



the cobalt salt became blue, DPPP (25 mg, 0.060 mmol) and anhydrous THF (1.0 mL) were sequentially added under argon. The mixture was stirred for 10 min at room temperature. 2-Bromo-2-methyldecane (**1a**, 0.12 g, 0.50 mmol) and allylmagnesium chloride (1.0 M solution in THF, 1.5 mL, 1.5 mmol) were successively added dropwise to the reaction mixture at -20°C . While the Grignard reagent was being added, the mixture turned reddish-brown. After being stirred for 2 h at -20°C , the reaction mixture was poured into saturated NH_4Cl solution, and the products were extracted with ethyl acetate (20 mL $\times$ 2). The combined organic layer was dried over Na_2SO_4 and concentrated. Purification of the crude oil by silica gel column chromatography (hexane) provided 4,4-dimethyl-1-dodecene (**2a**) and 2-methyl-1-decene (**3a**) (94 mg, 90% and 8% yields, respectively, as determined by ^1H NMR spectroscopy).

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Distal Cu Ion Protects Synthetic Heme/Cu Analogues of Cytochrome Oxidase against Inhibition by CO and Cyanide**

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Cytochrome c oxidase (CcO) is the enzyme that makes aerobic metabolism possible by catalyzing the final step in the respiratory electron-transfer chain—a four-electron ($4e^-$), four-proton (4H^+) reduction of O_2 to water.^[1,2] Catalysis proceeds at the heterobimetallic heme/ Cu_B site (Figure 1).^[3] The Fe center is the site of O_2 binding, and reduction of O_2 is coupled to oxidation of $\text{Cu}_\text{B}^{\text{I}}$, at least under single-turnover conditions.^[4,5] One of the poorly understood questions regarding the structure–activity relationship at the heme/ Cu_B site is how the heme reactivity is affected by the closely positioned, positively charged Cu_B center. Such effects are expected to manifest themselves in an attenuated affinity of the Fe center for small molecules. Among these, CO and the ions CN^- and N_3^- , are of particular interest, because the cytotoxicity of the CN^- and N_3^- ions arises from the inhibition of CcO and resultant respiratory shutdown,^[7] and CO is an endogenous inhibitor of ferrohemes. The mode of CN^- ion binding to the heme/ Cu_B site and the effect(s) of Cu_B on such binding remain controversial.^[8] Likewise, contradictory results have been reported for differences in the CO affinities of the wild-type and Cu_B -free mutants of terminal oxidases.^[9,10]

Previously, we demonstrated that the biomimetic complexes shown in Figure 1b quantitatively reproduce the key reactivity of the heme/ Cu_B site^[6,11] and thus allow the study of questions that are not easily addressed by working with the enzyme itself. Herein we report that the steady-state reduc-

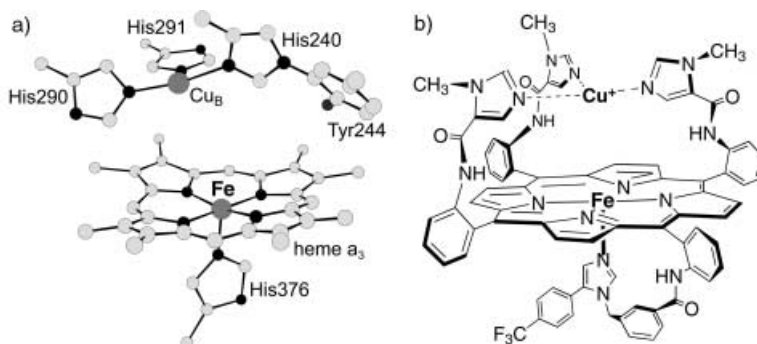


Figure 1. a) The heme/ Cu_B site of bovine cytochrome oxidase;^[3] the C atoms are light gray and the N and O atoms are black, b) a synthetic heme/ Cu_B analogue in the reduced form; exogenous ligands and counterion are omitted.^[6]

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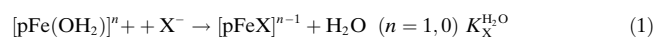
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tion of O₂ catalyzed by these biomimetic complexes is reversibly inhibited by both the CN[−] ion and CO, and the distal Cu center notably decreases the catalysts' susceptibility to inhibition. The possibility that Cu_B may protect against small-molecule inhibition has not been previously considered.

Inhibition was studied electrochemically in pH 7 buffered electrolytes with the water-insoluble catalysts deposited on the surface of an edge-plane graphite rotating-disk electrode to obtain rapid electron delivery from the electrode to the catalytic sites.^[13] Whereas the distal Cu center is critical for catalytic activity under diffusion-limited electron flux,^[12] the FeCu and Fe-only complexes catalyze O₂ reduction with identical kinetics, mechanism, and stability when electron flux is rapid.^[11] In this regime comparative voltammetry of the FeCu and Fe-only catalysts in the presence of the inhibitors yields data that are free of the ambiguities caused by the different catalytic behavior of the mono- and bimetallic catalysts.

Under anaerobic conditions, rapid equilibration among the [pFe^{III}(OH₂)]⁺, [pFe^{III}X], [pFe^{II}(OH₂)], and [pFe^{II}X][−] species (X = CN[−], N₃[−]; p represents the pentadentate porphyrin shown in Figure 1b) is observed by cyclic voltammetry of the Fe-only complex. Equilibrium constants for displacement of H₂O by X[−], $K_X^{H_2O}$, [Eq. (1), Table 1] have been derived from voltammetric titration experiments.^[13] The CN[−] ion has an appreciable affinity not only for Fe^{III} but also for Fe^{II} centers, whereas binding of the N₃[−] ion to Fe^{II} centers is only weakly favored over the coordination of H₂O. Anaerobic voltammetry of the FeCu complex in the presence of X[−] ions suggests the existence of more than four species and is complicated by the loss of Cu at [CN[−]] > 50 μM^[14,15] and at [N₃[−]] > 0.5 mM, which precluded quantitative characterization of this system.^[13]



Inhibition of O₂ reduction is more significant at higher potentials (Figure 2), at which CN[−] ions decrease the amount of the active catalyst both by directly binding to it (forming [pFe^{II}CN][−]) and by shifting the redox Fe^{III/II} equilibrium towards the ferric form, as [pFe^{III}CN] is a weaker oxidant than the [pFe^{III}(OH₂)]⁺ ion. This simple picture is sufficient to quantitatively model the catalytic half-wave potentials observed in a range of CN[−] concentrations.^[13]

At sufficiently reducing potentials the cyanide-ligated catalyst is so easily reduced that only a competition between O₂ and CN[−] ions for the Fe^{II} center determines the potential-

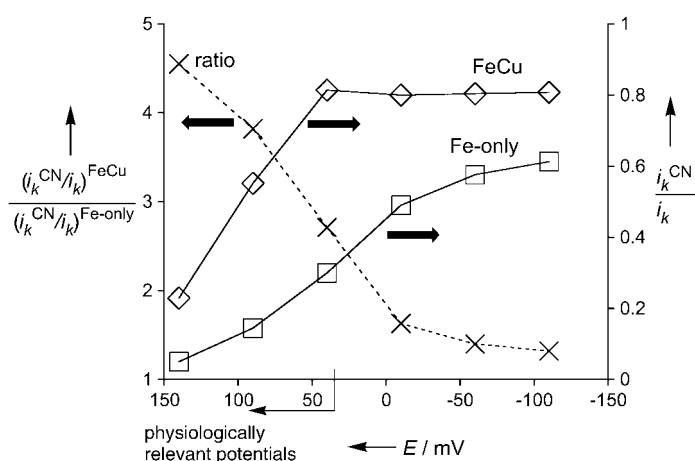
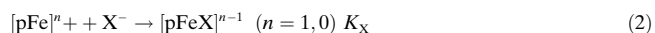


Figure 2. Susceptibility of the FeCu (◇) and Fe-only (□) catalysts to inhibition by CN[−] ions (3.5 μM) at pH 7 as a function of potential *E* (vs. the normal hydrogen electrode (NHE)). Right scale: the fraction of the retained catalytic activity, i_k^{CN}/i_k (i_k^{CN} : kinetic current in the presence of CN[−]; i_k : kinetic current in the absence of CN[−] ions). Left scale: the retained fraction for the FeCu catalyst relative to that for the Fe-only analogue (×). The physiologically relevant potentials are those of cytochrome c and ubiquinol, the physiological reductants of CcO and the related ubiquinol oxidase.

independent residual catalytic activity (Figure 2, $E < -0$ V). In accord with inhibition enzymatic kinetics, the ratio of the lost ($i_k - i_k^{CN}$) to retained (i_k^{CN}) catalytic activity is proportional to [CN[−]] (Figure 3). The proportionality coefficient K_X is the equilibrium binding constant of CN[−] ions to five-coordinate Fe^{II} centers [Eq. (2), Table 1]. The $K_{CN}/K_{CN}^{H_2O}$ ratio, equal to the affinity of [pFe^{II}] for H₂O, indicates that during steady-state catalysis in the absence of CN[−] ions < 1 % of the ferrous complex is five coordinate.



The data in Figure 3 show that, depending on the potential, the susceptibility of the Fe-only catalyst to CN[−] ion inhibition is up to five times that of the FeCu analogue. Relative to the Fe-only complex, the CN[−] ion affinity of the FeCu analogue is 2.6 and approximately two-times lower in the reduced and oxidized states, respectively, and its Fe^{III/II} potential in the aqua form is ~20 mV more oxidizing. Because at physiologically relevant potentials reduction of Fe^{III}CN unit is unfavorable, the higher Fe^{III/II} potential and the lower CN[−] ion affinity of the oxidized FeCu complex increase the difference in susceptibility of the mono and bimetallic catalysts at $E > -0$ V.

Table 1. Equilibrium constants, K [M^{−1}] for CN[−], N₃[−] ion, and CO binding to the synthetic analogues and the heme/Cu_B site, and the CO and O₂ on-rates, k_{on} , which are listed in square parentheses.

Catalyst	Redox and ligation state of Fe	Constant	CN [−]	N ₃ [−]	CO [k_{on} , M ^{−1} s ^{−1}]	O ₂ [k_{on} , M ^{−1} s ^{−1}]
Fe-only	[pFe ^{III} (OH ₂)] ⁺	$K_X^{H_2O}$	7×10^5 ^[a]	2×10^2 ^[a]		
	[pFe ^{II} (OH ₂)]	$K_X^{H_2O}$	3×10^3 ^[a]	2.5 ^[a]		
	[pFe ^{II}]	K_X	4×10^5 ^[b]			
FeCu	pFe ^{II}	K_X	1.5×10^5 ^[b]		1.9×10^6 [3.8×10^3]	$[1 \times 10^7]$ ^[11]
CcO	Fe ^{III} (OH ₂)		1×10^6 ^[16] – 10^8 ^[17]	1×10^2 ^[18] – 10^4 ^[19]	7.6×10^5 [3.3×10^3]	$[\sim 2.5 \times 10^7]$ ^[11]
	Fe ^{II} _{a3}		1×10^3 – 10^4 ^[17]		1×10^6 – 10^7 ^[17] [7×10^4] ^[19,20]	$[1-3 \times 10^8]$ ^[2]

[a] From anaerobic cyclic voltammetry.^[13] [b] From the data in Figure 3.

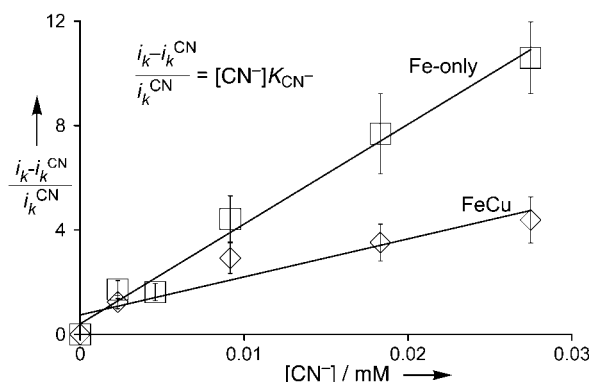


Figure 3. The ratio of the lost ($i_k - i_k^{\text{CN}}$) to retained (i_k^{CN}) catalytic activity at -100 mV (the potential-independent regime, see Figure 2) for the FeCu ($\diamond$) and Fe-only ($\square$) catalysts as a function of $[\text{CN}^-]$. The lines are the least-squares fits to the equation shown. The data were collected only at $[\text{CN}^-]$ where extraction of Cu was deemed to be insignificant, although a possibility of some Cu loss can not be excluded entirely.

Steric perturbations of CN^- ions within the more constrained binding pocket of the FeCu complex probably accounts for the lower CN^- ion affinity of this complex. The weaker CN^- ion binding to the oxidized FeCu catalyst relative to the Fe-only analogue is unexpected because the higher positive charge of the biometallic complex should favor anion binding. Possible CN^- ion binding to the Cu center, suggested by non-catalytic experiments,^[13] may compensate the extra charge and increase steric congestion around the Fe center. The weaker binding of CN^- ions particularly to the $\text{Fe}^{\text{III}}\text{Cu}^{\text{II}}$ catalyst is biologically significant because the tolerance of an organism to CN^- ion poisoning relates to the dissociative stability of the oxidized cyanide-ligated heme/ Cu_B site, which is not reducible by ferrocyanide c.

Because CO and the CN^- ion have similar stereoelectronic requirements as ligands, the steric origin of the weaker CN^- ion binding to the FeCu catalyst is supported by 2.5-fold lower affinity of the FeCu catalyst for CO. The effects of steric constraints on CO binding to heme units have been studied extensively and spectroscopic^[6] as well as structural^[3] evidence exists for Cu-induced perturbation of heme-bound CO in CO-ligated heme/ Cu_B site. The rates of CO binding to and dissociation off Fe centers (and hence K_{CO} , Table 1) in our heme/ Cu_B analogues were obtained by measuring the loss rate of catalytic O_2 reduction at a fixed potential in the presence of CO (Figure 4). A comparison of the FeCu and Fe-only catalysts reveals that Cu does not significantly affect the CO on-rate but accelerates the off-rate, which suggests Cu-induced destabilization of the CO adduct. In contrast to CN^- and N_3^- ions, equilibration between the free and CO-bound catalysts is slow. This situation results in cyclic voltammograms that display irreversible redox events from which relevant equilibrium constants, $K_{\text{CO}}^{\text{H}_2\text{O}}$, could not be determined.

Our biomimetic complexes reproduce the relative ligand affinities of the oxidized (o) and reduced (r) heme/ Cu_B site in CcO : $\text{CN}^-(\text{o}) \sim \text{CO}(\text{r}) > \text{CN}^-(\text{r}) > \text{N}_3^-(\text{o}) \geq \text{HCO}_2^-(\text{o})$.^[17] Quantitatively, the oxidized catalysts display lower and the reduced catalysts higher affinities for anionic ligands (CN^- , N_3^- , and formate^[13]) than the heme/ Cu_B site in the same redox

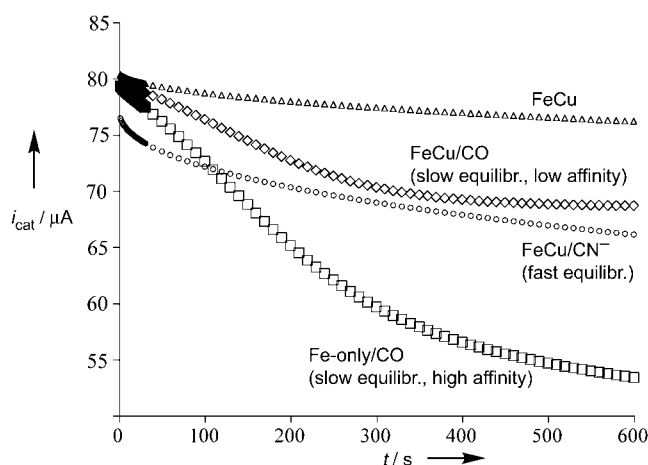


Figure 4. The catalytic current, i_{cat} , as a function of time, t , for O_2 reduction at -100 mV by the FeCu ($\diamond$) and Fe-only ($\square$) catalysts in the presence of CO (240 Pa) and, for comparison, by the FeCu catalyst in the absence of an inhibitor ($\triangle$) and in the presence of $10 \mu\text{M}$ CN^- ($\circ$).^[14] In air-saturated electrolytes at the electrode rotation frequency of 200 revolutions per minute. In the presence of CO, the rapid drop of i_{cat} at 100–300 s results from a slowly established equilibrium between the CO-bound and free catalysts. Least-square fits of the $i_{\text{cat}}(t)$ data^[13] yield the CO on- and off-rates.

state. This situation is consistent with more efficient charge stabilization (either $[\text{pFe}^{\text{III}}(\text{OH}_2)]^+$ or $[\text{pFe}^{\text{II}}\text{X}]^-$) in the biomimetic complexes in contact with an aqueous electrolyte relative to charge stabilization in the protein matrix.^[11] The rate of equilibration between CN^- (N_3^-) bound and free catalysts is rapid and qualitatively comparable to that of the “pulsed” oxidized cytochrome oxidase,^[21] but the on-rates of both CO and O_2 are approximately tenfold lower than those reported for the enzyme. The origin of this difference is uncertain.

In summary, we have observed that during the steady-state reduction of O_2 under physiologically relevant conditions Cu decreases the susceptibility of functional heme/ Cu_B analogues to inhibition by CN^- ions and CO. An up to fivefold-higher concentration of CN^- ions is required to inhibit the same fraction of the FeCu catalyst as the Fe-only analogue operating at physiologically relevant potentials. This results from a combination of weaker CN^- ion binding to both the $\text{Fe}^{\text{III}}\text{Cu}^{\text{II}}$ and $\text{Fe}^{\text{II}}\text{Cu}^{\text{I}}$ catalysts and an approximately 20 mV more oxidizing $\text{Fe}^{\text{III/II}}$ potential in the aqua FeCu complex.

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