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Red-edge-excitation fluorescence spectroscopy of single-tryptophan proteins

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Abstract. With the aim of finding non-equilibrium dipole-relaxational electronic excited states of tryptophan residues in proteins the dependence of the fluorescence emission maximum on excitation wavelength was studied for several proteins containing a single tryptophan residue per molecule. Spectral shifts upon red-edge excitation are not observed for short wavelength-emitting proteins (azurin, two-calcium form of whiting parvalbumin, ribonucleases C_2 and T_1). This may be because of the non-polar environment of the tryptophan residues in these proteins or because of the absence of dipole-orientational broadening of spectra. The effect was also not found for proteins emitting at long wavelengths (max. at 341–350 nm) – melittin at low ionic strength, IT-Aj1 protease inhibitor, myelin basic protein. In these proteins, the tryptophan residues are exposed to the rapidly relaxing aqueous solvent. Spectral shifts associated with red-edge excitation are observed for proteins emitting in the medium spectral range – human serum albumin in the *N* and *F* forms, IT-Aj1 protease inhibitor at pH 2.9, melittin at high ionic strength as well as the albumin-dodecylsulfate complex. This suggests the existence in these proteins of a distribution of microstates for tryptophan environment with various orientation of dipoles and of slow (on the nanosecond time scale) mobility of the field of these dipoles. As a result the emission proceeds from electronic excited states which are not at equilibrium.

Key words: Fluorescence of proteins, red-edge-excitation spectroscopy, nanosecond dynamics, dipole-reorientational relaxation

Introduction

The popularity of fluorescence spectroscopy in the study of proteins results from the simplicity or experiments and the high sensitivity of the position and intensity of spectra to structural changes in the protein. However, this method has not yet been developed to the extent where strict and unequivocal correlations between spectroscopic information and protein structure exist. In spite of much effort there are no final answers to the questions which faced investigators upon the introduction of this method (Vladimirov and Burstein 1960; Teale 1960; Konev 1967): What basic mechanisms are involved in spectral changes? What is the explanation for the large variations (up to 45 nm) exhibited by the fluorescence emission maximum of tryptophan in proteins? To what extent is the approach based on the analysis of models of tryptophan environment in isotropic media applicable in the study of proteins? Tryptophan fluorescence is sensitive to a number of factors: the environment polarity (Konev 1967; Longworth 1971; Burstein 1976, 1977), formation of complexes in the excited state (exciplexes) (Burstein 1976, 1977; Lumry and Hershberger 1978) and the rate of dipole-reorientational relaxation around the excited chromophore (Lakowicz et al. 1980; Lakowicz and Balter 1982). When studying protein spectra it is not always easy to identify the particular factor involved.

The analysis of dipole-relaxational properties is of special significance, since it allows one to obtain important information on protein dynamic properties. The application of nanosecond time-resolved spectroscopy allowed the observation in some cases (for example, Grinvald and Steinberg 1974) of spectroscopic behaviour in accord with the model of dipole-reorientational relaxation occurring at nanosecond times (Bakhshiev 1972). However, for many proteins there are no nanosecond shifts in the time-resolved fluorescence spectra of individual chromophores. This fact indicates that mobility of protein groups is outside the nanosecond time range but cannot resolve whether it is too fast or too slow. The recently proposed method of red-edge-excitation fluorescence spectroscopy (Demchenko 1981, 1982, 1984) is thought to identify

these two limiting situations. In several proteins studied it was shown that emission proceeded from chromophore excited states which were not at equilibrium with respect to orientation of surrounding dipoles. However, it is not clear how general this phenomenon is, and how it relates to other structural and spectroscopic properties of tryptophan residues in proteins.

The present communication is concerned with the results of studies on the red-edge-excitation shifts of fluorescence spectra of several proteins having a single tryptophan residue. This is the simplest case for the fluorescence study of proteins because the structural heterogeneity of tryptophan residues is partly excluded (with the exception of probable conformers at equilibrium, see Permyakov et al. 1985; Ludescher et al. 1985) and excitation energy homotransfer is eliminated. The aim of this paper is to correlate the red-edge-excitation fluorescence spectral shift with the positions of fluorescence spectra and with other spectroscopic properties of a number of single-tryptophan proteins.

Theory

The red-edge-excitation shift effect in fluorescence spectra is a phenomenon associated with the existence in the chromophore-environment system of a distribution in interaction energies and with the dynamic properties of the chromophore environment (Demchenko 1986). This effect was first observed by Rubinov and Tomin (1970) and Galley and Purkey (1970) in solid chromophore solutions. Its relation to structural relaxational properties of the excited-state chromophore environment was manifested in many subsequent publications (Macgregor and Weber 1981; Rubinov and Tomin 1984; Demchenko 1982) dealing with many chromophore-solvent systems including indole and tryptophan (Demchenko and Ladokhin 1988). Model experiments strongly suggest that though other processes in attaining intermolecular equilibrium in the excited state may be involved, inhomogeneity with respect to dipolar interactions and dipole-reorientational relaxation are the major factors responsible for this effect.

Assume that the absorption band contour is inhomogeneous and contains contributions of chromophores differing in their energy of interaction with the environment and whose individual absorption spectra are shifted to a different extent as a result of this interaction. On excitation at the red-edge one effects the photoselection of a particular part of the chromophore population within this distribution, namely that which possesses the lowest electronic transition energy. Their fluorescence spectrum (if there is no mobility of the chromophore environment prior to emission) differs from the average and is shifted to longer wavelengths. Thus the necessary conditions for observation of the shift upon red-edge-excitation are: substantial change of chromophore dipole moment on electronic excitation, the presence in the chromophore environment of strong dipoles in sufficient concentrations, their immobility or slow mobility on the time scale of electronic excitation.

Consider three specific cases which differ in conformity of the emission rates and chromophore environment mobility.

(i) Motions of polar molecules and groups in the chromophore environment do not occur at the time scale of emission. This is the case for a solid environment. The dipole-reorientational relaxation time τ_R is much longer than the fluorescence lifetime τ_F. The magnitude of the shift upon red-edge-excitation is very significant. It is dependent both on the chromophore properties (the magnitude and direction of the change in the dipole moment on excitation and the slope of the absorption spectrum at the red edge) and on the chromophore-environment interactions (the distribution of chromophore-environment interaction energies in the ground and excited states).

(ii) Relaxation occurs simultaneously with emission, $\tau_R \approx \tau_F$. This is the case for highly viscous environments. The shift upon edge-excitation depends on the relative contributions of early emitted quanta from non-relaxed states and the quanta emitted later from the relaxed states. The effect in this case should depend significantly on any factor that influences τ_R or τ_F; specifically, on temperature and fluorescence quenchers.

(iii) The mobility of the chromophore environment is rapid on the nanosecond time scale, and $\tau_R \ll \tau_F$. This is the case for a low-viscosity liquid environment. There is a rapid redistribution (equilibration) of interaction energies prior to emission, and fluorescence spectra are excitation-wavelength-independent.

There is a direct relation between the shift and other effects which are dependent on molecular relaxations in the excited state: relaxational temperature-dependent shift (which induced variations of τ_R and τ_F), lifetime-resolved shifts (variations of τ_F) and nanosecond time-dependent shifts (Lakowicz 1983). According to the Bakhshiev-Mazurenko theory of relaxation (Bakhshiev et al. 1966; Mazurenko and Bakhshiev 1970) the mean frequency of emission in steady-state spectra ν depends on τ_R and τ_F in the following way:

$$\frac{\nu - \nu_\infty}{\nu_0 - \nu_\infty} = \frac{\tau_R}{\tau_R + \tau_F}. \quad (1)$$

Here ν_0 is the emission frequency of the non-relaxed chromophore, which occurs at early times and is ob-

served in time-resolved spectra or in steady-state spectra at low temperatures, ν_∞ is the frequency of emission of already relaxed chromophore, that occurs at high temperatures or long times.

The dependence of the edge-excitation effect on τ_R and τ_F is similar to that of the relaxational shift (Demchenko and Ladokhin 1988). However, since relaxation results in the establishment of an equilibrium distribution, the effect vanishes and at infinite time the position of the fluorescence spectrum becomes independent of the excitation wavelength:

$$\nu_\infty - \nu_\infty^{\text{edge}} = 0. \tag{2}$$

Then if we assume that Eq. (1) is valid both for mid-band and for edge excitations, we obtain:

$$\frac{\nu - \nu^{\text{edge}}}{\nu_0 - \nu_0^{\text{edge}}} = \frac{\tau_R}{\tau_R + \tau_F}. \tag{3}$$

The important advantage of edge excitation spectroscopy follows from the fact that Eq. (3) does not contain ν_∞. Thus, determination of τ_R does not require knowledge of the spectrum of the completely relaxed state, which is often not possible in both steady-state and time-resolved experiments with proteins, because of small τ_F values and the fact that high temperatures may cause protein denaturation. $\nu - \nu^{\text{edge}}$ increases with τ_R and tends to a limiting value. According to Eq. (3) the ratio of the edge-excitation effect to its limiting value is a function of τ_R. The presence of the effect itself indicates that $\tau_R \geqq \tau_F$. Thus, a non-equilibrium excited state may be recognized from simple steady-state experiments without variations of any other parameter.

Materials and methods

Azurin from the microorganism *Pseudomonas aeruginosa*, with purity index $E_{625}/E_{280} = 0.63$ and isolated as described by Kamalyan and Nalbandyan (1977) was provided by M. G. Kamalian (Institute of Biochemistry, Armenian Academy of Science, Yerevan). Whiting (*Gadus Merlangus*) parvalbumin was a kind gift of E. A. Permyakov and L. P. Kalinichenko (Institute of Biological Physics, USSR Academy of Science, Pushchino, Moscow Region). Ribonuclease C_2 from *Aspergillus clavatus*, isolated as described by Bezborodova et al. (1983), was a gift of S. I. Bezborodova (VNII Genetika, Moscow). Melittin from bee venom was isolated in our laboratory by E. G. Kostrzhevska and T. L. Dibrova using conventional procedures including gel-filtration on a Sephadex G-25 column and DEAE-cellulose chromatography (Maulet et al. 1980). IT-Aj1 protease inhibitor, isolated from *Actinomyces junthinus* 118 using the procedure of Andreeva et al. (1978), was a gift of V. M. Grishchenko (Institute of Biochemistry and Physiology of Microorganisms, USSR Academy of Science, Pushchino, Moscow Region). β-casein from cow milk, pure according to electrophoretic and DEAE-chromatographic criteria, was isolated by D. S. Yankovsky (Institute of Meat and Milk Industry, Kiev). Human serum albumin was a crystalline preparation (Sigma) which was defatted by charcoal filtration and the monomer fraction was isolated by gel-filtration on Sephadex G-75. The data on ribonuclease T_1 from *Aspergillus clavatus* were communicated by E. A. Burstein (Institute of Biological Physics, USSR Academy of Science, Pushchino, Moscow Region).

Sodium dodecylsulfate was a product of Serva. Fluorescence spectra were recorded on an Hitachi MPF-4 spectrofluorometer equipped with a 150 W xenon lamp. Recording conditions were: excitation spectral bandwidth 2 nm, emission – 5 nm, ratio recording mode, scan speed 30 nm/min. Some experiments used an interference filter (cut off $\lambda > 320$ nm) to reduce stray light. Since the recorded spectra of indole and tryptophan agreed well with published corrected spectra, no correction for instrument response was employed. For protein samples rectangular 1 cm silica cells were used. The cell holders were thermostated by circulating water.

The experiments on the dependence of fluorescence spectra on excitation wavelength were conducted in two ways: either protein concentration was set constant or the protein concentration was varied in order to maintain nearly constant absorption (0.05 – 0.08) at the excitation wavelength. For this absorption range, the fluorescence intensity changes were practically linear with changes in protein concentration. With the proteins studied here no concentration-dependent effects in spectra were detected. In order to decrease light-scattering the protein preparations were centrifuged at 12,000 g for 10 – 15 min. The background emission of buffer solutions for each protein spectrum was recorded and subtracted.

Under the conditions of our experiment the standard error for the determination of $\lambda_{\max}^F$ was ± 0.5 nm. In order to increase the precision in determining the spectral shifts, the ratio of the intensities at the short and long wavelength wings of the spectrum, $I_{\lambda_1}/I_{\lambda_2}$, was obtained. In the case of a single emitting chromophore this parameter is a direct and (to a first approximation) linear function of $\lambda_{\max}^F$ (Filenko and Zyma 1981). The values of λ_1 and λ_2 were chosen at approximately half-maximal intensities and at a distance from the scattered light band. For red-edge excitation this band is increased in intensity and is superimposed on fluorescence spectra; this does not allow the precise determination of any parameter based on integration of emission band, e.g. the centre of mass. The error in determining $I_{\lambda_1}/I_{\lambda_2}$ was $\pm 4\%$ or better; this allows

the detection of shifts of λ^F_{max} by 0.3 – 0.4 nm. For the proteins emitting in the medium wavelength range $\lambda_1 = 320$ nm, $\lambda_2 = 360$ nm, the bandwidth of the spectrum $\Delta\lambda$ was determined as the distance in wavelengths between the points at half-maximum intensities.

Absorption spectra were recorded on "Specord UV-VIS" (Carl Zeiss, Jena, GDR) and Beckman, model 25 spectrophotometers.

Results

Proteins emitting in the short wavelength region

Figure 1 shows results for the dependence of the positions of the fluorescence maximum on excitation wavelength for several short wavelength emitting proteins: azurin with $\lambda^F_{max} = 307$ nm, whiting parvalbumin (calcium form) with $\lambda^F_{max} = 315$ nm and ribonucleases T_1 ($\lambda^F_{max} = 321$ nm) and C_2 ($\lambda^F_{max} = 323.5$ nm). Of the proteins studied azurin possesses the shortest wavelength fluorescence spectrum with fine structure, which is unique in proteins, but is typical of indole in hydrophobic environments. This served as a basis for classifying the azurin tryptophan residue as a representative of a very rare class A chromophore (Burstein 1977). The tryptophan residues of whiting parvalbumin and ribonucleases T_1 and C_2 are referred to by Burstein (1977) as class S chromophores (with a contribution from class I). The fluorescence spectra of class S possess some fine structure but have maxima at 316 nm (exciplex 1:1 emission, Burstein (1983)). The

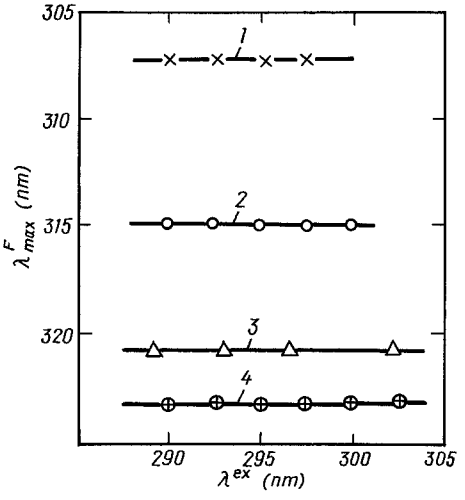

Fig. 1. Dependence of fluorescence maxima positions on excitation wavelength for single-tryptophan proteins emitting at short wavelength. *1:* azurin in 0.05 *M* ammonia-acetate buffer, pH 6.0. *2:* parvalbumin in 0.05 *M tris*-HCl buffer, pH 7.5 in the presence of calcium ions (0.1 m*M*). *3:* ribonuclease T_1 in 0.05 *M tris*-HCl buffer, pH 7.5. *4:* ribonuclease C_2 in 0.05 *M tris*-HCl buffer, pH 7.0, temperature 25 °C

relatively small Stokes shift and the superposition of the scattered light band on the spectra do not allow one to obtain fluorescence spectra in the excitation range 305–309 nm for these proteins. However, the results at shorter excitation wavelengths show the edge-excitation fluorescence effect to be absent, and the absorption contour behaves as a homogeneous one. That no excitation wavelength dependent shifts were present was also demonstrated by measuring the ratio of intensities at the two wings of the spectrum ($I_{\lambda_1}/I_{\lambda_2}$). No changes were found in the widths of the fluorescence bands.

The failure to detect inhomogeneity in chromophore – environment interactions may be explained in one of two ways:

(1) Rapid relaxational mobility may exist when $\tau_R \ll \tau_F$. If it occurred, the fluorescence spectra would be shifted significantly on freezing (at low temperatures τ_R increases). However, it is known that on lowering the temperature the fluorescence spectra of azurin (Burstein et al. 1977) and of class S proteins (Burstein 1977) are unchanged.

(2) Insignificant dipolar interactions and the absence of a substantial distribution in their energy. If so, selectivity of photoselection within a population of chromophores with different interaction energy is not sufficient and the effect of edge excitation may not be revealed even if the chromophore environment is rigid enough. The analysis of X-ray diffraction data for azurin (Turoverov et al. 1984) demonstrates that the environment of Trp-48 is actually hydrophobic with no contacts of this residue with polar groups and no hydrogen bonding. Probably, these are also the properties of class S proteins (Burstein 1977, 1983), their longer wavelength fluorescence spectra is due to a specific complex (exciplex 1:1) with one polar group, the formation of which may not require fast group mobility. Since whiting parvalbumin and ribonucleases T_1 and C_2 contain contributions of class I emission, there exists some possibility for exciplex formation with a second polar group (Burstein 1983). There is probably no other polar group to contact the indole ring, and protein dynamics is not so fast that these groups could approach it at the excited state lifetime.

Hershberger et al. (1980) in studies of phosphorescence and optically detected magnetic resonance spectra at liquid helium temperature observed inhomogeneous broadening of 0.4 nm for ribonucelase T_1. This effect is probably not sufficient to be detected in our experiments.

Thus, for proteins with short wavelength emission the effect of red-edge-excitation fluorescence shifts is not revealed. The suggested approach does not allow the width of the distribution of interaction energies of chromophore microstates and the rate of dipole-

reorientational mobility of groups in its environment to be evaluated.

Proteins emitting in the medium-wavelength range (325–341 nm)

Several proteins of this group were studied under different experimental conditions. IT-Aj1 protease inhibitor from microorganisms at pH 2.9, bee venom melittin at high ionic strength and neutral pH, human serum albumin at neutral and acid pH and its complex at neutral pH with dodecylsulfate (Fig. 2). A small fluorescence shift on changing excitation wavelength from 295 to 305 nm is observed for albumin at neutral pH – 2 nm ($\lambda_{max}^{ex295} = 340$ nm) and protease inhibitor at pH 2.9 – 3 nm ($\lambda_{max}^{ex295} = 337$ nm). It is more significant for melittin and amounts to 5 nm ($\lambda_{max}^{ex295} = 333.5$ nm). The acid *F*-form of serum albumin displays a fluorescence spectrum at shorter wavelengths than the *N*-form existing at neutral pH (Ivkova et al. 1971; Nyamaa et al. 1985). For the *F*-form with $\lambda_{max}^{ex295} =$ 329 nm the edge-excitation fluorescence shift is more significant, reaching 7 nm. The shifts of λ_{max}^{F} were confirmed by the substantial decrease of I_{320}/I_{360} values. With such substantial shifts of spectra, the bandwidths, $\Delta\lambda$, in the region 295–305 nm were increased by no more than 1 nm.

The fluorescence decay of human serum albumin is not exponential and at neutral pH is approximated by two lifetimes, 6.1 and 1.5 ns (Hazan et al. 1976). The mobility inside this protein is probably so low that the equilibrium distribution of dipolar groups at these times is not reached.

The ability of serum albumin to bind detergents at low (stoichiometric) concentrations is well-known. The tryptophan residue participates in the formation of a binding centre, and its fluorescence spectrum on binding is shifted substantially to shorter wavelengths (Ivkova et al. 1971; Eftink and Ghiron 1977). In studies of the albumin complex with dodecylsulfate we have observed a shift of 11 nm upon red-edge excitation ($\lambda_{max}^{ex295} = 323$ nm), the most significant for the proteins studied. Under the chosen experimental conditions the albumin molecule retains its native structure, and sites with high affinity for detergent are saturated. Both sulfo-groups and aliphatic chains of dodecylsulfate molecules participate in high-affinity interaction with the protein, their alkyl groups bind non-polar protein groups (Inoue et al. 1979). The substantial magnitude of the red-edge-excitation effect observed is supposed to originate not only from screening of the tryptophan residue from aqueous solvent and transition to a less polar environment, but also from immobilization of the environment, slowing down the relaxation of surrounding dipoles (Demchenko 1981).

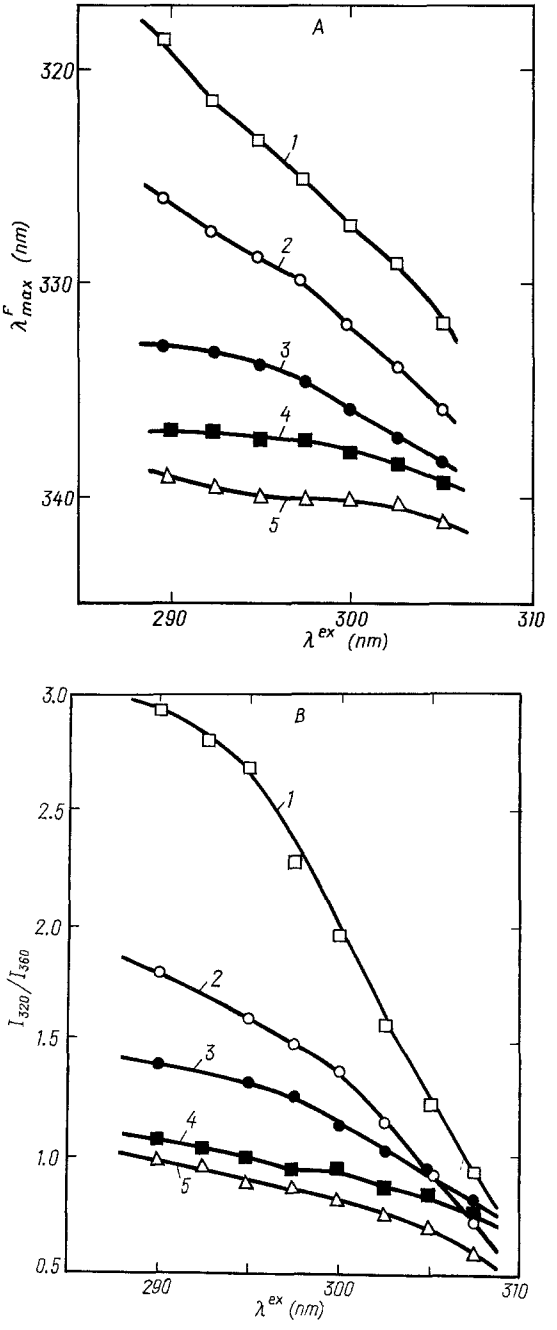

Fig. 2 A and B. Dependence of fluorescence maxima positions λ_{max} **(A)** and parameter I_{320}/I_{360} **(B)** on excitation wavelength for single-tryptophan proteins emitting in the medium wavelength range. *1:* albumin in 0.05 *M tris*-HCl buffer, pH 7.8 in the presence of 10^{-4} *M* sodium dodecyl sulfate. *2:* albumin in 0.1 *M* glycine buffer, pH 3.2. *3:* melittin in 0.05 *M tris*-HCl buffer, pH 7.5 with 0.15 *M* NaCl. *4:* protease inhibitor in 0.2 *M* glycine buffer, pH 2.9. *5:* albumin in 0.05 *M tris*-HCl buffer, pH 7,2, temperature 25 °C

It is possible to suggest that in the latter case, as well as in other cases where dependence on excitation wavelength is observed, that the red-edge-excitation fluorescence effects are directly relatred to incomplete relaxation of dipolar groups surrounding the trypto-

phan residue during emission. In the case of native albumin there may be both protein groups and immobilized water molecules. The tryptophan residue in native albumin is thought to have limited access to water since its fluorescence is quenched by ionic quenchers (Ivkova et al. 1971; Burstein 1977). The edge excitation effect increases significantly on transition of albumin to the *F*-form in acid media. This may be the result of an increase in rigidity of the tryptophan environment as well as of increased screening from the solvent. The latter is the traditional explanation for the short wavelength emission.

Melittin at high ionic strength forms tetrameric structures. According to X-ray diffraction data the tryptophan residues have partial access to solvent (Terwilliger et al. 1982). The position of the fluorescence spectrum at short wavelengths (relative to unfolded melittin) and the substantial red-edge effect indicates the nanosecond rigidity of its environment. It is interesting to note that when the fluorescence probe 2-*p*-toluidinonaphthalene-6-sulfonate (TNS) is bound to melittin in a site close to the tryptophan residue, the substantial fluorescence shift on edge excitation is also observed (Demchenko 1985).

The dependence of the fluorescence shift (differences in positions of fluorescence maxima on excitation at 305 and 295 nm) on temperature for serum albumin and protease inhibitor are presented in Fig. 3. The results indicate only a small influence of temperature in the range 0° to 42 °C on the magnitude of this effect. Quite different behaviour is observed for indole and tryptophan in model viscous solutions (Demchenko and Ladokhin 1988). In glycerol in this temperature range the dipolar relaxation time changes by one order of magnitude and with its value being about τ_F, and the observed variation of the edge-excitation effect magnitude is about half of its maximal value. Therefore, it may be suggested that in the case of the proteins studied the relaxation time is longer than the emission rate. This is not inconsistent with the observation that the albumin fluorescence spectrum in the *N*-form is shifted from 341 to 326 nm on freezing in water (Permyakov and Burstein 1975). The dependence of fluorescence shifts on the temperature on freezing in water differs substantially from that described by Eq. (1) and, probably, reflects the cooperative changes in protein conformational dynamics associated with solvent crystallization. It should be kept in mind that the tryptophan environment in a protein molecule is not expected to be homogeneous relative to its dipolar relaxational properties and individual relaxation rates of different groups may cover several orders. Therefore (even with the absence of any dependence of the edge effect on the temperature in the range 0°–40 °C) one cannot exclude the possibility that its magnitude reflects only a part of the whole effect which could be observed upon complete immobilization of chromophore environment.

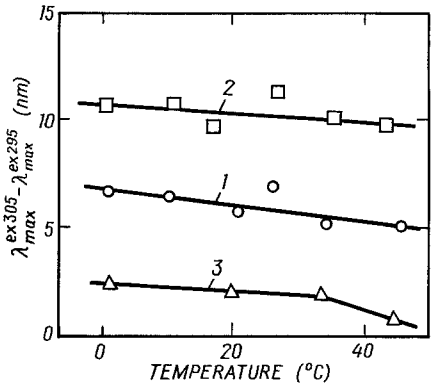

Fig. 3. Dependence of red-edge-excitation shifts of fluorescence spectra on temperature. *1:* albumin in 0.1 *M* glycine buffer, pH 3.2. *2:* albumin in 0.05 *M* *tris*-HCL buffer, pH 7.2 in the presence of 10^{-4} *M* sodium dodecylsulfate. *3:* protease inhibitor in 0.2 *M* glycine buffer, pH 2.9

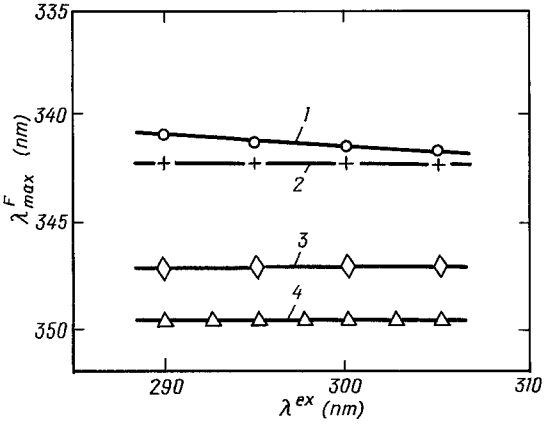

Fig. 4. Dependence of fluorescence maxima positions on excitation wavelength for single-tryptophan proteins emitting at long wavelength. *1:* β-casein in 0.04 *M* histidine buffer, pH 7.5. *2:* protease inhibitor in 0.05 *M* *tris*-HCL buffer, pH 7.0. *3:* myelin basic protein in 0.05 *M* *tris*-HCL buffer, pH 7.0. *4:* melittin in water. Temperature 25 °C

Thus, the shifts of fluorescence spectra upon red-edge excitation are observed for several proteins emitting in the medium wavelength range, they were classified by Burstein (1977) as proteins of classes I and II.

Proteins emitting in the longer-wavelength range (341–350 nm)

Of the proteins in this group we have studied β-casein, protease inhibitor at neutral pH, myelin basic protein and melittin at low ionic strength (Fig. 4). The data on the dependence of λ_{max}^{F} on λ^{ex} demonstrate that no shift upon red-edge excitation is observed for these proteins. These results are confirmed by observing no dependence of $I_{\lambda_1}/I_{\lambda_2}$ on excitation wavelength. The sig-

nificant Stokes shift and the absence of the red-edge effect indicate that the tryptophan residues in these proteins are located in a mobile rapidly relaxing dipolar environment, and $\tau_R \ll \tau_F$. According to Burstein (1977) the tryptophan residues emitting in the long wavelength range ($\lambda_{max} = 348-352$ nm) are surrounded by solvent water and are assigned to class III. They possess spectroscopic properties close to those of tryptophan in water, including high quenching rate by ionic quenchers. Our results agree with such an assignment. The dielectric relaxation time of free water is known to be $10^{-11}-10^{-12}$ s (Kuntz and Kauzmann 1974), which is substantially lower than τ_F. Differences in positions of fluorescence maxima between these proteins probably reflect the differences in interactions with neighbouring protein groups and water molecules whose motions are fast (sub-nanosecond). It may cause differences in effective dielectric constant and refractive index for the tryptophan environment. In the framework of Burstein's model of discrete classes of tryptophan residues, in β-casein and protease inhibitor at pH 7, besides class III there is a small contribution of class I, II or S chromophores.

Discussion

It is widely accepted that the fluorescence spectra of tryptophan residues in proteins do not depend on excitation wavelength. The choice of excitation wavelength in protein fluorescence studies is often arbitrary and ranges from the wavelength of the absorption maximum (275–280 nm) up to its far wavelength edge 305–310 nm. The choice of wavelength is sometimes determined by the necessity of excluding the contribution of tyrosine residues to protein emission (at $\lambda^{ex} >$ 290–295 nm) or of eliminating inter-tryptophan excitation-energy homotransfer due to Weber's red-edge effect (Weber and Shinitzky 1970) which may be achieved at $\lambda^{ex} > 295-300$ nm. Therefore the immediate conclusion from the results of this work is a warning: in proteins with sufficiently slow mobility of tryptophan environment the fluorescence spectra may depend substantially on the excitation wavelength which is a result of the effect of the red-edge-excitation shift. According to the data obtained for tryptophan in model solid and highly viscous solvents (Demchenko and Ladokhin 1988), this effect begins to be displayed at $\lambda^{ex} = 290$ nm and increases sharply as excitation shifts to longer wavelengths in the range 295–305 nm.

It should be noted that tyrosine emission can be observed in a number of proteins, and its contribution depends on excitation wavelength. According to our estimates for tyrosine and tryptophan in water the ratio of their absorption values is maximal at 275 nm ($\varepsilon^{Tyr}/\varepsilon^{Trp} = 0.26$), and decreases rapidly at longer wavelengths. At 280 nm, $\varepsilon^{Tyr}/\varepsilon^{Trp} = 0.21$, at 285 nm = 0.15, and at 290 nm = 0.025. It is lower than 0.01 at wavelengths longer than 295 nm. In proteins, the decrease of the tyrosine contribution to emission may cause the shift of fluorescence spectra to longer wavelengths. This shift takes place in the excitation range 275–290 nm and should be accompanied by a decrease in fluorescence bandwidth. Such a decrease was observed in studies of human serum albumin (Demchenko 1981), and no significant changes in bandwidth were found for excitation in the range 295–305 nm. These results demonstrate that in proteins with a substantial tyrosine contribution to emission the regions where this contribution is not observed and where the edge-excitation effect is observed partially overlap at 290–295 nm. In studies of proteins with no substantial tyrosine emission, 290 nm may be chosen as the excitation wavelength for routine recording of tryptophan fluorescence spectra.

In studies of indole and tryptophan in solid and viscous media (Demchenko and Ladokhin 1988) besides the postulated photoselection mechanism (Demchenko 1982) we have discussed other probable mechanisms which can result in the dependence of fluorescence spectra on excitation wavelength (the presence of any modified forms of tryptophans, photoselection between transitions to the ^1L_a and ^1L_b electronic states, specific complexes in the ground and excited states) and have concluded that they do not allow one to explain the whole effect. In proteins, in principle, there exists one more possibilities for generation of red-edge-excitation effects. Imagine two or several protein conformations with different tryptophan environments to be in equilibrium, and photoselection between them occurring on red-edge fluorescence excitation (there is a shift in absorption spectra between them). It should be noted that different factors can be responsible for shifts in absorption and emission spectra. The shifts of absorption spectra, as a rule, are determined by changes in electronic polarization of the medium, and on transition to a less polar medium with higher electronic polarization there is a shift no longer wavelengths (Demchenko 1986). The shifts of fluorescence spectra are to a greater extent (than the absorption ones) determined by dipolar interactions (Lakowicz 1983), and the fluorescence spectrum at longer wavelengths corresponds to higher polarity of environment. Thus, if on transferring melittin from a medium of low to one of high ionic strength, on complexing albumin with detergent and on changing to the acid form of protease inhibitor there occurs a shift of absorption spectra to longer wavelengths, which is actually the case, then the fluorescence spectra are shifted to shorter wavelengths. If two forms coexist in solution, red-edge excitation should photoselect the shorter wavelength-emitting form. However, we have

observed the opposite effect. Therefore in our case the chromophore microstates whose energy of dipolar interaction with the environment corresponds closest to the relaxed state are photoselected, and their emission is shifted to longer wavelengths with respect to the main emission.

One important area of protein research by fluorescence methods is the analysis of intramolecular dynamics (Burstein 1977, 1983; Lakowicz 1983; Demchenko 1986). One common way of extracting dynamic information from the steady-state spectra is to observe fluorescence shifts by inducing variations of τ_R and τ_F using fluorescence quenchers and variations of temperature or viscosity and then employing Eq. (1). In studies of native proteins the range of temperature variation is usually limited on the lower side by freezing of aqueous solvent and on the higher side by protein denaturation. Thus, for many proteins the range for studying the native state in solution is limited to 0°–60°, which corresponds to variations of relaxation times by one or two orders (Demchenko and Ladokhin 1988), and for some of them is more narrow. It follows from the theory presented above that the most significant dependence on temperature of the positions of emission maxima (Eq. 1) and of their red-edge excitation shifts (Eq. 3) should be observed when $\tau_R \approx \tau_F$. If spectra are independent of temperature, the cases $\tau_R \gg \tau_F$ and $\tau_R \ll \tau_F$ are difficult to distinguish by the positions of maxima alone. Nanosecond time-resolved spectroscopy is also ineffective in these cases since time-dependent shifts could not be observed. The shift upon red-edge excitation appears to be the most reliable criterion to resolve these two cases.

Employment of this criterion for our experimental data on single-tryptophan proteins shows, that the absence or incompleteness of reorientational relaxation of dipolar groups and, therefore, their slow dynamics in equilibrium is a rather general phenomenon in the studies of tryptophan residues in proteins emitting in the medium range (classes I and II according to Burstein (1977)). Fast relaxational mobility is typical of long-wavelength emitting proteins (class III). In the case of shorter wavelength emitting proteins (classes A and S), the dipole-orientational broadening of spectra is insufficient for photoselection of chromophores differing in their electronic transition energy and for the measurable red-edge-excitation effect, thus no definite result is obtained. The estimation of tryptophan environment polarity on the basis of the position of fluorescence spectra may be carried out only in limiting cases of very short or sufficiently long positions of spectra. In the intermediate cases the absence or incompleteness of dipole-orientational equilibrium of excited states should be considered, and simple experiments on the edge-excitation shifts of fluorescence spectra allow one to determine whether such a non-equilibrium situation exists in a specific case. Red-edge effects are easily observable in simple two-component chromophore solutions (Demchenko and Ladokhin 1988), and there is a need for systematic model studies on different environment properties (polarity, energy of dipolar interactions, the presence of specific bonding) in relation to these effects.

Red-edge-excitation spectroscopy is a method which supplements but does not substitute for other fluorescence approaches to protein dynamics. It can be combined with nanosecond time-resolved and lifetime (quenching)-resolved spectroscopy in order to gain a better understanding of the excited state phenomena and to differentiate dipole-reorientational relaxation from other reactions (Demchenko 1986). This can be easily done by applications of excitation-wavelength resolution into time-resolved and quenching experiments (Demchenko 1985; Demchenko and Shcherbatska 1985).

It should be stressed that the approximation of a protein interior by a homogeneous viscous medium on which the models of relaxation are based, is far from being realistic. In protein molecules the dynamic processes should be more complex in view of the variety of different mobile groups in size, correlation time and activation energy. There exists a hierarchy of motions, some of which are much faster and some are probably much slower than the excited state lifetime. Therefore there is an urgent need for methods that produce complementary information which will allow analysis of motions with different rates and testing of the relaxation models. Nanosecond time-resolved spectroscopy is of primary importance as a real-time method for studying dynamics in cases where the shifts of spectra occur on the nanosecond time scale. Red-edge excitation spectroscopy will probably prove to be advantageous in the analysis of relaxations beyond the nanosecond time range and especially in the detection of non-equilibrium excited states.

In conclusion we demonstrate that native proteins may show shifts of fluorescence spectra upon red-edge-excitation. This we interpret to indicate the existence, even within a single macro-conformation, of a population of microstates with different interaction energies of dipolar groups, and the existence of relaxational motion about an excited tryptophan residue occurring on the nanosecond or more slow time scale.

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References

Andreeva NA, Chermensky DN, Bezborodov AM (1978) Purification of extracellular trypsin inhibitor from Actinomyces janthinus 118. Appl Biochem Microbiol (Moscow) 14:25–31

Bakhshiev NG (1972) Spectroscopy of intermolecular interactions. Nauka, Leningrad

Bakhshiev NG, Mazurenko YuT, Piterskaya IV (1966) Luminescence decay in different portions of the luminescence spectrum of molecules in viscous solutions. Opt Spectrosc (USSR) 21:550–554

Bezborodova SI, Khodova OM, Stepanov VM (1983) Primary structure of *Aspergillus clavatus* ribonuclease C_2. Bioorg Chem (Moscow) 28:1136–1139

Burstein EA (1976) Luminescence of protein chromophores (Model studies). Ser Biophysica, vol 6. VINITI, Moscow

Burstein EA (1977) Intrinsic luminescence of proteins (origin and applications). Ser Biophysica, vol 7. VINITI, Moscow

Burstein EA (1983) Intrinsic protein luminescence as a method of studying rapid structural dynamics. Mol Biol (Moscow) 17:455–467

Burstein EA, Permyakov EA, Yashin VA, Burkhanov SA, Finazr-Argo A (1977) The fine structure of luminescence spectra of azurin. Biochim Biophys Acta 491:155–159

Demchenko AP (1981) Dependence of human serum albumin fluorescence spectrum on excitation wavelength. Ukr Biochim Z 53:22–27

Demchenko AP (1982) On the nanosecond mobility in proteins. Edge excitation fluorescence red shift of protein-bound 2(*p*-toluidinylnaphthalene)-6-sulfonate. Biophys Chem 15: 101–109

Demchenko AP (1984) Structural relaxation in protein molecules studied by fluorescence spectroscopy. J Mol Struct 114: 45–48

Demchenko AP (1985) Fluorescence molecular relaxation studies of protein dynamics. The fluorescence probe binding site of melittin is rigid on nanosecond timescale. FEBS Lett 182: 99–102

Demchenko AP (1986) Ultraviolet spectroscopy of proteins. Springer, Berlin Heidelberg New York

Demchenko AP, Ladokhin AS (1988) Red-edge-excitation fluorescence spectroscopy of indole and tryptophan. Eur Biophys J 15:369–379

Demchenko AP, Shcherbatska NV (1985) Nanosecond dynamics of the charged fluorescent probes at the polar interface of membrane phospholipid bilayer. Biophys Chem 22: 131–143

Eftink MR, Ghiron CA (1977) Exposure of tryptophanyl residues in proteins. Quantitative determination of fluorescence quenching studies. Biochemistry 16:5546–5551

Filenko AM, Zyma VL (1981) Two-wavelengths method for protein fluorescence small spectral shift registration. Mol Genet Biophys (Kiev) 6:126–135

Galley MC, Purkey RM (1970) Role of heterogeneity of the solvation site in electronic spectra in solution. Proc Natl Acad Sci USA 67:1116–1121

Grinvald A, Steinberg IL (1974) Fast relaxation processes in a protein revealed by the decay kinetics of tryptophan fluorescence. Biochemistry 13:5170–5177

Hazan G, Haas E, Steinberg IZ (1976) The fluorescence decay of human serum albumin and its subfractions. Biochim Biophys Acta 434: 144–153

Hershberger MV, Maki AH, Galley WC (1980) Phosphorescence and optically detected magnetic resonance studies of a class of anomalous tryptophan residues in globular proteins. Biochemistry 19:2204–2209

Inoue J, Sase S, Rhujo R, Nagaoka S, Sogami M (1979) Interaction between bovine plasma albumin and sodium dodecyl sulfate studied by means of ^{13}C-NMR spectra. Biopolymers 18:373–382

Ivkova MN, Vedenkina NS, Burstein EA (1971) Fluorescence of tryptophan residues of serum albumin. Mol Biol (Moscow) 5:214–224

Kamalyan MG, Nalbandyan RM (1977) Optical and magnetic properties of azurin from *Pseudomonas aeruginosa*. Biochimia (Moscow) 42:223–229

Konev SV (1967) Fluorescence and phosphorescence of proteins and nucleic acids. Plenum Press, NewYork

Kuntz ID Jr, Kauzmann W (1974) Hydration of proteins and polypeptides. Adv Protein Chem 28:239–345

Lakowicz JR (1983) Principles of fluorescence spectroscopy. Plenum Press, New York London

Lakowicz JR, Balter A (1982) Direct recording of the initially excited and the solvent relaxed fluorescence emission spectra of tryptophan by phase sensitive detection of fluorescence. Photochem Photobiol 36:125–132

Lakowicz JR, Cherek H, Bevan DR (1980) Demonstration of nanosecond dipolar relaxation in biopolymers by inversion of apparent fluorescence phase shift and demodulation lifetimes. J Biol Chem 255:4403–4406

Longworth JW (1971) Luminescence of polypeptides and proteins. In: Steiner RF, Weinryb I (eds) Excited states of proteins and nucleic acids. Plenum Press, New York London, pp 319–487

Ludescher RD, Volwerk JJ, de Haas GH, Hudson BS (1985) Complex photophysics of the single tryptophan of porcine pancreatic phospholipase A_2, its zymogen and an enzyme/micelle complex. Biochemistry 24:7240–7250

Lumry R, Hershberger M (1978) Status of indole photochemistry with special reference to biological application. Photochem Photobiol 27:819–840

Macgregor RB, Weber G (1981) Fluorophores in polar media. Spectral effects of the Langevin distribution of electrostatic interactions. Ann NY Acad Sci 366:140–154

Maulet Y, Mathey-Prevot B, Kaiser Y, Ruegy UT, Fulpius BW (1980) Purification and chemical characterization of melittin and acetylated derivatives. Biochim Biophys Acta 625: 274–280

Mazurenko YuT, Bakhshiev NG (1970) The influence of orientational dipolar relaxation on spectral, temporal and polarizational properties of luminescence in solutions. Opt Spectrosc (USSR) 28:905–913

Nyamaa D, Bat-Erdene O, Burstein EA (1985) The medium effects on functional and structural properties of serum albumin. III. Effect of temperature and ionic strength on the $N_1 - F$, $F_1 - F_2$ and $F_2 - E$ transitions of human serum albumin. Biophysica (Moscow) 19:833–840

Permyakov EA, Burstein EA (1975) Relaxation processes in frozen aqueous solution of proteins: temperature dependence of fluorescence parameters. Stud Biophys 51:91–103

Permyakov EA, Ostrovsky AV, Burstein EA, Pleashanov PG, Gerday Ch (1985) Parvalbumin conformers revealed by steady-state and time-resolved fluorescence spectroscopy. Arch Biochem Biophys 240:781–791

Rubinov AN, Tomin VI (1970) Bathochromic luminescence in low-temperature solutions of dyes. Opt Spectrosc (USSR) 29:1082—1086

Rubinov AN, Tomin VI (1984) Inhomogeneous broadening of electronic spectra of organic molecules in solid and liquid solutions. Preprint N 348, Institute of Physics, Minsk USSR

Teale FWJ (1960) The ultraviolet fluorescence of proteins in neutral solutions. Biochem J 76:381–388

Terwilliger TC, Weissman L, Eisenberg D (1982) The structure of melittin in the form I crystals and its application for melittin's lytic and surface activities. Biophys J 37:353–361

Turoverov KK, Kuznetsovy IM, Zaitsev VN (1984) Azurin UV-Fluorescence interpretation on the basis of X-ray data. Bioorgan Chem (Moscow) 10:792–806

Vladimirov YuA, Burstein EA (1960) Luminescence spectra of aromatic amino acids and proteins. Biophysica (Moscow) 5:385–392

Weber G, Shinitzky M (1970) Failure of energy transfer between identical aromatic molecules on excitation of the longwave edge of the absorption spectrum. Proc Natl Acad Sci USA 65:823–830