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A kinetic study on iron stimulation of the xanthine oxidase dependent oxidation of ascorbate

Rolf A. Løvstad
Department of Medical Biochemistry, Institute of Basic Medical Sciences, University of Oslo, PO Box 1112 Blindern, N-0317 Oslo, Norway (Tel: +47 22 85 11 01; Fax: +47 22 85 10 58)

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Abstract

Xanthine oxidase reduces molecular oxygen to H_2O_2 and superoxide radicals during its catalytic action on xanthine, hypoxanthine or acetaldehyde. Ascorbate is catalytically oxidized by the superoxide radicals generated, when present in the reaction solution (Nishikimi 1975). The present study shows that iron ions markedly stimulate the enzyme dependent ascorbate oxidation, by acting as a red/ox-cycling intermediate between the oxidase and ascorbate. An apparent K_m-value of 10.8 μM characterized the iron stimulatory effect on the reaction at pH 6.0. Reduced transition-state metals can be oxidized by H_2O_2 through a Fenton-type reaction. Catalase was found to reduce the effect of iron on the enzyme dependent ascorbate oxidation, strongly suggesting that H_2O_2, produced during catalysis, is involved in the oxidation of ferrous ions.

Introduction

Xanthine oxidase catalyzes the reaction between xanthine, hypoxanthine or acetaldehyde with molecular oxygen. It has been demonstrated that the enzyme is capable of both univalent and divalent reduction of molecular oxygen, thus releasing both superoxide radicals and H_2O_2 (McCord & Fridovich 1968; Knowles *et al.* 1969; Nakamura & Yamazaki 1969; Fridovich 1970). At pH 7 about 20% of the total electron flux through the enzyme could be accounted for in terms of univalent reduction of oxygen, the fraction decreasing with decreasing pH (Fridovich 1970). It has been suggested that the rapid enzymic generation of superoxide radicals and H_2O_2 might lead to severe tissue damage in hypoxic tissue during reoxygenation (McCord 1987).

Nishikimi (1975) reported that ascorbate was catalytically oxidized by xanthine oxidase during its interaction with xanthine. The apparent ascorbate oxidase activity was found to involve superoxide radicals generated in the process. It was suggested that ascorbate might function as a defense against superoxide radicals. The present kinetic study shows that the rate of the xanthine oxidase dependent oxidation of ascorbate is markedly increased in the presence of iron ions. A mechanism for the stimulatory effect of iron is proposed.

Materials and methods

Xanthine oxidase (EC 1.1.3.22), catalase (EC 1.11.1.6), conalbumin (iron-free), and ascorbate were purchased from Sigma Chemical Company (St. Louis, Missouri, USA); acetaldehyde, $(NH_4)_2Fe(SO_4)_2.6H_2O$ and H_2O_2 from E. Merck AG (Darmstadt, Germany).

Xanthine oxidase activity was determined by the rate of uric acid production at 295 nm (ε = 11.0 mM^{-1} cm^{-1}) with 0.1 mM xanthine at pH 7.5 at 25 °C (1 U = 1 μmole urate/min). The rate of ascorbate oxidation was obtained on the basis of its millimolar extinction coefficient, ε_{265} = 15.3 mM^{-1} cm^{-1}. Acetaldehyde was used as substrate for xanthine oxidase, since it does not absorb light at 265 nm. A saturating concentration of 10 mM was used in the experiments. Spectrophotometric mea-

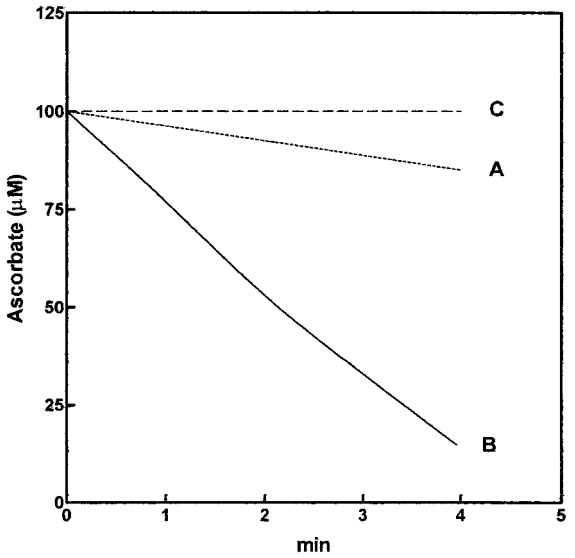

Fig. 1. Effect of xanthine oxidase on ascorbate oxidation in the absence (A) and presence (B) of 50 μM iron ions. A solution of 30 mU/ml xanthine oxidase was mixed with a solution of 10 mM acetaldehyde and 0.1 mM ascorbate (± Fe(II)) in 0.2 M sodium acetate buffer, pH 6.0 (T = 30 °C). C, Curve obtained when xanthine oxidase and iron were omitted from the system.

surements were performed in a Cecil 292 instrument connected to a Radiometer Rec 80 recorder.

Results and discussion

The time course curves in Figure 1 show that addition of xanthine oxidase to a solution of acetaldehyde and ascorbate increased the rate of ascorbate oxidation, as previously observed by Nishikimi (1975), due to the action of superoxide radicals generated in the process. Addition of 50 μM Fe(II)-ions further increased the rate of the enzyme dependent oxidation of ascorbate. When xanthine oxidase was omitted from the system no significant reaction took place (Figure 1).

The effect of pH on the rate of the iron stimulated reaction was measured. As shown in Figure 2 a maximum around pH 6 was obtained. The enzymic reaction taking place in the absence of iron was approx 10% of the total activity at this pH, increasing with pH, because the fraction of oxygen reduced to superoxide radicals increases with increasing pH (Fridovich 1970). The rate of ascorbate oxidation due to ferrous ion autoxidation was negligible in acetate buffer, pH 6.0. It was corrected for the ascorbate oxidation rate observed when iron was omitted from the system. At 6 < pH < 7 it was also necessary to correct for

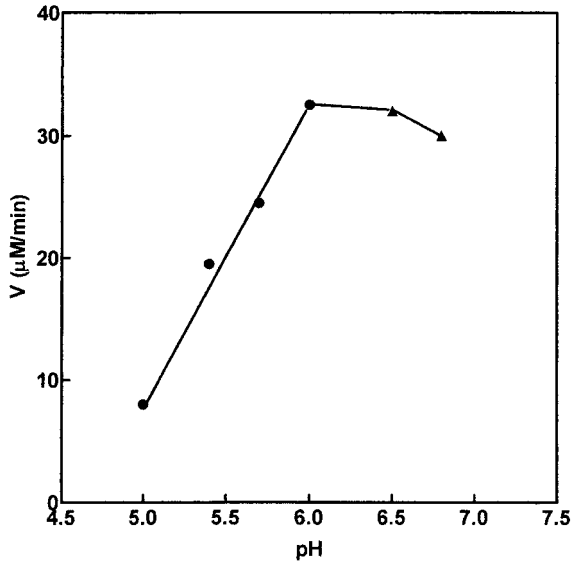

Fig. 2. Effect of pH on the stimulatory effect of iron on the xanthine oxidase dependent oxidation of ascorbate. The reaction solution contained 47 mU/ml xanthine oxidase, 10 mM acetaldehyde, 0.1 mM ascorbate and 0.1 mM iron ions in 0.2 M sodium acetate buffer (●), or 10 mM Hepes buffer (▲) (T = 30 °C).

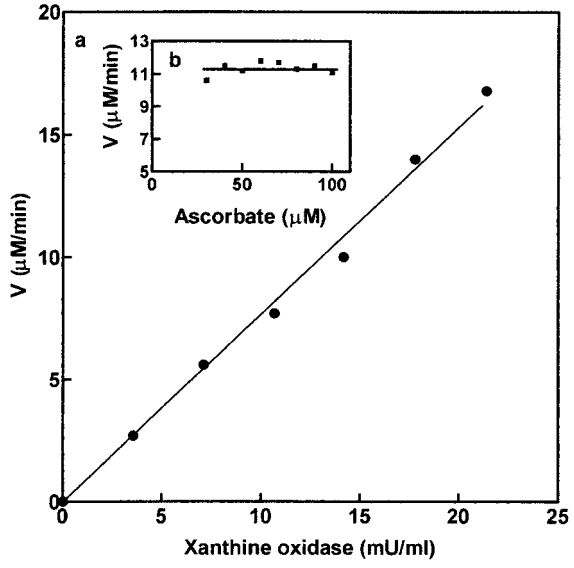

Fig. 3. a. Effect of xanthine oxidase concentration on the iron stimulated oxidation of ascorbate. The reaction solutions contained xanthine oxidase (3.6–21.4 mU/ml), 10 mM acetaldehyde, 0.1 mM ascorbate and 0.1 mM iron ions in 0.2 M sodium acetate buffer, pH 6.0 (T = 30 °C). b. Ascorbate oxidation rate at various ascorbate concentrations (30–100 μM) in the presence of 15 mU/ml xanthine oxidase, keeping conditions otherwise as decribed above.

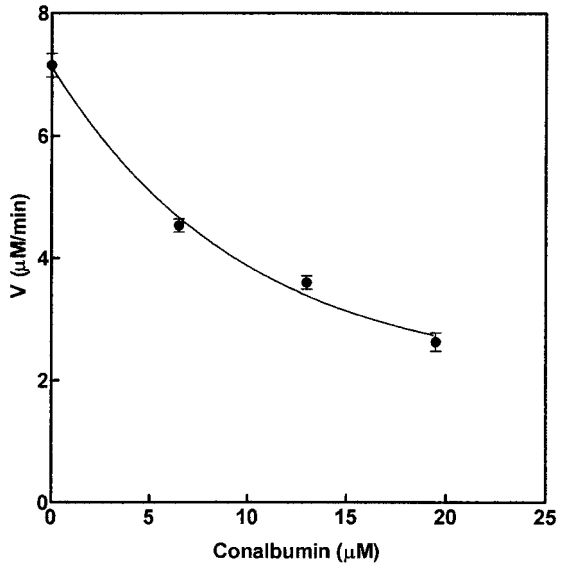

Fig. 4. Effect of conalbumin (iron-free) on the iron stimulated oxidation of ascorbate in the presence of xanthine oxidase. The reaction solutions contained 9 mU/ml xanthine oxidase, 10 mM acetaldehyde, 0.1 mM ascorbate, 50 μM iron ions and conalbumin (6.5–19.5 μM) in 0.2 M sodium acetate buffer, pH 6.0. (T = 30 °C). Mean values ± SEM (n = 3) are shown.

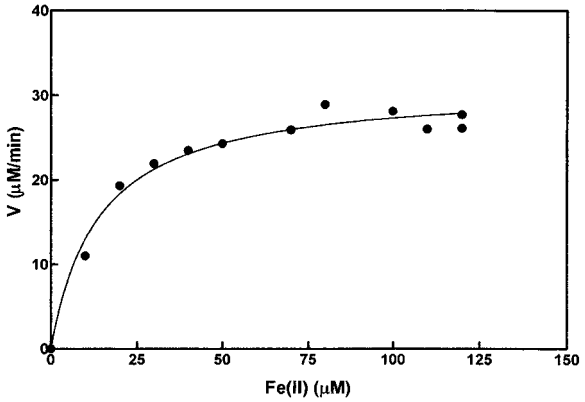

Fig. 5. Effect of iron ions, added as Fe(II) , on the rate of ascorbate oxidation in the presence of xanthine oxidase. The reaction solutions contained 27 mU/ml xanthine oxidase, 10 mM acetaldehyde, 0.1 mM ascorbate and iron ions (10–120 μM) in 0.2 M sodium acetate buffer, pH 6.0 (T = 30 °C).

ferrous ion autoxidation, representing about 3% and 11% of the total ascorbate oxidation rate at pH 6.5 and 6.8, respectively. At pH >7 formation of soluble iron polymer products was detected in the reaction solutions, and it was thus not possible to estimate the activating effect of iron at physiological pH. The following experiments were performed in acetate buffer, pH 6.0.

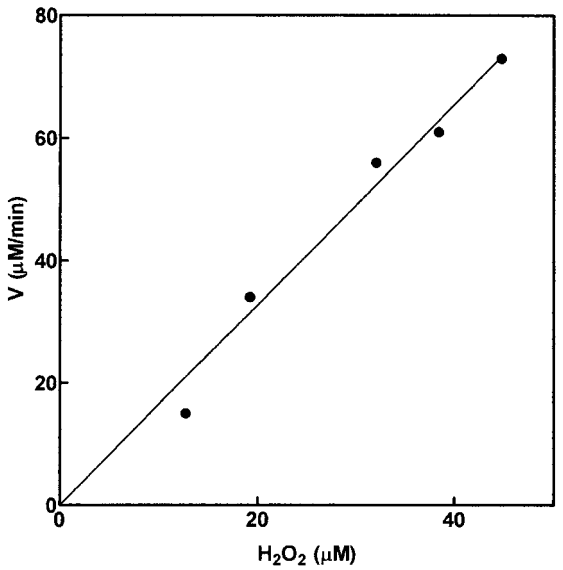

Fig. 6. Rate of Fe(II) oxidation as a function of H_2O_2 concentration. The reaction solutions contained 0.1 mM Fe(II), 65 μM conalbumin (iron-free) and H_2O_2 (13–45 μM) in 0.2 M sodium acetate buffer, pH 6.0 (T = 30 °C).

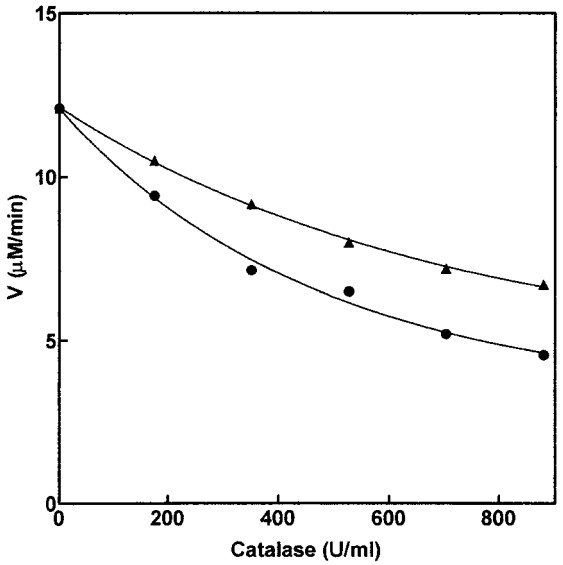

Fig. 7. Effect of catalase (●) on the iron ion stimulated oxidation of ascorbate in the presence of xanthine oxidase. The reaction solutions contained 16 mU/ml xanthine oxidase, 10 mM acetaldehyde, 0.1 mM ascorbate, 0.1 mM iron ions and catalase (175–880 U/ml) in 0.2 M sodium acetate buffer, pH 6.0 (T = 30 °C). ▲, Activity obtained when 0.5 mM azide was added to the reaction system.

The relationship between enzyme concentration and the rate of ascorbate oxidation, caused by the presence of 0.1 mM iron, was tested. A linear correlation between oxidation rate and xanthine oxidase concentration was obtained, as shown in Figure 3a. The reaction rate was found to be independent of ascorbate concentration, when it was kept higher than 30 μM (Figure 3b).

Topham *et al.* (1986) have demonstrated that xanthine oxidase promotes the incorporation of ferric ions into apotransferrin by catalyzing the oxidation of ferrous ions, thus functioning as a ferroxidase. The possibility therefore existed, that the stimulatory effect of iron could be due to interaction between ascorbate and ferric ions generated during the action of xanthine oxidase on acetaldehyde. Figure 4 shows that conalbumin, which strongly complexes two ferric ions per molecule, inhibited the iron enhanced oxidation of ascorbate; the observation supporting the suggestion that ascorbate is oxidized by ferric ions.

The rates of enzyme dependent ascorbate oxidation, as the result of iron stimulation, was measured at various concentrations of added Fe(II) (10–120 μM). As shown in Figure 5 a hyperbolical curve was obtained when the activity was plotted against iron concentration, the activity approaching a maximum value (V_{max}). From the data an apparent K_m-value of 10.8 ± 1.4 (SE) μM and a V_{max}-value of 33.4 ± 0.8 (SE) μM/min were calculated by means of a computer program published by Cleland (1967). From the V_{max}-value it is estimated that 1 U/ml xanthine oxidase oxidized 1.2 μmoles ascorbate per minute.

Most of the oxygen reduced by xanthine oxidase during catalysis at pH ≤ 7 is released as H_2O_2 (Fridovich 1970). Reduced transition-state metals can participate in a Fenton-type reaction, decomposing H_2O_2 to form the harmful hydroxyl radicals through an oxidation process, as shown in reaction 2 in the case of iron. Figure 6 shows a linear relationship between the rate of Fe(II) oxidation and the amount of added H_2O_2. Ferric ion formation was determined by adding conalbumin to the reaction solution and spectrophotometrically monitoring the appearance of the Fe(III)-conalbumin complex at 460 nm, as described by Johnson *et al.* (1967). From the plot in Figure 6 a second order rate constant, $k_2 = V/[Fe(II)][H_2O_2]$, of 16 mM^{-1} min^{-1} was estimated. Figure 7 shows that the stimulatory effect of iron on the xanthine oxidase dependent oxidation of ascorbate decreased in the presence of catalase, which transforms H_2O_2 to H_2O and oxygen. The action appeared to involve the catalytic activity of catalase since azide, an inhibitor, reduced the effect. Azide did not significantly affect the reaction in the absence of catalase. The results strongly suggest that H_2O_2, produced during catalysis, is indeed involved in the oxidation of ferrous ions. The following reaction mechanism is proposed:

$$O_2 \xrightarrow{\text{XO, Acetaldehyde}} H_2O_2 + {}^{\bullet}O_2^- \quad (1)$$

$$Fe(II) + H_2O_2 \longrightarrow Fe(III) + OH^{\bullet} + OH^- \quad (2)$$

$$Fe(III) + Asc \longrightarrow Fe(II) + {}^{\bullet}Asc^+ \quad (3)$$

(XO = xanthine oxidase, ${}^{\bullet}Asc^+$ = ascorbate radical) Iron functions as a red/ox-cycling intermediate between ascorbate and xanthine oxidase generated H_2O_2, thus promoting the enzyme catalyzed consumption of ascorbate, as well as increasing the formation of hydroxyl radicals. Iron has also been shown to dramatically enhance the reaction between ceruloplasmin (ferroxidase) and several organic diamines and ascorbate through its ability to pass electrons from substrate to enzyme (Curzon 1961; McDermott *et al.* 1967). Enzymic oxidation of the reduced form of transition-state metals may create pseudsubstrates, comprising numerous reducing agents that rapidly can reduce the oxidized form of the metal ion, as discussed by Frieden & Hsieh (1976).

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