CONVERSION OF FERROUS PEROXIDASE INTO COMPOUND III IN THE PRESENCE OF NADH.

I. Yamazaki and K. Yokota

Biophysics Division, Research Institute of Applied Electricity

Hokkaido University, Sapporo, Japan

Received February 19, 1965

The mechanism of enzymic activation of oxygen has engaged the attention of many investigators (Mason, 1957 a). Ferrous iron has been thought to be essential to the activity of oxidases containing iron as an active site. For example, in the case of heme enzymes Mason (1957 b) has suggested that they may exist in functionally active ferrous forms which have, like hemoglobin and myoglobin, the property of combining with molecular oxygen and this oxygen may be transferred to substrate, or be reduced in steps. In this respect Mason's suggestion that peroxidase Compound III has a ferroperoxidase-oxygen structure like oxymyoglobin is of great interest. However, experimental evidence which supports this idea has not been obtained.

The assumption that Compound III can be formed by reaction between ferroperoxidase and molecular oxygen has been supported (Mason, 1957 b) by (1) similarity of absorption spectra between ferroperoxidase-CO and Compound III, (2) appearance of CO-ferroperoxidase in the DHF-O_2-CO-peroxidase system, and (3) the same effective oxidation level between oxy-ferroperoxidase and Compound III (probably, Compound II + H_2O_2, George, 1953). Although we have recently presented evidence that Compound III is formed by addition of a suitable amount of dithionite to aerobic solutions of peroxidase, this reaction is sensitive to the amount of dithionite added and the method of the addition (Yamazaki et

al, 1963 ; Chance, 1965) and the reproducibility of the reaction is not so good. In this paper an evidence will be reported which shows that Compound III is formed directly from ferroperoxidase with the introduction of oxygen.

It was found (Yokota et al, 1965 b) that Compound III accumulates during the aerobic oxidation of NADH catalyzed by peroxidase, especially at acidic pH. Compound III disappears immediately after all oxygen has been consumed and then the peroxidase exists as a mixture of the ferric and ferrous forms in the presence of excess NADH. The ratio between the ferrous and ferric forms of the enzyme mainly depends on the pH (Fig. 1) and on the concentration of NADH. No reaction between peroxidase and NADH, however, can be detected immediately upon mixing of the two components under anaerobic conditions. The reduction has to be induced by the addition of a small amount of oxygen or H_2O_2. The reduction of peroxidase is, thus, demonstrated for the first time under the physio-

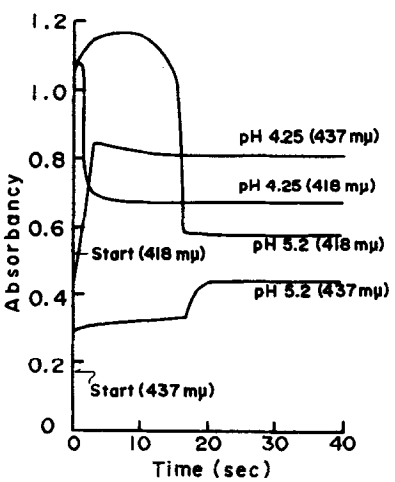

Fig. 1. Time course of the disappearance of Compound III (418 mμ) and the formation of ferroperoxidase (437 mμ) at pH 5.2 and 4.25. 1.5 mM NADH, 10 μM HRP and 0.1 M acetate. The initial oxygen concentration corresponds to air saturated solution (0.24 mM).

The steady absorbancy at 418 mμ was found to be due to the formation of Compound III by scanning the spectra over the visible range.

The peroxidase was prepared from wild horse-radish and the ratio of E_{403} to E_{278} for this enzyme was 3.16.

logical conditions without CO and this will represent a suitable experimental condition for demonstrating the conversion of ferroperoxidase to Compound III. When 1.2 mM of NADH is added to aerobic solutions of peroxidase at pH 4.1 about 88 per cent of the enzyme exists in the ferrous form after all of oxygen has been consumed and Compound III has disappeared. The introduction of oxygen to this solution rapidly converts the enzyme to Compound III again, but two problems obscure the analysis of the experimental result at this pH. First, Compound III can be also formed immediately after NADH is added to the aerobic solution of ferric peroxidase (Fig. 1) ; and second, Compound III thus formed is unstable under the experimental conditions. At neutral pH, on the other hand, Compound III accumulates very slowly when NADH is added to the aerobic solution of peroxidase and Compound III once formed is relatively stable ; half decay time being several minutes.

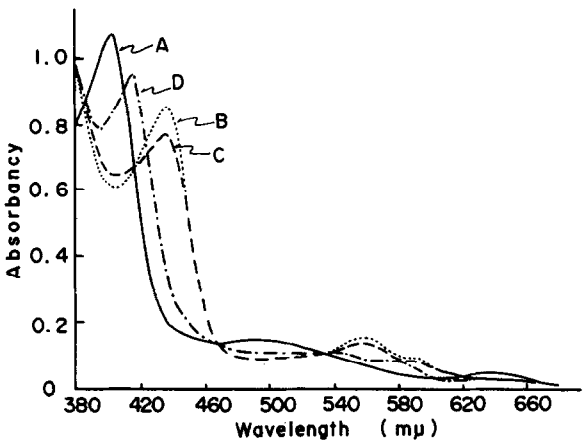

Fig. 2. The direct conversion of ferroperoxidase to Compound III by the introduction of molecular oxygen. A ; 10 μM peroxidase in 0.02 M acetate buffer (pH 4.1). B ; 0.04 ml of 0.09 M NADH was added to the aerobic solution of A (final concentration of NADH was 1.2 mM). There observed a rapid formation of Compound III, followed by reduction of the enzyme. The spectrum reached steady state within 20 seconds and was stable when nitrogen was passed on the surface of the solution. The enzyme then consisted of 85 % ferrous and 15 % ferric forms. C ; 0.045 ml of anaerobic solution containing 0.12 M Na_2HPO_4 and 1.5 M NaOH was added to the anaerobic solution of B (final pH was 7.2). At this stage the enzyme consisted of 75 % ferrous and 25 % ferric forms. D ; Instantaneous change in the spectrum was observed when oxygen was introduced to the solution of C. The enzyme then consisted of 67 % Compound III and 33 % ferric form.

An experiment is now carried out as follows (see Fig. 2). (1) 1.2 mM NADH is added to the aerobic solution of peroxidase at pH 4.1 and then one can obtain a mixture of 88 per cent ferrous and 12 per cent ferric peroxidase within about 20 seconds after the addition of NADH ; (2) the pH of the solution is then converted to neutrality by addition of mixed solution of sodium phosphate and sodium hydroxide under the anaerobic condition. Contamination by a trace amount of oxygen is inevitable under these conditions and causes a slight conversion of ferrous enzyme to the ferric form ; (3) the ferroperoxidase changes quantitatively to Compound III with the introduction of oxygen at neutral pH. The yield is almost 100 per cent (Table I). A sufficient amount of oxygen has to be added at once in this step. Otherwise, the yield of Compound III decreases. This conversion of ferroperoxidase to ferric in the presence

Table I. Stoichiometric conversion of ferroperoxidase to Compound III with the introduction of oxygen. Experimental conditions are described in Fig. 2. Final pH was adjusted by changing the amount of alkaline solution added.

per cent * of ferroperoxidase at pH 4.1 Step B in Fig. 2	Final pH	per cent of ferroperoxidase at each pH Step C in Fig. 2	per cent of Compound III Step D in Fig. 2	per cent of conversion
85	7.2	75	67	89
89	8.2	85	76	89
89	8.5	65	74	114
87	8.6	72	78	108
86	8.8	68	74	108
89	9.2	58	57	98

* The following values of milimolar extinction coefficient were used to calculate the amount of ferroperoxidase and Compound III.

$E_{437\ m\mu}$ = 20.5 for ferric and 97 for ferrous enzyme.

$E_{418\ m\mu}$ = 58 for ferric enzyme and 121 for Compound III.

The absorbancy for ferric enzyme at these wavelengths varies slightly from preparation to preparation.

of a small amount of oxygen which occurs also during step 2, can be explained by assuming that Compound III is unstable in the presence of free ferroperoxidase. The reaction between Compound III and ferroperoxidase may be as follows

$$\text{Compound III } (Fe_p^{2+} \cdot O_2) + Fe_p^{2+} + 2H^+ \longrightarrow 2\ Fe_p^{3+} + H_2O_2$$

where Fe_p^{2+} and Fe_p^{3+} are ferrous and ferric peroxidase, respectively. This seems to be the same kinetics that oxyhemoglobin and oxymyoglobin are most unstable at the oxygen concentration where a half of the hemoprotein exists in the free form (George, 1952). Although H_2O_2 induces the formation of Compound III at neutral pH its efficiency is very low. As can be seen in Fig. 3 the molar ratio of Compound III formed to H_2O_2 added is less than 0.16. So there seems to be no possibility of conversion of ferroperoxidase into Compound III through paths which involve reaction with H_2O_2. And now it is very likely that the stoichiometric conversion of ferroperoxidase to Compound III under the experimental

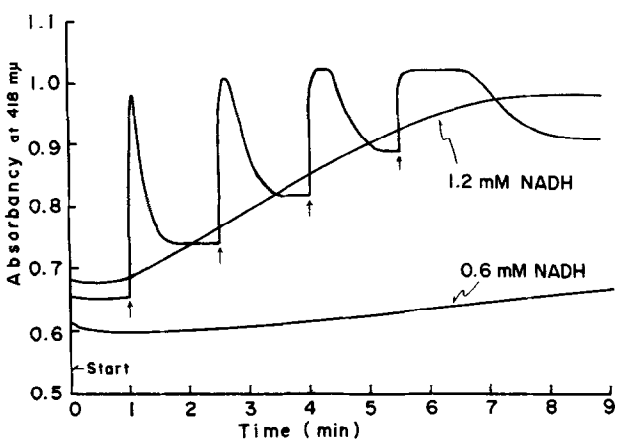

Fig. 3. Time course of the formation of Compound III and the effect of H_2O_2 on the reaction. At each arrow 0.03 ml of 1 mM H_2O_2 was added (final concentration was 10 μM) in the presence of 1.2 mM NADH. 10 μM peroxidase and 0.1 M phosphate, pH 7.2. 25°.

The initial rise in the absorbancy when NADH was added depended on ageing of the NADH solution. Sharp increases observed when H_2O_2 was added are due to the formation of Compound II. A part of the enzyme changed to Compound III after Compound II disappeared reacting with NADH.

conditions shown in Fig. 2 is due to the combination of ferroperoxidase with molecular oxygen. Molecular oxygen thus combined with ferrous enzyme is activated to some extent and reacts with certain hydrogen donors which are not capable of reacting directly with molecular oxygen (Yokota et al, 1965 a). Among these hydrogen donors indoleacetic acid has a remarkably high relative affinity for Compound III. It is of interest to note here that Compound III can also react with oxidizing agents as well as hydrogen donors. This fact suggests a hybrid structure for Compound III of ferro-oxygen and ferri-perhydroxyl complex. Free perhydroxyl radicals act as effective oxidants as well as reductants. The idea of hybrid structure is supported by the fact that ferroperoxidase-CO complex can not easily be derived from Compound III.

In spite of the effective conversion of ferroperoxidase into Compound III, the rapid accumulation of Compound III which occurs in the oxidation of NADH seems to depend mostly on the reaction of ferriperoxidase with perhydroxyl radical. And this radical is an active intermediate in the peroxidase-oxidase reaction (Yokota et al, 1965 b). The details will be reported elsewhere.

ACKNOWLEDGEMENTS

We wish to thank Dr. H. S. Mason for his helpful comments. This investigation has been supported by Research Grant AM - 06518 from the United State Public Health Service and by a research grant from the Department of Education of Japan.

REFERENCES

Chance, B., Discussion in "Oxidases and Related Redox Systems", Ed. by T. E. King, H. S. Mason and M. Morrison, John Wiley and Sons, Inc., New York City (In press).
George, P., Adv. in Catalysis IV, 367 (1952).
George, P., J. Biol. Chem., 201, 427 (1953).
Mason, H. S., Advances in Enzymology, 19, 79 (1957 a).
Mason, H. S., Proc. Intern. Symposium on Enzyme Chemistry, Tokyo and Kyoto, p 220 (1957 b).
Yamazaki, I. and Piette, L. H., Biochim. Biophys. Acta, 77, 47 (1963).
Yokota, K. and Yamazaki, I., Biochem. Biophys. Res. Comm., 18, 48 (1965 a).
Yokota, K. and Yamazaki, I., unpublished observersion (1965 b).