Effect of Solvent Diffusion on the Apomyoglobin−Water Interface

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ABSTRACT: Few techniques can identify interactions between proteins and individual water molecules when the protein is in solution. The present work has sought to bridge the gap between the molecular level studies and the search for a physical property of the solution (bathing the proteins) that would regulate the protein hydration level. The properties of the solution were varied by adding nondenaturing solutes and solvents to the protein solutions and then studying their effect on the intrinsic fluorescence of apomyoglobin. The resolution of the tryptophan emission into the two component spectra corresponding to tryptophans W7 (accessible to the solvent) and W14 (buried in the protein matrix) has allowed us to probe two specific parts of the protein. Whereas W14 is not affected when the medium is altered, the analysis of W7 fluorescence has shown that cosolvent diffusion plays a dominant role in the mobility of water molecules near the protein surface.

The X-ray structure of horse heart myoglobin is known (*1*). The extent of its hydration has also been extensively studied. X-ray and neutron diffractions (*1*, *2*) and computer simulations (*3*, *4*) suggest that the greater part of this protein is hydrated, with just the F and G helices remaining moderately dry. The study of the solvent effect on this single respiratory protein seems to be split between investigations of bulk solvent parameters [such as the dielectric constant (*5*), viscosity (*6*), or osmotic pressure (*7*)], which are likely to perturb structural and dynamic properties of the protein, and, on the other hand, the effort to provide molecular details on the protein−water contacts. This last approach describes the nature, the residence time, and the location of the water molecules at the protein−water interface (*8*). This search usually requires slightly hydrated protein powder.

Indeed, few techniques can identify individual water molecules when the protein is fully hydrated (in crystal or in solution), either because of the great excess of bulk water molecules or because the movement of water within the protein hampers their detection. The repartition of internal and surface water molecules and their interaction with the protein, when the protein is in full solution, are not yet experimentally known. What is more, the extent to which the constitution of the bulk solvent might influence the hydration level of the protein, or subdomains thereof, is not known.

This last question is addressed in the present study in which various chemical compounds were added to a solution of protein in buffer in order to alter the properties of the water (*9*). To avoid effects specific to a given cosolvent, while also avoiding any effects relating to varying chemical groups, three different polyols were used at varying concentrations. Cosolvents were limited to nondenaturing polyols without charge: glycerol, glucose, and sucrose. So as to remain as close as possible to the native molecular conformation, protein solutions were maintained at pH = 7.5.

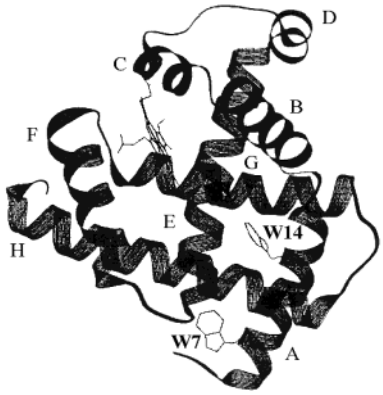

FIGURE 1: Ribbon representation of horse heart myoglobin, indicating the position of the two tryptophan residues, W7 and W14. This figure is reproduced with permission from ref *14*.

Horse heart myoglobin contains two tryptophan residues (Figure 1): one, W14, is in a buried region, and the other, W7, lies near the protein surface. The cosolvent effects on buried and exposed protein domains were investigated through the fluorescence perturbations of the two tryptophan residues in the apomyoglobin (apoMb)[1] molecule (myoglobin from which the prosthetic heme group was removed). Indeed, proteins exhibiting intrinsic tryptophan fluorescence are ideal for investigations of the relationships between dynamics and structure. Furthermore, at pH = 7.5, the tryptophan region of apomyoglobin retains the myoglobin conformation (*10*).

To measure how the bulk solvent acts on the hydration of W7 and W14, the total tryptophan fluorescence was resolved

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[1] Abbreviations: apoMb, apomyoglobin; Trp and W, tryptophan; TCE, trichloroethanol; DAS, decay-associated spectrum. ν is used rather than $\bar{\nu}$ to denominate wavenumbers (unit: cm^{-1}).

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to separate that of W7 (most accessible to solvent) from that of W14 (locked in the AGH junction). The fluorescence emissions of W7 and W14 were studied in terms of lifetime, relative quantum yield, and color (gravity center of the emission spectrum). Data show that the cosolvent molecules, by perturbing the mobility of the water molecules, shift the W7 fluorescence spectrum, thus offering a relevant unit for the strength of water−protein interactions.

MATERIALS AND METHODS

Salts and Organic Solvents. Tris(hydroxymethyl)aminomethane, bis-Tris {[bis(2-hydroxyethyl)amino]tris(hydroxymethyl)methane}, sucrose, glucose, and glycerol were purchased from Sigma.

Excluding the reference solution (20 mM phosphate buffer, pH = 7.5), three concentrations of each cosolvent were used in such a way that the molar concentrations varied in a similar proportion. Molality (Os, mol·kg^{-1}) was calculated using the equation:

$$\mathrm{Os} = (1000/M)(\%\ m/100)$$

where % *m* and *M* are respectively the percentage by mass and the molar mass of the cosolvent added. The above equation does not take into account volume variations (contraction or expansion) that will occur when cosolvent is added to water. Though the densities are not equal to unity, the molality will be taken to represent molarity in the rest of this work.

The maximum concentrations of the cosolvents used were limited by the maximum glucose concentration: saturation in pure water is around 47% by weight at 25 °C (Merck Index) but less at 9 °C, the temperature of the experiments described hereafter. In percentage by weight, were chosen 15%, 25%, and 35% glucose, 7%, 14%, and 21% glycerol, and 25%, 40%, and 50% sucrose. All percentages are given as weight of added compound per total weight of the final solution.

Protein Preparation. Lyophilized horse heart myoglobin was purchased from Sigma. Apomyoglobin is extracted from the holoprotein by acid acetone precipitation (*11*). The apomyoglobin precipitate is dissolved in bidistilled water at 4 °C and rid of the excess acetone by a 48 h dialysis, followed by lyophilization. The protein is renatured by dissolving it in 2 mM bis-Tris buffer, pH = 6; this solution is dialyzed at 5 °C against a 20 mM phosphate solution at pH = 7.5 for 24 h. The protein solution is then collected and centrifuged for 10 min at 29000*g*. The supernatant forms the mother solution. It holds less than 1% of heme, the limit being checked by the absorption of the Soret band. The apomyoglobin concentration is then adjusted to suit experimental needs using the optical density at 280 nm (ϵ_{280} = 13500 M^{-1}·cm^{-1}).

Any change in the native apomyoglobin conformation was monitored by two independent techniques. First, in all cases, the near-UV absorption spectra of apomyoglobin were not altered. Second, the chosen cosolvent conditions do not impede on apomyoglobin's capacity to rebind the heme group to yield anew the native holomyoglobin form. Therefore, the cosolvent conditions do not play on the apomyoglobin organization, and the samples were considered to be in their native state, typical of apomyoglobin at pH = 7.5.

Optical Absorption and Fluorescence Measurements. The UV optical absorption spectra were collected on a Cary 3E. The tryptophan fluorescence emission spectra were measured on a Perkin-Elmer LS 5B spectrofluorometer using bandwidths of 2.5 nm on both the excitation and emission beams. The optical cells had a 10 mm path length, and the optical density at 295 nm, excitation wavelength where the tyrosine absorption is very small (*12*), was maintained below 0.1. The apomyoglobin samples' concentrations were around 2 × 10^{-5} M. The relative fluorescence quantum yields of the tryptophans in apomyoglobin dissolved in the various cosolvents were obtained by comparing their emission intensity to that of the tryptophans in the apomyoglobin dissolved in the plain reference buffer. When the temperature rises from 9 to 25 °C, the emission spectra are very similar. The quantum yields decrease by less than 7%, in agreement with Laustriat and Gerard's observations on a large variety of tryptophan proteins (*13*).

The fluorescence emission of W7 was evaluated by the trichloroethanol (TCE) quenching method described previously (*14*). Tryptophan fluorescence was quenched by TCE, an apolar solute which dissolves in water as well as in the various cosolvents chosen. In apomyoglobin, TCE statically quenches the fluorescence of W7, without the W14 fluorescence being altered in any way. Briefly, this method considers that a fraction of the TCE concentration is complexed to the protein, following the relation:

$$\mathrm{apoMb} + \mathrm{TCE} \overset{K_D}{\longleftrightarrow} \mathrm{apoMbTCE}$$

Bringing this back to the fact that the change in the fluorescence emission upon addition of TCE is entirely due to the quenching of W7, one may say that

$$\Delta F = F_7^0 - F_7 = \frac{F_7^0}{1 + K_D/[\mathrm{TCE}]}$$

with F_7^0 as the initial fluorescence emission intensity of W7 and F_7 the residual fluorescence emission intensity of W7.

As $F_7^0 = f_a F^0$, where F^0 is the total fluorescence emission intensity of apomyoglobin in the absence of quencher and f_a is the fraction of its intensity that can be quenched by TCE, the above relation can be written:

$$\frac{F^0}{\Delta F} = \frac{1}{f_a} + \frac{K_D}{f_a[\mathrm{TCE}]}$$

The fluorescence lifetimes were obtained by measuring the phase shifts and the demodulation of the fluorescence emission intensity compared to the exciting beam, the intensity of which is modulated sinusoidally at frequencies varying from 4 to 200 MHz (*15*, *16*). The exciting beam's wavelength was 300 nm in order to avoid inter-tryptophan energy transfer. The entirety of the emitted light is detected at a right angle to the exciting beam through a polarizer set to the magic angle (54.7°). The fluorescence emission intensity is observed at different wavelengths selected by the emission monochromator (310, 320, 340, 360, 380, and 400 nm).

The barycenters of the emission spectra do not change with temperature (see above). So relaxation processes playing

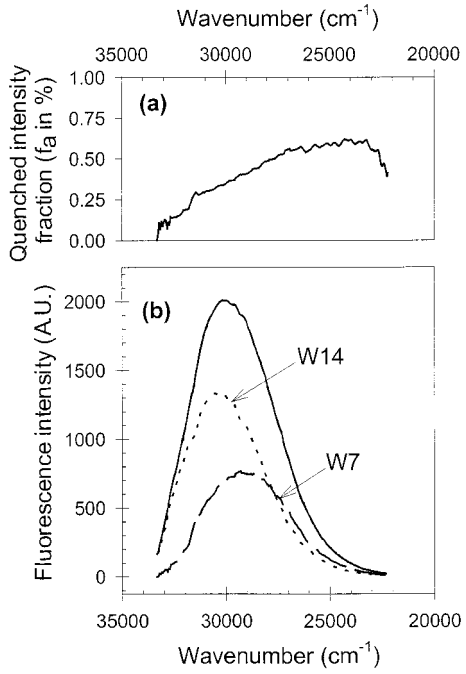

FIGURE 2: Resolution of the tryptophan fluorescence of apomyoglobin by static quenching with trichloroethanol (TCE). (a) Wavenumber distribution of the emission intensity fraction f_a, representing that part of the total fluorescence intensity that may be quenched by TCE. Only the fluorescence of W7 is quenched. (b) Resolved fluorescence emission of W7 and W14 residues within the total tryptophan emission from apomyoglobin. Multiplication of the total emission spectrum (—) by $f_a(\nu)$ yields the emission spectrum of W7 (− −). The complementary spectrum $\{[1 - f_a(\nu)]I(\nu)\}$ is that of W14 (- - -).

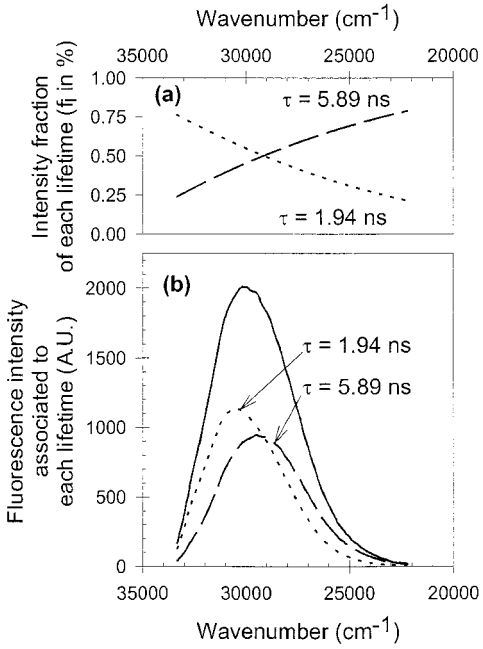

FIGURE 3: Resolution of the tryptophan fluorescence from apomyoglobin in terms of the component lifetimes. (a) Wavenumber distribution of the emission intensity fraction f_i corresponding to the long 5.89 ns (− −) and short 1.94 ns (- - -) lifetimes. (b) Decay-associated spectra of apomyoglobin showing two lifetimes: τ = 5.89 ns (− −) and τ = 1.94 ns (- - -). The total spectrum is also given (—) with the same scale as in Figure 2b.

gradually during the fluorescence decay time are highly improbable. It did not therefore seem necessary to carry out a TRES-type analysis (time-resolved emission spectra). The analysis was limited to the DAS-type (decay-associated spectra; *17*). Analysis of the data was carried through with the Globals software (LFD-Urbana) as described by Beechem (*17*). To compare the extracted data with the intensity of the emission spectra, the intensity fraction f_i was preferred to the population fraction α_i, when calculating τ_i, the different lifetimes. The fractional intensity f_i is related to the excited population fraction α_i of the ith component by

$$f_i = \frac{\alpha_i \tau_i}{\sum_i (\alpha_i \tau_i)}$$

RESULTS

(A) Fluorescence Characteristics of the W7 and W14 Residues in Apomyoglobin at pH = 7.5. (i) Component Resolution by Static Fluorescence Quenching of W7. As described in Materials and Methods, trichloroethanol (TCE) was used to selectively and statically quench the fluorescence of W7 in apomyoglobin. At each wavenumber ν of emission, the ratio $F^0/\Delta F$ of the total tryptophan emission intensity F^0 to the tryptophan emission intensity change ΔF due to the addition of quencher was calculated and plotted against $1/Q$, the inverse of the quencher concentration. The intercept on the y-axis is $1/f_a$, the inverse of the fraction of emission intensity that is quenched by TCE (*14*). This approach yields a curve $f_a(\nu)$ (Figure 2a). Multiplication of this curve by the emission spectrum $I(\nu)$ yields the spectrum of fluorescence emission that is quenched by TCE, i.e., the spectrum associated with W7 (Figure 2b). The complementary spectrum, $[1 - f_a(\nu)]I(\nu)$, is associated with the emission of W14. It appears that the fluorescence associated with W7 is redder than that associated with W14. This is in agreement with the fact that W14 is tightly buried within a rigid hydrophobic protein matrix, whereas W7, being at the protein surface, is more sensitive to solvent water molecules (*18*). The data also indicate that the fluorescence contribution of W7 to the total emission is about 38%.

(ii) Component Resolution through the Decay-Associated Spectra (Using Multifrequency Phase/Modulation Fluorometry). Analysis of time-resolved fluorescence intensity at pH = 7.5 and across the emission spectrum revealed two major lifetime components, 1.94 and 5.89 ns. In a few analyses, a third lifetime component appeared with a much longer lifetime ($\tau \approx 8.5$ ns) but a small to negligible value for the emission intensity fraction. The variability of this component, compounded with its very small fractional contribution, led us to ignore it in all the subsequent analyses.

Interpolating the f_i values obtained across the emission spectrum leads to a curve, $f_i(\nu)$, for each decay (Figure 3a). Multiplication of this curve by the corresponding emission intensities yields the spectra associated with each lifetime: $f_i(\nu)I(\nu)$ (Figure 3b). The spectrum associated with the long lifetime is the redder one and accounts for 49% of the total emission spectrum. The spectral position is identical to that of the spectrum associated with W7 above. It may therefore reasonably be said that the lifetime component of 5.89 ns corresponds primarily to emission from W7. There is, however, a discrepancy with regard to the fractional intensity (49% as opposed to 38%) that may only be understood if a

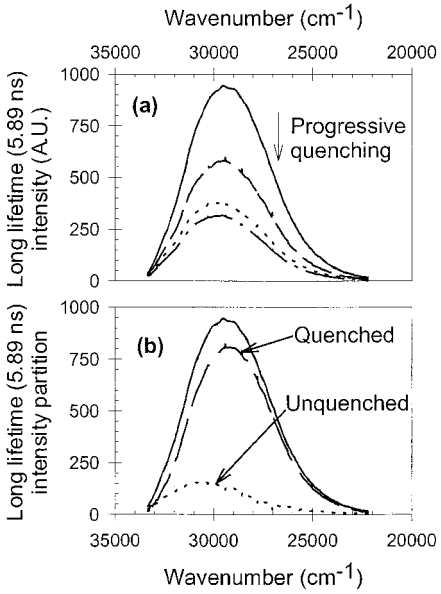

FIGURE 4: (a) Progressive quenching by TCE of the spectrum associated with the long lifetime (—). TCE concentrations are 8 mM (— —), 24 mM (- - -), and 48 mM (— - —). (b) Extrapolation to infinite TCE quencher concentration resolves the emission spectrum associated with the long lifetime (—) into its W7 (— —) and W14 (- - -) component spectra.

minor fraction of the spectrum associated with the long lifetime arises from emission from W14.

(iii) Spectra Attribution by TCE Quenching of the Spectrum Associated with the Long Lifetime. If there is a fraction of the spectrum associated with the long lifetime that belongs to W14, then this fraction will not be quenched by trichloroethanol. The TCE quenching of apomyoglobin was therefore combined with a DAS-type analysis. At various TCE concentrations, the lifetimes and their associated fractional intensities were measured across the emission spectrum. The lifetimes do not change, which confirms that TCE quenching is static. The spectrum associated with the short lifetime (1.94 ns) does not change with increasing amounts of TCE (data not shown). The decrease of the spectrum associated with the long lifetime (5.89 ns) is very large and follows the adjuncts of TCE (Figure 4a). It is also nonhomogeneous: the decrease is stronger on the red edge of the spectrum. Using the $F^0/\Delta F$ vs $1/Q$ plots, the extrapolation to infinite quencher concentration for each wavenumber yields the curve $f_a(\nu)$. This allows us to extract from the spectrum associated with the long lifetime the spectrum of the quenched portion (Figure 4b). This fraction forms nearly 82% of the spectrum associated with the long lifetime and 38% of the total fluorescence emission intensity. This is exactly the fraction of the total fluorescence emission intensity that was ascribed to the fluorescence of W7 by the TCE static quenching method alone. The remaining unquenched portion of the long-lived component exhibits a much more blue-shifted spectrum and likely arises from emission from W14.

To sum up, apomyoglobin fluorescence at pH = 7.5 may therefore be resolved into the fluorescence emission intensities of the two tryptophans: W7 is readily detected by the fact that its fluorescence emission is quenched by trichloroethanol. The W14 fluorescence emission is simply the complement. The emission of W7 has only one lifetime component, around 5.89 ns (38% of the total fluorescence emission intensity). The emission of W14 is more complex, with two lifetime components: one at 1.94 ns (51% of the total fluorescence emission intensity) and one near 5.89 ns (11% of the total fluorescence emission intensity). Thus the emission intensity of W14 accounts for 62% of the total fluorescence emission intensity.

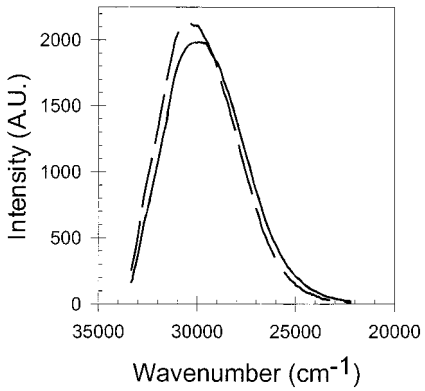

FIGURE 5: Cosolvent effect on the tryptophan fluorescence spectra of apomyoglobin at 8 °C before (—) and after (- - -) the addition of 50% sucrose.

It is quite clear that the population fraction α_i of each emitter does not follow the number of tryptophans present in apomyoglobin: W7 only appears as 18.6% of the total emitters, not 50%. This may be explained by the fact that the tryptophan excited-state populations do not mirror the ground-state populations. As the environments of the two tryptophans are very different, their fluorescence excitation and deexcitation are going to be different (*19*, *20*). The smaller relative quantum yield of W7 and its red shift could be due to the fact that W7 is more exposed (residue surface accessible to water is 11 Å^2 for W7 and 4 Å^2 for W14, compared to almost 300 Å^2 for a fully accessible tryptophan side chain) and subjected to stronger interactions with water than W14. Coupling of aqueous solvent molecules with W7 will reduce the excited-state population. The coupling of the excited state will in turn favor water relaxation and tryptophan deexcitation.

(B) Tryptophan Fluorescence Spectra of Apomyoglobin in the Presence of Cosolvent. After excitation at 295 nm, the total emission spectra of apomyoglobin vary when the various cosolvents are added. All the spectra shift to the blue, without changing quantum yield (e.g., 50% sucrose in Figure 5). The shifts are different on the blue and red edges of the spectrum.

Considering the above findings in pure buffer, static quenching by trichloroethanol was used to isolate the individual W7 and W14 fluorescence spectra in the presence of polyols. Multifrequency phase and modulation were used to determine the component lifetimes. The resolution of the total spectrum into its individual spectra by TCE quenching shows that the presence of these polyols does not alter the relative intensity of each tryptophan's emission either. The nonquenched W14 spectrum still accounts for 62% of the total; the quenched W7 emission constitutes the remaining 38%. Furthermore, the discrete lifetimes are not changed, and the spectra associated with each lifetime do not vary in relative intensity.

However, the presence of these polyols blue shifts the spectrum associated with the W7 fluorescence emission without modifying that of W14. To be sure that W14 is not

affected, one has to observe the spectra associated with both lifetime components. Using the decay-associated spectra of apomyoglobin in the presence of cosolvents, one can isolate the spectrum associated with the short lifetime ($\tau = 1.94$ ns) of W14. One can also isolate the spectrum associated with the long lifetime of W14 ($\tau \approx 5.89$ ns) by subtracting the W7 spectrum from the total spectrum associated with the long lifetime (data not shown). For both lifetimes of W14, the spectra are not shifted. This confirms that the W14 emission is not subjected to the medium surrounding the protein. It would seem then that the effects of the chosen cosolvents on the tryptophan fluorescence are limited to that part of the protein matrix which is nearest to the outside medium and that the protein interior does not "perceive" the effect of the cosolvent.

(C) Search for the Origin of the W7 Fluorescence Blue Shift. All of the cosolvents chosen blue shift the fluorescence of W7 without shifting that of W14. The position of the W7 spectrum's center of mass ν_G was determined by

$$\nu_G = \frac{\sum_\nu [I_\nu \nu]}{\sum_\nu I_\nu}$$

where I_ν is the intensity at each wavenumber ν. The shift of fluorescence emission is generally due to an influence of the medium on the emission dipole. Shifts of tryptophan fluorescence are usually interpreted either in terms of local polarity or in terms of local viscosity. However, it should be noted that the blue shifts due to the presence of these cosolvents at the given concentrations are not observed on *N*-acetyltryptophanamide (NATA) (data not shown); this would imply that the NATA−water interactions are not profoundly modified. Whatever explanation is given for the blue shifts of trypotphan W7 in apomyoglobin, it must take into account the very fact that W7 *is* an integral part of apomyoglobin and, furthermore, that W7 lies at the surface of the protein (as no effects were observed on tryptophan W14).

(i) Dielectric Effect. For many fluorophores, the cause for such a shift was sought in terms of an effect of the dielectric constant ϵ (*21*). However, in the case of tryptophan fluorescence, very large variations of ϵ are required to shift the emission spectrum (*22*); by comparison, the variations of the outside medium's ϵ are only very slight in the present work: $\epsilon = 78$ for water and $\epsilon = 73$ for 50% sucrose, the lowest value encountered in this study. So a direct dielectric effect of polar molecules around the fluorophore is improbable. Residue W7 is only partially exposed to the aqueous medium, so an indirect effect due to water molecules may be felt. A variation of the number of water molecules, due to the addition of cosolvent, translates as an osmotic pressure effect, rather than a dielectric moment effect.

(ii) Effect of Osmotic Pressure. Osmotic pressure Π is calculated from

$$\Pi = -\frac{RT}{V_w} \ln(a_w)$$

where R is the universal gas constant (8.2×10^{-2} dm^3·atm·K^{-1}·mol^{-1}), T is the absolute temperature (K), V is the molar volume of water (18×10^{-3} dm^3·mol^{-1}), and a_w is the activity of water, calculated from

$$a_w = X_w \exp^{-k(1-X_w)}$$

where X_w is the molar fraction of water [$n_w/(n_w + n_{solute})$, with n being the number of moles] and k is the Norrish constant (*23*).

Π is related to the number of particles in solution; the nature of these particles is taken into account through the Norrish constant, which, in this work, does not vary considerably. For glycerol, $k = 1.16$ (*24*); for glucose, $k = 2.25$ (*25*); for sucrose, $k \approx 7.1$ (estimated from osmotic pressure values previously reported; *26*). Assuming the constant for apomyoglobin is no more than 5 orders of magnitude larger, the number of protein molecules present in solution at the concentrations used will produce very little effect on the overall value of Π.

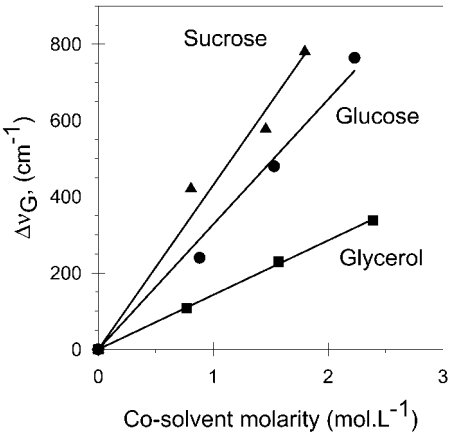

FIGURE 6: Displacement $\Delta\nu_G$ of apomyoglobin's tryptophan emission spectrum's center of gravity (ν_G) with the molarity of various cosolvents: glycerol (■), glucose (●), and sucrose (▲). The errors on ν_G are less than 15 cm^{-1}.

Considering that osmotic pressure and molarity are directly related, it was chosen to plot the observed blue shifts $\Delta\nu$ against the molarity of the compound added to form the given cosolvent (Figure 6). $\Delta\nu$ is linearly related to the molarity of each individual cosolvent, but molarity, as a parameter, cannot be considered as a single, unifying cause of the blue shifts observed. This conclusion does not preclude a variation in the number of water molecules around W7, but the extent of the variation depends on the cosolvent used.

(iii) Effect of Viscosity. This parameter refers to the mobility of molecules around the fluorophore. The color of the tryptophan fluorescence emission depends on the rigidity of the tryptophan's environment, so one might have reason to suspect the involvement of the cosolvent viscosity in the blue shift of the W7 fluorescence emission. The shift indeed increases uniformly with viscosity but not in a manner that is totally independent of the cosolvent used (Figure 7). Additionally, if the viscosity of the medium is directly related to the shifts of the fluorescence spectrum, then temperature should have an effect, as the viscosity of the external medium decreases with temperature. However, the spectrum is not red shifted when the temperature is raised from 9 to 25 °C (see Materials and Methods). This further shows that viscosity cannot be the fundamental parameter.

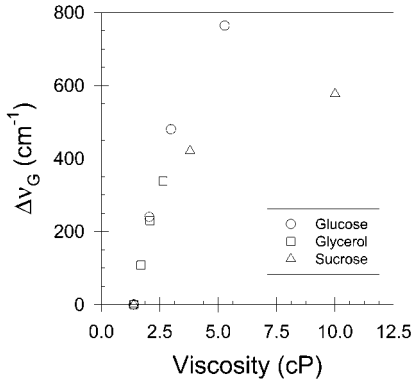

FIGURE 7: Displacement $\Delta\nu_G$ of apomyoglobin's tryptophan emission spectrum's center of gravity (ν_G) with the viscosity of various cosolvents: glycerol (□), glucose (○), and sucrose (△). The errors on ν_G are less than 15 cm^{-1}. The viscosity values for the different cosolvents were obtained from or by interpolation from past literature: glycerol (*46*), glucose (*47*), and sucrose (*48*).

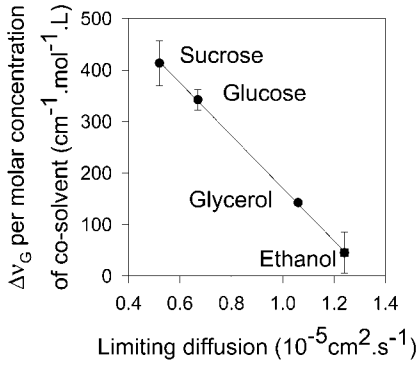

FIGURE 8: Correlation of the slopes obtained from the linear regressions in Figure 5 for the different cosolvents with the limiting diffusion of the said cosolvents. The line of best fit is extended to the diffusion value of 1.34, which corresponds to the value for ethanol. This point was added as a preliminary result, showing the quality of the correlation.

(iv) Normalization of the Fluorescence Blue Shift: Shift per Molar Concentration of Added Compound. It has been shown above that the cosolvent is a direct cause of the observed blue shift: the protein matrix does not carry the effect. It is therefore unnecessary to invoke internal protein viscosity, which has some currency in various theoretical and experimental works (*27*−*29*). Viscosity is a macroscopic quantity relating to the properties of the cosolvent solutions as a whole. At a molecular level, it is more correct to use the limiting diffusion of the added cosolvent molecules in water.

The limiting diffusion *D* is the diffusion value obtained by extrapolation to infinite dilution of cosolvent. So as to have a fluorescence parameter that is independent of cosolvent concentration, the blue shifts are normalized to the same molarity for all the cosolvents. Thus it is possible to determine a shift per molar concentration of added compound. The values thus extracted (Figure 8) have less inherent inaccuracy than the individual points (Figure 6). The plot of these values (the shifts per molarity for each cosolvent) against the diffusion of the added compound in water yields a straight line (Figure 8): the normalized displacements produced by the three cosolvents are well aligned. This demonstrates that limiting diffusion can be used as an overriding parameter of cosolvents, when studying their generalized effect on the position of the tryptophan fluorescence emission spectrum in apomyoglobin. It is no longer necessary to invoke the "nature" of the cosolvent.

DISCUSSION

The analysis and resolution of protein tryptophan emission spectra into their components are now possible by computer simulation (*30*). The analysis yields the fluorescence contribution and maximum position of each component. However, the particular effect of the external solvent on one of the components alone cannot be predicted yet by such theoretical calculations. Thus experiment remains the best approach for understanding the effect of the medium on proteins.

This work's first task was to experimentally isolate the fluorescence spectrum of W7 from the total fluorescence of apomyoglobin. This was achieved by using a quencher, trichloroethanol, that specifically and statically quenches the fluorescence of the outlying tryptophan, i.e., W7. Lifetimes were obtained by phase and modulation multifrequency measurements and DAS analysis.

Residue W7 in apomyoglobin is particularly well positioned to investigate the properties of crustal water, as it is at the interface between a protein "medium" and the solvent medium that the protein is immersed in. The advantage of this tryptophan residue in biophysical terms is that it is fluorescent, and its fluorescence emission position depends on the residue's environment: in this case, a combination of nearby amino acids and what is generally described as the first layer of water. Hydration water used to be regularly described as tightly bound water, endowed with little or no displacement. The present work can only be understood if this water is capable of displacement, the ability to diffuse being dependent on the physical properties of the medium immersing the protein. A similar conclusion was reached by theoretical simulations and diffraction experiments (*31*).

The relative quantum yield and lifetime of W7 and W14 do not vary with the cosolvent used or its concentration, but the position of the W7 spectrum (unlike that of W14) shifts to higher energy. The shift of the barycenter is directly proportional to the molarity of cosolvent used. Although for individual cosolvents the observed blue shifts appear related either to molarity or to viscosity, it would seem that, in a general sense, the position of the W7 fluorescence emission spectrum is sensitive to neither.

The blue shift of W7 is to be understood at a much more local level, in terms of a physical constant relevant to the molecular level: the limiting diffusion of the added cosolvent molecules in water. Indeed, for the three cosolvents chosen, the blue shift per concentration of cosolvent is linearly related to the limiting diffusion. However, the correlation is negative: the greater the limiting diffusion, the smaller the blue shift per concentration. It is then the residence time of the water molecules close to W7 that underlies the correlation. Because of their larger size, glycerol, glucose, and sucrose molecules cannot get near to individual amino acids in the same way. Thus, they have zero accessibility to either of the tryptophan residues.

Previously, it had been reckoned that the number of water molecules tightly bound and the strength of this binding do not vary; the present view is that the water−protein system

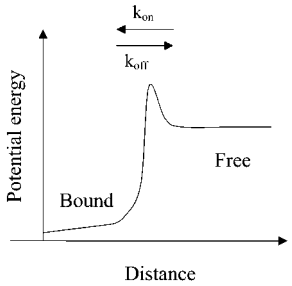

FIGURE 9: Schematic illustration of the dynamic equilibrium between protein-bound water molecules and free water molecules (bulk). The distance (*d*) given is that from the protein surface; i.e., at the protein surface $d = 0$. The figure gives the on and off rates (k_{on} and k_{off}, respectively) as discussed in the text. The modulation of k_{on} by the cosolvents is caused by a change in the potential energy of the bulk water molecules.

is in dynamic equilibrium (Figure 9). Evidence for this picture comes from the fact that, although myoglobin possesses 294 water-binding sites, only 164 such sites are actually occupied at any one time (*31*). A number of water molecules come within 4 Å of the protein surface but only interact with the protein's exposed surface areas for short time periods (≤2 ps) (*32*).

The weak interaction of these selected surface sites with the aqueous environment leads to a bulk-type solvation (*32*). Although the number of water molecules may not change greatly over time given a particular cosolvent condition, it is never the same hydrating molecules that are present: the system continuously exchanges water molecules with the bulk solvent. Given the dependence of the exchange rate on the bulk solvent physical properties, one would expect that the lower the diffusion constant, the slower the replacement of these water molecules near the protein surface.

At the opposite extreme, strong water−protein interactions determine interfacial regions with water residence times (≥35 ps) much longer than that of the water bulk (*33*, *34*). This lengthened time is not a result of increased hydrogen bond numbers, as, indeed, they remain unchanged (2 to 4). The geometry of the protein surface is a much more likely cause, as these long times are associated with water molecules resting in concave regions, grooves, and cavities (*31*). The water structure is rigid for a finite time, and this defines the protein hydration shell (*3*, *35*−*37*).

Given that the low concentrations of organic cosolvents used herein do not perturb the protein conformation, and also given the inability of the cosolvents to directly contact the protein surface, one must conclude that the rigid structure of the hydration shell is not affected. In the conditions described here, the residence time of the ordered water molecules trapped by the protein is exclusively controlled by the protein surface geometry (*31*). Therefore, the dissociation kinetics of water leaving this surface depend entirely on the number of ordered water molecules and the protein surface. This desorption process follows first-order kinetics, since it depends solely on the properties of the surface in question. Values are estimated to range from 4×10^7 to 3×10^9 s^{-1} (*38*).

In this work, we conjecture that the hydration layer (long protein−water interaction times) derives its water molecules from the solvation layer (short protein−water interaction times). We conjecture, furthermore that the diffusion-controlled arrival of water molecules from the bulk to the solvation layer limits the rate of arrival of water molecules to the hydration layer. The solvation layer would therefore be depleted in any one of two ways: return to the bulk or tight binding to the protein surface.

The number of water molecules on the hydration layer is controlled by adsorption and desorption. The addition of cosolvent plays on the former but not on the latter. The net effect on the hydration layer of these two processes, slower replacement plus equal release rate, is a decrease of the pressure of water molecules to bind to the protein surface. The weaker interactions between W7 and its aqueous environment lead to the observed blue shifts. The limiting diffusion of the cosolvent molecules proportionately influences the mobility of the water molecules and indirectly the color of the fluorescence emission, thus offering a relevant unit of measurement independently of the nature of the said cosolvent. This model has the advantage that it explains why these cosolvents at the given concentrations do not blue shift the fluorescence of *N*-acetyltryptophanamide (NATA): the protein surface is an integral part of the process.

Beyond these considerations, the correlation drawn between the blue shift per molar concentration of cosolvent and the limiting diffusion can lead to other conclusions: the range of limiting diffusions is restricted to values between 0.52×10^{-5} and 1.06×10^{-5} $cm^2 \cdot s^{-1}$ (all values taken from the Handbook of Chemistry). Other compounds can be used to increase the value of the limiting diffusion. Ethanol is such a compound ($D = 1.24 \times 10^{-5}$ $cm^2 \cdot s^{-1}$; value taken from the Handbook of Chemistry); it is also, unfortunately, a denaturing compound and must be used at low concentrations to avoid precipitation. First observations with nonprecipitating concentrations of ethanol (see Figure 8) indicate that the blue shift per molar concentration is a direct extrapolation of the straight line given in the present work, whatever effect the ethanol may have on the protein as a whole. This supports the present finding that the blue shift is due to a local, water mobility change.

This interpretation requires that the water lying on apomyoglobin's periphery be permeable to bulk water molecules. This is in apparent contradiction with the assumptions used, for instance, in the calculation of hydrodynamic volumes (*39*). This view is, however, supported by computer simulations (*3*, *4*, *40*) and by evidence from neutron diffusion experiments (*41*) which show that water molecules in myoglobin are far from fixed but diffuse within or through the protein (*42*).

The old view of a solid protein core surrounded by fixed water molecules, necessary to and resulting from a first-term analysis (*39*), is gradually being superseded by a model where the division is blurred: the protein has its own dynamics, and hydration concerns much of the protein to varying degrees depending on location (*40*). Accordingly, water molecules are found in varying degrees of order, from quasi-crystalline (*43*) to disordered (*44*). The residence time of such water is found to vary considerably from the pico- to the microsecond range (*45*). The present work shows that the limiting diffusion of the added cosolvent molecules modulates the mobility (and thus the residence time) of the water molecules close to the protein.

REFERENCES

1. Evans, S. V., and Brayer, G. D. (1990) *J. Mol. Biol. 213*, 885−897.
2. Cheng, X., and Schoenborn, B. (1990) *Acta Crystallogr. B46*, 195−208.
3. Lounnas, V., and Pettitt, B. M. (1994) *Proteins 18*, 133−147.
4. Lounnas, V., and Pettitt, B. M. (1994) *Proteins 18*, 148−160.
5. Dreyfus, M., Fries, J., Tauc, P., and Hervé, G. (1983) *Biochemistry 23*, 4852−4859.
6. Doster, W. (1983) *Biophys. Chem. 17*, 97−103.
7. Rishi, V., Anjum, F., Ahmad, F., and Pfeil, W. (1998) *Biochem. J. 329*, 137−143.
8. Bellissent-Funel, M.-C., Teixeira, J., Bradley, K. F., Chen, S. H., and Grespi, H. L. (1992) *Physica B 180, 181*, 740−744.
9. Lumry, R. (1995) in *Protein−solvent interactions* (Gregory, Ed.) pp 1−141, Dekker, New York.
10. Cocco, M. J., and Lecomte, J. T. J. (1990) *Biochemistry 29*, 11067−11072.
11. Rossi-Fanelli, A., Antonini, E., and Caputo, A. (1958) *Biochim. Biophys. Acta 30*, 608−615.
12. Lindqvist, L., Lopez-Campillo, A., and Alpert, B. (1978) *Photochem. Photobiol. 28*, 417−420.
13. Laustriat, G., and Gerard, D. (1976) in *Excited states of biological molecules* (Birks, J. B., Ed.) pp 388−399, Wiley, London.
14. Glandières, J.-M., Twist, C., Haouz, A., Zentz, C., and Alpert, B. (2000) *Photochem. Photobiol. 71*, 382−386.
15. Weber, G. (1981) *J. Phys. Chem. 85*, 949.
16. Jameson, D. M., Gratton, E., and Hall, R. D. (1984) *Appl. Spectrosc. Rev. 20*, 55−106.
17. Beechem, J. M. (1992) *Methods Enzymol. 210*, 37−54.
18. Burstein, E. A., Vedenkina, N. S., and Ivkova, M. N. (1973) *Photochem. Photobiol. 18*, 263−279.
19. Chen, Y., and Barkley, M. D. (1998) *Biochemistry 37*, 9976−9982.
20. Bohorquez, M., Cosa, J. J. Garcia, N. A., and Previtali, C. M. (1984) *Photochem. Photobiol. 40*, 201−205.
21. Brand, L., Seliskar, C. J., and Turner, D. C. (1971) in *Probes of structure and function of macromolecules and membranes* (Chance, B., Lee, C., and Blasie, J. K., Eds.) Vol. 1, pp 17−39, Academic Press, New York and London.
22. Van Dunren, B. L. (1961) *J. Org. Chem. 26*, 2954−2960.
23. Norrish, R. S. (1966) *J. Food Technol. 1*, 25−39.
24. Chirife, J., and Ferro-Fontan, C. (1980) *J. Food Sci. 45*, 1717−1719.
25. Labuza, T. P. (1984) *Moisture sorption: practical aspects of isotherm measurement and use*, American Association of Cereal Chemists, St. Paul, MN.
26. Berkeley, E., and Hartley, E. G. I. (1916) *Proc. R. Soc., Ser. A 92*, 477; reprinted in Timmermans, J. (1960) *The physical-chemical constants of binary systems in concentrated solutions*, Vol. 4, p 296, Interscience, New York.
27. Rholam, M., Scarlata, S., and Weber, G. (1984) *Biochemistry 23*, 6793−6796.
28. Lee, A. L., and Wand, A. J. (2001) *Nature 411*, 501−504.
29. Yedgar (1995) *Biophys. J. 68*, 665−670.
30. Reshetnyak, Y. K., Koshevnik, Y., and Burstein, E. A. (2001) *Biophys. J. 81*, 1735−1758.
31. Makarov, V. A., Andrews, B. K., Smith, P. E., and Pettitt, B. M. (2000) *Biophys. J. 79*, 2966−2974.
32. Pal, S. K., Peon, J., and Zervail, A. H. (2002) *Proc. Natl. Acad. Sci. U.S.A. 99*, 1763−1768.
33. Schoenborn B. P., Garcia A., and Knott B. (1995) *Prog. Biophys. Mol. Biol. 64*, 105−119.
34. Denisov, V. P., and Halle, B. (1996) *Faraday Discuss. 103*, 227−240.
35. Gu, W., and Schoenborn, B. P. (1995) *Proteins 22*, 20−26.
36. Rocchi, C., Bizzari, A. R., and Cannistraro, S. (1997) *Chem. Phys. 214*, 261−276.
37. Abseher, R., Schreiber, H., and Steinhauser, O. (1996) *Proteins: Struct., Funct., Genet. 25*, 366−378.
38. Nandi, N., and Bagchi, B. (1997) *J. Phys. Chem. B 101*, 10954−10961.
39. Cantor, C. R., and Schimmel, P. R. (1980) *Biophysical Chemistry. II. Techniques for the study of biological structure and function*, Freeman and Co.
40. Lounnas, V. (1999) in *Hydration processes in biology: theoretical and experimental approaches* (Bellissent-Funel, M.-C., Ed.) NATO Series A, Vol. 305, pp 261−290, IOS Press, Amsterdam.
41. Hanson, J. C., and Schoenborn, B. P. (1981) *J. Mol. Biol. 153*, 117−146.
42. Haouz, A., Zentz, C., and Alpert, B. (1997) *Chem. Phys. Lett. 279*, 79−84.
43. Temple Burling, F., Weis, W. I., Flaherty, K. M., and Brunger, A. T. (1996) *Science 271*, 72−77.
44. Yu, B., Blaber, M., Gronenborn, A. M., Clore, G. M., and Caspar, D. L. D. (1999) *Proc. Natl. Acad. Sci. U.S.A. 96*, 103−108.
45. Venu, K., Anders Svensson, L., and Halle, B. (1999) *Biophys. J. 77*, 1074−1085.
46. Neumann, A. A. (1968) in *Glycerol*, p 15, Morgan Grampian, London.
47. Powell, C. W. R. (1914) *J. Chem. Soc. (London) 105*, 1; reprinted in Timmermans (1960) *The physical-chemical constants of binary systems in concentrated solutions*, Vol. 4, p 285, Interscience, New York.
48. Bingham, E. C., and Jackson, R. F. (1918) *Bull. Bur. Stand. 14*, 59; reprinted in Timmermans (1960) *The physical-chemical constants of binary systems in concentrated solutions*, Vol. 4, p 309, Interscience, New York.
49. Timmermans, J. (1960) *The physicochemical constants of binary systems in concentrated solutions*, Vol. 4, Interscience, New York.

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