Binuclear Iron Centres in Proteins

This Review is dedicated to Dennis Darnall, who introduced the Author to haemerythrin. His recent tragic death saddens us.

Ralph G. Wilkins

Department of Chemistry, University of Warwick, Coventry CV4 7AL

1 Introduction

Iron is the fourth most prevalent element and the most abundant transition element in the Earth's crust. It is not surprising then that it occurs widely in biological materials which furthermore display a large variety of functions.[1]

Familiar to the reader is the iron–haem moiety which, in proteins, can transport and store dioxygen (haemoglobin and myoglobin), activate oxygen for insertion into a C–H bond (cytochrome P-450), and act as a conduit for electron transfer (cytochromes). It is probably less appreciated that there are a whole array of iron proteins, without a porphyrin ring, which can function in a similar way, albeit in simpler organisms. These are an emerging class of proteins which contain an oxygen bridged binuclear iron unit, designated Fe–O–Fe. The prominent members of this group and their general features are shown in Table 1. Intense interest in these proteins in the past twenty years is shown by a recent spate of reviews.[2-6] There is, in addition, a large number of enzymes containing iron bound to sulfur donors in bridging arrangements (the iron–sulfur proteins),[1] but we shall not be concerned with these in this Review.

The iron contents of all the proteins in Table 1 have been subjects of confusion and controversy, but the values are now established except perhaps for the case of methane monooxygenase. All contain at least one binuclear iron centre for which three states of oxidation might be anticipated, designated Fe_2^{III}, Fe_2^{II}, and $Fe^{III}Fe^{II}$. These entities are all known for each protein, although they are of variable stability. Most of the early investigations employed the fully oxidized species since these are usually the (stable) isolated forms. The proteins containing one and (especially) two Fe^{II} ions have been examined later because of their instability (sensitivity to air) and their lack of obvious spectral properties. Haemerythrin is the most studied and best characterized protein of the group in Table 1.[3] The various techniques which have been used to characterize the iron sites in these proteins were often first used with haemerythrin and so they will feature heavily in the discussions of that protein. Their applications to the other proteins will be detailed in the subsequent sections. Generally an historical sequence will be used in Section 2. Original literature references, except to the latest work, can be found in the reviews cited.

2 Structural Aspects

2.1 Haemerythrin

Haemerythrin is the oldest member of the group, having first been described in 1823. It usually occurs as an octamer in the coelomic fluid of the sipunculid (peanut worm) and as a monomer (myohaemerythrin) in the retractor muscle of the worm. Myohaemerythrin (13.9 kDa) is very similar to the octamer subunit, both structurally and in its properties. The relationship of the iron to the peptide chain has been known for some time and is shown diagrammatically in Figure 1. In deoxyhaemerythrin, both irons are in the + 2 oxidation state and this is the species which binds dioxygen reversibly to form oxyhaemerythrin. The latter slowly autoxidizes to methaemerythrin, in which both irons are in the + 3 oxidation state. This form, and a recently discovered mixed oxidation state containing an $Fe^{III}Fe^{II}$ unit do not bind O_2 but, typical of iron(III), they combine with a number of anions, the azide derivatives being particularly useful for examination.[3] The first inklings of the presence of a binuclear iron site with an oxide ion bridge in methaemerythrin and derivatives, as well as, significantly, in oxyhaemerythrin, came from an examination of their electronic spectra. The peak positions and relative intensities ($M^{-1}cm^{-1}$) for azidomethaemerythrin at 325 nm ($\epsilon = 6.8 \times 10^3$), 380 nm (sh) ($\epsilon = 4.3 \times 10^3$) and 446 nm ($\epsilon = 3.7 \times 10^3$) closely resemble those of an iron(III)–edta complex which has an *X*-ray crystallographically established oxo bridge $[Fe_2(edta)_2O]$, $\epsilon = 1.06 \times 10^4$ at 335 nm, 8.1×10^2 at 405 (sh) and 2.0×10^2 at 475 nm.

Two antiferromagnetically coupled high-spin iron(III) ions have low magnetic moments at 298 K (1.8—3.0 BM) and the magnetic susceptibility of the entity decreases with a decrease in temperature (anti-Curie behaviour). Such properties in simple metal complexes had been interpreted by Lewis and his coworkers in the 60s.[7] Their emergence in methaemerythrin (as well as the absence of an EPR signal) afforded further evidence for a bridged diiron site in the protein. A useful measure of the interaction between the two spins is the exchange coupling constant, J, defined by the spin-exchange Hamiltonian, $H_{exch} = -JS_1S_2$. Its value can be estimated from the temperature dependence of the magnetic susceptibility and is roughly a measure of the energy required to convert the system from a spin-paired into a spin-free state.[2] A negative value of J indicates antiferromagnetic coupling and the large value determined for azidomethaemerythrin and oxyhaemerythrin is typical for binuclear iron(III) complexes with μ-oxo bridges (Table 2). It was already clear, therefore, how valuable a comparison of the spectral and magnetic characteristics of this protein with iron complexes could be. These observations initiated the design and production of a very large number of model complexes[2,7] with interesting inorganic chemistry. These could be used also for comparisons with this and the other proteins of the group.

Mössbauer spectroscopy was another tool that was early used to probe the nature of the iron site in haemerythrin.[3,8] The ^{57}Fe isotope is particularly suitable for such study. The important parameters are the isomer shift (δ), which is the displacement of the doublet centre from zero, and the quadrupole splitting (ΔE_q), which is the energy difference between the two peaks (Figure 2).[8] Isomer shifts of about 0.5 mm s^{-1} and quadrupole splittings < 2 mm s^{-1} are typical of Fe^{3+} (Table 2). Lack of broadening of the spectra by an applied field at 4 K indicates that the irons are antiferromagnetically coupled. In addition *two* quadrupole doublets in oxyhaemerythrin (Figure 2 and Table 2) indicate that the two irons are inequivalent and that the species is best formulated as a met derivative containing the $Fe_2^{III}O_2^{2-}$ unit. The larger values of the isomer shift and quadrupole splitting for deoxyhaemerythrin (Figure 2 and Table 2) confirm that we are dealing with Fe^{2+} centres. A single quadrupole doublet does, however, falsely suggest that there are equivalent irons, which we now know is not the case.

Ralph G. Wilkins was born in Southampton and obtained his degrees while attending Southampton University College at the end of World War II. He has had a very enjoyable academic life in chemistry departments at the University of Sheffield, State University of New York at Buffalo, New Mexico State University, and the University of Warwick. He is Emeritus Professor at New Mexico State University and Honorary Professor at the University of Warwick.

He has had over forty years experience in research and teaching in the area of mechanisms of inorganic and bioinorganic reactions and some 150 publications.

Table 1 Proteins containing a binuclear iron unit and O-containing bridge(s)

Protein	Occurrence	Function	Ref.
Haemerythrin	Several phyla of marine invertebrates. Those from sipunculids (peanut worms) *Phascolopsis gouldii*, *Themiste zostericola*, and *Themiste dyscrita* most examined.	Stores and transports diooxygen.	2,3
Ribonucleotide reductase	Animals, bacteria, and virus-infected mammalian cells. Iron enzyme one of four types. That from *Escherichia coli* best characterized.	Catalyses formation of deoxyribonucleotide di- or tri-phosphates (first step in DNA synthesis).	2,4
Purple acid phosphatase	Glycoproteins from mammalian, plant, and microbiol sources. Those from bovine spleen and porcine uterine fluid (uteroferrin) most studied.	Unknown physiological role. Catalyses hydrolysis of phosphate esters in pH ~ 5—6.	2,5
Methane monooxygenase	Methanotropic bacteria. Those from Roman Baths (*Methylococcus capsulatus* (Bath), and *Methylosinus trichosporium* most studied.	Catalyses the oxidation of CH_4 to CH_3OH. Can also insert O into C—H bond of large variety of substrates.	2,6

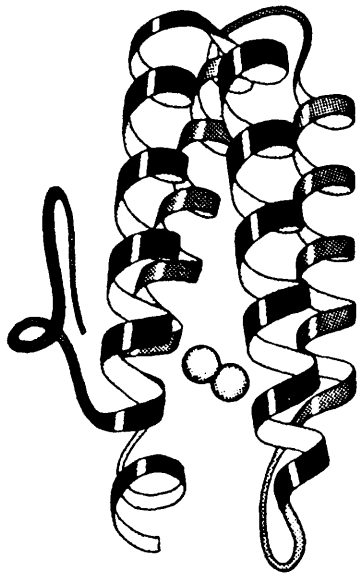


Figure 1 Tertiary structure of haemerythrin showing the relationship of the Fe_2 unit with the four α-helices in myohaemerythrin. (Reproduced by permission from J. S. Richardson, *Adv. Protein Chem.*, 1981, **34**, 167.)

In principle, infra-red measurements might yield information on vibrational modes at the chromophore site. Unfortunately there is substantial background due to water and protein so that resonance Raman is preferred, this being less sensitive to water and in addition resonance enhancement of vibrational modes is observed.[2] Resonance Raman peaks near 500 cm^{-1} and 800 cm^{-1} were assigned to ν_s(Fe—O—Fe) and ν_{as}(Fe—O—Fe) in methaemerythrin and oxyhaemerythrin. These assignments have been subsequently used to detect the presence of the Fe—O—Fe bridging unit in other proteins. An O—O stretch at 844 cm^{-1} in oxyhaemerythrin could be identified with that in peroxide. By using $^{16}O^{18}O$ it could be further shown that the two oxygen atoms were inequivalent, thus ruling out a symmetrically bound O_2 moiety. Spectral examination of polarized single crystals of oxyhaemerythrin and azidomethaemerythrin showed a similar position and mode of bonding for the O_2 and N_3^- moities, respectively. This was a timely finding which was shortly to be substantiated by *X*-ray crystallography.

The determination of the structure of oxidized haemerythrin by *X*-ray crystallography featured relatively early in the chemical history of this protein. This was a fortunate circumstance since it aided considerably the interpretation of the spectroscopic data with this protein and furthermore laid a firm basis for their applications to the other diiron proteins. The methaemerythrin azide adduct was chosen for study because of its stability. Early disagreement between two groups[9] working independently gave way in the early 80s to a consensus and the details of the iron site, roughly shown in Figure 1, now became clear (Figure 3).[9] Both irons are octahedrally coordinated and bridged, not only by the expected oxo group, but, quite surprisingly, also by an aspartate and a glutamate residue. These features are retained in oxyhaemerythrin, with the dioxygen bound to one iron(III) in an end-on-fashion, and with one oxygen close enough to the bridging oxygen atom to be hydrogen bonded to it, *i.e.* it is present as HO_2^- (Scheme 1). The lower value

Table 2 Magnetic parameters for iron-oxo complexes and proteins[a]

Species	$-J/cm^{-1}$	δ^b/mms^{-1}	$\Delta E_Q/mms^{-1}$
Oxidized forms			
Methaemerythrin	134	0.46[c]	1.57[c]
Oxyhaemerythrin	77	0.51	1.96
		0.52	0.95
Rubrerythrin	—	0.52	1.47
Ribonucleotide	108	0.55	1.62
reductase (Protein B2)		0.45	2.44
Uteroferrin[d]	⩽40	0.55	1.65
		0.46	2.12
Methane monooxygenase	32	0.50	1.07
$Fe_2O(OAc)_2(Me_3TACN)_2^{2+}$[e]	115	0.47	1.50
Reduced forms			
Deoxyhaemerythrin	13	1.14	2.76
Rubrerythrin	—	1.30	3.14
Ribonucleotide reductase (reduced Protein B2)	5	1.26	3.13
Uteroferrin (semi-reduced form)[d]	10	0.53[f]	1.78[f]
		1.22[g]	2.63[g]
Methane monooxygenase	—	1.30	3.14
$Fe_2O(OAc)_2(Me_3TACN)_2^{+}$[e]	15	0.48[f]	0.42[f]
		1.19[g]	3.38[g]
$Fe_2(OH)(OAc)_2(Me_3TACN)^{+}$[e]	13	1.16	2.83

[a] Mainly at 4 K. Data from L. Que, Jr., and A. E. True, *Prog. Inorg. Chem.*, 1990, **38**, 97, Tables I and II. [b] All isomer shifts are quoted relative to iron metal at room temperature. [c] Azide derivative shows two ΔE_Q values (1.47 and 1.95) and one δ value (0.51 mms^{-1}). [d] These values are modified on addition of phosphate. [e] Me_3TACN = 1,4,7-trimethyl-1,4,7-triazacyclononane. [f] High spin Fe^{3+}. [g] High spin Fe^{2+}.

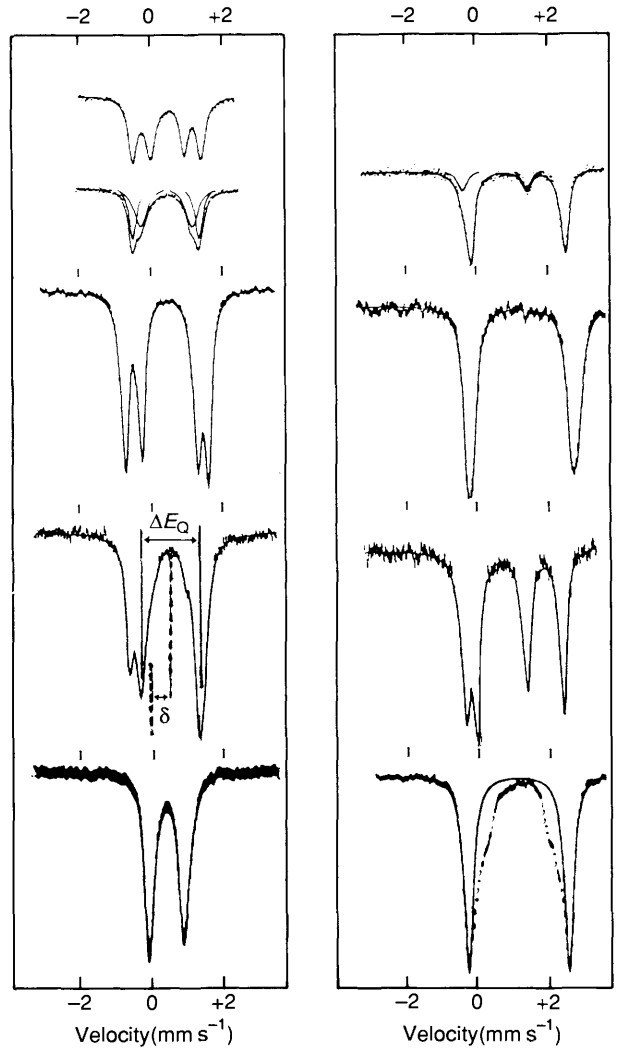


Figure 2 Mössbauer spectra of dinuclear iron proteins. Left, oxidized forms, from top, *Phascolosoma lurco* oxyhaemerythrin and azidohaemerythrin; *Escherichia coli* ribonucleotide reductase subunit B2; pig allantoic fluid purple acid phosphatase; *Methylosinus trichosporium* OB3b methane monooxygenase hydroxylase component. Right, corresponding fully reduced forms except pink (semi-reduced) acid phosphatase. Left ordinate represents absorption. All determinations at ~4 K except pink acid phosphatase (185 K).
[Adapted by permission from P. E. Clark and J. Webb, *Biochemistry*, 1981, **20**, 4628 (haemerythrin); J. B. Lynch, C. J.-Garcia, E. Münck, and L. Que, Jr., *J. Biol. Chem.*, 1989, **264**, 8091 (ribonucleotide reductase); P. G. Debrunner, M. P. Hendrich, J. De Jersey, D. T. Keough, J. T. Sage, and B. Zerner, *Biochim. Biophys. Acta*, 1983, **745**, 103 (purple acid phosphatase); B. G. Fox, K. K. Surerus, E. Münck, and J. D. Lipscomb, *J. Biol. Chem.*, 1988, **263**, 10553 (methane monooxygenase)].

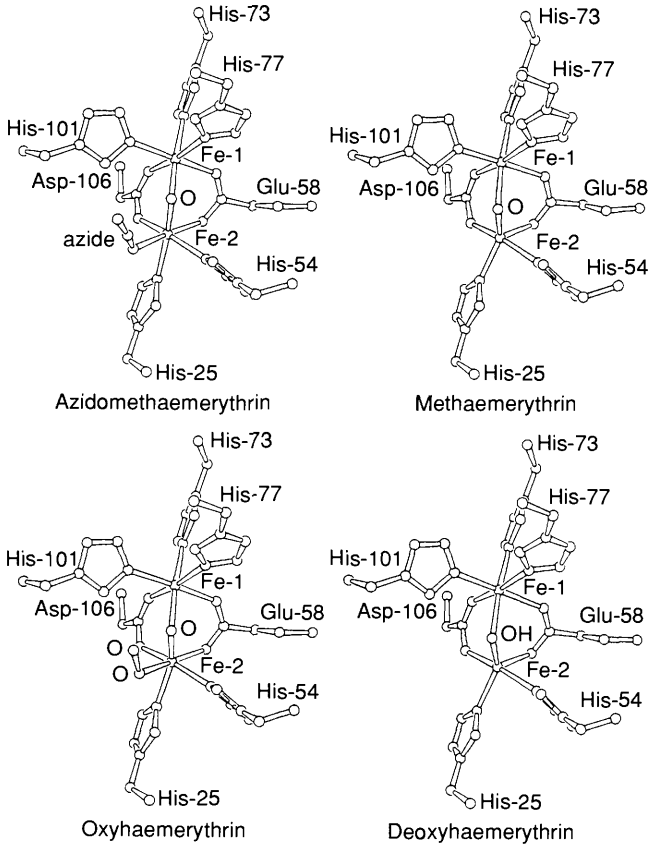
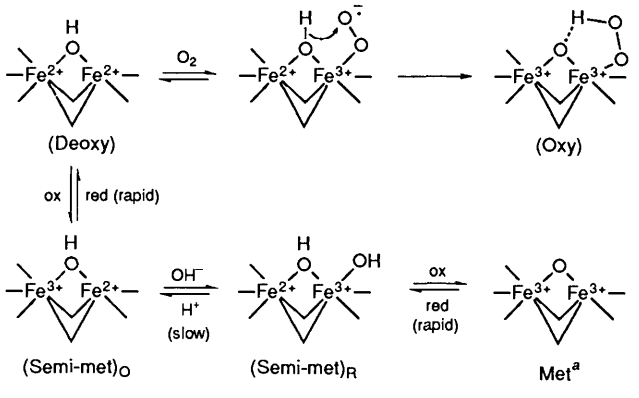


Figure 3 The binuclear iron complex in azidomethaemerythrin, methaemerythrin, oxyhaemerythrin, and deoxyhaemerythrin. In all cases octameric protein from *Themiste dyscrita* was used.



Scheme 1 Suggested mechanisms for interconversion of various forms of haemerythrin.
(Based on R. E. Stenkamp, L. C. Sieker, L. H. Jensen, J. D. McCallum, and J. Sanders-Loehr, *Proc. Natl. Acad. Sci. USA*, 1985, **82**, 713 and J. M. McCormick, R. C. Reem, and E. I. Solomon, *J. Am. Chem. Soc.*, 1991, **113**, 9066.)
[a]Species isolated in pH 6–7 region. ox = $Fe(CN)_6^{3-}$ and red = $S_2O_4^{2-}$. Within the bridge, Fe=Fe represents a glutamate and an aspartate linkage. Outside the bridge, Fe– represents a histidine residue.

of ν_s(Fe–O–Fe) for oxyhaemerythrin, by about 20cm^{-1}, than for the other met derivatives, had already been ascribed to hydrogen bonding of the O_2 moiety.

The structure of methaemerythrin (Figure 3) also produced a big surprise. It might have been anticipated that the azide in azidomethaemerythrin would be replaced by the water in anion-free methaemerythrin. In fact, the one iron(III) associated with N_3^- or O_2 in the met derivative becomes five-coordinated (Figure 3). The structure of deoxyhaemerythrin at 2.0 Å has been recently reported.[10] The longer Fe–O bond lengths and larger Fe–O–Fe angle (Table 3) support the idea, broached previously on the basis, for example, of a low $-J$ value (Table 2), that the bridging oxo group in deoxyhaemerythrin is protonated. The five- and six-coordinated irons in deoxyhaemerythrin and methaemerythrin confer bridge asymmetry (Figure 3). Bond distances and angles for all forms of haemerythrin are contained in Table 3.[10]

The application of electron paramagnetic resonance (EPR) to these systems has been very rewarding. New types of EPR signals were first observed for both the mixed oxidation state[11] and the fully reduced forms[12] of haemerythrin. As well as their intrinsic theoretical interest,[13] such signals have become import-

Table 3 Crystallographic data for various forms of haemerythrin[a]

Parameter	Azidomet[b]	Met	Oxy	Deoxy
3hisFe-O^{2-}(Å)	1.80	1.92	1.88	2.15
2hisFe-O^{2-}(Å)	1.79	1.66	1.79	1.88
Fe···Fe(Å)	3.23	3.25	3.27	3.32
Fe-O-Fe(°)	130	127	125	111

[a] From M. A. Holmes, I. Le Trong, S. Turley, L. C. Sieker, and R. E. Stenkamp, *J. Mol. Biol.*, 1991, **218**, 583. [b] Similar values for myohaemerythrin.

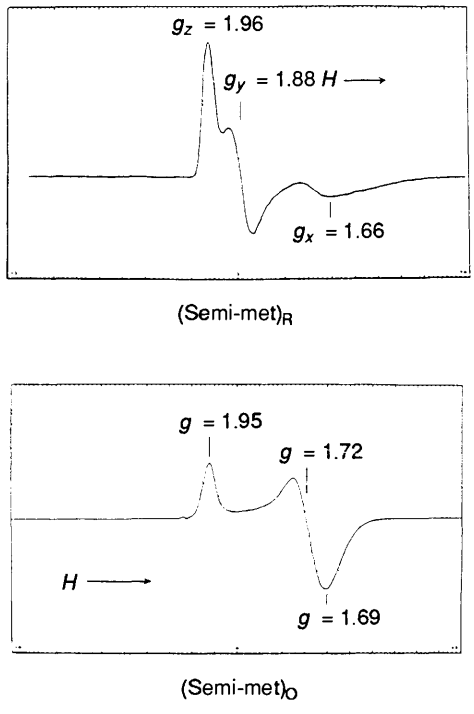


Figure 4 EPR spectra of (semi-met)$_O$ and (semi-met)$_R$ haemerythrin from *Phascolopsis gouldii*. Temperature = 10 K. (Reproduced by permission from B. B. Muhoberac, D. C. Wharton, L. M. Babcock, P. C. Harrington, and R. G. Wilkins, *Biochim. Biophys. Acta*, 1980, **626**, 337.)

ant clues for the existence of this type of binuclear iron unit in other proteins.

The species containing the mixed oxidation states (the so-called semi-methaemerythrin) escaped detection until the late 70s. One-electron reduction of methaemerythrin and one-electron oxidation of deoxyhaemerythrin gave two distinct species, termed (semi-met)$_R$ and (semi-met)$_O$ respectively, with different spectral characteristics and kinetic behaviour. These showed rhombic and axial EPR respectively only at liquid He temperatures with $g_{av} \sim 1.7$—1.8 (Figure 4 and Table 4).[11] These arise from an $S = \frac{1}{2}$ state with antiferromagnetic coupled high-spin Fe^{III} and Fe^{II} and $J \sim -8$ (Table 2), consistent with an endogenous bridging OH group. These signals resemble those found in iron–sulfur proteins which have a higher g_{av}, and g values greater than two (Table 4). Variable-temperature magnetic circular dichroism (MCD) and EPR spectroscopies have been interpreted in terms of structures for the two forms shown in Scheme 1.[14]

Quite surprisingly, strong adducts of deoxyhaemerythrin with certain anions are formed. The interaction has been detected by competition experiments with O_2[15] and directly by MCD as well as by the observation of a new type of EPR signal at $g \approx 16$ (with the azide adduct).[12] Weak ferromagnetic coupling between the

Table 4 Values of g for the EPR spectra near liquid helium temperatures of the mixed oxidation states of binuclear iron proteins

Species	g values
Themiste zostericola	
haemerythrin (semi-met)$_R$	1.96, 1.88, 1.66
(semi-met)$_O$	1.95, 1.72, 1.69
(semi-met)N_3^-	1.90, 1.82, 1.50
Desulfovibrio vulgaris	
rubrerythrin	
(semi-met)$_R$	1.98, 1.76, 1.57
Escherichia coli	
ribonucleotide reductase	1.93, 1.85, 1.64
Beef spleen acid phosphatase	
(pink) low pH form	1.94, 1.78, 1.65
high pH form	1.85, 1.73, 1.58
Methylococcus capsulatus (Bath)	
hydroxylase	
semi-reduced	1.92, 1.86, 1.71
Bacterial type ferredoxins	
$Fe_3^{III}Fe^{II}$form	2.12, 2.04, 2.03

irons and transitions within doublets of an integer spin system give rise to this signal, which is not observed in deoxyhaemerythrin but has since been detected in the reduced forms of ribonucleotide reductase and methane monooxygenase(*vide infra*) and other iron complexes and proteins.[2]

Finally *X*-ray absorption fine structure (EXAFS) has been the technique most recently applied to these proteins.[2a,16] The determination of accurate bond distances is possible and since concentrated solutions (mM) may be used, the method has particular value when the *X*-ray crystallographic approach has problems (lack of suitable crystals, high molecular weight protein). The iron-containing protein is subjected to high energy monochromatic *X*-rays. At a certain energy of incident radiation (around 7140 ev), transition of 1*s* electrons to unfilled 4*p* and 5*p* levels occurs and results in a pronounced absorption edge which is ~2—4 ev lower for Fe^{II} than Fe^{III}. At higher energies, photoelectrons are ejected and an absorption continuum results. Because of interference between the outgoing photoelectron wave from the iron and the returning wave from the backscattered ligand atom, or another iron, modulation of the continuum occurs. The degree and form of modulation can be used to give information on the distances and the types of neighbouring atoms involved,[2a,16] The use of simple model complexes for comparative purposes is essential and the selection of inappropriate models early resulted in, for example, longer values for the Fe···Fe distances for methaemerythrin than measured by *X*-ray crystallography. These difficulties have now been mainly overcome. Examination of Tables 3 and 5 shows generally good agreement (except for deoxyhaemerythrin) in the distances and angles at the diiron site measured by the two techniques. EXAFS experiments on semi-methaemerythrin show lengthening of the Fe–μ-O and Fe···Fe bonds, (Table 5) which, taken in conjunction with data from model complexes, suggests conversion of the bridging oxo into an hydroxo group as in deoxyhaemerythrin. Detailed information on the sites in purple acid phosphatase and methane monooxygenase has also been obtained from EXAFS measurements (*vide infra*).

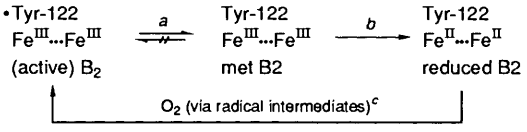
2.2 Ribonucleotide Reductase

Ribonucleotide reductase from *E. coli* (Table 1) consists of two dimeric non-identical proteins α_2 and β_2 known as protein B1 and protein B2 respectively.[4] The large α subunits contain the nucleotide binding site as well as redox-active cysteines. Protein B2 contains a tyrosyl radical near the diiron centre, the first

Table 5 EXAFS data for various forms of haemerythrin[a]

Parameter	Azidomet	Met	Oxy	Deoxy	Azidosemimet
Fe–μ-O(Å)	1.80	1.82	1.82	1.98	1.87
Fe···Fe(Å)	3.13—3.19	3.13	3.24	3.57	3.46
Fe–O–Fe(°)	126	118	128	128	135

[a] From L. Que, Jr. and A. E. True, *Prog. Inorg. Chem.*, 1990, **38**, 97.



Scheme 2 Redox chemistry of ribonucleotide reductase protein B2. [a] Mild reduction (hydroxyurea or enzymatically). [b] Stronger reductants ($S_2O_4^{2-}$/methyl viologen or enzymatically). [c] Stopped-flow and rapid freeze-quench EPR indicate two intermediates, J. M. Bollinger, Jr., D. E. Edmondson, B. H. Huynh, J. Filley, J. R. Norton, and J. Stubbe, *Science*, 1991, **253**, 292.

example of several proteins now known to contain a stable free radical.[1] This gives rise to an EPR ($g = 2.0047$) signal and a sharp absorption bond at 410 nm. These features disappear if B2 is reduced by hydroxyurea (or the tyrosine is replaced by phenylalanine by site-specific mutagenesis). The material, thus treated, termed met B2 (Scheme 2) now closely resembles methaemerythrin with iron-related spectral bands at 325 nm, 370 nm, 500 nm, and 600 nm.[17] The likelihood that B2 contained an iron site similar to that in oxy- and met-haemerythrin was strongly supported by variable-temperature magnetic susceptibility measurements (strong antiferromagnetically coupled high spin Fe^{III} signals) and resonance Raman spectra [ν_s(Fe–O–Fe) at 492 cm^{-1}].[4] It was long believed that there was only one binuclear cluster at the interface of the two identical polypeptide chains of protein B2. This would mean that the two irons would be expected to be indistinguishable. The observation of *two* pairs of Mössbauer quadrupole doublets of approximately equal intensity (Table 2 and Figure 2) was difficult to rationalize on this basis. This fact, together with improved iron analyses, led eventually to the courageous suggestion that the B2 dimer contained *two* diiron centres, bound to each of the polypeptide chains.[18] The δ and ΔE_Q values (Table 2) suggested that there was highly distorted high-spin Fe^{III}, while EXAFS experiments supported a coordination environment for the irons similar to that in haemerythrin, but with fewer nitrogen (histidine) ligands. Short Fe–O (1.8 Å), and Fe···Fe (~3.2 Å) bonds probably meant additional bridge or bridges to that provided by an oxo group.[2] All these features were satisfyingly confirmed in the recently reported three-dimensional structure of B2 at 2.2 Å resolution.[19] The iron site is shown in Figure 5.

There are indeed two diiron centres about 25 Å apart, each centre resting in the middle of four α-helices, reminescent of that of haemerythrin (Figure 1), although the Fe···Fe axis is parallel (not perpendicular) to the helices. The two irons are octahedrally coordinated but the irons have marked differences in the ligands used. There is an oxo and a single carboxylate bridge. The radical provided by Tyr-122 is ~5 Å from the closest iron and ~10 Å from the surface. Protein B2 can be reduced to a form containing two Fe^{II} ions (Scheme 2). Susceptibility and Mössbauer parameters of reduced B2 (Table 2) point to a structure similar to that in deoxyhaemerythrin, *i.e.* weak coupling of two high-spin Fe^{II} ions. It has been a puzzle for some time why a semi-reduced form of the protein has not been reported. Characteristic EPR signals (Table 4) have now, however, been observed when B2 is reduced anaerobically with diimide at pH 6.5 [20a] or reduced by γ-irradiation at 77 K.[20b]

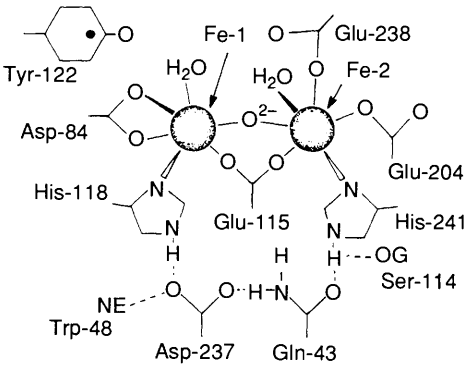


Figure 5 The coordination at the diiron site in *Escherichia coli* ribonucleotide reductase B2 protein.
(Reproduced by permission from P. Nordlund, B.-M. Sjöberg, and H. Eklund, *Nature*, 1990, **345**, 593.)

2.3 Purple Acid Phosphatase

Acid phosphatases have maximum hydrolytic activity towards orthophosphate monoesters in the slightly acid region (equation 1).[5] The metal-dependent subclass most studied contains iron and in particular, proteins isolated from porcine uterine fluid (uteroferrin, 35 kDa) and from bovine spleen (35 kDa). At first, as was often the case, the iron was not detected. Later, the iron content of uteroferrin became a controversial value. It was finally established when it was found that phosphate, which is tightly bound to the iron centre, interferes with the iron analysis.[5] Both proteins contain two irons per molecule. There is 90% homology in the structures of the beef spleen and porcine proteins and their properties are very similar.[5,21]

$$ROPO_3^{2-} + H_2O \rightarrow ROH + HPO_4^{2-} \quad (1)$$

The oxidized (purple) form is reduced in a one-electron process to the pink form, which is the enzymatically active mixed-valence form. Both forms have high absorptions ($\epsilon = 2 \times 10^3$ $M^{-1}cm^{-1}$/Fe) at 550 nm (purple) and 510 nm (pink). This is ascribed to one tyrosine coordinated to one of the Fe^{III} ions. This must therefore be the iron in the purple form which remains unreduced in the production of the pink form. Resonance Raman spectra of the purple and pink forms using visible excitation showed four resonance-enhanced peaks between 1600 cm^{-1} and 1160 cm^{-1} due to a coordinated tyrosine ring, but there is no evidence for an Fe–O–Fe symmetric stretch at ~500 cm^{-1} (Figure 6). Magnetic susceptibility and Mössbauer spectroscopy showed antiferromagnetically coupled iron centres, strong in the oxidized form and weak in the reduced species (Table 2). One of the most telling pieces of evidence for a haemerythrin-like binuclear iron site in the pink form is the low temperature EPR spectrum. The rhombic signal results from a mixture of a low pH form and a high pH form (Table 4) with a pK_a ~4.4. It is interesting that there are also two forms of semi-methaemerythrin interconverted by pH (Scheme 1, *vide infra*). Since suitable crystals of the protein have yet to be obtained for *X*-ray crystallographic examination, knowledge of the active site is still conjectural. Based on the experience with haemerythrin, and the EXAFS data (which suggest, because of a short Fe···Fe distance, that there is multiple bridging) as well as other spectral information, one that has been suggested is shown in Figure 7.[21]

The Fe_2 unit may also occur in some plant acid phosphatases.[2b] An interesting enzyme is present in red kidney beans. It contains one Zn^{2+} ion and one Fe^{3+} and has a similar site to that in the acid phosphatases. This is suggested by a visible absorption peak at 560 nm ($\epsilon = 3.4 \times 10^3 M^{-1}cm^{-1}$) and an EPR signal at $g = 4.3$, characteristic of high spin Fe^{III}. More significantly, replacement of (labile) Zn^{2+} by Fe^{2+} leads to an $Fe^{3+}Fe^{2+}$ form with a characteristic EPR ($g = 1.88, 1.76, 1.62$, and 1.49). Conversely, replacement of one iron(III) in purple

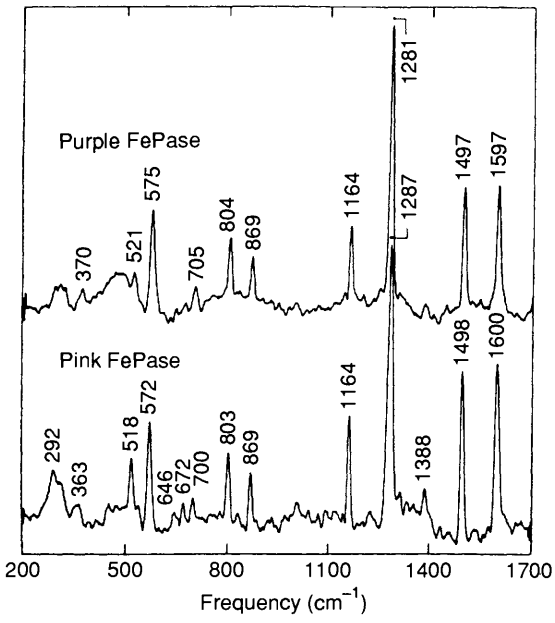


Figure 6 Resonance Raman spectra of purple and pink forms of beef spleen purple acid phosphatase, at pH 5.0 and 5 °C, using 514.5 nm excitation.
(Reproduced by permission from B. A. Averill, J. C. Davis, S. Burman, T. Zirono, J. S.-Loehr, T. M. Loehr, J. T. Sage, and P. G. Debrunner, *J. Am. Chem. Soc.*, 1987, **109**, 3760.)

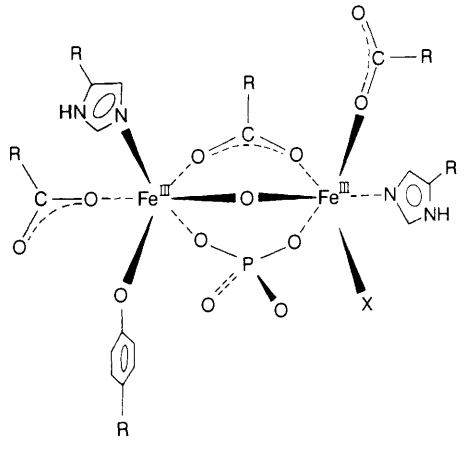


Figure 7 Proposed active site structure of purple acid phosphatase (phosphate complex).
(Reproduced by permission from J. B. Vincent and B. A. Averill, *The FASEB Journal*, 1990, **4**, 3009.)

uteroferrin by Zn^{2+} gives a protein which retains activity and has spectral characteristics (electronic, EPR) similar to those in kidney bean acid phosphatase.[2,5]

2.4 Methane Monooxygenase

The three types of respiratory proteins, which combine reversibly with dioxygen (haemoglobin, haemocyanin, and haemerythrin)[1] have corresponding oxygenases which use dioxygen (*e.g.* cytochrome P450, tyrosinase, and methane monooxygenase respectively).

There are two kinds of methane monooxygenase, the membrane bound enzyme containing copper and a soluble enzyme containing non-haem iron (s-MMO).[6] The soluble enzyme from *Methylococcus capsulatus* (Bath) and from other sources (Table 1) consists of three components: A, an hydroxylase (250 kDa) containing binuclear iron and substrate binding sites; B, a small (16 kDa) regulator protein with no metal chromophores; and C, a reductase (40 kDa) which accepts electrons from reduced nicotinamide adenine dinucleotide (NADH) and passes them to component A, one at a time. The whole entity catalyses reaction 2.

$$NADH + CH_4 + O_2 + H^+ \rightarrow NAD^+ + CH_3OH + H_2O \quad (2)$$

Protein A has been most studied but details of the iron site are the least understood of the four proteins considered in this section. Protein A has no absorption much above 350 nm and this poses a severe problem in its investigation. The magnetic parameters (Figure 2 and Table 2) hint at an antiferromagnetically coupled high spin Fe^{III} component with bridging provided by an OH on an OR group, but probably not an O group, because of the low J value. The best evidence for a binuclear iron site of the type considered in this Review is the appearance (low temperatures) of a rhombic EPR signal (Table 4) due to the production of the $Fe^{III}Fe^{II}$ form on mild reduction of methane monooxygenase,[22] and an integer spin EPR ($g = 16$) on strong reduction giving the $Fe^{II}Fe^{II}$ form.[23] A recent EXAFS study of protein A yields an Fe···Fe distance of 3.4 Å[24]

Further clues as to the nature of the iron site in the hydroxylase component of MMO emerge from a comparison of the amino-acid sequence for MMO and ribonucleotide reductase protein B2. As might be anticipated, the six amino-acids involved in the diiron site (Figure 5) are amongst the few amino-acid residues conserved in nine different sources of the B2 protein. In two separate subregions, the ligands Glu-115, His-118 and Glu-238, His-241 in B2 protein can be aligned with Glu-144, His-147 and Glu-243, His-246 in MMO.[6] It is not unreasonable then to suppose that these amino-acids in MMO are involved in metal binding in a similar type of diiron site as established in ribonucleotide reductase.

3 Reactivity of the Binuclear Iron Sites

This section must, of necessity, be brief but an attempt will be made to emphasize the important aspects.

3.1 Haemerythrin

The reaction of physiological importance is the reversible binding of dioxygen to deoxyhaemerythrin (equation 3, X = O_2), and this has been the focus of both structural and kinetic studies. The reaction is pictured as shown in Scheme 1. The high formation rate constants (k_f, Table 6), particularly for deoxymyohaemerythrin, prompted the investigators (Table 6, footnote *a*) to suggest that there was *addition* of O_2 to one of the Fe^{II} atoms and that Fe^{II}–H_2O replacement was not involved. This idea is supported by the now known structure of deoxyhaemerythrin (Figure 3). Binding by NO, but not CO, also occurs (Table 6).

Table 6 Kinetic and thermodynamic data for reaction of deoxyhaemerythrin with ligands X at 25 °C

X	$10^{-6} \times k_f$ $M^{-1}s^{-1}$	k_d s^{-1}	$10^{-6} \times K$ M^{-1}
O_2[a]	7.4(78)	51(315)	0.15(0.25)
NO[b]	4.2	0.84	5.0
HN_3[c]	0.03	0.1	0.3
HCNO[c]	0.058	0.012	4.8
HF[c]	0.005	0.01	0.50

[a] A. L. Petrou, F. A. Armstrong, A. G. Sykes, P. C. Harrington, and R. G. Wilkins, *Biochim. Biophys. Acta*, 1981, **670**, 377. Values in parenthesis for reaction of *Themiste zostericola* deoxymyohaemerythrin. [b] J. Springborg, P. C. Wilkins, and R. G. Wilkins, *Acta Chem. Scand.*, 1989, **43**, 967. [c] P. C. Wilkins and R. G. Wilkins, *Biochim. Biophys. Acta*, 1987, **912**, 48.

$$\text{deoxyhaemerythrin} + X \underset{k_d}{\overset{k_f}{\rightleftharpoons}} \text{deoxyhaemerythrin}\cdot X \qquad (3)$$

It was noted quite early that oxyhaemerythrin could be rapidly and reversibly bleached by anions such as azide, cyanate, and fluoride. This effect results from a surprisingly strong interaction of these anions with deoxyhaemerythrin. This has also been detected by MCD and EPR experiments.[12] The kinetics of formation and dissociation of the colourless anion adducts have been measured by the stopped-flow technique using the coloured oxyhaemerythrin as a competitive probe. The interesting results that emerge are that (a) binding is quite strong, comparable to that of O_2, (b) anion is introduced as the neutral HX adduct, and (c) k_f and k_d values (Table 6) are very small for an Fe^{II}–ligand interaction. These points emphasize the importance of the hydrophobic nature of the site on the dynamics of reactions which occur there.

The physiological significance of the semi-methaemerythrins, if any, is unknown. Some very interesting chemistry involving these forms has been reported. The two semi-met forms are in a pH-dependent equilibrium which is believed to involve a ligand-induced intramolecular electron transfer (Scheme 1). The interconversion is slow, and a first-order conformational change ($k \sim 10^{-3}\ s^{-1}$) is believed to control this and a number of other reactions involving haemerythrin.[3] The structures proposed explain the early observations of rapid interconversions of $(\text{semi-met})_O$ and deoxyhaemerythrin and of $(\text{semi-met})_R$ and methaemerythrin.[3]

3.2 Ribonucleotide Reductase

It is established that the tyrosine radical plays an essential role in the overall catalysis. The tyrosine radical might generate a protein radical on the B1 subunit and this in turn abstract a C-3′H for the nucleoside diphosphate substrate and initiate the deoxygenation reaction. What is quite unclear is the function of the iron centre, although it is suggested that it is necessary for the generation and stabilization of the radical. The redox chemistry of ribonucleotide reductase protein B2 (Scheme 2) shows that the tyrosyl radical, once reduced, can only be regenerated *via* the $Fe^{II}Fe^{II}$ form reacting with O_2. The reduced B2 form thus mediates the oxidation of Tyr-122. An oxidoreductase which may produce reduced B2 *in vivo* has been identified.[2c]

3.3 Purple Acid Phosphatase

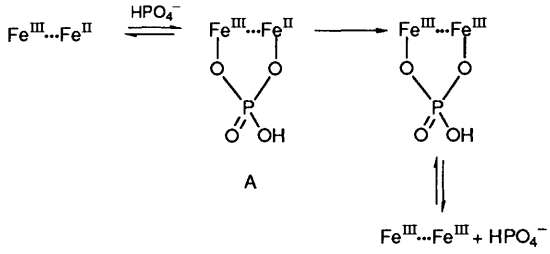
The physiological role of these proteins is uncertain. A number of oxyanions are inhibitors of the pink (reduced) enzyme. That most studied is phosphate (P_i) which is a competitive inhibitor and is indeed isolated with the enzyme. It is believed that a weak complex ($Uf_{red}\cdot P_i$) is reversibly formed and that this is then converted into the oxidized strong adduct ($Uf_{ox}\cdot P_i$) with loss of activity (equation 4).

$$Uf_{red} + P_i \rightleftharpoons Uf_{red}\cdot P_i \rightarrow Uf_{ox}\cdot P_i \qquad (4)$$

Evidence for the initially formed adduct was obtained by Mössbauer spectroscopy.[25] The EPR signals of $Uf_{red}\cdot P_i$ are broad and were finally detected only with difficulty, in the presence of those due to free Uf_{red}.[26] The proposed interaction of phosphate with the suggested diiron site in uteroferrin is shown in Scheme 3. Similar behaviour of a substrate ($ROPO_3H^-$) would facilitate hydrolysis and promote phosphate and phenolate formation.[26,27]

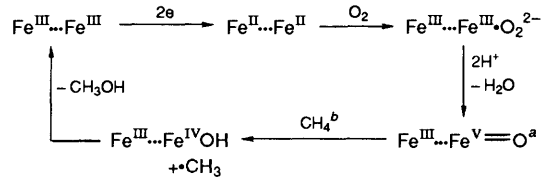
3.4 Methane Monooxygenase

Many of the reactions catalysed by methane monooxygenase are similarly catalysed by cytochrome P450. Parallelisms in their mechanism of action have been sought therefore, despite the profound differences in the coordination sites of the two proteins. Thus it is suggested that O_2 binds at, and substrate binds near, the iron centre with both proteins. A suggested substrate



Scheme 3 Proposed interaction of HPO_4^- with pink acid phosphatase. Production of A goes *via* a unidentate HPO_4^- binding to Fe^{III}.

radical mechanism is shown in Scheme 4.[6,28,29] A high-valent iron intermediate is postulated which abstracts a H atom from, for example, CH_4 to produce the methyl radical. Evidence for the $^{\bullet}CH_3$ radical has been obtained by recent exciting spin-trapping experiments.[30] Radicals have also been observed from the substrates methanol ($^{\bullet}CH_2OH$) and acetonitrile ($^{\bullet}CH_2CN$).[30] A similar type of mechanism to that shown in Scheme 4 has been suggested for the reaction of the diferrous forms of ribonucleotide reductase Protein B2 with O_2 and the concomitant production of tyrosine radicals.[31]



Scheme 4 Proposed mechanism for s-MMO-catalysed conversion of CH_4 into CH_3OH. An OH or OR bridge is represented by Fe···Fe. [a] The $Fe^{III}\cdots Fe^{V}=O$ entity may be in equilibrium with an $Fe^{IV}\cdots Fe^{IV}=O$ species. [b] It is likely that CH_4 is attached near the Fe···Fe entity during the whole sequence.

4 Replacement and Removal of Iron

The replacement of the metal in the native form of metalloprotein by a different metal is an engineering process which has long fascinated bioinorganic chemists. Invariably this conversion requires the production of the apo form, that is the protein stripped of the metal. This has to be effected sufficiently gently that the addition of the native metal ion to the apo form regenerates the original protein with substantially unchanged properties.

This process has been achieved with all the proteins listed in Table 1. The apo proteins are formed by dialysis of the metalloprotein against a chelating agent sometimes (haemerythrin and purple acid phosphatases) in the presence of a reducing agent, in which case it is the Fe_2^{II} form which is being treated. With uteroferrin, treatment with dithionite alone (no chelating agent) leads to the rapid loss of one iron and a slower (hours) removal of the second iron. The production of the apo form of uteroferrin containing no iron can be speeded up by the addition of denaturing agent. The presence of denaturing agent is also required for the successful preparation of apohaemerythrin,[32] while imidazole (probably) induces conformational changes and thus exposes the iron to attack (by 8-hydroxyquinoline) in the production of aporibonucleotide reductase protein B2. The chelating agents which are usually employed are catechols, 8-hydroxyquinoline, and 4,4′-bipyridine.

The revival of the native from the apo form can be quite difficult.[32] Usually the anaerobic addition of iron(II) salts in the presence of reducing agents produces the iron(II) form which can then be oxidized, usually by oxygen to the iron(III) form, which must have the native protein characteristics. If ^{57}Fe-enriched iron salts are used in the regeneration process, it is possible to produce ^{57}Fe-enriched protein which is useful for more sensitive

Mössbauer measurements.[32] Other metallo-derivatives may be prepared by treatment of the apo protein with the appropriate metal salt. In this way it may be determined how essential is the iron in the function of the protein. For example, the addition of Zn^{2+} to the 'half-apo' form of uteroferrin (in which only one of two irons is removed) produces an $Fe^{3+}Zn^{2+}$ form. This remains active and resembles the phosphatase from red kidney bean (Section 2.3). The $Zn^{2+}Zn^{2+}$ form generated from apo-uteroferrin and zinc salts is however inactive, thus demonstrating the necessity of one iron in the native protein. The distinctive properties of metal ions may also be exploited. Thus, the apo site in methane monooxygenase may be probed by titration with Mn^{2+} using the characteristic EPR spectra of Mn^{2+}, which is different from the Mn^{II}-containing enzyme.[33] It is clear that there are potential values in the use of the apo- and metallo-derivatives.

5 Future Developments

The detection of the binuclear iron site in biological materials has come slowly since its establishment in haemerythrin. The question arises as to whether there will be yet more examples. A realisation of the disparate nature of the proteins shown in Table 1 suggests that a diiron site should always be considered in newly found iron-containing proteins or, indeed, existing ones. Thus a novel non-haem protein isolated from the periplasmic fraction of *Desulfovibrio vulgaris* was recently shown to contain two rubredoxin-like $Fe(SR)_4$ centres. In addition, a binuclear iron centre was found. The latter must resemble that in haemerythrin since it has similar properties ascribable to that site (Tables 2 and 4) as well as those expected for a rubredoxin,[2b,c]. The physiological role for the protein (termed rubrerythrin) is unknown, a situation we have noted for the purple acid phosphatases.

Ferritin is an iron storage protein with a large number of irons, bridged by oxo and phosphate groups, inside a protein shell.[34] Ferritin, without the iron core, can be treated with a mixture of Fe^{2+} and Fe^{3+} ions in O_2 to regenerate the protein. In the early stages there is evidence for binuclear iron cluster formation, judging from Mössbauer, EPR, and EXAFS studies.[2b,35] Values of g for one of the initial transients, for example, are 1.95, 1.88, and 1.77 implicating a $Fe^{III}Fe^{II}$ oxo-bridged species.

The structural characterization of these proteins proceeds well apace. The mechanism of their action and particularly the varying roles of the diiron sites in these proteins are much less understood and it will require much ingenuity for their solution.

Acknowledgements. The author is grateful to Drs. H. Dalton and E. I. Solomon for preprints of publications and to Dr. R. E. Stenkamp for furnishing Figure 3.

6 References

1 J. J. R. Frausto da Silva and R. J. P. Williams, 'The Biological Chemistry of the Elements', Clarendon Press, Oxford, 1991, Chapter 12 and 13.
2 (*a*) J. Sanders-Loehr, in 'Iron Carriers and Iron Proteins', ed. T. M. Loehr, VCH Press, New York, 1989, 375; (*b*) J. B. Vincent, G. L. Olivier-Lilley, and B. A. Averill, *Chem. Rev.*, 1990, **90**, 1447; (c) L. Que, Jr. and A. E. True, *Prog. Inorg. Chem.*, 1990, **38**, 97.
3 (*a*) R. G. Wilkins and P. C. Harrington, *Adv. Inorg. Biochem.*, 1983, **5**, 51; *Coord. Chem. Rev.*, 1987, **79**, 195; I. M. Klotz and D. M. Kurtz, Jr., *Acc. Chem. Res.*, 1984, **17**, 16.
4 B.-M. Sjoberg and A. Graslund, *Adv. Inorg. Biochem.*, 1983, **5**, 87.
5 B. C. Antanaitis and P. Aisen, *Adv. Inorg. Biochem.*, 1983, **5**, 111; K. Doi, B. C. Antanaitis, and P. Aisen, *Struct. Bonding (Berlin)*, 1988, **70**, 1.
6 H. Dalton, D. D. S. Smith, and S. J. Pilkington, *FEMS Microbiol. Rev.*, 1990, **87**, 201; H. Dalton in 'Methane and Methanol Utilizers', ed. J. C. Murrell and H. Dalton, Plenum Press, New York. 1992, pp. 85.
7 D. M. Kurtz, Jr., *Chem. Rev.*, 1990, **90**, 585 discusses in detail the oxo and hydroxo-bridged diiron complexes.
8 A. Trautwein, *Struct. Bonding (Berlin)*, 1974, **20**, 101.
9 (*a*) R. E. Stenkamp, L. C. Sieker, and L. H. Jensen, *J. Am. Chem. Soc.*, 1984, **106**, 618; (*b*) S. Sheriff, W. A. Hendrickson, and J. L. Smith, *J. Mol. Biol.*, 1987, **197**, 273.
10 M. A. Holmes, I. L. Trong, S. Turley, L. C. Sieker, and R. E. Stenkamp, *J. Mol. Biol.*, 1991, **218**, 583.
11 B. B. Muhoberac, D. C. Wharton, L. M. Babcock, P. C. Harrington, and R. G. Wilkins, *Biochim. Biophys. Acta*, 1980, **626**, 337.
12 R. C. Reem and E. I. Solomon, *J. Am. Chem. Soc.*, 1984, **106**, 8323; 1987, **109**, 1216.
13 M. P. Hendrich, L. L. Pearce, L. Que, Jr., N. D. Chasteen, and E. P. Day, *J. Am. Chem. Soc.*, 1991, **113**, 3039; P. Bertrand, B. Guigliarelli, and C. More, *Nouv. J. Chem.*, 1991, **15**, 445.
14 J. M. McCormick and E. I. Solomon, *J. Am. Chem. Soc.*, 1990, **112**, 2005; J. M. McCormick, R. C. Reem, and E. I. Solomon, *J. Am. Chem. Soc.*, 1991, **113**, 9066.
15 P. C. Wilkins and R. G. Wilkins, *Biochim. Biophys. Acta*, 1987, **912**, 48.
16 C. D. Garner, *Adv. Inorg. Chem.*, 1991, **36**, 303.
17 C. L. Atkin, L. Thelander, P. Reichard, and G. Lang, *J. Biol. Chem.*, 1973, **248**, 7464.
18 J. B. Lynch, C. J.-Garcia, E. Munck, and L. Que, Jr., *J. Biol. Chem.*, 1989, **264**, 8091.
19 P. Nordlund, B.-M. Sjöberg, and H. Eklund, *Nature (London)*, 1990, **345**, 593.
20 (*a*) C. Gerez, M. Atta, M. Fontecave, J. Gaillard, C. Scheer, and J. M. Latour, *J. Inorg. Biochem.*, 1991, **43**, 536; A. Ehrenberg, R. Davydov, P. Allard, and S. Kuprin, *J. Inorg. Biochem.*, 1991, **43**, 535.
21 J. B. Vincent and B. A. Averill, *The FASEB Journal*, 1990, **4**, 3009.
22 M. P. Woodland and H. Dalton, *J. Biol. Chem.*, 1984, **259**, 53.
23 B. G. Fox, K. K. Surerus, E. Münck, and J. D. Lipscomb, *J. Biol. Chem.*, 1988, **263**, 10553.
24 J. G. Dewitt, J. G. Bentsen, A. C. Rosenzweig, B. Hedman, J. Green, S. Pilkington, G. C. Papaefthymiou, H. Dalton, K. O. Hodgson, and S. J. Lippard, *J. Am. Chem. Soc.*, 1991, **113**, 9219.
25 J. W. Pyrz, J. T. Sage, P. G. Debrunner, and L. Que, Jr., *J. Biol. Chem.*, 1986, **261**, 11015.
26 S. S. David and L. Que, Jr., *J. Am. Chem. Soc.*, 1990, **112**, 6455.
27 M. Dietrich, D. Münstermann, H. Suerbaum, and H. Witzel, *Eur. J. Biochem.*, 1991, **199**, 105.
28 J. Green and H. Dalton, *J. Biol. Chem.*, 1989, **264**, 17698.
29 B. G. Fox, J. G. Borneman, L. P. Wackett, and J. D. Lipscomb, *Biochemistry*, 1990, **29**, 6419.
30 N. Deighton, I. D. Podmore, M. C. R. Symons, P. C. Wilkins, and H. Dalton, *J. Chem. Soc., Chem. Commun.*, 1991, 1086.
31 L. Que, Jr., *Science*, 1991, **253**, 273. See however, J. M. Bollinger, D. E. Edmondson, B. H. Huynh, J. Filley, J. R. Norton, and J. Stubbe, *Science*, 1991, **253**, 292.
32 J. H. Zhang, D. M. Kurtz, Jr., Y.-M. Xia, and P. G. Debrunner, *Biochemistry*, 1991, **30**, 583.
33 M. Fontecave, P. C. Wilkins, and H. Dalton, unpublished experiments.
34 E. C. Thiel, *Adv. Enzymol. Relat. Areas Mol. Biol.*, 1989, **63**, 421.
35 P. M. Hanna, Y. Chen, and N. D. Chasteen, *J. Biol. Chem.*, 1991, **266**, 886.