DYNAMICAL DEDUCTIONS FROM NUCLEAR MAGNETIC RESONANCE RELAXATION MEASUREMENTS AT THE WATER-PROTEIN INTERFACE

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ABSTRACT Nuclear magnetic resonance (NMR) measurements provide both structural and dynamical information about the molecules in which nuclear resonances are observed. This manuscript addresses NMR relaxation of water protons in protein powder systems. Inclusion of magnetic communication between the water proton spins and protein proton spins leads to a clearer view of water molecule dynamics at the protein surface than has been previously available. We conclude that water molecule motion at the protein surface is somewhat slower than in the solute free solvent, but it is orders of magnitude faster than motions in a rigid ice lattice even in samples hydrated to levels well below what is generally thought to be the full hydration complement of the protein. The NMR relaxation data on lysozyme powders support a model that leaves adsorbed water very fluid at the protein surface with reorientational correlation times for the water shorter than nanoseconds.

An understanding of water-protein interactions is crucial to a detailed understanding of protein structure and catalysis. An important aspect of this interaction involves the solvent motion both in semisolid systems, such as tissues, and in the region immediately adjacent to a solute particle in a solution of a macromolecule. Our concepts of structure, however, draw most heavily from models that are based on crystalline low molecular weight solids. This basically sound strategy has been recently used to address structural aspects of water-peptide interactions (1). The structural information obtained from such systems is easy to visualize because the structure is static, usually geometrically simple, often highly symmetrical, and asthetically pleasing. There is therefore a great temptation to use the language and pictures associated with truly solid structures to describe structures in liquids or in liquids associated with solids. Some time ago Klotz (2) extended such an idea first proposed by Frank and Evans (3), who suggested that water adjacent to a protein be viewed as an "ice-like lattice." This approach conveys a structural picture, to be sure, but, as will be shown, errs by many orders of magnitude in implying the time scale for describing the motion of the oxygen atoms in the water under consideration. The consequences of such sometimes useful analogies involve semantic as well as conceptual problems that may be resolved to some extent by using the somewhat more cumbersome concepts of liquid structure characterization. NMR relaxation measurements provide dynamical information that is readily related to such descriptions.

The underlying ideas that have led to extensive applications of NMR relaxation to the study of surface systems (4–6) are apparent in Eq. 1.

$$1/T_1 = 3/2\gamma^4\hbar^2 I(I+1)[J(\omega) + J(2\omega)], \quad (1)$$

where T_1 is the time constant describing the recovery of magnetization parallel with the static magnetic field; γ, the nuclear magnetogyric ratio; $\hbar$, Planck's constant divided by 2π; I, the nuclear spin and $J(\omega)$, the density of fluctuations in the local fields at the frequency ω. The nature of the dynamical information derived from a measure of T_1 depends on the model used for several parts of the interaction contained in the spectral densities of Eq. 1. There are two major inputs: the source or strength of the field fluctuations, and their time dependence. For NMR relaxation in diamagnetic systems, the proton relaxation rate is usually dominated by dipole-dipole interactions with nearby proton magnetic moments. The motions of the adjacent magnetic moments is most often described statistically by an autocorrelation function. The simplest model, which is most often applied, assumes that the correlation function decays exponentially with a time constant, τ_c, usually called the correlation time. For the study of associated liquids such as water next to a protein surface, it is usually assumed that motion occurs in three dimensions and that translational and rotational motions are coupled. If reorientation of the interproton vectors is isotropic, the longitudinal relaxation rate takes the familiar form (7),

$$1/T_1 = (2/5)\gamma^4\hbar^2[I(I+1)/r^6][(\tau_c/1 + \omega^2\tau_c^2) + (4\tau_c/1 + 4\omega^2\tau_c^2)], \qquad (2)$$

where r is taken to be the interproton distance in the water molecule and the $J(\omega)$ have been evaluated as the Fourier transform of the exponentially decaying autocorrelation function describing reorientation of the interproton vectors that is also assumed to be isotropic. The anisotropic motion case has been treated (8) but has not generally been used in the surface systems because of the increased complication, though Woessner has presented experimental as well as theoretical approaches to the problem (9).

In general care must be taken to include both inter- and intramolecular contributions to relaxation. In associated liquids rotational reorientation is characterized by approximately the same time constant as translational reorientation and the distinction between intramolecular and intermolecular effects is more difficult to make experimentally (4–6, 10). For macromolecule-solvent interactions the situation is complicated further by there being several correlation times to consider: the slow motions of the large molecule and the faster motions of the solvent molecule. The problem is simplified in the work to be summarized here in that the systems studied are in all cases solids in the sense that the protein molecules are rotationally constrained; hence, whole protein molecule rotation makes no contribution to the spectral densities in the relaxation equation. With these assumptions there is direct access to a characterization of liquid motion; that is, knowledge of the interproton distance in the water molecule as well as the constants in Eq. 2 permits direct calculation of the correlation time from a measurement of T_1. A similar development (10) gives the tranverse relaxation rate, T_2^{-1}, as

$$1/T_2 = (1/5)\gamma^4\hbar^2[I(I+1)/r^6][3\tau_c + (5\tau_c/1 + \omega^2\tau_c^2) + (4\tau_c/1 + 4\omega^2\tau_c^2)]. \qquad (3)$$

The temperature dependence of the relaxation rates is usually used to characterize the system and test the relaxation hypothesis. Assuming a simple activation law for the correlation time leads to the temperature dependence represented schematically by the dotted lines in Fig. 1. Eqs. 2 and 3 predict that at the minimum in T_1, $\omega\tau_c = 0.616$ and $T_1/T_2 = 1.6$.

While the basic strategy outlined above is clear for relating the observable proton NMR

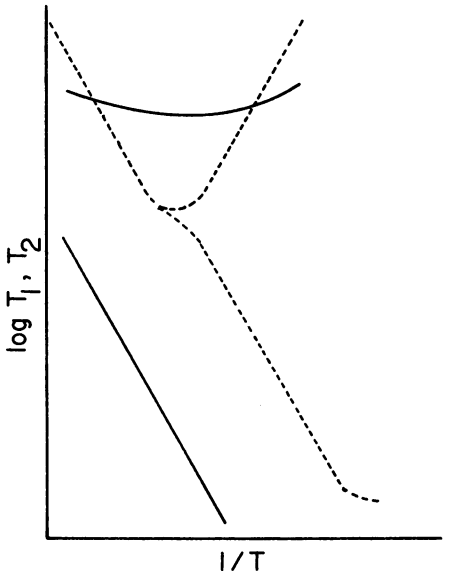

Figure 1 A schematic representation of the temperature dependence of the longitudinal and transverse relaxation times for water protons. The dashed lines are predicted by Eqs. 2 and 3, and the solid lines indicate the dependencies often observed for water on proteins.

relaxation rates to the correlation times for reorientation of the interproton vector in the water molecule, in practice it fails because the theory does not agree with experiments. Experiments on many protein systems may be summarized by the solid line drawn in Fig. 1 (11–15). The data are generally characterized by a value of T_1 at the minimum that is considerably larger than predicted by Eq. 2 and the ratio, T_1/T_2 at the T_1 minimum, is large.

One means to bring experiment closer to theory is to postulate that a distribution of correlation times is appropriate for the water molecule in the interfacial vicinity of the macromolecular surface (16, 17). Some critical subtleties that are involved in the application of this idea in the NMR case have been addressed by Resing (10). The concept does bring the theory more closely into line with experiments for some systems and usually involves a rather broad distribution of correlation times. This approach is undoubtedly of value for some surface systems; however, application in the present case neglects a critical feature of the NMR relaxation, namely, that there is a major contribution to the water relaxation rates from intermolecular effects that causes the assumptions of Eq. 2 to fail.

The assertion that intermolecular or cross-relaxation effects often dominate water proton NMR relaxation in protein systems is well-supported (20–22). The simplest and perhaps the most direct support is the observation that the water proton longitudinal relaxation is not described by a single time constant but by a sum of exponential time constants. The earliest report of this observation in protein systems (23) adopted a model prevalent in the discussions of liquids at surfaces based on the consequences of a slow chemical exchange of water molecules between two environments for the observed water protons, presumably bound and free in some sense. Application of such a chemical exchange model to the water-protein systems led to the requirement that the water molecule lifetimes in the protein associated state be long, on the order of at least tens of milliseconds. This is a remarkably long time

considering the weak interactions involved in the binding phenomenon. In the case of the water-protein systems such as lysozyme powders, crystals or collagen, the exchange model may be largely eliminated based on the observation that the observed relaxation curves are a sensitive function of the experimental details such as rf pulse widths used and isotopic substitution (22, 23). As shown in Fig. 2, the longitudinal NMR relaxation curve is a sensitive function of the rf pulse-widths used: an observation that is inconsistent with a chemical exchange model, but which is predicted by a mathematically similar but physically different process of magnetic exchange.

The time dependence of the proton magnetization in a hydrated protein system may be described by a pair of coupled equations involving three relaxation rates.

$$dM_W/dt = -(R_{1W} + R_T)M_W + R_T M_P, \tag{4}$$

$$dM_P/dt = (R_{1P} + R_T/F)M_P + R_T M_W/F, \tag{5}$$

where R_{1W} is the inherent water proton relaxation rate, R_{1P}, the protein proton relaxation rate, R_T, the transfer rate between the two spin populations, and F, the ratio of the number of protein protons to the number of water protons. M_W and M_P are the water and protein normalized, reduced magnetizations (22):

$$M_i(\tau) = [S_i(\infty) - S_i(\tau)]/nS_i(\infty), \tag{6}$$

where S is the free induction decay amplitude for the ith component after the second pulse of a 180-τ-90 experiment ($n = 2$) or after a 90-τ-90 experiment ($n = 1$). The solution of these equations is presented by Edzes and Samulski (22) if the substitutions k_w for R_T and k_m for R_T/F are made to achieve their notation. The roots of these equations correspond to the fast and slow components of the relaxation curves shown in Fig. 2. Several points are important: (*a*) The time constants that characterize the experimental decay are mixtures of the rate constants that appear in Eqs. 4 and 5. (*b*) The appearance of the relaxation curves is significantly pulse-width dependent, but the limiting slopes, $R_{1\text{fast}}$ and $R_{1\text{slow}}$, are not. (*c*) In

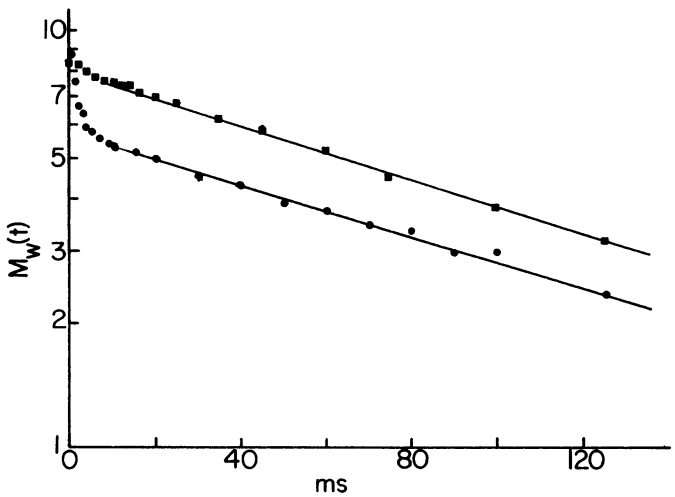

Figure 2 Water proton longitudinal magnetization in lysozyme powder containing 0.17 g water per gram lysozyme at 253 K measured at 57.5 MHz. The circles and boxes represent different strength rf pulses corresponding to 55 and 8.6 ms 180° pulses respectively.

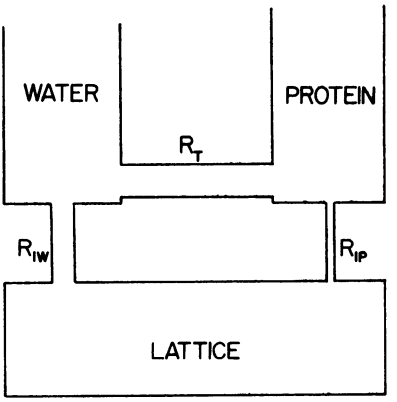

Figure 3 A schematic representation of the coupled relaxation problem for water-protein systems.

wet systems it is often not possible to observe the nonexponential decay because of the behavior predicted at large and small F; however, it has been demonstrated that cross-relaxation effects are very important even in the limit of a protein solution (24). (*d*) Interpretation of either $R_{1\text{fast}}$ or $T_{1\text{slow}}$ in terms of Eq. 2 is incorrect.

The situation may be visualized clearly by analogy to a fluid draining freely from the coupled reservoirs shown in Fig. 3. The pulse width dependence that is so apparent in Fig. 2 comes about because the length of the rf pulse determines the amount of magnetization delivered to each reservoir. Since the water signal is narrow and usually close to the carrier frequency, the water system is usually strongly affected by even a weak rf pulse; i.e. at $t = 0$, the water reservoir is filled to a high level. The protein spectrum is broad because the dipole-dipole interaction is unaveraged in the solid. A long weak pulse, which has a narrow spectral width, does not affect a significant fraction of the spins; i.e., the protein reservoir is filled to a low level relative to the water proton reservoir, depending of course also on F. If transfer between the water and protein system decreases rapidly while the protein reservoir actually fills for a time, only to drain through either the R_{1P} path or back through the water system path, R_T and R_{1W}.

The water proton relaxation data may be completely described by R_{1P}, R_{1W}, R_T, F, $M_W(0)$, and $M_P(0)$. A set of measurements at different pulse widths to vary $M_W(0)$ and $M_P(0)$ provides a means of extracting the basic relaxation rates. R_{1P} is small, even set to zero in an earlier treatment (21), so that the precision obtainable by extracting it together with the other two rates is poor. An alternative procedure is to measure R_{1P} directly in a protein system hydrated to the desired level with D_20, then extract R_{1W} and R_T directly from the nonexponential water proton relaxation curves on similar samples hydrated with H_2O. It is important to determine the temperature dependence of each relaxation rate contributing to the observed decay because Eq. 2, for example, does not yield a single value of the correlation time for particular value of T_1. It is also important to know how the several contributions affect $R_{1\text{slow}}$, because it is this parameter that is most often found in the literature of water adsorbed on surfaces including protein systems. The essence of the approach, then, is to identify R_{1W} with a relaxation rate dominated by water-proton interactions that is described at least approximately by Eq. 2. The separation of contributions leads to a value of R_{1W} that adequately accounts for the water proton-protein intermolecular interaction. R_{1W} still contains contributions from both intra- and intermolecular water-water proton interactions. Since rotational and translational motions are characterized by similar jump times, this rate

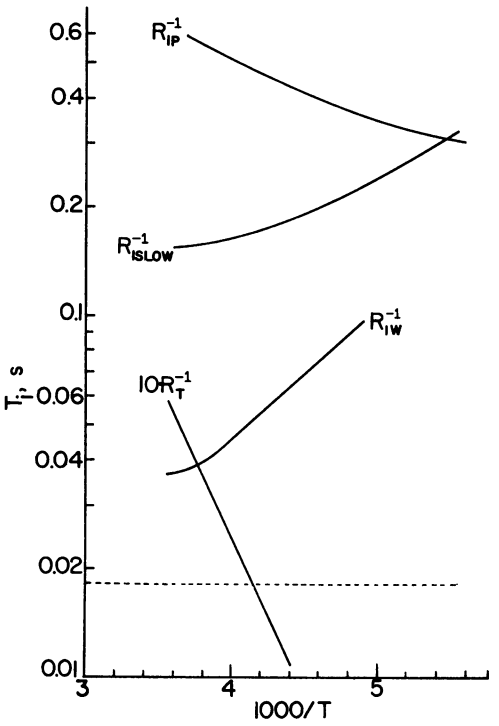

Figure 4 The temperature dependence of the several contributions to the water proton NMR relaxation at 57.5 MHz in a lysozyme sample hydrated to 0.17 g water per gram lysozyme (see reference 24). The dotted line represents the maximum value possible for the T_1 at the minimum calculated assuming only intramolecular dipole-dipole contributions. The value decreases to 10 ms if one uses the second moment for ice, which includes intermolecular contributions as well.

may be analyzed for a quantitative assessment of the water reorientation rate at the protein surface. Such an approach has been used by Bryant and Shirley (25) on data collected at 57.5MHz for protons. The results are represented schematically in Fig. 4 for lysozyme powder samples at 0.17 g water per gram lysozyme.

There are a number of interesting features of the relaxation summarized in Fig. 4, but this discussion will focus on only those aspects that bear directly on a statement about water molecule motion in the system. Inspection of Fig. 4 shows clearly that the directly observable, slowly decaying component of the water proton magnetization, R_{1slow}, which is most often reported, has a temperature dependence that results from changes in all of the more fundamental relaxation rates that contribute to it. The least dramatic changes are observed for R_{1P}. The present data for lysozyme protons agree well with similar measurements reported by Andrew and co-workers for lysozyme that is dry (26). The T_1 values of the protein protons are long even though they are part of a solid system and fall on the high temperature side of the T_1 minimum. The cause of this apparently anomolous finding has been clearly identified with a strong coupling of the protein protons to raidly rotating methyl groups in the solid protein; hence, relaxation of the protein spin system itself is complicated by internal cross-relaxation or spin diffusion to methyl relaxation sinks (26). Qualitatively, the addition of water that can move rapidly is equivalent to adding more rapidly rotating protons and thus provides additional relaxation paths for the protein protons. The lack of a large temperature dependence of the intrinsic protein proton relaxation times demonstrates that there are no

dramatic changes in the intrinsic protein proton relaxation rates that may be directly responsible for the minimum observed in the water longitudinal relaxation times. Therefore, motion of water molecules dominates the modulation of magnetic interactions that leads to relaxation of protons in hydrated lysozyme powders.

In contrast to the protein protons, R_{1W}^{-1} falls on the low temperature side of a minimum for most of the temperature range studied. To the extent that the effects of anisotropic motion may be neglected, this demonstrates that the water molecule motion at the dry protein surface is considerably slower than it would be in a sample of pure water. The values of R_{1W}^{-1} shown in Fig. 4 appear to approach a minimum at 57.5 MHz close to room temperature in this sample hydrated to a level of 0.16 g water per gram of lysozyme. Hence the correlation time associated with an isotropic model would be close to a nanosecond at this point. This correlation time is very short compared with that expected for any sort of solid structure. In addition, it must be appreciated that this sample is not completely hydrated; the normal complement of nonfreezable water associated with lysozyme is approximately twice the water content of the sample used (13, 27). There is good evidence that increasing water content leads to increased motion in the water adsorbed (21); therefore, the present case overestimates the correlation time for the water present in a fully hydrated protein sample. We may conclude then that the correlation time for the reorientation of the interproton vector of the water molecule changes by less than a factor of 100 in going from the liquid to an adsorbed state where the protein is constrained not to rotate. It is interesting to note that this correlation time for the water is considerably shorter than the correlation times for the rotation of the macromolecule as a whole when it is dissolved, i.e., 10^{-8} s or longer. These experiments on solid protein materials therefore strongly support conclusions deduced from measurements on solutions that, in the solvation domain of the protein in solution, there are very few if any water molecules rotating with the correlation time of the protein molecule (24, 28–30). We may not rule out 1% or so that may be nonexchangeable and rigidly a part of the protein structure.

Several aspects of the water relaxation and motion must be addressed further before a quantitative understanding of the water-protein dynamics is claimed. (*a*) Although we can eliminate very broad distributions of correlation times spanning three or four orders of magnitude for water molecule motion at the protein surface based on the present experiments, the possible existence of a much narrower distribution has not been eliminated. (*b*) Although the anisotropy of the water motion in the present system has not led to dipolar proton splittings that are sometimes observed in systems that have long range order, the extent to which there is an anisotropy in the water motion has not been estimated. Additional experiments with deuterium are in progress to assess its importance quantitatively. (*c*) While the relaxation rate R_{1W} is much closer to the transverse relaxation rate than $R_{1\text{slow}}$, the depression of the transverse relaxation time relative to the longitudinal relaxation time is not quantitatively understood. Nevertheless, the conclusion that the water motion at the protein interface is rapid, slowed at most by a factor of 100 relative to the solute free solvent, appears to be unavoidable.

The very fluid nature of water at the protein surface deduced from the present experiments is supported by a variety of experiments. Based on dielectric and thermodynamic measurements Hoeve and co-workers, for example, conclude that a continuous fluid model describes water adsorbed on collagen (31, 32), although certain details have been criticized (33). NMR among other methods has indicated that water molecule motion is fast even on rigid surfaces such as glass (34). Nevertheless, the NMR transverse relaxation measured in protein systems

remains a problem. Several laboratories have suggested that at least two types of water are required to explain the collagen data, for example (35, 38). Since many factors may affect the transverse relaxation rate that do not affect the longitudinal relaxation rate, interpretation of T_2 values is more hazardous. Woessner has pointed out again the possible importance of anisotropic motions that may not be simply resolvable (39) but this problem has not been addressed in all applications of T_2 data. Lauterbur and co-workers have published deuterium spectra that demonstrate a small residual anisotropy in the solvent motion in protein crystals (40). Indeed, some anisotropy is expected based on the x-ray results for small proteins which indicate that certain water molecule positions are reproducibly occupied (41).

In summary, the conclusion that the water molecule motion at the protein surface is fast is sound; however, a rigorous quantitative understanding of all aspects of the water relaxation in this environment is not presently at hand.

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DISCUSSION

Session Chairman: V. Adrian Parsegian *Scribe*: Thomas A. Gerken

PARSEGIAN: We have an extended written comment from Henry Resing.

RESING: Professor Bryant, as I see it, your aim is to determine the correlation time of water adsorbed on lysozyme powder with the ultimate aim of understanding the lifetime of the protein hydration envelope in solution. You wish to know if this hydration envelope is "ice-like" in structure or lifetime. From the data you present you conclude: (*a*) that there are no adsorbed molecules with a correlation time as long as the rotational correlation time of the lysozyme molecule in solution; (*b*) that the adsorbed water is not >100 times more viscous than bulk water; and (*c*) that the adsorbed water does not have an "ice-like" structure. I dispute these points, both on the basis of your data and on other grounds. Nevertheless I have no dispute with your experimental methodology and I have confidence that valuable estimates of water molecule mobility of the water protein interface will emerge from your studies. It is clear that cross-relation of the water protons with the protons of the immobile protein substrate will make the determination of the intrinsic NMR relaxation times less direct and much more time consuming than for substrates containing no protons. Fortunately, the intrinsic T_1 is still determinable. Thus, for general enlightenment, I wish to