# Protein-Bound Water Molecule Counting by Resolution of $^1$H Spin-Lattice Relaxation Mechanisms

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ABSTRACT Water proton spin-lattice relaxation is studied in dilute solutions of bovine serum albumin as a function of magnetic field strength, oxygen concentration, and solvent deuteration. In contrast to previous studies conducted at high protein concentrations, the observed relaxation dispersion is accurately Lorentzian with an effective correlation time of 41 ± 3 ns when measured at low proton and low protein concentrations to minimize protein aggregation. Elimination of oxygen flattens the relaxation dispersion profile above the rotational inflection frequency, nearly eliminating the high field tail previously attributed to a distribution of exchange times for either whole water molecules or individual protons at the protein–water interface. The small high-field dispersion that remains is attributed to motion of the bound water molecules on the protein or to internal protein motions on a time scale of order one ns. Measurements as a function of isotope composition permit separation of intramolecular and intermolecular relaxation contributions. The magnitude of the intramolecular proton–proton relaxation rate constant is interpreted in terms of 25 ± 4 water molecules that are bound rigidly to the protein for a time long compared with the rotational correlation time of 42 ns. This number of bound water molecules neglects the possibility of local motions of the water in the binding site; inclusion of these effects may increase the number of bound water molecules by 50%.

## INTRODUCTION

Solvation by water is fundamental to protein dynamics, thermodynamics, and function. Nuclear magnetic relaxation provides a powerful approach to characterizing molecular dynamics in general. In particular, the magnetic field dependence of spin-lattice relaxation rate constants provides a test of the usual theoretical models that are used to interpret the relaxation data and yields correlation times directly as inflection points in relaxation dispersion profiles. Magnetic relaxation dispersion (MRD) measurements on protein solutions have been studied in this context since 1969 (Koenig and Schillinger, 1969), but details of interpretation have been debated (Koenig et al., 1975; Bryant, 1978, 1996; Halle et al. 1981; Halle and Denisov, 1995; Kimmich, 1990a,b; Koenig and Brown, 1991; Koenig et al., 1993). There have been major advances using $^{17}$O MRD (Denisov et al., 1995; Denisov and Halle, 1995; Denisov et al., 1996; Denisov and Halle, 1996; Denisov et al., 1997; Denisov and Halle, 1998; Torres et al., 1998; Denisov et al., 1999; Langhurst et al., 1999; Baguet and Hennebert, 1999). The proton relaxation case has remained less clear. There are three main issues: The proton relaxation dispersion produces a correlation time for the rotational motion of the protein that is generally longer than that obtained from other measures of rotational mobility (Hallenga and Koenig, 1976). The usual models for nuclear spin relaxation predict that the dispersion should be Lorentzian, but most observations reported to date have not fit this simple model. Rather, the dispersion curves are broader and sometimes not symmetric, which may have various interpretations, including some form of correlation time distribution (Halle et al., 1998). Finally, the high-field portion of the relaxation dispersion often appears more highly broadened than the low-field portion, implying that an additional high-frequency process contributes to the relaxation events. This high-field tail has been attributed to a distribution of proton lifetimes at the protein–water interface (Bryant, 1996).

We have constructed a new MRD spectrometer that provides high sensitivity and high resolution measurements at much lower spin concentrations than previously detectable (Wagner et al. 1999). Measurement of water–proton spin-lattice relaxation rate constants under conditions of high deuteron substitution provides a significant expansion of the dynamic range of the MRD experiment by minimizing the intra- and intermolecular water–proton dipole–dipole interactions. That is, the limiting relaxation rate constant is lowered from approximately 0.3 $s^{-1}$ for pure $H_2O$ to about 0.03 $s^{-1}$ for the residual protons in 99.9% $D_2O$. This gain in dynamic range permits study of the relaxation dispersion profiles for protein solutions at much lower protein concentrations than was previously practical.

In the current study, we present MRD data obtained from dilute solutions of bovine serum albumin (BSA) in $H_2O$ and in $D_2O$. In contrast to previous MRD studies of protein solutions, the present data are easily fit with a simple Lorentzian function without recourse to proton exchange effects, distributions of exchange rates, protein aggregation, or other ad hoc assumptions. The inflection point in the relaxation dispersion curve is directly related to the rotational correlation time of the water–protein complex. Compared with studies of concentrated protein solutions, the

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rotational correlation time reported by the relaxation dispersion curve is in excellent agreement with fluorescence depolarization measurements and hydrodynamic calculations. These measurements suggest that the non-Lorentzian shape of the relaxation dispersion profile and the long rotational correlation times deduced from the inflection frequencies of previous studies resulted from the effects of high protein concentration that include protein aggregation. Furthermore, the effects of dissolved molecular oxygen are significant. In deoxygenated samples, the high-field distortion is eliminated. Finally, comparison of proton relaxation rate constants in $H_2O$ and in $D_2O$ permits assessment of the relative contributions of intra- and intermolecular proton dipole–dipole relaxation mechanisms in the protein-bound environment. This separation provides an assessment of the number of slowly exchanging water–molecule binding sites on the protein.

## THEORY

The proton spin-lattice relaxation rate constants for water that exchanges with protein sites in solution are a weighted average of relaxation interactions in bound and unbound environments. When the protein concentration is low, the observed relaxation may be written

$$R_{1,\mathrm{obs}} = P_{\mathrm{free}}(T_{1,\mathrm{free}})^{-1} + P_{\mathrm{bound}}(T_{1,\mathrm{bound}} + \tau_{\mathrm{ex}})^{-1}, \quad (1)$$

where $P_{\mathrm{free}} = 1 - P_{\mathrm{bound}}$ and $P_{\mathrm{bound}}$ is the probability that the water is bound; $T_{1,\mathrm{free}}$ and $T_{1,\mathrm{bound}}$ are the relaxation times of the free and bound environments, respectively; and $\tau_{\mathrm{ex}}$ is the mean residence time in the bound environment. The probability may be written simply as

$$P_{\mathrm{bound}} = \frac{[\mathrm{protein}]}{[\mathrm{water}]}, \quad (2)$$

where the brackets denote concentration.

The mean residence time, $\tau_{\mathrm{ex}}$ in Eq. 1, is often not known and may have a distribution of values reflecting a variety of binding sites. The effect of $\tau_{\mathrm{ex}}$ is negligible provided that it is longer than the rotational correlation time, $\tau_{\mathrm{rot}}$, of the complex (5–100 ns) and shorter than $T_{1,\mathrm{bound}}$, the relaxation time of the bound environment (>1–10 ms for proteins). This range of bound-state lifetimes thus provides an operational definition for a rotationally correlated spin pair as distinct from translationally correlated ($\tau_{\mathrm{ex}} < \tau_{\mathrm{rot}}$) or nonexchanging ($\tau_{\mathrm{ex}} \gg T_{1,\mathrm{bound}}$) spin pairs. There is convergence on the view that the observed magnetic spin relaxation in protein solutions is caused primarily by a relatively small number of water molecules that exchange slowly relative to the rotational reorientation of the protein (Koenig and Schillinger, 1969; Koenig, 1995; Bryant, 1996; Denisov and Halle, 1996).

Eq. 1 shows that the observed water–proton relaxation rate constant is the bound relaxation rate constant scaled by the probability that the water is bound. The bound relaxation rate constant, $1/T_{1,\mathrm{bound}}$, represents a sum of contributions from separate intra- and intermolecular dipolar interactions. In $H_2O$, both the intra- and the intermolecular interactions are homonuclear, and the relaxation is determined by a sum of terms of the form

$$R_{1\mathrm{D}} = \frac{2}{5}\frac{\gamma_{\mathrm{I}}^4 \hbar^2}{r_{\mathrm{IS}}^6} I(I+1)A^2\{J(\omega_{\mathrm{I}}) + 4J(2\omega_{\mathrm{I}})\}, \quad (3)$$

where $\gamma_{\mathrm{I}}$ is the proton magnetogyric ratio, $\hbar$ is Planck's constant divided by $2\pi$, $r_{\mathrm{IS}}$ is the distance between the two spins, $A^2$ is an order parameter that scales the dipolar interactions as a result of internal motions, and $J(\omega)$ is the spectral density that characterizes the magnetic noise caused by molecular motion (Abragam, 1961). The intra- and intermolecular interactions differ in the number and separation distance of the interacting spins. For protein-bound $H_2O$, there is only one intramolecular spin pair separated by 1.58 Å. There are also an unknown number of intermolecular proton–proton contacts characterized by larger intermoment distances. At a minimum, these spins are separated by the van der Waals proton–proton contact distance of approximately 2.2 Å, but they are more accurately represented by a sum over a number of separate interactions with unknown internuclear distances. Consequently, the relative importance of the intra- and intermolecular relaxation mechanisms to the observed spin is difficult to define. The problem is further complicated by contributions from labile protein protons associated with amides, amines, alcohols, etc. Labile protein sites with fast exchange rates contribute to the relaxation dispersion much as does the exchange of bound water molecules. In the present study, these effects are indistinguishable from intermolecular water–protein relaxation effects because they dilute with the isotope substitution of deuterium for hydrogen.

In $D_2O$ solutions, the detected residual protons are found primarily in HDO molecules for which the relaxation is somewhat different than in $H_2O$. The intramolecular dipolar interactions are heteronuclear and given by

$$R_{1\mathrm{D}} = \frac{2}{15}\frac{\gamma_{\mathrm{I}}^2 \gamma_{\mathrm{S}}^2 \hbar^2}{r_{\mathrm{IS}}^6} S(S+1) \times A^2\{J(\omega_{\mathrm{I}} - \omega_{\mathrm{S}}) + 3J(\omega_{\mathrm{I}}) + 6J(\omega_{\mathrm{I}} + \omega_{\mathrm{S}})\}, \quad (4)$$

where the symbols have the same meanings as above and $S = 1$ for deuterium. In an HDO molecule, the intramolecular relaxation rate constant is 24.3 times smaller than in $H_2O$. This scaling leads to $^1H$ relaxation dispersions in $D_2O$–protein solutions, which would be nearly undetectable if deuterons were the sole relaxation mechanism. However, a significant dispersion is detected because the intermolecular (H–H) homonuclear relaxation mechanism remains. By comparison of the observed relaxation dispersion detected in HDO with that in $H_2O$, it is possible to separate the

relative contributions of intra- and intermolecular relaxation mechanisms in protein solutions.

Two types of motion are important for the bound water molecules: the overall rotation of the protein molecule, and any motions of the water in the binding site or motions of the binding site components. The overall rotation of BSA is nearly isotropic, leading to Lorentzian spectral densities. For fast librational motions of the ligand in the binding site, the spectral densities are more difficult to determine and may differ for the intra- and intermolecular mechanisms. In particular, for the intermolecular interactions, the intermoment distance is also modulated with the correlation time of the relative motion, considerably complicating the relaxation model (Huang and Freed, 1975; Freed, 1978). In any case, the resolution of our experiment is currently insufficient to distinguish between the subtleties of different internal motions, so their effects are gathered in the order parameter, $A^2$. For H–H interactions in a bound water molecule, the order parameter may range from 1 to 0.25 depending on the type and range of the motion. For water twisting and slewing, the order parameter has been calculated as

$$A^2 = 1 - 3 \sin^2\phi \cos^2\phi, \quad (5)$$

where $\phi$ is the half angle of the libration (Venu et al., 1997)

The relaxation rate constant of the free environment, $1/T_{1,\text{free}}$, is also a sum of terms. The intramolecular H–H and H–D dipolar relaxation contributions for the free solvent molecules do not disperse within the frequency range of our experiment and appear only as shifts in the baseline rate constant. The free ligand is also relaxed by translational contacts with the protein surface ($\tau_c$ = 10–100 ps). These are homonuclear dipolar interactions in both solutions, but they also disperse beyond the range of accessible Larmor frequencies and provide only a baseline shift.

Small amounts of paramagnetic materials cause efficient nuclear spin relaxation because of the large electron magnetogyric ratio ($\gamma_e = 658\gamma_H$). Under normal atmospheric conditions, oxygen dissolves in water to approximately 0.27 mM. In the absence of protein, the observed MRD is very nearly Lorentzian with a correlation time of approximately 6 ps, which we attribute to the electron spin-lattice relaxation time, $T_{1e}$ (Teng et al. unpublished).

## EXPERIMENTAL

BSA (Fraction V, Sigma #3509A, St. Louis, MO) was used without further purification. Stock solutions of 5% wt/v protein were adjusted to pH or pH* of 6.95 and diluted to 1% wt/v, which corresponds to 0.15 mM concentration based on a 68 kDa molecular mass. Samples in equilibrium with air were used without further manipulations. Equilibration with other gases was achieved by bubbling either 100% $N_2$ or 100% $O_2$ through the sample at a rate of 2–3 bubbles/s for at least one hour. Samples were sealed in threaded Delrin sample holders with nylon screws that compressed silicone o-ring cord-stock plugs (Wagner et al., 1999).

The MRD data were obtained using a dual magnet spectrometer described elsewhere (Wagner et al., 1999). This system uses a superconducting 7.05T solenoid in tandem with a GMW 4-inch variable field electromagnet. The two magnets are isolated by an iron shield. The spin system is polarized in the high-field magnet, shuttled pneumatically to the remote electromagnet where the magnetization is allowed to decay for a variable time. The sample is then pneumatically returned to the high-field environment where the remaining magnetization is immediately quantified using a 90° pulse. A homebuilt dual resonance probe was used with a 300-W broadband (American Microwave Technology, Brea, CA) model 3304 rf amplifier. The experiments were performed at ambient laboratory temperature, maintained at approximately 294 K. Nonlinear fits to the data were performed using the Igor (WaveMetrics, Lake Oswego, OR) software package for the MacIntosh computing platform.

## RESULTS AND DISCUSSION

Data collected from a series of dilute solutions of BSA are shown in Fig. 1. The plots labeled *A*, *B*, and *C*, show the spin-lattice relaxation rate constants obtained from residual protons in dilute $D_2O$ solutions of BSA containing varying amounts of dissolved oxygen. Figure 1 *A* shows data collected from a sample in equilibrium with air (21% $O_2$ by volume). This relaxation dispersion profile has two distinct dispersion steps. The dispersion at the lower Larmor frequency occurs at 1.3 MHz, corresponding to a rotational correlation time of 42 ns, appropriate for this large protein. The high-frequency dispersion occurs at ~20 MHz, appropriate for an internal protein motion, motion of the bound water molecules, or relaxation of a paramagnetic electron spin. The dispersion is too large to be caused by a distribution of proton or water molecule exchange times.

In a dilute BSA sample in $D_2O$ equilibrated with 100% $N_2$, the relaxation data shown in Fig. 1 *C* is completely dominated by a single dispersion at low field. The high-field dispersion is significantly reduced, although not entirely removed. Figure 1 *B* shows data collected from a similar sample equilibrated against 1 atm of 100% $O_2$. Here, the high-field dispersion is significantly larger. We thus attribute the high-field dispersion in Fig. 1 *A* to the spin-lattice relaxation induced by the proton–oxygen–electron dipolar coupling that is modulated by the oxygen $T_{1e}$. The oxygen dispersion is small, 0.075 $s^{-1}$ in air, and it is only with the improved instrumentation of the sample shuttle HR-MRD that we are able to measure it accurately. Clearly, minute amounts of dissolved paramagnetics may have significant effects on the observed relaxation dispersion pro-

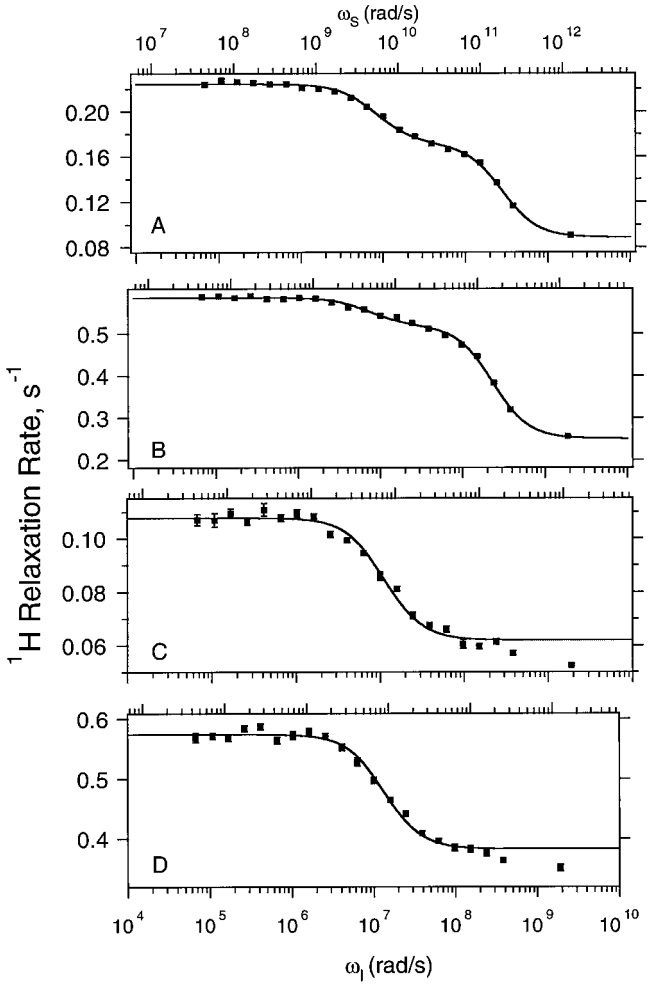


FIGURE 1 $^1$H spin-lattice relaxation rate constants for protons in aqueous solutions containing 1% (w/v) BSA at pH* = 7.0, and ambient laboratory temperature of 294 ± 1 K. (*A*) The protein was dissolved in $D_2O$ and the residual protons measured after equilibration with air. (*B*) The protein was dissolved in $D_2O$ and the residual proton measured after the solution was equilibrated with 1.0 atm oxygen gas. (*C*) The protein was dissolved in $D_2O$ and the residual protons measured after the solution was equilibrated with 1.0 atm nitrogen gas. (*D*) The protein was dissolved in $H_2O$ and the proton relaxation rate constants measured after the solution was equilibrated with 1.0 atm nitrogen gas. The top and bottom axes indicate the electron Larmor frequency and the lower axis indicates the proton Larmor frequency in units of rad/s.

files when baseline rate constants are small. These effects are not expected to be significant in $^2$H or $^{17}$O studies where the nuclei relax primarily by the nuclear electric quadrupole mechanism, and dipole–dipole effects are negligible by comparison.

In the absence of oxygen, as shown in Fig. 1, *C* and *D*, the MRD profiles may be fit with single Lorentzian dispersions that yield protein rotational correlation times of 41 ± 3 ns in $H_2O$ and 51 ± 4 ns in $D_2O$. The difference is proportional to the difference in the viscosities between the two solvents, and the result is in agreement with fluorescence depolarization measurements taken in extremely dilute solutions (Terpetschnig et al., 1995). These simple dispersion curves demonstrate that the broadening often observed in water proton dispersion curves, which has been the subject of considerable discussion, is the result of protein aggregation associated with the high protein concentrations previously required in such studies. In comparison with previous studies, this result represents a considerable simplification and allows a more detailed analysis of the data than was previously reasonable.

As described above, comparison of the data in Fig. 1, *C* and *D*, obtained from deoxygenated $D_2O$ and $H_2O$ solutions, respectively, allows separation of the relative magnitudes of the relaxation contributions from intra- and intermolecular dipolar mechanisms. The experimentally observed relaxation dispersion magnitudes are a function of the correlation time of the motion, which differs in the two solutions because of the difference in viscosities. This dependence can be removed by defining a parameter, $\beta = \Delta R_1/\tau_c$, in which $\Delta R_1$ is the magnitude of the observed dispersion (Denisov and Halle, 1996). If we assume that the water–protein binding equilibrium does not change upon isotopic substitution, the scale parameters for the relaxation dispersions observed in the two solvents are related by the simultaneous equations,

$$\beta(H_2O) = \beta_W(H_2O) + \beta_{WP}(HH), \quad (6)$$

$$\beta(D_2O) = \beta_W(HOD) + \beta'_{WP}(HH), \quad (7)$$

where $\beta_W$ is the relaxation component due to intramolecular dipolar interactions, $\beta_{WP}$ is the intermolecular relaxation component, and the prime indicates a reduction caused by deuteron substitution in the water binding site on the protein. These two equations can be related as

$$\beta(H_2O) = \rho\beta(D_2O), \quad (8)$$

$$\beta_W(H_2O) = \alpha\beta_W(HOD), \quad (9)$$

$$\beta'_{WP}(HH) = \kappa\beta_{WP}(HH), \quad (10)$$

where $\rho$ is the ratio of the observed dispersion scale factors in the two solvents, $\alpha$ is 24.3, the scale factor between H–H and H–D relaxation mechanisms, and $\kappa$ is the reduction in the intermolecular relaxation contribution due to deuterium substitution on the protein near the water binding site. The magnitudes of the observed dispersions are $9.35 \times 10^5$ s$^{-2}$ in $D_20$ and $4.44 \times 10^6$ s$^{-2}$ in $H_2O$. Neglecting the effects of deuteron substitution on the protein ($\kappa = 1$), we find that intermolecular relaxation mechanisms account for 83.9% of the relaxation dispersion in $D_2O$ solution, but only 17.7% in $H_2O$. These values represent the relative magnitudes of the intra- and intermolecular relaxation rate constants, averaged over all of the water binding sites in BSA with exchange times between 40 ns and ~1 ms. Reducing $\kappa$ causes the relative importance of the intermolecular interactions to decrease.

Using this result for the relative intra- and intermolecular relaxation contributions, we may draw a number of additional conclusions by analyzing the magnitude of the observed relaxation dispersions. In $H_2O$, the magnitude of the intramolecular relaxation contribution of protein-bound water molecules is $3.66 \times 10^6\ s^{-2}$. For a single, rigidly bound $H_2O$ molecule rotating with the observed protein rotational correlation time of 41 ns, the factor $\beta$ is $5.5 \times 10^{10}\ s^{-2}$. The proportion of bound water molecules may be calculated from the ratio of the protein and water concentrations, neglecting the equilibrium constant in Eq. 3, to get $2.64 \times 10^{-6}$. If only one water molecule were bound to each protein molecule, the observed relaxation dispersion factor $\beta$ due to the intramolecular0 dipolar interaction would be $1.45 \times 10^5\ s^{-2}$. Comparing this value with the observed intramolecular contribution leads to an estimate of $25 \pm 4$ water binding sites on the protein.

This result is close to previous reports based on different data. Denisov and Halle (1966) previously estimated $N_I$ for BSA as 30–60, whereas triple quantum filtered studies indicated approximately 40 bound waters in this protein. (Baguet, 1996). There are two assumptions made in arriving at the current number. First, we have assumed that the intermolecular relaxation does not change upon isotopic dilution with deuterons. Approximately 10% of the protein protons will exchange for deuterons in $D_2O$, primarily at backbone amide sites. This substitution effectively reduces the intermolecular dipolar interactions. Venu et al. (1997) calculated this reduction for a single water molecule binding site in BPTI and found a rather large decrease of 57% in this contribution to the relaxation. Using this value for $\kappa$ in Eq. 12 decreases the apparent number of binding sites to 16 which is much lower than the previous estimates.

The second assumption concerns the effects of rapid motions of the water molecule when bound to the protein. The bound water may have some orientational freedom in the binding site or binding site protein residues may suffer conformational fluctuations. In either case, such motions will lead to an order parameter, $A^2$, less than unity in Eqs. 4 and 5. The result is an increase in the apparent number of water binding sites. For BPTI, Venu et al. (1997) found one water bound essentially rigidly and three others with average order parameters of approximately 0.68. Combining these numbers leads to an average order parameter of 0.76, which increases the apparent number of water binding sites to 33 if $\kappa = 1$ or 21 if $\kappa = 0.43$. Moreover, since these two corrections have opposite effects on the apparent number of binding sites, incorporating them into the analysis does not significantly change the outcome. Because proton exchange effects appear in the intermolecular component of the relaxation, they do not interfere in this calculation, which is based on the intramolecular component of the dispersion. The relative scaling of $\kappa$ for these sites may be different for the different water sites, but this discussion suggests that the essential result does not change very much.

The separation of the intra- and intermolecular contributions to relaxation made here represents an average over the 25 or more water molecule binding site provided by serum albumin. To the extent that this average approximates well the binding site proton–proton contacts for protein sites in general, this measurement permits writing a general expression for relating the number of bound water molecules in terms of the correlation time and the relaxation dispersion amplitude:

$$N_I = b\,\frac{\Delta R_{1,obs}}{[\text{protein}]\tau_c}, \tag{11}$$

where $\Delta R_{1,obs}$ is the magnitude of the observed relaxation dispersion in $H_2O$, [protein] is the protein concentration in mol/L, $\tau_c$ is the correlation time associated with the dispersion, and $b$ is a constant equal to $8 \times 10^{-10}\ s^2$ mol/l, which includes the average fraction of intramolecular relaxation based on these BSA results. Furthermore, studies carried out in $D_2O$ will be much more sensitive to factors affecting relaxation of the protein protons than will studies in $H_2O$ because of the stronger dependence on intermolecular relaxation mechanisms.

The magnitude of the heteronuclear contribution to the relaxation reflects the binding site proton–proton contacts between the bound water molecule and the protein. Using the factor associated with intermolecular relaxation mechanisms and comparing it to the relaxation contribution expected from a van der Waals proton–proton contact ($r = 2.2$ Å), we may estimate the average number of protein protons in van der Waals contact with the bound water molecules. This calculation is somewhat sensitive to the value of $\kappa$, but the result varies from 1.57 proton–proton van der Waals interactions per bound water molecule when $\kappa = 1$ to 4.2 interactions when $\kappa = 0.4$. Not surprisingly, this latter value is more in line with the results of Venu et al. (1997), who found 11 proton–proton interactions with separation distances of 1.9 to 3.0 Å for a single bound water in BPTI.

In both solvent systems studied, a small high-field dispersion was observed even in deoxygenated solutions. This dispersion may be caused by residual oxygen; however, measurements on water in the absence of protein indicate that our deoxygenation methodology is sufficient for removal of the high-field dispersion caused by dissolved oxygen. Furthermore, the oxygen contribution to the proton relaxation rate constant is independent of the isotope composition. Therefore, the relaxation contribution to the $H_2O$ solution and the HOD solution should be the same if the source is oxygen. However, the contributions are significantly different in amplitude and in nearly the same ratio as the low-field dispersion amplitudes. We, therefore, tentatively attribute this residual high-field dispersion to internal motions involving the protein-bound water molecules or the protein, or both.

## CONCLUSIONS

These experiments demonstrate that the technique of observing the residual protons in $D_2O$ solutions effectively extends the dynamic range of the sample-shuttle HR-MRD technique by as much as two orders of magnitude. This extension increases the applicability of the technique to a wider range of physical conditions and sample compositions. The magnetic field dependence of proton spin-lattice relaxation rate constants in dilute oxygen-free protein solutions has a Lorentzian dispersion at an inflection frequency that is very simply related to the rotational correlation time of the molecule. In the absence of oxygen, there is no evidence of effects due to a distribution of exchange times. The observed rotational correlation times are in agreement with other measurements of rotational mobility, which implies that protein aggregation has been considerable in many previous studies. The spin-lattice relaxation in $H_2O$ solutions is dominated by intramolecular relaxation effects, but also contains a significant contribution from intermolecular relaxation and proton exchange. These ratios are considerably different in $D_2O$ solutions where the intramolecular relaxation mechanism is scaled and the observed relaxation dispersion is dominated by intermolecular effects including direct water–protein proton–proton interactions and the effects of proton exchange. Using this information, we determine that there are $25 \pm 4$ water binding sites on BSA with exchange times in the approximate range between 1 ms and the rotational correlation time of the protein. By analysis of the intermolecular fraction of the relaxation, we estimate that each water binding site has the equivalent of 2–4 van der Waals proton–proton contacts between the protein and the bound water molecule protons. This approach should be general and easily applied to a variety of proteins, which will permit characterization of water–protein dynamics and rotational correlation times at low protein concentrations. Because we have demonstrated that the relaxation dispersion is simply described by a Lorentzian shape, the changes in the relaxation dispersion shape with protein concentration may be used to study protein aggregation.

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## REFERENCES

Abragam, A. 1961. Principles of Nuclear Magnetism. Chap. 8. Clarendon Press, Oxford. 264–353.

Baguet, E., B. E. Chapman, A. M. Torres, P. W. Kuchel. 1996. Determination of the bound water fraction in cells and protein solutions using $^{17}O$-water multiple quantum filtered relaxation analysis. *J. Magn. Reson. Ser. B.* 111:1–8.

Baguet, E., and N. Hennebert. 1999. Characterization by triple-quantum filtered $^{17}O$ NMR of water molecules buried in lysozyme and trapped in a lysozyme-inhibitor complex. *Biophys. Chem.* 77:111–121.

Bryant, R. G. 1978. NMR relaxation studies of solute–solvent interactions. *Ann. Rev. Phys. Chem.* 29:167–188.

Bryant, R. G. 1996. The dynamics of water–protein interactions. *Ann. Rev. Biophys. Biomol. Struct.* 25:29–53.

Denisov, V. P., and B. Halle. 1995. Protein hydration dynamics in aqueous solution: a comparison of bovine pancreatic trypsin inhibitor and ubiquitin by oxygen-17 spn relaxation dispersion. *J. Mol. Biol.* 245:682–697.

Denisov, V. P., and B. Halle. 1996. Protein hydration dynamics in aqueous solution. *Faraday Discuss.* 103:227–244.

Denisov, V. P., and B. Halle. 1998. Thermal denaturation of ribonuclease A characterized by water $^{17}O$ and $^{2}H$ magnetic relaxation dispersion. *Biochemistry.* 37:9595–9604.

Denisov, V. P., and B. Halle. 1996. Protein hydration dynamics in aqueous solution. *Faraday Disc.* 103:227–244.

Denisov, V. P., B. Halle, J. Peters, H. D. Horlein. 1995. Residence times of buried water molecules in bovine pancreatic trypsin inhibitor and its G36S mutant. *Biochemistry.* 34:9046–9051.

Denisov, V. P., B. H. Jonsson, B. Halle. 1999. Hydration of denatured and molten globule proteins. *Nature Struct. Biol.* 6:253–260.

Denisov, V. P., J. Peters, H. D. Horlein, B. Halle. 1996. Using buried water molecules to explore the energy landscape of proteins. *Nat. Struct. Biol.* 3:505–509.

Denisov, V. P., K. Venu, J. Peers, H. D. Horlein, B. Halle. 1997. Orientational disorder and entropy of water in protein cavities. *J. Phys. Chem. B.* 101:9380–9389.

Freed, J. H. 1978. Dynamic effects of pair correlation functions on spin relaxation by diffusion in liquids. II. Finite jumps and independent $T_1$ processes. *J. Chem. Phys.* 94:2843–2847.

Halle, B., T. Anderson, S. Forsen, B. Lindman. 1981. Protein hydration from water oxygen-17 magnetic relaxation. *J. Am. Chem. Soc.* 103: 500–508.

Halle, B., and V. P. Denisov. 1995. A new view of water dynamics in immobilized proteins. *Biophys. J.* 69:242–249.

Halle, B., H. Johannesson, K. Venu. 1998. Model-free analysis of stretched relaxation dispersions. *J. Magn. Reson.* 135:1–13.

Hallenga, K., and S. H. Koenig. 1976. Protein rotational relaxation as studied by solvent $^{1}H$ and $^{2}H$ magnetic relaxation. *Biochemistry.* 15: 4255–4264.

Huang, L.-P., and J. H. Freed. 1975. Dynamic effects of pair correlation functions on spin relaxation by diffusion in liquids. *J. Chem. Phys.* 63:4017–4025.

Kimmich, R. 1990. Dynamic processes in aqueous protein systems. Molecular theory and NMR relaxation. *Makromol. Chem.* 34:237–248.

Kimmich, R., T. Gneiting, I. Kotitschke, G. Schnur. 1990a. Fluctuations, exchange processes, and water diffusion in aqueous protein systems: a study of BSA by diverse NMR techniques. *Biophys. J.* 58:1183–1197.

Kimmich, R., W. Nusser, T. Gneiting. 1990b. Molecular theory for NMR relaxation in protein solutions and tissue. Surface diffusion and free volume analogy. *Colloids Surf.* 45:283–302.

Koenig, S. H. 1995. Classes of hydration sites at protein–water interfaces: the source of contrast in magnetic resonance imaging. *Biophys. J.* 69: 593–603.

Koenig, S. H., K. Hallenga, M. Shporer. 1975. Protein–water interactions studied by solvent $^{1}H$, $^{2}H$, and $^{17}O$ magnetic relaxation. *Proc. Natl. Acad. Sci. USA.* 72:3283–3289.

Koenig, S. H., and R. D. Brown, III. 1991. Field cycling relaxometry of protein solutions and tissue: implications for MRI. *Prog. Nucl. Magn. Reson. Spectrosc.* 22:487–567.

Koenig, S. H., R. D. Brown, III, R. Ugolini. 1993. A unified view of relaxation in protein solutions and tissue including hydration and magnetization transfer. *Magn. Reson. Med.* 19:77–83.

Koenig, S. H., and W. E. Schillinger. 1969. Nuclear magnetic relaxation dispersion in protein solutions. *J. Biol. Chem.* 244:3283–3289.

Langhorst, U., R. Loris, V. P. Denisov, J. Doumen, P. Roose, D. Maes, B. Halle, J. Steyaert. 1999. Dissection of the structural and functional role of a conserved hydration site in RNase T1. *Protein Sci.* 8:722–730.

Terpetschnig, E., H. Szmacinski, H. Malak, J. R. Lakowicz. 1995. Metal–ligand complexes as a new class of long lived fluorophores for protein hydrodynamics. *Biophys. J.* 68:342–350.

Torres, A. M., S. M. Grieve, P. W. Kuchel. 1998. NMR triple-quantum filtered relaxation analysis of $^{17}$O-water in insulin solutions: an insight into the aggregation of insulin and the properties of its bound water. *Biophys. Chem.* 70:1–9.

Venu, K., V. P. Denisov, B. Halle. 1997. Water $^{1}$H magnetic relaxation dispersion in protein solutions. A quantitative assessment of internal hydration, proton exchange, and cross relaxation. *J. Am. Chem. Soc.* 119:3122–3134.

Wagner, S., T. J. R. Dinesen, T. Rayner, R. G. Bryant. 1999. Magnetic relaxation dispersion measurements using a dual magnet system. *J. Magn. Res.* 140:172–178.