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Effect of Nucleotide Replacements in $tRNA^{Phe}$ on Positioning of the Acceptor End in the Complex with Phenylalanyl-tRNA Synthetase

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Abstract—The effect of replacement of $tRNA^{Phe}$ recognition elements on positioning of the 3′-terminal nucleotide in the complex with phenylalanyl-tRNA synthetase (PheRS) from *T. thermophilus* in the absence or presence of phenylalanine and/or ATP has been studied by photoaffinity labeling with s^4U76-substituted analogs of wild type and mutant $tRNA^{Phe}$. The double mutation G34C/A35U shows the strongest disorientation in the absence of low-molecular-weight substrates and sharply decreases the protein labeling, which suggests an initiating role of the anticodon in generation of contacts responsible for the acceptor end positioning. Efficiency of photo-crosslinking with the α- and β-subunits in the presence of individual substrates is more sensitive to nucleotide replacements in the anticodon (G34 by A or A36 by C) than to changes in the general structure of $tRNA^{Phe}$ (as a result of replacement of the tertiary pair G19-C56 by U19-G56 or of U20 by A). The degree of disorders in the 3′-terminal nucleotide positioning in the presence of both substrates correlates with decrease in the turnover number of aminoacylation due to corresponding mutations. The findings suggest that specific interactions of the enzyme with the anticodon mainly promote the establishment (controlled by phenylalanine) of contacts responsible for binding of the CCA-end and terminal nucleotide in the productive complex, and the general conformation of $tRNA^{Phe}$ determines, first of all, the acceptor stem positioning (controlled by ATP). The main recognition elements of $tRNA^{Phe}$, which optimize its initial binding with PheRS, are also involved in generation of the catalytically active complex providing functional conformation of the acceptor arm.

Key words: acceptor end of tRNA, affinity modification, 4-thiouridine, phenylalanyl-tRNA synthetase, *Thermus thermophilus*, tRNA recognition

The accuracy in reproduction of a genetically prescribed protein structure depends on the ability of aminoacyl-tRNA synthetases (aaRS) to recognize and effectively aminoacylate the corresponding tRNA. The proper selection and aminoacylation of tRNA are provided by aaRS interactions with a certain set of tRNA nucleotide residues, which are called identity elements or recognition elements. At present, identity elements are known for all 20 systems of tRNA-aaRS from *E. coli* and for several systems from yeast, *Thermus thermophilus*, and higher eukaryotes including humans [1]. Aminoacylation of tRNA is a multistage process, which includes the initial binding of substrates, conformational rearrangement of the enzyme–substrate complex, chemical reaction in the active site, and release of products. Notwithstanding numerous studies on the problem of tRNA recognition mainly performed by *in vivo* genetic approaches and *in vitro* kinetic experiments with mutant tRNAs [1, 2], data on the discrimination of tRNA at the stage of binding and subsequent catalytic transformations are available only for a limited number of systems. Interactions of aspartyl-tRNA synthetase (AspRS) from yeast and of histidyl-tRNA synthetase (HisRS) from *E. coli* with the anticodon of cognate tRNAs mainly contribute to stability of the corresponding complexes and

Abbreviations: APM) *p*-(N-acryloylamino)phenylmercuric chloride; aaRS) aminoacyl-tRNA synthetase; XCA) X-ray crystallographic analysis; ps^4Up) 4-thiouridine-3′,5′-diphosphate; s^4U) 4-thiouridine; Phe-AMP) phenylalanyladenylate; PheRS) phenylalanyl-tRNA synthetase (similar abbreviation is used for other aminoacyl-tRNA synthetases, with their specificity indicated according to conventional names of amino acids).

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ensure the selection specificity at the binding stage [3, 4], whereas the tRNA^{Trp} identity mainly influences the rate of transfer of the activated amino acid [5]. Interactions of *E. coli* glutaminyl-tRNA synthetase with tRNA^{Gln} identity elements determine the efficiency of glutamine recognition [6]. Disorders in the tertiary structure of yeast tRNA^{Phe} influence the aminoacylation mechanism: dissociation of pyrophosphate becomes the rate-limiting stage [7]. Biochemical and structural data on interaction of the most studied yeast AspRS with homologous tRNA^{Asp} have shown that the substrate binding is initiated by recognition of the anticodon loop and that all recognition elements of tRNA^{Asp} are involved in formation of the catalytically active complex [8]. It has been shown for Asp- and Ser-specific enzymes of class II that the tRNA acceptor end is properly positioned for catalysis only in the presence of ATP and the amino acid [9-11]. Interaction of HisRS (of class II) with a stable histidyladenylate analog improves the discrimination of tRNA at the binding stage [4].

Phenylalanyl-tRNA synthetase (PheRS) is one of the most complicated aaRSs. All known cytoplasmic PheRSs have the rare subunit structure $(\alpha\beta)_2$ [12]. By *in vitro* kinetic studies, recognition elements of tRNA^{Phe} from various organisms have been detected [13-20]. The structure of *T. thermophilus* PheRS complex with tRNA^{Phe} has been studied in detail by X-ray crystallographic analysis (XCA) [21] and biochemical methods [22, 23]. Nucleotides of the tRNA^{Phe} anticodon are main specificity determinants in all systems. The contribution of other elements (nucleotides in the 20th and 73rd positions and nucleotide pairs A31-U39 and G30-C40) to the catalytic efficiency of aminoacylation strongly varies for different organisms and is minor in a thermophilic system. The proper conformation of tRNA^{Phe} is absolutely necessary for effective aminoacylation in all systems studied. As shown for *T. thermophilus* PheRS, the preferential recognition of tRNA^{Phe} at the binding stage is provided by specific (with involvement of bases) interactions with the anticodon and nonspecific (with involvement of ribose-phosphate groups) interactions with nucleotides of the central regions responsible for stabilization of the tertiary structure and conformational adaptation of the substrate [24]. Interaction of the tRNA^{Phe} acceptor end with the enzyme is not functional in the absence of other two substrates [25]. Effects of phenylalanine (Phe) and ATP on positioning of the tRNA^{Phe} acceptor end in the complex with PheRS [26, 27] have been shown using affinity modification with tRNA^{Phe} analogs containing different reactive nucleotides on the 3′-end. The productive binding of the terminal adenosine is determined by its specific contacts controlled by Phe-substrate; ATP modulates the acceptor arm conformation in the complete enzyme complex with all substrates. In the present work, photoaffinity labeling of *T. thermophilus* Phe RS with s^{4}U-containing tRNA^{Phe} derivatives and its mutants (with nucleotide substitutions in different regions of the structure) was compared. The comparison of labeling products of the protein subunits in the absence and in the presence of low-molecular-weight substrates revealed the role of different tRNA^{Phe} recognition elements in the productive binding of the acceptor end.

MATERIALS AND METHODS

Reagents used were as follows: ATP, UTP, GTP, CTP, L-phenylalanine, and a set of marker proteins with molecular weights from 36 to 193 kD from Sigma (USA); acrylamide, N,N′-methylenebisacrylamide, β-mercaptoethanol, Tris, and HEPES from Fluka (Switzerland); SDS from USB (USA); glycine and dithiothreitol from Serva (Germany). [α-^{32}P]ATP (30 TBq/mmol) was from ICN (USA). A preparation of *p*-(N-acryloylamino)phenylmercuric chloride (APM) was presented by Prof. G. Igloi (Freiburg University, Germany). 4-Thiouridine-3′,5′-diphosphate (ps^{4}Up) was synthesized by V. S. Bogachev from 4-thiouridine (Serva) as described in [27]. Plasmids with incorporated genes of wild type and mutant *E. coli* tRNA^{Phe} were presented by Prof. O. Uhlenbeck (University of Colorado, USA). The following enzyme preparations were used: alkaline phosphatase from calf intestine and recombinant T4 RNA ligase from Pharmacia Biotech (USA), benzonase from Merck (Germany), inhibitor of ribonucleases from Promega (USA). Phenylalanyl-tRNA synthetase (EC 6.1.1.20) was isolated from *T. thermophilus* HB8 as described earlier [28]. T7 RNA polymerase was isolated from *E. coli* BL21 cells carrying the pAR1219 plasmid.

Transcripts corresponding to the wild type and mutant *E. coli* tRNA^{Phe} sequence were synthesized *in vitro* using T7 RNA polymerase as described in [24]. **Labeled transcripts** were prepared by incorporation of [^{32}P]AMP into tRNA by addition of [α-^{32}P]ATP (0.2-1 MBq) into the reaction mixture (20 μl) for transcription.

tRNA^{Phe}-transcripts (wild type or mutant) containing s^{4}U residue on the 3′-end in the 76th position were synthesized as described in [27].

Modification of phenylalanyl-tRNA synthetase by s^{4}U-containing tRNA^{Phe} derivatives and analysis of labeling products. Reaction mixtures (10 μl) contained 50 mM Tris-HCl buffer (pH 8.5), 15 mM $MgCl_2$, the enzyme (0.05-10 μM), and ^{32}P-labeled tRNA^{Phe}-s^{4}U (0.05-0.4 μM). Samples were UV-irradiated using an HBO 200W high-pressure mercury lamp supplemented with a Bausch and Lomb monochromator (wavelength 365 nm) for 30-60 min at 25°C. The modification products were separated and analyzed as described in [27].

RESULTS

Mutant tRNA^{Phe}s with nucleotide replacements in different positions and synthesized by *in vitro* transcrip-

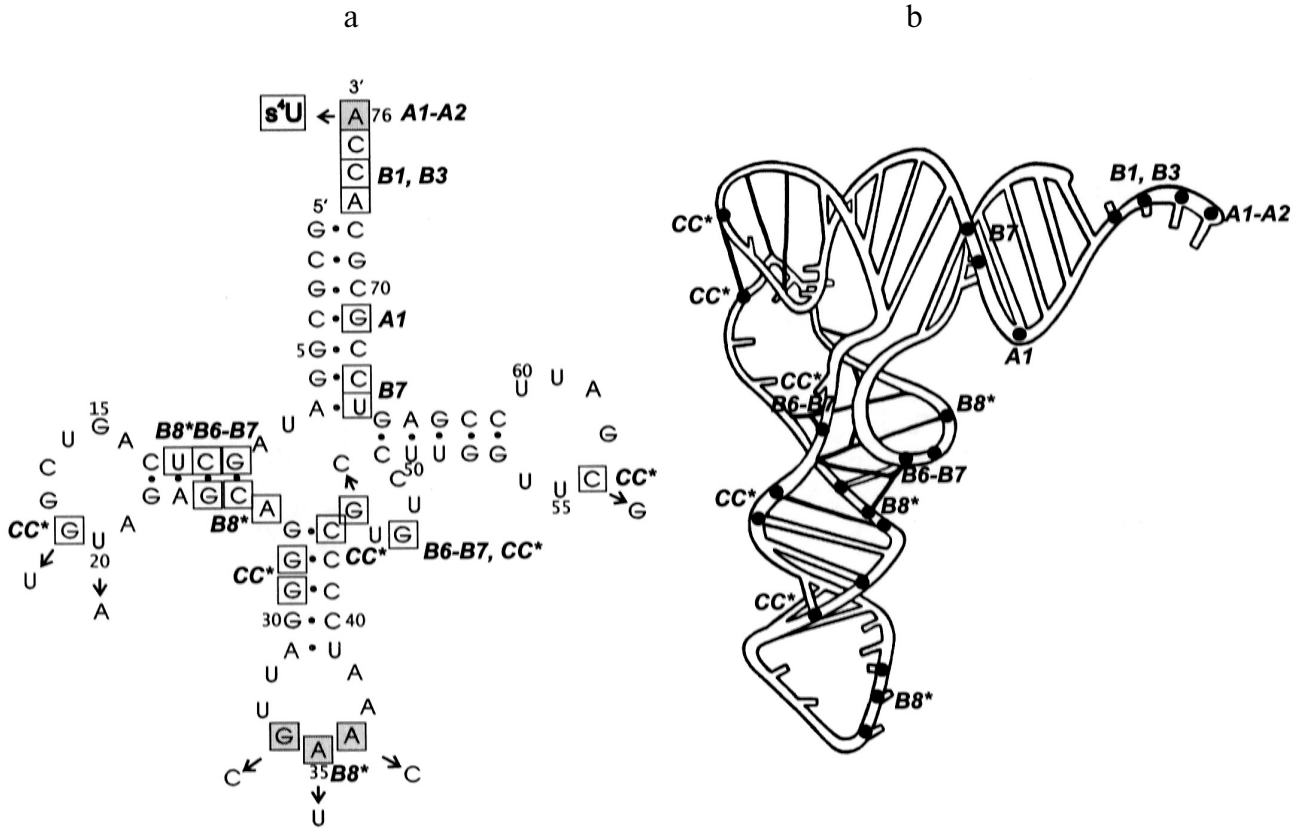

Fig. 1. a) Structure of $tRNA^{Phe}$ reactive analogs prepared on the basis of wild type or mutant *E. coli* $tRNA^{Phe}$ (synthesized by *in vitro* transcription). The s^4U residue is introduced in the 3′-terminal position instead of adenosine. The arrows show nucleotide replacements in $tRNA^{Phe}$ mutants. Nucleotides shown to contact PheRS in the crystal structure [21] are in rectangular frames; base-specific contacts are hatched. Domains of the protein α- (A1, A2, and the N-terminal coiled coil CC) and β-subunits (B1-B8) binding $tRNA^{Phe}$ are indicated; fragments marked with asterisks belong to the second monomer of the $(\alpha\beta)_2$-dimer. b) Nucleotides (marked with black circles) which form contacts with different structural domains of PheRS are shown on the three-dimensional structure of $tRNA^{Phe}$.

tion on the background of *E. coli* $tRNA^{Phe}$ were earlier used by us to determine the recognition elements of $tRNA^{Phe}$ and evaluate their roles in specific complexing with *T. thermophilus* PheRS [18, 19, 24]. The *E. coli* $tRNA^{Phe}$ transcript and *T. thermophilus* $tRNA^{Phe}$ (modified) have the same values of dissociation constants (K_d) of complexes with this enzyme and very similar values of kinetic parameters of aminoacylation [19, 24]. Six mutants of *E. coli* $tRNA^{Phe}$ contain replacements in different elements of the structure (Fig. 1a) and have different efficiency of the initial binding (in the absence of other substrates) and catalytic transformation (Table 1). The anticodon plays the leading role in provision of the specificity at all stages of the $tRNA^{Phe}$ interaction with PheRS; replacements of one-two nucleotides in its different positions (G34, A35, and A36) result in maximal losses in affinity and catalytic efficiency of aminoacylation. Three other mutants represent structural variants. The tertiary pair G19-C56 responsible for stabilization of the three-dimensional structure of $tRNA^{Phe}$ is significant for complexing and to lesser degree for effective catalysis. The U20 nucleotide and tertiary pair A26-G44 are involved in conformational adaptation of the enzyme and substrate [21-23]. Photoreactive derivatives of $tRNA^{Phe}$-transcripts (wild type and mutant), which contain the 4-thiouridine residue in the 3′-terminal position (Fig. 1a), were synthesized with RNA ligase by ligation of ps^4Up to the corresponding transcripts shortened by one nucleotide (from the 3′-end), with the subsequent removal of 3′-phosphate. s^4U-Containing tRNAs ($tRNA^{Phe}$-s^4U76) were isolated by affinity electrophoresis [29]. Photoaffinity labeling of PheRS with $tRNA^{Phe}$-s^4U76 (prepared on the basis of the wild type transcript) was an effective approach to study effects of low-molecular-weight substrates on positioning of the acceptor end in complex with the enzyme [27].

Electrophoresis in SDS-polyacrylamide gel of PheRS products of labeling with s^4U76-containing derivatives of different $tRNA^{Phe}$s shows (Fig. 2) that in each case a number of products of cross-linking to the α- and

Table 1. Kinetic parameters of aminoacylation of tRNA^{Phe} mutants and dissociation constants of their complexes with *T. thermophilus* PheRS

tRNA^{Phe} [a]	K_d, nM [b, c]	k_{cat} [b, d]	k_{cat}/K_m [b, d]
Wild type	5	1.0	1.0
G34A	1200(240)	0.1(10)	0.0053(190)
G34C/A35U	1600(320)	0.032(31)	0.00058(1720)
A36C	150(30)	0.24(4.2)	0.10(10)
U20A	40(8)	1.0	0.42(2.4)
G44C	20(4)	0.47(2.1)	0.35(2.8)
G19U/C56G	160(32)	0.59(1.7)	0.056(18)

[a] Prepared by *in vitro* transcription of the *E. coli* tRNA^{Phe} gene (wild type or its mutants). For tRNA^{Phe} mutants, positions and types of nucleotide replacements are indicated.

[b] Degree of increase in K_d or of decrease in kinetic characteristics k_{cat} and k_{cat}/K_m (compared to the corresponding parameters for the wild type transcript) is shown in parentheses.

[c] K_d values of complexes are determined by retardation in gel [24].

[d] Relative values of k_{cat} and k_{cat}/K_m are presented (normalized to the corresponding values for the wild type transcript).

β-subunits are generated. The subunits were identified in the products by hydrolysis with benzonase as described earlier [26, 27] (data not presented). The same products were recorded for the analogs of wild type tRNA^{Phe} (in the control experiment) and for those of mutants G34A, A36C, and G44C. Electrophoretic mobilities of three cross-linked products of analogs of structural mutants U20A and G19U/C56G were slightly different from the mobilities of the corresponding products in the control sample, and this most likely was due to structural differences of the tRNAs cross-linked. Nucleotide replacements in the indicated positions destabilize the overall folding of tRNA^{Phe} [17, 24], and under conditions of SDS-polyacrylamide gel electrophoresis, the denaturation degree of tRNA can affect the conformation and, consequently, migration of the covalent tRNA–protein complex. Such differences in mobilities of cross-linked products we have recorded for s^{4}U-substituted analogs of *E. coli* and human tRNA^{Phe} [27]. Positioning of the reactive nucleotide is most significantly affected by non-conservative (purine-pyrimidine) replacement of two nucleotides of the anticodon: the major fraction is constituted by light products of the α-subunit labeling (56-58 kD resolved incompletely) which fail to form the major fraction in the control experiment; other products (major and minor ones in the control experiment) have similar yields; an additional product of 104 kD of the β-subunit labeling appears and a minor product of 132 kD disappears (Table 2). These changes significantly decrease the labeling efficiency of the catalytic subunit. However, the conservative (purine-purine) replacement of one nucleotide in the anticodon (G34 by A) insignificantly influences relative yields of the products and slightly decreases the ratio of labeling levels of the α- and β-subunits. Noticeable changes in the relative levels of the subunit labeling in favor of the noncatalytic subunit occur only with the nucleotide replacement in the variable loop (G44 by C). Destabilization of the tRNA^{Phe} tertiary structure caused by replacement of the G19-C56 pair by U19-G56 slightly increases the relative efficiency of the analog cross-linking to the α-subunit. Mutations in the 20th and 36th positions only slightly influence relative yields of individual products and fail to change relative levels of the subunits labeling. All changes in the structure of tRNA^{Phe}-s^{4}U76, except the replacement of the 36th nucleotide, decrease the total labeling efficiency (determined as % of the reagent cross-linked to the protein from its total amount in the noncovalent complex). For the majority of mutations the decrease in labeling does not correlate with the destabilization degree of the complex (Tables 1 and 2). The findings suggest that mutations in different regions of tRNA^{Phe} have different effects on the binding strength and positioning of the 3′-end, which determine the cross-linking efficiency of the photoactivated nucleotide. The effect

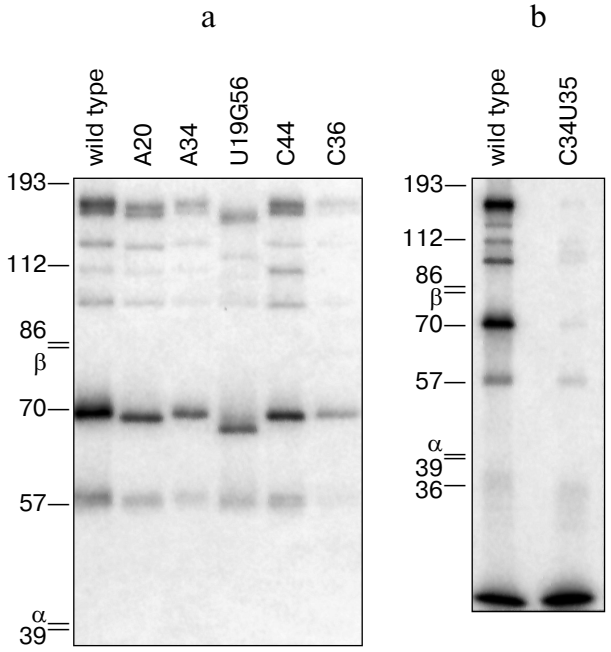

Fig. 2. Electrophoretic separation in SDS-polyacrylamide gel of products of covalent cross-linking to PheRS of ^{32}P-labeled s^{4}U76-substituted tRNA^{Phe} derivatives: a) wild type and mutants U20A, G34A, G19U/C56G, G44C, and A36C; b) wild type and mutant G34C/A35U. Concentration of the enzyme is 2 μM and those of tRNA reagents are 0.2 μM. Time of sample irradiation was 30 min. Positions of the enzyme α- and β-subunits and molecular weights of marker proteins are shown to the left.

Table 2. Affinity modification of *T. thermophilus* PheRS with s^4U76-substituted derivatives of $tRNA^{Phe}$ and its mutants

$tRNA^{Phe}$ [a]	Relative yield of products[b], %						α/β[c]	Efficiency of labeling[d], %
	α		β					
	56-58*	67*, 69**, 70	100, 104*	110	120*, 128**, 132	150*, 150-160**, 155-165		
Wild type	12	53	4.0	2.4	3.6	25	1.9	64
G34C/A35U	32	15	12; 14*	11		16	0.89	4
G34A	11	52	5.4	4.0	5.6	22	1.7	18
A36C	11	55	5.1	2.4	4.5	22	1.9	64
U20A	12	53**	3.3	2.5	5.2**	24**	1.9	40
G44C	9.0*	46	6.6	7.9	3.5	27	1.2	43
G19U/C56G	12*	56*	4.0	2.8	4.2*	21*	2.1	55

[a] Original $tRNA^{Phe}$s are given (Table 1) which were used for preparation of reactive analogs.
[b] The yield of the subunit labeling products from the total level of the enzyme labeling. Apparent molecular weights (kD) of the products are shown: the products with similar electrophoretic mobilities are grouped into the same column; the range of molecular weights is given for the incompletely resolved products (enlarged or doubled lane). The enzyme concentration is 2 μM, those of $tRNA^{Phe}$ analogs are 0.2 μM. Mean values of three determinations are presented; mean deviations of results were 6-8% for minor products (with the relative yield less than 10%) and 2-4% for other products.
[c] The ratio of labeling levels of the α- and β-subunits.
[d] Determined as the protein-cross-linked amount of the reagent normalized to the complex amount. The complex amount is calculated using K_d values for the original mutants (not considering the replacement of the 3′-terminal adenosine by 4-thiouridine).

of nucleotide replacements on the acceptor end positioning under conditions of nonfunctional complex (in the absence of small substrates) is clearly pronounced for G44C- and G34C/A35U-mutants.

By modification of PheRS with $tRNA^{Phe}$-s^4U76 derivatives with G34A, A36C, U20A, and G19U/C56G replacements in the presence of ATP and/or Phe we have studied the role of different structural elements of $tRNA^{Phe}$ in the functional binding of the acceptor end under the control of small substrates. Results of experiments with analogs of the wild type and two mutant $tRNA^{Phe}$s are presented in Fig. 3. Compared to the control experiment, the effects of Phe-substrate are the most different for the 3′-s^4U-substituted analog of the G34A mutant (Fig. 4a). In this case, the substrate effect on the generation of most of the products, including those of the α- and β-subunit labeling, differs quantitatively or qualitatively (increase instead of decrease) from its effect on modification of the enzyme by the wild type $tRNA^{Phe}$ analog. For other mutants qualitative and significant quantitative differences are manifested on minor products of labeling of the noncatalytic subunit. These findings suggest the leading role of the G34 from the anticodon in formation of the complex with the functional positioning of the 3′-terminal nucleotide, which is controlled by interaction of PheRS with Phe-substrate.

In the presence of ATP (Fig. 4b) relative yields of products of the β-subunit labeling increase and of those of the α-subunit decrease differently in the case of wild type $tRNA^{Phe}$-s^4U76 that, as a whole, decreases only 1.3-fold the total level of modification. All mutations suppress the β-subunit labeling and increase the inhibitory effect of ATP on the α-subunit labeling, and, as a result, the total efficiency of modification significantly (2.3-5-fold) decreases. In the case of analogs of structural mutants, ATP has similar effects on labeling of different residues of the β-subunit, while a significant selectivity is observed for analogs with replacements in the anticodon, with the prevalent effect on generation of the major products. The selectivity of ATP with regard to different products of the α-subunit labeling increases more markedly due to mutations in the anticodon than due to changes in the overall folding of $tRNA^{Phe}$-reagent: the generation of the major product is suppressed 1.9-fold stronger in the control experiment and 6.2-, 4.8-, 4.0-, and 2.7-fold stronger on modification with analogs of the mutants G34A, A36C, G19U/C56G, and U20A, respectively. These observations suggest that nucleotide replacements in the anticodon prevalently cause disorientation of the 3′-terminal nucleotide binding in the enzyme complex with ATP. We suggested [27] that the effect of ATP on the 3′-end of $tRNA^{Phe}$ positioning should be caused by conformational changes in the acceptor arm as a result of coordinated displacement of the acceptor stem with the motif 2 loop. The recorded effects of mutations on the PheRS modification in the presence of ATP characterize their dissimilar effects on the enzyme interactions with the stem and single-stranded fragments of the acceptor arm. Replacements of U20 or the tertiary pair G19-C56 most likely result in disorders in partial contacts with the stem,

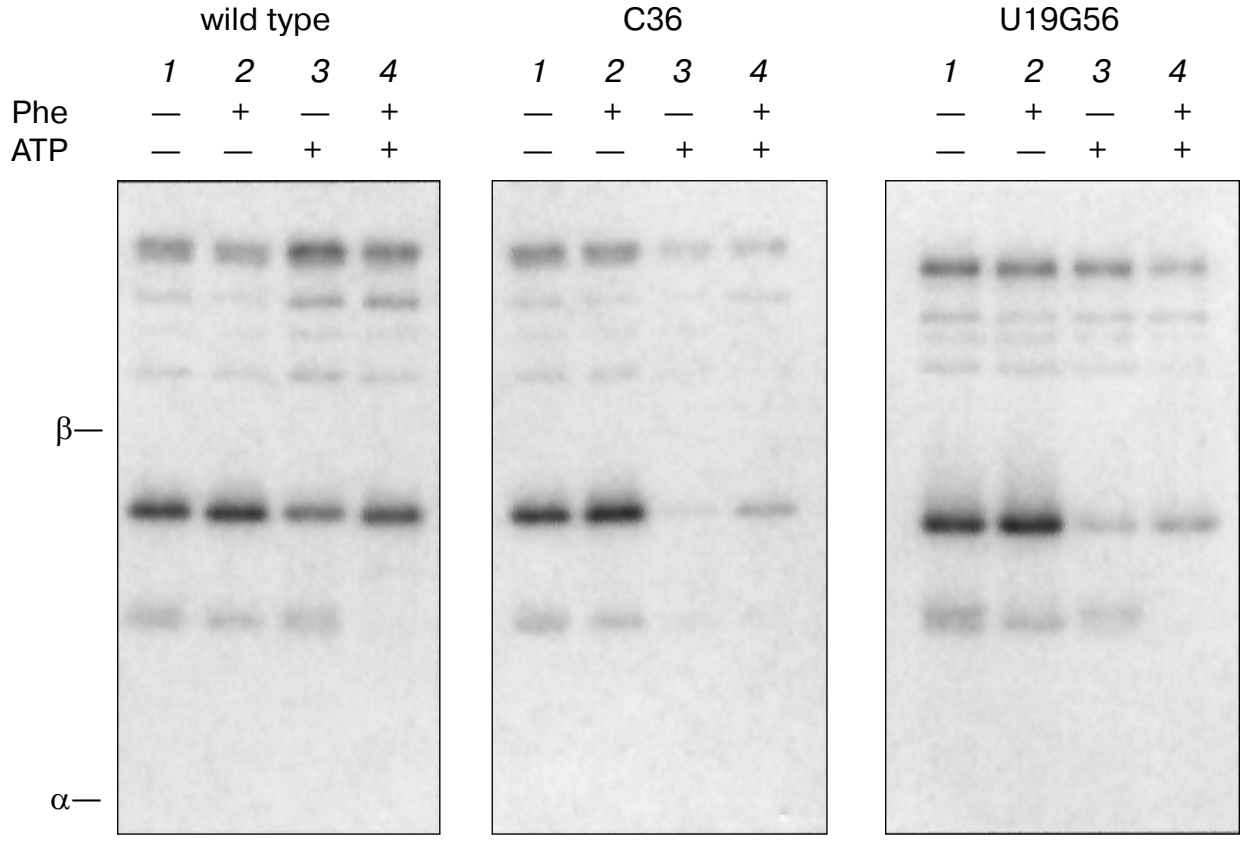

Fig. 3. The comparison of products of PheRS photoaffinity labeling with analogs of the wild type and mutant (G19U/C56G and A36C) tRNA^{Phe}s in the presence and in the absence of substrates. The concentrations are as follows: the enzyme, 2 μM; tRNA reagents, 0.2 μM; ATP, 5 mM; Phe, 50 μM. Samples were irradiated for 30 min. Positions of the subunits are shown to the left.

and this seems to mainly affect the strength of the arm binding. The sharper decrease in the protein labeling and disorientation of the reactive nucleotide as a result of G34A or A36C mutations can be due to disorders in interactions with the double- and single-stranded fragments because the acceptor end positioning is, first of all, determined by interactions with nucleotides in the 73rd-76th positions. Comparison of data on the PheRS modification in the presence of ATP with results of kinetic measurements (Fig. 4b and Table 1) shows that the relative yields of major products of the α- and β-subunit modification (67-70 and 150-165 kD, respectively) and the total efficiency of the protein labeling are decreased as a result of mutations in the same series (U20A, G19U/C56G, A36C, G34A) as the apparent constant of the catalytic rate of aminoacylation (k_{cat}). Thus, the effect of mutations on the efficiency of the 3′-terminal nucleotide cross-linking in the presence of ATP reflects disorders in the interactions responsible for the productive binding of the acceptor end.

In the presence of Phe and ATP (Fig. 4c) the total level of the protein labeling with the wild type tRNA^{Phe} analog is virtually the same as in the presence of ATP, only the labeling efficiency of the α- and β-subunits changes (13% increase and 19% decrease, respectively). In the case of analogs of G34A, G19U/C56G, and U20A mutants, the total labeling becomes still lower than in the presence of ATP because of decreased efficiency of the β-subunit labeling (by 8, 18, and 35%, respectively). On the contrary, analog of the A36C mutant attaches more effectively: labeling levels of the α- and β-subunits increase similarly (by 11-13%). Significant differences in the recorded effects suggest once more the differentiated role of the anticodon nucleotides and structural elements of tRNA^{Phe} (which determine the overall conformation of the ribose-phosphate chain) in determination of the 3′-terminal nucleotide position and conformation of the acceptor arm which are controlled by amino acid and nucleotide substrates, respectively. Relative yields of individual modification products in these experiments are the most demonstrative for evaluation of effects of different mutations on the functional positioning of the acceptor end (required for the productive interaction), which is ensured only in the presence of both small substrates. By

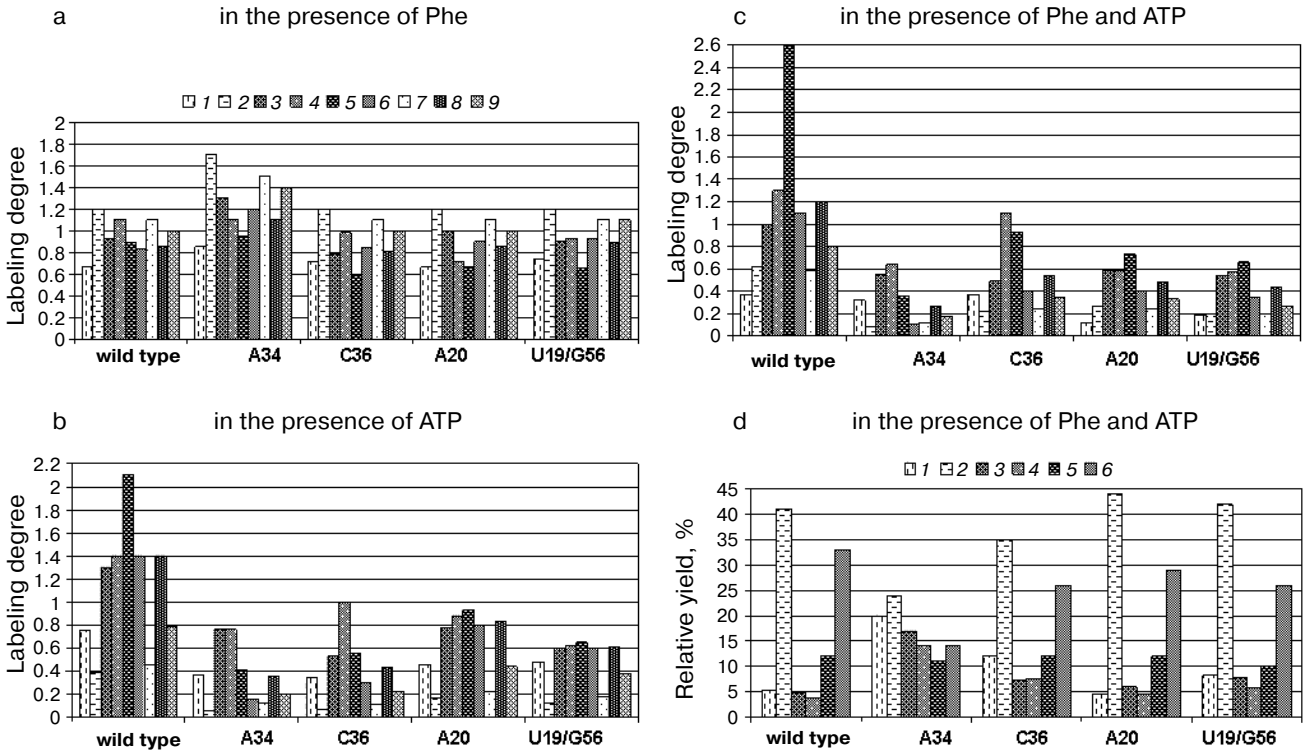

Fig. 4. Effects of low-molecular-weight substrates on labeling of PheRS with s^4U76-substituted derivatives of $tRNA^{Phe}$ and its mutants. The labeling degree is determined as the ratio of yield of individual products ((*1*) 56-58 kD, (*2*) 67, 69, 70 kD, (*3*) 100 kD, (*4*) 110 kD, (*5*) 120, 128, 132 kD, (*6*) 150, 150-160, 155-165 kD), or of all products of the subunit α (*7*) and β (*8*) labeling, or of total efficiency of the protein labeling (*9*) in the presence of substrates ((a) Phe, (b) ATP, (c) Phe and ATP) to corresponding values in the absence of substrates. d) Relative yields of the labeling products (% of the total labeling level of the protein) in the presence of Phe and ATP. Mean values of two experiments are presented; mean deviations were not more than 10%.

this parameter the replacement of G34 by A causes the maximum disorientation: the heaviest products of the α- and β-subunit labeling are the major ones in the control experiment and in the case of the mutant $tRNA^{Phe}$ analog their generation is comparable to that of other products (Fig. 4d). Minimal changes in the selectivity of labeling of the subunits and their individual residues are recorded in the case of U20A mutation. The A36 replacements by C and the tertiary G19-C56 pair by U-G cause disorientating effects intermediate between the above-mentioned mutations, but the first replacement is more crucial for the labeling selectivity of different residues of the α-subunit. Thus, the unfavorable effect of four mutations on positioning of the $tRNA^{Phe}$ 3′-terminal nucleotide in the PheRS complex with all functional ligands increases in the same series as the k_{cat} value decreases (Table 1). This correlation suggests that the catalysis of transfer of the $tRNA^{Phe}$ aminoacyl residue, which requires the acceptor end to be properly positioned, is the rate-limiting stage of aminoacylation for all mutants. The conservative replacement of the G34 nucleotide (by A) which is responsible for the maximal number of the anticodon specific contacts affects more unfavorably the functional positioning of the 3′-end than the non-conservative mutation A36C. The effect of the nucleotide replacement in the 36th position on the PheRS interaction with the 3′-terminal nucleotide is clearly manifested only in the presence of small substrates, and this suggests an important role of A36 in the conformational adaptation of the enzyme and $tRNA^{Phe}$ during formation of the productive complex. The preferential contribution of A36 compared to the contribution of G34 to the productive binding and not to the initial binding of $tRNA^{Phe}$ is supported by data on kinetic parameters of aminoacylation and stability of the complexes: the A36C mutation ranks 8-fold below the G34A mutation by changes in the K_d value and 2.4-fold by changes in the k_{cat} value (Table 1).

DISCUSSION

Changes in the overall folding of $tRNA^{Phe}$ have no such significant effect on the functional positioning of the 3′-terminal nucleotide as replacements of the anticodon nucleotides; the low cross-linking efficiency of analogs of structural mutants in the presence of Phe and ATP seems

to be mainly due to high conformational mobility of the acceptor arm because of partial disturbance of contacts with the acceptor stem. Binding of the tRNA^{Phe} acceptor stem occurs with involvement of the loop (residues α205-214) of motif 2 (specific for class II) and the loop fragment of the B7-domain (residues 536-539 of the β-subunit) [21]. The latter fragment is involved in intersubunit interactions that stabilize the conformation of the motif 2 loop which, in turn, is responsible for binding of ATP or its AMP moiety in adenylate [25]. The supposed partial disturbance of the enzyme interactions with the acceptor stem of tRNA^{Phe} structural mutants decreases the strength of binding of the acceptor arm as a whole. This destabilization can increase in the presence of nucleotide substrate, which induces the structural displacement of the motif 2 loop. An additional destabilization in the presence of ATP and Phe can be due to both the stronger binding of the AMP-moiety in adenylate produced and changes in the conformation of the motif 2 loop in the complex with adenylate. Replacements of G19 and C56 nucleotides responsible for nonspecific contacts with PheRS and significant stabilization of the tertiary structure of tRNA more strongly destroy the acceptor arm conformation (and this influences the cross-linking efficiency and positioning of the 3′-terminal nucleotide) compared to the U20A mutation. The nucleotide in the 20th position (lacking contacts with PheRS) contributes to tRNA^{Phe} recognition by determining the local structure of the D-loop whose conformation changes on complexing [21-23]. Its involvement in the conformational adaptation is supported by the following data. Different (three probable) types of U20 replacements are characterized by opposite effects on the initial (in the absence of other substrates) binding of tRNA^{Phe} and catalytic rate of aminoacylation: the more the complex stability decreases, the less the catalytic process is suppressed [24]. The U20A mutation most strongly destabilizes the complexing with PheRS but does not affect the k_{cat} value. The findings of the present work have shown that this replacement has the slightest effect on positioning of the 3′-terminal nucleotide in the presence of small substrates along with a significant decrease in the efficiency of its cross-linking that seems to be due to increased conformational mobility of the acceptor arm. A flexible conformation of the arm can be favorable for catalysis promoting a fine adjustment of the acceptor end in the productive complex. On the other hand, such a conformation can be involved in the control of other stages of aminoacylation: the acceptor arm conformation is modulated by ATP, and products of its hydrolysis (pyrophosphate and AMP) are released during the stage of phenylalanine activation and its transfer onto tRNA-substrate. The turnover number of aminoacylation (k_{cat}) measured by methods of steady-state kinetics is a complicated kinetic parameter determined by the rate not only of chemical reaction but also of formation of the productive complex and release of products. Hydrolysis of pyrophosphate by inorganic pyrophosphatase was earlier shown to significantly increase the aminoacylation efficiency of structural mutants (with replacements in the 20th position and in the tertiary pair G19-C56) of yeast tRNA^{Phe} and, affecting mainly the k_{cat} value, to significantly lesser degree increase aminoacylation of mutants with replacements in the anticodon [7]. To explain this phenomenon, a kinetic mechanism is proposed as follows: dissociation of pyrophosphate induced by tRNA-substrate is the main rate-limiting stage in aminoacylation of structural mutants. Another scheme is proposed for their aminoacylation in the presence of pyrophosphatase: tRNA^{Phe} is bound by the enzyme complex with adenylate only after dissociation of pyrophosphate. Although the activation mechanism remains unclear and unproved, these data suggest that the anticodon and proper conformation of tRNA^{Phe} should play different roles in determination of rates of individual stages of aminoacylation. Our findings have shown that interactions with the anticodon nucleotides are of fundamental importance for proper positioning of the 3′-terminal nucleotide required for the catalytic stage of aminoacylation of tRNA^{Phe}. Most likely, the catalysis itself is the main rate-limiting stage in aminoacylation of mutant yeast tRNA^{Phe} with replacements in the anticodon; therefore, the efficiency of their aminoacylation only slightly increases in the presence of pyrophosphatase. The totality of these data suggests a universal role of the anticodon in the productive interaction of tRNA^{Phe} with PheRS of pro- and eukaryotic origin; the anticodon seems to optimize contacts responsible for positioning of the CCA-end.

The data of the present work directly show the functional relation between different regions of tRNA^{Phe} and the acceptor end in the complex with PheRS: any replacement of recognition elements has effects on the positioning and/or binding efficiency of the 3′-terminal nucleotide. How does this interrelation occur? Of all structural fragments of the enzyme interacting with tRNA^{Phe}, only the B6-B7 domains are in contact with the D-stem, variable loop, and acceptor stem (Fig. 1). The C-terminal B8*-domain and the N-terminal coiled coil domain (CC*) of the symmetrically located αβ-heterodimer responsible for the majority of interactions with the anticodon and central regions of the substrate have no direct contacts either with the catalytic domain or with the acceptor arm of tRNA^{Phe}. Thus, information about the "recognition" of specificity determinants is transmitted into the active site through multistage interaction of the enzyme and tRNA^{Phe} accompanied by their conformational changes, including those induced by low-molecular-weight substrates. Our previous data on complexing *T. thermophilus* PheRS with different tRNA^{Phe}s, their mutants, and nonspecific tRNA^{Phe}s suggest the dominant role of the anticodon and the significant role of the proper tertiary structure in the optimal (strongest)

binding of $tRNA^{Phe}$ in the absence of other substrates [24]. The nature of nucleotides of the acceptor and anticodon stems, which form a network of nonspecific contacts with the protein, is not significant for the initial binding. Removal of the anticodon-binding domain of *T. thermophilus* PheRS decreases the binding strength to the level of nonspecific binding [30], and this also suggests the prevalent role of its interactions with $tRNA^{Phe}$ at the optimal complexing. These results of biochemical studies together with data of XCA allow us to suggest a concept of the $tRNA^{Phe}$ binding with PheRS. The acceptor arm, which fails to ensure the specificity, is bound last. Interactions with the anticodon initiate production of the specific complex. The close approaching of macromolecules to recognize the anticodon can occur due to electrostatic interactions of the ribose-phosphate chain of the central regions with the B7- and B8*-domains which are well structured in the native protein (in the absence of substrates) and exposed to the solution. The anticodon binding induces interactions of the N-terminal CC*-fragment of the small subunit (disordered in the absence of substrate) with ribose-phosphate residues of nucleotides of the loop portions of the central regions whose location is determined by the tertiary structure of $tRNA^{Phe}$. The process is accompanied by conformational changes in the enzyme and substrate and results in production of a strong complementary complex, with its acceptor end positioned into the active site. Such dynamics of interaction has been proposed for the simpler Asp-specific system where tRNA-substrate is bound with only three domains: the anticodon-binding, catalytic, and the so-called hinge domain [8]. The last domain is responsible for functional interrelations between different regions of $tRNA^{Asp}$: its insignificant displacement in the structure of the poorly active complex of *E. coli* AspRS with yeast $tRNA^{Asp}$ results in destruction of contacts only with one nucleotide of the acceptor arm but in a complete disorientation of the terminal adenosine [31]. Conformation of the $tRNA^{Phe}$ acceptor arm is stabilized by a network of interactions with two domains (A1 and A2) of the α-subunit and three domains (B1, B3, and B7) of the β-subunit (Fig. 1). The function similar to that of the AspRS hinge-domain can be realized by the B6-B7-module: it has contacts with the B8*-domain and catalytic module and is involved in binding of the central regions and acceptor stem of $tRNA^{Phe}$. The anticodon-binding B8*-domain forms additional contacts with the D-stem and A26 nucleotide, and the second nucleotide of the tertiary pair A26-G44 interacts with the N-terminal helix of the α-subunit which, in turn, binds the anticodon stem and other nucleotides stabilizing the tertiary structure of $tRNA^{Phe}$. All these interactions fix the angle between the anticodon and acceptor stems and, thus, establish the functional relation between the anticodon and the acceptor end. Earlier we obtained indirect data on involvement of the A26-G44 pair in conformational adaptation of the enzyme and substrate—mutations, which more strongly destabilize the complex, are less crucial for the catalytic efficiency of aminoacylation [24]. In the present work the G44 replacement by C which destroys the tertiary structure of $tRNA^{Phe}$ is shown to more markedly change the 3′-terminal nucleotide positioning in the absence of low-molecular-weight substrates, compared to replacement of the tertiary pair G19-C56 by U19-G56, although the latter affects significantly stronger the binding efficiency (Tables 1 and 2). These findings suggest the direct involvement of the tertiary pair A26-G44 in establishment of the functional relation between the central regions and acceptor end of $tRNA^{Phe}$ in the complex with PheRS. The maximal disorientation of the 3′-end and the sharp decrease in the labeling efficiency recorded on the double non-conservative mutation G34C/A35U confirm the initiating role of specific interactions of the enzyme with the anticodon nucleotides in formation of a complicated network of contacts providing the location of the acceptor end.

The findings suggest that structural elements of $tRNA^{Phe}$, which are responsible for optimization of its initial binding to PheRS, determine positioning of the acceptor end in the productive complex. Disturbance of specific interactions with the anticodon nucleotides is the most crucial for stability of the PheRS complex with $tRNA^{Phe}$ and for functional orientation of the acceptor end controlled by small substrates. Replacement of nucleotides, which determine the overall conformation of $tRNA^{Phe}$, has the lesser effect on the complexing efficiency and productive binding of the acceptor end. Mutual adaptation of the enzyme and $tRNA^{Phe}$ at the final stage of interaction preceding the catalytic stage requires the fine conformational adjustment of $tRNA^{Phe}$: any mutation affects more significantly interaction with the 3′-terminal nucleotide in the presence of Phe and ATP than in their absence. This process seems to be important for discrimination of nonspecific tRNAs, which form rather strong complexes with PheRS at the initial stage (in the absence of small substrates). Our experiments suggest that all major elements responsible for the $tRNA^{Phe}$ recognition play the universal role of the discriminating base (nucleotide in the 73rd position) in promotion of the 3′-end positioning [3, 6, 32, 33]. This role is likely to be common in all Phe-specific systems of bacterial origin, which were found to possess strictly conservative recognition elements [17, 19] and tRNA-binding structural domains [34].

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