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On the excited-state energy transfer between tryptophan residues in proteins: the case of penicillin acylase

Sebnem Ercelen[a], Dilek Kazan[a], Altan Erarslan[a,b], Alexander P. Demchenko[a,c,*]

[a] *TUBITAK Marmara Research Center, RIGEB, Gebze-Kocaeli 41470, Turkey*
[b] *Istanbul Technical University, Faculty of Science and Letters, Department of Molecular Biology and Genetics, 80626 Maslak, Istanbul, Turkey*
[c] *A.V. Palladin Institute of Biochemistry, Kiev 252030, Ukraine*

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Abstract

The problem, whether excited-state energy transfer occurs between Trp residues in a multi-tryptophan proteins and if it does, what kind of changes it induces in different parameters of protein fluorescence, is currently under active investigation. In our previous paper [Biophys. Chem. 72 (1998) 265], the energy transfer was found and studied in detail for Na,K-ATPase. It was shown that this transfer influences all parameters of fluorescence emission, which is detected at site-selective conditions (red-edge of excitation, blue and red edges of emission). Present experiments were performed on unusually tryptophan-rich protein, bacterial penicillin acylase (28 Trp per dimer of 82 kDa) and were aimed to extend these observations. They demonstrate substantial heterogeneity in the environments of tryptophan residues within the protein structure. This suggests, that in the present case, if the energy transfer exists, it should be directed from short-wavelength-emitting to long-wavelength-emitting tryptophan residues and thus could be easily observed by a number of time-resolved and steady-state fluorescence techniques. Unexpectedly, no signature of inter-tryptophan energy transfer was found.

Keywords: Protein fluorescence; Tryptophan environments; Excited-state energy transfer; Steady-state and time-resolved spectroscopy; Penicillin acylase

* Corresponding author. Tel.: +90-2626412300; fax: +90-2626412309.
E-mail address: dem@rigeb.gov.tr (A.P. Demchenko).

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1. Introduction

Fluorescence spectroscopy is one of the most popular methods in the studies of protein structure and dynamics, which is applicable to a wide variety of protein molecules and their complexes [1–4]. The experiments are focused on establishing the correlations between the parameters of fluorescence emission and structural or dynamic properties of proteins. Meanwhile, most of them have been performed on single-tryptophan proteins or genetically engineered single-tryptophan mutants [4–6]. This allows to simplify the parameters of tryptophan emission and to exclude the effects of positional heterogeneity of fluorophores. Application of fluorescence spectroscopy to multi-tryptophan proteins rises an additional important question. Is it always possible to consider fluorescence spectra, anisotropies and excited-state lifetime distributions additively, as a superposition of these parameters belonging to individual Trp residues?

The excited-state reaction, which results in the loss of such additivity, is well-known. It is the excited-state energy transfer (ESET) which allows one chromophore (donor) to absorb the energy of a light quantum and to transfer it to another chromophore (acceptor) which then can emit fluorescence [7,8] (see also ref. [2]). Since the energy transfer develops as a function of time, it may induce temporal effects in different parameters of fluorescence. The manifestation of ESET is easy when the donor and the acceptor are different chromophores with separated absorption and emission spectra. However, it is not the case for tryptophan residues in proteins, which may serve as both donors and acceptors of excited-state energy. Since fluorescence spectra of individual tryptophans in proteins are not well resolved, the ESET effects are not easily observed in these spectra and in spectral dependence of time-resolved emission decays. Moreover, in the studies of emission anisotropy they cannot be easily distinguished from rotational mobility effects [1,2]. This is the reason why the problem of Trp–Trp transfer in multi-tryptophan proteins is avoided by the majority of researchers.

The dipole–dipole inductive resonance mechanism of energy transfer [7,8] predicts, that the main factors on which the efficiency of transfer, E, depends are the sixth power of inter-chromophore distance and the overlapping integral between emission spectrum of the donor and absorption spectrum of the acceptor. Due to significant Stokes shift of Trp emission (separation of absorption and emission spectra), the Trp–Trp transfer is not observed in dilute amino acid solutions, but when these residues become proximal in a three-dimensional structure of a protein, it may become substantial. The estimates [9] show that effective energy transfer distance R_0 (the distance at which $E = 0.5$) between Trp residues in proteins may vary between roughly 7 and 12 Å depending on the polarity of Trp location and their quantum yield of fluorescence. The ESET should be higher if these residues are located in hydrophobic environments and their fluorescence spectra are at shorter wavelengths. Since Trp residues in different positions in protein structure as ESET donors and acceptors are usually not equivalent, their positional heterogeneity may result in a number of specific effects in spectral, polarization and time domains. These effects are especially pronounced at site-selective excitation and emission conditions, 'red edge' of excitation spectrum and 'blue' and 'red' edges of the spectrum of emission [2,10–12].

Recently, we performed a detailed fluorescence study of Na,K-ATPase, a membrane protein containing 16 Trp residues [13]. In line with predictions based on the models of ESET between identical chromophores in heterogeneous environments [2,10–12,14], we observed prominent features of this phenomenon: a strong variation of fluorescence lifetime distribution over fluorescence spectrum (with the appearance of negative exponential at longer wavelength edge); dependence of this effect on excitation wavelength; and the fast and emission-wavelength dependent decay of anisotropy. Since these effects were best pronounced at low temperatures, we excluded in low-temperature experiments the involvement of other excited-state reactions-dynamics of dielectric relaxations and intramolecular rotations of protein groups. Thus, the fact, that ESET can be essential in multi-tryptophan proteins and can

significantly influence their emission parameters, seems to be established. This arises a new question, how general is this phenomenon?

In the present study, we have focused on investigation of a protein with unusually high density of Trp residues, Penicillin acylase from *Escherichia coli*. This protein contains 28 Trp per heterodimer of molecular mass of 82 kDa (two chains consisting of 209 and 566 amino acids) [15]. Unexpectedly, we obtained the result which is quite different from that of Na,K-ATPase, no signatures of excited-state energy transfer have been detected.

2. Materials and methods

2.1. The protein

Penicillin acylase (penicillin amidohydrolase, EC 3.5.1.11) is the protein which is used on industrial scale for the production of 6-aminopenicillanic acid, the starting material for the synthesis of semi-synthetic penicillins. The protein used in our experiments was isolated from a chemical mutagenesis mutant of *E. coli* ATCC 11105 [16]. The cultivation conditions, isolation procedure for penicillin acylase, control for its purity and determination of enzyme activity were described elsewhere [16,17]. The specific activity of enzyme was 62 U/mg.

Protein concentration was determined using the data on molecular mass (82 kDa) and the molar extinction coefficient at 280 nm, $\varepsilon_{280} = 197\ 000\ M^{-1}\ cm^{-1}$. The latter was calculated from known tyrosine and tryptophan content (31 Tyr and 28 Trp) by the method described in ref. [2], according to the formula:

$$\varepsilon_{280} = 1250a + 5640b \quad (1)$$

where a and b are the contents of tyrosine and tryptophan in a protein, respectively.

All the experiments (except those for which the experimental conditions are indicated in the text) were performed in 50 mM phosphate buffer at pH 6.0.

2.2. Steady-state spectroscopic measurements

Fluorescence emission spectra were obtained by using the Quanta Master spectrofluorimeter (Photon Technology International, Canada) operating in quanta counting mode. The slits of excitation and emission monochromators were adjusted manually in the range 1–5 nm. The ratio of fluorescence intensities at the slopes of the spectra, I_{320}/I_{365} were obtained using the facility of this instrument to produce measurements of fluorescence intensity automatically at two wavelengths alternatively with automatic recording of their ratio. All the measurements were made in thermostated cell holders.

Fluorescence quantum yield was calculated using tryptophan solution in water with quantum yield 0.14 as a reference. The common method [18] was used consisting of calculating the ratio of the area under fluorescence spectra of protein sample and of the reference. The spectra were recorded on a wavenumber scale and their area were calculated using the Felix software of Quanta Master instrument. The absorbance values for penicillin acylase and tryptophan were equalized to 0.1 at 290 nm and their spectra were recorded at excitation 290 nm and essentially the same instrumental conditions. At this wavelength the correction for absorbance of tyrosine was unnecessary.

All the light absorption measurements were made on Cary 3 BIO spectrophotometer (Varian, Australia). Derivative spectra were recorded using software supplied by the manufacturer.

2.3. Steady-state fluorescence anisotropy

Steady-state anisotropies as a function of excitation (λ_{ex}) or emission (λ_{em}) wavelengths were obtained from the measured components of polarized fluorescence intensities using Quanta-Master instrument operating in L-format mode by manual rotation of excitation and emission polarizers which were supplied by the manufacturers. The anisotropy values were obtained from the equation:

$$r = \frac{I_{vv} - GI_{vh}}{I_{vv} + 2GI_{vh}} \quad (2)$$

where G is a correction factor defined as:

$$G = \frac{I_{hv}}{I_{hh}} \quad (3)$$

Here I_{vv}, I_{vh}, I_{hv} and I_{hh} are the polarized fluorescence intensities. The first and second subscripts refer to the orientation (vertical or horizontal) of the excitation and emission polarizers, respectively.

2.4. Time-resolved fluorescence measurements and data analysis

Fluorescence intensity and anisotropy decays were obtained on the experimental set-up installed on SB1 window of the synchrotron radiation machine Super-ACO (Anneau de Collision d'Orsay). Details of the measurement and data analysis were described in ref. [13] and references cited therein. Briefly, the excitation wavelength (band width 4 nm) was selected by a double grating monochromator. The time-correlated single-photon counting technique was used to record two polarized components of the decay, $I_{vv}(t)$ and $I_{vh}(t)$. Time resolution of the data acquisition system was approximately 20 ps and for each polarized component of fluorescence decay a total number of $2-4 \times 10^6$ counts have been collected. The fluorescence intensity decays $T(t)$ were reconstructed from polarized intensity decays by adding the parallel and twice perpendicular components with the account of the correction factor.

Analysis of fluorescence intensity decays $T(t)$ as sums of exponentials were performed by the Maximum Entropy method [19,20]. The method consists in the recovery of the lifetime distribution $\alpha(\tau)$ from $T(t)$ function

$$T(t) = I_{vv}(t) + 2\beta_{corr} I_{vh}(t) = \int_0^\infty \alpha(\tau)\exp(-t/\tau)d\tau \quad (4)$$

by maximizing the entropy function S in $\alpha(\tau)$ domain [19].

The mean excited state lifetime $\langle\tau\rangle$ is calculated as:

$$\langle\tau\rangle = \frac{\sum_j c_j \tau_j^2}{\sum_j c_j \tau_j} \quad (5)$$

where c_j is the fractional intensity of a component with lifetime τ_j.

Time-resolved decay of anisotropy was calculated from polarized emission decay components $I_{vv}(t)$ and $I_{vh}(t)$ using Eq. (2). It was analyzed by maximum entropy deconvolution into two components:

$$r = r_0[\beta\exp(-t/\theta_1) + \beta_2\exp(-t/\theta_2)] \quad (6)$$

where r is determined by Eq. (2), r_0 is the initial value of anisotropy, which decays in the course of two processes characterized by fractional amplitudes β_1 and β_2 and anisotropy decay times θ_1 and θ_2.

2.5. Trp–Trp distance measurement

The distance between Trps in penicillin acylase were measured using a computer program which was prepared for this propose. Three-dimensional co-ordinates of each atoms in every trp which were obtained from SWISSPDB were used for calculating mass center position of Trps. Mass centers of each trp were used as reference points in calculating the distances. They were measured by the program FORTRAN77 and sorted starting from the smallest values. In addition, relative orientation angels of trps which is also a criteria for energy transfer were calculated and taken into account. In our approximation the angels between Trp indole rings are the angels between the vectors located in the ring planes and oriented from C_2 to C_5 atoms.

3. Results

3.1. General characteristics of fluorescence emission of penicillin acylase

Fluorescence spectrum of penicillin acylase is characterized by a maximum at 334 ± 1 nm (Fig. 1), which is common for tryptophan emission of proteins. According to Burstein's classification [21,22], the spectrum with this position can be assigned to Class I of tryptophan fluorophores, which are thought as being in hydrophobic environments. It was shown, however, that the spectral shifts to 330 nm and below can be easily achieved even in highly polar solvents by just decreasing the temperature and thus decreasing the rates of dielectric relaxations of tryptophan environments [2,20,23,24]. This suggests the possibility to achieve the short-wavelength position of fluorescence spectra in relatively polar but low mobile protein sites [2,25]. Recent calculations of Callis [26] suggest that significant effect on this position can be produced by protein local electrostatic fields. The Burstein's classification is based on reference spectra of single-tryptophan proteins. Class I is characterized by the maxima at 330–332 nm and the spectral bandwidth at half-maximum height 48–50 nm. In our case, we observe the spectrum to be much broader (59–60 nm), which suggests a significant spectral and structural heterogeneity.

Penicillin acylase is characterized by a relatively high quantum yield of tryptophan fluorescence, 0.11. This demonstrates that the fluorescence emission is not significantly quenched compared to tryptophan derivatives in solutions and suggests that the excited state lifetime should be of the order of several nanoseconds.

3.2. Derivative absorption spectra

In order to characterize in more detail the heterogeneity of Trp environments, we analyzed the absorption spectra of penicillin acylase by recording their 1st and 2nd derivatives. The results are presented in Fig. 2. Since the contribution of Tyr absorption into $d\varepsilon/d\lambda$ and $d^2\varepsilon/d\lambda^2$ values at wavelengths longer than 290 nm is negligibly small [2], the derivative spectra in this wavelength range can be compared with that of tryptophan in solutions. They can characterize the broadening and shifts of Trp absorption spectra in proteins.

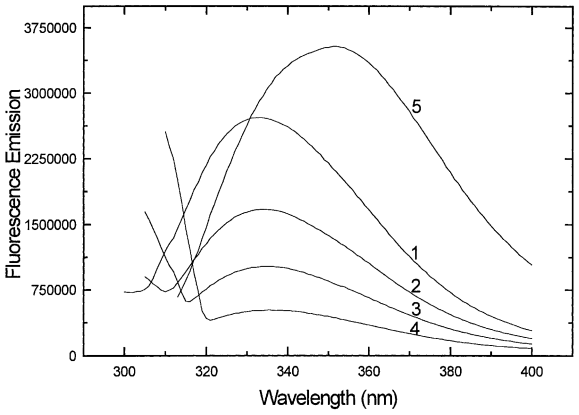

Fig. 1. Fluorescence emission spectra of penicillin acylase as a function of excitation wavelength. The spectra 1–4 are at excitation wavelengths 290, 295, 300 and 305 nm, respectively. The spectrum 5 for tryptophan in solution excited at 290 nm. $E_{290} = 0.1$, temperature 25°C.

We observe that in the first derivative the major negative peak is shifted from 290.5 to 294 nm, it is broader and smaller in amplitude by 2.5 times. In the second derivative, the long-wavelength positive peak shifts from 292.5 to 295 nm and exhibits three-fold decrease in intensity. Its major negative peak being 288 nm for tryptophan and 292 nm for protein shifts, with intensity decrease by 3.8 times. This result demonstrates the substantial shift of the parent absorption spectrum of penicillin acylase to longer wavelengths (by ~ 3–4 nm) and the increase of its width. The absorption spectrum, when it becomes broader, loses to a substantial extent its fine structure with an apparent decrease of resolution of its characteristic long-wavelength maximum. This is demonstrated by the fact that for our protein, in contrast to tryptophan, the minimum in the negative part of the first derivative, located at 289 nm, is much less noticeable and even does not reach its positive value.

The relation between the position of absorption spectrum and the polarity of Trp environ-

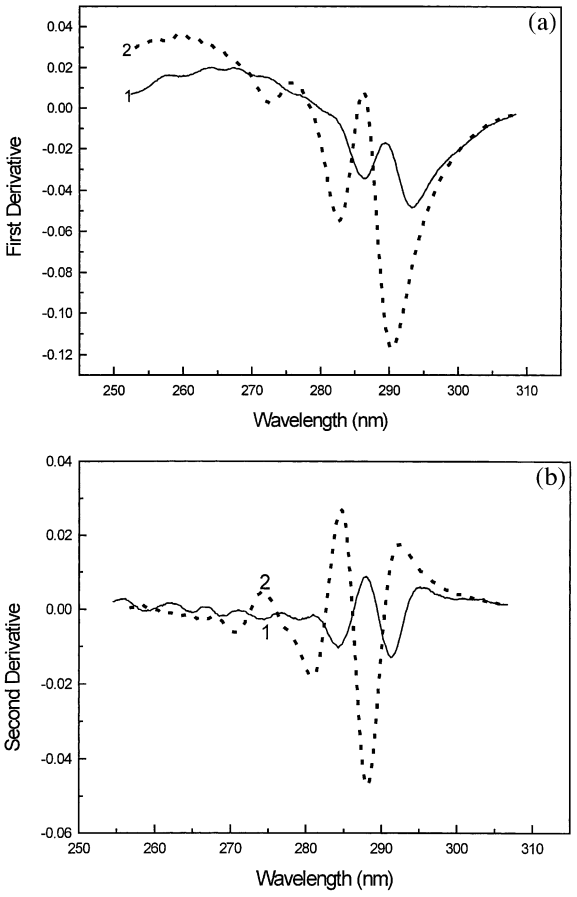

Fig. 2. Comparison of the first (a) and second (b) derivatives of absorption spectra of penicillin acylase with that of tryptophan in solution. Penicillin acylase (solid line), 2-tryptophan (dotted line). $E_{290} = 0.62$, temperature 25°C.

ment in a protein is not simple. In model systems when the tryptophan derivative is dissolved in hydrophobic solvent and the polar co-solvent is added, the absorption spectrum is shifted to longer wavelengths [27] due to formation of hydrogen bonds with imido group of indole ring [2]. However, since in the native proteins these bonds are to a significant extent saturated [28], the electronic polarization of the environment becomes most important and when the protein spectrum is compared with that of tryptophan in water, we always observe its longer wavelength position [2]. In the case of penicillin acylase, this position is highly variable giving rise to substantial spectral heterogeneity.

3.3. Red-edge effects

Red-edge effects are a common name for the dependencies of a number of fluorescence spectroscopic parameters on the excitation wavelength on its shift to long-wavelength edge [2,3,24,25]. They originate from the distribution on electronic transition energy (giving rise to inhomogeneous broadening) and require slow (compared to the excited-state lifetime) relaxations of these media to the change of the fluorophore dipole moment on electronic excitation. For proteins with emission maxima approximately 330 nm the observed long-wavelength shift of fluorescence spectra on red edge excitation (up to 305 nm) is usually significant, 5–8 nm [2,29].

The results presented in Fig. 1 demonstrate that indeed, we observe a significant dependence of fluorescence spectra on excitation wavelength. The maximum shifts to 334 nm at excitation 295 nm, to 335 nm at 300 nm and 338 nm at 305 nm. Thus, the red-edge-excitation shift of fluorescence spectrum is substantial, which witnesses to a slow (on nanosecond time scale) dynamics of the environment of tryptophan residues in this protein.

It may be noted that positional heterogeneity of tryptophan residues, if it is involved, is expected to act in the opposite direction to 'red-edge' shift let the light quanta of low energy excite selectively the tryptophans with absorption maxima shifted to longer wavelengths. Since these tryptophans occupy the positions screened from a polar aqueous environment and are surrounded by electronically polarizable groups, it is expected that their fluorescence spectra should be observed at shorter wavelengths. The resultant excitation wavelength dependence of fluorescence spectra may be a compensation of these two effects. It is noticeable that the 'red-edge' shift prevails in the present case.

3.4. Fluorescence quenching by heavy ions: manifestation of emission heterogeneity

Quenching of tryptophan fluorescence by ionic

quenchers (I^- or Cs^+) occurs due to heavy ion effect and requires direct collision of the ions with the fluorophore [30,31]. Therefore, for being quenched, the indole rings of Trp residues should be located at the molecular surface. In line with the previously published data [32], we observe that fluorescence emission of penicillin acylase is substantially quenched by ionic quenchers (by approx. 50% by KI and 30% by CsCl at concentrations close to saturating). Quenching is accompanied by short-wavelength spectral shift together with the decrease of the spectrum bandwidth, which indicates the selectivity of quenching towards tryptophans possessing fluorescence spectra at longer wavelengths. For more precise determination of the magnitudes of spectral shifts as a function of the quencher concentration, we determined the correlation between the relative decrease of fluorescence intensity and spectral shift. For increasing the precision in determining the position of the spectral maxima, two methods have been applied. One is the measurement of the ratio of intensities at two points in the regions of maximal slopes of the spectra (double-wavelength method [33]). The change in this intensity ratio is a precise measure of the spectral shift. The other is the transformation of the spectrum into its first derivative and finding the point of its crossing with the baseline. These methods gave identical results and in Fig. 3, we present the data obtained by ratiometric method.

The close superposition of these plots obtained for KI and CsCl as the quenchers suggest that the quenching of fluorescence on interaction with the ionic quenchers should be attributed to the Trp residues, which possess preferentially the long-wavelength-shifted fluorescence spectra. Decrease of their relative contribution on quenching results in the short-wavelength shift of the protein spectrum. It also shows that the Trp selectivity with regards to quenching does not depend substantially whether the quencher possesses positive or negative charge.

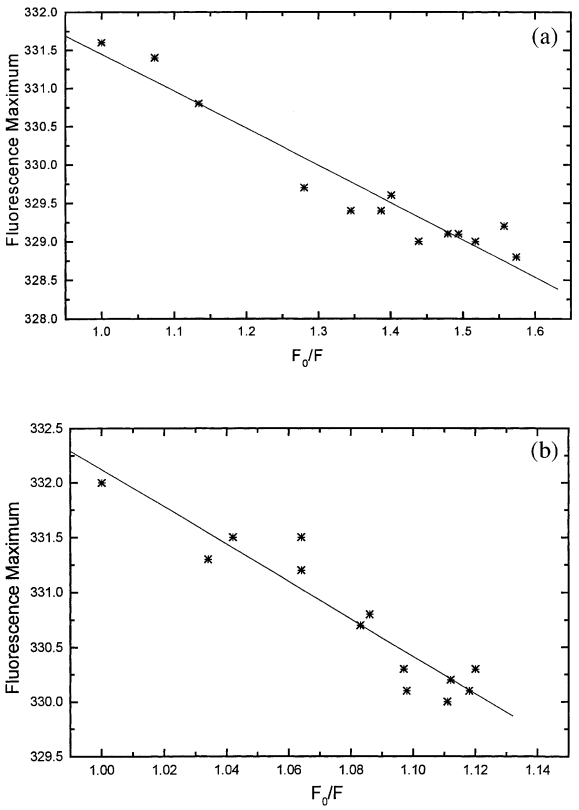

Fig. 3. The relative decrease of fluorescence intensity and spectral shift in the presence of quencher KI (a) and CsCl (b). The abscissa scale is the ratio of fluorescence emission intensities at the band maximum with the absence of quencher (F_0) and in its presence (F). The ordinate scale is the position of band maximum in nm. $E = 0.2$, the concentration ranges of the quenchers, 0–571 mM for both KI and CsCl.

3.5. Fluorescence lifetime distributions as a function of emission wavelength

The excited-state lifetime distributions obtained from the fluorescence decay curves by the Maximum Entropy method demonstrate the presence of four peaks centered at approximately 0.2, 0.8–0.9, 1.6–2.7 and 5–6 ns (Fig. 4). The variation in lifetime distribution at different emission wavelengths is observed. However, the total number of components remains the same and their positions are approximately the same (Table 1). We do not observe the characteristics for ESET features, an additional fast decaying component at short emission wavelength and negative (rising) component at long emission decays. The lifetimes of all four components do not depend significantly on emission wavelength and variation may be due to

Table 1
Positions of the excited-state lifetime components τ_1 (ns) and their relative surface areas (%) for penicillin acylase as a function of emission wavelength λ_{em} (nm). Excitation wavelength 295 nm. Temperature 21.5°C

λ_{em}	τ_1	τ_2	τ_3	τ_4
320	0.24 (31.5%)	0.92 (36.9%)	2.75 (19.6%)	5.90 (12.0%)
330	0.17 (30.1%)	0.89 (36.3%)	2.94 (20.4%)	6.13 (13.1%)
370	0.16 (30.4%)	0.77 (21.4%)	1.60 (24.8%)	5.02 (22.2%)

emission heterogeneity. Regarding relative amplitudes of these components, there is a tendency for decrease with the increase of emission wavelength of the shorter component τ_2 and of increase of the longer components τ_3 and τ_4. It should be mentioned that while we observe a more significant variation in position and intensity of long-lifetime components, which may be the result of heterogeneity of emitters, the first short-lifetime component demonstrates its independence on excitation wavelength.

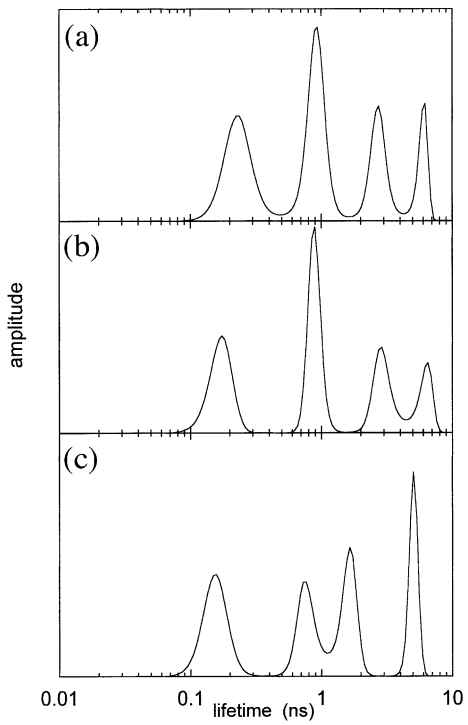

Fig. 4. Fluorescence lifetime distributions recovered by Maximum Entropy Method for penicillin acylase. Emission wavelengths: 320 nm (a); 330 nm (b); and 370 nm (c). Excitation wavelength 295 nm. Temperature 22°C. Chi-square values 0.93–1.03.

The presence in decay of only four sharply resolved components witness for the fact that instead of being broadly distributed, the lifetimes of 28 Trp residues of penicillin acylase are centered around only four most probable values. This result is surprising and needs a more detailed examination. It may add a new light to the discussion on the presence of structurally and spectroscopically discrete forms of tryptohan residues in proteins [22,26]. The presence of strongly dynamically quenched tryptophans with lifetimes shorter than 100 ps are not revealed.

Thus, the relative invariance of lifetime distribution over the fluorescence spectrum and in particular, the absence of differences in sign and number of its shortest component, may be considered as a witness against the inter-tryptophan energy transfer. This picture is entirely different from that observed for Na,K-ATPase [13], where this component at the long wavelength edge changed not only its intensity, but also the sign from positive to negative. This indicated the fast temporal decrease of emission intensity of ESET donors emitting at shorter wavelength and an increase of that of the acceptors emitting at longer wavelengths.

3.6. The studies of emission anisotropy

The dependence of fluorescence anisotropy on excitation wavelength is presented in Fig. 5. The data on penicillin acylase are compared with the data on NATA (*N*-acetyl-tryptophan-amide) in neat glycerol at −46°C [13]. They demonstrate that in the case of penicillin acylase, the characteristic fine structure with the minimum of

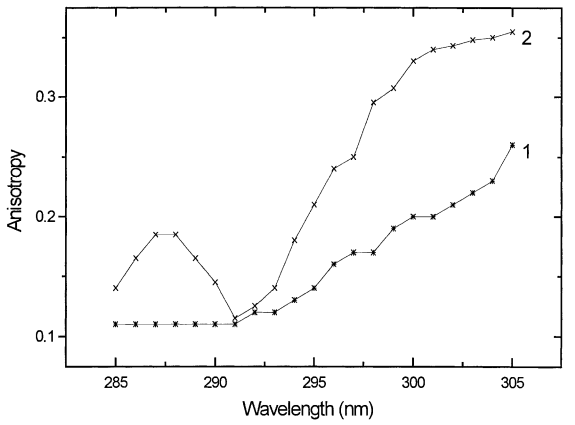

Fig. 5. Steady-state fluorescence anisotropy of penicillin acylase (1) and *N*-acetyl-tryptophan-amide (2) as a function of excitation wavelength. Penicillin acylase in 50 mM phosphate buffer pH = 6.0 at temperature 20°C. 2-*N*-Acetyl-tryptophan-amide in neat glycerol at −46°C. Emission wavelength = 334 nm.

anisotropy at 292 nm is not resolved. This is a clear indication of broad heterogeneity of emitting Trp residues, which is in line with the results of light-absorption spectral measurements and analysis of derivative spectra (see Section 3.2). In our protein, the emission anisotropy excited at the far red-edge (305 nm) does not reach the value observed for tryptophan derivative in rigid environment.

In the case of ESET in rigid environments, the transfer influences the observed anisotropy in a wavelength-dependent manner (the Weber's 'red-edge effect'). A dramatic decrease of emission anisotropy occurs with the excitation at the absorption band maximum, while at the red edge due to the failure of ESET, the anisotropy is increased up to the value that should be observed without transfer [2]. Therefore, our observations are not consistent with ESET model. The estimate of rotational correlation time θ for the whole protein based on spherical model and mol. wt. 82 000 results in the value 25 ns, which is far beyond the excited state lifetime value. Therefore, we can ascribe the lower value of anisotropy over the whole excitation spectrum to some kind of intra-molecular rotational mobility of tryptophans or tryptophan-containing segments.

The results of analysis of time-resolved anisotropy as a function of emission wavelength are presented in Table 2. They confirm the steady-state data. The anisotropy decay component in the time range of emission decay (0.2–6 ns) is negligible. Anisotropy decays on a much longer time scale, longer than 10^{-8} s and because the emission decays much steeper, the rotational correlation time values cannot be determined precisely. Comparison of these results with steady-state data suggests there is a very fast component of anisotropy that is not resolved in our experiment, it may be the local segmental motion.

In the case of homo-transfer, the rapid decay of anisotropy is usually considered as the major indication of ESET [7,8,34] and in model systems of concentrated solid dye solutions the transfer is observed throughout the excited-state lifetime [10]. Therefore, the absence of this decay on the time scale of emission is a strong witness against the inter-tryptophan energy transfer in our system.

Table 2
Parameters derived from analysis of time-resolved anisotropy as a function of emission wavelength, determined by Eq. (6). Excitation wavelength 295 nm. Temperature 21.5°C

Emission Wavelength (nm)	r_0	β_1	θ_1 (ns)	β_2	θ_2 (ns)
320	0.116	0.003	2.6	0.113	16.6
330	0.094	–	–	0.094	12.4
350	0.083	0.001	1.9	0.082	15.03
370	0.035	–	–	0.035	11.8

3.7. Analysis of inter-tryptophan distances

The whole range of 377 distances between all 28 Trp residues was analyzed (Fig. 6).

Most of these distances are outside of the range, which could allow the energy transfer between Trps. Meantime, there are Trps which are located at shorter distances. The cases of direct interaction between tryptophan residues are not detected. In principal, there are possibilities of sequential transfer in a chain of several Trp residues. That is why we selected several Trp–Trp pairs which display the shortest distance between them and analyzed in more detail with the account of their relative orientation (Table 3). Within this range of distances we have one cluster consisting of residues 65, 154, 163, 166 and 179. We have also one chain of closely located trptophan residues 240, 4, 283, 500 and 537. The residues 326, 337 and 180, 258 are represented as localized pairs. The fact, that energy transfer effects are not revealed in our experiments, may be due to insufficient contribution of these clusters to protein total emission. This may occur if one of the members in these clusters is interacting with a strong quenching group and serves a trap of excited state energy.

4. Discussion

4.1. Heterogeneity of tryptophan environments and expected ESET effects

Our data demonstrate that 28 Trp residues of penicillin acylase represent a system with substantial spectral heterogeneity. It produces the broadening of absorption spectrum of approximately 3–4 nm (500–700 cm^{-1}) which is added to common inhomogeneous broadening being of the order of 100 cm^{-1} [12]. This heterogeneity is displayed in fluorescence emission spectra, where we observe a broad bandwidth, the spectral shifts on addition of quenchers and a loss of resolution in excitation wavelength dependence of anisotropy. This behavior makes our system analogous to a concentrated solution of tryptophan fluorophores, for which the theory and results of the model studies demonstrate a number of ESET effects [2]. In these systems, the energy transfer should always be directed and occur from those fluorophores which emit at shorter wavelengths to those which emit at longer wavelengths (see chap.

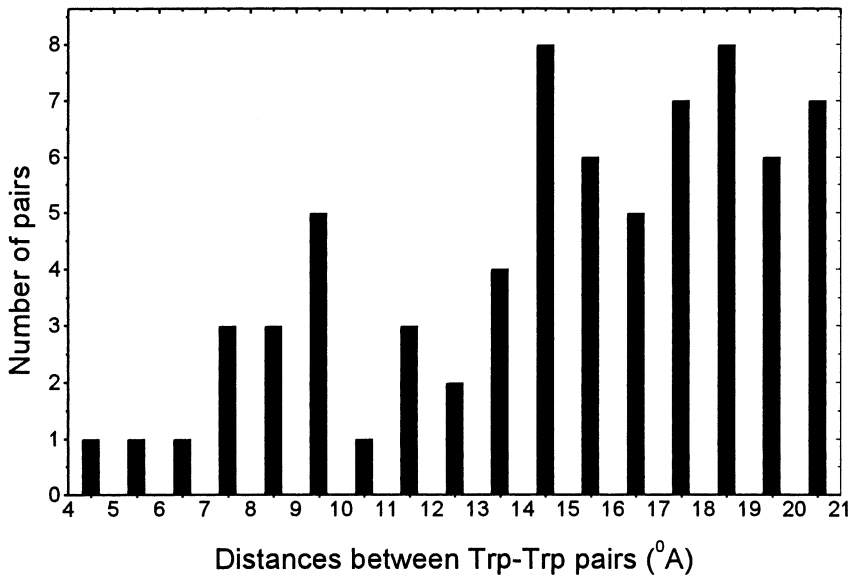

Fig. 6. Distribution of distances in Trp–Trp pairs in penicillin acylase.

Table 3
Distances and orientation angels between 14 closest (separated by less than 10 Å) tryptophan pairs in penicillin acylase. Trp.1 and Trp.2 are numbered according to their amino acid sequence

Trp.1	Trp.2	Distance (Å)	Orientation angels
154	179	4.8824	123.92
166	179	5.6795	125.22
163	166	6.7268	81.198
43	445	7.1858	120.17
65	166	7.5273	126.11
283	500	7.7472	89.114
65	179	8.5238	29.352
326	339	8.7897	14.404
4	240	8.8216	100.38
4	283	9.0869	81.753
65	154	9.0936	152.37
500	537	9.3962	48.368
180	258	9.9033	153.64
154	166	9.9445	53.882

9 of ref. [2] and refs. [10–12,14]). It temporarily depopulates the sub-population of short-wavelength-emitting fluorophores and reduces their lifetimes, while the time-dependent increase of the number of long-wavelength emitters (which are the excitation energy acceptors) should cause a substantial increase of effective lifetimes in the long-wavelength part of fluorescence spectrum. For Na,K-ATPase, this effect was very substantial-from 2 ns at 305 nm to more than 6 ns at 375 nm (at 19°C) [13]. For penicillin acylase we observe that this variation is insignificant. As we showed for Na, K-ATPase, ESET produced dramatic variation of lifetime distribution over the emission spectrum. Most spectacular in this respect are the short-lifetime components, which are strongly pronounced at the short-wavelength edge due to shortened emission of the ESET donors and which change their sign due to time-dependent re-population of the acceptors with fluorescence spectra shifted to longer wavelengths. The absence of significant emission-wavelength-dependent variation of individual lifetime components in the present case of penicillin acylase is a strong witness for the absence of ESET in this system.

The other feature of ESET in heterogeneous systems is the concentrational quenching of fluorescence, which is easily observed in concentrated liquid or solid (glasses, polymers) dye solutions [7]. In these systems the energy is circulating between the fluorophores which are both donors and acceptors of excited-state energy. If even a small amount of light-absorbing but not fluorescent species are present (e.g. dimers and aggregates of dye molecules), they produce dramatic quenching effect by serving the traps (radiationless deactivation channels) on ESET pathways. A number of protein groups being in contact with Trp indole ring may produce quenching effect [22,35] and thus generate these traps. It is known that among differently located Trp residues in proteins the distribution of quantum yield and lifetime values is much broader, than the distribution in positions of fluorescence maxima. Thus, the quantum yields vary within broad ranges from almost zero to 0.2–0.3 and the lifetimes—from tens of picos to 6–7 ns [5]. The fact that we observe a relatively high quantum yield and long lifetime of fluorescence could be the result of the absence of strongly quenched tryptophans or of the absence of ESET, in which they can participate.

Regarding the red-edge effect of shifts of emission spectra, the energy transfer may influence it in the following way. The spectra excited at the band maximum represent the increased contribution to total protein emission of ESET acceptors with long-wavelength shifted fluorescence spectra. They should be already shifted to longer wavelengths as a result of ESET and at the red-edge excitation this shift is removed because of ESET failure [2,10]. As a result, the red-edge effect may vanish or even disappear completely. This is not the case with penicillin acylase. The observation of a relatively strong red-edge effect witness against the possibility of ESET in the studied protein.

The model studies [36], demonstrate that ESET decreases the anisotropy over excitation spectrum and deforms it, the strongest change occurs at the band maximum (280–290 nm), while the high level of anisotropy is restored at the red edge (300 nm and longer). In contrast, rotational motions of Trp fluorophore should decrease emission anisotropy irrespective of excitation wave-

length [2]. For penicillin acylase we observe the decrease of anisotropy over the whole fluorescence excitation spectrum including its red edge, which is in line with some rotational mobility of Trp residues, but does not fit the energy transfer model. A strong argument against the directed energy transfer is the absence of emission wavelength effect on time-resolved anisotropy.

These results on the absence of ESET between Trp residues are in correspondence with the recently published three-dimensional structure of penicillin acylase at a resolution of 1.9 Å [22]. Unlike, e.g. trypsin or lysozyme, penicillin acylase exhibits the Trp residues being distributed randomly over the protein globule with no close proximity between them.

4.2. On the occurrence of inter-tryptophan energy transfer in native proteins

The problem of excited-state energy transfer between tryptophan residues remains one of the least resolved problems in protein fluorescence spectroscopy. On its solution depends the understanding of another very important problem, in what cases and to what extent we can consider the fluorescence spectrum of a multi-tryptophan protein as being composed of linear contributions of individual tryptophans. During last 50 years of active exploration of protein fluorescence, this problem was frequently discussed and the opinions ranged from complete neglecting of inter-tryptophan energy transfer to estimations of its significant values. Thus, Konev [1] estimated that the transfer may extend to the distances of 16–17 Å, which in small proteins is almost the size of the globule. The later estimates [9] of 7–12 Å seem more reasonable, but even they suggest that the transfer may frequently be observed dependent on positions of spectra, quantum yields and relative orientations of tryptophan partners. Ghiron and Longworth [37] based on substantial depolarization over excitation spectrum of trypsin, concluded about the significant extent of inter-tryptophan energy transfer in this protein. Meantime, based on their data, the rotational depolarization of fluorescence cannot be excluded. The idea to introduce into a protein molecule an additional spectroscopically different energy transfer acceptor and thus substitute the observation of homo-transfer by observation of hetero-transfer dependent sensitized emission was also explored. Selectively photooxidated tryptophan in lysozyme [38] and fluorescent inhibitor in the case of papain [39] served for this purpose. The results although not convincing, were strongly indicative for the presence of energy transfer effect. For α-chymotrypsin the results of steady-state and time-resolved experiments were compared with calculations of energy transfer efficiencies based on X-ray crystallographic data [40]. Three classes of tryptophans have been distinguished with efficient energy transfer within each class but not between the classes. The suggestions on ESET between tryptophans in different proteins were also made on the basis of steady-state polarization data [41], fast anisotropy decay [42], the absence of additivity in quantum yields [43–47] and spectral and lifetime data [48–50] in the comparison of wild type proteins with their Trp-substituted mutated forms. The possibility to explore spectral resolution in combination with time-resolved measurements was rarely used [51,52]. Thus, Gakamsky et al. [52], using methodology similar to ours, showed for a tryptophan-rich HLA-A2-peptide complex a nanosecond fluorescence spectral shift together with accelerated fluorescence depolarization at the red edge of emission spectrum, which is a strong indication of ESET.

The cited publications above, may be considered an exception in a broad literature on fluorescence spectroscopy of multi-tryptophan proteins. The latter usually ignores the possibility of energy transfer between tryptophans. The common argument for that is the goodness of fitting the experimental results into the models based on linear superposition of contributions of individual tryptophans. Often these models describe satisfactorily the protein spectra and emission decays and their validity is supported by the studies of single-tryptophan mutants [4,6,49]. In some cases, however, superposition of spectra or time-resolved decay curves of single-tryptophan mutants

give different results from that for intact protein and this difference may be due not only to the energy transfer but also to alteration of protein structure on site-directed substitutions [6]. Therefore, we need a general strategy, how to register the ESET effects and distinguish them from other excited-state reactions in proteins.

This strategy can be suggested on the basis of our previous [13] and present publications. It involves a combination of spectral-selective, time-resolved and anisotropy measurements. The witness of ESET is a dramatic variation in a number, intensity and sign of lifetime components as a function of emission wavelength. The presence of a component with negative amplitude at the long-wavelength edge of emission is a clear indication of the temporal increase of a number of emitters at particular wavelength. It demonstrates the increase of the number of excited tryptophans being ESET acceptors and possessing the long-wavelength fluorescence spectra. A dramatic change of this picture occurs when fluorescence is excited at the red edge of absorption band — the additional components at both edges of emission spectra are removed. In the study of steady-state and time-resolved anisotropy the witness for ESET is a strong time-dependent and excitation-wavelength-dependent depolarization. At short emission wavelengths and in early times we have to observe preferentially a less depolarized emission of ESET donors, while at longer times and longer wavelengths, we should observe a highly depolarized emission of the acceptors.

5. Conclusions

Penicillin acylase is an example of a protein, which is abundant in Trp residues, substantial heterogeneity in positions of these residues. We demonstrate the absence of excited-state energy transfer between them. This conclusion is made based on a combination of spectral, time-resolved and polarization measurements. The developed combined approach may be useful in the analysis of ESET in other multi-tryptophan proteins.

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