Multiple Conformational States in Myoglobin Revealed by Frequency Domain Fluorometry

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ABSTRACT: The tryptophanyl fluorescence decays of two myoglobins, i.e., sperm whale and tuna myoglobin, have been examined in the frequency domain with an apparatus which utilizes the harmonic content of a mode-locked laser. Data analysis was performed in terms of continuous distribution of lifetime having a Lorentzian shape. Data relative to sperm whale myoglobin, which possesses two tryptophanyl residues, i.e., Trp-A-5 and -A-12, provided a broad lifetime distribution including decay rates from a few picoseconds to about 10 ns. By contrast, the tryptophanyl lifetime distribution of tuna myoglobin, which contains only Trp-A-12, showed two well-separated and narrow Lorentzian components having centers at about 50 ps and 3.37 ns, respectively. In both cases, the χ^2 obtained from distribution analysis was lower than that provided by a fit using the sum of exponential components. The long-lived components present in the fluorescence decay of the two myoglobins do not correspond to any of those observed for the apoproteins at neutral pH. The tryptophanyl lifetime distribution of sperm whale apomyoglobin consists of two separated Lorentzian components centered at 2.25 and 5.4 ns, whereas that of tuna apomyoglobin consists of a single Lorentzian component, whose center is at 2.19 ns. Acidification of apomyoglobin to pH 3.5 produced a shift of the distribution centers toward longer lifetimes. The similarity between the values of the distribution centers of the long-lived components observed in the fluorescence decay of native myoglobins and those observed for apomyoglobins at acidic pH suggests that the long-lived component might arise from a conformational state (different from the native state) in which geometrical factors preclude energy transfer via Forster coupling from tryptophan to heme. This possibility has been further supported by the identification, in the steady-state emission spectrum of tuna myoglobin, of a component having an emission maximum similar to that observed at acidic pH, i.e., 335–337 nm. The difference between the tryptophanyl lifetime distributions of the two native holoproteins has been explained in terms of a higher degree of structural flexibility of tuna globin compared to that of sperm whale myoglobin.

The fluorescence lifetime of tryptophanyl residues in myoglobins differs from that observed in the corresponding apoproteins because of the quenching via Forster energy transfer with the heme group (Weber & Teale, 1959). The knowledge of the X-ray coordinates allowed the calculation of the transfer rate constants of the two tryptophanyl residues of sperm whale metmyoglobin, i.e., 0.125 and 0.031 ns for Trp-A-5 and Trp-A-12, respectively (Hochstrasser & Negus, 1984). In the case of tuna metmyoglobin, in which Trp-A-5 is missed, a single energy transfer limited decay of about 0.030 ns would be expected if one assumes that the basic folding of the protein is the same as that of sperm whale. The results so far reported using time-correlated single photon counting techniques have confirmed this picture except for the presence of a long-lived component of about 3 ns in the fluorescence decay. However, the contribution to the total fluorescence of this component has been estimated to be lower than 10% (Hochstrasser & Negus, 1984; Janes et al., 1987). A possible explanation is that the long-lived fluorescence arises from a small fraction of protein population, the tryptophanyl residues of which are not capable of energy transfer to the heme during the lifetime of the excited state, because of geometrical factors. However, energy-transfer calculations of Forster coupling as a function of the restricted rotation about the C(β)–C(γ) bond have excluded this possibility for the native sperm whale metmyoglobin (Hochstrasser & Negus, 1984). Moreover, the possibility that the long-lived component originates from some apoprotein present in solution was excluded since the addition of hemin did not reduce the contribution of the long-lived component (Hochstrasser & Negus, 1984; Janes et al., 1987).

Studies on the denaturation pattern of myoglobin induced by guanidine at neutral pH have indicated that this process involves the occurrence of a molecular intermediate (Bismuto et al., 1983). The molecular properties of this intermediate have been found similar to, although not identical with, those possessed by the acid-denatured form of the protein (Irace et al., 1986; Ragone et al., 1987). Both forms are not able to bind chromophores like 1-anilino-8-naphthalenesulfonic acid (ANS),[1] TNS, etc. (Balestrieri et al., 1976; Colonna et al., 1982; Irace et al., 1986). However, the presence of well-defined elements of secondary and tertiary structure suggests that these forms represent partially unfolded states, which may originate from either the unfolding of the two structural "halves" identified in the myoglobin molecule, i.e., segments 1–79 and 80–153, respectively (Wetlaufer, 1973; Kuntz, 1975; Wodak & janin, 1981), or the loss of the molecular interaction connecting the two structural units (Bismuto et al., 1983; Irace et al., 1986).

In the present work, we have reexamined the fluorescence decay of sperm whale and tuna myoglobins by frequency domain fluorometry using the harmonic content of a mode-

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[1] Abbreviations: ANS, 1-anilino-8-naphthalenesulfonic acid; TNS, 2-(*p*-toluidinyl)-6-naphthalenesulfonic acid.

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locked laser which is known to be a valid alternative to the use of time-correlated single photon counting techniques (Alcala et al., 1985). The fluorescence decay of metmyoglobins has been compared to those of the corresponding apoproteins. The data analysis was performed in terms of a continuous distribution of lifetime values with a Lorentzian shape. We have selected this type of distribution since a recently reported comparison among uniform, Gaussian, and Lorentzian distributions, for the analysis of the tryptophanyl emission data in several proteins, showed that Lorentzian distributions better describe the observed emission decay (Gratton et al., 1986; Alcala et al., 1987a–c). The results suggest that the long-lived component observed in the fluorescence decay of metmyoglobin might arise from a conformational state (different from the native) in which geometrical factors do not allow energy transfer via Forster coupling from tryptophan to heme.

Materials and Methods

Tuna myoglobin, prepared from the hearth ventricle of adult tuna (*Thunnus thynnus*) according to the method previously described (Balestrieri et al., 1978), and sperm whale myoglobin, purchased from Sigma, were processed in order to isolate the main component. The purification procedure involved gel filtration on a Sephadex G-50 Superfine column (2.5 cm × 100 cm) followed by ion-exchange chromatography on a Sepharose S column (2.5 cm × 40 cm). The Sepharose S column was developed by a linear salt gradient (0–0.5 M NaCl) added to 5 mM phosphate, pH 6.2 (500 mL for each vessel). All preparations of myoglobin were metmyoglobin and will be referred to as myoglobin. The final buffer was always 0.05 M sodium phosphate in 0.1 M NaCl at pH 7. 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 (Bismuto et al., 1985). The apoproteins were prepared by the butanone extraction technique (Teale, 1959) and purified in order to remove aggregated protein on a Sephadex G-25 Superfine column (1.5 cm × 50 cm). The molar extinction coefficients at 280 nm of apomyoglobins were calculated from tryptophan and tyrosine content by using extinction coefficients of 5500 and 1250 cm^2 mol^{-1}, respectively (Wetlaufer, 1962), and were found to be 8000 and 13 500 cm^2 mol^{-1} for tuna and sperm whale apomyoglobin, respectively.

Steady-state fluorescence spectra were recorded by using the photon counting spectrofluorometer constructed by Gratton and Limkeman (1983). The myoglobin fluorescence spectra were not corrected for the Raman contribution since the protein fluorescence is almost comparable to the Raman intensity because of the energy transfer from tryptophan to the heme. Lifetime measurements were performed by using a multifrequency cross-correlation phase and modulation fluorometer which uses the harmonic content of a high repetition rate mode-locked Nd-YAG laser. This laser is used to synchronously pump a dye laser whose pulse train is frequency doubled with an angle-tuned frequency doubler (Alcala et al., 1985). The ultraviolet light was continuously tunable over the range 280–310 nm. We chose excitation at 295 nm to eliminate the contribution of tyrosyl residues to the total fluorescence. The emission was observed through a long-wave pass filter (WG 330) with a cutoff wavelength at 330 nm to avoid Raman emission. The modulation frequency was variable from 4 to 300 MHz.

A solution of *p*-terphenyl (from Kodak) in cyclohexane was placed in the reference cell to correct for "color error" (Lakowicz et al., 1981). A lifetime of 1.000 ns was assigned to the reference solution. An excitation polarized at 55° with respect to the horizontal plane was used to eliminate polarization effects. 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 0.004, 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 (Alcala et al., 1987a). Phase and modulation data were also analyzed by using two or three exponentials by the nonlinear least-squares program described by Jameson et al. (1984).

Table I: Discrete Component Analysis of Myoglobin Emission Decay[a]

myoglobin	T_1	A_1	T_2	A_2	T_3	A_3
sperm whale	0.080	0.970	1.064	0.025	8.027	0.005
tuna	0.083	0.966	3.332	0.034		

[a] T = fluorescence lifetime in nanoseconds; A = preexponential factor.

Results

The tryptophanyl emission decay of most proteins can be satisfactorily described only by using several exponential components. Each component is usually associated with a specific indole residue. However, also for proteins containing a single tryptophan, the decay rate is better approximated by a sum of a discrete number of exponential components (Beechem & Brand, 1985). Recently, it has been shown that the analysis in terms of continuous Lorentzian lifetime distributions further improves the description of the tryptophanyl decay (Gratton et al., 1986; Alcala et al., 1987a–c). We have applied both the distribution approach and the exponential fit to the study of the tryptophanyl fluorescence decay of sperm whale and tuna myoglobins as well as their corresponding apoproteins. The decay parameters obtained by the exponential fit of data, collected at neutral pH and 10 °C, are shown in Table I. For both holoproteins, the decay could not be described as a single-exponential component. In the case of sperm whale myoglobin, the data were best fitted as a sum of three exponentials, two of which were in the nanosecond time scale, i.e., 1 and 8 ns, and the other in the subnanosecond range, i.e., 80 ps. The results are quite consistent with those recently reported for the same protein by Janes et al. (1987) using time-correlated photon counting techniques. The short-lived component, which is supposed to result from Forster energy transfer from tryptophans to heme, actually consists of two distinct fast components reflecting perhaps the different orientation of the two indole residues with respect to the heme moiety (Hochstrasser & Negus, 1984). The tryptophanyl fluorescence decay rate of tuna could be described by two exponential components, i.e., a short-lived component of 83 ps and a long-lived one of 3.3 ns (Table I).

Phase and modulation data relative to the holoproteins have also been analyzed in terms of continuous Lorentzian lifetime distributions. The Lorentzian distribution is characterized by two parameters, i.e., the center and the full-width at half-maximum (Bevington, 1969). The distributions were normalized and defined only in the positive lifetime domain. Figures 1A and 2A show the distributions obtained for sperm

Table II: Continuous Lorentzian Distribution Analysis of Myoglobin and Apomyoglobin Emission Decay[a]

protein	C_1	W_1	F_1	C_2	W_2	F_2	C_3	W_2
SW Mb (pH 7.5)	<0.01	0.16	0.609	<0.01	5.87	0.261	7.47	0.13
T Mb (pH 7.5)	0.05	0.05	0.610	3.37	0.06	0.390		
SW apoMb (pH 8.3)	2.48	0.17	0.001	2.24	0.05	0.719	5.43	0.07
T apoMb (pH 8.3)	2.88	0.19	1.000					
SW apoMb (pH 3.5)	0.10	0.10	0.004	2.31	0.17	0.248	6.17	0.05
T apoMb (pH 3.5)	0.01	0.06	0.010	1.63	0.09	0.260	5.29	0.24

[a] Abbreviations: C, distribution center in nanoseconds; W, full width at half-maximum in nanoseconds; F, fraction; SW, sperm whale; T, tuna.

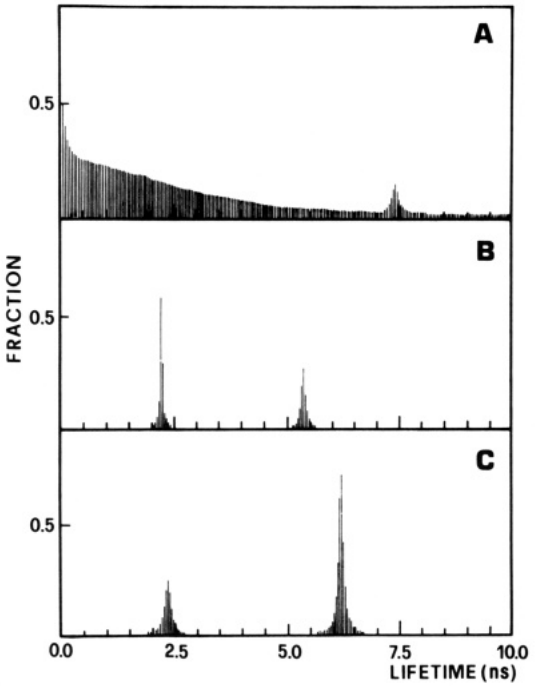

FIGURE 1: Tryptophanyl fluorescence lifetime distribution of sperm whale myoglobin and apoprotein at 10 °C. (A) Myoglobin at neutral pH; (B) apomyoglobin at pH 8.3; (C) apomyoglobin at pH 3.5. The parameters describing the distributions shown in figure are reported in Table II.

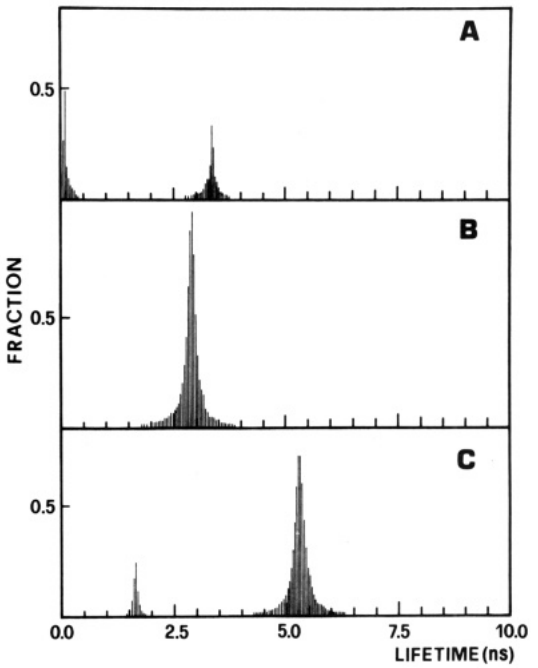

FIGURE 2: Tryptophanyl fluorescence lifetime distribution of tuna myoglobin and apoprotein at 10 °C. (A) Myoglobin at neutral pH; (B) apomyoglobin at pH 8.3; (C) apomyoglobin at pH 3.5. The parameters describing the distributions shown in figure are reported in Table II.

whale and tuna myoglobin, respectively. Table II shows the parameters relative to the continuous Lorentzian distribution analysis. In the case of sperm whale myoglobin, the data were fitted by using the superposition of three Lorentzian distributions. The center of the first two was found at very short lifetime values (<0.01 ns), whereas the third distribution was centered at about 7.5 ns. The superposition of the three Lorentzian distributions resulted very broad because of the width of one of the two short-lived distributions. The χ^2 obtained by using the superposition of three Lorentzian distributions, i.e., 1.35, was lower than that obtained by sum of three exponentials, i.e., 4.09. The distribution fit relative to tuna myoglobin required the superposition of two Lorentzian distributions, centered at 0.052 and 3.37 ns, respectively (Figure 2A). Moreover, the two Lorentzian distributions resulted well separated and narrow. Again, the χ^2 obtained from the distribution analysis was lower than that obtained from the exponential fit, i.e., 2.05 and 4.25, respectively.

The removal of the heme drastically changes the tryptophanyl lifetime distribution. Figure 1B shows the distribution recovered from phase and modulation data collected for sperm whale apomyoglobin at 10 °C and pH 8.3. The analysis of the fluorescence decay of this apoprotein still required the superposition of three Lorentzian components. The resulting fit shows two well-separated and narrow lifetime distributions having centers at 2.25 and 5.4 ns. Probably, each distribution reflects the contribution of a specific indole residue. This conclusion confirms the observation that the two tryptophanyl residues, although very close in the primary structure, are located in different microenvironments which affect the emission properties (Irace et al., 1981). In the case of tuna apomyoglobin, the distribution fit was performed by the use of a single Lorentzian component which resulted centered at 2.88 ns (Figure 2B). The use of two Lorentzian distributions did not improve the fit.

The acidification of sperm whale apomyoglobin from pH 8.3 to pH 3.5 shifted the tryptophanyl lifetime distribution pattern toward lifetime values slightly longer than those observed at neutral pH, i.e., the two distribution centers at pH 3.5 were at 2.31 and 6.17 ns, respectively. The lifetime distribution of sperm whale apomyoglobin at acidic pH confirms the picture that acids unfold the heme binding site but not the remainder of the globin molecule. In fact, the presence of two distinct Lorentzian components in the tryptophanyl emission decay indicates that the indole residues are located in distinct microenvironments even at acidic pH. If the protein were fully unfolded, the differences between the emission properties of the tryptophanyl residues would be absent.

The distribution fit of tuna apomyoglobin at acidic pH required the superposition of three Lorentzian components, the main component centered at 5.29 ns (Figure 2C). Therefore, even for this apoprotein, acidification shifts the distribution toward longer lifetime values. Probably, the requirement of more than one component in the analysis of the emission decay of tuna apomyoglobin at acidic pH reflects the

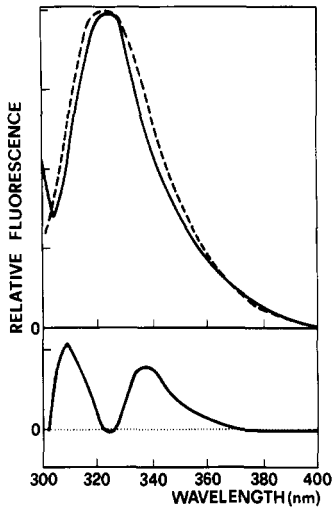

FIGURE 3: Steady-state emission spectra of tuna myoglobin at neutral pH and 10 °C. (—) Excitation at 295 nm; (---) excitation at 285 nm. The lower curve represents the difference between the two emission spectra.

heterogeneity generated by equilibrium among different conformational states.

The steady-state emission spectra of tuna myoglobin obtained at two different excitation wavelengths are shown in Figure 3. A broadening of the spectrum is observed on lowering the excitation wavelength. The difference between the spectra (uncorrected for the Raman contribution; see Materials and Methods), obtained upon excitation at 285 and 295 nm, consists of two positive peaks centered at 310 and 336 nm. The peak at 310 nm arises from a Raman shift whereas that centered at 335 nm originates from tryptophanyl residues selectively excited at a lower wavelength. It is interesting to observe that the emission maximum of the native apomyoglobin is centered at 321 nm whereas that of partially unfolded apoprotein occurs at 333–336 nm (Colonna et al., 1982; Irace et al., 1986).

Discussion

The fluorescence decay of tryptophanyl residues in proteins is affected by several factors including solvent exposure and interactions with other elements of the protein matrix. It has been reported that the tryptophanyl fluorescence lifetime varies over more than a factor of 100 in different proteins (Beechem & Brand, 1985). In the case of the heme proteins, the tryptophanyl emission decay is further influenced by the quenching via Forster coupling with the prosthetic group. The interpretation of the tryptophanyl fluorescence decay in terms of lifetime distributions is based on the assumption that lifetime heterogeneity arises from the multitude of different conformational states and substates that a protein may assume because of structural fluctuations.

A protein molecule can exist in more than one conformational state. Each state is usually related to the particular function exerted by the protein, e.g., the different conformational states that myoglobin assumes upon binding and release of oxygen. Moreover, the folding pathway of the protein molecule may involve the occurrence of conformational states which are in equilibrium with the functionally active conformation. 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., 1985, 1987; Frauenfelder & Gratton, 1985). Different substates have the same overall structure, but they differ in small details. Conformational changes are possible because of the relative weakness of the forces holding the covalently bounded backbone in the folded tertiary structure.

In previous works, we have shown that the molecular state of myoglobin at acidic pH is quite stable and may represent an intermediate in the unfolding–refolding pathway of the molecule (Bismuto et al., 1983; Irace et al., 1986; Ragone et al., 1987). The main characteristic of this state is the loss of structure occurred in the heme binding site. Since acidification of myoglobin results in a large increase of the tryptophanyl fluorescence (Shechter & Epstein, 1968), it is reasonable to admit that the indole residues are not able to transfer energy to the heme group at acidic pH as much as they do in the native conformation.

The observation that the lifetime distribution centers shift toward longer values upon acidification suggests that the long-lived components, observed in the tryptophanyl emission decay of native myoglobin, might arise from conformational states in which geometrical factors preclude the energy transfer from the indole residues to the heme, similarly to the acidic pH case.

The heterogeneity of the emission spectrum of native metmyoglobin at neutral pH further corroborates this hypothesis. In fact, the broadening of the emission spectrum, observed on lowering the excitation wavelength, can be explained in terms of selective excitation of a population of indole residues whose absorption is blue-shifted, i.e., indole residues which are more exposed to solvent (Demchenko, 1982). Moreover, the emission maximum of these selectively excited residues was found similar to that observed for the partially unfolded apoprotein, i.e., 333–336 nm (Irace et al., 1986).

There have been several observations indicating that the overall flexibility of tuna myoglobin is considerably higher than that of sperm whale myoglobin. Bismuto et al. (1987a) were able to detect different dynamic characteristics of the heme site of these two proteins at 20 °C. More recently, the analysis of the temperature dependence of phosphorescence performed on the two myoglobins revealed that the degree of internal flexibility of the tryptophanyl microenvironment of tuna myoglobin is higher than that of sperm whale globin (Bismuto et al., 1987b). The observation that the tryptophanyl fluorescence lifetime distribution of sperm whale myoglobin is much broader than that observed for tuna myoglobin (see Figures 1A and 2A) is consistent with the above reported conclusion. In fact, the interpretation of the fluorescence decay in terms of lifetime distributions is a consequence of protein flexibility (Alcala et al., 1987c). Because of protein flexibility, the tryptophanyl residues experience a variety of environments during the excited state, which results in a quasi-continuum distribution of lifetimes. In this respect, the factors, which affect the protein flexibility by varying the rate of interconversion among conformational states and substates, also influence the distribution width. A temperature increase produces a sharpening of the lifetime distribution because of the increased rate of interconversion. The same effect has been observed on decreasing the solvent viscosity (Alcala et al., 1987b,c). By contrast, protein denaturation induces a broadening of the lifetime distribution due to the wider variety of environments experienced by tryptophan during the excited state (Bismuto et al., 1988). Since the rate of interconversion influences the width of the lifetime distribution, a higher degree of internal flexibility will determine a narrowing of the lifteime distribution bands. On the other hand, if the rate of interconversion is smaller than the decay rate, the protein appears to be frozen for the duration of the excited state which cor-

respond to the time window of our observations. Finally, we may not exclude the possibility that the difference between the lifetime distributions of the two examined myoglobins has a static origin. In this case, the distribution of sperm whale myoglobin would be inhomogeneously broadened with respect to that of tuna myoglobin. However, this explanation appears to be less likely since denaturation studies have excluded the presence of significantive amounts of structural states of myoglobin different from that corresponding to the native form (Bismuto et al., 1983).

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