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MYELOPEROXIDASE OF THE LEUKOCYTE OF NORMAL BLOOD

I. REACTION OF MYELOPEROXIDASE WITH HYDROGEN PEROXIDE

TAKESHI ODAJIMA AND ISAO YAMAZAKI

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

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SUMMARY

Myeloperoxidase (donor:H_2O_2 oxidoreductase, EC 1.11.1.7; also known as verdoperoxidase, neutrophil peroxidase) prepared from normal peripheral blood leukocyte of pig reacted with hydrogen peroxide to form three compounds which correspond to Compounds I, II and III, well characterized in the reaction between hydrogen peroxide and peroxidases of horseradish and milk. The light absorption spectra of these compounds were found to be quite peculiar. Myeloperoxidase Compound III was formed during the aerobic oxidation of NADH and also from a direct reaction between ferrous myeloperoxidase and molecular oxygen. Ferrous myeloperoxidase formed a complex with CO under restricted conditions.

From the results obtained it is concluded that, in spite of the remarkable anomaly in the absorption spectra, the reaction mechanism of myeloperoxidase is similar to that of peroxidases of horseradish and milk.

INTRODUCTION

The enzyme of the leukocyte having peroxidase activity was prepared by AGNER[1] in 1941 from the pus of empyema of patients having tuberculosis and myeloid leukemia cells and was named verdoperoxidase. The enzyme was renamed myeloperoxidase by THEORELL AND AKESON[2]. Myeloperoxidase (donor:H_2O_2 oxidoreductase, EC 1.11.1.7; also known as verdoperoxidase, neutrophil peroxidase) has been purified from normal peripheral blood leukocytes[3], experimental chloroma[4], and bone marrow cells of guinea pig[5].

In 1941, AGNER[1] observed a H_2O_2 compound which had a light absorption band at 625 mμ. In 1951, CHANCE[6] reported that myeloperoxidase formed Compound I and Compound II upon the addition of H_2O_2. Recently, AGNER[7] has concluded that myeloperoxidase forms a dissociable H_2O_2 compound. These compounds, however, are not clearly identified as yet. The present paper will mainly describe the reaction products between myeloperoxidase and H_2O_2.

EXPERIMENTAL

Myeloperoxidase was prepared from leucocytes of normal blood by the method of SCHULTZ AND SHMUKLER[3] with a slight modification. 90 l of normal pig blood collected by arteries *carotis externa* puncture was mixed with 10 l of 3.8% sodium citrate solution and 25 000 units of heparin, and the mixture was allowed to stand for a couple of days. After the supernatant plasma was carefully removed, the buffy coat was collected and blended with 1 l of the plasma which had been removed. To this mixture an equal volume of 0.1 M phosphate buffer (pH 7.0) containing 1 mM of Versene was added, and the whole suspension was centrifuged for 15 min at 1000 rev./min. Washing with the same buffer was performed several times until the cells were nearly white. The cells were then suspended in 300 ml of 0.1 M phosphate buffer (pH 7.0). To each 100 ml of cell suspension 1 g of trypsin (1-300, Nutritional Biochemicals Corp) was added and digestion allowed for 4.5 h at 37°. Following digestion the solution was cooled to 2°, and ethanol precooled to −20° was added slowly until the concentration of alcohol was 50%. The suspension was centrifuged for 30 min at 17 000 rev./min. The precipitate was resuspended in a small volume of 0.1 M phosphate buffer and dialyzed for 24 h against the same buffer. Most of the myeloperoxidase was transferred to the supernatant when the dialyzed material was centrifuged for 30 min at 17 000 rev./min. The insoluble residues were saved and stored in a small volume of 0.1 M phosphate buffer. The supernatant was then chromatographed on Amberlite CG-50, previously equilibrated with 0.1 M phosphate buffer (pH 7.0). The green-colored resin was raked off the top of the column and washed into a chromatographic tube with 0.1 M phosphate buffer (pH 7.0). The myeloperoxidase was eluted with 0.7 M potassium phosphate (pH 7.0). The enzyme was again dialyzed for 24 h against 0.1 M phosphate (pH 7.0) and rechromatographed on Amberlite CG-50. The column was washed with 0.14 M potassium phosphate (pH 7.0) and then eluted with 0.7 M potassium phosphate (pH 7.0). The final enzyme preparation was ready after dialysis. All procedures were carried out below 5° except for the trypsin digestion. The enzyme used in this experiment had a ratio of $A_{430\ m\mu}/A_{280\ m\mu}$ of 0.70. The concentration of the enzyme was calculated according to AGNER[7,8], who reported that ε_{mM} at 430 mμ was 91 on the basis of iron concentration. Since EHRENBERG's[9] ESR data showed that Agner's enzyme gave a considerable absorption at $g = 4.3$ due to non-heme iron, ε_{mM} on the basis of heme iron should be higher than that value.

The recording spectrophotometers used were Hitachi instruments, Types EPS-2 and 124. The latter instrument was used for the estimation of absorbance in the expanded scale. The reaction and estimation were carried out in the sodium–potassium phosphate buffer at a solution temperature of 3° except when otherwise noted.

RESULTS

Fig. 1 demonstrates changes in the light absorption spectra of myeloperoxidase upon increases in the concentration of H_2O_2. It can be seen from the figure that a specific compound having absorption peaks at 456 and 630 mμ is formed in the reaction of myeloperoxidase with H_2O_2 of a concentration below 0.5 mM. Upon the further addition of 2.4 mM H_2O_2 to this compound, myeloperoxidase is converted into a new

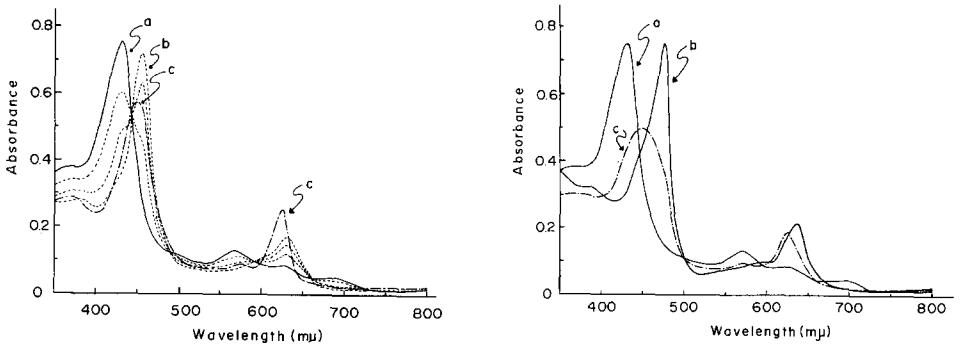


Fig. 1. Titration of myeloperoxidase with H_2O_2. Curve a; 8.2 μM myeloperoxidase in 0.05 M phosphate (pH 7.0). The dotted lines show that myeloperoxidase was converted into Compound II (Curve b) by the successive additions of H_2O_2 (0.12, 0.16 and 0.16 mM) and have isosbestic points. Curve c; 2.4 mM H_2O_2 was added to b.

Fig. 2. Formation of myeloperoxidase Compound III in the reaction between the ferrous enzyme and O_2. Curve a; 8.2 μM myeloperoxidase in 0.1 M phosphate buffer (pH 7.0). Curve b; ferrous myeloperoxidase in the presence of a slight excess of $Na_2S_2O_4$. Curve c; 0.1 ml water saturated with oxygen was added to b (2.5 ml). Curve c represents the mixture of predominantly Compound III with the ferric and ferrous enzymes.

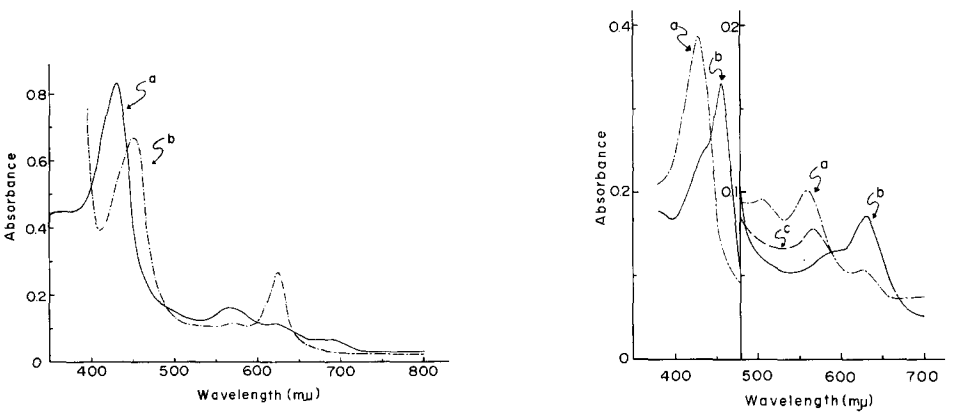


Fig. 3. Formation of myeloperoxidase Compound III from the ferric enzyme in the presence of NADH and oxygen. Curve a; 9.2 μM myeloperoxidase in 0.05 M phosphate buffer (pH 7.0). Curve b; 5 min after 4.4 mM NADH and 0.1 mM 2,4-dichlorophenol were added to a. 2,4-dichlorophenol is known to be an activator of the aerobic oxidation of NADH by peroxidase.

Fig. 4. Estimation of oxidizing capacity of myeloperoxidase Compound II with dimethyl-*p*-phenylenediamine. Compound II was prepared by adding 0.24 mM H_2O_2 to 10.2 μM myeloperoxidase in 0.05 M phosphate (pH 7.0) and was isolated from free H_2O_2 by Sephadex G-25 column chromatography. The reaction of H_2O_2 with the enzyme was carried out at 6° and the following experiments were at 3°. The enzyme (Curve b) then consisted of 3.2 μM Compound II and 1.0 μM ferric enzyme. When 0.28 mM dimethyl-*p*-phenylenediamine was added to the solution, the compound was reduced to the ferric enzyme forming 3.0 μM free radical of the added donor. Curve a represents the mixture of the ferric enzyme and the free radical. Curve c shows the spectrum of the ferric enzyme alone calculated from the Soret band of Curve a. Millimolar absorbance of dimethyl-*p*-phenylenediamine radical was 8.7 at 515 mμ and 0 around 400 mμ (ref. 12).

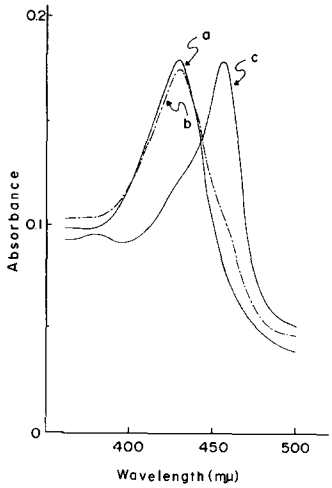
compound with absorption peaks at 452, 574 and 625.5 mμ. These two compounds are very similar to those observed by Agner. In his paper[7], however, two compounds were not clearly differentiated on a chemical basis. He concluded that a compound formed in the presence of H_2O_2 was dissociable, with a dissociation constant of 0.1 mM.

In Figs. 2 and 3, it is shown that the compound formed in the presence of a high H_2O_2 concentration will occur in quite different reaction systems. Fig. 2 demonstrates the formation of the compound in the reaction between ferrous myeloperoxidase and molecular oxygen. Myeloperoxidase is also transformed into this compound in the aerobic solution containing NADH and 2,4-dichlorophenol, as can be seen in Fig. 3. These phenomena are similar to those reported concerning the formation of Compound III of horseradish peroxidase and lactoperoxidase[10–12]. These Compounds III have been found to be at the three-equivalent oxidized level above the ferric enzyme[12]. The redox state of the myeloperoxidase compound with absorption peaks at 452, 574 and 625.5 mμ is also confirmed by the following experiments. The compound of myeloperoxidase is prepared from the NADH system (Fig. 3) and is separated from low molecular substances using a Sephadex G-25 column. Though this procedure is carried out around 3°, 41% of the compound has been converted into the ferric enzyme. An addition of excess dimethyl-*p*-phenylenediamine to the solution results in the transformation of the remaining compound into the ferric enzyme with concomitant formation of 2.5 moles of stable monodehydro compound of the added donor per mole of the compound (refer to Fig. 4). It may be concluded from the result that the compound which has absorption bands at 452, 574 and 625.5 mμ is at the three-equivalent oxidized level above the ferric enzyme and should be named myeloperoxidase Compound III.

The absorption spectrum of the compound formed in the presence of 0.5 mM H_2O_2 (Fig. 1, Curve b) is very similar to that of Agner's compound which was reported to be a dissociable one. This compound, however, is not converted into the ferric enzyme upon the addition of catalase. The compound is very stable even after excess H_2O_2 has been removed by Sephadex G-25 column chromatography. The determination of the redox state of this compound is again carried out with the use of dimethyl-*p*-phenylenediamine as a donor. From the experiment shown in Fig. 4 it is concluded that the compound is at the one-equivalent oxidized level above the ferric enzyme and should be named myeloperoxidase Compound II. The question now arises as to why the formation of myeloperoxidase Compound II needs an addition of such a excess amount of H_2O_2. In the case of horseradish peroxidase it has been well known that the stability of Compound II depends on the amount of endogenous donor which is inevitably present in the peroxidase preparation. The endogenous donor can be removed by pretreatment of the enzyme with a small amount of H_2O_2.

Fig. 5 demonstrates the formation of a new H_2O_2 compound when an equivalent mole of H_2O_2 is added to the endogenous donor-depleted myeloperoxidase. This compound is rather stable and is partially converted to Compound II by the addition of an equivalent mole of dimethyl-*p*-phenylenediamine. The compound which is formed by the addition of an equivalent mole of H_2O_2 is very likely to be Compound I, though the crucial confirmation has not yet been successful. The further addition of ten times more H_2O_2 to Compound I converts it into Compound II. This concentration of H_2O_2 is far less than those described in AGNER's paper[7] and in Fig. 1 of this paper.

It can been seen from the above results that myeloperoxidase behaves just like



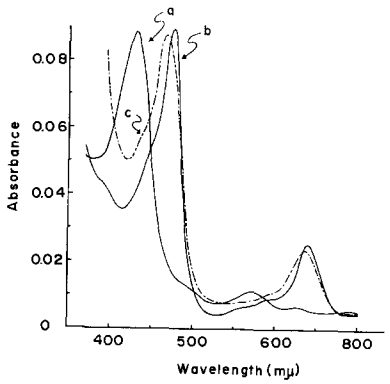


Fig. 5. Reaction of H_2O_2 with myeloperoxidase free of endogenous donor. Curve a; 2.0 μM myeloperoxidase in 0.05 M phosphate (pH 7.0). Curve b; 2.0 μM H_2O_2 was added to a. Curve c; 20 μM H_2O_2 was added to b.

Fig. 6. Formation of a complex between CO and ferrous myeloperoxidase. Curve a; 0.98 μM myeloperoxidase in 0.05 M phosphate (pH 7.0). Curve b; a slight excess of $Na_2S_2O_4$ was added to a. Curve c; CO was bubbled into b with a further addition of $Na_2S_2O_4$.

the peroxidases of horseradish and milk in spite of the great difference in the light absorption spectra. Since AGNER's first paper[1] it has been generally accepted[13] that ferrous myeloperoxidase does not combine with CO, with the exception of EHRENBERG's[9] suggestion of the formation of a CO complex. A CO complex can be observed under the restricted conditions described in Fig. 6. The result is consistent with Ehrenberg's description that upon the introduction of CO into ferrous myeloperoxidase the light absorption in the visible region of the spectrum is decreased by about 10% and shifts about 5 mμ in the direction of shorter wavelength.

DISCUSSION

The formation of a H_2O_2 compound of myeloperoxidase was described in the first paper on myeloperoxidase by AGNER[1]. The compound had a distinct absorption maximum at 625 mμ and was later classified by CHANCE[6] as peroxidase "Complex II". There was some confusion concerning two peroxide compounds formed in the presence of a large amount of H_2O_2. Chance observed a peroxide compound which had an absorption maximum approximately at 458 mμ in the Soret region, and he thought that this compound was the same as that reported by Anger. However, it is obvious from Fig. 1 and Table I that each absorption band is ascribed to different chemical species. These compounds are finally identified as Compounds II and III by the estimation of their oxidation equivalent. The assumed structure for myeloperoxidase Compound III is supported by additional evidence that the compound is formed in the same reaction system as was reported in the case of horseradish peroxidase[11] (Figs. 2 and 3).

TABLE I

THE ABSORPTION PEAKS OF MYELOPEROXIDASE DERIVATIVES

A number in parentheses shows a shoulder of the absorption spectrum.

Enzyme	*Wavelength (mμ)*
Ferric	430 (500), 570, 625, 690
Ferrous	475 (550), (585), 637
Compound I	430
Compound II	456 630
Compound III	452 574, 625.5
CO complex	468 634

Agner's "dissociable" peroxide compound is very likely to correspond to Compound II described in this paper. Approx. 0.3 mM H_2O_2 is necessary for half maximal formation of the compound in the experiment of Fig. 1. At room temperature this apparent dissociation constant becomes 0.1 mM, as was reported by AGNER[7]. However, due to the following reasons it is concluded that the compound is not dissociable: (1) the compound is fairly stable at low temperatures after H_2O_2 is removed by catalase or gel filtration, (2) much less H_2O_2 is sufficient for the formation of the compound when the enzyme is freed of endogenous donor, (3) the compound is formed from Compound I by the addition of hydrogen donor, and (4) only one-equivalent reduction of the compound recovers the free enzyme.

When the enzyme has been pretreated with a small amount of H_2O_2, an addition of an equivalent mole of H_2O_2 makes a slight but distinct change in the absorption spectrum. The spectrum differs distinctly from that of the ferric enzyme containing a small amount of Compound II. CHANCE[6] has reported the formation of myeloperoxidase "Complex I", having an absorption band approximately at 430 mμ. Though the details of his results are not available, these two compounds seem to be identical. The similarity of absorption spectra between the ferric form and Compound I of myeloperoxidase suggests that unlike the other peroxidases the replacement of H_2O with H_2O_2 in the sixth coordination position of the heme iron does not bring a big change in the electronic configuration of the heme.

When compared with other peroxidases, the marked characteristics of myeloperoxidase appear to be an anomaly in the absorption spectrum. And myeloperoxidase is only one peroxidase which is known to have two iron atoms per molecule[8,14], and AGNER[7] had suggested the cooperation of two iron sites in the catalase-like activity of the enzyme. It might be concluded from the present results that there is no essential difference in the reaction mechanism of the heme between myeloperoxidase and the peroxidases of horseradish and milk. Further study, however, is needed for the characterization of this peculiar peroxidase.

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