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Redox Reactivity of Animal Apoferritins and Apoheteropolymers Assembled from Recombinant Heavy and Light Human Chain Ferritins†

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ABSTRACT: The redox reactivities of air-oxidized apo horse spleen ferritin (HoSF) and apo rat liver ferritin (RaF) were examined by microcoulometry and reductive optical titrations. Microcoulometry on several independent lots of commercial HoSF revealed two distinct types of redox activity: one requiring 3−4 electrons and one requiring 6−7 electrons for full reduction of the protein shell. ApoRaF required 8−9 electrons to fully reduce the oxidized form. Reductive optical titrations confirmed the microcoulometric reduction stoichiometry and, in addition, showed that the spectra of both oxidized and reduced apoHoSF were distinct and possessed absorbances tailing into the visible region. The redox reactivity of both apoRaF and apoHoSF correlated with their H-subunit composition. Identical microcoulometric and optical experiments were conducted with recombinant apo human liver heavy (rHuHF) and light (rHuLF) ferritins, but neither was redox-active. These results suggest that the redox reactivity of native ferritins is due to their heteropolymeric nature. This was confirmed by mixing various proportions of rHuHF and rHuLF, dissociating the 24-mers into individual subunits with guanidine hydrochloride at pH 3.5, and renaturing to form heteropolymeric 24-mers. Microcoulometric measurements of these apoheteropolymers reassembled in vitro showed that they were redox-active like their native apoheteropolymer counterparts. The redox activity of these apoheteropolymers increased with H-subunit composition, reached a maximum near 12 H- and 12 L-subunits, and then declined to zero with increasing L-subunit composition. The decline in redox reactivity at high L-subunit concentrations indicates that both H- and L-subunits are involved in forming the observed redox centers. Apoheteropolymers formed from rHuLF and W93F (an H-chain mutant) were redox-inactive, suggesting that the conserved tryptophan is necessary for redox center formation.

Naturally occurring ferritins are 24-subunit iron storage proteins containing various proportions of L- ($M_r \sim 17\,500$) and H-subunits ($M_r \sim 18\,500$) assembled into nearly spherical molecules 130 Å in diameter with a hollow interior about 70 Å in diameter (*1*−*5*). When isolated from natural sources, ferritins typically contain 2000 iron atoms, but up to 4500 can be accommodated within their hollow interiors as iron-oxy-hydroxy cores containing variable amounts of associated phosphate. Three- and 4-fold channels formed at the boundaries where the subunits meet connect the interior cavity with the external solution and are likely pathways for iron entry and exit.

Iron deposition into ferritin and iron release from ferritin are each multistep processes involving protein-mediated redox reactions. Structural investigations initially suggested a site (ferroxidase center) in the H-subunit where iron is bound and oxidized (*6*−*8*). Extensive study using site-altered recombinant proteins has confirmed that ferroxidase activity is associated with the H-subunit (*2*, *3*, *9*), while the L-subunits may be responsible for structural integrity and core nucleation (*10*). Such results and hypotheses point to an important, dynamic, and specific role for each type of subunit present in heteropolymeric ferritins.

While the site of Fe^{2+} oxidation may be the ferroxidase center located within the H-subunit, the pathway by which electrons enter and leave this site, the process by which the protein mediates these redox reactions, and the roles of the two subunit types in redox processes remain poorly understood. The observation (*11*) that apo horse spleen ferritin is redox-active may suggest that amino acid side chains are involved in forming protein-specific redox centers. Initial evidence indicated (*11*) that the observed redox centers correlated with H-subunit composition and that the redox state of these centers influenced iron reactivity. The nature and specific location of these redox centers was not delineated and remains difficult to determine in naturally occurring heteropolymers because both H- and L-subunits are present.

A possible role for these endogenous redox centers in apoHoSF[1] may be inferred by comparing their redox proper-

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ties with those from heme-containing bacterial ferritins (BFRs). BFRs are also 24-subunit ferritins that contain up to 12 heme groups bound along 2-fold axes by novel bis-(methionine) ligation (*12*−*14*). BFR subunits are identical and possess relatively high sequence identity with animal H-subunits (*15*), including a closely related di-iron site in a similar location to that in the animal H-subunit (*16*, *17*). The iron deposition reaction with BFR shows that minor, but reproducible, heme perturbations (*16*) occur with EcBFR, but no evidence for electron transfer through the heme group was found. In contrast, the iron release reaction of AvBFR showed that significant heme reduction occurred (*18*) during iron release, suggesting that heme was involved in electron transfer from external reductants to the iron core of AvBFR. In BFRs, the iron release and deposition reactions appear to be distinct with regard to involvement of the heme in electron transfer. These BFR results suggest that there may be an analogy between the heme groups in BFRs and the redox centers present in apoHoSF, with the latter serving the same redox roles as the hemes in BFR. To address this possibility, it is necessary to better define the nature of the redox centers in apoHoSF and in other heteropolymeric animal ferritins so appropriate comparisons can be made.

To further explore the hypothesis that the H-subunit is the source of the previously observed redox reactivity in natural apoferritins (*11*), we have examined such reactivity in other native apoferritins as well as in rHuHF and rHuLF apohomopolymers and synthetic apoheteropolymers. Our results suggest that the H-subunit is indeed involved in formation of the observed protein-bound redox centers but only when associated with L-subunits in heteropolymers.

MATERIALS AND METHODS

Horse Spleen Ferritin. Apo- and holoHoSF were obtained from Sigma. Removal of iron from holoHoSF to form apoHoSF was carried out by the thioglycolic acid method (*19*) or by DT reduction with subsequent iron removal with bipy. ApoHoSF was oxidized by stirring in air for several hours at room temperature and then centrifuged to remove small amounts of denatured protein. Anaerobic, oxidized HoSF was reacted with a severalfold excess of DT or titanium(III) citrate (*20*) for 15 min to form completely reduced apoHoSF, which was separated from the reaction mixture on an anaerobic 1.0 × 10 cm Sephadex G-25 column equilibrated with 0.025 M TES, pH 7.5, in a Vacuum Atmospheres glovebox (O_2 < 1 ppm). Reductive optical titrations of oxidized, anaerobic apoHoSF in the 240−400 nm range were conducted with DT as reductant on a Hewlett-Packard 8453 diode array spectrophotometer. Second-derivative analysis of the optical absorption data was conducted with the Hewlett-Packard software.

AvFlp was prepared as previously described (*21*) and reduced to $AvFlpH_2$ with excess DT or Ti(III) followed by separation on an anaerobic 1.0 × 10 cm Sephadex G-25 column. $AvFlpH_2$ ($E_{1/2}$ = −520 mV) was loaded anaerobically into a 1.0 cm optical cell, aliquots of oxidized apoHoSF were added, and the spectrum was recorded. The reduction stoichiometry of HoSF was determined from the amount of the blue semiquinone radical ($AvFlp^{\bullet}$, ϵ_{580} = 9820 cm^{-1} M^{-1}) formed from the one-electron oxidation of $AvFlpH_2$.

Rat Ferritin. Two independent samples of holoRaF were purchased from Sigma and the iron was removed by the thioglycolic acid method. An additional step, utilizing DT reduction with bipy, removed iron to <1 Fe/RaF molecule. ApoRaF was stirred in air for several hours and centrifuged, and then microcoulometric and optical spectral measurements were collected as described for HoSF.

Recombinant Human Liver Heavy and Light Homopolymers. Purified rHuHF and rHuLF were prepared as previously described (*22*). One rHuLF sample was prepared from the human light ferritin gene supplied by Dr. J. Drysdale (Tufts University). Iron was removed with DT−bipy treatment followed by several concentration/dilution cycles with 0.025 M TES using an Amicon concentrator. Protein concentrations of rHuHF and rHuLF were determined by the Lowry method. Following the above treatment, apo-rHuHF and apo-rHuLF were stirred in air for several hours, made anaerobic, and reacted with DT while monitoring by optical spectroscopy. Identical samples were prepared for microcoulometric measurements.

rHuHF and rHuLF were each reconstituted with 120 Fe atoms/ferritin for microcoulometric measurement of the core reduction potential by adding 12 aliquots of 10 Fe^{2+} each to rHuHF and rHuLF while stirring in air.

Synthetic heteropolymers consisting of various proportions of H- and L-subunits were prepared from recombinant rHuHF, rHuLF, and site-altered rHuHF mutants as described (*22*).

Iron Analysis. Iron analyses on the various apoferritins described above were carried out by anaerobic reduction of a 0.05−0.2 mL portion of the ferritin in 1.0 mL of 0.025 M TES with DT in the presence of 1 mM bipy and measuring the absorbance of the $Fe(bipy)_3^{2+}$ (ϵ_{520} = 8400 cm^{-1} M^{-1}). In some cases iron content was also determined by use of a Perkin-Elmer inductively coupled plasma emission spectrophotometer. For the various apoproteins examined, iron analyses revealed that 0.2−2.0 Fe/ferritin molecule was present. The reliability of the iron determinations by both methods was typically 5−7%.

Microcoulometric Measurements. The number of electrons transferred to the various ferritins examined in this study was determined as previously described with 0.02 mM methyl and benzyl viologen as mediators (*11*, *23*). The redox potentials of reconstituted HoSF, rHuHF, and rHuLF containing 120 Fe atoms were determined at pH 8.0 and 9.0 as previously described (*24*). The reliability of this method in determining the number of electrons transferred to the various ferritins is 5−8% as judged by 8−12 replicate measurements.

Site-Altered Variants of HuHF. The C90E, W93F, and 222 mutants of rHuHF were prepared, purified, and characterized from *Escherichia coli* extracts as previously described (*8*). The recombinant H-subunits all contained the K86Q mutation that was originally made to promote crystallization (*7*).

[1] Abbreviations: HoSF, horse spleen ferritin; RaF, rat liver ferritin; rHuHF, recombinant human liver heavy ferritin; rHuLF, recombinant human liver light ferritin; BFR, heme-containing bacterial ferritin; EcBFR, *Escherichia coli* bacterial ferritin; AvBFR, bacterial ferritin; DT, dithionite; bipy, 2,2′-bipyridine; AvFlp, *Azotobacter vinlandii* flavoprotein; 222, E62K + H65G ferroxidase center mutant; PRC, protein redox center.

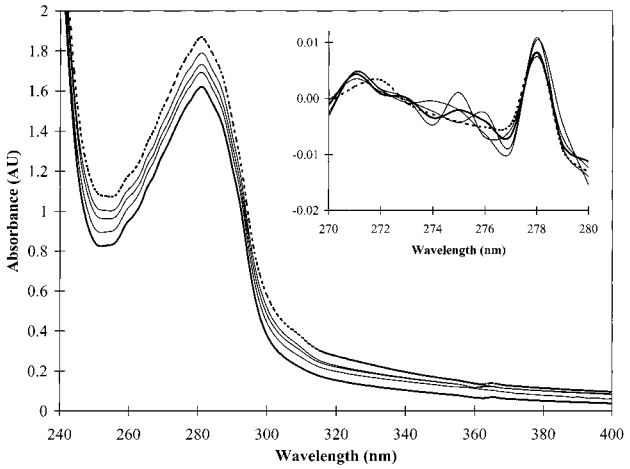

FIGURE 1: Reductive titration of apoHoSF by DT. Anaerobic, air-oxidized apoHoSF (solid dark line) was reduced with 1.50 electron equivalent aliquots of DT and the ascending spectra were recorded when all DT was reacted, as evidenced by the absence of a shoulder at 320 nm characteristic of unreacted DT. A total of 6.3 electrons were required for complete reduction. The inset shows the second derivative spectra of the absorbance spectra in the main figure. The heavy solid line is the second derivative spectrum of the initial oxidized apoHoSF, and the heavy dashed line is the spectrum of the reduced apoHoSF.

RESULTS

Reduction of ApoHoSF and ApoRaF. Figure 1 is a reductive titration of oxidized apoHoSF with DT with optical monitoring from 240 to 400 nm. Figure 1 was truncated at 400 nm, but the weak absorbance extends further into the visible region. The absorbance of HoSF increases with increasing DT concentration until complete apoHoSF reduction has occurred at approximately 6.3 electrons/HoSF. After each addition of DT (an anionic reductant), a shoulder at 320 nm transiently appears, characteristic of unreacted DT (*25*), indicating that apoHoSF reduction is not instantaneous. Reduction by reduced methyl viologen (a cationic reductant) is much faster, suggesting the slower reaction with DT may be due to unfavorable charge interaction between the protein and reductant, both of which are negative. Exposure of reduced apoHoSF to air after titration resulted in the absorbance decreasing to near its original value, demonstrating an overall reversible redox reaction with apoHoSF.

Close inspection of the individual spectra in Figure 1 shows that small spectral increases occur during the course of the titration. It is also seen that successive titration steps of 0.75 e^-/HoSF molecule (for clarity only steps of 1.5 e^-/HoSF are shown) produce irregular absorbance changes, suggesting that a complex mixture of chromophores is responsible for the absorbance change or that the environments of the chromophores are changing with the level of reduction. The inset displays the second derivative of the optical spectra over the 270−280 nm region and shows more clearly that stepwise reduction can cause both regular and irregular absorbance changes during titration. For example, the second derivative peak at 278 nm shows uniform changes as apoHoSF reduction proceeds, but at 280 and 275 nm rather irregular absorbance changes occur with reduction.

Figure 1 is typical of other apoHoSF optical titrations we have conducted showing that a total of six electrons is required for reduction; however, the observed optical changes do not totally reproduce those shown in Figure 1. If different chromophores are present in HoSF (or different environments are present), a given reductive titration may take different kinetic courses, giving different spectral changes during reduction that still result in a six-electron-reduced HoSF. Figure 1 shows optical changes in the 270−280 nm spectral region and suggests that the aromatic amino acids and/or their environments change during stepwise reduction.

Figure 1 and other experiments show a six-electron reduction of oxidized apoHoSF (designated as $HoSF_{(6)}$), a result consistent with earlier reports (*11*). However, some apoHoSF preparations gave optical titrations that required only three electrons for reduction (designated as $HoSF_{(3)}$), suggesting that apoHoSF was oxidized by only 3−4 electrons. Extensive exposure of $apoHoSF_{(3)}$ to air or oxidation with ferricyanide did not increase the level of oxidation beyond 3−4 electrons per apoHoSF, indicating that full oxidation had occurred. Both $apoHoSF_{(6)}$ and $apoHoSF_{(3)}$ are readily reconstituted to holoHoSF by oxidation of Fe^{2+} in air and appear to be identical except for the noted differences in redox behavior and spectroscopic properties. As will be discussed below, this variability in HoSF redox behavior parallels the H-subunit composition, suggesting the H-subunit is responsible for the observed redox behavior.

ApoRaF also undergoes reduction with DT similar to that shown in Figure 1 but requires 8−9 electrons for complete reduction. This higher level of reduction is also consistent with the presence of more H-subunits in RaF, as discussed below.

Reaction of $apoHoSF_{(6)}$ with Ti(III) resulted in reduction, as evidenced by a decrease in Ti(III) absorbance upon addition of $apoHoSF_{(6)}$. $AvFlpH_2$ readily reduced $apoHoSF_{(6)}$, as evidenced by formation and quantification of the blue $AvFlp^{\bullet}$ semiquinone produced by the one-electron oxidation of $AvFlpH_2$. The reducibility of $apoHoSF_{(6)}$ and $apoHoSF_{(3)}$ by the diverse reductants used clearly indicates that these natural apoferritins are redox-active. We have previously shown (*21*) that $AvFlpH_2$ is functional in mobilizing iron from the cores of holoHoSF, a result consistent with its ability to interact with and reduce $apoHoSF_{(6)}$. Direct and rapid transfer of electrons from the various reductants used, especially $AvFlpH_2$, suggests the redox centers in HoSF are readily accessible by being either on the surface or connected by an electron transfer network in the HoSF protein shell. In other experiments, cytochrome *c* oxidation of reduced apoHoSF readily occurs and similarly indicates facile electron transfer from the reduced centers in HoSF.

Table 1 confirms, by the more precise microcoulometric measurements, the reducibility of various ferritins. The results for apoRaF represent two separate preparations and those for $apoHoSF_{(3)}$ and $apoHoSF_{(6)}$ represent five and eight separate preparations, respectively. Table 1 clearly shows that apoRaF is more extensively oxidized than either type of apoHoSF, a result consistent with more H-subunits being present in the heteropolymers. HoSF nominally contains 15% H and 85% L and RaF contains 34% H and 66% L, giving 3−4 H-subunits in HoSF and 8−9 H-subunits in RaF. The number of H-subunits present in $HoSF_{(3)}$ and RaF is consistent with the level of $apoHoSF_{(3)}$ and apoRaF oxidation (3−4 and 8−9 e^-/ferritin molecule, respectively). The higher values for $apoHoSF_{(6)}$ likely arise from HoSF samples with higher H-subunit levels. This conclusion was qualitatively

Table 1: Microcoulometric Reduction of Various Types of Ferritins

ferritin type[a]	Fe present[b]	electrons/ferritin[c]	electrons/(ferritin-Fe)[d]
apoRaF	0.9	9.2 ± 0.5	8.3 ± 0.5
apoHoSF$_{(3)}$	0.3	3.8 ± 0.4	3.5 ± 0.4
apoHoSF$_{(6)}$	0.8	7.2 ± 0.5	6.4 ± 0.5
aporHuLF	1.6	2.0 ± 0.5	0.4 ± 0.5
holo-rHuLF	16.0	17.1 ± 2.0	1.1 ± 2.0
apo-rHuHF	3.5	2.8 ± 0.8	0.0 ± 0.8
holo-rHuHF	14.0	14.3 ± 1.0	0.3 ± 1.0

[a] Iron was removed by the DT−bipy method described in the Materials and Methods section and the resulting ferritin type is referred to as apo. The iron content of most holoferritins was that present after preparation. In some rHuHF where >50 Fe/rHuHF was present, partial iron removal was carried out to make microcoulometry more convenient. [b] Iron analysis was carried out immediately after the microcoulometric measurements. [c] This is the total measured reduction of the ferritin sample and includes both protein and iron reduction. [d] The value listed is the average of independent measurements made on several different samples. The indicated uncertainty represents the repeatability of 8−12 replicate microcoulometric measurements.

confirmed by scanning stained SDS−polyacrylamide gels, which showed that apoHoSF$_{(6)}$ contained roughly double the H-subunit composition of apoHoSF$_{(3)}$, but precise quantification was not possible.

rHuLF and rHuHF Homopolymers. Addition of DT to anaerobic apo-rHuLF or apo-rHuHF that had been stirred in air for several hours and then made anaerobic does not produce the same absorbance increases in the 270−280 nm spectral region as shown in Figure 1, indicating that neither apo-rHuHF nor apo-rHuLF homopolymer is redox-active. This result is confirmed by observing a stable peak in the aporHuHF and aporHuLF spectra at 320 nm, characteristic of unreacted dithionite.

Table 1 presents microcoulometric reduction data for aporHuLF and aporHuHF, as well as data for holo-rHuLF and holo-rHuHF containing the indicated small amounts of iron. The results show that apo-rHuLF has no significant redox reactivity by itself, a result consistent with the lack of reduction observed spectroscopically. This result represents the average of three independent apo-rHuLF preparations, two apo-rHuLF samples prepared at the University of Brescia, and one apo-rHuLF prepared at Brigham Young University from the rHuLF gene supplied by Dr. Drysdale. The indicated uncertainty is calculated from 10−12 replicates of each separate preparation. With low levels of Fe present, holo-rHuLF accepts electrons equivalent to the number of Fe atoms present, but there is no reduction attributable to protein redox sites induced by the presence of iron.

Microcoulometric measurements of two independent preparations of apo-rHuHF indicate that no reduction of air-exposed apo-rHuHF occurs except that attributable to the small amount of Fe present. This result is supported by the measurements on holo-rHuHF, which only show reduction equivalent to the Fe atoms present. The lack of spectral change upon addition of DT to anaerobic apo-rHuHF further supports the lack of reduction of apo-rHuHF. It is worth mentioning that while we were preparing our apoferritin samples, we observed that iron removal from ferritin was much slower for homopolymers than for heteropolymers, which may be attributable to their lack of redox activity.

Additional microcoulometric reduction measurements were carried out on recombinant rHuHF and rHuLF mutant apohomopolymers. The 222 mutant of the human H-subunit has an altered ferroxidase center that has a significantly slower rate of iron uptake, as previously described (*26*). The W93F mutant has an alteration in the sole conserved Trp residue on the H-subunit but has ferroxidase activity comparable to wild-type H-subunits (*27*). The C90E mutant alters a Cys (which is unique to the human H-subunit) to Glu that is present in the human L-subunit. It has been reported for galactose oxidase that C228 in conjunction with W272 functions as an electron acceptor (*28*). C90 is located near the end of the BC loop in the human H-subunit and is about 4 Å from the conserved W93 [comparable to 3.84 Å in galactose oxidase (*28*)]. Thus we wanted to determine if these two amino acids were responsible for the observed redox behavior in ferritin.

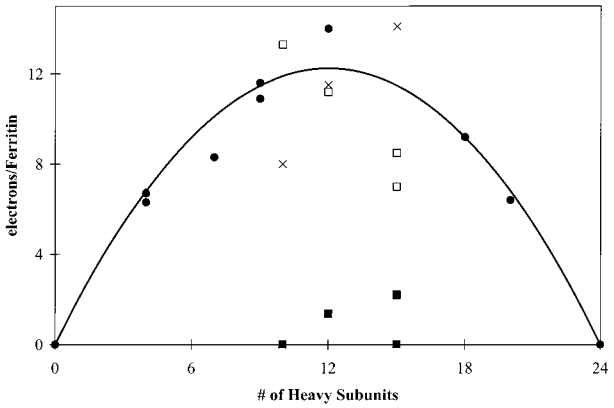

FIGURE 2: Microcoulometric reduction of apoheteropolymers prepared from apohomopolymers of rHuLF, rHuHF, and rHuHF mutants. The *y*-axis is the number of electrons transferred by microcoulometry to apoheteropolymers (●), formed from rHuHF and rHuLF apohomopolymers, containing the number of H-subunits shown on the *x*-axis. The solid line is drawn assuming preferential formation of H−L dimers and that H−L dimers form the redox center. Reduction of an apoheteropolymer formed by HCl denaturation with subsequent renaturation with phosphate buffer at a ratio of 4 H and 20 L, instead of the Gdn·HCl denaturation/renaturation process used in all other experiments, is coincident with the two points at the same ratio. Microcoulometric reduction of apoheteropolymers formed between rHuLF and the rHuHF mutants 222 (×), C90E (□) and W93F (■), respectively, is shown.

None of the apohomopolymers of these site-directed rHuHF or rHuLF mutants have any redox activity as measured by microcoulometry (data not shown, see later for heteropolymers). The results in Table 1 and the reduction measurements on rHuHF and rHuLF mutant apohomopolymers just discussed clearly indicate that natural apoheteropolymers are redox-active but that rHuHF and rHuLF apohomopolymers or selected site-altered apohomopolymers are not. These results raise the possibility that the redox reactivity of natural heteropolymeric ferritins is a consequence of H- and L-subunit interactions in heteropolymeric ferritins.

rHuHF and rHuLF Heteropolymers. Figure 2 summarizes microcoulometric measurements on apoheteropolymers formed from various combinations of redox-*inactive* rHuHF and rHuLF homopolymers. Measurements on apoheteropolymers formed by combining the redox-inactive rHuLF and site-altered H-chain mutants are also shown. Redox activity is clearly observed in all but one of the artificially produced apoheteropolymers. These results are important because none of the apohomopolymers used to form the apoheteropolymers is redox-active by itself. One possibility is that redox

reactivity is produced as a consequence of the aerobic denaturation/renaturation process that causes apo-rHuHF and apo-rHuLF to dissociate into individual subunits and then reassemble into apoheteropolymers. As control reactions, the same method performed with either apo-rHuHF or apo-rHuLF produced aporHuHF and aporHuLF that remained redox-inactive, indicating the aerobic denaturation process does not induce redox activity in the individual apo-homopolymers. For the more extensive data set involving H−L subunit interactions, the solid line is computed by assuming preferential formation of H−L dimers (*29*), which then produce the redox centers. This model gives a good fit to the H−L redox data and also fits the less extensive results for heteropolymers formed from mutant rHuHF and rHuLF. Figure 2 shows that the redox reactivity reaches a maximum near 12 electrons/heteropolymer, which then declines with increasing H-subunit composition. The formation of redox-active heteropolymers from redox-inactive homopolymers leads us to conclude that the redox centers are formed from H−L subunit interactions.

This conclusion raises questions regarding how these centers are formed and what amino acid side chains are involved. A partial answer is given by considering the results in Figure 2 involving formation of the heteropolymer formed between rHuLF and the W93F mutant. Only in this specific case is the redox reactivity of the corresponding heteropolymer far below that predicted by the line in Figure 2. A reduction stoichiometry near zero was observed for this heteropolymer in contrast to values near 10−12 electrons/heteropolymer observed for other heteropolymers of comparable H−L subunit composition. This result shows that if the apoheteropolymer formed from the W93F mutant and rHuLF is formed in the same manner as the other apoheteropolymers, then Trp at position 93 is essential for redox center formation. Since W93 is located on the outer surface of the ferritin molecule, it is unlikely that the structure of the W93F mutant would be significantly different. Only one Trp is present in native H-subunits and it is highly conserved in all known animal sequences. This conclusion and the observations from Figure 1 are consistent in supporting the view that aromatic amino acids are involved in redox center formation. It is possible, however, that other aromatic amino acids are also involved (Figure 1), and additional studies are needed to fully elucidate the amino acid composition of the redox centers in heteropolymers.

Figure 2 shows also the important result that the heteropolymers prepared from the 222 mutant at 222/rHuLF ratios of 10 (7.8), 12 (11.0), and 15 (14) are redox-active to the extent indicated in the parentheses. This result is significant because the 222 mutant homopolymers have been shown to have an inactive ferroxidase center (due to the alterations E62K and H65G) that is considerably less active than wild-type with respect to iron oxidation during iron uptake (*30*). The iron oxidation rate is comparable to that of rHuLF, which does not have a ferroxidase center. However, the 222 mutant still has Trp 93 present and forms a heteropolymer with rHuLF that is redox-active with similar numbers of redox centers as rHuHF−rHuLF heteropolymers. The retention of full redox reactivity in the absence of ferroxidase reactivity clearly demonstrates that the ferroxidase center and the redox centers are independent and have different functions in ferritin catalysis. While independent

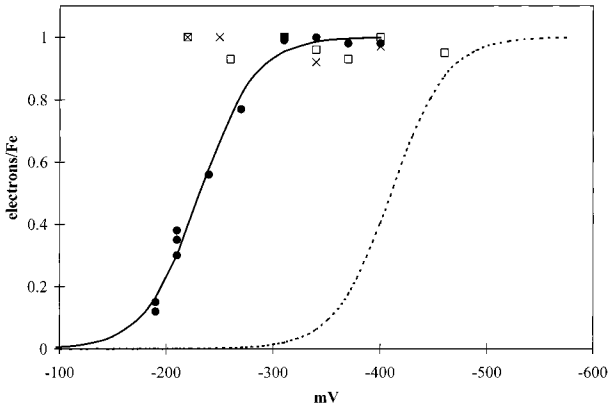

FIGURE 3: Reduction of reconstituted rHuHF, rHuLF, and synthetic heteropolymers. The *y*-axis shows the microcoulometric reduction at pH 7.8 as a function of potential for a reconstituted heteropolymeric ferritin (●) formed from 8 H and 16 L subunits containing 120 iron atoms. A Nernstian redox potential of −230 mV with an $n = 1$ value at pH 7.8 is obtained from the fit to the Nernst equation. Reduction of reconstituted rHuHF (×) and rHuLF (□) at pH 9.0 is shown. The dashed line is the Nernstian reduction curve of native HoSF at pH 9.0 (*24*).

of one another, they are within 12−16 Å of one another and could cooperate, perhaps by transfer of electrons through the richly aromatic region between the ferroxidase center and the redox center, in ferritin-mediated iron redox reactions.

The number of electrons transferred to the indicated apoferritins in Figure 2 has been corrected for any iron atoms present and should represent the actual number of redox centers formed. However, in some cases, the number of redox centers is up to 20% higher than the nominal H-subunit composition. The combined uncertainty in the microcoulometric and iron measurements does not accommodate this large an error, suggesting that Lowry protein measurements may underestimate the concentration of synthetic heteropolymers.

Reconstituted rHuHF, rHuLF, and rHuHF−rHuLF Heteropolymers. The redox potential of a heteropolymer composed of 8 H- and 16 L-subunits reconstituted with 120 Fe was measured at pH 7.8 as shown by the solid line in Figure 3. The data were fit to a well-defined Nernst reduction curve for an $n = 1$ process with a midpoint potential of −230 mV. This reduction potential is near that reported for native HoSF at pH 8.0 (*24*). The dashed line is the Nernstian reduction of native HoSF at pH 9.0. The redox potentials of native and synthetic holoheteropolymers at pH 8.0 are similar, and both show the same variation in reduction potential with pH variation. The results indicate that iron-reconstituted rHuHF−rHuLF heteropolymers behave similarly to native holoheteropolymers with regard to core reduction properties. In contrast, identical measurements carried out on reconstituted rHuLF and rHuHF homopolymers with 120 iron atoms at pH 9.0 showed that core reduction occurred but did not conform to a well-defined Nernstian reduction curve. This is readily seen for reduction of rHuHF and rHuLF at pH 9.0, which should be similar to the dashed redox curve but is not. The midpoint potential has shifted at least 300 mV more positive, which is beyond the limit of the mediators used. This result implies that the observed redox center in native or artificially produced heteropolymers may impart a specificity to the core reduction process by lowering the midpoint potential below that of the homopolymers.

DISCUSSION

In their presumed role as iron storage proteins, ferritins function catalytically during the oxidation of Fe^{2+} to form $Fe(OH)_3$ within their hollow interior and during iron mobilization by reduction of $Fe(OH)_3$ with subsequent release of Fe^{2+}. The mechanism by which these two processes are carried out by ferritins remains poorly understood. Extensive work on the iron deposition process has identified a ferroxidase center within the H-subunit that binds incoming iron and catalyzes its oxidation (*6*). This ferroxidase center is presumed functional during initial iron loading, but once an iron core has formed, a kinetic transition to oxidation on the iron core surface begins, which then serves as a catalytic Fe^{2+} oxidation site (*31*). Oxidation within ferritin by either process presents the interesting problem of how electrons are transferred from these two quite different sites to the protein exterior where redox reagents reside. With small oxidants such as O_2, oxidation presumably occurs by direct reaction with the two iron atoms bound at the ferroxidase site (*2*, *31*, *32*), although details are unclear whether a direct two-electron oxidation step occurs or two sequential one-electron oxidation steps occur. However, in the case of facile iron deposition by large or highly negatively charged oxidants (*21*), penetration into the ferroxidase site or contact with the mineral surface is not likely (*33*−*35*) and an alternate means for transport of electrons to remote oxidants is needed. Such considerations lead to the possibility that the protein may contain redox centers associated with electron transport pathways that function during iron redox reactions by transporting electrons from the ferroxidase site to external reagents.

This possibility was suggested when Watt et al. (*11*) reported that apoHoSF possessed inherent redox reactivity. The location and nature of these redox centers was not determined, nor could they be attributed to a specific subunit type because of the heteropolymeric nature of HoSF. Because redox-active metal ions were excluded and no other chromophore was identified, it was concluded that the observed redox centers were endogenous and formed from amino acid side chains. The unusual optical spectra tailing into the visible region, the anomalous fluorescence (*36*), and the presence of a number of conserved aromatic amino acids suggested that aromatic amino acids might be involved in forming these protein-bound redox centers. The involvement of Trp 93 in the H-subunit, as shown in Figure 2, is consistent with this hypothesis, but whether other aromatic amino acids are involved remains to be determined. It should be mentioned that Trp 93 is also conserved in the L-subunit and may be important in the formation of the redox centers. Although its role in the formation of the redox centers cannot be resolved at this time, future experiments will elucidate its involvement (for other proteins in which Trp residues play a redox role see refs *28* and *37*−*40*).

The results in Table 1 confirm redox activity in naturally occurring and synthetically derived human apoheteropolymers and suggest that the number of such centers correlates with the H-subunit content. However, homopolymeric apo-rHuHF, apo-rHuLF, and selected site-altered recombinant mutant apoproteins are not redox-active, implying that posttranslational modifications or H−L subunit interactions are responsible for the formation of redox centers in native apoheteropolymers. The latter is most likely the case as is demonstrated by formation of redox-active apoheteropolymers from redox-inactive apohomopolymers.

As initially suggested from measurements on naturally occurring apoferritins, the redox centers arise from the H-subunits, but this propensity is only expressed in heteropolymers through interaction with L-subunits as demonstrated in Figure 2. Such results suggest that the redox centers in heteropolymers are formed as a result of interactions along the 2-fold axis of the H−L subunit dimer, in analogy to the heme in BFRs. Trp 93 is located on the surface of the protein at the end of the BC loop and is only about 10 Å from the 2-fold channel. There are numerous aromatic and sulfur-containing amino acids both surrounding Trp 93 and intervening in the 28 Å between the two Trp residues on adjoining polypeptide dimers, which may participate in electron transfer. Combinations of sulfur-containing and aromatic amino acids have been found in other redox-active proteins such as rubredoxin, high-potential iron protein, ferrocytochrome *c*, flavodoxin, and the α-chain of deoxyhemoglobin (*41*). Morgan et al. (*41*) and Moore and Williams (*42*) have suggested possible advantages for the sulfur−π arrangement in redox proteins, two of which are the stepwise movement of electrons and the delocalization of charge on the sulfur. Met and Trp have been implicated as electron acceptors in cytochrome *c* peroxidase (*43*−*46*), while Cys and Trp function in galactose oxidase (*28*), as was mentioned earlier.

In the introduction, the possibility was presented that the redox centers characterized here in animal apoheteropolymers behave in an analogous way to hemes in BFRs, and here we consider this possibility further. In both cases, multiple low-potential redox centers are present, but the exogenous heme groups in BFRs are attached by bis(methionine) ligation and are labile, whereas in animal heteropolymers the redox centers appear to be endogenous and contain aromatic amino acids. The heme in EcBFR is not involved in electron transfer during iron deposition (*16*), but the heme in AvBFR is active in iron release (*18*). Reasoning by analogy, we conclude that the redox centers in animal heteropolymers are not required for iron deposition but might function in iron release. In support of this view, we note that iron deposition in rHuHF is rapid and efficient with O_2 as oxidant, but as we show here, rHuHF does not possess inherent redox centers. The results show that redox centers of the type discussed here are not required for the rapid iron deposition reaction in rHuHF, consistent with EcBFR behavior. Extending the analogy, the heme in AvBFR is directly involved in iron release, and consequently, we might expect that the redox centers in animal heteropolymers are involved in iron release. One observation that seems to support this view is the observation that during the preparation of apoferritin samples, iron release is much slower from ferritin homopolymers than from heteropolymers. Comparison of the iron release reaction has not been extensively studied in heteropolymers containing various levels of H-subunits, and only initial comparisons of iron release between holo-rHuHF and rHuLF have been reported (*26*). In the latter case, rHuHF releases its iron twice as fast as rHuLF, but a direct comparison of these results with heteropolymers containing redox centers has not been carried out; thus the question of redox center involvement in iron release remains unanswered. Furthermore, to dem-

onstrate the involvement of the redox centers during iron release in animal ferritins, as was done for AvBFR (*18*), is not readily accomplished because the weak, poorly defined spectroscopic characteristics of the redox centers in animal heteropolymers preclude such studies.

The above considerations suggest a means to unify ferritin redox reactions in terms of either the exogenous heme groups in BFRs or the endogenous redox centers reported here for animal ferritins. However, the suggested redox behavior may not be as simple as these analogies suggest because both iron deposition and iron release reactions catalyzed by ferritin are more complex than depicted. For example, rHuHF has a ferroxidase center and rapidly deposits iron up to 10 times faster than rHuLF, which does not have a ferroxidase center (*47*, *48*). In the absence of a ferroxidase center, rHuLF still accumulates sizable core sizes of >1000 but does so more slowly, suggesting that an alternate, but slower and perhaps independent, iron deposition pathway is present. The redox centers in heteropolymers may be involved in "gating" the reduction of the iron core. Figure 3 supports this conclusion by showing that the redox centers in heteropolymers are functional during iron reduction by apparently imparting specificity and control to the iron release reaction. The reconstituted synthetic heteropolymer (8 H- and 16 L-subunits) containing approximately eight redox centers produces well-behaved Nernstian redox reactions for core iron reduction, with reduction potentials and a pH dependency similar to those of native HoSF (*24*). On the other hand, the redox behavior of reconstituted rHuHF and rHuLF at pH 9.0 was not comparable to that of HoSF at the same pH nor to that at pH 8.0.

The further characterization of the redox activity of heteropolymer apoferritins may have some link to the ferroxidase center in the H-subunit. Sun et al. (*49*) observed that although rHuHF had a larger k_{cat} for iron oxidation than HoSF (16% H) or native human liver ferritin (4% H), the k_{cat} on a per H-subunit basis was just the opposite. They postulated that this result may be due to the L-chain being more efficient in core nucleation, but it is also possible that these results could be attributed to the inherent redox activity of ferritin heteropolymers. Sun et al. (*49*) also saw different Zn^{2+} inhibition behavior for the different ferritins studied. If the PRC is coupled to the ferroxidase center, the activity per H-subunit could increase due to increased efficiency of transporting electrons away from the ferroxidase center. In addition to the possible functions of these PRCs present in H−L heteropolymers during iron uptake and release, additional implications of this redox activity may apply to the in vivo behavior of ferritin (*2*). It will be interesting to see if the described redox activity has application to ferritin's reported roles in the regulation of oxidative stress (*50*), protein expression (*51*), cellular proliferation (*52*, *53*), and various diseases associated with the disruption of ferritin levels and iron metabolism (*54*, *55*).

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