Contribution of separate tryptophan residues to intrinsic fluorescence of actin. Analysis of 3D structure

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Received 18 March 1999

Abstract The location of tryptophan residues in the actin macromolecule was studied on the basis of the known 3D structure. For every tryptophan residue the polarity and packing density of their microenvironments were evaluated. To estimate the accessibility of the tryptophan residues to the solvent molecules it was proposed to analyze the radial dependence of the packing density of atoms in the macromolecule about the geometric center of the indole rings of the tryptophan residues. The proposed analysis revealed that the microenvironment of tryptophan residues Trp-340 and Trp-356 has a very high density. So these residues can be regarded as internal and inaccessible to solvent molecules. Their microenvironment is mainly formed by non-polar groups of protein. Though the packing density of the Trp-86 microenvironment is lower, this tryptophan residue is apparently also inaccessible to solvent molecules, as it is located in the inner region of macromolecule. Tryptophan residue Trp-79 is external and accessible to the solvent. All residues that can affect tryptophan fluorescence were revealed. It was found that in the close vicinity of tryptophan residues Trp-79 and Trp-86 there are a number of sulfur atoms of cysteine and methionine residues that are known to be effective quenchers of tryptophan fluorescence. The most essential is the location of SG atom of Cys-10 near the NE1 atom of the indole ring of tryptophan residue Trp-86. On the basis of microenvironment analysis of these tryptophan residues and the evaluation of energy transfer between them it was concluded that the contribution of tryptophan residues Trp-79 and Trp-86 must be low. Intrinsic fluorescence of actin must be mainly determined by two other tryptophan residues – Trp-340 and Trp-356. It is possible that the unstrained conformation of tryptophan residue Trp-340 and the existence of aromatic rings of tyrosine and phenylalanine and proline residues in the microenvironments of tryptophan residues Trp-340 and Trp-356 are also essential to their blue fluorescence spectrum.

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Key words: Actin; Tryptophan; 3D structure; Protein; Protein intrinsic fluorescence

1. Introduction

Intrinsic fluorescence of proteins is widely used to study their structure and dynamics (see e.g. [1–7]). However, the majority of proteins contain more than one tryptophan residue and thus the recorded values are averages. The value of the experimental data increases dramatically when the contribution of separate tryptophan residues can be estimated. To evaluate the contribution of separate tryptophan residues many approaches are used, namely, selective quenching [8–10], selective modification [11,12], selective excitation [13,14], separation of the integrated spectrum into components, assuming that there are a limited number of discrete forms of tryptophan residues [15,16], determination of decay associated spectra [17–19]. At the same time the successful determination of the contributions of individual tryptophan residues to the total fluorescence spectrum of protein, especially if it contains several tryptophan residues, is an exception (e.g. as in the case of lysozyme [11,20–22]) rather than a rule. In practice each of these methods has essential limitations and none of them provides reliable, unambiguous results. So the selectivity of selective modification and selective quenching is usually not high. The disruption of protein structure must not be ruled out using chemical modification. Selective excitation is possible only in special cases [13,14]. The assumption that there is a limited number of discrete forms of tryptophan residues [15,16] is not sufficiently justified in reality [23]. Determination of decay associated spectra also does not solve this problem, because not more than three (according to some authors four) time components can be detected in the fluorescence decay curve. However, some proteins with a single tryptophan residue have just the same number of time components in the fluorescence decay curve [24,25].

The most direct determination of the contribution of individual tryptophan residues to bulk fluorescence is the recording of the fluorescence characteristics of mutant proteins, in which tryptophan residues are mutated [26]. However, such studies are too costly and moreover it is known that even one amino acid change can lead to disruption of proper protein folding.

At the same time the existing concepts on the dependence of fluorescence characteristics of separate tryptophan residues upon their microenvironment allow predicting fluorescence characteristics if the microenvironment of the tryptophan residue is known. It can be done for proteins the structure of which is determined by X-ray analysis up to the co-ordinates of separate atoms. The main principles of the analysis of tryptophan residue microenvironment and peculiarities of their location in the protein macromolecule were developed in the course of analysis of the microenvironment of the single tryptophan residue of azurin [27]. Later it was used in the interpretation of fluorescence characteristics recorded for trypsin, trypsinogen, RNase C2 and some other proteins [27–30].

The intrinsic fluorescence of actin was used for studying its structure [30], conformational changes [31–33], process of polymerization [34], interaction with other proteins [35], etc. The actin macromolecule contains four tryptophan residues. The existence of the 3D structure of actin [36] permits a thorough investigation of the microenvironments of the tryptophan res-

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PII: S0014-5793(99)00574-8

idues. So the aim of this work was to estimate the contribution of separate tryptophan residues to the total fluorescence of actin, and with this example to demonstrate the potential of the proposed approach for other proteins and for the development of the theory of protein intrinsic fluorescence.

2. Materials and methods

Analysis of the characteristic features of the location of tryptophan residues in the actin macromolecule was done on the basis of the atom co-ordinates of actin: the DNase I complex 3D structure (file Pdb1atn.ent in the Protein Data Bank [36,37]). It was taken that the structures of free actin and actin in complex with DNase I are the same.

In this work a special analysis of the peculiarities of tryptophan residue location in protein that can affect its fluorescence characteristics is used [23,27]. In particular, this analysis includes the determination of the conformation of the tryptophan residue's side chain, identification of its nearest neighbors along the polypeptide chain and conformation of the part of the polypeptide chain where it is located. Special attention is paid to the analysis of the microenvironment of the tryptophan residue. The microenvironment of the tryptophan residue is determined as a set of atoms that are no more than r_0 distant from the geometric center of the indole ring. To take into account all atoms that can contact the indole ring, the value of r_0 was chosen as 7 Å [23,27]. For all atoms of the microenvironment the distance from the geometric center of the indole ring and their location relative to the indole ring are determined. The nearest atom of the microenvironment to each atom of the indole ring is specified and the distance between them is determined. One of the characteristics of the microenvironment is the packing density of atoms, that is determined as the number of atoms that compose the microenvironment, or the part of the microenvironment volume occupied by atoms. The volume of each atom is determined on the basis of its van der Waals radius and only the part of the volume that is inside the microenvironment is taken into account. This estimation is of course not exact because in reality atoms are incorporated by chemical bonds and occupy a smaller volume. Nonetheless, this is not significant for a comparative estimation of the packing density of the microenvironment of different tryptophan residues.

It is generally accepted that the fluorescent characteristics of tryptophan residues depend to a great extent upon their accessibility to the solvent. The exposure of a tryptophan residue to the solvent molecules depends not only on the packing density of its microenvironment, but also on its location in the protein macromolecule, i.e. whether it is located near the center of protein macromolecule or at the periphery. To estimate the accessibility of a tryptophan residue to the solvent molecules the dependence of the atom packing density vs. the distance from the geometric center of the indole ring is determined. For this purpose the atom packing density is calculated in sphere layers of identical thickness and different distance from the geometric center of the tryptophan residue.

Though it is considered that the characteristics of a tryptophan residue are mainly determined by its close vicinity, a long range effect cannot be excluded. In particular, the efficiency of non-radiative energy transfer from tyrosine to tryptophan residues and between tryptophan residues is evaluated [38]:

$$W = \frac{1}{1 + \frac{2/3}{k^2}\left(\frac{R}{R_0}\right)^6} \quad (1)$$

where R_0 is the so-called critical Förster distance, R is the distance between the geometric centers of the indole (phenol) rings of donor and acceptor, and k^2 is the factor of mutual orientation of donor and acceptor:

$$k^2 = (\cos\theta - 3\cos\theta_A \cos\theta_D)^2 \quad (2)$$

where θ is the angle between the directions of the emission oscillator of donor and absorption oscillator of acceptor; θ_A and θ_D are the angles between the said above oscillators and the vector connecting the geometric centers of donor and acceptor [39]. In this evaluation R_0 is taken from the literature, while other values are determined on the basis of atom co-ordinates [23,27]. The calculations were done under the assumption of rigid oscillators. In consequence of the uncertainty of donor quantum yield and overlap integral donor fluorescence and acceptor absorption the value of W for Trp-Trp energy transfer was calculated with two values of R_0, 7.8 and 8.7 Å [40,41].

3. Results and discussions

The fluorescence spectrum of native actin is a relatively blue one ($\lambda_{max} = 325$ nm, $A = 2.6$ [31,32]). Only some tryptophan containing proteins such as azurin *Pseudomonas aeruginosa* [42], RNase T1 [43], RNase C2 [29] and parvalbumin merlange [44] have a more blue fluorescence spectrum.

The position and the form of the protein fluorescence spectrum are determined by superposition of fluorescence spectra of its separate tryptophan residues, i.e. by their position in the wavelength scale and their relative contribution to the integrated protein radiation. Actin contains four tryptophan residues. One can try to explain the blue fluorescence spectrum of actin from the analysis of the microenvironments of each tryptophan residue. All four tryptophan residues of actin are located in subdomain 1 [36]. Tryptophan residues Trp-79, Trp-86 and Trp-340 are incorporated in the α-helix formed by Trp-79–Asn-92 and Ser-338–Ser-348. Tryptophan residue Trp-356 is situated in the unstructured region between the α-helices Ser-350–Met-355 and Lys–359–Ala-365 (Fig. 1).

The position of an individual tryptophan fluorescence spectrum in the wavelength scale depends upon the polarity of its microenvironment and its ability to relax during the tryptophan residue fluorescence lifetime (i.e. subnanosecond and nanosecond time scale). The blue spectrum in principle can appear in two cases – if the tryptophan residue is located in the hydrophobic microenvironment independently of microenvironment relaxation properties, or if the microenvironment is rigid even if it is polar. In the latter case the radiation appears from the unrelaxed state. The polarity of the tryptophan residue's microenvironment is determined by the accessibility of the tryptophan residue to the solvent molecules and

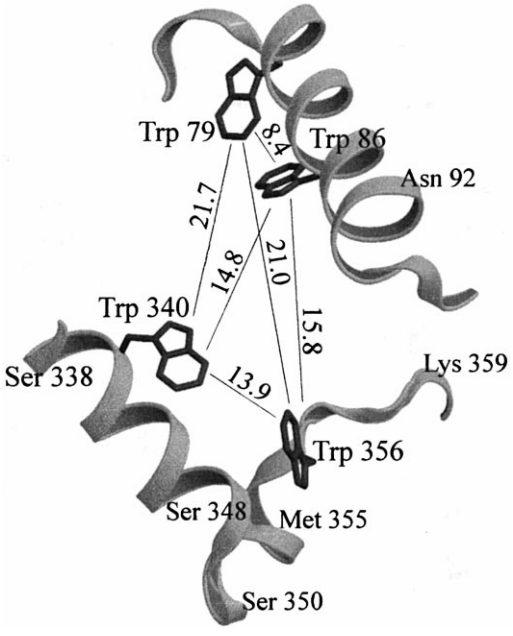

Fig. 1. Location of tryptophan residues in the actin macromolecule. The distances between the geometric centers of the indole rings of the tryptophan residues are given in Å.

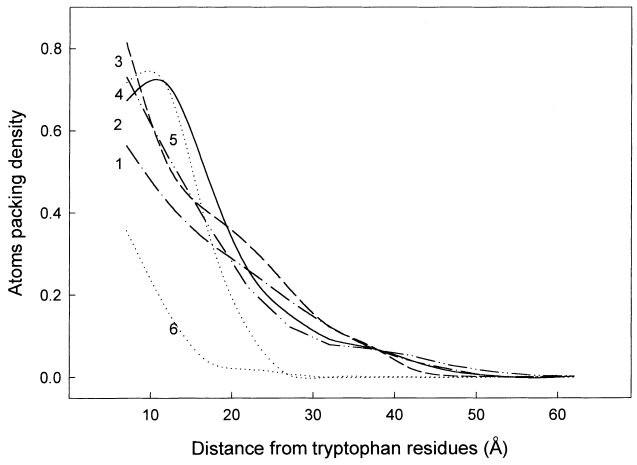

Fig. 2. Radial dependence of the packing density of atoms in the macromolecule about the geometric centers of the indole rings of the tryptophan residues in actin. Curves 1–4 are the dependences for Trp-79, Trp-86, Trp-340 and Trp-356 respectively. For comparison the dependences for Trp-48 of azurin (internal tryptophan residue) and Trp-19 of melittin (external tryptophan residue) are given (curves 5 and 6).

by the existence of polar groups of amino acid side chains of protein in its vicinity.

The analysis of the 3D structure of the actin macromolecule shows that the packing density of the microenvironments of separate tryptophan residues varies greatly (Table 1). Thus in a sphere with a radius of 7 Å, the center of which coincides with the geometric center of the indole ring of the analyzed tryptophan residue, there are 50, 61, 78 and 69 atoms of the protein for tryptophan residues Trp-79, Trp-86, Trp-340 and Trp-356 respectively. For comparison, there are 71 atoms in the microenvironment of the inner tryptophan residue of azurin [27], which has a unique blue fluorescence spectrum ($\lambda_{max} = 308$ nm [42]). Thus two tryptophan residues of actin, Trp-340 and Trp-356, have a very dense microenvironment ($d = 0.84$ and 0.76). And though they are located not in the center of the protein macromolecule, but closer to its periphery (the value of d decreases rapidly with the increase of r_0; Fig. 2), they are apparently inaccessible to the solvent. The packing density of the microenvironment of Trp-86 is lower than that of Trp-340 and Trp-356 (Table 1). At the same time Fig. 2 shows that this tryptophan residue is located far from protein's periphery, and it is evidently also inaccessible to the solvent. So the only tryptophan residue that can be regarded as exposed to the solvent is Trp-79.

The averaged experimental characteristic of tryptophan residue exposure can be obtained by fluorescence quenching by an external quencher [46]. Experiments on tryptophan fluorescence quenching of actin reveal the low accessibility of tryptophan residues to the molecules of the polar quencher acrylamide [47]. The quenching constant determined from the initial slope of the curve ($K = 1.7$ M^{-1}) [47] is practically as low as that for proteins such as RNase T1 ($K = 1.0$ M^{-1}) [48], which have blue, structural fluorescence spectra. The low value of the quenching constant indicates that tryptophan residues of actin are inaccessible to the solvent. It correlates with the very blue fluorescence spectrum of this protein. The blue emission spectrum of actin fluorescence and the low efficiency of fluorescence quenching by acrylamide can be explained if the contribution of tryptophan residue Trp-79 (that is exposed to the solvent) to the protein's intrinsic fluorescence is low.

The contribution of a separate residue in the total fluorescence of the protein is determined by its quantum yield. The quantum yield of tryptophan fluorescence primarily depends upon the existence of quenching groups of the amino acid side

Table 1
Characteristics of microenvironments and conformation of the side chains of tryptophan residues of actin

Conformation of the side chain of tryptophan residues and packing density of their microenvironments					Aromatic rings and proline residues involved in the microenvironment of tryptophan residue		Nitrogen, oxygen and sulfur atoms of amino acid side chains involved in the microenvironment of tryptophan residue			
Residue	N	d	χ_1 (deg)	χ_2 (deg)	Residue	R (Å)	Atom		R_1 (Å)	R_2 (Å)
Trp-79	50	0.60	295	95	Trp-86	6.4–10.6	ND2	Asn-115	5.4	3.8 (CZ2)
							OD1	Asn-115	5.6	4.1 (CZ2)
							NZ	Lys-118	5.2	4.5 (CG)
							SD	Met-119	7.0	4.9 (CH2)
Trp-86	61	0.70	282	325	Trp-79	6.7–8.9	SG	Cys-10	5.9	4.0 (NE1)
					Phe-90	6.2–8.0	ND2	Asn-12	5.2	4.0 (NE1)
					Phe-127	5.1–7.5	OD1	Asn-12	4.5	2.9 (NE1)
							SD	Met-82	5.1	4.9 (CZ2)
							OG1	Thr-89	6.8	4.5 (CD1)
							SD	Met-119	7.0	5.2 (CH2)
							SD	Met-123	5.1	5.1 (CD2)
Trp-340	78	0.84	190	89	Phe-21	8.5–9.4	OD2	Asp-24	6.6	4.6 (CH2)
					Pro-27	3.9–4.8	OG	Ser-344	5.3	3.9 (CZ3)
					Tyr-337	6.8–9.4				
Trp-356	69	0.76	282	115	Pro-102	4.1–6.2	OD1	Asp-3	6.9	5.6 (NE1)
					Pro-130	8.0–9.2	OD2	Asp-3	6.4	5.4 (NE1)
					Tyr-133	5.8–9.0				
					Phe-352	3.5–6.0				

N and d are the characteristics of the density of the tryptophan residue microenvironment (see Section 2); χ_1 and χ_2 are the angles that characterize the conformation of the tryptophan residue side chain [45]; R is the distance of the aromatic rings and proline residues involved in the microenvironment of the tryptophan residue from the geometric center of its indole ring (minimal and maximal values are given); R_1 and R_2 are the distances between the indicated atom and the geometric center of the indole ring and the nearest atom of the indole ring (given in parentheses).

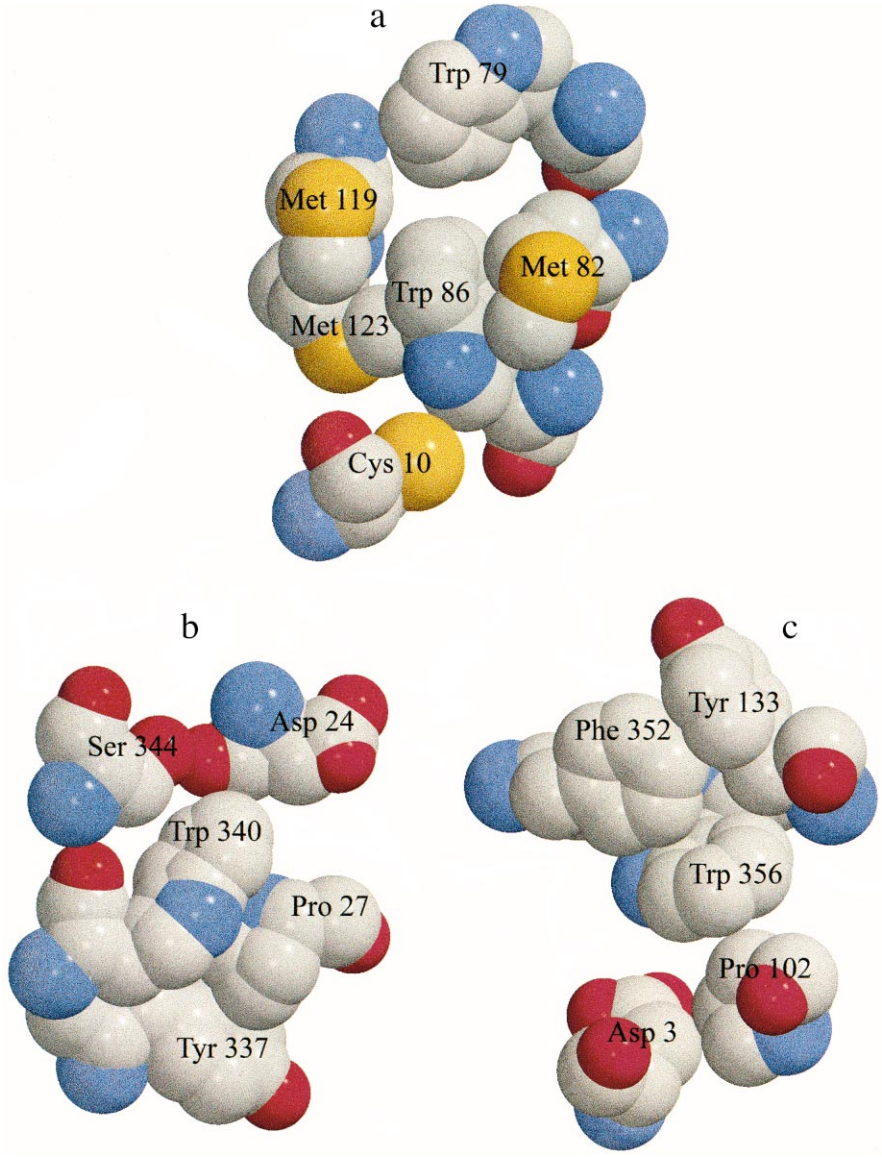

Fig. 3. Peculiarities of the microenvironments of the tryptophan residues of actin. Sulfur atoms of cysteine and methionine residues in the microenvironments of tryptophan residues Trp-79 and Trp-86 (a); aromatic residues and proline residues in the vicinity of tryptophan residue Trp-340 (b) and Trp-356 (c).

chains in its microenvironment and upon the efficiency of Trp-Trp non-radiative energy transfer. The conformation of tryptophan residue side chains, the polarity of its microenvironment and exposure to the solvent also can influence the value of the quantum yield.

The analysis of the actin structure reveals that microenvironments of both Trp-79 and Trp-86 tryptophan residues contain sulfur atoms (Table 1; Fig. 3a), which are known to be effective fluorescence quenchers [49]. These are the sulfur atoms of Met-119 in the vicinity of Trp-79 and the sulfur atoms of Met-82, Met-119, Met-123 and Cys-10 in the environment of Trp-86. The analysis of the dependence of the fluorescent quantum yield upon the peculiar features of microenvironments of tryptophan residues for a number of proteins revealed that the efficiency of quenching depends not only upon the proximity of the quenching group to the indole ring, but, to a great extent, upon the location of this group relative to the indole rings of the tryptophan residues [23]. A number of sulfur atoms in the vicinity of the indole ring of tryptophan residue Trp-86 and especially the immediate neighborhood of SG of Cys-10 to NE1 of the indole ring of Trp-86 (Fig. 3a) make it possible to regard this tryptophan residue as practically completely quenched. The determination of the distances between the geometric centers of the indole rings of tryptophan residues and their mutual orientation reveals the effective non-radiative energy transfer between tryptophan residues Trp-79 and Trp-86 (Fig. 1; Table 2). Consequently, even if tryptophan residue Trp-79 is not quenched by the SD atom of Met-119, it nonetheless must have a low quantum yield due to effective energy transfer to Trp-86. The calculations show that non-radiative energy transfer between other tryptophan residues is of low efficiency (Table 2).

Hence the intrinsic UV fluorescence of the native actin is mainly determined by tryptophan residues Trp-340 and Trp-356, which are practically inaccessible to the solute molecules. The microenvironments of these residues are formed mainly

Table 2
Non-radiative energy transfer between tryptophan residues in actin[a]

Residue	Trp-79	Trp-86	Trp-340	Trp-356
Trp-79		0.64–0.77	0.01–0.02	0.01–0.02
Trp-86	1.6		0.11–0.19	0.00–0.01
Trp-340	2.7	3.4		0.05–0.09
Trp-356	2.8	0.1	1.0	

[a]The table shows the values of the efficiency of non-radiative energy transfer W calculated with two values of R_0, 7.8 and 8.7 Å [40,41] (upper right part of the table) and orientation factors k^2 (lower left part of the table). These values were calculated according to Eqs. 1 and 2 (Section 2). The distances between the geometric centers of the indole rings of tryptophan residues, needed for the evaluation of non-radiative energy transfer, are given in Fig. 1.

by non-polar groups of protein and are closely packed (Fig. 3b,c). Though atoms OD2 of Asp-24, OG of Ser-324 and OD1 and OD2 of Asp-3 are incorporated in the microenvironment (Table 1), they are rather far away from the rings and it is unlikely that they can influence the fluorescent characteristics of these tryptophan residues. The distinctive feature of the microenvironments of tryptophan residues Trp-340 and Trp-356 is the existence of aromatic rings of the tyrosine and phenylalanine residues and rings of proline (Table 1; Fig. 3b,c). So there is phenol ring of Tyr-337 and the ring of Pro-27 in the close vicinity of tryptophan residue Trp-340, and the aromatic ring of Phe-352 and Tyr-133 and the ring of Pro-102 in vicinity of tryptophan residue Trp-356. Similar clusters of aromatic residues were found in proteins with one tryptophan residue, which have a blue fluorescent spectrum such as azurin, ribonuclease T1 and L-asparaginase [23]. The very high packing density of the microenvironment of Trp-340 can be explained by the location of proline residue Pro-27, which has immediate contact with it.

It is noteworthy that tryptophan residue Trp-340 is a t-conformer ($\chi_1 = 190°$, $\chi_2 = 89°$), unlike other tryptophan residues of actin and tryptophan residues of many other proteins [23]. The same conformation of the side chain was found for inner tryptophan residues with an extremely blue fluorescence spectrum, like Trp-48 of azurin and Trp-59 of ribonuclease T1, and for tryptophan residues completely exposed to the solvent, like Trp-25 of glucagon and Trp-19 of melittin [23]. Thus it can be the unstrained side chain conformation. At the same time the microenvironment of tryptophan residue Trp-340 is very closely packed. Its packing density is even higher than that of microenvironments of tryptophan residue Trp-48 of azurin ($d = 0.75$) and Trp-59 of ribonuclease T1 ($d = 0.80$). Furthermore, the oscillations of tryptophan residue Trp-340 are restricted by proline residue Pro-27, whose ring is practically parallel to the indole ring (Fig. 3a). It is not improbable that the unstrained conformation of the side chain of tryptophan residue Trp-340, as well as the existence of aromatic rings of tyrosine and phenylalanine residues and the proline residue, is essential for the formation of the blue fluorescence spectrum of actin.

Acknowledgements: This work was supported by RFBR Grants 96-04-49666, 96-03-40046 and INTAS Grant 94-1289.

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