Xanthine oxidase induced depolymerization of hyaluronic acid in the presence of ferritin

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Oxygen free radicals generated by xanthine oxidase are able to depolymerize hyaluronic acid in the presence of ferritin-bound iron. This suggests that ferritin can catalyse the Haber-Weiss reaction, leading to the formation of highly damaging hydroxyl radicals.

Haber-Weiss reaction *Fenton reaction* *Ferritin* *Iron* *Chelating agent* *Hyaluronic acid degradation*

1. INTRODUCTION

It has long been known that oxygen is toxic to aerobic cells when supplied in concentrations above the normal. Some biochemical mechanisms that have been suggested as explanations for this oxygen toxicity include participation of the superoxide radical (review [1]). This radical is not very reactive in itself, at least not in aqueous solutions (see discussion in [2]), and it has therefore been theorized that it interacts with hydrogen peroxide to form the highly reactive hydroxyl radical (the Haber-Weiss reaction); this reaction requires a catalyst such as copper or iron ions (Fenton-type catalysis; review [2]). Authors in [3] have proposed that phosphate-bound ferric iron is the physiological catalyst. Here we demonstrate that ferritin-bound iron is able to catalyse the formation of hydroxyl radicals from the superoxide generated by xanthine oxidase.

2. MATERIALS AND METHODS

The viscosity measurements were carried out in an Übbelohde viscometer (unpublished). Briefly, the reaction mixture contained 0.5 mg/ml hyaluronic acid (Healon®, $\bar{M}_w$ 3×10^6, Pharmacia AB, Uppsala), 0.2 mM hypoxanthine and 1.7 mUnits/ml xanthine oxidase in a 0.05 M phosphate buffer (pH 7.4). The xanthine oxidase was prepared from bovine milk as in [4]. Only one protein peak appeared on a Sephacryl G-200 chromatogram. One unit converts 1.0 μmol xanthine to uric acid per min at 25°C. The iron content of the reaction mixture was 0.06 μg/ml, as determined by atomic absorption; 0.04 μg/ml was derived from the hyaluronic acid preparation, only half of which could be removed by Chelex treatment and then at the expense of a considerable decrease in viscosity. The reagents were therefore not routinely treated with Chelex to remove iron.

Ferritin type I and apoferritin from horse spleen were purchased from Sigma (St. Louis, MO). The iron content of the ferritin was 22% and that of apoferritin 0.01%, as determined by atomic absorption. Ferritin and apoferritin were further purified by desalting on a molecular sieve column (PD-10, Pharmacia AB, Uppsala), either untreated or preincubated with 10 mM desferrioxamine (CIBA-Geigy AG, Basel) or 25 mM EDTA to remove loosely bound Fe ions.

The reduction in specific viscosity, n_{sp} (Fig.1), of the reaction mixture was a logarithmic function of time, i.e., it followed first-order reaction kinetics [3] at least for an experimental time, t, of up to 60 min. The n_{sp} was calculated as $A/B - 1$, where A is the flow time for the reaction mixture and B is the flow time for the buffer; after a prolonged

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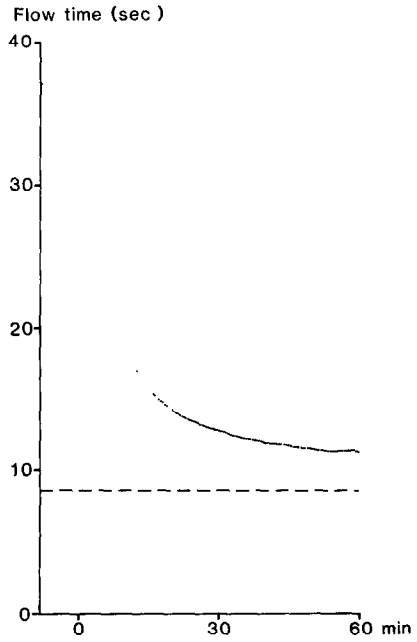

Fig.1. The decrease in hyaluronic acid viscosity. The graph illustrates an experiment in which 50 μM EDTA was added. The ordinate shows the flow time through the capillary viscometer. Enzyme was added at 0 min. Each point represents one measurement. The broken line indicates the flow time for the buffer.

experimental time, A would eventually reach the value of B. The rate constant, k, used to express the reaction velocity was calculated from a plot of $\ln(n_{sp})$ *vs* t, which has a slope of $-k$ according to the first-order reaction rate equation: $\ln(n_{sp})_t - \ln(n_{sp})_0 = -kt$. The coefficient of variation between multiplicate experiments was 10% or less.

3. RESULTS

Oxygen free radicals produced by xanthine oxidase in the absence of EDTA and ferritin caused only a very small decrease in the viscosity of hyaluronic acid. Addition of ferritin resulted in a decrease in viscosity, the rate of which was dependent upon the ferritin concentration. The effect observed was apparently not due to unbound iron ions in the ferritin preparation, as desalting of ferritin, either untreated or preincubated with the iron chelator EDTA or desferrioxamine, did not result in any significant change in the reaction rate (not shown). Apoferritin was considerably less potent than ferritin at the same protein concentration, although the minute amounts of iron present in the apoferritin had a comparatively high specific activity. Both the apoferritin and ferritin effects were iron-dependent, since they were abolished by desferrioxamine, which is known to form an iron complex which is unable to catalyse the Haber-Weiss reaction [5]. Furthermore, addition of $FeCl_3$ to the reaction mixture did not influence the reaction rate, indicating that apoferritin did not act simply by forming an iron complex analogous to that of EDTA. The apoferritin preparation contained a catalyst which could be removed by desalting chromatography.

Addition of EDTA to the reaction mixture resulted in a marked increase in the reaction rate due to the formation of a highly active catalytic complex between EDTA and traces of iron in the buffer. Addition of EDTA to a ferritin-containing reaction mixture caused an even more pronounced increase, although it may not be correct to attribute this to a ferritin–EDTA complex, since the iron contamination could not be removed from the other reagents.

4. DISCUSSION

The ability of protein-bound iron to catalyse the Haber-Weiss reaction in biological systems has been a matter of controversy. Transferrin [6–8] and lactoferrin [9,10] have been found to catalyse hydroxyl radical production induced by NADPH oxidase and xanthine oxidase. On the other hand, in [11] it was shown that the iron-containing proteins horseradish peroxidase, myeloperoxidase and ferritin did not catalyse the Haber-Weiss reaction; in [11] it was reported that lactoferrin was only a weak catalyst in the absence of EDTA, and in [13], in ESR studies, no hydroxyl radical formation from H_2O_2 was found in the presence of ferritin or transferrin. Authors in [3] and [14] presented evidence that only 'free' (not protein-bound) iron and, especially, iron chelated to molecules such as phosphate, ADP or ATP will function as physiological iron catalysts.

This study suggests that ferritin is able to catalyse the Haber-Weiss reaction leading to hydroxyl radical-dependent [15,16] depolymerization of hyaluronic acid. Our main argument in

Table 1

Effect of additives on the xanthine-oxidase-induced depolymerization of hyaluronic acid

Additive	Apoferritin/ ferritin (μg/ml)	Iron[a] (μg/ml)	Rate constant (h^{-1})
None	0	0	0.05
$FeCl_3$, 5 μM	0	0.3	0.05
Ferritin	216	48	1.12
Ferritin[b]	5	1.1	0.02
	12	2.6	0.15
	60	13	0.56
	100	22	1.10
	300	66	1.20
Ferritin + Desf, 0.1 mM	216	48	0.10
+ Desf, 1 mM	216	48	0.00
Ferritin[b] + Desf, 0.05 mM	60	13	0.21
+ Desf, 0.1 mM	60	13	0.13
Apoferritin	200	0.02	0.52
	400	0.04	0.49
Apoferritin[b]	50	0.005	0.04
	150	0.015	0.29
Apoferritin + Desf, 0.1 mM	200	0.02	0.00
Apoferritin[b] + Desf, 0.05 mM	150	0.015	0.02
Apoferritin + $FeCl_3$, 5 μM	200	0.35	0.59
EDTA, 50 μM	0	0	2.77
Ferritin + EDTA, 50 μM	60	13	7.5

[a] In addition to 0.06 μg/ml basically present in the reaction mixture
[b] Desalted by gel chromatography

Desf, desferrioxamine

favour of the idea that the catalyst is ferritin-bound iron, and not contaminating iron ions, is that EDTA was not required for the hyaluronic acid depolymerization. This is in contrast with the situation where iron added in the form of $FeCl_3$ or ferric iron contaminated the buffer. Furthermore, attempts to remove unbound iron by chelation and desalting chromatography did not indicate the presence of unbound iron.

Early studies showed that ferritin iron was reduced in the xanthine oxidase reaction and removed from the protein. Ferrous iron reacts with dissolved molecular oxygen, leading to the formation of hydroxyl radicals. Later studies have revealed, however [17], that only a very small amount of the total ferritin iron is released, making this mechanism of radical generation less likely.

Authors in [18] discussed a mechanism of hydroxyl radical formation by ferritin which may be of relevance to our observations. Ferritin-bound Fe(III) is first reduced to Fe(II) by a reductant, e.g., superoxide, and the following reactions then lead to hydroxyl radical formation:

$$Fe(II) + O_2 = Fe(II)...O_2...Fe(II) = Fe(III)\text{-}O\text{-}O\text{-}Fe(III)$$

$$Fe(III)\text{-}O\text{-}O\text{-}Fe(III) + Fe(II) + H_2O = 3Fe(III)\text{-}OH + OH^{\cdot}$$

Note that the iron is trapped in the protein during the entire reaction. This reaction scheme is compatible with the results in [19], where it was demonstrated that only iron chelates with at least one coordination site open to water will catalyse

the Haber-Weiss reaction. The ferritin molecule is composed of a protein shell, 2.7 nm thick, covering a 7.5 nm central cavity in which the iron is encapsulated; in the protein shell there are 6 channels, 1.0 nm wide, through which the iron is transported [20]. It is possible that the superoxide and hydroxyl radicals are also transported in these channels, thereby avoiding reactions with the protein shell.

Ferritin-bound iron is apparently a far less effective catalyst than iron complexed to EDTA, which may be partly explained by a loss of hydroxyl radicals that have reacted with the protein shell soon after their formation. However, as pointed out in [3], the iron–EDTA complex is an artifact of no physiological relevance, and the physiological iron chelator is most likely phosphate or phosphate esters such as ADP or ATP. Despite the use of a phosphate buffer in our experiments, we found only a very low reaction rate after addition of $FeCl_3$. This was apparently due to precipitation of Fe^{3+} by phosphate [19], which is prevented by EDTA.

The physiological importance of the catalytic function of ferritin is unknown. The amounts of ferritin in normal serum (up to 0.1 μg/ml) and tissues (up to 5 μg/ml) [18] may be too low to be significant. However, in pathological conditions, the serum ferritin level may reach 12 μg/ml [18], and in rheumatoid arthritis, a disease in which iron-catalysed hydroxyl radical formation might possibly influence the course of the disease [21], the synovial fluid contains ferritin in concentrations which correlate closely with other indices of intra-articular disease activity [22]. In addition, in [23], from experiments using ferritin in concentrations comparable to ours, it was proposed that ferritin-stimulated lipid peroxidation may be of significance in inflammation-induced tissue damage.

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