Characterization of the Copper−Sulfur Chromophores in Nitrous Oxide Reductase by Resonance Raman Spectroscopy: Evidence for Sulfur Coordination in the Catalytic Cluster

Marcela L. Alvarez,[†] Jingyuan Ai,[‡] Walter Zumft,[§] Joann Sanders-Loehr,*[,‡] and David M. Dooley*[,†]

Contribution from the Department of Chemistry and Biochemistry, Montana State University, Bozeman, Montana 59717, Lehrstuhl für Mikrobiologie, Universität Fridericiana, D-76228 Karlsruhe, Germany, and Department of Biochemistry and Molecular Biology, Oregon Graduate Institute of Science and Technology, Beaverton, Oregon 97006-8921

Received December 9, 1999. Revised Manuscript Received September 19, 2000

Abstract: Nitrous oxide reductase (N_2OR) from *Pseudomonas stutzeri*, a dimeric enzyme with a canonical metal ion content of at least six Cu ions per subunit, contains two types of multinuclear copper sites: Cu_A and Cu_Z. An electron-transfer role for the dinuclear Cu_A site is indicated based on its similarity to the Cu_A site in cytochrome *c* oxidase (CcO), a dicysteinate-bridged, mixed-valence cluster. The Cu_Z site is the catalytic site, which had long been thought to have novel spectroscopic properties. However, the low-energy electronic transitions and resonance Raman features attributable to Cu_Z have been difficult to reconcile with a lack of conserved cysteine residues in standard alignments of N_2OR sequences, other than those associated with the Cu_A site. Recent evidence indicates that nitrous oxide reductase contains acid-labile sulfide and that this sulfide is a constituent of the Cu_Z site (Rasmussen, T.; Berks, B. C.; Sanders-Loehr, J.; Dooley, D. M.; Zumft, W. G.; Thomson, A. J. *Biochemistry* **2000**, *39*, 12753−12756). We have used resonance Raman (RR) spectroscopy to selectively probe the Cu_A and Cu_Z sites of N_2OR in three oxidation states (oxidized, semireduced, and reduced) as well as Cu_A-only and Cu_Z-only variants. The Cu_A (mixed-valence, also designated as A_{mv}) RR spectrum exhibits 10 vibrational modes between 220 and 410 cm^{-1}, with >1-cm^{-1} ^{34}S isotope shifts that sum to −16.6 cm^{-1}. Many of these modes are also sensitive to ^{65}Cu and ^{15}N(His) and, thus, can be assigned to coupling of the Cu−S stretch, ν(Cu−S), with cysteine and histidine vibrations of the $Cu_2Cys_2His_2$ core. The RR spectrum of the Cu_Z site (Z_{ox}) reveals a novel Cu−sulfur chromophore with four S isotope-sensitive modes at 293, 347, 352, and 408 cm^{-1}, with a total ^{34}S shift of −19.9 cm^{-1}. The magnitude of the S isotope shifts and wide spread of perturbed frequencies are similar to those observed in Cu_A and therefore suggest a sulfur-bridged cluster in Z_{ox}. The Z_{ox} site has its ν(Cu−S)-containing modes at higher energy and exhibits less mixing with ligand deformations, compared to Cu_A. Reduction by dithionite produces a mixed-valence Cu_Z site (Z_{mv}) with six S isotope-sensitive RR modes between 282 and 382 cm^{-1} and a total ^{34}S-shift of −16.9 cm^{-1}. The observation of a nearly identical RR spectrum in the C622D variant of N_2OR, which lacks one of the conserved Cu_A Cys residues, establishes that Cu−S vibrations observed in this variant arise from the Z_{mv} site. Furthermore, none of the features assigned to Cu_Z are detected in a second variant that contains only Cu_A. Therefore the resonance Raman spectra reported here provide compelling evidence for a unique Cu−S cluster in the catalytic site of nitrous oxide reductase.

Introduction

Denitrification, the process by which nitrate is converted to dinitrogen by many soil and marine bacteria, is a key component of the global nitrogen cycle.[1−3] All four steps in this pathway (eq 1) are catalyzed by metalloproteins. In addition, the enzymatic reduction of nitrate, nitrite, and nitrous oxide is coupled to ATP synthesis via proton translocation and the formation of a membrane potential, thereby constituting anaerobic respiration in these microorganisms. Nitrous oxide reductase (N_2OR) catalyzes the terminal step of denitrification, which is the two-electron reduction of nitrous oxide to water and dinitrogen (eq 2).

$$2NO_3^- \rightarrow 2NO_2^- \rightarrow 2NO \rightarrow N_2O \rightarrow N_2 \quad (1)$$

$$N_2O + 2e^- + 2H^+ \rightarrow N_2 + H_2O \quad (2)$$

The recent X-ray crystal structure of *Pseudomonas nautica* nitrous oxide reductase[4] revealed the presence of two distinct multinuclear copper sites: a dinuclear, dithiolate-bridged cluster, essentially identical to the structurally characterized Cu_A site in cytochrome oxidase; and a novel tetranuclear copper cluster,

* To whom correspondence should be addressed: (phone) (406) 994-4801; (fax) (406) 994-5407; (e-mail) dmdooley@montana.edu.
[†] Montana State University.
[‡] Oregon Graduate Institute of Science and Technology.
[§] Universität Fridericiana.

(1) Zumft, W. G.; Viebrock, A.; Körner, H. In *The Nitrogen and Sulfur Cycles*; Cole, J. A., Ferguson, S. J., Eds.; Cambridge University Press: Cambridge, U.K., 1988; p 245.
(2) Berks, B. C.; Ferguson, S. J.; Moir, J. W. B.; Richardson, D. J. *Biochim. Biophys. Acta* **1995**, *1232*, 97−173.
(3) Zumft, W. G. *Microbiol. Mol. Biol. Rev.* **1997**, *61*, 533−616.
(4) Brown, K.; Tegoni, M.; Prudêncio, M.; Pereira, A. S.; Besson, S.; Moura, J. J.; Moura, I.; Cambillau, C. *Nat. Struct. Biol.* **2000**, *7*, 191−195.

10.1021/ja994322i CCC: $20.00
Published on Web 12/30/2000

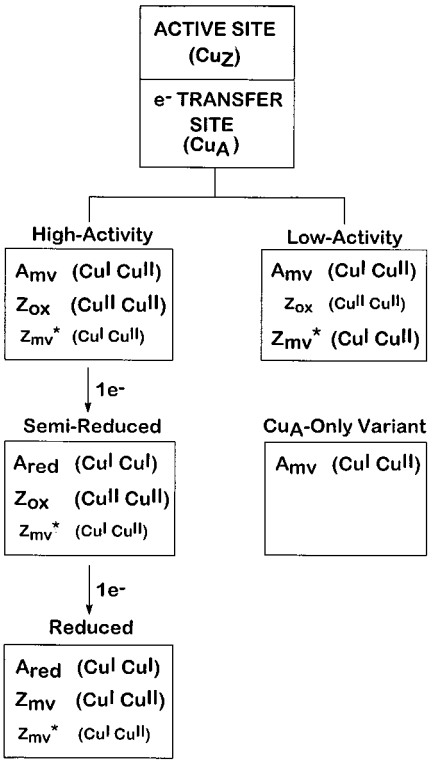

Figure 1. Oxidation states of Cu_A and Cu_Z sites in different forms of N_2OR. Only two of the four copper ions in the tetranuclear Cu_Z cluster are indicated. The dinuclear A species appear to have bridging cysteine thiolate ligands and are located at the electron-transfer site, whereas all of the multinuclear Z species are bridged by sulfide[22] and are located at the active site.

which is the first example of such a catalytic copper cluster in biology. The crystal structure is that of the reduced state, characterized by an absorption band at ∼640−650 nm at 100 K. Notably, the current crystallographic model for the catalytic cluster features seven histidine imidazole ligands and three ligands modeled as water or hydroxide distributed over the four copper ions.

Spectroscopic data, including multifrequency EPR,[5−9] ENDOR,[10] EXAFS,[11,12] magnetic circular dichroism (MCD),[7,11−13] and resonance Raman (RR) data[14,15] on the native, high-activity enzyme, had been interpreted in terms of two multinuclear copper sites: Cu_Z and Cu_A (Figure 1). The dinuclear Cu_A sites in nitrous oxide reductase and cytochrome *c* oxidase (CcO) are mixed-valence [Cu(1.5)·Cu(1.5)] units with two bridging cysteine thiolates, two terminal histidines, and a Cu−Cu distance of 2.5 Å.[16,17] In analogy to the established electron-transfer function of Cu_A in CcO, Cu_A in N_2OR is presumed to transfer electrons from the physiological donor to substrate bound in the catalytic site. The spectral features of the mixed-valence Cu_A in N_2OR have been documented and analyzed in considerable detail. In addition, an oxidized copper cluster has been detected in N_2OR that exhibits diamagnetic behavior, absorption bands at 570 and 650 nm, a copper−sulfur RR spectrum, and low-energy, intense circular dichroism (CD) and MCD transitions.[7,12,14,18,19] These spectral features were consistent with the presence of strongly antiferromagnetically coupled [Cu(II)·Cu(II)] centers with at least one coordinated sulfur anion (originally presumed to be cysteine) and were initially correlated to an oxidized form of the catalytic site, Cu_Z. Reduced N_2OR displays an absorption band at ∼650 nm and a broad $S = {}^1/_2$ EPR signal previously attributed to a mixed-valence state of Cu_Z. Cu_Z was formulated as an independent, multinuclear site responsible for the reduction of N_2O. However, standard alignments of N_2OR sequences from six sources reveal no fully conserved cysteines other than the two known Cu_A ligands.[20,21] A major step toward the resolution of the nature of Cu_Z is the very recent finding that N_2OR contains inorganic sulfide, at an apparent stoichiometry of $1S^{2-}$/subunit.[22] This finding led to the proposal that Cu_Z is the first example of a biological copper−sulfide cluster. Here we report detailed resonance Raman spectra that fully support this proposal and establish that Cu_Z is a copper−sulfur chromophore.

Elucidation of the copper centers in N_2OR has been facilitated by examining three oxidation states of the enzyme, i.e., native, semireduced, and reduced, as well as an enzymatically inactive Cu_A-only variant (previously designated N_2OR V), and a Cu_Z-only variant (produced by the C622D mutation). (Different forms of N_2OR from *P. stutzeri* have previously been abbreviated, in ref 4, as follows: N_2OR I, high-activity native enzyme obtained from anaerobic purification; N_2OR II, low-activity native enzyme obtained from aerobic purification; N_2OR III, dithionite-reduced enzyme; N_2OR V, Cu_A-only mutant obtained from a strain defective in the biosynthesis of the catalytic site.) Native N_2OR can be isolated anaerobically in a high-activity "purple" form (previously designated N_2OR I) or aerobically in a low-activity "pink" form (previously designated N_2OR II).[5] Both forms display a distinctive seven-line EPR signal that has been attributed to a delocalized unpaired electron in the Cu_A site (A_{mv}, Figure 1).[9] This mixed-valence site also dominates the absorption spectrum, with a prominent band at 540 nm, a shoulder at 480 nm, and a weaker transition at 780 nm (Figure 2). The Cu_Z site in the native enzyme appears to be present in two states, designated Z_{ox} and Z_{mv}* (Figure 1). While high-activity enzyme contains mainly diamagnetic Z_{ox}, evidence for the mixed-valence form (Z_{mv}*) is manifested as a weak, broad

(5) Riester, J.; Zumft, W. G.; Kroneck, P. M. H. *Eur. J. Biochem.* **1989**, *178*, 751−762.
(6) Neese, F.; Zumft, W. G.; Antholine, W. E.; Kroneck, P. M. H. *J. Am. Chem. Soc.* **1996**, *118*, 8692−8699.
(7) Farrar, J. A.; Thomson, A. J.; Cheesman, M. R.; Dooley, D. M.; Zumft, W. G. *FEBS Lett.* **1991**, *294*, 11−15.
(8) Antholine, W. A.; Kastrau, D. H. W.; Steffens, G. C. M.; Buse, G.; Zumft, W. G.; Kroneck, P. M. H. *Eur. J. Biochem.* **1992**, *209*, 875−881.
(9) Kroneck, P. M. H.; Antholine, W. A.; Riester, J.; Zumft, W. G. *FEBS Lett.* **1988**, *242*, 70−74.
(10) Neese, F.; Kappl, R.; Hüttermann, J.; Zumft, W. G.; Kroneck, P. M. H. *J. Biol. Inorg. Chem.* **1998**, *3*, 53−67.
(11) Scott, R. A.; Zumft, W. G.; Coyle, C. L.; Dooley, D. M. *Proc. Natl. Acad. Sci. U.S.A.* **1989**, *86*, 4082−4086.
(12) Dooley, D. M.; McGuirl, M. A.; Rosenzweig, A. C.; Landin, J. A.; Scott, R. A.; Zumft, W. G.; Devlin, F.; Stephens, P. J. *Inorg. Chem.* **1991**, *30*, 3006−3011.
(13) Farrar, J. A.; Neese, F.; Lappalainen, P.; Kroneck, P. M. H.; Saraste, M.; Zumft, W. G.; Thomson, A. J. *J. Am. Chem. Soc.* **1996**, *118*, 11501−11514.
(14) Dooley, D. M.; Moog, R. S.; Zumft, W. G. *J. Am. Chem. Soc.* **1987**, *109*, 6730−6735.
(15) Andrew, C. R.; Han, J.; de Vries, S.; van der Oost, J.; Averill, B. A.; Loehr, T. M.; Sanders-Loehr, J. *J. Am. Chem. Soc.* **1994**, *116*, 10805−10806.
(16) Tsukihara, T.; Aoyama, H.; Yamashita, E.; Tomizaki, T.; Yamaguchi, H.; Shinzawa-Itoh, K.; Nakashima, R.; Yaono, R.; Yoshikawa, S. *Science* **1995**, *269*, 1069−1074.
(17) Iwata, S.; Ostermeier, C.; Ludwig, B.; Michel, H. *Nature* **1995**, *376*, 660−669.
(18) Dooley, D. M.; Landin, J. A.; Rosenzweig, A. C.; Zumft, W. G.; Day, E. P. *J. Am. Chem. Soc.* **1991**, *113*, 8978−8980.
(19) Farrar, J. A.; Zumft, W. G.; Thomson, A. J. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95*, 9891−9896.
(20) Zumft, W. G.; Dreusch, A.; Lochelt, S.; Cuypers, H.; Friedrich, B.; Schneider, B. *Eur. J. Biochem.* **1992**, *208*, 31−40.
(21) McGuirl, M. A.; Nelson, L. K.; Bollinger, J. A.; Chan, Y.-K.; Dooley, D. M. *J. Inorg. Biochem.* **1998**, *70*, 155−169.
(22) Rasmussen, T.; Berks, B. C.; Sanders-Loehr, J.; Dooley, D. M.; Zumft, W. G.; Thomson, A. J. *Biochemistry*, **2000**, *39*, 12753−12756.

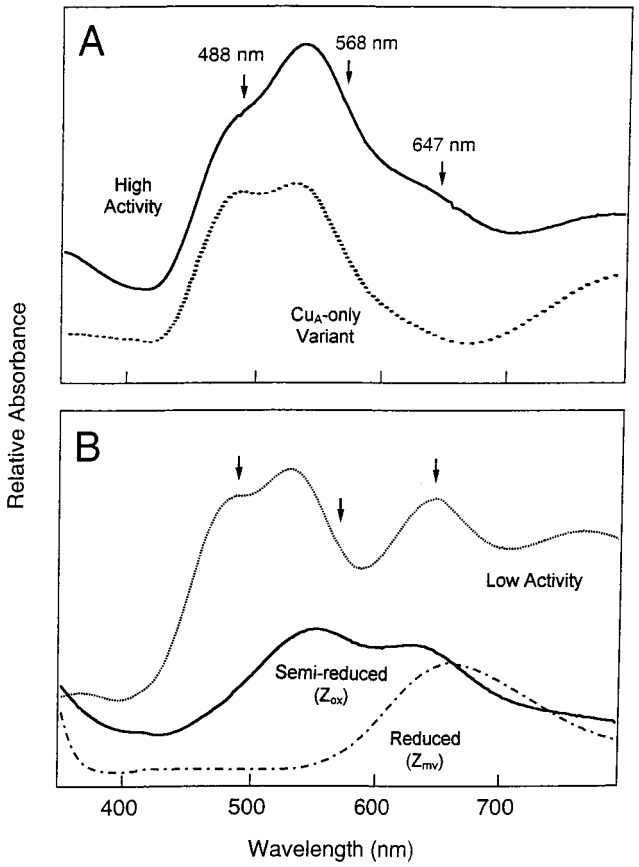

Figure 2. Absorption spectra of different forms of N_2OR. Arrows indicate excitation wavelengths. (A) High-activity (—) and Cu_A-only mutant (···). (B) Low-activity (···), semireduced by ascorbate (—), and reduced by dithionite (- · -).

signal at $g = 2.02$ in the EPR spectrum of native enzyme.[5,23] Further support for the presence of this moiety is exhibited in the absorption spectrum of low-activity N_2OR, which, in addition to the Cu_A-assigned bands, displays a distinct transition at 650 nm (Figure 2). On the basis of the absence of this feature in the spectrum of the Cu_A-only mutant (*5*), as well as its correlation to low enzymatic activity, Z_{mv}* has been ascribed to a blocked or improperly processed form of the Z_{ox} site.[7]

Addition of one electron-equivalent by ascorbate under anaerobic conditions produces semireduced N_2OR, in which the Cu_A site has been reduced to a spectroscopically silent [Cu(I)• Cu(I)] moiety (A_{red}, Figure 1).[7,24] The remaining absorption bands at 550 and 640 nm (Figure 2), which are not present in the analogous spectrum of the Cu_A-only variant, have been attributed to Z_{ox}. Addition of further electron-equivalents yields the blue, reduced N_2OR (previously designated N_2OR III), with a single absorption band at 650 nm (Figure 2). This feature, which is also absent in the absorption spectrum of the reduced Cu_A-only species, has been ascribed to the reduced form of Z_{ox}, characterized as, an $S = {}^1/_2$ EPR-active, mixed-valence cluster (Z_{mv}, Figure 1).[5] Attempts to completely reduce Z_{mv} have failed, despite the addition of large excesses of a variety of reductants.[5]

RR spectroscopy can selectively probe Cu−S vibrations in copper−sulfur chromophores and thus provides a sensitive means of monitoring Cu−S bonding and its contribution to the overall structure of a metal site.[25−27] In mononuclear Cu−cysteinate sites, excitation within the (Cys)S → Cu ligand-to-metal charge-transfer band typically results in the enhancement of at least five vibrational fundamentals between 250 and 500 cm^{-1}.[26] A single predominant Cu−S stretching mode, ν(Cu−S), can be distinguished in these spectra by its high intensity, its large frequency shift upon S or Cu isotope substitution, and its generation of high-frequency combination bands. The remaining weaker fundamentals are now attributed to modes arising from ground-state kinematic coupling of the Cu−S stretch with cysteine−ligand deformations.[28−30] The variations in ν(Cu−S) frequencies among different mononuclear Cu−cysteinate sites have been proposed to serve as sensitive indicators of Cu−S bond lengths and coordination geometries, ranging from trigonal planar (430−405 cm^{-1}) to tetrahedral (405−340 cm^{-1}) to tetragonal (365−300 cm^{-1}).[25] Copper sites containing one cysteine and two histidine ligands in a trigonal to tetrahedral array, as in azurin and stellacyanin, compose the type 1 site of cupredoxins. Also referred to as blue copper centers due to their intense absorbance near 600 nm, these electron-transfer sites are the mononuclear counterparts to the Cu_A site.

In contrast to mononuclear copper−cysteinate sites, the dinuclear Cu_A site exhibits two distinct Cu−S stretching modes, consistent with the presence of two bridging cysteine ligands in its symmetrical $Cu_2S_2N_2$ core.[25,31,32] Moreover, this center does not exhibit the continuum of coordination geometries observed for mononuclear sites but, rather, is a highly conserved, rigid structure, as evidenced by the striking consistency of the ν(Cu−S) frequencies among native Cu_A proteins and engineered constructs.[32] The two ν(Cu−S) modes experience significantly larger ^{34}S isotope shifts (−4 to −5 cm^{-1}) than the major ν(Cu−S) mode of mononuclear sites (−2 to −4 cm^{-1}). This distinction, in addition to differences in their overall ^{34}S isotope-dependence patterns, reflects the relatively pure nature of the $Cu_2S_2His_2$ vibrations and has made a normal coordinate analysis feasible. The results of these calculations[31] support the bridging thiolate structure and near-tetrahedral Cu coordination geometry revealed by X-ray crystal structures of Cu_A sites.[4,16,17]

The present RR study examines the low-energy absorption bands of all three redox states of *Pseudomonas stutzeri* N_2OR and two variants—one lacking Cu_A and the other lacking Cu_Z. Comparison of the Cu_A RR properties with those of Cu_A sites in CcO[15,31,32] and several engineered constructs[30] reveals the remarkable conservation of this rigid dinuclear copper structure, in agreement with the X-ray crystallographic findings.[4] Our ^{34}S isotope substitution studies also address the nature of the coordination in the Cu_Z site of N_2OR and provide the strongest evidence to date that a sulfur ligand is present in Cu_Z. Whereas the RR spectra of Z_{ox} and Z_{mv} reveal a multiplicity of sulfur

(23) Kroneck, P. M. H.; Riester, J.; Zumft, W. G.; Antholine, W. E. *Biol. Metals* **1990**, *3*, 103−109.
(24) Dooley, D. M.; Alvarez, M. L.; Rosenzweig, A. C.; Hollis, R. S.; Zumft, W. G. *Inorg. Chim. Acta* **1996**, *242*, 239−244.
(25) Andrew, C. R.; Sanders-Loehr, J. *Acc. Chem. Res.* **1996**, *29*, 365−372.
(26) Andrew, C.; Yeom, H.; Valentine, J. S.; Karlsson, B. G.; Bonander, N.; van Pouderoyen, G.; Canters, G. W.; Loehr, T. M.; Sanders-Loehr, J. *J. Am. Chem. Soc.* **1994**, *116*, 11489−11498.
(27) Dave, B. C.; Germanas, J. P.; Czernuszewicz, R. S. *J. Am. Chem. Soc.* **1993**, *115*, 12175−12176.
(28) Andrew, C. A.; Han, J.; den Blaauwen, T.; van Pouderoyen, G.; Vijgenboom, E.; Canters, G. W.; Loehr, T. M.; Sanders-Loehr, J. *J. Biol. Inorg. Chem.* **1997**, *2*, 98−107.
(29) Fraczkiewicz, R.; Fraczkiewicz, G.; Czernuszewicz, R. S., personal communication.
(30) Qiu, D.; Dasgupta, S.; Kozlowski, P. M.; Goddard, W. A., III; Spiro, T. G. *J. Am. Chem. Soc.* **1998**, *120*, 12791−12797.
(31) Andrew, C. R.; Fraczkiewicz, R.; Czernuszewicz, R. S.; Lappalainen, P.; Saraste, M.; Sanders-Loehr, J. *J. Am. Chem. Soc.* **1996**, *118*, 10436−10445.
(32) Andrew, C. A.; Lappalainen, P.; Saraste, M.; Hay, M. T.; Lu, Y.; Dennison, C.; Canters, G. W.; Fee, J. A.; Slutter, C. E.; Nakamura, N.; Sanders-Loehr, J. *J. Am. Chem. Soc.* **1995**, *117*, 10759−10760.

isotope-sensitive modes, their frequencies and intensities are markedly different from one another and from those of Cu_A. We propose that a single sulfide bridging up to four copper ions in Cu_Z, versus two thiolates bridging two copper ions in Cu_A, is responsible for the differences in the RR properties of the two mixed-valence sites (A_{mv} and Z_{mv}).

Materials and Methods

Native and Cu_A-only N_2OR were isolated and purified as described previously[5,33] from *P. stutzeri* ZoBell (ATCC 14405); the latter was expressed in the mutant strain MK402, which is defective in active-site biosynthesis.[5] The low-activity enzyme used for these studies was purified anaerobically from cells grown under sulfur limitation and anaerobic conditions. The Z_{mv}* sites in this enzyme and in enzyme that has been exposed to oxygen were confirmed to be equivalent on the basis of their identical 650-nm absorption bands and RR spectra. Fully labeled [^{34}S]N_2OR was obtained from cells grown on minimal medium containing 0.25 mM $Na_2{}^{34}SO_4$ (90 atom %, ICON). Incorporation of ^{63}Cu and ^{65}Cu (purity 99.7%) into N_2OR was achieved by the in vivo addition of the respective isotope into a growing culture of *P. stutzeri* ZoBell, as detailed previously.[6] Double-labeling of N_2OR with ^{65}Cu and [^{15}N]histidine (racemic D,L-[^{15}N $^{\alpha,\delta1,\epsilon2}$]histidine, 99% minimum ^{15}N, NIH National Stable Isotope Resource, Los Alamos, NM) was accomplished using an auxotrophic mutant generated by random transposon Tn5 mutagenesis.[6,34] The C622D and H583G variants were prepared and purified as described elsewhere.[35]

Protein samples were purified in either 25 or 50 mM Tris-HCl (pH 7.5) or 25 mM Tris-DCl (pD 7.5) buffers and were concentrated by ultrafiltration (Amicon, Microcon-50). Protein concentrations, which ranged from 0.5 to 2.5 mM protein depending on the availability of isotopic material, were determined spectrophotometrically ($\epsilon_{280} = 114$ mM^{-1} cm^{-1}). Enzymatic activity was monitored by gas chromatography as described previously.[33] D_2O-containing samples were prepared by diluting concentrated samples with D_2O buffer and reconcentrating; the exchange was repeated at least 5 times. Aside from a 5-cm^{-1} shift in the ice peak, no difference was observed in their RR spectra.

RR spectra were recorded on a custom McPherson 2061/207 spectrograph (0.67 m) equipped with a Princeton Instruments (LN-1100PB) liquid nitrogen-cooled CCD detector. Rayleigh scattering was attenuated using either a Kaiser Optical holographic supernotch filter or a McPherson 270 double-monochromator (600-groove grating) prefilter stage. Several lasers were used to obtain the desired excitation wavelength: Coherent Innova 302 Kr, Coherent Innova 90-6 Ar, and Coherent 599-01 dye (rhodamine 6G). Raman spectra were collected in an ~150° backscattering geometry from samples maintained at 15 K using a closed-cycle helium refrigerator (Air Products, Displex).[36] Air-sensitive samples were loaded and slowly frozen on the Displex in a glovebag filled with N_2 gas. Unless otherwise stated, spectra were obtained using an 1800-groove grating and a spectral resolution of 4 cm^{-1}. High-quality data were generally obtained with 10-min acquisitions. Calibration of the CCD spectrometer was achieved using aspirin as a frequency standard, and absolute frequencies are accurate to ±1 cm^{-1}. Isotope shifts, with an accuracy of ±0.5 cm^{-1}, were obtained from spectra recorded under identical experimental conditions and were evaluated by abscissa expansion and curve resolution of overlapping bands. The programs Grams 386 (Galactic Industries) and Origin (MicroCal) were used for data analysis and data presentation, respectively.

Results

Raman Signature of Cu_A Site. Excitation within the 480- or 540-nm absorption bands of native N_2OR yields a RR spectrum (Figure 3A) closely resembling those previously reported for the Cu_A sites (A_{mv}) in N_2OR from *P. stutzeri*[14] and *Achromobacter cycloclastes*,[15] cytochrome *c* oxidase (CcO),[15,31,32] and several engineered constructs.[32] The striking similarities of these spectra, as well as the spectrum obtained on the CcO Cu_A site with 850-nm excitation,[37] have verified that all three absorption bands (i.e., at 480, 540, and 780 nm) are associated with the same copper site and that the structure of this site is conserved among the different Cu_A-containing proteins. Furthermore, the presence of strongly resonance-enhanced Cu−S(Cys) stretching modes in all these spectra illustrates the substantial (Cys)S → Cu charge-transfer character of the three Cu_A absorption bands.

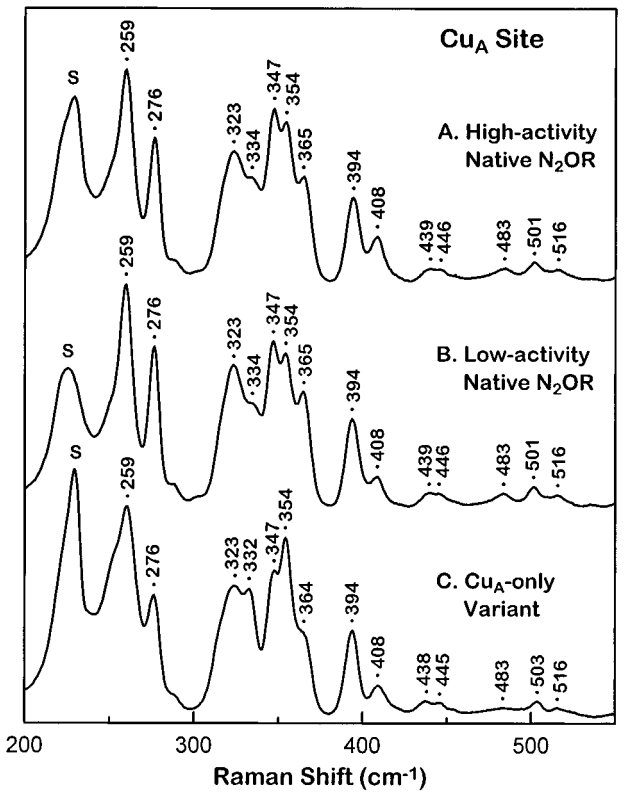

Figure 3. RR spectra of Cu_A site (A_{mv} cluster) obtained with 488-nm excitation. (A) High-activity N_2OR (0.8 mM). (B) Low-activity N_2OR (1.5 mM). (C) Cu_A-only variant of N_2OR (2.4 mM). S denotes 230-cm^{-1} ice mode from frozen solvent.

The 488-nm excitation spectrum of native N_2OR exhibits at least 11 fundamentals between 200 and 450 cm^{-1} (Figure 3A), two of which, at 259 and 347 cm^{-1}, are assigned as the distinctive Cu−S(Cys) stretching modes of A_{mv} (vide infra). As reported previously,[14] the Raman signature of A_{mv} in low-activity N_2OR is identical to that of native high-activity enzyme (Figure 3B). Differences between high- and low-activity N_2-OR have been attributed to the relative contributions of Z_{ox} and Z_{mv}*, which, as demonstrated by absorption spectroscopy (Figure 2), are not expected to influence A_{mv}. In contrast, loss of the catalytic site in the Cu_A-only variant does not alter the RR frequencies of A_{mv} but causes slight changes in their relative intensities (Figure 3C). These changes, which include an increase of intensity in the 354-cm^{-1} mode and a decrease in the features at 276, 323, 347, and 365 cm^{-1}, are suggestive of subtle perturbations in the A_{mv} structure. Anaerobic reduction of A_{mv} to an EPR-silent [Cu(I)·Cu(I)] moiety occurs im-

(33) Coyle, C. L.; Zumft, W. G.; Kroneck, P. M. H.; Körner, H.; Jakob, W. *Eur. J. Biochem.* **1985**, *153*, 459−467.
(34) Zumft, W. G.; Döhler, K.; Körner, H. *J. Bacteriol.* **1985**, *163*, 918.
(35) Charnock, J. M.; Dreusch, A.; Körner, H.; Neese, F.; Nelson, J.; Kannt, A.; Michel, H.; Garner, C. D.; Kroneck, P. M. H.; Zumft, W. G. *Eur. J. Biochem.* **2000**, *267*, 1368−1381.
(36) Loehr, T.; Sanders-Loehr, J. *Methods Enzymol.* **1993**, *226*, 431−470.
(37) Wallace-Williams, S. E.; James, C. A.; de Vries, S.; Saraste, M.; Lappalainen, P.; van der Oost, J.; Fabian, M.; Palmer, G.; Woodruff, W. H. *J. Am. Chem. Soc.* **1996**, *118*, 3986−3987.

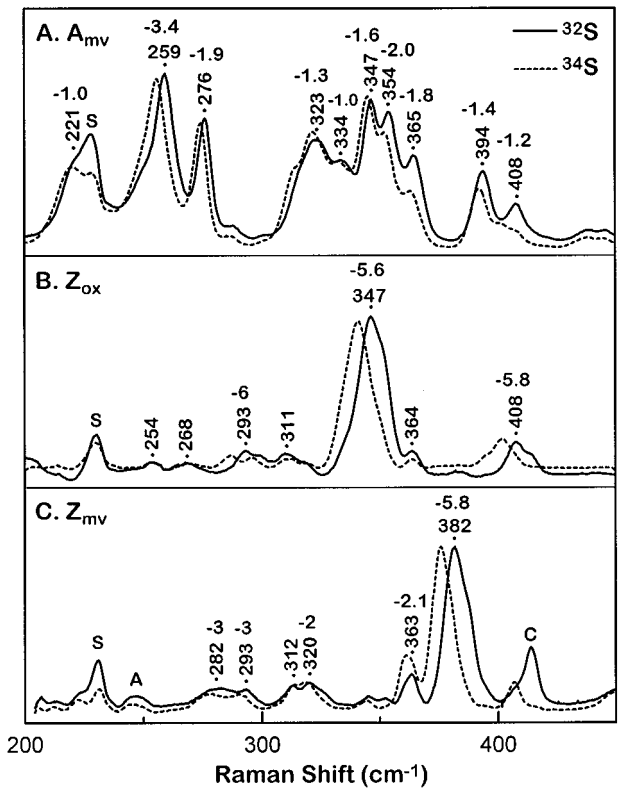

Figure 4. Sulfur isotope dependence of RR fundamentals. (A) A_{mv} cluster in high-activity N_2OR obtained with 488-nm excitation (2400-groove grating). (B) Z_{ox} in semireduced N_2OR probed with 568-nm excitation. (C) Z_{mv} in dithionite-reduced N_2OR probed with 647-nm excitation. Fully ^{34}S labeled N_2OR was obtained from cells grown on minimal medium containing $Na_2{}^{34}SO_4$. Sample concentrations were 0.8 mM for $[^{32}S]N_2OR$ and 1.0−1.3 mM for $[^{34}S]N_2OR$. Peak frequencies are listed for $[^{32}S]$ spectra (—) with frequency shifts for the $[^{34}S]$ spectra (- - -) indicated above. C denotes a band believed to arise from an altered Cu_A site (see text); its intensity is variable among preparations.

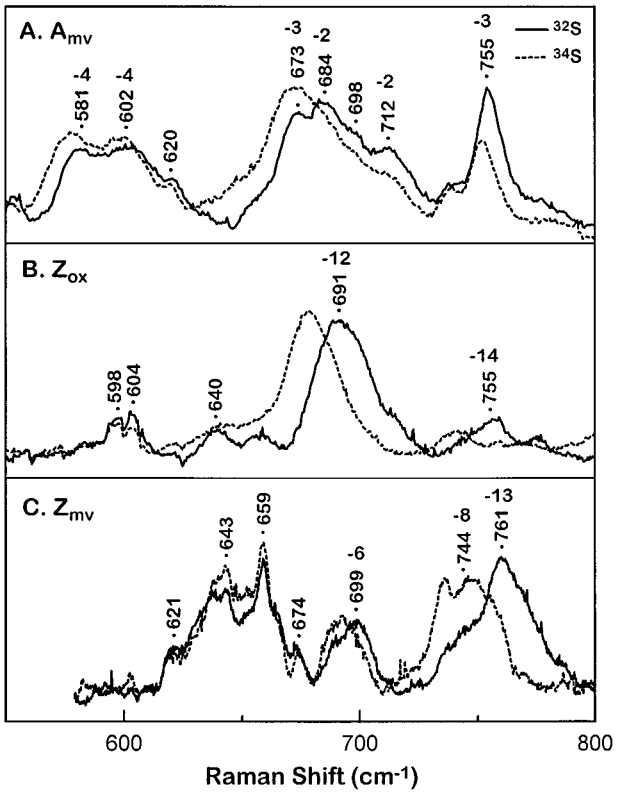

Figure 5. Sulfur isotope dependence of RR combination bands. (A) A_{mv} cluster in high-activity N_2OR obtained with 488-nm excitation. (B) Z_{ox} in semireduced N_2OR probed with 568-nm excitation. (C) Z_{mv} in dithionite-reduced N_2OR probed with 647-nm excitation. Sample and spectral conditions as in Figure 4.

mediately upon the addition of ascorbate or dithionite to produce the semireduced and reduced forms of the enzyme, respectively. Consistent with the disappearance of the (Cys)S → Cu charge-transfer bands at 480, 540, and 780 nm (Figure 2B), no vibrational modes attributable to A_{mv} are observed in either semireduced or reduced N_2OR.

Isotope Dependencies of Cu_A Raman Modes. The assignment of Cu−S(Cys) stretching vibrations can be resolved from the isotopic substitution of the coordinated cysteine sulfur atom. N_2OR is amenable to uniform ^{34}S substitution by growing cells in minimal medium containing $Na_2{}^{34}SO_4$ as the only sulfur source. While this preparation labels methionine residues as well as cysteine, previous studies have indicated negligible enhancement of the stretching vibrations associated with the weakly bound methionine sulfur.[38,39]

The two most prominent vibrational modes in the RR spectrum of A_{mv} occur at 259 and 347 cm^{-1} and, upon ^{34}S substitution, experience frequency shifts of −3.4 and −1.6 cm^{-1}, respectively (Figure 4A). While several other fundamentals at 276, 354, and 365 cm^{-1} exhibit substantial ^{34}S isotope shifts (−1.9, −2.0, and −1.8 cm^{-1}, respectively), the assignment of the 259- and 347-cm^{-1} modes as the principal ν(Cu−S) stretching vibrations is confirmed by their participation in high-frequency combination bands (Figure 5A; Table 1). Specifically, combination bands attributable to the 259-cm^{-1} fundamental are observed at 516 (2 × 259), 581 (259 + 323), and 602 (259 + 347) cm^{-1}. Similarly, the 347-cm^{-1} fundamental yields combination bands at 673 (347 + 323), 684 (2 × 347), 698

Table 1. Raman Frequencies and Isotope Shifts from Cu_A (A_{mv}) Cluster[a]

frequency	$\Delta^{34}S$	$\Delta^{65}Cu$	$\Delta^{15}N$(His)	assignment[b]
221	−1.0			
259	**−3.4**	**−0.5**	**−0.6**	ν(Cu−N)
276	−1.9	−0.8	−1.0	
323	−1.3	0	0	
334	−1.0	0	0	
347	**−1.6**	**−0.2**	**−0.4**	ν(Cu−S)
354	−2.0	0	−0.9	
365	−1.8	−0.3	−1.2	
394	−1.4	0	−0.4	
408	−1.2	−1.1	−0.3	
516				259 + 259
553	−2			
581	−4			259 + 323
602	−4			259 + 347
673	−3			347 + 323
684	−2			347 + 347
698				347 + 354
712	−2			347 + 365
755	−3			ν(S−C)

[a] A_{mv} in native N_2OR. Spectra obtained with 488-nm excitation. Frequencies (in cm^{-1}) are accurate to ±1 cm^{-1}. Boldface type indicates most intense peaks. Frequency shifts (Δ cm^{-1}) in the presence of heavier isotopes are accurate to ±0.5 cm^{-1} for intense fundamentals and ±1.0 cm^{-1} for weak fundamentals and combination bands. [b] Description of major contributing vibrational modes by analogy to Cu_A site in CcO.[25] Assignments of combination bands are based on frequencies and S isotope dependence.

(38) Thamann, T. J.; Willis, L. J.; Loehr, T. M. *Proc. Natl. Acad. Sci. U.S.A.* **1982**, *79*, 6396−6400.
(39) den Blaauwen, T.; Hoitink, C. W. G.; Canters, G. W.; Han, J.; Loehr, T. M.; Sanders-Loehr, J. *Biochemistry* **1993**, *32*, 12455−12464.

(347 + 354), and 712 (347 + 365) cm^{-1}. This phenomenon is reminiscent of the CcO Cu_A site, where the two ν(Cu−S) modes generate similar combination bands and have been assigned as A_g modes of the Cu_2Cys_2 core.[29] The RR spectrum of N_2OR displays an additional vibrational mode at 755 cm^{-1} (Figure 5A), which is not observed for the Cu_A site in CcO;[27] this S−C stretching mode is typical of mononuclear Cu−cysteinate proteins, where coupling between the Cu−S stretch and cysteine−ligand vibrations is significant.[28−30]

The effect of isotope substitution on the stretching vibrations of the N_2OR copper sites has also been examined using enzyme labeled with ^{65}Cu and double-labeled with ^{65}Cu and [^{15}N]-histidine. On the basis of the larger mass of copper relative to sulfur, the frequency shifts upon substitution of ^{63}Cu with ^{65}Cu are expected to be smaller than those observed with ^{32}S to ^{34}S substitution. For example, while type 1 cupredoxins exhibit Cu and S isotope sensitivities in the same vibrational modes, ^{65}Cu shifts are typically −1 cm^{-1} or less compared to ^{34}S shifts of −2 to −4 cm^{-1}.[28,40] The two ν(Cu−S) modes of the Cu_A site in N_2OR at 259 and 347 cm^{-1} experience only small ^{65}Cu shifts of −0.5 and −0.2 cm^{-1}, respectively, relative to their ^{34}S shifts of −3.4 and −1.6 cm^{-1} (Table 1). Similar behavior has been noted in the Cu_A site of CcO, which exhibits ^{65}Cu shifts of approximately −0.8 and −1.4 cm^{-1}, respectively, for the Cu−S stretching modes at 260 and 339 cm^{-1}, as compared to ^{34}S shifts of −4.1 and −5.1 cm^{-1} for these same fundamentals.[31] Only two other fundamentals of the Cu_A site in N_2OR at 276 and 365 cm^{-1} undergo Cu isotope shifts. The apparent lack in N_2-OR of any additional Cu isotope sensitivities in the Cu_A vibrational modes, which are expected to parallel those of S isotope dependencies, may be a consequence of the extensive coupling between the Cu−S stretching modes and the internal ligand coordinates. In this case, the corresponding ^{65}Cu shifts are most likely dispersed throughout a large number of fundamentals and may lie below the detection limit.

The Cu_A modes of N_2OR are also very sensitive to [^{15}N]-histidine substitution, with 6 of the 10 S-sensitive modes exhibiting ^{15}N shifts of −0.4 to −1.2 cm^{-1} (Table 1). Whereas the small isotope shifts and lower vibrational frequencies of the [^{15}N]histidine-sensitive modes are consistent with contributions from Cu−N(Im) vibrations, the higher energy modes, above 350 cm^{-1} are not observed in type 1 copper−cysteinate sites.[28,40,41] Nevertheless, the extensive [^{15}N]histidine dependence observed for the Cu_A Raman modes suggests a significant contribution from the histidine ligands.

No changes were observed in the RR spectrum of Cu_A equilibrated in D_2O, which is expected to affect both histidine and amide NH groups. However, imidazole, with only one exchangeable hydrogen, is expected to exhibit smaller deuterium shifts than the ^{15}N shifts resulting from [^{15}N]histidine substitution, and the deuterium effects may simply be too small to detect. Alternatively, a lack of deuterium shifts may reflect solvent inaccessibility of the exchangeable hydrogens. In the type 1 copper site of azurin, D shifts of −1 to −2 cm^{-1} are associated with seven vibrational modes between 372 and 476 cm^{-1} and are ascribed to the amide NH of cysteine and adjacent residues.[28] The failure to observe this type of D-dependence in A_{mv} or N_2OR suggests that the coupling of ligand vibrations does not extend as far into the polypeptide backbone.

Raman Signature of the Z_{ox} Site. The absorption bands at 550 and 640 nm that remain following the reduction of A_{mv} with ascorbate (Figure 2B, semireduced) have previously been attributed to an antiferromagnetically coupled [Cu(II)·Cu(II)] moiety, previously referred to as Z_{ox}.[7,24] On the basis of the crystallographic results, Z_{ox} may be formulated as an antiferromagnetically coupled Cu(II) tetramer or perhaps as a [Cu(II)·Cu(II)] dimer with a neighboring [Cu(I)·Cu(I)] dimer. In either case, bridging ligands among the Cu(II) ions would be required to mediate the magnetic coupling. Excitation within the 550-nm band yields a RR spectrum dominated by a single vibrational mode at 347 cm^{-1}, with several weaker features in the 200−450-cm^{-1} region (Figure 6B). The analogous RR spectrum of native N_2OR with 568-nm excitation is close to that of the semireduced enzyme in terms of both frequencies and intensities (Figure 6A), demonstrating that A_{mv} does not contribute to the 568-nm excitation spectrum and that its reduction does not affect Z_{ox}, which is expected given the separation between these centers observed in the crystal structure.

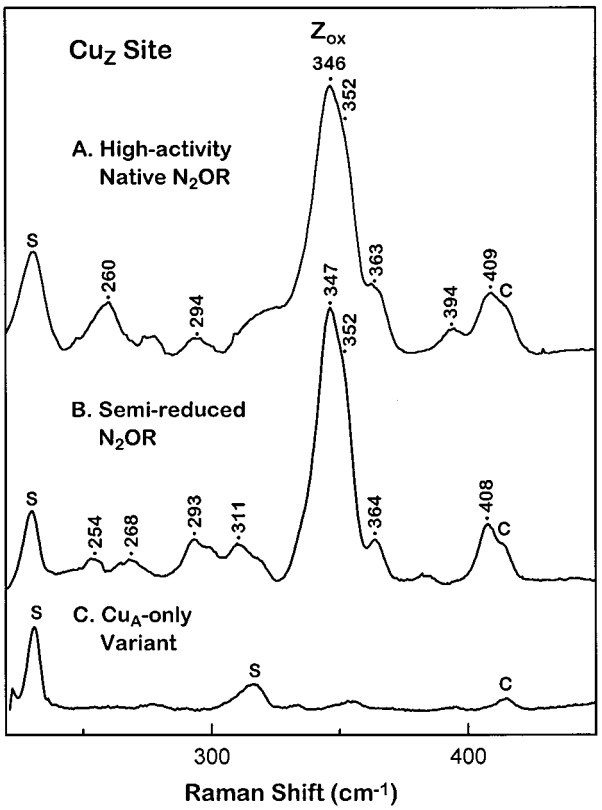

Figure 6. RR spectra of Cu_Z site (Z_{ox} cluster) obtained with 568-nm excitation. (A) Z_{ox} in high-activity N_2OR (0.8 mM). (B) Z_{ox} in N_2OR (0.8 mM) semireduced by ascorbate. (C) Cu_A-only variant of N_2OR (2.4 mM) probed with 578-nm excitation. C denotes 414-cm^{-1} peak from an altered-Cu_A contaminant (see text).

The variant N_2OR, which lacks the Cu_Z site, does not exhibit the Raman signature of Z_{ox} (Figure 6C), but displays a single fundamental at 414 cm^{-1} (labeled C), which is also present in the RR spectra of native, semireduced, and reduced enzyme, and displays an excitation maximum near 620 nm. The variable intensity of this mode in different sample preparations suggests that the contribution of this site is dependent on isolation conditions. On the basis of its continued presence after reduction with ascorbate or dithionite (Figure 7B and D), as well as its S isotope shift of −7 cm^{-1} (Figure 4C), this copper−sulfur vibration is assigned to an altered Cu site. The Cu−S species responsible for the 414-cm^{-1} feature most likely derives from modification of a Cu_A site because it is observed in the Cu_A-

(40) Qiu, D.; Dong, S.; Ybe, J. A.; Hecht, M. H.; Spiro, T. G. *J. Am. Chem. Soc.* **1995**, *117*, 6443−6446.

(41) Sanders-Loehr, J. In *Bioinorganic Chemistry of Copper*; Karlin, K. D., Tyeklár, Z., Eds.; Chapman Hall: New York, 1993; pp 51−63.

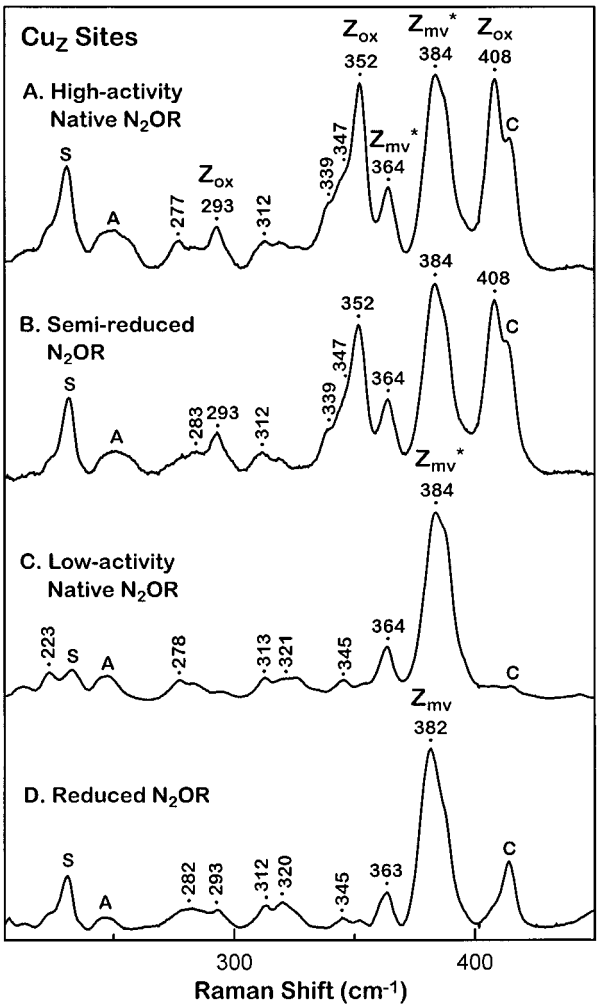

Figure 7. RR spectra of Cu_Z sites obtained with 647-nm excitation. (A) Mixture of Z_{ox} and Z_{mv}* in high-activity N_2OR; samples as in Figures 3A and 6A. (B) Mixture of Z_{ox} and Z_{mv}* in semireduced N_2OR; sample as in Figure 6B. (C) Z_{mv}* in low-activity N_2OR; sample as in Figure 3B. (D) Z_{mv} in dithionite-reduced N_2OR (0.8 mM). A denotes artifact attributable to 647-nm notch filter.

only variant (Figure 6C) but not in the Cu_Z-only variant (the C622D mutant, described below).

Excitation within the 640-nm band of native or semireduced N_2OR (Figure 7A and B, respectively) yields the same set of Raman frequencies at 347, 352, and 408 cm^{-1} that were attributed to Z_{ox} in the 568-nm excitation spectrum and thus establishes a common copper−sulfide ground-state structure for both absorption bands. However, the relative intensities of these fundamentals differ significantly at the two excitation wavelengths, indicating that the corresponding absorption bands must be associated with different excited-state structures, i.e., different electronic transitions. Specifically, when samples are excited at 647 nm, the 347-cm^{-1} fundamental loses intensity while that of the 352- and 408-cm^{-1} features are greatly enhanced. Further complexity in the interpretation of the 647-nm excitation spectrum arises from the presence of a second copper−sulfide species with features at 364 and 384 cm^{-1}; this species has been assigned to Z_{mv}*, a catalytically inactive variant of Z_{ox} (vide infra).

Isotope Dependencies of the Z_{ox} Raman Modes. An unusual feature of the RR spectrum obtained with 568-nm excitation is that three of the fundamentals at 293, 347, and 408 cm^{-1} have substantial Cu−S stretching character, as evidenced by their large S isotope shifts of −6.0, −5.6, and −5.8 cm^{-1}, respectively (Figure 4B). The atypical RR signature of Z_{ox} identifies this metal site as a novel copper−sulfur species, containing Cu(II) and S^{2-}, where the sulfide ligand bridges at least two Cu(II) ions. The attribution of three major Cu−S stretching modes to a single Cu(II)−sulfide chromophore is supported by their resonance enhancement upon excitation within both $S^{2-} \rightarrow$ Cu charge-transfer transitions, at 550 and 650 nm (Figures 6A and 7A,B), and by their involvement in combination bands (Figure 5B; Table 2). For example, 568-nm excitation produces combination bands at 691 (2 × 347) and 755 (347 + 408) cm^{-1}, with respective S isotope shifts of −12 and −14 cm^{-1}, and 647-nm excitation yields combination bands at 640 (352 +293), 700 (2 × 352), and 759 (352 + 408) cm^{-1}, with respective S isotope shifts of −5, −4, and −10 cm^{-1}. The behavior of the 293- and 352-cm^{-1} fundamentals is also unusual. Despite a large S isotope shift of −6.0 cm^{-1}, the intensity of the 293-cm^{-1} mode is relatively weak in both the 568- and 647-nm excitation spectra and contributes to only one combination band at 640 cm^{-1}. Conversely, the 352-cm^{-1} mode is intense in both spectra yet experiences a smaller S isotope shift of −2.5 cm^{-1}, suggesting that this vibration has less Cu−S stretching character. Nevertheless, this fundamental generates several combination bands, including an overtone at 700 (2 × 352) cm^{-1} and the combination band with the 293-cm^{-1} mode.

Table 2. Raman Frequencies and Isotope Shifts from Z_{ox} Cluster[a]

frequency	Δ^{34}S	Δ^{65}Cu	Δ^{15}N(His)	assignment
254	0			
268	0			
293	−6	−1	0	ν(Cu−S)
311	0	−1	0	
347	**−5.6**	**−1.9**	**0**	ν(Cu−S)
352[b]	−2.5	−1.2	−0.6	
364	0			
408[b]	−5.8	−1.8	−0.3	ν(Cu−S)
640[b]	−5	−2	0	352 + 293
691	−12	−4	0	347 + 347
700[b]	−4	−3	−1	352 + 352
755	−14	−5	0	347 + 408
759[b]	−10	−3	−1	352 + 408

[a] Z_{ox} site in semireduced N_2OR. Spectra obtained with excitation at 568 (S isotopes) or 578 nm (Cu and N isotopes). Frequencies and assignments as in Table 1. Identification of ν(Cu−S) are based on S isotope shifts. [b]Data obtained with 647-nm excitation.

The isotope shifts displayed by the RR modes of Z_{ox} upon ^{65}Cu substitution, although smaller in magnitude, parallel those resulting from ^{34}S substitution (Table 2). In addition, [^{15}N]-histidine substitution has almost no effect on the RR spectrum, suggesting only minor kinematic coupling between Cu−imidazole stretching modes and the Cu−S^{2-} chromophore. These results, which imply that the Cu isotope shifts originate mainly from Cu−S rather than Cu−N(Im) vibrations, are consistent with the weak intensity of the low-frequency Cu−N(Im) fundamentals (<290 cm^{-1}, which are only weakly enhanced), and the fairly pure nature of the Cu−S stretching modes at 293, 347, and 408 cm^{-1}.

Raman Signature of the Z_{mv} Site. Treatment of native N_2-OR with dithionite results in the conversion of A_{mv} to A_{red} and Z_{ox} to Z_{mv} and the appearance of a new absorption band at 650 nm characteristic of Z_{mv} (Figure 2B, reduced). Excitation of dithionite-reduced N_2OR at 647 nm produces a new RR spectrum with a dominant vibrational mode at 382 cm^{-1} (Figure 7D), proving that Z_{mv} has a distinctly different copper−sulfide ground-state structure from Z_{ox}, whose dominant vibrational modes are at 352 and 408 cm^{-1} with this excitation wavelength (Figure 7A,B). The 647-nm spectral pattern for Z_{mv} is somewhat

Table 3. Raman Frequencies and Isotope Shifts from Reduced Cu_Z Sites[a]

Z_{mv}					Z_{mv}*				
freq	$\Delta^{34}S$	$\Delta^{65}Cu$	$\Delta^{15}N$(His)	assignment	freq	$\Delta^{34}S$	$\Delta^{65}Cu$	$\Delta^{15}N$(His)	assignment
282	−3	0	0		278				
293	−3	−1	0		294				
312	0	0	0		313				
320	−2	−2	−1		321				
345	−1	0	0		345				
363	−2.1	−0.6	−0.6		364	−1.0	−0.4	−1.2	
382	**−5.8**	**−0.8**	**0**	ν(Cu−S)	**384**	**−4.7**	**−2.0**	**−0.6**	ν(Cu−S)
699	−6			382 + 320	701	−6			384 + 321
744	−8			382 + 363	747	−6			384 + 364
761	−13			382 + 382	766	−9			384 + 384

[a] Z_{mv} cluster from reduced N_2OR. Z_{mv}* cluster from low-activity N_2OR (frequencies) and high-activity N_2OR (isotope shifts). Spectra obtained with 647-nm excitation. Frequencies and assignments as in Tables 1 and 2.

similar to that of Z_{ox} (568-nm excitation) in that a single intense feature is observed at 382 (Figure 7D) and 347 cm^{-1} (Figure 6A), respectively, each with a high-frequency shoulder and a number of weaker features, primarily to lower frequencies. Although RR data were not collected on the reduced Cu_A-only mutant, previous work has verified that this form of the enzyme has no significant absorbance in the 400−800-nm region, consistent with the reduction of the Cu_A site and the absence of the Cu_Z site.[5]

Isotope Dependencies of the Z_{mv} Raman Modes. The sulfur isotope dependence of the Z_{mv} site is again similar to the Z_{ox} site in that the most intense feature at 382 cm^{-1} exhibits the largest ^{34}S shift of −5.8 cm^{-1} (Figure 4C). Smaller shifts of −2 to −3 cm^{-1} are seen in a number of the lower intensity modes. These assessments are confirmed by the appearance of three ^{34}S-sensitive combination bands at 699 (382 + 320), 744 (382 + 363), and 761 (2 × 382) cm^{-1}, with respective S isotope shifts of −6, −8, and −13 cm^{-1} (Figure 5C; Table 3). The role of the major ν(Cu−S) stretching mode as the generator of these combination bands is similar to the behavior observed for thiolate ligation, as illustrated by type 1 cupredoxins.[26] The RR modes of Z_{mv} experience ^{65}Cu and [^{15}N]histidine isotope sensitivities similar to those of Z_{ox}, in that ^{65}Cu shifts correlate well with those produced by ^{34}S substitution and almost no effect is observed upon [^{15}N]histidine substitution (Table 3). This finding suggests that, in contrast to the Cu_A site, the contribution of histidine ligands to the RR properties of the Cu_Z site is minor, regardless of its oxidation state. This is an interesting finding that warrants further investigation in light of the multiple histidine ligands in the Cu_Z cluster.

Raman Signature of the Z_{mv}* Site. The loss of enzymatic activity upon aerobic isolation and purification of N_2OR has previously been correlated to the amount of Z_{mv}*,[5,33] a mixed-valence form of the Cu_Z site whose presence, like that of Z_{mv}, is manifested as an absorption band at 650 nm (Figure 2). Excitation within this band yields a RR spectrum for Z_{mv}* (Figure 7C) that is remarkably similar to that of Z_{mv} (Figure 7D) in terms of fundamental frequencies, combination bands, and S isotope shifts (Table 3). Nevertheless, a distinction of 2 cm^{-1} is clearly evident in their dominant Cu−S stretching modes, i.e., 384 cm^{-1} in Z_{mv}* versus 382 cm^{-1} in Z_{mv}, perhaps indicating a minor structural difference, such as variation in the number or coordination of the solvent-derived ligands apparent in the structure.

As predicted from absorption, EPR, and MCD spectroscopy,[5,7,33] the contribution of Z_{mv}* to the 647-nm excitation spectrum of high-activity N_2OR is dramatically smaller (relative to the solvent peak, S) than that of low-activity enzyme, (Figure 7A and C, respectively). No changes are seen in the RR spectrum upon the addition of ascorbate to high-activity enzyme (Figure 7B), indicating that neither Z_{ox} nor Z_{mv}* is reduced by this reagent. This is apparently inconsistent with the observation by Farrar et al. that Z_{mv}*, like A_{mv}, can be reduced by ascorbate to an EPR-silent moiety.[7] Although these differences may simply reflect a slower reduction of Z_{mv}* compared to A_{mv}, attempts to reduce Z_{mv}* in low-activity N_2OR by addition of dithionite in this laboratory have also failed, as evidenced by a lack of change in the RR and absorption spectra (data not shown).

Raman Spectra of Copper Site Variants. Recently two variants of N_2OR were prepared and purified: C622D, in which one of the strictly conserved Cu_A cysteine ligands has been changed; and H583G, in which one of the Cu_A histidine ligands has been mutated.[35] Excitation at 488 nm reveals that a slightly perturbed Cu_A site is present in H583G but that Cu_A is absent in C622D, as expected (Figure 8A). These results clearly illustrate the critical importance of both cysteines in the assembly of the Cu_A center and the robustness of its Cu_2S_2 core. The RR frequencies of the Cu_A site in H583G are closely similar to those of the wild-type enzyme (Figure 3A), except for the lower frequency features at 265 and 275 cm^{-1}, which are shifted slightly and are somewhat less intense. These changes are consistent with greater Cu−histidine vibrational contributions in the low-frequency region (Table 1) and suggest that the loss or replacement of a histidine ligand does not perturb the remainder of the Cu_2S_2 core. A remarkably different picture emerges from the spectra collected with 647-nm excitation (Figure 8B). Notably, the RR spectrum of C622D is essentially identical to that of Z_{mv}* (Figure 7C), thereby providing strong support for the assignment of the absorption band and RR features to the Cu_Z site. Note that there is essentially no indication of a Z_{ox} center in the C622D spectrum (Figure 8B), whereas features attributable to *both* Z_{mv}* *and* Z_{ox} are evident in the spectrum of H583G. This suggests that the proper folding of the protein around the Cu_A site can influence the structure or redox properties (or both) of the Cu_Z site.

Discussion

Structure of the Cu_A Site. The Raman spectra of Cu_A in N_2OR from *P. stutzeri* (Figure 3A) and *A. cycloclastes*[15] are nearly identical in terms of the Cu−S stretching frequencies, their relative intensities, and the total number of fundamentals in the 200−450-cm^{-1} region. As previously noted,[25] the remarkable consistency of ±4 cm^{-1} for the two ν(Cu−S) modes at 260 and 343 cm^{-1} in all known Cu_A-containing moieties reflects the rigidity of this site imparted by the two bridging thiolates and is sharply contrasted by that of the single ν(Cu−S) mode near 390 cm^{-1} in mononuclear type 1 Cu−cysteinate sites, which can vary by as much as ±38 cm^{-1}. At the current

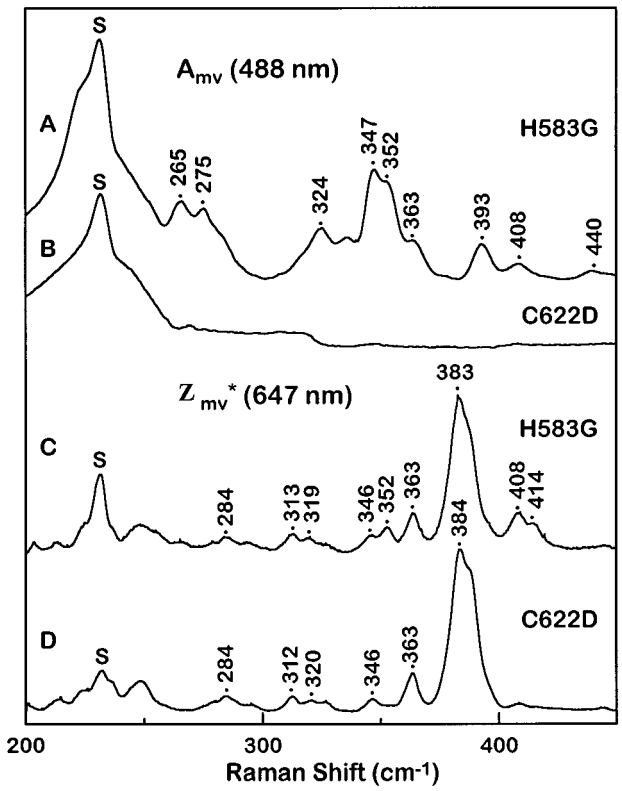

Figure 8. Resonance Raman spectra of H583G and C622D site-directed mutants of N_2OR. (A) A_{mv} in H583G N_2OR (0.7 mM) probed with 488-nm excitation. (B) A_{mv} in C622D N_2Or (1.2 mM) probed with 488-nm excitation. (C) and (D), Z_{mv}* sites in H583G and C622D, respectively, probed with 647-nm excitation.

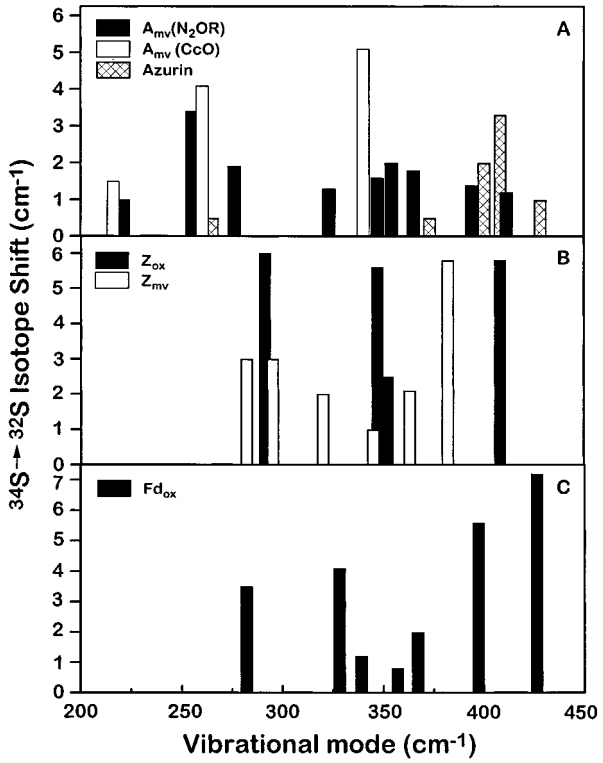

Figure 9. Sulfur isotope dependence of individual RR modes. (A) Cu_A site in N_2OR from *P. stutzeri* (data from Figure 4A); Cu_A site in CcO fragment from *P. denitrificans* (data from ref 25); and type 1 site in azurin from *Pseudomonas aeruginosa* (data from ref 41). (B) Z_{ox} in semireduced N_2OR (data from Figure 4B) and Z_{mv} in dithionite-reduced N_2OR (data from Figure 4C). (C) $Fe_2S_2(Cys)_4$ site in ferredoxin from *Prophyra umbilicalis* (data from ref 56).

resolution of the structures for CcO and N_2OR, the Cu_A sites in these two enzymes are practically identical. Minor structural variability in the Cu_A sites from N_2OR and CcO are detectable by RR spectroscopy and are manifested in the number of weaker fundamentals between 200 and 450 cm^{-1} and their distinctive ^{34}S dependence patterns (Figure 9A). The Cu_A spectra of CcO fragments from several sources reveal three well-resolved RR modes in this region, three of which are S isotope dependent and two of which are N isotope dependent.[31,32] A normal coordinate analysis (NCA) reveals that the RR spectrum of the Cu_A site in *Paracoccus denitrificans* CcO can be described as a set of coupled Cu−S and Cu−N vibrations arising from the Cu_2S_2 core and two terminal imidazoles with very little contribution from the cysteine ligand side chain.[31] In contrast, the RR spectrum of N_2OR Cu_A (Figure 9A) contains a much larger number of vibrational modes and more closely resembles that of the Cu_A construct from amicyanin,[32] a blue copper protein that has been converted to a Cu_A site by the addition of a cysteine-containing Cu-binding loop.[42] The observation of nine fundamentals in the Cu_A spectrum of N_2OR, all of which are S isotope sensitive, and seven of which are N isotope sensitive, suggests a greater degree of coordinate mixing of internal cysteine−ligand vibrations with Cu−S and Cu−N stretching. In addition, a cysteine−ligand S−C stretch is observed at 755 cm^{-1} in the RR spectrum of N_2OR (Figure 5A) but not in that of CcO. The presence of this mode, which is typical of mononuclear Cu−cysteinate proteins, where coupling between the Cu−S stretching mode and cysteine−ligand vibrations is significant,[24] further illustrates the extensive kinematic coupling present in the Cu_A site of N_2OR.

(42) Dennison, C.; Vijgenboom, E.; de Vries, S.; van der Oost, J.; Canters, G. W. *FEBS Lett.* **1995**, *365*, 92−94.

Table 4. Calculated and Observed Isotope Shifts for Copper−S(Cys) Sites[a]

species	Cu−^{34}S	^{65}Cu−(S,N)	Cu−^{15}N(His)
calculated shifts (sum)			
CuL[b]	−7.9	−5.3	−2.8
CuSCu, ($\nu_s + \nu_{as}$)[c]	−13.0		
$Cu_2S_2(Im)_2$, A_g modes[d]	−8.7	−4.8	−1.8
$Cu_2S_2(Im)_2$, all modes[d]	−29.3	−13.1	−4.5
observed shifts (sum)			
$CuS(His)_2$ in azurin[e]	−7.5	−2.4	−1.0
Cu_A (A_{mv}) in CcO[f]	−10.7	−3.8	−2.0
Cu_A (A_{mv}) in native N_2OR[g]	−16.6	−1.8	−4.5
Z_{ox} in native N_2OR[g]	−19.9	−6.9	−0.9
Z_{mv} in reduced N_2OR[g]	−16.9	−4.4	−1.6

[a] Sum of isotope shifts in all fundamentals between 210 and 450 cm^{-1}. Downshifts relative to frequencies for ^{32}S, ^{14}N, or ^{63}Cu. [b] Calculated for a diatomic oscillator with a copper−ligand (CuL) vibration at 400 cm^{-1}. Imidazole is treated as a point mass of 68 (^{14}N) or 70 (^{15}N). [c] Sum of shifts for ν_s(Cu−S−Cu) and ν_{as}(Cu−S−Cu) in a singly bridged cluster, calculated according to ref 39. [d] Sum of calculated shifts for CuL coordinates in a di-μ-S-bridged cluster with two terminal imidazoles (treated as point masses of 68). Based on NCA calculations in ref 25. [e] Azurin from *Pseudomonas aeruginosa*. Sum of ^{34}S shifts, based on curve-fitting, from ref 41 and Figure 8. Sum of ^{15}N and ^{65}Cu shifts for H117G mutant plus exogenous imidazole from ref 35. [f] Sum of isotope shifts for Cu_A-containing fragment in *P. denitrificans* CcO from ref 25. [g] Sum of isotope shifts for Cu_A, Z_{ox}, and Z_{mv} in *P. stutzeri* N_2OR from Tables 1−3, respectively.

Increased coupling in the Cu_A site of N_2OR is also evident in the comparison of their total S isotope shifts (Table 4). The total S isotope shift of the three S-sensitive fundamentals of *P. denitrificans* CcO Cu_A is −10.7 cm^{-1} (Table 4). These isotope shifts, as well as the large 79-cm^{-1} energy separation between the two predominant S-sensitive modes at 260 and 339 cm^{-1},

Table 5. Spectral Characteristics of Cu_A and Cu_Z Sites in N_2OR

property	A_{mv}	Z_{ox}	Z_{mv} (Z_{mv}*)
Cu valence[a]	+1.5, +1.5	+2, +2	+1.5, +1.5
absorption max (nm)	480, 540, 780	550, 640	650
magnetic properties	$S = {}^1/_2$	$S = 0$	$S = {}^1/_2$
EPR, $A_{\|\|}$	7-line[b]	none	
MCD (nm)[c]	−473, +525, −750	none	+625, −680,
ν(Cu−S) (cm^{-1})[d]			
488-nm excitation	**259**, 276, **347**, 354		
568-nm excitation		293, **347**, 352, 408	
647-nm excitation		293, 347, **352, 408**	363, **382**
sum of $\Delta^{34}S$[e]	−17 (±5)	−20 (±2)	−17 (±4)

[a] For the two copper ions in the Cu_A site and two of the four ions in the Cu_Z site. [b]Reference 8. [c]Reference 6. [d]This work. Identification of Cu−S stretching modes as in Tables 1−3. Boldface type denotes most intense Raman features. [e]Table 4.

have been fitted to a dinuclear copper model containing two bridging thiolates and two imidazoles trans-distorted from the Cu_2S_2 plane.[31] On the basis of these NCA calculations, three symmetric A_g modes are assigned to the Cu_A site: the 339-cm^{-1} mode is the totally symmetric stretch of the Cu_2S_2 rhombus; the 260-cm^{-1} mode has contributions from both Cu−S and Cu−N(Im) stretching modes; and a 138-cm^{-1} mode accounts for the motion of the two Cu atoms along the Cu−Cu axis, whose origin as either a Cu−S−Cu bend or a Cu−Cu stretch remains unclear.[31,43] The Cu_A site in N_2OR also displays a large energy separation between the two ν(Cu−S) modes of 88 cm^{-1}, but the S isotope pattern, with 10 S-sensitive modes, is significantly different (Figure 9A). While still consistent with the NCA for a Cu_A-type site, the larger total S isotope shift of −16.6 cm^{-1} and the increased number of S-sensitive modes suggest the participation of more than only A_g modes (Table 4). Such behavior most likely implies greater coupling of the Cu−S stretches with vibrations of the two cysteine backbones. Nevertheless, the RR spectroscopic data for A_{mv} in N_2OR strongly support a dithiolate-bridged cluster geometry as observed in the crystal structures of reduced N_2OR (where Cu_A is present as the [Cu(I)·Cu(I)] dimer) and of CcO (Table 5).

It is of interest that the Cu_A site in N_2OR also exhibits more vibrational coupling of the Cu−N(His) modes with the Cu−S(Cys) stretch, as evidenced by the larger total [^{15}N]histidine shift of −4.5 cm^{-1}, relative to −2.0 cm^{-1} in CcO (Table 4). Although this type of [^{15}N]His dependence is not observed in mononuclear Cu(Cys)$(His)_2$ sites.[37] a comparable total [^{15}N]-histidine shift of −4.3 cm^{-1} is observed for the dinuclear iron site in Rieske ferredoxins when the two histidine imidazoles of the $(Cys)_2FeS_2Fe(His)_2$ cluster are double-labeled with ^{15}N.[44] In addition, N-sensitive iron−sulfur modes are found between 320 and 360 cm^{-1}, due to coupling of the metal−imidazole and metal−sulfur stretches, as in N_2OR Cu_A. Evidently, this coordinate mixing does not require coplanarity because the two terminal histidines of Rieske proteins are tetrahedrally coordinated to one iron, and the imidazole rings are not coplanar with the Fe_2S_2 core. Precedence for the enhancement of metal−N(His) vibrations at higher vibrational frequencies is also found in oxyhemocyanin, a dicopper site with six histidine ligands, which displays six imidazole-dependent RR modes between 220 and 345 cm^{-1} upon excitation within its peroxide → Cu(II) CT band.[45] In this case, the copper atoms are bridged by a side-on bound (μ-η^2:η^2) peroxide, which allows coupling of Cu−N(His) and Cu−O(peroxide) stretches. Thus, it appears that histidine ligand modes are more likely to undergo coordinate mixing in sites where the metals are rigidly positioned by multiple bridging ligands, as in Cu_A, Rieske ferredoxins, and oxyhemocyanin or by heme groups as in myoglobin.[46]

Structure of the Z_{ox} Site. It is clear from the crystal structure that there are no thiolate ligands in the Cu_Z site, as predicted from the sequence analysis. As pointed out above, the RR spectrum of the Z_{ox} site in high-activity or semireduced N_2OR exhibits four sulfur isotope-sensitive modes at 293, 347, 352, and 408 cm^{-1} whose intensity is dependent on excitation wavelength (Figures 6A and 7A). Consequently, there must be a sulfur ligand present that is capable of generating low-energy charge-transfer transitions. The pattern of ^{34}S shifts in Z_{ox} is completely different from that in mononuclear cupredoxins such as azurin. The Cu−S stretch in azurin is coupled with cysteine ligand vibrations of *similar* energy such that most of the isotope-sensitive modes are found in a narrow frequency range of 370−430 cm^{-1} (Figure 9A) and the total ^{34}S shift is −7.5 cm^{-1} (Table 4). In contrast, the four isotope-sensitive modes in Z_{ox} exhibit a total ^{34}S shift of −19.9 cm^{-1} (Table 4), and these vibrational modes are separated over a much wider frequency range of 290−410 cm^{-1} (Figure 9B). We estimate that the error of the total ^{34}S isotope shift for Z_{ox} is ±2 cm^{-1}, based on the uncertainty of the weak features. Strikingly similar RR behavior is observed for the di-μ-sulfido-bridged $Fe_2S_2(Cys)_4$ clusters in oxidized ferredoxin where replacement with [^{34}S]sulfide bridges results in frequency shifts of −3 to −7 cm^{-1} for vibrations ranging from 280 to 425 cm^{-1} and a total ^{34}S shift of −24.4 cm^{-1} (Figure 9C). Moreover, oxidized adrenodoxin, which is analogous to 2Fe-ferrodoxin and Cu_A in having two bridging sulfurs for each metal ion, also exhibits a total ^{34}S shift of −23.9 cm^{-1}.[47] In contrast, 4Fe-ferrodoxin, with each metal ion coordinated to three bridging sulfurs, displays a total shift of −38.8 cm^{-1} upon replacement with [^{34}S]sulfide bridges.[48] Thus, the pattern of sulfur-isotope shifts in the RR spectrum of Z_{ox} (Figure 9B) is best explained by the presence of a bridging sulfide ligand. A terminal Cu − S^{2-} moiety appears unlikely for three reasons: first, the total ^{34}S isotope shift would probably be significantly smaller, on the order of −8 cm^{-1}, as in azurin; second, it would be more difficult to accommodate the extra electron density associated with S^{2-}, as opposed to OH^-, in the crystal structure; and third, there are currently no stable examples of terminal sulfides in late transition metal ion complexes.[49]

At least eight vibrational fundamentals between 200 and 450 cm^{-1} are resonance enhanced (Figure 7A). A three-atom Cu_2S structure can have only three normal modes of vibration, whereas a Cu_4S structure can have nine normal modes, suggesting that the sulfide ligand may bridge all the copper ions in the Cu_Z cluster. The observed electron density in the structure appears to be able to accommodate a sulfide ion, as opposed to

(43) Wallace-Williams, S. E.; James, C. A.; de Vries, S.; Saraste, M.; Lappalainen, P.; van der Oost, J.; Fabian, M.; Palmer, G.; Woodruff, W. H. *J. Am. Chem. Soc.* **1996**, *118*, 3986−3987.

(44) Rotsaert, F. A. J.; Pikus, J. D.; Fox, B. G.; Markley, J. L.; Sanders-Loehr, J., unpublished results.

(45) Ling, J.; Nestor, L. P.; Czernuszewicz, R. S.; Spiro, T. G.; Fraczkiewicz, R.; Sharma, K. D.; Loehr, T. M.; Sanders-Loehr, J. *J. Am. Chem. Soc.* **1994**, *116*, 7682−7691.

(46) Kincaid, J.; Stein, P.; Spiro, T. G. *Proc. Natl. Acad. Sci. U.S.A.* **1979**, *76*, 549−552.

(47) Han, S.; Czernuszewicz, R. S.; Kimura, T.; Adams, M. W. W.; Spiro, T. G. *J. Am. Chem. Soc.* **1989**, *111*, 3505−3511.

(48) Czernuszewicz, R. S.; Macor, K. A.; Johnson, M. K.; Gewirth, A.; Spiro, T. G. *J. Am. Chem. Soc.* **1987**, *109*, 7178−7187.

(49) Vicic, D. A.; Jones, W. D. *J. Am. Chem. Soc.* **1999**, *121*, 4070−4071.

hydroxide, in the predicted bridging location. Since the assembly of the catalytic Cu_Z site requires intracellular-processing enzymes,[3,50] it is possible that the sulfide ligand is inserted during enzyme biogenesis. An alternative explanation, recently proposed by Farrar et al.[19] is that the Z_{ox} cluster is an altered form of the A_{mv} cluster, such that the two cysteinate ligands conserved in standard sequence alignments are used in both the A and Z sites. Given the discovery of inorganic sulfide in N_2OR[22] and the possibility of other explanations for the results of Farrar et al., this hypothesis is unnecessary.

Structure of the Z_{mv} Site. In contrast to purple Cu_A sites, which typically display absorption bands at 480, 540, and 780 nm, the intensely blue Z_{mv} site in reduced N_2OR displays one strong absorption band at 650 nm. Excitation within this absorption band, as reported previously,[14] yields a RR spectrum with a dominant vibrational mode at 382 cm^{-1} and seven weaker features in the 250−400-cm^{-1} region (Figure 7D). The 382-cm^{-1} frequency of the most intense Cu−S vibrational mode in Z_{mv} appears to differ from the 352- and 408-cm^{-1} values for Z_{ox}, but there are a number of similarities in their Raman spectral patterns. Thus, Z_{mv} exhibits significant ^{34}S shifts (−2 to −5.8 cm^{-1}) in six vibrational modes that spread out over a frequency range of 282−382 cm^{-1} (Figure 9B) and can again be assigned to a structure in which the sulfide bridges multiple copper ions in the Cu_Z cluster. No S−C stretch is apparent, as expected. The total ^{34}S shift of −16.9 cm^{-1} for Z_{mv} (Table 4) is of a magnitude similar to the −19.9-cm^{-1} shift for Z_{ox} and is consistent with a bridged structure. As in the case of Z_{ox}, Z_{mv} exhibits a smaller degree of coordinate mixing than in the A_{mv} site of N_2OR, as evidenced by the smaller ^{15}N(His) shift of −1.6 cm^{-1} (Table 4). The C622D variant of N_2OR, which lacks one of the two conserved cysteine ligands, cannot form either A_{mv} or Z_{ox} but does form a Z_{mv}* species (Figure 8D), which displays an RR spectrum almost identical to that of Z_{mv} in wild-type N_2OR (Figure 7D).

Structure of the Z_{mv}* Site. An increase, relative to native N_2OR, in the absorption band at 650 nm in the aerobically isolated enzyme has previously been associated with a mixed-valence form of Cu_Z designated Z_{mv}*. The similarities between Z_{mv}* and Z_{mv} are striking (Table 5): identical absorbance maximums at 650 nm, nearly identical MCD features at 625 (+) and 660 nm (−), superimposable broad EPR signals, and RR spectra with nearly identical peak frequencies (±1 cm^{-1}) and intensities (Figure 7C,D). In light of these remarkable similarities, Z_{mv} and Z_{mv}* are considered to be the same spectroscopic chromophore rather than independent moieties. Minor differences in their spectroscopic properties, as well as differences in their apparent sensitivities to various reagents such as ascorbate, dithionite, and oxygen,[5,7] most likely reflect changes in the microenvironments of the catalytic copper cluster. Such protein perturbations may result from the different pathways by which the site is initially produced, e.g., reduction by dithionite versus aerobic enzyme purification.

A New Type of Copper Cluster in Biology. The data reported herein, along with the analytical data for sulfide, strongly indicate that Cu_Z is a copper−sulfide cluster, the first such active-site center reported. Importantly, our results provide a link between the designation of Cu_Z as the catalytic site[4,22,51]

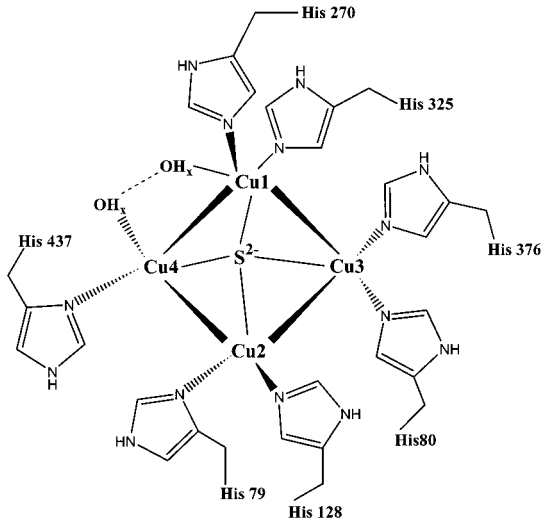

Figure 10. Model of the Cu_Z site in nitrous oxide reductase based on the X-ray crystal structure,[4] sulfur analyses,[22] and the spectroscopic data presented herein.

and the "spectroscopic" Cu_Z.[7] The significantly different vibrational frequencies and intensities in Z_{ox} and Z_{mv}, relative to those in Cu_A, are readily rationalized as reflecting the structural differences between the two types of clusters, e.g., sulfido bridging as compared to thiolate bridging. Moreover, several other aspects of the RR data argue strongly for substantial structural differences between Cu_A and Cu_Z: (1) the absence of a cysteine S−C stretch in the RR spectrum of Z_{ox} and Z_{mv} (Tables 1 and 2); (2) the smaller number of vibrational modes in Cu_Z (7 or 8 versus 10 in A_{mv}); (3) the total magnitude of the ^{15}N(His) shift, only −0.9 cm^{-1} in Z_{ox} and −1.6 in Z_{mv}, considerably smaller than the value of −4.5 cm^{-1} for the A_{mv} site in N_2OR (Table 4).

It is intriguing that an unprecedented type of copper site—a Cu_4S cluster—is utilized to catalyze one of the most difficult redox reactions in biology—the reduction of nitrous oxide to dinitrogen (eq 2). A schematic representation of Cu_Z based on the structure,[4] the analytical data,[22] and the results herein is shown in Figure 10. Clearly nitrous oxide reductase has again provided the inorganic biochemistry community a host of fascinating new questions to explore as we endeavor to elucidate the structure/reactivity correlations of the unique catalytic cluster and the molecular details of the structure, mechanism, and biosynthesis of N_2OR. These results also provide a very satisfying link between the crystal structure and the spectroscopy of N_2OR. We can now confidently assign the electronic transitions, vibrational frequencies, and EPR signals of N_2OR to either Cu_A or Cu_Z, thereby facilitating detailed studies of the relationships between active-site structure and function.

Role of the Cu_A Site in Electron Transfer. A low reorganization energy is important for minimizing the kinetic barrier of a redox reaction and thereby facilitating rapid electron transfer.[52] Multinuclear clusters such as Cu_A sites lower the reorganization energy by delocalizing the change in charge over multiple metal centers[53] and by maintaining a relatively rigid metal ion geometry that is favorable to both oxidation states. For example, EXAFS studies of CcO Cu_A have shown that very little change in Cu_2S_2 cluster geometry occurs upon one-electron reduction.[54] Resonance Raman studies have shown that all Cu_A-containing proteins and engineered constructs display common

(50) Zumft, W. G.; Kroneck, P. M. H. In *Mechanisms of Metallocenter Assembly*; Hausinger, R. P., Eichorn, G. L., Marzilli, L. G., Eds.; VCH Publishers: New York, 1996; pp 193−221.

(51) Prudêncio, M.; Pereira, A. S.; Besson, S.; Cabrito, I.; Brown, K.; Samyn, B.; Devreese. B.; Van Beeumen, J.; Rusnak, F.; Fauque, G.; Moura, J. J. G.; Tegoni, M.; Cambillau, C.; Moura, I. *Biochemistry* **2000**, *39*, 3899−3907.

(52) Ramirez, B. E.; Malmström, B. G.; Winkler, J. R.; Gray, H. B. *Proc. Natl. Acad. Sci. U.S.A.* **1995**, *92*, 11949−11951.

(53) Beinert, H. *Eur. J. Biochem.* **1997**, *245*, 521−532.

vibrational features reflective of a rigid $Cu_2Cys_2His_2$ core.[15,32] Kinetic studies of a Cu_A construct in azurin have shown that this engineered Cu_A site increases the rate of electron transfer 3-fold relative to the mononuclear Cu site in native azurin and lowers the reorganization energy by 50%.[55] The present RR data on the Cu_A site of N_2OR reveals a greater coupling of the Cu−S stretch with cysteine and histidine vibrations than in the Cu_A site of CcO. This coupling probably reflects an even greater rigidity for the Cu_A cluster in N_2OR, perhaps enhancing its effectiveness as an electron-transfer catalyst.

Loss of the N_2OR catalytic site, as in the case of the Cu_A-only variant (where Cu_A accounts for all the copper in the protein), causes subtle spectroscopic perturbations in the Cu_A site. These changes are manifested as slight differences in the relative intensities, but not frequencies, of the RR vibrational modes. EPR and ENDOR studies suggest a lower symmetry in the Cu_A site in the N_2OR variant, relative to that found in native enzyme.[10] The disturbance of this site, upon loss of the catalytic site, is understandable in terms of the proximity of the Cu_Z site in one subunit to the Cu_A in the other. As pointed out, the N_2-OR structure strongly suggests that Cu_A functions as the electron acceptor from physiological donors and likely undergoes two redox cycles during catalysis.

Abbreviations: N_2OR, nitrous oxide reductase; CcO, cytochrome *c* oxidase; RR, resonance Raman; EPR, electron paramagnetic resonance; ENDOR, electron nuclear double resonance; EXAFS, extended X-ray absorption fine structure; CD, circular dichroism; MCD, magnetic circular dichroism; NCA, normal coordinate analysis.

Acknowledgment. This work was supported by grants from the NSF [MCB-9723715 (D.M.D.)], the NIH [GM-18865 (J.S.-L.)], and the *Deutsche Forschungsgemeinschaft* and *Fonds der Chemischen Industrie* (W.Z.). We thank the Stable Isotope Resource at Los Alamos National Laboratory for providing the ^{15}N-enriched histidine (supported by NIH Grant RR02231, under the auspices of the U.S. Department of Energy). We also thank Drs. Roman Czernuszewicz, Jaqueline Farrar, and Andrew Thomson for sharing data prior to publication, and Thomas M. Loehr for numerous helpful discussions.

JA994322I

(54) Blackburn, N. J.; de Vries, S.; Barr, M. E.; Houser, R. P.; Tolman, W. B.; Sanders, D.; Fee, J. A. *J. Am. Chem. Soc.* **1997**, *119*, 6135−6143.
(55) Farver, O.; Lu, Y.; Ang, M. C.; Pecht, I. *Proc. Natl. Acad. Sci. U.S.A.* **1999**, *96*, 899−902.
(56) Han, S.; Czernuszewicz, R.; Kimura, T.; Adams, M. W. W.; Spiro, T. G. *J. Am. Chem. Soc.* **1989**, *111*, 3505−3512.