A PROTON NMR STUDY OF RELAXATION AND DYNAMICS IN POLYCRYSTALLINE INSULIN

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1. Introduction

The dynamical behaviour of protein molecules is believed to play an important role in the performance of their biological functions. Since the three-dimensional conformation of proteins is determined from X-ray and neutron diffraction methods applied to protein crystals, attention has recently been focussed on the dynamical behaviour of these biomolecules in the solid state through analysis of temperature factors in protein crystallography [1,2]. Such information complements that found in solution from high-resolution NMR spectroscopy [3–5], ab initio dynamics [6,7] and other studies [8]. Dynamical information concerning protein molecules in the solid state may also be obtained by NMR relaxation studies which is especially relevant now that there is NMR evidence supporting the view that the conformation of protein molecules is similar in the crystalline state and in solution [9]. This paper reports an investigation of proton relaxation and molecular dynamics in polycrystalline insulin.

2. Experimental

The work reported here was carried out on a high purity sample of solid bovine insulin kindly provided by the Research Department of the Boots Company, Nottingham. Assay by disc polyacrylamide gel electrophoresis showed <0.1% each of pro-insulin, arginine insulin, intermediate, mono- and di-desamidoinsulin; pro-insulin by radio-immunoassay 3.6 ± 0.6 ppm, high molecular weight impurity 0.33%; zinc 0.3%. The sample had been crystallized and dried in vacuo. It was stored at −20°C to minimize formation of high-molecular weight impurity. A second sample of bovine pancreatic insulin, obtained from Sigma, product no. 15500, gave results within experimental error the same as from the Boots sample. Specimens of ~1 g were pumped for 24 h at room temperature and sealed off.

Measurements of the proton NMR spin-lattice relaxation were made at 18, 30 and 60 MHz using a Bruker B-KR 322s variable-frequency pulsed NMR spectrometer in conjunction with an AEI RS2 electromagnet over 300–10 K. A $90°$-τ-$90°$ pulse sequence was used, the signals being recorded from the free induction decay ~10 μs after the exciting pulse. Recovery of the nuclear magnetization was exponential within experimental error, and could therefore be characterized by a single spin-lattice relaxation time T_1. The accuracy of T_1 values varied with the frequency and temperature of measurement, but was typically 5–10%.

3. Results and analysis

The experimental values of T_1 obtained at the 3 measuring frequencies over 40–300 K are shown in fig.1. The values of T_1 range over two decades from 85 ms to 5 s and display the minima which are highly characteristic of nuclear spin-lattice relaxation effected by molecular motions [10,11].

The data have been analyzed using the well-known Kubo-Tomita theory of nuclear dipolar relaxation [12]. As in the case of solid ribonuclease A [13] we have found that it is not possible to account for the

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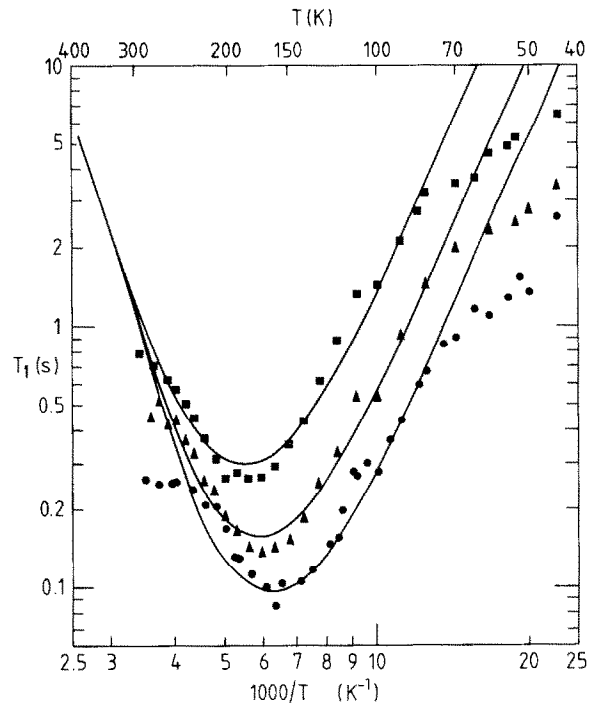

Fig.1. The variation with temperature of the proton spin-lattice relaxation time T_1 of solid insulin over 40–300 K: (●) 18 MHz; (▲) 30 MHz; (■) 60 MHz.

relaxation behaviour in a consistent manner at all 3 frequencies with the assumption that the molecular motion is characterized by a single correlation time τ_c, nor is this surprising. With simple molecules such as the monomeric amino acids in the solid state [14–16] one correlation time, or at most 2 or 3 distinct correlation times, suffice to characterize the well-defined molecular processes at each temperature. However the much more complex dynamical behaviour of a protein molecule is characterized by many degrees of freedom and a distribution of correlation times is needed to describe it.

Following the procedure adopted for solid ribonuclease A [13] the data have been least squares fitted to the generalized Kubo-Tomita theory using the Nottingham University ICL 1906A computer. This has yielded the theoretical curves shown as full lines in fig.1. It is to be noted in fig.1 that the experimental points fall systematically under the theoretical curves below 70 K, and for this reason experimental points have been included in the fitting process down to 80 K. The behaviour below 80 K is discussed later.

Table 1
Parameters characterizing molecular motion in solid insulin

Relaxation constant		$C = 2.0 \pm 0.2 \times 10^9$ s^{-2}
Mean activation energy		$E_A = 13.5 \pm 1.5$ kJ/mol
Pre-exponential factor	$\log_{10}$	$\tau_{0m} = -12.9 \pm 0.6$ s
Distribution parameter		$\beta_0 = 0.5 \pm 0.5$
Distribution parameter		$\beta_Q = 5.1 \pm 0.6$ kJ/mol

The same set of parameters characterizing the molecular motions has been used to fit the data at all 3 measuring frequencies. The RMS deviation of the points from the theoretical lines is 13%, which is very satisfactory over the wide range of experimental T_1 values. The best values of the parameters are given in table 1. In order to obtain the quoted estimates of accurary of the 5 parameters each was in turn held constant at a series of fixed values in the vicinity of the optimum value and the other 4 parameters were varied to minimize the RMA deviation. In this manner it was possible to explore the minimum in the 5-parameter space.

4. Discussion and conclusions

It has been recognized from work on amino acids [14–16], peptides [17] and homopolypeptides [18] in the solid state that an important source of proton spin-lattice relaxation arises from the hindered rotation of methyl groups in the sidechains of the residues alanine, isoleucine, leucine, methionine, threonine and valine. Three pieces of evidence support the view that this is the case in solid insulin also:

(i) The minimum value of T_1 at 60 MHz occurs at 175 K, in the vicinity of the minima found for the methyl groups in other related materials [13–18];

(ii) The spread of the Gaussian distribution of correlation times: The parameter β which characterizes that spread has a constant part β_0, which from table 1 turns out to be small, and a temperature-dependent part β_Q [19]. Evidently the distribution of correlation times arises essentially from a distribution of activation energies for the many methyl rotation sites on the insulin molecule, encompassing the range $E_A \pm \beta_Q$, namely 13.5 ± 5.1 kJ/mol (table 1). This is closely similar to the range 14.5 ± 8 kJ/mol found for the methyl groups in the solid amino acids [16];

(iii) The value of the relaxation constant C: We may first estimate the contribution that the methyl group reorientation can be expected to make to the relaxation constant in solid insulin. There are 28 methyl groups in the insulin molecule and 377 hydrogen atoms in the molecule. So each methyl group has to relax, on average, 13.46 protons. The relaxation constant for isolated methyl groups is 8×10^9 s^{-2} [16]. Consequently when the 3 protons in the methyl group are diluted to the 13.46 protons which it must now relax, the relaxation constant expected from the methyl groups in solid insulin is reduced to 1.78×10^9 s^{-2}. Comparing this with the measured value of C (table 1), we see that methyl group rotation can account for 89% of the spin-lattice relaxation of solid insulin over 80–300 K; no other plausible molecular motion could account for the measured value.

There remains some 11% of the relaxation to be accounted for by sidechain motions, NH_3 group rotation, segmental motion, reptation and whole body motions.

Below 70 K the values of T_1 fall systematically below the theoretical curves in fig.1. Here T_1 is 1–10 s and the correlation times for classical methyl group rotation by excitation over hindering barriers become too long for efficient relaxation. Other motional processes are evidently important here and we mention two classes:

(1) The motion of groups with weaker dipolar interactions, e.g., in CH_2 and N–H groups, by sidechain and backbone motions. Such motions are particularly important in polypeptides such as polyglycine which has no methyl groups and no sidechains and must depend on backbone motions to provide relaxation [18].

(2) Relaxation processes important at still lower temperatures are non-classical processes such as phonon-assisted methyl group tunnelling and phonon-scattered Raman processes [20,21].

Note the small deviation of T_1 below the theoretical curves at the high temperature end, over 270–300 K. This may arise from motion of the small proportion of water molecules in the solid protein, as was found in solid lysozyme [13], or from more hindered main-chain motions.

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