Effects of the Ligands of Beef Tryptophanyl-tRNA Synthetase on the Elementary Steps of the $tRNA^{Trp}$ Aminoacylation†

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ABSTRACT: Tryptophanyl-tRNA synthetase catalyzed formation of Trp-$tRNA^{Trp}$ has been studied by mixing $tRNA^{Trp}$ with a preformed bis(tryptophanyl adenylate)–enzyme complex in the 0–60-ms time range, on a quenched-flow apparatus. Analyzing the data gives an association rate constant $k_a = (1.22 \pm 0.47) \times 10^8$ M^{-1} s^{-1}, a dissociation rate constant $k_d = 143 \pm 73$ s^{-1}, and a dissociation constant $K_d = 1.34 \pm 0.80$ μM for $tRNA^{Trp}$. The maximum rate constant of tryptophan transfer to $tRNA^{Trp}$ is $k_t = 33 \pm 3$ s^{-1}. When starting the aminoacylation reaction with a mono(tryptophanyl adenylate)–enzyme complex, one obtains different kinetic profiles than when using a bis(tryptophanyl adenylate)–enzyme complex. Over a 0–400-ms time range, the monoadenylate–enzyme complex yields an apparent first-order reaction, while the bis-adenylate–enzyme complex yields a biphasic aminoacylation of $tRNA^{Trp}$. Analysis of Trp-$tRNA^{Trp}$ formation from both complexes according to simple reaction schemes shows that the dissociation of $tRNA^{Trp}$ from an enzyme subunit carrying no adenylate is 6.9-fold slower than from an enzyme subunit carrying an adenylate. The apparent rate constant of dissociation of nascent tryptophanyl-$tRNA^{Trp}$ is 4.9 s^{-1} in the absence of free tryptophan, which is much slower than its rate of formation (33 s^{-1}). Study of the concentration dependence of the enzyme-catalyzed hydrolysis of preformed Trp-$tRNA^{Trp}$ yields a K_d of 296 ± 44 nM for this derivative, corresponding to a rate constant of dissociation of the order of 36 s^{-1}, when it is assumed that its rate constant of association with the enzyme is the same as that of $tRNA^{Trp}$. These data are interpreted as showing that a rate-limiting conformation change of the enzyme–(nascent tryptophanyl-$tRNA^{Trp}$) complex follows the formation of Trp-$tRNA^{Trp}$ and precedes its dissociation. Study of the ATP–PP_i isotopic exchange catalyzed by the enzyme in the presence of $tRNA^{Trp}$ shows that $tRNA^{Trp}$ is a noncompetitive inhibitor of tryptophanyl adenylate formation. The maximum inhibition factor is either 33% when one considers that tRNA inhibits both subunits or 66% when one considers that it inhibits only the subunit on which it is bound. The data are discussed in light of the half-of-the-sites reactivity of the enzyme and of the activatory effect of tryptophan on the overall aminoacylation of $tRNA^{Trp}$ [Trezeguet, V., Merle, M., Gandar, J. C., & Labouesse, B. (1986) *Biochemistry 25*, 7125–7136]. They lead to the conclusion that at saturating substrate concentration there are four rate-determining steps, tryptophan activation, tryptophan transfer to $tRNA^{Trp}$, a conformation change following $tRNA^{Trp}$ aminoacylation, and Trp-$tRNA^{Trp}$ dissociation, while at low tryptophan concentration the conformation change following Trp-$tRNA^{Trp}$ aminoacylation becomes a major rate-limiting step.

The limiting event in a complex enzyme reaction is usually expected to be one of the chemical steps on the reaction pathway. In the case of the aminoacylation of $tRNA^{Trp}$ by the dimeric tryptophanyl-tRNA synthetase from beef, the activation of tryptophan into tryptophanyl adenylate [rate constant 40 s^{-1} per active site (Merle et al., 1984)], and its transfer from the adenylate to $tRNA^{Trp}$ [rate constant 35 s^{-1} per dimer (Trezeguet et al., 1986)], when individually studied, do not appear to be slow enough to account for the overall steady-state rate constant of $tRNA^{Trp}$ aminoacylation by the enzyme (6.5 s^{-1}; Merault et al., 1978). Therefore, another step has to be sought. Among possible steps, the association of $tRNA^{Trp}$ to the enzyme may be considered. However, at the $tRNA^{Trp}$ concentration used to obtain the maximum rate constant of aminoacylation (5 μM; Merault et al., 1978), it is likely that the association process is comparatively fast, when association rate constants such as observed in related systems as $tRNA^{Ser}$ or $tRNA^{Tyr}$ and their cognate synthetase (2×10^8 M^{-1} s^{-1}; Krauss et al., 1975) are taken into account. The dissociation of the reaction product, the aminoacyl-tRNA, may also be a limiting factor. This dissociation has been shown to be a multistep process in the case of valyl-tRNA synthetase (Kern & Gangloff, 1981). Another possibility is that a conformation change of either macromolecule be limiting. Such a conformational change has been observed during the aminoacylation reaction for several synthetases (Riesner et al., 1978; Kern & Gangloff, 1981; Holler et al., 1981; Baltzinger & Holler, 1982b; Beresten et al., 1983; Ferguson & Yang, 1986a) and for tRNA (Renaud et al., 1981; Lefevre et al., 1981; Yamashiro-Matsumara & Kawata, 1981; Ferguson & Yang, 1986b; Pelka & Schulman, 1986). It may be limiting (Baltzinger & Holler, 1982a). Finally, it is also conceivable that $tRNA^{Trp}$ perturbs the activation step strongly enough to make this step a partially limiting factor in the overall reaction. Modulations of the activation or of the transfer reactions by the substrates of the aminoacylation reaction have been previously observed for several synthetases (Kern & Gangloff, 1981; Fasiolo et al., 1981; Baltzinger & Remy, 1985; Trezeguet et al., 1986). When one keeps in mind that the specificity of the aminoacylation reaction has a dynamic character (Ebel et al., 1973; Von der Haar & Cramer, 1978; Okamoto & Savageau, 1984; Fersht & Dingwall, 1979; Lin et al., 1984),

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the presence of non purely chemical steps becomes important to characterize.

The present study was aimed at defining which step, if any, controls the overall rate of aminoacylation of tRNA^{Trp} by tryptophanyl-tRNA synthetase. This enzyme binds a single mole of tRNA^{Trp} per dimer (Fournier et al., 1987), but both subunits are active and catalyze the aminoacylation according to a half-of-the-sites mechanism (Trezeguet et al., 1986). A quenched-flow technique was used to follow the aminoacylation of tRNA^{Trp} under pre-steady-state conditions, in order to define the association and dissociation rate constants of tRNA^{Trp} to the enzyme and the dissociation rate constant of the newly formed tryptophanyl-tRNA^{Trp} from the enzyme, taking advantage of the possible use of a mono- or a bis-(tryptophanyl adenylate)–enzyme complex. The ATP–PP_i isotopic exchange reaction was used to examine the effect of tRNA^{Trp} on the activation step of the aminoacylation reaction, and the hydrolysis of preformed Trp-tRNA^{Trp} was used to determine the dissociation constant of this compound.

Materials and Methods

Enzymes. Tryptophanyl-tRNA synthetase from beef pancreas was prepared as in Merault et al. (1978). Its concentration was determined by absorbancy (ϵ = 90 000 cm^{-1} M^{-1}; Lemaire et al., 1969). Inorganic pyrophosphatase (500 units/mg) was from Sigma.

tRNA^{Trp}. Beef liver tRNA^{Trp} was prepared as in Fournier et al. (1976). Its amino acid acceptance (1500 pmol/260-nm absorbance unit) was used to calculate the tRNA^{Trp} concentration.

Labels. L-[^{14}C]Tryptophan (56 Ci/mol) was purchased from Amersham. Inorganic [^{32}P]pyrophosphate ([^{32}P]PP_i) and L-[U-^{14}C]tryptophan (615 Ci/mol) were purchased from New England Nuclear.

Standard Buffer. Except when otherwise stated, experiments were performed at 25 °C, in a standard buffer solution containing 100 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), pH 8, 0.1 mM ethylenediaminetetraacetic acid, 1 mM dithioerythritol, and $MgCl_2$. The total magnesium concentration was calculated in order to keep 1 mM free magnesium in the reaction medium as in Mazat et al. (1982) and Trezeguet et al. (1986).

Preparation of Tryptophanyl-tRNA^{Trp}. L-[^{14}C]Tryptophanyl-tRNA^{Trp} was prepared as follows: tRNA^{Trp} (15 μM) was aminoacylated by tryptophanyl-tRNA synthetase from beef (60 nM) with L-[^{14}C]tryptophan (100 μM) in the presence of ATP–Mg (10 mM) at pH 8, 25 °C. Under these conditions, tRNA^{Trp} aminoacylation was complete after 15 min. The reaction was stopped by decreasing the temperature from 25 to 0 °C and the pH from 8 to 5 by the addition of acetic acid. After phenolic extraction, L-[^{14}C]tryptophanyl-tRNA^{Trp} was purified by chromatography through a DEAE-cellulose column, equilibrated with 0.1 M sodium acetate, pH 5, and 0.3 M NaCl. After being washed with the same solution, tryptophanyl-tRNA^{Trp} was eluted with a salt gradient from 0.3 to 1.2 M NaCl and then precipitated with ethanol and stored at −20 °C.

Preformation of Bis- or Mono(tryptophanyl adenylate)–Enzyme Complexes. Labeled bis(Trp-AMP)–enzyme or mono(Trp-AMP)–enzyme complexes were preformed in situ by incubating at 25 °C (pH 8) tryptophanyl-tRNA synthetase (0.4 μM) with L-[^{14}C]tryptophan at twice the molar enzyme concentration or at equimolar concentration, respectively, in the presence of ATP–Mg (50 μM) and inorganic pyrophosphatase (1 unit/mL). Under these conditions, the complexes were synthesized within a few minutes (Mazat et al., 1982). Due to the anticooperative binding of tryptophan to the enzyme, K_{d1} = 1.6 and K_{d2} = 18.5 μM (Merle et al., 1984), the synthesis of tryptophanyl adenylate led to 74% of mono- and to 13% of bis(tryptophanyl adenylate)–enzyme complexes when enzyme and tryptophan were in stoichiometric amounts (0.4 μM each).

Kinetic Studies. (A) Kinetics of Transfer of Tryptophan to tRNA^{Trp}. The experiments were performed as in Trezeguet et al. (1986), in a quenched-flow machine built according to Gangloff et al. (1984).

(B) Kinetics of Enzyme-Catalyzed Hydrolysis of Tryptophanyl-tRNA^{Trp}. The hydrolysis of Trp-tRNA^{Trp} was studied by measuring the production of L-[^{14}C]tryptophan when tryptophanyl-tRNA synthetase (5–100 nM) was mixed with L-[^{14}C]tryptophanyl-tRNA^{Trp} (15 nM to 10 μM) at pH 8, 25 °C. Aliquots (100 μL) of the reaction mixture were withdrawn at different time intervals and added to 50 μL of 20% trichloroacetic acid to stop the reaction and to precipitate the nucleic acid. The low pH prevented the spontaneous hydrolysis of tryptophanyl-tRNA^{Trp}. To ensure a complete precipitation of aminoacyl-tRNA^{Trp}, 100 μL of a solution of a pool of tRNAs (3 absorbance units) was added. After 10-min centrifugation at 3000 rpm, aliquots of the supernatent (100 μL) were added to 5 mL of U50 scintillator solvent and counted for L-[^{14}C]tryptophan release with an Intertechnique SL 30 counter. Under these conditions, free tryptophan recovery was 84%.

(C) Kinetics of ATP–[^{32}P]PP_i Isotopic Exchange. The reaction was studied with variable ATP–Mg concentrations in the presence of tRNA^{Trp} or with variable PP_i concentrations in the presence of tryptophanyl-tRNA^{Trp}. In the first case, the experiments were carried out in the presence of high PP_i concentration (1 mM) and in the presence of AMP (5 mM) to avoid tRNA^{Trp} aminoacylation in the course of the isotopic exchange reaction; the reaction was started by addition of tryptophanyl-tRNA synthetase. In the second case, tryptophanyl-tRNA^{Trp} was synthesized in situ in the presence of 5 nM tryptophanyl-tRNA synthetase, 50 μM tryptophan, and 5 mM ATP–Mg; the isotopic exchange reaction was started by adding [^{32}P]PP_i at variable concentration after 15-min incubation. Aliquots (50 μL) of the reaction mixtures were removed after 1-, 2-, and 3-min reaction and flushed in 200 μL of 5% trichloroacetic acid to stop the reaction. Then, 500 μL of a 3% suspension of activated Norit A charcoal in 5% trichloroacetic acid containing 0.2 M sodium pyrophosphate was added to the samples. After filtration through Whatmann GF/c filters and washing with water (3 × 5 mL), the radioactivity retained by the charcoal was counted.

Data Analysis. The calculations were carried out with a Hewlett-Packard 9845 microcomputer using programs developed in the laboratory.

Results

Transfer Reaction of Tryptophan from Tryptophanyl Adenylate to tRNA^{Trp} under Pre-Steady-State Conditions. Pre-steady-state kinetics of tRNA^{Trp} aminoacylation are biphasic, when the reaction is started by mixing tRNA^{Trp} with a preformed bis(tryptophanyl adenylate)–enzyme complex, in which each subunit carries one adenylate (Trezeguet et al., 1986). Each phase corresponds to the formation of 1 mol of L-[^{14}C]tryptophanyl-tRNA^{Trp} per mole of enzyme, with two different time constants. The first phase directly reflects the transfer reaction of tryptophan from the adenylate to tRNA^{Trp}, while the second phase results from the combination of at least two processes, the dissociation of tryptophanyl-tRNA^{Trp} from the enzyme and a second transfer step. This biphasicity is due

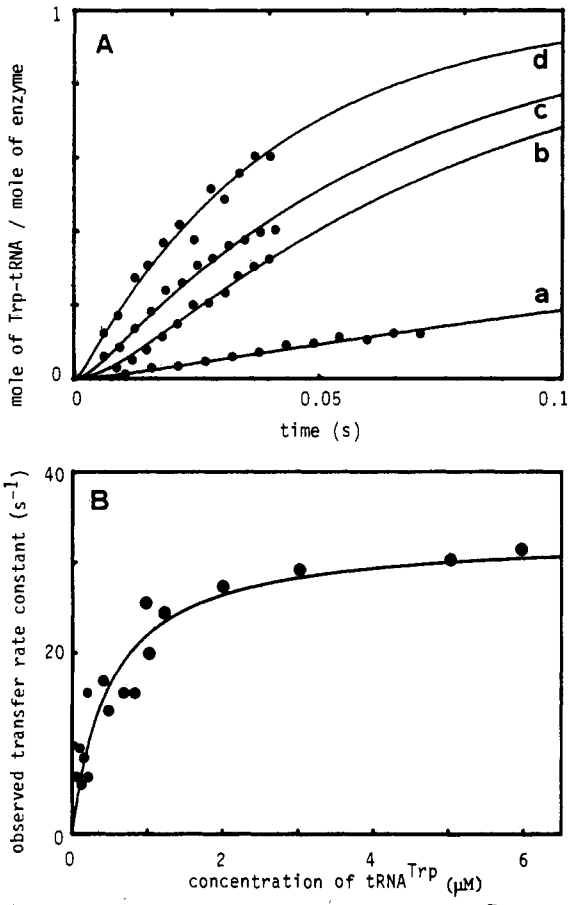
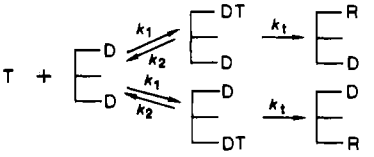


FIGURE 1: Kinetics of transfer of tryptophan to $tRNA^{Trp}$ from the preformed bis(tryptophanyl adenylate)–enzyme complex. (A) Kinetics of the transfer reaction under pre-steady-state conditions. The synthesis of L-[^{14}C]tryptophanyl-$tRNA^{Trp}$ (free or bound to the enzyme) was studied by a quenched-flow method, starting from a preformed bis-(L-[^{14}C]tryptophanyl adenylate)–enzyme complex. Thirty sets of enzyme and tRNA concentrations were used. This figure shows the data obtained with 35, 70, 150, or 200 nM enzyme–adenylate complexes and 100, 200, 400, or 3000 nM $tRNA^{Trp}$ (final concentrations), for curves a, b, c, and d, respectively. The enzyme–adenylate complexes were mixed with $tRNA^{Trp}$ at time 0. The theoretical curves which best fit the points were plotted according to eq 2, with values of k_1 = 0.6, 1.1, 1.8, and 1.1 × 10^8 M^{-1} s^{-1} and values of k_2 = 140, 50, 145, and 210 s^{-1}, for curves a, b, c, and d, respectively, and the value k_t = 33 s^{-1} for all curves. (B) Dependence of the observed transfer rate constant on $tRNA^{Trp}$ concentration. The observed transfer rate constant was determined from each curve with eq 1. The concentration of $tRNA^{Trp}$ varied from 50 nM to 6 μM and the concentration of the enzyme complex from 15 to 500 nM. The curve which fits the points corresponds to a Michaelian dependence with k_{max} = 33 s^{-1} and apparent K_M = 0.51 μM.

to the half-of-the-sites reactivity of the enzyme (Trezeguet et al., 1986). We have investigated, by quenched-flow technique, the dependence of the rate of the first phase on $tRNA^{Trp}$ concentration, by starting the reaction with a bis(L-[^{14}C]-tryptophanyl adenylate)–enzyme complex. The relative concentrations of the preformed complex and of $tRNA^{Trp}$ were such that less than 10% of the initial $tRNA^{Trp}$ was consumed at the end of the measurement, carried out over a 0–60-ms time range. In control experiments the stability of the preformed bis(L-[^{14}C]tryptophanyl adenylate)–enzyme complex was checked by measuring the percentage of L-[^{14}C]tryptophan transferred to $tRNA^{Trp}$ after a few seconds of reaction with 5 μM $tRNA^{Trp}$. For each concentration of the preformed enzyme–adenylate complex, in the range 15–500 nM, close to 100% of the initial tryptophan was esterified with $tRNA^{Trp}$.

Figure 1A shows that a lag phase preceded the synthesis of the aminoacyl-tRNA. The length of this lag phase decreased when the $tRNA^{Trp}$ concentration increased, suggesting that it reflected the diffusion-controlled association between tRNA and enzyme and that the aminoacylation reaction did not proceed after fast preequilibrium of tRNA binding to the enzyme. The apparent rate constant k_{obsd} of tryptophan transfer was derived from an analysis of the experimental points by the empirical expression

Scheme I



$$[R] = [E_0](1 - \exp[-k_{obsd}(t - t_0)]) \quad (1)$$

where [R] and [E_0] correspond to the aminoacyl-tRNA formed and to the initial enzyme concentrations, respectively, and where t_0 corresponds to the length of the lag phase. To determine k_{obsd} for each experimental set of enzyme and $tRNA^{Trp}$ concentrations, the lag time t_0 was iterated until the whole curve, analyzed as a simple exponential, yielded the lowest standard deviation. The dependence of k_{obsd} on $tRNA^{Trp}$ concentration is shown in Figure 1B. Analysis of this dependence, assuming that it was hyperbolic, gave the rate constant of transfer at tRNA saturation, k_t = 33 ± 3 s^{-1}.

In order to obtain informations on the association and dissociation rate constants of tRNA, the data of Figure 1A were more fully analyzed according to Scheme I. In this scheme, a single $tRNA^{Trp}$ at a time binds to the dimer, though both subunits of the bis(tryptophanyl adenylate)–enzyme complex are equivalent toward $tRNA^{Trp}$ binding. This behavior corresponds to the half-of-the-sites reactivity of the enzyme (Trezeguet et al., 1986). Scheme I only takes into account the first transfer of tryptophan, which spans the initial phase shown in Figure 1. In Scheme I, E, D, T, and R stand for enzyme, L-[^{14}C]tryptophanyl adenylate, $tRNA^{Trp}$, and L-[^{14}C]tryptophanyl-$tRNA^{Trp}$, respectively. k_1 and k_2 are the association and dissociation rate constants of $tRNA^{Trp}$, respectively, and k_t is the transfer rate constant of tryptophan.

The equation relating [R_t], the total amount of aminoacyl-tRNA formed (either complexed to the enzyme or free), to the rate constants k_1, k_2, and k_t and to the concentrations [E_0] and [T] is

$$[R_t] = 2k_1k_t[T][E_0]([\exp(R_1t) - 1]/R_1 - [\exp(R_2t) - 1]/R_2)/(R_1 - R_2) \quad (2)$$

with

$$R_1 = (1/2)(-2k_1[T] + k_2 + k_t + [(2k_1[T] + k_2 + k_t)^2 - 8k_1k_t[T]]^{1/2})$$

$$R_2 = (1/2)(-2k_1[T] + k_2 + k_t - [(2k_1[T] + k_2 + k_t)^2 - 8k_1k_t[T]]^{1/2})$$

Equation 2 was used to analyze the data obtained with a series of enzyme–adenylate complex concentrations, ranging from 15 to 500 nM, and of tRNA concentrations, ranging from 50 nM to 6 μM. An iterative least-squares procedure was followed to search for the best set of values for k_1 and k_2 for each experimental curve, with the value k_t = 33 s^{-1}, previously determined. This analysis led to k_1 = (1.22 ± 0.47) × 10^8 M^{-1} s^{-1} and k_2 = 143 ± 73 s^{-1}, respectively. The dissociation constant of $tRNA^{Trp}$, determined as the mean of the ratio k_2/k_1 obtained from each curve, was K_d = 1.34 ± 0.80 μM.

Asymmetry of the Subunits of the Mono(tryptophanyl adenylate)–Enzyme Complex toward $tRNA^{Trp}$ Binding.

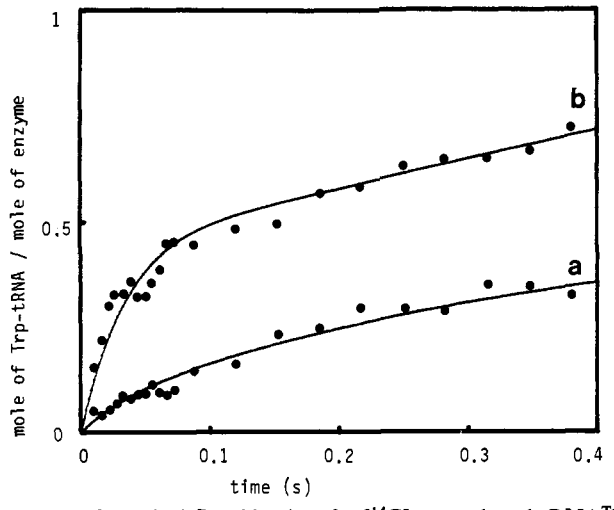


FIGURE 2: Quenched-flow kinetics of L-[^{14}C]tryptophanyl-tRNA^{Trp} synthesis from mono(L-[^{14}C]Trp-AMP)–enzyme and bis(L-[^{14}C]-Trp-AMP)–enzyme complexes. The complexes were preformed with 0.4 μM tryptophanyl-tRNA synthetase, 100 μM ATP, 1 unit/mL inorganic pyrophosphatase, and either 0.4 μM (curve a) or 0.8 μM (curve b) L-[^{14}C]tryptophan. The preformed complex was mixed at time 0 with 10 μM tRNA^{Trp} to give final concentrations of 0.2 μM complex and 5 μM tRNA^{Trp}. Curve a was plotted according to eq 3 and 4, taking into account the presence of 74% and 13% of mono- and bisadenylate–enzyme complexes, respectively, under conditions a. Curve b was plotted according to eq 4. The values used to plot both curves were k_t = 28 s^{-1}, k_2/k_3 = 6.9, and k_4 = 4.9 s^{-1}.

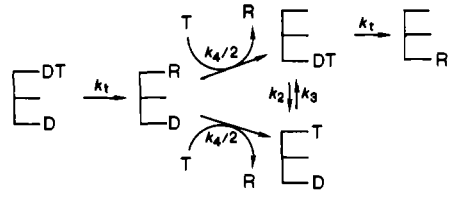
When tryptophanyl-tRNA synthetase was incubated with stoichiometric amounts of tryptophan and of enzyme subunit, in the presence of an excess of ATP–Mg and in the presence of inorganic pyrophosphatase, a bis(tryptophanyl adenylate)–enzyme complex is obtained (Graves et al., 1980). On the contrary, when equivalent molar concentrations of tryptophan and enzyme are used, the activation reaction can be carried out mainly on a single subunit of tryptophanyl-tRNA synthetase because tryptophan binds anticooperatively to the enzyme (K_{d1} = 1.6 μM and K_{d2} = 18.5 μM; Merle et al., 1984). This is achieved at tryptophan concentrations much lower than its second dissociation constant for the enzyme (Mazat et al., 1982). Under these conditions, a mono(tryptophanyl adenylate)–enzyme complex forms, along with small amounts of the bis(tryptophanyl adenylate)–enzyme complex. When concentrations of 0.4 μM enzyme and 0.4 μM tryptophan are used, the relative concentrations of free enzyme and of the mono and bis complexes are 0.13, 0.74, and 0.13, respectively. Both complexes are stable enough to be kept for several hours (Mazat et al., 1982). In the monoadenylate–enzyme complex, there is a chemical asymmetry of the enzyme subunits. One subunit bears an adenylate while the other subunit does not, and therefore, the two subunits may possibly present different affinities for tRNA^{Trp}.

Both these preformed mono(tryptophanyl adenylate)–enzyme and bis(tryptophanyl adenylate)–enzyme complexes were used to study the kinetics of L-[^{14}C]tryptophanyl-tRNA synthesis. With both complexes, the absence of a molar excess of tryptophan over the molar concentration of subunits did not allow the turnover of either tryptophanyl adenylate or tryptophanyl-tRNA^{Trp}. The tRNA^{Trp} concentration used (5 μM) yielded a maximum aminoacylation rate under steady-state conditions (apparent K_m = 0.27 μM; Dorizzi et al., 1977).

As shown in Figure 2, the observed initial rate of aminoacyl-tRNA^{Trp} synthesis was around 3 times lower when the aminoacylation was carried out with the mono(tryptophanyl adenylate)–enzyme complex (conditions a) than when the bis(tryptophanyl adenylate)–enzyme complex was used (conditions b). This result suggested that tRNA^{Trp} was able to bind to either one of the two subunits of the monoadenylate–enzyme complex, because a binding restricted to the subunit bearing the adenylate would result in very close initial rates under conditions a and b, keeping in mind that a single tRNA^{Trp} binds to the enzyme at a time.

Scheme II

Scheme III



The enzyme behavior was analyzed on the basis of Schemes II and III, for the experiments carried out with the monoadenylate- and the bisadenylate–enzyme complexes respectively, assuming that the binding step of tRNA^{Trp} to the enzyme was much faster than any other step of these schemes with 5 μM tRNA^{Trp} (600 s^{-1}, when an association rate constant of 1.2 × 10^8 M^{-1} s^{-1} is used). For this reason, in both Schemes II and III, the enzyme species E_{DT} and E_D^T are directly interelated through the dissociation rate constants of tRNA, instead of being interelated through the formation of the enzyme–adenylate complex bearing no tRNA. It was assumed that k_t, the rate constant of tryptophan transfer from an adenylate to tRNA by one subunit, was independent of the presence or absence of an adenylate on the other subunit.

In Schemes II and III, k_2 and k_3 are the rate constants of tRNA^{Trp} dissociation from a subunit bearing the adenylate and from a subunit bearing no adenylate, respectively, and k_4 is the dissociation rate constant of the nascent aminoacyl-tRNA. In E_{DT}, the tRNA molecule lies on a subunit bearing an adenylate, therefore, the species that can form the aminoacyl-tRNA are E_{DT} and E_D^{DT}. The species E_D^T does not bear an adenylate on the subunit where tRNA lies and cannot form the aminoacyl-tRNA.

According to Scheme II the aminoacyl-tRNA formed corresponds to the species E_R. The dissociation of E_R into E + R would not change the total amount of tryptophanyl-tRNA measured under the present experimental conditions (TCA precipitation) and was not specifically taken into account. Equation 3 expresses the concentration of tryptophanyl-tRNA as a function of time t:

$$[E_R] = (k_tC_1[\exp(R_3t) - 1]/R_3 + k_tC_2[\exp(R_4t) - 1]/R_4)[E_0] \quad (3)$$

with

$$C_1 = k_3(R_4 + k_t)/(R_4 - R_3)(k_2 + k_3)$$

$$C_2 = k_3(R_3 + k_t)/(R_3 - R_4)(k_2 + k_3)$$

$$R_3 = (1/2)[-(k_t + k_2 + k_3) + [(k_t + k_2 + k_3)^2 - 4k_tk_3]^{1/2}]$$

$$R_4 = (1/2)[-(k_t + k_2 + k_3) - [(k_t + k_2 + k_3)^2 - 4k_tk_3]^{1/2}]$$

According to Scheme III, the three species corresponding to the aminoacyl-tRNA are E_D^R, E_R, and R. Equation 4 expresses the total aminoacyl-tRNA concentration [R_t] obtained in Scheme III:

$$[R_t] = [E_D^R] + [E_R] + [R] \quad (4)$$

with

$$[E_D^R] = k_t[\exp(-k_t t) - \exp(-k_4 t)]/(k_4 - k_t)[E_0]$$

$$[E_R] = \{k_t C_3[\exp(R_3 t) - 1]/R_3 + k_t C_4[\exp(R_4 t) - 1]/R_4 - A[\exp(-k_t t) - 1] - Bk_t/k_4[\exp(-k_4 t) - 1]\}[E_0]$$

$$[R] = k_t k_4([\exp(-k_4 t) - 1]/k_4 - [\exp(-k_t t) - 1]/k_t)/(k_4 - k_t)[E_0]$$

where

$$C_3 = [k_t A + k_4 B + R_4(A + B)]/(R_3 - R_4)$$

and

$$C_4 = [k_t A + k_4 B + R_3(A + B)]/(R_4 - R_3)$$

In C_3 and C_4, coefficients A and B are

$$A = -k_4(2k_3 - k_t)/[2k_2(k_4 - k_t)]$$

$$B = -k_t k_4(2k_3 - k_4)/[2(k_4 - k_t) \times [k_4^2 - k_4(k_t + k_2 + k_3) + k_t k_3]]$$

Analyzing together the data of curve a with eq 3 for 74% of the enzyme population [corresponding to the mono(tryptophanyl adenylate)–enzyme complex] and with eq 4 for 13% of the enzyme population [corresponding to the bis(tryptophanyl adenylate)–enzyme complex] and the data of curve b with eq 4 showed that the values of k_t and k_4 and of the ratio k_2/k_3 could be determined. The individual value of the rate constants k_2 and k_3 could not be obtained independently. From a least-squares procedure, the set of values leading to a best fit of both curves a and b was $k_t = 28 \pm 5$ s^{-1}, $k_4 = 4.9 \pm 2.4$ s^{-1}, and $k_2/k_3 = 6.9 \pm 2.5$. The value obtained for the transfer rate constant, 28 s^{-1}, was actually in agreement with the value previously found (33 s^{-1}), when one takes into account that 5 μM tRNA was not saturating the enzyme under the present experimental conditions, with a K_d of 1.34 μM for the adenylate-bearing site. The relative values of k_2 and k_3 showed that, in the asymmetrical complex, the affinity of tRNA was greater for the subunit bearing no adenylate (empty subunit) than for the subunit bearing an adenylate. This ratio led to a K_d of 1.34/6.9 = 0.19 μM for the empty subunit, the same association rate constant of tRNA^{Trp} for the empty subunit and the adenylate bearing subunit being considered. This value is identical with that of the dissociation constant of tRNA for the free enzyme (Fournier et al., 1987).

Enzymatic AMP- and PP$_i$-Independent Tryptophanyl-tRNA^{Trp} Hydrolysis. In order to compare the affinity of tryptophanyl-tRNA^{Trp} to that of tRNA^{Trp}, the enzyme-catalyzed hydrolysis of tryptophanyl-tRNA^{Trp} (AMP and PP$_i$ independent) was used to determine the dissociation constant of this derivative. The hydrolysis reaction was studied in the absence of ATP and tryptophan, so that no aminoacylation could re-form the starting substrate from tRNA^{Trp}. Enzyme and aminoacyl-tRNA concentrations were such that less than 10% of Trp-tRNA^{Trp} was hydrolyzed during the time of the measurement, allowing us to neglect inhibition of the reaction by the end products, tryptophan and tRNA^{Trp}. The initial rate of tryptophanyl-tRNA^{Trp} hydrolysis was determined by measuring the release of L-[^{14}C]tryptophan from L-[^{14}C]-tryptophanyl-tRNA^{Trp} as function of time, for each set of enzyme and substrate concentrations. The rates were corrected for the spontaneous hydrolysis of tryptophanyl-tRNA^{Trp}, (rate constant 1.7×10^{-4} s^{-1}, determined in a control experiment).

Figure 3 shows the dependence of the initial rate, k, on Trp-tRNA^{Trp} concentration. Owing to the rather slow rate of the reaction, fast preequilibrium of aminoacyl-tRNA binding to the enzyme and fast release of the reaction product were considered to hold. Because the dependence appeared hyperbolic, the data were analyzed according to a simple Michaelian behavior, $k = k_h[R]/(K_h + [R])$, where k_h is the rate constant of the hydrolysis and K_h is the Michaelis constant of tryptophanyl-tRNA^{Trp} in the reaction. Tentative analyses according to the sum of two independent hyperbolas corresponding to the binding of 2 mol of Trp-tRNA^{Trp} per mole of enzyme did not lead to significant values for a second affinity constant. This finding disfavored the hypothesis that two molecules of tryptophanyl-tRNA bind to the enzyme at a time, except when one considers that both had the same binding constant. When one takes into account that a single Trp-tRNA^{Trp} binds to the enzyme under more favorable tRNA binding conditions (pH 5.8, 4 °C; Akhverdyan et al. 1977) and that a single tRNA is able to bind under the experimental conditions presently used (Trezeguet et al., 1986; Fournier et al., 1987), such a possibility was very unlikely. The data of Figure 3 gave $k_h = 3.6 \pm 0.1$ 10^{-2} s^{-1} and $K_h = 148 \pm 22$ nM.

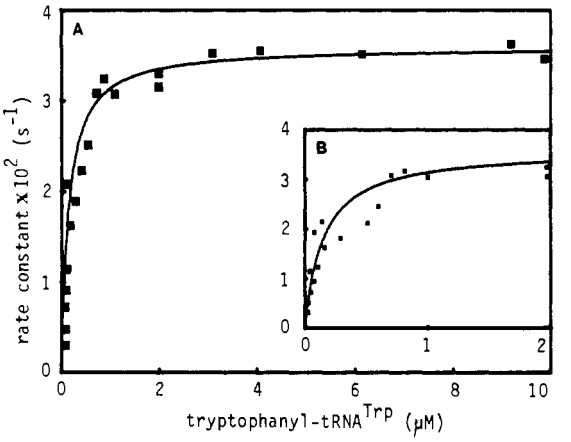


FIGURE 3: Dependence of the rate constant of enzyme-catalyzed tryptophanyl-tRNA^{Trp} hydrolysis with Trp-tRNA^{Trp} concentration. For each set of enzyme and substrate concentrations, the initial rate of hydrolysis was determined by measuring the release of L-[^{14}C]-tryptophan from L-[^{14}C]Trp-tRNA^{Trp} as a function of time. (Panel A) Variation of the hydrolysis rate constant in the 0–10 μM range of Trp-tRNA^{Trp} concentration. (Panel B) Variation of the rate constant in the 0–2 μM range. The experimental points were analyzed as a simple Michaelian process. The Michaelis constant was 148 ± 22 nM, and the maximal rate constant was $(3.6 \pm 0.1) \times 10^{-2}$ s^{-1}.

Effect of tRNA^{Trp} and Trp-tRNA^{Trp} on the ATP–[^{32}P]PP$_i$ Isotopic Exchange Reaction. In the absence of tRNA, tryptophanyl-tRNA synthetase catalyzes the ATP–[^{32}P]PP$_i$ isotopic exchange reaction (Knorre et al., 1974; Zinoviev et al., 1977). This reaction relies on the activation of tryptophan and on the pyrophosphorolysis of tryptophanyl adenylate. Because the activation reaction is significantly slower than the pyrophosphorolysis reaction (rate constants of 40 s^{-1} and 190 s^{-1}, respectively; Mazat et al., 1982), the isotopic exchange mostly reflects the activation process under nonlimiting PP$_i$ concentrations. It was used to examine the effect of tRNA^{Trp} on the activation of tryptophan.

Preliminary experiments were first carried out in order to define experimental conditions leading to no or little tRNA aminoacylation. The substrate concentrations were chosen to yield a rate of pyrophosphorolysis much faster than that of tryptophanyl adenylate synthesis. AMP was added to the reaction mixture to reverse the eventual synthesis of tryptophanyl-tRNA^{Trp}. With 5 mM ATP–Mg, 50 μM tryptophan, 5 mM AMP, 5 μM tRNA^{Trp}, and 3 nM enzyme, only 5% of tRNA^{Trp} aminoacylation was observed after 3-min incubation when the reaction was performed at 1 mM PP$_i$–Mg concen-

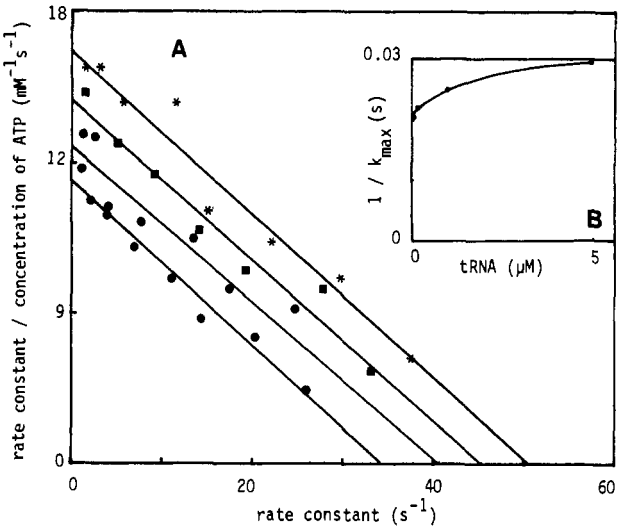


FIGURE 4: Eadie plots of the dependence of the rate constant of the ATP–[^{32}P]PP$_i$ isotopic exchange reaction with ATP–Mg concentration in the presence of tRNA^{Trp}. (A) The concentrations of the reactants were 5 nM enzyme, 5 mM AMP, 50 μM tryptophan, 1 mM [^{32}P]PP$_i$, and 0.2–5 mM ATP–Mg. Concentrations of tRNA^{Trp} were 0 (*), 0.2 (■), 1 (○), and 5 μM (●), respectively. Analysis of the plots led to k_{max}/K_M = 16.4 ± 0.4, 14.5 ± 0.4, 12.6 ± 0.4, and 11.3 ± 0.2 mM^{-1} s^{-1} and to $1/K_M$ = 0.326 ● 0.025, 0.319 ± 0.024, 0.277 ± 0.027, and 0.333 ± 0.020 mM^{-1}, respectively. The straight lines on the diagram were plotted with the mean value: K_M = 3.20 mM. The inserted plot (in panel B) shows the hyperbolic dependence of $1/k_{max}$ as a function of tRNA^{Trp} concentration.

tration, while 50% aminoacylation was measured at 50 μM PP$_i$–Mg concentration. Consequently, the effect of tRNA^{Trp} on the isotopic exchange was examined at 1 mM PP$_i$–Mg and variable ATP–Mg concentrations. These experimental conditions also disfavored the tryptophanyl adenylate–enzyme complex from being significantly present in the reaction medium (Mazat et al., 1982).

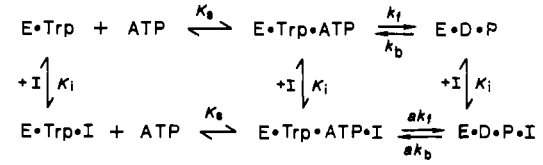
The dependence of the rate of isotopic exchange on ATP–Mg concentration is shown in Figure 4A. In the absence of tRNA^{Trp}, the maximal rate constant was 50 ± 8 s^{-1}. At 5 μM tRNA^{Trp}, the rate was 33% lower than in the absence of tRNA. The Michaelis constant for ATP–Mg was 3.2 mM.

In the absence of tRNA, the two enzyme subunits are equivalent toward tryptophanyl adenylate synthesis and toward tryptophanyl adenylate pyrophosphorolysis, at saturating tryptophan concentration (Merle et al., 1984). The linear Eadie plots in Figure 4 show that ATP–Mg presented the same Michaelis constant for each site of the dimer. The high value of the Michaelis constant of ATP–Mg (K_m = 3.2 mM), as compared to its dissociation constant [K_d = 1.4 mM (Merle et al., 1984)], could be accounted for by the presence of 5 mM AMP, which acted as a competitive inhibitor of ATP–Mg (Merault et al., 1978).

The partial noncompetitive inhibition pattern presented in Figure 4B was analyzed according to Scheme IV (Segel, 1975). In this scheme, I and D represent tRNA^{Trp} and tryptophanyl adenylate, K_s and K_i represent the apparent dissociation constants of ATP and tRNA, respectively, and a is the factor that modulates the overall rate constant k of the exchange reaction, $k = k_f k_b/(k_f + k_b)$ at substrate saturation, in the absence of tRNA. To obtain the inhibition pattern of Figure 4B, the same factor a had to be used for the forward and backward reactions in order to fit the data. According to this scheme, the expression of the maximum rate constant k_{max} in the presence of tRNA is

$$k_{max} = k(1 + a[I]/K_i)/(1 + [I]/K_i)$$

Scheme IV



The analysis of the data gave a = 0.67 ± 0.06 and K_i = 0.66 ± 0.32 μM. This latter value refers to an apparent inhibition constant and cannot be directly related to the true dissociation constant of tRNA for an empty or an adenylate-bearing subunit. The partial inhibition brought about by tRNA could be interpreted as reflecting an inhibition of either the enzyme subunit on which tRNA was bound or both subunits. Under the first assumption, 67% of the initial activity of both subunits remained in the presence of 5 μM tRNA^{Trp}. Under the second assumption, only 34% of the catalytic efficiency of one subunit remained after tRNA binding while the other subunit kept its full activity.

Similar experiments were carried out under conditions where tRNA^{Trp} was replaced by tryptophanyl-tRNA^{Trp}, as an inhibitor of the ATP–PP$_i$ isotopic exchange reaction. Under these conditions, AMP was omitted, ATP–Mg was 5 mM, and PP$_i$ was the variable substrate. These experiments led to a behavior similar to that observed under the previous conditions. In the presence of 5 μM tryptophanyl-tRNA^{Trp}, the overall rate of the exchange reaction was reduced to 69% of its value obtained in the absence of aminoacyl-tRNA.

Discussion

The Presence of Tryptophanyl Adenylate Weakens the Affinity of tRNA^{Trp} for Tryptophanyl-tRNA Synthetase. The affinities of tRNA^{Trp} for a free enzyme subunit (K_d = 0.19 μM; Fournier et al., 1987) and for an adenylate-bearing subunit [K_d = 1.34 μM (this study)] differ by a factor of 7. This sevenfold difference is again found when the dissociation rate constants of tRNA^{Trp} for the free subunit and for the adenylate-bearing subunit in the mono(tryptophanyl adenylate)–enzyme complex are compared (this study). Such a difference, obtained from quite different experimental approaches, is too large to lie within experimental errors. Therefore, one is led to conclude that, unexpectedly, tRNA^{Trp} binds better to the subunit that cannot yield an aminoacyl-tRNA than to the subunit that is potentially productive.

Several explanations may account for this difference. (i) It can be due to a simple steric effect from the adenylate. This hypothesis is not favored by the data of Leatherbarrow et al. (1985) and of Bedouelle et al. (1986), obtained on the tyrosine enzyme from *Escherichia coli*, which suggest that the adenylate and the tRNA binding regions of the enzyme are distinct. (ii) It can be due to conformational changes of the enzyme after adenylate formation. Such conformation changes were previously observed through perturbations of the absorbance spectrum, of the fluorescence emission of the protein, and of the ellipticity of its polypeptide backbone (Graves et al., 1980). (iii) It can be due to a restricted freedom of conformation of the enzyme after adenylate formation. Tryptophanyl-tRNA synthetase is known to be more resistant to thermodenaturation, mercuribenzoate reaction, and proteolysis when linked to tryptophanyl adenylate (Lemaire et al., 1967). These increased resistances likely rely on a more rigid structure of the protein, and such a rigid structure probably has less ability to adapt itself to the tRNA molecule while a larger freedom of conformation for the free enzyme is likely to increase the adaptability of its structure and to

increase its affinity for tRNA. Conformation changes of both $tRNA^{Trp}$ and the enzyme are known to occur on complex formation, suggesting a mutual adaptation of these molecules to each other (Scheinker et al., 1979; Beresten et al., 1983), as also suggested in the case of the yeast phenylalanine system (Renaud et al., 1981). The binding of tRNA to its cognate synthetase is known to proceed in two steps, a diffusion-controlled association step followed by a conformation rearrangement which is observed only with the cognate tRNA (Riesner et al., 1976; Krauss et al., 1977). Multistep binding processes were also suggested from inhibition of the aminoacylation reaction in the yeast valine and phenylalanine systems (Von der Haar & Cramer, 1978). Therefore, these different data suggest that $tRNA^{Trp}$ binds to the protein in a less favorable conformation when the enzyme bears the adenylate. This would also be true for noncognate tRNAs, which are known to bind to free tryptophanyl-tRNA synthetase (Fournier et al., 1987). The unexpected affinity decrease of the aminoacyl adenylate bearing enzyme for tRNA, which implies a priori a reduced ability to carry out the aminoacylation, may therefore participate in a discrimination process between $tRNA^{Trp}$ and other tRNAs.

The Presence of $tRNA^{Trp}$ Slows Down the Activation Step. The tryptophan activation reaction (rate constant 40 s^{-1}) is much slower than the tryptophanyl adenylate pyrophosphorolysis reaction (rate constant 190 s^{-1}) catalyzed by tryptophanyl-tRNA synthetase (Mazat et al., 1982; Merle et al., 1984). Therefore, with this enzyme the ATP–PP_i isotopic exchange reaction reflects mainly the amino acid activation step when the reaction is performed under conditions of limiting ATP–Mg concentrations and nonlimiting pyrophosphate concentrations. The fact that the isotopic exchange activity of tryptophanyl-tRNA synthetase was inhibited in a noncompetitive way by $tRNA^{Trp}$, under conditions of nonlimiting rate of pyrophosphorolysis, leads to the conclusion that $tRNA^{Trp}$ slows down the activation reaction of tryptophan and that ATP binding is not perturbed by tRNA. This is in agreement with the data of Leatherbarrow et al. (1985) and Bedouelle and Winter (1986) showing that in the case of the tyrosine enzyme of *E. coli* the tRNA and the ATP binding domains of the protein are distinct. The isotopic exchange data suggest that the enzyme inhibition brought about by $tRNA^{Trp}$ is linked to a conformational event occurring in the enzyme. Both subunits are simultaneously able to carry out the synthesis of tryptophanyl adenylate (Merle et al., 1984). Therefore, the question arises as whether the single bound tRNA inhibits the subunit on which it lies or whether it inhibits both subunits. Identical conformational events in both subunits would be necessary to induce the same inhibition level in both sites after binding of tRNA. This implies that the two subunits should keep a conformational symmetry after binding of a single ligand on the dimer. One may wonder whether this is the case, because several data rather suggest the contrary. The bindings of both tryptophan (Graves et al., 1980) and tRNA (Akhverdyan et al., 1977) are strongly anticooperative, suggesting a nonequivalence of the subunit's conformations after binding of a first mole of ligand. $tRNA^{Trp}$ has a large affinity difference for the enzyme subunits depending on whether they carry a tryptophanyl adenylate or not, also suggesting that these subunits are different from each other when one of them has bound this ligand. These data favor the idea that the conformational change following the binding of a ligand on one enzyme subunit is rather limited to this subunit. Such a behavior is consistent with the observation that 40–50% of the isotopic exchange activity of the enzyme remains after covalent binding of an analogue of tryptophanyl-tRNA (1 mol per mole), keeping in mind that the covalent modification is fully inhibited by the presence of bound adenylate (Akhverdyan et al., 1977). All these findings lead to the suggestion that the tRNA-bearing subunit is inhibited to the extent of 66% of its potential activity, while the free subunit remains normally active in the adenylate synthesis. Similar arguments allow the suggestion that the inhibition of the isotopic exchange reaction observed in the presence of tryptophanyl-$tRNA^{Trp}$ is due to the inhibition of the aminoacyl-tRNA-bearing subunit.

Nascent Trp-$tRNA^{Trp}$ Apparently Binds More Tightly to the Enzyme Than Preformed Trp-$tRNA^{Trp}$. Akhverdyan et al. (1977) have shown that at pH 5.8, 4 °C, 2 mol of tRNA can bind anticooperatively per mole of enzyme whereas a single mole of Trp-$tRNA^{Trp}$ can bind, suggesting that the affinity of Trp-$tRNA^{Trp}$ is weaker than that of $tRNA^{Trp}$ and that its binding is far more anticooperative. The binding of $tRNA^{Trp}$ has been shown to be strongly pH and temperature dependent and to shift from 2 mol per mole of dimeric enzyme at pH 6.0, 4 °C, to 1 mol per mole of enzyme at pH 8.0, 25 °C (Fournier et al., 1987). These different data make it unlikely that 2 mol of Trp-$tRNA^{Trp}$ bind to the enzyme under the present experimental conditions (pH 8.0, 25 °C). The dissociation constant derived from the Michaelis constant of Trp-$tRNA^{Trp}$ in the hydrolysis experiments, when binding of a single mole of this ligand per mole of enzyme is assumed, should have therefore twice the value of K_m, that is, should be of the order of 0.3 μM (2 × 0.148 μM), providing no kinetic step is interfering in such a determination. Using, as a first approximation, a binding constant of 0.3 μM and the association rate constant obtained for $tRNA^{Trp}$ (1.2×10^8 M^{-1} s^{-1}), one can calculate a dissociation rate constant of 37 s^{-1}. This value is so much larger than the rate constant of the enzyme-catalyzed hydrolysis of Trp-$tRNA^{Trp}$ (0.036 s^{-1}) that there is clearly no kinetically interfering step and the dissociation constant of this derivative is identical to twice the Michaelis constant. The apparent dissociation rate constant found for nascent Trp-$tRNA^{Trp}$ (4.9 s^{-1}) and that of preformed Trp-$tRNA^{Trp}$ (37 s^{-1}) imply that this ligand is more tightly held when it is just synthetized by the enzyme.

A Conformational Change Precedes the Dissociation of Nascent Tryptophanyl-$tRNA^{Trp}$. Taking into account that a large conformational change of the enzyme occurs upon formation of the enzyme–adenylate complex (Graves et al., 1980) and is followed by a further rearrangement on $tRNA^{Trp}$ binding (Scheinker et al., 1979; Beresten et al., 1983), the difference in the apparent dissociation rate constants of nascent and preformed Trp-$tRNA^{Trp}$ leads to the suggestion that the Trp-$tRNA^{Trp}$ dissociation step k_4 of Scheme III corresponds to at least two distinct processes. The first and slower process, which is not individually observable by quenched-flow techniques, must be a conformation rearrangement of the (newly formed aminoacyl-tRNA)–enzyme complex, leading to a conformation similar to that of the (preformed aminoacyl-tRNA)–enzyme complex. The second process, which is faster, is the dissociation of the reaction product itself. It is reasonable to assume that the dissociation of tryptophanyl-$tRNA^{Trp}$ is also accompanied by a conformational change restoring the initial state of the enzyme.

Is There a Limiting Step in $tRNA^{Trp}$ Aminoacylation? In the absence of free tryptophan, the overall dissociation process of tryptophanyl-$tRNA^{Trp}$ has a rate constant of 4.9 s^{-1}. Considering the dissociation rate constant of preformed Trp-$tRNA^{Trp}$, 37 s^{-1}, the rate constant of the conformational change is 5.7 s^{-1} [$4.9 = 5.7 \times 37/(5.7 + 37)$], i.e., lower than

Table I: Reaction Steps Leading to tRNA^{Trp} Aminoacylation

step	process	substrate or product	rate constant	ref[a]
1	association	Trp, ATP–Mg	10^8 M^{-1} s^{-1}	1
2	activation	Trp-AMP–enzyme complex	40 s^{-1} per site	2
3	association	tRNA^{Trp}	1.2×10^8 M^{-1} s^{-1}	3
4	transfer	Trp-tRNA–enzyme complex	33 s^{-1}	3
			35 s^{-1}	4
5	conformational change	Trp-tRNA–enzyme complex	5.7 s^{-1}	3
6	dissociation	Trp-tRNA^{Trp}	37 s^{-1}	3

[a] (1) Graves (1980); (2) Merle et al. (1984); (3) this study; (4) Trezeguet et al. (1986).

Table II: Effects of the Ligands of Tryptophanyl-tRNA Synthetase on Elementary Steps of the tRNA Aminoacylation Pathway

ligand	elementary step	effect	amplitude (%)[a]
tryptophan	Trp-tRNA^{Trp}–enzyme conformation change	activactory	440
ATP–Mg	tRNA^{Trp} aminoacylation	inhibitory	34
tRNA^{Trp}	tryptophan activation	inhibitory	33[b]
			66[c]

[a] The amplitudes of the effects are given for saturating concentrations of the ligands. [b] Considering the overall effect on both sites. [c] Considering an effect restricted to the tRNA^{Trp}-bearing subunit.

that of all other elementary steps so far evidenced (Table I). Therefore, it may appear as a potentially limiting step in the whole aminoacylation process. However, an activating effect of tryptophan on the overall aminoacylation reaction was observed by Trezeguet et al. (1986). This activation does not occur at the level of the transfer step of tryptophan to tRNA^{Trp}, which was not found to depend on the concentration of tryptophan (Trezeguet, unpublished data) and which is faster than 4.9 s^{-1} (Table I). The presently available data therefore suggest that tryptophan acts in promoting nascent Trp-tRNA^{Trp} dissociation or more likely in promoting the conformation rearrangement of the complex preceding that dissociation. This activation effect of tryptophan is of the order of threefold in the apparent overall dissociation process, which is therefore accelerated from a rate constant of 4.9 s^{-1} to a rate constant of the order of 15 s^{-1}. This overall dissociation process is in fact the result of the conformational change and of the dissociation itself (rate constant 37 s^{-1}); its rate constant in the presence of tryptophan, k_x, is such that $15 = 37k_x/(37 + k_x)$, i.e., of the order of 25 s^{-1}. Comparing 5.7 s^{-1} and 25 s^{-1} leads to an actual activating effect of tryptophan of 4.4-fold on the conformational change step. At contrast with this accelerating effect of tryptophan, tRNA^{Trp} and aminoacyl-tRNA^{Trp} have inhibitory effects on the tryptophan activation step, and due to binding competition between ATP and tRNA^{Trp}, ATP–Mg inhibits the transfer reaction (Trezeguet et al., 1986). This latter effect is supported by the observation that, in the absence of tRNA, ATP can bind to the enzyme–adenylate complex at the tRNA terminal adenosine binding site, leading to a transfer reaction which produces tryptophanyl ATP ester (Weiss et al., 1959). All these effects show that tryptophan activation, tRNA aminoacylation, and tryptophanyl-tRNA release are modulated by the substrates and products of the overall reaction.

Taking into account the different activating or inhibiting effects obtained with the three substrates, one finds an inhibitory effect of ATP on the transfer reaction of the order of 34%, an inhibitory effect of tRNA^{Trp} on the activation reaction of 33%, and an activatory effect of tryptophan on the overall Trp-tRNA^{Trp} dissociation of 300% (Table II). This set of

Table III: Saturation Fraction of the Enzyme by Its Substrates under Standard Conditions of Steady-State tRNA^{Trp} Aminoacylation[a]

substrate	dissociation constant	concn used in the reaction	saturation fraction
Trp	1.6 μM[b]	50 μM	0.99
	18.5 μM[b]		0.73
ATP–Mg	1.4 mM[b]	5 mM	0.78
tRNA^{Trp}	1.3 μM[c]	5 μM	0.80

[a] Dorizzi et al. (1977) and Merault et al. (1978). [b] Merle et al. (1984). [c] Dissociation constant for the tryptophanyl adenylate bearing subunit (this study). Under these concentration conditions and taking into account the inhibitory effects reported in Table II, the rates of tryptophan activation (for the two subunits) and tRNA aminoacylation are 35.5 s^{-1} and 18 s^{-1}, respectively.

interactions leads to rate constants of 53 s^{-1} ($2 \times 40 \times 0.66$, Tables I and II) for the activation step (mean value 26.5 s^{-1} per subunit), 22 s^{-1} (33×0.66, Tables I and II) for the transfer step, and 15 s^{-1} for the overall dissociation step of tryptophanyl-tRNA^{Trp}, respectively, when taking into account that both subunits of the enzyme are simultaneously active in the adenylate formation and present half-of-the-sites reactivity in the transfer reaction.

One may wonder whether, to a first approximation, the rate constants of the individual steps so far observed in the aminoacylation pathway of tRNA^{Trp} may be used to obtain a theoretical figure for the rate constant of the overall aminoacylation reaction carried out under steady-state conditions.

Assuming that the steps defined in Table I are linked in a purely linear way, the apparent rate constant of the steady-state aminoacylation is given by eq 5, where k'_2 and k'_4 refer

$$k_{ss} = k'_2k'_4k'_6/(k'_2k'_4 + k'_2k'_6 + k'_4k'_6) \quad (5)$$

to the rate constants of the activation step and of the transfer step, respectively, at the saturation coefficients linked to the different substrate concentrations shown in Table III, taking into account the effects shown in Table II, and where k'_6 refers to the overall Trp-tRNA^{Trp} dissociation. One obtains $k_{ss} = 6.7$ s^{-1}. This is in good agreement with the steady-state rate constant, 6.5 s^{-1}, experimentally obtained (Merault et al., 1978; Trezeguet et al., 1986).

Under the same assumption, the K_m of tRNA^{Trp} obtained under conditions of steady-state aminoacylation kinetics (0.27 μM; Dorizzi et al., 1977) may also be compared to that which should arise from the steps described in Table I. This K_m is given by eq 6, where $k' = k'_2k'_6/(k'_2 + k'_6)$ and where k''_4

$$K_m = K_dk'/(k' + k''_4) \quad (6)$$

is the rate constant of the transfer at tRNA saturation, taking into account the ATP inhibitory effect (Table II). One obtains $K_m = 0.44$ μM. This value, though higher than the experimental figure (0.27 μM), is lower than the K_d value for tRNA determined in this work (1.34 μM), as should be expected in a multistep mechanism. The present data afford an explanation for the difference between the experimental K_m value, 0.27 μM, and the observed K_d value of tRNA for the free enzyme, 0.19 μM (Fournier et al., 1987), which unexpectedly was found to be lower than K_m.

Both values for k_{ss} and K_m suggest that the different steps so far defined could grossly describe the aminoacylation process catalyzed by tryptophanyl-tRNA synthetase. This also suggests that, out of the six steps defined in Table I, there is no definite limiting step under conditions of saturating substrate concentrations. Due to the activatory or inhibitory effects of the substrates and products of the overall reaction (Table II), the four determining steps, tryptophan activation (mean value,

26.5 s^{-1} per subunit at Trp and ATP–Mg saturation), tRNA aminoacylation (22 s^{-1} at tRNA saturation), the conformational change preceding Trp-tRNA dissociation (25 s^{-1}), and the dissociation itself (37 s^{-1}), proceed with rather similar rate constants. On the contrary, at tryptophan concentrations low enough to bring about little or no acceleration of step 5, this step would become rate limiting.

However, the suggestion that the steps of Table I are linearly linked is clearly an oversimplification since the transfer step involves only one subunit while the enzyme is able to form 2 mol of tryptophanyl adenylate at a time. Therefore, a study of the overall aminoacylation under conditions allowing a test of a more complete model of the enzyme mechanism remains necessary to confirm the conclusions presently reached.

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