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Pseudomonas stutzeri N_2O reductase contains Cu_A-type sites

ROBERT A. SCOTT*, WALTER G. ZUMFT†, CATHERINE L. COYLE‡, AND DAVID M. DOOLEY§¶

*Departments of Chemistry and Biochemistry and the Center for Metalloenzyme Studies, University of Georgia, Athens, GA 30602; †Lehrstuhl fur Mikrobiologie, Universität Karlsruhe, D-7500 Karlsruhe 1, Federal Republic of Germany; ‡Exxon Research and Engineering, Annandale, NJ 08801; and §Department of Chemistry, Amherst College, Amherst, MA 01002

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ABSTRACT **N_2O reductase ($N_2O \rightarrow N_2$) is the terminal enzyme in the energy-conserving denitrification pathway of soil and marine denitrifying bacteria. The protein is composed of two identical subunits and contains eight copper ions per enzyme molecule. The magnetic circular dichroism spectrum of resting (oxidized) N_2O reductase is strikingly similar to the magnetic circular dichroism spectrum of the Cu_A site in mammalian cytochrome *c* oxidase [Greenwood, C., Hull, B. C., Barber, D., Eglinton, D. G. & Thomson, A. J. (1983) *Biochem. J.* 215, 303–316] and is unlike the magnetic circular dichroism spectra of all other biological copper chromophores obtained to date. Sulfur (or chlorine) scatterers are required to fit the copper extended x-ray absorption fine structure data of both the oxidized and reduced forms of N_2O reductase. Satisfactory fits require a Cu-N or Cu-O [denoted Cu-(N, O)] interaction at 2.0 Å, a Cu-(S, Cl) interaction at 2.3 Å and an additional Cu-(S, Cl) interaction at ≈2.6 Å (oxidized) or ≈2.7 Å (reduced). Approximately eight sulfur ions (per eight copper ions) at ≈2.3 Å are required to fit the extended x-ray absorption fine structure data for both the oxidized and reduced N_2O reductase. The 2.3-Å Cu-(S, Cl) distance is nearly identical to that previously determined for the Cu_A site in cytochrome *c* oxidase. A 2.6–2.7 Å Cu-(S, Cl) interaction is also present in resting and fully reduced cytochrome *c* oxidase. Comparison of the N_2O reductase sequence, determined by translating the structural *NosZ* gene, with cytochrome *c* oxidase subunit II sequences from several sources indicates that a Gly-Xaa-Xaa-Xaa-Xaa-Xaa-Cys-Ser-Xaa-Xaa-Cys-Xaa-Xaa-Xaa-His stretch is highly conserved. This sequence contains three of the probable ligands (two cysteines and one histidine) in a Cu_A-type site. Collectively these data establish that *Pseudomonas stutzeri* N_2O reductase contains Cu_A-type sites.**

N_2O reductase is the terminal enzyme in the metabolic pathway for denitrification ($2NO_3^- \rightarrow N_2$). This enzyme catalyzes the two-electron reduction of nitrous oxide:

$$N_2O + 2e^- + 2H^+ \rightarrow N_2 + H_2O. \quad [1]$$

N_2O reduction is coupled to ATP generation (1, 2) and appears to be linked to the formation of a membrane potential (3) and to proton translocation (4–6). N_2O reductase is composed of two identical subunits of 638 residues (M_r = 70,822) (7) and contains six to eight copper ions per enzyme molecule‖. At least some of these coppers form part of an unusual chromophore (λ_{max} = 540 nm, ε = 16,300 $M^{-1} \cdot cm^{-1}$) (8). Several gene products are required for assembly of the chromophore in the protein (7, 9–12). Resonance Raman spectroscopy has established that the principal electronic absorption bands of resting N_2O reductase are assignable as S → Cu(II) ligand-to-metal charge-transfer transitions, probably associated with [Cu(II)-S_2(Cys)$_2$N(His)] sites (13). Pulsed EPR results are also consistent with histidine ligation to the EPR-detectable coppers (14). The ground-state properties of at least some of the copper ions in N_2O reductase are unusual: from 10 to 77 K, a seven-line copper hyperfine pattern is observed, but at 100 K, $A_{||}$ is no longer resolved. Furthermore, between 50% and 80% of the copper is EPR nondetectable, depending upon the sample and pH (14, 15). Resting N_2O reductase can be isolated in different forms, as judged by variations in the absorption and EPR spectra and catalytic activity (8, 14–16). The high-activity and low-activity resting forms have been designated N_2OR I and N_2OR II, respectively (15). [Cu(II)-S_2(Cys)$_2$N(His)] sites are present in both the high- and low-activity resting forms (13), although the number of such sites in these two forms may be different. N_2O reductases isolated from other denitrifying bacteria are similar in many respects to the *Pseudomonas stutzeri*** enzyme (17, 18). For example, the enzyme from *Paracoccus denitrificans* also has two subunits with a total M_r of 144,000, eight copper ions per mole of protein, a similar visible absorption spectrum, and ≥70% EPR-nondetectable copper (17).

As implied above, there are at least two types of copper sites in N_2O reductase: [Cu(II)-S_2(Cys)$_2$N(His)] sites, which are presumably EPR detectable and are associated with the 540-nm absorption band (13), and EPR-nondetectable sites. Another type of copper site is observed in N_2OR II. This additional site is characterized by an absorption band at 650 nm, a broad EPR signal, and a resonance Raman spectrum that is similar to the spectra displayed by type 1 or "blue" copper sites (8, 13, 15). Reduced N_2O reductase displays these same spectroscopic features (8, 13, 15). The [Cu(II)-S_2(Cys)$_2$N(His)] sites in N_2O reductase (N_2OR I and II) are spectroscopically similar, in some respects, to the site in Cu(II)-substituted liver alcohol dehydrogenase (13, 19), where the ligands are two cysteine sulfurs, an imidazole (His) nitrogen, and water (19). There is considerable evidence to suggest that one of the copper sites in cytochrome *c* oxidase, the EPR-detectable Cu_A, also has cysteine sulfur and histidine imidazole ligands (20–22). In this paper we present results from x-ray absorption spectroscopy (XAS), magnetic circular dichroism (MCD) spectroscopy, and sequence comparisons that establish that resting N_2O reductase contains Cu_A-type sites.

EXPERIMENTAL PROCEDURES

Resting forms of N_2O reductase (N_2OR I and N_2OR II) were purified as described (8, 16). Samples were characterized by

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Abbreviations: XAS, x-ray absorption spectroscopy; MCD, magnetic circular dichroism; EXAFS, extended x-ray absorption fine structure.

¶To whom reprint requests should be addressed.

‖The copper stoichiometry is somewhat variable from one preparation to another. Generally, the more active preparations have higher copper contents, with a limiting stoichiometry of eight copper ions per enzyme molecule (dimer).

**Previously designated *Pseudomonas perfectomarina*; see Döhler, K., Huss, V. A. R. & Zumft, W. G. (1987) *Int. J. Syst. Bacteriol.* 37, 1–3.

specific activity, copper content, and A_{540}/A_{480} ratio (15). N_2OR I has at least 2–3 times the activity of N_2OR II and a higher A_{540}/A_{480} ratio. Absorption spectra were measured prior to and following XAS experiments to ensure sample integrity. Reduced N_2O reductase was prepared from a sample of N_2OR I by adding dithionite to a concentrated solution of the resting enzyme in the XAS cell. Reduction was monitored visually by using the characteristic color change from purple (N_2OR I, λ_{max} = 540 nm) to blue (N_2OR III, λ_{max} = 650 nm). XAS data collection and reduction are summarized in Table 1. Backscattering functions for curve-fitting analysis of Cu(II)-N, Cu(II)-S, Cu(I)-N, and Cu(I)-S interactions were empirically extracted from model compounds as detailed previously (23).

MCD spectra were obtained with a modified JASCO-J40A, equipped with a Morvue photoelastic modulator and a PAR lock-in amplifier. Spectra were recorded and processed with a Bascom-Turner digital recorder. An Alpha Scientific electromagnet provided fields of ≈1 tesla. Baseline corrections and the subtraction of natural circular dichroism were done digitally. The spectropolarimeter was calibrated with 10-camphorsulfonic acid and by using the MCD signal of $Fe(CN)_6^{3-}$ at 420 nm. Additional experimental details are given in the figure legends.

RESULTS

Visible absorption and MCD spectra of resting N_2O reductase are shown in Fig. 1. These spectra are very different from spectra typical of the well-characterized type 1, type 2, and type 3 sites (25). Much more striking is the comparison of the MCD spectra of N_2OR I and the Cu_A site in mammalian cytochrome *c* oxidase (Fig. 1*b*). Although the dispersion and polarizations of the Cu_A MCD are firmly established (24, 26), the absolute intensities are derived from a magnetization analysis and are more uncertain owing to partial overlap with signals from the heme centers (26). There is some uncertainty

Table 1. X-ray absorption spectroscopic data collection and reduction for N_2O reductase

Parameter	Edges	EXAFS
Facility	SSRL	SSRL
Beamline	IV-1	IV-1
Monochromator crystal	Si[220]	Si[220]
Detection method	Fluorescence	Fluorescence
Detector type	Ar ion chamber*	Ar ion chamber*
Scan length, min	14	23
Scans in average	2	10–11
Metal concentration, mM	6	6
Temperature, K	10	10
Energy standard	Cu foil (1st inflection)	Cu foil (1st inflection)
Energy calibration, eV	8980.3	8980.3
E_0, eV	9000	9000
Pre-edge background energy range, eV (polynomial order)	8700–8950 (2)	9050–9642 (2)†
Spline background energy range, eV (polynomial order)	9050–9382 (2)	9028–9180 (3) 9180–9400 (3) 9400–9642 (3)

SSRL, Stanford Synchrotron Radiation Laboratory.
*EXAFS Company, Seattle.
†The background was calculated from fitting this (EXAFS) region; then a constant was subtracted so that the background matched the data just before the edge.

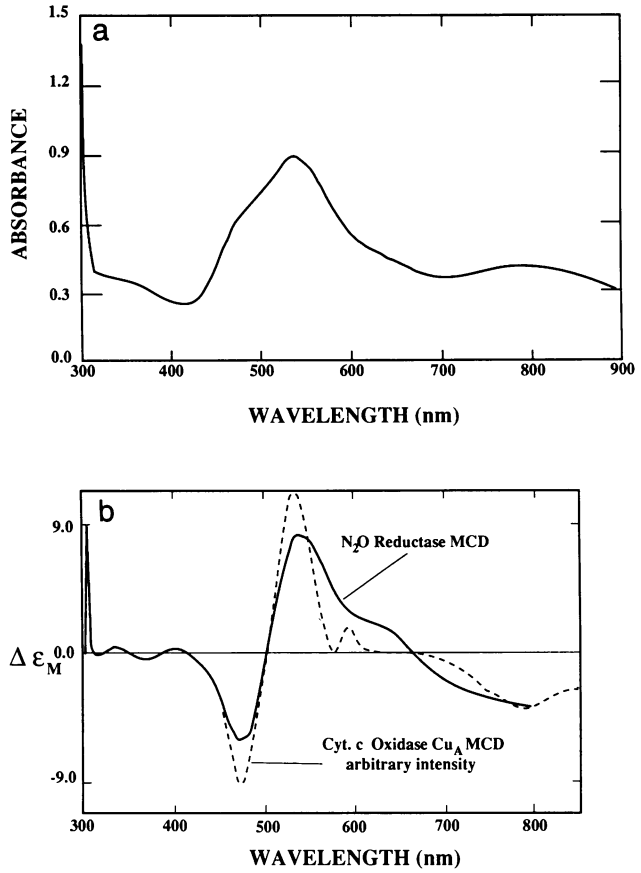

FIG. 1. Visible absorption (*a*) and MCD (*b*) spectra of resting N_2O reductase (N_2OR I, high activity) in 50 mM Tris buffer, pH 7.5, at 20°C. The Cu_A MCD spectrum also shown in *b* was adapted from ref. 24. Note that the N_2O reductase spectrum was obtained at 293 K, whereas the Cu_A MCD was determined at 4.2 K.

associated with the $\Delta\varepsilon_M$ values reported for N_2O reductase as the magnitude depends significantly on the A_{540}/A_{480} ratio (i.e., on the proportions of N_2OR I and N_2OR II in a given sample). Also complicating the comparison are the different temperatures at which the spectra were recorded and the fact that Cu(II) MCD is expected to be temperature dependent (see below). Nevertheless, the data suggest that at least one Cu_A-type site is present in N_2O reductase.

Copper x-ray absorption K-edge spectra for N_2OR I and mammalian cytochrome *c* oxidase are shown in Fig. 2. There is no detectable difference (in either the oxidized or reduced edges) between N_2OR I and N_2OR II (data not shown). In both cytochrome *c* oxidase and N_2O reductase, there is a relatively small edge-shift that accompanies reduction. Such

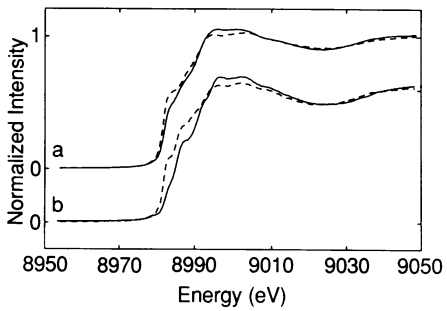

FIG. 2. Copper K-edge x-ray absorption spectra for high-activity N_2O reductase N_2OR I (traces a) and cytochrome *c* oxidase (traces b) (see ref. 27). In each set of edges, the solid line is for the oxidized (resting) enzyme, and the dashed line is for the dithionite-reduced enzyme.

behavior can be reproduced in model copper complexes with sulfur-donor ligands (27); the shapes of these absorption edges are also consistent with sulfur ligation. The copper edge of resting N_2OR I at pH 9.8 (data not shown) is nearly identical to the edge at pH 7.5 (Fig. 2). Thus the increase in specific activity (≈10-fold) at pH 10 compared to pH 7.5 is probably not associated with drastic changes in the oxidation state or ligation of the copper ions.

Copper extended x-ray absorption fine structure (EXAFS) data of resting and dithionite-reduced N_2OR I are shown in Figs. 3*a* and 4*a*, respectively. Curve-fitting results are displayed in Figs. 3*b* and 4*b* and are tabulated in Table 2. It must be emphasized that the coordination numbers are averaged over *all* the coppers in the enzyme. Sulfur (or chlorine) scatterers are required to fit the EXAFS of both the oxidized and reduced forms. Acceptable fits require a Cu-(N, O) interaction at ≈2.0 Å, a Cu-S or Cu-Cl [denoted Cu-(S, Cl)] interaction at ≈2.3Å, and a Cu-(S, Cl) interaction at ≈2.6 Å (oxidized, fit 3, Table 2) or ≈2.7 Å (reduced, fit 7, Table 2). The fits are slightly improved by introducing static disorder into the Cu-(N, O) distances (fits 4 and 8, Table 2). The 2.3-Å Cu-(S, Cl) distance is very similar to that determined for the Cu_A site in cytochrome *c* oxidase (27). The origin of the 2.6- to 2.7-Å Cu-(S, Cl) interaction is unknown. Recent analysis of Cu EXAFS data indicate the presence of similar Cu-(S, Cl) interaction in resting (≈2.6 Å) and fully reduced (≈2.7 Å) samples of cytochrome *c* oxidase (R.A.S., P. M. Li, S. I. Chan, unpublished observations). The presence of such a long-distance Cu-(S, Cl) interaction in the resting-state enzyme was first pointed out by George *et al.* (28). Our analysis

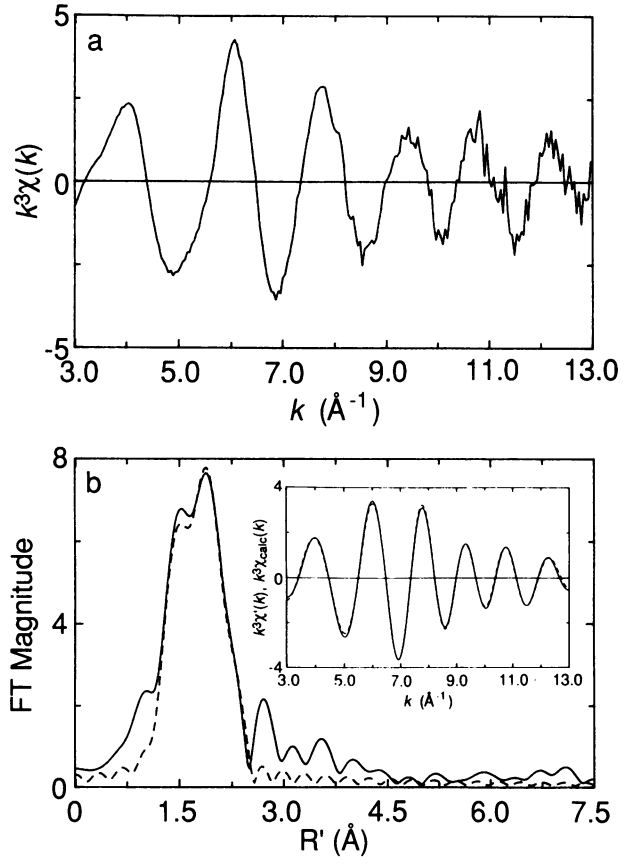

FIG. 3. Copper EXAFS data and curve-fitting simulation for oxidized high-activity N_2O reductase (N_2OR I). (*a*) EXAFS data. (*b*) The data in *a* were Fourier transformed over the k = 3.0–13.0 Å^{-1} range (k^3 weighting) to yield the solid line. (*Inset*) The first-shell peak (R' = 1.10–2.50 Å) of this Fourier transform data was back-transformed to yield the filtered EXAFS data shown as the solid line. The dashed lines in *b* and the *Inset* represent the best curve-fitting simulation (fit 3, Table 2).

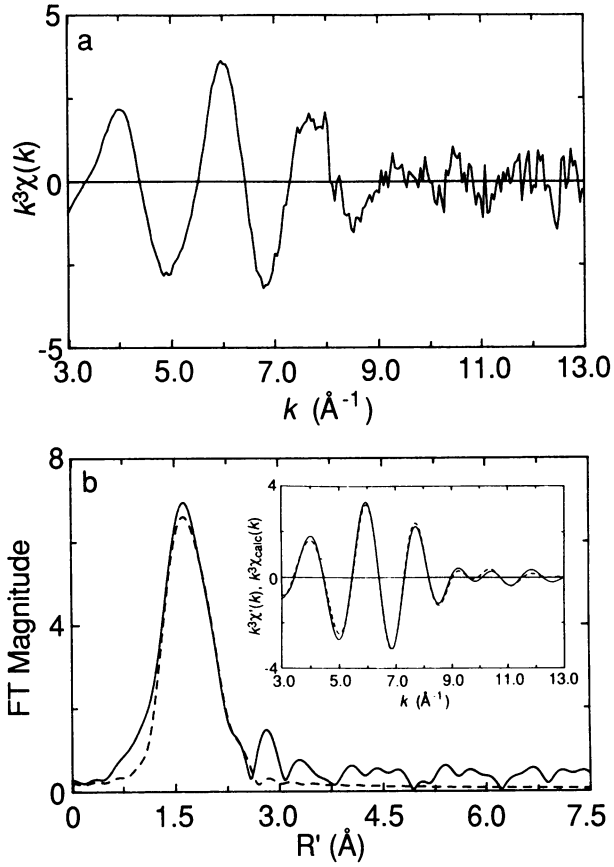

FIG. 4. Copper EXAFS data and curve-fitting simulation for dithionite-reduced N_2OR I. The data are displayed as described in the legend to Fig. 3. The Fourier filter range was R' = 1.05–2.55 Å and the dashed lines in *b* and the *Inset* represent fit 7 of Table 2.

does not preclude the possibility that significant copper–copper interactions may be present.

DISCUSSION

The MCD dispersion of $\Delta A/E$ of an electronic transition (A → J, from ground state A to some electronic excited state J) is the sum of three terms (A, B, and C) given by Eq. 2 (29):

$$\frac{\Delta A}{E}(\mathrm{A} \rightarrow \mathrm{J}) = 152.5\, HCl\left\{-A_1\left(\frac{\partial f}{\partial E}\right) + \left(B_0 + \frac{C_0}{kT}\right) f\right\}, \quad [2]$$

where **C** = concentration in mol/liter, l = pathlength in cm, and H = the magnitude of the magnetic field in tesla. $f(E)$ is the lineshape function (gaussian, for example) of the transition, and ΔA is the difference in absorption of left and right circularly polarized light induced by the magnetic field.

A_1, B_0, and C_0 are electronic parameters that determine the sign and magnitude of the corresponding A, B, and C terms. The sign and magnitude of these parameters depend upon magnetic field effects on the molecular energy levels and degeneracies of a chromophore and the selection rules for absorption of circularly polarized light (29–31). The important point is that MCD spectra reflect the electronic structure of a molecule and do not depend upon any inherent dissymmetry or coupling to a dissymmetric environment. Hence a close similarity in the MCD dispersion between two copper centers probably reflects similar electronic structures. For N_2O reductase, the observed electronic transitions are ligand-field (*d–d*) and ligand-to-metal charge-transfer transitions of Cu(II) sites. *B* and especially *C* terms compose the MCD spectra of low-symmetry Cu(II) complexes (31–33). At low temperatures the *C* terms dominate, owing to the inverse temperature dependence, so the Cu_A MCD spectrum in Fig.

Table 2. Curve-fitting results for the first coordination sphere of N_2O reductase derivatives

Fit	Cu(N, O) N_s^*	Cu(N, O) R_{as}, Å	Cu(N, O) $\Delta\sigma^2_{as}$, Å^2	Cu(S, Cl) N_s^*	Cu(S, Cl) R_{as}, Å	Cu(S, Cl) $\Delta\sigma^2_{as}$, Å^2	f'†
Oxidized sample (N_2OR I)							
1	3.0	1.99	+0.0054	1.0	2.27	−0.0004	0.059
2	1.0	1.86	+0.0027	1.0	2.27	−0.0018	0.052
	2.0	2.00	−0.0005				
3	2.5	1.99	+0.0046	1.0	2.26	+0.0002	0.011
				0.5	2.60	0.0000	
4	1.0	1.90	+0.0006	1.0	2.27	−0.0007	0.007
	1.5	2.03	−0.0011	0.5	2.60	+0.0006	
Reduced sample							
5	3.0	2.00	+0.0045	1.0	2.30	−0.0006	0.049
6	1.0	1.88	−0.0034	1.0	2.32	−0.0039	0.039
	2.0	2.02	−0.0040				
7	2.5	2.00	+0.0037	1.0	2.29	−0.0008	0.020
				0.5	2.70	−0.0022	
8	1.0	1.90	−0.0028	1.0	2.31	−0.0029	0.013
	1.5	2.03	−0.0043	0.5	2.69	−0.0005	

N_s, number of scatterers per copper ion; R_{as}, copper–scatterer distance; $\Delta\sigma^2_{as}$, relative mean square deviation in R_{as} [$\Delta\sigma^2_{as} = \sigma^2_{as}$(sample) − σ^2_{as}(model)]. The model compounds were Cu(II), $[Cu(imid)_4]^{2+}$ at 4 K; Cu(II)-S, $[Cu(mnt)_2]^{2-}$ at 4 K; Cu(I)-N, [Cu(BBDHp)]$^+$ at 4 K; Cu(I)-S, $[Cu(etu)_3]^+$ at 4 K. See refs. 22 and 27 for details.

*The values given were not varied during optimization.

†f' is a goodness-of-fit statistic normalized to the overall magnitude of the $k^3\chi(k)$ data (27):

$$f' = \frac{\{\sum[k^3(\chi_{obsd}(i) - \chi_{calc}(i)]^2/N\}^{1/2}}{(k^3\chi)_{max} - (k^3\chi)_{min}}.$$

2 is likely composed entirely of *C* terms with only a very minor (if not negligible) contribution from *B* terms. The similarity of the two spectra suggest that *C* terms also dominate the MCD spectrum of N_2O reductase. Scaling the N_2O reductase spectrum to 4.2 K gives a limiting $\Delta\varepsilon_M \approx 850$ $M^{-1}\cdot cm^{-1}$. Sequence comparisons (see below) suggest that one Cu_A-type site is present in each subunit of the N_2O reductase; since the native enzyme is a dimer, $\Delta\varepsilon \approx 400$–$500$ $M^{-1}\cdot cm^{-1}$ per Cu_A-type site. This is comparable to that inferred for Cu_A (≈ 2000 $M^{-1}\cdot cm^{-1}$). It should be noted that the N_2O reductase MCD spectra are at least an order of magnitude more intense than the MCD spectra of type 1, type 2, and type 3 copper sites (25, 31). If any of these sites were present in N_2O reductase, they would make practically no contribution to the MCD spectrum, which would therefore be dominated by the Cu_A-type sites. It follows that the 540-nm chromophore in N_2O reductase and Cu_A in cytochrome *c* oxidase are likely to have important structural features in common. Independent spectroscopic data indicate that a [Cu(II)-S_2(Cys)$_2$N(His)] model is responsible for both sites (13, 20–22, 27, 34, 35).

In particular, XAS data are consistent with such an interpretation. The Cu EXAFS curve-fitting results for N_2O reductase are strikingly similar to those for cytochrome *c* oxidase. Cu_A^{2+} of cytochrome *c* oxidase has been shown to have two (N, O)-containing ligands with an average Cu-(N, O) distance of 1.97 Å and 2 (S, Cl)-containing ligands with an average Cu-(S, Cl) distance of 2.28 Å (22). A Cu-(S, Cl) interaction at a very similar distance (2.27 Å) is absolutely required to simulate the Cu EXAFS data of oxidized N_2OR I. As with cytochrome *c* oxidase, reduction of the enzyme results in a lengthening of the Cu-(S, Cl) distance [to 2.31 Å for cytochrome *c* oxidase (36) and to 2.30 Å for N_2O reductase]. The "long" (≈2.6–2.7 Å) Cu-(S, Cl) interaction that is required to fit both oxidized and reduced N_2O reductase Cu EXAFS data (Table 2) has also been identified recently in both oxidized and reduced cytochrome *c* oxidase Cu EXAFS data (ref. 28; R.A.S., P. M. Li, S. I. Chan, unpublished observations). Further work is required to determine whether this feature is also associated with Cu_A-type sites. The amino acid sequence shows that 23 histidines and 9 cysteines are present per subunit, so multiple coordination by sulfur or imidazole or both is possible. Regardless of which fits best represent the average composition of the copper first-coordination shell, it should be emphasized that approximately eight sulfur ions (per eight copper ions) at 2.27 Å (oxidized) or 2.30 Å (reduced) are required to fit the data.

Additional evidence for the presence of a Cu_A site in each subunit of N_2O reductase comes from sequence comparisons. The Cu_A site of cytochrome *c* oxidase is proposed to be in subunit II (CoxII) (37, 38). A comparison of the N_2O reductase sequence [determined by translation of the *NosZ* gene (7), the native enzyme is a dimer of this gene product] with several CoxII sequences is shown in Fig. 5. The sequence Gly-Xaa-Xaa-Xaa-Xaa-Xaa-Cys-Ser-Xaa-Xaa-Cys-Xaa-Xaa-Xaa-His, containing three of the probable ligands in a Cu_A-type site, is highly conserved. Cysteines 196 and 200 have recently been implicated as Cu_A ligands in beef heart cytochrome *c* oxidase (39). The coordination numbers from the EXAFS analysis are certainly consistent with the implication from the sequences that N_2O reductase contains two Cu_A-type sites. No role for the conserved serine is yet known; one intriguing possibility is that it is also a copper ligand. The conserved Cys-618/Ser-619 pair resides in a conspicuous stretch of five aromatic residues. Positioned towards the amino terminus is a tyrosine pair that may be analogous to the conserved tyrosine/phenylalanine pair of cytochrome oxidase. Based on immunochemical crossreactivity, *Pseudomonas aeruginosa* has an N_2O reductase with properties similar to that of *P. stutzeri* (40). In addition, the finding that the structural gene *NosZ* of *P. stutzeri* hybridizes those of *P. aeruginosa, Alcaligenes eutrophus*, and *Paracoccus denitrificans* indicates the existence of a homologous enzyme in these organisms.

Hence we conclude that the [Cu(II)-S_2(Cys)$_2$N(His)] sites in resting N_2O reductase, previously identified by resonance Raman spectroscopy (13), and the Cu_A site in cytochrome *c* oxidase have closely similar structures. Recent EPR experiments (14, 15, 41) are consistent with this conclusion. It is intriguing that two terminal "oxidases," which couple small-

598	PQQTSSITFVADKPGLHWYYCSWFCHALHMEMVGRMMVEPA	NosZ	
176	PGRLNQTTFTATRPGVYYGQCSEICGANHSFMPIVLELIPL	CoxII	Human
176	PGRLNQTTLMSSRPGLYYGQCSEICGSNHSFMPIVLELVPL	CoxII	Bovine
224	PGRIAQLWFSVDQEGVYFGQCSELCGINHAYMPIVVKAVSQ	CoxII	*Paracoccus*
201	PGRLNQVSALIQREGVFYGACSELCGTGHANMPIKIEAVSL	CoxII	Yeast
204	PGRSNLTSISVQREGVYYGQCSEICGTNHAFTPIVVEAVTL	CoxII	Consensus

FIG. 5. Comparison of the amino acid sequence coded by the *NosZ* gene and the subunit II (Cox II) carboxyl-terminal regions of several cytochrome *c* oxidases. Identical residues in all sequences are indicated by boldface letters. Locations where the NosZ sequence matches at least one cytochrome *c* oxidase sequence are indicated by outlined letters. Aromatic residues vicinal to the central cysteines are underlined.

molecule reduction to ATP synthesis, both apparently contain a Cu_A-type site. Cu_A has been proposed to be a proton-pumping site in cytochrome *c* oxidase (42, 43) although there is some disagreement about this (44). The occurrence of a Cu_A-type site in these enzymes, and to date in no other type of enzyme to the best of our knowledge, suggests that Cu_A may have evolved to fulfill a particular role in energy-conserving electron-transport chains, whatever that role may turn out to be.

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