Tyrosyl Radical Formation during the Oxidative Deposition of Iron in Human Apoferritin†

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ABSTRACT: The radical chemistry of ferritin is incompletely understood. The present study was undertaken to investigate the production of radicals in H-chain recombinant human ferritin (HuHF) and mixed H/L-chain horse spleen ferritin (HoSF) and the potential role of radicals in the oxidative deposition of iron in these proteins. Radical production follows distinct pathways for the two proteins; an intact H-chain ferroxidase site is required for radical generation in both of them, however. With the H-chain HuHF, an EPR spectrum characteristic of a tyrosyl radical is seen following Fe^{2+} oxidation by O_2 and, based on measurements with site-directed variants, is suggested to arise from residue Tyr-34 located in the vicinity of the ferroxidase site. The observation of this radical correlates with the observation of a 400−600 nm absorbance seen in stopped-flow kinetics studies which seems to require the presence of Tyr-34 (Bauminger et al. (1993) *Biochem. J. 296*, 709−714). The data are inconsistent, however, with the Tyr-34 radical being critically important in the protein-catalyzed mechanism of iron oxidation. Unlike HuHF, the radicals observed in L-chain-rich HoSF appear to arise from hydroxyl radical damage to the protein through Fenton chemistry. These latter radicals also appear to be centered on aromatic amino acids and may be derived from histidine.

Ferritin is the principal reservoir for metabolic iron within the cell. The protein consists of a 24mer shell of two types of subunits, H and L, encapsulating a hydrous ferric oxide mineral core (Harrison et al., 1991). During the formation of ferritin from Fe^{2+}, O_2, and apoferritin, iron(II) oxidation takes place initially on dinuclear iron ferroxidase sites located on the H subunit (Hempstead et al., 1994; Lawson et al., 1991; Bauminger et al., 1993; Treffry et al., 1992; Sun et al., 1993; Treffry et al., unpublished experiments). At low fluxes of iron into the protein, hydrogen peroxide is the principal product of dioxygen reduction, with the overall iron(II) oxidation and core formation reaction being given by the following equation (Sun & Chasteen, 1992; Xu & Chasteen, 1991):

$$2Fe^{2+} + O_2 + 4H_2O \rightarrow 2FeOOH_{core} + H_2O_2 + 4H^+ \quad (1)$$

At high fluxes of Fe^{2+} into the protein or in the absence of the H-chain ferroxidase site, iron oxidation and hydrolysis take place directly on the mineral surface of the growing core according to the following reactions, where dioxygen is reduced to water and Fe^{3+} is incorporated into the core (Xu & Chasteen, 1991; Sun et al., 1993; Chen-Barrett, 1994):

$$4Fe^{2+} + O_2 + 4H^+ \rightarrow 4Fe^{3+} + 2H_2O$$

$$4Fe^{3+} + 8H_2O \rightarrow 4FeOOH_{core} + 12H^+ \quad (2)$$

sum:

$$4Fe^{2+} + O_2 + 6H_2O \rightarrow 4FeOOH_{core} + 8H^+$$

Early studies have shown that radicals are formed during iron oxidation in ferritin, but their functional roles, if any, are unknown (Chasteen et al., 1985; Grady et al., 1989). The functional importance of radicals in the redox chemistry of a number of metalloproteins has been increasingly recognized in recent years. Examples include the semiquinone radical of amine oxidase (McCracken et al., 1992) and the tyrosine radicals of galactose oxidase (Whittaker & Whittaker, 1990; Babcock et al., 1992), ribonucleotide reductase (Bender et al., 1992; Stubbe, 1989), prostaglandin synthase (DeGray et al., 1992; Lassmann et al., 1991, 1993), and photosystem II (Barry et al., 1990; Hoganson & Babcock, 1992). The generation of tyrosine radicals during the oxidative deposition of iron in ferritin is of special interest because of recent reports of a purple iron(III)−tyrosinate complex which is rapidly formed during the early stage of iron deposition in bullfrog ferritin (Waldo et al., 1993; Waldo & Theil, 1993). The complex exhibits a visible absorption at 550 nm characteristic of a Tyr → Fe^{3+} charge transfer transition and a resonance Raman spectrum typical of Fe^{3+}−phenolate coordination (Waldo et al., 1993).

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[1] Abbreviations: BES, *N,N*-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid; EPR, electron paramagnetic resonance; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; HoSF, horse spleen ferritin; HoLF, horse L-chain ferritin; HuHF, human H-chain ferritin; MOPS, 3-(*N*-morpholino)propanesulfonic acid; ShSF, sheep spleen ferritin; PBN, *N-tert*-butyl-α-phenylnitrone.

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In the present study, we have further examined radical production in ferritin with the goal of determining whether some of the radicals produced during iron oxidation are tyrosine radicals, at which sites on the protein they are formed, and whether they are important functionally. Previous spin trapping studies with horse spleen ferritin (HoSF) have provided evidence for the production of low levels of radicals in ferritin derived from Fenton chemistry (Grady et al., 1989). More recently, a transient early radical has been observed in HoSF and human H-chain ferritin by rapid-freeze quench EPR spectroscopy (Sun & Chasteen, 1994). This early radical does not appear to be the result of Fenton chemistry, however. It decays away within 2 min, and new, relatively stable, radicals slowly begin to form, with spectral features similar to those of tyrosine radicals. It is these latter radicals which are the subject of the present paper. Experiments were carried out with the recombinant human H-chain protein (HuHF) and various human site-directed variants including Y29F, Y32F, Y34F, Y137F, Q141E, W93F, and 222 (E62K,H65G) as well as with horse and sheep spleen ferritins. EPR signals were observed in HuHF which are attributable to a Tyr-34-based radical located in the vicinity of the H-chain ferroxidase site. Formation of this radical, however, does not appear to be an essential component of the mechanism of iron(II) oxidation. Different radical signals are observed for HoSF and probably arise from hydroxyl radical damage to the protein from Fenton chemistry. Both proteins, however, require intact ferroxidase sites for radical formation to occur.

MATERIALS AND METHODS

Horse spleen ferritin (HoSF) was purchased from Boehringer-Mannheim and sheep spleen ferritin (ShSF) isolated from frozen sheep spleens as described by Mertz and Theil (1983). Recombinant proteins were produced as previously described (Treffry et al., 1989; Levi et al., 1988). The recombinant HuHF and all variants also have the substitution K86Q, which facilitates protein crystallization but does not affect iron uptake (Lawson et al., 1991). Horse L-chain ferritin (HoLF) was a gift of Drs. K. Nagayama and S. Ebina of the Nagayama Protein Array Project, Tsukuba, Japan. Apoferritins were prepared by successive dialysis against dithionite or by reduction with sodium dithionite in an ultrafiltration cell (Bauminger et al., 1991) and typically contained 0.5 Fe/protein or less. The purity and subunit compositions of the ferritins were determined using 12.5% and 17% SDS PAGE (Arosio et al., 1978). All protein concentrations are expressed on a 24mer basis.

EPR measurements were performed on a Varian E-4 or E-9 spectrometer equipped with liquid nitrogen quartz Dewar inserts in TE_{102} or TE_{104} cavities, respectively. The spectrometers were interfaced to an ISA-standard Intel 80486-based computer with data acquisition hardware and software from Scientific Software Services, Bloomington, IL. For the power saturation studies, the leveled power output from the Varian E-101 microwave bridge was checked using a Narda Model 8441 power meter. The power distribution between the two halves of the critically coupled TE_{104} dual cavity, one half containing the quartz Dewar, was measured from the intensity of Varian strong pitch ($I \propto \sqrt{P}$). Eighty-eight percent of the power was distributed to the half of the cavity containing the Dewar.

Unless otherwise stated, protein radicals were generated in the following fashion. Moist Ar gas, freed of trace O_2 using a V(II) scrubber solution, was passed over a 8.8 or 17 μM 24mer apoferritin solution in 50 mM MOPS/0.15 M NaCl, buffer, pH 7.4, with constant stirring for 30−45 min (Chasteen et al., 1991). Microliter quantities of 0.10 M $FeSO_4$ solution, pH <2, were then added to the apoprotein solution to give an Fe^{2+}/protein ratio of 150:1. In the case of sheep spleen apoferritin, a ratio of 200:1 was used. Four minutes after the addition of the Fe^{2+}, moist O_2 (1 atm) was passed over the stirred solution for specified times varying from 40 s to 3 min for HoSF and 40 s to 1 min for HuHF to oxidize the Fe(II). Measurements with an oxygen electrode (Sun & Chasteen, 1992) showed that, after introduction of the 1 atm O_2, the concentration of O_2 in the protein solution reached 0.25 mM (the value for $P_{O_2} = 0.2$ atm) in only 67 s. The sample solution was then transferred to a quartz EPR tube and immediately frozen in a dry ice−acetone bath. EPR measurements were performed at 77 K. The concentration of observed protein radicals was calculated by comparing the double integral of the radical first derivative signal against that of the radical standard (2,2,5,5-tetramethyl-3-pyrrolin-1-yl)oxy-3-carboxamide (Eastman Kodak) of known concentration.

Protein radicals were also produced using the Fe^{2+}/H_2O_2, Fe^{2+}/EDTA/H_2O_2 and VO^{2+}/H_2O_2 hydroxyl radical generating systems. The concentrations of Fe^{2+} and EDTA (1:2) in the final solution were 1.66 and 3.32 mM, respectively. A VO^{2+}/protein ratio of 16/1 was employed with an apoHoSF 24mer concentration of 33 μM. In both cases, a 5 or 10 μL aliquot of 3% H_2O_2 was added as the oxidant to apoprotein (8.8 or 17 μM H_2O_2, respectively) in a MOPS buffer solution volume of 250 μL.

Reduced and oxidized forms of HoSF were prepared by incubating apoferritin with freshly prepared 4 mM sodium dithionite under argon or 4 mM potassium ferricyanide under air for 3−4 h according to the procedure of Watt et al. (1992). The reducing or oxidizing reagents were removed by ultrafiltration with buffer under dinitrogen gas before addition of Fe^{2+} followed by 1 atm O_2.

The free tyrosine radical was produced by irradiating a frozen solution of 10 mM L-tyrosine in 12.5 mM sodium borate buffer, pH 10, contained in a quartz EPR tube at 77 K (Sahlin et al., 1987). A Rayonet photochemical reactor lamp (Model RMR-500) with a maximum output at 254 nm was employed with continuous rotation of the sample in a liquid N_2 quartz Dewar for 2 min.

The spin trap radical samples were generated by incubating apoHoSF with 50 mM *N-tert*-butyl-α-phenylnitrone (PBN) in 0.15 M NaCl/50 mM MOPS buffer, pH 7.4, under an argon flow for 30 min prior to the addition of the Fe^{2+}, followed by either 1 atm O_2 or 10 μL of 3% H_2O_2 in a 250 μL volume. EPR measurements were performed at room temperature in an aqueous solution quartz flat cell. Protein spin trap adducts were separated using an Amicon 3 mL ultrafiltration cell fitted with either UM 5 or XM 300 membranes having MW exclusion limits of 500 and 300 000, respectively.

RESULTS

Figures 1 and 2 show the time course of the 77 K EPR spectra of apoHoSF (16% H-chain, 84% L-chain) and of

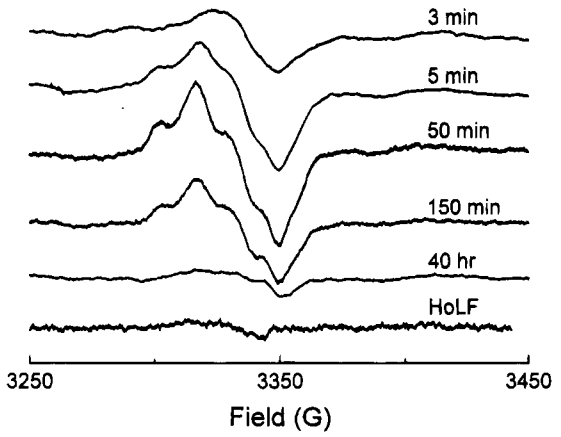

FIGURE 1: EPR spectra of horse spleen ferritin (HoSF) and horse L-chain ferritin (HoLF) radicals in frozen solution with O_2 as the Fe^{2+} oxidant. The same sample was repeatedly frozen and thawed to record the radical signal of HoSF as a function of time. The HoLF spectrum was measured at both 3 and 10 min after introduction of O_2. Conditions: [apoHoSF] = [HoLF] = 17 μM in 0.15 N NaCl/50 mM MOPS buffer, pH 7.4, [Fe^{2+}] = 2.55 mM (150 Fe/protein), [O_2] = 1 atm. Spectral parameters: Field set = 3350 G; scan range = 200 G; time constant = 3 s; power = 20 mW; modulation amplitude = 5 G; scan time = 16 min; temperature = 77 K; microwave frequency = 9.3712 GHz.

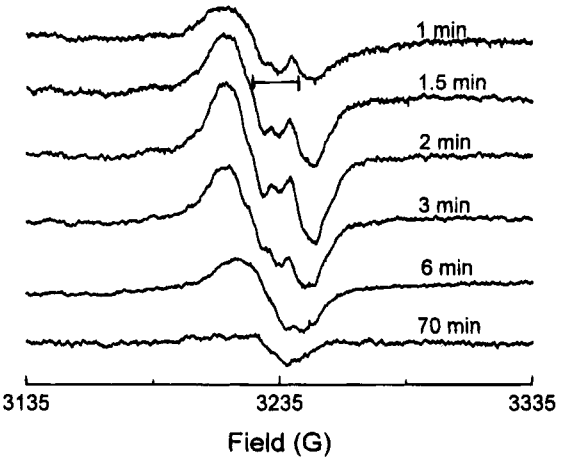

FIGURE 2: EPR spectra of recombinant human H-chain ferritin (HuHF) radical in frozen solution with O_2 as the oxidant. The conditions and spectral parameters are the same as in Figure 1 except [HuHF] = 8.8 μM, field set = 3235 G, and frequency = 9.0639 GHz.

apoHuHF (100% H-chain) when Fe^{2+} is added anaerobically to the protein (150 Fe/protein) in MOPS buffer, pH 7.4, followed by exposure to 1 atm O_2. The same EPR spectra were also obtained at lower Fe loadings of 48 Fe/protein and at pH 6.5 as in previous stopped-flow experiments (Treffry et al., 1993; Bauminger et al., 1993; Treffry et al., unpublished experiments), but the signals were not as intense. Nor was the maximum signal observed as intense when the solution was oxygenated before the addition of Fe^{2+}, implying that prior binding of Fe^{2+} to the protein is needed for radical production (as for apoferritin-catalyzed Fe(II) oxidation). The spectra were independent of buffer used, MOPS, HEPES, or BES. Good's buffers are known to have little effect on the rate of iron(II) autoxidation (Tadolini, 1987). Although HEPES buffer does produce a radical during autoxidation of iron(II) in the absence of protein (Grady et al., 1988), it is different from those observed here with ferritin.

The HoSF apoprotein shell has been shown to contain redox centers of unknown structure which can be cycled between oxidized and reduced states (Watt et al., 1992). To determine whether the redox state of the protein is related to the radicals observed here, the horse apoprotein was oxidized with ferricyanide or dioxygen, or reduced with dithionite (Watt et al., 1992) prior to introduction of Fe^{2+} aerobically. Spectra of the same intensity and lineshape as those shown in Figure 1 were obtained in all three instances; thus, formation of the radicals observed here is independent of the redox status of the apoprotein.

The EPR spectrum of HoSF evolves from the axial radical signal observed previously in rapid-freezing experiments (not shown) (Sun & Chasteen, 1994) to the relatively featureless signal seen at 3 min in Figure 1. Fine structure is evident at 5 min, becoming most resolved at 30−50 min, after which the signal decays. Therefore, in the time frame of the present experiment, at least two radicals are observed, a transient early radical with a featureless signal and a relatively more stable fine structure radical formed later (Figure 1). In the case of sheep spleen apoferritin (55% H-chain, 45% L-chain, ~40% cross-linked dimer), only the transient featureless signal is observed and no fine structure spectrum develops (not shown). The spectrum of HuHF shown in Figure 2 is likewise a composite of at least two types of signals and contains fine structure features also. The spectrum of HuHF also evolves in appearance with time from a fine structure spectrum to a featureless one and ultimately decays away (Figure 2). The EPR signals of both HuHF and HoSF radicals can be regenerated by a second addition of iron (150 Fe^{2+}/protein).

The fine structure spectrum of HuHF (Figure 2) has features similar to those reported for tyrosine radicals in other proteins (Hallahan et al., 1992; Hoganson & Babcock, 1992; Whittaker & Whittaker, 1990; Lassmann et al., 1991, 1993; Bender et al., 1989; King et al., 1967; DeGray et al., 1992; Barry et al., 1990). The spectrum of HuHF has $g = 2.0072 \pm 0.0008$ and partially resolved fine structure splittings of 7−8 G, which we ascribe to the 3,5 protons of the phenol ring of tyrosine. The large coupling of 17−18 G indicated by bars in Figures 2 and 3 is often seen with tyrosyl radicals and is assigned to the β proton of the methylene group (Hoganson & Babcock, 1992; Bender et al., 1989). While the spectrum of HoSF with $g = 2.0065 \pm 0.0005$ (Figure 1) looks similar to that of a tyrosine radical, it has larger fine structure splittings of 12−14 G unlike tyrosine. The spectra of both HoSF and HuHF have peak-to-peak linewidths of ΔH_{PP} = 29−31 G.

Figure 3 compares the EPR spectrum of HuHF with those for the variants Q141E and Y34F, which lack absorbancies in the 400−600 nm range in stopped-flow experiments (Treffry et al., unpublished experiments; Bauminger et al., 1993). The EPR signature of HuHF is absent in the variant Y34F, and only a weak signal is seen, implying that Tyr-34 is the site of radical formation in the human protein. Variants Y29F, Y32F, and Y137F have similar spectra to that of HuHF (Figure 3), precluding residues Tyr-29, Tyr-32, and Tyr-137 as sites of radical production. Variant W93F also exhibits the same spectrum (not shown) as HuHF, precluding Trp-93, which is the only tryptophan residue in ferritin, as the site of radical production. A strong radical signal is observed in variant Q141E, but it lacks the fine structure characteristic of the tyrosine radical of HuHF (Figure 3).

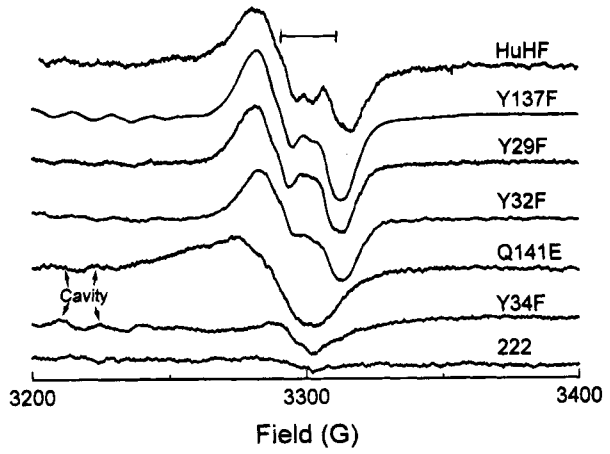

FIGURE 3: EPR spectra of human H ferritin (HuHF) and variants Y137F, Y29F, Y32F, Q141E, Y34F, and 222 (K86Q, E62K, H65G) radicals in frozen solution with O_2 as the oxidant. The solution conditions and spectral parameters are the same as in Figure 1 except field set = 3300 G, frequency = 9.2313 GHz, reaction time = 1.5 min, [HuHF] = 17 μM, [Y137F] = 10 μM (in 0.1 M MOPS, pH 7.1), and [222] = 3 μM with other protein concentrations at 8.8 μM.

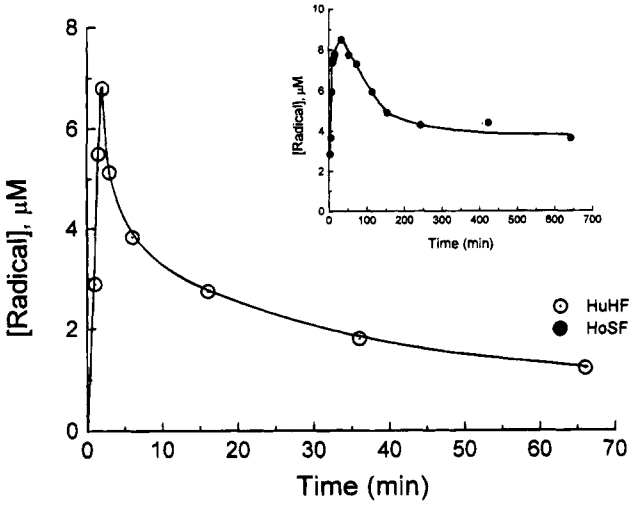

FIGURE 4: Time dependence of radical formation and decay for human H ferritin (HuHF) (⊙) and horse spleen ferritin (HoSF) (●) (inset). The conditions are the same as in Figure 1.

No fine structure features developed when the spectrum of Q141E was monitored as a function of time; the signal simply decayed away. Its origin is unknown. Variant 222 (K86Q, E62K, H65G) in which the putative ferroxidase center ligands Glu-62 and His-65 have been changed also fails to show the tyrosyl radical spectrum (Figure 3, bottom trace), indicating that an intact ferroxidase site is required for generation of radicals.

Figure 4 shows the EPR amplitude as a function of time for both HuHF and HoSF. The HuHF radical signal reaches a maximum in about 2 min and then decays over a period of 1 h, whereas under the conditions of the experiment, oxidation of the Fe^{2+} is complete within only 30 s in both HuHF and variant Q141E. In the case of the more slowly oxidizing HoSF, Fe^{2+} oxidation is complete within 2 min, but formation of the more stable fine structure radical continues for 30 min followed by its slow decay over a period of several hours (Figure 4, inset). For either HuHF or HoSF ferritin, the apparent yield of radicals is low. For HuHF and HoSF the maximal EPR intensity corresponds to 6.8 and

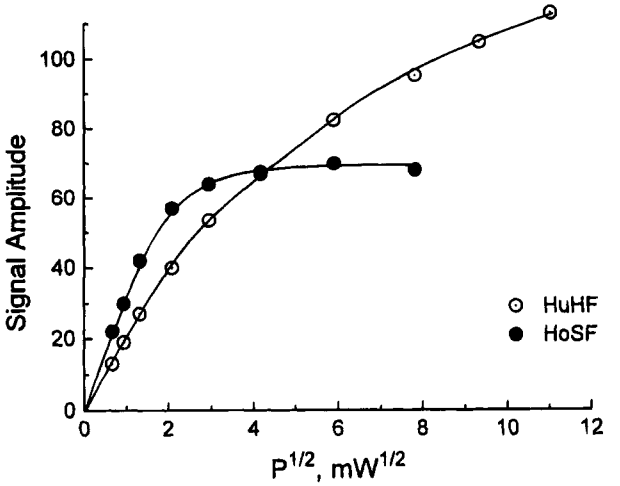

FIGURE 5: EPR microwave power saturation behavior of human H ferritin (⊙) and of horse spleen ferritin (●) radicals. Conditions and spectral parameters are the same as in Figure 1 except [HuHF] = 8.8 μM (150 Fe/protein), field setting = 3300 G, and frequency = 9.2559 GHz. Spectra were measured on samples frozen at 1.5 and 5 min for HuHF and HoSF, respectively, following introduction of 1 atm O_2 into the samples.

8.5 μM radical concentrations, respectively, at a 24mer protein concentration of 17 μM. Thus, only about one radical is formed per two protein molecules or per 300 Fe^{2+} oxidized (150 Fe^{2+}/protein). Because of possible magnetic interactions between iron and the radicals, which could generate EPR-silent species, the above radical concentrations should be considered lower limits to their true values.

The EPR spectrum of HoSF is sufficiently stable to enable ultrafiltration experiments to be done. The EPR signal is observed only with the protein fraction retained by an Amicon XM 300 membrane (MW exclusion limit 300 000), thus identifying the radical as a ferritin species. The radical spectrum is not seen with the horse L-chain homopolymer (Figure 1), demonstrating that the presence of the H-chain, which contains the ferroxidase site (Lawson et al., 1991), is required for its generation. Zn^{2+}, an inhibitor of Fe^{2+} oxidation in horse spleen apoferritin (Sun et al., 1992, 1993; Treffry et al., 1977), was found to decrease the fine structure signal by approximately 50% when added to the apoprotein at a ratio of 50 Zn^{2+}/protein prior to the addition of 50 Fe^{2+}.

Figure 5 shows the microwave power saturation behavior of the fine structure radical signals of HuHF at 1.5 min and HoSF at 5 min. The EPR amplitude (A) as a function of microwave power P (in mW) was fitted to the following equation (Styring & Rutherford, 1988) using the Levenberg–Marquardt algorithm for nonlinear regression:

$$A = KP^{0.5}/[1 + (P/P_{1/2})]^{0.5b} \quad (3)$$

Here $P_{1/2}$ is the microwave power at half-saturation of the EPR signal, K is a proportionality constant, and b is the inhomogeneity parameter (Styring & Rutherford, 1988; Sahlin et al., 1986; de Paula & Brudvig, 1985). A value of $b = 3$ represents a homogeneously broadened first-derivative line and $b = 1$ represents an inhomogeneous derivative line. Values of $P_{1/2} = 12.2 \pm 1.4$ mW and $b = 0.60 \pm 0.02$, and $P_{1/2} = 6.8 \pm 1.2$ mW and $b = 1.23 \pm 0.07$, at 77 K were obtained for HuHF and HoSF, respectively. The b parameters for the ferritins are similar to values reported for radicals in proteins (*e.g.*, Styring & Rutherford, 1988; Beck et al.,

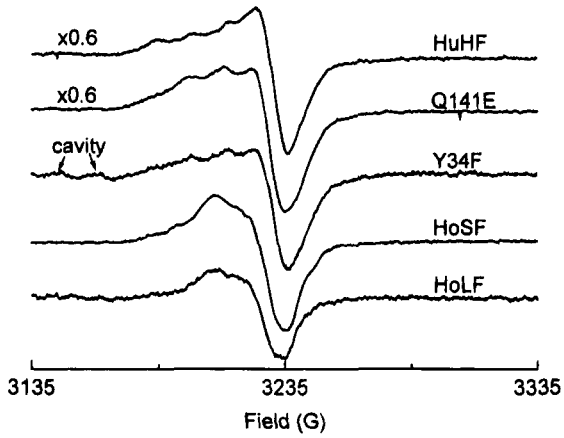

FIGURE 6: EPR spectra of human H ferritin (HuHF), variants Q141E and Y34F, horse spleen ferritin (HoSF), and horse L ferritin (HoLF) radicals in frozen solution with H_2O_2 as the Fe^{2+} oxidant. The conditions are the same as in Figure 1 except 5 μL of 3% H_2O_2 was used as the oxidant instead of O_2 in 250 μL, protein solution. All protein concentrations were 8.8 μM.

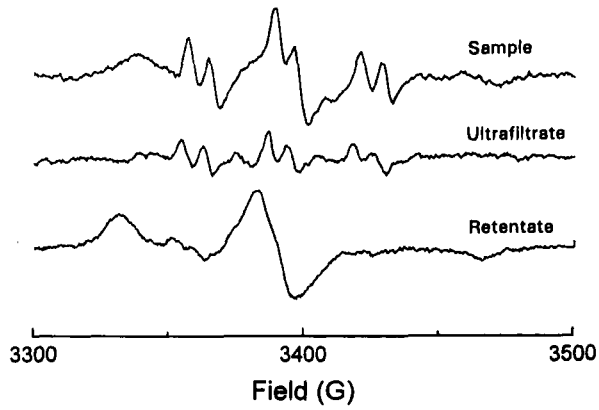

FIGURE 7: Room temperature EPR spectra of PBN spin trapped radicals separated by ultrafiltration. The conditions are the same as in Figure 6 except an anaerobic solution of 17 μM apoferritin was incubated with 50 mM PBN, 0.15 M NaCl, and 50 mM phosphate buffer, pH 7.4, for 30 min prior to the addition of 150 Fe^{2+}/protein for 4 min followed by 1 atm O_2. After 4 min the sample was transferred to an aqueous solution quartz flat cell for EPR measurement. The ultrafiltrate and retentate were separated on an Amicon XM 300 membrane.

1991) and indicate inhomogeneously broadened EPR lines in the frozen solution sample as expected. The $P_{1/2}$ values for HoSF and HuHF are about a factor of 10−20 larger than the value measured for the free tyrosine radical ($P_{1/2} = 0.64 \pm 0.19$ mW, $b = 1.48 \pm 0.17$) under the same conditions (graph not shown). Therefore, the electron spin relaxation rates of the radicals in the ferritins are considerably enhanced relative to that of the free tyrosine radical.

Experiments were conducted to establish whether the observed radicals in HuHF or HoSF are a consequence of Fenton chemistry (Graf et al., 1984; Grady & Chasteen, 1990; Grady et al., 1989). H_2O_2 produced during dioxygen reduction in ferritin (eq 1) can, in the presence of unreacted Fe^{2+}, lead to the Fenton reaction:

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^{\bullet} + OH^{-} \qquad (4)$$

The production of hydroxyl radical would then lead to the formation of secondary protein radicals (Davies et al., 1991). However, when H_2O_2 was used directly as the Fe^{2+} oxidizing agent under anaerobic conditions, the EPR spectra obtained with HuHF and variants Q141E, Y34F, and 222 (Figure 6) and with variants Y32F and Y29F (not shown) were all very similar to one another, with fine structure splittings of 12−14 G (as for HoSF), but distinctly different from those seen with O_2 as the oxidant (*cf.* Figures 2 and 6). In a control experiment, no radical signal was detected when H_2O_2 was added to the protein solution in the absence of iron. These observations suggest that the tyrosyl radicals observed with the human protein are not simply the products of Fenton chemistry.

In the case of the horse protein, however, Fenton chemistry does appear to play a role in radical production. In most experiments the HoSF spectra obtained with H_2O_2 contained fine structure features superimposable on those obtained with O_2 (*cf.* Figures 1 and 6). There is an underlying signal in Figure 6 which is not present in Figure 1; however, alternative use of H_2O_2 or O_2 at times resulted in *identical* fine structure spectra such as in Figure 1. Thus, H_2O_2 produced from O_2 during the ferroxidation reaction 1 appears to be the iron(II) oxidant involved in radical formation in HoSF through the Fenton reaction 4. Vanadyl ion, VO^{2+}, which is also known to promote Fenton chemistry (Carmichael, 1990) and bind to ferritin (Chasteen & Theil, 1982; Wardeska et al., 1986), likewise produces an H_2O_2 spectrum with apoHoSF similar to that seen with Fe^{2+} in Figure 6. When Fe^{2+} was added as the EDTA complex using either O_2 or H_2O_2 as the oxidant (Smith et al., 1990; Tadolini, 1987), only a weak EPR signal unlike those of either Figure 1 or 6 was observed (not shown), implying that Fe^{2+} binding to specific sites on the apoprotein is a requirement for formation of the HoSF-based radicals seen here.

Spin trapping experiments were also performed with HoSF to look for especially reactive radicals. Figure 7 shows the room temperature EPR spectrum obtained when Fe^{2+} was introduced to the apoprotein in the presence of the trapping reagent *N-tert*-butyl-α-phenylnitrone (PBN). The signals are enhanced 3−5-fold when H_2O_2 is used in place of O_2 as the oxidant, consistent with the trapped radicals being secondary radicals arising from hydroxyl radical damage to the protein. The top spectrum in Figure 7 is a superposition of two types of EPR signals, an anisotropic signal from a slowly tumbling PBN radical adduct, exhibiting an apparent anisotropic nitrogen splitting of $2a_{||} = 67.2$ G, and an isotropic signal from a rapidly tumbling of PBN adduct, exhibiting isotropic nitrogen and hydrogen splittings of $a_N = 16.28 \pm 0.09$ G, $a_H = 3.62 \pm 0.09$ G ($n = 5$). The two adducts can be separated by ultrafiltration using an Amicon XM-300 membrane, as shown by the spectra in Figure 7. The low molecular weight adduct is partially retained by a UM 5 membrane (MW exclusion limit ~500) (data not shown).

In an attempt to identify the radicals, the a_N and a_H splittings were checked against the parameters of known PBN adducts listed in the STDBII data base (Li et al., 1988), but no suitable match was found. Because of their high reactivity, the radicals captured by the spin trapping reagent probably are not the same as the relatively stable fine structure radicals observed directly by EPR (Figure 1).

DISCUSSION

Evidence for tyrosyl radical production during the oxidative deposition of iron in ferritin is presented here for the

first time. The central role of the H-chain ferroxidase site in radical formation in both HuHF and HoSF is underscored by the fact that neither the human H-chain variant 222 nor the horse L-chain homopolymer, both of which lack ferroxidase sites, produces radical spectra (Figures 1 and 3). The identification of Tyr-34 as the probable radical site in the human protein is consistent with its close proximity to the ferroxidase site (Bauminger et al., 1993) and with the retention of the characteristic tyrosyl radical EPR spectrum when other tyrosine residues, Tyr-29 and Tyr-32 nearby in the sequence as well as Tyr-137 also near the ferroxidase site, are changed.

The fine structure pattern, resolution, *g* factor, and time evolution of tyrosine radical spectra are known to vary considerably among proteins, depending on the protein environment of the radical and also on the rotational conformation of the phenolate ring relative to the β protons of the methylene carbon (Bender et al., 1989; Babcock et al., 1992; DeGray et al., 1992; Lassmann et al., 1991, 1993; Barry et al., 1990; Hoganson & Babcock, 1992). The time evolution of the EPR spectrum of HuHF in Figure 2 closely resembles that of the tyrosyl radical seen in the heme enzyme prostaglandin H synthase (PHS) with its substrate arachidonic acid (Lassmann et al., 1991). In both PHS and HuHF, the spectrum evolves from one having fine structure to a relatively featureless one. The fine structure of the HuHF spectrum is most pronounced at 2−3 min (Figure 2) and consists of overlapping signals from at least two radicals, both apparently associated with Tyr-34. Perhaps the two radicals only differ from one another in the orientation of the phenol ring relative to the benzylic hydrogens as proposed for PHS (Lassmann, et al., 1991). The radical in PHS does not appear to be kinetically important in the catalytic cycle of this enzyme (Lassmann et al., 1991). The same seems to be true for ferritin (*vide infra*).

Variants Y34F and Q141E, both of which catalyze the oxidation of Fe(II) at rates within a factor of 2 of that of the wild-type protein, do not show visible absorption spectra attributable to Fe(III)−tyrosinate complexes (Bauminger et al., 1993; Treffry et al., 1993; Treffry et al., unpublished experiments), nor do they produce the tyrosyl radical spectrum (Figure 3). Thus, the observed tyrosyl radical, rather than having a critical role in iron oxidation, seems more likely to be the result of a side reaction, perhaps involving reaction of superoxide with the protein. Some radical production might be anticipated in ferritin if, as proposed, iron(II) oxidation occurs in one-electron steps (Sun & Chasteen, 1992, 1994; Sun et al., 1993; Bauminger et al., 1989).

The presence of more than one pathway for radical production in the ferritins is illustrated by the different spectra seen for horse and human proteins and their distinctive time evolution profiles (Figures 1−4). The difference in radical chemistry between the two proteins may be related to the greater number of ferroxidase sites in the H-chain human protein (24 *vs* 3−4 for HoSF), thus minimizing nonspecific Fe^{2+} oxidation reactions such as the Fenton reaction which appears to be more prevalent in HoSF than in HuHF. The large number of L-chains (~20) in the horse protein may also participate in radical reactions not possible in HuHF. If tyrosyl radicals are formed in HoSF, their EPR signals must be obscured by the stronger signals of the Fenton chemistry-derived radicals.

One anticipates that the radical reaction pathway observed with HoSF will depend on the relative rates of production of O_2^- and H_2O_2 (from O_2 and Fe^{2+}) and their corresponding disproportionation reactions as well as their reactions with Fe^{2+}. The myriad of possible reactions between oxy radicals and ferritin may contribute to its conversion to hemosiderin. The radical chemistry observed here perhaps reflects another role for ferritin in the detoxification of oxy radicals and containment of iron-catalyzed radical reactions within the protein, thus minimizing radical damage to the cell (Grady & Chasteen, 1990; Grady et al., 1989). It is also possible that another oxidant, yet to be identified in the cell, plays a role in iron oxidation, thus diminishing some of the radical chemistry observed in the experiments with O_2 reported here.

The fine structure spectrum of HoSF with splittings of 12−14 G (Figure 1) is similar in shape to that of the radical seen for manganese protoporphyrin IX prostaglandin H synthase, which has splittings of ~11 G (Lassmann et al., 1991), although the two proteins differ in *g* factors, 2.0065 *vs* 2.0042. Such large splittings are inconsistent with those of known tyrosyl radicals (Lassmann et al., 1991). The extended lifetime of the HoSF radical (Figure 4) and its multilined hyperfine pattern are, however, in accord with it being an aromatic radical and, if not from tyrosine, then possibly from histidine, phenylalanine, or tryptophan. Histidine-based radicals have been reported to have similar EPR lineshapes to those of tyrosine radicals (King et al., 1967; Boussac & Rutherford, 1992).

It is interesting that, in both HuHF and HoSF, the radical EPR signal reaches a maximum well after oxidation is complete. Slow growth in the EPR spectrum, especially in the case of HoSF, may be due to a quenching of the EPR signal from nearby Fe^{3+} prior to its migration to core. The greater difficulty in saturating the radical spectra of HoSF and HuHF (Figure 5) relative to free tyrosyl radical suggests that some Fe^{3+} remains close to the radical center following oxidation (or that the radicals are formed near one another). Relaxation enhancement of EPR signals of tyrosine radical from nearby Fe^{3+} has also been observed with ribonucleotide reductase where weak coupling between the radical and the excited $S = 1$ state of the antiferromagnetically coupled $Fe^{3+}-Fe^{3+}$ dimer occurs (Sahlin et al., 1987; Beck et al., 1991). In HuHF a similar situation may exist between the tyrosyl radical and the dimeric $Fe^{3+}-Fe^{3+}$ complex of the ferroxidase site.

In conclusion, from the present and previous work it is evident that the radical chemistry of the ferritins is complex (Grady et al., 1989; Sun & Chasteen, 1994, Chasteen et al., 1985). While Fenton chemistry and other redox reactions, perhaps involving superoxide, appear to play a role in radical production in ferritin, no functionally essential radical has been identified to date. Possible functionally important radicals which remain to be investigated include the transient early radical seen in rapid-freeze quench EPR experiments (Sun & Chasteen, 1994) and strongly spin-coupled radicals such as $Fe^{3+}-O_2^-$ not observable by EPR.

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