NMR STUDIES OF HEMOPROTEINS: pH DEPENDENCE OF FERRIC HORSERADISH PEROXIDASE AND HORSE HEART MYOGLOBIN

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1. Introduction

N.m.r. spectroscopy has recently developed into a powerful tool for investigating structure and structure–function relationships of hemo-proteins and heme enzymes. Most of the n.m.r. studies on ferric hemoproteins so far reported have been performed with those in purely or nearly low spin state. Several of the ferric hemoproteins, which exhibit a pH-dependent spin state change, have been incompletely investigated [1,2].

In this report we concentrate on the n.m.r. measurements of pH-dependence for ferric horseradish peroxidase and horse heart myoglobin, covering the large hyperfine shift of heme ring methyl groups due to ferric high spin state. 220 MHz proton n.m.r. confirmed the result of magnetic susceptibility measurements by Theorell and Ehrenberg [3], and was, furthermore, able to distinguish the nature of the pH dependent spin state change between both hemoproteins.

2. Materials and methods

Horseradish peroxidase (Sigma Type VI) dissolved in ^2H_2O was used for n.m.r. measurements without further purification. Horse heart myoglobin (Sigma Type III) was dissolved in 10 mM potassium phosphate buffer (pH 6.0). After removing insoluble part by centrifuge, it was adsorbed on CM-cellulose (Whatman CM-52) column and then eluted by deuterated 0.5 M potassium phosphate buffer (p^2H 7.0). The elute was dialyzed against ^2H_2O just before p^2H adjustment. The heme concentration of each sample was about 1 mM.

p^2H value was adjusted by 4 M glycine–NaO^2H buffer, p^2H 12, and read out directly by the pH meter (Radiometer) equipped with micro-combination glass electrode (Ingold).

The proton n.m.r. spectra were measured with a Varian Associates HR-220 high resolution spectrometer connected to a Nicolet Pulsed Fourier Transform unit TT-100 at an ambient probe temperature of 20 ± 1°C. To observe largely hyperfine shifted signals of hemoproteins in ferric high spin state, the quadrature phase detection (QPD) technique was used along with short pulse width (20 μsec for 56° pulse). With an acquisition time of 0.1 sec for ± 20 KHz spectral width and ± 4K point transform, spectra were obtained in 2000~4000 scans, taking a total time of 4~7 min. The details of n.m.r. measurement for ferric high spin hemoproteins will be reported else where [4].

3. Results and discussion

p^2H-dependent behaviors of ferric horseradish peroxidase and horse heart myoglobin were monitored by the hyperfine shifted proton signals of heme ring methyl groups.

Fig.1(a) illustrates the p^2H dependence of 220 MHz proton n.m.r. spectra for ferric horse-radish peroxidase

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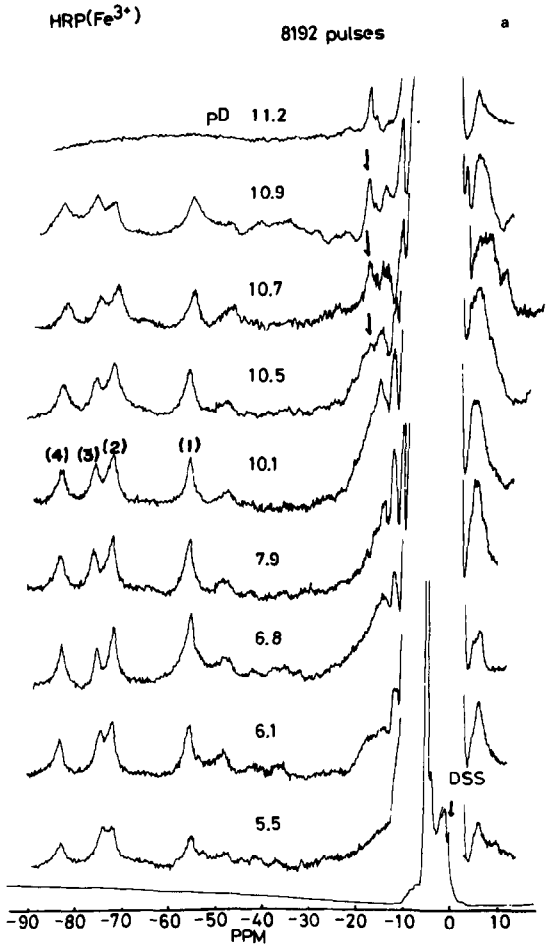

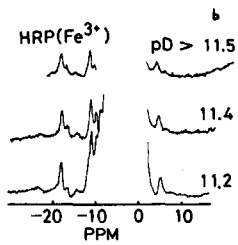

Fig.1. (a) 220 MHz proton FT-n.m.r. spectra of ferric horseradish peroxidase between p^2H 5.5 and 11.2 at 21°C. Signals of heme peripheral methyl groups are indicated by (1)~(4). (b) 220 MHz proton FT-n.m.r. spectra of ferric horseradish peroxidase above p^2H 11.2. A hyperfine shifted signal at −18 ppm is considered to be one of the four heme ring methyl groups.

at 21°C. In the p^2H region between 5.5 and 10.9, there exist four proton signals (1)~(4) with equivalent intensity, which do not exhibit any p^2H-dependent shift. Since these signals are greatly hyperfine-shifted down to −85 ppm, they are considered to be due to four heme-ring methyl groups effected by the paramagnetism of ferric high spin iron ($S = 5/2$). Above p^2H 10.5 new signals, which may be assigned to those from ferric low spin state, appear in higher field region between −10 and −20 ppm, separately from signals (1)~(4). Fig.1(b) indicates the n.m.r. signals above p^2H 11.2. These results suggest that the spin state of ferric horseradish peroxidase transits from high spin to low spin ones with raising p^2H value and that the midpoint of the transition is located around p^2H 11. The profile of this transition corresponds well to the magnetic susceptibility measurements by Theorell and Ehrenberg [3], who reported that the horseradish peroxidase changes from predominantly high spin state (effective Bohr magneton number n_{eff}=5.44) to low spin state (n_{eff}=2.66) with pK 11.

Through the n.m.r. measurements, the p^2H dependent behavior of peroxidase was found drastically different from that of myoglobin, as follows. In fig.2 is illustrated the acid-base transition of horse heart ferrimyoglobin observed with 220 MHz proton n.m.r. at 21°C. In neutral p^2H region, myoglobin has four hyperfine shifted methyl signals (1)~(4) between −50 and −90 ppm, which are characteristic of acid metmyoglobin in ferric high spin state. By raising the p^2H value, these methyl signals shift to higher field side with pK 9.1, and at p^2H 11.2

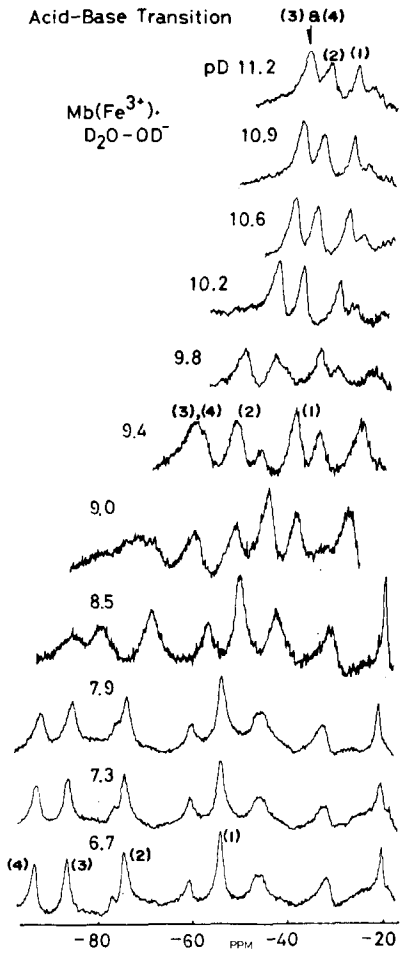

Fig.2. 220 MHz proton FT-n.m.r. spectra of ferric horseheart myoglobin at various p^2H values. Hyperfine shifted signals of heme ring methyl groups (1)~(4) gradually shift to high field with raising p^2H value. A part of this p^2H dependence (above p^2H 10) has been analyzed previously [1].

they are located between −25 and −40 ppm, indicating the presence of alkaline mytmyoglobin in spin equilibrium state [1]. N.m.r. signals at p^2H 9.4 appear to be the average of acidic and alkaline forms, which suggests that the exchange time between both forms is shorter than 10^{-4} sec on 220 MHz proton n.m.r. time scale. Consequently, it is reasonable that the rapid exchange between acidic and alkaline forms of myoglobin occurs through the $^2H^+$ dissociation and association of solvent side)NH of distal histidine (E7) imidazole [1].

On the contrary to the case of myoglobin, the exchange time between high spin and low spin forms of horseradish peroxidase is longer than 10^{-4} sec and so the exchange is fairly slow, because 220 MHz proton n.m.r. can separate two forms as shown in fig.1. In the case of ferric myoglobin incompletely saturated with cyanide ion at p^2H 7, the low spin form of cyanide complex was clearly distinguished from the high spin form of residual acid metmyoglobin by 220 MHz proton n.m.r. because of the slow exchange of 6th ligand between CN^- and ^2H_2O. The slow exchange between high spin and low spin forms of horse radish peroxidase suggests that the transition around p^2H 11 is attributable not to the proton dissociation and association of heme-linked amino acid residue but to the direct exchange of the 6th ligand on ferric heme iron. Some internal nitrogeneous ligand such as histidine or lysine might replace the 6th ligand of native horse radish peroxidase and yield low spin state in alkaline region.

Although several discussions have been performed on the 6th ligand of peroxidase based on the proton relaxation method [5,6], more direct method should be applied to the determination of the 6th ligand.

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