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Conformational Flexibility of Cytokine-Like C-Module of Tyrosyl-tRNA Synthetase Monitored by Trp144 Intrinsic Fluorescence

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Abstract The non-catalytic COOH-terminal module formed after proteolytic cleavage of full-length mammalian tyrosyl-tRNA synthetase displays dual function: tRNA binding ability and cytokine activity. With the aim to explore the intramolecular dynamics of C-module in solution we used fluorescence spectroscopy to study conformational changes of isolated protein. We used information from fluorescence spectra and computational model for characterization of a microenvironment of a single tryptophan residue (Trp144). Its fluorescence parameters and protection from quenching by Cs^+ ions indicate the internal localization—buried into protein globule. The fluorescence quenching of Trp144 by acrylamide suggests rapid conformation dynamics of the C-module in nanosecond time scale. The temperature-induced conformational changes in the C-module were monitored by the fluorescence measurements of Trp144 emission and by red-edge excitation shift. An emission maximum shift up to ∼349 nm and significant decrease of 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.

Keywords C-module of tyrosyl-tRNA synthetase · Protein intrinsic fluorescence · Protein dynamics

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Introduction

Mammalian tyrosyl-tRNA synthetase is composed of two structural modules: NH_2-catalytic module (miniTyrRS) and non-catalytic cytokine-like C-terminal module separated from each other by flexible peptide linker [1–3]. In full-length enzyme C-module has an affinity for tRNA as *cis*-factor. After proteolytic cleavage from the synthetase catalytic body it displays the cytokine-like function [3–6]. In order to explain the C-module functioning mechanism, we proposed a conformational switching hypothesis, which suggests that two functions could be realised in different conformations. Such conformational changes may be governed by protein intramolecular dynamic, which has not yet been explored for isolated C-module of TyrRS.

Fluorescence spectroscopy is a sensitive approach for investigating structural and molecular dynamic properties of proteins [7–9]. The intrinsic protein fluorescence is caused mainly by tryptophan residues—convenient natural probes in protein structure. A valuable feature of intrinsic protein fluorescence is the high sensitivity of tryptophans to their local environments. Using such protein probes we can gather information about the fluorophore microenvironment properties, protein dynamics in solution or protein conformational transitions. Tryptophan residues also appear to be uniquely sensitive to the collisional quenching by external reagents [7]. Quenching of protein intrinsic fluorescence can be used to probe the relative solvent exposure of tryptophan residues in proteins under a specified set of conditions.

In the present paper, we have characterised the intrinsic C-module fluorescence and investigated temperature-induced conformational changes in the protein using the methods of fluorescence spectroscopy. C-module of tyrosyl-tRNA synthetase contains a unique tryptophan residue (Trp144) which

is an intrinsic probe in its structure [10, 11]. This, in turn, allows the investigation of C-module intramolecular dynamics and monitoring of local conformational transitions in protein structure.

Experimental

Materials and methods

Chemicals

L-tryptophan and acrylamide were obtained from Sigma Chemical Company (St. Louis, MO, U.S.A). All other chemicals were of analytical grade. The buffer solution for fluorescence measurements contained 20 mM TrisHCl, 150 mM NaCl at pH 7.5.

Expression and purification of recombinant C-module of the bovine tyrosyl-tRNA synthetase

Recombinant C-module of bovine tyrosyl-tRNA synthetase (Fig. 1) was expressed in *Escherichia coli* BL21(DE3) cells harbouring pET30a-YCD3 plasmid. The cells were grown at 37°C in LB-kanamicin (30 μg/ml) medium with vigorous shaking up to the cell density corresponding to 0.5 at A_{600}. The expression of C-module was induced by addition to cell culture of isopropyl-thio-β-galactopyranoside to a final concentration of 1 mM. The culture was grown for an additional 4h. Cells were harvested by centrifugation (6000 g × for 5 min), supernatant was discarded, and the cell pellet was frozen at $-20\,^{\circ}$C. Then the cell pellet was thawed on ice and resuspended in lysis buffer (20 mM TrisHCl, pH 8.0, 500 mM NaCl, 10 mM imidazole, 5 mM β-mercaptoethanol), and disintegrated by sonication. After centrifugation at 10000 × g for 30 min at $+4\,^{\circ}$C, the supernatant was loaded on a Ni-NTA column equilibrated with lysis buffer. The column was washed with ten column volume washing buffer (20 mM TrisHCl, pH 8.0, 500 mM NaCl, 20 mM imidazole, 5 mM β-mercaptoethanol). Proteins bound to the column were eluted with a five-column volume linear gradient of imidazole from 40 to 100 mM in elution buffer (20 mM TrisHCl, pH 8.0, 150 mM NaCl, 5 mM β-mercaptoethanol). The purity of the protein was detected by SDS-polyacrylamide gel electrophoresis and was about 95%. Protein and tryptophan concentrations were determined using the extinction coefficients $\varepsilon_{280}^{\mathrm{C-module}} = 9650\mathrm{M}^{-1}\mathrm{cm}^{-1}$ and $\varepsilon_{280}^{\mathrm{Trp}} = 5690\,\mathrm{M}^{-1}\,\mathrm{cm}^{-1}$, respectively. UV absorbance measurements were performed using a Specord UV VIS spectrophotometer (VEB Carl Zeiss, Jena, Germany).

Fluorescence spectroscopy.

All experiments were carried out using a Hitachi Model 850 fluorescence spectrophotometer (Hitachi, Ltd.,

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    1          11         21         31         41         51
    |          |          |          |          |          |
  1 MHHHHHHSSG LVPRGSGMKE TAAAKFERQH MDSPDLGTDD DDKAMDPNSE PEEVIPSRLD     60
 61 IRVGKVISVD KHPDADSLYV EKIDVGEAEP RTVVSGLVQF VPKEELQDRL VVVLCNLKPQ    120
121 KMRGVKSQGM LLCASVEGVN RKVEPLDPPA GSAPGERVFV KGYEKGQPDE ELKPKKKVFE    180
181 KLQADFKISD EYIAQWKQTN FMTKMGSVSC KSLKGGNIS                          219
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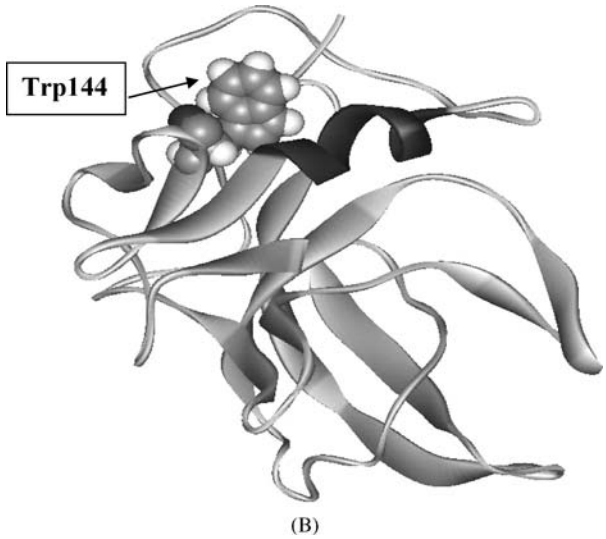
(A)



(B)

Fig. 1 (A) Amino acid sequence of recombinant C-module of bovine TyrRS (B) 3D structure model of C-module of bovine TyrRS (cm5 model)

Tokyo, Japan) and Cary Eclipse (Varian Inc., Mulgrave, Victoria, Australia). The fluorescence spectra were highly reproducible and equipment independent. In order to maintain constant temperature in the cell holder, Hitachi Model 850 fluorescence spectrophotometer was fitted with circulating water bath. The sample temperature was controlled by chromel-alumel thermocouple with an accuracy of $\pm$ 0.2 °C. A 0.5 × 0.5 cm quartz cuvette was used for all experiments. The excitation and emission slits of 5 nm were used in fluorescence experiments. The protein fluorescence quantum yield was determined as $q = 0.14\, S_{\mathrm{C-module}} / S_{\mathrm{Trp}}$, where 0.14 is a quantum yield of Trp fluorescence at 25 °C [12], $S_{C-\mathrm{module}}$ and S_{Trp} are the areas under fluorescence spectra normalized to the molar concentration of C-module and tryptophan respectively. The excitation wavelength was set at 296 nm for all experiments that monitor changes of emission maximum with temperature. For the experiments that monitor changes of emission maximum with excitation wavelength at the temperature range from 20 °C to 60 °C, the excitation wavelengths were 290, 292, 294, 296, 298, 300, 302 and 304 nm. All emission spectra were corrected for Raman scattering by subtraction of corresponding blank spectra. We fitted the Gaussian function to the maximum region of fluorescence spectrum to determine its emission maximum.

The fluorescence quenching experiments were performed with acrylamide and Cs^+ (CsCl) quenchers. C-module protein solution was titrated at 20 °C with 5 M acrylamide and CsCl to a final concentration of 0.3 M, the excitation wavelength was 296 nm. Steady state intensity emission of C-module was corrected for the absorption of acrylamide (A_Q^{296}) at the excitation wavelength and for the dilution of C-module during its titration. A correction coefficient for absorption, $10^{A_Q^{296} L/2}$ (where L is the pathlength of the cuvette), was applied. The fluorescence quenching data were analyzed according to the Stern-Volmer equation [7, 8]: $I_o / I = 1 + K_q [Q]$; where I_o and I are the fluorescence intensities in the absence and presence of the quencher, respectively; and [Q] is the quencher concentration.

Computational structure analysis

Visualization and analysis of Trp144 local microenvironment have been performed for C-module 3D structure using the SwissPDB-Viewer 3.7(b2) program. The spatial model of C-module (cm5) was built previously, using the comparative homology modeling approach [13]. Its sequence of 169 a. a. (Pro360-Ser528 in the sequence of *Bos taurus* tyrosyl-tRNA synthetase) was found to have 92.9% identity in comparison with the sequence of C-terminal domain of human TyrRS [14] (Protein Data Bank code 1NTG). Homology modeling was performed using Swiss-Model web-server 3.5 [15] and

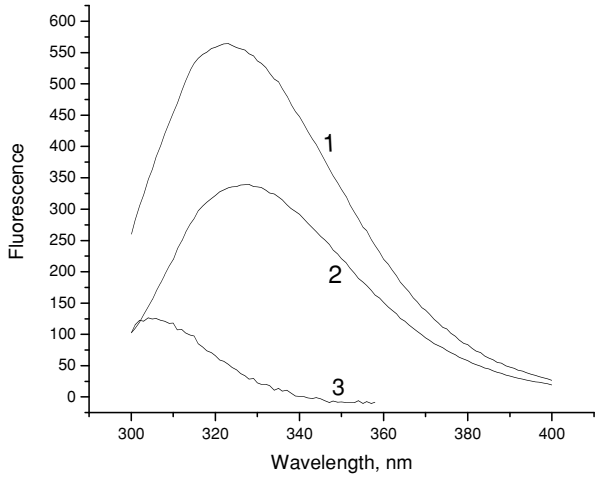


Fig. 2 Fluorescence emission spectra of C-module at 20°C in buffer containing 20 mM TrisHCl, 150 mM NaCl at pH 7.5 at excitation wavelength 280 nm (1), 296 nm (2). Tyrosine fluorescence spectrum (3) of C-module was obtained by subtraction of normalized at 370 nm emission C-module spectra determined upon excitation at 280 and 296 nm

12 isolated amino acid substitutions were replaced using "First approach mode."

Results and discussion

Fluorescence spectra

The fluorescence emission spectra of recombinant C-module of tyrosyl-tRNA synthetase at 20 °C upon excitation at 296 and 280 nm are presented at Fig. 2. The difference between emission spectra on excitations at 296 and 280 nm is due to the contribution of tyrosine fluorescence at excitation wavelength of 280 nm. Tyrosine fluorescence spectrum of C-module was obtained by subtraction of emission spectra determined upon excitation at 280 and 296 nm, which were normalized at 370 nm, and revealed a maximum position at 305 nm. The contribution of tyrosine fluorescence amounts to ~10% of the total fluorescence emission spectrum. The structure of C-module includes a unique tryptophan residue (Trp144) and three tyrosine residues (Tyr27, Tyr111, and Tyr140) [10, 11]. Hence, the intrinsic protein fluorescence emission upon excitation at 296 nm is determined solely by Trp144. On excitation at 296 nm, the fluorescence emission maximum of C-module Trp144 was at 327 nm, the Trp144 quantum yield −0.09, and the Trp144 emission spectrum bandwidth at the half maximal amplitude –49 nm. These data indicate that according to the model of discrete states of tryptophan fluorophores in proteins, Trp144 belongs to the class I of tryptophan residues [16]. According to this model the Trp144 is the internal residue buried into protein globule. The total accessibility of its indole ring to the solvent molecules is very small (about 6%) [16].

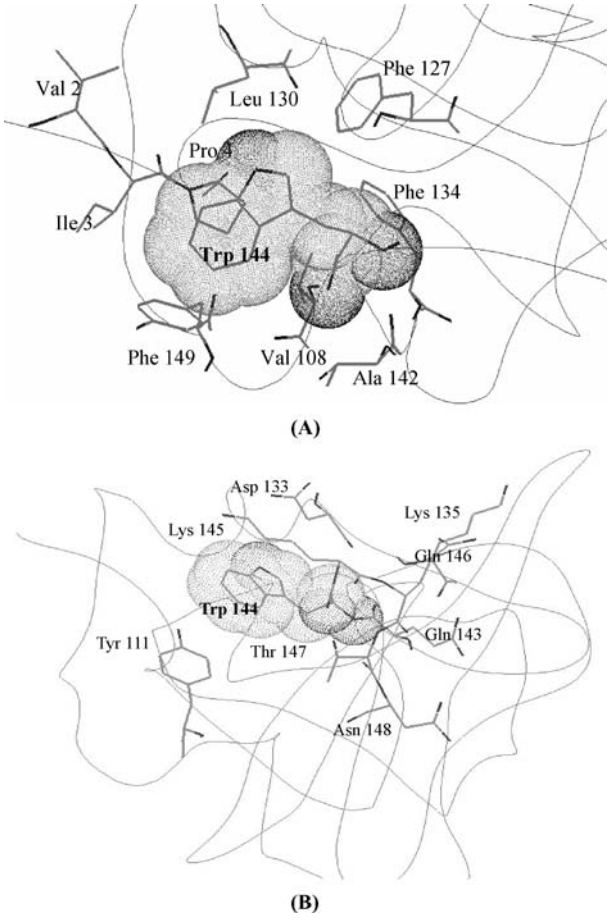


Fig. 3 The hydrophobic (**a**) (Val2, Ile3, Pro4, Val108, Leu130, Phe134, Ala142 and Phe149) and hydrophilic(**b**) (Tyr111, Asp133, Lys135, Gln143, Lys145, Gln146, Thr147 and Asn148) amino acid residues of Trp144 microenvironment

Analysis of Trp144 microenvironment

Independent of spectroscopic studies, the accessibility of Trp144 to the solvent was estimated from the 3D structure model of C-module (Fig. 1B) using *SwissPDB-Viewer 3.7(b2)*. It was found that the estimated value of the total accessibility of Trp144 indole ring was about 1–2% (model cm5). This value is in good agreement with the experimental fluorescence spectroscopy data and indicates buried localization of Trp144 residue in protein globule.

The microenvironment of Trp144 was visualized and analyzed in the 5 Å layer around tryptophan residue (Figure 3). There are 16 amino acid residues in this region, among which the following are hydrophobic (Val2, Ile3, Pro4, Val108, Leu130, Phe134, Ala142, Phe149) and hydrophilic (Tyr111, Asp133, Lys135, Gln143, Lys145, Gln146, Thr147, Asn148). It is important to mention that the side chains of charged residues (Asp133, Lys135 and Lys145) can quench Trp144 fluorescence [17].

Quenching of Trp144 fluorescence by external quenchers

In order to probe the relative exposure of Trp144 in C-module, we used the quenching of protein intrinsic fluorescence by external agents: acrylamide and Cs^+ ions. The quenching efficiency was characterized by the ratio $(\Delta I / I_o)\cdot 100\%$, where $\Delta I = I_o - I$ is the fluorescence intensity decrease in the presence of the quencher concentration at 0.3 M, and I_o is the initial protein fluorescence intensity at the absence of quencher. Fluorescence quenching data for C-module are presented in Fig. 4 as plots of I_o / I versus [\it Q]. No noticeable effect was observed with CsCl indicating the inaccessibility of Trp144 for cationic quencher; and at 0.3 M acrylamide the degree of quenching was $\sim 38\%$. Quenching constant K_q for acrylamide was calculated from the slope of Stern-Volmer plot (Fig. 4), according to Stern-Volmer equation. It gave us the K_q value of 1.7 ± 0.2 M^{-1}.

These results suggest that Trp144 residue is buried in the protein globule, and while being accessible to acrylamide avoids contact with Cs^+. A lack of Trp144 fluorescence quenching with Cs^+ cations may also be due to the unfavorable electrostatic interactions with the positively charged groups of Lys135 and Lys145 laying around the fluorophore.

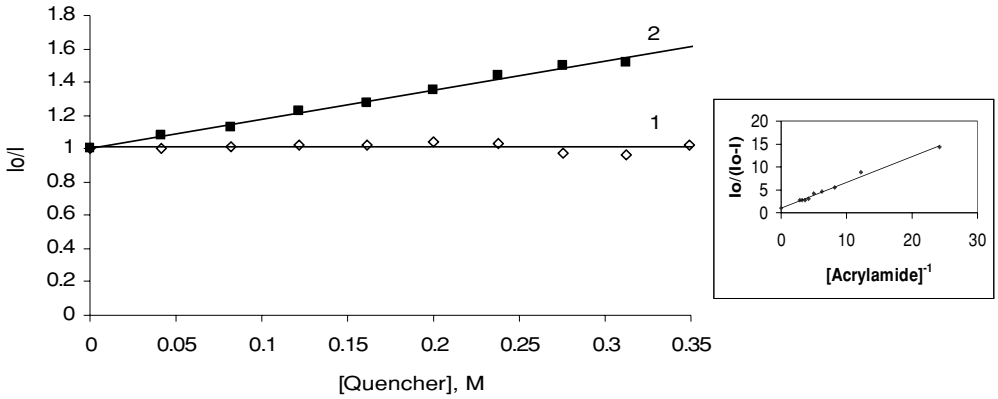


Fig. 4 The Stern-Volmer plot of quenching C-module fluorescence by Cs^+ (1) and acrylamide (2). The inset is the Lehrer plot for C-module

It is known that acrylamide molecules preferably quench the exposed tryptophan residues in proteins, but at the same time acrylamide may penetrate into protein globule. Generally, the amino acids in a static protein structure are tightly packed, however the free space for penetration of acrylamide molecules into the protein structure may be formed as a result of dynamical fluctuations of protein conformation and formation of local cavities.

Temperature-induced conformational change of the C-module Trp144 microenvironment

We used fluorescence measurements of Trp144 emission in the temperature range from 20°C to 60°C to investigate temperature-induced conformational change of tyrosyl-tRNA synthetase C-module. As seen from Fig. 5 and Fig. 6, both the emission maximum and the quantum yield of Trp144 fluorescence reveal considerable temperature-dependent changes which can be divided into two steps. In the temperature range of 20–37°C the Trp144 fluorescence emission maximum shifts by 3 ± 0.3 nm (from 327 to 330 nm at 20°C and 37°C, respectively) into long wavelength region (Fig. 5). These data indicate an increase of Trp144 microenvironment mobility at 37°C comparing to that at 20°C.

In the range from 37°C to 52°C the Trp144 fluorescence maximum was shifted by ∼20 nm into long wavelength region with the significant decrease of the emission intensity. The emission maximum at 52°C was at 348.5 ± 0.5 nm. This indicates that according to the model of discrete classes of tryptophans in proteins, Trp144 belongs to the class III of fully exposed tryptophan residues [16]. Such emission maximum shifts of the tryptophan fluorescence are caused by the conformational rearrangement of the Trp144 environment that leads to the exposure of fluorophore into rapidly relaxing water environment.

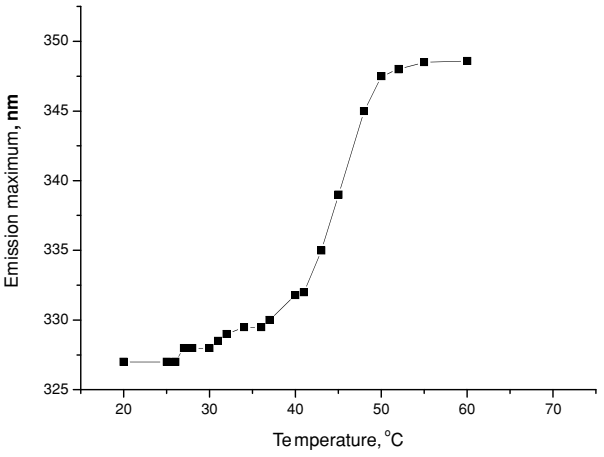


Fig. 5 The temperature dependence of fluorescence emission maximum of C-module in buffer containing 20 mM TrisHCl, 150 mM NaCl at pH 7.5

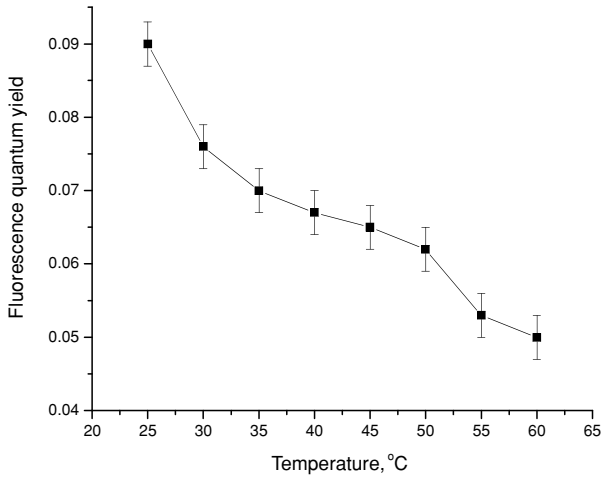


Fig. 6 Fluorescence quantum yield of C-module in buffer containing 20 mM TrisHCl, 150 mM NaCl at pH 7.5 as a function of temperature

Red-edge excitation of the C-module tryptophan fluorescence

We also performed an independent study of the Trp144 microenvironment dynamics by the red-edge excitation fluorescence spectroscopy. Red-edge shifts occur because of long-wavelength fluorophore excitation at the red edge of the absorption spectrum selectively excites fluorophores interacting more strongly with solvent molecules in the excited state. The solvent molecules surrounding these fluorophores are oriented in a way similar to that found in the relaxed-state solvent and the emission from this state will be red-shifted [17–19]. Consequently, the magnitude of the excitation red shifts depends on dynamic properties of fluorophore environment.

We measured fluorescence spectra of C-module in the excitation wavelength range from 290 to 304 nm at 20°C and detected a gradual red-edge shift effect (Fig. 7). These data indicated the absence of the fast structural relaxation of Trp144 microenvironment at 20°C. Hence, the Trp144 microenvironment is rigid at this temperature and the fluorophore is shielded from the rapidly relaxing water molecules. In other words, dipole relaxation time of this environment, τ_r, is smaller than the fluorophore excited-state lifetime, τ_{em}, $(\tau_r < \tau_{em})$. In order to monitor C-module conformational changes we measured the temperature dependence of red-edge excitation fluorescence shift. At higher temperatures the dipole relaxation rate of tryptophan environment will increase because the Trp144 environment mobility enhances. As a result of temperature increase the magnitude of the red-edge excitation shift (λex304-λex290) is expected to decrease. Our experimental data confirm this

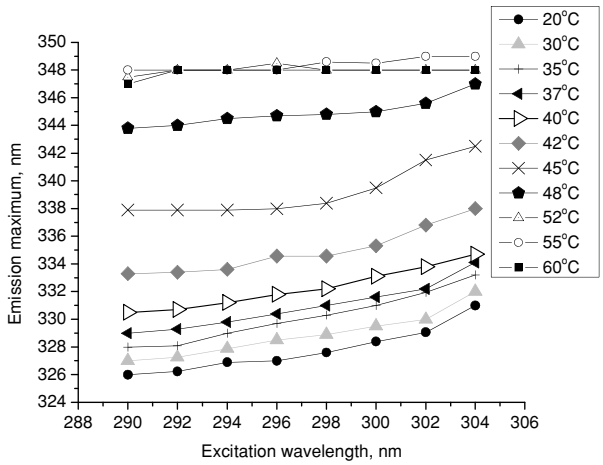


Fig. 7 Fluorescence emission maximum of C-module plotted as a function of excitation wavelength at different temperatures

assumption (Fig. 8). As seen from Fig. 8, when temperature climbed above 37°C the magnitude of the red-edge excitation shift decreased and almost disappeared at 52–60°C. This effect indicates the disruption of Trp144 specific microenvironment in the native globule at high temperatures.

Conclusions

From the analysis of our data acquired from C-module fluorescence spectra, quenching of Trp144 C-module fluorescence and computational analysis of Trp144 microenvironment we can summarize that Trp144 residue of C-module is buried inside the protein globule. At the same time, Trp144 is partially accessible to acrylamide molecules. This fact indicates the penetration of the quencher through fluctuating protein matrix, or in the other words, the presence of fast intramolecular protein dynamics in nanosecond time scale.

Monitoring of temperature-induced conformational changes of Trp144 microenvironment in C-module detects minor conformational changes at 20–37°C which indicate the increase of Trp144 microenvironment mobility in this temperature range. We detected the major conformational transition of Trp144 from buried native state into polar solvent environment at 37–52°C both by emission maximum shift to 348.5 ± 0.5 nm and significant decrease and disappearance of the red-edge excitation shift effect.

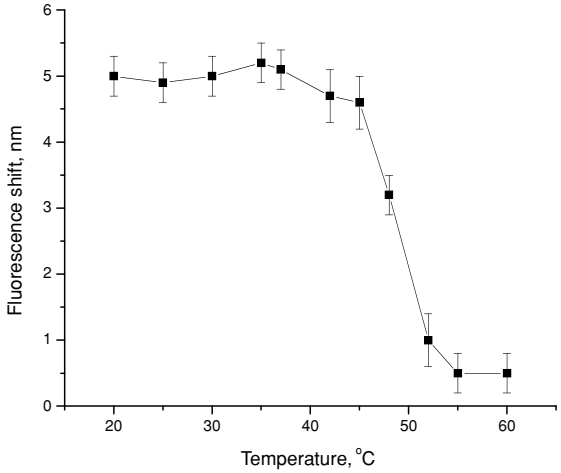


Fig. 8 The influence of temperature on the red-edge excitation shift magnitude of Trp144 fluorescence in C-module

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