

The isolation of histidine tRNA from *Thermus thermophilus* and the study of its primary structure and interaction sites with homologous aminoacyl-tRNA synthetase

O.I. Gudzera, I.A. Krikliiviy, A.D. Yaremchuk, M.A. Tukalo

The Institute of Molecular Biology and Genetics, NAS of Ukraine
150, Academician Zabolotny Str., Kyiv, 03143, Ukraine

mtukalo@imbg.org.ua

*The structure of histidine tRNA (tRNA^{His}) is different from tRNA structures of other amino acids specificities by the presence of an extra nucleotide on the 5'-end (G-1). To study the molecular mechanism of interaction of tRNA^{His} with histidyl-tRNA synthetase the purification method of *T.thermophilus* tRNA^{His} was elaborated and the primary structure of isoacceptor form of tRNA₁^{His} was studied. Individual tRNA^{His} was isolated from the crude tRNA, using the combination of low pressure chromatography on benzoyl-DEAE-cellulose (BD-cellulose) and DEAE Toyopearl 650 with high pressure liquid chromatography on DEAE 5PW and Ultrapore C8 columns. The primary structure of *T.thermophilus* tRNA₁^{His} was determined using the method of rapid gel-sequencing. The studied structure is different from *Escherichia coli* tRNA^{His} in 23 positions. The interaction sites of *T.thermophilus* tRNA^{His} with histidyl-tRNA synthetase were investigated using the method of chemical modification by ethylnitrosourea. It was shown that histidyl-tRNA synthetase protects the following phosphates of tRNA^{His} from alkylation by ethylnitrosourea: 8 – between acceptor and D-stem; 27, 28, 29 – from 5'-side of anticodon stem; phosphate 34 in anticodon and phosphates 67, 68 from 3'-side of acceptor stem. All the revealed sites of tRNA^{His} are localized on one side of three-dimensional structure of tRNA in the same one where the variable stem side is. D-stem is located on the opposite side and does not interact with the enzyme.*

Key words: tRNA, aminoacyl-tRNA synthetase, RNA-protein recognition, chemical modification

Introduction. The fidelity of protein biosynthesis depends on correct aminoacylation of tRNA by aminoacids. This reaction is catalyzed by aminoacyl-tRNA synthetases which recognize the

aminoacid and tRNA specifically. At present there is considerable progress in solving the problem of how aminoacyl-tRNA synthetase can specifically recognize cognate tRNAs from a pool of various tRNA species sharing a similar tertiary structure [1, 2]. Histidine tRNA is unique in possessing an extra nucleotide G-1 at the 5'-end, which forms the additional base-pair

with the discriminatory residue 73, thus the acceptor stem of tRNA^{His} is one nucleotide base-pair longer than other tRNAs. tRNA^{His} of prokaryotes and organelles have C73 as their discriminator base, while eukaryotes have A73 and G73 [3].

The study of aminoacylation of *Escherichia coli* tRNA^{His} transcripts *in vitro* proved that the G1 – C73 base-pair is the most important determinant of the recognition by histidyl-tRNA synthetase, while anticodon plays a less expressed role [4]. Contrariwise, the investigation of aminoacylation of tRNA transcripts from yeast proved that the contribution of anticodon into tRNA recognition by histidyl-tRNA synthetase is bigger in comparison with the role of discriminatory basis [5]. At present the crystals of *E.coli* and *T.thermophilus* tRNA synthetase complexes are obtained with low molecular substrates – histidine and histidyl - adenylate, using the X-ray structural analysis their structure is determined, and the mechanism of histidine activation is suggested [6-9]. On the basis of charges distribution investigation on the surface of *T.thermophilus* histidyl-tRNA synthetase and the comparison of these results with the data for other aminoacyl-tRNA synthetases of IIa class, the model of tRNA recognition by enzyme is suggested [8]. Also the crystals of tRNA^{His} complex with *T.thermophilus* histidyl-tRNA synthetase were obtained [7], but the structure of this complex was not defined yet. Thus, the study of tRNA^{His} interaction with histidyl-tRNA synthetase in the solution is of significant interest.

This work describes the isolation of *T.thermophilus* tRNA^{His}, the definition of its primary structure and the study of interaction sites with histidyl-tRNA synthetase in the solution.

Materials and Methods. The following materials were used in the work: [-³²P]ATP, [-³²P]ATP, [5'-³²P]pCp with specific activity of 2000-3000 Cu/mmol ("Amersham", UK); T1-ribonuclease from *Aspergillus orizae* ("Sankyo", Japan); alkaline phosphatase *E.coli* ("Worthington", USA); T4-polynucleotidekinase ("Boheringer", Germany); nuclease P1 from *Penicillium citrinum* ("Calbiochem", USA); ribonuclease VI from cobra venom, PhyM from *P.polycephalum*, U2 from *Usilago sphaerogena*, CL3 from chicken liver, ribonuclease from *Bacillus cereus* – ("Pharmacia" production, Sweden). Ethylnitrosourea

was synthesized by A.G. Terentiev (IMBG of NAS of Ukraine).

The cells of extreme thermophile *T.thermophilus* HB-27 were grown in the fermenter with the volume of 300 liters in the medium, containing peptone (2%), yeast extract (1%) and NaCl (0.2%) at the temperature of 74-76°C and intensive aeration with the speed of 200 rpm. The cells were collected by centrifugation.

Crude tRNA was obtained by phenol extraction of RNA from the biomass with the subsequent deproteinization by mixtures of phenol:chlorophorm (1:1) and chlorophorm:isoamyl alcohol (9:1) [10]. To purify the RNA from proteins and contaminations of nucleotide and polysaccharide nature, chromatography was used on DEAE-cellulose, equilibrated by 0.0125 M tris-HCl, pH 7.5. At first, the contaminations were eluated by 0.15 M NaCl, and then by 0.25 M NaCl in the same buffer, tRNA from the column was eluated by 1 M NaCl, precipitated by 2.5 of the volume of cooled ethanol in the presence of 2% of potassium acetate. The sediment of tRNA was collected by centrifugation, then washed by 70% etanol and dried.

The individual tRNA^{His} was isolated from crude tRNA, using chromatography on benzoyl-DEAE-cellulose (BD-cellulose), DEAE Toyopearl 650 and high pressure liquid chromatography (HPLC) on the columns of DEAE 5PW and Ultrapore C8 ("Gold System" equipment of "Beckman" company, USA).

T. thermophilus histidyl-tRNA synthetase was obtained in the homogeneous state using the method described before [7].

The acceptor activity of tRNA^{His} on different stages of purification was defined according to the maximum level of forming ¹⁴C-histidyl-tRNA. Standard reaction mixture in the volume of 0.25 ml contained 0.1 tris-HCl, pH 7.6, 10 mM MgCl₂, 5 mM ATP, 0.02 mM ¹⁴C-histidine, 0.4 mg of crude preparation of *T. thermophilus* aminoacyl-tRNA synthetases and 0.05 ml of the tRNA solution from the investigated fractions. The reaction mixture was incubated for 7 minutes at 65°C, the reaction was stopped by adding 0.5 ml of cooled 10% TCA. The sediments of aminoacyl-tRNA were applied to GFC filters, washed by 2% TCA, dried and the radioactivity was measured by liquid scintillation spectroscopy.

Labelling of tRNA^{His} by radioactive phosphorus at 3'- and 5'- ends was accomplished as described in [12, 13, 14].

The primary structure of tRNA^{His} was studied, using two methods of sequencing the labeled tRNA: specific chemical degradation [17] and enzymatic sequencing, based on the hydrolysis of tRNA by specific endonucleases [16, 17].

Enzymatic hydrolysis of *T. thermophilus* tRNA^{His} was performed for 15 minutes at 55°C in 5 l of 20 mM Na-citrate, pH 5.0, 1 mM EDTA, 7 M urea were used for ribonucleases of T1 and PhyM; 20 mM Na-citrate, pH 3.5, 1 mM EDTA, 7 M urea were used for U2; 20 mM Na-citrate, pH 5.0, 1 mM EDTA were used for ribonuclease from *B. cereus*; 10 mM Na-phosphate, pH 6.5 and 4.2 M urea were used for ribonuclease CL3. tRNA fragments were separated by electrophoresis in 12.5% and 20% PAAG in the presence of 8 M urea.

The alkylation of labeled tRNA^{His} and its complex with histidyl-tRNA synthetase was performed using ethylnitrosourea under conditions, stabilizing three-dimensional structure of tRNA and at the same time favouring the formation of specific complex tRNA^{His} – histidyl-tRNA synthetase [18]. The reaction mixture in the final volume of 25 l contained 50 mM tris-HCl, pH 7.9, 5 mM MgCl₂, 2.5 mM 2-mercaptoethanol, 0.8 M tRNA^{His}, 3.2 M histidyl-tRNA synthetase. Ethylnitrosourea was added to the reaction mixture as a saturated ethanolic solution (2.5 l). In control experiments, pure ethanol was used in place of the ethylnitrosourea solution.

The modification reaction was carried out at 37°C for 2 hours and stopped by adding 3 l 3 M sodium acetate, pH 5.5. The aminoacyl-tRNA synthetases were removed by phenol extraction and tRNA was precipitated by adding three volumes of ethanol. For more complete precipitation, 10 g of glycogen were added to the mixture.

The alkylation of tRNA under denaturing conditions was performed in 25 l 0.3 M Na-cacodylate buffer, pH 8.0, containing 2 mM EDTA at 80°C for 2 min.

The cleavage of the polynucleotide chain at the modified residues was carried out by incubation in 10 l 0.1 M tris-HCl pH 9.0 at 55°C for 5 min. The samples of hydrolyzed tRNA were analyzed by gel-

electrophoresis in 12.5% PAAG in 5 mM tris-borate buffer, pH 8.3, containing 1 mM EDTA and 8 M urea. The electrophoretic bands were assigned by comparison with the T1 –ribonuclease partial digest of tRNA^{His}. Autoradiograms were scanned on the "UltraScan XL" densitometer ("LKB", Sweden).

Results and Discussion. *The isolation of individual tRNA^{His} from T. thermophilus.* tRNA^{His} was isolated from crude tRNA, using the combination of low pressure chromatography on BD-cellulose and DEAE Toyopearl 650 with HPLC on columns DEAE 5PW and Ultrapore C8.

Chromatography on BD-cellulose. 4 g of crude tRNA from *T. thermophilus* were applied to the BD-cellulose column (5 x 60 cm), equilibrated by 50 mM sodium-acetate buffer, pH 4.5, containing 0.35 M NaCl and 10 mM MgCl₂. Elution was carried out with concave gradient of the NaCl from 0.45 M (buffer A, 4.0 liter) to 1.5 M (buffer B, 2.0 liter). Concave form of the salt gradient was produced using gradient vials of different diameter ($D_A/D_B = 2/1$). The application of such a gradient form results in the decrease of the total gradient volume in 40% and in the corresponding speeding of the process. Moreover, the advantages of this gradient in the comparison with the linear one are in the fact that in the beginning of chromatography, when the main mass of material is eluted from the column, small steep slope of gradient is created, and in the end of chromatography, when "tail" drags on, the gradient becomes steeper and the material is eluted more compactly. NaCl concentration in the mixer after the output of volume $V(C_v)$ was calculated according to the formula [19].

$$C_V = C_A + ((C_A - C_B) (1 - V) / (V_A - V_B))^{(S_A/S_B)},$$

where C_A and C_B – initial concentrations of eluent in the reservoir and mixer; V_A, V_B – initial volumes of the liquid in vessels; S_A, S_B – squares of vessels cross-section.

Elution speed was 120 ml/h and fractions with volume of 16 ml were collected. After reaching 1.5 M salt concentration the elution was continued with .5 M NaCl in 10% ethanol. Acceptor activity of tRNA for histidine was assayed as described above. Fig.1 presents typical chromatography on BD-cellulose,

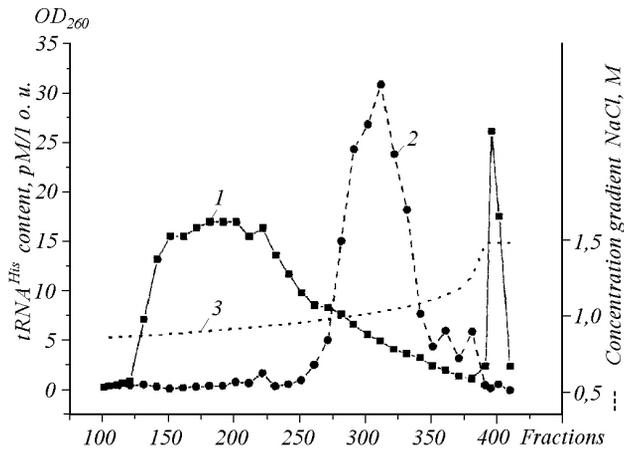


Fig. 1. The chromatography of tRNA from *T. thermophilus* on the column with BD-cellulose: 1- optic density at 260 nm; 2 – activity of tRNA^{His}; 3 – gradient of NaCl concentration.

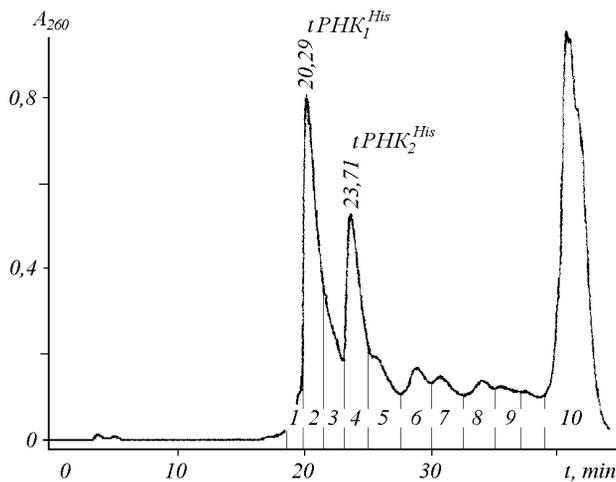


Fig. 2. The purification of tRNA^{His} from *T. thermophilus* on the Ultrapore C8 column. The vertical lines show the fractions which were collected in the course of chromatography. The output time of tRNA^{His} from the column is 20.3 minutes (fraction 2, purification degree 95%) and 23.7 minutes (fraction 4, purification degree 80%).

which shows that tRNA^{His} was found in the fractions 270-340. These fractions were pooled and precipitated with 2.5 volumes of ethyl alcohol, dried and kept at -20 C.

Chromatography on DEAE Toyapearl 650. 350 mg of tRNA, obtained after the chromatography on BD-cellulose, were applied to the column of DEAE

Toyapearl 650 (2.6 x 25 cm), equilibrated with 10 mM sodium-acetate buffer, pH 4.5, containing 0.2 M NaCl, 10 mM MgCl₂ and 10% isopropanol. Elution was carried out with linear gradient of NaCl from 0.2 to 0.3 M (2 x 800 ml). Elution speed was 150 ml/h, and fractions volume - 15 ml. The fractions containing tRNA^{His} were pooled, precipitated by ethanol and dried. The obtained preparation contained about 30% of tRNA^{His}.

Further purification of tRNA^{His} was carried out using HPLC Gold System ("Beckman", USA).

Chromatography on the column of DEAE 5PW. The following buffer solutions were used to perform chromatography: buffer A - 50 mM Tris-HCl, pH 7.5, 0.2 M NaCl, 10 mM MgCl₂ and 10% isopropanol; buffer B - the same, but with 1 M NaCl. The increase of NaCl concentration in the chromatography process was in agreement with the curve number 4, programmed in Gold System. The chromatographic fractions were manually collected, and acceptor activity tRNA^{His} was assayed. Retention time of tRNA^{His} on the column was 41 minutes.

Chromatography on the Ultrapore C8 column. The last step of tRNA^{His} purification was the chromatography on the high-pressure column Ultrapore C8. Buffer A contained 50 mM ammonium acetate, and 10 mM MgCl₂, buffer B - 10% isopropanol. The gradient was performed in the same way as in case of chromatography on DEAE 5PW column. The results of tRNA^{His} chromatography on Ultrapore C8 column are shown in Fig.2. On this purification step two isoacceptor tRNA^{His} were isolated with yield 2.5 and 1.7 mg for RNA₁^{His} and RNA₂^{His}, respectively. Purity of obtained preparations, checked by electrophoresis in 8% PAAG with 8 M urea, was not less than 98%. Thus, the method of isolating isoacceptor tRNA^{His} developed by us, allows obtaining highly purified tRNAs, suitable both for biochemical researches and structural work.

The study of the primary tRNA₁^{His} structure. Before studying the interaction sites of *T. thermophilus* tRNA₁^{His} with cognate aminoacyl-tRNA synthetase, it was necessary to define the primary structure of this tRNA. For this aim two complementary methods were used: the method of rapid gel-sequencing according to Pitty [15] and the method of specific nuclease

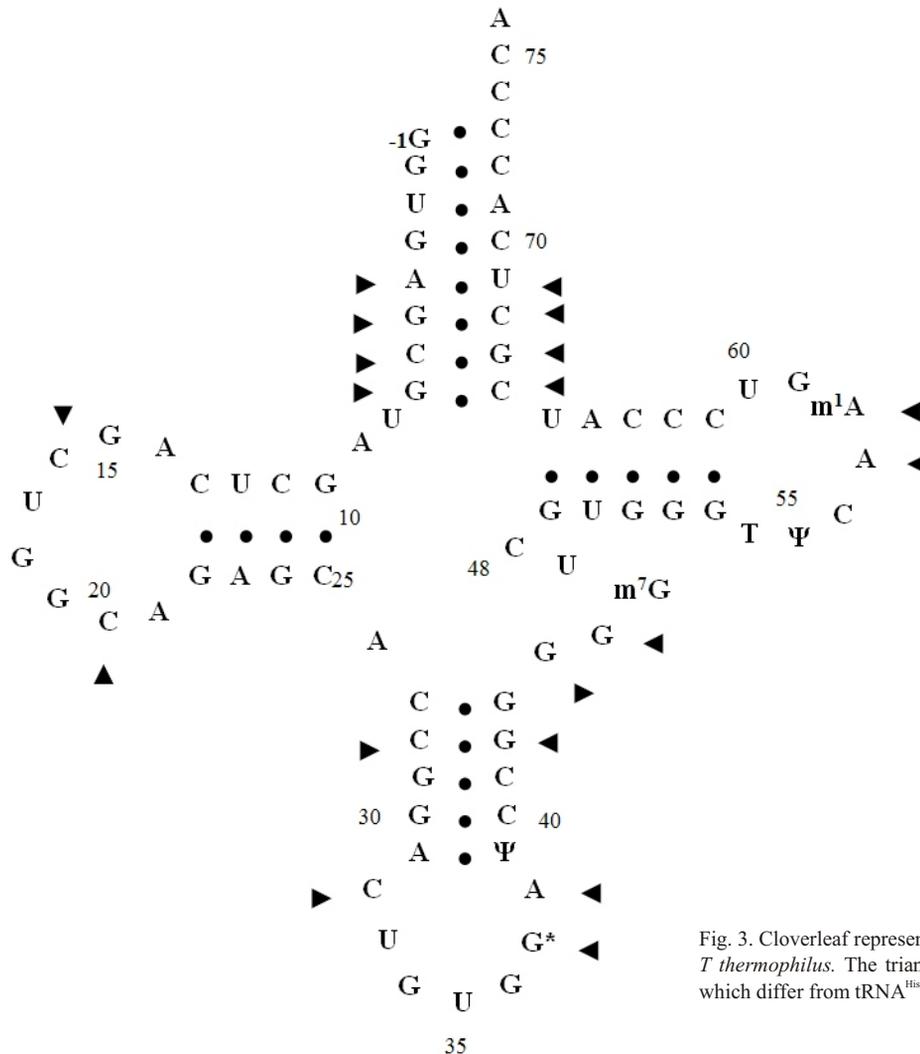


Fig. 3. Cloverleaf representation of tRNA^{His} from *T. thermophilus*. The triangles mark nucleotides which differ from tRNA^{His} from *E. coli*.

hydrolysis [17]. As a result of conducted researches the complete nucleotide sequence of *T. thermophilus* tRNA₁^{His} was defined (Fig.3). The comparison of primary structure of the studied tRNA₁^{His} with the structure of *E. coli* tRNA^{His} reveals rather a great similarity between these macromolecules. Differences were found in 23 positions: eight – in the acceptor stem, two – in D-loop, one – between D-stem and anticodon stem, three – in the anticodon stem, three – in the anticodon loop, two – in the T-loop (Fig.3). The obtained data of the primary structure of *T. thermophilus* tRNA₁^{His} show that it has extra G-1 nucleotide on the 5'-end and extra base pair G-1 – C73 in the acceptor stem. These features of tRNA structure are typical for all the known tRNA^{His} from prokaryotes [3].

The study of tRNA₁^{His} interaction with histidyl-tRNA synthetase by the method of chemical modification. The interaction sites of *T. thermophilus* tRNA₁^{His} with the cognates synthetase were investigated using the method of chemical modification by ethylnitrosourea. It was established previously that the alkylation of tRNA phosphate groups in the contact sites with the enzyme is complicated [18].

Typical experiment with 3'-labeled *T. thermophilus* RNA₁^{His} is shown in Figure 4. In the presence of histidyl-tRNA synthetase, several bands are nearly suppressed or are strongly reduced, suggesting that the corresponding phosphate groups are protected by the enzyme from alkylation.

The extent of phosphate alkylation was measured by densitometry of the autoradiograph and relative

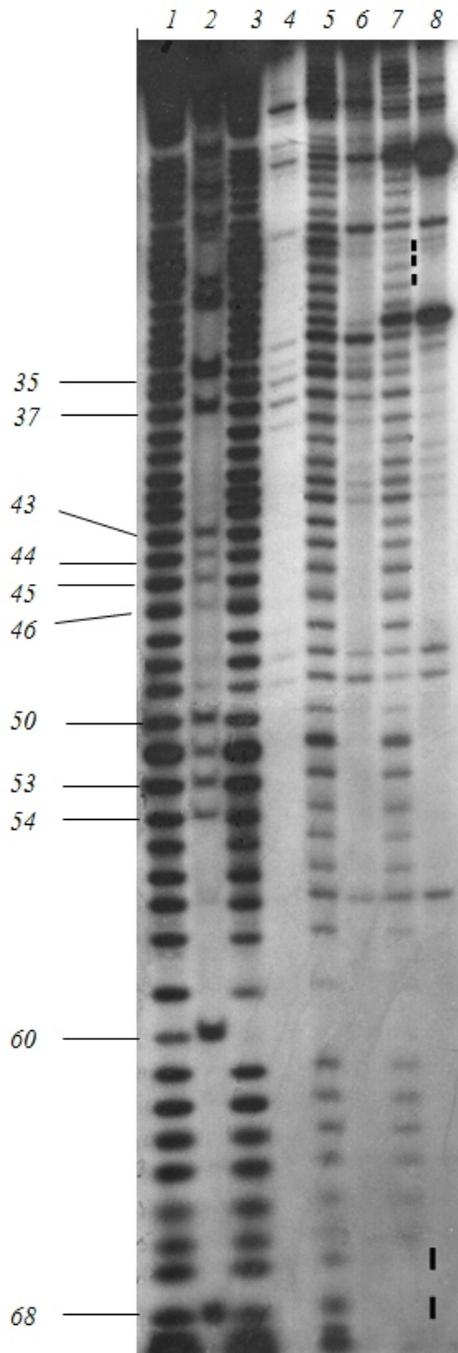


Fig. 4. Separation in 12. % PAAG of the hydrolysis products of 3'-labeled tRNA^{His} from *T.thermophilus*, alkylated by ethylnitrosourea in the conditions, denaturing (1) and stabilizing (3, 5) three dimensional tRNA structure, as well as in the presence of histidyl-tRNA synthetase (7); brackets mark the phosphates, the level of modification of which decreases significantly in the presence of histidyl-tRNA synthetase. Corresponding control incubations (4, 6, 8) were carried out in the absence of ethylnitrosourea; 2 – tRNA^{His}, partially hydrolyzed by T1 nuclease. The stripes enumeration corresponds to the phosphates in the tRNA^{His} structure.

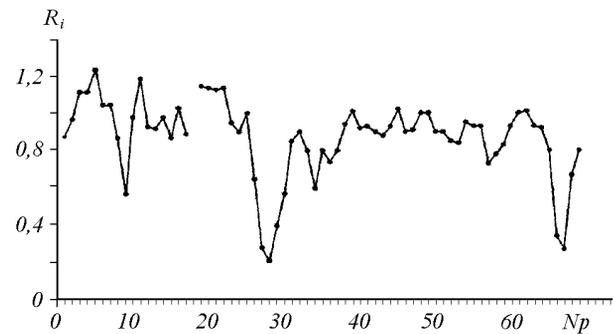


Fig. 5. Relative reactivities of the tRNA₁^{His} from *T.thermophilus* phosphates at the alkylation by ethylnitrosourea in the presence of histidyl-tRNA synthetase. R_i are the ratios between the intensities of the corresponding electrophoretic bands of the tRNA^{His} alkylation patterns in the presence of the enzyme and free tRNA^{His}; Np – phosphates numbers.

reactivities of phosphates of tRNA₁^{His}, which is in the complex with the enzyme, were calculated in comparison with the free tRNA. The data of these experiments are shown in Figure 5 and summarized on cloverleaf representation of tRNA in Figure 6. Presence of histidyl-tRNA synthetase resulted in strong protection (40 – 70 % decrease in band density) of phosphates in the acceptor stem (67 and 68) and in the anticodon stem (27, 28, 29). Weaker protection (20 – 40 % decrease in band density) was observed in the corner of the acceptor and D-stems (phosphate 8) and in the anticodon loop (phosphate 34).

It is worth mentioning that all the defined tRNA^{His} sites are on one side of three dimensional tRNA structure, namely, on the side of the variable loop, while D-stem is located on the opposite side of the molecule and it does not interact with the enzyme. Thus, obtained data suggest that the interaction of histidyl-tRNA synthetase with the homologous tRNA shows a recognition pattern characteristic of class 11 aminoacyl-tRNA synthetases [20].

A footprinting technique using phosphorotioate-containing RNA transcripts has been applied to identify contacts between *E.coli* tRNA^{His} and its cognate aminoacyl-tRNA synthetase [21]. However, in the mentioned work the protection of some sites which evidently do not coincide with the expected complex structure on the basis of X-ray structural

protected by histidyl-tRNA synthetase from alkylation, according to the model, should interact with the amino acid residues of the loop 96-98. The largest interaction zone of tRNA^{His} with the enzyme is the anticodon arm where, according to our biochemical data, the phosphates 28-30 in the anticodon stem and a phosphate 34 in the anticodon interact with the protein. For the interaction with the phosphates of anticodon stem in the complex model, there is a cluster of positively charged amino acids [8], formed by the amino acid residues of the loop 13- 14 and the residues, which are included in the composition of alpha-helix 14. To obtain a more complete picture of contacts of C-end domain with anticodon stem tRNA^{His}, as well as to get clear details of the interaction of these macromolecules on the atomic level it is necessary to obtain crystallographic data of the structure of their complex.

О. И. Гудзера, И. А. Крикливый, А. Д. Яремчук, М. А. Тукало

Выделение гистициновой тРНК из *Thermus thermophilus* и изучение ее первичной структуры и участков взаимодействия с гомологичной аминоксил-тРНК синтетазой

Резюме

Структура гистициновых тРНК (тРНК^{His}) отличается от структур тРНК других аминокислотных специфичностей наличием на 5'-конце дополнительного нуклеотида (G-1). Для изучения молекулярного механизма взаимодействия тРНК^{His} с гистицил-тРНК синтетазой разработан метод очистки тРНК^{His} из *T. thermophilus* и изучена первичная структура изокцентронной формы тРНК^{His}. Индивидуальную тРНК^{His} выделяли из суммарной тРНК, используя комбинацию хроматографии низкого давления на бензоилированной ДЭАЭ-целлюлозе (БД-целлюлоза) и ДЭАЭ Тоугорепл 650 с жидкостной хроматографией высокого давления на колонках DEAE 5PW и Ultrapore C8. Первичную структуру тРНК^{His} из *T. thermophilus* определяли с использованием методов быстрого гель-секвенирования. Изученная структура отличается от тРНК^{His} из *Escherichia coli* по 23 положениям. Участки взаимодействия тРНК^{His} из *T. thermophilus* с гистицил-тРНК синтетазой исследовали с помощью метода химической модификации нитроэтилмочевинной. Показано, что гистицил-тРНК синтетазой защищает от алкилирования этилнитрозомочевинной следующие фосфаты тРНК^{His}: 8' между акцепторным и D-стеблем; 27, 28, 29 ? с 5'-стороны антикодонного стебля; фосфат 34 в антикодоне и фосфаты 67, 68 с 3'-стороны акцепторного стебля. Все обнаруженные участки тРНК^{His} локализованы на одной стороне пространственной структуры тРНК ? там же, где и вариабельный стебель. D-стебель находится с противоположной стороны и не взаимодействует с ферментом.

Ключевые слова: тРНК, аминоксил-тРНК синтетазы, РНК-белковое узнавание, химическая модификация.

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