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Decay of superoxide catalyzed by ferritin

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Ferritin iron can be reduced by $O_2^{\cdot-}$, released, and form a Fe(II)–chelator complex. However, the thermodynamic influence of the chelator may disturb the reaction balance. We therefore excluded the chelator and measured instead the effect of ferritin on the decay of $O_2^{\cdot-}$, monitored by direct spectrophotometry at pH 9.5. Ferritin, but not apoferritin, accelerated the decay of $O_2^{\cdot-}$. Ferritin iron was apparently the responsible agent. The effect of ferritin was maintained after several bursts of $O_2^{\cdot-}$, and the ratio degraded $O_2^{\cdot-}$/released Fe(II) greatly exceeded one, consistent with a catalytic reaction.

Ferritin; Superoxide; Spectrophotometry; Kinetics; Catalysis

1. INTRODUCTION

The cytotoxic effect of $O_2^{\cdot-}$ and H_2O_2 can be strongly aggravated by iron ions and small molecular weight iron complexes. Being reduced and oxidized by $O_2^{\cdot-}$ and H_2O_2, respectively, iron acts as a catalyzing agent in the Haber-Weiss reaction which generates the extremely injurious hydroxyl radical [1].

Several in vitro studies indicate that $O_2^{\cdot-}$ may react with the core of ferritin and release Fe(II) [2–4]. These and other studies have suggested a pathogenetic role for ferritin at the cellular level, for instance, in inflammation with activated, $O_2^{\cdot-}$ producing neutrophils and in reperfusion injury of ischemic tissues [5–8].

In the iron release studies, however, only very few Fe(II) atoms/ferritin molecule could be released by $O_2^{\cdot-}$, as judged from the sensitivity of the reaction to superoxide dismutase. This was the case even after repeated exposures of ferritin to $O_2^{\cdot-}$ [9,10]. Furthermore, release of Fe(II) was assessed by using an artificial Fe(II) specific chelator with a redox potential typically in the range 0.8–1 V (e.g. bathophenanthroline disulphonic acid; BPS). The thermodynamic driving force introduced by the non-physiological chelator, may favour the formation of the Fe(II)–chelator complex [11,12]. Therefore, one should be careful to interpret the pathophysiological significance of these studies.

In the present study, we excluded the chelator from the medium, and instead of using release of iron as a measure for the interaction between ferritin and $O_2^{\cdot-}$, we monitored the rate of decay of $O_2^{\cdot-}$ by a direct spectrophotometric technique.

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2. EXPERIMENTAL

2.1. *Materials*

Horse spleen ferritin (Calbiochem AG, Luzern, Switzerland) with an average of 800 Fe(III)/molecule was incubated with EDTA for one hour followed by chromatography on a Sephadex G-100 column before use. Apoferritin (Sigma Chem. Co., St. Louis, MO, USA) contained up to 10 Fe(III)/molecule after chromatography. Catalase (EC 1.11.1.6) (Sigma) was passed through a Sephadex G-100 column to remove superoxide dismutase activity. Catalase activity was defined according to Aebi at pH 6.8 [13], and was essentially the same within the pH-range used in this study. Cu,Zn-superoxide dismutase (EC 1.15.1.1) (from bovine erythrocytes), 7.8 U/pM when assayed as in [14], bovine albumin, 2-amino-2-methyl-1-propanol and Tris were from Sigma. KO_2 (Fluka AG, Buchs, Switzerland) is a reactive reagent which should be handled very carefully [14]. Other chemicals were of the highest purity commercially available.

Protein was determined as in [15]. The iron content of ferritin, water and other chemicals was determined by atomic absorption spectrophotometry. Water was purified in the Milli-Q Water Purification System (Millipore Corp., Bedford, MA, USA) and was free of iron and copper. As a routine, all glassware was washed with acid. Buffers were filtered by a Millipore 0.22 μm (pore-size) filter prior to use.

2.2. *Assays*

The decay of $O_2^{\cdot-}$ was monitored in a Hewlett-Packard HP 8450A diode array spectrophotometer at 23°C, using a medium composed of 50 mM 2-amino-2-methyl-1-propanol, pH 9.5, 0.35 U/ml of catalase and 0.08 μM of ferritin protein (unless otherwise stated). From a solution of KO_2 dissolved in 50 mM NaOH, aliquots of approx. 15 μl were transferred to the medium and the declining concentration of $O_2^{\cdot-}$ was monitored as the decrease in absorbance ($A_{250} - A_{360}$) [14,16]. The rate of $O_2^{\cdot-}$ decay was generally expressed as the apparent pseudo-first-order rate constant calculated from the concentration interval 16 to 4 μM of $O_2^{\cdot-}$ [14]. The low concentration of ferritin gave no disturbing background absorbance.

$O_2^{\cdot-}$-dependent release of iron from ferritin was determined essentially as described previously, using xanthine oxidase as the source of $O_2^{\cdot-}$ and BPS as the Fe(II) chelator [9].

3. RESULTS

The decrease in absorbance ($A_{250} - A_{360}$) caused by

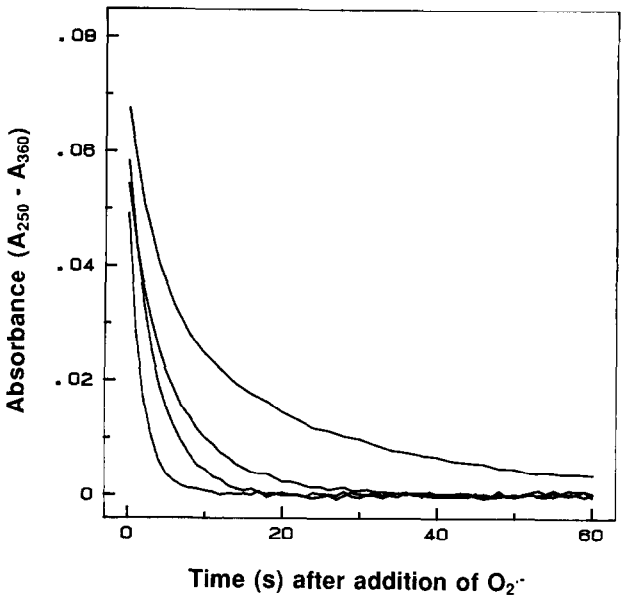

Fig. 1. Effect of ferritin on the decay of $O_2^{\cdot-}$. $O_2^{\cdot-}$ (from KO_2) was added to the incubation medium at time 0. Upper curve, without ferritin; below, with 0.04, 0.08 and 0.16 μM of ferritin, respectively.

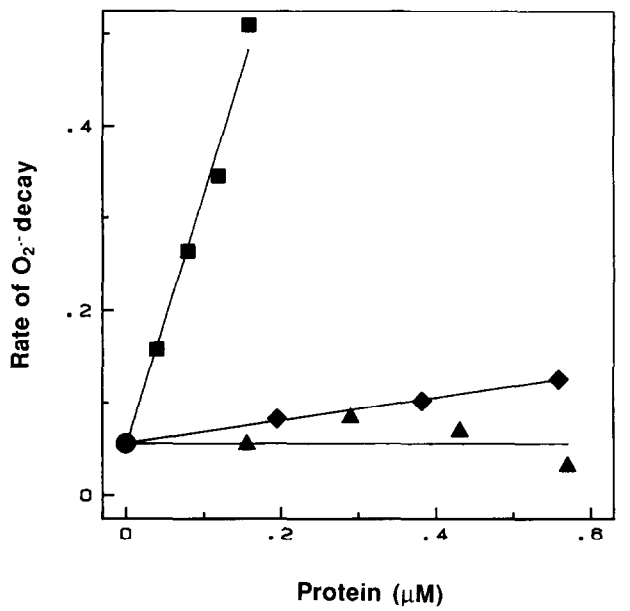

Fig. 2. Rate of $O_2^{\cdot-}$ decay in the presence of ferritin and albumin. The rate of $O_2^{\cdot-}$ decay was calculated as described in section 2. ■, ferritin; ◆, apoferritin; ▲, albumin.

the decay of $O_2^{\cdot-}$, and the stimulating effect of ferritin, is shown in Fig. 1. At the start of the reaction, the concentration ratio ($O_2^{\cdot-}$:ferritin) was about 300:1. By analyzing the $O_2^{\cdot-}$ decay curves as described in [16], we found that the ferritin-induced decay of $O_2^{\cdot-}$ was 1st-order in $O_2^{\cdot-}$, i.e. it appeared as the addition of a 1st-order reaction to the spontaneous 2nd-order dismutation of $O_2^{\cdot-}$. That means that ferritin behaved as a catalyst [16]. The rate of $O_2^{\cdot-}$ decay increased linearly with increasing amounts of ferritin (Fig. 2). In comparison, apoferritin had only a slight effect, and albumin had no effect on the $O_2^{\cdot-}$ decay.

When ferritin was exposed to repeated bursts of $O_2^{\cdot-}$ with approximately 5 min intervals, the rate of $O_2^{\cdot-}$ decay was about the same after each burst, although there was a slight decline after the third burst and onwards (Fig. 3). By analyzing the decay curves, we found that ferritin still acted catalytically after several bursts of $O_2^{\cdot-}$. The same pattern, but at approximately 25% higher rates, was obtained when ferritin was supplied with a single dose of Fe(III) (as $FeCl_3$) before the first burst of $O_2^{\cdot-}$ (Fig. 3). In comparison, addition of $FeCl_3$ to apoferritin or albumin had no effect on the $O_2^{\cdot-}$ decay, neither had $FeCl_3$ alone.

Because the direct spectrophotometric assay could not detect rate changes beyond the high spontaneous $O_2^{\cdot-}$ dismutation rate at a physiological pH [16], pH 9.5 was generally used. To get an idea of the importance of pH, we did the following. After demonstrating that replacing the buffer with Tris did not alter the results, pH was reduced step-wise from 9.5 towards 8.0. By compensating for the spontaneous dismutation, $O_2^{\cdot-}$ decay caused solely by ferritin was determined as the bimolecular rate constant for the reaction between ferritin and $O_2^{\cdot-}$ (Fig. 4). The reaction was about 15-fold faster at pH 8.0 than at pH 9.5.

In comparison, with xanthine oxidase as source of $O_2^{\cdot-}$

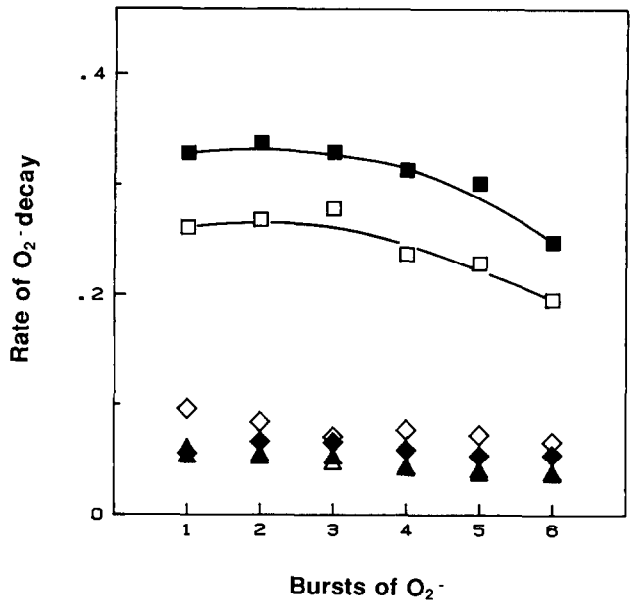

Fig. 3. Effect of repeated bursts of $O_2^{\cdot-}$. The incubation medium was exposed to repeated bursts of $O_2^{\cdot-}$. The protein concentration was 0.08 μM. The rate of $O_2^{\cdot-}$ decay is shown in the presence of: □, ferritin; ◇, apoferritin; △, albumin. Corresponding closed symbols, $FeCl_3$ added to a final concentration of 60 μM.

and BPS as chelator [9], $O_2^{\cdot-}$-dependent release of iron from ferritin was not more than 1.6-fold higher at pH 7.0 than at pH 9.5. The production of $O_2^{\cdot-}$ was kept unchanged, so that $O_2^{\cdot-}$ was not rate limiting in the iron release reaction (data not shown).

4. DISCUSSION

Ferritin accelerated the decay of $O_2^{\cdot-}$ in a dose-dependent manner (Figs. 1, 2), and iron was apparently responsible since the metal-free proteins had no effect.

Metal complexes (M) which catalyze the decay of $O_2^{\cdot-}$, do so after the following scheme where the reaction rates of (1) and (2) are equal [17–19]:

$$M^{n+} + O_2^{\cdot-} \rightarrow M^{(n-1)+} + O_2 \quad (1)$$

$$M^{(n-1)+} + O_2^{\cdot-} + 2H^+ \rightarrow M^{n+} + H_2O_2 \quad (2)$$

The total metal-induced reaction will (unless saturated) be first-order in $O_2^{\cdot-}$ when it is differentiated from the spontaneous dismutation. That was just what we found for the ferritin-induced decay of $O_2^{\cdot-}$. Reaction (2) explains why the reaction rate increased at lower pH. By extrapolating the pH-curve (Fig. 4), the bimolecular rate constant for the reaction between ferritin and $O_2^{\cdot-}$ was estimated to be about 10×10^6 $M^{-1} \cdot s^{-1}$ at pH 7.0. In comparison, in a pulse radiolysis study using competition kinetics with cytochrome *c*, Buettner et al. [20] found a value of $2 \pm 1 \times 10^6$ $M^{-1} \cdot s^{-1}$. The difference is most likely explained by different experimental conditions, as they used an indirect assay for the detection of $O_2^{\cdot-}$, and the concentration of ferritin was 5–60 times higher than in our study.

To be biologically active in aqueous fluids at physiological and alkaline pH, iron must bind to a ligand to avoid precipitation as insoluble Fe(III) hydroxide [21,22]. In vitro, loading of apoferritin with iron is most efficiently carried out by using Fe(II) and an oxidant [10,23]. However, Treffry and Harrison [22] showed that ferritin, but not apoferritin, was able to bind up to 200 extra iron atoms/ferritin molecule when iron was added as Fe(III). In line with this, the capacity to eliminate $O_2^{\cdot-}$ was permanently increased when ferritin was supplemented with a single dose of ferric iron (Fig. 3). The fact that iron added to apoferritin or albumin, had no effect, indicated a cooperation between the extra Fe(III) and the native iron already present in ferritin.

In general, the interaction between Fe(III) in ferritin and a reducing agent is carried out one-to-one [20]. However, in this study there was apparently no one-to-one relationship between the number of degraded $O_2^{\cdot-}$ molecules and the number of Fe(II) ions *released* by $O_2^{\cdot-}$. On the contrary, from the data of Fig. 3 it can be calculated [16] that at each burst of $O_2^{\cdot-}$, about 200 molecules of $O_2^{\cdot-}$ reacted with each ferritin molecule, which amounts to a total of about 1,000 $O_2^{\cdot-}$/ferritin molecule after 5 bursts. Obviously, a corresponding amount of iron was not released from the ferritin which contained about 800 Fe(III)/molecule. Even at the 6th burst the catalytic activity of ferritin was significant. We have previously shown in experiments, designed to detect reductive release of iron, that not more than 1–2 Fe(II) atoms/ferritin molecule were released after 5 bursts of $O_2^{\cdot-}$ [9]. This result was obtained with BPS as the Fe(II) chelator. The obvious divergence between $O_2^{\cdot-}$ decay and Fe(II) release is in agreement with the findings of [24,25], who reported that in the absence of a chelator, Fe(II) formed by reduction of ferritin-bound Fe(III), can be retained by the ferritin.

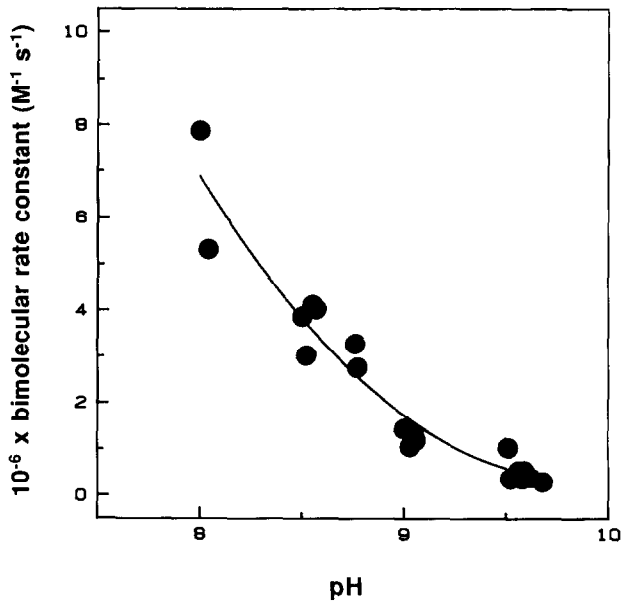

Fig. 4. Effect of pH on the reaction between $O_2^{\cdot-}$ and ferritin. The bimolecular rate constant for the reaction between ferritin (as protein) and $O_2^{\cdot-}$ at pH 8–9.5. The incubation medium was as described in section 2, except that 50 mM Tris buffer was used.

Like the rate of $O_2^{\cdot-}$ decay, reductive release of iron from ferritin is slowed down at increasing pH [24]. However, the decrease in the rate of Fe(II) release from ferritin by $O_2^{\cdot-}$, was only about one-tenth of the corresponding decrease in the rate of decay of $O_2^{\cdot-}$ effectuated by ferritin.

In conclusion, our results show that ferritin catalyzed the decay of $O_2^{\cdot-}$. The concomitant loss of ferritin iron, if any, was *not* stoichiometrically related to the amount of $O_2^{\cdot-}$ molecules degraded. We propose that Fe(II) formed in reaction (2), is not released from ferritin, but reoxidized in situ according to reaction (2). In consequence, our results add a new dimension to the antioxidant attribute of ferritin.

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