TEMPERATURE DEPENDENT MOLECULAR MOTION OF A TYROSINE RESIDUE OF FERROCYTOCHROME *C*

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1. Introduction

The mobility of the side chains of amino acid residues of proteins in solution has recently been detected and studied by nuclear magnetic resonance (NMR) spectroscopy. The most readily observed mobility is that of tyrosine and phenylalanine residues, which has been detected in lysozyme [1], cytochrome *c* [2,3], bovine pancreatic trypsin inhibitor protein (BPTI) [4], parvalbumin [5], and alkaline phosphatase [6]. This motion, which involves rotation about the Cβ–Cγ bonds of the aromatic side chains, has also been the subject of theoretical calculations for BPTI [7]. Detailed knowledge of the rates of rotation of specific residues provides information concerning the nature of protein structure, and may be important in elucidating mechanisms of protein action. This paper is concerned with NMR techniques which can provide accurate measurements of the rates of rotation of amino acid side chains of proteins. These techniques, which involve spectra simulation and cross-saturation, are illustrated for a tyrosine residue of ferrocytochrome *c*.

2. Materials and methods

Horse cytochrome *c* (Grade VI) was obtained from Sigma Chemical Co. Samples were prepared as previously described [3]. The NMR spectra were obtained using a Bruker 270 MHz spectrometer, operating in the Fourier transform mode, with an Oxford Instrument Co. magnet. Free induction decays were collected in a Nicolet 1085 computer. The selective saturation experiments were performed by applying a long, low power radio-frequency pulse (~2 s) immediately prior to the observation pulse. Acetone and dioxan were used as internal standards but all shifts are quoted in parts per million (ppm) downfield from 2,2-dimethyl-2-silapentane-5-sulphonate.

3. Results and discussion

3.1. *The spectrum of ferrocytochrome* c

The aromatic region of the proton NMR spectrum of horse ferrocytochrome *c* at various temperatures is shown in fig.1. As described previously [3] the spectrum at 57°C consists of a number of well resolved resonances. These correspond to the meso protons, the protons of two of the histidine residues and the tryptophan residue, and to the protons of three of the tyrosine and phenylalanine residues. The pattern of the resonances of these tyrosine and phenylalanine residues shows that there is rapid rotation about the Cβ–Cγ bonds. For some of the other tyrosine and phenylalanine residues this is not the case. We are concerned in this paper with the behaviour of the resonances of one such tyrosine residue. However, we first consider the general features of the temperature dependence of the proton NMR spectrum of ferrocytochrome *c*. The temperature effects involving CH resonances are completely reversible. There is a decrease in linewidth of most of the resonances with increasing temperature corresponding to an increase in T_2 values. In addition, a number of resonances

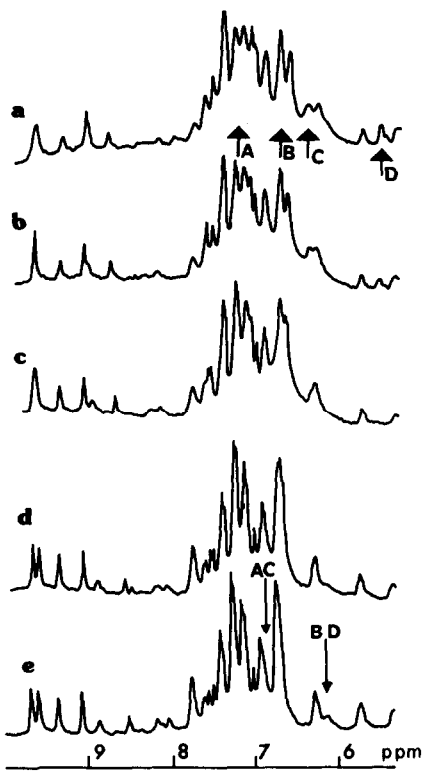

Fig.1. The aromatic region of the ^1H spectrum of horse ferrocytochrome *c* in D_2O, pH 5.3 at: (a) 22°C (b) 37°C (c) 57°C (d) 77°C (e) 97°C. The four resonances labelled A,B,C and D at low temperatures are assigned to a tyrosine residue. Because of increasing rotation rates resonances A and C, and B and D, coalesce to form resonances AC and BD respectively at high temperatures.

experience small changes in chemical shift with variation of temperature. However, no significant change in the coordination of the iron occurs over this temperature range for the resonance positions of the 5th and 6th iron ligands, histidine 18 and methionine 80 are little affected by increasing temperature. For example, the methionine 80 *S*-methyl resonance shifts by only 0.03 ppm over the range 3–97°C. We now turn to the tyrosine resonances of immediate interest.

Below 37°C, four one-proton intensity resonances are observed; at 7.19 ppm (resonance A), 6.78 ppm (resonance B), 6.33 ppm (resonance C) and at 5.59 ppm (resonance D). Resonance D is resolved separately whilst the other three resonances have been detected by double-resonance methods (see below). As the temperature is increased resonance D broadens and disappears. At temperatures above 77°C two new resonances are observed at 6.78 ppm and 6.18 ppm which sharpen as the temperature is increased. These resonances are designated AC and BD since they occur at approximately the average position of A and C, and B and D respectively. In horse ferrocytochrome *c* these resonances cannot be clearly resolved even at 97°C. However in tuna ferrocytochrome *c* similar resonances are observed with analagous behaviour and here the two resonances observed at high temperature can be seen to be two-proton intensity doublets. Double resonance experiments show that these are coupled to each other, and that they therefore arise from a tyrosine residue.

The changes with temperature are interpreted as follows. At low temperatures four resonances arise from the four tyrosine protons, represented in fig.2. This requires that the flipping rate about the Cβ–Cγ bond is slow compared to the difference in chemical shift between the two ortho proton resonances, and between the two meta proton resonances. As the temperature is increased, the rate of flipping increases and exchange broadening occurs. At still higher temperatures, the fast exchange condition occurs, where a single resonance arises from the two ortho protons, and from the two meta protons.

3.2. *Cross-saturation measurements*

At temperatures ⩽ 37°C irradiation of resonance D causes changes in the intensity of the three other resonances of the tyrosine residue. These effects can be observed clearly using difference spectroscopy as shown in fig.3. Resonance B has been reduced in intensity considerably more than resonances A and C. These effects are interpreted as follows, with reference to fig.2. Irradiation of the resonance of H_a causes a large

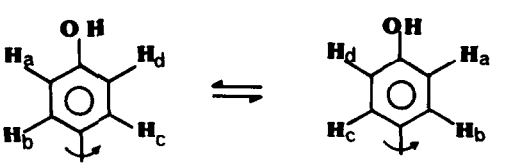

Fig.2. Rotation of a tyrosine side chain about the Cβ–Cγ bond. In the nomenclature used here the protons H_a, H_b, H_c and H_d, give rise to four resonances A,B,C and D below 37°C. A correspondence between H_a and resonance A etc. is not inferred.

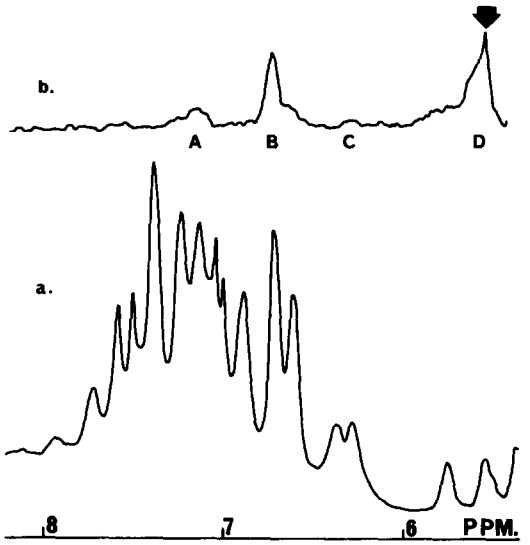

Fig.3. (a) The spectrum at 27°C, as in fig.1, and (b) the difference between (a) and a spectrum accumulated using a pre-saturation pulse on resonance D. A large effect is observed on resonance B, with secondary effects on resonances A and C.

decrease in the intensity of the resonance of H_d. This occurs because a flip about the Cβ–Cγ bond interchanges the H_a and H_d positions transferring the magnetization of H_a, which is zero because of the irradiation, to H_d [8,9]. The secondary effects on the resonances of H_b and H_c are caused by the nuclear Overhauser effect (from H_a to H_b, and from H_d to H_c following transfer of magnetization) which causes a reduction in intensity of ^{1}H-resonances of proteins [10].

It is possible to measure the rate of tyrosine flip by measuring the reduction in intensity of the cross-saturated resonance, if there is no nuclear Overhauser effect. We have assumed here that there is no nuclear Overhauser effect between the two resonances H_a and H_d, since the separation between the protons is large.

It can be shown [9] that:

$$\frac{I_a}{I_b} = \frac{1/T_1}{1/T_1 + k}$$

where I_a and I_b are the intensities after and before irradiation of the resonance from which magnetization transfer occurs.

T_1 is the spin-lattice relaxation time, measured by non-selective 180° and 90° pulses, and k is the rate of flip. The T_1 value of resonance D is 0.6 ± 0.1 S over the temperature range 4–37°C. Using the cross-saturation data in conjunction with this value of T_1, the values of k at various temperatures were calculated. These values are plotted in fig.5.

3.3. *Spectral simulation and activation energy of tyrosine rotation*

Simulated spectra of the tyrosine proton resonances were calculated on the Oxford University ICL 1906A computer using the program DNMRS originated by Kleier and Binsch [11] and made available by Dr P. Anstey, SRC Computer Library, School of Chemical Sciences, University of East Anglia, Norwich, NR4 4TJ, England. The program input requires the intrinsic transverse relaxation time T_2, the spin–spin coupling constants (J), chemical shifts (δ) and coupling pattern.

There are two possibilities for the coupling pattern; resonance A is coupled either to resonance B or to resonance D. These cannot be distinguished experimentally by spin-decoupling because of the large linewidths of the resonances. The nuclear Overhauser

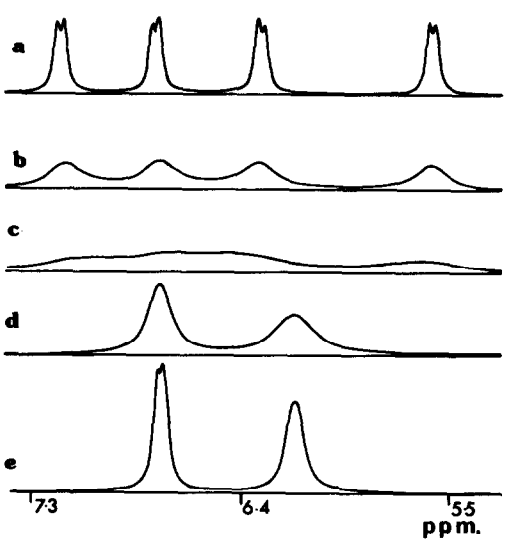

Fig.4. Computer simulation of the resonances of tyrosine residue undergoing rotation of rates (k): (a) 5 s^{-1} (b) 120 s^{-1} (c) 320 s^{-1} (d) 3400 s^{-1} (e) 10 000 s^{-1}. These rates correspond approximately to the temperatures shown in fig.1. The other parameters used in the simulations, δ, J and T_2 were measured as explained in the text.

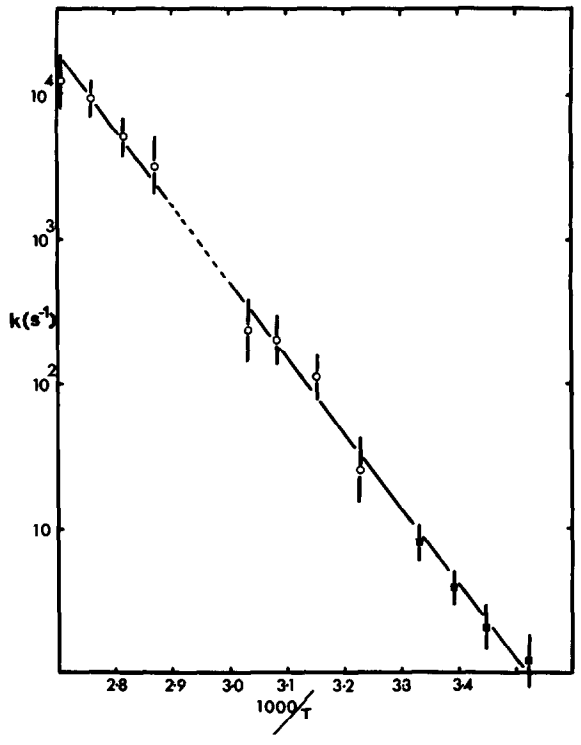

Fig.5. Plot of k versus $1000/T$; (○) calculated from spectral simulation; (■) from cross-saturation.

effect observed on irradiation of resonance D (fig.3) appears to be larger for resonance A than for resonance C, implying that the proton of A is adjacent to that of D, hence that A and D are coupled. This coupling pattern is used in fig.4. The values of δ were taken from the low temperature spectra and J was taken to be 8.5 Hz. The intrinsic T_2 values were obtained by measuring the decay of the transverse magnetization of non-exchanging aromatic resonances using a train of 180° pulses [12]. These T_2 values were found to be temperature dependent with a temperature variation described by an activation energy of about 17 kJmol^{-1}. The value at 25°C was 45 ms.

Using the above parameters, a simple iterative procedure was used to obtain the simulated spectra shown in fig.4. The values of k obtained from these fits are shown in fig.5 together with the estimated errors. These errors are large when the exchange contribution is small or large. The region with an immeasurably large exchange contribution (intermediate exchange region) is illustrated by a dotted line in the figure. Because of the uncertainty in the coupling pattern, the alternative possibility (i.e. A coupled to B) was also simulated. The values of k were found to be the same as those given, within the experimental error of the procedure.

These results together with the values of k obtained using cross-saturation give a good straight line Arrhenius plot over the whole temperature range, within experimental error. The values of k, $\Delta H^{\neq}$ and $\Delta S^{\neq}$ from this plot are calculated to be 8 s^{-1}, 97 kJ mol^{-1} and 96 JK^{-1} respectively at 25°C.

Conclusions

The temperature variation of the resonances A,B,C,D, AC and BD assigned to a tyrosine residue of ferrocytochrome c, is completely consistent with rotation about the Cβ–Cγ bond (fig.3). This is confirmed not only by a computer simulation of the tyrosine resonances but also by double resonance experiments at temperatures $\leqslant$ 37°C. The values of rate constant obtained from these two complementary methods lie on a good straight line Arrhenius plot. The high activation energy observed is consistent with the rotation of this tyrosine being hindered by the packing of other groups about it. The rotation rate is thus a sensitive probe for the overall stability and conformation of the protein. The straight line Arrhenius plot thus indicates a remarkable integrity of the structure of horse ferrocytochrome c over a very wide temperature range. The analogous resonances in rabbit, beef, chicken, pigeon, tuna and donkey ferrocytochrome c show a very similar temperature dependence. Thus despite changes in the primary sequences of these proteins the packing of groups around this tyrosine is probably similar.

Acknowledgements

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