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THE MECHANISM OF AEROBIC OXIDASE REACTION CATALYZED BY PEROXIDASE

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SUMMARY

1. The peroxidase–oxidase reaction has been studied, using an electron-paramagnetic-resonance spectrometer and a recording spectrophotometer, in order to adduce additional evidence to support a mechanism involving the formation of free radicals.

2. It has been found that peroxidase catalyzes the formation of free radicals of hydrogen donors in the presence of H_2O_2.

3. The free radicals derived from dihydroxyfumarate, triose reductone and indoleacetic acid can reduce molecular oxygen and peroxidase itself.

4. One equivalent reduced molecular oxygen, perhydroxyl radical, is supposed to be the active intermediate in the peroxidase–oxidase reaction.

5. Compound III and oxygenated ferroperoxidase are one and the same peroxidase derivative which can be produced from the three different systems, ferroperoxidase + O_2, ferriperoxidase + perhydroxyl radical, and Compound II + H_2O_2. Compound III is not an active intermediate for dihydroxyfumarate oxidation.

INTRODUCTION

Since SWEDIN AND THEORELL[1] found that HRP catalyzes the aerobic oxidation of DHF, the mechanism of the reaction has been investigated by many workers. Several substances have been found which are oxidized by the peroxidase–oxidase system and Table I summarizes the cases in which peroxidases act as oxidases. The properties of the peroxidase–oxidase reaction are not always the same for each hydrogen donor but typical features of the reaction can be summarized as follows.

H_2O_2 necessity

The reactions listed in Table I proceed without the addition of H_2O_2, but are strongly inhibited by catalase (EC 1.11.1.6). Therefore, it may be concluded that a

Abbreviations: HRP, horse-radish peroxidase; DHF, dihydroxyfumarate; IAA, indole acetic acid; EPR, electron paramagnetic resonance; DKS, diketosuccinate.

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TABLE I

PEROXIDASE–OXIDASE REACTIONS

Substrates	*Sources*	*References*
DHF	Horse radish	1, 18
DHF	Turnip	9
DHF	Japanese radish	34
IAA	Horse radish	32, 35
IAA	*Omphalia flavida*	36, 37
IAA	Turnip	4
IAA	Wheat-germ	38
Indole propionate, indole butyrate	Horse radish	39
Oxalate, oxalacetate, keto-malonate, dihydroxytartrate	Horse radish	40
Phenylacetaldehyde	Horse radish	41
Phenylpyruvate	Lupine seedlings	42
Triose reductone	Turnip	9
DPNH, TPNH	Horse radish	8

trace amount of H_2O_2, which has been accumulated by the autoxidation of hydrogen donors, is necessary to cause the reaction to proceed since H_2O_2 is formed as a consequence of the reduction of oxygen. Some contradictory cases have been reported in the oxidation of IAA. WAYGOOD, OAKS AND MACLACHLAN[2] reported that catalase catalyzes the oxidation of IAA in the presence of manganese and resorcinol or 2,4-dichlorophenol. On the other hand, KENTEN AND MANN reported that catalase inhibits the reaction completely at pH 6.5–7, but causes only a short lag period at pH 6, after which the oxidation proceeds with increasing velocity and finally reaches a velocity slightly greater than that of the control from which catalase is omitted.

Inhibition by CO

Probably the most controversial results have been reported on the effect of CO. SWEDIN AND THEORELL[1] reported that the HRP–oxygen–DHF system is inhibited by CO and that the inhibition is released by light. Although CHANCE observed a small inhibition by CO in the same system, he concluded that CO has essentially no effect on the reaction since he was unable to observe the CO-complex of ferroperoxidase.[18] The same negative result was reported by MASON AND ANAN[3]. YAMAZAKI AND SOUZU[4] also could not confirm inhibition by CO in the turnip peroxidase–oxygen–IAA system. However, RAY[5] has recently observed the light-sensitive inhibition by CO in the *Omphalia flavida* enzyme–oxygen–IAA system; the inhibition is affected by oxygen concentration. MORITA AND KAMEDA[6] and NICHOLLS[7] also have independently observed the inhibition by CO in the oxidation of DHF by peroxidase; the inhibition is affected by the concentration of oxygen and depends upon the type of enzyme preparation.

Effects of metals

Promotive effects of manganese on the peroxidase–oxidase reaction have been

observed by several people and there appears to be an optimum concentration of manganese of about 10–100 μM which is necessary in order to observe the maximum enhancement. A strongly activating effect has been shown in the cases of DHF, DPNH and IAA, and a slight one in the case of triose reductone. Only Co^{2+} has been found to substitute to some extent for manganese in the oxidation of DPNH (see ref. 8) and of triose reductone[9]. Cu^{2+} is a highly efficient inhibitor when DHF, DPNH and triose reductone are used as hydrogen donors.

Effects of other peroxidase substrates

Strongly promotive effects by monophenols and resorcinol have been observed in the peroxidase–oxidase reaction, while the other peroxidase substrates, hydroquinone and ascorbic acid are efficient inhibitors. According to the oxido-reductive characteristics of the intermediates formed in the peroxidase reaction, YAMAZAKI[10] proposed the tentative division of the peroxidase substrates into redogenic and oxidogenic groups. Oxidogenic substances all activate the reaction, while redogenic substances are invariably inhibitory.

Effects of cyanide

The compounds such as KCN and hydroxylamine, which are known to affect enzymes containing iron, are generally inhibitory although NICHOLLS[11] observed a stimulating effect of HCN in the HRP–oxygen–DHF system under certain conditions. Some irregular effect of cyanide was also reported by MORITA AND KAMEDA[6]. SWEDIN AND THEORELL[1] observed the formation of a Compound III-like substance during DHF oxidation by HRP. This has been confirmed by many others, but only in the peroxidase–oxygen–DHF system.

Several mechanisms have already been proposed to explain the above properties of the peroxidase–oxidase reactions, which will be discussed later. We have also proposed a free-radical mechanism for the reaction[4,10,12] and we shall report some additional evidence to support such a mechanism in this paper.

EXPERIMENTAL

HRP (R.Z.,$E_{403}/E_{278} = 3.0$) was purified and crystallized by the method of KENTEN AND MANN[13]. A Varian EPR spectrometer and a Cary recording spectrometer were used throughout this experiment. The flow apparatus which is attached to the EPR spectrometer and the estimation of free radical concentrations have been described elsewhere[15].

RESULTS

EPR spectra of free radicals have been observed during the peroxidatic reaction of redogenic substrates with the exception of IAA, but not of the oxidogenic substrates such as resorcinol and phenol. These are listed in Table II with additional information about the intermediates formed during the peroxidatic reaction. The free radicals derived from oxidogenic substrates seem to be very unstable and react with each other instantaneously to form dimerized or further complicated oxidation

TABLE II

VARIOUS FEATURES OF PEROXIDASE SUBSTRATES

	Reducing activity of the intermediates formed in peroxidase reaction					*Effects on the aerobic oxidation***	*EPR signal at steady state of the reaction****
	H-acceptor						
	O_2	*Methylene blue**	*Cytochrome c*	Fe^{3+}	*Peroxidase*		
Redogenic substrates (YH_2)							
Triose reductone	+	+	+		+	Substrates	+ (3)
DHF	+	+	+		+	Substrates	+ (1)
IAA	+	−	+	+	+	Substrates	−
Ascorbic acid	−	−	+		−	i	+ (2)
Reductic acid	±	−	+		−	i	+ (5)
Hydroquinone	−	−	+		−	i	+ (5)
Pyrogallol	−	+	+			i	+
p-Phenylenediamine	−	−	−			i	+
Catechol	−	−	−			si	+ (9)
Oxidogenic substrates (XH_2)							
p-Cresol	−	−	−			a	
m-Cresol	−	−				a	
Guaiacol	−	−				a	
Resorcinol	−	−				a	−
m-Phenylenediamine	−	−				a	
Aniline	−	−				a	
Phenol	−	−				a	−
Uric acid	−	−				a	

* In the case of methylene blue a negative result does not mean that the intermediates can not reduce methylene blue, since reduced methylene blue is very easily reoxidized by the same peroxidase system.

** i, inhibitory; si, slightly inhibitory; a, activating. There is usually an optimal concentration to give maximum stimulation by oxidogenic substrates.

*** The line numbers of the EPR signal of the free radical are shown in parentheses.

products. It might be expected that these free radicals are strong one-electron oxidizing agents. As can be seen in Fig. 1, an addition of resorcinol greatly accelerates the oxidation of ascorbic acid by the HRP–H_2O_2 system and also increases the concentration of the ascorbic acid free-radical in the steady state by about four times that of the control without resorcinol. The same acceleration can be observed when phenol is added. The increased free radical signal is identified as ascorbic acid free radical from its typical two-line hyperfine pattern at $g = 2.0043$. No signals are observed around $g = 2$ in the absence of ascorbic acid.

As reported in the previous paper[14,15], the DHF free radical cannot be observed under the aerobic conditions. Fig. 2 shows this phenomenon in more detail. During the peroxidatic oxidation of DHF, 0.15 μM of free radical is generated and observed under anaerobic conditions. Although very rapid oxidation of DHF must take place in the initial stages of the reaction when oxygen is present, one cannot observe an EPR signal around $g = 2$ during this time. An EPR signal corresponding to 0.15 μM of free radical of DHF suddenly appears when almost all of the oxygen

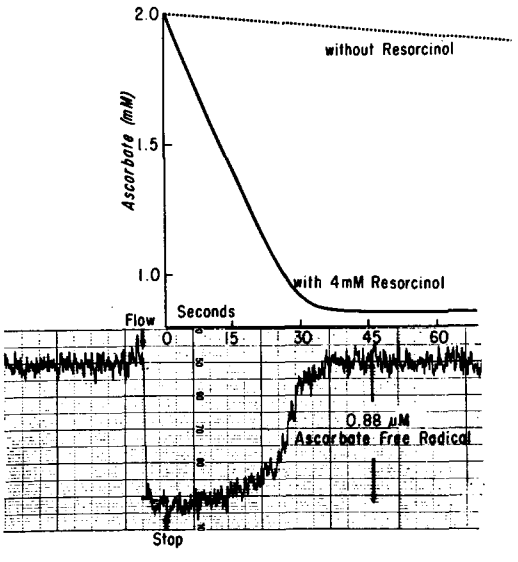

Fig. 1. Effect of resorcinol on the rate of disappearance of ascorbate and on the free radical of ascorbate in the steady state during the peroxidatic oxidation of ascorbate. Ascorbate (2 mM); H_2O_2 (1 mM); HRP (0.1 μM); acetate buffer (0.1 M, pH 4.8). The upper and lower diagrams show the disappearance of ascorbate and its free radical, respectively. In the experiment with the free radical, the magnetic field was adjusted so as to obtain the maximum of the derivative curve[24]. Acetate-buffered solutions of HRP and ascorbate (with or without resorcinol), and of H_2O_2, were forced by compressed N_2 into the two respective arms of the mixing chamber. The time of reaction during steady flow in the lower diagram is about 0.1 sec at the centre of the cavity and the time at stopping the flow is almost simultaneous with that of the start of the reaction in the upper diagram. The concentrations of ascorbate free radical at the steady state were calculated from their whole signal and were 0.88 μM and 0.23 μM in the presence and absence of resorcinol, respectively. In the latter case, 0.23 μM free radical persisted for a long time.

in the cuvette has been consumed and at a time in which slow oxidation, which is observed under anaerobic conditions, sets in again. This is reasonable since the amounts of DHF and H_2O_2 consumed during the aerobic conditions are extremely small compared with the initial concentrations of these substances. No appreciable change in the signal is observed in the presence, or absence, of oxygen when triose reductone is used as the hydrogen donor.

As mentioned previously, the most ambiguous results which have been reported in this oxidase reaction are those dealing with inhibition by CO and the formation of a CO–ferroperoxidase complex. In spite of the observation of SWEDIN AND THEORELL that the peroxidase–oxygen–DHF system is inhibited by CO and that the inhibition is released by light, many people have been unable to confirm these observations. However, RAY[5], MORITA AND KAMEDA[6] and NICHOLLS[7] have recently confirmed the inhibition by CO again, so it appears that perhaps this controversy over the effect of CO may be due merely to differences in the reaction system studied. We have been able to verify this dependence upon experimental conditions in the following reactions. We have found that reproducible formation of the CO–ferroperoxidase complex is obtained when H_2O_2 is added, little by little, through a capillary with the continuous passage of CO into the solution containing HRP and a hydrogen donor,

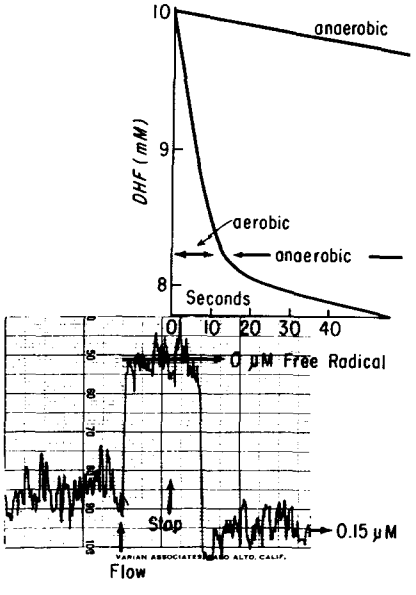

Fig. 2. Free radical of DHF during the peroxidatic reaction. DHF (10 mM); H_2O_2 (10 mM); HRP (0.2 μM) ;acetate buffer (0.1 M, pH 4.8). In the upper diagram, the disappearance of DHF was observed spectrophotometrically at 342 mμ. The anaerobic reaction started from a N_2-saturated solution, and the aerobic from an O_2-saturated solution. Aerobic reaction changes to anaerobic after rapid oxidation of about 2 mM of DHF. The lower diagram shows the fluctuation of the free radical of DHF during the oxidation of DHF. Flow experiments were carried out by passing the aerobic solution of DHF and H_2O_2 into one arm of the mixing chamber and the aerobic solution of HRP into the other arm. The time at stopping the flow is almost simultaneous with that of the start of the reaction in the upper diagram. The magnetic field was adjusted so as to obtain the maximum of the derivative curve of the free radical of DHF.

such as IAA, DHF and triose reductone, but not hydroquinone or ascorbic acid. Without the addition of H_2O_2, the complex hardly appears, while the rapid addition of H_2O_2 seems to inhibit the formation of complex. The order of effectiveness of hydrogen donors to reduce peroxidase is IAA, DHF and triose reductone. Peroxidase is inactivated and precipitates in the presence of IAA and H_2O_2 and so the spectrum of the CO–ferroperoxidase complex derived from the IAA system can be observed only in the initial stage of the reaction and becomes obscure several minutes after the addition of H_2O_2. There is no important change in the absorption spectrum for 10 min in the presence of IAA without H_2O_2 addition (see Fig. 3). When CO is gassed through a solution of HRP and DHF, a very small amount of HRP changes to the CO–ferroperoxidase complex without the addition of H_2O_2 but a small addition of H_2O_2 greatly increases the rate of formation of the complex. The sluggish formation of the complex in the absence of added H_2O_2 is possibly due to the autoxidation of DHF which occurs in the presence of a trace amount of oxygen (see Fig. 4).

Another interesting feature of the reaction is the appearance of a Compound III-type spectrum, especially in the oxidation of DHF. Compound III has been known as the peroxidase–H_2O_2 complex which forms in the presence of high concentrations of H_2O_2. Although one would expect the H_2O_2 concentration during DHF oxidation to be too low to produce this compound, it is believed that a Com-

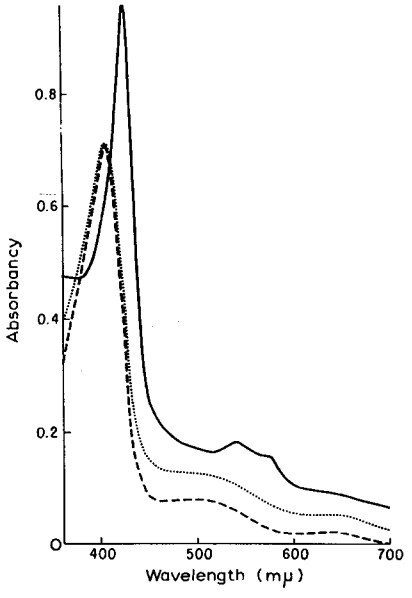

Fig. 3. Formation of the CO–ferrous HRP complex. IAA (2 mg/3 ml); HRP (7 μM); acetate buffer (0.1 M, pH 4.8). H_2O_2 (0.1 M) was added continuously through a capillary at the rate of 0.07 ml per min while gassing with CO. — — —, HRP only; ———, 2 min with H_2O_2; ....., 10 min without H_2O_2.

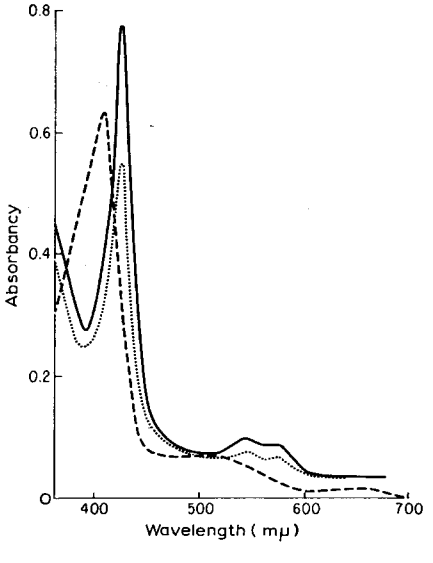

Fig. 4. Formation of CO–ferrous HRP complex. DHF (0.01 M); HRP (7 μM); acetate buffer (0.1 M, pH 4.8). H_2O_2 (0.1 M) was added at the rate of 0.07 ml/min while gassing with CO and the absorption spectrum was recorded 3 min after the addition of H_2O_2 had started. — — —, HRP only; ———, with H_2O_2; ....., with H_2O_2 and 1 mM Mn^{2+} added.

pound III-like substance does appear during the oxidation of DHF and that it is the same compound as that formed at high concentrations of H_2O_2. No experiments have been done to elucidate the mechanism of Compound III formation during the

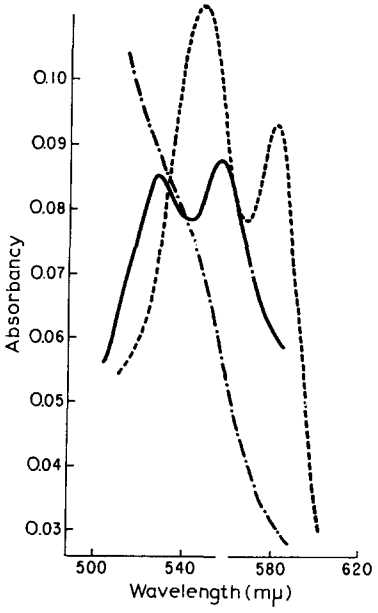

Fig. 5. Absorption spectra of HRP (—·—·—), Compound II (———) and Compound III (— — — —).

oxidation of DHF. Figs. 6 and 7 show the effects of H_2O_2 on the formation of Compound III in the HRP–oxygen–DHF system. As can be seen in Fig. 6, sluggish formation of Compound III occurs without the addition of H_2O_2. Direct trans-

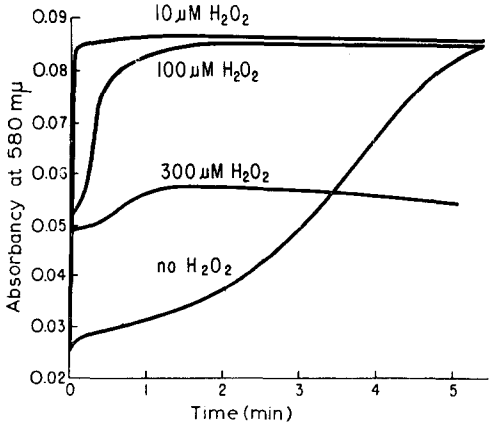

Fig. 6. Effect of concentration of H_2O_2 on the formation of Compound III during the aerobic oxidation of DHF. DHF (250 μM); HRP (10 μM); acetate buffer (0.05 M, pH 4.75); aerobic (saturated with air). condition

formation of HRP to Compound III can be confirmed by the successive observations of spectra between 500 and 600 mμ with a Cary recording spectrometer. The typical spectra of free HRP, Compound II and Compound III are shown in Fig. 5. Compound II is not present in appreciable amount during the course of the reaction. When an equimolar amount of H_2O_2 is added to HRP, almost all of the HRP changes instantly into Compound III which then persists for a long time. If ten times more H_2O_2 is added at once, one can see the slower formation of Compound III after the initial formation of Compound II. A slightly higher concentration of H_2O_2 than of DHF changes HRP to Compound II but not to Compound III. The stoichiometric relationship between the disappearance of DHF and H_2O_2 and the formation of Compound III is shown in Fig. 7. 4 μmoles of H_2O_2 consumes 10 μmoles of DHF and forms 6.3 μmoles of Compound III. One can see another interesting finding in Fig. 7, namely, that the oxygen-consuming oxidation of DHF is almost inhibited during

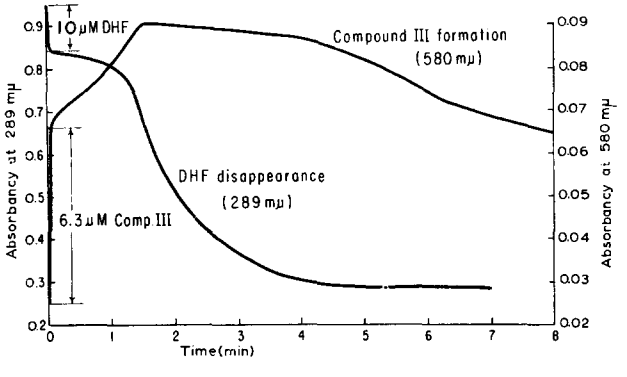

Fig. 7. Stoichiometric relationship between the disappearance of DHF and H_2O_2 and the formation of Compound III. H_2O_2 (4 μM) was added to HRP (10 μM) in the presence of DHF (65 μM) under aerobic conditions. Acetate buffer (0.05 M, pH 4.75).

the formation of Compound III and suddenly appears after the concentration of Compound III reaches its maximum. This might be consistent with the additional observation that the initial rate of DHF oxidation is much slower in the presence of a high concentration of enzyme (above 5 μM). In other words, the higher enzymic concentration elongates the lag phase of the reaction, which corresponds to the period of formation of Compound III.

According to the result of HARBURY[16], the autoxidation of ferroperoxidase was so fast that an oxygenated intermediate could not be detected spectrophotometrically. If it is true, the subsequent intermediate product may be the HO_2 radical and this is not consistent with our results that the HO_2 radical has a strong affinity for ferriperoxidase. In our experiment, as can be seen in Fig. 8, an intermediate is observed during the autoxidation of ferroperoxidase which is reduced by sodium hydrosulphite. The absorption spectrum of the intermediate has maxima at 546 and 582 mμ and is indistinguishable from that of oxymyoglobin, oxyhemoglobin and Compound III of peroxidase. The intermediate decomposes to free peroxidase spontaneously with a half-life of several minutes. The rate of the autoxidation of ferroperoxidase is influenced by the experimental conditions. The intermediate

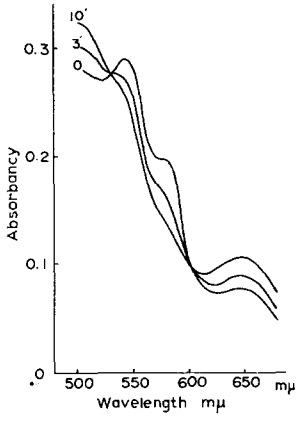

Fig. 8. Formation of an intermediate compound during the autoxidation of ferroperoxidase. The absorption spectrum was taken (in this experiment only) with a Hitachi recording spectrophotometer and scanning from 500 to 680 mμ in about 20 sec. The time when scanning started at 500 mμ was recorded after the addition of 2 or 3 small pieces of solid $Na_2S_2O_4$ and shown in the figure. HRP (33 μM), R.Z. = 2.0 (only in this experiment); acetate buffer (0.01 M, pH 4.7). Similar results were also obtained at pH 3.7 and 7.0.

which is formed by the second addition of sodium hydrosulphite is very unstable and one half of it decays within a minute. When an excess amount of sodium hydrosulphite is added, reduced peroxidase is reoxidized instantaneously as soon as oxygen is introduced to the solution of the enzyme and then one can hardly observe the intermediate.

DISCUSSION

According to the findings of SWEDIN AND THEORELL based on the inhibition of the enzyme by CO which is released by light, and on the necessity of H_2O_2, LEMBERG AND LEGGE[17] proposed the mechanism by which ferroperoxidase was considered to act as the oxygen-binding oxidase and H_2O_2 was involved in the formation of ferroperoxidase, the first phase of the reaction. CHANCE[18], however, was unable to confirm the formation of a CO–ferroperoxidase complex and proposed an alternative mechanism in which the activation of oxygen is brought about by a manganese–enzyme–substrate complex. From the observation that peroxidase is rapidly transformed into Compound III or a substance spectroscopically like it, MASON AND ANAN[3] suggested that this Compound III might be the active intermediate compound in the peroxidase–oxidase reaction.

Another series of experiments has been made on the aerobic oxidation of IAA by peroxidase. For the reaction, MACLACHLAN AND WAYGOOD[19] proposed a free-radical mechanism in which peroxidase catalyzes the oxidation of the manganese and the manganic ions in turn initiate the chain-oxidation of IAA. KENTEN AND MANN[20] have shown that manganese is oxidized by peroxidase in the presence of resorcinol or monophenols, and MACLACHLAN AND WAYGOOD[19] have shown that manganic ions act catalytically in IAA oxidation. This hypothesis offers a reasonable explanation for the features of the oxidation of IAA in the presence of manganese and monophenols, but it does not account for the peroxidase–oxidase reaction in the absence of such activators.

Another type of free-radical mechanism was proposed by YAMAZAKI[10] who concluded that peroxidase catalyzes the formation of free radicals of hydrogen donors in the presence of H_2O_2, and that these free radicals derived from redogenic substrates, can reduce cytochrome *c*, ferric ion, methylene blue, or molecular oxygen. The possibility of forming such free radicals has been suggested by GEORGE[21,22], who found that the peroxidase–H_2O_2 intermediate, Compound II, is in a one-electron oxidized state compared with free peroxidase. CHANCE[23] has also observed one-electron reduction from the primary intermediate of peroxidase–H_2O_2, Compound I to Compound II. This assumption of one equivalent oxidation and reduction has recently been confirmed by the observation of EPR signals during the peroxidase reaction[14,15,24]. The stoichiometric reduction of ferric ion[4] and methylene blue[12] by peroxidase-generated free radicals has been observed, while chain reactions might be expected when molecular oxygen is used as electron acceptor for free radicals, as is shown in Fig. 9.

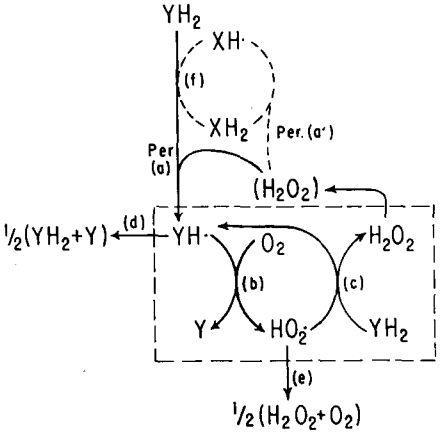

Fig. 9. Tentative scheme for O_2-consuming oxidation of YH_2 (triose reductone, DHF and IAA) catalyzed by peroxidase. Reaction a is confirmed by the observation of EPR signals during peroxidatic reaction[14,24]. Reaction b is assumed from the observation that the free radical of DHF disappears in the presence of oxygen (Fig. 2). We have no direct evidence to confirm Reaction c but it is supported from many indirect results. Reaction d is the main decay reaction of the free radical of YH in the simple peroxidatic reaction[24]. Reaction e is the dismutation reaction of the perhydroxyl radical and supposed to be very fast. Reactions a' and f represent another possible pathway for the formation of free radicals of YH when resorcinol or phenol is added (Fig. 1). Broken line shows chain reaction.

Now, it is worthwhile to discuss the reaction between these substrate free radicals and molecular oxygen or peroxidase from the stand point of their redox potential. According to MICHAELIS[25], if one distinguishes three oxidation levels, the reduced from *(R)*, the semi-oxidized form *(S)* and the totally oxidized form *(T)* in a bivalent reversible system, which is not disturbed by any complications such as dimerization of any of the molecular species concerned, or by any subsequent irreversible process, the potentials of the three systems, $T = R$, $S = R$ and $T = S$ are, $E = E_m + RT/2F \ln. t/r$, $E = E_1 + RT/F \ln. s/r$, $E = E_2 + RT/F \ln. t/s$,

where r, s and t designate the molar concentration of R, S and T. Since there is an equilibrium, there can be only one common value of E. Then,

$$E_m = \frac{E_1 + E_2}{2}$$

This mean normal potential, E_m, has been determined for many bivalent systems and E_1 and E_2 can be estimated when the constant for semiquinone formation, $k = s^2/rt$, is known. Then, the following equations can be derived:

$$E_1 = E_m - RT/2\,F \ln. k$$
$$E_2 = E_m + RT/2\,F \ln. k$$

The order of the three normal potentials is the "natural sequence", $E_1 < E_m < E_2$ when $k > 1$ and is the "reversed order", $E_1 > E_m > E_2$ when $k < 1$. As can be seen from the above equations, the smaller the k value, the larger the difference between E_1 and E_2 in the "reversed-order" system. Since the constant for semiquinone formation of redogenic substrates listed in Table II is usually smaller than unity, the potential E_2 is lower than the mean normal potentials of bivalent redox systems (these are generally between + 0.2 and + 0.4 V in the case of ascorbic acid, DHF, triose reductone or hydroquinone at pH 5–7) and sometimes as low as −0.3 V which has been reported by HARBURY[16] as the normal potential of peroxidase. Thus, it might be possible that the free radicals of DHF, IAA and triose reductone can reduce HRP to the ferrous form. Although the free radical itself seems to be in an activated state, it is not always true that the free radical can reduce systems which have a higher redox potential than that of the free radical. In other words, the fact that the free radicals of ascorbic acid and hydroquinone are unable to reduce HRP can be attributed to two main reasons; the first is the simple barrier of the redox potential of the reaction, and the second is the steric hindrance of the reaction between the free radicals and the active centre of the enzyme.

According to the review by GEORGE AND GRIFFITH[26], the most reliable value of the potential of the first one-equivalent reduction of molecular oxygen is −0.9 V which does not change over at pH 4.0 because $HO_2\cdot$ is supposed to be a strong acid. The disappearance of the free radical of DHF under aerobic conditions strongly suggests the existence of an interaction between the free radical and molecular oxygen. This might mean that the potential of the DHF·/DKS system is comparable to, or less than, −0.9 V, provided that the value of −0.9 V is correct for the system O_2/HO_2. Since the mean normal potential of DHF is around +0.2 to +0.3 V, E_1 must, therefore, be 1.4–1.5 V in order to give an E_2 value of −0.9, and similarly the formation constant, k, for the free radical must be of the order of 10^{-40} at 30°. These are very unlikely values since Compound II can easily oxidize DHF by one equivalent; Compound II + DHF → HRP + DHF· and the potential of Compound II/HRP is around +1.0 V (see ref. 22). This means that the potential of DHF·/DHF must be lower than +1.0 V. This is probably the most uncertain aspect of this free-radical mechanism and should be reconsidered after more reliable values of the potentials of the systems, $O_2/HO_2\cdot$ and Compound II/HRP are known. This inconsistency is also borne out in HARBURY's findings that ferroperoxidase can be easily oxidized by molecular oxygen as well as some cytochrome b, the redox potential

of which is around 0. These inconsistencies would not exist if the potential of O_2/HO_2· turned out to be between 0 and −0.3 V instead of −0.9 V.

HO_2· acts as a strong, one-equivalent oxidant in the presence of suitable reducing substances and a chain reaction may proceed in so far as it reacts with DHF to produce a free radical of DHF as shown in Fig. 9. The dismutation of the HO_2 radical, however, is extraordinarily fast so that the concentration of the free radical in the steady state is below the sensitivity of the EPR spectrometer. Reaction e, chain-termination, is usually dominant over Reaction c, chain-maintenance, especially when formation of the HO_2 radical is very fast. The activating effect of manganese seems to depend on the elimination of the dismutation Reaction e, which is schematized in Fig. 10. The essential point of the mechanism is the transfer of the one-electron oxidizing ability from a HO_2 radical to a manganic ion which will efficiently oxidize DHF and produce the free radical of DHF. In the presence of monophenols, an alternative path of manganese oxidation may be expected as was suggested by KENTEN AND MANN[20] and MACLACHLAN AND WAYGOOD[19].

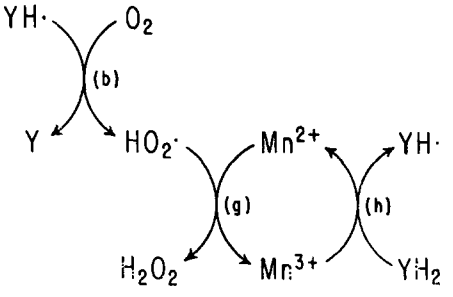

Fig. 10. Promotive effect of manganese on the peroxidase–oxidase reaction. The presence of manganese promotes the chain reaction in Fig. 9 by reducing Reaction e.

The results in Figs. 3 and 4 clearly shows that peroxidase is reduced by the free radicals of DHF and IAA. It is very important to note that slow addition of H_2O_2 is necessary to produce ferroperoxidase under anaerobic conditions and rapid addition is inhibitory to the ferroperoxidase formation. Since the species necessary to form ferroperoxidase are the free radicals of DHF and IAA and free peroxidase, high H_2O_2 concentration must be inhibitory since Compound II is now dominant instead of free peroxidase in the solution. Under aerobic conditions, most of the free radicals of DHF react with oxygen and only a small portion of them reduce peroxidase to the ferro form. When CO exists in this system in the dark, ferroperoxidase is converted to the CO-complex and thus removed from the reaction system. Now, the controversy of inhibition by CO can be explained in two ways, the difference in the experimental conditions and the nature of the peroxidases. The most critical condition is the amount of H_2O_2 in the reaction-solution required to produce the free radicals of hydrogen donors while still keeping most of the enzyme in the free state. The reduction of peroxidase by the free radical under anaerobic conditions is influenced slightly by manganese as shown in Fig. 4, but manganese completely removes the effect of CO under aerobic conditions by increasing the production of H_2O_2 which in turn increases the formation of Compound II during the reaction. The

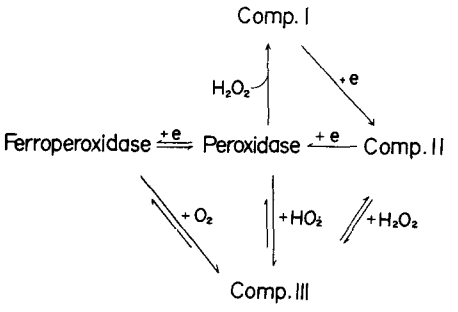

Fig. 11. Tentative scheme for the relationship between peroxidase derivatives which appear during peroxidase–oxidase reaction.

presence of a high level of oxygen makes it difficult to produce CO–ferroperoxidase complex because ferroperoxidase has a strong affinity toward oxygen which competes with the CO for ferroperoxidase. Recently, MORITA AND KAMEDA[6] pointed out the possibility that HRP I and Japanese radish peroxidase *c* might have higher redox potentials than HRP II (normal HRP) and Japanese radish peroxidase *a* and might therefore be more easily reduced by the free radical. They also suggested an alternative mechanism of the aerobic oxidase activity of peroxidase which involves ferroperoxidase in the reaction of HRP I and Japanese radish peroxidase *c*. It must be noticed here, however, that inhibition by CO does not always suggest the mechanism which involves ferroenzyme as an activator of oxygen in the oxidase reaction. Fig. 12 shows that inhibition by CO does occur in the oxidase mechanism of peroxidase where ferro-species are not active.

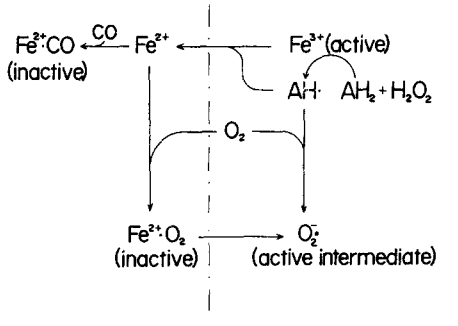

Fig. 12. A possible scheme for the inhibition by CO in the peroxidase–oxidase reaction. Refer to Figs. 9 and 11.

A similar mechanism for ferroperoxidase activity has been suggested by TANAKA AND KNOX[27] in their tryptophane peroxidase–oxidase reaction and they rename this enzyme as tryptophane pyrrolase because this enzyme remains as the ferro-form during the reaction and acts as an oxygen-transferring enzyme. It is interesting to note that H_2O_2 is essential to reduce this enzyme in the first stage of the reaction. The necessity of H_2O_2 to reduce the enzyme seems to be possitive evidence to support the participation of a free radical in the reduction of haem

iron, but it still remains to be shown whether the ferro-form of the enzyme can be reoxidized by oxygen or whether it acts as an O_2-transferring catalyst in the reaction of Japanese radish peroxidase *c* and tryptophane pyrolase.

In the presence of more than 1 μM of HRP, there appears to be a distinct lag period which becomes longer as the concentration of HRP increases. This lag phase stops as soon as Compound III reaches its maximum concentration. This sharp change in the oxidation of DHF can be explained easily by assuming that the active intermediate species, the HO_2 radical, is effectively trapped by free peroxidase. H_2O_2 is also essential in the production of Compound III and the stoichiometry of the reaction is consistent with the following mechanism:

$$2\ \mathrm{DHF} + \mathrm{H_2O_2} \xrightarrow{\mathrm{HRP}} 2\ \mathrm{DHF\cdot} + 2\ \mathrm{H_2O}$$
$$\mathrm{DHF\cdot} + \mathrm{O_2} \longrightarrow \mathrm{DKS} + \mathrm{HO_2\cdot}$$
$$\mathrm{HO_2\cdot} + \mathrm{HRP} \longrightarrow \text{Compound III}$$

and the overall reaction would be:

$$2\ \mathrm{DHF} + \mathrm{H_2O_2} + 2\ \mathrm{O_2} + 2\ \mathrm{HRP} \rightarrow 2\ \mathrm{DKS} + 2\ \text{Compound III} + 2\ \mathrm{H_2O}$$

The actual ratio of DHF:H_2O_2:HRP found experimentally is 2.5:1.0:1.57. The loss of the small excess of DHF may be due to Reaction c in Fig. 9.

Peroxidase Compound III was sometimes supposed to be a ferroperoxidase–O_2 complex and to be an active intermediate in the peroxidase–oxidase reaction. Although the discrepancy between HARBURY's experiments and ours has not been elucidated, it is very likely that oxygenated ferroperoxidase is an intermediate during the autoxidation of ferroperoxidase. Judging from the similarity of the absorption spectra and oxidation levels, the peroxidase derivatives which are called Compound III, or oxygenated ferroperoxidase, might be the same as MASON[28] has suggested already; and they might be produced from the three different systems, ferroperoxidase + O_2, ferriperoxidase + perhydroxyl radical, and Compound II + excess H_2O_2, as schematized in Fig. 11. There are two paths of formation of Compound III during the peroxidase–oxidase reaction. Firstly, peroxidase is reduced by the free radicals of hydrogen donors and then it combines with O_2; secondly, oxygen receives one electron from the free radicals of hydrogen donors and reacts with ferriperoxidase. The latter must be dominant in the DHF–O_2–peroxidase system because the formation of Compound III in the DHF–H_2O_2–O_2–peroxidase system is much faster (Fig. 6) than the reduction of peroxidase in the DHF–H_2O_2–CO–peroxidase system (Fig. 4).

From these and other observations, peroxidase Compound III is characterized by the following features: (a) According to the strong electron-donating capacity of the ferroperoxidase iron, the electronic localization of the ferroperoxidase–O_2 complex must be slightly different from that of myoglobin and haemoglobin complexes with O_2 and it can be easily split into ferriperoxidase and perhydroxyl radical rather than into ferroperoxidase and O_2. This might be the reason why the ferroperoxidase–CO complex has not been observed after gassing CO into the solution of Compound III formed in the presence of a high level of H_2O_2, (CHANCE[28] and by us (unpublished observation)). (b) In spite of its ferroperoxidase–O_2 type of structure, Compound III is not an active intermediate at least for DHF oxidation. For, as

shown in Fig. 7, the oxygen-consuming oxidation of DHF scarcely occurs during the formation of Compound III and suddenly appears after the concentration of Compound III reaches its maximum. The oxygen in the Compound III may become an effective oxidant after it takes one electron from ferroperoxidase and is reduced to the perhydroxyl radical. The sluggish H_2O_2 destruction by peroxidase which was reported by MORITA AND KAMEDA[29] can be also explained by the mechanism shown in Fig. 11.

The activating effect of cyanide at low concentration seems to be related to the abnormal dependency of the rate of reaction upon the peroxidase concentration and it is suggested that rapid reversibility of combination of ferriperoxidase with cyanide keeps the active ferri-enzyme at a suitable concentration for the constant production of the free radical of DHF, and prevents the reaction of the HO_2 radical with ferri-peroxidase.

Although the oxygen-consuming oxidation of triose reductone, DHF and IAA by peroxidase is likely to happen according to the same mechanism as shown in Fig. 9, the features of the reaction are not always the same for each substrate. These are summarized in Table III. When Reaction a is slow as in the case of DHF and IAA, the addition of the oxidogenic group (XH_2) enhances the overall oxidase reaction by raising the concentration of the YH free radical through the mechanism

TABLE III

REACTION TYPES OF OXIDATION OF DHF, IAA AND TRIOSE REDUCTONE

	DHF	*IAA*	*Triose reductone*
Reaction a in Fig. 9	slow	slow	fast
Activity of free radicals			
O_2^- reduction (Reaction b)	fast	fast (?)	slow
Reduction of peroxidase	fast	very fast	slow
Activation by			
Mn^{2+}	+++++	++	+
Oxidogenic substrates	++	++	−
Main termination of the chain-reaction, in Fig. 9	Reaction e	Reaction e	Reaction d

shown in Fig. 9. When Reaction b is fast, the main termination of the chain reaction is Reaction e and Mn^{2+} will accelerate the overall reaction through the mechanism shown in Fig. 10. Oxidation of IAA shows a particular feature compared with that of DHF because of the strong activity of the free radical of IAA which inactivates peroxidase in the absence of a suitable hydrogen-acceptor. In the case of triose reductone oxidation, activation by oxidogenic group and Mn^{2+} is not significant since Reaction a is very fast and Reaction b is slow. Here the main termination of the chain-reaction is Reaction d.

Irregularity of the effect of catalase on the peroxidase-oxidase reaction might be due also to the complication of the chain mechanism as shown in Fig. 9. It is true that catalase is invariably a strong inhibitor when it is incubated from the

time at which the reaction commences and sometimes the reaction occurs after a long lag phase. This is explained by the fact that catalase has a strong affinity towards H_2O_2 tending to form Compound I (see ref. 30) but it may not be so effective on the decomposition of H_2O_2 at extremely low concentrations of H_2O_2. Furthermore the affinity of HRP toward H_2O_2 is also strong enough to compete with the decomposition of H_2O_2 by catalase.

Now, it is worthwhile to summarize the assumed features of the HO_2 radical. (1) It has a strong affinity for free HRP. The resultant compound has the same absorption spectrum and is in the same oxidation level as that formed in the presence of an excess amount of H_2O_2 alone ($Per^{4+} + H_2O_2$). (2) It effectively oxidizes Mn^{2+} to Mn^{3+}, Reaction g. So Mn^{2+} inhibits the formation of Compound III and hydroxylation (see 5). (3) Its dominant decay is by dismutation in the absence of excess HRP or Mn^{2+}, Reaction e. (4) It oxidizes DHF, IAA and triose-reductone to produce their free radicals, Reaction c. (5) It forms oxygenated compounds with certain aromatic substances.

$$HO_2\cdot + RH \rightarrow RO\cdot + H_2O$$

This is our postulated mechanism for the hydroxylating activity of peroxidase which was found by MASON *et al.*[31,33]. (6) The redox potential of $O_2/HO_2\cdot$ must be between 0 and -0.3 V.

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