Release of iron from ferritin by xanthine oxidase

Role of the superoxide radical

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Mobilization of iron from ferritin by xanthine oxidase was studied under aerobic and anaerobic conditions. Aerobic iron release amounted to approx. 3.7 nmol/ml in 10 min. This amount was decreased by approx. 30% under anaerobic conditions. Aerobic iron mobilization involved two mechanisms. About 70% was released by $O_2^{\cdot-}$ generated by xanthine oxidase. The rest was released by $O_2^{\cdot-}$ -independent mechanisms, which also accounted for the total iron release when O_2 was absent. A possible transfer of reducing equivalents directly from xanthine oxidase to ferritin is discussed. The results imply that, in pathological conditions with increased formation of $O_2^{\cdot-}$, iron may be released from ferritin. Furthermore, in hypoxic tissues xanthine oxidase can release iron from ferritin by an $O_2^{\cdot-}$ -independent process. Free iron is liable to catalyse the formation of the extremely reactive and damaging $OH^{\cdot}$ radical.

INTRODUCTION

Iron is an effective catalyst for the formation of radicals from $O_2^{\cdot-}$ and H_2O_2 . In the overall Haber–Weiss reaction, Fe^{3+} is reduced by $O_2^{\cdot-}$ to Fe^{2+} , which in a second step (the Fenton reaction) reacts with H_2O_2 to generate the extremely reactive hydroxyl radical ($OH^{\cdot}$). In biological tissues $OH^{\cdot}$ can attack adjacent molecules and cause irreparable damage (Halliwell & Gutteridge, 1986).

In vivo most of the iron is bound to haem proteins and non-haem proteins, and is therefore not directly available to catalyse the Fenton reaction. The existence of an intracellular mobile pool of reactive low- M_r iron complexes has been postulated, but not directly demonstrated (Jacobs, 1977; Crichton, 1984). If the pool exists, it seems to be so small and rigorously regulated that it may be of minor importance.

On the other hand, under certain circumstances iron-binding proteins may release iron and facilitate the formation of $OH^{\cdot}$. An obvious candidate is the widely distributed iron-storage protein ferritin. There is evidence that ferritin can stimulate peroxidation of liposomes (O'Connell *et al.*, 1985) and of liver microsomal fractions (Koster & Slee, 1986), most probably by increasing the production of $OH^{\cdot}$ (Carlin & Djursæter, 1984).

Fe^{2+} can be released from ferritin by reductants small enough to pass through channels in the protein shell that surrounds the central iron core of the ferritin molecule (Jones *et al.*, 1978; Ford *et al.*, 1984). Biemond *et al.* (1984) and Thomas *et al.* (1985) have presented diverging results with respect to the capability of $O_2^{\cdot-}$ to effect the release of Fe^{2+} from ferritin. Both found that iron was mobilized from ferritin by xanthine oxidase. Finding that iron release could be completely inhibited by SOD, Thomas *et al.* (1985) concluded that $O_2^{\cdot-}$ was the active iron-mobilizing species. Biemond *et al.* (1984), however, found no blocking effect of SOD on the release of iron from ferritin by xanthine oxidase. They therefore suggested that probably electrons were transferred from

the enzyme–substrate complex to ferritin via an unknown mechanism. On the other hand, using stimulated granulocytes as a source of $O_2^{\cdot-}$, Biemond *et al.* (1984) also demonstrated release of ferritin iron that could be partly inhibited by SOD.

Although remaining a matter of debate, there is some evidence suggesting a possible specific role for xanthine oxidase in the mobilization and utilization of ferritin iron in the body, at least in hypoxic or anaerobic tissues (Mazur *et al.*, 1958; Grace *et al.*, 1970; Duggan & Streeter, 1973; Topham *et al.*, 1982). Xanthine oxidase should therefore not be considered solely as a neutral $O_2^{\cdot-}$ -generating system.

The aim of the present study was to show if $O_2^{\cdot-}$ can mobilize iron from ferritin and to emphasize the significance of $O_2^{\cdot-}$ in the mobilization of iron from ferritin by xanthine oxidase.

MATERIALS AND METHODS

Reagents

Xanthine (grade III), xanthine oxidase (milk, grade IV), SOD (from bovine erythrocytes), cytochrome *c* (horse heart), catalase (bovine liver), uricase (type 1, pig liver), horse spleen apoferritin and BPS were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). KO_2 came from Fluka A.G., Buchs, Switzerland.

No cytochrome oxidase activity was found in the cytochrome *c* preparation when it was tested for as recommended by Schacter (1985).

No catalase activity could be detected in the SOD preparation when it was tested for as described by Bergmeyer (1974).

One unit of xanthine oxidase activity was defined as 1 μ mol of uric acid produced/min at 25 °C at pH 7.0.

Reagents were passed through a Chelex 100 chelating ion-exchange resin (Bio-Rad Laboratories, Richmond, CA, U.S.A.), and all glassware was washed with acid to remove contaminating iron and other metals.

Abbreviations used: SOD, superoxide dismutase; BPS, bathophenanthrolinesulphonate.

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Preparation of ferritin

Fractions of ferritin with different contents of Fe^{3+} were prepared by the addition of various amounts of $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2$ to a solution containing 1 mg of apo-ferritin/ml, 20 mM- KIO_3 , 80 mM- $\text{Na}_2\text{S}_2\text{O}_3$ and 10 mM-Hepes/NaOH buffer, pH 7.4 (Harrison *et al.*, 1975; Ulvik & Romslo, 1978). After the formation of ferritin, the samples were passed through a Sephadex G-50 column and thereafter through a Chelex 100 chelating ion-exchange resin to remove excess reagents and non-specifically bound iron.

The iron content of ferritin was determined by atomic absorption spectrophotometry, with iron wire dissolved in 0.1 mM-HCl as a standard. Protein was determined by the method of Bradford (1976), with bovine serum albumin as standard.

Assay of uric acid, superoxide and iron released from ferritin

Uric acid was determined by increase in the absorbance at 292 nm ($\epsilon = 12.5 \text{ mM}^{-1} \cdot \text{cm}^{-1}$) (Hochstein *et al.*, 1984).

$\text{O}_2^{\cdot -}$ was determined as the SOD-sensitive reduction of cytochrome *c* measured as the change in absorbance at 550 nm (McCord & Fridovich, 1969) (Fig. 1). The absorption coefficient $18.5 \text{ mM}^{-1} \cdot \text{cm}^{-1}$ was the difference between the absorbances of reduced and oxidized cytochrome *c* (Margoliash & Frohwirt, 1959).

Release of ferrous iron from ferritin was determined by measuring the increase in $\Delta(A_{530} - A_{700})$ caused by the formation of the $\text{Fe}^{2+}(\text{BPS})_3$ complex with $\epsilon = 22.14 \text{ mM}^{-1} \cdot \text{cm}^{-1}$ (Ulvik & Romslo, 1979).

The incubations were performed either in a thermostatically controlled Varian 2300 instrument or in a Hewlett-Packard 8450A diode-array spectrophotometer.

Incubations

Under standard conditions at 37 °C, the following concentrations were used in a volume of 1 ml: 10 mM-Hepes/NaOH buffer, pH 7.0, 0.02 unit of xanthine oxidase/ml, 100 μM -xanthine, 60 μM -cytochrome *c*, 0.2 mg of catalase/ml, 0.08 mg of SOD/ml, 1 mM-BPS and 0.8 μM -ferritin protein with an iron load as indicated in legends to the Figures. Changes in the incubation mixture were as described in the legends to the Figures.

A near-anaerobic condition was obtained in a Thunberg cuvette by first heating the incubation medium without enzyme to 60 °C. Thereafter the cuvette was purged with 99.9%-pure N_2 for 10 min and then cooled to 37 °C before the reaction was started by adding enzyme from the side arm. The efficiency of this process was confirmed in separate experiments where the absorption spectrum for haemoglobin was changed from that of oxyhaemoglobin to that of deoxyhaemoglobin during the procedure (Winterhalter, 1974).

The intact function of xanthine oxidase after this process was controlled in separate experiments where the rate of uric acid production was measured after addition of O_2 to the medium.

RESULTS

Superoxide formation

Fig. 1 shows the time course of the xanthine oxidase reaction, including consumption of O_2 , formation of uric acid and reduction of cytochrome *c*. Catalase led to an

Fig. 1. Consumption of O_2 , formation of uric acid and reduction of cytochrome *c* by xanthine oxidase: effect of catalase and SOD

The incubation mixture contained 0.02 unit of xanthine oxidase/ml, 100 μM -xanthine and 60 μM -cytochrome *c* in 10 mM-Hepes buffer, pH 7.0, at 37 °C. Uric acid and reduced cytochrome *c* were determined as described in the text. The O_2 concentration was measured with a vibrating platinum electrode. ·····, Consumption of O_2 ; —, formation of uric acid; — — —, reduction of cytochrome *c*; — — — —, reduction of cytochrome *c* in the presence of 0.4 mg of catalase/ml; — · — · —, reduction of cytochrome *c* in the presence of 0.08 mg of SOD/ml.

increase in reduced cytochrome *c*, most probably by preventing reoxidation by H_2O_2 formed simultaneously with $\text{O}_2^{\cdot -}$. This interpretation was confirmed in separate experiments without xanthine oxidase, where reduced cytochrome *c* was readily oxidized by H_2O_2 , an effect that was inhibited by catalase (results not shown).

Increasing concentrations of ferritin had no significant effect on the constant rate of cytochrome *c* reduction (results not shown).

SOD-sensitive release of iron from ferritin

When ferritin and BPS were mixed with xanthine/xanthine oxidase, about $3 \mu\text{M}$ - $\text{Fe}^{2+}(\text{BPS})_3$ was formed during the first 5–6 min of the incubation (Fig. 2a). After this rapid phase the release of iron from ferritin continued linearly and at a much lower rate, with about $0.12 \mu\text{M}$ - $\text{Fe}^{2+}(\text{BPS})_3$ formed per min. In the presence of SOD the rapid initial phase of iron mobilization almost completely disappeared, although the linear steady-state reaction rate was maintained. Catalase did not change the time course of ferritin iron release, but increased the amount of chelated Fe^{2+} by 12–14%.

The importance of xanthine oxidase was evident from the nearly complete absence of $\text{Fe}^{2+}(\text{BPS})_3$ formation when either the enzyme or its substrate xanthine was omitted from the incubation medium.

Chelation of 'background iron' in the absence of ferritin depended strictly on the complete xanthine/xanthine oxidase reaction (Fig. 2a), and was decreased to a minimum by SOD (results not shown).

Fig. 2. Mobilization of iron from ferritin (a) by xanthine oxidase in aerobic conditions and (b) by KO_2 : effect of catalase and SOD

(a) The incubation mixture contained 0.02 unit of xanthine oxidase/ml, 100 μM -xanthine, 1 mM-BPS and 0.8 μM -ferritin (approx. 1400 Fe^{3+} atoms/molecule) in 10 mM-Hepes buffer, pH 7.0. Iron release was determined as the formation of the $\text{Fe}^{2+}(\text{BPS})_3$ complex (see the text). ●, Release of iron from ferritin; △, release of iron in the presence of 0.4 mg of catalase/ml; ○, release of iron in the presence of 0.08 mg of SOD/ml; □, control without ferritin; ▲, control without xanthine oxidase. (b) The incubation mixture contained 0.8 μM -ferritin (approx. 1000 Fe^{3+} atoms/molecule) and 1 mM-BPS in 10 mM-Hepes buffer, pH 7.0. Arrows indicate addition of a few grains of KO_2 . —, Release of iron from ferritin; ----, release of iron in the presence of 0.3 mg of SOD/ml.

The lack of effect of uricase on the formation of $\text{Fe}^{2+}(\text{BPS})_3$ ruled out any possible activity of uric acid generated by xanthine oxidase (results not shown).

Azide, a scavenger of singlet oxygen (Cadenas & Sies, 1984), had no influence on $\text{Fe}^{2+}(\text{BPS})_3$ formation.

Fig. 3. Mobilization of iron from ferritin by xanthine oxidase in aerobic and anaerobic conditions: effect of SOD

Near-anaerobiosis was obtained as described in the text. The incubation mixture contained 0.02 unit of xanthine oxidase/ml, 100 μM -xanthine, 1 mM-BPS and 0.8 μM -ferritin (approx. 1400 Fe^{3+} atoms/molecule) in 10 mM-Hepes buffer, pH 7.0. Release of iron from ferritin was determined in an aerobic (●) and in a near-anaerobic (■) incubation mixture. Aerobic (○) and anaerobic (□) release of iron was also measured in the presence of 0.08 mg of SOD/ml. ▲, Anaerobic control without xanthine oxidase.

Generation of singlet oxygen by xanthine oxidase has been denied by Nagano & Fridovich (1985). However, according to others, it might result from disproportionation of $\text{O}_2^{\cdot-}$ or from reactions between intermediates in the Haber-Weiss reaction (Cadenas & Sies, 1984).

When KO_2 was used as source of $\text{O}_2^{\cdot-}$ instead of xanthine oxidase, the $\text{Fe}^{2+}(\text{BPS})_3$ complex was formed very rapidly, but its formation was effectively inhibited by SOD (Fig. 2b).

Iron release under anaerobic conditions

Under near-anaerobic conditions there was a time-dependent, almost linear, release of iron from ferritin (Fig. 3). After 10 min the amount of released iron was about 74% of the total release obtained in parallel aerobic experiments. Addition of SOD to eradicate residual $\text{O}_2^{\cdot-}$ led to some decrease in the mobilization of iron from ferritin. Anaerobic release of iron from ferritin was about 2 times that of the $\text{O}_2^{\cdot-}$ -independent iron release under aerobic conditions.

Omission of either xanthine or xanthine oxidase from the medium led to a considerable decrease in the amount of iron mobilized from ferritin, demonstrating the importance of a complete substrate-enzyme complex under anaerobic conditions also.

Effect of increasing ferritin concentrations and the iron load of ferritin

The amount of iron mobilized from ferritin with a fixed content of about 1400 Fe^{3+} atoms/molecule was increased with increasing concentrations of ferritin and

Fig. 4. Effect of increasing concentrations of ferritin on iron mobilization by xanthine oxidase

The incubation mixture contained 0.02 unit of xanthine oxidase/ml, 100 μM-xanthine and 1 mM-BPS in 10 mM-Hepes buffer, pH 7.0. The incubation time was 10 min. The concentration of ferritin (approx. 1400 Fe³⁺ atoms/molecule) was increased as indicated. Iron release was determined as described in the text.

Fig. 5. Effect of ferritin iron content on mobilization of iron by xanthine oxidase

The incubation mixture contained 0.02 unit of xanthine oxidase/ml, 100 μM-xanthine, 0.8 μM-ferritin and 1 mM-BPS in 10 mM-Hepes buffer, pH 7.0. The incubation time was 10 min. Mobilization of iron from ferritin fractions with different iron contents was determined as described in the text.

reached a plateau at about 1.2 μM (Fig. 4). Repeated experiments at each concentration of ferritin gave a variation coefficient of 1–5%.

At equimolar concentrations with respect to protein there was a positive correlation between the ferritin iron load and amount of iron mobilized. Approx. 40% less iron was removed from a ferritin containing 70 Fe³⁺ atoms/molecule compared with a ferritin containing 20 times more iron (Fig. 5).

DISCUSSION

We have shown that iron can be released from ferritin and chelated as Fe²⁺(BPS)₃ in the presence of xanthine/xanthine oxidase and O₂. The parallel occurrence of the most vigorous iron release (Fig. 2a), the formation of O₂^{•-} by xanthine oxidase (Fig. 1) and the marked inhibition of Fe²⁺(BPS)₃ formation by SOD (Fig. 2a) in the first minutes of the incubation period was strong evidence that this iron was mobilized from ferritin by O₂^{•-}.

The slight stimulating effect of catalase on iron release could be explained by the elimination of H₂O₂-dependent oxidation of Fe²⁺ (Thomas *et al.*, 1985), but possibly also by a higher sustained concentration of O₂ and O₂^{•-} resulting from stimulated breakdown of H₂O₂.

Topham *et al.* (1986) have shown that xanthine oxidase has a considerable ferroxidase effect that is independent of O₂^{•-} and H₂O₂. We do not know if this had any influence on the formation of Fe²⁺(BPS)₃ in our system, but, if so, our results would be underestimated.

The capability of O₂^{•-} to remove iron from ferritin was verified in experiments with KO₂ as source of O₂^{•-} (Fig. 2b).

Despite careful precautions to avoid contamination,

limited amounts of non-ferritin iron were chelated by the O₂^{•-}-dependent reaction (Fig. 2a). Some of this might be non-haem iron that originated from the enzyme. It is not clear if non-specific iron was chelated to the same extent when ferritin was present and competed for O₂^{•-}.

We conclude that O₂^{•-} can mobilize iron from ferritin, most probably by means of the generally accepted redox mechanism required for this process (Jones *et al.*, 1978; Watt *et al.*, 1985). In agreement with this, Biemond *et al.* (1984) reported that O₂^{•-} produced by the respiratory burst in stimulated granulocytes could release iron from ferritin. However, when they used xanthine oxidase or KO₂ as a source of O₂^{•-}, release of iron from ferritin could not be inhibited by SOD, which is a fundamental criterion for assignment of a function of O₂^{•-} (McCord & Fridovich, 1969). On the contrary, and in agreement with our results, Thomas *et al.* (1985) found that mobilization of iron from ferritin by xanthine oxidase and by KO₂ was sensitive to SOD. Similar results were reported by Williams *et al.* (1974). However, whereas they found that aerobic iron release by xanthine oxidase was inhibited by more than 95% by SOD, we found only about 70% inhibition in the presence of excess SOD (Fig. 2a). The aerobic SOD-insensitive iron release suggested a second, O₂^{•-}-independent, mechanism for the release of iron from ferritin by xanthine oxidase. In older studies xanthine oxidase has been assigned a role for the mobilization of iron from ferritin, but only under strict anaerobic conditions (Mazur *et al.*, 1958; Duggan & Streeter, 1973). In our study the course of the O₂^{•-}-independent iron release under aerobic conditions was linear right from the beginning of the reaction (Fig. 2a), as was the reaction pattern when iron was released from ferritin by xanthine oxidase under anaerobic conditions (Fig. 3).

We conclude from these experiments that iron can be mobilized from ferritin by xanthine oxidase in two different ways. In the presence of O_2 most of the iron is mobilized by a $O_2^{\cdot-}$ -dependent mechanism. As the $O_2^{\cdot-}$ -concentration declines iron is to an increasing extent released by another mechanism, which ultimately accounts for the total iron release when anaerobiosis is obtained. There is evidence that electrons can be transferred from xanthine oxidase to artificial electron acceptors such as Methylene Blue, Nitro Blue Tetrazolium and phenazine methosulphate and thus bypass the $O_2^{\cdot-}$ -forming flavin centres of the enzyme (Topham *et al.*, 1986). Indirectly our results support the presence of a similar pathway of electrons between xanthine oxidase and ferritin iron. The same was suggested by Koster *et al.* (1985), who in preliminary experiments showed that xanthine oxidase devoid of its flavin centres, and therefore incapable of generating $O_2^{\cdot-}$ and H_2O_2 , still retained 34% of its original ferritin-iron-releasing capacity.

The rather weak stimulating effect of the ferritin iron content on the amount of iron mobilized by $O_2^{\cdot-}$ (Fig. 5) agreed with a similar modest effect of the iron content on the generation of $OH^{\cdot}$ radicals (Bannister *et al.*, 1984) and on lipid peroxidation (Gutteridge *et al.*, 1983) when ferritin was used as iron source. Together with the saturation kinetics involved in the interaction between ferritin and xanthine oxidase (Fig. 4), this suggests that only a small fraction of the iron deposited in the ferritin molecule can be rapidly removed by $O_2^{\cdot-}$.

The capability of $O_2^{\cdot-}$ to mobilize iron from ferritin may have pathophysiological consequences. *In vivo*, $O_2^{\cdot-}$ that escapes from the immediate disproportionation by SOD may react with adjacent ferritin molecules, release Fe^{2+} and initiate radical-producing reactions. For instance, such events can be expected in inflammation with stimulated, $O_2^{\cdot-}$ -producing, granulocytes (Babior, 1984; Biemond *et al.*, 1984), and in tissues that are reperfused after hypoxia (White *et al.*, 1985; McCord, 1985). In hypoxia the NAD^+ -dependent xanthine dehydrogenase is readily converted into xanthine oxidase (McCord & Roy, 1982). With regard to the two mechanisms described in the present study, xanthine oxidase may be able to mobilize iron from ferritin both in the initial hypoxic or anaerobic phase during ischaemia, and later during reperfusion and reoxygenation of the tissue. The result will be an increase in the amount of reactive iron, which could explain the reperfusion injury (White *et al.*, 1985) of the affected tissue.

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