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Water–protein interactions in the molten-globule state of carbonic anhydrase b: An NMR spin-diffusion study

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Abstract

We have used the homonuclear Overhauser effect (NOE) to characterize a model protein: carbonic anhydrase B. We have obtained NOE difference spectra for this protein, centering the on-resonance signals either at the methyl-proton or at the water-proton signals. The spin-diffusion spectra obtained as a function of protein concentration and temperature provide direct evidence of much greater protein–water interaction in the molten-globule state than in the native and denatured states. Furthermore, although the protein loses its gross tertiary structure in both the molten-globule and denatured states, it remains almost as compact in its molten-globule state as it is in the native state. The spin-diffusion spectra, obtained as a function of a variable delay time after the saturation pulse, allowed us to measure the relaxation times of several types of proton in the solution. These spectra contain enough information to distinguish between those water molecules solvating the protein and the free ones present as bulk water.

Keywords: molten globule; NMR; protein folding; protein hydration; spin diffusion

For more than a decade, many researchers have undertaken the challenging task of investigating the mechanisms of interaction between water molecules and globular proteins (Ooi & Oobatake, 1988; Otting et al., 1991a, 1991b; Holak et al., 1992; Levitt & Park, 1993; Willams et al., 1994; Alonso & Daggett, 1995; Denisov et al., 1995, 1999; Ernst et al., 1995; Bhattacharjya & Balaram, 1997; Takano et al., 1997; Mesgarzadeh et al., 1998; Shortle, 1999). Some of these authors have examined the possible role of water as a stabilizing (or destabilizing) force in the maintenance of the native structure of proteins (Ooi & Oobatake, 1988; Willams et al., 1994; Ernst et al., 1995; Takano et al., 1997). It is, however, more difficult to study water–protein interactions for nonnative, partially folded conformations (so-called molten globules) than it is for native, folded proteins. Biophysical methods applied to the characterization of native proteins often fail to be very informative when studying the molten-globule (MG) state. Nevertheless, the introduction of multiparametric methods, which are capable of monitoring several different properties during the same experiment, may represent an important step forward in the study of such protein conformations. Multidimensional NMR can provide significant information about where the interaction between water and protein molecules takes place at the protein surface, the quantities of water molecules and protein cavities and lifetimes of the water molecules, among other dynamic characteristics (Willams et al., 1994; Alonso & Daggett, 1995; Denisov et al., 1995; Ernst et al., 1995; Otting, 1997; Mesgarzadeh et al., 1998). NMR allows us to see almost all the spectral compounds in the sample and to measure nuclear Overhauser effects (NOE), relaxation times, spin–spin couplings, and so on. Nonetheless, it should be pointed out that the NMR data obtained for various different nuclei may not always agree with that obtained by other methods (Otting et al., 1991a; Denisov et al., 1995; Otting, 1997). This may be due to the complexity of nonnative protein states and to the differing ability of various methods to measure the characteristics of distinct parts of the molecule. For example, magnetic relaxation dispersion of water ^{17}O measures short relaxation times, and thus characterizes only very fast processes (in the NMR timescale). This is not to imply, however, any real contradiction between this and other methods, but rather a different perspective (Denisov et al., 1999; Shortle, 1999). The rigidity parameter (G) was introduced to measure quantitatively the remaining structure and compactness of a protein molecule after undergoing the effects of temperature, denaturing agents, changes in pH, or any other variable (Kutyshenko, 1990, 1991a, 1993; Griko & Kutyshenko, 1994). G is defined as the ratio between the integral intensities measured in spin diffusion (SD) and in normal ^1H-NMR spectra for a given spectral region of a protein (6.5–7.5 ppm for aromatic and 1.4–2.7 ppm for aliphatic

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Abbreviations: G, rigidity parameter; MG, molten globule; NI, normalized integral intensities; NOE, nuclear Overhauser effect; SD, spin diffusion; ST, saturation time; VD, variable delay applied after the saturation pulse.

regions), under exactly the same experimental and instrumental conditions. G values of ~0.1 have been measured for several denatured proteins while values of ~0.5 have been recorded for native structures (Kutyshenko, 1990, 1993). The G values for the MG state of some proteins are only slightly less than for the native state (Kutyshenko, 1993), implying that the MG is still significantly compact despite its loss of native conformation. Experiments carried out by other authors have revealed the existence of extensive NOEs between aromatic and aliphatic protons in the molten-globule state of some proteins (Balbach et al., 1997), although attempts to detect such NOEs directly by using two-dimensional NMR experiments have on the whole been unsuccessful (Alexandrescu et al., 1993). Carbonic anhydrase B is a well-known protein and its native, molten-globule, and denatured states are well characterized (Wong & Tanford, 1973; Dolgikh et al., 1984; Semisotnov et al., 1987, 1996; Ptitsyn et al., 1990). We have studied the molten-globule state of bovine carbonic anhydrase B by measuring its spin-diffusion spectra under several experimental conditions.

Results

High-resolution, proton NMR spectra for the native, denatured and MG states of carbonic anhydrase B are shown in Figures 1A, 1B, and 1C. There are no secondary chemical shifts for either the denatured or MG states, indicating a loss of the gross tertiary structure of the protein in both states. SD experiments using several variable delay (VD) times (see Materials and methods) were performed with the protein in all three states at several temperatures and concentrations (between 0.10 and 0.50 mM). Figures 1D, 1E, and 1F show the SD spectra obtained for the protein at a concentration of 0.50 mM in its three states, centering the on-resonance sequences at the methyl proton frequency and using a variable delay (VD) of 100 ms, applied before the reading pulse. It is clear in this figure that the MG state is still considerably rigid, while this is not the case with the denatured protein, an observation which agrees with our previous work on other proteins (Kutyshenko, 1991a, 1993; Kutyshenko & Khechinashvili, 1989b). The effects of temperature upon the rigidity parameter of the aliphatic protons of the protein in its native, molten-globule and denatured states are shown in Figure 2. The G values measured are similar to those observed with other proteins studied elsewhere in these three states (Kutyshenko, 1990, 1991b, 1993). The G values for the MG state are closer to the values for the native state than to those for the denatured state, indicating that the MG state is still quite compact despite its loss of native conformation. Parameter G depends upon molecular weight (Kutyshenko, 1991b) and the packing density of a protein (Kalk & Berendsen, 1976; Akasaka, 1979). It also depends upon the macromolecular rotational motion determined by its correlation time τ, which in turn alters with temperature (Kalk & Berendsen, 1976). If we accept as a starting premise that the volume of the protein is constant, then, according to the Stokes–Einstein relationship, the correlation time should be proportional to the ratio η/T (where η is the viscosity of the medium). Thus, theoretical G values for the protein in the MG state can be calculated as a function of temperature by using as reference the G value measured at 293 K and taking the water viscosity values from Lide (1998). The calculated values for G do not agree with the experimental ones, suggesting that other phenomena may be involved.

Significant water signals were only observed in the SD spectra of the protein in the MG state but not in the other two states (see Fig. 1), perhaps indicating the presence of strong and long-lasting (in the NMR timescale) interactions between protein and water molecules in this MG state. We confirmed this by repeating the experiments under the same conditions, but centering the on-resonance sequences at the water frequency instead of the methyl-proton frequency, and obtaining similar spectra to those given in Figure 1F (result not shown). The frequency of protein Cα protons is close to that of water and there is a chance that the diffusion spectra obtained by excitation at the water signal might be due to the simultaneous excitation of Cα protons rather than to the excitation of the water molecules themselves. To discriminate between these possibilities, we carried out experiments as a function of delay time. The logarithms of some of the NI intensities obtained (normalized as described in Materials and methods) are given in Figure 3. The relaxation of the aliphatic protons to their equilibrium distribution is better described by double exponential equations, leading to slightly concave or convex curves. The results obtained for the aliphatic protons (exciting at the methyl frequency) and for the water protons (exciting at the water frequency) show such small curvatures that they can also be described by single exponential equations. The relaxation times obtained by fitting these results to either single or double exponential equations agree within experimental uncertainty. Nevertheless, the NI intensities obtained for the aliphatic protons by excitation at the water frequency or for the water protons by excitation at the methyl frequency (see below) do not fit to single exponential curves. Figure 3 also shows that the relaxation times for the aliphatic signals were very different when the SD spectra were obtained by centering the on-resonance sequences at the methyl (about 1 s) or at the water (nearly 10 s) frequencies. This confirms that the SD spectra obtained by excitation at the water signal were not due to any possible simultaneous excitation of protein protons. Moreover, the relaxation times of the water and aliphatic protons were practically equal (about 10 s) when the SD spectra were obtained by excitation at the water frequency. Thus, it appears that the energy applied to the water protons was transferred to the protein protons, and that the energy applied to the protein protons was similarly transferred to the water molecules.

The relaxation times for the aliphatic protons obtained at several temperatures and at two protein concentrations are given in Table 1. These values agree within experimental uncertainty with those measured directly by inversion-recovery experiments, although the latter values appear to be slightly higher at low temperature. The standard deviations of the relaxation times obtained from the non-linear, least-squares fittings were between 1 and 4% with either method, although their standard errors may well be higher. The relaxation times do not appear to depend upon temperature, within experimental uncertainty. There is a decrease of about 10% in the relaxation times between 0.10 and 0.50 mM protein concentration, which is higher than that allowed by experimental error. The values given in Table 1 also agree with the relaxation times of the protein aliphatic protons obtained from the SD spectra by excitation at the water frequency, at both protein concentrations (0.96 and 0.81 s at 0.10 and 0.50 mM, respectively, at 20 °C). These results may be explained by some protein association in the MG state at these temperatures and within this concentration range. It is worth mentioning that the relaxation times measured for the methyl and aliphatic protons (about 1.0 and 1.5 s, respectively) in the native and denatured states (by excitation at the methyl frequency) are also practically independent of temperature.

Our results obtained at different temperatures concerning the dependence of the water-signal intensity upon delay time, ob-

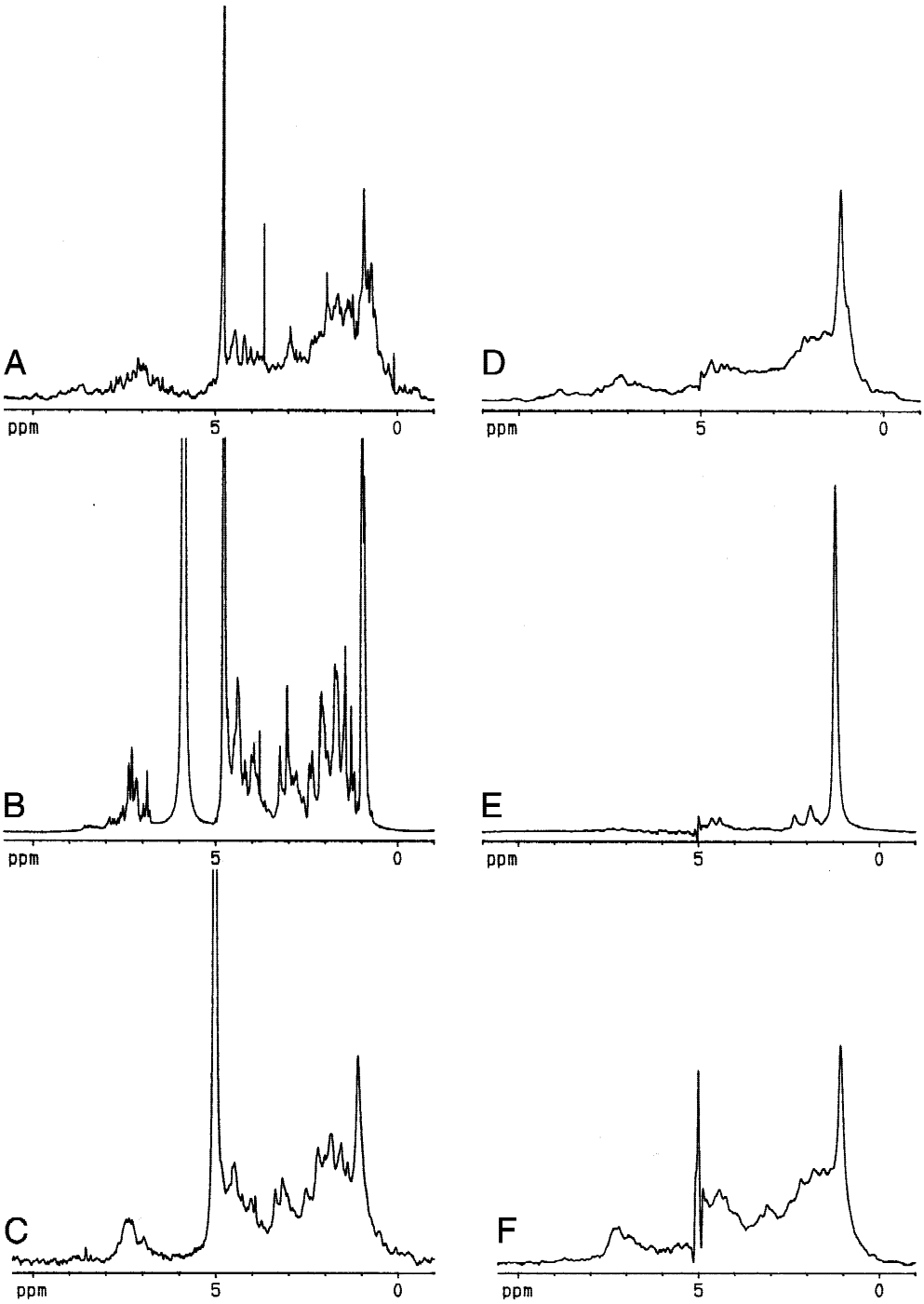

Fig. 1. NMR spectra of carbonic anhydrase B at concentrations of around 0.50 mM and 293 K. The spectra (**A**, **B**, **C**) are ^{1}H NMR spectra, while **D**, **E**, and **F** are spin-diffusion spectra obtained by centering the on-resonance signal at the methyl proton frequency with a delay time of 100 ms: (**A**,**D**) native state, pH 7.2; (**B**,**E**) denatured state, pH 7.2 and 7.2 M urea; (**C**,**F**) molten globule state, pH 3.9. The intensity of all the spectra has been normalized to obtain equal intensities of the methyl proton signals.

served in the spin-diffusion spectra of the MG state of the protein upon excitation of its methyl protons, are set out in Figure 4. These intensities initially increased with delay time, reaching a maximum at about 2 s and then decreased at higher delay times. The protein-signal intensities measured in the same spectra were shown above (see Fig. 3). The behavior observed in Figure 4 suggests that the dependence of the normalized intensities (NI) upon delay time (t) could be adjusted to a double exponential. We began a nonlinear, least-squares fitting with an equation containing five adjustable parameters, $\mathrm{NI} = a * \exp(-t/\tau_1) - b * \exp(-t/\tau_2) + c$, from which we always obtained very high correlation coefficients ($\sim$0.9998). The adjustments were slightly worse when we used

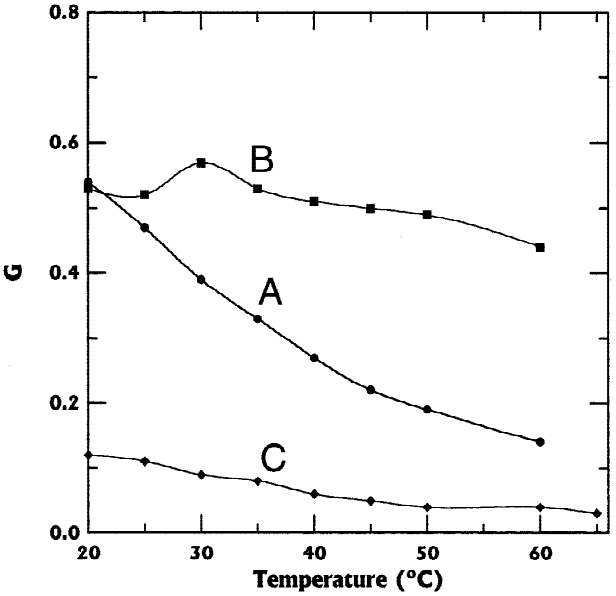

Fig. 2. Temperature dependence of the rigidity parameter G for carbonic anhydrase B in (**A**) the native, (**B**) MG, and (**C**) denatured states. The lines are spline curves provided only as a guide for the eye.

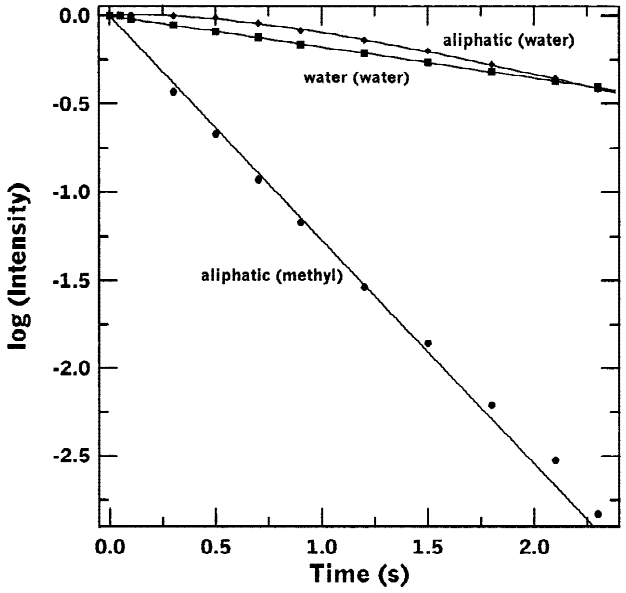

Fig. 3. Influence of the delay time on the logarithm of the normalized intensities NI of aliphatic and water protons measured in the SD spectra of the carbonic anhydrase B molten globule. The on-resonance signal was centered at the proton frequencies depicted in parenthesis. The spectra were taken at 284 K and $c = 0.50$ mM. The symbols stand for the experimental values and the lines are the best fitting to a double exponential equation with three parameters: $\mathrm{NI} = a * \exp(-k_1 t) + (1 - a) * \exp(-k_2 t)$. The values of the parameters found by nonlinear least-squares adjustments are: water (water), $a = 1.003$, $k_1 = 0.177$, $k_2 = 0.466$; aliphatic (water): $a = 1.353$, $k_1 = 0.30$, $k_2 = 1.34$; and for aliphatic (methyl): $a = 1.195$, $k_1 = 1.273$, $k_2 = 1.279$.

only four adjustable parameters, thus obtaining some higher χ^2 values. It is logical that we should achieve better fittings and smaller deviations around the fitted regression function when using more parameters. The statistic of the fittings may, however, dictate the minimum number of parameters required to describe the system correctly. We tested this number therefore by using the method described by Neter et al. (1990). We began by testing the fittings to the above equation and also to a reduced equation in which $c = 0$, our intention being to determine statistically whether *Ho*: $c = 0$ or *Ha*: $c \neq 0$ holds true. We calculated the error sum of squares in the fittings for both the full and reduced models (with five and four parameters, respectively) and taking into account their respective degrees of freedom we calculated the corresponding F values, which were always lower than 10^{-2}, implying that *Ho* holds true ($p < 0.001$). We similarly tested whether *Ho*: $a = b$ or *Ha*: $a \neq b$ holds true. The F values obtained were between 0.02 and 0.19, implying that the hypothesis *Ho* is also valid ($p < 0.01$). Finally, we also tested our fittings to a double exponential equation with only three parameters (i.e., $c = 0$ and $a = b$). We found F values of between 5 and 31, according to the temperature, implying that the null hypothesis is refused ($p < 0.99$). All these statistical results indicate that our data should be fitted to a double exponential with four parameters and that it cannot be decided whether $c = 0$ or $a = b$ upon statistical grounds alone. We prefer to choose the equation in which $c = 0$ for several reasons: (1) its F values determined in the previous tests are smaller; (2) the agreement between the theoretical and experimental values appears to be better, according to plots such as that in Figure 4, especially at high temperature, where the experimental error is greater; (3) the variations vs. temperature in parameter τ_1, which has always the greater standard deviation, appear to be less erratic; (5) the values of τ_1 obtained by fitting to this equation are closer to those obtained by fitting to the equation with five parameters and also to those measured directly in inversion-recovery experiments. The parameter values obtained for the best fittings to this equation are given in Table 2 at several temperatures and two protein concentrations. They have been used to calculate the theoretical curves in Figure 4. It can be seen that at the lower protein concentration τ_2 is practically constant at about 1 s while τ_1 in-

Table 1. *Relaxation times, τ (in seconds), for aliphatic protons (1.4–2.7 ppm) of carbonic anhydrase B at pH 3.9 (MG state) and several temperatures*

	τ^a	τ^b		τ^b
T (K)	$c = 0.50$ mM[c]	$c = 0.50$ mM	T (K)	$c = 0.10$ mM
284	0.902 (0.019)	0.758 (0.012)	—	—
294	0.878 (0.012)	0.745 (0.013)	293	0.871 (0.026)
306	0.931 (0.014)	0.836 (0.011)	298	0.923 (0.016)
310	0.852 (0.032)	—	303	0.941 (0.021)
316	0.840 (0.016)	0.843 (0.015)	323	0.915 (0.011)
Mean values	0.880 (0.037)	0.795 (0.051)		0.913 (0.030)

[a] Values measured from inversion-recovery experiments

[b] Values measured from SD spectra centering the on-resonance sequences at the methyl frequencies

[c] Values in parenthesis are standard deviations of the fittings; "$c =$" stands for protein concentration.

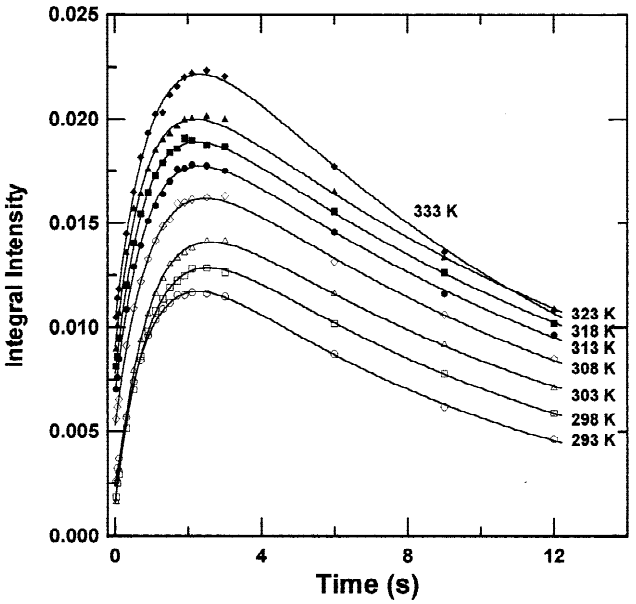

Fig. 4. Influence of the delay time on the normalized intensities NI of the water protons measured in the SD spectra of the carbonic anhydrase B molten globule at the temperatures indicated. The on-resonance signal was centered at the methyl proton frequency. The symbols are experimental values and the lines are the theoretical values calculated with the parameters given in Table 2 and the equation $NI = a * \exp(t/\tau_1) - b * \exp(t/\tau_2)$.

creases with temperature from 9 to 15 s (except for the value obtained at the highest temperature used, 60 °C). The values of parameters *a* and *b* are very close, although *a* is always greater than *b* and this difference increases concomitantly with temperature. At the higher protein concentration, the temperature-dependency trend for all four parameters is similar to that observed at the lower concentration, although both relaxation times are now shorter.

Discussion

The absence of secondary chemical shifts in the NMR spectra of globular proteins is characteristic of both the denatured and MG states. Nevertheless, the evidence provided by our spin-diffusion experiments points to the idea that these two states of the protein behave very differently: the spin-diffusion spectrum of the MG state is similar to its ^{1}H–NMR spectrum, while the denatured protein yields a "weak" spin-diffusion spectrum, where practically only the methyl-group signals are visible. The packing of the polypeptide chains appears to remain largely compact in the MG state, as it does in the native one (Ptitsyn & Semisotnov, 1991; Christensen & Pain, 1994), and spin-diffusion can be initiated and propagated because the condition $\omega^2\tau^2 \gg 1$, where ω is the spectrometer frequency and τ is the protein correlation time, holds good for interproton distances smaller than 4 Å (Kalk & Berendsen, 1976; Akasaka, 1979). When the protein is denatured it becomes less compact, which leads to an increase in both intermolecular mobility and the distance between protons belonging to different spin systems. Thus, the spin-diffusion effect might decrease significantly and energy excitation by the NOE may only occur inside the same spin system (Kutyshenko & Khechinashvili, 1989a, 1989b; Derome, 1991).

Table 2. *The experimental NI values of the water signal obtained from SD spectra by excitation at the methyl frequency were fitted by nonlinear least-squares methods to the equation* $NI = a * \exp(-t/\tau_1) - b * \exp(-t/\tau_2)$*, thus obtaining the parameters given here as a function of temperature*

T (K)	100 a[a]	τ_1 (s)[a]	100 b[a]	τ_2 (s)[a]	τ_1 (s)[b]
293	1.67 (0.02)[c]	9.26 (0.15)	1.43 (0.02)	0.96 (0.02)	9.19 (0.06)
298	1.80 (0.02)	10.72 (0.18)	1.64 (0.02)	1.01 (0.02)	10.42 (0.08)
303	1.89 (0.03)	12.40 (0.32)	1.72 (0.03)	0.98 (0.03)	11.12 (0.09)
308	2.12 (0.03)	13.03 (0.32)	1.59 (0.03)	1.00 (0.03)	11.80 (0.10)
313	2.24 (0.03)	14.06 (0.41)	1.55 (0.03)	0.92 (0.03)	12.29 (0.14)
318	2.35 (0.02)	14.47 (0.27)	1.57 (0.02)	0.87 (0.02)	12.33 (0.15)
323	2.47 (0.04)	14.72 (0.59)	1.56 (0.04)	0.87 (0.03)	12.21 (0.19)
333	2.97 (0.06)	11.75 (0.36)	1.93 (0.05)	1.07 (0.05)	10.85 (0.20)

T (K)	100 a[d]	τ_1 (s)[d]	100 b[d]	τ_2 (s)[d]
294	4.58 (0.06)	6.00 (0.12)	3.28 (0.06)	0.70 (0.02)
306	4.23 (0.06)	7.74 (0.14)	3.22 (0.05)	0.84 (0.02)
316	4.90 (0.06)	6.93 (0.12)	3.52 (0.05)	0.73 (0.02)
327	4.54 (0.06)	6.73 (0.16)	2.85 (0.06)	0.54 (0.02)
343	3.73 (0.04)	8.04 (0.19)	2.31 (0.04)	0.54 (0.02)
362	2.89 (0.04)	11.64 (0.34)	1.91 (0.04)	0.59 (0.03)

[a] From SD experiments at $c = 0.10$ mM.
[b] From inversion-recovery experiments.
[c] Values in parenthesis are standard deviations obtained from the fittings.
[d] From SD experiments at $c = 0.50$ mM.

Parameter G measures quantitatively the existence of NOEs and will depend upon the relaxation of the rapidly rotating methyl groups attached to the protein, which is itself subject to a slower rotational diffusion (Kalk & Berendsen, 1976). G values under conditions such as those described above ($\omega^2\tau^2 \gg 1$) will be directly proportional to the correlation time of the protein τ and to r^{-6}, where *r* represents the average interproton distance (Kalk & Berendsen, 1976). An estimation of the correlation time can be made by considering the protein to be a rigid sphere and using the Stokes–Einstein relationship, $\tau = V\eta/kT$. We have already mentioned in Results that the experimental *G* values did not agree quantitatively with the expected behavior for a constant-volume sphere. In Figure 5, therefore, we have plotted the ratio TG/η calculated as a function of temperature, which should be proportional to the protein volume if the Stokes–Einstein relationship is fulfilled. These results show that the apparent protein volume increases linearly vs. temperature. Similar calculations for other proteins [apomyoglobin, lactalbumine, and ribonuclease A (Kutyshenko, 1993)] are also plotted in the same figure, showing in this case that their apparent volumes decrease slightly vs. temperature. We believe that protein association may be responsible for the variation depicted for carbonic anhydrase B in Figure 5, although the model used was fairly crude: the protein in the MG state is in fact far from being a rigid and impermeable sphere; its rigidity is rather lax and it is considerably solvated by water. G remains practically constant at concentrations lower than 0.1 mM, probably because the protein is a single monomer under these conditions. Our results at the higher protein concentration can, however, easily be explained on the basis of protein monomer, dimers, and trimers in equilibrium.

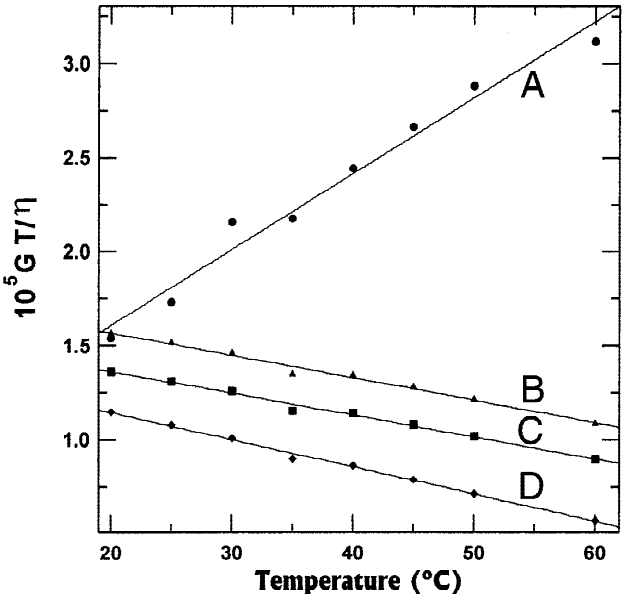

Fig. 5. Temperature dependence of GT/η for several proteins in the MG state. **A:** Carbonic anhydrase B (pH 3.9). **B:** Apomyoglobin (pH 3.7). **C:** Lactalbumine (pH 4.5). **D:** RNAse (pH 3.4). The G values have been taken from this work and from Kutyshenko (1993). Viscosity values of water in $kg\,m^{-1}\,s^{-1}$ come from Lide (1998).

The association of carbonic anhydrase B in the MG state has been shown by the SAXS technique (Semisotnov et al., 1996). It is now widely accepted that proteins in the molten-globule state have a great tendency to aggregate, especially at low pH, where they are "sticky" (Filimonov et al., 1993; Christensen & Pain, 1994).

Our data given in Table 1 and Figures 1 and 3 strongly support the idea put forward by other authors (Kalk & Berendsen, 1976; Ernst et al., 1995) that methyl groups provide an effective relaxation sink for proteins larger than 20 kD at high NMR frequencies. Ernst et al. (1995) put this down to their favorable spectral properties, i.e., narrow lines and high intensities (due to the presence of three protons). The relaxation times of the aliphatic protons of carbonic anhydrase B in the MG state found in both spin-diffusion and inversion-recovery experiments are very close (Table 1), an observation made more than 20 years ago for some native and denatured proteins (Kalk & Berendsen, 1976). We obtained practically the same relaxation times when we analyzed the signals belonging to the aromatic protons when the methyl groups were excited (results not shown), thus giving extra credence to the existence of such a relaxation sink.

Also noteworthy is the presence of water signals in the spin-diffusion spectra in Figure 1, obtained by the excitation of the protein methyl protons. This implies the existence of water molecules (either at the protein surface or in cavities) at distances of <4 Å from the protein protons. What is especially interesting is that the spectrum for the MG state shows the highest water signal compared to the spectra for the other two protein states. This may indicate that there are a large number of water molecules in close contact with some protein protons, i.e., that the protein is largely solvated in the MG state. Spin-diffusion experiments performed by excitation at the water signal confirm this conclusion. We have already shown that it is not due to a simultaneously excitation of some protein protons, because the relaxation times of the aliphatic protons are close to those of water molecules when the on-resonance signal is centered at the water frequency. They are, however, close to those of the protein protons when the on-resonance signal is centered at the frequency of the protein protons (see Fig. 3; Table 1).

That disordered water exists in large hydrophobic cavities of native proteins is now generally accepted, although much of it is not detectable by X-ray diffraction. Many of these and other bound water molecules are in fast exchange with bulk solvent water on the timescale of the chemical shift (see, for example, Otting, 1997 and references quoted therein). The MG state of proteins is usually assumed to be less compact than the native state (Ptitsyn & Semisotnov, 1991) with nonpolar groups being highly exposed to the solvent (Christensen & Pain, 1994). Thus, greater hydration might be expected in this state, as we have mentioned above (see Fig. 1). The MG state could then make a good model to study this hydration, although it is more difficult to observe specific NOEs in this state than in the native state of the protein.

To the best of our knowledge, all experiments made to date have found that the chemical shifts of bound water protons, detectable as NOE cross peaks with protein protons, are identical to those of bulk water. This might be due either to the fact that the chemical shifts of both types of water molecule are indeed identical or that the bound water protons experience conformation-dependent shifts that are canceled by proton exchange or by the exchange of intact water molecules. Some evidence does in fact exist to confirm this latter hypothesis (Otting et al., 1991a). Since there is almost no chemical-shift dispersion of different (in terms of involvement in protein–water interactions) water proton signals of carbonic anhydrase B in the MG state, they can only be distinguished by their kinetic profiles. Spin-diffusion propagation times for water molecules in a protein–water complex should be close to the protein relaxation times. Water molecules involved in protein–water interactions should have relaxation times similar to protein relaxation times (about 1 s), while water molecules situated at a distance from the protein should have relaxation times similar to bulk water relaxation times (from 9 to 14 s).

We show in Results that the water-signal intensities in the spin-diffusion spectra of the MG of carbonic anhydrase B could not be adjusted to single-exponential equations when the excitation was centered at the methyl protons (Fig. 3). This was also true for the aliphatic-proton signals when the spin-diffusion spectra were recorded by excitation at the water frequency (Fig. 4). The relaxation of a system of coupled spins has been extensively studied both theoretically and experimentally, leading to the conclusion that the relaxation can be approximated by a single exponential when the effects due to the correlation of motion of different dipolar pairs, or the so-called cross-correlation, is negligible (Kalk & Berendsen, 1976). It has also been shown that the equations describing the longitudinal relaxation of magnetization are in fact the sums of two exponentials when the cross-relaxation mechanism becomes active (Kalk & Berendsen, 1976; Bothner-By et al., 1984; Olejniczak et al., 1986). Nevertheless, in the theoretical equations obtained the two pre-exponential terms have always been equal to each other, although with opposite signs (Bothner-By et al., 1984; Olejniczak et al., 1986). The data in Figure 3 reveal such a small variation vs. the delay time that it is impossible to determine whether the small differences between the two pre-exponential terms of the fitted equations were, in fact, significant or merely due to experimental error. This can be done however with the data set out in Figure 4, which are better fitted by a double exponential

with four significantly different parameters (see Results; Table 2). This cannot be explained merely by taking into account spin diffusion, either a two-spin or a multispin system, given that we are working under the condition $\omega^2\tau^2 \gg 1$. Edzes and Samulski (1977) assumed two populations of water molecules with very different relaxation rates to explain the different values found for the two pre-exponential parameters obtained when fitting the data for hydrated collagen, but their equations do nothing to explain our results because they lead to absurd consequences, such as negative values for the relaxation times. Neither does the idea of water molecules both bound and unbound to the protein in equilibrium, such as Akasaka applied to the case of myosin (Akasaka, 1979) explain our results. It may well be, in fact, that several of these factors are involved and we are now engaged in determining the source of such small but significant differences between the two pre-exponential parameters given in Table 2.

Materials and methods

Bovine carbonic anhydrase B was generously given to us by Prof. O. Ptitsyn's laboratory (Pushchino). The protein was dissolved in a solution containing 30–40 mM sodium acetate-D3 or sodium phosphate, 99.9% D_2O (99.9% from SDS, France) at the desired pH values. The urea used in these experiments was recrystallized twice from D_2O and its concentration was calculated from the refraction index of the solution (Pace et al., 1989). pH values were measured directly with a pH-meter and subject to no further correction. NMR spectra were collected at 500 MHz using a Bruker AMX-500 spectrometer with 160 scans, a spectral width of 6,000 Hz and 16K FID. SD spectra were obtained by using the sequence: $\{\text{D1-ST-VD-}\pi/2\text{-FID}\}_{\text{on-resonance}} - \{\text{D1-ST-VD-}\pi/2\text{-FID}\}_{\text{off-resonance}}$, where D1 is the relaxation delay (~40 s), ST (saturation time) is the time for the spin-diffusion excitation (500 ms, 40 dB of 5 W) and VD is a variable delay (0.02–12 s) applied before the 6 μs 90-deg reading pulse. SD excitation was centered at either the methyl proton frequency (~0.9 ppm) or at the water frequency (4.76 ppm) for the on-resonance sequences and at −1.5 ppm (far away from the spectral signals) for the off-resonance sequences. Chemical shift values were normalized to DSS. The relaxation times were measured with the inversion-recovery sequence 180-τ-90, as described in Farrar and Becker (1971). The integral intensities (I) for water and protein aliphatic protons (1.4–2.7 ppm) in the SD spectra were normalized by dividing them by the I values for the same signals in the normal ^{1}H-NMR spectra and these normalized values are referred to as NI. The NI values obtained were often expressed as relative values, considering that the NI values obtained with a VD of 20 ms has a reference value equal to one. The G values were calculated as explained in Griko and Kutyshenko (1994).

All mathematical calculations and the nonlinear, least-squares fitting of the experimental results to the theoretical equations were made with programs written by the authors in IDL (Interactive Data Language, Research Systems Inc., Boulder, Colorado). Practically identical parameters were obtained by using the Prisma package.

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