Biochimica et Biophysica Acta, 546 (1979) 520—538
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BBA 47669

THE NATURE OF THE COPPER ATOMS OF CYTOCHROME *c* OXIDASE AS STUDIED BY OPTICAL AND X-RAY ABSORPTION EDGE SPECTROSCOPY

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(Received September 19th, 1978)
(Revised manuscript received November 11th, 1978)

Key words: Cytochrome c oxidase; X-ray absorption edge spectroscopy; Copper

Summary

X-ray absorption edge spectroscopy has been used to study the copper of 1—2 mM cytochrome *c* oxidase in the resting oxidized, mixed-valence, and fully reduced states. A comparison was made of this protein with copper complexes and with natural and artificial copper proteins. Spectra were obtained with synchrotron radiation from the SPEAR storage ring using highly sensitive fluorescence detectors. Temperatures of −80 to −120°C were employed further to improve the stability of the samples and to avoid the possibility of either auto- or photon-induced reduction of the materials, which might have occurred in previous studies. In order to characterize the valence states of the Cu and Fe components, the samples were monitored by infrared and visible spectroscopy before and after irradiation by the X-ray beam. The combination of the optical and X-ray absorption techniques has afforded a deconvolution of the four species of copper in the various states of cytochrome *c* oxidase and the tentative assignment of Cu_a, the copper redox coupled to the heme *a* of cytochrome *a*, as a highly covalent type of copper and Cu_{a3}, the copper of cytochrome a_3, as a more ionic 'blue' type I copper. The implications of these findings upon the mechanism of action of cytochrome oxidase are briefly outlined.

Abbreviation: TMPD, tetramethyl-*p*-phenylenediamine.

Introduction

Three major classifications of copper sites in copper proteins can be made on the basis of spectroscopic properties [1]. Type I or 'blue' copper sites exhibit absorption bands near 450 nm ($\epsilon \sim 0.3$–$1\ cm^{-1} \cdot mM^{-1}$) and 600 nm ($\epsilon \sim 3$–$10\ cm^{-1} \cdot mM^{-1}$) and a near infrared band approximately at 800 nm ($\epsilon \sim 0.3$–$3\ cm^{-1} \cdot mM^{-1}$). EPR spectra of type I sites exhibit a narrow nuclear hyperfine structure ($A_{\parallel}$ between 4 and $9 \cdot 10^{-3}\ cm^{-1}$) [2], and they have oxidation-reduction potentials of about 0.3—0.4 V. Type II or 'non-blue' copper sites have optical properties that are not as clearly defined, but a weak absorption band is observed in the region of 600—700 nm ($\epsilon \sim 0.3$–$0.4\ cm^{-1} \cdot mM^{-1}$) [1]. A broadly spaced hyperfine structure in the EPR spectrum ($A_{\parallel}$ between 12 and $22 \cdot 10^{-3}\ cm^{-1}$) [2] is characteristic. In the case of proteins having both type I and type II copper sites, the 'non-blue' copper has a high affinity for anions, while the 'blue' copper is more quickly reduced. Artificial and natural type II copper proteins contain nitrogen and oxygen as ligands (sulfur is ruled out completely) [2], while the type I sites in plastocyanin [3] and in azurin (Jensen, L., personal communication) have been shown to contain cysteinyl sulfur.

Type III sites are 'EPR non-detectable' and are thought to be diamagnetic [1]. These sites consist of either two Cu^{+} or a spin-paired Cu^{2+}-Cu^{2+} which exhibit a near ultraviolet absorption band near 330 nm ($\epsilon \sim 2.5$–$5\ cm^{-1} \cdot mM^{-1}$). Oxidation-reduction potentials are greater than 0.5 V [1].

Blue copper oxidases contain each of the different types of copper sites described above and contain at least four copper atoms/molecule. These proteins participate in the single electron oxidation of substrates and four electron reduction of O_2. Each of the copper sites has been assigned a function, although there is not unanimous agreement on this point. Other enzymes containing one or two copper atoms form hydrogen peroxide as the product of molecular oxygen reduction (for a summary see [1]).

Another copper-containing oxidase that catalyzes the reduction of oxygen to water and the single electron oxidation of substrate is cytochrome *c* oxidase. This protein contains only two copper atoms but contains two molecules of heme *a* as well.

An understanding of the nature and function of the copper atoms of cytochrome oxidase has been rendered difficult because of their enigmatic responses to a variety of physical techniques. Few physical methods are responsive to both redox states of both copper atoms. For example, EPR is responsive to one of the four forms. The copper atom associated with oxidized cytochrome *a* exhibits an EPR absorption near $g = 2$ [4,5] which lacks the characteristic hyperfine structure associated with Cu^{2+} and thus is often difficult to distinguish from some free radical signals [2]. The second copper atom associated with cytochrome a_3 is EPR silent, probably because of proximal location to the heme.

The optical absorption of cytochrome *c* oxidase near 600 nm contains contributions from both heme *a* groups [6] making the copper contribution difficult to determine. Also, different results have been obtained at low temperature or room temperature for the near infrared absorption band of oxidized

cytochrome oxidase at 830 nm ($\epsilon \sim 2\ cm^{-1} \cdot mM^{-1}$) [6]. The suggestion that these differences were in large part due to heme *a* absorptions seem to be largely discredited because of the lack of distinctive infrared absorption bands of heme *a* in reduced cytochromes *a* and a_3, in spite of the very strong α, β, and γ transitions, together with the lack of distinctive responses in the infrared region due to heme ligation of cytochrome a_3 in the reduced and oxidized states [1,7,8]. This is especially notable in disruption of the redox equilibrium by CO ligation of heme *a* in cytochrome a_3 at the midpoint of the redox titration [6]. Thus, the implied 'total' assignment of the 830 nm (or 860 nm) absorption to the copper associated with cytochrome *a*, Cu_a *, seems inappropriate, in spite of the apparent correlations with the midpotential of the EPR titrations at $g = 2$. Recently, attention has been called to the possibility that the copper associated with the heme *a* of cytochrome a_3 has absorbance at 744 nm [9] in intermediate valence states. The 744 nm band 'may belong to a blue copper protein' [9]. For these reasons, we have sought other more incisive approaches to the nature of the copper atoms of cytochrome oxidase.

A technique by which both copper atoms can be observed in different oxidation states is X-ray absorption edge spectroscopy. The absorption spectrum near the K-edge absorption is characterized by structural features containing information about the molecular energy levels of the absorbing atom. In ionic systems, identification of the $1s \rightarrow 3d$, $1s \rightarrow 4s$, and $1s \rightarrow 4p$ transitions has been made for several metal atoms using atomic levels and the one electron character of 1s transitions. In more covalent systems, mixing due to vibronic interactions must be considered [10]. Likewise, ligand field mixing due to ligand geometry produces s-p mixing. Therefore only in ionic cases can pure transitions (e.g. $1s \rightarrow 4s$) be considered. In all of these studies, it is clear that the X-ray photon energy at which these transitions occur depends on the charge density of the absorber, the degree of covalency of the bonds, and the coordination geometry. The effects of each of these parameters on the observed transitions have recently been investigated by Blumberg et al. (Blumberg, W.E., Peisach, J. and Powers, L.S., manuscript in preparation) in copper complexes and in natural and artificial proteins for which corresponding EPR measurements were made [2]. This technique, then, could provide a method for investigation of the local electronic environment of the copper atoms in cytochrome oxidase.

X-ray absorption edge spectra of copper and iron in cytochrome *c* oxidase in the oxidized and reduced states have been previously observed by Hu et al. [11,12]. These authors conclude that one of the copper atoms is in the Cu(I) state in the fully oxidized protein. This conclusion is examined in detail in this paper.

The participation of the copper atoms in the function of this protein is not yet fully understood, and the present work addresses this question for the protein in the fully oxidized, fully reduced and the two-mixed-valence states, before attempting further studies of copper in the enzymatically interesting intermediates discussed by Chance et al. [13].

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* The terms Cu_a and Cu_{a3} refer to the copper atoms that are redox coupled with the hemes *a* in cytochromes *a* and a_3, respectively.

Experimental methods

Cytochrome oxidase preparations

Three types of cytochrome oxidase preparations were employed and were made by the methods described in Refs. 14—17. They will be denoted K, H, and V for their donors, T.E. King, J. Harmon, and S. Vik. In the case of K preparation, the authors assayed the copper and found that the 'adventitious copper' was less than 10% [17]. An assay made on preparation V indicated less than 10% 'adventitious copper' by atomic absorption relative to heme *. The preparation did not exhibit a detectable EPR signal near $g = 12$ (Blum, H., personal communication). No assays were made of preparation H, but the edge absorption data suggested that this preparation had 'adventitious copper' to the extent of 10—20%. The results of this paper are chiefly focused on preparation V and include some edge studies of preparation K.

Samples were prepared from preparation V, which contained 160 mg protein/ml (12 nmol heme *a*/mg protein) corresponding to 1 mM each of Cu_a and Cu_{a3}, buffered in 20 mM Tris-HCl, containing 90 mM NaCl at pH 7.4. In the experiments described below, no additional buffer was added, and the maintenance of pH at low temperatures depended upon the protein concentration itself [18]. 90 mM NaCl is used in preparation V to maintain high ionic strength for cytochrome *c* oxidase-lipid reconstitution [19]. No spectral shifts due to Cl^- were detected.

The special conditions for the particular samples are as follows:

Sample I — oxidized (Fig. 1): Sample I was undiluted cytochrome *c* oxidase (preparation V, as received) (trace C, before 30 min X-irradiation; trace B, after X-irradiation). 400 μM ferricyanide (trace A) was added to the material to ascertain if any reduction had occurred during the period of irradiation, and none was found.

Sample II — reduced + CO (Fig. 1, trace E): Reduction of the heme and copper was effected by the addition of ascorbate (7 mM), tetramethyl-*p*-phenylenediamine (TMPD, 3.6 μM), and cytochrome *c* (3.6 μM). Ethylene glycol (29% final concentration) was added to permit the subsequent addition of ferricyanide or oxygen at low temperatures. These latter reagents, contained in a total volume of 35 μl, were saturated with 1 atm CO for 15 min. The were then added to an equal volume sample of cytochrome oxidase and allowed to stand for 30 min in a CO atmosphere at 23°C. Then, the sample was frozen at —196°C and maintained at —78°C in the dark. The dilution factor was 0.50.

Sample III — mixed valence state + CO (Fig. 1, trace D): Sample III was prepared from sample I by additions of 7.1 mM ascorbate, 3.6 μM cytochrome *c*, 3.6 μM TMPD and 15% ethylene glycol. The resulting solution was exposed to CO for 30 min at room temperature as before and thereafter cooled to -15°C for 30 min under an atmosphere of CO. At this point, 5.7 mM ferricyanide was added with vigorous mixing and allowed to react for 30 s, and then the sample was rapidly frozen at —196°C and subsequently maintained at

* Determination of the Cu/Fe content by atomic absorption of preparations K and V indicates that the copper content exceeds the iron content by no more than 10% (the edge data in Fig. 2 refer to preparations K and V) (Scarpa, A., personal communication).

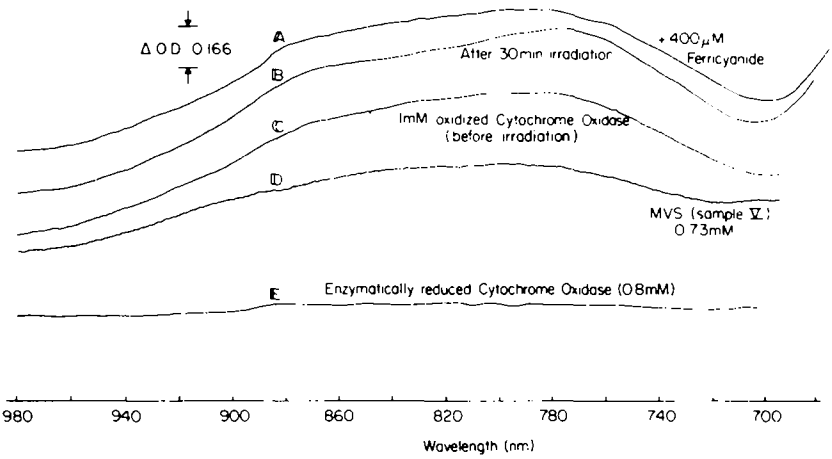

Fig. 1. Representative examples of infrared absorbance of cytochrome oxidase samples used in these experiments. The sample designation of chemical conditions of traces A—F is indicated in the text.

--78°C. The protein dilution factor was 0.71.

Sample IV — reduced + CO (not illustrated): The preparation procedure was similar to that of sample II, except that no ethylene glycol was added. The dilution factor for this preparation was 0.63.

Sample V — mixed valence state + CO (not illustrated): Sample V was prepared with 8 mM ascorbate, 7.2 μM TMPD, 3.6 μM cytochrome *c*, and 30% ethylene glycol. As before, this mixture was saturated with CO for 30 min at room temperature. The oxidase was then added and subsequently equilibrated at room temperature for 16 min. The temperature was then lowered to --15°C, and in this case 40 min was allowed for equilibration with CO. At the end of this interval, 11 mM ferricyanide was added, and the solution was stirred for 30 s and then rapidly chilled to --78°C. The dilution factor was 0.67.

Sample VI — converse mixed valence state: The converse mixed valence state was obtained by pretreating the oxidase with 30 mM formate for 30 s at 23°C, and then adding 80 μM diaminodurene and 8 μM phenozine methosulfate and 6 mM NADH. 30 s thereafter the sample was freeze-trapped at --78°C. The absorption in the infrared region was 26% of that in the fully oxidized state, indicating that a mixed-valence state was obtained.

Sample VII. Another sample, similar to sample V, was prepared with 30 mM formate, 60 μM diaminodurene, 6 μM phenozine methosulfate and 3 mM NADH, and 28% of the infrared absorption remained.

Sample VIII. The same mixed-valence state was made by partial reduction of the cyanide adduct. The reaction with cyanide is slow, and reaction overnight in the presence of 3 mM KCN at 4°C was employed. Thereafter, diaminodurene, phenozine methosulfate, and NADH were added in the same concentrations as in sample VII. The residual infrared absorption was 23% in this case.

Sample IX. In order to ensure a completely oxidized state in sample VIII, not only was ferricyanide added as described above (2 mM) but diaminodurene (200 μM) was added to ensure electron equilibration in all oxidase molecules.

No increase of infrared absorption over untreated controls was observed in the samples treated in this manner.

Stellacyanin. Stellacyanin (3 mM) was prepared according to the method of Mims and Peisach [20]. The A_{604}/A_{280} was 0.197. Reduced stellacyanin was prepared by adding excess solid sodium dithionite to the oxidized protein until no trace of blue color remained. Both oxidized and reduced stellacyanin samples were frozen and maintained at −78°C.

Spectrophotometer for optical studies

A styrofoam-insulated cuvette holder accomodating the 22 X 2 X 1.2 mm cuvettes used for the edge spectra was connected to a thermostatically controlled stream of N_2 gas maintained at temperatures from −120 to −80°C and was coupled to the spectrophotometer and the photomultiplier (RCA 7102 infrared-sensitive S-1 surface). The split beam spectrophotometer consisted of a 250 mm focusing Bausch and Lomb grating monochromator (600 lines/mm) [21]. The second-order overlap was eliminated by a Wratten 15 gelatin filter (blocking below 500 nm), thus permitting a wavelength scan from 980 to 540 nm. The light beam was oscillated from reference to sample by a synchronous motor operating at 60 Hz. The 120 Hz signal was amplified, peak detected and phase demodulated from phase adjustable gates derived from the line frequency. An adjustable RC filter and log converter (0–1.5) were provided.

Data analysis

The turbid-frozen reaction medium (30% ethylene glycol buffer) was matched with a 'black tape' slit of the same light transmission as a reference sample, and the absorption difference spectrum of the two (960—520 nm) was read out in terms of the shift of dynode voltage necessary to give a null output of the phase-sensitive demodulator over the spectral range. These corrections were stored by an A to D converter and an eight bit 1024 address memory. The base-line correction was stored in a second memory.

The sample containing only buffer was replaced by the oxidase or stellacyanin sample and the spectrum was stored in the computer memory and corrected for the stored base-line. Any number of averages of these spectra could be obtained; however, the signal to noise ratio in a single scan was adequate for data analysis.

An X-Y recorder displayed the difference of the oxidase spectrum and the base-line error spectrum to give the results shown in Figs. 1, 2 and 3.

Experimental design

The near infrared absorption of Cu^{2+} in cytochrome c oxidase. We assign the near infrared bands of cytochrome *c* oxidase to oxidized copper. It is generally agreed that the near infrared absorption bands are largely due to copper [1,6–8]. We may now add two more pieces of evidence. The first is the lack of any absorption band change on photolysis of CO bound to cytochrome a_3, and the second is the featureless spectrum in the infrared region of reduced oxidase under conditions where the electronic transitions elsewhere (e.g. the α, β, and γ peaks) are maximal. Also there are a variety of copper proteins that absorb prominently in the 740—840 nm region when oxidized, and negligible when

reduced [22]. Thus the extinction coefficients of the heme transitions in the infrared region appear to be a small fraction of the observed 1 $cm^{-1} \cdot mM^{-1}$, and account for 0.1 $cm^{-1} \cdot mM^{-1}$ or less. Therefore, to a first approximation we may use the infrared absorption as an indicator of the oxidation state of cytochrome oxidase copper.

The current assignment of the 830 nm absorption band to Cu_a is based largely upon the correlation of the midpoint potentials measured in redox titration (+240 mV) [23] with the EPR in the region of $g = 2$ and the optical data at 840–1000 nm. In fact, in the titration of cytochrome oxidase with reductant either in the presence or absence of CO, Wever et al. [6] find that the heme *a* and copper of cytochrome a_3 are completely reduced by two electron equivalents/cytochrome oxidase molecule. At the same time about 40% of the absorption at 830 nm attributable to copper disappears. Furthermore, at this point in the titration and beyond, the addition of CO causes a redistribution of electrons in favor of reduction of $a_3 \cdot Cu_{a_3}$ concomitant with the oxidation of $a \cdot Cu_a$. At the same time about 15% of the 830 nm band disappears, in contrast to the expectation based upon the 100% assignment of the near infrared absorption band exclusively to Cu_a and in support of our assignment of considerable absorption to Cu_{a_3}.

This leads us to the useful conclusion that when there is no infrared absorption (i.e. the base-line is flat in the reduced spectra of Fig. 1) all the copper in cytochrome *c* oxidase is reduced. When no further increase in the infrared absorption band can be obtained by the addition of oxidizing agents, such as ferricyanide, the copper can be considered to be oxidized. Similarly, the absorption at 605 nm ascribed to heme *a* is maximal when the protein is in the reduced state and minimal (but non-zero) in the oxidized state.

The procedure we have followed involves the measurement of the identical samples used in the edge absorption study in a dual beam (split beam) spectrophotometer [21] adapted for the same low temperatures and high concentrations as required by the edge spectrometer. Following these procedures, we obtained the four redox states employed for the edge spectrometry.

The oxidized state. We have employed frozen samples of cytochrome *c* oxidase and have measured the infrared absorption of the protein before and after irradiation. We have also ensured the oxidation of the oxidase by the addition of ferricyanide in two-fold excess of any possible endogenous reductant (400 μM for 1 mM oxidase). Thus, our assay procedure for the elimination of possible reduced components in the oxidase preparation, depends upon an experiment with an initially aerated oxidase preparation, freeze-trapping in that state, optical and edge absorption evaluation, thawing aerobically, addition of ferricyanide and rerunning of optical spectra. We found that there is no further oxidation of the protein observed as evaluated above. This procedure ensures that no components not monitored optically are in the reduced state.

The reduced state. Similar precautions are necessary to ensure that the oxidase is in the reduced state, especially because of the possible heterogeneous nature of the purified protein. Enzymatic reduction in an atmosphere of CO should eliminate the infrared absorption bands and enhance the visible absorption at 605 nm. The possibility that this procedure fails to reduce all of the cytochrome a_3 can be evaluated from an assay based on photolysis of CO for

reduced cytochrome a_3. This assures that the ferrous state had been achieved. Inactive and slowly reduced components of the solubilized oxidase are unlikely to be present in preparations that are maintained frozen at −78°C. The heme components are assayed optically by the fraction of the reducible oxidase that binds CO. Unreduced copper components can be clearly delineated by their near infrared absorption spectra. Thus, we can identify preparations in either the fully oxidized or fully reduced states.

The mixed-valence states. This $a_3 \cdot Cu_{a_3}$ reduced mixed-valence state is prepared by the addition of 1—10 mM ferricyanide to the CO-ligated preparation at −25°C which converts cytochrome *a* to the oxidized state but leaves cytochrome a_3 reduced. The infrared absorption spectrum is now about half as intense as in the fully oxidized protein. We do not take this result to mean 100% conversion to the mixed-valence state since the extinction coefficients of Cu_{a_3} and Cu_a in the infrared region have not as yet been ascertained. However, we can conclude that our redox states are similar to those of Wever et al. [6] and that we have reached essentially the same redox state as they did but from the opposite direction of titration (see their Fig. 1A).

Stellacyanin. After X-irradiation the infrared band lost 30% in intensity after 30 min. The A_{604}/A_{280} ratio, which was 0.197 before irradiation, was determined to be 0.178 when measured about a month after the X-ray experiments.

X-ray absorption methods

Measurements. The absorption edges were recorded at the Stanford Synchrotron Radiation Laboratory using the broadband radiation from the SPEAR storage ring. The spectrometer, described by Kincaid [24], employs a 220 channel cut silicon crystal monochromator giving approx. 1 eV resolution at copper (8980 eV). Incident intensity, I_0, was measured using an ion chamber and fluorescence, F, was recorded with NaI scintillation counters. All data were collected at temperatures between −120°C and −80°C, depending on the oxidation state of the sample, in a specially designed temperature-controlled cell.

Two standards were used for energy calibration of the edge spectra. The first, an external standard, $CuCl_2 \cdot 2H_2O$, is a compound for which the energy of the rather narrow $1s \rightarrow 4p$ transition is known. The second, an internal standard, is a sharp intensity change (glitch) observed in I_0 and F (although not necessarily in the ratio of F/I_0) that is caused by another Bragg reflection of the crystal monochromator whose position in energy is known. From these, relative changes in energy are known to ±0.1 eV.

Data analysis. A straight line background was fitted to the data well below the absorption edge and subtracted from the edge data. This linear background is caused by Compton and elastic scattering in the dilute samples. The intensities of the edges were normalized to that of the completely oxidized state spectra since the dilution factors in the chemistry of producing the reduced and mixed-valence states were known. Thus it was possible to compare any set of data with any other as in the case of Figs. 6 and 7. The positions in energy of $1s \rightarrow 3d$ and $1s \rightarrow 4s$ (or 4s-4p mixture) transitions were determined by subtracting a smooth curve fitted to the respective $1s \rightarrow 4p$ transitions, giving the position to ±0.1 eV.

Results

Optical data

Oxidized stellacyanin (3 mM) was transferred to a 70 μl cuvette having a 1.2 mm pathlength and was frozen at −196°C. The infrared and absorption traces showed an absorbance of 0.6 at 840 nm (Fig. 2). The reduced stellacyanin showed no significant absorption in the infrared region. For cytochrome *c* oxidase, (1 mM) preparation V (sample I), the oxidized state shows an infrared absorption of A = 0.60 at 840 nm (Fig. 1c).

In the 'mixed-valence state + CO' sample III, the infrared absorption was 0.33 and when corrected for dilution the absorption was 0.47, 78% of that of sample I. Sample V had similar optical properties.

Reduced sample II exhibited in three tests near infrared absorption <10% of that observed for the oxidized state. Sample IV showed <15%.

Traces A, B, and C of Fig. 1 are near infrared absorption for oxidized cytochrome *c* oxidase (sample I, preparation V). Trace D, is that for the mixed-valence state (sample III) and E, that for the enzymatically reduced oxidase (sample III). The traces presented are those of the identical samples used in the EXAFS experiment without any subsequent treatment. The concentrations are, respectively, 1 mM for A—C, 0.73 mM for D, and 0.80 mM for E. The low temperature spectrum of copper of cytochrome oxidase has not been presented previously and shows features which resemble the room temperature spectra except that the region 680--750 nm has a broader maximum than in the room temperature study. The amplitudes of the spectra can be measured with respect to the flat base-line of the enzymatically reduced oxidase by taking 980 nm as a point of 'zero' absorption. On this basis, traces A—C can be considered to be identical to within experimental error (≤10%). Thus, the 30 min irradiation causes no change of the near infrared spectrum of the fully oxidized protein, suggesting that there was little damage to the copper sites. Evidence that cytochrome oxidase is oxidized is provided by the negligible change caused by the addition of 400 μM ferricyanide (the dilution factor for the ferricyanide addition is negligible). Reduction of cytochrome oxidase by cytochrome *c*-ascorbate-TMPD gives a flat base-line as indicated by trace E. At low temperatures the spectral difference between the oxidized and reduced states is even more evident than at room temperature.

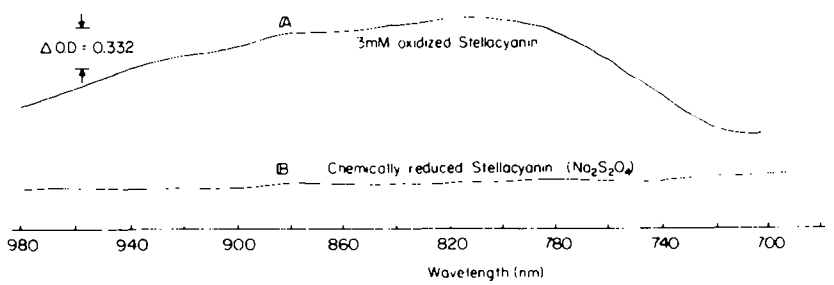

Fig. 2. Oxidized and reduced infrared absorption spectra for 3 mM stellacyanin.

Fig. 2 illustrates the near infrared spectrum of a typical type I copper compound, stellacyanin. In this case, trace A is the absorption spectrum of 3 mM oxidized stellacyanin and trace E that of dithionite-reduced stellacyanin.

The significance of the comparison of the infrared absorption spectra of stellacyanin and cytochrome oxidase is best elucidated by an examination of the difference between oxidized cytochrome oxidase and the carbon monoxide-bound mixed-valence state of the protein where the copper absorbs (Fig. 3, trace A). The difference is then a computed spectrum of the oxidized copper site associated with cytochrome a_3 (trace C). In trace B, the oxidized stellacyanin spectrum has been normalized to the same scale as trace C. Here again the greater breadth of the stellacyanin spectrum is noteworthy.

The low temperature enhancement factors [25] may be calculated from the room temperature and low temperature extinction coefficients, the concentration of copper, and the optical pathlength of 1.2 mm. We measured the factor for the stellacyanin, and it is 4-fold. Calculation for cytochrome oxidase is based upon enzyme molar concentration and involves contribution from both Cu_a and Cu_{a_3} to the 830 nm band. Thus the extinction coefficient calculated from the titration of Wever et al. [6] is 2.0 $cm^{-1} \cdot mM^{-1}$. The enhancement factor for the copper absorption at low temperature is calculated to be 2-fold. The factor of 2 difference in enhancement for the two copper proteins is attributable to the differing temperature dependence of Cu_a, the major contributor to the 830 nm band of cytochrome oxidase, and the type I copper of stellacyanin.

X-ray absorption edge spectroscopy

Blumberg et al. (Blumberg, W.E., Peisach, J. and Powers, L.S., manuscript in preparation) reports results of edge studies on a series of Cu(II) complexes and natural and artificial copper proteins for which EPR measurements have been made [2]. Fig. 4 is taken from their data and lists the observed transitions. Although the results of their studies are discussed elsewhere, it is important to note two important points. The first is that as covalency increases the 1s → 3d transition ceases to be observed. The second is that as covalency increases the 1s → 4s (or 4s-4p mixture) transition systematically moves to lower energy. In fact, when their data for Cu(II) compounds are compared with data for

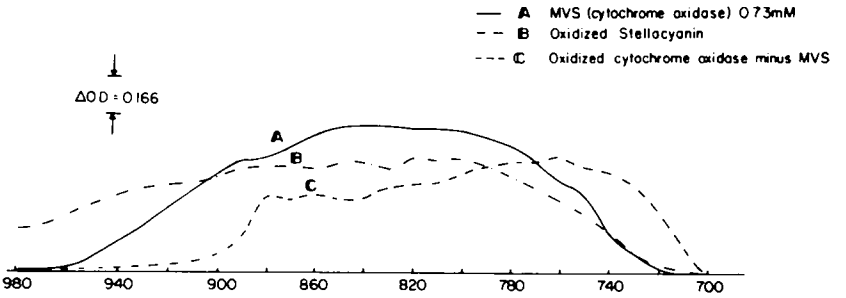

Fig. 3. Comparison of shapes of infrared absorption bands: (A) the infrared absorption of cytochrome oxidase in the mixed-valence state (as in Fig. 1); (B) oxidized stellacyanin (as in Fig. 2); (C) the difference between oxidized cytochrome oxidase and the mixed-valence state. The traces are normalized to identical maxima.

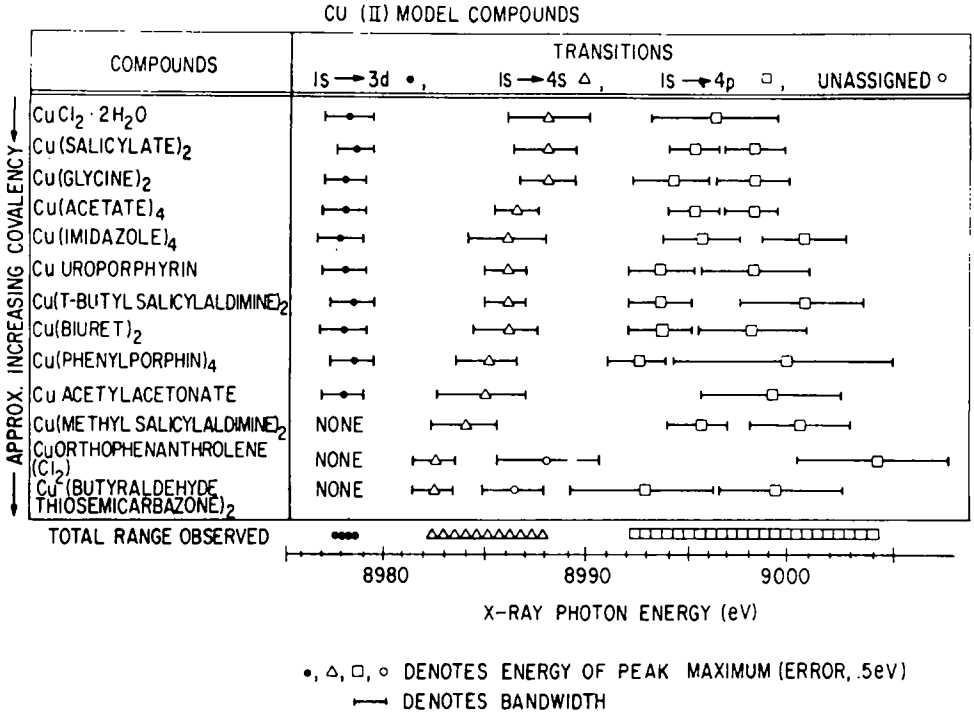

Fig. 4. Comparison of observed transitions for Cu(II) model compounds [12]. Note that the 1s → 4s transition may be 1s → 4s-4p mixture in some cases.

Cu(I) model compounds from their studies and those reported by Eccles [26] and Hu et al. [11] it is clear that the formal valence state (i.e. Cu(I) or Cu(II)) cannot be determined simply by the observation of the energy at which a single transition (e.g. 1s → 4s or 4s-4p mixture) occurs. As can be seen from Fig. 5, the energy ranges for this transitions overlap. Only when a 1s → 3d transition is unambiguously observed can a Cu(II) be differentiated from a Cu(I) by observation of a single transition. If no 1s → 3d transition is observed, the valence state cannot be deduced since this transition in covalent Cu(II) complexes is not found. Further, the regions in energy where ionic and covalent compounds of both Cu(I) and Cu(II) are observed are indicated in Fig. 5.

The absorption edges for the fully oxidized and enzymatically reduced + CO-ligated states of cytochrome *c* oxidase are similar to those observed by Hu et al. [11] (Fig. 6). However, the peak they reported at 8983.7 eV in the oxidized state and assigned to Cu(I) varied in our preparations (K, H, and V) from approx. 8% to approx. 20% of the peak at 8983.1 eV for the reduced state but was never observed to be as much as 50%. This peak is similar to that reported for oxidized plastocyanin (and the spectra in this region are comparable), and Hu et al. [11] assigned it to 'autoreducible protein'. It is difficult to reconcile that the peak at 8983.7 eV which we observe in the oxidized state is the same as the 8983.1 eV peak in the reduced state, as they postulated [11], since these peaks differ in energy by 0.6 eV and our relative error is ±0.1 eV. As

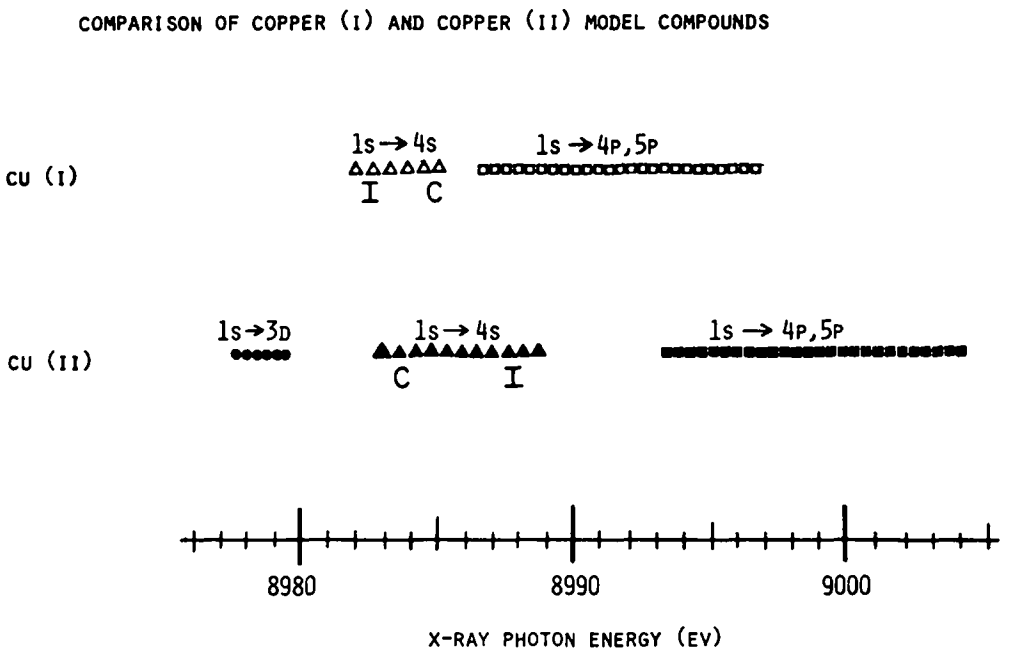

Fig. 5. Comparison of absorption energy ranges observed for transitions in Cu(I) [11,12,26] and Cu(I) [27] model compounds. I and C denote ionic and covalent, respectively.

stated above, the observations of the energy of a single transition (e.g 1s → 4s or 4s-4p mixture) does not support their conclusion [11] as these energies overlap across valence states (Fig. 5). Considering these arguments, the slight inflection we observe at 8983.7 eV in the oxidized state will be assigned to 'advantitious copper' which we believe is not catalytically functional.

The edge spectrum for the carbon monoxide-bound mixed-valence state of cytochrome *c* oxidase is also shown in Fig. 6. The lower energy portion is

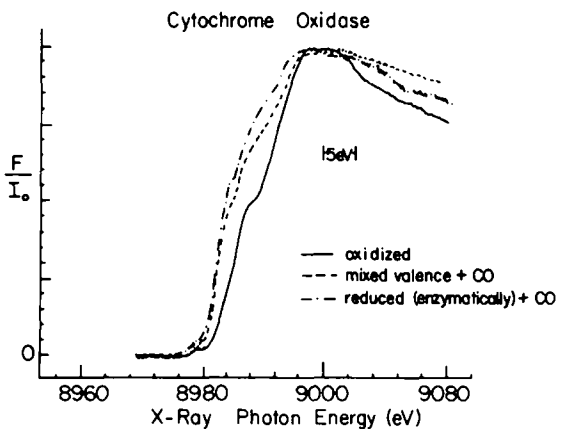

Fig. 6. X-ray absorption edges of cytochrome oxidase in the oxidized, mixed-valence + CO, and reduced + CO states.

similar to that of the reduced state +CO while the region of the 4p transition (approx. 9000 eV) is more like that of the oxidized state. In each state, the observed spectrum is a sum of the absorption edges of the different types of copper in the protein.

In order to gain insight into the structure and valence states of the individual copper atoms of cytochrome *c* oxidase, it is useful to compare edge spectra with those for various model compounds. One such model compound, stellacyanin, exhibits an edge spectrum which is almost identical to that of cytochrome oxidase (oxidized state, Fig. 7) in the higher energy portion (1s → 4p transition region). This 'blue' or type I copper protein contains a single copper atom that has properties similar to type I copper found in blue copper oxidases [1]. Fig. 7 shows the comparison between reduced stellacyanin and reduced cytochrome oxidase in the presence of CO. The lower energy portions of both are identical. As was observed in model compounds (Fig. 5) (Blumberg, W.E., Peisach, J. and Powers, L.S., manuscript in preparation), the ionic Cu(II) compounds (having transitions at higher energies) showed larger changes to lower energy upon reduction than did covalent Cu(II) compounds. Thus if cytochrome *c* oxidase contains such a copper atom, it would be the more ionic * of the two types of copper and upon reduction the lower energy portion (1s → 4s transition region) of the stellacyanin spectrum should then be identical to that of cytochrome oxidase. Indeed it is. Therefore, stellacyanin appears to be a good model for one of the copper atoms in cytochrome oxidase, i.e. the more ionic one. The EPR spectra of stellacyanin and Cu_a are quite different, and therefore we conclude that this more ionic copper may be the one associated with cytochrome a_3 (denoted Cu_{a3}) which is 'EPR non-detectable'.

Since the cytochrome *c* oxidase edge spectra are the sum of edges from both types of copper present, subtraction of the contribution of the copper we attribute to that associated with cytochrome a_3, for which stellacyanin is the model, (in one-half the intensity of the cytochrome oxidase edge) will leave the contribution of the copper associated with cytochrome *a*. Fig. 8 shows the results. These assumptions can be tested for consistency since the mixed-valence states of cytochrome oxidase were also measured. Fig. 9 shows the comparison of the experimentally determined mixed-valence, CO-ligated state and the result of adding the edge spectra of the Cu_a in the oxidized state to that for Cu_{a3} (stellacyanin) in the reduced state. The edge for the mixed-valence state with formate (identical to that with CN) compared to the result of adding the model edge for Cu_{a3} (stellacyanin) in the oxidized state to Cu_a in the reduced state is also shown. The agreement is rather good with the only variance being in the width in the higher energy (or 1s → 4p transition) region for the mixed-valence state + CO. This broadening might occur because the geometry of the mixed-valence state of cytochrome oxidase may be altered from that of the fully oxidized or reduced states. This agreement is slightly better when the added result is a composite based on 35% of the edge of oxidized Cu_a and 55% of the edge of reduced Cu_{a3} (the remaining 10% being

* The covalency for Cu^{2+} complexes varies from highly ionic for the fluoride complexes to most covalent for solid oxides and sulfides. Although the copper in stellacyanin is highly covalent when compared to water-soluble complexes, it is nearer to the ionic end than to the covalent end of the total scale. Thus, the term 'covalent' must be used relative to the specific compounds under comparison in a given study.

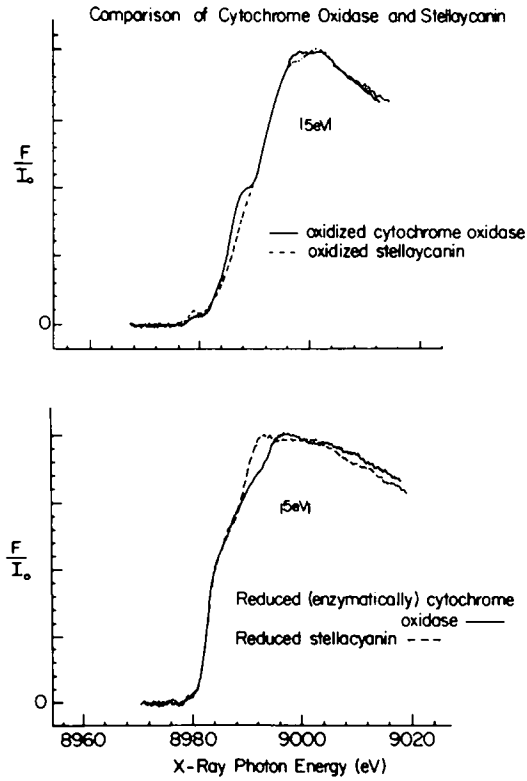

Fig. 7. Comparison of X-ray absorption edges in the oxidized and reduced states of cytochrome oxidase with those of stellacyanin.

that of 'adventitious copper'). Other combinations decrease this agreement significantly. In addition, it should be noted that the spectra of the mixed-valence states (Figs. 6 and 9) could not be reproduced by adding one-half of the spectral intensity for the fully oxidized protein to one-half the intensity

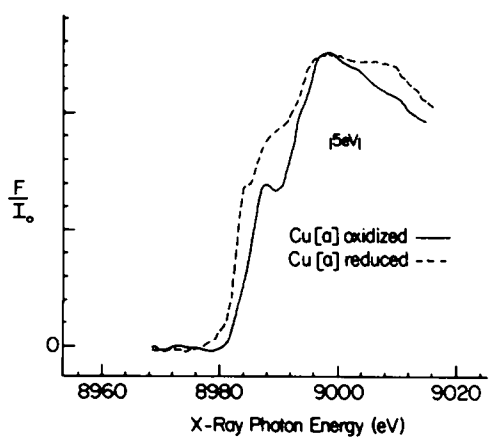

Fig. 8. Edge spectrum deduced for Cu_a in the oxidized and reduced states of cytochrome oxidase.

for the reduced protein. This is not surprising since the mixed-valence state is not merely an equal mixture of fully oxidized and fully reduced protein. These measurements constitute a structural proof, other than that based on optical spectral signatures, that the mixed-valence state is a unique compound and not a mixture. The energy difference upon reduction is greater for Cu_{a_3} than for Cu_a (Figs. 7 and 8).

Finally, we ask whether the edges deduced for Cu_a are reasonable or similar to those for other copper compounds (Blumberg, W.E., Peisach, J. and Powers, L.S., manuscript in preparation and Refs. 11 and 26). Fig. 10 shows a comparison of the edges deduced for Cu_a in the oxidized and reduced states with those for two model compounds, Cu(II)oxalate(imidazole)$_2$ and Cu(II)-(diethyldithiocarbamate)$_2$. The $1s \rightarrow 4s$, 4p transition intensity is larger for Cu_a oxidized than that for Cu(II)oxalate(imidazole)$_2$ indicating that the Cu_a geometry is more tetrahedral. Comparison with the edge for Cu(II)(diethyl-thiocarbamate)$_2$ shows that the two edge spectra are very similar. In the reduced state Cu_a and Cu(I)oxalate(imidazole)$_2$ are again very similar, but the edge spectrum of Cu(II)(butyraldehyde thiosemicarbazone)$_2$, containing 2 N- and 2 S- does not compare favorably with the edge shape of Cu_a although the transition energies are similar.

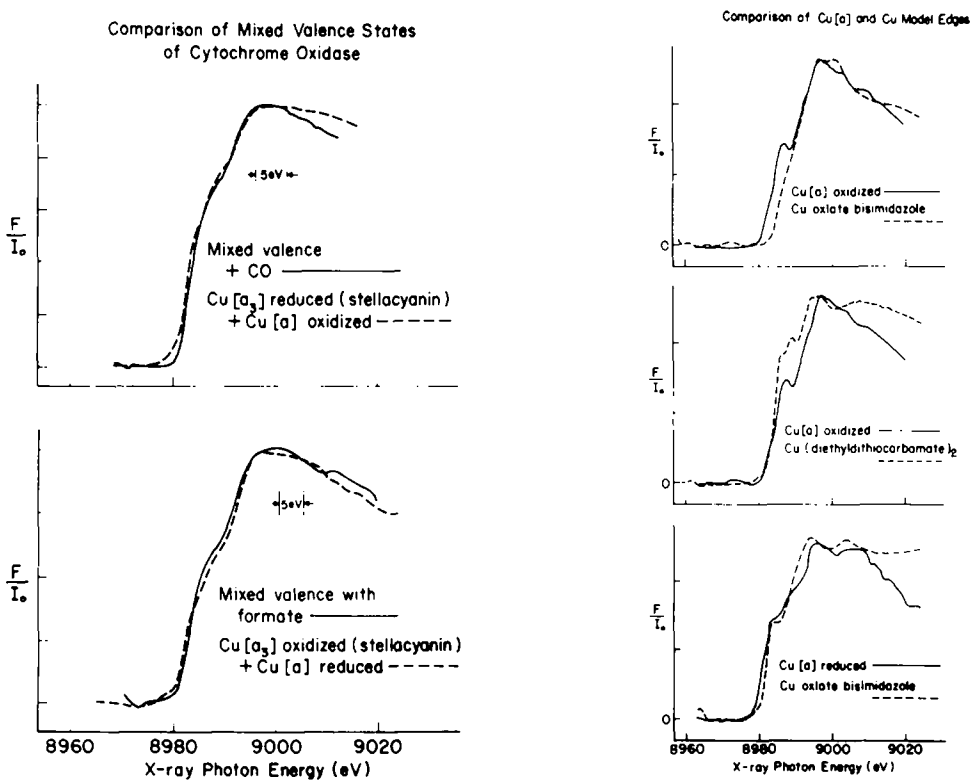

Fig. 9. Comparison of the X-ray absorption edge for mixed-valence states of cytochrome oxidase with the sums of the model edges for Cu_a and Cu_{a_3} in the respective redox states.

Fig. 10. Comparison of model edges for Cu_a in cytochrome oxidase with other Cu model compounds.

Discussion

Cytochrome *c* oxidase when isolated is usually in the oxidized state. It does, however, contain endogenous reducing substances equivalent to 0.3—0.5 electron/molecule [6,28]. Since it is well recognized that a portion of the preparation can be autoreducible, we have employed special means to monitor this possibility. The highly concentrated cytochrome oxidase solutions, standing for a number of hours at room temperature, may indeed be subject to such autoreduction processes, and reduction of the copper might occur. Thus, it is difficult to accept statements relevant to the oxidation state of copper by Hu et al. [11] concerning the resting state of the enzyme [6,9,29] where the autoreduction has not been taken into consideration. We find it difficult to accept that the optical spectra measured on diluted oxidase verify the state of reduction of the concentrated oxidase since transfer of the protein in an atmosphere of gaseous nitrogen (in which oxygen is usually present) is an ineffective procedure.

X-ray absorption edge spectroscopy

In the previous sections, methods and techniques were presented that were used to ensure purity and the desired oxidation state of our preparations. The results of the optical and X-ray edge absorption studies suggest chemical models for the copper atoms.

The Cu_{a_3} in cytochrome *c* oxidase is assigned as type I or 'blue' copper. This assignment was made even though no EPR spectrum for this copper atom is observed since it is spin coupled to the iron in cytochrome a_3. The classification of Cu_{a_3} as type I copper is suggested from the edge studies of our model compound, stellacyanin, which has a similar electronic environment, although not necessarily having the same ligating groups. It should also be noted that edge spectra for plastocyanin [11], another protein containing type I copper, are almost identical to those observed for stellacyanin. The width of the $1s \rightarrow 4p$ transition region, however, is slightly broader, possibly due to the fact that plastocyanin has an axial distortion from tetrahedral symmetry while the distortion in stellacyanin is more rhombohedral. The copper ligation is also different.

The optical absorption spectrum of cytochrome *c* oxidase in the oxidized state exhibits bands in the 800—780 nm region and near 600, 440, and 340 nm at room temperature. Although heme *a* has strong absorptions near 600 and 440 nm the extinction coefficient at 800—780 nm is only approx. 2.0 $cm^{-1} \cdot mM^{-1}$ and is comparable to those reported for type I copper containing proteins in this region. The oxidation-reduction potential of the 840 nm band of Cu_a is 0.24 V [23], while that of cytochrome a_3 is higher (approx. 0.395 V) [30]. Presumably the potential of Cu_{a_3} is near to that of heme *a* in cytochrome a_3 and thus nearer that of type I copper of laccase.

Thus, we may summarize the properties of type I 'blue' copper that are in common with the observed or deduced properties of Cu_{a_3}: (1) a high extinction coefficient in the infrared region; (2) a midpoint potential considerably more positive than that of Cu_a; (3) in the configuration of compound C [9] as discussed below, a strong absorption band at 609 nm. Additional information

suggests that this copper exists in a binuclear iron-copper configuration which has no EPR spectrum and in which the 340 nm absorption is attributed to a charge transfer band.

Cu_a has a more covalent environment than Cu_{a3}, since its edge shifts less upon reduction. The shift to lower energy by approx. 2—3 eV can be interpreted as either a formal change in valence from Cu(II) to Cu(I) or as a ligand change. The fact that the edge shape upon reduction (i.e. the intensity of the deduced transitions) is very similar and somewhat characteristic of Cu(I) model compounds [11,12,16] suggests a possible change in formal valence; however, further studies are required for verification. The fact that this copper has a more covalent environment is also suggested by the oxidation-reduction potential. This may well be an important consequence of its function as an electron 'reservoir' in the electron transfer mechanism.

Optical results

Other spectroscopic methods support the assignment made on the basis of the X-ray absorption edge data. Both Cu_a^{2+} and Cu_{a3}^{2+} absorb light in the near infrared region ($\epsilon \sim 1\ cm^{-1} \cdot mM^{-1}$). For example, in the configuration of compound C_2 the absorption maximum of Cu_{a3}^{2+} is at 740 nm while in the configuration of compound B_2 it is at 790 nm [9]. In compound C_2 a further absorption band can be observed at 609 nm, which can be assigned to a type I blue copper since the oscillator strengths are similar (Brill, A., personal communication) although the absorption band is much sharper than in models such as stellacyanin. In the oxidized or resting state there are two absorption bands that could be associated with oxidized copper, a strong 597 nm and a weak 340 nm band. While these absorption bands are consistent with those in laccase (and, with the exception of the 340 nm absorption band, with stellacyanin and plastocyanin [11] as well), their intensities are not quantitatively in agreement, and, most importantly, much of the 597 nm absorption and a part of the 340 nm absorption may be due to the oxidized heme *a* and Cu_{a3} [31]. It is not possible at present to resolve further the separate contributions of copper and iron to the optical spectra of the resting enzyme since there are difficulties in separating the subunits of cytochrome oxidase into fractions containing the heme and copper in their intact environments [32].

King [33] found an amino acid sequence in subunit 2 of cytochrome *c* oxidase which was somewhat homologous with the sequence for azurin, and Buse et al. [34] confirmed this and located bound copper in this subunit as well. Our results suggest that this may be Cu_{a3} and that another copper-binding site, that of cytochrome *a*, should be found in this or another subunit. This binding site, however, might not be homologous to any known blue copper protein as it is much more covalent in nature.

Interactions of iron and copper occur which, although they may be at too long a range to alter the nature of the X-ray absorption edge, may be pivotal in their effects upon the EPR and optical transitions. A type I Cu^{2+} (typically $\epsilon_{830} \sim 1\ cm^{-1} \cdot mM^{-1}$) usually exhibits a much stronger absorption band ($\epsilon_{600} \sim 5\ cm^{-1} \cdot mM^{-1}$) [1,22], but this is not recognized as such in the oxidized or resting cytochrome oxidase. This difficulty may be attributed to interactions in the above-mentioned binuclear complex in which an $a_3^{3+} \cdot Cu_{a3}^{2+}$ interaction

shifts the 610 nm band to shorter wavelength and merges it with the α bands of the two heme components of the cytochrome oxidase.

Interestingly enough, a small absorption band is observed in cytochrome *c* oxidase which is reminiscent of the band which is characteristic of binuclear Cu(II) complexes in copper oxidases which exhibit no EPR signal. Dimeric copper (II)acetate also has an absorption in this region (at 330 nm) which is attributed to a charge transfer transition [35]. The 340 nm absorption in oxidized cytochrome *c* oxidase could be due to the interaction of oxidized heme and copper [36]. The high midpotential characteristic of type III copper may be reached by Cu_{a3}.

As has been recently reviewed by Peisach [37], there are two distinctly different functions for the two portions of cytochrome oxidase. Cytochrome a_3 participates in the binding of oxygen to iron, the formation of highly reactive intermediates with tightly bound oxygen, and the formation of reduction products by means of short-range electron transfer reactions between the metal atoms and oxygen. The function of cytochrome *a* is a complementary one: no binding of oxygen is necessary, no intermediate compounds are formed. Instead these components provide an electron donor pool for maintaining a minimal concentration of reactive intermediates of oxidized cytochrome a_3 and a maximal concentration of the species directly reactive with oxygen, reduced cytochrome a_3 . In the oxidase functioning under physiological conditions, the electron donor pool also includes cytochromes *c*, c_1, and Rieske's iron-sulfur protein.

In conclusion, the problem of the identity of the copper atoms of cytochrome oxidase and of some model compounds is considerably advanced by the edge absorption studies, but further studies over a wider range of models seem necessary to explain the complex nature of the Fe-Cu interactions in this remarkable enzyme.

Acknowledgement

The portion of this research carried out at SSRL was approved under Project 105. The portion of this investigation carried out at the University of Pennsylvania was supported in part by grants from NIH HL 17826 and NIGMS 12202. The portion of this investigation carried out at Albert Einstein College of Medicine was supported in part by U.S. Public Health Service Grant HL-13399 from the Heart and Lung Institute. This is Communication No. 393 from the Joan and Lester Avnet Institute of Molecular Biology. Many thanks are due for preparations received from R. Capaldi, T.E. King, and J. Harmon.

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