Dioxygen and Hemerythrin

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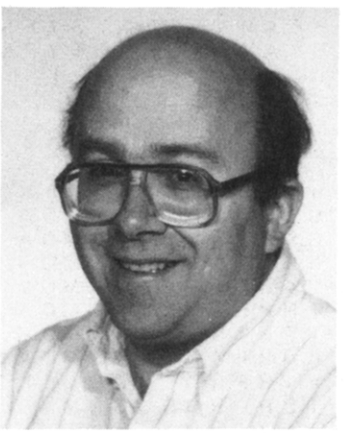
Contents



Ronald Stenkamp obtained his B.A. degree from the University of Oregon in 1970 and his Ph.D. from the University of Washington in 1975. After 2 years as a postdoctoral fellow at Yale University, he returned to the University of Washington, where he is now a member of the faculty in the Department of Biological Structure. His research interests are centered on the use of X-ray crystallographic methods to determine the three-dimensional structures of biologically interesting molecules ranging from small peptides and metal complexes to proteins.

I. Introduction

Hemerythrin is one of the three major metalloproteins capable of reversibly binding dioxygen. Found in animals of several marine invertebrate phyla, it differs fundamentally from hemoglobin and hemocyanin by possessing an active site containing two iron atoms linked by carboxylate groups and a μ-oxo bridging atom. Figure 1 summarizes the protein's reaction with dioxygen and the change from deoxy- to oxyhemerythrin when the two iron atoms bridged by a μ-hydroxyl group bind dioxygen to yield a μ-oxo bridge and a protonated peroxide. This review will focus on the structural aspects of the oxygenation reaction. Recent review articles provide more complete discussions of the chemistry of hemerythrin and related non-heme iron proteins.[2-6]

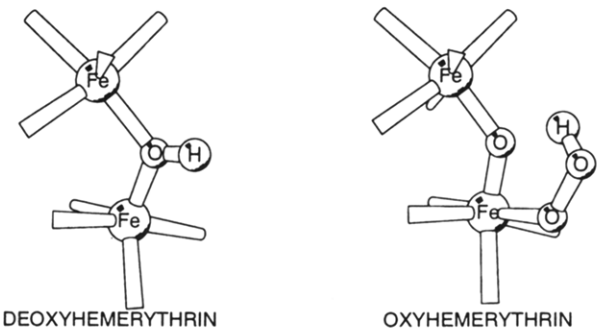


Figure 1. Summary of dioxygen binding by hemerythrin: (a) deoxyhemerythrin, (b) oxyhemerythrin. (Figure drawn using MOLSCRIPT.[1])

II. Protein Characterization

Throughout the 1950s and 1960s extensive physical and chemical research into the structure and reactivity of hemerythrin was carried out to determine how the polypeptide and iron atoms combined to yield a functional metalloprotein. Hemerythrin from the coelomic cavity of the sipunculid worm *Phascolopsis gouldii* was the subject of most of these studies, and thus it has become the reference molecule for subsequent analyses. Considerable structural and functional information, especially for met-, deoxy, and oxyhemerythrin, has also been obtained for the octamer from *Themiste dyscrita*. To date, no significant functional or spectroscopic differences have been found between these two hemerythrins.

One of the first questions addressed was the determination of the iron/dioxygen stoichiometry. Early analytical experiments[7] indicated that multiple iron atoms were involved in binding each dioxygen molecule. As analytical techniques and protein purifications improved, the ratio that emerged was two iron atoms per dioxygen molecule. Using *o*-phenanthroline extractions of the protein and optical spectroscopy, Klotz et al.[7] concluded that deoxyhemerythrin contained ferrous iron while oxyhemerythrin contained a peroxo anion bound to two ferric iron atoms. This view still holds.

The molecular weight of the protein was determined using a variety of techniques. Love,[8] in 1957, obtained a molecular weight of 120 000 using X-ray crystallographic methods. Subsequently it was shown that *P.*

0009-2665/94/0794-0715$14.00/0

Table 1. Amino Acid Sequences for Hemerythrins and Myohemerythrins

1–38		39–45	
1 2 3		4	
1 2 3 4 5 6 7 8 9 0 1 2 3 4 5 6 7 8 9 0 1 2 3 4 5 6 7 8 9 0 1 2 3 4 5 6 7 8		9 0 1 2 3 4 5	*Td* numbering
L			iron ligands
h h h h h h h h h h h h h h h h h h		h h h h h	helices
G F P I P D P Y V W D P S F R T F Y S I I D D E H K T L F N G I F H L A I D		D N A D N L G	*Pg* hemerythrin
G F P I P D P Y G W D P S F R T F Y S I I D D E H K T L F N G I F H L A I D		D N A D N L G	*Tz* hemerythrin[a]
G F P I P D P Y C W D I S F R T F Y T I V D D E H K T L F N G I L L L S Q A		D N A D H L N	*Td* hemerythrin
G F P V P D P F I W D A S F K T F Y D D L D N Q H K Q L F Q A I L T Q G N V		G G A T A G D	*Sc* hemerythrin
G W E I P E P Y V W D E S F R V F Y E Q L D E E H K K F K G I F C D I R D		N S A P N L A	*Tz* myohemerythrin
P F D I P E P Y V W D E S F R V F Y D N L D D E H K G L F K G V F N C A A D	M	S S A G N L K	*Pg* myohemerythrin I
G F E V P E P F K W D E S F Q V F Y D K L D E E H K Q I F N A I F A L G G G		N N A D N L K	*Nd* myohemerythrin
V K V P E P F A W N E S F A T S Y K N I D L E H R T L F N G L F A L S E F		N T R D Q L L	*Lu* hemerythrin, alpha
M K I P V P Y A W T P D F K T T Y E N I D S E H R T L F N G L F A L S E F		N T Q H Q L N	*Lu* hemerythrin, beta
										invariant residues

46–64		65–67		68–89	
5 6				7 8	
6 7 8 9 0 1 2 3 4 5 6 7 8 9 0 1 2 3 4		5 6 7		8 9 0 1 2 3 4 5 6 7 8 9 0 1 2 3 4 5 6 7 8 9	*Td* numbering
L L				L L	iron ligands
h h h h h h h h h h h h h h h h h h h				h h h h h h h h h h h h h h h h h	helices
E L R R C T G K H F L N E Q V L M Q A		S Q Y		Q F Y D E H K K E H E G F I H A L D N W K G	*Pg* hemerythrin
E L R R C T G K H F L N Q E V L M Q A		S Q Y		Q F Y D E H K K A H E E F I R A L D N W K G	*Tz* hemerythrin[a]
E L R R C T G K H F L N E Q Q L M Q A		S Q Y		A G Y A E H K K A H D D F I H K L D T W D G	*Td* hemerythrin
N A Y A C L V A H F L F E E A A M Q V		A K Y		G G Y G A H K A A H E E F L G K V K G G S A	*Sc* hemerythrin
T L V K V T T N H F T H E E A M M D A		A K Y		S E V V P H K K M H K D F L E K I G P V D A	*Tz* myohemerythrin
H L I D V T T T H F R N E E A M M D A		A K Y		E N V V P H K Q M H K D F L A K L G G L K A	*Pg* myohemerythrin I
K M I D V T A N H F A D E E A M M L A	S	A A Y	K	S E H P G H K K K H E D F L A V I R G L S A	*Nd* myohemerythrin
A C K E V F V M H F R D E Q G Q M E K		A N Y		E H F E E H R G I H E G F L E K M G H W K A	*Lu* hemerythrin, alpha
A A I E V F T L H F H D E Q G Q M I R		S N Y		V N T K E H T D I H N G F M D T M R G W Q S	*Lu* hemerythrin, beta
| | | |		|		| | |	invariant residues

	90–113		
	1 1		
	9 0 1		
	0 1 2 3 4 5 6 7 8 9 0 1 2 3 4 5 6 7 8 9 0 1 2 3	*T. dyscrita* hemerythrin numbering	
	L L	iron ligands	
	h h h h h h h h h h h h h h	helices	refer.
	D V K W A K S W L V N H I K T I D F K Y K G K I	*P. gouldii* hemerythrin	13,14,15
	D V K W A K S W L V N H I K T D F K Y K G K I	*T. zostericola* hemerythrin	16
	D V T Y A K N W L V N H I K T I D F K Y R G K I	*T. dyscrita* hemerythrin	17
	D A A Y C K D W L T Q H I K T I D F K Y K G K L	*S. cumanense* hemerythrin	18
P V D A K	N V D Y C K E W L V N H I K G T D F K Y K G K L	*T. zostericola* myohemerythrin	19
P L D Q G	T I D Y A K D W L V Q H I K T T D F K Y K G K L	*P. gouldii* myohemerythrin, type I	20
P V P N D	K L L Y A K D W L V N H I K G T D F T Y K G K L	*N. diversicolor* myohemerythrin	21
P V A Q K	D I K F G M E W L V N H I P T E D F K Y K G K L	*L. unguis* hemerythrin, alpha subunit	22
P V P Q K	A L K D G M E W L A N H I P T E D F K Y K G K L	*L. unguis* hemerythrin, beta subunit	23
	| | | | | | | | |	invariant residues	

[a] Portions of the sequence of *T. zostericola* hemerythrin are based on similarities to that of *P. gouldii* hemerythrin. Revisions of the *P. gouldii* sequence have not been confirmed for *T. zostericola*. Accordingly, it is probable that Q58 in *T. zostericola* (one of the iron ligands) is actually E58 as in the other hemerythrins.

gouldii hemerythrin was an octamer[9] with a subunit molecular weight near 13 500 (108 000 for the octamer). Hemerythrins from other tissues and species have been isolated in other oligomeric forms. Myohemerythrin is a monomeric protein usually isolated from the muscles of sipunculids[10] and is very similar to the hemerythrin subunit, both in structure and function, just as myoglobin is similar to the hemoglobin subunits. Dimeric, trimeric, and tetrameric hemerythrins have also been found in other species of sipunculids,[11,12] and their subunits are also similar to those of the octameric hemerythrins. Because of the existence of these different oligomers, the molecule of chemical interest for much of the remainder of this review will be the subunit containing one dioxygen binding site per polypeptide chain.

The amino acid sequences for a number of hemerythrins have been determined.[13-23] Table 1 summarizes the aligned sequences. There are several conserved residues beyond those involved in iron ligation, and they will be briefly discussed later with respect to their possible roles in the functioning of the protein.

Table 2 lists the percentages of identical amino acids found in pairwise comparisons of the sequences. The myohemerythrins and the *Lingula unguis* hemerythrin subunits are slightly longer than the other hemerythrins and have only about 40% sequence identity with them. The sequence differences appear to have no significant effect on the chemical properties of the metal centers, but they are important for intermolecular interactions involved in forming the different oligomers and in the cooperative oxygen binding exhibited by *L. unguis* hemerythrin.[24,25] Similar sequence differences will likely be seen in the two subunits found in the hemerythrin from the brachiopod *L. reevii*[26] since cooperative oxygen binding is also exhibited by this protein.

III. Three-Dimensional Structure of Hemerythrin

The three-dimensional molecular structures of myohemerythrin and three hemerythrins from different

Table 2. Percentage of Identities between the Amino Acid Sequences

		1	2	3	4	5	6	7	8	9
1	*P. gouldii* hemerythrin	100	95	78	44	48	51	46	45	43
2	*T. zostericola* hemerythrin	95	100	76	46	47	50	46	42	40
3	*T. dyscrita* hemerythrin	78	76	100	47	46	48	44	40	39
4	*S. cumanense* hemerythrin	44	46	47	100	44	46	49	37	30
5	*T. zostericola* myohemerythrin	48	47	46	44	100	69	63	42	38
6	*P. gouldii* myohemerythrin, type 1	51	50	48	46	69	100	57	44	38
7	*N. diversicolor* myohemerythrin	46	46	44	49	63	57	100	43	38
8	*L. unguis* hemerythrin, α subunit	45	42	40	37	42	44	43	100	65
9	*L. unguis* hemerythrin, β subunit	43	40	39	30	38	38	38	65	100

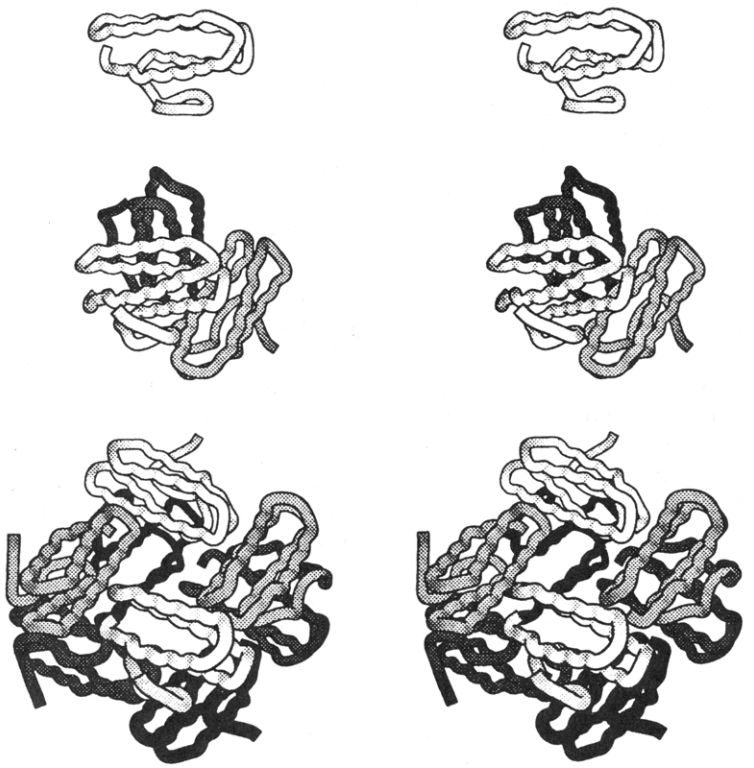


Figure 2. Stereoviews of the quaternary structures of (a, top) monomeric myohemerythrin, (b, middle) trimeric hemerythrin, and (c, bottom) octameric hemerythrin. (Figure drawn using MOLSCRIPT.[1])

species have been obtained using X-ray crystallographic methods.[27-30] Azidometmyohemerythrin from *T. zostericola* is a monomer,[27] azidomethemerythrin from *P. gouldii* is an octamer,[28] azidomethemerythrin from a species of *Siphonosoma* is a trimer,[29] and azidomet-, met-, deoxy- and oxyhemerythrin from *T. dyscrita* are all octamers.[30] No crystallographic structures for the dimeric and tetrameric hemerythrins are yet available. The quaternary structures showing the subunit packing arrangements are summarized in Figure 2. Slight amino acid changes in the polypeptides change the subunit interactions and alter the structure of the oligomers. The role the different oligomers play in the physiological functioning of hemerythrins is not known. Also, detailed analysis of the relationship between the amino acid sequence and the subunit–subunit interactions is complicated by the lack of sequence information for the dimeric, trimeric, and tetrameric molecules and structural information for the dimer and tetramer.

Each subunit is 40 Å long and 20 Å across and thick. The octamer, with 422 (D_4) symmetry, is about 60 Å across and 40 Å high. The large channel running through the center of the octamer is interesting, but considering the existence of the other oligomeric forms, the channel is unlikely to have functional significance.

The folding of the polypeptide chain in the monomeric molecules (subunits) is shown in Figure 3. The structure is very simple at this level with the polypeptide folding into four α-helices that pack roughly parallel to one another.[31] Myohemerythrin and hemerythrin were among the first proteins identified with the parallel α-helical tertiary structure, but it has now been found in a number of molecules including several cytochromes,[32,33] tobacco mosaic virus coat protein,[34,35] ferritin,[36] and various cytokines and hormonelike proteins.[37-44] One characteristic of this packing of α-helices is that while the helices appear to run parallel to each other, there is in fact only one small close-contact region, and the helices slightly diverge from each other away from the contact region. One result of this is the generation of a wedge-shaped molecule.[45] The divergence of the helices also produces a cavity or cleft between the helices that can be filled with a heme group in the cytochromes or an iron complex in the hemerythrins. The binding environment in the cavity, both for metals and for ligands, can be altered by lining the surfaces of the helices with appropriate amino acid side chains. In the case of hemerythrin, in addition to the

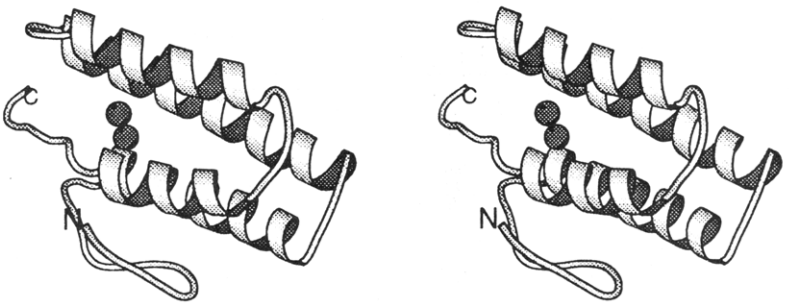


Figure 3. Stereoview of the structure of the deoxyhemerythrin subunit. The ribbon drawing shows the secondary and tertiary structure of the subunit with the two iron atoms located between the helices. (Figure drawn using MOLSCRIPT.[1])

amino acids bound to the iron atoms, the cleft is lined with hydrophobic amino acids making a suitable binding environment for exogenous ligands such as dioxygen.

IV. Structure of the Iron Center

The novelty of the metal center in hemerythrin complicated its structure determination and led to its examination using a large array of experimental methods. Chemical modifications of the protein,[46–49] various spectroscopic approaches (absorption,[50–53] Mossbauer,[54–56] resonance Raman,[53,57–63] circular dichroism,[50–53] NMR,[64,65] EXAFS[66–69]), magnetic susceptibility measurements,[70,71] and X-ray crystallographic techniques[27–30,72–74] have all been applied to hemerythrin in attempts to determine what the metal center looks like, i.e., which atoms are connected to produce the complex. None of the methods were sufficient alone to answer the basic structural questions concerning even the identities of the amino acids bound to the iron atoms. There have been several structural models proposed for the metal complex, and they have evolved as the various research groups studying hemerythrin have interacted with each other, sharing their results and their conclusions. Given the amount of experimental evidence consistent with the models to be presented here, it seems unlikely that major surprises remain concerning the structure of the active site in hemerythrin.

The metal complex consists of two iron atoms, roughly 3.25–3.5 Å apart, bound to the protein ligand by way of seven amino acid side-chain residues (see Figure 4). There are five histidine and two carboxylic acid residues (aspartic acid and glutamic acid) bound to the iron atoms. Three of the histidine residues (residues 73, 77, and 101 in *P. gouldii* and *T. dyscrita* hemerythrins) bind to one iron, and two (25 and 54) bind to the other. The glutamic and aspartic acid residues (58 and 106) bridge between the metals using each oxygen atom of their carboxylates to bind to separate iron atoms.

In addition, in all forms of hemerythrin, an oxygen atom derived from water is bound to both irons. Resonance Raman investigations of oxyhemerythrin generated in $H_2{}^{18}O$ provide strong evidence that the bridging oxygen is derived from water.[60–62] In met-, azidomet-, and oxyhemerythrin, the bridging oxygen is unprotonated, and thus is a μ-oxo bridge. A number of pieces of evidence support this conclusion. First, the absorption spectra of met-, azidomet-, and oxyhemerythrins contain bands similar to those of small molecule iron complexes containing μ-oxo bridges.[50] Second, the iron atoms in hemerythrin are antiferromagnetically coupled with a coupling constant similar to those of other μ-oxo bridged compounds (J = −77 cm^{-1} (oxy) and −134 cm^{-1} (met)).[71] Third, the Fe–O bond distances from EXAFS and crystallographic studies are similar to the bond lengths found in appropriate model compounds.[66,67,74]

In deoxyhemerythrin, the bridge is believed to be protonated. The magnetic coupling constant is much smaller, as would be expected for a μ-hydroxo bridge[51,64] (J = −15 cm^{-1}), and the Fe–O bond distances observed in deoxyhemerythrin using EXAFS[69] and crystallographic techniques[74] are slightly longer than those in met-, azidomet-, or oxyhemerythrin. This elongation is consistent with the increase in Fe–O distances in going from μ-oxo to μ-hydroxo bridged complexes. Table 3 contains a summary of a selected set of reported bond lengths and angles for the metal complexes in hemerythrins and shows the significant lengthening of the bridging Fe–μO bonds in deoxyhemerythrin.

Recently, the three-dimensional structures of two other metalloproteins containing binuclear oxo-bridged iron complexes have been solved. Figure 5 compares the metal complexes in ribonucleotide reductase[75] and methane monooxygenase[76] with that of hemerythrin. While all three proteins make use of a μ-oxo or μ-hydroxo bridge between the iron atoms, there are interesting differences in the other iron ligands. Two carboxylic acid side chains bridge between the two iron atoms in hemerythrin, while only one carboxylate bridge is found in ribonucleotide reductase. The complex in methane monooxygenase contains two carboxylate bridges, but one is an acetate and only one is provided by the protein. The enzymes (ribonucleotide reductase and methane monooxygenase) also have fewer nitrogen ligands in the complexes and utilize more non-protein ligands (water and acetate).

There are also interesting differences in the mode of binding of histidine in the complexes in these three proteins. In hemerythrin, all of the histidine residues bind through their ϵ nitrogen atoms, while in the other two proteins, the δ nitrogen is bound to the metal. Until the structures of the latter two were determined, there had been no observations of iron atoms binding to the δ nitrogen of histidine.[77] The functional significance of this is not clear, but Chakrabarti reported that histidine binds metals through the ϵ nitrogen about 75% of the time. Histidine binds to copper and zinc using either nitrogen atom. For example, in nitrite reductase, one of the copper atoms is bound to histidine through the ϵ nitrogen while the other is bound by

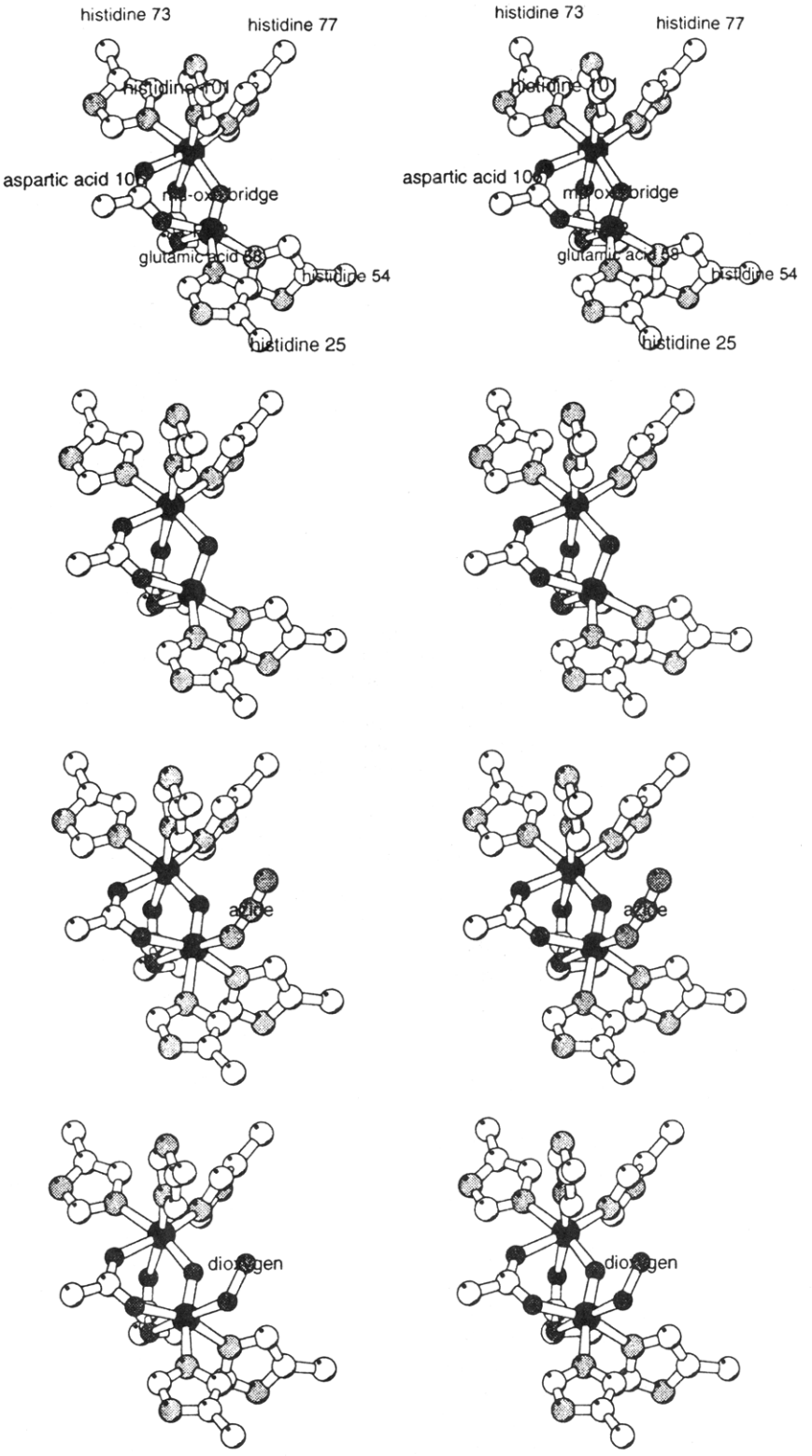


Figure 4. Stereoviews of the iron complexes in (a, first) *T. dyscrita* methemerythrin, (b, second) deoxyhemerythrin, (c, third) azidomethemerythrin, and (d, fourth) oxyhemerythrin. (Figure drawn using MOLSCRIPT.[1])

histidine through the δ nitrogen.[78] Also, in carbonic anhydrase, zinc is found bound to histidines through both nitrogen atoms.[79] The folding of a polypeptide into a globular protein could easily give rise to conformational restrictions that would dictate whether the ϵ or δ nitrogen atoms were available for metal binding. Whether differences in the mode of binding are associated with chemical and functional differences in metalloproteins is still an unanswered question.

V. Unliganded Complexes: Met- and Deoxyhemerythrin

The geometric structures of the metal centers in met- and deoxyhemerythrin are quite similar. Figure 4 contains stereoscopic views of the complexes in met- and deoxyhemerythrin. One of the iron atoms is octahedrally bound to six ligands: three nitrogen atoms from histidine residues, two oxygen atoms from the

Table 3. Bond Lengths (Å) and Angles (deg) for the Binuclear Iron Centers[a]

	azidomet				met		deoxy		oxy	
method	X-ray cryst	X-ray cryst	EXAFS	EXAFS	X-ray cryst	EXAFS	X-ray cryst	EXAFS	X-ray cryst	EXAFS
ref	72	73	66	69	73	69	74	69	74	69
Fe1–Fe2	3.23	3.23	3.38	3.24	3.25	3.13	3.32	3.57	3.27	3.24
Fe–N (av)	2.16	2.21	2.13	2.17	2.16	2.14	2.22	2.25	2.20	2.22
Fe–O (av)	2.10	2.17	1.71–1.76	2.08	2.12	2.07	2.20	2.12	2.17	2.11
Fe–μ-O (bridge) (av)	1.79	1.80		1.80	1.79	1.82	2.02	1.98	1.84	1.82
Fe1–Oε1 (Glu 58)[b]	2.05	2.17			2.26		2.33		2.20	
Fe1–Nε2 (His 73)	2.25	2.24			2.22		2.23		2.22	
Fe1–Nε2 (His 77)	2.11	2.20			2.15		2.21		2.18	
Fe1–Nε2 (His 101)	2.13	2.22			2.15		2.24		2.21	
Fe1–Oδ1 (Asp 106)	2.10	2.15			2.08		2.17		2.13	
Fe1–μ-O (bridge)	1.80	1.80			1.92		2.15		1.88	
Fe2–Nε2 (His 25)	2.22	2.21			2.07		2.15		2.14	
Fe2–Nε2 (His 54)	2.15	2.24			2.23		2.28		2.25	
Fe2–Oε2 (Glu 58)	2.18	2.25			2.04		2.14		2.20	
Fe2–Oδ2 (Asp 106)	2.05	2.09			2.08		2.14		2.15	
Fe2–μ-O (bridge)	1.77	1.79			1.66		1.88		1.79	
Fe2–azide	2.11	2.17								
Fe2–O2									2.15	
Fe–μ-O–Fe	130.	128.3	165	127	130.1	118	110.6	128	125.4	125

[a] Comparison of the bond lengths and angles obtained for different proteins using different techniques is complicated by several technical considerations. First, the values reported from EXAFS studies are averages for types of bonds. Information about individual bonds [for instance, the Fe–Oε1 (Glu 58) bond] is not available from the EXAFS studies of hemerythrin. However, in refs 44, 66, and 69, error estimates of about 0.05 Å are reported for the iron–ligand distances. Second, no good estimates of the standard deviations in the bond lengths and angles are available from the crystallographic studies. This is mainly due to the limited resolution of the crystallographic investigations and the compensating application of restraints in refinement. Any statistical comparison of bond lengths and angles from such refinements will likely reflect more on the fit of the values to the restraints rather than on the accuracy of the results. It should be pointed out that some crystallographic methods used for smaller mols. have not been successfully applied in macromol. structure refinements. In particular, approximations used in calculating the normal matrix in the least-squares refinement of proteins make standard deviations derived from that source extremely suspect. In the crystal structure of *T. dyscrita* hemerythrin, there are multiple copies of the subunits in the asymmetric unit, so we averaged among them to improve the precision of the bond lengths and angles.[74,75] We also calculated the standard deviation in the mean values, but since restraints were still being applied, the resulting numbers (0.01–0.03 Å) are somewhat low estimates of the errors. [b] Residue numbering from *P. gouldii* and *T. dyscrita* hemerythrins.

carboxylic acids, and the bridging oxygen. The other iron atom is pentacoordinate in a distorted trigonal bipyramidal environment. Here the ligands are two nitrogens (histidine rings) and three oxygens (the other carboxylate oxygens and the bridge).

Refinements of the crystal structures of methemerythrin[73] and deoxyhemerythrin[74] have been carried out and have resulted in the bond lengths and angles in Table 3. On the basis of their coordination numbers and geometry, the two iron atoms are not equivalent. Several Mossbauer spectra of met- and deoxyhemerythrin suggested that the iron atoms should be in equivalent environments,[54,55] and this interpretation governed the types of complexes suggested for the metal center before crystallographic views became available. Mossbauer spectroscopy seems to be insensitive to the types or extent of the structural asymmetry found in the unliganded complexes, although it should be noted that additional Mossbauer measurements on hemerythrin from *Phascolosoma lurco* indicate that the iron atoms are inequivalent.[56]

While the overall coordination geometry is the same for met- and deoxyhemerythrin, the iron atoms are in different oxidation states in the two forms: ferric for methemerythrins and ferrous for deoxyhemerythrin. The bond lengths in deoxyhemerythrin are lengthened somewhat relative to methemerythin, but basically, the unliganded forms of hemerythrin are very similar in the overall structures of their metal sites.

It should be noted that the major deviations in Table 3 are associated with met- and deoxyhemerythrin. The

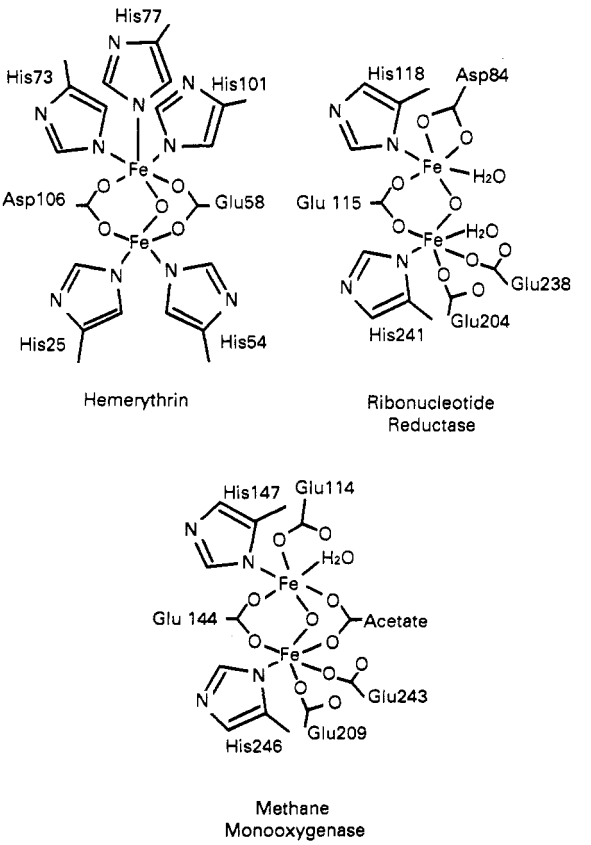


Figure 5. Dinuclear iron complexes in (a, top) hemerythrin, (b, middle) ribonucleotide reductase,[75] and (c, bottom) methane monooxygenase.[76]

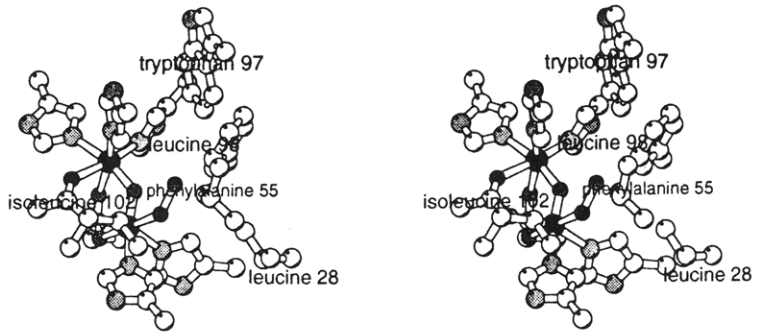


Figure 6. Stereoview of the hydrophobic dioxygen binding site in hemerythrin. (Figure drawn using MOLSCRIPT.[1])

longer Fe–O distances in deoxyhemerythrin fit with other chemical information indicating a hydroxo bridge, but the asymmetric Fe–μ-O bond lengths found in the crystallographic refinement of met- and deoxyhemerythrin are not supported by resonance Raman measurements.[80,81] The relative intensities of the symmetric and asymmetric Fe–O stretches have been correlated with asymmetries in the Fe–μ-O bond lengths in a number of small compounds, but the asymmetry observed crystallographically in met- and deoxyhemerythrin[73,74] is much larger than that expected from the vibrational spectroscopy. Currently, the asymmetry is seen only in crystallographic studies of met- and deoxyhemerythrin, the forms containing penta- and hexacoordinate iron atoms in each complex. While the differences in coordination number might lead to inequivalent Fe–μ-O distances, it remains unsettling that the crystallographic results are inconsistent with the resonance Raman measurements for the proteins.

Another item in Table 3 deserving attention is the large Fe–Fe distance found in deoxyhemerythrin using EXAFS techniques.[53] It is possible that additional model compounds with μ-hydroxo bridges would affect the EXAFS derivation of that Fe–Fe distance.

In spite of these small discrepancies, the values obtained by EXAFS and X-ray crystallography presented in Table 3 agree fairly well with each other. Of course, the fact that similar restraints were applied in the crystallographic refinements would cause the resulting distances to be similar. In this and other metalloprotein crystal structure determinations, the choice and application of restraints in refinement significantly affect the resulting structural models. This point should be kept in mind when characterizing novel complexes since the use of inappropriate restraints can obscure important structural information.

VI. Liganded Complexes: Azidomet- and Oxyhemerythrin

Hemerythrin functions by binding a small exogenous ligand, dioxygen, to its metal center, but the protein can also bind a variety of other ligands, both to the iron center and elsewhere. Small ligands such as azide, thiocyanate, and nitric oxide bind to the metal center,[50,82–85] oxyanions such as perchlorate and phosphate bind,[86–88] but not to the active site, and sulfide derivatives have been generated,[89–93] presumably with sulfur replacing the μ-oxo bridge.

The liganded forms of hemerythrin best characterized structurally are azidomet- and oxyhemerythrin, where small molecule ligands are bound with both iron atoms in the Fe(III) oxidation state. Views of the azidomet- and oxyhemerythrin iron centers are included in Figure 4. Liganded complexes of deoxy (Fe(II)–Fe(II)) and semi-met mixed valence (Fe(II)–Fe(III)) states have also been formed and are expected to be similar in structure to azidomet and oxy states, although small significant differences in the geometry of the complex are likely.

In azidomet- and oxyhemerythrin, the small ligands bind end-on to the iron atom that was pentacoordinate in the unliganded derivatives. Resonance Raman experiments[59] on azidomet- and oxyhemerythrin formed using isotopically labeled azide and dioxygen narrowed the possible models for binding to the complex to two: an end-on model and a bridging one. The bridged model was consistent with evidence obtained from Mossbauer spectroscopic studies, but end-on binding is that seen directly in several crystallographic investigations. This binding mode makes both iron atoms hexacoordinate with the ligands arranged roughly octahedrally.

The amino acid residues surrounding the dioxygen binding site that are not bound to the iron atoms are all hydrophobic. There are five residues with atoms within 4 Å of the dioxygen molecule in *T. dyscrita* oxyhemerythrin: two leucines, one isoleucine, one phenylalanine, and one tryptophan. Figure 6 shows how these residues surround the bound dioxygen and isolate it from the surface of the protein.

Four of these five residues are invariant across the sequences reported in Table 1. (Leucine 28 is replaced by an isoleucine in one sequence.) In addition to these four residues and the seven bound to the iron atoms, there are 13 other invariant residues identified in Table 1. As Figure 7a shows, all 24 invariant residues are located at the end of the protein containing the iron complex. Most of them are buried residues and presumably contribute to the structural integrity of the metalloprotein. Several of the residues (proline 5, proline 7, tryptophan 10, phenylalanine 14, phenylalanine 29, and phenylalanine 107) are involved in maintaining the conformation of the N-terminal tail and provide a hydrophobic core to hold the tail up against the α-helical bundle.

Most of the other invariant residues are packed around the iron complex and the active site and contribute to the overall hydrophobicity of this part of the structure and help stabilize the tertiary structure of the C-terminal part of the protein. Several of the invariant tyrosine residues and lysine 112 create a hydrogen-bonding network that stabilizes the distorted helical structure near aspartic acid 106, one of the iron ligands.

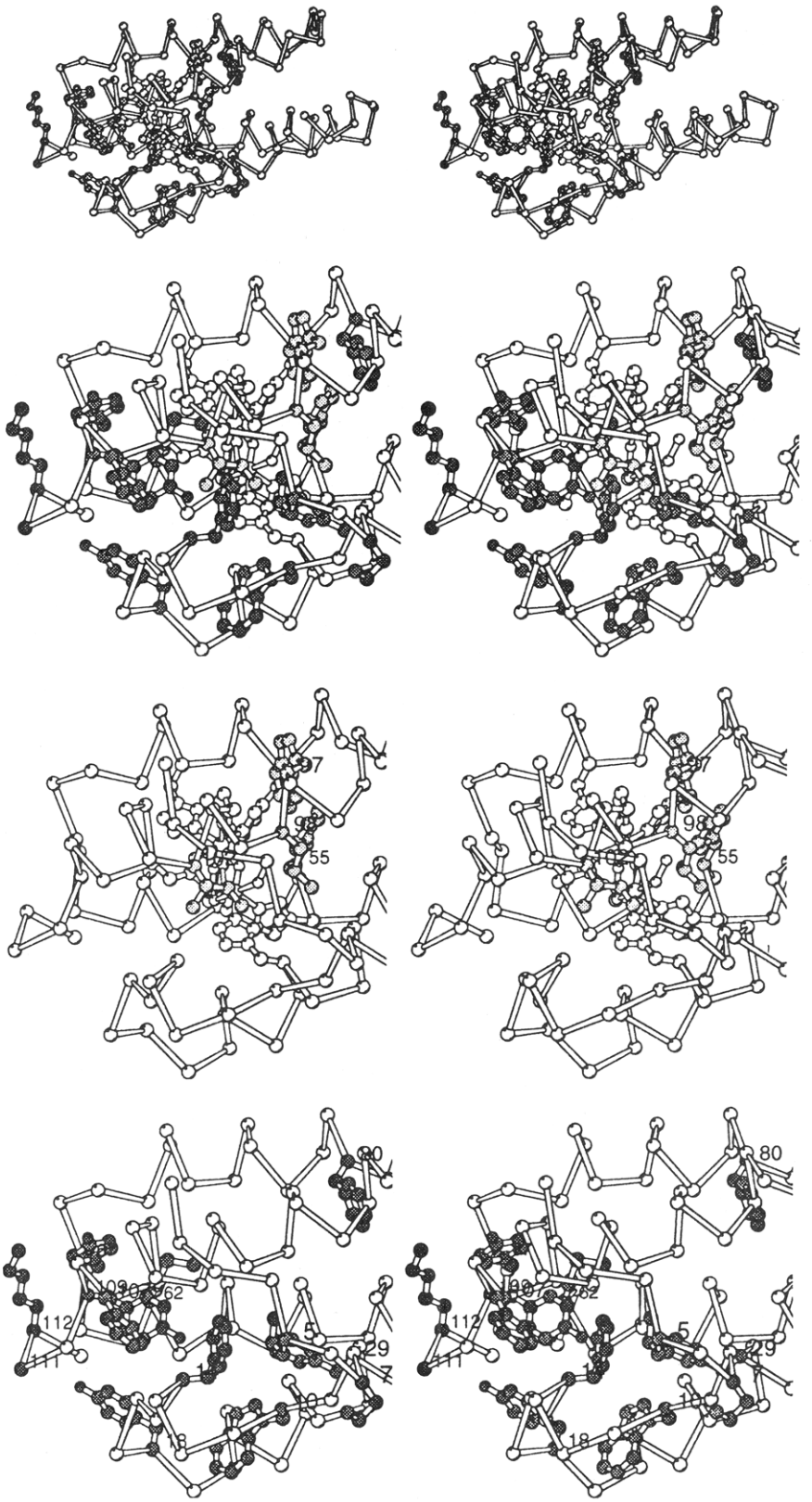


Figure 7. Stereoview of the hemerythrin subunit showing the location of the invariant residues: (a, first) α carbon plot showing the relationship between the invariant residues and the overall subunit structure (Residues bound to the iron atoms are white; other invariant residues are gray.), (b, second) Close-up of the invariant residues (c, third) the iron complex and the invariant residues forming the dioxygen site, (d, fourth) the remaining invariant residues.

Of the three invariant tyrosine residues, tyrosine 109 is closest to the metal complex and early on was considered as a possible iron ligand. Its hydroxyl is within 3 Å of the oxygen atoms of glutamic acid 58 and aspartic acid 106, the atoms bound to the iron atoms in the carboxylate bridges. A similar interaction is seen in azidometmyohemerythrin,[72] but the orientation of the hydroxyl with respect to the carboxylate planes in the *T. dyscrita* hemerythrins is not optimal for a strong hydrogen bond. Tyrosine 109 might be invariant because it fits in the packing of the side chains in this region and provides a suitable transition between the

polar iron liganding groups and the core of the protein.

Given our present knowledge of protein structure and function, it is easy to believe that the invariant residues listed in Table 1 are important in the functioning of hemerythrin. Much of the above discussion however is simply speculation, and careful mutagenesis and structural and functional studies will be necessary to fully sort out the roles the invariant residues play in oxygen transport and storage.

The views of the iron complex shown in Figure 4 are derived largely from X-ray crystallographic studies and display the strengths and limitations of that kind of structural information. While many techniques have been used to probe the structure of the metal center, crystallographic studies provided the first detailed models of the complex. These were sufficient to give a general view of the groups bound to the metal atoms and were useful for assessing the large amount of experimental information available for hemerythrin. They also guided the synthesis of model compounds for this class of iron compounds.[94-100] Kurtz has recently reviewed the chemistry of oxo-bridged iron complexes and its relevance to biological systems.[101] Detailed structural and spectroscopic studies of model compounds containing the Fe–μ-O–Fe unit have been invaluable in interpreting results obtained for hemerythrin and the other iron proteins, but attempts to synthesize small complexes capable of reversibly binding dioxygen have not yet succeeded. Presumably the functional differences between hemerythrin and the model complexes are associated with the environmental effects of the polypeptide on the complex. It will be interesting to see what creative synthetic approaches will be necessary to obtain reversible dioxygen binding in small Fe–μ-O–Fe complexes.

VII. Reactions of Hemerythrin

As important as crystallographic studies of proteins are for obtaining overviews of the molecular structures, they are limited in their precision by the relatively low resolution of the diffraction patterns, and they generally do not provide much information about the dynamics or reactivities of macromolecules. Other kinds of information (spectroscopic, kinetic, etc.) need to be combined with the structural models to allow a better understanding of the reactivity and functionality of the protein.

Many chemical transformations of hemerythrin (metal exchange, redox reactions, coordination changes)[2,102] have been discovered and manipulated in trying to understand its biological function. Recent progress in reconstituting the protein with iron and other metals[102] should prompt further investigations of the chemistry of metalloproteins containing similar metal complexes. The review article by Wilkins and Wilkins[2] provides a good summary of the range of reactivities of hemerythrin. Information relevant to the binding of dioxygen will be summarized here.

The kinetics of dioxygen binding have been investigated *in vitro* using temperature jump, stopped flow, and laser photolysis techniques.[2,103-105] In the case of *P. gouldii* hemerythrin, the species most studied *in vitro*, the protein shows no cooperativity or pH dependence in its dioxygen binding.[103] In one kinetic study of *P. gouldii* hemerythrin, the rate constant for binding is 7.4×10^6 M^{-1} s^{-1} while the rate constant for dissociation is 51 s^{-1}. The equilibrium constant then is 1.5×10^5 M^{-1}, indicative of strong binding to the iron complex. A more recent investigation of *P. gouldii* hemerythrin in which the effects of deuterated solvent were also studied gave similar values ($k_{on} = 1.2 \times 10^{-7}$ M^{-1} s^{-1}, $k_{off} = 43.1$ s,[1] and $K_{eq} = 2.8 \times 10^{-5}$ M^{-1}).[106] The on and off rates are more rapid for *Sipunculus nudus*) hemerythrin and *T. zostericola* myohemerythrin,[103,104] but the equilibrium constants are very similar to those reported for *P. gouldii* hemerythrin. It is not yet clear how the kinetic properties of hemerythrin can be correlated with the protein structure. Further experiments probing how dioxygen arrives at the active site will be necessary before observations such as the influence of D_2O on the dioxygen dissociation rate (but not the association rate) can be fully understood. Given the limitations of crystallographic investigations for studying the details of protein dynamics, other techniques and approaches will be needed to generate a structural base for understanding the kinetics of the oxygenation reaction and the competition between oxygenation and autooxidation.

Figure 1 is consistent with the kinetic, spectroscopic, and structural data currently available for deoxy- and oxyhemerythrin and provides a good summary of what is known about the hemerythrin–dioxygen interaction. Dioxygen binds to one of the iron atoms in the metal center as a peroxo anion, the strongest evidence for which is found in the resonance Raman spectra of oxyhemerythrin,[57] where an O–O stretching frequency of 844 cm^{-1} characteristic of peroxide is found.

As stated above, the binding pocket in hemerythrin is quite hydrophobic, and this type of environment presents a problem for stabilizing the peroxo anion. One solution for this would be to protonate the peroxo anion. Because dioxygen binding is pH independent, the proton must be provided by the protein and not the solvent. The only functional group in the deoxyhemerythrin binding pocket capable of donating a proton to dioxygen is the μ-hydroxo bridge since hydrophobic amino acid residues form the rest of the surface of the ligand-binding cavity and no internal water molecules have been found in the O_2 binding site in either deoxy- or oxyhemerythrin.

Experimental evidence concerning the protonation of the peroxo anion has been provided by resonance Raman, circular dichroism, and single-crystal absorbance spectroscopies. Isotope effects on the vibrational spectra of the bound oxygen provide the strongest evidence for the transfer of the proton to the peroxo anion and the existence of a hydrogen bond between the μ-oxo bridge and the protonated dioxygen species.[62,63] In addition, the experimentally observed electronic transitions are supportive of a μ-hydroxo bridge in deoxyhemerythrin and a protonated peroxo group in oxyhemerythrin. Reem et al.[52] also pointed out that the bent Fe–O–Fe unit aids in the transfer of the proton to the dioxygen molecule and that the bridging carboxylate groups are likely important for stabilizing this geometry. While the location of the bound dioxygen seen in the crystal structure of oxyhemerythrin is consistent with the hydrogen-bonding model, the resolution limit of the diffraction study (2.0

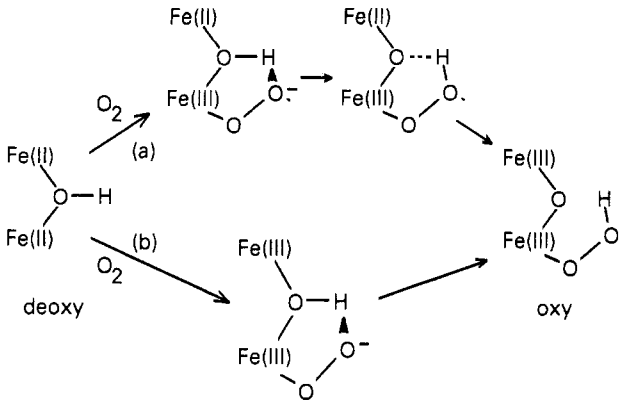


Figure 8. Models for oxygenation of hemerythrin (adapted from ref 5).

Å) rules out any possibility for locating the hydrogen atom in the hydrogen bond.

Direct structural evidence for the μ-hydroxo bridge in deoxyhemerythrin is not overly strong, but the crystallographic and EXAFS investigations of deoxyhemerythrin indicate that the Fe–O bond distances are slightly longer than in oxy- or methemerythrin[69,74] just as is found in model compounds with μ-hydroxo bridges.[96] Finally, it should be noted that a hydrogen bond between the bound dioxygen and the distal histidine is thought to stabilize oxyhemoglobin.[107-110]

Figure 1, while serving as a description of the beginning and final structures of deoxy- and oxyhemerythrin, leaves open several possible mechanisms for the order in which the proton and electrons are transferred from the metal center to the dioxygen species. Que and True[4] and Howard and Rees[5] have proposed alternative mechanisms, the options presented by Howard and Rees consisting of two one-electron transfers bracketing proton transfer from the metal center to the dioxygen or a mechanism with proton transfer following a concerted two-electron transfer (see Figure 8). Strong experimental evidence differentiating between these possibilities is not yet available, but Howard and Rees point out that resonance Raman studies of the NO derivative of deoxyhemerythrin indicate the presence of a hydrogen bond to the ligand in the resulting mixed-valence compound that would be consistent with the initial step of the first mechanism.[84]

VIII. Allostery and Cooperativity

Allosteric effectors and cooperative dioxygen binding play major roles in the biological functions of hemoglobin and hemocyanin. The octameric hemerythrins best characterized chemically, those from *P. gouldii* and *T. dyscrita*, are noncooperative in their dioxygen binding, but oxy anions such as perchlorate and phosphate are allosteric effectors for binding of ligands to the iron complex. Binding of oxy anions at a site on the surface of the protein,[111] 10–15 Å away from the active site, destabilizes several complexes of methemerythrin[86,87] as well as oxyhemerythrin.[88] Perchlorate does this by increasing the off-rate for dioxygen.[103] A structural base for understanding the effect is not available due to the low resolution of the crystallographic study of perchlorate binding.[111] Phosphate affects oxygenation at nonphysiological concentrations, and searches for other physiological allosteric effectors of *P. gouldii* hemerythrin have not been successful. ^{31}P NMR experiments on *P. gouldii* hemerythrocytes have shown that large amounts of 2-aminoethyl phosphorate ($O_3^{2-}POCH_2CH_2NH_3^+$) and (2-aminoethyl)phosphonate ($O_3^{2-}PCH_2CH_2NH_3^+$) are present, but while the first compound affects azide binding to hemerythrin, neither compound has any significant effect on dioxygen binding.[112]

While the most extensively studied hemerythrins show no large allosteric effects or cooperative binding, this is not a general characteristic of all hemerythrins. The dioxygen affinity and cooperativity of hemerythrin isolated from the tentacles of the sipunculid *T. zostericola* is modulated by Ca^{2+}, Cl^-, and CO_2.[113] The octameric hemerythrins from *P. gouldii* and *T. dyscrita* are isolated from the coelomic cavities, so these differences in allosteric and cooperative behavior might indicate differences associated with compartmentalization within an organism or they might be due to differences between species.

There are also significant differences between the hemerythrins found in sipunculids and those found in brachiopods. Hemerythrin from *L. unguis* binds dioxygen cooperatively at pH 7.6 while it does so noncooperatively at pH 6.8.[24] Spectroscopic investigations of this hemerythrin[114] and the one isolated from *L. reevii*[115] indicate that their diiron complexes are similar to those in the sipunculids so the differences in dioxygen binding are not associated with a fundamentally different metal site. Cooperativity is not shown in the binding of ligands other than dioxygen, implying that the oxidation change in the iron center upon addition of dioxygen is important for the intersubunit interactions involved in the cooperative binding.[115] Recently, Zhang and Kurtz[26] showed that *L. reevii* hemerythrin also contains two distinct subunits, making it an $\alpha_4\beta_4$ octamer. Crystallographic investigations are underway in this laboratory to determine the structural basis for cooperativity in this molecule.

IX. Comparative Chemistry, Biochemistry, and Biology

Hemerythrin's limited distribution in the animal kingdom implies that it is an evolutionary development that failed to provide the best means of solving the reversible oxygen-binding problem. Hemoglobin and hemocyanin are much more successful in this regard. The inappropriateness of hemerythrin as an oxygen-binding protein for other organisms is not because it binds dioxygen poorly, since the iron complex binds dioxygen effectively.

However, hemerythrin and the other dioxygen binding proteins differ significantly in their allosteric effects and cooperative dioxygen binding. Presumably these have provided evolutionary advantages to organisms making use of hemoglobin and hemocyanin. A small amount of structural similarity among all three dioxygen binding proteins has been observed.[116] Two of the helices binding iron and copper atoms in hemerythrin and hemocyanin can be superposed with two of the helices that provide the histidine ligands in hemoglobin. A case can be made for the three proteins being related via an ancestral metal-binding protein,[116] but it is also

possible that the similar structures are a reflection of the properties of amino acid sequences capable of binding metal atoms and not necessarily evidence for evolutionary connections between the dioxygen-binding proteins. It might then be that the proteins developed independently as different molecular solutions for the transfer or storage of dioxygen in natural systems.

Hemerythrin appears to be an important molecule for the animals that use it for dioxygen storage or transfer, but the physiological function of the protein is not completely understood. Sipunculids and brachiopods are sedentary animals and have moderate metabolic needs for dioxygen. They are able to live under anaerobic conditions for 5–6 days, and hemerythrin might carry out a storage function to supply dioxygen when the animals are unable to obtain it from sea water.[117] A complication associated with the biological role of hemerythrin is that the various forms of hemerythrin found in the muscles, the tentacles, and the coelomic cavity of the sipunculids have different dioxygen-binding affinities. In some species, the coelomic hemerythrin has a higher affinity for dioxygen than does the tentacular hemerythrin; thus these species extract dioxygen from their tentacles. In other species the pattern is reversed, and the animals appear to respire through their body wall.[25] Whether hemerythrin functions as simply an oxygen-storage molecule in these organisms or is associated with more complicated control mechanisms has yet to be clearly investigated.

X. Conclusion

At this point, it appears that the major questions concerning the fundamental chemical properties of hemerythrin have been largely solved although it is dangerous to make such a claim since it is possible that someone might investigate the molecule with a new technique or new theory and initiate another round of progress in understanding its properties. More research probing the interactions between metal atoms and the protein ligand can be expected, especially now that reconstitution of the protein is possible and changes can be introduced into the protein using site-directed-mutagenesis techniques. Likewise, additional investigations of the allosteric and cooperative binding properties of the brachiopod hemerythrins will likely provide interesting comparisons between cooperative and noncooperative proteins, and the control processes such as those found in brachiopod hemerythrins might serve as models for ways to engineer specific properties into proteins of biotechnical interest.

Amazing progress has been made over the past 40 years in understanding hemerythrin and its functions. There have been many results reported, some confusion as to what they meant, and a few mistakes made in probing this interesting metalloprotein. It has been exciting to watch investigators develop flashes of insight, apply an array of experimental techniques, recover from mistaken conclusions, compete with one another, and transform and rephrase the questions being addressed.

XI. Acknowledgement

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XII. References

(1) Kraulis, P. J. *J. Appl. Cryst.* **1991**, *24*, 946.
(2) Wilkins, P. C.; Wilkins, R. G. *Coord. Chem. Rev.* **1987**, *79*, 195.
(3) Sanders-Loehr, J. In *Iron Carriers and Iron Proteins*; Loehr, T. M., Ed., VCH: Weinheim, 1989; p 373.
(4) Que, L., Jr.; True, A. E. *Progress in Inorganic Chemistry: Bioinorganic Chemistry*; Lippard, S. J., Ed.; John Wiley & Sons, Inc.; New York, Vol 38 1990; p 97.
(5) Howard, J. B.; Rees, D. C. *Adv. Protein Chem.* **1991**, *42*, 199.
(6) Wilkins, R. G. *Chem. Soc. Rev.* **1992**, 171.
(7) Klotz, I. M.; Klotz, T. A.; Fiess, H. A. *Arch. Biochem. Biophys.* **1957**, *68*, 284.
(8) Love, W. E. *Biochim. Biophys. Acta* **1957**, *23*, 465.
(9) Klotz, I. M.; Keresztes-Nagy, S. *Biochemistry* **1963**, *2*, 445.
(10) Klippenstein, G. L., Van Riper, D. E.; Oosterom, E. A. *J. Biol. Chem.* **1972**, *247*, 5959.
(11) Sieker, L. C.; Bolles, L.; Stenkamp, R. E.; Jensen, L. H.; Appleby, C. A. *J. Mol. Biol.* **1981**, *148*, 493.
(12) Addison, A. W.; Bruce, R. E. *Arch. Biochem. Biophys.* **1977**, *183*, 328.
(13) Klippenstein, G. L. *Biochemistry* **1972**, *11*, 372.
(14) Klippenstein, G. L.; Holleman, J. W.; Klotz, I. M. *Biochemistry* **1968**, *7*, 3868.
(15) Gormley, P. M.; Loehr, J. S.; Brimhall, B.; Hermodson, M. A. *Biochem. Biophys. Res. Commun.* **1978**, *85*, 1360.
(16) Ferrell, R. E.; Kitto, G. B. *Biochemistry* **1971**, *10*, 2923.
(17) Loehr, J. S.; Lammers, P. J.; Brimhall, B.; Hermodson, M. A. *J. Biol. Chem.* **1978**, *253*, 5726.
(18) Uchida, T.; Yano, H.; Satake, K.; Kubota, I.; Tsugita, A. *Protein Seq. Data Anal.* **1990**, *3*, 141.
(19) Klippenstein, G. L.; Cote, J. L.; Ludlam, S. E. *Biochemistry* **1976**, *15*, 1128.
(20) Long, R. C.; Zhang, J.-H.; Kurtz, D. M., Jr.; Negri, A.; Tedeschi, G.; Bonomi, F. *Biochim. Biophys. Acta* **1992**, *1122*, 136.
(21) Takagi, T.; Cox, J. A. *FEBS Lett.* **1991**, *285*, 25.
(22) Yano, H.; Satake, K.; Ueno, Y.; Kondo, K.; Tsugita, A. *J. Biochem.* **1991**, *110*, 376.
(23) Yano, H.; Satake, K.; Ueno, Y.; Tsugita, A. *Protein Seq. Data. Anal.* **1991**, *4*, 87.
(24) Zimmer, J. R.; Tachi-iri, Y.; Takizawa, H.; Handa, T.; Yamamura, T.; Kihara, H. *Biochim. Biophys. Acta* **1986**, *874*, 174.
(25) Manwell, C. *Comput. Biochem. Physiol.* **1960**, *1*, 277.
(26) Zhang, J. H.; Kurtz, D. M., Jr. *Biochemistry* **1991**, *30*, 9121.
(27) Hendrickson, W. A.; Klippenstein, G. L.; Ward, K. B. *Proc. Natl. Acad. Sci., U.S.A.* **1975**, *72*, 2160.
(28) Ward, K. B.; Hendrickson, W. A.; Klippenstein, G. L. *Nature* **1975**, *257*, 818.
(29) Smith, J. L.; Hendrickson, W. A.; Addison, A. W. *Nature* **1983**, *303*, 86.
(30) Stenkamp, R. E.; Sieker, L. C.; Jensen, L. H. *Proc. Natl. Acad. Sci., U.S.A.* **1976**, *73*, 349.
(31) Presnell, S. R.; Cohen, F. E. *Proc. Natl. Acad. Sci., U.S.A.* **1989**, *86*, 6592.
(32) Mathews, F. S.; Bethege, P. H.; Czerwinski, E. W. *J. Mol. Biol.* **1979**, *254*, 1699.
(33) Finzel, B. C.; Weber, P. C.; Hardman, K. D.; Salemme, F. R. *J. Mol. Biol.* **1985**, *186*, 627.
(34) Bloomer, A. C.; Champness, J. N.; Bricogne, G.; Staden, R.; Klug, A. *Nature* **1978**, *276*, 362.
(35) Stubbs, G.; Warren, S.; Holmes, K. *Nature* **1977**, *267*, 216.
(36) Banyard, S. H.; Stammers, D. K.; Harrison, P. M. *Nature* **1978**, *271*, 282.
(37) Bazan, J. F. *Science* **1992**, *257*, 410.
(38) McKay, D. B. *Science* **1992**, *257*, 412.
(39) Smith, L. J.; Redfield, C.; Boyd, J.; Lawrence, G. M. P.; Edwards, R. G.; Smith, R. A. G.; Dobson, C. M. *J. Mol. Biol.* **1992**, *224*, 899.
(40) Powers, R.; Garrett, D. S.; March, C. J.; Frieden, E. A.; Gronenborn, A. M.; Clore, G. M. *Science* **1992**, *256*, 1673.
(41) Wlodawer, A.; Pavlovsky, A.; Gustchina, A. *FEBS Lett.* **1992**, *309*, 59.
(42) Diederichs, K.; Boone, T.; Karplus, P. A. *Science* **1991**, *254*, 1779.
(43) Abdel-Meguid, S. S.; Shieh, H.-S.; Smith, W. W.; Dayringer, H. E.; Violand, B. N.; Bentle, L. A. *Proc. Natl. Acad. Sci., U.S.A.* **1987**, *84*, 6434.
(44) Vox, A. M.; Ultsch, M.; Kossiakoff, A. A. *Science* **1992**, *255*, 306.
(45) Weber, P. C.; Salemme, F. R. *Nature* **1980**, *287*, 82.
(46) Rill, R. L.; Klotz, I. M. *Arch. Biochem. Biophys.* **1971**, *147*, 226.
(47) York, J. L.; Roberts, M. P. *Biochim. Biophys. Acta* **1976**, *420*, 265.
(48) Klippenstein, G. L. *Biochem. Biophys. Res. Commun.* **1972**, *49*, 1474.
(49) Klippenstein, G. L. *Am. Zool.* **1980**, *20*, 39.
(50) Garbett, K.; Darnall, D. W.; Klotz, I. M.; Williams, R. J. P. *Arch. Biochem. Biophys.* **1969**, *103*, 419.
(51) Reem, R. C.; Solomon, E. I. *J. Am. Chem. Soc.* **1987**, *109*, 1216.
(52) Reem, R. C.; McCormick, J. M.; Richardson, D. E.; Devlin, F. J.; Stephens, P. J.; Musselman, R. L.; Solomon, E. I. *J. Am. Chem. Soc.* **1989**, *111*, 4688.
(53) Dunn, J. B. R.; Addison, A. W.; Bruce, R. E.; Loehr, J. S.; Loehr, T. M. *Biochemistry* **1977**, *16*, 1743.

(54) Okamura, M. Y.; Klotz, I. M.; Johnson, C. E.; Winter, M. R. C.; Williams, R. J. P. *Biochemistry* **1969**, *8*, 1951.
(55) Garbett, K.; Johnson, C. E.; Klotz, I. M.; Okamura, M. Y.; Williams, R. J. P. *Arch. Biochem. Biophys.* **1971**, *142*, 574.
(56) Clark, P. E.; Webb, J. *Biochemistry* **1981**, *20*, 4628.
(57) Dunn, J. B. R.; Shriver, D. F.; Klotz, I. M. *Proc. Natl. Acad. Sci. U.S.A.* **1973**, *70*, 2582.
(58) Dunn, J. B. R.; Shriver, D. F.; Klotz, I. M. *Biochemistry* **1975**, *14*, 2689.
(59) Kurtz, D. M., Jr.; Shriver, D. F.; Klotz, I. M. *J. Am. Chem. Soc.* **1976**, *98*, 5033.
(60) Kurtz, D. M., Jr.; Shriver, D. F.; Klotz, I. M. *Coord. Chem. Rev.* **1977**, *24*, 145.
(61) Freier, S. M.; Duff, L. L.; Shriver, D. F.; Klotz, I. M. *Arch. Biochem. Biophys.* **1980**, *205*, 449.
(62) Shiemke, A. K.; Loehr, T. M.; Sanders-Loehr, J. *J. Am. Chem. Soc.* **1984**, *106*, 4951.
(63) Shiemke, A. K.; Loehr, T. M.; Sanders-Loehr, J. *J. Am. Chem. Soc.* **1986**, *108*, 2437.
(64) Maroney, M. J.; Kurtz, D. M., Jr.; Nocek, J. M.; Pearce, L. L.; Que, L., Jr. *J. Am. Chem. Soc.* **1986**, *108*, 6871.
(65) Que, L., Jr.; Maroney, M. J. In *Metal Ions in Biological Systems*; Sigel, H., Ed.; Marcel Dekker, Inc.: New York, Vol. 21 1987; p 87.
(66) Hendrickson, W. A.; Co, M. S.; Smith, J. L.; Hodgson, K. O.; Klippenstein, G. L. *Proc. Natl. Acad. Sci., U.S.A.* **1982**, *79*, 6255.
(67) Elam, W. T.; Stern, E. A.; McCallum, J. D.; Sanders-Loehr, J. *J. Am. Chem. Soc.* **1982**, *104*, 6369.
(68) Elam, W. T.; Stern, E. A.; McCallum, J. D.; Sanders-Loehr, J. *J. Am. Chem. Soc.* **1983**, *105*, 1949.
(69) Zhang, K.; Stern, E. A.; Ellis, F.; Sanders-Loehr, J.; Shiemke, A. K. *Biochemistry* **1988**, *27*, 7470.
(70) Moss, T. H.; Moleski, C.; York, J. L. *Biochemistry* **1971**, *10*, 840.
(71) Dawson, J. W.; Gray, H. B.; Hoenig, H. E.; Rossman, G. R.; Schredder, J. M.; Wang, R.-H. *Biochemistry* **1972**, *11*, 461.
(72) Sheriff, S.; Hendrickson, W. A.; Smith, J. L. *J. Mol. Biol.* **1987**, *197*, 273.
(73) Holmes, M. A.; Stenkamp, R. E. *J. Mol. Biol.* **1991**, *220*, 723.
(74) Holmes, M. A.; Le Trong, I.; Turley, S.; Sieker, L. C.; Stenkamp, R. E. *J. Mol. Biol.* **1991**, *218*, 583.
(75) Nordlund, P.; Sjoberg, B.-M.; Eklund, H. *Nature* **1990**, *345*, 593.
(76) Rosenzweig, A. C.; Frederick, C. A.; Lippard, S. J.; Nordlund, P. *Nature* **1993**, *366*, 537.
(77) Chakrabarti, P. *Protein Eng.* **1990**, *4*, 57.
(78) Godden, J. W.; Turley, S.; Teller, D. C.; Adman, E. T.; Liu, M. Y.; Payne, W. J.; LeGall, J. *Science* **1991**, *253*, 438.
(79) Kannan, K. K.; Ramanadham, M. *Int. J. Quantum. Chem.* **1981**, *20*, 199.
(80) Sanders-Loehr, J.; Wheeler, W. D.; Shiemke, A. K.; Averill, B. A.; Loehr, T. M. *J. Am. Chem. Soc.* **1989**, *111*, 8084.
(81) Gomez-Romero, P.; Witten, E. H.; Reiff, W. M.; Backes, G.; Sanders-Loehr, J.; Jameson, G. B. *J. Am. Chem. Soc.* **1989**, *111*, 9039.
(82) Keresztes-Nagy, S.; Klotz, I. M. *Biochemistry* **1965**, *4*, 919.
(83) Nocek, J. M.; Kurtz, D. M., Jr.; Sage, J. T.; Debrunner, P. G.; Maroney, M. J.; Que, L. Jr. *J. Am. Chem. Soc.* **1985**, *107*, 3382.
(84) Nocek, J. M.; Kurtz, D. M., Jr.; Sage, J. T.; Xia, Y.-M.; Debrunner, P.; Shiemke, A. K.; Sanders-Loehr, J.; Loehr, T. M. *Biochemistry* **1988**, *27*, 1014.
(85) Springborg, J.; Wilkins, P. C.; Wilkins, R. G. *Acta Chem. Scand.* **1989**, *43*, 967.
(86) Garbett, K.; Darnall, D. W.; Klotz, I. M. *Arch. Biochem. Biophys.* **1971**, *142*, 455.
(87) Garbett, K.; Darnall, D. W.; Klotz, I. M. *Arch. Biochem. Biophys.* **1971**, *142*, 471.
(88) DePhillips, H. A., Jr. *Arch. Biochem. Biophys.* **1971**, *144*, 122.
(89) Bayer, E.; Krauss, P.; Schretzmann, P. *Z. Naturforsch.* **1970**, *B25*, 327.
(90) Freier, S. M.; Duff, L. L.; Van Duyne, R. P.; Klotz, I. M. *Biochemistry* **1979**, *18*, 5372.
(91) Lukat, G. S.; Kurtz, D. M., Jr.; Shiemke, A. K.; Loehr, T. M.; Sanders-Loehr, J. *Biochemistry* **1984**, *23*, 6416.
(92) Pearce, L. L.; Utecht, R. E.; Kurtz, D. M., Jr. *Biochemistry* **1987**, *26*, 8709.
(93) Maroney, M. J.; Scarrow, R. C.; Que, L., Jr.; Roe, A. L.; Lukat, G. S.; Kurtz, D. M., Jr. *Inorg. Chem.* **1989**, *28*, 1342.
(94) Armstrong, W. H.; Lippard, S. J. *J. Am. Chem. Soc.* **1983**, *105*, 4837.
(95) Wieghardt, K.; Pohl, K.; Gebert, W. *Angew. Chem.* **1983**, *22*, 727.
(96) Armstrong, W. H.; Lippard, S. J. *J. Am. Chem. Soc.* **1984**, *106*, 4632.
(97) Spool, A.; Williams, I. D.; Lippard, S. J. *Inorg. Chem.* **1985**, *24*, 2156.
(98) Lippard, S. J. *Angew. Chem., Int. Ed. Engl.* **1988**, *27*, 344.
(99) Hagen, K. S.; Lachicotte, R. *J. Am. Chem. Soc.* **1992**, *114*, 8741.
(100) Dong, Y.; Menage, S.; Brennan, B. A.; Elgren, T. E.; Jang, H. G.; Pearce, L. L.; Que, L., Jr. *J. Am. Chem. Soc.* **1993**, *115*, 1851.
(101) Kurtz, D. M., Jr. *Chem. Rev.* **1990**, *90*, 585.
(102) Zhang, J.-H.; Kurtz, D. M., Jr. *Proc. Natl. Acad. Sci., U.S.A.* **1992**, *89*, 7065.
(103) De Waal, D. J. A.; Wilkins, R. G. *J. Biol. Chem.* **1976**, *251*, 2339.
(104) Petrou, A. L.; Armstrong, F. A.; Sykes, A. G.; Harrington, P. C.; Wilkins, R. G. *Biochim. Biophys. Acta* **1981**, *670*, 377.
(105) Alberding, N.; Lavalette, D.; Austin, R. H. *Proc. Natl. Acad. Sci. U.S.A.* **1981**, *78*, 2307.
(106) Armstrong, G. D.; Sykes, A. G. *Inorg. Chem.* **1986**, *25*, 3135.
(107) Phillips, S. E. V.; Schoenborn, B. P. *Nature* **1981**, *292*, 81.
(108) Shaanan, B. *Nature* **1982**, *296*, 683.
(109) Kitagawa, T.; Ondrias, M. R.; Rousseau, D. L.; Ikeda-Saito, M.; Yonetani, Y. *Nature* **1982**, *298*, 869.
(110) Jameson, G. B.; Drago, R. S. *J. Am. Chem. Soc.* **1985**, *107*, 3017.
(111) Stenkamp, R. E.; Sieker, L. C.; Jensen, L. H. *J. Mol. Biol.* **1978**, *126*, 457.
(112) Robitaille, P.-M. L.; Kurtz, D. M., Jr. *Biochemistry* **1988**, *27*, 4458.
(113) Mangum, C. P.; Burnett, L. E. *J. Exp. Zool.* **1987**, *244*, 59.
(114) Kaminaka, S.; Takizawa, H.; Handa, T.; Kihara, H.; Kitagawa, T. *Biochemistry* **1992**, *31*, 6997.
(115) Richardson, D. E.; Emad, M.; Reem, R. C.; Solomon, E. I. *Biochemistry* **1987**, *26*, 1003.
(116) Volbeda, A.; Hol, W. G. J. *J. Mol. Biol.* **1989**, *206*, 531.
(117) Edmonds, S. J. *Proceedings of the International Symposium on the Biology of the Sipuncula and Echiura*; Rice, M. E., Todorivic, M., Eds.; National Museum of Natural History, Smithsonian Institution: Washington DC, 1976; Vol. II, p 3.