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REDUCTION OF CYTOCHROME c BY HYDROGEN PEROXIDE AND ITS INHIBITION BY SUPEROXIDE DISMUTASE

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Ferricytochrome c is slowly converted by hydrogen peroxide to an equilibrium mixture of ferricytochrome c and ferrocytochrome c, and in the process, the hydrogen peroxide is decomposed. The reductant appears to be superoxide anion, produced from the reaction of hydrogen peroxide with oxygen. Because the reduction of ferricytochrome c by hydrogen peroxide is inhibited by superoxide dismutase, we propose that the enzyme acts by converting superoxide anion to a dimerized product that is less active as a reductant.

Aerobic solutions of hydrogen peroxide appear to contain an equilibrium mixture of several species (1,2) that contribute to the reactivity of H_2O_2. For example, the reducing ability of H_2O_2 may be mediated, in part, by its content of O_2^-, while its oxidizing ability may be mediated, in part, by its content of HO_2 and OH. We have used ferricytochrome c to detect the presence of O_2^- in solutions of H_2O_2 and found that the cytochrome is indeed reduced by H_2O_2 in a manner that is inhibited by superoxide dismutase. Although ferrocytochrome c accumulates slowly, the actual rate of its formation is considerably greater because it is also oxidized by H_2O_2. Measurement of the acid that is liberated from hydrogen peroxide simultaneously with superoxide indicates that the generation of superoxide is a continual process. The driving force for this reaction may be the dimerization of superoxide, a process that appears to be catalyzed by superoxide dismutase, which greatly enhances the liberation of acid from H_2O_2.

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MATERIALS AND METHODS

Acid Production - pH was determined with a Metrohm, Model E-512 pH meter and EA-147 electrode (Brinkmann) in a water-jacketed glass vessel kept at 30°C by the circulation of water. Recordings were made with a Linear, Model 555 recorder (Linear Instruments Corp.). The formation of acid under pH-Stat conditions was measured as previously described (3), using NaOH prepared daily by diluting standardized, 1 M NaOH with 0.15 M NaCl. The NaOH was protected from atmospheric CO_2 with Mallcosorb (Mallinckrodt). See legends to figures for other conditions.

Spectrophotometry - Reduction and oxidation of cytochrome c were followed at 550 nm in 10-mm lightpath cuvettes using a Gilford, Model 222 spectrophotometer equipped with thermospacers kept at 30°C by the circulation of water. Recordings were made with a Linear, Model 555 recorder. Absorption spectra were obtained with a Beckman, Acta C-III spectrophotometer at ambient temperature (23°C). See legends to figures for other conditions.

Chemicals - Superoxide dismutase, Type I, from bovine blood (Sigma) was dissolved in water (10 mg/ml, 30,000 units/ml) and stored frozen. Cytochrome oxidase from bovine heart (Sigma) was reconstituted with water (40 mg/ml, 1,000 units/ml) and stored frozen. Cytochrome c, Type VI, from horse heart (Sigma) was dissolved (200 mg, 16 µmol) in 1 ml of 0.15 M NaCl, treated with 1 equivalent of potassium ferricyanide, placed on a 1.5 x 27-cm column of Sephadex G-25 (medium), and eluted with 0.15 M NaCl at 25 ml/hr. The peak fractions (4 x 1 ml), were pooled and stored frozen. Ferrocytochrome c was prepared by treating 100 mg of cytochrome c with 4 equivalents of sodium hydrosulfite and purifying as above, except that the mobile phase was purged with N_2. Other chemicals were the best commercial grade available, and solutions were prepared with Pyrex-distilled water that was previously deionized.

RESULTS

Reduction of Cytochrome c - Fig. 1 illustrates the reduction of ferricytochrome c by a 10-fold excess of H_2O_2. The accumulation of ferrocytochrome c is slow and reaches a stable equilibrium after about 8 hr. Absorption spectra of equilibrium mixtures (not shown) indicate the presence of only ferricytochrome c and ferrocytochrome c in a ratio of 3 to 1. H_2O_2 is apparently degraded during this process, for addition of a second aliquot of H_2O_2 causes an immediate oxidation of the ferrocytochrome c, followed by a second cycle of reduction (Fig. 1). A similar response occurs when H_2O_2 is added to a solution of purified ferrocytochrome c (not shown). However, the addition of cytochrome oxidase to H_2O_2-reduced ferrocytochrome c results in a permanent oxidation (not shown).

Effect of Superoxide Dismutase - The nature of the reducing agent that is derived from H_2O_2 is most likely to be the superoxide anion, produced according to Equation 1.

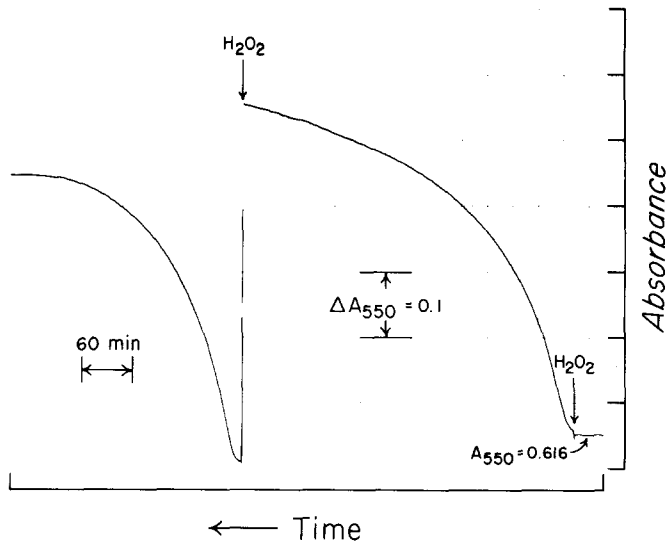

Figure 1 - Reduction and oxidation of cytochrome c by H_2O_2. The initial reaction mixture contained 144 µmol of NaCl, 20 µmol of BES[1], 20 µmol of HEPES (final pH 7.3), and 0.07 µmol of ferricytochrome c in a volume of 1.0 ml and had an A_{550} of 0.616 vs. a reference lacking ferricytochrome c. At the times indicated, H_2O_2 (10 µl, 0.7 µmol) was added. The reaction was conducted at 30°C and followed at 550 nm with the spectrophotometer offset by 0.565 O.D. The recorder pen was inactivated during additions.

$$H_2O_2 + O_2 \rightarrow 2O_2^- + 2H^+ \quad (1)$$

Inasmuch as superoxide dismutase is known to inhibit the reduction of cytochrome c by superoxide (4-6), we tested the ability of superoxide dismutase to inhibit the reduction of cytochrome c by H_2O_2. As shown in Fig. 2, superoxide dismutase decreases both the rate and degree of ferrocytochrome c formation, indicating that ferricytochrome c and superoxide dismutase compete for the superoxide anion generated from H_2O_2.

The conventional view of superoxide dismutase is that it catalyzes the reverse of the reaction depicted in Equation 1 (4-6). Nevertheless, the present work and other recent studies with horse metmyoglobin (7,8) suggests that superoxide dismutase may be removing superoxide by catalyzing the formation of the more stable protonated dimer (Equations 2 and 3).

$$2O_2^- \rightarrow O_4^{2-} \quad (2)$$

$$O_4^{2-} + H^+ \rightarrow HO_4^- \quad (3)$$

[1]Abbreviations used are: BES, 2-[bis(2-hydroxyethyl)amino]ethanesulfonic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

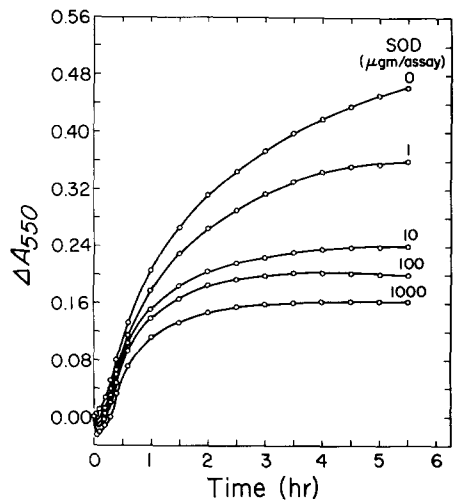

Figure 2 - Effect of superoxide dismutase (SOD) on reduction of ferricytochrome c by H_2O_2. Reaction mixtures contained 144 μmol of NaCl, 20 μmol of BES, 20 μmol of HEPES (final pH 7.3), 0.07 μmol of ferricytochrome c, and 0 to 1 mg of SOD, as noted, in a starting volume of 1.1 ml. The initial A_{550} was 0.562, and at zero time, H_2O_2 (10 μl, 0.7 μmol) was added. The reaction was conducted at 30°C and followed at 550 nm.

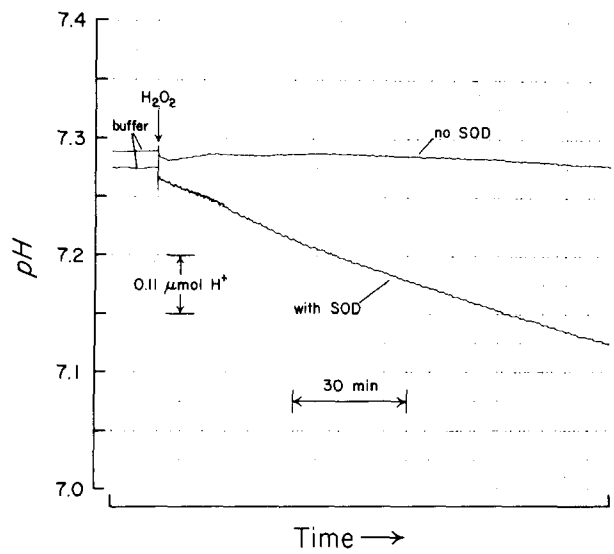

Figure 3 - Decomposition of H_2O_2 as determined by changes of pH. Reaction mixtures contained 180 μmol of NaCl, 2.5 μmol of BES, 2.5 μmol of HEPES, and 1 mg of superoxide dismutase (SOD), as noted, in a volume of 1.0 ml. The initial pH was 7.29 in the absence of SOD and slightly less in its presence. At zero time, H_2O_2 (100 μmol, 100 μl) was added as shown. Reactions were conducted at 30°C in an atmosphere of humidified N_2 (0.2 lit/min); however, similar results were obtained in an atmosphere of O_2. After 2.5 hr, the reaction mixture containing SOD was back-titrated with standardized 10 mM NaOH to determine the relationship between pH change and acid release.

Thus, the overall breakdown of H_2O_2 promoted by superoxide dismutase would be described by the sum of Equations 1 to 3 (Equation 4).

$$H_2O_2 + O_2 \rightarrow HO_4^- + H^+ \quad (4)$$

Liberation of Acid from H_2O_2 - The above considerations suggest that H_2O_2 can degrade spontaneously according to Equation 4 and that this breakdown could be measured by means of the acid released. The liberation of acid from H_2O_2 in the absence and presence of superoxide dismutase is shown in Fig. 3. The enhanced release of acid in the presence of superoxide dismutase indicates that the enzyme does indeed promote the breakdown of H_2O_2 as described by Equation 4.

While spontaneous liberation of acid from H_2O_2 also occurs in the absence of superoxide dismutase, it is barely noticeable under the conditions used in Fig. 3. However, long-term incubation of H_2O_2 under conditions of constant pH clearly demonstrates the spontaneous release of acid as well as the enhancement of this reaction by superoxide dismutase (Fig. 4). As shown in Fig. 4, protons are liberated from 0.1% of the H_2O_2 in 2.5 hr in the absence of superoxide dismutase, and in 20 min in the presence of superoxide dismutase. These observations are consistent with the idea that H_2O_2 gradually liberates superoxide anion, which can subsequently dimerize or be utilized for reduction of cytochrome c. Hodgson and Fridovich (9) and Symonyan and Nalbandyan (10) have previously demonstrated the generation of superoxide from H_2O_2 at high pH.

DISCUSSION

The results presented in this communication demonstrate that H_2O_2 spontaneously liberates both protons and a reducing agent, and that superoxide dismutase promotes the release of acid while decreasing the level of reductant. These observations may be rationalized by the scheme shown in Fig. 5, which assumes an oxygen-dependent equilibrium between peroxide and superoxide.

In the absence of cytochrome c and superoxide dismutase, the superoxide arising from H_2O_2 is thought to gradually dimerize; this would account for the slow accumulation of acid in H_2O_2 solutions. The enhanced production of acid

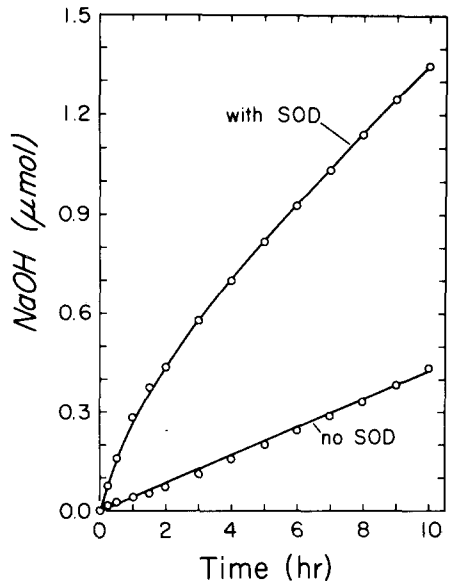

Figure 4 - Superoxide dismutase-dependent liberation of acid from H_2O_2. Reaction mixtures contained 150 μmol of NaCl, 2.5 μmol of BES, 2.5 μmol of HEPES, and either 1 mg (0.1 ml) of SOD or 0.1 ml of 0.15 M NaCl in an initial volume of 1.1 ml. Before each experiment, the initial pH (7.25) was adjusted to 6.95 with 8 μl of 0.1 M HCl, then brought to the set point (pH 7.00) with 1 mM NaOH in 0.15 M NaCl. Reactions were initiated with H_2O_2 (100 μmol, 0.1 ml, in 0.15 M NaCl), and the pH maintained at 7.00 with the above NaOH. A small amount of excess acidity associated with the H_2O_2 (about 0.01 μmol) was titrated immediately and subtracted from all other values. Reactions were conducted at 30°C in atmospheres of either humidified N_2 or O_2 (0.1 lit/min). The data obtained using N_2 and O_2 were pooled as no differences were apparent. Each set of points represents the mean of 4 experiments.

in the presence of superoxide dismutase would thus result from catalysis of the dimerization reaction (Fig. 5).

The present work also shows that H_2O_2 causes both reduction of ferricytochrome c (Equation 5) and oxidation of ferrocytochrome c (Equation 6). Thus,

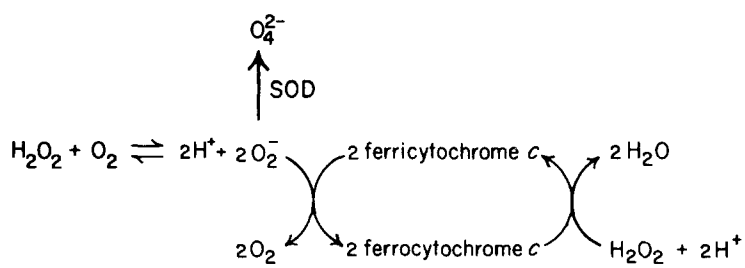

Figure 5 - Scheme showing cytochrome c-induced decomposition of H_2O_2 brought about by reduction of ferricytochrome c by superoxide and oxidation of ferrocytochrome c by hydrogen peroxide. The net reaction is: $H_2O_2 \rightarrow H_2O + \frac{1}{2}O_2$. Inhibition by superoxide dismutase (SOD) of ferricytochrome c reduction by H_2O_2 is postulated to occur via SOD-induced dimerization of superoxide.

when these components are allowed to react, the H_2O_2 gradually disappears (Equation 7), leaving at equilibrium the oxidized and reduced forms of cytochrome c. The oxidation of ferrocytochrome c by H_2O_2 was previously described by Mochan and Degn (11).

$$2\text{cyto-}c^{3+} + H_2O_2 + O_2 \rightarrow 2H^+ + 2O_2 + 2\text{cyto-}c^{2+} \quad (5)$$

$$2\text{cyto-}c^{2+} + H_2O_2 + 2H^+ \rightarrow 2H_2O + 2\text{cyto-}c^{3+} \quad (6)$$

$$\text{Sum:} \qquad H_2O_2 \rightarrow H_2O + \tfrac{1}{2}O_2 \quad (7)$$

Because superoxide reacts rapidly with ferricytochrome c (12-13), it is clear in the present case that its formation from peroxide is rate-limiting. The slowness of superoxide formation is not surprising, considering that the reaction is not favored thermodynamically (12,13). Alternatively, the slow rate of superoxide formation may represent a requirement for singlet oxygen (14,15), a possibility that merits further investigation. It should be noted that attempts to demonstrate a requirement for oxygen by means of differences in reactivity in N_2 and O_2 atmospheres were not successful. This apparently is the result of the small amount of oxygen required and the likelihood that this requirement is met by oxygen liberated from the degradation of H_2O_2.

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