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 × 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).

Received: August 8, 2002 [Z19929]

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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 (C*c*O) 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 Cu_B^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 C*c*O 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-

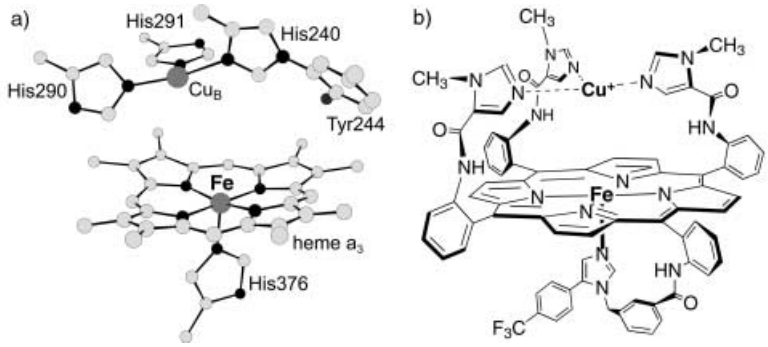

Figure 1. a) The heme/Cu_B site of bovine cytochrome oxidase;[3] the C atoms are light gray and the N and O atoms are black, b) a synthetic heme/Cu_B analogue in the reduced form; exogenous ligands and counterion are omitted.[6]

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[**] This work was supported by NIH and a Stanford Graduate Fellowship (R.B.).

Supporting information for this article is available on the WWW under http://www.angewandte.org or from the author.

  0044-8249/02/4121-4139 $ 20.00+.50/0

tion of O_2 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 O_2 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 $[pFe^{III}(OH_2)]^+$, $[pFe^{III}X]$, $[pFe^{II}(OH_2)]$, and $[pFe^{II}X]^-$ species ($X = CN^-$, N_3^-; p represents the pentadentate porphyrin shown in Figure 1b) is observed by cyclic voltammetry of the Fe-only complex. Equilibrium constants for displacement of H_2O 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 N_3^- ion to Fe^{II} centers is only weakly favored over the coordination of H_2O. 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 $[CN^-] > 50\ \mu M$[14,15] and at $[N_3^-] > 0.5$ mM, which precluded quantitative characterization of this system.[13]

$$[pFe(OH_2)]^n + + X^- \rightarrow [pFeX]^{n-1} + H_2O \quad (n = 1, 0)\ K_X^{H_2O} \qquad (1)$$

Inhibition of O_2 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 $[pFe^{III}(OH_2)]^+$ 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 O_2 and CN^- ions for the Fe^{II} center determines the potential-independent residual catalytic activity (Figure 2, $E < \sim 0$ 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 H_2O, indicates that during steady-state catalysis in the *absence* of CN^- ions $< 1\%$ of the ferrous complex is five coordinate.

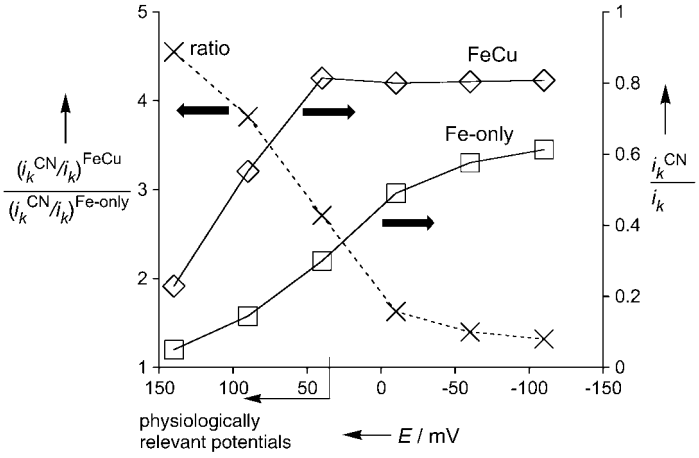

Figure 2. Susceptibility of the FeCu (◇) and Fe-only (□) catalysts to inhibition by CN^- ions (3.5 μM) at pH 7 as a function of potential E (vs. the normal hydrogen electrode (NHE)). Right scale: the fraction of the retained catalytic activity, i_k^{CN}/i_k (i_k^{CN}: kinetic current in the presence 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 C*c*O and the related ubiquinol oxidase.

$$[pFe]^n + + X^- \rightarrow [pFeX]^{n-1} \quad (n = 1, 0)\ K_X \qquad (2)$$

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 $E > \sim 0$ V.

Table 1. Equilibrium constants, K [M^{-1}] for CN^-, N_3^- ion, and CO binding to the synthetic analogues and the heme/Cu_B site, and the CO and O_2 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]		1.9×10^6 [3.8×10^3]	[1×10^7][11]
FeCu	pFe^{II}	K_X	1.5×10^5[b]		7.6×10^5 [3.3×10^3]	[$\sim 2.5 \times 10^7$][11]
C*c*O	$Fe_{a3}^{III}(OH_2)$		1×10^6[16]–10^8[17]	1×10^2[18]–10^4[19]		
	Fe_{a3}^{II}		1×10^3–10^4[17]		1×10^6–10^7[17] [7×10^4][19,20]	[1–3×10^8][2]

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

  0044-8249/02/4121-4140 $ 20.00+.50/0 *Angew. Chem. Int. Ed.* **2002**, *41*, No. 21

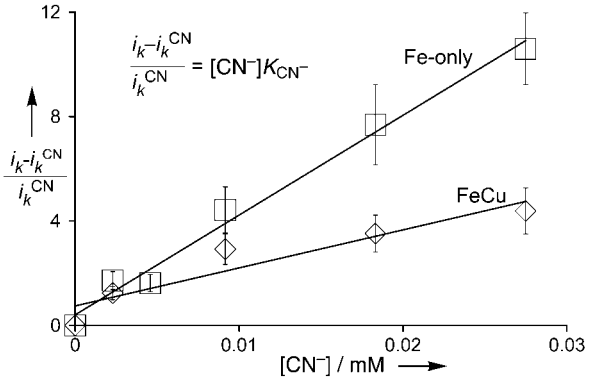

Figure 3. The ratio of the lost ($i_k - i_k^{CN}$) to retained (i_k^{CN}) catalytic activity at −100 mV (the potential-independent regime, see Figure 2) for the Fe-Cu (◇) and Fe-only (□) catalysts as a function of [CN⁻]. The lines are the least-squares fits to the equation shown. The data were collected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

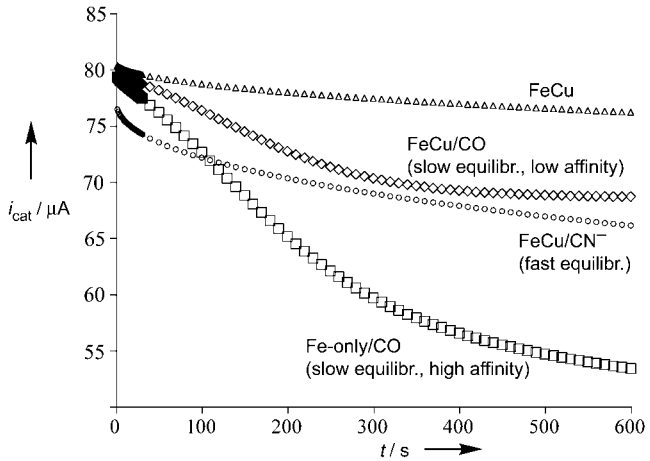

Figure 4. The catalytic current, i_{cat}, as a function of time, t, for O_2 reduction at −100 mV by the FeCu (◇) and Fe-only (□) catalysts in the presence of CO (240 Pa) and, for comparison, by the FeCu catalyst in the absence of an inhibitor (△) and in the presence of 10 μM CN^- (○).[14] In air-saturated 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 $i_{cat}(t)$ data[13] yield the CO on- and off-rates.

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 $Fe^{III}Cu^{II}$ 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 reducable by ferrocytochrome 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, $K_{CO}^{H_2O}$, could not be determined.

Our biomimetic complexes reproduce the relative ligand affinities of the oxidized (o) and reduced (r) heme/Cu_B site in *Cc*O: $CN^-(o) \sim CO(r) > CN^-(r) > N_3^-(o) \geq HCO_2^-(o)$.[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 state. This situation is consistent with more efficient charge stabilization (either $[pFe^{III}(OH_2)]^+$ or $[pFe^{II}X]^-$) 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 $Fe^{III}Cu^{II}$ and $Fe^{II}Cu^{I}$ catalysts and an approximately 20 mV more oxidizing $Fe^{III/II}$ potential in the aqua FeCu complex.

Received: July 30, 2002 [Z19851]

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