Biophysical Chemistry, 48 (1993) 39–48
Elsevier Science Publishers B.V., Amsterdam

BIOCHE 1783

Intramolecular dynamics in the environment of the single tryptophan residue in staphylococcal nuclease ☆

Alexander P. Demchenko [a], Ignacy Gryczynski [b], Zygmunt Gryczynski [b], Wieslaw Wiczk [b], Henryk Malak [b,*] and Mayer Fishman [b]

[a] *A.V. Palladin Institute of Biochemistry, Ukrainian Academy of Sciences, 9 Leontovich Street, Kiev 252030 (Ukraine)*
[b] *Center for Fluorescence Spectroscopy, University of Maryland School of Medicine, Department of Biochemistry, 108 N. Greene Street, Baltimore, MD 21201 (USA)*

(Received 24 April 1992; accepted in revised form 21 April 1993)

Abstract

The dipole relaxational dynamics in the environment of a single tryptophan residue Trp-140 in staphylococcal nuclease was studied by time-resolved (multi-frequency phase-modulation) spectroscopy and selective red-edge excitation. The long-wavelength position of the fluorescence spectrum (at 343 nm) and the absence of red-edge excitation effects at 0 and 20°C indicate that this residue is surrounded by very mobile protein groups which relax on the subnanosecond time scale. For these temperatures (0–20°C) the steady-state emission spectra did not show the excitation-wavelength dependent shifts (red-edge effects) for excitation wavelengths from 295 to 308 nm; however, the anisotropy decay rate is slow (tens of nanoseconds). This suggests that the spectral relaxation is due to mobility of the surrounding groups rather than the motion of the tryptophan itself. The motions of the tryptophan surrounding are substantially retarded at reduced temperatures in viscous solvent (60% glycerol). The temperature dependence of the difference in position of fluorescence spectra at excitation wavelengths 295 and 305 nm demonstrate the existence of red-edge effect at sub-zero temperatures, reaching a maximum value at −50°C, where the steady-state emission spectrum is shifted to 332 nm. The excitation and emission wavelength dependence of multi-frequency phase-modulation data at the half-transition point (−40°C) demonstrates the existence of the nanosecond dipolar relaxations. At −40°C the time-dependent spectral shift is close to monoexponential with the relaxation time of 1.4 ns.

Keywords: Tryptophan; Intramolecular dynamics; Staphylococcal nuclease; Time-resolved fluorescence

1. Introduction

It is now well established that protein molecules have significant internal motional freedom. The motions are activated by temperature and exist at equilibrium [1–3]. The characteristic times of these motions extend over a very broad interval from picoseconds to seconds. Every protein should have a specific pattern of intramolecular mobility which depends on its structure as a whole and on the local environment of its moving segments. This pattern can be influenced by both thermodynamic parameters (temperature, pressure) and specific factors such as association of subunits or binding of ligands.

The dynamic picture of a protein is thought to be of no less importance than its three-dimensional structure for understanding the mecha-

☆ Dedicated to Professor Franciszek Kaczmarek on the occasion of his 60th birthday.

* Corresponding author.

nisms of biological functions. The concept of dynamics is the basis of many theories for the mechanisms of enzyme catalysis [4,5], ligand binding by heme proteins [6] and selective transmembrane transport of ions [7]. The function of proteins probably requires a specific pattern of motions ("functionally important motions" [8]) which are determined by molecular design and selected by evolution for optimal functional performance. However, at present the experimental evidence is insufficient to make predictions about the correlation between protein structure and the functionally important (or unimportant) mobility. Therefore, protein dynamics a field of active research.

Staphylococcal nuclease is a useful protein for spectroscopic and biophysical studies. Its three-dimensional structure is known to a resolution of 1.65 Å [9], and its structure and dynamics in solution was extensively studied by NMR spectroscopy [10,11]. It has been successfully used in studies of protein folding–unfolding mechanisms [12], rotational mobility of the tryptophan residue [13], the origin of the multi-exponentiality of emission decay [14], and the structure and stability of mutant forms [15–17].

The aim of the present study is to investigate the local intramolecular mobility in the environment surrounding single tryptophan residue Trp-140. For this purpose we applied the methods of molecular relaxation fluorescence spectroscopy [18,19] which include steady-state temperature-dependent [20] and selective red-edge-excitation [19,21–23] measurements. Additionally, we use the time and wavelength-dependent emission to calculate time-resolved emission spectra for the tryptophan residue [23–26], which in turn were used to calculate the relaxation time of dipoles surrounding the tryptophan residue. We are interested in demonstrating different complementary approaches to analyze protein dipole-relaxational dynamics. The relaxation should not only change the excited-state energy with time, but also should produce transitions between different microscopic environments (sub-states) (Fig. 1). These environments can be photoselected at the red-edge excitations [19,22], and their difference from the mean-excited chromophore environments vanish in the course of relaxation. It is interesting therefore to correlate this approach with the direct determination of time-resolved shifts.

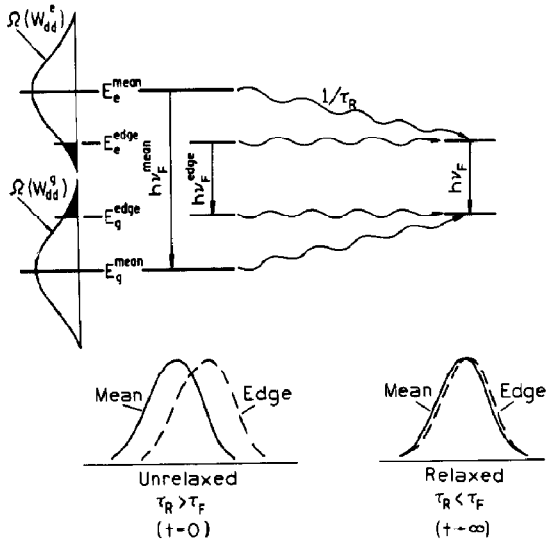

Fig. 1. The scheme of energy levels (above) and the spectra (below) illustrating the site-photoselection model of relaxation. The ground-state distribution of chromophore–environment interaction energy $\Omega(W_{dd}^g)$ is different from that in the excited state, $\Omega(W_{dd}^e)$, which allows to produce photoelection at the red edge. On dielectric relaxation of the dipoles surrounding the chromophore with the relaxation time τ_R there is not only a decrease of the mean excited-state energy level (the fluorescence spectra shift to longer wavelengths), but also a disappearance of the excitation-wavelength dependence of spectra.

2. Materials and methods

Staphylococcal nuclease, isolated from a wild strain of *Staphylococcus aureus*, was a generous gift from Dr. Ludwig Brand (Johns Hopkins University, Baltimore, MD). Protein concentrations were determined using the value of $A_{280} = 0.93$ for 1 mg/ml solution. For all experiments we used a constant protein concentration of 0.5 mg/ml, in 0.05 *M* tris-acetate buffer, pH 7.0. In experiments with water–organic co-solvent mixtures, the co-solvents (glycerol, 60% by weight or sucrose, 40% by weight) were added to aqueous buffer before the experiment.

The steady-state spectra were recorded on a SLM spectrofluorometer. The samples were thermostated in the range from 20°C to −50°C using a circulating bath. The temperature was measured directly in the spectrofluorometric cell with a thermistor. A quartz dewar was used for the studies at liquid nitrogen temperatures.

The time-resolved data were obtained on a multifrequency phase-modulation spectrometer [27,28] at the Center of Fluorescence Spectroscopy (University of Maryland, Baltimore, MD). The excitation source was a 3.79-MHz train of pulses, about 7 ps wide, obtained from the cavity-dumped output of a synchronously pumped rhodamine 6G dye laser. The dye laser output was frequency-doubled to 295 nm or 302 nm. This source is intrinsically modulated to many gigahertz and is used to directly excite the sample. All intensity decays were measured using rotation-free polarization conditions, with the donor emission selected by an interference filter, 10-nm bandwidth, in the range from 310 to 400 nm. For all analyses the uncertainties in the phase ($\delta\phi$) and modulation (δm) values were taken as 0.2° and 0.005, respectively.

Fluorescence intensity decays were analyzed in terms of the multi-exponential model

$$I(\lambda, t) = \sum_i \alpha_i(\lambda) \exp(-t/\tau_i(\lambda)) \qquad (1)$$

where λ is the emission wavelength, $\alpha_i(\lambda)$ the pre-exponential factor and $\tau(\lambda)$ the wavelength-dependent lifetime, as described previously [29,30]. Time-dependent emission spectra and emission centers-of-gravity were calculated as previously described in detail [24]. Anisotropy decays were obtained from frequency response of the polarized emission [31–32]. The data were fit to a single-exponential anisotropy decay law

$$r(t) = r_0 \exp(-t/\theta_r) \qquad (2)$$

where r_0 is the initial anisotropy and θ_r is the correlation time.

3. Results

The steady-state fluorescence spectrum of staphylococcal nuclease (Fig. 2) is centered at

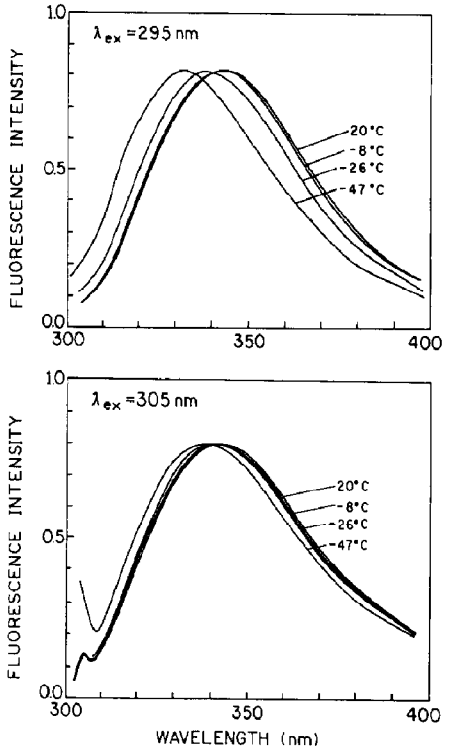

Fig. 2. Steady-state fluorescence spectra of staphylococcal nuclease at different temperatures and the excitation wavelengths 295 nm and 305 nm. Solvent: 60% glycerol, pH 7.0.

longer wavelengths compared to many single-tryptophan proteins [19]. The position of the maximum is at 343 nm in 0.05 *M* Tris-acetate buffer, pH 7.0 at 20°C. This suggests a polar and mobile environment for the tryptophan residue [32]. It is known however that Trp-140 in the protein crystals is positioned at the C-terminus and is not exposed to aqueous solvent [9] (see discussion). Hence, the environment of the indole ring is formed by the surrounding amino acid residues. Consequently, the local mobility of these groups is primarily responsible for attaining the equilibrium of dipolar interactions at the times which are much faster than the emission.

At 20°C in aqueous buffer the variation of the excitation wavelength in the range 295–308 nm does not reveal any change in position or bandwidth of the fluorescence spectrum. This result demonstrates the absence of a red-edge excitation effect, which in turn suggests that the motions of the dipoles surrounding the tryptophan

residue are very fast compared with the excited-state lifetime [3,19,22]. Prior to emission there occurs the equilibration and averaging of the local environment produced by the surrounding protein matrix. This is consistent with the value of the dielectric relaxation time of the chromophore environment $\tau_R = 0.8$ ns at 20°C obtained earlier [25].

The addition of viscogenic co-solvents does not produce any shift in the fluorescence spectrum at room temperature. This indicates the absence of any significant direct contact of the indole ring with the solvent and of any substantial perturbation of protein structure by the organic co-solvents. When studied in the temperature range from 20°C to 0°C we observed no red-edge effect, similarly to the result found in the absence of viscogenic co-solvents. Thus even in viscous media the motions of dipoles are on the subnanosecond time scale.

From earlier studies [33] it was known that the tryptophan residue in staphylococcal nuclease was rigid in a sense that no rotational motion was observed independent of rotation of the whole protein. Subsequently, a small component with a 0.2 ns correlation time was detected by frequency-domain fluorometry [34], which probably reflects the motions of the indole ring within a small cone angle. We tested the effects of glycerol and sucrose on the single-exponent approximation of the anisotropy decay time. The results (Table 1) demonstrate an increase in anisotropy decay time which is the result of retarding the rotation of the whole molecule. The independent motions of the tryptophan itself appear to be small in this protein under our experimental conditions. Hence, the subnanosecond dipolar relaxation should reflect the dynamics of surrounding

Table 1

The effect of viscous solvents on time decay of anisotropy (excitation 295 nm, emission 340 nm, 20°C)

Condition	r_0	$-\theta_r$ (ns)	χ^2_R
Aqueous buffer, pH 7.0	0.245	11.4	4.56
60% glycerol	0.242	86.5	24.0
40% sucrose	0.251	66.3	7.30

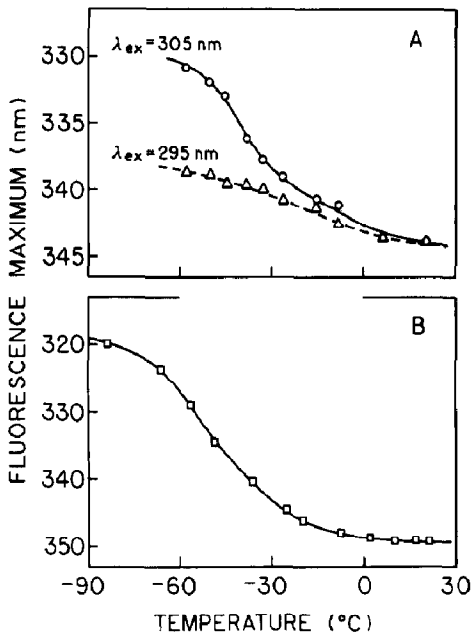

Fig. 3. (a) Dependence of the fluorescence emission maxima of staphylococcal nuclease on temperature with excitation wavelengths at 295 and 305 nm in 60% glycerol at pH 7.0. (b) The temperature dependence of the position of the maximum of fluorescence spectrum of tryptophan in 60% glycerol.

dipoles rather than the rotation of the indole ring relative to the protein matrix.

When the protein solution in 60% glycerol is cooled to temperatures below 0°C the fluorescence spectra shift to shorter wavelengths (Fig. 2). The half-transition point is observed at −40°C. This shift is accompanied by the appearance of the red-edge effect. In the range 0 to −50°C the spectra excited at 295 nm shift by 11 nm, reaching the value of 332 nm. A further decrease of temperature to 77 K does not increase this shift. If the spectra are excited at 305 nm the temperature-dependent blue shift is much smaller. The difference between the spectra excited at these wavelengths reaches 7 nm (Fig. 3a). According to the theory of spectroscopy of molecular relaxations (see Section 4) the values of the excited state lifetime τ_F and the relaxation time at the temperature −40°C should attain equal magnitude. These results can be compared with the data on temperature-dependent studies of tryptophan in 60% glycerol (Fig. 3b). In this case the similar sigmoid function is observed in the same

interval of temperature. However, tryptophan demonstrates a much larger spectral shift, the maximum changes from 346 nm at 20°C to 318 nm at −100°C. This difference should be due to different polarity of the tryptophan environment in solution and in the protein and also, probably, the difference in hydrogen bonding. Meantime, the half-transition point is the same and situated at −40°C.

The staphylococcal nuclease was subjected to detailed time-resolved spectroscopic studies using the multi-frequency phase-modulation technique [13,34]. As expected, the frequency-depended on phase and modulation values changes as a function of emission wavelength in a manner indicating dipolar relaxation (Fig. 4). That is, the phase angles are larger and the modulation smaller on the red-edge of the emission spectrum. Additionally, the amplitude of the shorter lifetime component decreases with increasing emission wavelength (Table 2). However, these data did not display the expected unique features of an excited state process, there being phase angles

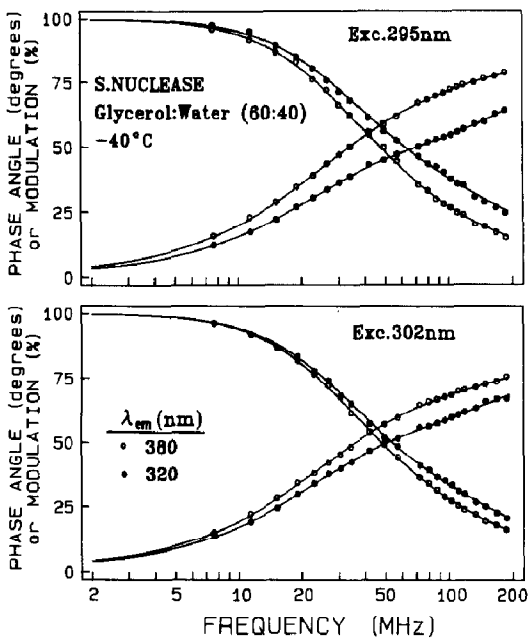

Fig. 4. Frequency-response of emission on the short-wavelength (320 nm) long-wavelength (380 nm) edges of the fluorescence spectra. The excitation wavelengths were 295 nm (above) and 302 nm (below).

Table 2

Time-decay of fluorescence intensity at selected excitation and emission wavelengths for staphylococcal nuclease in 60% glycerol at −40°C

Emission wavelengths (nm)	α_1 (λ)	f_1 (λ)	α_2 (λ)	f_2 (λ)	$\langle\tau_F\rangle$ (ns)
Excitation 295 nm [a]					
313	0.640	0.250	0.360	0.750	4.69 [b]
30	0.569	0.198	0.431	0.802	4.94
340	0.303	0.075	0.697	0.925	5.53
360	0.209	0.047	0.791	0.953	5.66
380	0.128	0.027	0.872	0.973	5.76
Excitation 302 nm [c]					
320	0.520	0.166	0.480	0.834	5.14
340	0.315	0.078	0.685	0.922	5.61
360	0.261	0.061	0.739	0.939	5.69
380	0.258	0.060	0.742	0.940	5.70

[a] $\tau_1(\lambda) = 1.10$ ns, $\beta_2(\lambda) = 5.89$ ns.
[b] $\langle\tau_F\rangle = \Sigma_i f_i \tau_i$.
[c] $\tau_1(\lambda) = 1.09$ ns, $\tau_2(\lambda) = 5.99$ ns.

greater than 90° indicative of a negative pre-exponential factor. As expected, the effect is much smaller upon red-edge excitation at 302 nm. This indicates that 302 nm excitation results in the photoselection of chromophore-environment microstates which correspond more closely to the relaxed state [3,19,22].

The multi-frequency phase-modulation data were collected at a series of emission wavelengths (Table 2), and were used to reconstruct the time-resolved spectra (Fig. 5). We observe a significant temporal shift of the spectra which is much smaller for the red-edge excitation. If the shift is plotted as the gravity-center $\bar{\nu}_{cg}$ of the spectrum versus time, we observe decay kinetics which are very close to single-exponential functions (the semi-logarithmic plots are almost linear). The relaxation time is 1.4 ns (Fig. 6). No difference was found between the relaxation times observed at excitation wavelengths 295 and 302 nm. Thus the combination of time-resolved and steady-state red-edge-excitation methods of fluorescence spectroscopy demonstrate that dielectric relaxation of the environment of the tryptophan residue at −40°C occurs on the nanosecond time.

In contrast to the low-temperature data, the spectral relaxation in glycerol–water at 20°C ap-

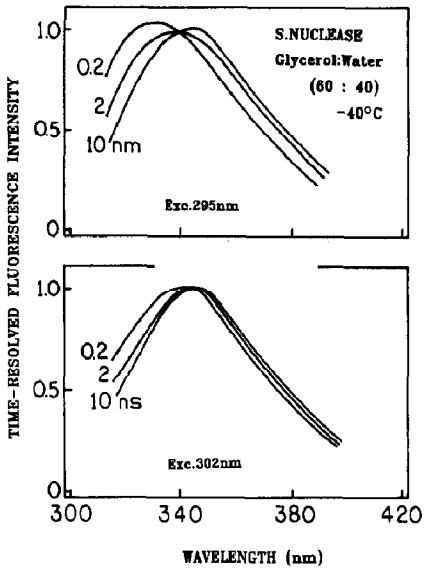

Fig. 5. Time-resolved emission spectra of staphylococcal nuclease at excitation wavelengths 295 nm (top) and 302 nm (bottom) in 60% glycerol at −40°C.

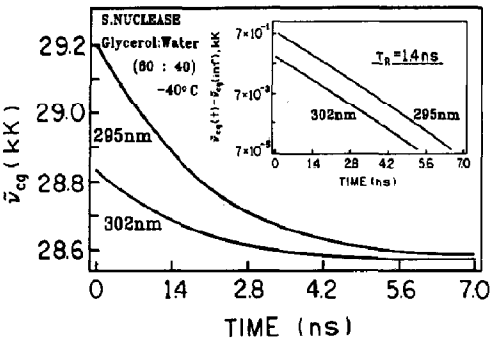

Fig. 6. Time-resolved centers-of-gravity of the staphylococcal nuclease fluorescence spectra for excitation at 295 and 302 nm. Also shown are the logarithmic transforms of the difference $\nu - \nu_\infty$ (insert) in 60% glycerol at −40°C.

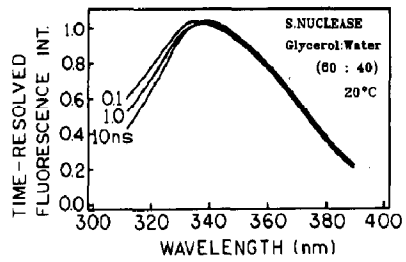

Fig. 7. Time-resolved emission spectra of staphylococcal nuclease in glycerol–water (60:40) at 20°C.

Table 3

The effect of viscous solvents on time decay of fluorescence intensity at selected emission wavelengths. (Temperature 20°C, excitation wavelength 295 nm)

Emission wavelengths (nm)	$\alpha_1(\lambda)$	$f_1(\lambda)$	$\alpha_2(\lambda)$	$f_2(\lambda)$
Aqueous buffer, pH 7.0 [a]				
340	0.603	0.467	0.397	0.533
60% Glycerol [b]				
320	0.216	0.043	0.784	0.957
340	0.087	0.015	0.913	0.985
380	0.001	0.000	0.999	1.000
400	−0.001	–	0.999	–
40% Sucrose [c]				
320	0.199	0.052	0.801	0.948
340	0.095	0.023	0.905	0.977
380	0.079	0.019	0.921	0.981
400	0.063	0.015	0.937	0.985

[a] $\tau_1(\lambda) = 3.98$ ns, $\tau_2(\lambda) = 6.87$ ns.
[b] $\tau_1(\lambda) = 0.85$ ns, $\tau_2(\lambda) = 5.23$ ns.
[c] $\tau_1(\lambda) = 1.18$ ns, $\tau_2(\lambda) = 5.29$ ns.

pears to be substantially faster. The emission-wavelength dependence of fluorescence intensity decay and the time-resolved spectra (Fig. 7) demonstrate the picosecond spectral relaxations in aqueous sucrose and glycerol solutions at 20°C, which are very similar to those in aqueous solutions (Table 3). This is evident from the more rapid decay of $\bar{\nu}_{cg}$, as calculated from the multi-exponential analysis (Table 3). It is interesting, however, that in viscous solvents the emission becomes more homogeneous (short-living component increases and the long-living component decreases) with no substantial change in mean fluorescence lifetime.

4. Discussion

4.1. Location of the tryptophan residue

The results of protein fluorescence spectroscopic studies are often interpreted in terms of molecular dynamic properties without a detailed understanding the structure of the system in question. It is important however, to consider the available data on staphylococcal nuclease structure in order to analyze whether the tryptophan

environment is actually polar, whether it consists of groups of substantial segmental mobility and whether tryptophan is close enough to the surface to allow the influence of dipolar motions by the solvent. Hence, we questioned what dipoles could contribute to the effect of dielectric relaxation in the environment of the excited indole chromophore of Trp-140?

The X-ray crystallographic data for staphylococcal nuclease crystals (solved by Dr. Eaton E. Lattman, The Johns Hopkins University, Baltimore, MD, [9]) show that Trp-140 belongs to the exposed C-terminal part of the polypeptide chain and is oriented inside the protein interior with no substantial access area to the solvent. These results demonstrate that the oxygen atom of one entrapped water molecule (distance 2.89 Å) is closest to indole nitrogen of Trp-140. This water molecule probably forms hydrogen bonds with the indole nitrogen and also with the backbone carbonyl of Ala-109. The bulky shape of the indole ring and this bonding can be responsible for slower mobility of indole ring with respect to surrounding groups. Within a 5 Å distance from the indole nitrogen there are several other polar groups: Lys-133 and Glu-129 side chains which are capable of segmentary motions, and the backbone carbonyls of Ala-109 and Leu-137. Several more polar residues are observed within a 7 Å distance: side chains of Lys-1 10, -134 and -136, Ser-128, Asn-138 and Gln-106. Also found are the carbonyl groups of the main chain of Val-104, Gly-106, Ser-128, Glu-129, Ala-130, Glu-131, Lys-134, Asn-138, Ile-139 and tryptophan itself, as well as a number of amide groups. Thus the protein groups surrounding Trp-140 are polar and appear to posses substantial mobility.

The distance from the indole ring to the protein surface is about 7 Å, which may be sufficient for the dielectric influence of solvent dipoles [35,36]. Since we do not observe any perturbation of the spectra in glycerol or sucrose solutions (and for tryptophan dissolved in 60% glycerol the fluorescence spectra shift from 353 to 347 nm), the dielectric influence of solvent on Trp-140 cannot be very significant. The coupling of dynamics between solvent dipoles and protein groups can be important, however, [37] and this could be a subject of future research. Since we observed that the temperature range of relaxations in the protein is superimposed on the relaxation range for the solvent, this coupling in our case should be very significant.

It is known that in aqueous solution native protein structures are stabilized by the addition of glycerol or sucrose [38,39]. Moreover, these agents are extensively used for cryoconservation of tissues and cells with complete or almost complete retention of functional activity [40]. This is because the direct interaction of these co-solvents with the protein is unfavorable and protein is preferentially solvated by water. This leads to increased thermodynamic stability of protein conformations with the minimum of solvent-accessible area. Since our results do not reveal any indication of conformational change on addition of glycerol or sucrose, and no effect of cold-denaturation [41] (which would produce the shift of fluorescence spectrum to longer wavelengths which is opposite to that we observe), we believe that within the wide low-temperature interval studied the protein is in its native state and we actually observe the effects of dipole relaxation, in this case slowed by the viscogenic experimental conditions.

4.2. *Dipole-relaxational dynamics*

The relaxational dynamics of dipoles that surround the excited chromophore is the major component of the temporal shift of spectra in polar environments. In liquid solvents this dynamics is observed in the picosecond, and in viscous solvent in the nanosecond time range. It was observed, however, that many native protein molecules in liquid water environment behave as microscopic viscous and solid media. Their relaxations are in the nanosecond and slower range [3,19,32]. The relaxation pattern should be determined by protein conformation.

Briefly, the principle of molecular relaxation spectroscopy consists in the following. Electronic excitation results in a change of chromophore dipole moment in both magnitude and direction. Therefore when initially excited the chromophore is not at equilibrium with its dielectric environ-

ment. Equilibrium can be reached by relaxation of the electric field with time which can be achieved by rotation and/or displacement of surrounding dipoles. The relaxation results in the decrease in the energy level of the excited state and thus the shift of fluorescence spectra to longer wavelengths.

The application of fluorescence spectroscopy allows the dielectric relaxation time τ_R to be obtained in several ways. One is the observation of temporal shifts of fluorescence spectra. In the simplest case of the Debye relaxation model (single τ_R), the time-dependent shift is an exponential function of time:

$$\bar{\nu}_t = \bar{\nu}_{t\to\infty} + (\bar{\nu}_{t=0} - \bar{\nu}_{t\to\infty}) \exp(-t/\tau_R) \qquad (3)$$

where $\bar{\nu}$ is the position in wavenumbers of the gravity centrum of the spectrum [20]. The advantage of this approach that it requires the observations of emission decays only and it does not require to produce variation of any external parameter (temperature, pressure or high concentrations of fluorescence quenchers) which can result in perturbation of protein structure. Its application at room temperatures resulted in subnanosecond relaxation times [3,19,22]. The relaxations slow down to the nanosecond range at the decrease of temperature to −40°C. The relaxation time 1.4 ns, obtained from these data, is in reasonable agreement with the results of temperature-dependent steady-state and red-edge excitation experiments.

Another possibility to estimate τ_R is suggested by the analysis of time-integrated (steady-state) spectra. If the mean excited state lifetime (τ_F) is known, it provides an intrinsic time marker, which allows observation of time-dependent events. The observed emission is an average of the time-resolved spectra over the decay time. The steady-state spectral shifts can then be expressed as a function of τ_R and τ_F [25]:

$$\frac{\bar{\nu} - \bar{\nu}_{t\to\infty}}{\bar{\nu}_{t=0} - \bar{\nu}_{t\to\infty}} = \frac{\tau_R}{\tau_F + \tau_R}. \qquad (4)$$

One can change τ_R by variation of temperature, and/or viscosity, whereas τ_F can be changed by collisional fluorescence quenching which allows calculation of τ_R [26] with suitable assumptions about the spectra at $t = 0$ and infinity. The limiting value at low temperatures can be taken as the reference value for the case of $\tau = 0$ (unrelaxed environment). The reference value at high temperatures, when the relaxation is complete, can be taken as the reference value for the case $\tau \to \infty$. In our case these values correspond to temperatures −50°C and 20°C. From eq. (4) it follows that τ_R should be equal to τ_F at the half-transition point, which we observe at −40°C. This estimate results in relaxation time of about 5 ns.

A complimentary approach to obtain relaxation times, τ_R, is to apply the site-photoselective red-edge excitation. Excitation by the low-energy quanta (at the red edge of absorption spectrum) results in a shift of the fluorescence spectra to longer wavelengths (Fig. 1). The difference between the emission spectra excited at the mean ($\bar{\nu}$) and edge ($\bar{\nu}^{ed}$) excitation is also a function of τ_R and τ_F [3,19,21]:

$$\bar{\nu} - \bar{\nu}^{ed} = (\bar{\nu}_0 - \bar{\nu}_0^{ed}) \frac{\tau_R}{\tau_R + \tau_F} \qquad (5)$$

where $\bar{\nu}_0$ and $\bar{\nu}_0^{ed}$ are the fluorescence spectra of completely unrelaxed fluorophores (at low temperatures or at short observation times). The results demonstrate that the disappearance of the red-edge effect occurs essentially in the same temperature interval as the main-band-excitation spectral shifts, which should result in similar values of the relaxation rates (5 ns).

The discrepancy between the time-resolved and steady-state determinations of the τ_R values (1.4 ns and 5 ns) is not unexpected in view of the fact that the time-resolved experiment is performed at a single temperature, and for the determination of τ_R from the shifts of the steady-state spectra the temperature dependence in an extended range should be analyzed. It is possible therefore that some dynamics modes are inactive at low temperatures and are activated as the temperature is increased. The contribution of relaxation components which are much slower or faster than the value determined in nanosecond time resolved experiment may be undetected if these

experiments performed at a single temperature. But the existence of these components may produce deformation of the temperature dependence of the steady-state spectra. The fact that the discrepancy between the τ_R values does not exceed one order of magnitude indicates that such contributions in our case are not substantial.

The positions of ultraviolet fluorescence spectra in proteins depend both on polarity and on dipole-relaxational properties of the environments of tryptophan residues.

Electronic excitation of a chromophore results in a change of Its dipole moment in both magnitude and direction. The initially excited chromophore is not at equilibrium with its dielectric environment; however, equilibrium can be reached with time by rotation and/or displacement of the surrounding dipoles. Thus, protein dynamics can be studied by observation of temporal shifts of the fluorescence spectra as well as of other spectroscopic effects of dipolar relaxation. According to Mazurenko and Bakhshiev [42] the spectroscopically observed dielectric relaxation time (τ_R), which is the relaxation time of the reactive field surrounding chromophore and the relaxation time obtained in direct dielectric measurements (τ_d) are related by

$$\tau_R \approx \frac{n^2+2}{\varepsilon+1}\tau_d. \qquad (6)$$

According to the classification of single-tryptophan proteins on the position of the fluorescence spectra and existence of red-edge effects [32], the environment of Trp-140 in staphylococcal nuclease should be considered to be polar and relaxed. The difference from other similar cases (monomeric melittin, casein, myelin basic protein) is that in these proteins the tryptophan residues were exposed to aqueous solvent and Trp-140 in staphylococcal nuclease is buried. The temperature-dependent spectral shifts due to dipole relaxations were observed for melittin in a tetrameric form and increased with temperature in the range 30–50°C [43]. In our knowledge staphylococcal nuclease is the first case where the nanosecond dipolar relaxation in the interior of a globular protein are observed at decreased temperatures.

Acknowledgements

The work has been done in the Center of Fluorescence Spectroscopy, NSF DIR-8710401 with support from NIH grant GM-39617. We thank Professor Ludwig Brand for providing the sample of staphylococcal nuclease used in this study and Professor Eaton E. Lattman for providing us the information on X-ray coordinates of atoms. We thank also Professor Joseph R. Lakowicz for helpful discussion and suggestions.

References

1 G. Careri, P. Fasella and E. Gratton, Annu. Rev. Biophys. Bioeng. 8 (1979) 69–97.
2 G.A. McCammon, Rep. Prog. Phys. 47 (1984) 1–46.
3 A.P. Demchenko, Luminescence and dynamics of protein structure (Naukovca Dumka, Kiev, 1988).
4 G.R. Welch, B. Somogyi and S. Damjanovich, Prog. Biophys. Mol. Biol. 39 (1982) 109–146.
5 B. Somogyi, G.R. Welch an dS. Damjanovich, Biochim. Biophys. Acta 768 (1984) 81–112.
6 H. Frauendfelder and R.D. Young, Comments Mol. Cell. Biophys. 3 (1986) 347–372.
7 A.P. Demchenko, N.N. Kositsky and V.I. Teslenko, Biophys. Chem. 35 (1990) 25–35.
8 H. Frauenfelder and E. Gratton, Methods Enzymol. 127 (1986) 207–217.
9 P.J. Loll and E.E. Lattman, Proteins: Struct. Funct. Genet. 5 (1989) 183–201.
10 L.E. Kay, D.A. Torchia and A. Bax, Biochemistry 28 (1989) 8972–8979.
11 D.A. Torchia, S.W. Sparks and A. Bax, Biochemisry 28 (1989) 5509–5524.
12 C.B. Anfinsen, A.N. Schecter and H. Taniuchi, Cold Spring Harbor Symp. Quant. Biol. 36 (1971) 249–255.
13 J.R. Lakowicz, I. Gryczynski, H. Szmacinski, H. Cherek and N. Joshi, in: Time-resolved spectroscopy in biochemistry, SPIE vol. 909 (SPIE, Bellingham, WA, 1988) pp. 178–185.
14 M.R. Eftink, C.A. Ghiron, A. Kautz and R.O. Fox, Biophys. J. 55 (1989) 575–579.
15 M.R. Eftink, C.A. Ghiron, I. Gryczynski, W. Wiczk, G. Laczko and J.R. Lakowicz, in: Time-resolved laser spectroscopy in biochemistry II SPIE, vol. 1204 (SPIE, Bellingham, WA, 1990) pp. 137–143.
16 D. Shortle, A.K. Meeker and E. Freire, Biochemistry 27 (1988) 4761–4768.
17 G. Wright and R.B. Freedman, Protein Eng. 2 (1989) 583–588.
18 J.R. Lakowicz, Principles of fluorescence spectroscopy (Plenum Press, New York, 1983).

19 A.P. Demchenko, Ultraviolet spectroscopy of proteins (Springer, Berlin, 1986).
20 Yu.T. Mazurenko and N.G. Bakhshiev, Opt. Spect. (USSR) 28 (1970) 905–913.
21 J.R. Lakowicz and S. Keating-Nakamoto, Biochemistry 23 (1984) 3013–3021.
22 A.P. Demchenko, Essays Biochem. 22 (1986) 120–157.
23 J.R. Lakowicz, E. Gratton, H. Cherek, B.P. Maliwal and G. Laczko, J. Biol. Chem. 259 (1984) 10967–11972.
24 J.R. Lakowicz, H. Cherek, G. Laczko and E. Gratton, Biochim. Biophys. Acta 777 (1984) 183–193.
25 H. Szmacinski, J.R. Lakowicz and M.L. Johnson, in: Time-resolved laser spectroscopy in biochemistry, SPIE vol. 909 (SPIE, Bellingham, WA, 1988) pp. 293–277.
26 J.R. Lakowicz and D. Hogen, Biochemistry 20 (1981) 1366–1373.
27 J.R. Lakowicz and B.P. Maliwal, Biophys. Chem. 21 (1985) 61–78.
28 J.R. Lakowicz, G. Laczko, and I. Gryczynski, Rev. Sci. Instrum. 57 (1986) 2499–2506.
29 J.R. Lakowicz, E. Gratton, G. Laczko, H. Cherek and M. Linkemann, Biophys. J. 46 (1984) 463–477.
30 E. Gratton, J.R. Lakowicz, B. Maliwal, H. Cherek, G. Laczko and M. Linkemann, Biophys. J. 46 (1984) 479–486.
31 J.R. Lakowicz, H. Cherek, B. Maliwal and E. Gratton, Biochemistry 24 (1985) 376–383.
32 A.P. Demchenko, Eur. Biophys. J. 16 (1988) 121–129.
33 J. Munro, J. Pecht and L. Stryer, Proc. Natl. Acad. Sci. USA 76 (1979) 56–60.
34 I. Gryczynski, H. Szmacinski, H. Cherek, N. Joshi and J.R. Lakowicz, Eur. Biophys. J. 19 (1991) 125–140.
35 A.P. Demchenko and A.S. Ladokhin, Eur. Biophys. J. 15 (1988) 369–379.
36 L.I. Krishtalik, Biophysika (USSR) 30 (1985) 768–772.
37 G.P. Singh, F. Parak, S. Hunklinger and K. Dransfeld, Phys. Rev. Let. 47 (1981) 685–688.
38 J.F. Back, D. Oakenfull and M.B. Smith, Biochemistry 18 (1979) 5191–5196.
39 K. Gekko and S.N. Timasheff, Biochemistry 20 (1981) 4667–4777.
40 P. Dozon, Cryobiochemistry. An introduction (Academic Press, London, 1977).
41 J.M. Sturtevant, and S.U. Venyaminov. Proc. Natl. Acad. Sci. USA 85 (1988) 3343–3347.
42 Y.T. Mazurenko and Bakhshiev, Opt. Spectrosc. 28 (1970) 490–494.
43 A.P. Demchenko and A.S. Ladokhin, Biochim. Biophys. Acta 955 (1988) 352–360.