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CALCIUM BINDING BY HORSERADISH PEROXIDASE C AND THE HEME ENVIRONMENTAL STRUCTURE

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SUMMARY

The hyperfine shifted proton NMR spectrum of isoenzyme c of horseradish peroxidase indicated that one calcium ion is essential to the enzyme in maintaining the protein structure in the heme vicinity.

Haschke and Friedhoff reported that isoenzyme c of horseradish peroxidase contains two moles calcium per mole enzyme [1]. Although they suggested from the effect of calcium removal on decreasing specific activity and thermal stability of the enzyme that calcium ion functions in maintaining the protein structure of the enzyme, further spectroscopic experiment appears necessary to prove the binding of calcium ion by the enzyme.

Recently, we obtained the hyperfine shifted proton NMR spectra of native horseradish peroxidase and its reaction intermediates, compounds I and II [2, 3]. These spectra exhibited well resolved heme peripheral methyl proton resonances, which reflect quite sensitively differences in the electronic states of the heme iron atom of these horseradish peroxidase derivatives. In this study, we present a conclusive evidence showing that one calcium ion is essential to the enzyme in maintaining the protein structure in the heme environment.

MATERIALS AND METHOD

Isoenzyme c of horseradish peroxidase purchased from Toyobo (Type G-C-I) was used without further purification. The

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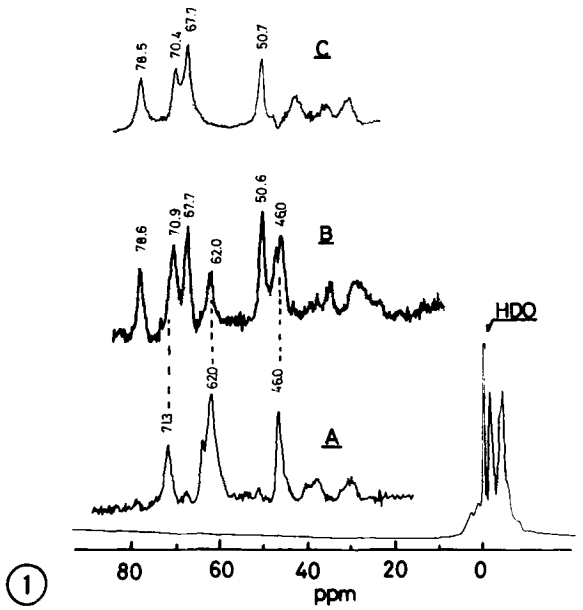
purity index, RZ value, of the enzyme was 3.4. The concentration of the enzyme was determined spectrophotometrically at 403 nm by using an absorptivity of 102 cm^{-1} mM^{-1} at pH 7.0. Deuterium oxide (99.7 atom % ^2H) was purchased from Merck.

Removal of the bound calcium from the enzyme was achieved by a method of Haschke and Friedhoff [1]. The concentration of calcium was determined with an atomic absorption spectrometer.

Proton NMR spectra were recorded at 220 MHz on a Varian HR-220 spectrometer equipped with a Nicolet TT-100 pulse Fourier transform operation unit. Typically, 1024 transients were collected and transformed using a 27 μs 55° pulse with a pulse repetition time of 0.051 s, and a series of approximately 20 such blocks was added to yield the final spectrum. Proton chemical shift is referenced with respect to the proton signal of the residual water in the enzyme solution, assigning a positive value for lowfield resonance.

RESULTS AND DISCUSSION

Calcium analysis of the isoenzyme c from Toyobo showed that this enzyme contains 4.0 moles of calcium ion per mole of the enzyme. These bound calcium ions were removed by incubation of the enzyme with guanidine hydrochloride and EDTA [1]. The proton NMR spectrum of the calcium-free enzyme in 50 mM phosphate buffer at p^2H 7.0 was recorded at 24° C and is illustrated in Figure 1A. Figure 1C shows the spectrum of the native enzyme at p^2H 7.0 and 24° C. The spectrum of the calcium-free enzyme is different from that of the native enzyme in the hyperfine shifted region; the four heme peripheral methyl proton signals of the native enzyme are observed at 78.5, 70.4, 67.7 and 50.7 ppm, and those of the calcium free enzyme are at 71.3, 62.0, 62.0 and 46.0 ppm downfield from HDO resonance. When calcium ion was titrated to the solution of the calcium-free enzyme, the heme methyl proton peaks of the enzyme decreased in intensities with a concomitant appearance and increase in intensities of the peaks of the native enzyme. This suggests that calcium ion binds to the enzyme at a rate slower than the proton NMR time scale (10^{-4}s). In Figure 1B, the proton signals from the calcium-free enzyme and the native enzyme are simultaneously seen in the presence of a half-saturating amount



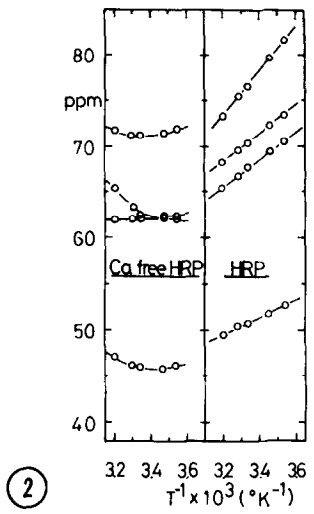


Fig. 1. Proton NMR spectra of isoenzyme c of horseradish peroxidase at pH 7.0 and 24° C. (A) Spectrum of the calcium-free enzyme. (B) Spectrum of the calcium-free enzyme in the presence of a half-saturating amount of calcium ion. The ratio of the concentrations of the heme and calcium ion is 1 : 0.6. The enzyme concentration is 1.02 mM. (C) Spectrum of the native enzyme.

Fig. 2. Temperature dependence (9-39 °C) of the four heme peripheral methyl proton signals of the calcium-free and the native enzymes of horseradish peroxidase at pH 7.0.

of calcium ion. This spectral change of the enzyme upon addition of calcium ion was saturated when one mole of calcium was added to one mole of the enzyme. Although our calcium analysis showed that this enzyme contains 4 moles calcium per mole enzyme, the addition of calcium ion more than one molar equivalent per mole enzyme does not produce any further spectral shift. The above results indicate that conformation at the heme group of the calcium-free enzyme is different from that of the native enzyme, and that one calcium ion is essential to the enzyme in maintaining the structure in the heme vicinity.

An additional support for the structural difference in the heme vicinity between the calcium-free and the native enzymes

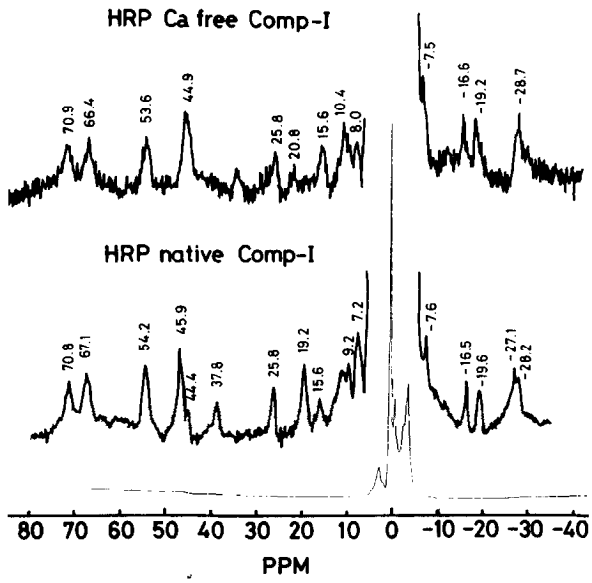


Fig. 3. Proton NMR spectra of the compound I of the calcium-free enzyme and that of the native enzyme of horseradish peroxidase at pH 7.0 and 24 °C.

was provided by the temperature dependence of their proton NMR spectra. Figure 2 compares the temperature dependences of the four heme peripheral methyl proton peaks between the calcium-free and the native enzymes. The signals of the native enzyme shifted upfield with increasing temperature, obeying the normal Curie law, whereas those of the calcium-free enzyme were almost insensitive to change in temperature within the range 9-39 °C, suggesting that calcium-free enzyme differs in the electronic state of the heme iron from the native enzyme.

Having confirmed that one calcium ion contributes to the enzyme in maintaining the protein structure in the heme environment, we have studied the effect of calcium ion removal from the enzyme on the proton NMR spectrum of the compound I. As Figure 3 shows, the calcium-free enzyme reacts with H_2O_2 to form the compound I and to give a slightly different NMR spectrum from the compound I of the

native enzyme. Furthermore, the decay of the compound I of the calcium free enzyme to the ferric form was much faster than that of the compound I of the native enzyme, as indicated by the NMR spectral time progression of these transient species. Although kinetic study on the peroxidase reaction of the calcium-free enzyme is needed to elucidate the effect of calcium removal on the specific activity of the enzyme, the present NMR study of the compound I from the calcium-free enzyme showed that calcium ion is necessary for the enzyme to maintain the protein structure, which stabilizes the higher oxidation state of the compound I.

In summary, the present study firmly established that one calcium ion is essential to isoenzyme c of horseradish peroxidase to maintain the protein structure in the heme vicinity.

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