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Chemical approaches to bridged biological metal assemblies

R. H. Holm

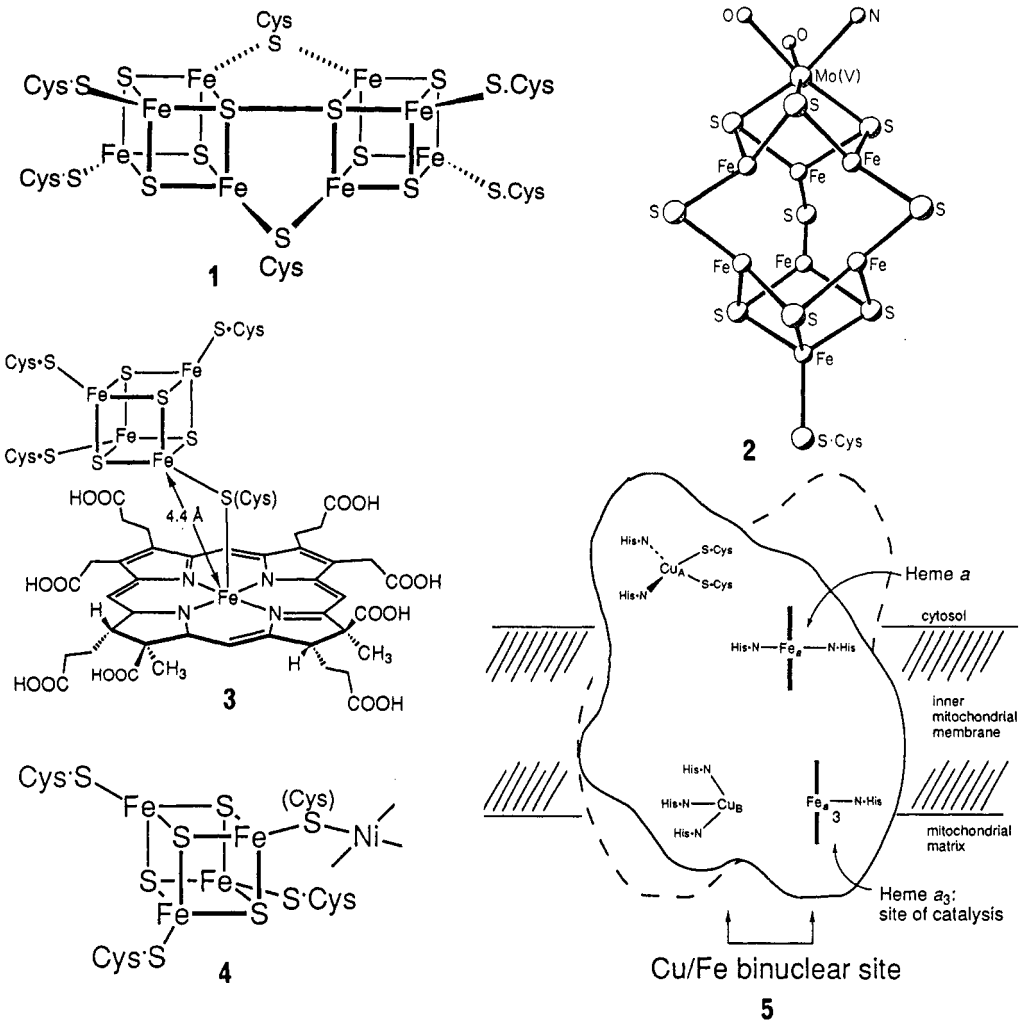
Department of Chemistry, Harvard University, Cambridge, Massachusetts 02138, U.S.A.

Abstract. Biological metal centers that consist of two fragments covalently connected by one more bridging atoms or groups are becoming increasingly recognized by physicochemical properties and protein crystallography. As a class, bridged biological metal assemblies pose challenging problems in chemical synthesis; at least some are potentially subject to further structural, electronic, and reactivity characterization provided the assembly itself or a close molecular simulation thereof can be prepared. Synthetic analogue approaches to three bridged assemblies are summarized here: nitrogenase FeMo/V cofactor, the catalytic site of sulfite/nitrite reductase, and the heterometal CuFe site in cytochrome *c* oxidase. The cofactor cluster is approached by the clusters $[MFe_4S_6(PEt_3)_4L]$ (M = Mo, V; L = Cl^-, RS^-), in which a cuboidal Fe_4S_3 unit is linked to the M site by three μ_2-S atoms. The sulfite/nitrite site analogue consists of an Fe_4S_4 cluster linked to a heme group through an unsupported μ_2-S bridge. The binuclear site in the oxidized form of cytochrome *c* oxidase has been investigated by synthesis of the unit $[Fe^{III}\text{-}O\text{-}Cu^{II}]$ in a molecular heme complex. The cyanide-inhibited form of the enzyme has been simulated by the preparation of a series of heme complexes containing the bridge unit $[Fe^{III}\text{-}CN\text{-}Cu^{II}]$ in which the Fe atom is six-coordinate and low-spin.

INTRODUCTION

Metal centers in metallobiomolecules serve one or more functions of protein structure configuration, metal storage and release, dioxygen binding, electron storage and transfer, and catalysis. The large majority of such centers are mononuclear coordination units or individual metal clusters in which two or more metal atoms are tightly bridged and, if paramagnetic, are usually exchange-coupled. Over the last decade, an additional general type of metal center has been structurally identified or inferred from spectroscopic and magnetic properties. Those of interest here are termed *bridged biological metal assemblies*, or simply *assemblies*, and consist of two discrete fragments that are juxtaposed wholly or in part by one or more covalent bridges. Crystallographically authenticated examples include the binuclear iron centers in the dioxygen-carrying protein hemerythrin (1) and the enzymes ribonucleotide reductase (2) and methane monooxygenase (3), which share the common feature of bridging oxo/hydroxo and carboxylate groups. Similarly, two copper atoms in the trinuclear copper array of ascorbate oxidase are bridged by hydroxide (4). An imidazolate-bridged heterometallic pair has been established in CuZn superoxide dismutase (5). Those of immediate interest in this laboratory are listed below (X = unestablished bridge). Known or possible structures are illustrated as **1-5** Here we summarize progress toward satisfactory synthetic analogues of assemblies **2**, **3**, and **5**.

- nitrogenase P-clusters (**1**)
  $[(Fe_4S_4)\text{-}(\mu_2\text{-}S\cdot Cys)_2\text{-}(Fe_4S_4)]$ (core S-S) (6)
- nitrogenase cofactor (**2**)
  $[(Fe_4S_3)\text{-}(\mu_2\text{-}S)_3\text{-}(MFe_3S_3)]$ (M = Mo (6), V)
- sulfite/nitrite reductase catalytic site (**3**)
  $[(Fe_4S_4)\text{-}(\mu_2\text{-}X)\text{-}(\text{siroheme})]$ (7,8)
- carbon monoxide dehydrogenase catalytic site (**4**)
  $[(Fe_4S_4)\text{-}X\text{-}Ni]$, $[(Fe_4S_4)\text{-}X\text{-}Ni\text{-}X\text{-}(Fe_4S_4]$ (9)
- cytochrome *c* oxidase Cu/Fe binuclear site (**5**)
  $[Cu\text{-}(\mu_2\text{-}X)\text{-}Fe]$ (X = O^{2-}, OH^-, Cl^-, S^{2-}, RS^-) (10,11)

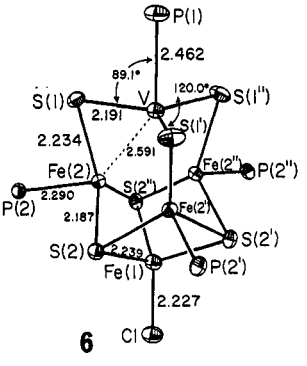


THE NITROGENASE COFACTOR

The catalytic component of the nitrogenase protein complex is the FeMo protein ($\alpha_2\beta_2$ subunits, $M_r \approx 215$ kD) which contains two copies each of the P-cluster **1** and the cofactor cluster **2.** The former appears to be an intramolecular electron donor in catalysis of the overall enzymatic reaction $N_2 + 8H^+ + 8e^- \rightarrow 2NH_3 + H_2$. Properties of the cofactor have been delineated prior to knowledge of its structure (12). Determination of the structure of the FeMo protein and of the Fe protein, the ultimate electron donor to the former, by Kim, Rees, and coworkers (6) has been enormously influential for it has provided an atomic-level view of what previously could only be conceived as a substrate-processing machine whose complexity was matched only by the mystery of its structure and mechanism. The structure of the cofactor is identical in the FeMo proteins from *Azotobacter vinelandii* and *Clostridium pasteurianum*. It consists of Fe_4S_3 and $MoFe_3S_3$ cuboidal fragments bridged by three μ_2-S atoms, each of which is part of a previously unencountered planar $Fe(\mu_3\text{-}S)_2(\mu_2\text{-}S)$ coordination unit. Indeed, this stereochemistry is required for the observed bridge disposition; tetrahedral, octahedral, or 5-coordinate arrangements force the bridging atoms inward toward one another at unacceptably short distances. The cofactor is held in the protein by binding to cysteinate at the tetrahedral Fe site and to an imidazole group of histidine at the Mo site; coordination at the latter site is completed by a bidentate homocitrate ligand. There is general agreement that the cofactor cluster is the site of substrate binding, activation, and catalysis, but the precise postion(s) at which these events transpire is presently a matter of speculation. Alternative nitrogenases contain vanadium instead of molybdenum. EXAFS results indicate that the Mo and V sites are very similar (12); consequently, V-Fe-S clusters are also pertinent to the problem of cofactor synthesis.

Synthesis of suitable analogues of **1-5** can be conceived as the coupling of dissimilar fragments through the desired bridge. This approach requires the fragments and the development of reactions that minimize or eliminate undesired coupling products. In the case of cofactor cluster **2**, neither of these prerequisites is yet available. The cuboidal clusters $[Fe_4S_3(NO)_7]^{1-}$ (13) and $[Fe_4S_3(NO)_4(PPh_3)_3]^{0,1+}$ (14) are known but are unsuitable because of low oxidation states and the difficulty of removing ligands with retention of core structure. The topology of **2** has been realized in $[Ni_8S(S\mathit{t}Bu)_9]^{1-}$ (if the central μ_6-S atom is ignored), a compound formed by self-assembly (15). Our initial efforts have utilized the cluster self-assembly approach. Reaction system 1 in THF affords the vanadium and molybdenum clusters **6** and **7**, respectively, in 40-70% yield (16). The two clusters are isostructural with C_3 symmetry. They consist of an Fe_4S_3 cuboidal fragment

$$[MCl_3(THF)_3] + 4.5(Me_3Si)_2S + 2FeCl_2(PEt)_2 \rightarrow [MFe_4S_6(PEt_3)_4Cl] \quad (1)$$



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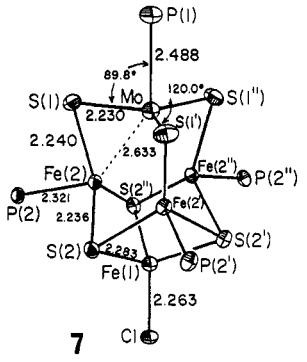

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bridged by three μ_2-S atoms to a heterometal M. The unique Fe atom has the usual tetrahedral geometry found in cubane-type Fe_4S_4 clusters while the symmetry-related Fe atoms display an unusual but precedented (17) trigonal pyramidal FeS_3P coordination which allows the triply bridging interaction with M = V and Mo, whose trigonal pyramidal stereochemistry is without precedent. Comparison of **6/7** with **2** makes evident the structural similarity; the synthetic clusters present ten atoms ($Fe_4(\mu_3\text{-}S)_3(\mu_2\text{-}S)_3$) with bond connectivity and spatial disposition analogous to the native cluster. Clusters **6** and **7** and their derivatives currently provide the closest structural approach to the 17-atom core of the cofactor cluster.

Investigation of the electronic and reactivity features of clusters **6** and **7** and their derivatives is at an early stage (16). Reaction 2 has been demonstrated, permitting the introduction of ligands at the tetrahedral Fe site which simulate cysteinate binding and provide a structural and electronic probe. Clusters **8** and **9** retain the core structures and metric features of their precursors. The five independent bonded Fe-S distances are all longer

$$[MFe_4S_6(PEt_3)_4Cl] + RS^- \rightarrow [MFe_4S_6(PEt_3)_4(SR)] + Cl^- \quad (2)$$
$$M = V\ (\mathbf{8}),\ Mo\ (\mathbf{9})$$

in the Mo vs. the V clusters, indicating that the additional valence electron in **7** and **9** is antibonding and largely delocalized over the Fe_4S_3 cuboidal fragment. The Fe-(μ_2-S) distances are not experimentally distinguishable in the V and Mo clusters, consistent with this description which is further supported by ^{57}Fe isomer shifts and proton isotropic shifts of thiolate groups in **8** and **9** (16). The isomer shifts indicate somewhat more reduced Fe sites and larger paramagnetism compared to V clusters **6** and **8**, which have a diamagnetic ground state and a thermally accessible triplet state. Perhaps the most significant potential advantage of **6-9** lies in manipulation of the heterometal fragment: alteration or removal so as to afford a magnetically isolated Fe_4S_3 fragment, allowing the first electronic examination of such a cluster in physiologically relevant oxidation state(s). A remote possibility is its utilization in synthesis of the MFe_3S_3 fragment, thereby affording the actual core topology of cofactor cluster **2**. Further investigation of the reactivity properties of clusters **6-9** is in progress.

SULFITE REDUCTASE CATALYTIC SITE

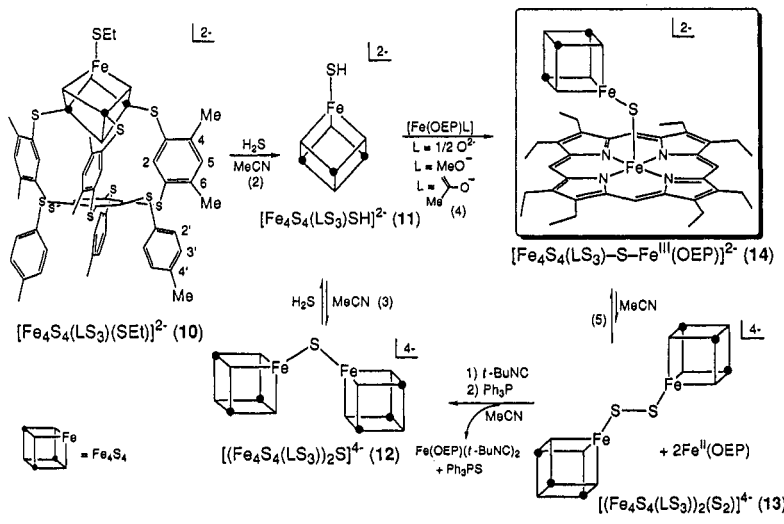
This site is the only demonstrated instance of an Fe_4S_4 cluster bridging to another prosthetic group. The original crystal structure of the assimilatory sulfite reductase (SiR) of *E. coli* established the array **3**, but was not of sufficient resolution to demonstrate a cysteinate bridge which, however, was regarded as probable (7).

An explicit model involving this bridge and consistent with the X-ray and all spectroscopic data has been proposed by Siegel and coworkers (18). As isolated, the enzyme is in the $[Fe_4S_4]^{2+}$ (S = 0)/Fe^{3+} (S = 5/2) state; in all oxidation states the cluster and siroheme are exchange-coupled (19). Another SiR species is found in assimilatory-type enzymes from anaerobic bacteria. That from *Desulfovibrio vulgaris* (Hildenborough) (M_r ≈ 27 kD) has been the most thoroughly investigated. It also contains a magnetically coupled Fe_4S_4 cluster and siroheme (20), but the detailed structure is unknown. Chemical analysis indicating an atom ratio S:Fe ≈ 1:1 (20) and radiosulfur labeling showing that one sulfur atom exchanges more rapidly than the others (21) have led to the suggestion of a sulfide bridge in **3**. Attempts to prepare stable assemblies in which Fe(II/III) is thiolate-bridged to an Fe_4S_4 cluster have been unsuccessful, but have demonstrated the utility of Fe-site-differentiated clusters (22) in forming bridges to external Fe species (23).

Our approach to *sulfide*-bridged assemblies is summarized in part in Figure 1 (24). Site-differentiated cluster **10**, derived from the semirigid tridentate cavitand ligand LS_3^{2-}, is readily converted in reaction 2 to the hydrosulfide-functionalized cluster **11**. This is the key precursor to the desired product; its concentration is maximized by control of equilibrium 3. In a set of directed acid-base reactions 4 utilizing Fe^{III}(OEP) species with strongly basic axial ligands, the desired assembly **14** was produced. The reaction systems **11**/[Fe(OEP)($OClO_3$)]/Et_3N, $[Fe_4S_4(LS_3)(SSiEt_3)]^{2-}$/[Fe(OEP)F], and the reverse of equilibrium reaction 5 also afford **14**. In the latter, [Fe(OEP)] undergoes an oxidative addition reaction with the persulfide-bridged double cubane **13**. Assembly **14** has not yet been obtained as diffraction-quality crystals. However, it is formed to the extent of ≥90% in six different reactions, each designed to have the same unambiguous outcome. In addition, the assembly $[Fe_4S_4(LS_3)(\mu_2\text{-}S)Fe^{III}(salen)]^{2-}$ has been prepared by acid-base coupling (24).

Proof of the structure of **14** follows from its synthesis and from ^1H NMR properties. The assembly exhibits an isotropically shifted spectrum in which the ratios of the methine and ethyl group isotropic shifts of **14** to those of the typical high-spin complex [Fe(OEP)(OMe)] are 0.5-1.1 at 298 K. This ensures that the Fe^{III}(OEP)S fragment of the assembly is high-spin. The situation is quite different for the cluster ligand. Shifts of the ring substituents of the coordinated phenylthiolate portion of the LS_3 ligand are acutely sensitive to the

Fig. 1. FORMATION OF A BRIDGED Fe_4S_4–S–HEME ASSEMBLY



identity of the ligand at the unique site (22), allowing detection of ligand substitution. Shift differences between reactant and product are typically ≤0.3 ppm. However, the ratios of isotropic shifts at the 2- and 4,5,6-positions of the ligand (Figure 1) in **14** vs. precursor **11** are 7.2-11.5. The zero-field Mössbauer spectra of the two clusters are essentially identical and require the $[Fe_4S_4]^{2+}$ oxidation state. The enhanced isotropic shifts in the assembly, which are mainly contact in origin, are, therefore, consistent only with extensive spin delocalization from the high-spin Fe(III) fragment to the diamagnetic cluster portion of **14** (24). This in turn mandates a covalent bridge between heme and cluster. The cluster isotropic shifts are a manifestation of magnetic hyperfine interactions at the Fe sites, which have been observed in the Mössbauer spectra of $[Fe_4S_4]^{2+}$

clusters in site **3** (19,20). This effect is one defining feature of exchange coupling between bridged components in native and synthetic heme-cluster assemblies. The influence of thiolate vs. sulfide bridging on the extent of coupling remains to be determined. In the context of analogues, a desirable next step is to link cluster and heme covalently by attaching to the porphyrin three thiol ligands in the correct spatial disposition, followed by cluster binding and insertion of the desired bridge in the void between the cluster and heme. Such a construction would obviate equilibrium 5, which is undesirable in functional studies of **14** and related assemblies where the heme Fe(II) state is required to bind substrate.

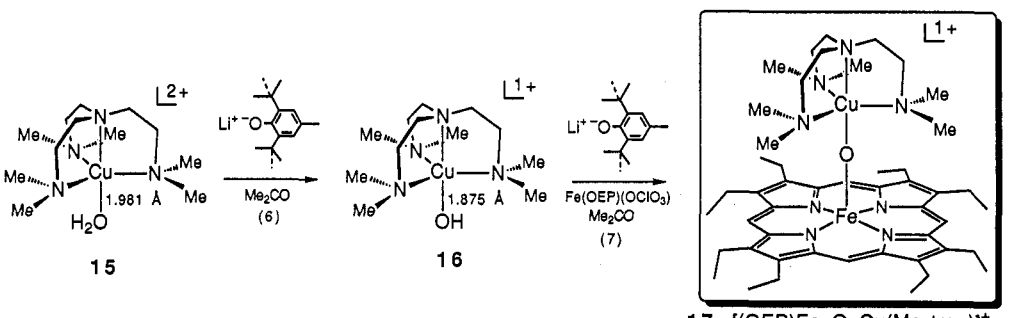
THE Cu/Fe BINUCLEAR SITE OF CYTOCHROME *c* OXIDASE

The oxidized ("as-isolated") form

Cytochrome *c* oxidase (CcO) catalyzes the dioxygen/water redox reaction $O_2 + 4H^+ + 4e^- \rightarrow 2H_2O$ which links the biochemical pathways of respiration and photosynthesis (10). Together with the quinol oxidases, these enzymes form a superfamily of heme-Cu oxidases. Schematic rendering **5** (10b) shows the various metal centers in CcO; although not an object of study here, we note the evidence that the Cu_A site may actually be binuclear (25). Simulation of the Cu/Fe binuclear site presents the initial problem of linking two labile heterometals across a bridge in the unit [Fe^{III}-X-Cu^{II}] corresponding to the oxidized form of the enzyme in which the likely bridge X = O^{2-}, OH^-, S^{2-}, RS^-,or Cl^-. The two metal centers are antiferromagnetically coupled through the bridge to produce an S = 2 ground state. Endeavors to achieve a synthetic representation of the site have proceeded apace for *ca.* 15 years; the insightful pioneering efforts of Gunter, Murray and coworkers (26) notwithstanding, structurally well defined analogues have not been achieved until very recently.

Our approach to an *oxo*-bridged assembly is summarized in Figure 2 (27). Deprotonation of Cu(II) aquo complex **15** with a hindered base in reaction 6 affords the hydroxo species **16**. Both complexes have trigonal bipyramidal stereochemistry; note the shorter Cu–O bond in **16** consistent with formation of an anionic ligand. Deprotonation of **16** in the presence of axially labile [Fe(OEP)($OClO_3$)] affords the desired bridge assembly **17** in 70% yield by means of coupling reaction 7. The structure of **17** has been determined in three different crystalline environments; the indicated dimensions are essentially constant and thus intrinsic to this particular combination of ligands. The Fe(III) atom is displaced from the porphyrin plane toward the bridging oxo atom

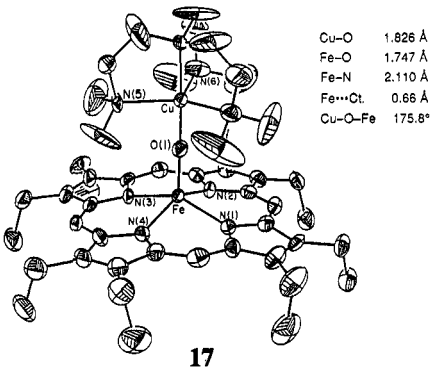
Fig. 2. **SYNTHESIS OF HEME-Cu(II) OXO-BRIDGED ASSEMBLY**



15 **16** **17** [(OEP)Fe–O–Cu(Me_6tren)]$^+$

17: Cu–O, 1.826 Å, Fe–O 1.747 Å, Cu–O–Fe 175.8°

and the Fe^{III}-O-Cu^{II} bridge is virtually linear; the Cu-Fe separation is 3.57 Å. Bond distances at and Mössbauer parameters of the Fe site demonstrate the presence of high-spin Fe(III). As shown in Figure 3, the magnetism of **17** closely follows the Curie-Weiss law at 4.2-300 K with a Curie constant consistent with S = 2 (27), thereby establishing this assembly as a coupled system with a quintet ground state, the first such example in CcO analogue chemistry. Note the difference in magnetic behavior between **17** and the [Fe^{III}-F-Cu^{II}] species in Figure 3, which has uncoupled S = 5/2 and 1/2 centers. Subsequently, Nanthakumar *et al.* (28) have reported a second heme-based assembly with a linear [Fe^{III}-O-Cu^{II}] bridge and an S = 2 ground state. Hence, this state is now established for linear bridges of this type involving trigonal bipyramidal Cu and tetragonal high-spin Fe^{III} configurations.



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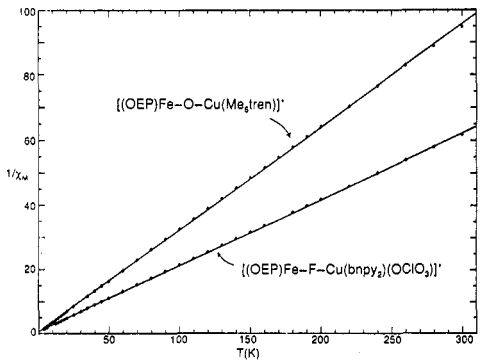


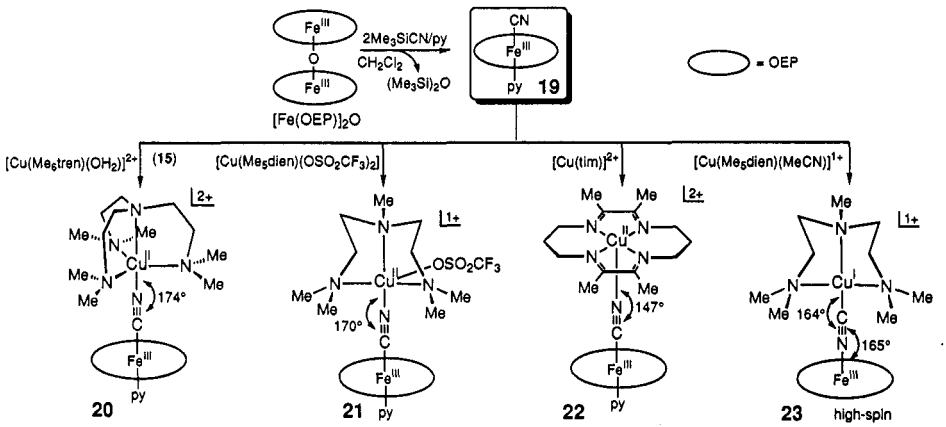
Fig. 3. Temperature dependence of the magnetism of **17** and an uncoupled [Fe^{III}-F-Cu^{II}] bridged assembly

The most recent EXAFS results of a binuclear site, that in the cytochrome aa_3 quinol oxidase of *Bacillus subtilis* (which lacks Cu_A), have been analyzed in terms of an asymmetric bridge structure with bridge atom X = S or Cl, Fe–X = 2.36 Å, Cu–X = 2.21 Å, and Fe···Cu = 3.70 Å (11). These results became available after completion of our work on **17**. Because this enzyme does not exhibit an EPR signal from Cu_B, its binuclear site is presumably coupled in an S = 2 ground state, similar to the situation in bovine heart CcO. While oxo-bridged assembly **17** achieves the most idiosyncratic electronic feature of the oxidized binuclear site, it must be considered that other bridges may mediate exchange coupling with the same ground state outcome (but different coupling constants). Lastly, oxidase preparations are often heterogeneous, and the possibility that there is not a unique bridge in the oxidized binuclear site must be entertained. Assemblies with other bridges are now an urgent priority in the synthetic analogue approach to the oxidized binuclear site.

The cyanide-inhibited form

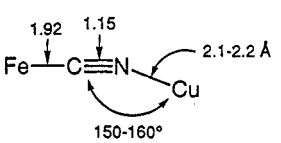
Oxidized and reduced heme-Cu oxidases bind a number of exogenous anions, including cyanide (29). While cyanide can disrupt the function of a variety of metal sites in proteins, it is known to be a rapid and irreversible inhibitor of CcO. Indeed, its lethal toxicity has been traced to the inhibition of CcO (30). The view that cyanide binds in the binuclear site, thereby preventing the reduction of dioxygen, is familiar. Palmer (31) has recently raised the issue with regard to a structural interpretation of bound cyanide stretching frequencies. We have initiated research to synthesize Fe–CN–Cu bridged species and explore any relationship between bridge structure and values of ν_{CN} (32). The synthetic method shown in Figure 4 has afforded the first structurally authenticated heme-Cu(II,I) cyanide-bridged assemblies. The nitrogen end of bound cyanide in readily prepared heme complex **19** is sufficiently nucleophilic to displace labile ligands (water, acetonitrile, acetone, triflate) from Cu complexes. In this way, some ten assemblies including **20-23** have been prepared and structurally characterized. Because the stereochemistry of the Cu_B site is unknown, the structures at the Cu(II) subsites in [Fe^{III}-CN-Cu^{II}] bridges were varied across trigonal bipyramidal, square pyramidal, and tetragonal (32). The

Fig. 4. **SYNTHESIS OF HEME-Cu(II,I) CYANIDE-BRIDGED ASSEMBLIES**



cyanide ligand is N-bound in an axial position in each case and in an equatorial position in several distorted square pyramidal complexes including **21**. In each molecule, the Fe-C-N angles are essentially linear but the Cu-N-C angles θ (140-174°) and Cu–NC distances *d* (1.88-2.45 Å) vary; **20** (1.74°, 1.90 Å) and **22** (147°, 2.17 Å) are nearly limiting cases in the latter respects. The largest departure from linearity has been found in the doubly bridged tetragonal octahedral assembly $\{[(py)(OEP)Fe\text{-}CN]_2Cu(cyclam)\}^{2+}$ (140°, 2.45 Å). Values of θ decrease with increasing *d* in an approximately linear fashion (θ = -66.9*d* + 300).

Cyanide stretching frequencies (ν_{CN}) of oxidized enzymes have been reported for bovine heart CcO and a quinol oxidase from *E. coli*; these occur in the narrow interval 2152-2146 cm^{-1} (29). The synthetic assemblies with [Fe^{III}-CN-Cu^{II}] bridges range over 2181-2120 cm^{-1} in the solid state; solution values are nearly the same (32). Values of coordinated cyanide in an M-CN group are always higher than in the free or ionically bound form (2080 cm^{-1} in NaCN) because covalent bonding removes electron density from an sp σ* MO; at constant geometry frequencies can be even higher in doubly bridged M-CN-M'. On the other hand, π-bonding places electron density in π* MO's and decreases frequencies. As seen in Figure 5, ν_{CN} tends to increase as θ increases and *d* decreases; a decrease in bond angle will tend to diminish cyanide bond order, especially when the bridge contains a π-donor such as low-spin Fe(III). From our investigations of cyanide vibrational frequencies, and also magnetic and Mössbauer spectroscopic features, in bridged assemblies of known structure (32), we have arrived at the following conclusions. (i) The low-spin Fe(III) and Cu(II) sites are ferromagnetically coupled to yield an S = 1 ground state, as in the enzymes. (ii) A relatively "tight" bridge is formed, as opposed to others with weaker interactions including hydrogen bonding ([Fe-CN···Cu], [Fe···NC-Cu], [Fe/Cu···H-]). (iii) Bridge structure **24** is consistent with structure/ν_{CN} correlations. (iv) Given the structure of assembly **23** (ν_{CN} 2100 cm^{-1}), the linkage isomer structure [Fe^{III}-NC-Cu^{I}] must be considered for one-electron reduced bridges. (v) On the basis of near-convergence of birational and electronic properties, the [Fe^{III}-CN-Cu^{II}] bridge in enzymes is considered as established; i.e., the classical view of bridge formation in cyanide-inhibited heme-Cu oxidases is correct. These conclusions are offered within the limitations of the database (32), one of which is that it does not include all reasonable stereochemistries at the Cu(II) site. They are justified in the sense that the ν_{CN} values are derived from *known* structures, and frequencies and structural parameters correlate.



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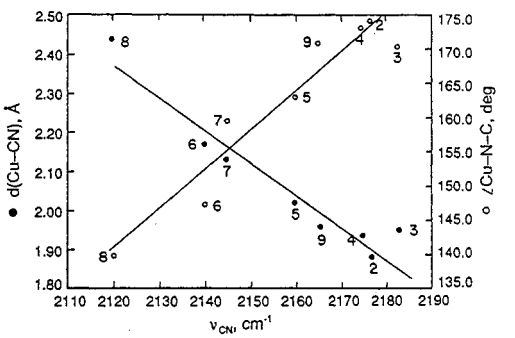

Fig. 5. Correlation of ν_{CN} with θ and *d* in the [Fe^{III}-CN-Cu^{II}] bridge.

Acknowledgments

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