OXIDATION OF ASCORBIC ACID WITH SUPEROXIDE ANION GENERATED BY THE XANTHINE-XANTHINE OXIDASE SYSTEM

Morimitsu Nishikimi

Roche Institute of Molecular Biology
Nutley, New Jersey 07110

Received January 29,1975

Summary: Ascorbic acid was found to be oxidized by O_2^- which was generated by the xanthine-xanthine oxidase system. From a kinetic analysis of the inhibition of this reaction by superoxide dismutase, the second-order rate constant for the reaction between ascorbic acid and O_2^- at pH 7.4 was estimated to be $2.7 \times 10^5\ M^{-1}\ sec^{-1}$. A function of ascorbic acid as a defense against O_2^- is presented.

Molecular oxygen is reduced to O_2^- in a large number of reactions of biological importance, in both enzymic and nonenzymic processes (1). Fridovich and his co-workers (1-4) proposed that the toxicity of oxygen relates to its conversion to O_2^- which is detrimental to cells, and that this radical is scavanged by superoxide dismutase which catalyzes the reaction, $2\ O_2^- + 2\ H^+ \rightarrow O_2 + H_2O_2$. Since O_2^- is a strong oxidant, naturally occurring reducing agents are also likely to scavenge O_2^- generated in vivo. Ascorbic acid may be one such substance. In fact, there have been a number of reports suggesting the oxidation of ascorbic acid by O_2^- or its conjugate acid $HO_2^\cdot$ (5-7). To understand better the possible function of ascorbic acid as a defense against O_2^- it was decided to evaluate the rate constant for the reaction between ascorbic acid and O_2^-, using the xanthine-xanthine oxidase system as an O_2^- generating system (8).

Materials and Methods: Xanthine oxidase (grade 1) was purchased from Sigma, and was dialyzed against 0.02M potassium phosphate buffer (pH 7.0) before use. Catalase was obtained from Calbiochem, and superoxide dismutase from Miles-Seravac. The activity of superoxide dismutase was assayed by its ability to inhibit the reduction of cytochrome c by the xanthine-xanthine oxidase system (8). The molar concentration of superoxide dismutase was determined from its activity, assuming that the enzyme causes 50% inhibition of the cytochrome c reduction at a concentration of 3.1×10^{-9}M (8). Sodium ascorbate was a product of Sigma and other reagents were analytical grade.

The oxidation of ascorbic acid coupled to the xanthine-xanthine oxidase system in 0.1M potassium phosphate buffer (pH 7.4) at 25° was followed by absorbance change at 249.6 nm in a 1.0 cm cuvette using a Beckman ACTA CIII spec-

trophotometer. No absorbance change was observed for the oxidation of xanthine to uric acid at this wavelength. Catalase (22 μg/ml) was added to the reaction mixture to prevent the oxidation of ascorbic acid by H_2O_2 which was formed in the xanthine oxidase reaction. The rate of oxidation of ascorbic acid was obtained on the basis of its molar extinction coefficient at 249.6 nm (ε = 8,430 M^{-1} cm^{-1}). This value was determined, assuming ε_{265} = 15,300 M^{-1} cm^{-1}, at neutral pH (9). Protein was determined by the Lowry method (10).

Results and Discussion: When ascorbic acid (4.6×10^{-5}M) was added to a solution containing xanthine (4.0×10^{-5}M), EDTA (1.0×10^{-4}M), catalse (22 μg/ml), and 0.1M potassium phosphate buffer (pH 7.4), slight, spontaneous oxidation of ascorbic acid was observed. Addition of xanthine oxidase (10 μg/ml) accelerated the rate of oxidation of ascorbic acid for a short period of time (curve I in Fig. 1A) which was found to correspond to the interval during which xanthine was oxidized by xanthine oxidase (Fig. 1B). Xanthine oxidase acting on xanthine produces $O_2^{\cdot-}$ (8). It was predicted that the $O_2^{\cdot-}$ oxidized ascorbic acid in the system containing xanthine oxidase and xanthine. This was found to be the case, as evidenced by the complete inhibition of the oxidation of ascorbic acid by the addition of superoxide dismutase, which catalyzes the dismutation of $O_2^{\cdot-}$ (curve II in Fig. 1A).

Ascorbic acid is a ubiquitous substance present in rather high amounts in both animal and plant tissues. It is worthwhile to compare the rates of reaction of $O_2^{\cdot-}$ with ascorbic acid and with superoxide dismutase, an enzyme which is thought to function as a potential defense against $O_2^{\cdot-}$. The oxidation of ascorbic acid with $O_2^{\cdot-}$ may occur in the following fashion:

$$O_2^{\cdot-} + AH_2 + H^+ \xrightarrow{k_A} H_2O_2 + AH^{\cdot} \qquad (1)$$

$$O_2^{\cdot-} + AH^{\cdot} + H^+ \rightarrow H_2O_2 + A \qquad (2)$$

Here AH_2, $AH^{\cdot}$ and A indicate ascorbic acid, its semiquione radical and dehydroascorbic acid, respectively. These reactions are thermodynamically possible, since the redox potential of the couple $2H^+$, $O_2^{\cdot-}/H_2O_2$ (+ 0.94 V at pH 7.0) is considerably higher than those of the couples H^+, $AH^{\cdot}/AH_2$ (+ 0.31 V at pH 7.5) and H^+, $A/AH^{\cdot}$ (-0.21 V at pH 7.5) (11, 12). Since the redox potential of the couple

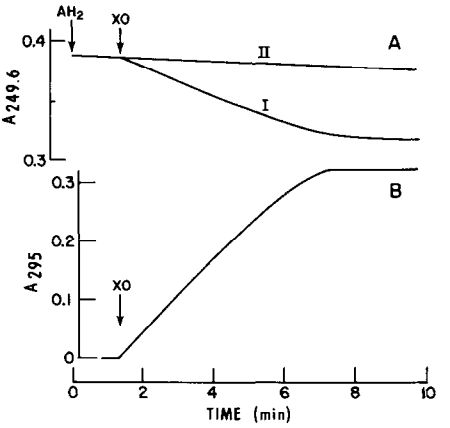

Fig. 1 Oxidation of ascorbic acid coupled to the xanthine oxidase reaction.

A. I, To 1.0 ml of a solution containing xanthine (40 nmoles), EDTA (100 nmoles), and catalase (22 µg), in 0.1M potassium phosphate buffer, pH 7.4, 46 nmoles of sodium ascorbate (AH_2^-) in 1 µl was added, then 10 µg of xanthine oxidase (XO) in 15 µl was added, as indicated by the arrows. The change in absorbance at 249.6 nm was recorded. II, The same reaction was performed in the presence of superoxide dismutase (0.25 nmole).

B. Oxidation of xanthine to uric acid by xanthine oxidase under the same conditions as above except for the absence of ascorbic acid. The change in absorbance at 295 nm was recorded.

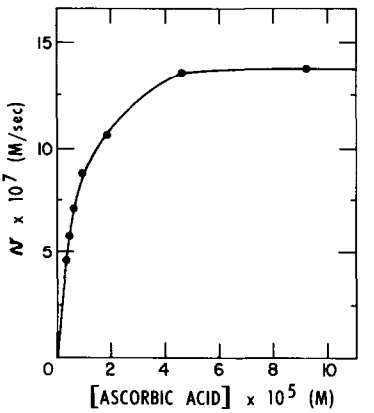

Fig. 2 The rate of oxidation of ascorbic acid as a function of its concentration.

The oxidation of ascorbic acid was measured under the same conditions as specified in Fig. 1A for curve I except for the variations in ascorbic acid concentration. The rate of ascorbic acid oxidation coupled to the xanthine oxidase reaction (v) was obtained by subtracting the rate of spontaneous oxidation from the rate observed in the presence of the xanthine-xanthine oxidase system.

$O_2^{\cdot-}/O_2$ (-0.33 V) is lower than that of the couple H^+, A/AH$^\cdot$; AH$^\cdot$ radical is unable to reduce O_2 to $O_2^{\cdot-}$, as indicated by Yamazaki et al. (12). Assuming that reaction 2 proceeds faster than reaction 1 under the experimental conditions, the rate of disappearance of $O_2^{\cdot-}$ (V_A) is two times the rate of reaction 1, and the latter rate is equal to the observed rate of oxidation of ascorbic acid to dehydroascorbic acid, and V_A can be formulated as follows:

$$V_A = 2\, k_A\, [AH_2]\, [O_2^{\cdot-}] \qquad (3)$$

Figure 2 shows the initial rate of oxidation of ascorbic acid accelerated by the xanthine oxidase system as a function of the ascorbic acid concentration. It may be noted that the rate shows saturation with respect to the concentration of ascorbic acid. At a saturating level of ascorbic acid, the rate of spontaneous dismutation of $O_2^{\cdot-}$ becomes negligible compared with that of disappearance of $O_2^{\cdot-}$ through reactions 1 and 2. In the presence of superoxide dismutase under such a condition, $O_2^{\cdot-}$ disappears mostly through reactions 1 and 2 and superoxide dismutase-catalyzed reaction.

$$2\, O_2^{\cdot-} + 2\, H^+ \xrightarrow{k_{SOD}} O_2 + H_2O_2 \qquad (4)$$

Therefore, the rate of generation of $O_2^{\cdot-}$ (V) is the sum of the rate of disappearance of $O_2^{\cdot-}$ through reactions 1 and 2 and that of reaction 4 (V_{SOD}).

$$V = V_A + V_{SOD} \qquad (5)$$

Since the catalytic reaction of superoxide dismutase is first order with respect to enzyme and to $O_2^{\cdot-}$ (13, 14), the rate of this reaction is represented as:

$$V_{SOD} = k_{SOD}\, [SOD]\, [O_2^{\cdot-}] \qquad (6)$$

The following equation can be derived from equations 3, 4 and 6.

$$\frac{V}{V_A} = 1 + \frac{k_{SOD}}{2\, k_A\, [AH_2]}\, [SOD] \qquad (7)$$

A similar equation was previously derived for the reduction of cytochrome c in the xanthine oxidase reaction (15). The effect of varied concentrations of

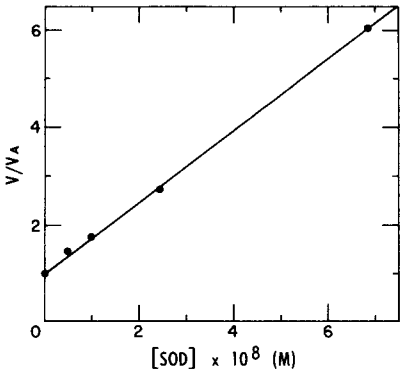

Fig. 3 Relationship between V/V_A and [SOD].

The oxidation of ascorbic acid was measured under the same conditions as specified in Fig. 1A for curve I except for the addition of varying concentrations of superoxide dismutase (SOD). For the definitions of V and V_A, see the text.

superoxide dismutase on the rate of oxidation of ascorbic acid at a saturating level of the latter (4.6×10^{-5}M) was examined. When the data were plotted according to Eq. 7, a straight line, intersecting the ordinate at a point of $V/V_A = 1$, was obtained. From its slope, the value of k_A was calculated to be 2.7×10^5 M^{-1} sec^{-1}, assuming $k_{SOD} = 1.9 \times 10^9$ M^{-1} sec^{-1} (13). Although k_A is fairly small compared to k_{SOD}, it seems likely that V_A is comparable to V_{SOD} in view of the fact that the tissue content of ascorbic acid is fairly high. For example, the amounts of ascorbic acid and superoxide dismutase in human adrenal gland are approximately 0.5 mg/g (16) and 50 μg/g (17), respectively. If we assume that both are uniformly distributed in cells, their concentrations are of the order of 10^{-3}M and 10^{-6}M, respectively. Therefore, V_A and V_{SOD} become comparable to each other in a tissue like adrenal gland whose content of ascorbic acid is high. It should be noted that the semiquinone of ascorbic acid does not reduce O_2 to $O_2^{\cdot-}$ like other semiquinones of naturally occurring compounds such as flavin and NADH (18, 19). Thus it is likely that ascorbic acid functions as a defense against $O_2^{\cdot-}$ in addition to superoxide dismutase. It has been reported that ascorbic acid protects against oxygen poisoning (20, 21). This effect of the vitamin seems to be related to its role as a scavenger of $O_2^{\cdot-}$.

ACKNOWLEDGMENT

The author thanks Dr. Sidney Udenfriend for his helpful comments on the preparation of this manuscript.

REFERENCES

1. Fridovich, I. (1974) in Molecular Mechanism of Oxygen Activation, (Hayaishi, O., Ed.) pp. 453-477, Academic Press, New York.
2. McCord, J.M., Keele, B. B. Jr., and Fridovich, I. (1971) Proc. Natl. Acad. Sci. 68, 1024-1027.
3. Gregory, E. M., and Fridovich, I. (1973) J. Bacteriol. 114, 1193-1197.
4. Gregory, E. M., Goscin, S. A., and Fridovich, I. (1974) 117, 456-460.
5. Barr, N. F., and King, C. G. (1956) J. Am. Chem. Soc. 78, 303-305.
6. Michelson, A. M. (1973) Biochimie 55, 465-479.
7. Elstner, E. F., and Kramer, R. (1973) Biochim. Biophys. Acta 340-353.
8. McCord, J. M., and Fridovich, I. (1969) J. Biol. Chem. 244, 6049-6055.
9. Carpéni, G. E. (1938) Helv. Chim. Acta 21, 1031-1036.
10. Lowry, O. H., Rosenbrough, N. J., Farr, A. L., and Randall, R. J. (1951) J. Biol. Chem. 193, 265-275.
11. Wood, P. M. (1974) FEBS Lett. 44, 22-24.
12. Yamazaki, I., Yamazaki, H., Tamura, M., Ohnishi, T., Nakamura, S., and Iyanagi, T. (1968) in Oxidation of Organic Compounds, Vol. 3 (Gould, F. G., Ed.) pp. 290-306, American Chemical Society, Washington, D. C.
13. Rotilio, G., Bray, R. C., and Fielden, M. (1972) Biochim. Biophys. Acta 268, 605-609.
14. Klug-Roth, D., Fridovich, I., and Rabani, J. (1972) J. Am. Chem. Soc. 95, 2786-2790.
15. Sawada, Y., and Yamazaki, I., (1973) Biochim. Biophys. Acta 327, 257-265.
16. Yanovsky, M., Almaden, P., and King. C. G. (1934) J. Biol. Chem. 106, 525-529.
17. Hartz, J. W., Funakoshi, S., and Deutsch, H. F. (1973) Clin. Chim. Acta 46, 125-132.
18. Vaish, S. P., and Tollin, G. (1971) Bioenergetics 2, 61-72.
19. Land, E. J., and Swallow, A. J. (1971) Biochim. Biophys. Acta 234, 34-45.
20. Benbough, J. E. (1969) J. Gen. Microbiol. 56, 241-250.
21. Jamieson, D., and Van den Brenk, H. A. S. (1964) Biochem. Pharmacol. 13, 159-164.