INACTIVATION OF THE Tu-GTP RECOGNITION SITE IN AMINOACYL-tRNA BY CHEMICAL MODIFICATION OF THE tRNA

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SUMMARY: Modification of $tRNA^{Phe}$ of Brewer's yeast by the technique of periodate oxidation, borohydride reduction to the diol, and enzymatic charging with phenylalanine yielded a $Phe\text{-}tRNA^{ox\text{-}red}$ whose 2',3' carbon-carbon bond at the 3'-terminal ribose was absent. This modified Phe-tRNA was unable to form a ternary complex with Tu-GTP when assayed by the Millipore filter technique and was unable to bind to ribosomes at either the T factor dependent or independent sites. This represents the first example of a modification at a specific locus in the tRNA molecule which affects the Tu-GTP recognition site.

INTRODUCTION: The role of the T factors, Tu and Ts (1) in bacterial protein synthesis has become clear in recent years through the efforts of several laboratories (2-8), and is summarized in Fig. 1. The central role played by the Tu-GTP-AA-tRNA[1] complex in the selection of a proper AA-tRNA for polypeptide synthesis has been emphasized by studies which show how this mechanism operates to exclude unwanted tRNAs and AA-tRNAs from the protein synthetic machinery. Deacylated or N-acyl AA-tRNAs are prevented from binding at the T factor requiring ribosomal site and hence from interfering with normal peptide bond formation because they cannot form the initial complex with Tu and GTP (9-13), and the same is true for AA-tRNAs whose amino acid group has been removed by nitrous acid treatment (13). Two additional studies show that certain aspects of tRNA structure are also required for recognition by the Tu-GTP complex in addition to the presence of an unmodified amino acid linked to the tRNA. First, $Met\text{-}tRNA^{fMet}$ does not interact with Tu-GTP (14) although it carries a normal amino acid, and second, the denatured form of

[1] Abbreviations used are: AA-tRNA, aminoacyl-tRNA; $Met\text{-}tRNA^{fMet}$, methionyl-tRNA species which can be formylated; Leu-tRNA, leucyl-tRNA; $tRNA^{Phe}$ and Phe-tRNA, tRNA species accepting phenylalanine and phenylalanyl-tRNA, respectively; $Phe\text{-}tRNA^{ox\text{-}red}$, phenylalanyl-tRNA chemically modified as described in the text.

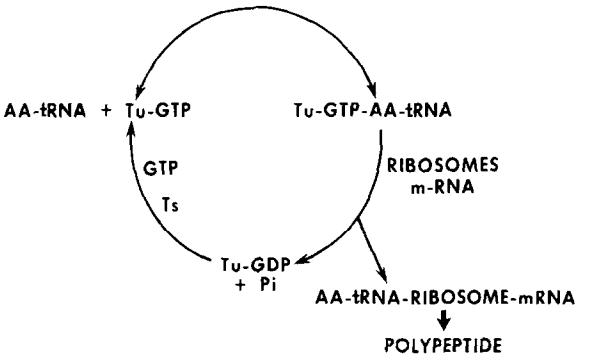

Fig. 1. The Tu-GTP-AA-tRNA cycle in protein synthesis adapted from Weissbach et al. (7).

Leu-tRNA of yeast interacts only very poorly (13, 15) although it too possesses an unmodified amino acid.

As part of our studies on the Tu-GTP recognition site in tRNA, we report in this communication a third case in which a normal amino acid esterified to a modified tRNA cannot interact with the Tu-GTP complex. When $tRNA^{Phe}$ of yeast is treated first with periodate and then with borohydride, the resulting diol can still be charged with phenylalanine (16). However, this Phe-tRNA in which the 2',3' carbon-carbon bond of the ribose of the terminal adenylate moiety is absent cannot react with the Tu-GTP complex.

EXPERIMENTAL: Phe-tRNA and $Phe\text{-}tRNA^{ox\text{-}red}$ - Brewer's yeast $tRNA^{Phe}$ (1200 pmoles phenylalanine acceptance/A_{260} unit) was obtained from Boehringer, and a crude yeast Phe-tRNA synthetase was prepared as described by Lindahl et al. (17). Phe-tRNA was synthesized by scaling up the standard assay procedure for measuring phenylalanine acceptance, and was isolated by phenol treatment, ethanol precipitation, and Sephadex G-25 gel filtration (18). Periodate oxidation of both $tRNA^{Phe}$ and $Phe\text{-}tRNA^{Phe}$ was performed in a solution containing 0.1 M NaOAc buffer, pH 5.4; 2-200 A_{260} units per ml of tRNA, and 1-4 mM $NaIO_4$ for 30 min at room temperature in the dark. Oxidation was terminated by addition of a 1.5-fold excess of glucose to periodate. The oxi-

dized tRNA was isolated and washed once by ethanol precipitation, and dissolved in water. Reduction of the dialdehyde to the diol was performed essentially as described by Cramer et al. (16). A 5 mg/ml solution of $NaBH_4$ in 0.2 M phosphate buffer, pH 7.8, was adjusted to pH 8.1 at room temperature with acetic acid. One volume of this solution was added to one volume of oxidized tRNA (25-120 A_{260} units/ml) and incubated for 110 min at room temperature in the dark. The tRNA was isolated and washed once by ethanol precipitation, and dissolved in water. Since the reduction reaction was sufficiently alkaline to hydrolyze 70% or more of the phenylalanine from the Phe-tRNA, it was necessary to recharge both the tRNA and Phe-tRNA which had been exposed to the oxidizing and reducing conditions. Recharging and reisolation was carried out as described above, both preparations being charged to almost the same extent (Table 1). This procedure is summarized in Fig. 2.

Other materials - Uniformly labeled ^{14}C-L-phenylalanine was obtained from Amersham/Searle and used at the same specific activity of 36.8 mc/mmole in all experiments. [^{3}H]GTP (1.16 Ci/mmole) was purchased from Schwarz Bio-Research. Poly U was obtained from Miles Laboratories, Inc. 95% pure Tu and Ts were generously supplied by Drs. D. Miller, J. Hachmann, and H. Weissbach. Ribosomes were prepared from late log phase E. coli Q13 by the procedure described by Ofengand and Henes (18) except that ribosomes used for enzymatic or nonenzymatic ^{14}C-Phe-tRNA binding were additionally washed 3 times with 0.5 M NH_4Cl. E. coli Q13 100,000 x g supernatant protein was kindly supplied by Mr. Z. Hussain.

Phenylalanine acceptance was assayed in the absence of C^{12} amino acids as previously described (18) except the ^{14}C-phenylalanine concentration was 20 μM, and reactions were stopped by TCA precipitation on Millipore filters (19). The filters were dissolved in naphthalene-dioxane solution (20) and counted.

The formation of Tu-GTP and Phe-tRNA-Tu-GTP complexes was assayed by retention of the Tu-[^{3}H]GTP complex on Millipore filters and the reduction

in this retained radioactivity due to the passage of the Phe-tRNA-Tu-GTP ternary complex through the filter as described previously (7). Tu-GDP was first converted to Tu-GTP by incubation at 37° for 10 min in assay buffer containing 7 mM phosphoenolpyruvate and 20 μg of pyruvate kinase (7), then chilled to 0°; Phe-tRNA was added, and the mixture incubated for 5 min at 0°. The reaction was stopped and samples processed as described previously (7). No hydrolysis of Phe-tRNA or $Phe\text{-}tRNA^{ox\text{-}red}$ occurs under these conditions.

Enzymatic and nonenzymatic binding of Phe-tRNA to ribosomes was measured in a reaction mixture containing in a volume of 0.15 ml, 50 mM Tris-Cl, pH 7.4; 80 mM KCl; 80 mM NH_4Cl; 15 mM or 8 mM $MgCl_2$ as indicated; 1 mM dithiothreitol; 2 mM phosphoenolpyruvate; 0.7 mM GTP; 0.04 μg pyruvate kinase; 60 units Tu (8); 1800 units Ts (8); 1.1 A_{260} units poly U; 3.3 A_{260} units of 0.5 M NH_4Cl washed 70S ribosomes from E. coli Q13 and various amounts of ^{14}C-Phe-tRNA. For nonenzymatic binding, Tu, Ts and GTP were omitted from the reaction mixture. Incubations were for 15 min at 30°, and terminated by the addition of 1.0 ml of a wash buffer containing 50 mM Tris-Cl, pH 7.4; 160 mM NH_4Cl, and 12 mM $MgCl_2$. After passing the diluted incubated mixture through a Millipore filter, the filter was washed three times with 2 ml of cold buffer, dissolved in naphthalene-dioxane (20) and counted.

TABLE 1

PHENYLALANINE ACCEPTANCE ACTIVITY AFTER OXIDATION AND REDUCTION OF $tRNA^{Phe}$

tRNA Preparation	Specific Activity (pmoles/A_{260})			
	RNA^{Phe}		$Phe\text{-}RNA^{Phe}$	
	Prep. A	Prep. B	Before Recharging	After Recharging
(1) Original	1242	1159	1165	1330
(2) Periodate-treated	< 7	< 6	1010	1040
(3) $NaBH_4$ reduction of (2)	916	908	317	1135

Preparation of modified tRNA and assay of acceptor activity was as described in Experimental.

RESULTS: Preparation of Phe-tRNA^{ox-red} - Table 1 summarizes the acceptance activity of the tRNA at each stage of modification. In order to confirm that the only relevant modifications were those occurring at the 3'-terminal adenosine group, a pre-charged control Phe-tRNA was also carried through the treatment since oxidation at the 3'-end of this molecule is prevented by the presence of the amino acid. Line (2) of the table shows that periodate oxidation was complete since no acceptance activity was left in the tRNA^{Phe} preparation, and line (3) shows that subsequent reduction almost completely restored the acceptance activity (16). The slight difference in the amount of phenylalanine bound before and after the recharging of Phe-tRNA^{Phe} in the activity assay probably is due to some deacylation during the preparation of this material since after periodate treatment, this difference was abolished. The product of the oxidation-reduction reaction is assumed to be as indicated in Fig. 2 because (a) the above functional effects are in agreement with those previously

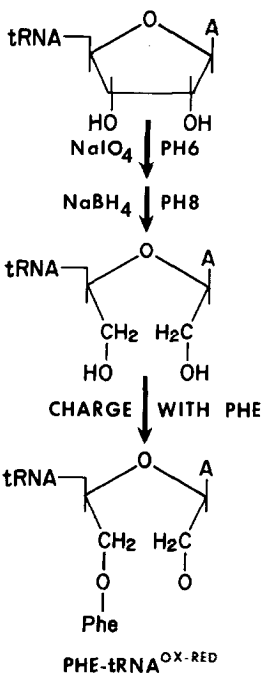

Fig. 2. Preparation of Phe-tRNA^{ox-red}.

reported by Cramer et al. (16) who characterized the product of this reaction, and (b) the half-life for chemical hydrolysis of Phe-tRNA^{ox-red} at 37° in 0.1 M Tris buffer, pH 8.6, was 52 min compared to 16 min for normal Phe-tRNA and the reaction obeyed strict first order kinetics. This decrease in reactivity is in the direction expected for an unoriented diol ester vs. a cis-diol ester (21, 22) and confirms that a modification has occurred at the aminoacyl-end of the tRNA. These observations do not exclude modification reactions at other sites in the tRNA. However, if present, they must have occurred with the Phe-tRNA control preparation also, and since this material remained functional, they cannot be involved in the changes discussed here.

Reaction with Tu-GTP - The failure of Phe-tRNA^{ox-red} to form a ternary complex with Tu-GTP is illustrated in Fig. 3. In this experiment, the amount of complex formed was almost stoichiometric with the amount of AA-tRNA added,

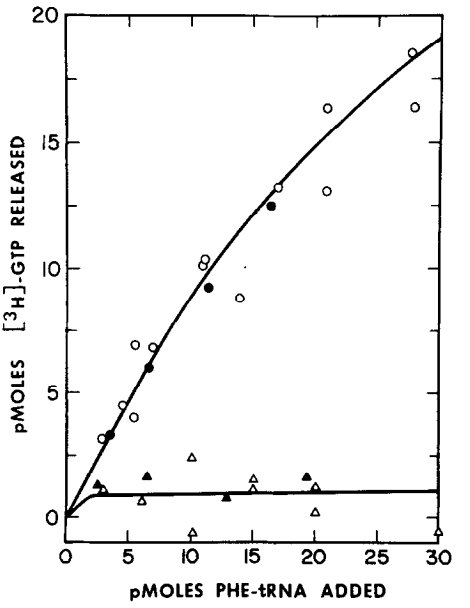

Fig. 3. Ability of Phe-tRNA and Phe-tRNA^{ox-red} to react with Tu-GTP complex. The formation of Phe-tRNA-Tu-GTP ternary complex was assayed by the Millipore filter technique as described in Experimental. In this composite of three experiments, 100% GTP retained on the filter ranged from 20.6 to 34.0 pmoles. Untreated Phe-tRNA, ○; Phe-tRNA exposed to the oxidation and reduction conditions, ●; and Phe-tRNA^{ox-red} (two different preparations), △ and ▲.

as shown by the open and filled circles, representing untreated Phe-tRNA and Phe-tRNA carried through the chemical treatment, respectively. Phe-tRNA^{ox-red}, however, is clearly unable to make a filtrable complex with Tu-GTP. To see if this failure was due to some inhibitor in the Phe-tRNA^{ox-red} preparation, 0, 7.2, 15, and 22.5 pmoles of Phe-tRNA^{ox-red} were added to 9.8 pmoles of normal Phe-tRNA and these mixtures then reacted with 15.2 pmoles of Tu-GTP. 7 pmoles of [^{3}H]GTP was released whether the Phe-tRNA^{ox-red} was present or not, indicating that no inhibitory substances were present. Moreover, it is unlikely that a Phe-tRNA^{ox-red}-Tu-GTP complex formed which was not filterable, since it can be calculated that if the modified Phe-tRNA bound to Tu-GTP with the same affinity as the control but made a non-filterable complex, the addition of 22.5 pmoles of Phe-tRNA^{ox-red} to 9.8 pmoles of normal tRNA should have reduced the released [^{3}H]GTP from 7 to 4.6 pmoles or less. This amount of reduction would have been readily detected.

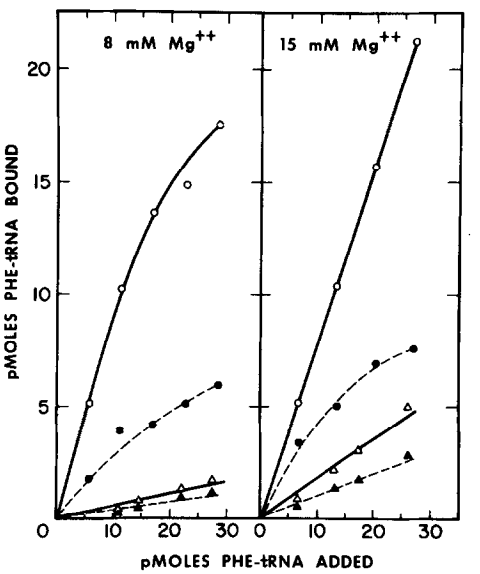

Fig. 4. The binding of Phe-tRNA and Phe-tRNA^{ox-red} to ribosomes with and without Tu, Ts and GTP. Each of the 0.15-ml reaction mixtures contained the components indicated under Experimental. (A) at 8 mM Mg^{++}, (B) at 15 mM Mg^{++}. ○ and ●, Phe-tRNA exposed to the oxidation and reduction conditions; △ and ▲, Phe-tRNA^{ox-red}. Open symbols, with Tu, Ts and GTP; filled symbols, without Tu, Ts and GTP.

Ability to bind to ribosomes and make polypeptide - Although no stable AA-tRNA-Tu-GTP complex could be detected by the filter assay, it was considered possible that the transient formation of such a complex might allow the transfer of Phe-tRNA^{ox-red} into a stable Phe-tRNA-ribosome-poly U complex, especially since the area of the tRNA responsible for Tu-GTP recognition might be quite different from that for binding to the ribosomes. However, as shown in Fig. 4, this was not the case. Not only was T factor dependent binding abolished, but T factor independent binding, thought to represent binding to the peptidyl-tRNA site (4), was also blocked. Note that the active control Phe-tRNA was the preparation described in Experimental that had been carried through the oxidation-reduction treatment in the precharged state.

When tested in a polyphenylalanine synthesizing system dependent on added Phe-tRNA, Phe-tRNA^{ox-red} was unable to function (data not shown).

DISCUSSION: The features of the tRNA molecule that are recognized by the Tu-GTP complex must be distinctly different from those utilized by aminoacyl-tRNA synthetases, since in the former case, different AA-tRNAs must not be discriminated against, while in the latter case, these same differences are essential for selecting cognate tRNAs out of a mixture of similar molecules. In other words, the Tu-GTP complex seeks out the common features of AA-tRNAs, while the synthetases search out the differences among individual tRNA molecules. Our finding that cleavage of the 2',3' carbon-carbon bond of tRNA^{Phe} was able to block interaction with the Tu-GTP complex but did not effect recognition by its cognate synthetase only emphasizes the difference between these two classes of recognition sites.

The Tu-GTP recognition site in AA-tRNA can now be said to require at the minimum (a) a free amino group on the esterified amino acid, (b) a correct overall tertiary structure, and (c) an intact ribose ring of the 3'-terminal adenosine group.

It should be emphasized that all of the fully active control Phe-tRNA used in these experiments had been exposed to the same chemical treatment as

the test tRNA except that the 3'-terminus was blocked by enzymatic charging of phenylalanine. Therefore, the only chemical change that can be relevant to the activity differences between the two preparations must be related to modifications at the 3'-end. While we have so far presented only indirect evidence that such a change occurred, preliminary chromatographic characterization of the phenylalanyl-adenosine derivatives liberated from the Phe-tRNAs by pancreatic RNase does confirm that a structural change took place. Experiments are now underway to replace the modified terminal adenylate group by a normal one in order to demonstrate more directly that this chemical treatment affected the Tu-GTP complex forming ability only by modifying the 3'-terminus of the tRNA.

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