Biophysical Chemistry 25 (1986) 315–323
Elsevier



# WHAT CAUSES THE DEPOLARIZATION OF TRYPSIN AND TRYPSINOGEN FLUORESCENCE

## INTRAMOLECULAR MOBILITY OR NON-RADIATIVE ENERGY TRANSFER?

K.K. TUROVEROV and I.M. KUZNETSOVA

*Institute of Cytology, Academy of Sciences of the U.S.S.R., 4, Tikhoretsky Avenue, 194064 Leningrad, U.S.S.R.*

Received 18th March 1986
Revised manuscript received 2nd September 1986
Accepted 29th September 1986

*Key words: Trypsin; Intrinsic fluorescence; Energy transfer; X-ray data analysis; Intramolecular mobility*

Analysis of protein data bank information about the coordinates of definite atoms of protein macromolecules provides an opportunity to evaluate the efficiency of non-radiative resonance energy transfer within the model of fixed, strictly oriented oscillators. Such evaluations for trypsin and trypsinogen (and also for trypsin complex with a pancreatic inhibitor) show that the efficiency of energy transfer among each pair of tryptophan residues is negligibly small. It is also shown that a fairly effective energy transfer from tyrosine to tryptophan residues is possible. The conclusions have been made that the Tyr-Trp energy transfer is one of the factors determining the shape of the trypsin polarization spectrum, and that upon fluorescence excitation at the long-wavelength edge of the absorption spectrum, the depolarization of trypsin fluorescence in aqueous solution at ambient temperature – compared to model compounds (tryptophan, *N*-acetyltryptophan, glycyltryptophan, etc.), under the conditions of infinite viscosity – is due to the Brownian rotational motion of the macromolecules as a whole as well as the intramolecular mobility. The differences in the level and character of intramolecular mobility of trypsin and trypsinogen are discussed.

## 1. Introduction

Non-radiative energy transfer among tryptophan residues and from tyrosine to tryptophan residues is one of the factors responsible for depolarization of the intrinsic fluorescence of proteins. Since the contribution of tyrosine residues to the absorption depends upon the wavelength of excitation, the efficiency of Tyr-Trp energy transfer affects the form of the polarization spectrum. It is generally accepted that upon excitation at the long-wavelength edge of the absorption spectrum, there is neither Tyr-Trp energy transfer (the contribution of tyrosine residues in this spectral region is negligibly small [1]), nor Trp-Trp transfer (the so called 'red edge' effect [2]), which is very important for the study of the intramolecular mobility of proteins by means of rotational depolarization [3].

However, the existence of the red edge effect in proteins, under the conditions of a relaxing microenvironment of chromophores, was questioned [4] and became the subject of discussion (for opposite opinions see refs. 3 and 4). Therefore, although we believe that the red edge effect should necessarily take place in proteins, it is reasonable to evaluate the efficiency of Trp-Trp energy transfer on the basis of the distances between tryptophan residues and their mutual orientation, whenever possible.

In the present report, such an attempt at evaluating the Trp-Trp energy transfer is made for trypsin and trypsinogen. We have undertaken this study since the conclusion drawn by Ghiron and Longworth [5] about the high efficiency of energy transfer among tryptophan residues in trypsin (even upon excitation at the long-wavelength edge of the absorption band) is contrary to our idea [6] of what are the main factors responsible for fluo-

rescence depolarization in this protein.

Research data on the efficiency of energy transfer from tyrosine to tryptophan residues in trypsin and trypsinogen are very controversial, ranging from very high efficiencies (close to unity) in aqueous solutions at ambient temperature according to Kronman and Holmes [7] to the absence of transfer under the same conditions according to Arrio et al. [8]. The intermediate values $W = 0.78$ and 0.274 were obtained by Longworth [9] and Lerner and Lami [10]. According to Ghiron and Longworth [5], there is no energy transfer in the wide region of pH in aqueous and glycerol/water solutions at ambient temperature and a considerable increase in energy transfer is observed with decreases in temperature.

As far as we know, the efficiency of energy transfer among tyrosine residues in these proteins has not been discussed in the literature at all, although this question is also very important for the interpretation of spectral and polarizational characteristics.

The purpose of this work therefore was to determine the efficiency of Tyr-Tyr, Tyr-Trp and Trp-Trp energy transfer by using the protein data bank information about the structure of trypsin and trypsinogen, to evaluate the influence of these processes on the spectral and polarizational characteristics of the proteins analysed and to study the intramolecular mobility of their tryptophan residues.

## 2. Materials and methods

All luminescence measurements were made with the spectrofluorimeter described earlier [11]. The methods for measurement of spectral and polarizational characteristics and Perrin plots are specified in refs. 12 and 6, respectively. The average precision of the polarization measurements was 0.002.

Trypsin and trypsinogen (Spofa, Czechoslovakia) were additionally purified by chromatography on CM-cellulose. Information on the spatial structure of trypsin [13], trypsinogen [14] and the trypsin complex with pancreatic inhibitor [15] was taken from the protein data bank. Details of data processing have been described in ref. 16.

## 3. Results and discussion

The spectral and polarizational characteristics of trypsin and trypsinogen, generally having a form typical of tryptophan-containing proteins, indicate that the contribution of tyrosine residues in emission is negligibly small [12]: the value of parameter $A = I_{320}/I_{365}$ ($I_{320}$ and $I_{365}$: fluorescence intensities registered at 320 and 365 nm respectively) that characterizes the form and position of the fluorescence spectrum [17], and the value of the ratio of the emission anisotropy recorded at 320 and 365 nm ($r_{320}/r_{365}$) remain practically constant across the excitation spectrum (fig. 1). Taking into account that the tyrosine residues of trypsin at the maximum ($\lambda_{ex} = 278$ nm) absorb nearly 35% of the light absorbed by all of the chromophores, the absence of the tyrosine component in emission may be explained by: (1) quenching of the fluorescence of all or most of the tyrosine residues by the nearest quenching groups; (2) high efficiency of energy homotransfer among tyrosine residues, some of which are being quenched or transferring their energy of excitation to tryptophan residues; (3) high efficiency of energy transfer from tyrosine residues to tryptophan ones, and by various combinations of the above ways of tyrosine deactivation.

Analysis of the spatial structure of trypsin and trypsinogen has provided us with information about the microenvironment of each tyrosine residue. This, unfortunately, is not enough for the evaluation of their quantum yields. We can only classify Tyr 20 and Tyr 172 with a great deal of certainty as the entirely quenched residues because of the presence nearby of such effective

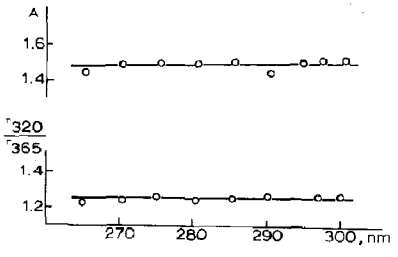


Fig. 1. The dependence of the parameter $A = I_{320}/I_{365}$ (top) and the ratio $r_{320}/r_{365}$ (bottom) upon $\lambda_{ex}$ for trypsin.

quenchers as S–S bonds [18] (Cys 22–Cys 157 and Cys 168–Cys 182). Although, near a number of other tyrosine residues, there also exist groups that are potentially able to quench, at least partially, their fluorescence (Lys 60 and 188 near Tyr 39 and 184, Met 104 near Tyr 59, His 57 and 91 near Tyr 94 and 234, Asp 189 near Tyr 228), the analysis of the microenvironment of tyrosine residues does not warrant the conclusion that the absence of a tyrosine component in trypsin fluorescence is caused by the complete quenching of all tyrosine residues. The role of Tyr-Tyr and Tyr-Trp energy transfer in the deactivation of tyrosine residues will be examined below.

### *3.1. Calculation of the efficiency of non-radiative resonance energy transfer*

The efficiency of non-radiative resonance energy transfer can be calculated according to ref. 19 as follows:

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

where $R$ is the actual distance between donor and acceptor of energy and $R_0$ the distance between donor and acceptor at which the probability of energy transfer equals 0.5 (the so-called Förster's radius of energy transfer). According to Förster [19], this value is given by

$$R_0 = 8.8 \times 10^{-25} \times q_D k^2 n^{-4} J, \qquad (2)$$

where $q_D$ is the quantum yield of donor in the absence of acceptor, $n$ the refractive index of the intervening medium in the range of frequencies where the fluorescence spectrum of donor $F_D(\nu)$ overlaps the absorption spectrum of acceptor $E_A(\nu)$, while $J$ is the overlap integral:

$$J = \int_0^\infty F_D(\nu)\epsilon_A(\nu)\nu^{-4}d\nu, \qquad (3)$$

$k^2$ is the orientation factor reflecting the mutual orientation of the donor's emission and the acceptor's absorption transition moments (see, e.g., ref. 20):

$$k^2 = (\cos\theta - 3\cos\theta_A \cos\theta_D)^2. \qquad (4)$$

In eq. 4, $\theta$ is the angle between the emission and absorption oscillators of donor and acceptor; $\theta_A$ and $\theta_D$ are the angles between the directions of the mentioned oscillators and the vector linking these molecules.

While calculating $W$ the distance $R$ between respective tryptophan and tyrosine residues and the value of $k^2$ can be determined on the basis of protein data bank information. The simplest model for such a calculation can be that of fixed oscillators strictly oriented in space. It is natural to accept for the donor-acceptor linking vector the one between the geometrical centres of their phenol and indole rings, although, strictly speaking, the points of its application are unclear.

Data on the orientation of the oscillators responsible for the long-wavelength absorption and fluorescence of tyrosine and tryptophan residues are available in the literature: the oscillator of the long-wavelength band of tyrosine is oriented along the symmetry axis of the phenol ring [21]; the $^1L_a$ and $^1L_b$ oscillators, responsible for the long-wavelength absorption band of tryptophan residues [22], are located in the plane of the indole ring, $^1L_a$ being oriented at an angle of 60° to the $C_\beta$–$C_\lambda$ bond [23–25] (see, also, fig. 2 in ref. 3) and $^1L_b$ oriented perpendicularly to $^1L_a$ [22].

For evaluating $W$, one should necessarily determine (or know) the value $R_0$, which depends not only on $k^2$, but also on the quantum fluorescence yield $q_D$, refractive index of the intervening medium $n$ and overlap integral $J$. Two procedures can be used for this purpose. First, the direct calculation of this value according to eq. 2; in this case one should determine experimentally the overlap integral $J$ for the analysed system, and accept the values of $n$ and $q_D$. It should be pointed out that the value of the refractive index $n$ of the intervening medium hardly affects the value of $R_0$: the substitution of the maximum value of $n$ for the minimum one ($n_{max} = 1.6$, $n_{min} = 1.33$ [26]) will change the value of $R_0$ only $(1.6/1.33)^{-4} = 1.047$-times. A far more significant error in the determination of $R_0$ arises because of the fact that the value of the quantum yield $q$ of individual tyrosine and tryptophan residues in proteins is usually unknown and may differ greatly from that

of amino acids in aqueous solution [1].

The second way of evaluating $R_0$ for the analysed system presupposes the use of the previous estimates known from the literature (designated here as $R'_0$):

$$R_0^6 = \frac{q_D}{q'_D} \cdot \frac{k^2}{(k^2)'} \cdot \left(\frac{n}{n'}\right)^{-4} \cdot \frac{J}{J'} \cdot (R'_0)^6, \qquad (5)$$

where all values with a prime relate to the system for which $R'_0$ was determined. If $R'_0$ was evaluated for the system in which donor and acceptor participate in the rapid and chaotic Brownian motion (liquid solutions: $(k^2)' = \langle (k^2)' \rangle = 2/3$ [19,26]), and there are grounds to believe that $J/J' \approx 1$ and $q_D/q'_D \approx 1$, then

$$W = \frac{1}{1 + \frac{2/3}{k^2}(R/R'_0)^6}. \qquad (6)$$

As follows from the above, during calculations of the efficiency of energy transfer in proteins with the use of protein data bank information, a significant error may arise first of all due to the impossibility of accurate determination of $R_0$. Such evaluations of $W$ can nonetheless be very useful, especially when $R \gg R_0$ or $R \ll R_0$, because in these cases the error in evaluation of $R_0$ is not essential for the conclusion about the absence or high efficiency of energy transfer. In this study the evaluation of $W$, based on the use of protein data bank information, is employed for the determination of the efficiency of Tyr-Tyr, Tyr-Trp and Trp-Trp energy transfer in trypsin and trypsinogen.

### 3.2. Tyr-Tyr and Tyr-Trp energy transfer

The research data on the efficiency of energy transfer from tyrosine to tryptophan residues in trypsin vary greatly. In order to evaluate Tyr-Trp energy transfer efficiency in trypsin, Kronman and Holmes [7], Arrio et al. [8] and Longworth [9] compared the quantum yields of tryptophan fluorescence, excited in the region of maximum absorption of tyrosine residues and at 295 nm, where tyrosine absorption is negligibly small. On the basis of the experimentally obtained quantum yield independence of the excitation wavelength, Kronman and Holmes [7] have drawn the conclusion that the Tyr-Trp energy transfer efficiency in trypsin and trypsinogen is very high: $W = 1.07 \pm 0.07$ and $0.91 \pm 0.18$, respectively. According to the data in ref. 8, on the other hand, the values of the quantum yield at 278 and 295 nm differ significantly ($q_{278} = 0.09$ and $q_{295} = 0.12$). Proceeding from these, the authors have concluded that there is no Tyr-Trp energy transfer in trypsin. However, in actual fact, the Tyr-Trp energy transfer efficiency, evaluated on the basis of these experimental data according to the methods given in ref. 7, is nearly 0.35. According to Longworth [9] $W$ equals 0.78. Lerner and Lami [10] also used the method based on the demonstration of sensitization of tryptophan emission by tyrosine, though modified in such a way that the differences of the spectral properties of internal and external tyrosine and tryptophan residues and the dependence of tryptophan quantum yield upon $\lambda_{ex}$ are taken into account. According to these authors $W$ equals 0.274. Comparison of all the literature data indicates the existence of a large error in the determination of $W$ by the method of demonstration of sensitization of tryptophan emission by tyrosine.

For trypsin, *N*-acetyltryptophanamide in aqueous solution and glycerol/water mixtures (1 : 1) at pH 3 and 8, at ambient temperature, Ghiron and Longworth [5] obtained similar values of the ratio $r_{270}/r_{300}$ (table 1) here used to characterize the polarization excitation spectrum, and concluded that there is no Tyr-Trp energy transfer in trypsin under such conditions. At the same time, at low temperatures (−45 °C) the value of this ratio for trypsin proved to be much lower. According to our data, the value of ratio $r_{270}/r_{300}$ for trypsin and trypsinogen (pH 3, 25 °C, aqueous solutions) is somewhat lower than that for tryptophan and its derivatives in viscous solutions (table 1). One should bear in mind, however, that Tyr-Trp energy transfer is not the only factor that determines the form of the polarization spectrum [27]. This means that the existence of Tyr-Trp energy transfer cannot be concluded on the basis of our polarization measurements. Our data only call into question the conclusion of Ghiron and Longworth about the absence of Tyr-Trp energy transfer [5].

Table 1

The ratio $r_{270}/r_{300}$ for trypsin, trypsinogen and model compounds of low molecular weight

Data published by Ghiron and Longworth [5] are given in parentheses.

| | |
|---|---|
| Trypsin | 0.55 (0.52–0.58) |
| Trypsinogen | 0.57 |
| DL-Tryptophan | 0.76 |
| *N*-Acetyl-DL-tryptophan | 0.75 |
| Glycyl-DL-tryptophan | 0.76 |
| *N*-Acetyl-DL-tryptophanamide | (0.60–0.63) |

Since evaluation of the efficiency of Tyr-Trp and Tyr-Tyr energy transfer is essential for clarifying the absence of a tyrosine component in the fluorescence spectrum, while the published research data on the Tyr-Trp energy transfer are very controversial and the polarization measurements do not give any definitive answer, we have determined these values using the information about the distances between tyrosine and tryptophan residues and their mutual orientation obtained from the protein data bank.

The tyrosine fluorescence spectrum depends very little upon the polarity of the medium and is practically the same for both tyrosine residue in protein and free tyrosine in aqueous solution [1,18]. This is why, when evaluating the efficiency of Tyr-Tyr and Tyr-Trp energy transfer in proteins, the value of $J/J'$ can be accepted as being equal to unity. With respect to $R'_0$ the values given in ref. 28 for some possible values of $q_D$ of tyrosine residues can be taken.

Calculations show that the non-radiative energy transfer between any pair of tyrosine residues in trypsin (table 2), and also in trypsinogen and the complex of trypsin with pancreatic inhibitor is low. Even if we assume that $q_D = 0.2$ ($R'_0 = 8.8$ Å [28]) the efficiency of energy transfer will be greater than 0.05 only for the pairs Tyr 59-Tyr 94, Tyr 39-Tyr 151, Tyr 20-Tyr 184-Tyr 172 and Tyr 172-Tyr 228, but even in these cases it will not exceed 0.27. These evaluations of $W$ are certainly exaggerated because the quantum yield of tyrosine residues most probably must be much smaller than 0.2. Thus, the absence of a tyrosine component in the trypsin fluorescence cannot be explained by energy transfer to the quenched tyrosine residues.

Calculation of the efficiency of Tyr-Trp energy transfer was performed for three values of $R'_0$ (14.7, 13.1 and 11.9 Å) that correspond to quan-

Table 2

Efficiency of non-radiative energy transfer among tyrosine residues of trypsin

The given values of $W$ correspond to $R'_0$ 7.9 and 8.9 Å.

| No. of tyrosine residue | No. of tyrosine residue | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | 20 | 29 | 39 | 59 | 94 | 151 | 172 | 184 | 228 |
| 29 | 0.17 | | | | | | | | |
| | 0.27 | | | | | | | | |
| 39 | 0.00 | 0.00 | | | | | | | |
| 59 | 0.00 | 0.00 | 0.00 | | | | | | |
| | | | 0.01 | | | | | | |
| 94 | 0.00 | 0.00 | 0.01 | 0.11 | | | | | |
| | | | 0.02 | 0.20 | | | | | |
| 151 | 0.01 | 0.00 | 0.11 | 0.00 | 0.01 | | | | |
| | | | 0.19 | 0.01 | 0.02 | | | | |
| 172 | 0.00 | 0.00 | 0.00 | 0.00 | 0.01 | 0.01 | | | |
| 184 | 0.05 | 0.02 | 0.00 | 0.00 | 0.00 | 0.00 | 0.04 | | |
| | 0.10 | 0.04 | | | | 0.01 | 0.07 | | |
| 228 | 0.00 | 0.01 | 0.00 | 0.00 | 0.00 | 0.00 | 0.14 | 0.00 | |
| | | 0.03 | | | | | 0.25 | | |
| 234 | 0.00 | 0.00 | 0.00 | 0.00 | 0.09 | 0.00 | 0.01 | 0.00 | 0.02 |
| | | 0.01 | | 0.01 | 0.16 | | | | 0.03 |

tum yields of tyrosine residues of $q_D = 0.2$, 0.1 and 0.05, respectively [28]. It is evident that sufficient energy transfer is possible from some tyrosine to tryptophan residues: Trp 215-Tyr 94-Trp 237, Tyr 172-Trp 215, Tyr 228-Trp 215, Trp 215-Tyr 234-Trp 237 (table 3). Average values of the efficiency of Tyr-Trp energy transfer for each tyrosine residue $< W >_i$ (a system of one donor and several acceptors) were determined as follows [29]:

$$\langle W \rangle_i = \frac{\sum_j \frac{W_j}{1 - W_j}}{1 + \sum_j \frac{W_j}{1 - W_j}}, \quad (9)$$

where $W_j$ is the efficiency of energy transfer from the given ($i$) donor to one of the acceptors ($j$) under the assumption that this acceptor is the only one. Then $\langle W \rangle_i$ were averaged all over tyrosine residues $\langle W \rangle = \sum_i \langle W \rangle_i / n$ ($n$, number of tyrosine residues). For three values of $R'_0$, given above, $\langle W \rangle$ was found to be equal to 0.60, 0.47 and 0.32, respectively. Similar results were obtained for trypsinogen. In the trypsin complex with pancreatic inhibitor – besides the effective energy transfer between the mentioned pairs of residues – the energy transfer from Tyr 10 of inhibitor to Trp 215 of enzyme ($W = 0.74$ for $R'_0 = 13.1$ Å) can also be very effective. Because there are no data about the quantum yields of tyrosine residues under the conditions of the absence of energy transfer ($q_D$) there is a significant uncertainty in the estimations of $W_j$ and $\langle W \rangle$. At the same time the distances between tyrosine and tryptophan residues and their mutual orientations are such that sufficiently effective energy transfer is possible. The absence of tyrosine residues' contribution to the fluorescence appears to be caused both by low quantum yields of certain tyrosine residues (due to the close vicinity of quenching groups) and by Tyr-Trp energy transfer.

Table 3

Efficiency of non-radiative energy transfer from tyrosine to tryptophan residues in trypsin

The given values of $W$ correspond to $R'_0$ 11.9 Å ($q = 0.05$) and 15.0 Å ($q = 0.2$).

| No. of tyrosine residue | Trp 51 | Trp 141 | Trp 215 | Trp 237 |
|---|---|---|---|---|
| 20 | 0.00–0.02 | 0.02–0.07 | 0.01–0.05 | 0.01–0.03 |
| 29 | 0.15–0.41 | 0.00 | 0.00 | 0.01 |
| 39 | 0.00 | 0.06–0.21 | 0.01 | 0.01–0.05 |
| 59 | 0.23–0.54 | 0.10–0.31 | 0.00–0.01 | 0.02–0.06 |
| 94 | 0.01–0.02 | 0.05–0.17 | 0.43–0.75 | 0.38–0.78 |
| 151 | 0.00–0.07 | 0.01 | 0.16–0.48 | 0.00 |
| 172 | 0.00–0.08 | 0.02–0.08 | 0.98–1.00 | 0.00 |
| 184 | 0.00–0.02 | 0.01–0.04 | 0.04–0.15 | 0.00–0.01 |
| 228 | 0.02–0.07 | 0.04–0.15 | 0.83–0.95 | 0.11–0.33 |
| 234 | 0.19–0.36 | 0.00–0.01 | 0.68–0.83 | 0.99–1.00 |

### *3.3 Trp-Trp energy transfer*

Relying on the experimentally measured anisotropy of trypsin fluorescence in aqueous solutions and in equivolume glycerol/water solvent at 23 and −45°C, Ghiron and Longworth [5] have drawn the conclusion that the non-radiative energy transfer among tryptophan residues is virtually the only cause of fluorescence depolarization in these proteins. Some reasons make this conclusion doubtful.

After comparing the anisotropy of trypsin fluorescence in aqueous and equivolume glycerol/water solvent at ambient temperature, Ghiron and Longworth have concluded that the Brownian rotational motion contributes very little to the depolarization of trypsin, compared to *N*-acetyltryptophanamide in rigid media. However, the change in fluorescence anisotropy registered on the addition of glycerol (see table 1 and ref. 5) is fairly significant, and to explain it one should necessarily assume the existence of the intramolecular mobility which depends upon the viscosity (see below). Moreover, the authors of ref. 5, when interpreting the experimental data, do not consider the Weber red edge effect [2], according to which the energy homotransfer does not take place upon excitation at the long-wavelength edge of the absorption spectrum, even though it does at shorter wavelength excitation. Later, it is true, the probability of the red edge effect in proteins was questioned [4]. The author of this study has used for proteins the so-called theory of inhomogeneous orientational broadening of spectra which is

valid for solutions (see refs. 30–33). According to this theory, the red edge effect can take place only in rigid, non-relaxing media and does not occur in a relaxing medium. At the same time, at least some experiments in ref. 5 were carried out in 50% glycerol at −45°C, i.e., under conditions when there are absolutely no reasons to ignore the red edge effect. Moreover, as we mentioned earlier [3], even under the conditions of a relaxing microenvironment the radiative centres of proteins preserve the inherent heterogeneity. Nevertheless, since the question of the red edge effect has not been definitely solved, it is expedient, when possible, to evaluate the Trp-Trp energy transfer efficiency on the basis of distances between tryptophan residues and of their mutual orientation.

The evaluation of Trp-Trp energy transfer is complicated by the fact that the fluorescence spectra of proteins are shifted toward shorter wavelengths as compared to free tryptophan in aqueous solution. This is why the value of the overlap integral $J$ and, consequently, the value of $R_0$ can be far greater for proteins than for free amino acid in solution ($R'_0 = 5.8$–$7.2$ Å [18,26]).

To determine the efficiency of Trp-Trp energy transfer in proteins one can use the value $R'_0 = 7.8$ Å obtained by Eisinger et al. [28] according to eq. 2. In this work the overlap integral was determined experimentally, while the quantum yield, refractive index and orientation factor were tentatively accepted as $q_D = 0.1$, $N = 1.5$ and $k^2 = 2/3$. At these values of $R'_0$ and $(k^2)'$, with the presumption that only oscillator $^1L_a$ participates in emission, we have obtained an efficiency of energy transfer in trypsin between Trp 51 and Trp 237 equal to 0.053. The distances between other pairs of tryptophan residues are so large ($R = 19.4$–$26.1$ Å) that the efficiency of energy transfer between them does not exceed 0.01 (table 4). Since the quantum yield of trypsin, according to different authors (see the summarized data in ref. 1), varies within 0.08–0.15 (mean value 0.12), and the fluorescence spectrum is even of a somewhat longer wavelength position ($\lambda_{max} = 334.5$ nm) compared to that of chymotrypsinogen ($\lambda_{max} = 331.5$–$333$ nm) [1], there is every reason to believe that $R'_0$ for trypsin does not exceed 7.8 Å. Still, even if we assume that $R'_0 = 8.7$ Å (this value corresponding to $q_D = 0.2$ is considered to be maximum for proteins [28]), the efficiency of energy transfer between $^1L_a$ Trp 51 and $^1L_a$ Trp 237 will not exceed 0.1 and will be lower than 0.02 for other pairs.

In principle, not only oscillator $^1L_a$, but also $^1L_b$ can participate in the Trp-Trp energy transfer. The latter does not irradiate in proteins [12] and thus cannot donate energy. Nevertheless, it can be an acceptor of energy. We cannot exclude the possibility that in some cases the orientation of the $(^1L_a)_D$, $(^1L_a)_A$ and $(^1L_b)_A$ oscillators will be more favourable for the $(^1L_a)_D \rightarrow (^1L_b)_A$ energy transfer than for the $(^1L_a)_D \rightarrow (^1L_a)_A$ transition. It is just this situation that is observed for Trp 51-Trp 237 energy transfer in trypsin and trypsinogen (table 5): the value of $k^2$ for ($^1L_a$ Trp 51)$_D \rightarrow$ ($^1L_b$ Trp 237)$_A$ and ($^1L_a$ Trp 237)$_D \rightarrow$ ($^1L_b$ Trp 51)$_A$ transitions are higher than the respective value for $^1L_a$ Trp 51-$^1L_a$ Trp 237 transition. At the same time it must be remembered that the long-wavelength edge of the $^1L_b \leftarrow A$ absorption band of indole is some nanometres shorter in comparison with the $^1L_a \leftarrow A$ band [34]. The intensity of the $^1L_b \leftarrow A$ band at least does not exceed [34] (according to ref. 23, much lower) the intensity of the $^1L_a \leftarrow A$ absorption band. Consequently, the $^1L_b \leftarrow A$ absorption band contributes only a small portion (see refs. 28 and 34) to the overlap integral of the absorption and emission spectra of tryptophan residues in proteins. For this reason the value of $R'_0$ for $(^1L_a)_D \rightarrow (^1L_b)_A$ energy transfer must be lower than for the $(^1L_a)_D \rightarrow (^1L_a)_A$ transition. This gives grounds for neglecting the $^1L_b$ oscillator when calculating Trp-Trp energy transfer in proteins.

Table 4

Efficiency of non-radiative energy transfer among tryptophan residues in trypsin

The given values of $W$ correspond to $R'_0$ 7.8 Å.

| No. of tryptophan residue | No. of tryptophan residue | | |
|---|---|---|---|
| | 51 | 141 | 215 |
| 141 | 0.003 | | |
| 215 | 0.002 | 0.002 | |
| 237 | 0.053 | 0.004 | 0.009 |

Table 5

Characteristics of energy transfer among different transition moments of Trp 51 and Trp 237 in trypsin and trypsinogen

| Object | $R$ (Å) | Donor and acceptor transition moments | $R_0$ (Å) | $k^2$ | $W$ |
|---|---|---|---|---|---|
| Trypsin | 10.17 | $(^1L_a)_D$–$(^1L_a)_A$ | 7.8 | 0.183 | 0.053 |
| | | $(^1L_a$ Trp 51$)_D$–$(^1L_b$ Trp 237$)_A$ | – | 1.210 | – |
| | | $(^1L_a$ Trp 237$)_D$–$(^1L_b$ Trp 51$)_A$ | – | 0.856 | – |
| Trypsinogen | 10.27 | $(^1L_a)_D$–$(^1L_a)_A$ | 7.8 | 0.221 | 0.059 |
| | | $(^1L_a$ Trp 51$)_D$–$(^1L_b$ Trp 237$)_A$ | – | 1.239 | – |
| | | $(^1L_a$ Trp 237$)_D$–$(^1L_b$ Trp 51$)_A$ | – | 0.813 | – |

The efficiency of energy transfer between each pair of tryptophan residues in trypsin has thus been proved to be low. The uncertainty in determination of the efficiency of energy transfer in this case is minimum because the low efficiency of this energy transfer is due to the large distances between tryptophan residues. All other parameters that determine the efficiency of energy transfer can only slightly influence the result in this case. Similar results have been obtained for trypsinogen and complex of trypsin with an inhibitor.

### *3.4. Intramolecular mobility of tryptophan residues in trypsin and trypsinogen*

The measurements of Perrin plots (i.e., the dependences of the reciprocal value of fluorescence polarization $1/P$ upon $T/\eta \cdot \tau/\tau_0$, where $T$ and $\eta$ are the absolute temperature and viscosity of the solvent, and $\tau_0$ and $\tau$ the mean lifetimes of the excited states in aqueous and glycerol/water solvents) for trypsin and trypsinogen (fig. 2) made it possible to reveal the differences in the level and character of the intramolecular mobility in enzyme and zymogen. As follows from this figure, the absolute value of the polarization is higher for trypsin than for trypsinogen in aqueous solutions, as well as for extrapolation to infinite viscosity. For trypsin, as also for trypsinogen, the value of the intercepts of the Perrin plots on the $Y$-axis ($1P_0' = 4.9$ and 5.4 for trypsin and trypsinogen, fig. 2) is higher than the respective value for tryptophan, glycyltryptophan and $N$-acetyltryptophan under conditions of infinite viscosity ($1/P_0 = 3.9$ [6]). Moreover, the slope of the reduced Perrin plot, $\tan \alpha = [(1/P_0 - 1/3)/(1/P_0' - 1/3) - 1]/T$, for trypsin exceeds the theoretical value $\tan \alpha_0 = 3\eta/T \cdot \tau_0/\rho_0$ calculated for a chaotic orientation of oscillators: $\tan \alpha = 5.1 \times 10^{-6}$; $(\tan \alpha_0)_{min} = 2.4 \times 10^{-6}$ under the conditions where the oscillators responsible for fluorescence are orientated along the long axis of the macromolecule and $(\tan \alpha_0)_{max} = 5.5 \times 10^{-6}$ at their orientation along the short axis ($\rho_0$ correlation time for the rigid ellipsoid with mass and asymmetry equal to those of trypsin) (see ref. 6).

The validity of the ratio $\tan \alpha > \tan \alpha_0$ demonstrates that the existence of a slow mobility depending upon solvent viscosity is highly probable in this protein. Worthy of note is the fact that the relaxation time of the slow motion in the enzyme is essentially shorter than that in its inactive predecessor ($\tan \alpha_{trypsin} > \tan \alpha_{trypsinogen}$). At the same time, the level of the high-frequency intramolecular mobility (or intramolecular mobility not depending on solvent viscosity [6]) is higher in trypsinogen than in trypsin ($1/P_0'$ $_{trypsinogen} > 1/P_0'$ $_{trypsin}$).

Comparative analysis of the structure of trypsin and trypsinogen macromolecules, based on the

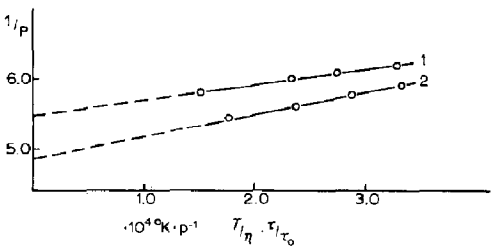


Fig. 2. Perrin plots. (1) Trypsinogen, (2) trypsin; $\lambda_{ex} = 296.8$ nm, $\lambda_{reg} = 365$ nm.

information about the coordinates of individual atoms, stored in the protein data bank, shows that the activation of trypsinogen causes a noticeable change in the microenvironment of three out of four tryptophan residues. Root mean square deviations of the atoms of the microenvironment (those located at a distance of less than 7 Å from the geometrical centre of indole ring [16]) of tryptophan residues Trp 141, Trp 51, Trp 215 and Trp 237 are 1.31, 0.61, 0.56 and 0.22 Å respectively. The $O_{\delta 1}$, $O_{\delta 2}$ and $C_{\delta}$ atoms of Asp 194 that are included in the microenvironment of Trp 141 undergo respective maximal deviations of 6.4, 4.8 and 4.8 Å. $O_{\epsilon 1}$ and $N_{\epsilon 2}$ atoms of Glu 30 deviate 2.0 and 2.4 Å. Deviations of yet another thirteen atoms of protein and three atoms of bound water, forming the microenvironment of this tryptophan residue, range from 1.0 to 1.5 Å. In the microenvironment of Trp 51, the larger deviations are characteristic of $O_{\delta 1}$ and $N_{\delta 2}$ atoms of Asn 48 and of $C_{\delta 1}$ atoms of Ile 242 ($R = 2.25$, 2.26 and 2.63 Å); deviations of another three atoms range from 1.0 to 1.5 Å. In the case of Trp 215 only three atoms deviate more than 1 Å ($N_{\epsilon 2}$ and $O_{\epsilon 1}$ of Gln 175 deviate 3.2 and 1.2 Å, 0 Gly 216, 1.6 Å).

Thus, the use of X-ray data makes it possible to conclude that the efficiency of Trp-Trp energy transfer in trypsin is very low and that the depolarization at the long-wavelength excitation is determined exclusively by the change in orientations of tryptophan residues during the lifetime of their excited states. Comparison of the Perrin plots for enzyme and its inactive predecessor and the structures of these proteins allowed the conclusion that the differences in the level and character of the intramolecular mobility of their tryptophan residues are caused by differences in the structures of their microenvironments.

## References


1 E.A. Burstein, Itogi Nauki Tekh. Ser. Biofiz. 7 (1977) 187.
2 G. Weber and M. Shinitsky, Proc. Natl. Acad. Sci. U.S.A. 65 (1970) 823.
3 K.K. Turoverov and I.M. Kuznetsova, Mol. Biol. 17 (1983) 468.
4 E.A. Burstein, Mol. Biol. 17 (1983) 455.
5 C.A. Ghiron and J.W. Longworth, Biochemistry 18 (1979) 3828.
6 I.M. Kuznetsova and K.K. Turoverov, Mol. Biol. 17 (1983) 741.
7 M.J. Kronman and L.G. Holmes, Photochem. Photobiol. 14 (1971) 113.
8 B. Arrio, M. Hill and C. Parquet, Biochimie 55 (1973) 283.
9 J.W. Longworth, in: Excited states of proteins and nucleic acids, eds. R.F. Steiner and I. Weinryb (Plenum Press, New York, 1971) p. 319.
10 J. Lerner and H. Lami, in: Excited states of biological molecules, ed. J.B. Birks (Wiley, New York, 1976) p. 601.
11 E.V. Gusev, K.K. Turoverov, Yu.M. Rozanov and N.S. Volosov, in: Functional cellular morphology, genetics and biochemistry, ed. A.S. Troshin (Nauka, Leningrad, 1974) p. 364.
12 K.K. Turoverov and I.M. Kuznetsova, Mol. Biol. 19 (1985) 1321.
13 H. Fehlhammer, W. Bode and P. Schwager, Protein Data Bank (1977) 1PTN.
14 W. Bode, H. Fehlhammer and R. Huber, Protein Data Bank (1979) 1TGA.
15 A. Ruchmann, D. Kukla, P. Schwager, K. Bertels, R. Huber, W. Bode, J. Deisenhoffer, W. Steigemann, W. Kohl and C.A. Ryan, Protein Data Bank (1976) 1PTC.
16 K.K. Turoverov, I.M. Kuznetsova and V.N. Zaitsev, Biophys. Chem. 23 (1985) 79.
17 K.K. Turoverov and B.V. Schelchkov, Biofizika 15 (1970) 965.
18 E.A. Burstein, Itogi Nauki Tekh. Ser. Biofiz. 6 (1977) 213.
19 T. Förster, Radiat. Res. Suppl. 2 (1960) 326.
20 R.E. Dale and J. Eisinger, Biopolymers 13 (1974) 1573.
21 T.B. Truong and A.J. Petit, Phys. Chem. 83 (1979) 1300.
22 G. Weber, Biochem. J. 75 (1960) 335.
23 Y. Yamamoto and J. Tanaka, Bull. Chem. Soc. Jap. 45 (1972) 1362.
24 V.N. Umetskaya and K.K. Turoverov, Opt. Spectrosc. 44 (1978) 1090.
25 T. Yamane, T. Andou and T. Ashida, Acta Crystallogr. B22 (1977) 1650.
26 I. Steinberg, Annu. Rev. Biochem. 40 (1971) 83.
27 I.M. Kuznetsova, I.I. Kirik and K.K. Turoverov, Mol. Biol. 15 (1981) 989.
28 J. Eisinger, B. Feuer and A.A. Lamola, Biochemistry 8 (1969) 3908.
29 R.B. Gennis and C.R. Cantor, Biochemistry 11 (1972) 2509.
30 I.M. Gulis, A.I. Lomyak and V.I. Tomin, Izv. Akad. Nauk SSR, Ser. Fiz. 42 (1978) 307.
31 E.N. Bodunov, E.V. Kolobkova and V.L. Ermolaev, Opt. Spectrosc. 44 (1978) 252.
32 V.T. Koyava and V.I. Popechits, J. Prikl. Spectrosc. 31 (1979) 982.
33 B.I. Stepanov, A.N. Rubino and V.I. Tomin, Izv. Akad. Nauk SSR, Ser. Fiz. 46 (1982) 380.
34 B. Valeur and G. Weber, Photochem. Photobiol. 25 (1977) 441.