The Catalytic Center in Nitrous Oxide Reductase, Cu_Z, Is a Copper−Sulfide Cluster†

Tim Rasmussen,‡,§ Ben C. Berks,§ Joann Sanders-Loehr,|| David M. Dooley,⊥ Walter G. Zumft,# and Andrew J. Thomson*,‡

Centre for Metalloprotein Spectroscopy and Biology, School of Chemical Sciences, and School of Biological Sciences, University of East Anglia, Norwich NR4 7TJ, U.K., Department of Biochemistry and Molecular Biology, Oregon Graduate Institute of Science and Technology, Beaverton, Oregon 97006-8921, Department of Chemistry, Montana State University, P.O. Box 173400, Bozeman, Montana 59717-3400, and Lehrstuhl für Mikrobiologie, Universität Fridericiana, D-76228 Karlsruhe, Germany

Received August 2, 2000

ABSTRACT: The crystal structure of nitrous oxide reductase, the enzyme catalyzing the final step of bacterial denitrification in which nitrous oxide is reduced to dinitrogen, exhibits a novel catalytic site, called Cu_Z. This comprises a cluster of four copper ions bound by seven histidines and three other ligands modeled in the X-ray structure as OH^- or H_2O. However, elemental analyses and resonance Raman spectroscopy of isotopically labeled enzyme conclusively demonstrate that Cu_Z has one acid-labile sulfur ligand. Thus, nitrous oxide reductase contains the first reported biological copper−sulfide cluster.

Nitrous oxide reductase (N_2OR),[1] which catalyzes the final step in bacterial denitrification, the reduction of nitrous oxide to dinitrogen (*1*, *2*), plays a critical environmental role in preventing release into the atmosphere of the potent greenhouse gas nitrous oxide. The enzyme-catalyzed reaction is particularly challenging since nitrous oxide is both kinetically inert to reduction and an extremely poor transition metal ligand (*2*). The structure of the catalytic site has remained unknown until the recent report of the crystal structure of nitrous oxide reductase from *Pseudomonas nautica* (*3*). The enzyme is a functional homodimer in which a dinuclear Cu_A center, similar to that found in cytochrome *c* oxidase of mitochondria and bacteria (*4*, *5*), transfers electrons from an external electron donor to the catalytic center, designated Cu_Z. The latter is a copper cluster of unprecedented structure in which four copper ions are liganded by seven histidine residues and three additional ligands (one bridging and two terminal) modeled as either OH^- or H_2O (*3*). The four copper ions form a highly distorted tetrahedron around the bridging ligand, OH^-, which makes bonding contacts to all four copper ions with distances ranging from 2.15 to 2.38 Å. It is difficult to reconcile the structure of the Cu_Z center with spectroscopic data that strongly suggested sulfur ligation. Specifically, in the reduced state, Cu_Z generates intense magnetic circular dichroism (MCD) transitions in the visible region with a pair of perpendicularly polarized bands centered at 630 nm, and the EPR signals show very low unpaired electron spin density on the copper ions, indicating substantial metal−ligand covalency (*6*−*8*). These spectroscopic signatures are those expected of copper ions with bridging sulfur ligands but would be very surprising in a copper cluster containing only histidine and oxygen ligation (*9*).

One possible source of sulfur coordinated to Cu_Z that might not be unambiguously assigned in the structure at medium resolution is inorganic or acid-labile sulfur. We have therefore carried out sulfide and metal analyses of native N_2OR isolated from two bacterial sources, *Paracoccus pantotrophus* (formerly *Thiosphaera pantotropha*) and *Pseudomonas stutzeri.* Two mutant forms of the enzyme isolated from *P. stutzeri* have also been studied, one a site-directed variant C622D lacking copper in the Cu_A site but containing an intact Cu_Z site (*10*) and the other, N_2OR V, a variant produced in a mutant defective in cofactor assembly, which lacks Cu_Z but contains intact Cu_A. The resonance Raman (RR) spectra have also been determined from the reduced state of the enzyme by excitation at 647 nm, giving resonant enhancement only of vibrational modes coupled to electronic excitations within the Cu_Z cluster. Shifts in vibrational modes on isotopic substitution of S and Cu have also been recorded which identify both the chemical nature of the element and its metal ligation.

MATERIALS AND METHODS

Chemical Analysis. Sulfur analysis was performed using the method described by Beinert for determination of S^{2-}

† This work was supported by BBSRC Grants 43/B07079 to B.C.B., V. Fülöp, and S. J. Ferguson and 83/B08413 to A.J.T. and B.C.B. and 83/B111958 to the CMSB, by NIH Grant GM18865 to J.S.L., by the NFS to D.M.D., and by the DFG (Zu29/14-1) and Fonds der Chemischen Industrie to W.G.Z.

* To whom correspondence should be addressed: Centre for Metalloprotein Spectroscopy and Biology, School of Chemical Sciences, University of East Anglia, Norwich NR4 7TJ, U.K. E-mail: a.thomson@uea.ac.uk. Phone: +44 1603 592023. Fax: +44 1603 592003.

‡ School of Chemical Sciences, University of East Anglia.

§ School of Biological Sciences, University of East Anglia.

|| Oregon Graduate Institute of Science and Technology.

⊥ Montana State University.

Universität Fridericiana.

[1] Abbreviations: N_2OR, nitrous oxide reductase; MCD, magnetic circular dichroism; EPR, electron paramagnetic resonance; RR, resonance Raman; CT, charge transfer; EXAFS, extended X-ray absorption fine structure.

10.1021/bi001811i CCC: $19.00 © 2000 American Chemical Society
Published on Web 09/30/2000

Table 1: Determination of Copper and Inorganic Sulfur of Nitrous Oxide Reductase from *Pa. pantotrophus* and *P. stutzeri*

nitrous oxide reductase	Cu atoms/protein dimer	inorganic sulfurs/protein dimer	Cu/S ratio
wild type from *Pa. pantotrophus*	10.5 ± 2.6 (8)[a]	1.7 ± 0.4 (10)[b]	6.2
wild type from *P. stutzeri*	9.9 ± 1.7 (2)	1.6 ± 0.2 (6)[c]	6.2
N_2OR V from *P. stutzeri* (strain MK 402)	3	0.10 ± 0.04 (4)	30

[a] Results ± the standard deviation (based on *n* samples). [b] From four separate protein preparations. [c] From two separate protein preparations.

and S^0 in stable ferredoxins (*11*). The copper content was determined by inductively coupled-plasma emission spectrometry using a Thermo Jarrell Ash Polyscan 61e apparatus. The protein was quantified with the bicinchoninic acid method using bovine serum albumin as the standard (*12*).

Protein Purification and Characterization. N_2OR was purified from *P. stutzeri* ZoBell (ATCC 14405) as described previously (*13*). N_2OR V was obtained from the mutant strain MK402 which is defective in active site biosynthesis (*8*). Fully labeled [^{34}S]N_2OR was obtained from cells grown on minimal medium containing 0.25 mM $Na_2{}^{34}SO_4$ (90 at. %, ICON). Incorporation in vivo of ^{63}Cu and ^{65}Cu (99.7% pure) into N_2OR was achieved by addition of the isotope into a growing culture of *P. stutzeri* as detailed previously (*14*). Double labeling of N_2OR with ^{65}Cu and [^{15}N]histidine (racemic D,L-[^{15}N]histidine, 99% minimum, NIH National Stable Isotope Resource, Los Alamos, NM) was accomplished using an auxotrophic mutant generated by random transposon Tn5 mutagenesis (*15*, *16*). The C622D variant was prepared and purified as described elsewhere (*10*). Purification of N_2OR from *Pa. pantotrophus* was essentially as described previously (*17*) with the exception that the entire preparation was carried out under an anoxic atmosphere at 18 °C. Enzymatic activity was assayed spectrophotometrically with dithionite-reduced methyl viologen as the electron donor in 50 mM sodium Hepes (pH 7.5) (*17*) or by photochemical reduction of deazaflavin (*13*). The specific activities were as follows: *Pa. pantotrophus*, 6.9−12 μmol min^{-1} mg^{-1}; *P. stutzeri* WT, 5.5 μmol min^{-1} mg^{-1}; and C622D and N_2OR V, no activity. Resonance Raman (*18*, *19*), EPR (*7*), and MCD (*20*) spectra were measured as previously described.

RESULTS

The Cu_Z Center Contains a Cu−S Bond. The RR spectrum of dithionite-reduced *P. stutzeri* N_2OR exhibits a prominent band at 382 cm^{-1} (Figure 1a). Since the excitation wavelength of 647 nm corresponds to the absorption maximum of the reduced form of Cu_Z, the 382 cm^{-1} mode is associated with the Cu_Z cluster. On substitution of the natural sulfur isotope ^{32}S with ^{34}S, this band undergoes an isotope shift of −5.8 cm^{-1}. Substitution of ^{63,65}Cu with ^{65}Cu, and of histidine with ^{15}N (His), gives isotope shifts of −0.8 and 0 cm^{-1}, respectively (data not shown). Thus, the 382 cm^{-1} RR band arises from a stretching vibration involving copper−sulfur motion. This Cu−S vibrational mode is also detected at 384 cm^{-1} in the RR spectrum of the Cu_A deficient mutant C622D (Figure 1b), confirming that the sulfur atom detected is associated with Cu_Z.

N_2OR Contains Acid-Labile Sulfide When Cu_Z Is Present. Sulfide and copper analyses on native N_2OR isolated from *Pa. pantotrophus* and *P. stutzeri* both show copper levels close to 12 per dimer as anticipated from the crystal structure (Table 1). Inorganic sulfur, at 1.7 ± 0.4 sulfur atoms per protein dimer in one case and 1.6 ± 0.2 in the other, was also found, giving a Cu/S ratio of 6.2 in both proteins. Therefore, the native enzyme contains one inorganic sulfur atom per monomer. This atom can be associated with the Cu_Z cluster because an insignificant amount of sulfur, <0.1, is present in the N_2OR V, a variant which lacks Cu_Z (*8*). Furthermore, no Cu−S vibrational modes characteristic of Cu_Z are seen in the RR spectrum of the N_2OR V variant (*21*). The intensities of the RR, MCD, and UV−vis spectra of Cu_Z in mutant C622D point to the presence of one Cu_Z center per monomer (*10*). However, analysis of the sulfide content leads to greater variability and lower absolute values (0.46−0.85 sulfur atom per dimer) than for the wild-type protein, suggesting that the sulfide ion may be more susceptible to handling losses in this form of the enzyme which lacks Cu_A.

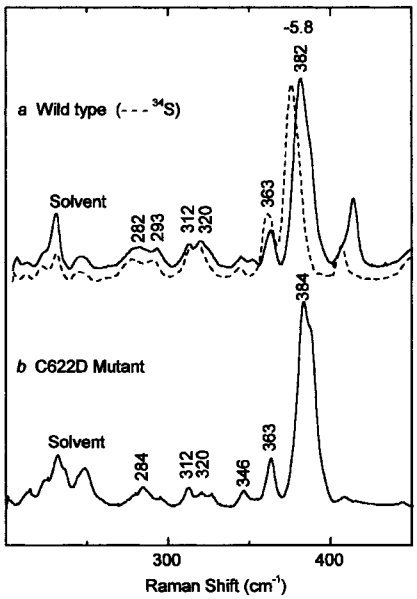

FIGURE 1: Resonance Raman spectra of Cu_Z obtained with 647 nm excitation (a) from dithionite-reduced N_2OR (~1 mM) obtained from cells grown on minimal medium with ^{32}S (—)- or ^{34}S-labeled (- -) sulfate. Peak frequencies are for the ^{32}S species with ^{34}S frequency shifts listed above. (b) RR spectra of the C622D mutant protein of N_2OR (1.2 mM).

Optical and Magnetic Properties Indicate a Bridging Sulfur Ligand in Cu_Z. The crystal structure of the "blue" form of the enzyme from *P. nautica* was determined in which both Cu_A and Cu_Z are reduced (*22*). In this state, Cu_A is EPR silent and lacks optical absorption, whereas the Cu_Z center is paramagnetic, giving an $S = {}^1/_2$ EPR signal, and has broad absorption maxima at 630 and 760 nm typical of reduced N_2OR. The crystal absorption spectrum of this form of the enzyme frozen at 100 K exhibited a prominent absorption peak at 635 nm and a broad shoulder at ca. 750 nm (*3*), demonstrating the similarity of the Cu_Z cluster between proteins from different sources, and also proving that the crystalline form that was studied contained the reduced, *S*

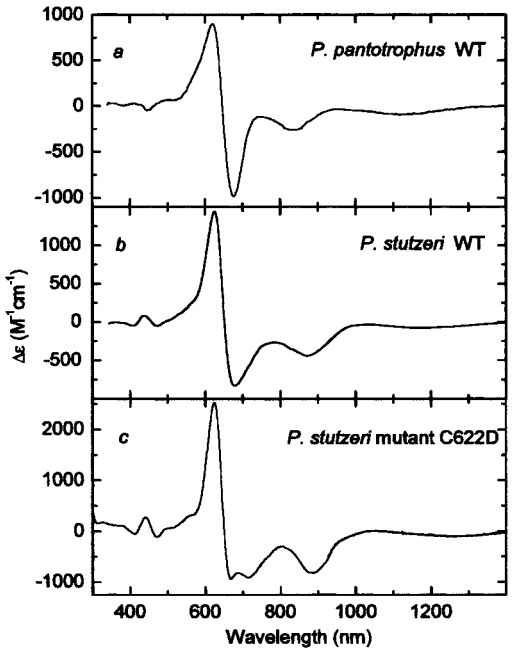

FIGURE 2: MCD spectra of Cu_Z from (a) 0.067 mM dimer N_2OR from *Pa. pantotrophus* reduced with dithionite, (b) 0.5 mM N_2OR from *P. stutzeri* reduced with dithionite, and (c) 0.036 mM N_2OR from *P. stutzeri* mutant C622D as isolated. Spectra were recorded at 4.2 K and a field strength of 5 T.

$= {}^1/_2$ form, of Cu_Z. Hence, reduced Cu_Z in the crystalline form has the same molecular structure as the solution forms of the enzyme from *Pa. pantotrophus* and *P. stutzeri*.

Magnetic circular dichroism (MCD) spectra measured at 4.2 K detect optical transitions only from paramagnets. The broad absorption spectrum of reduced Cu_Z ($S = {}^1/_2$) is resolved into at least nine optical transitions in the low-temperature MCD spectrum (*7*). This provides a diagnostic fingerprint of the reduced Cu_Z center, showing it to be structurally similar in the native enzyme from *Pa. pantotrophus* and *P. stutzeri* and in mutant C622D (Figure 2). The MCD signals are intense and likely arise from sulfide−copper(II) and copper(I)−copper(II) intervalence charge transfer (CT) transitions (*23*−*26*). The oppositely signed MCD bands centered at 650 nm come from two perpendicularly polarized transitions into excited states lying close in energy. The near degeneracy of these excited states may arise from the high symmetry of the metal−sulfur cluster. Since only one labile sulfide per Cu_Z has been detected, the sulfur ligand must occupy a bridging position between at least two copper ions to meet the symmetry requirement. The X-band EPR signal from the $S = {}^1/_2$ ground state of Cu_Z shows no resolved copper nuclear hyperfine interaction (*6*, *13*, *22*, *27*). This is atypical of the EPR spectra of mixed-valence copper clusters with N and O ligation (*28*) but shows the high degree of covalence expected from a covalent Cu−S interaction. Hence, the MCD and EPR spectra support the conclusion drawn from metal analyses and RR in showing the presence of sulfur ligation to copper in Cu_Z.

DISCUSSION

The analytical evidence together with the RR data shows that the Cu_Z cluster contains one sulfide ion per four copper ions and proves that the sulfide ion is part of a cluster with significant Cu−S bonding. These data, together with previous spectroscopic studies, strongly suggest that the bridging ligand in the Cu_Z cluster, which has been modeled in the 2.4 Å resolution crystal structure as an oxygen atom, is inorganic sulfide. The large total RR isotope shift of −17 cm^{-1} in the RR spectrum upon substitution of ^{34}S for ^{32}S points to a bridging sulfide with two or more Cu−S bonds (*21*). The vibrational frequency of 382 cm^{-1} for ν(Cu−S) can be compared with values of ν(Fe−S) in Fe−S clusters. These lie in the range of 335−445 cm^{-1} for the symmetric μ_3-S stretch in [3Fe-4S] and [4Fe-4S] cores and in the range of 280−290 and 390−395 cm^{-1} for the μ_2-S stretching modes in [2Fe-2S] (*29*). Analysis of the Cu K-edge EXAFS suggested a contribution from sulfur to the backscattering from Cu_Z (*10*). A schematic model of Cu_Z is given in Figure 3 in which the sulfide ion is proposed to bridge the four copper ions. Cu_Z represents the first example in biology of a copper−sulfide cluster, although a synthetic copper−sulfide cluster made by insertion of Cu(I) into a [3Fe-4S] core cluster to form [3FeCu-4S] cubane cluster has been reported (*30*). Cu_Z is a copper-based analogue of the iron−sulfur clusters commonly encountered in biological redox proteins. It remains to be seen what role a copper−sulfide cluster plays in the catalytic reduction of N_2O. One can speculate that the bridging sulfide ion could accept the oxygen atom from N_2O, forming a sulfoxide intermediate prior to reduction to water, and releasing N_2.

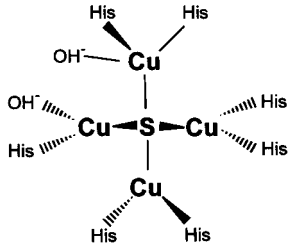

FIGURE 3: Schematic diagram of Cu_Z based on the crystal structure (*3*) and spectroscopic data found in the work presented here.

ACKNOWLEDGMENT

We are grateful to B. Schreckenberger, J. Nuttall, C. Bouchard, R. Jones, and C. Robinson for assistance with protein purification, M. L. Alvarez, J. Ai, and H. Seward for help with spectroscopy, and J. Farrar, R. R. Eady, and C. Andrew for helpful discussions.

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BI001811I