Mössbauer and EPR Studies of the Binuclear and Trinuclear Antiferromagnetically Coupled Iron(III)-Binding Sites in Ferreascidin

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Previous studies of the interaction of iron(III) with ferreascidin, a glycoprotein isolated from the blood cells of the stolidobranch ascidian *Pyura stolonifera*, have shown that the iron's coordination sphere involves two (3,4-dihydroxyphenyl)alanine (DOPA) residues and possibly one tyrosine (Taylor, S. W.; Hawkins, C. J.; Winzor, D. J. *Inorg. Chem.* **1993**, *32*, 422). Herein we report variable-field (0−11.1 T) low-temperature Mössbauer spectra which reveal the existence of at least three distinct iron(III)-binding sites. In conjunction with electron paramagnetic resonance spectroscopy of ferreascidin and model complexes, site 1 is found to be a strongly antiferromagnetically coupled ($|J| > 100$ cm^{-1}) binuclear iron(III) center which may possess a μ-oxo bridging group, while site 2 is also an antiferromagnetically coupled ($\sim 20 < |J| < \sim 80$ cm^{-1}) binuclear iron(III) center. Site 3 is a linear trinuclear iron cluster in which the three high-spin iron(III) ions are antiferromagnetically coupled with $J_{12} \simeq J_{23} > J_{13}$ producing a paramagnetic $S = {}^5/_2$ ground state. The absence of sulfur and sulfide in ferreascidin indicates that the bridging atoms in these three clusters (sites) must be oxygen and/or nitrogen atoms. Site 3 represents the first example of a trinuclear iron(III) cluster with a paramagnetic $S = {}^5/_2$ ground state to be found in metalloproteins. Structures for the metal ion binding sites are proposed.

Introduction

Ferreascidin is a novel protein containing the highest aromatic amino acid content (42% tyrosine, 17% (3,4-dihydroxyphenyl)-alanine (DOPA),[2] and 8% phenylalanine) of any naturally occurring protein yet characterized and is isolated from the blood cells of the stolidobranch ascidian *Pyura stolonifera*.[3−6] It has a molecular mass of 10 000 Da and contains two glucose units as well as a 360 nm chiral chromophore which is, as yet, uncharacterized. There are no sulfide or sulfur-containing amino acids present in the protein. Recently the N-terminal sequence was identified as Leu-DOPA-X (where X is none of the known amino acids).[7] However, protein sequence information is largely lacking because the protein has an apparent lack of susceptibility to proteolytic enzymes, probably as a result of extensive posttranslational modification rendering the usual sites for proteolytic attack unidentifiable. Ferreascidin is a basic protein, soluble in acid but exhibiting a broadness of precipitation at neutral to alkaline pH values, isoelectric precipitation being brought on by ionization of one or more histidine residues (pK_a' ~7.5) and also by any oxidation or modification of catechol (pK_a' ~8.7 for the first hydroxyl group) that affects the pI.[6] Solubility is also greatly affected by the type of ionic species present in the buffer solutions; for instance phosphate induces rapid precipitation at pH 7.0 and sulfur containing compounds, SDS, ammonium sulfate and ammonium persulfate precipitate ferreascidin at pH 5.0.[6,8]

Despite being isolated free of other protein contaminants by comparatively simple procedures, ferreascidin preparations exhibit microheterogeneity[3,5,6] which manifests itself as a continual variation in the ratio of absorbances of the 277 nm band (due to the aromatic residues) to those of an unidentified 360 nm chromophore across the elution profile. The nature of this microheterogeneity is still unknown, but three possible sources have been suggested:[6] (i) the degree or position of hydroxylation, (ii) variation in the structure, number, or position of covalently attached unknown group or groups which are responsible for the 360 nm chromophore, and (iii) internal cross-linking involving Michael condensation of amines with DOPA.[9] A combination of any two, or all three, of the above factors is, of course, also possible. However, the discovery that the DOPA composition is fairly constant for all forms of the protein[7] suggests that (ii) is the most likely cause of the microheterogeneity and furthermore provides a means of quantitating the protein by assaying for DOPA, thus overcoming the difficulties encountered in establishing absorbance/weight relationships using various protein assays.[5]

Although ferreascidin as isolated does not contain any metal ions, ferreascidin's name derives from its ability to form stable

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(2) Abbreviations. Bistris, 2,2-bis(hydroxymethyl)-2,2′,2″-nitrilotriethanol; cat, catechol; DOPA, (3,4-dihydroxyphenyl)alanine; EPR, electron paramagnetic resonance; NTA, nitrilotriacetate; OAc, acetate; salen, *N*,*N*′-ethylenebis(salicylideneaminate); SDS, sodium dodecyl sulfate; T1,4DMIP, tris(1,4-dimethylimidazol-2-yl)phosphine; THF, tetrahydrofuran.

(3) Dorsett, L. C.; Hawkins, C. J.; Grice, J. A.; Lavin, M. F.; Merefield, P. M.; Parry, D. L.; Ross, I. L. *Biochemistry* **1987**, *26*, 8078.

(4) Lavin, M. F.; Watters, D.; Wynne; M.; Ross; I. L.; Taylor; S. W.; Hawkins, C. J. *Proc. 9th. Aust. Biotech. Assoc. Conf.* 1990.

(5) (a) Taylor, S. W. Ph.D. Thesis, The University of Queensland, 1993. (b) Taylor, S. W. Honours Thesis, The University of Queensland, 1987.

(6) Ross, I. L. Ph.D. Thesis, The University of Queensland, 1990.

(7) Waite, J. H.; Wynne, M. Personal communication, 1991.

(8) Since these compounds have no effect on the electronic absorption spectrum of ferreascidin, the most likely explanantion for the precipitation of ferreascidin is the formation of a stable neutral complex which falls out of solution.

(9) Waite, J. H. *Comp. Biochem. Physiol.* **1990**, *97B*, 19.

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complexes with iron(III).[10] An electronic absorption spectral titration of ferreascidin (66.3 μM) with ferric chloride at pH 7.0 reveals the development of a ligand (DOPA) to metal charge transfer band at 520 nm.[11] Replacement of ferric chloride with iron nitrilotriacetate (NTA) (0−40 μM) produced similar results. At higher concentrations of iron-NTA the 520 nm band shifted toward 570 nm, which is characteristic for the catecholate to iron(III) charge transfer band found in the bis(catecholato)iron-(III) complex.[12] This wavelength shift was suggested to arise from the displacement of a coordinated tyrosine ligand by NTA.[11] A similar shift is also observed upon lowering the pH to 5.0. Although peaks at 1265−1271, 1327−1341, and 1481−1489 cm^{-1} attributable to catecholate coordinated to iron(III) were observed in the resonance Raman spectrum of ferreascidin, there was no evidence for tyrosine coordination.[5a,13] A detailed analysis of the spectral titrations revealed a one to two stoichiometric ratio for the binding of iron to DOPA in ferreascidin with a binding constant of 4×10^{17} M^{-1} at pH 7.0, μ 0.2.[11] Thus the authors proposed one or more mononuclear iron(III)-binding sites each with two DOPA ligands and possibly a tyrosine ligand. The iron(III) complex formed *in vitro* does not appear to be present in intact blood cells, as they are yellow. Thus the iron and ferreascidin are probably separately compartmentalized in the blood cells. However, the complex does appear to form upon cell lysis and has been suggested to be important in the process of wound repair in *P. stolonifera*.[11]

In this paper we report Mössbauer and electron paramagnetic resonance (EPR)[14] spectra of the iron(III) ferreascidin complex which reveal the presence of two antiferromagnetically coupled binuclear iron(III) clusters and an antiferromagnetically coupled linear trinuclear iron(III) cluster. This research was recently presented at the 6th International Conference on Bioinorganic Chemistry.[15]

Experimental Section

Materials: Ferreascidin was extracted and isolated from the blood cells of *Pyura stolonifera* collected from Hastings Point, Northern New South Wales, Australia.[3,4]

Iron(III) ferreascidin (^{56}Fe) was prepared for EPR measurements from ferreascidin that had been titrated with ferric chloride as described previously.[11] At the completion of the titration, the iron(III) ferreascidin solution was split equally into two portions. The pH of one of these solutions was adjusted to 5.0 with dilute HCl, and then each solution was concentrated by ultrafiltration through an Amicon YM5 membrane to a volume of approximately 2 mL. The solutions were stored at −80 °C. The A_{277}/A_{360} ratios for the pH 5.0 and 7.0 solutions were 2.37 and 2.39, respectively.

Iron(III) ferreascidin (^{57}Fe) for Mössbauer measurements was required at a considerably higher concentration than for other spectroscopic measurements. Ferreascidin from a number of preparations was pooled and concentrated by ultrafiltration to a final volume of approximately 10 mL ([cat] = 0.55 mM, measured with the Arnow assay[16]). The A_{277}/A_{360} ratio was 2.44. This solution was dialyzed against 1 L of outgassed 5 mM 2,2-bis(hydroxymethyl)-2,2′,2″-nitrilotriethanol (Bistris) buffer (pH 7.0, 0.2 M NaCl) overnight. $^{57}Fe_2O_3$, 95% enrichment (Oak Ridge National Laboratories, Oak Ridge, TN), 1.0508 mg, was dissolved in a minimum volume of concentrated HCl (with heating), the solution cooled on ice and NaOH added dropwise until the pH of the solution was approximately 3. The resultant yellow ^{57}Fe-enriched ferric chloride solution (approximately 1.5 mL) was added dropwise with stirring to the solution of ferreascidin and the pH not permitted to drop below 5 by readjustment to 7 with dilute NaOH. The protein solution turned deep purple/black. The solution was slowly stirred for about 20 min; then excess iron was removed by dialysis against two 1 L changes of outgassed 5 mM Bistris buffer (pH 7.0, 0.2 M NaCl) (8 h each). The dialyzed solution was concentrated to about 1 mL by ultrafiltration, and ~0.3 mL of the concentrate was transferred to an EPR tube. The remaining portion of the concentrate was transferred to a perspex Mössbauer absorber holder which was sealed with liquid perspex under nitrogen and the sample stored at −80 °C prior to measuring the Mössbauer spectra. The remaining iron(III) ferreascidin concentrate (in the ultrafiltration cell and dialysis sac) was diluted and reconcentrated with 5 mM Bistris buffer, previously adjusted to pH 5, and was used for EPR and electronic spectral measurements. The electronic spectra of the iron(III) ferreascidin complex had visible absorption bands at 520 nm for the pH 7.0 sample and at 570 nm for the pH 5.0 sample, consistent with published spectra.[11]

Electron Paramagnetic Resonance Spectroscopy. X-band (9−10 GHz, TE_{102} rectangular cavity) EPR spectra of ^{56}Fe and ^{57}Fe ferreascidin at both pH 5.0 and 7.0 were measured on a Bruker ESP300E spectrometer. A flow-through Oxford Instruments ESR910 cryostat in conjunction with an Oxford Instruments ITC-4 variable-temperature controller was employed for temperatures as low as 2 K at the sample position in the cavity. Spectrometer tuning, signal averaging, and subsequent data manipulation were performed with version 3.01 of Bruker's esp300e software. For all of the EPR spectra, the modulation frequency was 100 kHz and the modulation amplitude was set to a maximum of one-tenth of the line width at half-height. The microwave frequency and magnetic field were calibrated using an EIP 548B microwave frequency counter and a Bruker ER035M gaussmeter, respectively.

Mössbauer Spectroscopy. Mössbauer spectra were measured in the Physics Department at Monash University with a standard electromechanical transducer operating in a symmetrical constant-acceleration mode. A conventional helium bath cryostat was employed for temperature control with the sample maintained in exchange gas. Data were collected with an LSI based 1000 channel multichannel analyzer. Velocity calibration was made with respect to iron foil. Spectra were fitted to a Voigtian profile (or Doppler integral[17]). This line shape appears if there is a Gaussian distribution of a particular hyperfine parameter which modifies the absorption from Lorentzian. The Voigtian profile as a function of velocity (v) is given by:[15]

$$Y(v) = \frac{1}{\beta_M\sqrt{\pi}}\int_{-\infty}^{\infty}\frac{dx}{1+x^2}\exp[-(x+v_0-v)^2/\beta_M]^2 \quad (1)$$

and its limiting forms are pure Lorentzian or Gaussian as the mixing coefficient (β_M) tends to zero or infinity, respectively.[17] Both lines within a doublet in the observed Mössbauer spectrum, and the corresponding lines in a sextet, were constrained to have equal line widths and intensities, while the relative line positions in a sextet were defined by the hyperfine field and the nuclear g values. The area of lines 1 and 3 within a sextet were constrained to the ratio 3:1 but the relative area of line 2 was allowed to be a free parameter. The value of the parameter β_M is independent for the three pairs of lines in a sextet.

Results

EPR Spectroscopy. X-band EPR spectra of iron(III) ferreascidin at 2 K are displayed in Figure 1 for both the ^{56}Fe- and ^{57}Fe-enriched samples at pH values of 5.0 and 7.0. Spectra obtained are consistent with those previously observed for iron-(III) ferreascidin prepared with both ferric chloride and iron-

(10) Hawkins, C. J.; Parry, D. L; Wood, B. J.; Clark, P. *Inorg. Chim. Acta* **1983**, *78*, L29.
(11) Taylor, S. W.; Hawkins, C. J.; Winzor, D. J. *Inorg. Chem.* **1993**, *32*, 422.
(12) Avdeef, A.; Sofen, S. R.; Bregante, T. L.; Raymond, K. N. *J. Am. Chem. Soc.* **1978**, *100*, 5362.
(13) Taylor, S. W.; Nugent, K.; Hawkins, C. J. Personal communication, 1992.
(14) The International EPR Society is adopting and recommending that "EPR" be used to describe this technique.
(15) Taylor, S. W.; Hanson, G. R.; Cashion, J. D.; Brown, L. J.; Hawkins, C. J. *J. Inorg. Biochem.* **1993**, *51*, 493.
(16) Waite, J. H.; Tanzer, M. L. *Anal. Biochem.* **1981**, *111*, 131.
(17) Price, D. C. *Aust. J. Phys.* **1981**, *34*, 51.

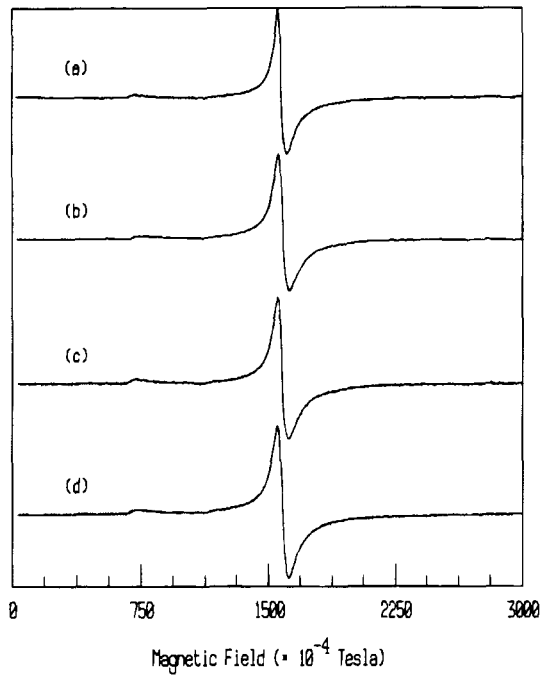

Figure 1. X-band EPR spectra of iron(III) ferreascidin complexes: (a) ^{56}Fe ferreascidin (pH 5.0), (b) ^{57}Fe ferreascidin (pH 5.0), (c) ^{56}Fe ferreascidin (pH 7.0), (d) ^{57}Fe ferreascidin (pH 7.0). Conditions: temperature = 2 K, ν = 9.444 GHz.

NTA complexes.[5,11] In all spectra, resonances attributable to an $S = {}^5/_2$ spin ground state were observed at $g = 9.32 \pm 0.02$, $g = 4.27 \pm 0.01$, and $g = 0.57$ (weak; results not shown). Apart from resonances around $g \approx 2$ (estimate $g_{\|} = 2.18$, $g_{\perp} = 2.06$, $A_{\|} = 225 \times 10^{-4}$ cm^{-1}) due to a minor copper impurity (results not shown), no other resonances were observed between 0.005 and 1.4 T.

The resonances arising from the $S = {}^5/_2$ spin state were interpreted in terms of the spin Hamiltonian[18]

$$\mathcal{H} = D[S_z^2 - {}^1/_3 S(S+1)] + E(S_x^2 - S_y^2) + \beta \mathbf{S}\cdot\mathbf{g}\cdot\mathbf{B} \quad (2)$$

where D and E are the axial and rhombic zero field splitting parameters. From rhombograms[19] for an $S = {}^5/_2$ spin state a value for $|E/D| = 0.3$ was obtained. Only very small broadening was observed in the ^{57}Fe ($I = {}^1/_2$) EPR spectra relative to the ^{56}Fe ($I = 0$) spectra (~0.6 mT), indicating that the hyperfine coupling to the ^{57}Fe nucleus is less than the line width of the $g = 4.27$ resonance.

Mössbauer Spectroscopy. Mössbauer spectra of the ^{57}Fe-enriched ferreascidin (pH 7.0, μ 0.2) sample in the absence and presence of applied magnetic fields (0.5, 5, and 11.1 T) are shown in Figures 2 and 3, respectively. The Mössbauer parameters (Table 1) and the subspectra (Figures 2 and 3) were obtained using the fitting methods described in the Experimental Section.

In the absence of a magnetic field, the Mössbauer spectrum (Figure 2a) consists of two features, the first a quadrupole doublet and the second a magnetically split sextet corresponding to 24% and 76% of the area, respectively. The isomer shifts and quadrupole and magnetic splittings for these features are typical of high-spin iron(III) complexes in which iron(III) is five- or six-coordinate.[20-23]

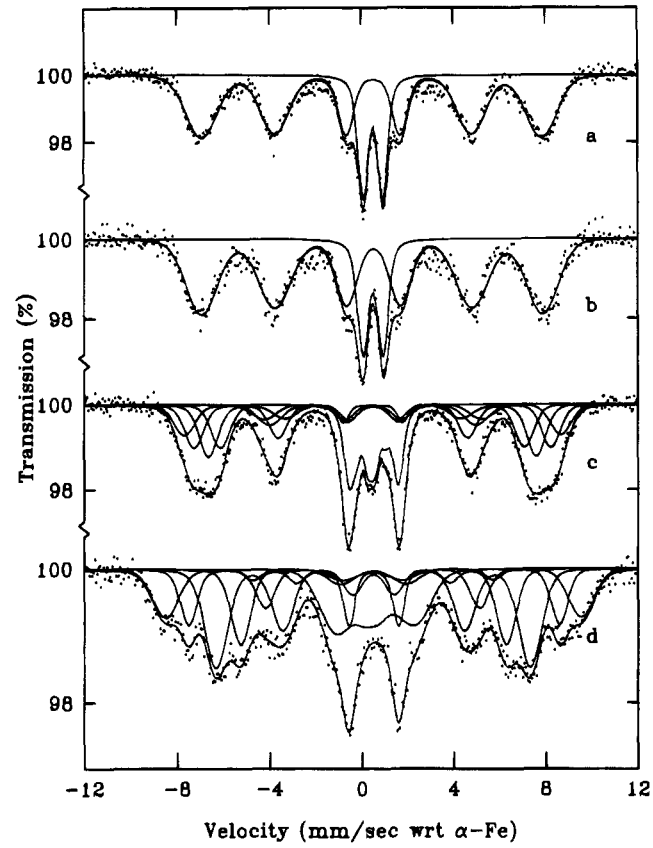

Figure 2. Experimental and fitted (—) Mössbauer spectra of ^{57}Fe-enriched ferreascidin (pH 7.0, μ = 0.2) at 4.2 K: (a) zero field, (b) B_0 = 0.5 T, (c) B_0 = 5 T, (d) B_0 = 11.1 T. The magnetic field was applied parallel to the γ-radiation beam.

Application of magnetic fields up to 11.1 T (Figures 2b–d and 3) shows a number of interesting features. The splitting of the quadrupole doublet arises only from the external magnetic field and is not modified by any hyperfine field such as would result from unpaired electrons associated with a mononuclear high-spin iron(III) site (Figure 4). Consequently, the species giving rise to this doublet (site 1, Table 1) must have a magnetic moment close to or equal to zero, i.e. $S = {}^1/_2$ or 0.

The magnetic component in the absence of a magnetic field was fitted to a single broad sextet (Figure 2a, Table 1). Application of a magnetic field up to 5 T (Figure 2b,c) results in the broadening of this sextet. This broadening arises from the summation of the applied (B_{app}) and hyperfine magnetic field (B_{hf}) vectors where the nuclear magnetic moments are precessing around the nuclear spin quantization axis (I_z) with a range of θ values. This situation corresponds to antiferromagnetically coupled iron(III) sites.[23]

At 11.1 T the broad sextet becomes partially resolved, producing a multiline spectrum. This spectrum has been fitted to four sextets arising from two sites (sites 2 and 3, Table 1, Figures 2d and 3a–e) and the zero field doublet (site 1, Table 1, Figures 2d, 3a,f). The relative areas for these sites are 20%: 31%:49% for sites 1–3, respectively. The behavior of each site in an applied magnetic field is illustrated in Figure 4. Sites 2 and 3 are separate antiferromagnetically coupled clusters which are not resolved in zero field and have a mean hyperfine field of 46.0 T. In an applied field of 11.1 T, these sites are resolved having hyperfine fields of 56.1 and 35.9 T and of 51.1 and 41.7 T respectively.

(18) Smith, T. D.; Pilbrow, J. R. In *Biological Magnetic Resonance*; Berliner, L., Reuben, J., Eds.; Plenum Press: New York, 1980; p 85.
(19) Wickman, H. H.; Klein, M. P.; Shirley, D. A. *J. Chem. Phys.* **1965**, *42*, 2113.
(20) Murray, K. S. *Coord. Chem. Rev.* **1974**, *12*, 1.
(21) Kurtz, D. M., Jr. *Chem. Rev.* **1990**, *90*, 585 and references therein.
(22) Que, L., Jr.; True, A. E. *Prog. Inorg. Chem.* **1990**, *38*, 97.
(23) Gibb, T. C. *Principles of Mössbauer Spectroscopy*; Chapman and Hall: London, 1976. (a) *Ibid.*; p 41.

Table 1. Mössbauer Parameters for ^{57}Fe Ferreascidin.[a]

	site 1, doublet					site 2, B(av) = 46.0								site 3, B(av) = 46.6							
						sextet 1				sextet 2				sextet 3				sextet 4			
B_{app}	δ	ΔE_Q	B_{hf}	$\Gamma/2^b$	A%	δ	B_{hf}	$\Gamma/2^b$	A%	δ	B_{hf}	$\Gamma/2^b$	A%	δ	B_{hf}	$\Gamma/2^b$	A%	δ	B_{hf}	$\Gamma/2^b$	A%
0	0.50	0.85	0	0.18	24	0.48	46.0	0.3–0.7	76			[c]				[c]				[c]	
0.5	0.51	0.87	0	0.20	19	0.51	46.1	0.4–0.7	81			[c]				[c]				[c]	
5.0	0.48	0	7.0	0.16–0.21	20	0.49	41.1	0.3–0.5	18	0.49	50.8	0.3–0.5	16	0.49	44.3	0.3–0.5	25	0.49	48.2	0.3–0.5	20
11.1	0.50	0	12.0	0.38–0.88	20	0.52	35.9	0.32–0.41	17	0.52	56.1	0.32–0.58	14	0.52	42.5	0.21–0.51	33	0.51	50.1	0.34–0.64	16

[a] Units: magnetic field (B)—T; isomer shift (δ), half-width at half-height ($\Gamma/2$), and quadrupole splitting (ΔE_Q)—mm/s. Error estimates: δ and ΔE_Q ±0.02, mm/s, B_{hf}, ±0.5 T. [b] The line width was allowed to vary for each pair of lines in the sextet (see Experimental Section). Consequently a range of line widths is given. [c] Data were fitted to a single sextet (sextet 1), as the sextet was unresolved at 0 and 0.5 T.

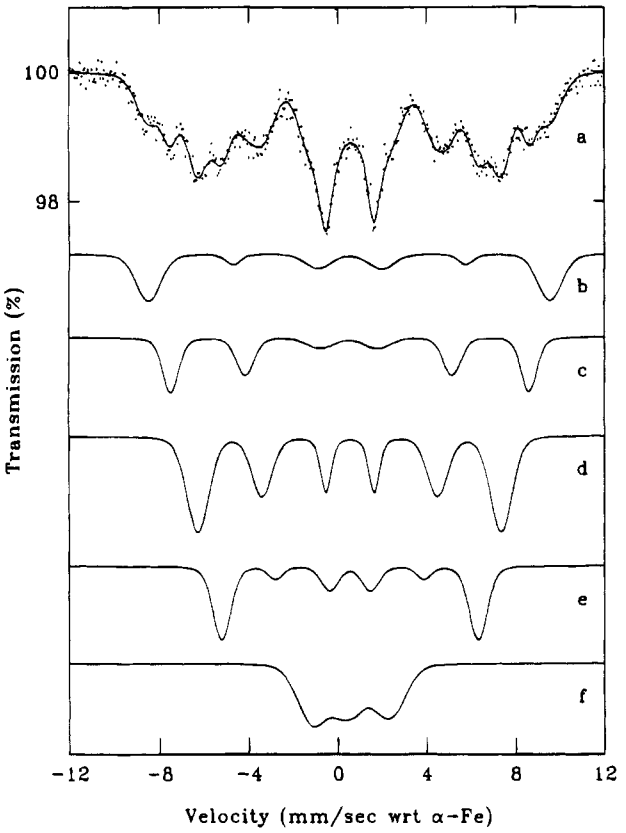

Figure 3. Expansion of the Mössbauer spectrum of ^{57}Fe-enriched ferreascidin (pH 7.0, μ = 0.2) at 4.2 K and B_0 = 11.1 T (Figure 2d) revealing the fitted subspectra: (a) experimental spectrum, (b) sextet 2, B_{hf} = 56.1 T, (c) sextet 4, B_{hf} = 50.1 T, (d) sextet 3, B_{hf} = 42.5 T, (e) sextet 1 B_{hf} = 35.9 T, (f) zero-field doublet.

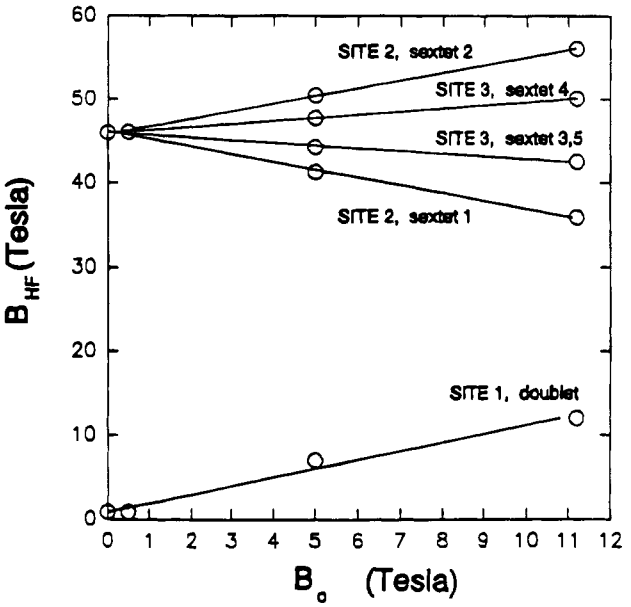

Figure 4. Plot of hyperfine magnetic field versus applied field for the different iron sites in ^{57}Fe ferreascidin.

An examination of the relative areas (Table 1) of the sextets in sites 2 and 3 reveals that the ratio of sextets 1 and 2 in site 2 is 1:1 and that for sextets 3 and 4 in site 3 is 2:1. The simplest interpretation of these results is that site 2 contains an antiferromagnetically coupled binuclear high-spin iron(III) cluster, and site 3 may be either an antiferromagnetically coupled trinuclear high-spin iron(III) cluster or two sites (sites 3 and 4), comprising an antiferromagnetically coupled pair of high-spin iron(III) ions (site 3) and a mononuclear high-spin iron(III) site (site 4).

If we assume that sextets 3 and 4 arise from two sites (3 and 4), then site 4 would have to have an identical isomer shift and applied field dependence of the hyperfine field to be coincident with sextet 3 of site 3. In addition, site 4 must also have the same population in order to give the observed overall doubling of the relative area, when compared with the other sextets (Table 1). The field dependence of this sextet (Figure 4) (decreasing with increasing applied magnetic field as the hyperfine and applied fields are diametrically opposed) is not inconsistent with a mononuclear site. However, the coordination environment of the iron(III) ion (site 4) would have to closely resemble that of one of the antiferromagnetically coupled pair of iron atoms to produce very similar hyperfine fields, a situation that is unlikely, given that a mononuclear site has at least one different coordinating ligand at the analogous position of the bridge in the binuclear site. The presence of this bridging ligand should enhance the exchange interaction between the iron atoms and hence the transferred hyperfine field. Clearly, the Mössbauer data indicate that site 3 is most probably a single antiferromagnetically coupled trinuclear high-spin iron(III) cluster.

If we assume that the hyperfine field, B_{hf}, on an individual iron atom within a cluster is oppositely directed to the spin on that atom and that B_{hf} and the applied field, B_{app}, add vectorially to give the total observed field at that nucleus, B_{obs}, then it is possible to obtain both the angle θ between the applied field and the electronic spin directions and the angle ψ between the applied field and B_{obs} from each of the sextets in sites 2 and 3. The geometry is shown in Figure 5, where we note that, in the presence of B_{app}, the spins make different angles θ_A and θ_B. Because the direction of B_{obs} is also the nuclear quantization direction, an independent evaluation of ψ_A and ψ_B can be obtained from the relative intensities of the lines (1,6):(2,5):(3,4) which are proportional to $[9(1 + \cos^2 \psi)/4]:[3 \sin^2 \psi]:[3(1 + \cos^2 \psi)/4]$.[23a] Rewriting this in the form $3:x:1$ allows the calculation of the ψ_i $\{\psi_i = \cos^{-1}[\sqrt{((4 - x)/(4 + x))}]\}$ from the average relative area of lines 2 and 5. The results are shown in Table 2, and it can be seen that the two sets of results for the ψ_i are quite consistent, indicating the basic correctness of the geometry. We see that the spins on site 2 are aligned antiparallel and reasonably closely to being parallel and antiparallel to the applied field, while those on site 3 are antiparallel and much

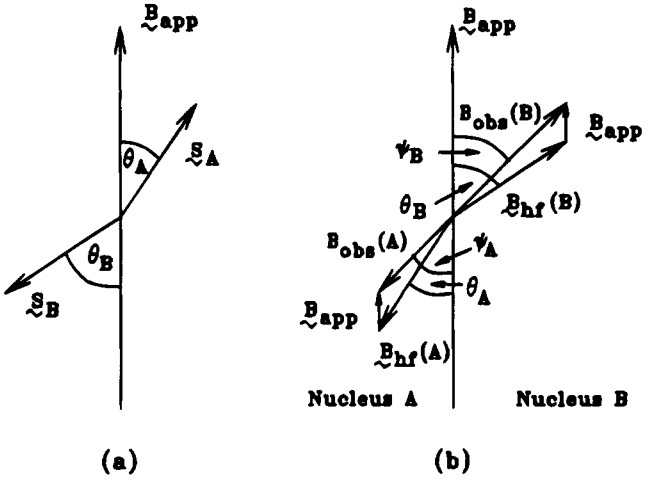

Figure 5. Orientations of the principal fields for the two different atoms in sites 2 and 3 relative to the applied magnetic field, B_{app}, of (a) the mean electronic moment directions, S_A and S_B, and (b) the vector addition of B_{hf} and B_{app} to give the total resultant field B_{obs}. B_{hf} is oppositely directed to its respective S in (a).

Table 2. Values of the Angles Defined in Figure 5, As Derived from the Mössbauer Spectrum at 11.1 T

site	sextet	ψ^a (deg)	ψ^b (deg)	θ^b (deg)
2	1	27	28	21
2	2	26	22	27
3	3	65	79	65
3	4	60	62	75

[a] Obtained from the intensities of lines 2 and 5. [b] Obtained from the observed hyperfine field using Figure 5.

more nearly perpendicular to the applied field. This would seem to indicate a very small anisotropy for site 3 and a relatively strong exchange coupling when one takes into account the fact that there is a strong uncompensated moment on this site. For the trinuclear cluster (site 3), the absence of any additional broadening in sextet 3 indicates that the two atoms contributing to this are probably identical and thus the antiferromagnetic exchange constants J_{12} and J_{23} are the same. Although atoms 1 and 3 are parallel, little can be said about J_{13} at present since it could be ferromagnetic and any size up to being comparable with the other exchange constants, or antiferromagnetic as long as it is much weaker than them. The latter possibility is the likely situation if the cluster is linear. We were unable to draw any conclusions about the orientations of the moments for sites 2 and 3 relative to the line joining the atoms. The moments could be on planes perpendicular to this line, in a plane which includes this line, or even in a plane unrelated to the line.

Discussion

Models for the Iron(III)-Binding Sites in Ferreascidin. Previous electronic absorption spectral titrations of ferreascidin with either ferric chloride or iron-NTA identified two DOPA ligands and possibly one tyrosine ligand in the coordination sphere of the iron(III) ion.[11] The analysis of these spectral titrations assumed independent binding of iron(III) to DOPA in ferreascidin. This is only consistent with the present results if DOPA is coordinated in a bidentate mode to each iron(III) center and is not involved in bridging the two ions. If DOPA were to provide one or two bridging oxygen atoms, then we would expect cooperative binding. X-ray crystallographic studies of the bis(catecholato)iron(III)[24] and piperidinium bis-(μ-1,1′-biphenyl-2,2′-diolato-O,μ-O′)-(1,1′-biphenyl-2,2′-diolato-O,O′)ferrate(III)−ethanol (1/2)[25] complexes show that the ligand provides the bridging oxygen atoms, as opposed to a μ-oxo bridge. The isotropic exchange coupling constants, J for these complexes are $J = -9.7$ and -14 cm^{-1} ($\mathcal{H} = 2JS_1 \cdot S_2$), respectively.[24,25] EPR spectra of these complexes in the solid and frozen solution states reveal a large number of resonances ranging from $g \sim 16$ to $g \sim 1$.[24,25] In the strong exchange limit the $g \sim 16$ resonance observed in the frozen solution EPR spectrum of bis(catecholato)iron(III) arises from a $\Delta M_S = \pm 4$ transition within the $|\pm 2\rangle$ doublet of an $S_T = (S_1 + S_2) = 2$ thermally populated spin state.[24] The absence of these resonances in the EPR spectrum of the iron(III) ferreascidin complex suggests that catecholate bridging of the iron(III) centers is not present in the protein (sites 1, 2 and 3), and secondly that $|J|$ for sites 1 and 2 must be greater than ~ 20 cm^{-1}.[26] The absence of catecholate bridging of the iron(III) centers is consistent with previous iron(III)-binding studies of ferreascidin.[11]

Site 1. Mössbauer spectra show that this species has a magnetic moment close to or equal to zero, $S_T = {}^1/_2$ or 0. The absence of any resonances around $g = 1.5-3$ in the EPR spectrum of ferreascidin rules out the possibility that this species can be attributed to a low-spin iron(III) ($S = {}^1/_2$) center. The most likely species is an antiferromagnetically coupled binuclear high-spin iron(III) complex in which the exchange coupling constant is significantly greater than the temperature at which the Mössbauer spectrum was recorded (4.2 K) such that the excited magnetic spin states ($S_T = 1, 2, 3, 4, 5$) cannot be thermally populated. For $J = -100$ cm^{-1}, significant population of the $S_T = 1$ spin state only occurs at temperatures greater than 15 K.[27] The isomer shifts and quadrupole splittings for site 1 are similar to those for antiferromagnetically coupled μ-oxo-bridged binuclear iron(III) complexes.[20-23] For instance, the binuclear [Fe$_2$(μ-O)(salen)$_2$] complex has $\delta = 0.56$ mm/s, $\Delta E_Q = 0.92$ mm/s, and $J = -89$ to -95 cm^{-1}.[21] Although the [2Fe-2S]$^{2+}$ clusters in plant ferredoxins ($\delta = 0.34$ mm/s, $\Delta E_Q = 0.64-0.81$ mm/s;[28] $-J \geq 200$ cm^{-1} [29]) have quadrupole splittings similar to those observed in site 1 (Table 1) and [Fe$_2$(μ-O)(salen)$_2$], the isomer shifts are somewhat lower, a consequence of changing the coordination number around the iron(III) from five- or six-coordinate (octahedral) to four-coordinate (tetrahedral).

The strong antiferromagnetic coupling between the two iron(III) ions and the absence of sulfur-containing amino acids or sulfide in the protein is indicative of at least one μ-oxo bridging ligand as depicted in Figure 6a. Although a *cis*-arrangement of the DOPA ligands is illustrated, the Mössbauer results cannot rule out the possibility of a *trans*-configuration.

Site 2. The presence of a sextet in the zero field Mössbauer spectrum of the iron(III) ferreascidin complex, which splits into sextets 1 and 2 in an applied field, indicates that the antiferromagnetic coupling ($|J|$) between the two iron(III) ions is not as strong as in site 1 but larger than ~ 20 cm^{-1}. A binuclear iron-

(24) Grillo, V. A.; Hanson, G. R.; Wang, D. M.; Hambley, T. W.; Gahan, L. R.; Murray, K. S.; Moubaraki, B.; Hawkins, C. J. *Inorg. Chem.*, in preparation.
(25) Ainscough, E. W.; Brodie, A. M.; McLachlan, S. J.; Brown, K. L. *J. Chem. Soc., Dalton Trans.* **1983**, 1385.
(26) The absence of EPR signals from a thermally populated spin state (e.g. $S = 2$) may be a consequence of significant J strain broadening, though a comparison of the X- and Q-band EPR spectra of bis(catecholato)iron(III)[24] indicates minimal strain broadening.
(27) Owen, J. *J. Appl. Phys. Suppl.* **1961**, *32*, 213S.
(28) Dunham, W. R.; Palmer, G.; Sands, R. H.; Bearden, A. J. *Biochim. Biophys. Acta* **1971**, *253*, 134. Isomer shifts reported in this reference were calibrated with respect to ^{57}Co diffused into platinum. These values were modified for an iron foil reference using a conversion shift of 0.349 mm/s.
(29) (a) Petersson, L.; Cammack, R.; Rao, K. K. *Biochim. Biophys. Acta* **1980**, *622*, 18. (b) Palmer, G. In *Iron sulfur proteins, Vol. 2*; Lovenberg, W., Ed.; Academic Press: New York, 1973; p 285.

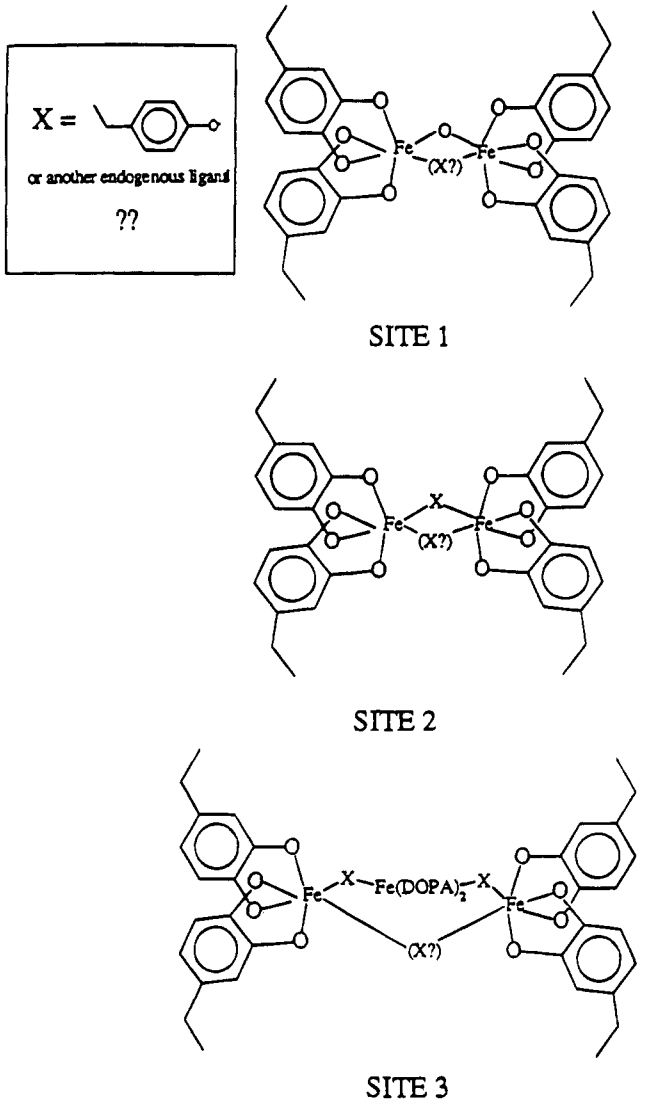

Figure 6. Models for the Iron(III)-Binding Sites in Ferreascidin.

(III) center with one or two bridging ligands is proposed for the structure of site 2 (Figure 6b). μ-Hydroxo or μ-phenoxo groups would furnish this intermediate exchange coupling between the centers.[21] A μ-phenoxo bridge is a more likely candidate, as tyrosine constitutes 42% of ferreascidin's amino acid composition[3] and the proton on a hydroxy bridge would be expected to readily dissociate at neutral pH, leaving a μ-oxo bridge, which would produce stronger antiferromagnetic coupling. Other amino acids potentially capable of coordination and/or bridging are far less ubiquitous in the structure of the protein (aspartic acid (2%), threonine (2%), serine (2%), glutamine (1%), methionine (1%), lysine (3%), and histidine (7%)).[3] μ-Phenoxo bridging (formation or dissociation) may also conveniently explain the wavelength shift, observed in the electronic absorption spectral titration of ferreascidin with iron(III)-NTA which was attributed to the displacement of tyrosine from the iron(III)'s coordination sphere.[11] The 360 nm chromophore of ferreascidin, which is of unknown structure, is another potential candidate for a bridging ligand.

Site 3. Mössbauer spectra reveal two sextets (3 and 4) with isomer shifts and hyperfine splittings consistent with iron(III) and having relative areas in the ratio of 2:1 (Table 1), indicating the presence of a trinuclear iron(III) cluster. EPR data show that this cluster is paramagnetic with an $S = {}^5/_2$ ground state.[30] Possible structures for this site include (a) a cubane-like cluster, (b) a triangular cluster, or (c) a linear cluster. A brief discussion of these three possibilities is given below.

(a) Cubane-like Cluster. Mössbauer, EPR, and low-temperature MCD have provided considerable insight into the magnetic coupling within the trinuclear $[3Fe\text{-}4S]^+$ clusters in iron–sulfur metalloproteins, for example the inactive form of aconitase and oxidized ferredoxin I from *Azotobacter vinelandii*.[31,32] The $[3Fe\text{-}4S]^+$ cluster in aconitase has a cubane type structure with an iron atom missing from one of the apices.[33] EPR and Mössbauer spectroscopies have shown that two of the high-spin iron(III) centers (S_1 and $S_2 = {}^5/_2$) are antiferromagnetically coupled ($S' = S_1 + S_2$) producing $S' = 0, 1, 2, 3, 4$ and 5 spin states.[34] The excited spin states $S' = 2$ and 3 can then antiferromagnetically couple with the third high-spin iron(III) center ($S_3 = {}^5/_2$), producing an EPR-active $S_T = {}^1/_2$ ground state, provided that $-J_{23} > -J_{12} > -J_{13} > 0$, $0.5 < J_{13}/J_{23} < 1$, and $0.6 < J_{12}/J_{23} < 1$.[34] However, EPR studies of iron(III) ferreascidin revealed resonances at $g = 9.32$ and 4.27 arising from an $S = {}^5/_2$ ground state and not at $g = 2.01$ as found for the $[3Fe\text{-}4S]^+$ clusters in metalloproteins,[35,36] thus eliminating this structural motif.

(b) Triangular Bridged Cluster.[37] Another possibility is that of antiferromagnetic interactions between the three iron atoms, which occur in triangular bridged complexes, for example $[Fe_3O(OOCR)_6L_3]^-$ (R = methyl, fluoromethyl, and chloro-, dichloro-, and trichloromethyl; L = aqua). At room temperature the EPR spectra consist of a large number of overlapping resonances, which simplify to a single asymmetric resonance around $g \approx 2$ at 4.2 K. The Heisenberg, Dirac, and Van Vleck scheme for triangular iron(III) complexes reveals that the lowest spin state at 4.2 K is $S' = {}^1/_2$, which has 4-fold degeneracy. The complex room-temperature spectra were assumed to arise from thermally populated spin states.

The room-temperature zero field Mössbauer spectra show a quadrupole doublet ($\Delta E_Q = 0.45\text{–}0.75$ mm s^{-1}) and are consistent with three iron(IIII) ions in an equilateral triangle. The hyperfine field for each of the three iron(III) ions, assuming ideal trigonal symmetry, is 3.67 T; however, if the degeneracy of the ground state is raised by lowering the symmetry, then the hyperfine fields for each ion will be different. These small hyperfine fields result from magnetic (spin) frustration. The EPR and Mössbauer results for this type of cluster are clearly inconsistent with the $S = {}^5/_2$ ground state and the large hyperfine fields found for site 3.

(c) Linear Cluster. The $[3Fe\text{-}4S]^+$ cluster in the inactive form of aconitase can be converted into a linear purple form by increasing the pH to 10.5.[35] In contrast to the native protein,

(30) Spin concentration measurements have not been performed, as the calculations require a knowledge of the transition probability for the reference and unknown samples. Although the transition probability can be calculated from the exchange coupling constants and the rhombic and axial zero field splitting parameters, these values are unknown for site 3 in ferreascidin, and consequently any estimate of the number of spins would be meaningless.

(31) Münck, E. In *Iron-Sulfur Proteins*; Spiro, T. G., Ed.; John Wiley and Sons: New York 1982; p 147.
(32) Johnson, M. K.; Robinson, A. E.; Thomson, A. J. In *Iron-Sulfur Proteins*; Spiro, T. G., Ed.; John Wiley and Sons: New York, 1982; p 367.
(33) Robbins, A. H.; Stout, C. D. *Proc. Natl. Acad. Sci. U.S.A.* **1989**, *86*, 3639.
(34) Kent, T. A.; Huynh, B. H.; Münck, E. *Proc. Natl. Acad. Sci. U.S.A.* **1980**, *77*, 6574.
(35) Kennedy, M. C.; Kent, T. A.; Emptage, M.; Merkle, H.; Beinert, H.; Münck, E. *J. Biol. Chem.* **1984**, *259*, 14463.
(36) (a) Kuchta, R. D.; Hanson, G. R.; Holmquist, B.; Abeles, R. H. *Biochemistry* **1986**, *25*, 7301. (b) Townson, S. M.; Hanson, G. R.; Upcroft, J. A.; Upcroft, P. *Eur. J. Biochem.* **1994**, *220*, 439.
(37) Cannon, R. D.; White, R. P. *Prog. Inorg. Chem.* **1988**, *36*, 195.

the purple form shows a Mössbauer spectrum consisting of three subspectra (δ_i = 0.28, 0.28, and 0.31 mm/s; B_{hfi} = 33, 32, and 24 T; i corresponds to sextets 1−3) (ΔE_Q could not be determined at 200 K because of the slow relaxation of the $S = {}^5/_2$ site) and an EPR spectrum (g = 9.6, g = 4.3) arising from an $S_T = {}^5/_2$ ground state in which S_1 and S_2 are ferromagnetically coupled producing $S' = 5$ which is then antiferromagnetically coupled with $S_3 = {}^5/_2$ for $J_{12} \sim J_{23} > J_{13}$.[35] In the high magnetic fields employed in our experiments, we would expect that in such a situation the nuclei associated with S_1 and S_2 would see different total fields, resulting in, at least, broadened lines in sextet 3 even if the sites did not give completely resolved subspectra.

Mössbauer and magnetic susceptibility studies of the $[Fe_3S_4(SR)_4]^{3-}$ (R = ethyl, phenyl) clusters isolated by Girerd *et al.*[38] yield data (δ = 0.23−0.29 mm/s at 77 K, ΔE_Q = 0.52−0.63 mm/s, $J_{12} = J_{23} = -300$ cm^{-1}, and $-100 < J_{13} < 100$ cm^{-1}) which are vitually identical to those found for the purple form of aconitase.[35] Interestingly, for both the $[Fe_3S_4(SR)_4]^{3-}$ cluster and the purple form of aconitase, the Mössbauer subsite spectra are different, while two of the three subsite spectra are identical for the trinuclear cluster (site 3) in the iron(III) ferreascidin complex.

Recently, the trinuclear iron(III) complexes $[Fe_3(\mu\text{-}OH)_2(\mu\text{-}OAc)_4(T1,4DMIP)_2](PF_6)_3$·THF[39] and $[Fe_3(\mu\text{-}O)(\mu\text{-}OH)(\mu\text{-}OAc)_4(HB(3,5\text{-}iPr_2pz)_3)_2]$·MeCN[40] were synthesized and characterized with X-ray crystallography, Mössbauer spectroscopy, and EPR spectroscopy. These complexes share many but not all of the characteristics of site 3. The X-band EPR spectra of these complexes display resonances at g = 9.46, 4.29[39] and 9.7, 4.3[40] which are characteristic of an $S = {}^5/_2$ ground state. Magnetic susceptibility measurements show that adjacent high-spin iron(III) ions are antiferromagnetically coupled ($J_{12} \approx J_{23}$) while the terminal iron(III) ions are weakly ferromagnetically coupled ($J_{13} \approx 0$ cm^{-1}). The room-temperature Mössbauer spectrum of $[Fe_3(\mu\text{-}OH)_2(\mu\text{-}OAc)_4(T1,4DMIP)_2](PF_6)_3$·THF consists of a single quadrupole doublet at zero field (δ = 0.41 mm/s, ΔE_Q = 0.74 mm/s)[39] while two quadrupole doublets (δ = 0.47, 0.52 mm/s, ΔE_Q = 1.71, 0.52 mm/s) in the ratio of 2:1 are observed for $[Fe_3(\mu\text{-}O)(\mu\text{-}OH)(\mu\text{-}OAc)_4(HB(3,5\text{-}iPr_2pz)_3)_2]$·MeCN.[40] At low temperatures multiline spectra were observed, indicating a paramagnetic ground state, though no attempt at this stage was made to analyze the spectra.

Of the proteins and model complexes discussed above, the structure most consistent with the trinuclear iron(III) cluster (site 3) in ferreascidin is a linear cluster similar to the bis(μ-hydroxo)-bridged model complex[39] in which each pair of iron(III) ions is antiferromagnetically coupled ($J_{12} \approx J_{23}$) and there is weaker coupling between the terminal iron(III) ions (Figure 6c). Conclusive identification of the bridging groups is impossible at this stage, as there is insufficient information (J_{12}, J_{23}, and J_{13}) to determine whether the bridging groups are μ-oxo, μ-hydroxo, μ-phenoxo, the chromophore, an unknown group, or a combination of the above.

Formation of Sites 1−3. A ferric chloride solution (0.1 M, pH 3.0) was added to ferreascidin buffered at pH 7.0 with Bistris. The fact that NaOH was required to maintain the pH at 7.0 (see Experimental Section) indicates that 5 mM Bistris was insufficient to maintain the pH at 7.0. At no point during the addition was the pH allowed to drop below 5.0. Ferric chloride solutions (0.1 M) in the presence of $NaClO_4$ (3 M) are known to contain the following species:[41] Fe(III), $Fe(OH)^{2+}$, $Fe(OH)_2^+$, $Fe(OH)_3$, $Fe(OH)_4^-$, $Fe_2(OH)_2^{4+}$, and $Fe_3(OH)_4^{5+}$, the predominant species at pH 3.0 being the trinuclear cluster. It is possible that the binuclear and trinuclear moieties have been directly added to ferreascidin, with the remaining ligating sites on iron(III) being filled by two DOPA ligands. At an iron concentration of 10^{-5} M, which was employed in the spectral titrations of iron(III) with ferreascidin, only mononuclear iron hydroxo species are present.[41] The similarity of the electronic absorption and EPR spectra for the iron(III) ferreascidin complex prepared herein and that in the spectral titration study[11] indicates that the iron(III)-binding sites 1−3 were also present in the spectral titration studies. Interestingly, at low concentrations of iron, mononuclear iron may bind to ferreascidin with subsequent formation of the bi- and trinuclear species with the addition of bridging ligands, while at higher iron concentrations the bi- and trinuclear moieties may be added directly to ferreascidin. In the latter case the bridging ligand(s) (μ-oxo, μ-hydroxo) may be replaced by a functional group from the protein, for instance a μ-phenoxo group from tyrosine.

Previous Mössbauer and EPR Studies on Iron(III) Ferreascidin. Hawkins *et al.*[10] have reported the formation of an iron sulfur cluster complex "ferriascid" when ferreascidin is treated with iron(II) sulfate. In the reduced form, ferriascid was reported to be a yellow precipitate which turned blue in the presence of oxygen. Ferreascidin may be stored in the reduced form *in vivo*, as the blood cells have a yellow appearance, while the iron(III) ferreascidin complex is purple at neutral−alkaline pH and blue in slightly acidic media. Given current results, however, this conclusion should be taken with caution since (i) sulfate readily precipitates the yellow protein, (ii) oxidation of iron(II) would produce iron(III), which would be readily bound by the protein, and (iii) other properties of the oxidized form of the iron sulfur cluster such as its electronic absorption and EPR spectra are fully explained by the results reported herein and the iron-binding studies.[11] Current results cannot explain the reported liberation of CO_2 on addition of iron(II) sulfate nor of H_2S on addition of concentrated HCl to the oxidized form of "ferriascid" observed by Hawkins *et al.*[10]

A comparison of the Mössbauer parameters for "ferriascid"[10] with those for the ^{57}Fe ferreascidin sample studied herein reveals a number of similarities. The zero-field spectrum at 4.2 K had a single, slightly asymmetric quadrupole doublet with δ = 0.53 mm/s and ΔE_Q = 0.91 mm/s. No evidence of a magnetically split sextet was observed at zero field, in contrast with the current sample. With an applied longitudinal field of 5 T, the spectrum was composed of a sextet (δ = 0.5 mm/s, B_{hf} = 54.2 T), with broadened inner edges of lines 1 and 6, and a nonmagnetic component (δ = 0.98 mm/s, B_{hf} = 1.8 T). The difference between the iron(III) ferreascidin complex enriched with ^{57}Fe and "ferriascid" is either related to the degree of purity or microheterogeneity present in each preparation or to how the sample for Mössbauer measurements was prepared (frozen solution versus a freeze-dried solid mixed into boron nitride[10]). Ferreascidin used in this study was purified to homogeneity using the method described by Dorsett *et al.*[3] with modifications described by Lavin *et al.*[4] The extent of the microheterogeneity in this sample was ascertained from the ratio of A_{277} to A_{360} and was found to be 2.44. Fereascidin with A_{277}/A_{360} greater

(38) Girerd, J. J.; Papaefthymiou, G. C.; Watson, A. D.; Gamp, E.; Hagen, K. S.; Edelstein, N.; Frankel, R. B.; Holm, R. H. *J. Am. Chem. Soc.* **1984**, *106*, 5941.

(39) Vankai, V. A.; Newton, M. G.; Kurtz, D. M. *Inorg. Chem.* **1992**, *31*, 341. Applying an isomer shift correction of 0.14 mm/s for the thermal red shift yields a value of 0.55 mm/s for δ at 4.2 K.

(40) Kitijima, N.; Amagai, H.; Tamura, N.; Ito, M.; Moro-oka, Y.; Heerwegh, K.; Penicaud, A.; Mathur, R.; Reed, C. A.; Boyd, P. D. W. *Inorg. Chem.* **1993**, *32*, 3583.

(41) Baes, C. F., Jr.; Mesmer, R. E. *The Hydrolysis of Cations*; R. E. Kreiger: FL, 1986, p 237.

than 3 was found to have undergone considerable oxidation of the DOPA residues. A value of 2.44 for A_{277}/A_{360} indicates that this sample of ferreascidin exhibits minimal oxidation. Unfortunately, the corresponding data for ferreascidin used in the previous Mössbauer study are unavailable, though the ratio of A_{277} to A_{360} would certainly have been greater than 3, as the purity of ferreascidin preparations has increased significantly since that time.

Concluding Remarks

Mössbauer spectroscopy has revealed that the binding of iron(III) to ferreascidin is much more complicated than first thought, with evidence for three sites. Although the precise values of the isotropic exchange coupling constants are not yet obtainable, Mössbauer and EPR spectra provide estimates for the ranges of values that J may have for each of the sites: site 1—a strongly antiferromagnetically coupled ($-J \geq 100$ cm^{-1}) binuclear iron(III) site; site 2—an antiferromagnetically coupled binuclear iron(III) site with an exchange coupling constant of intermediate magnitude, $\sim 20 < -J < \sim 80$ cm^{-1}; site 3—a trinuclear iron cluster in which the three high-spin iron(III) centers are antiferromagnetically coupled with $J_{12} \approx J_{23} > J_{13}$ producing a paramagnetic $S = {}^5/_2$ ground state.

Identification of the iron-binding site responsible for the spectral shift observed in the electronic absorption spectrum (associated with the displacement of tyrosine[11]) of iron(III) ferreascidin should be obtainable from a pH-dependent study of the Mössbauer and EPR spectra. Although the EPR spectra of the iron(III) ferreascidin complex, measured at pH 5.0 and 7.0, have identical line shapes and $|E/D|$ ratios, it is still possible that there is a change in the axial and rhombic zero field splitting parameters D and E (providing that both D and E are greater than the microwave quantum, 0.3 cm^{-1}) which could account for the spectral shift observed in the electronic absorption spectrum.[11] Alternatively, this spectral shift may involve at least one of the EPR-silent sites (sites 1 and/or 2) observed in the Mössbauer spectra.

Unequivocal identification of the bridging ligands present in sites 1−3 of iron(III) ferreascidin remains the subject for further investigation. Although the molecular mass of the protein is quite small (10 000 Da), the microheterogeneity of the protein eliminates the possibility of structural determination by X-ray crystallography. A combination of spectroscopic methods and spectral comparisons with results obtained for well-characterized model complexes presents the best opportunity to further investigate the iron(III)-binding sites in ferreascidin. In particular, the determination of the exchange coupling constant (J) from magnetic susceptibility, Mössbauer spectroscopy, or magnetic circular dichroism experiments should allow determination of which of the possible bridging ligands (μ-oxo, μ-hydroxo, or μ-phenoxo) are present in the three antiferromagnetically coupled bi- and trinuclear high-spin iron(III) clusters. Examination of the near edge X-ray absorption spectrum may also provide evidence for the existence of a μ-oxo bridge.[42]

It is unlikely that all the **three** different sites would be found in one molecule of protein, as accommodation of the three proposed sites would require 14 unmodified DOPA residues.[3,6] This number of DOPA residues, when combined with 35 tyrosines (amino acid analysis data predict 2.47 tyrosine/DOPA), would give an extinction coefficient at 280 nm of ~70 000 M^{-1} cm^{-1} (without consideration of a 42% contribution of the 360 nm chromophore to the absorbance at 280 nm[6]) compared with the experimentally observed value[5b] of 29 000 M^{-1} cm^{-1}. A more likely possibility is that the different sites reflect the microheterogeneity present in ferreascidin preparations, with different forms of the protein favoring different clusters. Thus the stability constant reported by Taylor *et al.*[11] may reflect an average value for the different sites.

The biological implications of ferreascidin interacting with, or forming, these iron(III) clusters are unclear. It is interesting to speculate that ferreascidin may act as a nucleating agent for ferritin-like iron(III) oxide deposits, especially in view of the question of metal accumulation in ascidians. However, such mineral-like deposits have not, as yet, been identified in *Pyura stolonifera*'s blood cells by analytical electron microscopy and, as stated previously, there is no evidence for the formation of these complexes in intact cells. The observation of a "transient purplish gelatinous mass" upon the addition of dilute sodium hydroxide to the corpuscles of this ascidian[43] suggests that iron(III) ferreascidin complexes might constitute part of the hemostat of injured *Pyura stolonifera* individuals after ferreascidin and iron are concomitantly released from the cells on lysis.

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(42) (a) Penner-Hahn, J. E. In *Metal Clusters in Proteins*; Que, L., Ed.; American Chemical Society: Washington, DC, 1988; p 28. (b) Gorun, S. M. In *Metal Clusters in Proteins*; Que, L., Ed.; American Chemical Society: Washington, DC, 1988; p 152.

(43) Endean, R. *Aust. J. Mar. Freshwater Res.* **1955**, *6*, 35.