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Isolation and purification of *Thermus thermophilus* $tRNA^{Lys}$ and determination of its modified nucleotides

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Lysyl-tRNA synthetase along with aspartyl- and asparaginyl-tRNA synthetase belongs to subclass IIb and has a number of specific features. All subclass IIb synthetases have N-terminal anticodon-binding domains (~140 residues), function as homodimers $_2$, and anticodon triplets for each ARSase are the main but insufficient identity elements. On the other hand, all tRNA anticodons, corresponding to each synthetase, contain a central U and discrimination between them often depends on one nucleotide only. Thus, anyone can ask a question about the structural basis and mechanisms of discrimination between these anticodons by homologous structurally similar ARS binding domains, as well as about the possible role of modified bases in this respect. In this paper we described the methodological approach for purification to homogeneity of Thermus thermophilus $tRNA^{Lys}$ and determination of its modified nucleotides.

Keywords: $tRNA^{Lys}$, liquid chromatography, extreme thermophyle Thermus thermophilus, benzoylated diethylaminoethyl cellulose, modified nucleotides

Introduction. Aminoacylation of tRNA is a key stage of expression of genetic information, which ensures accurate translating of nucleotide sequence into the corresponding sequence of amino acids. This reaction is catalyzed by aminoacyl-tRNA synthetases (ARS), which recognize specifically the homologous amino acid and tRNA. According to modern classification [1, 2] lysyl-tRNA synthetase belongs to subclass IIb and, unlike subclass IIa ARSases, is specific for the existence of N-terminal anti-codon binding domain (~140 b.p. long).

A number of researches [3–7] revealed that specific features of subclass IIb aminoacylating systems (lysine, asparagine, and aspartic acid) consist in their close structural similarity; they function as homodimer $_2$and for each of them anticodon triplets of homologous tRNA are the main, but not sufficient, elements of recognition.

On the other hand, all tRNAs homologous to mentioned ARSases, contain uridine as the central anticodon base – $tRNA^{Asp}$ (34-G/QUC, codons GAU and GAC), $tRNA^{Asn}$ (34-G/QUU, codons AAU and AAC), and $tRNA^{Lys}$ (34-UUU and 34-CUU, codons AAA and AAG). Thus, the discrimination of abovementioned anticodons depends on the first letter of anticodon, and, in this respect, the obvious question arises: what are the structural bases and the mechanisms of discrimination of these anticodons by structurally similar homologous binding domains?

The presence of modified nucleosides in tRNALys was shown to be very important for its recognition by

the homologous lysyl-tRNA synthetase. The specificity of recognition of non-modified $tRNA^{Lys}$ transcripts decreases 140–2700 times [5, 8], however, no correlation towards any specific modification has been revealed. For instance, crystallographic structure data for the complex of *T. thermophilus* lysyl-tRNA synthetase with *Escherichia coli* $tRNA^{Lys}$ demonstrate that modifications of bases of antocodon loop U34 and A37 do not occur in the places of direct contacts with the enzyme [9]. At the same time, it has been established that effective aminoacylation of $tRNA^{Glu}$ by glutaminil-tRNA synthetase *E. coli* requires modification of mnm5s2U-34 [10]. Modifications in anticodon $tRNA^{Lys}$ moiety are of great importance for binding to ribosome [11–13], correct codon-anticodon recognition [14, 15]; they were also shown to influence the translation rate [16] and to be necessary for HIV reverse transcription initiation [17].

To clarify the structural role of modified $tRNA^{Lys}$ nucleosides in the aforementioned processes, it is necessary to study $tRNA^{Lys}$ complexes with corresponding macromolecules. Therefore, the isolation of individual $tRNA^{Lys}$ from *T. thermophilus*, containing natural minor nucleotides, is of great importance and absolute necessity.

Materials and Methods. *The following materials* have been used: benzoylated DEAE-cellulose (BD-cellulose) (Serva, Germany); Sepharose 4B (Pharmacia Fain Chemicals, Sweden); NaCl, $MgCl_2$, (Fisher, USA); tris, ammonium sulfate, phenyl methyl sulfonyl fluoride (Calbiochem, USA); ammonium acetate, 2-mercaptoethanol, dithiotreitol (Merk, Germany); filters GF/C; diethylaminoethyl (DEAE)-cellulose (Whatman, UK); isopropyl alcohol, ^{14}C-lysine (300 Ci/mol); ^{14}C-proline (239 Ci/mol) (Amersham, UK). All reagents were of analytical grade and were used without any additional treatment. All solutions were prepared with bidistilled water.

Equipment: centrifuge K-70 (Germany); Specord UVVIS spectrophotometer (Germany), chromatographic equipment: Gold-System and high-efficiency chromatography columns Spherogel TSK DEAE 5PW 21.5x150 mm (Beckman, Japan); Ultrapore RPMS C8 10.0x250 mm (Beckman, USA); Ultrapore ODS C18 4.6x250 mm (Beckman, USA); scintillation counter RackBeta (LKB, Sweden).

Isolation and purification of *T. thermophilus* $tRNA^{Lys}$ were carried out in several steps, including total *T. thermophilus* tRNA preparation, BD-cellulose column chromatography, reverse-phase chromatography on Sepharose 4B and high-efficiency liquid chromatography on Spherogel-TSK DE 5PW (21.5x150 mm) and Ultrapore RPMS C8 (10.0x250 mm).

The cultivation of *T. thermophilus* HB-8 strain, total tRNA preparation, chromatography on BD-cellulose column, and enzyme isolation for tRNA acceptor activity testing were done as previously described [18, 19].

Modified nucleotides of $tRNA^{Lys}$ were identified using HPLC system with Ultrasphere C18 column (4.6x250 mm) according to Gehrke's recommendations [20]. To define the nature of modified bases complete hydrolysis of 10–20 мg of *T. thermophilus* $tRNA^{Lys}$ was carried out. Reaction mixture in the final volume of 50 мl contained: 80 mM of sodium formate, pH 7.8, 0.5 mM $MgCl_2$, 10–20 мg of tRNA, 0.8 мg of RNAase A, 2.4 мg of bovine bowel phosphomonoesterase, 2.4 мg of phosphodiesterase of snake venom. The hydrolysis was performed during 18 hours at 37 C. Nucleosides obtained were separated on C18 column using HPLC Gold System with simultaneous registration of absorption spectra using scan detector 167. Three buffer solutions were used for chromatography: A – 10 mM $NH_4H_2PO_4$, pH 5.3; 2.5% methanol; B – 10 mM $NH_4H_2PO_4$, pH 5.1; 20% methanol; C – 10 mM $NH_4H_2PO_4$, pH 4.9; 35% acetonitryl. The solutions were thoroughly filtered (pore diameter- 0.2 мm) and degassed.

Results and Discussion. To perform BD-cellulose chromatography (column 5x60 cm) at the first stage of purification we used 4 g of total tRNA. It is noteworthy that fractions with $tRNA^{Lys}$ acceptor activity were eluted from column in the high optical density region (Fig.1), which contains also tRNAs of other specificities (leucine, proline, alanine etc.). $tRNA^{Lys}$ and $tRNA^{Pro}$ were shown to overlap the most (fractions 236–350). The attempts for further $tRNA^{Lys}$ purification on DE 5PW and C8 (HPLC) columns, similarly to $tRNA^{Ser}$ purification [19], were not successful. Thus we performed additional purification step – reverse-phase chromatography on the column with 4B sepharose

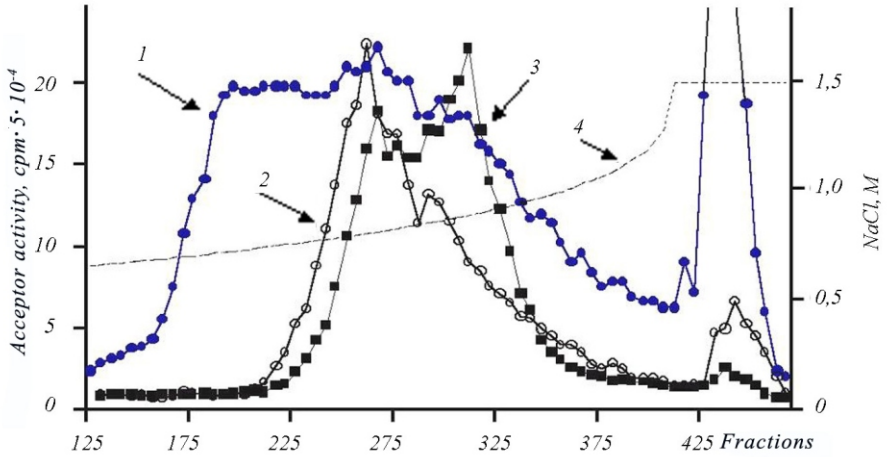

Fig.1 Chromatography of total tRNA from *T. thermophilus* on the column with benzoylated DEAE-cellulose: 1 – optical density at 260 nm; 2 – incorporation of ^{14}C-lysine; 3 – incorporation of ^{14}C-proline; 4 – concentration of NaCl.

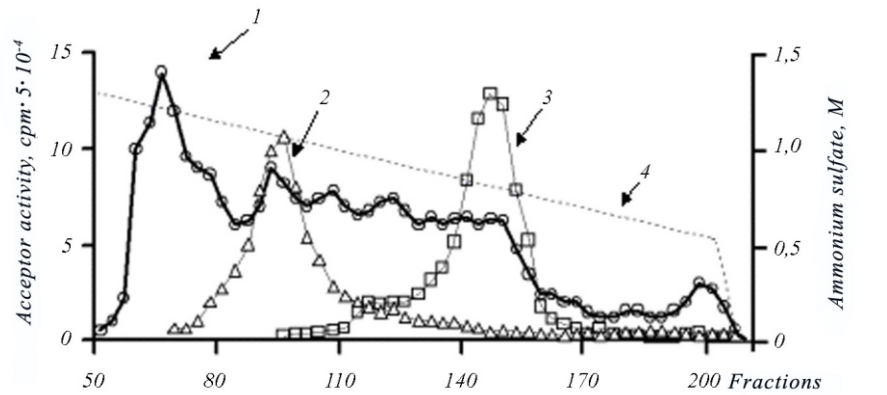

Fig.2 Purification of tRNA^{Lys} from *T. thermophilus* on the column with sepharose 4B in the reverse gradient of ammonium sulfate concentration: 1 – optical density at 260 nm; 2 – incorporation of ^{14}C-lysine; 3 – incorporation of ^{14}C-proline; 4 – gradient of ammonium sulfate concentration.

using ammonium sulfate gradient. Physical background of this chromatography lies in the property of tRNA to interact at high ammonium sulfate concentration with column matrix by hydrophobic bonds, which weaken and disappear along with decreasing salt concentration.

Dried tRNA (430 mg) was dissolved in water and added to buffer to maintain tRNA in the solution, which contained 0.01 M of sodium acetate (pH 4.5), 0.01 M $MgCl_2$, 1.6 M of ammonium sulfate, and 6 mM 2-mercaptoethanol. This solution (30 ml) was cooled down to 4 C and applied on the column (2.6x70 cm) with sepharose 4B, previously equilibrated with the same buffer and pre-cooled at 4 C. tRNA chromatography was carried out by reverse linear gradient of ammonium sulfate concentration (from 1.55 to 0.6 M). Elution rate was 42 ml/hr, gradient volume 2x1.5 L. Fractions of 15 ml were collected and analyzed for lysine and proline acceptor activities in reactions of aminoacylation (Fig.2). As shown on Fig.2, tRNA^{Lys} is well separated from tRNA^{Pro} and in some extent from tRNAs of other specificities. The disadvantage of sepharose 4B chromatography with the reverse gradient of ammonium sulfate concentration is that the latter prevents direct precipitation of tRNA from the solution upon alcohol addition. Thus prior to alcohol precipitation, fractions 81–106, containing tRNALys, were combined, diluted 2–3 times with water, and applied on the column with DEAE-cellulose or DEAE-Toyopearl 650 M. The column was washed with 0.1 M NaCl to get rid of ammonium sulfate, and then tRNA was eluted by 1 M NaCl.

Further purification of tRNA^{Lys} was performed on high-pressure columns using HPLC Gold System.

Chromatography on Spherogel-TSK DE 5PW (21.5x150 mm) The chromatography was conducted using following buffer solutions: A – 0.05 M tris-Cl, pH 7.5; 0.2 M NaCl, 0.01 M $MgCl_2$, and 10%

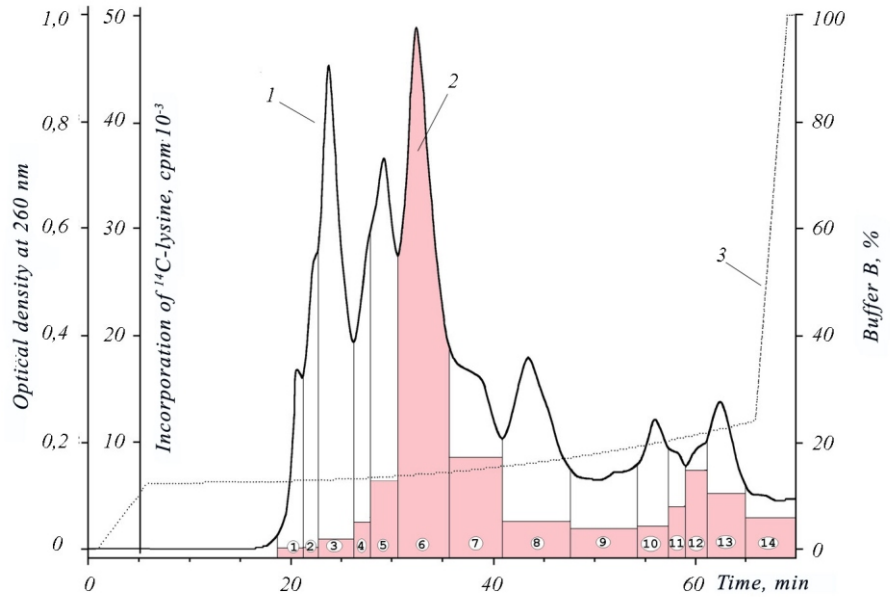

Fig.3 Purification of $tRNA^{Lys}$ from *T. thermophilus* on Spherogel TSK DEAE 5PW column: 1 – optical density at 260 nm; 2 – incorporation of ^{14}C-lysine; 3 –gradient of buffer B concentration.

2-isopropanol; buffer B differs from A in NaCl content – 1.0 M.

Dried tRNA was dissolved in water and mixed with equal volume of buffer A. 15–20 mg of tRNA were applied on the column, washed with buffer A and for 5 min the portion of buffer B was increased linearly to 17%, then the elution was carried out by gradient concentration of buffer B, according to curve 4, programmed by Gold System, that allows increasing the portion of buffer B to 30% during 60 min. Flow rate was 5 ml/min at room temperature (20–25 C). Optical density profile was registered at 260 nm. The fractions collected during the chromatography were later analyzed for the presence of $tRNA^{Lys}$. Fig.3 shows the typical chromatography profile, demonstrating that tRNAs with lysyl acceptance leave the column as a single peak. Fractions 6 and 7 were selected for further isolation of individual $tRNAs^{Lys}$. The purity of obtained $tRNA^{Lys}$ preparation was about 50%.

Final purification of $tRNA^{Lys}$ was performed on high-pressure column Ultrapore C8 (1x20 cm) using Gold System. Buffer A contained 0.05 M of ammonium acetate and 0.01 M $MgCl_2$; buffer B – 10% 2-isopropanol. 4–5 mg of tRNA dissolved in buffer A was applied on the column, which was developed by linear concentration gradient of buffer B. At the beginning during 10 min buffer B concentration was increased to 3.5%, then to 9% during next 60 min. Flow rate was 4 ml/min at room temperature (20–25 C). Fig.4 shows that after chromatography individual $tRNA_1^{Lys}$ (fraction 5) and $tRNA_2^{Lys}$ (fraction 7) were isolated.

The purity of preparations obtained was verified by electrophoresis of intact or ^{32}P-labeled tRNA in 8% PAGE in the presence of 8M urea and also by the estimation of lysine acceptance activity. One optical unit A_{260} (40 мg) of *T. thermophilus* $tRNA^{Lys}$ accepted 1520 pmol of ^{14}C-lysine, which corresponds to the purity level of 95%. From 4 mg of total tRNA applied at the first step on BD-cellulose column we obtained 7.3 mg of $tRNA_1^{Lys}$ and 3.5 mg of $tRNA_2^{Lys}$ after the last step of purification.

Minor nucleotides of *T. thermophilus* tRNA were identified using HPLC on Ultrasphere C18 column with the registration of absorption spectra using 167 HPLC Gold System scan detector. It is worth mentioning that the content of modified bases in tRNA sequence is very low (one residue per one molecule) comparing to the main nucleotides (A, G, U, C), that complicates their identification and requires taking care about chromatography conditions.

Components of buffer solutions should have the lowest possible molar coefficients of extinctions in the UV region of spectrum in order to minimize the influence on the base line. We used 10 mM $NH_4H_2PO_4$ (molar extinction coefficient <0.01 at 195 nm). The

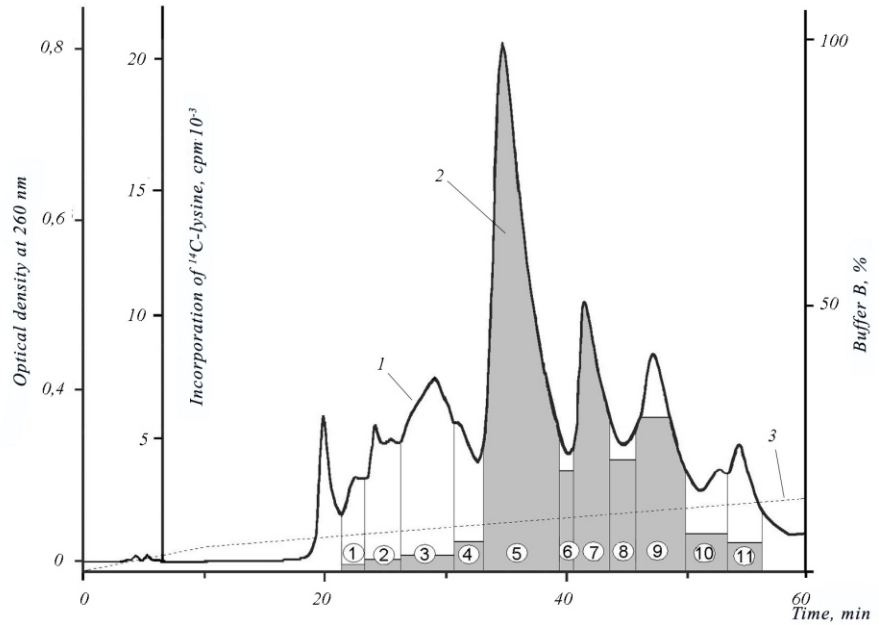

Fig.4 Purification of tRNA^{Lys} from *T. thermophilus* on Ultrapore C8 column: 1 – optical density at 260 nm; 2 – incorporation of ^{14}C-lysine (grey area); 3 – gradient of buffer B concentration.

application of higher concentration of this salt decreases the life-time of columns and resins. Higher concentrations of other salts – 0.05–0.2 M (ammonium acetate, ammonium phosphate) ensure the obtaining more symmetrical peaks and reproducibility of main nucleotides retention time on the column, neutralizing the active centers of the chromatography matrix. From another hand, the application of these salts is very convenient, since it is possible to obtain nucleosides in low salt solution and dry them directly in the vacuum. It is obvious, that increasing of buffer solution pH value decreases the positive charge of main nucleotides and increases the negative charge of acidic nucleotides. Therefore, the main nucleotides become less ionized and their mobility slows down, while acidic nucleosides become more ionized and their mobility increases. The mobility of neutral nucleosides does not change. On the contrary, insignificant decrease in pH value results in the opposite effect. As eluent, acetonitrile is 2 times stronger than methanol, but slightly differs in the selectivity. It should be mentioned, that long-term storage of acetonitrile (3 months or more) results in the formation of acrylic polymers, causing the damage of pumps and valves. Fig.5 shows that besides 4 main nitrogen bases (U, C, G, A), tRNA1Lys contains also minor bases Y, m^{1}A, m^{7}G, I, , s^{4}U, Gm, ms^{2}t^{6}A. Control chromatographic analysis of individual minor nucleotides at the same conditions has been performed to verify the reliability of this identification. Almost all minor nucleosides present in tRNA$_1^{Lys}$ structure were eluted from column in the gradient created by mixing of buffer solutions A and B. To remove ms^{2}t^{6}A from the column only buffer B was used.

On the basis of data obtained, using the methods of fast gel-sequencing and determination the nature of minor nucleosides by HPLC, we were able to define the complete nucleotide sequence of tRNA$_1^{Lys}$ from *T. thermophilus*. tRNA$_1^{Lys}$ was shown to consist of 76 nucleotide residues and, thus, belongs to the class of tRNA with short variable stem (data not shown). Seven modified bases were found in this tRNA located in anticodon, variable, T and D-loops and anticodon stem. Unlike tRNA^{Lys} from *E. coli*, anticodon structure of which contains 34-mnm^{5}s^{2}U, tRNA$_1^{Lys}$ from *T. thermophilus* possesses anticodon 34-CUU (Fig.6). It has been anticipated earlier, that modified bases in anticodon loop play the crucial role in the formation of specificity elements of *E. coli* tRNA^{Lys} [5]. On the other hand, the study on spatial structure of *T. thermophilus* lysyl-tRNA synthetase complexes with tRNA^{Lys} (mnm^{5}s^{2}U-34) from *E. coli* (2.75 A) and non-modified transcript of tRNA^{Lys} (34-CUU) from *T. thermophilus* (2.9 A) showed that anticodons of tRNA^{Lys} from *E. coli* as well as transcript were located in the identical conformations, but modified groups of bases 34 and 37

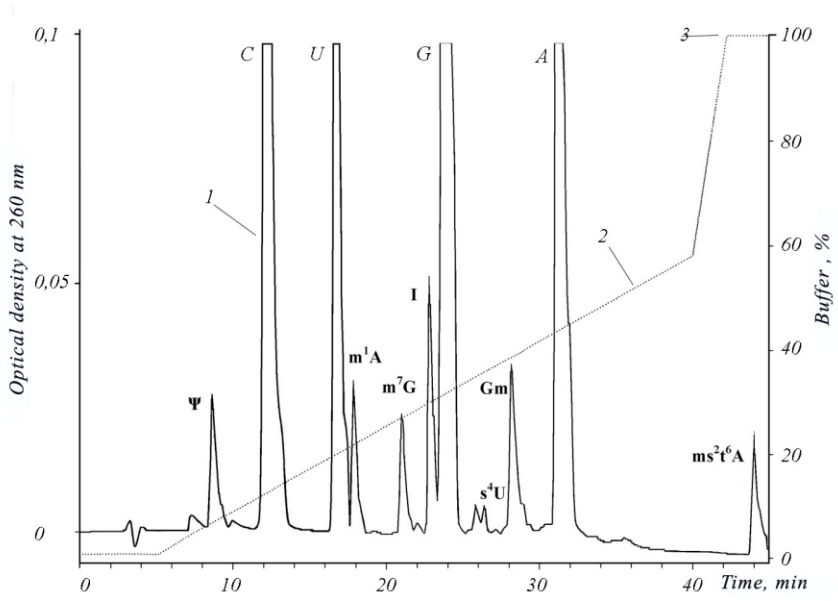

Fig.5 Distribution of nucleosides, obtained after hydrolysis of $tRNA^{Lys}$ from *T. thermophilus*, on Ultrapore C18 column: 1 – optical density at 260 nm; 2 – content of the gradient, formed by buffers A and B; 3 – buffer B.

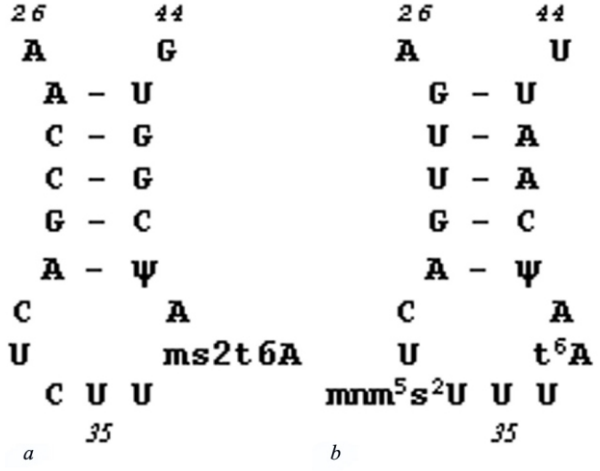

Fig.6 Comparison of anticodon stems primary structures of $tRNA^{Lys}$ from *T. thermophilus* (a) and *E. coli* (b).

didn't not have any contacts with synthetases [9]. Moreover, using *E. coli* $tRNA^{Lys}$ variant without U34 modification for activity measurements it was demonstrated that the absence of modification in the anticodon loop had insignificant effect on the k_{cat}/K_M value [21].

Thus, other modifications of $tRNA^{Lys}$ nucleotides, located outside of anticodon, might play important role ensuring the specificity of aminoacylation reaction. One possible candidate is 39 in anticodon stem [8]. To solve this problem completely we need to perform further structural and functional studies using various $tRNA^{Lys}$ transcripts and native naturally modified tRNAs as well.

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Виділення і очищення індивідуальної $тРНК^{Lys}$ із *Thermus thermophilus* та визначення її мінорних основ

Резюме

Лізил-тРНК синтетаза разом з аспартил- і аспарагіл-тРНК синтетазами належить до IIб підкласу і має низку характерних особливостей. Всі АРСази IIб підкласу включають N-кінцеві антикодон-зв'язувальні домени (близько 140 амінокислотних залишків), функціонують як гомодимер $_2$ і для кожної АРСази антикодонові триплети гомологічних тРНК є головними, але недостатніми елементами упізнавання. При цьому для всіх тРНК, гомологічних АРСазам IIб підкласу, центральною основою антикодону є уридин, і дискримінація цих антикодонів залежить лише від їхньої першої літери. Виникають закономірні запитання щодо структурних основ і механізмів дискримінації цих антикодонів структурно близькими зв'язувальними доменами АРСаз та можливої ролі мінорних основ тРНК у цих процесах. Наведено розроблені методичні підходи до отримання високоочищеної $тРНК^{Lys}$ із T. thermophilus і визначення мінорних основ у її структурі.

Ключові слова: $тРНК^{Lys}$, хроматографія, екстремальний термофіл Thermus thermophilus, бензоїльована ДЕАЕ-целюлоза, модифіковані нуклеотиди.

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

Выделение и очистка индивидуальной $тРНК^{Lys}$ из *Thermus thermophilus* и определение ее минорных оснований

Резюме

Лизил-тРНК синтетаза вместе с аспартил- и аспарагил-тРНК синтетазами принадлежит к IIб подклассу и имеет ряд характерных особенностей. Все АРСазы подкласса IIб имеют N-концевые антикодон-связывающие домены (около 140 аминокислотных остатков), функционируют в виде гомодимера $_2$ и для каждой АРСазы антикодоновые триплеты являются главными, но недостаточными элементами узнавания. При этом для всех тРНК, гомологичных этим АРСазам, центральным основанием антикодона является уридин, и дискриминация этих антикодонов зависит только от первой буквы антикодона. Возникают закономерные вопросы относительно структурных основ и механизмов дискриминации этих антикодонов структурно близкими связывающими доменами АРСаз и возможной роли минорных оснований в этих процессах. Приведены разработанные методические подходы к получению высокоочищенной тРНК^{Lys} из T. thermophilus и определению минорных оснований в ее структуре.

Ключевые слова: тРНК^{Lys}, хроматография, экстремальный термофил Thermus thermophilus, бензоилированная ДЭАЭ-целлюлоза, модифицированные нуклеотиды.

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