A Solid-State NMR Study of Protein Hydration and Stability

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Purpose. The mobility of protein in powders at different hydration levels was studied in relation to aggregation and activity.

Methods. Magic angle spinning ^{13}C, ^{15}N, ^{1}H, ^{2}H, and ^{17}O NMR techniques were used to determine changes in the mobility of surface residues in proteins as a function of hydration and related to changes in activity. NMR relaxation measurements of high frequency (ω_0, T_1) and low frequency (ω_1, $T_{1\rho}$) motions have been carried out on lyophilized DNase, insulin and lysozyme stored at different relative humidities. Moisture-induced aggregation and enzymatic activity of the lyophilized proteins was determined by high performance size exclusion chromatography and bioassays.

Results. There was little change in $T_{1\rho}$ observed with increasing humidity. The results show, however, that there is a decrease in T_1 for DNase, insulin and lysozyme at relative humidities ranging from 0–98%, and we propose that the reduction in T_1 is related to the aggregation susceptibility of proteins during storage at different humidities. The water mobility was determined directly using ^{17}O NMR experiments. We found that as the amount of weakly-bound water increases, the protein surface mobility decreases and is coupled with increased aggregation. Aggregation measurements at different humidities were correlated with bioassays for lysozyme and found to be consistent with the hydration data.

Conclusions. Mobility of protein molecules was determined by solid-state NMR over a wide range of % RH and it was found that water content leads to a change in mobility of protein molecules. The aggregation and activity of proteins were strongly correlated to change in molecular mobility.

KEY WORDS: NMR relaxation time; protein mobility; bound water; activity; aggregation.

INTRODUCTION

Proteins have recently been found increasingly useful in therapeutics, diagnostics and biochemical applications (1). However, proteins in the dried form often have limited shelf life. The residual water on the surface of charged and polar groups and on the peptide backbone can cause the protein to be unstable and degrade (2,3). Aggregation, deamidation and oxidation are among the most common pathways for protein degradation. Evidence in a recent report on insulin suggests that moisture-induced aggregation of freeze-dried proteins in the solid-state results in loss of activity (4). Thus it is important to understand the mobility and reactivity of protein molecules with increasing water content.

Solid-state NMR is a technique that allows us to study the mobility of proteins as dry powders. NMR relaxation measurements allow us to measure the mobility of protein molecules, bound water molecules, surface groups and exchangeable groups with deuterium; and relate protein mobility to its stability and aggregation (5). Relaxation times such as T_1, spin-lattice or longitudinal (ns-μs timescale), $T_{1\rho}$, spin-lattice in rotating frame (μs-ms) and T_2, spin-spin or transverse (ms-s) have been measured for solid polymers (6). The correlation time for molecules with low mobility is difficult to relate to the relaxation measurement. However, T_1 is dominated by dynamics on the ω_0 timescale (MHz since ω_0, the Larmor frequency is proportional to the magnetic field), $T_{1\rho}$ is related to the spin-locking field ω_1 (kHz), and T_2 reports on the slow molecular motions (primarily 0 Hz and also ω_0).

We have chosen to study the mobility of three important proteins: insulin, lysozyme, and DNase, by solid-state NMR. Insulin serves as a stimuli to both cell growth and metabolism and is involved in control of blood glucose (7). Lysozyme controls the growth of susceptible bacteria and modulates host immunity against infections and depression of immune response (8). Recombinant Human Deoxyribonuclease type 1 (DNase) is used for the treatment of cystic fibrosis (9). The NMR relaxation parameters of these proteins were measured at various relative humidities, and these results correlated with bioactivity data.

MATERIALS AND METHODS

Materials

Recombinant Human Deoxyribonuclease type 1 (DNase) was a gift from Genentech, South San Francisco, CA. Insulin (Sigma I-5500) and lysozyme (Sigma L-6876) were used as purchased. The DNase and insulin contained no buffer salts but the lysozyme contained 5% weight buffer salts of sodium acetate and sodium chloride. Relative humidities (RH) used in this project were 0% RH (P_2O_5, to dry the protein) and 20, 38, 53, 69, 79, and 98% using saturated salt solutions (to control the % RH at room temperature) of CH_3COOK, NaI, $Mg(NO_3)_2$, KI, NH_4Cl, and K_2SO_4 (10) respectively. The water used to dissolved the salts included H_2O (distilled water), D_2O (with 99.9% ^{2}H) and $H_2{}^{17}O$ (with 20% ^{17}O, purchased from CIL Inc., Andover, MA).

Preparation of Lyophilized Proteins

The water content of the lyophilized proteins after storing at 0% RH for 8 days, was determined by Karl Fischer (KF) titration and cross-checked using both thermogravimetric analysis and water desorption measurement under dry nitrogen. The mean water content of each of the proteins was less than 0.5% weight which was not distinguishable from the background variation of the KF titration. Protein samples were equilibrated to different water contents by storing over saturated salt solutions at different % RH in a dessicator for 5–6 days. The results indicated that sample weight increased until equilibrium was achieved in ~3 days.

Proteins powders were then packed into Si_3N_4 rotors (Doty, Columbia, SC) with sample weights of ~54 mg DNase, ~50 mg insulin and ~74 mg lysozyme.

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Determination of Protein Aggregation and Enzymatic Activity

Aggregation of each of the proteins was determined by size exclusion HPLC on a Protein-Pak 125 column (Waters) at an isocratic flowrate of 0.5 ml/min. The samples were reconstituted in deionized water by gentle shaking for 10–20 mins to ensure dissolution. After centrifugation for 15 mins at 15,000 rpm, the supernatants were taken for HPLC analysis. The mobile phase was 50 mM sodium phosphate buffer at pH 6.24 with 150 mM sodium sulfate for lysozyme; 0.5 mM HEPES buffer, 150 mM NaCl and 1 mM $CaCl_2$ titrated to pH 7.0 for DNase; 50 mM sodium phosphate buffer at pH 3.0 with 300 mM sodium chloride for insulin. Absorbance was monitored at 280 nm. The percent of soluble aggregates was calculated by comparing the aggregate peak area with that obtained from a standard at the same concentration. The percent insoluble aggregates was estimated from the difference in the total peak area between the sample and the standard of each protein.

Lysozyme activity against *M. luteus* was measured turbidimetrically at 450 nm in 65 mM potassium phosphate buffer at pH 6.24 (11). The bacterial cell lysis was carried out in a reaction volume of 2.6 ml at 25°C. For each analysis a three-point standard curve was obtained to cover the linear range of 20–40 units of enzymatic activity.

^1H, ^2H, ^{13}C, ^{15}N, and ^{17}O NMR Measurements

Solid state NMR spectroscopy was carried out on an Inova 300 Varian spectrometer (Varian, Palo Alto, CA), operating at 75.453 MHz for ^{13}C, 300.040 MHz for ^1H, 46.051 MHz for ^2H, 30.398 MHz for ^{15}N and 40.670 MHz for ^{17}O. Acquisition and processing of data were performed using Varian software. The dynamics of protein molecules were measured by ^{13}C and ^{15}N CPMAS (Cross Polarization Magic Angle Spinning) NMR techniques to measure high frequency (ω_0, T_1) and low frequency (ω_1, $T_{1\rho}$) motions. Cross polarization is used to enhance the signal intensity of low abundance and magnetic moments nuclei by a factor of up to 4 (12). It can also decrease the signal acquisition time because the repeat cycle of the experiment is determined by the proton relaxation time, which is faster than carbon relaxation (13). Magic Angle Spinning is a technique that is used to remove chemical anisotropy (6,12) and suppress the dipole-dipole interaction (6,13). The ^{13}C and ^{15}N NMR spectra were obtained using cross polarization (CP) from protons. Samples were spun at the magic angle, at spinning speeds of 6–7 kHz.

The acquisition time used in ^{13}C and ^{15}N $T_{1\rho}$ experiments was 0.068 s, delay time 2.5 s and 2 s with a proton $\pi/2$ pulse width of 4.4 μs and 7.5 μs for ^{13}C and ^{15}N respectively, and number of scans 1024. The $T_{1\rho}$ relaxation times were determined by fitting an exponential curve of the signal intensity versus various τ values (where τ is the variable delay time and is the length of the proton spin-locking pulse). The equation that is used to then determine the relaxation time $T_{1\rho}$ is $I = I_0 \cdot e^{-\tau/T_{1\rho}}$.

T_1 was measured using the inversion-recovery (π-τ-$\pi/2$) pulse sequence with $\pi/2$ (pw) = 4.4 μs, 7.5 μs for ^{13}C and ^{15}N respectively, and number of scans = 2048. T_1 values were then determined by fitting an exponential curve to a plot of signal intensity against τ. The signal intensity and τ are exponentially related by the equation, $I = I_\infty (1 - 2e^{-\tau/T_1})$ or $\ln (A_\infty - A_\tau) = \ln 2A_\infty - \tau/T_1$, where A is the height of the spectral line, and A_∞ is the limiting value of A, measured using a very long interval between the π and $\pi/2$ pulse (13,14).

Single pulse experiments were used for ^2H, ^1H, ^{13}C, and ^{17}O spectra to study the bound water molecules, with $\pi/2$ pulse = 9, 5, 4.4, and 6.5 μs respectively, and repetition time = 0.5 s (except for ^{13}C 2 s). The ^{17}O spectra were acquired using water to reference the chemical shift position (0 ppm) of ^{17}O.

RESULTS AND DISCUSSION

Dynamics of Protein

The mobility of proteins and protein surface groups were determined by $T_{1\rho}$ and T_1 experiments using ^1H, ^{13}C, and ^{15}N nuclei. Both $T_{1\rho}$ and T_1 experiments were carried out over a wide range of relative humidities. For the same % RH, ^{15}N relaxation times, T_1 and $T_{1\rho}$, were found to be similar to those for ^{13}C (e.g., for lysozyme at 0% RH, ^{15}N and ^{13}C relaxation times were 0.68 s and 0.80 s for T_1; and 6.54 and 6.95 ms for $T_{1\rho}$, respectively). This confirms that relaxation (of whole protein molecules and surface groups on the proteins) is mainly due to the protons in CP experiments. In a CP experiment, magnetization is transferred from the abundant nuclei (protons) to the rare nuclei (^{13}C or ^{15}N) and thus the relaxation properties of the protons are primarily reported by the measurement. The CP experiment combined with MAS resolves the resonances from different groups giving better resolution than direct proton NMR experiments. Direct ^1H relaxation results, shown in Table I for insulin 0.7 s at 98% RH, were found to be similar to those for direct ^{13}C T_1 measurements which confirms that the relaxation is mainly due to "immobilized" protons. The similar T_1 results obtained for different nuclei, suggest relaxation is due to the motion of nuclei (protons) from different environments (interface of the protein and the water surface).

^{13}C CPMAS T_1 and $T_{1\rho}$ of protein were measured over a wide range of % RH. A $T_{1\rho}$ experiment is illustrated in Figure 1. The peak heights of each resonance were measured for the carboxylated group (C=O) region (175 ppm), the methine (CH) region (50 ppm) and methyl (CH_3) region (15 ppm) and plotted versus the delay times. It was found that as the % RH increased, $T_{1\rho}$ remained relatively constant, suggesting that the molecular motion on the kHz timescale, most likely due to motion of whole protein molecules or molecular segment, is not greatly affected by the number of water molecules that are bound on the surface up to 98% RH. This is shown in Figure 2 where the average $T_{1\rho}$ for C=O, CH, CH_3 groups versus % RH was 5–7 ms.

The T_1 experiments were measured for a range of different % RH values. The peak heights (intensity) of each resonance versus τ were plotted to derive the value of the relaxation time (Figure 3). With increasing hydration of the proteins it was

Table I. Direct ^1H and ^{17}O T_1 Relaxation Measurements at 98% RH

Protein	^1H T_1 (s) ± 5%	^{17}O T_1 (ms) ± 5%
DNase	0.85	0.33
Insulin	0.71	1.69
Lysozyme	0.80	0.19

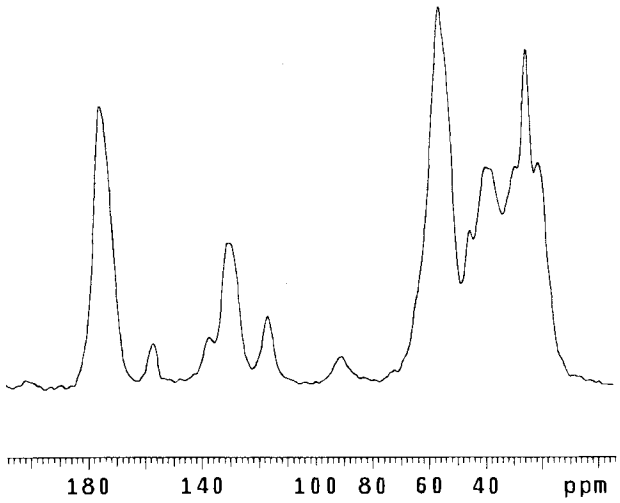

Fig. 1. ^{13}C CPMAS $T_{1\rho}$ spectra of insulin at 0% RH with delay time (τ) 0.5 ms.

found that T_1 decreased, as shown in Figure 4. The decrease in T_1 values of the proteins as a function of RH (%) was similar with gradient $\sim 4.2 \times 10^{-3}$ s (% RH)$^{-1}$ for a wide range of molecular weight proteins (DNase ~ 31,000 molecular weight, insulin ~ 6,000 and lysozyme ~ 14,400 (15).

It was found that as the amount of water increases, T_1 is decreased which is most likely due to changes in dynamics of surface groups on the protein. Water is mobile and has a fast relaxation rate (R) 0.39 sec^{-1} ($R = 1/T_1$), i.e., T_1 of 2.56 s (16) compared with the proton T_1's for dry protein 0.8 to 1 s found in this study. Water bound to protein surfaces may cause the protein surfaces groups to relax more rapidly as the number of protons increase. When water molecules bind to the protein molecules, their motion is restricted resulting in decrease in the overall mobility of the water. As the amount of water molecules bound to protein increases, the T_1 is decreased as the mobility of protein surface groups is altered, possibly due to an increase in hydrogen bonding on the surface of the protein that restricts the mobility of the proteins on the MHz timescale.

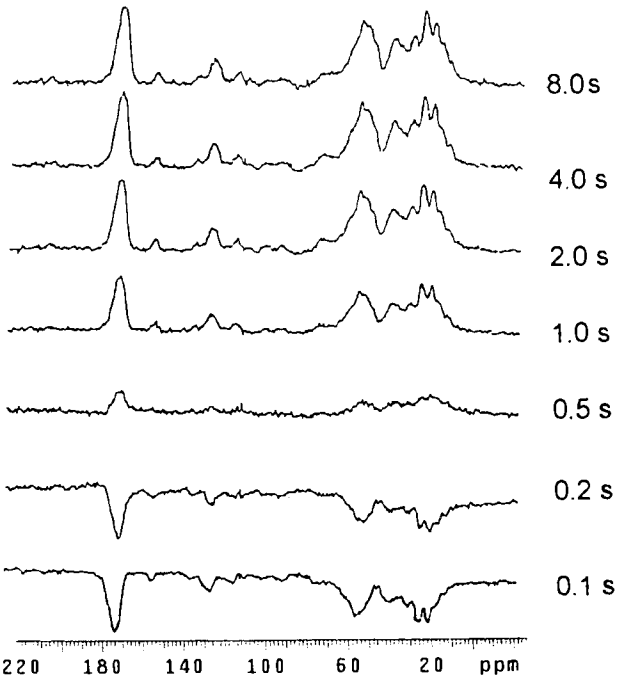

Fig. 3. ^{13}C CPMAS inversion recovery T_1 spectra of DNase at 98% RH with delay times (τ) 0.1, 0.2, 0.5, 1, 2, 4, 8 s with a spectral frequency range of 225 ppm to −25 ppm.

Effect of Isotopic Substitution in Water on Relaxation

T_1 and $T_{1\rho}$ values depend on many factors, including the state of the sample (solid, liquid or gas), molecular size, mobility, temperature, solvent and the presence and nature of impuri-

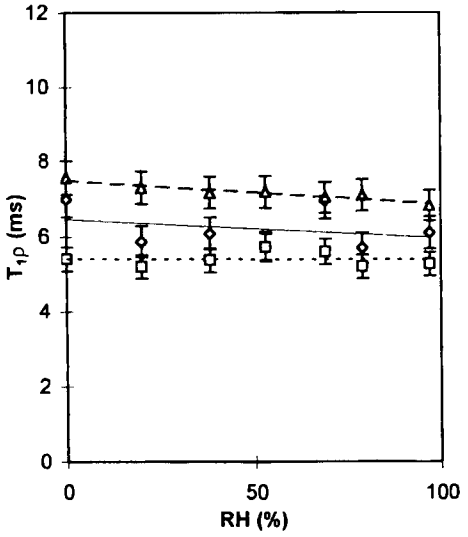

Fig. 2. Average $T_{1\rho}$ for C=O, CH, CH_3 groups for three proteins (---▲---DNase, . . .■. . . insulin and —♦— lysozyme) versus % RH.

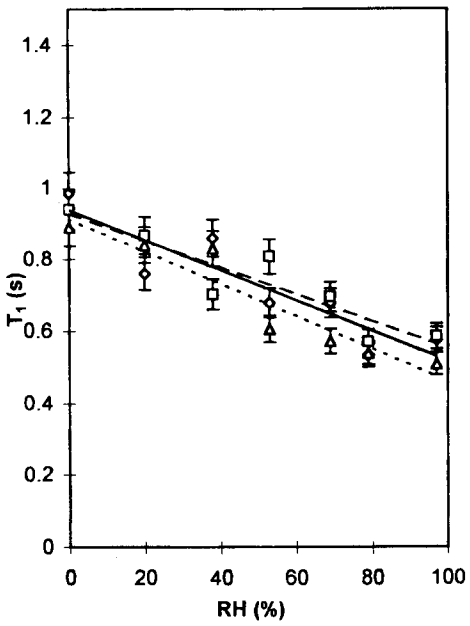

Fig. 4. Average T_1 for C=O, CH, CH_3 groups for three proteins (---▲---DNase, . . .■. . . insulin and —♦— lysozyme) versus % RH.

ties (12). The most important factors contributing to T_1 relaxation are dipole-dipole interaction, paramagnetic relaxation, and quadrupolar relaxation. Other potential contributions to T_1 relaxation are other small molecules or groups of high symmetry, rapid rotation of the molecules (12), or segmental motion. By using D_2O and $H_2{}^{17}O$, the importance of exchangeable protons, and hence sidechain mobility, on relaxation can be assessed. Both nuclei (^2H, ^{17}O) have spin number greater than $^1/_2$, and possess an additional relaxation process via interactions with a fluctuating electric field (17). The quadrupole effect occurs for spin $>^1/_2$ with a non-spherically symmetrical nuclear charge distribution that gives rise to quadrupole moments Q. T_1 for quadrupolar nuclei has been found to be as small as 10^{-4} sec. The relationship of T_1 and Q is $1/T_1 \propto Q^2$ (18). Deuterium has spin = 1, Q = 0.002875 cm^2 and ^{17}O has spin = 5/2, Q = −0.026 cm^2. Q^2 for deuterium is much smaller than for ^{17}O, therefore, ^2H T_1 will be longer compared to that for ^{17}O.

The ^{13}C CPMAS $T_{1\rho}$ measurements of protein hydrated with 98% RH salt solution using D_2O and $H_2{}^{17}O$ are shown in Table II. There is some change in $T_{1\rho}$ observed. Both deuterium water and ^{17}O water result in longer $T_{1\rho}$ most likely due to a decrease in dipole-dipole relaxation.

The ^{13}C CPMAS T_1 measurements of protein hydrated with 98% RH salt solution using D_2O and $H_2{}^{17}O$ are shown in Table II also. T_1 for ^{13}C–D is longer than that obtained for ^{13}C–H due to a decrease in dipole-dipole relaxation with deuterium nuclei. The exchangeable protons, (−NH and −OH) in protein molecules are replaced by deuterons which have a weaker dipolar interaction with the ^{13}C and hence T_1 is longer. The longer T_1 with deuterons also suggests that the contribution from the sidechains to relaxation is important. A small increase in T_2 has been observed for protons in protein powders when lyophilized from D_2O (5). The change in T_2 was ascribed to the mobility of the sidechains. The increase in T_1 seen when protons are exchanged with deuterons is primarily a result of the decrease in dipolar relaxation mechanism but could be due to the relative mobility of the sidechains compared to the rest of the protein molecule. The exchangeable −NH groups in the backbone would be less mobile than the sidechains and thus have a shorter relaxation time compared to the sidechains. Following deuterium exchange, mainly the sidechains contribute to proton relaxation and a longer T_1 would result.

T_1 for ^{13}C CPMAS using $H_2{}^{17}O$ is generally shorter than for normal water due to more efficient quadrupolar relaxation from the ^{17}O to the protons involved in CP. Insulin has a longer ^{13}C CP T_1 using ^{17}O water, possibly due to extra "free" water molecules (see discussion in next section). The ^{17}O relaxes the protons involved in CP and leads to a shorter T_1 compared to proteins hydrated with normal water. These protons are most likely on groups close to water molecules and possibly surface groups and sidechains.

Dynamics of Bound Water Molecules

The relaxation times of water bound to the protein molecules were measured at 98% RH. 1D ^1H spectra gave 3 types of protons: amide proton in the region of 6–7 ppm, aliphatics in the region 1–2 ppm, and "free" and bound water in the region of 4–5 ppm.

The ^{17}O T_2 was too short (~ μs) to measure and, therefore, only T_1 relaxation values were obtained. Direct ^{17}O T_1 was shorter using 20% ^{17}O labeled water than normal water (~6 ms versus 20 ms). The T_1 relaxation times of ^{17}O are shown in Table I indicating that the relaxation of water bound to protein powders is very fast compared to free water (~8 ms, (19)). Another observation is that insulin has longer ^{17}O T_1 (1.69 ms) than lysozyme (0.19 ms) and DNase (0.33 ms). Insulin has more H_2O per unit mass (in insulin there is 1 H_2O/81 Da compared with DNase 1 H_2O/101 Da and lysozyme 1 H_2O/131 Da). Longer T_1 results for insulin were also observed at 98% RH with D_2O and $H_2{}^{17}O$ (Table II), which supported the view that water content has a strong effect on relaxation properties. This suggests that insulin has more mobile water molecules associated with it per unit mass than the other proteins. Since insulin is more mobile than lysozyme and DNase in the presence of water, it may be more reactive, and therefore, less stable compared to the other proteins.

As discussed earlier, the presence of water molecules affects the faster motions of protein sidechains possibly due to hydrogen bonding causing the protein relaxation times (T_1) to decrease. Similarly, this could result in a decrease in the water relaxation times (T_1). The amount of water in insulin is more (on a molar weight basis) compared to lysozyme and DNase, thereby insulin has a longer water T_1 compared to lysozyme and DNase since insulin has more mobile water molecules. The correlation of ^{17}O T_1 relaxation with number of 'free' water molecules maybe worth studying further.

Dynamics of Exchangeable Protons

Protons that have the propensity to hydrogen bond (for example, amide protons and protons taking part in intra- and inter-molecular bonds), will undergo proton exchange easily in a 'wet' environment. Therefore, the exchangeable protons will be amide protons and hydrogen bonding groups on the protein surface.

Our ^2H T_1 results show that the exchangeable protons (water, amide) in proteins possess shorter relaxation times (insulin 9 ms, DNase 8 ms and lysozyme 7 ms) compared to "free water" ~450 ms (20,10). The effect is bigger for ^2H T_1 (~8 ms c.f. ~450 ms) compared to ^{17}O T_1 (~1 ms c.f. ~8ms). Both nuclei report on water molecules but the ^2H results are due mainly to water exchangeable groups (amide protons) and H-bonding groups (including C=O and surface groups possibly involved in aggregation). The ^{17}O reports only on the water, and the water is restricted in mobility. However, the restriction is greater for the deuterium exchangeable groups on the protein molecules.

As noted above, the relaxation times of protein surface groups and the protein as a whole are mainly due to protons. Direct ^2H T_1 was used to determine the relaxation times primar-

Table II. Average $T_{1\rho}$ (ms) and T_1 (s) ± 5% for ^{13}C CPMAS Experiments Using Water of Different Nuclear Spin

Proteins	98% RH D_2O		98% RH in H_2O		98% RH in $H_2{}^{17}O$	
DNase	8.75 ms	0.88 s	6.83 ms	0.51 s	8.56 ms	0.46 s
Insulin	6.79 ms	0.73 s	5.70 ms	0.59 s	9.36 ms	0.82 s
Lysozyme	12.7 ms	0.91 s	6.10 ms	0.58 s	6.12 ms	0.46 s

Table III. Activity and Aggregation Results for Lysozyme Stored at 20°C for 60 Days

RH (%)	Remaining activity (%)	Soluble aggregates (%)	Insoluble aggregate (%)
0	92.1 ± 2.7	0	0
53	89.5 ± 6.8	8.1 ± 0.2	0
98	67.0 ± 3.3	7.3 ± 0.6	23.6 ± 3.7

ily of the exchangeable groups of the proteins. However, deuterium will not only be exchanged on the surface –NH and –OH groups, but also in intra- and inter-molecular hydrogen bonds. The ^2H relaxation times are not reporting on just the surface groups of the protein (that are restricted by hydrogen bonding), but also on the mobility of whole protein molecules/aggregates. As a result, a larger effect on ^2H T_1 was observed as compared to ^{17}O T_1 since ^{17}O reports only on the water molecules, whereas the ^2H reports on the mobility of the protein. The increase in water content may lead to a loss in protein activity, and an increase in aggregation. As aggregation increases, relaxation rates would increase, leading to shorter T_1 relaxation times. The changes in T_1 with hydration after a few days storage may be reporting on a propensity for aggregation in the longer term.

Stability Data on Hydrated Proteins

Proteins used for this study were equilibrated for 5–6 days at each % RH and no significant aggregation occurred (<3%, except for DNase at 98% RH where there was 6% aggregation). Thus the NMR experiments largely measured the non-aggregated proteins. The proteins were then stored for a longer period of 60 days and further results indicated that the aggregation is strongly dependent on the storage humidity (Tables III & IV). The changes in molecular mobility were correlated with an increase in aggregation, and the increase in aggregation was related to a decrease in enzyme activity for lysozyme. The changes in T_1 may be acting as a reporter for the propensity to aggregate. However, the correlation of aggregation and relaxation time was observed for lysozyme and DNase only. For insulin, the aggregation-relaxation time relationship was bell-shaped with the highest aggregation found at 53% RH (instead of 98% RH as for the other proteins). This could be due to a dilution of the reacting insulin molecules by the free water at the high humidity, as has been reported for a number of proteins including bovine insulin (2,5,22–24). This appears to be supported by the earlier observation that insulin has more mobile water molecules associated with it then the other proteins. However, the HPLC assays show a broadening of the peak for the insulin monomer, suggesting that other low MW soluble aggregates might have been co-eluted. Thus, further detailed characterization of aggregation is needed to draw an unequivocal conclusion whether the relaxation-time-aggregation relationship is also applicable to insulin.

Table IV. Percent Soluble Aggregates of DNase and Insoluble Aggregates of Insulin After the Powders Were Stored at Different RH at 20°C for 60 Days

Sample	RH (%)	% Aggregates
DNase	0	0.5 ± 0.1
	53	3.2 ± 1.2
	98	18.2 ± 0.4
Insulin	0	7.5 ± 2.5
	53	27.7 ± 2.8
	98	9.7 ± 2.7

The aggregation susceptibility of proteins was previously found to relate to their molecular mobility as indicated by protein proton spin-spin relaxation times (T_2) (5). For bovine serum albumin and γ-globulin (5), the proton T_2 was found to increase from 10 to 20 μs with increasing hydration, indicating an increase in mobility on the slow timescale. The present data indicate that the spin-lattice relaxation time (T_1), which reports on faster timescale dynamics, decreases with increasing hydration whereas the intermediate timescale relaxation measurement $T_{1\rho}$ remains relatively constant. The rapid motions (T_1), most likely due to surface groups and sidechains, appear to be restricted and may be reporting on the aggregation propensity of the protein. The slow motions (T_2), due to motion of whole molecules or molecular segments, increase with hydration; while the intermediate mobility ($T_{1\rho}$) showed little change. The slightly larger T_2 observed using D_2O (5) indicate that the side chains of protein molecules are more mobile than the backbone and this mobility may be accessible to T_1 measurements. It has been shown that water increases the low frequency mobility of protein powders (5) and, since thermal energy drives molecular motion, this may lead to a decrease in mobility at other frequencies of the spectral density function. An increase in T_2 may manifest itself as a decrease in a higher frequency motion such as T_1. The higher frequency motions of the sidechains may be decreased or slowed and the low frequency motions may be increased with the addition of water. This could lead to increased reactivity and aggregation of the protein molecules.

FTIR studies have shown that freeze drying leads to conformational changes in lyophilized protein (25,26) and that this may be prevented by the addition of stabilizers. It would be of interest to further study these proteins with the addition of stabilizers and measure their effect on relaxation properties.

CONCLUSIONS

Mobility of protein molecules was determined by solid-state NMR over a wide range of % RH and it was found that water content leads to a change in mobility of protein molecules. The aggregation and activity of proteins were correlated to changes in molecular mobility. As relative humidity increased from 0% RH to 98% RH, $T_{1\rho}$ remained constant since the slower molecular motion of whole protein molecules is not greatly affected by the number of water molecules that is bound to the surface. T_1 decreased possibly due to the restriction in higher frequency motions caused by an increase in hydrogen bonding on the surface of proteins. ^1H, ^2H, and ^{17}O T_1 measurements indicated that relaxation is mainly through protons, primarily the exchangeable protons that take part in aggregation. The shorter T_1 relaxation times at higher RH most likely resulted from a change in higher frequency mobility of surface groups and report on the propensity of the protein to aggregate. A decrease in T_1 was associated with changes in aggregation and enzymatic activity.

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