THE INFLUENCE OF THE ATP, AMINO ACIDS AND THEIR ANALOGS ON THE KINETICS OF THE AFFINITY LABELLING OF THE PHENYLALANYL-tRNA SYNTHETASE

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1. Introduction

Up to now the affinity labelling approach has been used only for the investigation of the functional topography of the synthetases [1].

It seems to us that some additional useful information may be obtained from the kinetic investigation of the affinity labelling. One may expect that slight conformational changes, without any significant influence on the total affinity of the reagent towards enzyme, may result in rather severe changes of the mutual orientation of the reacting group and the enzyme site to be modified. These changes may cause significant alteration in the rate of the modification.

In the present paper the kinetics of the affinity labelling of the phenylalanyl-tRNA synthetase from *E. coli* MRE-600 with *N*-chlorambucilyl-[^{14}C]phenylalanyl-tRNA was investigated and it was found that the reaction rate is significantly influenced by phenylalanine. ATP and some ATP analogs.

2. Materials and methods

Unfractionated tRNA was obtained from *E.coli* MRE-600 according to [2]. Purified phe-RSase* was obtained according to Stulberg [3]. [^{14}C]Phenylalanine, 220 Ci/mol ('Chemapol'), α-[^{32}P]ATP, 15 Ci/mmol ('Amersham'), HUFS ultrafilters ('Chemapol'), ATP, ADP, AMP, deoxyadenosine ('Reanal'), L-phenylalanine, L-tyrosine ('Reakhim', USSR), GTP, UTP (SKTB BAV, Novosibirsk, USSR) were used.

Preparative scale aminoacylation of unfractionated tRNA was performed according to [4]. Chb-tRNA was obtained as described earlier [5]. The extent of *N*-acylation was about 90–95%. The specific radioactivity of chb-tRNA was 14 000 cpm per A_{260} unit; For [^{14}C]phe-tRNA, 9000 cpm per A_{260} unit.

To obtain the complex, the enzyme (1.25×10^{-7} M) and chb-tRNA (3.75×10^{-7} M) were incubated in a final vol 200 μl for 1 min at 25°C in buffer A (0.025 M sodium acetate, pH 5.8–0.005 M $MgSO_4$–0.0001 M EDTA). The complex of synthetase with chb-tRNA was tested by measuring radioactivity retained after protein sorption onto a nitrocellulose filter (HUFS). The unbound radioactivity was washed off with buffer A (3 × 3 ml) saturated with chlorambucil to decrease the nonspecific absorption of chb-tRNA on the filters. The amount of nonspecific absorption of chb-tRNA, in a control experiment, was about 30% of the total radioactivity absorbed.

The kinetics of alkylation was tested by measuring radioactivity bound to the enzyme, retained after protein sorption onto HUFS filters at pH 7.5. Enzyme (1.25×10^{-7} M) and chb-tRNA (3.75×10^{-7} M) were incubated in buffer A or in buffer B (0.1 M Tris–HNO_3, pH 7.5–0.005 $MgSO_4$–0.0001 M EDTA) at 25°C in the presence or in the absence of L-phenylalanine, ATP and other ligands (see table 1). At definite time intervals 200 μl samples of the reaction mixture were diluted with Tris–HCl buffer (pH 7.5) to a total vol 3 ml. The solution was then passed through filters saturated with non-labelled

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** *Abbreviations* used: chb-tRNA, *N*-chloroambucilyl-[^{14}C] phenylalanyl-tRNA; [^{14}C]phe-tRNA, [^{14}C]phenylalanyl-tRNA; phe-RSase, phenylalanyl-tRNA synthetase (E.C. 6.1.1.).

Table 1
Influence of ligands on the rate of phe-RSase alkylation with chb-tRNA

pH	Ligand	Concentration M X 10^{-3}	Rate of alkylation %
5.8	–	–	100%
	L-phe	0.8	93
	L-phe	1.6	68
	L-phe	3.3	36
	L-tyr	3.3	100
	L-val	3.3	100
	ATP	2.5	80
	ATP	5.0	43
	ATP	10.0	21
	ATP + L-phe	10.0 + 3.3	0
	dA	10.0	100
	AMP	10.0	18
	PP	10.0	6
	ADP	10.0	87
	GTP	10.0	23
	UTP	10.0	100
7.5	–	–	100
	L-phe	3.3	50
	L-phe + ATP	3.3 + 10.0	0

chlorambucil prior to use. Filters were washed with the buffer (3 X 3 ml), dried and counted with a toluene scintillator. It was found that the reversible complexes of chb-tRNA or [^{14}C]phe-tRNA with the enzyme were not retained by the filter. At the same time the mean value of the efficiency of the absorption of the alkylated enzyme was 85%.

The reaction mixture used to estimate the rate of tRNA aminoacylation contained in 0.5 ml: 50 μmol Tris, 1.2 μmol ATP, 5 μmol $MgSO_4$, 2.4 X 10^{-3} μmol [^{14}C]phenylalanine, 10^{-2} μmol tRNA and 0.005–0.01 mg enzyme. pH of the reaction mixture was 7.5. The reaction was run at 25°C. The yield of [^{14}C]phe-tRNA was determined as described earlier [6].

3. Results and discussion

To prove the identity of the binding centers for tRNA and its analog we investigated the competition of tRNA and chb-tRNA for binding with phe-RSase

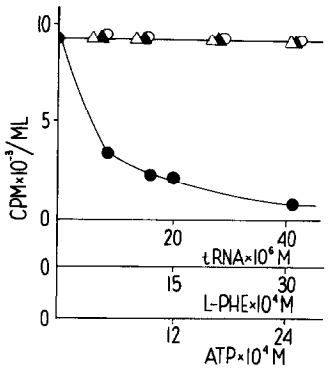

Fig.1. Dependence of Phe-RSase complex formation with chb-tRNA on the concentrations of tRNA (•), L-phenylalanine (▲), ATP (△), L-phenylalanine + ATP (○).

(see fig.1, curve 1). It is seen that tRNA competes with the analog in complex formation. At the same time the presence of L-phenylalanine or ATP or a mixture of them does not decrease the amount of complex formed between chb-tRNA and the enzyme (see fig.1, curves 2, 3, 4). A similar situation exists

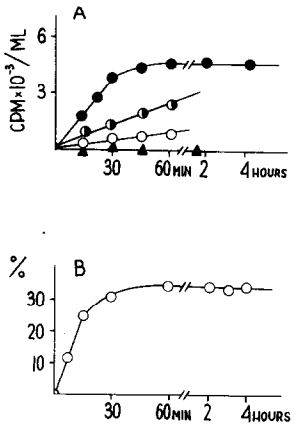

Fig.2 A) Kinetics of Phe-RSase alkylation with chb-tRNA in the absence of substrates (•), in the presence of L-phenylalanine 3.3 X 10^{-3} M (•), ATP 10^{-2} M (○), L-phenylalanine 3.3 X 10^{-3} M + ATP 10^{-2} M (▲). B) Dependence of the extent of Phe-RSase inactivation on the time of incubation with chb-tRNA. The extent of Phe-RSase inactivation was calculated as the ratio of the initial aminoacylation rates with aliquots of the incubation mixtures of the enzyme with chb-tRNA and with tRNA. The conditions of the incubations were the same as for alkylation. 0.01 ml aliquots were used for catalytic activity measurements as described in Materials and methods.

for all amino acids and ATP analogs indicated in table 1.

A typical time course of phe-RSase alkylation with chb-tRNA is shown in fig.2A (curve 1). It is seen that the reaction is completed within 1 hr.

The degree of inactivation of the enzyme in aminoacylation (see fig.2B) parallels the degree of covalent binding of chb-tRNA to enzyme.

Typical time courses of alkylation of phe-RSase in the presence of L-phenylalanine and ATP separately or together are also shown in fig.2A (curves 2, 3, 4). It is seen that the presence of either phenylalanine or ATP decreases the rate of modification.

To test the possibility of the ATP alkylation with chb-tRNA in the complex with the enzyme we carried out the procedure of phe-RSase alkylation in the presence of α-[^{32}P]ATP. There was no detectable [^{32}P]radioactivity bound to chb-tRNA during gelfiltration of the reaction mixture on Sephadex G-100 at pH 5.8 (see fig.3).

In the presence of a mixture of L-phenylalanine and ATP no modification takes place. These observations are in agreement with the results of other authors indicating the coupling of the reactions of amino acid and ATP with the enzyme [7–9]. A similar effect of these substrates on the alkylation rate was also observed at pH 7.5 (see table 1). At this pH value, however, a much greater excess of chb-tRNA over the enzyme is necessary to obtain measurable modification. It seems that this effect is due to decreased stability of the complex of synthetase with tRNA under these conditions.

The effects of a number of amino acids and of ATP analogs on the modification was also investigated. The results obtained are summarized in table 1. It is seen that the inhibitory action of the amino acid is specific for phenylalanine since comparable concentrations of L-valine and L-tyrosine do not alter the alkylation rate.

The inhibitory effect of ATP analogs is greatly influenced by the structure of the ligand. ATP, AMP and pyrophosphate exhibit the inhibitory effect without any influence on the amount of chb-tRNA in the complex with enzyme. ADP and deoxyadenosine are inactive in the same concentrations. UTP is inactive, while GTP rather unexpectedly decreases the rate of modification. The latter result permits us to suggest that GTP may be an effector in the reaction catalysed by phe-RSase. Therefore we investigated the influence of GTP on aminoacylation of tRNA^{Phe}. The kinetic curves of aminoacylation of tRNA^{Phe} with [^{14}C] phenylalanine in the presence of GTP are represented in fig.4. It is seen that the aminoacylation rate decreases as the concentration of GTP is increased.

The most likely reason for the inhibitory effect of the amino acid, ATP and some analogs on the phe-RSase alkylation seems to be the competition between them and chb-[^{14}C] phe-residue for the site of alkylation on the enzyme. The other reason appears to be the change of the mutual orientation of the reacting group and the enzyme site to be modified as a result of some conformational change induced by the interaction of the enzyme with added ligands.

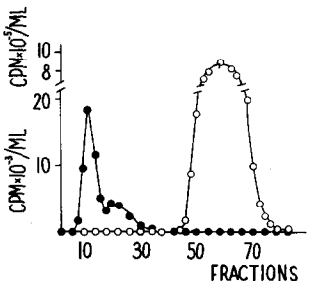

Fig.3. Gel-filtration of the mixture of Phe-RSase with chb-tRNA in the presence of α-[^{32}P]ATP on Sephadex G-100, at pH 5.8, 25°C. The reaction mixture contained 3.75×10^{-7} M chb-tRNA, 1.25×10^{-7} M Phe-RSase, 5×10^{-3} M ATP. It was incubated preliminarily in buffer A for 1.5 hr. The dimensions of the column were 34 × 0.8 ml, the fraction volume 0.4 ml. The elution was performed in buffer A. (•) ^{14}C-radioactivity, (○) ^{32}P-radioactivity.

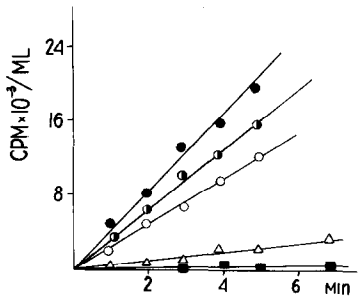

Fig.4. Kinetics of aminoacylation of tRNA with [^{14}C]phenylalanine at 25°C (•) without GTP, (◉) 10^{-3} M GTP, (○) 2.5×10^{-3} M GTP, (△) 5.0×10^{-3} M GTP, (■) 10^{-3} M GTP in the absence of ATP.

The results obtained demonstrate that the kinetics of affinity modification is highly sensitive to the action of small ligands even if the ligands have no apparent influence on the stability of the complex between enzyme and the reactive analog of tRNA. Therefore, it may be used as a method of investigation of the interaction between the binding sites of different substrates.

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