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^1H Nuclear Magnetic Resonance Relaxation of Water on Lysozyme Powders

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Abstract: Nuclear magnetic resonance transverse and longitudinal relaxation rates are reported at 30 MHz for water protons adsorbed on lysozyme powders from the gas phase as a function of temperature and water content. The free induction decay amplitude accounts for all of the water protons in the sample over the range of water concentrations studies. Two types of model are considered to interpret the transverse NMR relaxation data. It is shown that a multisite fast exchange model which assumes water concentration independent protein binding sites is inconsistent with the data. A crude model that includes the possibility that water molecule motion at all sites may be influenced by subsequent addition of water does account for the observations. A chemical exchange model and a cross-relaxation model are considered as sources of the nonexponential decay observed in the longitudinal relaxation data throughout the temperature and concentration range studied. It is shown that when cross-relaxation between protein protons and water protons is included, long water molecule residence times at protein sites are not required and that the usual approaches to the analysis of water relaxation at surfaces must be altered. The data demonstrate that the rate of water molecule motion decreases with decreasing water content, even though some distribution of motional correlation times may be appropriate. The water in the immediate vicinity of the protein surface appears to be best characterized as a viscous liquid but not as a solid.

An understanding of the interactions between water and proteins is central to an understanding of the many relationships between protein structure, catalysis, activation, function, and decomposition. In spite of the importance of water in maintaining the structural integrity of proteins, the details of the water-protein interaction are poorly understood. Even in the relatively dry state, the water-protein interaction appears to be of practical importance for problems such as the storage of foods, organism drought survival, cold or heat hardiness in plants, and the mechanical properties of natural products derived from primarily protein sources. The present study was undertaken to investigate the water-protein interaction in dry protein systems. For physical reasons we have chosen to examine lyophilized lysozyme powders rehydrated through the gas phase.

Most previous work relevant to the present study has been summarized in reviews.[1-3] Papers of particular importance to protein powder systems were contributed by the laboratories

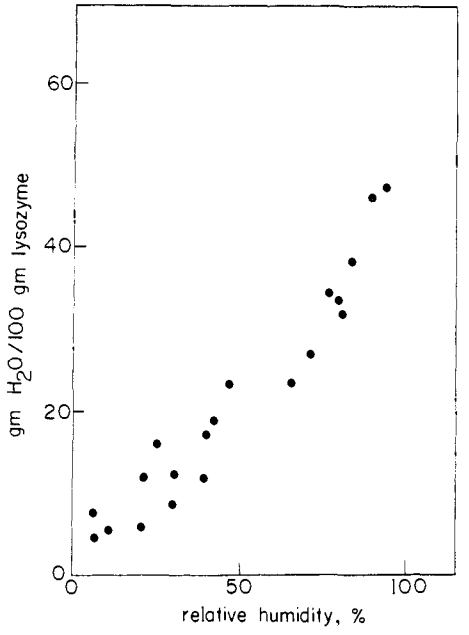

Figure 1. Water content of rehydrated lysozyme powders determined by Karl Fischer titration as a function of the relative humidity over water-glycerol mixtures.

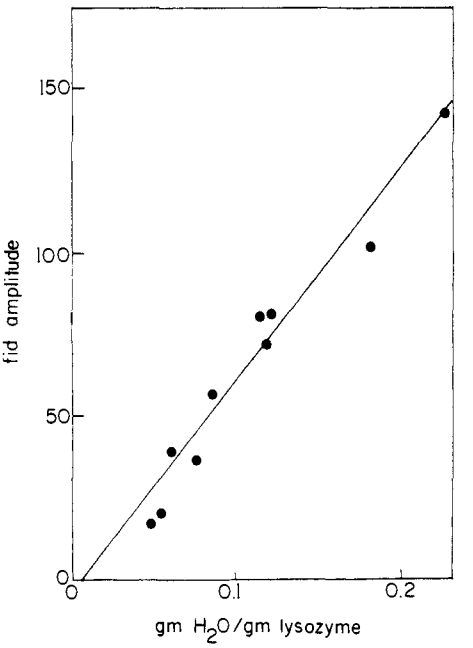

Figure 2. ^1H NMR free induction decay amplitude for water adsorbed on lysozyme powders as a function of water content at room temperature.

of Brey[4,5] and Danyluk.[6] These investigators reported a dramatic decrease in the water proton line width as water content of a protein system is increased and they attempted to measure both longitudinal and transverse NMR relaxation rates using conventional continuous wave (CW) methods. Kruger reported a similar study on lysozyme crystals using time domain spectroscopy which has considerable advantage over the CW approaches for obtaining dynamic information.[7] The most general features of the earlier work are reflected in the present data; however, significant differences in detail are apparent which may be due to the limitations of some earlier instrumentation.

Experimental Section

Lysozyme was obtained from Worthington Biochemical Corporation as the salt-free powder. Diphenylthiocarbazone, carbon tetrachloride, and 1,10-phenanthroline were obtained from Eastman Kodak. Na_2EDTA was purchased from Allied Chemical Co. and Karl Fischer reagent and spectral grade methanol were purchased from Mallinckrodt.

Glassware and other instruments coming in contact with proteins after purification were treated to remove metal impurities by immersion in a 1:1 mixture of nitric and sulfuric acids for not less than 24 h followed by immersion in a solution 0.01 in EDTA and 0.001 M in 1,10-phenanthroline at pH 5–6 for not less than 48 h. All water used was passed through a Continental deionized water supply. Lysozyme was dissolved in deionized water and dialyzed for at least 24 h against a pH 5.0 solution of 0.01 M EDTA and 0.01 M 1,10-phenanthroline. Dialysis solutions were changed four times during this period and the resulting protein solutions lyophilized to yield the powder for the gas-phase hydration.

The dry lysozyme powder was gently ground in a mortar and placed in a plastic weighing tray above a glycerol–water solution of selected composition in a wide-mouth screw-top jar. Samples were allowed to stand in the jar from 1 to 4 days after which they were transferred to NMR tubes for the relaxation measurements. Following the NMR measurements the samples were destroyed in determining the water content accurately from the total sample weight and the total content given by Karl Fischer titration. Additional details of this procedure are discussed elsewhere.[8]

The NMR measurements were made at 30 MHz on a pulsed NMR spectrometer assembled in this laboratory employing a 12-in. Varian high-resolution electromagnet. The 90° pulse width was 3 μs and the receiver recovery time about 15 μs. Temperature was controlled using a Varian variable temperature controller with nitrogen as the cryogenic fluid. Temperatures were calibrated with a diode thermometer. Longitudinal relaxation times were determined using the 180–90° pulse sequence, while the transverse relaxation times were measured using the Gill–Meiboom modification of the Carr–Purcell pulse sequence.[9] The 180° pulse repetition rate in the Carr–Purcell sequence was varied in the vicinity of 100 μs but these variations caused no change in the measured transverse relaxation rate. At the shortest transverse relaxation times, no error is introduced by using the free induction decay of the magnetization as a measure of T_2.

Results

Representative results of the hydration procedure are shown in Figure 1. The NMR free induction decay amplitude for lysozyme powder samples is shown as a function of water content in Figure 2. A linear least-squares fit gives an intercept of 0.15 ± 0.38 g/100 g of protein with a linear correlation coefficient of 0.996. The goodness of fit is not altered if the origin is included in the calculation. There is considerable difficulty in preparing NMR samples of protein powders because the physical properties of the powder change with water content making sample packing difficult to reproduce. We attribute the scatter in Figure 2 primarily to this source.

NMR transverse relaxation times are exponential, within experimental error, throughout the water content and temperature range studied. Transverse relaxation times are shown as a function of water content in Figure 3 for rehydrated lysozyme powders; the low water content region is expanded in Figure 4. A linear least-squares fit to the T_2 data in Figure 4 yields a slope of 117 μs (100 g of protein)(g of water)$^{-1}$. The x-axis intercept of 0.042 g of water per g of protein corresponds to about 25 water molecules per protein molecule. The data of Figures 2 and 4 do not provide any evidence that there is any "NMR invisible" water in the present experiments. Although the protein protons contribute to the net magnetization of the sample, we may choose not to observe them directly by observing the magnetization after the transverse components of the protein proton signal have decayed to zero.

Longitudinal proton relaxation data are shown in Figure 5 for a representative protein powder sample containing 0.24 g of water/g of protein. In general the longitudinal NMR relaxation for water adsorbed on proteins is nonexponential but is adequately described by two time constants throughout the

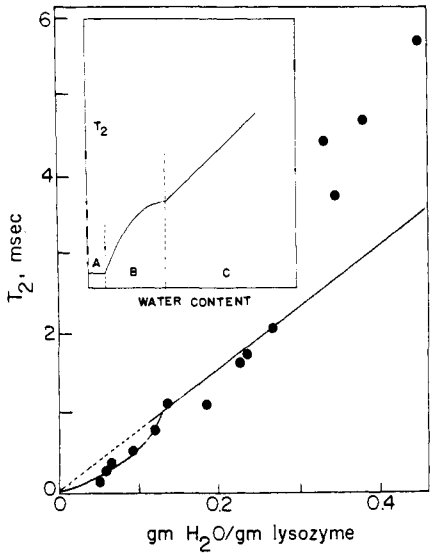

Figure 3. ^1H NMR transverse relaxation time at 30 MHz for water adsorbed on lysozyme powders at room temperature as a function of water content. The inset represents a plot of T_2 as a function of water content assuming a static model.

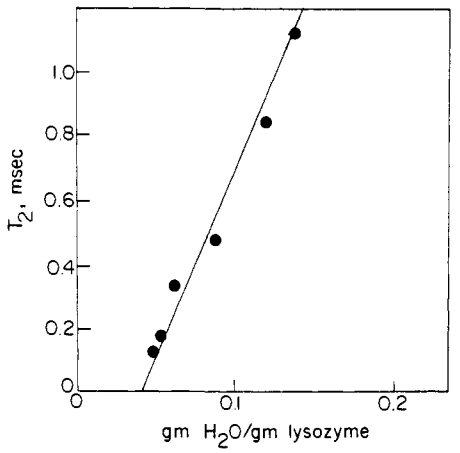

Figure 4. ^1H NMR transverse relaxation time at 30 MHz for water adsorbed on lysozyme powders at room temperature as a function of water content in the low water content region.

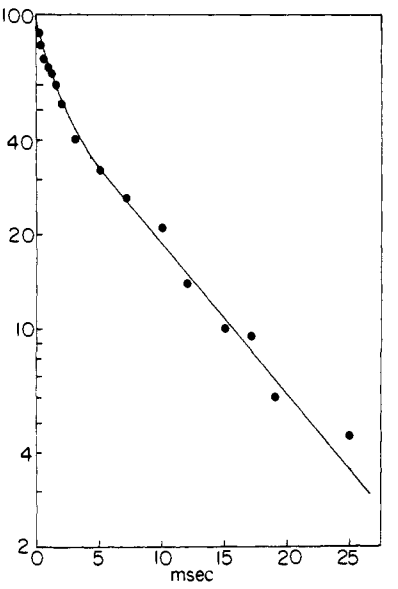

Figure 5. The log of the ^1H longitudinal magnetization for water as a function of time for a lysozyme powder sample containing 0.24 g of water per g of lysozyme. The solid line is calculated from a cross-relaxation mechanism described in the text.

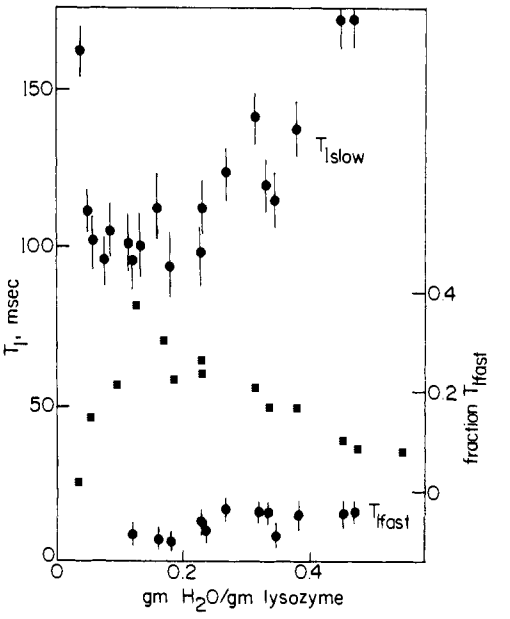

Figure 6. The ^1H longitudinal NMR relaxation times at 30 MHz for water adsorbed on lysozyme powders at room temperature as a function of water content. The solid boxes represent the fraction of the longitudinal magnetization which relaxes with the shorter time constant.

water concentration and temperature range studied. The longitudinal relaxation times are shown as a function of water content in Figure 6. Also shown is the fraction of the total water proton signal that relaxes with the shorter time constant. The slow time constant plotted in Figure 6 appears to pass through a minimum as water content is decreased while the faster relaxation time changes relatively little. At the lowest water contents, the amplitude of the fast relaxing signal decreases substantially and the difficulty of accurately estimating the faster time constant becomes prohibitive.

The NMR free induction decay amplitude for the water protons is shown as a function of temperature for representative protein powder samples at two different water contents in Figure 7. The spectrometer was gated so that the protein protons do not contribute to the observed signal and these data have been corrected for the increase in signal intensity resulting from changes in the Boltzmann factor with temperature. It is clear from the data in Figure 7 that there is a well-defined freezing event for the wet protein powder sample; however, none is apparent in the low water content sample. The longitudinal and transverse NMR relaxation times are shown as a function of reciprocal temperature in Figure 8. The activation energy appropriate to the slope of the linear transverse relaxation data is 5.9 kcal/mol of water; however, the interpretation of this number is hazardous because a complete understanding of the factors that influence the transverse relaxation is lacking at present. The slower longitudinal relaxation component of the water signal passes through a minimum at the lowest temperatures reached; however, the errors are sufficiently large and the minimum sufficiently broad that the precise position of the minimum is difficult to assign. The temperature dependence of the fast relaxing longitudinal component is difficult to define because the errors attending the extrapolation involved in measuring it are large and because it represents a smaller fraction of the total signal. There is no clearly resolved shoulder in the T_1 or T_2 data that could be identified with a

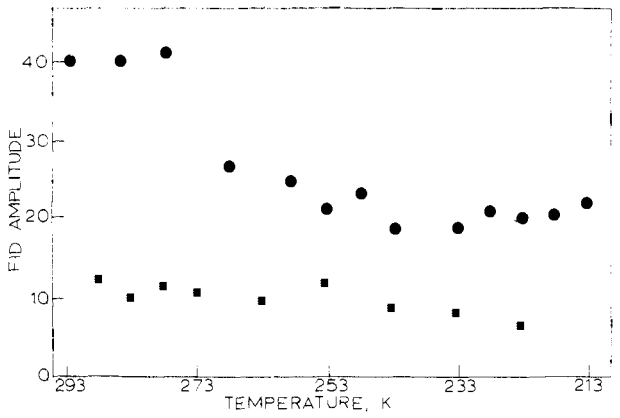

Figure 7. NMR free induction decay amplitude in arbitrary units measured at 30 MHz for water protons on lysozyme powder with a water content of 0.69 g of water per g of lysozyme (●) and 0.25 g of water per g of lysozyme (■).

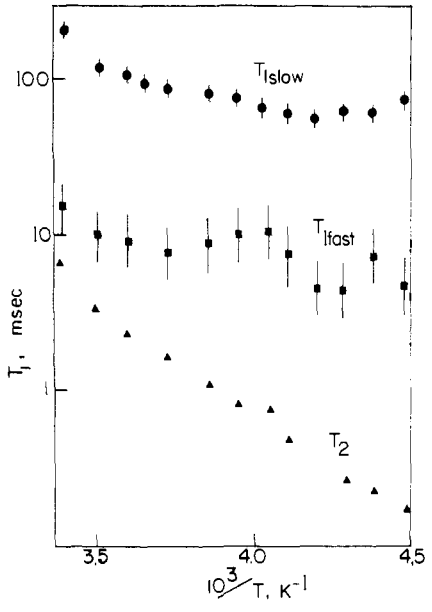

Figure 8. Longitudinal and transverse NMR relaxation times at 30 MHz for water protons in a lysozyme powder sample containing 0.69 g of water per g of lysozyme as a function of the reciprocal temperature: (▲) the transverse NMR relaxation time; (■) the fast longitudinal relaxation time obtained by graphically subtracting the slowly relaxing component; (●) from the total signal.

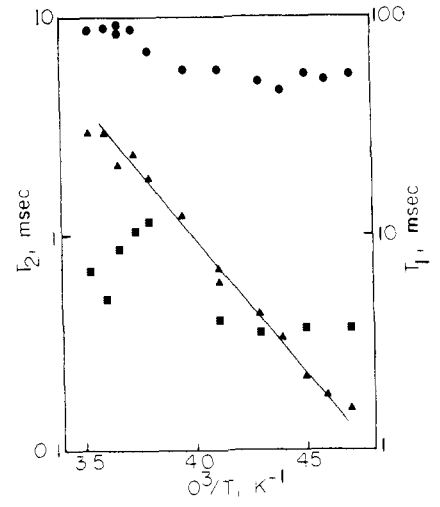

Figure 9. Longitudinal and transverse NMR relaxation times at 30 MHz for water protons in a lysozyme powder sample containing 0.25 g of water per g of lysozyme as a function of the reciprocal temperature: (▲) the transverse NMR relaxation time; (■) the fast longitudinal relaxation time obtained by graphically subtracting the slowly relaxing component; (●) from the total signal.

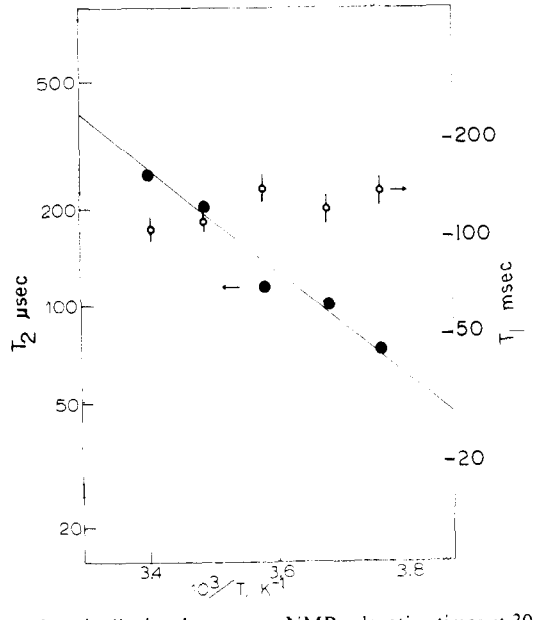

Figure 10. Longitudinal and transverse NMR relaxation times at 30 MHz for water protons adsorbed on lysozyme powder at 0.06 g of water per g of lysozyme. The fast and slowly relaxing longitudinal relaxation components could not be accurately resolved at this water content.

chemical exchange contribution such as has been observed in some liquids adsorbed on solids.[10]

Data for a lysozyme sample containing 0.25 g of water per g of lysozyme are shown as a function of reciprocal temperature in Figure 9. Two longitudinal relaxation times are resolved and the greater one passes through a broad minimum similar to that in Figure 8. The activation energy corresponding to the slope of the T_2 plot is 5.7 kcal/mol of water.

Longitudinal and transverse relaxation times are shown as a function of reciprocal temperature in Figure 10 for a dry sample containing only 0.06 g of water per g of lysozyme. The activation energy corresponding to the slope of the transverse relaxation data is 7.4 kcal/mol of water. At this low water content only a small fraction of the total water magnetization relaxes with a short time constant. Although it is clearly present, we have not plotted this fast component because the errors in extracting this number are very large.

Discussion

Several approaches to the interpretation of NMR line widths and relaxation rates have been suggested.[1-6] The simplest model is to assume that the macromolecule provides binding sites for the water molecules which significantly alter the relaxation properties of the water protons. The details of the relaxation mechanism are not important to the gross features of the model. If water exchanges rapidly among the various sites in the system including unbound sites, the observed water proton T_2 will be a weighted average of those for each site.[11] Two types of model are possible: a static model where the protein sites remain unchanged as additional water is added, or a fluid model where the properties of the water molecule sites are a function of the water content.

The static model requires that: (1) the sites which are occupied by water molecules first are also those with the shortest

relaxation times; (2) the number of water molecules associated with each site may be specified; (3) the chemical exchange rate among the different sites is rapid compared with the relaxation rates measured. We may then write:

$$(1/T_2)_{obsd} = \sum_{sites} P_i/T_{2i} \quad (1)$$

where P_i and T_{2i} are the probability and relaxation time associated with the ith site. For simplicity it is often assumed that the transverse relaxation rate may be given by an expression of the form:

$$1/T_{2i} = K_i{}^2\tau_{ci} \quad (2)$$

where K_i and τ_{ci} are the van Vleck second moment and the correlation time appropriate to site i. This expression represents a significant approximation because it assumes that the motions of the different water molecules are uncorrelated, that extreme narrowing approximations are valid, and that the second moment may be known or is the same for every site. Of these the most critical appears to be the assumption of extreme narrowing, which must certainly break down at low temperatures and low water coverages or both. Provided that the relaxation of the water protons is dominated by a dipole–dipole mechanism, the extreme narrowing approximation may be lifted and the frequency dependence included in eq 2. At constant frequency and temperature, eq 2 would then still be approximately correct to within a constant factor.

If we now assume that there are two populations, A and B, the observed transverse relaxation time will be given by:

$$(T_2)_{obsd} = wT_{2B}/(w_A((T_{2B}/T_{2A}) - 1) + w) \quad (3)$$

where w_A/w has been substituted for the population of protons at the A environment and eq 1 rearranged for the relaxation time rather than the rate. A typical plot of this equation is shown in the insert to Figure 3 where regions A, B, and C are indicated. Throughout region A the A sites are populated and the observed transverse relaxation time is that appropriate to that site alone. In region B, $w \ll w_A((T_{2B}T_{2A}) - 1)$ and a close approximation to a straight line is obtained. In region C the contribution of the fast relaxing population is diluted and the relaxation time approaches T_{2B}. If we extend this model slightly by assuming that there are several populations in the A group, then the A region may have a definite slope but represents an average of several rapidly exchanging populations. The remainder of the plot would be identical with that shown and there would still be a limiting linear region.

At first glance the data of Figure 3 appear to fit this model provided that the presumed fastest relaxing water component is not observed, that is, provided that T_{2A} is significantly less than 50 μs, a very generous instrument response time. The apparent x axis of the Figure 4 intercept is 4 g of water per 100 g of protein, which should correspond to w_A. Since the straight line portion of Figure 3 extends beyond 2 ms, $T_{2B}/50$ μs is much greater than 1. Substitution of this limiting information into eq 3 permits calculation of the maximum slope permitted by the model which is 13 μs (g of water)$^{-1}$ (100 g of protein). This value is more than a factor of 10 smaller than that observed of 117 μs (g of water)$^{-1}$(100 g of protein). Therefore, a static two-population model does not account for the data.

There are two primary objections to this treatment. (1) The population implied by the x-axis intercept may not all exchange with the observed protons. (2) More than one type of A site is needed. The first objection is not consistent with the observation that the free induction decay amplitude accounts for all of the water protons in the sample down to the lowest water contents measured. The only nonobserved protons in the experiment are those of the protein. While resonant spin exchange between protein and water protons is certainly possible, chemical exchange is in general not possible at the rates required for the vast majority of protein protons.

This static model may be extended to include an arbitrarily large number of populations since the fast exchange assumption permits rapid averaging of all groups of sites populated. When water is first added to a new population, x, the slope of the observed relaxation time vs. water content plot may be represented as in eq 3 provided that T_{2A} is replaced by T_{2av}, w_A by $w - w_x$, and T_{2B} by T_{2x}. Therefore extension to any number of populations will not explain the data of Figure 3 with the assumptions of a static model.[7]

A fluid model may be generated in a variety of ways which somehow includes the idea that the addition of successive water molecules to the dry protein has a cooperative effect in catalyzing motions of the water or protein or both. This idea seems intuitively sensible when the polar character of both the protein surface and the water molecule is considered. We may construct such a model using the following assumptions. (1) Isolated water molecules on the protein surface are firmly affixed, and hence have $T_2 < 50$ μs, a typical protein proton T_2. (2) The correlation time of a water molecule at the protein surface is inversely proportional to the number of water molecules coordinated to it. (3) The number of water molecules coordinated to a given water molecule is determined strictly on a statistical basis; i.e., as the amount of adsorbed water molecules increases, a state of complete saturation is eventually reached in which all water molecules at the protein surface are coordinated once to the protein and three times to other water molecules. (4) Beyond the saturation concentration additional water molecules adsorbed by the protein have properties like those of bulk water, but may exchange with protein sites. (5) The lifetime of water in any environment is sufficiently short that the fast exchange conditions apply.

The observed transverse relaxation rate may be expressed in terms of D, the fraction of surface binding sites occupied by water molecules. The parameter D was arbitrarily set so that it is unity when the water content is 13 g/100 g of protein. Although this choice fits the data well, it need not have any fundamental physical significance. We may then write the observed transverse relaxation rate when D is less than 1 as:

$$(1/T_2)_{obsd} = \frac{(1-D)^3}{T_{2,1}} + \frac{3D(1-D)^2}{T_{2,2}} + \frac{3D^2(1-D)}{T_{2,3}} + \frac{D^3}{T_{2,4}} \quad (4)$$

where $T_{2,i}$ refers to the average T_2 of the water molecules coordinated $i-1$ times to other water molecules. When D is greater than 1 there are at most two states for the purposes of the calculation; a state with $T_2 = T_{2,4}$ associated with the water–protein interaction, and a state associated with the remainder of the water molecules in the sample with T_2 appropriate to the bulk liquid. Table I summarizes one choice of parameters. The transverse relaxation times assigned to each environment listed in Table I are arbitrary and the solid line in Figure 3 is calculated from eq 4 using these values. In spite of the obviously crude choice of probability factors in addition to other oversimplifications, the model fits the data well up to about 30 g of water per 100 g of lysozyme. At higher water contents the measured T_2 is longer than that predicted by the model and it seems necessary to include additional features. Several physical changes at this water content are obvious: the powders become hard to handle, sticky, and appear inhomogeneous. Perhaps at this point sufficient water is present to allow large scale protein conformational changes which expose new binding sites and bury others.

The increase in the longitudinal relaxation time T_{1S} with increasing water content shown in Figure 6 clearly supports the conclusion based on the transverse relaxation data that

Table I. Fluid Model Parameters for Transverse Relaxation[a]

Environment type	No. of H bonds To protein	No. of H bonds To H_2O	Calcd probability	Assumed T_2, μs
1	1-3	0	$(1-D)^3$	50
2	1-3	1	$3(1-D)^2D$	333
3	1-2	2	$3(1-D)D^2$	500
4	1	3	D^3	1 000
5	0	4	If $D < 1.0$; if $D \geq 1$, $x_T - y_T/x_T$	1 000 000

[a] Definitions: x_T = number of water molecules adsorbed; y_T = available sites; $D = x_T/y_T$ or 1, whichever is less.

water molecule motion increases with increasing water content. More specific conclusions, however, require understanding the nonexponential character of the data in Figure 5. Two approaches may account for the nonexponential decay of longitudinal magnetization observed in these systems: (1) a chemical exchange model, which has been widely used in the treatment of liquids adsorbed on solids; (2) a cross-relaxation model which looks mathematically similar, but which does not require any exchange of material between the two populations with different relaxation properties.

The chemical exchange approach was developed by Zimmerman and Brittin[11] and applications of it to adsorbed liquids have been reviewed and extended by Resing[12] and Pfeifer[10] among others. The essence of the idea is that if there are two environments in the sample which provide very different relaxation times, then one expects to resolve separate relaxation rates for the different environments provided that the chemical exchange between them is slow. In the limit of fast exchange between the two environments only a single line is resolved corresponding to a single relaxation time or a simple exponential decay of magnetization. In earlier work on proteins we have pointed out that the exchange model leads to two major problems. (1) Uncomfortable constraints on the exchange rates are required. That is, the lifetimes for the water molecules in the protein environment have to be on the order of tens of milliseconds to explain the clear resolution of two longitudinal relaxation components. Such long lifetimes for water in the protein environment do not seem reasonable, although in other contexts such a model appears to be well justified. It is difficult to imagine that the water molecule exchange rate on and off the protein surface changes by many orders of magnitude when the crystal or the protein powder is dissolved with the addition of very little water. Evidence for such slow exchange rates in solution is lacking in the light of Koenig's recent work.[13] (2) If water molecule exchange rates are sufficiently slow on the time scale appropriate to longitudinal relaxation to account for the two time constants resolved, then the exchange rates are certainly sufficiently slow to resolve two transverse relaxation components. However, only one transverse relaxation rate has been resolved in the protein powder systems studied. To make the exchange model fit the data, it is necessary to postulate that the transverse relaxation rates in the different environments are equal or at least sufficiently similar that experimental resolution of two rates is not possible. We may not eliminate the chemical exchange model completely based on these data; however, a much simpler explanation is available which has strong support from other systems.

Since the ^1H NMR lines are broad for both the water and the protein, the two proton populations are simultaneously on resonance. Magnetization may therefore be transferred between one spin population and another even though no mass transfer is involved. We may assume three relaxation rates: one for the water spins, R_w, one for the protein spins, R_s, and a transfer rate, R_t, which couples the two populations. A Bloch type equation describing this situation may be written as.[14-18]

$$d(I)/dt = -(R_w + R_t)(I - I_0) + (R_t/F)(S - S_0)$$
$$d(S)/dt = -(R_s + R_t/F)(S - S_0) + R_t(I - I_0) \quad (5)$$

where F is the ratio of I to S spin population, and I_0 and S_0 are the equilibrium magnetizations for the I and S spins, respectively. These equations integrate to a sum of exponentials which may easily fit the relaxation data in the present experiments. For example, the solid line through the data in Figure 5 was calculated using the constants $R_w = 33\ s^{-1}$, $R_s = 5\ s^{-1}$, and $R_t = 50\ s^{-1}$.

There are several important features of these results. (1) The quality of the fit is not very sensitive to the choice of R_s since R_s is much smaller than the other rates involved. (2) The relaxation time, R_w^{-1}, that corresponds approximately to the water intramolecular relaxation time is much shorter than the dominant experimentally observed parameter, $T_{1,slow}$, indicated in Figure 2. This result may be interpreted to indicate that the protein influences the water molecule motion much more than has been deduced from the large value of $T_{1,slow}$ in many systems. In very crude terms the relaxation rate for the water protons has decreased by about a factor of 100 compared to that in the pure liquid. The hydration domain of the protein powder then looks like a viscous liquid but not like a solid. (3) The cross-relaxation mechanism will not make the transverse relaxation nonexponential; however, it will contribute directly to the magnitude of the transverse relaxation rate.[15] Therefore, the transverse relaxation rates for water in such systems are generally depressed by an intermolecular relaxation mechanism which makes any direct comparison of T_1 and T_2 values hazardous. (4) The severity of the nonexponential longitudinal decay will depend on the relative amounts of water and protein protons as well as the ratio of the relative relaxation rates for the two spin populations. (5) Since the long protein proton relaxation times may be seriously altered by traces of paramagnetic impurities, the details of the nonexponential behavior may be a sensitive function of sample preparation. This possibility also makes interpretation of the fractions of fast and slowly relaxing longitudinal relaxation components very hazardous at best. (6) It is clear that the fraction of fast or slowly relaxing water protons need not be any measure of the population of water molecules associated with a particular group of nonexchanging water molecules. (7) Since the protein proton resonance is broad, the effectiveness of a 180° radiofrequency pulse in inverting the magnetization of the protein protons will be a function of the pulse width. Therefore, the fraction of fast and slow component observed is expected to vary with the effective spectral width of the spectrometer transmitter.

The cross-relaxation model represented by eq 5 was applied to the longitudinal relaxation data for each point represented in Figure 6 using a nonlinear least-squares fitting program. The goodness of fit was not sacrificed by arbitrarily setting R_s equal to zero, thus decreasing the number of variables fit. The cross-relaxation parameters derived in this way are shown as a function of water content in Figure 11. The derived relaxation times are approximately a linear function of water content. As in the transverse relaxation data there appears to be a discontinuity at approximately 0.35 g of water per g of protein which we do not understand in detail. The derived longitudinal relaxation times, T_{1w}, shown in Figure 11 as solid circles, should be most closely associated with intramolecular or water-water intermolecular relaxation mechanisms. These relaxation times are significantly shorter than those usually reported for water in protein systems, such as protein crystals, frozen protein solutions, or tissues.[19-22] The longer experimental longitudinal relaxation time apparent in Figure 5 is

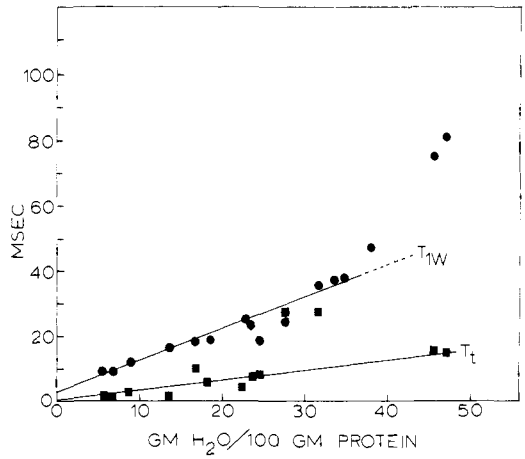

Figure 11. ^1H longitudinal NMR relaxation parameters at 30 MHz for water adsorbed on lysozyme powders derived from the cross-relaxation model after setting the protein proton relaxation rate equal to zero. T_{1w} is the water proton relaxation time and T_t is the time constant characterizing spin transfer between the protein protons and the water protons.

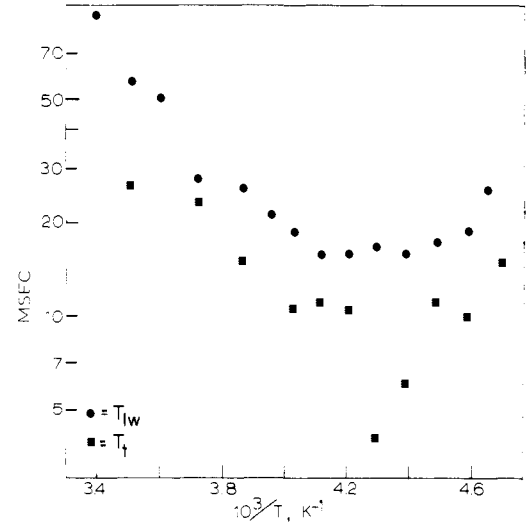

Figure 12. Longitudinal NMR relaxation time parameters derived from the cross-relaxation model assuming $R_{1S} = 0$ as a function of reciprocal temperature. Standard errors for $T_{1w} = R_{1w}^{-1}$ are approximately 10 and 50% for $T_t = R_t^{-1}$. The original data are shown in Figure 3.

determined by R_t and R_s, not R_w. The water molecule dynamics associated with the much shorter, derived longitudinal relaxation time, T_{1w}, are therefore generally slower than suggested by simply identifying the longer experimental relaxation time with some sort of unbound or noninteracting water.

The data of Figure 7 show that there is a clear freezing event in wet protein powder systems even though it is not obvious that there should be "free" water present to freeze. The amount of water remaining observable in the wettest lysozyme sample is approximately 0.35 g of water per g of protein. This value is not very precise because of difficulties of uniformly packing an NMR tube with such a protein powder; however, it is consistent with an earlier finding that 0.34 g of water/g of lysozyme remains unfrozen in frozen lysozyme solutions.[19] It is not absolutely necessary that the unobserved water be in the form of ice. If a wide distribution of correlation times for water molecule motion is assumed, one might predict that a decrease in signal intensity should attend a decrease in temperature when part of the water molecule population falls into the slow motion limit for the NMR experiment. This is equivalent to failing to meet the fast chemical exchange condition. Such an explanation has been offered and developed extensively by Resing for other systems and applied more recently to protein systems.[23-25] With this approach the decrease in signal amplitude should not be sudden; however, the data of Figure 1 show a sudden drop close to 273 K. Therefore such a correlation time argument cannot account for the data close to 273 K. Such an explanation may be appropriate for the slight decrease in the free induction decay amplitude with temperature between 265 and 233 K, but then it is difficult to understand why the free induction decay amplitude remains approximately constant between 233 and 213 K. One possibility is that the presumed simple distribution of correlation times is inappropriate and that a bimodal distribution is required.[13] If a bimodal distribution were assumed, only the slow moving fraction of the total water population could move into the slow motion limit at the higher temperatures. A second decrease in signal amplitude should then be observed at much lower temperature when the mode of the fast moving water population approaches the slow motion cutoff. On the other hand, it is also possible that there is a distribution of water-protein interaction enthalpies so that some of the unfrozen water associated with the protein close to 272 K freezes at a lower temperature. The absence of a clear freezing event and a significant temperature dependence of the free induction decay amplitude for the drier sample supports this simpler explanation.

The temperature dependence of the NMR relaxation parameters was initially studied to determine whether there were significant changes in the water molecule motion on the protein surface as temperature decreased. The most direct indicator of this should be the position of the minimum in T_1 for the water protons. However, a simple analysis is complicated by the fact that the longitudinal relaxation may be influenced significantly by the cross-relaxation mechanism. The amplitude vs. time data for the longitudinal magnetization of each temperature in Figure 8 were fit to the model in eq 5 assuming again that R_s was 0. The derived parameters are plotted as a function of reciprocal temperature in Figure 12. Both the water relaxation parameter and the transfer parameter pass through a minimum at approximately the same temperature. The dominant motion responsible for this dependence is ascribed to the water molecules. If there were significant protein motions on the time scale of nanoseconds implied by the position of the T_1 minimum, the protein proton spectrum would be observable, but is not with our gating conditions. Therefore, there must be components of the water molecule motion at a frequency higher than $2\pi \times 30$ MHz at temperatures down to the position of the T_1 minima in Figures 8, 9, and 12. On this basis we may conclude that the average water molecule moves rapidly on the protein surface. There is little difference between the low-temperature portions of Figures 8 and 9 which suggests that the water motion in these two systems is similar. At temperatures above the freezing event shown in Figure 1, the water proton relaxation shown in Figure 8 is clearly different from that in Figure 9. The source of these differences is likely to be the onset of a variety of chemical exchange events involving the water protons and protein ionizable groups which are expected to become increasingly important at higher temperatures.[26]

The similarity in the data for Figures 8 and 9 below the freezing event might be attributed to the freezing event forcing the remaining liquid water content of the two samples to be very similar. This idea is not supported by the quantitative aspects of Figure 7. After the freezing event in the wetter protein powder sample, approximately 0.35 g of water per g of protein remains observable. This value is considerably larger than the total water content of the sample used for the exper-

iment in Figure 9. Therefore, either the basic water molecule motions at the protein surface are not particularly sensitive to water contents in the range between 0.25 and 0.35 g of water per g of protein or the real differences in water molecule motions do not significantly affect the appearance of the very broad T_1 minimum. On the other hand, the data for the very dry sample shown in Figure 10 are clearly different from either of the wetter samples. The value of the T_1 for the driest sample measured increases with decreasing temperature suggesting that water motion is sufficiently slow to place the spectrum on the low-temperature side of the T_1 minimum. The conclusion that water molecule motion slows down with decreasing water content is supported by the water concentration dependence for the longitudinal and transverse NMR relaxation rates in Figure 6.

The shapes of the graphs in Figures 8, 9, and 12 suggest that some distribution of correlation times may be required to adequately characterize the data. However, contributions of cross-relaxation to both longitudinal and transverse relaxation imply that we may have little faith in the nature of the distribution derived for the correlation times by a fairly simple comparison of T_1 and T_2 values.[20-23] Although a distribution of correlation times may still be required to describe the details of the NMR relaxation data, the distribution will necessarily be less broad than previously supposed. In addition, any distribution proposed must consider the effects of the low-frequency dispersion in T_1 which is implied by $T_{1\rho}$ measurements and NMR dispersion measurements on protein solutions below their freezing temperatures.[27,28] Partial inclusion of this effect is implied by the recent work of Zipp et al. where a bimodal distribution was used to fit NMR relaxation data.[29]

Conclusions

We have not discussed or made significant assumptions about the mechanistic details of the relaxation mechanism for the water protons at the protein surface. We have only assumed that relaxation may be described by a set of relaxation times that are controlled by dipole-dipole interactions. With these limitations the simple static model most often applied to interpret the NMR relaxation of water in semisolid systems fails to account for the observations of transverse relaxation in important ways. If the adsorbed water molecules are permitted to influence the motional characteristics of those previously adsorbed, a crude model accounts for the observations. The nonexponential longitudinal NMR relaxation of water protons observed in these protein systems need not be discussed in terms of a chemical exchange model that requires very long residence times for water molecules at protein binding sites. A cross-relaxation model, which has been previously used extensively for understanding relaxation in polymers among other systems, accounts well for the water proton longitudinal relaxation. This result suggests significant modification of previous approaches to understanding the NMR relaxation water molecules at a protein surface. The water content dependence and the temperature dependence of the water proton relaxation rates support the conclusion that water molecule motion on the protein surface becomes slower with decreasing water content and temperature.[30]

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(30) NOTE ADDED IN PROOF. A reviewer brought to our attention that a spin-diffusion explanation for proton relaxation on lysozyme powders had been presented by Dr. Gottfried J. Kruger of the Magnetic Resonance Laboratory, Physics Division, EURATOM, Ispra, Italy, at the Fourth International Symposium on Magnetic Resonance held at the Weizmann Institute of Science, Rehovot, Israel, in August of 1971 (Paper B14d). Dr. Kruger has not as yet published these results including the cross-relaxation model which appears to be essentially identical with that used in the present paper (G. J. Kruger, private communication).