PROTON NUCLEAR MAGNETIC RESONANCE STUDY OF FORMATE BINDING TO METMYOGLOBIN

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1. Introduction

Hershberg and Chance [1] have investigated the binding of formate ion to metmyoglobin and catalase by nuclear magnetic resonance (NMR) relaxation technique. From the analysis of the bulk formate proton relaxation rate, T_{1M}^{-1}, they determined the minimum distance between the paramagnetic heme iron and formate proton in metmyoglobin to be 9 Å long. Hence they concluded that formate does not directly bind to the heme iron.

Recently we have obtained the hyperfine-shifted proton NMR spectra of native metmyoglobin and its formate complex both in ferric high spin (S 5/2) state. Both of them exhibited well resolved hyperfine-shifted heme methyl resonances, which sensitively reflect subtle difference in electronic structure of the heme iron [2]. In this communication we present a conclusive indication of the direct formate binding to the heme iron in metmyoglobin using the hyperfine-shifted heme methyl resonance as a probe.

2. Materials and methods

Horse heart metmyoglobin purchased from Sigma (type III) was dissolved in 10 mM phosphate buffer, pH 6.0. After removing insoluble part by centrifuge the soluble portion was applied to a CM-cellulose column (Whatman, CM 52) equilibrated with the same buffer and then eluted by deuterated 0.5 M phosphate buffer, p^2H 7.0. The elute was dialyzed against ^2H_2O. The concentration of the protein was about 2 mM for each sample. All the external ligands added were reagent grade and the molar ratio to metmyoglobin was about 50 : 1.

The p^2H was adjusted by careful addition of deuterated NaO^2H (CEA isotope) aqueous solution and the p^2H value was the direct reading of a pH meter, Radiometer Model 28, equipped with a micro combination electrode (Ingold).

Proton NMR measurements were performed on a Varian HR-220 spectrometer equipped with a Fourier transform accessary, Nicolet TT-100, at 220 MHz and 21°C. The spectra were obtained by 4 k points transform of 40 kHz spectral width after about 10 000 pulses. The chemical shifts in parts per million (ppm) were referred with respect to an internal reference, 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) with positive sign to lower field resonances.

3. Results and discussion

Figure 1 shows the hyperfine-shifted heme methyl resonances of some metmyoglobin derivatives in ferric high-spin state. The largely shifted heme methyl resonances, which have been assigned from their integrated intensities to three proton resonances, were observed at 93.4 ppm, 86.9 ppm, 73.7 ppm and 53.2 ppm from DSS for native metmyoglobin. They clearly reflect the weak interaction between the water molecule and the paramagnetic heme iron, which allows distribution of large electron spin densities from the paramagnetic center to the periphery of the heme [2]. Other weak ligand complexes, $metMbF^-$ and $metMbHCOO^-$, also exhibited well resolved heme methyl resonances which were markedly different

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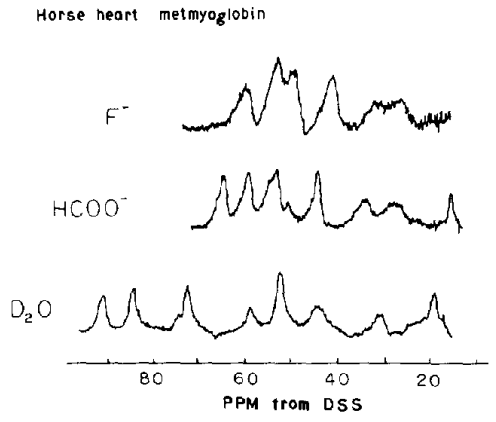

Fig.1. The hyperfine-shifted heme methyl resonances of metmyoglobin derivatives at 21°C and neutral p^2H.

from those of native metmyoglobin in their chemical shifts. Thus the hyperfine-shifted heme methyl resonance are sensitively reflecting the difference in the ligand–heme iron interactions among the high spin complexes. This NMR evidence supports the direct binding of formate to the heme iron. Yet such a question may be evoked that the external ligand might not directly coordinate to the heme iron, instead it is only perturbing the mode of interaction between the unsubstituted water molecule and the heme iron, and that the changes in the hyperfine-shifted heme methyl resonance and visible absorption spectrum of the formate complex [3] might be secondary responses. However, such a possibility can be ruled out by the following analysis. In native horse metmyoglobin the acid–base transition is known to be a very rapid process with pK_a 8.9. The proton NMR observation of the acid–base transition of metmyoglobin has also substantiated such a mechanism [4,5]. With raising the p^2H value the four heme methyl resonances of native metmyoglobin gradually shifted to higher field and gave a distinct alkaline type spectrum at the high p^2H extreme.

The alkaline titration of the formate complex, in contrast, showed a quite different behavior as shown in fig.2. In the region between p^2H 7 and p^2H 9 the hyperfine-shifted heme methyl resonances of the formate complex were observed at 69.1 ppm, 61.7 ppm, 54.4 ppm and 43.3 ppm, which were significantly different from those of native metmyoglobin.

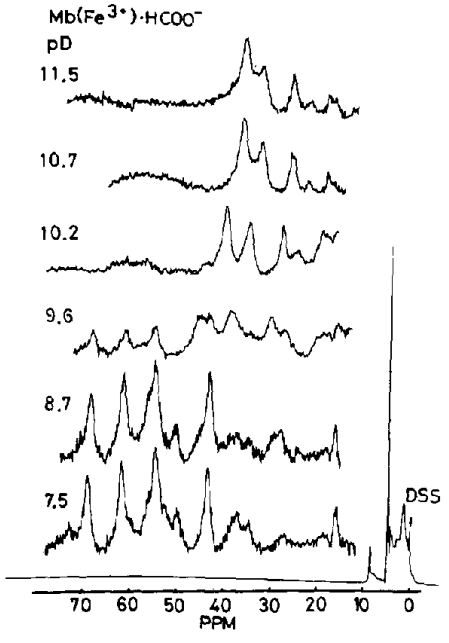

Fig.2. The alkaline titration behavior of metmyoglobin–formate complex at 21°C.

With raising p^2H the formate type resonance disappeared gradually and the alkaline type resonances appeared concomitantly. Above p^2H 10 only the resonance of alkaline form remained. This NMR evidence implies that the acid–alkaline transition behavior is slow enough on the NMR time scale, ca. 10^{-4} s, and the slow process reasonably corresponds to the slow ligand exchange process from $HCOO^-$ to O^2H^- at high alkaline p^2H region. Thus the acid–alkaline transition behavior of the formate complex is different from that of native metmyoglobin and shows that the formate ion directly coordinates to the heme iron because if water molecule is not substituted by formate ion, the normal acid–base transition should be observed.

The NMR spectral characteristics of the formate complex of metmyoglobin is manifested in the proton NMR spectrum of an abnormal hemoglobin, hemoglobin M Milwaukee (β67E11 Val → Glu) [6]. Hemoglobin M Milwaukee is a naturally occurring valency hybrid containing two permanently oxidized hemes in the β subunits. The X-ray structural analysis of this hemoglobin by Pertz et al. [8] showed that the oxygen

atom of the carboxylate of β67E11 residue is located close enough to bind to the heme iron. In the proton NMR spectra of hemoglobin M Milwaukee reported by Fung et al. [6] and Lindstrom et al. [7], the hyperfine-shifted heme methyl resonances, which came from the β subunits alone, were observed in the paramagnetic region between ca. 40 ppm and 60 ppm from H^2HO. Noticing that the residual water proton resonance is observed about 5 ppm downfield from DSS at room temperature, the hyperfine-shifted heme methyl resonances of the ferric β subunits of hemoglobin M Milwaukee are observed in much the same region as that of formate–metmyoglobin complex.

Thus it is quite evident that formate is bound to the heme iron in metmyoglobin formate complex. The distance from the iron to formate proton is estimated to be about 3 Å long, if the negatively charged carboxylate oxygen is coordinated to the heme iron in the same way as in the β subunits of hemoglobin M Milwaukee. The minimum distance of 9 Å between the iron and formate proton obtained from NMR relaxation study using Solomon-Bloembergen equations [1] seems overestimated. The investigation of the hyperfine-shifted heme methyl resonances, therefore, is more convenient for the studies of ligand binding to the heme iron of hemoprotein.

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