INVESTIGATION OF THE PHENYLALANYL-tRNA SYNTHETASE MODIFICATION WITH γ-(*p*-AZIDOANILIDE)-ATP

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1. Introduction

Photoreactive compounds containing azido group are widely used for the affinity labelling of proteins [1–4]. Recently ATP-γ-*p*-azidoanilidate* was found to be competitive inhibitor of ATP for phenylalanyl-tRNA synthetase (EC 6.1.1.) and binding of the ATP moiety to the enzyme under irradiation of the mixture of the enzyme with azido-ATP_1 was demonstrated [5]. However these data were insufficient to prove that affinity labelling of the enzyme takes place.

In the present paper it is shown that binding of the ATP moiety of azido-ATP_1 to Phe-RSase, up to 20 mol per mol of enzyme, does not result in enzyme inactivation and cannot be prevented by addition of excess of either ATP or other substrates. Therefore in spite of the affinity of the photoreagent for enzyme the modification in this case is not specific to the main ATP binding center. At the same time another ATP analog of similar structure with disrupted conjugation between ATP and the azido group, azido-ATP_2, was found to inactivate the same enzyme under irradiation.

2. Materials and methods

E. coli MRE 600 unfractionated tRNA was purchased from Special Design Bureau of BAS (Novosibirsk, USSR), [^{14}C]phenylalanine (330 Ci/mol) and nitrocellulose ultrafilters HUFS (0.24 μm) were from Chemapol (Czechoslovakia), ATP was from Reanal (Hungary). [^{3}H]ATP (0.07 mCi/mM) was from Isotope (USSR). All other preparations were analytical grade reagents.

Phe-RSase from *E. coli* MRE 600 was purified using a published procedure to specific activity 200 units/mg [6]. The purity of the enzyme (80–85%) was estimated also by means of polyacrylamide gel electrophoresis. Protein concentration was determined spectrophotometrically at 280 nm ($A\,\frac{\text{mg/ml}}{\text{cm}} = 0.9$).

p-Azidoaniline and *N*-methyl, *N*-(*p*-azidobenzyl)-amine were obtained according to [7]. [^{3}H]γ-(*p*-azidoanilidate) of ATP was synthesized using *p*-azidoaniline according to a general method for preparing γ-amides of ATP [8].

Azido-ATP_2 was synthesized using the active ATP-derivative prepared by the reaction of ATP with dicyclohexylcarbodiimide in a manner similar to [9]. Azido-ATP_1 and Azido-ATP_2 were characterised by UV and IR-spectroscopy.

Photoinduced modification of Phe-RSase was performed by irradiation at 14°C using an SVD-120A mercury lamp and a cut-off filter transmitting wavelengths greater than 300 nm. The intensity of UV-irradiation was determined using chemical actinometry [10] and was 1.9×10^{17} quant/sec. The reaction mixtures contained enzyme (7×10^{-7}–1×10^{-6} M) and azido-ATP (10^{-5}–10^{-3} M) in a final volume 1–2 ml of Tris–HCl 0.05 M, pH 7.5, with 10^{-3} M $MgSO_4$. The reaction was complete in 10 min under irradiation conditions used**.

Aliquots of irradiated and control samples were

*Abbreviations: Azido-ATP_1-γ-(*p*-azidoanilide)-ATP, Azido-ATP_2-*N*-methyl, *N*-(*p*-azidobenzyl)-γ-ATP-amide; Phe-RSase-phenylalanyl-tRNA synthetase (AMP) (EC 6.1.1.); BSA-bovine serum albumin.

**In the previous paper [5] a significantly lower lamp intensity was used thus resulting in a longer reaction time.

taken to measure the level of the covalent attachment of the reagent and the initial rate of tRNA aminoacylation.

The covalent attachment of [^{3}H]azido-ATP to the enzyme was determined by measuring radioactivity retained with the protein on the nitrocellulose filters at pH 7.5 [5].

The amount of [^{14}C]Phe-tRNA formed was determined by measuring the radioactivity precipitated by cold 5% trichloroacetic acid on FN-16 paper filters [12]. The radioactivities were counted using a Mark II Nuclear Chicago Scintillation counter.

3. Results and discussion

The following conditions must be fulfilled in order to prove that the interaction of some reactive analog of the substrate with protein is affinity labelling: (1) the analog has to be a competitive inhibitor or substrate; (2) the limiting number of moles of the analog covalently bound to enzyme in the presence of excess reagent has to be equal to the number of sites specific for substrate; (3) covalent binding of the analog has to inactivate the enzyme; (4) the covalent attachment must be prevented in the presence of a sufficient excess of substrate; (5) for a multisubstrate reaction, with mutual coupling of the active centers, addition of other substrates must influence the course of the affinity labelling.

The fulfilment of the first criterion was already demonstrated [5]. Azido-ATP$_1$ was found to be a strong competitive inhibitor of tRNA aminoacylation catalyzed by Phe-RSase, the K_1 value being only two times greater than K_M for ATP.

A typical dependence of the number of the [^{3}H]-azido-ATP$_1$ molecules attached to enzyme under irradiation on the reagent concentration is shown in fig.1. It is seen that as many as 20 mol of the reagent may be covalently bound per mol of the enzyme at an analog concentration of 10^{-3} M. No inactivation of the enzyme was found after covalent attachment of either 1 or 20 mol of the analog (fig.2). As may be seen from table 1 the excess of ATP does not prevent the reaction of azido-ATP$_1$ with the enzyme. In a separate experiment it was demonstrated that nearly an equal number of moles of the reagent may be attached to BSA under the same reaction conditions.

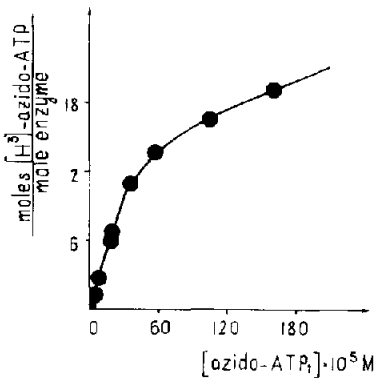

Fig.1. Dependence of the number of [^{3}H]azido-ATP molecules bound to Phe-RSase on the concentration of azido-ATP$_1$. The reaction mixtures contained 0.4 mg/ml enzyme, 0.05 M Tris–HCl, pH 7.5, 10^{-3} M $MgSO_4$. The time of irradiation was 10 min.

The results obtained mean that there is no ATP-site-specific photoinduced reaction between *E. coli* Phe-RSase and azido-ATP$_1$ in spite of rather high affinity of the reagent for enzyme.

The mutual interactions of ATP, amino acid and tRNA binding centers of ARSases were demonstrated earlier [13–15]. We attempted to enhance specificity

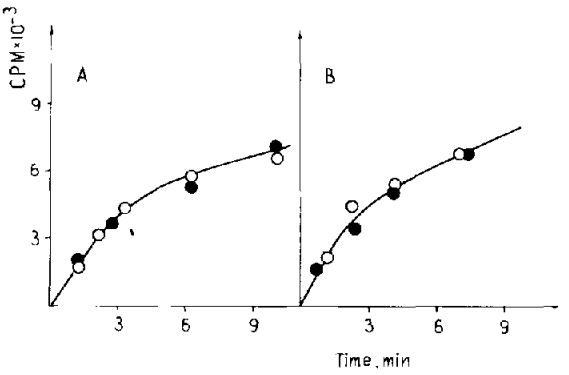

Fig.2. Kinetics of tRNA-aminoacylation in the presence of modified enzyme (○) and in the presence of enzyme preincubated in the dark (●) (A) 1 mol [^{3}H]azido-ATP$_1$ bound to 1 mol Phe-RSase at 2.65×10^{-5} M [^{3}H]azido-ATP$_1$ concentration; (B) 20 mol [^{3}H]azido-ATP bound to 1 mol Phe-RSase. The reaction mixtures for tRNA aminoacylation contained 0.05 M Tris–HCl, pH 7.5, 10^{-3} M ATP, 5×10^{-3} M $MgSO_4$, 3×10^{-6} M [^{14}C]phenylalanine, 2×10^{-5} M tRNA and 5–7 μg Phe-RSase in a total vol 0.3 ml. Reaction was performed at 25°C.

Table 1
The influence of the presence of Phe-RSase substrates on the reaction of [^{3}H]azido-ATP$_1$ attachment to Phe-RSase on irradiation

Ligand	Concentration (M)	The attachment of [^{3}H]azido-ATP to Phe-RSase (moles per mole of enzyme)
without substrates	–	4.43
ATP	1.0×10^{-4}	5.56
phenylalanine	1.0×10^{-5}	5.18
ATP + phenylalanine	1.0×10^{-4} + 1.0×10^{-5}	6.17
tRNA^{Phe}	6.4×10^{-6}	2.23

of the reaction with azido-ATP$_1$ by doing the reaction in the presence of other substrates. The results are presented in table 1. It is seen than the level of covalent attachment even slightly increases in the presence of phenylalanine, ATP or both. In the presence of tRNA the number of reagent molecules attached decreases approx. by 2.

A possible explanation of this result is the absence of a suitable reactive group of the protein in the vicinity of a nitrene radical formed from azido-ATP under irradiation. An additional reason may be strong conjugation between triphosphate and azidophenyl moieties of the reagent which restricts the area of the protein amenable to the attack of the nitrene radical formed.

Therefore, we may expect specific reaction to occur in the case of some other proteins as well as with reagents of other dimensions and lacking strong conjugation between affine and reactive moieties of the reagent.

It was demonstrated that azido-ATP$_1$ does inactivate tryptophanyl-tRNA-synthetase from beaf pancreas (these results obtained in collaboration with V.Z. Akhverdyan and L.L. Kisselev will be published separately).

In the present work we have prepared another photoreactive ATP analog namely azido-ATP$_2$, containing N-(CH$_3$)-CH$_2$-C$_6$-H$_4$-N$_3$ grouping instead of N-H-C$_6$-H$_4$-N$_3$ in the γ-position of ATP. In this reagent besides somewhat greater dimension there is a freedom of rotation around N–C and C–C bonds due to

Table 2
The influence of ATP and phenylalanine on the Phe-RSase inactivation on irradiation in the presence of azido-ATP$_2$.

Ligands	Phe-RSase inactivation (%)
Without substrates	95
ATP (2.5×10^{-3} M) + phenylalanine (1.0×10^{-4} M)	50

The reaction mixtures contained 0.05 M Tris–HCl, pH 7.5, 8×10^{-3} M $MgSO_4$, 2×10^{-5} M azido-ATP$_2$, 0.096 mg/ml enzyme in total vol 0.6 ml. The time of irradiation was 1 h at 14°C. For conditions of aminoacylation reaction see in the legend to fig.3.

disrupted conjugation between triphosphate and phenylazido moieties. It was demonstrated that the irradiation of Phe-RSase with azido-ATP$_2$ results in an inactivation of the enzyme (fig.3). Moreover the presence of the mixture of ATP (2.5×10^{-3} M) and phenylalanine (1.0×10^{-4} M) decreases the degree of inactivation of Phe-RSase by azido-ATP$_2$ (2×10^{-5} M), irradiation during 1 h at 14°C from 95% to 50%. These results indicate that photoinduced reaction of Phe-RSase with azido-ATP$_2$ is really affinity labelling. The decrease of the extent of inactivation by azido-ATP$_2$ in the presence of azido-ATP$_1$ was also demonstrated thus suggesting that both reagents bind

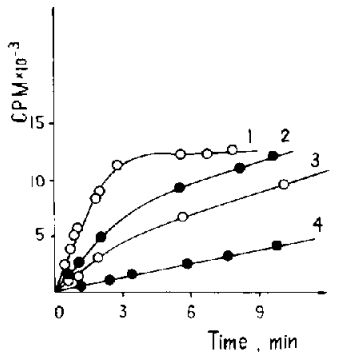

Fig.3. Kinetics of tRNA-aminoacylation catalysed of enzyme irradiated in the presence of azido-ATP$_2$. (1) Without irradiation (2) irradiation for 30 min; (3) irradiation for 1 h (4) irradiation for 1 h 30 min. The reaction mixtures contained 0.05 M Tris–HCl, pH 7.5, 3.6×10^{-3} M $MgSO_4$, 4.55×10^{-5} M azido-ATP$_2$. 0.16 mg/ml enzyme in total vol 0.6 ml. 0.005 ml aliquots of these mixtures were used for measuring the aminoacylation rate.

to the same site of the enzyme. The structural pecularities of one of them prevent it being an affinity label of Phe-RSase.

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References

[1] Fleet, G. W. J., Porter, R. R. and Knowles, J. R. (1969) Nature, 224, 511–512.
[2] Converse, G. A. and Richards, F. P. (1969) Biochemistry, 8, 4431–4436.
[3] Girshovich, A. S., Bochkareva, E. S. and Pozdnjakov, V. A. (1974) Acta Biol. Med. Germ. 33, 639–648.
[4] Ruoho, A. E., Kiefer, H., Roeder, P. E. and Singer, S. I. (1973) Proc. Nat. Acad. Sci., USA, 70, 2567–2571.
[5] Budker, V. G., Knorre, D. G., Kravchenko, V. V., Lavrik, O. I., Nevinsky, G. A. and Teplova, N. M. (1974) FEBS Lett., 49, 159–162.
[6] Stulberg, M. P. (1967) J. Biol. Chem. 242, 1060–1064.
[7] Silberrad, O. and Smart, D. I. (1906) J. Chem. Soc. 89, 170–171.
[8] Babkina, G. T., Zarytova, V. F. and Knorre, D. G. (1975) Bioorg. Khim. 1, No 5, 611–615.
[9] Zarytova, V. F., Knorre, D. G., Kurbatov, V. A., Lebedev, A. V., Samukov, V. and Shishkin, G. V. (1975) Bioorg. Khim. 1, No 6, 793–799.
[10] Calvert, J. G. and Pitts, J. N. Jr. (1968) Photochemistry, p.p. 625–627, 'Myr', Moskva.
[11] Kelmers, A. D., Novelli, G. D. and Stulberg, M. P. (1965) J. Biol. Chem. 240, 3979–3983.
[12] Holler, E., Rainey, P., Orme, A., Bennet, E. L. and Calvin, M. (1973) Biochemistry, 12, 1150–1159.
[13] Blanquet, S., Payat, G., Poiret, M. and Waller, J-P. (1975) Eur. J. Biochem., 51, 567–571.
[14] Maelicke, A., Engel, C. and Cramer, F. (1974) Eur. J. Biochem. 42, 311–314.