Decay of Oxyperoxidase and Oxygen Radicals: A Possible Role for Myeloperoxidase

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The formation of superoxide anion during the decay of oxyperoxidase to ferric peroxidase was detected by using a spectrophotometric assay based on the use of adrenaline. The finding that peroxidase is a potential source of superoxide suggests a possible role for myeloperoxidase in leucocytes.

Two potential sources of superoxide anion radical ($O_2^{\cdot-}$), the single-electron reduction product of molecular oxygen, have been recognized in the cell. One is the reoxidation of reduced molecules, namely flavins (Massey *et al.*, 1969), ferredoxins (Orme-Johnson & Beinert, 1969) and metabolites such as xanthine (Knowles *et al.*, 1969), by molecular oxygen to yield H_2O_2 via two single-electron steps and consequent dismutation of two $O_2^{\cdot-}$ ions. The second source would be provided by the oxygenated adducts of haem proteins, which were proposed to have the structure of a superoxo-ferrihaem, $Fe^{3+}O_2^{\cdot-}$ (Weiss, 1964), from which $O_2^{\cdot-}$ could directly dissociate, leaving the oxidized form of the haem iron (see Antonini & Brunori, 1971, for review). Evidence that production of $O_2^{\cdot-}$ actually occurs during autoxidation of haem proteins has been produced in cases where the autoxidation is fast, such as fish haemoglobin (Misra & Fridovich, 1972*b*), bovine haemoglobin in 1M-potassium phosphate buffer at pH 6.0 (Wever *et al.*, 1973) and human α and β chains of haemoglobin (Brunori *et al.*, 1975).

Demonstration of actual production of $O_2^{\cdot-}$ by potential sources is important in view of the possible effects of the radical on various components of the cell. Superoxide has been involved in peroxidation of membrane lipids (Zimmermann *et al.*, 1973) and in the aetiology of pathological phenomena such as haemolysis (Fee & Teitelbaum, 1972), so that the enzyme superoxide dismutase (McCord & Fridovich, 1969), present in the erythrocytes and in the parenchymal cells, would play a critical role in the defence mechanism operative against free $O_2^{\cdot-}$ produced by one of the various potential sources. In leucocytes, liberation of $O_2^{\cdot-}$ was shown to be enhanced during phagocytosis (Babior *et al.*, 1973), thus suggesting that radical production may have a bactericidal role. In view of the correlation between antimicrobial activity and peroxidase content of leucocytes (Brune *et al.*, 1973), it was decided to see whether production of $O_2^{\cdot-}$ by oxyperoxidase could be demonstrated. In fact it is known that both horseradish peroxidase and myeloperoxidase form an oxygen adduct, called

oxyperoxidase or Compound III, which may be obtained either when O_2 reacts with ferrous (Fe^{2+}) peroxidase or when $O_2^{\cdot-}$ is added to the ferric (Fe^{3+}) form (Wittenberg *et al.*, 1967; Odajima & Yamazaki, 1972). Oxyperoxidase decays to ferric peroxidase through a mechanism which depends on the presence or absence of free ferrous peroxidase (Phelps *et al.*, 1974). Demonstration that $O_2^{\cdot-}$ is produced in the decay of oxyperoxidase to ferric peroxidase may be taken as an indication for the fact that, through the production of oxygen radicals, myeloperoxidase may be involved in killing of bacteria by leucocytes.

Materials and methods

Peroxidase from horseradish (type VI) was obtained from Sigma Chemical Co. (St. Louis, Mo., U.S.A.) and used without further purification. Adrenaline was obtained from E. Merck (Darmstadt, Germany). Catalase from horse liver (purified powder) was obtained from Sigma. Superoxide dismutase was prepared from bovine erythrocytes as described by McCord & Fridovich (1969). Catalase and superoxide dismutase were purified from each other by passage through a Sephadex G-75 column (1cm $\times$ 60cm), equilibrated with 0.1M-potassium phosphate buffer, pH 7.4. Peroxidase was transformed into the CO derivative by dithionite reduction under a CO atmosphere, and then passed through a Sephadex G-25 column (2cm $\times$ 20cm) equilibrated at pH 7.4 to remove excess of dithionite and CO. The concentration of CO-peroxidase was calculated by using a molar extinction coefficient of 10 at 578nm (Wittenberg *et al.*, 1967). Oxyperoxidase was obtained from the CO derivative by photodissociating the CO adduct with a photographic flash in the presence of $270\mu M$ O_2 . Decay to the Fe^{3+} form was followed in the visible or in the Soret regions of the spectrum in 0.1M-potassium phosphate buffer, pH 7.4, at 10°C. $O_2^{\cdot-}$ production was measured by the adrenaline co-oxidation test (Misra & Fridovich, 1972*a*). The adrenochrome formed was monitored at 462nm, a wavelength which is isosbestic for oxyperoxidase and ferric peroxidase at pH 7.4.

Fig. 1. Effect of adrenaline on the decay of oxyperoxidase

The concentration of peroxidase was $30 \mu\text{M}$. Other conditions: pH 7.4, 0.1 M-potassium phosphate buffer; temp. = 10°C ; observations at 578 nm. k is the rate constant for first-order decay and was calculated from $t_{1/2}$ values.

Results and discussion

The decay of oxyperoxidase to ferric peroxidase at pH 7.4 and 10°C was very slow (half-time of several minutes), in agreement with previous findings (Wittenberg *et al.*, 1967). The rate of decay was practically unaffected by addition of superoxide dismutase or catalase. On the other hand, addition of adrenaline accelerated the decay of oxyperoxidase, as shown in Fig. 1, where the rate constant for first-order decay is reported as a function of adrenaline concentration (from 0.3 to 2.5 mM). This rather unexpected finding parallels a similar effect observed in the autoxidation of the isolated α and β chains from human haemoglobin (Brunori *et al.*, 1975). It may be recalled that the decay of oxyperoxidase is accelerated in the presence of ferrous peroxidase, indicating co-oxidation of ferrous iron with the decay of oxyperoxidase (Phelps *et al.*, 1974).

The formation of adrenochrome, followed at 462 nm (isosbestic for oxyperoxidase and ferric peroxidase), parallels in time the decay of oxyperoxidase.

Fig. 2 reports the percentage inhibition of the extent of formation of adrenochrome for a series of experiments performed at constant peroxidase concentration and variable concentrations of superoxide dismutase and catalase. The inhibition observed is independent of catalase and superoxide dismutase concentration above about $1 \mu\text{M}$, indicating that a saturation of the effect is reached with catalytic amounts of both enzymes.

These results show that co-oxidation of adrenaline parallels the decay of oxyperoxidase. The effect of catalase indicates that a portion of the co-oxidation of adrenaline occurs via H_2O_2 ; the largest fraction, however, seems to involve superoxide anions, as shown by the effect of superoxide dismutase, which

Fig. 2. Dependence of co-oxidation of adrenaline on the concentration of catalase (○) or superoxide dismutase (●)

Co-oxidation of adrenaline was followed at 462 nm, which is isosbestic between oxyperoxidase and ferric peroxidase. The percentage inhibition was computed on the basis of the observed extent of reaction. The effect of superoxide dismutase was tested in the presence of saturating amounts of catalase (i.e. $1.5 \mu\text{M}$), and the percentage inhibition calculated by reference to the experiments containing saturating amounts of catalase and no superoxide dismutase. Peroxidase concentration was $25 \mu\text{M}$, and adrenaline concentration 2 mM. Other conditions: pH 7.4, 0.1 M-potassium phosphate buffer; temp. = 10°C .

removes free $\text{O}_2^{\cdot-}$ from the system via the dismutation reaction (McCord & Fridovich, 1969). In conclusion, the data presented above indicate that oxyperoxidase is a source of $\text{O}_2^{\cdot-}$. In view of the capability of both horseradish peroxidase and myeloperoxidase to form an oxygen adduct, these results may provide a clue to the role of myeloperoxidase in leucocytes, where the decay of oxy-(myelo)peroxidase, with concomitant production of $\text{O}_2^{\cdot-}$, may play a crucial role in the attack on bacterial membranes.

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