$^{14}N^{1}H$ AND $^{2}H^{1}H$ CROSS-RELAXATION IN HYDRATED PROTEINS

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ABSTRACT The frequency dependence of the proton spin-lattice relaxation time T_1 of solid hydrated bovine serum albumin and α-chymotrypsin has been measured over 4.5 decades in the range 10^4 to $3 \cdot 10^8$ Hz mainly by the aid of the field-cycling technique. The comparison between H_2O- and D_2O-hydrated samples permitted the distinction of exchangeable and unexchangeable protons. In all cases the $^{14}N^{1}H$ cross-relaxation dips due mainly to the amide groups have been observed. In addition, in the case of the deuterium exchanged proteins a $^{2}H^{1}H$ quadrupole dip appears. The amide groups act as relaxation sinks due to the coupling of the amide proton to ^{14}N and adjacent protons. Outside of the dip regions the proton-proton coupling dominates. The fluctuations of the $^{14}N^{1}H$ and $^{1}H^{1}H$ interactions are of a different type. The unexchangeable protons show a T_1 dispersion outside of the quadrupole dip regions given by the exceptional power law $T_1 \sim \nu^{0.75 \pm 0.05}$. It is shown that apart from structural information of the ^{14}N spectra, $^{14}N^{1}H$ cross-relaxation spectroscopy permits the determination of correlation times in the range 10^{-7} s $< \tau < 10^{-4}$ s.

INTRODUCTION

Proton spin-lattice relaxation in protein systems normally is mediated by the coupling to neighboring dipole nuclei. We have to consider the protons themselves, nitrogen-14 nuclei and, in the case of partially deuterated systems, deuterons. The latter two simultaneously are quadrupole nuclei, so that the interaction to them reveals itself by the characteristic quadrupole dips in the proton T_1 dispersion (1, 2). These cross-relaxation phenomena are experimentally accessible by the field-cycling technique (e.g., 3), which nowadays permits field changes fast enough to cover a range of frequencies far above the relevant nuclear quadrupole resonance (NQR) frequencies.

Several aspects must be considered before performing this experiment. First, the quadrupole dips help to identify the locations at which the main spin-lattice relaxation mechanism takes place. On the other hand, the analysis of the dip shape and of the overall frequency dependence outside of the dip regions should provide valuable information concerning the relevant fluctuations. In addition, the spectral NQR parameters (quadrupole coupling constant and asymmetry parameter of the electric-field gradient) reflect features of the molecular structure.

The first point should not be misinterpreted in the sense that, for example, dipolar $^{14}N^{1}H$ interaction per se completely determines the relaxation mechanism. The $^{14}N^{1}H$ groups within proteins naturally are part of multi-spin systems and, hence, $^{14}N^{1}H$ protons are additionally relaxed by dipolar $^{1}H^{1}H$ coupling. The cross-relaxation phenomena, however, permit us to judge the influence and to identify the location of these relaxation sinks. Above all, this is crucial to the understanding of relaxation contrasts in biomedical nuclear magnetic resonance (NMR) tomography (4).

The goal of the present work is related more to the dynamics of the molecules. We want to clarify how the proton T_1 dispersion of hydrated protein systems can be interpreted with respect to backbone fluctuations. To prevent any influence of rotational tumbling of the molecules we have chosen completely hydrated but still solid protein systems.

EXPERIMENTAL

Bovine serum albumin (BSA) (dry, purest, electrophoretic purity of 100%) was purchased from Behring-Werke (Marburg/Lahn, Federal Republic of Germany). α-Chymotrypsin (α-Ch) from bovine pancreas was provided in lyophilized form from Fluka (Buchs, Switzerland). The protein was exposed to a humid atmosphere until a homogeneous degree of hydration of 16% H_2O was reached. Deuterium exchange was reached by dissolving the lyophilized material in D_2O. After 2 wk the samples were lyophilized again and rehydrated in a D_2O-saturated atmosphere. The D_2O content was then again 16%. The sample temperature in all cases was (15 ± 1)°C. The field-cycling apparatus was an improved version of that described previously (5). A detailed description will be published elsewhere. The data above 20 MHz have been measured with conventional pulsed NMR spectrometers using the saturation-recovery pulse sequence.

THEORETICAL BACKGROUND

^{14}N and ^{2}H are quadrupole nuclei in contrast to ^{1}H. This means that at low magnetic fields a deviation from the linear relationship between resonance frequency and flux density arises. The interaction of the

quadrupole with electric-field gradients within the molecules becomes the dominating mechanism in the low-field limit.

The consequence is a resonance crossing of protons and quadrupole nuclei (compare Fig. 1). Energy conserving flip-flop transitions then become possible. As the quadrupole nuclei are strongly coupled to the lattice, their spin-lattice relaxation is much faster than that of the protons. Therefore, a net flow of energy occurs from the protons to the quadrupole nuclei, which reveals itself as "quadrupole dips" in the proton T_1 dispersion (1).

The question is now which mechanisms determine the shape and width of the dips arising by this cross-relaxation phenomenon. As a first point we will discuss the broadening by the orientation dependence of the quadrupole frequencies that arises in the absence of rotational symmetry and motional averaging.

Previously (1) we have used ^{14}N frequencies calculated in the low-field approximation. We now prefer an exact formalism (compare 6) on the basis of the energy eigenvalues $E_i = E_i$ (C, η, θ, Ψ, B_0), which can be calculated by solving the secular equation of the complete Hamiltonian as a function of the magnetic flux density B_0 and of the Euler angles θ and Ψ between the molecular and the laboratory frame. C and η are the quadrupole coupling constant and the asymmetry parameter, respectively. Thus the orientation dependence of the ^{14}N frequencies can be evaluated numerically. The results are represented by Fig. 1. In rigid samples with powder geometry, the inhomogeneous broadening of the ^{14}N resonance by the orientational distribution of the amide groups relative to the magnetic field leads to certain minimum widths of the quadrupole dips.

The dips will be further broadened by the intensity function of the flip-flop transitions. The total width at half height of the cross-relaxation lines is plotted in Fig. 2 as a function of the correlation time τ. Obviously the widths of the dips will be governed by the spectral density if $\tau < 10^{-5}$ s (ν_1, ν_2) and $\tau < 10^{-4}$ s (ν_3). These limits in reality may be even less stringent due to partial motional averaging of the orientational broadening. The opposite limit of the τ ranges accessible by cross-relaxation spectroscopy is given by the disappearance of the dips, which will be smeared out for $\tau < 10^{-7}$ s. Within these limits it is possible to evaluate safely the correlation times of the dipolar $^{14}N^1H$ interaction.

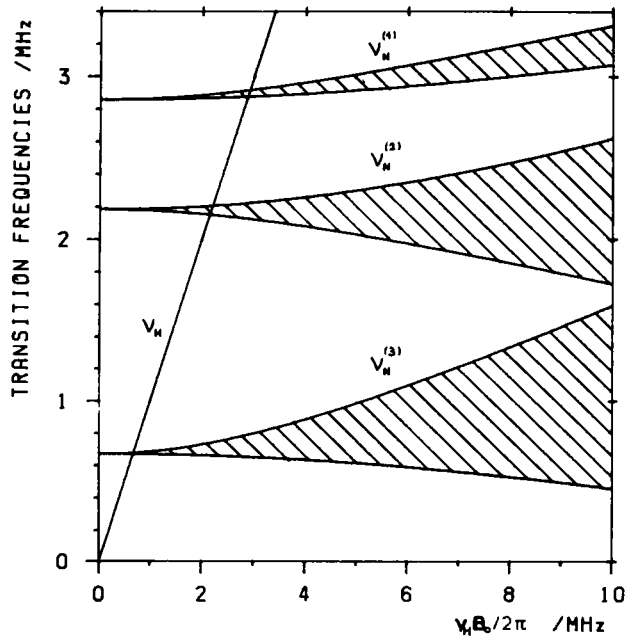

FIGURE 1 ^1H and ^{14}N transition frequencies vs. magnetic flux density. The nitrogen frequencies have been calculated with $\eta = 0.4$ and $e^2qQ/h = 3.36$ MHz. (η, asymmetry parameter; e, elementary charge; q, field gradient; Q, quadrupole moment.) The hatched areas indicate the maximum spread of the ^{14}N frequencies due to the orientational distribution. The overlap ranges of the ^1H and ^{14}N resonance are 2.876 MHz $< \nu_H <$ 2.917 MHz, 2.150 MHz $< \nu_H <$ 2.207 MHz, and 0.6709 MHz $< \nu_H <$ 0.6791 MHz, respectively.

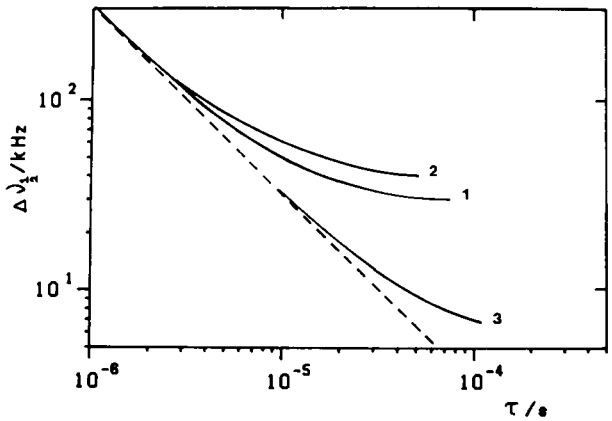

FIGURE 2 Widths at half height of the three $^{14}N^1H$ flip-flop rate lines (Fig. 1) vs. the correlation time. $\Delta\nu_{1/2}$ has been calculated on the basis of the flip-flop term of Eq. 1 with a Lorentzian intensity function. The dashed line represents the τ dependence without orientational broadening, whereas the solid curves are derived from the powder cross-relaxation dips without motional averaging.

Other potential sources of dip broadening such as a distribution of quadrupole coupling constants, broad proton resonance lines, or an inhomogeneous or unstable magnetic field are negligible in the present investigation. One finds especially that the ^{14}N coupling constant of the amide groups of proteins is equal to that of the homopolypeptide polyalanine, which also shows identical dip shapes (Winter, F., and R. Kimmich, unpublished results).

For the description of the data presented in this work, we refer to the theory published in a previous paper (1, see Eq. 7). From Fig. 2 it follows that in the case of our systems the orientation dependence of the quadrupole frequencies can be neglected, so that the ^{14}N frequencies can be replaced by their powder averages. Then the proton T_1 dispersion can be described by

$$1/T_1 \sim A/\omega_H^a + \sum_{i=1}^{3} B_i \{I[\omega_H - \omega_N^{(i)}] + I[\omega_H + \omega_N^{(i)}]\} \quad (1)$$

(T_1, proton spin-lattice relaxation time; ω_H, circular proton resonance frequency; $\omega_N^{(i)}$, circular nitrogen resonance frequencies; $I(\omega)$ spectral density; a, A, and B_i parameters to be fitted). As the nitrogen frequencies $\omega_N^{(i)}$ are taken independent of the orientation, we can form the powder average of the parameters B_i leading to the equality $B_1 = B_2$. The $^{14}N^1H$ single-flip intensity function (which eventually provides a nonnegligible contribution in the high-field case) and the proton-proton contribution have been approximated by an effective power law assuming the spin-diffusion limit (first term). In the presence of deuterons, a further set of intensity functions referring to this quadrupole nucleus has to be added to Eq. 1.

Let us finally mention that the quenching of the dipole moments of the quadrupole nuclei (7) is irrelevant. It would be relevant only for magnetic fields so low that the heteronuclear relaxation mechanism anyhow is negligible (see below).

RESULTS

Figs. 3 and 4 show the proton T_1 dispersions of BSA and α-Ch hydrated with H_2O and D_2O. In contrast to our previous work (1) the improved apparative conditions

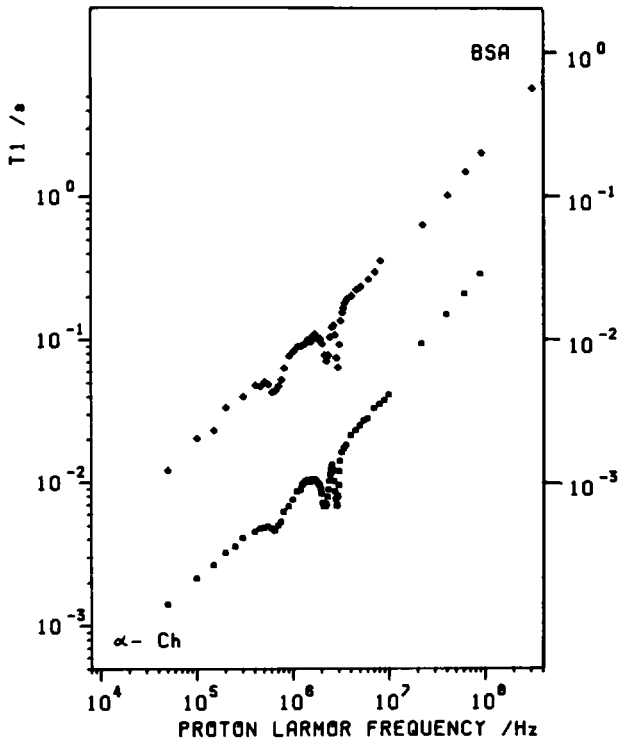

FIGURE 3 Proton T_1 dispersion of H_2O-hydrated BSA and α-Ch at 15°C. The *left* axis of ordinates refers to α-Ch, the *right* to BSA.

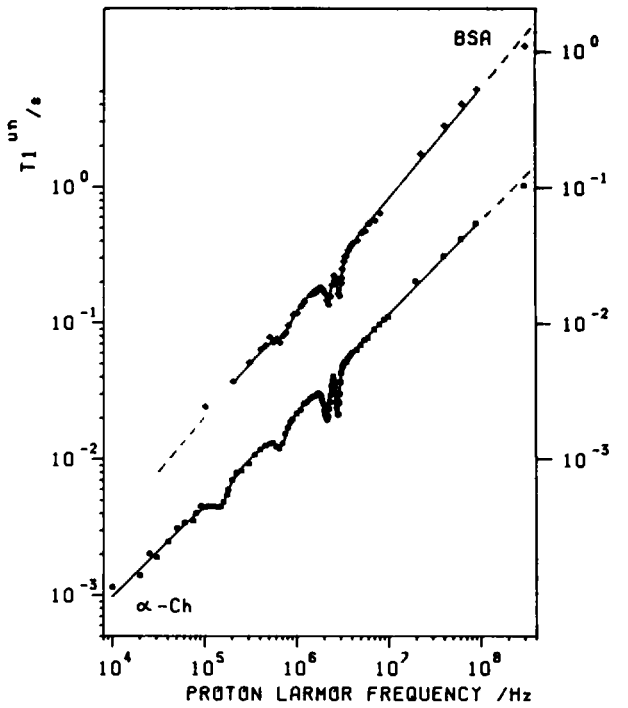

FIGURE 4 Proton T_1 dispersion of D_2O-hydrated BSA and α-Ch at 15°C. The data are identified with T_1^{un} (see Eq. 2). The *left* axis of ordinates refers to α-Ch, the *right* to BSA. The slopes of the two curves outside of the dip regions represent the limits within which the slopes of the T_1-dispersions of several other proteins have been found (Winter, F., and R. Kimmich, unpublished results). In the case of BSA the $^2H^1H$ dip has not been recorded.

permitted us to resolve clearly all three $^{14}N^1H$ quadrupole dips.

The protein system can be subdivided into exchangeable and unexchangeable proton phases. As a crude approach we neglect any direct interaction between these phases. We then can assume the rapid spin-diffusion formula for the total relaxation rate of the H_2O-hydrated system

$$1/T_1 = p/T_1^{ex} + (1 - p)/T_1^{un}, \qquad (2)$$

where p is the fraction of the exchangeable protons, T_1^{ex} their spin-lattice relaxation time, and T_1^{un} that of the unexchangeable protons. Neglecting the proton-deuterium interaction, T_1^{un} can be identified with the relaxation time of the deuterium exchanged system, so that T_1^{ex} can be evaluated from the two experimental data sets. The result is plotted in Fig. 5. Note that the consideration of the interaction between the two phases would formally lead to modified T_1^{ex} and T_1^{un} values, which therefore should be regarded as effective ones (11).

Obviously $^{14}N^1H$-quadrupole dips appear in both proton phases. This means that the protons of the hydration shells as well as the unexchangeable protein protons sense the influence of the amide nitrogens. In the case of α-Ch, the $^2H^1H$-quadrupole dip of the D_2O-exchanged system could also be observed due to the high density of data points. In this case only a single dip can be resolved indicating a nearly rotational symmetry of the field gradient. The $^2H^1H$ dip must be due mainly to deuterons in amide groups because correlation times obeying the dip condition $\omega_{dip}\tau > 1$ can be expected predominantly there (12). Hence the

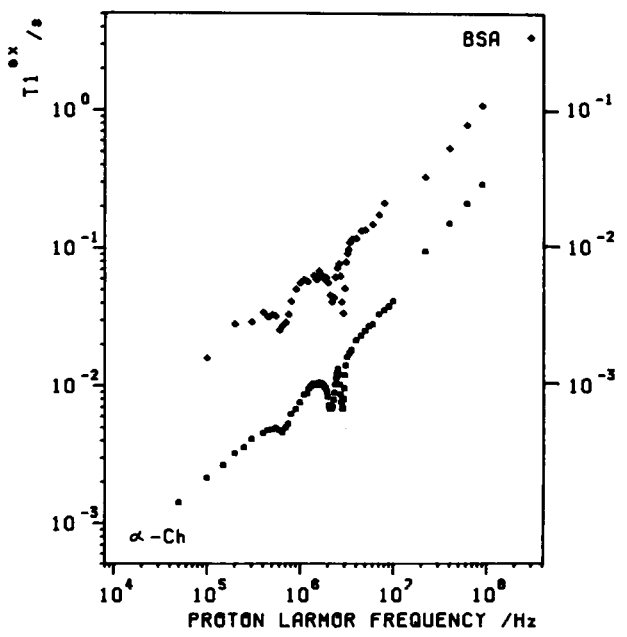

FIGURE 5 Proton T_1 dispersion of the exchangeable protons evaluated according to Eq. 2 with $p = 0.40$ (BSA) and $p = 0.39$ (α-Ch). p is the fraction of H_2O, NH_x, OH, and SH protons (8–10). The *left* axis of ordinates refers to α-Ch, the *right* to BSA.

$^2H^1H$ dip proves that the amide hydrogen nuclei act as interaction partners to nonamide protons.

The solid curves in Fig. 4 represent fits of Eq. 1 to the data points of the D_2O-hydrated samples. The parameters for BSA are $\nu_N^{(1)} = 2.87$ MHz, $\nu_N^{(2)} = 2.18$ MHz, $\nu_N^{(3)} = \nu_N^{(1)} - \nu_N^{(2)}$, $a = 0.8$, $A = 2.1 \cdot 10^7\ s^{-a-1}$, $B_1 = B_2 = 1.6 \cdot 10^7\ s^{-2}$, $B_3 = 1.2 \cdot 10^7\ s^{-2}$, $\tau = 1.0 \cdot 10^{-6}$ s. In this fit a single fluctuation process with a Lorentzian spectral density has been assumed for the $^{14}N^1H$ interaction. The ^{14}N frequencies correspond to $e^2qQ/h = 3.3$ MHz and $\eta = 0.4$.

The parameters of the fit to the data of D_2O-hydrated α-Ch are $\nu_N^{(1)} = 2.81$ MHz, $\nu_N^{(2)} = 2.13$ MHz, $\nu_N^{(3)} = \nu_N^{(1)} - \nu_N^{(2)}$, $a = 0.7$, $A = 2.1 \cdot 10^6\ s^{-a-1}$, $B_1^N = B_2^N = 1.0 \cdot 10^7\ s^{-2}$, $B_3^N = 0.9 \cdot 10^7\ s^{-2}$, $\tau_N = 1.3\ \mu s$, $\nu_D^{(1)} = \nu_D^{(2)} = 0.15$ MHz, $B_1^D = B_2^D = 2.9 \cdot 10^6\ s^{-2}$, $\tau_D = 5.7\ \mu s$. The indices N and D refer to the $^{14}N^1H$- and $^2H^1H$-quadrupole dips, respectively. In this case Eq. 1 has been supplemented by terms analogously taking into account the $^2H^1H$ interaction. As the asymmetry parameter η for the amide deuterons is rather low (13), no attempt has been made to resolve the $^2H^1H$ dip into the three transitions principally occurring with spin 1 nuclei in the low-field case. Hence, the fitted τ_D value is of a more formal nature and will not be discussed further. The ^{14}N frequencies correspond to the same quadrupole coupling constant and asymmetry parameter as with BSA.

The quadrupole resonance data fit well to the results of direct NQR investigations (13). The parameter a has also been determined with unpublished data of other protein and polypeptide systems. The average value was found to be $a = 0.75 \pm 0.05$ in accordance with the above values.

CONCLUSIONS AND DISCUSSION

The appearance of quadrupole dips permits us to draw conclusions concerning the location of the relaxation mechanisms. As the $^{14}N^1H$ dips have been found both with the exchangeable and unexchangeable protons, the amide groups are good candidates to form relaxation centers effective for the whole system at frequencies near the $^{14}N^1H$ dips. Below this region the $^{14}N^1H$ coupling becomes irrelevant for the proton relaxation because of the negligible spectral densities of the $^{14}N^1H$ interaction (1). Nevertheless the amide groups contribute to the relaxation process due to the homonuclear coupling between amide and nonamide protons. The experimental evidence for that is given by the $^2H^1H$ dip (Fig. 4), which indicates the central role of the amide hydrogen nucleus for all kinds of couplings relevant for proton relaxation.

The description of the data of the deuterium-exchanged samples on the basis of Eq. 1 appears to be quite successful (Fig. 4). There is, however, a series of remarkable features that must be discussed in detail.

The T_1 data of the deuterium exchanged α-Ch indicate a uniform slope (outside of the dip regions) that extends over more than 4 decades. The slope is given by the parameter a in the first term of Eq. 1. An intensity function yielding the fitted value $a = 0.7$ in the spin-diffusion limit is not compatible with the shape of the quadrupole dips. These can best be described with Lorentzian spectral densities, which would lead to $a = 2$. Also, the correlation time $\tau = 1.3\ \mu s$, which has been fitted to the width of the $^{14}N^1H$ dips, is not compatible with the observation of the spin-diffusion limit down to 10^4 Hz. The quadrupole splitting of the amide deuterons in other protein systems (14, 15) also indicates that the reorientation of these deuterons is characterized by a time constant much greater than the τ value given above. We conclude that the $^1H^1H$ and $^{14}N^1H$ interactions are governed by different mechanisms.

Fluctuations of the dipolar interaction among protons mainly arise from molecular rearrangements. Hence a nonLorentzian intensity function of the observed type can easily be imagined. The coupling between ^{14}N and protons, on the other hand, can additionally be modulated by ^{14}N spin flips provided that the ^{14}N spin-lattice relaxation time is short enough. Such a spin flip fluctuation mechanism is well known with solutions of paramagnetic ions, where extremely short relaxation times of the ion spins frequently occur as a consequence of additional spin-lattice couplings (16).

In the case of ^{14}N, a coupling much more effective than dipolar interaction is mediated by the quadrupole interaction with the electric-field gradient. Any modulation of the field gradient will strongly perturb the ^{14}N spin states. The most probable mechanism leading then to ^{14}N spin flips is the Raman or indirect process (17, 18). The principle of this mechanism is that a vibronic quantum is transferred to another one, where the frequency difference corresponds to the spin transition energy. The time dependence of the perturbation of the quadrupole interaction therewith is given by the vibrations themselves.

In contrast to these weak perturbations a strong collision mechanism is occasionally assumed in context with quadrupole nuclei (19). In this case the nitrogen spin-lattice relaxation time should be in the order of magnitude of the mean interval between the collision events. If these events consist of molecular reorientations, the $^1H^1H$ interaction should also be affected and we would again arrive at the discrepancy described above. Strong collisions arising by purely electronic events, on the other hand, to our knowledge have never been proven for proteins.

Clearly, if the correlation function of the $^{14}N^1H$ interaction is identical to the spin-lattice relaxation function of the ^{14}N nuclei, we have to deal with exponential decays and, hence, with Lorentzian spectral densities. In conclusion, the correlation times of the $^{14}N^1H$ interaction should reflect the protein backbone dynamics via the spin-lattice relaxation of the amide nitrogens. A more detailed theory of the nitrogen relaxation in proteins would certainly deserve further efforts because it must be so intimately related to the dynamical properties of the backbones.

The uniform slope $a = 0.75 \pm 0.05$ outside of the dip regions over more than 4 decades is a further outstanding

result found with the deuterium exchanged proteins. No other experiment with any other system is known to us which yields this slope over such an extreme frequency range (compare 3). Lyophilized proteins and homopolypeptides show the same uniform slopes within the same limits irrespective of the conformation (Winter, F., and R. Kimmich, unpublished results; note, however, that the absolute values of T_1 of lyophilized samples strongly depend on conformational properties). The mechanism that is responsible for this slope must be connected with motions of the backbone structure visible in its pure form only with the nonexchangeable protons or in the absence of a hydration shell.

The generality with which the $a = 0.75$ slope has been found with deuterium exchanged or lyophilized proteins and polypeptides contradicts the assumption of a distribution of correlation times. For instance, BSA has an α-helix content much higher than that of α-Ch (20). The backbone protons of purely α-helical polyalanine also led to the same slope (Winter, F., and R. Kimmich, unpublished results). This striking insensitivity of the slope to conformational properties favors a general mechanism. As an explanation we suggest that the protein molecules experience a sequence of conformational jumps (compare 21) leading to a suitable type of statistics of the fluctuations of the $^1H^1H$ couplings. A corresponding model calculation is in preparation.

Interestingly, the exchangeable protons did not show the straight T_1 dispersion observed with T_1^{un} (Fig. 5). Possibly this indicates a separate mechanism taking place within the hydration shells. One could think of a motion relative to the protein surface (22). In this study we have investigated hydrated but still solid proteins. The solid state is an essential prerequisite for the appearance of the reported phenomena. In liquid solutions other motions such as the rapid rotational tumbling of the whole molecules will at least partially conceal these processes (23, 24). This should be taken into account when interpreting the T_1 dispersion of such systems. Thus, models leading to quite different frequency dependences have been discussed in reference 25 and in work by Rorschach, H. E., and C. F. Hazlewood (manuscript submitted for publication).

We would like to thank Dr. Foerster, Bruker Analytische Messtechnik, Rheinstetten, Federal Republic of Germany for carrying out the 300-MHz measurements.

This work has been supported by the Deutsche Forschungsgemeinschaft.

Received for publication 31 July 1984 and in final form 9 April 1985.

REFERENCES

1. Winter, F., and R. Kimmich. 1982. Spin lattice relaxation of dipole nuclei (I = 1/2) coupled to quadrupole nuclei (S = 1). *Mol. Phys.* 45:33–49.
2. Kimmich, R., W. Nusser, and F. Winter. 1984. In vivo NMR field-cycling relaxation spectroscopy reveals $^{14}N^1H$ relaxation sinks in the backbones of proteins. *Phys. Med. Biol.* 29:593–596.
3. Kimmich, R. 1980. Field cycling in NMR relaxation spectroscopy: Applications in biological, chemical and polymer physics. *Bull. Magn. Reson.* 1:195–218.
4. Mansfield, R., and P. G. Morris. 1982. NMR Imagining in Biomedicine. Academic Press, Inc., New York. 354 pp.
5. Voigt, G., and R. Kimmich. 1980. Chain fluctuations in the amorphous regions of polyethylene as indicated in proton relaxation spectroscopy. *Polymer.* 21:1001–1008.
6. Muha, G. M. 1982. The Zeeman effect in spin = 1 systems. *J. Magn. Reson.* 49:431–443.
7. Leppelmeier, G. W., and E. L. Hahn. 1966. Nuclear dipole field quenching of integer spins. *Phys. Rev.* 141:724–731.
8. Dayhoff, M. O., editor. 1976. Atlas of Protein Sequence and Structure. Vol. 5, Suppl. 3. National Biomedical Research Foundation, Silver Spring, MD. 414 pp.
9. Dayhoff, M. O., editor. 1972. Atlas of Protein Sequence and Structure. Vol. 5. National Biomedical Research Foundation, Silver Spring, MD. 418 pp.
10. Birktoft, J. J., and D. M. Blow. 1972. Structure of crystalline α-chymotrypsin. *J. Mol. Biol.* 68:187–240.
11. Koenig, S. H., R. G. Bryant, K. Hallenga, and G. S. Jacob. 1978. Magnetic cross-relaxation among protons in protein solutions. *Biochemistry.* 17:4348–4358.
12. Lynch, L. J. 1983. Water relaxation in heterogeneous and biological systems. *In* Magnetic Resonance in Biology. J. S. Cohen, editor. John Wiley & Sons, Inc., New York. 248–304.
13. Edmonds, D. T. 1977. Nuclear quadrupole resonance. *Phys. Reports.* 29:233–290.
14. Spohn, K.-H., and R. Kimmich. 1983. Characterization of the mobility of various chemical groups in the purple membrane of *Halobacterium halobium* by ^{13}C, ^{31}P, and ^2H solid state NMR. *Biochem. Biophys. Res. Commun.* 114:713–720.
15. Schramm, S., and E. Oldfield. 1983. Nuclear magnetic resonance studies of amino acids and proteins. Rotational correlation times of proteins by deuterium nuclear magnetic resonance spectroscopy. *Biochemistry.* 22:2908–2913.
16. Hausser, R., and F. Noack. 1964. Kernmagnetische Relaxation und Korrelation in Zwei-Spin-Systemen. *Z. Physik.* 182:93–110.
17. Woessner, D. E., and H. S. Gutowsky. 1963. Nuclear pure quadrupole relaxation and its temperature dependence in solids. *J. Chem. Phys.* 39:440–456.
18. van Kranendonk, J., and M. B. Walker. 1968. Theory of spin-lattice relaxation in anharmonic crystals. *Can. J. Phys.* 46:2441–2461.
19. Alexander, S., and A. Tzalmona. 1965. Relaxation by slow motional processes. Effect of molecular rotations in pure quadrupole resonance. *Phys. Rev.* 138:A845–A855.
20. Walton, A. G. 1981. Polypeptides and Protein Structure. Elsevier/North-Holland, New York. 393 pp.
21. Englander, S. W., and N. R. Kallenbach. 1984. Hydrogen exchange and structural dynamics of proteins and nucleic acids. *Q. Rev. Biophys.* 16:521–655.
22. Andrew, E. R., D. J. Bryant, and T. Z. Rizvi. 1983. The role of water in the dynamics and proton relaxation of solid proteins. *Chem. Phys. Lett.* 95:463–466.
23. Kimmich, R., and F. Noack. 1970. Zur Deutung der kernmagnetischen Relaxation in Proteinlösungen. *Z. Naturforsch.* 25a:1680–1684.
24. Koenig, S., and W. E. Schillinger. 1969. Nuclear magnetic relaxation dispersion in protein solutions. *J. Biol. Chem.* 244:3283–3289.
25. Escanye, J. M., D. Canet, and J. Robert. 1983. Frequency dependence of water proton longitudinal NMR relaxation times in mouse tissues around the freezing transitions. *Biochim. Biophys. Acta.* 762:445–451.