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Study on interaction of *Thermus thermophilus* prolyl-tRNA synthetase with cognate $tRNA_{CGG}^{Pro}$ by methods of chemical modification in solution

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Two isoacceptors Thermus thermophilus HB8 $tRNA^{Pro}$ were isolated by the chromatography methods with purity about 95 and 97 %. The primary structures of isoacceptors $tRNA_{GGG}^{Pro}$ and $tRNA_{CGG}^{Pro}$ were studied by the gel-sequencing method, and differences between them were found in 18 positions. Our results show that in solution the cognate prolyl-tRNA synthetase protects the phosphates located in D-stem (9, 10, 13), 5'-end of anticodon-stem (26–29), anticodon-loop (34, 35, 37–39) and acceptor-stem (67, 68) of $tRNA_{CGG}^{Pro}$ from alkylation by ethylnitrosourea,.

Keywords: prolyl-tRNA synthetase, $tRNA^{Pro}$, Thermus thermophilus, chromatography, autoradiography, ethylnitrosourea.

Introduction. The prolyl-tRNA synthetase (ProRS) catalyzes the aminoacylation of cognate $tRNA^{Pro}$ by two-stage mechanism, which includes the amino acid activation with formation of prolyl-adenylate and the further proline transference onto 3'-end adenosine of cognate tRNA.

According to the chemical structure proline (pirrolydine-2-carboxylic acid) is imino acid. A distinguishing feature of proline among 20 aminoacids, detected in the proteins, is the atom of Nitrogen, forming the peptide bond, which also participates in the formation of proline pyrrolidine ring. This leads to the sharp bend of polypeptide chain in the place of peptide bond formed by proline. The bend determines to a great extent the spatial structure of a protein molecule. Thus, the accuracy of aminoacylation of cognate tRNA by proline is of special importance. Though in the cases when amino acids have slight structural differences (for example differs by methyl group), ProRS, as well as other aaRSs, is not able to achieve a complete discrimination and can make an error: it can either activate a similar noncognate amino acid (alanine, cysteine) [1, 2], or aminoacylate tRNA with noncognate amino acid.

ProRS along with GlyRS, HisRS, SerRS and ThrRS belongs to the class IIa [3, 4] and functions as $_2$-homodimer. The distinctive feature of the class IIa synthetases is that they all (except SerRS) have cognate C-terminal anticodon-binding domain, consisting of more than 100 amino acid residues. A spatial localization of this domain varies significantly relative to a catalytic domain. In HisRS it is connected with the cata-

lytic domain by a long peptide and located exclusively opposite the active centre of another subunit. The question is how the recognition and binding of tRNAs by class IIa synthetases occur and how they interact with tRNA anticodon?

The determination of spatial structures of *Escherichia coli* and *T. thermophilus* HisRS [5, 6] and *T. thermophilus* GlyRS [7] by the method of X-ray crystallography has provided us with a possibility to build only hypothetical model of binding tRNA by the IIa class synthetases, based on the research of surface electrostatic potential. In addition, the phylogenetic analysis of amino acid sequences of prolyl-tRNA synthetases of various origins has shown that they can be referred to 2 structural forms, which have diverged early during the evolution [8, 9]. The prokaryote-like ProRSs have a large insertions between structural motifs 2 and 3, and their C-terminal domain is absent whole the eukaryote/archaeon-like ProRSs contain a unique C-terminal domain.

We have obtained the crystals of $tRNA_{CGG}^{Pro}$ and $tRNA_{GGG}^{Pro}$ complexes with *T. thermophilus* ProRS (ProRSTT) and their structures have been determined [9]. That was the first structural data, showing, how the class IIa synthetase recognizes tRNA anticodon. A several novel features for the IIa class synthetases have been revealed by the comparison of received data for native ProRSTT structure (0.243 nm) and its complex with proline (0.29 nm) [10]. The C-terminal domain is a Zn-binding structure. It has been found that there is only one molecule of tRNA per dimer of the enzyme in a crystal. The anticodon of tRNA is well structured, anticodon-loop is compact and only 3 main anticodon bases (G35, G36, and G37) interact with the enzyme.

Watson-Crick pairs of bases U32-U38 ($tRNA_{GGG}^{Pro}$) or U32-A38 ($tRNA_{CGG}^{Pro}$), absent in free tRNA, are formed upon tRNA binding to the enzyme. The upper part of anticodon stem is cross-contacted with another subunit of the dimmer. The tRNA 3' and 5' ends are disordered and the acceptor end does not enter the enzyme active site. A possible reason for may be an 'incorrect' conformation of the active site due to the absence of proline, since the essential conformational changes of the enzyme occur during the binding of proline. The data obtained have confirmed the results of biochemical research according to which the bases G35 and G36 are the elements of tRNA recognition by the synthetase [11]. However, the additional enzyme contacts with tRNA, including D- and acceptor-stem, have to be present for the efficient aminoacylation. Besides, some doubts concerning the identity of macromolecular structures in crystal and solution always arise. To understand the mechanism of tRNA aminoacylation by the class IIa synthetases it is necessary to define how the anticodon-binding domain positions the acceptor end of tRNA and ensures the effective aminoacylation.

The aim of this work is to study the interaction between $tRNA^{Pro}$ and *T. thermophilus* prolyl-tRNA synthetase in solution by the method of chemical modification. At present time ethylnitrosourea is one of the best reagents to investigate tRNA interaction with aminoacyl-tRNA synthetase, equally modifying tRNA bases in loop and helical regions.

Materials and methods. The following materials and instruments were used in the current research: benzoyl DEAE-cellulose (BD-cellulose) ('Serva', Germany); 4B-sepharose ('Pharmacia Fain Chemicals', Sweden); NaCl, $MgCl_2$ ('Fisher', USA); diethylpyrocarbonate, phenylmethylsulfonyl fluoride ('Calbiochem', USA); 2-mercaptoethanol, dithiothreitol (DDT) ('Merk', Germany); GF/C filters; DEAE cellulose ('Whatman', England); isopropyl alcohol, ^{14}C-prolyl (239 Cu/mole), [-^{32}P]ATP, [-^{32}P]ATP with specific activity 2000-3000 Cu/mole ('Amersham', England); dimethyl sulfate, hydrazine ('Fluka', Switzerland); tris, sodium-hydrobromide ('Serva', Germany); snake venoms phosphodiesterase, alkaline phosphatase from *E. coli* ('Sigma', USA); T1-ribonuclease ('Sankyo', Japan); snake venom phosphodiesterase ('Worthington', USA); polynucleotide kinase of phage T4, ('Pharmacia', Sweden); tRNA-nucleotidetransferase of yeast (Institute of molecular biology and genetics of National Academy of Sciences of Ukraine). Ethylnitrosourea was synthesized by A. G. Terentyev (Institute of molecular biology and genetics of National Academy of Sciences of Ukraine). X-ray film ('Codac', USA) was used.

The solutions have been prepared on redistilled water. The list of used instruments: centrifuge K-70 (Germany), centrifuge Jouan MR 14.11 ('Jouan', France), spectrophotometer Specord UVVIS (Germany), chro-

matographic equipment Gold-System and highly-efficient chromatographic columns Spherogel TSK DE 5PW 2.0 15 cm, Ultrapore RPMS C8 1.0, 25 cm ('Beckman', USA), electrophoresis Macrophor 2010, scintillation counter Rackbeta, densitometr UltraScan XL ('LKB' Sweden).

The *T. thermophilus* cells HB-8 strain was grown as previously described [12, 13] in order to obtain prolyl-tRNA synthetase and crude tRNA.

tRNA isolation and purification from *T. thermophilus* was performed in several stages, including the isolation of crude tRNA, chromatography on columns with BD-cellulose, reversed-phase chromatography on 4B-sepharose and highly-efficient liquid chromatography (HPLC) on the columns Spherogel-TSK DE 5PW and Ultrapore RPMS C8 as described [12, 14, 15].

$tRNA_{CGG}^{Pro}$ and $tRNA_{GGG}^{Pro}$ from *T. thermophilus* were labeled with radioactive phosphorus on the 3'-end, using [5'-^{32}P]pCp and RNA-ligase [16], [-^{32}P]ATP and tRNA-nucleotidiltransferase [17], received as described [18]. The 5'-end of tRNA was labeled using [-^{32}P] ATP in phosphorylation reaction [19].

The *T. thermophilus* $tRNA_{CGG}^{Pro}$ and $tRNA_{GGG}^{Pro}$ nucleotide sequences have been determined by two methods of tRNA sequencing: specific chemical degradation [20, 21] and hydrolysis of tRNA with specific endonucleases [21, 22].

The alkylation of ^{32}P-labeled $tRNA_{CGG}^{Pro}$ and its complex with prolyl-tRNA synthetase was performed with the use of ethylnitrosourea under the conditions stabilizing a spatial structure of tRNA, and at the same time they promote the formation of the specific complex of $tRNA^{Pro}$-prolyl-tRNA synthetase [23]. A reaction mixture of 25 l included: 50 mM of tris-HCl (pH 7.9); 5 mM of $MgCl_2$; 2.5 mM of 2-mercaptoethanole; 0.8 M of $tRNA^{Pro}$; 3.2 M of prolyl-tRNA synthetase and 2.5 l of saturated solution of ethylnitrosourea in ethyl alcohol. The reagent concentration was 75 mM. The alkylation of tRNA in the presence of cognate ProRSTT was curried out for 2 hours at 37 °C and stopped by adding 3 l of 3 M sodium acetate (pH 5.5). In control experiments the equivalent amount of ethanol has been added in stead of ethylnitrosourea; instead of prolyl-tRNA synthetase, heterological aminoacyl-tRNA synthetase (3.2 M) has been added. After the reaction had been finished, enzymes have been removed by phenol extraction, tRNA has been precipitated by 10 g glycogen and 3 volumes of ethyl alcohol added.

Under denaturating conditions the tRNA alkylation was curried out in 25 l of

0.3 M Na-cacodylic buffer (pH 8.0), containing 0.1 mM of EDTA, for 2 minutes at 80 °C. Polynucleotide chain was split under modified bases in 10 l of 0.1 M tris-HCl (pH 9.0) for 5 minutes at 55 °C.

The obtained fragments of tRNA were separated by electrophoresis in 12.5% polyacrylamide gel in 5 mM tris-borate buffer (pH 8.3), which contained 1 mM EDTA and 7M urea with further autoradiography of a gel. The electrophoretic bands were identified, comparing a mobility of fragments with that of fragments, obtained by partial hydrolysis of tRNA with ribonuclease T1. The intensity of electrophoretic bands on the gel radioautographs, which represented the phosphate modification degree, was estimated by scanning densitometer UltraScan XL from 'LKB' (Sweden).

Results and discussion. *Isolation of individual $tRNA_{CGG}^{Pro}$ and $tRNA_{GGG}^{Pro}$ from T. thermophilus.* Because of the existence of several $tRNA^{Pro}$ isoacceptors , which are present in entire pool of a total tRNA preparation, the isolation of individual isoacceptor tRNA is a quite complicated task. $tRNAs^{Pro}$ from *T. thermophilus* have been isolated and purified in several chromatographic stages. During the column chromatography on BD-cellulose $tRNA^{Pro}$ from *T. thermophilus* was eluted in a zone of high optical density and contained a lot of contaminations consisting of other tRNAs. Therefore, the reversed-phase chromatography on the 4B-sepharose column was used as the second stage, which allowed removing most of contaminations and what is very important allowed a separation of $tRNA^{Pro}$ from $tRNA^{Lys}$ [15]. The further purification was performed by the HPLC methods on the Spherogel-TSK DE 5PW column. Final purification of tRNA was curried out on the Ultrapore C8 column, that resulted in obtaining 2 isoacceptors tRNA($tRNA_{CGG}^{Pro}$ and $tRNA_{GGG}^{Pro}$) from *T. thermophilus* (Figure 1). The purity of obtained preparations was about 95 and 97% (1505 and 1545 mole/one optical unit) respectively.

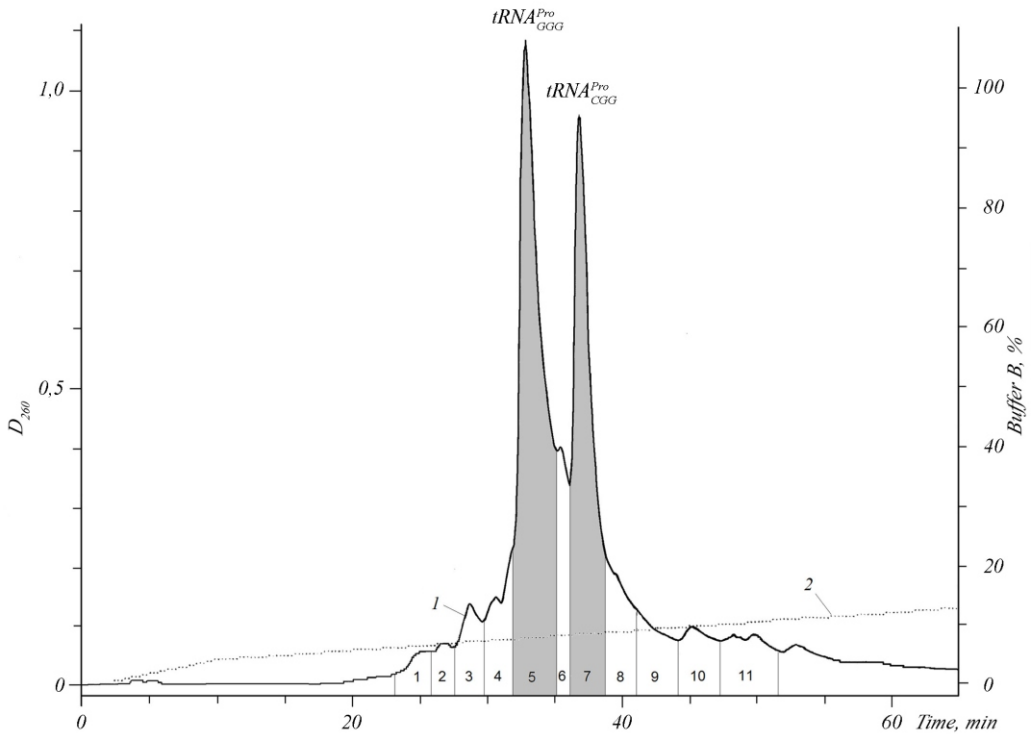

Fig. 1. Purification of *T. thermophilus* isoacceptor $tRNA_{GGG}^{Pro}$ and $tRNA_{CGG}^{Pro}$ on reversed-phase Ultrapore column C8: *1* – optical density at 260 nm; *2* –gradient of buffer B concentration. Stained peak contains $tRNA_{GGG}^{Pro}$ and $tRNA_{CGG}^{Pro}$ with the purity of ~ 95% and 97%, respectively.

Determination of nucleotide sequences of T. thermophilus $tRNA_{CGG}^{Pro}$ *and* $tRNA_{GGG}^{Pro}$**.** Nucleotide sequences of *T. thermophilus* $tRNA_{CGG}^{Pro}$ and $tRNA_{GGG}^{Pro}$ have been studied using two methods of labeled tRNA sequencing: specific chemical degradation [20, 21] and tRNA hydrolysis by specific endonucleases [21, 22]. As a result the full nucleotide sequences of T. thermophilus $tRNA_{CGG}^{Pro}$ and $tRNA_{CGG}^{Pro}$ have been determined. In the figure 2 the primary structures are represented in the form of clover leaf. There are 77 nucleotide bases included into the composition of each $tRNA^{Pro}$. 18 distinguishing nucleotides have been revealed by comparison of their structures (they are marked out with bold italic type). The most significant difference is noticed in the anticodon stem (8) and T-stem (4), 2 distinctions have been found in both anticodon loop and acceptor stem (8) and one in both D-loop and acceptor end. $tRNA^{Pro}$s from *T. thermophilus* have typical features of prokaryotic $tRNA^{Pro}$: the first CG-pair in the acceptor stem and the specific structure of D-stem. The homology of $tRNA_{CGG}^{Pro}$ primary structure with corresponding isoacceptor $tRNA_{CGG}^{Pro}$ from *E. coli* is 78%.

Thus, the proline system in *T. thermophilus* is unique considering that ProRSTT belongs to the eukaryote/archaeon-like type according to its structural features while its cognate $tRNA^{Pro}$ belongs to the prokaryotic group of $tRNA^{Pro}$. Therefore, from the evolutional point of view to research $tRNA^{Pro}$ recognition by its aminoacyl-tRNA synthetase is of great interest.

Study on the T. thermophilus prolyl-tRNA synthetase interaction with cognate $tRNA_{CGG}^{Pro}$ *in solution***.** The selection of *T. thermophilus* tRNA is based on the results of the study of ProPCTT complexes with both isoacceptor $tRNA^{Pro}$s by the methods of X-ray crystallography [9]. The crystals of *T. thermophilus* ProRSTT-$tRNA_{CGG}^{Pro}$ had better resolution and appeared to be more informative. The obtained results allowed constructing a spatial model of the complex. The results of X-ray diffraction analysis of the *T. thermophilus* ProRSTT-$tRNA_{GGG}^{Pro}$ complex entirely correspond to the model, built for the ProRSTT-$tRNA_{CGG}^{Pro}$ complex. The regions, where $tRNA_{CGG}^{Pro}$ contacts with prolyl-tRNA synthetase, were investigated by the ethylnitrosourea alkylation of free $tRNA^{Pro}$ in the presence of cognate aminoacyl-tRNA synthetase. The tRNA alkylation reaction was performed as described in “Materials and methods”.

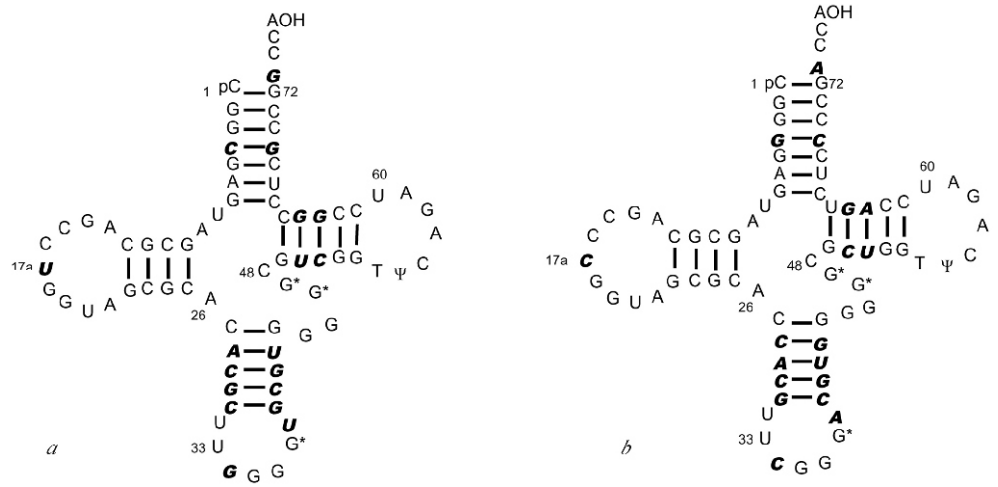

Fig.2. Cloverleaf structure of *T. thermophilus* $tRNA_{GGG}^{Pro}$ (a) and $tRNA_{CGG}^{Pro}$ (b). Nucleotides, common for $tRNA_{GGG}^{Pro}$ and $tRNA_{CGG}^{Pro}$ are in italics bold

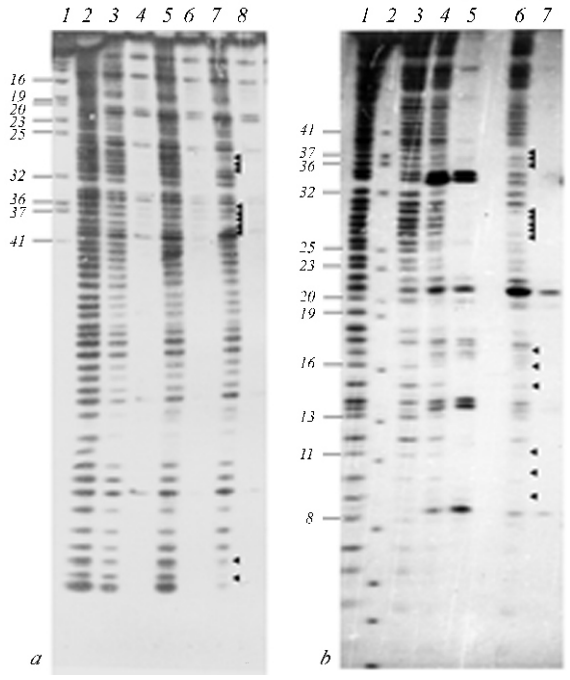

Fig. 3. Autoradiogramms of 12,5 % polyacrylamide gels obtained with 3'-labeled (A) and 5'-labeled (B) *T. thermophilus* $tRNA_{CGG}^{Pro}$ in ethylnitrosourea mapping experiments.

A: alkylation of tRNA alone under the native (3, 5) and denaturating (2) conditions, and alkylation of tRNA in the presence of cognate prolyl-tRNA synthetase (7); corresponding control incubations (4, 6, 8) without ethylnitrosourea treatment. 2 - $tRNA^{Pro}$(CGG), partial ribonuclease T1 digest.

B: alkylation of tRNA alone under the native (3, 4) and denaturating (1) conditions, and alkylation of tRNA in the presence of cognate prolyl-tRNA synthetase (6); corresponding control incubations (5, 7) without ethylnitrosourea treatment. 2 - $tRNA^{Pro}$(CGG), partial ribonuclease T1 digest.

Numbering of bands corresponds to the phosphate position. Triangles indicate phosphates from alkylation in the presence of prolyl-tRNA synthetase.

Polyacrylamide gel radioautographs, obtained after fragments separation, are given in the figure 3. These fragments have been obtained by the separation of 3'-^{32}P-labeled (a) and 5'-^{32}P-labeled $tRNA_{CGG}^{Pro}$ (b), alkylated by ethylnitrosourea with and without prolyl-tRNA synthetase. One can see that some phosphate modification occurs to lesser extent in the presence of cognate enzyme in comparison with modification of a free $tRNA^{Pro}$ or in the presence of heterological synthetase. It is important to note that the bands, corresponding to the phosphates, located closer to the end of tRNA molecule, have less intensity both in the presence of enzyme and its absence. The reason is a disadvantage of the method which requires precipitation of the obtained fragments by ethanol. In such conditions short fragments precipitate worse than long ones. However, the study of intensity of electrophoretic bands by scanning densitometer and the estimation of relative intensity levels of corresponding bands in the presence and absence of the cognate ProRSTT (Fig. 4) provide us with a reliable information on the phosphate protection with the enzyme.

The location of protected phosphates in the presence of prolyl-tRNA-synthetase in the structure of $tRNA^{Pro}$ is represented in the figure 5. It can be seen that the $tRNA_{CGG}^{Pro}$ phosphates, which have low ability for alkylation in the presence of ProRSTT, are located in positions on the 5'-end of D-stem and D-loop (9, 10, 13, 16, 17, 17a), on the 5'-end of anticodon stem (26, 27, 28, 29) and anticodon loop (34, 35, 37, 38) , on the 3'-end of acceptor stem (67, 68). The participation of anticodon bases, bases on 5'-side of anticodon stem

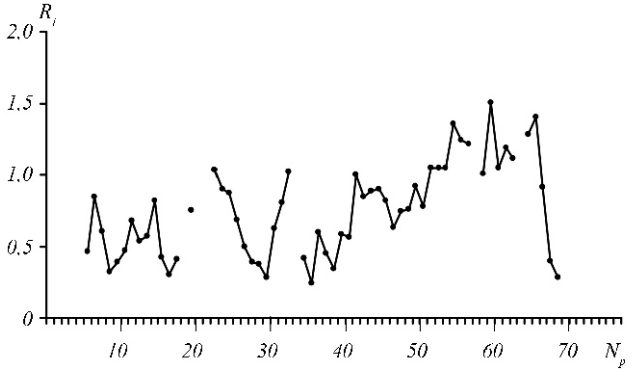

Fig. 4. Pattern of phosphate reacivities towards ethylnitrosourea in *T. thermophilus* $tRNA_{CGG}^{Pro}$ in the presence of prolyl-tRNA synthetase as compared to the free native molecules. R_i values are the ratios between the intensities of the corresponding electrophoretic bands of the tRNA alkylation patterns in the presence of the enzyme and free tRNA; Np – phosphates numbers.

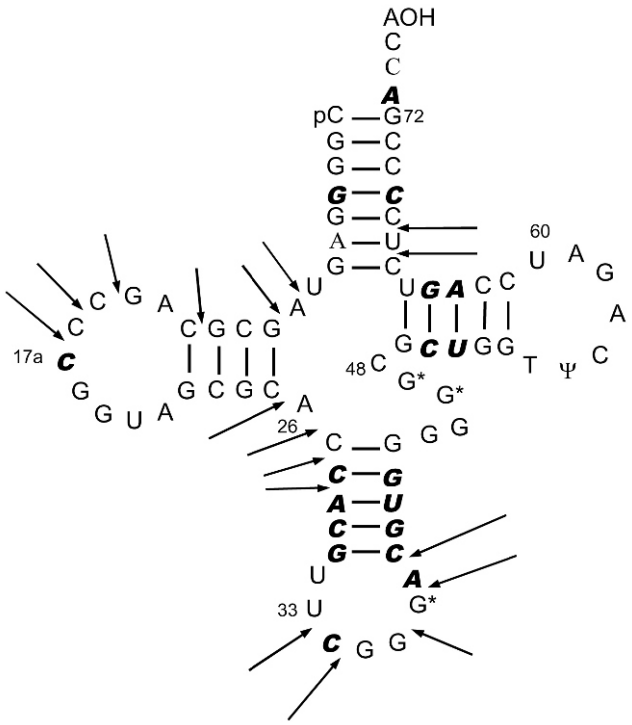

Fig. 5. Cloverleaf structure of *T. thermophilus* $tRNA_{CGG}^{Pro}$. The arrows show phosphates protected by prolyl-tRNA synthetase against alkylation by ethylnitrosourea. Differences between $tRNA_{GGG}^{Pro}$ end $tRNA_{CGG}^{Pro}$ are indicated in italics bold.

and 3'-side of acceptor stem in the interaction with prolyl-tRNA synthetase is proved as well by the method of tRNA protection from ribonuclease hydrolysis (data are not shown). However, it is necessary to note there are no direct contacts of D-loop with the enzyme in crystal structure, therefore the decrease in phosphate 16, 17 and 17a reactivity is connected with their participation in the stabilization of corresponding D-loop conformation, induced by tRNA interaction with the enzyme. We suggest that such D-loop conformation is more stable due to the complex formation with the synthetase. The data obtained indicate that the contacting points of $tRNA^{Pro}$ with the synthetase are located at the side of a major groove of anticodon stem and anticodon is key-important in the $tRNA^{Pro}$ interaction with cognate prolyl-tRNA synthetase.

These results well agree with the results of crystal complexes research by the crystallographic methods [9]. The main contacts between the anticodon-binding domain of ProRSTT and $tRNA^{Pro}$ are created by a -sheet and two -helixes of the enzyme, directed to the anticodon loop of tRNA from the side of major groove. A hydrophobic patch, which specifically binds the anticodon base G35 and G36, is formed by the amino acid residues Ile295, Pro322 and Phe336. In the recognition of anticodon $tRNA^{Pro}$ Lys353, Asp254, Glu349, Lys297 and Thr331 also participate, creating hydrogen bonds with anticodon bases or interacting with phosphates. It is worth noting that almost all pointed out residues are conservative in all known eukaryote/archaeon-like ProRS.

The bases G35 and G36 of $tRNA^{Pro}$ are the recognition elements for ProRS. As it has been shown by biochemical research, these bases substitution for others leads to the aminoacylation efficiency decrease by 9-164 times for the prokaryotic ProRS and by 300-1100 times for the eukaryotic one [11].

The results obtained designate an important function of the ribose-phosphate backbone and spatial configuration of $tRNA^{Pro}$ in the processes of tRNA recognition by the cognate enzyme. The interaction of enzyme residues with the bases G35 and G36 of tRNA has also been shown. The protection of the phosphates 67 and 68 in the acceptor stem from modification is believed to be an important result of this study. According to the crystallographic data, the acceptor stem of $tRNA^{Pro}$ has got no direct contacts with the enzyme and a spatial localization of the acceptor end has not been defined. However, the phosphates 67 and 68 can interact with Arg146 and Arg148 and fix the acceptor stem in the position, providing efficient aminoacylation, as a result of conformational changes during the proline binding. It

is necessary to note that Arg102 corresponds to the position of Arg146 in a spatial structure of *Entercoccus faecalis* ProRS (ProRSEF) and according to the computer model of ProRSEF-tRNA complex these amino acids take part in the interaction with ribose-phosphate backbone of the $tRNA^{Pro}$ acceptor stem around of the 68 phosphate [24]. In the latter model the enzyme interaction with ribose-phosphate backbone also occurs around the phosphates 10 and 13 at the 5'-side of anticodon stem and anticodon loop. This agrees with the data, obtained by us for *T. thermophilus* $tRNA^{Pro}$. It is clear that both the primary structure of *T. thermophilus* $tRNA^{Pro}$ and its interaction with the cognate synthetase have peculiarities of prokaryotic $tRNA^{Pro}$ type.

Thus, several new details of ProRS interaction with $tRNA^{Pro}$ have been revealed as a result of the performed research, which together with the crystallographic data, help to understand the mechanism of the class II synthetases interaction with tRNA and how anticodon-binding domain of ProRS interacts with tRNA anticodon. For further research it is important to study a triple complex of prolyl-ProRS-$tRNA^{Pro}$ which promotes fixation of the tRNA acceptor end in the catalytic centre of enzyme.

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Вивчення взаємодії проліл-тРНК синтетази *Thermus thermophilus* з гомологічною $тРНК_{CGG}^{Pro}$ методами хімічної модифікації в розчині

Резюме

Методами хроматографії виділено дві ізоакцепторні $мРНК^{Pro}$ з T. thermophilus HB8 чистотою біля 95 і 97 % відповідно. Вивчено первинні структури $мРНК_{CGG}^{Pro}$ і $мРНК_{GGG}^{Pro}$ з T. thermophilus, які відрізняються між собою у 18 положеннях. Показано, що в розчині гомологічна проліл-мРНК синтетаза захищає від алкілування етилнітрозосечовиною фосфати $мРНК_{CGG}^{Pro}$, розташовані в D-стеблі (9, 10 і 13), на 5'-кінці антикодонового стебла (26, 27, 28 і 29), в антикодоновій петлі (34, 35, 37 і 38) і з 3'-боку акцепторного стебла (67, 68).

Ключові слова: проліл-мРНК синтетаза, $мРНК^{Pro}$, Thermus thermophilus, хроматографія, алкілування етилнітрозосечовиною, авторадіографія.

С. П. Егорова, И. А. Крикливый, О. П. Коваленко, А. Д. Яремчук, М. А. Тукало

Изучение взаимодействия пролил-тРНК синтетазы *Thermus thermophilus* с гомологичной $тРНК_{CGG}^{Pro}$ методами химической модификации в растворе

Резюме

Методами хроматографии виделены две изоакцепторные $мРНК^{Pro}$ из T. thermophilus HB8 чистотой около 95 и 97 % соответственно. Изучены первичные структуры $мРНК_{CGG}^{Pro}$ и $мРНК_{GGG}^{Pro}$ из T. thermophilus, отличающиеся между собой в 18 положениях. Показано, что в растворе гомологичная пролил-тРНК синтетаза защищает от алкилирования этилнитрозомочевиной фосфаты $мРНК_{CGG}^{Pro}$, расположенные в D-стебле (9, 10 і 13), на 5'-конце антикодонового стебля (26, 27, 28 и 29), в антикодоновой петле (34, 35, 37и 38) и с 3'-стороны акцепторного стебля (67 и 68).

Ключевые слова: пролил-мРНК синтетаза, $мРНК^{Pro}$, Thermus thermophilus, хроматография, алкилирование этилнитрозомочевиной, авторадиография.

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