Covalent complex of phenylalanyl-tRNA synthetase with 4-thiouridine-substituted $tRNA^{Phe}$ gene transcript retains aminoacylation activity

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Abstract s^4U-containing transcripts of $tRNA^{Phe}$ gene have been prepared by complete substitution of 16 U residues or by random incorporation of s^4U residues followed by affinity electrophoresis isolation of s^4U-monosubstituted tRNA transcripts. Both analogs have been cross-linked to *Thermus thermophilus* phenylalanyl-tRNA synthetase (PheRS) and the specificity of the cross-linking has been demonstrated. Functional activity of the covalent complex of PheRS with the s^4U-monosubstituted transcript has been shown by aminoacylation of 60% of the enzyme-cross-linked tRNA. This is the first instance in which biological activity of aminoacyl-tRNA synthetase and cross-linked tRNA in a specific complex has been revealed.

Key words: Protein-nucleic acid recognition; Phenylalanyl-tRNA synthetase; Affinity labeling; 4-Thiouridine; *Thermus thermophilus*

1. Introduction

Recognition and specific aminoacylation of tRNA by aminoacyl-tRNA synthetases (aaRSs) is a key step that governs the accuracy of protein synthesis. Recently, the study of tRNA aminoacylation has mainly focused on determination of the tRNA structural elements required for specific recognition by cognate synthetases (reviewed in [1,2]). High-resolution X-ray crystallographic data have been obtained for some complexes of aaRSs with tRNAs [2–5]. From affinity labeling and site-directed mutagenesis of a number of aaRSs the peptide regions interacting with the tRNA recognition elements have been identified [2].

PheRS is a $\alpha_2\beta_2$ heterotetramer that belongs to class II of aaRSs. The three-dimensional structure of *Thermus thermophilus* PheRS cocrystallized with its cognate tRNA has been resolved [5]. The amino acid residues responsible for specific interaction with three nucleotides of the anticodon and with the 3′-terminal CCA sequence of $tRNA^{Phe}$ have been defined. These nucleotides are critically important for efficient aminoacylation with this enzyme [6,7]. Little contribution of some nucleotides involved in the $tRNA^{Phe}$ tertiary interactions and the nucleotide in position 20 to $tRNA^{Phe}$ recognition has been shown by in vitro kinetics measurement for mutant $tRNA^{Phe}$ gene transcripts [6]. Additional contacts with tRNA backbone in the anticodon stem, the D-arm, the acceptor stem and the variable loop have been found by iodine footprinting [8]. Several nucleotides of these regions have been proposed to be in close contact with PheRS in the crystal structure, but their interaction with the enzyme has not been examined in detail.

In the present work we have used s^4U-containing transcripts of $tRNA^{Phe}$ gene for affinity labeling of *T. thermophilus* PheRS. Modified polynucleotides containing photoreactive s^4U residues have been extensively used for investigating RNA-protein interactions in different systems other than aaRSs [9,10]. The most important among many advantages of such RNA derivatives discussed in [9,10] is the zero length of the reactive group providing identification of the structural regions of two macromolecules, which are in close contact. It was of particular interest to examine the functional activity of the $tRNA^{Phe}$-PheRS covalent complex. No data on aminoacylation of tRNA derivatives cross-linked to aaRSs have been reported so far. The results of these experiments provided direct evidence for high specific labeling of the tRNA binding site of PheRS.

2. Materials and methods

2.1. Materials

ATP, GTP, CTP, UTP and spermidine were purchased from Sigma. Acrylamide, *N*,*N*′-bisacrylamide and tris(hydroxymethyl)aminomethane were obtained from Fluka and dithiothreitol from Serva. Sephadex G-200 (10–40 μm) was purchased from Pharmacia Fine Chemicals. L-Phenyl[2,3-^{3}H]alanine (1.96 TBq/mmol) was a product of St. Petersburg Institute of Applied Chemistry. [U-^{14}C]ATP (17 GBq/mmol) and L-[U-^{14}C]phenylalanine (13 GBq/mmol) were purchased from UVVVR. 4-Thiouridine-5′-triphosphate (s^4UTP) was synthesized from 4-thiouridine-5′-monophosphate (Serva) by V.S. Bogachev (Novosibirsk Institute of Bioorganic Chemistry). (*N*-Acryloylamino)phenylmercuric chloride (APM) was kindly provided by G.L. Igloi (Universitat Freiburg, Germany). Plasmid DNA containing the *Escherichia coli* $tRNA^{Phe}$ gene under the control of the phage T7 promoter was a generous gift of O.C. Uhlenbeck (University of Colorado, USA).

PheRS with a specific activity of 350 units/mg was isolated from *T. thermophilus* HB8 as described [11]. One unit of PheRS activity is defined as the amount of enzyme which catalyzes phenylalanylation of 1 nmol of $tRNA^{Phe}$ for 1 min at 37°C. T7 RNA polymerase was isolated from *E. coli* BL21 harboring the plasmid pAR1219. *T. thermophilus* $tRNA^{Phe}$ with acceptor activity of 1700 pmol of phenylalanine/A_{260} unit was isolated as described [12,13]. $tRNA^{Phe}$ lacking the

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Abbreviations: aaRS(s), aminoacyl-tRNA synthetase(s), E.C. 6.1.1; APM, (*N*-acryloylamino)phenylmercuric chloride; PAG(E), polyacrylamide gel (electrophoresis); PheRS, phenylalanyl-tRNA synthetase; s^4U, 4-thiouridine; [1s^4U]-$tRNA^{Phe}$ and [16s^4U]-$tRNA^{Phe}$, $tRNA^{Phe}$ gene transcripts containing 1 or 16 s^4U residues; $tRNA^{Phe}$(-pA76), $tRNA^{Phe}$ lacking the 3′-terminal nucleotide

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3′-terminal nucleotide, tRNA^{Phe}(−pA76), was prepared by subjecting tRNA^{Phe} to one cycle of the Whitfeld degradation procedure [14].

2.2. Preparation of thiolated tRNA^{Phe} gene transcripts

Run-off transcription with T7 RNA-polymerase of *Bst*NI-linearized DNA was performed as described [15] with some modifications. The completely thiolated tRNA^{Phe} gene transcript was synthesized in the presence of s^{4}UTP without UTP and isolated by a denaturing 10% PAGE. To prepare s^{4}U-monosubstituted (monothiolated) transcript, the transcription was carried out in the presence of s^{4}UTP and UTP mixed in a 1:4 molar ratio. tRNAs with different s^{4}U content were fractionated on a denaturing 10% PAG containing 2 μg/ml APM according to described procedures [16,17]. The s^{4}U content of the separated fractions was determined from the absorbance ratio at 330 and 260 nm as described [18]. ^{14}C-labeled transcripts (specific activity 2000 cpm/pmol) were prepared in the presence of 0.2 mM [^{14}C]ATP added to 0.8 mM ATP in the transcription reaction mixture.

2.3. Cross-linking of thiolated tRNA^{Phe} gene transcripts to PheRS and isolation of the covalent tRNA^{Phe}-PheRS complex

The 40–50-μl reaction mixture contained 1 μM PheRS, 2–3 μM [1s^{4}U]-tRNA^{Phe} or 2–7 μM [16s^{4}U]-tRNA^{Phe}, 15 mM $MgCl_2$, 50 mM Tris-HCl, pH 8.5. Irradiation of the samples was performed with 337 nm light of a nitrogen laser (model LGI-21) at 25°C for 30 min. Control experiments were carried out with the enzyme irradiated alone or in the presence of 2 μM wild-type transcript of the tRNA^{Phe} gene. After irradiation 1-μl aliquots were withdrawn for measurement of a residual enzyme activity. The remaining irradiated mixture was applied on a 3.5×220-mm Sephadex G-200 column equilibrated with 50 mM Tris-HCl, pH 8.5. The sample was eluted with the same buffer at a flow rate of 1 ml/h. The absorbance at 260 nm and 280 nm was monitored with a Millichrome detector. The peak containing protein was pooled, 10- and 20-μl aliquots were taken for measurement of ^{14}C radioactivity. The remainder of the pooled material was assayed for aminoacylation activity as described below. Protein concentration was measured in the control sample with the enzyme irradiated in the absence of tRNA.

2.4. Aminoacylation of tRNA

PheRS activity in aminoacylation reaction was determined as described [6,11]. The 100-μl reaction mixture contained 10 nM PheRS, 2 μM tRNA^{Phe} (K_m 0.12 μM), 10 μM L-[^{14}C]phenylalanine, 5 mM ATP, 9 mM $MgCl_2$, 50 mM Tris-HCl, pH 8.5. Kinetic parameters for tRNA^{Phe} gene transcripts were determined as described [6]. All aminoacylation kinetics of transcripts were carried out in a buffer containing 15 mM $MgCl_2$. Initial rates for phenylalanylation were measured in the presence of 10 nM PheRS. The phenylalanylation level (charging capacity) was determined with a concentration range of 50–200 nM tRNA^{Phe} analog and 10–100 nM PheRS. All aminoacylation kinetics presented in this work were carried out at 37°C. Aminoacylation of tRNA^{Phe} analog cross-linked to PheRS was performed in the following way. The enzyme was separated from uncoupled tRNA by gel filtration (see Section 2.3). 25- and 50-μl aliquots of the pooled protein were taken for aminoacylation assay. $MgCl_2$, ATP and [^{3}H]phenylalanine at the concentrations used for aminoacylation reaction were added to the enzyme in a total volume of 0.1 ml. 15-μl aliquots were taken at various times to measure [^{3}H]phenylalanine incorporation into the trichloroacetic acid-precipitable fraction as described [11]. The effect of the unchargeable analog tRNA^{Phe}(−pA76) on aminoacylation of the PheRS-cross-linked tRNA^{Phe} was determined in a concentration range of 1–6 μM tRNA^{Phe}(−pA76) and of 15–40 nM cross-linked tRNA. In parallel, the enzyme-cross-linked tRNA was replaced with the same amount of free wild-type tRNA^{Phe} transcript.

3. Results and discussion

s^{4}U-containing analogs of tRNA^{Phe} were synthesized by in vitro transcription of *E. coli* tRNA^{Phe} gene with T7 RNA polymerase in the presence of s^{4}UTP. The completely thiolated transcript [16s^{4}U]-tRNA^{Phe} prepared in the absence of UTP contains 16 residues of s^{4}U per molecule (Fig. 1). The monothiolated analog [1s^{4}U]-tRNA^{Phe} was prepared by random incorporation of s^{4}U in the presence of both UTP and s^{4}UTP followed by isolation of s^{4}U-monosubstituted tRNA fraction on affinity gel. The sulfur-containing nucleic acids were retarded by specific interaction with an organomercurial derivative (APM) present in the gel, which made it possible to separate transcripts with different s^{4}U content [16,17].

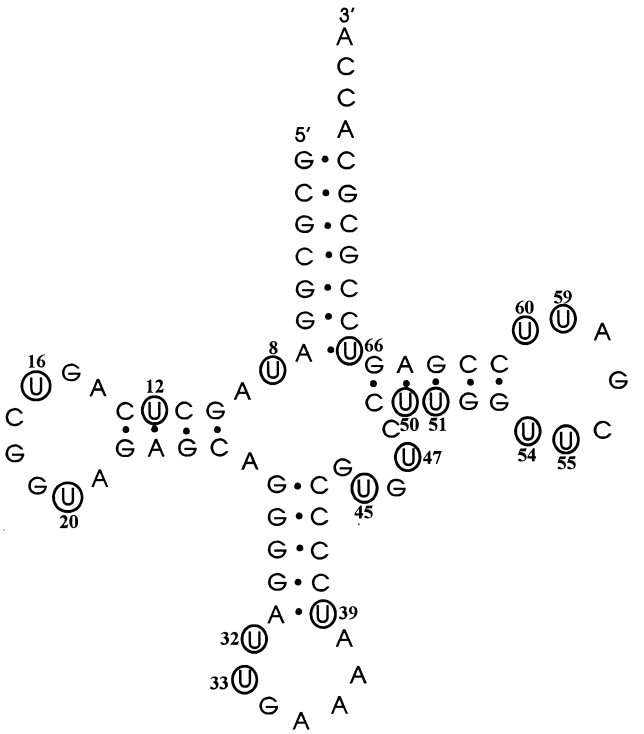

Fig. 1. Secondary structure of the tRNA^{Phe} gene transcript. Circled positions were substituted in the completely thiolated analog [16s^{4}U]-tRNA^{Phe}. The monothiolated analog [1s^{4}U]-tRNA^{Phe} contained one s^{4}U residue randomly distributed between the numbered positions.

Kinetic parameters for aminoacylation with *T. thermophilus* PheRS of thiolated transcripts were determined (Table 1). The data were compared with K_m and k_{cat} values for the wild-type *E. coli* tRNA^{Phe} transcript known to be the efficient heterologous substrate for *T. thermophilus* PheRS with catalytic efficiency similar to that of *T. thermophilus* tRNA^{Phe} [6]. No differences in aminoacylation kinetic parameters for monothiolated and unthiolated transcripts were observed. The substitution of 16 U residues by s^{4}U reduced the relative catalytic efficiency 14-fold primarily due to an increased K_m.

s^{4}U-containing tRNA^{Phe} analogs were cross-linked to PheRS by UV irradiation at 337 nm. To determine the yield of cross-linking reaction, the irradiated mixtures were separated by SDS-PAGE (data not shown). 17% of the input [1s^{4}U]-tRNA^{Phe} and 34% of [16s^{4}U]-tRNA^{Phe} were cross-

Table 1
Kinetic parameters of aminoacylation with *T. thermophilus* PheRS for s^{4}U-containing tRNA^{Phe} gene transcripts

tRNA^{Phe} (transcript)	K_m (μM)	k_{cat}*	k_{cat}/K_m[a]
Wild-type	0.19	(1.0)	(1.0)
[1s^{4}U]-tRNA^{Phe}	0.20	1.0	0.95
[16s^{4}U]-tRNA^{Phe}	2.3	0.85	0.070

[a] k_{cat} and k_{cat}/K_m are normalized to the wild-type transcript.

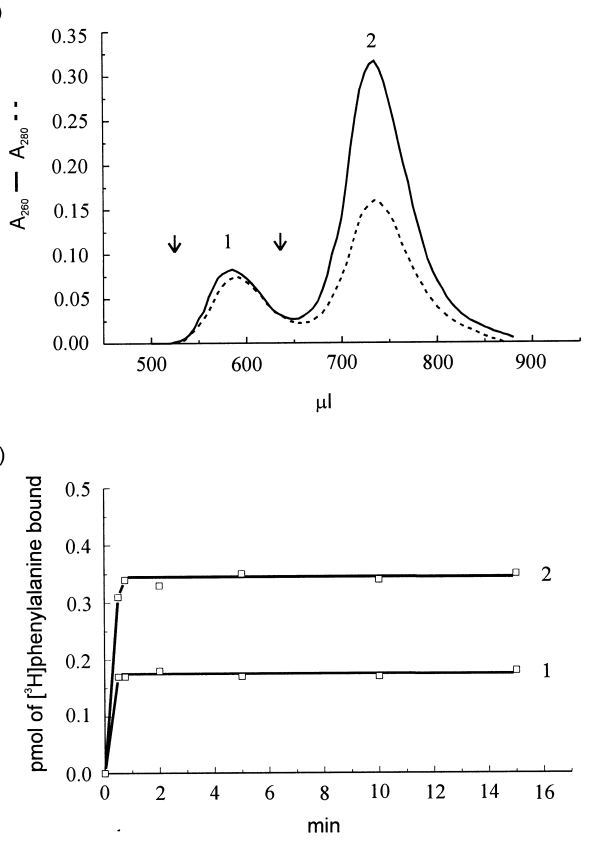

Fig. 2. Gel filtration of the covalent complex of PheRS and [1s^{4}U]-tRNA^{Phe} on Sephadex G-200 column (a). 30 μl of the irradiated mixture was applied on a column. The protein material indicated with arrows was pooled. Aminoacylation of [1s^{4}U]-tRNA^{Phe} cross-linked to PheRS (b). 25-μl (1) and 50-μl (2) aliquots of the pooled material of peak 1 (a) were mixed with [^{3}H]phenylalanine and $MgCl_2$-ATP. Each aliquot taken for measurement of [^{3}H]phenylalanine incorporation contained 0.28 pmol (1) or 0.56 pmol (2) of the enzyme-cross-linked tRNA^{Phe} analog.

linked to PheRS after irradiation of the enzyme with a 2-fold molar excess of each tRNA analog over the protein. The covalent coupling of the enzyme and the tRNA analog was accompanied by a loss of the enzyme activity. One mol of PheRS was completely inactivated by the incorporation of 1.8 mol of [16s^{4}U]-tRNA^{Phe}. The stoichiometry of labeling suggests that *T. thermophilus* PheRS is a functional dimer like *E. coli* PheRS [19]. Cross-linking was completely inhibited by addition of the excess of *T. thermophilus* tRNA^{Phe} but not by addition of a non-cognate tRNA, indicating the specificity of the labeling reaction.

We examined aminoacylation activity of the covalent complexes of PheRS with s^{4}U-containing tRNA^{Phe} analogs. Enzyme-cross-linked tRNA^{Phe} was separated from unbound

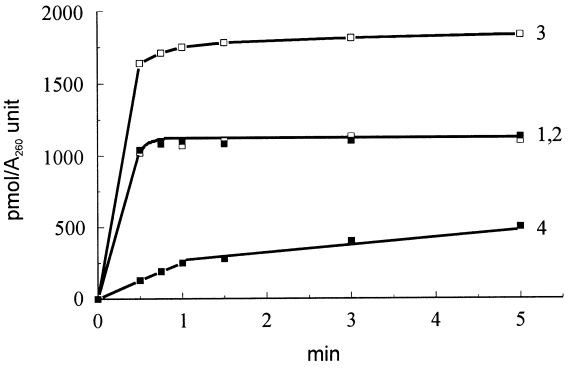

Fig. 3. Effect of tRNA^{Phe}(−pA76) on aminoacylation of [1s^{4}U]-tRNA^{Phe} cross-linked to PheRS (1, 2) or free (the wild-type transcript) tRNA^{Phe} (3, 4). (□) No addition, (■) plus 4 μM tRNA^{Phe}(−pA76). The concentrations of PheRS (in the experiment with the enzyme-cross-linked tRNA the total concentration of free and cross-linked PheRS) and tRNA^{Phe} (the PheRS-cross-linked analog or the wild-type transcript) were 40 nM and 15 nM, respectively.

tRNA^{Phe} analog by gel filtration on Sephadex G-200. Fig. 2a shows the elution profile of the irradiated mixture of PheRS and ^{14}C-labeled analog [1s^{4}U]-tRNA^{Phe}. Peak 1 contains the covalent tRNA-enzyme complex (or complexes differing in cross-linked position of tRNA) and a free enzyme that cannot be separated by this procedure. Peak 2 corresponds to the free tRNA analog. The amount of the bound tRNA^{Phe} was determined by measuring ^{14}C-radioactivity of the pooled material of peak 1. The data on residual enzyme activity and level of cross-linking are presented in Table 2. Addition of [^{3}H]phenylalanine and Mg^{2+}-ATP to the mixture of covalent complex and free PheRS (the pooled material of peak 1, Fig. 2a) led to incorporation of ^{3}H-radioactivity into the cross-linked tRNA^{Phe} (Fig. 2b). In the control experiment with PheRS irradiated in the presence of the wild-type tRNA^{Phe} gene transcript neither ^{14}C-labeled transcript nor [^{3}H]phenylalanine incorporated into the trichloroacetic acid-insoluble fraction was detected in the protein peak, indicating that the isolated enzyme was not contaminated with the free tRNA. The data of the experiments with varying concentrations of the analog showed that about 60% of the enzyme-cross-linked monothiolated tRNA^{Phe} is phenylalanylated. It should be mentioned that the activity of PheRS after the cross-linking reaction was determined as an initial rate of aminoacylation of tRNA^{Phe} added at sub-saturating concentration with catalytic amount of the enzyme (see Section 2). The contribution of the phenylalanylated enzyme-cross-linked tRNA analog to the total amount of phenylalanyl-tRNA^{Phe} formed at these conditions did not exceed 1%. The loss of the enzyme activity is presumably due to the blockage of the tRNA binding site by the cross-linked tRNA.

Table 2
Cross-linking of s^{4}U-containing tRNA^{Phe} gene transcripts to PheRS and aminoacylation of the cross-linked tRNA^{Phe}

tRNA^{Phe} gene transcript (μM)	Residual enzyme activity (%)	Mol of cross-linked tRNA^{Phe}/mol of the enzyme	Mol of [^{3}H]Phe incorporated/mol of cross-linked tRNA^{PHe}
Wild-type (2)	100	0	0
[1s^{4}U]-tRNA^{Phe} (2)	80	0.36	0.61
[1s^{4}U]-tRNA^{Phe} (3)	79	0.38	0.57
[16s^{4}U]-tRNA^{Phe} (4)	38	1.10	0.01
[16s^{4}U]-tRNA^{Phe} (7)	16	1.50	0.01

The observed aminoacylation of [1s^{4}U]-tRNA^{Phe} cross-linked to PheRS might be catalyzed either by the cross-linked enzyme or by the significant amount of free PheRS. To exclude the second possibility, aminoacylation was tested in the presence of tRNA^{Phe} lacking the 3′-terminal nucleotide (Fig. 3). Aminoacylation kinetics of the enzyme-cross-linked tRNA was not affected by addition of the excess of the unchargeable tRNA^{Phe} analog, while aminoacylation of free tRNA^{Phe} was considerably inhibited at identical conditions. Thus, aminoacylation in the covalent complex was not abolished by suppression of the activity of free enzyme in the same mixture, indicating functional activity of the cross-linked enzyme.

For completely thiolated transcript, negligible level of phenylalanylation of the PheRS-cross-linked tRNA was observed (see Table 2). This loss of activity is probably associated with inactivation of the bound tRNA analog, caused by cross-linking reactions. [16s^{4}U]-tRNA^{Phe} irradiated but not linked to PheRS was also examined for the acceptor activity and was not aminoacylated at all conditions used. Previously, *E. coli* tRNA^{Phe} containing a single intramolecular cross-link between s^{4}U8 and C13 has been shown to retain 75% of the acceptor activity of the native tRNA^{Phe} [20]. Many RNA-RNA cross-links that can form the completely thiolated transcript, both free and enzyme-cross-linked, are likely to distort tRNA conformation, resulting in inactivation.

The observed biological activity of the covalent complex of PheRS with the s^{4}U-monosubstituted tRNA^{Phe} analog strongly suggests that the cross-linked tRNA is located exactly at the tRNA binding site, with the acceptor end of the bound tRNA being properly positioned toward the catalytic site of the enzyme. s^{4}U-containing transcripts of tRNA^{Phe} should be useful for identifying the contact regions of PheRS and tRNA^{Phe} bound in a specific complex in solution.

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