}$$

(*c*) *Fit with Experimental Data.* Assuming a single binding site on the enzyme allows us the calculation of $T^*$ (eq A9 and A10) and $V_0$ (eq A11) as a function of $E_t$. This was done in the conditions of Figure 6 and is shown in the upper panel of Figure 10. The figure shows that the two curves are very similar in shape and, in a first approximation, can be derived from each other by a simple transformation. This indicates that $T^*$ and $V_0$ are closely related.

The lower panel shows the $V_0 = f(E_t)$ curve in the case of the anticooperative model. If only the "strong" site is involved in protection, it can be observed that the curves are indistinguishable from the one obtained with the single site model. On the other hand, if the "weak" site is involved in protection, the dependence is strikingly altered. The good agreement obtained between the experimental values of $T^*$ and those obtained theoretically on the basis of a single binding site (Figure 6) suggests protection only by the strong binding site. When applied to the classical sequential model where the $E_1^1$ form is symmetric, the analysis above indicates that the tRNAs fixed on $E_1^1$ have a new rate constant $k_b'$, $k_b' = (k_b + k_f)/2$, where $k_f$ and $k_b$ applied respectively to the free tRNA and to the tRNA bound to the $E_0^1$ form.

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