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### Journal
Protein Science, 5(1)

### ISSN
0961-8368



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### Publication Date
1996

### DOI
10.1002/pro.5560050115

*Protein Science* (1996), 5:121-126. Cambridge University Press. Printed in the USA.


# Pressure-induced perturbation of ANS-apomyoglobin complex: Frequency domain fluorescence studies on native and acidic compact states

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(RECEIVED July 5, 1995; ACCEPTED October 26, 1995)

**Abstract**

The pressure dependence of the flexibility of the 8-anilino-1-naphthalene sulfonate (ANS)-apomyoglobin complex was investigated in the range between atmospheric pressure and 2.4 kbar by frequency domain fluorometry. We examined two structural states: native and acidic compact. The conformational dynamics of the ANS-apomyoglobin complex were deduced by studying the emission decay of ANS, which can form a noncovalent complex with the apoprotein in both the native and the acidic compact forms. Because the free fluorophore has a very short lifetime (less than 75 ps), its contribution can be separated from the long-lived emission. The latter arises from ANS molecules bound to the protein and provides information on the structural and dynamic characteristics of the macromolecule. The fluorescence emission decay of the ANS-apomyoglobin complex at neutral pH has a broad fluorescence lifetime distribution (width at half-maximum = 4.1 ns). The small changes in the fluorescence distribution parameters that occur with changes in pressure indicate that the ANS-apomyoglobin complex at neutral pH holds its compactness even at 2.4 kbar. A small contraction of molecular volume has been detected at low pressure, followed by a slight swelling with an increase in flexibility at higher pressures. The heterogeneity of ANS fluorescence in the acidic compact state of apomyoglobin is even greater than that in the native form (distribution width = 10 ns); moreover, the acidic compact state appears more expanded and accessible to solvent molecules than the native state, as suggested by the distribution center, which is 11 ns for the former and 19 ns for the latter. The lifetime distribution center remains constant with increasing pressure, which suggests that no other binding site is formed at high pressure.

**Keywords:** ANS-apomyoglobin complex; apomyoglobin; conformational dynamics; frequency-domain fluorometry; molten globule; protein flexibility

A protein in a given structural state has a wide variety of nearly isoenergetic conformational substates, which all perform the same function but at different rates (Frauenfelder et al., 1988). This large number of conformational substates gives rise to a plurality of fluorescence emission rates, which may be modeled by a quasicontinuous fluorescence lifetime distribution (Alcalà et al., 1987; Bismuto et al., 1988; Bismuto & Irace, 1989), although plausible alternative explanations have been reported that do not invoke multiple conformations of protein molecules (Sienicki et al., 1991; Baizer & Prendergast, 1993). The distribution center is related to the average environment of the excited fluorophore; the width of the lifetime distribution characterizes the strength and variety of the mechanisms influencing the microenvironmental properties of the fluorophore in the excited state. The distribution width depends not only on the number of different environments or conformational substates, but also on the interconversion rate among substates during the lifetime of the excited state.

Myoglobin was the first protein in which hierarchically organized conformational substates were detected, and it remains the best-studied example of the phenomenon (Austin et al., 1975). Recently, Frauenfelder et al. (1990) studied the effects of pressure on myoglobin using visible and Fourier transform infrared (FTIR) techniques. They concluded that pressure alters both the relative substate populations and the functional properties of the individual substates and, thus, can produce large changes in the static and dynamic properties of a protein ensemble. The effect of pressure on protein structure also has been described in terms

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of cavity reduction inside the protein matrix as well as changes in the hydration of mainly peripheral regions of the polypeptide chain (Heremans, 1982; Gavish et al., 1983; Nölting & Sligar, 1993; Silva & Weber, 1993; Cioni & Strambini, 1994; Jonas & Jonas, 1994).

For this paper, we investigated the effect produced by hydrostatic pressure on the lifetime distribution of 8-anilino-1-naphthalene sulfonate (ANS) bound to horse apomyoglobin at neutral pH and in acidic, high-salt conditions in the range from atmospheric pressure to 2.4 kbar. ANS has been employed in studies on apomyoglobin since the original report of Stryer (1965) showing that apomyoglobin binds ANS with strong affinity in the nonpolar site normally occupied by heme. The complex exhibits an ORD spectrum similar to that of the free apoprotein. Moreover, the histidine residues show the same net ionization behavior in the complex as in the free apoprotein (Breslow et al., 1967). More recently, NMR studies confirmed that the binding of the dye does not perturb the structure of apomyoglobin (Cocco & Lecomte, 1994).

We also studied the effects of pressure on the dynamic properties of the compact structural state formed by apomyoglobin in acidic, high-salt conditions (0.3 M NaCl, pH 2.0). This compact state has some similarities with the molten globule state, a supposed universal intermediate having great flexibility because of the weakness of the tertiary interactions among the residues constituting the polypeptide chain (Ptitsyn et al., 1990; Lin et al., 1994). It is well documented that the compact state of many proteins is able to bind ANS because the fluctuating tertiary structure creates accessible apolar pockets where the fluorophore can bind (Semisotnov et al., 1991).

The advantage of using ANS is that the free fluorophore in aqueous solution has a very short lifetime compared to that of the bound fluorophore. Therefore, examination of the ANS emission decay yields information on the structural states that retain the ability to bind the fluorophore. When native apomyoglobin is subjected to increasing pressure, it undergoes a conformational change that causes it to lose the ability to bind ANS, although it retains elements of organized tertiary architecture (Bismuto et al., 1996). The acidic compact state, by contrast, retains the ability to bind the fluorophore even at relatively high pressures. Starting from this observation, we undertook a frequency-domain emission decay study to specifically characterize the dynamics of these two structural states at increasing pressures.

## Results

ANS is virtually nonfluorescent in aqueous solution. Its fluorescence is strongly enhanced when it binds to apomyoglobin, and the emission maximum is shifted toward the blue region, with maxima located at 450 and 480 nm for the native and acidic, high-salt proteins, respectively (Stryer, 1965; Bismuto et al., 1992). In both cases, neither the emission maximum nor the fluorescence polarization of the extrinsic fluorophore depends on the molar ratio of ANS to apomyoglobin.

A pressure increase from atmospheric to 2.4 kbar causes a strong decrease in the fluorescence intensity with no change in the emission maximum. This finding suggests that the ANS-apomyoglobin complex undergoes dissociation. Conversely, in the case of the high-salt, acidic protein, this pressure change causes a rather small increase in the fluorescence yield of bound ANS. There is again no change in the position of the emission maximum, which remains at 480 nm (Bismuto et al., 1996).

The pressure dependence of the emission decay of ANS bound to apomyoglobin was investigated in two different conditions—at neutral pH and at acidic pH with a high salt concentration—by frequency domain fluorometry. The excitation was at 350 nm and the emission was collected through an interferential filter (Corion P-10-480) in the frequency interval between 5 and 200 MHz. Protein samples were subjected to hydrostatic pressures ranging from atmospheric to 2.4 kbar. The bottom graph of Figure 1 shows the phase shifts and modulation factors of the ANS-apomyoglobin complex at atmospheric pressure and neutral pH. For ANS bound to native apomyoglobin, the observed frequency response is complex and does not correspond to what would be expected for a single emitting fluorophore. An even greater heterogeneity was observed at pH 2.0 in the presence of 0.3 M sodium chloride (bottom graph of Fig. 2). The data shown in Figures 1 and 2 were analyzed by the nonlinear least-square routines as indicated in the Materials and methods using algo-

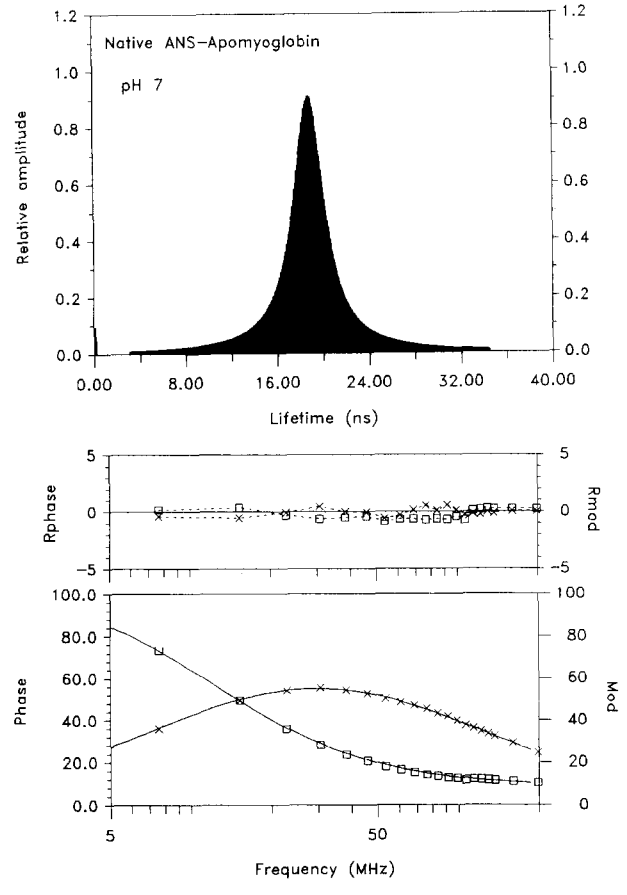


**Fig. 1.** ANS bound to native apomyoglobin at neutral pH and atmospheric pressure. Bottom graph: frequency dependence of the phase shift and demodulation factors. Top graph: the result obtained by fitting the emission decay data in terms of a lifetime distribution having Lorentzian shape. Center graph: the frequency dependence of the difference between the observed and calculated phase shifts and demodulation factors. Excitation was at 350 nm. Emission was collected through an interferential filter (Corion P-10-485). Protein concentration was 5.0 $\mu$M; ANS-apomyoglobin molar ratio was 1:1. The solution contained 5 × $10^{-3}$ M sodium phosphate, pH 7.0. Temperature was 20 °C.

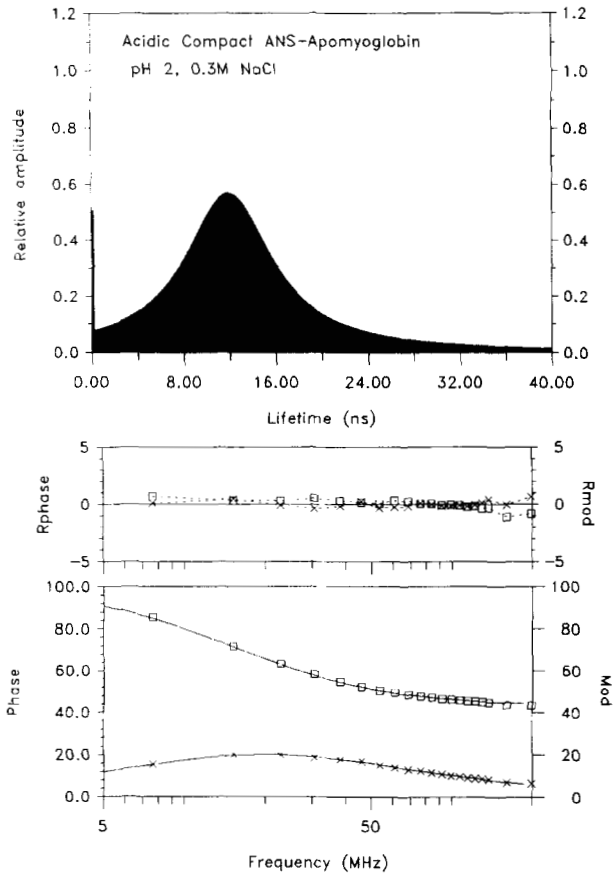


**Fig. 2.** ANS bound to apomyoglobin at acidic pH in the presence of 0.3 M NaCl and at atmospheric pressure. Bottom graph: frequency dependence of the phase shift and demodulation factors. Top graph: the result obtained by fitting the emission decay data in terms of a lifetime distribution having Lorentzian shape. Center graph: the frequency dependence of the difference between the observed and calculated phase shifts and demodulation factors. Excitation was at 350 nm. Emission was collected through an interferential filter (Corion P-10-485). Protein concentration was 5 nM; ANS-apomyoglobin molar ratio was 1:1. The solution contained $5 \times 10^{-3}$ M sodium phosphate, 0.3 M NaCl, pH 2.0. Temperature was 20 °C.

rithms for multiexponential as well as distributional analysis. In this paper, we show the data analysis obtained by fitting the experimental values to continuous lifetime distributions. Although an analysis based on three or four discrete components could be substituted for the distributional analysis, our point of view about protein structure is founded on the existence of conformational substates, and we will discuss the results from distribution analysis. The top graphs of Figures 1 and 2 show the unimodal Lorentzian lifetime distributions obtained by fitting. The middle graphs show that the differences between the observed and calculated values for the phase and modulation are statistically distributed around zero. Both fits required the addition of a discrete component with a very short lifetime (of about 75 ps); the origin of this component might be related to scattered light as well as the free fluorophore emission. The distributions are very broad, the widths being 4.1 and 10.0 ns for ANS bound to the native and acidic, high-salt proteins, respectively. The distribution centers are also different (18.9 and 11.9 ns for neutral and acidic apomyoglobin, respectively), probably owing to differences in the accessibility of the ANS fluorophore to solvent in the two apomyoglobin structural forms and to differences in dipolar relaxation rates (Bismuto et al., 1987, 1993).

Figures 3 and 4 show the frequency dependence of phase and modulation at increasing pressure for neutral and acidic, high-salt ANS-apomyoglobin complexes respectively. For the native protein, the phase shift detected at each frequency increases up to 1,200 bar; thereafter, a large decrease is observed. The modulation factor is independent of pressure except at the highest applied pressure, which caused an increase at each modulation frequency. For the acidic protein in the presence of 0.3 M NaCl, only a small increase in the phase shift and a corresponding decrease in the modulation were observed.

Table 1 summarizes the results of the distributional analysis of the emission data shown in Figures 3 and 4. The contribution from the very short component increases slightly with increasing pressure, probably because of the dissociation of ANS at neutral pH. Conversely, in acidic, high-salt conditions, the contribution of the short component decreases with increasing pressure, possibly owing to a pressure-induced enhancement of ANS binding (Bismuto et al., 1996). Figure 5 compares the pressure dependence of the distribution center and width for ANS-apomyoglobin at pH 7 and at pH 2 in the presence of 0.3 M NaCl. For the native ANS-apomyoglobin complex, the center of the lifetime distribution increases from atmospheric pressure to 800 bar and then decreases slightly. By contrast, for ANS bound to the acidic compact apoprotein, the center of the lifetime distribution remains constant. The width slowly increases with pressure for neutral apomyoglobin, whereas for the acidic, high-salt protein, it decreases monotonically.

Figure 6 shows the pressure dependence of the steady-state fluorescence polarization resulting from 350-nm excitation of ANS bound to apomyoglobin in the native and acidic compact states. For native apomyoglobin, the ANS polarization decreases from 1 to 1,200 bar and then increases. For apomyoglobin in the acidic compact state, the emission polarization decreases monotonically with increasing pressure.

## Discussion

The emission decay of ANS bound to native apomyoglobin is dependent on the number of conformations among which the

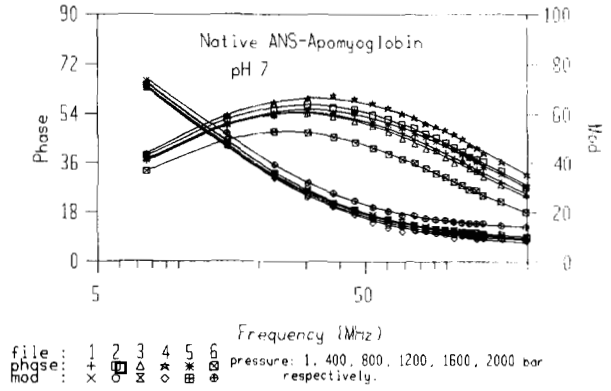


**Fig. 3.** Frequency dependence of phase shift and demodulation factor of ANS bound to apomyoglobin at pH 7.0 at the indicated pressures. Other experimental conditions are as in Figure 1.

**Table 1.** *Lorentzian lifetime distribution analysis of ANS-apomyoglobin under increasing pressures*[a]

| | Pressure (bars) | | | | | | |
|---|---|---|---|---|---|---|---|
| | 1 | 400 | 800 | 1,200 | 1,600 | 2,000 | 2,400 |
| **pH 7** | | | | | | | |
| Fraction | 0.913 | 0.920 | 0.933 | 0.918 | 0.908 | 0.870 | 0.850 |
| Center(ns) | 18.9 | 20.5 | 19.3 | 18.8 | 18.5 | 18.2 | 17.9 |
| Width (ns) | 4.1 | 4.1 | 4.2 | 4.3 | 4.6 | 5.5 | 5.8 |
| Chi-square | 1.5 | 1.4 | 1.6 | 2.1 | 2.3 | 2.9 | 1.5 |
| **pH 2 + salt** | | | | | | | |
| Fraction | 0.572 | 0.599 | 0.615 | 0.619 | 0.629 | 0.647 | 0.652 |
| Center(ns) | 11.9 | 11.7 | 11.9 | 12.1 | 12.1 | 12.0 | 11.9 |
| Width (ns) | 10.0 | 10.1 | 9.0 | 8.6 | 7.8 | 7.4 | 7.2 |
| Chi-square | 1.02 | 2.3 | 1.5 | 1.6 | 1.03 | 1.2 | 1.4 |

[a] The fit required the addition of a discrete component with a very short lifetime (less than 75 ps).

heme-binding site may fluctuate, on the mobility around each conformation, and on the interconversion rate among these conformations (Bismuto et al., 1989). The heterogeneity of the microenvironments generated by the interaction of the noncovalently bound fluorophore and the heme binding site produces a wide distribution of fluorescence lifetimes, which at atmospheric pressure is centered at about 18 ns and has a width of 4.5 ns. The center of the lifetime distribution is related to the average environment surrounding the ANS molecule. The distribution center observed for native apomyoglobin is determined by the hydrophobicity of the heme-binding site, where the fluorophore is known to be bound (Stryer, 1965). The width of this lifetime distribution is a measure of the plurality of conformational substates with different decay times existing in the apomyoglobin at neutral pH (Bismuto & Irace, 1989).

Increasing pressure causes the heme pocket to undergo destructuration, with a consequent dissociation of ANS molecules (Bismuto et al., 1996). It is possible to correlate ANS dissociation with structural events in this site, because the fluorophore

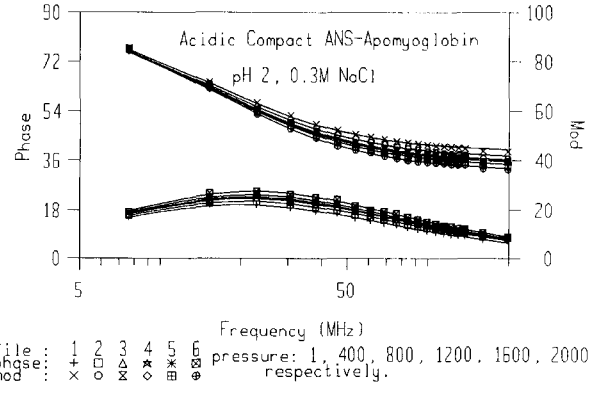


**Fig. 4.** Frequency dependence of the phase shift and demodulation factor of ANS bound to apomyoglobin at pH 2.0 in the presence of 0.3 M NaCl at the indicated pressures. Other experimental conditions are as in Figure 2.

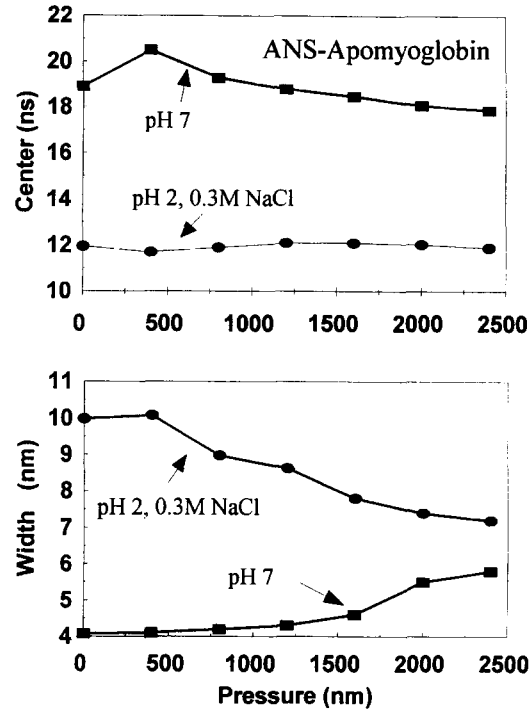


**Fig. 5.** Pressure dependence of the lifetime distribution center (top) and width (bottom) of ANS bound to apomyoglobin at pH 7.0 (■) and at pH 2.0 in the presence of 0.3 M NaCl (●). Experimental conditions are those reported in Figures 3 and 4.

binds only to the heme site. The emission maximum of ANS ranges from 520 nm (in water) to 450 nm or less (in organic solvents and proteins), and the fluorescence lifetime ranges from a few picoseconds to about 20 ns (Bismuto et al., 1985; Moore et al., 1985). The fact that both parameters are independent of increasing pressure does not support the possibility that other, nonspecific binding sites appear. The long-lived fluorescence emission observed at each examined pressure arises from the fraction of apomyoglobin molecules that retain the ability to bind ANS. Therefore, it provides information on the dynamic properties of the ANS-apomyoglobin complex. The data reported in Table 1 and Figure 4 show that the ANS fluorescence lifetime depends only weakly on pressure. The small observed increase (from 19 to 20 ns) can be correlated with a small contraction in molecular volume undergone by apomyoglobin between 1 and 800 bar, which improves the protein packing and reduces the internal cavities. This interpretation is supported by the observation that the efficiency of energy transfer from tryptophans to ANS increases from 1 to 800 bar (Bismuto et al., 1995). The decrease in the steady-state emission polarization (Fig. 6) observed in the low-pressure range is consistent with this reduction in molecular volume. In fact, the small magnitude of the lifetime variation of ANS bound to apomyoglobin suggests that the polarization decrease depends mainly on the reduction of the rotational correlation time, which is related to the protein molecular volume (Weber, 1953). At higher pressures, the center of the ANS lifetime distribution is shortened and the width of the distribution broadened, probably owing to a swelling of the apomyoglobin molecule. At constant temperature, it is reasonable to suppose that a pressure increase would reduce

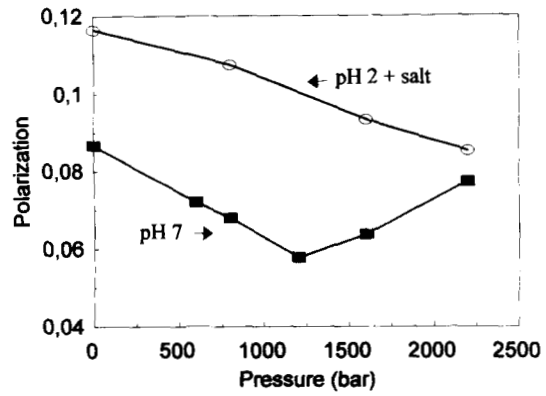


**Fig. 6.** Pressure dependence of ANS fluorescence polarization of apomyoglobin at pH 7.0 (■) and at pH 2.0 in the presence of 0.3 M NaCl (○). Excitation and emission wavelengths were 350 and 480 nm, respectively.

the distances among the amino acid residues, with a consequent increase in the Born repulsion. Rotation around the bonds that link the amino acid residues to the polypeptide backbone would work to reduce the repulsion. This effect would create larger free volumes, which the solvent could then comfortably occupy. The result would be a swelling of the protein molecule and the replacement of many of the short-range interactions among protein components by interactions with water molecules interspersed in the protein matrix (Weber, 1992). This model explains the small decrease shown by the lifetime center with a further increase in pressure. The increase of the emission polarization might be due to the positive contribution to the specific molar volume of apomyoglobin of the hydration of new solvent-exposed residues (Gekko & Hasegawa, 1986; Iqbal & Verrall, 1988; Kharakoz & Sarvazyan, 1993).

The acidic compact form of apomyoglobin binds ANS in a manner quite different from that seen at neutral pH. This idea is corroborated by the large difference in the center of lifetime distribution at atmospheric pressure for the native and acidic compact forms of the protein. The emission decay of ANS bound to the acidic, high-salt apomyoglobin has a distribution center value of 11 ns, which indicates that the ANS-binding site is much less buried than at neutral pH (Fig. 5). The lifetime distribution is very wide, the width at half maximum being 10 ns. The large fluorescence heterogeneity is due to the greater flexibility of the acidic compact state. Because of the fluctuating nature of the protein matrix in high-salt, acidic conditions, the ANS molecule experiences a large variety of microenvironments during the lifetime of the excited state. However, the possibility cannot be excluded that the fluorescence heterogeneity may also arise from the appearance of multiple binding sites. This explanation is less likely because of the independence of the emission maximum and fluorescence polarization on the protein and/or ANS concentration. The lifetime distribution center remains constant with increasing pressure, which suggests that no other binding site is formed at high pressure. The lifetime distribution width and the steady-state fluorescence polarization decrease linearly with increasing pressure, indicating that the average environment becomes more homogeneous and more compact. This hypothesis is corroborated by the recently reported studies on the adiabatic compressibility of some molten globules at atmospheric pressure, showing that the molten state is softer than native protein (Nöltig & Sligar, 1993).

## 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 × 25 cm) from Pharmacia equilibrated with 0.05 M phosphate, pH 7.0. The protein homogeneity was controlled by SDS gel electrophoresis with 15% gels and 5% stacking gels. Myoglobin concentration was determined spectrophotometrically in the Soret region using 157,000 $cm^{-1}$ $M^{-1}$ as 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 content (Dahyoff, 1972) by using molar absorption coefficients of 5,500 and 1,250 $cm^{-1}$ $M^{-1}$, respectively (Wetlaufer, 1962). The apomyoglobin solution in absence of salt was 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 an HPLC reverse-phase procedure. The ANS concentration was determined spectrophotometrically using 5,000 $cm^{-1}$ $M^{-1}$ as the molar absorption coefficient (Weber & Young, 1964).

### *High-pressure steady-state fluorescence experiments*

The steady-state measurements were 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, whereas the excitation was at 350 nm for ANS. The temperature of the sample compartment was controlled with an external bath circulator (Neslab Model LT50). The sample temperature was measured in the compartment surrounding the bottle-like cuvette prior to each measurement 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 before and after application of the pressure were compared as a test of reversibility. Pressure-induced spectral changes were found to be reversible. Steady-state emission polarization measurements were performed in L-Format configuration 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 that 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; Alcalà et al., 1987; Beechem, 1992). The quality of the fit was assessed by chi-square 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). The light scattering of the sample at 350 nm (interference filter, Corion P-10-350) was used as reference. The same results were obtained when measurements were performed at atmospheric pressure inside the pressure bomb or in a regular cuvette and using *p*-terphenyl in cyclohexhane in the reference cell.

## Acknowledgments

We thank Professor Gregorio Weber for helpful suggestions and discussion. We are also indebted to Drs. T. Hazlett and M. Van de Ven for their suggestions and assistance. This paper has been supported by C.N.R. grants 93.00087.CT04 and 93.01344.CT14.

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