AFFINITY MODIFICATION OF PHENYLALANINE: tRNA-LIGASE OF *E. COLI* MRE-600 WITH *N*-CHLORAMBUCILYL-[^{14}C]-PHENYLALANYL-tRNA

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1. Introduction

Recently, alkylating tRNA derivatives have been proposed in our laboratory for the affinity labelling of ribosomes [1] and for intramolecular alkylation of tRNA [2,3]. It was found [1] that an *N*-acyl-phenylalanyl-tRNA whose *N*-acyl residue was an aromatic β-chloroethylamine chlorambucil* may serve as a normal substrate in the ribosomal system and that it alkylates rRNA within the specific complex with ribosomes and poly U.

Affinity modification of an amino acid:tRNA-ligase with a reactive moiety bound to tRNA has been described for the first time by Bruton and Hartley [4]. The purpose of the present communication is the application of *N*-chlorambucilyl-phenylalanyl-tRNA for the affinity modification of phenylalanine:tRNA-ligase.

2. Materials and methods

Partially purified phenylalanine:tRNA-ligase of *E. coli* MRE-600 has been obtained according to ref. [5]. *E. coli* MRE-600 unfractionated tRNA was obtained as described earlier [6]. Preparative scale aminoacylation of unfractionated tRNA with [^{14}C]phenylalanine (220 Ci/mole, Chemapol. CSSR) was performed according to ref. [7]. *N*-Chlorambucilyl- and *N*-acetyl-[^{14}C]phenylalanyl-tRNA were obtained from unfractionated [^{14}C]phenylalanyl-tRNA as described earlier [1,8]. *N*-Acetyl-[^{14}C]-phenylalanyl-tRNA* had a specific radioactivity 7000 cpm per A_{260} unit, *N*-chlorambucilyl-[^{14}C]-phenylalanyl-tRNA** – 15 000 cpm per A_{260} unit. The extents of *N*-acylation as found according to ref. [9] were about 90–95%.

To obtain the complexes of *N*-acyl-aminoacyl-tRNAs with phenylalanine:tRNA-ligase, the components were incubated in 0.025 M sodium acetate, pH 5.8–0.005 M $MgSO_4$–10^{-4} M EDTA [10]. The affinity alkylation of the ligase was performed in the same buffer. The complex of the enzyme with *N*-acetyl-[^{14}C]-Phe-tRNA was assayed by filtration through hitrocellulose filters (HUFS, Synpor) [11], this method, however, was inaccplicable in the case of *N*-chb-[^{14}C]-Phe-tRNA [1] because of its strong absorption to the filters. The complex of the enzyme with *N*-chb-[^{14}C]-Phe-tRNA was assayed by gel-filtration on Sephadex G-100. To destroy tRNA within the complex, it was treated with pancreatic ribonuclease at RNAse: tRNA molar ratio 1:8 (3 hr at 25°C). The radioactivity bound by the protein was counted after precipitation with 5% trichloroacetic acid. The reaction mixtures prepared to evaluate the rate of tRNA aminoacylation contained in 0.5 ml: 50 μmoles Tris; 1.2 μmoles ATP; 5 μmoles $MgSO_4$; 2.4×10^{-3} μmoles [^{14}C]phenylalanine, 1×10^{-2} μmoles tRNA and 0.01–0.05 mg protein. The reaction was run at 25°C. The yield of aminoacyl-tRNA was determined by applying aliquots of reaction mixtures into paper discs impregnated with 5% trichloroacetic acid followed by washing the discs

* γ-[*p-bis*(2-Chloroethyl)-aminophenyl]-butyric acid.

* Below abbreviated as *N*-acetyl-[^{14}C]-Phe-tRNA.
** Below abbreviated as *N*-chb-[^{14}C]-Phe-tRNA.

Table 1
The formation of complexes between phenylalanine:tRNA-ligase and *N*-acetyl-[^{14}C]phenylalanyl-tRNA at pH 5.8 (25°C).

	Radioacitivy of the complex, cpm per 1 A_{260} unit of protein
1. E:N-Acetyl-[^{14}C]-Phe-tRNA 1:2* M/M	20 000
2. E:N-Acetyl-[^{14}C]-Phe-tRNA:tRNA 1:2:10 M/M/M	3640

* The components ratios correspond to the concentrations of the individual phenylalanine:tRNA-ligase and tRNA^{Phe}.

with cold 5% trichloroacetic acid [12]. The radioactivities were counted by means of Mark II Nuclear Chicago scintillation counter.

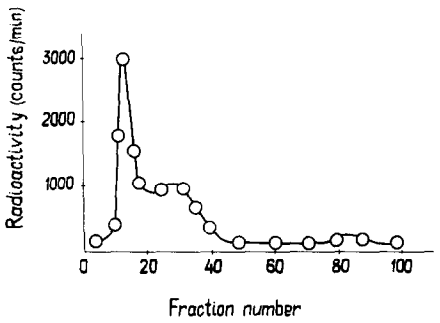

Fig. 1. Gel-filtration of the mixture of phenylalanine:tRNA-ligase with *N*-chb-[^{14}C]-Phe-tRNA on Sephadex G-100 at pH 5.8, 25°C. The gel-filtration conditions are given in 'Materials and methods'. The reaction mixture (0.48 ml) contained 0.33 A_{260} units of protein and 3.72 A_{260} units of tRNA which corresponded to ligase/tRNA^{Phe} molar ratio 1:2. The dimensions of the column were 34 × 0.8 ml, the fraction volume 0.4 ml.

3. Results and discussion

To achieve maximum affinity modification, it was necessary to run the reaction of *N*-chb-[^{14}C]-Phe-tRNA with the ligase under the conditions which favour the formation of a complex between them [1]. These conditions were found using a non-reactive analog of *N*-chb-[^{14}C]-Phe-tRNA, namely, *N*-acetyl-[^{14}C]-Phe-tRNA [13]. It was found that a stable complex of the latter with phenylalanine:tRNA-ligase is formed at pH 5.8, 25°C. *N*-Acetyl-[^{14}C]-Phe-tRNA was displaced from the complex by addition of intact tRNA (see table 1).

The complex of *N*-chb-[^{14}C]-Phe-tRNA with phenylalanine:tRNA-ligase was obtained at pH 5.8, 25°C, and isolated by gel-filtration on Sephadex G-100 [10] (see fig. 1). It was found that repeated gel-filtration under the same conditions does not destroy the complex.

To study the selectivity of the affinity alkylation, a partially purified preparation of phenylalanine:tRNA-ligase was employed which contained about 5% of the enzyme along with other ligases. This preparation was incubated with *N*-chb-[^{14}C]-Phe-tRNA at pH 5.8, 25°C at phenylalanine:tRNA-ligase/*N*-chb-[^{14}C]-Phe-tRNA molar ratio 2:1. Aliquots of the incubation mixture were assayed for the capacity to acylate tRNA with phenylalanine, lysine and valine. In a control experiment, the enzyme was incubated similarly with *N*-acetyl-[^{14}C]-Phe-tRNA. The extents of the ligases inactivation were found from the ratio of the rates of aminocylation catalyzed by enzyme preparations incubated with *N*-chb-[^{14}C]-Phe-tRNA and with *N*-acetyl-[^{14}C]-Phe-tRNA. The kinetic curves of the aminoacylation of tRBA with [^{14}C]phenylalanine after 7 hr incubation are shown in fig. 2. A typical time–course of the inactivation of the enzyme caused by incubation with *N*-chb-[^{14}C]-Phe-tRNA is shown in fig. 3. It is seen that the activity of phenylalanine:tRNA-ligase drops to 40% of the

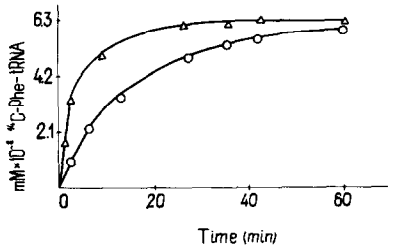

Fig. 2. Kinetics of the acylation of tRNA with [^{14}C]phenylalanine at 25°C. (△–△–△) Acylation by enzyme preincubated with *N*-acetyl-[^{14}C]-Phe-tRNA; (○–○–○) Acylation by enzyme preincubated with *N*-chb-[^{14}C]-Phe-tRNA. The time of the preincubations was 7 hr.

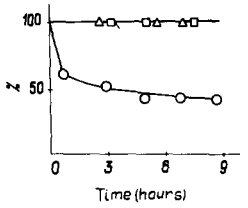

Fig. 3. Dependence of the extent of ligases inactivation on the time of incubation with *N*-chb-[^{14}C]phenylalanyl-tRNA. (○–○–○) Phenylalanine:tRNA-ligase; (△–△–△) Valine:tRNA-ligase; (□–□–□) Lysine:tRNA-ligase.

starting value in 8 hr of incubation. The activities of lysine: and valine:tRNA-ligases remain unchanged. Hence, *N*-chb-[^{14}C]-Phe-tRNA does selectively inactivate phenylalanine:tRNA-ligase.

To demonstrate the formation of a covalent bond between *N*-chb-[^{14}C]-Phe-tRNA and phenylalanine: tRNA-ligase the reaction mixture after affinity alkylation was subjected to gel-filtration on Sephadex G-100 under the conditions which destroy the complex between the ligase and intact tRNA [14]. It is seen that *N*-acetyl-[^{14}C]-Phe-tRNA dissociates from the ligase in 0.3 M KCl as revealed by gel-filtration whereas *N*-chb-[^{14}C]-Phe-tRNA after incubation with the ligase remains bound to it under these dissociating conditions; *N*-chb-[^{14}C]-Phe-tRNA/the ligase ratio within this presumably covalently bound complex is equal to 0.6 (see table 2). The presence of a covalent bond is confirmed by the fact that about 40% of the radioactivity remains in the protein fraction after the treatment of the complex with pancreatic RNAse. The radioactivity is not removed also by treatment under the conditions which ensure complete hydrolysis of the ester bond of *N*-chb-[^{14}C]-Phe-tRNA (pH 8.7, 8 hr) (see table 2). All these experiments were run in parallel with those with *N*-acetyl-[^{14}C]-Phe-tRNA; it is noteworthy that *N*-acyl-aminoacyl-tRNA in our experiments is not protected by the ligase from pancreatic RNAse action at high concentrations of the latter.

All the above evidence leads to the conclusion that *N*-chb-[^{14}C]-Phe-tRNA does efficiently alkylate phenylalanine:tRNA-ligase. At present there is not enough data to identify the grouping of the enzyme molecule which is involved in the affinity alkylation. This subject will be studied in more detail in the future.

Table 2

The method of determination	Amount of the reagent bound by protein*			
	N-chb-[^{14}C]-Phe-tRNA		*N*-Acetyl-[^{14}C]-Phe-tRNA	
	cpm per 1 A_{260} unit	moles per mole of the ligase	cpm per 1 A_{260} unit	moles per mole of the ligase
1. Gel-filtration of the mixture of the ligase with *N*-acyl-[^{14}C]-Phe-tRNA (1:2) in a buffer containing 0.3 M KCl	47 600	0.60	280	0.007
2. RNAase-hydrolysis after incubation *N*-acyl-[^{14}C]-Phe-tRNA with the enzyme at pH 5.8	32 600	0.40	284	0.007
3. RNAase-hydrolysis after incubation *N*-acyl-[^{14}C]-Phe-tRNA without the enzyme, at pH 5.8** (9 hr, 25°C)	489	0.006	300	0.008
4. RNAase-hydrolysis after incubation with the enzyme at pH 8.7 (5 hr, 25°C)	31 400	0.39	304	0.008
5. Hydrolysis with 5% trichloroacetic acid (90°C, 15 min)	1590	0.02	–	–

* The reaction mixtures treated were after 50% inactivation of phenylalanine:tRNA-ligase.
** Enzyme was added after RNAase hydrolysis.

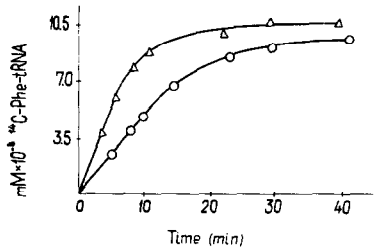

Fig. 4. Kinetics of the acylation of tRNA with [^{14}C]phenylalanine. (△–△–△) 0.05 M Tris–HCl pH 7.5; (○–○–○) 0.025 M Acetate pH 5.9. Concentrations of the other components of the reaction mixture are given in 'Materials and methods'.

However, the data available seem to rule out the possibility of the attack at histidine residues, at the SH-group of cysteine and at the ϵ-amino group of lysine or terminal amino group because the bond formed is labile in 5% trichloroacetic acid (see table 2). Hence the most probable candidate for the affinity alkylation site is the carboxyl grouping.

Finally it could appear that the medium employed for the affinity alkylation (pH 5.8) completely inhibits the normal catalytic function of the enzyme, and this would make doubtful the validity of the data obtained. However, it was found that this is not the case (see fig. 4), suggesting that the covalent complex obtained is similar to the functionally active complex of the ligase with tRNA. Our recent experiments revealed that the modification of phenylalanine:tRNA-ligase with *N*-chb-[^{14}C]-Phe-tRNA inhibits the ligase catalyzed deacylation of [^{14}C]-Phe-tRNA to the same extent as the acylation reaction. This suggests that the deacylation recognition site of the ligase is identical to the acylation recognition site.

The covalent complex obtained seems to be an interesting model for the studies of the mechanisms involved in tRNA aminoacylation as well as for investigations aimed at elucidating the tertiary structure of tRNA bound by the enzyme.

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