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THE KINETICS OF THE REACTION OF FERRITIN WITH SUPEROXIDE

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Using pulse radiolysis and competition kinetics with cytochrome *c*, the reaction of superoxide with horse spleen ferritin was investigated. The second-order rate constant is estimated to be $2 \pm 1 \times 10^6\,dm^3\,mol^{-1}\,s^{-1}$.

KEY WORDS: Superoxide, ferritin, kinetics.

INTRODUCTION

It is now realized that superoxide may play a significant role in the production of cellular damage associated with inflammation,[1] hyperoxia[2] and ischemia.[3] In addition, superoxide has been implicated as having a role in certain disease states such as cancer,[4,5] muscular dystrophy,[6] sickle cell anemia,[7] and perhaps even aging.[8] Superoxide also appears to be involved in redox cycling of certain drugs.[9]

However, superoxide is not a good direct initiator of oxidative damage. To be effective it requires the presence of transition metals as catalysts, iron being the most prevalent and logical catalyst in a biological system. In humans most of the iron in the body is continuously recycled, with 70% being present in hemoglobin and 20–25% present as storage iron. Thus, the most likely candidates as sources for catalytic iron are hemoglobin, ferritin, transferrin, and perhaps hemosiderin.

Ferritin is a large iron storage protein (for reviews see [10–13]). It can store within the central core up to 4500 Fe(III) ions as a micro-crystalline ferric-oxide-phosphate. Access to the core by small cations, anions and neutral species can be gained by six channels in the protein shell. The hydrous ferric oxide core of horse spleen ferritin was shown to be reduced by one electron per iron atom with $E_{1/2} = -190\,mV$ at pH 7.0 and $E_{1/2} = -310\,mV$ at pH 8.0 (versus the NHE).[14] Thus, at physiological pH cellular reducing agents such as ascorbate and superoxide can, on thermodynamic grounds, reduce the iron to Fe(II), releasing the iron. Indeed, ferritin-ascorbate has been shown to stimulate lipid peroxidation,[15,16] and oxygen free radicals generated by xanthine oxidase were shown to depolymerize hyaluronic acid in the presence of ferritin.[17] In addition, $O_2^{\cdot -}$ was shown to release iron from ferritin[3,18–20] and bring about ferritin-promoted superoxide-dependent lipid peroxidation.[20]

Here we report the kinetics of the reaction of $O_2^{\cdot -}$ with ferritin as investigated by pulse radiolysis.

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MATERIALS AND METHODS

Horse spleen ferritin was from Boehringer Mannheim GmbH. The analysis indicated 24.6% Fe and 75.6% protein by weight. Using 4500 Fe(III)'s/ferritin molecule as saturation, and a molecular weight of 445 kDa for the apoprotein, the ferritin was estimated to be 58% Fe(III) saturated (2600 Fe(III)'s/ferritin molecule). A solution estimated to be 100 nM in ferritin yielded an A_{380}/A_{420} of 1.91, consistent with a Fe/N of 1.8–1.9.[21] Using ε_{380} (ml mg Fe^{-1} cm^{-1}) of 19.0,[21] the analytical and spectroscopic estimates of the iron content of the ferritin agreed to within 5%. Horse heart cytochrome *c* was from Sigma (Type III).

The pulse radiolysis experiments were done with a Febetron 705 accelerator and kinetic UV/VIS spectroscopy system.[22] Pulses of 1.6–1.8 MeV electrons (50 ns pulse duration) were delivered to a quartz cuvette (5 mm depth, 20 mm optical path length) containing the sample. The doses were 40–50 Gy, which in the presence of 10 mM formate, yielded a superoxide concentration of approximately 25 μM at the end of the pulse. The time-resolved absorbance of cytochrome *c* at 550 nm was digitized and used for evaluation as outlined by Bors *et al.*[23]

Due to the strong absorbance of ferritin at 245 nm, the λ_{max} of $O_2^{\cdot -}$, it was not possible to achieve a high enough concentration of ferritin to use standard pseudo first-order kinetic techniques. Thus, kinetic competition experiments with cytochrome *c* as a competitor for $O_2^{\cdot -}$ was performed. Kinetic analysis was done using the data collected within 30 ms after the pulse. The rate constant for the reaction of $O_2^{\cdot -}$ with cytochrome *c* at pH 7.0 and pulse radiolysis conditions was taken as 1.1×10^6 dm^3 mol^{-1} s^{-1}.[24]

The pulse radiolysis solutions contained sodium formate, 10 mM, in oxygen-saturated 10 mM phosphate buffer, pH 7.00. The ferritin concentration varied from 0.5–4 μM while the cytochrome *c* varied from 5–10 μM. To remove trace impurities of transition metals Chelex 100 resin (100–250 mesh, sodium form, Bio-Rad Laboratories, Richmond, CA) was added to the prepared phosphate-formate solutions, approximately 10 ml/l. After stirring for one hour, the resin was removed by filtration. The removal of catalytic metals was verified with ascorbate.[25] Chelex 100 precipitates cytochrome *c*, thus it was added to the filtered buffer. To insure that no loosely bound metals were present in the ferritin, approximately 1 ml of Chelex resin was added to 5 ml of the ferritin solution and after stirring was removed by filtration. No change in the UV-VIS absorbance spectrum of the ferritin solution was observed as a result of the Chelex treatment.

RESULTS AND DISCUSSION

In the kinetic competition experiments, ferritin was found to compete effectively with cytochrome *c* for the radiolytically generated superoxide. From the competition plot (Figure 1) the second-order rate constant for the reaction of $O_2^{\cdot -}$ with ferritin at pH 7.0 was found to be $2 \pm 1 \times 10^6$ dm^3 mol^{-1} s^{-1}. This represents a "rate constant" of approximately 3×10^5 dm^3 mol^{-1} s^{-1} $channel^{-1}$ for each of the six channels of the ferritin molecule. This rate is equal to that of $O_2^{\cdot -}$ reacting with Fe(III)-EDTA,[26,27] an excellent promoter of iron-dependent lipid peroxidationn as well as being an excellent catalyst for the Haber–Weiss reaction.[28] As most of the intracellular iron is located in the ferritin molecule, it would appear that iron could always be available as an oxidation catalyst, especially when abnormal fluxes of superoxide might appear, such

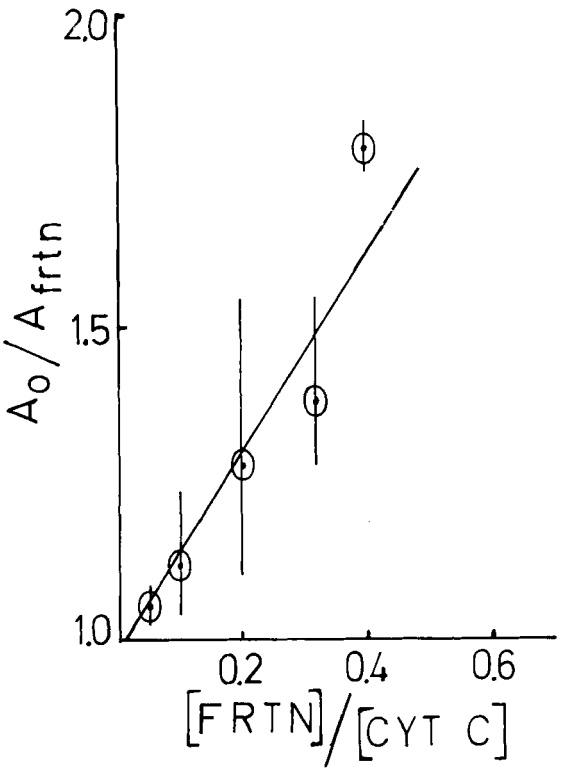

FIGURE 1 Competition plot for ferritin and cytochrome *c*. The initial rates (within 30 ms after the pulse) as reflected by the absorbance of cytochrome *c* at 550 nm were used. The error bars represent the range for separate experiments. The points represent the average of three separate experiments. Signal averaging of four to eight pulses was done to arrive at the result for an experiment. Using a value of $1.1 \times 10^6\,dm^3\,mol^{-1}\,s^{-1}$ for the reaction of $O_2^{\cdot-}$ with cytochrome *c* at pH 7.0,[24] the rate constant for the reaction of superoxide with ferritin was determined to be $2 \pm 1 \times 10^6\,dm^3\,mol^{-1}\,s^{-1}$.

as in cases of infiltration by granulocytes or macrophages or in reperfusion of ischemic tissue.

The reduction of Fe(III) to Fe(II) appears to be the most likely mechanism for iron release from ferritin *in vivo*. It would appear that superoxide can serve as a reductant for ferritin iron.

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