

Structural and Dynamical Examination of the Low-Temperature Glass Transition in Serum Albumin

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ABSTRACT The nuclear magnetic transverse decay and the proton second moment of bovine serum albumin samples dry and hydrated with different water isotope compositions show that at temperatures around 170 K, there is a dramatic change in the dynamics of the water associated with the protein interface. By comparison, observation of the protein protons when hydrated with deuterium oxide provides no evidence for significant dynamical changes near 170 K. The proton second moment of the hydrated protein shows that the protein structure becomes more open with increasing hydration from the lyophilized condition and that the side chains extend from the protein surface into the solvent in the hydrated but not the dry cases. The proton second moment of serum albumin hydrated with H₂O increases dramatically with decreasing temperature near 170 K, demonstrating that the water forms a rigid solid around the protein which effectively fills the surface irregularities created by the protein fold. Solvation with dimethyl sulfoxide yields small effects compared with water.

INTRODUCTION

Protein motions are essential for function, yet details concerning functional dynamics remain elusive. Experimental (1–9) and computational (10–18) studies show that proteins exhibit a transition that is characterized by a dramatic change in the amplitude and physical character of atomic fluctuations at temperatures between 160 and 220 K. These changes have several names, including the dynamical transition and the glass transition (17–19). Various arguments have connected the transition to solvent participation (5,7,13,15,17); however, it remains unclear how the solvent affects the protein energy landscape and which motions are activated or quenched with increasing or decreasing temperature.

Water-protein interactions as characterized by nuclear magnetic resonance have been reviewed by several authors (20–26). Long-lived water molecules that are bound sufficiently long to experience the rotational motion of the protein are rare, of the order of 1–3 molecules/10 kD protein. Nevertheless, these long-lived molecules exchange with bulk water molecules in a time that is short compared with 100 μ s and are responsible for carrying the dynamical information about the protein to the bulk water. It is these long-lived water molecules that are critical for determination of the T_1 based contrast in magnetic imaging of tissues (27–29). The vast majority of water interacts with the protein at the surface and these surface water molecules are dynamically very mobile. The local translational diffusion constant is difficult to measure accurately, but spectroscopic approaches that provide a measurement integrated over the first 10 Å from the surface give a value that is approximately three times smaller

than that of the bulk water (30). Further, the general absence of water-proton to protein-proton nuclear Overhauser effects for surface water demonstrates that the surface dynamics are characterized by correlation times ≤ 100 ps at room temperature (31–33).

For all samples studied here, the protein is best described magnetically as a solid in that the global rotational motion of the protein is stopped. Under these conditions, the proton dipolar couplings are not averaged by rapid rotational reorientation and the ¹H NMR spectrum consists of a single line ~ 30 kHz wide. Although high-resolution spectroscopy is lost, by exploiting the difference in the protein and solvent transverse magnetization decay rates, as well as controlling the isotopic composition of the solvent, the dynamic behavior of the solvent and the protein may be monitored separately to provide new and complementary information to that available from earlier NMR measurements (4) neutron scattering (1–3,5,7), Mossbauer spectroscopy (34), x-ray diffraction (35), and steady-state optical studies (6). Here we report proton NMR studies of immobilized bovine serum albumin (BSA) over the temperature range 150 K to 302 K for dry protein and protein solvated with H₂O, D₂O and dimethylsulfoxide (DMSO)-d₆. As temperature decreases, the proton NMR spectrum reports significant structural and dynamical changes associated with the water resonance in the temperature range usually associated with the protein dynamical transition. Similar experiments in D₂O show that a number of protein side chains become trapped in an extended conformation in the D₂O glass and are magnetically distinguishable from the main protein-proton population. We also report results of solvating the protein with DMSO-d₆ that are qualitatively different from those in the presence of water.

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EXPERIMENTAL PROCEDURE

Bovine serum albumin was obtained from Sigma Chemical (St. Louis, MO) and dialyzed against at least five changes of deionized water. The protein was lyophilized using a mechanical vacuum at 337.8 K. Solvated samples were prepared by adding the desired mass of solvent, such as deionized water, deuterium oxide (99.9 atom % D, Cambridge Isotope Laboratories, Andover, MA) or dimethyl- d_6 sulfoxide (99.9 atom % D, Sigma Chemical), to a known mass of protein. Solvated protein samples were allowed to equilibrate for at least three days at 310 K. The amount of moisture in the hydrated BSA sample was additionally checked by a Karl Fischer titrator (Aquatest 8, Photovolt Instruments, Indianapolis, IN). The solvated protein samples used in this study were prepared to contain 0.32 g water/g of total sample mass.

For BSA samples prepared with D_2O , 1 g of BSA was dissolved in 20 mL of D_2O and stirred at 325 K for 4 h, then transferred to a Centricon filter (30,000-mol wt cutoff, Millipore, Bedford, MA) and concentrated to 5 mL in the centrifuge. The concentrated solution was diluted again to 20 mL with D_2O and the procedure repeated four times to minimize the number of exchangeable protons remaining on the protein. Finally, the protein was lyophilized and dried at 337.8 K using a mechanical vacuum.

The nuclear magnetic resonance data were recorded using a fast-field cycling NMR spectrometer FFC-2000 (Stelar, Mede, Italy). The Stelar spectrometer provides temporal control of the magnetic field that permits the measurement of the magnetic field dependence of spin-lattice relaxation rate constants (36), which will be described in a separate publication. Here we focus on the implications of the transverse decay data. In these experiments, the field-switching time used was 3 ms. Spins were polarized at 30 MHz and free induction decays were recorded after a single (6.7- μ s) 90° excitation pulse applied at 15.8 MHz. Temperature was varied from 150 K to 302 K using the Stelar VTC90 variable temperature controller, which was calibrated using an external thermocouple inserted into a surrogate sample at the resonance position in the probe. Based on repeated calibrations, the temperature in all NMR experiments was controlled to 0.5 K. Samples were allowed to equilibrate for at least 20 min at each temperature before data acquisition.

RESULTS AND DISCUSSION

Transverse decay

The transverse magnetization decay, or free induction decay (FID), of solvated serum albumin samples at 302 K is multi-component, as illustrated in Fig. 1. In the solvated samples, the component with the shortest decay-time constant is characteristic of the solid protein, and similar decays are detected on the dry protein.

The components that decay more slowly include contributions from the water protons and protein-side-chain pro-

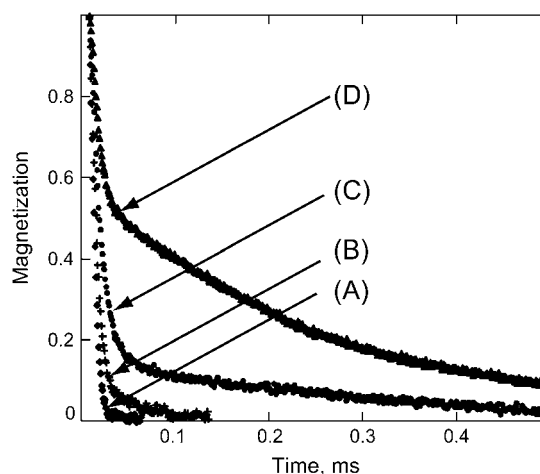


FIGURE 1 The proton transverse magnetization, normalized to the initial point in the decay, as a function of time for bovine serum albumin samples at 302 K: (A) lyophilized dry BSA (\blacklozenge); (B) BSA solvated with 32 mass % DMSO- d_6 (+); (C) BSA hydrated with 32 mass % D_2O (\bullet); (D) BSA hydrated with 32 mass % H_2O (\blacktriangle).

tons that are sufficiently mobile to experience significant motional narrowing. The fast motions of some protein side chains partly average the local dipolar couplings and increase the decay time significantly so that these side-chain protons are not easily separated from the water protons in the hydrated BSA sample. However, the side-chain-proton contribution is clearly apparent when the solvent is D_2O . A small amount of adventitious water in deuterated BSA- D_2O and BSA-DMSO- d_6 samples cannot be excluded, but it is estimated to be $<0.2\%$ of the solvent content and essentially unimportant as a contribution to the slowly decaying transverse magnetization. At ambient temperature, the observed slow component of the decay for BSA rehydrated with D_2O accounts for 10% of total magnetization (Fig. 1); for BSA solvated with DMSO- d_6 , the slow component accounts for 5% of total magnetization. In both cases, these slow components reflect the motional averaging of dipolar interactions caused by some side-chain flexibility.

It is important to note that, although the slowly decaying components in the hydrated samples are dominated by essentially liquid water and some motionally averaged protein-side-chain protons, the transverse decay may be accelerated because of local magnetic-field inhomogeneity caused by the imperfect packing of the protein in the sample and inhomogeneity of the applied magnetic field. Thus, the slow decay constants for these samples do not reflect the usual transverse decay time, T_2 , which could be recovered using a spin-echo experiment. Rather, the decay constants may be denoted T_2^* ; however, none of the conclusions would change if an echo experiment were used to detect the slow components instead.

The components of the decay may be easily separated and the contributions from slowly decaying, or “liquid”, and nonrotating, or “solid”, protons characterized independently.

Although there are well-documented procedures for handling spectral components such as Gaussian and Lorentzian components for a single spectral line (37,38), the present samples generally are sums of Gaussian components and not directly amenable to these methods. Because of the solid character of the samples and the fact that they are both dynamically and magnetically inhomogeneous, we treated the transverse decay of the solvated samples as a sum of Gaussian components. The more slowly decaying or liquid component of each FID was analyzed and fitted first using the equation

$$M(t)_{xy}^{\text{liquid}} = M(0)_{xy}^{\text{liquid}} \times e^{-\frac{(\Delta_G^{\text{liquid}})^2 t^2}{4}} = M(0)_{xy}^{\text{liquid}} \times e^{-\frac{M_2 t^2}{2}}. \quad (1)$$

Here, M_{xy}^{liquid} is the transverse magnetization of the liquid component, t is time, and Δ_G^{liquid} characterizes the width of the Gaussian line, and M_2 is the second moment of the line. The fit of the slow component was subtracted from the total FID to extract the rapidly decaying or solid-protein decay, which was fitted in the same manner (Eq. 1). The second moment was extracted directly from the magnitude of the coefficient of t^2 in the fit of the transverse decay.

As illustrated in Fig. 1, the dry albumin sample yields a single fast Gaussian decay at 302 K, which is a manifestation of the relative rigidity of the dry protein. The transverse relaxation time constant for dry BSA at this temperature is $8.8 \mu\text{s}$, whereas that for the solid component of the hydrated albumin sample is $12.6 \mu\text{s}$. These numerical values are consistent with the previously reported results for dry and hydrated protein systems (20,39–42).

The relative amount of liquid and solid component in different BSA samples was calculated from the zero-time amplitudes of the transverse decay components, $M(0)_{xy}^{\text{liquid}}$ and $M(0)_{xy}^{\text{solid}}$, obtained from the procedure described above. The solid proton fraction is shown as a function of temperature for BSA hydrated to 32% by mass with H_2O and D_2O in Fig. 2. For the sample hydrated with H_2O the liquid component at 302 K accounts for 40% of the total magnetization intensity. For this sample, there is a dramatic decrease in the fraction of liquid protons with decreasing temperature; at ~ 170 K, the slowly decaying or liquid component of the transverse decay is no longer observable. This signal loss is attributed to a large decrease in the molecular motions of water and the formation of a disordered solid phase, or glass. All protons in the solid dephase rapidly following an excitation pulse, and the transverse decay is characterized by a single Gaussian function, as shown in Fig. 3. Below this transition, the water protons and the protein protons are magnetically indistinguishable; there is a single transverse decay and longitudinal decay, which requires that the hydrogen spins are similarly immobilized and form a solid of essentially uniform hydrogen-atom density in which the spins are strongly coupled. Below 170 K there is no evidence for residual water molecule motion in any environment around the protein that causes motional averaging of the

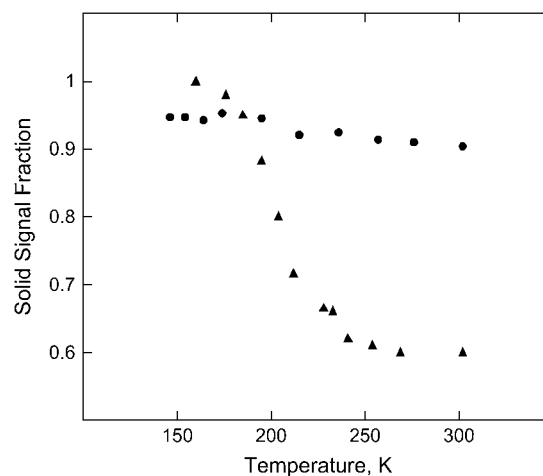


FIGURE 2 The fraction of the total proton transverse magnetization decay that is rapid as a function of temperature for BSA samples hydrated to the level of 32 mass % H_2O (\blacktriangle) and D_2O (\bullet). For the dry protein, the solid fraction is uniformly one throughout this temperature range.

^1H - ^1H dipolar couplings. An estimate of the time scale over which this rigidity is sensed is the magnitude of the transverse decay time constant, which is somewhat less than $6 \mu\text{s}$.

The temperature dependence of the observable proton transverse decay is different when the protein is hydrated with D_2O instead of H_2O . In the deuterated solvent, only the protein protons are observable. At 302 K, the sample hydrated with D_2O has a liquidlike or slowly decaying transverse component which accounts for $\sim 10\%$ of the magnetization. This component is ascribed to protein-side-chain protons that have sufficient local motion to cause motional narrowing of the line. Within the experimental error, this

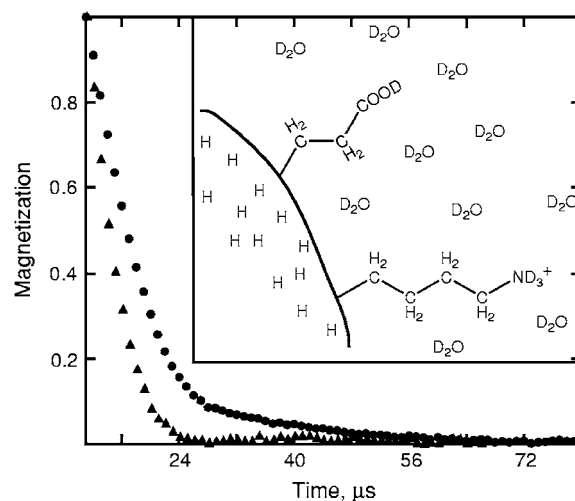


FIGURE 3 The proton transverse magnetization of bovine serum albumin normalized to the initial point in the decay as a function of time for BSA samples at 154 K: \bullet , 32 mass % D_2O ; \blacktriangle , 32 mass % H_2O . (Inset) Schematic representation of protein-surface-side-chain protons magnetically isolated in the D_2O glassy matrix.

measurement is consistent with BSA-32% H₂O data, where slowly decaying fraction of the magnetization consists of 32% H₂O and ~8% of motionally averaged protein-side-chain protons. Thus, above the glass transition, some of the protein side chains are sufficiently mobile to contribute to the slowly decaying proton population. Although not isotropically averaged, as in a solution, there is considerable side chain motion that is facilitated by the solvent. We note here that previous studies (21,43) have shown that there are 25 ± 4 molecules of water bound to BSA for a time longer than the rotational correlation time of 41 ± 3 ns of serum albumin in the dilute protein solutions. If these sites are occupied at 32 wt % hydration and their local reorientational motions are minimal, i.e., the order parameter is close to unity, these water molecule signals will appear in the solid fraction of the BSA-H₂O transverse magnetization decay; however, when D₂O is used, there will be no contribution from these sites. This effect can be a source of a small quantitative difference when solid and liquid fractions of the samples hydrated with H₂O versus D₂O are considered.

The temperature dependence of the proton transverse decay for serum albumin samples hydrated with D₂O differs from that of the samples hydrated with H₂O in a fundamental way, although the thermodynamic and dynamic state of the system is practically identical. As shown in Figs. 2 and 3, unlike the H₂O case, a slowly decaying component in the D₂O sample persists even at 154 K. This slowly relaxing component is not caused by residual HOD in the D₂O, which contributes only a very small fraction to the total observed signal at a much higher temperature, as noted earlier. Rather, the magnitude is consistent with a contribution from protein side chains that extend into the D₂O glass and are magnetically isolated from other protons, as shown schematically in the inset to Fig. 3. This point may be understood by considering the second moment for the observed protons, which, in the case where the decay is Gaussian, is directly related to the parameters of the transverse magnetization as shown in Eq. 1 (44).

Second moment

The second moment, M_2 , arising from dipole-dipole coupling of nuclear spins may be written (44) as

$$M_2 = M_2^{\text{like}} + M_2^{\text{unlike}}$$

$$= \frac{3}{4} \gamma_I^4 \hbar^2 I(I+1) \frac{1}{N} \sum_{j < k} \frac{(1 - 3\cos^2 \theta_{jk})^2}{r_{jk}^6} + \frac{1}{3} \gamma_I^2 \gamma_S^2 \hbar^2 S(S+1) \frac{1}{N} \sum_{j < k} \frac{(1 - 3\cos^2 \theta_{jk})^2}{r_{jk}^6}, \quad (2)$$

where γ is the magnetogyric ratio, \hbar is Planck's constant, N is the number of nuclei per unit cell, \vec{r}_{jk} is the radius vector between nuclei j and k , and θ_{jk} is the angle between the applied magnetic field and radius vector \vec{r}_{jk} . In Eq. 2, the contributions from both like and unlike spins are included.

The symbol I is used for the reference spins and S for the ones that influence the reference spins. Each term in sums of the Eq. 2 describes a contribution of the k th spin to the local field at spin j . The larger the sum in Eq. 2, the larger the second moment becomes and, therefore, the shorter the transverse decay time becomes. If a protein side chain extends into the water glass at low temperature, the side-chain protons have contributions to the second moment dominated by nearest neighbor side-chain protons as well as immobilized water protons, which are present at essentially the same packing density as the protein protons in the remaining solid protein. However, if the protein-side-chain protons are surrounded by water deuterons, the contributions to the sums in Eq. 2 are much smaller because the deuteron has a much smaller magnetic moment than the proton. Thus, comparison of the transverse decay for the lowest-temperature hydrated protein samples shows that the protein side chains in the hydrated cases extend into the solvent, which becomes a solid glass at low temperature. However, in the dry protein, the transverse decays of the side-chain protons are not distinguishable from the remaining protein protons. Therefore, unlike the hydrated case, the second moment of the side-chain protons is the same as that for the bulk of the protein protons, which means that the side chains are collapsed onto the surface. If the side chains extended away from the surface in the absence of a protonated solvent matrix, they would be magnetically isolated and yield a slowly decaying component, as seen in the D₂O sample at low temperature.

In this context, it is interesting to treat the protein with a nonaqueous solvent. Data are shown in Fig. 1 for serum albumin solvated with dimethylsulfoxide- d_6 at 32 mass %, which corresponds to significantly fewer solvent molecules per protein than in the hydrated cases. Nevertheless, this low level of solvation permits observation of a small, slowly decaying transverse magnetization at 302 K, as shown in Fig. 1. Unlike the D₂O case, this slowly decaying signal is lost at ~200 K. Therefore, at this level of solvation by DMSO, the protein-surface side chains are not magnetically well isolated, and therefore, are not extended from the protein surface below 200 K.

The proton second moments obtained from the transverse decays for dry BSA and the rapidly decaying components of the BSA samples solvated with 32% H₂O, D₂O, and DMSO- d_6 are shown as a function of temperature in Fig. 4. The second moment of the dry BSA sample exhibits weak monotonic temperature dependence, increasing ~25% as the temperature is changed from 302 to 154 K. This increase must result from a decrease in the local motion of the protein protons or a slight contraction of the total effective structure that affects the sums in Eq. 2. Upon addition of 32 mass % DMSO- d_6 to the protein, the second moment of the solid-protein protons is reduced slightly at 302 K as compared to the dry BSA, which is consistent with minor changes in the average local flexibility in the protein. However, at temperatures below ~200 K, the slow transverse magnetization component is lost and the second moment is practically

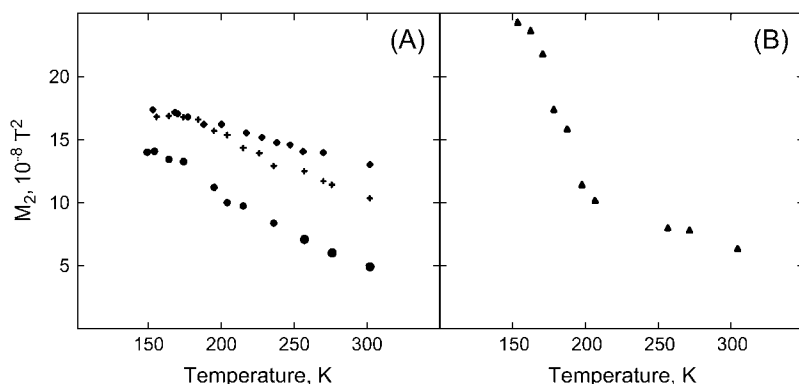


FIGURE 4 The proton second moment as a function of temperature for bovine serum albumin samples. (A) lyophilized BSA (\blacklozenge); BSA solvated with 32 mass % DMSO- d_6 ($+$); BSA solvated with 32 mass % D $_2$ O (\bullet). (B) BSA solvated with 32 mass % H $_2$ O (\blacktriangle).

identical with that of the dry protein. Therefore, structural changes in the protein are not large at this level of DMSO solvation because the sums in Eq. 2 do not change significantly.

When BSA is hydrated with either H $_2$ O or D $_2$ O, however, the second moment decreases by a factor of >2 at 302 K as compared to the dry sample. There may be two contributions to the second moment change. Local motional averaging of the dipolar couplings are well known to reduce second moments in solids, as noted for the dry case (44). In addition, the second moment may change if the structure of the protein changes upon hydration, because the sums in Eq. 2 depend on the inverse sixth power of internomment distances. There is compelling evidence from the magnetic-field dependence of the proton spin-lattice relaxation rate constant that the proton distribution in space changes when the protein is hydrated (29), and our observations of the second moment changes are consistent with contributions from hydration-induced structural changes. On hydration, the second moment decreases relative to the dry solid even at 150 K in the D $_2$ O glass. Therefore, the hydrated structure is more open, leading to smaller sums in Eq. 2. In addition, water is a good solvent for the protein and permits some side chains to extend from the surface as discussed above. The added local mobility may also propagate into the protein structure and reduce the second moment by increased local motion. However, this motion is reduced with decreasing temperature and is unimportant below 170 K. The temperature dependence in the protein-proton second moment shown for the sample in D $_2$ O demonstrates that these changes are not large.

The second moment for the albumin sample hydrated with H $_2$ O shows a dramatic transition at ~ 170 K, as the liquid fraction of the transverse decay is lost (Fig. 4 B). The second moment of the albumin samples hydrated with H $_2$ O at 150 K is larger than that for the dry protein at the same temperature, even though the D $_2$ O sample shows that the protein-proton second moment isolated in solvent deuterons is actually somewhat smaller than for the dry case. This observation results because when the H $_2$ O becomes immobilized at the protein surface, the water spins fill in the spaces and add immobilized protons to the sum in the second moment. The transition in the proton second moment is then largely

associated with the water spins, which become rotationally immobilized with decreasing temperature. We note that this water immobilization occurs at a temperature well below the freezing point of water. Indeed, it is an old observation that frozen protein solutions at 250 K exhibit a well-characterized liquid-water component in the NMR spectrum, even though the proteins are locked in an ice matrix and do not rotate (45–49). The water in protein crystals behaves similarly, as indicated previously by the NMR spectrum at 298 K and 250 K (50,51).

The dynamical behavior observed for the hydrated BSA is in agreement with previous reports by Doster et al. (2,3) on the temperature dependence of the mean-square displacement, although the temperature dependence of the second moment for the protein in D $_2$ O does not show a significant change in the region of the glass transition. One could make a case for a very small change, which is on the order of experimental error near 170 K; however, we are reluctant to argue that this is significant, even though the neutron diffraction experiments show that the dependence of the mean-square displacement with temperature changes with this transition. In part, this apparent discrepancy between the two methods derives from the long time average of the atom positions that is sensed by the dipolar couplings of Eq. 2. An increase in the mean-square displacement does not change the time-average internomment distance that enters Eq. 2 for the second moment. The increase in the mean-square displacement changes a little bit the vibrational averaging of the dipolar coupling, which is a complex issue because the dipolar coupling is not a linear function of distance (52,53). Thus, no dramatic changes in the second moment in Fig. 4 are expected, even though there are clear changes in the mean-square displacements with temperature. Therefore, a major factor in the dynamics of the protein solvated with water is the formation of a water glass at the interface over the range of temperatures associated with the dynamical transition.

CONCLUSIONS

Measurements of NMR transverse magnetization decay in dry and variously solvated protein systems provide a simple

and efficient means of examining structural and dynamical changes in the protein-solvent system as a function of temperature. The use of isotope substitution permits separation of contributions from the protein and the solvent protons in the system that is largely characterized as a solid. Comparisons of transverse magnetization decays show that the solvation by water supports some protein side chains in the solvent and releases them from the dense packing on the surface experienced in the dry state. The temperature dependence of the proton second moment in the dry protein shows that there are small incremental changes in the local dynamics and structure of the protein as the temperature is lowered to 150 K; however, there is no direct evidence for large dynamical changes in the dry protein. Even though some side chains of bovine serum albumin solvated with DMSO- d_6 exhibit an increased mobility at temperatures above 200 K, this mobility is lost below 200 K. In addition, the proton second moment is the same as that of the dry protein, so that structural changes on solvation by DMSO are not detectable by this measure. On the other hand, the magnitudes of the proton second moment show that the structure of the hydrated protein is different from that of the dry protein and that the effective interproton distribution in space is less dense in the hydrated state compared with the dry state. In hydrated protein systems at room temperature, there is a significant liquid component that is lost only when the temperature is well below 273 K. The significant change in the proton second moment at low temperatures shows that water forms a disordered solid at the protein interface below ~ 170 K which reduces the effective local motions of both main-chain and side-chain portions of the protein.

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