Effect of Hydrostatic Pressure on the Fluorescence of Indole Derivatives

Paulo Roberto F. Louzada, Jr.,[1] **Marcelo E. Scaramello,**[1] **Clarissa Maya-Monteiro,**[1] **Alex W. M. Rietveld,**[1] **and Sérgio T. Ferreira**[1]

Effects of hydrostatic pressure on the fluorescence emission of L-tryptophan, N-acetyl-L-trytophanamide and indole were investigated. An increase in pressure ranging from 1 bar to 2.4 kbar results in reversible red-shifts of the emission of the three fluorophores. The pressure-induced red-shift amounts to about 170 cm^{-1} at 2.4 kbar, and appears related to changes in Stokes shift of the fluorophores caused by pressure effects on the dielectric constant and/or refractive index of the medium. As the pressure range investigated here is the range commonly used in studies of protein subunit association and/or folding, these observations raise the need for caution in interpreting pressure-induced spectral shifts. The significance of these observations to pressure studies of proteins is illustrated by investigation of pressure effects on human Cu,Zn superoxide dismutase (SOD) and azurin from *Pseudomonas aeruginosa*. A reversible 170 cm^{-1} red-shift of the emission of SOD was observed upon pressurization to 2.4 kbar. This might be interpreted as pressure-induced conformational changes of the protein. However, further studies using SOD that had been fully unfolded by guanidine hydrochloride, and fluorescence anisotropy measurements indicated that the observed red-shift was likely due to a direct effect of pressure on the fluorescence of the single tryptophan residue of SOD. Similar pressure-induced red-shifts were also observed for the buried tryptophan residue of azurin or for azurin that had been previously denatured by guanidine hydrochloride. These observations further suggest that the effective dielectric constant of the protein matrix is affected by pressure similarly to water.

INTRODUCTION

In the past 15 years an increasing number of reports have appeared on the use of hydrostatic pressure as a reversible thermodynamic variable in the study of protein-protein and protein-ligand interactions (for recent reviews, see[1-4]). Advantages in the use of hydrostatic pressure (as compared to experiments involving changing temperature or pH, or addition of chaotropic agents) arise from the possibility of perturbing chemical equilibria without changing the energy content or chemical composition of the systems under study. Although monomeric proteins are generally unaffected by pressure up to 2–3 kbar (i.e., in the range used to produce subunit dissociation of oligomeric proteins[5]), recent work has expanded the applications of pressure to studies of protein denaturation and folding,[6-8] in particular when high pressure is used in combination with low temperatures.[9,10]

Several techniques including UV-Vis absorption, light scattering, Raman and infrared spectroscopies and

[1] Departamento de Bioquímica Médica, Instituto de Ciências Biomédicas, Universidade Federal do Rio de Janeiro, Rio de Janeiro, RJ 21941-590, Brazil

NMR have been used in high pressure studies of proteins ([2] and references therein). However, due to its high sensitivity and relative ease of use, fluorescence spectroscopy has been the method of choice for monitoring pressure effects on proteins.[2] The intrinsic fluorescence of proteins (notably tryptophan emission) is very sensitive to changes in physicochemical environment surrounding the fluorophores, and has been extensively used to follow conformational changes produced by subunit dissociation and/or unfolding. A survey of the literature reveals that the fluorescence parameter most frequently used to follow pressure-induced conformational changes is the red-shift of the average fluorescence emission to lower energies.[1,2] In most cases, subunit dissociation or denaturation result in increased exposure of tryptophan residues to a polar (aqueous) medium, which produces a red-shift of the fluorescence.[11]

The basic assumption in studies using intrinsic fluorescence measurements to monitor pressure-induced conformational changes is that spectral shifts are caused by pressure effects on the proteins, rather than by direct effects of pressure on tryptophan emission. In this report we show that fluorescence emission spectra of L-tryptophan, indole and N-acetyl-L-tryptophanamide (NATA) undergo red-shifts of about 70 cm^{-1} per kbar of hydrostatic pressure applied, which can be significant compared to pressure-induced red-shifts of some proteins. This increase in Stokes shift of the fluorophores could be erroneously interpreted as a pressure-induced protein conformational change. The significance of these observations to pressure studies of proteins is illustrated by examination of the effects of pressure on human Cu, Zn superoxide dismutase (SOD) and azurin from *Pseudomonas aeruginosa*.

MATERIALS AND METHODS

Materials: Indole, L-tryptophan, NATA and azurin from *P. aeruginosa* were purchased from Sigma. Ultrapure GdnHCl was from Schwarz/Mann. Cu, Zn superoxide dismutase was purified from human erythrocytes as described in,[12] and was > 99% pure by SDS-PAGE analysis. Spectroscopic grade dioxane was from Merck. All other reagents were of the highest analytical grade available.

Fluorescence measurements: All measurements were performed on an automated ISS (ISS Inc., Champaign, IL) GREG-200 spectrofluorometer. Samples were excited at 285 nm (8 nm bandpass for both excitation and emission). Measurements under pressure were carried out using either the high pressure vessel originally

described in[13] or a newly developed vessel designed by ISS Inc., equipped with sapphire or quartz windows, respectively. Similar results were obtained with both types of pressure vessel or window materials. Temperature of the pressure cell was kept constant at 23 °C by means of a temperature jacket connected to a circulating temperature bath, and was monitored by a telethermometer attached to the cell. All experiments were carried out in 50 mM Tris-Cl, pH 7.0. Spectral centers of mass (ν_{av}, average emission wavenumber, in cm^{-1}) were calculated with software provided by ISS Inc., as:

$$\nu_{av} = \Sigma \; \nu \; I(\nu)/\Sigma \; I(\nu) \tag{1}$$

where $I(\nu)$ is the fluorescence intensity at wavenumber ν. Fluorescence anisotropy measurements were carried out on the same instrument with polarizers in excitation and emission ports. Anisotropy was measured in the pressure bomb equipped with quartz windows, and data were corrected for birefringence of the windows according to.[13]

RESULTS

Figure 1A shows fluorescence emission spectra of L-tryptophan at atmospheric pressure or at 2.4 kbar of pressure. Application of pressure promoted a red-shift of the emission. Figure 1B shows a progressive red-shift of the average emission wavenumber to lower energies with increasing hydrostatic pressure. Similar shifts (*ca.* 170 cm^{-1}) were observed in the emission of indole, L-tryptophan and NATA (Fig. 1B) when pressure was increased from 1 bar to 2.4 kbar. Spectral shifts were observed immediately following application of pressure and were promptly and completely reversible upon pressure release (Fig. 1B, filled symbols). Experiments with NATA showed that pressure effects on the fluorescence emission were not dependent on the concentration of fluorophore (ranging from 4.5 to 20 μM, corresponding to 0.025-0.113 OD units at 281 nm; data not shown).

In an investigation of pressure effects on human Cu, Zn superoxide dismutase, we found that pressures up to 2.4 kbar promoted a reversible red-shift of approximately 170 cm^{-1} of the fluorescence emission of this protein (Fig. 2A). The similarity between the shifts observed for indole analogs (see above) and SOD led us to carry out pressure experiments with SOD under conditions which promoted complete unfolding of the protein. SOD was incubated in the presence of 6.5 M GdnHCl for 24 hours at 4 °C[14] prior to the pressure experiment. Under these conditions SOD is known to be fully unfolded[14] and any effect of pressure on its fluo-
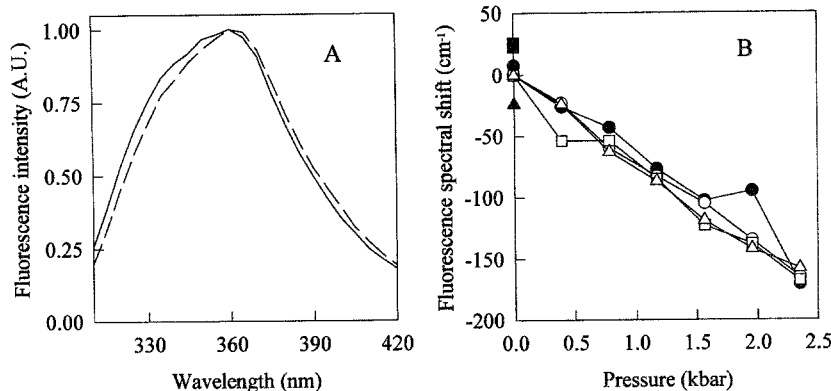
Fig. 1. Pressure-induced spectral red-shift of indole and derivatives. *Panel A*: Fluorescence emission spectra of L-tryptophan at atmospheric pressure (continuous line) or at 2.4 kbar of pressure (dashed line). *Panel B*: Spectral centers of mass were calculated as described in "Materials and Methods" and the difference relative to the center of mass at atmospheric pressure is plotted as a function of pressure. 2.8×10^{-5} M Indole ($\triangle$), 2×10^{-5} M L-tryptophan ($\square$), 2×10^{-5} M NATA ($\bigcirc$), 2×10^{-5} M NATA in a solution of 90% (v/v) dioxane in aqueous buffer ($\bullet$). Filled symbols represent spectra measured immediately after pressure release.

rescence is not likely to originate from conformational changes of the protein. The emission of SOD in the presence of 6.5 M GdHCl at atmospheric pressure was red-shifted by 420 cm^{-1} relative to the emission of native protein (compare spectra at 1 bar in Figs. 2A and B), as expected from a more solvent-exposed environment of the tryptophan residue in the unfolded protein. The interesting finding, however, is that similar pressure-induced red-shifts of the fluorescence of SOD were observed with unfolded or native protein (Fig. 2C).

Fluorescence anisotropy measurements under pressure showed that for two distinct concentrations of SOD (2 or 10 μM) the anisotropy remained approximately constant up to 2.4 kbar (Fig. 3). This indicates that the average mobility of the single tryptophan residue in each subunit of dimeric SOD was not affected by increasing pressure. A decrease in anisotropy would be expected upon unfolding or subunit dissociation of SOD. Thus, these results further suggest that in this case the spectral shift does not correlate with protein structural changes induced by pressure.

The effects of pressure on tryptophan emission described above were observed either with free fluorophores in aqueous solution (Fig. 1) or with a solvent-exposed tryptophan residue in SOD[15] (Fig. 2). To determine whether such direct effects of pressure were also relevant in proteins containing tryptophan residues not exposed to the aqueous medium the following experiments were conducted. First, NATA in 90% dioxane solution was used as a model system that mimics the effective environment of tryptophan residues in the protein matrix.[16] Figure 1B (filled circles) shows that the pressure-induced fluorescence red-shift of NATA in

dioxane solution was the same as tryptophan derivatives in completely aqueous buffer. Secondly, we investigated pressure effects on the fluorescence emission of azurin. Azurin is a monomeric protein of 16 kDa containing a single tryptophan residue deeply buried in the protein matrix (located at approximately 8 Å from the surface.[17]) Figure 4 shows that, as in the case of SOD, for both native azurin and azurin previously denatured by incubation in 6.5 M GdnHCl for 24 hours similar red-shifts were observed upon pressurization.
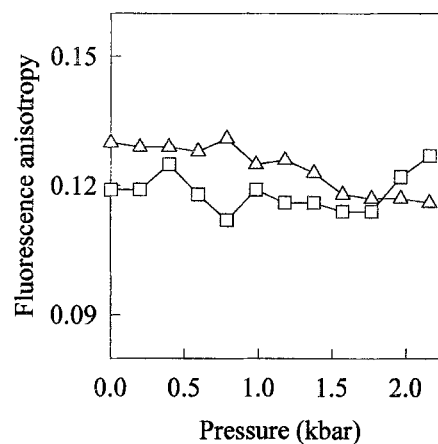
DISCUSSION

In this report we show that the fluorescence emission of indole derivatives (including tryptophan) undergoes a shift to lower energies with increasing pressure. These observations are in line with early reports from the groups of Drickamer and Weber showing a red-shift in the peak and an increase in fluorescence emission intensity of tryptophan at much higher pressures (10 kbar).[18,19] However, in those early reports no further explanations were proposed for these observations. Since we have observed shifts as large as 170 cm^{-1} for indole derivatives in the pressure range up to 2.4 kbar (a range commonly used in studies involving proteins), this "direct" effect on the emission of the fluorophores should be taken into account when analyzing pressure-induced fluorescence emission shifts in proteins. This can be particularly relevant for proteins that exhibit small spectral shifts upon pressurization. In such cases, independent confirmation of the existence of pressure-induced subunit dissociation or denaturation should be
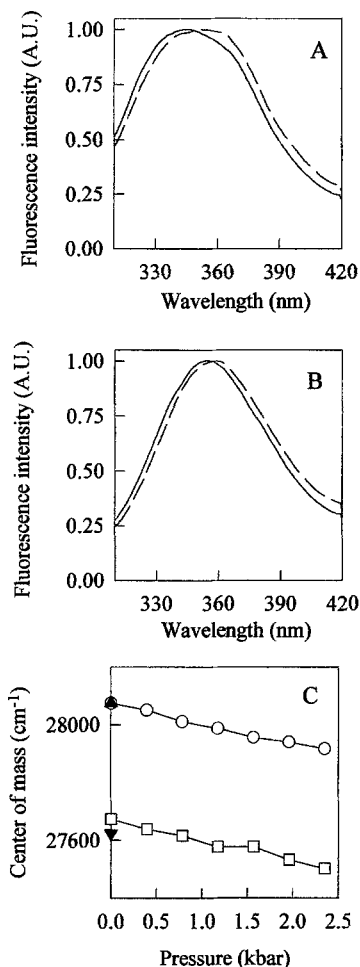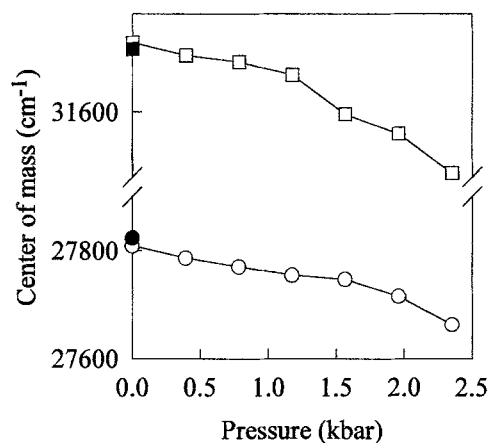
Fig. 2. Pressure-induced spectral red-shift of native or unfolded SOD. *Panel A*: Fluorescence emission spectra of SOD (5 μM) at atmospheric pressure (continuous line) or at 2.4 kbar of pressure (dashed line). *Panel B*: Fluorescence emission spectra of SOD (5 μM) in the presence of 6.5 M GdnHCl at atmospheric pressure (continuous line) or at 2.4 kbar of pressure (dashed line). *Panel C*: Spectral centers of mass (calculated as described in "Materials and Methods") as a function of pressure: (○, ▲) native SOD; (□, ▼) SOD + 6.5 M GdnHCl. Filled symbols represent spectra measured immediately after pressure release.

Fig. 3. Fluorescence anisotropy measurements of SOD under pressure. Fluorescence anisotropy measurements were carried out for SOD at the indicated pressures, and data were corrected for birefringency of the quartz windows according to.[11] (□) 2 μM SOD; (△) 10 μM SOD.

Fig. 4. Pressure-induced red-shift of native or unfolded azurin. Spectral centers of mass (calculated as described in "Materials and Methods") as a function of pressure: (□) native azurin; (○) azurin + 6.5 M GdnHCl. Filled symbols represent spectra measured immediately after pressure release.

obtained (for example, through fluorescence anisotropy measurements;[20]).

It is of interest to consider the possible origins of the effect of pressure on tryptophan fluorescence. The difference in energy between the absorption and emission of a fluorophore (i.e., the Stokes shift) results from interactions of the dipole moment of the fluorophore with the reactive fields induced in the surrounding solvent during the excited state.[11] In some cases, the Stokes shift may also be due to specific interactions (such as hydrogen bonding or formation of charge trans-

fer complexes) between the fluorophore and solvent molecules.[11] Pressure effects on specific interactions between fluorophore and solvent cannot be easily predicted. On the other hand, the effect of pressure on the Stokes shift can be estimated from an analysis of the dependence of the general solvent effect on physicochemical properties of the medium. Several theories have been presented to account for the dependence of the Stokes shift on the refractive index (n) and dielectric constant (ε) of the medium.[21-26] According to the general theory forwarded by Lippert,[22] the Stokes shift is given by:

$$\nu_2 - \nu_f = \frac{2\,(\mu^* - \mu)^2}{hca^3}\left[\frac{\varepsilon - 1}{2\varepsilon + 1} - \frac{n^2 - 1}{2n^2 + 1}\right] \quad (2)$$

where ν_a and ν_f are the absorption and emission frequencies in cm^{-1}, respectively; μ^* and μ are the dipole moments of the fluorophore in the excited and ground states, respectively; h is Planck's constant, c is the speed of light, and a is the Onsager radius of the cavity within which the fluorophore resides. Inspection of Eq. (2) shows that an increase in refractive index should result in a decrease in energy loss between absorption and emission, whereas an increase in dielectric constant should result in an increase in Stokes shift (i.e., a red-shift of the emission relative to absorption). Although we have not been able to find data on the refractive index of water at high pressures, data on pressure effects on the dielectric constant are available in the range from 1 bar to 3 kbar, and show a monotonic increase in ε of about 14 % in this pressure range.[27,28] This could at least partially explain the increased Stokes shift observed under pressure. The Stokes shift for indole in water has been shown to deviate appreciably from the value that would be predicted solely from the Lippert equation.[29] This has been attributed to specific interactions between indole (or its derivatives) and water, including exciplex formation and hydrogen bonding.[29] Thus, a complete description of the effect of pressure on the Stokes shift of indole derivatives in water would require knowledge of how pressure affects these specific interactions. Pressure is not expected to significantly affect hydrogen bonding, as volume changes involved in these interactions are nearly zero.[4]

In order to investigate the significance of these findings in relation to protein fluorescence, we have utilized two systems: SOD and azurin. SOD is a homodimer containing a single tryptophan residue per subunit. This tryptophan residue lies on the outside of the β-barrel of SOD, exposed to the solvent[15], and one side of the indole ring lies against C$^\beta$ and C$^\gamma$ of asparagine residue 19. Upon complete unfolding by GdnHCl contact of the tryptophan residue with asparagine 19 is presumably lost, possibly explaining the further red-shift of the emission of denatured SOD relative to native protein (Fig. 2A and B). For both native and denatured SOD similar pressure-induced red-shifts were observed (Fig. 2C). Together with the fact that fluorescence anisotropy measurements indicated that the mobility of the tryptophan residue of SOD was not affected by pressure (Fig. 3), this result seems to indicate that the effect of pressure on the fluorescence of SOD resulted from a direct effect on tryptophan emission.

One interesting question that remained to be investigated was whether the "direct" effect of pressure on tryptophan emission would also be significant in proteins containing buried tryptophan residues. This is of special interest since the explanation presented above for the pressure effect on the Stokes shift includes pressure-induced changes in dielectric constant and/or refractive index of the medium surrounding the fluorophore. Thus, if the same pressure effects were observed for buried or water-exposed tryptophan residues in proteins, this could mean that the effective dielectric constant of the protein matrix is affected by pressure in a manner similar to the dielectric constant of water. In order to investigate this point, pressure effects on the fluorescence of native and denatured azurin were studied. In native azurin, the single tryptophan residue is very much buried in the protein matrix,[17] being at approximately 8 Å from the surface and displaying a very blue-shifted emission (emission maximum at 308 nm; data not shown). Upon incubation for 24 hours at 4°C in the presence of 6.5 M GdnHCl azurin is completely denatured (emission maximum at 351 nm; data not shown). Boiling of the same sample that had been previously treated with GdnHCl caused no further red-shift of the fluorescence (data not shown), indicating that unfolding by GdnHCl was complete. Interestingly, for both native and denatured azurin pressure-induced fluorescence red-shifts (Fig. 4) were similar to the red-shifts observed for native or denatured SOD (Fig. 2) or for free fluorophores (Fig. 1). It is important to note that previous phosphorescence studies of pressure effects on apoazurin concluded that pressure did not unfold the protein, but rather stabilized its structure.[17] Pressure stabilization of the structure of apoazurin was proposed to be due to stronger intramolecular hydrogen bonding that follows from a close packing of the polypeptide.[17] Therefore, the fluorescence red-shift we have observed cannot be explained by pressure-induced unfolding of azurin. These results support the idea that the effective dielectric constant of the protein matrix is affected by pressure similarly to water.

An additional model system investigated was NATA in 90 % dioxane solution. This condition was chosen to mimic the lower effective dielectric constant environment in the protein matrix.[16] As shown in Figure 1 (filled circles), NATA displayed identical red-shifts when subjected to pressure in this lower dielectric constant medium or in completely aqueous buffer.

An alternative possibility that might also be considered is that the red-shift of tryptophan emission could be due to preferential stabilization under pressure of the ^1L_a or ^1L_b electronic transitions of indole. This possibility has been raised to explain pressure-induced changes in

fluorescence polarization of lysozyme.[30] Since pressure-effects on the polarization spectrum cannot be easily separated from other effects,[30] at the moment we cannot evaluate whether this mechanism could be (partly) responsible for the observed pressure-induced red-shift.

Regardless of the precise origins of the spectral shift of tryptophan under pressure, our results with SOD and azurin show that caution should be used in the interpretation of small shifts of protein fluorescence under pressure.

ACKNOWLEDGMENTS

NOTE ADDED IN PROOF: After this manuscript was in press it came to our attention that Politis and Drickamer (1981)[31] had also examined the effect of pressure on indole luminescence. Those authors[31] showed a red-shift of the fluorescence emission peak of indole and tryptophan upon application of pressure to solutions of the fluorophores in alcohols or organic solvents. These effects were generally related to changes in dielectric constant of the solvent. However, in Tris-buffered aqueous solutions little or no changes in fluorescence peak location were reported. This is in contrast with our observations made in Tris-buffered aqueous solutions of indole, tryptophan or NATA. While the reasons for this apparent discrepancy are not completely clear, we feel that our determination of spectral shifts by calculation of spectral centers of mass (average emission wavenumbers) offers a significant increase in precision relative to the observation of small changes in peak position alone.[31]

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