CONFORMATIONAL MOBILITY WITHIN THE STRUCTURE OF MUSCULAR PARVALBUMINS. AN NMR STUDY OF THE AROMATIC RESONANCES OF PHENYLALANINE RESIDUES

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1. Introduction

X-ray crystallography has enabled three-dimensional models of the structure of many globular proteins to be constructed. However, there is increasing evidence that, at least in solution, protein molecules do not behave as rigid assemblies. Instead, conformational fluctuations of very different kinds and with very different rate constants take place and these may be detected by several techniques. The extent and the mechanism of these motions remain largely unknown and it is not clear how widely these motions vary from one protein to another.

In 1971, optical studies (UV and CD) of a globular protein (the parvalbumin major component from hake muscle) suggested the existence of conformational mobility for the side-chains of the phenylalanine residues owing to rotational isomerism about the $C^{\alpha}-C^{\beta}$ and $C^{\beta}-C^{1}$ bonds [1]. A ^{13}C n.m.r. study of one parvalbumin from carp muscle provided further evidence that the aromatic rings of the phenylalanine residues possess greater motion than the overall molecular rotation can provide [2].

Recently, improved ^{1}H n.m.r. techniques have allowed the resolution and the assignment of a great number of resonances of individual groups in small proteins [3]. By applying these techniques to lysozyme, a detailed comparison of the solution and X-ray structures of this protein is being carried out [4]. This has provided direct evidence for several types of molecular motion in this protein. One particular type of motion is relatively easy to define. This is the specific rotational motion (flipping) of individual aromatic residues (tyrosine and phenylalanine) about the $C^{\beta}-C^{1}$ bonds. This type of motion has been shown to occur in lysozyme [5] in cytochrome *c* [6] and in the bovine pancreatic tryptic inhibitor [7,8].

Parvalbumins form a class of homologous globular proteins of low molecular weight (approx. 11 500), characterized by a great affinity for the calcium ion and a composition with a high content of phenylalanine [9]. They contain 8–10 phenylalanine residues, while tryptophan and tyrosine are, when found, only present as a few residues. X-ray diffraction studies of parvalbumin p*I* 4.25 from carp muscle allowed the description of a compact globular structure, where the phenylalanine residues are mainly internal [10]. Owing to all these properties, parvalbumins in solution are defined by very characteristic high-resolution ^{1}H n.m.r. spectra [11]. Comparison between the solid and the dissolved states appears therefore possible for these proteins, and this has already been undertaken [11].

In this work, a detailed analysis of the down-field region of the ^{1}H n.m.r. spectra (between 5.5 and 8.0 ppm) of several parvalbumins has been achieved. Most of the aromatic resonances of the 10 phenylalanine residues of carp parvalbumin p*I* 4.25 have been resolved using multiple resonance techniques. It is clearly established that most, if not all, of the internal phenylalanine residues of parvalbumins are undergoing rapid flips about $C^{\beta}-C^{1}$ bonds. Some evidence for more extensive conformational mobility within the structure of these proteins is provided.

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2. Materials and methods

The four major parvalbumins (p*I*s: 4.47, 4.37, 4.25 and 3.95) from carp *(Cyprinus carpio)* were prepared as described previously [16]. The component p*I* 4.25 was characterized by its calcium content. A value of 2.2 Ca^{2+}/protein was determined by atomic absorption spectroscopy.

N.m.r. spectra were recorded by the Fourier transform method at 270 MHz on a Bruker spectrometer employing an Oxford Instrument Company magnet and a Nicolet 1084 computer fitted with a 293 pulse controller and a disc unit. The n.m.r. techniques have been described elsewhere [3–6,12–14].

Parvalbumin samples were dissolved in 99.8% D_2O–ND_4OD. Exchangeable protons were replaced by deuterons by keeping the solution at ambient temperature, for several hours at pH 10. Solutions were lyophilized.

One 8 mM solution of carp 4.25 parvalbumin, used for the decoupling and thermal studies, was made up by dissolving the deuterium-exchanged protein in a slightly basic (pH 10) D_2O–ND_4OD solution, affording a final pH value of 6.8. Other solutions of different parvalbumins were made up by dissolving deuterium-exchanged samples in D_2O-phosphate buffer at pH 7.4, as previously described [11]. Protein concentrations between 3 mM and 10 mM were used. In some cases dithiothreitol (Sigma reagent) was added to prevent polymerization. Chemical shift values are quoted in parts per million (ppm) and positive numbers indicate shifts to low field from the reference resonance, sodium 2,2,3,3-tetradeuterio-3-(trimethylsilyl) propionate (Merk reagent), which was usually used as an internal reference at the concentration of 10^{-4} M. The atoms in the phenylalanine and histidine residues are numbered according to the 1974 recommendations on the nomenclature of α-amino acids (1975) Biochemistry, 14, 449.

3. Results and discussion

The ^1H n.m.r. spectra of all the parvalbumins are very well resolved. At 270 MHz, using convolution difference techniques for resolution enhancement [13], the multiplet structure and spin–spin coupling is readily observed. In this work, spin-decoupling experiments were performed for the aromatic resonances of several parvalbumins. These were carried out by means of time-shared double resonance, which was performed by varying the irradiation frequency throughout the range of the aromatic proton resonances, and the resulting decoupling observed either directly or by means of difference spectroscopy [3,5]. This is illustrated in figs.1 and 2 for the resonances of the five protons of one phenylalanine of carp 4.25 parvalbumin. Irradiation at a triplet resonance of two proton area (at 6.25 ppm) caused decoupling at two positions in the spectrum. At one of these was a one proton triplet resonance (at 5.95 ppm) which decoupled to a singlet (the observed area decreasing because of a negative Overhauser effect, as described previously [14]). The other resonance (at 7.21 ppm) was a doublet, of area two protons, which was detected by using difference spectroscopy (fig.1). Irradiation separately at each of these affected resonances caused decoupling of the two proton area triplet resonance to a doublet in each case (fig.2). Similar experiments were performed for other aromatic resonances in the spectrum of this protein. Six complete sets of coupled resonances of the same pattern (a two proton area doublet coupled to a two proton area triplet coupled also to a one proton area triplet) were discovered. The remaining resonances (20 aromatic phenylalanine protons in the region from 6.9 to 7.4 ppm) were severely overlapping, but two further sets of two proton area doublets coupled to two proton area triplets were identified. In no case was there any evidence for a coupling pattern other than that given above. The two singlet resonances of the single histidine 26 were identified at 6.8 ppm (C^4–H) and 7.6 ppm (C^2–H). The resonance at 5.7 ppm (one proton area singlet) and those at approx. 5.2 ppm (presumably two protons) were identified as arising from non-aromatic protons. Full details of these experiments will be published separately.

It follows from the observed coupling pattern that in all the observed cases, the resonances of the two ortho and of the two meta protons of each phenylalanine residue are equivalent under the conditions used here. This is true even at 5°C. In order to obtain this result, it is merely necessary for each aromatic group to rotate rapidly about the C^β–C^1 bond. This motion need not to be free, for flips through 180° around the C^β–C^1 bond between the two

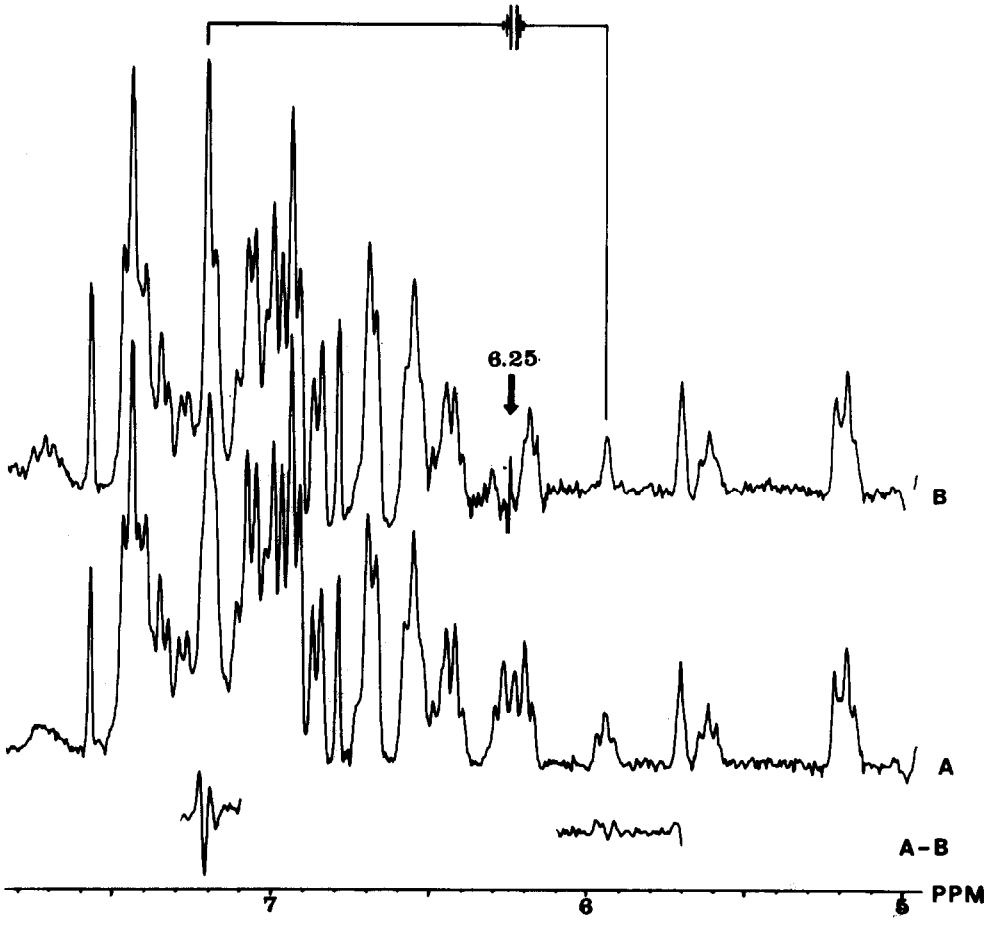

Fig.1. (A) The convolution difference spectrum (aromatic part) of carp 4.25 parvalbumin at pH 6.8 at 50°C. (B) The effect of time shared irradiation at 6.25 ppm. Part of the difference spectrum (A–B) is shown below.

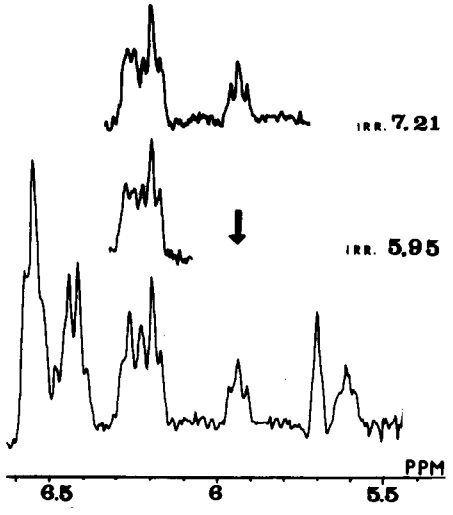

Fig.2. The effect of time shared irradiation at 5.95 ppm and at 7.21 ppm on the resonance at 6.25 ppm. Conditions as Fig.1.

identical conformers of the symmetric aromatic ring suffice, if the rate of interconversion is rapid enough on the n.m.r. time scale. Without this motion, the observed equivalence would only arise by coincidence, and for the number of resonances observed here this can be ruled out. Because of the asymmetric environment of each phenylalanine residue, it may be anticipated that the chemical shifts of all the five aromatic protons will be different for a nonrotating

phenylalanine ring. Calculations, based on the rigid model of carp 4.25 parvalbumin derived from the X-ray studies [10], afforded in general very different chemical shift values for the two ortho and the two meta protons belonging to the same phenylalanine residue [11]: the largest calculated difference was about 1.9 ppm for the two meta protons of the phenylalanine residues 29 and 85. Therefore the rate of flips around the C^{β}–C^{1} bond may be in some cases much larger than 1000 Hz. The observation of fast exchange in all cases means however that no precise value, or upper limit, can be set for any residue. A very interesting feature of the present n.m.r. analysis of carp 4.25 parvalbumin is the observation of several resonances from para and meta protons which are very upfield shifted from their unperturbed position, i.e. signals at 5.6 ppm (one para proton), at 5.95 ppm (one para proton), at 6.2 ppm (two meta protons) and at 6.25 ppm (two meta protons) belonging to three different phenylalanine residues. This result suggests that many of the phenylalanine residues are in close proximity and that the respective orientation between residues is such that para and/or meta hydrogens of one residue are pointing towards the π electron distribution of another residue. This situation essentially precludes $\pi - \pi$ stacking between phenylalanine aromatic rings within the protein. A similar conclusion was previously derived from the X-ray studies, which described the internal core of the protein as formed by a cluster of 8 phenylalanine residues with no $\pi - \pi$ stacking [17].

Evidence for more extensive molecular motion is derived from a study of the temperature dependence of chemical shift values. On raising the temperature, it is found that all ring current shifted resonances (both aromatic and CH_3 resonances) move back towards their unperturbed positions. There is no indication of any conformational transition in the explored temperature range (from 5°C to 80°C). This implies that the whole protein alters gradually, e.g. it expands, as the temperature is increased. A similar result has been observed for lysozyme [15]. It is likely that, in association with this expansion, a gradual perturbation of the equilibria between different side-chain conformations as well as of the kinetics of their interconversion could occur. The presence of different conformers (including C^{α}–C^{β} rotations) was already inferred for the phenylalanine residues of the parvalbumins on the basis of optical methods. [1].

Comparative experiments with the four major carp parvalbumins as well as with parvalbumins from other species (e.g. hake) have shown that the n.m.r. equivalence of the ortho and of the meta resonances of most if not all phenylalanine residues is a general feature of this class of proteins. Similarities in the coupling patterns of the ring current shifted resonances are observed, although the overall appearances of the spectra could differ markedly. Thus it seems that there are similar conformations for these different proteins, especially with regard to the organization of the internal hydrophobic core. During the present study, it was observed that some parvalbumin samples were characterized by n.m.r. spectra which could not be understood by assuming the presence of a single molecular species. For instance the spectrum of carp 4.25 parvalbumin appears more complex than that previously described under the same conditions [11]. However an identical spectrum is re-established when a disulfide reducing reagent, dithiothreitol, is added. Recently, a light-scattering study indicated the existence of a polymerisation process in the carp parvalbumin solutions, which was shown to involve the formation of a dimer on the basis of gel electrophoresis analysis [18]. It is likely that the dimer results from the formation of an S–S bond between two molecules of these proteins possessing a single cysteine residue [18].

4. Conclusions

The n.m.r. experiments described here have shown that 8, if not all 10, of the phenylalanine residues of carp parvalbumin p*I* 4.25 are undergoing rapid rotation or flipping about the C^{β}–C^{1} bond, despite the fact that all are internal residues. This equivalence is also found in related parvalbumins. Conformational mobility of a more extensive nature is also proposed from more indirect n.m.r. evidence.

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