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Publication Date
1977-10-01
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October 12, 1977

Prepared for the U. S. Department of Energy under Contract W-7405-ENG-48
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The Binding of Copper to Ferritin and Apoferritin as Studied by Electron Paramagnetic Resonance and Its Possible Physiological Role as a Catalyst in Iron Release Kinetics

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by
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Running Title: Binding of Copper to Ferritin
Footnotes

1. Abbreviations used:

   - epr  - electron paramagnetic resonance
   - HEPES - N-2-hydroxyethylpiperazine-N'-ethane sulfonic acid
   - FMN  - flavin mononucleotide
   - NADH - nicotine adenine dinucleotide
   - tris  - tris-(hydroxymethyl)aminomethane
ABSTRACT

We have investigated the binding of copper by ferritin and apoferritin. We find these proteins to possess a site which strongly and specifically binds copper. Both cupric and cuprous ions are bound by the proteins. Electron paramagnetic resonance spectra of the cupric complexes are reported. Addition of imidazole results in epr spectral changes which reveal the copper to lie in a site of rhombic symmetry with at least four nitrogenous ligands. These ligands probably are histidine and lysine residues.

The kinetics of iron release from both ferritin and ferritin/Cu have been examined. Rates are dramatically enhanced for the copper-containing protein. The half-time for release by ascorbic acid under our conditions is a factor of $10^3$ less for the copper-containing protein relative to the copper-free protein. These observations are discussed in light of the substantial clinical evidence that copper and ascorbic acid are important for normal iron metabolism in mammals.
Ferritin is the principal iron storage protein in mammals. That protein isolated from equine spleen has been found to consist of a spherical polypeptide shell of molecular weight 440,000 composed of 24 identical subunits (Harrison and Hoy, 1973; Crichton, 1973). The protein contains a variable amount of iron, with a maximum of about 4500 iron atoms, in the form of a ferric oxyhydroxide microcrystal located in its inner cavity (Granick, 1946). The iron-free protein, apoferritin, can be prepared by reduction and chelation of the ferric ion in the microcrystalline core.

Ferritin formation apparently involves the oxidation of ferrous ion to ferric ion in the presence of a suitable oxidizing agent (Bielig and Bayer, 1955). Apoferritin has been shown to catalyze this oxidation in vitro with the subsequent formation of the ferric oxyhydroxide core (Niederer, 1970; Macara et al., 1972, 1973a; Crichton and Bayer, 1972; Bryce and Crichton, 1973), but this catalytic oxidation is inhibited by a variety of divalent transition metal ions, particularly Zn^{2+} (Niederer, 1970; Macara et al., 1973b).

As part of a study of the interactions of a variety of transition metal ions with both apoferritin and ferritin using epr, we have examined the binding of copper ions by the proteins. We have also examined the kinetics of iron release from the ferritin/Cu complex.

Materials and Methods

The samples of equine spleen ferritin used in these studies were primarily obtained from Polysciences, Inc., Warrington, Pennsylvania. Generally, these were obtained as solutions of protein twice recrystallized with CdSO_{4}. Ferritin samples used in kinetic studies were cadmium-free, however.
Samples of ferritin were also prepared from fresh horse spleen in a method similar to that of Granick (1946), except that solutions of the protein were maintained at higher pH than normally used. Specifically, 300 g of spleen was homogenized with 750 ml of 100 mM Tris buffer (final pH 7.4). This homogenate was heated to 80° for ten minutes and denatured proteins were removed by filtration and centrifugation. Ferritin was then further purified from the mother liquor (pH 7.4) by centrifugation techniques (Penders et al., 1968) or by crystallization from 5% CdSO₄ (final pH 6.6).

Apoferitin was prepared from the ferritin by reduction and removal of iron with mercaptoacetic acid at a pH of 5.2 (Crichton, 1973). Apoferritin concentrations were determined by observation of the absorbances at 280 nm where apoferritin has an extinction, \( \text{A}_{1%\text{cm}} = 9.0 \) (Macara et al., 1973b).

Copper was bound to both ferritin and apoferritin by one of two methods. In the first, the protein was dialysed against 100 mM acetate buffer at pH 5.5 overnight. The copper (as cupric sulfate) was then added in excess to the dialysis vessel. After copper concentrations had equilized, the protein/Cu²⁺ solution was dialyzed against several changes of buffer at the pH of interest. This procedure prevented the precipitation of Cu(OH)₂ inside the dialysis bag, even for a final pH greater than 8. In the second procedure, copper (as cupric sulfate solution) was added directly to a solution of protein buffered at the pH of interest. No precipitation of copper hydroxide was noted at any pH if this addition was slow enough and in small enough quantities (vida infra).
To prevent interference from other metal ions, polyethylene containers were employed as much as possible. Any glassware used was first soaked in 6M HCl for three hours or longer and then thoroughly rinsed with water. All water used was twice distilled - the second time with a Corning AG-1b automatic still containing vicor elements.

Amounts of metal ions bound to the protein were determined by atomic absorption by staff of the University of California, College of Chemistry analytical laboratory.

Electron Paramagnetic Resonance. EPR spectra were obtained with a Varian model V4502 epr spectrometer operating in the range 9.0-9.5 GHz (X band) employing a Hall device regulated magnet. Microwave frequencies were measured directly and magnetic fields were measured by detecting proton magnetic resonance. A varian V4531 microwave cavity was used. Low temperatures (to -120°) were obtained by passing current through a resistor immersed in liquid nitrogen and passing the boil off over the sample which was contained in a dewar in the microwave cavity.

EPR spectra of frozen solutions were simulated with a modification of the program RHOM (Venable, 1965), which allows simulation of glass spectra of magnetically dilute species in crystal fields of rhombic symmetry. This program was run on the Control Data Corporation 7600 computer of the Lawrence Berkeley Laboratory.

Kinetic Studies. Kinetics of iron release from ferritin were investigated at 22° in the presence and absence of copper bound to the protein. The ferritin used was all from one lot of cadmium-free protein. A sample of ferritin/Cu was prepared by the first method described above, while a second sample of ferritin was subjected to the same dialysis procedures,
but without addition of copper. These samples were both prepared in 50 mM HEPES buffer at pH 7.5. Each solution was diluted with buffer to give an absorbance of 1.0 at 420 nm (the absorbance at this wavelength is due almost entirely to the ferric oxyhydroxide core). A sample of freshly prepared reductant solution (either ascorbic acid or sodium dithionite) was added to the cuvette by syringe. The cuvette was then capped, inverted three times to mix the contents and placed in the spectrometer (a Cary 14). The decay of absorption was then followed as a function of time. In other experiments, the effects of other metal ions on iron release were investigated. In these cases, metal ion solutions were added to the cuvette containing buffered ferritin solution before reductant was added.

Results

EPR Spectra. Spectra of copper complexes of both ferritin and apoferritin are shown in Fig. 1. As shown, cupric ion binds to both the protein and the apoprotein in an identical manner. Measured parameters in the room temperature spectra are $g_|| = 2.29$, $g_\perp = 2.07$ and $A_\parallel = -145$ G, where the negative sign is assumed. A frozen solution of apoferritin/Cu$^{2+}$ gives the epr spectrum shown in Fig. 1c. As can be seen the lines sharpen somewhat and we now measure $g_|| = 2.29$, $g_\perp = 2.07$ and $A_\perp = -161$ G.

A solution of the apoferritin/Cu$^{2+}$ complex is a light green color. Addition of imidazole results in an immediate color change to light blue. An epr spectrum of a frozen solution of the resulting apoferritin/Cu$^{2+}$/imidazole complex is shown in Fig. 2b. The extra splittings observed in this spectrum are ligand hyperfine, or superhyperfine, splittings and result from the interaction of the unpaired hole on the cupric ion with
the magnetic moments of $^{14}$N nuclei coordinated to the metal ion. If imidazole is titrated into the solution of apoferritin/Cu$^{2+}$, this spectrum is obtained after the addition of one imidazole molecule per cupric ion. Further addition of imidazole produces no further changes. A computer simulation of this spectrum is shown in Fig. 2a. The parameters used in the simulation are: $g_x = 2.05$, $g_y = 2.06$, $g_z = 2.26$; $A_x = -30$ G, $A_y = -30$ G, and $A_z = -175$ G. Also included is the effect of four equivalent coordinated nitrogen nuclei with an isotropic $^{14}$N coupling constant $A_N = -15$ G. Neither the assumption of an axial g tensor nor the assumption of superhyperfine interaction with less than four nitrogeneous ligands results in a reasonable simulation of our observed experimental spectrum. Both the extent of superhyperfine splittings and their position in the spectrum are sensitive to the number of nitrogens and the symmetry of the g tensor. In the perpendicular region of the spectrum the number of nitrogens cannot be obtained by simply counting the observed superhyperfine splittings. We also should note that the spectrum shown in Fig. 2 is of the naturally occurring copper isotopes. Spectra of apoferritin/$^{63}$Cu$^{2+}$ are not significantly different.

Properties of the Complex. The amount of copper bound by apoferritin is quite dependent on the pH of the medium. At pH 5.5 we find only 2.1 cupric ions to bind per apoferritin molecule. At pH 7.5, however, we find 47.5 cupric ions per molecule, or about 2 ions per protein subunit. We have also observed that if apoferritin is loaded with copper at high pH and the pH is lowered by addition of HCl, the excess copper is released from this binding site.
On the other hand, the apoferritin/Cu complex is quite stable and dialysis against daily changes of buffer for up to a week does not significantly reduce the amount of copper bound to the protein. Furthermore, binding of copper is quite specific. In other experiments in which reagent grade CoSO$_4$ was titrated against apoferritin, we obtained the same epr spectrum as Fig. 1. The copper impurity in the reagent grade cobalt sulfate is 0.002%. Similarly, titrations with ferric ion (as ferric nitrate) produce the spectra of Fig. 1, even though the copper impurity is only 0.003%. Thus, even in the presence of many orders of magnitude higher concentrations of divalent or trivalent ions, this site will selectively bind cupric ion.

If reducing agents are added to the apoferritin/Cu$^{2+}$ complex, the color of the solution changes from light green to light reddish-brown. Also, the epr signal vanishes. Addition of excess oxidizing agent will restore both the color and the epr spectrum of the cupric complex. Furthermore, dialysis of the reddish-brown solution against buffer does not affect the results of an oxidation.

It is of interest to note that commercially supplied samples of ferritin can contain copper bound to this site. For instance, a sample obtained from Calbiochem (La Jolla, California) contained 10 copper atoms per molecule of protein, a typical sample from Polysciences (Warrington, Pennsylvania) contained one copper atom per molecule, and a sample from Miles Laboratories (Kankakee, Illinois) contained less than one copper atom per ferritin molecule.

The method for purification of ferritin presented by Granick (1946) contains steps at which the pH of the protein solution is reduced to less
than 5.5 and recent variants of the procedure (Harrison et al., 1974) include a step in which the pH is reduced to 4.8. Since any copper bound to ferritin would be released and removed during these purification procedures, we isolated and purified ferritin in such a way that the pH was never less than 6.6 in any step as explained above. Analysis showed such ferritin prepared from a single spleen by centrifugation techniques to contain about one copper atom per protein molecule and ferritin prepared by crystallization with CdSO₄ to contain 2.2 copper atoms per molecule. The discrepancy can be entirely accounted for by assuming that the ferritin prepared by the second method bound all of the cupric ion impurity present in the cadmium sulfate used.

Iron Release Kinetics. The results of our investigations of the kinetics of iron release from both ferritin and ferritin/Cu are presented in Fig. 3. Several features of these results should be noted. First, the initial rate of release of iron from ferritin is significantly different from that of ferritin/Cu if the reducing agent is ascorbate, but the initial rate is essentially the same for the two proteins if dithionite is employed as reducing agent. For the case in which ascorbate is used to reductively release the iron, the half-time for release decreases from about 100 hours to about seven minutes when copper is bond in the ferritin "tight-binding" site. When the reducing agent is dithionite, the release of iron appears to be a two-step process, with an initial fast step and a later slow one. When copper is present only a single fast rate of release is observed for dithionite.

If all iron is released from the ferritin solutions used in these studies the final ferrous ion concentration is about 1.7 mM (using \( \frac{1}{\text{lcm}} = 100 \) at 420 nm, Macara et al., 1972). Addition of similar amounts of ferrous ion
(as ferrous ammonium sulfate) to these solutions changes the kinetics as seen in Fig. 4. It therefore appears that the second step in the release of iron from copper-free ferritin by dithionite is a result of inhibition of release by the free ferrous ion which accumulates in solution. It is of interest to note that this ferrous ion does not seem to be an inhibitor of release from ferritin/Cu.

Discussion

EPR Spectra. There have been previous reports that the interaction of copper with ferritin is unique. In an investigation of metal ion-ferritin interactions, Rüssel (1970) examined the interactions of Cd$^{2+}$, Zn$^{2+}$, Pb$^{2+}$, Cu$^{2+}$, Tl$^{3+}$ and Ag$^{+}$ with both ferritin and synthetic ferric oxyhydroxide micelles which serve as analogues for the ferritin core (Spiro and Saltman, 1969). Of the ions studied, only cupric ion displayed different binding properties in the two systems. This means that only cupric ion was observed to bind to the polypeptide chain of ferritin. Macara et al. (1973b) have also investigated metal ion binding to ferritin. They studied Zn$^{2+}$, Cd$^{2+}$, Mn$^{2+}$, Cu$^{2+}$ and Tb$^{3+}$ binding by observing the displacement of protons by these ions at pH 5.5. Cupric ion was again unique in that it displaced two protons per ion bound. They interpreted the data as showing ferritin to contain two binding sites for copper per subunit. This compares with our observations that copper is very selectively bound to a tight-binding site, of which there are two such sites per subunit.

From the computer simulation of Fig. 2, we find that the cupric ion binds in such a way as to have four nearly equivalent nitrogeneous ligands which must lie nearly in a plane about the ion. Since the spectrum is split by the ligand hyperfine interaction only after the addition of imidazole, and
since the addition of imidazole to solutions of apoferritin/Cu\(^{2+}\) is accompanied by a color change, we know that at least one of these ligands must be an imidazole nitrogen. However, the titration of imidazole against apoferritin/Cu\(^{2+}\) shows that only one of the cupric ligands is the added imidazole. We are therefore left with three nitrogenous ligands per cupric ion which must arise from amino acid residues. Considering the pH dependence of the copper binding, it is most likely that either an imidazole nitrogen of a histidine residue or an N-terminal nitrogen of the polypeptide is involved in the binding. The possibility that an N-terminal nitrogen is involved rests on the outcome of the current debate on the heterogeneity of the apoferritin. If the observed presence of two polypeptide chains per subunit is a result of thermal degradation during the heat treatment step during isolation of the protein (as Collet-Cassart and Crichton, 1975, have suggested), then the fact that at least one N-terminal nitrogen is acetylated (Saran, 1966; Mainwaring and Hofmann, 1968) removes this group from consideration as a possible ligand for copper. On the other hand, if indeed the apoferritin subunit is composed of two polypeptide chains, one of which has an N-terminal nitrogen with \(pK_{\text{a}} = 7.5\) (Silk and Breslow, 1976), then such a nitrogen is a possible ligand. However, since only one such group would exist per 18,500 daltons, and we observe that two cupric ions bind for that molecular weight, some asymmetry in these two binding sites would be implied if such a group coordinated the metal ion. We have never observed any evidence that the copper binding sites are inequivalent. Nonetheless, cupric ion epr can be somewhat insensitive to some types of changes in binding sites, so we cannot rule out this possibility.
The imidazole nitrogens of histidine residues may also bind the copper. It has been found that there are three titrable histidine residues per molecular weight of 18,500. Two of these are found to titrate between pH 5.6 and pH 7.3, while one titrates at higher pH (Silk and Breslow, 1976). Furthermore, these three histidine residues have identical reactivities with bromacetate in both ferritin and apoferritin. This is of importance since we observe the copper to bind identically to the protein and the apoprotein. Here, again, we see an implied asymmetry in the copper binding sites if we assume all three of these histidine residues to be involved in copper binding. Again, however, we note that although the epr spectrum shows no such asymmetry, it may be simply a result of the insensitivity of epr to such an effect.

From the above, we can account for no more than four of the six nitrogeneous cupric ligands. The most probable source of the others is the ε-amino nitrogens of lysine residues. In apoferritin, seven of these are titratable per molecular weight of 18,500 with pKₐ = 9.6 (Silk and Breslow, 1976). Of these, 3 to 4 are susceptible to chemical modification in ferritin and such modification of these residues does not affect the catalytic activity of apoferritin (Wetz and Crichton, 1976). Consequently, we have three or four lysine residues that could provide a nitrogen as a ligand in both ferritin and apoferritin. In spite of the fact that the pKₐ of the ε-amino nitrogen is high, it can complex cupric ion at pH < 8 (Hatamo et al., 1971). In fact, a study of complexes of Cu²⁺ with the side chains of Nα-dodecaneyl-L-lysine at the interface of an oil microemulsion gave spectra very similar to those obtained here with g∥ = 2.26, g⊥ = 2.07, A∥ = -170 G and AN = -15 G with four coordinated ε-amino nitrogens. At room
temperature the limited motion of these complexes resulted in an averaged spectrum which was still powder-like but with an apparent reduction in $A_{ii}$ and a loss of superhyperfine structure - exactly what we observe with ferritin/Cu$^{2+}$/imidazole (Smith et al., 1977). This model system also demonstrates that the ε-amino nitrogen is a strong ligand for cupric ion. It is also of interest to note the ε-amino nitrogens have been suggested as ligands in copper metalloproteins in which the metal ion undergoes oxidation and reduction because the ε-amino group with its long, flexible hydrocarbon connection to the polypeptide backbone should behave as a unidentate ligand and thus allow rearrangements in the coordination sphere of the copper and so accommodate the different coordination preferences of cuprous and cupric ions (Österberg, 1974).

To some extent the nature of the ligands of cupric ion can be determined by performing simple molecular orbital calculations using the spin Hamiltonian parameters. The procedure is that of Maki et al. (1964). In this procedure the basis set is the set of real 3d orbitals. First-order perturbation calculations using a perturbation term consisting of the spin-orbit Hamiltonian allow determination of the elements of the $g$ tensor ($\mathbf{g}$) and the hyperfine interaction tensor ($\mathbf{A}$) in terms of a number of parameters. The final equations are:

\[
\begin{align*}
g_{xx} &= 2 - 2 \alpha_3 , \\
g_{yy} &= 2 - 2 \alpha_2 , \\
g_{zz} &= 2 - 8 \alpha_1 , \\
\end{align*}
\]

and

\[
\begin{align*}
A_{xx} &= P[-2\alpha_3 - \kappa + \frac{2}{7} + \frac{3}{7} \alpha_2] , \\
A_{yy} &= P[-2\alpha_2 - \kappa + \frac{2}{7} + \frac{3}{7} \alpha_3] , \\
A_{zz} &= P[-8\alpha_1 - \kappa - \frac{7}{7} - \frac{7}{7}(\alpha_2 + \alpha_3)] .
\end{align*}
\]
In these equations the $\alpha_1$, $\alpha_2$ and $\alpha_3$ are parameters related to the separation of the various 3d orbital energy levels, $\kappa$ is the Fermi contact term, and $P = g_g n \beta_n <r^{-3}>$ where $g$ and $g_n$ are the electron and the nuclear $g$ factors, $\beta$ and $\beta_n$ are, respectively, the electron and nuclear magnetons and $r$ is the electron-nuclear distance. Because the parameter $P$ is a function of the distance of the unpaired electron from the cupric ion nucleus it is a measure of the amount of delocalization of the valence electron density of the ion onto the ligands. Thus it could be expected that this parameter has values which are characteristic of different types of ligands, as Maki et al. (1964) have pointed out.

For the apoferritin/Cu$^{2+}$/imidazole complex we calculate $P = 0.0257$ cm$^{-1}$ and $\kappa = 0.449$. For the apoferritin/Cu$^{2+}$ complex we find $P = 0.0247$ cm$^{-1}$ and $\kappa = 0.442$. We compare these values with that of cupric ion in galactose oxidase which has $P = 0.0263$ cm$^{-1}$ and which has been shown to have four nitrogenous ligands (Cleveland et al., 1975). We also note that $N,N'$-ethylene bis(trifluoroacetylacetoniminato) copper(II):

![Chemical Structure]

is reported to have a value of $P = 0.0257$ cm$^{-1}$ (Giordano and Bereman, 1974). In other words, the ligands of the apoferritin/Cu$^{2+}$/imidazole complex do not appear to differ greatly from those of the apoferritin/Cu$^{2+}$ complex. The value of $P$ for the latter species is consistent with coordination by either a nitrogeneous or oxygen ligand which is displaced by imidazole. It seems most likely that the fourth ligand is a molecule of water which would be easily displaced by an imidazole molecule.
Iron Release Kinetics. At the present time the only system reported to release iron from ferritin on a physiological time scale is a combination of flavin mononucleotide (FMN) and nicotine adenine dinucleotide (NADH) (Sirivech et al., 1974; Dognin and Crichton, 1975; Crichton et al., 1975). The proposed route of electron transport for iron reduction is:

\[
\begin{align*}
\text{NAD}^+ \quad \text{FMN} \quad 2\text{Fe}^{3+} \quad \text{O}_2 \\
\text{NADH} + \text{H}^+ \quad \text{FMNH}_2 \quad 2\text{Fe}^{2+} \quad ?
\end{align*}
\]

The FMN-FADH system will also rapidly reduce ferric ion in solutions of ferric chloride. One feature of the system is that low oxygen concentrations are required (<3 µM, according to Sirivech et al., 1974) to depress the direct reaction of FMNH₂ with molecular oxygen. Normal sera concentrations of oxygen in humans are in the range 50-120 µM (40-90 Torr partial pressure) (Osaki et al., 1966). A study of ferritin iron release in dog liver perfusate at a controlled oxygen partial pressure of 30-40 Torr has shown that at this O₂ concentration iron release from ferritin to transferrin is normal, however, (Osaki and Johnson, 1969). When copper is bound in the tight binding sites on ferritin, we see iron release in physiologically reasonable times with a physiological reducing agent, namely ascorbate. Furthermore, mobilization of iron begins immediately upon addition of reductant; that is, it goes at normal oxygen partial pressures. Finally, reduction occurs by some novel route since ferrous ion build-up does not inhibit the release.

Apparently, we observe the reduction of iron by a process in which this reduction is coupled to the oxidation of cuprous ion bound to the ferritin polypeptide. Cuprous ion results from the reduction of cupric
ion by a reducing agent in the protein medium (see Martell and Kham, 1973) as follows:

\[
\begin{align*}
\text{dehydroascorbic acid} & \quad \text{Cu(II)} & \quad \text{Fe(III)} \\
\text{ascorbic acid} & \quad \text{Cu(I)} & \quad \text{Fe(II)}
\end{align*}
\]

The implication of these studies is that iron released from ferritin \textit{in vivo} may be dependent on the presence of copper bound to the protein. This is especially interesting because of a substantial amount of clinical evidence that copper is indeed important for normal iron metabolism. Lee \textit{et al.} (1968) found that copper deficient swine absorbed iron from their diet into the duodenal lumen at normal rates, but that the subsequent transfer of iron to the plasma was impaired. If iron supplements were injected, iron was found to accumulate in the reticuloendothelial system and the hepatic parenchymal cells. Goodman and Dallman (1969) observed similar effects in copper-deficient rats and noted that iron would also accumulate in the ferritin of the erythroid cells. A further observation was that administration of copper to the copper-deficient animals resulted in the rapid release of iron from ferritin and a subsequent rise in the plasma iron concentration. Lee attributed these results to a failure of serum ferroxidase activity (in particular, the activity of ceruloplasmin, see below) preventing oxidation of ferrous ion so that it could be incorporated into apotransferrin. The accumulation of iron in the ferritin of the erythroid cells led Goodman and Dallman to postulate that besides a defect in the plasma transport of iron, some defect in the intracellular transport of the element must also result.
The ferroxidase activity of ceruloplasmin is well established (Osaki et al., 1966) and it is known to contain eight copper atoms. However, it is not the only source of plasma ferroxidase activity. Another copper-containing protein can provide some ferroxidase activity (Topham and Sung, 1973). A study by Lee et al. (1969) found that citrate is yet another source of serum ferroxidase activity, accounting for 15% of such activity in normal patients and almost 100% in patients with Wilson's disease; a condition in which ceruloplasmin is absent and yet iron mobilization is unimpaired.

If we postulate that copper is essential for the release of iron from ferritin, we can explain the build-up of iron stores in copper-deficient animals with two assumptions: (1) iron in the mucosal cells passes through ferritin and (2) there is some mechanism by which copper can be transferred to ferritin to release iron (or some mechanism by which copper can be removed from protein to store iron). At this time we have no evidence to support the second assumption. The first assumption deals with the question of the presence or absence of iron binding proteins besides ferritin in the mucosal cells. This is a matter of much debate in the literature with several such proteins being reported (see, for example, Turnball, 1974; Halliday et al., 1976). A study by Linder and Munro (1975) has shown that unless extreme precautions are taken to exclude pancreatic enzymes from mucosal cell preparations the ferritin in them will undergo proteolysis, giving rise to several iron binding ferritin fragments. They found that if one does exclude the pancreatic enzymes, the mucosal cell is found to contain only ferritin-bound iron as well as some low molecular weight iron species (mimicked by FeCl₃) which contains iron in equilibrium with the ferritin iron.
Thus it would appear that the assumption that iron in the mucosal cells will pass through ferritin is reasonable. As to the build-up of iron in ferritin at other points, we propose that lack of copper precludes its delivery to the ferritin and thus iron release is inhibited. Consequently, both effects of copper deficiency on iron metabolism can be explained without the necessity of postulating two separate mechanistic defects.

There is also clinical evidence that ascorbic acid is important in the release of iron from ferritin in vivo. Wapnick et al. (1970) in a study of Bantu tribesmen in South Africa found that although these subjects often suffer from iron overload, their plasma iron levels may be normal or even depressed. At the same time ferritin stores of iron are greatly increased. Administration of ascorbic acid resulted in a release of iron from cellular stores and an increase in plasma iron concentrations in scrobutic individuals but produced no effects in individuals with normal serum ascorbate levels. Similar effects have also been observed in guinea pigs (Lipschitz et al., 1971).

In summary, we have shown ferritin and apoferritin to selectively and tightly bind copper. It appears that the protein supplies three nitrogeneous ligands arising from histidine and lysine residues with the possible involvement of an N-terminal nitrogen. Copper bound in this site greatly increases the rate of reductive release of iron from ferritin under physiological conditions and in the presence of physiological reducing agents. Clinical evidence would seem to indicate that copper is important to the mobilization of iron from the protein in vivo.
Acknowledgments:

We would like to thank Professor W. L. Hubbell for help with our biochemical procedures, and early correspondence with Dr. P. M. Harrison was greatly appreciated. This work was supported by the United States Energy Research and Development Administration.
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cont'd.
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Figure Legends

Figure 1.

a) EPR spectrum of ferritin/Cu$^{2+}$ in 50 mM HEPES buffer, pH 7.5; temperature is 22°; modulation amplitude is 6.0 G. Parameters measured are:

\[ g_\parallel = 2.29, g_\perp = 2.07 \text{ and } A_\parallel = -145 \text{ G}, \]

where negative A is assumed.

b) EPR spectrum of apoferritin/Cu$^{2+}$ in 50 mM HEPES buffer, pH 7.5; temperature is 22°; modulation amplitude is 6.0 G. Parameters measured are the same as those of spectrum a.

c) EPR spectrum of apoferritin/Cu$^{2+}$ in 50 mM HEPES buffer, pH 7.5; temperature is -105°; modulation amplitude is 6.0 G. Parameters measured are:

\[ g_\parallel = 2.29, g_\perp = 2.07 \text{ and } A_\parallel = -161 \text{ G}. \]

Figure 2

a) Simulated epr spectrum using the program RHOM with the parameters:

\[ g_x = 2.05, g_y = 2.06, g_z = 2.26, A_x = -30 \text{ G}, A_y = -30 \text{ G}, \text{ and } A_z = -175 \text{ G}. \]

Also included is the coupling to four equivalent nitrogen ligands with 

\[ A_N = -15 \text{ G}. \]

b) Experimental epr spectrum of apoferritin/Cu$^{2+}$/imidazole in 50% 19 mM imidazole buffer, pH 7.5; 50% glycerine. Temperature is -125°; modulation amplitude is 3.0 G.

Figure 3

Kinetics of iron release from ferritin. Absorbance is at 420 nm.

\[ \Delta \text{ iron release from copper-free ferritin by } 43 \text{ mM ascorbate} \]

\[ + \text{ iron release from ferritin/Cu}^{2+} \text{ by } 43 \text{ mM ascorbate} \]

\[ \circ \text{ iron release from copper-free ferritin by } 43 \text{ mM dithionite} \]

\[ \ast \text{ iron release from ferritin/Cu}^{2+} \text{ by } 43 \text{ mM dithionite.} \]

All are in 50 mM HEPES, pH 7.5 at 22°.

cont'd.
Fig. Legends, cont'd.

Figure 4
Kinetics of iron release from ferritin under the conditions of Fig. 3.

- iron release from ferritin by 47 mM dithionite
- iron release from ferritin by 47 mM dithionite in the presence of 1.0 mM Fe\(^{2+}\)
Fig. 3
Fig. 4
This report was done with support from the Department of Energy. Any conclusions or opinions expressed in this report represent solely those of the author(s) and not necessarily those of The Regents of the University of California, the Lawrence Berkeley Laboratory or the Department of Energy.