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Ability of modified forms of phenylalanine tRNA to stimulate guanosine pentaphosphate synthesis by the stringent factor-ribosome complex of E. coli

James Ofengand and Richard Liou

Roche Institute of Molecular Biology, Nutley, NJ 07110, USA

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ABSTRACT

$tRNA^{Phe}$ of E. coli, modified at its 4-thiouridine (^{4}Srd) and 3-(3-amino-3-carboxypropyl)uridine ($nbt^{3}Urd$) residues, was tested for its ability to induce (p)ppGpp synthesis. The ^{4}Srd residue was derivatized with the p-azidophenacyl group, cross-linked to Cyd_{13}, and the borohydride reduction product of the cross-link was prepared. The $nbt^{3}Urd$ residue was derivatized with the N-(4-azido-2-nitrophenyl)glycyl group. None of these derivatives had more than a minor effect on the affinity of the tRNA for the stringent factor-ribosome complex, and no effect at all on the maximum velocity of (p)ppGpp synthesis, either at 2 or 82 mM NH_4Cl. These two regions of the tRNA which are on opposite faces of the tRNA molecule do not appear to be structurally important for recognition by the stringent factor-ribosome complex. They may provide useful sites, therefore, for the introduction of photoaffinity or fluorescent probes with which to study tRNA-stringent factor recognition.

INTRODUCTION

The stringent factor-dependent synthesis of guanosine penta- and tetra phosphates, (p)ppGpp, from ATP and GTP or GDP, is strongly stimulated when stringent factor is bound in a complex consisting of ribosomes, mRNA, and the appropriate unacylated tRNA in the ribosomal A site (reviewed in 1). Under appropriate conditions, the rate of synthesis is directly proportional to the concentration of tRNA added (2-5). Chemically modified tRNA species vary in their activity in this system both with respect to their relative affinities for the ribosome-stringent factor complex and in the maximum rate of synthesis that they can induce (4). Consequently, a study of the activity of modified tRNAs can help to delineate those features of the tRNA molecule which are recognized by stringent factor. Previous studies have shown that none of the uridine or uridine-derived nucleotides (such as pseudouridine) are essential, since they can all be replaced by 5-fluorouridine (3), but that the exact structure of the 3'-end is very important (4,5). Except for a preliminary report that substitution of uridine for ribothymidine had no effect, and that replacement of N,N-dimethyl guano-

sine by guanosine did inactivate (6), no other tRNA modifications have been studied.

In this report, we describe the effect of modification at two distinct sites in the body of the tRNA molecule, one in the vicinity of 4-thiouridine (residue 8), and the other in the extra loop, specifically at the nbt^{3}Urd residue. The introduction of photoaffinity and fluorescent probes at these two sites have only a minimal effect on the stimulatory activity for (p)ppGpp synthesis.

MATERIALS AND METHODS.

Modified tRNAs.

E. coli tRNA^{Phe} (11-1200 pmoles phenylalanine acceptance per A_{260} unit) was obtained from Boehringer. S-(p-azidophenacylacetyl)-tRNA^{Phe} (APAA-tRNA) was prepared by reacting tRNA^{Phe} with p-azidophenacyl bromoacetate (APAA-Br) as described by Ofengand *et al.*, (7). The extent of reaction of the ^{4}Srd was monitored by the loss of photochemical crosslinking to C_{13} which was measured by the fluorescence of the reduced $^4S_8$-C_{13} binucleotide (8). Since there is always some crosslinking in untreated tRNA which prevents complete derivatization, determination of the extent of prior crosslinking and derivatization was accomplished by analyzing samples of both control and derivatized tRNA before and after irradiation to the maximum extent of crosslinking. The percent crosslinking was calculated from the control tRNA sample as F(0 irradiation) x 100 divided by F(maximum irradiation) where F is fluorescence, and appropriate solvent blanks were subtracted. The F per A_{260} unit for unirradiated control or derivatized tRNA was the same. The percent derivatization was calculated as [$F_{control}$(plus irradiation)-$F_{modified}$(plus irradiation)] x 100 divided by [$F_{control}$(plus irradiation)-$F_{control}$(minus irradiation)]. Reaction of only the ^{4}Srd residue was confirmed by using the closely analogous compound [*carbonyl*-^{14}C] p-azidophenacyl bromide (APA-Br) (7), under identical reaction conditions. This preparation was the kind gift of S. S. and S. H. Hixson, University of Massachusetts and Mount Holyoke College. Stoichiometric incorporation of the APA residue was completely blocked by prior formation of the $^4S_8$-C_{13} crosslink (7) showing that only ^{4}Srd had reacted. N-(4-azido-2-nitrophenyl)glycyl-tRNA (NAG-tRNA) was prepared by reacting the N-hydroxysuccinimide (NOS) ester of NAG with tRNA^{Phe} as described previously (7). The activated carboxyl group reacts only with free aliphatic amino groups such as in the nbt^{3}Urd residue of *E. coli* tRNA^{Phe} and several other tRNAs. The extent of incorporation was determined by use of [^{3}H]NAG-NOS and purification

of the modified tRNA to constant specific radioactivity (7). As expected, only a single radioactive spot was found on fingerprint analysis of an RNase T_1 digest. XL-tRNA, $tRNA^{Phe}$ whose $^4S_8$ and C_{13} residues are crosslinked, was prepared by irradiation to a maximum yield according to published procedures (8). XLR_{20}- and XLR_{60}-tRNA was prepared by treating 2.3 A_{260} units/ml of XL-tRNA with 2 mg/ml of $NaBH_4$, 0.1 M sodium borate buffer pH 9.5, 0.1 M KCl, and 0.02 M EDTA as described previously (8) for 20 and 60 minutes respectively. As measured by the fluorescence assay (8), full reduction was reached by 20 min.

Other materials.

High salt washed "loose couple" ribosomes, stringent factor, and phenylalanyl-tRNA synthetase were prepared as described (3,4) as were APAA-Br and [^{3}H]NAG-NOS (7).

Methods.

The phenylalanine acceptance of the modified tRNAs were measured by the standard and dimethysulfoxide (DMSO) assays as previously described (9) except that only [^{14}C] phenylalanine at 10-20 μM was used, 1 mM DTT was present, bovine serum albumin was absent, and a purified Phe-tRNA synthetase from E. coli was used. Synthesis of (p)ppGpp was measured as described (3) using 0.22 A_{260} units of "loose couple" ribosomes, tRNA, NH_4Cl, and incubation at 30° as indicated in each figure legend. Reactions were linear with time at least to 50-60% conversion of GTP to (p)ppGpp. Reaction of [carbonyl-^{14}C] APA-Br with $tRNA^{Phe}$ was assayed by TCA precipitation onto paper discs as described by Ofengand et al., (7).

RESULTS

Properties of the modified tRNAs.

Four different modifications of E. coli $tRNA^{Phe}$ were examined. The ^{4}Srd residue was (a) reacted with APAA-Br (Fig. 1A) or (b) crosslinked photochemically to Cyd_{13} (Fig. 1C, I) and also converted to the fluorescent binucleotide (Fig. 1C, II). On the other side of the molecule, the nbt^{3}Urd residue was reacted with NAG-NOS (Fig. 1B). The extent of modification and retention of acceptor activity is summarized in Table 1.

APAA-Br reacted with 96% of the available ^{4}Srd so that this preparation actually consisted of 13.5% XL, 3.6% unmodified, and 82.9% APAA-^{4}Srd containing tRNA. The acceptor activity was somewhat lower than the untreated control tRNA, but the use of special acylating conditions improved the yield. Most of the decrease in specific activity was also found when control tRNA

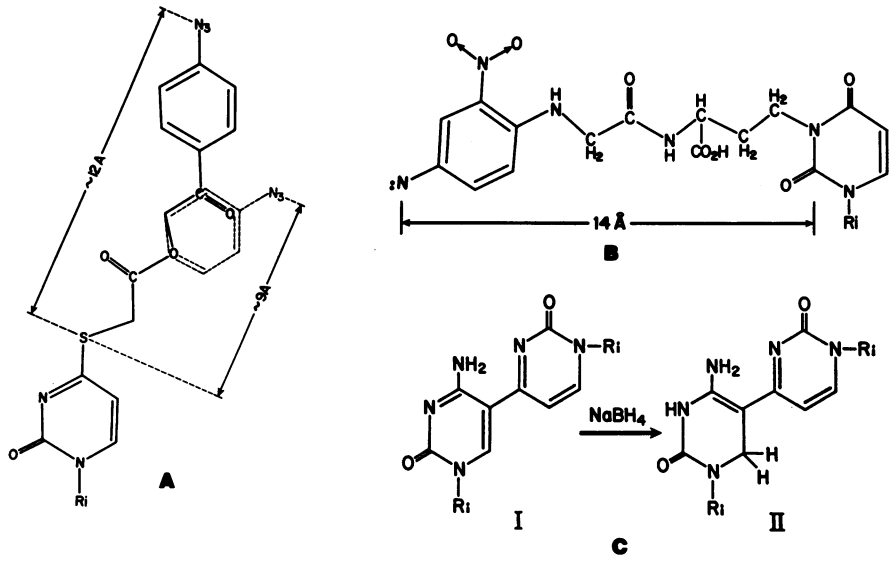

Fig. 1. Structure of the modified tRNA residues examined.
Panel A. The adduct of 4-thiouridine with p-azidophenacyl bromoacetate (solid line) and p-azidophenacyl bromide (dashed line).
Panel B. The adduct of 3-(3-amino-3-carboxypropyl)uridine (nbt^3U) with the N-hydroxysuccinimide ester of N-(4-azido-2-nitrophenyl)glycine.
Panel C. The binucleotide (I) formed between $^4S_8$ and C_{13} of tRNA by irradiation, and its reduction product (II).

TABLE I

PROPERTIES OF THE MODIFIED $tRNA^{Phe}$ SPECIES

$tRNA^{Phe}$ Species	Specific Activity		^{4}Srd Modified		
	Standard Assay	DMSO Assay	Cross-linked	Derivatized	[^{3}H]NAG Incorporated
	pmole/A_{260}unit		percent		pmole/A_{260}unit
Unmodified	1253	1227	6.6	-	-
APAA	773	971	13.5	82.9	-
NAG	860	901	-	-	1042
XL	232	1010	100	-	-
XLR_{20}	-	1209	100	-	-
XLR_{60}	-	1153	100	-	-

Definition, preparation, and assay of the various tRNA species were as described in Materials and Methods.

was exposed to the same reaction conditions in the absence of APAA-Br (981 and 1073 pmol/A_{260} unit in the standard and DMSO assays, respectively). On the other hand, modification by crosslinking ^{4}Srd to Cyd_{13} (8) was strikingly more inhibitory (10) when using the standard assay even with a large excess of enzyme and incubation time. Virtually full acylation could be obtained, however, by the use of special acylation conditions (DMSO assay). Reduction to the fluorescent binucleotide (II) did not affect the yield of aminoacylation in the DMSO assay, even when the reduction time was extended to 60 minutes.

Inhibition of acylation by crosslinking is not a general effect, being known only for Phe and Pro-tRNA synthetases (11). Moreover, it is not simply related to modification or loss of ^{4}Srd, since modification with APAA-Br (Table 1) or NEM (12) had little or no effect, while conversion to uridine by several methods (11,12) also does not block acylation. Inhibition is probably due to some specific steric requirement of Phe-tRNA synthetase for the 5-base region of the molecule spanned by the crosslink rather than for the ^{4}Srd itself. This may be a general feature of Phe-tRNA synthetases as similar results were found for misacylation of crosslinked Val-tRNA with yeast Phe-tRNA synthetase (13). The stimulation of aminoacylation by methylation of G_{10} (14) is also consistent with this view.

The extent of derivatization of the nbt^{3}Urd residue with NAG was consistent with its acceptor activity. In this case, activity was unaffected by the assay conditions used. As above, most of the decrease in activity from untreated tRNA was due to the reaction conditions. The activity of NAG-$tRNA^{Phe}$ contrasts with the finding (15) that similar modification of the nbt^{3}Urd residue of E. coli $tRNA^{Phe}$ with dimethylaminonapthalene-5-sulfonyl glycine and N-methyl anthranilic acid inhibited phenylalanine acceptor activity by 100 and 95%, respectively, but is consistent with the observation that phenoxyacetylation of this residue does not affect acceptor activity (16,17). No doubt, the specific steric and charge properties of the particular modifying group are important factors which influence acylating capability.

Stimulation of (p)ppGpp synthesis.

The activity of the various modified tRNAs is shown in fig. 2 (2-3 mM NH_4Cl) and fig. 3 (81-82 mM NH_4Cl). The low and high NH_4Cl concentrations were chosen on the basis of prior work (4) which showed that a greater degree of specificity among related modifications could be obtained at the high salt concentration. At low NH_4Cl, only small differences were noted for the XLR

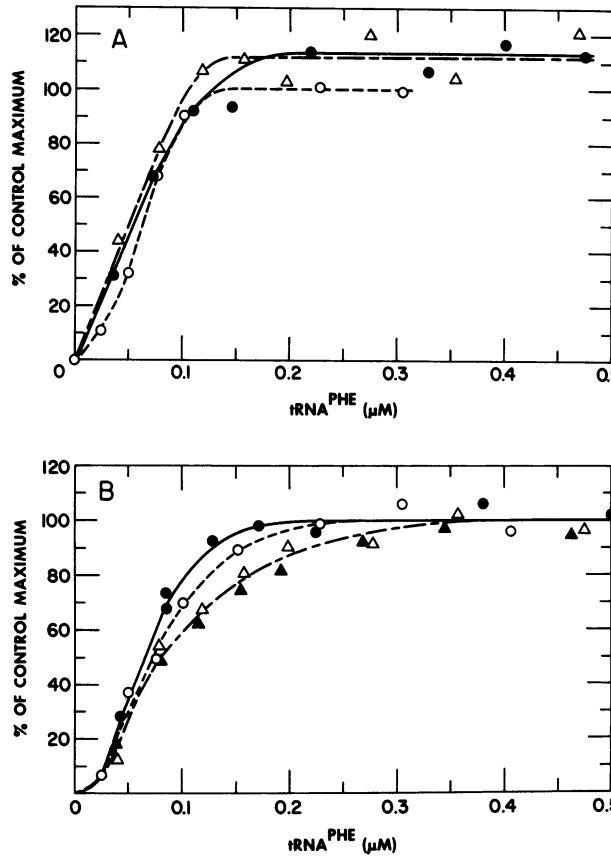

Fig. 2. Stimulation of (p)ppGpp synthesis by modified $tRNA^{Phe}$ at low salt.
Panel A. Reaction mixtures contained 2.9 mM NH_4Cl, tRNA as indicated, and were incubated for 55 min. 100% activity corresponds to 6.2 nmoles synthesized. 0, unmodified tRNA; ●, APAA-tRNA; Δ, NAG-tRNA.
Panel B. Reaction mixtures contained 2.2 mM NH_4Cl, tRNA as indicated, and were incubated for 65 min. 100% activity corresponds to 8.2 nmoles synthesized. 0, unmodified tRNA; ●, XL-tRNA; Δ, XLR_{20}-tRNA; ▲, XLR_{60}-tRNA. Other assay conditions were as described in Materials and Methods.

series and none for the other modifications in the apparent affinity (estimated as the tRNA concentration needed for half-maximal rate of reaction). There was no effect on the maximum velocity. At high NH_4Cl, differences became more apparent. Although the same maximum velocity was reached in all cases, it is clear that APAA-tRNA had a lowered affinity, as did the XL and XLR species, although to a lesser extent. It is interesting that the relative effects on stringent factor recognition are quite different from

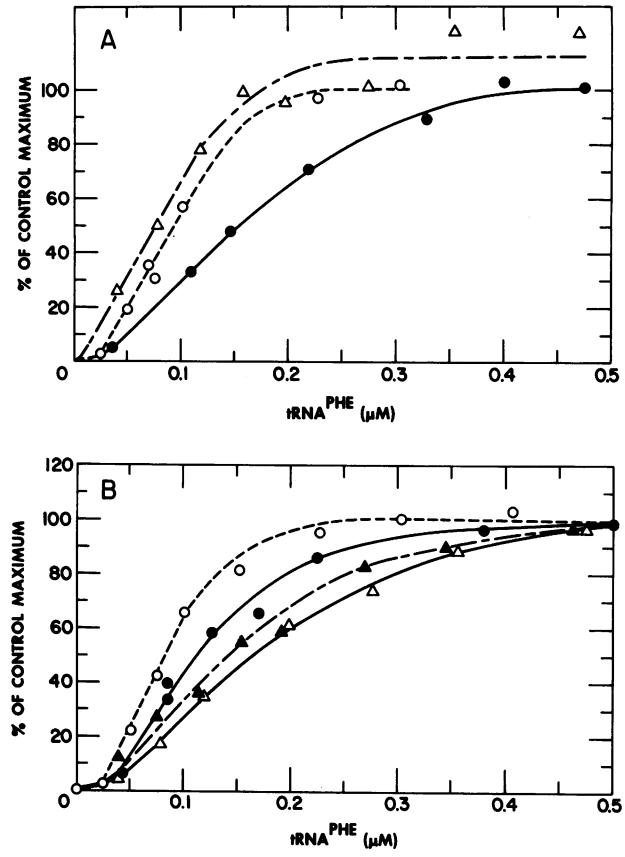

Fig. 3. Activity of modified $tRNA^{Phe}$ at high salt.
Panel A. Reaction mixtures contained 81.4 mM NH_4Cl, tRNA as indicated, and were incubated 60 min. 100% activity corresponds to 9.5 nmoles synthesized. 0, unmodified tRNA; ●, APAA-tRNA; Δ, NAG-tRNA.
Panel B. Mixtures contained 82.2 mM NH_4Cl, tRNA as indicated and were incubated 60 min. 100% activity corresponds to 8.6 nmole synthesized. 0, unmodified tRNA, ●, XL-tRNA; Δ, XLR_{20}-tRNA; ▲, XLR_{60}-tRNA.

those with synthetase, where the APAA modification had little effect but crosslinking was severely inhibitory.

DISCUSSION

These results are useful from two standpoints. First, chemical modification of tRNA can help to delineate recognition sites for its various interacting proteins, especially if the modifications are without effect (18). Fig. 4 summarizes those sites that have been modified with retention of ac-

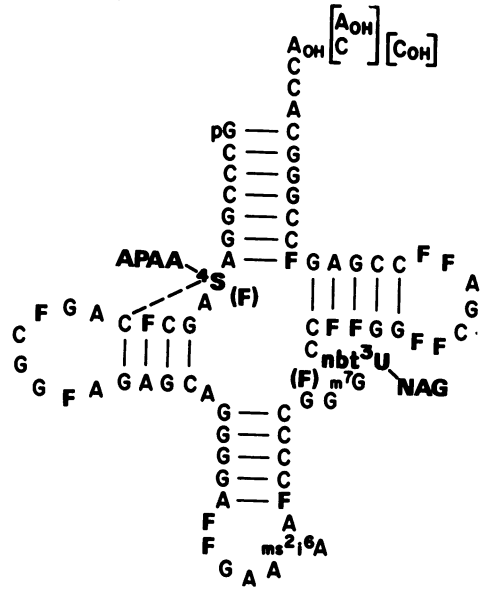

Fig. 4. Primary sequence of E. coli $tRNA^{Phe}$ showing the sites and nature of modifications (in bold face type) which still allow effective stimulation of (p)ppGpp synthesis. The dashed line signifies both crosslinked (XL) and crosslinked-reduced (XLR) tRNAs. Each modification was made separately except that all of the 5-fluorouridines, designated F, which replace U, hU, Ψ, rT, ^{4}S, and possibly nbt^{3}U, were substituted simultaneously. Modifications to the 3'-end were as shown in brackets.

tivity in this and previous work from this laboratory (3,4). In addition, Sprinzl and Richter (5) have shown that the 3'-terminal adenine can be replaced by formycin, and that the 2'-OH of the 3'-terminal ribose can be replaced by H. These modifications have essentially no effect on (p)ppGpp synthesis stimulated by tRNA, except for the replacement of the 3'-terminal A by C or by formycin. In these two cases, the activity was reduced 3 and 6 fold, respectively.

Since the fluorine atom is only 1.13 times larger than H, substitution of 5-fluorouridine for uridine and uridine-derived nucleosides is not expected to have a marked effect, unless the modified uridine being replaced has a specific functional role. As pointed out elsewhere (3), Ψrd in particular does not appear to have any such specific role in (p)ppGpp synthesis, and replacement of ^{4}Srd by F is also in agreement with the lack of effect of the modifications discussed here. The two bulky modifying groups introduced, APAA and NAG, are, in the three-dimensional model of tRNA, attached

to residues quite close to each other in space (19), although the modifying groups in fact point essentially in opposite directions, more or less perpendicular to the plane defined by the L-shape of the tRNA. Since these residues are 10-14Å long when maximally extended, it is reasonable to conclude that an extremely close contact with the stringent factor-ribosome complex does not occur at the sites of these particular modified bases. This does not preclude contact with part of the ribosome or stringent factor, however, since among several possibilities, the APAA and/or NAG residues have considerable latitude for reorientation. In fact, we have already shown that APA and APAA-$tRNA^{Phe}$ can be covalently crosslinked to the ribosome when bound in the A site by EFTu (20,21). It may also be possible to photo-affinity label the A site with a stringent factor-bound tRNA.

Joining of $^{4}Srd_{8}$ and Cyd_{13} limits somewhat the breathing modes of tRNA (19), but reduction of the binucleotide does not further affect the stereochemistry (Fig. 1C). Consequently, the lack of effect indicates that there is no substantial opening of the tRNA molecule in this region at any step of the process of induction of (p)ppGpp synthesis. This result is consistent with all previous studies on crosslinked tRNAs which show that synthetase and tRNA-nucleotidyl transferase recognition (11), formation of a ternary complex with EFTu-GTP (22), ribosomal A and P site binding (23), and initiation factor recognition (24) are all unaffected. Since the reduced binucleotide (Fig. 1C-II) is fluorescent, it may be possible to use XLR-tRNA as a fluorescent probe of tRNA interaction with the stringent factor-ribosome complex. This type of probe which involves a minimal disturbance of the native structure should be preferable to ones where foreign fluorescent groups are added.

Finally it should be noted that induction of (p)ppGpp synthesis is the only assay currently available for measuring the interaction of unacylated tRNA at the ribosomal A site, and will be a useful corollary to EFTu-directed binding of aminoacyl-tRNA. The only other assay for binding of unacylated tRNA to ribosomes (25) is specific for the ribosomal P site.

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