THE NO-PROBED DETECTION OF THE HEME-LINKED IONIZATION GROUP OF MYOGLOBIN.

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SUMMARY

The heme-linked ionization group having pK=7 is detected with NO-ferromyoglobin complex using electron paramagnetic resonance spectroscopy with second derivative display. The possible participation of the distal histidine is discussed for this ionization group.

INTRODUCTION

The heme-linked ionization group has been characterized mostly by the pH-dependence of the affinity towards various ligands, together with the direct proton titrations and oxidation-reduction potential(1-4). With myoglobin(Mb), the absence of Bohr effect suggests the lack of the tightly coupled heme-linked ionization group with ligation in the ferrous states, in contrast to the ferric state, where the pH-dependent changes of the dissociation constants have been well analyzed with various ligands(1,2).

In this communication, the detection of the heme-linked ionization group is attempted with NO-ferromyoglobin complex, where the pH-dependence of the hyperfine structure of the electron paramagnetic resonance(EPR) spectra is examined in detail over the wide pH-range.

MATERIALS AND METHOD

Sperm whale oxymyoglobin was prepared from the meat by the method of Yamazaki et al(5). All other reagents were

obtained commercially with the analytical grade.

Ferrous NO-complex of myoglobin was obtained by use of nitrite and dithionite according to Yonetani et al(6) or NO gas treatment(7).

EPR absorption spectra were measured by the Varian E-line spectrophotometer with 100KHz-field modulation. The operation was X-band(9.301 GHz) and microwave power was 5 mW. The second derivative display was obtained by use of the 1 KHz frequency modulation unit. The measurements were performed at liquid nitrogen temperature using the Dewar flask inserted into the cavity. The pH values were those at room temperature(8).

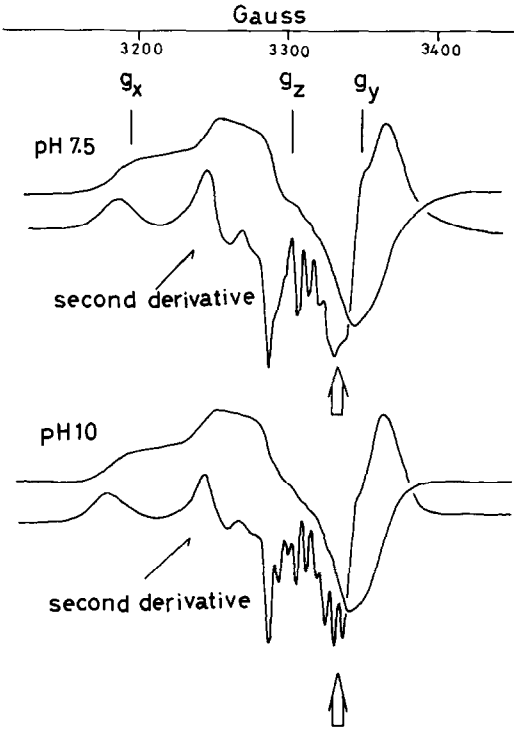

Fig. 1
The EPR absorption spectra of NO-myoglobin at pH 7.5 and 10. Mb; 500μM. 0.2 M phosphate buffer at pH 7.5 and 0.2 M Tris-HCl buffer at pH 10. First derivative spectra: Modulation Frequency; 100KHz, Modulation amplitude; 2 gauss. Second derivative spectra; Second modulation frequency and modulation amplitude are 1KHz and 5 gauss.

RESULTS

Fig. 1 shows the EPR spectra of NO-myoglobin at pH 7.5 and 10 with both first and second derivative display. Though the hyperfine structure is hardly observed with the first

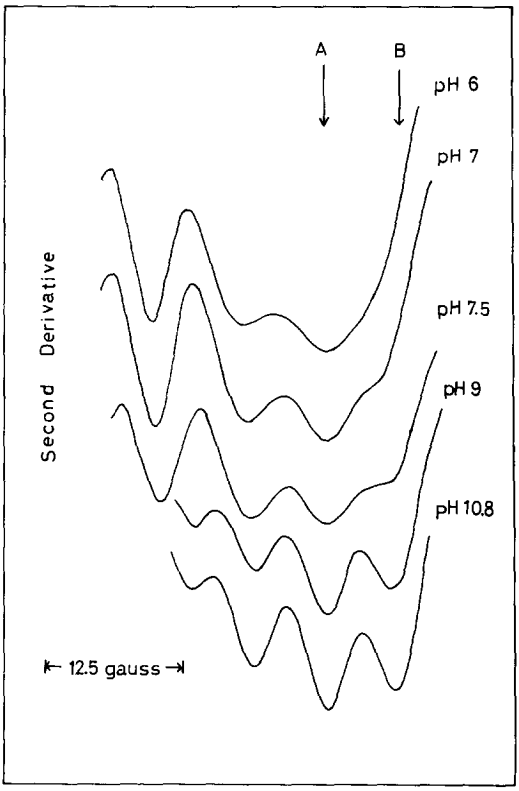

Fig. 2
The pH-dependence of the second derivative spectra of NO-myoglobin. The region arrowed in Fig.1 is recorded in the expanded scale. Buffer concentration; 0.2 M Glycine-HCl(pH 6,7). 0.2 M phosphate(pH 7.5). 0.2 M Tris-HCl(pH 9). 0.2 M Glycine-NaCl(pH 10.8). All the experimental conditions of EPR are same as to those of Fig.1.

derivative spectrum, 9 lines of the fine structure centered at g_z is clearly seen in the second derivative display. At pH 7.5, such hyperfine structure is rather unclear as compared with that of pH 10. The 9-lined hyperfine structure can be easily attributed to the coupling of nitrogen atom(nuclear spin, I=1) of the imidazole group of proximal histidine and NO molecule through the heme-iron(6,7).

The pH-dependent changes of the spectra of NO-myoglobin are shown in Fig. 2, where the region of high magnetic field

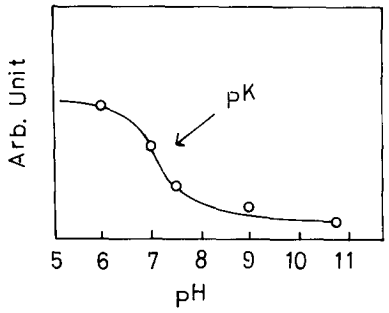

Fig. 3
The pH-titration of the hyperfine structure of NO-myoglobin. The ratio of the absorption at (A) and (B) in Fig.2 is plotted against pH.

in Fig. 1(arrowed in the figure) is recorded with the expanded scale. With increasing the pH, the absorption peaks become more distinctly than those of acidic pH's.

The relative EPR signal amplitude is plotted in Fig. 3 with the function of pH, where the data are taken from Fig. 2. The pK value of this transition is found to be approximately 7. In the region lower than pH 6 and above 11, the reproducible results were not obtained because of the denaturation of myoglobin.

DISCUSSION

The high resolution of the second derivative display is well demonstrated in the present study. Here, it is noted that the absorption in the second derivative display corresponds to the slope of the first derivative spectra, which can not be related directly to the spin density. However, the above does not conflict with the following discussion, since the pH-dependent relative changes of the absorption are only taken account(cf. Fig. 2). The details of this method is discussed elsewhere(9).

As is seen in Figs. 1 and 2, the pH-dependent changes of hyperfine structure gives the direct evidence of the presence of heme-linked ionization group, which affects the electronic structure of heme-iron and NO bond. In the ferrous ligated state, the possibility has been proposed time to time that the hydrogen bond might be presented between nitrogen atom of imidazole ring of distal histidine and ligated molecule(10-13). The value of pK=7 obtained from the present study may support the above possibility. The other possibility of the proximal histidine seems to be unlikely, since the pK value of this proton dissociation is much more alkaline pH($\sim$12) as shown in the model system of heme-imidazole complex(14).

The absence of Bohr effect, however, may conflict with the present results, if affinity of NO is also pH-independent, since pH-invariance of the affinity corresponds to the absence of the interaction between the ionization group and heme-iron. The possible interpretation, at present, is that the alteration of the electronic state of heme-iron perturbed by this proton dissociation is very small as compared with the overall ligation. The further characterization of this heme-linked ionization group is now under extensive investigations. The applications of the present method into other hemoproteins will be appeared very soon in elsewhere.

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