

Decay of superoxide catalyzed by ferritin

Bjørn J. Bolann and Rune J. Ulvik

Laboratory of Clinical Biochemistry, University of Bergen, Haukeland Hospital, N-5021 Bergen, Norway

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Ferritin iron can be reduced by O_2^- , 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^- , monitored by direct spectrophotometry at pH 9.5. Ferritin, but not apoferritin, accelerated the decay of O_2^- . Ferritin iron was apparently the responsible agent. The effect of ferritin was maintained after several bursts of O_2^- , and the ratio degraded O_2^- /released Fe(II) greatly exceeded one, consistent with a catalytic reaction.

Ferritin; Superoxide; Spectrophotometry; Kinetics; Catalysis

1. INTRODUCTION

The cytotoxic effect of O_2^- and H_2O_2 can be strongly aggravated by iron ions and small molecular weight iron complexes. Being reduced and oxidized by O_2^- 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^- 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^- 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^- , as judged from the sensitivity of the reaction to superoxide dismutase. This was the case even after repeated exposures of ferritin to O_2^- [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^- , we monitored the rate of decay of O_2^- by a direct spectrophotometric technique.

Correspondence address: B.J. Bolann, Laboratory of Clinical Biochemistry, University of Bergen, Haukeland Hospital, N-5021 Bergen, Norway. Fax: (47) (5) 97 3115.

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 μ m (pore-size) filter prior to use.

2.2. Assays

The decay of O_2^- 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 μ M of ferritin protein (unless otherwise stated). From a solution of KO_2 dissolved in 50 mM NaOH, aliquots of approx. 15 μ l were transferred to the medium and the declining concentration of O_2^- was monitored as the decrease in absorbance ($A_{250} - A_{360}$) [14,16]. The rate of O_2^- decay was generally expressed as the apparent pseudo-first-order rate constant calculated from the concentration interval 16 to 4 μ M of O_2^- [14]. The low concentration of ferritin gave no disturbing background absorbance.

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

3. RESULTS

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

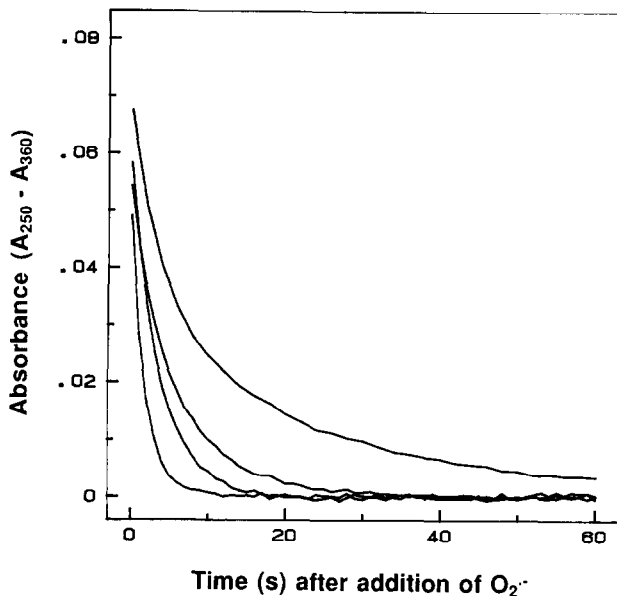


Fig. 1. Effect of ferritin on the decay of O₂⁻. O₂⁻ (from KO₂) was added to the incubation medium at time 0. Upper curve, without ferritin; below, with 0.04, 0.08 and 0.16 μM of ferritin, respectively.

the decay of O₂⁻, and the stimulating effect of ferritin, is shown in Fig. 1. At the start of the reaction, the concentration ratio (O₂⁻:ferritin) was about 300:1. By analyzing the O₂⁻ decay curves as described in [16], we found that the ferritin-induced decay of O₂⁻ was 1st-order in O₂⁻, i.e. it appeared as the addition of a 1st-order reaction to the spontaneous 2nd-order dismutation of O₂⁻. That means that ferritin behaved as a catalyst [16]. The rate of O₂⁻ 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 O₂⁻ decay.

When ferritin was exposed to repeated bursts of O₂⁻ with approximately 5 min intervals, the rate of O₂⁻ 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 O₂⁻. The same pattern, but at approximately 25% higher rates, was obtained when ferritin was supplied with a single dose of Fe(III) (as FeCl₃) before the first burst of O₂⁻ (Fig. 3). In comparison, addition of FeCl₃ to apoferritin or albumin had no effect on the O₂⁻ decay, neither had FeCl₃ alone.

Because the direct spectrophotometric assay could not detect rate changes beyond the high spontaneous O₂⁻ 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, O₂⁻ decay caused

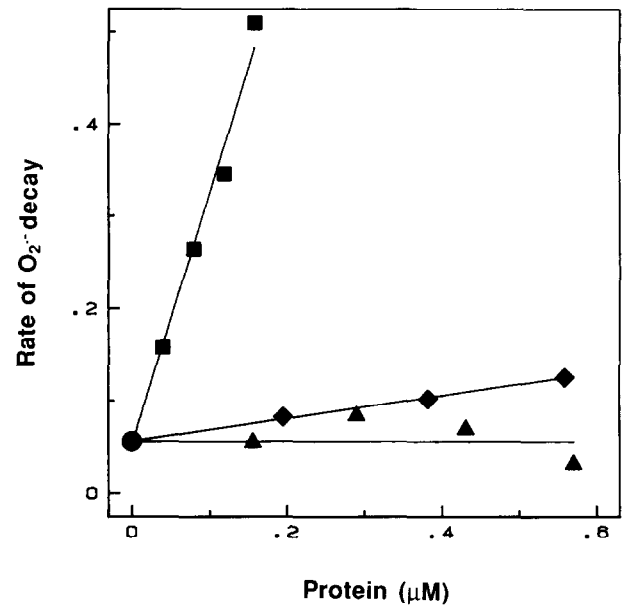


Fig. 2. Rate of O₂⁻ decay in the presence of ferritin and albumin. The rate of O₂⁻ decay was calculated as described in section 2. ■, ferritin; ◆, apoferritin; ▲, albumin.

solely by ferritin was determined as the bimolecular rate constant for the reaction between ferritin and O₂⁻ (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 O₂⁻

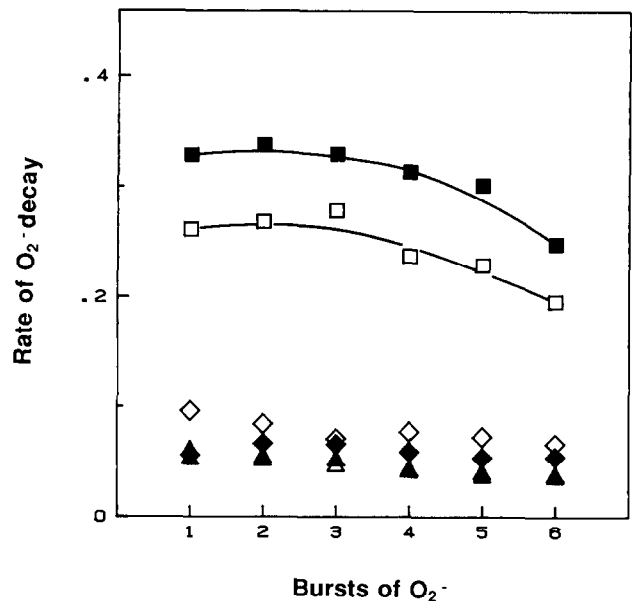


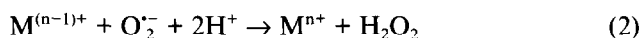
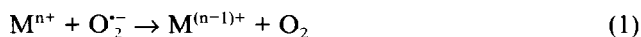
Fig. 3. Effect of repeated bursts of O₂⁻. The incubation medium was exposed to repeated bursts of O₂⁻. The protein concentration was 0.08 μM. The rate of O₂⁻ decay is shown in the presence of: □, ferritin; ◇, apoferritin; ▲, albumin. Corresponding closed symbols, FeCl₃ added to a final concentration 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]:



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 $10 \times 10^6 M^{-1} \cdot s^{-1}$ 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

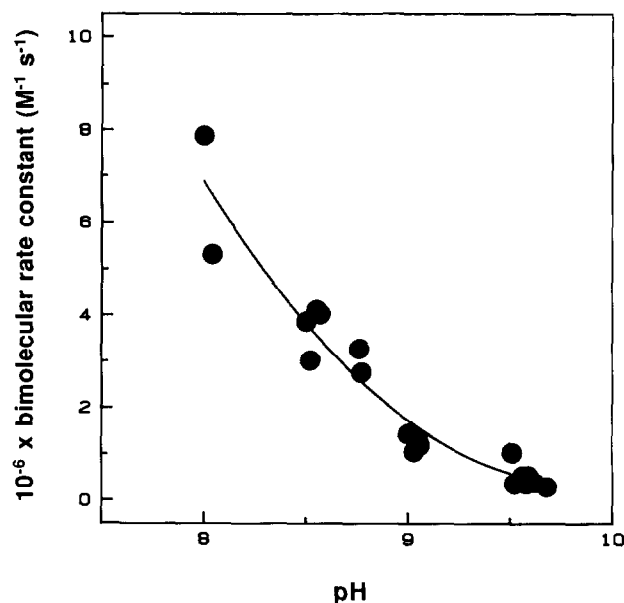


Fig. 4. Effect of pH on the reaction between $O_2^{\cdot-}$ and ferritin. The bimolecular rate constant for the reaction between ferritin (as protein) and $O_2^{\cdot-}$ at pH 8-9.5. The incubation medium was as described in section 2, except that 50 mM Tris buffer was used.

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.

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 $O_2^{\cdot-}$ effected 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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