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Redox Reactions of Apo Mammalian Ferritin†

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ABSTRACT: Apo horse spleen ferritin undergoes a 6.3 ± 0.5 electron redox reaction at −310 mV at pH 6.0–8.5 and 25 °C to form reduced apoferritin ($apoMF_{red}$). Reconstituted ferritin containing up to 50 ferric ions undergoes reduction at the same potential, taking up one electron per ferric ion and six additional electrons by the protein. We propose that apo mammalian ferritin (apoMF) contains six redox centers that can be fully oxidized forming oxidized apoferritin ($apoMF_{ox}$) or fully reduced forming $apoMF_{red}$. $ApoMF_{red}$ can be prepared conveniently by dithionite or methyl viologen reduction. $ApoMF_{red}$ is slowly oxidized by molecular oxygen but more rapidly by $Fe(CN)_6^{3-}$ to $apoMF_{ox}$. Fe(III)–cytochrome *c* readily oxidizes $apoMF_{red}$ to $apoMF_{ox}$ with a stoichiometry of 6 Fe(III)–cytochrome *c* per $apoMF_{red}$, demonstrating a rapid interprotein electron-transfer reaction. Both redox states of apoMF react with added Fe^{3+} and Fe^{2+}. Addition of eight Fe^{2+} to $apoMF_{ox}$ under anaerobic conditions produced $apoMF_{red}$ and Fe^{3+}, as evidenced by the presence of a strong $g = 4.3$ EPR signal. Subsequent addition of bipyridyl produced at least six $Fe(bipyd)_3^{2+}$ per MF, establishing the reversibility of this internal electron-transfer process between the redox centers of apoMF and bound iron. Incubation of $apoMF_{red}$ with the Fe^{3+}–ATP complex under anaerobic conditions resulted in the formation and binding of two Fe^{2+} and four Fe^{3+} by the protein. The various redox states formed by the binding of Fe^{2+} and Fe^{3+} to $apoMF_{ox}$ and $apoMF_{red}$ are proposed and discussed. The yellow color of apoMF appears to be an integral characteristic of the apoMF and is possibly associated with its redox activity.

Mammalian ferritin (MF) is a 24-subunit iron storage protein (Ford et al., 1984) that can contain up to 4500 iron atoms isolated within its hollow interior in the form of an FeOOH mineral core. The release of iron from the ferritin mineral core is thought to occur by a two-step process initially requiring electron transfer from external reductants to the Fe^{3+} ions in the mineral core, followed by chelation and removal of the resulting Fe^{2+} [for reviews see, Thiel (1987) and Crichton and Charloteaux-Wauters (1987)]. Of importance to both iron mobilization and deposition in MF is the process by which electrons are transported through the 20–30-Å-thick protein shell that sequesters the mineral core. A low potential, pH-dependent, mineral core reduction process has been shown to occur in MF, producing stable, mixed Fe^{2+}/Fe^{3+} core states which can then release Fe^{2+} in the presence of chelators (Watt et al., 1985; Jacobs et al., 1989). It has also been established that large chemical redox reagents and even proteins are effective in reducing and oxidizing the ferritin core, presumably, without actually entering the ferritin interior (Watt et al., 1988). Thus, while it is conceivable that small oxidizing and reducing molecules such as oxygen and dithionite could diffuse into the ferritin interior and make direct contact with the mineral core, there must also be processes by which electrons are transferred from external redox agents to iron atoms in the mineral core. These processes could involve localized redox centers in the protein shell. Indeed, preliminary evidence for redox activity of apoMF has been reported (Watt & Frankel, 1990).

A potentially related problem concerns the color of apoMF. Granick and Michaelis (1943) originally reported that apoferritin is pale yellow in color. This characteristic of apoMF has been noted on occasion since then, but never properly explained. If apoMF is indeed redox active, the yellow color could be related to the redox activity of the protein.

In this paper, we present detailed evidence on the redox activity of apoMF from horse spleen. The results suggest that apoMF has six redox-active centers. The location of the redox centers in the protein has not been determined, but we suggest that tyrosines near the six hydrophobic channels are involved. We also report on how iron atoms bound to the protein interact with the redox centers. Finally, we have studied the yellow color of apoMF and conclude that it is an inherent property of the protein, possibly connected with aromatic amino acids that could be involved in the redox activity.

MATERIALS AND METHODS

Horse spleen ferritin was obtained from Sigma, Calbiochem, and Boehringer-Mannheim, and the iron cores were removed by the thioglycolic acid dialysis method (Treffry & Harrison, 1978). The resulting apoferritin typically contained 1–5 iron atoms per ferritin molecule. This remaining iron was removed in a second step which consisted of adding reduced methyl viologen (MV) to 0.5 mM, incubating for 30 min in a Forma Glove Box (10% H_2, 90% N_2, <5 ppm O_2), adding bipyridyl to 1.0 mM to chelate the Fe^{2+}, and passing the mixture through a 1.0 cm × 15 cm Sephadex G-25 column to separate reduced ferritin from excess MV and chelated Fe^{2+}. Iron analysis on the resulting apoferritin revealed <0.3 Fe/apoferritin. The resulting reduced apoferritin ($apoMF_{red}$) was concentrated to 30 mg/mL, kept anaerobic, and used in experiments described below.

Oxidized apo mammalian ferritin ($apoMF_{ox}$) is formed when $apoMF_{red}$ is vigorously stirred in air. This oxidation was found to be a slow process with residual $apoMF_{red}$ existing as long

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as several days after initial O_2 exposure. In order to quantitate the amount of oxidation, a procedure was developed to rapidly and completely oxidize $apoMF_{red}$ to $apoMF_{ox}$ by reacting $apoMF_{red}$ with excess $Fe(CN)_6^{3-}$ for 30 min followed by chromatographic separation of the protein.

Many of the reactions described below involve reactions of air-sensitive reagents with MF and the isolation and characterization of air-sensitive MF intermediates. To maintain complete anaerobic conditions, all air-sensitive reactions were conducted in Vacuum Atmospheres glove boxes containing argon or nitrogen atmospheres (<1 ppm O_2). Microcoulometry was carried out as previously described (Watt, 1979), and all potentials are reported relative to the normal hydrogen electrode (NHE).

The iron core of MF was also removed by two other reductive/chelation processes which did not involve the thioglycolic acid treatment described above. The first required the reduction of MF with excess $Na_2S_2O_4$ for several hours in the presence of the bipyridyl chelator followed by anaerobic Sephadex G-25 chromatography to remove the excess $Na_2S_2O_4$ and the chelated Fe^{2+}. The second required reduction of MF with excess Zn in the presence of 0.1 mM methyl viologen (MV) as mediator in the presence of excess bipyridyl followed by repeated dialysis or by Sephadex G-25 chromatography as above.

Fractionation of ApoMF. ApoMF was fractionally precipitated by adding selected amounts of saturated ammonium sulfate to protein solutions initially 57 mg/mL to produce solutions containing 85%, 65%, 33%, and 16% of the original apoMF protein concentration. The precipitated protein was separated from protein remaining in solution by centrifugation at 10 000 rpm for 20 min in a clinical centrifuge. Both the protein precipitate and its supernatant were separately dialyzed against 0.025 M Tes ([*N*-tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid), pH 7.5, to remove the ammonium sulfate and centrifuged to remove small amounts of denatured protein.

The pH of a separate apoMF sample (37 mg/mL) was slowly lowered with 0.05 M HCl to near the isoelectric point to precipitate 10%, 47%, 67%, and 88% of the initial protein concentration. The cloudy solutions were centrifuged to separate the protein precipitate from that remaining in the supernatant, and the protein precipitate was washed with cold water adjusted to pH 4.5. The precipitate was dissolved in 0.025 M Tes, pH 7.25, and the supernatant was adjusted to pH 7.25 with 0.05 M NaOH. Both the redissolved protein precipitate and its corresponding supernatant solution were filtered through 0.45-μm Millipore filters prior to optical measurements. ApoMF solutions were adjusted to pH values near 12 and chromatographed on G-25 and then adjusted to pH 7.0 prior to optical measurements.

MF solutions at 20 mg/mL were precipitated by addition of $CdSO_4$ to a concentration of 5%; the protein precipitate was separated by centrifugation and redissolved in 0.05 M Tes containing 0.01 M EDTA.

Optical absorbance measurements at 252, 280, and 340 nm of the supernatant and redissolved protein pellets prepared by the three methods just outlined were made on a Varian 2200 spectrophotometer. The A_{280}/A_{340} and A_{280}/A_{252} ratios were determined for each solution and compared.

Sedimentation velocity measurements at protein concentrations ranging from 15 to 25 mg/mL in 0.05 M Tes, 0.1 M NaCl, at pH 8, were conducted in a double-sector cell using an An-D rotor at 48 000 rpm (168000*g*) in a Beckman Model E ultracentrifuge equipped with a UV scanner set at 400 nm. A Beckman Model L8 preparative ultracentrifuge equipped with a Ti 70 rotor was used to examine the sedimentation behavior at 60 000 rpm (265600*g*) of apoMF at 50–100 mg/mL.

Microcoulometric reduction of $apoMF_{ox}$ was conducted as previously described (Watt, 1979) using apoMF previously oxidized either by air, $Fe(CN)_6^{3-}$, or Fe(III)–cytochrome *c*. ApoMF, first reacted anaerobically with 8, 16, 24, and 32 Fe^{2+} ions for 30 min was then exposed to air for several hours to convert all Fe^{2+} to Fe^{3+}. These solutions were then made anaerobic under argon and microcoulometric measurements made on the reconstituted Fe^{3+} ferritin samples.

Reduced ApoMF. Optical spectra at room temperature and EPR spectra at 4.2 and 77 K were recorded on anaerobic solutions of $apoMF_{red}$ at 10–15 mg/mL and repeated on these same solutions after a 2–12-h exposure to air at 25 °C to produce $apoMF_{ox}$. The Fe(III)–ATP complex reported by Mansour et al. (1985) was reacted anaerobically with $apoMF_{red}$ and as a control with $apoMF_{ox}$ for 30 min and the MF proteins were separated by anaerobic G-25 chromatography. Excess bipyridyl was added to the protein fraction, and the resulting $Fe(bipyd)_3^{2+}$ complex was quantitated at 520 nm to establish the concentration of Fe^{2+} produced by the reaction. Excess dithionite was then added, and the process was repeated to determine the total iron present in the protein fraction.

RESULTS

Watt and Frankel (1990) initially reported that apoMF is redox active and undergoes a six-electron redox reaction. This initial observation has been confirmed, examined in more detail, and extended to other conditions in an attempt to understand and to more precisely quantitate the nature of the redox centers present. As isolated from the thioglycolic acid core removal step or from subsequent steps involving MV, Zn, or $S_2O_4^{2-}$ reduction, the redox centers in the protein shell of apoMF are fully reduced. However, upon prolonged exposure to air under typical storage conditions, the reduced centers gradually undergo oxidation. The oxidation by O_2 is quite slow and residual $apoMF_{red}$ is present several days after preparation. Thus, many preparations of apoMF are usually mixtures of the reduced and oxidized forms of MF, and to maintain MF in the reduced state, anaerobic conditions must be observed. Cytochrome *c* or $Fe(CN)_6^{3-}$ rapidly oxidizes $apoMF_{red}$, and the use of these reagents offers the most convenient method we have found for preparing fully oxidized apoMF. In what follows, we first describe the redox properties of apoMF and then present results which relate to the nature of these redox centers.

Microcoulometry of Oxidized ApoMF. Figure 1 shows the result of controlled potential microcoulometric reduction of $apoMF_{ox}$ as a function of applied potential. $ApoMF_{ox}$ undergoes a methyl viologen-mediated six-electron reduction with a midpoint reduction potential ($E_{1/2}$) of −310 mV relative to the normal hydrogen electrode (NHE). With seven independently prepared, air-oxidized apoMF samples, the overall reduction stoichiometry varied from 4.0 to 7.1 electrons/apoMF with most values near 6.0 e/apoMF as shown in Figure 1. Two effects contribute to this irreproducible stoichiometric behavior for air-oxidized apoMF. ApoMF has a high affinity for metal ions commonly contaminating glassware, buffers, reagents, etc., such as copper and iron, which, when bound, undergo reduction in the potential range studied in Figure 1 and increase the reduction stoichiometry. However, a more important contributing factor arises from the method of preparation of apoMF. To remove all iron

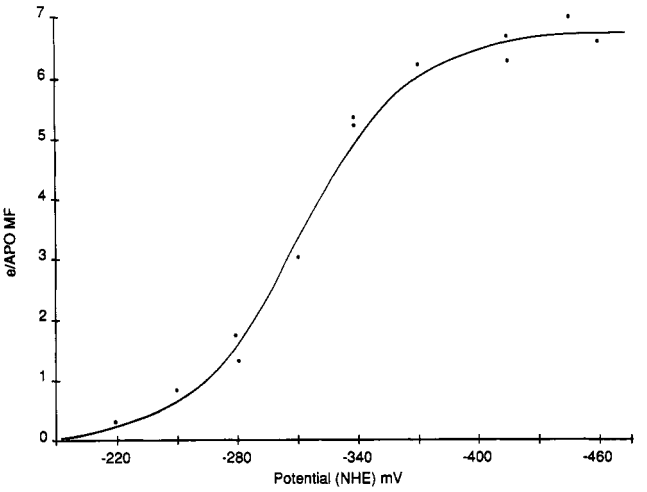


FIGURE 1: Microcoulometric reduction of oxidized apo horse ferritin as a function of applied potential. Apoferritin was oxidized either by $Fe(CN)_6^{3-}$ or air oxidation and made anaerobic under nitrogen, and the extent of reduction was measured as a function of applied potential by microcoulometry. Reduction was carried out in 0.05 M Tes, 0.1 M NaCl containing 0.1 mM methyl viologen at pH 8.0. The solid line is a Nernst equation fit for $E_{1/2} = -310$ mV vs the normal hydrogen electrode (NHE) and $n = 1$.

Table I: Microcoulometric Reduction of Reconstituted Horse Spleen Ferritin[a]

Fe^{3+}/ApoMF	equiv added/ApoMF	equiv added – Fe^{3+}
0	6.4	6.4
8.0	15.1	7.1
16.0	22.4	6.4
24.0	29.6	5.6
32.0	39.7	7.7

[a] The samples in column 1 were prepared by stoichiometrically adding the indicated numbers of Fe^{2+} to apoMF, followed by air oxidation for 30 min. These samples were then made anaerobic and the reducing equivalents transferred to each at −560 mV vs NHE were determined by microcoulometry and listed in column 2. Column 3 contains the number of reducing equivalents transferred to the protein shell in excess of the Fe^{3+} present. These values were determined by subtracting the Fe^{3+} present in column 1 from the total reducing equivalents transferred to the protein presented in column 2.

following the thioglycolic acid dialysis step, a MV reduction step is utilized for converting residual Fe^{3+} to Fe^{2+}, which is then easily chelated and removed. These conditions also produce $apoMF_{red}$ (as shown by the reduction curve in Figure 1), which in the presence of air is oxidized back to $apoMF_{ox}$. This air oxidation is not always complete even after several hours of O_2 exposure, resulting in apoMF slightly reduced (incompletely oxidized), an effect which contributes to the lower reduction stoichiometries. Reaction with excess $Fe(CN)_6^{3-}$ or oxidized cytochrome *c*, followed by separation of the oxidized ferritin by chromatography, produces completely oxidized apoferritin. Samples prepared in this manner gave a more reproducible reduction value near six, as shown in Figure 1. The $E_{1/2}$ value was invariant with pH over the range 6–9, indicating that no proton exchange occurs during reduction, a result verified by observing no pH change as apoMF samples undergo MV reduction at pH 8.0. These results exclude the presence of redox centers in the MF protein shell that require concomitant proton transfer with electron transfer and indicate that the MF redox centers only transfer electrons.

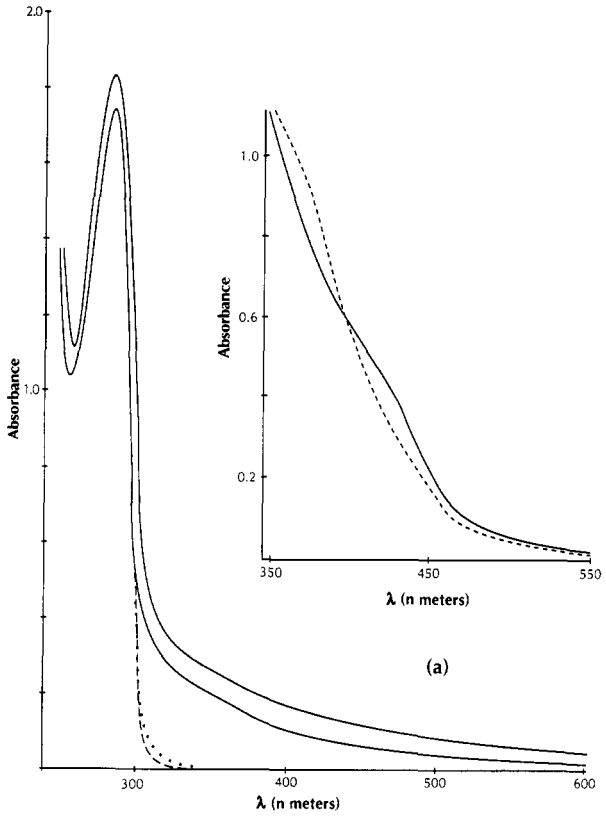
Table I presents results for complete reduction at −560 mV of reconstituted horse spleen ferritin containing 8, 16, 24, and 32 Fe^{3+} ions produced by air-oxidizing Fe^{2+} added initially to either reduced or oxidized ferritin. In the presence of the developing iron core, air oxidation of the protein shell seems to be faster and more complete than for $apoMF_{red}$ alone. Reduction values corrected for the Fe^{3+} present yield the reduction stoichiometry for the protein shell itself and are seen to be constant at about 6–7 electrons/apoMF, a value consistent with the results in Figure 1. This latter correction assumed that the initial Fe^{3+} was reduced by one electron to Fe^{2+}, an assumption verified by showing that all initial iron was recovered as the Fe^{2+}–bipyridyl chelate. The results show that the apoMF protein shell still undergoes the reduction shown in Figure 1 even in the presence of a small Fe^{3+} mineral core. Experimental errors due to subtracting two similar, large numbers (the total number of electrons transferred minus the Fe^{3+} present) prevented meaningful examination of this effect to core sizes larger than about 50 Fe^{3+} ions using microcoulometry. Results identical to those in Table I were obtained whether Fe^{2+} was initially added to $apoMF_{red}$ or $apoMF_{ox}$ prior to oxidation to Fe^{3+}, indicating that Fe^{2+} oxidation occurred independently of the redox status of the apoferritin redox state.

Results identical to those discussed above were obtained with horse spleen ferritins obtained from three independent suppliers, indicating that the observed redox behavior for apoMF is not source dependent. Identical results also were obtained with ferritin samples whose iron cores were removed by the thioglycolic acid method, by $S_2O_4^{2-}$ reduction, or by Zn/MV reduction followed by bipyridyl chelation and removal of the resulting Fe^{2+}. Such independent methods of apoMF preparation tend to eliminate chemical artefacts, such as the presence of disulfide bonds formed between thiol groups of the protein and thioglycolic acid or protein bound sulfite groups, as being responsible for the observed redox behavior. We conclude from these control reactions that the results in Figure 1 and Table I arise from inherent redox properties of the apoMF protein shell.

Redox Reactivity of Reduced ApoMF. Figure 2b shows that the addition of oxidized cytochrome *c* to $apoMF_{red}$ results in the transfer of electrons to the heme group of this oxidizing protein. Electron transfer between these two proteins is rapid and indicates that the electron centers on $apoMF_{red}$ readily interact with cytochrome *c*. Titration of a known amount of $apoMF_{red}$ with oxidized cytochrome *c* demonstrates that about 6 electrons are transferred to cytochrome *c* from $apoMF_{red}$, results consistent with Figure 1. The exact number of electrons transferred is difficult to quantitate due to the absorbance increase when apoferritin changes oxidation states between $apoMF_{red}$ and $apoMF_{ox}$ as shown in Figure 2a. Similar results were obtained using air and $Fe(CN)_6^{3-}$ as oxidants.

The functionality of the reduced centers toward iron was suggested by observing the transfer of electrons from $apoMF_{red}$ to iron when reacted with the Fe^{3+}–ATP complex (Mansour et al., 1985) forming 2 Fe^{2+} and 4 Fe^{3+}, which remain bound to the apoprotein. The bound Fe^{2+} must have formed from reduction of Fe^{3+} by the reduced protein centers because no other reductant was present. The 4 bound Fe^{3+} were inferred by first reducing the protein with dithionite and measuring the total iron present and then subtracting the number of previously observed Fe^{2+} ions. The addition of the Fe^{3+}–ATP complex to $apoMF_{ox}$ was used as a control reaction and showed that 6 Fe^{3+} ions bound to the oxidized protein.

The cytochrome *c* oxidation of $apoMF_{red}$ clearly establishes that external oxidants are capable of interacting with the redox centers present in $apoMF_{red}$. The formation of Fe^{2+} bound to the protein upon addition of the Fe^{3+}-ATP complex to $apoMF_{red}$ indicates that an internal process occurs to bind



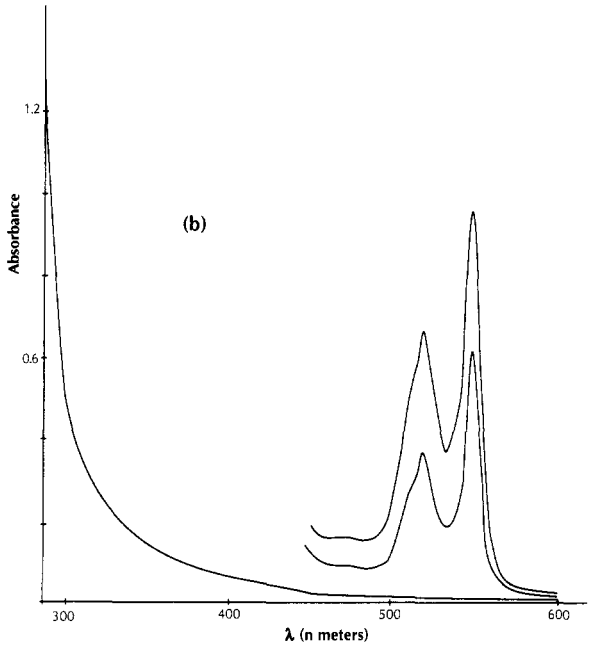


FIGURE 2: Optical spectroscopic properties of apo horse ferritin. (a) The main figure shows the spectrum of reduced apoferritin prepared anaerobically (bottom spectrum) at 2 mg/mL in 0.05 M Tes, pH 7.5. The upper spectrum is reduced apoferritin oxidized either for 12 h in air or by $Fe(CN)_6^{3-}$ at the same protein concentration. The dashed line (- - -) is twice-crystallized chymotrypsin or three-times-crystallized pepsin at concentrations giving the same absorbance at 280 as the apoferritin. The dotted spectrum (···) is twice-crystallized bovine serum albumin under the same conditions. The insert is oxidized apoferritin (- - -) at 18 mg/mL in 0.05 M Tes, pH 8.0, and the same concentration of apoferritin dissociated into subunits in 67% acetic acid (—). (b) The lower spectrum is 1.0 mL of 5.48 μM reduced apoferritin to which 24.0 μmol (lower spectrum on the right) and 40 μmol (upper spectrum on the right) of oxidized cytochrome *c* were added anaerobically.

and reduce the iron from the Fe^{3+}–ATP complex. These two reactions may be sub-reactions of a cycle carried out by the

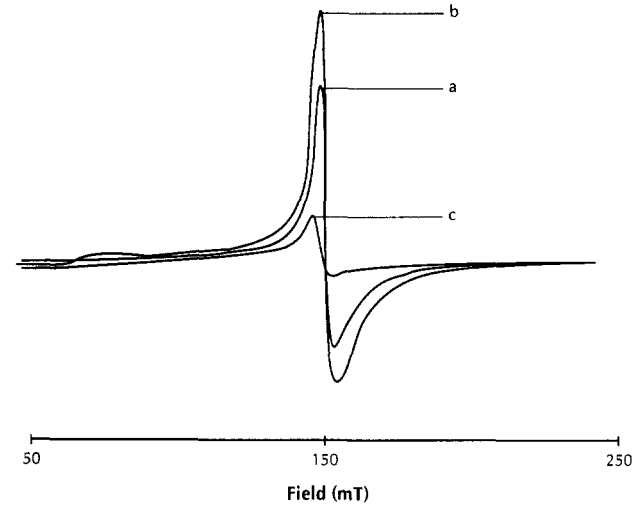


FIGURE 3: EPR spectra of Fe^{2+} added to oxidized apo horse ferritin. (a) Oxidized apo horse ferritin at 24.2 mg/mL was made anaerobic and 8 Fe^{2+} reacted anaerobically for 30 min. Nearly all iron can be removed as the Fe^{2+}–bipyridyl complex following this treatment, showing that the iron has not been inadvertantly oxidized and that the observed Fe^{3+} arises from internal oxidation of Fe^{2+}. (b) Same as (a) except the Fe^{2+}–apoferritin mixture was stirred in air for 30 min to oxidize all Fe^{2+} to Fe^{3+}. (c) Control reaction prepared by adding 8 Fe^{2+} to $apoMF_{red}$. All samples were prepared in 3-mm-i.d. quartz EPR tubes, and the spectra were recorded at 4.2 K and 50 μW.

redox centers present in apoMF which when combined could explain the results reported by Watt et al. (1988) that cytochrome *c* and other oxidizing proteins are capable of functioning as external oxidants to added Fe^{2+} in depositing Fe^{3+} into apoMF. That only 2 Fe^{2+} are formed during the reaction between $apoMF_{red}$ and added Fe^{3+}–ATP may indicate that optimal conditions have not been found for this reaction to go to completion or that the redox equilibrium between the Fe^{3+}–ATP complex and $apoMF_{red}$ is only partially favorable toward formation of Fe^{2+} and $apoMF_{ox}$. This latter possibility is supported by experiments described in the next section involving the interaction of Fe^{2+} with $apoMF_{ox}$.

In the initial report of the redox reactivity of apoMF (Watt & Frankel, 1990), the presence of a $g = 2$ EPR signal was noted. Similar $g = 2$ signals have been reported by Grady et al. (1989) and Hanna et al. (1991). We have examined a number of apoMF samples that were fully reduced, were fully oxidized, or were in between these two redox extremes. EPR signals near $g = 2$ are observed which integrate to about one spin or less per MF, but their variable intensity and spectroscopic structure have not allowed us to determine whether or not they are relevant to the redox states of apoMF.

Redox Reactivity of Oxidized ApoMF. Figure 1 clearly establishes that $apoMF_{ox}$ undergoes a six-electron reduction, indicating the presence of six redox-active centers in the protein. The functionality of these centers is demonstrated in Figure 3 which shows that the addition of 8 Fe^{2+} ions to $apoMF_{ox}$ under carefully controlled anaerobic conditions results in the formation of Fe^{3+} ions, as measured by EPR spectroscopy. When this sample was oxidized by air, the resulting $g = 4.3$ signal only increased slightly, indicating that most of the iron was already in the Fe^{3+} state. The addition of 8 Fe^{2+} to $apoMF_{red}$ as a control reaction showed only a small $g = 4.3$ signal which results from residual iron present in apoferritin (Cheng & Chasteen, 1991). These results suggest that an internal redox reaction has occurred in which the incoming Fe^{2+} is oxidized by the oxidized protein-bound redox centers forming Fe^{3+} and reduced protein redox

centers. The anaerobic addition of bipyridyl to such protein samples shows that 6.5 of the original 8 Fe^{2+} ions initially added to six-electron-oxidized $apoMF_{ox}$ are recovered as the Fe^{2+}–bipyridyl complex. This result establishes that the redox reactivity within the protein which is responsible for Fe^{3+} formation is reversible and that the formation of Fe^{3+} is not a result of inadvertant oxidation. The redox behavior just noted may explain an earlier observation (Jacobs et al., 1989b) that when $^{57}Fe^{2+}$ was bound to apoMF under anaerobic conditions, up to 25% of the added Fe^{2+} was observed by Mössbauer spectroscopy to be Fe^{3+}. The apoMF used in the Mössbauer measurements had been freshly prepared but handled without regard to anaerobic conditions (the oxidation phenomenon was unknown at that time) and was probably 25% oxidized and 75% reduced before it was made anaerobic prior to the $^{57}Fe^{2+}$ addition. Upon Fe^{2+} addition, 25% of the added iron became oxidized by internal electron transfer from Fe^{2+} to the oxidized protein centers yielding the 25% Fe^{3+} observed by Mössbauer spectroscopy. The results reported in Figure 3 indicate that the internal oxidation of Fe^{2+} to Fe^{3+} by the protein centers is essentially complete but not irreversible. Apparently, the high driving force for formation of the $Fe(bipyd)_3^{2+}$ chelate is able to reverse the internal redox equilibrium to an extent that allows Fe^{2+} to be removed by chelation. The nearly complete oxidation of Fe^{2+} to Fe^{3+} by $apoMF_{ox}$ indicates that the redox equilibrium favors Fe^{3+} and $apoMF_{red}$ formation and is consistent with the results in the previous section which showed that only partial conversion of the Fe^{3+}–ATP complex to Fe^{2+}-bound MF occurs when Fe^{3+}–ATP and $apoMF_{red}$ react.

Spectroscopy and Properties of ApoMF. At concentrations greater than 20 mg/mL, iron-free, apoMF possesses a distinctly yellow color which results from the aromatic amino acid peak, shown in Figure 2a, tailing into the visible region of the spectrum. The insert in Figure 2a compares native apoMF at pH 8 with dissociated subunits at the same total protein concentration in 67% acetic acid and shows that subunit dissociation decreases the intensity of the weak shoulder near 365 nm and shifts its position toward the visible region of the spectrum. This behavior was observed for apoMF samples prepared from holoferritin obtained from three independent suppliers and prepared by different reduction–chelation methods. Dialysis of apoMF against 0.05 M Tes, pH 7.5, or 67% acetic acid for 22 h does not diminish the optical absorption, indicating that the yellow color remains protein associated in both native and subunit-dissociated samples and excludes the possibility of a labile, small molecular weight contaminant either weakly protein bound or trapped within the native protein interior as the source of the yellow color. The absorbance of chymotrypsinogen, pepsin, and bovine serum albumin in the 300–350-nm range is also shown in Figure 2a to demonstrate the spectroscopic behavior of typical aromatic amino acid side chains and to show that the aromatic amino acid "tail" in apoferritin extends into the visible spectral range. Figure 2a also compares the optical spectra of $apoMF_{ox}$ and $apoMF_{red}$ at identical protein concentrations. These spectra were taken by first preparing $apoMF_{red}$ and then allowing it to oxidize in the air for 12 h until the absorbance attained a constant value. Identical spectra were obtained by $Fe(CN)_6^{3-}$ oxidation. $ApoMF_{ox}$ has a higher extinction than $apoMF_{red}$ all across the spectral region investigated. Addition of sodium dithionite or reduced MV to $apoMF_{ox}$ causes rapid reduction to occur, forming $apoMF_{red}$ with the accompanying absorbance shown in Figure 2a. These results demonstrate the reversibility of the redox centers in apoMF and also show that the redox behavior is linked to the yellow color present in apoMF.

Chromatography of apoMF at pH 12 gave a single protein band containing a yellow color. Optical measurements of the solution adjusted to pH 7 shows the yellow color to still be present at the same intensity. Selective precipitation by (1) ammonium sulfate addition, (2) pH adjustment with HCl to near the isoelectric point of apoMF or (3) $CdSO_4$ precipitation fractionated apoMF to varying degrees between precipitate and supernatant solution. Comparison of A_{280}/A_{254} and A_{280}/A_{340} ratios was used to evaluate whether the yellow color tracked with or was independent of the protein during these precipitation treatments. The results show that the A_{280}/A_{252} values remained essentially invariant at 1.85 ± 0.10 (the range for several independently prepared samples) and was independent of the degree of precipitation. However, the A_{280}/A_{340} value near 10.5 for native apoMF tended to decrease to values as low as 8.0 in the protein precipitate and increase to about 13.0 in the supernatant for both the ammonium sulfate, $CdSO_4$, and acid precipitation treatments. A decrease in the A_{280}/A_{340} ratio (at a constant A_{280} value) indicates that the A_{340} value (and hence the intensity of the yellow color) increases in the precipitated protein and decreases slightly in the solution but demonstrates that the yellow color appears to be quite strongly protein associated.

Sedimentation velocity measurements at 48 000 rpm at protein concentrations of 15–25 mg/mL, following protein movement at 400 nm, established the presence of a major (95% of total protein) and a heavier, minor band (3–5% of total protein). The heavier band sedimented with a velocity suggesting the presence of a contaminating protein dimer (Bjork, 1973). The sedimentation coefficient of the major band was 15 s, a value smaller than that typically found (Bjork, 1973; Stefanini et al., 1982) for apoMF (16–18 s), at zero protein concentration, but perhaps smaller due to the nonideality of these solutions at the high protein concentrations used to monitor protein movement at 400 nm. When monitored at 60 000 rpm over 2–5 h in a preparative centrifuge, apoMF at 70–110 mg/mL sedimented as a yellow front behind which was a colorless solvent zone with little protein present. When allowed to completely sediment, a bright yellow, homogeneous pellet was observed, demonstrating that the yellow color sediments with the protein. Taken together, the dialysis, the fractional precipitation, and centrifugation results indicate that the yellow color is an inherent property of apoMF.

DISCUSSION

The experimental results reported here clearly show that apoMF undergoes a well-defined, pH-independent, redox reaction involving six electrons. The presence of six centers in apoMF which communicate readily with large redox proteins and yet are able to change the redox status of added iron seems very relevant to ferritin function, and at this point little is known regarding the structure and location of these centers within the apoMF molecule. From the presence of six such centers, we suggest that the observed redox behavior may be associated with or occurs near the hydrophobic channels on the 4-fold symmetry axes. Indeed, the reported structure of mammalian ferritin (Ford et al., 1984) clearly indicates a number of tyrosine groups closely associated with the 4-fold channels. These tyrosine groups are conserved in all mammalian ferritins and other ferritins so far examined (Artymiuk et al., 1990) but are not conserved in the few bacterial ferritin sequences reported (Smith et al., 1990; Grossman et al., 1992) to date. It is possible that the redox centers observed

here for MF are related to the conserved tyrosine groups and fulfill the role in mammalian ferritin that heme groups satisfy in bacterial ferritin. Investigation of the nature and location of these centers within the MF protein shell is continuing as is comparison of the redox behavior in mammalian ferritin with that of the heme in bacterial ferritin.

The observed redox reactivity of apoMF shown in Figure 1 is likely to be physiological because (1) it nearly coincides with the reduction potential required for Fe^{2+} release (Watt et al., 1985); (2) protein reduction as well as Fe^{3+} reduction occurs in the presence of developing core (Table I and Figure 1); (3) the EPR results show that when Fe^{2+} interacts with $apoMF_{ox}$, Fe^{3+} is formed and the protein centers are presumably reduced; and (4) the addition of Fe^{3+} in the form of its ATP complex is partially reduced to Fe^{2+} which binds to apoferritin. Such conclusions lead us to propose that there are at least three separate but interconvertible redox states which are formed during the sequential development of stable Fe^{3+}-bound apoMF species arising from the interaction of Fe^{2+} with apoMF in the presence of a suitable oxidant. These interconversions are summarized by Scheme I for one of the possible six identical sites present in apoMF.

Scheme I: Possible Combinations of Redox States in ApoMF

$$[Fe^{3+}\text{–}MF_{ox}] \underset{}{\overset{-e}{\rightleftharpoons}} [Fe^{3+}\text{–}MF_{red}] \underset{+e}{\rightleftharpoons} [Fe^{2+}\text{–}MF_{red}]$$

According to Scheme I, the state on the extreme left is present upon complete oxidation by oxygen or other oxidants of Fe^{2+}–apoMF combinations and corresponds to those samples in Table I, where both the iron and apoferritin centers are fully oxidized. The middle state is observed when Fe^{2+} is anaerobically added to $apoMF_{ox}$ and is exemplified by the results in Figure 3 which show that the iron is oxidized and the protein is reduced by internal electron transfer. The state on the extreme right corresponds to the binding of Fe^{2+} by completely reduced apoferritin and probably corresponds to previously reported results (Jacobs et al., 1989) for Fe^{2+} binding to apoMF. The results reported in this study and interpreted by Scheme I suggest that the redox status of apoMF-bound iron can be changed by altering the redox status of the protein shell. For example, Figure 3 shows that Fe^{2+} is converted to Fe^{3+} during reaction with $apoMF_{ox}$. It will be of interest in future experiments to determine the outcome of adding one electron equivalent to or removing one electron equivalent from the states on the extreme left and right of Scheme I, respectively. In both cases, the middle state is predicted to form by transfer of electrons through the protein centers and not by direct interaction of the reductant at the metal atom. The possibility of forming $[Fe^{2+}\text{–}MF_{ox}]$ from $[Fe^{2+}\text{–}MF_{red}]$ by a single-electron oxidation of the apoprotein redox center is not possible because the proposed $[Fe^{2+}\text{–}MF_{ox}]$ species is redox unstable with respect to $[Fe^{3+}\text{–}MF_{red}]$ and corresponds to the conditions occurring in Figure 3 which clearly shows that the protein center is a strong enough oxidant to oxidize Fe^{2+} to Fe^{3+} forming the middle species.

The initial proposal of a ferroxidase center (Bakker & Boyer, 1986) and the recent reports (Lawson et al., 1990; Artymuik et al., 1990) extending this view and suggesting details of the location and mode of operation of the ferroxidase center in human H subunit ferritin suggest that a connection might exist between the ferroxidase site and the redox centers reported here and their reactivity toward both Fe^{2+} and Fe^{3+}. Horse spleen ferritin is composed of approximately 90% L subunit and 10% H subunit and according to the recent identification of the ferroxidase center (Lawson et al., 1990) would contain 2–3 H subunits and therefore 2–3 ferroxidase centers per molecule. The number of ferroxidase centers predicted from these calculations does not at present correlate with the six redox centers reported here. Because the variability of the subunit composition ranges from 85% to greater than 90% for the L subunit (Harrison et al., 1991; Yang et al., 1987), electrochemical measurements coupled with the determination of the actual H subunit content present in the sample may provide a correlation between the ferroxidase center and the redox center. Electrochemical measurements may also be performed on ferritins composed entirely of H or L subunits using recombinantly prepared ferritins. The examination of these ferritins using microcoulometry would determine if the redox behavior is specific to one of the subunit types or if it is a property created by the quaternary structure of subunit assembly.

Other differences between the ferroxidase center hypothesis and the redox centers reported here remain to be reconciled. For example, most ferroxidase reactions are examined in the presence of excess O_2 and represent the formation of species to the far left of Scheme I, but most of our experiments have been carried out under anaerobic conditions and represent species toward the right and middle of Scheme I. The ferroxidase center hypothesis focuses on the amino acids responsible for the iron-binding sites, whereas the redox reactions of apoMF reported here focus more on the electron-transfer properties of the MF protein. At this point, we see no direct connections between the ferroxidase centers and the redox centers reported here for MF, but connections might become apparent with further study.

The optical spectra in Figure 2a clearly show that the yellow color of apoMF is due to an elevated absorption at wavelengths longer than about 340 nm in comparison with other proteins having typical UV optical absorption near 280 nm. At very high concentrations, a weak shoulder near 365 nm is sometimes discernible. The various centrifugation and precipitation procedures used to fractionate the MF protein have clearly established that the yellow color is not separable under a variety of conditions and, therefore, represents an inherent property of the apoferritin molecule. This same conclusion was reached by Otsuka and Listowsky (1980) using a number of other separation procedures. The results in Figure 2a along with the known (Stefanini et al., 1976, 1982) anomalous fluorescence (for native apoMF but not for dissociated subunits) and other reported spectroscopic behavior (Listowsky et al., 1972) are consistent with the explanation that the aromatic amino acids in mammalian ferritin either are in an abnormal environment or are in an altered structural state. For example, the structure of horse ferritin (Ford et al., 1984) shows a series of tyrosine groups in a nearly linear array with Tyr-28 on antiparallel subunits forming a face to face structural feature. The observed changes in absorbance with a corresponding change in the redox state of the protein as shown in Figure 2 link the yellow color with the redox centers present in apoMF. Since we and others (Otsuka & Listowsky, 1980; Maruyama & Listowsky, 1982) have not been able to spectroscopically identify or physically separate any impurities which might serve as redox centers and since there are no known centers present from the three-dimensional structure of horse ferritin, we conclude that the redox behavior seen in Figure 1 must be associated in some way with the amino acids (more specifically the aromatic amino acids) which comprise the apoferritin shell. Such a proposal is consistent with the

results of Maruyama and Listowsky (1982), who have studied the color and unusual fluorescence of apoMF and suggested the presence of an "integral component of ferritin that could be involved in redox mechanisms that may play a pivotal role in iron deposition and mobilization mediated by the protein". Such a center has been reported here, and future study should reveal its nature and location.

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