Inorg. Chem. **2006**, *45*, 3187–3190

Inorganic Chemistry
Article

Characterization of a Bimetallic-Bridging Intermediate in the Reduction of NO to N_2O: a Density Functional Theory Study

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Received June 17, 2005

We have applied density functional theory to characterize the bimetallic-bridging intermediate in the reduction of NO to N_2O by heme–copper oxidoreductases. The present study provides information of the electronic and vibrational structure of the putative intermediate, and thus, it forms the basis for a quantitative accurate atomic-level description of the key steps in the N–N bond formation and the N–O bond cleavage mechanism.

Introduction

The family of heme−copper oxidoreductases (heme Fe−Cu_B) is ubiquitous in bioenergetics, playing a key role in understanding the common evolution of aerobic respiration and bacterial denitrification.[1,2] It has been proposed that in bacterial NO reductases (Nor) and in heme-copper oxidoreductases the mechanisms of O_2 and NO reduction share common features and that NO and O_2 may be used as alternative substrates by both enzyme families. Of the heme−copper oxidoreductases known, the *ba*$_3$ and *caa*$_3$ from *Thermus thermophilus* and the *cbb*$_3$ from *Pseudomanas stutzeri* are known to reduce O_2 and NO to H_2O and N_2O, respectively.[2−4] Nor consists of a high-spin heme *b* and a non-heme Fe, whereas that of the oxidoreductases consists of either a high-spin heme a_3 or a heme *b* and a Cu_B atom. Both Nor and heme Fe−Cu_B oxidoreductases insert two NO molecules in their active site which are specificity determined by the nature of the protein active-site cavity and, with a mechanism which has not been characterized yet, produce N_2O. Elucidation of this mechanism is of fundamental importance in understanding the chemistry of NO in biological systems and necessary for insight in the proposal of possible co-evolution of aerobic respiration and denitrification.

The midpoint redox potentials of each of the metal centers in Nor have been measured, and from the unexpectedly low midpoint redox potential of heme b_3 ($E_m = 60$ mV), it was suggested that full reduction of the dinuclear center is thermodynamically unfavorable.[3] On the basis of resonance Raman (RR) experiments, it was proposed that under physiological conditions NO activation in Nor occurs with a mixed-valence form of the enzyme in which the low-spin hemes *b* and *c* and the non-heme Fe are reduced and heme b_3 is in the oxidized form.[5] Binding of two NO molecules to the ferric dinuclear center was excluded, and thus, it was concluded that heme b_3 has higher affinity for NO than the non-heme Fe. In the heme−copper oxidoreductases, however, the reduced center (heme Fe^{2+}−Cu_B^{1+}) would be the catalytically active redox state.[2,6a−b]

Because the reduction of NO to N_2O is a $2e^-/2H^+$ process, the formation and cleavage of the N−N and the N−O bonds, respectively, can take place in a reduced heme Fe^{2+}−Cu_B^{1+} center. Recently, the primary His−heme a_3^{2+}−NO/Cu_B^{1+} species were identified by RR spectroscopy, demonstrating its characteristic Fe−NO and N−O stretching frequencies

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10.1021/ic050991n CCC: $33.50

at 539 and 1620 cm^{-1}.[7] However, our understanding of the NO chemistry is relatively premature compared with the O_2 chemistry,[8a-d] mostly because of the lack of structural information on transient reaction intermediates by RR spectroscopy or density functional theory (DFT) calculation. DFT calculations on heme−copper binuclear center models provide insight into the structural, energetics, and electronic structure of key intermediates involved in the catalytic O_2 reduction mechanism.[9a-c]

Here we employed a DFT method to provide a quantitative accurate atomic-level description of the key steps in the N−N bond formation and the N−O bond cleavage mechanism. Due to the difficulty in detecting transient, short-lived intermediates, no information is available in the literature on the chemical nature of the bound hyponitrite intermediate or the cleavage mechanism of the N−O bond. A complete analysis should address the contribution of the protein that is supposedly responsible for the proton donation, as well as the rate-limiting step in the N−O bond cleavage mechanism. Quantitatively, the goal is to understand how the binuclear center stabilizes the transition state for proton donation and subsequent N−O cleavage to form N_2O. Such knowledge is essential in building quantitative models for the activation of two NO molecules by the binuclear heme Fe−Cu_B and dinuclear heme Fe−Fe centers, and in addition, it establishes the computational framework for the study of highly reactive NO intermediates in both oxidases and the bona fide Nor.

Experimental Section

Computational Details. All computations were carried out by using the Gaussian98 program package. We have used hybrid density functional B3LYP calculations for analyses of the NO reduction on heme−copper bimetallic model compounds containing 98−100 atoms. The 6−31g* basis set was used for geometry optimization and subsequent evaluation of the Hessian of optimized model complexes, and computed vibrational frequencies are listed without scaling. Restricted closed-shell singlet calculations were performed for the computationally expensive frequency analyses of optimized models. This might be justified because optimization of a key reaction intermediate of hyponitrite intermediate I under the unrestricted open-shell singlet and triplet spin state conditions did not make significant difference from the spin-singlet closed-shell system (the bond length difference is 0.001−0.02 Å). The triplet spin state optimized model was computed as 6 kcal/mol higher in energy than the open-shell singlet spin state. In addition, the NO reduction by the heme−copper bimetallic centers is expected to occur along a singlet spin state potential energy surface, as will be discussed later on. However, since the restricted closed-shell calculation cannot localize atomic spins over bimetallic centers, the broken-symmetry method[10a-b] was applied to analyze such spin delocalization. The broken-symmetry methodology is based on unrestricted Hartree−Fock or DFT calculations for spin-singlet open shell systems in which spin-up and spin-down electrons are allowed to localize on different atomic centers. The broken-symmetry state is not a pure spin state described by a singlet determinant, but rather a weighted average of pure spin states to give a spin-coupling pattern in an antiferromagnetic alignment. The energy of a broken-symmetry state is usually above but close to the true ground-state energy. Thus, the energetics of the spin-singlet closed shell models of reaction intermediates and a transition state was improved by the broken-symmetry computation using the 6−311g* basis set.

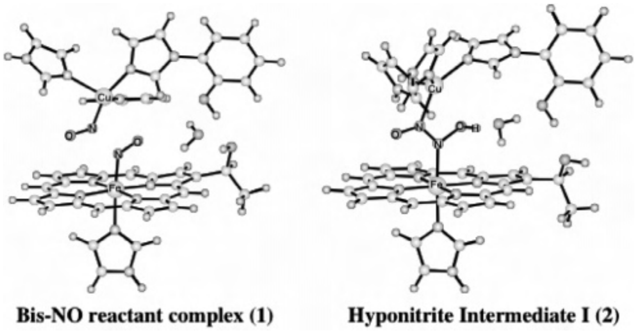

Figure 1. Computational models of bis-nitrosyl reactant complex (**1**) and hyponitrite intermediate I (**2**).

Results and Discussion

NO Reduction by the Heme-Copper Bimetallic Center. The catalytic NO reduction process can be considered to take place through coupling of a second NO molecule to the primary Fe^{2+}−NO intermediate, yielding a bis-nitrosyl complex (**1**), as shown in Figure 1. The heme side chains of *ba*$_3$-oxidereductase was simplified as shown in Figure 1. While it is not present in the X-ray structure,[11] we introduced a water molecule that provides a hydrogen-bonding network from the tyrosine residue and heme side chain to the heme-bound ligand. This type of a water molecule was also proposed theoretically to play an important role in the O−O bond cleavage mechanism of cytochrome *c* oxidase.[9]

The spin state of the bis-nitrosyl reactant complex (**1**) is either triplet or singlet because a six-coordinate ferrous heme-ligand adduct usually adopts a low-spin state and the cuprous (Cu_B^{1+}) center is diamagnetic, thereby locating two spins on the NO ligands. For the formation of the N−N bond between the two NO molecules, however, diamagnetic coupling of the two spins would be favorable in the reactant complex. The calculated Fe···Cu_B separation of 4.64 Å demonstrates reasonable agreement with the distance of 4.4 Å determined by the X-ray crystal structural analyses of the oxidized form of *ba*$_3$ oxidoreductase.[11] At this stage, the N−N bond formation would not be completed due to the N···N separation of 1.8 Å, as shown in Figure 2.

The spin density in complex **1** obtained by the broken-symmetry method is listed in Table 1. Nearly one spin is

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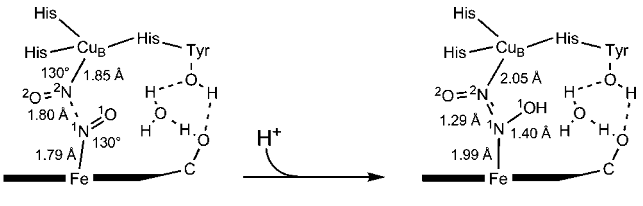

(1) **(2)**

Figure 2. Geometry of bis-nitrosyl reactant complex (**1**) and hyponitrite intermediate (**2**).

Table 1. Atomic Spin Densities (Charges) of Complex **1** and **2**[a]

atom[b]	1	2
Fe	−0.93 (1.30)	0.99 (1.42)
Cu	−0.03 (1.16)	−0.56 (1.26)
^{1}N	0.42 (−0.09)	−0.01 (−0.09)
^{1}O	0.30 (−0.27)	0.00 (−0.54)
^{2}N	0.09 (−0.18)	−0.10 (−0.05)
^{2}O	0.10 (−0.17)	−0.07 (−0.31)

[a] Obtained by single-point calculation by 6-311G* basis set. [b] Atoms are shown in Figure 2

located on the heme iron, indicating oxidation of the heme iron (Fe^{3+}). The total charge of heme-bound NO is −0.36, indicating that the iron electron density is partly delocalized to the ligand. The O atom of heme-bound NO contains more negative charge (−0.27) than that of the N atom (−0.09), which is also reflected by the 0.3 spin density on the O atom in parallel with the 0.48 spin density on the N atom. Thus, the O atom of heme-bound NO would be susceptible to protonation, as will be demonstrated below. On the other hand, Cu$_B$ is the cuprous state as judged from almost zero spin density and the Cu$_B$-bound NO contains 0.35 negative charge that is the same amount as that of the heme-bound NO. However, the Cu$_B$-bound NO contains almost zero spin, indicating that the two π* orbitals of NO are diamagnetically and almost equally occupied with electron densities. The description of a metal−NO bond is theoretically of particular importance, and a detail analysis of the electronic structure should be presented soon.

Upon protonation of the OH group of the side chain of the heme (to which so-called K proton channel is connected in the *ba*$_3$ oxidoreductase), the geometry of the model complex was optimized accompanying with proton migration from the model geranyl group to the terminal O atom of heme-bound NO, yielding a hyponitrite bridged bimetallic center (complex **2**), as shown in Figure 2. Complex **2**, termed as hyponitrite intermediate I, includes dimerized NO with a N−N distance of 1.29 Å and a Fe⋯Cu$_B$ distance at 4.73 Å.

The α- and β-spin orbitals of complex **2** are drawn in Figure 3. The spin-orbitals are localized on the Cu$_B$ and heme iron centers as indicated by the spin densities listed in Table 1. The π* orbitals of the Cu$_B$ bound NO are occupied with α- and β-spin densities, thereby yielding diamagnetic NO.

Since the hyponitrite intermediate is the key species in the NO reduction mechanism,[6,7] its characterization is important to form a basis for future experimental studies. Hence, the normal modes and the vibrational frequencies of the hyponitrite intermediate I associated with the ligand vibration are shown in Figure 4. There are six normal modes

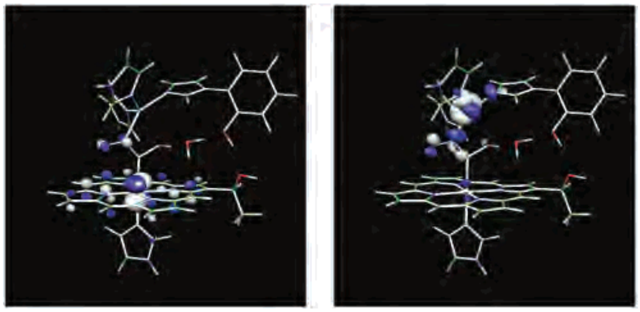

Figure 3. α- and β-spin orbitals of complex **2**.

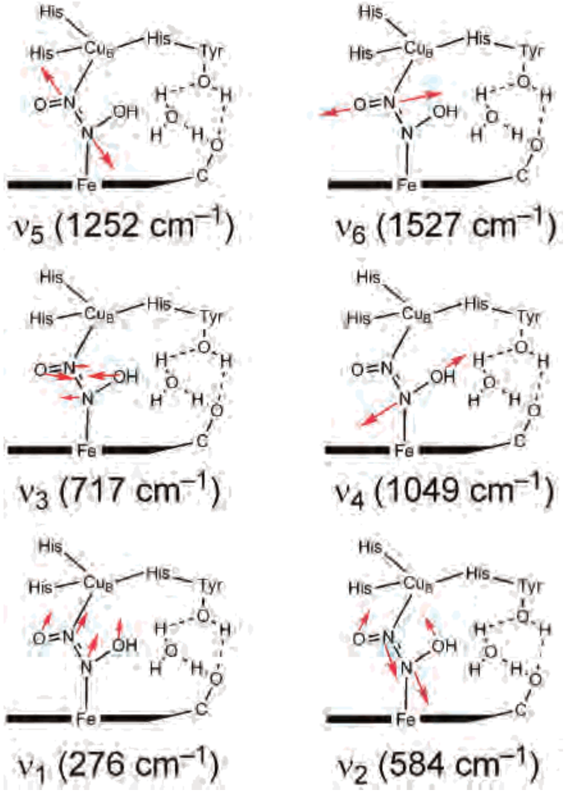

Figure 4. Normal modes and frequencies of metal-bridging ligand in 2.

Table 2. Vibrational Frequencies of Metal Bridging Ligand and Isotope Sensitivity

mode	$^{14}N^{16}O$	$^{15}N^{16}O$	$^{14}N^{18}O$	$^{15}N^{18}O$
ν_6	1527	1500	1500	1465
ν_5	1252	1215	1249	1212
ν_4	1049	1029	1034	1005
ν_3	717	712	689	685
ν_2	584	571	573	561
ν_1	276	270	269	268

($3N - 6$; $N = 4$) associated with the bridging ligand atoms vibration. The direct evidence of the N−N bond formation would be provided by the detection of the N−N stretching mode (ν_5). The calculated ν_5 frequency is 1252 cm^{-1}, exhibiting ^{15}N isotope sensitivity, and the $^{14}N^{18}O$ isotope did not affect the frequency significantly, as listed in Table 2. The intrinsic ligand vibration of Cu$_B$-bound NO (ν_6) is 1527 cm^{-1}, which is significantly lower than the value of the intrinsic ligand vibration in complex **1** (ν_{FeN-O}, 1624 cm^{-1}; ν_{CuN-O}, 1723 cm^{-1}) due to electron density delocalization to Cu$_B$-bound-NO π* orbitals. The same isotope sensitivity of ν_6 to $^{15}N^{16}O$ and $^{14}N^{18}O$ is ascribed to a varied relative

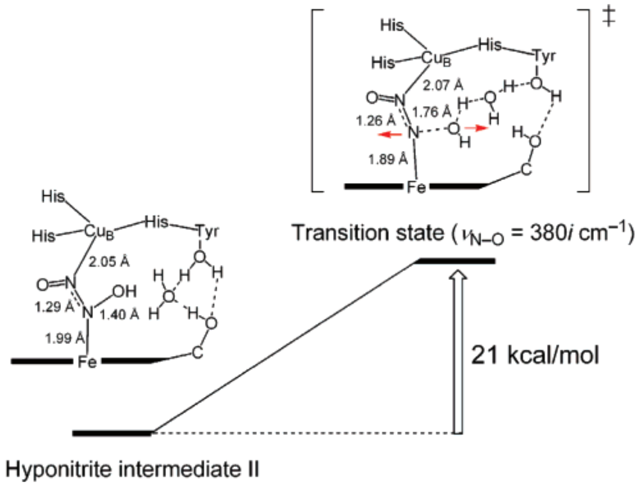

Figure 5. Geometry and energetics of hyponitrite intermediate II and the transition state.

weight of N and O nuclear displacements in those isotope bound complexes; the relative displacement of the O atom against that of the N atom in $^{15}N^{16}O$- and $^{14}N^{18}O$-bound complexes are 67% and 49%, respectively. Thus, the ^{18}O isotope sensitivity would be reduced in the $^{14}N^{18}O$-bound form. Upon protonation of the terminal O atom of the heme-bound NO, the N−O bond becomes a single bond due to the electron density delocalization to the π* orbital, and thus the N−OH stretching (ν_4) frequency is lowered significantly to 1049 cm^{-1}. The isotope sensitivity of ν_4 is a zigzag pattern, and this might be due to large displacement of the N atom (the displacement of the O atom is 50% of that of the N atom) in the normal mode. The ν_2 and ν_3 modes are deformational motion with respect to the O−N−N−OH moiety, and the ν_3 involves large displacement of the O atom moieties while ν_2 has large motion of the N atoms, which are reflected in the isotope sensitivity. The lowest frequency of ν_1 is associated with the Cu_B−N stretching and coupled with a heme out-of-plane deformational mode.

The hydroxyl group of the hyponitrite ligand would be subsequently protonated to release H_2O. Thus, we built a protonated hyponitrite intermediate I model (hyponitrite intermediate II). Using this protonated model, the activation barrier for the N═N bond formation and concomitant N−O bond scission was estimated. The estimation of the Hessian of an optimized transition state (TS) candidate, which demonstrated a negative eigenvalue of 380i cm^{-1} along the N−O bond axis, indicated that the optimized structure is the TS for the N−O bond scission and thereby N═N double bond formation. The barrier that must be overcome for the TS was computed to be 21 kcal/mol at the B3LYP/6−311g* theory level using the broken-symmetry method that is twice the activation energy calculated for O−O bond activation on a heme−copper binuclear center.

Summary

In summary, we have characterized possible key reaction intermediates in the catalytic reaction cycle of NO reduction by the heme−copper oxidase model. The present study provides information of the electronic and vibrational structure of the putative intermediate in the NO reduction on the heme−copper binuclear center, and thus, it forms the basis for future vibrational spectroscopic studies.

Acknowledgment. This work was supported by JSPS Research Fellowship for Young Scientists (T.O.), by the Greek Secretariat of Research (0191AP to C.V.), and by Grant-in-aids for Specifically Promoted Research from the Ministry of Education, Science, Sports and Culture, Japan (T.K., 14001004). The computations were partly carried out at the Research Center for Computational Science of the Okazaki Research Facilities.

IC050991N