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Mechanisms of Electron Transfer from Sulfite to Horseradish Peroxidase–Hydroperoxide Compounds†

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ABSTRACT: Using a rapid-scan spectrophotometer equipped with a stopped-flow apparatus, reactions of sulfite with compounds I and II of two horseradish peroxidase isoenzymes A and C were investigated. The direct two-electron reduction of peroxidase compound I by sulfite occurred at acidic pH but the mechanism gradually changed to the two-step reduction with the intermediate formation of compound II as the pH increased. The pH at which the one- and two-electron changes occurred at the same speed was 4.5 for peroxidase A and 7.7 for peroxidase C. A new peroxidase intermediate was found in the reaction between peroxidase compound II and sulfite. The sulfite compound showed a characteristic absorption band at 850 nm and the optical spectrum was similar to that of isoporphyrins but was quite different from that of sulfhemoproteins. The rate (k) of conversion from the sulfite–compound II complex to the sulfite compound was proportional to the concentration of H^+ and the log k vs. pH plot for peroxidase A moved to the acidic side by 1.1 pH unit from that for peroxidase C.

It has been established by George (1952), Chance (1952a), and Yamazaki et al. (1960) that in the presence of hydrogen peroxide horseradish peroxidase catalyzes the one-electron oxidation of various bivalent redox molecules through the following catalytic cycle of the enzyme

$$\text{peroxidase} \xrightarrow{H_2O_2} \text{compound I} \xrightarrow{e^-} \text{compound II} \xrightarrow{e^-} \text{peroxidase} \quad (1)$$

On the other hand, Björkstén (1970) and Roman and Dunford (1972) have observed that there is a direct two-electron transfer from iodide to peroxidase compound I, thus forming the ferric enzyme without the intermediate formation of peroxidase compound II.

The definition and classification of one- and two-electron transfer mechanisms in enzymatic oxidation–reduction reactions have been reviewed by Yamazaki (1971). Various reactions between enzymes and substrates are grouped into two typical types: one- and two-electron transfers, while a few reactions are classified as a mixed type. The reaction of peroxidase compound I with sulfite is likely to belong to the mixed type (Roman and Dunford, 1973; Yamazaki and Yokota, 1973). This reaction appears to be suitable for studying the electron transfer mechanism on the basis of structure–function relation because a considerable amount of information has been given upon the structure of the catalytic site of horseradish peroxidase and the mechanism of electron transfer from sulfite to peroxidase compound I can be altered by the pH change.

In the course of the above experiment, a peculiar intermediate compound was found in the reaction of peroxidase compound II with sulfite. The detailed analysis of the reaction would also provide useful information concerning the relation between the mechanism of electron transfer and the structure of catalytic site of horseradish peroxidase.

Materials and Methods

Horseradish peroxidase was purified from wild horseradish roots by the method of Shannon et al. (1966) with slight modification. Enzyme preparations used in this experiment were horseradish peroxidase C and horseradish peroxidase A, a mixture of isoenzymes A_1 and A_2, according to nomenclature by Paul (1958) and Shannon et al. (1966).

Buffer systems used were sodium acetate for pH 4.05–5.45, potassium phosphate for pH 6.00–7.60, and Tris-HCl for pH 8.01–8.40. The ionic strength in reaction solutions was adjusted to 0.1. Spectrophotometric measurements were performed with a Hitachi recording spectrophotometer, Model EPS-3T for ordinary time-scale experiments and with a Union Giken rapid reaction analyzer, RA-1300 for rapid reactions. The latter instrument combined with a flow apparatus can be used either as a rapid wavelength-scan spectrophotometer or as a sensitive photometer at a fixed wavelength. The absorption spectra are measured by means of a image dissector with a maximum speed of 300 nm/ms and are memorized in a digital computer system. The analogue replica is afterwards obtained in a X–Y recorder. The dead time of the flow apparatus at an N_2 gas pressure used in this experiment was less than 1 ms. For si-

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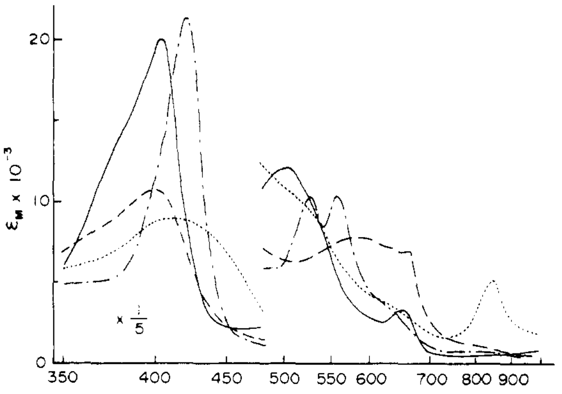

FIGURE 1: Absorption spectra of horseradish peroxidase C. Free peroxidase (solid line). Compound I (broken line); 6 μM enzyme plus 6 μM H_2O_2. Compound II (chain line); 6 μM enzyme, 25 μM H_2O_2, and 25 μM ascorbate. Sulfite compound (dotted line); the Soret and visible spectra were measured at pH 5.05 and 25 °C using the rapid-scan method (see the legend of Figure 2) and correction was made to subtract spectrum contributed from the ferric enzyme. All other experiments were conducted at pH 6.0 and 2 °C. For near-infrared spectra between 700 and 1000 nm, 32 μM enzyme was used.

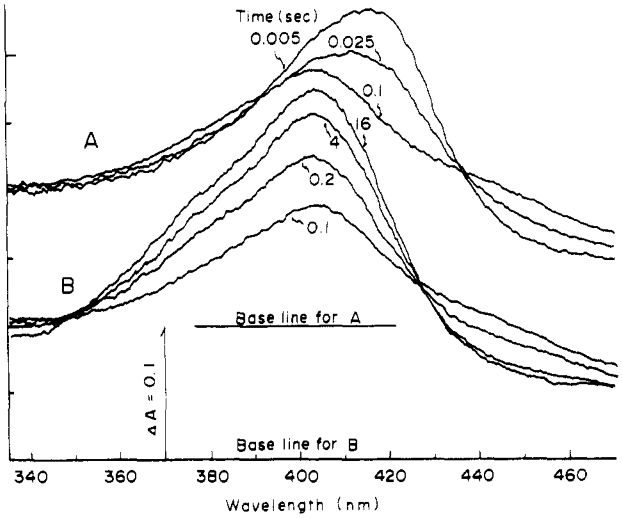

FIGURE 2: Rapid-scan spectrophotometric measurement for the reaction of peroxidase compound II with sulfite. Two solutions of 6.6 μM peroxidase C (5.4 μM compound II and 1.2 μM ferric enzyme) and 1 mM sulfite were mixed at pH 5.05. The enzyme solution was prepared by addition of 6 μM H_2O_2 and 3 μM ascorbate to 6.6 μM peroxidase C. The dead time was about 1 ms. Reaction times indicated in the figure corresponded to the time interval from the stop of the flow to the end of the wavelength scan. The wavelength was scanned from 485 to 335 nm in 5 ms. A and B show formation and decomposition of the sulfite compound, respectively. Because of limitation of the number of memory channels two groups of traces were given from separate experiments.

multaneous measurements of absorbance changes at two wavelengths a Hitachi two-wavelength double-beam spectrophotometer Model 356 was used.

Results

As briefly reported previously (Roman and Dunford, 1973; Yamazaki and Yokota, 1973), horseradish peroxidase was converted to a new spectral species when sulfite was added to peroxidase compound II (Figure 1). Particularly, a drastic decrease in absorbance occurred in the Soret region. No characteristic absorption band was observed in the visible region but unlike other peroxidase derivatives a distinct absorption band (850 nm) was observed in the near-infrared region. This sulfite compound spontaneously decomposed to generate the free enzyme (Roman and Dunford, 1973). The formation and decomposition of the sulfite compound could be analyzed with use of a rapid-scan spectrophotometer. Figure 2 shows spectral changes during the reaction of compound II of peroxidase C with sulfite. The decomposition was relatively slow as compared with the formation and each reaction occurred in separated time ranges under the experimental conditions given in Figure 2. Stopped-flow measurements at a fixed wavelength were conducted using the same instrument and time courses of the absorbance change are illustrated in Figure 3. The wavelength of 411 nm was isosbestic of peroxidase compound II and the ferric enzyme. The first-order rate constant for the decomposition was independent of pH and the value of about 0.2 s^{-1} is well consistent with the result reported by Roman and Dunford (1973).

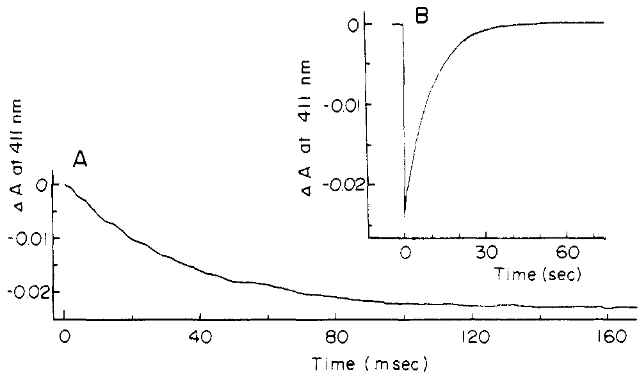

FIGURE 3: Time courses of formation (A) and decay (B) of the sulfite compound. Peroxidase C (2.2 μM) (80% compound II and 20% ferric enzyme) was mixed with 1 mM sodium sulfite at pH 5.05 and 25 °C. The wavelength of 411 nm was an isosbestic point between free peroxidase and compound II.

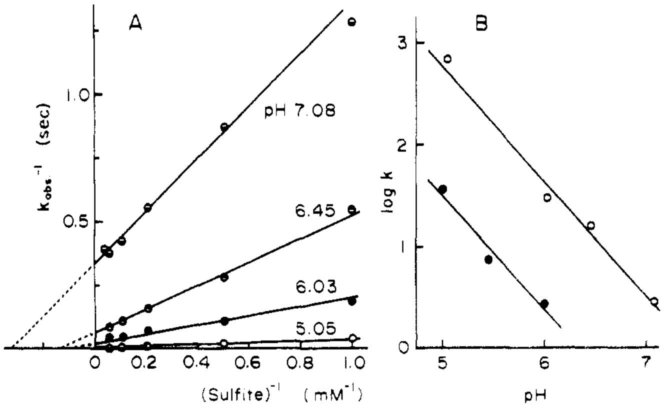

FIGURE 4: Dependence of the rate of formation of the sulfite compound upon concentrations of sulfite and H^+. (A) Double-reciprocal plots of the rate and the concentration of sodium sulfite at various values of pH. The experimental procedures are as described in Figure 3. (B) Dependence of the maximum rate upon pH. Data of peroxidase C (open circles) are replotted from Figure 4A. Data (solid circles) were obtained from similar experiments with peroxidase A.

The rate of formation of the sulfite compound was greatly dependent on the concentrations of sulfite and hydrogen ion. Double-reciprocal plots of apparent first-order rate constant and sulfite concentration at various pH values are shown in Figure 4A. The logarithms of the maximum rate constants obtained from Figure 4A are plotted against pH in Figure 4B. Similar experiments were conducted with peroxidase A and the results are also plotted in the same figure.

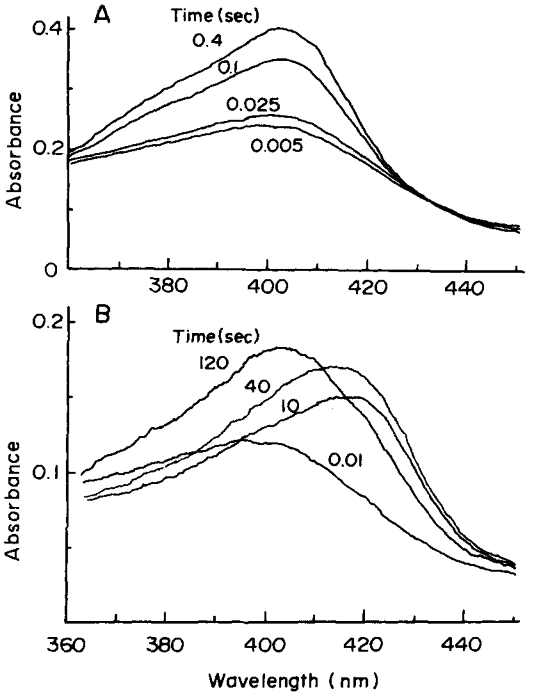

FIGURE 5: Rapid-scan spectrophotometric measurement for the reaction of peroxidase compound I with sulfite. (A) Two solutions of 8 μM compound I (8 μM peroxidase C + 8 μM H_2O_2, 15 °C) and 20 mM sodium sulfite (25 °C) were mixed at pH 6.03. (B) Two solutions of 4 μM peroxidase C plus 4 μM H_2O_2 (15 °C) and 10 mM sodium sulfite (25 °C) were mixed at pH 8.01. Reaction times indicated in the figure are as described in Figure 2. The temperature of 15 °C was used so as to diminish the decomposition of compound I.

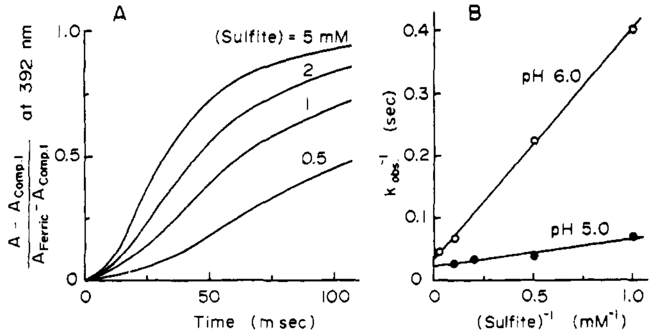

FIGURE 6: Dependence of the rate of reduction of peroxidase compound I by sulfite upon the concentrations of sulfite and H^+. (A) Time courses of two-electron reduction of peroxidase compound I by sulfite. An enzyme solution (4 μM peroxidase C + 4 μM H_2O_2) was mixed with sodium sulfite solutions at pH 5.00 and 20 °C. Concentrations of sodium sulfite in reaction mixtures are indicated in the figure. The ordinate scale was normalized for convenience of computer simulation. The wavelength of 392 nm, an isosbestic point between peroxidase Compound II and the sulfite compound, was monitored so as to cancel an absorbance change due to formation of the sulfite compound from compound II present in several percent in the starting enzyme solution. (B) Double-reciprocal plots of the pseudo-first order rate constant and the concentration of sodium sulfite. The rate constants at pH 5.0 were calculated from data in Figure 6A according to the procedure described in the text. The values at pH 6.0 were obtained from similar experiments.

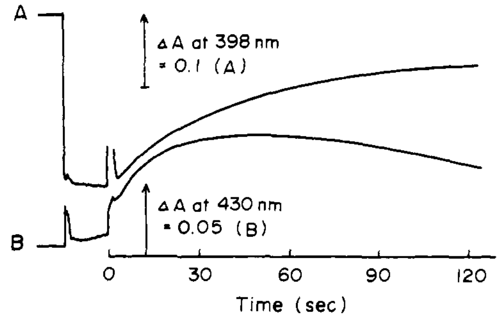

FIGURE 7: Time courses of reduction of peroxidase compound I by sulfite at pH 7.67. The reaction was started by addition of 20 μl of 10 mM sodium sulfite to 2 ml of 6 μM compound I (peroxidase C) at 20 °C. Absorbance changes at two wavelengths were simultaneously followed by a Hitachi two-wavelength double-beam spectrophotometer. (A) Formation of free peroxidase was followed at 398 nm (isosbestic of compounds I and II). (B) Formation of compound II at 430 nm (isosbestic of free peroxidase and compound I).

Björkstén (1970) has observed that the reduction of peroxidase compound I by iodide is a one-step reaction without the intermediate formation of compound II. The mechanism of catalase reaction is believed to involve one-step reduction of catalase compound I (Chance, 1952b; Keilin and Nicholls, 1958). In the reaction of compound I of peroxidase C with sulfite, as reported by Roman and Dunford (1973), the one-step reduction could be also demonstrated in the acidic pH region by the use of the rapid-reaction analyzer (Figure 5A). Neither peroxidase compound II nor the sulfite compound was detected from the experimental data. However, when the reaction was conducted at pH 8.01, compound I of peroxidase C was reduced to the ferric enzyme apparently via Compound II (Figure 5B).

The kinetic stopped-flow trace of absorbance at 392 nm at acidic pH (Figure 6A) indicated the existence of a lag period in the conversion from compound I of peroxidase C to the ferric enzyme. Although no intermediate of the enzyme was spectrophotometrically detectable in Figure 5A, the existence of the lag phase would suggest the following reduction process of compound I.

$$\text{compound I} + \text{sulfite} \overset{K}{\rightleftharpoons} \text{compound I}\cdots\text{sulfite} \xrightarrow{k_a}$$

$$\text{compound I-sulfite} \xrightarrow{k_b} \text{free peroxidase} + \text{sulfate} \qquad (2)$$

As the latter 70% of the spectral change in Figure 6A followed first-order kinetics, the apparent rate constant was tentatively calculated. Figure 6B shows double-reciprocal plots of the rate constant and the concentration of sulfite at pH 5.0 and 6.0. Assuming that the equilibrium in the formation of compound I· · ·sulfite was achieved fast, the equilibrium constant was calculated as 1.6 mM at pH 5.0 and 10 mM at pH 6.0. It appeared that the pH change affected the K value but not so much the rate constant. By computer simulation of the data in Figure 6A approximate values of k_a and k_b were estimated to be 50 and 100 s^{-1} at pH 5.0, and 25 and 100 s^{-1} at pH 6.0, respectively. For the calculation, the two complexes of compound I with sulfite were assumed to have the same absorbance at 392 nm as that of free compound I.

At neutral pH, two mechanisms of one- and two-electron reductions of compound I of peroxidase C were found to be mixed. In order to determine the ratio of two reactions, changes in absorbance at 398 and 430 nm were followed simultaneously, as shown in Figure 7. The wavelengths of 398 and 430 nm were isosbestic between compounds I and II and between compound I and free peroxidase, respectively. Consequently, the rate of two-electron reduction could be measured at 398 nm and that of one-electron reduction at 430 nm. The lag period was not taken into consideration for measurement of these slow reactions. Above pH 7.0, a considerable amount of compound II was formed as the product of one-electron reduction of compound I but accumulation of the sulfite com-

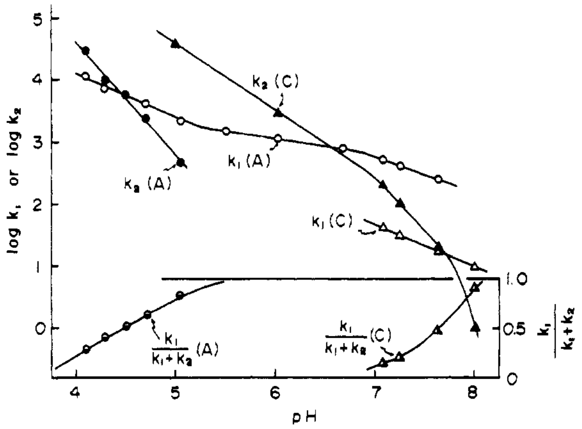

FIGURE 8: Dependence of rate constants of compound I reduction by sulfite upon pH. Rate constants, k_1 and k_2 are second-order rate constants ($M^{-1}\ s^{-1}$) for one- and two-electron reductions of compound I by sulfite, respectively. Isoenzymes are designated in parentheses. The ratio $k_1/(k_1 + k_2)$ is also plotted against pH (right ordinate). For peroxidase C, k_1 and k_2 were measured at low concentrations of sulfite, where the initial rates were regarded to be proportional to the sulfite concentration (Figure 6A). For peroxidase A, since the reduction of compound I to compound II obeyed first-order kinetics with respect to sulfite up to 10 mM and from the reasons described in the text, k_2 and $k_1/(k_1 + k_2)$ were measured with the use of the rapid-reaction analyzer at 439 and 455 nm, respectively. The absorbance change at 455 nm was attributable to the formation of the sulfite compound (see Figure 1). A compound I solution (8.8 μM peroxidase A + 8.8 μM H_2O_2) was mixed with a 10 mM sodium sulfite solution at 20 °C.

pound was negligibly small. Spontaneous formation of compound II from compound I by endogenous donor was less than 10% of the formation of compound II caused by sulfite (Figure 7). The one-electron reduction of compound I by an impurity in the sulfite solutions could be neglected because recrystallization of the reagent gave no effect on the result shown in Figure 7. The rate constants and their ratios are plotted against pH in Figure 8. The $k_2(C)$ plot is consistent with the result of Roman and Dunford (1973) in the overlapped pH region.

Discrimination between one- and two-electron reductions of compound I became more difficult for peroxidase A than for peroxidase C. In the case of peroxidase A, competition between the two reactions occurred at markedly acidic pH. Around the pH, compound II formed from compound I reacted with sulfite forming the sulfite compound. The rate of one-electron reduction of compound I was measured from absorbance changes at 439 nm. This wavelength was isosbestic between compound II and the sulfite compound and a little difference in absorbance at 439 nm between compound I and the free enzyme could be neglected. Since the sulfite compound is not formed from the two-electron reduction of compound I, the ratio of one- and two-electron changes should be reflected on the amount of the sulfite compound accumulated during the reduction of compound I provided that formation of the sulfite compound is relatively fast as compared with its decomposition. This assumption appeared to be valid in the pH range between 4 and 5. The rate constant for the two-electron reduction of compound I was calculated from the data of the above two measurements. In Figure 8 the data are compared with those of peroxidase C.

Discussion

The reaction between peroxidase compound II and sulfite appears to be analogous to that of sulfhemoprotein formation reported by Nicholls (1961), Nichol et al. (1968), and Berzofsky et al. (1972). For sulfhemoproteins Nicholls (1961) has proposed various structures that include methene-bridge thioketone, methene-bridge thiol, and methene bridge or pyrrole-ring cyclic thioether. From the similarity of spectral data with ferrochlorin proteins, pyrrole ring cyclic thioether has been considered to be the most likely (Nichol et al., 1968; Berzofsky et al., 1972).

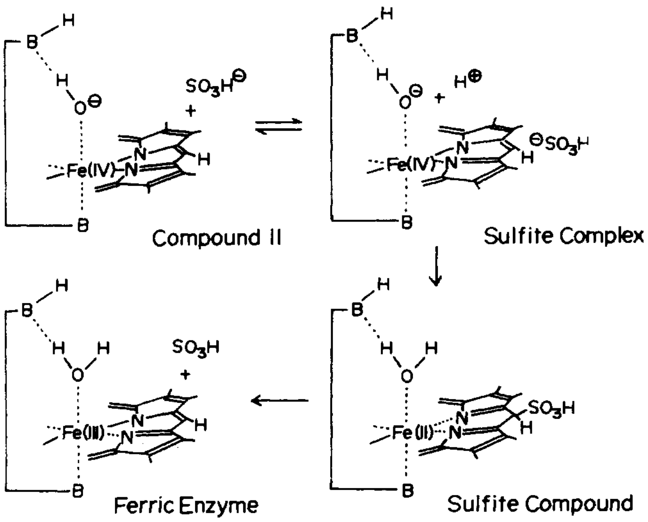

FIGURE 9: Mechanism proposed to account for the reaction of peroxidase compound II with sulfite. The sixth ligand in peroxidase compound II is assumed to be a hydroxide anion, which stabilizes the ferryl form. The model is inferred from pH titration experiments (Yamada and Yamazaki, 1974). Coordination of one oxygen atom to the iron has been justified by the works of Hager et al. (1972) and Schonbaum and Lo (1972).

The peroxidase sulfite compound differs from sulfhemoproteins by having a relatively weak Soret band and a characteristic near-infrared band. Contrary to the previous suggestion of Yamazaki and Yokota (1973), the peculiar spectrum of the sulfite compound can not be attributable to the cation radical of porphyrin reported by Dolphin et al. (1971), because of the difference in the visible and near-infrared spectra between the sulfite compound and peroxidase compound I (Figure 1). The whole spectrum of the sulfite compound is rather similar to that of an isoporphyrin formed from the reaction of the dication of zinc *meso*-tetraphenylporphyrin with methanol (Dolphin et al., 1970). Unlike the case of catalase and myoglobin, horseradish peroxidase compound II is reduced by hydrogen sulfide to free peroxidase without the intermediate formation of sulfperoxidase (Nicholls, 1961). It seems likely that sulfide attacks a pyrrole ring of compound II of catalase and myoglobin and sulfite a methene bridge of compound II of horseradish peroxidases. Figure 9 shows a mechanism proposed to account for the reaction of peroxidase compound II with sulfite. It is shown in this scheme that the two reducing equivalents of sulfite are transferred to the iron atom and the reaction is accompanied by a proton addition. The pK_a values for the distal bases of reduced peroxidases A and C are reported to be 5.8 and 7.3, respectively, and their base strengths are considered to affect the basicity at the oxygen atom coordinated to the heme iron (Yamada et al., 1975). Thus, it seems that the shift of 1.1 pH unit in the log k vs. pH plot in Figure 4B is attributable mainly to the difference of basicity at the oxygen atom between the two isoenzymes. The structure proposed for the sulfite compound is similar to that of "ROX" once proposed for compound I by Brill and Williams (1961). The rate of decomposition of the sulfite compound is known to be independent of pH (Roman and Dunford, 1973). The presence of an iron-bound water molecule in the sulfite compound is not clear. The structures of heme sites for free peroxidase and compound II are drawn from the paper of Yamada and Yamazaki (1974).

It is interesting to note that a spectrum analogous to isoporphyrins has been observed during the heme degradation of horseradish peroxidase in the presence of excess hydrogen peroxide (Bagger and Williams, 1971) and of indoleacetic acid (Yamazaki and Yamazaki, 1973). It would be also interesting to compare the sulfite compound with an intermediate found by Chance (1965) when a limited amount of sodium dithionite was added to an aerobic solution of horseradish peroxidase. He has reported that the compound is characteristic of the high-spin peroxidase and the Soret spectrum resembles that of peroxidase compound I. Since compound II and sulfite are probably formed in his experimental conditions his compound is likely to be the sulfite compound reported in this paper.

It has been shown by Björkstén (1970) and Roman and Dunford (1972) that the reaction of horseradish peroxidase compound I with iodide proceeds without the intermediate formation of compound II. This would be the first example of direct two-electron reduction of horseradish peroxidase compound I. The reduction of compound I by sulfite at low pH provides the second example. In this case, according to the definition of Yamazaki (1971), the mechanism changes from the two-electron type to the one-electron type as the pH is increased. Similar pH dependence of the electron-transfer mechanism has been reported in a NADH,[1] xanthine oxidase, and *p*-benzoquinone system (Nakamura and Yamazaki, 1973). As seen in Figure 8, the rate of two-electron reduction of compound I is much dependent upon the pH compared to that of one-electron reduction. The results may be related to the fact that the two-electron reduction of compound I needs two protons but the one-electron reduction needs only one proton in the pH range tested in this experiment (Schonbaum and Lo, 1972; Yamada and Yamazaki, 1974). From a comparison between the data in Figure 4B and Figure 8 it seems likely that the site of the sulfite attack in the two-electron reduction is a methene bridge of porphyrin, as suggested in the reaction between compound II and sulfite. Since the rate of the one-electron reduction of compound I of peroxidase A is higher than that of peroxidase C, the one-electron reduction appears to occur through a mechanism different from that of formation of the sulfite compound. It would be permissible to propose a working hypothesis that there are two inherent sites in a molecule of compound I that are respectively responsible for the one- or two-electron reduction of the compound.

Functional difference between catalase and peroxidase appears to be related to the electron-transfer mechanism. For instance, catalase compound I oxidizes nitrite in a direct two-electron transfer (Chance, 1952b), while peroxidase compound I in a one-electron transfer (Chance, 1952a; Roman and Dunford, 1973). Participation of exogenous protons in determining the mechanism of electron transfer is interesting in this respect.

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[1] Abbreviation used: NADH, reduced nicotinamide adenine dinucleotide.