A Functional Model Related to Cytochrome c Oxidase and Its Electrocatalytic Four-Electron Reduction of O_2

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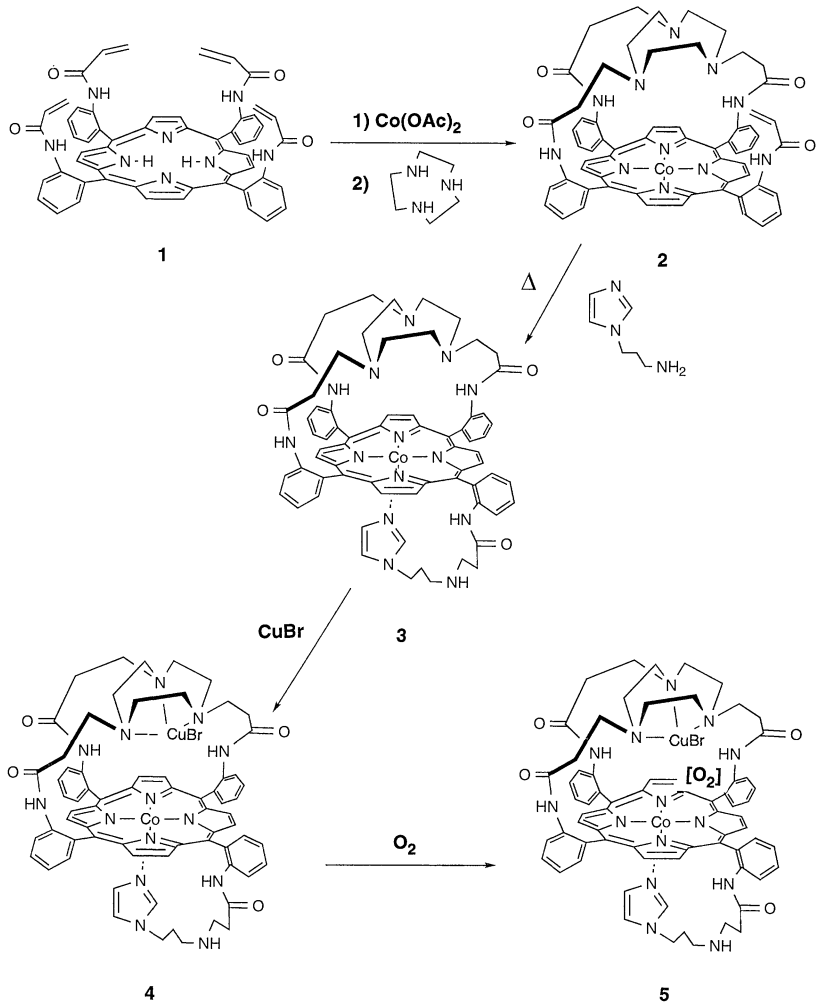
A cytochrome c oxidase model that consists of a cobalt(II) porphyrin with a copper(I) triazacyclononane macrocycle fastened on the distal face and an imidazole covalently attached to the proximal face has been synthesized and characterized. Redox titrations with molecular oxygen (O_2) and cobaltocene were carried out, and O_2 was found to bind irreversibly in a 1:1 ratio to the model compound. This O_2 adduct (a bridged peroxide) can be fully reduced to the deoxygenated form with four equivalents of cobaltocene. The model compound was adsorbed on an edge-plane graphite electrode, and rotating ring-disk voltammetry was used to monitor the electrocatalytic reduction of O_2. Four-electron reduction of O_2 was observed at physiological pH.

Oxidative phosphorylation, a process required by all respiring organisms, involves four-electron ($4e^-$) reduction of O_2 to H_2O by the cytochrome c oxidase enzyme family (*1–4*). The active site of cytochrome c oxidase has recently been structurally defined by x-ray diffraction (*5*). These structures demonstrate that, as previously surmised from indirect evidence, the O_2 binding-activating site in cytochrome c oxidase is composed of a myoglobin-like heme (heme a_3) and a copper atom (Cu_B) coordinated to three imidazoles from histidine residues on the "distal" side.

Many issues still remain controversial, however, namely the role of Cu in O_2 binding and the nature of the O_2-bound intermediates that may be present during the catalytic cycle. The mechanism by which cytochrome c oxidase effects the $4e^-$ reduction of O_2 is not fully understood. Most investigators have proposed that the cycle passes through a peroxide intermediate—the result of two sequential $1e^-$ reductions of O_2 as it binds to Fe (*2, 3*). There is disagreement concerning whether peroxide is bound only to Fe or bridges both Fe and Cu. Time-resolved resonance Raman spectroscopy has failed to identify bound peroxide during catalytic turnover (*6*). It is essential that neither superoxide nor peroxide leak during catalytic reduction; these partially reduced O_2 species are toxic. The limiting reduction potential of the biological system, 0.3 V versus the normal hydrogen electrode (NHE), is controlled by the reductant, cytochrome c, and this potential is near the thermodynamic potential connecting O_2 with H_2O_2 at pH 7. If the two metal centers have an affinity for peroxide similar to that of protons, the enzyme would waste little energy getting to the peroxide stage. Subsequent reduction of H_2O_2 to water (0.8 V) at physiological pH is exergonic and provides energy for the formation of adenosine triphosphate and the generation of heat, which are the primary functions of this enzyme (*7*).

These unresolved issues are the focus of our study of synthetic models that mimic O_2 activation and the catalytic overall $4e^-$, $4H^+$ reduction performed by cytochrome c oxidase (*8–11*). We have developed a Co(II) porphyrin with a Cu(I) triazacyclononane macrocycle fastened on the distal face and an imidazole covalently attached to the proximal face. This model compound has structural and functional features similar to those of the O_2 binding site of enzymes in the naturally occurring oxidase family. In our complex, both metals are redox-active and can bind O_2. We describe here the synthesis of this synthetic model (**4**, Scheme 1), characterize its O_2 adduct, and demonstrate a stoichiometric cycle that reduces O_2 by $4e^-$. We also report a catalytic $4e^-$ reduction of O_2 to H_2O at physiological pH when **4** is adsorbed on an edge-plane graphite (EPG) electrode.

This cytochrome c oxidase model com-



Scheme 1. Scheme for the synthesis of the oxy cytochrome c oxidase model (**5**) from the Michael acceptor (**1**). Cobalt acetate [$Co(OAC)_2$] is added before the triaza cap to place cobalt(II) selectively into the porphyrin. Only then is copper inserted in the cap to form the bimetallic deoxy cytochrome c oxidase model (**4**). This is oxygenated to form **5**, irreversibly.

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pound was synthesized by the "congruent multiple Michael addition" method (*12*) according to Scheme 1. The Michael acceptor, **1**, was metallated with Co and then combined with 1,4,7-triazacyclononane to form **2**, which was isolated by chromatography and then allowed to react with 1-(3-aminopropyl) imidazole to form a covalently linked imidazole-tailed compound, **3**. The model compound, **4**, was then obtained by the metallation of **3** with CuBr. The method of synthesis and the chemistry exhibited by **4** are completely consistent with the proposed structure (*13*).

The Co(II)-Cu(I) complex, **4**, strongly binds O_2 in a precise 1:1 stoichiometry (Fig. 1). We believe that this very stable oxygenated adduct, **5**, contains a bridging peroxide on the basis of three lines of evidence. First, this very stable adduct, **5**, exhibits a single spot on thin-layer chromatography (TLC); its mass spectrum shows a single parent ion 32 mass units above that of the deoxygenated complex, **4**. Second, the oxygenated adduct, **5**, exhibits a peroxide-like $^{16}O_2$ infrared (IR) band at 804 cm^{-1} ($^{18}O_2$ 756 cm^{-1}). Third, quantitative reduction with four equivalents of the $1e^-$ reducing agent cobaltocene (Cp_2Co) cleanly regenerates the deoxygenated Co(II)-Cu(I) complex, **4**.

The ease of binding of O_2 to **4** was demonstrated by the lack of displacement of bound O_2 under a continuous purge with pure Ar. This reactivity differs dramatically from that of the Cu-free compound (**3**), which shows reversible O_2 binding that results in a Co-myoglobin–O_2-like adduct. The difference in reactivity between **3** and **4** demonstrates that the Cu plays a crucial role in O_2 binding to **4**.

It is notable that the Cp_2Co spectral titration exhibits an isosbestic point between the band maxima for **4** (at 418 nm) and **5** (at 444 nm). Apparently the first electron reduction is rate-limiting because no intermediate redox states were observed. This titration also suggests that the intermediates are both kinetically and thermodynamically unstable with respect to the bridged peroxide **5** and the deoxy complex **4**. We repeated the quantitative titration sequence (i) 1 O_2 and (ii) 4 Cp_2Co four times, and each time the sequence showed the identical spectral peaks and isosbestic point.

When adsorbed on an EPG electrode, the bimetallic complex **4** catalyzes the $4e^-$ reduction of O_2 in aqueous solution at pH 7.3. We demonstrated this overall catalytic $4e^-$ reduction by using rotating ring-disk voltammetry (*14*). Between 0.3 and 0.16 V versus NHE, at pH 7.3 O_2 is catalytically reduced at the graphite disk; the Pt ring shows no evidence of H_2O_2 as a by-product. The intermediacy of free H_2O_2 in a two-step process was also ruled out by an experiment that demonstrated that the adsorbed catalyst does not reduce H_2O_2 under N_2. The $4e^-$ reduction was further confirmed by the slope of a Koutecky-Levich plot (*15*) (Fig. 2). These results are consistent with the Cp_2Co titration described above.

Whereas a direct $4e^-$ reduction pathway is followed at pH 7.3, a $2e^-$ pathway occurs in acidic solution below pH 1. This difference is probably due to the protonation of the imidazole tail at low pH. In our experiments with **4** on EPG at lower, more reducing potentials, some H_2O_2 was produced; our earlier $4e^-$ catalysts exhibited a similar phenomenon (*9*).

Both the redox-active Cu complex and an internal axial ligand are required by the $4e^-$ reduction of O_2; separate experiments—one in which we used the Cu-free complex **3** and another in which we used a Co-Cu complex lacking the appended imidazole ligand—exhibited only $2e^-$ reduction of O_2 to H_2O_2. This result strongly suggests that Cu and a bridging peroxide intermediate are directly involved in the catalytic $4e^-$ reduction of O_2 by compound **4** (*16*).

There are striking parallels between cytochrome c oxidase and the functional model **4**. Both natural and synthetic systems effect $4e^-$ reduction of O_2 at physiological pH; neither leaks H_2O_2 during catalytic O_2 reduction. Each has two, different, redox-active, neighboring metal centers that can collectively bind and reduce O_2 by $2e^-$. Both the natural Fe and synthetic Co porphyrins are fitted with a "proximal" imidazole, which directs O_2 binding to the "proximal," intermetallic face. It has been proposed that the enzyme passes through a peroxide stage, although the observation of a peroxide intermediate in natural systems is controversial (*2*, *3*). The model **4** forms a bridged peroxide complex, **5**, which is undoubtedly a catalytic intermediate. In the enzyme, addition of the third electron is thought to induce peroxide O–O bond cleavage; similarly, the model-bridging peroxide is decomposed on addition of $1e^-$. In neither case is the rate-limiting step during catalysis known. The model complex **4** has Co rather than Fe, but the behavior of Co is known to be similar to that of Fe in binding and activating O_2. The present study is an important first step in developing models from which the catalytic function of cytochrome c oxidase may be inferred.

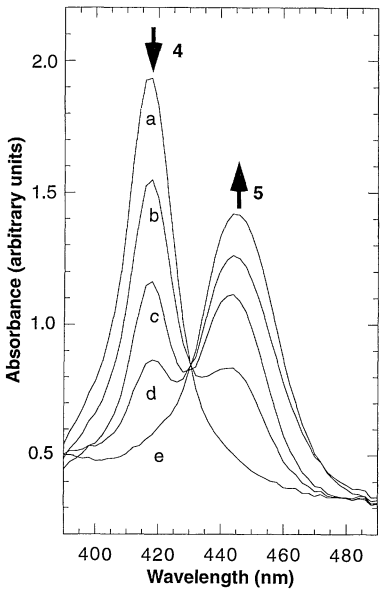

Fig. 1. Titration of O_2. In the titration, measured quantities of pure O_2 were added by syringe to a sealed flask containing 3×10^{-6} M **4** in toluene. Curve a, No O_2 added; curve b, total of 1/4 equivalent of O_2 added; curve c, total of 1/2 equivalent of O_2 added; curve d, total of 3/4 equivalent of O_2 added; curve e, total of 1 equivalent of O_2 added.

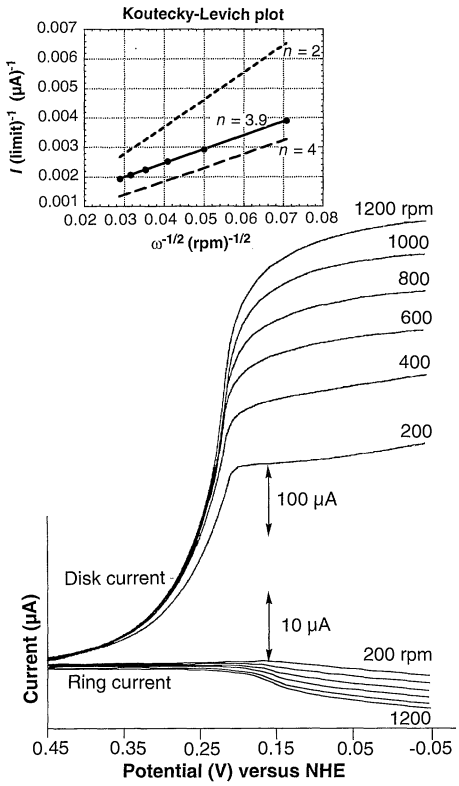

Fig. 2. The $4e^-$ reduction of O_2 in a pH 7.3 buffer solution. Buffer solution, 0.1 M $NaClO_4$ + 0.025 M phosphate buffer; disk area, 0.46 cm^2; scan rate, 100 mV/s; collection efficiency, 0.12; ring potential, +1.1 V versus NHE. The disk potential where limiting currents were measured was 0.15 V versus NHE. The ring and disk current scales are different. In the Koutecky-Levich plot, ω refers to the rotation rate of the EPG electode, I is the limiting current at a disk potential of 0.15 V, and n is the number of electrons involved in the reduction of O_2.

REFERENCES AND NOTES

1. For recent reviews on cytochrome c oxidase, see G. Palmer, *J. Bioenerg. Biomembr.* **25**, 145 (1993), and (*2–4*).

2. G. T. Babcock and M. Wikström, *Nature* **356**, 301 (1992).
3. B. G. Malmström, *Chem. Rev.* **90**, 1247 (1990).
4. ———, *Arch. Biochem. Biophys.* **280**, 233 (1990); S. I. Chan and P. M. Li, *Biochemistry* **29**, 1 (1990); R. A. Capaldi, *Annu. Rev. Biochem.* **115**, 569 (1990). For a general reference, see L. Stryer, *Biochemistry* (Freeman, New York, ed. 4, 1995), pp. 529–558.
5. T. Tsukihara *et al.*, *Science* **269**, 1069 (1995); S. Iwata, C. Ostermeier, B. Ludwig, H. Michel, *Nature* **376**, 660 (1995); T. Tsukihara *et al.*, *Science* **272**, 1136 (1996).
6. R. S. Blackmore, C. Greenwood, Q. H. Gibson, *J. Biol. Chem.* **266**, 19245 (1991); W. H. Woodruff *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **88**, 2588 (1991); M. Oliveberg and B. G. Malmström, *Biochemistry* **31**, 3560 (1992); T. Ogura *et al.*, *J. Am. Chem. Soc.* **115**, 8527 (1993).
7. G. B. Jameson, in *Metal-Containing Polymeric Materials*, C. Carraher, M. Zeldin, J. Sheats, B. Culbertson, C. U. Pittman Jr., Eds. (Plenum, New York, 1996), pp. 421–468.
8. We have invented and examined a series of metal porphyrin catalysts that effect overall $4e^-$, $4H^+$ reduction of O_2 to 2 H_2O when adsorbed onto an EPG electrode (*9, 10*). Indirect evidence suggests that there is an essential association between the metal and an uncharacterized ligand derived from the EPG electrode (*11*). Hitherto, none of these oxygen electrode catalysts have contained an intramolecular axial ligand or an electroactive Cu ion. Furthermore, electrocatalytic $4e^-$ reduction of O_2 by these synthetic compounds has been limited to a pH below 3.5 and occurred at a limiting potential close to that connecting O_2 with H_2O_2 at those pH values.
9. For a review, see J. P. Collman, P. S. Wagenknecht, J. E. Hutchison, *Angew. Chem. Int. Ed. Engl.* **33**, 1537 (1994).
10. J. P. Collman, L. L. Chng, D. A. Tyvoll, *Inorg. Chem.* **34**, 1311 (1995).
11. J. P. Collman *et al.*, in preparation.
12. J. P. Collman *et al.*, *J. Am. Chem. Soc.* **116**, 2681 (1994).
13. The previously reported compound **1** (*12*) has been characterized by elemental analysis, ^1H and ^{13}C nuclear magnetic resonace (NMR) spectroscopy, and mass spectrometry (MS). Complex **2** was prepared in two steps; both the metallated intermediate and **2** were shown to be homogeneous by TLC and were characterized by MS. Several examples of this capping reaction were reported in earlier work (*8*). The formation of **3** by a Michael addition and rotation has been modeled for the metal-free analog and characterized by TLC, MS, and ^1H NMR. Metallated complexes **3** and **4** were found to be homogeneous by TLC and have been characterized by MS. The quantitative titration of O_2, converting **4** to **5**, and the quantitative $4e^-$ reduction of **5** to **4** are described in the text, as is the characterization of the bridged peroxide **5**. Addition of more O_2 does not affect **5**, nor does the addition of more Cp_2Co affect **4**.
14. The technique of rotating ring-disk voltammetry permits the quantitative measurement of (unwanted) H_2O_2 production and allows discrimination between the formation of free H_2O_2 as an intermediate or merely as a minor side product. The ring-disk electrode assembly consists of a pyrolytic graphite disk inside a concentric Pt ring. The porphyrin to be tested as a reduction catalyst is applied to the graphite disk by irreversible adsorption. As the assembly is rotated, fresh O_2-saturated electrolyte is drawn vertically toward the disk surface and ejected radially across the disk and ring. The disk potential is controlled by a potentiostat, and the disk current-potential profile records the O_2 reduction process. At the same time, the ring is held at a potential where any H_2O_2 reaching it is rapidly oxidized to O_2. The ring current response thus monitors H_2O_2 production; and the ratio of disk to ring current, normalized for the collection efficiency, defines the relative contributions of the $4e^-$ and $2e^-$ reduction processes.
15. J. Koutecky and V. G. Levich, *Zh. Fiz. Khim.* **32**, 1565 (1958). The slope obtained from the Koutecky-Levich plot closely matches that established from known $4e^-$ catalysts under the same conditions.
16. Our preliminary cyclic voltammetric results with this Cu-Co–imidazole system in tetrahydrofuran under N_2 show two reversible redox couples: Cu(I)-(II) and Co(II)-(III) at a potential $E_{1/2}$ = 0.26 V and 0.6 V versus NHE, respectively. The presence of O_2 should shift both potentials to higher values. Our electrocatalytic reduction of O_2 begins at ~0.3 V versus NHE.
17. Supported by NIH (grant 5R37 GM-17880-26) and NSF (grant CHE9123187-A2). P.C.H. and X.Z. are the recipients of Franklin Veatch fellowships from the Stanford University Chemistry Department. The Mass Spectrometry Facility, University of California, San Francisco, is supported by NIH (grants RR 04122 and RR 01614). We thank D. A. Tyvoll, S. Harford, and M. Rapta for assistance with electrochemical studies and for helpful discussions and T. A. Eberspacher for help with IR studies.

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Enantiomeric Excesses in Meteoritic Amino Acids

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Gas chromatographic–mass spectral analyses of the four stereoisomers of 2-amino-2,3-dimethylpentanoic acid (DL-α-methylisoleucine and DL-α-methylalloisoleucine) obtained from the Murchison meteorite show that the L enantiomer occurs in excess (7.0 and 9.1%, respectively) in both of the enantiomeric pairs. Similar results were obtained for two other α-methyl amino acids, isovaline and α-methylnorvaline, although the α hydrogen analogs of these amino acids, α-amino-*n*-butyric acid and norvaline, were found to be racemates. With the exception of α-amino-*n*-butyric acid, these amino acids are either unknown or of limited occurrence in the biosphere. Because carbonaceous chondrites formed 4.5 billion years ago, the results are indicative of an asymmetric influence on organic chemical evolution before the origin of life.

The origin of homochirality, that is, the almost exclusive one-handedness of the chiral molecules found in terrestrial organisms, is a key problem of the origin of life. Both biotic and abiotic theories of homochirality have been proposed (*1*). According to the former, life was initially based on achiral molecules or racemates, and the use of specific enantiomers came about through evolution. In the latter, a tendency toward homochirality is presumed to have been inherent in chemical evolution, and thus the asymmetry preceded the origin of life.

Meteorites, specifically the carbonaceous chondrites, carry a record of the organic chemical evolution of the early solar system (*2*). It is reasonable to suppose that if some asymmetric process influenced the formation or degradation of organic compounds in the parent molecular cloud, the solar nebula, or the prebiotic solar system, then enantiomeric excesses would have resulted and might still be observable in the organic compounds of carbonaceous chondrites. Evidence for such an effect has been sought in the form of net optical rotation by meteorite extracts (*3*), as well as by directly measuring enantiomer ratios of specific chiral compounds (*4–6*). The results have been either negative or unconvincing, the latter largely because of the suspicion of terrestrial contamination when small excesses of the L enantiomers have been reported in meteoritic amino acids that are also common in the biosphere (*7*). Collectively, these results have given rise to the widely held view that the chiral compounds of meteorites occur as racemic mixtures. In contrast, we report here the detection of enantiomeric excesses in four amino acids indigenous to the Murchison meteorite.

We initially targeted for study 2-amino-2,3-dimethylpentanoic acid (2-a-2,3-dmpa), an amino acid with two chiral centers and, consequently, four stereoisomers (*8*) (Fig. 1). This amino acid meets two important criteria: (i) It is present in the Murchison meteorite (*9*) but has not been reported to occur in terrestrial matter, and (ii) its two chiral centers are resistant to epimerization because one (C-2) lacks a hydrogen atom and the other (C-3) has a methine hydrogen atom of low acidity. Consequently, it is likely that the chiral centers retained their original configurations through the aqueous and mild thermal processing experienced by the meteorite parent body (*10*) and that the original enantiomer ratios have not been compromised by contamination.

We synthesized 2-a-2,3-dmpa in the laboratory as a mixture of the four stereoisomers (*9*) and analyzed them individually by gas chromatography–mass spectrometry (GC-MS) of their *N*-fluoroacyl isopropyl esters on Chirasil-L-Val and Chirasil-D-Val capillary columns. The four stereoisomers are well resolved on both phases (Fig. 2), although this requires the use of *N*-pentafluoropropionyl (PFP) isopropyl esters with the L phase and *N*-trifluoroacetyl (TFA) isopropyl esters with the D phase. The two diastereomeric pairs were separated on Chirasil-L-Val but overlap

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