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Determination of the Active Form of the Tetranuclear Copper Sulfur Cluster in Nitrous Oxide Reductase

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Supporting Information

ABSTRACT: N_2OR 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 N_2OR 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 N_2O.

Nitrous oxide reductase (N_2OR) 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] N_2O 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 N_2O binds and reduction occurs. Defining the tetranuclear copper site and its interaction with N_2O is key to understanding the catalytic properties of N_2OR.

Structural and spectroscopic characterization of N_2ORs 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 N_2O 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) state,[6] and a slow reductive activation using the reductant reduced methyl viologen (MV, also used for steady-state turnover assays of N_2OR) is required to access the $4Cu^I$ (fully reduced) redox state of Cu_Z*, which is its active state in N_2O 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 this 4Cu2S 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* N_2OR, 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.

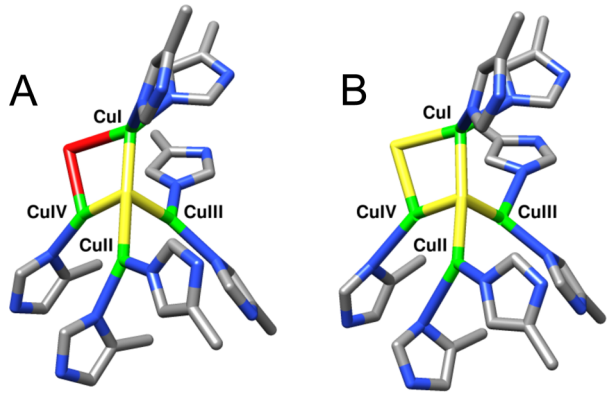

Figure 1. Crystal structures of (A) Cu_Z* derived from aerobically prepared N_2OR and (B) Cu_Z derived from anaerobically prepared N_2OR. Copper shown in green, sulfur yellow, carbon gray, oxygen red, and nitrogen blue.

Determining the relative reactivity of Cu_Z and Cu_Z* with N_2O is complicated by the fact that purified samples of N_2OR 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* N_2OR, preparations of N_2OR containing exclusively Cu_Z* at full occupancy have been obtained from broken cells that have been stored frozen,

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followed by purification in the presence of oxygen (see SI).[12] N_2OR samples purified with this procedure have spectral features characteristic of 1-hole Cu_Z* (an absorption band at 640 nm) and oxidized (i.e., $2Cu^{1.5}$) 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 oxygen[13] 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* N_2OR 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 $2Cu^{II}2Cu^{I}$ (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 were 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)

Table 1. Redox States of the Cu_A, Cu_Z, and Cu_Z* Sites Present and the Reductant Used

	redox and spin state present		
reductant	Cu_A	Cu_Z	Cu_Z*
ascorbate	$2Cu^{I}$, $S = 0$	2-hole, $S = 0$	1-hole, $S = 1/2$
dithionite	$2Cu^{I}$, $S = 0$	1-hole, $S = 1/2$	1-hole, $S = 1/2$
methyl viologen	$2Cu^{I}$, $S = 0$	1-hole, $S = 1/2$	fully reduced, $S = 0$

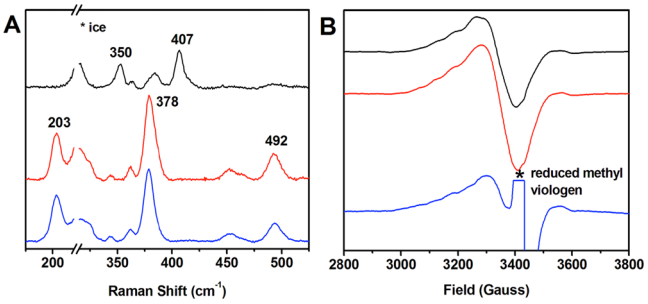

Figure 2. Comparison of N_2OR containing 65% Cu_Z and 35% Cu_Z* after reduction by ascorbate (black), sodium dithionite (red), and methyl viologen (blue). (A) Resonance Raman at 77 K obtained by laser excitation at 676 nm. (B) EPR at 77 K.

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* N_2OR (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 MV, 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 MV 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 4Cu2S 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 MV).

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 MV) 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 h^{-1} (Figure S3), indicating a slow reaction with N_2O (Figure 3A). In the course of the reaction, the absorption band at 14 700 cm^{-1} that is dominantly due to 1-hole Cu_Z (Figure 3A inset, blue) decays

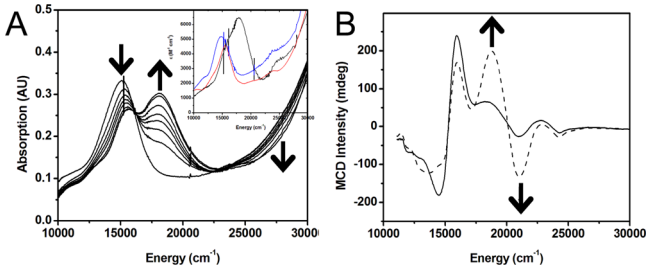

Figure 3. (A) Absorption spectra showing the progress of the single-turnover reaction of N_2O with dithionite-reduced N_2OR containing 65% Cu_Z and 35% Cu_Z*. Inset: Absorption spectra of 2-hole Cu_Z (black), 1-hole Cu_Z (blue), and 1-hole Cu_Z* (red). (B) MCD spectra at 5 K and 7 T, showing the oxidation of Cu_A after 0.5 min (solid) and 3 h (dashed) of the reaction of N_2O with dithionite-reduced N_2OR containing 65% Cu_Z and 35% Cu_Z*.

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and an absorption feature forms at 17 850 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 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 cm^{-1}. The MCD spectrum early (after 30 s, Figure 3B, solid) and late (after 3 h, 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 time scale 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 *6 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 MV 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 MV 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_Z^o (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 MV. Since 1-hole Cu_Z is not reduced by MV (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 nonphysiological, 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 is 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 μ_2S in Cu_Z.[18]

Table 2. Cu_Z* and Cu_Z Percentage, Specific Activity, and Normalized Specific Activity for 100% Cu_Z* for Two N_2OR samples, the First Prepared via Rapid Chromatography and the Second via Standard Aerobic Purification[a]

% Cu_Z*	% Cu_Z	specific activity (μmol N_2O 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

[a]The percentages of Cu_Z* and Cu_Z were determined by EPR.

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 4Cu2S 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 μ_2S 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 μ_2S ligand in the different redox states of the Cu_Z cluster, which is presently underway.

■ ASSOCIATED CONTENT

Supporting Information

Experimental details, time course of the MV reduction, resonance Raman of N_2O reduction by 1-hole Cu_Z, kinetic fits for N_2O reduction, absorption showing no N_2O reaction with 2-hole Cu_Z, and modeling of the reaction of MV-reduced N_2OR and N_2O. 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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