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NUCLEAR MAGNETIC RELAXATION DISPERSION IN MONOCLINIC LYSOZYME CRYSTALS

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Nuclear magnetic relaxation measurements are reported as a function of field strength corresponding to the frequency range from 0.01 to 20 MHz for water protons in monoclinic lysozyme crystals at 278 and 298 K. Though the instrumentation used selects only a portion of the total magnetization to sample, the data clearly indicate a field dependence of the relaxation rate that signals the presence of slow motions characterized by time constants in the range of tenths of microseconds and slower. The data support, but do not uniquely prove, the hypothesis that this time scale is that appropriate to the isotropic averaging of locally anisotropic water molecule motion at the protein surface.

1. Introduction

Understanding the influence of water on the structure and dynamics of proteins and other macromolecules is crucial not only to fundamental biophysical chemistry but also to many practical problems including macromolecule storage, stability and organism response to stresses such as drought and cold. The systems studied most often are protein solutions; these, however, offer a very complex dynamical problem in that in addition to surface interactions one has to take detailed account of the coupling of translational and rotational motions of the protein to the water molecule motions. These protein-water interactions influence the rotational relaxation of the water molecules and can be sensed by observing the magnetic relaxation of the water nuclei, the spin-lattice relaxation rate of the proton spins, for example. The various correlation times that determine these rates also cause the rates to be magnetic field dependent, so that more detailed information on the various relaxation processes can be obtained in a nuclear magnetic relaxation dispersion (NMRD) experiment [1–3]. The dynamical problem may be simplified considerably by immobilizing the protein so that neither translation nor rotation of the protein molecules is possible; the remaining dynamical variables then involve the motions of the water relative to the protein or intramolecule motions of the protein molecule itself. Several choices are possible for the state of the immobilized protein: dry lyophilized protein powder, wet protein powder, frozen solution and protein crystal. The present study focuses on the last: protein crystals that contain a full complement of water.

The usual NMRD characterization of solvent-surface dynamics involves the study of various NMR relaxataion times at constant and usually high field (expressed as Larmor frequency) as a function of temperature [4,5]. This approach is often the only one possible, but suffers from several difficulties including those associated with freezing events in aqueous systems, changes in the stability or structure of the macromolecule with temperature, and changes in the shape of any

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distribution that may be required to characterize the motions present in the systems studied. On the other hand, measurement of the NMR relaxation rate at constant temperature over a wide range of field (the dispersion of the relaxation) provides the same type of information about the dynamics without these difficulties at the price of performing a technically much more difficult experiment. The present study was undertaken because of the desire to understand the full range of water-protein interactions and the suspicion that low-frequency processes make important contributions to the water-protein interaction as monitored by NMRD.

2. Experimental background

The NMRD spectrometer has been described previously [1,6]. Monoclinic lysozyme crystals were grown as previously described [7] and contained in 10-mm glass tubes for measurement. Sample temperature was maintained by a system that circulated liquid dichlorodifluoroethylene thermostatically controlled in a Neslab RTE-4 regulated bath through the sample chamber. Each longitudinal relaxation rate reported is the result of a series of measurements involving a minimum of 30 points on the decay curve which were fitted to an exponential with a least-squares program.

The proton NMR signal from a protein crystal is complicated [7] by the nonexponential character of both the longitudinal and transverse relaxation decays. There is a very rapid decay in the transverse direction due to the solid protein protons with an average T_2 of about 10 μs. In addition, with crystals saturated with water at room temperature there are two additional decay constants; one of about 3 ms and the other considerably longer. This longest decay is lost if the crystals experience a freezing event or if the crystals are dried below 0.23 g H_2O/g protein. We may therefore associate this component with the packing of large molecules and the spaces that must be filled with liquid in order to maintain the uniformity of the crystal. In contrast, the longitudinal relaxation of the water protons is characterized by two time constants at all water contents and temperatures. At either low temperatures or water contents, the nonexponential decay is pronounced and caused by the magnetic coupling or exchange between water and protein protons in the system, each with rather different relaxation rates. Detailed analysis of the coupled proton relaxation is presented elsewhere [8,9]. Above the freezing event at high water contents the nonexponential longitudinal relaxation persists, still characterized by two time constants. Since the addition of more water to the system cannot eliminate the cross-relaxation important at lower temperatures or water contents [3], the existence of only two decay times implies that the water in the intermolecular spaces of the crystal mixes with that at the surface on the time scale of the long relaxation times even if it does not mix on the scale of approx. 10 ms as implied by the resolution of two slow transverse decay times.

The field-cycling NMRD apparatus discriminates against several of the relaxation components detailed above because of the way it samples the magnetization and the time required for the field to be switched and to settle. A schematic plot of the field strength as a function of time during one cycle of the relaxation measurement is shown in fig. 1. T_1 at a desired measure field is obtained by polarizing the spins at a high-soak field, dropping

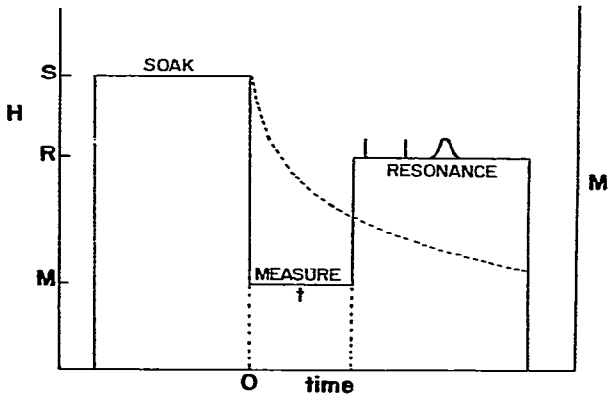

Fig. 1. Schematic representation of the magnetic field strength as a function of time during one cycle of a relaxation time measurement at the measure field H_m. A 90–180° radio-frequency pulse sequence is applied 12 ms after the field has nominally achieved the resonance field.

the field strength rapidly to the desired measured field, letting them relax there for a time t_m, raising the field to the convenient sample or measure field (4 MHz) at which an echo resulting from a 90–180° echo pulse pair is integrated. For simple exponential decay, a semilogarithmic plot of the echo amplitude or integral vs. time is then linear with slope $-1/T_1$. This sampling technique provides an easy signal to integrate and eliminates difficulties associated with receiver recovery following strong radiofrequency pulses. The practical consequences of this measuring technique for the present experiments is that the slowness of the sampling strategy makes the longer longitudinal relaxation time the only one observable. In addition, the 180° pulse echo is generated about 12 or more ms after the initial 90° pulse, thus effectively eliminating the 3 ms transverse component from observation. The spectrometer therefore measures the T_1 value associated with only the slower relaxing transverse proton population. We have tentatively identified this signal with water not intimately associated with the protein surface based on its freezing event and the relatively long longitudinal and transverse decay times. This identification is supported by loss of the signal on freezing.

3. Results

T_1 values for water protons of the slow transverse relaxation component in monoclinic lysozyme crystals are shown in fig. 2 as a function of field indicated as the proton Larmor frequency at 298 and 278 K. Several features are obvious: (1) There is very little difference in the data taken at the two different temperatures. (2) The magnitudes of the relaxation rates observed are small. (3) The curvature of the semilogarithmic plot over the observed frequency range is small. (4) The shape of the dispersion curve eliminates a single correlation time or a Markovian autocorrelation for the internal field. Though trace paramagnetics are always potential problems in relaxation experiments, care was taken to exclude them. Moreover, paramagnetic impurities have completely differently shaped NMRD spectra.

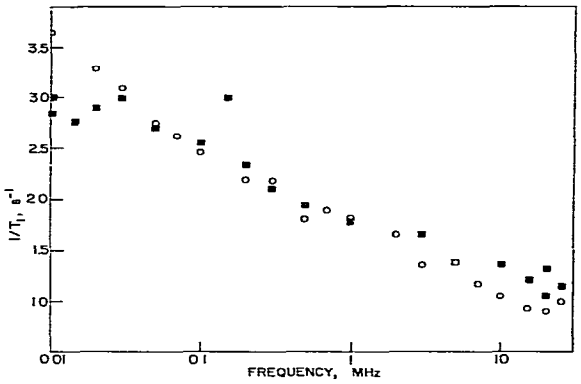

Fig. 2. ^{1}H-NMR longitudinal relaxation rates for water protons in monoclinic lysozyme crystals as a function of field at 278 (○) and 298 K (■).

4. Analysis and discussion

The longitudinal NMR relaxation rates observed in the present experiments represent a particular average of several relaxation rates that could be extracted from these samples. The water populations mix efficiently on the time scale of the relaxation time measurements; i.e., the water at the surface mixes with the water population that is directly observed, and these two populations have different characterizations. The longitudinal relaxation of water at the lysozyme surface has been studied independently in powder samples and requires at least three time constants for its characterization [8,9]: one for water relaxation, R_w; one for the protein proton relaxation, R_p; and one for the transfer of magnetization between these two groups of spins, R_t. These coupled spin systems yield a biexponential longitudinal relaxation where the observable decay constants are a mixture of these R_i. At room temperature, high water contents, and at the long times as fixed by the spectrometer sampling scheme, only the slowest longitudinal relaxation rates can be observed for this surface region which we will call $R_{s,slow}$. However, at high temperatures with the justified assumption that R_t is large compared with the other two rates, $R_{s,slow}$ becomes simply the weighted average of R_w and R_p:

$$R_{s,slow} = P_w R_w + (1 - P_w) R_p \quad (1)$$

where P_w is the number of water protons in the protein interface region divided by the sum of this and the number of protein protons. From the earlier work on high-frequency proton relaxation in lysozyme crystals [7], we may estimate P_w, since there are 0.23 g water/g lysozyme or 0.385 mol water protons/mol lysozyme protons that should contribute in this way. This gives P_w as 0.28. R_p has been measured at higher fields [9,10] for dry lysozyme so that knowledge of R_s permits calculation of R_w with these approximations once $R_{s,slow}$ is known.

$R_{s,slow}$ is not observed directly in the present experiments, since it averages with the relaxation rate of the remainder of the water present in the crystal. Since this averaging is fast we may write

$$R_{obs} = P_f R_f + P_s R_{s,slow} = \left(\frac{17}{40}\right) R_f + \left(\frac{23}{40}\right) R_{s,slow} \quad (2)$$

where the coefficients of R are derived from the resolution of two components in the relaxation observed at 30 MHz [7]. That is 23/40 of the water signal is identifiable with the surface region and the remainder not. We have no independent knowledge of R_f, the rate for the nonsurface water, but may estimate it as 0.5 s^{-1}. Substitution of this approximation and evaluation of the above expression for $R_{s,slow}$ gives

$$R_{obs} = 0.575 R_{s,slow} + 0.21 \quad (3)$$

The last term is small and a modest error in estimating R_f will not have a large effect on the subsequent analysis. Combining this result with eq. 1 gives

$$R_{obs} = 0.16 R_w + 0.415 R_p + 0.21 \quad (4)$$

Substitution of estimated values of R_p and R_w obtained from data taken at 30 MHz on drier lysozyme powders suggests that the analysis is at least consistent with the foregoing, although R_{obs} calculated in this way is about a factor of 2 larger than that shown in fig. 2. This error is in the direction expected, as drying the sample will increase R_w. This agreement is remarkable given the difference and frequencies in the water contents of the samples used in estimating these parameters.

The basic problem is summarized in eq. 4 regardless of the errors involved in assigning numerical values to the coefficients of R_w and R_p. The NMRD observed as $R_{obs}(\nu)$ may arise from the field dependence of either R_w or R_p or both. To date, experiments have not been reported that clearly separate the field dependence of these two quantities, as even the rotating-frame relaxation measurements usually made at lower temperatures suffer from the same mixture of relaxation parameters. The present field-cycling equipment does not permit direct observation of R_p as a function of field. The data of fig. 2, however, clearly demonstrate the existence of a dispersion in the NMR relaxation rate of the water protons in the protein crystal that signals the onset of motions beginning at about 10 MHz. This frequency range covered is sufficiently low that significant changes in R_p arising from fluctuation in the structure of the protein molecule may not be excluded. The motions implied by the onset of dispersion between 1 and 10 MHz fall in the time scale of tenths of microseconds to hundredths of microseconds. This is also exactly the time scale that has been suggested for the onset of averaging of water molecule anisotropic motion at the protein surface [9]. It should be noted that a possible source for additional dispersion in the crystal samples is the diffusion of the bulk water in the anisotropic environment of the crystal interior. This source is apparently responsible for the observation of ^2H quadrupole doublet structure in lysozyme crystals while it is not observed in powder or frozen solution samples [11].

In summary, the present experiments have shown clearly the onset of important motions in the protein crystal reported by the proton nuclear relaxation corresponding to time scales of the order of tenths of microseconds or faster. This result is consistent with but does not uniquely prove the hypothesis offered earlier that this time scale is that appropriate to isotropic averaging of locally anisotropic water molecule motions at the protein surface [9].

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