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 that between compound III and native enzyme at 440 nm. The rate of decay was faster at alkaline pH values and also in the presence of superoxide. Compound III decayed to native enzyme without any detectable intermediate. The rate of decay was faster at alkaline pH values and also in the presence of superoxide. The reaction was determined spectrophotometrically using an extinction coefficient of 430 nm of 178 $\text{mM}^{-1}\text{cm}^{-1}$/molecule of enzyme. The superoxide dismutase (Sigma) was used without further purification. Its concentration was calculated using an absorbance coefficient of 15.9 $\text{mM}^{-1}\text{cm}^{-1}$ at 285 nm. Xanthine and xanthine oxidase (0.095 units/ml) were also purchased from Sigma.

**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 RZ values ($A_{450}/A_{360}$) of 0.80 or greater. The myeloperoxidase concentration was determined spectrophotometrically using an extinction coefficient of 430 nm of 178 $\text{mM}^{-1}\text{cm}^{-1}$/molecule of enzyme. The superoxide dismutase (Sigma) was used without further purification. Its concentration was calculated using an absorbance coefficient of 15.9 $\text{mM}^{-1}\text{cm}^{-1}$ at 285 nm. Xanthine and xanthine oxidase (0.095 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. Pseudofirst 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 nor the rate of compound III formation.

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
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.

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 6.5 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 $(4.0 \pm 0.1) \times 10^4$ 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).
Role of Ascorbic Acid in Myeloperoxidase Function

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 

\[ \text{O}_2^- \]

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 

\[ \text{O}_2^- \]

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).

**FIG. 4.** Spectral changes accompanying the decay of compound III formed using the xanthine-xanthine oxidase system. Compound III was formed by adding 10 µl of xanthine oxidase to a reaction mixture containing 1 µM myeloperoxidase, 0.5 mM xanthine, 0.1 M phosphate buffer, pH 7.1. The first scan was started 10 min and the last scan 75 min after initiation of the reaction. The arrows indicate the direction of absorbance changes with increasing time.

**FIG. 5.** Effect of pH on the stability of compound III. Compound III was prepared by adding 100 µM DHF and 1 µM H2O2 to 1 µM spleen myeloperoxidase in 0.1 M buffer. Repetitive scans were taken for a period of 3 min and 30 s each. A, citrate-phosphate buffer, pH 4.7. B, phosphate buffer, pH 7.1. C, bicarbonate buffer, pH 9.2.
Role of Ascorbic Acid in Myeloperoxidase Function

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, 5.5 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.

its DHF oxidation by horseradish peroxidase (40). Our results indicate that myeloperoxidase has a very high affinity for O$_2^-$ because compound III formation was only partially inhibited by up to 3 eq of superoxide dismutase. When superoxide dismutase was added after compound III has been formed the rate of decay to the native form was faster suggesting that superoxide dismutase is able to scavenge O$_2^-$ from the oxyperoxidase complex.

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, 0.625 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 O$_2^-$ and that sufficient O$_2^-$ 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 phagocyte.

$$\text{Compound III} + O_2^- \rightarrow \text{compound I} + O_2$$

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
Role of Ascorbic Acid in Myeloperoxidase Function

bactericidal agent HOCl. The active enzyme intermediate for this reaction is compound I, which is formed when a stoichiometric equivalent of H2O2 reacts with the enzyme. H2O2 is produced from the dismutation of O2. H2O2 production was never observed without O2 production (43). When a large quantity of O2 is generated, dismutation is faster and more H2O2 is produced. In such an event, both compounds II (native enzyme + excess H2O2) and III (native enzyme + O2) 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

Reaction of compound III of myeloperoxidase with ascorbic acid.
L A Marquez and H B Dunford