DEUTERIUM NMR OF WATER IN IMMOBILIZED PROTEIN SYSTEMS

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ABSTRACT Deuterium NMR spectra are reported for lysozyme crystals, powders, and frozen solutions. At high water contents the spectrum is a superposition of a narrow central component and a quadrupole doublet. The quadrupole splitting and the relaxation rates of both components, monitored as a function of water content and temperature, are discussed in terms of models for the water-protein interaction. The anisotropy of the water molecule motion is clearly demonstrated by the deuterium quadrupole splitting observed in the protein single crystal, but such splittings were not found in protein powders and frozen protein solutions. We therefore suggest that the most useful view of such data is to consider the water-protein interactions at the surface to be mixed rapidly and that a distribution of interactions be invoked rather than an oversimplified view often taken of a two or *n*-site mixing where *n* is small.

INTRODUCTION

The water-protein interaction is critical to our understanding of many aspects of protein chemistry. Nuclear magnetic resonance (NMR) techniques, which provide both structural and dynamic information, have been widely applied to test various concepts about the water-protein interaction (1–3). Although ^{1}H resonance is often studied because of its sensitivity and the wide availability of instrumentation specifically designed for ^{1}H spectroscopy, ^{2}H NMR spectroscopy offers the advantage that relaxation is dominated by the nuclear electric quadrupole mechanism. Relaxation is therefore controlled by this intramolecular mechanism rather than by intermolecular interactions that may severely complicate ^{1}H relaxation spectroscopy. The nuclear quadrupole moment of the deuterium is also sufficiently large that the first-order quadrupole effect is significantly larger than a first-order dipole-dipole interaction that can be observed for interacting protons. A net anisotropy of the water molecule reorientation in the sample is therefore more clearly reflected in the deuterium spectrum than in the proton spectrum (4). Lauterbur et al. (5) have reported deuterium quadrupole splittings in large crystals of lysozyme suspended in a medium that attempted to match the magnetic susceptibility of the suspending fluid and the crystal. Deuterium quadrupole splittings are well known in many protein systems where long range order persists; e.g., in fibrous materials such as collagen (4) or muscle (6).

Although previous studies have demonstrated that the motion of water at the protein surface is rapid (7), the question of the degree or extent of the motional anisotopy at the surface is largely unresolved. This issue is critical to an understanding of water at protein surfaces and ^{1}H NMR relaxation studies of the water-protein system in particular, because small net anisotropy, which may not be resolved in first-order spectra, may contribute significantly to transverse relaxation rates (8). Failure to take this anisotropy into account would lead to an incorrect estimate of the motional time scale and perhaps also lead to an overestimation of the width of any distribution assumed for the correlation times appropriate to the interproton vector. The present study was undertaken to investigate further the possibility of net anisotropic motions of water molecules at the protein surface and examine further the earlier deuterium result on protein crystals (5). We have examined here deuterium spectra and relaxation rates in lysozyme powders, frozen solutions, and crystals. In solid systems such as these, completely immobilized deuterons will give very broad lines and very large quadrupole splittings. The protons on the protein that have been exchanged with solvent deuterons fall into this category, and we have chosen a spectrometer configuration to suppress these signals. In doing this we may also discriminate against completely immobilized and nonexchanging water molecules, but previous proton and deuterium data require that the vast majority of water in these crystals be mobile (9). The focus of this work is therefore to characterize more completely the motional anisotropy of the very liquid water present in these crystals. The results lead to a better understanding of the dynamics at the protein surface, but leave open several questions about the microdynamical behavior of water in closely confined spaces.

EXPERIMENTAL PROCEDURES

^{2}H NMR spectra were obtained at 9.2 MHz with a Varian Associates, Inc. (Palo Alto, CA) 12-in electromagnet and a spectrometer assembled in the laboratory and described elsewhere (10, 11). The ^{2}H NMR probe used the tapped series tuned circuit of single coil design. A 3-Ω resistor placed in series with the 11 × 19 mm 24-turns inductor reduced the Q to ~46, which resulted in an effective ring-down time of 33 μs. The low Q required a 90° pulse width of 24 μs, and a software delay of 50 μs was routinely used before data acquisition except as indicated. T_1 measurements were made with the Nicolet NTC software (Nicolet Instruments, Mountain View, CA) and the usual inversion recovery experiment using at least 15 different τ values. The estimated error for the T_1 values is ~4%.

Three-times crystallized hen egg-white lysozyme was obtained from Sigma Chemical Company (St. Louis, MO), dialyzed against a solution 0.01 M in each EDTA and 1,10-phenanthroline at pH 5, dialyzed against deionized water, and then lyophilized. The resulting powder was exchanged twice with deuterium oxide, lyophilized again, then brought to final water content by means of equilibration with deuterium oxide through the vapor phase.

Monoclinic lysozyme crystals were grown as previously described (9), and cross-linked with gluteraldehyde (12) to permit easy exchange of the deuterium for the hydrogen in the water. Water contents were determined gravimetrically by drying the lysozyme powder in a drying pistol at 337 K with a mechanical vacuum pump.

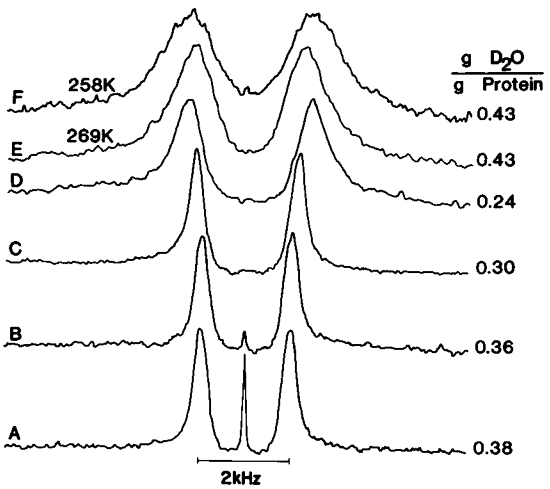

FIGURE 2 ^{2}H spectra, obtained at 9.2 MHz for a gluteraldehyde cross-linked lysozyme crystal of ~8 mm in length and ~2 mm in thickness, showing quadrupole splitting as a function of water content and temperature. (*A–D*) single crystal spectra at room temperature hydrated to different water content levels as shown. (*E–F*) spectra at 269 and 258 K for the single crystal with 0.43 g water/g lysozyme.

RESULTS

^2H_2O deuterium spectra taken from samples of polycrystalline, hydrated powders, and frozen solutions of lysozyme are shown in Fig. 1 as a function of sample and as a function of the software delay between the exciting 90° r.f. pulse and onset of data acquisition. We observed that there is a significant apparent increase in the "resolution" of the spectra in only the polycrystalline samples as the Fourier transform is begun with data at later times in the free induction decay. The expedient of changing the software delay is simpler than an echo experiment as a means of selecting or sorting data as a function of transverse relaxation rate and it requires significantly less precision in pulses and timers (13).

The spectra for the polycrystalline sample show a splitting, although the shape is complicated by the distribution of transverse relaxation rates implied by the dependence of the line shape on the delay, as shown in Fig. 1. Similar distortions of the line shape are not observed for the hydrated powders or the frozen solutions. Fig. 2 shows ^{2}H spectra for a large single crystal of monoclinic lysozyme as a function of water content and temperature. In this case the difficulty of overlapping powder line shapes is avoided and the spectrum is clearly resolved into two components, a narrow central line, apparent only at higher water content, and a broader component split into a crystalline quadrupole doublet. These spectra were collected with the

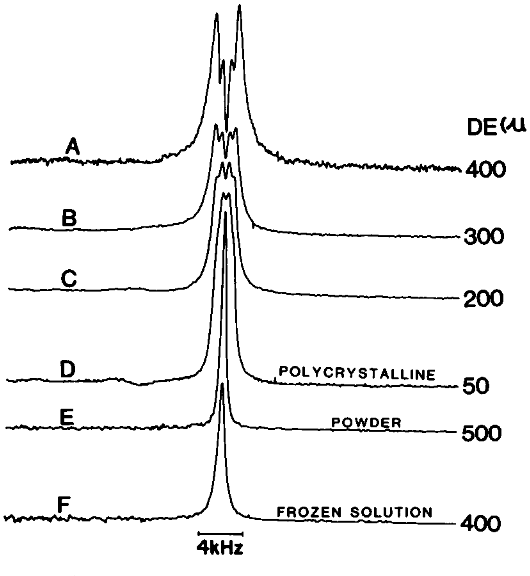

FIGURE 1 ^{2}H spectra for different lysozyme samples obtained at 9.2 MHz as a function of DE, the software delay between the exciting 90° r.f. pulse and onset of data acquisition. (*A–D*) Polycrystalline sample with 0.31 g water/g lysozyme, obtained at 300 K. (*E*) Hydrated lysozyme powder with 0.32 g water/g lysozyme. (*F*) Frozen lysozyme solution at 263 K.

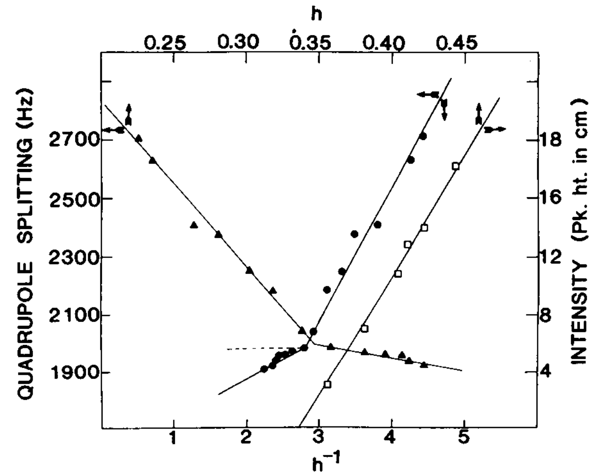

FIGURE 3 The measured ^{2}H quadrupole splitting in Ha for the single crystal as a function of h(▲) and h^{-1}(●), where h is the water content expressed in grams of water per gram of protein. The straight lines are drawn only to guide the eye. The dashed lines are explained in the text. □, intensity in peak heights of the central component in the high water-content region. The arrows indicate the relevant axes for the data points.

long axis of the monoclinic crystal perpendicular to the static field at a rotational angle of maximum splitting. No attempt was made to obtain the complete rotational pattern for the crystal in the present experiments. As shown in Fig. 3, the central narrow component decreases in intensity with decreasing water content, *h*, expressed throughout this paper in grams of water per gram of protein. The quadrupole splitting is also a function of water content, and is shown as a function of both *h* and h^{-1} in Fig. 3.

The central component of the single crystal spectrum is lost in a freezing event at ~273 K, but the quadrupole doublet remains as the temperature is decreased below this freezing temperature. Some super cooling was noted at 269 K, and the spectrum obtained at 269 K shown in Fig. 2 was obtained on the warming portion of the temperature cycle. As indicated in Fig. 2, the quadrupole splitting increases with decreasing temperature below the freezing event. To judge from the apparent signal-to-noise ratio, it is possible that the signal intensity also decreases with decreasing temperature below the freezing event, but accurate intensity measurements were not attempted.

The longitudinal relaxation rates of the central component and the observed quadrupole doublet were measured as a function of water content (Fig. 4). Several features are noteworthy: (*a*) There is apparently an abrupt change in slope in the plot of ^{2}H longitudinal relaxation rates of the quadrupole doublet vs. water content at the concentration where the central component is lost. (*b*) Within the estimated errors, the ^{2}H NMR relaxation rate for the high and low water content regions of the outer doublet is linear with h^{-1}. (*c*) The central line in the spectrum is relaxed much less efficiently than the outer lines and the data do not extrapolate linearly to the protein free value. (*d*) All the data presented were taken at water contents that are at or less than that required to saturate the crystal with water (14). The crystals were stabilized through the dehydration process by crosslinking with gluteraldehyde, a reagent of choice in the present case because of the supporting crystallographic studies (12).

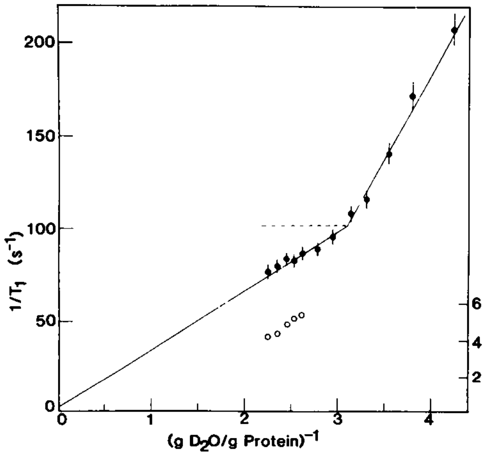

FIGURE 4 ^{2}H NMR longitudinal relaxation rates of the doublet component (●) (left-hand axis) and the central component (○) (right-hand axis) measured at 9.2 MHz as a function of h^{-1}. The dashed line is explained in the text.

DISCUSSION

The spectra shown in Fig. 1 demonstrate that a quadrupole splitting is apparent only in the crystalline samples of lysozyme. Although the crystal form is different, this finding is in agreement with earlier reports by Lauterbur et al. (5). The deuterium line shape in the polycrystalline sample is not that characteristic of a simple spin one powder line. However, one reason for the distortion is shown by the dependence of the spectrum on the starting point in the free induction decay for the Fourier transform. If the line were simple and characterized by a single transverse decay time, the line shape, but not the signal-to-noise ratio, would be independent of the delay. On the other hand, in the present spectra a marked dependence on the delay is observed, which demonstrates that the observed line is a superposition of lines characterized by different transverse decay rates and possibly quadrupole splittings. This simple method of testing the line for homogeneity appears to be a very useful application of T_2 resolved spectroscopy in cases like this, where dynamic but not necessarily clear spectral resolution is possible. That the lines shown in Fig. 1, spectra *A-D*, are heterogeneous is well supported in this case by the single-crystal spectra of Fig. 2, which provide a clearer view of the spectroscopic problem.

For a cross-linked lysozyme crystal saturated with deuterium oxide, the deuterium spectrum is clearly resolved into two components, a narrow central peak and the quadrupole doublet that responds in the usual way to reorientation of the crystal. In these experiments, no attempt was made to match the bulk magnetic susceptibility of the crystal to any supporting medium; the crystals were simply placed in a NMR tube and supported with pieces of pyrex glass. The well known effect associated with a susceptibility-induced splitting that behaves like a dipole or quadrupole doublet splitting when the sample geometry is the same as that used here, is not expected to be nearly as large as the splitting observed (15). Indeed, the proton dipolar splitting has not been reported for similar crystals, and the effects of susceptibility differences should be larger for protons because of the larger magnetogyric ratio. Thus, we obtain similar results to those found by Lauterbur et al. (5) without the possible difficulty of the supporting medium interacting significantly with the crystal and the protein structure.

The appearance of a narrow central line in the deuterium spectrum is not surprising, since it is well known that the protein crystal contains sufficient spaces for reorientation of the water molecule about all axes (16). The observation of the well resolved sharp deuterium line in these monoclinic crystals is consistent with the existence of two components in the transverse relaxation of water protons in earlier studies on lysozyme crystals (9, 17, 18). Like the present central component, the slowly relaxing transverse water proton decay was also eliminated at lower water contents in the crystal and was eliminated by the freezing event. Thus, we may associate this narrow component in the deuterium spectrum with water that simply fills holes in the protein crystal and does not interact strongly with the protein.

The observation of the quadrupole doublet may lead to the conclusion that there is a clear and specific interaction at the protein surface that so constrains the water molecules that the first-order quadrupole interactions fail to average. Caution is required, however, because the observed quadrupole splitting is a property of the whole crystal, in that the width of the splitting depends on the orientation of the whole crystal. The splitting observed is also a small fraction of the total quadrupole coupling constant for water (19, 20), so that a very significant motional averaging has occurred in the crystal. The observed splitting cannot arise from protein specific interactions in which the water is constrained to a particular region of a single protein molecule's surface for a long time because if it were, similar splittings would arise in frozen solutions and powders; but none is observed. In addition, it is likely that in that case the single crystal spectrum would be a sum of such very local specific interactions and thus that it would be a powder pattern of sorts and not respond as it does to simple rotation. Thus, we conclude that the observed splitting is a result of water motion anisotropy that is induced by the morphology of the spaces available to the water in the protein crystal. The absence of such splittings in other samples where the protein is immobilized, such as the frozen solution or the hydrated powder, implies either that the water molecule motion on the surface is sufficiently rapid that the spectrum averages to an isotropic limit, or that the local anisotropy and the resultant splitting in these cases are small compared with the relaxation rates that determine the overall line width.

The quadrupole splitting, and the longitudinal relaxation data all show dramatic changes in slope at water contents between 0.3 and 0.35 h. The intensity of the central component also decreases to zero between these water contents. These different aspects of the deuterium resonance all apparently monitor in different ways the same event; namely, the disappearance of the central component. The water content at which the central component is lost is close to the value at which the protein has a full complement of water. The best estimate for a full complement of water presently appears to be 0.38 h for lysozyme (21), but in the crystal this value should decrease because of protein-protein contacts. Thus, loss of the central component is consistent with the loss of what must be considered as non-"bound" water; but it cannot be without effect on the water-protein interaction as monitored by the doublet spectrum, since both the magnitude of the quadrupole splitting and the longitudinal relaxation time change in response to the dehydration from the central component. The simplest explanation for the loss of the central component with dehydration or freezing is that the water molecules producing this signal are simply removed from observation by loss from the sample in the dehydration experiment or by freezing. In the freezing case, the deuterium spectrum of the frozen water would remain as a very broad doublet spectrum of the ice that would be very difficult to separate from the superposition of similar spectra arising from the deuterium atoms placed in the protein at exchangeable positions in the preparation of the sample. No attempt, therefore, has been made to observe these broader components. One may also hypothesize that these physical changes somehow convert or mix the water contributing to the central component with that causing the doublet, the primary evidence for which would be intensity comparisons. Although we feel this possibility is unlikely, intensity measurements on the present spectra cannot be sufficiently precise to confirm or deny such a suggestion.

It is tempting to analyze the data of Figs. 3 and 4 in terms of a chemical exchange model, because the data are linear in h^{-1} in two regions. Several problems arise, however, in application of this model and simple modifications of it. The simplest model that has been extensively applied to water-protein systems is a two or n-site exchange model (22) in which it is assumed that there are two types of water: that bound in some intimate way with the protein, and that which is free or not bound to the protein. The present data may be approached from this point of view. If two environments are assumed, a simple equation results for either $1/T_1$ or the quadrupole splitting:

$$\frac{1}{T_1} = \frac{1}{T_{1A}} + \frac{K}{h}\left(\frac{1}{T_{1B}} - \frac{1}{T_{1A}}\right),$$

where K contains some constants and the number of strongly bound molecules, and h is the water content in grams of water per gram of protein. Provided that there is not some peculiarity in the concentration of the two environments assumed, the observable will be a linear function of h^{-1}. If the number of sites is increased so that there is an n-site exchange, linearity still results as long as the water dehydrates from the protein in order of increasing nuclear relaxation rate. It is clear from the data in Figs. 3 and 4 that the linear dependence of the observable on h^{-1} implied by this model is observed on either side of the change in slope. Further consideration leads to difficulty, however.

If we were to assume that all water which appears in the doublet spectrum is bound, we would expect the properties of the water in this bound domain to be a function of water content until the bound domain is saturated. Thus, in the strict sense of a bound-free exchange model, we should predict that the quadrupole splitting and the relaxation rate of the "bound" phase would be independent of water content once the bound phase was filled; i.e., the data in Figs. 3 and 4 should follow the dotted lines according to this view. But as they clearly do not, we must therefore conclude that water most intimately associated with the protein is altered in some way by the addition of water to the central component of the spectrum. Since the two spectra are well resolved, however, the two populations do not mix in the time scale of the reciprocal of the quadrupole coupling observed. Poor exchange mixing between the two components is also supported by the small relaxation rate for the central component. A simple exchange model is not consistent with the details of the behavior at high water contents. Although the relaxation rate data for the doublet spectrum shown in Fig. 4 behave linearly in the high water content regions, the exchange model predicts that the relaxation rate extrapolates to the value appropriate to the protein-free system. Although a line may be drawn through the data to satisfy this constraint, the line so drawn is not close to a best fit to the data. In addition, such an extrapolation fails entirely for the central component data also shown in Fig. 4. Finally, a simple model that envisions exchange of water between a protein surface and a bulk phase is not consistent with the absence of similar quadrupole splitting in the frozen solution and protein powder case. Thus, if any multiple site exchange model is assumed, it must be appreciated that the two or *n* participating environments are complex indeed, and must represent rather significant mixtures of many contributions.

An alternative way of viewing the data is to consider a continuous distribution of interactions that dehydrate in the approximate order of the relaxation rates but that mix or average completely in the time of the measurement. In this case, the dehydration process would shift the mean of the distribution, and hence the observable, linearly with h^{-1}. An important feature of the present crystal data is that the addition of water to the central component of the deuterium spectrum, the intensity of which increases linearly with the total water content above a certain minimum value, affects the quadrupole coupling constant represented in the spectrum and the relaxation rate for the doublet. As has been pointed out, it is also dangerous to interpret this result strictly in terms of a shell exchange model of water-protein interactions. It appears that a very reasonable interpretation includes the known response of the crystal structure to dehydration. That is, as the water is removed from the larger spaces of the crystal, the crystal must strain with the appearance of the void. Hence, protein-protein contacts may adjust and the protein itself may change shape somewhat as the aqueous support is removed. Indeed some structural rearrangement is implied by loss of x-ray data quality in dehydrated crystals. Therefore, we suggest that the changes in slope evident in Figs. 3 and 4 result from indirect interactions that include response of the whole crystal as a structural entity to the loss of density attending loss of the solvent, water.

CONCLUSIONS

The present data confirm earlier ^{1}H NMR evidence for the existence of resolvable water signals in protein crystals. It points out clearly the dynamic response of water to the anisotropic environment in the crystalline interior that is not readily apparent in other protein-water systems, such as the hydrated powder at the same water contents. Therefore, the anisotropy in water motion, which is clearly demonstrated in the present crystal spectra, arises because of the nature of the spaces available for water after protein molecules are packed into the crystal, and is not attributable to long-lived, specific, water-single protein molecule interactions. Finally, we suggest that although the simplest two or *n*-site exchange model may be applied to the data, it represents an oversimplified view of the situation. We suggest that a more reasonable model is a rapidly mixed distribution of interactions between water and the protein. The rapid mixing at the protein surface is consistent with present and earlier NMR relaxation studies.

The assistance of Mr. Timothy Knoblauch in crystal preparation and helpful discussions with Dr. William Shirley are gratefully acknowledged.

This work was supported by the National Institutes of Health (grant GM 18719) and by the Chemistry Department, University of Minnesota.

Received for publication 29 January 1981 and in revised form 10 September 1981.

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