the ether layer separated and dried. To this ether solution was added 2 μL of cyclopentanone as an internal standard, and quantitative analysis by GC gave the concentrations of CyOH and CyONE with time.

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Contribution from the Department of Chemistry, Amherst College, Amherst, Massachusetts 01002, Departments of Chemistry and Biochemistry and Center for Metalloenzyme Studies, University of Georgia, Athens, Georgia 30602, Lehrstuhl für Mikrobiologie, Universität Karlsruhe, D-7500 Karlsruhe 1, Federal Republic of Germany, and Department of Chemistry, University of Southern California, Los Angeles, California 90089

Spectroscopic Studies of the Copper Sites in Wild-Type *Pseudomonas stutzeri* N_2O Reductase and in an Inactive Protein Isolated from a Mutant Deficient in Copper-Site Biosynthesis

David M. Dooley,*[1] Michele A. McGuirl,[1] Amy C. Rosenzweig,[1] Judith A. Landin,[1] Robert A. Scott,[2] Walter G. Zumft,[3] Frank Devlin,[4] and Philip J. Stephens[4]

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Pseudomonas stutzeri N_2O reductase is a complex multicopper enzyme (approximately 8 Cu ions/protein molecule). Two copper sites appear to be closely similar to the Cu_A-type site in cytochrome *c* oxidase, but relatively little is known about the other copper sites in N_2O reductase. In this paper circular dichroism, magnetic circular dichroism, and X-ray absorption and fluorescence spectroscopy have been used to further characterize the copper sites in native N_2O reductase and in a "mutant" protein isolated from a strain deficient in the biosynthesis of the N_2O reductase copper sites that contains only 2 copper ions/protein molecule. Both magnetic circular dichroism and X-ray absorption (Cu K-edge and EXAFS) data are consistent with the presence of (on average) one Cu_A-type site per protein in the mutant N_2O reductase. Comparisons of the near-infrared circular dichroism spectra of the oxidized native and "mutant" N_2O reductases suggest that transitions at 7200 and 9500 cm^{-1} in the native enzyme are associated with copper sites other than the Cu_A-type sites. To the best of our knowledge, these are the first electronic spectral features that can be attributed to non-Cu_A-type sites in N_2O reductase. The near-infrared bands are significantly less intense in preparations of N_2O reductase that display lower specific activities. Several electronic transitions are resolved in the circular and magnetic circular dichroism spectra of dithionite-reduced N_2O reductase. Notably a near-infrared band is observed in the circular dichroism spectrum at 8200 cm^{-1}. The data are plausibly attributed to a highly covalent [Cu(II)-S^-(cys) ↔ Cu(I)-$S^•$(cys)] site in the *reduced* enzyme. Copper removal from N_2O reductase enhances the tryptophan fluorescence intensity about 3-fold; most of the quenching appears to be associated with occupation of the Cu_A-type sites.

Denitrifying organisms can couple nitrate, nitrite, and nitrous oxide reduction to ATP synthesis,[5] via proton translocation and the formation of a membrane potential.[6] N_2O reductase is generally the terminal enzyme in a complete denitrification pathway; this enzyme catalyzes the two-electron reduction of nitrous oxide (eq 1). The physiological electron donors for various N_2O reductases have not yet been definitively identified, but *c*-type cytochromes are considered likely candidates.[7]

$$N_2O + 2e^- + 2H^+ \rightarrow N_2 + H_2O \qquad (1)$$

All N_2O reductases isolated to date are complex multicopper enzymes. The enzymes from *Pseudomonas stutzeri*[8-12] and *Paracoccus denitrificans*[13] are the best characterized. Multiple forms of the *P. stutzeri* N_2O reductase have been isolated or prepared; key features of these various forms have been summarized in the literature.[8,9] The native enzyme may be isolated in high-activity or low-activity forms (N_2OR I and N_2OR II, respectively) and contains a maximum of eight copper ions per protein molecule. The *P. stutzeri* protein is a dimer of two identical subunits, and the amino acid sequence has been obtained by translation of the *nosZ* gene in this organism.[14] Comparison of the N_2O reductase sequence to published cytochrome oxidase sequences,[11] together with spectroscopic data,[11,12] strongly indicates that N_2O reductase contains Cu_A-type sites. These sites are prominently reflected in the visible absorption spectrum of the resting enzyme (Figure 1). Resonance Raman spectroscopy has shown that the principal electronic absorption band at 540 nm is a RS^-(cys) → Cu(II) ligand-to-metal charge-transfer (LMCT) transition.[10] A [Cu^{II}(cys-*S*)$_2$(his-*N*)$_2$] site is most consistent with the resonance Raman data;[10] a similar coordination environment has been suggested for Cu_A in cytochrome oxidase.[15] Cu_A is believed to be located in subunit II of cytochrome oxidase (coxII);[16]

* To whom correspondence should be addressed.

(1) Amherst College.
(2) University of Georgia.
(3) Universität Karlsruhe.
(4) University of Southern California.

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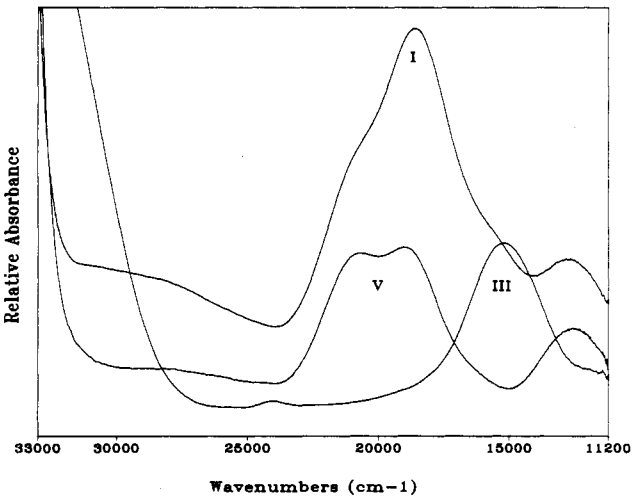

Figure 1. Electronic absorption spectra of N_2O reductase, normalized to protein concentration: I, high-activity, oxidized (resting) form, designated N_2OR I; III, N_2OR I following anaerobic reduction by dithionite, designated N_2OR III; V, modified N_2O reductase isolated from mutant strain MK402, designated N_2OR V.

the sequence Gly-X_5-Cys-Ser-X_2-Cys-X_3-His is highly conserved in coxII sequences and is also present in the N_2O reductase sequence.[11] Recently, a conserved methionine residue in this sequence has been identified.[17] Thus, it seems likely that N_2O reductase contains two Cu_A-type sites (one per subunit, on the basis of the sequence).

In contrast, relatively little is known about the other copper sites in this enzyme. Only between 20 and 50% of the copper in resting N_2O reductase is EPR detectable,[8,9,12] suggesting that Cu(I) sites or antiferromagnetically coupled Cu(II) sites, or both, are present in the oxidized (resting) enzyme. Multiple-frequency EPR spectra are consistent with mixed-valent [Cu(II)–Cu(I)] sites in oxidized N_2O reductase.[18,19] Interestingly, the dithionite-reduced form of N_2OR I (designated N_2OR III) displays an unusually (for copper) broad and unstructured EPR signal, which has about 50% of the total integrated intensity of the initial N_2OR I form. N_2OR III also displays an intense (ϵ = 6100 M^{-1} cm^{-1}) absorption band at 650 nm (Figure 1); the resonance Raman spectrum of this chromophore is similar to blue (type 1) copper spectra.[10] A 650-nm absorption band, associated with a resonance Raman spectrum that is essentially identical with that of N_2OR III, is also observed in low-activity (N_2OR II) forms of the resting enzyme. The relationship between the 650-nm chromophores in N_2OR II and III and the copper sites in the native, high-activity resting form, N_2OR I, is not known.

Several mutant *P. stutzeri* strains have been isolated that are deficient in N_2O reductase biosynthesis.[20,21] Three genes, presumably within a single transcriptional unit, have been shown to be required for proper copper incorporation into apo N_2O reductase. None of these three genes is operative in a transposon Tn5-induced mutant (strain MK402), where it appears that the promoter region has been altered by insertional mutagenesis.[21] An inactive N_2O reductase, designated N_2OR V, with an average copper content of only 2 Cu ions/protein molecule, has been purified from this strain.[9,20] Approximately 50% of the copper in N_2OR V is EPR-nondetectable.[9] The absorption spectrum of N_2OR V is shown in Figure 1 and suggests that the Cu_A-type sites might be partially occupied in the "mutant" enzyme.

In this paper we describe the results of spectroscopic studies on various forms of the *P. stutzeri* N_2O reductase. New electronic transitions have been observed in the near-infrared region that are probably not associated with the Cu_A-type sites. X-ray absorption (edge and EXAFS) and CD/MCD spectroscopy have been used to probe the nature of the copper sites in the mutant N_2O reductase.

Experimental Procedures

Materials and Enzymes. Wild-type N_2O reductase from *P. stutzeri* ATCC 14405 was purified under anaerobic conditions by procedures previously described[8] and stored in liquid nitrogen until used. Preparations used for the experiments described herein generally had a copper content of 7 ± 1 ions/protein molecule. The purity was checked periodically by SDS and gradient gel electrophoresis and by isoelectric focusing on a Pharmacia PhastSystem. Mutant strain MK402 served as the source of the derivative enzyme, N_2OR V, which was purified by the same procedure as that for the wild-type enzyme except aerobic conditions were maintained. Owing to its lack of enzymatic activity, N_2OR V was monitored by immunochemical methods throughout the purification. N_2OR V was stored in liquid nitrogen prior to use. All chemicals were reagent grade and were used without further purification. Ar gas was purified by passage through an oxygen trap (Chemical Research Supplies). Buffers were prepared from distilled, deionized water or from D_2O (99.8 atom % D, Aldrich).

Sample Preparation. N_2O reductase samples for spectroscopic experiments in the visible region were in 50 mM Tris buffer, pH 7.5. Generally, beads of the enzyme were removed from liquid-nitrogen storage, thawed, and diluted with ice-cold buffer to a concentration of 0.06–0.12 mM immediately before use. Samples were then allowed to warm to ambient temperature prior to recording their spectra. Samples for near-infrared spectroscopy were in 50 mM Tris buffer, pD 7.9–8.0, prepared by dissolving Tris base in D_2O and adding DCl (Aldrich) until the measured pH, pH*, = 7.5–7.6. Thawed beads of N_2O reductase were diluted with deuterated buffer and further equilibrated by at least three concentration/dilution cycles in an ultrafiltration cell (Amicon, YM 30 membrane). Final protein concentrations were as follows: N_2OR I, 0.30 mM; N_2OR II, 0.51 mM; N_2OR III, 0.32 mM; N_2OR V, 1.08 mM. N_2OR III was prepared under Ar by adding a 1/30 v/v dithionite solution to give a final dithionite concentration of approximately 9.0 mM. Care was taken to ensure that the copper-depleted N_2OR V was not exposed to adventitious copper. All buffers were purified over Chelex columns, and glassware (or plasticware) was thoroughly acid-washed. In our experience phosphate buffers are generally preferable to Tris buffers for low-temperature X-ray absorption spectroscopy. Hence, the N_2OR V sample was dialyzed against 0.1 M potassium phosphate buffer, pH 7.2, and concentrated to approximately 2 mM protein. Samples for fluorescence spectroscopy were 0.025–0.035 mM ($A_{295} \approx 0.1$) in either 25 or 50 mM Tris, pH 7.5. Copper-depleted N_2O reductase was prepared as follows. A sample of N_2OR I was reduced anaerobically with 15 equiv (over protein) of dithionite and then dialyzed under Ar against three changes of 25 mM Tris buffer (pH 7.5) containing 10 mM KCN. Cyanide was removed by dialysis (again under Ar) against three changes of Tris buffer. All procedures were carried out at 4 °C.

Spectroscopy. Visible CD and MCD spectra were recorded with JASCO J-500 or modified J-40 instruments, as described earlier.[11] A 2-nm spectral bandwidth was maintained. An OLIS Cary-14 and a Cary 219 spectrophotometer interfaced to an IBM PC/AT were used to obtain visible and near-infrared absorption spectra. Near-infrared CD spectra were obtained with a laboratory-constructed instrument that has been described in the literature.[22] Experimental details for the X-ray absorption (edge and EXAFS) measurements were as described in ref 11, except that the copper concentration was 4 mM. The spectral resolution was 0.5 and 1.0 eV for the edge and EXAFS regions, respectively. A Spex Fluorolog photon-counting spectrometer was used for fluorescence spectroscopy. Standard conditions were 1.8-nm band-pass on the excitation and emission monochromators and 0.5-nm steps with 0.5-s integration times. Fluorescence counts were divided by a reference signal generated via a beam splitter and reference photomultiplier to compensate for fluctuations in the lamp output.

Results

CD spectra of N_2OR I, N_2OR III, and N_2OR V in the visible region are shown in Figure 2. The CD spectrum of N_2OR II (not shown) is closely similar to that of N_2OR I. The inset in Figure 2 shows the CD spectrum of another, more concentrated,

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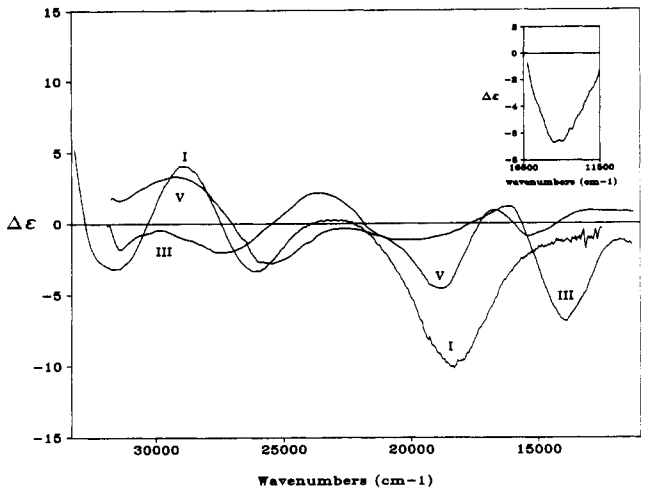

Figure 2. Circular dichroism spectra of N_2O reductase: I, spectrum of the resting, high-activity form, N_2OR I; III, spectrum of the dithionite-reduced form of N_2OR I, designated N_2OR III; V, spectrum of the inactive protein isolated from mutant strain MK402, designated N_2OR V. Inset: CD spectrum of N_2OR I in the red region, showing the transition observed by using a more concentrated sample.

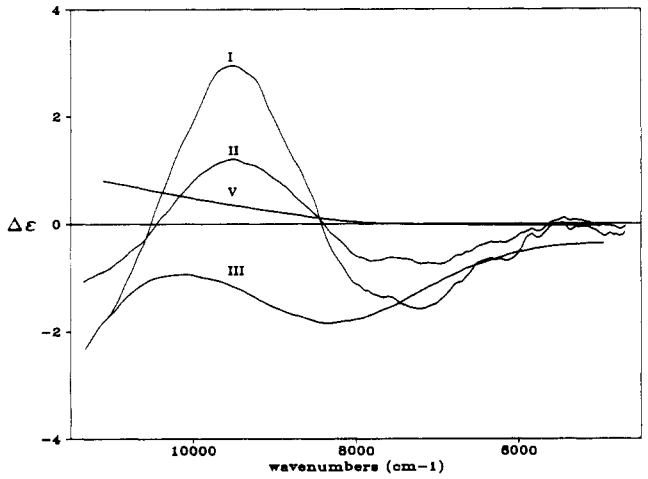

Figure 3. Near-infrared circular dichroism spectra of N_2O reductase: I, N_2OR I; II, low-activity resting form, designated N_2OR II; III, N_2OR III; V, N_2OR V.

sample of N_2OR I in the 600–900-nm region (16 667–11 110 cm^{-1}). There are several notable features of these spectra. First, the intensities of the N_2O reductase CD transitions (Figure 2) are quite comparable to those associated with blue (type 1) copper sites,[23] although the dispersions are distinct. Further, these spectra must reflect a number of low-energy electronic transitions in the near-UV and visible regions, consistent with the presence of $[Cu^{II}(cys\text{-}S)_2(his\text{-}N)_2]$ sites or other types of $[Cu^{II}(cys\text{-}S)]$ chromophores in N_2O reductase.[23,24] Finally, N_2OR I and N_2OR V have transitions at similar energies throughout the visible region, although the rotational strengths or signs of the bands differ in some cases. Additional transitions are observed in the near-infrared region (Figure 3). The negatively signed intensity above 10 000 cm^{-1} in Figure 3 is associated with the lowest energy features evident in Figure 2 (and the inset there). Bands are observed in the near-infrared region at the following energies: N_2OR I and II, 9500 and 7200 cm^{-1} (~1050 and 1390 nm);

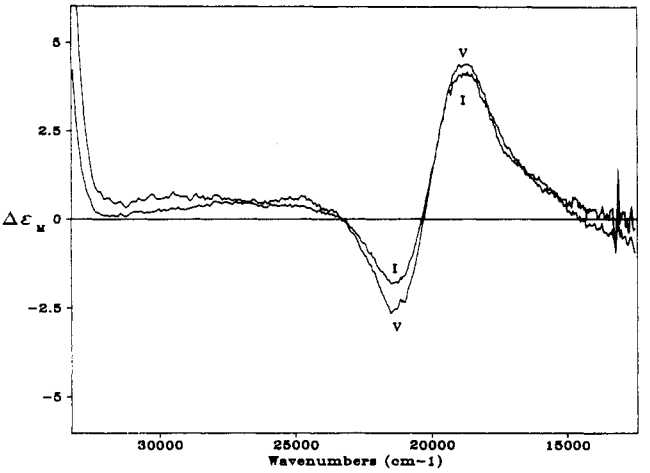

Figure 4. Comparison of the room-temperature, visible-region MCD spectra of native N_2O reductase (N_2OR I) and the mutant enzyme (N_2OR V): I, N_2OR I; V, N_2OR V. Each spectrum has been normalized to the paramagnetic copper content of the sample.

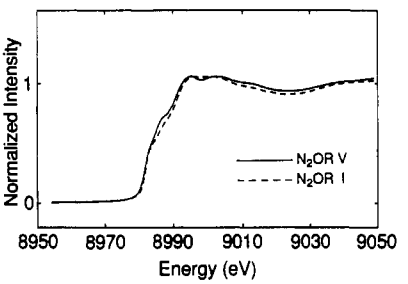

Figure 5. Copper K-edge X-ray absorption spectra of the native (N_2OR I) and mutant (N_2OR V) N_2O reductases, normalized to unit edge jump.

N_2OR III, 8200 cm^{-1} (~1220 nm). The intensities of the N_2OR II bands are approximately half those of N_2OR I. Unexpectedly, no near-infrared transitions were detected for N_2OR V. Numerous studies have established that Cu(II) complexes with near-tetrahedral geometries display ligand field transitions in the near-infrared region.[23,24] For example, type 1 Cu(II) sites typically display near-infrared transitions at 5000–6000 cm^{-1} (2000–1667 nm), 8000–10 000 cm^{-1} (1250–1000 nm), and 11 000–14 000 cm^{-1} (~900–700 nm). Therefore, the bands observed in the near-infrared CD spectra of N_2O reductase samples may be plausibly assigned as Cu(II) d–d transitions, at least for N_2OR I and N_2OR II.

Figure 4 compares the visible MCD spectra of N_2OR I and N_2OR V. N_2OR I contains two Cu_A-type sites, although these sites may not be fully occupied. Recent low-temperature MCD[25] and magnetic susceptibility[26] measurements indicate that the Cu_A centers, which dominate the MCD spectrum,[11,25] can account for all the observed paramagnetism of native N_2O reductase at pH 7.5. Therefore, the MCD spectra in Figure 4 have been normalized to the paramagnetic copper content of the samples. Clearly, the normalized MCD spectra of N_2OR I and N_2OR V are closely similar.

The copper sites in the "mutant" N_2O reductase have also been characterized by X-ray absorption spectroscopy (XAS). Copper K-edge spectra of N_2OR V and N_2OR I are shown in Figure 5. These spectra are clearly very similar, but minor differences are apparent, especially a slight shift to lower energy in N_2OR V compared to N_2OR I. Copper EXAFS data for N_2OR I and N_2OR V as well as the associated Fourier transforms are displayed in Figure 6. Much greater differences between N_2OR V and N_2OR I are apparent, particularly in the k = 7–11 Å range, where N_2OR V has considerably more amplitude. This is reflected in the FT, where it is clear that the R' = 1.9 Å peak has increased

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Table I. Curve-Fitting Results for the First Coordination Sphere of N_2O Reductase Derivatives[a]

		Cu(N,O)			Cu(S,Cl)			
sample	fit	N_s	R_{as}, Å	$\Delta\sigma_{as}^2$, Å^2	N_s	R_{as}, Å	$\Delta\sigma_{as}^2$, Å^2	f'[b]
N_2OR I	1	(3.0)[c]	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	
N_2OR V	5	1.99	2.01	(+0.0046)	1.47	2.27	(+0.0002)	0.009
					0.83	2.59	(0.0000)	
	6	0.35	1.90	(+0.0006)	1.47	2.27	(−0.0007)	0.006
		1.15	2.02	(−0.0011)	0.83	2.59	(+0.0006)	

[a] N_s is the number of scatterers per copper; R_{as} is the copper–scatterer distance; $\Delta\sigma_{as}^2$ is a relative mean-square deviation in R_{as}, with $\Delta\sigma_{as}^2 = \sigma_{as}^2$(sample) − σ_{as}^2(model). The model compounds are as follows: Cu(II)–N, $[Cu(imid)_4]^{2+}$ at 4 K; Cu(II)–S, $[Cu(mnt)_2]^{2-}$ at 4 K. [b] f' is a goodness-of-fit statistic normalized to the overall magnitude of the $k^3\chi(k)$ data:

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

[c] Numbers in parentheses were not varied during optimization.

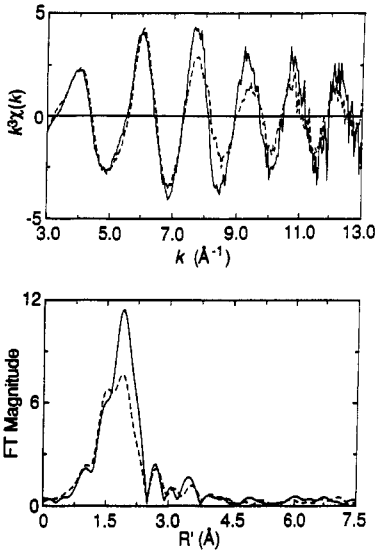

Figure 6. Copper EXAFS data and Fourier transforms for oxidized high-activity N_2O reductase (N_2OR I) and the oxidized mutant protein (N_2OR V): (A, top) EXAFS data for N_2OR V (—) and N_2OR I (---) (B, bottom) Fourier transforms over the k = 3.0–13.0 Å^{-1} range (k^3 weighting) of the data in A for N_2OR V (—) and N_2OR I (---).

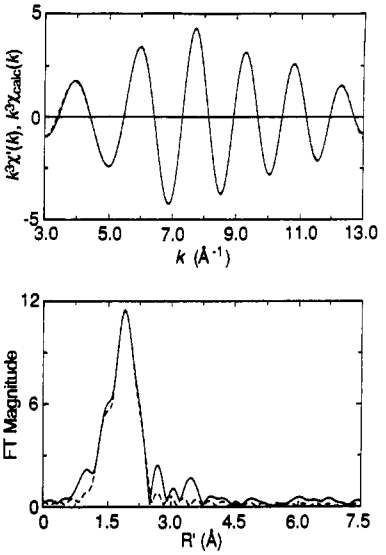

Figure 7. Curve-fitting simulation for the N_2OR V EXAFS. The first shell peak (R' = 1.10–2.50 Å) of the Fourier transform data was back-transformed to yield the filtered EXAFS data shown as the solid line. The dashed line represents the best curve-fitting simulation (fit 6, Table I).

in amplitude. EXAFS curve-fitting analyses for both N_2OR I and N_2OR V are set out in Table I, and the best fit is compared to the filtered data in Figure 7. N_2OR V curve-fitting was performed by assuming that the same types of scatterer shells are present as in N_2OR I; Debye–Waller factors for each type of scattering shell were fixed at the N_2OR I values for that shell. The number, N_s, of scattering atoms in each shell was varied in the optimizations for N_2OR V. As previously established for N_2OR I,[11] Cu–(S,Cl) interactions at 2.27 and 2.59 Å are required to fit the N_2OR V EXAFS. Note, however, that the number of (S,Cl) scatterers is increased, and the number of (N,O) scatterers decreased, in N_2OR V relative to N_2OR I. Another interesting feature of these fits is that the 2.59-Å Cu–(S,Cl) interactions appear to scale with the 2.27-Å Cu–(S,Cl); i.e., there are consistently about half as many of the longer scatterers. At this time we have not attempted to fit any of the features attributable to second- and third-shell scattering with a Cu–Cu interaction, primarily because we wish to obtain data on an independent sample to ensure that all such features are fully reproducible. Hence, the current EXAFS analysis does not preclude the possibility that significant Cu–Cu interactions may be present.

Fluorescence spectroscopy has been used to check possible interactions between aromatic residues and the copper centers in

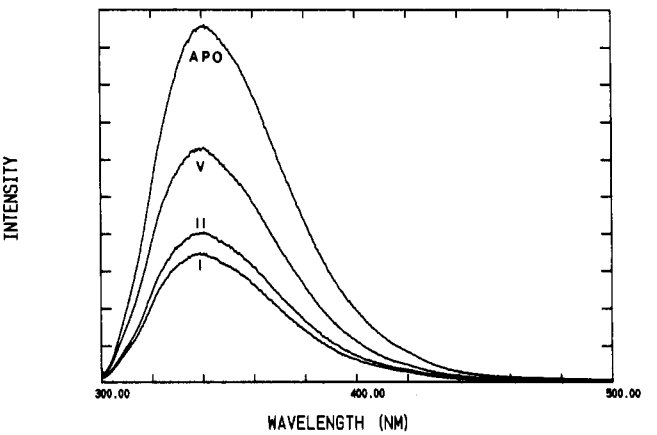

Figure 8. Fluorescence spectra of various forms of N_2O reductase, obtained with 295-nm excitation: I, N_2OR I; II, N_2OR II; V, N_2OR V; Apo, copper-depleted N_2OR I.

N_2O reductase. Fluorescence spectra, obtained with 295-nm excitation, of various forms of N_2O reductase are displayed in Figure 8. Because the excitation wavelength was 295 nm, tyrosine

residues make practically no contribution to the protein fluorescence spectra shown in Figure 8. Copper binding dramatically quenches the tryptophan fluorescence of apo N_2O reductase. The N_2OR V fluorescence intensity is approximately midway between that of N_2OR I and the apo protein, which roughly correlates with its Cu_A content (vide infra). Much of the quenching might therefore be associated with copper binding at the Cu_A-type site. If so, this implies that the neighboring tryptophan residues are efficient energy acceptors. N_2OR I and N_2OR II have similar tryptophan fluorescence spectra, but the somewhat greater intensity of N_2OR II is consistently observed and maintained when the fluorescence is excited at 280 nm (data not shown).

Discussion

We have previously described evidence from X-ray absorption and MCD spectroscopy that is consistent with the presence of Cu_A-type sites in native N_2O reductase.[11] Comparisons to published cytochrome oxidase subunit II (cox II) sequences revealed significant homology between a 15-residue N_2O reductase sequence and the cox II sequences containing the Cu_A binding site from several organisms.[11] Thorough studies of N_2O reductase by cw-EPR spectroscopy[18,19] and pulsed-EPR spectroscopy[12] have provided independent evidence for Cu_A-type sites in resting N_2O reductase. Some key spectroscopic or physical signatures for Cu_A include (1) the MCD spectrum, which is conspicuously different from the MCD spectra of other types of Cu(II) sites,[23] (2) the copper X-ray absorption edge, (3) the 2.27- and 2.6-Å Cu–(S,Cl) interactions and (4) the electron spin-echo envelope modulation pattern, which also appears unique among Cu(II) proteins.[12] The association of the seven-line EPR signal, which is characteristic of N_2OR I, with the Cu_A site has been more controversial,[27] principally owing to the emerging evidence that the seven-line signal is most consistent with a mixed-valence $S = {}^1/_2$ [Cu(1.5)···Cu(1.5)] center.[19] This view is at odds with widely accepted models for Cu_A.[27]

Collectively the data presented in Figures 4–6 and Table I strongly suggest that the "mutant" enzyme, N_2OR V, also contains an occupied Cu_A-type site. The intensity of the MCD spectrum suggests that, on average, the N_2OR V protein contains one Cu_A-type Cu(II).[28] EXAFS curve-fitting analyses (Table I, Figure 6) are fully consistent with a higher percentage of the total copper in N_2OR V being Cu_A type. Riester et al. have previously pointed out that the EPR parameters, particularly the **g** values, of N_2OR V suggest it has an electronic environment similar to that of Cu_A.[9] N_2OR V also displays the characteristic multiline EPR signals at S-, C-, and X-band observed for N_2OR I.[19] Kroneck et al. have recently suggested that the mixed-valence site may be the main copper site in N_2OR V.[19]

It is important to recognize that the primary structure of N_2OR V is identical with that of the native enzyme; the structural gene (*nosZ*) is transcribed normally in strain MK402, from which N_2OR V has been isolated. During purification N_2OR V behaves very similarly to the wild-type enzyme. Furthermore, N_2OR V is immunochemically and electrophoretically identical with the copper-depleted form of N_2OR I. N_2OR V is also translocated across the cytoplasmic membrane into the periplasmic space, where the wild-type enzyme functions. Hence, it is plausible that the tertiary structures of N_2OR V and N_2OR I are at least similar. Recent evidence indicates that full copper incorporation into *P. stutzeri* N_2O reductase requires three, possibly four, genes in addition to *nosZ*.[21] The proteins coded by these genes are probably involved in copper processing or insertion and appear to be specific for N_2O respiration. To our knowledge, N_2O reductase is the first copper enzyme for which a strong case can be made that metal binding to the apo protein is enabled, assisted, or catalyzed by additional proteins. In strain MK402 three of these genes, those downstream of *nosZ*, are not functional so that only copper incorporation that is independent of these proteins can occur. In this context, it may be significant that purified N_2OR V does not bind added copper.[9] For these reasons we believe that it is important to characterize the copper sites in the purified "mutant" protein, N_2OR V.

The similarity of the room-temperature MCD spectra, the XAS features, and the EPR spectra suggests that the electronic structures of the Cu_A-type site in N_2OR I and the paramagnetic center in N_2OR V are also similar. On the other hand, the absorption spectra of N_2OR I and N_2OR V are not identical (Figure 1).[28] In fact, the N_2OR V spectrum resembles that of wild-type N_2O reductase following copper removal and reconstitution;[9] the reconstituted protein is also catalytically inactive. Moreover, the visible CD spectra of N_2OR V and N_2OR I differ with regard to rotational strength and sign below 20 000 cm^{-1}. Such effects may be attributed to differences in the coupling between the dissymmetric protein matrix and the Cu(II) electronic states, especially since the energies of the various transitions in the spectra of N_2OR I and N_2OR V do not appear to be very different. Alternatively, the differences between the absorption and CD spectra of N_2OR I and N_2OR V may reflect the contributions from copper sites that simply are not occupied in the mutant protein. On balance the evidence indicates that a Cu_A-type site (possibly somewhat perturbed) is present in N_2OR V, but the structural basis for any differences in the Cu_A-type sites in N_2OR I and N_2OR V cannot yet be defined.

The conclusion that N_2OR V probably contains an occupied Cu_A-type site may have important consequences for interpreting the near-infrared CD spectra of both N_2OR V and N_2OR I. Clearly the signal-to-noise ratio in Figure 3 is sufficient to exclude the possibility that the bands at 7200 and 9500 cm^{-1} (~1390 and 1050 nm) were not observed in the N_2OR V spectrum owing to the lower absolute content of Cu_A-type sites in N_2OR V. Since the **g** values of N_2OR I and N_2OR V are close in magnitude, the ligand-field transitions of the paramagnetic sites in these proteins should be observed at approximately the same energies. This idea is supported by the close correspondence in the energies of the LMCT transitions between N_2OR I and N_2OR V; for example, the principal LMCT transition in N_2OR I is at 18 620 cm^{-1} (537 nm) compared to 18 900 cm^{-1} (526 nm) in N_2OR V (Figure 1). It follows that the presence of the near-infrared CD bands in N_2OR I and N_2OR II, and their absence in N_2OR V, suggest that Cu(II) sites *other than the Cu_A-type sites* are associated with the transitions at 7200 and 9500 cm^{-1}.[29] There are few, if any, other electronic spectral features that may be attributed to the non-Cu_A-type sites in N_2O reductase. This result, together with the observation of a near-infrared transition for N_2OR III, suggests that near-infrared CD spectroscopy will be an informative probe of the active-site structure and reactions of N_2O reductase.

The structures of the paramagnetic blue (λ_{max} = 650 nm) chromophores in N_2OR III and N_2OR II are problematic. As noted previously,[8] the EPR spectrum of N_2OR III is very unusual, particularly for a Cu(II) site. This signal is present to a variable extent in N_2OR II as well. However, the resonance Raman spectrum of the 650-nm chromophore is similar to the resonance Raman spectra of blue copper proteins.[10] For this reason it was suggested that N_2OR II and N_2OR III contained a copper site (or sites) that is (are) similar to a classical blue copper site and perhaps very inaccessible to external reductants.[10] Riester et al. showed that the blue chromophore in N_2OR III could not be

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(28) The intensity of the principal electronic absorption band (λ_{max} = 540 nm) of N_2O reductase varies significantly from preparation to preparation and is sensitive to a variety of laboratory factors. Hence, it is a less reliable measure of the Cu_A content. The oscillator strengths of ligand-to-metal charge-transfer transitions depend upon ground-state and excited-state covalency[23,24] and can readily vary by a factor of 2 or more in response to minor structural perturbations. This seems, for example, to be the case in blue copper (type 1) proteins.

(29) It is possible that the rotational strengths of the near-infrared transitions in N_2OR V are much smaller than those in N_2OR I. In order for the transitions to go undetected under our conditions, the difference in rotational strengths in the near-infrared region would have to be significantly greater than the differences evident in the visible region (Figure 2).

reduced by any of several strong reducing agents with varied structures and net charges.[9] They suggested that the spectroscopic and chemical properties of the blue chromophore in N_2OR III might be consistent with its formulation as a [Cu(I)–SR•] site "stabilized" by additional sulfur ligation.[9] These suggestions concerning the nature of the blue N_2O reductase center are not necessarily inconsistent, since [Cu(II)–SR^-] and [Cu(I)–SR•] may simply be resonance forms describing a highly covalent paramagnetic site. Such considerations have been raised in discussions of the Cu_A site in cytochrome oxidase.[15] Certainly the number, intensities, and energies of the transitions observed in the visible and near-infrared CD spectra of N_2OR III are consistent with formulating the blue chromophore as a near-tetrahedral [Cu(II)–SR^-] site, most probably with pronounced delocalization. On the basis of our data, we cannot unequivocally distinguish between the limiting possibilities, since too little is known about the electronic properties of copper(I)–thiyl radicals.

N_2O reductase contains nine tryptophans per subunit; five of these are in the carboxyl-terminal half of the protein, which contains the Cu_A-type site. Two tryptophan residues are located within two or three residues of a conserved cysteine (cys-618), which is part of the presumed Cu_A site. The tryptophan fluorescence quenching (Figure 8) in N_2OR I and N_2OR V, relative to the apo protein, is pronounced and seems to correlate with the Cu_A content rather than the total copper content. This suggests that the Cu_A-type sites may form a "fluorescence-energy sink" when occupied. Such a result would not be unexpected given the paramagnetism and the number of low-energy electronic states associated with these sites. It remains to be seen if the fluorescence is sensitive to the redox state of the Cu_A-type sites or other copper sites in N_2O reductase. The initial results do suggest that fluorescence spectroscopy will be useful in studies of N_2O reductase and that additional studies are warranted.

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Contribution from the Department of Chemistry and Biochemistry, Utah State University, Logan, Utah 84322-0300, and Departments of Chemistry and Nutrition and Food Sciences, University of Arizona, Tucson, Arizona 85721

Stoichiometry of Electron Uptake and the Effect of Anions and pH on the Molybdenum and Heme Reduction Potentials of Sulfite Oxidase

Jack T. Spence,[1a] Cary A. Kipke,[1b,†] John H. Enemark,*[,1b] and Roger A. Sunde[1c,‡]

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Microcoulometric titrations of chicken liver sulfite oxidase under varied conditions at 25 °C have shown that the enzyme requires three electrons for complete subunit reduction. Reduction potentials (vs NHE) for the molybdenum site at pH 9.00 in the absence of coordinating anions (Mo(VI/V) = 0.057 V, Mo(V/IV) = −0.233 V) are considerably more negative than at pH 6.00 in 0.10 M KCl (Mo(VI/V) = 0.131 V, Mo(V/IV) = −0.086 V), consistent with the formation of chloride complexes of the Mo(V) and Mo(IV) states. Phosphate ($H_2PO_4^-$) competes effectively with chloride for the Mo(V) site of the enzyme at lower concentrations (0.020 M phosphate vs 0.10 M KCl, pH 7.00) and complexes the Mo(VI) state, as seen in the significant negative shifts of the reduction potentials (Mo(VI/V) = 0.038 V, Mo(V/IV) = −0.239 V). Dissociation constants for proton and phosphate complexes of the Mo(VI) state and the chloride complex of the Mo(IV) state have been estimated. The heme reduction potentials decrease with increase in pH and are relatively unaffected by anions. The results are consistent with the results of recent EPR and EXAFS studies of the enzyme regarding active-site structure.

Introduction

Sulfite oxidase (sulfite: ferricytochrome *c* oxidoreductase EC 8.1.2.1) is a dimeric enzyme that contains one molybdopterin center and one cytochrome b_5 type heme in each monomeric subunit.[2,3] The enzyme catalyzes the physiologically important oxidation of sulfite to sulfate, with the oxidation of substrate occurring at the molybdenum center.[4-6] The postulated catalytic cycle for the enzyme requires a two-electron change in the oxidation state of the molybdenum site (Mo(VI)/(Mo(IV)) and a one-electron change in the oxidation state of the heme site (Fe(III)/Fe(II)).[4,7] Data for the electron uptake of sulfite oxidase have not been previously reported. Electron uptake results for both nitrate reductase (*Chlorella vulgaris*) and milk xanthine oxidase, obtained by microcoulometry, show that their molybdenum centers are reduced by two electrons.[8,9]

The heme reduction potentials for the chicken liver and beef liver enzymes have been previously measured at room temperature by spectroelectrochemical titration at pH 7.00 in the presence of chloride and phosphate ions.[10,11]

The molybdenum reduction potentials for the beef liver enzyme have been previously determined by potentiometric titration using low-temperature EPR spectroscopy at pH 7.00 and 9.00 in the

* To whom correspondence should be addressed.
† Current address: CDI/3M Health Care, Tustin, CA.
‡ Current address: University of Missouri, Columbia, MO.

(1) (a) Utah State University. (b) Department of Chemistry, University of Arizona. (c) Department of Nutrition and Food Sciences, University of Arizona.
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