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# Effect of copper(II) on the aerobic oxidative uptake of iron(II) by horse spleen apoferritin ‡

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The interaction of copper(II) with horse spleen apoferritin has been investigated and approximately 16–22 $Cu^{II}$ ions are shown to bind relatively tightly to the 24 subunit protein. The bound $Cu^{II}$ is shown to have a catalytic effect on the aerobic oxidative uptake of $Fe^{II}$ rather than the inhibitory effect that $Zn^{II}$ has. Possible reasons for this are discussed.

Ferritins are multimeric proteins characterised by their ability to accumulate deposits of non-haem iron within a protein shell consisting of 24 subunits.[1–3] In mammalian ferritins the iron is deposited as a hydrated iron(III) oxide usually described as ferrihydrite, $Fe_2O_3{\cdot}9H_2O$, and there is sufficient space within a single ferritin shell for 4500 $Fe^{III}$ ions to be contained as ferrihydrite.[1] The mechanisms by which the mineralised $Fe^{III}$ core is deposited have generated much interest.[2–4] To achieve rapid core formation the incoming iron has to be $Fe^{II}$, and this requires there to be a site or sites of $Fe^{II}$ oxidation within the protein.[4–8] The growing $Fe^{III}$ mineral represents one potential set of oxidation sites and additional possible sites include the so-called ferroxidase sites within the protein shell.[7,8] These latter sites are contained by some types of ferritin subunits, (heavy) H-chains, but not by (light) L-chains, and their role in oxidizing $Fe^{II}$ rapidly in the presence of $O_2$ has been established by site-directed mutagenesis experiments.[3,8] However, L-chain ferritins do build up $Fe^{III}$ cores indicating that there are other oxidation sites available.[5,6] Release of iron from the mineral core is of equal biological importance to its formation but possible release processes have not been well characterised. However, as with core formation, rapid iron release requires the iron to be $Fe^{II}$.[3,4,9] Possible physiologically relevant reductants for iron release include ascorbate and the superoxide radical ($O_2^{-}$).[10–12] In characterising these reactions it was found that the presence of $Cu^{II}$ increases the rate at which iron is released by ascorbate[10] and copper(II)–ferritin mixtures catalyse the decay of superoxide.[13] Both of these effects could be due to reactions between $Cu^{II}$ and reductant independent of ferritin, though the latter study suggested that horse spleen ferritin contains an unspecified number of high-affinity $Cu^{II}$ binding sites that are the catalytic centres. Whether there is any physiological significance to the effect $Cu^{II}$ has on the reductive release of $Fe^{III}$ from ferritin remains to be established. However, it has long been clear that iron and copper metabolism are related in eukaryotes,[14] and recently it has been shown that: high affinity copper uptake by the yeast *Saccharomyces cerevisiae* requires a copper-containing oxidase,[15] mutations in the gene for the copper-containing protein ceruloplasmin are associated with iron overload disease in humans,[16] and people with low levels of ceruloplasmin have increased iron deposits in various tissues.[17]

The effect of $Cu^{II}$ on the rate of iron release from ferritin, and the observation that ferritin can bind $Cu^{II}$ tightly, raises questions concerning the possible involvement of $Cu^{II}$ in uptake of $Fe^{II}$. This is the subject of the present paper which demonstrates that $Fe^{II}$ oxidation by horse spleen ferritin is enhanced by the presence of $Cu^{II}$.

## Experimental

All reagents were analytical reagent grade and used without further purification unless otherwise stated, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) and 4-morpholinepropanesulfonic acid (MOPS) were purchased from Sigma Chemical Co. Protein concentrations were determined by the Lowry method,[18] and iron and copper determined spectrophotometrically, as their ferrozine {disodium salt of 4,4′-[3-(2-pyridinyl)-1,2,4-triazine-5,6-diyl]bis(benzenesulfonic acid)} and bicinchoninic acid (2,2′-biquinoline-4,4′-dicarboxylic acid) complexes respectively, or by atomic absorption spectrophotometry.

Horse spleen holoferritin was purchased from Boehringer Mannheim. Apoferritin was prepared by extensive dialysis of holoferritin against 1% thioglycolic acid (sulfanylacetic acid) solution and NaCl (0.15 M) at pH 6.0 (HEPES, 0.05 M) at 4 °C and under argon, followed for some samples by dialysis for 2–4 d against NaCl (0.15 M) in the presence of the ion-exchange resin chelex-100 (30 g $l^{-1}$, Bio-Rad Laboratories). Sample 4 in Table 1 was not dialysed against chelex-100 but sample 5 was. The final step for all apoferritin preparations was dialysis against NaCl (0.15 M) at pH 7 (MOPS, 0.05 M). Iron analysis showed that the apoferritin preparations with and without chelex-100 treatment contained less than one iron per ferritin molecule. Copper-treated apoferritin was prepared by the addition of a 100-fold molar excess of $CuCl_2$ to solutions of apoferritin (4 mg $cm^{-3}$) and NaCl (0.15 M) at pH 7 (MOPS, 0.05 M) and at 4 °C. After 4 h the mixture was spun at 5000 *g* for 15 mins to remove the small amount of precipitated material, the supernatant applied to a Sephadex G-25 column (HEPES, 20 mM, pH 7) to separate the apoferritin from excess $CuCl_2$, and the eluate concentrated to 8 mg $cm^{-3}$ in a Centricon-30 device (Amicon) with NaCl (0.15 M) at pH 7 (MOPS, 0.05 M). Some samples of copper(II)-treated apoferritin that had been passed through a sephadex G25 column were chromatographed on freshly prepared columns of chelex-100 at pH 7 (MOPS, 0.05 M). Comparative studies to investigate the effect of dialysis against chelex-100 and the effect of added $Cu^{II}$ on the rate of $Fe^{II}$ oxidation were carried out with ferritin from the same Boehringer batch.

The aerobic oxidative uptake of $Fe^{II}$ by apoferritin was monitored by following the absorbance increase at 310 or 420 nm on the addition of 0.1 $cm^3$ aliquots of $(NH_4)_2Fe(SO_4)_2$ solutions to

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**Table 1** Effect of $Cu^{II}$ on the rate of aerobic oxidative $Fe^{II}$ uptake by horse spleen ferritin

| Sample number | Sample treatment (see text) | Number of $Cu^{II}$ ions per ferritin molecule | Initial rate of $Fe^{II}$ oxidation with 1 mM $Fe^{II}$ added ($\Delta A_{420}$/min) |
|---|---|---|---|
| 1 | Sample 5 plus $Cu^{II}$ after G25 and centricon-30 treatment | 32 ± 2 | 0.26 |
| 2 | Sample 1 after passage through chelex-100 column | 22 ± 2 | 0.23 |
| 3 | Sample 2 after further passage through chelex-100 column | 16 ± 2 | 0.11 |
| 4 | Apoferritin without dialysis against chelex-100 (see Experimental section) | 17 ± 2 | 0.10 |
| 5 | Apoferritin after dialysis against chelex-100 (see Experimental section) | 0–2 | 0.09 |

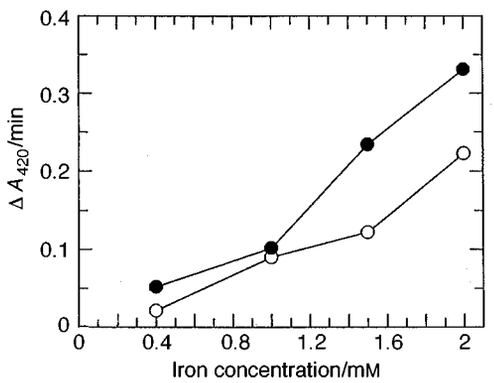


**Fig. 1** Initial rate for $Fe^{II}$ oxidation by apoferritin prepared without dialysis against chelex-100 (●) and apoferritin prepared with dialysis against chelex-100 (○). The assays were carried out with solutions of apoferritin at a concentration of 1 mg $cm^{-3}$ and at this concentration, 2 mM $Fe^{II}$ is equivalent to ≈1000 $Fe^{II}$ ions per ferritin molecule

0.9 $cm^3$ aliquots of apoferritin at pH 6.5 (HEPES, 0.15 M) to give final concentrations of 1 mg $cm^{-3}$ ferritin and $Fe^{II}$ (0.15–2 mM). The rate of iron uptake was determined by measuring the initial rate of change of the absorbance. All rate measurements were made at 25 °C. Haem-binding assays using haemin chloride [a chloro(porphyrinato)iron(III) co-ordination complex] were carried out by the method described by Kadir and Moore.[19]

## Results and Discussion

Commercially available horse spleen ferritin is of variable quality, with some having considerably altered properties to freshly prepared ferritin. We used a haem-binding assay as a guide to sample quality: damaged ferritin binds haem whereas undamaged ferritin does not.[20] The ferritin obtained from Boehringer used in this work was undamaged by this criterion. However, different batches of protein gave slightly different rates of $Fe^{II}$ oxidation, though the general trends reported in this paper were common to all batches. The rates of $Fe^{II}$ oxidation by ferritin given in Fig. 1 and Table 1 were determined from the increase in absorbance at 420 nm as described in the Experimental section. Iron(III) bound to ferritin has a broad absorbance band and some workers use the change in absorbance at 310 nm to monitor $Fe^{II}$ oxidation (*e.g.* see refs. 21 and 22) and others use 420 nm (*e.g.* ref. 6). In a series of experiments using 310 nm to monitor $Fe^{II}$ oxidation (data not shown) we found the same relationships between samples as we found using 420 nm. The absorbance assays used monitor $Fe^{II}$ oxidation but not whether the resulting $Fe^{III}$ or $Fe^{II}$ awaiting oxidation is encompassed within the ferritin shell. Previous studies have indicated that after a relatively short period of time the majority of added $Fe^{II}$ up to the levels used in this work is inaccessible to 1,10-phenanthroline and that the $Fe^{III}$ product is contained within the protein.[3–8,23,24] In our work, oxidation of the added $Fe^{II}$ was not accompanied by turbidity, which would have been indicative of the formation of iron(III) hydroxide precipitates outside the ferritin shell, and the amber colour of the $Fe^{III}$ ions travelled with the ferritin on gel filtration columns (data not shown) confirming that the $Fe^{III}$ was associated with the ferritin.

Apoferritin prepared without extensive dialysis against chelex-100 consistently had faster rates of $Fe^{II}$ oxidation than apoferritin prepared with dialysis against chelex-100 (Fig. 1). The only consistent difference found between samples prepared with and without the chelex-100 dialysis step was their copper content: samples not dialysed against chelex-100 contained copper whilst those dialysed did not.

The influence of $Cu^{II}$ on the rate of oxidation of $Fe^{II}$ by apoferritin was investigated in a series of experiments summarised in Table 1. Apoferritin was prepared with and without dialysis against chelex-100 (samples 5 and 4 respectively), and to the sample that had been dialysed a large excess of $Cu^{II}$ was added and the $Cu^{II}$ not bound to the ferritin removed by gel filtration (sample 1). Sample 1 was then subjected to two rounds of chromatography on a chelex-100 column with aliquots taken after each round; samples 2 and 3. Sample 4, the apoferritin prepared with dialysis against chelex-100, contained only 0–2 $Cu^{II}$ ions per ferritin molecule on average and had the lowest rate of $Fe^{II}$ oxidation of all the samples. The effect of the gel filtration and chelex-100 chromatography was to progressively remove $Cu^{II}$ from the copper(II)-treated ferritin, and this was accompanied with a reduction in the rate of $Fe^{II}$ oxidation (Table 1). We conclude from these data that the enhanced rate of oxidation of $Fe^{II}$ by apoferritin results from the presence of tightly bound $Cu^{II}$.

Our finding that $Cu^{II}$ is not readily removed by chromatography on chelex-100, and therefore is relatively tightly bound to apoferritin, is consistent with other reports. Thus, Bolann and Ulvik[13] observed that even after six passages through a chelex-100 column their apoferritin contained an average of ≈9 $Cu^{II}$ ions per molecule, and Pead *et al.*[25] showed by gel filtration that horse spleen apoferritin at pH 7.5 can bind 32–33 $Cu^{II}$ ions with a dissociation constant $< 10^{-7}$ M. This latter study also showed that $Cu^{II}$ displaces $Fe^{II}$ from sites on ferritin under anaerobic conditions, a result that is expected if the binding sites are the same and the relevant binding affinities follow the Irving–Williams order.[26]

Zinc(II) also binds relatively tightly to horse spleen apoferritin, displacing $Fe^{II}$ under anaerobic conditions.[25] However, unlike $Cu^{II}$, $Zn^{II}$ inhibits the oxidative uptake of $Fe^{II}$.[24,27] This difference between the effect of $Zn^{II}$ and $Cu^{II}$ could be due to the ability of $Cu^{II}$ to take part in redox reactions or to differences between the manner in which $Zn^{II}$ and $Cu^{II}$ bind to the ferritin shell. One possibility is that the proposed dinuclear ferroxidase site within the H-chains of the ferritin[3,7,8] is the binding site for the inhibitory $Zn^{II}$ or catalytic $Cu^{II}$. In the absence of any other metal ions, two $Fe^{II}$ ions have been proposed to bind at this site, becoming oxidised by $O_2$ and forming a μ-oxo or μ-hydroxo bridged diiron(III) centre, perhaps transiently.[3,7] There is no firm structural data in support of this proposal however. A similar centre has been suggested to form in the related bacterial protein bacterioferritin but, again, there is no firm evidence for this though there is clear structural and spectroscopic evidence for the formation of a dimanganese(II) or dicobalt(II) centre, and thus by implication a diiron(II) centre, in each subunit of *Escherichia coli* bacterioferritin.[28,29] Kinetic

and mutagenesis studies show that the *E. coli* bacterioferritin centre does catalyse the oxidation of iron(II)[30] though it appears to become blocked after one cycle of loading and oxidation.[31] The explanation advanced for this is that one of the product $Fe^{III}$ ions moves away from the site with the other remaining in place. Returning to the contrasting effects of $Zn^{II}$ and $Cu^{II}$ in horse spleen ferritin, if these ions are bound so that they occupy one end of each site, an incoming $Fe^{II}$ occupying the other end might be oxidised by $Cu^{II}$ but not by $Zn^{II}$. The product $Fe^{III}$ could then migrate from the site to allow another $Fe^{II}$ to take its place alongside the product $Cu^{I}$. At this point a two-electron reduction of $O_2$ would generate $Cu^{II}$ and $Fe^{III}$ and, with the migration of $Fe^{III}$ away from the site, the cycle could be repeated. Alternatively, since the ferritin molecule contains a plethora of potential binding groups, including some on the surface of the protein that help form the wall of the central cavity[3,5] and some in inter-subunit channels that occur at the eight three-fold symmetry axes of the ferritin shell,[24,32] the effect of $Cu^{II}$ may be from binding at sites other than the ferroxidase centre. Studies of $Cu^{II}$ binding to mutants of H-chain and L-chain ferritins and mutants of bacterioferritin are needed to help resolve this matter.§

## Conclusion

Copper(II) binds tightly to horse spleen apoferritin and has a catalytic effect on the aerobic oxidative uptake of $Fe^{II}$. Whether this is physiologically significant remains to be determined but it does provide a useful mechanistic tool for characterising the complex mechanisms by which the non-haem iron cores of ferritin are laid down.


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§ *Note added at proof*. During the Dalton Discussion meeting it became clear that $Cu^{II}$ had a dramatic catalytic effect on the ferroxidase activity of *E. coli* bacterioferritin[33] and discussions centred on whether $Cu^{II}$ bound at the carboxylate-rich dinuclear sites of ferritin and bacterioferritin would have redox properties that allowed it to oxidise $Fe^{II}$. This can only be answered by further experimentation. It may be relevant that a recent X-ray study of human ceruloplasmin[34] located $Fe^{II}$ at sites very similar to the ferritin ferroxidase sites, and relatively close to a trinuclear copper centre. The trinuclear copper centre was proposed to oxidise the $Fe^{II}$.

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