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Heme/Copper Assembly Mediated Nitrite and Nitric Oxide Interconversion

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Supporting Information

ABSTRACT: The heme$_{a3}$/Cu$_B$ active site of cytochrome *c* oxidase is responsible for cellular nitrite reduction to nitric oxide; the same center can return NO to the nitrite pool via oxidative chemistry. Here, we show that a partially reduced heme/Cu assembly reduces NO_2^- ion, producing nitric oxide. The heme serves as the reductant, but the Cu^{II} ion is also required. In turn, a μ-oxo heme-Fe^{III}–O–Cu^{II} complex facilitates NO oxidation to nitrite; the final products are the reduced heme and Cu^{II}–nitrito complexes.

Nitrogen oxides (NO_x) are components of great interest in both biological and environmental sciences. Nitric oxide (NO) is an important cellular signaling molecule and a powerful vasodilator involved in many physiological and pathological processes.[1] Nitrite (NO_2^-) is the one-electron-oxidized product of endogenous NO metabolism. Recent studies indicate that nitrite plays a critical biological role by serving as a biochemical circulating reservoir for NO, in particular under conditions of physiologic hypoxia (low O_2 tensions; see also below) and ischemia. The nitrite-to-NO conversion represents an important alternative source of NO to the classical oxygen-dependent L-arginine-derived generation of NO catalyzed by nitric oxide synthase (NOS).[2] Subsequently, suggested conserved roles for the NO_2^-/NO pool in cellular processes include oxygen sensing and oxygen-dependent modulation of intermediary metabolism.[3] It is now considered that in order to stimulate NO signaling, nitrite reductase activity occurs widely in differing cellular environments and is effected by a variety of proteins/enzymes, including hemes, those with molybdenum,[4] and what draws our current interest, cytochrome *c* oxidases (C*c*O's).[3,5]

The link between nitrite/NO redox interconversion and O_2 sensing is thought to occur in mitochondria at the C*c*O binuclear heme$_{a3}$/Cu$_B$ center; C*c*O is the terminal enzyme of the mitochondrial respiratory chain. Here, molecular oxygen consumption (i.e., O_2 reduction to water) is down-regulated in hypoxia by increased NO generation via C*c*O nitrite reductase activity, as reduced heme/Cu centers dominate when the O_2 concentration is low.[3a,4,6] The NO thus generated inhibits C*c*O activity by reversibly binding to heme$_{a3}$ in place of O_2, resulting in cellular O_2 accumulation (Figure 1). Some of the NO produced also participates in hypoxic signaling, the up-regulation of nuclear genes needed in response to the inherent dangers of low cellular O_2 concentrations.[3]

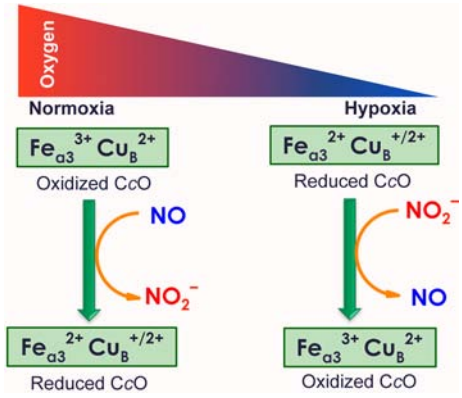

Figure 1. Cytochrome *c* oxidase (C*c*O) functioning in nitrite (NO_2^-)/nitric oxide (NO) interconversion as part of its role in regulation of O_2 balance. The availability of O_2 influences the redox state of the C*c*O containing the heme$_{a3}$/Cu$_B$ binuclear center.

In turn, in normoxia, high local O_2 concentrations do not allow nitrite to compete as an oxidant at the C*c*O binuclear center; thus, NO/O_2 binding is noncompetitive,[7] and heme$_{a3}$/Cu$_B$ oxidizes NO back to nitrite (Figure 1) to rejoin the storage pool. NO is thought to first attack oxidized Cu$_B$, formally giving Cu^I–NO^+; the latter hydrolyzes to nitrite.[8,9]

In this report, we describe a chemical system involving a heme/Cu assembly-mediated interconversion of these important nitrogen oxides. A partially reduced/oxidized state, with reduced heme and oxidized copper ion (i.e., $Fe^{II}\cdots Cu^{II}$) efficiently converts nitrite to NO. When a fully oxidized $Fe^{III}\cdots Cu^{II}$ heme/Cu complex is employed, NO is readily oxidized to nitrite. The overall reactions are represented by eqs 1 and 2:

$$(P)Fe^{II}Cu^{II} + NO_2^- \rightarrow (P)Fe^{III}\text{–}O\text{–}Cu^{II} + NO \quad (1)$$

$$(P)Fe^{III}\text{–}O\text{–}Cu^{II} + NO \rightarrow (P)Fe^{II}Cu^{II} + NO_2^- \quad (2)$$

The nitrite reductase chemistry,[10] here however in a heme/Cu chemical system, consisted of the iron(II) complex $(F_8)Fe^{II}$ [F_8 ≡ tetrakis(2,6-difluorophenyl)porphyrinate(2−)][11] and a preformed copper(II)–nitrito complex $[(tmpa)Cu^{II}(NO_2)][B(C_6F_5)_4]$ [tmpa ≡ tris(2-pyridylmethyl)amine]; the latter was synthesized by adding $AgNO_2$ to a chloride precursor $[(tmpa)Cu^{II}(Cl)][B(C_6F_5)_4]$, and its X-ray structure revealed an O-bound nitrito ligated Cu(II) ion (Scheme 1).[12]

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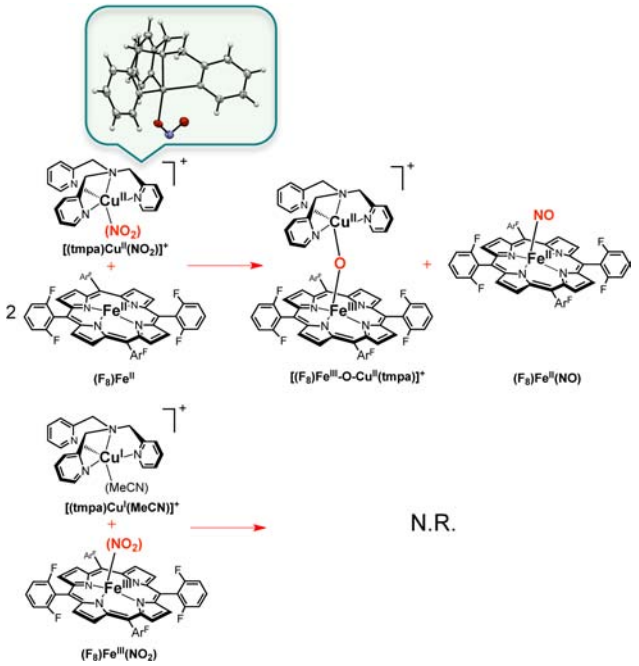
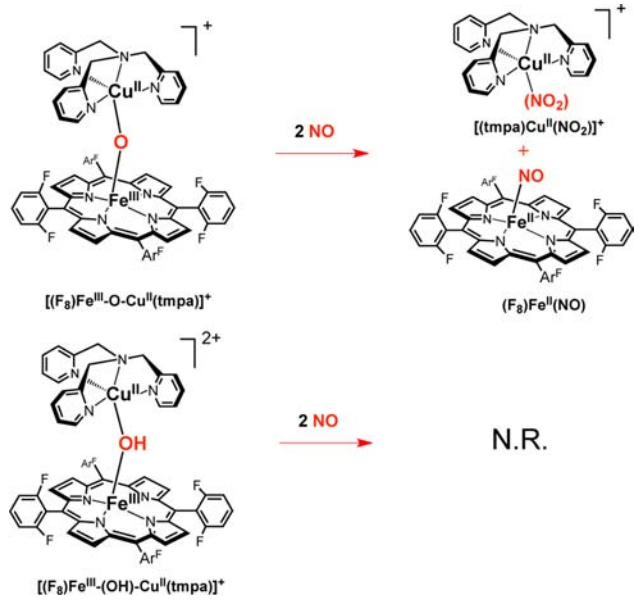
Scheme 1. Heme/Copper Assembly-Mediated Reduction of Nitrite to Nitric Oxide



Scheme 2. Heme/Copper Assembly-Mediated Oxidation of Nitric Oxide to Nitrite



When 2 equiv of $(F_8)Fe^{II}$ were mixed with 1 equiv of $[(tmpa)Cu^{II}(NO_2)]^+$ under a N_2 atmosphere in acetone at room temperature (RT), a reaction ensued, and on the basis of UV−vis, electron paramagnetic resonance (EPR), and IR spectroscopies, a 1:1 mixture of the heme−nitrosyl species $(F_8)Fe^{II}(NO)$ and the μ-oxo complex $[(F_8)Fe^{III}-O-Cu^{II}(tmpa)]^+$ were produced.[12] These products were readily identified, having been previously thoroughly characterized.[13] To determine whether the heme or the copper ion is the reductant in this one-electron process ($NO_2^- \rightarrow NO$), we also carried out the reaction in which nitrite was added to the oxidized heme complex $[(F_8)Fe^{III}]SbF_6$[13b] (binding of nitrite to the ferric heme[14] was indicated by a large UV−vis change)[12] and then the reduced complex $[(tmpa)Cu^{I}(MeCN)]^+$ [12,15] was added. In this case there was no reaction (Scheme 1), even over a period of days.[12] Control experiments showed that nitrite reacts only very slowly with $(F_8)Fe^{II}$ and not at all with $[(tmpa)Cu^{I}(MeCN)]^+$. Moreover, no nitrite reductase activity was observed for the fully reduced metal combination, nitrite plus $(F_8)Fe^{II}$ and $[(tmpa)Cu^{I}(MeCN)]^+$.[12]

These observations indicate that the heme is the reductant in this heme/Cu nitrite reductase chemistry. The need for 2 equiv of $(F_8)Fe^{II}$ is due to the well-known high affinity of NO to bind ferrous hemes.[16] The initially formed NO reacts very rapidly with $(F_8)Fe^{II}$; thus, if the reaction were carried out with equimolar quantities of $(F_8)Fe^{II}$ and $[(tmpa)Cu^{II}(NO_2)]^+$, only half of the iron would be available to reduce nitrite, and the rest would trap the NO as $(F_8)Fe^{II}(NO)$. The role of the Cu^{II} ion appears to be to provide a Lewis acid interaction with nitrite, facilitating NO_2^- (N−O) bond cleavage and stabilization of the resulting oxo anion via eventual formation of $[(F_8)Fe^{III}-O-Cu^{II}(tmpa)]^+$.

To demonstrate that heme/copper assemblies can mediate NO oxidation to nitrite, as occurs biologically in order to remove excess NO when it is not needed and return it to the nitrite pool (see above), we employed $[(F_8)Fe^{III}-O-Cu^{II}(tmpa)]^+$. Addition of NO to this fully oxidized heterobinuclear complex led to rapid reaction (Scheme 2) and formation of nitrite, which bound to Cu(II); the $(F_8)Fe^{II}$ formed[17] in this redox reaction was trapped by a second equivalent of NO to give $(F_8)Fe^{II}(NO)$. UV−vis (Figure 2) and IR (ν_{NO} = 1688 cm^{-1})[12] spectroscopies directly indicated nitrosyl complex formation. Nitrite analysis employing capillary electrophoresis revealed that this ion was produced in 95% yield.[12] EPR spectroscopy confirmed that a copper(II)−nitrito complex was produced (Figure 2); a sample taken from the reaction mixture was identical in all regards to that of an authentic sample of a 1:1 mixture of $(F_8)Fe^{II}(NO)$ and $[(tmpa)Cu^{II}(NO_2)]^+$.

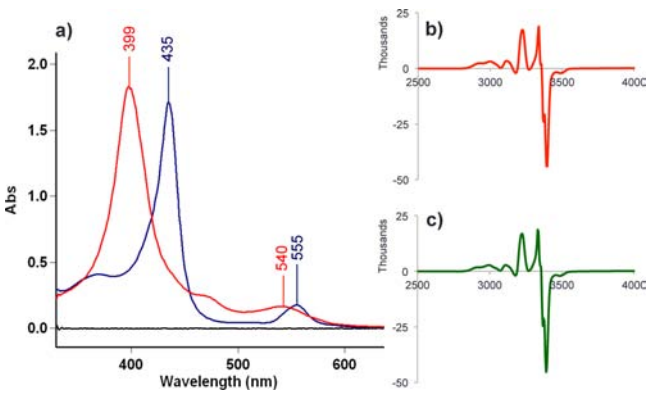


Figure 2. (a) UV−vis spectra of $(F_8)Fe^{III}-O-Cu^{II}(tmpa)][B(C_6F_5)_4]$ (**1**) (blue) and $(F_8)Fe^{II}(NO)$ generated from **1** + NO(g) (12 μM in acetone at RT) (red). (b, c) EPR spectra of (b) the products of the reaction of $(F_8)Fe^{III}-O-Cu^{II}(tmpa)][B(C_6F_5)_4]$ and NO (red) and (c) an authentic sample of a 1:1 mixture of $F_8Fe^{II}(NO)$ and $[(tmpa)Cu(NO_2)][B(C_6F_5)_4]$ (green). The EPR spectra were recorded at 20 K (1 mM in MeTHF).

It is important to explain why the reaction required 2 molar equiv of NO (Scheme 2). The second equivalent was not involved in the redox chemistry but was needed to trap the free $(F_8)Fe^{II}$ produced by the NO oxidase chemistry. When $(F_8)Fe^{II}$ is present, it effects the reverse reaction, namely, reduction of Cu(II)-bound nitrite to give NO (Scheme 1). This was

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demonstrated as follows: When 25 mL of a 10 μM solution containing a mixture of $(F_8)Fe^{II}(NO)$ and $[(tmpa)Cu^{II}(NO_2)]^+$ produced by the reaction of $[(F_8)Fe^{III}-O-Cu^{II}(tmpa)]^+$ with excess NO (green spectrum in Figure 3) was

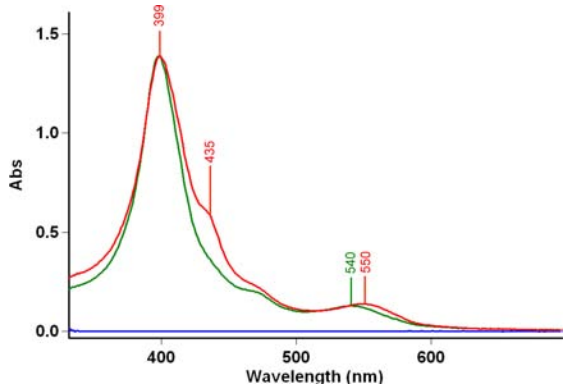

Figure 3. UV−vis spectra of a mixture of $(F_8)Fe^{II}(NO)$ (λ_{max} = 399 nm) and $[(tmpa)Cu^{II}(NO_2)]^+$ derived from the NO oxidase chemistry before (green) and after (red) the addition of 2 equiv of $(F_8)Fe^{II}$. The red spectrum shows the presence of a 2:1 mixture of $(F_8)Fe^{II}(NO)$ and $[(F_8)Fe^{III}-O-Cu^{II}(tmpa)]^+$ (435 nm, sh). The new second equivalent of $(F_8)Fe^{II}(NO)$ was derived from the nitrite reductase chemistry described by Scheme 1. See the text for further explanation.

titrated with 25 mL of a 20 μM solution (i.e., 2 equiv) of $(F_8)Fe^{II}$, the product solution (red spectrum in Figure 3) contained ~5 μM $[(F_8)Fe^{III}-O-Cu^{II}(tmpa)]^+$ along with 10 μM $(F_8)Fe^{II}(NO)$; the spectral intensity was equivalent to that observed in the starting mixture because of dilution. This proves that the backward reaction can and does occur, that is, that $[(tmpa)Cu^{II}(NO_2)]^+$ reacts first with $(F_8)Fe^{II}$ in a 1:1 stoichiometry to give NO, which is then trapped by the second equivalent of $(F_8)Fe^{II}$.

We also tested the μ-hydroxo complex $[(F_8)Fe^{III}-(OH)-Cu^{II}(tmpa)]^{2+}$ for "NO oxidase" chemistry, but upon addition of NO, there was no nitrite production (Scheme 2).[12] Instead, very slow (hours) reductive nitrosylation[18] occurred, and all of the heme present was converted to $(F_8)Fe^{II}(NO)$. It is thus clear that the μ-oxo complex ($[(F_8)Fe^{III}-O-Cu^{II}(tmpa)]^+$ is efficient or at least special in its ability to effect a redox reaction (formally $Fe^{III} \rightarrow Fe^{II}$) that includes oxo transfer.[19]

In summary, this report has described new chemistry with heme/Cu assemblies and nitrogen oxide interconversion: nitrite reduction to nitric oxide can readily be effected with our heme/copper chemistry. The reduced heme is the source of the one electron required. The presence of Cu^{II} ion as a Lewis acid is crucial. While nitrite reduction to NO is well-known to occur via heme proteins such as hemoglobin and myoglobin,[20] bacteria/fungal heme cd_1[21] or copper nitrite reductases,[22] and certain copper(I) complexes,[22a,23] it appears that the transformation with heme/Cu synthetic complexes has not been examined to date. We have shown here that both heme and Cu are required, at least in our system. It is notable that the heme is the reductant on the basis of the observed products; however, cyclic voltammetric determination of the redox potentials for the separate complexes $(F_8)Fe$ (−0.20 V vs Fc^+/Fc)[12] and Cu(tmpa) (−0.42 V vs Fc^+/Fc)[12] indicate the latter is a better reductant. For C*c*O, the opposite appears to be the case, as the $heme_{a3}$ has a lower redox potential than does Cu_B.[24]

On the other hand, the heme is also the redox entity, as $[(F_8)Fe^{III}-O-Cu^{II}(tmpa)]$ effects oxidation of NO to nitrite by oxo transfer (see above)[19] with reduction of $(F_8)Fe^{III}$. The closely related species $[(F_8)Fe^{III}-(OH)-Cu^{II}(tmpa)]^+$ and/or heme-only complexes do not enable this reaction. However, as already mentioned, it is the Cu_B in C*c*O that is thought to oxidize NO,[9] and it is well-known that NO can react with Cu^{II} complexes,[25] affording nitrite. This may imply that in C*c*O it is a particular coordination environment, a specific structural and/or redox state, that is required for mediation of the NO oxidase chemistry.

Further investigations will include our probing of the mechanisms of the reactions described in this report. For nitrite reduction, critical mechanistic components will certainly include the heme reductive capability and the nitrite−Cu binding mode (e.g., O- vs O,O′- vs N-bound). As our heme and Cu centers have switched redox capabilities compared with C*c*O, we also wish to change the heme or the Cu ligand to make the Cu center a better oxidant than the heme. For our heme/Cu NO oxidation chemistry, we are uncertain about which metal is the real oxidant,[18] so further investigations are required; as is often the case, "the devil is in the details".

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Synthetic and analytical details; UV−vis, IR, and EPR spectra; cyclic voltammetry and capillary electrophoresis results; and X-ray structural details and CIF files. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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(17) By analogy to what has in the past been suggested for the enzyme reaction,[8b] nitric oxide attacks at the cupric center. Formally, this affords Cu^I-NO^+, to which oxo transfer occurs, along with electron transfer from Cu^I to Fe^{III} to give the Cu^{II}−nitrito complex and Fe^{II}, which is then trapped by a second NO molecule. Separately, we can demonstrate that with the present complexes $[(tmpa)Cu^I(MeCN)]^+$ and $[(F_8)Fe^{III}]SbF_6$, the Cu^I-to-Fe^{III} electron transfer readily occurs in acetone (see the Supporting Information).
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