PHOTOAFFINITY REAGENTS FOR MODIFICATION OF AMINOACYL-tRNA SYNTHETASES

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1. Introduction

Up to now, a few derivatives of aminoacyl-tRNA with a reactive group attached to the aminoacyl residue [1–4] and a reactive analog of an amino acid [4] have been used for the affinity labeling of aminoacyl-tRNA synthetases (EC 6.1.1).

For detailed investigation of the active centers of these enzymes, it is necessary to prepare tRNA analogs bearing reactive group at some other sites of the tRNA molecule, as well as reactive ATP analogs.

The present communication describes ATP and tRNA analogs containing a photoreactive azido group, namely ATP-γ-*p*-azidoanilide and tRNA containing the $N_3C_6H_4NH$-CO-CH_2-group attached to a 4-thiouridine residue.

The possibility of using the photoreactivity of the 4-thiouridine residue of tRNA for photoaffinity labeling of phe-RSase* is also reported; a similar approach has been proposed by other workers [6] who described a light induced reaction of 4-thiouridine triphosphate with RNA polymerase.

2. Materials and methods

Unfractionated tRNA was obtained from *E. coli* MRE-600 according to ref. [6]. Purified phenylalanyl-tRNA synthetase was obtained according to [8]. [^{14}C]-Phenylalanine (220 Ci/mole, 'Chemapol', Czechoslovakia), HUFS ultrafilters ('Chemapol'), α-[^{32}P]-ATP (1950 Ci/mole, 'Amersham', England), ATP ('Reanal', Hungary) were used. [^{14}C]Phe-tRNA of specific activity 10 000 cpm per A_{260} unit was prepared according to ref. [9]. *p*-Azidoaniline was obtained from *p*-amino-acetanilide according to [10], m.p. 60–62°C. γ-(*p*-Azido-anilide) of ATP was synthesized using the active ATP derivative, prepared by the reaction with *N*-cyclohexyl-*N'*-β-(4-methylmorpholinium)-ethylcarbodiimide [11]. The details of the synthesis and the identification of this product will be described elsewhere.

To prepare *p*-azidobromacetanilide 0.2 mmole bromoacetic acid were mixed with 0.2 mmole dicyclohexylcarbodiimide in 3 ml dry THF. Dicyclohexylurea was filtered off and the solution evaporated to dryness. The oil formed was dissolved in tetrahydrofuran (THF) This procedure was repeated 3–4 times. The product was isolated as an oil; it was stored at 4°C in the dark. The presence of the azido group in the product was proved using IR-spectroscopy.

tRNA containing an azido group was obtained by treatment of tRNA (3 mg/ml) with *p*-azidobromacetanilide (13 mg/ml) in 50% THF at room temperature for 24 hr. In the course of the reaction, there takes place a decrease of the absorbance at 334 nm characteristic of 4-thiouridine residues in tRNA. The product was stored in the dark at low temperature.

Modification of the tRNA 4-thiouridine residues with iodoacetamide was performed in 0.01M Tris HCl buffer, pH 8 (0.5 mg/ml tRNA and 5 mg/ml iodoacetamide). The reaction was run for 6 hr at 20°C. The absorbance at 334 nm typical of 4-thiouridine decreased after the modification to 15% of the initial value.

Photoinduced modification of the phe-RSase was performed by irradiation at 10°C of 1–2 ml of the

* *Abbreviations*: ARSases, aminoacyl-tRNA synthetases; phe-RSase, phenylalanyl-tRNA synthetase; TCA, trichloroacetic acid; THF, tetrahydrofuran; [^{14}C]phe-tRNA, [^{14}C]-phenylalanyl-tRNA.

enzyme solution using SVD-12A mercury lamp and a cut-off filter transmitting wave lengths greater than 300 nm. The covalent attachment of the reagents to the enzyme was determined by measuring radioactivity retained after protein sorption on the nitrocellulose filters at pH 7.5 [12]. The unbound radioactivity was washed off with Tris–HCl, pH 7 (3 × 3 ml). It was found that the reversible complexes of [^{14}C]phe-tRNA and ATP-γ-*p*-azidoanilide with the enzyme are not retained by the filter. The enzyme activity was determined by measuring the initial rates of amino-acylation and of the ATP-^{32} P-pyrophosphate exchange. The reaction mixture for the aminoacylation of tRNA contained 0.05 M Tris–HCl, pH 7.5, 10^{-3} M ATP, 3.5×10^{-3} M $MgSO_4$, 2.5×10^{-6} M [^{14}C] phenyl-alanine, 10^{-5} M tRNA, and 0.01–0.04 mg of the enzyme in total volume 0.3 ml. Reaction was performed at 25°C. The amount of [^{14}C]phe-tRNA formed was determined by measuring the radioactivity precipitated by cold 5% trichloroacetic acid (TCA) on FN-16 paper filters (3 × 3 cm) pre-impregnated with TCA solution [13]. The reaction mixture for the ATP-^{32}P-pyrophosphate exchange measurements contained 4 mM ATP, 25 mM $MgSO_4$, 0.2M sodium pyrophosphate (12 Ci/mole), 50 mM Tris–HCl pH 7.5 and 0.005 mg of the enzyme in total volume 0.5 ml. The amount of [^{32}P]ATP formed was measured according to [14]. The radioactivities were counted by means of a Mark II Nuclear Chicago scintillation counter.

3. Results and discussion

The main advantage of photoreactive compounds for the affinity labeling is the possibility of decoupling completely the stage of the non-covalent binding and the modification reaction. Specific modification may be performed by these reagents under very mild conditions. Formation of specific irreversible complexes of tyrosyl-tRNA synthetase with $tRNA_1^{tyr}$ and $tRNA_2^{tyr}$ *E. coli* takes place under irradiation with the total light of mercury lamp [15]. Several points of covalent binding of tRNA to the enzyme were identified in the product of such an irradiation. However, we found that irradiation of phe-RSase from *E. coli* MRE-600 for the time necessary for such binding results in complete inactivation of the enzyme. Therefore this approach seems to be dubious for the functional activity investigations with this enzyme.

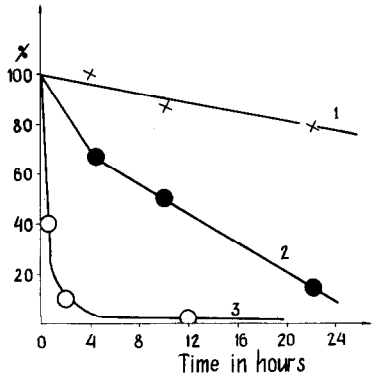

Fig. 1. The time course of the inactivation of phenylalanyl-tRNA synthetase (% of the initial activity in aminoacylation reaction) in the presence of [^{14}C]phe-tRNA pretreated with iodoacetamide (1); in the presence of [^{14}C]phe-tRNA (2); in the presence of tRNA treated with *N*-(*p*-azidophenyl)-iodoacetamide (3). Both irradiated and control mixtures contained 0.5×10^{-6} M phe-RSase, 0.4×10^{-4} M tRNA (1×10^{-6} M $tRNA^{phe}$) in 2 ml of 0.025 M acetate buffer, pH 5.8, 0.05 M $MgSO_4$. 0.01 ml aliquots were used for catalytic activity measurements as described in Materials and methods. The degree of inactivation was calculated as the ratio of the initial aminoacylation rates with aliquots of irradiated and control samples.

We attempted to use for the purpose the photo-reaction of the tRNA 4-thiouridine residues [16,17] which absorb at 334 nm. It was anticipated that irradiation at this wavelength would not induce photo-reactions of any other base residues. It is known that the chemical modification of the *E. coli* $tRNA^{phe}$ at its single 4-thiouridine residues does not change the acceptor activity of tRNA [18]. We confirmed this finding by treatment of tRNA with iodoacetamide. Unfractionated tRNA modified with this reagent retained the same acceptor activity towards phenylalanine as the control sample.

The time course of the decrease of the amino-acylating activity of *E. coli* phe-RSase under UV-irradiation in the presence of unfractionated tRNA of *E. coli* is shown in fig. 1; 95% inactivation takes place in 22 hr. The inactivation of the enzyme irradiated without tRNA under identical conditions does not exceed 15%. The same figure shows the time course of the inactivation of the enzyme in the presence of unfractionated tRNA modified at 4-thiouridine residues

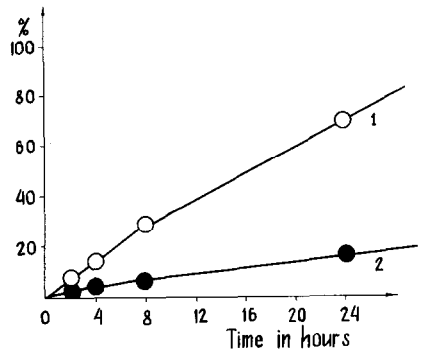

Fig. 2. Kinetic curves of the attachment of [^{14}C]phe-tRNA to phe-RSase on irradiation. The composition of the reaction mixture the same as in fig. 2. 1) irradiated sample; 2) control sample (incubated in the dark).

with iodoacetamide. It is seen that such modified tRNA lacking 4-thiouridine residue induces inactivation of the enzyme to a significantly lower extent. Therefore, it may be concluded that the photoreaction of the 4-thiouridine with the enzyme is the main reason of the inactivation.

Fig. 2 demonstrates the time course of radioactivity binding to phe-RSase irradiated in the presence of [^{14}C]phe-tRNA. The binding of radioactivity to the enzyme coincides with the decrease of aminoacylating activity (fig. 1, curve 1).

The irradiation of phe-RSase in the presence of [^{14}C]phe-tRNA for 18 hr resulted in 83% inactivation of the enzyme as measured by aminoacylation of tRNA. However, the modified enzyme catalyzed ATP-^{32}P-pyrophosphate exchange, the activity being equal to 60% of the starting value. Therefore, the catalytic center of the enzyme should not be severely damaged after attachment of tRNA to the enzyme at the thiouridine residue. The slight decrease of the catalytic activity towards ATP-[^{32}P]pyrophosphate may reflect some changes due to the modification as well as the effect of covalently bound tRNA on the first steps of the activation of amino acid in agreement with [19].

The azido derivative of tRNA was obtained by modification of tRNA with *p*-azidobromoacetanilide. This photoreactive derivative is very efficient in inducing photo-inactivation of tRNA^{phe} (*E. coli*); 90% decrease of the aminoacylating activity takes place in 2 hr of irradiation under conditions similar to the above described. ATP-γ-anilide was shown earlier [20] to be a competitive inhibitor in the enzymatic aminoacylation of tRNA^{phe}. In the course of the present studies we prepared a photoreactive analog of ATP. This compound exhibited no substrate activity in the aminoacylation reaction. However, it is a strong competitive inhibitor of phe-RSase (see fig. 3) with K_i only two times greater than K_m of ATP.

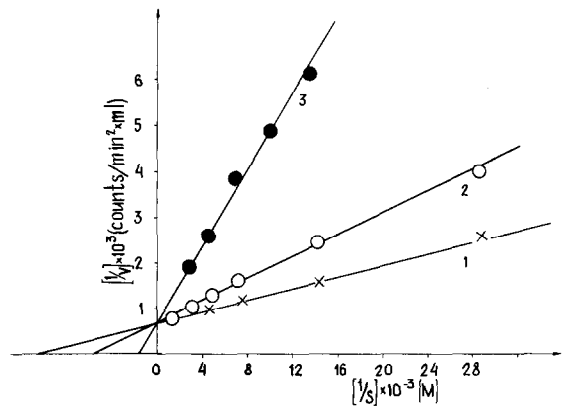

Fig. 3. Lineweaver-Burk plot of the dependence of the initial rate of the aminoacylation of tRNA^{phe} in the ATP concentration range $3.5 \times 10^{-5} - 2.8 \times 10^{-4}$ M. 1) without ATP-γ-*p*-azidoanilide; 2) with 0.44×10^{-4} M of azido analog; 3) with 0.87×10^{-4} M of the analog.

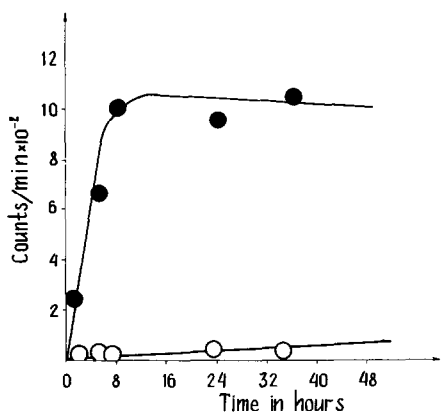

Fig. 4. The time course of the attachment α-[^{32}P]ATP-γ-*p*-azidoanilide to phe-RSase at irradiation (●–●–●) and in the dark (○–○–○). The reaction mixture contained phe-RSase 0.6×10^{-6} M, α-[^{32}P]ATP-γ-*p*-azidoanilide 7.5×10^{-4} M, Tris–HCl buffer, pH 7.5 0.02 M, $MgSO_4$ 0.05 M. Aliquots were put on the nitrocellulose filters and analyzed as described in Materials and methods.

Covalent binding of α-[^{32}P]ATP-γ-(*p*-azidoanilide) to phe-RSase is induced by UV-irradiation of the mixture of the enzyme with the reagent. The results are shown in fig. 4. It is seen, that the reaction is completed within 8 hr. 2.5 moles of the ATP analog are bound per mole of phe-RSase, suggesting that the analog binds at several centers.

The data obtained demonstrate that the derivatives of phe-RSase substrates described may be used as affinity reagents for functional and structural investigation of the enzyme.

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