Protein Dynamics

NMR Spectroscopic Detection of Protein Protons and Longitudinal Relaxation Rates between 0.01 and 50 MHz**

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Nuclear magnetic relaxation data of water nuclei at variable fields provide valuable information on the dynamics of water–solute interactions.[1–3] However, information can be collected only within certain field ranges in which the nuclear relaxation rates are field-dependent owing to the dispersion of the spectral density, $J(\omega,\tau)$. The dispersion depends on the type of motion and on the observed nucleus. The most informative ^{1}H NMR spectroscopic frequency range for rotational motions is centered around 10 MHz for small proteins ($M_W \approx 10^4$ Da) and smaller frequencies for larger proteins and protein aggregates. Of course, resolution is low at these fields and in practice only one signal (or an unresolved signal envelope) can be detected. Furthermore, only the abundant water protons can be conveniently studied under such low sensitivity.

Relaxation of the isotopes ^{17}O and ^{2}H in enriched water can also be studied;[1] in these cases the centers of the informative field ranges increase by a factor ≈ 7 with respect to ^{1}H as a result of the magnetogyric ratios of ^{17}O and ^{2}H. Because water interacts with proteins, such relaxation studies provide direct information on the nature of water–protein dynamics, but give only indirect information on the protein itself.[4–6] It would be highly desirable to have complementary information directly from the protons of the protein, but this has been impractical so far owing to the low sensitivity of the available instrumentation. Protein ^{1}H relaxation data have been reported only on protein solutions of concentrations $\geq 35\%$ by mass,[7,8] far from physiological conditions. Improvements in field-cycling technology[9–12] have led to the production of instrumentation[13] with a $\approx$10-fold increase in the signal-to-noise ratio,[14] with which direct protein ^{1}H detection can be attempted for protein concen-

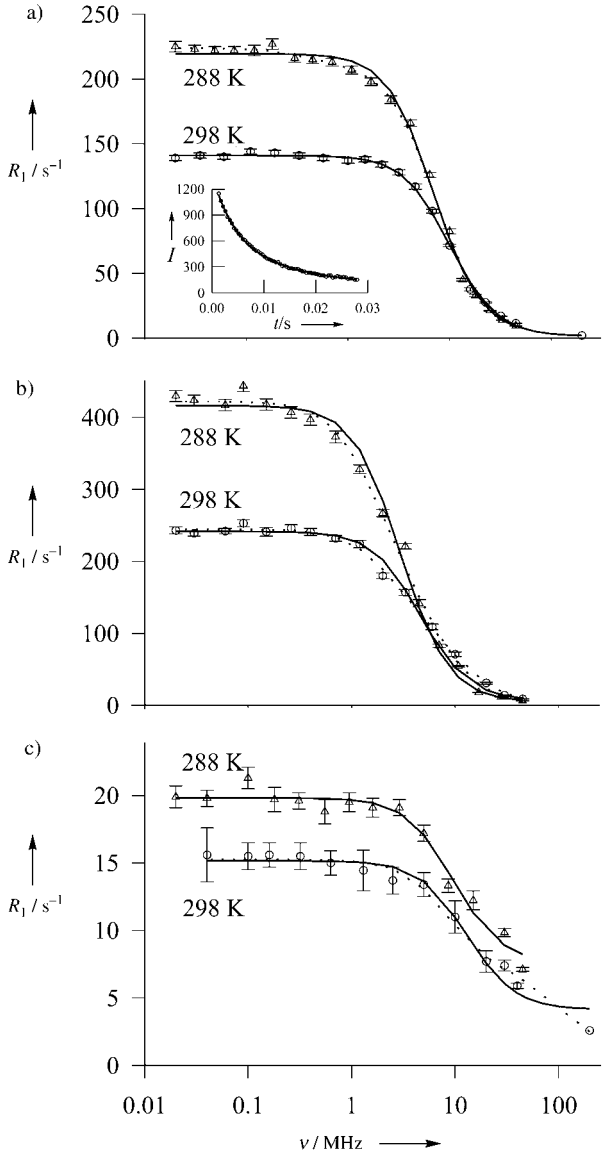

Figure 1. Protein ^{1}H relaxation dispersions for lysozyme solutions (2.8 mM in D_2O) at a) pH* 3.5, and b) pH* 9.0 (pH=pH-meter reading in D20 solution). The time decay of the collective protein ^{1}H magnetization at 0.1 MHz and its single-exponential fit are shown in the inset of part a). Theoretical relaxation rates were obtained through single-exponential fits of time decays of collective protein ^{1}H magnetization calculated from the known protein structure of lysozyme[17] and a complete relaxation matrix (CORMA) analysis.[18] Only exchangeable NH protons from secondary structure elements ($\approx$50% of total) were included, whereas all other exchangeable proton positions were assumed to be deuterated. Inclusion of either all or none of the NH protons affected the calculated rates by $\pm$2%. The theoretical low-field $\langle E^2 \rangle$ values obtained in this way were used to fit Equation (1) to the data (solid lines). The dotted lines represent data fits with two $J(\omega,\tau)$ terms in Equation (1) (Table 1). Panel c) shows the protein ^{1}H relaxation dispersion for a solution of α-synuclein in D_2O (1.4 mM, pH* 7.1). The solid and dotted lines represent fits with one or two $J(\omega,\tau)$ terms in Equation (1), respectively (Table 1). Points at 200 MHz were obtained from inversion-recovery experiments performed on a standard spectrometer.

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trations of ≈ 1 mM. Notably, the field-shuttling technique cannot be applied for these purposes, as it is limited by the time of sample transfer which does not permit detection of relaxation rates greater than $\approx 20\ s^{-1}$.[15,16]

The inset in Figure 1a shows the collective proton-magnetization decay at 0.1 MHz for a solution of lysozyme (2.8 mM, pH* 3.5 in D_2O; pH* = pH-meter reading in D_2O solution) previously treated with D_2O for deuteration at exchangeable-proton positions. For a rigid protein, at all frequencies of interest herein, CH_2 protons relax approximately two to three times faster than CH and nonexchanged NH protons; internal rotation causes CH_3 protons to relax as slowly as CH protons.[18] The magnetization decay should therefore be nonexponential, as it is a superposition of different decays. In practice, however, the decay can be fit with a single relaxation rate, R_1, in the time range from 1 ms to five times the value of R_1^{-1}. The root-mean-square deviation from the calculated decay is always below 1% of the maximum intensity of the signal. Residual water protons (estimated always to be fewer in number than protein protons by the peak amplitudes observed at 200 MHz) do not contribute appreciably to the observed decay in magnetization, as the polarization field was applied for a duration much shorter than the proton-relaxation time of water. Figure 1a shows the relaxation dispersion of lysozyme in D_2O. Note that the R_1 values at low field are on the order of $10^2\ s^{-1}$, whereas they decrease to essentially zero at high field. Relaxation can be interpreted with an equation of the type[1,6]

$$R_1 = S^2\langle E^2\rangle J(\omega,\tau_R) \qquad (1)$$

in which $J(\omega,\tau_R) = \frac{0.8\tau_R}{1+4\omega^2\tau_R^2} + \frac{0.2\tau_R}{1+\omega^2\tau_R^2}$, τ_R is the rotational correlation time, S^2 is an effective order parameter, and $\langle E^2\rangle$ is the squared average interaction energy among protein protons, whose theoretical value can be predicted (Figure 1) if the protein structure is known.

The best-fit τ_R and S^2 values are shown in Table 1. The τ_R values are as expected for monomeric lysozyme[19] and are in agreement with ^{15}N-relaxation measurements.[20] The S^2 parameter accounts for local motions that occur at times shorter than τ_R. The S^2 values are somewhat smaller than those found at high field according to the model-free approach for NH protons,[20] as for the measurements reported herein, the side chains contribute to the sub-τ_R mobility. Side-chain order parameters were estimated earlier to range between 0.05 and 0.9, depending on their position.[20] This effective S^2 parameter can be viewed as a measure of the overall rigidity of the protein.

Lysozyme is known to aggregate at high pH values, and is proposed[19] to be dimeric at pH 9.0. Measurements at pH* 9.0 (Figure 1b) demonstrate the sensitivity of this technique to aggregation. The R_1 values are markedly higher than those at low pH values; this results primarily from an increase in τ_R (Table 1).

Confirmation that this technique is also sensitive to the effective S^2 parameter comes from the dispersion of α-synuclein in its native, largely unfolded state[21] (Figure 1c). The low-field R_1 values of α-synuclein are only about 10% of those of lysozyme. A dispersion is observed with a correlation time similar to the τ_R of lysozyme (Table 1). As the two proteins have similar molar masses, the observed correlation time would correspond roughly to the τ_R value of hypothetically folded α-synuclein. As the $\langle E^2\rangle$ value is dominated by short-range interactions and is therefore modestly sensitive to the extent of protein folding, the small R_1 value essentially originates from a small S^2 value [Eq. (1)].

Information regarding protein rigidity (S^2) and aggregation (τ_R) can thus be easily obtained from the direct relaxation dispersion of protein protons. With respect to water nuclei dispersions, the analysis is neither complicated by the unknown number of long-lived water molecules and exchangeable protein protons, nor by the presence of water protons that exchange faster than τ_R (Table 1, footnote [a]).

Expected improvements in sensitivity will allow an increased complexity in analysis; for example, the detection of equilibria between different protein aggregates should be possible as well as the determination of protein folding in more quantitative terms. The quality of the present data may already permit further semiquantitative considerations. Inspection of Figure 1a and 1b reveals that a fit with a single $J(\omega,\tau)$ term in Equation (1) is satisfactory at low pH values at 298 K, reasonably good at low pH values at 288 K and at high pH values at 298 K, and definitely less satisfactory at high pH values at 288 K. A fit with two $J(\omega,\tau)$ terms in Equation (1) was thus attempted (Table 1). Largely aggregated protein at 0.3% is sufficient for the dramatic improvement of the data fit for low pH values at 288 K, whereas at high pH values a mixture of monomers and higher aggregates would fit the data much better and would yield more reasonable S^2 values. The fit with a single $J(\omega,\tau)$ term is good for α-synuclein, but tends to overestimate the height of the high-field-relaxation plateau. A much better fit of the data

Table 1: Fitting parameters for the relaxation dispersions of the protons of lysozyme and α-synuclein.

			One spectral density			Two spectral densities					
	pH*	T [K]	τ_R [ns]	S^2	α [%][a]	τ_R [ns]	%	τ'_R [ns]	%	S^2	α [%][a]
lysozyme	3.5	298	9.0 ± 0.4	0.77 ± 0.04	1.2 ± 2.5						
	3.5	288	12.9 ± 1.5	0.85 ± 0.09	1.2 ± 4.1	12.4 ± 0.1	99.7 ± 0.9	231 ± 447	0.3	0.85[b]	1.5 ± 1.8
	9.0	298	21.0 ± 3.7	0.53 ± 0.10	7.1 ± 6.7	9.0[b]	75 ± 7	35 ± 7	25	0.77[b]	2.2 ± 2.7
	9.0	288	31.7 ± 5.4	0.64 ± 0.12	3.3 ± 6.0	12.9[b]	64 ± 13	47 ± 13	36	0.85[b]	0.2 ± 3.5
α-synuclein	7.1	298	7.0 ± 1.8	0.08 ± 0.02	28 ± 10	10 ± 3		0.6 ± 0.2		0.08[b]	0 ± 6
	7.1	288	9.5 ± 2.5	0.06 ± 0.02	38 ± 10						

[a] Percent value of the high-field plateau, related only to fast local motions. Note that in nuclear magnetic relaxation dispersion for water nuclei, this term is dominated by the relaxation contribution from an unknown number of water molecules exchanging rapidly with respect to τ_R. [b] Fixed at the monomer value.

at 298 K is obtained by using a model-free approach under the assumption of segmental motions[22] of the unfolded protein to account for the missing $(1-S^2)$ term. An inflection with a τ value of 10 ns and a second inflection with a τ value of 0.6 ns is in good agreement with the experimental data (Figure 1c and Table 1).

In conclusion, we have shown that data from direct protein ^{1}H relaxation dispersion yield information on: 1) the lack of rigidity through S^2, which is directly related to the range of folded states of the protein (from fully folded to the extreme state of complete unfolding) and 2) protein aggregation through a safe estimate of τ_R.

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