Reaction of Compound III of Myeloperoxidase with Ascorbic Acid*

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A relatively pure and stable compound III of bovine spleen myeloperoxidase was prepared from native enzyme using the aerobic oxidation of dihydroxyfumarate to generate $O_2^-$. Spectral scans show well defined peaks at 450 and 625 nm and an isosbestic point between compound III and native enzyme at 440 nm. Compound III decayed to native enzyme without any detectable intermediate. The rate of decay was faster than between compound III and native enzyme at 440 nm. Ascorbic acid reduces compound III at alkaline pH values and also in the presence of superoxide dismutase. The rate of decay was faster than detectable intermediate. The rate of decay was faster than between compound III and native enzyme at 440 nm. Ascorbic acid reduces compound III to the ferric native enzyme using the aerobic oxidation of dihydroxyfumarate.

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Materials and Methods

Bovine spleen myeloperoxidase was isolated and purified using a combination of published procedures with some minor modifications (15-17). The enzyme preparations used in this study exhibited $A_280$ values ($A_{440}/A_{280}$) of 0.80 or greater. The myeloperoxidase concentration was determined spectrophotometrically using an extinction coefficient of 430 nm of 178 $m^{-1}cm^{-1}/molecule$ of enzyme (18). Superoxide dismutase (Sigma) was used without further purification. Its concentration was calculated using an absorbance coefficient of 15.9 $m^{-1}cm^{-1}$ at 265 nm (19). Xanthine and xanthine oxidase (0.005 units/ml) were also purchased from Sigma.

1-Ascorbic acid and DHF (Sigma) were reagent grade. Aqueous solutions were freshly prepared before each day experiments were performed. DHF was dissolved in argon-saturated water immediately prior to use. Hydrogen peroxide (30% solution, BDH Chemicals) concentration was determined using the horseradish peroxidase assay (20). All chemicals used for the buffers were reagent grade and used without further purification. Solutions were prepared using deionized water obtained from the Milli Q system (Millipore).

All experiments were carried out at 25.0 ± 0.5°C and an ionic strength of 0.1 M due entirely to the contribution of the buffer. The pH was measured using a Fisher microprobe electrode and a Fisher digital pH meter.

Optical absorption spectra were obtained with a Cary 219 recording spectrophotometer. Rapid scan spectral and kinetic measurements were conducted using the Photal (formerly Union Giken) RA 601 rapid reaction analyzer equipped with a 1-cm observation cell. One reservoir contained a stable preparation of compound III (approximately 1 μM) in 0.2 M (ionic strength) phosphate buffer, and the other reservoir contained ascorbic acid in aqueous solution. Pseudo-first order rate constants were determined by a nonlinear least squares computer analysis of exponential traces.

Results

Compound III of myeloperoxidase from bovine spleen was prepared by reacting the ferric enzyme with $O_2^-$ generated from both (a) aerobic oxidation of DHF and (b) the xanthine-xanthine oxidase reaction system. The visible spectra of compound III formed using the DHF reaction system is shown in Fig. 1. The Soret peak of the native enzyme disappears, and a new one at 450 nm appears. Also, the broad shoulder at 625 nm becomes a sharp peak.

Fig. 2 shows that compound III formation was not instantaneous. Moreover, a large excess (100-fold) of DHF was required for full formation of this enzyme intermediate. The order of addition of DHF and $H_2O_2$ to the native enzyme had no effect on the amount of compound III formed.

The compound III prepared using DHF was very stable as shown by the decay spectra in Fig. 3. The stability of compound III prepared using the DHF system was compared with that using the xanthine-xanthine oxidase system. Compound III prepared using the latter method exhibited a broad band at about 446 nm, remained stable only for about 10 min, and...
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Fig. 1. Spectra of compound III formed using the DHF system. A, native enzyme; the reaction cuvette contains 1.1 μM spleen myeloperoxidase in 0.1 M phosphate buffer, pH 7.1. B, compound III; 100 μM DHF and 1 μM H₂O₂ were added to the native enzyme in A.

Fig. 2. Effect of amount of DHF added on compound III formation. A, to 1.2 μM native enzyme in 0.1 M phosphate buffer, pH 7.1, was added 62 μM DHF and 1 μM H₂O₂. Spectra 1 and 2 were taken 3 min and 30 s after each other. B, 38 μM more DHF was added to A. Scan times for spectra 1, 2, 3, and 4 are the same as in A. The arrows indicate the direction of absorbance changes with increasing time.

Fig. 3. Spectral changes accompanying the decay of compound III formed using DHF. Compound III was prepared as described in Fig. 1. Each scan takes 12 min and 30 s. The last scan was taken after 4 h and 10 min. The arrows indicate the direction of absorbance changes with increasing time.

then started to decay back to the native state with a well defined isosbestic point at 440 nm (Fig. 4).

Fig. 5 shows the effect of pH on the stability of compound III. At pH 4.7 compound III remains virtually unchanged for 15 min while at pH 9.2 considerable reversion back to the native state has occurred within the same time span.

The addition of superoxide dismutase to compound III caused an acceleration of its decay to the ferric form (Fig. 6). The isosbestic point at 440 nm was maintained. When 6.8 μM superoxide dismutase was added to 1 μM native enzyme prior to the addition of DHF and H₂O₂, compound III formation was completely inhibited. However, when a lower concentration of superoxide dismutase was added (i.e. 3 μM), some compound III still formed.

In an attempt to accelerate the decay of compound III to the ferric state, ascorbic acid, which is both a reducing agent and a free radical scavenger, was added to the reaction system. Fig. 7 shows rapid scan spectra of the reaction between compound III and ascorbic acid. Compound III decays back to native state within 8 s, the scans exhibiting an isosbestic point at 440 nm.

Fig. 8 shows stopped flow traces of the reaction between compound III and ascorbic acid. Pseudo-first order conditions were maintained by using at least a 100-fold excess of ascorbic acid. The reaction was followed at 430 nm, corresponding to the formation of the native enzyme. The reaction of ascorbic acid with compound III obeyed first order kinetics. From the slope of the plot of rate constant versus ascorbic acid concentration the second order rate constant for the reaction is found to be (4.0 ± 0.1) × 10⁷ M⁻¹ s⁻¹.

DISCUSSION

Compound III (also known as oxyperoxidase) is one of the oxidized intermediates of peroxidases. It may be represented as a resonance hybrid between the complexes ferroperoxidase-oxygen and ferriperoxidase-superoxide (i.e. Fe(II) O₂ ↔ Fe(III)-O₂⁻) (21–24). The formation of compound III can occur through three possible reaction pathways: ferrous enzyme and dioxygen, ferryl enzyme (compound II) and H₂O₂, or ferric enzyme (native) and superoxide (14, 25). In the case of myeloperoxidase, compound III has been prepared through several methods (1, 3, 7, 24, 26, 27).
The reaction between ferric myeloperoxidase and superoxide anion radical has gained a wide degree of attention due to its possible involvement in the enzyme’s microbicidal activity. In leukocytes O$_2^-$ production was observed to be enhanced in the presence of latex particles (6). The respiratory burst oxidase is the enzyme involved in the reduction of molecular oxygen to O$_2^-$ at the expense of NAD(P)H. This enzyme is dormant in resting phagocytes but becomes activated when the cells are stimulated by various agents. The oxidase has been purified and characterized, and the mechanism by which substances activate it has also been the subject of several recent investigations (28-31).

Compound III is reportedly formed in intact phagocytosing granulocytes (7). Earlier studies suggest that myeloperoxidase’s bactericidal activity was due to the production of O$_2^-$ accompanying the decay of compound III (32). On the other hand, other authors who studied the same process concluded on the basis of available spectral data that compound II was formed (33). The methods used to prepare compound III have always been plagued with the presence of compound II as an impurity. Moreover, since the Soret spectra of these two intermediates are very similar it is often difficult to discriminate between them.

In an attempt to obtain a relatively pure preparation of compound III, we generated a perhydroxyl radical (HO; the protonated form of O$_2^-$) via the aerobic oxidation of DHF in the presence of H$_2$O$_2$ and reacted it with ferric myeloperoxidase. In this work, we used bovine spleen myeloperoxidase which has been demonstrated to be identical with granulocyte myeloperoxidase (34).

Two criteria have been proposed to evaluate the purity of compound III preparations: first, symmetry of the Soret band and second, the ratio $A_{625nm}/A_{456nm}$. For compound II, this ratio is 0.17 whereas for compound III, the value is 0.52. In mixtures, the value lies between these two extremes (3). Fig. 1 shows that the first criteria is well satisfied. The sharp peaks at 450 and 625 nm and the broader one at 575 nm are also consistent with the reported spectra of compound III (1, 3, 35). The ratio $A_{625nm}/A_{456nm}$ obtained is 0.44, which is better than the values obtained using other methods of preparation (1, 3, 7, 24, 26). Compound III prepared using DHF was also found to be very stable as shown by the decay spectra (compare Figs. 3 and 4). An isosbestic point at 440 nm observed in the decay spectra of both preparations suggests that the conversion from compound III to native enzyme does not pass through any other intermediate.

The stability of myeloperoxidase compound III relative to the superoxide adduct of other peroxidases have been documented (7, 24). In this work we also find that a greater proportion of native enzyme was converted to compound III at lower pH. Moreover, the compound III formed at lower pH was most stable (Fig. 5). It would also be expected that compound III formation will be faster at low pH because HO; reacts with the ferric enzyme more rapidly than O$_2^-$ (36, 37). Since myeloperoxidase-catalyzed halogenation has a pH optimum at 5 (38, 39), it would seem that the accumulation of compound III at acid pH would limit the microbicidal activity of the enzyme. How then does the phagocytic system prevent this from happening?

Previously it was reported that superoxide dismutase inhibi-
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FIG. 6. Effect of superoxide dismutase on compound III decay. A, 1.1 μM myeloperoxidase in 0.1 M phosphate buffer, pH 4.7. B, compound III was formed by adding 100 μM DHF and 0.5 μM H2O2 to A. C, 6.5 μM superoxide dismutase added to B. Arrows show direction of absorbance changes with time.

FIG. 7. Rapid spectral scans of the reaction between compound III and ascorbic acid. One reservoir contained compound III prepared as described in Fig. 1 in 0.2 M phosphate buffer, pH 7.1. The other reservoir contained 1 mM ascorbic acid in aqueous solution. Spectra a was taken at 650 ms and b at 8.3 s after mixing.

FIG. 8. Typical time course traces of the reaction between compound III and ascorbic acid. Absorbance changes were followed at 430 nm. One reservoir contained compound III prepared as described in Fig. 7 while the other reservoir contained at least 100-fold excess ascorbic acid. A, 0.025 mM; B, 0.250 mM; C, 0.125 mM ascorbic acid. The broken lines are exponential curve fits.

If less than 100 eq of DHF is added to the enzyme there is incomplete formation of compound III. Considering myeloperoxidase's strong affinity for O2− and that sufficient O2− was generated in the system, conversion to compound III should have been quantitative. The explanation we can offer for our results is based on the finding that compound III has superoxide dismutase activity (35). It was suggested that the following reaction occurs in the phagocytes.

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\text{Compound III} + O_2^- \rightarrow \text{compound I} + \text{O}_2\n\]

Moreover, it has been reported that the apparent superoxide dismutase activity observed in azurophil granules was attributable not to superoxide dismutase itself but to myeloperoxidase (41). Evidence of the one-electron reduction shown above is given by the reaction between compound III and one electron donors such as p-cresol and ferrocyanide (21). Compound I is the active intermediate in the chlorination reaction. Thus, the above reaction would provide a pathway to ensure that the enzyme is in an active form.

There are other reducing agents present in the phagocyte. Ascorbic acid is present in substantially high concentration. In the presence of a large excess of ascorbic acid compound III is rapidly converted back to the native state without formation of any detectable intermediate (Fig. 7). Interestingly, the time course traces of the reaction showed a biphasic character (Fig. 8). We attribute the faster initial phase to the reaction between ascorbic acid and compound II (12, 13) inadvertently formed in the preparation. The pseudo-first order rate constants obtained from the second phase of the reaction correspond to the reaction between compound III and ascorbic acid. The conversion of compound III back to the native state makes the enzyme available for HOCl production via compound I. Recently, the rate constants for human myeloperoxidase compound I and compound III formation were determined using pulse radiolysis (42). Although compound III formation is an order of magnitude slower than compound I formation, the former reaction is still sufficiently rapid to influence HOCl production by the myeloperoxidase system in phagocytes. Thus, the ability of ascorbate to reduce compound III to native enzyme is significant.

The result of this work, as well as other documented studies (12, 13), provides another possible physiological function of ascorbic acid in leukocytes. The primary function of myeloperoxidase present in these cells is to oxidize Cl− to the
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bactericidal agent HOCl. The active enzyme intermediate for this reaction is compound I, which is formed when a stoichiometric equivalent of $H_2O_2$ reacts with the enzyme. $H_2O_2$ is produced from the dismutation of $O_2^-$. $H_2O_2$ production was never observed without $O_2^-$ production (43). When a large quantity of $O_2^-$ is generated, dismutation is faster and more $H_2O_2$ is produced. In such an event, both compounds II (native enzyme + excess $H_2O_2$) and III (native enzyme + $O_2^-$) may form. The enzyme thus becomes inactive in the chlorination reaction. However, in the presence of reducing agents like ascorbic acid compounds II and III are converted back to the native state and made available for HOCl formation. Thus, the bactericidal activity of the enzyme is maintained.

REFERENCES

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