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Modeling Coordination Sites in Metallobiomolecules

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Summary. Synthetic metal complexes can closely approach the properties of metal ions in proteins and yield useful information concerning biological structure and function.

Metallobiomolecules are natural products which in their biologically active forms contain integral stoichiometries of one or more metallic elements. A partial classification scheme for some of the more extensively investigated types of metallobiomolecules is presented in Fig. 1. Here the principal distinction is between enzymes, catalysts of bond-breaking and bond-making transformations, and the other types of metallobiomolecules which themselves are noncatalytic in this sense. The metals specified are included in the 11 most common biological elements (H, C, N, O, Na, Mg, K, Ca, S, P, Cl) and the 7 next most abundant elements (Mn, Fe, Co, Cu, Zn, Mo, I), which are found in living organisms. This classification identifies metals involved in some fundamental biological processes such as electron storage and transfer (Fe, Cu), dioxygen binding, storage, and activation (Fe, Cu), and substrate activation and catalysis (Mg, Mn, Fe, Co, Cu, Zn, Mo). Various estimates place at one-fourth to one-third the fraction of proteins and enzymes that on purification to homogeneity contain metals or that otherwise require metal ions for biological activity (*1*).

Investigations of metallobiomolecules have increased markedly in the last decade. High-resolution x-ray crystallographic results, in particular, have facilitated detailed considerations of structural, electronic, and reactivity properties at the molecular level. These metallobiomolecules are highly elaborated coordination complexes whose metal-containing sites (coordination units), comprising one or more metal atoms and their ligands, are usually the loci of electron transfer, binding of exogenous molecules, and catalysis and in this article are termed "active sites." The demonstrated or potential relation between the properties of these sites and those of synthetic coordination complexes has contributed significantly to the emergence of the interdisciplinary field of bioinorganic chemistry. In this article we focus on one aspect of this field which is complementary to investigations of biological molecules themselves: the current status of synthetic chemical approaches to the active sites of selected biomolecules. Quoting from our earlier account (*2*): "When reduced to practice, this approach necessitates the synthesis of relatively low molecular weight complexes, which, ideally, are obtainable in crystalline form and approach or duplicate the biological unit in terms of composition, ligand types, structure, and oxidation level(s). Such models, or synthetic analogues, of course, cannot simulate the environmental effects of and whatever structural constraints are imposed by the normal protein conformation. Indeed, this may be considered an advantage of synthetic analogues, for, being unencumbered by the protein, they should reflect the intrinsic properties of the coordination unit unmodified by the protein milieu." This approach has several significant potential capabilities (*2*, *3*): (i) deduction of minimal site structure (where unknown) based on coincident model-biomolecule properties, or (where known) required for execution of biological function; (ii) detection of the influence of the biomolecular environment on the intrinsic properties of the site as represented by the model.

In the absence of models that realize the first capability, the structures of metal coordination units in biomolecules have been inferred from spectroscopic results or have been more directly established by x-ray crystallography. Selected coordination units whose structures have been deduced from crystallographic investigations (*4*–*36*) are collected in Table 1 [note exclusions (*37*–*40*)] in order to provide recognition of the compositional and structural diversity of metal ion binding sites. These units are classified by a scheme introduced earlier (*2*, *3*). Structural representations (**1** to **11**) of a number of these units are presented in Fig. 2. With very few exceptions, none of these structures has been refined to the stage where metrical information concerning metal-ligand interactions approaches the precision normally forthcoming from x-ray diffraction investigations of low-molecular-weight coordination complexes. Nonetheless, these results, most of which were obtained at a resolution poorer than 2.5 Å, usually suffice to define the coordinated atoms or groups and the overall coordination geometry. At the present stage in the development of protein crystallography little credence should be given to metrical results on proteins if such results appear to be in serious disagreement with those from spectroscopically congruent model compounds.

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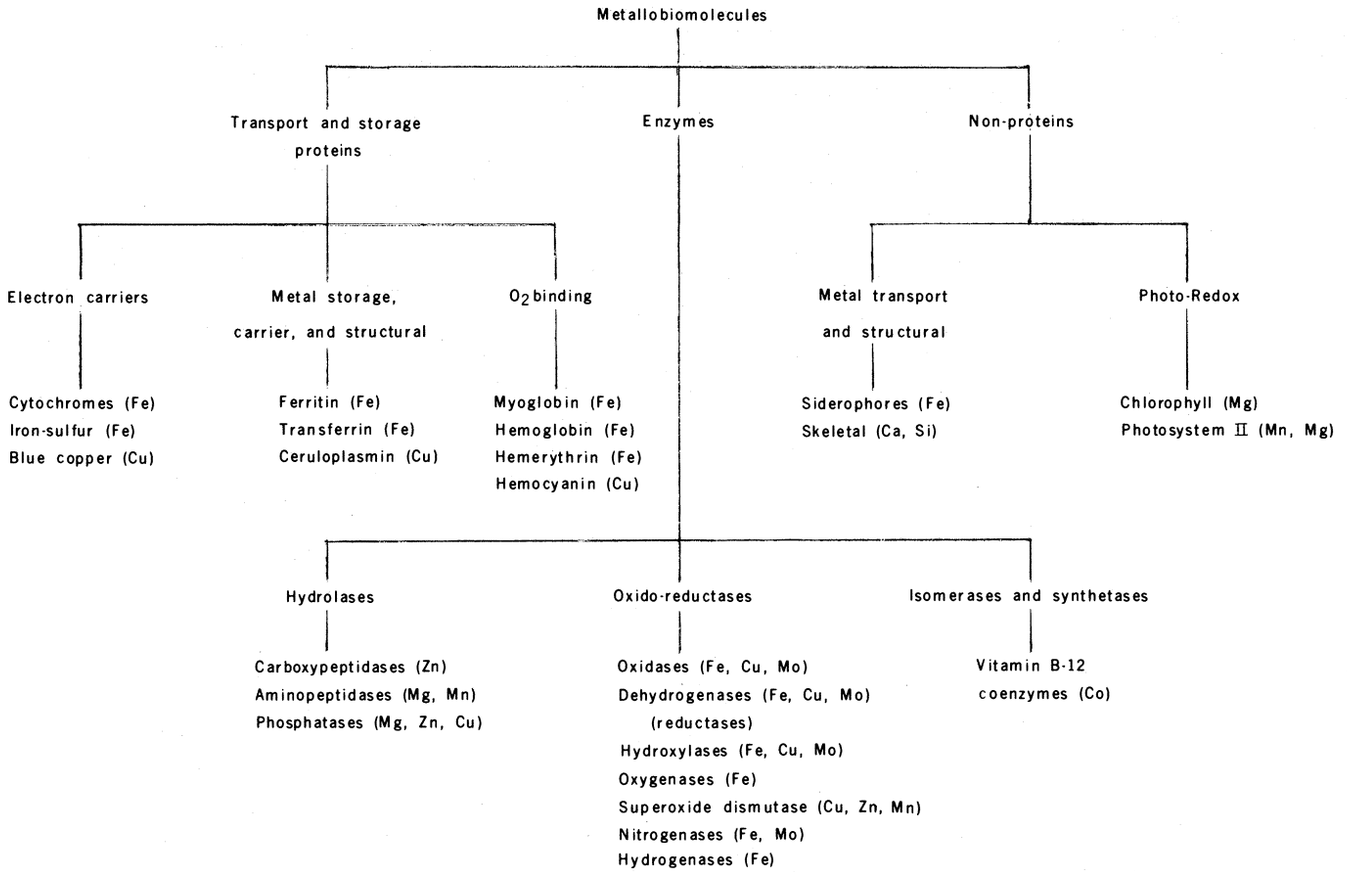
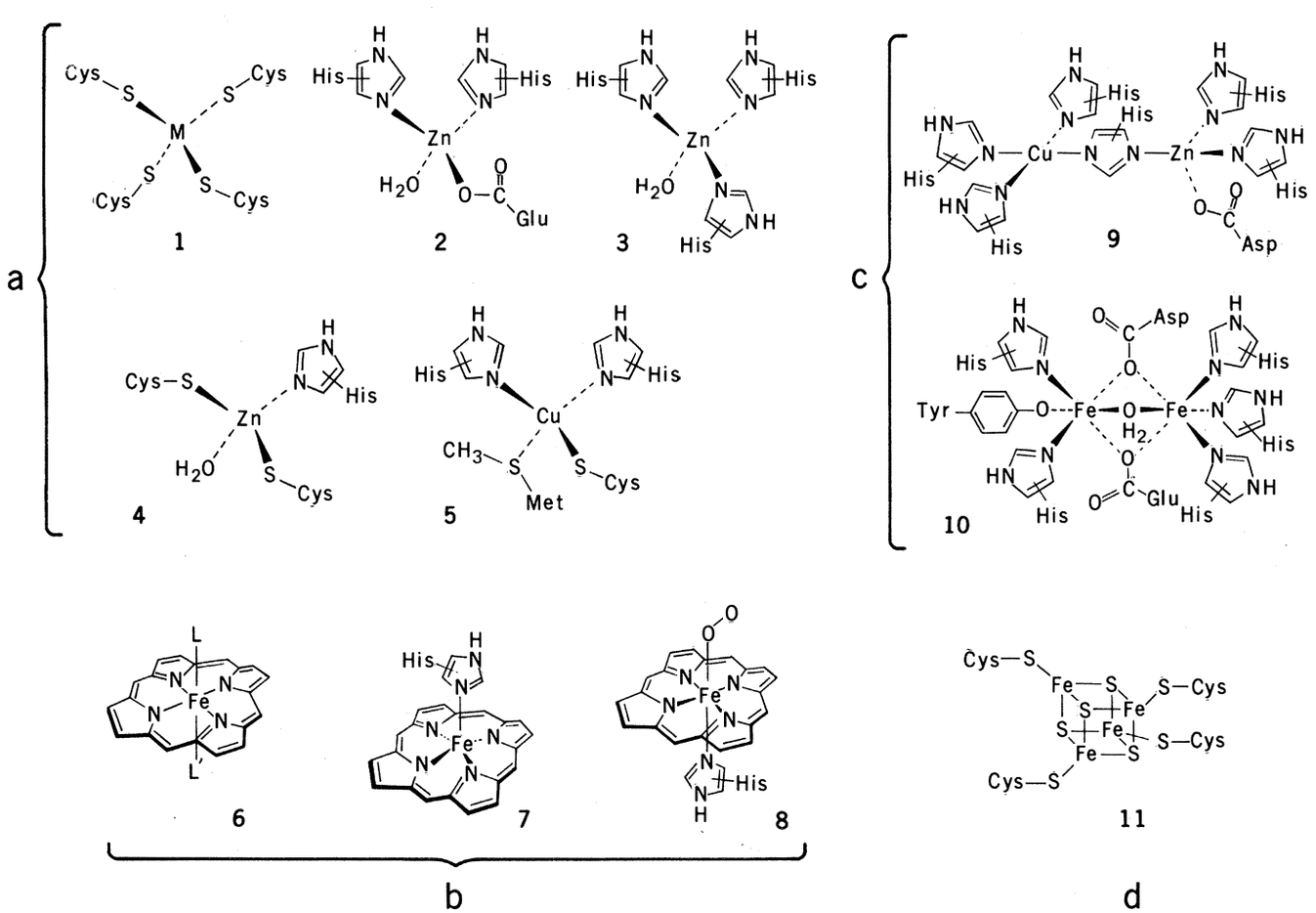


Fig. 1. Partial classification of metallobiomolecules in terms of biological function. Examples rather than complete tabulations for each class are given.



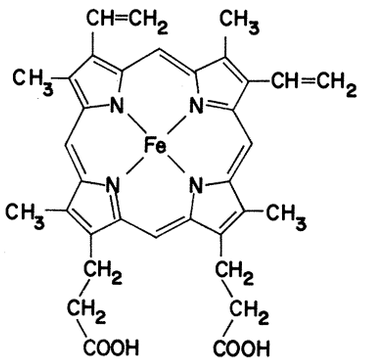
A developing new technique in biomolecular structural determination, which does not require crystalline materials, is x-ray absorption spectroscopy (*41*). Analysis of extended fine structure (EXAFS) at energies above the absorption edge of the metal atom whose spectrum is excited can provide both nearest-neighbor distances from that atom to about ≤ 0.03 Å and information concerning the number and types of atoms coordinated. No angular details are obtainable. This technique has been applied to representatives of several of the classes in Fig. 1; results relevant to models of several different types of coordination units are discussed below.

Inspection of Table 1 and Fig. 2 reveals that types *a* to *d* of coordination units may be the objects of synthesis. Of these the deceptively simple mononuclear sites, type *a*, and the binuclear sites, type *c*, have been the most difficult to model effectively owing to the presence of dissimilar ligands bound to a common metal atom and the pronounced substitutional lability of ions such as Fe(III), Mn(II), Cu(II), and Zn(II). The most significant progress in modeling of active sites has occurred with types *b* and *d*, which contain integral substructures (heme groups, cubic Fe_4S_4 cores) readily assembled and subject to elaboration by variation of terminal ligands. We assess (below) the contributions to selected biological systems afforded by active site models, some of the difficulties and limitations of the approach, and its future prognosis. As will be seen, this approach to the study of active sites can be applied to systems with known and unknown active site structures. Certainly those in Fig. 2 constitute an incomplete evolutionary set, and other structures may be discovered initially by chemical synthesis.

Dioxygen Transport and Storage: Myoglobin and Hemoglobin

In natural systems, all species known to bind the dioxygen molecule for subsequent transport, storage, or activation in enzymic reactions are metallobiomolecules. Those with a transport and storage function include the mammalian proteins myoglobin and hemoglobin, hemerythrin (*31–34*), a nonheme iron protein found in the coelomic fluids of invertebrates, and hemocyanin (*42*), which is a copper protein occurring in the blood of arthropods and molluscs. Structural data for the first three proteins are summarized in Table 1. We restrict consideration to the heme proteins myoglobin and hemoglobin, which contain type *b* coordination units.

Deoxymyoglobin consists of the heme [(protoporphyrinato-IX)iron(II), (**12**)] and



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the connected globin (polypeptide chain). Hemoglobin is closely related to myoglobin, but is a tetramer consisting of four heme groups and four globin groups. The interactions among these four units of the tetramer, interactions grouped under the general term of allosteric properties, are physiologically essential but are poorly understood at the molecular level.

The major function of these heme proteins is the reversible binding of molecular oxygen, which occurs at the iron (II) site. Here the ferrous ion is surrounded by five nitrogen atoms, four from the porphyrin and one from the terminal imidazole group of the proximal histidine residue, the point of attachment between heme and globin. Inorganic chemists have for many years interested themselves in the seemingly simple task of producing an FeN_5 unit that would bind O_2 reversibly. Until recently all such efforts had met with failure. If O_2 is added at room temperature to a ferrous porphyrin, Fe(Por), and a nitrogenous base, B, in a suitable solvent, $Fe(O_2)(Por)(B)$, the desired analog of oxymyoglobin is not formed but instead the irreversibly oxidized Fe(III) product (Por)Fe-O-Fe(Por), the "μ-oxo dimer," is formed. A lesson learned by inorganic chemists is that one of the functions of the globin is to protect the heme group from irreversible oxidation, a fact that was far from obvious to the biochemists as well until the model syntheses were attempted.

While inorganic chemists were unsuccessfully attempting to model the heme group, protein biochemists were making progress in the characterization of myoglobin and hemoglobin. In a recent article Ladner, Heidner, and Perutz (*23*) end with the statement: "This concludes the x-ray analysis of this structure [horse hemoglobin] which M. F. Perutz began in J. D. Bernal's Crystallographic Laboratory at Cambridge in the autumn of 1937." The progress in those 40 years represents one of the great epics in molecular science. It is estimated (*23*) that the root-mean-square errors in atomic positions in horse hemoglobin are about 0.32 Å and that the displacements of the iron atoms from the porphyrin planes are known to an error of about 0.06 Å, remarkable results for a protein of molecular weight 64,000. [For comparison, the corresponding errors in the small molecule bis(1-methylimidazole)(protoporphyrinato-IX)-iron(III) · methanol · water are about 0.001 Å for the Fe position and 0.007 Å for the C, N, and O positions (*43*).]

In many instances the relation of hemoglobin structure to function is well understood and it has been possible to correlate altered hemoglobin function in abnormal hemoglobins with structural change (*44*). But these results have been obtained with data based on roughly known stereochemistry about the iron atoms in deoxyhemoglobin and methemoglobin and the related myoglobins, and with no information for the corresponding oxyhemes (*45*). The estimated errors in atomic positions given above translate into an error of about 25° in an Fe-X-Y angle, where XY is O_2 or CO. With errors of this magnitude we still lack precise knowledge of the mode of attachment of molecular oxygen to the heme site, even though structural results on oxyhemes are now available (*18, 19*).

In addition, we have only indirect information on the changes that occur at the iron site on oxygenation. Yet such information is crucial to an understanding of the relationships of hemoglobin structure to function at the molecular level and especially for an understanding of the allosteric properties of hemoglobin. In hemoglobin, oxygen affinity increases with increasing oxygen pressure but in a sigmoidal manner, rather than with the hyperbolic dependence expected if there were no interactions among the four heme groups. The physiological implications of such interactions are profound. For example, in the change from arterial oxygen pressure of about 95 mm-Hg under physiological conditions to a venous pressure of about 40 mm-Hg only about 1 ml of O_2 per 100 ml of blood would be

Fig. 2 (facing page). Structures of some metal coordination units in proteins classified as in Table 1: (a) **1** (M = Fe, Zn) to **5**; (b) **6** (L = L′ = N-His, cytochrome *b*; L = N-His, L′ = S-Met, cytochrome *c*) to **8**; (c) **9**, **10**; (d) **11**. See (*143*) for a presentation of the myohemerythrin (metazide) active site.

released during circulation through the tissues if hemoglobin were a monomer (as is myoglobin) devoid of allosteric properties; instead about 4.5 ml of O_2 are released.

A number of other enzymatic systems, including phosphorylase and glutamate dehydrogenase (*46*), are allosteric proteins, and therefore in a sense understanding allosteric properties in hemoglobin is an initial step in understanding the working of multicenter proteins in general.

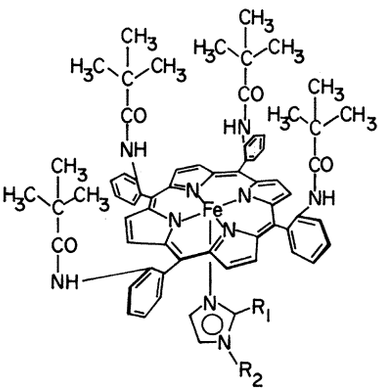
Several thermodynamic models have been proposed to explain the basis of allosteric properties (*47*, *48*). Calculations of strain energy in various hemoglobins (*49*, *50*), together with a detailed comparison (*51*) of their crystal structures, have yielded a structural model for allosteric properties (*51*). These properties may arise from possible conformational changes that are initiated at and about the iron site on ligation. Because these changes at the iron site have been imprecisely defined for the proteins themselves, they have been extrapolated from spectroscopically congruent models for these sites, models amenable to more precise structural characterization.

Such models have been synthesized by several groups of chemists who have found ways of subverting the irreversible oxidation of the ferrous porphyrins. Wang (*52*) approached the problem by immobilizing an iron complex in a polystyrene matrix. He found that the FeN_5 unit would bind O_2 reversibly but could not, because of immobilization, dimerize. Such a system is not amenable to detailed structural study. Chang and Traylor (*53*) prepared the first reversible oxygen carrier of an iron porphyrin. They found that a porphyrin system containing a covalently attached imidazole ligand reacts reversibly with O_2 in solution at −45°C. Subsequent studies have shown that reversibility does not result from the presence of the attached base, but from the low temperature used. Indeed, irreversible oxidation may be prevented at low temperatures, and a number of studies of reaction 1 have been completed (*54*). Such studies provide important assessment of the role of the globin in reactions of the protein systems. But again these low-temperature systems are not amenable to direct structural study.

$$Fe(Por)(B)_2 + A \rightarrow Fe(Por)(B)(A) + B \quad (1)$$

where A is O_2, CO, or NO.

Two systems that are accessible to structural study have now been produced. Collman and his co-workers (*55*) have synthesized a "picket fence" porphyrin (**13**), while Baldwin and his co-



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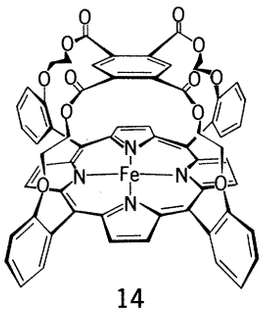
workers (*56*) have produced a "capped" porphyrin (**14**). Each of these porphyrins is sterically encumbered on one side, thereby directing the binding of a nitrogenous base to the other side while providing a sterically protected binding site for dioxygen. In the presence of a base, the Fe(II) complexes bind dioxygen reversibly at room temperature. Virtually all of the structural studies have been

Table 1. Metal coordination units obtained from x-ray crystallography of selected biomolecules, excluding siderophores (*37*), vitamin B_{12} coenzymes (*38*), bacteriochlorophyll (*39*), calcium-binding proteins (*40*). Abbreviations of amino acids: Asp, aspartate; Cys, cysteine; Glu, glutamate; His, histidine; Met, methionine; Tyr, tyrosine. For each amino acid the atom bound to the metal precedes the abbreviation; thus S-Cys implies coordination of the cysteine group via its S atom.

Molecule	Molecular weight	Coordination unit	Reference
		(Type a) Mononuclear, side-chain binding	
Rubredoxin (Rd_{ox})	6,000	$[Fe(S\text{-}Cys)_4]$*	(*4*, *5*)
Carboxypeptidase A,B	34,500	$[Zn(N\text{-}His)_2(O\text{-}Glu)(OH_2)]$*	(*6*)
Thermolysin	34,600	$[Zn(N\text{-}His)_2(O\text{-}Glu)]$*, $4[Ca\text{-}O_{6,7}]$†‡	(*7*)
Concanavalin A	102,000 (4)§	$[Mn(N\text{-}His)(O\text{-}Asp)_2(O\text{-}Glu)(OH_2)_2]$, $[Ca\text{-}O_6]$†‡	(*8*)
Carbonic anhydrase B,C	~29,000	$[Zn(N\text{-}His)_3(OH_2)]$*	(*9*)
Insulin	36,000 (6)	$[Zn(N\text{-}His)_3(OH_2)]$*	(*10*)
Liver alcohol dehydrogenase	80,000 (2)	$[Zn(S\text{-}Cys)_2(N\text{-}His)(OH_2)]$*, $[Zn(S\text{-}Cys)_4]$*	(*11*)
Azurin	14,000	$[Cu(N\text{-}His)_2(S\text{-}Cys)(S\text{-}Met)]$*	(*12*)
Plastocyanin	10,500	$[Cu(N\text{-}His)_2(S\text{-}Cys)(S\text{-}Met)]$*	(*13*)
Aspartate carbamoyltransferase	306,000 (5)	$[Zn(S\text{-}Cys)_4]$*	(*14*)
		(Type b) Mononuclear, porphyrin + side-chain axial binding	
b-type cytochromes	~11,000	$[Fe(N_4\text{-porphyrin})(N\text{-}His)_2]$	(*15*)
c-type cytochromes	~12,000	$[Fe(N_4\text{-porphyrin})(S\text{-Met})(N\text{-}His)]$	(*16*)
Myoglobin, hemoglobin	17,800, 64,500‖ (4)		(*17*)
Oxy		$[Fe(N_4\text{-porphyrin})(N\text{-}His)(O_2)]$	(*18*, *19*)
Deoxy, Met		$[Fe(N_4\text{-porphyrin})(N\text{-}His)]$	(*20*, *21*) (*22*, *23*)
MetX (X = CN^-, N_3^-)		$[Fe(N_4\text{-porphyrin})(N\text{-}His)(X)]$	(*24*)
CO		$[Fe(N_4\text{-porphyrin})(N\text{-}His)(CO)]$	(*25*, *26*)
		(Type c) Binuclear, side-chain bridging and terminal binding	
Superoxide dismutase#	32,000 (2)	$[(His\text{-}N)_3Cu\text{-}\mu(N\text{-}His)$¶$\text{-}Zn(N\text{-}His)_2(O\text{-}Asp)]$*	(*27*)
Hemerythrin (aquomet)**	108,000 (8)	$[(His\text{-}N)_2(Tyr\text{-}O)Fe\text{-}\mu(Asp,Glu,OH_2)\text{-}Fe(N\text{-}His)_3]$†	(*28*, *29*, *141*)
Methemerythrin (ligand complexes)			(*30*)
		(Type d) Bi-, polynuclear, inorganic bridging plus terminal side-chain binding	
Myohemerythrin (metazide)††	13,900	$[(His\text{-}N)_2(Tyr\text{-}O)Fe\text{-}O\text{-}Fe(O\text{-}Tyr)(N\text{-}His)_2]$	(*31–33*, *143*)
Ferredoxin ($8Fe\text{-}Fd_{ox}$)	6,200	$2[Fe_4S_4(S\text{-}Cys)_4]$	(*34*, *35*, *87*)
"High-potential" protein ($HP_{red,ox}$)	9,300	$[Fe_4S_4(S\text{-}Cys)_4]$	(*35*, *36*)

*Distorted tetrahedral. †Distorted octahedral. ‡Sidechain plus H_2O ligation. §Number of subunits is given in parentheses. ¶Distorted planar. ‖Mammalian, references to hemoglobins from other sources included. #Eukaryotic (bovine erythrocyte). **_Thermiste dyscritum_. ††_Thermiste zostericola_.

carried out on the various "picket fence" porphyrin systems (*57*). The first question to be answered, and one that has interested chemists for years, is the nature of the attachment of O_2 to the Fe



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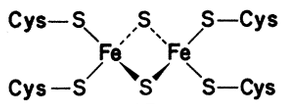
atom in the heme proteins. The synthesis and crystallization of $Fe(O_2)$(TpivPP)(1-MeIm) [TpivPP indicates *meso*-tetra ($\alpha,\alpha,\alpha,\alpha$-orthopivalamidephenyl)porphyrinato or "picket fence porphyrin"; and 1-MeIm indicates 1-methylimidazole] has finally permitted this question to be answered. The O_2 molecule is bound to the iron atom in the end-on, bent geometry (*58*) as in **8**. Essential congruence of this model complex with the iron site in oxyhemoglobin has been established by spectroscopic techniques (*55*). We thus have a dramatic illustration of the use of structural information on model systems to provide fundamental information about the more complex protein systems. The O-O distance in this model and related ones corresponds to that in the superoxide (O_2^-) ion, from which a formal description of oxyhemoglobin as an $Fe^{3+}O_2^-$ system may be proposed (*59*). Unfortunately, crystals of the unoxygenated model, Fe(TpivPP)(1-MeIm), suitable for diffraction studies, have not been isolated, and therefore no direct comparison of the changes at the iron center on oxygenation has been possible. However, suitable crystals of Fe(TpivPP)(2-MeIm) have been obtained (*60*) and these can be oxygenated to yield crystalline $Fe(O_2)$ (TpivPP)(2-MeIm) (*61*).

The results of these crystal structure determinations have important implications for biological systems, although caution must be observed in making comparisons, as 2-MeIm is a sterically bulky base and systems containing it model some of the abnormal hemoglobins [those containing "T-state" oxyhemoglobin (*47*)]. The core in these model systems is surprisingly flexible, with the Fe-porphyrin plane distance a sensitive function of the steric demands of the base. The diminished oxygen affinity in some of the abnormal hemoglobins may result from the inability of the O_2 molecule to approach the Fe atom closely, as is the case in $Fe(O_2)$(TpivPP)(2-MeIm). This lengthened Fe-O distance may be contrasted with the T-state adduct of hemoglobin with NO wherein the Fe-N (imidazole) bond is apparently ruptured (*62-64*). This difference between NO and O_2 may reflect their relative binding and *trans*-labilizing properties. In this regard, although we have stressed the structural results, various equilibrium and thermodynamic measurements on these model systems have provided important comparisons with the native systems on the binding of O_2 and CO (*55*).

In a related model system, the displacement of the Fe(II) ion (0.55 Å) from the mean porphyrin plane in Fe(TPP)(2-MeIm) (*65*) (TPP indicates 5,10,15,20-tetraphenylporphyrinato) is essentially the same as the assessment of this distance in deoxy hemoglobin (*21*). This result indicates that there are no major effects of the globin on the iron geometry, a point of previous contention (*66*). Indeed, the various proposals (*65, 67*) of a "trigger mechanism" for change in the quaternary structure of hemoglobin on oxygenation, based on optimistic assessment of errors inherent in the protein structure determinations and on stereochemical changes in earlier, less relevant model systems, are undoubtedly too simplistic in view of the diversity of iron geometries in these recent models and in view of our knowledge of the corresponding geometries in analogous cobalt porphyrin systems (*68*). Similarly, the congruence of the Fe-N(imidazole) bond lengths in Fe(TpivPP)(1-MeIm), Fe(TpivPP)(2-MeIm), and $Fe(O_2)$(TpivPP)(2-MeIm) suggests that the proposal (*69*) that this bond is under "tension" in T-state oxyhemoglobin needs revision (*70*). These quaternary changes in hemoglobin and the molecular basis for the allosteric properties are poorly understood. However, if such an understanding is achieved, it will rest not only on experiments on the proteins themselves, but also on the inventive synthesis and characterization of congruent model systems.

Electron Carriers

Iron-sulfur proteins as universal electron carriers? The physiological function of iron-sulfur proteins, where it is known or can be inferred, is that of electron transport. A number of small soluble proteins, known generically as ferredoxins (Fd) (whose molecular weights are 6,000 to 25,000), serve as components of electron transfer chains coupled to various oxidoreductases. These proteins constitute one of the three major types of metal-containing electron carriers (Fig. 1). Because they have been isolated from diverse bacterial, plant, and mammalian sources, iron-sulfur proteins are the most widely dispersed carriers of this type in biology. Virtually all properties of these proteins have been reviewed (*2, 71, 72*). With respect to modeling biological coordination units, iron-sulfur proteins have presented an interesting challenge. No other group of metallobiomolecules contains at least three distinct units. The mononuclear unit **1** (M = Fe), found in rubredoxin (Rd), and the tetranuclear cluster **11** (Fig. 2) have been directly demonstrated by x-ray diffraction; the same is now true of the binuclear cluster structure **15** (type *d*)



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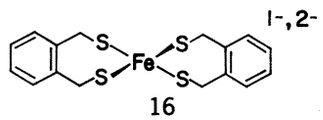
(*73*) which is also fully consistent with extensive physicochemical data (*72*).

In a program initiated in 1971 with our co-workers, synthetic analogs of these three types of Fe-S units have been obtained and their structural and physicochemical properties have been established (*2, 3, 74*). Species **16** (*2, 75*), **17** (*2, 76*), and **18** (*2*) are analogs of protein sites



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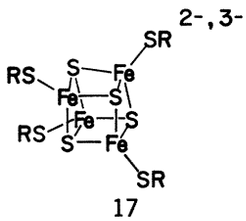

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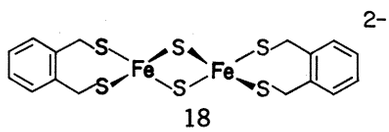

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1, **11**, and **15**, respectively. Virtually all of their properties closely approach or duplicate those of protein sites in corresponding oxidation levels. In particular, the structures of **16** and **17** can be fairly described as high-resolution versions of **1** and **11**. Comparison of results for the 8-Fe Fd_{ox} (containing two clusters) and HP_{red} proteins (Table 1) with several $[Fe_4S_4(SR)_4]^{2-}$ structures (*2*) is especially convincing in this respect; the cubane-type Fe_4S_4 cores of all species are essentially congruent. EXAFS analyses (*77, 78*) have shown that mean Fe-S distances in the three types of protein units and the

$$[Fe(SR)_4]^{2-} \rightleftharpoons [Fe(SR)_4]^{1-}$$

$$Rd_{red} \rightleftharpoons Rd_{ox} \quad (2)$$

(a) (b)

$$[Fe_2S_2(SR)_4]^{4-} \rightleftharpoons [Fe_2S_2(SR)_4]^{3-} \rightleftharpoons [Fe_2S_2(SR)_4]^{2-}$$

$$[Fd_{s\text{-}red}] \quad Fd_{red} \rightleftharpoons Fd_{ox} \quad (3)$$

2Fe(II) Fe(II)+Fe(III) 2Fe(III)

(a) (b) (c)

$$[Fe_4S_4(SR)_4]^{4-} \rightleftharpoons [Fe_4S_4(SR)_4]^{3-} \rightleftharpoons [Fe_4S_4(SR)_4]^{2-} \rightleftharpoons [Fe_4S_4(SR)_4]^{1-}$$

$$Fd_{red} \rightleftharpoons Fd_{ox} \leftrightarrow [Fd_{s\text{-}ox}]$$

$$[HP_{s\text{-}red}] \leftrightarrow HP_{red} \rightleftharpoons HP_{ox} \quad (4)$$

4Fe(II) 3Fe(II)+Fe(III) 2Fe(II)+2Fe(III) Fe(II)+3Fe(III)

(a) (b) (c)

Scheme 1

mean Fe · · · Fe distances in **11** and **15** differ by $\lesssim 0.03$ Å from those of their analogs in the same oxidation level. Taken together, the crystallographic and EXAFS results indicate that the protein units closely approach the essentially unconstrained structures of their analogs.

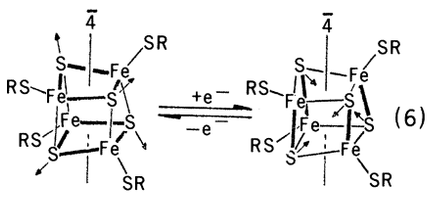
Of the various properties presented by synthetic analogs, the simplest, net charges, are of prime importance inasmuch as they define oxidation levels and the formal oxidation states of the metal atoms therein. For the proteins, such information is obtainable only indirectly from spectroscopic properties. Comparison of physical properties has established the oxidation level equivalencies referred to above. These are presented in scheme 1, columns a, b, and c of the electron transfer series (reactions 2 to 4); presumed nonphysiological protein oxidation levels are given in brackets. All members of the analog series have been detected electrochemically. Of the seven physiologically significant oxidation levels, analogs of five have been obtained as crystalline salts of structures **16** to **18**; in addition $[Fe(SPh)_4]^{2-}$ (*79*) is an acceptable Rd_{red} analog. $[Fe_2S_2(SR)_4]^{3-}$ and $[Fe_4S_4(SR)_4]^{1-}$, both of which are unstable, remain as objectives of synthesis. Of the various synthetic procedures that afford analogs (*2, 80*), those yielding the clusters **17** merit special comment. The reactions 5, involving simple inexpensive reagents, produce clusters in good yield. The product of these reactions, as in the initial syntheses of most inorganic clusters, was not deliberately predestined.

$$FeCl_3 \begin{cases} + HS^- + RS^- \\ + S_8 + RS^- \end{cases} \xrightarrow{CH_3OH} [Fe_4S_4(SR)_4]^{2-} \quad (5)$$

The formation of these clusters results from their being the most thermodynamically stable soluble reaction product, a matter that could not have been assured beforehand. Consequently, these reactions lead to cluster formation by a process of spontaneous self-assembly. Their success raises the more general possibility that other clusters in metallobiomolecules might be produced by similar means, as discussed below.

Synthetic access to the analogs **16** to **18** has led to a number of investigations whose results bear directly on the structure and function of Fe-S coordination units in proteins. One of the earliest findings was a convincingly similar set of spectroscopic and magnetic properties of 2-Fe Fd_{ox} proteins and the dianion **18**, thereby providing substantial evidence of the correctness of the proposed structure **15**. Electrochemical studies of analogs, summarized in reactions 2 to 4, scheme 1, have shown the total number of oxidation levels, related by one-electron transfer reactions, which are inherently accessible to each type of unit. Inasmuch as the principal biological function of iron-sulfur proteins is electron transfer, this information is of considerable importance. The demonstration of 3-, 2-, and 1-oxidation levels in reaction 4 transposes from hypothesis to fact the "three-state" description of the protein clusters **11** (*81*), which was advanced to rationalize the observations that in the isolated condition Fd proteins could only be reduced and HP (high potential) proteins could only be oxidized. These forms of the proteins contain sites isoelectronic with each other and with $[Fe_4S_4(SR)_4]^{2-}$; consequently, reduction and oxidation, which occur at very different potentials (about -0.42 and $+0.35$ V, respectively), result in units that differ by two electrons. A major unanswered question is why, in their normal aqueous solution configurations, all proteins with 4-Fe clusters cannot be made to traverse the same three oxidation levels at comparable potentials. Given the structural and electronic similarities of the species in column b of reaction series 4, the answer appears to lie in the effects of protein structure and environment extrinsic to the clusters and whatever structural constraints are imposed by a protein on other cluster oxidation levels. Some current work on these matters is briefly considered.

As was indicated earlier, one significant attribute of comparative protein-analog studies is that analog properties are those intrinsic to a given coordination unit in a protein. Deviations from these properties signal an influence of the protein matrix. In one comparison, aqueous potentials of the Fd_{ox}/Fd_{red} couple of a prototypic ferredoxin (-0.43 V) and of two $[Fe_4S_4(SR)_4]^{2-,3-}$ couples (-0.49, -0.51 V), measured under identical conditions, differ by < 0.1 V (*82*). The reported range of protein potentials is from -0.28 to -0.49 V, indicating in other cases larger influences that are, however, almost always such as to shift these potentials to less negative values than the intrinsic potential of about -0.5 V (*83*). Cluster structural differences between the members of protein couples are expected; unfortunately, no Fd_{red} structure is available and EXAFS results, while suggesting a small increase in mean Fe-S distances in passing to the Fd_{red} form (*78*), are insufficiently sensitive to localize structural changes. The recent synthesis of reduced clusters $[Fe_4S_4(SR)_4]^{3-}$ (*80*) has facilitated definition of structural changes pursuant to electron transfer (*76, 84*). Four synthetic cluster dianions and the isoelectronic units in Fd_{ox} and HP_{red} proteins exhibit the idealized compressed tetragonal structure **19** with eight long Fe-S bonds



(6)

Compressed D_{2d} Elongated D_{2d}

19 20

(dark lines) roughly perpendicular to the $\bar{4}$ axis. Such structural uniformity is a persuasive indicator that **19** is the inherently stable form of this oxidation level. In contrast, reduced clusters do not exhibit uniform solid-state structures but in

solution do adopt the idealized elongated tetragonal configuration **20** or ones closely related thereto. Structure **20** is observed in the crystalline state for $[Fe_4S_4(SPh)_4]^{3-}$ (*76*) but not for $[Fe_4S_4(SCH_2Ph)_4]^{3-}$ (*85*); the physicochemical properties in solution of these and other reduced clusters are nearly coincident (*76, 84*). Hence, the picture that emerges for the *unconstrained* structural changes upon electron transfer is reaction 6 whose essential feature is expansion and contraction of the Fe_4S_4 core by about 0.08 Å along the $\bar{4}$ axis. Any protein structural features that alter core dimensional changes could conceivably shift Fd_{ox}/Fd_{red} potentials from their intrinsic value. A more provocative possibility is that, in the native form of HP_{red} protein, molecular structural forces may provide a sufficient barrier to the anisotropic core dimensional change that the HP_{red}/HP_{s-red} potential (reaction 4) is displaced to a decidedly negative value, for example, below that for H_2 evolution. One can reduce HP_{red} in a mixture of 80 percent dimethyl sulfoxide and water, a medium capable of unfolding protein structure, at an estimated potential of $\lesssim -0.6$ V (*86*), which is in the range of certain Fd_{ox}/Fd_{red} and $[Fe_4S_4(SR)_4]^{2-,3-}$ potentials in the same medium (*82*). Protein structural and environmental effects, including different extents of peptide-cluster hydrogen bonding in Fd_{ox} and HP_{red} proteins (*87*), doubtless act in concert to set protein potentials. The properties of analogs provide the essential baseline against which the effects of protein structure may be assessed.

Synthetic analogs exhibit a diverse reaction chemistry, one aspect of which has resulted in the development of a new method of identifying 2-Fe and 4-Fe coordination units in proteins. The clusters **21** (n = 2, 4) easily undergo thiolate substitution reactions upon treatment with thiols (*2, 74*) and other electrophiles XY (*88*) (see reactions 7 and 8).

$$[Fe_nS_n(SR)_4]^{2-} + m\ R'SH \rightarrow \underset{\mathbf{21}}{} [Fe_nS_n(SR)_{4-m}(SR')_m]^{2-} + m RSH \quad (7)$$

$$[Fe_nS_n(SR)_4]^{2-} + mXY \rightarrow \underset{\mathbf{21}}{} [Fe_4S_4(SR)_{4-m}X_m]^{2-} + mYSR \quad (8)$$

The occurrence of reaction 7 suggested a related process, reaction 9, in which Fe_2S_2 and Fe_4S_4 core structures are

$$\text{Holoprotein} \xrightarrow[\text{Unfolding solvent}]{\text{RSH}} \underset{\mathbf{21}}{[Fe_nS_n(SR)_4]^{2-}} + \text{apoprotein} \quad (9)$$

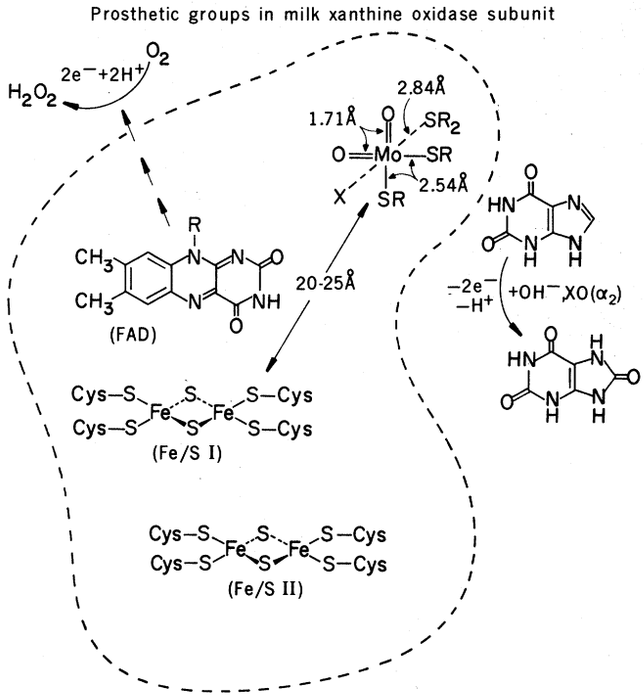


Fig. 3. Schematic depiction of the prosthetic groups in one subunit of milk xanthine oxidase (α_2 subunit structure). The indicated distance was obtained from EPR results; core extrusion results do not identify the cysteinate ligands of the two Fe_2S_2 units.

removed from a protein in the form of the analogs **21**, whose appropriate spectroscopic properties (obtained from independently synthesized compounds) allow their identification and quantitation. The process, which we describe as core extrusion [alternatively, cluster displacement (*89*)], has been developed in several laboratories. The usual experimental conditions employ a solvent medium capable of unfolding protein structure and an aromatic thiol in large excess as the extrusion reagent. Units of known structure (**11**, **15**) in small proteins are readily extruded and assayed by absorption spectrophotometry (*89-92*); quantitation of species **21** to within 10 percent of values based on protein composition and concentration is usual. The extrusion method has provided the first demonstration of the nature of Fe-S centers in hydrogenase, an enzyme found in bacteria and algae which catalyzes H_2 evolution or uptake. Different preparations of a clostridial enzyme contain three (*90*) and one (*93*) Fe_4S_4 units. Subsequently, hydrogenase from another bacterium has yielded three such units upon extrusion (*94*).

Because many enzymes with unestablished Fe-S units contain visible chromophores (for example, flavins, heme) which interfere with spectrophotometric examination of reaction 9, newly developed methods for detection of extrusion products include ^{19}F NMR (*92*) and EPR spectroscopy (*91, 95*). Application of these procedures has yielded these results; milk xanthine oxidase, $4Fe_2S_2$ (*92*); succinate dehydrogenase, $2Fe_2S_2 + 1Fe_4S_4$ (*96*); FeMo protein of nitrogenase, $\sim 4Fe_4S_4$ (*95, 97*).

The extrusion method at present identifies only two types of core structures in Fe-S coordination units and provides no direct information as to terminal ligands or other structural and environmental features of these units. Despite these limitations, this method, alone or in conjunction with other physical methods, can supply useful structural identification of prosthetic groups in an enzyme. In the absence of a full protein crystallographic determination, this is the minimum information required to frame a mechanism of action, however tentative. Xanthine oxidase (*98*) is a case in point. Of the prosthetic groups in one subunit of this enzyme (Fig. 3), the flavin had been identified earlier, the two Fe-S sites have been shown to contain Fe_2S_2 units (consistent with earlier evidence), and the Mo coordination environment in the oxidized enzyme has been partially defined by EXAFS analysis (*99*). The latter is the catalytic site; the two electrons removed from substrate are likely funneled through the Fe-S sites to the flavin and then to dioxygen as the physiological acceptor (*98, 100*). For hydrogenase, which contains no other prosthetic groups, the extrusion method appears to demonstrate the presence of Fe_4S_4 units, which must be the site of catalysis. Replacement of one thiolate group in **17** by a more labile X ligand using reaction 8 may afford a purely synthetic hydrogenase system. Reduced substituted clusters are able to effect at least one reductive substrate transformation, acetylene to *cis*-

$1,2\text{-}C_2H_2D_2$ (*101*), a reaction also exhibited by nitrogenase. The hydrogenase case, together with evidence that in one bacterial monooxygenase (*102*) a Fe_2S_2 unit may serve as a functional component of the catalytic site, raises the general possibility that the physiological function of Fe-S sites may not be exclusively that of outer-sphere electron transfer. The analogs **16** to **18**, or suitably modified versions thereof, should be of prime importance in any eventual development of these sites as reaction centers.

Blue copper proteins. Many copper-containing proteins and enzymes (*103*) possess highly distinctive coordination units, containing "type 1" or "blue" copper, which imparts a beautiful, intense blue color to these substances in their oxidized [Cu(II)] forms. Exhibited by these units, which are of type *a* (Table 1), is a set of properties not yet reproduced in its entirety in a single synthetic species. These properties include an absorption band near 600 nm, responsible for the blue color and one or two orders of magnitude more intense than visible bands of conventional Cu(II) complexes, unusually small copper hyperfine coupling constants ($A_{\parallel}$) in EPR spectra, and potentials for the couple (reaction 10) which are appreciably higher than that for Cu(II)/Cu(I) in aqueous solution (− 0.15 V). The latter property

$$\text{Oxidized protein [Cu(II)]} \underset{-e^-}{\overset{+e^-}{\rightleftharpoons}} \text{reduced protein [Cu(I)]} \quad (10)$$

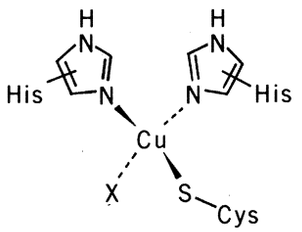
(blue, paramagnetic) (colorless, diamagnetic)

reflects enhanced stabilization of Cu(I) in the protein matrix. Blue copper proteins are widely dispersed in nature, occurring in bacterial, plant, and mammalian systems. An electron-transfer function has been established for at least two proteins, plastocyanin (in photosynthesis) and azurin, and for this reason we classify the blue copper proteins as electron carriers (Fig. 1). Since a biological role is not known for many of these proteins, this classification scheme may be too restrictive. Some copper-containing oxidases (Fig. 1) also contain blue Cu sites.

A most significant development in the study of blue copper proteins is the recent crystallization and x-ray structural characterization of oxidized plastocyanin from poplar leaves (Table 1). At a resolution of 2.7 Å (*13*) the coordination unit **5** is established, containing Cu(II) in an approximately tetrahedral environment. Moreover, the unit in oxidized azurin, examined both by x-ray crystallography (*12*) and EXAFS (*104*), appears to be similar.

Although tetrahedral coordination geometry and binding by thiolate ligands are entirely plausible for Cu(I), their appearance in unit **5** is contrary to the great bulk of experience in Cu(II) chemistry. By far the most stable coordination geometries for Cu(II) are planar or tetragonally distorted octahedral (*105*); nonplanar stereochemistry usually can be achieved only by steric enforcement by ligand structure (*106*) or by solid-state effects. Moreover, Cu(II) generally oxidizes thiolates to disulfides. In short, the normal chemical and structural preferences of Cu(I) and Cu(II) are distinctly different. It may be that the Cu(I) site in the blue copper proteins is similar to the Cu(II) site: this site may have evolved as a compromise between the requirements of Cu(I) and Cu(II) ions so that without gross structural rearrangements (Franck-Condon barrier) rapid electron transfer can occur.

Clearly, the protein milieu is of paramount importance in imposing a copper coordination environment that is exceptional. The challenge posed in modeling this and other type *a* units is different from that in the porphyrin and Fe-S areas: integral substructures are absent, and a self-assembly reaction of Cu(I,II) with individual imidazole, thiolate, and thioether ligands will certainly fail. The only type *a* unit that has been successfully reproduced synthetically is **1** (M = Fe), which is a much simpler problem owing to the presence of four identical ligand atoms. The challenge is increased by the fact that there cannot be a binding site unique to all blue copper proteins. Physicochemical evidence supports the conservation of three ligands in the unit **22**. However, homology in



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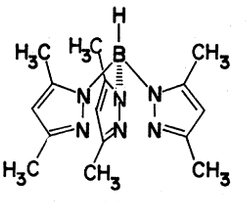
amino acid sequences of all blue proteins is not highly developed (*107*). As one example, the protein stellacyanin lacks methionine (*108*); hence, the environment around copper in this protein must differ from that in poplar plastocyanin; that is, ligand X in **22** cannot be a thioether.

The considerable challenge that arises in modeling the active sites in blue copper proteins requires, at a minimum, the synthesis and characterization of stable nonplanar Cu(I,II)-N_2SX systems. One might ask if this objective is worth meeting, inasmuch as the plastocyanin site has been approximately defined from diffraction studies. We reply in the affirmative for several reasons. Variation of ligand X in **22** might allow its identification in at least stellacyanin, thereby realizing capability (i) of the model system approach. If the difficulty in obtaining suitable crystals of plastocyanin and azurin is typical, one cannot expect a plethora of diffraction results in the near future. Although there exists a mass of data on the spectroscopically rich oxidized proteins, there are few correlations between these data and copper complexes of well-characterized structure and behavior. Even less is known about the equally important reduced forms of the proteins in reaction 10. Finally, there are as yet few, if any, clear connections between the characteristics of the copper sites and the details of structure. How these characteristics respond to controlled modifications of the composition and structure of the coordination unit (such as the effect of variant X in **22**) is of fundamental importance in interpreting the chemical, spectroscopic, redox, and structure-function relationships among blue copper proteins.

For these reasons, there have been many recent attempts to provide simple isolable, characterizable, low-molecular-weight models, or presumed models, for the Cu(I) and Cu(II) binding sites in the blue copper proteins (*109*). It is our opinion that to date none of these attempts has been entirely successful, but some useful information has been obtained. For example (*109*, *110*), the following systems have been synthesized; $[Cu(I)(SR)L]^-$, Cu(II)(SR)L, Cu(II)(OR)L, and Co(II)(SR)L, where L = **23**, hydrotris(3,5-dimethyl-l-pyrazolyl)borate. All have been characterized spectroscopically. In addition, crystal structures have established the coordination geometries in a Cu(I) and a Co(II) species. From these results and those on the native systems the following



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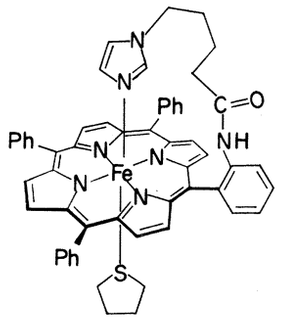
conclusions were reached. (i) The strong band at approximately 600 nm common to all blue copper proteins and displayed by Cu(II)(SR)L may unequivocally be assigned to thiolate sulfur-to-metal charge transfer. (ii) The resonance Raman spectra of the proteins can be assigned on the basis of metal-ligand stretching vibrations in the 350 to 450 cm^{-1} region (Cu-N) and at about 270 cm^{-1} (Cu-S). (iii) The $g_{\|}$ parameters from the EPR spectra of synthetic and native systems are similar, suggestive of reasonable correspondence in ligating atoms. (iv) The magnitudes of the $A_{\|}$ parameters in the synthetic systems do not duplicate the unusually low values in the native systems because of significant differences in the Cu(II) coordination geometries. The ligand L forces a trigonally distorted coordination on the Cu(II) ion that must be absent in the native systems. (v) The two essential characteristics of the oxidized blue copper binding sites appear to be the Cu-S-Cys linkage and an approximately tetrahedral coordination about the Cu(II) ion. The Cu(II)*S*-Cys bond does not in itself guarantee a small value for $A_{\|}$ and, in fact, the intense blue color is a property that is separable from the low value of $A_{\|}$. (vi) Finally, on the basis of plots of $A_{\|}$ versus $g_{\|}$ for models and proteins it is possible to group the blue copper centers into three different regions: stellacyanin, umecyanin, and *Rhus vernicifera* laccase; most azurins; plastocyanins, most laccases, ascorbate oxidases, and human ceruloplasmin. Whether or not these regions arise from differing X ligands in the $Cu(His)_2(Cys)X$ unit remains to be sorted out through studies of the proteins in conjunction with the synthesis and characterization of more elaborate and meaningful models. Indeed, the species whose properties constitute the basis for these conclusions must be regarded as approximations to, rather than true synthetic analogs of, the blue copper binding sites. Accurate models of the sites **5** and **22** (with, for example X = RS^-, RO^-, R_2S), replete with distorted tetrahedral stereochemistry, dissimilar donor atoms, and stability toward spontaneous internal electron transfer in the oxidized form, remain formidable synthetic objectives.

Cytochrome c. These proteins constitute the third major class of metal-containing electron carriers and have been isolated from mammals, insects, plants, and bacteria (*16*). In all eukaryotes, cytochrome *c* is a member of the mitochondrial respiratory chain and is the immediate electron donor to cytochrome oxidase. All members of this class are heme proteins whose porphyrin structure differs from **12** in that the two vinyl groups are replaced by CH_3CH-S-Cys units which covalently link the ring to the polypeptide chain. The coordination unit of all cytochromes *c* in both the reduced [Fe(II)] and oxidized [Fe(III)] forms is **6** with L = N-His and L′ = S-Met (*16*). Precise structural details of these units have not been reported.

Although unit **6** has been established crystallographically for nearly a decade, an appropriate synthetic analog has been prepared only recently. Complex **24**, Fe(TPP-Im)(THT) (*111*), containing as axial ligands to Fe(II) a tetrahydrothiophene (THT) molecule and the imidazole



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group of a pendant side chain ("tail") attached to a porphyrin ring substituent, is an admirably close match to **6**. Its synthesis and properties are illuminating in several respects. (i) The greater affinity of heme iron for imidazole over thioethers is subverted by inclusion of the imidazole tail, a stratagem similar to that employed in the design of **13**, **14**, and imidazole-tailed dioxygen carriers. (ii) The near constancy of Fe-N and Fe-S distances in **24** and several related $[Fe(TPP)(SR_2)_2]^{0,+}$ species (*111*) is indicative of a minimal structural reorganization barrier to electron transfer. If the geometry of unit **6** is unconstrained in both oxidation states of the cytochromes, a similar property should apply to the proteins. (iii) The redox potential of **24** is within 100 mV of that of many common cytochromes *c* (about +260 V), which is about 460 mV more positive than the potentials of cytochromes with two axial N-His ligands. The difference between the potentials of **24** and Fe(TPP-Im)(Im) is 160 mV, which is a good estimate of the intrinsic effect of replacing N-His with S-Met in the proteins. The remaining 300 mV represents the contribution from protein environment (*111*) extrinsic to the redox site. The results in (ii) and (iii) are clear examples of information critical to interpretation of protein structure and function which are best, or at least more readily, achieved by the model system approach. Lastly, the synthetic flexibility inherent in the tailed porphyrin concept (*55, 111, 112*) presages its increasing use in providing porphyrin complexes with dictated sets of axial ligands.

Active Site Analogs: Selected Unfulfilled Goals

The foregoing accounts testify to the substantial progress that has been made in modeling active sites of oxygen-carrying and electron-transfer proteins. In addition, there are numerous metalloenzymes whose active sites are potentially subject to structure-function clarification by the synthetic analog approach. Some selected examples are considered.

1) Multielectron oxidoreductases catalyze substrate transformations in processes involving the transfer of four or six electrons.

Cytochrome oxidase:

$$O_2 + 4H^+ + 4e^- = 2H_2O \quad (11)$$

Sulfite reductase:

$$SO_3^{2-} + 8H^+ + 6e^- = H_2S + 3H_2O \quad (12)$$

Nitrite reductase:

$$NO_2^- + 7H^+ + 6e^- = NH_3 + 2H_2O \quad (13)$$

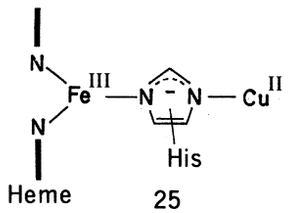
Nitrogenase:

$$N_2 + 6H^+ + 6e^- = 2NH_3 \quad (14)$$

Reactions 11 to 14 are in general not readily accomplished in the laboratory under ambient conditions using homogeneous solutions of synthetic catalysts. The challenge in modeling the sites of such enzymes is especially formidable if one accepts the restrictions that catalysts have a physiologically realistic composition and that intermediate products of substrate reduction not be released during a catalytic cycle. The latter restriction in particular may be difficult to meet inasmuch as the above enzymes contain elaborate intramolecular electron transfer apparatus which presumably control electron flux to the catalytic sites. Clearly the initial step is to obtain viable models of these sites in the resting forms of the enzymes.

Cytochrome oxidases. The minimal functional unit of these enzymes, which are the terminal oxidases in mitochondrial respiration and catalyze the reduction of dioxygen to water with electrons supplied by cytochrome *c* (*113*) (reaction

11), consists of two hemes (a, a_3) and two copper ions. Four electrons are required to convert the oxidized enzyme to the fully reduced form, consistent with 2 Fe(III)-heme $\xrightarrow{2e^-}$ 2 Fe(II)-heme and 2 Cu(II) $\xrightarrow{2e^-}$ 2 Cu(I). A number of features of the metal-containing units are unusual, but none more so than that which renders one Fe(III)-heme and one Cu(II) ion undetectable by EPR spectroscopy (*113, 114*). The suggestion that the EPR-invisible components exist as a magnetically coupled high-spin Fe(III) · · · Cu(II) complex (*115*) has been elaborated in the form of structural proposal **25** (*114*), which has occasioned



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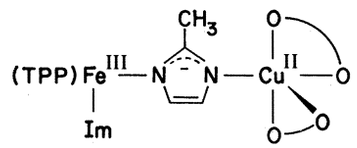
much current interest. Several synthetic species containing Fe and Cu bridged by nitrogen heterocycles (*116, 117*), including **26** (*117*), have been produced as have



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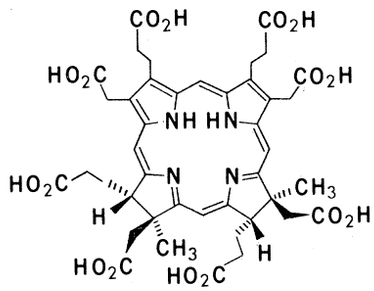
porphyrin complexes with a Fe(II)-Im$^-$-Fe(II) bridge (*118*). None of these is fully appropriate for testing **25**, but their properties are mildly suggestive of another type of bridge structure in the enzyme. The identity of the group modulating the rather strong magnetic coupling [$-J \sim 200$ cm^{-1} (*114*)] remains unestablished, but may well yield to a model system approach with obligatory variation of bridge structure in binuclear Fe/Cu complexes.

Sulfite and nitrite reductases. These remarkable enzymes (*119, 120*) catalyze the reduction reactions 12 and 13, which are integral parts of the sulfur and nitrogen biological cycles on earth. Beautiful enzymological work has shown that both types of enzymes possess a common active site group (*121*), designated as siroheme, and has culminated in the structure proof of the metal-free group, sirohydrochlorin, as **27** (*122*). This group differs from those in other heme proteins and enzymes (**12**) in that peripheral carbon atoms in two adjacent rings are saturated. The macrocyclic structure is of the isobacteriochlorin type, the few synthetic examples of which should now enjoy substantial utility as models of **27**, which itself has not yet been synthesized. Stimulated by the role of siroheme as the catalytic site of sulfite and nitrite reduction (*119–121, 123*), investigations have begun in several laboratories on the properties and structures of isobacteriochlorins and their metal complexes (*124, 125*). Octaethylisobacteriochlorin and its metal complexes afford chromophores nearly identical with those of **27** and its complexes. Further, the Fe(III) complex **28** with an axial thiolate ligand



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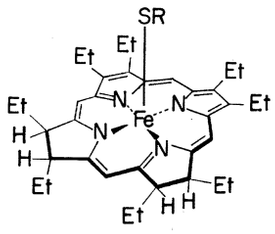

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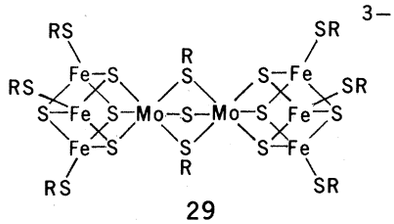
(*125*) exhibits an EPR spectrum suggestive of an Fe-S-Cys interaction in the latter. Compared with other multielectron oxidoreductases, sulfite and nitrite reductase are particularly advantageous for model system studies because certain of the structural features at the active site are now known. Species such as **28** should prove to be useful vehicles for probing properties of the resting and substrate-bound enzyme states and, perhaps, of the mechanism of complete reduction of substrates which is largely unknown.

Nitrogenase. The requirement for fixed nitrogen in the world's food economy, a matter needing no elaboration here (*126*), together with the complexity of the enzyme and its mechanism of substrate reduction (reaction 14), has precipitated research in many disciplines. Of all metallobiomolecules that have been purified to (or near to) homogeneity, none has provided structural and mechanistic features more challenging to untangle than nitrogenase. The enzyme is a molecular complex consisting of an electron-carrying Fe protein and an Fe-Mo protein rich in metal content (~ 2 Mo, ~ 28 to 32 Fe/220,000 daltons) and containing the catalytic site. About half the iron content of Fe-Mo proteins (*127*) is organized into Fe_4S_4 centers (*95, 97, 128*), which may serve as intramolecular electron transfer sites. Most or possibly all of the remaining iron is associated with molybdenum in one or two units, the Fe-Mo cofactor, which has been dissociated from the native protein and examined separately (*129*). Inferential evidence, but not proof, that the catalytic apparatus is contained in this unit derives in part from the abiological reduction of dinitrogen in the presence of molybdenum complexes (*130, 131*).

Proposals for the catalytic site structure and mechanism of action of nitrogenase abound. The only incisive current evidence for the site structure follows from EXAFS analysis of several Fe-Mo proteins, which points to a cluster structure in which Mo is surrounded by Fe and S atoms at < 3 Å (*132*). Other spectroscopic results (*133*) are not inconsistent with this possibility and indicate the presence of a cluster structure not previously encountered in synthetic or natural systems. In view of the cluster proposition and the spontaneous assembly of the clusters (**17**) by reaction 5, recent research has been directed toward the synthesis of Mo-Fe-S clusters from elementary reagents (*134*). The system MoS_4^{2-}/$FeCl_3$/RS^- has yielded $[Mo_2Fe_6S_9(SR)_8]^{3-}$ (**29**), $[Mo_2Fe_6S_8(SR)_9]^{3-}$, and $[Mo_2Fe_7S_8(SR)_{12}]^{3-,4-}$, all of which have been shown to contain $MoFe_3S_4$ units with a cubane-type geometry not unlike that of the Fe_4S_4 core in **17**. Independent work by Christou *et al.* has provided additional $[Mo_2Fe_6S_8(SR)_9]^{3-}$ species by similar means (*135*). The Mo EXAFS of **29** bears the closest resemblance to that



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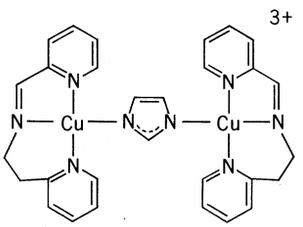
of the enzyme, suggesting similar Mo environments in both cases but not proving the existence of a complete $MoFe_3S_4$ core structure or any sort of Mo · · · Mo bridged unit in the latter. Nonetheless, the comparative EXAFS spectra offer encouragement to further synthetic pursuit of the Mo site in nitrogenase, either through the use of cluster self-assembly or perhaps by linking Mo-Fe-S clusters or Mo complexes to Fe_4S_4 clusters in order to provide a composition close to

that (seven to eight Fe per Mo) of the Fe-Mo cofactor. The latter property and labile coordination positions for binding of dinitrogen are lacking in Fe-Mo-S clusters thus far synthesized.

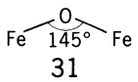
2) Superoxide dismutases are enzymes that catalyze the dismutation of O_2^- to dioxygen and hydrogen peroxide, thereby apparently preventing the deleterious effects of the highly reactive superoxide ion on those enzymes (for example, xanthine oxidase) (Fig. 3) which metabolize O_2 (*136*). Dismutases containing manganese or iron have been isolated, but the bovine enzyme containing copper and zinc (Table 1) has been the most thoroughly studied. This enzyme presents the type *c* active site structure **9** containing the Cu-Im$^-$-Zn bridge in which copper, alternating between Cu(I) and Cu(II), is the catalytic site (*136*); zinc is not essential for enzyme activity. The primary goal in modeling the active site is not necessarily simulation of enzyme action, for Cu(II) ion in aqueous acid will dismutate superoxide at rates as fast or faster than the enzyme (*137*). Instead, model system studies have been directed at a more precise structural description of the bridge unit than is possible from the protein data at 3 Å resolution (*27*), and elucidation of the chemical stability of the bridge (postulated to break during enzyme turnover) and of details of magnetic interactions modulated by the bridge. The existence of an active form of the enzyme with Cu(II) substituted for native Zn(II) ion (*138*) has prompted synthesis of a number of previously unknown discrete Cu(II)-Im$^-$-Cu(II) complexes (*139, 140*). One example is **30**



30

(*140*), in which the bridge group forms a 90° dihedral angle with the other ligands. Such species exhibit Cu(II) · · · Cu(II) magnetic interactions (*139, 140*) comparable with that ($-J \sim 26$ cm^{-1}) in the Cu(II)-substituted enzyme, providing excellent evidence that a bridge unit closely related to that of **30** is present in the latter. Additional work on model complexes has provided useful information on *p*H-dependent bridge stability and electronic details from EPR spectroscopy (*140*). A remaining objective is the synthesis of a species appropriate to accurate determination of the unconstrained structure of the Cu(I,II)-Im$^-$-Zn(II) bridging unit. It is noted that some license has been taken in the depiction **9** of the active site inasmuch as the x-ray results do not establish the existence of Cu-N and Zn-N bonds to the interposed imidazole ring (*140*).

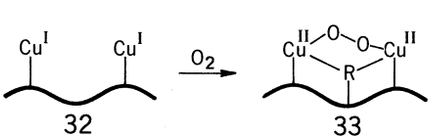
3) Alternative dioxygen carriers, a term coined by others (*32*), describes hemerythrin and hemocyanin, which have been mentioned earlier. Despite their names these proteins contain no heme and only hemerythrin contains iron. Together with myoglobin and hemoglobin they constitute the known members of the group of dioxygen binding and transport proteins (Fig. 1). The properties and structure of hemerythrin have been reviewed recently (*32, 33, 141, 142*); structural information is provided in Table 1. The structure of azidometmyohemerythrin at 2.0 Å has been refined (*143*); it involves an Fe · · · Fe separation of 3.44 (5) Å, not inconsistent with the presence of the μ-oxo group **31**. The active site



31

structure of aquomethemerythrin, obtained at 2.5 Å resolution (*28, 29*), is proposed to be **10** with a Fe · · · Fe separation of $\lesssim 3$ Å (*141*). As has been remarked (*141*) "... at least one of the models must be incorrect. If either of the present models had been the only one proposed, it would likely have been accepted as 'the structure.' ... The present differences in the Fe complexes in hemerythrin and myohemerythrin must certainly raise questions concerning the possible extent of errors in many protein models arising from single source studies." Unfortunately, active site modeling of hemerythrin is sufficiently challenging so that no one has yet developed reversible dioxygen binding systems devoid of heme or involving binuclear Fe centers.

Hemocyanin, a copper-containing protein (*42*), bears certain superficial similarities to hemerythrin. Although no structural information is available from protein crystallography, the active site is binuclear and binds one dioxygen molecule resulting in oxidation of Cu(I) to Cu(II) (**32** → **33**). Spectroscopic results point to an equivalence of oxygen atoms in a μ-peroxo structure (*144*) and the presence of a bridging group R of protein origin (*145*) in oxyhemocyanin (**33**).



32 33

As for hemerythrin, active site modeling of hemocyanin presents an imposing challenge. No one has yet developed reversible dioxygen binding systems involving binuclear Cu centers. Recently a promising system has been described in which two Cu(I) complexes react with a single dioxygen molecule, but with ~ 20 percent loss in activity through each cycle (*146*). Conceivably, rather elaborate ligand systems, such as cryptates or cyclic peptides, may be necessary for effective models. Optimal ligands should provide intramolecular metal-metal separations capable of stabilizing, but not irreversibly, the synthetically elusive μ-peroxo binuclear fragment while also affording close approach of metal ion spectroscopic properties to those of the oxy- and met-forms of the proteins.

Conclusions

Synthetic approaches to metallobiomolecular active sites have provided valuable information concerning the structure and function of these sites. Both corroborative and speculative structural models (*147*) have provided much information that has not been or cannot be obtained from investigations of the natural systems themselves. Examples include the influence of protein structure and environment on site properties and the determination of precise unconstrained site structures.

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Crustacean Eye Fine Structure Seen with Scanning Electron Microscopy

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Summary. The internal fine structure of crustacean compound eyes has been reexamined with scanning electron microscopy. Several different preparative techniques were used in a comparative study of crab, crayfish, shrimp, and stomatopod eyes. The three-dimensional pattern of photoreceptive, dioptric, and screening components of these eyes has been directly demonstrated, and new insight has been gained into their functional organization. Particularly interesting in apposition eyes is the elaborate array of boundary membranes and protoplasmic strands linking the photoreceptive microvilli to their parent cell cytoplasm across the large intracellular vacuoles surrounding the axial rhabdom. Quantitative application of scanning electron microscopy to this system promises to advance our understanding of its proven high rate of receptor membrane turnover.

If success can be measured by the number of arthropod individuals and species dependent on compound eyes, they are the most successful light receptor organs ever evolved. Consequently, an understanding of their functional organization is important to an understanding of vision in general. Yet several critical features of their fine structure remain unresolved despite outstanding light microscopy beginning as early as the 1890's (*1*) and many subsequent light microscopic and electron microscopic studies (*2*). As a result, current research on their optics, electrophysiology, photochemistry, cell biology, and behavioral consequences is hampered by persistent morphological ambiguities. Particularly acute is the need for precise knowledge of receptor cell patterns and connectivity. These obviously determine first-order input for visual discrimination and information channeling.

To reduce such deficiencies in the field of crustacean visual physiology (*3*), we have recently turned to scanning electron microscopy (SEM) (*4*). This technique overcomes the inadequate resolution of light microscopy and the internal membrane face limitations of freeze fracture electron microscopy. It also directly demonstrates three-dimensional relationships at intermediate scale ranges usually requiring many sections and laborious reconstruction with transmission electron microscopy. Modest application of SEM has previously been made to internal structures of insect compound eyes, but little as yet to those of crustaceans (*5*). We are developing this approach specifically to apply new SEM data to quantitative experimental analyses especially of the photoreceptor membrane. The main technical problems are exposing appropriate cellular elements [like the eighth retinular cell (*6*, *7*)] and determining their precise relations (Figs. 1 and 2).

So far, we have applied a variety of well-known fixative and preparative techniques (*8*). Some eyes have been macerated in boric acid while still fresh; others, after fixation and dehydration, have been broken or cut open at room temperature. Additonal specimens were fractured while frozen in ethanol at liquid nitrogen temperatures. All were then

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