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THE ENVIRONMENT OF THE TRYPTOPHAN RESIDUE IN *PSEUDOMONAS AERUGINOSA* AZURIN AND ITS FLUORESCENCE PROPERTIES

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Special analysis of the tryptophan residue localization in the structure of the macromolecule of *Pseudomonas aeruginosa* azurin made it possible to prove many explanations in the existing literature of the extraordinary fluorescence properties of this protein, to choose between various contradictory conclusions and in some cases even to make new interpretations of the known experimental data. It has been revealed that the microenvironment of the tryptophan residue is in principle formed by non-polar hydrocarbon groups. The density of the microenvironment is not very high and there are cavities around the ring. The conformation of the side chain of the tryptophan residue is unstrained. These results have been analysed in connection with available data on the unique short-wave fluorescence spectrum position and the existence of the high-frequency indole ring mobility with significant amplitude. Judging by the distance between tryptophan and tyrosine residues and their mutual orientation, the conclusion was made that there is no energy transfer from Tyr 72 to tryptophan and that the efficiency of the energy transfer from Tyr 108 to tryptophan is about 0.5. The mechanism of the dramatic increase in fluorescence efficiency when the copper atom is removed has been discussed with due regard to the fact that the 'blue' copper centre is displaced from the indole ring by more than 10 Å.

1. Introduction

The fluorescence parameters of the single tryptophan residue of azurin from *Pseudomonas aeruginosa* are unusual in many respects and until now have not been unequivocally and completely interpreted. Authors of the studies concerned with the fluorescence parameters of azurin tend to explain the unique short-wavelength position (λ_{max} = 308 nm [1]) of the fine structured [2] fluorescence spectra of this protein by a highly hydrophobic environment of its tryptophan residue. According to Ugurbil et al. [3], the medium which surrounds the tryptophan residue in azurin must be non-polar and have a low polarizability, i.e., it must be hydrocarbon like. Burstein et al. [2], however, consider that the requirement of a strongly rigid environment of the indole chromophore is of equal importance for the existence of such a spectrum. By contrast, time-resolved fluorescence polarization measurements suggest the conclusion that the tryptophan side chain exhibits very rapid oscillations within a large angular range [4]. Recently, the existence of the nanosecond internal rotational mobility of the tryptophan residue in azurin was confirmed by Limkeman and Gratton [5]. In our opinion, the existence of the nanosecond mobility of the side chain of tryptophan residue does not appear to be strange. The mobility of tryptophan residues in highly hydrophobic environments has been proved for some other proteins by means of fluorescence rotational depolarization, using steady-state excitation [6]. Nonetheless, the question of intramolecular mobility in azurin still remains open [7,8].

The mechanism of the influence of the 'blue'

copper centre on the fluorescence parameters of azurin has not yet been elucidated. Removal of the copper ion causes a dramatic increase in fluorescence efficiency [9]. According to later research [10–12] the enhancement in the fluorescence yield of the apo form is 6-fold. At the same time, copper removal is accompanied by no changes in the spectral position [9–12] and leads to insignificant alterations of the mean radiative lifetime [10–12]. In order to explain the quenching of fluorescence by the copper ion in holoazurin, Finazzi-Agrò et al. [1,9] suggested that the tryptophan residue is in direct contact with the copper centre. The proximity of the tryptophan residue and the copper ligands was suggested by Szabo et al. [12]. According to Ugurbil et al. [3,13], copper is a neighbour of the tryptophan residue, though it is not its ligand. At the same time X-ray data presented by Adman and Jensen [14] make the validity of these assumptions doubtful.

There is no consensus of opinion on the participation of tyrosine residues in emission and on

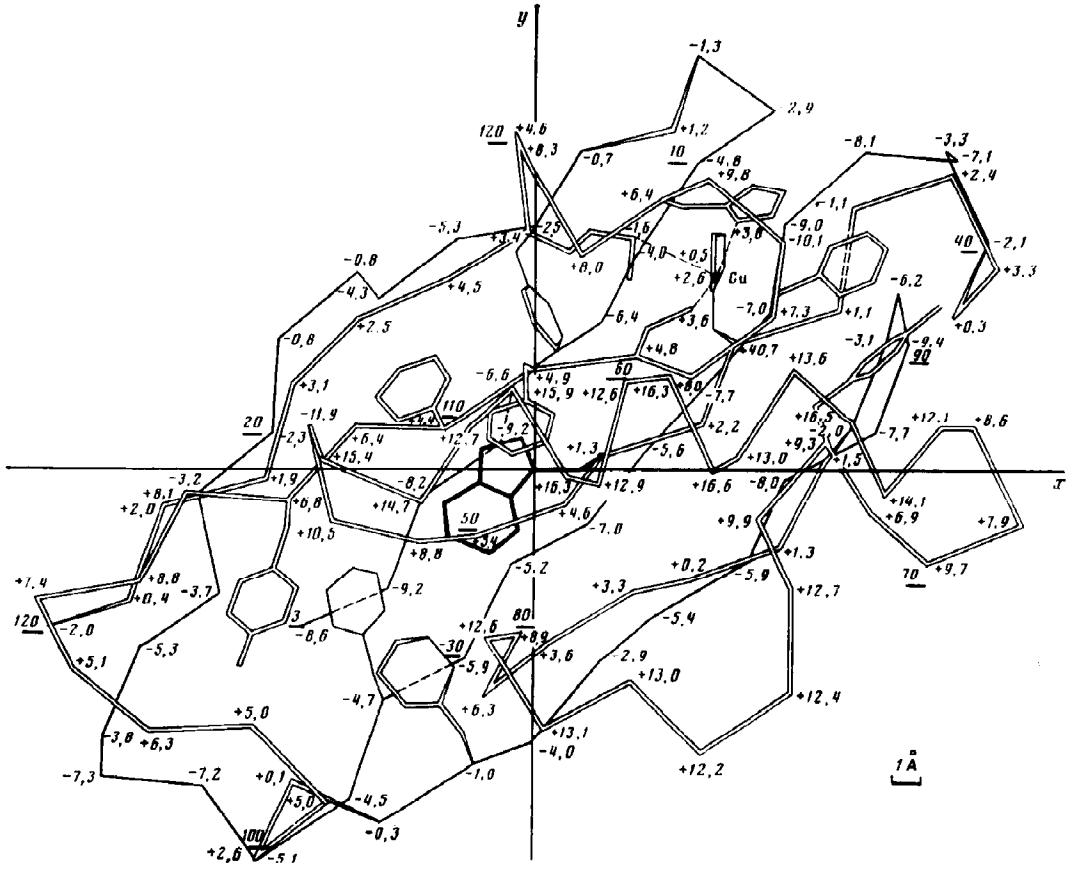

Fig. 1. Structure of azurin in the tryptophanocentric coordinate system. Origin of the coordinates, the point of the localization of the C_γ atom; *OX* axis, the direction of the C_β–C_γ bond; plane *XY*, the plane of the indole ring of Trp 48. Shown are all atoms of tryptophan, tyrosine and phenylalanine residues; all atoms of the residues of the copper centre and only C_α atoms of all others; values of *Z* coordinates for all C_α atoms and the numbers of every tenth residue are given. This and all other figures are based on the data obtained by Adman and Jensen [14] and stored in the Protein Data Bank.

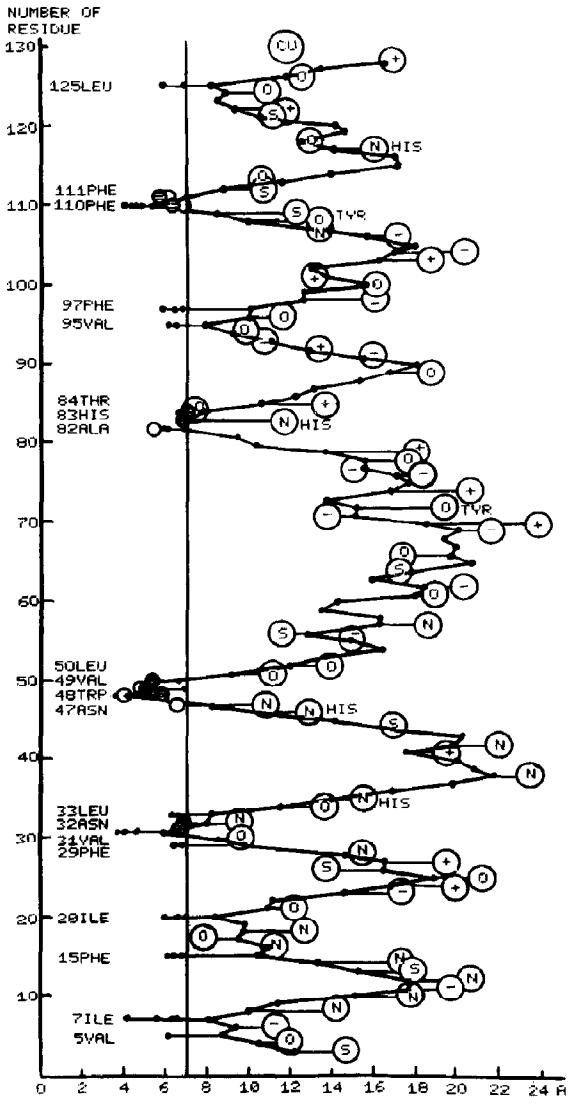

Fig. 2. Distance of the polypeptide chain from the centre of Trp48 of the indole ring in azurin. ⊖, $O_{\delta 2}$ and $O_{\epsilon 2}$ atoms of carboxylic groups of Asp and Glu residues; ⊕, N_{ζ} and N_{H2} atoms of amino and guanidylic groups of Lys and Arg residues; Ⓝ, $N_{\delta 2}$ and $N_{\epsilon 2}$ atoms of amide groups of Asn and Gln residues; Ⓞ, O_{γ} and $O_{\gamma 1}$ atoms of hydroxyl groups of Ser and Thr residues; Ⓢ, S_{γ} and S_{δ} atoms of Cys and Met residues; Ⓞ Tyr, O_{H} atoms of Tyr residues; Ⓝ His, $N_{\delta 1}$ atoms of His residues; ○ and ⊜, O and N atoms of peptide bonds of the microenvironment of the tryptophan residue.

the efficiency of energy transfer from tyrosine residues to that of tryptophan. Burstein et al. [2] reported that there is a complete tyrosine-tryptophan energy transfer. Szabo et al. [12], in contrast, drew the conclusion that there is no tyrosine-tryptophan energy transfer and that the contribution of tyrosine residues to emission is significant.

Determination of the protein structure to a resolution that yields coordinates of definite atoms [14,15] enables us to test the validity of a number of interpretations of the extraordinary fluorescence characteristics of this protein, to choose between controversial conclusions and, sometimes, to give a new explanation of the known experimental data. Descriptions of the protein structure given in crystallographic works [14,15] were not sufficient for this purpose. A special analysis and a transition to various tryptophanocentric systems were therefore urgently needed.

2. Analysis of protein data bank information: Constructing tryptophanocentric drawings of the protein structure

In order to obtain a general idea of the localization of the tryptophan residue in the structure of a protein macromolecule, the proposed analysis of X-ray data ensures the construction of drawings of protein in tryptophanocentric coordinate systems (see, e.g., fig. 1). The diagrams of the distances between the polypeptide chain and the centre of the indole ring of the residue under test (fig. 2) provide information on the localization of polar and charged groups of the side chains, peptide bonds, sulphur atoms of methionine, cysteine and S–S bonds, imidazolic rings of histidine residues, etc., which, as is known [16], can affect different characteristics of tryptophan fluorescence. In order to analyse the peculiarities of the microenvironment of the tryptophan residue, drawings of all atoms (with their van der Waals radii), which are no greater than r_0 distant from the centre of the indole ring, were constructed (fig. 3). To take into account all atoms that can contact the indole ring, the value of r_0 chosen was 7 Å. The determination of distances between each atom of the ring and all

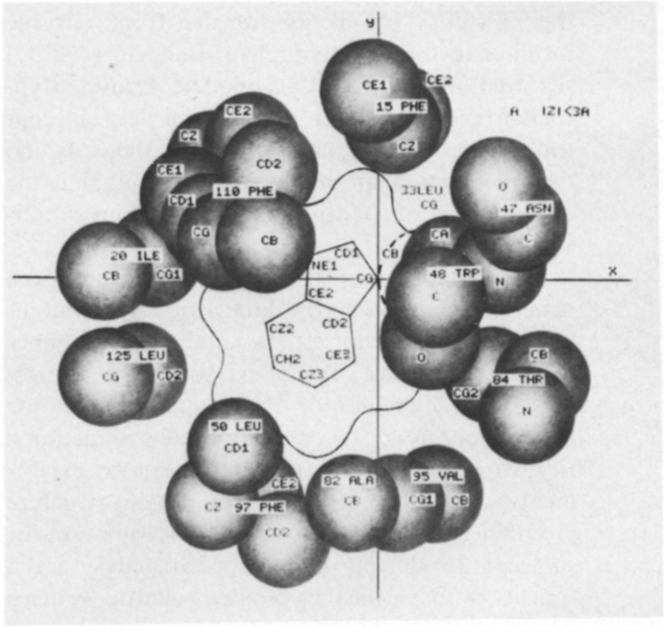

Fig. 3. Microenvironment of the tryptophan residue in azurin. All atoms that are no further than 7 Å from the centre of the indole ring are shown. Van der Waals radii were taken as equal to 1.5 Å. (A) Projection on the XY plane (atoms with $|Z| \leqslant 3$ Å are shown only), (B and C) projections on the plane YZ for atoms with $X < X_0$ and $X > X_0$, respectively (X_0, geometrical centre of the indole ring in the tryptophanocentric system, $X_0 = -1.7$ Å).

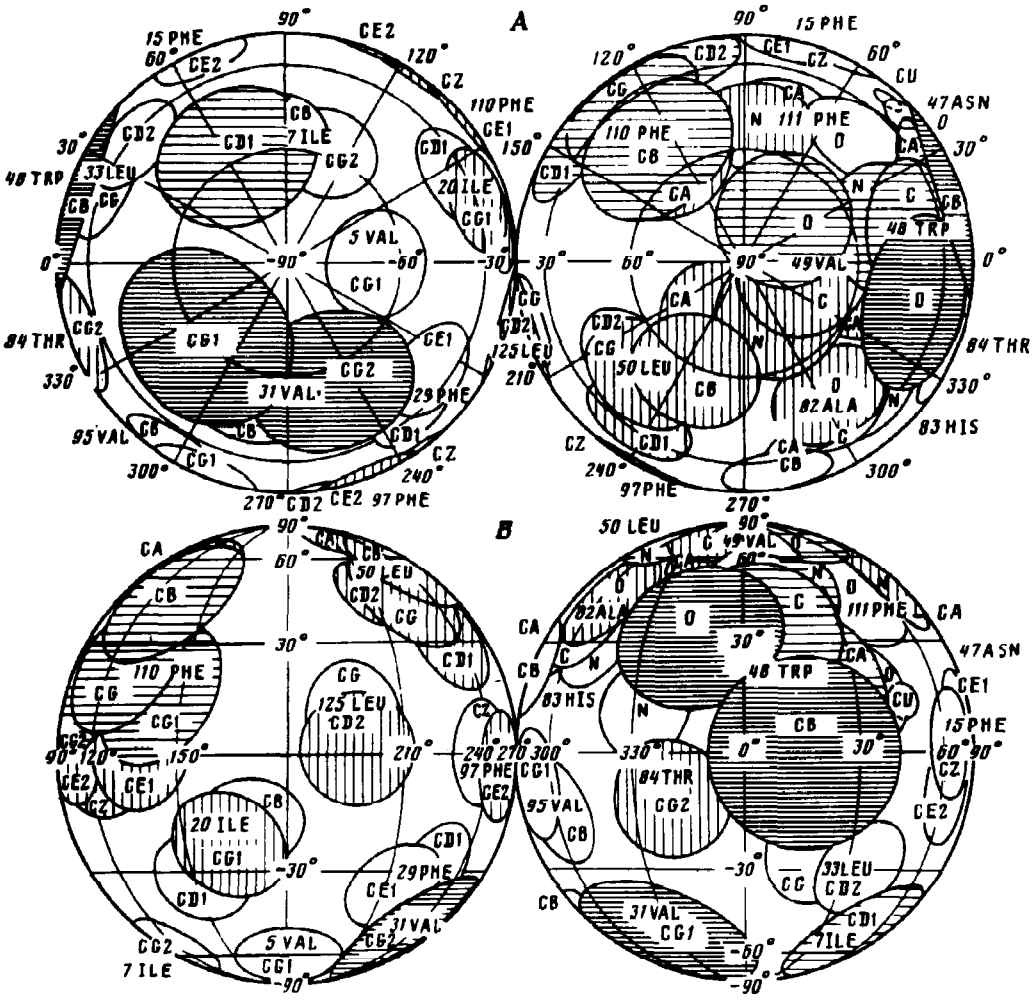

Fig. 4. Drawing of the unit sphere with the centre coinciding with the centre of the tryptophan residue onto which atoms of the microenvironment of the tryptophan residue are projected. (A, B) Transverse and normal orthographical projections. On the network are given the values of the azimuth and polar angles φ and Θ. Equatorial plane, the plane of the indole ring; zero direction of azimuth angle, the direction parallel to the direction of the C_β–C_γ bond. Atoms distances from the centre of the ring of $r \leqslant 4$, $4 < r \leqslant 5$, $5 < r \leqslant 6$ and $6 < r \leqslant 7$ Å are given by different hatching ($r \leqslant 4$ Å, the most dense hatching; $6 \leqslant r \leqslant 7$ Å, unhatched).

atoms of the microenvironment enabled detection of all atoms that have immediate contacts with the indole ring. The proposed analysis also permits the determination of the torsional angles χ_1 and χ_2, which characterize the conformation of the side chain of the residue under test.

Some criteria for estimating the packing density of the atoms of the microenvironment have been determined. They include determination of the number of atoms in the sphere with $r = 7$ Å, and of the part of the volume of this sphere, that is occupied by these atoms; the construction of the orthographical projections of the unit sphere onto which all atoms of the microenvironment are projected (fig. 4). Such evaluations characterize the accessibility of the residue under test to the solvent molecules and the potential ability of the indole ring to participate in intramolecular mobility.

Determination of the distances between the geometrical centres of the ring of tryptophan and

Table 1

Distances between atoms of the microenvironment and each atom of the indole ring of the tryptophan residue in azurin

Data are given for atoms that are no further than 4.4 Å from one of the atoms of the ring, i.e., for the largest distance at which OH, NH, CH, CH_2 and CH_3 groups can make contact with the indole ring via their hydrogen atoms.

Amino acid residue	Atom	Indole ring centre (in Å)	Distance from atoms of indole ring (in Å)								
			C_γ	$C_{\delta 1}$	$C_{\delta 2}$	$N_{\varepsilon 1}$	$C_{\varepsilon 2}$	$C_{\varepsilon 3}$	$C_{\zeta 2}$	$C_{\zeta 3}$	C_{H2}
Ile 7	$C_{\delta 1}$	4.1	3.7	3.4	4.2	3.6	4.2	5.1	5.1	5.8	5.8
Phe 15	$C_{\varepsilon 2}$	6.4	4.9	4.1	6.2	5.1	6.2	7.5	7.4	8.5	8.5
	C_ζ	6.1	4.6	3.7	5.8	4.7	5.8	7.1	7.0	8.1	8.1
Val 31	C_β	4.5	5.2	5.9	4.6	5.6	4.9	4.2	5.1	4.2	4.7
	$C_{\gamma 1}$	3.7	3.9	4.7	3.6	4.6	4.2	3.4	4.7	4.0	4.6
	$C_{\gamma 2}$	4.0	5.2	5.7	4.3	5.0	4.3	4.0	4.1	3.7	3.7
Trp 48	N	5.8	4.0	4.7	5.0	6.0	6.1	5.4	7.4	6.7	7.6
	C_α	4.3	2.6	3.3	3.6	4.5	4.6	4.2	5.9	5.5	6.2
	C	4.2	3.0	3.9	3.5	4.8	4.6	3.8	5.6	4.9	5.7
	O	4.0	3.2	4.3	3.3	5.0	4.5	3.1	5.4	4.2	5.2
	C_β	3.4	1.6	2.6	2.7	3.8	3.8	3.4	5.1	4.8	5.5
Val 49	N	5.0	4.0	4.5	4.4	5.4	5.2	4.7	6.1	5.6	6.3
Leu 50	C_β	5.2	6.3	6.8	5.3	6.2	5.3	4.7	4.8	4.1	4.1
	C_γ	5.2	6.7	7.0	5.5	6.2	5.2	5.0	4.4	4.1	3.7
	$C_{\delta 1}$	5.6	7.3	7.7	6.0	6.8	5.7	5.2	4.7	4.0	3.7
Ala 82	O	5.4	5.7	6.7	5.0	6.8	5.8	4.1	6.0	4.3	5.3
	C_β	6.1	7.1	8.1	6.0	7.8	6.6	4.7	6.3	4.1	5.1
Val 95	$C_{\gamma 1}$	6.1	6.7	7.9	5.8	7.8	6.7	4.5	6.7	4.4	5.6
Phe 97	$C_{\varepsilon 2}$	5.8	7.5	8.2	6.2	7.4	6.1	5.1	5.2	3.8	4.0
	C_ζ	6.5	8.3	8.9	7.0	7.2	6.7	6.0	5.6	4.7	4.4
Phe 110	C_β	4.0	4.6	4.0	4.4	3.3	3.5	5.2	3.6	5.2	4.6
	C_γ	4.3	4.9	3.9	4.8	3.1	3.7	5.8	3.8	5.9	5.0
	$C_{\delta 1}$	4.5	5.5	4.6	5.1	3.4	3.8	6.0	3.5	5.8	4.7
	$C_{\delta 2}$	4.8	4.8	3.6	5.1	3.2	4.1	6.3	4.7	6.6	5.9
	$C_{\varepsilon 1}$	5.3	6.2	5.1	5.9	4.0	4.6	6.9	4.3	6.7	5.6
	$C_{\varepsilon 2}$	5.5	5.5	4.2	5.9	3.8	4.9	7.1	5.3	7.4	6.6
	C_ζ	5.5	6.0	4.8	6.1	4.0	4.9	7.2	5.0	7.3	6.3
Leu 125	$C_{\delta 2}$	5.8	7.7	7.6	6.6	6.2	5.5	6.5	4.1	5.3	4.1

tyrosine residues and the angles characterizing the mutual orientation of their oscillators facilitates an estimation of the orientational factor k^2 and the efficiency of energy transfer Tyr ↔ Tyr, Tyr → Trp and Trp ↔ Trp.

For obtaining the necessary information and carrying out the aforesaid analysis, some programs have been developed. If there are several tryptophan residues in a protein, this analysis should be performed for each tryptophan residue separately; if necessary the same analysis can be made for tyrosine and phenylalanine residues.

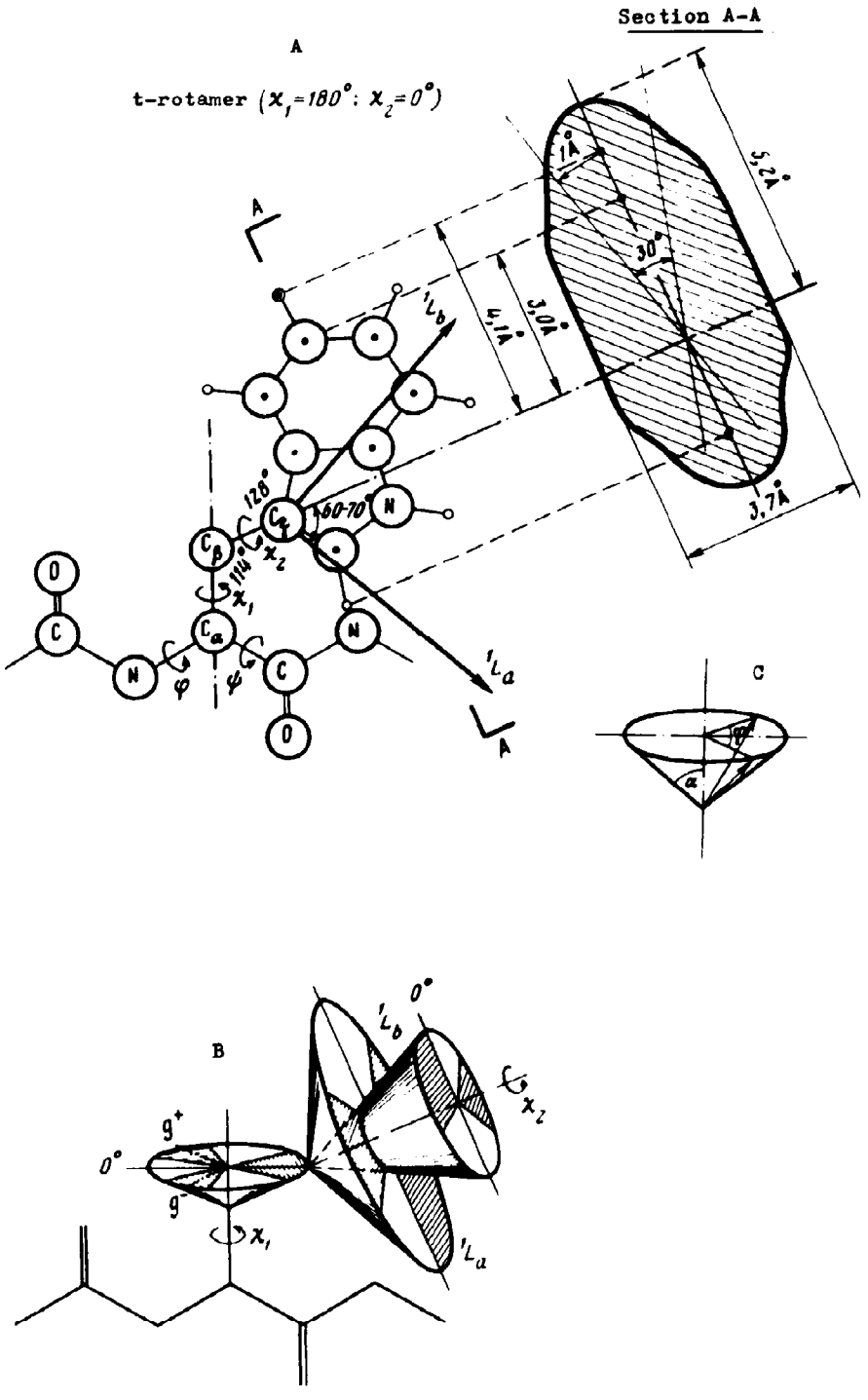

Fig. 5. Illustration of the tryptophan side chain mobility. Explanations are given in the text.

3. Properties of the microenvironment of the tryptophan residue of azurin: Position of the fluorescence spectrum and mobility of the indole ring

The distances between atoms of the microenvironment and each atom of the indole ring of the tryptophan residue of azurin are given in Table 1. With the exception of the two oxygen and two nitrogen atoms of the peptide bonds, the nearest microenvironment of tryptophan residue is formed by hydrocarbon groups. Besides hydrocarbon groups, only the one oxygen atom of the tryptophan residue is in direct contact with the ring. The $C_{\varepsilon 2}$ and C_Z atoms of Phe 15, C_β of Ala 82, $C_{\varepsilon 2}$ of Phe 97 and $C_{\delta 2}$ of Leu 125 contact the ring at its periphery and do not block its rotation around the C_β–C_γ bond. $C_{\delta 1}$ atom of Ile 7, C_γ and C_β of Leu 50, C_β, C_γ and $C_{\delta 2}$ of Phe 110, C_β, $C_{\gamma 1}$ and $C_{\gamma 2}$ of Val 31 come into immediate contact with the plane of the indole ring. High-frequency mobility of the indole ring with a significant amplitude, detected by time-resolved fluorescence anisotropy [4], may be only due to the mutual displacement of these atoms. Munro et al. [4] gave the values of some angles that in the authors' opinion may characterize the amplitude of tryptophan mobility. Analysing the data within the limits of the model of free rotation, the authors determine the angle of the orientation of the emission oscillator about the axis of rotation. But this angle is in fact strictly determined by the rigid orientation of the emission oscillator ^1L_a [17] about the indole ring, and according to Yamamoto and Tanaka [18] and Umetskaya and Turoverov [19], is determined to be 60–70° (fig. 5A). Moreover, there is no doubt that the motion of the tryptophan residue in the protein cannot be free. Fig. 5 illustrates the possible motions of the side chain of the tryptophan residue in the protein – isomerization and torsional oscillations of the indole ring about the C_α–C_β and C_β–C_γ bonds. The areas of torsional oscillations are hatched in section B of fig. 5 according to ref. 22. The geometry of the tryptophan residue is taken from that of Yamane et al. [20]. The length of the covalent bonds, van der Waals radii and the 'thickness' of the indole ring in section A-A (fig. 5) are given according to Pauling and Pauling [21]. The orientation of the oscillators ^1L_a and ^1L_b, that determine the long-wavelength absorption band [17], relative to the direction of the C_β–C_γ bond, is based on the data given in refs. 18 and 19. The most probable mechanism of indole ring motion in the protein that leads to depolarization of the fluorescence is torsional oscillations about the C_β–C_γ bond [8]. The mean value of the azimuth angle φ of these oscillations can be determined on the basis of the equations given in refs. 23 and 24 that connect the amplitude of the high-frequency mobility with the depolarization caused by it. Our calculations, analogous to those made for other proteins [8] and based on the data of Munro et al. [4], show that this angle is about 30°. It may be obtained by joint displacement of the nearest microenvironment atoms by 1 Å. Given below are some arguments showing that such mobility is quite possible.

The distances between the geometrical centre of the indole ring and all polar groups that can influence the tryptophan fluorescence are given in fig. 2. It follows that, no polar groups of the side chains exist in the indole ring microenvironment. Only hydroxyl groups of Thr 17, 30, 84 and Ser 34 and amide groups of Asn 32 are located at a distance of $r < 10$ Å from the indole ring. However, these atoms, except the hydroxyl group of Thr 17, are located in such a way that the indole ring is shielded from them by the nearer non-polar hydrocarbon groups. These data are in agreement with the conclusion that the tryptophan residue has an extraordinarily hydrophobic microenvironment, a conclusion based on the phenomenon of the extremely short-wavelength position of the fluorescence spectrum ($\lambda_{max} = 308$ nm [1]). At the same time, it must be pointed out that among the microenvironment atoms, there are some nitrogen and oxygen atoms of the polar polypeptide groups of the backbone (9 nitrogen and 6 oxygen atoms are located within the radius of 7 Å from the centre of the indole ring, see fig. 2). As mentioned above, some of them are located at a distance of $r < 4.4$ Å from the indole ring. This result is of interest, since we cannot exclude the fact that peptide groups alongside the polar groups of the side chains may affect the position of the fluorescence spectrum and other fluorescence characteristics of tryptophan residues.

In our opinion, the existence of the large hydrophobic region also explains the significant mobility of the tryptophan side chain. Hydrophobic interconnections, in contrast to polar ones, are not paired and the substitution of one connecting group for another does not require much energy. Hence, considerable intramolecular mobility in hydrophobic regions of protein macromolecules can occur. Some experimental results obtained from the rotational depolarization of intrinsic ultraviolet fluorescence [6,8] confirm this assumption.

It is also most likely that the unstrained conformation of the side chain of the tryptophan residue is the condition indispensable to such a short-wavelength spectrum position and, possibly, to the existence of intramolecular mobility with a significant amplitude. Precisely such a case of the conformation of a tryptophan residue occurs in azurin ($\chi_1 = 177°$, t-isomer and $\chi_2 = 73°$).

Orthographical projections of the unique sphere, onto which atoms of the nearest microenvironment of the tryptophan residue are projected (Fig. 4), indicate that the packing of the indole ring microenvironment is not very dense, and that there are cavities around the indole ring. A comparison of the packing of the microenvironment of the tryptophan residue in azurin and other proteins confirms this conclusion. There are thus 72 atoms of protein and 5 atoms of bound water in the microenvironment of Trp 141 of trypsinogen, much more than in the microenvironment of Trp 48 of azurin (69 atoms). The existence of cavities is in agreement with the effect of azurin fluorescence quenching by oxygen, with a quenching constant similar to that of tryptophan residues possessing a much longer wavelength spectrum position [25]. Low constants of azurin fluorescence quenching by KI [26] and acrylamide [25], and also the inaccessibility of the tryptophan residue to water molecules are governed both by a greater size of these molecules, as compared to O_2, and by a much lower affinity of H_2O, KI and acrylamide molecules to the hydrophobic hydrocarbon matrix around the indole ring. The existence of cavities around the tryptophan residue of azurin can be regarded as a factor that favours essential intramolecular mobility in the azurin macromolecule.

4. Non-radiative energy transfer from tyrosine to tryptophan residue

The efficiency of non-radiative resonance energy transfer between donor and acceptor molecules, separated by a distance R, can be evaluated as follows:

$$W = \frac{1}{1 + (R/R_0)^6 \frac{2}{3k^2}}, \qquad (1)$$

which is easy to obtain from the corresponding equations given by Förster [27]. R_0 is the distance between donor and acceptor for which, under the condition of fast chaotic Brownian rotational motion, energy transfer $W = 0.5$. In this case $k^2 = \overline{k^2} = 2/3$, where k^2 is the factor of mutual orientation of donor and acceptor (see, e.g., ref. 28):

$$k^2 = (\cos\Theta - 3\cos\Theta_A \cos\Theta_D)^2. \qquad (2)$$

Here Θ is the angle between the direction of the donor and acceptor oscillators, and Θ_A and Θ_D the angles between the directions of the appropriate oscillator and the vector, connecting donor and acceptor.

When calculating the efficiency of energy transfer from a tyrosine to a tryptophan residue, it was assumed that the oscillator of the long-wavelength absorption band of the tyrosine residue is directed along the symmetry axis of the phenol ring [29]. The direction of the ^1L_a oscillator, which is responsible for the long-wavelength side of the tryptophan residue absorption band [17], makes an angle of 60° with that of the C_β–C_γ bond (fig. 5). Calculations were made in the range of the model of fixed, strictly directed oscillators. The value R_0 was chosen to be the same as for the pair of free tyrosine and tryptophan residues in aqueous solution, i.e., 13–15 Å [30]. The values of R, Θ, Θ_A and Θ_D were determined from X-ray data and those of k^2 and W were calculated from them. All these values are given in table 2. It is evident (see figs. 1 and 3) that the assumption, according to which the indole ring of the tryptophan residue is pressed between the phenol rings of tyrosine residues [2], is not valid. The calculations of W have shown that the conclusion made in ref. 2 about the

Table 2

The calculated values of the parameters that characterize the efficiency of the non-radiative energy transfer from tyrosine residues to the tryptophan residue in azurin

Number of tyrosine residue	R (Å)	k^2	W [a]
72	17.2	0.19	0.05–0.11
108	11.2	0.09	0.26–0.44

[a] Two values of W correspond to $R_0 = 13$ and 15 Å, respectively.

100% efficiency of energy transfer from tyrosine to the tryptophan residue is erroneous. The energy transfer from Tyr 72 is very low. The efficiency of non-radiative energy transfer for the pair Tyr 108–Trp 48 is 26–44%. This result differs from the conclusion about the absence of tyrosine-tryptophan energy transfer in azurin made by Szabo et al. [12], and is close to the evaluation of energy transfer made by Ugurbil et al. [3,31].

It must be pointed out that although the given estimates of energy transfer efficiency reflect the real situation, they are rather approximations for the following reasons: (1) the crystal structure of azurin is assumed to be exactly the same as that in aqueous solution; (2) the donor-acceptor separation vector was determined between the geometrical centres of these molecules, despite the fact that their application points are not known; (3) the accuracy of determination of the direction of ^1L_a in the indole ring is not high; (4) participation of the ^1L_b oscillator of the tryptophan residue in energy transfer is not clear; (5) intramolecular mobility of the tryptophan residue (see above) that may significantly influence the value of k^2 (see, e.g., ref. 28) was not taken into account when the efficiency of energy transfer was determined; (6) it is impossible to define and utilize the real value of the refractive index n of the medium between donor and acceptor, although the probability of energy transfer depends upon it [27]; (7) the quantum yield of tyrosine residues and, consequently, the real value of R_0 is not known. As for azurin the uncertainty of W due to the reason in the above-mentioned point seems to be small, because rough estimates based on the data given by Szabo et al. [12] show that the quantum yield of the tyrosine residue in azurin is similar to that of free tyrosine in solution.

5. The mechanism of quenching tryptophan fluorescence in azurin by its copper centre

The analysis of the X-ray data shows that the distance between the copper ion and the indole ring geometrical centre is 11.8 Å (fig. 2). The copper ligands are also significantly removed from the indole ring: S_γ of Cys 112 and S_δ of Met 121 – at distances of 10.6 and 11.0 Å; $N_{\delta 1}$ of His 46 and 117 – at 11.8 and 13.9 Å; and the geometrical centres of histidine residues – at 12.1 and 15.0 Å. It is quite clear that the suggestion that 6-fold quenching of tryptophan fluorescence in holo-azurin, as compared to its apo form, may be explained by direct contact of the copper ion or one of its ligands with the indole ring [1,9,12] is in conflict with the X-ray data.

Some authors reported a small difference between the absorption spectra of the apo and holo forms of azurin [10,12]. They associated it with the absorption band of the copper centre in this spectral region. However, it must be taken into account that the intensity of this band and, consequently, the value of the overlap integral of the tryptophan (donor) emission spectrum with the acceptor absorption spectrum, determining the probability of energy transfer, is small. As mentioned above, the distance between the tryptophan residue and the copper centre is great enough. Hence, even if the copper centre has an absorption band in the ultraviolet region of the spectrum, the energy transfer does not explain the 6-fold quenching of tryptophan fluorescence in holoazurin.

There are assumptions in the literature that as a result of excitation of the indole ring, charges are redistributed so that a high electron density is localized near one of the atoms and that, under certain conditions, the loss of an electron can occur, thus leading to the quenching of tryptophan fluorescence [16,32]. It might similarly be suggested that the electrostatic field of the positive charge, that is localized on the copper atom, induces the loss of an electron from the indole ring after excitation. There are also examples in the

literature of an electron being removed at a large distance from donor to acceptor molecules [33]. In this connection, it is interesting to mention that the analysis of the X-ray data has revealed that, in spite of the great distance between the copper centre and the indole ring, the latter is practically unshielded from the copper ion by any protein group. This means that there is a long cavity between the tryptophan residue and the copper ion (see fig. 4). In the case of azurin, it is not necessary to assume an electron transfer from donor to acceptor in order to explain the quenching of fluorescence. It would be sufficient to assume a very rapid loss of an electron from the indole ring. Since the mean value of the lifetimes of the apo and holo forms of azurin are very similar, one could suggest that the constant of the loss of the electron, accompanying the deactivation of the tryptophan residue, is very large (the loss is practically instantaneous). The reduction of copper does not affect significantly the quantum yield of azurin fluorescence [11]. Thus, the loss of the electron from the excited indole ring also seems to occur when copper bears a single positive charge.

The application of the aforesaid X-ray data analysis to a great number of proteins with a known structure will provide the opportunity to check, make more precise and sometimes even to revise present concepts of interconnections of the protein intrinsic ultraviolet fluorescence characteristics and the properties of a particular tryptophan, as well as of a certain tyrosine or phenylalanine, residue microenvironment. This work is now being carried out in our laboratory.

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