PHENYLALANYL-tRNA SYNTHETASE FROM *ESCHERICHIA COLI* MRE-600

Activation by nucleotides and affinity modification of the effector binding sites

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1. Introduction

Some nucleotides and their analogs were shown [1–5] to interact with aminoacyl-tRNA synthetases as uncompetitive and non-competitive inhibitors. Therefore, it seems reasonable to suggest the existence of some additional nucleotide binding centers for these enzymes.

It was shown in [6] that some nucleotide mono-, di- and triphosphates as well as β-anilidates of diphosphates and γ-anilidates of triphosphates exhibit some activation properties at low concentration in the reaction catalyzed by tryptophanyl-tRNA synthetase from beef pancreas.

Here we demonstrate that activation in the presence of some nucleotides and their derivatives is observed also for other aminoacyl-tRNA synthetases. We describe the enhancement of the enzymatic activity as well as of the affinity for ATP of phenylalanyl-tRNA synthetase in the presence of nucleotides and their analogs. This effect disappears after photoaffinity labelling of Phe-RSase with β-(*p*-azidoanilide)–GDP and *N*-methyl,*N*-(*p*-azidobenzyl)-γ-amide–ATP. These results suggest that modification takes place at the effector sites of the enzyme.

2. Materials and methods

Phe-Rsase from *E. coli* MRE-600 was purified using the procedure in [7] with some modifications.

Abbreviations: Phe-RSase, phenylalanyl-tRNA synthetase (EC 6.1.1 20); azido-ATP_1, γ-(*p*-azidoanilide)–ATP; azido-ATP_2, *N*-methyl, *N*-(*p*-azidobenzyl)-γ-amide–ATP, azido-GDP, β-(*p*-azidoanilide-)–GDP

E. coli MRE-600 unfractionated tRNA was purified according to [8]. [^{14}C]Phenylalanine (330 Ci/mol), [^{14}C]ATP (30 Ci/mol), [^{14}C]GDP (30 Ci/mol) and nitrocellulose ultrafilters (0.24 μm pore sizes) were from 'Chemapol' (Czechoslovakia). FN-16 paper filters were from 'Filtrak' (FRG). Nucleotides were from 'Reanal' (Hungary). Nucleotides containing <0.5% impurities were used. All other reagents were analytical grade.

p-Azidoaniline and *N*-methyl, *N*-(*p*-azidobenzyl)-amine were obtained according to [9]. All β- and γ-anilidates of nucleotides were obtained by condensation of nucleotides with amines in water in the presence of water-soluble carbodiimide. The synthesis and characterisation of these compounds were detailed in [6].

Photo induced modification of Phe-RSase was performed by irradiation at 10°C of the enzyme solution using a CVD-120 A mercury lamp and a cut-off filter transmitting wavelengths >300 nm. The reaction mixture contained 0.05 M Tris–HCl buffer (pH 7.5), 10^{-3} M $MgCl_2$, 10^{-5}–2×10^{-4} M photoreactive analog and 0.2–0.5 mg Phe-RSase in 0.1–0.5 ml final vol. Protein concentration was determined spectrophotometrically at 280 nm ($A_{1\,cm}^{mg/ml} = 0.9$) [10].

Irradiated aliquots and samples kept in the dark were taken to measure the level of the covalent attachment of the reagent and the initial rate of tRNA aminoacylation. The covalent attachment of the reagents to the enzyme was determined by measuring the radioactivity retained after protein adsorption on the nitrocellulose filters. The unbound radioactivity was washed off with 0.05 M Tris–HCl buffer (pH 8) containing 10^{-3} M ATP.

The amount of [^{14}C]phenylalanyl-tRNA formed was determined by measuring the radioactivity pre-

cipitated by cold 5% trichloroacetic acid on FN-16 paper filters. The reaction mixtures contained: 0.05 M Tris–HCl buffer (pH 7.5), $5-1 \times 10^{-3}$ M $MgCl_2$, 5×10^{-6} M [^{14}C]phenylalanine, $10^{-4}-10^{-3}$ M ATP, $10^{-8}-10^{-3}$ M nucleotides or their β,γ-amidates, 1–2 mg/ml tRNA and 1–2 μg/ml Phe-RSase. The radioactivities were counted using a Mark II Nuclear Chicago Scintillation Counter.

3. Results and discussion

γ-(*p*-Azidoanilide)–ATP was demonstrated to be a competitive inhibitor of ATP in the reactions catalyzed by aminoacyl-tRNA synthetases [11,12]. However, at low concentration this analog exhibits some activation properties. In fig. 1 the dependence of the rate of tRNA aminoacylation on the concentration of azido-ATP_1 is presented. It is seen that the activation proceeds at an analog concentration 2 orders of magnitude lower than the K_i value (1.2×10^{-3} M).

The influence of some other nucleotides and their β- and γ-anilidates is shown in table 1. The analog concentration providing maximum activation significantly depends on the nucleotide structure. The level of activation varies for different enzyme preparations. At high concentrations of nucleotides and their derivatives ($10^{-3}-10^{-2}$ M) inhibition of tRNA aminoacylation is observed. The inhibition could not occur through single chelation of free $MgCl_2$ by the investigated nucleotides. The inhibitory effects of nucleotides were about the same at 10^{-3} M and at 5×10^{-3} M $MgCl_2$ and the remarkable inhibitory action of the adenine nucleotides is also observed at 10^{-4} M. The results obtained suggest the existence of some additional noncatalytic nucleotide binding centers. A set of nucleotides and their derivatives may interact with these centers as effectors of the reactions catalysed by aminoacyl-tRNA synthetases. If this is the case β- and γ-anilidates of nucleotides containing the photoreactive groups should modify noncatalytic binding centers.

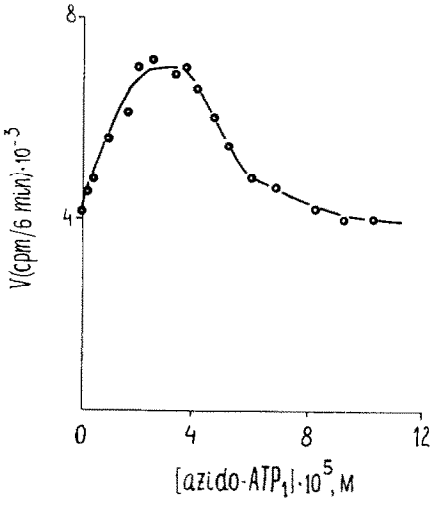

Fig.1. Dependence of the initial rate of the aminoacylation of $tRNA^{Phe}$ on the concentration of azido-ATP_1.

Table 1
The influence of nucleotides and their β- and γ-amidates on the rate of tRNA aminoacylation catalysed by phenylalanyl-tRNA synthetase

Nucleotide	Level (%) of activation	Analog concentration (μM) at maximum activation
GMP	25–30	1.0
CMP	25–30	70
NAD	25–30	60
Azido-ADP	30–40	100
Azido-GDP	30–40	70
Azido-ATP_1	40–80	30

In table 2 the results of photoattachment of β-(*p*-azidoanilide)–GDP to Phe-RSase are presented. GMP has a strong protective action against covalent attachment of β-(*p*-azidoanilide)–GDP. GMP appears to

Table 2
The influence of ligands on the level of covalent attachment of the β-(*p*-azidoanilide)–[^{14}C]GDP (10^{-4} M) to Phe-RSase

Ligand	Concentration (mM) of ligands	Decrease of the level of covalent attachment of azido-GDP[a] (mol/mol enzyme)
ATP	1.0	0
Phenylalanine	0.1	0
ATP + Phe	1.0 + 0.1	0.3–0.4
GMP	1.0	1.4–1.5
GMP + Phe	1.0 + 0.1	1.4–1.5
GMP + ATP	1.0 + 1.0	1.4–1.5
GMP + ATP + Phe	1.0 + 1.0 + 0.1	1.5–1.6

[a] The level of the covalent attachment of analog in the absence of ligands was 1.8 mol/mol enzyme

Time of irradiation was 40 min

protect 2 sites in the enzyme against the reaction with the analog. There is no effect of either ATP or phenylalanine when taken separately. However the mixture of these substrates has a slight protective action probably due to binding of ATP to the effector site in the presence of phenylalanine.

The modification of the effector sites with azido-GDP leads to the loss of enzyme sensitivity to the effectors (mono, di-, triphosphates of nucleotides and their amidates). The results are presented in fig.2. The presence of GMP in the irradiated mixture permitted us to obtain the enzyme with the unoccupied effector sites. The addition of nucleotides in this case results in enzyme activation (see fig.2).

The dependence of the tRNA-aminoacylation rates on ATP concentration for the enzyme irradiated in the presence of azido-GDP is presented in fig.3. The occurrence of β-(*p*-azidoanilide)–GDP covalently linked to the enzyme leads to the biphasic double reciprocal plot. K_m value for ATP calculated from the right part of the biphasic curve 2 decreases 5–7 times.

A decrease of the app. K_m value for ATP is observed also in the presence of effectors without their covalent binding to the enzyme. The double reciprocal plots of tRNA-aminoacylation on the concentration of ATP at different concentrations of azido-GDP, azido-ADP, CMP and NAD are presented in fig.4.

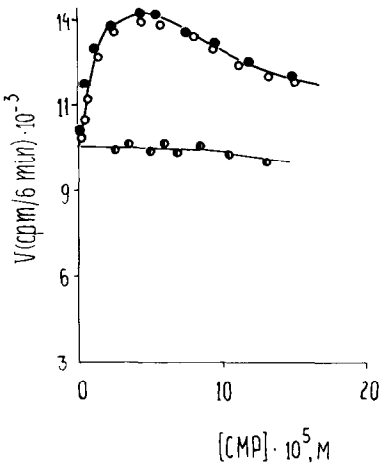

Fig.2. Dependence of the initial rate of aminoacylation of $tRNA^{Phe}$ on the concentration of CMP for the different preparations of Phe-RSase: (•) enzyme preincubated with azido-GDP in the dark; (○) enzyme irradiated in the presence of azido-GDP and GMP (10^{-3} M); (•) enzyme irradiated with azido-GDP. Azido-GDP was 10^{-4} M; incubation was for 1 h.

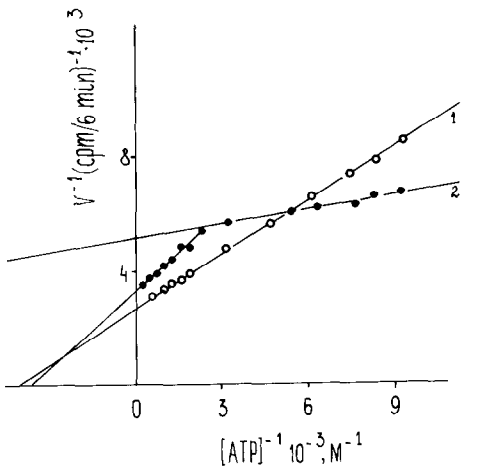

Fig.3. Double reciprocal plot of the dependence of the initial rate of aminoacylation of $tRNA^{Phe}$ on ATP concentration: 1, enzyme incubated with azido-GDP in the dark; 2, enzyme irradiated in the presence of azido-GDP. Azido-GDP was 10^{-4} M; irradiation was for 1 h.

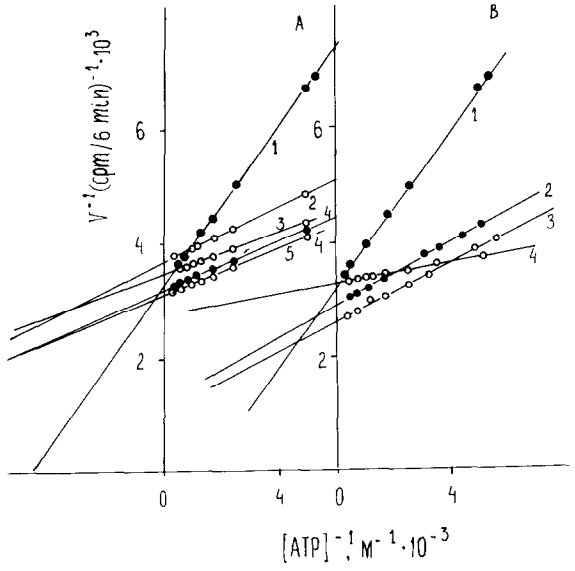

Fig.4. Double reciprocal plot of the dependence of the initial rate of $tRNA^{Phe}$-aminoacylation on the concentration of ATP. (A): 1, in the absence of nucleotides; 2, in the presence of azido-GDP (1.5×10^{-3} M); 3, azido-GDP (2.1×10^{-3} M); 4, in the presence of azido-ADP (3.6×10^{-3} M); 5, azido-ADP (5×10^{-3} M). (B): 1, in the absence of nucleotides, 2, in the presence of CMP (4×10^{-3} M); 3, CMP (2×10^{-3} M); 4, 2.5×10^{-3} M NAD.

The modification of the effector sites has been performed also with *N*-methyl, *N*-(*p*-azidobenzyl)-γ-amide-ATP. Azido-ATP$_2$ is a competitive inhibitor of Phe-RSase with respect to ATP and phenylalanine in the reaction of aminoacylation with K_i values of 3×10^{-5} M and 6×10^{-5} M, respectively. It was shown earlier that the ultraviolet irradiation of this analog with Phe-RSase results in inactivation of the enzyme [13]. It is worth mentioning that there is no protection in the presence of ATP. However the presence of phenylalanine and ATP or phenylalanine alone decreases the level of enzyme inactivation [14]. These results suggest that azido-ATP$_2$ should modify both the catalytic site and the noncatalytic nucleotide binding center.

The data concerning the influence of the ligands on covalent attachment of azido-ATP$_2$ to the enzyme are presented in table 3. Phenylalanine alone and in the presence of ATP has the same protective action and really protects two sites in the enzyme. The mixture of GMP and phenylalanine provides additive protection against the covalent attachment of the azido-ATP$_2$.

Ultraviolet irradiation of Phe-RSase with azido-ATP$_2$ in the presence of phenylalanine as protective ligand for the active sites results in the enzyme insensitivity to the effector action of nucleotides. The irradiation of the enzyme in the presence of GMP, phenylalanine and ATP leads to the enzyme derivative which is sensitive to the presence of nucleotides. (The results are presented in fig.5.)

The data obtained enable one to suggest that the sites blocked by azido-GDP and azido-ATP$_2$ are the effector centers of Phe-RSase. Centers of this kind were found also in tryptophanyl-tRNA synthetase from beef pancreas [6].

Table 3
The influence of ligands on the level of covalent attachment of *N*-methyl, *N*-(*p*-azidobenzyl)-γ-amide-[^{14}C]ATP (10^{-4} M) to Phe-RSase

Ligand	Concentration (mM) of ligands	Decrease of level of covalent attachment of azido-ATP$_2^a$ in the presence of ligands (mol/mol enzyme)
Phenylalanine	0.1	1.4–1.5
Phe + ATP	0.1 + 1.0	1.4–1.5
GMP	1.0	1.7–1.8
GMP + Phe	1.0 ± 0.1	3.2–3.3
GMP + Phe + ATP	1.0 ± 0.1 + 1.0	3.2–3.3

[a] The level of the covalent attachment of analog in the absence of ligands was 4 mol/mol enzyme

Time of irradiation was 2 h

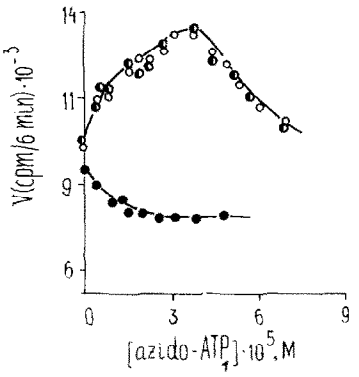

Fig.5. Dependence of the tRNA^{Phe} aminoacylation rate on the concentration of azido-ATP$_1$: (○) enzyme preincubated with azido-ATP$_2$ in the dark; (●) enzyme irradiated with azido-ATP$_2$ in the presence of phenylalanine (10^{-4} M); (◉) enzyme irradiated in the presence of GMP (10^{-3} M) phenylalanine (10^{-4} M) and ATP (10^{-3} M).

We observed activation in the presence of nucleotides of tRNA aminoacylation catalyzed by other aminoacyl-tRNA synthetases, e.g., valyl- and methionyl-tRNA synthetases from *E. coli* MRE-600 (data no presented). Therefore it may be concluded that the occurrence of effector centers is a feature general for this type of enzymes. However to understand the biological regulatory significance of these centers additional experiments should be carried out.

Acknowledgements

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