Nitrous-oxide reductases (N2ORs) catalyze the two-electron reduction of \( \text{N}_2\text{O} \) to \( \text{N}_2 \). The crystal structure of N2ORs from *Pseudomonas nautica* (Pn) and *Paracoccus denitrificans* (Pd) were solved at resolutions of 2.4 and 1.6 Å, respectively. The Pn N2OR structure revealed that the catalytic CuZ center belongs to a new type of metal cluster in which four copper ions are liganded by seven histidine residues. A bridging oxygen moiety and two other hydroxide ligands were proposed to complete the ligation scheme. The exact structure of the CuZ center remained unknown, however, and its binuclear nature has been questioned because twice the number of electrons expected are needed for complete reduction.

The first structure of N2OR shed light on the elusive CuZ center. The structure of Pn N2OR was solved by multiple wavelength anomalous dispersion phasing using the copper anomalous signal and was refined to 2.4 Å resolution. It is a dimer in the crystal, as in solution, in which each N2OR monomer is composed of two domains formed by contiguous segments in the amino acid sequence: the N-terminal domain, a β-propeller, and the C-terminal domain with a cupredoxin fold. The CuZ center of N2OR comprises four copper ions arranged in a distorted tetrahedron and seven histidine residues. CuI, II, and III are ligated by two histidines, whereas CuIV is ligated by only one, thereby forming a putative substrate binding site.

The bridging ligand has recently been proposed to be an inorganic sulfur ion, which would reconcile the previous spectroscopic interpretations with the novel CuZ cluster structure. We have reinvestigated this question by three means: (i) direct observation of a sulfur atom in the 1.6 Å electron density map of the recently solved N2OR from Pd; (ii) introduction of a bridging sulfur in the Pn N2OR electron density map, followed by refinement; and (iii) direct chemical determination of the inorganic sulfur content in Pn and Pd N2ORs. These studies, reported here, confirm the presence of a bridging sulfur in the CuZ cluster.

**MATERIALS AND METHODS**

Purification and Inorganic Sulfur Characterization—Pn N2OR was purified and characterized as described elsewhere. Sulfur determination was done by the method of Fogo and Popowsky (8) with the alterations suggested by Beinert (9). This method is based on the precipitation of protein in alkaline medium containing zinc hydroxide. After acidification, the condensation occurs between the H\(_2\)S formed and two molecules of N,N’-dimethylphenyldiamine forming methylene blue that absorbs at 670 nm. As sulfur can be oxidized by oxygen and can be partially lost by acidification, it is therefore very important to remove air from all the solutions and to perform all the manipulations.
under anaerobic conditions. We have used [3Fe-4S] ferredoxin II from Desulfovibrio gigas to calibrate the titration method. Copper was determined by a spectrophotometric method using cuproine as a copper ligand. The Cu-Cu and Cu–His bond energy constants were determined by a spectrophotometric method using cuproine as a copper ligand. The CuZ center, the first at 3.5 Å resolution. All four molecules in the Pd N2OR asymmetry were observed in the region of the CuZ center, the first at +8 σ, allowing an S2– ion to be manually placed bridging the four copper ions, and a second difference density. The CuZ center from Pd N2OR, including ligating His but oxygen ligands, was then used as an initial model for the Pn enzyme. The CuZ geometry was optimized by manual repositioning, removing the NCS restraints and introducing water molecules. At this stage, the Rfree remained blocked at a value of 27.6%. It became apparent that, whereas molecules A and B in the first dimer were well defined, in the second dimer molecule D was disordered and C displayed a high agitation. To model the disorder of molecule D, a high B-factor of molecule C compared with those of molecules A and B in the first dimer was well defined. The Pd N2OR data were collected on BW7A (DESY, Hamburg), and the Pd N2OR data were collected on ID14-4 (ESRF, Grenoble), and the Pn N2OR data were collected on BW7A (DESY, EMBL-Hamburg). Data were indexed and integrated with DENZO/SCALEPACK (12) or with MOSFLM/SCALA (13). Molecular replacement was performed with AMoRe (14). The model building used TURBO-FRODO (15), and refinement was performed with CNS 0.9 (16). Statistics on data collection and refinement are presented in Table I. The coordinates have been deposited with the Protein Data Bank with codes 1NQI and 1FWX for Pn N2OR and Pd N2OR, respectively.

RESULTS AND DISCUSSION

Inorganic Sulfur Determination—Copper and sulfur were determined for the Pn N2OR in three different samples. The copper content per monomer was found to be 6.0, close to the value published previously (in Ref. 6). The sulfur content was 1.05 ± 0.05 sulfur atoms/monomer, corresponding to a copper/sulfur ratio of 6.3. For the Pd N2OR, only one sample was measured. The values obtained per monomer were 5.5 copper atoms and 0.7 sulfur atom, corresponding to a copper/sulfur ratio of 7.8.

Three-dimensional Structure—Pn N2OR structure was solved and refined at 2.4 Å resolution (5). A Pn N2OR monomer was used as a starting model to solve, by molecular replacement, the structure of the Pd N2OR, using data between 10 and 3.5 Å resolution. All four molecules in the Pd N2OR asymmetric unit were located successively (more detailed data will be presented elsewhere).3 Preliminary minimization and B-factor refinements were performed with bulk solvent correction and NCS restraints but with neither water molecules nor the three OH− ions of the CuZ center. Two regions of discrete Fo−Fc SigmaA difference density were observed in the region of the CuZ center, the first at +8 σ, allowing an S2− ion to be manually placed bridging the four copper ions, and a second difference density at +5 σ, in which an oxygen ligand (OH− ion or oxygen atom) was positioned, bound to CuI but also close to CuIV. The chemical nature of these ions was confirmed after iterative rounds of refinement and graphical remodeling in which the Cu-Cu and Cu–His bond energy constants were

![](https://www.jbc.org/doi/abs/10.1074/jbc.2007.07.11344)

**FIG. 1.** Pn N2OR, the N2O reductase from *P. nautica*. An overall view of the crystal structure of the N2O dimer is shown; one monomer is uniformly colored gray, whereas for the other monomer the propeller domain β-strands are dark green and the α-helices light green, the linker is purple, and the β-strands of the cupredoxin domain are blue or red according to the β-sheet they form; the short 310 helix adjacent to the CuA center is dark blue (figure prepared with MOLDRAW (19)).

<table>
<thead>
<tr>
<th>TABLE I Data collection and refinements statistics for <em>P. nautica</em> and <em>P. denitrificans</em> N2ORs</th>
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<td><strong>Refinement statistics for <em>P. nautica</em> N2OR</strong></td>
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<td>Mean B-factors (Å²): molecules A, B, C, D r.m.s.d. of B-factors: main chain/side chain r.m.s.d. of bonds (Å)</td>
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3 K. Djinic-Carugo, manuscript in preparation.
constants were increased, and rounds of cartesian minimization and unrestrained B-factor refinement were performed with NCS restraints. In the revised CuZ cluster of Pn N2OR, the bridging sulfur and unique oxygen moiety refined to give B-factors similar to those of their associated molecules and no \( F_o - F_r \) difference Fourier density.

The CuZ Clusters from \( P. \) denitrificans and \( P. \) nautica \( N_2O \) Reductases—The Pd N2OR CuZ cluster has a very well defined electron density (Fig. 2). At 5 and 10 \( \sigma \) levels, the \( 2F_o - F_r \) SigmaA electron density map shows individual spheres centered on the sulfur and copper atoms, respectively (Fig. 2, A and B). The volume of the \( 2F_o - F_r \) SigmaA map contoured at the 4.5 \( \sigma \)
level for the CuZ cluster sulfur is comparable with that of other sulfurs in the neighborhood, such as Met\textsuperscript{470} (Fig. 2, B and C). An oxygen moiety, located on CuIV, also has a discrete density that disappears under the 4 σ contour level. The cluster is very similar to that published for Pn N2OR (5), with some exceptions: the presence of an inorganic sulfur bridging the four copper ions, and the presence of a unique oxygen moiety bound to CuIV, but very close also to CuI (Figs. 2, A and B, and 3).

In the revised CuZ cluster from Pn N2OR, a bridging inorganic sulfur and a unique oxygen moiety resisted refinement. Its geometry is slightly different, however, from that previously published; the CuI position has moved slightly outward because of a histidine coordinated to this site, was previously attributed to an oxygen moiety bound to CuI. The positive electron density bulb attributed to an oxygen ligand was previously attributed to an oxygen moiety bound to CuIV. The lengthening of 0.25 Å of the CuI–CuIV/CuIV distances, and the deviations are given in parentheses, C, the CuZ center of Pd N2OR. The interatomic distances within the CuZ cluster of the best defined monomers are indicated in Ångströms for CuZ (OH or O\textsuperscript{2−}), and the deviations are given in parentheses, C, the CuZ center of Pd N2OR.

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Spectroscopic Studies—In addition to the EPR results reported previously (6), preliminary ENDOR data\textsuperscript{4} indicate that no exchangeable protons were detected at the CuZ site. These data indicate also that the spin density (Cu\textsuperscript{2+} d9) must reside on copper IV, because an intense \textsuperscript{14}N ENDOR resonance, most probably due to the histidine coordinated to this site, was identified. These results are compatible with the proposed structure of the Pn N2OR CuZ coordination (5).

CONCLUSION

Based on spectroscopic data (mainly magnetic circular dichroism), Farrar et al. (3) have concluded that the properties attributed to CuZ chromophore are due to a thiolate bridged dinuclear copper center. These authors also conclude that N2OR contains three forms of copper thiolate center, CuA, CuZ, and CuZ\textsuperscript{*}, each able to perform a single redox cycle (3). Resonance Raman studies indicated that there is a highly covalent (Cu(II)-S-Cu(I)-S(Cys)) thiolate site on the reduced form of N2OR (17, 18). The crystal structure of Pn N2OR showed there to be no cysteine thiolate ligation in the CuZ cluster, the only ligating residues being histidines (5). However, the high resolution Pd N2OR structure revealed that the CuZ cluster has an inorganic bridging sulfur atom instead of an oxygen as has been proposed for Pn N2OR (5, 6). The results of inorganic sulfur titration performed on Pd and Pn N2OR were consistent with this observation. The coordinates of the revised CuZ center, issued from the Pd N2OR structure, could be successfully transplanted in the Pn enzyme model and refined, which further confirms the presence of a bridging sulfur in Pn N2OR. These observations appear to be consistent with recent results obtained with Pseudomonas stutzeri N2OR (7), indicating that the presence of a bridging sulfur in the CuZ cluster might be a general feature of N2ORs. However, the consequences of the presence of an inorganic sulfur ion for the reduction mechanism of N\textsubscript{2}O remain unclear.

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Revisiting the Catalytic CuZ Cluster of Nitrous Oxide (N₂O) Reductase: EVIDENCE OF A BRIDGING INORGANIC SULFUR
Kieron Brown, Kristina Djinovic-Carugo, Tuomas Haltia, Ines Cabrito, Matti Saraste, José J. G. Moura, Isabel Moura, Mariella Tegoni and Christian Cambillau

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