Affinity labeling of phenylalanyl-tRNA synthetase from E.coli MRE-600 by E.coli tRNA^{phe} containing photoreactive group.

I.I. Gorshkova, D.G. Knorre, O.I. Lavrik and G.A. Nevinsky

Institute of Organic Chemistry, Siberian Division of the Academy of Sciences,
Novosibirsk 630090, USSR

Received 26 April 1976

ABSTRACT

The photoinduced reaction of phenylalanyl-tRNA synthetase (E.C. 6.1.1.20) from E.coli MRE-600 with tRNA^{phe} containing photoreactive p-N₃-C₆H₄-NHCOCH₂-group attached to 4-thiouridine sU₈ (azido-tRNA^{phe}) was investigated. The attachment of this group does not influence the dissociation constant of the complex of Phe-tRNA^{phe} with the enzyme, however it results in sevenfold increase of K_m in the enzymatic aminoacylation of tRNA^{phe}. Under irradiation at 300 nm at pH 5.8 the covalent binding of [¹⁴C]-Phe-azido-tRNA^{phe} to the enzyme takes place 0.3 moles of the reagent being attached per mole of the enzyme. tRNA prevents the reaction. Phenylalanine, ATP, ADP, AMP, adenosine and pyrophosphate (2.5 x 10⁻³ M) don't affect neither the stability of the tRNA-enzyme complex nor the rate of the affinity labelling. The presence of the mixture of either phenylalanine or phenylalaninol with ATP as well as phenylalaninol adenylate exhibits 50% inhibition of the photoinduced reaction. Therefore, the reaction of [¹⁴C]-Phe-azido-tRNA with the enzyme is significantly less sensitive to the presence of the ligands than the reaction of chlorambucilyl-tRNA with the reactive group attached to the acceptor end of the tRNA studied in ¹. It has been concluded that the kinetics of the affinity labelling does permit to discriminate the influence of the low molecular weight ligands of the enzyme on the different sites of the tRNA - enzyme interaction.

INTRODUCTION

It was found earlier ¹ that the affinity alkylation of Phe-RSase from E.coli MRE-600 by chb-tRNA is rather sensitive to a variety of the low molecular weight ligands specific to the enzyme. The effects were supposed to be due to some local conformational changes induced by the ligands. These changes being without any influence on the total affinity of the tRNA derivative to the enzyme may result in rather severe changes of the mutual orientation of the reacting group and the enzyme site to be modified. However in the case of chb-tRNA with the reactive group attached to aminoacyl moiety of phe-tRNA the inhibitory effects of the ligands may be in some cases interpreted as the result of the competition of the ligand and the reactive group for the modification sites.

In the present paper the kinetics of the affinity labelling of phe-RSase and its sensitivity to low molecular weight ligands is studied using another tRNA derivative with the reactive group attached to 4-thiouridine residue sU₈. This derivative containing p-N₃-C₆H₄-NHCHCH₂-group was demonstrated earlier to inactivate Phe-RSase under UV-irradiation ².

MATERIALS AND METHODS

DEAE-cellulose DE-52 "Whatman", [¹⁴C]-phenylalanine (220Ci/mole) "Chemapol", HUFs, AUFS ultrafilters "Chemapol", L-phenylalanine, L-tryptophane "Reakhim", USSR were used. ATP, ADP, AMP, adenosine, GTP "Special Design Bureau of BAS", Novosibirsk, USSR were 95-99% purity.

Abbreviations: Phe-RSase - phenylalanyl-tRNA synthetase (E.C. 6.1.1.20);

[¹⁴C]-phe-tRNA - [¹⁴C]-phenylalanyl-tRNA^{phe};

azido-tRNA - tRNA^{phe} with p-N₃-C₆H₄-NHCOCH₂- group attached to the 4-thiouridine sU₈ residue;

BSA - bovine serum albumine;

chb-tRNA - γ-[p-N,N-bis-(2-chloroethyl)]-aminophenyl-butyryl-phenylalanyl-tRNA (chlorambucil-phenylalanyl-tRNA);

pp - sodium pyrophosphate;

TCA - trichloroacetic acid.

L-phenylalaninol was obtained according to ³. L-phenylalaninol-AMP was obtained according to ⁴.

E.coli MRE-600 unfractionated tRNA was purchased from "Special Design Bureau of BAS", Novosibirsk, USSR. tRNA enriched in tRNA^{phe} species up to 13% used in the most part of experiments. The enrichment was performed using BDEAE-cellulose chromatography according to ⁵.

Phe-RSase from E.coli MRE-600 with specific activity up to 200 units/mg was obtained as described in ⁶. The initial rate of [¹⁴C]-Phe-tRNA formation was measured by counting the radioactivity precipitated by cold 5% TCA on FN-16 paper filters ⁷. The reaction mixture for the aminoacylation of tRNA contained in 0.5 ml : 50 μmole tris HCl pH 7.5, 1.2 μmole ATP, 10 μmole MgSO₄, 2.4 x 10⁻³ μmole [¹⁴C]-phenylalanine, 10⁻² μmole tRNA, 0.65 mg BSA, 1 μmole β-mercaptoethanol and 0.5 to 5 μg Phe-RSase.

The reaction was performed at 25° C. The activity of the other aminoacyl-tRNA synthetases tested using 7 amino acids (valine, lysine, leucine, isoleucine, proline, tyrosine, serine) were negligible. Protein concentration was determined spectrophotometrically using an absorption coefficient at 280 nm of $A_1^{1\text{ mg/ml}} = 0.9$.

p-Azidobromoacetanilide was obtained according to ⁸. The radioactive compound was obtained by similar way using [¹⁴C]-bromoacetic acid (6 mCi/mMole).

Azido-tRNA was obtained according to technique ². The extent of reaction was tested by measuring the decrease of the typical of the 4-thiouridine residues absorbance at 334 nm. The extent of tRNA modification was 70%.

Preparative scale aminoacylation of tRNA and of azido-tRNA was performed according to ⁹. The preparations of [¹⁴C]-Phe-azido-tRNA with specific activity no less 35000 cpm per A₂₆₀ unit were used.

To obtain the complex the enzyme (1.6 x 10⁻³ μmole) and [¹⁴C]-Phe-azido-tRNA (3.3 x 10⁻³ μmole) were incubated in a final volume 0.2 ml for 1 min at 4°C in buffer A (0.025 M sodium acetate pH 5.8 - 0.005M MgSO₄ - 0.0001M EDTA). The complex of the enzyme with [¹⁴C]-Phe-azido-tRNA was tested

by measuring radioactivity retained after protein sorption onto a nitrocellulose filter (HUF5 0.24 μ). The unbound radioactivity was washed off with buffer A (3 x 1.5 ml) at 4°C. The amount of nonspecific absorption of [14 C]-Phe-azido-tRNA in a control experiment was about 10% of the total radioactivity.

The efficiency of [14 C]-Phe-azido-tRNA sorption on nitrocellulose filters in the presence of Phe-RSase excess was 30%. The binding constant of [14 C]-Phe-azido-tRNA with Phe-RSase were determined taking into account this value.

Photoinduced modification of Phe-RSase was performed by irradiation at 14°C using SVD-120A mercury lamp and a cut-off filter transmitting wave lengths greater than 300nm. The intensity of UV-irradiation was determined using chemical actinometry ¹¹.

The kinetics of the covalent attachment of [14 C]-Phe-azido-tRNA was tested by measuring radioactivity bound to the enzyme, retained after protein sorption onto nitrocellulose filters at pH 7.5. Enzyme (2.3×10^{-3} μ mole), [14 C]-Phe-azido-tRNA (7.3×10^{-3} μ mole) and other substrates at corresponding concentrations (See Table 3) were incubated in the final volume (0.9 ml) of buffer A. The incubation was performed under and without irradiation (control mixture). The aliquots of these mixtures (0.075 ml) were carried in 1.5 ml 0.05 M tris HCl buffer pH 7.5 (buffer B). After incubation during 1 min the solution was filtered through the nitrocellulose filter previously washed with buffer B. The unbound radioactivity was washed off with buffer B (3 x 1.5 ml). The extent of [14 C]-Phe-azido-tRNA covalent attachment was determined taking into account the enzymatic deacylation of [14 C]-Phe-azido-tRNA. The degree of [14 C]-Phe-azido-tRNA deacylation was determined by measuring the radioactivity from the aliquot of the same mixture precipitated by cold 5% TCA on FN-16 paper filters (2.5 x 2.5 cm) pre-impregnated with TCA ¹².

To estimate extent of the enzyme inactivation under irradiation the enzyme (2.3×10^{-3} μ mole) in the buffer A was irradiated for 30 min at 14°C. The irradiation was carried out both in the presence and in the absence of substrates and

their analogs. The aliquots of experimental and control (without irradiation) mixture were taken to measure the aminoacylation rate. It is seen (Fig.1) that the enzyme inactivation at irradiation both in the presence and in the absence of the low molecular weight substrates is negligible.

RESULTS AND DISCUSSION

It was shown earlier ² that the attachment of $N_3C_6H_4NHCCH_2$ group to sU_8 residue of $tRNA^{phe}$ does not change the extent of the limit tRNA aminoacylation. In the present paper more detailed quantitative characteristics of the azido-tRNA interaction with Phe-RSase were obtained.

To compare the affinities of $tRNA^{phe}$ to Phe-RSase concentration of the complex of the enzyme with either $[^{14}C]$ -Phe-tRNA or $[^{14}C]$ -azido-tRNA was measured as a function of tRNA concentration using nitrocellulose membrane technique. The typical binding curve for $[^{14}C]$ -Phe-azido-tRNA is repre-

Figure 1

The kinetics of the aminoacylation of tRNA catalysed by Phe-RSase: o -enzyme preincubated without irradiation, ● - preincubated under irradiation in the absence of the ligands, △▲ - preincubated under irradiation in the presence of 10^{-3} M of ATP and 3.3×10^{-3} M of phenylalanine.

sented in fig.2. The limit binding is as high as 0.7 moles of $[^{14}\text{C}]$ -Phe-azido-tRNA per mole of the enzyme.

The dissociation constant K_s of the complex was calculated using equation:

$$\frac{1}{[E S]} = \frac{K_s}{E_o \cdot [S]} + \frac{1}{E_o}$$

where E_o is the total enzyme concentration, $[E S]$ - the complex concentration, $[S]$ - the concentration of the nonbound $[^{14}\text{C}]$ -Phe-azido-tRNA or $[^{14}\text{C}]$ -Phe-tRNA. K_s values obtained are quite similar being $(1.9 \pm 0.9) \times 10^{-8}$ M for $[^{14}\text{C}]$ -Phe-tRNA and $(1.8 \pm 0.9) \times 10^{-8}$ M for $[^{14}\text{C}]$ -Phe-azido-tRNA. These values are several times greater than K_s for chb-tRNA obtained in ¹³ (0.5×10^{-8} M). Due to significant correction of $[E S]$ values introduced by taking into account the low efficiency of the complex sorption on the nitrocellulose filters these values may be regarded only as approximate. The values calculated without correction are several times greater. In any case it may be concluded that phenylalanyl derivatives of tRNA^{phe} and azido-tRNA^{phe} have approximately equal affinities to Phe-RSase.

Figure 2

The dependence of the Phe-RSase complex formation with $[^{14}\text{C}]$ -Phe-azido-tRNA at pH 5.8 on the concentration of $[^{14}\text{C}]$ -Phe-azido-tRNA.

Fig.3 demonstrates the influence of tRNA on the formation of the complex of $[^{14}\text{C}]$ -Phe-azido-tRNA with Phe-RSase. It may be seen that tRNA competes with $[^{14}\text{C}]$ -Phe-azido-tRNA thus indicating that the latter binds to the same site of the enzyme.

FIGURE 3

The dependence of the Phe-RSase complex formation with $[^{14}\text{C}]$ -Phe-azido-tRNA at pH 5.8 on the concentration of the added tRNA. The mixture contained in 0.2 ml: 0.6×10^{-3} μmole $[^{14}\text{C}]$ -Phe-azido-tRNA, 0.2×10^{-3} μmole Phe-RSase and different concentrations of crude tRNA.

The low molecular weight ligands - ATP, AMP, pyrophosphate and phenylalanine as well as some analogs (ADP, GTP, adenosine, phenylalaninol) don't influence significantly $[^{14}\text{C}]$ -Phe-azido-tRNA binding to the enzyme (fig.4). The mixture of L-phenylalanine with ATP is also without any measurable influence. The slight decrease of the $[^{14}\text{C}]$ -Phe-azido-tRNA binding was found in the presence of ATP, GTP and pyrophosphate at concentrations exceeding 3×10^{-3} M.

The double reciprocal plot of the dependence of the initial aminoacylation rates of tRNA^{phe} and azido- tRNA^{phe} on

Figure 4

The dependence of the relative amount of the $[^{14}\text{C}]$ -Phe-azido-tRNA - enzyme complex on the ligands concentration. The amount of the complex formed in the absence of the ligands is taken as 100%.

their concentrations are represented in fig.5. It is seen that the modification results in a seven-fold increase of K_m values for tRNA ($3.3 \times 10^{-7} \text{ M}$ for tRNA and $2.5 \times 10^{-6} \text{ M}$ for azido-tRNA). The $V_{\max}/E$ ratio changes from 27.6 min^{-1} for tRNA to 1.7 min^{-1} for azido-tRNA. Therefore, in spite of the similarity of the K_s values for both tRNA's the kinetic parameters differ significantly.

Figure 5

The double reciprocal plot of the dependence of the initial rate of the aminoacylation of tRNA^{phe} (1) and azido- tRNA^{phe} (2) on the tRNA concentration. The reaction mixture contained $7.5\mu\text{g/ml}$ of the enzyme in the case of tRNA^{phe} and $75\mu\text{g/ml}$ of the enzyme in the case of azido- tRNA^{phe} .

Figure 6

The time course of the attachment of $[^{14}\text{C}]$ -Phe-azido-tRNA to Phe-RSase under irradiation (1), under irradiation in the presence of $1.4 \times 10^{-5} \text{ M}$ tRNA (2) and without irradiation (3).

Fig.6 represents the kinetic curve of the photoinduced $[^{14}\text{C}]$ -Phe-azido-tRNA attachment to Phe-RSase at pH 5.8 (curve 1). The limit amount of the reagent covalently bound is as great as 0.3 moles per mole of the enzyme. The attachment without irradiation is negligible (curve 3). The presence of tRNA significantly decreases the modification rate (curve 2). This indicates that photoinduced attachment proceeds in the vicinity of tRNA binding site.

T a b l e

The influence of different ligands on the relative affinity modification rate of Phe-RSase with $[^{14}\text{C}]$ -Phe-azido-tRNA and chb-tRNA

Ligand	Concentration $\text{M} \times 10^{-3}$	The rate of the enzyme alkylation with chb-tRNA (%)	The rate of the photoinduced modification of Phe-RSase with $[^{14}\text{C}]$ -Phe-azido-tRNA (%)
-	-	100	100
L-Phe	3.3	36	100
ATP	2.5	80	100
ATP	5.0	43	85
ATP	10.0	21	-
L-Phenylalaninol	3.3	-	100
ADP	2.5*	87	100
AMP	2.5*	18	100
PP	2.5*	6	150
GTP	2.5*	23	150
A	2.5*	100	100
ATP + L-Phe	2.5*+3.3	0	48 $\pm$ 8
ATP + L-Phe-ol	2.5*+3.3	-	54 $\pm$ 8
L-Phenylalaninoladenylate	2.5*	-	58 $\pm$ 8

* - in the case of chb-tRNA the concentration was 10^{-2}M .

To check once more the specificity of the reaction under investigation the modification of BSA with azido-tRNA containing $\text{N}_3\text{-C}_6\text{H}_4\text{NHCO-}[^{14}\text{C}]\text{H}_2$ group was estimated in the same condition. It was found to be very low (0.01 moles/mole of BSA).

The data concerning the influence of the low molecular weight ligands on the kinetics of the photoaffinity labelling of Phe-RSase with [^{14}C]-Phe-azido-tRNA are presented in the table. The data obtained earlier ^{1,13} for affinity alkylation of the same enzyme with chb-tRNA are given for comparison.

It is seen that all ligands investigated do not decrease the photoinduced reaction rate. The slight decrease of the reaction rate in the presence of $5.0 \times 10^{-3}\text{M}$ ATP may be explained as to be due to decreasing affinity of the reagent in the presence of high ATP concentrations (fig.4). The presence of GTP and pyrophosphate slightly enhances the reaction rate although these ligands decrease significantly the rate of the affinity alkylation of Phe-RSase with chb-tRNA.

However, approximately two-fold decrease of the photoinduced modification rate was found in the presence of the mixture of ATP+ either L-phenylalanine or L-phenylalaninol as well as in the presence of L-phenylalaninol adenylate.

Two main reasons may be suggested to explain the influence of the ligands on the photoaffinity modification rate. The first is the direct interaction of the photoreactive group with the ligand due to overlapping of the site of modification with the ligand binding site or the competition between the reactive group of the enzyme and some group of the ligand for enzyme and photoreactive group of the reagent.

The first reason seems to be highly improbable. The dimension of the group (the distance between S atom of SU_8 and the end atom of the azido group) may be estimated as $11.6\text{\AA}$.

In the same time the distance between sU_8 and acceptor end of $tRNA^{phe}$ may be estimated as great as 41 Å according to three-dimensional structure of $tRNA^{phe}$ from yeast¹⁴ which is proposed to be common for all elongator $tRNA$ 's¹⁵. Aminoacyladenylate and consequently, its analog aminoalkyladenylate has to be placed in the vicinity of the acceptor end to provide the possibility of the aminoacyl residue transfer from adenylylate to OH-group of the terminal adenosine. The same has to be the case for phenylalanine and ATP if the concerted mechanism¹⁶ operates in the $tRNA$ aminoacylation. This distance is too great to permit any interaction between photo-reactive group and the ligands.

Therefore, it may be concluded that same conformational changes are induced by phenylalaninol adenylylate or by the mixture of ATP with either phenylalanine or phenylalaninol which have some measurable consequences rather far from the catalytic site of the enzyme. The severe effect of methioninyl adenylylate on the methionyl- $tRNA$ synthetase from *E. coli* was found in¹⁷ namely it was demonstrated that methioninyl adenylylate induced the formation of an additional $tRNA$ recognizing site on the enzyme. The effect of the mixture of ATP with amino acid or amino alcohol may be regarded as a result of the coupling between ATP and phenylalanine sites of the enzyme. The coupling of ATP and amino acid sites was demonstrated by several authors¹⁸⁻²⁰.

The effects obtained in this paper are significantly lower than those in the case of the affinity alkylation by chb- $tRNA$ with reactive group attached to aminoacyl residue of $tRNA$. Therefore, it may be concluded that the kinetics of the affinity labelling does permit to discriminate the influence of the low molecular weight ligands of the enzyme on the different sites of the $tRNA$ - enzyme interaction.

ACKNOWLEDGEMENTS

The authors are thankful to M.M.Bikkina for the assistance in experiments and V.N.Ankilova for the preparation of the enzyme.

REFERENCES

1. Gorshkova, I.I. and Lavrik, O.I. (1975) FEBS Letters **52**, 135-138.
 2. Budker, V.G., Knorre, D.G., Kravchenko, V.V., Lavrik, O.I., Nevinsky, G.A. and Teplova, N.M. (1974) FEBS Letters **49**, 159-162.
 3. Ikehara, M. and Uesugi, S. (1969) Chem. Pharm. Bull. **17**, 348-354.
 4. Cassio, D., Lemoin, F., Waller, J.P., Sandrin, E. and Boissonnas, R.A. (1967) Biochemistry **6**, 827-835.
 5. Roy, K.I. and Söll, D. (1968) Biochem. Biophys. Acta **161**, 572-574.
 6. Stulberg, M.P. (1967) J. Biol. Chem. **242**, 1060-1064.
 7. Kelmers, A.D., Novelli, G.D. and Stulberg, M.P. (1965) J. Biol. Chem. **240**, 3979-3981.
 8. Sibberad, O. and Smart, B.J. (1906) J. Chem. Soc. **89**, 170-171.
 9. Knorre, D.G., Sirotyuk, V.Y. and Stephanovich, L.E. (1967) Molecul. Biol. **1**, 837-841.
 10. Yarus, M. and Berg, P. (1970) Anal. Biochem. **35**, 450-466.
 11. Calvert, J.G. and Pitts, Jr. J.N. (1968) Photochemistry, p.p. 625-527, Myr, Moskva.
 12. Schreiner, A.A. and Schimmel, P.L. (1972) Biochemistry, **11**, 1582-1586.
 13. Gorshkova, I.I. and Lavrik, O.I. (1975) Molecul. Biol. **9**, 887-892.
 14. Rich, A. personal communication.
 15. Kim, S.H., Susmann, J.L., Suddath, F.L., Quigley, G.L., McPherson, A., Wang, A.H.J., Seeman, N.C. and Rich, A. (1974) Proc. Nat. Acad. Sci. USA **71**, 4970-4974.
 16. Loftfield, R.B. and Eigner, E.A. (1969) J. Biol. Chem. **6244**, 1746-1754.
 17. Blanquet, S., Iwatsubo, M. and Waller, J.-P. (1973) Europ. J. Biochem. **36**, 213-226.
 18. Kosakowsky, M.M. and Holler, E. (1973) Eur. J. Biochem. **38**, 274-282.
 19. Penzer, G.R. and Plumbridge, J.A. (1974) Eur. J. Biochem. **45**, 291-295.
 20. Blanquet, S., Fayat, G., Waller, J.-P. and Iwatsubo, M. Eur. J. Biochem. **24**, 461-469.
-