PHOTOAFFINITY LABELING OF 23 S rRNA IN *ESCHERICHIA COLI* RIBOSOMES WITH POLY(U)-CODED ETHYL 2-DIAZOMALONYL-PHE-tRNA

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1. Introduction

Reactive substrate analogues may label the vicinity of ribosomal tRNA binding sites including their active centers. Recently, reactive Phe-tRNA derivatives [1–3] as well as analogues of chloramphenicol [4,5] have been used for labeling proteins located close to active sites involved in peptidyl-transfer.

Photolysis of diazo-ketones and -esters yields highly-reactive carbenes [6], capable of insertion even into aliphatic side chains [7]. The nearly universal reactivity of such compounds in photoaffinity labeling [8,9] facilitates reaction with the physically most adjacent residue(s) in the active site; labeling may thus not depend on the existence of a nucleophilic group.

This paper demonstrates that by use of an ultraviolet-reactive peptidyl-tRNA analogue, ethyl-2-diazomalonyl-Phe-tRNA (fig. 1), covalent binding occurs to 23 S ribosomal RNA rather than to ribosomal proteins.*

2. Materials and methods

Ethyl-2-diazomalonyl-[^{3}H]Phe-tRNA^{Phe}(Yeast) was synthesized by acylation of [^{3}H]Phe-tRNA^{Phe} (Yeast) with ethyl-2-diazomalonyl-*N*-hydroxysuccinimide ester according to a modified procedure [10]

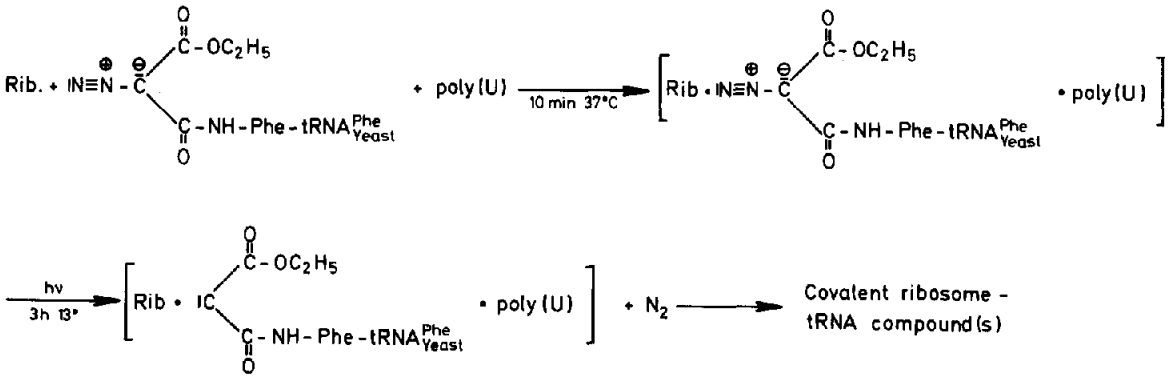

fig. 1. Scheme of reactions in photoaffinity labeling with ethyl-2-diazomalonyl-Phe-tRNA: poly(U)-coded binding, ultraviolet-activation and formation of covalent bond(s).

* Results reported at the 9th International Congress of Biochemistry, July 2nd, 1973.

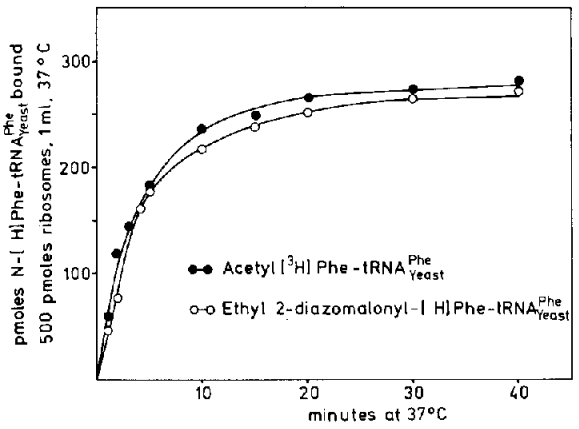

Fig. 2. Kinetics of binding of ethyl-2-diazomalonyl-Phe-tRNA and acetyl-Phe-tRNA to ribosomes. 1 ml reaction mixtures contained 10 mM $MgCl_2$, 10 mM Tris–HCl pH 7.4, 60 mM KCl (buffer A), 1.4 nmoles of acetyl-[^{3}H] Phe-tRNA^{Phe} or ethyl-2-diazomalonyl-[^{3}H] Phe-tRNA^{Phe}, 100 μg poly(U) and 500 pmoles of *E.coli* ribosomes. Reaction mixtures were incubated at 37°C. 100 μl-aliquots were taken at the times indicated and filtered through nitrocellulose filters. Filters had been treated overnight with saturated tetra-sodium diphosphate solution to prevent adsorption of Phe-tRNA derivatives to the filter.

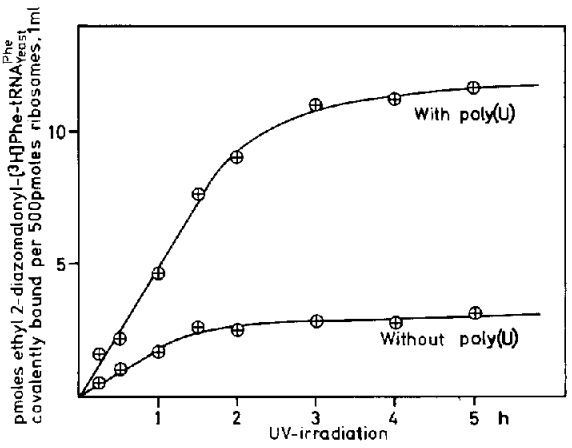

Fig. 3. Kinetics of irreversible binding of ethyl-2-diazomalonyl-[^{3}H] Phe-tRNA^{Phe}(Yeast) to ribosomes during ultraviolet-irradiation. Five hundred pmoles of *E.coli* ribosomes were incubated with ethyl-2-diazomalonyl-[^{3}H] Phe-tRNA^{Phe} (Yeast) for 10 min at 37°C in the absence of light, cooled in ice and then photolyzed. At the times indicated, 100 μl-aliquots were withdrawn. 5 μl 0.5 M K–EDTA pH 7.0 and 0.2 μg RNAaseA were added and the aliquots were incubated for 30 min at 37°C. 75 μl aliquots were plated on GF/A filters, which were placed in 10% trichloroacetic acid at 4°C for precipitation. The filters were washed with 5% trichloroacetic acid, ethanol/ether (1:1) and ether, dried and counted in 2 ml toluene containing 0.4% diphenyloxazole.

of Lapidot et al. [11]. Ethyl-2-diazomalonyl-*N*-hydroxysuccinimide ester was obtained from ethyl-2-diazomalonyl chloride and *N*-hydroxysuccinimide as follows: 4 ml anhydrous pyridine in 20 ml tetrahydrofuran was added dropwise to a stirred, ice-cooled solution of ethyl-2-diazomalonyl chloride (0.05 mole, prepared by the method of Vaughan and Westheimer [12]), and *N*-hydroxysuccinimide (10 g, 0.85 mole) in 30 ml tetrahydrofuran. The mixture was stirred overnight at ambient temperature, filtered, and the solvent was removed under reduced pressure. The residual solid was crystallized from isopropanol–petrol ether. Recrystallization from dichloromethane–petrol ether gave 6.5 g (51%) of the pure diazo compound: mp 114°C; ir (KBr) 2118 cm^{-1} (diazo stretching band); λ max (methanol) 253 nm (disappeared under ultraviolet irradiation); *Anal.* calculated for $C_9H_9O_6N_3$: C, 42,36; H, 3,56; N, 16,47; 0, 37,62. *Found*: C, 42,42; H, 3,54; N, 16,46; 0, 37,56. Acetyl-[^{3}H] Phe-tRNA^{Phe} (Yeast) was obtained by acetylation of [^{3}H] Phe-tRNA^{Phe}(Yeast) with acetyl-*N*-hydroxysuccinimide ester [11,13]. Yields of acylation varied between 80–100%, as determined by kinetics of hydrolysis catalyzed by Zn^{2+} in the presence of Tris–HCl pH 7.6 [10,14] as well as by thin layer chromatography of alkaline hydrolysates.

tRNA^{Phe}(Yeast) (Boehringer) was charged with [^{3}H] Phe (770 Ci/mole, Radiochemical Centre, Amersham) with a purified enzyme fraction obtained from baker's yeast, as described by Von der Haar [15]. Ribosomes of *E.coli* MRE 600 were isolated and purified as described [16]. Photolysis was performed at 13°C in Pyrex tubes (eliminated short wavelength radiation) using a quartz-glass tungsten lamp (Quarzlampen GmbH, Hanau) held at a distance of 5 cm. rRNA was prepared from 70 S ribosomes according to Traub et al. [17]. Proteins were extracted with acetic acid as described by Hardy et al. [18]. Other methods are given in figure legends.

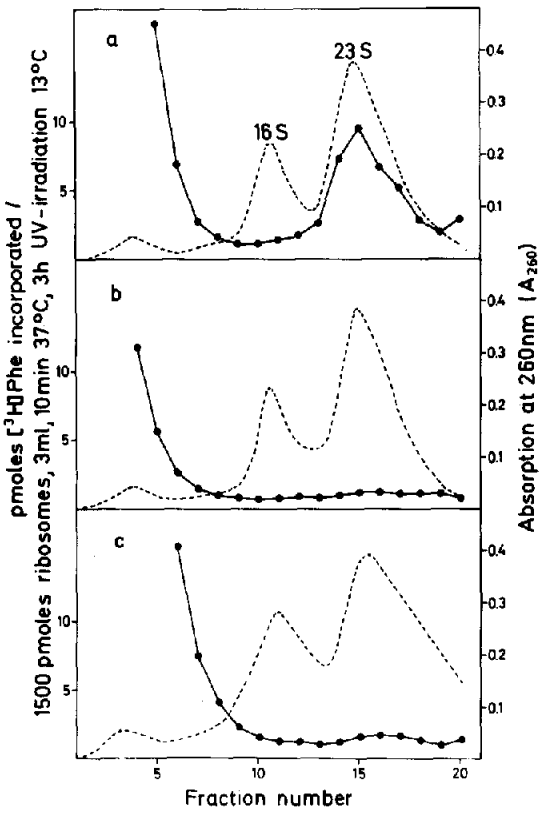

Fig. 4. Demonstration of poly(U)-dependent covalent binding of ethyl-2-diazomalonyl-[^{3}H] Phe-tRNA^{Phe}(Yeast) to 23 S ribosomal RNA. (a) 1.5 nmoles *E.coli* ribosomes were incubated for 10 min at 37°C with 3 nmoles ethyl-2-diazomalonyl-[^{3}H] Phe-tRNA^{Phe} and then photolyzed for 3 hr at 13°C. The complex was recovered by centrifugation, the pellet was resuspended in buffer A and rRNA was prepared by phenol extraction as described by Traub et al. [17]. Two hundred µl-samples were layered onto a 38 ml 5–20% sucrose gradient and centrifuged in a SW 27 rotor at 27 000 rpm for 16.5 hr. 2.0 ml fractions were collected. Radioactivity was monitored by adding 10 ml Unisolve to 1 ml of each fraction. (b) Same as (a) but poly(U) was omitted from the incubation mixture. (c) Ethyl-2-diazomalonyl-[^{3}H] Phe-tRNA^{Phe} was replaced by the same amount of acetyl-[^{3}H] Phe-tRNA^{Phe} in the incubation mixture. (•-•-•), Radioactivity in pmoles [^{3}H] Phe incorperated. (—) Absorption at 260 nm (A_{260}).

3. Results

Both in the kinetics and extent of poly(U)-coded complex formation with *E.coli* ribosomes, ethyl-2-diazomalonyl-Phe-tRNA behaved like acetyl-Phe-tRNA (fig. 2). Under standard binding conditions in the absence of light, the diazomalonyl derivative was not reactive within the complex. This was demonstrated by the absence of radioactivity in the macromolecular material, precipitated by cold trichloroacetic acid after a mild RNAase digestion. Ultraviolet-irradiation, however, resulted in a high molecular weight product, due to covalent attachement of the carbene intermediate.

Table 1
Extent of covalent attachment of ethyl-2-diazomalonyl-[^{3}H] Phe-tRNA^{Phe} to ribosomal proteins and rRNA.

pmoles Ethyl-2-diazomalonyl-[^{3}H] Phe-tRNA^{Phe} covalently bound/500 pmoles ribosomes, 1 ml, 10 min 37°C, 3 hr photolysis at 13°C		
	Protein fraction	rRNA
With poly(U)	0.5	9.4
Without poly(U)	0.2	2.5

The kinetics of irreversible binding under ultraviolet-irradiation, performed after complex formation is shown in fig. 3. Acetic acid extraction of protein after treatment with RNAase revealed very little (about 5%) radioactivity in the protein fraction. Nearly all the labeled product remained in the RNA pellet (table 1). Therefore, the rRNA from modified 70 S ribosomes was isolated and separated by sucrose gradient centrifugation (fig. 4). Most of the radioactivity remained at the top of the gradient, representing previously complex-bound tRNA; a considerable amount of radioactivity, however, could be detected in the region of 23 S RNA, thereby indicating specific reaction(s) with RNA of 50 S ribosomal subunits. In a control experiment, using acetyl [^{3}H] Phe-tRNA^{Phe} instead of the photo-sensitive label, very little radioactivity was found in the 23 S peak.

Recently it has been suggested that a close relationship exists between 5 S rRNA and peptidyl transferase [19]. So the question arose whether 5 S rRNA might have been labeled in our experiments. Fractions containing either 5 S RNA or 23 S RNA were collected from the gradient and precipitated with ethanol. Pellets were dissolved in 0.3 M Tris–HCl pH 9.0 and incubated for 2 hr at 37°C. The treatment effectively removes amino acids esterified to tRNA without affecting phosphodiester bonds of RNA [11]. After such treatment no remaining trichloroacetic acid-

insoluble radioactivity was found in 5 S RNA fractions, but the amount of label attached to 23 S RNA fractions was not reduced. Therefore, we concluded that 5 S RNA was not covalently labeled.

4. Discussion

Many investigators are looking for neighborhoods and possible functional roles of individual ribosomal proteins; but so far rather little attention has been given to the function of ribosomal RNA in protein synthesis.

Pellegrini et al. [2] described another peptidyl-Phe-tRNA analogue, *N*-bromoacetyl-Phe-tRNA^{Phe}, which seemed to form similar amounts of covalent bonds with both 50 S proteins and 23 S rRNA. The authors suggested, however, that the radioactivity cosedimenting with 23 S rRNA might be due to modification of a protein, which remained tightly bound to the RNA. By means of iodoacetyl-Phe-tRNA^{Phe} we achieved labeling of proteins L 2, L 20 and S 4 (Bispink and Matthaei, unpublished data), which seem to be located directly on 23 S and 16 S rRNA, respectively [20].

The results presented in this report indicate that during photolysis of complex bound ethyl-2-diazomalonyl-Phe-tRNA^{Phe} covalent binding occurs preferentially to 23 S rRNA rather than to ribosomal proteins. This observation might imply a close physical proximity of the peptidyl-moiety of bound peptidyl-tRNA and 23 S rRNA, and thereby a possible involvement of this RNA in one or several tRNA binding sites, most likely in the peptidyl transferase center. Localization of the site of attachment within 23 S rRNA could be helpful in answering the question whether the binding site is made up of a composite structure including both rRNA and protein(s), as discussed earlier by Noller et al. [21] for the tRNA binding site on the 30 S subunit.

References

[1] Bochkareva, E.S., Budker, V.G., Girshovich, A.S., Knorre, D.G. and Teplova, N.M. (1971) FEBS Letters 19, 121.
[2] Pellegrini, M., Oen, H. and Cantor, C.R. (1972) Proc. Natl. Acad. Sci. U.S. 69, 837.
[3] Czernilofsky, A.P. and Kuechler, E. (1972) Biochim. Biophys. Acta 272, 667.
[4] Bald, R., Erdmann, V.A. and Pongs, O. (1972) FEBS Letters 28, 149.
[5] Sonenberg, N., Wilchek, M. and Zamir, A. (1973) Proc. Natl. Acad. Sci. U.S. 70, 1423.
[6] Kirmse, W. (1964) Carbene Chemistry, New York, N.Y., Academic.
[7] Vaughan, R.J. and Westheimer, F.H. (1969) J. Am. Chem. Soc. 91, 217.
[8] Kiefer, H., Lindstrom, J., Lennox, E.S. and Singer, S.J. (1970) Proc. Natl. Acad. Sci. U.S. 67, 1688.
[9] Brunswick, K.J. and Cooperman, B.S. (1971) Proc. Natl. Acad. Sci. U.S. 68, 1801.
[10] Bispink, L. (1973), Diplomarbeit Göttingen.
[11] Lapidot, Y., de Groot, N., Rappoport, S., Hamburger, A.D. (1967) Biochim. Biophys. Acta 149, 532.
[12] Vaughan, R.J. and Westheimer, F.H. (1969) Analyt. Biochem. 29, 305.
[13] Lapidot, Y., de Groot, N., Fry-Shafrir, I. (1967) Biochim. Biophys. Acta 145, 292.
[14] Matthaei, J.H., Voigt, H.P., Heller, G., Neth, R., Schöch, G., Kubler, H., Amelunxen, F., Sander, G. and Parmeggiani, A. (1966) Cold Spring Harbor Symp. Quant. Biol. 31, 25.
[15] Von der Haar, F. (1973), Eur. J. Biochem. 34, 84.
[16] Matthaei, J.H. and Nirenberg, M.W. (1961) Proc. Natl. Acad. Sci. U.S. 47, 1580.
[17] Traub, P., Mizushima, S., Loury, C.V. and Nomura, M. (1971) The Enzymes 20, 391.
[18] Hardy, S.J., Kurland, C.G., Voynow, P. and Mora, G. (1969) Biochemistry 8, 2897.
[19] Chladek, S. (1971) Biochem. Biophys. Res. Commun. 45, 695.
[20] Stöffler, G., Kaya, L., Rak, K.H. and Garrett, R.A. (1971) J. Mol. Biol. 62, 411.
[21] Noller, H.F. and Chaires, J.B. (1972) Proc. Natl. Acad. Sci. U.S. 69, 3115.