KINETICS OF FORMATION OF TRYPTOPHANYL-ADENYLATE BY TRYPTOPHANYL-tRNA SYNTHETASE FROM BEEF PANCREAS

Guy MERAULT, Julie LABOUESSE, Pierre-Vincent GRAVES and Bernard LABOUESSE
Institut de Biochimie Cellulaire et Neurochimie du CNRS, 1 rue Camille Saint-Saëns, 33000 Bordeaux, France

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1. Introduction

The formation of an aminoacyl-adenylate has been shown to occur for most aminoacyl-tRNA synthetases as a first step in the tRNA aminoacylation reaction [1,2]. In the particular case of tryptophanyl-tRNA synthetase from beef pancreas a tryptophanyl–adenylate–enzyme complex has been evidenced by gel filtration [3] and spectroscopic changes [4]. Two moles of adenylate can be bound per mole of dimeric enzyme, though under some conditions it has been suggested that tryptophan can also be convalently linked to the protein in a 1:1 ratio when the enzyme is obtained after fast purification procedure [5]. In the case of the non-covalent complex of the enzyme with L-tryptophan 2 mol tryptophan are bound in an apparent anti-cooperative way to the protein [6]. The formation of the adenylate–enzyme complex has not been studied under prestationary conditions which could show whether or not it is rate determining in the overall ATP–PP_i isotope exchange and in the tRNA aminoacylation reactions. Since the formation of the adenylate–enzyme complex can be evidenced by spectroscopic changes of the enzyme [4], this reaction can be followed kinetically using the fluorescence changes of the system. This formation can also be studied by the depletion of [^{32}P]ATP according to [7] and by the measurement of the stoichiometry of appearance of [^{14}C]tryptophanyl–adenylate [8] in order to ascertain that the recorded variations of the spectroscopic signal correspond really to the chemical step of carboxyl activation of tryptophan. This paper presents a preliminary report of the study of such a pre-stationary phase.

2. Materials and methods

Tryptophanyl-tRNA synthetase was prepared as in [9]. Its concentration was determined spectrophotometrically ($\epsilon_{280} = 9 \times 10^4\ M^{-1} . cm^{-1}$). The ATPase activity of the enzyme was negligible in the absence of L-tryptophan under the conditions of this study.

Chemicals were reagent grade (Merck). Yeast inorganic pyrophosphatase (spec. act. 500 units/mg) was from Boehringer. [γ-^{32}P]ATP (5000 Ci/mmol), [^{14}C]-ATP (60 mCi/mmol) and L-[^{14}C]tryptophan (52 mCi/mmol) were from the Radiochemical Centre, Amersham. All the experiments were performed in 50 mM Tris buffer (pH 8.0) containing 0.1 mM EDTA, 1 mM mercaptoethanol (or 1 mM dithioerythritol) and 1 mM magnesium acetate at 25°C (buffer A). Fluorescence intensities were measured with an Aminco SPF 500 spectrofluorimeter. In most cases the excitation wavelength was 295 nm and the emission wavelength 340 nm.

2.1. *Active site titration*

The active site stoichiometry of adenylate formation was determined by measuring the 'burst' of ATP depletion as in [7].

The stoichiometry was calculated using the following equation:

$$n = \frac{(cpm)_o - (cpm)_{extr.}}{(cpm)_o - (cpm)_\infty} \times \frac{(ATP)_o}{(E)_o}$$

where n is the no. mol ATP consumed/mol dimeric enzyme; $(cpm)_o$, $(cpm)_{extr.}$ and $(cpm)_\infty$ correspond to the radioactivity at time zero, after extrapolation back to time zero of the linear part of the curve of the time

course of the overall ATP depletion and after 2 h, respectively (fig.1).

The isolation of an enzyme–tryptophanyl–adenylate complex on DEAE-cellulose filters was carried out as in [8].

3. Results and discussion

Fig.1A shows the ATP consumption as a function of time when tryptophanyl-tRNA synthetase was mixed with a small excess of ATP and tryptophan, in the presence of inorganic pyrophosphatase. This consumption showed two distinct parts: the first part was over within ~1 min and was non-linear with time. The second part was slower and linear with time up to the end. The extrapolation to zero time of this linear part showed up the consumption of 2 mol ATP/mol dimeric enzyme during the first rapid part of the reaction. The semilogarithmic representation of this first part calculated from the overall ATP decrease by substraction of the contribution of the second part, showed a biphasic process (fig.1B, curve a). This plot could be decomposed into two distinct linear portions corresponding to two first-order reactions whose rate constants, under the experimental conditions of fig.1, were respectively, 0.06 and 0.01 s^{-1}. The consumption of ATP for each reaction corresponded to 1 mol ATP as seen from the extrapolation back to zero time of each linear portion of the semilogarithmic plot. The slow linear phase of the depletion was attributed to the hydrolysis of the adenylate bound to the enzyme, seen as a hydrolysis of ATP in the presence of tryptophan. In the absence of inorganic pyrophosphatase, the ATP depletion of the slowest part extrapolated back to zero time to <2 mol ATP/mol enzyme. In this later case only a single first order process could be evidenced after correction for the slow part (not shown).

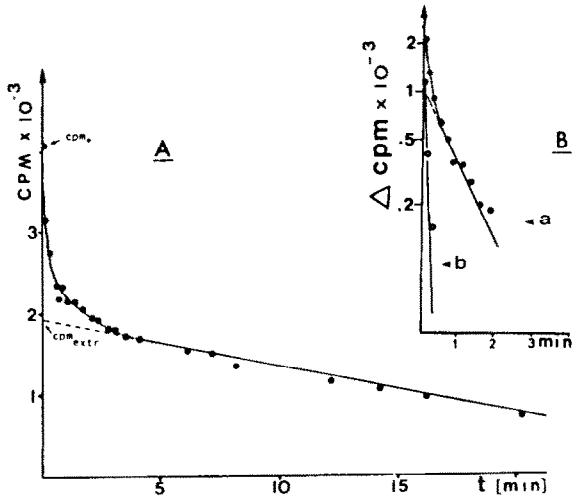

Fig.1. (A) Direct plot of the kinetics of [γ-^{32}P]ATP disappearence. The reaction mixture (600 μl) contained 5 μM [γ-^{32}P]ATP, 7 μM L-tryptophan, 12 μM tryptophanyl-tRNA synthetase, 0.4 U/ml of inorganic pyrophosphatase in buffer A. The reaction was initiated by addition of the enzyme solution. Just before initiation triplicate 20 μl aliquots were withdrawn for the determination of the zero time radioactivity (cpm_o). After incubation for different lengths of time 20 μl aliquots were pipetted and diluted 5% trichloroacetic acid. These aliquots swirled into 200 μl of a 2% suspension of activated charcoal (Norit) in 5% trichloroacetic acid containing 0.2 M phosphate. The samples were filtered through Whatman GF/C glass-fibre filters. The filters were washed, dried and the radioactivity determined in a Nuclear Chicago Mark I gas-flow counter. (B) Curve (a): semilogarithmic plot of curve (A). The extrapolation to time zero of the linear part of curve (A) was taken as the asymptote of its non-linear initial decrease. The extrapolation to time zero of the linear part of curve (a) was then taken as a secondary asymptote to obtain curve (b).

When L-[^{14}C]tryptophan was used in increasing concentration, at a fixed ATP level of 5 μM, a radioactive derivative could be retained on DEAE-cellulose filters. The total amount of bound tryptophan levelled off at a ratio of 2 mol tryptophan/mol enzyme (fig.2). The same result was obtained when [^{14}C]ATP was

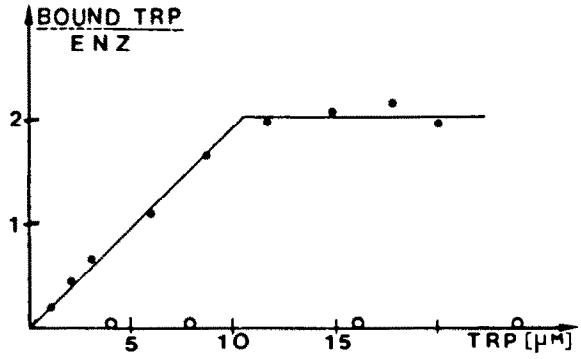

Fig.2. Stoichiometry of binding of L-[^{14}C]tryptophan to tryptophanyl-tRNA synthetase on DEAE-cellulose filters in the presence of ATP. The reaction mixture (100 μl) contained 5 μM tryptophanyl-tRNA synthetase deprived of bound tryptophan according to [10], either 50 μM ATP (•——•) or no ATP (o——o) and variable concentrations of L-[^{14}C]tryptophan. The mixtures were incubated 20 min in the presence of 2 U/ml of pyrophosphatase at 25°C then cooled to 4°C. 80 μl portions were applied on DEAE filters pre-soaked in buffer A, washed with 20 ml of the same buffer, dried and counted.

used instead of labelled tryptophan, at a fixed tryptophan concentration, and increasing ATP concentrations: 2 mol AMP/mol enzyme were retained on the filters (not shown).

In [4], the adenylate formation catalyzed by tryptophanyl-tRNA synthetase was associated with fluorescence changes of the system, which can be observed at 340 nm. Fig.3A shows the decrease in fluorescence of the system when ATP, tryptophan and enzyme were mixed in the presence of inorganic pyrophosphatase. The semilogarithmic plot of this process (fig.3B) was biphasic and could be resolved into two linear portions. The rate constants of these two phases were found to be the same as those determined for the rapid ATP depletion (fig.1) suggesting that the

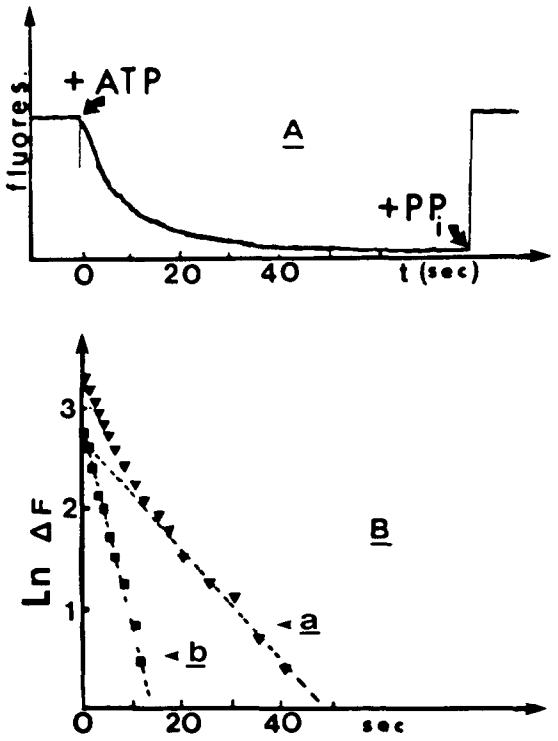

Fig.3. (A) Kinetics of the fluorescence change of the tryptophanyl-tRNA synthetase + tryptophan mixture after addition of ATP. Concentrations were 0.3 μM enzyme, 5 μM enzyme, 5 μM L-tryptophan, 50 μM ATP and 4 U/ml inorganic pyrophosphatase in buffer A. At the time indicated by the arrow 100 μM sodium pyrophosphate was added to the mixture. No fluorescence quenching was observed after addition of pyrophosphate in the absence of ATP. (B) Curve (a): semilogarithmic plot of curve (A). The linear part of curve (a) was used as secondary asymptote to obtain curve (b). The ordinate is expressed as natural logarithm of the change observed in (A).

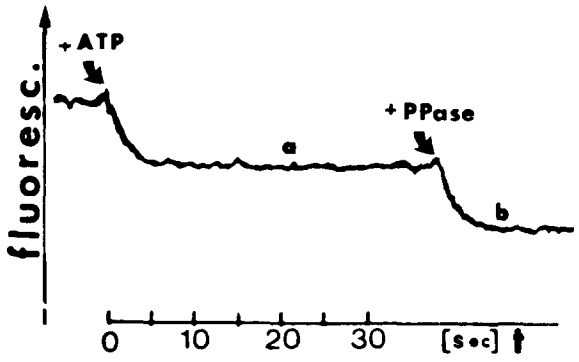

Fig.4. Fluorescence change of the enzymes tryptophan mixture after addition of ATP in the absence (a) and in the presence (b) of 0.4 U/ml inorganic pyrophosphatase (added at the time indicated by the arrow). Concentrations were 1 μM tryptophanyl-tRNA synthetase, 10 μM L-tryptophan and 50 μM ATP.

same phenomenon was being observed. In the absence of inorganic pyrophosphatase the same reaction mixture led to a much smaller variation of fluorescence (fig.4, curve a). Full change comparable to the one observed in fig.3A could be obtained by addition of pyrophosphatase to the reaction mixture (fig.4, curve b). Addition of PP_i after full change of fluorescence (fig.3A) brought about an immediate rise of fluorescence back to its initial value. The time course of this rise was too fast to be recorded on the spectrophotometer. The experiment described in fig.3 was repeated in the presence of only traces of inorganic pyrophosphatase (0.1 U/ml) using $^{32}PP_i$. After isolation of ATP on charcoal the incorporation of 1.9 mol $^{32}PP_i$/mol enzyme into the trinucleotide could be measured. This result indicated that close to 2 mol ATP/mol enzyme had been synthesized. It was not compatible with the existence under our experimental conditions of a covalent tryptophanyl–enzyme intermediate which would not be able to lead to ^{32}P-labelling of ATP from $^{32}PP_i$.

These observations confirm that upon addition of ATP and L-tryptophan, tryptophanyl-tRNA synthetase from beef pancreas leads to the formation of tryptophanyl–adenylate. This formation shows the following characteristics:

(i) It follows a biphasic process. Each phase leads to the formation of one tryptophanyl–adenylate on each subunit of the enzyme. The apparent rate of formation of the first adenylate is much larger than that of the second under the experimental conditions used here (by a factor of $\leqslant 5$). If this

were not the case the two exponentials of fig.1B and 3B could not be resolved in semilogarithmic plots as two definite straight lines extrapolating to the same amplitude at zero time. This biphasic process is likely to be related to the anti-cooperative binding of tryptophan to the dimeric enzyme [6]. The fluorescence change and the depletion method give the same rate constants.

(ii) It is fully reversible when pyrophosphate is added.

(iii) It does not go to completion when inorganic pyrophosphatase is omitted from the incubation mixture. The small amount of pyrophosphate formed is sufficient to prevent full formation of 2 adenylates/enzyme molecule, suggesting a high affinity of pyrophosphate for the enzyme–adenylate complex.

(iv) The adenylate is unstable in the presence of an excess of tryptophan over the amount of enzyme subunits as shown by the slow hydrolysis of ATP after the initial formation of 2 adenylates/dimer.

The major observation of this study is that at non-saturating concentration of tryptophan the rate of formation of the adenylate on each subunit does not appear to be identical. This suggests that these subunits are not kinetically equivalent. Such non-equivalence was observed for several oligomeric aminoacyl-tRNA synthetases [11,12] and might be a common feature of this class of enzymes, though in one case, the two subunits could not be kinetically distinguished [13,14].

The formation of tryptophanyl–adenylate and its pyrophosphorolysis are currently under study by stopped-flow techniques.

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