Kinetics of Anticooperative Binding of Phenylalanyl-tRNA^{Phe} and tRNA^{Phe} to Phenylalanyl-tRNA Synthetase of *Escherichia coli* K10†

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ABSTRACT: The kinetics of binding of Phe-tRNA^{Phe} to phenylalanyl-tRNA synthetase were investigated by stopped-flow techniques employing 2-(*p*-toluidinyl)naphthalene-6-sulfonate as a reporter group. The kinetics were found to follow the concentration of Phe-tRNA^{Phe} in terms of a saturation function. When added, tRNA^{Phe} inhibited both the kinetics of association and the kinetics of dissociation. The kinetics of Phe-tRNA^{Phe} binding have been analyzed to be a superposition of two reactions, the association to the phenylalanine-specific binding site and the association to the tRNA-specific binding site of the enzyme. The two modes are mutually exclusive. When tRNA^{Phe} is added, the binding of Phe-tRNA^{Phe} at the tRNA-specific site is suppressed by competitive inhibition. In binding to the tRNA-specific site, the unacylated tRNA affects the rates of association and dissociation of Phe-tRNA^{Phe} binding at the Phe-specific site in terms of an antagonistic mechanism. The values of both rate constants are decreased depending on the degree of saturation of the enzyme with the unacylated tRNA before and after the association of Phe-tRNA^{Phe}. A model is presented, and kinetic and equilibrium constants are determined.

Phenylalanyl-tRNA synthetase of *Escherichia coli* K10 has been shown to associate with Phe-tRNA^{Phe} via two, mutually exclusive modes (Güntner & Holler, 1979). One involves binding of the phenylalanyl moiety to the amino acid specific site, and the other involves binding to the tRNA-specific site of the enzyme. The association kinetics have previously been attributed to a sequential mechanism, an initial binding to the tRNA-specific site followed by a rearrangement with binding to the amino acid specific site (Holler, 1976). By investigating the inhibitory effect of tRNA^{Phe} on the binding kinetics, I conclude now that the sequential mechanism is incorrect because tRNA^{Phe} cannot inhibit competitively the formation of the complex at the amino acid specific site. The kinetic analysis is complicated by the mutual antagonistic binding of Phe-tRNA^{Phe} and tRNA^{Phe} in a ternary complex with the enzyme.

Materials and Methods

Phenylalanyl-tRNA synthetase (EC 6.1.1.20) was prepared from *E. coli* K10 as described (Hanke et al., 1974). The preparation had 2.0 mol of active sites per mol of enzyme (Bartmann et al., 1975a) and a specific activity of 53 000 nmol mg^{-1} h^{-1}. tRNA^{Phe} (1400 pmol per A_{260} unit in water) and ATP were purchased from Boehringer (Mannheim). Phe-tRNA^{Phe} was prepared as described (Güntner & Holler, 1979). L-[^{14}C]Phenylalanine was a product of Amersham Buchler (Braunschweig). TNS[1] was obtained from Serva (Heidelberg), and all other reagents of the highest possible grade were from Merck (Darmstadt).

The kinetics of complex formation of phenylalanyl-tRNA synthetase and Phe-tRNA^{Phe} were followed at 25 ± 1 °C in the presence of the fluorescent dye TNS as has been described (Bartmann et al., 1975b). The method uses the fact that TNS, L-phenylalanine, and Phe-tRNA^{Phe} (but not tRNA^{Phe}) all compete for the same binding site on the enzyme and that TNS exhibits an increase in its fluorescence intensity upon binding. In the present method, we follow the binding of Phe-tRNA^{Phe} to this site via the decrease in fluorescence intensity that accompanies the loss of enzyme·TNS complexes. Corrections of physical parameters due to competition by the dye have been omitted because of low concentrations of TNS in comparison with the dissociation constant of the enzyme·TNS complex (0.02 mM; Pimmer & Holler, 1979).

A Durrum-Gibson stopped-flow apparatus equipped with a Durrum 16400 fluorescence attachment was used. The drive syringes contained enzyme plus tRNA^{Phe} separated from Phe-tRNA^{Phe} plus tRNA^{Phe}. The solutions were mixed in a 1:1 ratio. Unless otherwise noted, all solutions contained 50 mM Tris-HCl buffer (pH 7.5), 10 mM $MgCl_2$, 0.1 mM EDTA, 0.2 mM dithioerythritol, and 6 μM TNS. Association between enzyme and Phe-tRNA^{Phe} was followed by recording the decrease in fluorescence intensity on a Tektronix 7623A storage oscilloscope equipped with a 7B53A dual-time base and with a 7A18 dual-trace amplifier.

The rate constant k(obsd) evaluated from first-order plots was determined as a function of Phe-tRNA^{Phe} concentration at various fixed concentrations of tRNA^{Phe}. The dissociation constant of Phe-tRNA^{Phe} binding to the enzyme and enzyme·tRNA^{Phe} complex, respectively, was evaluated from the reaction amplitude, which corresponds to the overall change in fluorescence intensity during the time of reaction. No intensity change occurred during the dead time (2.9 ms) of the apparatus. The dissociation constants were evaluated according to known procedures (Güntner & Holler, 1979).

The rate constant of dissociation of Phe-tRNA^{Phe} from the enzyme·tRNA^{Phe} complex was measured by the same technique in the presence of TNS but employing a rapid 6- or 18-fold dilution of a mixture of 0.09 μM enzyme, 0.19 μM Phe-tRNA^{Phe}, and varying concentrations of tRNA^{Phe} into buffer that contained tRNA^{Phe} at the concentrations applied in the other syringe. Because of this dilution, the preformed enzyme·Phe-tRNA^{Phe}·tRNA^{Phe} complex had to equilibrate at the new equilibrium by a release of Phe-tRNA^{Phe}. Thus, dilution is accompanied by an increase in fluorescence intensity. The final concentration of free Phe-tRNA^{Phe} was sufficiently low so that the kinetics of reassociation did not need to be

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[1] Abbreviations used: EDTA, (ethylenedinitrilo)tetraacetic acid; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol; TNS, 2-(*p*-toluidinyl)-naphthalene-6-sulfonate; Phe-tRNA^{Phe}, L-phenylalanyl ester of tRNA^{Phe}.

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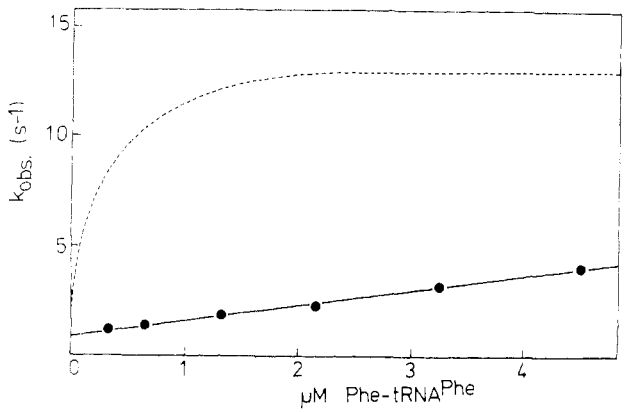

FIGURE 1: Observed rate constants for the formation of enzyme·Phe-tRNA^{Phe} complexes as a function of [Phe-tRNA^{Phe}] in the absence (---) or presence (—) of 10 μM tRNA^{Phe}. The curve in the absence of tRNA^{Phe} has been computed on the basis of eq 3 with the parameters $k_f = 32 \times 10^6$ M^{-1} s^{-1}, $k_b = 2.2$ s^{-1}, and $K_1 = 0.4$ μM calculated from published work (Holler, 1976). The rate constants in the presence of 10 μM tRNA^{Phe} are k_b(obsd) = 0.8 s^{-1} and k_f(obsd) = 0.7×10^6 M^{-1} s^{-1}. For experimental conditions, see the text. The concentration of phenylalanyl-tRNA synthetase was 0.04 μM.

considered. In other experiments, Phe-tRNA^{Phe} was rapidly displaced from the complex by mixing equal volumes of buffer and TNS containing 0.14 μM enzyme plus 0.8 μM Phe-tRNA^{Phe} in one syringe and varying amounts of tRNA^{Phe} in the other syringe, respectively. Because of hydrolysis, the time of incubation of Phe-tRNA^{Phe} and enzyme was not allowed in these experiments to exceed 2 min. At most, 15% of the total Phe-tRNA^{Phe} was hydrolyzed. As expected, the dissociation of Phe-tRNA^{Phe} from the complex was expressed as an increase in fluorescence intensity.

Results and Discussion

The binding of Phe-tRNA^{Phe} to phenylalanyl-tRNA synthetase is seen as a time-dependent decrease in fluorescence intensity of TNS known to occupy the phenylalanine-specific binding site of the enzyme (Kosakowski & Holler, 1973). The mechanism leading to the observed intensity decrease is likely to be the intrusion and binding of the phenylalanyl moiety of Phe-tRNA^{Phe} in the Phe-specific binding site of the enzyme and thereby the displacement of the dye which becomes nonfluorescent when surrounded by water (Güntner & Holler, 1979).

The values of the observed rate constants follow the concentration of Phe-tRNA^{Phe} in terms of a saturation function (Holler, 1976). tRNA^{Phe} present at higher concentrations ($\gtrsim$0.5 μM) tends to eliminate saturation, supporting a linear dependence (Figure 1). The observed rate constants become smaller as [tRNA^{Phe}] is increased. This suggests that uncharged tRNA^{Phe} acts as an inhibitor of the association of Phe-tRNA^{Phe} to the enzyme.

Since it is actually the fluorescence of TNS that is observed (Kosakowski & Holler, 1973), possible complications by the association kinetics of the dye·enzyme complex have to be considered. For the following reasons, such an interference is unlikely. (1) The association and dissociation of TNS interacting with the enzyme exhibit rate constants $\gtrsim$200 s^{-1} faster than those of the reactions observed here (<30 s^{-1}) (Pimmer & Holler, 1979). Mixing TNS with a solution of enzyme·Phe-tRNA^{Phe} complex in the absence or presence of tRNA^{Phe} does not give rise to a reaction in the time range investigated. (2) The size of the reaction amplitude depends in a predictable way on the nature of the aminoacyl side chain of the acyl-tRNA^{Phe} and can be explained by a competition between this side chain and TNS to associate with the Phe-specific binding site of the enzyme (Güntner & Holler, 1979). Uncharged tRNA by itself has no effect on the fluorescence.

The kinetics in Figure 1 are reconciled with the alternative models in Figure 2A. The models are based on the previous finding that Phe-tRNA^{Phe} can form two kinds of mutually exclusive complexes (Güntner & Holler, 1979). One involves the tRNA^{Phe}-specific binding site [complex $(EL)_1$], and the other involves the phenylalanine-specific binding site [complex $(EL)_2$] of the enzyme and the corresponding parts of the Phe-tRNA^{Phe} molecule. The sequential kinetic model describes the reaction as first forming a complex at the tRNA-specific site, being followed by a rearrangement of this complex into a second complex having Phe-tRNA^{Phe} bound to the phenylalanine-specific site of the enzyme. Contrary to this model where the Phe-specific site is accessible only by the way of the tRNA-specific site, the parallel mechanism considers both binding sites to be directly accessible from the solvent. The corresponding eq 3 and 6 (eq 1–9 are shown in Figure 2) for the experimental rate constant, k(obsd), are derived in a straightforward manner assuming that Phe-tRNA^{Phe} is in excess of enzyme and that the complex at the tRNA-specific site is in preequilibrium with the formation of the complex at the Phe-specific binding site (Gutfreund, 1972). In this derivation, the degree of the observed decrease in fluorescence intensity is proportional to the concentration of enzyme·Phe-tRNA^{Phe} formed. The rate by which the decrease appears is then formulated as a function of the concentration of the enzyme·Phe-tRNA^{Phe} complex, $(EL)_2$, and on the basis of the reaction equations in Figure 2A finally as a function of the rate constants k_f and k_b, the dissociation constant K_1, and the concentration of the free Phe-tRNA^{Phe}, [L]. The substitution for the concentration $[(EL)_2]$ during derivation takes into account that the complex $(EL)_2$ is formed in the rate-limiting step via complex $(EL)_1$ for the sequential model whereas complex $(EL)_2$ in the parallel model forms directly from free E and L in the presence of competitive formation of $(EL)_1$. The eq 3 and 6 for the apparent rate constants can be obtained from the integrated rate equations. The definitions of rate and equilibrium constants used here and subsequently are given in Figure 2A.

Within the frame of the proposed models, the assumption of preequilibrium is supported by the finding that the reaction amplitude resembles the amount of decrease in fluorescence intensity measured by titration at equilibrium. It has to be mentioned that the models in Figure 2 and the treatment of the kinetic equations consider independent, single sites. However, it has been demonstrated that phenylalanyl-tRNA synthetase contains two active sites which interact in an anticooperative manner (Bartmann et al., 1975a). The approximation followed here resembles a minimal reaction scheme to account for all kinetic observations.

The sequential and the parallel models cannot be distinguished on the basis of eq 3 and 6. They are, however, recognized in the presence of an inhibitor such as tRNA^{Phe}. In the sequential model, the association of Phe-tRNA^{Phe} to form the complex at the Phe-specific site is blocked by the binding of tRNA^{Phe} to the tRNA-specific site of the enzyme [$(EL)_1$ cannot form]. Such a competitive inhibition would not be seen in the parallel model (Figure 2B). These facts were taken into account by introducing the competition for the formation of the complex $(EL)_1$ and by considering the enzyme·tRNA^{Phe} complex incapable of binding Phe-tRNA^{Phe} in the case of the sequential model but independent association of Phe-tRNA^{Phe} (at the Phe-specific site to form IEL) in the case of the parallel

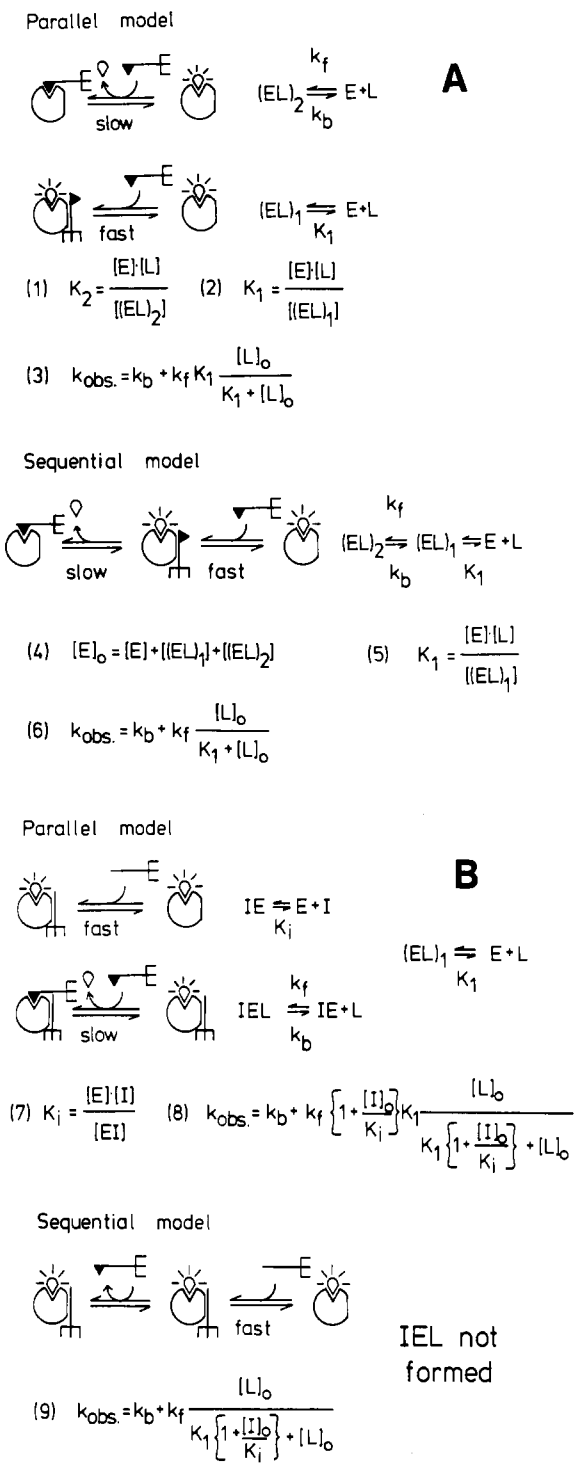

FIGURE 2: Alternative kinetic models describing the formation of the enzyme·Phe-tRNA^{Phe} complexes in the absence (A) and in the presence (B) of tRNA^{Phe}. For simplification, only a single active site of phenylalanyl-tRNA synthetase is considered. Symbols are as follows: ☼ = TNS occupying the Phe-specific site, fluorescent; ♀ = TNS after displacement, nonfluorescent; —E = tRNA^{Phe} functioning as inhibitor, I; ▾—E = Phe-tRNA^{Phe}, short symbol L; $(EL)_1$ = the enzyme·Phe-tRNA^{Phe} complex at the tRNA-specific site of the enzyme; $(EL)_2$ = the enzyme·Phe-tRNA^{Phe} complex at the Phe-specific site of the enzyme; IE = the enzyme·tRNA^{Phe} complex; IEL = the enzyme·tRNA^{Phe}·Phe-tRNA^{Phe} complex. tRNA^{Phe} binds exclusively at the tRNA-specific binding site of the enzyme. K_1, K_2, and K_i are dissociation constants; k_f and k_b are rate constants of association and dissociation, respectively. The ternary complex enzyme·tRNA^{Phe}·Phe-tRNA^{Phe} of the parallel model is supposed to bind tRNA^{Phe} and Phe-tRNA^{Phe} independently. Rate constants k(obsd) refer to the formation of complexes $(EL)_2$ and IEL, respectively. Zero subscripts refer to absolute concentrations.

model (Figure 2B). The calculated expressions for the observed rate constants are eq 8 and 9. It can be seen that K_1 in eq 3 and 6 (in the absence of competitive inhibitor) has been replaced by $K_1(1 + [I]_0/K_i)$, a feature typical for competition. For practical purposes, the use of eq 8 and 9 was adapted to the employment of small concentrations:

$$[\text{Phe-tRNA}^{Phe}]_0 << K_1(1 + [I]_0/K_i)$$

Equations 8 and 9 are simplified for the parallel model to

$$k(\text{obsd}) = k_b + k_f[L]_0 \quad (10)$$

and for the sequential model to

$$k(\text{obsd}) = k_b + k_f\frac{[L]_0}{K_1(1 + [I]_0/K_i)} \quad (11)$$

Plots of k(obsd) vs. $[L]_0$ should be linear for both models. Lines with best fits to the experimental points should intersect the ordinate at k_b(obsd) = k_b and have slopes k_f(obsd) which are not affected by the presence of tRNA^{Phe} in the case of the parallel model but which should become smaller for increasing concentrations of tRNA^{Phe} in the sequential model.

An inspection of Figures 1 and 3 reveals that both the intercept k_b(obsd) and slope k_f(obsd) become smaller as [tRNA^{Phe}] increases, indicating that neither the parallel nor the sequential model holds, at least in the assumed, simple form (Figure 2). Clearly, the sequential model is ruled out because in this case k_b(obsd) could never be a function of the inhibitor concentration. The sequential model does not take into account a ternary enzyme·Phe-tRNA^{Phe}·tRNA^{Phe} complex while this is the case in the parallel model (Figure 2B). To account for the observed dependence of the slope and intercept in Figure 3 on [tRNA^{Phe}], one may assume anticooperativity between binding tRNA^{Phe} and binding Phe-tRNA^{Phe}. Indeed, this assumption would be in agreement with the [tRNA^{Phe}] dependence seen for k_b(obsd) and k_f(obsd). Half-saturation concentrations of k_f(obsd) and k_b(obsd) vs. [tRNA^{Phe}] can be tentatively ascribed to the dissociation constant of tRNA^{Phe} binding to free enzyme and to the enzyme·Phe-tRNA^{Phe} complex, respectively. Then, because of anticooperativity between the two ligands, the dissociation constant for the ternary complex should have a higher value than the dissociation constant of the binary enzyme·tRNA^{Phe} complex. The actual values of [tRNA^{Phe}] at half-saturation are ~0.4 μM for k_f(obsd) and ~5 μM for k_b(obsd) from Figure 3, in agreement with this consideration. The dissociation constant of the E·tRNA^{Phe} complex has been reported to be 0.2 μM (Bartmann et al., 1975b).

I prefer the term "antagonism" instead of "anticooperativity" in order to distinguish the case of binding of unlike ligands to unlike sites from the case of binding alike (or different) ligands to sites which are identical before the addition of the ligands. A reaction scheme (eq 12) which takes into account antagonistic binding is depicted in Figure 4. An analogous scheme has been recently presented to describe synergistic binding (Kosakowski & Holler, 1973). The kinetic equations used for the analysis of the data in Figure 3 are derived as follows.

The decrease in fluorescence intensity ΔF monitors the formation of both complexes E·Phe-tRNA^{Phe} and tRNA^{Phe}·E·Phe-tRNA^{Phe} (Figure 4):

$$\frac{d\Delta F}{dt} = \frac{d\{[(EL)_2] + [IEL]\}}{dt} = k_f[E][L] + k_f^i[IE][L] - k_b[(EL)_2] - k_b^i[IEL] \quad (13)$$

Concentration terms [E] and [IE] in eq 13 are substituted by

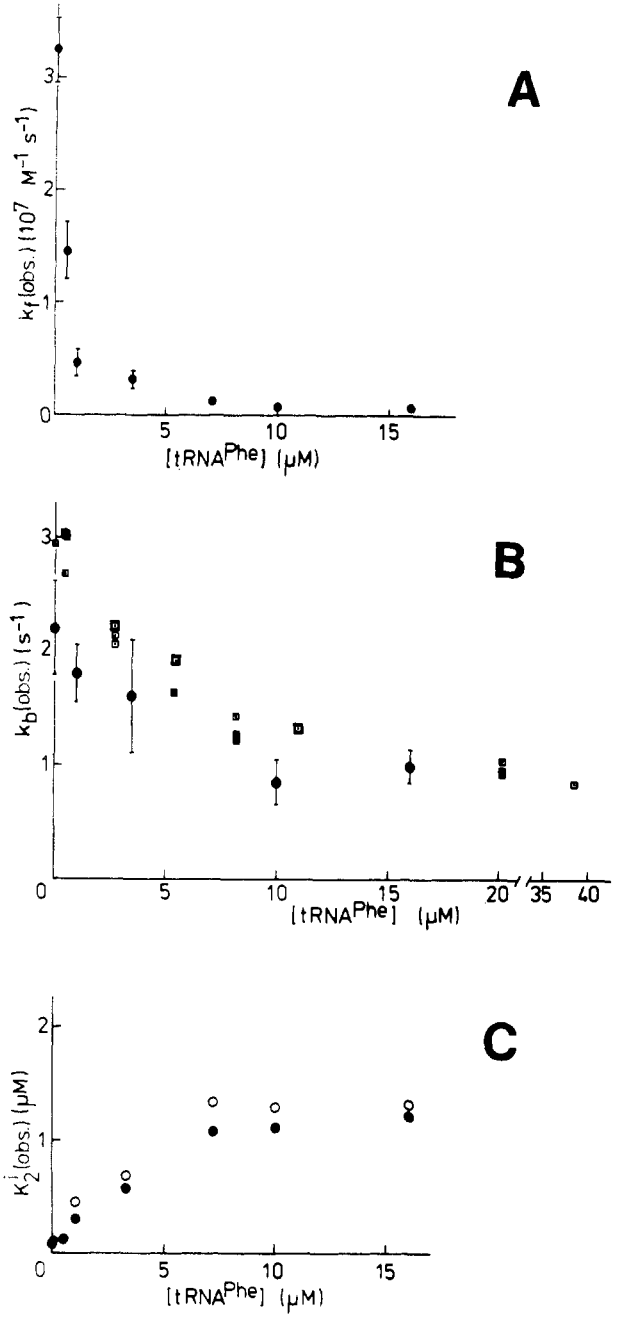

FIGURE 3: Rate and equilibrium constants for the binding of Phe-tRNA^{Phe} to phenylalanyl-tRNA synthetase as a function of tRNA^{Phe} concentration. Apparent rate constants k_f(obsd) and k_b(obsd) for association and dissociation were determined from the slope and intercept of k(obsd) vs. [Phe-tRNA^{Phe}] according to eq 16 at various fixed [tRNA^{Phe}]. Dissociation rate constants were also measured by the dilution or displacement method (▣). Dissociation constants K_2^i(obsd) denote apparent dissociation constants of Phe-tRNA^{Phe} binding to the Phe-specific site of the enzyme in the presence of tRNA^{Phe} and are either calculated as k_b(obsd)/k_f(obsd) (○) or have been evaluated from the reaction amplitude as a function of [Phe-tRNA^{Phe}] (●).

expressions containing $[(EL)_2]$ and [IEL] by employing the conservation equation

$$[E]_0 = [E] + [(EL)_1] + [(EL)_2] + [IE] + [IEL]$$

and the definition of the dissociation constants

$$K_1 = \frac{[E][L]}{[(EL)_1]} \qquad K_i = \frac{[E][I]}{[IE]} \qquad K_i^L = \frac{[(EL)_2][I]}{[IEL]} \quad (14)$$

The resulting rate equation is simplified for the experimental

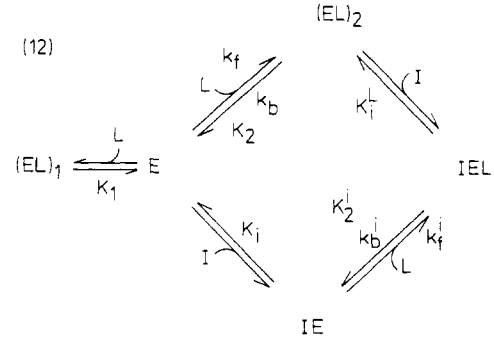

FIGURE 4: Modification of the parallel kinetic model including antagonistic binding of Phe-tRNA^{Phe} (to the Phe-specific site) and tRNA^{Phe} (to the tRNA-specific site). The dissociation constants K_i and K_2 refer to binary complexes and K_i^L and K_2^i refer to ternary complexes. Rate constants k_f^i and k_b^i belong to the association and the dissociation of Phe-tRNA^{Phe} from the ternary complex. L denotes Phe-tRNA^{Phe} and I denotes tRNA^{Phe}. $(EL)_1$ is the enzyme·Phe-tRNA^{Phe} complex at the tRNA-specific binding site of the enzyme.

approach of using low concentrations of Phe-tRNA^{Phe} but in excess of total enzyme:

$$[E]_0 << [I]_0, [L]_0$$

$$[L]_0 << K_1(1 + [I]_0/K_i) \quad (15)$$

The expression for the observed rate constant was obtained from the integrated form of the rate equation:

$$k(\text{obsd}) = k_b(\text{obsd}) + k_f(\text{obsd})[L]_0 \quad (16)$$

$$k_b(\text{obsd}) = k_b\frac{1 + \alpha([I]_0/K_i^L)}{1 + [I]_0/K_i^L}$$

$$k_f(\text{obsd}) = k_f\frac{1 + \beta([I]_0/K_i)}{1 + [I]_0/K_i}$$

$$\alpha = \frac{k_b^i}{k_b} \qquad \beta = \frac{k_f^i}{k_f}$$

A fundamental assumption is again that the binding of tRNA^{Phe} as well as the formation of the complex $(EL)_1$ is at preequilibrium with the formation of the complexes $(EL)_2$ and IEL, in which Phe-tRNA^{Phe} occupies the phenylalanine-specific binding site of the enzyme. As a control, the rate constants which were obtained by rapid dilution and displacement (Figure 3B) turned out to be compatible with the values of k_b(obsd), which had been obtained from k(obsd) vs. [Phe-tRNA^{Phe}] plots by extrapolation.

The procedures for evaluation of the parameters in reaction scheme 12 are as follows. (1) Rate constants k_b and k_f were determined by the linearization technique described by Holler (1976) except that the computation of k_f was based on eq 3 for the parallel model in the absence of tRNA^{Phe}. By the same method, the value of the dissociation constant, K_1, for binding of Phe-tRNA^{Phe} to the tRNA^{Phe}-specific site has been determined to be 0.4 μM (Holler, 1976). (2) When tRNA^{Phe} was present at various fixed concentrations, observed rate constants k(obsd) were plotted as a function of Phe-tRNA^{Phe} concentration according to eq 16. Values of k_b(obsd) and of k_f(obsd) were obtained from the intercept and slope, respectively. They are plotted in Figure 3A,B as a function of the inhibitor (tRNA^{Phe}) concentration. Rate constants k_b^i and k_f^i at saturating [tRNA^{Phe}] were taken from plots of k_b(obsd) and k_f(obsd), respectively, vs. $[\text{tRNA}^{Phe}]^{-1}$ by extrapolation to infinite [tRNA^{Phe}] (not shown). (3) The dissociation constant K_2 (Figure 4) was calculated as k_b/k_f. (4) The dissociation

Table I: Rate and Equilibrium Constants of the Enzyme·Phe-tRNA Complex That Involves the Phe-Specific Site[a]

tRNA^{Phe} absent	tRNA^{Phe} present
$k_f = (32 \pm 5) \times 10^6$ M^{-1} s^{-1}	$k_f^i = (0.4 \pm 0.1) \times 10^6$ M^{-1} s^{-1}
$k_b = 2.2 \pm 0.4$ s^{-1} [b]	$k_b^i = 0.6 \pm 0.1$ s^{-1}
$k_b = 3 \pm 0.2$ s^{-1} [c]	$K_i = 0.4 \pm 0.1$ μM
$K_2 = 0.07$–0.09 μM	$K_2^i = 1.9 \pm 0.6$ μM

[a] Conditions are 50 mM Tris-HCl buffer (pH 7.5), 10 mM $MgCl_2$, 0.1 mM EDTA, 0.2 mM dithioerythritol, and 25 °C. Parameters are defined by eq 12 and other equations in the text. Most parameters have been evaluated from the data in Figures 1 and 3. [b] Evaluated by extrapolation of k_{obsd} vs. [Phe-tRNA^{Phe}] → 0 (Holler, 1976). [c] By dilution and displacement (see the text).

constants K_i and K_i^L were computed as follows from the [tRNA^{Phe}] dependence of the dissociation constant $K_2^i(obsd) = k_b(obsd)/k_f(obsd)$. The same apparent dissociation constant can be obtained for various fixed concentrations of tRNA^{Phe} from the dependence of the reaction amplitude on the concentration of Phe-tRNA^{Phe}. They have been measured previously (Güntner & Holler, 1979) and are given together with the kinetically determined values in Figure 3C for comparison. The computation of K_i and K_i^L is based on eq 17 for the case

$$K_2^i(obsd) = \frac{K_2 K_i^L}{K_i} - K_i^L \frac{K_2^i(obsd) - K_2}{[tRNA^{Phe}]} \qquad (17)$$

that the concentration of complex $(EL)_1$ can be neglected. From previous results and under the conditions of eq 15, this concentration is indeed negligible against that of other complexes. An equation analogous to eq 17 has been derived previously for a reaction scheme representing that in Figure 4 provided complex $(EL)_1$ is omitted (Güntner & Holler, 1979). The reader is referred to this publication for further information. For computation, $K_2^i(obsd)$ was plotted against $[K_2^i(obsd) - K_2]/[tRNA^{Phe}]$ (not shown). The slope and the intercept of the line with the best fit to the experimental points were used for the calculation of K_i^L and K_i as is deducible from eq 17. (5) In omitting the formation of $(EL)_1$, the reactions of Figure 4 come to equilibrium at $K_i K_2^i = K_2 K_i^L$. When the known values for K_i, K_2, and K_i^L were employed, that of the dissociation constant K_2^i is calculated.

The values of the kinetic and equilibrium constants are summarized in Table I. They deserve some comments. (1) Antagonistic binding of tRNA^{Phe} and Phe-tRNA^{Phe} may be characterized by a coupling constant

$$a = K_i^L/K_i = K_2^i/K_2 \simeq 18$$

This value might be compared with a coupling constant of 13 for anticooperative binding of tRNA^{Phe} (Bartmann et al., 1975b). Whether similar conformational transitions are involved in antagonistic binding of tRNA^{Phe} and Phe-tRNA^{Phe} in regard to anticooperative binding of tRNA^{Phe} is unknown at present. The *E. coli* Tyr- and Met-specific enzymes are thoroughly investigated in their kinetics of binding tRNAs (Pingoud et al., 1975; Blanquet et al., 1976). It has been demonstrated in these cases that anticooperative binding operates with enhanced rates of dissociation of enzyme·$(tRNA)_2$ complexes in comparison to those of enzyme·tRNA complexes. If this is generalized, our present system is peculiar in following the opposite effects. (2) The magnitude of the rate constant $k_f = 3.2 \times 10^7$ M^{-1} s^{-1} cannot be reconciled with that of a diffusion-controlled collision [$(0.9$–$2.7) \times 10^8$ M^{-1} s^{-1}; Riesner et al., 1976]. Some rearrangement of the enzyme·Phe-tRNA^{Phe} probably contributes to the slower rate observed here. This rearrangement is affected by the binding of tRNA^{Phe}, leading to the observed 80-fold smaller value of k_f^i in comparison with k_f. The nature of the rearrangement is again unknown at present. (3) We have previously argued that the complex of Phe-tRNA^{Phe} which involves the Phe-specific binding site of the enzyme could be on the catalytic route (Holler, 1976). A comparison of the steady-state rate of aminoacylation with the rate of the dissociation of this complex suggested that the putative rearrangement by which the phenylalanyl moiety leaves the Phe-specific binding site could be rate limiting. Because of its antagonistic effect, binding of tRNA^{Phe} would be expected to inhibit aminoacylation at high concentrations where the value of $k_b = 3$ s^{-1} ultimately becomes $k_b^i = 0.1$ s^{-1}. Such a phenomenon expresses itself as substrate inhibition if events are similar during catalytic aminoacylation. I have performed steady-state aminoacylation experiments under conditions of 0.21 nM enzyme, 2–20 μM tRNA^{Phe}, 20 μM L-[^{14}C]phenylalanine (20 μCi/μmol), 2 mM ATP, 10 mM $MgCl_2$, 0.1 M Tris-HCl (pH 7.5), 10 mM KCl, 2 mM reduced glutathione, and 5-min reaction time at 25 °C. The catalytic rate constant 3.8 ± 0.3 s^{-1} was independent of [tRNA^{Phe}], indicating the absence of substrate inhibition. The failure to produce substrate inhibition could mean that tRNA^{Phe} and Phe-tRNA^{Phe} do not bind simultaneously to the same active site. Such a behavior would fit into the concept of half-of-the-sites reactivity put forward recently for phenylalanyl-tRNA synthetase of yeast (Fasiolo et al., 1977).

Conclusion

The stopped-flow experiments in the presence of tRNA^{Phe} as an inhibitor have confirmed recent findings that tRNA^{Phe} and Phe-tRNA^{Phe} can bind side by side to phenylalanyl-tRNA synthetase although both being present at saturating concentrations (Güntner & Holler, 1979). The lack of substrate inhibition (tRNA^{Phe} being the substrate) seems to rule out, however, simultaneous binding of tRNA^{Phe} and Phe-tRNA^{Phe} at the same active site while catalytic aminoacylation continues.

The results of competition between Phe-tRNA^{Phe} and tRNA^{Phe} via the tRNA specific binding site of the enzyme complement those obtained for the competition between Phe-tRNA^{Phe} and L-phenylalanine at the Phe-specific site (Güntner & Holler, 1979). The absence of competitive inhibition by tRNA^{Phe} to prevent complex formation of Phe-tRNA^{Phe} at the amino acid specific binding site indicates that a prior association of Phe-tRNA^{Phe} to the tRNA-specific binding site is not obligatory.

The association of Phe-tRNA^{Phe} and tRNA^{Phe} with the enzyme is antagonistic. The effect is a 80-fold decrease in the rate of association and a 5-fold decrease in the rate of dissociation of Phe-tRNA^{Phe} from the complex. Antagonistic binding probably does not occur during catalysis. The reasons for these phenomena are presently unknown.

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Overproduction, Purification, and Subunit Structure of *Escherichia coli* Glycyl Transfer Ribonucleic Acid Synthetase†

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ABSTRACT: Glycyl-tRNA synthetase has been purified from an overproducing *Escherichia coli* strain carrying a hybrid *ColE1* plasmid containing the *glyS* locus. A novel scheme making extensive use of blue dextran–Sepharose affinity chromatography has yielded two forms of purified enzyme. Extensive physical and kinetic studies, however, have revealed no significant differences between them. The purified enzyme was found to have a molecular weight near 205 000 and is composed of two α and two β polypeptide chains with molecular weights near 40 000 and 65 000, respectively. Analyses of sedimentation velocity and sedimentation equilibrium data indicate that the enzyme is nonspherical and possesses considerable structural anisotropy. The native enzyme was found to dissociate in the presence of 0.5 M NaSCN, and the α and β subunits were readily separated by gel filtration. Following removal of NaSCN, hydrodynamic experiments showed that isolated β protein existed as a monomer in solution while isolated α protein displayed a reversible, self-association. Neither subunit showed significant catalytic activity. The subunits are not irreversibly denatured by NaSCN dissociation, since a complex indistinguishable from the native enzyme with virtually full catalytic activity is formed when the subunits are mixed in a 1:1 molar ratio. From these data, we infer that α–α interactions contribute to the stability of the native enzyme while β–β interactions do not. In addition, it appears that a functional catalytic site requires participation by both types of subunits, either directly via the contribution of amino acid residues by both subunits or indirectly via conformational stabilization.

Aminoacyl-tRNA synthetases catalyze the esterification of a specific amino acid to a cognate tRNA with the concomitant hydrolysis of ATP to AMP and pyrophosphate. Each of these enzymes, therefore, is essential to the synthesis of cellular proteins. Approximately 50 enzymes of this class, largely from procaryotes and yeast, have been isolated and studied from a number of viewpoints [for reviews, see Söll & Schimmel (1974) and Kisselev & Favorova (1974)]. Glycyl-tRNA synthetase from *Escherichia coli* was first isolated by Ostrem & Berg (1970, 1974), who described some of the enzyme's basic properties including an $\alpha_2\beta_2$ subunit structure, rare within this class of enzymes.

Work in our laboratory began with the aim of investigating the functional contribution made by each type of subunit to the native enzyme and to assess the enzyme's potential for regulation. Initial studies demonstrated the existence of two separable forms of enzymatic activity in partially purified preparations (Francis & Nagel, 1976). Significantly, one enzyme form showed a sigmoidal kinetic response to increasing tRNA concentration, suggesting it had the capacity to act as a regulatory element.

As is the case with many of these enzymes, the small amounts of enzyme available from wild type *E. coli* strains made rapid progress on this work difficult. The colony bank assembled by Clarke & Carbon (1976), however, has provided a solution to this problem. In particular, the isolation of a hybrid plasmid containing *glyS*, the structural gene for glycyl-tRNA synthetase, allowed the enzyme to be overproduced in *E. coli*. Some of our data which contributed to the characterization of the *glyS* plasmid are reported here.

Overproduction of the enzyme has allowed us to obtain sufficient quantities of the enzyme to investigate the molecular details of its structure and function. This paper describes the first results we have obtained with enzyme from the overproducing strain. A preliminary report of some of this work has been presented (McDonald et al., 1979).

Experimental Procedure

Materials. Unfractionated *E. coli* B tRNA was purchased from Plenum Scientific Research, Inc. [1-^{14}C]Glycine was obtained from ICN Pharmaceuticals, Inc., or Amersham Corp. Enzyme grade ammonium sulfate was purchased from Schwarz/Mann, Inc. Bovine pancreatic ribonuclease A, deoxyribonuclease I, bovine glutamic dehydrogenase, aldolase, bovine serum albumin, and blue dextran were purchased from Sigma Chemical Co. Sepharose 4B, Sepharose 6B, and Sephadex G-150 were products of Pharmacia Fine Chemicals, Inc. Ultrogel AcA44 was purchased from LKB. Cyanogen bromide and 1-methyl-2-pyrrolidinone were products of Eastman Kodak. Phosphorylase A and soybean trypsin inhibitor were obtained from Worthington Biochemicals Corp. Other chemicals were reagent or higher grade.

Bacterial Strains and Growth. Experiments were performed with the *E. coli* K12 derivative CH754 (*argH metE xyl trpA36 recA56*). This strain, as well as clones carrying four hybrid *ColE1* plasmids containing the *xyl* locus, was obtained

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