PROTON MAGNETIC RESONANCE SPECTROSCOPY OF HISTIDINE RESIDUES IN PROTEINS

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In a previous publication Bradbury and Scheraga (1966) showed that the proton magnetic resonance of the C-2 protons of the four histidine residues in ribonuclease could be observed. In addition, it was found that there was a considerable downfield shift of the resonances on charging of the imidazole rings, from which it was possible to obtain an approximate estimate of the apparent acid dissociation constants of the histidine residues in the protein. We have now extended this work to a representative range of proteins and find that, in general, two types of behaviour are possible. Either the C-2 proton resonances can be observed as in ribonuclease and lysozyme or cannot be visualized at all (using a 60 Mc high-resolution instrument with spectrum accumulation) due to structural broadening of the resonance (Kowalsky, 1962; Mandel, 1965). For lysozyme, the C-2 proton resonance of the single histidine residue is readily observed, and its pK has been determined. Trypsin, chymotrypsin and their zymogens fall in the latter category and it therefore seems probable that the histidine residues are held rigidly, by an interaction of the imidazole rings with another group in the molecule.

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EXPERIMENTAL

Materials. Lysozyme (twice crystallised), α-chymotrypsin (three times crystallised) chymotrypsinogen A (three times crystallised), trypsin (twice crystallised) and trypsinogen (once crystallised) were obtained from Worthington Biochemicals, insulin (crystalline, bovine pancreas) and cytochrome-c (horse heart, type III) from Sigma Chemical Co., and myoglobin (sperm whale) from Mann Research Labs. Trifluoroacetic acid (Fluka, purum) and D_2O (99.8%, Australian Atomic Energy Commission) were used as solvents.

Methods. Solutions of the proteins in D_2O at various values of pD were prepared according to the methods described previously (Bradbury and Scheraga, 1966) and pD was determined by the relationship pD = pH meter reading + 0.40 (Glascoe and Long, 1960; Li, Tang and Mathur, 1961). The pH (accuracy $\pm$ 0.02) was measured by a Beckman Research pH Meter No. 101901 fitted with micro electrodes. The solutions were examined using either a Perkin Elmer 60 Mc spectrometer, Model R-10, at 33.4°C or a Varian Model A-60 spectrometer at 25 $\pm$ 2°, each of which was equipped with a computer of average transients (CAT). In D_2O the narrow HDO resonance was used both to trigger the CAT and also as a reference peak in accurate measurements of chemical shift (Bradbury and Scheraga, 1966). In trifluoroacetic acid solution, tetramethylsilane (TMS) was added and used for these purposes. The relative intensities of various resonances were determined by cutting out and weighing the appropriate peak areas.

RESULTS AND DISCUSSION

In Figure 1 that region of the NMR spectrum which includes the resonances of tyrosine, phenylalanine, tryptophan and histidine is reported for some typical proteins (see e.g., Kowalsky, 1962; Mandel, 1965; Bradbury and Scheraga, 1966).

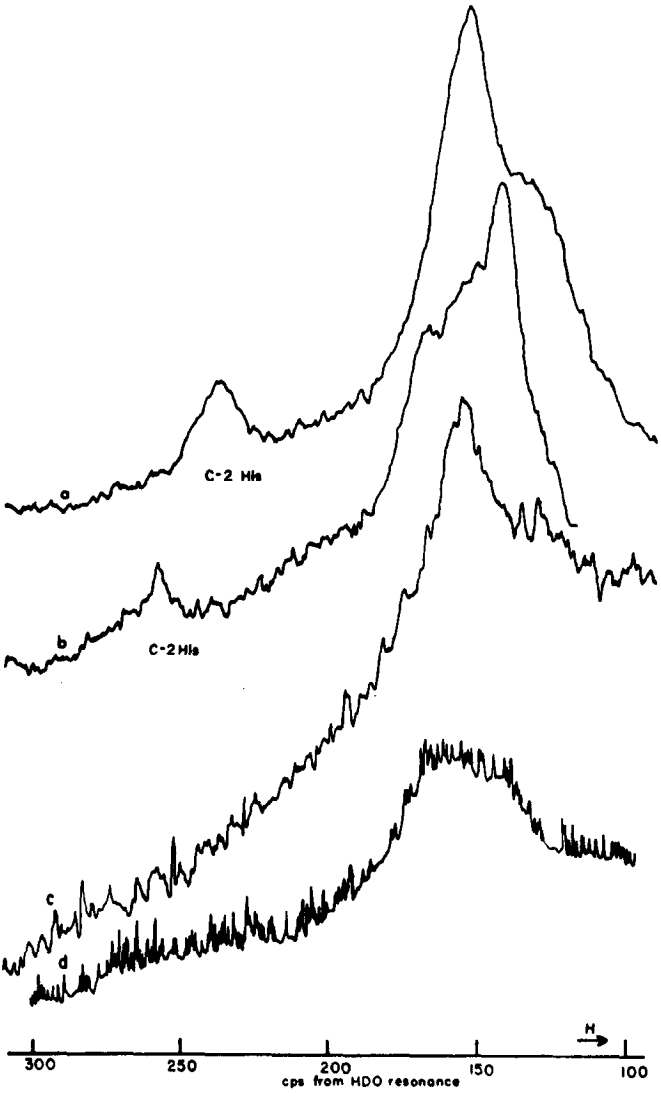

Figure 1. Computed NMR spectra (100 traces) in D_2O of (a) 10% insulin, pD 2.84, (b) 10% lysozyme, pD 3.54, (c) 15% α-chymotrypsin, pD 6.84, (d) 15% trypsin, pD 4.41.

In the case of insulin and lysozyme the C-2 protons of the histidine resonances are identified both by their position with respect to the HDO resonance and their intensities compared with that of the aromatic peak. The single histidine residue in lysozyme gives a single peak in the pD

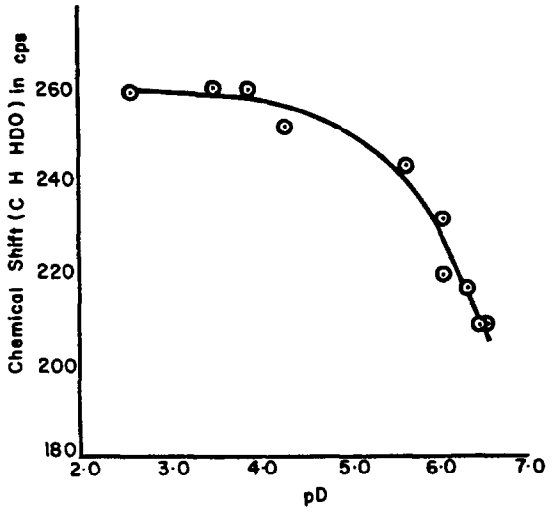

Figure 2. The effect of pD on the chemical shift of the C-2 histidine proton of lysozyme (5% in D_2O containing 0.2 M NaCl, such that ionic strength = 0.2-0.25).

range 2.5-6.5, but above this pD the protein becomes insoluble at 5% concentration and also the histidine resonance tends to be obscured by the broad tryptophan resonance. Since the S shaped curve in Figure 2 is incomplete it is only possible to obtain an approximate estimate of the apparent dissociation constant (pK') of histidine, from the centre of the region of maximum gradient of the graph (pK' = 6.3 $\pm$ 0.2 in D_2O). The figure in water is about 0.50 less than that in D_2O (Bradbury and Scheraga, 1966) and hence pK' = 5.8 $\pm$ 0.2 in H_2O. The intrinsic dissociation constant for histidine in lysozyme pK_{int} is calculated (using the treatment and the results of Tanford and Wagner (1954)) to be 6.3 $\pm$ 0.3 in water. This figure is in reasonable agreement with their results (pK_{int} = 6.5 to 7.0) and is indicative of a "normal" histidine residue.

It is noted in Figure 1 that α-chymotrypsin and trypsin do not give any evidence of a C-2 histidine resonance and this type of behaviour has also been noted for the corresponding zymogens even when spectra are computed on solutions of concentrations of 25% (W/V). The amount of

histidine present on a molar basis is less in lysozyme (which gives a readily observable, computed histidine resonance even with a 5% (W/V) solution in D_2O) than in any of the four proteins listed above. Indeed, the concentration of histidine residues in 25% solutions of the four proteins which do not give a C-2 histidine resonance is more than five times that of a 5% solution of lysozyme. It is also noteworthy that C-2 histidine resonances have been observed in this work on computed spectra from insulin (see Figure 1), ribonuclease (see also Bradbury and Scheraga, 1966), cytochrome-c and myoglobin. However, the C-2 histidine resonance is observable in the total acid hydrolysate of a 15% solution of α-chymotrypsin and in a 10% solution of α-chymotrypsin in trifluoroacetic acid (see Figure 3).

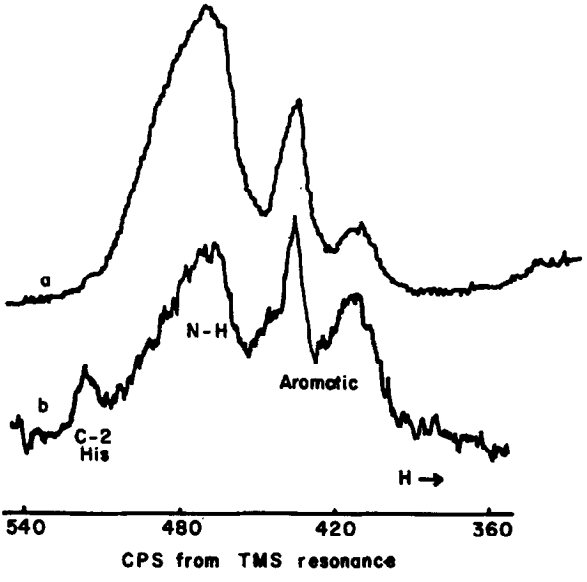

Figure 3. NMR spectra in trifluoroacetic acid of (a) 10% chymotrypsin, computed 300 traces, (b) 15% myoglobin.

The C-2 histidine resonances are not observed for α-chymotrypsin, α-chymotrypsinogen, trypsin and trypsinogen in D_2O because of broadening of the resonances to give an estimated line width of >30-40 cps. A line

width of 30-40 cps is observed for the α C-H proton resonance in a poly-L-amino acid in the α-helical form (Markley, Meadows, and Jardetzky, 1967; Bradbury and Fenn, unpublished results). It is considerably higher than the figure of 5 cps obtained for the line width of the C-2 resonance in poly-L-histidine (molecular weight 8000) in the partial α-helical form at pD 6.2 and also for the histidine in lysozyme which occurs in position 15, at the end of an α-helical section (Blake et al., 1965). The four other proteins which give C-2 resonances (see above) have line widths of 7-16 cps. At the present stage there is insufficient data available on line widths to be dogmatic, but it would appear probable that the histidine residues in trypsin, α-chymotrypsin and their corresponding zymogens are held rather rigidly in position by an interaction between each imidazole ring and another group in the molecule. Alternatively, a less likely explanation is that the line broadening is due to aggregation in the aqueous solutions used.

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