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Metal–Metal Bonding in Biology: EXAFS Evidence for a 2.5 Å Copper–Copper Bond in the $\mathrm{Cu_A}$ Center of Cytochrome Oxidase[†]

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ABSTRACT: Evidence for a direct Cu–Cu bond in the $\mathrm{Cu_A}$ center of cytochrome oxidase is reported. Simulation of the X-ray absorption spectrum of a recombinant $\mathrm{Cu_A}$-binding domain of *Bacillus subtilis* cytochrome oxidase, and comparison with a structurally characterized directly-bonding Cu(1.5)...Cu(1.5) inorganic complex, suggests that a Cu–Cu interaction of 2.5 ± 0.1 Å together with a short 2.2 Å Cu–S interaction may be present in the $\mathrm{Cu_A}$ site. In light of these data, previous interpretations of the EXAFS of a number of cytochrome oxidase and nitrous oxide reductase enzymes which modeled the 2.6 Å interaction as a long Cu–S(methionine) bond are possibly incorrect. A structural model based on the new data is presented which suggests that the $\mathrm{Cu_A}$ sites in cytochrome oxidase and N_2O reductase are likely composed of a pair of modified type 1 copper centers with one histidine, one cysteine, and one weakly bound ligand (Met and/or Gln) joined by a Cu–Cu bond.

Since the early discovery of transition metal complexes containing metal–metal bonds, metal–metal bonding has become recognized as a common phenomenon (Cotton, 1983). In biological systems, however, despite numerous examples of multinuclear clusters of metal ions at the active sites of proteins, direct metal–metal bonding has not been suggested. Instead, metal–metal interactions such as exist have been presumed to be weak and/or mediated by bridging ligands. Here we present the first evidence for a direct metal–metal bond in a biological system, the $\mathrm{Cu_A}$ center of cytochrome *c* oxidase.

Cytochrome *c* oxidase is the terminal oxidase of the mitochondrial respiratory chain in mammals and of the energy-transducing electron transfer chains of certain bacteria (Malmstrom, 1990; Babcock & Wikstrom, 1992). The enzyme is responsible for harnessing the energy of dioxygen reduction into the synthesis of ATP, and catalyzes the four-electron reduction of molecular oxygen to water:

$$O_2 + 4H^+ + 4e^- \rightarrow 2H_2O$$

Two one-electron centers [designated cytochrome *a* (Fe_a) and $\mathrm{Cu_A}$] and one two-electron center [an antiferromagnetically coupled cytochrome a_3–$\mathrm{Cu_B}$ (Fe_{a3}–$\mathrm{Cu_B}$) pair] accept electrons from cytochrome *c* and furnish the four electrons required to accomplish this reaction, with the oxygen molecule being bound and reduced at the Fe_{a3}–$\mathrm{Cu_B}$ site. Fe_a and the Fe_{a3}–$\mathrm{Cu_B}$ pair are known to be located in subunit 1 of the 13-subunit complex of the mammalian enzyme, while $\mathrm{Cu_A}$ is located in subunit 2.

Extensive spectroscopic data have provided a satisfactory description of Fe_a and the Fe_{a3}–$\mathrm{Cu_B}$ pair. The former is believed to be a 6-coordinate (low-spin) *a*-type cytochrome with bishistidine axial coordination. The Fe_{a3}–$\mathrm{Cu_B}$ center, although less well characterized, appears to be composed of a 5-coordinate (high-spin) heme center coupled to the $\mathrm{Cu_B}$ center via a ligand bridge, and $\mathrm{Cu_B}$ is likely coordinated by two or three histidines. The potential metal-binding residues in subunit 1 have recently been identified by site-directed mutagenesis, and the reaction dynamics of exogenous ligand binding have been extensively probed by laser photolysis of the Fe_{a3}–CO complex, indicating that dioxygen binds first at the $\mathrm{Cu_B}$ center and is then efficiently directed to the Fe_{a3} center via an elaborate mechanism which may involve shuttling of the bridging ligand between the two metal centers (Shapleigh et al., 1992; Woodruff et al., 1991).

The nature of the $\mathrm{Cu_A}$ center has been more enigmatic (Malmstrom & Aasa, 1993; Chan & Li, 1990). $\mathrm{Cu_A}$ has long been recognized as being unusual, largely because of its unique EPR spectrum, with small and unresolved Cu hyperfine splitting in the $g_\parallel$ region, and an absorption band at 830 nm associated with a characteristic MCD signature. However, the presence of intense overlapping bands from the heme centers always complicated the interpretation of the spectral data. Nevertheless, synthesis of spectroscopic data from EPR, ENDOR, EXAFS, and MCD spectroscopy led to the original formulation of $\mathrm{Cu_A}$ as a mononuclear $\mathrm{(His)_2(Cys)_2Cu(II)}$ center, where the unpaired electron on the Cu(II) was believed to be delocalized onto one or more sulfur ligands in order to explain the unusually small Cu hyperfine splitting (Stevens et al., 1982; Martin et al., 1988; Li et al., 1987; Gurbiel et al., 1993; Greenwood et al., 1983; Thompson et al., 1986).

More recently, it has been recognized that $\mathrm{Cu_A}$ bears a close resemblance to the A center of nitrous oxide reductase, a copper enzyme which catalyzes the conversion of N_2O to N_2 in denitrifying bacteria. In particular, both enzymes share

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the unique intense far-red MCD signature (Farrar et al., 1991; Scott et al., 1989), and have very similar EXAFS, indicating the presence of 2 O/N ligands at 2.0 Å, about 1.5 S/Cl ligands at 2.3 Å, and an unusual shell of between 0.5 and 1.0 S/Cl ligands at 2.6 Å (Scott et al., 1989; Dooley et al., 1991; George et al., 1993). Furthermore, both proteins contain a homologous region of primary sequence extending over at least 50 residues, which contains as conserved and thus potential Cu_A-binding residues 2 cysteines, 2 histidines, and a methionine in a cupredoxin (blue-copper)-like fold (Buse & Stevens, 1991; Zumft et al., 1992). The EPR spectrum of the A center of N_2O reductase exhibits a unique 7-line hyperfine splitting of the g_z region, which has been interpreted as arising from a mixed-valence Cu(1.5)...Cu(1.5) dinuclear cluster (Riester et al., 1989; Kroneck et al., 1988, 1990; Antholine et al., 1992). At X-band, the EPR spectrum of Cu_A in cytochrome oxidase is not obviously similar to this mixed-valence EPR signal, but nearly 3 decades ago, Beinert had made the particularly inciteful suggestion that it might in fact arise from a dinuclear Cu(I)...Cu(II) center (Beinert et al., 1962). In more recent work, Antholine, Kroneck, and co-workers have shown by meticulous studies at three different frequencies that the unusual EPR spectrum of Cu_A is in fact best interpreted in terms of a Cu(1.5)...Cu(1.5) site, but with somewhat smaller hyperfine coupling, and which is only partially resolved even at low frequency (Antholine et al., 1992). This work has challenged the traditional view of Cu_A as a mononuclear center, and nicely explains the fact that high-activity preparations of cytochrome oxidase always appear to contain three Cu atoms per two Fe atoms (Buse & Stevens, 1991; Pan et al., 1991).

Further confirmation of the dinuclear nature of Cu_A has come from studies on bacterial cytochrome *c* oxidases (Van der Oost et al., 1992; Kelly et al., 1993). An engineered Cu_A-type purple center constructed in the CyoA subunit of the *Escherichia coli* cytochrome *bo* quinol oxidase (which lacks the Cu_A consensus sequence in the wild-type enzyme) has been shown to contain dinuclear copper with similar EPR properties to the mammalian Cu_A, but with better resolved Cu hyperfine splittings on the $g_\parallel$ line, and an absorption spectrum [λ_{max} 358(s), 536(s), and a flat peak between 750 and 780 nm] which closely resembles that of N_2O reductase. Mutagenesis experiments on this engineered Cu_A site have confirmed two cysteines, two histidines, and a methionine as ligands to the dinuclear site (Kelly et al., 1993). This line of approach has culminated in the expression of soluble Cu_A-binding domains from *Paracoccus denitrificans* (Lappalainen et al., 1993) and *Bacillus subtilis* (Wachenfeldt et al., 1994), thus allowing the Cu_A center of cytochrome *c* oxidase to be studied without masking from the overlapping heme bands. These preparations have provided unambiguous confirmation of the dinuclearity of the Cu_A center, and its unique associated absorption spectrum.

If Cu_A is indeed a dinuclear center, what is the nature of the Cu-Cu interaction? In this report, we provide evidence that in fact it contains a short 2.5 Å Cu-Cu bond, and thereby is the first biological system where true metal-metal bonding has been identified. This results in a unique one-electron redox center which allows efficient delocalization of the one electron over the two copper nuclei.

MATERIALS AND METHODS

The water-soluble Cu_A domain was prepared and characterized using procedures described in detail elsewhere (Wachenfeldt et al., 1994; Saraste et al., 1990; van der Oost et al., 1992). Samples for EXAFS were concentrated to approximately 1 mM in total copper (20 mM Tris, pH 8.0, 20% glycerol, v/v). EXAFS data were collected on beamline X9 at NSLS, Brookhaven National Laboratory, with an electron beam energy of 2.5 GeV and a maximum stored current of 220 mA. Data were collected with a Si(111) double-crystal monochromator and a grazing incidence mirror to reject harmonics. The protein sample was measured as a frozen glass in 20% glycerol at approximately 20 K in the fluorescence mode using a 13-element Ge detector, whereas the model complex was measured in transmission mode at the same temperature. To avoid detector saturation, the count rate of each detector channel was kept below 35 kHz by adjusting the hutch entrance slits. Under these conditions, no dead-time correction was necessary. Fifteen scans were collected for the protein, and summed individually for each detector. The summed data for each detector were then inspected, and only those channels that gave high-quality backgrounds free from glitches or scatter peaks were included in the final average. Raw data were averaged, background-subtracted, and normalized to the smoothly varying background atomic absorption using standard procedures (Blackburn et al., 1991). The experimental energy threshold ($k = 0$) was chosen as 8985 eV. Data analysis was carried out by least-squares curve-fitting utilizing full curved-wave calculations as formulated by the SRS library program EXCURV (Binsted et al., 1988; Gurman et al., 1984, 1986; Gurman, 1989), using methodology described in detail in previous papers from this laboratory (Strange et al., 1987; Blackburn et al., 1991). The parameters refined in the fit were as follows: E_0, the photoelectron energy threshold; R_i, the distance from Cu to atom *i*; $2\sigma^2_i$, the Debye–Waller term for atom *i*. For the model complex, the coordination numbers were fixed at their crystallographic values. For the protein fits, the coordination numbers were allowed to vary, but were constrained so as to produce Debye–Waller factors within reasonable limits (first shell, $0 < 2\sigma^2 < 0.012$; second shell, $0.005 < 2\sigma^2$). The quality of the fits was determined using a least-squares fitting parameter, *F*, defined as

$$F^2 = (1/N)\sum k^6(\chi_i^{\text{theor}} - \chi_i^{\text{exp}})^2$$

referred to as the fit index.

RESULTS AND DISCUSSION

In the absence of a crystal structure, EXAFS spectroscopy offers a useful approach to determining the nature of the Cu–Cu interaction at the Cu_A site. The intact cytochrome oxidase has been extensively studied by EXAFS, but the presence of Cu_B and its interaction with the Fe_{a3} center have complicated the interpretation of the scattering due to Cu_A (Scott et al., 1986; Scott, 1989). For the spectroscopically similar A center in N_2O reductase, EXAFS studies have been unable to locate any Cu–Cu interaction in the 3.0–4.0 Å region (Scott et al., 1989; Dooley et al., 1991; George et al., 1993; SooHoo et al., 1991) where weak, nonbonded metal–metal interactions are traditionally found in metalloprotein systems. On the other hand, both enzymes exhibit an unusual 2.6 Å Cu–X interaction which has been simulated as Cu–S, and assigned to Cu–S(methionine) (Dooley et al., 1991; George et al., 1993). The availability of the soluble Cu_A domain has enabled us to reexamine the EXAFS of the Cu_A site, without the complication of the Cu_B contribution. Our data show that there is a strong likelihood that the 2.6 Å Cu–S interaction was misassigned, and that it fits better to Cu–Cu at 2.5 Å.

The key to unravelling the nature of the Cu–Cu interaction in Cu_A has come from the synthesis, spectroscopy, and

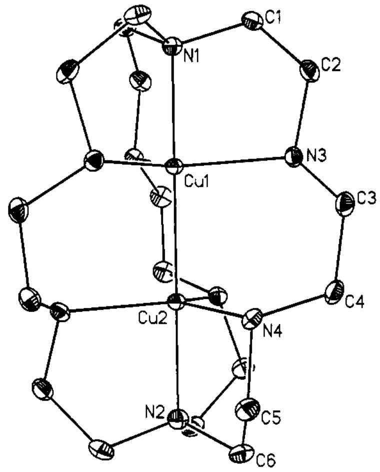

FIGURE 1: ORTEP diagram showing the three-dimensional structure of the cationic portion of the mixed-valence [Cu(1.5)...Cu(1.5)] octaazacryptand acetate complex with a Cu1–Cu2 distance of 2.415 Å. A 3-fold crystallographic axis of symmetry runs through the Cu–Cu bond such that for each of the numbered atoms which lie off the axis, two other entirely equivalent atoms exist. The atoms that contribute to the EXAFS are identified as follows. The first shell is comprised of three equivalent Cu1–N3 bonds (2.085 Å) and one Cu–N1 (2.060 Å) for Cu1, and three equivalent Cu2–N4 (2.045 Å) and one Cu2–N2 (2.068 Å) for Cu2. These distances are too close to resolve by EXAFS and give an average Cu–N first-shell distance of 2.065 Å. In addition to the Cu–Cu interaction, other atoms shown in the figure also contribute to the EXAFS, and can be collected into shells of six C atoms per copper at R_{av} = 2.85 Å (Cu1–C1, Cu1–C2, Cu2–C5, and Cu2–C6 plus the other two sets of each required by symmetry), and nine C/N atoms per copper at R_{av} = 3.24 Å (Cu1–C3, Cu1–C4, Cu1–N4, Cu2–C4, Cu2–C3, and Cu2–N3 plus the two other sets of each required by symmetry).

crystallography of an unusual mixed-valence dicopper complex of a macrobicyclic octaazacryptand ligand (L = $N[CH_2CH_2NHCH_2CH_2NHCH_2CH_2]_3N$) (Barr et al., 1993), as shown in Figure 1. The close Cu–Cu-bonding interactions (2.364 Å in the nitrate salt and 2.415 Å in the acetate salt) and the 7-line EPR spectrum (one electron delocalized over two copper nuclei) result in the formulation of this dicopper center as an electron-delocalized Cu(1.5)...Cu(1.5) moiety. The unusually sharp, intense visible absorbance bands [λ_{max} = 622 (ϵ = 2900 M^{-1} cm^{-1}) and 736 nm (ϵ = 4500 M^{-1} cm^{-1}) in methanol] exhibited by this complex are too low in energy to be ascribed to Cu–N(amine) LMCT transitions, are are most likely associated with transitions within this Cu(1.5)...Cu(1.5) center. The EPR and electronic spectra of this model compound are reminiscent of those observed for the purple Cu_A-type centers in the bacterial oxidase subunit II preparations, and the A cluster of N_2O reductase, although the anisotropy of the EPR spectra is clearly different. Thus, in the model, the unpaired electron resides in the d_{z^2} orbital which is co-linear with the Cu–Cu bond, whereas in Cu_A itself, g_z is located along the parallel direction, suggesting that the unpaired electron is in an orbital more closely aligned with the *xy* plane, with the two coppers of the dinuclear unit interacting largely within this plane (Barr et al., 1993; Antholine et al., 1992; von Wachenfeldt et al., 1994; Beinert et al., 1962). Nevertheless, despite the differences in ligand environment and electronic structure between enzyme and model, the possibility exists that the intense low-energy visible absorbance bands exhibited by the enzymes could also be the signature of a mixed-valence Cu–Cu bond, rather than pure Cu–S charge transfer.

We have carried out X-ray absorption studies on the model complex, and compared the results with XAS data on the *Bacillus* Cu_A domain. Figure 2a and Figure 2c show the EXAFS and Fourier transforms, respectively, of the model complex. The first shell in the transform is seen to be split into two well-resolved peaks. The first peak at ~2.0 Å is derived from the 4-coordinated N atoms while the second peak at ~2.4 Å corresponds to the Cu–Cu bond. We have carried out a detailed simulation of the spectrum using the full curved-wave least-squares fitting program EXCURV88, and have obtained an excellent fit to the data as shown by the dashed lines in Figure 2a,c. Reference to the crystal structure indicates that there are four shells of atoms which could contribute to the EXAFS: the 4-coordinated N atoms at R_{av} = 2.065 Å, Cu–Cu at 2.415 Å, a shell of six C per copper at R_{av} = 2.85 Å,[1] and a more distant shell of nine C and N atoms distributed about a mean of 3.24 Å from each copper.[2] The best-fit distances and their associated Debye–Waller terms are compared with the crystallographic distances in Table 1, where it can be seen that the agreement is excellent. The EXAFS-derived distances for the shell of coordinated N atoms agree within 0.02 Å of the crystallographic value. More significantly, the simulation accurately reproduces the oscillations at higher k ($k > 7$ $Å^{-1}$), which are due predominantly to the short Cu–Cu interaction, and the data nicely show the characteristic maximum of the Cu–Cu amplitude envelope at k = 8–9 $Å^{-1}$. An important result extracted from these data is that the simulated Cu–Cu distance is ~0.05 Å shorter than the crystallographic value, which is probably due to small inaccuracies in the atomic potentials used to calculate the phase shifts. However, it allows us to calibrate our estimates of Cu–Cu distances in the enzyme system, which are therefore also expected to be ~0.05 Å shorter than their true values.

We have also investigated the EXAFS of the recombinant soluble Cu_A domain of *Bacillus subtilis* expressed in *E. coli* (Wachenfeldt et al., 1994). The preparation used in this study exhibited the characteristic UV/vis spectrum of the purple Cu_A center (λ_{max} 365, 480, and 530 nm and a broad absorption between 770 and 800 nm). EPR spectra at both X-band and S-band showed the multiline Cu_A-type spectrum in the g_z region, but also showed signals characteristic of type 2 copper, tightly bound in an adventitious site. The extraneous Cu^{2+} accounted for ~30% of the total copper in the sample. The EXAFS spectrum of the protein is shown in Figure 2b. The Fourier transform (Figure 2d) clearly shows at least two resolved peaks in the first shell at ~2.0 and 2.5 Å, corresponding closely to the peaks observed in the model complex. In view of the evidence for N and S coordination at the Cu_A site (Kelly et al., 1993), the 2.0 Å peak is expected to be due to Cu–N (2.0 Å) and Cu–S (2.2 Å) interactions. The resolved peak at ~2.5 Å is unexpectedly intense for a weak Cu–S(methionine) as suggested by George et al. (1993), and in view of the results from the model, we have fit this to a Cu–Cu interaction. The least-squares fitting converged rapidly to give the simulations of EXAFS and Fourier transform shown as the dashed lines in Figure 2b,d, with the parameters given in Table 2, *viz.*, 2 N at 1.93 Å, 0.7 S at 2.18 Å, and 0.7 Cu atom at 2.47 Å. The Debye–Waller factor (DW) for Cu–Cu (0.008 $Å^2$) is of the same magnitude as found in the model compound. In view of the fact that 30% of the total copper was bound adventitiously (with an expected coordination number of 3–4 O/N-type ligands), this equates to ap-

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[1] A shell of six C per Cu is found at the average distance of 2.85 Å, comprised of Cu1–C3, Cu1–C2, Cu2–C5, Cu2–C6, and two other sets of two Cu–C interactions per copper related to these by the 3-fold crystallographic symmetry.

[2] A shell of 9 C/N per Cu is found at an average distance of 3.24 Å comprised of Cu1–C3, Cu1–C4, Cu1–N4, Cu2–C4, Cu2–C3, Cu2–N3, and two other sets of three atoms per copper related by the 3-fold crystallographic symmetry.

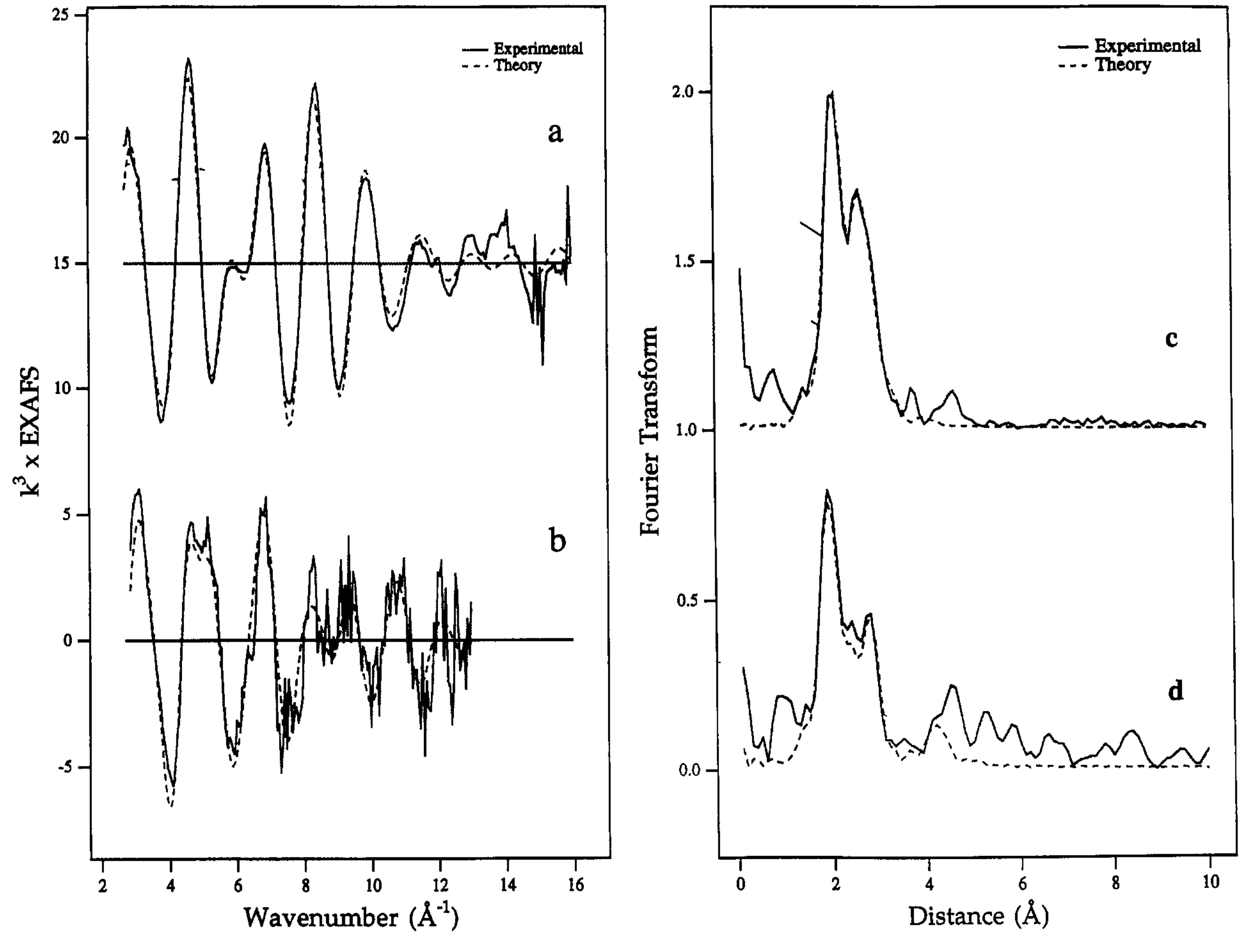

FIGURE 2: Background-subtracted EXAFS and associated Fourier transforms (phase-corrected using the N phaseshift) for the mixed-valence model complex and *Bacillus subtilis* soluble Cu_A domain. Panel a shows the experimental (solid) and simulated (dashed) EXAFS spectra for the model complex, with the associated Fourier transforms shown in panel c. Panel b shows the experimental (solid) and simulated (dashed) EXAFS spectra for the Cu_A domain, with associated Fourier transforms in panel d. The simulations for both the model and the enzyme data contain a Cu–Cu interaction to account for the resolved shell at 2.4–2.5 Å in the Fourier transforms. The metrical parameters used to produce these simulations are given in Tables 1 and 2.

Table 1: Metrical Parameters Used in the Simulation of EXAFS Spectra of the Mixed-Valence [Cu(1.5)···Cu(1.5)] Complex of the Octaazacryptand Ligand Shown in Figure 1, and Comparison with Crystallographic Distances

shell	distance[a] (Å)	Debye–Waller (Å^2)	R_{av}(crystal) (Å)
4 N	2.05 ± 0.02	0.010	2.065
1 Cu	2.35 ± 0.07	0.011	2.415
6 C	2.79 ± 0.05	0.016	2.85
9 C	3.27 ± 0.05	0.051	3.24

[a] Uncertainties in the distances arise primarily from errors in the calculation of phase shifts. The estimates given in the table are determined from comparisons between EXAFS and crystallography of copper–ligand distances, from an accumulated library of model complexes analyzed in our laboratory. Thus, first-shell Cu–C/N/O distances are typically accurate to ±0.02 Å, second and third shell Cu–C/N/O to ±0.05 Å, and metal–metal interactions to ±0.07 Å.

Table 2: Metrical Parameters Used in the Simulation of EXAFS and Fourier Transforms of the *Bacillus subtilis* Soluble Cu_A Domain, As Shown in Figure 2b and Figure 2d, Respectively

shell[a]	distance (Å)	Debye–Waller (Å^2)	fit index[b]
2 N	1.93 ± 0.02	0.005	3.45
0.7 S	2.18 ± 0.03	0.005	
0.7 Cu	2.47 ± 0.07	0.008	
4 C[c]	2.82 ± 0.05	0.016	
4 C[c]	4.19 ± 0.05	0.016	

[a] Coordination numbers are generally considered accurate to ±20–25%. [b] The fit index (*F*) is a measure of the goodness of fit in the least-squares analysis and is defined as $F^2 = (1/N)\sum k^6(\chi_i^{theor} - \chi_i^{exp})^2$, where χ_i is the *i*th EXAFS data point and *N* is the number of points in the simulated spectrum. [c] Additional shells of 4C atoms at 2.82 and 4.19 Å are included in the fit. These almost certainly arise from the C_β and C_γ shells of the imidazole rings which are expected at this distance. However, the multiple scattering which is generally associated with the EXAFS of imidazole rings [see Strange et al. (1987)] is not observed, suggesting that the copper–histidine outer-shell interactions are uncorrelated: such a situation often occurs for histidine coordination to dinuclear sites in which copper–imidazole outer-shell interactions on different metal centers become uncorrelated from each other.

proximately one N, one S, and one Cu per Cu in Cu_A, or two N, two S, and one Cu–Cu bond in the dinuclear Cu_A center. The values of the Cu–N and Cu–S distances are reminiscent of those found for type 1 copper sites, and the rather short Cu–S bond is noteworthy. The apparent Cu–N distance must be an average of the N coordinated to Cu_A and the adventitious copper, with the latter typically in the range 1.96–2.00 Å. This suggests that the actual Cu–N distance in Cu_A should be considerably shorter than the 1.93 Å average determined by EXAFS, *i.e.*, 1.90 Å or shorter. While short, such a distance would be quite consistent with the presence of only three strong ligands to Cu_A (N–His, S–Cys, Cu), and with the short 2.2 Å Cu–S distance, but preparations lacking the adventitious copper are necessary before a precise value for the Cu_A–N distance can be obtained.

It is important to stress that as regards simulation, this fit is not unique. The previous studies on Cu_A in cytochrome oxidase and N_2O reductase also identified a 2.6 Å Cu–X interaction and assigned it to a long Cu–S/Cl. On the basis

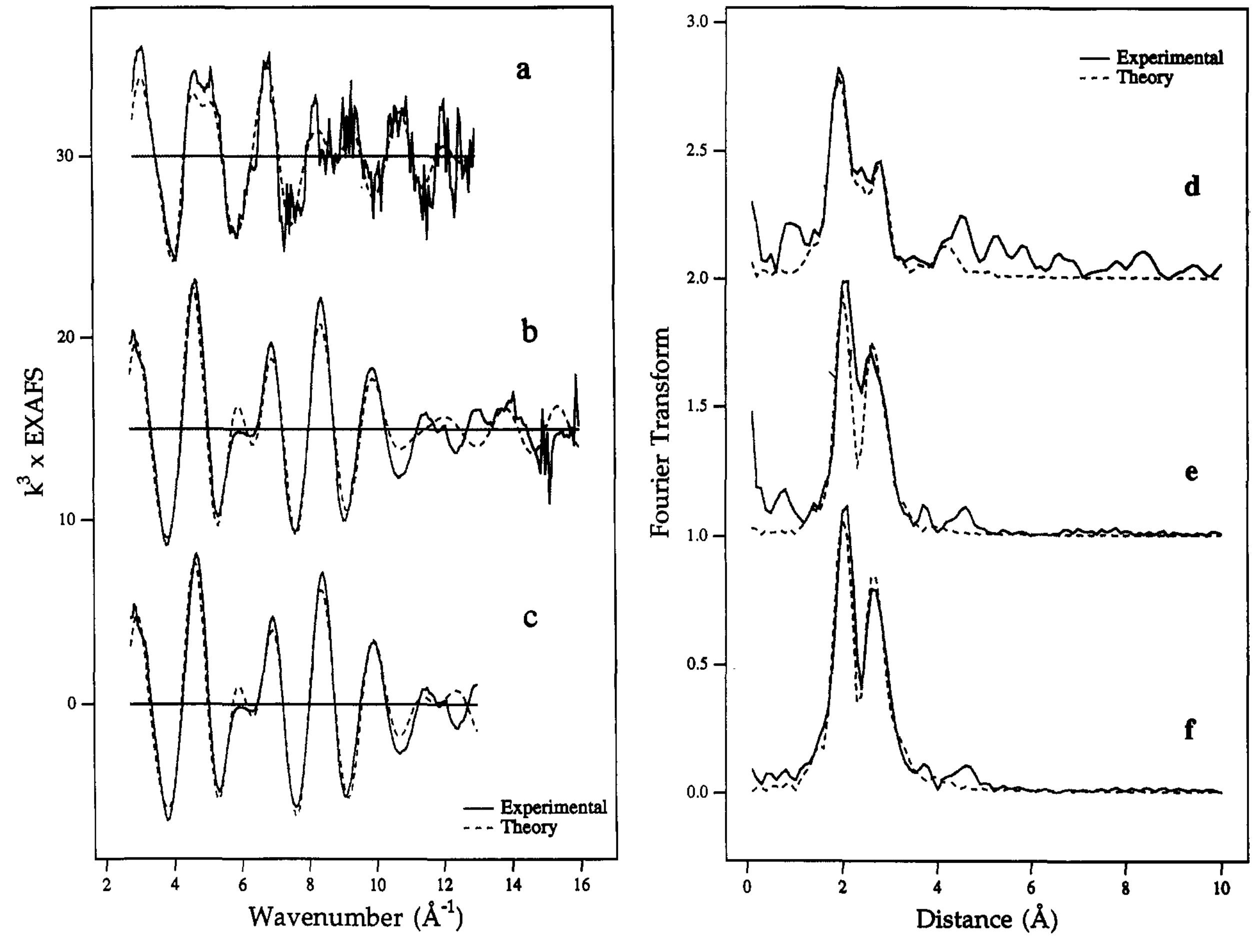

FIGURE 3: Background-subtracted EXAFS and associated Fourier transforms (phase-corrected using the N phaseshift) for the mixed-valence model complex, and *Bacillus subtilis* soluble Cu$_A$ domain, showing the effect of simulating the data with Cu–S instead of Cu–Cu. Panel a shows the experimental (solid) and simulated (dashed) EXAFS spectra for the Cu$_A$ domain with the associated Fourier transforms shown in panel d. Panel b shows the experimental (solid) and simulated (dashed) EXAFS spectra for the model complex using data to k = 16 Å^{-1}, with associated Fourier transforms in panel e. Note that both fits are reasonable at lower k but the model complex Cu–S simulation becomes poor above k = 11 Å^{-1}, and the simulated Fourier transform does not reproduce the first shell satisfactorily. Panels e and f show essentially the same fit as in panels b and e, but with a restricted data range to k = 13 Å^{-1}, indicating that Cu–Cu can easily be misinterpreted as Cu–S over the range of data usually obtainable for enzyme samples. The metrical parameters used to produce these simulations are given in Table 3.

Table 3: Metrical Parameters Used in the Simulation of EXAFS and Fourier Transforms of the *Bacillus subtilis* Soluble Cu$_A$ Domain and the Octaazacryptand Model Complex with Cu–S in Place of Cu–Cu at 2.5–2.6 Å[a]

fit	shell	distance (Å)	Debye–Waller (Å^2)	fit index	k_{max} (Å^{-1})
A	2 N/O	1.93	0.003	3.54	13
	0.7 S	2.21	0.008		
	0.7 S	2.65	0.000		
	4 C	2.81	0.021		
	4 C	4.20	0.017		
B	4 N/O	2.04	0.008	2.25	16
	1.1 S	2.54	0.006		
	6 C	2.80	0.015		
	9 C	3.28	0.048		
C	4 N/O	2.04	0.007	1.38[b]	13
	1.0 S	2.55	0.002		
	6 C	2.79	0.019		
	9 C	3.28	0.046		

[a] The fits correspond respectively to the simulations shown in Figure 3 as follows: fit A, *Bacillus subtilis* Cu$_A$ data (Figure 3a,d); fit B, model complex data to k = 16 Å^{-1} (Figure 3b,e); fit C, model complex data using a restricted data range to k = 13 Å^{-1} (Figure 3c,f). [b] The improvement in the value of the fit index is due entirely to restricting the data range, so as to exclude the k = 13–16 Å^{-1} region when the Cu–S wave fits poorly.

of polarized XAS studies of oriented samples of cytochrome oxidase, George and co-workers have noted that the Cu–X is oriented along the membrane normal, and they have assigned it to a Cu–methionine, probably Met-210 (George et al., 1993). In the present study, if instead of the Cu–Cu bond a longer Cu–S wave is included, the fit also converges to approximately the same value of the fit index (least-squares residual) with one S per Cu and a Cu–S distance of 2.65 Å. The simulated EXAFS and Fourier transforms corresponding to this fit are shown in Figure 3a,d with the metrical parameters listed in Table 3, fit A. However, chemical considerations argue forcibly against the Cu–S interpretation. The Debye–Waller term for the 2.65 Å Cu–S is close to zero, compared with the much larger value of 0.008 Å^2 for the *shorter* 2.2 Å Cu–S interaction. This is totally unreasonable, especially in view of the observation that the long (>2.7 Å) Cu–S(Met) interactions known to be present in type 1 copper sites are never observed by EXAFS, due to very large Debye–Waller terms, in contrast to the short (<2.2 Å) Cu–S(Cys) interactions which dominate the EXAFS spectra of the type 1 proteins (Adman et al., 1991; Blackburn, 1989). The only way to increase the DW of the 2.65 Å Cu–S shell is to increase its coordination number above 1 per Cu, which results in a deterioration of the fit and is inconsistent with the consensus sequence data (Buse & Stevens, 1991; Zumft et al., 1992).

We have also investigated whether the model complex could be simulated with a Cu–S instead of the Cu–Cu. The best

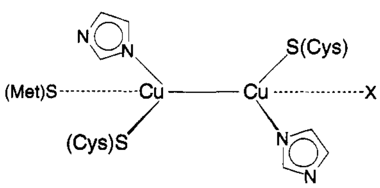

FIGURE 4: Possible structure for the dinuclear mixed-valence Cu_A site of cytochrome oxidase and N_2O reductase. Note that neither ligand depicted with dashed lines is detected by EXAFS, and their presence must be considered speculative at this time.

fit obtained is shown in Figure 3b (and Figure 3e), with metrical parameters given in Table 3, fit B. It can be seen that in this case, the fit is poorer for Cu–S than for Cu–Cu but the mismatch between experiment and theory occurs predominantly at high k (k = 11–16 Å^{-1}). If on the other hand the data range is restricted to k_{max} = 13 Å, the fits shown in Figure 3c,f (Table 3, fit C) are obtained, which are acceptable. This fitting exercise illustrates that it is unlikely that Cu–Cu can be unambiguously distinguished from Cu–S unless the data range extends to at least k = 14 Å^{-1} or above, and even then, the overlap between 2.2 Å Cu–S and 2.5 Å Cu–Cu may still prevent unambiguous assignment. On the other hand, like the protein data, the Debye–Waller factor required to force Cu–S to fit the model compound (Table 3, fit C) is also unreasonably small (0.002 Å^2) for a weak 2.6 Å Cu–S bond, and substantiates the assignment of the Cu–Cu in the protein data.

CONCLUSIONS

In conclusion, we believe that the data have provided strong evidence for the unprecedented existence of a Cu–Cu bond at the Cu_A site of the *Bacillus subtilis* cytochrome oxidase. The simulations give a Cu–Cu distance of 2.45 Å, but as indicated by the model complex data, this is probably underestimated by ~0.05 Å such that the true Cu–Cu distance is probably 2.50 ± 0.07 Å. Previous assignments of this interaction to Cu–S were in all probability incorrect. Other ligands to the Cu_A center appear to include at least one N probably from histidine at about 1.93 Å, and a short 2.18 Å Cu–S. The data cannot exclude the presence of an additional weak Cu–S (methionine) as suggested by sequence and mutagenesis studies, but by analogy to type 1 copper, this is not expected to be detectable by EXAFS.

Our data are thus entirely consistent with the results from ENDOR (Martin et al., 1988; Gurbiel et al., 1993; Hansen et al., 1993) and mutagenesis (Kelly et al., 1993) which predict two histidines, two cysteines, and one methionine per dinuclear Cu_A center, with no coordinated water (Hansen et al., 1993). However, the discovery of the Cu–Cu bond solves the dilemma that there are insufficient ligands to coordinate both coppers, since each copper has the other copper as an additional ligand. On the basis of all of the data, we propose a model for the Cu_A site as shown in Figure 4. We have included two additional weakly-bound ligands, a methionine and a hitherto undetermined ligand X, even though these are not detected by EXAFS, in order to preserve the analogy to the type 1 copper center, and our intuitive belief that the Cu centers must be at least pseudo-4-coordinate in order to function efficiently in electron transfer. Since there is only one conserved methionine in the consensus Cu_A sequence, X is not expected to be methionine. On the other hand, a peptide carbonyl (as in the case of azurin) or a glutamine (as suggested for stellacyanin) would be possible candidates (Adman, 1991; Guss et al., 1988).

We believe this model explains many of the puzzles associated with the nature of Cu_A. The two copper centers are not identical, which may explain the differences in the N coupling constants observed in the recent ENDOR studies of Hoffman and co-workers (Gurbiel et al., 1993). Nevertheless, the two copper centers apparently have close enough redox potentials to stabilize a fully delocalized valence. Further studies are necessary to fully delineate the structure, spectroscopy, and electrochemistry of this novel site.

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REFERENCES

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

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

Babcock, G. T., & Wikstrom, M. (1992) *Nature 356*, 301–308.

Barr, M. E., Smith, P. H., Antholine, W. E., & Spencer, B. (1993) *J. Chem. Soc., Chem. Commun.* **1993**, 1649–1652.

Beinert, H., Griffiths, D. E., Wharton, D. C., & Sands, R. H. (1962) *J. Biol. Chem. 237*, 2337–2346.

Binsted, N., Gurman, S. J., & Campbell, J. W. (1988) SERC Daresbury Laboratory EXCURV88 Program.

Blackburn, N. J. (1989) in *Synchrotron Radiation and Biophysics* (Hasnain, S. S., Ed.) pp 63–103, Ellis Horwood, Chichester, U.K.

Blackburn, N. J., Hasnain, S. S., Pettingill, T. M., & Strange, R. W. (1991) *J. Biol. Chem. 266*, 23120–23127.

Buse, G., & Steffens, G. C. (1991) *J. Bioenerg. Biomembr. 23*, 269–289.

Chan, S. I., & Li, P. M. (1990) *Biochemistry 29*, 1–12.

Cotton, F. A. (1983) *J. Chem. Educ. 60*, 713.

Dooley, D. M., McGuirl, M. A., Rosenzweig, A. C., Landin, J. A., Scott, R. A., Zumft, W. G., Devlin, F., & Stephens, P. J. (1991) *Inorg. Chem. 30*, 3006–3011.

Farrar, J. A., Thompson, A. J., Cheesman, M. R., Dooley, D. M., & Zumft, W. G. (1991) *FEBS Lett. 294*, 11–15.

George, G. N., Cramer, S. P., Frey, T. G., & Prince, R. C. (1993) *Biochim. Biophys. Acta 1142*, 240–252.

Greenwood, C., Hill, B. C., Barber, D., Eglington, D. G., & Thompson, A. J. (1983) *Biochem. J. 215*, 303–316.

Gurbiel, R. J., Fann, Y., Surerus, K. K., Werst, M. M., Musser, S. M., Doan, P. E., Chan, S. I., Fee, J. A., & Hoffman, B. M. (1993) *J. Am. Chem. Soc. 115*, 10888–10894.

Gurman, S. J. (1989) in *Synchrotron Radiation and Biophysics* (Hasnain, S. S., Ed.) pp 9–42, Ellis Horwood Ltd., Chichester, United Kingdom.

Gurman, S. J., Binsted, N., & Ross, I. (1984) *J. Phys. C 17*, 143–151.

Gurman, S. J., Binsted, N., & Ross, I. (1986) *J. Phys. C 19*, 1845–1861.

Guss, J. M., & Freeman, H. C. (1983) *J. Mol. Biol. 169*, 521–563.

Hansen, A. P., Britt, R. D., Klein, M. P., Bender, C. J., & Babcock, G. T. (1993) *Biochemistry 32*, 13718–13724.

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

Kroneck, P. M. H., Antholine, W. E., Riester, J., & Zumft, W. G. (1988) *FEBS Lett. 242*, 70–74.

Kroneck, P. M. H., Antholine, W. E., Kastrau, D. H. W., Buse, G., Steffens, G. C., & Zumft, W. G. (1990) *FEBS Lett. 268*, 274–276.

Lappalainen, P., Aasa, R., Malmstrom, B. G., & Saraste, M. (1993) *J. Biol. Chem. 268*, 26416–26421.

Li, P. M., Gelles, J., Chan, S. I., Sullivan, R. J., & Scott, R. A. (1987) *Biochemistry 26*, 2091–2095.

Malmstrom, B. G. (1990) *Arch. Biochem. Biophys. 280*, 233–241.

Malmstrom, B. G., & Aasa, R. (1993) *FEBS Lett. 325*, 49–52.

Martin, C. T., Scholes, C. P., & Chan, S. I. (1988) *J. Biol. Chem. 263*, 8420–8429.

Nanthakumar, A., Fox, S., Murthy, N. N., Karlin, K. D., Ravi, N., Huynh, B. H., Orosz, R. D., Day, E. P., Hagen, K. S., & Blackburn, N. J. (1993) *J. Am. Chem. Soc. 115*, 8513–8514.

Pan, L. P., Li, Z., Larsen, R., & Chan, S. I. (1991) *J. Biol. Chem. 266*, 1367–1370.

Riester, J., Zumft, W. G., & Kroneck, P. M. H. (1989) *Eur. J. Biochem. 178*, 751–762.

Saraste, M., Metso, T., Nakari, T., Jalli, T., Lauraeus, M., & van der Oost, J. (1990) *Eur. J. Biochem. 195*, 517–525.

Scott, R. A. (1989) *Annu. Rev. Biophys. Chem. 18*, 137–158.

Scott, R. A., Schwartz, J. R., & Cramer, S. P. (1986) *Biochemistry 25*, 5546–5555.

Scott, R. A., Zumft, W. G., Coyle, C. L., & Dooley, D. M. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 4082–4086.

Shapleigh, J. P., Hosler, J. P., Tecklenburg, M. M. J., Kim, Y., Babcock, G. T., Gennis, R. B., & Ferguson-Miller, S. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 4786–4790.

SooHoo, C. K., Hollocher, C. K., Kolodziej, A. F., Orme-Johnson, W. H., & Bunker, G. (1991) *J. Biol. Chem. 266*, 2210–2218.

Stevens, T. H., Martin, C. T., Wang, H., Brudvig, G. W., Scholes, C. P., & Chan, S. I. (1982) *J. Biol. Chem. 257*, 12106–12113.

Strange, R. W., Blackburn, N. J., Knowles, P. F., & Hasnain, S. S. (1987) *J. Am. Chem. Soc. 109*, 7157–7162.

Thompson, A. J., Greenwood, C., Peterson, J., & Barrett, C. P. (1986) *J. Inorg. Chem. 28*, 195–205.

Van der Oost, J., Lappalainen, P., Musacchio, A., Warne, A., Lemieux, L., Rumbley, J., Gennis, R. B., Aasa, R., Pascher, T., Malmstrom, B. G., & Saraste, M. (1992) *EMBO J. 11*, 3209–3217.

Von Wachenfeldt, C., de Vries, S., & van der Oost, J. (1994) *FEBS. Lett. 340*, 109–113.

Woodruff, W. H., Einarsdottir, O., Dyer, R. B., Bagley, K. A., Palmer, G., Atherton, S. J., Goldbeck, R. A., Dawes, T. D., & Kliger, D. S. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 2588–2592.

Zumft, W. G., Dreusch, A., Lochelt, S., Cuypers, H., Friedrich, B., & Schneider, B. (1992) *Eur. J. Biochem. 208*, 31–40.