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ON THE NANOSECOND MOBILITY IN PROTEINS

EDGE EXCITATION FLUORESCENCE RED SHIFT OF PROTEIN-BOUND 2-(*p*-TOLUIDINYLNAPHTHALENE)-6-SULFONATE

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The fluorescence spectra of 2-(*p*-toluidinylnaphthalene)-6-sulfonate associated with β-lactoglobulin, β-casein, and bovine and human serum albumins are shown to depend on excitation wavelength. A long-wave shift of the spectra is observed at the long-wave edge excitation, reaching 10 nm and above. A similar phenomenon is found in glucose glass and in glycerol at +1°C, i.e., in systems with delayed dipolar solvent relaxation, but not in liquid solutions. This phenomenon is proposed to be based on relaxation processes in the excited state. There exists a distribution of chromophore microstates with different interactions with surrounding groups which results in heterogeneous broadening of the electronic spectra and allows photoselection of a part of this distribution, being characterized by a low transition energy. The fast structural relaxation results in an altered distribution and, if this is the case, the effect of edge excitation of fluorescence spectra is not observed. If the structural relaxation during the excited state lifetime is absent, this effect is maximal. This interpretation is in agreement with results on the influence of red edge excitation on the low-temperature fluorescence spectra of dyes and with the data on time-resolved nanosecond fluorescence spectroscopy. The results of this work strongly support the significant dye fluorescence spectral shifts on protein binding, being determined not only by polarity changes in their environment, but also by relaxation properties of protein groups in this environment. These results also indicate that on the nanosecond time scale, the structural relaxation around the excited chromophore in proteins may be incomplete.

1. Introduction

The functional properties of protein molecules appear to depend upon their dynamic properties on the nanosecond time scale [1–3]. Fluorescence spectroscopic methods that allow the study of protein nanosecond dynamics are widely used. They are based on different phenomena which occur during the lifetime of the excited chromophore: rotation of the chromophore group itself, relaxation of surrounding dipolar and fluorescence-quenching groups, diffusion of low molecular weight quenchers through the protein matrix [2,4]. One of the most developed trends in these studies is the application of extrinsic fluorescent probes (reporter groups) which are associated with proteins and provide information about the physical properties of binding centers [5].

The fast (compared to the excited state lifetime) motions of protein groups result in dipolar relaxation around chromophore dipoles, the magnitude and direction of which alter on excitation. The above-mentioned relaxation results in the long-wave shift of the fluorescence spectrum. If the environment of an excited chromophore is rigid enough, the dipolar relaxation time τ_R is comparable with or longer than the excited state lifetime τ_F. In this case, emission proceeds from the excited

Abbreviation: 2,6-TNS, 2-(*p*-toluidinylnaphthalene)-6-sulfonate.

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state which is not in equilibrium with respect to dipolar solvent relaxation, and the emission is blue-shifted [6,7]. The application of time-resolved emission spectroscopy allows direct observation of the long-wave shift of fluorescence spectra with time in cases when dipolar relaxation proceeds during the excited state lifetime [5,8–12].

The delayed dipolar relaxation may be responsible for another phenomenon—the red shift of steady-state fluorescence spectra if excitation is performed at the red edge of the absorption spectrum (edge excitation red shift) [13–22]. Such a shift was observed in the low-temperature fluorescence spectra of different dyes, both in solid solutions and in polymer matrix.

This paper presents observations on the edge excitation red shift in the emission spectra of a fluorophore at ambient temperature on its association with protein molecules. The edge excitation red shift of a fluorescent probe for protein structure indicates a decreased mobility of protein groups in the probe-binding center and may be used in the study of this phenomenon.

2. Materials and methods

Crystallized preparations of bovine serum albumin (Koch-Light), human serum albumin (Sigma) and β-lactoglobulin from cow's milk (Serva) were used without additional purification. A chromatographically and electrophoretically homogeneous preparation of β-casein from cow's milk was kindly provided by D.S. Yankovsky, Ukrainian Institute of Meat and Milk Industry, Kiev. Molar protein concentrations were determined from the molecular weight and specific extinction values: bovine serum albumin (M_r = 69000; $E^{1mg/ml}_{278nm} = 0.67$), human serum albumin ($M_r = 70000$, $E^{1mg/ml}_{279nm} = 0.53$), β-lactoglobulin ($M_r = 35500$, $E^{1mg/ml}_{280nm} = 0.95$ and β-casein (M_r = 23000; $E^{1mg/ml}_{280nm} = 0.46$).

2,6-TNS (crystalline, pure) was produced by Serva. 2,6-TNS concentrations were determined from the absorbance at 366 nm ($\epsilon_{366nm} = 4.08 \times 10^3$ mol^{-1} [23]).

Chemically pure glycerol (Reakhim, U.S.S.R.) was redistilled. Glucose was of reagent purity (Reakhim, U.S.S.R.). Glucose glasses were prepared as described elsewhere [24].

Excitation and fluorescence spectra were recorded on a Hitachi MPF-4 fluorescence spectrophotometer. The spectral bandwith of the excitation monochromator was 2 nm and that of the fluorescence monochromator 2–5 nm. The scan speed was 30 nm/min. The spectra were not corrected. The fluorescence spectra of solutions were recorded in rectangular silica cells in a thermostatically controlled cell holder. Glucose glass samples were studied using a solid sample holder (Hitachi) with frontal geometry of excited and fluorescence beams.

3. Results

3.1. 2,6-TNS complexes with β-lactoglobulin, serum albumins and β-casein

The influence of excitation wavelength on the fluorescence spectra of 2,6-TNS complexes with β-lactoglobulin, β-casein, and bovine and human serum albumins was studied (table 1). The binding of the hydrophobic probes 2,6-TNS and 8-anilinonaphthalene-1-sulfonate by these proteins is known to be very strong and results in a dramatic increase in quantum efficiency and a significant short-wave shift of the dye fluorescence spectra [23,25]. The proteins were studied under conditions of stability in their native conformation. The molar protein/dye ratio was selected to be sufficiently high to guarantee the complete binding of the probe, but not to distort significantly the excitation spectrum due to absorption of intrinsic protein chromophores.

The emission spectra of 2,6-TNS associated with β-lactoglobulin (fig. 1) and human serum albumin (fig. 2) depend significantly on the excitation wavelength λ_{ex}. This dependence is complex. No differences are observed if the fluorescence spectra excited at the excitation spectrum maxima 280, 322 and 360 nm are compared. There is a small red shift at λ_{ex} 340–350 nm and a significant gradual red shift at $\lambda_{ex} > 360$ nm. If λ_{ex} is increased to 400–405 nm this shift increases and fails to reach any definite limit. The studies at

Table 1

The changes in the emission spectra of 2,6-TNS bound to different proteins at the mean (360 nm) and edge (390–400 nm) excitation

Protein	Protein/dye molar ratio	λ_{ex}^{360nm} (max) (nm)	λ_{ex}^{400nm} (max) − λ_{ex}^{360nm} (max) (nm)	$\Delta\lambda_{ex}^{390nm} - \Delta\lambda_{ex}^{360nm}$ (nm)
β-Lactoglobulin [a]	3.7	416	14	−8
β-Casein [b]	3.3	432	7	−3
Bovine serum albumin [a]	4.5	432	8	3
Human serum albumin [a]	3.8	432	13	−2

[a] In 0.05 M Tris-HCl, pH 7.0.
[b] In 0.02 M histidine, pH 7.5.

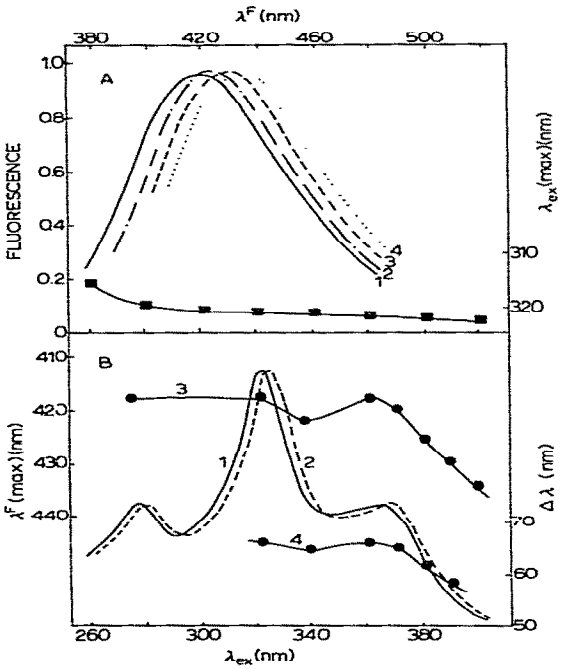

Fig. 1. The influence of excitation wavelength on the fluorescence spectra of 2,6-TNS associated with β-lactoglobulin. (A) The peak-normalized fluorescence spectra at different excitation wavelengths λ_{ex} [(1) 360 nm, (2) 380 nm, (3) 390 nm, (4) 400 nm] and the dependence of the excitation maximum on emission wavelength (■———■). (B) The excitation spectra at emission wavelengths 420 nm (1) and 520 nm (2). The dependence of maxima λ(max) (3) and bandwidth Δλ (4) of fluorescence spectra on λ_{ex}. Protein concentration, 0.4 mg/ml; 2,6-TNS, 3×10^{-5} M. 0.05 M Tris-HCl, pH 7.0; 20°C.

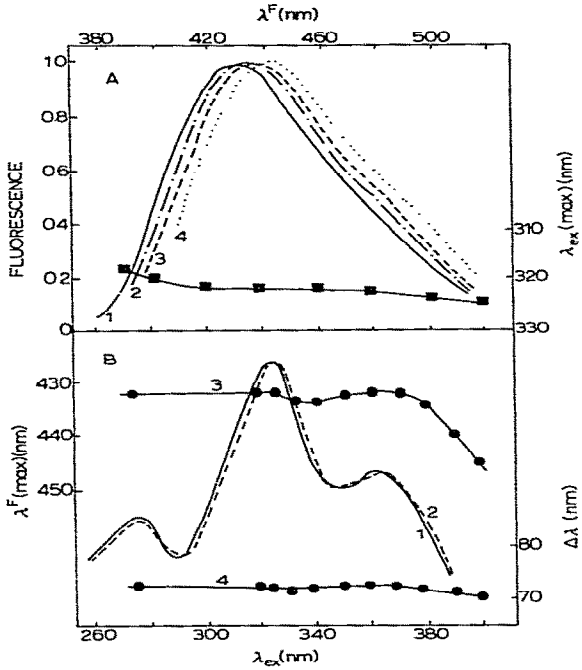

Fig. 2. The influence of excitation wavelength on the fluorescence spectra of 2,6-TNS associated with human serum albumin. (A) The peak-normalized fluorescence spectra at different excitation wavelengths λ_{ex} [(1) 360 nm, (2) 380 nm, (3) 390 nm, (4) 400 nm] and the dependence of the excitation maximum on emission wavelength (■———■). (B) The excitation spectra at emission wavelengths 430 nm (1) and 510 nm (2). The dependence of maxima λ(max) (3) and bandwidth Δλ (4) of fluorescence spectra on λ_{ex}. Protein concentration, 2.6 mg/ml; 2,6-TNS, 10^{-5} M. 0.05 M Tris-HCl, pH 7.0; 20°C.

$\lambda_{ex} > 400$ nm are complicated by scattered light and low fluorescence intensity. Therefore, the difference was chosen between the positions of the fluorescence maxima on excitation at 400 and 360 nm λ_{ex}^{400nm} (max) $- \lambda_{ex}^{360nm}$ (max) as a simple parameter to characterize the magnitude of the effect. The data presented in table 1 show that the edge excitation red shift in the proteins studied is of the order of 10 nm.

The fluorescence spectrum half-maximum bandwidth $\Delta\lambda$ of 2,6-TNS associated with β-lactoglobulin and human serum albumin decreased on edge excitation (figs. 1 and 2). The same is observed for β-casein (table 1). In the case of bovine serum albumin, on shifting of the main fluorescence band its width tends to decrease, but the spectrum long-wave shoulder is observed which appears to increase the total bandwidth.

Could the effect of edge excitation be the result of probe binding at different protein sites and resulting heterogeneity of absorption and emission? The photoselection of one of the emitting components at the edge excitation requires significant differences in the absorption (and excitation) spectra of emitting species, which is not the case. In our experiments, the differences between the absorption spectra of free and protein-bound 2,6-TNS are not great (the shift being 1–1.5 nm and the extinction ratio at the wavelengths of fluorescence excitation not exceeding 1.1). The spectral differences between chromophores bound at different sites are expected to be even smaller than those between bound and unbound ones and are insufficient for the induction of edge excitation photoselection of significant magntiude.

An important finding is that not only the fluorescence spectral shift on edge excitation, but also the excitation spectra are shifted at the short-wave and long-wave edge emission (figs. 1 and 2). The latter shift is much smaller but quite distinct. The shift is to the red at the long-wave edge emission and to the blue at the short-wave edge. It is not followed by changes in shape of the excitation spectrum. At the short-wave edge emission it reaches several nanometers. It is much greater than the spectral difference between the excitation (or absorption) spectra of free and bound 2,6-TNS. Thus, the edge emission shift of the excitation spectra can hardly be considered as the result of photoselection of chromophores bound at different sites and possessing different absorption properties.

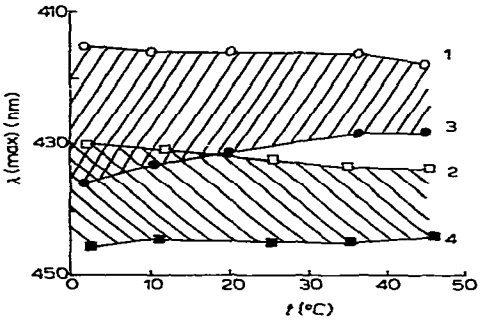

Fig. 3. The dependence of the maxima of the fluorescence spectra of 2,6-TNS associated with β-lactoglobulin (1, 3) and human serum albumin (2, 4) at excitation 360 nm (1, 2) and 400 nm (3, 4) on temperature.

The edge excitation fluorescence red shift is temperature dependent. In the studies of β-lactoglobulin and human serum albumin the effect decreases as the temperature is elevated (fig. 3). Both the long-wave shift of fluorescence spectra excited at the absorption maximum and the short-wave shift at the edge excitation are observed as the temperature rises. The serum albumin binding constant of 8-anilinonaphthalene-1-sulfonate, which is similar to that of 2,6-TNS, is temperature independent within a wide range of protein dye ratios [25]. Thus, the observed effects of temperature may be considered to result specifically from changes in the environmental properties of the bound dye.

3.2. Model studies of 2,6-TNS in isotropic solvents

Fig. 4 presents results of 2,6-TNS studies in glucose glass. On excitation in the maxima of the excitation spectrum the maximum of the fluorescence spectrum is at 421–421.5 nm. Fluorescence excitation at the red edge of the spectrum results in a significant long-wave fluorescence shift (λ_{ex}^{400nm} (max) $- \lambda_{ex}^{360nm}$ (max) $= 12$ nm). Furthermore, in a region of excitation wavelengths that corresponds to the red edge of the 322 nm band (340–350 nm)

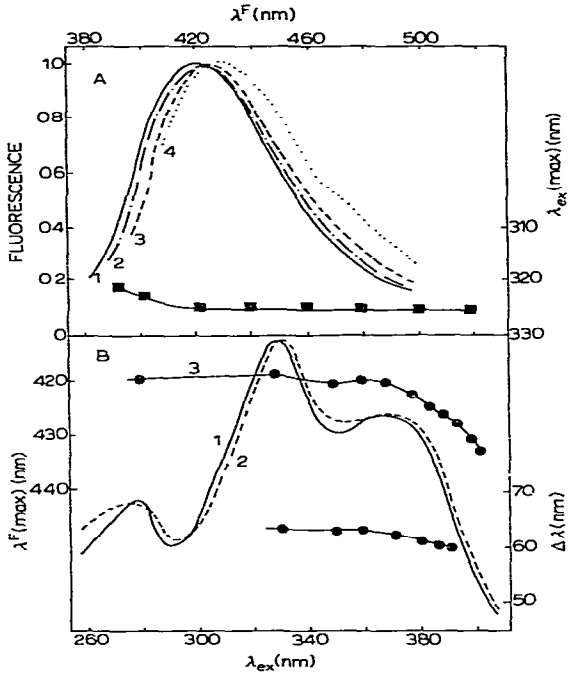

Fig. 4. The influence of excitation wavelength on the fluorescence spectra of 2,6-TNS in glucose glass at 20°C. (A) The peak-normalized fluorescence spectra at different excitation wavelengths λ_{ex} [(1) 360 nm, (2) 380 nm, (3) 390 nm, (4) 400 nm] and the dependence of the excitation maximum on emission wavelength (■———■). (B) The excitation spectra at emission wavelengths 430 nm (1) and 510 nm (2). The dependence of maxima λ(max) (3) and bandwith (4) of fluorescence spectra on λ_{ex}. 2,6-TNS concentration, 10^{-4} M.

a small fluorescence red shift is also observed.

The decrease in fluorescence bandwidth on red edge excitation was found in the spectra of 2,6-TNS in glucose glass. ($\Delta\lambda_{ex}^{310nm} - \Delta\lambda_{ex}^{360nm} = -4$ nm). On cooling and glass formation of glucose, the structural and particularly dipolar relaxation time is known to increase significantly, which is supported by a 5-fold decrease in the dielectric constant [26] and a large short-wave shift of the fluorescence spectra of dissolved dyes [27]. Thus, in this system the equilibrium orientation of dipoles around the excited chromophore dipole is not reached in the excited state lifetime. This property of chromophore environment is apparently responsible for the observed fluorescence red shift at the edge excitation.

The data presented in fig. 5 show that the long-wave fluorescence shift on edge excitation is observed in the studies of 2,6-TNS in glycerol at +1°C (λ_{ex}^{400nm} (max) − λ_{ex}^{360nm} (max) = 10 nm). The shift is followed by a decrease in fluorescence bandwidth ($\Delta\lambda_{ex}^{390nm} - \Delta\lambda_{ex}^{360nm} = -4$ nm). In glycerol at 36°C the 2,6-TNS fluorescence spectrum is significantly shifted to the red (λ(max) = 453 nm), and no edge excitation shift or change of bandwidth is observed. These results are in agree-

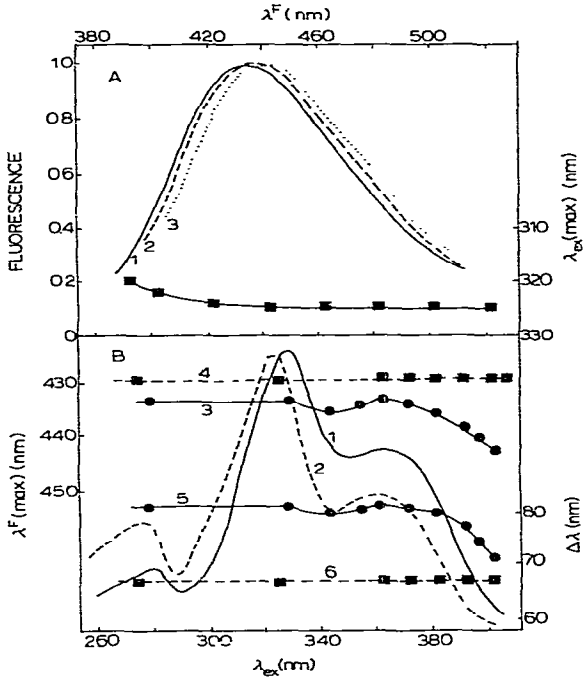

Fig. 5. The influence of the excitation wavelength on fluorescence spectra of 2,6-TNS in glycerol and ethanol. (A) The peak-normalized fluorescence spectra at different excitation wavelengths λ_{ex} [(1) 360 nm, (2) 390 nm, (3) 400 nm] and the dependence of excitation maximum on emission wavelength in glycerol at +1°C. (B) The excitation spectra at emission wavelength 430 nm in glycerol at +1°C (1) and 80% aqueous ethanol at 20°C (2). The dependence of maxima λ(max) (3, 4) and bandwidth (5, 6) of fluorescence spectra of 2,6-TNS in glycerol at +1°C (3, 5) and 80% aqueous ethanol (4, 6) on λ_{ex}. 2,6-TNS concentration, 3×10^{-5} M.

ment with published data [8,10,11,20] showing rapid dipolar solvent relaxation in glycerol at elevated temperature, but the decrease in temperature to 0°C causes, the delayed dipolar solvent relaxation that influences the fluorescence spectroscopic data. During the excited state lifetime, the energetic equilibrium in the excited state with respect to solute-solvent dipolar interactions is not attained. The edge excitation fluorescence shift in the studies of 2,6-TNS solutions at ambient temperature in alcohols, glycols and esters, i.e., in solvents that differ significantly in their dielectric constant and refractive index and therefore are commonly used for the determination of 'polarity scales' relating solvent polarity and positions of maxima of fluorescence spectra [21,26], is not observed. As an illustration, the data on 2,6-TNS in 80% aqueous ethanol are presented in fig. 5. In a wide range of excitation wavelengths both the position of the maximum and the bandwith of the fluorescence spectrum are not altered.

The position of the maximum of the 2,6-TNS fluorescence spectrum in 80% ethanol almost corresponds to that of 2,6-TNS on binding with β-casein, bovine and human serum albumins (table 1). The existence of an edge excitation fluorescence shift in the latter case shows that the spectral properties of a fluorescent probe bound to proteins and in liquid isotropic solvents are not equivalent, and that the solvent polarity scale [21,28,29] is insufficient to describe the spectral properties of a fluorescent probe associated with proteins.

4. Discussion

In general, fluorescence spectra in a condensed medium do not depend on the excitation wavelength owing to fast and effective internal conversion and vibrational relaxation processes in the excited state. On emission, the distribution of chromophores on vibrational levels is determined by thermal equilibrium with the medium and does not depend on the excitation wavelength. The edge excitation fluorescence red shift is a special case that depends on a specific chromophore and its environment [13–22]. Our own and literature data suggest that the following conditions are required for this to occur: chromophore-environment dipolar interactions and distribution of chromophores according to different energies of interaction resulting in heterogeneous broadening of the spectra; change in chromophore dipolar moment on excitation; absent or small dipolar relaxation during the excited state lifetime. These requirements appear to be met by 2,6-TNS binding to the proteins studied. The dipolar moment of 2,6-TNS changes significantly on excitation—by 44 debye [5]. The orientation of the polar groups of a protein structure, rigid on the nanosecond scale, as well as the firmly bound water molecules that are relatively immobile may exist in the probe binding center.

Rubinov and Tomin [14] and Itoh and Azumi [17] suggested the mechanism of edge excitation fluorescence shift to be based on the distribution of chromophores with respect to electronic excitation energy. Both in the ground and excited states there exists a statistical distribution of chromophores on their dipolar interactions with the environment. The differences in the field of chromophore environment cause the shifts of their absorption and fluorescence excitation spectra and allow photoselection of certain chromophores with chromophore-environment interactions divergent from the mean. In particular, this may be realized at the red edge of the excitation spectrum where the dependence of fluorescence properties on excitation wavelength may appear.

The model of this phenomenon, presented here, extends the ideas of Rubinov and Tomin [14], Itoh and co-workers [17,18] and others [19–22]: the excited state distribution on excitation is determined by the ground state distribution of the magnitude and configuration of the field of dipoles. If the dipolar relaxation time τ_R is less than the excited state lifetime τ_F, the equilibrium excited state distribution is attained as a result of relaxation and no edge excitation effect is observed. In the case $\tau_R > \tau_F$, the former distribution is 'frozen' and does not alter the excited state lifetime. Fluorescence proceeds from this population of nonequilibrium states and depends on excitation energy.

The scheme presented in fig. 6 illustrates the

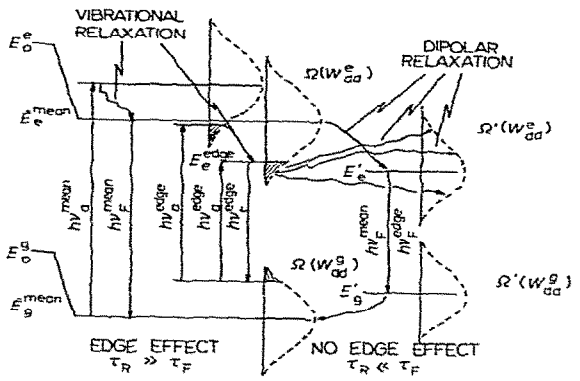

Fig. 6. Diagram of energy levels illustrating the influence of relaxation phenomena on fluorescence spectra and the origin of edge excitation fluorescence red shift.

model and helps discussed some of its consequences in the light of our experiments. It is a conventional diagram of energy levels which takes account of relaxational phenomena and statistical distribution of chromophores on their interaction energy with dielectric environment. The energy of any ground or excited vibronic level may be determined from $E = E_0 + \Omega(W_{dd})$, where E_0 is the energy of the unperturbed level and $\Omega(W_{dd})$ the distribution of perturbations on the energy of dipolar interactions with the environment. In the ground state, $W_{dd}^g \propto (\bar{\mu}_g \cdot \langle \bar{\mu}_m \rangle)$ and in the excited state $W_{dd}^e \propto (\bar{\mu}_e \cdot \langle \bar{\mu}_m \rangle)$, where $\bar{\mu}_g$ and $\bar{\mu}_e$ are the chromophore dipolar moments in the ground and excited states, respectively, and $\langle \bar{\mu}_m \rangle$ is the mean effective dipolar moment of the medium.

In the ground and excited states the chromophore dipolar moment vectors $\bar{\mu}_g$ and $\bar{\mu}_e$ may differ not only in their magnitude, but also in their direction. Edge excitation is the case for which the absolute value of W_{dd} is minimal for the ground level E_g^{edge} and maximal for the excited level E_e^{edge}. In this situation the chromophores are photoselected with such configurations of their environment dipoles that their dipolar interactions with the environment are minimal in the ground and maximal in the excited state.

At ambient temperature, the characteristic times for vibrational relaxation are 10^{-11}–10^{-12} s, and solvent dipolar relaxation is the slowest energetic process in the excited state. Let us consider the excited level $\bar{E}_e$ which is at vibrational equilibrium, but not at equilibrium with respect to dipolar interaction of chromophore with dielectric environment. On excitation by a quantum of sufficient energy ($h\nu_a^{mean} \geqslant E_e^{mean} - E_g^{mean}$), the whole population of chromophores that differ in interaction energy with the environment is excited. If excitation by the low energy quanta $h\nu_a^{edge}$ (at the red edge) is performed, the chromophores with the ground state energy above E_g^{edge} and the excited state energy under E_e^{edge} are photoselected (dashed zones, fig. 6). In the absence of dipolar relaxation at the time τ_F, the energy of the emitted quanta is also low, less than $h\nu_F^{mean}$, and the edge excitation fluorescence red shift is observed. If the structural dipolar relaxation is a fast process compared to the excited state lifetime, the redistribution in the chromophore-environment interaction energy will also be fast. As a result, the chromophore, excited even by low-energy quanta, 'forgets about it', and the distribution of emitted quanta on their energy becomes independent on excitation wavelength.

The model presented is at variance with that of Azumi et al. [18]: there is no ground to believe that the limiting energy value of emitted quantum at the edge excitation $h\nu_F^{edge}$ at $\tau_R \gg \tau_F$ is the energy of a quantum emitted on transition between the two most probable solvent dipolar interaction-dependent energy states $\bar{E}_e'$ and $\bar{E}_g'$ at $\tau_R \ll \tau_F$. Unlike $\bar{E}_e'$, E_e^{edge} is a virtual level, the displacement of which alters the rigidity of photoselection. It follows that in the case of ideal selection of one vibrational sublevel, the shift of the fluorescence spectrum as λ_{ex} increases will approach, but never exceed, the shift of λ_{ex}. Such significant unlimited shifts were observed recently [21] in the spectra of anthracene derivatives at 77 K.

The correlation between the magnitude of fluorescence shifts at mean excitation on melting of solid chromophore solution (transition from $\tau_R \gg \tau_F$ to $\tau_R \ll \tau_F$ environment conditions) [7,27] and on transition to edge excitation is not simple. The former shift is determined by the magnitude of change in the chromophore dipolar moment on excitation [31]. The latter one may depend, to a greater extent, on the change in its direction. As a

result, the distributions $\Omega(W_{dd}^{g})$ and $\Omega(W_{dd}^{e})$ differ and within these distributions there are the chromophores whose energy of interaction with the environment alters from minimal to maximal on excitation. It was shown recently that the edge excitation shift in the laser-excited fluorescence spectra of rhodamines G and 4C, which are known to change slightly the magnitude of their dipolar moments on excitation, is much greater than the mean excitation fluorescence shift on softening and melting of solvent glass [22]. In this case E_e^{edge} is situated lower than $\bar{E}_e'$.

It follows from the scheme presented that on edge excitation, only part of the original distribution participates in emission. Thus, a decrease in spectral bandwidth should be expected. In fact, this phenomenon is observed in experiments with 2,6-TNS in model media and on association with proteins, and was indicated by others [17,19,20] in different chromophore-solvent systems.

Apart from the changes in position and half-width of fluorescence spectra at edge excitation, small changes in these parameters are observed at 340–350 nm, i.e., on the long-wave edge of the vibrational spectral component at 322 nm. Being very fast compared to dipolar relaxation, the vibrational relaxation does not alter the distribution of chromophores on their interaction energy with the environment, and on excitation at the long-wave edge of a higher vibrational sublevel of the vibronic band it results in a transition to the long-wave edge of the lowest sublevel with subsequent emission of quanta. A similar phenomenon was found in anthracene spectra in polymer films [19].

The selection of the high and low interaction energy part of the distribution $\Omega(W_{dd}^{e})$ may be performed in fluorescence, and a dependence of excitation spectra on the emission wavelength may appear. Pavlovich [30] observed the short-wave shift of excitation spectra of phthalimide derivatives using short-wave fluorescence detection. This phenomenon is also found in our studies on 2,6-TNS, both in solvents with slow structural relaxation and on association with proteins. According to the model, the short-wave fluorescence emission selects the lowest (high interaction energy) part of the ground state distribution and the highest (small interaction energy) part of the excited state distribution. The long-wave emission selects the opposite parts of these distributions. The steep, short-wave part of the fluorescence spectrum allows higher selectivity. Therefore, the shift of excitation is larger at the short-wave emission.

For the sake of simplicity, the model considers only the extreme cases $\tau_R \gg \tau_F$ and $\tau_F \ll \tau_F$. In the intermediate case $\tau_R \approx \tau_F$, the edge-excitation and edge-emission effects also exist, as indicated by their presence in glycerol at +1°C. Studies of the magnitude of the effects in this case may in principle be applied for the determination of τ_R.

The edge excitation fluorescence shift is related to the time-dependent spectral shift observed by means of nanosecond time-resolved emission spectroscopy [5,8–12] and reflects the same phenomenon, i.e., the dynamic orientation of surrounding groups around the excited chromophore. A time-dependent shift may be noticed if τ_R is of the order of τ_F (not exceeding $3\tau_F$ [8]). If $\tau_R \gg \tau_F$, there is no time-dependent shift, but the edge excitation shift is expected to reach its maximum. In glycerol at about 0°C for different chromophores, τ_R and τ_F are of similar magnitude. This allows the observation of both the time-dependent shift for aminophthalimides [8], 8-anilinonaphthalene-1-sulfonate [10] and 2,6-TNS [10,11], and the edge excitation shift for phthalimides [20] and 2,6-TNS (fig. 5 of this work). A time-dependent shift was observed in 2,6-TNS associated with bovine serum albumin [11]. For this protein-dye complex the edge excitation is demonstrated here. Both methods shows that in the associates of 2,6-TNS with proteins, in contrast to fluid solutions of this dye, the solvent relaxation is not sufficiently rapid and fluorescence does not reflect the energetics of the excited state in equilibrium with the dielectric environment. On association with proteins the fluorophores behave as they do in glycerol or glucose glass.

Fluorescent probes are widely used in studies of proteins, protein associates and membranes [5,29]. Probes such as 2,6-TNS and 8-anilinonaphthalene-1-sulfonate are often referred to as 'hydrophobic probes' beause of the similarity in the probe fluorescence parameter changes (the short-wave shift and increase in quantum efficiency) on binding

and on transition to solvents of decreased polarity and thus higher 'hydrophobicity'. However, similar alterations of spectra may be observed in the case of delayed dipolar relaxation of surrounding groups. Studies on time-dependent spectral shifts [5,11,12] indicate that the delayed dipolar relaxation is the key process in producing the spectral effect on binding. In this study, we demonstrate that polarity shift and 'rigidity shift' may, in principle, be distinguished if excitation is performed at the red edge. More work is required to establish quantitative correlations between the fluorescence parameters at the 'mean' and 'edge' excitation, and rigidity and polarity of the binding center. However, one may argue that the environment of 2,6-TNS associated with β-lactoglobulin is more hydrophobic than that with human serum albumin because in the former case the position of the fluorescence maximum is more short-wave and there is no significant difference in the edge excitation effect between them.

The edge excitation fluorescence red shift may also be observed for the intrinsic tryptophan fluorescence in proteins. This phenomenon was found to exist in the fluorescence spectra of the sole tryptophan residue of human serum albumin. The effect increases on transition of this protein from N- to F-form and especially on sodium dodecyl sulfate binding [32],

In conclusion, the edge excitation fluorescence red shift is related to excited state processes on the nanosecond time scale. It may represent a new approach in the study of dipolar relaxation in proteins and may be a source of information on the polarity and rigidity of probe environment.

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