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Understanding the reaction that powers this world: Biomimetic studies of respiratory O_2 reduction by cytochrome oxidase*

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Abstract: Cytochrome *c* oxidase (CcO) is an enzyme that catalyzes efficient, selective, and fast reduction of molecular oxygen to water as a means of respiratory energy generation. The biomimetic approach provides a valuable alternative to traditional biochemical methods to unravel the structural and electronic properties of the CcO's catalytic (heme/Cu_B) site that endow the enzyme with its unique reactivity. However, the contribution of biomimetic studies of CcO to our understanding of CcO's biochemistry has been complicated by the lack of convincing evidence that the reactivity of the biomimetic analogs is relevant to that of CcO. Recently reported porphyrin-based compounds are the first analogs that reproduce key aspects of the reactivity of CcO toward O_2. Extensive data collected with these biomimetic analogs demonstrate that the bimetallic nature of the CcO's catalytic site may be an adaptation to reduction of O_2 under turnover-limiting electron flux; a monometallic heme-only site appears sufficient for rapid O_2 reduction under physiologically relevant conditions of pH and electrochemical potential, provided that electron flow to the heme is not kinetically limited. These biomimetic data suggest that in CcO the distal Cu ion (Cu_B) may serve as an electron-preloading site to allow the enzyme to accumulate a sufficient number of external reducing equivalents before it even binds O_2. This mechanism minimizes the population of enzymatic species containing partially reduced oxygen species.

WHY O_2 REDUCTION IS SO IMPORTANT IN OUR WORLD

Reduction of molecular oxygen (O_2) to "oxides" (including water), either via sequential delivery of four electrons and four protons (electrochemical reduction), or via a series of atom transfers, truly makes this world go round. Aerobic metabolism, wherein molecular oxygen serves as the terminal electron acceptor in respiration, is the most efficient terrestrial form of energy metabolism [1–2]. Technology would be impossible without aerobic combustion as the major means of obtaining energy in a form (heat) that we can convert into useful work.

There is enormous and ever-expanding literature devoted to understanding the reactivity of O_2 from a fundamental viewpoint, in the context of aerobic biochemistry and in technological applications. I know of no convincing examples for reduction of O_2 into oxides that bypass any partially reduced intermediates. These intermediates are extremely important in determining O_2 chemistry. Whereas molecular oxygen is thermodynamically a powerful four-electron oxidant, under ambient conditions it is kinetically inert. The substantial kinetic barrier to O_2 reduction originates from its being a weak nucleophile, a poor H-atom abstractor [3] and a weak 1e reductant, particularly in neutral aqueous solution.

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Even though one-electron reductions of the other dioxygen species (O_2^-/HO_2 and H_2O_2) are thermodynamically favorable, they suffer from high intrinsic barriers to charge transfer; 2e reduction (atom transfer) of H_2O_2 requires extensive bond rearrangement [3].

$$O_2 + H^+ \rightleftharpoons HO_2^+ \qquad \Delta G^o \sim 95 \text{ kcal/mol, K} \sim 10^{-70} \text{ (est.)}^*$$

$$O_2 + {}^1\!/_2H_2 \rightleftharpoons HO_2 \qquad \Delta G^o = 3 \text{ kcal/mol, K} = 6 \times 10^{-3}$$

$$O_2 + e^- \rightleftharpoons O_2^- \qquad \Delta G^o = 8 \text{ kcal/mol, K} = 3 \times 10^{-6}, \Delta E^o = -0.33 \text{ V (vs. NHE)}^{**}$$

The biosphere benefits greatly from this inertness of O_2 as it allows the existence of highly reduced organic matter in an atmosphere rich in a powerful oxidant. But such inertness also means that rapid aerobic oxidation will occur only if energy is put into the system to overcome the intrinsic kinetic barriers, or the reaction is catalyzed (i.e., the kinetic barriers are lowered by stabilizing otherwise high-energy intermediates). The energy of the reactants can be increased by raising the temperature, and indeed combustion is the most technologically important means for the utilization of the oxidizing potential of O_2. Combustion proceeds via oxygen atoms, which are produced in dissociation of O_2 at sufficiently high temperatures [4]. This pathway, however, is inaccessible to living organisms due to their tolerance of only a narrow temperature range. As a result, organisms activate O_2 at ambient con-

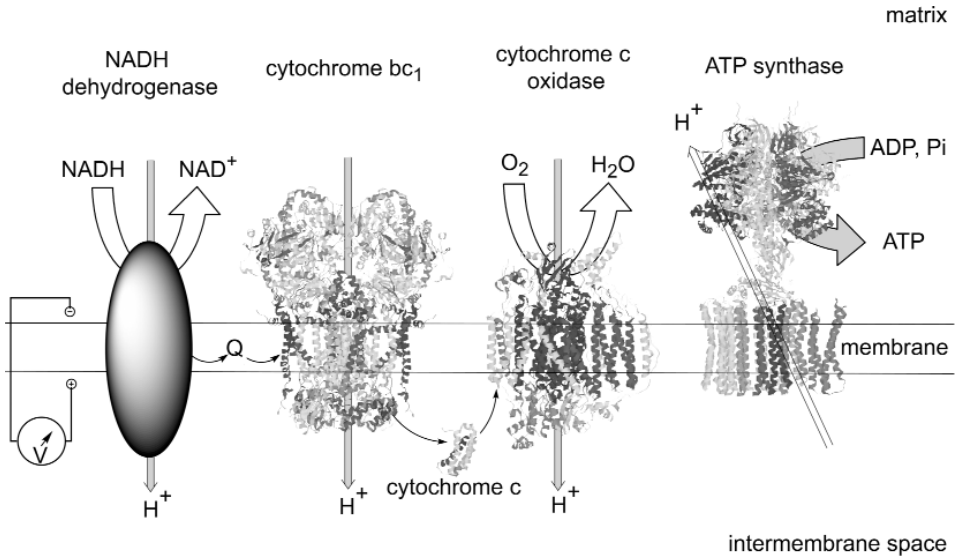

Fig. **1** The mitochondrial respiratory electron-transfer chain and ATP synthase. White and gray arrows depict spontaneous (favorable or exergonic) and nonspontaneous (unfavorable or endergonic) processes, respectively. The electron-transfer path is shown by narrow arrows. Q represents the quinone pool. NADH dehydrogenase, cytochrome bc_1 complex, and cytochrome *c* oxidase maintain the electrochemical transmembrane gradient (depicted by the voltmeter) by coupling the thermodynamically unfavorable H^+ transfer to spontaneous oxidation of NADH, quinol, and ferrocytochrome c, respectively. The electrons are ultimately removed from the chain by reducing O_2 to H_2O. A spontaneous relaxation of the electrochemical H^+ gradient through ATP synthase powers the energetically uphill synthesis of ATP. Representations of cytochrome bc_1, cytochrome c, CcO, and ATP synthase were generated from coordinates deposited in PDB (codes: 1BGY [5], 1A7V [6], 1OCC [7], 1C17 (F0) [8], 1E79 (F1) [9]).

* The thermodynamic values are referenced to 1 atm partial pressure or 1 M aqueous solution at 298 K for gases and H^+, HO_2^+, HO_2, respectively, as the standard states.

** The values are for neutral (pH 7) solution in equilibrium with 1 atm partial pressure of O_2.

ditions relying on metalloenzymes to lower the energies and the reactivities of partially reduced oxygen intermediates (such as O_2^-, H_2O_2, and •OH) to accelerate, and to increase the efficiency of, O_2 reduction, and to prevent generation of these very reactive species in a vicinity of reduced organic substances, such as proteins, lipids, and nucleic acids.

In mammals, 90 % of consumed molecular oxygen is reduced to H_2O by a mitochondrial-membrane enzyme, cytochrome *c* oxidase [1]. This enzyme belongs to the superfamily of heme/Cu terminal oxidases [10] and is the final (terminal) enzyme of aerobic respiration. Foods that we consume undergo anaerobic catabolism [1], generating highly reducing electron carriers, such as NADH. Spontaneous electron transfer from more- to less-reducing electron carriers in the mitochondrial electron transfer chain of eukaryotes maintains the protonmotive force (pmf), a charge and proton-concentration gradients across the inner mitochrondrial membrane (Fig. 1). The pmf, which is a biological equivalent of a river just upstream of a dam, is utilized to power endergonic synthesis of ATP from ADP and inorganic phosphate. For the chain to operate continuously, the electron-accepting state of each electron carrier must be constantly regenerated. Cytochrome *c* oxidase catalyzes the regeneration (oxidation) of the least-reducing electron carrier, cytochrome c, by molecular oxygen, thereby completely removing low-energy (spent) electrons from the electron-transfer chain in the form of H_2O.

$$O_2 + 8H^+_{\text{matrix}} + 4\,\textit{ferro}\text{-Cc} \rightarrow H_2O + 4H^+_{\text{IMS}} + 4\,\textit{ferri}\text{-Cc}$$

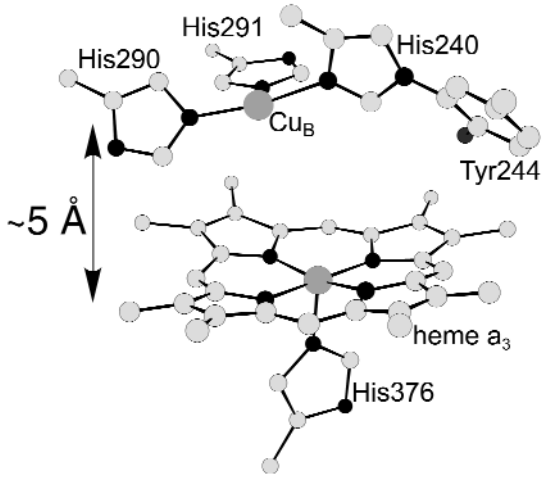

Fig. 2 The catalytic, O_2-reducing, site of CcO from the X-ray structure of bovine heart CcO [7]. The peripheral substituents of the heme are omitted for clarity. C atoms are light gray, O and N atoms are black, and Fe and Cu ions are dark gray.

CcO is a large multi-subunit enzyme containing four redox-active centers. The binuclear Cu_A and six-coordinate heme a centers, both of which contain coordinatively saturated metal ions, are involved in the transport of electrons from ferrocytochrome c to the catalytic site of CcO, the heme a_3/Cu_B site (Fig. 2) [11]. The latter consists of a 5-coordinate, imidazole-ligated heme (an Fe protoporphyrin derivative [12]), which is common in O_2 biochemistry, and an unusual, tris-imidazole coordinated Cu ion [7]. At the Fe···Cu distance of ~5 Å, a bridging diatomic ligand, such as O_2^{2-}, can be accommodated, but whether such a species ever forms during catalytic turnover is highly controversial [11]. A covalent link between one of the Cu-coordinating imidazoles and a phenol of a tyrosine is formed post-translationally [12], and is speculated to arise from a phenoxyl radical generated during the first turnover of the enzyme [13].

Dioxygen reduction by CcO is (1) highly selective toward complete 4e reduction (as opposed to incomplete reduction to O_2^-, H_2O_2, and/or •OH, i.e., partially reduced oxygen species), (2) efficient (e.g., the fraction of the free energy of reduction lost as heat relative to that used to maintain the pmf is small), and (3) fast. The importance of minimizing the release of partially reduced oxygen species during catalytic O_2 reduction lies both in maximizing the energy efficiency of the reduction as these species all have $\Delta G^o_f > 0$ and in minimizing the collateral damage as they rapidly oxidize and oxygenate biological matter [14]. The selectivity of O_2 reduction by CcO is not known precisely, but it is probably >99 %. About 1–2 % of O_2 consumed by a normally functioning mitochondrion is converted into O_2^-; however, most, if not all, of it comes from a reaction between O_2 and highly reducing electron carriers, such as NADH (see Fig. 1), and not from CcO [1].

Under biologically relevant conditions, the reversible potential of the O_2/H_2O couple is ~800 mV (all potentials are cited vs. the normal hydrogen electrode, NHE), whereas CcO uses electrons at ~250 mV (the $Fe^{III/II}$ potential of cytochrome c) to reduce O_2. (The functionally and structurally related ubiquinol oxidase, which couples oxidation of ubiquinol to reduction of O_2 operates at ~50 mV [15].) However, in contrast to a cathode of a fuel cell, wherein the overpotential (~550 mV or ~50 kcal/mol) is wasted as heat, CcO utilizes a portion of this overpotential (~25 kcal/mol O_2) to translocate 4 protons per each O_2 reduced across the inner membrane, performing work against the pmf [16,17]. The rest is released as heat, keeping us warm.

In vivo, the turnover frequency (TOF) of CcO is limited by the frequency of collisions between CcO and its biological electron donor, ferrocytochrome c, which diffuses freely in the transmembrane space (Fig. 1). Under normal physiological conditions, cytochrome c receives and donates an electron every 5 to 20 ms [1]. In contrast, single-turnover experiments indicate that CcO, containing fully reduced heme/Cu_B site, binds O_2 and reduces it to the redox level of water within ~170 μs (compound R to P_M, Fig. 3) [15,18,21,22]. As a result, under normal physiological conditions, CcO appears to operate under a constant dearth of electrons. Hence, one of the more fascinating questions of CcO bio-

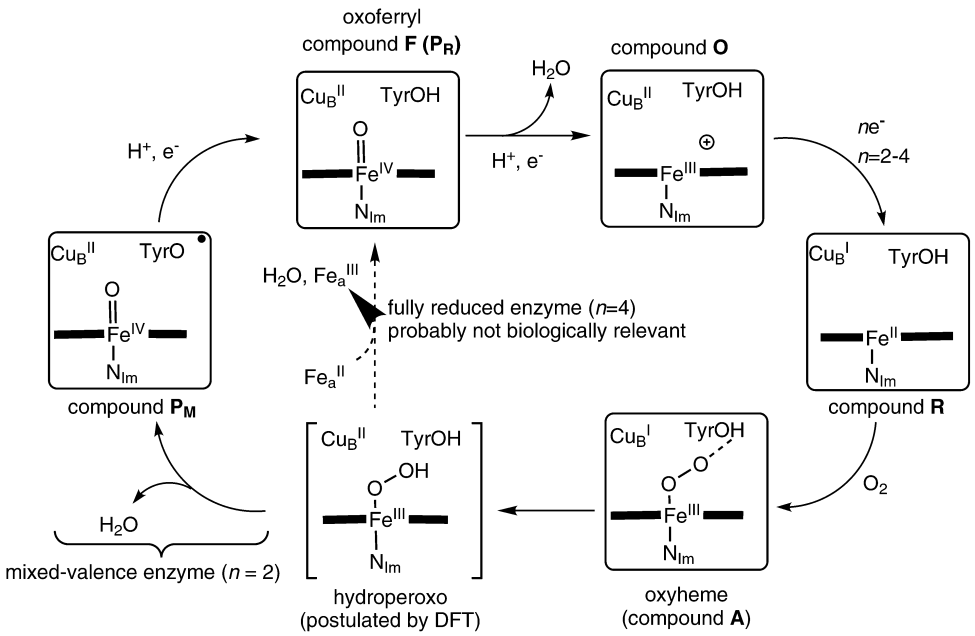

Fig. 3 The plausible mechanism of O_2 reduction at the heme/Cu_B site in CcO as a function of the initial redox state of the enzyme; shown are the spectroscopically observed intermediates in single turnover experiments with their names [11,17,18] and the ferric-hydroperoxo species whose intermediacy was proposed from DFT calculations [19,20]. See Fig. 2 for the structure of the catalytic site. TyrOH is tyrosine 244.

chemistry is the identification of mechanisms, as well as chemical and structural features that enable the enzyme to affect a multielectron reduction on a time scale 1–2 orders of magnitude faster than the arrival of external reducing equivalents.

Dioxygen reduction by CcO has been studied extensively, and we understand some of the strategies that allow the enzyme to perform the feat of rapid, complete, and efficient O_2 reduction complicated by slow electron arrival and excess of O_2. One strategy involves the dependence of the electron affinities of CcO's redox cofactors on the overall redox state of the enzyme [23], which prevents O_2 binding to CcO until the enzyme has accumulated enough electrons from ferrocytochrome c to reduce O_2 to "oxides". Dioxygen is an unusual ligand in that its binding to a metal ion always requires substantial transfer of electron density onto the bound O_2 [10]. Consequently, only Fe^{II} and Cu^{I} bind O_2, whereas Fe^{III} and Cu^{II} are aerobically inert. In both the resting (fully oxidized) and the 1e-reduced forms of CcO, the catalytic site contains Fe^{III} and Cu^{II} ions and the enzyme does not bind O_2. However, as soon as 1e-reduced CcO gets another electron from ferrocytochrome c, the electron affinities of the redox cofactors change so that both electrons localize mainly on the heme/Cu_B site [23] and O_2 binds rapidly to ferroheme a_3 by way of Cu^{I}_{B} [11,18]. Although this "mixed-valence" form of CcO has only two external electrons, it reduces O_2 by four electrons, by temporarily transferring oxidizing equivalents from O_2 to the protein matrix, generating a phenoxyl radical [24] and ferryl (compound P_M, Fig. 3). This mechanism minimizes the lifetime of any catalytic intermediates that have bound partially reduced oxygen species (such as ferric-hydroperoxo, Fig. 3), which may dissociate and damage the cell.

BIOMIMETIC APPROACH TO UNDERSTANDING THE O–O BOND REDUCTION BY CcO

Although spectroscopic studies of the kinetics of O_2 reduction by wild-type and mutant CcO have been extremely valuable in elucidating many aspects of the O_2 reduction mechanism [11], understanding how the most intriguing transformation, reductive cleavage of the O–O bond, occurs has been precluded by the lack of observable intermediates in the corresponding steps (compounds A to P, Fig. 3). Based on DFT calculations [19,20] this step was proposed to proceed via a slow generation of a peroxo-level intermediate (ferric-hydroperoxo/Cu_B^{II} wherein Cu_B^{II} may [20] or may not [19] interact with the distal O atom) followed by rapid reduction of the peroxo O–O bond to the redox level of water with 2e coming from 1e oxidations of (a) the heme (from ferric to ferryl states) and (b) the imidazole-linked tyrosine (to the phenoxyl radical). Such kinetics leads to a vanishingly small steady-state concentration (or very short lifetime in single-turnover experiments) of the peroxo-level intermediate, which makes it spectroscopically invisible. This calculation, therefore, suggests that Cu_B reduces Fe_{a3}-bound superoxide to hydroperoxide by donating a H atom from the Cu_B^{I}-OH_2 moiety. The resulting Cu_B^{II} may or may not facilitate the reduction of the peroxo O–O bond by polarizing the bound hydroperoxide (which would be analogous to the role of the H bond in the catalase mechanism [25]). This sequence must be viewed as only tentative not only because the heme/Cu_B site is quite complex for computational studies, but also because populations of various catalytic intermediates may depend strongly on the availability of proton donors in the vicinity of the catalytic site [26]. The exact number and position of H_2O molecules at and around the catalytic site is not yet known. Several Cu_B-less mutants of heme/Cu terminal oxidases have been prepared and studied in an attempt to probe the role of Cu_B in CcO [27–29]. Whether the lack of the catalytic activity of such mutants is due to the intrinsic inability of the monometallic heme-only site to reduce O_2, or arises from rapid degradation of the Cu_B-less forms remains a fascinating question.

The biomimetic approach provides an alternative to the traditional biochemical studies of this issue [10,30]. With CcO, biomimetic chemists have been mainly concerned with understanding the role of Cu_B in O_2 reduction at the heme/Cu_B site and the existence of bridged peroxo-level intermediates in a stereoelectronic environment similar to that of heme/Cu_B. Biomimetic studies are generally predicated on an assumption that important aspects of the reactivity of a specific enzyme can be reproduced in a much smaller molecule, and in the context of CcO they have developed along one of three routes:

the first two utilize small-molecule synthetic analogs of the catalytic site and the third is based on engineered proteins, such as myoglobin.

In electrocatalytic biomimetic studies [10,30], a heme/Cu_B analog is deposited on the surface of an electrode, which serves as a source of electrons (the ferrocytochrome c analog), and the assembly is exposed to an aqueous buffered electrolyte containing dissolved O_2. This set-up allows studying CcO analogs under both biologically relevant conditions and steady-state turnover. The efficiency, kinetics, and selectivity of the catalysis as well as their dependence on O_2 tension, pH, the presence of an inhibitor, etc. can be quantified. The most comprehensive biomimetic investigation of the role of Cu_B in CcO to date has come from such studies [31–34]. However, spectroscopic characterization of a catalytic system confined to the electrode has not yet been possible.

In a second approach, the O_2 reactivity of a reduced heme/Cu_B analog is studied spectroscopically usually at low temperature and in an aprotic solvent [10,30]. This methodology resembles that of nonaqueous enzymology [35]: by suppressing acid-base reactions and utilizing lower temperatures than are accessible for aqueous media it becomes possible to stabilize intermediates that are too short-lived under biologically relevant conditions for spectroscopic detection or even isolation. This method has allowed isolation and spectroscopic characterization of the first biomimetic analog of compound A (Fig. 3) [33] as well as several Fe^{III}/Cu^{II} peroxo-bridged compounds [10,36,37]. The major limitation of this tactic is the uncertainty as to the extent to which the reactivity of the heme/Cu_B analogs is altered by the nonbiological medium and hence the relevance of the findings to O_2 reactivity of CcO.

The final approach, which involves engineering a heme/Cu_B-like site into a simple protein, such as myoglobin [38], has yet to be widely employed. Such engineered heme/Cu_B sites most closely reproduce the stereoelectronic environment of the Fe-Cu core in CcO; and the proteins can be studied in aqueous solution under homogeneous conditions. However, adequate structural characterization of such analogs is difficult, and the stereochemistry of the engineered catalytic site is often unknown. In addition, delivery of protons and electrons to the catalytic site appears to be a problem, and protein-based CcO analogs have not yet been shown to reduce O_2 under catalytic turnover.

Several aspects of the biomimetic studies of O_2 reduction at the heme/Cu_B site have been recently reviewed [10,30,39]. The present paper is limited mainly to analyzing most recent results (published after 2002) in the context of both their contribution to our understanding of the mechanism of biological O_2 reduction, and the lessons that can be applied to developing more successful strategies for studying enzymatic mechanisms using the biomimetic approach.

Ensuring biological relevance of biomimetic data

Probably the most critical aspect of a biomimetic project aimed at helping to unravel an enzymatic mechanism is to establish that the data obtained with the biomimetic analog are relevant to the enzymatic system. The paradigm of the biomimetic approach is to simplify a biological entity to the point where it can be studied more productively. The simplification is achieved by ignoring structural features of the original system that are deemed unimportant for the realization of a specific reactivity pattern and often by modifying reaction conditions under which the catalysis is studied.

Mechanistic conclusions drawn from studying a biomimetic heme/Cu_B analog can be assumed to be reasonably relevant to CcO if this analog can be demonstrated to reproduce the O_2 reactivity of the heme/Cu_B site. The most critical aspect of such reactivity is the capacity of the heme/Cu_B site to reduce O_2 to H_2O at ~250 mV while converting <1 % of the consumed O_2 into partially reduced oxygen species. Similar $Fe_{a3}^{III/II}$ and $Cu_B^{II/I}$ potentials appear important for this reactivity (see above). The heme/Cu_B site is also unique in the apparent lack of any interaction between O_2 bound at the heme a_3 and Cu_B (compound A, Fig. 3) despite the ~5 Å Fe···Cu distance.

The chemical and structural composition of the heme/Cu_B site is extraordinarily challenging to reproduce in a small molecule. The generally difficult synthetic chemistry of imidazoles is compounded

by the apparently chelating nature of the imidazole ligands at the heme/Cu_B site (Fig. 2): the specific conformation of the protein backbone (rather than direct chemical bonds between the ligands) likely increases significantly the effective affinity of these imidazoles to the metal sites and thus the dissociative stability of the resulting complexes. We have developed a methodology to covalently link both proximal and distal imidazoles to the porphyrin core so as to reproduce this chelating effect (Fig. 4) [40]. As a result of this structural similarity, our heme/Cu_B analogs reproduce the key reactivity of the catalytic site (see below). One structural feature of the heme/Cu_B site yet to be included in a biomimetic analog is the imidazole-phenol assembly, which would dramatically increase both the structural fidelity and the synthetic complexity of the resulting heme/Cu_B analog. Such a molecule is a target in several labs around the world.

Because of the synthetic difficulties posed by chelating imidazole superstructures, several simplified heme/Cu_B analogs have been prepared and studied [10]. These simplified compounds lack the proximal imidazole ligand and/or substitute the tris-imidazole environment of Cu_B with a chelate con-

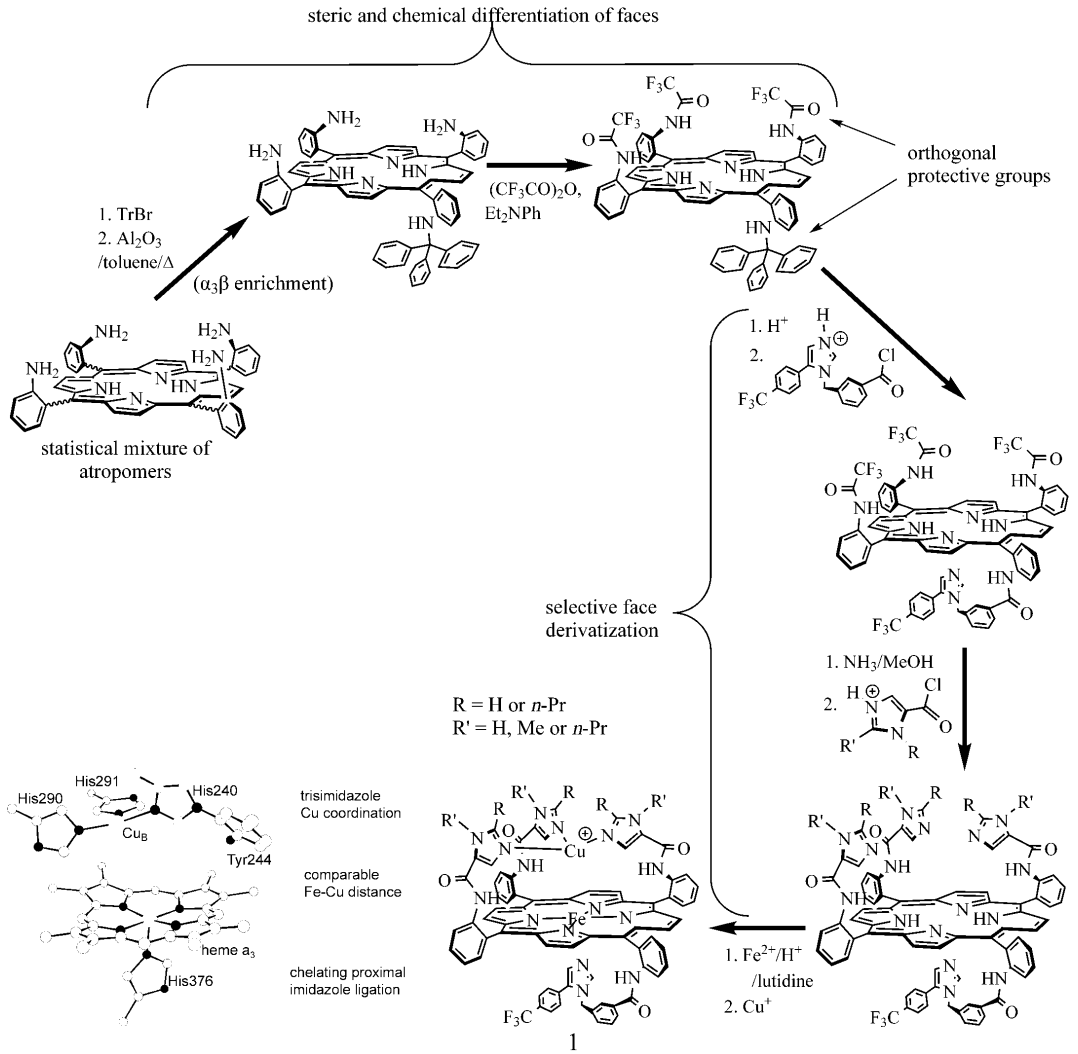

Fig. 4 A general synthetic route to biomimetic heme/Cu_B analogs reproducing the effectively chelating nature of the proximal and distal imidazole ligation of Fe and Cu_B [40]. The structure of the heme/Cu_B site is shown for comparison.

taining aliphatic or pyridine nitrogen donors. In many studies, the imidazole ligation of heme a_3 was approximated by adding pyridine to a solution of the biomimetic analog lacking a covalently linked proximal ligand. Whether this ligand remains coordinated throughout the reaction is not clear. Four-coordinate Fe^{II} centers are even more often used in such work, because elimination of a σ-basic ligand trans to Fe-bound O_2 diminishes the propensity of the dioxygen adducts to undergo irreversible oxidation [10]. The consequences of these simplifications are deviations from the reactivity pattern observed for CcO. For example, under anhydrous conditions, stoichiometric binding of O_2 to simplified heme/Cu_B analogs invariably yields Fe^{III}/Cu^{II} peroxo-bridged adducts [10], not the ferric-superoxo/Cu^{I} form of compound A (Fig. 3). Naruta and coworkers beautifully demonstrated [36] the side-on binding of the O_2^{2-} ligand in such a compound (Fig. 5), which is unprecedented in biological heme/O_2 chemistry. The absence of the chelating proximal imidazole also appears to eliminate catalytic activity of such heme/Cu_B analogs toward O_2 reduction in the physiologically relevant potential range (>50 mV) [10]. In biomimetic electrocatalytic O_2 reduction, the non-imidazole ligation of the distal Cu substantially decreases the lifetime of the heme/Cu_B analog, possibly by inducing homolytic O–O bond cleavage either in a peroxo-level intermediate or free H_2O_2 generated as a byproduct of O_2 reduction [10]. As important as such studies are for understanding the basic dioxygen chemistry of transition-metal ions, particularly Fe^{II} and Cu^{I}, it remains uncertain whether the phenomena observed with such compounds would be expected to exist in CcO.

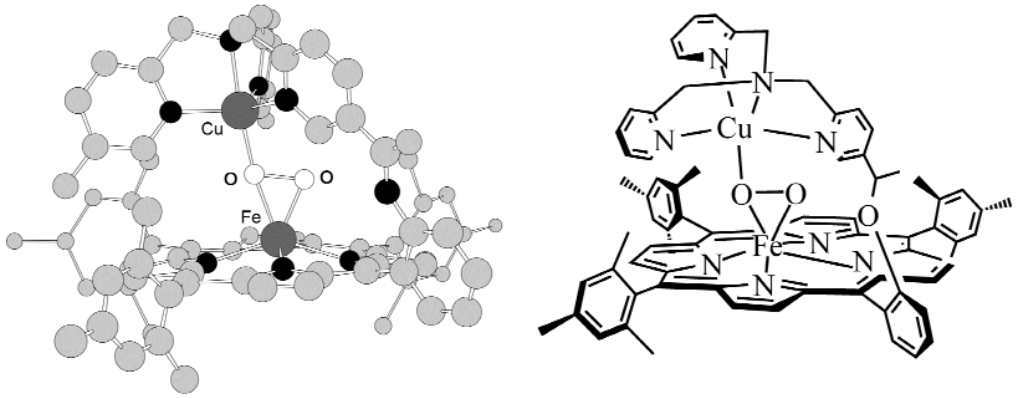

Fig. 5 The crystal and chemical structures of an O_2 adduct of a simplified heme/Cu_B analog, which lacks the proximal imidazole to Fe and the tris-imidazole coordination environment of Cu [36]. Coordinated O_2^{2-} is white; C atoms are light gray, O and N atoms of the ligand are black, and Fe and Cu ions are dark gray. The illustration was generated from coordinates in CCDC.

Biomimetic studies of O_2 reduction by CcO also require a methodology to carry out catalytic O_2 reduction under physiologically relevant conditions of electrochemical potential, pH, and electron flux. Reduction of O_2 by CcO can be treated as a redox half-reaction and as such it is well suited for electrochemical studies, particularly by rotating ring-disk voltammetry [41]. In this method, a water-insoluble biomimetic heme/Cu_B analog is deposited on the disk electrode of the ring-disk electrode, which is subsequently brought into contact with a buffered aqueous electrolyte (Fig. 6). Rotation of the electrode results in a continuous and readily quantifiable flux of reagents (O_2 and H^+) to the disk-confined catalyst as well as transport of the products to the ring electrode. The disk electrode supplies electrons to the catalyst for O_2 reduction (i.e., it fulfills the role of ferrocytochrome c in vivo, or of $Ru(NH_3)_6^{2+}$ in some potentiometric assays of CcO activity in vitro). Disk potential, and hence the potential of the electrons it supplies, is varied. Whereas this has no biological analogy (the potential of ferrocytochrome c is fixed at ~250 mV), studying the catalysis at various potentials is important mechanistically. The ring elec-

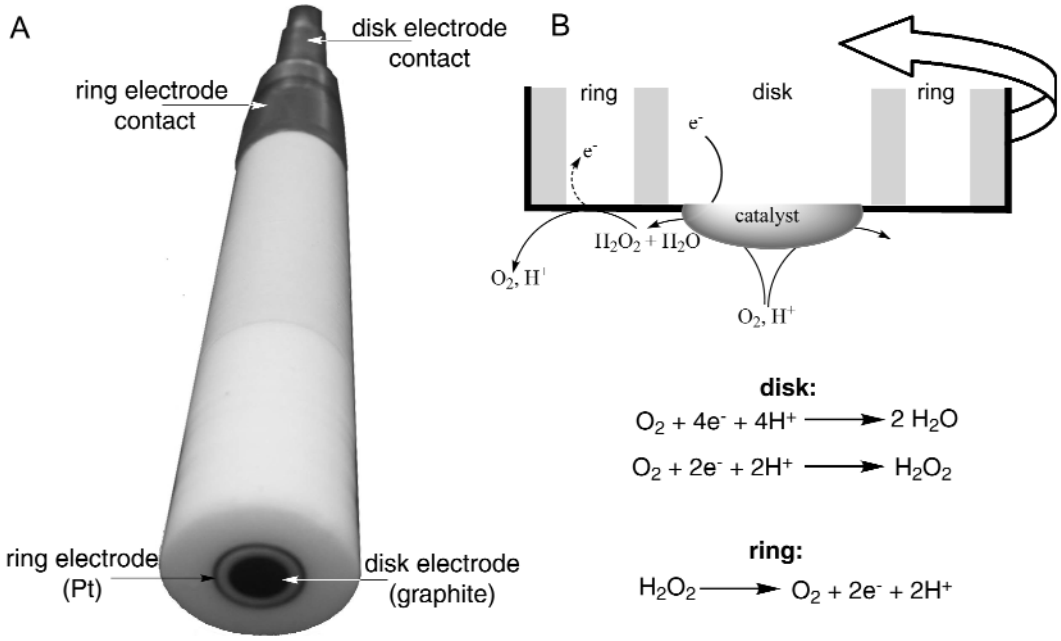

Fig. 6 Rotating ring-disk electrode [41]. **A**. A photograph of a typical RRDE showing the arrangement of the disk and ring electrodes. **B**. A schematic of an RRDE in operation: rotation of the electrode (block arrow) generates a flow of the electrolyte to the electrode, which delivers the reactants (solid arrows) to the disk-confined catalyst. The products of the catalytic reduction are transported to the ring electrode where H_2O_2 and/or O_2^- (if any are produced by the catalysts) are oxidized and the generated current (broken arrow) is measured.

trode is held at a fixed oxidizing potential to rapidly oxidize to O_2 partially reduced oxygen species, such as H_2O_2 and O_2^-, which may be produced by the catalyst. This oxidizing ring current is compared with the disk current to determine the fraction of the electron flux that the catalyst converts into partially reduced oxygen species. With additional data, or assumptions, this quantity can be converted into the fraction of O_2 that the catalyst reduces only to cytotoxic byproducts. The ring-disk electrode provides a unique advantage for studying O_2 reduction by enabling the detection of partially reduced oxygen species in a system where such species have only transient existence due to their susceptibility to disproportionation, and catalytic and stoichiometric reduction. The disk current contains kinetic information about the overall catalytic turnover.

Adsorbing a small amount of pure catalyst at the graphite disk electrode generates a catalytic film wherein electrons distribute rapidly among catalytic sites and the electrode (i.e., the catalyst is in a redox equilibrium with the electrode) [10,31,42]. While this is different from the conditions under which CcO operates, where the turnover is limited by electron arrival, eliminating kinetic limitations of electron transport allows kinetic mechanistic studies of the catalytic reaction itself. Of course, one must be aware that biologically relevant conclusions cannot be drawn from these data alone, and catalysis under physiologically relevant slow electron flux must be examined. We have developed a set-up to reproduce this slow electron flux while utilizing the advantages of the rotating ring-disk electrode. Incorporating a biomimetic analog into a few-μm thick lipid film confined to the disk electrode surface yields isolated freely diffusing catalytic sites [32]. Although this generates a reverse situation in respect to the mitochondrial membrane, with the catalyst mobile and the reductant stationary, the electron flux is still governed by the frequency of collisions between the catalyst and the electron donor (which may be either the electrode or a more reduced form of another catalyst molecule).

Evidence that catalysts 1 reproduce O_2 reactivity of the heme/Cu_B site

To establish that catalysts **1** (Fig. 4) are adequate functional analogs of the heme/Cu_B site, the redox behavior of the complexes directly adsorbed on an electrode was studied in the absence and presence of O_2. The results are summarized and compared with the pertinent data for CcO in Table 1. The reactivity of **1** toward both O_2 and H_2O_2 reduction is comparable to that of CcO. A kinetic mechanism of O_2 reduction catalysis by **1** (Fig. 7) was derived by analyzing the pH dependence of the potential-dependent turnover frequency. This mechanism is unique among any metalloporphyrins yet studied. Most notably, **1** catalyzes the O–O bond reduction by a reaction sequence that is similar to that proposed for CcO, wherein a formally ferric-peroxo intermediate is generated in the turnover determining step. This is significant because: (1) such kinetics ensure highly selective 4e reduction as the steady-state concentration of the ferric-hydroperoxo intermediate, which is the most likely source of H_2O_2 in metalloporphyrin-catalyzed O_2 reduction, is low; and (2) it is the first metalloporphyrin system wherein 2e reduction of the peroxo intermediate is faster than the preceding 1e reduction of the bound superoxide [10]. Both the presence of a σ-basic proximal ligand and of a distal environment capable of polarizing the O–O bond are known to be important in accelerating the rate of the O–O bond reduction in ferriheme-peroxo systems [25]. The facile O–O bond reduction by **1** suggests that these biomimetic catalysts adequately reproduce the distal and proximal stereoelectronic environment of the heme/Cu_B site.

Table 1 Reactivity of biomimetic analogs **1** (Fig. 4) toward O_2 and H_2O_2 and the relevant data for CcO.

	FeCu[a]	CcO[b]
E^o, mV (no substrate)	120	≥250[c]
% of the 4e O_2 reduction pathway	>96–98	>99 (?)
TOF of O_2 reduction at 250 mV, s^{-1}	>2	>50
Intermediate generated in the turnover-limited step	(por)Fe^{III}-OOH	(por)Fe^{III}-OOH (?)[d]
$(k_{app})_{max}$ of O_2 reduction, $M^{-1}s^{-1}$	1.2×10^5	$1.5 - 2.5 \times 10^5$
k_{O_2}, $M^{-1}s^{-1}$	$>1.5 \times 10^7$	$1 - 3 \times 10^8$
Redox state of the O_2 adduct	$Fe^{III}(O_2^-)/Cu^I$ [33]	$Fe^{III}(O_2^-)/Cu^I$
k_{app} of H_2O_2 reduction at 450 mV, $M^{-1}s^{-1}$	800	700 [17]
$(k_{app})_{max}$ of H_2O_2 reduction, $M^{-1}s^{-1}$	1.7×10^4	2×10^4 [e]

[a]Data from ref. [31] unless indicated otherwise.
[b]Data from ref. [11] unless indicated otherwise.
[c]The heme/Cu_B potential depends both on the site's protonation state [26] and on the redox states of heme a and Cu_A [23] and it may be higher or lower than indicated.
[d]Based on DFT calculations [19,20].
[e]Single-turnover experiment, ref. [17].

Both the $Fe^{III}Cu^{II}/Fe^{II}Cu^I$ redox potentials (under anaerobic conditions) and the TOF at potentials >50 mV of catalysts **1** are lower than those of the heme/Cu_B site. This was suggested to originate from the more polar, hydrophilic environment in which **1** were studied relative to the surroundings of the heme/Cu_B site [33]. The more polar environment stabilizes the oxidized, charged Fe^{III}/Cu^{II} state making reduction of the catalysts to the active $Fe^{II}Cu^I$ state less favorable. The higher water content increases the population of the 6-coordinate, aqua-ligated (por)Fe^{II}-OH_2 intermediate. Both processes decrease the activity of the catalysts in the physiologically relevant potential range by suppressing the steady-state concentration of the catalytically active, 5-coordinate, (por)$Fe^{II}\cdots Cu^I(Im)_3X$ form (X is an exogenous ligand, if any). This hypothesis was supported computationally [42] as well as by the electrochemical behavior of **1** in a hydrophobic lipid membrane [32].

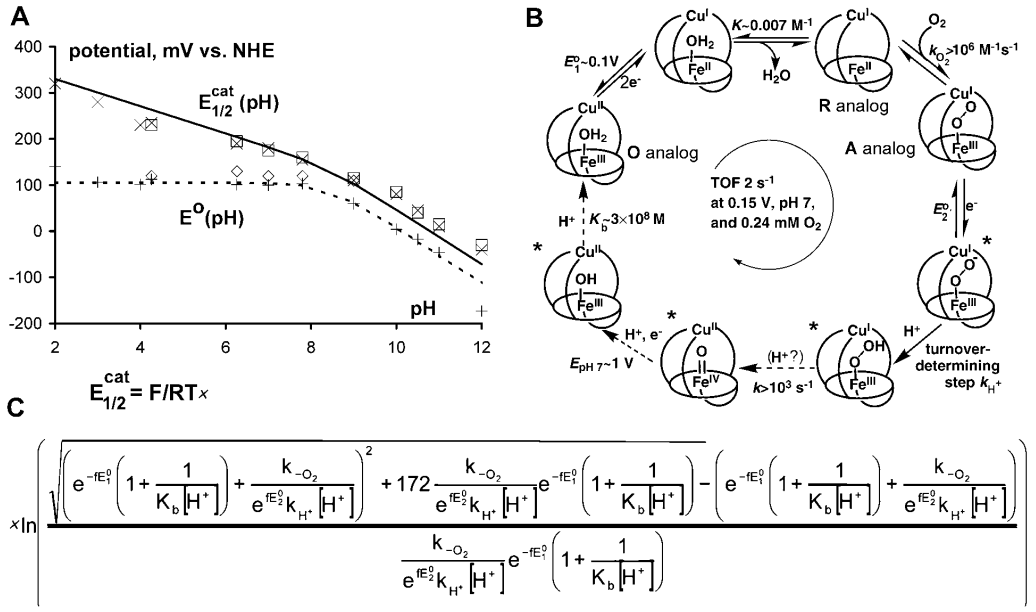

Fig. 7 The mechanism of O_2 reduction by biomimetic catalysts **1** (Fig. 4). **A**. The pH dependence of the standard potentials in the absence of O_2, E^o (◊, the $Fe^{III}Cu^{II}/Fe^{II}Cu^{I}$ potential of the FeCu complex; +, the $Fe^{III/II}$ potential of the Cu-free analog), and of the catalytic half-wave potentials, $E^{o}_{1/2}$ (□, the FeCu catalyst; ×, the Cu-free catalyst,). Note that the dependences are identical for both the Cu-free and FeCu catalysts. The least-squares fit (LSF) of the E^o vs. pH data (**A**, broken line) indicates a water molecule (with pK_a ~8.4) as an exogenous 6th ligand at the Fe^{III}. The $E^{o}_{1/2}$ vs. pH data reveals two reversible electron-transfer steps and a protonation as components of the turnover-determining part of the catalytic cycle. The mechanism in **B** is most consistent with these data (analogs of compounds O, R, and A of the CcO cycle, Fig. 3, are indicated). Based on these data, a rate law was derived (**C**) which fits well the experimental data (**A**, solid line) using a single adjustable parameter, $k_{-O_2}/e^{fE^0_2}k_{H^+}$ (F and R are the Faraday and gas constants, respectively; other symbols are defined in **B**). The reaction steps depicted with broken arrows are kinetically invisible at pH 7; the structures of intermediates indicated with the asterisks (**B**) are unknown; the other intermediates were prepared independently and characterized spectroscopically [31,33].

The structure of the oxygenated form of **1** was also determined spectroscopically [31]. Unlike all other heme/Cu_B analogs, oxy-**1** exists as a ferric-superoxide/Cu^I redox isomer, with little if any interaction between the bound O_2 and Cu^I, despite the accessible Fe···Cu distance and $Cu^{II/I}$ potential, thereby mimicking oxygenated heme/Cu_B (compound A, Fig. 3). The stereoelectronic origin of the preference for the O_2^-/Cu^I vs. O_2^{2-}/Cu^{II} isomer is not known in either case.

Dioxygen reduction by the FeCu vs. Cu-free forms of 1: The role of Cu

The reactivity of **1** reviewed above suggests that these complexes are suitable for probing the role of distal Cu in O_2 reduction at the heme/Cu_B site of CcO. The latter objective was accomplished by comparing the catalytic properties of **1** in the FeCu and Cu-free forms under a variety of conditions. NMR studies indicate that the conformations of the distal structure in the FeCu and Fe-only forms of **1** are similar [40] so that the steric properties of the O_2 binding pocket in these two catalysts are comparable and the difference between them is mainly electronic. In addition, a derivative containing Zn^{2+} in place of Cu^{2+}, **1**FeZn, allows probing electrostatic influence of the distal metal on the reactivity of the heme without redox activity associated with the $Cu^{I/II}$ couple [31,40]. Profound electrostatic effects were observed in CO derivatives of **1**FeCu, **1**Fe-only, and **1**FeZn [40].

In the regime of rapid electron flux (catalysts directly adsorbed on the electrode), no significant differences in the TOF, mechanism, and stability of the FeCu and Cu-free forms were observed, although the FeCu catalyst produced less partially reduced oxygen byproducts at potentials >50 mV (see below) [31]. This similarity was interpreted as an indication that in the regime of readily accessible external reductants Cu is not essential for O_2 reduction, which is catalyzed efficiently by the porphyrinatoiron(II) moiety alone. Comparable stability of the catalysts, which was not dependent of the presence of •OH scavengers, suggests that **1**Fe-only, unlike simpler Fe porphyrins [25], does not induce O–O bond homolysis (Fenton chemistry).

In contrast, under the biologically relevant electron-flux limited turnover (3 % mol catalyst in a lecithin film), **1**FeCu catalyzes O_2 reduction without generating detectable quantities of partially reduced oxygen species, whereas the activity of the Cu-free derivatives is very low and disappears quickly [32]. Similarly, Cu-free mutants of CcO are catalytically inactive [26–28]. Faster decomposition of the Cu-free forms of **1** was rationalized in the context of the catalytic mechanism determined for the adsorbed catalyst (Fig. 8). However, the FeCu catalyst operating under the turnover-limiting electron flux loses its activity ~100-fold faster than the same catalyst reducing O_2 with an excess of electrons. Therefore, it appears that a fraction of **1**FeCu undergoes oxidation of the organic ligand (which is the most likely mechanism of the degradation). Formation of an organic radical (most likely phenoxyl, Fig. 3) is thought to be a part of the normal O_2 reduction mechanism at the heme/Cu_B site. Catalysts **1**, however, lack phenol and oxidation of the $Fe^{IV}Cu^{II}$ state, if it happens, can only yield an imidazole or porphyrin cation-radical. Because the phenoxyl radical is a less reactive species than imidazole or porphyrin cation-radicals, the incorporation of a phenol into **1** can be reasonably expected to produce a catalyst which would be more stable when operating under turnover-limiting electron flux.

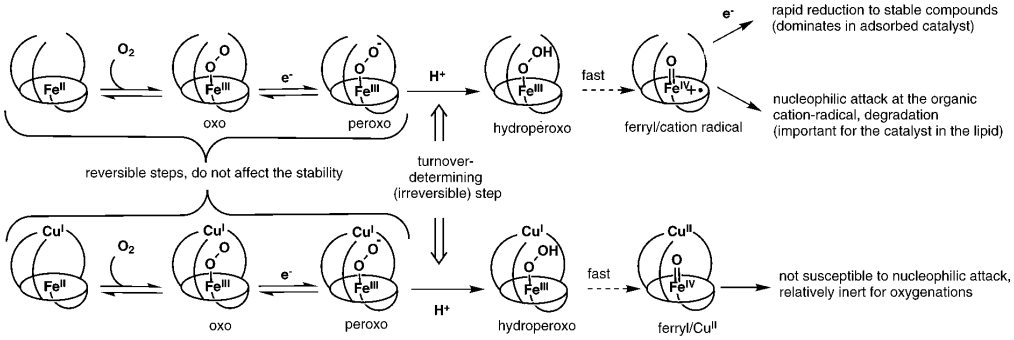

Fig. 8 A plausible set of reactions accounting for the lower stability of the **1**Fe-only catalyst relative to that of the FeCu analog only when electron flux is slow (freely diffusing catalyst in a lipid film).

Although the **1**FeCu and **1**Fe-only catalysts appear to catalyze O_2 reduction via a similar mechanism and with the same kinetics when electrons are in excess, the FeCu catalyst releases notably less partially reduced oxygen species (Fig. 9). The origin of this phenomenon appears to be >3-fold lower rate of autooxidation of oxy-**1**FeCu relative to oxy-**1**Fe-only. This conclusion was based on the identification of O_2^- as the major partially reduced oxygen species released by **1**Fe-only (but not by **1**FeCu) during O_2 reduction at potentials >50 mV [31]. To quantify the fractions of H_2O_2 and O_2^-/HO_2 that contribute to the observed ring current, O_2 reduction by **1**FeCu and **1**Fe-only was studied in the presence of fast selective O_2^- reductants, such as N-(2-mercaptopropionyl)glycine (MPG) and trolox. These compounds rapidly convert superoxide into peroxide without drawing any current from the disk electrode. Two-electron oxidation of H_2O_2 to O_2 at the ring electrode generates twice as much current as oxidation of the same molar amount of O_2^- (1e oxidation). If free superoxide is being produced by the

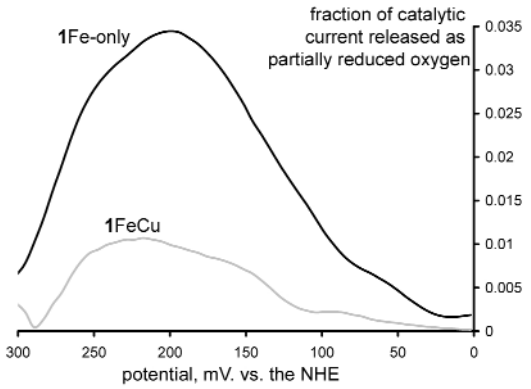

Fig. 9 In the regime of rapid electron flux, the FeCu catalyst reduces O_2 without releasing as much partially reduced oxygen species as does the Fe-only analog. The reason is slower autooxidation of oxy-**1**FeCu [31]. The potential dependence of the catalytic selectivity is consistent with the catalytic mechanism (Fig. 7) if the major side-reaction is autooxidation.

catalyst during turnover, a fraction of this O_2^- will get reduced to H_2O_2 by the reductant (MPG or trolox) before reaching the ring; as a result, the ring current and the ring-to-disk currents ratio will increase in the presence of the superoxide reductants relative to their absence. The magnitude of the increase depends both on the fraction of O_2^- in the mix of partially reduced oxygen species released by the catalyst, and the relative significance of secondary O_2^- sinks, such as disproportionation, redox reactions with other components of the catalytic film and/or electrode, etc. Because of these effects, the magnitude of the increase should correlate with the rate constants for the reduction of O_2^- to HO_2^- by the added reductants as well as their molar fraction in the catalytic film. Such correlation was indeed observed [31]. Catalytic films of **1**Fe-only containing 25 % molar fraction of MPG, which is the fastest reductant studied, produced approximately twice as much ring current as an identical film containing an inert additive, suggesting that O_2^-/HO_2 is the major partially reduced oxygen species generated by the Cu-free catalyst. Ferrous porphyrins generate O_2^- (and a ferric porphyrin) under aerobic conditions by autooxidation, the heterolytic cleavage of the Fe–O bond in the O_2 adduct, which is electronically close to a Fe^{III}–(O_2^-) limit. The apparent rate of autooxidation in neutral aqueous solutions are 10^{-6}–0.01 s^{-1} for O_2 adducts of heme proteins and can be as high as ~0.2 s^{-1} for some synthetic Fe^{II} derivatives [43]. The apparent autooxidation rate constant for **1**Fe-only on the electrode surface was ~0.01 s^{-1} [31]. A similar difference in the stabilities of oxy-**1**FeCu and oxy-**1**Fe-only was observed in nonaqueous medium as well, but the mechanism whereby Cu suppresses autooxidation is not known [33].

CONCLUSIONS

Biomimetic studies may have several objectives. One is to demonstrate that a specific enzymatic reactivity pattern can be achieved in a rationally designed small (relative to the enzyme) molecule, via a biologically relevant mechanism. For example, this objective dominated early work of Collman and coworkers on ferrous porphyrins as reversible O_2 carriers [44]. Another is to use broad structure/activity relationships learned from enzymes to develop synthetically useful catalysts, which may have no structural and/or mechanistic similarity to the enzymatic sites (Breslow's original definition of "biomimetic" [45]). A third objective is to help understand the structure/activity relationship at the enzymatic site by studying the reactivity of a structurally similar biomimetic analog. All three objectives have been invoked over the 25 years of the biomimetic work on CcO. It seems instructive to analyze how close we are now to meeting those objectives.

CcO carries out two major processes: it catalyzes oxidation of ferrocytochrome c by O_2 and it couples the free energy of this reaction to translocation of H^+ against the pmf. The latter process is so complicated and poorly understood that no attempts have been made to mimic it. Although numerous molecular catalysts of 4e O_2 reduction are known, few, if any, even among those designed specifically as biomimetic heme/Cu_B analogs, truly reproduce the O_2 reactivity of the heme/Cu_B site. It is reasonable to accept an electrode as a substitute of ferrocytochrome c. However, most biomimetic heme/Cu_B analogs fail because: (1) they require substantially more-reducing potentials than CcO does to reduce O_2 to H_2O; (2) even with an excess of external reducing equivalents, 4e reduction seems to require more than a single Fe center and proceeds via the intermediacy of free H_2O_2 and/or •OH because the stereoelectronic properties of these catalysts do not make reduction of the peroxo O–O bond efficient enough to compete with hydrolysis of the peroxo intermediate(s) and O–O bond homolysis [10]; (3) they bind O_2 as bridged peroxide not as the O_2^-/Cu^I form of compound A (Fig. 3) [10]. The fact that it has taken ~25 years and dozens of compounds to finally design one (compounds **1**, Fig. 4) that approaches the heme/Cu_B site in its O_2 reactivity is an indication as much of the challenging nature of O_2 reduction by CcO as of the failure of the biomimetic community to clearly define what "reproducing O_2 catalysis by CcO" means. Even compounds **1** fall short of CcO in their maximum catalytic activity at physiologically relevant potentials and in their lifetime under turnover-determining electron flux. We understand the plausible origins of these failings, which are the insufficiently hydrophobic environment around the Fe–Cu site and the lack of the phenol residue, respectively, and we can, albeit at a significant synthetic cost, incorporate these features into future analogs. Therefore, reproducing the O_2 reactivity of the heme/Cu_B site requires meticulously precise replication of many of its structural aspects. This illustrates our lack of the true understanding of what endows the heme/Cu_B site with its remarkable reactivity: although we know how to assemble a molecule containing the same moieties as the enzymatic site, we have no idea what (if any) alternative moieties/architectures may give us a similar reactivity.

The second objective, the design of a practical catalyst based on the structure of the heme/Cu_B site, does not appear to be realistic. A practical process most relevant to the physiological function of CcO is O_2 reduction in a fuel cell. The heme/Cu_B site is optimized to reduce O_2 under conditions which are significantly different from those of the fuel cell cathode. First, the stereoelectronic organization of the heme/Cu_B site seems to have been significantly influenced by the CcO's function of O_2 reduction under the turnover-limiting electron flux, something which is unlikely to exist at an electrode. Second, heme/Cu_B operates at an overpotential of ~550 mV to have free energy for endergonic translocation of protons. Such arrangement is advantageous for a living organism, which can utilize only the concentration gradient, not an electrical current, but is wasteful for an electrical current generator. Finally, although heme/Cu_B and hemes in general are very efficient at reducing the peroxo O–O bond, which is arguably the most difficult step in O_2 reduction by many other catalysts [10], this efficiency is achieved by elaborate and not particularly robust moieties, for which at present no alternative can be rationally designed. It seems that laccase and related multi-Cu oxidases [46,47] may be more suitable prototypes for practical molecular O_2 reduction catalysts.

Understanding the structure/activity relationship at an enzymatic site using the biomimetic approach requires a compound that sufficiently closely reproduces the structure and the reactivity of the site for the findings to be biologically relevant. Compounds **1** probably satisfy this criterion. They were used to understand the role of Cu in O_2 reduction at the heme/Cu_B site. The substantial amount of data collected on these compounds suggest that Cu_B may play a role of an electron-preloading site, involved in accumulation of reducing equivalents prior to O_2 binding to CcO. The bimetallic nature of the heme/Cu_B site of CcO thus appears to be a means to minimize generation of partially reduced oxygen species under conditions of O_2 reduction with a turnover-limiting electron flux. This conclusion is partly based on the observation that an Fe porphyrin with the distal and proximal environment similar to those of the heme/Cu_B site (less Cu_B) is capable of efficient and selective reduction of O_2 to H_2O under physiologically relevant potential and pH provided that electron flux is faster than the chemical steps.

The latter observation has implications beyond the O_2 chemistry of CcO. For ~25 years, two paradigms affected the field of molecular O_2 reduction: (1) complete 4e reduction of O_2 without the intermediacy of free H_2O_2 at a monometallic site is unlikely, and (2) two-electron reduction of the metal-bound peroxo species is slower (and often cannot kinetically compete with dissociative release of H_2O_2) than reduction of the metal-O_2 adduct (often viewed as containing a 1e-reduced dioxygen ligand, superoxide). Enzymes are not subject to these limitations: for example, cytochromes P450 reduce O_2 at a monometallic heme site, provided that the substrate (a source of two electrons for O_2 reduction) is in the catalytic pocket. Compounds **1** represent the first synthetic molecules that can be regarded with reasonable confidence to overcome both limitations. This property is probably due to adequate replication in **1** of the stereoelectronic attributes that are important for O–O bond activation, such as a strong σ-basic proximal ligand, a distal superstructure capable of polarizing bound O_2 substrate and a protective, hydrophobic catalytic pocket. Whether compounds can be designed that achieve similar efficiency without utilizing the same structural moieties as do the O_2-activating enzymes remains to be seen.

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