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

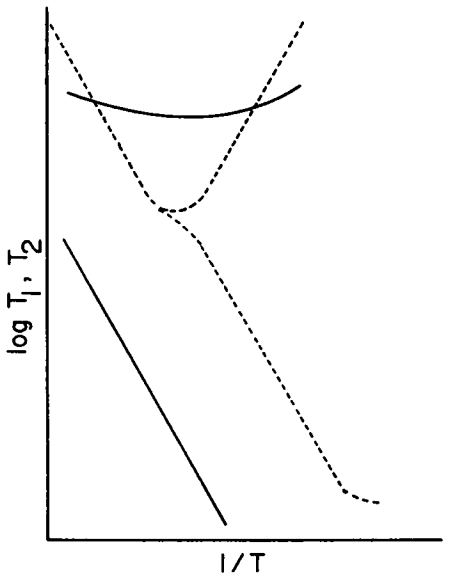

Figure 1 A schematic representation of the temperature dependence of the longitudinal and transverse relaxation times for water protons. The dashed lines are predicted by Eqs. 2 and 3, and the solid lines indicate the dependencies often observed for water on proteins.

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_{1fast} and R_{1slow}, are not. (*c*) In

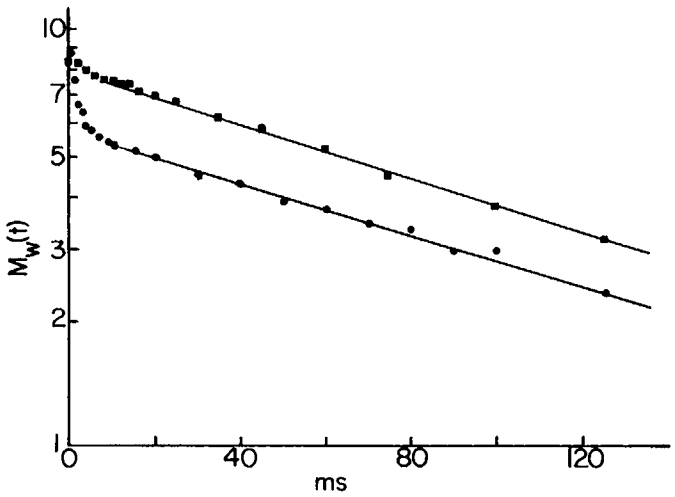

Figure 2 Water proton longitudinal magnetization in lysozyme powder containing 0.17 g water per gram lysozyme at 253 K measured at 57.5 MHz. The circles and boxes represent different strength rf pulses corresponding to 55 and 8.6 ms 180° pulses respectively.

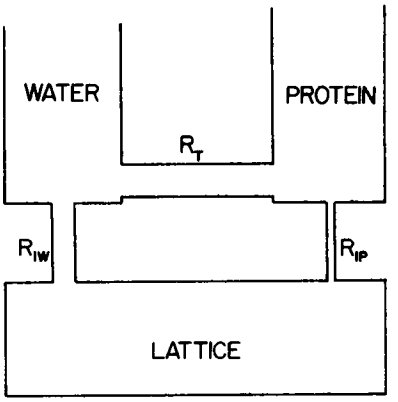

Figure 3 A schematic representation of the coupled relaxation problem for water-protein systems.

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_{1fast} or T_{1slow} 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_{1slow}, 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

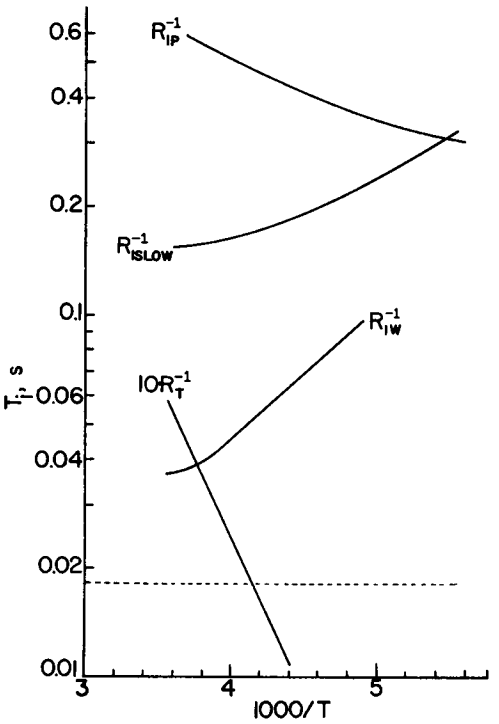

Figure 4 The temperature dependence of the several contributions to the water proton NMR relaxation at 57.5 MHz in a lysozyme sample hydrated to 0.17 g water per gram lysozyme (see reference 24). The dotted line represents the maximum value possible for the T_1 at the minimum calculated assuming only 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

pose some questions concerning your treatment of the experimental data, and to estimate errors which might arise in the data and its treatment.

Edzes and Samulski (1) find relaxation and exchange parameters comparable to yours in their study of H_2O on collagen. From a very rich data suite they find a statistical error for R_{1W} of ~15% at room temperature. In Fig. 4 *a* (which I have modified from your Fig. 4) I attribute an error band to your data based on the result of Edzes and Samulski and on the law for the propagation of errors. Since you find magnetic exchange to be fast, i.e. $R_T >> R_{1W}$, R_{1P}, the fast exchange approximation should be valid (1, 2).

$$R_{1W} = P_W^{-1} R_{1\text{slow}} - (1 - P_W) R_{1P}. \qquad \text{(A)}$$

Eq. (A), when used to estimate errors, suggests that the error should increase as $R_{1\text{slow}}$ and R_{1P} become closer in value; hence the increasing width of the error band of lower temperatures in Fig. 4 *a*.

The conditions for fast exchange appear to be met. There is rapid flow of magnetization betwen the reservoirs of Fig. 3, as the value of R_T indicates, and Eq. (A) should apply. But using the observed $R_{1\text{slow}}$, R_{1P}, and P_W in Eq. (A) yields a value for R_{1W} of 52 ms at $10^3/T = 4.5$, rather than the value of 70 ms you plot (see point in Fig. 4 *a*). Why do you find Eq. (A) to be invalid?

Much of your reasoning on the value of the correlation time for the adsorbed water at room temperature hinges on the interpretation that there is a minimum in R_{1W}^{-1} there. In light of the discussion of errors above, how firm is this conclusion? The location of the minimum (on the temperature scale of R_{1W}^{-1}, i.e., of the intrinsic T_1 of the adsorbed water, is quite crucial in using the theory of Edzes and Samulski (1). Loosely, for $\tau_W > \omega^{-1}$ ($\omega = 2\pi \times 60 \times 10^6\ s^{-1}$ in your work) cross-relaxation is possible, while for $\tau_W < \omega^{-1}$ cross-relaxation is not possible. Thus, on sweeping through the temperature range including the T_1 minimum one should go from a condition of no exchange at high temperature to one of effective exchange at lower temperature. If only the water magnetization is observed (observation at $t >> T_{2P}$) one should pass from exponential relaxation to relaxation as the sum of two exponentials; there is fragmentary evidence of this phenomenon (3). Evidently you are well into the region of $\tau_W > \omega^{-1}$ in these lysozyme studies.

You determine T_{1P} from T_1 measurements of protons of the protein hydrated with D_2O. I believe that the protons in the protein surface will relax due to the relative motion of the adsorbed-molecule protons and that this should change T_{1P} from that observed in the D_2O case. Have you considered such an effect?

In Fig. 4 *a* there is also juxtaposed to your data a plot of your Eq. 2 for the relaxation time T_{1W} with a unique correlation time at each temperature. For the plot I used an intramolecular proton-proton dipolar, second moment of $1.6 \times 10^{10}\ s^{-2}$ and an intermolecular second moment of $0.6 \times 10^{10}\ s^{-2}$. The intermolecular second moment is

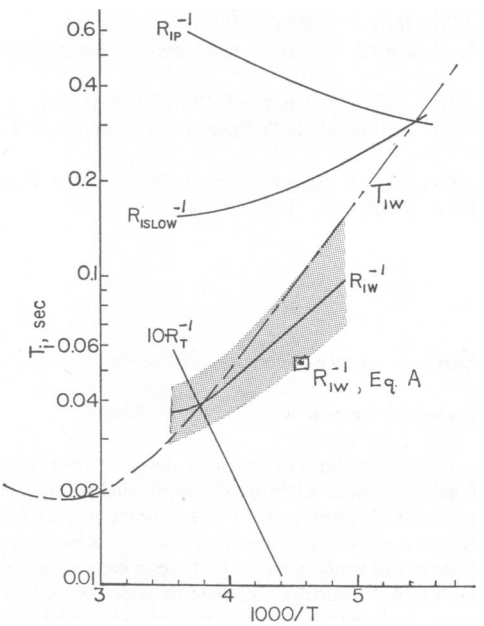

Figure 4 *a* Overlay on Fig. 4 of Bryant's text. Relaxation-times and transfer time vs. reciprocal temperature for water on lysozyme. The additions are a theoretical relaxation time T_{1W} (dashed line), an error band (shaded area) on the reported R_{1W}^{-1}, and one point of R_{1W}^{-1} reworked (square).

comparable to those estimated for other adsorbed water systems (4); for the intermolecular contribution a correlation time of $\tau/2$ was used. As a function of temperature

$$\tau = 5 \times 10^{-11} \exp(2700/RT) \qquad \text{(B)}$$

where R is in cal/mol deg). The parameters were chosen to pass the curve through the point at which $R_{1P} = R_{1slow} = R_{1W}$ (the last equality flows from Eq. [A]), and to keep the plot roughly within the error band. I assert that the fit uses the least number of assumptions and free parameters and is therefore, in our present state of knowledge, the best fit to the data (Occum's razor). Thus, τ at room temperature is about 5×10^{-9}s; the adsorbed water is ~4,000 times as viscous as bulk water (rather than 100 times); and the mobility of the adsorbed water is ~ half way between that of water at room temperature and the proton mobility in ice at 273 K. Further, τ is getting quite close to that found for proteins in solution, $\sim 3 \times 10^{-8}$s (5).

On the other hand, note that the minimum value of T_{1W} (see Fig. 4 *a*), ~19 ms, is almost half of the least value observed for R_{1W}^{-1}, as shown in your Fig. 4. If, as you assert, that least observed value is taken as a true T_1 minimum value, then some adjustments in the theory are necessary, and the candidates are perhaps anisotropic motion (no evidence yet), or perhaps a distribution of correlation times. In comparison with similar adsorption systems involving proton free substrates (6), I judge that a log-normal distribution in τ with full width at half maximum of ~1.2 orders of magnitude in τ will raise the theoretical minimum to that observed. This strategy will unfortunately allow a considerable probability density of τ at τ values greater than or equal to the protein rotational correlation time, contrary to what you believe.

Finally you imply a contradiction between "ice-like" structure and fast molecular motion. There are, of course, ordered solids in which molecular or atomic motion is fast: platinum hydrides, super-ionic conductors, and hydrated zeolites. In zeolite-A, where water molecule motion is fast (7), the water molecule positions are beginning to show up in x-ray studies (8).[1] In clathrate hydrates, such as tetrahydrofuron hydrate, THF.17 H_2O, the rotational correlation time for the water molecules approaches 5×10^{-8}s and that for diffusion 2×10^{-7}s at the melting point of the clathrate. I don't know if there are theoretical grounds for claiming that fast motion implies lack of order. Certainly, NMR relaxation tells us about the lifetime of a local structure, but I feel it tells very little about long, or even short range order. In your mind, is an "ice-like" or clathrate hydration sphere for proteins clearly forbidden?

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BRYANT: I appreciate Dr. Resing's comments, as he has made substantial contributions to our understanding of NMR relaxation at surfaces. There is no question that the analysis of sums of exponentials may be difficult. While our present spectrometer, which is better than the one used to collect the data on which this discussion is based, gives us precision of a per cent in amplitude vs time, the errors in T_{1S} or T_{1F} are easily several percent. I do not believe we may presently measure T_{1W} to within an error of 20%. Certainly Fig. 4 is not any better than that, and there we may have underestimated R_{1W} or overestimated R_{1W}^{-1}.

Now to answer Dr. Resing's question about a failure of the equation $R_{1W} = P_W^{-1}[R_{1slow} - (1 - P_W)R_{1P}]$, which is predicted by $R_{1T} >> R_{1W} = R_{1P}$, since we find clearly that $R_T >> R_{1W} >> R_{1P}$, there is a rapid mixing of magnetization and spin lattice rates. It is possible then to write, $R_{1S} = P_W R_{1W} + P_P R_{1P} = P_W R_{1W} + (1 - P_W)R_{1P}$, which when solved for R_{1W} gives Dr. Resing's equation. The difficulty is now to define P_W and P_P in terms of accurately known quantities. If the spin systems may be assumed to be in equilibrium within themselves, then the P_i are simply ratios of populations or concentrations of protons in each system. As motions slow down at low temperature, the distinction between spin systems may become difficult to make and the model will fail. Nevertheless, it is interesting and informative to approach this question from several limiting cases. If we assume R_T is very large

[1] L. B. McCuster and K. Seff. 1978. Private communication.

compared to R_{1W} and R_{1P}, and that R_{1P} is zero, it is easy to set limits on R_{1W} based on the directly measured R_{1S}. This situation corresponds to connecting the two reservoirs of Fig. 3 by an arbitrarily large pipe and shutting off the R_{1S} connection to the lattice. Then all magnetization drains through the R_{1W} path, and R_{1W} is related directly to R_{1S} and F, the ratio of the number of protein protons to water protons by the relation, $R_{1W} = R_{1S}(1 + F)$. In the present case, F is ~3.5 so that R for this limit R_{1W} is 4.5 times R_{1S} or T_{1W} is 4.5 times shorter than T_{1S}. That is well below our line in Fig. 4. R_{1P} is measured and is small compared to R_{1W}, which is clear even from the data in Fig. 2. Since the inequalities associated with this model are reasonably well satisfied, the R_{1W}^{-1} curve shown in Fig. 4 is perhaps an underestimate of R_{1W}. The problem has to do with the propogation of errors and the way that the R_{1W}^{-1} curve was extracted from the data. With the newer data now available we expect to resolve the discrepancy. In summary we have no evidence that the equation Dr. Resing suggests fails.

Dr. Resing also asked, "How firm is the conclusion of a minimum in R_{1W}?" The conclusion that R_{1W} passes through a minimum is well supported by both earlier and more recent data as well as the qualitative arguments about the relationship between R_{1W} and R_{1S} made above.

Another question was that based on Edzes and Samulski's work, suggesting that cross-relaxation is expected for $\tau_\omega > \omega_0^{-1}$ but not for $\tau_\omega < \omega^{-1}$. Thus, cross-relaxation should go from a condition of no cross-relaxation exchange at high temperature to one of efficient exchange at low temperature. We do not have data that support this expectation. I do not have any confidence in the arguments which lead to the prediction, either. We have made no attempt to interpret R_T which in the present phenomonological model characterizes the rate of spin transfer between a pair of spin ensembles. The theories of which I am aware are based on relaxation in an ensemble of spin pairs. I am not confident of the extrapolation. I should comment in passing that cross-relaxation is clearly observed even in solutions of chloroform in benzene—a nonviscous liquid limit if you like—while cross-relaxation is also well known in solids, indeed even within the protein proton spin system itself.

With respect to Dr. Resing's proposed fit to the R_{1W} curve, I have no argument with fitting R_{1W} to an equation of the form of Eq. (2), but that effort does neglect the effects of motional anisotropy. We have not done that yet because of our lack of confidence in the precision of R_{1W}. Even taking his fit, which gives a value τ_{H_2O} at room temperature of 5 nsec, I suggest that bringing the water content up to saturation, i.e. twice as much water, will increase the motion at the surface substantially so that τ_{H_2O} at room temperature will be on the order of 10^{-10} s. That is two orders of magnitude faster than τ_{ROT} for proteins. My point is that water at the protein surface, the great bulk of it anyway, moves about faster by a large margin than the protein rotates. Whether the viscosity is a 100 or 500 times that for water as a pure solvent is not worth arguing over at present partly because interpretation of τ_c for H_2O in terms of a simple viscosity is uncertain.

I have two comments about Dr. Resing's concern for a distribution of correlation time. First, I should remind you that in the powder samples studied, there is no protein rotation, and the system is very dry relative to a solution. Thus the dynamics deduced must represent a slow motion bound on the situation in a solution. Secondly, the data analysis of Koenig and collaborators on the frequency dependence of ^{17}O, ^2H and ^1H relaxation times in aqueous protein solutions suggests that a significant number of immobilized water molecules is inconsistent with their data. In the present case, if all the assumptions made by Dr. Resing are correct—in particular that the motion is largely independent of H_2O concentration, then our data cannot describe his assertion. We are presently analyzing data obtained at much higher water contents and this data will undoubtedly be able to address the question more definitely. I think that it is very unlikely that we will be able conclusively to support the view that a substantial fraction of the water at the protein surface is moving with $\tau_c \sim \tau_{ROT}$ for the protein.

As for Dr. Resing's last question, the relation between the motion of water and the structure is often clouded. I do not see a contradiction yet between the x-ray results and the NMR results. As I understand the scattering experiment, reproducible occupancy of particular oxygen atom positions is all that is required to produce density ascribable to water. Since the x-ray scattering event is very fast compared to the motional times we are talking about, the water can jump about rapidly, as long as some positions on the protein are reproducibly occupied.

The limit, however, that I think is inconsistent with the NMR data is the idea that a protein carries around with it a sheath of water in the same sense that a metal ion does, that is, with a well-defined first coordination sphere where the lifetime is long compared with rotation and translational jumps of the ion. My problem with "ice-like" is perhaps semantic. "Ice-like" conveys to me a rigid structure for water next to a protein for which I have no evidence since the correlation times we find are essentially those for a fluid.

KUNTZ: Bob, I don't disagree at all with your picture of a very fluid hydration shell. However, let's explore what kind of limits you can place on the slower processes going on. The fact that the T_1 does not go all the way down to the expected limit suggests that there are some other motions, but as you say maybe only within a factor of ten. On the other hand the fact that the transverse relaxation time (T_2) in water-protein systems is perhaps fiftyfold different from what you expect at your T_1 minimum suggests that there are some other events occurring, maybe not well characterized physical motions but events of some kind that are much slower than the nanosecond, or faster, time scale of which you have spoken. What are your feelings about this?

BRYANT: We don't understand transverse relaxation in detail. Since T_2 relaxation is so much more efficient than

longitudinal relaxation, this clearly implies that there is a slow process or a slow modulation of a magnetic interaction. It isn't clear that it must come from slow water molecule tumbling or the mixing of water molecules, some of which move very slowly. I think the best hypothesis at present is that this depression of transverse relaxation times comes about from a contribution to transverse relaxation from a small component of motional anisotropy in the water molecule but that is a little bit difficult to prove beyond a shadow of doubt. We are trying to do that, and we have seen in systems like this using deuteron magnetic resonance, quadrupole doublet spectra of water on cellulose, which clearly demonstrate an anisotropy. In the globular protein systems so far, with one exception, the line-widths for deuterium become broad before we resolve clearly the quadrupole splitting. I think it is quite possible that the slow process has to do with fast motions. That is, it may take a lot longer for the whole anisotropy to be completely averaged than it does for the molecule to wiggle. So for a water orientation to average over a sphere may take a lot longer than for it to hop about on the surface of a protein molecule. We can't prove this, but that would account for the depression transverse relaxation.

KUNTZ: Do you feel that the anisotropy would then reflect something of the "ice-like" model: that some residual structure has to be averaged over a far slower time scale than that in which the hopping motion of the water molecules is seen?

BRYANT: I am not sure what I can say to that.

FINNEY: What is the form of your sample? You are dealing with a hydrated powder, but do you have any real idea about where the water is being absorbed? How intimately is it associated with the protein molecular surfaces? I ask this partly in connection with some work of Ian Golton, David Davis, and myself which involves, in addition to NMR, infrared and differential scanning calorimetry of dehydrated films that are homogeneous uniform glasses. From neutron scattering we have a handle on the dispersion of the lysozyme molecules in the glass samples and how the water goes in to swell the system with increasing hydration. In such a condensed concentrated glass there are, in contrast to the crystal, a variety of close contacts. In this system we observe the time behavior of the water protons in positions where several protein surfaces meet. We obtain similar coorelation times to your powder samples. Is this equivalent to something you are looking at and, if so, how do you think in fact it will extrapolate to a state of solution where these confining surfaces are removed?

BRYANT: No, I don't know precisely where the water molecules go when you hydrate a protein through the gas phase. The proteins are obviously to some extent in contact, but I am not sure if any of us knows whether or not there are excluded surface regions. The picture you draw is not unreasonable and indeed the situation may change somewhat as you dissolve the protein and/or transfer it directly to solution. However, I am not aware of any solution data in contradiction to the essence of the conclusion that the great bulk of motion at the surface of the protein is very fast, particularly compared to the rotation of the protein. By doing the experiment as we have done it, we eliminate the possibility of free rotation of the protein, which is important in understanding the physics of the situation.

MINTON: One of your major conclusions is that you can rule out a broad distribution of correlation times. You present a model which is able to account for your data without invoking a broad distribution of correlation time. I don't think these two are exactly identical. If you want to rule out the broad distribution, you would have to show in addition that a model based on a combination of a broad distribution with the spin-exchange could not account for your data. If you could do this with one relaxation time plus spin-exchange, a more complicated model would probably do as well by just manipulating parameters.

BRYANT: The situation is roughly as follows: After accounting for cross relaxation, which contributes in a major way to the observable R_{1S}, the rates derived from the model for the intrinsic water relaxation, R_{1W} in our present notion, are much faster than the R_{1S}. If you wish to apply an analysis to R_{1W} now based on Eq. (2), a distribution of correlation times may still be required. However, the R_{1W}^{-1} is much closer to the expected values for T_1 at the minimum. Therefore a distribution of correlation times which might be invoked must be much narrower than has been presumed previously based on a now clearly incorrect analysis of R_{1S}. Referring to Fig. 1, the effect of properly accounting for the cross-relaxation is to move the solid line down significantly toward the dashed curve so that the discrepancy is only about a factor of two rather than a factor of ten or more. Thus, application of a broad distribution of correlation times to the derived R_{1W}^{-1} that would account for an analysis of R_{1S}^{-1} is inconsistent with the values of R_{1W}. To account for a factor of two rather than ten or more, a much narrower distribution is required.

LLINÁS: My question has to do with the accuracy of your T_1 measurements. You can get very different T_1's depending on the method you use—inversion recovery or saturation recovery or partial angle recovery. How do your results agree among themselves?

BRYANT: Your point is well taken. We have measured the basic rates in several ways: 180-τ-90, 90-τ-90 or using

both a soft 180° and a hard 180° pulse and looking at both the water protons and the protein protons. The several approaches yield the same relaxation parameters. You may wonder whether we completely irradiate the solid spin system, but if the solid spin system achieves thermal equilibrium with itself in the time of the experiment, (an important assumption of the analysis we have used) an imperfect 180° pulse will not matter. The fact that we get the same rates of several approaches supports this assumption.