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Registry No. CAT, 9040-07-7; CM, 56-75-7; Ser, 56-45-1; Ala, 56-41-7; Gly, 56-40-6; Cys, 52-90-4; Asn, 70-47-3; acetyl-CoA, 72-89-9.

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Reactions of the NAD Radical with Higher Oxidation States of Horseradish Peroxidase†

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ABSTRACT: The reactions of the NAD radical ($NAD^{\bullet}$) with ferric horseradish peroxidase and with compounds I and II were investigated by pulse radiolysis. $NAD^{\bullet}$ reacted with the ferric enzyme and with compound I to form the ferrous enzyme and compound II with second-order rate constants of 8×10^8 and 1.5×10^8 M^{-1} s^{-1}, respectively, at pH 7.0. In contrast, no reaction of $NAD^{\bullet}$ with native compound II at pH 10.0 nor with diacetyldeutero-compound II at pH 5.0–8.0 could be detected. Other reducing species generated by pulse radiolysis, such as hydrated electron (e_{aq}^-), superoxide anion (O_2^-), and benzoate anion radical, could not reduce compound II of the enzyme to the ferric state, although the methylviologen radical reduced it. The results are discussed in relation to the mechanism of catalysis of the one-electron oxidation of substrates by peroxidase.

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

$$Fe^{3+} \xrightarrow{H_2O_2} \text{compound I} \underset{AH^{\bullet}}{\overset{AH_2}{\rightleftharpoons}} \text{compound II} \underset{AH^{\bullet}}{\overset{AH_2}{\rightleftharpoons}} Fe^{3+} \quad (1)$$

An important feature of this process is the formation of free radical intermediates, as clearly demonstrated by EPR techniques (Yamazaki et al., 1960; Yamazaki & Piette, 1963; Piette et al., 1964). The free radicals thus formed are very reactive and act as strong reductants or oxidants (Ohnishi et al., 1969). In the presence of suitable electron donors or acceptors, electron transfer occurs between the free radical and the added molecule (Yamazaki & Ohnishi, 1966; Ohnishi et al., 1969; Nakamura et al., 1985). Therefore, it might be expected that the free radicals would react directly with the peroxidase. If so, two-electron oxidation of the substrate would occur without formation of free radicals. However, no such reaction between substrate free radicals and enzyme has been detected, and the only radical decay mechanism observed has been dismutation or dimerization. Indeed, Nakamura et al. (1985) reported that the one-electron flux for hydroquinone oxidation by horseradish peroxidase is nearly 100%. One of the interesting questions to be answered is why the radical does

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not transfer an electron to the enzyme during the catalytic cycle.

Among the oxidations of substrates by peroxidase, the aerobic oxidation of NADH is very peculiar and exhibits oscillatory responses of reactions (Yokota & Yamazaki, 1965a, 1977; Yamazaki & Yokota, 1967). The one-electron oxidized form of NADH (NAD•) formed in this process is a very strong reductant (Farrington et al., 1980; Anderson, 1980) and reduces the ferric hemes of cytochrome *c* (Simic et al., 1975) and cytochrome P-450 (Debey et al., 1979). This raises the possibility that NAD• might reduce peroxidase directly through the enzyme catalytic cycle. In order to investigate whether NAD• reduces the heme of various oxidation states of horseradish peroxidase, we have now studied the time course of absorption changes of peroxidase after pulse radiolysis in the presence of NAD^+. Some advantages of the pulse radiolysis technique for determining the spectral and kinetic behavior of NAD• have been demonstrated previously (Land & Swallow, 1968, 1971; Kosower et al., 1978; Farrington et al., 1980; Anderson, 1980). We have been unable to detect the reaction of NAD• with compound II of peroxidase, though the radical was found to reduce both the ferric form of peroxidase and compound I.

Materials and Methods

Horseradish peroxidase was purified from the crude material from Sigma (*RZ* = 0.1) by DEAE- and CM-cellulose column chromatography according to the method of Schannon et al. (1960). The enzyme used (*RZ* = 3.2) was a main fraction absorbed on the CM-cellulose column. Diacetyldeuteroperoxidase was prepared by recombination of apoperoxidase with diacetyldeuteroheme, followed by DEAE- and CM-cellulose column chromatography by the method of Tamura et al. (1972).

Compound I of peroxidase was prepared by the addition of equimolar hydrogen peroxide to the ferric enzyme. Compound II was prepared by the addition of equimolar ferrocyanide to compound I at pH 10: the compound II thus formed was passed through a Sephadex G-25 column to eliminate ferricyanide and H_2O_2. Unlike that of the native enzyme, compound II of the diacetyldeuteroheme enzyme is stable over a wide range of pH (Makino et al., 1985). The compound II of diacetyldeuteroperoxidase was prepared by the addition of 2–3-fold excess amounts of H_2O_2 to the ferric enzyme, and the compound thus formed was passed through a Sephadex G-25 column to eliminate H_2O_2. The purities of the samples were checked by the optical absorption spectra in the Soret region. Contamination of other enzymatic species was negligible.

Samples of peroxidase for pulse radiolysis were prepared as follows. Solutions containing 50 mM phosphate buffer (pH 5–8) or 10 mM borate buffer (pH 8–10), 1 mM NAD^+, and *tert*-butyl alcohol (for scavenging OH•) were deaerated by bubbling with argon for at least 30 min. Concentrated solutions of the enzyme prepared by the above-mentioned method were separately deoxygenated by repeated evacuation and flushing with argon and then added to the deaerated solutions anaerobically. To study the reaction of superoxide anion (O_2^-), samples of peroxidase were saturated with oxygen by bubbling with oxygen gas. Bottles containing approximately 100 mL of solution were connected to the flow cell (1-cm light path) in the front of the accelerator. A fresh solution was used for each pulse.

Pulse radiolysis experiments were performed with an electron linear accelerator in the Institute of Scientific and Industrial Research of Osaka University (Kobayashi et al., 1988), or in the Paterson Laboratories (Keene, 1964). The pulse width was 10 ns. Photolysis by the analyzed light was minimized by means of optical shutters and selected filters.

Optical absorption spectra were measured by using a Shimadzu MPS-2000 spectrophotometer.

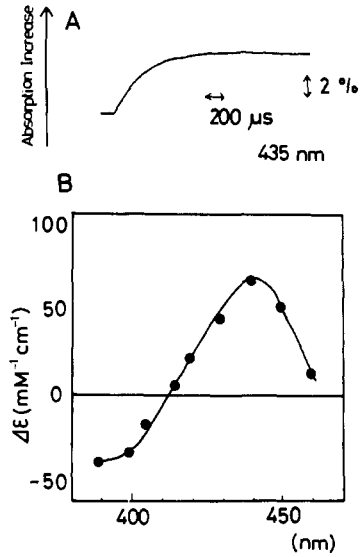


FIGURE 1: (A) Oscilloscope trace of the absorption change after pulse radiolysis of ferric horseradish peroxidase in the presence of NAD^+. The reaction medium contained 8 μM peroxidase, 1 mM NAD^+, 0.1 M *tert*-butyl alcohol, and 50 mM phosphate buffer at pH 7.0. (B) Difference between the spectra of the ferrous and the ferric forms of peroxidase. Experimental points are obtained from the absorbance changes after reduction by 0.5 μM NAD•. The line shows the change expected for production of ferrous peroxidase as calculated from the change produced by the addition of sodium dithionite.

Results

Reactions of NAD•. In the presence of 1 mM NAD^+, a transient spectrum with an absorption maximum at 400 nm was observed at 100 ns after pulse radiolysis. This spectrum is identical with that obtained upon pulse radiolysis of free NAD^+ in aqueous solution (Land & Swallow, 1968). Under the conditions used, the hydrated electron (e_{aq}^-)[1] reacts with NAD^+ to yield NAD• nearly quantitatively, since the concentration of NAD^+ (1 mM) is much higher than that of peroxidase. In the absence of peroxidase, the lifetime of NAD• is longer than 6 ms, whereas it decreased with the increase of the concentration of peroxidase under the condition that [peroxidase] ≫ [NAD•]. The decay of NAD• was accompanied by an absorption increase at 435 nm and a decrease at 390 nm. Figure 1A shows the absorption change at 435 nm after pulse radiolysis of ferric peroxidase in the presence of NAD^+. When 0.5 μM NAD• was generated in 8 μM ferric enzyme, 0.48 μM ferric enzyme was reduced to form the ferrous enzyme. These values were estimated from the absorption changes at 400 and 435 nm due to NAD• and the formation of the ferrous enzyme, respectively. This shows that NAD• reduces the ferric enzyme nearly quantitatively. Figure 1B shows the difference between the spectra of the solution before and after the pulse. The figure also shows the changes which are calculated from the concentration of NAD• and the absorbance changes corresponding to formation of the ferrous form from the ferric enzyme. The correspondence between the changes shows that the product is ferrous peroxidase, in 90–100% yield. The yield does not depend on pH in the range from 5 to 10.

[1] Abbreviations: MV, methylviologen; e_{aq}^-, hydrated electron.

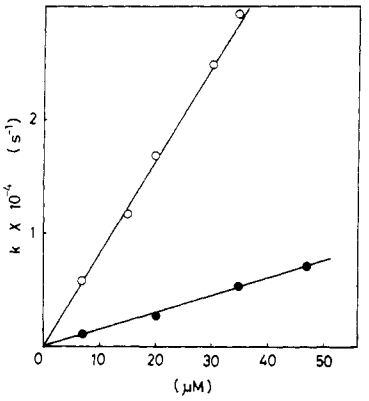


FIGURE 2: Concentration dependence of the pseudo-first-order rate constants for reaction of NAD• with the ferric peroxidase (○) and compound I (●). The reaction medium contained 0.1 M *tert*-butyl alcohol, 50 mM phosphate buffer, and 0.5 μM NAD•.

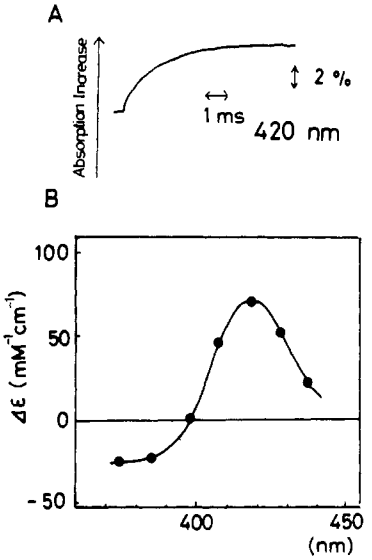


FIGURE 3: (A) Oscilloscope traces of the absorption change after pulse radiolysis of compound I of peroxidase in the presence of NAD^+ and 50 mM phosphate buffer at pH 7.0. (B) Difference spectrum of compound II minus compound I of peroxidase. Experimental points are obtained from the absorbance change after reduction by 0.5 μM NAD•. The line shows the change expected for production of compound II as calculated from changes when compound I and compound II of peroxidase were prepared, respectively, by the addition of equimolar hydrogen peroxide, and equimolar hydrogen peroxide and ferrocyanide to the ferric peroxidase at pH 10.0.

The absorption change in Figure 1A obeyed first-order kinetics. In Figure 2, pseudo-first-order rate constants for such reactions are plotted against the concentration of ferric enzyme (pH 7.0). In the same figure, corresponding constants for the reaction of NAD• with compound I of the enzyme are also shown (see below). From the slope of the plot for ferric enzyme in Figure 2, the second-order rate constant for the reaction of NAD• with the ferric enzyme is calculated to be 8×10^8 M^{-1} s^{-1}.

The reduction of compound I of the enzyme with NAD• was performed. Figure 3A shows the absorption change at 420 nm after pulse radiolysis of compound I in the presence of NAD^+. The absorption of the enzyme was found to increase with a half-time of 700 μs. The difference spectrum obtained 2 ms after pulse is identical with the difference between the spectra of compound II and compound I of the enzyme, as shown in Figure 3B. This suggests that NAD• also reduces compound I of the enzyme nearly quantitatively. Figure 2 shows the dependence of the apparent first-order rate constant on the concentration of compound I. From the slope, the second-order rate constant for the reaction of NAD• with compound I is calculated to be 1×10^8 M^{-1} s^{-1}.

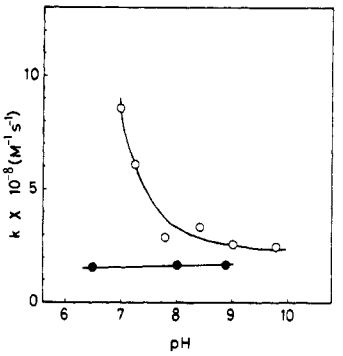


FIGURE 4: pH dependence of the second-order rate constants for the reaction of NAD• with ferric peroxidase (○) and compound I (●). Buffer systems were 50 mM phosphate buffer (pH 6–8) and 5 mM borate (pH 8–10).

The second-order rate constants for the reaction of NAD• with the ferric form of the enzyme and compound I are plotted against pH in Figure 4. The rates for the reduction of the ferric enzyme increase with the decrease of pH, whereas those for the reduction of compound I are almost constant. The pH dependence of the rate constants is similar to pH–E_0 curves of the ferric enzyme and compound I, respectively (Hayashi & Yamazaki, 1979).

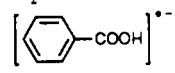
The reaction of NAD• with compound II of peroxidase could not be performed at neutral and acidic pH values, since compound II is only stable at alkaline pH (Hayashi & Yamazaki, 1979; Hewson & Hager, 1979). Thus, the reduction was attempted at pH 10.0. However, we could not detect any absorption changes corresponding to reduction of compound II. Under our conditions, NAD• decayed by second-order kinetics to form a dimer with an absorption maximum at 350 nm (Land & Swallow, 1968). This suggests that NAD• radicals hardly react with compound II at pH 10.0 but rather react with each other, as observed in the absence of the enzyme.

In order to investigate whether NAD• reacts with compound II at neutral and acidic pH values, compound II of diacetyldeutero-HRP was used. In contrast to native peroxidase, compound II of artificial peroxidase containing 2,4-diacetyldeuterohemin in place of protohemin IX is very stable over a wide range of pH (Makino et al., 1986). On the addition of hydrogen peroxide to the ferric diacetyldeuteroperoxidase, compound II was formed spontaneously via compound I. The reduction of compound II of this enzyme by NAD• was attempted from pH 5.0 to 8.0. However, we could not detect any absorption change attributable to reduction.

Reactions with Hydrated Electrons and Other Radicals. The reactions of the ferric, compound I, and compound II forms of peroxidase with e_{aq}^- were studied. The lifetime of e_{aq}^-, measured at 700 nm, indicated a rapid reaction with the enzyme in the each case. The second-order rate constant for the reaction of e_{aq}^- with the ferric enzyme at pH 7.1 was estimated to be 3×10^{10} M^{-1} s^{-1} and was not different for the compound I and compound II forms. However, the yields of heme reduction were found to depend on the oxidation state. The yields for the ferric enzyme and compound I were 40–60% and 7–10% of the e_{aq}^- reacting, respectively. In contrast, the yields for compound II of native peroxidase at pH 10.0 and diacetyldeuteroperoxidase from pH 5.0 to 8.0 were found to be less than 1%.

Table I compares the rate constants for the reaction of various radicals with the ferric, compound I, and compound

Table I: Rate Constants for Reaction of Various Radicals with the Ferric Form and Compound I of Native Peroxidase and with Compound II of Diacetyldeuteroperoxidase[a]

radical	Fe^{3+}	compound I	compound II[b]
NAD•	8×10^8	1.5×10^8	nd[b]
$MV^{\bullet+}$	5×10^7	5×10^8	8.5×10^7
O_2^-	9×10^5[c]	1.6×10^6[d]	nd
	2×10^9	8×10^7	nd
d_{aq}^-	3×10^{10}	3×10^{10}	3×10^{10}
	40–60%[e]	7–10%[e]	<1%[e]

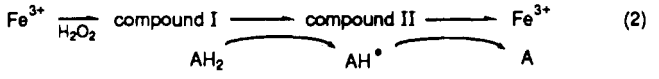
[a] All values were determined under the condition of [peroxidase] ≫ [radical] at pH 7.0. [b] The values were determined in the reaction of native peroxidase at pH 10.0 and diacetyldeuteroperoxidase at pH 7.0. [c] The formation of compound III (Shimizu et al., 1989). [d] The value is comparable to that determined by Bielski and Gebicki (1974). [e] Reduction yield in the reaction with e_{aq}^-. [f] Not determined.

II forms of the enzyme, determined under the condition of [peroxidase] ≫ [radical].

Of the various radicals examined, only $MV^{\bullet+}$ could reduce compound II to form the ferric enzyme. The second-order rate constant was found to be 8.5×10^7 M^{-1} s^{-1} at pH 7.0. Figure 5A shows the absorption change at 430 nm after pulse radiolysis of compound II of diacetyldeuteroperoxidase at pH 7.0 in the presence of MV. The absorption decrease of 430 nm was accompanied by the decay of $MV^{\bullet+}$ at 600 nm and an increase at 410 nm. When the 0.8 μM MV^+ was generated in the presence of 12 μM compound II, the reduction of compound II, as determined from the absorption change in the Soret region, was stoichiometric with the concentration of $MV^{\bullet+}$ consumed, as measured at 600 nm ($\epsilon_{MV^{\bullet+}}$ = 13.3 mM^{-1} cm^{-1}) (Farrington et al., 1973). The kinetic difference spectrum obtained 3 ms after the pulse is shown in Figure 5B and is similar to the difference spectrum of the ferric form minus compound II of diacetyldeuteroperoxidase. On the other hand, no reactions of the radicals with compound II of native peroxidase at pH 10.0 or diacetyldeuteroperoxidase from pH 5.0 to 8.0 could be detected.

Discussion

The oxidation of organic molecules (AH_2) by peroxidase occurs by way of one-electron transfer through reaction 1. If the free radical AH• were to react with compound II as a reducing agent, the following reaction might be expected:



(2)

In this case, the enzyme would be catalyzing a two-electron oxidation. However, no such reaction has been observed, except for the peroxidase reaction of iodine (Björkstén, 1970; Roman & Dunford, 1972) and the thyroid peroxidase reaction (Nakamura et al., 1985). An explanation is that the concentration of the free radical is too low to react with the enzyme in the steady state of the catalytic cycle. The present finding shows that radicals such as NAD• do not react with compound II. From Table I, similar results are expected in the reactions of other free radicals. The lack of reduction by radicals, in contrast to compound I and the ferric enzyme, reflects the unique character of compound II. The particular protein moiety of compound II to which the heme is bound opposes the reduction of the heme by the radical. It is especially striking that the powerful reductant e_{aq}^- could not reduce compound II, although it reduced compound I and the ferric enzyme. The reaction by e_{aq}^- is assigned mainly to a direct reaction proceeding via the exposed edge of the heme. This

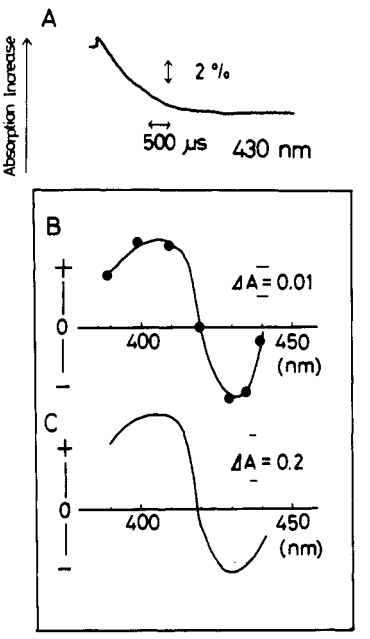


FIGURE 5: (A) Oscilloscope trace of the absorption change after pulse radiolysis of compound II of diacetyldeuteroperoxidase in the presence of methylviologen. The reaction medium contained 12 μM compound II, 1 mM methylviologen, 0.1 M *tert*-butyl alcohol, and 50 mM phosphate buffer at pH 7.0. (B) Kinetic difference spectrum of compound II of diacetyldeuteroperoxidase at 5 ms after pulse radiolysis. (C) Difference spectrum of the ferric form minus compound II of diacetyldeuteroperoxidase. Compound II was formed by the addition of 32 μM hydrogen peroxide to the ferric peroxidase at pH 7.0.

suggests that the heme in compound II may be well buried in a hydrophobic pocket, so that e_{aq}^- reacts with amino acid residues of compound II instead. The difference in the reduction yield of the oxidation states of peroxidase by e_{aq}^- could be related to differences in protein conformation. In contrast to e_{aq}^-, the reaction of radicals with amino acid residues is negligible, since this process is energetically unfavorable (Butler et al., 1982). Radicals decay prior to access to the heme pocket of compound II, whereas electron transfer from radicals to the ferric enzyme or compound I occurs at a porphyrin edge exposed to the solvent. The reducibility of compound II by $MV^{\bullet+}$ may be explained by the stability of the radical: $MV^{\bullet+}$ is stable in a deaerated solution and can approach the active site of compound II within its lifetime. If NAD• can reduce compound II slowly, the rate constant must be several orders of magnitude smaller than 5.6×10^7 M^{-1} s^{-1} (Land & Swallow, 1968). In an exceptional case, Piette et al. (1964) observed that the chloropromazine radical generated by the peroxidase reaction further reacts with compound II. In this case, the stability of the radical seems to allow further oxidation of the free radical.

Similar considerations apply to differences in the rates of oxidation of substrates by compound I and compound II. It has been reported that compound I oxidizes the substrate about 10–100 times faster than compound II (Chance, 1952; Chance & Fergusson, 1954; Cormier & Pritchard, 1968; Cotton & Dunford, 1973; Yamazaki et al., 1973; Hayashi & Yamazaki, 1979). This difference cannot be explained by the redox potential of E_0(compound I/compound II) and E_0(compound II/ferric) (Hayashi & Yamazaki, 1979). It seems likely that peroxidase has an active-site structure which accepts the organic substrate to give a stable complex. The formation of this complex might precede the substrate oxidation by compound II. In contrast, the one-electron transfer from donor to compound I may occur at a porphyrin edge exposed to the solvent without the formation of a stable complex.

In preliminary experiments, results similar to those reported here were obtained in the reaction of radicals with the oxygenated form of peroxidase (compound III).[2] No reaction between radicals such as $NAD^{\bullet}$ and $MV^{\bullet+}$ and compound III could be detected, in contrast to oxymyoglobin, although compound III is reactive against a number of hydrogen donors (Tamura & Yamazaki, 1972; Makino et al., 1976; Yokota & Yamazaki, 1965b; Smith et al., 1982). The mechanism of one-electron transfer from donors to compound III can be explained by similar considerations to that discussed here.

The anomalous role of compound II of peroxidase in the mechanism of substrate oxidation has now become evident. The influence of protein conformation, iron accessibility, and disposition of other specific reactive sites on the reduction of peroxidase by free radicals is being further investigated.

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Registry No. $NAD^{\bullet}$, 82642-62-4; $MV^{\bullet+}$, 25239-55-8; O_2^{-}, 11062-77-4; $C_6H_5COOH^{\bullet-}$, 766-76-7; H_2O_2, 7722-84-1; peroxidase, 9003-99-0.

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