

# Pressure-Induced Perturbation of Apomyoglobin Structure: Fluorescence Studies on Native and Acidic Compact Forms<sup>†</sup>

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**ABSTRACT:** The nature of the structural changes that apomyoglobin undergoes when subjected to hydrostatic pressure, ranging from atmospheric pressure to 2.4 kbar, has been investigated by steady-state fluorescence and frequency domain fluorometry. In particular, we have examined the intrinsic tryptophanyl emission and that of the extrinsic probe 1-anilino-8-naphthalenesulfonate (ANS) bound to apomyoglobin at neutral pH, as well as at strongly acidic high-salt conditions. Apomyoglobin at neutral pH undergoes a pressure-induced structural transition, which causes the disorganization of the heme binding region with a consequent ANS dissociation; a concomitant increase in solvent accessibility to the N-terminus of the macromolecule in which tryptophans are located is also observed. At 2.4 kbar, the tryptophanyl emission is not coincident with that of a fully solvent exposed residue, thus suggesting that the N-terminal region of the apomyoglobin molecule retains elements of organized structure. The spectroscopic properties of the structural state attained at 2.4 kbar and neutral pH are different from those of the acidic compact state. The acidic compact state of apomyoglobin undergoes a pressure-induced structural change that brings the tryptophanyl residues in contact with the solvent, but does not affect the ability to bind ANS.

The folding of small proteins into the native three-dimensional structure occurs spontaneously, despite the astronomically great number of accessible conformations (Levinthal, 1968). It has been suggested that structural intermediates involved in the folding pathway may play a crucial role in effectively limiting the search of the native structure to only a small region of the available conformational space (Wetlaufer, 1973). Recently, a universal intermediate was hypothesized: the so-called “molten globule” (Oghgushi & Wada, 1983; Ptitsyn, 1990; Ptitsyn & Uversky, 1994). Its properties, i.e., compactness, secondary structure, and overall architecture, are similar to those of native protein; moreover, the molten globule possesses only fluctuating tertiary structural interactions.

The experimental identification and structural characterization of intermediate forms appear to be of fundamental importance for the understanding of the folding process of proteins. However, the supporting evidence for the existence of intermediates is very arduous to obtain in native conditions because only the native state of the protein is substantially populated (Ptitsyn, 1987). The addition of acid, salt, or organic solutes such as urea or guanidine hydrochloride causes structural transitions from the native state to unfolded or partly denatured forms of proteins (Tanford, 1970). Apomyoglobin provides a good model for studying the structure of partly folded states (Colonna et al., 1982; Irace et al., 1986; Ragone et al., 1987; Barrick & Baldwin, 1992; Lin et al., 1994). The conformation of apomyoglobin depends on both pH and salt content. Specifically, it is native

at neutral pH and fully unfolded at pH 2 in the absence of salt; the addition of salt such as 0.3 M NaCl to fully unfolded apomyoglobin at pH 2 produces a partly folded form, which has been called the “acidic compact state” (Goto et al., 1990; Hughson et al., 1990; Bismuto et al., 1992; Sirangelo et al., 1994). The experimental evidence reported so far has shown that the acidic compact state of apomyoglobin shares some of the characteristics expected for the molten globule state. However, dynamic fluorescence studies performed recently on the acid-induced unfolding of apomyoglobin in the presence and absence of salt suggested that the acidic compact form could not be an intermediate in the folding process and, therefore, should not be identified with a molten globule (Bismuto & Irace, 1994).

The study of the denaturation process of proteins is complicated by the observation that different denaturing agents often leads to different end points. Moreover, it is arduous to discriminate between the intrinsic factors that stabilize a structural state of a protein and the specific effects caused by the binding of the denaturant molecules. In this respect, the use of pressure to affect protein conformation seems to be a more suitable tool, as documented by numerous articles (Weber & Drickamer, 1983; Silva & Weber, 1993; Prevelige et al., 1994).

The effects of hydrostatic pressure in the range of 1 atm to 10 kbar have been studied in about a dozen proteins made up of a single peptide chain (Weber, 1992). The pressure limit is dictated by the freezing of water at room temperature, which occurs at about 12 kbar. The methods of study have been virtually limited to optical spectroscopy, i.e., absorption and emission of light by intrinsic chromophores, and, more recently, Raman scattering (Heremans & Wong, 1985) and NMR (Jonas & Jonas, 1994). The emission of light can be accurately measured at protein concentrations much lower than those required for Raman scattering or NMR, although

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these latter methods can yield information on protein features at the atomic level.

In this paper, we report the effect of relatively high pressures on the structure of horse apomyoglobin in the native state, as well as in the acidic compact form, by means of intrinsic fluorescence emission and that of the extrinsic probe 1-anilino-8-naphthalenesulfonate (ANS). The results show that the increase of pressure on native apomyoglobin induces a molecular transition that causes the dissociation of the ANS molecule because of the disorganization of the heme region. However, the transition is preceded by a small reduction in the intramolecular ANS-tryptophan distance, as suggested by energy transfer studies. Instead, the N-terminal region of the apomyoglobin molecule at neutral pH maintains some structural elements. The acidic compact form of apomyoglobin subjected to increasing pressures undergoes a structural transition, at the end of which the ability to bind ANS is retained.

## MATERIALS AND METHODS

**Myoglobin.** Horse myoglobin was purchased from Sigma; the protein was used after purification by fast liquid chromatography using a Superdex-75 column (10 mm  $\times$  25 cm) from Pharmacia equilibrated with 0.05 M phosphate (pH 7.0). The protein homogeneity was controlled by sodium dodecyl sulfate gel electrophoresis with 15% gels and 5% stacking gels (Laemmli, 1970). Myoglobin concentration was determined spectrophotometrically in the Soret region, using  $157\,000\text{ cm}^{-1}\text{ M}^{-1}$  as the molar absorption coefficient at 410 nm (Harrison & Blout, 1965).

**Apomyoglobin.** The heme was removed from myoglobin by the 2-butanone extraction procedure (Teale, 1959). The heme contamination of apoprotein was assessed spectrophotometrically. In all cases no significant absorption was observed in the Soret region. The concentration of apomyoglobin was determined by absorption at 280 nm. The molar absorption coefficient was calculated from the tryptophan and tyrosine contents (Dayoff, 1976) by using molar absorption coefficients of  $5500\text{ cm}^{-1}\text{ M}^{-1}$  and  $1250\text{ cm}^{-1}\text{ M}^{-1}$ , respectively (Wetlaufer, 1962). The apomyoglobin solutions in the absence of salt were obtained by exhaustive dialysis against deionized water.

**Chemicals and Solutions.** All common chemicals were reagent grade and were purchased from Sigma. ANS was from Molecular Probes and was tested by the HPLC reverse-phase procedure. ANS concentration was determined spectrophotometrically using  $5000\text{ cm}^{-1}\text{ M}^{-1}$  as the molar absorption coefficient (Weber & Young, 1964).

**High-Pressure Steady-State Fluorescence Experiments.** Steady-state measurements have been made with a Greg PC fluorometer from ISS (Champaign, IL). The excitation was at 295 nm for the tryptophanyl residues to exclude the tyrosine contribution to the fluorescence emission, while the excitation was at 350 nm for ANS. The temperature of the sample compartment was controlled by using an external bath circulator (Neslab Model LT50). The sample temperature was measured in the compartment surrounding the bottle-like cuvette prior to each measurement by using a digital thermometer (Omega Model 410 B-TC). The high-pressure cell was that previously described by Paladini and Weber (1981). The emission spectra were acquired at each pressure after 20 min of pressure equilibration. The spectra

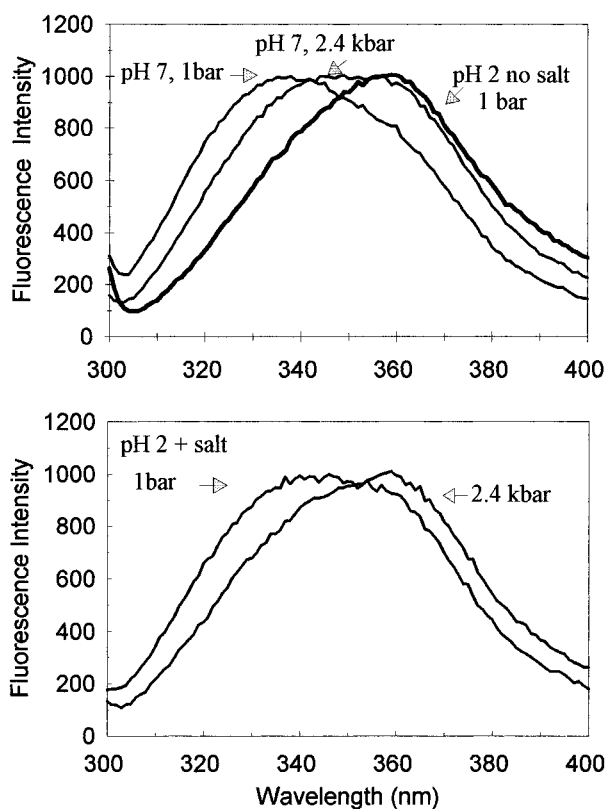


FIGURE 1: Tryptophanyl fluorescence emission spectra of horse apomyoglobin at the indicated pH's and pressures. The spectra were normalized for intensity at the emission maximum. The temperature was 20 °C and the excitation wavelength was 295 nm. Protein concentration was 5  $\mu\text{M}$ .

before and after pressure application were compared as a test of reversibility. Pressure-induced spectral changes were found to be reversible. Steady-state emission polarization measurements were performed in the L format with excitation and emission at 350 and 480 nm, respectively.

**Frequency Domain Emission Decay Measurements.** Lifetime measurements were performed by a multifrequency cross-correlation phase and modulation fluorometer, which uses 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 (Alcalà et al., 1985). The data were analyzed as described previously (Gratton et al., 1984; Lakowicz et al., 1984; Beechem et al., 1991). The quality of the fit was assessed by  $\chi^2$  values and by plots of weighted residuals. Excitation was at 350 nm, and the emission was observed through an interferential filter (Corion P-10-485). Light scattering of the sample at 350 nm (interference filter, Corion P-10-350) was used as a reference. The same results were obtained when the measurements were performed at atmospheric pressure inside the pressure bomb or in a regular cuvette utilizing *p*-terphenyl in cyclohexane in the reference cell.

## RESULTS

**Tryptophanyl Steady-State Emission.** The fluorescence spectrum of horse apomyoglobin upon excitation at 295 nm is due to the contribution of the two tryptophanyl residues, which are located in the N-terminal region at positions A-5 and A-12 (Dayoff, 1976). The emission maximum of the native protein at neutral pH is 337 nm. Figure 1 shows the

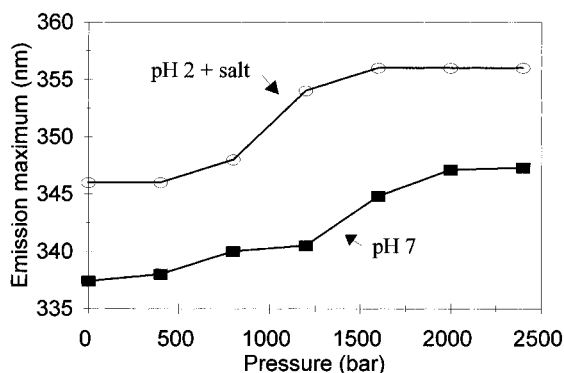


FIGURE 2: Pressure dependence of the tryptophanyl fluorescence emission maximum of horse apomyoglobin at pH 7.0 and 2.0 in the presence of 0.3 M NaCl. The other experimental conditions are as reported for Figure 1.

spectral effect produced by the application of high pressure (2.4 kbar) to apomyoglobin at neutral pH and at pH 2.0 in the presence and absence of 0.3 M sodium chloride; for a better comparison, the spectra shown in the figure are normalized for the intensity. The pressure increase causes a shift in the fluorescence spectrum of native apoprotein toward the red region, i.e., from 337 to 345 nm, with a concomitant 40% increase in fluorescence quantum yield, as detected by comparing the areas under the emission spectra. In the range of pressures studied, the fluorescence intensity of the monomeric tryptophanyl residue, i.e., *N*-acetyltryptophanamide (NATA), increases by about 10% and no significant spectral shift is observed (Thomas et al., 1976). The spectra shown in the upper part of Figure 1 indicate that the microenvironment of the tryptophanyl residues of apomyoglobin becomes more polar at 2.4 kbar. However, the emission maximum is still far from being similar to that of the fully exposed indole residues. In fact, the emission of the fully unfolded apomyoglobin, i.e., at pH 2.0 and in the absence of salt, is at 355 nm similar to that obtained for NATA. The lower part of Figure 1 shows the pressure effect on the spectrum of apomyoglobin at pH 2 in the presence of 0.3 M NaCl. At atmospheric pressure, the emission band is broad with a maximum at 345 nm. At 2.4 kbar, the emission spectrum shifts from 345 to 355 nm, i.e., the same emission maximum observed for the acidic apoprotein in the absence of salt.

Figure 2 shows the pressure dependence of the position of the emission maxima of native and acidic high-salt apomyoglobin. Transitions are observed for both conditions. For neutral apomyoglobin below 1200 bar, the emission maximum is almost constant, but at higher pressure, a sigmoidal increase is observed with an apparent midpoint at 1.6 kbar. For the acidic compact apomyoglobin, the midpoint of the transition is about 900 bar.

**ANS–Apomyoglobin Emission.** In aqueous solution, the quantum yield of ANS is about 0.004 and the emission maximum is 520 nm (Stryer, 1968). At 2.4 kbar, ANS shows only a small displacement in the emission maximum toward longer wavelengths. The fluorescence yield of the ANS bound to apomyoglobin is strongly enhanced and the emission maximum is shifted toward the blue region, with maxima located at 460 and 480 nm for the native and acidic high-salt proteins, respectively. Although ANS shows binding affinity for both native and high-salt acidic apomyoglobin (Stryer, 1965; Bismuto et al., 1992), the binding site might

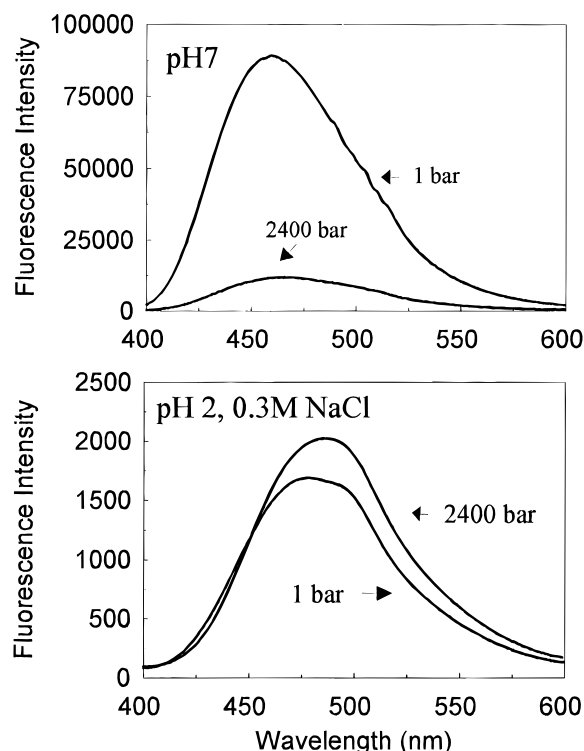


FIGURE 3: Fluorescence emission spectra of the ANS–apomyoglobin complex at the indicated pH's and pressures. The temperature was 20 °C and the excitation wavelength was 350 nm. Protein concentration was 5  $\mu$ M; ANS:apomyoglobin molar ratio was 1:1.

be different. In fact, it is well documented that the compact state of many proteins is able to bind ANS because of its fluctuating tertiary structure, which allows the formation of accessible apolar pockets where the fluorophore can be bound (Semisotnov et al., 1991). Therefore, there might be no correlation between the specific binding site existing in the native protein, i.e., coincident with the heme pocket, and that of the acidic compact form.

The upper part of Figure 3 compares the ANS emission spectra of neutral apomyoglobin resulting from excitation at 350 nm obtained at atmospheric pressure and at 2.4 kbar. A strong decrease in the fluorescence intensity is observed, suggesting that the dissociation of the ANS–apomyoglobin complex occurs upon increasing the pressure. The lower part of Figure 3 shows the pressure effect on the emission of ANS fluorescence of the high-salt acidic apoprotein. An increase in pressure causes a 20% increase in fluorescence intensity. The possibility that protein aggregation could be responsible for the appearance of ANS fluorescence at acidic pH in the presence of salt was excluded on the basis of the following observations: (i) the protein concentration is sufficiently low, i.e.,  $5 \times 10^{-6}$  M; (ii) the fluorescence polarization of the bound chromophore is constant upon varying the protein concentration; and (iii) the pressure increase from 1 to 2400 bar determines a monotonic decrease in fluorescence polarization from 0.116 to 0.094. Apomyoglobin, in the absence of salt at pH 2.0, does not bind ANS, with the fluorescence intensity being very low and resembling that of the free fluorophore even under pressure (data not shown). Figure 4 shows the pressure dependence of ANS fluorescence intensity at 460 and at 480 nm for native and high-salt acidic apomyoglobin, respectively. The apparent transition midpoint for neutral apomyoglobin is coincident

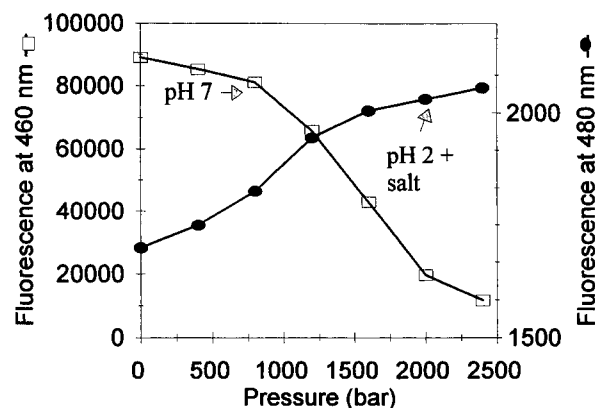


FIGURE 4: Pressure dependence of fluorescence intensity of the ANS-apomyoglobin complex at the indicated pH's. The experimental conditions are identical to those reported for Figure 3.

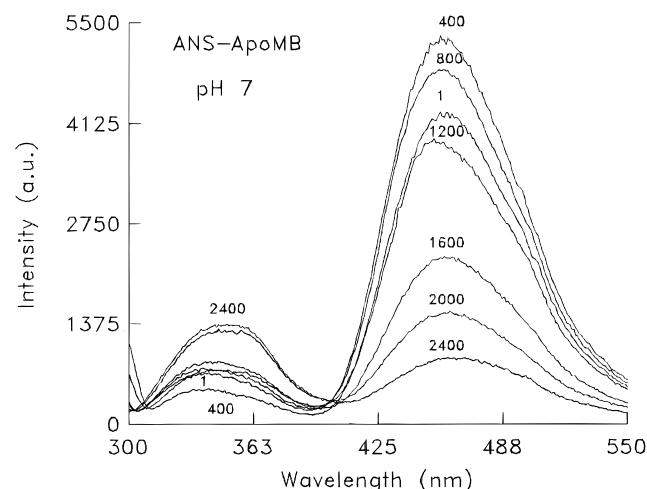


FIGURE 5: Effect of pressure on the tryptophanyl and ANS emissions of the ANS-apomyoglobin complex at neutral pH. Excitation was at 295 nm. The other experimental conditions are identical to those described for Figure 3.

with that observed for the pressure dependence of tryptophanyl emission, i.e., 1.6 kbar.

**Fluorescence Energy Transfer of the ANS-Apomyoglobin Complex.** Figure 5 shows the emission spectra of the ANS-apomyoglobin complex at neutral pH obtained with excitation at 295 nm at increasing pressures. Two emission bands are observed in each spectrum with maxima centered in correspondence with tryptophanyl and ANS emissions, respectively. The fluorescence intensity of the ANS band increases from 1 to 800 bar, and then it decreases on further pressure increase. The opposite is observed for the tryptophanyl band, which decreases in the range 1–800 bar and then increases. This complex spectral behavior may be explained in terms of variations in energy transfer efficiency from tryptophan to ANS. From 1 to 800 bar, the transfer becomes more efficient probably because of a reduction in the distance between the N-terminus and the ANS binding site. A further increase of pressure causes the loss of transfer as a consequence of the dissociation of the ANS-apomyoglobin complex. Figure 6 shows the pressure dependence of the tryptophanyl emission of the ANS-apomyoglobin complex at neutral pH in comparison with that recorded in the absence of ANS. At low pressure, the quenching of the tryptophanyl emission is concomitant with the increase in ANS emission, thus indicating the occurrence of an energy transfer between

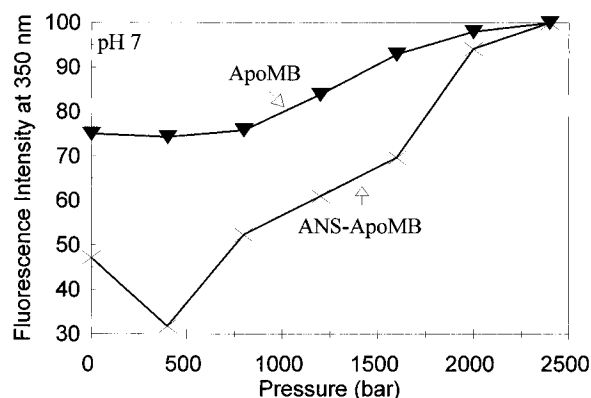


FIGURE 6: Pressure dependence of the tryptophanyl emission intensity of apomyoglobin and the ANS-apomyoglobin complex at neutral pH. The experimental conditions are the same as those described for Figure 5.

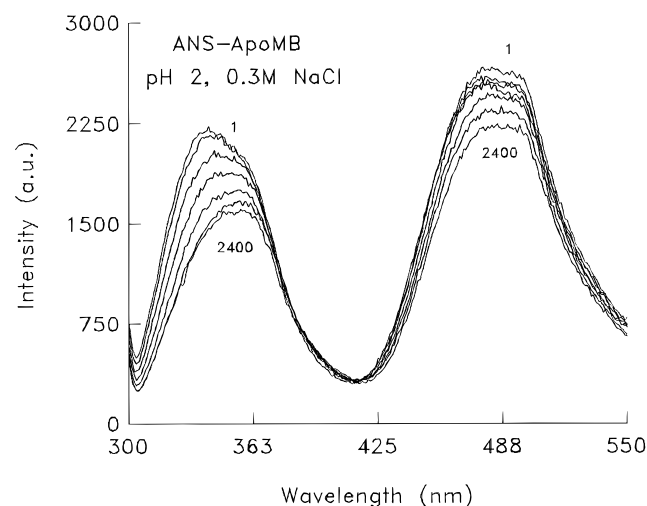


FIGURE 7: Effect of pressure on tryptophanyl and ANS emissions of ANS-apomyoglobin complex at pH 2.0 in the presence of 0.3 M NaCl. Excitation was at 295 nm. The other experimental conditions are similar to those described for Figure 3.

the two fluorophores. At high pressure, the dissociation of ANS determines that the emissions in the presence and absence of ANS become similar.

Figure 7 shows the pressure dependence of the fluorescence spectrum of the ANS-apomyoglobin complex at acidic high-salt apomyoglobin upon excitation at 295 nm. The intensity variations of the two emission bands, i.e., tryptophanyl and ANS, do not show the same complex pattern observed at neutral pH (Figure 5). In fact, both bands decrease upon increasing pressure.

**Effect of 0.5 M Glucose on Fluorescence Emission of Salt-Free Acidic Apomyoglobin.** The addition of 0.5 M glucose to apomyoglobin at pH 2 in the absence of salt does not affect the steady-state tryptophanyl fluorescence, the emission spectrum being superimposed on that recorded in the absence of glucose. The pressure dependence is also similar: the 2.4 kbar causes a 15% increase in fluorescence intensity without a shift in the wavelength emission maximum (data not shown). The acidic salt-free protein is unable to bind ANS even after the addition of 0.5 M glucose: the emission spectrum resembles that of the free fluorophore in the solvent, with an emission maximum over 510 nm. A pressure increase does not induce the appearance of the typical protein-bound ANS fluorescence spectrum.

Table 1: Emission Decay Analysis of ANS–Apomyoglobin at Neutral pH

	pressure	
	atmospheric pressure	2.4 kbar
center (ns)	18.9	17.9
width (ns)	4.1	5.8
$\chi^2$	1.5	1.5

*Emission Decay of ANS–Apomyoglobin at Neutral pH.*

The effect of 2.4 kbar on the emission decay of ANS bound to apomyoglobin at neutral pH was investigated by excitation at 350 nm in the frequency range 5–200 MHz. The data were analyzed by the nonlinear least-squares routine indicated in Materials and Methods by using algorithms for multiexponential as well as distributional analysis. Better fits were obtained by modeling the data to a Lorentzian distribution of fluorescence lifetime with the addition of a very short discrete component of 75 ps corresponding to the lifetime of ANS free in the solvent. The results in Table 1 show that the center, as well as the width, of the lifetime distribution of the ANS bound to apomyoglobin at neutral pH is essentially unaffected by hydrostatic pressure action.

**DISCUSSION**

In this paper, we describe pressure-induced transitions of the apomyoglobin structure at neutral pH as well as at acidic high-salt conditions. Usually, at neutral pH, the application of hydrostatic pressures below 4 kbar does not produce protein unfolding (Heremans, 1982). For metmyoglobin the characteristic absorption bands in the 400–700 nm region of the spectrum undergo significant changes when the protein is subjected to hydrostatic pressure higher than 5 kbar, in a manner similar to that observed for the denaturation induced by urea and other organic solutes (Kauzmann et al., 1973). However, some proteins undergo a considerable change in structure at relatively low pressure as a consequence of a contraction of the molecular volume (Cioni & Strambini, 1994). The small reduction in protein size seems to be due to the elimination of internal cavities. At higher pressure, the protein compressibility seems to be determined by the hydration of the peripheral region of the polypeptide chain (Hasegawa, 1989). Apomyoglobin at neutral pH, as well as at high-salt acidic conditions, evidences large changes in the emission properties that suggest structural transitions to intermediate forms induced by pressure in a moderate range from atmospheric pressure to 2.4 kbar. The stronger susceptibility of apomyoglobin to pressure action is attributable to the lack of stabilizing interactions with the prosthetic group (Schechter & Epstein, 1968); moreover, the acidic compact form is less stable than neutral apomyoglobin because of the destructurant effect induced by the protonation of numerous residues in the polypeptide chain (Bismuto et al, 1983; Sirangelo et al., 1994).

The application of 2.4 kbar to apomyoglobin at pH 7 produces a shift of the steady-state tryptophanyl fluorescence emission from 336 to 345 nm, a value that is different from that relative to the complete unfolded structure (355 nm). This suggests that the N-terminal region of the molecule is still structured at 2.4 kbar. In the structural state attained at 2.4 kbar, the protein does not seem able to bind ANS as documented by the large decrease in the emission intensity

of the extrinsic fluorophore, while the emission maximum remains unchanged (Figure 3). This conclusion is corroborated by the observation that the fluorescence lifetime distribution parameters, i.e., center and width, are not pressure dependent. It must be noted that hydrophobic fluorescence probes, such as ANS, bind accessible apolar regions of proteins, and therefore, correlation between ANS association or dissociation and structural events in a specific site might not be stated unless exclusive binding to such a specific site is observed. This is the case of native apomyoglobin at neutral pH and atmospheric pressure, which is known to bind one molecule of ANS in the same nonpolar site of the heme with strong affinity (Stryer, 1965). Moreover, there is no experimental evidence about the appearance of multiple binding sites for ANS to apomyoglobin upon increasing hydrostatic pressure. The emission maximum of ANS ranges from 520 (in water) to 450 nm or less (in organic solvent and proteins), and the fluorescence lifetime ranges from a few picoseconds to about 20 ns (Moore et al., 1985; Bismuto et al., 1985). Since the ANS emission mainly arises from molecules bound to protein, the free fluorophore being virtually nonfluorescent, the lack of dependence of fluorescence parameters on pressure suggests that the fluorescence decrease is due to the loss of the binding capacity of apomyoglobin for ANS and not to the appearance of other nonspecific binding sites. The loss of binding also determines an abrupt reduction in the Förster resonance energy transfer from tryptophans to ANS (Figure 5).

The simultaneous occurrence of two molecular events, independently detected by tryptophanyl and ANS fluorescence emissions (Figures 2 and 4), indicates that pressure induces a structural change responsible both for the disorganization of the heme binding site and the increase in solvent accessibility to the indolic residues located in the N-terminus. The pressure-induced transition seems similar to the transition that apomyoglobin undergoes at neutral pH in the presence of low guanidine hydrochloride concentrations (Irace et al., 1986; Bismuto & Irace, 1988); this transition causes the disorganization of the heme pocket, while the N-terminal branch, containing the tryptophanyl residues, and the C-terminal region of the molecule, where tyrosyl residues are located, remain structured. These residues become fully exposed to the solvent only at denaturant concentrations greater than 2.0 M.

The question that arises is whether the intermediate obtained by relatively high hydrostatic pressure on native protein is similar to the acidic compact state that apomyoglobin adopts at acidic pH in the presence of high salt at atmospheric pressure. The data reported in this paper show a similarity between the tryptophanyl spectrum obtained at high pressure and neutral pH and that relative to the acidic compact state at atmospheric pressure (Figure 1). However, the acidic high-salt protein is able to bind ANS. The binding of ANS to the acidic compact state has been interpreted as being due to the electrostatic shield exerted by salt, which minimizes the repulsion among charged residues at acidic pH and, thus, allows contact among hydrophobic regions of the unfolded protein molecule. Comparable concentrations of uncharged solute molecules such as glucose at acidic pH are unable to induce the formation of compact region of apomyoglobin, even under high pressure such as 2.4 kbar. It must be pointed out that a pressure increase at acidic pH in the presence of high salt does not affect the binding

capacity for ANS, although it determines further structural disorganization of the N-terminal region as documented by the observation that the tryptophanyl residues become completely solvent exposed. The emission spectrum at 2.4 kbar corresponds to that of the monomeric tryptophan residue. The persistence of the ANS binding site indicates that, in these conditions, the polypeptide chain is not in the fully unfolded state, thus suggesting that the whole protein behaves as a molten globule. The possibility that ANS originates from protein-protein association was excluded on the basis of the pressure dependence of fluorescence polarization. The observation that at pH 2.0, in the presence of salt, the ANS fluorescence polarization monotonically decreases upon increasing the pressure suggests that pressure-induced compaction might occur.

In conclusion, the data reported in this paper suggest that the acidic compact form, at atmospheric pressure, differs from a classical molten globule and confirm the hypothesis that it is composed of a subdomain formed by A, G, and H helices, while the remainder of the apoprotein is in the molten state (Hughson et al., 1990; Bismuto et al., 1992; Sirangelo et al., 1994). At 2.4 kbar the whole protein, in the presence of salt at acidic pH, becomes molten as documented by the increased solvent accessibility to tryptophanyl residues and the persistence of ANS binding. However, it must be considered that no data are actually available on the secondary structure and size at relatively high pressure.

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