Nitrous oxide reductase (N₂OR) catalyzes the two-electron reduction of N₂O to N₂ and H₂O in the last step of the bacterial denitification process.1-4 It is a dimeric protein. The recently solved crystal structure of N₂O indicates that in each subunit there is a Cu₄ center, which is the electron-transfer site, and a CuZ center, which is the catalytic site. The neighboring Cu₄ and CuZ centers are from different subunits.5,6 The CuZ center has a strikingly new structural motif consisting of a μ₄-sulfide bridged tetranuclear cluster. The whole CuZ cluster is coordinated by seven His ligands with a weakly bound water at the Cu/Cu₄ edge (atom numbering is defined in Figure 2A, vide infra), which is the substrate access site. Our previous studies have shown that the CuZ center in dithionite-reduced N₂OR (the resting form) is a partially delocalized S = 1/2, 1Cu²/3Cu¹ cluster.7 This cluster provides a mechanism for overcoming the reaction barrier of N₂O reduction by a simultaneous two-electron reduction pathway to the substrate bound in a μ₁-3-bridging mode.7,8 In this study, we experimentally determine that the redox active form of the CuZ cluster in enzymatic turnover is the all-reduced 4Cu¹ form by using a combination of activity determinations and EPR spectroscopy. This is the first demonstration that the S = 1/2 form of CuZ can be further reduced. DFT calculations were performed to provide insight into the nature of N₂O binding to and activation by this all-reduced 4Cu¹ form relative to the 1Cu²/3Cu¹ resting form of the CuZ site.

Activity studies of the enzyme were performed spectrophotometrically, 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.5,12 The activity of the Pseudomonas nautica N₂OR 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) of the resting 1Cu²/3Cu¹ CuZ 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 CuZ is a one-hole system (1Cu²/3Cu¹),7,8 the gradual decrease of the EPR signal on incubating with methyl viologen and dithionite indicates that the CuZ cluster is reduced to the 4Cu¹ form. Therefore, the direct correlation of enzyme activity with the reduction of the CuZ (k = 0.07 min⁻¹ for both fits. Inset: Decrease in the CuZ EPR signal, measured at different incubation times in reducing conditions. EPR spectra measured at 77 K. 9,319 GHz. (**) Methyl viologen radical signal.

CuZ is a one-hole system (1Cu²/3Cu¹),7,8 the gradual decrease of the EPR signal on incubating with methyl viologen and dithionite indicates that the CuZ cluster is reduced to the 4Cu¹ form. Therefore, the direct correlation of enzyme activity with the reduction of the CuZ (k = 0.07 min⁻¹ for both fits) indicates that the redox active form of the enzyme is the 4Cu¹ state. It should be further noted that the slow rate of reduction of the resting CuZ center (1Cu²/3Cu¹) indicates that it cannot be in the catalytic cycle, and the rate of reoxidation of the CuZ center must be fast in enzymatic turnover.

Density functional calculations were performed to study the N₂O binding to and activation by the CuZ cluster. The CuZ 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 CuZ cluster (Cu₁ and Cu₄b), and the N₂O ligand has several coordination modes.8,13 The lowest-energy structures of the CuZ-N₂O complex are shown in Figure 2. The 4Cu¹ form prefers the μ₁-3-N₂O bridging mode. In the inactive 1Cu²/3Cu¹ form, N₂O is coordinated through the N atom to Cu₄ in an end-on fashion. The binding energy (at the BP86/6-311G* level of theory) of N₂O to CuZ is higher for the 4Cu¹ form (∆Eₙ = −18.5 kcal mol⁻¹) than that for the 1Cu²/3Cu¹ form (∆Eₙ = −15.7 kcal mol⁻¹).

In the CuZ(1Cu²/3Cu¹)-N₂O complex, the geometric structure of the N₂O ligand is very similar to the gas-phase structure of N₂O.14 The situation is quite different in the CuZ(4Cu¹)-N₂O complex, where the N-N=O bond decreases 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

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the free $\text{N}_2\text{O}$ molecule and in the CuII/3CuI

Figure 3. (A) Contributions of $\text{N}_2\text{O}$, CuI, and CuIV to the density of states (DFT-optimized geometries of the $\text{N}_2\text{O}$ complexes with all-electron charges of the $\text{N}_2\text{O}$ ligand in the CuII/3CuI)

Figure 2. (A) Contributions of $\text{N}_2\text{O}$, CuI, and CuIV to the density of states (DFT-optimized geometries of the $\text{N}_2\text{O}$ complexes with all-electron charges of the $\text{N}_2\text{O}$ ligand in the CuII/3CuI)

the free $\text{N}_2\text{O}$ molecule and in the CuII/3CuI form. The very bent geometry of $\text{N}_2\text{O}$ in the complex causes a 2 eV splitting of the degenerate LUMO of $\text{N}_2\text{O}$ into two nondegenerate LUMO* orbitals and stabilizes the resultant in-plane LUMO by $\sim 3$ eV due to loss in antibonding character (Figure 3B). This shifts the LUMO closer to the fully occupied d orbitals of the 4CuI cluster and makes the $\text{N}_2\text{O}$ ligand a better electron acceptor.

The LUMO of the CuII (4CuI) – $\text{N}_2\text{O}$ complex (Figure 3A) is 54% $\pi^*$ orbital of $\text{N}_2\text{O}$ and contains 12 and 10% d orbital contributions from CuI and CuIV, respectively. The Mulliken and natural electron charges of the $\text{N}_2\text{O}$ ligand in the CuII (4CuI) – $\text{N}_2\text{O}$ complex are both around $-0.53$ a.u., indicating a very substantial back-donation from the Cu d orbitals to the $\pi^*(\text{N}_2\text{O})$ orbitals. In comparison, the charge of $\text{N}_2\text{O}$ in the CuII (1CuI/3CuI) – $\text{N}_2\text{O}$ complex is only around $-0.1$ a.u. The large charge transfer from the CuII (4CuI) cluster to $\text{N}_2\text{O}$ in the complex plays a crucial role in $\text{N}_2\text{O}$ activation, and it is clear that the all-reduced form of CuII is better for back-bonding than the 1CuI/3CuI form.

With respect to possible reaction mechanisms, the O atom of $\text{N}_2\text{O}$ in the CuII (4CuI) – $\text{N}_2\text{O}$ 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$_2$O $\pi$ orbital, which has 66% O character with the occupied Cu d orbitals. This reduced state of CuII can thus serve as an efficient two-electron reducing agent. The created electron holes on CuI and CuIV by the 2 e$^-$ reduction of $\text{N}_2\text{O}$ can be efficiently delocalized through the bridging sulfide $\sigma$ 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 CuII forms.

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Supporting Information Available: Procedure for activity determinations, quantification of EPR signal intensities, calculation details and DFT-optimized structures of CuII – $\text{N}_2\text{O}$ complexes, orbital decomposition analysis data for the frontier molecular orbitals of CuII (4CuI) – $\text{N}_2\text{O}$, and detailed density-of-states plots (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

References
(13) The possible coordination modes of $\text{N}_2\text{O}$:
(14) The optimized $\text{N}_2\text{O}$ structure:
(15) The O atom charge in the CuII (4CuI) – $\text{N}_2\text{O}$ complex is only $0.2$ e$^-$, which makes this complex deactivated for proton attack relative to the free $\text{N}_2\text{O}$ molecule where the O atom charge is $-0.3$ e$^-$.

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