THE ACTIVITY OF THE HORSERADISH PEROXIDASE COMPOUND III.

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Of the three well-known peroxidase compounds with hydrogen peroxide, Compounds I, II and III, the last one has been the most ambiguous with respect to oxidation level and reactivity. Keilin and Mann (1936) reported that Compound III is decomposed in the presence of donors and Chance (1951) concluded that Compound III reacts rapidly with donors to give the free enzyme and that as a consequence no measurable amount of this compound accumulates to inhibit the activity of peroxidase under the usual experimental conditions. Compound III is formed not only in the presence of excess hydrogen peroxide but also upon the addition of dihydroxyfumarate (DHF) directly to the aerobic peroxidase solution (Theorell et al, 1940 ; Chance, 1952). Because of the appearance of Compound III during the peroxidase-oxidase reaction, Mason (1958) suggested the participation of this compound as an oxygen activating intermediate in the oxidase reaction. In spite of these suggestions by Chance and Mason, no direct and quantitative results have been reported on the reactivity of compound III because of the difficulty in isolating the reactions of Compound III with various reagents. As can be seen in Fig. 1, the DHF system represents a suitable experimental condition for estimating the reactivity of Compound III. It has been found (Yamazaki et al, 1963) that a stoichiometric amount of DHF and hydrogen peroxide is needed to convert peroxidase to Compound III, which decays gradually after DHF disappears; the half time for decay is several minutes. No

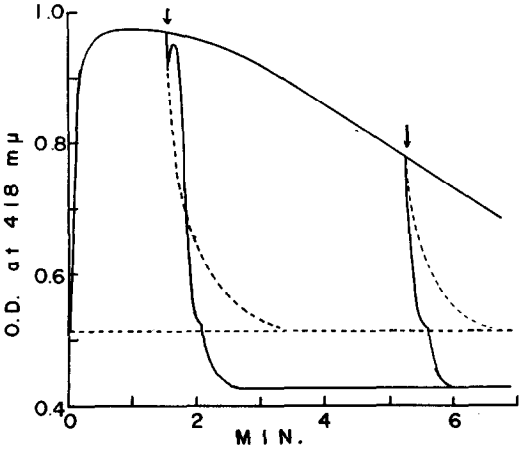

Fig. 1. Effect of electron donors on the stability of Compound III. 0.05 M acetate, pH 5.0, 25°. Compound III was produced by the addition of 0.2 mM dihydroxy-fumarate (DHF) and 10 μM hydrogen peroxide to the aerobic solution of horse radish peroxidase (9.3 μM). The value E_{403} = 107.7 $mM^{-1}cm^{-1}$ for horse radish peroxidase was used according to Paul's data (1963). The ratio of E_{403} to E_{278} for this peroxidase was 3.0. 0.1 mM indoleacetic acid (solid line) and 0.1 mM hydroquinone (dotted line) were added at arrows. In the control experiment DHF disappeared completely 4 minutes after DHF and hydrogen peroxide were added. Hydroquinone reduced Compound III to free peroxidase but indoleacetic acid converted the enzyme to a compound similar to Compound IV via free enzyme. The latter is a exceptional case and the details will be reported elsewhere.

intermediates such as Compound II are detected during the conversion of Compound III to free peroxidase. This is inconsistent with George's result (1953), in which he showed that removal of the excess H_2O_2 by catalase brings about a decomposition of Compound III, yielding Compound II with a reaction half time of about 36 seconds. Moreover, 0.2 μM catalase had no effect on the decomposition of Compound III under the experimental conditions shown in Fig. 1.

The addition of indoleacetic acid greatly accelerates the decomposition of Compound III to give free peroxidase (Fig. 1). Such acceleration of the decomposition was observed in the presence not only of electron donors but also of electron acceptors (Fig. 2). Since no intermediates of peroxidase can be detected during the decomposition it may be concluded that the rate limiting step is the first reaction of Compound III with the donors or acceptors themselves. The rate constants for the

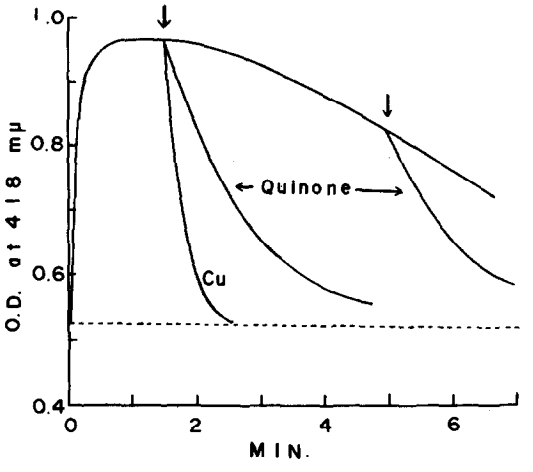

Fig. 2. Effect of electron acceptors on the stability of Compound III. Experiemntal conditions were same as those described in Fig. 1. 0.1 mM p-benzoquinone and 0.1 mM Cu^{2+} were added at arrows.

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reactions, which can now be estimated, are shown in Table I. Extensive kinetic experiments upon peroxidase reactions were carried out by Chance (1951, 1953), who estimated rate constants for the reactions between Compound II and many electron donors. Some of these are also shown in Table I. There is no linear relationship between the two rate constants but Compound II is very roughly 10^3 times more active than Compound III toward the usual hydrogen donors. It can be easily seen from Table I that indoleacetic acid has a remarkably high relative affinity for Compound III. This specificity, which has also been found in other reactions between indoleacetic acid and peroxidase, may imply a role of peroxidase in the metabolism of indoleacetic acid in plant tissue. This will be discussed elsewhere.

Peroxidase Compound III has sometimes been thought to have an oxymyoglobin type structure and Mason (1958) used this theory in his mechanism for the peroxidase-oxidase reaction. Some more direct evidence was recently presented by us to make the ferroperoxidase-oxygen structure for Compound III plausible (Yamazaki et al, 1963) and to predict a possible role of Compound III in the peroxidase-oxidase reaction (Yamazaki

Table I. Rate constants for the reaction of peroxidase derivatives with reductants and oxidants. pH 7.0 for Compound II (except as noted) and pH 5.0, 25° for Compound III.

Reductants	Compound II ($M^{-1} \times sec.^{-1}$) (Chance, 1951 and 1953)	Compound III ($M^{-1} \times sec.^{-1}$)
Hydroquinone	3×10^6	5.3×10^2
Catechol	2×10^6	4.1×10^2
Resorcinol	3×10^5	2.1
p-Cresol		7.0×10
Pyrogallol	3×10^5	5.5×10^2
Phenol	4×10^5	6.7
Guaiacol	3×10^5	9.0×10^2
o-Phenylenediamine	5×10^7	1.9×10^3
p-Phenylenediamine	3×10^7	1.7×10^4
Ferrocyanide		7.8×10
Reductone	1×10^6 (pH 4.2)	3.4×10^3
Dihydroxyfumaric acid	1×10^4 (pH 4.6)	very slow
Ascorbic acid	2×10^4 (pH 4.6)	4.6×10
Indoleacetic acid	1.8×10^4 * (pH 5.0)	2.2×10^3
Oxidants		
p-Benzoquinone		1.3×10^2
Ferricyanide		3.3×10
Cu^{++}		1.2×10^3

* This was estimated from the velocity of iron reduction accompanying the peroxidatic oxidation of indoleacetic acid under anaerobic conditions (Yamazaki et al, 1960)

et al, 1964). Consideration may now be given to the problem whether Compound III accepts only one electron or two electrons simultaneously in the first reaction with donor molecules. Although it is not easy to

reach a firm conclusion, it may be safely said that the first step of compound III decomposition probably corresponds to a one equivalent reduction because one-electron donors such as p-cresol and ferrocyanide can react with Compound III as well as two-electron donors (as seen in Table I). If one assumes the ferroperoxidase-oxygen structure for Compound III the following mechanisms may be suggested for the decomposition of Compound III in the presence of donors and acceptors, respectively.

$$\underset{(Fe_p^{2+}O_2)}{\text{Compound III}} \xrightarrow[(+2H^+)]{\text{+ electron}} \underset{(Fe_p^{3+})}{\text{Peroxidase}} + H_2O_2 \ (\longrightarrow \text{Compound I}) \quad (1)$$

$$\underset{(Fe_p^{2+}O_2)}{\text{Compound III}} \xrightarrow{\text{- electron}} \underset{(Fe_p^{3+})}{\text{Peroxidase}} + O_2 \quad (2)$$

The rapid conversion of oxymyoglobin to metmyoglobin in the presence of ferricyanide or quinones seems to be analogous to Reaction (2). This might suggest a hybrid structure for Compound III of ferro-oxygen and ferri-perhydroxyl complexes.

Compound III is not an active intermediate in the hydroxylation of salicylic acid catalyzed by peroxidase because salicylic acid has no effect on the stability of compound III. This is consistent with the previous conclusion by Mason and Buhler (1961) that an active intermediate for the hydroxylation might be perhydroxyl ion. It might be worthwhile to note here that DHF is a very poor donor for Compound III, as reported by Chance (1952) and ourselves (1963). This is the reason why Compound III accumulates effectively in the DHF reaction. Tryptophane, tyrosine, uric acid and xanthine have no detectable effect on the decomposition of Compound III. These negative results have been confirmed by observing the absorption spectra in the visible region.

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