A Synthetic Analogue for the Oxygen Binding Site in Cytochrome c Oxidase

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Prodigious progress[1] has been made in the synthesis and characterization of both structural and functional analogues of the oxygen-binding heme proteins hemoglobin (Hb) and myoglobin (Mb). Recently, widespread attention has been directed toward the preparation of models[2] of the oxygen binding site within the cytochrome *c* oxidases, the terminal enzymes in the electron transfer chain.[3] These mitochondrial membrane bound, multimetallic enzymes seize O_2 in a binuclear center formed by a heme (heme a_3) and a copper atom (Cu_B). Electron transfer, along with addition of four protons, consummates the overall four-electron reduction of O_2 to two H_2O molecules.[4] Toxic intermediates such as H_2O_2 and HO_2^- are not released during this exergonic reaction. The energy produced by the O_2 reduction is coupled to proton translocation, contributing to the protonmotive force which drives conversion of ADP to ATP.[5,6] Thus, cytochrome *c* oxidases are the master enzymes which play an essential role in oxidative phosphorylation, a process required by all mitochondrial respiring organisms.[3] It is important to understand the detailed mechanism of O_2 activation and its four-proton, four-electron reduction by this system.

Considerable progress has been made in unraveling the catalytic reaction cycle.[4,7] Time-resolved resonance Raman spectroscopy using oxygen isotopes has been indispensable in identifying reaction intermediates.[8] Nonetheless, many issues remain controversial, for example, the role of copper in O_2 binding, and the nature of the O_2-bound intermediates that must be present during the catalytic cycle.[9]

All cytochrome oxidases are membrane bound. For this reason it has not been possible as yet to define the structure at atomic resolution.[10] Nevertheless, the relationship between the iron and the copper atoms in the binuclear center which forms the active site of the enzyme has been partially characterized by spectroscopic and other structural studies. MCD[11] and EPR[12] studies have shown that, in the oxidized form [Fe(III), Cu(II)] of the enzyme, the iron and the copper are strongly spin coupled. This finding and EXAFS measurements[13,14] suggest an Fe–Cu distance of 3.5–5 Å in the oxidized enzyme, but the distance between the reduced centers is not known.

Our interest in this subject stems from the conjunction of three paths: the invention of molecular catalysts for the four-electron reduction of O_2,[15] the synthesis of MbO_2 models,[16] and the discovery of a powerful, general method for introducing macrocycles over porphyrins in a single "congruent multiple Michael addition" step.[17] Herein we report the synthesis and partial characterization of a synthetic model of the cytochrome *c* oxidase O_2 binding site, preliminary characterization of its O_2 adduct, and demonstration of a stoichiometric cycle which reduces O_2 by four electrons.

This cytochrome *c* oxidase model was synthesized according to Scheme 1. The Michael acceptor (**1**) was metalated with iron and then combined with 1,4,7-triazacyclononane. The product (**2**) was isolated by chromatography and then metalated with CuBr to form the cytochrome *c* oxidase model (**3**). Compound **3** has structural features similar to the proposed oxygen binding site of the native enzymes. Like the native enzymes, **3** contains an iron(II) porphyrin fastened in close proximity to a copper-(I).[18] Addition of excess 1,5-dicyclohexylimidazole results in axial iron(II) ligation similar to that in the naturally occurring family.[19] The copper is coordinatively bound to three tertiary amines that act in place of the supporting ligands chelated to the copper in the native enzymes.[20]

A solution of **3** with 500 equiv of 1,5-dicyclohexylimidazole in toluene was titrated with dioxygen while being monitored by UV–vis spectroscopy. An *irreversible* adduct of dioxygen and **3** *rapidly* ($t_{1/2} \approx 5$ min) forms in a measured 1:1 ratio, indicated by a shift in the Soret band from 442 nm to 428 nm. The irreversibility of O_2 binding was demonstrated by the lack of displacement of the bound O_2 unit under both CO and Ar purges. This is in marked contrast to the copper-free compound **2**, which *slowly* ($t_{1/2} \approx 10$ h) forms a *reversible*, MbO_2-like adduct under the same conditions. The difference in behavior of compounds **2** and **3** implies that the copper plays a definite role in dioxygen binding.

The oxygenated adduct (**4**) was titrated with cobaltocene, whose disappearance was followed by ^1H NMR. The reduction **4** → **3** was monitored by UV–vis spectroscopy.[21] Four equivalents of cobaltocene (4 e^-) were required for the complete reduction of the dioxygen adduct (**4**). This requirement of 4 equiv of cobaltocene

(1) (a) Momenteau, M.; Reed, C. A. *Chem. Rev.* **1994**, *94*, 659. (b) Collman, J. P.; Zhang, X. In *Comprehensive Supramolecular Chemistry*; Lehn, G.-M., Atwood, J. L., Suslick, K. S., Eds.; Pergamon Press: Oxford, U.K.; Vol. 5, submitted for publication.

(2) (a) Lee, S. C.; Holm, R. H. *J. Am. Chem. Soc.* **1993**, *115*, 11789. (b) Karlin, K. D.; Nanthakumar, A.; Fox, S.; Murthy, N. N.; Ravi, N.; Huynh, B. H.; Orosz, R. D.; Day, E. P. *J. Am. Chem. Soc.* **1994**, *116*, 4753.

(3) For a general reference on cytochrome *c* oxidase, see: Stryer, L. *Biochemistry*, 3rd ed.; W.H. Freeman: New York, 1988; pp 406–407.

(4) (a) Babcock, G. T.; Wikström, M. *Nature* **1992**, *356*, 301. (b) Chan, S. I.; Li, P. M. *Biochemistry* **1990**, *29*, 1. (c) Malmström, B. G. *Chem. Rev.* **1990**, *90*, 1247. (d) Capaldi, R. A. *Annu. Rev. Biochem.* **1990**, *59*, 569.

(5) Wikström, M. K. F. *Nature* **1977**, *266*, 271.

(6) Mitchell, P. *Nature* **1961**, *191*, 144.

(7) Malmström, B. G. *Acc. Chem. Res.* **1993**, *26*, 332.

(8) (a) Han, S.; Ching, Y.-C.; Rousseau, D. L. *Nature* **1990**, *348*, 89. (b) Ogura, T.; Takahashi, S.; Hirota, S.; Shinzawa-Itoh, K.; Yoshikawa, S.; Appelman, E. H.; Kitagawa, T. *J. Am. Chem. Soc.* **1993**, *115*, 8527. (c) Varotsis, C.; Zhang, Y.; Appelman, E. H.; Babcock, G. T. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 237.

(9) (a) Blackmore, R. S.; Greenwood, C.; Gibson, Q. H. *J. Biol. Chem.* **1991**, *266*, 19245. (b) Woodruff, W. H.; Einarsdóttir, O.; Dyer, R. B.; Bagley, K. A.; Palmer, G.; Atherton, S. J.; Boldbeck, R. A.; Dawes, T. D.; Kliger, D. S. *Proc. Natl. Acad. Sci. U.S.A.* **1991**, *88*, 2588. (c) Oliveberg, M.; Malmström, B. G. *Biochemistry* **1992**, *31*, 3560.

(10) Yoshikawa, S.; Tera, T.; Yoko, T.; Tsukihara, T.; Caughey, W. S. *Proc. Natl. Acad. Sci. U.S.A.* **1988**, *85*, 1354.

(11) Thomson, A. J.; Greenwood, C.; Gadsby, P. M. A.; Peterson, J.; Edlinton, D. G.; Hill, B. C.; Nicholls, P. *J. Inorg. Biochem.* **1985**, *23*, 187.

(12) Brudvig, G. W.; Blair, D. F.; Chan, S. I. *J. Biol. Chem.* **1984**, *259*, 11001.

(13) (a) Powers, L.; Chance, B.; Ching, Y.-C.; Angiolillo, P. *Biophys. J.* **1981**, *34*, 465. (b) Scott, R. A. *Annu. Rev. Biophys. Biophys. Chem.* **1989**, *18*, 137.

(14) Powers, L.; Lauraeus, M.; Reddy, K. S.; Chance, B.; Wickström, M. *Biochim. Biophys. Acta* **1994**, *1183*, 504.

(15) Collman, J. P.; Wagenknecht, P. S.; Hutchison, J. *Angew. Chem., Int. Ed. Engl.* **1994**, *33*, 1537.

(16) Collman, J. P.; Zhang, X.; Wong, K.; Brauman, J. I. *J. Am. Chem. Soc.* **1994**, *116*, 6245.

(17) Collman, J. P.; Zhang, X.; Herrmann, P. C.; Uffelman, E. S.; Boitrel, B.; Straumanis, A.; Brauman, J. I. *J. Am. Chem. Soc.* **1994**, *116*, 2681.

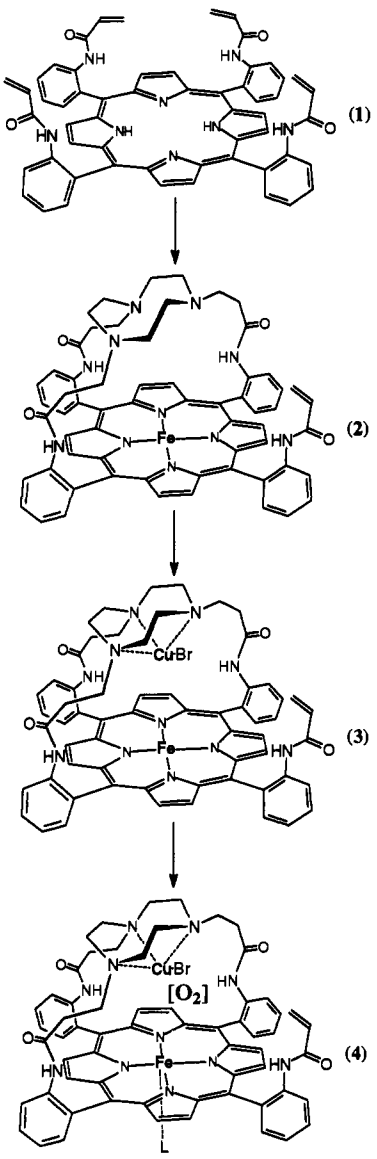
(18) Shapleigh, J. P.; Hosler, J. P.; Tecklenburg, M. M. J.; Kim, Y.; Babcock, G. T.; Gennis, R. B.; Ferguson-Miller, S. *Proc. Natl. Acad. Sci. U.S.A.* **1992**, *89*, 4786.

(19) Stevens, T. H.; Chan, S. I. *J. Biol. Chem.* **1981**, *256*, 1069.

(20) Cline, J.; Reinhammer, B.; Jensen, P.; Venters, R.; Hoffman, B. M. *J. Biol. Chem.* **1983**, *258*, 5124.

0002-7863/94/1516-9783$04.50/0

Scheme 1



to completely reduce the oxygen adduct implicates further a 1:1 O_2 to porphyrin stoichiometry. The process of alternately adding 1 mol of dioxygen and 4 equiv cobaltocene per mole of **3** was repeated five times with no apparent compound degradation.

Electrospray mass spectroscopy performed on the dioxygen adduct (**4**) shows a distribution of peaks centered at $m/z = 1248$. The isotopic distribution of this envelope of peaks is consistent with a 1:1 dioxygen:porphyrin adduct (**4**), after loss of the axial ligand.

Resonance Raman spectra of the models were obtained. No MbO_2-like bands in the 570 cm^{-1} region were present in the resonance Raman spectrum of **4** under equilibrium conditions. Instead, an isotope dependent peak was observed at 758 cm^{-1} for the $^{16}O_2$ adduct, which shifted to 740 cm^{-1} for $^{18}O_2$. The frequency of the line for the $^{16}O_2$ adduct is similar to the O–O stretching mode of the Cu–O–O–Cu bridged peroxo intermediate in hemocyanin.[22] The magnitude of the isotope shift is smaller than expected, but studies of the $^{17}O_2$ adduct suggest that coupling between the O–O stretching mode and some other mode may be occurring, complicating the isotope shifts. No oxygen isotope sensitive line in this 750 cm^{-1} region was detected in the resonance Raman spectrum of the copper-free compound **2**.

A solution of **3** in pyridine-d_5, which served as the axial ligand as well as the solvent, yielded a paramagnetic ^1H NMR spectrum with β-pyrrolic proton signals at 50–55 ppm indicative of five-coordinate ($S = 2$) iron(II).[23] Addition of exactly 1 equiv of O_2 results in a diamagnetic ^1H NMR spectrum. Possible explanations for the apparent diamagnetism of the adduct include an antiferromagnetic coupling of Cu(II) to Fe(III) through a peroxo bridge, or an MbO_2-like species[24] with increased stabilization through a dipolar interaction with Cu(I), but the Raman data exclude this latter possibility. Other studies to confirm the presence of a possible peroxo intermediate are ongoing.

In conclusion, we have synthesized a cytochrome *c* oxidase model which forms an adduct with dioxygen in 1:1 ratio. The adduct oxidizes 4 equiv of cobaltocene and, to the best of our knowledge, is the first such cytochrome *c* oxidase model O_2 adduct reported. We are currently investigating the exact nature of the oxygen adduct, which at this moment appears to be a bridged peroxo species.

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Supplementary Material Available: Synthetic details for the preparation of compounds **1–4** (4 pages). This material is contained in many libraries on microfiche, immediately follows this article in the microfilm version of the journal, and can be ordered from the ACS; see any current masthead page for ordering information.

(21) Upon addition of 2 equiv of cobaltocene to **4** the UV–vis spectrum returned to that observed for **3**. An additional 2 equiv of Cp_2Co were consumed by the resulting solution as determined by following the titration with ^1H NMR (the characteristic signal of Cp_2Co does not appear until more than 4 equiv of cobaltocene are added). The initial 2 e^- reduction apparently proceeds in a concerted process; addition of 1 equiv of Cp_2Co results in a mixture of compounds **4** and the 2 e^- reduced product. A 1 e^- reduced product is not observed. To further establish the 4 e^- requirement in the reduction **4** → **3**, the 4 e^- reduced product (**3**) was mixed with a solution of the peroxide complex (**4**). Both species (**3**, **4**) remained unchanged; they do not react.

(22) Loehr, T. M.; Shiemke, A. K. In *Biological Applications of Raman Spectroscopy*; Spiro, T. G., Ed.; Wiley: New York, 1988; pp 439–490.

(23) (a) LaMar, G. N.; Eaton, G. R.; Holm, R. H.; Walker, F. A. *J. Am. Chem. Soc.* **1973**, *95*, 63. (b) LaMar, G. N.; Walker, F. A. *J. Am. Chem. Soc.* **1973**, *95*, 1782. (c) Goff, H.; LaMar, G. N.; Reed, C. A. *J. Am. Chem. Soc.* **1977**, *99*, 3641. (d) Goff, H.; LaMar, G. N. *J. Am. Chem. Soc.* **1977**, *99*, 6599. (e) Collman, J. P.; Brauman, J. I.; Collins, T. J.; Iverson, B. L.; Lang, G.; Pettman, R. B.; Sessler, J. L.; Walter, M. A. *J. Am. Chem. Soc.* **1983**, *105*, 3038.

(24) Collman, J. P.; Brauman, J. I.; Doxsee, K. M.; Halbert, T. R.; Bunnenberg, E.; Linder, R. E.; LaMar, G. N.; DelGaudio, J.; Lang, G.; Spartalian, K. *J. Am. Chem. Soc.* **1980**, *102*, 4182.