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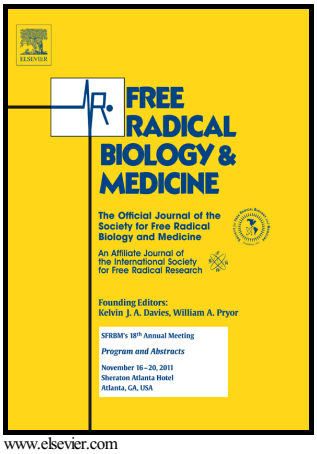

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Labile Iron Potentiates Ascorbate-Dependent Reduction and Mobilization of Ferritin iron.

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Abstract

Ascorbate mobilizes iron from equine spleen ferritin by two separate processes. Ascorbate alone mobilizes ferritin iron with an apparent $K_{m\ (ascorbate)}$ ≈ 1.5 mM. Labile iron > 2 μM, complexed with citrate (10 mM), synergises ascorbate-dependent iron mobilization by decreasing the apparent $K_{m\ (ascorbate)}$ to ≈ 270 μM and raising maximal mobilization rate by ≈ 5-fold. Catalase reduces the apparent $K_{m(ascorbate)}$ for both ascorbate and ascorbate + iron dependent mobilization by ≈ 80%. Iron mobilization by ascorbate alone has a higher activation energy (E_a = 45.0 ± 5.5 kJ/mole) than when mediated by ascorbate with labile iron (10 μM) (E_a = 13.7 ± 2.2 kJ/mole); also mobilization by iron-ascorbate has a three-fold higher pH sensitivity (pH range 6.0-8.0) than with ascorbate alone. Hydrogen peroxide inhibits ascorbate's iron mobilizing action.

EPR and autochemiluminescence studies show that ascorbate and labile iron within ferritin enhances radical formation, whereas ascorbate alone produces negligible radicals. These findings suggest that iron catalysed single electron transfer reactions from ascorbate, involving ascorbate or superoxide and possibly ferroxidase tyrosine radicals, accelerate iron mobilization from the ferroxidase centre more than EPR silent, bi-dentate two-electron transfers. These differing modes of electron transference from ascorbate mirror the known mono and bidentate oxidation reactions of dioxygen and hydrogen peroxide with di-ferrous iron at the ferroxidase centre. This study implies that labile iron, at physiological pH, complexed with citrate, synergises iron mobilization from ferritin by ascorbate (50-4000 μM). This autocatalytic process can exacerbate oxidative stress in ferritin-containing inflamed tissue.

Abbreviations.

Ascorbic acid, Asc; ascorbic acid oxidase, Asc Ox; ascorbate anion, $AscH^-$; ascorbate dianion, Asc^{2-}; ascorbyl radical, $AscH^{\bullet}$; ascorbate radical, AFR or $Asc^{\cdot-}$; catalase, Cat. ; chemiluminescence, CL; dehydroascorbate, DHA; electron paramagnetic resonance, EPR; ferritin heavy chain, FHC; ferritin light chain, FLC; ferroxidase centre, FOC; Michaelis Menten coefficients in mM and nmol mg^{-1} ferritin min^{-1}, respectively, K_m, $V_{max\ (ascorbate)}$; milliTesla, mT; 3-(N-morpholine)propanesulfonic acid, MOPS; non-transferrin bound iron, NTBI; reactive oxygen species, ROS; superoxide dismutase, SOD; xanthine oxidase, XO.

Introduction

Iron is an essential mineral required for synthesis of iron-containing proteins, including haemoglobin, myoglobin and cytochromes [1]. In a typical 70 kg adult human male, around 30% of his total iron (6 g) is sequestered in ferritin. Free "labile" iron activity within body fluids is normally very low and present mainly as hydrated complexes in proteins, such as albumin, or as low molecular weight chelates with citrate or glutathione [2]. In the plasma of patients suffering from iron overload, the amount of redox active and chelatable non-transferrin bound iron (NTBI) is in the range of 1-20 µM [3–5]. Ascorbic acid is highly concentrated in a few tissues, e.g. brain, adrenal glands, and cartilage, [6] but low in others, e.g. liver and pancreas [7]. Under these circumstances, a complex tissue-differentiated regulation of the iron storage/release from ferritin must be considered.

Ferritin is a multimeric iron storage protein consisting of a mixture of 24 heavy (FHC) and light (FLC) polypeptide chains with combined weights of 474 kDa that form a spherical shell with the capacity to store oxidised ferric iron. Only the FHC chains possess ferroxidase activity (i.e. the capacity to catalyse oxidation of Fe(II) to Fe(III)); FLC-rich ferritins, such as equine spleen ferritin, have slower iron oxidation rates, but promote nucleation and storage of ferric iron into the ferritin core [8]. The numbers of iron ions stored as crystalline solid per ferritin cage normally vary from 500 to 2500. In iron-overload conditions, this number can reach 3000 to 4000 [9] .

Nonpolar channels, predominantly lined with leucine lie within the six four-fold subunit intersections of the ferritin shell [10,11]; whereas hydrophilic channels, lined predominately with anionic aspartate and glutamate side-chains, are present in all eight of the three-subunit

intersections. Ferrous iron Fe(II) enters and leaves ferritin through these 3-fold hydrophilic channels and a gateway iron binding site at the channel entrance [12] Iron(II) entry via the threefold channels can be blocked by Zn(II) [13].

Iron uptake into ferritin via multiple oxidation processes.

The initial reaction of ferrous iron with ferritin involves dioxygen reacting with two vicinally bound ferrous ions at the ferroxidase centre (FOC), present only in FHC, in a sequential electron transfer process to reduce dioxygen to hydrogen peroxide [14–16] to produce diferric-peroxo precursor (2FeOOH), protons and hydrogen peroxide (Equations 1 and 2) and then to the diferric oxo product. Similar processes occur in other members of the ferritin-like superfamily, e.g. ribonucleotide reductase, R2 proteins, where a di-iron ligand pair is bridged by glutamate carboxyl residues [17,18]**.**

$2Fe(II)\text{-}P + O_2 + 2H^+ \dashrightarrow 2Fe(III)(O_2) + 2H^+ \dashrightarrow 2Fe(III)\text{-}P + H_2O_2$ Eq 1

where Fe(II)2-P represents the protein complex with iron [19,20]

The unstable bridged oxy-diferric complex undergoes hydrolysis, which results in proton release and is the basis of the mineralization process. As iron loading proceeds, a higher proportion of oxygen from water takes part in the reaction and net hydrogen peroxide production is reduced to zero:

$4Fe(II) + O_2 + 6H_2O \rightarrow 4FeOOH\text{-}P + 8H^+$ Eq 2

At still higher iron loads,[11] hydrogen peroxide is consumed in a two-electron transfer reaction to convert the diferrous to diferric complex at the FHC, thereby consuming the potentially toxic hydrogen peroxide

$2Fe(II) + H_2O_2 + 2H_2O \rightarrow 2FeOOH\text{-}P + 4H^+$ Eq 3

This differs from the Fenton reaction in which water solvated ferrous iron is oxidised by hydrogen peroxide to produce ferric iron and hydroxyl radical.

$Fe(II) + H_2O_2 \rightarrow Fe(III) + HO^{\bullet} + OH^-$ [21] Eq 4

The one-electron reduction of O_2 by Fe(II) produces superoxide [18]

$Fe(II) + O_2 \rightarrow Fe(III) + O_2^{\bullet -}$ Eq 5

The sum of reactions in equation 4 and the reversed reaction in equation 5 [22] in aqueous solutions is termed the Haber-Weiss reaction [22,23] equation 6,

$$H_2O_2 + O_2^{\bullet-} \xrightarrow{Fe(II)/Fe(III)} HO^{\bullet} + OH^- + O_2 \quad \text{Eq 6}$$

This process is facilitated when ferric iron is chelated with citrate or adenine nucleotides or EDTA [5] and citrate is used as the iron buffer in all experiments described in this paper. At aqueous interfaces, the most favoured reaction appears to be a two-electron oxidation [24].

$$Fe(II) + H_2O_2 \rightarrow Fe(IV)O^{2+} + H_2O \quad \text{Eq 7}$$

Or

$$Fe(II) + O_3 \rightarrow Fe(IV)O^{2+} + O_2 \quad \text{Eq 8}$$

and this reaction may also occur within the ferritin core.

It has been reported that ferric citrate complexes can auto-reduce and take part in redox cycling [25].

Release of iron from ferritin

Although lysosomal proteolytic degradation of the ferritin shell with consequent iron release occurs [26], this is an uncontrolled and potentially toxic process; normally, a more controlled mechanism is thought to occur [26,27]. Elucidating this buffering role by ferritin of cytoplasmic labile iron and reactive oxygen species (ROS) is the current priority. Iron mobilization from the ferritin core involves reduction of the insoluble ferric to soluble ferrous state. Electron transfer between the cytosol and the core is essential to this release process. The liberated core ferrous ions are hydrated, solubilised and exit via ferritin's threefold polar channels with water [18]. Electron donors, such as superoxide ($O_2^{\bullet-}$) or ascorbate anion ($AscH^-$), or ascorbate radical, $Asc^{\bullet-}$ (AFR) [28], may pass directly via the wall channels to the ferritin core to reduce iron by one-electron transfer [29–31]

$$AscH^- + \text{Citrate-Fe(III)} \rightarrow Asc^{\bullet-} + \text{Citrate-Fe(II)} + H^+ \quad \text{Eq 9}$$

A subsequent one-electron reduction of ferric to ferrous iron and also producing dehydroascorbate (DHA) can be obtained by oxidation of AFR [4,23].

$$Asc^{\bullet-} + \text{Citrate-Fe(III)} \rightarrow \text{DHA} + \text{Citrate-Fe(II)} \quad \text{Eq 10}$$

An alternative mechanism may involve electron transference by tunnelling through the protein via an electron transport chain. Haem, too large to pass into the ferritin via the pores, can act as an electron donor [29,32]. Electrons can also be transferred to ferritin by passing an electric current directly from electrodes [33].

The spontaneous rate of ascorbate oxidation

$Asc^{2-} + O_2 \rightarrow Asc^{\bullet-} + O_2^{\bullet-}$ Eq 11

is slow (300 $M^{-1} s^{-1}$), but in the presence of traces of iron or copper ascorbate oxidation is notoriously rapid [21,34,35]. Ascorbate can readily donate electrons to metal ions such as iron and copper[36–39] as seen in equations 9 and 10.

Ascorbate is generally considered as an efficient antioxidant of nearly every oxidizing radical, e.g. $R^{\bullet} + AscH^{-} \rightarrow Asc^{\bullet-} + RH$ [40] .

This reaction can be either retarded or accelerated by complexation with reagents such as desferrioxamine or citrate, respectively [23,41].

The mechanisms by which combinations of metal ion, dioxygen, and ascorbate produce superoxide, hydrogen peroxide, and hydroxyl radicals are contentious [21,37–39,42] . Metal ion catalysis of superoxide production results in extensive redox cycling when iron, oxygen, and ascorbate are together in solution, (see equations 5-11) [43]. The review paper by Frei and Lawson [37] indicates that metal ion-catalysed oxidation of ascorbate produces ascorbate radicals as a consequence of the transient intermediate formation of superoxide radicals that disproportionate to hydrogen peroxide and dioxygen. In turn, the hydrogen peroxide formed can react with superoxide in the Haber-Weiss reaction (Equations 5 and 6) to produce hydroxyl radical and may be the basis of ascorbate's observed pro-oxidant effect.

These secondary reactions initiated by ascorbate, oxygen, and metal ions can be inhibited by superoxide dismutase and catalase and thereby can alter the rates of ferritin iron mobilization [44] .

Many studies have shown that ascorbate increases the rate of iron release from ferritin by reducing core ferric to ferrous iron [45–47]. The apparent K_m for this complex ascorbate-dependent iron mobilization process from ferritin is in the range 1.5-2.0 mM [44,47,48] . In this paper the apparent $K_{m\ (ascorbate \pm Fe)}$ refers to the half-maximal concentration at which ascorbate activates the entire iron mobilization process and thus may be affected by the local concentrations of competitors such as hydrogen peroxide or activators such as ascorbate radical. This is apparent $K_{m(ascorbate)}$ is determine by non-linear least squares regression analysis of the entire data set using the Levenberg-Marquardt algorithm included with Kaleidagraph (Synergy Software.com).

However, ascorbate-dependent release of iron from ferritin is not considered to provide a major contribution to pathological oxidation reactions because its concentration is deemed generally to be too low to generate high iron mobilization rates [49] . Nevertheless, it is evident that labile iron has an important role in ascorbate-mediated iron mobilization from ferritin [44,50–53]. Our primary goal was to explore more thoroughly this potentially toxic relationship.

Our second aim was to observe how hydrogen peroxide, superoxide, and ascorbate anion and radical affect rates of iron mobilization from ferritin [52,54–56] . Hydrogen peroxide acts as an oxidant for iron mineralisation and therefore retards iron mobilization [14,57]. Interestingly hydrogen peroxide has been observed to stimulate ascorbate oxidation catalysed by copper ion [36]. This suggests that hydrogen peroxide has differing roles in ferritin iron and ascorbate oxidation in aqueous solution.

Materials and Methods

The following chemicals and enzymes were obtained from Sigma-Aldrich Chemical Co., UK. Superoxide dismutase (SOD) bovine recombinant, expressed in *E. coli*, lyophilized powder, ≥2500 units/mg protein, thus 10 units/ml is equivalent to 4 µg SOD or 120 nM and 50 units /ml is equivalent to 600 nM. Catalase (Cat.), obtained as a lyophilized powder, 2000-5000 units/mg, 60 kDa mol wt., i.e. catalase at 10 units.ml^{-1} is equivalent to 5 nM; ascorbic acid oxidase (Asc Ox) from *Cucurbita sp.*, 1000-3000 unit/mg protein, mol wt. 70 kDa/monomer, i.e. 0.25 unit/ml ≡ 2 nM; xanthine oxidase (XO) from bovine milk and horse spleen ferritin in saline solution. Ferrozine iron (3-(2-pyridyl)-5, 6-diphenyl-1, 2, 4-triazine-4', 4''-disulfonic acid sodium salt reagent, hydrate) was obtained from Acros Organics – Fisher Scientific (www.fisher.co.uk). Citric acid, hydrogen peroxide solution, ascorbic acid, ferrous sulphate, and zinc chloride were obtained from BDH Ltd, Dorset, UK. All solutions were prepared freshly with double-distilled water.

Determining effects of ferrozine in citrate MOPS buffered medium on iron mobilization from horse spleen ferritin.

Ferrozine produces a strongly coloured complex with ferrous iron that is stable over normal physiological pH ranges [58] . Ferrozine (75 µM) was added to a mixture containing final concentrations of horse-spleen ferritin (50 or 75 nM) diluted in NaCl (150 mM), sodium citrate (10mM), ascorbic acid (Asc) (0-10 mM), 3-(N-morpholine)propanesulfonic acid MOPS (50 mM) buffer at pH 7.0 and varying concentrations of ferrous iron ($FeSO_4$). Sodium citrate

(10 mM) gives stable Fe-buffering and reduces iron activity to approximately 3 orders lower than its nominal concentration. This permits iron release from ferritin to be monitored over 2-3h. Reagents were added to Nunc 96-well plates (www.thermoscientific.com/en/about-us/) with final volumes of 100 µl per well and absorption was monitored at 550 nm with a Molecular Devices Spectra Max 190 plate reader (GMI-inc.com). All the presented data are means ± SEM shown as error bars. Each point is the average of triplicate or quadruplicate assays, **Figure 2A**. The linear and non-linear fits ± SEM to the data were obtained using the Levenberg-Marquardt algorithm included with Kaleidagraph (Synergy Software.com).

EPR spectra of ascorbate ferritin solutions

The EPR spectra of ascorbate free radical (AFR) were recorded with Bruker spectrometers (ER200D and Elexsys 500 (Bruker Spectrospin, Wissembourg, France)) operating at 9.85 GHz and equipped with a high-sensitivity cavity. The ascorbate radical doublet (A_H = 0.18 mT) is measured at g ≅ 2.00. Typical settings used were: microwave power, 10 mW; modulation frequency, 100 kHz; modulation amplitude, 0.2 mT; receiver gain, 60 dB; time-filter constant 40.96 ms; conversion time, 41.04 ms; data points, 1024; sweep width, 0.8 mT; sweep time 21.01 s. EPR spectra were recorded sequentially during the whole reaction at 24˚C. Data acquisition was performed using Bruker Xepr software, then analysed and presented using Microsoft Excel 2013 with Microsoft Chart 2013.

The reagent solutions contained in a sealed sample cell comprised ascorbate, labile iron and equine spleen ferritin in citrate buffer (1 mM, pH 7.0). Spectra were recorded immediately after mixing with or without superoxide dismutase and/or catalase. When indicated, the ferritin solution was deoxygenated either by a cycle of vacuum-argon gas bubbling at room temperature before transferring to the sample cell, or with nitrogen flushed around gas permeable PTFE tubing (Extruded Sub-Lite-Wall, inside diameter 0.635 mm, wall thickness 0.051 mm; Zeus Industrial Products, Ireland [59]).

The ascorbate radical concentration was estimated using coarse quantification of the EPR signal with 4-hydroxy-tetramethylpiperidin-1-oxyl (TEMPOL) as an external standard and accurate simulation of the individual spectra with the program developed by Rockenbauer and Korecz [60].

Simulation of ascorbate radical reaction with ferritin.

The rates of ascorbate radical production from ascorbate (250 μM) with ascorbic acid oxidase (8 nM) and its disproportionation to ascorbate and dehydroascorbate and simultaneous reaction with varying concentrations of ferritin (0, 200 nM, and 500 nM) in citrate buffer pH 7.0 at 24°C were simulated using the differential equation modelling program Berkeley Madonna Jmadonna version 9 (http://www.berkeleymadonna.com/), see **supplementary text**. The simulations were fitted to the observed EPR data by least square estimates using Excel Solver 2013 add-in and using the Levenberg-Marquardt algorithm included with Kaleidagraph (Synergy Software.com).

Chemiluminescence (CL) studies.

Solution samples in citrate buffer at pH 7.0 and 24˚C were prepared and luminescence monitored in a Perkin Elmer luminescence spectrometer, model LS50B with the excitation beam switched off. Chemiluminescence detection of radical products released from ferritin was performed with 500 nM ferritin and 0-25 μM $FeSO_4$. Ascorbate was varied in a buffer solution of 50 mM MOPS and 10 mM citrate at pH 7.

Data analysis: Unpaired Student's t-tests were used to obtain the statistical significance of comparisons. All the data reported show results obtained from at least three independent sets of data in which each point is a replicate of at least three points. Where multiple tests were done, Bonferroni's correction was used. Data displayed in graphs show means ± standard error of the means (S.E.M.). The effects of ascorbate, iron, and citrate on the latency of iron release from ferritin were analysed using two-way ANOVA.

Results

Study of ascorbate and labile iron on iron release from ferritin in vitro

Ascorbate (0-10 mM) in buffer solution, as previously demonstrated [47,48,61] , released iron from ferritin (**Figure 1A**). In the absence of ferritin, negligible changes in ferrozine absorbance were recorded over the time course of the assay.

Release rates of iron from ferritin increase as ascorbate concentration increases from 0-10 mM (*apparent* K_m 1.4 ± 0.2 mM) **(Figures 1B and 1C)** [44,47,48]. Increasing buffer Fe(II) concentration increased the sensitivity of ascorbate-dependent iron release. The apparent K_m of ascorbate-dependent iron mobilization fell as a hyperbolic function of iron concentration

from 1.4 ± 0.2 mM with zero iron to ≈ 200 ± 20 μM with [$FeSO_4$] = 10 μM. The V_{max} of ascorbate-dependent iron mobilization, with [ascorbate] = 10 mM, in citrate buffer 10 mM at pH 7.0, increased on raising [$FeSO_4$] from 0 to 10 μM as an exponential function of [$FeSO_4$]; the V_{max} increased by approximately fivefold.

The iron sensitivity of ferritin is controlled by the complexation with citrate. With citrate (10 mM), iron activity is approximately 3 orders lower than its nominal solution concentration, i.e. for iron citrate (10 μM), the Fe(II) activity is ≅ 10 nM [62] . Raising citrate to 20 mM substantially reduces the iron sensitivity of ferrozine to iron release (see **supplement Figure S1A).**

Effects of pH change on ascorbate (10 mM) and iron (10 μM) -dependent release of ferritin iron.

Whilst changing buffer pH from 6.0 to 8.0, with zero ascorbate, there were negligible effects on ferritin iron release. Also, Fe(II) 10 μM, without ascorbate, had negligible effects on iron mobilization **(Figure 2A).** With ascorbate (10 mM) present, there was a large pH-sensitive iron release rate. Whilst with both 10 mM ascorbate and $FeSO_4$ 10 μM present, iron release from ferritin was three-fold more sensitive to pH than with Ascorbate (10 mM) alone, ($P < 0.001$ of both ascorbate and ascorbate + Fe effects).

Effects of temperature change on ascorbate and iron-dependent release of ferritin iron

Ferritin iron release was monitored at 4°, 24° and 37° C with Ascorbate (10 mM) ± $FeSO_4$ 10 μM. There was a 4-fold increase in the temperature coefficient of ferritin iron mobilization rate with $FeSO_4$ 10 μM and Ascorbate (10 mM), compared with that of Ascorbate (10 mM) alone. The activation energy with ascorbate alone ($E_{a\ (ascorbate)}$ = 45.0 ± 5.5 kJ/mole) is three-fold higher than with ascorbate and iron together {$E_{a\ (ascorbate\ +\ Fe(II))}$ = 13.7 ± 2.2 kJ/mole, ($P<0.01$)} **Figure 2B.**

Evidence of autocatalysis of ferritin iron mobilization by combined external ascorbate and iron.

The synergistic iron and ascorbate activity in mobilizing ferritin iron implies that with ascorbate present, the mobilized iron, will further auto-catalyse ferritin iron mobilization. A longer latency prior to attaining a steady state rate of iron release is likely to be more evident with low ascorbate and iron concentrations. This prediction is confirmed in **Figures 3A and 3B**. Increasing either ascorbate with low iron (Fe(II) < 10 μM) ($P < 0.0001$), or iron with low

ascorbate ($P < 0.0001$), reduce the latency period before mobilization rates are observed (**Figure 3B)**. Exposing ferritin to catalase abolished the latency ($P < 0.01$) **(Figure 3C)**, indicating that endogenous hydrogen peroxide stabilizes the FOC iron.

Effects of zinc on ascorbate-dependent iron release from ferritin.

As mentioned in the introduction, Zn(II) blocks iron flow via the three-fold ferritin channels, [14] ; increasing $Zn(II)Cl_2$ from 0 to 10 μM decreased ascorbate-dependent release of iron from ferritin in the absence of external Fe(II). The $I.C._{50}$ of Zn(II) on the ascorbate-dependent iron release is 2.2 ± 0.4 μM **(Figure 4A).** Additionally, Zn(II) 20 μM fully blocks iron release stimulated by ascorbate (10 mM) + Fe(II) (35 μM) ($P < 0.001$ (**Figure 4B**) that). Both Zn(II) and iron(II) concentrations were raised to generate full activation and inhibition of iron mobilization. This experiment demonstrates that iron released by ascorbate originates from the ferritin core, rather than from displacement from the ferritin's external surface [63,64]

Hydrogen peroxide promotes Fe(II) oxidation within ferritin

Hydrogen peroxide (300 μM) has been observed to inhibit ascorbate-dependent iron mobilization from ferritin by up to 40% [44] . The effects of hydrogen peroxide on iron mobilization, without interference from any concurrently generated superoxide were observed by addition of superoxide dismutase to the incubation solutions (see equations 5 and 6). Hydrogen peroxide has a much stronger inhibitory effect on ascorbate-dependent iron release in the conditions used here, than previously reported [24]. This inhibition is apparently non-competitive and follows sigmoid kinetics consistent with Hill coefficient $n \approx 3$ for exogenous hydrogen peroxide $\geq$ 0.1 mM **(Figure 5A)**. In the absence of exogenous hydrogen peroxide, the Hill coefficient of ascorbate-dependent iron mobilization equals 1. Raising H_2O_2 from 0 to 0.25 mM reduced the V_{max} of ascorbate-dependent iron mobilization by ≈ 4-fold i.e. 80% inhibition ($P < 0.001$) **(Figure 5A).** Hydrogen peroxide also reduced the apparent affinity of ascorbate; *apparent* $K_{m\ (ascorbate)}$ increased from 1.8 ± 0.2 mM without added H_2O_2, to 6.2 ± 1.4 mM when H_2O_2 was raised to 0.25 mM ($P < 0.01$) **(Figure 5A).**

Labile $FeSO_4$ 10 μM counteracts the inhibitory effect of hydrogen peroxide on ascorbate's mobilizing activity on ferritin iron **(Figure 5B);** the *apparent Km* $_{(ascorbate)}$ on ferritin iron mobilization with H_2O_2 (0.25 mM) fell from 6.2 ± 1.4 mM, without iron (**Figure 5A**) to 2.1 ± 0.2 mM ($P < 0.015$) with $FeSO_4$ 10 μM (**Figure 5B**). Raised hydrogen peroxide also increased the Hill coefficient of ferritin iron mobilization with ascorbate and iron present from n=1 without

H_2O_2, to n=1.8 with hydrogen peroxide (0.25 mM), and to n=3.0 with hydrogen peroxide (1.0 mM).

Sigmoid kinetics have been reported for ferritin Fe(II) oxidation of mutant forms of human H-chain ferroxidase and nucleation sites of human mitochondrial ferritin [19,65] . Oxo-anions such as phosphate, arsenate, or vanadate have been implicated in sigmoidal kinetics of iron oxidation [66] . An explanation for the sigmoid kinetics is that once the centre is saturated with iron, deposition occurs at additional lower affinity sites to the ferroxidase centre [67]. It is possible that a similar explanation for the sigmoidal curves seen in **Figures 5A and 5B** is that hydrogen peroxide may differentially stabilize iron depending on the sites within the ferritin core.

The inhibitory effects of hydrogen peroxide on ascorbate dependent ferritin iron mobilization with varying labile iron concentrations are corroborated in **Figure 5C**. Raising iron(II) from zero to 5 µM increased the $I.C._{50}$ of hydrogen peroxide inhibition of ascorbate-dependent ferritin iron mobilization from 0.11 ± 0.03 mM to 0.8 ± 0.2 mM ($P < 0.005$). These findings indicate that the previously reported small effect of H_2O_2 on ascorbate-dependent ferritin iron mobilization might have been due to trace metal contamination [24] .

Thus, labile iron increased the apparent efficacy of ascorbate as a mobilizer of ferritin iron, **Figures 1B and 1C**, whilst reducing the efficacy of H_2O_2 as a stabilizer of iron within the ferroxidase centre **(Figure 5C)**. An explanation for both these phenomena is that the presence of labile iron prevents the bidentate competitive interactions of ascorbate and hydrogen peroxide with di-iron at the ferroxidase centre (see **Discussion, Graphic, and Figure 6A).**

Ascorbate-dependent iron mobilization from ferritin by superoxide.

Superoxide dismutase is expected to suppress the influence of superoxide anion on redox cycling of Fe(II/III) catalysed by ferritin. The effects of ascorbate (0-20 mM) together with SOD (600 nM) are shown in **Figure 5A**, or with ascorbate (0-20 mM) and Fe(II) (10 µM) in **Figure 5B**, or with varying SOD (0-120 nM) in **Figure 7A.** SOD was added was to discriminate between potential effects of superoxide from those more specifically due to ascorbate-dependent iron release from ferritin, (see equations 5, 6, 11).

The ascorbate-dependent *apparent* K_m of iron release was unaffected with superoxide dismutase (SOD) (600 nM) present, apparent K_m 1.8 ± 0.2 mM (**Figure 5A,** *continuous line, open circles***),** compared with zero SOD **(Figure 1B).** Similarly there was no significant effect on

the apparent $K_{m\ (ascorbate+\ Fe(II))}$ of iron mobilization (250 ± 30 μM) with SOD (120 nM) **(Figure 5B** *continuous line, open circles***)** compared to the apparent $K_{m\ (ascorbate+\ Fe(II))}$ = 200 ± 20 μM with zero SOD **(Figure 1B).** It may be deduced from these results that any superoxide production by ascorbate interactions with ferritin or iron does not alter the apparent $K_{m\ (ascorbate)}$ of ferritin iron mobilization, either in the absence or presence of $FeSO_4$ 10 μM.

Superoxide dismutase (SOD) caused a concentration-dependent inhibition of ascorbate-dependent iron release from ferritin, as has been previously observed [42] (**Figure 7A)**. With ascorbate (10 mM), the SOD-dependent fractional inhibition of iron mobilization from ferritin i.e. *{rate of iron release with varying concentrations of SOD/rate of iron release without SOD}* was reduced as $FeSO_4$ concentration was raised from zero. With $FeSO_4$ (1 μM), SOD (120 nM) reduced ascorbate (10 mM)-dependent ferritin iron mobilization by 55%, whereas with $FeSO_4$ (10 μM), SOD (120 nM) reduced ferritin iron mobilization only by 15% ($P < 0.005$). This indicates that the combination of Fe(II) (10 μM) + ascorbate (10 mM) is a SOD-insensitive ferritin iron mobilizing factor.

The effects of superoxide produced by oxidation of xanthine by xanthine oxidase on ascorbate ± $FeSO_4$ 10 μM mobilization of ferritin iron.

Superoxide generated by xanthine oxidase oxidation of xanthine or hypoxanthine is known to mobilize ferritin iron [45,53,68]]. To test whether the apparent loss of SOD efficacy as an inhibitor of ascorbate-dependent ferritin iron mobilization with raised labile iron concentration is due to incomplete superoxide eradication, or to another species of free radical capable of mobilizing ferritin iron, xanthine oxidase (XO) generated effects were examined. XO activity with ascorbate (1 mM) alone increased iron mobilization rates by 100% ($P < 0.01$) **(Figure 7C).** Xanthine oxidase had a relatively smaller effect on iron mobilization by ascorbate (1 mM) + $FeSO_4$ (10 μM), (16% increase, $P < 0.05$).

The smaller relative increase in iron mobilization by ascorbate (1 mM) + $FeSO_4$ (10 μM) suggests that superoxide is already being generated by this combination and that at least one additional factor besides superoxide contributes to the higher rate iron mobilization. The larger relative increase in mobilization rate with ascorbate alone suggests nevertheless that superoxide is an important component to iron mobilization.

Effect of catalase on ascorbate and iron dependent iron mobilization from ferritin

Catalase catalyses two-electron disproportionation of hydrogen peroxide to water and oxygen and has been observed previously to increase slightly the rate of ferritin iron mobilization by superoxide generated by xanthine oxidase [69] . Here too it is shown that catalase (5 nM) enhances iron-ascorbate-dependent ferritin iron mobilization without apparently affecting iron mobilization by ascorbate alone **(Figure 7B).** This finding indicates that hydrogen peroxide produced mainly by iron and oxygen interaction with ferritin (Equation 1) retards ascorbate and iron-dependent ferritin iron mobilization **(Figure 3C),** as has already been demonstrated with exogenously added hydrogen peroxide (**Figure 5C).**

Catalase (10 nM) effects on ascorbate-dependent iron mobilization were further investigated. Catalase reduced the apparent $K_{m\ (ascorbate)}$ of ferritin iron mobilization from control 1.2 ± 0.14 mM to 260 ± 30 μM (P< 0.001) without affecting the $V_{max\ (catalase)}$ = 10.9 ± 0.3 and $V_{max\ (control)}$ = 9.8 ± 0.3 nmoles min^{-1} mg^{-1} ferritin **(Figure 8A)**. Similarly, catalase reduced the apparent K_m of ascorbate + Fe (10 μM)-dependent ferritin iron mobilization from 390 ± 31 μM to 77.6 ± 10.5 μM (P< 0.001) (two-tailed Student's n= 12 independent assays per point, two experiments), without significantly altering V_{max} (control rate 65 ± 2; catalase rate 60 ± 2 nmoles min^{-1} mg^{-1} ferritin) **(Figure 8B).**

These results signify that ferritin's ferroxidase centre is shielded from the reducing activity of low ascorbate concentrations ≤ 1 mM by endogenous hydrogen peroxide. They also indicate that the estimates activation energy difference and the pH sensitivity difference between Ascorbate (10 mM) ± $FeSO_4$ 10 μM **(Figures 2A and 2B)** will be unaltered by endogenous hydrogen peroxide concentrations, as the of ascorbate-dependent ferritin iron mobilization apparent K_m s are 10-50 fold below the ascorbate (10 mM) used in these experiments.

Ascorbic acid oxidase increases iron release

XO, in addition to generating superoxide, also produces hydroxyl radical, uric acid, a radical scavenger, and urate (uric acid) radical. [70,71]. These other reaction products create ambiguities in interpreting the role of XO in inducing iron mobilization from ferritin [44,53,61]. To reduce this uncertainty, the effect of ascorbic acid oxidase (Asc Ox), which produces ascorbate free radical ($Asc^{\cdot-}$) directly from ascorbate [72], was examined. As the ascorbyl radical ($AscH^{\cdot}$)/ascorbate radical ($Asc^{\cdot-}$) couple has a pK_a of -0.86, the deprotonated form, ascorbate radical, predominates at neutral pH (**Figure 6B**) [38]. Asc Ox (2 nM), with ascorbate 1 mM and both SOD (120 nM) and catalase (5 nM) added to avoid contamination from extraneous superoxide and hydroxyl radical, increased the rate of iron mobilization by

approximately 100% (P <0.001) (**Figure 9A).** These results support the view that in addition to ascorbate anion, ascorbate radical is also a potent agent of single electron ferritin iron release. Ascorbic acid oxidase (1 nM) reduced *apparent* $K_{m\ \text{ascorbate}}$ of ascorbate-dependent iron release from 1.52 ± 0.52 mM (without Asc Ox) to 0.59 ± 0.13 mM (P < 0.01) **(Figure 9B).** This finding supports the view that single electron transfer reactions from ascorbate radical can mobilize ferritin iron more readily and with a much lower *apparent* $K_m \approx$ (200-500 μM) than ascorbate alone (1.5-2.0 mM), (**see Discussion**).

Ferritin ascorbate interactions monitored with chemiluminescence

Much controversy exists as to the chemical species monitored by the chemiluminescence reporters Lucigenin [73–75] and L-012, a Luminol derivative [76–78]. To circumvent the ambiguities ascribed to luminescence reporters, which may themselves generate redox reactions, the effects of ascorbate and iron on ferritin-generated autochemiluminescence signals (CL) were used. The signal is optimal in the blue-green colour range of the spectrum (490 nm), as shown in **Figures 10**.

In the absence of ferritin, background autochemiluminescence is negligible. Ferritin prolongs CL, so the signals were observed for periods of 20-30 minutes in citrate buffer at pH 7.0. No CL signal was obtained with ferritin in the absence of ascorbate. Ascorbate alone with ferritin (500 nM) generated only a small CL signal increasing steadily with ascorbate (0.1-10 mM) **(Figure 10C)**. The maximal luminescence emission peak occurred at 470 nm (**Figure 10A).** Addition of iron(II) greatly enhanced the CL signal. With ferritin and ascorbate + $FeSO_4$ (25 μM), the optimal CL signal is between 470-490 nm. Increasing above ascorbate (100 μM) with ferritin (500 nM) and $FeSO_4$ (25 μM) reduced the CL signal to approximately 50% of maximum with ascorbate in the concentration range > 250 μM **(Figure 10C)**. Catalase alone, or together with SOD, enhanced the optimal CL signal of iron + ascorbate + ferritin by approximately 50% **(Figure 10D)**. The finding that both catalase alone or in combination with SOD enhances the CL signal, suggests that hydrogen peroxide produced by ascorbate and iron interaction with ferritin suppresses CL, as it does for iron mobilization **(Figures 5C).** *EPR signals of ascorbate iron ferritin interactions.*

EPR methodologies are commonly regarded as the "gold standard" for detection of radicals in biological systems[79,80]. To be detectable by EPR, short-lived superoxide and hydroxyl radical require spin traps and like chemiluminescence reporters, EPR spin trap radical adducts may produce falsely negative results; they may be subject to reducing agents [79] and are

often unstable [81]. However unlike chemiluninescence reporters do not generate false positive signals. No spin traps were used here to observe catalysis of ascorbate radical formation, as long-lived ascorbate radicals are readily detected by EPR without spin traps, and its reaction with ferritin at room temperature [[47,82] .

Steady state ascorbate radical concentration is dependent on its formation by ascorbate oxidation in the presence of dioxygen, as catalyzed by free iron, or copper ions [32,80] chelated with citrate [2,3,23,42] ; or by the action of ascorbic acid oxidase [83,84] (see equations 10 and 11). Its decay rate depends both on availability of electron acceptors, such as ferritin and its disproportionation reaction as a second order function of ascorbate radical concentration. , equation 12, [42,72,84,85] .

$H^+ + 2Asc^{-\bullet} \rightarrow AscH^- + DHA$ Eq 12

Electrons donated by either $AscH^-$ or $Asc^{-\bullet}$ can reduce ferritin iron [86]. With ferritin present there are possible radical electron transfer from both $AscH^-$ and $Asc^{-\bullet}$ as follows (**See graphic**).

$AscH^- + Fe(III)\text{-}P \rightarrow Asc^{-\bullet} + H^+ + Fe(II)\text{-}P$ Eq 13A

$Asc^{-\bullet} + Fe(III)\text{-}P \rightarrow DHA + Fe(II)\text{-}P$ Eq 13B

The EPR ascorbate radical signal with iron (10 μM) increases by tenfold following addition of ferritin, from approximately 5 nM to 50 nM **(Figure 11A).** Thus, approximately 90% of the ascorbate radical EPR signal in the ferritin-ascorbate iron citrate pH 7.0 solution is generated by the additional presence of ferritin**.** The results shown here demonstrate that free iron increases the ferritin-induced ascorbate-radical signal as a linear function of iron ($P < 0.001$) **(Figure 11C).** The ascorbate radical signal has a $t_{½}$ of $\cong$ 40 min with ascorbate 250 μM, $FeSO_4$ (10 μM), and 500 nM ferritin (not shown). The relatively long half-life of this reaction is due in part to the slow disproportionation rate of ascorbate radical (Equation 12) that results in partial ascorbate regeneration.

Raising the initial ascorbate concentration from 0-1000 μM with ferritin (500 nM) and $FeSO_4$ (20 μM) increases ($P < 0.001$) ascorbate free radical concentration from zero to 120 nM as a hyperbolic function of ascorbate with a small linear component **(Figure 11E)**.

As has been previously observed [86] , superoxide dismutase (120 nM) reduces the AFR signal ($P < 0.01$) and anoxic conditions almost completely abolish the AFR signal **(Figure 11D).**

Interactions between ferritin and ascorbate radical.

Ferritin's reactivity with ascorbate radical was determined by observing the altered rates of ascorbate radical decrease [87] . High ascorbate radical concentrations were produced by ascorbic acid oxidase (1 unit ml^{-1} ≡ 8 nM) acting on ascorbate (250 μM) in citrate (1 mM) buffer pH 7.0 at 24˚C. The ascorbate radical signal initially increased at a rate dependent on ascorbate oxidase conversion of ascorbate to ascorbate radical. However, this is increase is too rapid to be captured by the protocols used in **(Figures 12A, 12B).** Loss of ascorbate radical is dependent on the combined rate of ferritin-induced AFR signal decay, which increases as a linear function of horse spleen ferritin concentration **(Figure 12C),** and of AFR disproportionation ($5x10^5$ M^{-1} s^{-1}) measured at 37°C, pH 7.4 [23]. The data are consistent with a large second order rate constant of ascorbate radical reaction with ferritin (k = $1.2x10^7$ M^{-1} s^{-1}). This increases the rate of ascorbate radical loss. Addition of iron also increased the overall rate of ascorbate radical decrease with ferritin (200 nM) **(Figure 12D)**.

Discussion.

Ascorbate-dependent iron release is potentiated by external iron

The following discussion aims to interpret the results reported here in terms of mono- and di-electron transfers to diferric iron 2Fe(III) at the ferroxidase centre of ferritin. The reduction of FOC2Fe(III) is taken as the limiting step for iron release from ferritin. The redox potential E_m is assumed to consist of several stepwise redox reaction, E_m = [{Fe(III)−Fe(III)}/{Fe(III)−Fe(II)}]= +210 mV and E_m [{Fe(III)−Fe(II)}/{Fe(II)−Fe(II)}] = +50 mV; a metastable Fe(III) in the ferroxidase centre mediates electrons transference from an external reducing agent to the Fe(III) in the mineral core [88].

One electron transfers

This study corroborates previous work showing that ascorbate increases the rate of iron mobilization from ferritin (*apparent Km* $_{(ascorbate)}$ ≈ 1.5 mM, pH range 6-8)[46,47,61]. The main findings uncovered are that the rate of iron release from ferritin is synergised by the presence of pathophysiological concentrations of both labile iron (2.5-25 μM) and ascorbate (50-250 μM) and that the chemical mechanism of this synergism differs from that induced solely by ascorbate. The difference between the two mechanisms of mobilization is apparently related to the dissimilar processes controlling one and two electron transfer reactions to oxo-bridged di-iron at the ferroxidase centre in the ferritin core.

Superoxide radical

Superoxide is a well-documented agent of single electron iron release from ferritin [46,93]. However, nominally "superoxide-induced" mobilization of ferritin iron is incompletely inhibited by SOD [[44,53,89] as confirmed in **(Figure 7A)**. Superoxide generated by xanthine oxidase/xanthine (XO/X) has previously been observed to release iron from ferritin [53,90–92]. Doubts have been expressed as to whether superoxide is the principal agent of single electron transference and reduction of the ferritin core iron [53]. Iron mobilization by superoxide on its own is a rather slow process [38]. This is ascribed to its relatively low redox potential E° $O_2/O^{\bullet-}$ -180mV, close to that of Fe(III/II) within the ferroxidase centre [93,94] .

Ascorbate and ascorbate radical.

One electron transfers by iron-catalysed formation of ascorbate radical.

It has been suggested that, at neutral pH, AscH- undergoes oxidation synergised by iron ($E^\circ Asc^\bullet/AscH^-$ = 174 mV) [94]. The ascorbate free radical (AFR) produced [40,95] can transfer single electrons to the FOC. Thus, excess AFR production promoted by iron accelerates ascorbate-dependent iron mobilization from ferritin. Additionally, iron may also accelerate the rate of interaction between ascorbate radical and ferritin.

Ascorbate radical is formed, slowly, by spontaneous oxidation of ascorbate (equation 11), or more rapidly with iron catalysis, (equations 9 and 10), or enzymatically by ascorbate oxidase. It is apparent from the above discussion that, in addition to superoxide radical and ascorbate, ascorbate radical, generated in the reaction with ferritin, also mobilizes ferritin iron**.**

One electron transfers from ascorbate radical to ferritin iron

Ascorbic acid oxidase in the presence of dioxygen oxidizes ascorbate to form AFR [3,34,47,96] **(Figures 12A,12B).** Increased AFR production, by ascorbic acid oxidase or by reaction of ascorbate with labile iron complexed with citrate [23,97] increased the rate of iron mobilization from ferritin and both these processes reduced the *apparent* $K_{m\ (ascorbate)}$ of ascorbate-dependent ferritin iron mobilization significantly **(Figures 1B, 9B and S1A)**. These processes were only partially inhibited by SOD. The *apparent* $K_{m\ (ascorbate)}$ obtained with ascorbic acid oxidase (0.59 ± 0.13 mM) **(Figure 9B)** is closer to that observed with ascorbate and iron and SOD (*apparent* $K_{m\ (ascorbate)}$ = 0.25 ± 0.03 mM) **(Figure 5B)** and significantly lower than that observed without iron and with SOD (600 nM) (*apparent* $K_{m\ (ascorbate)}$ = 1.8 ± 0.2 mM) ($P < 0.001$) **(Figure 5A)**, or without iron or SOD, *apparent* $K_{m\ (ascorbate)}$ = 1.52 ± 0.52 mM ($P < 0.05$), (**Figure 1C**)**.**

Both exogenous and endogenous hydrogen peroxide slow the rate of iron mobilization by one electron transfer from ascorbate, as is evident from **Figures 5C and 8B**.

The autoluminescence studies illustrated in **Figure 10** revealingly show large differences between luminescence induced by ascorbate alone and in combination with $FeSO_4$ (25 μM). The contrast between the very low autoluminescence signals of ferritin with ascorbate alone and the much higher chemiluminescence seen with ascorbate + iron indicates that in this latter case luminescence is mainly elicited by single electron transfers.

The results observed with EPR methods corroborate the view that single electron transport from ascorbate radical occurs rapidly with ferritin **(Figures 11A, 11B and 11D).** Also, that superoxide, ascorbate anion, and ascorbate radical all react with ferritin to promote iron reduction at the ferroxidase centre that lead to its mobilization. It is evident that hydrogen peroxide suppresses iron mobilization both from direct inhibitory effects following exogenous addition of hydrogen peroxide and from the increased mobilization rates observed with catalase.

As well as being a promoter of ascorbate radical generation (**Figure 11A**), ferritin evidently is an extremely effective promoter of ascorbate radical decay **(Figures 12B and 12C)** and as a hydrogen peroxide scavenger **(Figure 5C)** [98].

Two electron transfer processes in relation to ferritin iron mobilization

In addition to single electron transfer reactions, two electrons can be transferred from appropriate donors to acceptor sources [38,99–101] Where hydrogen peroxide is used as a direct two-electron acceptor, dual electron transfer reactions or bidentate reactions are viewed as an alternative means of oxidizing the vicinal di-ferrous ions within the FOC [100]. This circumvents intermediate ROS formation [19] Two electron transfer processes are used in other oxo-bridged di-iron complexes e.g. catalases [101–103].

These reactions are dependent on either dioxygen or hydrogen peroxide as two electron oxidizing agents that bridge di-iron or di-copper complexes, as illustrated diagrammatically in the **introductory graphic**, **Figure 6A,** and Eq. 1 and are evidently obscured from chemiluminescence or EPR detection.

The nature of ferritin's chemiluminescent signal elicited by ascorbate and iron

During iron uptake by apoferritin a "blue" intermediate is formed with an absorption maximum at 625 nm. It has been suggested that a highly conserved tyrosine residue in the vicinity of the ferroxidase centre [104] forms a tyrosine radical and acts as a one electron capacitor [105–107]. Tryptophan fluorescence is also activated by iron citrate uptake into soybean ferritin [[87]. No equivalent absorption or luminescence studies on ferritin iron reduction and mobilization have been reported to date; however, during electrolysis or horseradish peroxidase-catalysed oxidation of tyrosine, a blue-green chemiluminescence signal at 490 nm [88] similar to that observed here with ferritin in **Figure 10B,** was attributed to the formation of an exciplex between tyrosine cation radical $Tyr^{\cdot+}$ and superoxide, probably originating from compound III ($Fe(III)\cdots O_2^{\cdot-}$): $[Tyr^{\bullet+}\, O_2^{\bullet-}]^* \rightarrow h\nu$ (490 nm). SOD quenched this reaction with horseradish peroxidase [108,109], but catalase was ineffective. It has been suggested that aromatic radicals in metalloproteins can act as intermediate staging posts in electron transfer reactions [12,88,107,110]. In the present study, SOD alone did not reduce the chemiluminescence signal and SOD + catalase enhanced the signal **(Figure 10D)** suggesting that that ascorbate radical or an ascorbate iron citrate complex may activate the chemiluminescence signal.

The biphasic nature of the chemiluminescence response to raised ascorbate with Fe(II) (25 μM) is consistent with the view that citrate-Fe(II)-$Asc^{\bullet-}$ complex, rather than citrate-Fe(II)-$AscH^-$ generates the chemiluminescence signal as higher $AscH^-$concentrations may displace $Asc^{\bullet-}$ from the complex **(Graphic, Figure 10C).**

The enhancement of the ferritin chemiluminescence signal by catalase **Figure 11D**, corroborates the deduction from the experiments that endogenous hydrogen peroxide stabilizes the diferric ions at the ferroxidase centre from reduction by ascorbate-dependent electron donation. Hence removal of this shield promotes both iron mobilization and electron transference between ascorbate ferritin iron within the ferroxidase centre and a neighbouring tyrosine group[88].

The experimental support for the view that two-electron reductive reactions of iron mobilization from ferritin differ from single-electron reactions is summarised as follows:

- The *apparent* K_m for ascorbate-dependent ferritin iron mobilization is eightfold higher and V_{max} approximately fivefold lower in the absence of labile iron **(Figures 1B and 1C).**

- The pH sensitivity of iron mobilization in the presence of ascorbate is approximately fourfold lower with iron present than without iron **(Figure 2A)**. This suggests that two-electron reductions are less proton-dependent than the one-electron transfer reactions [19] (**Figure 6A**).
- The activation energy E_a of iron mobilization with ascorbate alone is threefold higher than with iron present **(Figure 2B)**.
- The $I.C._{50}$ of hydrogen peroxide inhibition of ascorbate-dependent mobilization of ferritin iron is at least sevenfold lower with ascorbate alone than with iron present ($P < 0.01$) **(Figure 5C)**.
- Catalase reduces the apparent K_m of ascorbate-dependent mobilization by approximately 80%, both with iron absent **(Figure 8A)** or iron present (10 μM) **(Figure 8B).** This indicates that endogenous hydrogen peroxide shields ferritin iron against reduction by ascorbate. Even with catalase present, the ferritin iron mobilization apparent $K_{m\ (ascorbate + Fe(II))}$ is much lower than the apparent $K_{m\ (ascorbate)}$ ($P < 0.0001$).
- Even high ascorbate concentrations (10 mM) generate a very low chemiluminescent signal of ferritin, whereas the maximal ferritin chemiluminescence signal with ascorbate (100 μM) and iron (25 μM) is twenty-fold higher than with ascorbate alone**.**
- Labile iron (40 μM) increases the ferritin-induced ascorbate radical EPR signal with ascorbate (250 μM) by 400% (**Figure 11C)**. This is mainly due to enhanced intramolecular formation of ascorbate radical within ferritin [21,27,111].

The results reported here show a synergy between low concentrations of labile iron and ascorbate as reducing and mobilizing agents of iron from the ferritin core. This explains why ascorbate alone is not notably pro-oxidant [34].

Raised levels of total iron have been detected in synovial tissue and fluid from patients with rheumatoid arthritis [112–115] with EPR of ascorbate free radicals [49,113,116]. In inflammation and with cancer therapy raised ascorbate levels can have a pro-oxidant effect [21,49,54,117–120]. This can be ascribed to interactions with metal ions and may also relate to enhanced iron release from ferritin [21,54,121,122].

Since autocatalytic iron release from ferritin by ascorbate occurs within the pathophysiological concentration ranges of ascorbate and labile iron and is retarded by effective chelation therapy such as desferrioxamine [43,123] or quercetin [56] that are known to inactivate Fenton reactions, this therapeutic approach to treatment of inflammatory disease might be worth exploring more vigorously.

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Supplementary Figure S1A Effect of varying $FeSO_4$ concentration in citrate buffer (10 and 20 mM) with MOPS (50 mM) at pH 7.0 on the rates of iron mobilization from ferritin (500 nM) with ascorbate (5 mM) at 24°C. Apparent $K_{m\ Fe}$ = 3.4 ± 2.0 μM with citrate (10 mM); apparent $K_{m\ Fe}$ = 43 ± 15 μM with citrate (20 mM) ($P < 0.025$). **B Simulated and EPR observed changes in ascorbate free radical (right Y scale) and simulated ascorbate concentration changes (left Y scale) in incubations of ascorbate with ascorbic acid oxidase with or without ferritin at 24°C**. Ascorbate (250 μM); Asc Ox (8 nM) (i.e. 1 unit ml^{-1}); ferritin (0-200 nM); citrate (1 mM) pH 7.0.

Supplementary Figure S2 Plots of time course of ascorbate radical decrease with ascorbic acid oxidase and varying ferritin at 24°C. Ascorbate (250 μM); Asc Ox (8 nM) (i.e. 1 unit ml^{-1}); ferritin (0-500 nM); no $FeSO_4$; citrate (1 mM) pH 7.0. Mono-exponential fits are overlaid on EPR experimental data points.

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Figure 1 Chemical reactions involved in ascorbate and iron induced redox cycling. Equation numbers refer to equations in text. **A Effect of ferritin and labile iron on typical time courses monitoring the development of ferrous-ferrozine complex using absorption spectrophotometry at 550 nm at 24°C**. Ascorbate (10 mM), ferritin 0-50 nM; $FeSO_4$ 0-2.5 μM; ferrozine (75 μM); citrate (10 mM); MOPS (50 mM); pH 7.0. Each plotted point is the average of triplicate readings, the errors on each point are too small to be plotted. Rates of ferritin iron mobilization, calibrated to nmole mg^{-1} ferritin min^{-1}, are derived from the difference between the slopes of linear regression analysis with and without ferritin. **B Effects of varying ascorbate and $FeSO_4$ concentration on the rate of iron(II) release from ferritin at 24°C as determined from ferrozine absorption changes.** Ascorbate 0-10 mM, ferritin (50 nM); $FeSO_4$ 0-10 μM; ferrozine (75 μM); citrate (10 mM); MOPS (50 mM); pH 7.0. Values are mean ± S.E.M. of triplicate experiments. **C Effect of $FeSO_4$ concentration on apparent K_m and V_{max} values of ascorbate-dependent release of Fe(II) from ferritin.** Values are mean ± S.E.M. of triplicate experiments. Raising labile iron from 0 to 10 μM results in a hyperbolic decrease in apparent $K_{m\ ascorbate}$ from 1.5 mM to approximately 200 μM (broken line, open squares); (*apparent* $K_{m\ Fe}$ 0.8 ± 0.2 μM). The V_{max} of ascorbate-dependent iron mobilization from ferritin increases exponentially with labile iron (continuous line, open circles).

Figure 2A Effects of altering pH on ascorbate-dependent release of Fe(II) from ferritin at 24°C as determined from ferrozine absorption changes. Ascorbate 0-10 mM, ferritin (50 nM); $FeSO_4$ (0-10 μM); ferrozine (75 μM); citrate (10 mM); MOPS (50 mM) pH 5.5-8.5. There is negligible Fe(II) release when ascorbate is absent. The sensitivity of ascorbate-dependent iron

release to [H^+] is enhanced in the presence of $FeSO_4$ (10 μM). Values are mean ± SEM, n= 4 observations per point (P<0.01). **B Arrhenius plots showing the rate of Fe(II) loss from ferritin;** plotted as Log_N(rates of Fe(II) release in moles iron (II) per mole ferritin and per second). Ascorbate (10 mM), ferritin (50 nM); $FeSO_4$ (0-10 μM); citrate (10 mM); MOPS (50 mM); pH 7.0. Temperatures were 4°C, 24°C and 37°C. Values are mean ± SEM, n= 6 observations per point. There is a 3-fold decrease in activation energy (E_a) with ascorbate and 10 μM $FeSO_4$ compared to Fe(II) absent. (P<0.01).

Figure 3A Ferrozine absorbance changes following exposure of ferritin to varying $FeSO_4$ concentrations. Ascorbate (10 μM), ferritin (50 nM); $FeSO_4$ (0.1-10 μM); ferrozine (75 μM); citrate (10 mM); MOPS (50 mM); pH 7.0. Each plotted point is the average of n = 4 readings, the errors on each point are too small to be plotted. Raising labile iron reduces the latency of iron mobilization to reach steady state. **B. Effects of ascorbate and iron on the latency of iron mobilization from ferritin determined from ferrozine absorption changes**. Ascorbate (10 μM - 5 mM); ferritin (50 nM); $FeSO_4$ 0.1-10 μM; ferrozine (75 μM); citrate (10 mM); MOPS (50 mM); pH 7.0. Two-way ANOVA shows independent effects of iron (P < 0.0001) and of ascorbate (P < 0.0001). **C Effect of catalase on the initial release of iron(II) from ferritin measured by ferrozine absorption changes**. Ascorbate (100 μM); ferritin (50 nM); $FeSO_4$ (10 μM); catalase (0-10 nM); ferrozine (75 μM); citrate (10 mM); MOPS (50 mM); pH 7.0. Catalase raised the initial rate during the first 15 min (P < 0.001).

Figure 4A Zn(II) inhibits ascorbate-dependent iron release from ferritin as determined from ferrozine absorption changes. Ascorbate (10 mM); ferritin (50 nM); $ZnCl_2$ (0-10 μM); no $FeSO_4$; ferrozine (75 μM); citrate (10 mM); MOPS (50 mM); pH 7.0. Values are mean ± SEM of 2 experiments, n = 6 replicates per point. Inhibition occurred in a concentration dependent manner. Best fit hyperbolic regression line is shown $y = I.C._{50}.\ Vmax/(I.C._{50} + [Zn])$, with $I.C._{50\ Zn(II)} = 2{,}2 \pm 0{,}4$ μM. **B Zn(II) inhibits ascorbate + Fe(II) tandem iron mobilization from ferritin as determined from ferrozine absorption changes.** Ascorbate (10 mM); ferritin (50 nM); $ZnCl_2$ (0-20 μM); $FeSO_4$ (35 μM); ferrozine (75 μM); citrate (10 mM); MOPS (50 mM); pH 7.0. Values are mean ± SEM of 2 experiments, n = 6 replicates per point (p < 0.001).

Figure 5 Influence of hydrogen peroxide on the rate of ascorbate-dependent iron release from horse spleen ferritin as determined from ferrozine absorption changes. Ferritin (50 nM); ferrozine (75 μM); citrate (10 mM); MOPS (50 mM) pH 7.0. Values are mean ± SEM, n= 4

replicates per point. **(A)** Ascorbate (0-20 mM) and H_2O_2 (0-0.25 mM) were varied in the presence of SOD (600 nM, ie. 50 units.ml^{-1}) alone. H_2O_2 non-competitively inhibits ascorbate-dependent release in the absence of external Fe (II). **(B)** Ascorbate (0-20 mM) and H_2O_2 (0-1 mM) were varied in the presence of SOD (120 nM, i.e. 10 units.ml^{-1}) and $FeSO_4$ (10 μM). H_2O_2 has a negligible effect on ascorbate-dependent release in the presence of both Fe (II) and SOD. **(C)** H_2O_2 (0-5 mM) and $FeSO_4$ (0-5 μM) were varied in the presence of SOD (120 nM) and ascorbate (10 mM). In the presence of increasing external Fe, K_i of H_2O_2 is increased from its initial value of 150 μM, i.e. a reduction of H_2O_2 inhibition is observed ($P < 0.001$). The Hill equation[128] was applied empirically to obtain line fits to the data in **Figures 5A** and **5B**, where rate of iron release $y = \frac{\text{Vmax.}[ascorbate]^{n}}{\text{apparent Km} + [ascorbate]^{n}}$; where n is the Hill coefficient of ligand cooperativity. The fits and error estimates of the parameters were obtained using the Levenberg-Marquardt algorithm as outlined in Methods. The fits in **Figure 5C** were obtained using the equation $y = \frac{\text{I.C.50 .Vmax}}{\text{I.C.50} + [ascorbate]}$.

Figure 6 A Proposed chemical structures involved in two electron transfers to the diferrous ferroxidase centre. Electron transfer from the diferrous ferroxidase centre to hydrogen peroxide, yielding an oxo-bridged diferric ferroxidase complex and avoiding hydroxyl radical production via the Fenton reaction as envisaged by Bou-Abdallah *et al.* [105], is shown in the upper panels and is mirrored in the lower panels by the reverse two-electron flow from ascorbate to the oxo-bridged diferric ferroxidase complex thus avoiding ascorbate radical formation. **B Chemical structures involved in one-electron transferences between ascorbate, ascorbate radical, and ferroxidase centre.** The graphic illustrates the sequential single electron processes between ascorbate and the di-ferric ferroxidase centre in which ascorbate radical is generated as an intermediate which may in turn reduce another ferric atom in a different ferroxidase centre of ferritin.

Figure 7 A Effect of superoxide dismutase on labile iron-dependent iron release from ferritin as determined from ferrozine absorption changes. Ascorbate (10 mM); FeSO4 (0-10 μM); SOD (0-120 nM); ferritin (50 nM); ferrozine (75 μM); citrate (10 mM); MOPS (50 mM); pH 7.0. Values are mean ± SEM, n = 3 replicates per point. The rates of ferritin iron mobilization are expressed as fractional value of the values without SOD. Increasing the external Fe(II) reduces the fractional inhibition of iron mobilization by SOD (120 nM) from 58% with zero

iron to 15% with $FeSO_4$ 10 μM ($P < 0.01$). **B Effect of catalase on iron release from ferritin in the presence of ascorbate and $FeSO_4$ as determined from ferrozine absorption changes.** Ascorbate 250 μM; $FeSO_4$ (0-10 μM); Catalase (5 nM; ferritin (50 nM); ferrozine (75 μM); citrate (10 mM); MOPS (50 mM) pH 7.0 (n= 8 for each condition). Catalase enhances ascorbate + iron tandem iron mobilization from ferritin ($P < 0.01$) but has no significant effect on ascorbate-dependent iron mobilization without iron. **C Effect of xanthine oxidase (XO) on iron mobilization in the presence of ascorbate and $FeSO_4$ as determined from ferrozine absorption changes.** Ascorbate (1 mM); $FeSO_4$ (0-10 μM); xanthine (0.15 mM); XO (0-0.4 milliunits mL^{-1}); ferritin (50 nM); ferrozine (75 μM); citrate (10 mM); MOPS (50 mM) pH 7.0 (n= 4 replicates per point). XO activity increased the rate of ascorbate-dependent iron release without labile iron by 100% (++ $P < 0.01$), but the rate of ascorbate + iron tandem iron release from ferritin only by 16% (*** $P < 0.001$).

Figure 8 A Effect of catalase on ascorbate-dependent iron mobilization from ferritin as determined from ferrozine absorption changes. Ascorbate 0-10 mM; no $FeSO_4$; Cat (0-10 nM); ferritin (50 nM); ferrozine (75 μM); citrate (10 mM); MOPS (50 mM); pH 7.0. The apparent K_m of ascorbate-dependent iron release decreases with addition of catalase (10 nM) from 1.20 ± 0.14 mM to 0.26 ± 0.03 mM ($P < 0.0001$), while V_{max} increases from 9.80 ± 0.33 to 10.9 ± 0.3 nmoles mg^{-1} ferritin min^{-1}.**B Effect of catalase on ascorbate + iron-dependent iron mobilization from ferritin as determined from ferrozine absorption changes**. Ascorbate (0-2.5 mM); $FeSO_4$ (10 μM); Catalase (0-10 nM); ferritin (50 nM); ferrozine (75 μM); citrate (10 mM); MOPS (50 mM) pH 7.0 (n= 12 per point two experiments). The apparent K_m of ascorbate + iron-dependent iron release decreases with addition of 10 nM catalase from 390 ± 31 μM to 77.6 ± 10.5 μM ($P < 0.0001$), while V_{max} increases from 120 ± 4 to 125 ± 3 nmoles mg^{-1} ferritin min^{-1}.

Figure 9 A Effect of ascorbic acid oxidase (Asc Ox) in combination with SOD + Catalase on ascorbate-dependent iron mobilization from ferritin as determined from ferrozine absorption changes. Ascorbate (1 mM); SOD (0-120 nM); Catalase (0-5 nM); Asc Ox (0-2 nM); ferritin (50 nM); ferrozine (75 μM); citrate (10 mM); MOPS (50 mM) pH 7.0 (n= 8 replicates per condition). Asc Ox increases ascorbate-dependent iron mobilization by ascorbate 1 mM**.** The effect of Asc Ox is significant with the enzyme at 2 nM ($P < 0.05$) and even at 1 nM ($P < 0.001$) (not shown). Asc Ox (2 nM)-dependent iron mobilization is unaffected by SOD (120 nM) and catalase activity (10 nM) ($P < 0.001$). **B Effect of ascorbic acid oxidase (Asc Ox) on ascorbate-dependent iron mobilization from ferritin as determined from ferrozine absorption changes**. Ascorbate (0-2 mM); Asc Ox (0-1 nM); ferritin (50 nM); ferrozine (75

μM); citrate (10 mM); MOPS (50 mM); pH 7.0. Each point is average of two separate experiments (each point replicated 3-4 times). Asc Ox decreases the *apparent* $K_{m\ ascorbate}$ of iron mobilization from ferritin ($P < 0.01$).

Figure 10 A Effect of ascorbate on scans of autochemiluminescence emission of ferritin at 24°C. Autochemiluminescence of the incubation without ferritin was subtracted. Ascorbate (50-10000 μM); ferritin (500 nM); citrate (10 mM); MOPS (50 mM); pH 7.0. There is a small increase in luminescence with peak at 475 nm as ascorbate is increased from zero to 10 mM. **B Effect of ascorbate on scans of autochemiluminescence emission of ferritin in the presence of iron (II) at 24°C.** Autochemiluminescence of the incubation without ferritin was subtracted. Ascorbate (25-1000 μM); ferritin (500 nM); $FeSO_4$ (25 μM); citrate (10 mM); MOPS (50 mM); pH 7.0. The ascorbate-dependent increase in luminescence at 475 nm is larger in the presence of iron (II).**C**. **Luminescence of ferritin at 480 nm as a function of ascorbate with (broken line, open squares) or without (continuous line, open circles) addition of $FeSO_4$ at 24°C.** Autochemiluminescence of the incubation without ferritin was subtracted. Ascorbate 25-10000 μM; ferritin (500 nM); $FeSO_4$ 0-25 μM; citrate (10 mM); MOPS (50 mM); pH 7.0. **D Comparison of effects of catalase, SOD, and catalase + SOD on the maximal luminescence emission of solutions of ferritin (500 nM), $FeSO_4$ (25 μM), and ascorbate (250 μM) at 24°C.** Citrate (10 mM); MOPS (50 mM); pH 7.0. Catalase (5 nM) or Catalase (5 nM) + SOD (120 nM) together increase autochemiluminescence, whereas SOD alone has no effect.

Figure 11 A Comparison of EPR spectra of ascorbate radical observed in 1 mM citrate buffer at pH 7.0 and 24°C upon addition of ferritin (500 nM) on $FeSO_4$ (10 μM) + ascorbate (250 μM). B Comparison of EPR spectra of ascorbate radical observed in 1 mM citrate buffer at pH 7.0 and 24°C upon varying ascorbate from 0 to 1000 μM in the presence of ferritin (500 nM) + $FeSO_4$ (20 μM). C. Effect of varying $FeSO_4$ concentration on the ascorbate radical concentration measured by EPR in the presence of ferritin (500 nM) + ascorbate (250 μM) at 24˚C in citrate buffer (1 mM) at pH 7.0. There is a linear increase in AFR concentration with Fe(II). **D Comparison of relative EPR signal of control {ascorbate (250 μM), iron (20 μM), ferritin (500 nM)} upon addition of SOD (600 nM) and deoxygenation at 24˚C in citrate (1 mM) buffer at pH 7.0.** SOD reduces the ascorbate radical amplitude with ferritin by 58% and anoxia by 90 %. **E Effect of varying ascorbate on the ascorbate radical concentration measured by EPR in the presence of ferritin (500 nM) + $FeSO_4$ (20 μM) at 24˚C in 1 mM citrate buffer at pH 7.0.** Signal amplitude increases as a hyperbolic function of ascorbate.

Figure 12A Effect of ascorbic acid oxidase on the time course of the ascorbate radical EPR signal decline at 24°C. Ascorbate 250 µM; Asc Ox (8 nM) (i.e. 1 unit ml^{-1}); ferritin (200 nM); $FeSO_4$ (20 µM); citrate (1 mM) pH 7.0. Each scan takes 21 s and is immediately followed by the next.

Figure 12 B. Plots of time course of ascorbate radical decrease with ascorbic acid oxidase with varying ferritin at 24°C. Ascorbate (250 µM); Asc Ox (8 nM) (i.e. 1 unit ml^{-1}); ferritin (0-500 nM); citrate (1 mM) pH 7.0. Experimental data (broken lines) are overlaid with mono-exponential decay simulations (continuous lines). The lines are fitted to the reaction scheme provided in the supplementary information.**C Effect of varying ferritin on the pseudo-first order rate constant of ascorbate radical decrease at 24°C.** Ascorbate (250 µM); Asc Ox (8 nM) (i.e.1 unit ml^{-1}); ferritin (0-500 nM); citrate (1 mM) pH 7.0. The rates of ascorbate radical decrease as a linear function of ferritin concentration. **D Effect of varying $FeSO_4$ concentrations on the pseudo-first order rate constant of ascorbate radical decrease at 24°C.** Ascorbate (250 µM); $FeSO_4$ (0-40 µM); ferritin (500 nM); citrate (1 mM) pH 7.0.

Figure 1

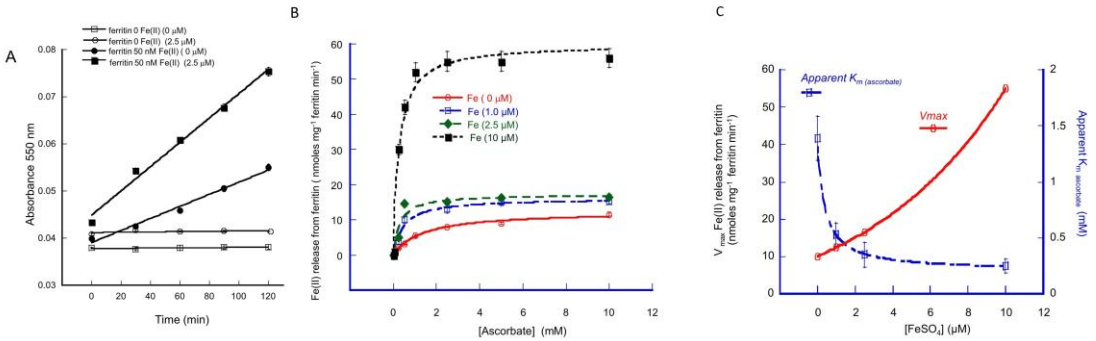


Figure 2

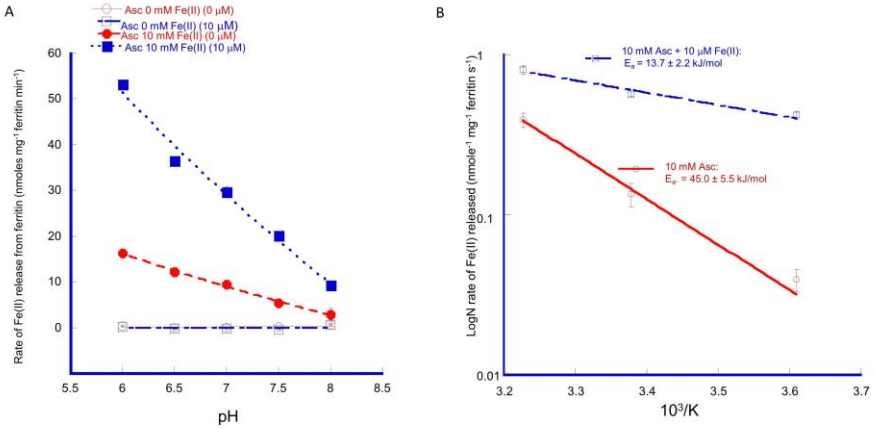
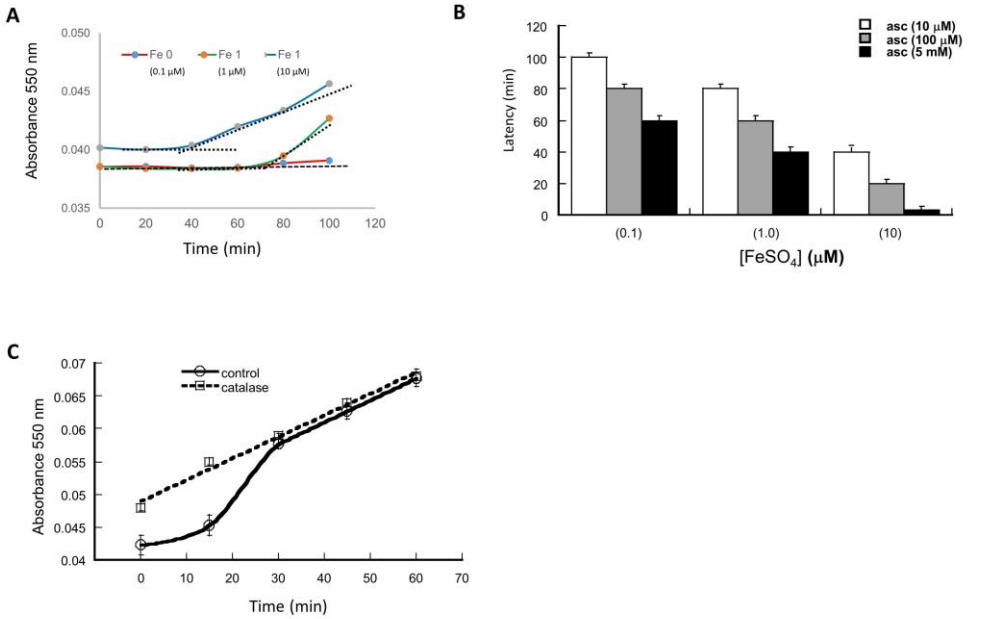


Figure 3



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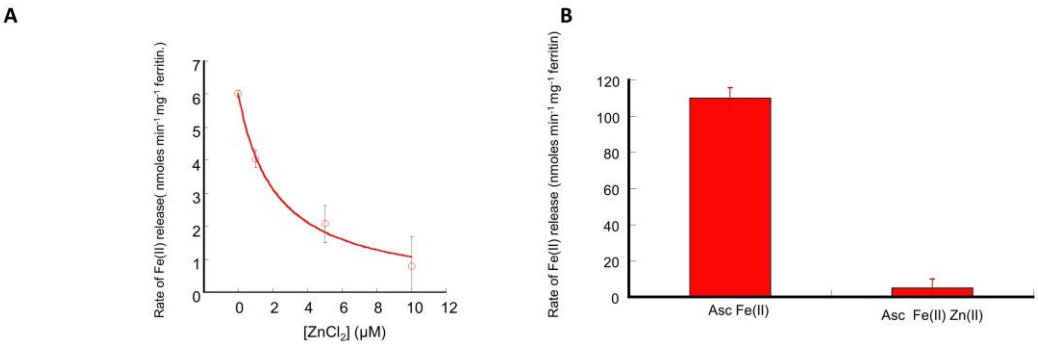

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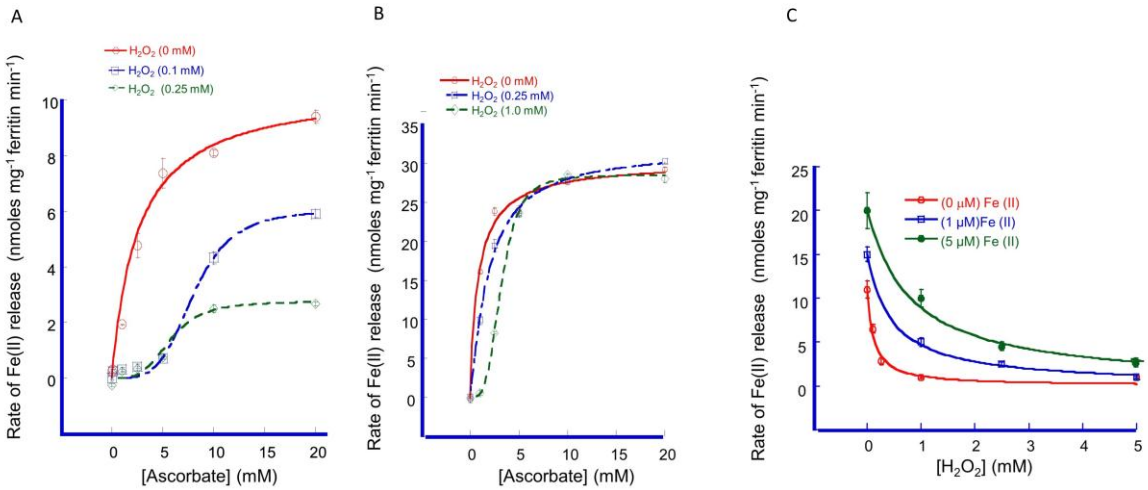
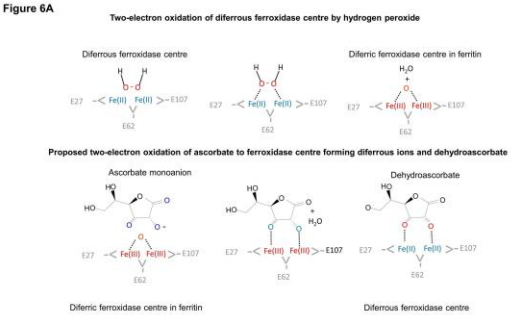


Figure 6A



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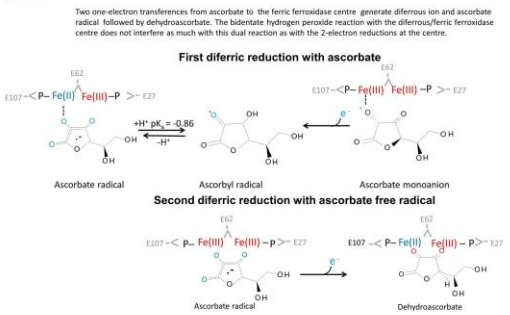

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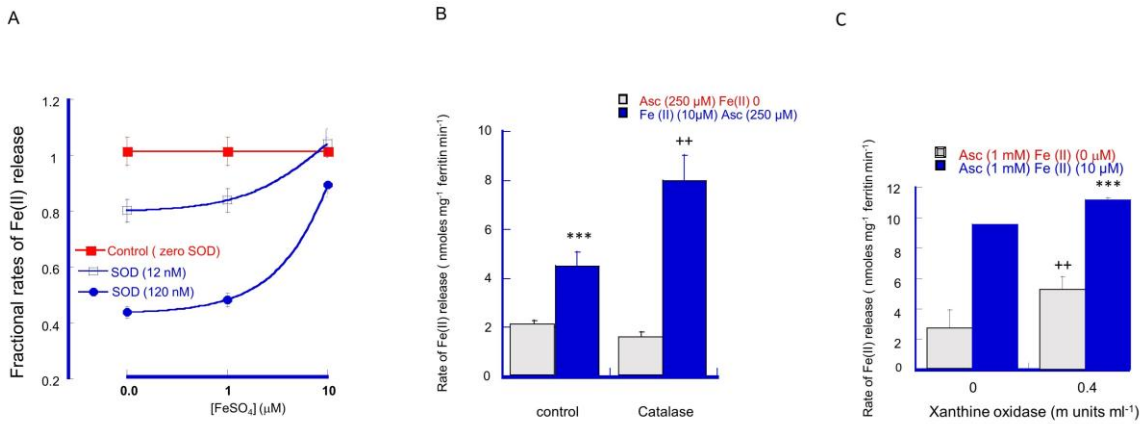

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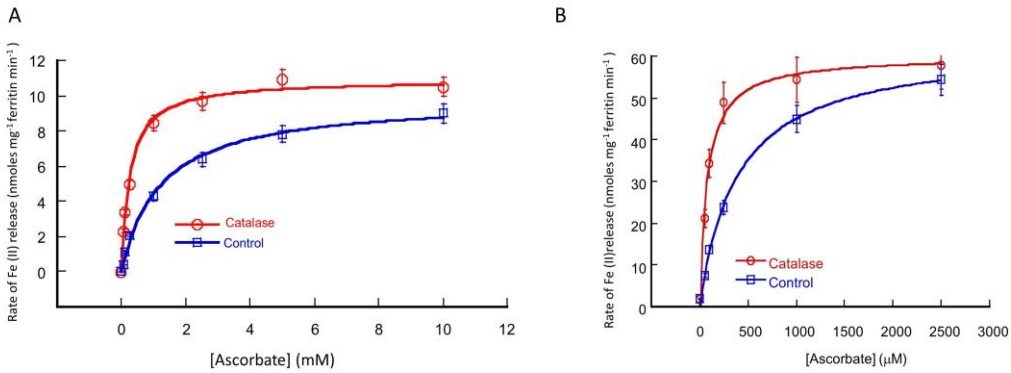

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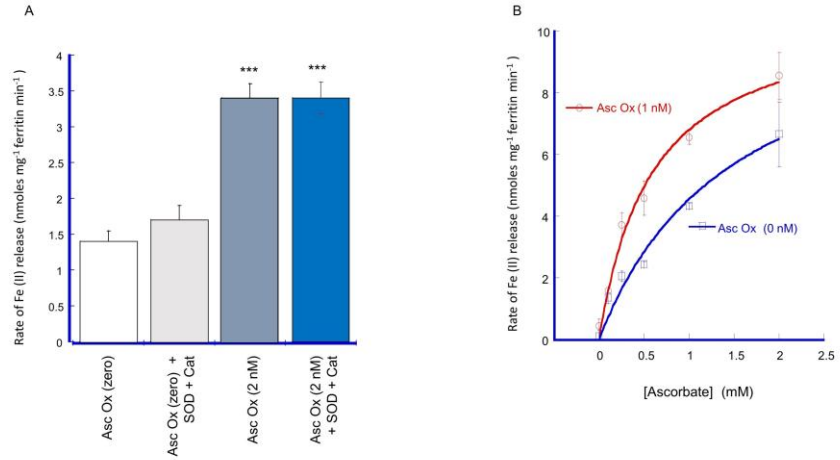

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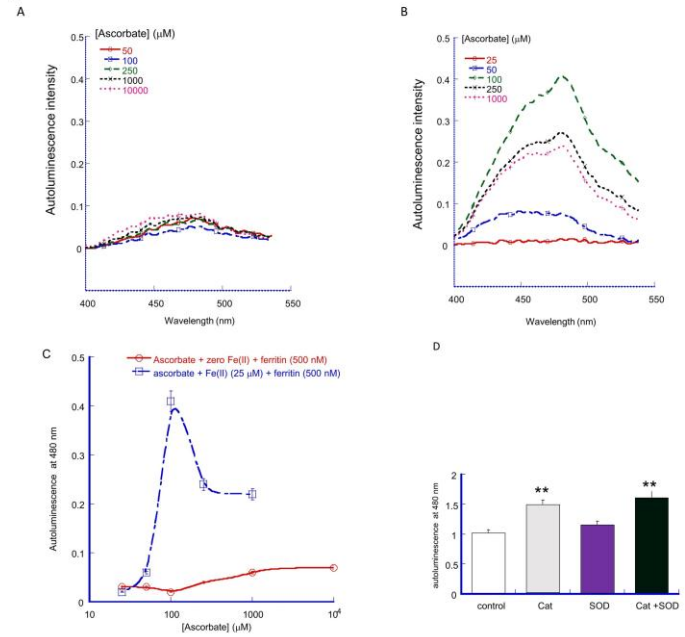

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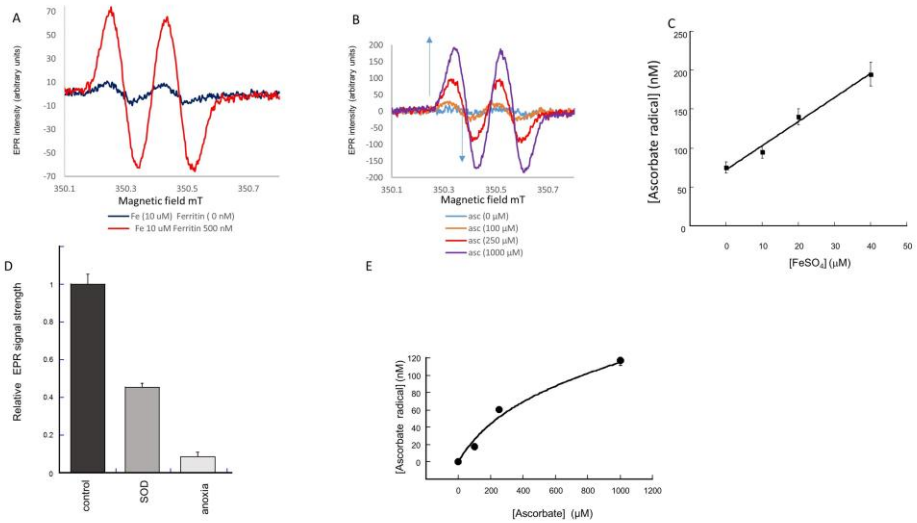

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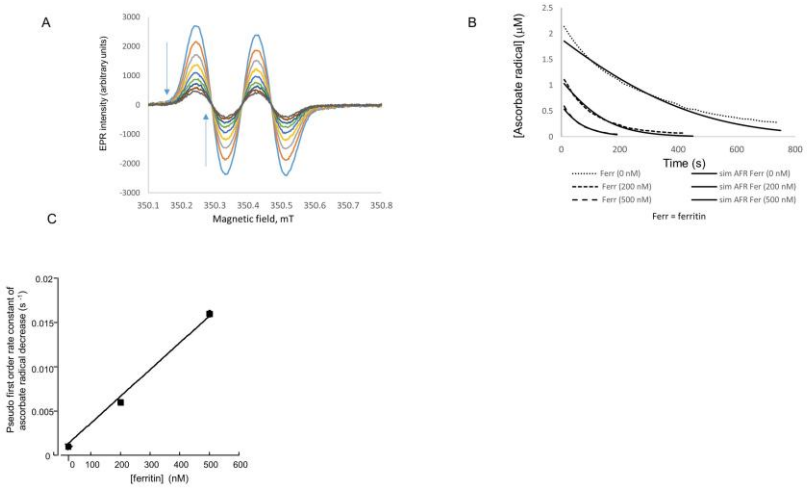
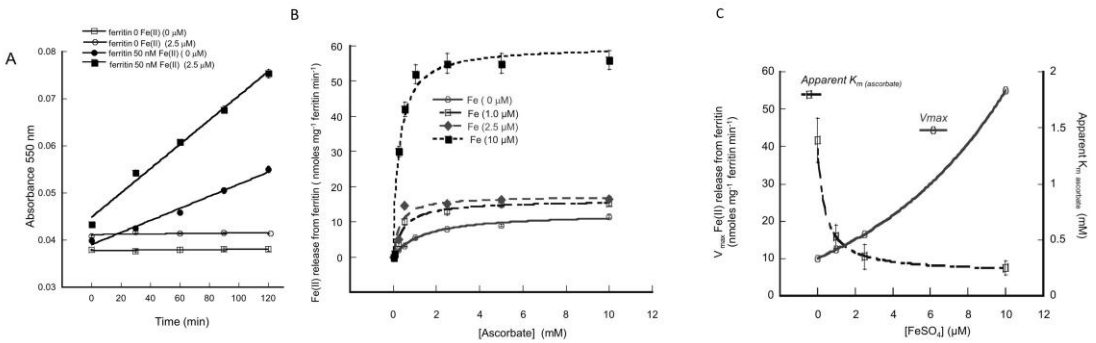


Figure 1



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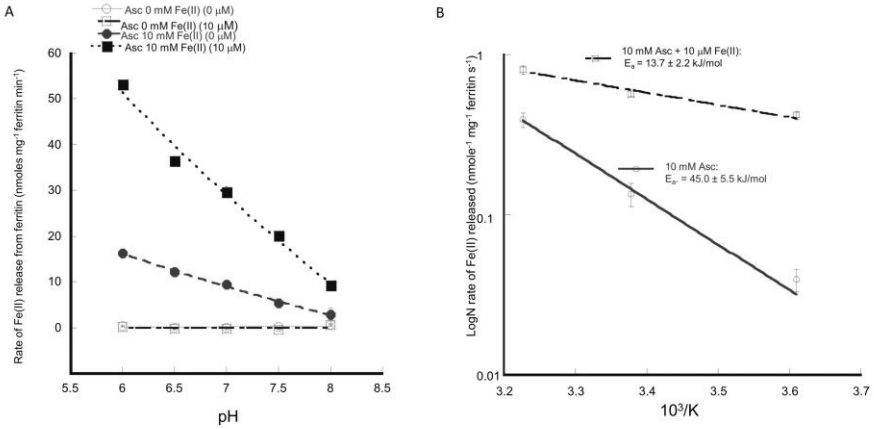

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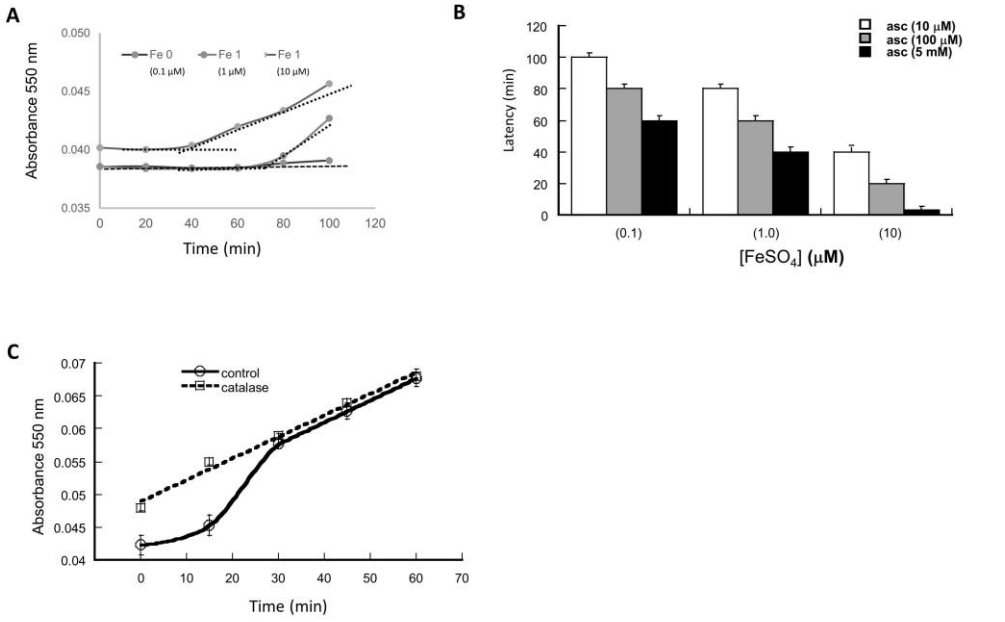

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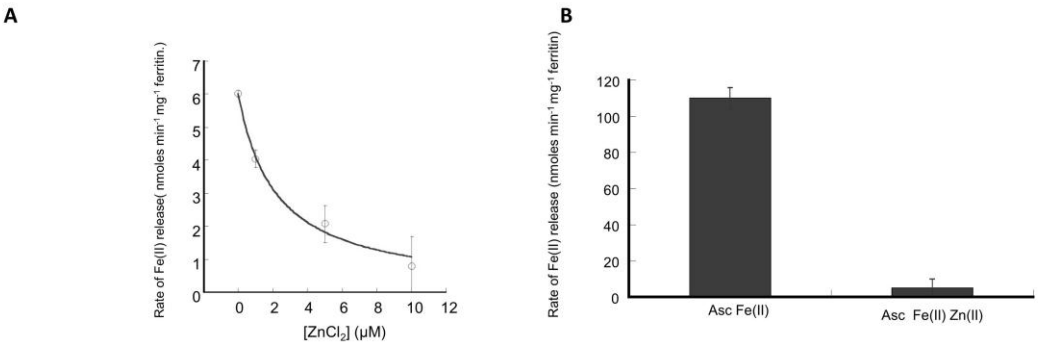

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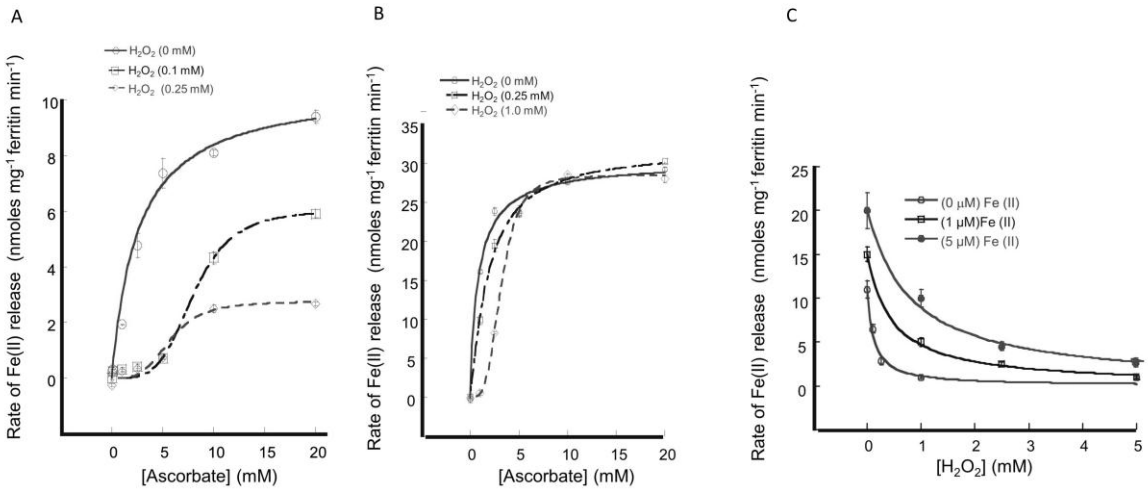

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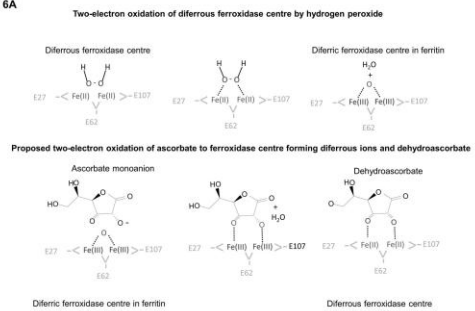

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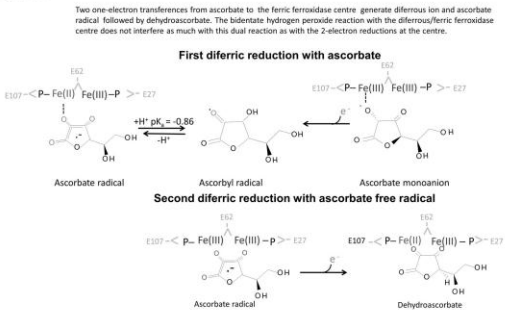

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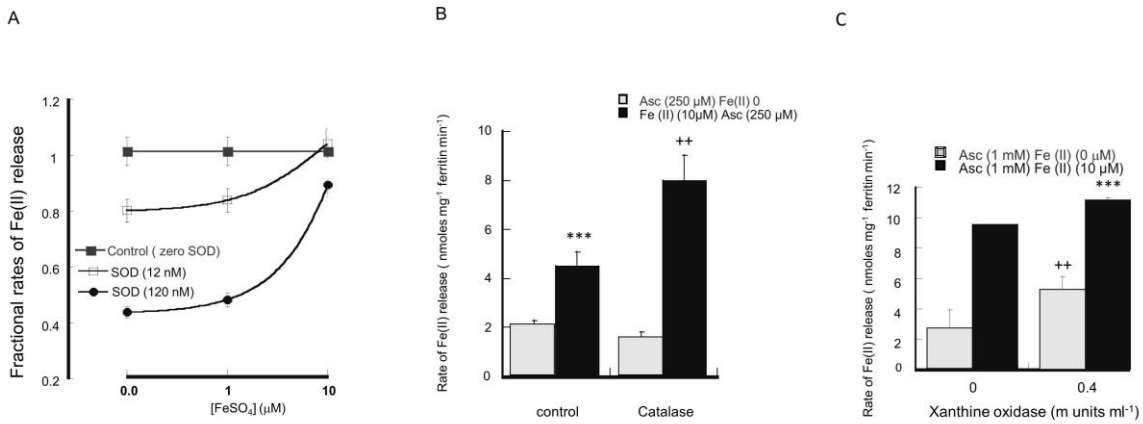

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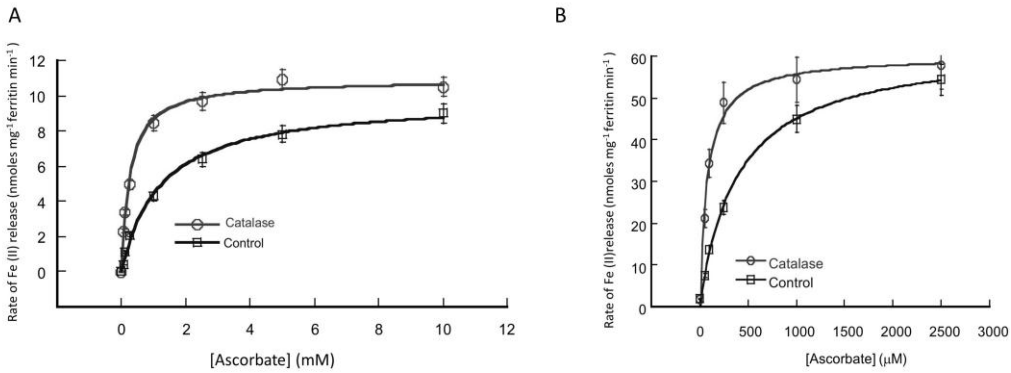

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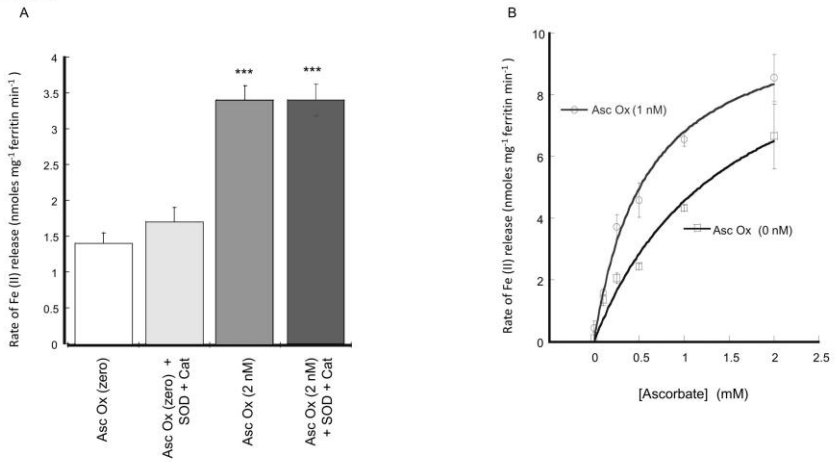

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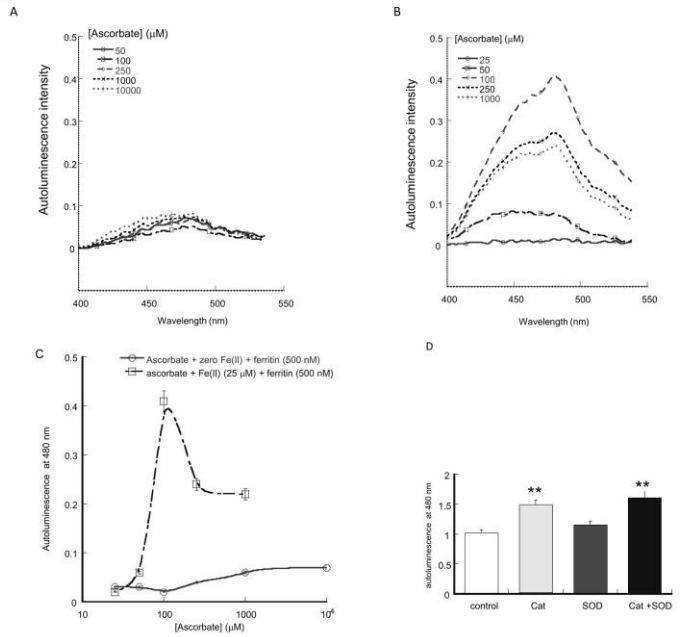

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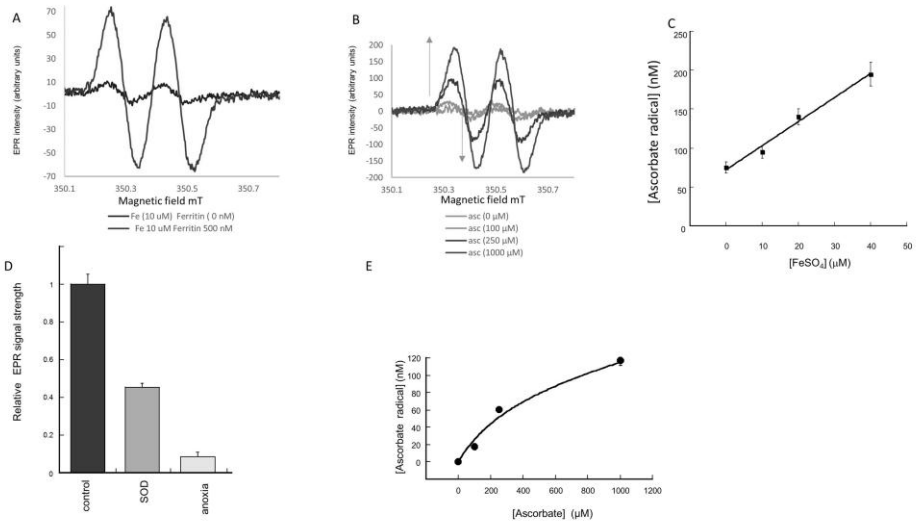

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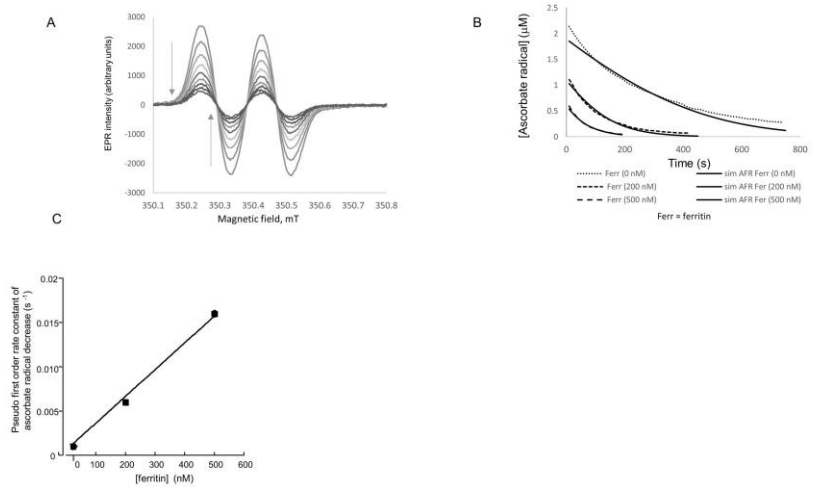
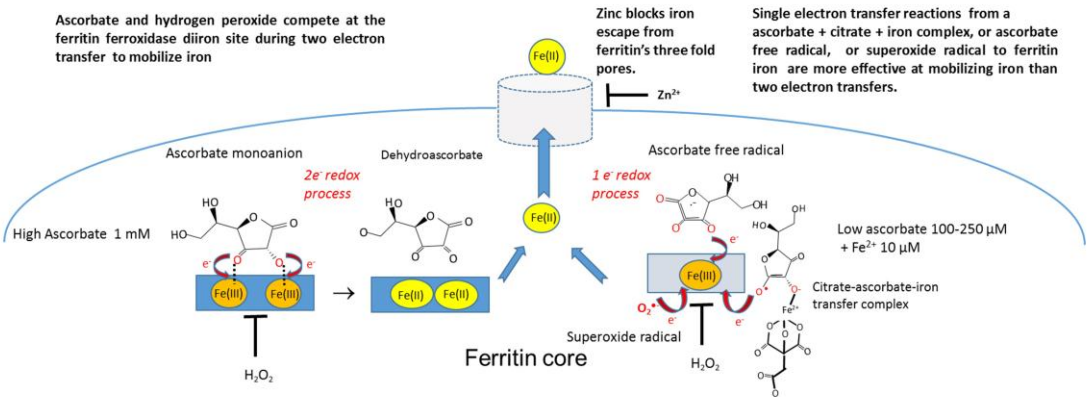


graphical abstract



Highlights

- Labile iron (LI) synergises ascorbate-dependent ferritin iron mobilization (ADFIM).
- LI reduces the K_m and increases the V_{max} of ADFIM.
- Catalase eradicates H_2O_2 shielding of ferritin's ferroxidase centre from reducing agents.
- ADFIM with LI is more pH sensitive and has a lower activation energy.
- Ascorbate radical (AFR) generated by ascorbate oxidase increases ADFIM.
- LI changes ADFIM from a slow dual to a fast bi-mono electron transfer reaction.