

BBA 65229

## REACTION OF PEROXIDASE WITH REDUCED NICOTINAMIDE-ADENINE DINUCLEOTIDE AND REDUCED NICOTINAMIDE-ADENINE DINUCLEOTIDE PHOSPHATE

K. YOKOTA AND I. YAMAZAKI

*Biophysics Division, Research Institute of Applied Electricity, Hokkaido University, Sapporo (Japan)*

(Received February 3rd, 1965)

---

SUMMARY

1. Peroxidase (donor: $\text{H}_2\text{O}_2$  oxidoreductase, EC 1.11.1.7) catalyzes the aerobic oxidation of NADH and NADPH at acidic pH in the absence of cofactors such as  $\text{Mn}^{2+}$  and monophenols.

2. Peroxidase Compound III appears during the reaction and disappears when almost all the oxygen in the reaction solution has been consumed.

3. A part of peroxidase is rapidly reduced with consumption of  $\text{H}_2\text{O}_2$  or  $\text{O}_2$  in the presence of NADH and NADPH.

4. It is suggested that the free radicals derived from NADH and NADPH are active intermediates in the formation of Compound III and the reduction of peroxidase.

5. The different types of peroxidase-oxidase reaction possible when dihydroxyfumarate, triose reductone, indole acetate and NADH are used as hydrogen donors are discussed.

---

## INTRODUCTION

The aerobic oxidation of reduced nicotinamide nucleotides has been found to be catalyzed by peroxidases of horse radish<sup>1-4</sup> and of uterus<sup>5,6</sup> in the presence of  $\text{Mn}^{2+}$  and certain phenols. It was suggested<sup>1,6</sup> that a free radical mechanism might be involved in the NADH oxidation catalyzed by peroxidase but the relation between peroxidase (donor: $\text{H}_2\text{O}_2$  oxidoreductase, EC 1.11.1.7) activity and oxygen consumption has not been explained.

Since SWEDIN AND THEORELL<sup>7</sup> found that horse-radish peroxidase catalyzes the aerobic oxidation of dihydroxyfumaric acid, the mechanism of peroxidase-oxidase reaction has been investigated by many workers<sup>8</sup>. We have proposed a free radical mechanism for this reaction<sup>9-11</sup> and suggested that peroxidase catalyzes the formation of free radicals from the hydrogen donor and these radicals are then capable of reduc-

ing molecular oxygen. The present study shows that such a mechanism is also involved in the aerobic oxidation of NADH and NADPH catalyzed by peroxidase.

#### EXPERIMENTAL

Horse-radish peroxidase was purified and crystallized by the method of KENTEN AND MANN<sup>12</sup>. The enzyme was then passed through a DEAE-cellulose column at pH 7.0. The ratio  $A_{403}:A_{278}$  of the horse-radish peroxidase used was 3.16. The molar concentration of horse-radish peroxidase was calculated by assuming that the value,  $A_{403}$  is  $107.7 \text{ mM}^{-1} \text{ cm}^{-1}$  (ref. 13). NADH and NADPH were obtained from Boehringer (Germany).

A Hitachi recording spectrophotometer was used and reactions were carried out at  $25^\circ$  throughout this experiment. Anaerobic conditions were obtained by gassing the cuvettes 10 min with nitrogen which was deoxygenated by passage first through alkaline solution of pyrogallol and then over heated copper.

#### RESULTS

It has been reported<sup>1,3,5</sup> that the aerobic oxidation of NADH and NADPH at neutral pH is catalyzed by peroxidases only in the presence of both  $\text{Mn}^{2+}$  and certain phenols. At acidic pH, however, the peroxidase-catalyzed oxidation of NADH oc-

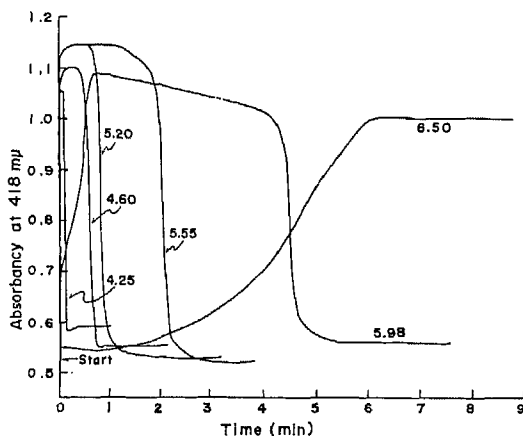


Fig. 1. Changes in the absorbance at  $418 \text{ m}\mu$  as a function of time at different pH values (shown in the figure). The rise in the absorbance at  $418 \text{ m}\mu$  is due to the formation of Compound III (see Fig. 2).  $1 \text{ mM}$  NADH,  $9.3 \text{ }\mu\text{M}$  horse-radish peroxidase,  $0.1 \text{ M}$  acetate or phosphate. The initial oxygen concentration corresponded to the air-saturated solution.

curred at a fairly high speed without the addition of any cofactors. At high concentrations of horse-radish peroxidase most of the enzyme was found in the form of Compound III during the reaction. When almost all of the oxygen in the solution had been consumed Compound III suddenly disappeared and the enzyme was then found to consist of a mixture of the ferric and ferrous forms. The mode of reaction greatly depended on the pH of the reaction solution as shown in Fig. 1. By scanning

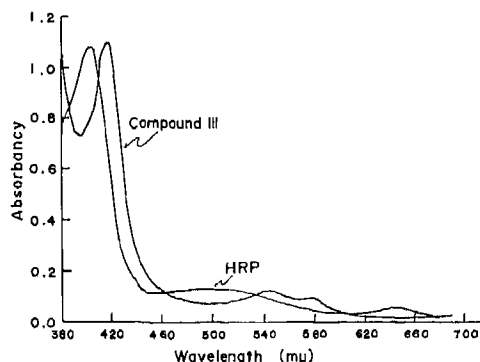


Fig. 2. Absorption spectrum of the intermediate of peroxidase formed during the oxidation of NADH. 1 mM NADH, 10  $\mu$ M horse-radish peroxidase (HRP) and 0.1 M acetate (pH 5.2).

the absorption spectrum over the visible range (Fig. 2) the increase in light absorption at 418 m $\mu$  was identified to be due to formation of Compound III. The velocity of NADH oxidation or O<sub>2</sub> consumption can be roughly estimated by measurement of the time required for the concentration of Compound III to fall to half its maximal value. But the kinetics of formation of Compound III do not seem to be similar to that of formation of an active intermediate described by CHANCE<sup>14</sup>. Fig. 3 shows the dependency of formation of Compound III upon the concentration of NADH to be irregular. Further, the relationship between the concentration of NADH and the rate of O<sub>2</sub> consumption was found to be non-linear. Fig. 3 shows that the increase in the concentration of NADH from 1 mM to 1.5 mM increased the rate of O<sub>2</sub> consumption by almost a factor 4. In the presence of 0.46 mM NADH the oxidation started after a short lag time and then continued at a fairly high speed until almost all of the NADH

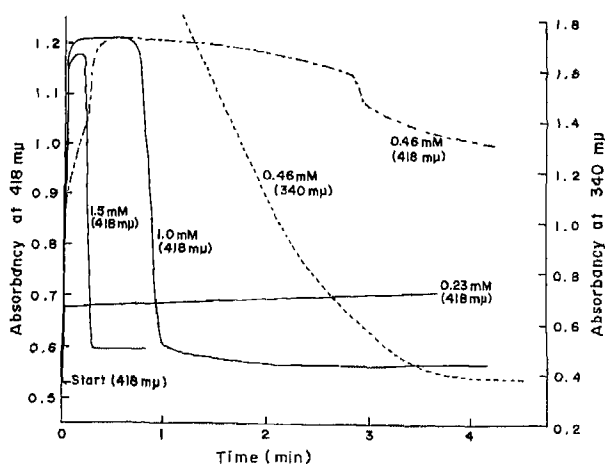


Fig. 3. Effect of NADH concentration (shown in the figure) on the formation and the decay of Compound III. 10  $\mu$ M horse-radish peroxidase and 0.1 M acetate (pH 5.2). The oxidation of NADH was measured at 340 m $\mu$  in the case of 0.46 mM NADH. The absorbancy of 10  $\mu$ M horse-radish peroxidase at 340 m $\mu$  was 0.3. The initial oxygen concentration corresponded to the air-saturated solution.

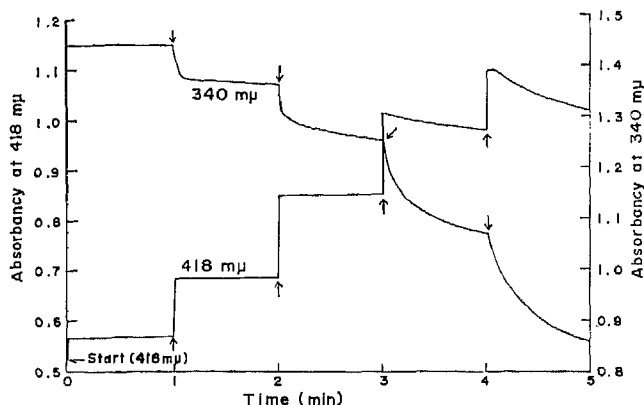


Fig. 4. Requirement of  $\text{H}_2\text{O}_2$  for the formation of Compound III and the oxidation of NADH at low concentrations of NADH. Each arrow indicates the addition of 0.04 ml of  $\text{H}_2\text{O}_2$  (final concentration,  $4 \mu\text{M}$ ). 0.2 mM NADH,  $10 \mu\text{M}$  horse-radish peroxidase and 0.1 M acetate (pH 5.2). The oxidation of NADH was measured at  $340 \text{ m}\mu$ . Air-saturated conditions.

was oxidized. This observation is apparently inconsistent with the fact that no appreciable oxidation of NADH was observed in the presence of 0.2 mM NADH (Fig. 4). However, when a small amount of  $\text{H}_2\text{O}_2$  corresponding to a final concentration of  $4 \mu\text{M}$  was added to the solution a part of the enzyme changed instantaneously into Compound III with the concomitant oxidation of NADH. Successive such identical additions of  $\text{H}_2\text{O}_2$  clearly showed that the amount of  $\text{O}_2$  consumed during the peroxide-induced oxidation of NADH increased with increasing concentrations of Compound III.

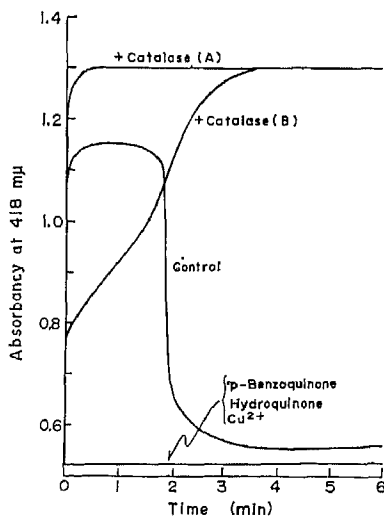


Fig. 5. Effect of  $\text{Cu}^{2+}$  (0.1 mM), catalase ( $1.2 \mu\text{M}$ ), hydroquinone (0.1 mM) and *p*-benzoquinone (0.1 mM) on the formation and the decay of Compound III at pH 5.2. The reaction was started by the addition of 0.04 ml NADH solution (final concentration, 1 mM). Catalase had been incubated with peroxidase solution (A) and with NADH solution (B) before the reaction started. The increase in absorbance due to the addition of catalase was 0.22.

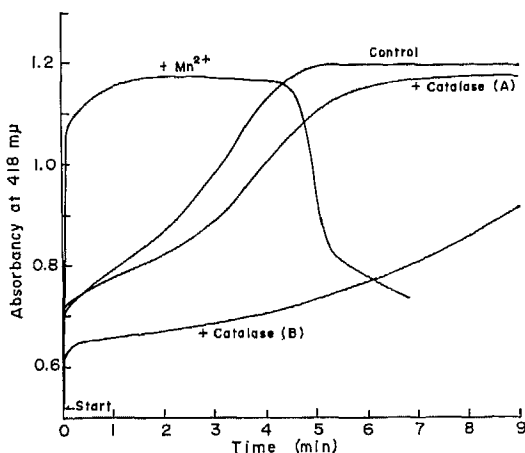


Fig. 6. Effect of  $Mn^{2+}$  ( $0.1 \text{ mM}$ ) and catalase ( $0.24 \mu\text{M}$ ) on the formation and the decay of Compound III at pH 6.4. The reaction was started by the addition of  $0.04 \text{ ml}$  NADH solution (final concentration  $1 \text{ mM}$ ). Catalase was preincubated with peroxidase solution (A) and with NADH solution (B) before the reaction was initiated. The increase in absorbancy due to catalase was  $0.044$ .

The requirement of  $H_2O_2$  for the formation of Compound III suggests that a peroxidase cycle is involved in the reaction as reported in the case of the dihydroxy-fumaric acid- $O_2$ -horse-radish peroxidase system<sup>11</sup>. When  $1 \text{ mM}$  NADH was added at pH 5.2, an instantaneous transformation of horse-radish peroxidase to Compound III was observed without the previous addition of  $H_2O_2$ . Hydroquinone, *p*-benzoquinone and  $Cu^{2+}$  strongly inhibited the formation of Compound III (Fig. 5). Catalase (EC 1.11.1.6) was found to inhibit the  $O_2$  consumption but affected the formation of Compound III only to a small extent. However, this effect could be greatly increased by preincubation of the NADH solution with catalase. As can be seen from Fig. 6, the formation of Compound III in time at pH 6.4 may be divided into two distinctly

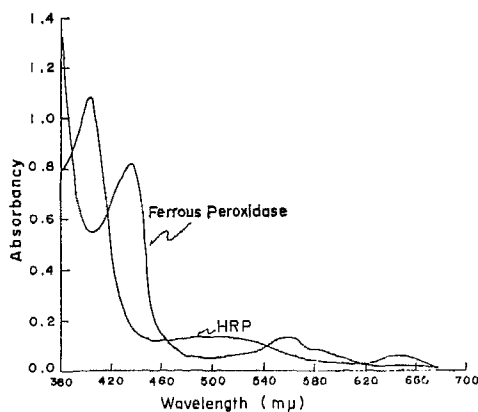


Fig. 7. Absorption spectrum of reduced peroxidase in the presence of NADH. Absorption spectrum was obtained from Expt. A in Fig. 9 after the second addition of  $H_2O_2$ . HRP, horse-radish peroxidase.

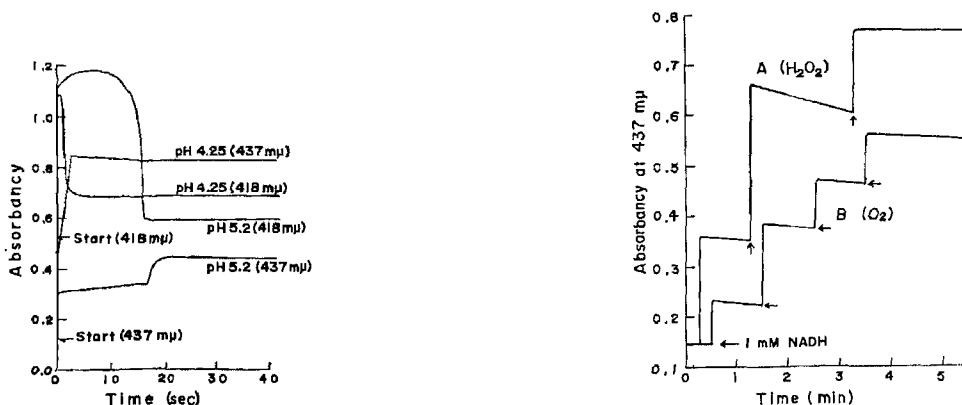


Fig. 8. Time course of the disappearance of Compound III (418  $m\mu$ ) and the formation of ferrous peroxidase (437  $m\mu$ ) at pH 5.2 and 4.25. 1.5 mM NADH, 10  $\mu$ M horse-radish peroxidase and 0.1 M acetate. The initial oxygen concentration corresponded to the air-saturated solution.

Fig. 9. Reduction of peroxidase induced by  $H_2O_2$  or  $O_2$ . Each arrow indicates the addition of 0.04 ml  $H_2O_2$  (A) or 0.1 ml water saturated with air (B). The final concentrations were 10  $\mu$ M  $H_2O_2$  and 8.3  $\mu$ M  $O_2$ , respectively. 1 mM NADH, 10  $\mu$ M horse-radish peroxidase and 0.1 M acetate (pH 4.25). Anaerobic conditions. The difference in the initial rise observed when 1 mM NADH was added depended on ageing of the NADH solution.

separated phases. Firstly, a small but instantaneous formation and secondly, a slow but complete formation. The former rapid reaction was found to depend on ageing of the NADH solution and was negligible when NADH was preincubated with catalase. The formation of Compound III could still be observed when catalase was added separately.

The participation of reduced peroxidase in the aerobic oxidation catalyzed by peroxidase has been discussed by many workers. The formation of reduced peroxidase in the presence of CO has been confirmed<sup>7,11,16-18</sup>. However, all attempts to demon-

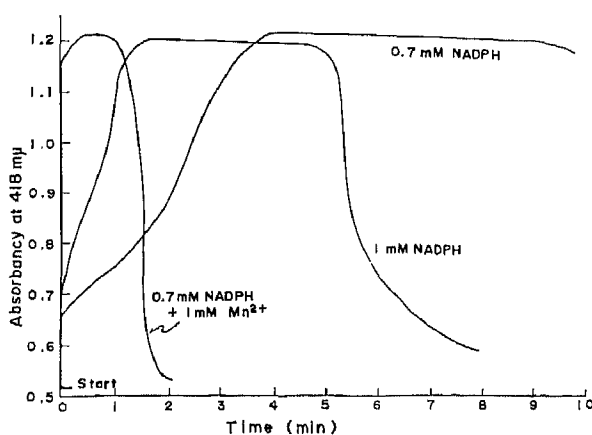


Fig. 10. Formation of Compound III in the presence of NADPH. 10  $\mu$ M horse-radish peroxidase and 0.1 M acetate (pH 5.2). The initial oxygen concentration corresponded to the air-saturated solution.

strate formation of ferrous peroxidase in the absence of this compound have so far proved unsuccessful. As shown in Fig. 8, at acidic pH a rapid reduction of peroxidase occurred in the NADH-horse-radish peroxidase system immediately after all  $O_2$  had been consumed and Compound III disappeared. Under the anaerobic conditions Compound III completely disappeared and the horse-radish peroxidase was found to consist of a mixture of the ferric and ferrous forms. The ratio between the ferrous and ferric forms of the enzyme mainly depended on the pH and on the concentration of NADH. In spite of the rapid appearance of reduced peroxidase after the  $O_2$  had been consumed, no reaction between peroxidase and NADH could be detected immediately upon mixing of the components (Fig. 9). The reduction had to be induced by the addition of a small amount of  $O_2$  or  $H_2O_2$ .

Formation of Compound III and ferrous peroxidase was also observed when NADPH was substituted for NADH in the system described above (Fig. 10).

## DISCUSSION

The mechanism of the aerobic oxidation of NADH catalyzed by peroxidases has previously been discussed on basis of the reaction system kept at neutral pH when  $Mn^{2+}$  and certain phenols are required as cofactors<sup>1,3,6</sup>. In the present paper, however, the simple reaction system of acidic pH in which horse-radish peroxidase catalyzes the aerobic oxidation of NADH without the addition of such cofactors will mainly be discussed. The characteristic feature of the reaction under these conditions seems to be the remarkable accumulation of Compound III during the reaction and the sudden transformation from Compound III to ferric and ferrous forms of the enzyme which occurs upon disappearance of the  $O_2$  even in the absence of CO.

We have already presented the mechanism for the peroxidase-oxidase reaction

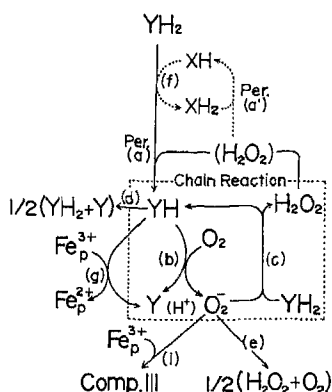


Fig. 11. Tentative scheme for the oxygen-consuming oxidation of  $YH_2$  (dihydroxyfumaric acid, triose reductone, indoleacetic acid and NADH) catalyzed by peroxidase. The reaction is composed of an enzymic formation of free radical (Reaction a) and a chain reaction (Reactions b and c). Chain termination may occur by Reactions d and e. The relative importance of Reactions g and i to the chain reaction cycle depends on the activity of Compound III toward  $YH_2$  (see Fig. 13).  $Mn^{2+}$  promotes Reaction c (ref. 11) and certain monophenols promote the enzymic formation of free radicals by Reactions a' and f.

in the presence of dihydroxyfumaric acid, triose reductone and indoleacetic acid<sup>11,19</sup>. The reaction mechanism has been slightly revised and is shown in Figs. 11 and 12. Although the mechanism seems to be essentially the same, the individual features of the reaction are not always identical for each of the substrates mentioned above. Differences may be encountered in the following six elementary steps. (1) Reaction of  $\text{YH}_2$  with peroxidase- $\text{H}_2\text{O}_2$  compound resulting in the enzymic formation of YH radicals (Reaction a). (2) Reduction of  $\text{O}_2$  to perhydroxyl radicals by YH radicals

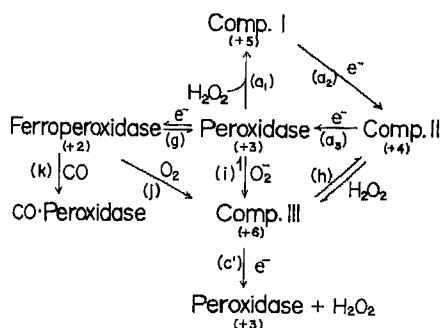


Fig. 12. Tentative scheme for the relationship between peroxidase derivatives which appear during the peroxidase-oxidase reaction. Numbers in parentheses show the effective oxidation level of the iron of peroxidase derivatives. A complete peroxidase cycle is composed of Reactions  $a_1$ ,  $a_2$  and  $a_3$ . Biological reagents can reduce peroxidase only in their semiquinone forms.

(Reaction b). (3) One-equivalent oxidation of  $\text{YH}_2$  by perhydroxyl radicals (Reaction c). (4) Dismutation of YH radicals (Reaction d). (5) Reduction of peroxidase by YH radicals (Reaction g). (6) Reaction of  $\text{YH}_2$  with Compound III (Reaction c'). Compound III decomposes to free enzyme in the presence of certain hydrogen donors<sup>19,20</sup>. Dissimilarities exhibited by the four hydrogen donors in these elementary reaction steps will bring about differences in the individual features of the overall reaction, such as the accumulation of Compound III, the reduction of the enzyme and the requirement of cofactors. In Table I reaction constants or relative reaction velocities for the six elementary steps as well as the relative degree of accumulation of Compound III and the requirement of cofactors are given for each of the four redogenic<sup>9</sup> substrates mentioned.

#### Accumulation of Compound III

There are two possible paths of formation of Compound III during the peroxidase-oxidase reaction<sup>11</sup>. Firstly, peroxidase is reduced by an YH radical (Reaction g) and then combines with  $\text{O}_2$  and secondly,  $\text{O}_2$  receives one electron from an YH radical (Reaction b) and reacts with ferric peroxidase (Reaction i). The latter process is dominant in the dihydroxyfumaric acid- $\text{O}_2$ -horse-radish peroxidase system because Reaction g is slow compared with the rapid formation of Compound III (ref. 11). On the other hand the former path may contribute to some extent in the NADH- $\text{O}_2$ -horse-radish peroxidase system because Reaction g is rather fast in this case as can be seen from Figs. 8 and 9. It should be noticed that Compound III accumulates during the oxidation of  $\text{YH}_2$  only if  $\text{YH}_2$  is a poor substrate for Compound III. As shown in



TABLE I

REACTION TYPES OF OXIDATION OF DIHYDROXYFUMARIC ACID, TRIOSE REDUCTONE, INDOLEACETIC ACID AND NADH

See Figs. 11, 12 and 13. Rate-limiting step in Reaction a is the reaction of  $\text{YH}_2$  with Compound II. Approximate values are given for the second-order rate constant. The values for dihydroxyfumaric acid and triose reductone are quoted from CHANCE's results. The number of plus signs indicates the relative velocity of the reaction for each hydrogen donor.

Hydrogen donor	Dihydroxy-fumaric acid	Triose reductone	Indoleacetic acid	NADH	
pH of reaction	Acidic	Acidic	Acidic	Acidic	Neutral
Reaction a, $\text{M}^{-1}, \text{sec}^{-1}$ (Compound II + $\text{YH}_2$ )	$10^4$	$10^8$	$2.0 \cdot 10^4$	$10^4$	$10^8$
Reaction b ( $\text{YH} + \text{O}_2$ )	+++++	+	+++	+++	?
Reaction c ( $\text{YH}_2 + \text{O}_3^-$ )	+	+++++	++	+++++	±
Reaction d (dismutation of $\text{YH}$ )	+++	++	+++++	+++++	?
Reaction c', $\text{M}^{-1}, \text{sec}^{-1}$ (Ref. 20)	<10	$3.4 \cdot 10^8$	$2.2 \cdot 10^8$	$10^2$	<10
(Compound III + $\text{YH}_2$ )					
Reaction g (reduction of horse-radish peroxidase)	++	+	++++	+++++	?
Accumulation of Compound III	+++++	—	—	++++	+++*
Activation by $\text{Mn}^{2+}$	+++++	+	++	++	+++++
Activation by phenols	++	±	++	++	+++++

\* In the presence of  $\text{Mn}^{2+}$ .

Table I, dihydroxyfumaric acid is almost inactive toward Compound III but indoleacetic acid and triose reductone will rapidly decompose this compound. NADH reacts with Compound III at a moderate speed at acidic pH but almost not at all at neutral pH.

#### Catalase effect

In order that the reaction in which  $\text{H}_2\text{O}_2$  participates as an active intermediate is to be inhibited by catalase, there must be a competition between the decomposition of  $\text{H}_2\text{O}_2$  and peroxidatic formation of the  $\text{YH}$  radical (Reaction a) for  $\text{H}_2\text{O}_2$ . As the affinity of horse-radish peroxidase for  $\text{H}_2\text{O}_2$  is high the amount of catalase to inhibit the reaction has to be large. The instantaneous formation of Compound III when NADH is added may be due to a trace amount of  $\text{H}_2\text{O}_2$  accumulated during the ageing of the NADH solution. The initial rapid formation of Compound III observed in the control experiment of Fig. 6 may be eliminated if the NADH solution is preincubated with catalase, whereas no effect is observed upon pretreatment of the peroxidase.

#### Reduction of peroxidase

This effect seems to be very remarkable since for the first time the reduction of peroxidase has been found to be caused by biological substances in the absence of

CO. Further, NADH and NADPH are evidently capable of reducing the heme protein at a high rate in the absence of specific dehydrogenase. The oxidation-reduction potential of horse-radish peroxidase has been reported by HARBURY<sup>21</sup> to be  $-0.27$  V at pH 7 and  $-0.10$  V at pH 4. As judged from these values the potentials of NADH and NADPH are, thus, low enough to permit a reduction of the peroxidase. From Fig. 9 it is, however, evident that horse-radish peroxidase is not reduced by direct reaction with the reduced nicotinamide nucleotide. Ferrous peroxidase can be observed in  $O_2$ -free solutions but a small amount of  $O_2$  is necessary to cause the reduction of the enzyme.  $H_2O_2$  can effectively replace it. From these results it appears that the operation of a peroxidase cycle (Reaction a) is essential for the reduction of the enzyme and the direct reductant is a semi-oxidized form of NADH or NADPH.

The conversion from ferrous peroxidase to Compound III by the introduction of  $O_2$  has been confirmed in further experiments and the details will be reported elsewhere<sup>20</sup>.

#### *Mn<sup>2+</sup> and phenols*

The requirement of cofactors at neutral pH can be explained as follows. In the presence of NADH Reaction a is slow at neutral pH and the addition of an oxidogenic donor<sup>9</sup> will promote the formation of the YH radical by Reaction a' in Fig. 11. The perhydroxyl radical exists in its dissociated form ( $O_2^-$ ) above pH 4.0 and at neutral pH NADH occurs as a negative ion. The reaction between the two anionic molecules is found to be slow because of the electrostatic repulsion, and  $Mn^{2+}$  may promote the electron transfer (Reaction c) by acting as a bridge between the anions. The mechanism for such bridged electron transfer has been discussed in detail by GEORGE AND GRIFFITH<sup>22</sup>. The chain reaction shown in Fig. 11 is a set of non-enzymic reactions which operates effectively at acidic pH but not at neutral pH. At neutral pH the chain reaction of the NADH system can operate only in the presence of  $Mn^{2+}$  (ref. 19), which is an effective accelerator for the formation of Compound III. This is, however, inconsistent with the observation that Compound III accumulates in the dihydroxyfumaric acid-horse-radish peroxidase- $O_2$  system whereas only Compound II accumulates in the dihydroxyfumaric acid-horse-radish peroxidase- $O_2$ - $Mn^{2+}$  system<sup>16</sup>. This discrepancy may be explained as follows. In the dihydroxyfumaric acid-horse-radish peroxidase- $O_2$ - $Mn^{2+}$  system the chain reaction-cycle described in Fig. 11 will operate effectively so that the formation of Compound III becomes negligible. Furthermore a stoichiometric accumulation of  $H_2O_2$  occurs, which results in the conversion of the enzyme to Compound II. On the other hand, as  $O_2^-$  ions are required for the formation of Compound III there will, clearly, be no accumulation of Compound III without propagation of the chain reaction. It can be said that the chain reaction operates in such a way that Compound III is accumulated only to a moderate degree in the NADH-horse-radish peroxidase- $O_2$ - $Mn^{2+}$  system.

#### *Participation of ferrous peroxidase and Compound III*

Consideration may now be given to the problem of the participation of ferrous peroxidase in the peroxidase-oxidase reaction. In Fig. 11 the reduction of peroxidase is shown to be initiated by the formation of the free radical, YH, which then either starts the chain reaction by reaction with  $O_2$  or, alternatively acts as a reducing agent by reaction with ferric peroxidase. It seems likely that the non-enzymic chain reaction

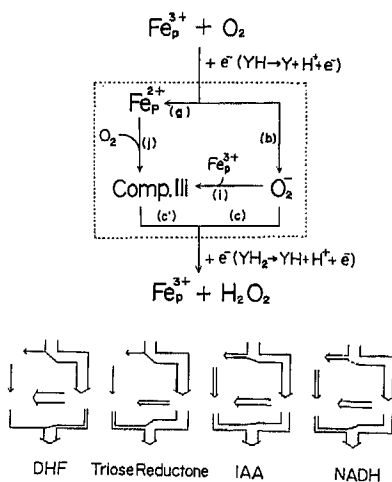
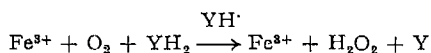


Fig. 13. Mixed mechanism of peroxidase-oxidase reaction. The participation of Compound III is shown in the four diagrammatical figures for each substrate. This participation is negligible small in the oxidation of dehydroxyfumaric acid (DHF) and triose reductone and less than 10% even in the case of indoleacetic acid (IAA). Addition of  $\text{Mn}^{2+}$  promotes Reaction c and decreases the degree of participation of Compound III.

accounts for the main part of the  $\text{O}_2$  uptake observed in the peroxidase-oxidase reaction, but the possibility can not be ruled out that ferrous peroxidase participates in the reaction to some extent. Now, Reactions b and c can be modified as shown in Fig. 13. Although it is not easy to reach a firm conclusion it is likely that the first step in the decomposition of Compound III involves a one-equivalent reduction<sup>20</sup>. Independent of reaction path the overall reaction schematized in Fig. 13 will be formulated as follows,



The results presented in Table I together with other observations suggest that the degree of participation of ferrous peroxidase in the reaction is greater for NADH and indoleacetic acid than what is the case with triose reductone and dihydroxyfumaric acid. Even in the case of indoleacetic acid the contribution of the Compound III path to the reaction of  $\text{O}_2$  consumption seems to be less than 10% (ref. 23). In the NADH system the ferrous peroxidase path might be involved in the reaction with  $\text{O}_2$  to some extent at acidic pH but is almost of no importance at neutral pH in the presence of  $\text{Mn}^{2+}$ .

LEMBERG AND LEGGE<sup>24</sup> and MASON<sup>25</sup> have postulated a sequence of reactions in which peroxidase remains ferrous after the initial activation. Mason suggested the ferrous peroxidase- $\text{O}_2$  structure for Compound III, which has recently been supported by our experimental evidence<sup>11,26</sup>. Compound III, however, is not a typical active intermediate. In the presence of an excess of peroxidase the aerobic oxidation of NADH does occur only when most of the enzyme has been converted into Compound III. Compound III, thus, seems to be a kind of regulator of free radical reaction in biological system. The details of the mechanism will be discussed elsewhere.

## ACKNOWLEDGEMENTS

We wish to thank Dr. R. NILSSON for his helpful comments. This investigation has been supported by research grants from the National Institute of Health (AM 06518) and the Scientific Research Fund of Ministry of Education of Japan.

## REFERENCES

- 1 T. AKAZAWA AND E. E. CONN, *J. Biol. Chem.*, **232** (1958) 403.
- 2 H. G. WILLIAMS-ASHMAN, M. CASSMAN AND M. KLAVINS, *Nature*, **184** (1959) 427.
- 3 S. J. KLEBANOFF, *J. Biol. Chem.*, **234** (1959) 2480.
- 4 M. H. KLAPPER AND D. P. HACKETT, *J. Biol. Chem.*, **238** (1963) 3736.
- 5 V. P. HOLLANDER AND M. L. STEPHENS, *J. Biol. Chem.*, **234** (1959) 1901.
- 6 J. BEARD AND V. P. HOLLANDER, *Arch. Biochem. Biophys.*, **96** (1962) 592.
- 7 B. SWEDIN AND H. THEORELL, *Nature*, **145** (1940) 71.
- 8 P. NICHOLLS, in O. HAYAISHI, *Oxygenases*, Academic Press, New York and London, 1962, p. 273.
- 9 I. YAMAZAKI, *Proc. Intern. Symp. Enzyme Chem. Tokyo Kyoto, 1957*, p. 224.
- 10 I. YAMAZAKI AND H. SOUZU, *Arch. Biochem. Biophys.*, **86** (1960) 294.
- 11 I. YAMAZAKI AND L. H. PIETTE, *Biochim. Biophys. Acta*, **77** (1963) 47.
- 12 R. H. KENTEN AND P. J. G. MANN, *Biochem. J.*, **57** (1954) 347.
- 13 K. G. PAUL, *Acta Chem. Scand.*, **12** (1958) 1312.
- 14 B. CHANCE, *Arch. Biochem. Biophys.*, **71** (1957) 130.
- 15 B. CHANCE, *J. Biol. Chem.*, **197** (1952) 577.
- 16 P. M. RAY, *Arch. Biochem. Biophys.*, **87** (1960) 19.
- 17 Y. MORITA AND K. KAMEDA, *Mem. Res. Inst. Food Sci., Kyoto Univ.*, **23** (1961) 1.
- 18 M. H. KLAPPER AND D. P. HACKETT, *J. Biol. Chem.*, **238** (1963) 3743.
- 19 I. YAMAZAKI, K. YOKOTA AND R. NAKAJIMA, in T. E. KING, H. S. MASON AND M. MORRISON, *Oxidases and Related Redox Systems*, John Wiley, New York City, in the press.
- 20 K. YOKOTA AND I. YAMAZAKI, *Biochem. Biophys. Res. Commun.*, **18** (1965) 48.
- 21 H. A. HARBURY, *J. Biol. Chem.*, **225** (1957) 1009.
- 22 P. GEORGE AND J. S. GRIFFITH, in P. D. BOYER, H. LARDY AND K. MYRBÄCK, *The Enzymes*, Academic Press, New York, 1959, p. 347.
- 23 R. NAKAJIMA AND I. YAMAZAKI, unpublished observation.
- 24 R. LEMBERG AND J. W. LEGGE, *Hematin Compounds and Bile Pigments*, Interscience, New York, 1949, p. 419.
- 25 H. S. MASON, *Proc. Intern. Symp. Enzyme Chem., Tokyo Kyoto, 1957*, p. 220.
- 26 I. YAMAZAKI AND K. YOKOTA, *Biochem. Biophys. Res. Commun.*, **19** (1965) 249.

*Biochim. Biophys. Acta*, **105** (1965) 301-312