On the mechanism of $tRNA^{Trp}$ aminoacylation catalysed by beef tryptophanyl-tRNA synthetase using presteady-state kinetics

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The dimeric tryptophanyl-tRNA synthetase from beef pancreas has been found to activate 2 tryptophans/mol enzyme [Eur. J. Biochem. (1982) 128, 389–398]. By using quenched-flow and stopped-flow methods under presteady-state conditions, we show that only one enzyme subunit operates at a time in the aminoacylation of $tRNA^{Trp}$ and that the transfer reaction is not the rate-limiting step in the overall aminoacylation process.

tRNA *Aminoacylation* *Aminoacyl-tRNA synthetase* *Presteady-state kinetics*

1. INTRODUCTION

Tryptophanyl-tRNA synthetase from beef pancreas, an α_2-dimeric enzyme [1,2], has the following binding and kinetic properties:

(i) It binds non-covalently 2 mol tryptophan in an anticooperative way (K_d = 1 and 20 μM [3]). However, it can also bind 1 mol tryptophan covalently [4].

(ii) It binds non-covalently 2 mol tryptophanyl adenylate [5,6].

(iii) From the pyrophosphate concentration dependence of tryptophanyl adenylate pyrophosphorolysis it has been inferred that 2 pyrophosphate molecules can be bound, both having the same affinity for the enzyme adenylate complex and that these 2 adenylates are bound in an equivalent way [7]. However, a covalent monopyrophosphoryl enzyme has also been described [8].

(iv) It binds 2 molecules of $tRNA^{Trp}$ [9] in an anticooperative way [10] (K_d ~300 nM and 1 μM [10]).

Both subunits therefore appear to be able to bind all substrates and products of the aminoacylation reaction.

(v) Kinetically it makes the 2 tryptophanyl adenylates with an anticooperative dependence on tryptophan concentration that closely follows the binding of tryptophan [7] and with a Michaelian dependence on ATP-Mg^{2+} concentration [7]. The anticooperative kinetics of adenylate synthesis have been alternatively attributed to the slow release of one high affinity pyrophosphate molecule from the enzyme [11].

The aminoacylation reaction when carried out under steady state conditions shows a Michaelian dependence of the rate on tRNA concentration [12]. It is not known whether a single site or two sites are involved in this reaction though both adenylates are thought to be identically available. On the grounds of chemical modifications of the enzyme by tRNA derivatives [10] and ATP derivatives [13] half-of-the-sites reactivity has been suggested.

The aminoacylation steady state rate constant (6.5 s^{-1} for the dimer [9]) is much lower than the amino acid activation rate constant measured in the absence of tRNA (40 s^{-1}/subunit [7]). This suggests that the transfer reaction of tryptophan from the adenylate to tRNA or the dissociation of tryptophanyl-tRNA from the enzyme should be strongly rate limiting, providing the adenylate formation is not slowed down by tRNA. Since no at-

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tempt has yet been made to study this transfer reaction no definite conclusion has emerged about the role of each subunit in the aminoacylation reaction.

The present study has been aimed at the following questions:

(1) Are both subunits active in the aminoacylation reaction as they are in the activation reaction?

(2) Is the transfer of tryptophan the rate-limiting step in the overall aminoacylation?

Here, we study the aminoacylation reaction of beef $tRNA^{Trp}$ by tryptophanyl-tRNA synthetase from beef pancreas under prestationary conditions, using quenched-flow and stopped-flow methods.

2. MATERIALS AND METHODS

Tryptophanyl-tRNA synthetase was prepared as in [12]. Its concentration was determined by absorbancy ($\epsilon_{280} = 9 \times 10^4\ M^{-1}.cm^{-1}$) [14]. $tRNA^{Trp}$ from beef liver was prepared as in [15]. The amino acid acceptance was 1400 pmol/260 absorbancy unit. The $tRNA^{Trp}$ concentration was calculated taking this value into account.

PP_i (500 units/mg) was from Sigma, L-[^{14}C]tryptophan (58 mCi/mol) was from Amersham. All other chemicals were from Merck.

The experiments were performed in 100 mM Tris–HCl buffer (pH 8) containing 0.1 mM EDTA and 1 mM DTE, at 25°C. Mg^{2+} was 1 mM based on $K_d = 15\ \mu M$ for ATP-Mg^{2+} [16]. The quenched-flow experiments were carried out in a machine built according to [17]. The reaction was started by mixing the enzyme and substrate solutions (0.5 ml/syringe) and stopped by addition of 5% trichloroacetic acid after different reaction times. The incubation time varied from 5–400 ms. Twelve different fractions corresponding to equally-spaced incubation times were obtained for each run. The [^{14}C]tryptophanyl-tRNA coming from [^{14}C]tryptophan was counted in a Bray scintillation mixture. The stopped-flow experiments were carried out with a Durrum-Gibson apparatus equipped with a fluorescence detector. A Corning C S 0.54 filter was used in the emission beam. The excitation wavelength was 295 nm. The presteady-state kinetics of aminoacylation of tRNA was studied under the experimental conditions of aminoacylation shown to give maximum steady state rate of reaction (10 mM ATP, 50 μM tryptophan, 5 μM tRNA, 1 mM free Mg^{2+}, pH 8) at 25°C [12]. These concentrations are nearly saturating for all substrates ($K_d = 1.6\ \mu M$ and 18.5 μM for tryptophan, $K_d = 1.4$ mM for ATP-Mg^{2+} [18], $K_m = 0.3\ \mu M$ for $tRNA^{Trp}$ [5,6]).

3. RESULTS AND DISCUSSION

3.1. *Quenched-flow experiments*

(*a*) The quenched-flow experiments were first carried out with tryptophanyl-tRNA synthetase preincubated with a stoichiometric amount of [^{14}C]tryptophan and with an excess of ATP-Mg^{2+} in the presence of PP_i, without tRNA (conditions *a*). Under these conditions a complex carrying 2 tryptophanyl adenylates/mol enzyme has been shown to form [19]. Upon mixing this complex with a large excess of tRNA (5 μM) over the enzyme (0.3 μM) in the presence of 10 mM ATP-Mg^{2+} and 50 μM [^{14}C]tryptophan an initial burst of [^{14}C]tryptophanyl-tRNA was observed, followed by a slow formation of labelled tryptophanyl-tRNA in a pseudo steady-state reaction (fig.1a).

The appearance of a burst indicated that the reaction followed either a 2 step process; for example, that the 2 catalytic sites were carrying on the transfer of tryptophan at quite different rates; or, that there was a fast transfer of [^{14}C]tryptophan followed by a steady state aminoacylation reaction.

(*b*) The quenched-flow experiments were then carried out with tryptophanyl-tRNA synthetase preincubated under the same conditions as in *a* except that tryptophan was unlabelled (conditions *b*). In this case the tryptophanyl–adenylate enzyme complex was initially cold. When mixed with tRNA it could not lead to labelled tryptophanyl-tRNA. Fig.1b shows that when this complex was added as in *a* to tRNA in the presence of 10 mM ATP-Mg^{2+} and 50 μM [^{14}C]tryptophan a lag phase was observed, preceding the formation of [^{14}C]tryptophanyl-tRNA in a pseudo steady-state similar to that previously obtained. Considering that the linear parts of curves a and b (fig.1) are likely to show the same process these data were not in agreement with the hypothesis that these linear

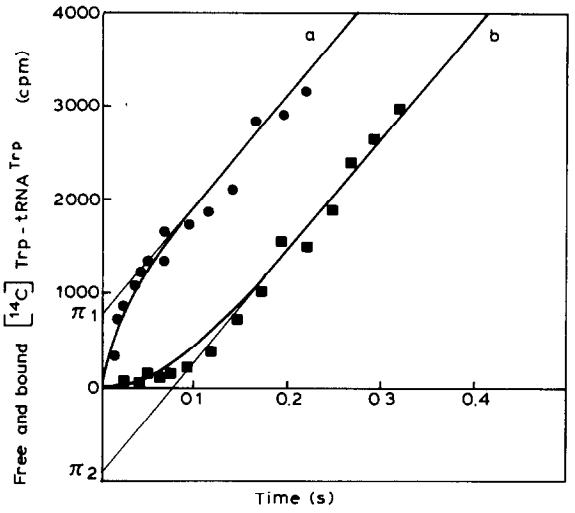

Fig.1. Presteady-state kinetics of tRNA aminoacylation under quenched-flow conditions. The first syringe (0.5 ml) contained 0.6 μM enzyme, 20 mM ATP-Mg^{2+}, 1.2 μM L-[^{14}C]tryptophan (●), or unlabelled L-tryptophan (■) and 0.1 unit/ml inorganic pyrophosphatase. The second syringe (0.5 ml) contained 100 μM L-[^{14}C]tryptophan, 10 μM $tRNA^{Trp}$. The third syringe (1 ml) contained 5% trichloroacetic acid. The mixture contained in the first syringe was allowed to incubate for 10 min at 25°C to ensure the formation of the tryptophanyl adenylate–enzyme complex.

parts could correspond to a slow transfer following the fast one: under conditions *b* the unlabelled amino acid would not generate a labelled aminoacyl-tRNA. Furthermore, the amount of aminoacyl-tRNA formed in the linear part of the curve exceeded what could be expected from the enzyme concentration.

The analytical expressions corresponding to curves *a* and *b* were therefore written on the basis of the following hypotheses:

(1) The fast phase obtained under conditions *a* was the transfer of tryptophan.

(2) The linear phase observed after 100 ms did correspond to a steady state formation of aminoacyl-tRNA.

Scheme 1 depicts these hypotheses:

$$E_i.W\sim AMP.tRNA \xrightarrow{k_1}$$

$$E_i.W\text{-}tRNA + AMP \xrightarrow[(+\,W + ATP + tRNA)]{k_2}$$

$$E_i.W\sim AMP.tRNA + PP_i + W\text{-}tRNA$$

E_i refers to subunits 1 or 2 of the enzyme, W~AMP represents tryptophanyl adenylate and W-tRNA represents tryptophanyl-tRNA. In this scheme, k_1 corresponds to the rate of the transfer reaction and k_2 corresponds to the rate resulting from all steps preceding or following the transfer, including the aminoacyl-tRNA dissociation from the enzyme and the adenylate formation. In such a scheme all substrates are expected to undergo fast pre-equilibrium with the enzyme and are considered as saturating.

Depending on the tryptophan (labelled or cold) used in the preincubation of the enzyme with ATP-Mg^{2+} the formation of total radioactive aminoacyl-tRNA (free and bound to the protein) should follow eq. (1) or (2).

When tryptophan was labelled during the preincubation (conditions *a*):

$$[W^*\text{-}tRNA] = n[E] \times \left[\frac{k_1k_2t}{k_1+k_2} + \frac{k_1^2}{(k_1+k_2)^2}(1-\exp(-k_1-k_2)t)\right] \quad (1)$$

When tryptophan was unlabelled during the preincubation (conditions *b*):

$$[W^*\text{-}tRNA] = n[E]\left[\frac{k_1k_2}{k_1+k_2}t + \frac{k_1^2}{(k_1+k_2)^2} \times (1-\exp(-k_1-k_2) + \exp(-k_1t) - 1\right] \quad (2)$$

In eq. (1) and (2) *n* refers to the number of subunits carrying out the synthesis of tryptophanyl-tRNA and [E] to the total enzyme concentration.

According to eq. (1) and (2) the straight lines taken as the steady state formation of aminoacyl-tRNA in curves *a* and *b* can be extrapolated back to time zero and define the positive burst π_1 and the negative burst π_2:

$$\pi_1 = n\,[E]\,\frac{k_1^2}{(k_1+k_2)^2} \qquad \text{(conditions } a\text{)}$$

$$\pi_2 = -n\,[E]\,\frac{2k_1k_2 + k_2^2}{(k_1+k_2)^2} \qquad \text{(conditions } b\text{)}$$

The amplitude between the two bursts is $\pi = \pi_1 - \pi_2 = n[E]$. The determination of this amplitude should then lead to the titration of the active sites actually carrying out the transfer process, independently of any consideration about the

chemical or conformational nature of the steps of rate k_1 and k_2. In particular k_2 can be the combination of the rates corresponding to several processes: activation of the amino acid, release of the aminoacyl-tRNA, isomerisation of the enzyme.

The analysis of curves a and b from fig.1 allowed us to obtain $n = 0.95 \pm 0.15$ and the rate constants k_1 and k_2: $k_1 = 25 \pm 5\ s^{-1}$, $k_2 = 11 \pm 3\ s^{-1}$. The step of rate k_1 is much faster than k_{cat}, the steady state rate of formation of aminoacyl-tRNA. This latter rate is $k_{cat} = k_1k_2/(k_1 + k_2) = 7.6\ s^{-1}$, in reasonable agreement with that obtained under normal steady state conditions ($6.5\ s^{-1}$ [12]). k_1 is also significantly faster than the rate constant k_2.

These results suggest that only one site could be titrated under the present conditions ($n = 0.95$) and that the transfer of tryptophan is not the limiting step in the overall aminoacylation reaction.

3.2. *Stopped-flow experiments*

The formation of the tryptophanyl–adenylate enzyme complex is associated with a quenching of the fluorescence of the enzyme [19]. The reverse reaction (pyrophosphorolysis) restores the initial fluorescence [20]. The transfer reaction can also be expected to bring about an increase of the enzyme fluorescence since it corresponds to the destruction of the adenylate–enzyme complex. On the other hand, the formation of an ester bond on the carboxylic group of tryptophan is known to strongly decrease the fluorescence of this amino acid [21]. Therefore, the pseudo steady-state aminoacylation of tRNA with tryptophan can be expected to give rise to a fluorescence quenching, preceded in the prestationary phase by a fluorescence increase when the protein is initially under the form of a tryptophanyl adenylate–enzyme complex.

In the first stopped-flow experiments the aminoacylation was started by mixing the free enzyme (1 μM) with all substrates (40 μM tryptophan, 5 mM ATP-Mg^{2+} and 5 μM tRNA) at time zero. Fig.2a shows that a continuous decrease of the fluorescence of the mixture was observed. This fluorescence levelled off after 5 s. This continuous decrease can be interpreted as showing the steady state formation of tryptophanyl-tRNA since its rate was $6\ s^{-1}$ until the tRNA concentration became rate limiting (after 2 s). This rate was obtained under steady state conditions in [12].

In the second stopped-flow experiments the enzyme was initially preincubated in the presence of tryptophan, ATP and traces of inorganic pyrophosphatase (under conditions of adenylate formation [7]). It was then mixed at time zero with tRNA and with the same excess of tryptophan and ATP as in fig.2a. Fig.2b shows that an initial fluorescence rise was visible before the same quenching as in fig.2a eventually occurred. This initial rise was interpreted as showing the destruction of the adenylate–enzyme complex; i.e., as corresponding to the transfer of tryptophan from the adenylate to tRNA. The absence of such an initial fluorescence rise when all substrates were added to

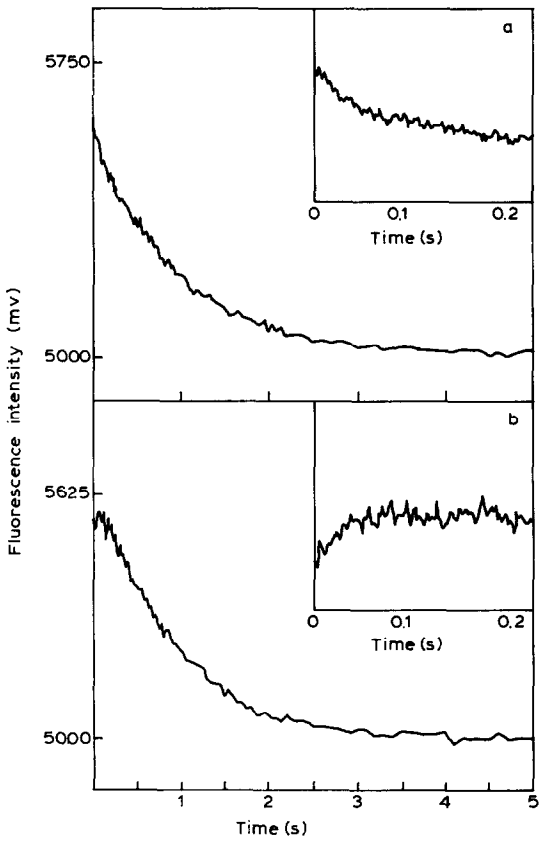

Fig.2. Presteady-state kinetics of tRNA aminoacylation under stopped-flow conditions. The first syringe (2 ml) contained 2 μM enzyme (a) or 2 μM enzyme, 100 μM ATP-Mg^{2+}, 5 μM L-tryptophan and 0.1 units/ml inorganic pyrophosphatase (b). The second syringe (2 ml) contained 80 μM L-tryptophan, 10 mM ATP-Mg^{2+} and 10 μM $tRNA^{Trp}$. In (b) the syringe mixture was allowed to incubate as in fig.1. The time course of the fluorescence change was recorded with two time bases. Rise time was 1 ms.

the free enzyme suggests two conclusions:
(1) This rise was specifically linked to the preformation of the adenylate;
(2) There exists a step slower than the transfer, which occurs before it.

The conclusions of the quenched-flow and of the stopped-flow data appear therefore to be consistent.

A burst in the formation of the aminoacyl-tRNA preceding the establishment of the stationary state has already been observed for the yeast arginyl enzyme [22], the *B. stearothermophilus* methionyl enzyme [23] and the yeast valyl enzyme [17] and is implicit in eq. (1). The finding that a single tryptophan molecule is transferred from the $(adenylate)_2$–enzyme complex supports the observation in [10] suggesting half-of-the-sites reactivity in the aminoacylation reaction. The single K_m of the aminoacylation under steady state conditions [9,12] should then correspond to the functioning of one site at a time in this reaction even though both tryptophanyl–adenylates were made by the enzyme [19].

In conclusion, these data are interpreted as showing that tryptophanyl-tRNA synthetase from beef pancreas presents the two following characteristics in the aminoacylation reaction:
(1) Only one subunit operates at a time in the transfer of the amino acid;
(2) The transfer step is not the limiting step in the overall aminoacylation.

Since the adenylate synthesis in the absence of tRNA is much faster than the aminoacylation it seems necessary to conclude that a third process (isomerisation steps or something else) plays a determinant role in the rate of catalysis.

Isomerisation steps have already been shown to constitute essential processes in the valine enzyme from yeast [34].

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