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Intrinsic fluorescence of single-tryptophan form of tyrosyl-tRNA synthetase catalytic module with the replacements of Trp 87 and Trp 283 by alanine

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Aim. Mammalian tyrosyl tRNA synthetase (TyrRS) is composed of N-terminal catalytic miniTyrRS and non-catalytic C-terminal domain. After cleavage both domains of TyrRS reveal non-canonical cytokine functions. It is important to study the conformational changes of miniTyrRS in the course of ligands binding in different nanocomposite complexes. Fluorescence spectroscopy is a very powerful method to detect the local conformational changes of proteins. The study of single-tryptophan form of the protein can provide important information about flexibility and local conformational changes of the protein functional sites. **Methods.** Site-directed mutagenesis, bacterial expression, fluorescence spectroscopy. **Results.** Intrinsic fluorescence characteristics of single-tryptophan Trp40-mini TyrRS were measured, a spectral maximum at 332 nm was revealed, which corresponds to the buried state of Trp40 fluorophore in protein globule. Fluorescence quenching of Trp40 by acrylamide revealed the existence of conformational flexibility of mini TyrRS. **Conclusions.** Fluorescence studies of the single-tryptophan form of tyrosyl-tRNA synthetase revealed a buried state of Trp40 fluorophore but high conformational flexibility of the enzyme at the nanosecond time scale.

K e y w o r d s: tyrosyl-tRNA synthetase, fluorescence spectroscopy, mutant form of miniTyrRS, active site, conformational flexibility.

Introduction

Aminoacyl-tRNA synthetases are the key enzymes of protein biosynthesis. These enzymes catalyze the activation of amino acids and their covalent attachment to transfer RNAs thus performing the first stage of the genetic information translation into protein structure [1–3]. Tyrosyl tRNA synthetase of *Bos taurus* (528 aa, 59.2 kDa) is composed of two structural modules, N-terminal catalytic mini TyrRS (342 aa, 39 kDa) and C-terminal cytokine-like (166 aa, 20 kDa) modules connected by a flexible linker [4, 5]. N-terminal module (mini

TyrRS) possesses full catalytic function, whereas C-terminal module corrects and stabilizes the placement of tRNA in the active center of the enzyme. After cleavage of human tyrosyl-tRNA synthetase by elastase into mini TyrRS and C-terminal module, both separate structural modules revealed different cytokine activities [6–8].

Fluorescence spectroscopy is one of the most informative methods for studying the conformational features and intramolecular dynamics of proteins [9–11]. The intrinsic fluorescence of proteins is mainly due to the emission of tryptophan residues, which are the local structural probes in 3D structures of proteins [12–13]. These probes provide information about the local properties of the microenvironment of fluorophores, protein dynamics in solution and allow monitoring the conformational changes of protein, which may have functional significance. Earlier, we successfully applied fluorescence spectroscopy to study the structure and dynamics of mammalian leucyl-tRNA synthetase [14] and tyrosyl-tRNA sytnthetase [15–16].

In the structure of the catalytic module *Bt*TyrRS there are 3 tryptophan residues, which are located in the active center of the enzyme (Trp40), near the dimerization region of mini *Bt*TyrRS subunits (Trp87) and at the binding site of cognate $tRNA^{Tyr}$ anticodon triplet (Trp283). The different arrangements of Trp40, Trp87 and Trp283 residues in mini TyrRS make promising the monitoring of the local conformational changes, provided only 1 Trp fluorophore at each of these functional sites. Using site-directed mutagenesis, we have replaced the Trp87 and Trp283 codons with alanine codons in the mini *Bt*TyrRS cDNA cloned in plasmid pET30a-39KYRS, leaving only one Trp40 near the active site of the enzyme [17].

The main aim of this study was to investigate the structural characteristics and local dynamics of a single Trp40 form of the catalytic mini *Bt*TyrRS by fluorescence spectroscopy.

Materials and Methods

The recombinant protein-producing strain was obtained on the basis of the BL21 (DE3) pLysE recipient (Stratagene, USA) transformed with a suitable construct according to the generally accepted method [18]. Plasmid construct pET30a-39KYRSW40 was created on the basis of the vector pET-30a (+) (Novagen, USA) and contained cDNA encoding the synthesis of Trp40-TyrRS under the control of the T7 phage promoter. A selective marker of plasmid pET30a (+) was the *kan* gene, which provides the resistance of transformed cells to the kanamycin. Plasmid DNA was isolated using the Gene JET Plasmid Miniprep Kit from Thermo Scientific. The concentration of plasmid DNA was determined on a spectrophotometer NanoDrop 2000 ("Thermo Scientific"). The genetically engineered strains of E. coli DH5α and BL21 (DE3) pLysE were used, respectively, to transform the recombinant plasmid pET30a-39KYRSW40 into E. coli cells and to express the cDNA of the *Bos taurus* Trp40-TyrRS. Competent *E. coli* cells were obtained according to the methods of Nishimura *et al.* [18]. All procedures for the transformation of plasmid DNA encoding the sequence of the mutant mini *Bt*TyrRS were performed according to [19]. Plasmid constructs were analyzed by electrophoresis in 0.7–1 % agarose gel.

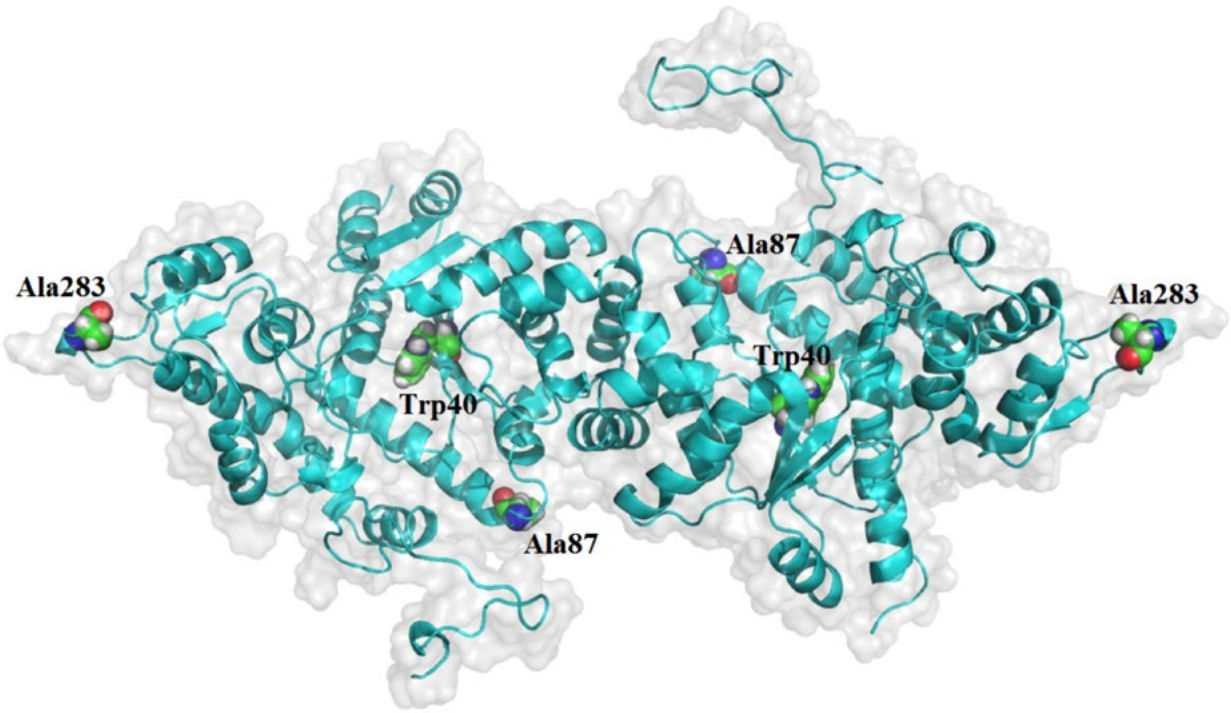

Fig. 1. Localization of Trp40 residues in the active sites of both subunits of mini-TyrRS mutant form with the replacements of Trp87 and Trp283 by alanine.

Cultivation of E. coli and induction of expression of recombinant mini BtTyrPC in bacterial culture were performed in Luria-Bertani (LB) medium with 30 μg/ml kanamycin. The transformed with recombinant plasmid pET30a-39KYRSW40 competent cells E. coli BL21 (DE3) pLysE were grown on a shaker (Environmental Shaker Incubator ES-20) at 37 °C to an optical density A_{600} = 0.7. The synthesis of the target protein was induced by adding 1M propropyl thiogalactopyranoside (IPTG) up to 1 mM concentration followed by incubation at 37 °C for 4 hours. The biomass collected from 100 ml of culture was resuspended in 12 ml of cell lysis buffer (50 mM sodium phosphate buffer, pH 8.0, 500 mM NaCl, 10 mM imidazole, 5 mM β-mercaptoethanol). Lysis was performed using ultrasonic disintegrator with ultrasound (6 cycles of 20s, intervals of 20s). The lysate was clarified by centrifugation at 13,000 rpm for 30 minutes at 4 °C. The supernatant was applied on a 1 ml Ni-NTA agarose column equilibrated with the cell lysis buffer, washed with 10 ml of the wash buffer (50 mM sodium phosphate buffer, pH 8.0, 500 mM NaCl, 20 mM imidazole, 5 mM β-mercaptoethanol). The recombinant protein was eluted with 5 ml of the elution buffer (50 mM sodium phosphate buffer, pH 8.0, 150 mM NaCl, 200 mM imidazole, 5 mM β-mercaptoethanol). All fractions, in which the protein was detected were pooled and dialyzed against 200 ml of the dialysis buffer (500 mM sodium phosphate

buffer pH 8.0, 150 mM NaCl) for 20 hours at +4 °C. Electrophoretic analysis of the recombinant protein in 12 % polyacrylamide gel was performed under denaturing conditions in the Laemmley buffer system [20]. The gels were stained with Coomassie blue R250. Concentration of the purified mutant mini TyrRS protein was determined spectrophotometrically with BioMate 5 spectrophotometer using an extinction coefficient of 9970 $M^{-1}cm^{-1}$ at a wavelength of 280 nm. The optical absorption coefficient was determined from amino acid analysis using the ProtParam program (http://expasy.ch/cgibin/protparam).

Fluorescence spectra were recorded on a Hitachi M850 spectrofluorimeter (Japan), which was equipped with a thermostated cuvette holder. The temperature in the cuvette was determined with an accuracy of +0.2 °C. Measurements were performed in a quartz cuvette with an optical path length of 0.5 cm. The spectral width of the slits for the monochromator of the exciting light and the recording system was 5–10 nm. The wavelength of the excitation light was 296 nm, the wavelength range for the fluorescence spectra was 300–400 nm, the fluorescence was recorded at an angle of 90° to the direction of the excitation light beam.

Results and Discussion

Recombinant single-tryptophan form of mini-TyrRS was expressed in *E. coli* strain BL21 (DE3) and retained full tRNA aminoacylating activity inherent in [the] native enzyme. Since the final yield of the target recombinant protein in bacterial systems largely depends on the culture conditions, the optimal expression parameters of mini *Bt*TyrRS in *E. coli* were experimentally determined. It was shown that the highest level of recombinant mini *Bt*TyrRS expression in *E. coli* was achieved by adding IPTG at the second hour of cultivation (OD_{600} = 0.7–0.9) and incubation time was 4 hours at 37 °C. We used these conditions in order to obtain one-tryptophan mutant form of *B. taurus* TyrRS in preparative quantities. After chromatographic purification of mutant mini-TyrRS on Ni-NTA agarose the yield of recombinant protein was about 2.5 mg from 100 ml of bacterial culture.

The fluorescence spectrum of the mutant form of the catalytic modulus of Bos taurus tyrosyl-tRNA synthetase at the 296 nm excitation wavelength is shown in Fig. 2. Intrinsic fluorescence characteristics, in particular, the position of the fluorescence maximum, λ_m, and the half-width of the fluorescence spectra, $\Delta\lambda$, are 332 nm and 56 nm, respectively. According to the three spectral classes model of tryptophan residues in protein structure, the tryptophan residue at position 40 refers to the spectral class I, which is characterized by emission of Trp fluorophore localized inside the protein globule without contact with free water [12]. It should be pointed that the polarity of the tryptophan residue microenvironment is determined not only by its accessibility to the solvent molecules, but also by its own polar protein groups, which are the parts of its microenvironment [12, 13]. Characteristics of tryptophan fluorescence of Trp40 mini TyrRS clearly indicate the immobilization of the fluorophore inside the synthetase.

When comparing the tryptophan fluorescence spectra for the native and denatured by 6M guanidine hydrochloride forms, we observed a significant decrease in the fluores-

cence intensity and shift of the emission maximum to the long-wavelength part of the spectrum (Fig. 2). This indicates a significant change in the microenvironment of the Trp40 residue during unfolding of protein.

The effects of mutant Trp40 form of mini-TyrRS denaturation were monitored with the gradient addition of guanidine hydrochloride (Fig. 3). The shift of fluorescence maximum and a decrease of the emission intensity at the increasing of guanidine hydrochloride concentration were observed indicating a gradual unfolding of the protein structure.

We analyzed the fluorescence intensity and maximum position of Trp40 mini TyrRS during the thermal melting (Fig. 4). It was found that the temperature dependence of the maximum position of tryptophan fluorescence reveals a sigmoid shape with transition in the region of about 55 °C. This indicates a significant temperature stability of mutant Trp40 mini TyrRS. Although the conformational mobility increases uniformly with increasing temperature, the significant structural changes of protein were observed only at temperatures above 50 °C.

At the next step we investigated the external quenching of protein fluorescence with neutral acrylamide molecules and charged Cs^+ ions. The quenching plots are usually represented as the dependence of the relative fluorescence intensity F_0/F on the quencher concentration (Stern-Volmer plots). The fluorescence intensities of Trp40 mini TyrRS at different quencher concentrations were measured. Based on these data, fluorescence quenching plots were constructed in Stern-Volmer coordinates (Fig. 5).

The linear character of tryptophan fluorescence quenching plots in Stern-Volmer coordinates with neutral and ionic quenchers indicates the dynamic process of quenching. Stern-Volmer constants were determined as 5.39 and 0.67 for acrylamide and Cs^+, respectively. A high value of the Stern-Volmer constant for acrylamide quenching was due to the ability of this quencher to diffuse through the protein matrix which reveals its conformational flexibility at the nanosecond time scale. This effect clearly indicates the existence of conformational fluctuations of this protein in solution, as well as the dynamic availability of buried Trp40 residue to solvent molecules. In turn, a low efficiency of fluorescence quenching by Cs^+ ions can be explained by the presence of positively charged groups, e.g. Lys37 at the surface of protein near Trp40 fluorophore.

Earlier, we performed detailed fluorescence studies of single-tryptophan non-catalytic C-terminal domain of *Bos taurus* TyrRS, which also revealed cytokine activity [21–22]. Fluorescence quenching of Trp144 by acrylamide suggested a rapid conformation dynamics of the C-module in nanosecond time scale [21]. The temperature-induced conformational changes of the C-module have been monitored by the fluorescence measurements. Fluorescence emission maximum shift up to ~349 nm and the red-edge shift effect at 37–52 °C indicated a major conformational transition of Trp144 from buried native state into highly relaxing polar solvent environment [22].

For years, tryptophan fluorescence of proteins is widely used as a tool to monitor the changes in proteins and to make inferences regarding local structure and dynamics [9–12, 23–24]. The interpretation of fluorescence data from multi-Trp proteins is generally complicated due to complex fluorescence processes and environ-

ment-sensitive nature of individual Trp residues [25]. Most effective approach is the use of single-tryptophan mutants of proteins in order to study the structural details, local conformational flexibility and rearrangements, especially at the protein functional sites [26–29].

Hence, in this work we studied in details some fluorescence characteristics of the single-tryptophan form of Trp40-mini TyrRS. The single-tryptophan N-terminal mini TyrRS and C-terminal domain may be extensively used in future investigations of cytokine activities

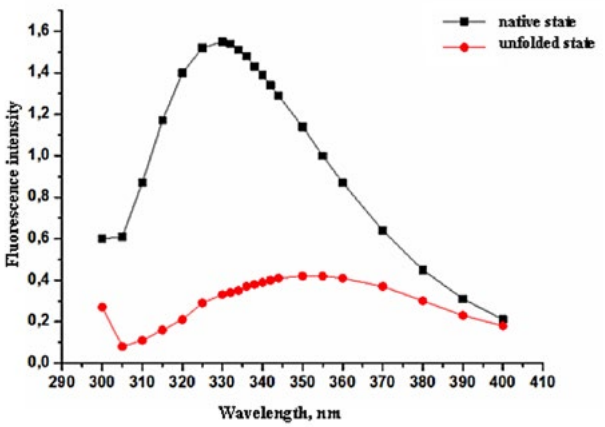

Fig. 2. Fluorescence spectra of the mutant Trp40 form of *B.taurus* miniTyrRS in the native and unfolded state in 6M guanidine hydrochloride at the excitation wavelength of 296 nm.

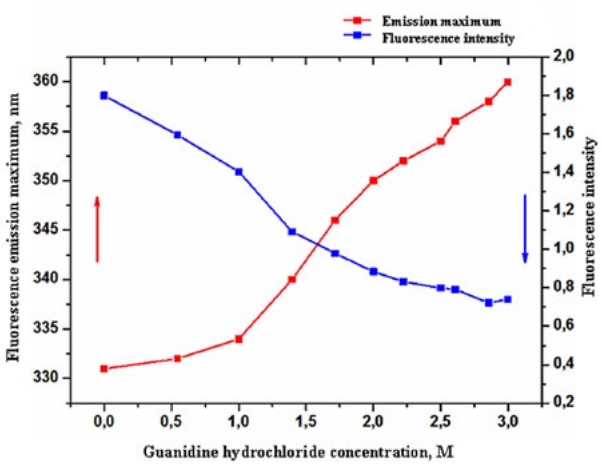

Fig. 3. Changes in fluorescence intensity and emission maximum of the Trp40 mutant form of *B.taurus* mini TyrRS with increasing guanidine hydrochloride concentration

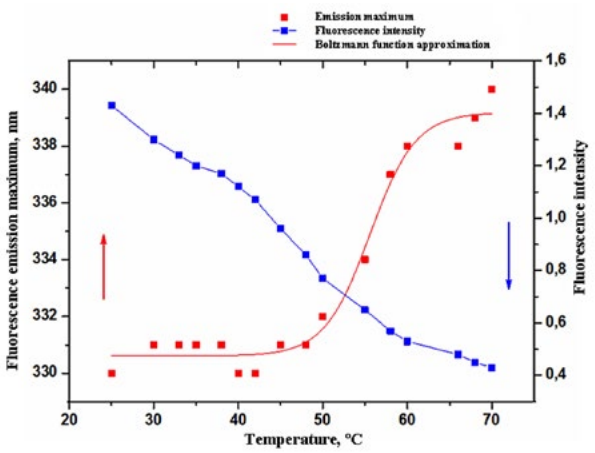

Fig. 4. Changes in fluorescence intensity and emission maximum of the Trp40 mutant form of *B.taurus* mini TyrRS during the protein melting.

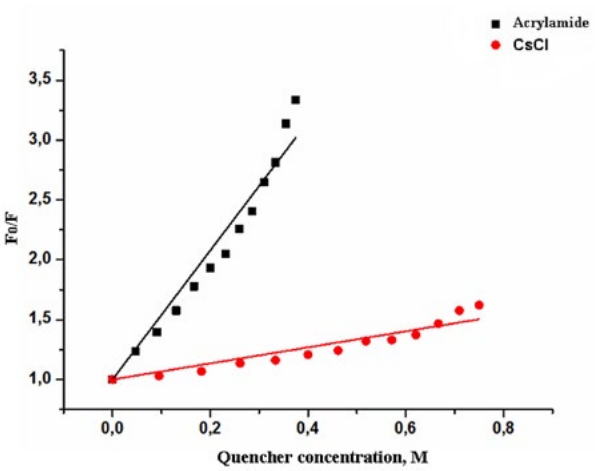

Fig. 5. Stern-Volmer plots of the Trp40 mutant form of *B.taurus* mini TyrRS fluorescence quenching with neutral acrylamide and Cs^+ ions at 25 °C .

both as isolated proteins and their nanocomposite complexes.

Conclusions

Fluorescence characteristics of the single-tryptophan Trp40 mini TyrRS form revealed a buried state of the Trp40 fluorophore, which is located near the enzyme active site. At the same time, the high level of quenching of Trp40 fluorescence quenching by acrylamide revealed conformational flexibility of the protein and dynamic accessibility of the fluorophore to non-charged quenchers on a nanosecond timescale.

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Власна флуоресценція однотриптофанової форми каталітичного модуля тирозил-тРНК синтетази із замінами Trp 87 та Trp 283 на аланін

І. О. Блащак, В. Заєць, Л. А. Коломієць, О. І. Корнелюк

Мета. Тирозил-тРНК-синтетаза ссавців (TyrRS) складається з N-кінцевого каталітичного модуля miniTyrRS та некаталітичного С-кінцевого домену. Після розщеплення обидва домени TyrRS виявляють неканонічні цитокінові функції. Важливо вивчити конформаційні зміни miniTyrRS як у процесі розпізнавання субстратів, особливо тРНК, так і інших лігандів у різних нанокомпозитних комплексах. Флуоресцентна спектроскопія — дуже потужний метод виявлення локальних конформаційних змін ферментів. Вивчення однотриптофанової форми білка може надати важливу інформацію про локальну гнучкість та конформаційні зміни активного центру ферменту. **Методи.** Сайт-спрямований мутагенез, бактеріальна експресія, флуоресцентна спектроскопія. **Результати.** Характеристики власної флуоресценції однотриптофанової форми Trp-40 miniTyrRS були виміряні та виявили спектральний максимум при 332 нм, що відповідає екранованому стану флуорофору Trp40. Гасіння флуоресценції Trp-40 акриламідом показало наявність високої конформаційної рухливості активного центру. **Висновки.** Флуоресцентні дослідження однотриптофанової форми тирозил-тРНК синтетази виявили екранований стан флуорофору Trp40, але високу конформаційну рухливість активного центру ферменту в часовому наносекундному інтервалі.

Ключові слова: тирозил-тРНК синтетаза, флуоресцентна спектроскопія, мутантна форма miniTyrRS, активний центр, конформаційна рухливість.

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