Activation of N$_2$O Reduction by the Fully Reduced μ_4-Sulfide Bridged Tetranuclear Cu$_Z$ Cluster in Nitrous Oxide Reductase

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Nitrous oxide reductase (N$_2$OR) catalyzes the two-electron reduction of N$_2$O to N$_2$ and H$_2$O in the last step of the bacterial denitrification process.[1–4] It is a dimeric protein. The recently solved crystal structure of N$_2$OR indicates that in each subunit there is a Cu$_A$ center, which is the electron-transfer site, and a Cu$_Z$ center, which is the catalytic site. The neighboring Cu$_A$ and Cu$_Z$ centers are from different subunits.[5,6] The Cu$_Z$ center has a strikingly new structural motif consisting of a μ_4-sulfide bridged tetranuclear cluster. The whole Cu$_Z$ cluster is coordinated by seven His ligands with a weakly bound water at the Cu$_I$/Cu$_{IV}$ edge (atom numbering is defined in Figure 2A, vide infra), which is the substrate access site. Our previous studies have shown that the Cu$_Z$ center in dithionite-reduced N$_2$OR (the resting form) is a partially delocalized $S = 1/2$, 1Cu^{II}/3Cu^I cluster.[7] This cluster provides a mechanism for overcoming the reaction barrier of N$_2$O reduction by a simultaneous two-electron reduction pathway to the substrate bound in a μ-1,3-bridging mode.[7,8] In this study, we experimentally determine that the redox *active form* of the Cu$_Z$ cluster in enzymatic turnover is the all-reduced 4Cu^I form by using a combination of activity determinations and EPR spectroscopy. This is the first demonstration that the $S = 1/2$ form of Cu$_Z$ can be further reduced. DFT calculations were performed to provide insight into the nature of N$_2$O binding to and activation by this all-reduced 4Cu^I form relative to the 1Cu^{II}/3Cu^I resting form of the Cu$_Z$ site.

Activity studies of the enzyme were performed spectrophoto-metrically, following the oxidation of dithionite-reduced methyl viologen at 600 nm using standard literature protocol under anaerobic conditions at room temperature.[9–11] Past studies indicate that higher activity can be achieved by preincubation with dithionite-reduced methyl viologen.[9,12] The activity of the *Pseudomonas nautica* N$_2$OR was measured after periods of incubation of the enzyme in excess methyl viologen and dithionite solution. The activity vs incubation time profile is shown in Figure 1 (blue curve). It was observed that enzyme activity is dependent on the incubation time in the presence of reductant. It initially increases rapidly and then saturates slowly after about 40 min, when highest activity is obtained.

Parallel EPR measurements of the sample in the same reducing conditions were performed at periods of similar incubation times at which activities were measured to correlate with the activity assays. The characteristic EPR signal ($g_{||} = 2.16$)[7] of the resting 1Cu^{II}/3Cu^I Cu$_Z$ cluster decreases with increasing incubation time (Figure 1, inset).

The EPR signal intensity vs incubation time profile (Figure 1, red curve) shows a very similar decay rate to the growth rate of the enzymatic activity vs incubation time profile, indicating that the two processes are directly correlated. Since the resting form of
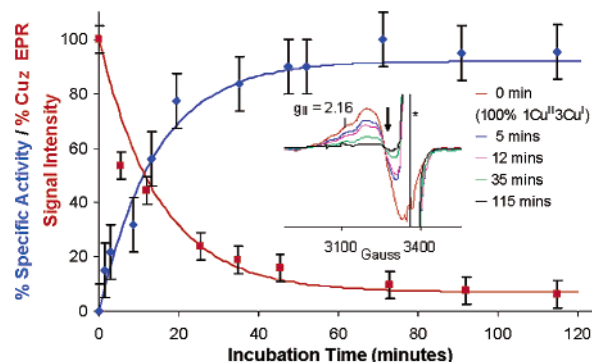
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Figure 1. N$_2$OR activity vs time (100% corresponds to 275 μmols N$_2$O-reduced min^{-1} mg^{-1} of enzyme) (blue ◆) and Cu$_Z$ EPR signal intensity vs time (100% corresponds to the dithionite-reduced resting 1Cu^{II}3Cu^I form at $t = 0$) (red ■). Solid lines are exponential fits, $k = 0.07$ min^{-1} for both fits. Inset: Decrease in the Cu$_Z$ EPR signal, measured at different incubation times in reducing conditions. EPR spectra measured at 77 K, 9.319 GHz. (∗) Methyl viologen radical signal.

Cu$_Z$ is a one-hole system (1Cu^{II}/3Cu^I),[7,8] the gradual decrease of the EPR signal on incubating with methyl viologen and dithionite indicates that the Cu$_Z$ cluster is reduced to the 4Cu^I form. *Therefore, the direct correlation of enzyme activity with the reduction of the resting Cu$_Z$ ($k = 0.07$ min^{-1} for both) indicates that the redox active form of the enzyme is the 4Cu^I state.* It should be further noted that the slow rate of reduction of the resting Cu$_Z$ center (1Cu^{II}/3Cu^I) indicates that it cannot be in the catalytic cycle, and the rate of rereduction of the N$_2$O-oxidized Cu$_Z$ center must be fast in enzymatic turnover.

Density functional calculations were performed to study the N$_2$O binding to and activation by the Cu$_Z$ cluster. The Cu$_Z$ crystal structure coordinates were used as the initial structure for geometry optimization. The His ligands were modeled as 1H-imidazoles, and their positions were kept frozen during optimizations.

There are two available coordination sites in the Cu$_Z$ cluster (Cu$_I$ and Cu$_{IV}$), and the N$_2$O ligand has several coordination modes.[8,13] The lowest-energy structures of the Cu$_Z$–N$_2$O complex are shown in Figure 2. The 4Cu^I form prefers the μ-1,3-N$_2$O bridging mode. In the inactive 1Cu^{II}/3Cu^I form, N$_2$O is coordinated through the N atom to Cu$_I$ in an end-on fashion. The binding energy (at the BP86/6-311G* level of theory) of N$_2$O to Cu$_Z$ is higher for the 4Cu^I form ($\Delta E_f = -18.5$ kcal mol^{-1}) than that for the 1Cu^{II}/3Cu^I form ($\Delta E_f = -15.7$ kcal mol^{-1}).

In the Cu$_Z$(1Cu^{II}/3Cu^I)–N$_2$O complex, the geometric structure of the N$_2$O ligand is very similar to the gas-phase structure of N$_2$O.[14] The situation is quite different in the Cu$_Z$(4Cu^I)–N$_2$O complex, where the N–N and N–O bond distances are elongated relative to the gas-phase structure by 0.03 and 0.07 Å, respectively. The N–N–O bond angle is 139°, in contrast to the linear geometry in
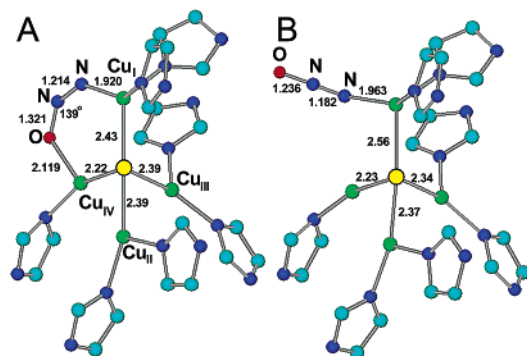
Figure 2. DFT-optimized geometries of the N_2O complexes with all-reduced $4Cu^I$ Cu_Z (A) and one-hole $1Cu^{II}/3Cu^I$ Cu_Z (B). Hydrogen atoms are not shown.
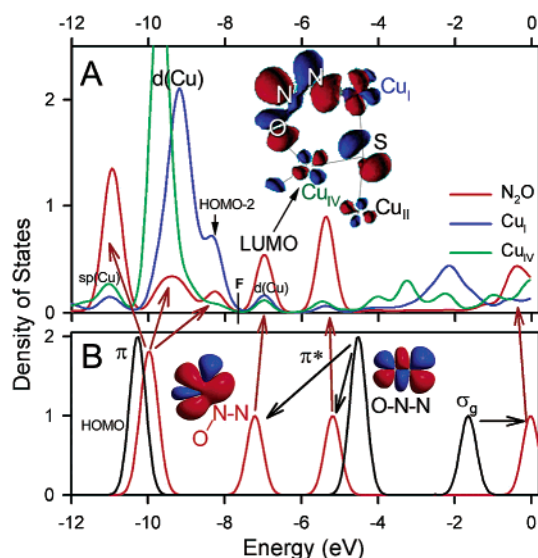

Figure 3. (A) Contributions of N_2O, Cu_I, and Cu_{IV} to the density of states of the $Cu_Z(4Cu^I)-N_2O$ complex. The Fermi level is indicated by F. Inset: LUMO of $Cu_Z(4Cu^I)-N_2O$. (B) Density of states of the N_2O molecule with a linear geometry and with the N_2O ligand geometry in the same Coulombic potential as in the $Cu_Z(4Cu^I)-N_2O$ complex. Inset: the LUMO of bent (left) and linear (right) N_2O.

the free N_2O molecule and in the $Cu_Z(1Cu^{II}/3Cu^I)-N_2O$ complex. The very bent geometry of N_2O in the complex causes a 2 eV splitting of the degenerate LUMO of N_2O into two nondegenerate π^* orbitals and stabilizes the resultant in-plane LUMO by ~3 eV due to loss in antibonding character (Figure 3B). This shifts the LUMO closer to the fully occupied d orbitals of the $4Cu^I$ cluster and makes the N_2O ligand a *better electron acceptor*.

The LUMO of the $Cu_Z(4Cu^I)-N_2O$ complex (Figure 3A) is 54% π^* orbital of N_2O and contains 12 and 10% d orbital contributions from Cu_I and Cu_{IV} atoms, respectively. The Mulliken and natural electron charges of the N_2O ligand in the $Cu_Z(4Cu^I)-N_2O$ complex are both around −0.53 a.u., indicating a very substantial back-donation from the Cu d orbitals to the $\pi^*(N_2O)$ orbitals. In comparison, the charge of N_2O in the $Cu_Z(1Cu^{II}/3Cu^I)-N_2O$ complex is only around −0.1 a.u. The large charge transfer from the $Cu_Z(4Cu^I)$ cluster to N_2O in the complex plays a crucial role in N_2O activation, and it is clear that the all-reduced form of Cu_Z is *better for back-bonding* than the $1Cu^{II}/3Cu^I$ form.

With respect to possible reaction mechanisms, the O atom of N_2O in the $Cu_Z(4Cu^I)-N_2O$ complex is activated for electrophilic attack by a proton. The O charge is −0.5 e⁻, and the HOMO−2, which lies close to the HOMO and has 6% O contribution, can serve as the donor frontier molecular orbital. This O contribution comes from the mixing of the N_2O π orbital, which has 66% O character with the occupied Cu d orbitals.[15] This and other possible reaction channels are presently being evaluated.

In summary, the $4Cu^I$ form of Cu_Z is the active state in the catalytic reduction of N_2O. N_2O binding in a bent μ-1,3-bridging mode to the $4Cu^I$ form is most efficient due to strong back-bonding from Cu_I and Cu_{IV}. This reduced state of Cu_Z can thus serve as an efficient two-electron reducing agent. The created electron holes on Cu_I and Cu_{IV} by the 2 e⁻ reduction of N_2O can be efficiently delocalized through the bridging sulfide σ superexchange pathways, contributing to the thermodynamics and kinetics of this process by lowering the geometric and electronic reorganization energies between the all-reduced and oxidized Cu_Z forms.

Supporting Information Available: Procedure for activity determinations, quantification of EPR signal intensities, calculation details and DFT-optimized structures of Cu_Z-N_2O complexes, orbital decomposition analysis data for the frontier molecular orbitals of $Cu_Z(4Cu^I)-N_2O$, and detailed density-of-states plots (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Zumft, W. G. *Microbiol. Mol. Biol. Rev.* **1997**, *61*, 533.
(2) Zumft, W. G.; Kroneck, P. M. H. *Adv. Inorg. Biochem.* **1996**, *11*, 1993.
(3) Rasmussen, T.; Berks, B. C.; Butt, J. N.; Thompson, A. J. *Biochem. J.* **2002**, *364*, 807.
(4) Dooley, D. M.; McGuirl, M. A.; Rosenzweig, A. C.; Landin, J. A.; Scott, R. A.; Zumft, W. G.; Devlin, F.; Stephen, P. F. *Inorg. Chem.* **1991**, *30*, 3006.
(5) Brown, K.; Tegoni, M.; Prudêncio, M.; Pereira, A. S.; Besson, S.; Moura, J. J. G.; Moura, I.; Cambillau, C. *Nat. Struct. Biol.* **2000**, *7*, 191.
(6) Brown, K.; Djinovic-Carugo, K.; Haltia, T.; Cabrito, I.; Saraste, M.; Moura, J. J. G.; Moura, I.; Tegoni, M.; Cambillau, C. *J. Biol. Chem.* **2000**, *275*, 41133.
(7) Chen, P.; George, S. D.; Cabrito, I.; Antholine, W. E.; Moura, J. J. G.; Moura, I.; Hedman, B.; Hodgson, K. O.; Solomon, E. I. *J. Am. Chem. Soc.* **2002**, *124*, 744.
(8) Chen, P.; Cabrito, I.; Moura, J. J. G.; Moura, I.; Solomon, E. I. *J. Am. Chem. Soc.* **2002**, *124*, 10497.
(9) Prudêncio, M.; Pereira, A. S.; Tavares, P.; Besson, S.; Cabrito, I.; Brown, K.; Samyn, B.; Devreese, B.; VanBeeumen, J.; Rusnak, F.; Fauque, G.; Moura, J. J. G.; Tegoni, M.; Cambillau, C.; Moura, I. *Biochemistry* **2000**, *39*, 3899.
(10) Kristjansson, J. K.; Hollocher, T. C. *J. Biol. Chem.* **1980**, *255*, 704.
(11) Alvarez, M. L.; Ai, J.; Zumft, W.; Sanders-Loehr, J.; Dooley, D. M. *J. Am. Chem. Soc.* **2001**, *123*, 576.
(12) Ferretti, S.; Grossmann, J. G.; Hasnain, S. S.; Eady, R. R.; Smith, B. E. *Eur. J. Biochem.* **1999**, *259*, 651.
(13) The possible coordination modes of N_2O: μ-1,3-N_2O, μ-3,1-N_2O, μ-1,1-N, μ-1,1-O, η^2, N end-on, O end-on.
(14) The optimized N−N and N−O distances are 1.180 and 1.247 Å (BP86/LanL2DZ) and 1.143 and 1.192 Å (BP86/6-311G*), respectively.
(15) The O atom charge in the $Cu_Z(1Cu^{II}/3Cu^I)-N_2O$ complex is only −0.2 e⁻, which makes this complex deactivated for proton attack relative to the free N_2O molecule where the O atom charge is −0.3 e⁻. In addition, the $1Cu^{II}/3Cu^I$ form has a greater Coulombic barrier for proton attack due to its higher positive charge.

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