THE BINDING OF Phe-tRNA^{Phe} AND Gly_2Phe-tRNA^{Phe} TO RETICULOCYTE RIBOSOMAL PEPTIDYL SITES BY A MECHANISM NOT INVOLVING TRANSLOCATION

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Received 25 January 1972

1. Introduction

Incubation of *E. coli* ribosomes with Phe-tRNA and poly U results in the formation of ribosomal bound diPhe-tRNA both under enzymatic and non-enzymatic binding reaction conditions [1, 2]. It has been shown that enzymatic translocation was not involved in the diPhe-tRNA formation [1, 2] indicating a direct binding of Phe-tRNA to the ribosomal P site. In addition it was found that peptidyl-tRNA such as Gly_2Phe-tRNA binds directly to the P site of *E. coli* ribosomes [3].

Studies using ribosomes from rat liver [4] and reticulocytes [5] gave different results. Little or no diPhe-tRNA was formed when these ribosomes were incubated with Phe-tRNA and poly U. Moreover, acetylPhe-tRNA when bound to rat liver ribosomes reacted with puromycin only upon addition of T-2, the rat liver translocase [6]. These results seemed to indicate that Phe-tRNA and N-substituted Phe-tRNA do not bind directly to the P site of 80 S type ribosomes.

We would like to report here that incubating rabbit reticulocyte ribosomes with purified Phe-tRNA^{Phe} in the absence of elongation factors and GTP resulted in the formation of considerable amounts of diPhe-tRNA. Moreover, purified peptidyl-tRNA^{Phe} when bound to these ribosomes reacted directly with puromycin in the absence of translocase.

These results provide strong evidence for the binding of Phe-tRNA and peptidyl-tRNA to the P site of reticulocyte ribosomes by a mechanism which does not involve T-2 mediated translocation.

2. Methods

Crude ribosomes from rabbit reticulocytes [7] were purified by incubation in an amino acid incorporation mixture, puromycin treatment and centrifugation through an 0.88 M KCl containing medium essentially according to Martin and Wool [8]. In order to eliminate any possible residual T-2 activity, the ribosomes were treated with N-ethylmaleimide (NEM) [9]. Purified ^{14}C-Phe-tRNA^{Phe} and Gly_2 ^{14}C-Phe-tRNA^{Phe} were prepared in the following way: uncharged tRNA (*E. coli*) was chromatographed on a BD-cellulose column [10]. The partially purified tRNA^{Phe} (0.42 nmoles/A_{260} unit) was charged with ^{14}C-Phe and part of the ^{14}C-Phe-tRNA was converted to Gly_2 ^{14}C-Phe-tRNA [11]. The ^{14}C-Phe-tRNA and the Gly_2 ^{14}C-Phe-tRNA were purified by rechromatography on BD-cellulose column (purity: 1.35 and 1.12 nmole/A_{260} unit, respectively). The purified preparations were dialyzed against 1 mM ammonium formate pH 5.0 and lyophilized. The liophilized powder was dissolved in 0.01 M acetate buffer pH 5.0 a short time before use.

Binding assays were performed by the Millipore filter technique of Nirenberg and Leder [12]. The extent of the puromycin reaction was determined from the difference in TCA precipitable material before and after puromycin addition to a binding reaction mixture. For analysis, the ribosomal bound radioactive material was eluted from the Millipore filters with SDS (sodium dodecyl sulphate) as described by Siler and Moldave [4]. Chromatography of the alkaline hydrolyzates of

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the ribosomal bound radioactive material was performed in a solvent system containing *n*-butanol–acetic acid–H_2O (78:5:17). Purified $tRNA^{Phe}$ was a generous gift from Dr. Kelmers (Oak Ridge National Laboratories) and Fusidic acid from Dr. Barbara Stearns (Squibb Inst. for Medical Research, New Brunswick, N.J., USA).

3. Results

It can be seen from fig. 1A that diPhe-tRNA is formed when Phe-$tRNA^{Phe}$ is added to reticulocyte ribosomes at 10 mM magnesium ion concentration. The possibility of translocation was excluded by the pretreatment of the ribosomes with NEM [9] and omission of GTP from the incubation mixture. In experiments not presented here, Fusidic acid, an inhibitor of translocation [13], was added to the incubation mixture at a final conc. of 10^{-3} M and the extent of diPhe-tRNA formation did not decrease.

Fig. 1B shows that substitution of purified Phe-$tRNA^{Phe}$ by an unpurified preparation of Phe-tRNA in an otherwise identical reaction mixture abolished almost completely the formation of diPhe-tRNA (Phe from this Phe-tRNA preparation could be incorporated into polyPhe-tRNA in a ribosomal incorporation system).

The formation of diPhe-tRNA was also strongly inhibited when $tRNA^{Phe}$ was added to an incubation mixture containing Phe-$tRNA^{Phe}$ (fig. 1C).

In experiments reported in table 1 the possibility of binding peptidyl-tRNA such as Gly_2Phe-tRNA to the ribosomal P site was investigated. The interaction of bound Gly_2Phe-tRNA with puromycin was used as an indication of the binding of peptidyl-tRNA to the P site.

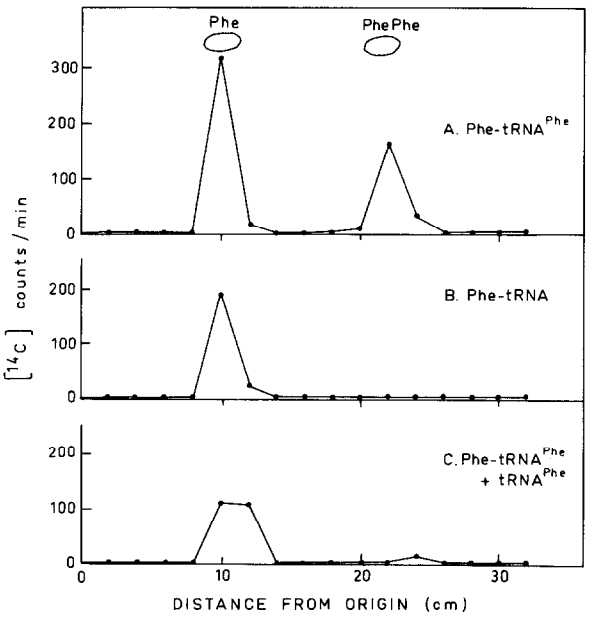

Fig. 1. Paper chromatography of the alkaline hydrolysate of the binding reaction products. 0.1 ml (final vol) of the incubation mixture contained: 60 mM Tris-HCl pH 7.4; 70 mM KCl; 60 μg poly U; 6.5 pmoles ^{14}C-Phe-$tRNA^{Phe}$ or ^{14}C-Phe-tRNA; 11 pmoles $tRNA^{Phe}$ (when added); 0.8 A_{260} units of NEM-treated ribosomes; 10 mM $MgCl_2$. After incubating for 15 min at 37° the ribosomal bound radioactive material was analyzed as indicated in Methods.

Table 1
The binding of Gly_2Phe-tRNA to the P site of reticulocyte ribosomes.

Substrate	Mg^{2+} concentration (mM)	Binding (pmoles)	Released by puromycin (pmoles)	Released by puromycin (pmoles)
Gly_2-^{14}C-Phe-$tRNA^{Phe}$	10	1.94	1.77	91
Gly_2-^{14}C-Phe-tRNA	10	1.62	0.06	3
Gly_2-^{14}C-Phe-$tRNA^{Phe}$ 3 $tRNA^{Phe}$	10	1.30	0.38	29

Incubation mixture: 60 mM Tris-HCl pH 7.4; 70 mM KCl; 10 mM $MgCl_2$; 75 μg poly U; 1.5 A_{260} units NEM treated ribosomes; 8.4 pmoles Gly_2-^{14}C-Phe-$tRNA^{Phe}$ or 12.6 pmoles Gly_2-^{14}C-Phe-tRNA; $tRNA^{Phe}$ (when added) 33 pmoles. After the binding reaction at 37° for 15 min puromycin (final conc. 10^{-3} M) was added. The puromycin reaction was determined after an additional incubation for 20 min at 37°.

The results in table 1 show that at 10 mM magnesium ion concentration over 90% of the bound $Gly_2Phe\text{-}tRNA^{Phe}$ from the purified preparation reacted with puromycin. On the other hand, when unpurified Gly_2Phe-tRNA was used in the binding reaction, no significant puromycin reaction was observed although the binding was nearly as high as the binding of the purified substrate. The addition of $tRNA^{Phe}$ to the purified $Gly_2Phe\text{-}tRNA^{Phe}$ inhibited the extent of puromycin reaction considerably under the same conditions. The binding was inhibited to a much smaller extent.

4. Discussion

The results presented here prove that contrary to previously accepted views [14], the peptidyl site of 80 S type ribosomes can be occupied by aminoacyl- or peptidyl-tRNA by a binding mechanism not involving T-2 and GTP. The results of other investigators who obtained neither significant yields of diPhe-tRNA nor a significant puromycin reaction, can be explained in view of the results presented here, by the presence of uncharged tRNAs in their preparation of tRNA derivatives and probably in their ribosomal preparations. The method used for the preparation of ribosomes in this investigation ensured the effective removal of both the peptide and its tRNA moiety from the ribosomes [15]. Substitution of the puromycin treated ribosomes by ribosomes washed only in high salt resulted in drastic reduction in the amount of diPhe-tRNA formation (unpublished results). Contrary to *E. coli* ribosomes, Phe-tRNA or Gly_2Phe-tRNA from the unpurified preparations bind only to a small extent to the P site of reticulocyte ribosomes. This difference in binding characteristics very likely reflects the greater sensitivity of Phe- and Gly_2Phe-tRNA binding to reticulocyte P site to inhibition by uncharged tRNA as compared to *E. coli* ribosomes. The extent of this inhibition seems to be Mg^{2+} ion-dependent; it decreases with increasing Mg^{2+} ion concentration (to be published).

Acknowledgement

This investigation was supported in part by a grant from the Stiftung Volkswagenwerk, Germany, No. 11 1604.

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