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Determination of the active form of the tetranuclear copper sulfur cluster in nitrous oxide reductase

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Abstract

N₂OR has been found to have two structural forms of its tetranuclear copper active site, the 4CuS Cu_Z^{*} form and the 4Cu₂S Cu_Z form. EPR, resonance Raman, and MCD spectroscopies have been used to determine the redox states of these sites under different reductant conditions, showing that the Cu_Z^{*} site accesses the 1-hole and fully reduced redox states while the Cu_Z site accesses the 2-hole and 1-hole redox states. Single turnover reactions of N₂OR for Cu_Z and Cu_Z^{*} poised in these redox states and steady state turnover assays with different proportions of Cu_Z and Cu_Z^{*} show that only fully reduced Cu_Z^{*} is catalytically competent in rapid turnover with N₂O.

Nitrous oxide reductase (N₂OR) is a copper containing metalloenzyme that performs the last step of bacterial denitrification, the two electron reduction of nitrous oxide to dinitrogen and water. This reaction completes the nitrogen cycle and prevents the release of nitrous oxide, a potent greenhouse gas and ozone-depleting agent, into the atmosphere.^{1,2} N₂O reduction is thermodynamically favorable, but a catalyst is required to overcome the 59 kcal/mol activation barrier for this reaction in the gas phase.³ Nitrous oxide reductase contains two multinuclear copper sites, a binuclear Cu_A electron transfer site and a tetranuclear copper-sulfide cluster, Cu_Z, the active site, at which N₂O binds and reduction occurs. Defining the tetranuclear copper site and its interaction with N₂O are key to understanding the catalytic properties of N₂OR.

Structural and spectroscopic characterization of N₂ORs before 2011 showed that the active site is a unique tetranuclear copper site with a μ_4 bridging sulfide ligand and an accessible edge where a solvent-derived ligand is coordinated, proposed to be the site of N₂O binding (Cu_I-Cu_{IV} in Figure 1A).^{4,5} The electronic structure and reactivity of this cluster, known as Cu_Z^{*}, have been extensively characterized. The resting redox state of Cu_Z^{*} is the 3Cu^I1Cu^{II} (1-hole) state⁶ and a slow reductive activation using the reductant reduced methyl viologen (also used for steady-state turnover assays of N₂OR) is required to access the 4Cu^I (fully reduced) redox state of Cu_Z^{*}, which is its active state in N₂O reduction.^{7,8} However, a recent X-ray crystal structure has revealed a different structural form of the active site in enzyme purified in the absence of oxygen.⁹ This form, known as Cu_Z,¹⁰ contains a second sulfur ligand bound on the Cu_I-Cu_{IV} edge of the tetranuclear copper site (Figure 1B). Since

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Supporting Information. Experimental details, time course of the MV reduction, resonance Raman of N₂O reduction by 1-hole Cu_Z, kinetic fits for N₂O reduction, absorption showing no N₂O reaction with 2-hole Cu_Z and modeling of the reaction of MV reduced N₂OR and N₂O. This material is available free of charge via the Internet at <http://pubs.acs.org>.

this $4\text{Cu}_2\text{S}$ Cu_Z cluster is present in an anoxic isolation, it was proposed to be relevant for physiological N_2O reduction. However, the direct reaction of the Cu_Z cluster with N_2O has yet to be demonstrated and compared to that of Cu_Z^* . In this study, using *Marinobacter* (*Ma.*) *hydrocarbonoclasticus* N2OR, we report the redox states and single turnover reactivity of Cu_Z with N_2O to elucidate which redox state of the Cu_Z site reacts with N_2O and to compare this reactivity with the previously reported single turnover reactivity of fully reduced Cu_Z^* .⁷ We determine that 1-hole Cu_Z (along with reduced Cu_A) reacts with N_2O at a very slow rate and only the fully reduced state of Cu_Z^* reacts with N_2O at a rapid rate required for catalytic turnover.

Determining the relative reactivity of Cu_Z and Cu_Z^* with N_2O is complicated by the fact that purified samples of N2OR generally contain a mixture of Cu_Z and Cu_Z^* and in some cases the cluster was not present at full occupancy.^{10,11} In the case of *Ma. hydrocarbonoclasticus* N2OR, preparations of N2OR containing exclusively Cu_Z^* at full occupancy have been obtained from broken cells that have been stored frozen followed by purification in the presence of oxygen (see SI).¹² N2OR samples purified with this procedure have spectral features characteristic of 1-hole Cu_Z^* (an absorption band at 640 nm) and oxidized (i.e. $2\text{Cu}^{1.5}$) Cu_A . Enzyme preparations containing high amounts of Cu_Z have previously been obtained from the periplasmic extract of fresh cells followed by purification in the absence of oxygen¹³ or by rapid chromatographic purification in the presence of O_2 using only two chromatographic separation steps.¹⁴ The latter method was used to obtain the Cu_Z -containing samples of *Ma. hydrocarbonoclasticus* N2OR used in this study (see SI). Under both sets of conditions, the isolated enzyme has full cluster occupancy and spectral features characteristic of Cu_Z in the $2\text{Cu}^{\text{II}}2\text{Cu}^{\text{I}}$ (2-hole) redox state^{10,15} (an absorption band at 540 nm; 1-hole Cu_Z has an absorption band at 680 nm) and some oxidized Cu_A .

The amounts of Cu_Z and Cu_Z^* present in a sample could be determined by EPR spin quantitation after addition of a reductant chosen to select specific redox states of the Cu_A , Cu_Z and Cu_Z^* sites (Table 1).¹⁰ The specific redox states present with a given reductant in Table 1 was determined by resonance Raman spectroscopy. Excitation at 676 nm, a wavelength where 2-hole Cu_Z , 1-hole Cu_Z and 1-hole Cu_Z^* all have absorption intensity, yields a resonance Raman spectrum for an ascorbate-reduced sample (Figure 2A black) that shows the characteristic vibrations of 2-hole Cu_Z (350 and 407 cm^{-1}) and 1-hole Cu_Z^* (378 cm^{-1}).¹⁵ In this state Cu_A is reduced (2Cu^{I}) and 2-hole Cu_Z is diamagnetic, so spin quantitation of the EPR spectrum (Figure 2B black) yields the amount of 1-hole Cu_Z^* . Subsequent reduction by sodium dithionite results in an increase in the EPR spin intensity (Figure 2B red) as the 2-hole Cu_Z state is reduced to the 1-hole redox state. This is accompanied by the appearance of three characteristic vibrations for 1-hole Cu_Z in the resonance Raman spectrum (Figure 2A red) at 203 , 378 , and 492 cm^{-1} (the vibrations characteristic of 1-hole Cu_Z^* are still present but overlap those of 1-hole Cu_Z). Spin quantitation of a dithionite reduced sample thus yields the total tetranuclear cluster concentration (the sum of 1-hole Cu_Z and 1-hole Cu_Z^*). Comparison of the ascorbate reduced and dithionite reduced spin intensities further yields the amount of Cu_Z . In this manner, comparison of the EPR spin intensities of a preparation of *Ma. hydrocarbonoclastes* N2OR (purified by rapid chromatography in the presence of O_2) after ascorbate reduction and dithionite reduction (Figure 2B black and red spectra, respectively) shows that this purification yields enzyme containing 35% Cu_Z^* and 65% Cu_Z .

Further reduction of this sample by excess reduced methyl viologen, which is known to reduce Cu_Z^* to the fully reduced state,⁷ can be monitored by both EPR (Figure 2B blue) and resonance Raman spectroscopies (Figure 2A blue) to determine the redox state of Cu_Z present under steady-state assay conditions. After prolonged methyl viologen reduction (Figure S1), EPR spin quantitation shows that 65% of the copper sites remain oxidized

(Figure 2B blue). The resonance Raman spectrum continues to exhibit the characteristic vibrations of 1-hole Cu_Z (Figure 2A blue). Thus, the $4\text{Cu}_2\text{S}$ Cu_Z site does not access the fully reduced redox state and is present in the 1-hole redox state under steady-state assay conditions (excess reduced methyl viologen).

Since the redox states of Cu_Z and Cu_Z^* under different reduction conditions are now defined (Table 1), reduction followed by a single turnover reaction with N_2O can be used to determine the intrinsic reactivity of the accessible redox states of Cu_Z with N_2O . In these experiments N_2OR containing 65% Cu_Z was incubated with one of the three reductants (ascorbate, dithionite, or reduced methyl viologen) to select the redox states of the Cu_Z and Cu_Z^* sites (Table 1). Subsequently the reductant was removed and the single turnover reaction of the reduced enzyme with a stoichiometric amount of N_2O was monitored by UV/Vis absorption spectroscopy. When ascorbate is used as the reductant to select the 2-hole redox state of Cu_Z , no reaction with N_2O occurs (Figure S2), indicating that neither the 2-hole redox state of Cu_Z nor the 1-hole redox state of Cu_Z^* can react with N_2O even in the presence of reduced Cu_A . In contrast, when dithionite reduction is used to select the 1-hole redox state of Cu_Z (and reduce Cu_A), the N_2O reaction results in changes in the absorption spectrum that occur with a pseudo first order rate constant of 0.6 hr^{-1} (Figure S3), indicating a slow reaction with N_2O (Figure 3A). In the course of the reaction, the absorption band at $14,700 \text{ cm}^{-1}$ that is dominantly due to 1-hole Cu_Z (Figure 3A inset, blue), decays and an absorption feature forms at $17,850 \text{ cm}^{-1}$, characteristic of 2-hole Cu_Z . The formation of 2-hole Cu_Z is also supported by the appearance of resonance Raman vibrations at 350 and 407 cm^{-1} (see Figure S4).

The absorption of 2-hole Cu_Z overlaps with the region where absorption features of Cu_A would contribute at $18,500$ and $20,800 \text{ cm}^{-1}$, so it is not clear from only the absorption spectrum whether Cu_A is also oxidized in this reaction. Magnetic circular dichroism (MCD) was used to determine this, since 2-hole Cu_Z is diamagnetic and MCD silent,¹⁰ while oxidized Cu_A has an intense MCD pseudo-A feature centered at $20,000 \text{ cm}^{-1}$. The MCD spectrum early (after 30 seconds, Figure 3B solid) and late (after 3 hours, Figure 3B dashed) in the single turnover reaction of dithionite reduced N_2OR shows that Cu_A is indeed oxidized in this N_2O reduction on the same timescale as Cu_Z oxidation. Thus the reaction of 1-hole Cu_Z containing N_2OR with N_2O is a two electron process in which 1-hole Cu_Z is oxidized to 2-hole Cu_Z and reduced Cu_A is oxidized by one electron. N_2O reduction by 1-hole Cu_Z and reduced Cu_A occurs with a rate which is six orders of magnitude slower than the turnover number of 321 s^{-1} determined for this enzyme.¹⁶ This strongly indicates that while 1-hole Cu_Z (with Cu_A) does react with N_2O , it is not kinetically competent in catalysis under steady state conditions.

To verify this, reduction with methyl viologen was used to select the redox states of Cu_Z and Cu_Z^* present under steady-state assay conditions, 1-hole Cu_Z and fully reduced Cu_Z^* (Table 1). After removal of methyl viologen and addition of N_2O , a complicated single turnover reaction occurs, with contributions from a fast process and two slower processes (see Figure S5). These data are well simulated by adding a 35% contribution from the fast single turnover reaction of fully reduced Cu_Z^* with N_2O to a 65% contribution from the slow reaction of 1-hole Cu_Z with N_2O observed with dithionite reduced N_2OR . Thus, the fast phase is due to rapid oxidation of fully reduced Cu_Z^* and Cu_A upon N_2O reduction by Cu_Z^* . A lower limit for the rate of this rapid oxidation is 200 s^{-1} (see Figure S6), which is comparable to the k_{cat} of active N_2OR .¹⁶ The two slower processes observed are the decay of Cu^{O}_Z (an intermediate in the single turnover reaction of fully reduced Cu_Z^* with N_2O)¹⁷ to resting 1-hole Cu_Z^* and the slow reaction of 1-hole Cu_Z with N_2O with formation of 2-hole Cu_Z .

Further support for this determination that fully reduced Cu_Z^* is the active form of the cluster under steady state assay conditions was obtained by comparing the steady-state activities of *Ma. hydrocarbonoclasticus* N_2OR obtained from the two preparation methods described above, the first using fresh cells with rapid chromatographic purification to maximize Cu_Z content and the second using standard aerobic purification conditions. The relative amounts of Cu_Z and Cu_Z^* determined from EPR spin integration and the steady state activities (assayed according to previously published procedures) for these enzyme samples are reported in Table 2. A clear correlation is observed between the steady-state activity of N_2OR and the amount of Cu_Z^* but not Cu_Z present. This is consistent with the observation that high steady-state activity for N_2OR is only observed after reductive activation by prolonged incubation with methyl viologen. Since 1-hole Cu_Z is not reduced by methyl viologen (Table 1 and Figure 2), the only new species formed during the reductive activation is fully reduced Cu_Z^* , which is responsible for the high activity.

The single turnover and steady-state reactivity results presented here demonstrate that under steady-state assay conditions fully reduced Cu_Z^* is the active form of the tetranuclear cluster in N_2OR . While 1-hole Cu_Z will reduce N_2O , only fully reduced Cu_Z^* is kinetically competent to be involved in steady-state turnover. While the conditions used for steady-state assays of N_2OR are non-physiological, the reaction of 1-hole Cu_Z with N_2O would be an essential step in any catalytic cycle involving Cu_Z . The slow rate of this process in single-turnover experiments indicates that 1-hole Cu_Z is likely not the active species *in vivo*. High reactivity for the tetranuclear copper cluster with N_2O thus reflects contributions from both the ability of Cu_Z^* to access the fully reduced redox state and the presence of an open coordination site at the Cu_I - Cu_IV edge; neither are available for Cu_Z . The importance of this open edge is consistent with iodide inhibition of turnover, where crystallography shows that iodide bridges the Cu_I - Cu_IV edge in a similar fashion to the $\mu_2\text{S}$ in Cu_Z .¹⁸

While it is not kinetically competent in turnover, the 1-hole redox state of Cu_Z does react with N_2O , a two electron process which appears to involve one electron from 1-hole Cu_Z and one electron from the reduced Cu_A site. This is consistent with the crystal structure of N_2O bound to anaerobic N_2OR which shows an N_2O molecule localized between the Cu_Z and Cu_A sites.⁹

The reduction experiments described here show that the $4\text{Cu}_2\text{S}$ Cu_Z site is not reduced past the 1-hole state under the reductant conditions used in turnover (Table 1). Thus, the Cu_Z site has two accessible redox states, the 2-hole resting state and the 1-hole reduced state. This differs from the redox properties of the Cu_Z^* site, where the 1-hole state is the resting form and the fully reduced state is accessible under the assay conditions in Table 1. These different redox properties likely relate to the additional $\mu_2\text{S}$ ligand. This highly covalent sulfide donor ligand would stabilize the more oxidized redox state of Cu_Z , while the solvent derived edge ligand in Cu_Z^* is a poorer donor and would enable further Cu_Z^* reduction. A full analysis of the different redox properties of the Cu_Z site requires the determination of the protonation state of its $\mu_2\text{S}$ ligand in the different redox states of the Cu_Z cluster, which is presently underway.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Crystal structures of (A) Cu₂* derived from aerobically prepared N₂OR and (B) Cu₂ derived from anaerobically prepared N₂OR. Copper shown in green, sulfur yellow, carbon grey, oxygen red, and nitrogen blue.

Figure 2. Comparison of N₂OR containing 65% Cu_Z and 35% Cu_Z* after reduction by ascorbate (black), sodium dithionite (red), and methyl viologen (blue). A) Resonance Raman at 77 K obtained by laser excitation at 676 nm. B) EPR at 77 K.

Figure 3.

A) Absorption spectra showing the progress of the single turnover reaction of N_2O with dithionite reduced N2OR containing 65% Cu_Z and 35% Cu_Z^* . Inset: Absorption spectra of 2-hole Cu_Z (black), 1-hole Cu_Z (blue) and 1-hole Cu_Z^* (red). B) MCD spectra at 5 K and 7 T, showing the oxidation of Cu_A after 0.5 min (solid) and 3 hours (dashed) of the reaction of N_2O with dithionite reduced N2OR containing 65% Cu_Z and 35% Cu_Z^* .

Table 1

Redox states of the Cu_A, Cu_Z, and Cu_Z* sites present and the reductant used.

Reductant	Redox and Spin State Present		
	Cu _A	Cu _Z	Cu _Z *
ascorbate	2Cu ^I , S=0	2-hole, S=0	1-hole, S=½
dithionite	2Cu ^I , S=0	1-hole, S=½	1-hole, S=½
methyl viologen	2Cu ^I , S=0	1-hole, S=½	fully reduced, S=0

Table 2

Cu_Z* and Cu_Z percentage, specific activity and normalized specific activity for 100% Cu_Z* for two N₂OR samples, the first prepared via rapid chromatography and the second via standard aerobic purification. The percentages of Cu_Z* and Cu_Z were determined by EPR.

% Cu _Z *	% Cu _Z	Specific Activity (μmol N ₂ O min ⁻¹ mg ⁻¹)	Specific Activity Normalized to 100% Cu _Z *
35±5%	65±5%	54 ± 3	154 ± 9
96±14%	4±14%	141 ± 7	147 ± 7