# Protein backbone fluctuations and NMR field-cycling relaxation spectroscopy

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# LETTERS

## Protein Backbone Fluctuations and NMR Field-Cycling Relaxation Spectroscopy

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Proton and deuteron field-cycling relaxation spectroscopy has been employed for the characterization of fluctuations in proteins and in their hydration shells. The nature of the fluctuations is shown to be different. Deuteron relaxation dispersion of water in particular does not reflect the dynamics specific for protein backbones. Protein backbone fluctuations are characterized by simple power laws describing the overall frequency dependence of the spin–lattice relaxation time over several decades. The exponent changes at about 200 K from a constant value above this temperature to values decreasing with decreasing temperatures. This may be interpreted by a transition from ergodic to nonergodic behavior in the time scale of the experiments.

### Introduction

The purpose of this Letter is to demonstrate that, apart from protein overall tumbling, the processes governing nuclear magnetic relaxation of protein nuclei and of protein hydration water experience different fluctuation mechanisms. In particular it is to be shown that interpretations of water relaxation data on the basis of intramolecular protein dynamics alone are misleading. A further aim is to verify phenomena of protein dynamics which are analogous to observations obtained with other techniques.

Two experiments are reported supplementing our previous work on protein backbone[1,2] and hydration water[3-5] dynamics. Based on studies with diverse NMR techniques, the following conclusions can be stated:

1. Protein backbone fluctuations near room temperature are characterized by an anomalous behavior of the spectral density $I(\nu)$. Five different protein or polypeptide systems with or without heavy hydration water have been studied. The overall frequency dependence of the proton spin–lattice relaxation time $T_1$ (without $^{14}N^1H$ quadrupole dips and partially after separating the influence of methyl group rotation) can be described in a wide range of at least $10^4 < \nu < 10^8$ Hz by power laws

$$\frac{1}{T_1(\nu)} \propto [I(\nu) + 4I(2\nu)] \propto \nu^{-a}, \quad 0.67 < a < 0.83 \qquad (1)$$

The exponent $a$ averaged over eight independent sets of data of the diverse protein or polypeptide samples with and without hydration water is $\langle a \rangle = 0.75$. The dispersion slope of hydrated proteins tends to be slightly lower than that of lyophilized samples. There are indications for a weak correlation to the $\alpha$ helicity.

2. At low temperatures the $T_1$ dispersion tends to be weaker.

3. Proton relaxation of hydration water is slightly affected by backbone fluctuations (via cross relaxation), but there is no such observation for deuteron relaxation. As the dominating mechanism in the absence of macromolecular tumbling, rather, surface diffusion has been concluded. It is a process mediating reorientations of the hydration water molecules as a consequence of translational displacements at the protein/water interface.

In this study, we present additional experimental data the interpretation of which supplements the above findings. As a

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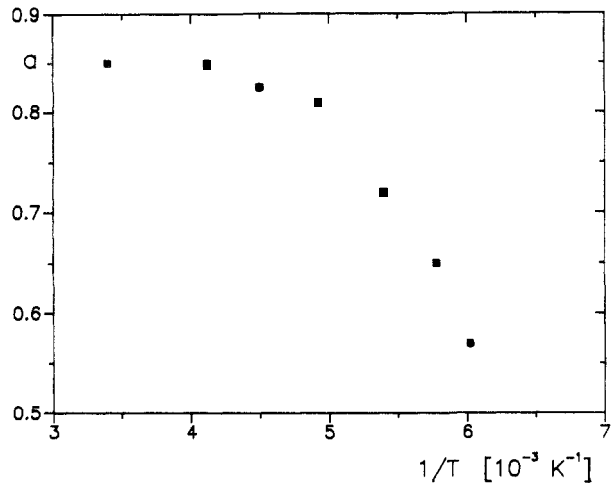


**Figure 1.** Temperature dependence of the exponents of the power law eq 2 measured in lyophilized bovine serum albumin by the aid of proton field-cycling relaxation spectroscopy.

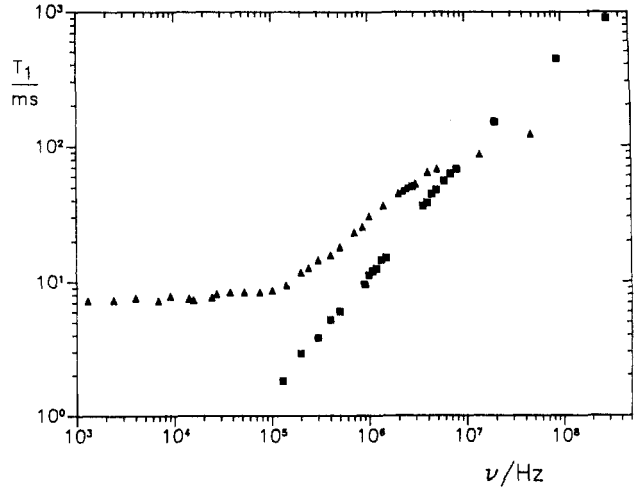


**Figure 2.** Frequency dependences of the proton (■) and deuteron (▲) spin-lattice relaxation times $T_1$ of a $D_2O$ solution of bovine serum albumin (65% water by weight). Proton relaxation data in the regions of the $^{14}N$ quadrupole dips are not shown.[2] The different $T_1$ dispersions indicate different fluctuation processes dominant in the water (deuterons) and in the protein (protons).

protein example we have studied bovine serum albumin (BSA) lyophilized or dissolved in $D_2O$. In the latter case, protein and water fluctuations can be studied separately by proton and deuteron relaxation, respectively. The measuring method is NMR field-cycling relaxation spectroscopy[6,7] which directly probes the spectral densities of the fluctuations. A version of the home-built apparatus used for the measurements has been described elsewhere.[8] The frequency dependence of the deuteron spin–lattice relaxation times in particular was measured by the aid of a switchable superconducting magnet coil.

## Results and Discussion

The frequency dependence of the protein proton spin–lattice relaxation time can be described over several decades by simple power laws

$$T_1 \propto \nu^a \qquad (2)$$

Figure 1 shows the temperature dependence of the exponents $a$ of lyophilized BSA. The values vary from 0.85 at room temperature to about 0.57 at 166 K. Above 200 K a plateau is visible suggesting that the nature of the fluctuations, which are responsible for the relaxation process, is temperature independent. Below 200 K a strong decrease of the exponents occurs. Previously we have already shown that the slope of the $T_1$ dispersion at low frequencies is not determined by side-group motions.[1] Thus the change at 200 K indicates a different dynamic behavior of the backbone.

The exponents plotted in Figure 1 were measured with lyophilized samples. The same protein proton $T_1$ dispersion was found in $D_2O$ solutions at room temperature.[2] Figure 2 shows further data for solutions with about 65% $D_2O$ and about 35% protein. The concentration was chosen so that the water content exceeds the saturation concentration of the hydration shells (about 30% water), but is low enough with respect to the critical concentration where macromolecular tumbling becomes possible.[4] The influence of tumbling motions therefore is negligible.

The spin–lattice relaxation rate directly reflects (compare eq 1) the spectral density of the fluctuations modulating the spin interactions in the frequency range of the experiment. The combined application of proton and deuteron field-cycling relaxation spectroscopy therefore permits a direct comparison of the fluctuation behavior of the protein backbones (proton NMR) and of the surrounding water (deuteron NMR). With deuterons spin diffusion is negligible. Also modulation of nonsecular interactions by exchange processes is not effective for spin–lattice relaxation in our frequency range. Exchange deuterons in the protein therefore are not expected to contribute to spin–lattice relaxation of the water deuterons (in contrast to the case of transverse relaxation).

Hydration water molecules often are imagined to be attached at the polar sites of the protein surface so that rotational or translational degrees of freedom are suppressed. One then might assume that the hydration water molecules experience the same type of fluctuations as the protein nuclei just as if they were integral constituents of the macromolecule. The comparison of the two $T_1$ dispersion data sets, however, unambiguously shows that the intensity function as seen from the $T_1(\nu)$ data is different for water deuterons and for protein protons, respectively. While the $T_1$ dispersion of the latter is characterized in our frequency range by $T_1 \propto \nu^{0.85}$, water shows low- and high-frequency plateaus and a frequency dependence at medium frequencies roughly given by $T_1 \propto \nu^{0.6}$. Fluctuations of the protein structure therefore cannot be dominating for water molecules attached to the protein surface, and water relaxation cannot be considered to be indicative of the nature of protein backbone fluctuations. Only with $H_2O$ solutions a relatively weak contribution from the proteins to the relaxation of water can be stated as a consequence of cross relaxation mechanisms.[2]

The problem of the deviating fluctuation patterns can be solved unconstrainedly by assuming that hydration water molecules underlie surface diffusion as an independent process modulating the interactions of spins in water. The notion is that water molecules are translationally displaced on the *rugged* protein/water interface. Reorientations thus are mediated by translations. In comparison to this mechanism, the influence of protein fluctuations on water relaxation is expected to be weak because of the comparatively low fluctuation amplitudes in the absence of tumbling motions.

Surface diffusion requires a high hydration water mobility. A direct verification of translational degrees of freedom has been demonstrated by the aid of NMR field-gradient diffusion measurements[5] showing that even in myoglobin crystals water diffusion coefficients are of the same order of magnitude as in bulk water.

The peculiar slope of the $T_1$ dispersion of the protein protons, on the other hand, can be interpreted by the anomalous diffusion of dilating defects or excitations along the backbone chains.[1] Adopting this model, the change at 200 K then should indicate a kind of trapping of the defects at low temperatures.

W. Doster (private communication) has suggested that a process of that kind is an example of the transitions between "class a" and "class c" processes in the nomenclature by Alexander et al.[9]

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Tahir-Kheli and Holzhey[10] consider a similar model but assume a mutual (repulsive) interaction of the diffusing excitations so that the diffusion properties depend on the temperature-dependent concentration. The changing $T_1$ dispersion may generally be considered as a manifestation of a transition between an ergodic behavior at high temperatures to a nonergodic situation connected with frozen-in states in the time scale of the experiments at low temperatures. Thus a certain analogy to the glass transition of glass-forming materials can be stated.

Other experiments provide similar findings indicating changes at 200 K. An NMR example is the temperature dependence of the second moment of the proton line.[1] The Mössbauer resonance absorption of the heme iron in myoglobin shows a crossover at 200 K.[11] Flash photolysis experiments for ligand binding indicate the freezing of ligand transport at about 200 K.[12] Recently an interesting absorption and scattering study using Mössbauer radiation has been reported.[13] The temperature dependence of the mean square displacements of myoglobin crystals was found to show a clear break at about 200 K. Above this temperature, the displacements were interpreted by intramolecular short-range modes, which could be identical with those that are responsible for the peculiar backbone fluctuation.

The fact that findings of NMR and non-NMR experiments can be projected on each other reduces the variety of possible interpretations considerably. The conclusion is in particular that the low-frequency NMR relaxation times of protein protons (in the absence of overall tumbling) are governed by backbone dynamics. The NMR data thus reveal the peculiar spectral density of these fluctuations.

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# Scanning Tunneling Microscopy of Polythiophene, Poly(3-methylthiophene), and Poly(3-bromothiophene)

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Scanning tunneling microscope (STM) images were obtained from polythiophene, poly(3-methylthiophene), and poly(3-bromothiophene) doped with tetrafluoroborate. Images of polythiophene contained both helical and chainlike structures. The distance between thiophene units was about 4 Å, and imaging may have occurred at the sulfur atoms. The poly(3-methylthiophene) had an ordered zigzag structure in 350-Å$^2$ images, but less regular structure in 30-Å$^2$ images. The poly(3-bromothiophene) surface images gave indications of ridges in 500-Å$^2$ images, but an almost regular dislocation in its linear chains in 20-Å$^2$ images. It would also appear that the sulfur atoms are involved in the tunneling process.

There has been a great deal of interest in the surface structure and electronic structure of conducting organic polymers.[1,2] Recently, STM was used to image polypyrrole to give evidence of semicrystalline growth and helical polymer growth.[3] Helical conformations have been predicted for polythiophene by using MNDO for geometrics and EHT for band-gap calculations.[1] Polypyrrole-coated platinum has been imaged in an acetonitrile solution,[4] which could have a different structure than dry films. The morphology of the conducting films has been shown to be dependent upon both the dopant anion incorporated and the conditions of polymer formation.[5] The insoluble nature of some electrochemically deposited conducting polymers has made their characterization more difficult. CP-MAS NMR experiments have been used to characterize organic conducting films[6] as have X-ray diffraction and transmission electron microscopy[4] and IR and XPS.[7,8]

STM images have been cited as direct evidence in support of a helical structure in doped polypyrrole.[3] This type of structure has also been calculated for highly doped 3-methylthiophene.[9] While helical structures have been imaged on solid surfaces,[3] rodlike anti planar configurations may be preferred on the neutral molecule in solution. This is supported by SANS measurements, and in solution doping can change the configuration.[10]

STM images[11,12] were obtained of electrochemically deposited thick films of polythiophene, poly(3-methylthiophene), and poly(3-bromothiophene).[13] Platinum was used as a substrate material. Images were taken on various parts of the film; in some

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(11) The STM used in these studies was a custom ultra high vacuum (UHV) compatible design, employing a UHV inchworm for coarse sample approach and a single-tube element for $x$, $y$, and $z$ scanning. Atomic resolution images of highly oriented pyrolytic graphite were used for calibration.
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(13) Deposition was done galvanostatically on a EGG-PAR Model 362 scanning potentiostat. The plating surface was platinum foil 4 mm × 10 mm. This size was necessary to make the necessary electrical contacts in the STM. The electroplating solutions contained 3.0 mL of acetonitrile (Aldrich HPLC Grade), 0.1 mL of the thiophene compound, and 50.0 mg of lithium tetrafluoroborate. Five milliamperes of current was passed through for 240 s. Large plates formed this way; covering of the entire surface was somewhat uneven, being thickest at the edges. The foil was mounted for STM imaging by using conducting glue to attach it to the metal strip that fit the contacts of the STM.

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