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X-ray absorption edge studies on oxidized and reduced cytochrome *c* oxidase

(copper oxidation states/absorption edge fine structure/model copper compounds/core electronic transitions/synchrotron radiation)

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ABSTRACT **The x-ray absorption edge spectra of the Cu and Fe centers in oxidized and reduced cytochrome *c* oxidase (ferrocytochrome *c*:oxygen oxidoreductase; EC 1.9.3.1) have been obtained using synchrotron radiation from the SPEAR storage ring at the Stanford Linear Accelerator Center. In addition, oxidized and reduced plastocyanin as well as a number of model copper compounds in various oxidation states were also examined. A comparison of the absorption edge fine structure of cytochrome oxidase with those of the models indicates that one of the two coppers in the oxidized protein is in the +1 oxidation state. Upon reduction of the protein with dithionite, the second copper becomes Cu(I). The shift in the Fe K-edge of cytochrome oxidase upon reduction is small (about 2 eV or 3×10^{-19} J) and is comparable to that previously observed for the reduction of the heme iron of cytochrome *c*.**

Cytochrome *c* oxidase (ferrocytochrome *c*:oxygen oxidoreductase; EC 1.9.3.1) is the membrane-bound enzyme involved in the terminal step of respiration, that is, the reduction of molecular oxygen to water. It is of central importance to all aerobic organisms since the exothermic reaction of the enzyme with oxygen is coupled with energy conservation in the form of ATP, which is the "biological currency" for almost all energy-requiring biological reactions. In its functional form, the oxidase contains two heme irons and two copper ions, each of which appears to be distinct from the other, yet interacting. Electron paramagnetic resonance (EPR) studies on the oxidized protein suggest the presence of a very unusual Cu(II) and a low-spin Fe(III) (1–4). The other Cu and heme iron atoms appear to be EPR silent. One possible explanation for this is that the silent Cu and heme iron are antiferromagnetically coupled to each other. An alternate explanation would be the presence of Cu(I) or low-spin Cu(III) and Fe(II). The strongest argument against Cu(I) and Fe(II) in the fully oxidized state of the protein is provided by the results of coulometric titrations which show that the oxidase can be titrated reversibly with four equivalents of electrons generated under anaerobic conditions (5). This argument involves the assumption that the only redox-active centers in the protein are the metal ions. Upon reduction, the protein exhibits no EPR spectrum, implying full reduction of the metal ions to diamagnetic Cu(I) and Fe(II) (4, 6).

Thus, the current picture of cytochrome oxidase is one in which the oxidized protein contains two ferric irons and two cupric coppers, and the reduced protein two ferrous irons and two cuprous coppers. Unfortunately, this picture is based mainly on indirect evidence, and the question of the oxidation states of some of the metal centers (particularly of the coppers) in the oxidized protein must still be considered unsettled.

In an effort to resolve this question, we have applied the technique of x-ray absorption edge spectroscopy to examine the charge states of the Cu and Fe centers in cytochrome *c* oxidase. X-ray absorption spectroscopy in the neighborhood of the K-shell absorption edge of the metal can provide information about the charge density, degree of covalency, and the point symmetry of the metallic site. In the present work, we have used the intense broadband synchrotron radiation from the SPEAR storage ring at the Stanford Linear Accelerator Center. The advantage of this source is that the x-ray flux is high over a continuum of energies (down to the critical cutoff), thus permitting x-ray absorption spectroscopy to be done on relatively dilute samples (e.g., <1 mM). Thus, this method provides a means of studying the previously elusive Cu atoms in cytochrome oxidase and a probe into the electronic environments about the Fe atoms. In addition to the cytochrome oxidase spectra, we have also obtained edge spectra of another copper protein (plastocyanin) and of a number of model copper compounds. These spectra have been analyzed in terms of peak positions in the different oxidation states, and are used as a starting point in the analysis of the oxidase spectra.

MATERIALS AND METHODS

Cytochrome *c* Oxidase. Bovine heart cytochrome *c* oxidase was the generous gift of Tsoo E. King and coworkers at the State University of New York at Albany. It was suspended in a 0.5% sodium cholate/50 mM sodium phosphate buffer at pH 7.4, at concentrations of either 0.5 mM or 1.25 mM in total heme A. The reduced protein was prepared by dissolving a 50-fold excess of solid sodium dithionite in a solution of the oxidized oxidase under nitrogen. About 200 μl of sample was required for each measurement. The samples were kept frozen in liquid nitrogen until spectroscopic measurements were made. The x-ray absorption was measured at room temperature.

Plastocyanin. Bean plastocyanin (molecular weight 10,800), at a concentration of 1 mM in 0.05 M ammonium acetate, pH 6.0, was the gift of David Dooley and Harry Gray. It contains one Cu per functional unit and no other metal atoms. The reduced protein was prepared by adding 0.01 ml of 0.2 M nitrogen-flushed dithionite solution to 0.2 ml of the oxidized protein under nitrogen. The blue color of the oxidized form immediately disappeared upon addition of the dithionite, indicating

Abbreviations: EPR, electron paramagnetic resonance; Me_2PIMI, 1,3-bis[2-(4-methylpyridyl)imino] isoindoline; $HB(pz)_3$, hydrotris (1-pyrazolyl) borate; $(PAA)_2en$, the Schiff base complex of ethylenediamine with 1-phenyl-1,3,5-hexanetrione; dien, diethylenetriamine; tren, triethylenetetraamine; Im, imidazole; bi, biuret; $H_{-3}G_4$, tetraglycine; binuclear Cu(II) complex, the binuclear Cu(II) complex of the macrocyclic ligand derived from the Schiff base condensation of 5-methyl-2-hydroxyisophthalaldehyde and 1,3-diaminopropane.

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Table 1. X-ray absorption edge data for Cu(I) compounds

Compound	Transition energies, eV		
	$1s \rightarrow$ "4s"	$1s \rightarrow 4p$	$1s \rightarrow 5p$
$Cu(Me_2PIMI)CO$	8982.2	8988.4	8996.0
CuCN	8982.9	8988.3	8997.0
CuCl	8984.0	8987.8	—
CuI	8984.0	8986.6	—
$[Cu(HB(pz)_3)]_2$	8984.8	—	8996.7
$CuCO(HB(pz)_3)$	8985.2	—	8997.0

reduction of the Cu center. The samples were kept frozen in liquid nitrogen until the measurements were made.

Model Compounds. CuCl, CuI, CuCN, $Cu(Me_2PIMI)CO$, $[Cu(HB(pz)_3)]_2$, and $CuCO(HB(pz)_3)$ were used as representatives of the +1 oxidation state of copper. (Me_2PIMI is 1,3-bis[2-(4-methylpyridyl)imino]isoindoline; $HB(pz)_3$ is hydrotris(1-pyrazolyl) borate.) Reagent grade CuCl and CuCN were used. The latter three compounds were kindly donated by Robert Gagné.

Of the Cu(II) compounds, $Cu_2(PAA)_2en$ and the binuclear Cu(II) complex [dinuclear Cu(II) complex of the macrocyclic ligand derived from the Schiff base condensation of 5-methyl-2-hydroxyisophthalaldehyde and 1,3-diaminopropane] were gifts from R. Gagné. $Cu(dien)(NO_3)_2$, $CuCl(HB(pz)_3)$, $Cu(tren)Im(PF_6)_2$, and Cs_2CuCl_4 were contributed by Harry Gray and coworkers. $CuCl_2 \cdot 2H_2O$ was commercially obtained. Data for $K_2Cu(bi)_2$, CuO, $Cu(II)(H_{-3}G_4)^{2-}$, and Cu(II)-periodate were kindly made available to us by William E. Blumberg (personal communication). [$(PAA)_2en$ is the Schiff base complex of ethylenediamine with 1-phenyl-1,3,5-hexanetrione; dien is diethylenetriamine; tren is triethylenetetraamine; Im is imidazole; bi is biuret; and $H_{-3}G_4$ is tetraglycine.]

W. E. Blumberg also contributed data on the Cu(III) compounds: Cu_2O_3, $KCu(bi)_2$, $Cu(III)(H_{-3}G_4)^-$, and Cu(III) periodate.

X-ray Absorption Measurements. The x-ray absorption measurements were performed at the Stanford Synchrotron Radiation Project. The collimated, broadband synchrotron radiation from the SPEAR storage ring passed through a channel-cut silicon crystal monochromator (220 planes). The transmitted beam then passed through a 15-cm nitrogen-filled ion chamber and onto the sample (7). The Cu or Fe fluorescence $K\alpha$ radiation from the target was detected by a nine-element array of NaI scintillation counters. Data were collected by accumulating counts from the NaI detectors into individual channels for 1 sec. A weighted sum of the counters, as well as the output of the voltage controlled oscillator, I_0, was stored in the computer. The monochromator was advanced by approximately 0.2 eV, and the cycle was repeated. For the model compounds, a single scan on the neat solid afforded sufficiently precise data. For the protein data, four or more individual spectra were recorded and then later summed.

Data Analysis. The transition metal x-ray fluorescence radiation emerging from the sample is proportional to the photoabsorption cross section (8). For dilute samples, there is also present an unavoidable background due to compton and elastic scattering, which has a very nearly linear dependence upon the energy over the 100 eV (160×10^{-19} J) range studied. This linear background was removed by fitting a straight line to the data below the absorption edge and subtracting the fit from the data set.

Since the $1s \rightarrow 3d$ and $1s \rightarrow 4s$ transitions fall on the rapidly rising slope of the dominant $1s \rightarrow 4p$ transition, their positions and intensities are difficult to determine visually. Therefore, their positions and intensities were determined by subtracting from the data a smooth background curve representing the $1s \rightarrow 4p$ contribution to the absorption spectrum. The background function was taken to be a polynomial over a ±4.0 eV range centered about the apparent peak position, and the fitting criterion forced the polynomial to fit the data relatively well at the extremes of the 4 eV range. Thus, the polynomial subtraction tends to effectively remove the $1s \rightarrow 4p$ spectral contribution with a minimum distortion of the signal. This assumption was tested by successively subtracting polynomials of order 1, 2, 3, and 4, and by varying the region of the polynomial subtraction. The transition energies were determined by fitting a parabola to the remaining data, and taking the energy (in eV) to be the center of the parabola. Finally, the transition strengths were calculated by integrating the data over a ±1.0 eV range about the calculated transition energy. This integral was normalized to the photoabsorption coefficient at 9020 eV, where the absorption coefficient is insensitive to the details of the bound-to-bound transitions but is somewhat modulated by the so-called extended fine structure (EXAFS). This modulation is typically 10% or less and is the chief uncertainty in this normalization procedure. The integration procedure was found to be sensitive to the limits of integration, but the relative strengths of corresponding transitions among different compounds were found to be independent of the integration limits, to a precision of ±20%.

Table 2. A comparison of the ($4s - 4p$) energy level separations for the Zn(II) ion and the Cu(I) compounds studied

Compound	Description	Energy level separation, eV ($4s - 4p$)
Zn(II)	Free ion	6.1
$Cu(Me_2PIMI)CO$	Square planar	6.2
CuCN	—	5.4
CuCl	Tetrahedral-ionic	3.9
CuI	Tetrahedral-ionic	2.7
$[Cu(HB(pz)_3)]_2$	Tetrahedral-covalent	~0
$CuCO(HB(pz)_3)$	Tetrahedral-covalent	~0

Optical Spectra. The optical spectra of the irradiated and unirradiated cytochrome oxidase samples were obtained on a Beckman Acta CIII UV-visible spectrophotometer using a specially constructed air-tight optical cell. The reduced oxidase was diluted appropriately with nitrogen-flushed buffer for the visible absorption measurements and was handled exclusively under nitrogen. The spectra obtained verified the state of reduction of the samples as compared to previously published spectra (9) and indicated that no changes occur in the heme chromophores upon prolonged exposure to x-rays.

RESULTS

Model Compounds. The K-absorption edge spectral data of the Cu(I) compounds are presented in Table 1. The lowest energy absorption in all of the compounds except $[Cu(HB(pz)_3)]_2$ and $CuCO(HB(pz)_3)$ has been assigned to the forbidden $1s \rightarrow 4s$ transition. The next absorption is much stronger than the first and occurs between 2.7 and 6.2 V to higher energy. It is attributed to the fully allowed $1s \rightarrow 4p$ transition. In Table 2, the energy separation between these assigned transitions for each compound is compared with the energy separation obtained from spectroscopic tables (10) for the corresponding excited states of the Z + 1 or, in this case, the Zn(II) ion. The validity of this comparison is discussed in detail by Shulman *et al.* (11). We see that as the ligand geometry proceeds from square planar to tetrahedral, the $4s$-$4p$ separation decreases,

Table 3. X-ray absorption edge data for Cu(II) compounds

Compound	Transition energies, eV		
	$1s \rightarrow 3d$	$1s \rightarrow 4s$	$1s \rightarrow 4p$
$K_2Cu(bi)_2$	8979.8	8986.2	?
CuO	8979.4	8986.2	9000.0
$Cu_2(PAA)_2en$	8979.9	8986.5	9000.0
$Cu(II)(H_{-3}G_4)^{2-}$	8978.1	8986.8	8997.9
$Cu(tren)Im(PF_6)_2$	8979.2	8987.0	8997.8
Cs_2CuCl_4	8978.5	8987.1	8993.7, 8997.0
$Cu(dien)(NO_3)_2$	8979.4	8987.6	8995.0
$CuCl(HB(pz)_3)$	8979.1	8988.1	8996.5
$CuCl_2 \cdot 2H_2O$	8978.8	8988.2	8995.9
Cu(II)-periodate	Weak	Weak	8998.0
Binuclear Cu(II) complex	8979.3	Weak	9000.0

which is what one would expect for increased *s-p* mixing. In the tetrahedrally coordinated covalent pyrazolyl borate complexes, the 4*s*-4*p* splitting seems to have disappeared altogether, indicating complete *s-p* mixing resulting in a single hybrid state. Note that the energy of the transition in this case is between that of the $1s \rightarrow 4s$ and $1s \rightarrow 4p$ transitions of the other Cu(I) complexes. Thus, for these two compounds, it is not valid to speak of the $1s \rightarrow 4s$ or the $1s \rightarrow 4p$ transition separately. However, for the sake of tabulation, the energy of this $1s \rightarrow (4s\text{-}4p)$ transition is listed together with the $1s \rightarrow 4s$ transitions of the other Cu(I) compounds. For this reason, we denote this set of transitions as $1s \rightarrow$ "4*s*". The spectral features above the 4*p* transition are presumably due to transitions from the 1*s* to 5*p* and higher *p* orbitals.

Table 3 shows the edge data of a number of Cu(II) compounds. The lowest energy absorption is very weak and has been assigned to the symmetry-forbidden $1s \rightarrow 3d$ transition. The second peak or shoulder at higher energies corresponds to the $1s \rightarrow 4s$ transition, and the maximum corresponds to the $1s \rightarrow 4p$ transition. The energy differences among these transitions are compared with the energy level separation among the 3*d*, 4*s*, and 4*p* levels of the Zn(III) ion in Table 4. We see that the most ionic of the Cu(II) compounds, $CuCl_2 \cdot 2H_2O$, bears the closest fit to the ionic Zn(III) levels, since atomic level comparisons are only relevant to very ionic compounds.

The Cu(III) spectral data are shown in Table 5. In the biuret, oxide, and periodate complexes, the $1s \rightarrow 3d$ transitions are extremely weak and occur roughly 7–9 V below the $1s \rightarrow 4s$ transition. In the tetraglycine, the $1s \rightarrow 3d$ peak is stronger but shifted about 2 eV to higher energy with a similar shift in the $1s \rightarrow 4s$ peak. The $1s \rightarrow 4p$ peaks occur 20–22 eV above the

Table 4. A comparison of the $(3d - 4s)$, $(4s - 4p)$, and $(3d - 4p)$ energy level separations for the Zn(III) ion and the Cu(II) compounds studied

Compound	Energy level separation, eV		
	$(3d - 4s)$	$(4s - 4p)$	$(3d - 4p)$
Zn(III)	10.0	7.9	17.8
$K_2Cu(bi)_2$	6.4	—	—
CuO	6.8	13.8	20.6
$Cu_2(PAA)_2en$	6.6	13.5	20.1
$Cu(II)(H_{-3}G_4)^{2-}$	8.7	11.1	19.8
$Cu(tren)Im(PF_6)_2$	7.8	10.8	18.6
Cs_2CuCl_4	8.6	6.6(9.9)*	15.2(18.5)*
$Cu(dien)(NO_3)_2$	8.2	7.4	15.6
$CuCl(HB(pz)_3)$	9.0	8.4	17.4
$CuCl_2 \cdot 2H_2O$	9.4	7.7	17.1
Binuclear Cu(II) complex	—	—	20.7

* Values in parentheses if the 8997.0 peak is used.

Table 5. X-ray absorption edge data for Cu(III) compounds

Compound	Transition energies, eV		
	$1s \rightarrow 3d$	$1s \rightarrow 4s$	$1s \rightarrow 4p$
Cu_2O_3	8978.6	8985.7	8998.5
$KCu(bi)_2$	8978.4	8986.4	8998.6
Cu(III)-periodate	8978.0	8987.0	9000.0
$Cu(III)(H_{-3}G_4)^-$	8980.6	8987.8	9001.0

3*d* peak and the spectra are fairly featureless above this peak. Zn(IV) spectroscopic data are not available for comparison.

Cytochrome *c* Oxidase. The Cu K-edges of both the oxidized and the reduced cytochrome *c* oxidase are shown in Fig. 1. upon comparison of the spectra of the oxidized and the reduced protein, one immediately notices a net shift in intensity towards lower energies upon reduction as well as a change in the shape of the edge. Five peaks or shoulders are observed in the oxidized spectrum, and at least four are observed in the reduced. The assignment of the peaks will be discussed below.

Fig. 2 compares the results obtained with the model compounds and cytochrome oxidase. The data from the model compounds establish an energy range for each of the transitions in the various oxidation states of copper. The peaks occurring in the cytochrome oxidase spectra are represented as discrete lines. A fair amount of overlap is seen among the transitions of the different oxidation states, which gives rise to some ambiguity in the assignment of peaks in the oxidase spectra. In particular, all the transitions for Cu(II) are indistinguishable from those of Cu(III). However, the $1s \rightarrow$ "4*s*" transitions of the Cu(I) compounds, at about 8983 eV, do not overlap any transitions of the Cu(II) or Cu(III) compounds. Thus, the existence of a transition in this range is strong evidence for the presence of Cu(I).

In the spectrum of reduced cytochrome oxidase, we see that the lowest energy peak falls at 8983.1 eV, which is well within the range for the $1s \rightarrow$ "4*s*" transition of Cu(I). The next few peaks fall in the range for $1s \rightarrow 4p$ and $1s \rightarrow$ higher *p* transitions of Cu(I) compounds. No attempt is made to make separate peak assignments for the two coppers in cytochrome oxidase.

The oxidized oxidase spectrum exhibits a barely visible $1s \rightarrow 3d$ transition at 8979 eV. According to the model compound data, this may be attributed to either a Cu(II) or a Cu(III). The next transition at 8983.7 eV, however, clearly corresponds to

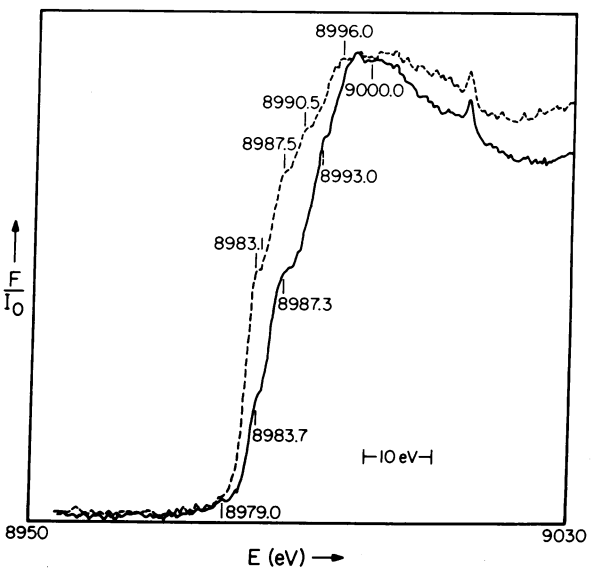

FIG. 1. The copper K-edge spectra of oxidized (—) and reduced (- - -) cytochrome *c* oxidase.

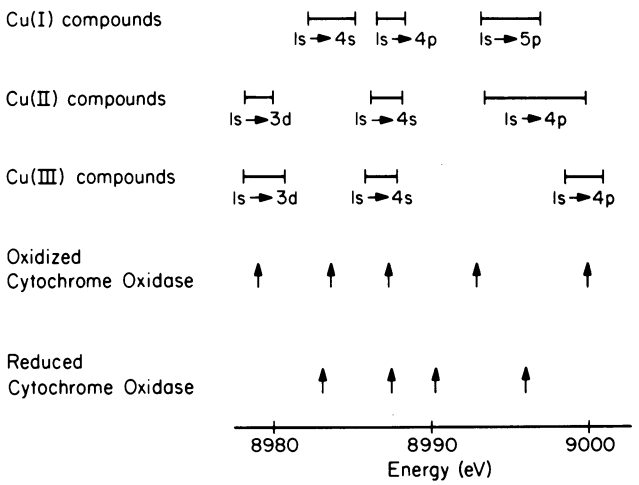

FIG. 2. Comparison of the transition energy data for the Cu(I), Cu(II), and Cu(III) model compounds with the transitions observed in oxidized and reduced cytochrome *c* oxidase.

a $1s \rightarrow$ "$4s$" transition of a Cu(I). Intensity measurements on properly normalized spectra show that it is half the intensity of the corresponding $1s \rightarrow$ "$4s$" peak in the reduced oxidase spectrum. The remaining peaks fall in the region of overlap between the $1s \rightarrow 4p$ transition of Cu(I) and the $1s \rightarrow 4s$ and $1s \rightarrow 4p$ transitions of Cu(II) and Cu(III). Thus, it is not possible to make unambiguous assignments for these peaks.

Plastocyanin. The K-edge absorption spectra of oxidized and reduced bean plastocyanin are shown in Fig. 3. In both oxidized and reduced states, the plastocyanin spectra are quite similar to the Cu K-edges of the oxidase in the oxidized and in the reduced state, respectively, especially in the high energy regions. The prominent exception is that the low energy peak assigned to the $1s \rightarrow$ "$4s$" transition of Cu(I) in oxidized cytochrome oxidase is not present in the spectrum of oxidized plastocyanin.¶ This peak, however, is present in the spectrum of the reduced protein and is assigned to the $1s \rightarrow$ "$4s$" transition of Cu(I). Note that the lowest energy transition in the oxidized spectrum, which is assigned to the $1s \rightarrow 3d$ transition of Cu(II), apparently disappears upon reduction. This is consistent with Cu going from Cu(II) to Cu(I).

Fe K-Edge of Cytochrome Oxidase. The iron absorption edge spectra of cytochrome oxidase were also obtained for the oxidized and the reduced protein. As shown in Fig. 4, there is a small shift of about 2 eV to lower energy in going from the oxidized to the reduced state. This is considerably less than the shift observed by others for ionic Fe complexes but of comparable magnitude for shifts obtained with covalent Fe complexes and the heme Fe of cytochrome *c* (11). However, the edge spectra due to the two individual heme irons are not resolved here. In fact, the edge is fairly narrow.

DISCUSSION

The data on the model compounds show that the energies of the $1s \rightarrow 3d$ and/or $1s \rightarrow 4s$ transitions for each oxidation state of copper fall within a very narrow range. Considering the diversity of the ligands involved, this is remarkable. In comparing

¶ Intensity measurements of the very slight inflection in the edge spectrum of oxidized plastocyanin in the region of the $1s \rightarrow$ "$4s$" transitions show that the strength of this transition is less than 10% of the $1s \rightarrow$ "$4s$" transition in the corresponding region of the spectrum of reduced plastocyanin. This small component of Cu(I) intensity in the oxidized spectrum is attributed to the small fraction of autoreducible protein that has been detected by other means (H. B. Gray, personal communication).

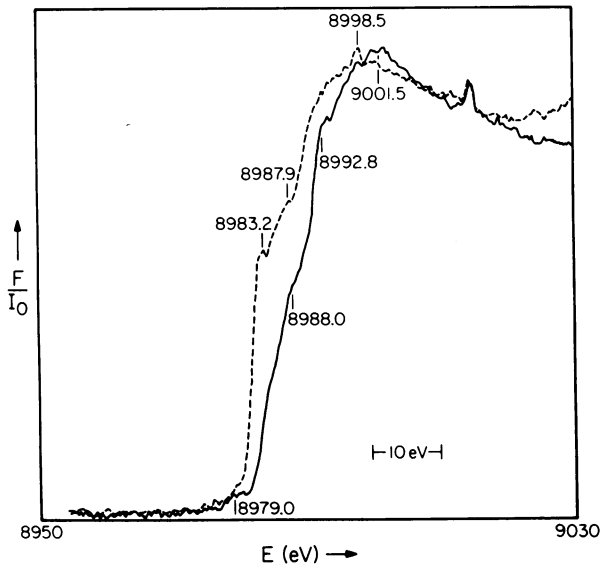

FIG. 3. The copper K-edge spectra of oxidized (—) and reduced (- - -) bean plastocyanin.

the data from the Cu(I) and Cu(II) compounds, we immediately note the appearance of the $1s \rightarrow 3d$ transition in the d^9 system. In addition, we see an overall shift of the $1s \rightarrow 4s$ and $1s \rightarrow 4p$ transitions towards higher energies. This is expected since it requires more energy to move an electron away from a nucleus with a greater positive charge. However, in going from Cu(II) to the analogous Cu(III) compounds, there is no general upward shift in the energies for the three transitions. This suggests that although the Cu in these compounds carries a formal charge of +3, the electron density about the nucleus is essentially the same as for Cu(II). This may be due to enhanced $L \rightarrow M\pi$ bonding. Thus, on the basis of the x-ray absorption data alone, it is not possible to distinguish between formal +2 and +3 oxidation states. It is possible, however, to identify a Cu(I) compound by its very characteristic $1s \rightarrow$ "$4s$" transition. Since there is no overlap between this transition and any of the transitions of Cu(II) and Cu(III), a Cu(I) assignment for a peak occurring in this region is fairly straightforward.

Comparison of the absorption edge fine structure of cytochrome *c* oxidase with that of the model compounds indicates

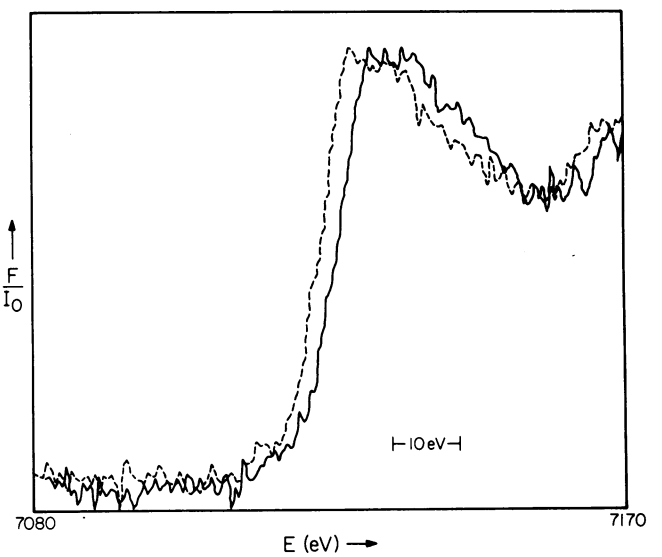

FIG. 4. The iron K-edge spectra of oxidized (—) and reduced (- - -) cytochrome *c* oxidase.

that Cu(I) is present not only in the reduced, but also in the oxidized protein. The normalized intensity of the $1s \rightarrow 4s$ signal in the spectrum of the reduced oxidase is 0.12, twice that of the Cu(I) $1s \rightarrow 4s$ signal in the spectrum of the oxidized protein. For the model Cu(I) compounds, the normalized intensity of the $1s \rightarrow 4s$ transitions is within the range of 0.08–0.14; the strength of the corresponding transition in reduced plastocyanin is 0.12 (unpublished). These results strongly suggest that one of the two Cu ions in oxidized cytochrome *c* oxidase is Cu(I).

Considering the importance of this conclusion, we have considered and rejected several alternative explanations of the transition at 8983.7 eV. One might argue that radiation damage in the sample might partially reduce the oxidized sample. However, no change in the x-ray absorption spectra was observed with time. Also, the optical spectra of the irradiated samples agreed well with the published data. Finally, the data from three separate experiments on two different batches of cytochrome oxidase are identical, the ratio of Cu(I) in the reduced to oxidized protein being 2:1 in each case. Thus, we feel that we can reasonably exclude the possibility of Cu(I) being merely an experimental artifact.

We also considered the possibility that our model compounds were not sufficient representatives of copper in a protein environment. For this reason, we have examined the Cu edge spectra of another copper protein, bean plastocyanin. The x-ray absorption edge spectra of oxidized and reduced plastocyanin bear a close resemblance to the respective spectra of oxidized and reduced cytochrome oxidase, as a comparison of Figs. 1 and 3 will show. Note, however, that oxidized plastocyanin does not contain a transition in the energy range that encompasses the $1s \rightarrow$ "$4s$" transitions of Cu(I). A further comparison of the plastocyanin data with that of the model compounds shows that the transition energies in oxidized and reduced plastocyanin are well within the energy ranges established by Cu(II) and Cu(I) model systems, respectively. Thus, we see that the electronic environment of copper in a protein is not significantly different from that of copper in the inorganic models studied. This argues in favor of our assignment of the 8983.7 eV transition in the spectrum of oxidized cytochrome oxidase to Cu(I) since a transition in this region is characteristic of all of the Cu(I) models and present in none of the Cu(II) models.

Thus, of the two coppers in cytochrome *c* oxidase, only one of the coppers formally undergoes a change in oxidation state between Cu(I) and Cu(II) or Cu(III), while the other is always effectively Cu(I). Reductive titration experiments show that four electron equivalents can be reversibly taken up by the protein, and it is generally assumed that the four metal atoms are involved in oxidation and reduction (5). The notion that the redox-active sites are restricted to the metal ions would be compatible with our findings if the reducible Cu in the oxidized protein were Cu(III). However, our x-ray absorption data is ambiguous with respect to the oxidation state of this second copper and there is no other experimental evidence that even suggests the possible occurrence of Cu(III) in oxidized cytochrome oxidase. On the other hand, there is no direct evidence that both coppers partake in the oxidation–reduction reactions. The EPR signal at approximately $g = 2$, which has been attributed to Cu(II) in the oxidized protein, at most accounts for only half of the total copper in cytochrome oxidase (1, 4). In addition, there is a question whether this signal is actually due to copper or to a sulfur radical (12). Thus, there is a serious possibility that both coppers are EPR silent and that one of the four electron acceptors is really a protein ligand. In fact, it has been previously postulated that a Cu(I)-disulfide system might exist in the protein such that the complex as a whole participates in electron transfer with the ligand being the prime reversible electron acceptor (13). Such model systems are discussed by Hemmerich (14). The results obtained in our x-ray absorption experiments are not inconsistent with such a model. However, since none of the ligands to copper are known, a clarification of this point awaits future investigation.

In summary, on the basis of x-ray absorption edge studies of cytochrome oxidase, plastocyanin, and a host of model compounds, evidence is presented that one of the coppers in oxidized cytochrome *c* oxidase is in the +1 oxidation state. The implications of this finding are that it calls for a reinterpretation of some of the existing data on the protein, perhaps a redefinition of "invisible copper," and a reexamination of the mechanisms proposed for electron transfer mediated by cytochrome oxidase.

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