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MYELOPEROXIDASE OF THE LEUKOCYTE OF NORMAL BLOOD

III. THE REACTION OF FERRIC MYELOPEROXIDASE WITH SUPEROXIDE ANION

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SUMMARY

Myeloperoxidase (donor:H_2O_2 oxidoreductase, EC 1.11.1.7) Compound III was formed by the addition of superoxide anions (O_2^-), which had been produced from the electrolytic reduction of molecular oxygen in an aprotic solvent, to a solution of ferric myeloperoxidase. The formation of Compound III was inhibited by superoxide dismutase from pig erythrocytes. It is concluded that myeloperoxidase Compound III can be formed from the direct reaction between ferric myeloperoxidase and O_2^-. By the use of electron spin resonance the concentration of O_2^- in a cathodic chamber was found to reach approx. 10 mM.

INTRODUCTION

In a previous paper[1] it was reported that myeloperoxidase (donor:H_2O_2 oxidoreductase, EC 1.11.1.7) Compound III can be formed during aerobic oxidation of NADH and by the reactions of ferrous myeloperoxidase with O_2 and of myeloperoxidase Compound II with H_2O_2. It was also found[2] that the conversion of myeloperoxidase into Compound III in an aerobic solution of NADH is inhibited by superoxide dismutase, which decomposes superoxide anions.

The result substantiates the involvement of O_2^- in the formation of peroxidase Compound III during peroxidase–oxidase reactions, which has been suggested by Yamazaki and Piette[3].

The present paper describes the direct reaction between ferric myeloperoxidase and O_2^- produced electrochemically in an aprotic solvent.

MATERIALS AND METHODS

Myeloperoxidase was prepared from leukocytes of normal pig blood by the method reported in a previous paper[1]. The concentration was calculated on the basis

of a value of 95 $mM^{-1} \cdot cm^{-1}$ for the extinction coefficient at 430 nm. The $A_{430\,nm}/A_{280\,nm}$ ratio for the present enzyme was about 0.7. Superoxide dismutase was prepared from pig erythrocytes using the method of McCord and Fridovich[4]. The concentration of superoxide dismutase was estimated from the molar extinction coefficient of 350 at 665 nm[5]. All other materials were obtained from commercial sources at the highest available states of purity.

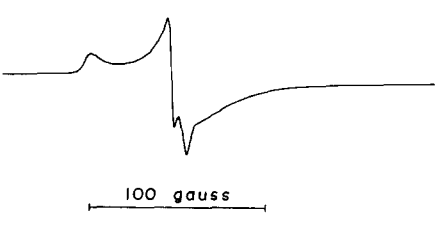
The power supply for the electrochemical reaction used was either a M and S (Type 20 T) or a Mitsumi (SJ-1055) instrument. Electron spin resonance (ESR) was investigated with a Varian V-4500 X-band instrument, utilizing 100 kcycles field modulation. The recording spectrophotometers used were Hitachi instruments, Types 124 and EPS-3T.

EXPERIMENTAL AND RESULTS

The method of electrolytic production of superoxide anions in *N,N*-dimethylformamide is a modification of the procedure reported by Maricle and Hodgson[6] and McCord and Fridovich[4]. The cell used in this experiment is the same as that used by McCord and Fridovich[4]. Both chambers of the cell were filled with the same solution, 0.1 M tetra-*n*-butylammonium bromide in *N,N*-dimethylformamide. This electrolytic glass cell holds about 100 ml. The two chambers are connected by a glass tube (1 cm × 4 cm) which holds a sintered glass disc in the middle. The cathode is a pool of mercury and the anode is a coil of platinum wire (diameter, 0.3 mm and length, about 12 cm). O_2, which had been dried by bubbling concentrated H_2SO_4, was continuously bubbled through the cathode chamber. A direct current of 20 mA was applied with a power supply, Type SJ-1055 (Mitsumi, Co.) which had been used for disc electrophoreses.

Fig. 1 shows an ESR spectrum of the superoxide anion radical accumulated during 3 h electrolysis. This ESR spectrum is very similar to that of superoxide anions formed in a 0.1 M tetra-*n*-butylammonium perchlorate–*N,N*-dimethylformamide solution[6] and in a glassy $^2H_2O_2$–^2H_2O (ref. 7).

Fig. 2 shows the increase in the concentration of superoxide anion radicals in



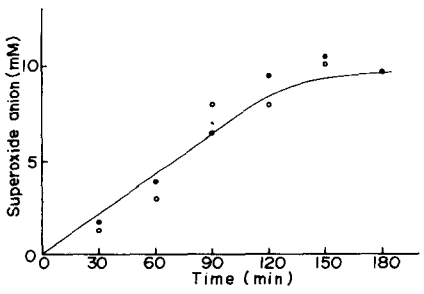


Fig. 1. ESR spectrum of superoxide anion radical in a dimethylformamide solution with 0.1 M tetrabutylammonium bromide. The spectrum was measured at 100 °K. The sample was a cathodic solution after a current of 20 mA was maintained for 120 min.

Fig. 2. Time course of the superoxide radical formation by electrolysis. The concentration of superoxide anion was measured by ESR with use of $CuSO_4$ solution containing EDTA as a standard of spin concentration. A current was 20 mA and the experiment was repeated twice. ESR measurements were carried out at 100 °K (○) and 77 °K (●).

the cathodic chamber as a function of the time of electrolysis. A cupric EDTA solution was used as a standard of the spin concentration. The concentration of superoxide anion radical was found to reach 9.5 mM when a current of 20 mA was maintained for 150 min. The reduction of O_2 could also be measured with a Clark electrode, after 0.5 ml of cathodic solution was mixed with 2.5 ml 0.1 M phosphate buffer (pH 7.5). The superoxide anions protonate and dismutate to form O_2 and H_2O_2. The O_2 evolved in this reaction partially escaped from the solution and it was difficult to measure it quantitatively. Since the oxygen evolution was very fast the O_2 concentration soon reached a constant level. So, the H_2O_2 could be measured as the amount of O_2 evolved when catalase (EC 1.11.1.6) was added. If H_2O_2 is formed only from superoxide anions one mole of O_2 will be formed at the expense of 4 moles of superoxide anions. Based upon this assumption, the concentration of the superoxide anion accumulated after 150 min electrolysis was measured as 7.2 mM. This value is consistent with the ESR data (Fig. 2) and it may thus be concluded that the reduced form of O_2 in the *N,N*-dimethylformamide solution is mostly the superoxide anion.

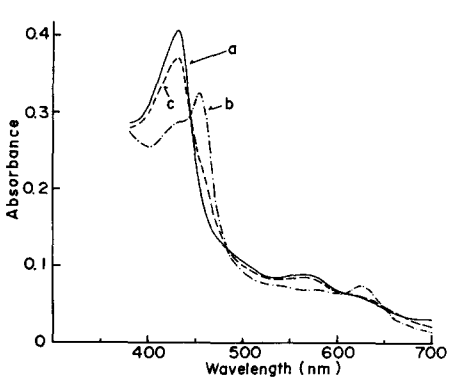


Fig. 3. Inhibition of myeloperoxidase Compound III formation by superoxide dismutase. Curve a, 4.4 μM myeloperoxidase in 0.1 M phosphate buffer (pH 7.5). 0.05 ml cathodic solution was added to the solution of a (2.5 ml) in the absence (Curve b) and presence (Curve c) of superoxide dismutase. The final concentration of superoxide anion was 170 μM. The experiments were carried out at 3 °C.

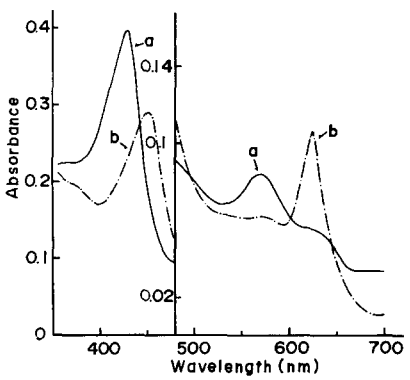


Fig. 4. Formation of myeloperoxidase Compound III by the addition of a large amount of cathodic solution. Curve a, 4.4 μM myeloperoxidase in 0.1 M phosphate buffer (pH 7.5); Curve b, 0.5 ml cathodic solution was added to the solution of a (2.5 ml). The final concentration of superoxide anion was 1.7 mM.

Fig. 3 shows that when superoxide anion at a final concentration of 170 μM was added to a solution of ferric myeloperoxidase, approx. 75% of the enzyme was converted to Compound III. This conversion was completely inhibited by 0.5 μM superoxide dismutase and the enzyme (Spectrum c) then consisted of the ferric form and a small amount of Compound II. The addition of 1.7 mM superoxide anions caused a complete conversion of the enzyme into Compound III as shown in Fig. 4. However, in this case the 0.85 mM of H_2O_2 formed from 1.7 mM superoxide anions is high enough to convert the enzyme partially into Compound III through the reaction of Compound II with H_2O_2 (ref. 1).

DISCUSSION

The mechanism of myeloperoxidase Compound III formation was found to be quite similar to the case of horseradish peroxidase[1]. The reactions responsible for Compound III formation have been extensively studied for the horseradish enzyme and it has been suggested that peroxidase Compound III is formed from the reactions of Compound II with H_2O_2 (refs 8 and 9), of ferrous peroxidase with O_2 (refs 10 and 11) and of ferric peroxidase with superoxide anion[3]. It was, however, difficult to confirm directly an involvement of superoxide anion in the formation of Compound III. Recently, the utility of superoxide dismutase has been reported by McCord and Fridovich[4] in confirming an involvement of superoxide anion in enzymatic[4] and non-enzymatic[12] reactions. By the use of superoxide dismutase they also demonstrated that cytochrome *c* is reduced by superoxide anion which has been generated by the electrolytic reduction of O_2 in an aprotic solvent, *N*,*N*-dimethylformamide. This principle has been successfully applied to the analysis of the peroxidase reactions.

The concentration of superoxide anion generated by the electrolytic reduction of O_2 was measured directly with an ESR spectrometer. It was found that the concentration can be also evaluated from the amount of H_2O_2 formed when an aliquot of cathode solution is mixed with water. The end of electrolysis can be judged roughly from the colour of the solution. The superoxide anion seems to be stabilized as a complex with a tetrabutylammonium cation in *N*,*N*-dimethylformamide and its concentration is found to reach 10 mM. The superoxide anion complex dissociates to its free anion state immediately after the solution is mixed with water at neutral pH and then dismutates into O_2 and H_2O_2 with a rate constant of 3.10^5 $M^{-1} \cdot s^{-1}$ at pH 7.5 (ref. 13). This rate is not so fast and a considerable amount of the radical can react with myeloperoxidase to form Compound III. Acceleration of the dismutation by superoxide dismutase indeed inhibits the formation of Compound III.

The use of myeloperoxidase has an advantage for confirmation of the reaction of ferric hemoprotein with superoxide anion since the addition of less than 0.1 mM H_2O_2 affects the absorption spectrum of the enzyme only slightly at 3 °C (ref. 1) and a marked difference can be seen in the presence and absence of superoxide dismutase. Quantitative studies, however, have been unsuccessful because of the technical difficulty of mixing instantaneously a small amount of aprotic solvent with a water solution.

Unlike other peroxidases, myeloperoxidase contains two heme groups. Agner[14] suggested that both iron atoms take up H_2O_2 at high peroxide concentration and the formation of a compound with two H_2O_2 molecules might result in the catalase-like reaction of myeloperoxidase. It is concluded from the previous[1] and present results that there is no difference in the mechanism of Compound III formation between myeloperoxidase and other peroxidases containing one heme group per molecule. So, it seems unlikely that myeloperoxidase Compound III formed at higher peroxide concentration is a compound which has two peroxide molecules per enzyme. In a previous paper[1] it was reported that myeloperoxidase Compound III has three oxidizing equivalents more than the ferric enzyme per molecule of the enzyme. Accordingly, the present result would suggest that one of the two heme groups is converted to the state of Compound III while the rest remains in the ferric form. The difference in reactivity between the two iron atoms will be discussed elsewhere[15].

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