Coordination Chemistry Reviews, 79 (1987) 195–214
Elsevier Science Publishers B.V., Amsterdam – Printed in The Netherlands

THE COORDINATION CHEMISTRY OF THE BINUCLEAR IRON SITE IN HEMERYTHRIN

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(Received 4 July 1986)

CONTENTS

A. INTRODUCTION

Hemerythrin is the iron containing respiratory protein found in four marine phyla. The usual oligomeric form of the protein is octameric, but in the retractor muscle of *Themiste zostericola* it is monomeric (myohemerythrin) [1,2]. Other species contain the very unusual trimeric [3–6] as well as tetrameric and dimeric hemerythrins [7]. The molecular weight of the octamer is 108,000 with each subunit weighing 13,500—about the same as myohemerythrin (13,900).

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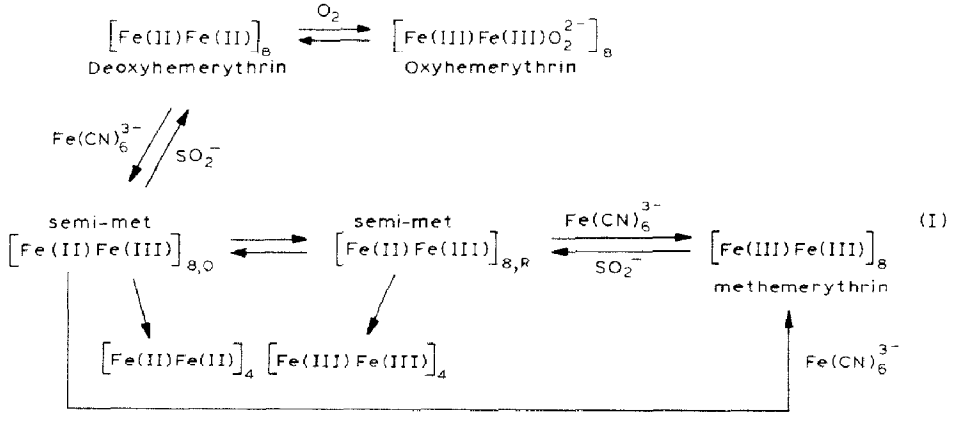
0010-8545/87/$7.00

In spite of its name, hemerythrin contains no porphyrin ring. Instead its active site consists of a pair of iron atoms which in the Fe(III)Fe(III) oxidation state are bridged by an oxo group and two amino acid residues [8–10]. This met (Hr^+) form of the protein does not react with oxygen, but does form complexes with anions such as N_3^-, SCN^-, CNO^-, etc. [11–13]. Deoxyhemerythrin (Hr°), in which both irons are in the +2 state, reacts with one mole of oxygen to form oxyhemerythrin, HrO_2. There is no cooperativity among the eight subunits, nor is there a Bohr effect observed during oxygen uptake. Two exceptions to this general observation are the brachiopodal hemerythrins from *Lingula unguis* [14] and *Lingula reevii* [15]. Distinct semi-met species where the irons are in the intermediate (II, III) state have been described [16–18]: semi-met_R results from the one-electron reduction of methemerythrin (Hr^+), semi-met_O from the one-electron oxidation of deoxyhemerythrin and a semi-met form also arises from the reaction of met with sulfide [19]. Scheme I shows the relationships among the various redox-linked states of hemerythrin. Although recent results of Sykes and coworkers [18] suggest that other species exist in the reduction path (see below), the ones indicated in Scheme I are the only ones that have so far been thoroughly characterised.

Those involved in the study of hemerythrin have benefited from the very nice X-ray crystallographic work of two different research groups. Early differences in their findings [20,21], which have now been resolved, led to extensive refinement of the data and thus on to a wealth of detail regarding the structure of the protein [10,22,23]. The folding of the polypeptide chain, whether in a subunit of the *Themiste dyscritum* or *Phascolopsis gouldii* octameter, or trimer [6] or in myohemerythrin, is the same in spite of the fact that the octamers and myo are only 42% homologous and that there are five additional residues in the latter. There are four nearly parallel sections



SCHEME 1.

of α-helix which contain 70–75% of the amino acids, confirming earlier CD predictions [2, 24]. The two iron atoms in each subunit are coordinated to amino acid residues from at least three of the four helices resulting in a very non-labile metal center [21]. Removal of the iron atoms from hemerythrin has proved possible but is usually accompanied by at least a partial unfolding of the protein structure. Regeneration of the "native" protein has however not been accomplished, possibly because of the difficulty in reforming the extremely rigid Fe–O–Fe site [25].

Hemerythrin is the Cinderella of respiratory proteins, hemoglobin and hemocyanin being the you-know-whats. Several reviews have compared and contrasted the three biological oxygen carriers [21,26–34]. Recently, however, there has been a resurgence of interest in hemerythrin because of the proposed existence of similar diiron sites in the B2 subunit of ribonucleotide reductase [35,36], purple acid phosphatases from pig allantoic fluid [37,38] and beef spleen [39], component A of methane monooxygenase from *Methylococcus capsulatus* (Bath) [40,41] and in the early stages of the formation of the ferritin core [42]. Hemerythrin provides an opportunity to "try out" structural techniques on a protein with an established and unique Fe_2 center. This has been particularly true of EXAFS experiments which have been helpful in corroborating the X-ray results [43].

Most hemerythrin reviews have been concerned with the structural aspects of the protein [44–53]. In this review we will emphasize the chemical reactions which occur at the iron–iron site and how the structural data help in their interpretation, with particular attention being paid to investigations carried out in the ten years since the last review of hemerythrin in this journal [54]. Most of the discussion will refer to results obtained with octameric protein except where chemically produced monomeric or myohemerythrin is specifically indicated.

B. METHEMERYTHRIN

(i) The Fe(III)Fe(III) site

Methemerythrin is the most stable form of the protein and as such has been the subject of numerous physical measurements. The iron site is unusual in that there are three bridging groups between the two high spin Fe(III) ions; an aspartate, a glutamate and a μ-oxo. The μ-oxo bridge was proposed early on by workers who compared the spectral and magnetic properties of hemerythrins to those of model complexes [11,55,56]. The remaining coordination sites are occupied by three histidine groups around one iron atom and two histidine groups around the other [10]. At any pH below ~ 6.8 and in the absence of added anions the iron bound to only two

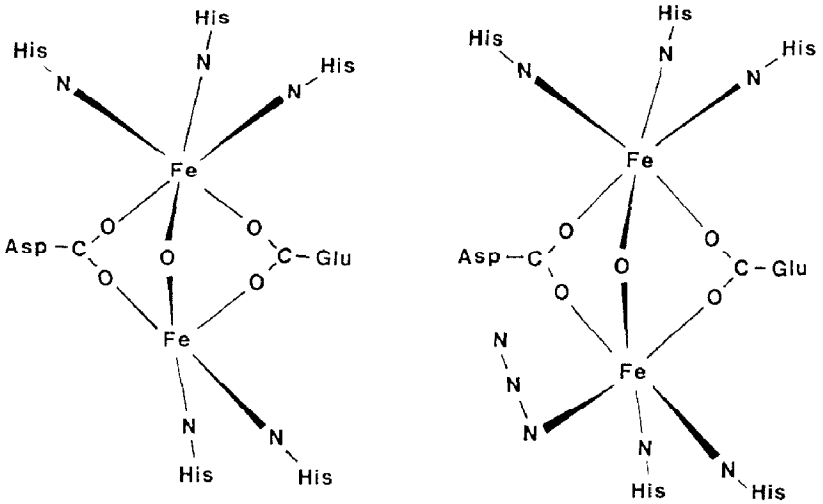


Fig. 1. Structures of the binuclear iron sites in met and azidomethemerythrin [10].

histidines has a vacant coordination site. At any pH above the pK ~ 7.8 hydroxide ion is believed to occupy the sixth site [57,58]. Deuterium effects seen in the resonance Raman spectra of hydroxomethemerythrin suggest the OH^- is hydrogen bonded to the oxo bridge either directly or through an intervening water molecule [59]. In the met anionic adducts such as azidomethemerythrin the exogenous ligand occupies the sixth coordination site. The structures of the iron atom sites in met- and azidomethemerythrin are shown in Fig. 1 [10].

Synthetic models of the met site have only recently been prepared [60–63]*. The structures and properties (especially the strong antiferromagnetic coupling of the Fe(III) centers) of the model compounds are quite close to those of methemerythrin. However, none of the reactions of the protein, beyond precipitation of certain SCN^- and N_3^- adducts [64], have been duplicated as yet. The strategy used in the recent syntheses of models of the iron atom site has led to the preparation of a series of novel polynuclear iron complexes [60a].

(ii) The reactions

(a) Anations

A number of monovalent anions react with methemerythrin to form complexes with a range of stabilities (Table 1) [12,13,65,66]. The formation

* The model in ref. 63(b) is a monobridged structure and not as good for hemerythrin as the others.

TABLE 1

Rate and stability constants for $Hr^+ + X^-$ at pH 6.3 and 25 °C [12,13]

Anion	k_f (M^{-1} s^{-1})	k_d (s^{-1})	K (kinetic) (M^{-1})	K (spectral) (M^{-1})
Br^-	0.016	0.002	8	—
HCO_2^-	0.012	0.0015	8	—
F^-	0.035	3.9×10^{-4}	90	95
Cl^-	0.006	7.5×10^{-5}	80	—
CNO^-	0.52	1.3×10^{-4}	—	4×10^{3}
SCN^-	82	0.0069	1.2×10^{4}	0.9×10^{4}
N_3^-	7.0	$\leqslant 5\times10^{-5}$	$\geqslant 1.4\times10^{5}$	1.0×10^{6}

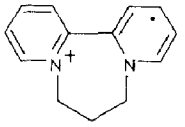
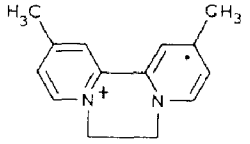
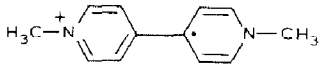
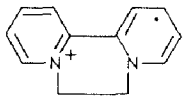
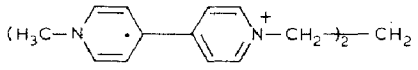
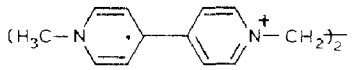
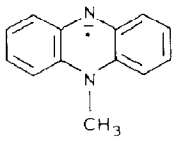
rate-constants are of the same order as those for $[Fe(H_2O)_5X^-]^{2+}$ complexes, but the rates of dissociation are generally smaller, so that the Hr^+X^- adducts are more stable than their simple iron(III) counterparts [12,13]. The stabilities and rates of formation of met-anion complexes decrease in the presence of perchlorate and similar anions [65,66]. X-ray crystallography shows that a ClO_4^- anion bound ~ 12 Å from the iron atoms has some structural effect at the anion binding-site [44,67]. The rates of formation of Hr^+X^- adducts decrease with increasing pH for most of the anions due to competition with OH^- [12,13]. The reaction of thiocyanate ion with met becomes biphasic at high pH and this is the only anion for which this behavior has been thoroughly investigated [57,68]. The reaction of hydroxide ion with methemerythrin is particularly interesting as it is slow and appears to be conformationally controlled (see section E) [57]. The discussion so far has been confined to octameric hemerythrin. Similar reactions occur with myohemerythrin and chemically produced monomers, the main difference being the faster rates observed with the monomers.

(b) Reductions

One electron reduction of methemerythrin produces an Fe(II)Fe(III) species that is referred to as $(\text{semi-met})_R$. The properties and reactions of the semimets will be discussed separately. The reduction of met is usually rapid (e.g. $k_{SO_2^-} = 10^6$ M^{-1} s^{-1}), pH independent and can be accomplished by reagents ranging from the hydrated electron to bisviologen type radicals [18,51,69]. Table 2 shows that there is a nice correlation between reducing power and the rate at which met is reduced. Where simple Marcus theory [70] can be applied, the observed and calculated rate constants agree nicely [69]. The reduction rates for $Co(sep)^{2+}$ and $Cr(bipy)_3^{2+}$ have also been found to be reasonably consistent with their E^0 values [18]. Superoxide ion does not reduce methemerythrin within the time that O_2^- disproportionation

TABLE 2

Data for methemerythrin reduction at 25 °C

	$E°$ (volts)	k(obs) [a] ($M^{-1}\,s^{-1}$)	k(calc) [b] ($M^{-1}\,s^{-1}$)
e^-_{aq}	−2.9	1.0×10^9	—
CO_2^-	−2.0	6.8×10^7	—
	−0.55	6.2×10^6	1.4×10^7
	−0.49	5.4×10^6	6.2×10^6
 MV	−0.45	3.8×10^6	3.7×10^6
 DQ	−0.35	1.1×10^6	8.7×10^5
SO_2^-	−0.26	1.5×10^5	2.1×10^5
$[Cr(bipy)_3]^{2+}$	−0.26	2.5×10^5 [c]	9.3×10^5
$[Co(sep)]^{2+}$	−0.26	255 [c]	60
 PTQ	−0.19	8.0×10^5	7.1×10^4
 Riboflavin (fully reduced)	−0.19	7.2×10^4	7.1×10^4
	−0.12	7.2×10^4	2.1×10^4
	+0.2	9.9×10^3	79

[a] Data from ref [69]. [b] Calculated on basis of the simple Marcus expression [70] as detailed in ref [69]. [c] Data from ref [18].

occurs at pH 9, which requires that the rate constant for reduction must be $\leqslant 10^3\ M^{-1}\ s^{-1}$ [68]. This result is consistent with the finding that for a number and variety of oxidants, the ratio of the reduction rates of SO_2^- to

O_2^- is 10^2–10^3 [71]. When sulfide ion is used, reduction to semi-met is accompanied by μ-S^{2-} replacement of the μ-oxo bridge [19,72]. Autoreduction of μ-S^{2-}-met to μ-S^{2-}-semi-met occurs via dissociation of the bridge, the released sulfide ion acting as reductant. The autoreduction is slowed by perchlorate ion but is accelerated by anions such as azide [73,74].

The components of a methemerythrin reductase system have been found in the hemerythrocytes of *P. gouldii* and five Australian sipunculids [75,76]. Cytochrome b_5 isolated from *P. gouldii* reduces met to semi-methemerythrin ($k = 650\ M^{-1}\ s^{-1}$) with little build up of deoxy during the reaction. A similar reaction between deoxymyoglobin and methemerythrin is more than 100 times slower presumably because myoglobin is not specifically an electron transfer protein [77].

Dithionite reductions of met-anion adducts are relatively slow, apparently consisting of single first-order reactions. This slowness is explained by the reduction being controlled by dissociation of the anion and the observed rate constants agree very well with those expected on this basis [13]. Only in the case of the SCN^- ion can each participant (Hr^+SCN^-, semi-met and Hr°) be separately analysed and accounted for completely in terms of the scheme comprising (1)–(3) [69]

$$Hr^+SCN^- \rightleftharpoons Hr^+ + SCN^- \qquad k_1 = 7 \times 10^{-3}\ s^{-1};\ k_{-1} = 7.0\ M^{-1}\ s^{-1} \quad (1)$$

$$Hr^+ \xrightarrow{SO_2^-} \text{semi-met} \qquad k_2 = 1.5 \times 10^5\ M^{-1}\ s^{-1} \quad (2)$$

$$\text{semi-met} \xrightarrow{SO_2^-} Hr° \qquad k_3 = 2.1 \times 10^{-3}\ s^{-1} \quad (3)$$

C. DEOXYHEMERYTHRIN

(i) The Fe(II)Fe(II) site

The Fe(II)Fe(II) site in deoxyhemerythrin is nearly invisible to many spectroscopic techniques and the crystals required for X-ray crystallography are for obvious reasons difficult to handle. As a result, not nearly as much is known in detail (compared to met) about this site in the physiologically important form of the protein. Until recently it was thought that the μ-oxo bridge must be missing in deoxy because of very weak antiferromagnetic coupling [7,45,52]. However, difference electron density maps of deoxy (at low resolution) and methemerythrin indicate no major differences in the iron atom sites of the two species [23,78]. One iron atom is pentacoordinate as in met and there is no evidence to suggest a broken μ-oxo bridge. The $Fe–O_{oxo}$ bonds are lengthened relative to those in met indicating protona-

tion of the bridge. This agrees very well with the results of Reem and Solomon who proposed an OH bridge in deoxy on the basis of their MCD studies [79]. A deoxy model has been synthesized in which both irons are +2 and the μ-oxo bridge is protonated [80]. In addition, there is a diiron(III) complex in which the bridge can be protonated and deprotonated, and in each of these cases the Fe–O bonds are longer in the protonated form [81]. No deoxy model has been prepared as yet via reduction of a met model-complex.

(ii) The reactions

(a) With oxygen

This is of course what a respiratory protein is designed to do—react reversibly with oxygen with little net oxidation of the active site. Deoxyhemerythrin does this quite well, forming the very stable oxy species ($K = 1.3 \times 10^5$ M^{-1}) extremely rapidly ($k_f = 7.5 \times 10^7$ M^{-1} s^{-1}) [50,82–84]. The X-ray difference electron density maps showing one five-coordinate iron in deoxy concur with the kinetic results which suggest addition of O_2 rather than substitution of even a weakly held ligand [83]. The rates of formation and dissociation of HrO_2 are virtually independent of pH and ionic strength effects [83]. Perchlorate ion has little effect on the formation rate in *P. gouldii* protein, but reduces the rate of dissociation of O_2, and thus the stability constant, by 3-fold [82]. The effect of ClO_4^- and other additives on the stability of *T. zostericola* HrO_2 is not very great, but it does cause an increase in the stability of oxymyohemerythrin [82,85]. The sulfhydryl reagent *p*-hydroxymercuribenzoate greatly increases the oxygen affinity of *T. zostericola* deoxymyohemerythrin when one or both of the cysteines is bound to Hg [85]. The complex octameric structure of hemerythrin suggests the presence of an allosteric modulator in hemerythrocytes analogous to organic phosphate regulators of hemoglobin oxygenation [1,45,51,53]. No such effector for oxygen binding to deoxyhemerythrin has as yet been demonstrated [53].

Oxyhemerythrin is best described as a met-peroxy species, the ferrous irons being oxidized as the oxygen is reduced [78]. Resonance Raman spectra and X-ray difference density profiles of met and oxyhemerythrin indicate protonation of the O_2^{2-} and formation of a hydrogen bond between the hydroperoxy group and the μ-oxo bridge (Fig. 2) [23,55,59,78,86]. The O_2 dissociation rate constant is reduced by 20% when H_2O is replaced by D_2O and this is ascribed to transfer of H^+ of the μ-OH group to the bound O_2 in the dissociation process [87]. Autoxidation of oxy to met is, in the absence of anions, a very slow process ($t_{1/2} \sim 18.5$ h, pH 7, 25 °C): added anions catalyse this process [88]. With N_3^- and CNO^- the reaction is biphasic and

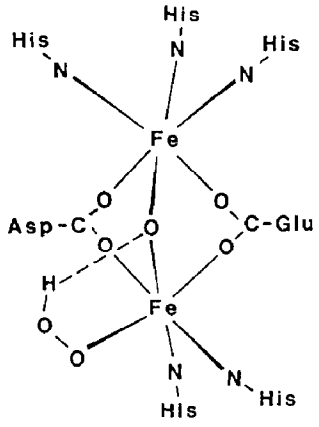


Fig. 2. Structure of the binuclear iron site in oxyhemerythrin showing the proposed hydroperoxy, μ-oxo hydrogen bond [23,78,86].

can be interpreted as a combination of either steps (4), (5) and (7) or steps (4), (5) and (6). With the latter the $Hr^{\circ}X^{-}$ adduct is a dead-end complex.

$$HrO_2 \rightleftharpoons Hr^{\circ} + O_2 \tag{4}$$

$$Hr^{\circ} + X^{-} \rightleftharpoons Hr^{\circ}X^{-} \tag{5}$$

$$HrO_2 + X^{-} \rightarrow Hr^{+}X^{-} + O_2^{2-} \tag{6}$$

$$Hr^{\circ}X^{-} + O_2 \rightarrow Hr^{+}X^{-} + O_2^{2-} \tag{7}$$

The two combinations are equivalent kinetically. Autoxidation of oxyhemerythrin in the presence of SCN^{-} probably does not proceed via a semi-met intermediate (not detected spectrally during the first half-life of reaction at pH 6.1 [89]) and for this reason the combination (4), (5) and (6) is preferred, with step (6) being simply a nucleophilic displacement O_2^{2-} by X^{-}.

The second-order rates of recombination of oxygen with deoxy and deoxymyohemerythrin after photodissociation of O_2 agree very with those obtained by temperature-jump methods [51,83,84]. If an unstable deoxy form is produced by laser photolysis it must either revert to the stable form very quickly (< 50 μs) or react with O_2 at the same rate as the stable form.

(b) Oxidations

Only hydrogen peroxide takes deoxyhemerythrin directly to a methemerythrin without detectable accumulation of semi-met. This met form and its azide adduct have visible spectra, especially in the region above 400 nm, which are different from those of normal $Hr^{+}OH^{-}$ and $Hr^{+}N_3^{-}$

[90a], although this has been disputed [90b]. The only other reagents found to react cleanly with deoxy are $Fe(CN)_6^{3-}$, a series of $Fe(CN)_5X^{n-}$ complexes and $Co(terpy)_2^{3+}$. In each case the product is $(semi\text{-}met)_O$. The ferricyanide oxidation is the only reaction studied thus far where *T. zostericola* deoxy reacts at a rate significantly different (700 times faster) from that of *P. gouldii* [17]. The $(semi\text{-}met)_O$/deoxy potential of 310 mV has been measured using the series of $Fe(CN)_5X^{n-}$ complexes whose potentials vary from 300–600 mV. Oxyhemerythrin is not oxidized directly by iron-cyano complexes or H_2O_2, but instead the reaction occurs through the deoxy species.

(c) Ligand interactions

The observation that N_3^- and CNO^- bleach oxyhemerythrin (see eqns. (4) and (5)) led to the study of the kinetics of binding of these anions and of F^- to deoxy via competition with O_2 [69,88,89]. From these data collected over pH ~5.5–9.0, it appears that HX is the reactive species in each case. Overall rate and thermodynamic constants for eqn. 2

$$Hr^\circ + HX \rightleftarrows Hr^\circ HX \quad k_f,\ k_d,\ K \tag{2}$$

assuming the proton is associated with X^- and not the protein, are collected in Table 3. The extent of binding found for N_3^-, CNO^- and F^- at pH 7.7 is in good agreement with these data [79]. No interaction between deoxy and Cl^-, Br^-, SCN^-, CN^-, ClO_4^-, HCO_3^-, HCO_2^-, $CH_3CO_2^-$, NCN^{2-}, N_2O or imidazole was observed [79,89].

The reaction of deoxy with NO is reported to result in the formation of an adduct of formulation [Fe(II)Fe(III)NO^-] [91]. The study of the kinetics of the interchange reaction (3)

$$HrNO + O_2 \leftrightarrows HrO_2 + NO \tag{3}$$

in which NO is replaced by O_2 in the deoxy adduct, leads to values of k_f and k_d in (4)

$$Hr^\circ + NO \leftrightarrows HrNO \quad k_f,\ k_d \tag{4}$$

TABLE 3

Rate and stability constants for $Hr^\circ HX$ at 25 °C, $I = 0.5$ M [89]

Acid	k_f (M^{-1} s^{-1})	k_d (s^{-1})	K (M^{-1})	ΔH (kcal mol^{-1})	ΔS (eu)
HN_3	3.0×10^4	0.1	3.0×10^5	−6.07	+4.5
HCNO	5.8×10^4	0.012	4.8×10^6	−6.0	+13.4
HF	5.2×10^3	0.01	5.3×10^5	−7.5	+0.7

of 2.3×10^6 M^{-1} s^{-1} and 0.45 s^{-1} respectively (pH 7, $I = 0.5$ M, 25°C). These values give rise to a formation constant (5×10^6 M^{-1}) some 30 times greater than that of the oxygen adduct [89]. Reaction of deoxy with nitrite produces semi-met · NO_2^- with no further oxidation to met [92].

D. SEMI-METHEMERYTHRIN

(i) The Fe(II)Fe(III) site

An exciting recent find in hemerythrin chemistry has been the semi-met forms [16,17,93,94]. In light of the fact that the met-deoxy electron density difference maps show little alteration in the iron site in the two forms [23], both semi-met O and R almost certainly must be structurally quite similar to one another and to met and deoxy. This suggestion has also been made on the basis of an analysis of the g values in the EPR spectra of the semi-mets [95]. A very small distortion at the iron atom site could account for the rhombic to axial change seen in going from $(\text{semi-met})_R$ to $(\text{semi-met})_O$. The two semi-mets are, however, chemically quite different, especially in their redox behavior (see Section (ii) (b) below). The values of g_x and g_y being far from $g = 2$ are the consequence of an octahedral Fe(II) according to the model used [95]. The proton NMR spectrum of semi-met azide indicates binding of three histidines to the Fe(II), supporting octahedral coordination for this iron [96]. Accumulated EPR data and the electronic and resonance Raman spectra of semi-met azide led to a similar conclusion with regard to the geometry of the Fe(II) [52,97]. Also, the bridge in semi-met is suggested to be a μ-hydroxo rather than a μ-oxo based on the observation that sulfide ion substitutes at the semi-met but not at the met oxidation level [74]. Linear electric field effect experiments also show charge localization on one of the two irons in semi-met [98].

Low temperature EPR spectroscopy has proved invaluable in the identification of Fe(II)Fe(III) sites in hemerythrin [19,94,99] and in other proteins [37–42]. Integration of the signal for semi-met azide has shown there is indeed 1 spin/2Fe [100]. Similar g values to those for semi-met hemerythrin are observed in the EPR spectra of the pink forms of uteroferrin [37,38] and beef spleen purple acid phosphatase [39], in reduced component A of methane monooxygenase from *Methylococcus capsulatus* (Bath) [40,41] and in the early stages of iron build-up in ferritin [42]. These spectra are shown in Fig. 3. In the case of the purple acid phosphatases it is the Fe(II)Fe(III) form which is enzymatically active as a phosphatase [37–39].

A semi-met model has recently been synthesized in which the irons in the Fe(II)Fe(III) site are believed to be bridged by two phenoxo (but not an oxo or hydroxo) groups [101].

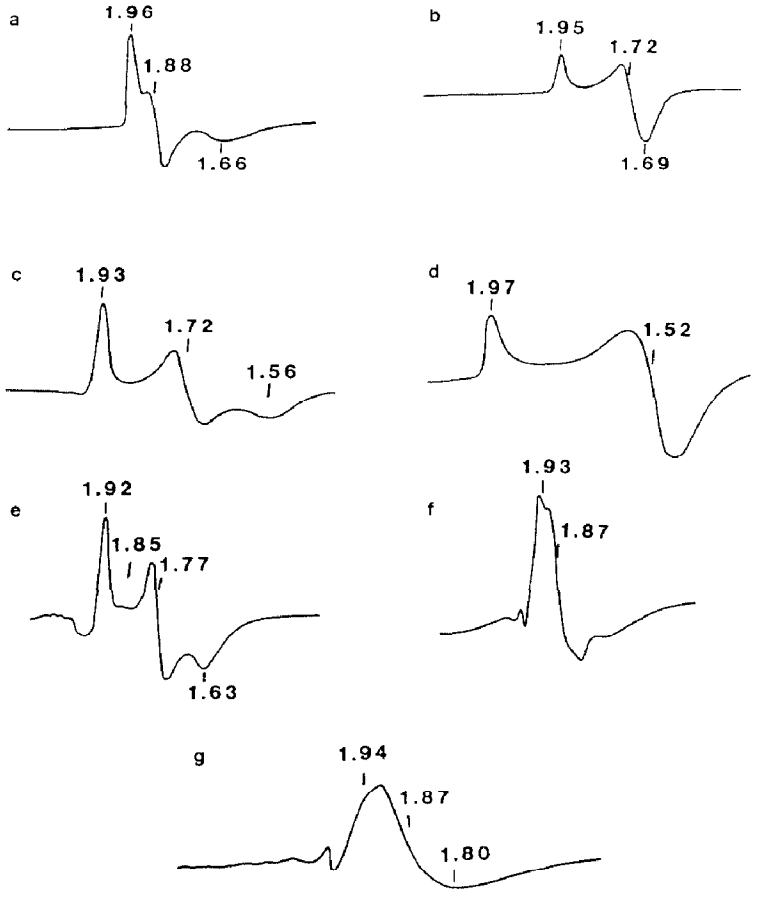


Fig. 3. EPR spectra of the Fe(II)Fe(III) sites in: (a) (semi-methemerythrin)$_R$ [94]; (b) (semi-methemerythrin)$_O$ [94]; (c) porcine uteroferrin (pink form) [37]; (d) molybdate treated porcine uteroferrin (pink form) [38]; (e) beef spleen acid phosphatase (pink form) [39]; (f) component A of methane monooxygenase (reduced form) [40]; and (g) apoferritin + 0.5 Fe^{3+} and 5.0 Fe^{2+}/subunit [42]. Spectra were recorded at 3–10 K; principal g values are shown in each spectrum.

(ii) The reactions

(a) Anations

Anion addition to the semi-met forms is to be expected since there is one Fe(III) atom in each site. The only species for which no adducts form is the μ-sulfide bridged semi-met [19,72,73]. The Fe(II)Fe(III) $\cdot$ X^- complexes are much less stable and more labile (larger rates of formation and dissociation) than the corresponding Hr^+X^- species [102]. EPR spectra of several of the adducts have been recorded [50]. Where the complexes could be generated from either semi-met R or O, a single semi-met $\cdot$ X^- appeared to result.

(b) Redox reactions

The oxidation of $(\text{semi-met})_R$ by $Fe(CN)_6^{3-}$ and H_2O_2 to met is rapid and direct, as is the reduction of $(\text{semi-met})_O$ to deoxy by dithionite and other reducing agents. This behavior shows that although the semi-mets are structurally similar they are chemically quite distinct. The product of the H_2O_2 oxidation of $(\text{semi-met})_R$ is normal met with its familiar Hr^+OH^- and $Hr^+N_3^-$ visible spectra and rate of reaction with azide. However, like deoxy, $(\text{semi-met})_O$ produces an unusual met form (Section. C (ii) (a)) which does not change to normal met overnight [90]. Further reduction of $(\text{semi-met})_R$ to deoxy (or oxidation of $(\text{semi-met})_O$ to met) is a more complicated process. The reaction is believed to involve disproportionation of two Fe(II)Fe(III) sites to one Fe(II)Fe(II) and one Fe(III)Fe(III) site, followed by reduction of the Fe(III)Fe(III) unit etc. until all the irons are in the +2 state [94,102,103]. The electron transfer required for disproportionation within the octamer is over a distance of 28–30 Å and is another reaction which appears to be conformationally controlled (see Section E). The electron transfer rate itself is then almost certainly faster than the $\sim 10^{-3}$ s^{-1} observed for disproportionation. In the absence of excess reductant (or oxidant) the semi-mets spontaneously disproportionate cleanly via the same conformational change to four Fe(III)Fe(III) and four Fe(II)Fe(II) units which behave like an equimolar mixture of met and deoxy hemerythrin, (eqn. 5).

$$[\text{Fe(II)Fe(III)}]_8 \rightarrow [(\text{Fe(II)Fe(II)})_4(\text{Fe(III)Fe(III)})_4] \quad (5)$$

The quaternary structure of the protein showing the location of the iron sites and the distances between nearest neighbors (the distance travelled by the electron) is pictured in Fig. 4. *P. gouldii* $(\text{semi-met})_R$ does not disproportionate completely (only ~ 10–20%) unlike both semi-mets from *T. zostericola* and *T. dyscritum* [94]. Two $(\text{semi-metmyo})_R$ molecules disproportionate to a $(\text{semi-metmyo})_R$, metmyo and deoxymyo equilibrium mixture, and adding metmyo to an equal concentration of deoxymyo results in the same three component equilibrium mixture, (eqn. 6) [50,99].

$$2\text{Fe(II)Fe(III)} \rightleftarrows \text{Fe(II)Fe(II)} + \text{Fe(III)Fe(III)} \quad (6)$$

A quarter met species, $[\text{Fe(II)Fe(II)}]_4[\text{Fe(III)Fe(II)}]_4$, has been proposed as an intermediate during the reduction of $(\text{semi-methemerythrin})_R$ to deoxy by certain metal ion reductants using *T. zostericola* protein [18]. The species has not been observed in the dithionite reduction of *T. zostericola* or *P. gouldii* $(\text{semi-met})_R$, where all the protein could be accounted for by semi-met (via EPR or N_3^- uptake) and deoxy (via HrO_2) during the whole reaction [51,69].

Reduction of $(\text{semi-metmyo})_R$ is much more difficult to understand than the corresponding reaction in the octamer [99]. If it were disproportionation

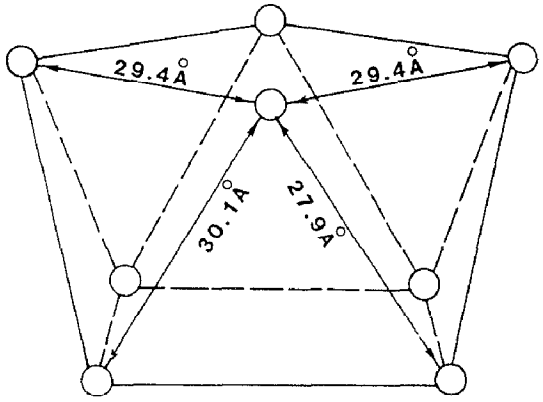


Fig. 4. Distances between nearest neighbor diiron sites in octameric hemerythrin. The circles represent the iron units which describe a nearly regular square antiprism [104].

controlled, as with $(\text{semi-met})_R$, second-order kinetics would be expected. However, the spectral changes associated with production of deoxymyo are neither first nor second order and are very dependent on the "freshness" of the protein. Furthermore, the EPR signal due to $(\text{semi-metmyo})_R$ disappears before the reduction to deoxymyo is ~ 25% complete.

Ferricyanide oxidation of *P. gouldii* $(\text{semi-met})_O$ to met at pH 6.3 is direct, in contrast to its behavior at pH 8.2 and that of *T. zostericola* and *T. dyscritum* at all pH values [17]. The $Fe(CN)_6^{3-}$ oxidation of semi-met azide is biphasic, (eqn. 7)

$$[\text{Fe(II)Fe(III)}N_3^-]_8 \xrightarrow[-8N_3^-]{+8Fe(CN)_6^{3-}} [\text{Fe(III)Fe(III)}] \xrightarrow{+8N_3^-} [\text{Fe(III)Fe(III)}N_3^-]_8 \tag{7}$$

In the first step N_3^- is lost during the oxidation to met which then recombines with the azide in the second step [17].

Sulfide ion reacts with $(\text{semi-met})_R$ to produce μ-S^{2-}-semi-met, but under the same conditions $(\text{semi-met})_O$ is reduced to deoxy. Ferricyanide oxidation of μ-S^{2-}-semi-met produces μ-S^{2-} met with retention of the sulfide bridge [73]. The μ-S^{2-}-met/μ-S^{2-}-semi-met potential of ~ 300 mV was measured using $Fe(CN)_6^{3-}$ and cytochrome c as oxidant partners [74].

E. CONFORMATIONALLY CONTROLLED REACTIONS

The interconversion $Hr^+ \rightleftarrows Hr^+OH^-$ is neither a simple acid–base reaction nor the straightforward addition of hydroxide ion to a vacant coordination site. The rate of the reaction in met octamer *(T. zostericola* and *P. gouldii)* and metmyohemerythrin is controlled by a first order conforma-

TABLE 4

Hemerythrin reactions controlled by a first-order conformational change.

Protein form	Reaction (pH)	Rate constant (s^{-1})	Ref.
methemerythrin	acid–base (8.2)	3.3×10^{-3}	57,68
	SCN^- addition (9.0)	5.0×10^{-3}	57,68
	$S_2O_4^{2-}$ reduction (9.0)	2.7×10^{-3}	57,68
	thiol reagents (9.0)	2.0×10^{-3}	105
	SDS (7.8)	$2\text{–}11\times10^{-3}$	25
semi-methemerythrin	O → R (8.2)	1.3×10^{-3}	106
	$Fe(CN)_6^{3-}$ oxidation (8.2)	1.3×10^{-3}	102
	intramolecular disproportionation (8.2)	2.2×10^{-3}	102
metmyohemerythrin	acid–base (7.0)	7.2×10^{-3}	57,68
	SCN^- addition (8.2)	3.2×10^{-3}	57,68
	$S_2O_4^{2-}$ reduction (8.2)	3.5×10^{-3}	57,68
	unstable metmyo → metmyo (8.2)	4.6×10^{-3}	99
semi-metmyohemerythrin	O → R (8.2)	1.0×10^{-2}	99

tional change [50,57]. Dithionite reduction of Hr^+ is rapid ($k_{SO_2^-} = 10^6\ M^{-1}\ s^{-1}$) but at high pH reduction must await the "base to acid" slow conversion [57,68]. When Hr^+ is quickly plunged to high pH (8.6) followed by dithionite addition the met behaves as if it were being reduced at low pH (6.3) and no additional $\sim 10^{-3}\ s^{-1}$ change is observed [89]. Perchlorate ion is found to have a similar effect in altering the pK values of *T. zostericola* met and metmyohemerythrin, [50,68] as it did with *P. gouldii* [57] met, in contrast to recent findings [85]. Several other reactions of hemerythrin, not involving electron transfer, appear to be controlled by this conformational change [105]. The disproportionation-dependent redox reactions have already been discussed (Section D (ii)(b)). Since transformation of $(semi\text{-}met)_O$ to $(semi\text{-}met)_R$ occurs with a rate constant ($1.3 \times 10^{-3}\ s^{-1}$) identical to that of oxidation of $(semi\text{-}met)_O$ by $Fe(CN)_6^{3-}$ [106], it is apparent that the conformational change occurs at the semi-met level also. Since the associated rate constants for monomer (myo) and octamer fall within the range 10^{-3}–$10^{-2}\ s^{-1}$ (Table 4) the conformational change cannot be solely a function of the octameric structure.

F. THE LAST TEN YEARS

Great strides have been made in our understanding of the structure and chemistry of hemerythrin since it was last the subject of a review in this

journal [54]. Most of the additional structural detail has come from refinement of the X-ray crystallographic data and comparisons of electron density profiles. The detection of the completely unexpected five-coordinate iron atom in met and probably in deoxyhemerythrin (where it was certainly suggested by kinetic measurements [83]) were results of these studies [10,23]. There is also no longer any doubt that the O_2 in HrO_2 and anions in Hr^+X^- complexes bind at this five-coordinate iron atom site and not between the two iron atoms [8,10,23]. The two different proposals of arrangement of ligands bound to the irons [20,21] have coalesced and aspects of both have been substantiated. The currently accepted diiron site has a μ-oxo and two amino acid bridges and Tyr 109(114 in myo) is no longer regarded as a ligand (Figs. 1 and 2) [8, 10, 23]. It is now thought that the μ-oxo bridge remains in deoxy and that it is protonated [23,78,79]. In semi-met it is believed that the Fe(II) is bound to three histidines (octahedral coordination) [52,95–97]. Evidence for hydrogen bond formation between the μ-oxo bridge and hydroperoxy in HrO_2 and hydroxy in Hr^+OH^- has been found [23,55,58,59,78,86]. The suggestion that hydroxy ion binds to met at high pH is the result of spectral investigations since Hr^+OH^- has not as yet been crystallized. The helical arrangement of the polypeptide chain in the subunit or in myohemerythrin, the so called hemerythrin fold, has been observed in other proteins with entirely different sequences and functions [10,47,49,51].

Characterization of two semi-met forms was a bit surprising and the differences between them remain a question [16,17,52,93–98,102]. The EPR spectra of the semi-mets have been particularly useful in identifying similar sites in other proteins [37–42] and in semi-met sulfide [19]. A semi-met model with two phenoxo bridges has been reported [101], and simple diiron complexes containing a μ-oxo and two carboxylate bridges which are structurally (but not chemically) similar to met and deoxy have been synthesized [43, 60–64]. The semi-met and met sulfides contain a μ-S^{2-} rather than a μ-oxo bridge and neither adds anions [19,72–74]. It has been known for a long time that carbon monoxide will not replace O_2 in HrO_2 [51], but it has now been shown that N_3^-, CNO^-, F^- and NO will, and that the NO adduct is significantly more stable than the oxygen adduct [69, 89]. Several examples of conformationally controlled reactions have been observed in hemerythrin chemistry [50,51,57,68,69,94,102,103,105], two of the more interesting being an acid–base transformation [57,68] and certain electron transfer reactions [94,102,106].

In the future, we would hope to see further structural efforts on deoxy and semi-methemerythrins, although these present substantial practical problems. In addition, the replacement of iron by other metals would lead to very interesting chemistry and an insight into the features that make the

diiron site in hemerythrin such a wonderful oxygen carrier. Finally, there will certainly be a good deal more chemistry revealed for a protein which has not been shy in yielding unexpected results–both an aggravation and a delight to the investigator.

ACKNOWLEDGEMENTS

We thank Professor Joann Sanders-Loehr for a pre-publication manuscript. We acknowledge the efforts of a small talented group of colleagues and the National Institutes of Health which made possible our work on hemerythrin.

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