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Tryptophanyl Fluorescence Heterogeneity of Apomyoglobins. Correlation with the Presence of Two Distinct Structural Domains[†]

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ABSTRACT: The individual fluorescence of the two tryptophan residues (Trp-7 and Trp-14) of mammalian apomyoglobins has been resolved by comparing the fluorescence properties of these proteins to those of bluefin tuna apomyoglobin, which contains only Trp-14. The two tryptophan residues have been found to have different emission maxima, i.e., 321 for Trp-14 and 333 for Trp-7. The fluorescence of Trp-14 depends on the protonation of a sterically related histidyl residue in the pH range between 8.3 and 5.6, where no conformational change was detected. This residue has been identified as His-119. At pH 8.3 the quantum yield of Trp-7 is lower than that of Trp-14. An increase of the fluorescence intensity of Trp-7 occurs when the heme binding site of apomyoglobin is destroyed by acid or a low concentration of guanidine hydrochloride. An independent unfolding of the N-terminal district of the apomyoglobin molecule occurs on increasing the guanidine concentration. The two distinct structural transitions have been discussed in terms of two domains of tertiary structure.

The luminescence properties of tryptophan residues are, in general, dependent upon their microenvironments. The widespread location of chromophores into the proteic matrix is responsible of the great diversity in emission behavior for the single residues. This fact leads into the consideration that the observed protein fluorescence arises from unequal contributions of individual residues to the total emission. The resulting luminescence heterogeneity is detectable by various fluorescence techniques (Kronman, 1976; Longworth, 1977), but the discrimination and the study of the fluorescence properties characterizing each residue always present difficulties.

Most of the myoglobins have two tryptophans which fill the invariant positions 7 and 14 (Dayhoff, 1972; Jones, B. N., et al., 1976; Lehman et al., 1977). Few attempts have been made to distinguish the fluorescence behavior of the two tryptophans.

Kirby & Steiner (1970) reported that the two tryptophan residues of sperm whale apomyoglobin differ, one of the two being more fluorescent than the other one, but they were not able to distinguish the individual contribution. An interesting

Table I: Aromatic Amino Acid Composition and Some Fluorescence Properties of Apomyoglobins

protein source	mol of residue/mol of ApoMb			emission max[f] (nm)	quantum yield[g]	extent of perturbation by ethylene glycol (%)
	Trp	Tyr	Phe			
buffalo[a]	2	2	4	325	0.070	9
beef[b]	2	2	6	326	0.065	10
sperm whale[b]	2	3	6			
minke whale[c]	2	2	7	328	0.085	10
bottlenose dolphin[d]	2	2	7	327	0.090	8
bluefin tuna[e]	1	2	7	321	0.140	0

[a-e] The aromatic amino acid compositions are taken from [a]Balestrieri et al. (1973), [b]Dayhoff (1972), [c]Lehman et al. (1977), [d]Jones et al. (1976), and [e]Balestrieri et al. (1978). [f] Determined in 0.05 M potassium phosphate and 0.15 M KCl, pH 8.3; excitation = 295 nm. [g] Determined by using N-acetyltryptophanylamide as a quantum yield standard ($Q = 0.14$). [h] Determined by comparing the fluorescence increase upon addition of 20% ethylene glycol to native and guanidine-unfolded apomyoglobin.

work was done by Anderson et al. (1970) that showed that the two tryptophanyl residues of Aplysia apomyoglobin are differently exposed to the solvent and hence subject to different environmental influences. However, it should be noted that the Aplysia myoglobin contains the tryptophans in different positions, i.e., 14 and 126 (Kovacs et al., 1977).

A correct approach to the identification of heterogeneous species should lie on the selective chemical modification of proteins where the fluorescence features of a given tryptophan residue could be studied by elimination of its contribution to the spectrum. We have achieved the same goal by using tuna apomyoglobin, with the specific requisite residue replacement. In fact, this protein contains only one tryptophan, the indole residue in position 7 being missed (Balestrieri et al., 1977, 1978).

In the present work we have compared the fluorescence properties of tuna apomyoglobin with those of other species containing two tryptophans. The individual behavior of the two tryptophans resulting from acid or guanidine perturbation has been depicted. The results are explained in terms of two distinct structural domains.

Materials and Methods

Myoglobins. Bluefin tuna, buffalo, and beef myoglobins were prepared according to the methods previously described (Balestrieri et al., 1973, 1978). All preparations of myoglobin were metmyoglobin and will be referred as myoglobin. The homogeneity of the preparations was tested by disc gel electrophoresis at pH 8.6 on 7% polyacrilamide, as reported by Davis (1964). A single protein band was always observed by this technique. Sperm whale myoglobin was purchased from Sigma Chemical Co. Myoglobin from Atlantic bottlenosed dolphin (*Tursiops truncatus*) and from Arctic minke whale (*Balaenoptera acutorostrata*) was a gift of Dr. F. R. N. Gurd (Department of Chemistry, Indiana University, Bloomington, IN).

Apomyoglobins. The heme was removed from myoglobin by the 2-butanone extraction procedure of Teale (1959). This method was preferred to that of cold acid–acetone extraction (Rossi-Fanelli et al., 1958), since it produces a minor denaturation of the globin. The contamination of the apoprotein by myoglobin was assessed spectrophotometrically. In all cases no significant absorption was observed in the Soret region.

Protein Concentration. The concentrations of apomyoglobins were determined by absorbance at 280 nm on a Perkin-Elmer Model 575 spectrophotometer. The molar extinction at 280 nm of bluefin tuna, buffalo, beef, minke whale, and bottlenosed dolphin was calculated from the tryptophan and tyrosine content (see Table I) by using molar extinction coefficients of 5500 and 1250, respectively (Wetlaufer, 1962).

Chemicals and Solutions. All common chemicals were reagent grade and were purchased from British Drug Houses.

Ultrapure Gdn·HCl[1] was obtained from Schwarz/Mann.

Acid titrations were performed by careful addition of small amounts of concentrated HCl to buffered solutions (5×10^{-2} M phosphate, 1×10^{-2} M acetate, and 0.15 M KCl) from an Agla syringe while the solutions were stirred magnetically. Measurements of pH were carried out by using combined electrodes with either Radiometer Model 26 or Methrom Herisau 405 pH meters.

In denaturation experiments the protein was added to buffered solutions of Gdn·HCl; 0.15 M KCl was present in all solutions. The fluorescence was then followed in time until an apparent equilibrium was reached. The reversibility of Gdn·HCl denaturation was confirmed by diluting samples in concentrated denaturant with buffer.

Ultraviolet Fluorescence. Fluorescence measurements were made in the range where emission was linear with protein concentration. The absorbance of all solutions was <0.1 A at the excitation wavelength. The temperature of the solution cell was maintained at 22 °C.

The relative quantum yields of tryptophan emission were calculated by comparing the absorbance (Abs) at 295 nm and the areas (A) of the emission spectra of the protein with a standard of known quantum yield, according to the equation

$$q_{prot} = q_{st} \frac{A_{prot}}{A_{st}} \frac{Abs_{st}}{Abs_{prot}}$$

The standard was a twice recrystallized preparation of N-acetyltryptophanamide. A quantum yield of 0.14 has been reported for this compound in aqueous solution (Eisinger, 1969).

Polarization Measurements. Polarization measurements were performed on a Perkin-Elmer MPF2A spectrofluorometer. The polarization was calculated from $P = (I_{VV} - GI_{VH})/(I_{VV} + GI_{VH})$ where $G = I_{HV}/I_{HH}$, I is the intensity, and the first and the second subscripts refer to the plane of polarization of the excitation and emission beams, i.e., V, vertical, and H, horizontal (Chen et al., 1969).

Results

The molecular structure of a large number of apomyoglobins from different animal species, i.e., buffalo, beef, sperm whale, minke whale, bottlenose dolphin, and bluefin tuna, has been examined by following the effects produced by perturbing agents, such as acid and/or guanidine hydrochloride, on the spectral properties of this class of homologous proteins. Table I shows the aromatic amino acid composition and some fluorescence properties of all examined apomyoglobins. The

[1] Abbreviations used: Gdn·HCl, guanidine hydrochloride; ANS, 1-anilino-8-naphthalenesulfonate; Trp, tryptophan; His, histidine; Lys, lysine; Glu, glutamic acid; CD, circular dichroism; q, quantum yield.
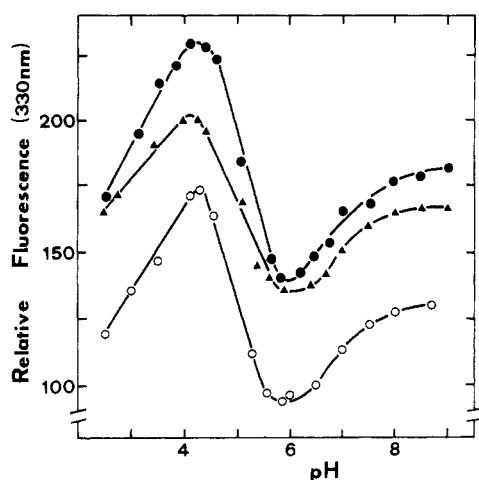
FIGURE 1: Acid pH dependence of tryptophanyl fluorescence at 330 nm of beef (O), bottlenosed dolphin (●), and minke whale (▲) apomyoglobins in 0.05 M potassium phosphate, 0.01 M acetate, and 0.15 M KCl. Excitation was at 295 nm. Samples were prepared by diluting 1:20 concentrated solutions of each protein (optical density at 280 nm = 1.0).

Table II: Properties of Apomyoblobins at Different pHs

protein source	emission max (nm) pH 8.3	emission max (nm) pH 5.6	$(q_{max} - q_{min})/q_{max}$
buffalo	325	327	0.26
beef	326	328	0.22
sperm whale	330[a]	330[a]	0.23
minke whale	328	330	0.29
bottlenose dolphin	327	328	0.19
bluefin tuna	321	321	0

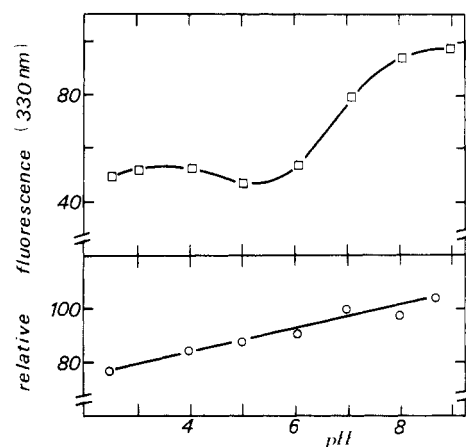[a] Data from Kirby & Steiner (1970).

FIGURE 2: Acid pH dependence of tryptophanyl fluorescence at 330 nm of bottlenosed dolphin (O) and minke whale apomyoglobins (□) in 0.05 M potassium phosphate, 0.01 M acetate, 0.15 M KCl, and 6.0 M guanidine hydrochloride. Excitation was at 295 nm. Protein samples were prepared as reported in Figure 1.

most significant difference among the proteins listed in Table I is that tuna apomyoglobin contains a single tryptophan residue in position 14 (Balestrieri et al., 1978) whereas the other ones contain two tryptophanyl residues located in position 7 and 14 (Dayhoff, 1972; Jones, B. N., et al., 1976; Lehman et al., 1977). Moreover, the emission maximum of tuna apomyoglobin occurs at a wavelength slightly lower than that observed for the other apomyoglobins. In addition, the quantum yield of tuna apomyoglobin resulting from 295-nm excitation is much higher than that observed for mammalian apomyoglobins.

The accessibility of fluorescence perturbants such as ethylene glycol (20% v/v) to the indole rings of apomyoglobins was evaluated by comparing the extent of perturbation in phosphate buffer, pH 8.3, and in 6.0 M guanidine hydrochloride. The perturbation values were found to range between 5% and 10% depending on animal source as shown in Table I. The results reported in this paper are consistent with those obtained by Kirby & Steiner (1970) on sperm whale apomyoglobin using iodate ion as a fluorescence quencher. In contrast to the mammalian apomyoglobins, tuna apomyoglobin was not perturbed by 20% ethylene glycol under the same experimental conditions.

(a) Effects of Acid. The acid pH dependence of tryptophanyl fluorescence at 330 nm of beef, minke whale, and dolphin apomyoglobin is reported in Figure 1. The acid titration patterns of the three globins resemble each other rather closely, and they are almost completely superimposable on those found for sperm whale (Kirby & Steiner, 1970) and buffalo apomyoglobin (Colonna et al., 1978). The differences observed at pH 8.3 in the intensity of tryptophanyl fluorescence are due to the slightly different quantum yield values of the three globins. The acid titration curves can be divided into three distinct regions: the first going from 8.3 to 5.6 will be indicated as the neutral region; the remaining ones will be indicated as acidic (from 5.6 to 4.0) and as strongly acidic (from 4.0 to 2), respectively.

(1) Neutral Region. Lowering the pH from 8.3 to 5.6 resulted in a quenching of the tryptophanyl fluorescence with no significant shift of the emission maximum (Table II). According to Kirby & Steiner (1970), the fluorescence quenching observed from pH 8.3 to pH 5.6 could be due to the formation of a charge-transfer complex between the indole

ring of a tryptophan residue (donor) and the imidazolium ring of a nearby protonated histidyl residue (acceptor). The probability of a charge-transfer complex being formed may be quantitatively estimated by the ratio of the difference between the quantum yields at lower and upper plateaus of the fluorometric titration curve and the maximal quantum yield (Shinitzky & Goldman, 1967). The probability values determined for all examined apomyoglobins are reported in Table II. The values were found to be around 0.30, lower than those found by Shinitzky & Goldman (1967) for model compounds, which ranged between 0.52 and 0.78.

The low values of $\Delta q/q_{max}$ may be justified by the distance between the two residues involved in the charge-transfer complex formation, which need not to be adjacent in the primary sequence. Beef and bottlenosed dolphin apomyoglobins do not contain any histidine residue close to the tryptophans in their primary structure: in fact, the closest histidine is 10 residues away from Trp-14, i.e., at position 24 (Dayhoff, 1972; Jones, B. N., et al., 1976).

On the other hand, minke whale apomyoglobin contains a histidine residue (His-8) adjacent to tryptophan (Lehman et al., 1977). Nevertheless, the acid titration pattern as well as the probability value found for minke whale apomyoglobin is very similar to those obtained for the other examined proteins. This observation suggests that the charge-transfer complex with tryptophan in minke whale apomyoglobin is not formed with His-8, but it takes place with a different sterically related residue.

Figure 2 shows the fluorometric titration of minke whale and bottlenose dolphin apomyoglobin in 6.0 M guanidine hydrochloride. The latter protein showed a monotonic decrease of tryptophanyl fluorescence between pH 8.3 and 5.6, indicating that the loss of tertiary structure removes the sterically interacting histidine from the indole. In contrast to this result,
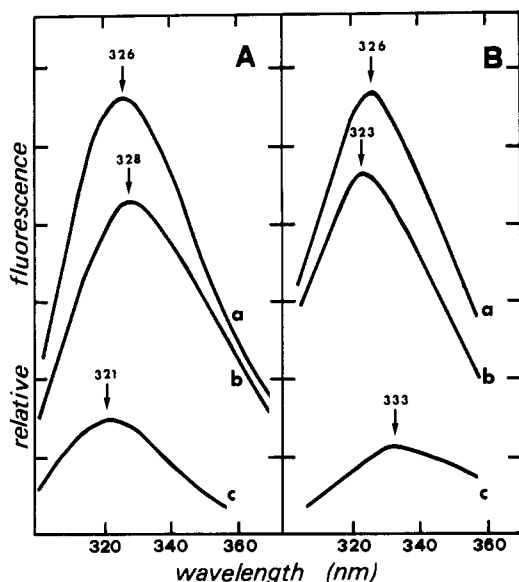
FIGURE 3: Effect of acid (A) and of iodide (B) on the fluorescence spectrum of beef apomyoglobin. (A) (a) pH 8.0; (b) pH 5.6; (c) indicates the computed difference spectrum (a) − (b). (B) (a) pH 8.0 without iodide; (b) pH 8.0 with 0.235 M iodide; (c) indicates the computed difference spectrum (a) − (b). The solvent was 0.05 M potassium phosphate, 0.01 M acetate, and 0.15 M KCl. Excitation was at 295 nm. Protein samples were prepared as indicated in Figure 1.

FIGURE 4: Acid pH dependence of tryptophanyl fluorescence at 320 (O) and at 360 (▲) nm of beef apomyoglobin in 0.05 M potassium phosphate, 0.01 M acetate, and 0.15 M KCl. Excitation was at 295 nm. Samples were prepared as indicated in Figure 1. The lower curve is the difference between the values at 320 and 360 nm.

a large change of tryptophanyl fluorescence was observed in the titration of the former apomyoglobin under the same experimental conditions. The probability value determined for minke whale apomyoglobin in 6.0 M guanidine hydrochloride, i.e., 0.45, was larger than that observed in phosphate. Moreover, a significant difference was also observed in the pK value determined in phosphate, i.e., pK = 7.1, and in guanidine, i.e., pK = 6.7. The result reported above may be explained by assuming that the folded structure of minke whale apomyoglobin does not allow any interaction between the two adjacent residues. Only in denaturing solvents His-8 becomes free from other steric interactions and, if protonated, interacts with the adjacent tryptophan forming a charge-transfer complex. In fact, the extent of fluorescence change observed in 6.0 M guanidine hydrochloride was almost similar to that obtained by Shinitzky & Goldman (1967) on histidyl-tryptophan dipeptides.

Another possible explanation for the fluorescence quenching observed as pH lowers from 8.3 to 5.6 is that a conformational change takes place and affects the electrostatic microenvironment of tryptophanyl residues. This possibility seems to be less likely since no change of CD activity in the far-UV as well as of tryptophanyl fluorescence polarization was observed in the same pH range. Moreover, the emission maximum of all the examined mammalian apomyoglobins was red shifted at pH 5.6 only 1 or 2 nm.

Figure 3A shows the fluorescence difference spectrum generated upon acidification of beef apomyoglobin from pH 8.3 to 5.6. Since very little spectral shift occurs in this pH range, the difference spectrum would correspond rather closely to the spectrum of the tryptophan quenched at acid pH. The fluorescence difference spectrum reported in Figure 3A shows the presence of a maximum centered at 321 nm. Dolphin and whale apomyoglobin gave similar fluorescence difference spectra with maxima at the same wavelength. The correspondence between the acid fluorescence difference maximum of mammalian apomyoglobin and the emission maximum of tuna globin suggests that the acid-quenched tryptophan in
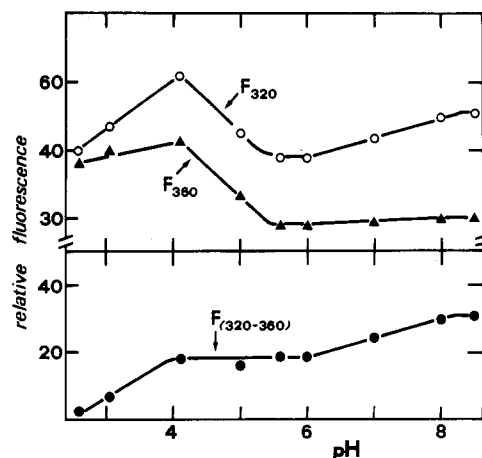
mammalian apomyoglobins could be the one located in position 14, Trp-7 being missed in tuna apomyoglobin.

Since the emission maximum of unperturbed mammalian apoprotein, which reflects contributions from both indole residues, has its maximum around 326 nm, the emission maximum of Trp-7 should be at wavelengths higher than 326 nm. We have verified this possibility by examining the fluorescence difference spectrum generated upon addition of iodide to apomyoglobin solutions. It is well-known that iodide is a selective quencher of the fluorescence of the tryptophanyl side chains of proteins which are exposed to some extent to the solvent (Lehrer, 1971). The effect of 0.235 M iodide on the emission spectrum of beef apomyoglobin at neutral pH is shown in Figure 3B. In presence of iodide the emission maximum of beef apomyoglobin is blue shifted 3 nm, and it occurs at 323 nm; on the other hand, the resulting fluorescence difference spectrum which would correspond to the spectrum of iodide-quenched tryptophan shows the presence of a maximum at 333 nm. Therefore the two tryptophanyl residues of mammalian apomyoglobins are not equivalent in their luminescent properties since their emission maxima occur at different wavelengths; more precisely, the emission of Trp-14 occurs at ∼321 nm whereas that of Trp-7 is at a longer wavelength, i.e., 333 nm. Similar findings were obtained by Lee & Richards (1971), who were able to calculate the solvent accessibility of the two tryptophan residues of sperm whale myoglobin from X-ray data.

Figure 4 shows an attempt made to isolate the contributions of the two different emitting chromophores upon acidification of beef apomyoglobin. The variations of the fluorescence intensity at 360 nm should reflect the contribution of the more exposed tryptophanyl residue, i.e., the one located in position 7, while the intensity at 320 nm should be indicative of the behavior of the more buried residue, i.e., Trp-14. The result reported in Figure 4 shows that the fluorescence change at 360 nm between pH 8.0 and pH 5.6 is very low (<8%) compared to the extent of the change observed at 320 nm (25–30%).

The acid pH dependence of tryptophanyl fluorescence of tuna apomyoglobin is shown in Figure 5. In contrast to the mammalian apomyoglobins, the fluorescence intensity as well as the emission maximum of tuna globin was unchanged on lowering the pH from 8.3 to 5.6. This result, which appears to be in contrast with the above-reported conclusion, may be justified by the fact that tuna myoglobin as well as the globins isolated from several tuna species contains half of the histidine
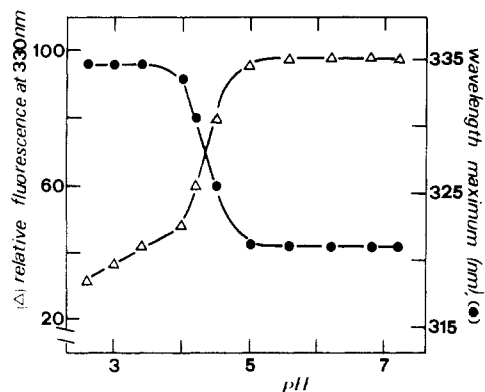
FIGURE 5: Acid pH dependence of tryptophanyl fluorescence at 320 nm (Δ) and of the emission maximum ($\bullet$) of bluefin tuna apomyoglobin in 0.05 M potassium phosphate, 0.01 M acetate, and 0.15 M KCl. Excitation was at 295 nm. Samples were prepared as indicated in Figure 1.

residues normally present in mammalian myoglobins (Balestrieri et al., 1978; Fosmiri & Brown, 1976; Konosu et al., 1958). Therefore, it is likely that the tryptophan-interacting histidine is missed in tuna globins.

(2) *Acidic and Strongly Acidic Regions*. Further acidification of mammalian apomyoglobins to pH 4.0 produced a large increase of the quantum yield (Figure 1) and a red shift of the emission maximum from 325 to about 330–332 nm. These changes result from a conformational change which produces a 50% loss in helical structure (Kirby & Steiner, 1970; Colonna et al., 1978) and makes the apoprotein unable to bind ANS (Shen & Hermans, 1972). We have examined the acid-induced molecular transition of apomyoglobin by following the tryptophanyl fluorescence polarization in the pH range from 8.3 to 2.0. Between pH 8.3 and 5.6 the degree of polarization was unchanged, and it was found to be around 0.14 for all the examined apoproteins. Acidification to pH 4.0 produced a decrease of the tryptophanyl fluorescence polarization from 0.14 to 0.10. No further change was observed on lowering the pH from 4.0 to 2.0. The polarization change indicates that the internal flexibility of the globin matrix is enhanced; however, the value of tryptophanyl polarization observed at pH 4.0 is significantly higher than that observed for low molecular weight model compounds in water or for fully denatured proteins (Alexander et al., 1978). This observation suggests that at pH 4.0 the tryptophans are still embedded into a structural environment possessing a large amount of organized structure. Moreover, the emission maximum at pH 4.0 (330–332 nm) is still far from that observed for tryptophan in water (350–360 nm) (Teale, 1960). The fluorescence quenching observed at pH lower than 4.0 (Figure 1) could be due to a protonation of a nearby carboxyl group since no further conformational change seems to take place, as documented by the changes in the far-ultraviolet CD activity (Colonna et al., 1978) and in the tryptophanyl fluorescence polarization.

The acid titration behavior of the tryptophanyl fluorescence of tuna apomyoglobin differs from that observed for mammalian apomyoglobins. Acidification to pH 4.0 (Figure 5) resulted in a large decrease ($\sim$50%) of tryptophanyl fluorescence with a concomitant red shift of the emission maximum from 321 to 335 nm. Further acidification to pH 2.0 did not change the emission maximum although the fluorescence intensity was reduced by 16%.

(b) *Effects of Guanidine Hydrochloride*. The effects of increasing the concentration of guanidine hydrochloride on the fluorescence properties of beef, minke whale, and bottlenosed
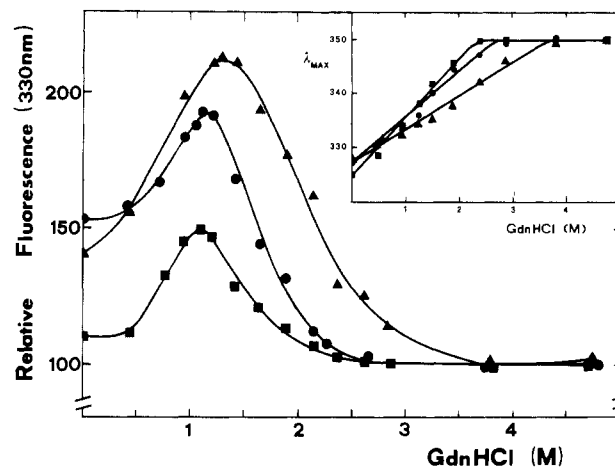

FIGURE 6: Effect of guanidine hydrochloride on the tryptophanyl emission intensities of beef ($\blacksquare$), bottlenosed dolphin ($\bullet$), and minke whale ($\blacktriangle$) apomyoglobins at pH 8.0 in 0.05 M potassium phosphate and 0.15 M KCl. The inset shows the effect of guanidine hydrochloride on the emission maximum. Excitation was at 295 nm. Protein samples were prepared as described in Figure 1.
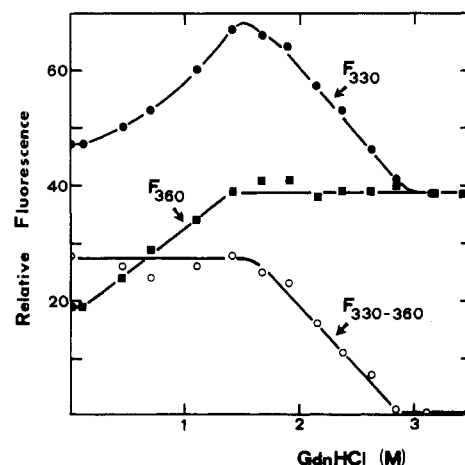

FIGURE 7: Effect of guanidine hydrochloride on the tryptophanyl fluorescence emission of minke whale apomyoglobin at 330 ($\bullet$) and 360 ($\blacksquare$) nm at pH 8.0 in 0.05 M potassium phosphate and 0.15 M KCl. (O) is the difference between the fluorescence intensities at 330 and 360 nm. Protein samples were prepared as reported in Figure 1.

dolphin apomyoglobins at pH 8.0 are reported in Figure 6. The increase of denaturant concentration produces changes both in fluorescence magnitude and in the position of emission maximum. The guanidine unfolding patterns of the three globins resemble each other rather closely, and they are almost completely superimposable on the one previously reported for buffalo apomyoglobin (Colonna et al., 1978).

The emission intensity change at 330 nm is rather complex: between 0 and 1.0–1.2 M guanidine hydrochloride the fluorescence intensity of the examined mammalian apomyoglobins increases almost linearly whereas at higher denaturant concentrations the fluorescence drops rather sharply, reaching the minimum at 2.5–3.0 M Gdn·HCl. However, the emission maximum of the three globins is linearly shifted to a higher wavelength upon increasing the concentration of guanidine hydrochloride from 0 to 2.5–3.0 M; at these concentration values the fluorescence maximum is similar to that observed for low molecular weight model compounds in aqueous solution (Teale, 1960). It is difficult to explain why the effect of guanidine hydrochloride on the tryptophanyl emission is biphasic, whereas the red shift of the emission maximum is linearly related with denaturant concentration. An attempt
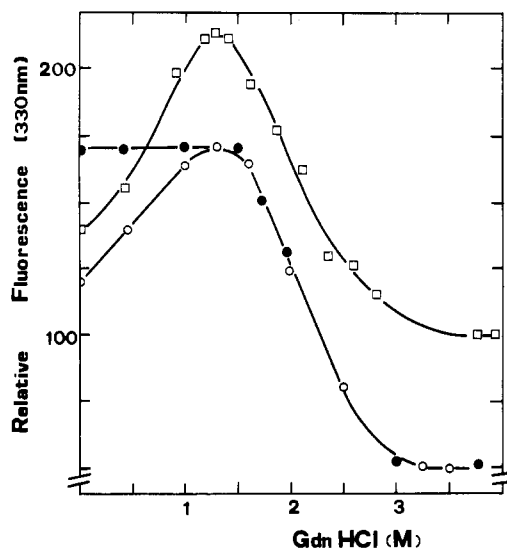
FIGURE 8: Effect of guanidine hydrochloride on the tryptophanyl fluorescence at 320 nm of minke whale apomyoglobin at different pH values: (□) pH 8.0; (O) pH 6.0; (●) pH 4.0. The solvent was 0.05 M potassium phosphate, 0.01 M acetate, and 0.15 M KCl. Excitation was at 295 nm. Protein samples were prepared as indicated in Figure 1.

made to understand the biphasic behavior of the fluorescence intensity is shown in Figure 7. The change of the fluorescence intensity of minke whale apomyoglobin at 360 nm should reflect the contribution of the more exposed tryptophanyl residue, which we have shown to be the one in position 7. On the other hand, the fluorescence difference between the intensities at 330 and 360 nm may be indicative of changes occurring in the microenvironment of the more hidden indole chromophore.

The data reported in Figure 7 show that the changes in the luminescence properties of the two major emitting chromophores occur as distinct processes. Between 0 and 1.5 M guanidine hydrochloride the fluorescence intensity at 360 nm increases linearly with denaturant concentration, whereas at higher Gdn·HCl concentration no further changes are observed. The question arises whether the biphasic behavior reflects two distinct molecular transitions, having midpoints at 0.8 and 2.2 M guanidine hydrochloride, respectively. Balestrieri et al. (1976a) have demonstrated by equilibrium data obtained at several guanidine concentrations that a loosening of the tight folding of the native structure, leading to abolition of the capacity to bind ANS molecules, occurs as a separate process preceding the exposure of the tryptophan side chains to the solvent. Following this observation, the transition observed at low denaturant concentration should reflect the conformational change occurring in the ANS binding site which has been found to be the same as that of the heme (Stryer, 1965).

Shen & Hermans (1972) have demonstrated that the acid-induced conformational change of sperm whale apomyoglobin occurring between pH 5.6 and 4.2 also unfolds the ANS binding site. Figure 8 shows the transition curves of minke whale apomyoglobin obtained by increasing the concentration of Gdn·HCl at three different pH values, i.e., 8.0, 6.0, and 4.0. At pH 6.0 the transition curve is similar to that obtained at pH 8.0 except for the fluorescence intensity which is quenched at pH 6.0.

At pH 4.0 the biphase behavior is lost, and the transition curve resembles that obtained by fluorescence difference between the intensities at 330 and 360 nm (see Figure 7). Thus, the acid-induced molecular transition which takes place be-

tween pH 5.6 and 4.0 makes the apomyoglobin not susceptible to the fluorescence change observed at low guanidine concentrations.

Discussion

The results reported in this paper can be fitted into the following general picture. Mammalian apomyoglobins contain two tryptophanyl residues in positions 7 and 14 (Dayhoff, 1972; Jones, B. N., et al., 1976; Lehman et al., 1977) which are not equivalent in their luminescence properties. Although the two indole chromophores are very close in the primary sequence, the heterogeneity of tryptophanyl emission depends upon differences in the specific microenvironment of each emitting chromophore.

The acid pH dependence of tryptophanyl fluorescence of mammalian apomyoglobins between pH 8.3 and 5.6 results from the quenching of Trp-14 by a sterically related histidine residue with pK in the same pH range. Two histidyl residues may be involved in forming a charge-transfer complex with Trp-14 as indicated by the X-ray structure of sperm whale myoglobin, i.e., His-24 and His-119 (Takano, 1977). His-24 is completely buried among the B, E, and G helices, whereas His-119 is partially exposed in the GH loop between the G and H helices (Matthew et al., 1978).

The coplanar geometry of charge-transfer complexes requires a full ring face of the donor to be available for electron sharing with the acceptor (Slifkin, 1971); in this respect His-119 appears to be the most probable candidate for charge-transfer complex formation because of its orientation (Watson, 1968; Takano, 1977). Moreover, the fluorometric titration pattern of Trp-14 is consistent with the pK of His-119 which has been found to range between 6.5 and 7.0 by several authors (Shire et al., 1974a,b; Cohen et al., 1972; Hayes et al., 1975).

More recently the pK of His-119 has been found to be 5.55 by proton NMR (Botelho & Gurd, 1978; Botelho et al., 1978). In this case the discrepancy between the fluorometric titration and the pK of His-119 could be due to a minor structural change occurring in the neutral pH range. Evidences for such a change have been previously reported (Jones, W. C., et al., 1976; Colonna et al., 1978). However it should be taken into account that the removal of the heme decreases the helical content of the molecule (Breslow et al., 1965) and changes its shape (Crumpton & Polson, 1965) and its surface charge (Shire et al., 1974a,b). All these changes certainly produce environmental modification which can affect the pK of certain groups.

The lack of His-119 could make understandable the different fluorometric titration pattern observed for bluefin tuna apomyoglobin; this is not unlikely since it is known that His-119 as well as His-24 is missed in the myoglobins of some marine species like shark and conch (*Busicon caniculatum*) (Castillo et al., 1977).

The fluorescence enhancement observed on lowering the pH from 5.6 to 4.3 results from a conformational change which produces loss of secondary and tertiary structure. However, the helical content, as well as the degree of tryptophanyl fluorescence polarization and the position of the emission maximum, indicates that a large amount of residual organized structure is still present at pH 4. Shen & Hermans (1972) found that apomyoglobins lose the capacity to bind ANS in the pH range 6-4. The observation that ANS binds the apoprotein in the same nonpolar moiety of heme (Stryer, 1965) leads to the conclusion that the acidic molecular transition unfolds the molecular district containing the heme pocket but not the N-terminal region of the molecule where both tryp-

tophans are located. Similar findings were obtained by Balestrieri et al. (1976a) following guanidine unfolding of apomyoglobins from several animal species. They observed that the molecular transition destroying the heme pocket occurs as separate process preceding the exposure of the tryptophanyl residues to the solvent. Both the acid- and guanidine-induced unfolding of the heme pocket occur with enhancement of tryptophanyl fluorescence without much change of the emission maximum.

The transition curves of mammalian apomyoglobins, obtained by following the fluorescence intensities at different wavelengths, show that the unfolding of the heme pocket increases the quantum yield of a tryptophan residue, whose contribution to the total emission is more evident at the red-wavelength edge of the emission spectrum. Since Trp-14 has its emission maximum centered at 321 nm, we must admit that the increase of quantum yield upon unfolding of the heme pocket depends predominantly on Trp-7, whose emission is at longer wavelength, i.e., 333 nm.

The Trp-7 seems to be quenched to some extent by a sterically related group. Unfolding of heme pocket by acid or guanidine should remove the quencher from Trp-7 as documented by the increase of fluorescence intensity observed on lowering the pH to 4.3 or increasing guanidine hydrochloride concentration to 1.0–1.2 M.

Native tuna apomyoglobin has a much higher value of quantum yield, if compared to that of mammalian apoproteins (see Table I). If we assume that tryptophanyl residues of mammalian apomyoglobins behave independently, then the measured quantum yield, Q_{obsd}, can be considered as a number-average yield of the individual residue yields, Q_i, i.e., $Q_{obsd} = Q_i/n$, where Q_i is the yield of the ith tryptophan residue and n is the number of residues. Therefore, the quantum yield of Trp-7 might be calculated as

$$Q_{Trp-7} = 2Q_M - Q_T$$

where Q_M and Q_T are the observed quantum yield of mammalian and tuna apomyoglobin, respectively. Such a calculation obtained by using the data reported in Table I confirms our feeling that the fluorescence of Trp-7 is strongly reduced by a sterically interacting quencher group in native apomyoglobins. The X-ray analysis of sperm whale myoglobin (Takano, 1977) revealed that Trp-7 is close to Lys-79 which forms a salt bond with Glu-4 to stabilize the N-terminal region of the A helix. Since both the charged and uncharged ϵ-amino groups of lysine are potential quenchers of tryptophan in proteins (Brand & Witholt, 1967), we can reasonably identify Lys-79 as the quencher group.

The low extent of fluorescence perturbation by ethylene glycol or by iodide ion is also in agreement with the above reported conclusion. In fact, the perturbing agents would mostly act on the more exposed tryptophan residue, i.e., the one in position 7, whose contribution to the total fluorescence emission is very low. However, other possible explanations could be represented by the presence of an energy transfer between tryptophanyl residues or by excimer formation(s). In fact, it has been suggested that the low quantum yield observed for lysozyme can be explained in terms of excimer formation(s) (Longworth, 1966).

The above-reported data can be explained by assuming the presence in the apomyoglobin molecule of two domains which represent the product of independent folding processes. In fact, our data indicate that acid or low guanidine concentration only produces unfolding of the molecular district containing the heme binding site. Higher guanidine concentration unfolds the residual protein structure of the N-terminal region. This

conclusion is consistent with a large number of data existing in the literature (Puett, 1972; Kuntz, 1975; Wetlaufer, 1973).

Ptitsyn & Rashin (1975) were able to simulate the folding process of sperm whale apomyoglobin by taking in account the initial presence of nucleation centers. The most important feature of their analysis is the presence of two structural "halves", the function of which is to trap the heme molecule by a subsequent domain association step. This conclusion is also supported by the fact that the size of the apomyoglobin molecule is larger than that of myoglobin (Crumpton & Polson, 1965). The collapse of the two halves around the heme appears to be due to those residues which are in nonregular conformations (Epand & Sheraga, 1968). Binding of heme increases the protein stability by a few kilocalories per mole (Puett, 1973; Balestrieri et al., 1976b), and the whole structure becomes tightly connected and therefore behaves as one cooperative region. The removal of the heme increases the mobility of the two folded units and makes them susceptible to an independent unfolding process.

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Nuclear Magnetic Resonance Characterization of Aromatic Residues of α-Lactalbumins. Laser Photo Chemically Induced Dynamic Nuclear Polarization Nuclear Magnetic Resonance Studies of Surface Exposure[†]

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ABSTRACT: The α-lactalbumins are involved as modifier proteins in lactose biosynthesis. Lactalbumins from different mammalian species are cross-reactive with the galactosyltransferases from others because of their homologous primary and tertiary structure. We have studied the surface exposure of several α-lactalbumins (bovine, goat, human, guinea pig, and rabbit) by the laser photo-CIDNP technique, an NMR method which measures the access of a photoexcited flavin dye to surface exposed Tyr, Trp, and His residues. An exposed histidine-68 residue exists in the bovine and goat species but is missing in other species and is replaced with a His-10 in the guinea pig species. The only exposed tryptophan residue was Trp-104, which cross-relaxed to nearby Trp-60. Cross-polarization to the latter residue was proven by taking free induction decays after extremely short light pulses (0.1 s). Furthermore, this cross-polarized Trp-60 resonance was absent in the guinea pig species, which has a substitution at position 60. The chemical shifts of these Trp residues were extremely similar in all species but rabbit, which has substituted His for a Tyr at position 103, situated close to both Trp-104 and -60. Three of the four tyrosines in bovine, goat, human, and guinea pig α-lactalbumin were exposed (the latter species has an extra Tyr in its sequence which was apparently not accessible). The rabbit species contains only two Tyr residues, the one which was not accessible (Tyr-50) being common to all species.

α-Lactalbumin, a principal milk whey component, functions as a "modifier" protein in lactose biosynthesis. The protein-enzyme lactose synthase complex between αLA[1] and galac-

tosyltransferase (UDPgalactose:D-glucose 4-β-galactosyltransferase, EC 2.4.1.22) exhibits over a 1000-fold stronger affinity for glucose than in the absence of α-lactalbumin. Thus, the unique structural changes which accompany the association of one molecule each of galactosyltransferase and of αLA manifest themselves in an altered specificity from N-acetylglucosamine to glucose acceptors. The primary structures for several αLA species have been reported, in-

[1] Abbreviations used: αLA, α-lactalbumin; CIDNP, chemically induced dynamic nuclear polarization; DSS, 2,2-dimethyl-2-silapentane-5-sulfonate; FID, free induction decay.