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Dynamic fluorescence of extrinsic fluorophores as a tool for studying protein conformational substates

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Summary. The fluorescence lifetime distribution of 2-*p*-toluidinyl-6-naphthalene sulfonic acids (TNS) bound to the heme site of apomyoglobin has been examined. The results were compared to those observed for the free fluorophore in isotropic nonviscous solvent. Two different excitation wavelengths were used, i.e. 290 and 350 nm. The results showed that the distribution of TNS bound to apomyoglobin is wider than that of the free fluorophore, thus indicating the existence of a large number of conformational substates originating from the interaction between TNS and the protein matrix. The comparison of the distribution obtained at two different excitation wavelengths allowed the emission arising from conformational substates, in which the excited state of fluorophore moiety has a higher probability to be populated by Forster energy transfer mechanism, to be distinguished.

Key words: Dynamic fluorescence - Protein conformational dynamics - Apomyoglobin - Conformational substates - Naphthalene sulfonate fluorescence

Introduction

Protein molecules are not rigid, solid-like entities but can fluctuate among a very large number of conformational substates differing from each other by small structural details. The substates are separated by energetic barriers that can be overcome by increasing the temperature (Frauenfelder et al. 1988). Because of the existence of substates, the emission decay of fluorescent molecules incorporated into proteins are interpreted better in terms of a continuous lifetime distribution (Alcala et al. 1987). A continuous lifetime distribution is characterized by two parameters: center and width. The center depends on the average surroundings of the emitting molecule whereas the distribution width is influenced by two opposing factors, i.e. the extent of conformational substates and the rate of interconversion among them (Bismuto et al. 1988; 1989b). More specifically, the increase of the former causes the widening of the distribution, the converse occurs on increasing the interconversion rate.

The fluorescence characteristics of naphthalensulfonates make these dyes useful probes for examining the structural as well as the dynamic properties of certain regions of the protein matrix, especially those forming hydrophobic pockets. Globins are known to bind the naphthalensulfonates in the same non-polar site of the heme in the molar ratio of 1:1 (Stryer 1965; McGregor and Weber 1981). When the dye is bound to the globins, its fluorescence properties, i.e. fluorescence lifetime, emission maximum and quantum yield, change dramatically.

We have examined the fluorescence lifetime distribution of 2-*p*-toluidinyl-6-naphthalene sulfonic acid (TNS) bound to the heme site of apomyoglobin. The results were compared to those observed for the free fluorophore in isotropic nonviscous solvent.

Materials and methods

The main component of sperm whale myoglobin was prepared according to the method previously described (Bismuto et al. 1989a). Myoglobin concentrations were determined spectrophotometrically in the Soret region using 157000 $cm^2\ mol^{-1}$ as the absorption coefficient (Bismuto et al. 1989a). The apoprotein was prepared by butanone extraction and purified on a Sephadex G-25 Superfine column (1.5 cm × 50 cm) in order to remove aggregated protein. The molar absorption coefficient at 280 nm, calculated from tryptophan and tyrosine content, was 13500 $cm^2\ mol^{-1}$.

TNS was purchased from Molecular Probes (Junction City, OR) and recrystallized twice from 0.2 M magnesium chloride cold solution. TNS concentration was determined spectrophotometrically by using a molar absorption coefficient of 6640 $cm^2\ mol^{-1}$ at 350 nm (Bismuto et al. 1989b). The TNS aproprotein molar ratio was maintained below 1:4 to minimize any contribution from nonspecifically bound fluorophore. The fluorescence background from a comparable unlabeled sample was insignificant. All experiments were carried out by using freshly prepared solutions of apomyoglobin.

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Lifetime data were obtained by using the multifrequency cross-correlation phase and modulation fluorometer described by Gratton and Limkeman (1984) with modulation frequencies ranging between 5–140 MHz. The samples were excited with a 300-W xenon lamp and the exciting - light wavelength selected by a Jobin Yvon monochromator. The emission was observed through a long-wave-pass filter with a cutoff wavelength at 400 nm (Corion LG-400F). In phase flurometry, a lifetime measurement consists of a set of values of the emission phase shift (P) and demodulation (M) with respect to the exciting light at several different modulation frequencies. Usually, 20 different modulation frequencies were used, and the data were collected until the standard deviations for each measurement of phase and modulation were below 0.2° and 0.002, respectively.

Results and discussion

The measurements of phase delay and demodulation factor of the emission with respect to the excitation, i.e. 290 and 350 nm, were performed using several modulation frequencies ranging between 5–140 MHz. The data were analyzed using different functions to describe the fluorescence decays, i.e. exponential fit and probability-density functions of different shape (Alcala et al. 1987). In the case of TNS in ethanol at 10° C, the monoexponential fit provided a chi-squared value substantially identical to that obtained from the bi-exponential fit, i.e. 1.5, thus indicating that the decay is well represented by a single lifetime, i.e. 7.5 ns. For TNS bound to the heme site of apomyoglobin, the data could not be fitted with single, bi-, or three-exponential model. The fit was considerably improved using a continuous symmetric lifetime distribution having a Lorentzian shape. The same type of analysis was also carried out for TNS in ethanol. The results are shown in Table 1. In the case of TNS in ethanol, the center of the distribution is independent of the excitation wavelength and the very narrow width (0.05 ns) is that expected for a monoexponential-emitting molecular species. This finding indicates that TNS emission occurs from the same excited electronic level irrespective of the excitation wavelength. For TNS bound to sperm whale apomyoglobin, the emission lifetime distribution resulting from 350-nm excitation was found centered at 8.1 ns, a value which is consistent with that expected for the fluorophore located in a hydrophobic protein region. The large distribution width (0.86 ns) indicates a large conformational freedom of the protein matrix surrounding the excited fluorophore. This further confirms that the heme pocket has a large flexibility, as was supposed from knowledge of the crystal structure of myoglobin. The width of the lifetime distributions of the TNS-apomyoglobin complex obtained with excitation at 290 nm is 0.49 ns. Such a drastic reduction of the width was attributed to the selective mechanism of nonradiative energy transfer, which takes place in this protein (Balestrieri et al. 1976) from the intrinsic protein chromophores, i.e. tryptophan residues which are the species absorbing predominantly at 290 nm, to the TNS fluorophore. In fact, the large number of conformational substates in the protein produces a quasi-continuous range of distances between the donor and the acceptor group. The transfer rate constant, which influences the observed lifetime, depends on the inverse of the sixth power of the donor–acceptor distance. In conclusion, the lifetime distribution observed on excitation at 290 nm arises from substates having higher probabilities to be populated by Förster energy transfer mechanism.

Table 1. Continuous unimodal Lorentzian lifetime distribution parameters of TNS in ethanol and TNS bound to sperm whale apomyoglobin[a]

	Excitation (nm)	Center (ns)	Width (ns)	Chi-square
TNS in ethanol	290	7.590	0.050	2.150
	350	7.503	0.050	1.980
TNS bound to apoMb	290	7.928	0.492	1.783
	350	8.137	0.860	2.890

[a] Determination made at 10° C

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