N.m.r. of solid biopolymers

E. R. Andrew
Departments of Physics, Radiology and Nuclear Engineering Sciences, University of Florida, Gainesville, Florida 32611, USA
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The dynamical behaviour of four solid polypeptides (α-chymotrypsin, insulin, lysozyme, ribonuclease A) has been investigated by proton magnetic relaxation. The interpretation was supplemented by measurements on the constituent amino acids, and on dipeptides, tripeptides and homopolypeptides. The following motions were identified and characterized: methyl group reorientation, segmental motion, sidechain motion, proline ring puckering, reorientation of water molecules in the structure. Slow motions were investigated through measurements of the dipolar relaxation time T_{1D}.

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From the very earliest days of n.m.r. it has been possible to study the motion of molecules in the solid state, first from the narrowing of the proton dipolar-broadened spectrum, and in more detail from the measurements of the proton spin-lattice relaxation time T_1. So we wondered how much we could learn about the dynamical behaviour of larger molecules such as proteins from n.m.r. in the solid state. This has the interest that we are examining the molecules in the same environment as that in which their X-ray structures were determined and it would also complement n.m.r. studies on these molecules in solution.

One advantage of working in the solid state is the very wide temperature range which is accessible from room temperature down to quite low temperatures and this gives the chance to characterize the motions well with activation parameters. In solution often only a small temperature range is accessible before denaturing and degradation occur.

A second advantage of working in the solid state is that the large protein molecule is essentially fixed in the crystal lattice so that we examine directly the intramolecular motions. In solution on the other hand the intramolecular motions are superposed on tumbling of the whole molecule, which complicates the analysis, and of course it can only be applied to those groups whose spectral lines are separately resolved and assigned.

In the present work we are looking at the relaxation of the whole collective assembly of dipolar-coupled protons. So we cannot be specific about the details of particular individual groups, but as we shall see we can determine the types of motion which contribute most and we can characterize them. A protein contains several hundred protons, maybe a thousand or more, each with its own molecular environment, which makes it a complicated system to investigate. So the philosophy of our work was to start with simpler precursors and having established some types of behaviour and understood them quantitatively we could then move on to protein molecules themselves with more confidence.

So we began by looking at the proton relaxation time T_1 of all the classical 20 amino acids of which proteins are composed, in the solid state from 100 to 500 K. This work has been reported in a series of three papers[1-3]. Analysis using the well-known Kubo–Tomita theory of dipolar relaxation[4] developed from the original relaxation theory of Bloembergen, Purcell and Pound[5], quantitatively identified reorientation of methyl and NH_3 groups and motions of other side groups. At each temperature a single correlation time τ_c characterized each motion and its temperature-variation followed a simple Arrhenius activation law, from which an activation energy E_A and pre-exponential factor τ_0 was determined. The whole relaxation–temperature curve was fitted by computer, not just the asymptotes. Measurements were made at two frequencies and the same parameters were used in fitting the results from both. A comparison of the measured and calculated dipolar coupling constants provided a strong test of the correct identification of the motions. This work on the solid amino acids is a subject in itself and some of the amino acids exhibited most interesting side chain motions, particularly arginine, cysteine, methionine, phenylalanine and valine.

These measurements were extended to polycrystalline dipeptides and tripeptides[6], for example alanylglycine and glycylalanine and the series glycine, diglycine, triglycine, polyglycine. We usually found the features of the monomeric amino acids carried over with minor modifications into the peptides. Next we examined a series of homopolypeptides[7] and by then felt we had enough experience to help us tackle some solid proteins.

The four proteins examined in polycrystalline form were α-chymotrypsin, insulin, lysozyme and ribonuclease A, selected because they are well-characterized, their X-ray structures have been determined, they are readily available in pure form and are not too expensive. We used about 0.5 g for each specimen, and pumped them for 24 h at room temperature to remove oxygen and most of the water. Measurements were made at 18, 30 and 60 MHz on each protein from 300 K down to 10 K. Results for ribonuclease A are shown in *Figure 1*.

The results[8-11] exhibited altogether broader shallower curves than those found for the monomeric amino acids and the simple peptides, and could only be accounted for assuming a distribution of correlation times τ_c. This is

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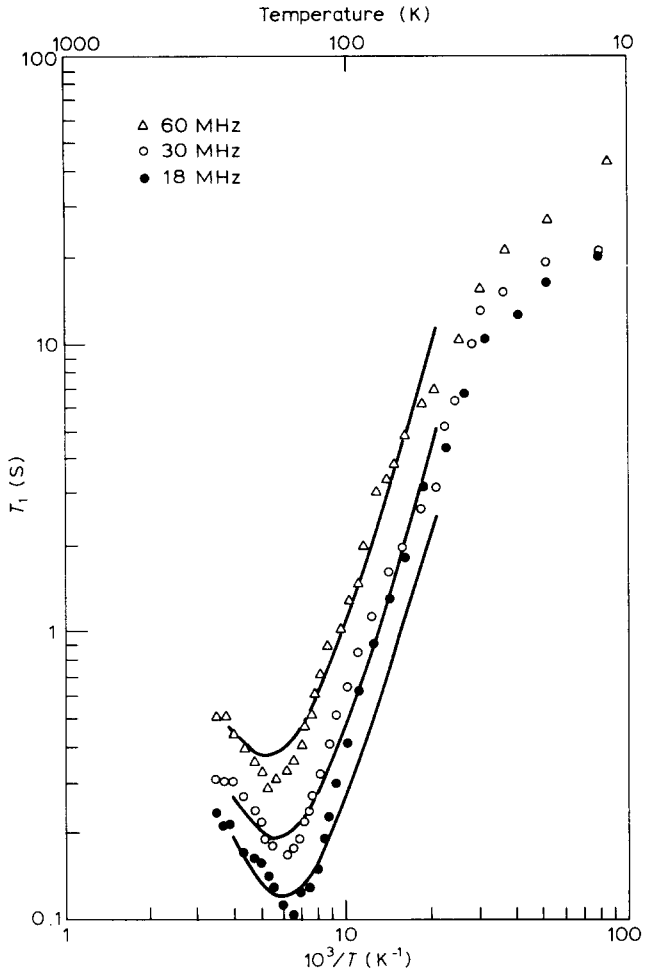

Figure 1 Temperature dependence of the proton spin-lattice relaxation time T_1 in solid ribonuclease A between 10 and 300K. The full lines are theoretical curves calculated as described in the text: (△) 60 MHz; (○) 30 MHz; (●) 18 MHz

really not surprising. In the pure amino acids we sometimes resolved two or even three correlation times from two or three independent molecular group motions at each temperature. In a biopolymer such as these protein molecules, with very many different proton sites and degrees of freedom, many correlation times are needed. We should note that we could fit any one of the relaxation curves measured at a single measuring frequency with a single thermally activated correlation time, but not all three curves for the three measuring frequencies simultaneously. This illustrates how important it is to work at several measuring frequencies.

Four well-known distribution functions were tried: Gaussian (log-normal), Cole–Cole, Cole–Davidson, Fuoss–Kirkwood. It was found that the Gaussian distribution provided the best characterization of the data for all four polycrystalline proteins over the range 70–250 K.

We can go a long way towards identifying the motions responsible. The earlier work on precursors leads us to expect methyl groups and their reorientation to be important in the side chains of six amino acids, namely alanine, isoleucine, leucine, methionine, threonine and valine. The relaxation minima of the proteins occur in approximately the same temperature range as in most precursors. The activation energies are very similar to those of the amino acids; we do not expect them to be exactly the same, but it is satisfactory that they are similar and it suggests that most of the restrictions to reorientation are intramolecular. Moreover we can calculate the expected relaxation constant, assuming that rapid spin diffusion maintains a common spin temperature and that the methyl protons have to relax all protons in the protein molecules. This analysis shows that methyl reorientation in the side chains accounts for about 70% of the measured relaxation constants, leaving some 30% to be accounted for by side chain motions, segmental motion, reptation and whole-body motions.

Since we are dealing with an inhomogeneous system with methyl and other groups in a wide variety of environments, the assumption of a distribution of values of τ_c, each characterizing an exponential correlation function, seems physically more appropriate than use of a single suitably chosen non-exponential correlation function such as that of Williams and Watts[12].

Above 250 K and below 60 K the experimental values of T_1 fall below the calculated curves for all four proteins and evidently other processes become important here. At low temperatures there seems to be a weaker but less-hindered process. To cast light on this we examined two homopolypeptides devoid of methyl groups, namely polyproline and polyglycine.

Polyproline showed a surprisingly strong proton relaxation[13]. Analysis of the relaxation constant showed that this could be attributed to conformational motion of the proline rings. There are a number of proline residues in proteins, for example there are nine in α-chymotrypsin. This motion can therefore be expected to contribute significantly to proton relaxation over the whole temperature range.

By contrast the proton relaxation in polyglycine is much weaker[13], arising from segmental motions of the chains which modulate the dipolar interactions of the methylene and imino groups. Below 60 K the relaxation of solid proteins and polyglycine tend to become equal; this supports the view that segmental motions of the protein chain make important contributions to relaxation in solid proteins at low temperatures.

Above 250 K the deviation of T_1 below the computed curves is attributed to additional relaxation contributions of water molecules in the structure. We have investigated this with measurements on polycrystalline lysozyme and α-chymotrypsin progressively hydrated with H_2O and D_2O up to 60% by weight[14]. The amount of water added was measured by increase of weight and by Karl–Fischer titration. The Karl–Fischer titration showed that our normal specimens pumped at room temperature retained about 2% water, strongly bound.

Two main features were noticed[14], see *Figure 2*. Above 180 K increase of hydration progressively decreased T_1 exhibiting a characteristic minimum which shifted to progressively lower temperatures. Below 180 K increase of hydration progressively increased T_1. The increased relaxation rate above 180 K is due to reorientation of the water molecules. Additional water is bound less strongly causing the T_1 minimum to shift to lower temperatures. Analysis shows[14] that the activation energy for this motion decreases from 30 kJ mol^{-1} for the first increments of water to 20 kJ mol^{-1} for the last increments. It is noteworthy that the last increments of water contribute to relaxation even down to 180 K. The relaxation constant for the water reorientation turns out to be about 56% of that for isotropic intramolecular dipolar relaxation of pure water. This reduced value can be attributed to three

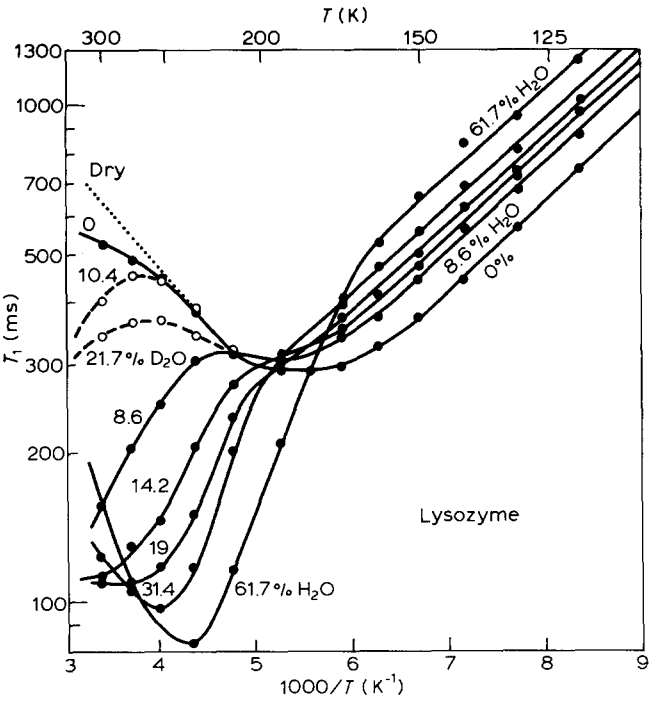

Figure 2 Variation with temperature of the proton spin-lattice relaxation time T_1 at 60 MHz is polycrystalline lysozyme with various degrees of hydration. Full circles and full lines refer to hydration with H_2O; open circles and dashed lines refer to hydration with D_2O

causes: (1) anisotropy of the reorientation of the water molecules on their sites, (2) a distribution of activation parameters on the various sites of attachment, (3) a lengthening of the interproton distance due to hydrogen bonding of the water molecules on their sites.

Below 160 K T_1 increased monotonically and approximately linearly with successive additions of water. At these lower temperatures the water molecules are frozen in the structure and contribute nothing to relaxation, but they do add to the load of protons needing to be relaxed by other protein motions.

When D_2O is added instead of H_2O we have similar effects, but very much weaker because of the relative inefficiency of deuterons in relaxing the load of protons; indeed calculations show that it is 4.2% smaller which approximately accounts for what is observed.

This study confirms that the presence of water in proteins causes extra relaxation at higher temperatures due to motions of the water molecules themselves, but there is no evidence of any change in the protein molecules induced by the presence of the water.

All the work described so far is based on measurements of T_1 which are particularly responsive to dynamic behaviour in the frequency range 10^5–10^{10} Hz. Recently we have extended our studies to measurements of the dipolar relaxation time T_{1D} using the Jeneer–Broekaert pulse sequence[15], since this is responsive to dynamical behaviour in the spectral region 1 to 10^5 Hz, enabling a wide spectral range 1 to 10^{10} Hz to be investigated overall.

Extending the expression for T_{1D} for a distribution of correlation times in the same manner as we did previously for T_1 and using the activation parameters previously obtained from measurements of T_1, we predict that a minimum in T_{1D} should be found at about 85 K, about 100 K lower than for T_1. Experiment bore this prediction out closely for both α-chymotrypsin and lysozyme[16]. This extrapolation was a severe test of the parameters obtained from T_1 measurements and gave some confidence to the description and characterization of the motions obtained through T_1. The shift of the T_{1D} minimum 100 K lower, where the motions are some 10^4 times slower gives the opportunity to examine new motions at higher temperatures. The minimum value of T_{1D} is 210 times shorter than that of T_1 at 60 MHz for lysozyme, but theory predicts a ratio of 3000. A similar discrepancy was found for α-chymotrypsin, and is attributed to a spin-diffusion bottleneck.

In some cases the use of T_{1D} enables relaxation mechanisms and motions to be resolved which are not resolved in T_1. An example was provided by solid polyvaline. In addition to a deep minimum in T_{1D} attributed to methyl group reorientation in the monomer side chains, there is a weaker minimum at about 200 K which is attributed to a more hindered reorientation of the whole side chain which modulates a much smaller fraction of the overall dipolar second moment.

So summarizing, it has been possible through the study of proton relaxation in solid proteins and related molecules to identify and characterize the following molecular motions which contribute to relaxation:

(1) methyl group reorientation
(2) segmental motion
(3) side chain motion
(4) proline ring puckering
(5) water molecule reorientation

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