Conformational Substates of Myoglobin Detected by Extrinsic Dynamic Fluorescence Studies

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ABSTRACT: The extent of conformational substates of two apomyoglobins, i.e., sperm whale and tuna apomyoglobin, was investigated by examining the fluorescence decay in the frequency domain of the extrinsic fluorophore TNS [6-(*p*-toluidino)-2-naphthalenesulfonic acid] bound to the heme binding site. Data analysis was performed in terms of a continuous, unimodal lifetime distribution having a Lorentzian shape. The results were compared with those for the free fluorophore in an isotropic nonviscous solvent. The incorporation of TNS into the protein matrix resulted in a broadening of the lifetime distribution due to the microenvironmental heterogeneity generated by structural fluctuations. The larger width of lifetime distribution observed for TNS bound to tuna apomyoglobin was related to a more extended conformational space accessible to the fluorophore in this protein compared to sperm whale myoglobin. A temperature increase from 15 to 40 °C produced a further broadening of the lifetime distributions of TNS bound to both proteins. This result can be explained by assuming the existence of conformational substates at high energy content or separated by high energy barriers, which are not populated at low temperature. The overall picture emerging from the reported data is that the lifetime distributions of TNS bound to apomyoglobins are determined largely by the number of conformational substates accessible to the protein matrix and, to a lesser extent, by the interconversion rates among these states.

The dynamic aspects of protein structure have been extensively investigated in the past 10 years, and a picture of increasing complexity has emerged (Careri et al., 1975, 1979; Karplus & McCammon, 1981; McCammon & Karplus, 1983; Frauenfelder & Debrunner, 1983). It is now well established that a protein molecule can exist in more than one conformational state. Each state is related to the particular function exerted by the protein (Frauenfelder, 1985; Frauenfelder & Gratton, 1985) or to the complex folding–unfolding pathway of the molecule (Bismuto et al., 1989). However, even in a given conformational state, a protein molecule will not remain in a unique conformation but will fluctuate among a large number of conformational substates (Austin et al., 1975; Frauenfelder et al., 1979; Ansari et al., 1987). Different substates have the same overall structure, but they differ in small details. Recently, a hierarchical model for such substates has been proposed, each level of the hierarchy being characterized by a specific interconversion time scale and energy (Ansari et al., 1985, 1987; Frauenfelder & Gratton, 1985; Kuriyan et al., 1986).

The fluorescence characteristics of naphthalenesulfonates make these dyes useful probes for examining the structural as well as the dynamic properties of certain regions of the protein matrix, expecially those forming hydrophobic pockets. Globins are known to bind the naphthalensulfonates in the same nonpolar site of the heme in the molar ratio of 1:1 (Weber & Young, 1964; Stryer, 1965; Gafni et al., 1977; MacGregor & Weber, 1981). When the dye is bound to the globins, its fluorescence properties, i.e., fluorescence lifetime, emission maximum, and quantum yield, change dramatically (Stryer, 1965). We have examined the fluorescence decay of 6-(*p*-toluidino)-2-naphthalenesulfonic acid (TNS),[1] a fluorophore included in this class of compounds, bound to different apomyoglobins, i.e., tuna and sperm whale apomyoglobins, which possess different dynamic as well as structural properties of the heme binding site (Bismuto et al., 1985; Bismuto et al., 1987a,b).

The emission decay of the apomyoglobin-bound TNS is supposed to be strictly dependent on the number of conformations among which the heme binding site may fluctuate, on the mobility around each conformation, and on the interconversion rate among these conformations. The usual analysis of the fluorescence decay consists of determining the number and the relative amplitude of the exponential components which contribute to the total emission. In the case of protein containing a single fluorophore, each component is then associated with a different conformation or subconformation. Recently, Gratton et al. (1986) have demonstrated that the emission decay of tryptophanyl residues is better resolved by a continuous distribution of decay rates rather than by the sum of a discrete number of exponential components. We have used the same approach to analyze the emission decay of TNS bound to apomyoglobin. Static as well as dynamic considerations justify the use of continuous distributions in the analysis of the fluorescence decay rates of proteins (Alcalà et al., 1987a,b). The results show that the heterogeneity of microenvironmental substates generated from the interaction between the fluorophore and the heme binding site is different for the two examined apomyoglobins.

Materials and Methods

The main component of tuna and sperm whale myoglobin was prepared according to the method previously described (Bismuto et al., 1989). Myoglobin concentrations were determined spectrophotometrically in the Soret region using the following extinction coefficients: 139 000 and 157 000 cm^2 mol^{-1} for tuna and sperm whale myoglobin, respectively

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[1] Abbreviation: TNS, 6-(*p*-toluidino)-2-naphthalenesulfonic acid.

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(Bismuto et al., 1985). The apoproteins were prepared by a butanone extraction technique (Teale, 1959) and purified on a Sephadex G-25 Superfine column (1.5 cm × 50 cm) in order to remove aggregated protein. The molar extinction coefficients at 280 nm, calculated from tryptophan and tyrosine content (Wetlaufer, 1962), were 8000 and 13 500 cm^2 mol^{-1} for tuna and sperm whale apomyoglobin, respectively.

TNS, from Molecular Probes (Junction City, OR), was recrystallized twice according to the procedure of Gafni et al. (1977). TNS concentration was determined spectrophotometrically by using a molar extinction coefficient of 6640 cm^2 mol^{-1} at 350 nm (Gafni et al., 1977). The TNS:apoprotein molar ratio was maintained at less than 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.

Lifetime data were obtained by using the multifrequency cross-correlation phase and modulation fluorometer described by Gratton and Limkeman (1983) with modulation frequencies ranging between 17 and 150 MHz. The samples were excited with a 150-W xenon lamp, and the exciting light was passed through an interference filter with a center wavelength transmittance at 350 nm and a full width at half-maximum of 10 nm (Corion Co., Holliston, MA; Model P10-350). The emission was observed through a long-wave-pass filter with a cutoff wavelength at 400 nm (Corion LG-400F). In phase fluorometry, 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, at least 10 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. The temperature of the sample compartment was controlled by using an external bath circulator (Neslab Model LT50). The sample temperature was measured prior to and after each measurement in the sample cuvette using a digital thermometer (Omega, Model 410 B-TC). The observed phase shifts and modulation values were analyzed in terms of Lorentzian lifetime distributions by using the algorithm described elsewhere (Alcalã et al., 1987a). Phase and modulation data were also analyzed by using one, two, or three exponentials by the nonlinear least-squares program described by Jameson et al. (1984).

Results

The phase shifts and modulation values, collected at several frequencies ranging between 5 and 140 MHz, have been analyzed in terms of continuous unimodal Lorentzian lifetime distributions. The distributions were normalized and defined only in the positive lifetime domain. Figure 1 shows the unimodal Lorentzian distributions obtained for TNS in ethanol at 10 and 30 °C. In both cases, the distributions were very narrow, the full width at half-maximum being equal to 0.05 ns, a value which is that expected when an emission decay consisting of a single-exponential component, such as that of TNS in an isotropic nonviscous solvent (Bismuto et al., 1987), is analyzed in terms of the continuous Lorentzian lifetime distribution. Moreover, the χ^2 value obtained from the distribution approach, i.e., 1.8, was similar to that obtained from the monoexponential fit. Therefore, the analysis in terms of unimodal distribution does not appear to improve the fit in the case of the free fluorophore dissolved in ethanol.

The incorporation of TNS in the heme binding site of apomyoglobin resulted in a broadening of the distribution

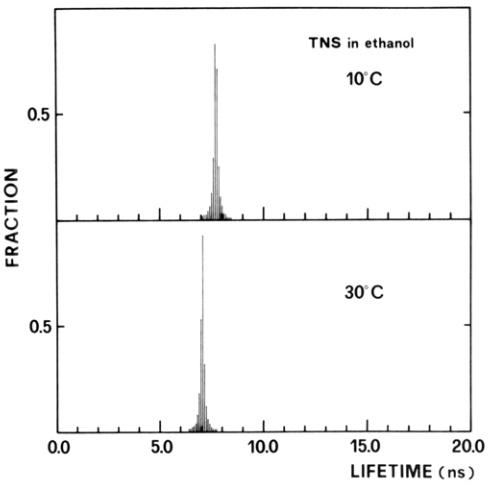

FIGURE 1: Lifetime analysis using a single Lorentzian distribution for TNS in ethanol at the indicated temperatures. TNS absorbance at the excitation wavelength (350 nm) was lower than 0.1. Emission was observed through a LG 400 filter.

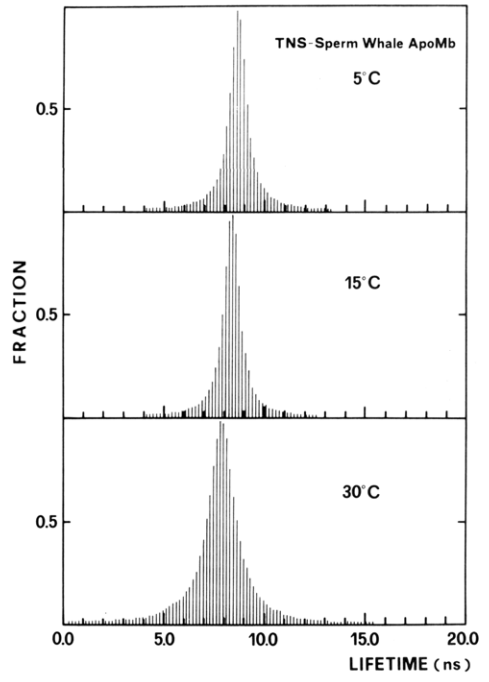

FIGURE 2: Lifetime analysis using a single Lorentzian distribution for TNS–sperm whale apomyoglobin at the indicated temperatures. All solutions contained 0.01 M phosphate/0.15 M NaCl, pH 7.0. TNS absorbance at the excitation wavelength (350 nm) was lower than 0.1. Protein:TNS molar ratio was less than 0.2. Emission was observed through a LG 400 filter.

pattern as shown in Figures 2 and 3 for sperm whale and tuna apomyoglobin, respectively. In both cases, the quality of the fits obtained from the distribution analysis was improved in comparison with that from the mono- as well as the biexponential fit. This observation suggests that a continuous Lorentzian distribution better represents the decay rate of an extrinsic fluorophore incorporated in the protein matrix. The large increase of the distribution width observed for TNS bound to apomyoglobin, i.e., from 0.05 ns in ethanol to 0.86 and 1.62 ns when bound to sperm whale and tuna apomyoglobin, respectively, reveals a great extent of emission heterogeneity, probably generated by fluctuations of the protein matrix. However, the microenvironmental heterogeneity of TNS bound to tuna apomyoglobin appears to be even greater than that observed for the same fluorophore bound to sperm

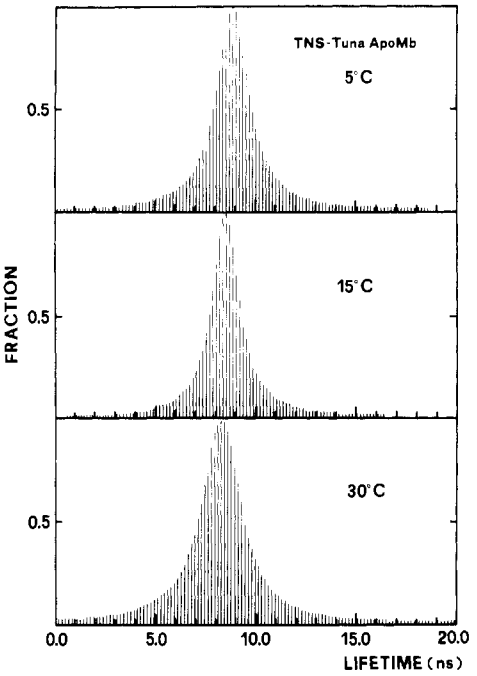

FIGURE 3: Lifetime analysis using a single Lorentzian distribution for TNS–tuna apomyoglobin at the indicated temperatures. The experimental conditions are those reported in Figure 2.

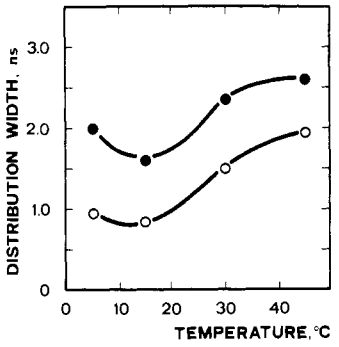

FIGURE 4: Temperature dependence of the width of the unimodal Lorentzian lifetime distribution of TNS–apomyoglobin complexes: (●) TNS–tuna apomyoglobin; (○) TNS–sperm whale apomyoglobin. The experimental conditions are those reported in Figure 2.

whale apomyoglobin as far as the distribution width is concerned. The small shifts observed for the distribution centers, i.e., from 7.5 ns for TNS in ethanol to 8.3 and 8.5 ns for TNS bound to sperm whale and tuna apomyoglobin, respectively, can be related to changes in the average surroundings of the emitting fluorophore.

The temperature dependence of the lifetime distribution of TNS bound to the different apomyoglobins was also investigated. The unimodal Lorentzian lifetime distributions at three different temperatures are shown in Figures 2 and 3 for sperm whale and tuna apomyoglobin, respectively. The dependence of the distribution width on temperature is shown in Figure 4. The χ^2 values obtained at different temperatures were almost similar, the actual value ranging between 1.3 and 1.6. Moreover, the χ^2 values were lower than those obtained from mono- as well as two- or three-exponential fits. For both proteins, two opposite thermal effects on the width of the lifetime distribution are observed. Between 5 and 15 °C, the full width at half-maximum decreases, whereas in the thermal range between 15 and 40 °C a broadening of the lifetime distribution occurs, reaching a plateau at temperatures above 45 °C. By contrast, the distribution center monotonically decreases on increasing temperature (Figure 5), thus indicating

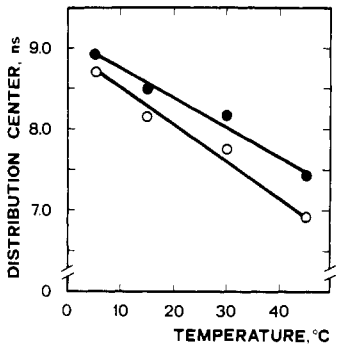

FIGURE 5: Temperature dependence of the center of the unimodal Lorentzian lifetime distribution of TNS–apomyoglobin complexes: (●) TNS–tuna apomyoglobin; (○) TNS–sperm whale apomyoglobin. The experimental conditions are those reported in Figure 2.

that no gross conformational change occurs in the heme binding site between 5 and 45 °C.

Discussion

The interpretation of the emission decay of a fluorophore incorporated in the protein matrix in terms of continuous lifetime distribution is a direct consequence of structural fluctuations (Alcalà et al., 1987a,b). In fact, the flexibility of the protein structure generates a great variety of environments which the fluorophore may experience during the excited state. In this respect, the width of lifetime distribution depends on the number of subconformations that the polypeptide chain can assume and on the interconversion rate among these subconformations. If the interconversion rate is smaller than the decay rate, the protein matrix is virtually frozen for the duration of the excited state so that the fluorophore emission arises from subconformations which differ from each other.

It has been demonstrated that the factors, which affect the number of substates and the interconversion rate, also influence the distribution width. Bismuto et al. (1988) reported that the broadening of the tryptophanyl lifetime distribution observed on protein denaturation is related to the larger number of conformational substates existing in the unfolded state. Alcalà et al. (1987c) explained the sharpening of the tryptophanyl lifetime distribution of proteins, observed on increasing temperature, as due to the augmented rate of interconversion among conformational substates. A similar conclusion was drawn for the distribution sharpening observed on decreasing the solvent viscosity (Alcalà et al., 1987b,c).

In this paper, we have investigated the extent of conformational substates resulting from the interaction between the extrinsic TNS fluorophore and the heme binding site of two different apomyoglobins by means of unimodal Lorentzian lifetime distributions. The observation that the lifetime distribution of TNS bound to tuna apomyoglobin is broader than that relative to sperm whale globin suggests that the fluorophore experiences, in the former protein, a larger variety of environments. This could be due to a more extended conformational space region accessible to TNS in tuna apomyoglobin. This conclusion is consistent with the observations that tuna apomyoglobin is more swollen than sperm whale apomyoglobin and that the extent of solvent accessibility to the heme pocket of tuna apomyoglobin is larger than that observed for sperm whale globin (Bismuto et al., 1985). Alternatively, if we assume that the number of substates is similar for the two proteins, it must be admitted that the differences observed between tuna and sperm whale apomyoglobin are related to different dynamic properties. In this respect, the broader lifetime distribution, i.e., that observed in the case of tuna

apomyoglobin, would correspond to the lower interconversion rate. This explanation seems to be less likely since there have been several observations in the literature which suggest that the heme binding site of tuna apomyoglobin may have more flexibility than the corresponding site in sperm whale apomyoglobin (Bismuto et al., 1985). For example, the rotational strength of the dichroic bands acquired by the heme in the native structure of tuna myoglobin is lower than that observed for sperm whale myoglobin. This indicates that the aromatic side chains which interact with the heme moiety in the tuna myoglobin are in a less rigid environment than those responsible for the Soret circular dichroism of sperm whale globin (Bismuto et al., 1985). Additionally, the analysis of the temperature dependence of phosphorescence performed on the two apomyoglobins revealed that the internal flexibility of the tryptophanyl microenvironment of tuna globin is higher than that of sperm whale myoglobin (Bismuto et al., 1987b).

The conclusion that the lifetime distribution width of TNS bound to apomyoglobin is influenced by the number of substates rather than differences in their dynamic properties is further corroborated by the thermal dependence of the distribution width. In fact, the distribution width of TNS lifetime increases on going from 15 to 40 °C, whereas that relative to the tryptophanyl emission decreases as expected because of the augmented interconversion rate induced by the temperature increase (Bismuto et al., 1988). Presumably, the increase of the TNS lifetime distribution width reflects the existence of many other conformational substates which cannot be populated at low temperature because of their high energy content and/or high energy barriers. Therefore, the distribution of microenvironmental states becomes large enough on increasing temperature that it cannot be balanced by the augmented rate of interconversion among them. Finally, the observation that protein unfolding results in a broadening of the tryptophanyl lifetime distribution (Bismuto et al., 1988) might support that the effect observed on increasing the temperature on the TNS lifetime distribution could be due to denaturation effects. This hypothesis must be excluded since the unfolding of apomyoglobin results in the loss of the ability to bind TNS, which, when released in aqueous solution, becomes virtually not fluorescent. Moreover, the unfolding of the heme binding site has been demonstrated to be highly cooperative without the occurrence of intermediate structures having different conformations of the heme binding site and, hence, different emissive properties of the fluorescent probe (Bismuto & Irace, 1988).

References

Alcala, R., Gratton, E., & Prendergast, F. (1987a) *Biophys. J. 51*, 587–596.

Alcala, R., Gratton, E., & Prendergast, F. (1987b) *Biophys. J. 51*, 597–604.

Alcala, R., Gratton, E., & Prendergast, F. (1987c) *Biophys. J. 51*, 925–936.

Ansari, A., Berendzen, J., Bowne, S. F., Frauenfelder, H., Iben, I. E., Sauke, T. B., Shyamsunder, E., & Young, R. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 5000–5004.

Ansari, A., Berendzen, J., Braunstein, D., Cowen, B. R., Frauenfelder, H., Hong, M. K., Iben, I. E. T., Johnson, J. B., Ormos, P., Sauke, T. B., Schol, R., Schulte, A., Steinbach, P. J., Vittitow, J., & Young, R. D. (1987) *Biophys. Chem. 26*, 337–355.

Austin, R. H., Beeson, K. W., Eisenstein, L., Frauenfelder, H., & Gunsalus, I. C. (1975) *Biochemistry 14*, 5355–5365.

Bismuto, E., & Irace, G. (1988) *Int. J. Pept. Protein Res. 32*, 321–325.

Bismuto, E., Colonna, G., Savy, F., & Irace, G. (1985) *Int. J. Pept. Protein Res. 26*, 195–207.

Bismuto, E., Irace, G., Colonna, G., Jameson, D. M., & Gratton, E. (1987a) *Biochim. Biophys. Acta 913*, 324–328.

Bismuto, E., Strambini, G. B., & Irace, G. (1987b) *Photochem. Photobiol. 45*, 741–744.

Bismuto, E., Gratton, E., & Irace, G. (1988) *Biochemistry 27*, 2132–2136.

Bismuto, E., Irace, G., & Gratton, E. (1989) *Biochemistry 28*, 1508–1512.

Careri, G., Fasella, P., & Gratton, E. (1975) *CRC Crit. Rev. Biochem. 3*, 141–164.

Careri, G., Fasella, P., & Gratton, E. (1979) *Annu. Rev. Biophys. Bioenerg. 8*, 69–97.

Frauenfelder, H. (1985) *Structure and Motion: Membranes, Nucleic Acids and Proteins* (Clementi, E., Corongiu, G., Sarma, M. H., & Sarma, R. H., Eds.) pp 205–218, Adenine, Guilderland, NY.

Frauenfelder, H., & Debrunner, P. (1983) *Annu. Rev. Phys. Chem. 33*, 283–303.

Frauenfelder, H., & Gratton, E. (1985) *Methods Enzymol. 127*, 471–492.

Frauenfelder, H., Petsko, G. A., & Tsernoglou, D. (1979) *Nature (London) 280*, 558–563.

Gafni, A., DeToma, R. P., Manzow, R. E., & Brand, L. (1977) *Biophys. J. 17*, 155–168.

Gratton, E., & Limkeman, M. (1983) *Biophys. J. 44*, 315–324.

Jameson, D. M., Gratton, E., & Hall, R. (1984) *Applied Spectrosc. Rev. 20*, 55–106.

Karplus, M., & McCammon, J. A. (1981) *CRC Crit. Rev. Biochem. 9*, 293–349.

Kuriyan, J., Wilz, S., Karplus, M., & Petsko, G. A. (1986) *J. Mol. Biol. 192*, 133.

MacGregor, R. B., & Weber, G. (1981) *Ann. N.Y. Acad. Sci. 366*, 140–153.

McCammon, J. A., & Karplus, M. (1983) *Acc. Chem. Res. 16*, 187–202.

Stryer, L. (1965) *J. Mol. Biol. 13*, 482–495.

Teale, F. W. S. (1959) *Biochim. Biophys. Acta 35*, 543–549.

Weber, G., & Young, L. B. (1964) *J. Biol. Chem. 239*, 1415–1423.

Wetlaufer, D. (1962) *Adv. Protein Chem. 17*, 303–390.