Cu_A of Cytochrome *c* Oxidase and the A Site of N_2O Reductase Are Tetrahedrally Distorted Type 1 Cu Cysteinates

Colin R. Andrew,[1a] Jane Han,[1a] Simon de Vries,[1b]
John van der Oost,[1c] Bruce A. Averill,[1d]
Thomas M. Loehr,[1a] and Joann Sanders-Loehr*,[1a]

Department of Chemistry, Biochemistry, and Molecular Biology
Oregon Graduate Institute of Science and Technology
Portland, Oregon 97291-1000
Department of Molecular and Cellular Biology
Vrije Universiteit
1081 HV Amsterdam, The Netherlands
Department of Microbiology and Enzymology
Technical University of Delft
2628 BC Delft, The Netherlands

Received July 25, 1994

Cytochrome *c* oxidase catalyzes the reduction of dioxygen to water in the final step of the respiratory chain in mitochondria and many aerobic bacteria.[2] The enzyme utilizes reducing equivalents from cytochrome *c* and stores them in acceptor sites that include a Cu_A center, a low-spin heme Fe_a, and finally a dinuclear heme Fe_{a3}/Cu_B site where the reduction of O_2 to H_2O takes place. Studies of the Cu_A center have previously been hampered by heme spectral interference, a problem now remedied by the creation of water-soluble Cu_A-containing fragments from *Bacillus subtilis*[3] and *Paracoccus denitrificans*.[4] These Cu_A fragments have optical and EPR spectra which are remarkably similar to those of nitrous oxide reductase (N_2OR), an enzyme that catalyzes the conversion of N_2O to N_2 in denitrifying bacteria.[5] The Cu_A-containing B2 fragment from *B. subtilis* cytochrome *c* oxidase[3] and the A site of *Achromobacter cycloclastes* N_2OR[5,6] exhibit intense (Cys)S → Cu(II) charge transfer bands which have enabled us to obtain resonance Raman (RR) spectra of these two proteins. We find that the RR, EPR, and optical properties are indicative of tetrahedrally distorted type 1 Cu sites.

The purple Cu_A-containing B2 fragment of *B. subtilis* cytochrome *c* oxidase has absorption bands at 360, 480, 530, and 780 nm and an unusual seven-line EPR hyperfine splitting.[3] The latter has led to the conclusion that Cu_A is actually a delocalized mixed-valence [Cu(1.5)−Cu(1.5)] species.[5] A comparison of EXAFS data for the B2 fragment and a macrocyclic [Cu(1.5)−Cu(1.5)] complex further suggests that the copper ions of Cu_A are linked through a Cu−Cu bond of 2.50 Å, thus offering the first example of a metal−metal bond in a biological system.[7] The A site of N_2OR is also purple in color with absorption bands at 481, 534, 630, and 780 nm[6] and a seven-line EPR spectrum typical of a mixed-valence dinuclear Cu center.[5] The similarity of its EXAFS spectrum[8] with that of Cu_A raises the possibility that N_2OR also contains a Cu−Cu bond.[7] Furthermore, the B2 fragment and N_2OR share a homologous amino acid sequence in which two cysteines, two histidines, and a methionine are conserved in a cupredoxin-type fold.[9] These residues have been implicated as Cu ligands since incorporation of this Cys_2His_2Met ligand set into cytochrome *bo* quinol oxidase generates a purple Cu_A-type site.[10]

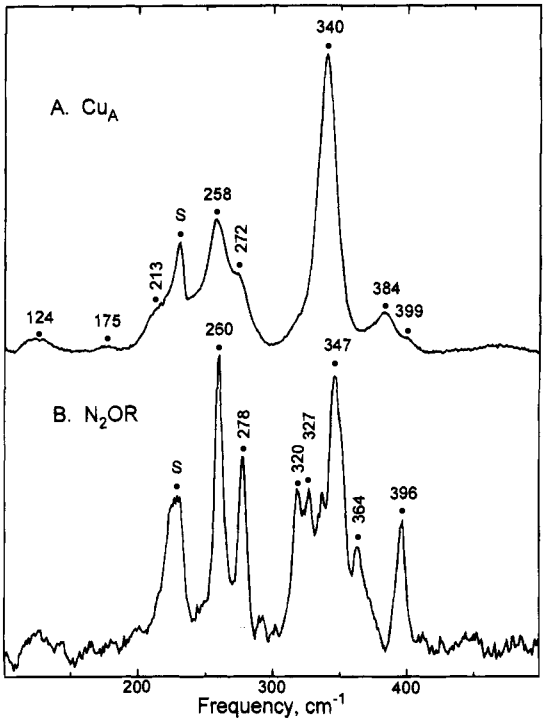

Figure 1. Resonance Raman spectra of Cu_A and N_2OR upon 488-nm excitation at 15 K (obtained as in ref 12). (A) *B. subtilis* B2 fragment (1.5 mM in Cu_A) in 20 mM Tris-Cl (pH 8.0): spectrum with 150 mW and 4-cm^{-1} resolution. (B) *A. cycloclastes* N_2OR (1.0 mM in Cu A) in 50 mM MES buffer (pH 6.4): spectrum with 50 mW and 5-cm^{-1} resolution. S = frozen solvent.

The RR spectra of Cu cysteinate proteins are dominated by a Cu−S(Cys) stretching vibration, ν(Cu−S), that can be identified by its large S- or Cu-isotope shift, its high RR intensity, and its role as the generator of high-frequency combination bands.[11] Excitation of *B. subtilis* Cu_A within its 480-nm absorption band yields a RR spectrum with a strong peak at 340 cm^{-1} and a number of weaker features between 124 and 399 cm^{-1} (Figure 1A). This pattern is typical of Cu cysteinate centers where multiple RR modes arise from coupling of ν(Cu−S) with cysteine ligand deformations.[11,12] Substitution of ^{63}Cu with ^{65}Cu produces shifts of −1.7, ca. −1.0, and −1.0 cm^{-1}, respectively, in the peaks at 340, 272, and 258 cm^{-1}. The band at 340 cm^{-1} is assigned as the predominant Cu−S(Cys) stretch on the basis of its large isotope shift, its high intensity, and its generation of a combination band at 598 (340 + 258) cm^{-1} and an overtone at 679 (340 × 2) cm^{-1}.

* Author to whom correspondence should be sent.

(1) (a) Oregon Graduate Institute. (b) Technical University of Delft. (c) Vrije Universiteit. (d) Present address: E. C. Slater Institute, University of Amsterdam, 1018 TV Amsterdam, The Netherlands.

(2) (a) Babcock, G. T.; Wikström, M. *Nature* **1992**, *356*, 301−308. (b) Malmström, B. G.; Aasa, R. *FEBS Lett.* **1993**, *325*, 49−52.

(3) von Wachenfeldt, C.; de Vries, S.; van der Oost, J. *FEBS Lett.* **1994**, *340*, 109−113.

(4) Lappalainen, P.; Aasa, R.; Malmström, B. G.; Saraste, M. *J. Biol. Chem.* **1993**, *268*, 26416−26421.

(5) Antholine, W. E.; Kastrau, D. H. W.; Steffens, G. C. M.; Buse, G.; Zumft, W. G.; Kroneck, P. M. H. *Eur. J. Biochem.* **1992**, *209*, 875−881.

(6) Hulse, C. L.; Averill, B. A. *Biochem. Biophys. Res. Commun.* **1990**, *166*, 729−735.

(7) Blackburn, N. J.; Barr, M. E.; Woodruff, W. H.; van der Oost, J.; de Vries, S. *Biochemistry* **1994**, *33*, 10401−10407.

(8) (a) 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. (b) George, G. N.; Cramer, S. P.; Frey, T. G.; Prince, R. C. *Biochim. Biophys. Acta* **1993**, *1142*, 240−252.

(9) (a) Buse, G.; Steffens, G. C. *J. Bioenerg. Biomembr.* **1991**, *23*, 269−289. (b) Zumft, W. G.; Dreusch, A.; Lochelt, S.; Cuypers, H.; Friedrich, B.; Schneider, B. *Eur. J. Biochem.* **1992**, *298*, 31−40.

(10) Kelly, M.; Lappalainen, P.; Talbo, G.; Haltia, T.; van der Oost, J.; Saraste, M. *J. Biol. Chem.* **1993**, *268*, 16781−16787.

(11) Andrew, C. R.; 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.*, in press.

(12) Han, J.; Loehr, T. M.; Lu, Y.; Valentine, J. S.; Averill, B. A.; Sanders-Loehr, J. *J. Am. Chem. Soc.* **1993**, *115*, 4256−4263.

0002-7863/94/1516-10805$04.50/0

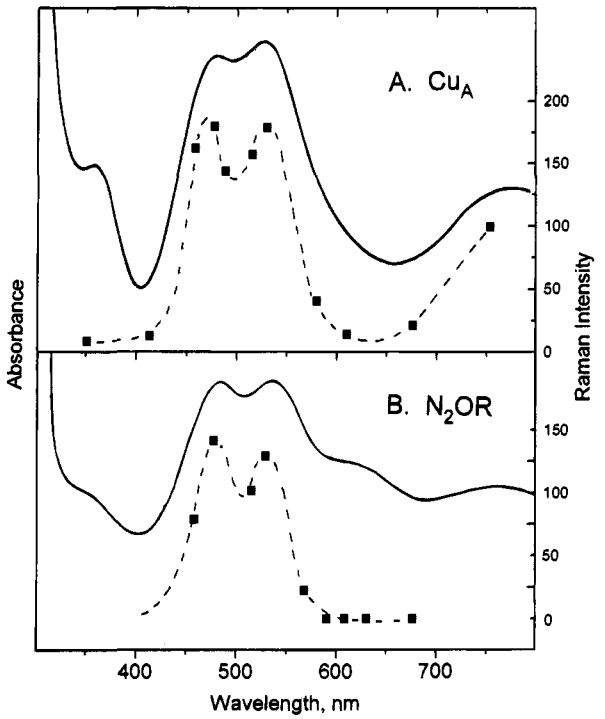

Figure 2. Absorption spectra (—) and Raman excitation profiles (— — —) for ν(Cu−S) modes of (A) Cu_A (340 cm^{-1}) and (B) N_2OR (347 cm^{-1}). At each excitation wavelength, the height of the ν(Cu−S) mode was measured relative to the height of the 230-cm^{-1} ice mode, and the molar intensity per mixed-valence site was normalized to the 980-cm^{-1} mode of sulfate (ref 12).

Application of Badger's rule[13] leads to a calculated Cu−S(Cys) bond length of ~2.22 Å, which is in good agreement with the EXAFS value of 2.18 Å.[7] The Cu-isotope dependence of the 272-cm^{-1} band is consistent with its identification as a Cu−N(His) stretch, its frequency and isotope shift being close to the values of 284 and −2 cm^{-1}, respectively, for ν[Cu−N(His)] in azurin.[12] The Cu_A fragment from *P. denitrificans* cytochrome *c* oxidase shows an almost identical RR spectrum with its most intense feature at 340 cm^{-1},[4,14] whereas mitochondrial cytochrome *c* oxidase exhibits the analogous ν(Cu−S) mode at 330 cm^{-1}.[15]

Excitation of *A. cycloclastes* N_2OR within its 481-nm absorption band produces a set of vibrational modes between 260 and 396 cm^{-1} (Figure 1B) that closely resembles the previously reported RR spectrum of *Pseudomonas perfectomarina* N_2OR.[16] The RR spectrum of N_2OR has its two most intense features at 260 and 347 cm^{-1}, similar to the peaks at 258 and 340 cm^{-1} in the RR spectrum of Cu_A (Figure 1A). By analogy, the 347-cm^{-1} peak in N_2OR is assigned as the predominant ν(Cu−S) mode. The unusually intense peak at ~260 cm^{-1} in both Cu_A and N_2OR may arise from an additional Cu−S stretch of the $Cu_2(Cys)_2$ moiety, similar to the 282-cm^{-1} feature of $Fe_2S_2(Cys)_4$ ferredoxins.[17]

The absorption and RR excitation profiles of Cu_A and N_2OR are remarkably similar to one another (Figure 2). In both cases, the intensity of the ν(Cu−S) mode tracks the 480- and 530-nm absorption bands, denoting significant (Cys)S → Cu CT character in each band. Since excitation within either absorption band produces the same set of RR frequencies, the 480- and 530-nm absorption bands can both be assigned to the same Cu−Cys chromophore. In the case of Cu_A, the 780-nm absorption band also leads to enhancement of the 340-cm^{-1} ν(Cu−S) mode and, thus, represents yet another electronic transition of this Cu−Cys moiety (Figure 2A). This observation explains the ability to obtain a RR spectrum for Cu_A in mitochondrial cytochrome *c* oxidase using 840-nm excitation.[15] Although the 780-nm absorption band may contain a contribution from a Cu(I)−Cu(II) intervalence transition, no evidence for a Cu−Cu vibration in *B. subtilis* Cu_A was obtained in the present study. Furthermore, a (Cys)S → Cu CT transition in the 700−800-nm region is also found in *mononuclear* Cu proteins. For example, the type 1 Cu site in nitrite reductase (NiR) from *A. cycloclastes* has absorption maxima at 458, 585, and 695 nm, all of which lead to RR enhancement of Cu−Cys vibrational modes, and another 385-nm absorption maximum which is RR-inactive.[12] This behavior is closely similar to that of Cu_A and N_2OR.

The X-ray crystal structure of *A. cycloclastes* NiR shows that it has a tetrahedrally distorted type 1 Cu site in which the Cu is pulled ~0.5 Å out of the His_2Cys ligand plane and toward the axial methionine, giving a Cu−S(Met) distance of 2.62 Å.[12,18] Spectroscopic parameters of NiR which are also indicative of a tetrahedrally distorted structure[12] are its rhombic EPR signal ($A_{||}$ = 73.8 G),[6] its elongated Cu−S(Cys) bond with a ν(Cu−S) of 364 cm^{-1}, its Raman intensity of ~200 (relative to a sulfate standard), and its large $\epsilon_{458}/\epsilon_{585}$ value of 1.34. In contrast, type 1 sites with a trigonal planar geometry[11,12] have an axial EPR signal, a shorter Cu−S(Cys) bond with a ν(Cu−S) of ~420 cm^{-1}, a relative Raman intensity of ~500, and an $\epsilon_{460}/\epsilon_{600}$ value of ~0.10. The spectroscopic properties of *B. subtilis* Cu_A and *A. cycloclastes* N_2OR place them in the tetrahedrally distorted type 1 category. These include a narrow EPR hyperfine splitting of 76.4 G per Cu(II),[3] an elongated Cu−S(Cys) bond with a ν(Cu−S) of ~340 cm^{-1} (Figure 1), a relative Raman intensity of ~150 per Cu(II) (Figure 2), and an $\epsilon_{480}/\epsilon_{530}$ value of ~1.0 (Figure 2). The low ν(Cu−S) frequencies of 330−347 cm^{-1} for Cu_A and N_2OR compared to the ~360-cm^{-1} values for tetrahedrally distorted, mononuclear type 1 sites[11] correlate with the lower average valence of the [Cu(1.5)—Cu(1.5)] state.

In conclusion, the striking similarity of the optical and RR spectroscopic properties of the Cu_A site in fragment B2, the A site in N_2OR, and the type 1 site in NiR indicates the presence of tetrahedrally distorted type 1 Cu cysteinates in all three proteins. The detection of only one Cu−S(Cys) stretching vibration in Cu_A and N_2OR implies that the two Cys ligands are spectroscopically equivalent or that only one Cu−Cys is resonance enhanced. The presence of two equivalent Cys ligands seems more likely in view of the near identity of the redox potentials of the two Cu ions, which gives rise to complete electron delocalization. We propose that the type 1 character of Cu_A in cytochrome *c* oxidase and the A site in N_2OR is derived from a trigonal ligand set about each Cu consisting of a His N, a Cys S (at a distance of ~2.22 Å), and a second Cu. A similar model for a (His,Cys)Cu−Cu(His,Cys) has been proposed on the basis of EXAFS measurements.[7]

Acknowledgment. This work was supported by grants from the National Institutes of Health (GM-18865 to T.M.L. and J.S.-L.), the National Science Foundation (DMB-8917427 to B.A.A.), and a fellowship from the Royal Dutch Academy of Science (to J.v.d.O.).

(13) Herschbach, D. R.; Laurie, V. W. *J. Chem. Phys.* **1961**, *35*, 458−463. The change in bond distance, *r*, relative to azurin was calculated from the relationship $(r_1 - d_{ij})/(r_2 - d_{ij}) = (\nu_2/\nu_1)^{2/3}$. For a Cu−S bond, d_{ij} was estimated to be 1.5. For azurin, r_1 and ν_1 were taken as 2.13 Å and 414 cm^{-1}, respectively (ref 11), thus giving r_2 = 2.22 Å for Cu_A.

(14) Andrew, C. R.; Sanders-Loehr, J.; Lappalainen, P.; Saraste, M. Unpublished results.

(15) Takahashi, S.; Ogura, T.; Shinzawa-Itoh, K.; Yoshikawa, S.; Kitagawa, T. *Biochemistry* **1993**, *32*, 3664−3670.

(16) Dooley, D. M.; Moog, R. S.; Zumft, W. G. *J. Am. Chem. Soc.* **1987**, *109*, 6730−6735.

(17) Han, S.; Czernuszewicz, R. S.; Kimura, T.; Adams, M. W. W.; Spiro, T. G. *J. Am. Chem. Soc.* **1989**, *111*, 3505−3515.

(18) Adman, E. T. *Adv. Protein Chem.* **1991**, *42*, 145−197.