Identification of the EPR-Active Iron–Nitrosyl Complexes in Mammalian Ferritins†

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ABSTRACT: This study was undertaken to characterize the nitric oxide complexes of mammalian ferritin and their EPR properties to gain a better understanding of the interaction of NO with non-heme iron proteins within the cell. Measurements were made with horse spleen apo- and holoferritins, with chemically modified proteins, and with recombinant human H-chain apoferritin and its site-directed mutants. Three types of EPR signals (A, B, and C) have been identified and attributed to iron–nitrosyl complexes at imidazole groups of histidine, thiol groups of cysteine, and carboxylate groups of aspartate and glutamate, respectively. The C-type axial spectrum has features at $g_\perp' = 4$ and $g_\parallel' = 2$ characteristic of a paramagnetic Fe^{3+}–NO^- complex with total spin $S = {}^3/_2$ and probably arises from nonspecific binding to carboxylate groups on the protein. The $S = {}^1/_2$ axial B-type signal ($g_\perp' = 2.033$ and $g_\parallel' = 2.014$) is formed at Cys-130 (human H-chain sequence numbering). His-128 and possibly His-118 are sites of formation of the rhombic $S = {}^1/_2$ A-type complex ($g_x' = 2.055$, $g_y' = 2.033$, and $g_z' = 2.015$); the former residue perhaps plays a role in the conformational stability of the protein as well as in iron binding. The data reveal that the residues Cys-130 and His-128 in the vicinity of 3-fold channels leading to the interior of the protein shell are important in iron–nitrosyl complex formation in mammalian ferritins.

Nitric oxide (NO) is produced in a variety of mammalian cells and may serve as a cytotoxic effector, neurotransmitter, immune regulator, and endothelium-derived relaxing factor (EDRF)[1] (Henry et al., 1991; Stamler et al., 1992; Culotta & Koshland, 1992; Traylor & Sharma, 1992; Stadler et al., 1993). This small molecule has one unpaired electron and can bind Fe(II) to form an iron–nitrosyl complex that is EPR-active. EPR signals from such complexes have been detected in both normal and tumor cells (Lancaster & Hibbs, 1990; Pellat et al., 1990; Drapier et al., 1991; Henry et al., 1991; Lancaster et al., 1992; Kosaka et al., 1992; Stadler et al., 1993; Henry et al., 1993). The EPR spectra of liver cells and of ferritin in the presence of NO are strikingly similar (Drapier et al., 1991; Stadler et al., 1993), and it seems likely that some EPR signals in whole cells are due to the formation of iron–nitrosyl complexes with non-heme iron proteins, such as ferritin. Ferritin is the major reservoir for non-heme iron in the cell (Theil, 1989; Harrison & Lilley, 1989). It has also been reported that nitric oxide may mediate iron release from ferritin (Reif & Simmons, 1990) and that NO may modulate the RNA-binding activities of the iron regulatory factor (IRF) (Drapier et al., 1993; Weiss et al., 1993), which regulates the translation of mRNA for ferritin and destabilizes the mRNA for the transferrin receptor (Theil, 1993). Therefore, the characterization of the iron–nitrosyl complexes formed in ferritin is of considerable interest.

Ferritins are a class of iron storage and detoxification proteins found widely in bacteria, fungi, plants, and animals (Theil, 1989; Harrison & Lilley, 1989; Harrison et al., 1991). Horse spleen ferritin is the most frequently studied ferritin, and the three-dimensional structures of the horse spleen apoferritin and the human recombinant H-chain protein have been determined by X-ray crystallography (Rice et al., 1983; Ford et al., 1984; Lawson et al., 1991). The protein consists of a hollow apoprotein shell with a molecular weight of about 480 000 with a central iron core in the form of a ferric hydroxy phosphate. Up to 4500 iron atoms can be stored in the central cavity of ferritin (Clegg et al., 1980a,b). The apoferritin shell has 24 subunits, assembled as 12 dimers, arranged in a nearly spherical structure with the space group *F*432 in the cubic crystal system (Harrison, 1959; Banyard et al., 1978). This molecular architecture provides six 4-fold hydrophobic channels and eight 3-fold hydrophilic channels leading to the interior of the shell (Rice et al., 1983; Ford et al., 1984; Harrison et al., 1986). The 24 subunits can be further classified as H-subunits (heavy) and L-subunits (light) with apparent molecular weights of about 21 000 and 19 000, respectively, on SDS–PAGE (Arosio et al., 1978). Putative ferroxidase and nucleation sites involved in the formation of the iron core have been identified [Lawson et al. (1991) and references cited therein].

A $g' = 2$ EPR spectrum of iron–nitrosyl complexes of horse spleen ferritin and $g' = 2$ and $g' = 4$ spectra of bacterioferritin have been reported recently (Drapier et al., 1991; Le Brun et al., 1993a). However, the nature of the iron–nitrosyl complexes responsible for the signals is poorly understood. In the present article, we further characterize the EPR spectra of mammalian ferritin and elucidate the sites of iron–nitrosyl complex formation in the protein. The results show the presence of both $S = {}^1/_2$ and $S = {}^3/_2$ complexes in mammalian ferritin. Iron–nitrosyl complexes with the thiol groups of

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[1] Abbreviations: EDRF, endothelium-derived relaxing factor; HoSF, horse spleen apoferritin; rHF, recombinant human liver H-chain apoferritin; IRF, iron regulatory factor; EDTA, ethylenediaminetetraacetic acid; TGA, thioglycolic acid; PMB, *p*-(chloromercuri)benzoate; DEP, diethyl pyrocarbonate; MOPS, 3-(*N*-morpholino)propanesulfonic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid.

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cysteine and the imidazole groups of histidine residues in the vicinity of the 3-fold channels are responsible for the two types of $S = {}^1/_2$ species observed; one with an axial **g**-tensor (the Cys sites) and the other with a rhombic tensor (the His sites). Nonspecific binding of Fe^{3+}–NO^- to the carboxylate groups of aspartate and glutamate residues of the protein gives rise to the $S = {}^3/_2$ species.

MATERIALS AND METHODS

Materials. All chemicals were reagent grade and used without further purification unless otherwise indicated. Horse spleen ferritin (cadmium-free) was obtained from Boehringer Mannheim. Thioglycolic acid (TGA), sodium acetate, sodium chloride, sodium nitrite, ascorbic acid, *p*-(chloromercuri)-benzoate (PMB), diethyl pyrocarbonate (DEP), and glycerol were purchased from Aldrich Chemical Company, Inc.; ferrous sulfate heptahydrate, sulfuric acid, and disodium EDTA dihydrate were from J. T. Baker Inc.; copper nitrate atomic absorption standard and ethylenediamine were from Fisher Scientific Company; MOPS and MES buffers were from Research Organics, Inc.; imidazole was from Sigma Chemical Co.; and acetic acid was from VWR Scientific.

Preparation of Apoferritin and Recombinant H-Chain Ferritin and Its Mutants. The apoferritin was prepared by dialysis against TGA as described previously (Hanna et al., 1991a). The concentration of horse spleen apoferritin was determined by its absorbance at 280 nm, with molar absorptivity $\epsilon = 1.95 \times 10^4$ cm^{-1} M^{-1} per subunit (Heusterspreute & Crichton, 1981). The amounts of H- and L-subunits in horse spleen ferritin were determined to be 15% H and 85% L by scanning the Coomassie Blue stained SDS–PAGE gels with a densitometer (Arosio et al., 1978; Sun et al., 1993).

Recombinant H-chain ferritin and its mutants were prepared as described previously (Levi et al., 1988). The iron was then removed by dialysis against 1% TGA in 0.1 M sodium acetate at pH 5.5 for 24 h followed by dialysis against 0.1 M MOPS/0.1 M NaCl solution, pH 7.0 (Levi et al., 1988). The protein concentrations were determined by Bio-Rad protein assay using bovine serum albumin as a standard. The absorbance maximum of Coomassie Brilliant Blue G-250 when binding to protein was measured at 595 nm on a Cary 219 spectrophotometer.

Additions of Fe(II) and Gaseous Nitric Oxide. Ferrous ion was added anaerobically to apoferritin as 50 or 150 mM $FeSO_4 \cdot 7H_2O$ (pH 2.0). The protein solution was placed in a microcentrifuge tube or a glass vessel fitted with a septum and stirred under an argon atmosphere. Nitric oxide gas was produced by dripping 1 M ferrous sulfate heptahydrate in 2 N H_2SO_4 solution into a 1 M sodium nitrite stock solution under argon (Blanchard, 1946). The evolved NO gas was passed over the stirred anaerobic protein solution. All containers and EPR tubes were flushed with argon and fitted with septa. Solution transfers were made with gas-tight syringes, and EPR samples were immediately frozen in a dry ice/acetone bath.

Chemical Modification. The thiol groups of cysteine residues in apoferritin were modified by the addition of 1 equiv of PMB per subunit to 20 μM apoferritin in 0.15 M MOPS/0.1 M NaCl (pH 7.0) buffer. After a 2-h incubation period, the concentration of bound PMB was determined by the absorbance at 232 nm ($\epsilon = 1.65 \times 10^4$ cm^{-1} M^{-1}) (Stefanini et al., 1989). Modification of histidines was performed by adding a 4.8-fold molar excess of DEP over the histidine content of the L-subunit to 20 μM apoferritin in 0.15 M MOPS/0.1 M NaCl buffer at pH 7.0. After a 30-min incubation, the number of modified histidines was determined from the molar absorption difference at 242 nm with $\epsilon = 3.2 \times 10^3$ cm^{-1} M^{-1} (Ovádi et al., 1967). The concentration of stock DEP was determined by the absorbance at 230 nm, using the molar absorptivity 3.0×10^3 cm^{-1} M^{-1} (Melchior & Fahrney, 1970).

EPR Spectroscopy. EPR spectra were obtained at 77 K using a Varian E-4 spectrometer with a TE_{102} rectangular cavity and a quartz liquid nitrogen Dewar insert. All EPR measurements were performed with calibrated quartz EPR tubes. Data manipulations and EPR spectral simulations were conducted on a 486 computer using the programs EPRWare (Scientific Software Services) and EPRPOW (White & Belford, 1976), respectively. The *g*-factors were determined from the microwave frequency and magnetic field at resonance and relative to the known *g*-factor of strong pitch ($g_s = 2.0028$).

The spin concentration of EPR-active iron–nitrosyl complexes in apoferritin was determined by comparing the EPR double integral with that of 1 mM copper nitrate/3 mM EDTA in a 25% glycerol/75% water mixture (pH 3.5). The appropriate *g*-factor correction was made to the EPR intensities (Aasa & Vänngård, 1975).

RESULTS

Iron–Nitrosyl Complexes in Ferritin. The EPR spectra of horse spleen apoferritin (HoSF) with 120 Fe^{2+}/molecule added to the 24-mer protein under argon followed by flushing with gaseous nitric oxide for 5 min at pH 6.0 and 7.0 are shown in Figure 1a,b, respectively. Three types of EPR signals, A, B and C, are evident. The A- and B-type signals occur near $g' \approx 2$ and overlap with one another; the C-type signal occurs at $g' = 4$ with a weaker component at $g' = 2$, the latter being obscured by the A- and B-type signals.[2] The EPR amplitude of the $g' = 4$ signal is twice as large at pH 6.0 (Figure 1a) as at pH 7.0 (Figure 1b). In contrast, the $g' = 2$ A- and B-type signal is favored by the higher pH and is 5 times more intense at pH 7.0 (note that the instrument gain is different). Both the $g' = 2$ and $g' = 4$ signals increase with further incubation of the protein with NO and reach a maximal intensity after 10–15 min. A 5-min incubation period was typically used for most experiments since longer incubation periods often were observed to cause some precipitation in the protein sample.

The C-Type Signal. The C-type spectrum has features at $g_\perp' = 4$ and $g_\parallel' = 2$ and is characteristic of an $S = {}^3/_2$ paramagnetic complex. The transfer of one electron from Fe(II) to NO causes the formation of an Fe^{3+}–NO^- complex as evidenced by X-ray absorption edge spectra (XAS) and normal coordinate calculations based on resonance Raman vibrations for FeEDTA–NO and other model complexes (Zhang et al., 1992), by Mössbauer data from the putida-monooxin·4-methoxybenzoate·NO complex (Bill et al., 1985), and by the result that the addition of ferricyanide under anaerobic conditions to the former complex does not change the EPR spectrum, which indicates that the iron in the complex cannot be further oxidized and remains in the Fe(III) state

[2] In control experiments when Fe^{2+} was added to apoferritin under aerobic conditions, no $g' = 4$ and $g' = 2$ signals (A-, B-, and C-type) were observed, and only a single resonance at $g' = 4.3$ from mononuclear Fe^{3+} was seen (data not shown). The $g' = 4$ and $g' = 2$ signals can be generated by the addition of ascorbate to the deaerated protein solution in the presence of NO, reducing the Fe(III) back to Fe(II) with concomitant loss of the $g' = 4.3$ signal.

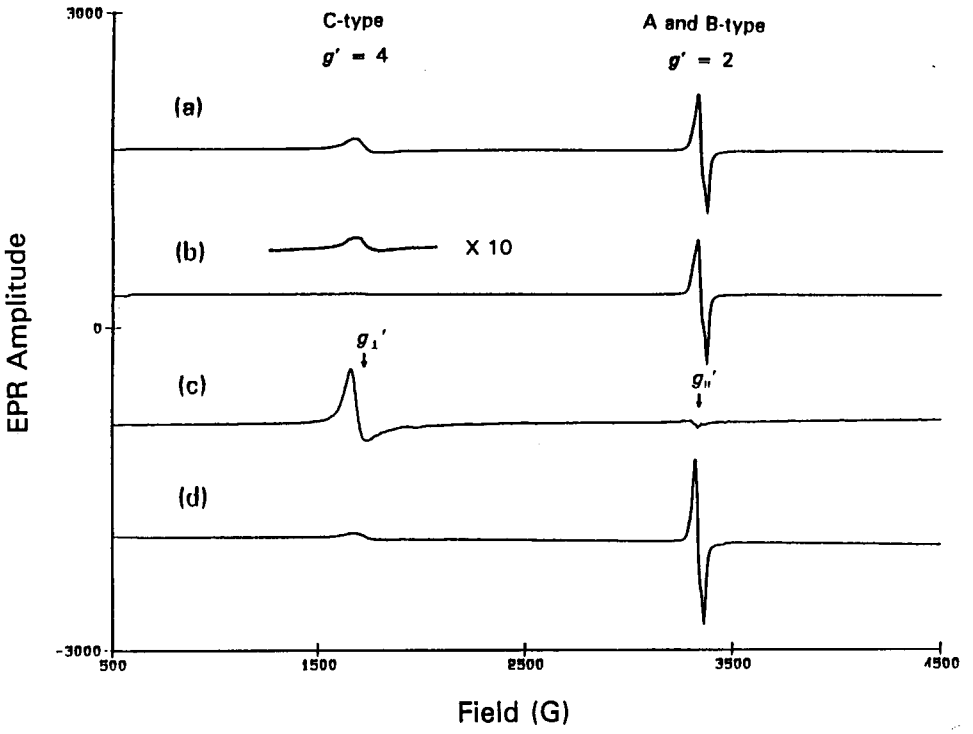

FIGURE 1: (a) EPR spectrum of HoSF (20 μM) with 120 Fe^{2+}/molecule flushed with NO for 5 min under anaerobic conditions in 0.1 M MES/0.1 M NaCl buffer, pH 6.0. (b) Same as a except in 0.15 M MOPS/0.1 M NaCl buffer, pH 7.0. (c) EPR spectrum of 0.5 M acetate with 120 Fe^{2+}/molecule flushed with NO for 5 min under anaerobic conditions at pH 6.0. (d) EPR spectrum of horse spleen holoferritin (20 μM) with 60 Fe^{2+}/molecule flushed with NO for 5 min under anaerobic conditions at pH 6.0. Spectrometer settings: microwave power, 10 mW; frequency, 9.373 GHz; modulation amplitude, 5 G; time constant, 0.3 s; receiver gain, 500 (a), 125 (b), 2000 (c), and 620 (d); temperature, 77 K.

(Twilfer et al., 1985). The strong antiferromagnetic coupling of the high-spin Fe(III) ($S = {}^5/_2$) with NO^- ($S = 1$) yields a total spin of $S = {}^3/_2$. The $S = {}^3/_2$ spin Hamiltonian for such a system is given by

$$\hat{H} = g_0\beta\vec{B}\cdot\vec{S} + D(S_z^2 - {}^5/_4) + E(S_x^2 - S_y^2) \qquad (1)$$

where $g_0 = 2.0$, and D and E are axial and rhombic zero-field splitting constants, respectively. When $h\nu \ll 2D$, $2D$ being the energy separation between the $|\pm{}^3/_2\rangle$ and $|\pm{}^1/_2\rangle$ Kramers doublets, the transitions within the $|\pm{}^1/_2\rangle$ Kramers doublet in the axial ligand field give rise to signals at $g_\perp' = 4$ and $g_\parallel' = 2$ (Salerno & Siedow, 1979), as observed here (Figure 1). From the EPR line width, we estimated $g_y' - g_x' \leq 0.14$ and, using the first-order perturbation equation $g_y' - g_x' = 12E/D$, a rhombicity parameter $E/D \leq 0.012$ (Arciero et al., 1985; Petrouleas & Diner, 1990; Trautwein et al., 1991). Thus, the $S = {}^3/_2$ metal center has axial or nearly axial magnetic symmetry.

Quantification of the EPR-active Fe^{3+}–NO^- species responsible for the C-type spectrum is complicated by the fact that part of the spectrum overlaps with the A- and B-type signals near $g' \approx 2$. Therefore, the $S = {}^3/_2$ EPR spectral envelope was quantified by spectral simulation, as has been done for $S = {}^5/_2$ soybean and human lipoxygenases to determine the concentration of spins in these Fe(III) proteins (Slappendel et al., 1981; Chasteen et al., 1993). The total spin population, N_{total}, of the two Kramers doublets, $|\pm{}^3/_2\rangle$ and $|\pm{}^1/_2\rangle$ of the $S = {}^3/_2$ spin manifold of states, is given by the Boltzmann factor:

$$N_{total} = N_{\pm 1/2}[1 + \exp(-2D/kT)] \qquad (2)$$

At 77 K, the axial zero-field splitting constant D is much smaller than kT, and a Boltzmann factor of 2 ($N_{total} = 2N_{\pm 1/2}$) was used to calculate the total spin concentration responsible for the C-type spectrum. A typical spin concentration equivalent to 25% of the total iron in the sample was found for the C-type spectrum at pH 6.0. A titration of the spin concentration of the C-type species against the total Fe^{2+} added to the sample was made at pH 6.0. A linear relationship was obtained up to 480 Fe^{2+} added per 24-mer apoferritin, corresponding to 120 EPR-observable Fe^{3+}–NO^- species per 24-mer protein molecule. The relatively high level of EPR-active Fe^{3+}–NO^- species (120 $S = {}^3/_2$ spins/24-mer apoferritin molecule) detected under these experimental conditions indicates the presence of multiple binding sites for the Fe^{3+}–NO^- complex in apoferritin.

The C-type spectrum observed in Figure 1a with apoferritin appears to be due to the binding of Fe^{3+}–NO^- at carboxylate groups on the protein. This assignment is supported by our observation that the C-type spectrum is readily observed at pH 5, which is above the pK_a typical of carboxylate groups, and by the report of an EPR spectrum with g values of 4.11, 3.95, and 2.00 from the FeEDTA–NO complex (Rich et al., 1978). Moreover, the simple addition of Fe^{2+} to an anaerobic sodium acetate solution at pH 6.0 under NO also generates a C-type spectrum with signals at $g_\perp' = 4$ and $g_\parallel' = 2$ (Figure 1c). No C-type signals were observed in similar experiments with *non*-carboxylate ligands such as ethylenediamine and imidazole or with the buffers employed in the protein experiments (data not shown). The amplitude of the $g' = 4$ signal decreased by approximately 2-fold when either protein or acetate solution (pH 6.0) was exposed to air for 1 min, but no change in line shape was observed, implying that the Fe^{3+}–NO^- complex was rendered an EPR-silent species in the presence of dioxygen, perhaps through replacement of NO^- by O_2^- (Twilfer et al., 1985) or oxidation of the Fe^{3+}–NO^-

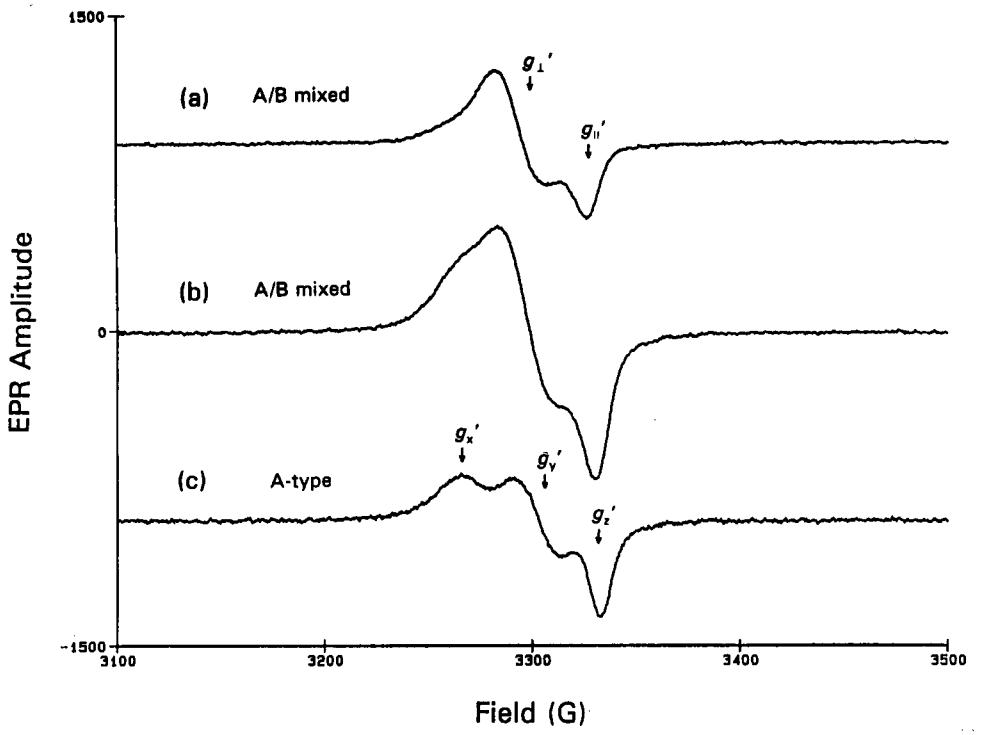

FIGURE 2: (a) EPR spectrum of HoSF (20 μM) with 120 Fe^{2+}/molecule flushed with NO for 5 min under anaerobic conditions at pH 6.0 ($g_\perp' = 2.033$ and $g_\parallel' = 2.014$). (b) Same as a except flushed with NO for 15 min. (c) EPR difference spectrum, b – a (1:1), $g_x' = 2.053$, $g_y' = 2.029$, and $g_z' = 2.011$. Other conditions are given in the legend to Figure 1. Spectrometer settings: microwave power, 10 mW; frequency, 9.372 GHz; modulation amplitude, 1 G; time constant, 0.3 s; receiver gain, 1500; temperature, 77 K.

complex followed by formation of oligonuclear Fe^{3+} species. In the case of the Fe^{3+}–NO^- protein complex, no $g' = 4.3$ signal from mononuclear Fe^{3+} is generated when O_2 is admitted to the solution.

Figure 1d shows the EPR spectrum of a mixture of holoferritin and Fe^{2+} under NO at pH 6.0. A $g' = 4$ signal with essentially the same amplitude as that seen in the spectrum of the apoprotein plus Fe^{2+} and NO (Figure 1a) is observed.[3] These results indicate that the binding sites associated with carboxylate groups are accessible to the Fe^{2+} in this instance, implying that the presence of an iron core does not block the formation of C-type Fe^{3+}–NO^- complexes in the holoprotein.

Site-directed mutants were employed to identify the region(s) of the protein responsible for the C-type spectrum. Four recombinant human H-chain apoferritin (rHF) mutants were examined: mutant 222 in which the putative ferroxidase site ligands Glu-62 and His-65 are altered (E62K, H65G, and K86Q); mutant A2 in which all three carboxylate groups of the putative nucleation site are changed (E61A, E64A, and E67A); mutant S1 in which both the putative ferroxidase and nucleation site ligands are changed (E61A, E62K, E64A, H65G, E67A, and also D42A and K86Q); and mutant 206 in which the carboxylate groups inside the 3-fold hydrophilic channels are changed (D131A and E134A). The $g' = 4$ signal was observed in all of the mutants. On the basis of these findings, we conclude that neither the ferroxidase site, the nucleation site, nor the sites within the 3-fold channels are solely responsible for the C-type spectrum. It seems likely the C-type spectrum is due to nonspecific binding of the $S = {}^3/_2$ iron–nitrosyl complex to carboxylate groups on the protein in general. This assignment is supported by the fact that the $g' = 4$ signal is present in almost all non-heme Fe(II)-containing proteins (Henry et al., 1991) and is observed in this work with human H-chain and horse spleen ferritins. The high stoichiometry of binding, >120 Fe^{3+}–NO^-/24-mer HoSF (*vide supra*), is also consistent with nonspecific binding.

The A- and B-Type Signals. The A and B-type EPR signals show time dependence in their formation. The EPR spectra of apoferritin with Fe^{2+} in the presence of NO after 5- and 15-min incubations are illustrated in Figure 2a,b, respectively. The difference spectrum obtained by subtracting one spectrum from the other is shown in Figure 2c. The B-type species is formed first and displays an axial EPR signal with $g_\perp' = 2.033$ and $g_\parallel' = 2.014$ (Figure 2a). As the incubation time increases, the A-type species develops and has the rhombic EPR features shown in the difference spectrum (Figure 2c), with resonances at $g_x' = 2.053$, $g_y' = 2.029$, and $g_z' = 2.011$. A similar A-type spectrum has been reported for bacterioferritin (Le Brun et al., 1993). The A- and B-type EPR signals identified here for HoSF are similar to those reported for the iron–nitrosyl complexes of histidine and cysteine residues, respectively, in free amino acids and proteins (Woolum et al., 1968). They remain stable when air is admitted to the sample (up to 10 min), whereas in the absence of NO, the Fe^{2+} is rapidly oxidized.

Spin quantification of the composite A-type His and B-type Cys EPR signal yields values of 12–29 spins/24-mer, depending on the sample preparation (120–360 Fe^{2+} added per 24-mer apoferritin at pH 7–7.5) and assuming $S = {}^1/_2$ systems. The spin concentration is divided approximately equally between the A and B signals. A similar value of 22 spins/24-mer protein has been reported for the A-type signals

[3] No C-type spectrum is observed in the mixture of holoprotein with Fe^{2+} at pH 7.0 (data not shown). In control experiments, no signals above background at $g' = 4$ and $g' = 2$ can be detected in holoferritin with NO in the absence of added Fe^{2+} or ascorbate at either pH 6.0 or 7.0 after a 5-min incubation. However, after a 1-h incubation, a weak $g' = 2$ A-/B-type signal (about 0.5% as intense as that in Figure 2a) is observed, suggesting that either a small amount of Fe^{2+} is present in holoferritin or NO is capable of reducing the ferric core of the protein as previously reported (Reif & Simmons, 1990).

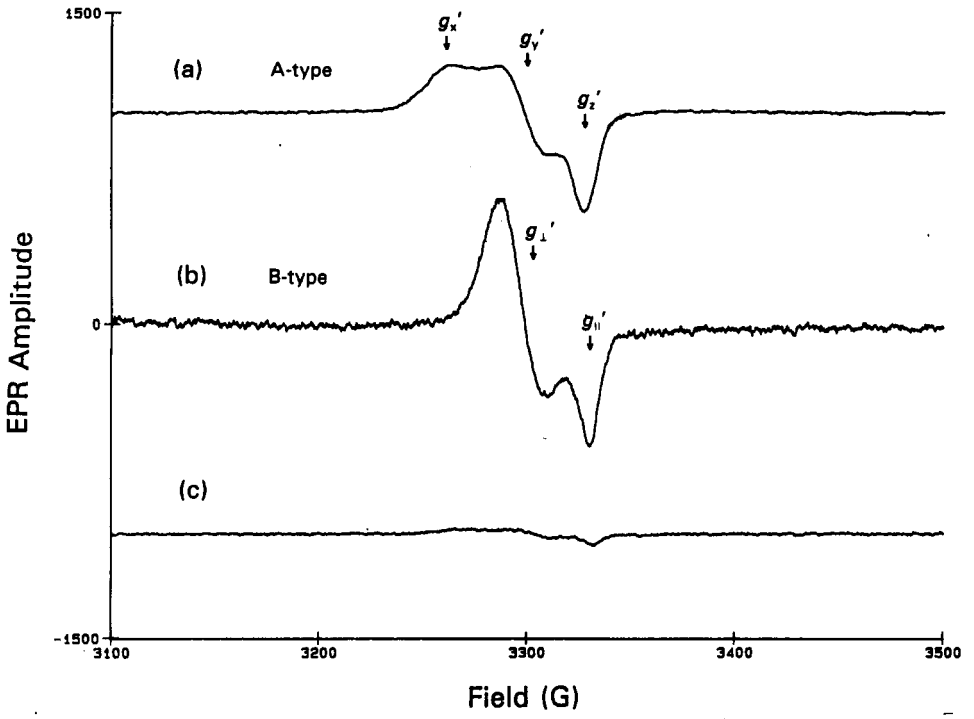

FIGURE 3: (a) EPR spectrum of PMB-treated HoSF (20 μM) with 48 Fe^{2+}/molecule flushed with NO for 5 min under anaerobic conditions at pH 7.0, $g_x' = 2.055$, $g_y' = 2.033$, and $g_z' = 2.015$. (b) EPR spectrum of DEP-treated HoSF (20 μM) with 120 Fe^{2+}/molecule flushed with NO for 5 min under anaerobic conditions at pH 7.0, $g_\perp' = 2.033$ and $g_\parallel' = 2.014$. (c) EPR spectrum of HoSF (20 μM) treated with both PMB and DEP with 48 Fe^{2+}/molecule flushed with NO for 5 min under anaerobic conditions at pH 7. Other conditions are given in the legend to Figure 1. Spectrometer settings: microwave power, 10 mW; frequency, 9.372 GHz; modulation amplitude, 1 G; time constant, 1 s; receiver gain, 2500 (a and c) and 8000 (b); temperature, 77 K.

observed in bacterioferritin (Le Brun et al., 1993b).

To further establish the involvement of histidine and cysteine residues in iron–nitrosyl complex formation, chemical modification studies were carried out on HoSF. When the thiol groups of cysteine are modified with PMB, the B-type Cys signal is not seen, and only the A-type His signal is observed with resonances at $g_x' = 2.055$, $g_y' = 2.033$, and $g_z' = 2.015$ (Figure 3a). A total of 1.3 thiol groups of cysteine per subunit was found to be modified in agreement with the fact that one thiol group (Cys-126) per subunit can be modified in HoSF, the other thiol group (Cys-48) being unaffected (Stefanini et al., 1989). On the other hand, when the imidazole groups of histidine are modified with DEP,[4] the A-type His signal is not seen and only the B-type Cys signal is observed ($g_\perp' = 2.033$ and $g_\parallel' = 2.014$) (Figure 3b).[5] Chemical modification of the protein with *both* PMB and DEP causes both the A- and B-type signals to disappear (*cf.* Figure 3c).

The $g' = 2$ (A and B-type) signals are observed at pH 7.0 with both HoSF (85% L-subunits) and rHF (100% H-subunits) and have essentially the same intensities in both proteins. This result implies that either the residues involved in iron–nitrosyl complex formation are conserved between both H- and L-subunits or the binding is nonspecific. Direct evidence for specific binding comes from the EPR spectra of cysteine and histidine mutants of rHF. There are three cysteine residues in human H-chain ferritin, namely, Cys-90, Cys-102, and Cys-130 (human H-chain sequence numbering; Boyd et al., 1985). As shown in Figure 4b,c, respectively, the EPR intensities of mutants S3 (C90E and K86Q) and S4 (C90E, C102A, and K86Q) are essentially the same as that of rHF (*cf.* Figure 4a), indicating that neither Cys-90 nor Cys-102 is an important iron binding site. In contrast, the B-type Cys signal is totally absent from mutant S5 (C90E, C102A, C130A, and K86Q) and only the A-type His signal remains (Figure 4d), indicating that Cys-130 is involved in the formation of the B-type complex. Cys-130 is conserved between H- and L-subunits, but Cys-90 and Cys-102 are not (Harrison & Lilley, 1989; Harrison et al., 1991). These findings are in accord with the chemical modification studies with HoSF mentioned above, implicating Cys-126 (Cys-130 in human) in iron–nitrosyl complex formation.

The origin of the A-type His signal is not as evident as that of the B-type Cys signal. Two histidine residues (His-118 and His-128) are conserved between H- and L-subunits (Harrison & Lilley, 1989; Harrison et al., 1991). The corresponding rHF mutants were therefore examined. Only a B-type Cys signal (intensity 79% of that of rHF) is observed after a 5-min incubation with NO in mutant S9 (H118A and K86Q); however, upon purging with NO for a longer time (10 min), an A-type His signal slowly develops (*cf.* Figure 5a,b). Surprisingly, in mutant S10 (H128A and K86Q), the A-type His signal is *totally* absent while the B-type Cys signal involving Cys-130 is greatly attenuated (intensity is 5% that of rHF) (Figure 5c). Thus, mutation of His-128 influences *both* the A- and B-type signals. Both A- and B-type signals are observed in mutant 206 (D131A and E134A), indicating that the carboxylate groups inside the 3-fold hydrophilic channels are not involved in the formation of either the A- or B-type

[4] There are six histidine residues per L-subunit in HoSF (Heusterspreute & Crichton, 1981). A total of 7.8 histidines per subunit have been found to be modified, as described in Materials and Methods. The overestimation of the number of modified histidines is due to the higher absorbance at 240 nm of the dicarbethoxyhistidyl derivative, as shown previously for horse liver alcohol dehydrogenase (Morris & McKinley-McKee, 1972).

[5] Upon purging the histidine-modified sample with NO for a longer time (15 min), a very weak but visible A-type signal slowly develops (data not shown). This weak A-type signal probably arises from either incomplete chemical modification of the protein or slow reversal of the modification reaction, which is known to occur (Eyzaguirre, 1987; Lundblad, 1991).

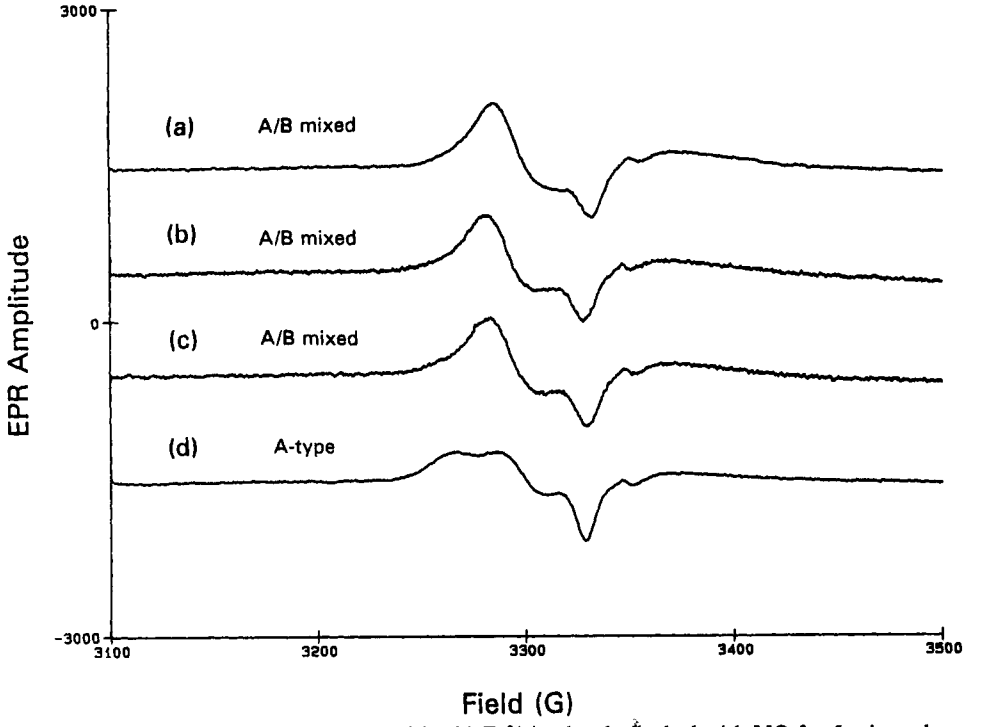

FIGURE 4: EPR spectra of rHF and its mutants at 2.6 μM with 120 Fe^{2+}/molecule flushed with NO for 5 min under anaerobic conditions at pH 7.0: (a) rHF; (b) mutant S3 (C90E and K86Q); (c) mutant S4 (C90E, C102A, and K86Q); (d) mutant S5 (C90E, C102A, C130A, and K86Q). Other conditions are given in the legend to Figure 1. Spectrometer settings: microwave power, 10 mW; frequency, 9.372 GHz; modulation amplitude, 5 G; time constant, 1 s (a and d) and 0.3 s (b and c); receiver gain, 4000; temperature, 77 K.

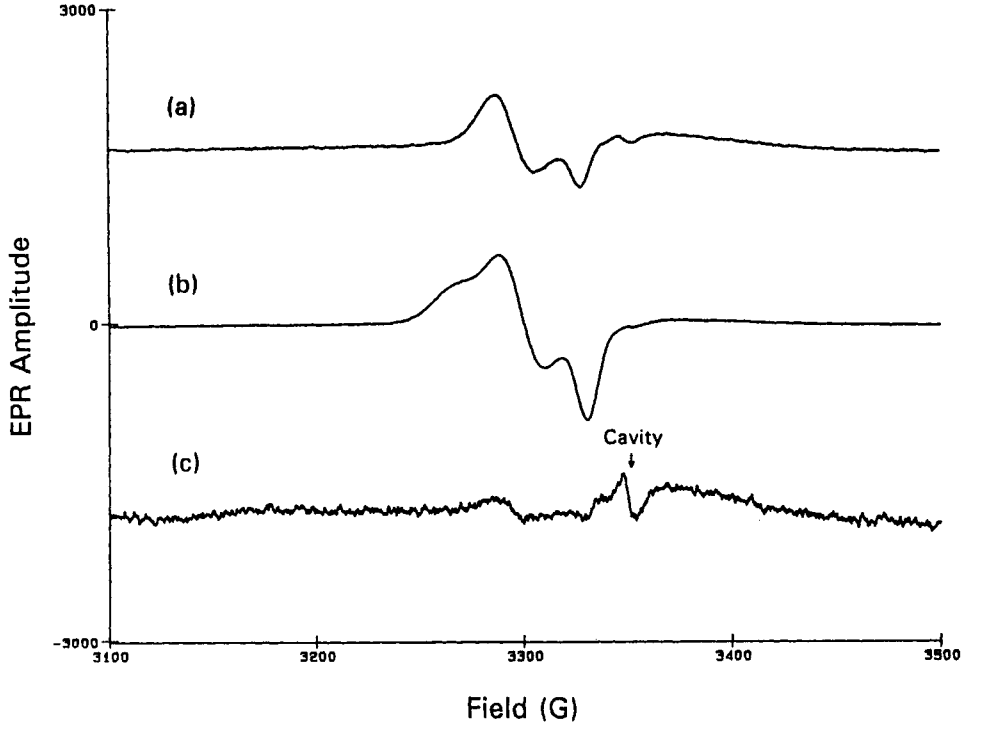

FIGURE 5: (a) EPR spectrum of rHF mutant S9 (H118A and K86Q) (2.6 μM) with 120 Fe^{2+}/molecule flushed with NO for 5 min under anaerobic conditions at pH 7.0. (b) Same as a except flushed with NO for an additional 5 min. (c) Same as a except for rHF mutant S10 (H128A and K86Q). The signal at $g' = 2.002$ is due to the cavity. Other conditions are given in the legend to Figure 1. Spectrometer settings: microwave power, 10 mW; frequency, 9.372 GHz; modulation amplitude, 5 G; time constant, 1 s; receiver gain, 4000 (a), 1250 (b), and 20 000 (c); temperature, 77 K.

complex. The results of the chemical modification and mutant studies are summarized in Table 1.

DISCUSSION

The results of the present work provide new insight into the EPR-observable iron–nitrosyl complexes of ferritin. Three types of EPR signals (A, B, and C) have been identified from the spectra of apoferritin and holoferritin in the presence of Fe^{2+} and NO under anaerobic conditions (Figures 1 and 2). The observation of A-, B-, and C-type EPR signals in both the presence and absence of an iron core indicates that the core must not be involved in binding at the sites of EPR-

Table 1: Effects of Chemical Modification of Cysteine and Histidine Residues in HoSF and rHF Mutants on the Formation of the Iron–Nitrosyl Complexes (A- and B-Type EPR Signals)

sample	mutation[a]	EPR signal	
HoSF		A	B
PMB–HoSF[b]		A	
DEP–HoSF[c]			B
PMB–DEP–HoSF[d]			
rHF		A	B
rHF mutant S3	K86Q+C90E	A	B
rHF mutant S4	K86Q+C90E+C102A	A	B
rHF mutant S5	K86Q+C90E+C102A+C130A	A	
rHF mutant S9	K86Q+H118A	A[e]	B
rHF mutant S10	K86Q+H128A		B[f]
rHF mutant 206	D131A+E134A	A	B

[a] Human H-chain sequence numbering. [b] Cysteine residues modified. [c] Histidine residues modified. [d] Cysteine and histidine residues modified. [e] A-type signal cannot be seen at the beginning but appears later (see text and Figure 5). [f] Signal only 5% as intense as for rHF.

visible iron–nitrosyl complex formation. However, the presence of EPR-silent iron–nitrosyl species in ferritin, perhaps involving core iron, is not precluded by the present data.

A C-type spectrum due to an $S = {}^3/_2$ species is reported here for mammalian ferritin for the first time and appears to be due to nonspecific binding of the iron–nitrosyl complex to carboxylate groups on the protein. The C-type spectrum is similar to the spectra of $S = {}^3/_2$ spin systems reported previously for non-heme iron proteins, although the line shape of the $g_\perp' = 4$ resonance is different for different proteins because of varying degrees of rhombicity (Galpin et al., 1978; Salerno & Siedow, 1979; Arciero et al., 1983; Twilfer et al., 1985; Chen et al., 1989; Petrouleas & Diner, 1990; Diner & Petrouleas, 1990; Le Brun et al., 1993). Bacterioferritin shows two $S = {}^3/_2$ signals with one of greater rhombicity than seen here for mammalian ferritin (Le Brun et al., 1993a,b). The appearance of an $S = {}^3/_2$ EPR spectrum implies that the iron–nitrosyl C-type complex is most likely in the mononitrosyl form. Carboxylate binding to the Fe^{3+}–NO^- complex is probably either monodentate with another anionic ligand from the protein or bidentate involving both oxygens of the carboxylate group as proposed by others for such complexes (Pearsall & Bonner, 1982; Diner & Petrouleas, 1990).

The chemical modification and site-directed mutagenesis experiments further show that the two types of signals (A and B) near $g' = 2$ are due to iron–nitrosyl association with histidyl and cysteinyl ligands, respectively. Woolum et al. (1968) have reported two types of EPR signals from $S = {}^1/_2$ iron–nitrosyl complexes, depending on the amino acid composition of the protein. It was concluded that the axial signal with g values of 2.036 and 2.013 is due to an iron–nitrosyl complex of thiol groups and that the rhombic signal with g values of 2.055, 2.039, and 2.014 is due to an iron–nitrosyl complex of imidazole groups (Woolum et al., 1968; Henry et al., 1991). The A-type His and B-type Cys signals identified here with horse and human ferritins are in accord with these results.[6]

Each iron–nitrosyl complex producing the B-type Cys signal most likely binds to only one Cys-130, as for Hg^+ (PMB) bound to cysteine shown previously (Harrison et al., 1986; Stefanini et al., 1989). In ferritin the distance between two cysteines related by 3-fold symmetry (Cys-130–Cys-130′)[7] is too large (10.5 Å) for them to bind the same iron ion (Table 2). Both Cys-130 and His-118 face the 3-fold channel and point toward the outer surface. Although the distance between Cys-130 and His-118 in the same subunit is only 4 Å (Table 2), mutant S9 (H118A and K86Q) shows that His-118 is not involved in the formation of the B-type Cys complex.

Table 2: Distance between Atoms from the Cysteine and Histidine Residues near the 3-fold Channels[a]

atoms from the residues[b]	distance (Å)
N_ϵ(His-118)–N_ϵ(His-128)	12.4
N_ϵ(His-118)–N_ϵ(His-118′)[c]	9.8
N_ϵ(His-128)–N_ϵ(His-128′)[c]	19.4
S_γ(Cys-130)–S_γ(Cys-130′)[c]	10.5
S_γ(Cys-130)–N_δ(His-118)	4.6
S_γ(Cys-130)–N_ϵ(His-118)	4.0
S_γ(Cys-130)–N_δ(His-128)	9.3
S_γ(Cys-130)–N_ϵ(His-128)	10.4

[a] See footnote 8. [b] Human H-chain sequence numbering. [c] Prime refers to the adjacent subunit of the same 3-fold channel.

The $S = {}^1/_2$ B-type Cys complex is most likely in the dinitrosyl form, as for iron–dinitrosyl model complexes (McDonald et al., 1965; Burlamacchi et al., 1969). However, a mononitrosyl complex cannot be ruled out. Such compounds displaying axial EPR signals at $g \approx 2$ are known to exist. In these instances, strong ligand fields are required to produce low-spin ($S = {}^1/_2$) octahedral coordination about the Fe(I) (Goodman & Raynor, 1970; Glidewell & Johnson, 1987; Butler et al., 1987; Danon et al., 1964; McNeil et al., 1965; Goodman et al., 1966; Griffith et al., 1958), a situation that probably does not occur in ferritin.

The precise roles of His-118 and His-128 in the formation of the A-type His complex are unclear. Mutation of His-118 to Ala somewhat slows the formation of the A-type His complex relative to the wild-type protein (*cf.* the low-field shoulder on Figures 4a and 5a) but has little effect on the B-type Cys complex, suggesting that His-118 is a potential site of A-type His complex formation but that there are other sites as well (*e.g.*, His-128). In contrast, mutation of His-128 abolishes the A-type His signal altogether and also nearly eliminates the B-type Cys signal (Figure 5c). While His-128 is probably the site of A-type His complex formation, it may also be important in maintaining the conformation of the protein in the vicinity of the 3-fold channels. His-128 faces the 3-fold channel, but is on the cavity side and is exposed. It is also involved in intra- and interchain interactions; it has a charge-induced dipole interaction with Arg-76 of the same subunit and forms a hydrogen bond with Asn-139 of the adjacent subunit.[8] The α-carbons of His-128 and Cys-130 are only 4.8 Å apart. Thus, the mutation of His-128 may induce a conformational change that influences both A and B iron binding sites. His-118 and Cys-130 are on different helices (C and D, respectively), which are divided by a sharp turn (Boyd et al., 1985), and thus may be more sensitive to a conformational change due to the mutation of His-128.

It is known that $S = {}^1/_2$ iron–nitrosyl complexes can have more than one imidazole group of histidine as a ligand (Woolum et al., 1968). However, the distances between two histidine residues in ferritin (His-118–His-128, His-118–His-

[6] The B-type signal shown in Figure 3b has features at $g = 2.040$ and 2.014 using the g-labeling system from previous reports (Drapier et al., 1991; Henry et al., 1991); the A-type signal shown in Figure 3a has features at $g = 2.055$, 2.039, and 2.015.

[7] The prime refers to the symmetry-related adjacent subunit of the same 3-fold channel.

[8] The coordinates (entry 1FHA of the December 1990 version) were obtained from the Protein Data Bank (Lawson et al., 1991) at the Brookhaven National Laboratory.

118′, and His-128–His-128′)[7] are larger than 10 Å (Table 2), implying that in the absence of a major structural rearrangement there is only one histidyl ligand in each binding site that is responsible for the A-type His signal.

The A-type rhombic EPR signal reported for bacterioferritin has been suggested to arise from an Fe^{2+}–Fe^{2+}–NO binuclear center with $S = {}^1/_2$ (Le Brun et al., 1993a,b). Our data provide no information about the nuclearity of the A-type complexes observed here. However, the paramagnetic $S = {}^1/_2$ species observed years ago with iron–nitrosyl model complexes have been proposed to be one iron atom bound with two NO groups and two anionic ligands X (*e.g.*, hydroxyl, phosphate, cysteine, etc.) in a tetrahedral structure, $Fe(NO)_2X_2$ (McDonald et al., 1965; Burlamacchi et al., 1969). The iron in such a complex is postulated to be in the Fe(I) state with a low-spin ($S = {}^1/_2$) d^7 configuration. An octahedral coordination with acetate and hydroxyl as ligands has also been considered for the dinitrosyl complex (Pearsall & Bonner, 1982).

An EPR spectrum near $g' = 2$ of horse spleen holoferritin treated with $NaNO_2$ and ascorbate has been reported by Drapier et al. (1991). In their experiment, the Fe(III) in holoferritin was reduced by ascorbate to Fe(II), and the latter reacted with nitric oxide to form an $S = {}^1/_2$ iron–nitrosyl complex, which yielded an EPR signal with g values of 2.038 and 2.011 similar to those reported here for the axial B-type Cys signal.[6] They also noticed that the broad signal at $g = 2.038$ is asymmetric with a line width of 28 G. This broadening is most likely due to the rhombic A-type His signal identified in this work. Ascorbate and $NaNO_2$ were used to generate NO *in situ* in previous experiments (Woolum et al., 1968; Drapier et al., 1991), whereas in the present work gaseous nitric oxide was used directly to avoid possible complications of having ascorbate present in the protein solution. Both methods were tested, however, and gave the same spectral results.

EPR studies on the binding of a variety of metal ions including Fe^{2+} to HoSF have implicated the binding of one or two metal ions near or in the 3-fold channels of HoSF (Wardeska et al., 1986). Residues His-118, His-128, and Cys-130 are all located in the vicinity of the 3-fold channels. Previous chemical modification and spin-labeling studies suggest that the Cys-126 (horse sequence numbering, Cys-130 in rHF) on the outer surface near the entry to the 3-fold channel is involved in initial iron uptake in horse spleen ferritin (Stefanini et al., 1989; Desideri et al., 1991). EPR, ESEEM, and ENDOR studies show that VO^{2+} and Fe^{2+} compete 1:1 for the same protein binding site(s) involving a nitrogen donor ligand, presumably from the imidazole group of either histidine 118 or 128 (Gerfen et al., 1991; Hanna et al., 1991b). Thus, the present study of iron–nitrosyl complex formation is in accord with this earlier work implicating residues in and near the hydrophilic channels in metal binding and uptake by ferritin. Recent studies with site-directed mutants confirm the importance of the 3-fold channels in Fe^{2+} uptake (Treffry et al., 1993).

The functional role, if any, for nitric oxide in ferritin biochemistry, however, remains unclear. One proposal for the identity of EDRF is a non-heme iron–dinitrosyl complex with thiol groups (Vanin, 1991). It is reasonable to speculate that NO is produced, stored, and transferred in cells as a dinitrosyl–iron(I)–dithiol complex, since free NO can be oxidized in living cells (Vanin, 1991; Mülsch et al., 1991). In this regard, the chemical form of NO within the cell may well be important in influencing its lifetime, transport, and targeted delivery to sites of action (Stamler et al., 1992). It has been suggested that iron released from the cells treated with NO is from the degradation of active centers of Fe–S proteins (Hibbs et al., 1984, 1988; Lancaster & Hibbs, 1990; Pallet et al., 1990). Ferritin perhaps stores NO, as well as iron, in the form of the iron–nitrosyl complexes observed here.

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