Heme and Cysteine Microenvironments of Tuna Apomyoglobin. Evidence of Two Independent Unfolding Regions†

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ABSTRACT: The heme and cysteine microenvironments of bluefin tuna apomyoglobin have been investigated by examining the fluorescence properties of two extrinsic chromophores, i.e., ANS and 1,5-AEDANS. 1,5-AEDANS was covalently bound to the single cysteine residue found in the primary structure of tuna apomyoglobin. Recombination experiments with hemin showed that tuna apomyoglobin does not bind 1,5-AEDANS in the same binding site of the heme, although the fluorescence properties of the covalently bound 1,5-AEDANS strongly suggest that the dye is embedded in a rather nonpolar microenvironment. ANS was selected because of its ability to bind the apomyoglobin in the same nonpolar moiety of the heme. Acidification of apoMb–AEDANS to pH 3.0 produced an increase of 1,5-AEDANS fluorescence intensity and a shift of its emission maximum from 475 to 470 nm. In the same pH range apomyoglobin lost its ability to bind ANS. Two independent transitions were observed with increasing concentrations of guanidine. Low guanidine concentration (less than 1.0 M) unfolded the heme binding site as indicated by the disappearance of ANS fluorescence, whereas higher denaturant concentration was required to produce full normalization of 1,5-AEDANS emission spectrum.

It has been suggested that myoglobins contain two independent unfolding regions, which are tightly connected by the heme in the native structure (Balestrieri et al., 1976; Colonna et al., 1978; Irace et al., 1981). The X-ray analysis of the myoglobin structure (Wetlaufer, 1973) as well as the diagonal plot analysis of Kuntz (1975) revealed the possibility of such an occurrence, but the identification of domain regions was complicated by the presence of both the heme and many long-range intramolecular interactions, which made it difficult to recognize the domain borderlines. A correct approach would be the labeling of strategic protein sites with structural probes having peculiar characteristics. In this way, disarraying the whole structure by denaturants, one can independently follow the molecular district in which each probe is embedded.

We have used intrinsic, i.e., tryptophan, and extrinsic fluorophores, i.e., ANS[1] and 1,5-AEDANS, as structural probes to demonstrate the presence in the apomyoglobin molecule of two structural regions having independent globular properties. ANS is known to bind apomyoglobin in the same nonpolar moiety of the heme (Stryer, 1965), whereas 1,5-AEDANS is a fluorescent reagent which combines the reactivity of iodoacetate toward sulfhydryl groups with the spectral properties of the naphthalenesulfonic acids. The spectroscopic properties of 1,5-AEDANS and some of its derivatives, including conjugated proteins, have been extensively investigated by Hudson & Weber (1973).

Bluefin tuna apomyoglobin was selected as reference protein because of some peculiar characteristics. In contrast to mammalian apomyoglobins which contain two tryptophan residues, i.e., Trp-7 and Trp-14, tuna apomyoglobin contains a single tryptophan residue which has been identified to be that in position 14 (Balestrieri et al., 1977, 1978). Moreover, the single cysteinyl residue found in the primary structure of tuna apomyoglobin (Balestrieri et al., 1978; Watts et al., 1980) provides the specific strategic site for 1,5-AEDANS labeling.

Materials and Methods

Myoglobin. Bluefin tuna myoglobin was 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% polyacrylamide, as reported by Davis (1964). A single protein band was always observed by this technique.

Apomyoglobin. 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 the Soret region. In all cases no significant absorption due to the heme was observed.

Protein Concentration. The concentrations of apomyoglobin were determined by absorbance at 280 nm on a Perkin-Elmer Model 575 spectrophotometer. The molar extinction at 280 of bluefin tuna apomyoglobin was calculated from the tryptophan and tyrosine content (Balestrieri et al., 1978) 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 was obtained from Schwarz/Mann. ANS was a product of Merck Co., its Mg^{2+} salt was recrystallized twice with the method described by Weber & Young (1964), and the molar extinction at 350 nm in 0.05 M phosphate buffer, pH 7.0, was observed to be $(4.97 \pm 0.1) \times 10^3$ cm^2 mM^{-1}. ANS binding constants were determined as described by Stryer (1965). *N*-AcCysAEDANS was prepared according to the method described by Hudson & Weber (1973). *N*-AcCys was allowed to react with 1,5-IAEDANS in the molar ratio of 1:1 for 2 h in the dark at 0 °C. The pH was kept constant at 7.5 by small additions of concentrated NaOH. The reaction was terminated by acidification at pH 2.0, and the reaction mixture was left overnight at 4 °C to allow the precipitation of *N*-AcCysAEDANS. The precipitate was collected by centrifugation and washed several times with

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[1] Abbreviations: ANS, 1-anilino-8-naphthalenesulfonate; 1,5-AEDANS, *N*-[(acetylamino)ethyl)]-5-naphthylamine-1-sulfonic acid; 1,5-IAEDANS, *N*-(iodoacetylaminoethyl)-5-naphthylamine-1-sulfonic acid; *N*-AcCys, *N*-acetylcysteine; apoMb, apomyoglobin; Gdn·HCl, guanidine hydrochloride; CD, circular dichroism.

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glass-distilled water and ethanol. ApoMb–AEDANS was prepared according to the method described by Kang et al. (1979). The reaction was performed in 0.01 M phosphate buffer, pH 7.5, containing 0.1 M KCl. The yield of reaction was controlled by varying the molar ratio between the reagents and/or the reaction times. The reaction was terminated by adding an excess of 2-mercaptoethanol. Excess unreacted 1,5-IAEDANS was removed by repeated dialyses against 0.05 M phosphate buffer, pH 7.5, or by gel filtration.

The capacity of apoMb–AEDANS to recombine with hemin was tested by following the optical density at 411 nm as function of the amount of hemin added to a fixed quantity of apoMb–AEDANS. ApoMb–AEDANS protein samples with an extent of 98% of AEDANS labeling were used in the hemin recombination experiments. Apomyoglobin samples with a lower extent of AEDANS labeling, i.e., 15–20%, were used in denaturation experiments.

Acid titrations were performed by careful addition of small amounts of concentrated HCl to buffered solutions (0.05 M phosphate, 0.01 M acetate, and 0.15 M KCl) from an Agla syringe while the solutions were stirred magnetically.

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.

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

Quantum yields were determined by comparing the relative yield of a dye solution with that of a solution of quinine sulfate in 0.5 M H_2SO_4 (quantum yield = 0.70) having the same optical density.

Fluorescence measurements in hemin recombination experiments were made by using 0.2-cm quartz cuvettes in order to reduce the screening effect due to the ligand. The emission spectra of 1,5-AEDANS–apoMb in presence of increasing concentration of hemin were corrected by a factor corresponding to the fluorescence decrease observed upon titration of 1,5-AEDANS–apoMb with hemin in 6.0 M Gdn·HCl. In fact, in the denaturant solvent no fluorescence change is to be expected if screen effects are absent. However, the extent of fluorescence quenching by hemin observed in 6.0 M Gdn·HCl was less than 10% of that observed in nondenaturant solvent.

Results

Fluorescence. The labeling of 1,5-AEDANS to the single –SH group of tuna apomyoglobin does not change the overall protein structure as indicated by the close similarity between the far- and the near-ultraviolet CD spectra of the labeled and unlabeled apomyoglobin (Colonna et al., 1980).

The comparison between the fluorescence properties of 1,5-AEDANS bound to the –SH group of tuna apomyoglobin and those of the simplest model compound *N*-AcCysAEDANS was thought to provide information about the microenvironment of the thiol group. As shown in Table I the emission maximum of the fluorophore bound to the apomyoglobin occurs at shorter wavelength (475 nm) than that of the free chromophore in water (496 nm). Moreover, the quantum yield of the apomyoglobin-bound chromophore (0.55) is much higher than that of the model compound in water (0.26). These findings indicate that 1,5-AEDANS, when bound to apomyoglobin, is in a rather nonpolar microenvironment even though not completely shielded from any contact with the more polar solvent. In fact, the emission of 1,5-AEDANS in ethanol is further blue shifted and occurs at 460 nm.

Table I: Fluorescence Properties of *N*-AcCysAEDANS and ApoMb-AEDANS in Different Solvents

compound	solvent	λ_{max} (nm)	quantum yield
N-AcCysAEDANS	phosphate, pH 7.0	496	0.26
N-AcCysAEDANS	6.0 M Gdn·HCl	496	0.26
N-AcCysAEDANS	ethanol	460	0.70
apoMb-AEDANS	phosphate, pH 7.0	475	0.55
apoMb-AEDANS	6.0 M Gdn·HCl	496	0.28
apoMb-AEDANS	phosphate, pH 7.0, + saturating hemin concn	482	0.12

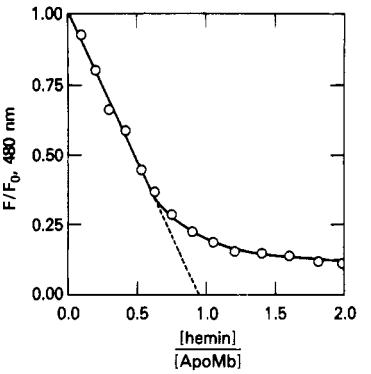

FIGURE 1: Degree of fluorescence quenching of apoMb–AEDANS as a function of hemin binding. Solvent: 0.05 M phosphate buffer, pH 7.0, and 0.15 M KCl; Excitation was at 336 nm. Protein concentration: 2.1×10^{-6} M.

Hemin Binding. The addition of hemin to 1,5-AEDANS-labeled apomyoglobin resulted in a red shift of the emission maximum from 475 to 482 nm and in a concomitant decrease of the fluorescence intensity at 480 nm (Figure 1). The fluorescence quenching is quite well related to the increasing saturation of the heme binding site of the apomyoglobin by hemin as observed by the concomitant absorbance increase in the Soret region. Both results seem to be those expected if one assumes that the 1,5-AEDANS is bound to the apomyoglobin in the same nonpolar moiety of heme. In fact, the addition of hemin would displace the extrinsic chromophore from the heme binding site because of its higher affinity (Stryer, 1965). However, the fluorescence maximum of the bound 1,5-AEDANS upon binding of hemin is at shorter wavelength if compared with that of the free chromophore in water and that of the apomyoglobin-bound 1,5-AEDANS in 6.0 M Gdn·HCl. Therefore, it appears that 1,5-AEDANS is also far from being completely exposed to the solvent upon hemin saturation.

Effect of Acid. Apomyoglobins are well-known to undergo an acid-induced conformational change which produces ~50% loss in helical structure (Kirby & Steiner, 1970; Colonna et al., 1978) and makes the apoprotein unable to bind ANS (Shen & Hermans, 1972). Since ANS binds the apomyoglobin in the same nonpolar moiety of the heme (Stryer, 1965), the acid molecular transition can be considered as resulting from the unfolding of the molecular district containing the heme pocket. This conclusion is supported by the fact that the N-terminal region of the molecule, which is not involved in the formation of the heme pocket, still retains elements of secondary and tertiary structure (Irace et al., 1981). We have examined the acid-induced molecular transition of tuna apomyoglobin by

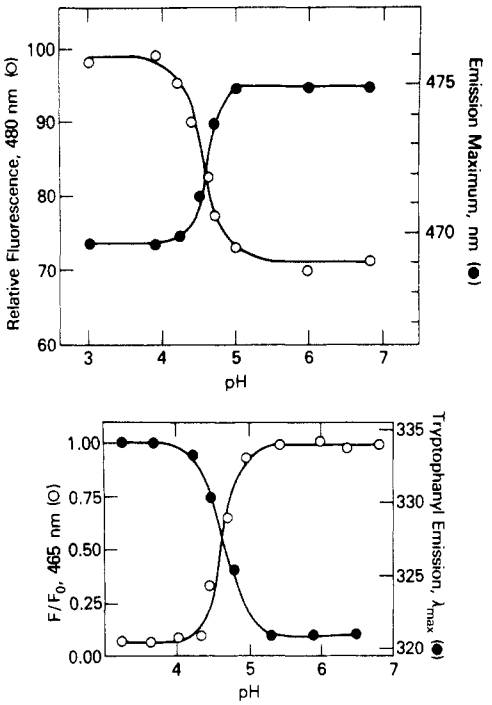

FIGURE 2: (Top) Acid pH dependence of AEDANS fluorescence intensity (○) and emission maximum (●) of apoMb–AEDANS. Protein concentration: 2.1×10^{-6} M in 0.05 M phosphate and 0.15 M KCl. Excitation was at 336 nm. (Bottom) Acid pH dependence of the tryptophanyl emission maximum (●) and ANS fluorescence intensity (○) of tuna apoMb and apoMb–ANS, respectively. Protein concentration: 2.1×10^{-6} M in 0.05 M phosphate buffer and 0.15 M KCl. Excitation was at 280 and 350 nm for apoMb and apoMb–ANS, respectively.

examining the changes occurring in the fluorescent properties of 1,5-AEDANS bound to its thiol group. The results have been compared with those obtained by following the intrinsic protein fluorescence and that of the ANS–apomyoglobin conjugate. The results are shown in Figure 2. Between pH 7.0 and pH 5.2 neither the ANS fluorescence intensity and maximum nor the tryptophanyl emission was modified; acidification to pH 4.0 produced disappearance of ANS fluorescence and a concomitant red shift of the tryptophanyl emission from 321 to 335 nm. The observation that the emission maximum of apoMb–ANS remains constant at 465 nm suggests that the polarity of the microenvironment of the dye is invariant during the course of the acid unfolding; therefore, the fluorescence intensity changes observed at 465 nm between pH 5.2 and pH 4.0 only depend on the amount of protein molecules which retain the ability to bind ANS. In the same pH range the fluorescence intensity of the bound 1,5-AEDANS increased by 35% and its emission maximum shifted from 475 to 470 nm. Both the intensity increase and the emission shift of 1,5-AEDANS fluorescence are indicative of changes occurring in the microenvironment of the bound chromophore, which becomes less polar. Therefore, it appears evident that 1,5-AEDANS, when bound to apomyoglobin, does not lie in the same hydrophobic binding site of the heme. In fact, the heme pocket is destroyed by acid as documented by the loss of ANS binding capacity.

Effect of Guanidine. Exposure of tuna apomyoglobin to increasing guanidine concentration produced loss of ANS binding ability and decrease of 1,5-AEDANS fluorescence intensity. The results are shown in Figure 3.

The transition curves obtained with the two apomyoglobin derivatives, i.e., ANS- and 1,5-AEDANS–apomyoglobin conjugates, were not superimposible, the midpoints being 0.33

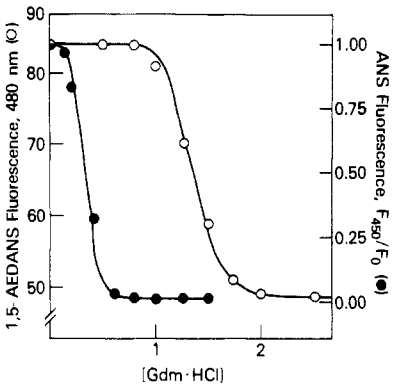

FIGURE 3: Effect of Gdn·HCl on the AEDANS fluorescence intensity (○) and ANS fluorescence intensity (●) of apoMb–AEDANS and apoMb–ANS, respectively. Protein concentration: 2.1×10^{-6} M in 0.05 M phosphate buffer and 0.15 M KCl, pH 8.0. Excitation was at 336 and 350 nm for apoMb–AEDANS and apoMb–ANS, respectively.

and 1.4 M Gdn·HCl for the ANS and the 1,5-AEDANS conjugates, respectively. The possibility that the fluorescence decrease at concentration below those producing unfolding might be due to a salt effect affecting binding was excluded since the ANS binding constants at 0 and 0.4 M Gdn·HCl were essentially similar, i.e., 8.9×10^{-6} and 9.2×10^{-6} M, respectively. No change in the emission maximum of ANS was observed during the guanidine unfolding. Therefore, the changes observed in the ANS fluorescence intensity reflect the equilibrium between molecules which retain the native structure and those which have lost their ability to bind ANS. The quantum yield of the bound 1,5-AEDANS in 6.0 M Gdn·HCl as well as its emission maximum in the same solvent was rather similar to those observed for the free chromophore in aqueous solution (Table I).

In contrast to the transition curves reported in Figure 3, those obtained by following the tryptophanyl fluorescence intensity (Balestrieri et al., 1978) and the emission maximum (Balestrieri et al., 1976) of tuna apomyoglobin with increasing Gdn·HCl showed midpoints at 1.2 M Gdn·HCl without revealing the existence of two molecular transitions. However, the denaturation profiles were much broader than those reported in this paper, probably because the changes in the fluorescence properties of the intrinsic chromophore, i.e., red shift of the tryptophanyl emission and decrease of the fluorescence intensity, were in the same direction in both the molecular events. A biphasic behavior of the tryptophanyl fluorescence has been observed for mammalian apomyoglobins (Colonna et al., 1978; Irace et al., 1981), which contain two indole residues, i.e., Trp-7 and -14. In this case the biphasic transition profile is due to the different behavior of the two tryptophanyl residues. In fact, in the native apomyoglobin at pH 8.0, Trp-7 is quenched by Lys-79. Unfolding of the heme pocket by acid or low guanidine concentration removes the quencher from the indole residue, thus determining an increase of fluorescence intensity. Further increase of denaturant concentration produces unfolding of the N-terminal region with subsequent decrease of the fluorescence intensity.

Discussion

The fluorescence characteristics of naphthalensulfonates make these dyes useful probes of the polarity of protein binding sites. In our studies we have used two naphthalensulfonate compounds, i.e., 1,8-ANS and 1,5-AEDANS, to probe the chemical and physical properties of hydrophobic sites of tuna apomyoglobin when subjected to acid- and guanidine-induced

unfolding. Since 1,8-ANS is known to bind apomyoglobin in the same nonpolar site of the heme (Stryer, 1965), the changes observed in the fluorescence quantum yield and emission band of this chromophore, when bound to apomyoglobin, can be related to structural modifications occurring in the heme binding site. As far as 1,5-AEDANS is concerned, the use of this fluorescent probe was suggested by the fact that tuna apomyoglobin contains a single cysteinyl residue. This residue is located in position A11 (Cys-13) as indicated by homology of bluefin tuna (*Thunnus thynnus*) with the sequence of yellowfin tuna (*Thunnus albacares*) (Watts et al., 1980). This site, which is adjacent to the earlier used fluorescent probe, i.e., Trp-14 (Irace et al., 1981), is probably largely buried in the A/HE helix contact as suggested by analogy with the well-known molecular structure of sperm whale myoglobin (Kendrew et al., 1961). This places 1,5-AEDANS bound to apomyoglobin distant from the ANS binding site in the porphyrin pocket. The data reported in this paper agree with the above-reported conclusion; in fact, the changes observed in the emission properties of the apomyoglobin-bound 1,5-AEDANS are not related to the unfolding of the heme pocket. Therefore, it seems reasonable to admit that the covalently bound 1,5-AEDANS is able to detect structural modifications occurring in the N-terminal region of the molecule. Moreover, a close similarity between the behavior of 1,5-AEDANS and that of the single tryptophan residue (Trp-14) has been observed in the guanidine unfolding of tuna apomyoglobin, i.e., rather similar midpoints (1.4 and 1.2 M for 1,5-AEDANS and Trp, respectively).

The data reported in this paper support the idea that the apomyoglobin structure results from the interaction of two or more distinct structural units, which represent the product of independent folding processes (Irace et al., 1981; Kuntz, 1975; Wetlaufer, 1973; Puett, 1973). Two intervening sequences (introns) have been found in all globin genes so far examined. In the α- and β-globin genes, the three coding sequences (exons) correspond to amino acid residues 1–31, 32–99, and 100–141 and 1–30, 31–104, and 105–146, respectively (Nishioka & Leder, 1979; Konkel et al., 1978, 1979). A structural as well as functional role for the three segments encoded by exons has been recently proposed by Go (1981). A further interruption between codons 68 and 69 has been found by Jensen et al. (1981) in the leghemoglobin gene. This intron splits the central segment in two units, the function of which is to fix the heme group in its functional position. This result agrees with the original observation of Go (1981) that the central segment should have been coded by separate exons. Probably, the intervening sequence which splits the central exon of ancestral globins disappeared because of selection pressure. We have now shown that exposure to acid unfolds the heme binding site of the molecule, which is encoded by the central coding sequence, but not the N-terminal region of the molecule, the structure of which seems to become more compact. In fact, the increase of the fluorescence intensity and the blue shift of the emission maximum of the apomyoglobin-bound 1,5-AEDANS observed on lowering the pH from 5.0 to 4.0 can be explained by assuming that the hydrophobic site containing the extrinsic chromophore becomes less accessible to solvent molecules. Moreover, the α-helix content as well as the degree of tryptophanyl polarization and the position of the emission maximum indicates that a large amount of organized structure is still present at pH 4.0 (Irace et al., 1981).

A slight unfolding of the N-terminal region occurs upon binding of the hemin to apomyoglobin as indicated by the red shift (7 nm) observed in the emission of 1,5-AEDANS. Therefore, the accessibility of the solvent molecules to 1,5-AEDANS seems to be closely related to the structure of the heme binding site, the N-terminal region being less structured when the hemin is bound to the apomyoglobin. The converse holds for the acid-induced unfolding of the heme binding site which results in a refolding of the N-terminal moiety.

Two independent cooperative transitions were observed when the tuna apomyoglobin was exposed to increasing concentrations of guanidine. Low guanidine concentration (less than 1.0 M) unfolds the heme binding site as indicated by the disappearance of ANS fluorescence, whereas higher denaturant concentration is required to unfold the residual globular structure.

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