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Method Development and Results of Protein Thermal Denaturaton Analysis by ^{1}H NMR

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NMR spectroscopy is a powerful technique for probing protein structure, stability, function and folding. Any expansion of NMR to other fields, such as engineering, could only be a benefit. Since protein thermal stability studies can provide useful information for protein processing in the biopharmaceutical industry, the applicability of one-dimensional proton (1D ^{1}H) NMR for thermal denaturation midpoint (T_m) and longitudinal relaxation times (T_1) for thermal stability data were investigated for the globular protein lysozyme. The considerations for protein NMR spectral analysis through temperature changes are elaborated upon. These considerations may also be of great value for protein, peptide, carbohydrate and oligonucleotide spectral analysis through changes other than temperature, such as pressure, pH, concentration and solute interactions. Since the experimental considerations elaborated upon were used for both T_1 and T_m analysis, they should also apply to the determination of other NMR-derived data. The denatured and native H15 C2H hen lysozyme protons were used to estimate T_m to within 2.3%. The native H15 C2H ^{1}H T_1 is greater than the denatured ^{1}H T_1 at all temperatures ascertained in the presence or absence of polyethylene glycol 1000 (PEG 1000). The difference in the native and denatured H15 C2H T_1 values indicates that the surface-located histidine residue experiences a differing local native conformation than the denatured local averaged confomation state. The H15 C2H denatured ^{1}H T_1 is shorter in 20% PEG 1000 than at 0% PEG 1000. The lower denatured T_1 in the presence of PEG indicates that PEG may interact with the denatured protein. © **1997 John Wiley & Sons, Ltd.**

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INTRODUCTION

Thermal stability studies of globular proteins may provide valuable information for drug delivery, production, purification, storage and transportation of proteins. Although there are several methods for analyzing protein stability,[1–3] NMR techniques offer both rapid and detailed information on function, folding, stability and structure. Since NMR provides the atomic coordinates of molecules in solution, it is very sensitive to native structural characteristics and thus offers potential insights into product characteristics previously unavailable from macroscopic analysis techniques such as circular dichroism (CD) and differential scanning calorimetry (DSC), separation-based analysis techniques such as electrophoresis and high-performance liquid chromatography, or pass/fail techniques such as activity assays. In fact the search for better production processes and the rectification of damaged products must start with better analysis techniques. This leads to strong demands for better analysis techniques in both academia and industry.

NMR is an extremely powerful probe of protein structure and folding, since individual nuclei may be monitored for conformational,[4] dynamic[5] and kinetic[6] details at specific sites in the protein structure. Protein structural variations may result in chemical shift, coupling and intensity changes. These changes may be used to follow various parameters. For instance, protein denaturation spectra often yield both resolved denatured and native peaks which change intensity or peak location during denaturation.[7,8] Intensity changes may be readily measured and for a two-state folding equilibrium the folding rate may be slow enough that exchange broadening is not observed,[7] allowing monitoring of denaturation from melting temperature (T_m) and longitudinal relaxation time (T_1) determinations.

The T_m values provide protein stability information under varying conditions. It can be obtained by DSC, hydrogen exchange, fluorescence, UV absorbance, CD, optical rotatory dispersion or NMR. Although NMR-determined T_m values are less common, for an observed two-state equilibrium, NMR may provide a simple and rapid T_m determination, since the denatured to native proton peak area ratio can be used to calculate the percentage denatured.[7] In fact, if a two-state equilibrium is observed then only a few spectra may be needed to approximate the melting temperature after the temperature is adjusted around the estimated T_m.[9] Further, in production or process development it could be possible to monitor the T_m of an unstable biomolecule with a single 1D NMR spectrum, where a single NMR spectrum at the known T_m temperature could be acquired. If a selected native to denatured peak ratio was a value

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other than 1.0, then something has gone astray with the product, process or production. The use of NMR might provide information that would otherwise be difficult to obtain in a production settings even when the use of NMR to determine T_m values was unnecessary.

Actually, NMR is not necessarily the method of choice for measuring T_m. NMR currently requires a relatively large quantity of protein, at least 0.1 mM or greater, compared with other techniques such as DSC and CD. In addition, proteins with molecular weights greater than 60 kDa do not yield ^{1}H NMR spectra. However, NMR techniques are improving rapidly such than superconducting probes, field gradient probes, magnetic field size, electronic hardware, computing capacity and spectral processing techniques, such as linear prediction, are all decreasing the quantity of protein required for analysis and increasing the resolution of peaks. Additionally, the multi-dimensional NMR techniques have greatly increased peak resolution and allowed the continued increase in molecule size that can be analyzed. Further, techniques such as CD and DSC yield a more macroscopic result rather than the microscopic insight given by NMR, since NMR allows particular nuclei in various amino acid residues to be studied. For instance, if CD or DSC were used to determine T_m, the value may be an average for that molecule, whereas the molecule could have regions with similar yet different T_m values. If NMR was used, the T_m of two amino acid residues, in different molecular regions, could be determined simultaneously.

The T_1 values provide details pertaining to the local and global state of the protons. For protons T_1 is largely determined from the energy exchange between the spins and lattice incurred through molecular dynamics, which is mediated by molecular tumbling, molecular size, intramolecular mobility, viscosity and distance to neighboring protons. The interaction distance to the neighboring fluctuating field of the nuclear magnetic dipole is small but may include both intramolecular and intermolecular protons. Changes in any of these parameters will alter the T_1 and could therefore indicate changes in distance and number of neighboring protons.

This paper examines the suitability of 1D ^{1}H Fourier transform (FT) NMR spectra for protein T_m determinations and the relevance of T_1 values for protein stability details. The experimental NMR issues addressed should not only pertain to T_m or T_1 determinations for small globular proteins, such as lysozyme, that form two-state folding equilibria, but these considerations elaborated upon in determining quantitative NMR spectral data for a series of temperatures should also be applicable to other biopharmaceutically relevant molecules such as peptides, large organic molecules, oligonucleotides, carbohydrates and any subunit of the larger multi-subunit proteins and oligonucleotides. In fact, the successful isolation of both the denatured and native H15 C2H proton peaks in a crowded spectral region by using D_2O rather than H_2O indicates a great potential for the analysis of many smaller molecules with less crowded spectra (note: C, H, I, L, M, N, V and W refer to the amino acid residues cysteine, histidine, isoleucine, leucine, methionine, asparagine, valine and tryptophan, respectively). The considerations elaborated upon should also apply to the analysis of any NMR spectral parameter through many different changing conditions, such as pressure, pH, cosolvents, concentration, agitation and solute addition, such as salts, substrates and inhibitors. Finally, the T_m of lysozyme using the H15 C2H proton in D_2O was determined, while the T_1 of both denatured and native protons in the presence and absence of polyethylene glycol (PEG) were determined and are discussed.

EXPERIMENTAL

Materials

Hen egg white lysozyme (three times crystallized, dialyzed and lyophilized), and Spectrapor dialysis tubing, molecular weight cut-off 12 000–14 000, were purchased from Sigma Chemical (St Louis, MO, USA, deuterium oxide of 99.9% and 99.96% D and deuterium chloride from Cambridge Isotope Laboratories (Andover, MA, USA), PEGs 1000, 1500, 2000, 3400 and 8000 from Aldrich Chemical (Milwaukee, WI, USA) and ethylene glycol (EG) standards from Wilmad Glass (Buena, NJ, USA).

Sample preparation

Lysozyme was purified by extensive dialysis at pH 2.0–3.0 and then lyophilzed.[10] Stock solutions were prepared to remove H_2O by dissolving 300 mg of lyophilized lysozyme in 99.9% D_2O (pH 4.0, 3.5 mM). The protein solution was then incubated at 81 °C for 25 min, immediately frozen at −70 °C, lyophilized, sealed in a small bottle and stored at 4 °C (desiccated) until used. Protein prepared by this method had a minimal amount of entrained H_2O and most labile potons were exchanged for deuterium.

All protein samples were adjusted to 3.5 mM by weight. The pH was adjusted to ±0.05 pH units with 1.0 M DCl in D_2O using a Corning glass electrode. All pH values reported are uncorrected for deuterium isotope effect. PEG–D_2O solutions were prepared by weight to weight percentage of PEG. Approximately 1.65 ml of PEG–D_2O was prepared by mixing a stock solution of 50% (w/w) PEG 1000 in 99.9% D_2O with the relevant weight of 99.96% D_2O. PEG was dried by placing the PEG in a glass bottle in an oven at 81 °C for 24–48 h.

NMR parameters

All spectra were accumulated with a Bruker ^{19}F ^{1}H or ^{13}C ^{1}H dual probe and a 16-bit digitizer on a Bruker AM 500 spectrometer. Spectra were processed and analyzed using the Bruker Aspect 3000 program. Temperature calibrations were performed on Bruker AM 500 and JEOL FX90Q spectrometers. Temperatures were calculated from the EG hydroxyl to methyl peak separation (ppm) using the equation

$$T\ (\mathrm{K}) = 465.6 - 101.317\Delta\ (\mathrm{ppm}) \qquad (1)$$

The fresh sealed EG sample was calibrated as appears elsewhere.[9]

Protein 1D spectra were recorded with 1–8K of time domain points, a sweep width of 8333 or 7246 Hz, 32–200 scans and a 90° observe pulse. The water resonance in D_2O was suppressed with a presaturation pulse of minimum power. For protein in a PEG–D_2O solution a time-shared, 0.05 s pulse presaturation sequence was used to suppress all the PEG and water signals.

Processing of spectra

The free indication decays were zero filled to 16K data point and multiplied by a cosine-bell squared function of eliminate truncation artifacts. Spectra were zero and first-order phase corrected and baseline roll was adjusted using a cubic spline interpolation program. Peaks were integrated in regions, shown to be devoid of denatured protein peaks at 75 °C,[8] including the region upfield of the denatured peak of V29 γCH3 at 0.74 ppm and the region between the denatured peaks of N46 αCH at 4.79 ppm and W108 C5H at 6.96 ppm. The spectrum downfield of 7.6 ppm is also devoid of non-labile peaks other than the H15 C2H in D_2O.

Chemical shifts were referenced to the C64 αCH at 5.83 ppm (35 °C)[10] and 5.82 ppm (75 °C)[8] at temperatures below and above 70 °C, respectively. Resonances were referenced to a resolved lysozyme peak since accurate peak locations were not required,[11–13] it has minimal peak temperature migration[8] and it is well resolved. The C64 αCH may have minimal peak migration due to conformational restraint from the disulfide bond and the adjacent aromatic residues, W62 and W63, may cause local non-tertiary shifts in the resonance of C64 αCH.

Determination of thermal denaturation curve

Protein solutions were incubated for 20 min at 81 °C immediately before experiments to eliminate residual labile proton peaks and then cooled to a native protein temperature. The EG sample was placed in the probe to determine the temperature. The sample was then placed in the probe and a spectrum was taken. The temperature was raised and allowed to equilibrate for 10 min or longer if it was raised more than 10 °C, and a spectrum was accumulated at this temperature. Next, this second temperature was determined with the EG sample in the probe. The temperature was then raised again and the third temperature was determined. The EG was replaced with the sample and a spectrum was taken. This procedure for determining each sample temperature was repeated until all spectra were obtained. Shimming, pulse width and carrier and presaturation frequencies were adjusted with temperature as required.

Two situations may occur for proteins that undergo a two-state equilibrium: either both or only one, either the denatured or native, NMR peaks are resolved. Often in 1D NMR only one peak is resolved and the fraction denatured must be determined from the ratio of the area ratio of the resolved protein peak to a reference peak at a denaturation temperature $(I_i/I_r)_j$ to the area ratio of the same protein peak to the reference peak at a temperature at which the protein is fully native or denatured $(I_i/I_r)_0$:

$$\text{fraction denatured} = (I_d/I_r)_j/(I_d/I_r)_0 = 1 - (I_n/I_r)_j/(I_n/I_r)_0 \quad (2)$$

Subscript i refers to subscripts d or n which refer to denatured or native protein, respectively, subscripts j and 0 refer to denaturation and standard temperatures respectively, and I_d, I_n and I_r refer to denatured, native and reference peak areas, respectively. This requires an internal or external intensity reference compound, due to the intrinsic NMR peak change with temperature, from concentration, Boltzmann distribution and general temperature effects.[14] This peak must not overlap any protein peaks for calibration of the intensity at each temperature and must be heat stable. Internal references must not interact with the protein or other solution components.

If both the denatured and native peaks are fully resolved, then the fraction denatured is the ratio of the denatured peak area (I_d) to the sum of the native and denatured peak areas ($I_d + I_n$) at each temperature:

$$\text{fraction denatured} = I_d/(I_d + I_n) = r/(1 + r) \quad (3)$$

where $r = I_d/I_n$.

All denatured protein percentages were determined from the ratio of the resolved H15 C2H denatured and native peak areas, since this does not require an extra intensity reference. Although the sum of the native and denatured H15 C2H peaks would provide an internal reference, this proton exchanges with deuterium at increasing rates above about the T_m temperature[7] and thus does not represent an accurate reference, although this has been shown to provide an accurate reference under certain circumstances.[11]

Progressive saturation Fourier transform T_1 determinations

Standard progressive saturation Fourier transform (PSFT) T_1 determinations were followed.[15] After the spectrometer temperature had stabilized, the sample was placed in the spectrometer and allowed to reach thermal equilibrium. Several spectra at different t_r were then obtained. For each t_r the first four free induction decays were removed to give steady-state saturation. For the H15 C2H proton at high temperature the equilibrium intensity was determined midway through the T_1 determination, after several short t_r time spectra, to minimize hydrogen–deuterium exchange (HX) losses on the final equilibrium peak area. Spectra were accumulated after the sample had returned to equilibrium after the previous spectrum. The T_1 were calculated from non-linear regression analysis of the peak area (I) and t_r using the equation

$$I/I_0 = 1 - \exp(-t_r/T_1) \quad (4)$$

and a non-linear regression program (SAS Institute, Cary, NC, USA), giving the T_1 and equilibrium peak area (I_0).

RESULTS AND DISCUSSION

Considerations for analyzing NMR spectral series

Peak intensity is a function of many variables, including magnetic field homogeneity and linewidth. These discrepancies are minimized if peak areas rather than

heights are determined. For peak location determinations peak area is not important, although the signal-to-noise ratio is, while peak area determinations are still a function of sample concentration, signal-to-noise ratio, temperature, processing errors and methods, nuclear Overhauser effects (NOEs), HX, T_1 and t_r.[15] The signal-to-noise ratio can be improved by using more sample, more scans, better hardware and probes, higher magnetic fields and adjusting t_r to account for T_1 effects, but this has traditionally been a major limitation of NMR methods. In fact the following investigation is mainly made necessary in order to overcome the poor signal-to-noise ratio of NMR methods.

NMR peak intensity is affected by both concentration and temperature. In fact, from 35 to 80 °C, D_2O increases in volume by about 3%[16] while intensity, which is proportional to the inverse of temperature,[14] decreases by about 14%, which combine to reduce the intrinsic signal by 17%. An 18% experimental decrease in the αCH_2 peak area from 38–74 °C occurred for a diglycine sample in D_2O, verifying that peak intensity should be calibrated at each temperature.

Baseline roll can be minimized by adjusting the gate delay,[15,17] but some baseline roll may always exist, requiring a baseline correction. For the T_m and T_1 measurements a cubic spline interpolation program was found sufficient, using spectral regions of lysozyme known to be devoid of peaks[8,10] as the baseline points and no baseline distortions were observed. Additionally, proper phase correction, prone to small errors, is made more difficult by baseline roll, but a 5% phase error has been reported to only cause a 1% intensity error.[15] Errors due to analog to digital converter non-linearities were absent in four otherwise identical spectra other than having been accumulated with four different receiver gains, since all these spectral intensities were within $\pm 1\%$ of each other after adjustment for the different gains.

Presaturation of solvent peaks may alter protein peak intensities, owing to saturation transfer between the denatured and native peaks when either overlap the solvent peaks or intensity may increase due to NOE. Water (4.66 ± 0.4 ppm) and PEG (3.8 ± 0.5 and 5.3 ± 0.2 ppm) resonances overlap both denatured and native lysozyme peaks.[8,10] In this study, the H15 C2H proton at 8.7 ± 0.2 ppm, does not overlap solvent peaks but an NOE from nearby ring NH protons could develop; however, the histidine ring NH protons are almost 100% exchange in D_2O so the effect should be very small. The NOE influences cannot be completely eliminated since it is propagated through space and the teritary protein structure may cause presaturated protons to be close in space; however, an NOE can be reduced with minimal presaturation power. The presaturation power can be reduced further with a 16-bit digitizer since the improved dynamic range allows solvent peaks to be larger than the solute peaks. Only 0.2% of the peak area was lost for a 10 dB presaturation power increase in the range of this study.

Presaturation was used for solvent suppression despite discrepancies. Tailored excitation techniques[15] are less reliable since the excitation pulse must follow the PEG and water peaks as they shift with temperature. The migrating excitation pulse causes intensities to vary with temperature, which is not a factor for a single-temperature determination if the denatured and native proton have similar chemical shifts, but is less reliable for denaturation curve determinations. Progressive saturation is unreliable if the solute T_1 are comparable to the solvent T_1, when fully relaxed solute protons are quantitatively required. Selective inversion of the solvent peak is especially tricky in dual-solvent studies. Actually, pulsed field gradients[18,19] are possibly the best scheme in a multi-solvent system.

Accurate sample temperature determinations must be ensured. An electrically heated gas stream flowing around the sample may be difficult to control and placing a thermocouple at the sample location is not feasible. However, the use of a temperature-calibrated EG sample allows an accurate sample temperature (± 0.25 °C) determination.[9] The heating device may drift over several hours by ± 1.0 °C owing to instabilities. Temperature stability can be improved by using higher gas glow rates,[20] minimal adjustments, waiting for thermal equilibrium or by using better temperature systems. Measuring the temperature before and after a spectrum can document stability.

Quantitative NMR usually necessitates the use of a 30° observe pulse, a $t_r \geqslant 5T_1$ of the slowest relaxing proton and as many scans as time permits. Longitudinal relaxation changes with temperature so t_r should be determined from the longest T_1 for the temperature transition, which can be laborious to determine accurately. Thus, a t_r of $5T_1$ of the longest estimated T_1 with a shorter pulse angle is best, if accurate quantitative data are required, although this will reduce the signal-to-noise ratio and, as discussed below, may need to be avoided.

Determination of T_m

The H15 C2H proton is the only proton for which the denatured and native peaks are fully resolved in D_2O at 500 MHz by 1D NMR. It was the only proton monitored for denatured percentages. The denatured peaks of all the remaining resolved native lysozyme peaks occur in unresolved regions of the 1D spectrum. For these peaks only native peak areas can be monitored as the temperature changes, causing a loss of accuracy from the two additional reference peak integrations.

The protein must be heated prior to experimentation to eliminate the labile protons in the H15 C2H spectral region or the calculated percentage denatured may increase by up to 25%. Preheating the sample may cause H15 C2H proton peak loss both from HX and irreversible denaturation. However, heating the sample for 25 min at 81 °C caused less than a 1.2% irreversible loss of the resolved native peaks. The initial HX H15 C2H peak loss is more difficult to estimate since the initial spectrum contains overlapping labile protons that disappear upon heating. However, during a 13 h denaturation curve determination, 88.7% of the H15 C2H peak disappeared, mostly after the T_m, largely from HX, since only a 14.4% irreversible decrease in non-labile peaks occurred over the 13 h. The difference in the two decreases indicates minimal HX loss of the H15 C2H for short heatings.

The H15 C2H protons have a relatively long T_1, probably a manifestation of being in an isolated ring structure.[6] They also exchange with D_2O at high temperatures such that above 75 °C they disappear in a few hours or even quicker.[7] Thus, although the H15 C2H protons ratio gives an accurate estimate of the T_m, assuming that the protein is fully cooperative, the relatively long t_r and the hydrogen exchange combine to reduce the signal-to-noise ratio, especially at high temperatures. This can be avoided by obtaining the data from a large protein stock solution, and using a new sample at every temperature,[7,21] but this is more expensive and time consuming, and may effect homogeneity in high-field magnets unless high-quality tubes are used. In order to speed a T_m determination, it would be advantageous to use one sample and to determine the shortest t_r and the longest observe pulse allowable, within the required accuracy of the investigation.

The apparent T_1 reported for the native and denatured H15 C2H protons are 1.5 ± 0.2 and 1.7 ± 0.3 s, respectively,[6] suggesting similar native and denatured H15 C2H relaxation. This could be due to few interactions with other protons to slow the relaxation pathway, since it is in an isolated ring. Since only the denatured to native peak area ratio is required for a denatured percentage calculation, at t_r shorter than $5T_1$ of the H15 C2H could be possible if the denatured and native T_1 are similar, since the intensities would be similarly affected, giving rise to negligible errors and quicker experiments.

The hypothesis that a shorter t_r could yield a similar result was tested by obtaining two consecutive spectra with a t_r of 7.24 s and then 1.0 s, at temperatures from 35–81 °C (Fig. 1). However, the percentage denaturation calculated from the H15 C2H peak areas is markedly different for the two delays. The shorter t_r yields a quicker onset of denaturation at all temperatures (Fig. 2), yielding a T_m of 70.8 °C, which is 3 °C lower than the 73.7 °C for the 7.24 s delay, which is more comparable to the literature T_m.[2,6,8,21] The T_m was determined from the ratio of the enthalpy (ΔH) to entropy (ΔS) changes of denaturation, where the equilibrium constant of unfolding (K) is unity. ΔH and ΔS were calculated from the Gibbs free energy change (ΔG) and the experimental K from Eqn (5) and a least-squares non-linear regression fit (Fig. 2):

$$\Delta G = -\ln K = (\Delta H/RT) - (\Delta S/R) \quad (5)$$

where R is the universal gas constant.[22]

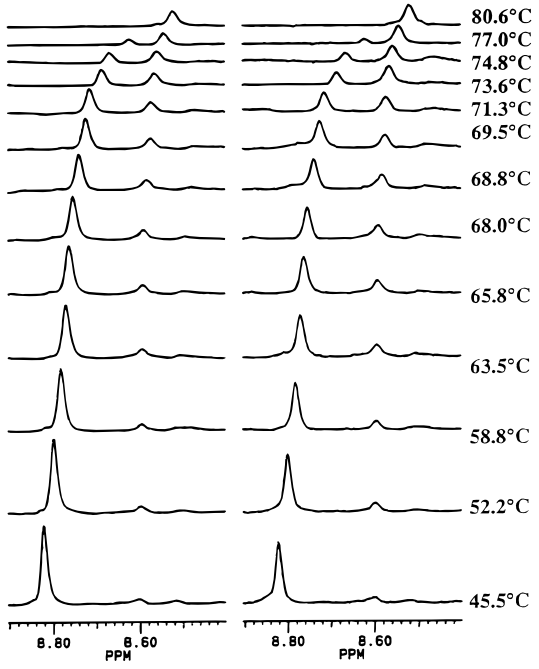

Figure 1. H15 C2H region of the 500 MHz 1D ^{1}H NMR spectrum of lysozyme at 3.5 mM, pH 3.8 in 99.96% D_2O. The t_r for the spectra on the left and right are 7.24 and 1.0 s, respectively. The right and left peaks are the denatured and native peaks, respectively.

Determinations of T_1

Since the T_m results indicate that the T_1 may be sensitive to protein stability, PSFT T_1 studies were performed with $12t_r$ at 76.7 and 72.5 °C, where the T_1 were previously determined to be similar.[6] The denatured peak rapidly reaches its true intensity, while the native peak does not reach maximal intensity until after 5 s (Figs 3 and 4). Additionally, a plot of the percentage denaturation *vs.* t_r shows a drastic reduction in the apparent denaturation, from 70% to 51% between 1 and 20 s at 76.6 °C, respectively (Fig. 5) and from 37% to 18% between 1 and 20 s at 72.5 °C, respectively (Fig. 5). The native T_1 at 72.5 and 76.7 °C are almost identical, whereas the denatured T_1 at 72.5 °C is slightly larger than at 76.7 °C (Table 1). The native T_1 is longer than the denatured proton in all cases studied. The H15 C2H HX was minimized by using a PSFT T_1 determination technique rather than other slower techniques[14] and only 80 scans were used. This procedure is less accurate than others but the correlations of linearity (r^2) for linearized T_1 calculations are close to unity.

Although the calculated T_1 values listed in Table 1 are not of the absolute highest accuracy, the two H15 C2H peaks do not increase in unison as the delay lengthens (Fig. 3). In fact, using the calculated native

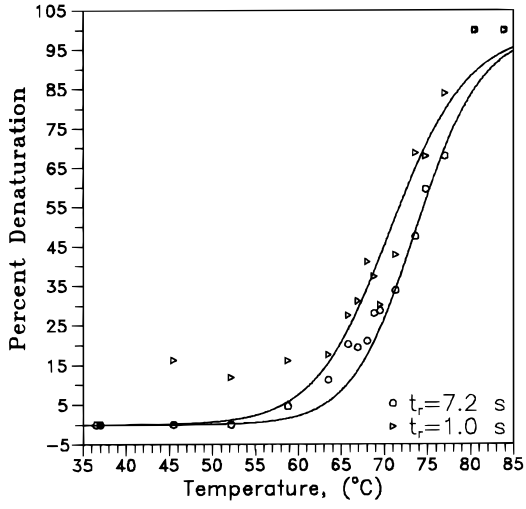

Figure 2. Thermal denaturation of lysozyme followed by the H15 C2H protons at 3.5 mM, pH 3.8 in 99.96% D_2O; △, $t_r = 1.0$ s; ○, $t_r = 7.24$ s; solid line represents the non-linear regression fit to $RT \ln K = T\Delta S - \Delta H$.

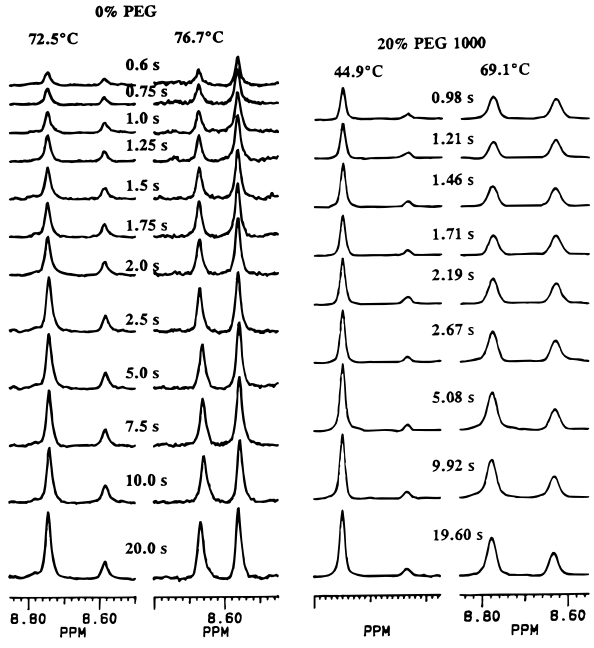

Figure 3. 500 MHz 1D ^{1}H NMR PSFT T_1 spectra of the H15 C2H lysozyme proton as a function of t_r at 3.5 mM, pH 3.8 in 99.96% D_2O. At 76.7 °C the denatured and native peaks are at 8.57 and 8.67 ppm, respectively, and at 72.5 °C they are at 8.58 and 8.75 ppm, respectively.

and denatured T_1 values and the non-linear predicted equilibrium areas (I_0), the theoretical percentage denaturation versus t_r plot follows the experimental data (Fig. 5). Actually, the phenomena may be due to

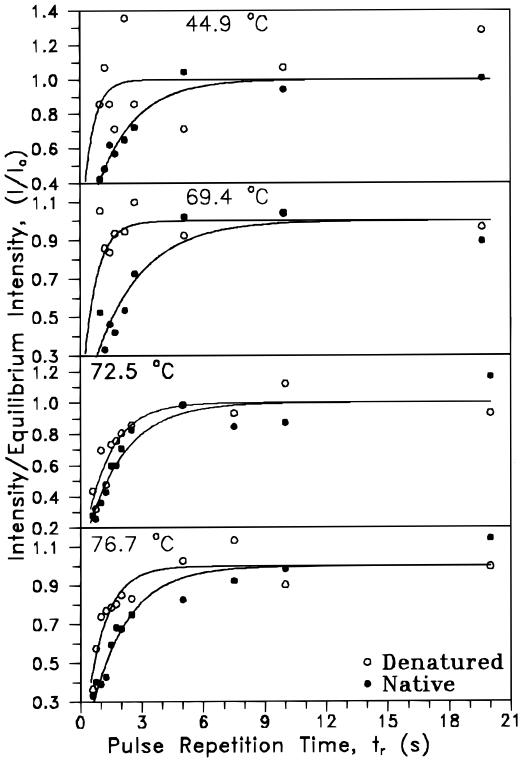

Figure 4. Dimensionless peak intensity (I/I_0) as a function of t_r for the H15 C2H lysozyme proton at 3.5 mM, pH 3.8 in D_2O at 76.7 and 72.5 °C and in 20% PEG 1000 at 69.4 and 44.9 °C. ●, Native; ○, denatured; I_0 = non-linear regression determined equilibrium intensity; solid line represents fit to $I/I_0 = 1 - \exp(-t_r/T_1)$ using calculated T_1.

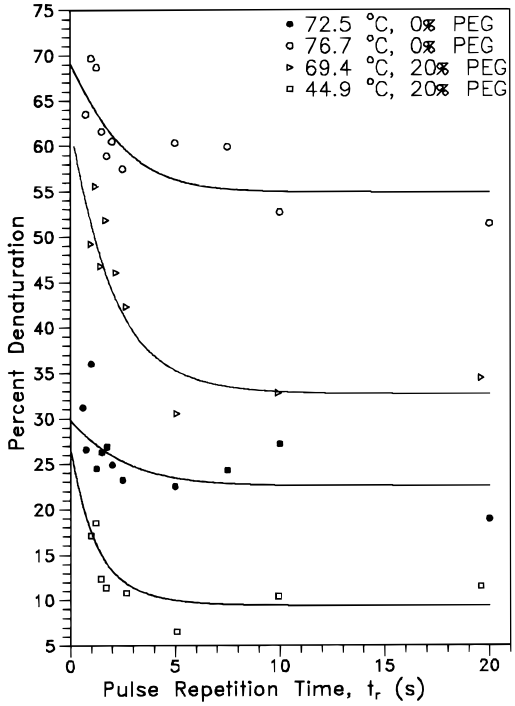

Figure 5. Percentage lysozyme denaturation from the H15 C2H proton denatured to native peak area ratio as a function of t_r at 3.5 mM, pH 3.8 in D_2O and 0% PEG at 76.7 and 72.5 °C, and at 20% PEG 1000 at 69.4 and 44.9 °C. Solid line represents fit to $I_d/(I_d + I_n)$ using calculated T_1 and equilibrium intensity.

the denatured proton sampling a conformationally acessible space that includes contact with more solvent or protein protons than are available in the native state. This implies for this native surface residue[4] that the ring structure interacts with fewer solvent and protein protons, since relaxation should increase in the absence of other protons owing to fewer dipolar interactions. The ring orientation or shielding could be analyzed from solution structure modeling.[4]

T_1 in a cosolvent

The T_1 were determined by PSFT experiments at different temperatures, in 20% (w/w) PEG 1000, which has been reported to destablize lysozyme and other proteins.[22] At both temperatures investigated, 44.9 and 69.4 °C, the denatured T_1 is significantly less than that of the native proton (Table 1). At 44.9 °C the denatured H15 C2H T_1 calculation is plagued by a poor signal-to-noise ratio since the denatured peak is still of low intensity because of a low percentage of denatured protein,

Table 1. ^{1}H PSFT-determined T_1 (s) of the lysozyme H15 C2H at 3.5 mM, pH 3.8 in D_2O, non-linear regression fit to $I = I_0[1 - \exp(-t_r/T_1)]$

[PEG 1000] (%, w/w)	Temperature (°C)	H15 C2H Denatured	H15 C2H Native
0	38.1		2.34 ± 0.09
0	72.5	1.27 ± 0.14	1.86 ± 0.14
0	76.7	0.97 ± 0.12	1.79 ± 0.11
20	44.9	0.52 ± 0.76	1.84 ± 0.10
20	69.4	0.67 ± 0.20	2.28 ± 0.19

as indicated by the large error (Table 1). The T_1 at 45 °C can still be considered to have merit, since the peak area did not follow any significant trend to increase (Fig. 3), which the native peak actually did, suggesting that the T_1 is confined to the calculated value or less, especially since the T_1 at 44.9 and 69.4 °C are similar (Table 1).

Actually, the T_1 of the denatured proton in 20% PEG 1000 is less than that in the absence of PEG (Table 1), indicating that PEG may interact with the denatured protein. The effect is less likely to be due to viscosity effects since the native H15 C2H T_1 in 0% and 20% PEG at different temperatures is not similarly affected. A non-linear regression fit of the lysozyme native H15 C2H PSFT data in 0% PEG yielded T_1 of 2.34, 1.86 and 1.79 s at 38.1, 72.5 and 76.7 °C, respectively, which may indicate a decrease in the native T_1 as the temperature increases. However, in 20% PEG the T_1 increased from 44.9–69.4 °C by the opposite amount. However, the native H15 C2H T_1 is similar to 0% and 20% PEG and a plot of the percentage denatured and t_r in 20% PEG 1000 at 69.1 and 44.9 °C (Fig. 5) both show that the percentage denatured levels off after about 5 s, similar to 0% PEG (Fig. 5), suggesting that a t_r of 5–7.5 s could be sufficient for T_m or other quantitative studies.

T_1 of other native lysozyme protons

The above PSFT data were also used to determine the T_1 values of some of the resolved native protons which could be useful for studies at other sites in the protein if an internal or external reference is used. Most of the resolved protons have $T_1 \leqslant T_1$ of the H15 C2H proton, but higher than previously reported[6] (Table 2). Since the percentage denatured levels off after 5 s, reasonable T_m estimations may be possible from spectra accumulated with a t_r of 5 s or more. A plot of I/I_0 against t_r for all the resolved residues, using the non-linear predicted equilibrium intensities, shows that after 5–7 s the intensity is within ±10% and ±20% of the equilibrium intensity for 0% and 20% PEG 1000, respectively (Figs 6 and 7). Spectra were also accumulated with t_r of 7.5, 10 and 20 s, at 35, 72 and 75 °C and 0% PEG, at better signal-to-noise ratios, and the intensity leveled off after 7 s again (Fig. 6).

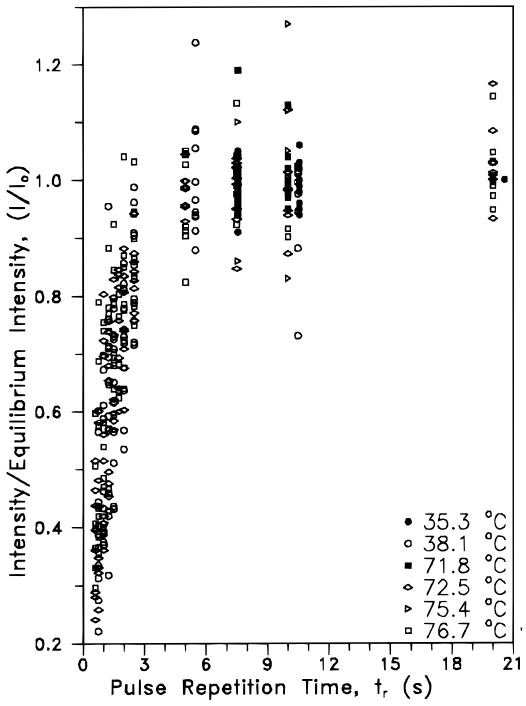

Figure 6. Dimensionless peak intensity (I/I_0) as a function of t_r at 3.5 mM, pH 3.8 in 99.96% D_2O with 0% PEG. ●, 35.3 °C (100 scans); ○, 38.1 °C (32 scans); ■, 71.8 °C (200 scans); ◇, 72.5 °C (80 scans); △, 75.4 °C (150 scans); □, 76.7 °C (80 scans); I_0 determined by non-linear regression.

Determination of T_m error

The actual error in the percentage denatured from using less than $5T_1$ can be calculated from the calculated T_1 using the equation

$$\frac{\text{observed percentage denatured}}{\text{true percentage denatured}} = \frac{(1 + I_{0,\,nat}/I_{0,\,den})}{1 + \dfrac{I_{0,\,nat}[1 - \exp(-t_r/T_{1,\,nat})]}{I_{0,\,den}[1 - \exp(-t_r/T_{1,\,den})]}} \quad (6)$$

The native denatured peak equilibrium area are $I_{0,\,nat}$ and $I_{0,\,den}$, respectively, and $T_{1,\,nat}$ and $T_{1,\,den}$ are the native and denatured peak longitudinal relaxation times, respectively. The error calculations show a

Table 2. PSFT-determined T_1 (s) of some resolved lysozyme protons at 3.5 mM, pH 3.8 in D_2O non-linear regression fit to $I = I_0[1 - \exp(-t_r/T_1)]$

	0% PEG			20% PEG 1000	
Proton	38.1 °C	72.5 °C	76.7 °C	44.9 °C	69.1 °C
I98 γCH	1.21 ± 0.15	NM[a]	NM	NM	NM
M105 βCH	1.02 ± 0.32	NM	NM	NM	NM
L17 δCH$_3$	0.98 ± 0.04	0.88 ± 0.06	0.93 ± 0.05	1.67 ± 0.14	1.25 ± 0.17
N39 αCH	1.50 ± 0.05	NR[a]	NR	NR	NR
N65 αCH	2.12 ± 0.10	NR	NR	NR	NR
N59 αCH	1.97 ± 0.21	1.88 ± 0.05	1.58 ± 0.06	2.44 ± 0.18	2.67 ± 0.17
C64 αCH	1.60 ± 0.16	1.77 ± 0.02	1.86 ± 0.08	1.80 ± 0.11	1.72 ± 0.18
W28 C5H	0.94 ± 0.21	1.33 ± 0.06	1.38 ± 0.05	1.67 ± 0.16	1.34 ± 0.15
W108 C5H	1.02 ± 0.11	1.31 ± 0.14	1.31 ± 0.05	1.70 ± 0.13	1.51 ± 0.20

[a] NM = not measured; NR = not resolved.

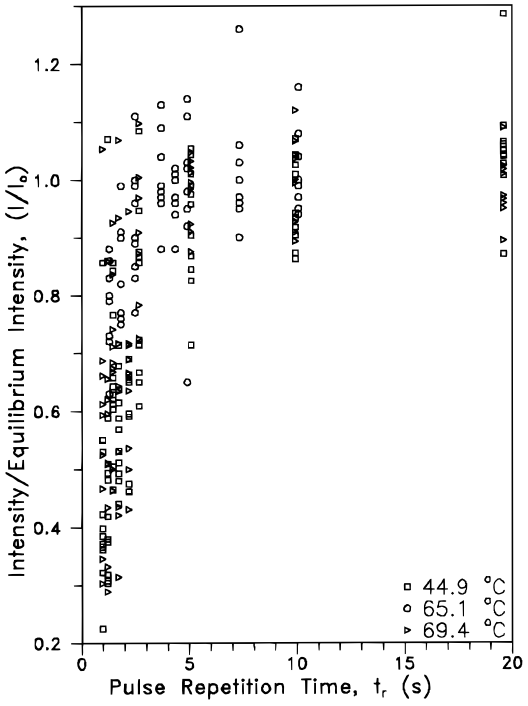

Figure 7. Dimensionless peak intensity (I/I_0) as a function of t_r at 3.5 mM, pH 3.8 in 99.96% D_2O with 20% (w/w) PEG 1000. □, 44.9 °C (64 scans); ○, 65.1 °C (32 scans); △, 69.4 °C (64 scans); I_0 determined by non-linear regression.

maximal difference in the true and observed percent denatured at about the T_m (Table 3). The difference error at 95% and 1% is less than 0.5 °C in all cases. The difference error does not go above 1.1 °C even when a t_r of $3T_1$ is used with the denatured T_1 in 0% PEG (Table 3). Thus, a t_r of 7.24 s yields minimal error, totalling no more than 2.3% (Table 3). Similar error calculations could be performed for other parameters determined from NMR data, extending the applicability of this T_m and T_1 study.

CONCLUSIONS

Considerable improvements in NMR data can be accomplished when using older NMR technology through the use of many clever techniques that exist mainly to offset the major limitation of NMR, i.e. sensitivity. Processing and acquisition methods and parameter selection, solvent peak removal, temperature stabilization and determination can all combine to improve NMR data. A wide assortment of these were discussed and then employed in the determination of T_m and T_1 values.

The use of D_2O rather than H_2O allowed the isolation of the H15 C2H denatured and native lysozyme ^1H peaks in an otherwise crowded 1D NMR spectral region. Accurate temperature determination and stabilization, reduced pulse repetition times, single samples and the thermodynamic equations explaining unfolding equilibrium, allowed the lysozyme melting curve and T_m to be determined with values similar to those determined by others,[2,6,8,21] even though the H15 C2H proton exchanges with deuterium at denaturation temperatures. For greater accuracy, shorter observe pulses, longer t_r and more scans should be used; however, the rapid loss of the lysozyme H15 C2H proton due to HX and irreversible losses required shorter t_r and longer pulse angles to improve the signal-to-noise ratio.

An equation was used to estimate the error incurred from using less than equilibrium NMR acquisition parameters for the melting curve denaturation percentages. The error is greatest at the T_m value but even with a t_r value of $3T_1$ the error is contained to 2.3% or 1.1 °C. A conformation of destabilization would thus be possible when the change in T_m is greater than the combined error, e.g. the 5 °C[22] lysozyme T_m decrease in 20% (w/v) PEG 1000.

A non-linear regression fit of PSFT data was used to determine the T_1 of several lysozyme protons for several conditions. The native and denatured H15 C2H ^1H T_1 were determined at 38.1, 72.5 and 76.7 °C in D_2O at 3.5 mM. The native T_1 were larger than the denatured for both denaturation temperatures. This T_1 difference indicates fewer protein to H15 C2H proton interactions than in the denatured state, since there are drastically fewer H_2O solvent molecules in D_2O to provide an alternative relaxation source in both states.

The H15 C2H ^1H T_1 were also determined in the presence of 20% (w/w) PEG 1000 in D_2O at 44.9 and 69.4 °C. Again the native T_1 were larger than the denatured. Additionally, the denatured T_1 in the presence of PEG were greater than in just D_2O. Since the native T_1 in the presence and absence of PEG were similar, viscosity is unlikely to be the cause of the denatured T_1 differences. Thus the smaller H15 C2H denatured T_1 in the presence of 20% PEG is possibly an indication of the proximity of the proton to PEG, an interaction with PEG or an alteration in the denatured conformation in PEG. The general decrease of the H15 C2H T_1 from the native to denatured states indicates that the position of the H15 ring at the native lysozyme surface is a different local conformation than the denatured or random conformationally averaged state. The T_1 of several other resolved native lysozyme ^1H peaks were also determined and indicate that relaxation reaches equilibrium after 5–7 s for a many lysozyme protons.

This study indicates the applicability of NMR technology for investigations of parameters such as T_m and

Table 3. Errors when using $t_r < 5T_1$ of the H15 C2H native proton of lysozyme

t_r 7.2 s	Denatured T_1 (s)	Native T_1 (s)	True percentage denatured (D_{eq})	Observed percentage denatured (D)	Percentage error[a]	$D - D_{eq}$
$4T_1$	0.97	1.8	95.0	95.1	0.1	0.1
			50.0	50.4	0.8	0.4
			1.0	1.0	1.7	0.0
$3T_1$		2.3	95.0	95.2	0.2	0.2
			50.0	51.1	2.3	1.1
			1.0	1.0	4.6	0.0
$3.1T_1$	0.67	2.3	95.0	95.2	0.2	0.2
			50.0	51.1	2.1	1.1
			1.0	1.0	4.3	0.0

[a] $(100 \times D/D_{eq}) - 100$.

T_1. The results presented should provide a guide in determining parameters other than T_m and T_1 using NMR data. Although NMR is limited to smaller macromolecules, such as proteins less than 60 kDa, there is certainly a wide assortment of biopolymers and biomolecules in the biopharmaceutical industry to be analyzed. In fact, NMR is used widely in the research, development and production of complex pharmaceutical organics. Further NMR sample quantities required are decreasing steadily and resolution is improving. NMR provides the actual monitoring of nuclei rather than the macroscopic parameters determined by other techniques. For instance, electrophoresis is fundamentally a separation and does not indicate atomic level changes in a product, but rather size and charge chracteristics of the whole or fragmented product. NMR could easily test a functional modification in production with regard to the extent and accuracy of the modification. Thus the rapid improvements in NMR technology should allow for a wider use of NMR in industry.

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