PROTON MAGNETIC RESONANCE STUDY OF THE HISTIDINE RESIDUES OF HORSE MYOGLOBIN

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1. Introduction

In recent years proton magnetic resonance methods have proven useful in the study of protein structure, particularly in the investigations of histidine residues which have led to conclusions about the active sites of the enzymes ribonuclease A [1–3] and ribonuclease T_1 [4, 5]. In this communication we report the results of a proton magnetic resonance investigation in ^2H_2O of the histidine residues of horse myoglobin with the heme iron in the reduced state. Myoglobin, a monomeric oxygen transporting protein in mammalian muscle, has been widely studied and has been characterized structurally by X-ray diffraction studies [6–8]. Proton magnetic resonance studies [9–12] have been made previously on myoglobin, but the titration behaviors of the histidine residues of reduced myoglobin have not before been reported. The results of an NMR-monitored, acid-base titration of horse CO-Mb (II)* reported here show that at least 6 histidine residues have 'pK' values** between 6.7 and 7.2. Three others have 'pK' values considerably lower than this. The remaining 2 histidine residues, perhaps numbers 36 and 93, are not revealed in the C-2 imidazole proton resonance spectral region and appear to be 'masked' by interactions with other parts of the myoglobin. From the relatively upfield positions of at least 2 C-2 proton resonance signals of protonated imidazoles, it is deduced that interactions of imidazole rings with carboxylate and/or carbonyl groups exist, as reported by Kendrew et al. [6–8] for crystalline sperm whale myoglobin. It was also found that the environment of one histidine residue changes significantly when the iron-bound CO is replaced by O_2 at a 'pH' of 10.

* (II) and (III) here refer to the iron oxidation state. The ligand is indicated by CO– or O_2– before Mb (Myoglobin).

** pH readings and pK estimates were not corrected for deuterium isotope effects on the electrode and on equilibrium respectively and are therefore designated as 'pH' and 'pK'.

2. Materials and methods

At each 'pH' value for which a spectrum was made, a separate sample was prepared, the following procedure being used: 100 mg of horse Mb(III) (Miles Laboratories Inc., Kankakee, Ill.) was taken up at room temperature in 1.0 ml of ^2H_2O (Merck, Darmstadt, Germany) which was 0.2 M in NaCl. The solution was then shaken for 15 minutes with 0.5 mg of $Na_2S_2O_4$ in order to reduce the myoglobin to the Fe^{2+} state. After this the 'pH' (measured with a Radiometer Type PHM 26 pH meter in conjunction with an Ingold Type

405-M 3/2 combination electrode) was raised to 10 by adding 2% NaO^2H (in 0.2 M $NaCl/^2H_2O$). Under these conditions all the myoglobin became dissolved. Then the 'pH' was lowered with 2% ^2HCl (in 0.2 M NaCl/ $NaCl/^2H_2O$) to the desired value. At 'pH' values below 8.5 this resulted in the formation of a precipitate of myoglobin, which was removed by centrifugation. The 'pH' was measured again, and a portion of the clear solution was transferred to an NMR sample tube 5 mm in diameter. A coaxial external standard of TMS was inserted and the tube sealed. Proton magnetic resonance spectra were carried out with a 90 MHz HFX-3 Bruker Physik nuclear magnetic resonance spectrometer equipped with a Fabritek Model SD 1 computer of average transients. The temperature of the probe was 30°. Between 60 and 250 sweeps were averaged for each spectral determination. For the experiments in which CO was bound to the heme, the above procedure (dissolution, pH adjustment, etc.) was carried out in the presence of CO in a glovebox which had been previously purged of all air by N_2. Absorption spectra carried out in the range 450–650 nm on the CO–Mb(II) and O_2–Mb(II) samples with a Zeiss Model PMQ II spectrophotometer were normal, having the known characteristics [13] for native, reduced myoglobin.

3. Results and discussion

Typical spectra are shown in fig. 1. Those obtained at high pH are of best comparative quality, due to the higher solubility and consequent higher concentration of myoglobin at high pH. The peaks between 9.2 and 7.9 parts per million (ppm) from tetramethylsilane (TMS) result from the C-2 protons of histidine residues [1–5], of which there are 11 in horse myoglobin [14]. The ring protons of aromatic amino acids, including the C-4 protons, of the histidine residues make up the large peak seen in the spectra at around 7.6 ppm from TMS. Porphyrin methine proton signals are expected to appear at around 9.7 ppm from TMS [12, 15] and therefore do not interfere with the results reported here.

Protonation of imidazole nitrogen generally results in an increased downfield shift (a change of about 1 ppm) for the C-2 proton [16], and thus the peak position of the latter is a good parameter for monitoring

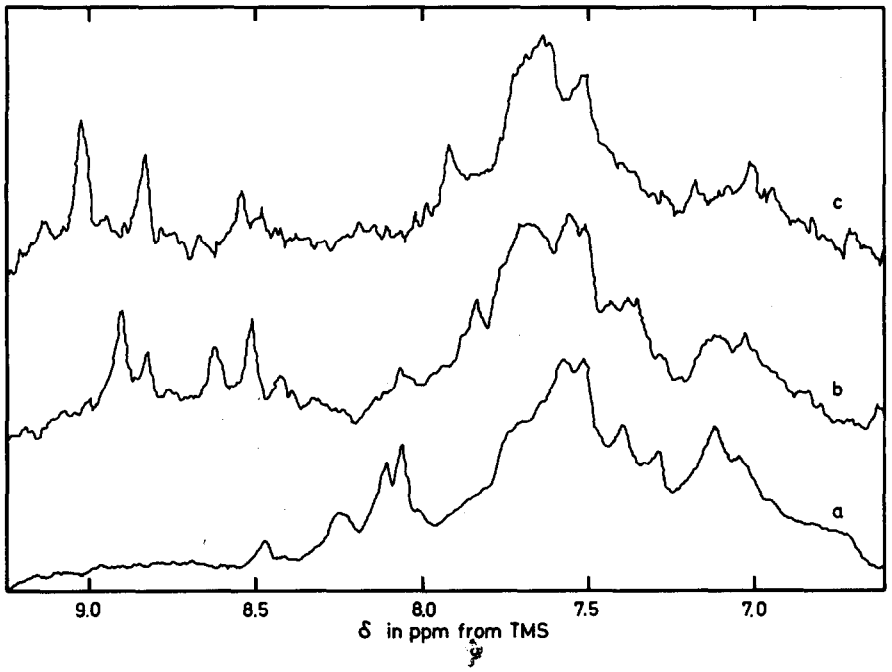

Fig. 1. Proton magnetic resonance spectra of CO-Mb(II) in 0.2 M $NaCl/^2H_2O$ at 'pH' values of 10.00 (a), 6.12 (b) and 5.48 (c).

the titration behavior of histidine residues [1–5]. A plot of the chemical shifts of the observable histidine C-2 proton peaks vs. 'pH' was made and is shown in fig. 2. Nine such signals were observed, and with this number it is not possible to assign unambiguously all point connections. The lines were drawn on the basis of relative peak size and breadth and assuming no gross irregularities in the shape of the titration curves (such as for example maxima). Many of these point connections could be made with reasonable and even absolute certainty and are shown as solid lines in fig. 2. The dotted lines indicate connections which were to varying degrees uncertain as a result of mergings and/or possible crossings-over of signals. Because of these uncertainties we report not 'pK' values but rather 'pK' ranges for histidine residues.

It is interesting to observe that, in their protonated form, at least 2 histidine residues have chemical shifts which are between 8.5 and 8.6 ppm from TMS, this being a range considerably upfield from the C-2 proton signals of protonated free imidazole or histidine which occur at about 9.2 ppm from TMS. This could be a result of shielding caused by interactions of imidazole with the oxygens of carboxyl groups and/or peptide bonds. Indeed Kendrew and coworkers have found [6–8] from X-ray diffraction studies that a number of such interactions exist in crystalline, sperm whale myoglobin. The participating groups in all of these interactions occurring in sperm whale myoglobin are present in horse myoglobin with the one exception of glutamic acid 109 which is replaced in the latter by aspartic acid 109 [14]. Thus it is likely that the same types of interactions occur in horse CO-Mb(II) and are responsible for the relatively upfield positions of some of the signals seen here. The relatively high 'pK' values of these histidine residues (see fig. 2) are also in line with this interpretation.

One histidine C-2 proton signal occurs at high pH at an unusually downfield position (8.47 ppm from TMS) which does not show any pH dependence above 'pH' 7.1. Below this 'pH' it is difficult because of signal crossovers to determine by how much the peak moves, but a spectrum made at 'pH' 6.12 reveals quite clearly (see fig. 1) that a peak no longer exists at this position. Assuming that there is no pH-induced conformational change shifting the position of the signal, this indicates that the corresponding imidazole is being protonated at 'pH' 6.12. This would

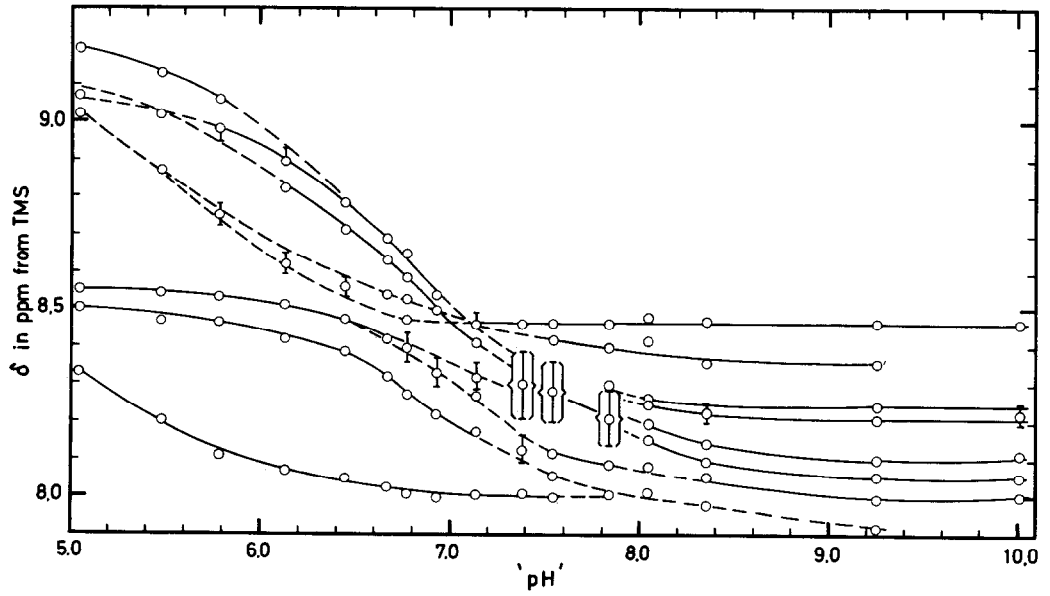

Fig. 2. Chemical shifts of the histidine C-2 protons plotted against 'pH'. The symbols ⨎, δ, and ⨏ represent signals which were broad and signals having downfield and upfield shoulders respectively. Dotted lines indicate uncertainties in peak-movement assignments, as described in the text.

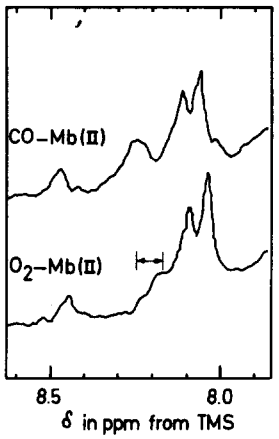

Fig. 3. Histidine C-2 proton region of magnetic resonance spectra of CO–Mb(II) and O_2–Mb(II) at pH = 10.05 ± 0.05. The arrow indicates the 0.072 ppm peak movement described in the text.

indicate that the peak does not correspond to the iron-bound residue number 93, as might otherwise be expected from the downfield position.

At least 6 histidine residues have 'p*K*' values between 6.5 and 7.1, and 3 have 'p*K*' values below 6. It should be noted that Breslow and Gurd [17] have concluded, from studies on sperm whale Mb(III) that 6 histidine residues are accessible and that the others become unmasked only below pH 4.5 as the protein denatures. More recently Takenaka et al. [18] have found that, in horse Mb(III), 8–9 histidine residues are accessible.

The question arises as to why the two remaining histidine C-2 protons are not revealed in the nuclear magnetic resonance spectra, and it is possible that these peaks are moving with others. It appears more likely however that restriction of mobility of the imidazole groups by interaction with other parts of the molecule leads to line broadening extensive enough to render undetectable the magnetic resonance signals. A similar observation and explanation has been reported by King and Roberts [19] in their work on carbonic anhydrase B. The 2 corresponding histidine residues may be numbers 93 and 36. The former is bound by iron and the latter forms interactions with the carboxyl group oxygens of *two* glutamic acids, 38 and 109 in sperm whale myoglobin [6–8].

The C-2 histidine proton regions of the spectra of CO–Mb(II) and O_2–Mb(II) at 'pH' 10.00 ± 0.05 are shown in fig. 3. A peak seen at 8.23 ppm in the former moves 6.5 Hz (0.072 ppm) upfield when the CO ligand is replaced by O_2, indicating that the environment of at least one histidine residue, perhaps the 'distal' histidine number 64 [6–8] is thereby significantly changed.

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