Infrared Evidence of Cyanide Binding to Iron and Copper Sites in Bovine Heart Cytochrome c Oxidase

IMPLICATIONS REGARDING OXYGEN REDUCTION*

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Cyanide binding to bovine heart cytochrome c oxidase at five redox levels has been investigated by use of infrared and visible-Soret spectra. A C–N stretch band permits identification of the metal ion to which the CN⁻ ion is bound and the oxidation state of the metal. Non-intrinsic Cu, if present, is detected as a cyanide complex. Bands can be assigned to CuCN at 2093 cm⁻¹, Cu²⁺CN at 2151 or 2185 cm⁻¹, Fe³⁺CN at 2131 cm⁻¹, and Fe⁴⁺CN at 2058 cm⁻¹. FeCN is found only when the enzyme is fully reduced whereas the reduced CuCN occurs in 2-, 3-, and 4-electron reduced species. A band for Fe⁴⁺CN is not found for the complex of fully oxidized enzyme but is for all partially reduced species. Cu⁺CN occurs in both fully oxidized and 1-electron-reduced oxidase. CO displaces the CN⁻ at Fe³⁺ to give a C–O band at 1963.5 cm⁻¹ but does not displace the CN⁻ at Cu⁺. Another metal site, noted by a band at 2042 cm⁻¹, is accessible only in fully reduced enzyme and may represent Zn²⁺ or another Cu⁺. Binding of either CN⁻ or CO may induce electron redistribution among metal centers. The extraordinary narrowness of ligand infrared bands indicates very little mobility of the components that line the O₂ reduction site, a property of potential advantage for enzyme catalysis. The infrared evidence that CN⁻ can bind to both Fe and Cu supports the possibility of an O₂ reduction mechanism in which an intermediate with a cc-peroxo bridge between Fe and Cu is formed. On the other hand, the apparent independence of Fe and Cu ligand-binding sites makes a heme hydroperoxide (Fe–O–O–H) intermediate an attractive alternative to the formation of an Fe–O–O–Cu linkage.

More than 90% of the oxygen consumed by an animal involves cytochrome c oxidase (CcO),¹ a complex protein of the inner mitochondrial membrane (1). This enzyme catalyzes Reaction 1, a key reaction in aerobic energy production, and functions as a proton pump coupled to electron transfers from O₂ + 4 cytochrome c²⁺ + 4H⁺ → 2H₂O + 4 cytochrome c³⁺ (1) cytochrome e to O₂ (2, 3). The dioxygen reduction site structure appears widely conserved in animals, plants, and microorganisms (4) and contains a unique iron porphyrin prosthetic group, heme A (5). The enzyme transfers four electrons to bound O₂ obtained at a time from cytochrome c²⁺; four O–H bonds are formed with high efficiency without release of incompletely reduced O₂ species such as O₂, H₂O₂, or HO·. How the structure at the O₂ reduction site enables CcO to catalyze the conversion of O₂ to water so effectively remains poorly understood.

The enzyme obtained from bovine heart is the most extensively studied CcO preparation. The heart enzyme, as a monomer, is a complex of 13 polypeptide subunits (6, 7), phospholipids, two heme A chromophores (a and aa), coppers, zinc, and magnesium (3, 5, 8–10). The importance of the two hemes and two copper atoms in electron transfers from cytochrome c²⁺ to O₂ is well recognized but roles for the other metals remain unclear. The stoichiometries of the metals as well as x-ray diffraction data for crystals of our CcO preparation support the existence of CcO as a dimer containing 5 Cu, 4 Fe, 2 Zn, and 2 Mg in the native state (9–11). However, the minimal unit for reduction of O₂ by cytochrome c²⁺ is apparently a monomer containing only two hemes (8, 12). Elucidation of the structure of the O₂ reduction site and the chemical steps by which O₂ is reduced to water has been limited by difficulties associated with purification and crystallization of such a complex membrane protein (13–15) and by a rate of O₂ reduction too high to permit readily detectable amounts of intermediate species under physiologically relevant turnover conditions (16, 17).

Infrared spectroscopy with infrared active ligands provides a direct means of exploring the O₂ reduction site (18). Carbon monoxide, long known as a potent respiratory inhibitor that binds to and inhibits CcO (8), exhibits extremely narrow C–O stretch bands at 1964 and 1959 cm⁻¹ when bound to the iron of reduced heme a₁(19). The insensitivity of these bands to changes in pH, temperature, external medium, and the oxidation states of metals other than heme a₁ ion indicates an unusually immobile O₂ reduction site well isolated from the external medium (20). Based on these findings a novel mechanism was proposed for O₂ reduction within a reaction pocket which is closed to reactants from the outside and experiences little change in conformation during the hydrogen and electron transfer steps (20, 21).

Cyanide is another infrared active ligand that binds to CcO and is a potent inhibitor of respiration. We recently demon-
Cyanide Binding to Fe and Cu Sites in Cytochrome c Oxidase

Cyanide was found to bind to iron and copper sites in cytochrome c oxidase. The authors used infrared spectra to study the binding of cyanide to the enzyme. They used three different isotopes of carbon (12C, 13C, and 14N) and observed changes in the infrared spectra. The cyanide bands near 2093 cm\(^{-1}\) were found to be more pronounced in deuterium oxide (D\(_2\)O) than in water (H\(_2\)O), which suggests that the spectral changes are due to hydrogen bonding.

The authors confirmed that the cyanide bands near 2093 cm\(^{-1}\) are due to cyanide bound to an intrinsic metal center by analyzing the infrared spectra of samples in D\(_2\)O and H\(_2\)O. The signal-to-noise ratio was significantly higher in D\(_2\)O, which is consistent with the idea that the hydrogen bonding is responsible for the spectral changes.

The authors also investigated the effect of metal cyanide bands near 2090 cm\(^{-1}\) on the infrared spectra. They found that these bands are more readily examined in D\(_2\)O than in H\(_2\)O, which suggests that the spectral changes are due to hydrogen bonding.

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Cyanide Binding to Fe and Cu Sites in Cytochrome c Oxidase

**Table I**

Visible-Soret spectral data for unliganded and cyanide species of cytochrome c oxidase at five redox levels

<table>
<thead>
<tr>
<th>Protein 0.9 - 2.2 mM in the absence and presence of cyanide (10 - 45 mM) in 10 mM sodium phosphate buffer, pH 7.4, at 20 °C.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Redox level</td>
</tr>
<tr>
<td>IV</td>
</tr>
<tr>
<td>III</td>
</tr>
<tr>
<td>II</td>
</tr>
<tr>
<td>I</td>
</tr>
<tr>
<td>0</td>
</tr>
</tbody>
</table>

*These values are somewhat variable.

**Figure 2.** Infrared spectra of fully oxidized cytochrome c oxidase liganded with \(^{12}\text{C}^{14}\text{N}\), \(^{13}\text{C}^{14}\text{N}\), and \(^{15}\text{N}^{13}\text{N}\). The oxidase was incubated with each cyanide isotope for 20 h in 10 mM sodium phosphate buffer, pH 7.4, at 4 °C. Top, 0.9 mM oxidase and 30 mM total cyanide as \(^{12}\text{C}^{14}\text{N}\). Center, 1.46 mM oxidase and 30 mM total cyanide as \(^{13}\text{C}^{14}\text{N}\). Bottom, 1.46 mM oxidase and 30 mM total cyanide as \(^{15}\text{N}^{13}\text{N}\). The top spectrum represents an average of nine accumulated single scans. The center and bottom spectra are each the average of 20 accumulated single scans. The reference cell for each measurement contained water.

Metal. Enzyme preparations that do contain Cu could show greater intensity in this region of the spectrum than is shown in Fig. 7.

Exposure of the solution of CcO(O)CN to CO results in a visible-Soret spectrum characteristic of CcO(O)CO (Fig. 4). There is also a loss of the 2058 cm\(^{-1}\) band in the infrared and the appearance of a new band at 2063.5 cm\(^{-1}\) (Fig. 8), a band characteristic of the C-O stretch band of CO bound to Fe\(^{3+}\).

**Table II**

Infrared parameters for CN stretch bands of cyano cytochrome c oxidase at five redox levels in D\(_2\)O buffer

<table>
<thead>
<tr>
<th>Redox level</th>
<th>(\nu) (cm(^{-1})) (\pm 1.0)</th>
<th>(\Delta\nu) (cm(^{-1})) (\pm 2.0)</th>
<th>(c) (mm(^{-1}) cm(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>IV</td>
<td>2151</td>
<td>9</td>
<td>0.13</td>
</tr>
<tr>
<td>III</td>
<td>2165</td>
<td>14</td>
<td>0.11*</td>
</tr>
<tr>
<td></td>
<td>2151</td>
<td>9</td>
<td>0.10</td>
</tr>
<tr>
<td></td>
<td>2101</td>
<td>9</td>
<td>0.14</td>
</tr>
<tr>
<td></td>
<td>2090</td>
<td>11</td>
<td>0.06*</td>
</tr>
<tr>
<td>II</td>
<td>2131</td>
<td>8</td>
<td>0.16</td>
</tr>
<tr>
<td></td>
<td>2093</td>
<td>12</td>
<td>0.15</td>
</tr>
<tr>
<td>I</td>
<td>2131</td>
<td>10</td>
<td>0.18</td>
</tr>
<tr>
<td></td>
<td>2093</td>
<td>10</td>
<td>0.16</td>
</tr>
<tr>
<td>0</td>
<td>2092</td>
<td>9</td>
<td>0.15</td>
</tr>
<tr>
<td></td>
<td>2058</td>
<td>9</td>
<td>0.22</td>
</tr>
<tr>
<td></td>
<td>2042</td>
<td>18</td>
<td>0.29*</td>
</tr>
</tbody>
</table>

*These values are somewhat variable.

**Table III**

Effects of \(^{13}\text{C}\) and \(^{15}\text{N}\) on C-N stretch frequencies for cytochrome c oxidase cyanides at five redox levels

<table>
<thead>
<tr>
<th>Redox level</th>
<th>(\nu\text{C}^{12}\text{CN})</th>
<th>(\nu\text{C}^{13}\text{CN})</th>
<th>(\nu\text{C}^{12}\text{CN})</th>
<th>(\nu\text{C}^{15}\text{CN})</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>2092</td>
<td>2094</td>
<td>43</td>
<td>44</td>
</tr>
<tr>
<td></td>
<td>2058</td>
<td>2015</td>
<td>43</td>
<td>43</td>
</tr>
<tr>
<td></td>
<td>2042</td>
<td>1998</td>
<td>44</td>
<td>44</td>
</tr>
<tr>
<td>I*</td>
<td>2131</td>
<td>2086</td>
<td>45</td>
<td>44.5</td>
</tr>
<tr>
<td>II*</td>
<td>2131</td>
<td>2086</td>
<td>45</td>
<td>44.5</td>
</tr>
<tr>
<td>III*</td>
<td>2165</td>
<td>2120</td>
<td>45</td>
<td>45</td>
</tr>
<tr>
<td></td>
<td>2150</td>
<td>2105</td>
<td>45</td>
<td>45</td>
</tr>
<tr>
<td></td>
<td>2131</td>
<td>2084</td>
<td>46</td>
<td>44.5</td>
</tr>
<tr>
<td>IV</td>
<td>2191</td>
<td>2109</td>
<td>46</td>
<td>40</td>
</tr>
</tbody>
</table>

*Band hidden under strong H\(^{14}\text{C}^{12}\text{N}\) band.

*Isotopic shifts of the 2093 cm\(^{-1}\) band in these oxidation states have not been determined.

*Isotopic shift of the band was determined for the band in CcO(MV)CN.

ND, not determined.
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FIG. 3. Effects of EDTA on the infrared spectrum of fully oxidized cytochrome c oxidase cyanide in D$_2$O. CcO(IV) was incubated with 19 mM sodium cyanide at 4°C for 20 h in 30 mM potassium phosphate buffer, pH 7.4. A, 1.85 mM CcO(IV) washed three times with the buffer containing 50 mM EDTA. B, 1.67 mM CcO(IV) without EDTA treatment. Each spectrum represents an average of 25 single scans. The base line of each spectrum was slope-corrected.

(19). This evidence for the replacement of CN$^-$ at heme iron by CO demonstrates that the 2058 cm$^{-1}$ band is due to heme bound $^{13}$C$^{15}$N$^-$ (Fig. 5). The CO stretch band parameters are the same whether CN$^-$ is present or absent. Furthermore, the 2093 cm$^{-1}$ band is not affected by exposure of CcO(0)CN to CO. The intensity of the 2042 cm$^{-1}$ band for $^{13}$C$^{15}$N$^-$ also remains the same with CO present. The 2042 cm$^{-1}$ band appears only when the enzyme is fully reduced, varies in intensity with different preparations of enzyme, and is not removed by EDTA treatment. The frequency and width of this band are comparable to those found for ferrocyanide (22), but, since the enzyme contains very low levels of non-heme iron, the 2042 cm$^{-1}$ band appears due to CN$^-$ bound to an intrinsic metal which is accessible to cyanide only when the enzyme is fully reduced.

Visible-Soret and CN$^-$ Infrared Spectra for Cyanide Complexes of Partially Reduced Species of Cytochrome c Oxidase—The oxidation states of individual metals within protein at overall oxidation states (IV) and (0) may be represented as Fe$_2$Cu$_2$Fe$_2$Cu$_2$ and Fe$_2$Cu$_2$Fe$_2$Cu$_2$, respectively. Fe$_2$, and Cu$_2$ represent ligand-binding metals at the O$_2$ reduction site and Fe$_2$ and Cu$_2$, the two other metals nearer to the site of electron transfer from cytochrome c (19). However, at the intermediate overall redox levels (III), (II), and (I) the electron distribution among the four metal centers is not uniquely defined, e.g. in level (III) any one of the four metals may be reduced with the other three oxidized.

Treatment of levels (I), (II), and (III) for 2.2 mM CcO in anaerobic 10 mM sodium phosphate buffer, pH 7.4, obtained by the NADPH/PMS reduction system described earlier (19), with neutralized KCN solution to give 10.7 mM cyanide results in rapid changes in visible-Soret spectra (Fig. 9). The infrared spectrum of CcO(III)CN in H$_2$O reveals two bands at 2151 and 2131 cm$^{-1}$ (Fig. 10, Table II) which isotopic shifts confirm are due to metal cyanides (Fig. 10, Table III). A band is also found at 2131 cm$^{-1}$ for both CcO(II)CN and CcO(0)CN. Bands found near 2058 and 2042 cm$^{-1}$ with CcO(0)CN were not detected in levels (I), (II), and (III). Each spectrum of Fig. 10 represents an average of 16 accumulated single scans and was not processed with smoothing routines. The reader is in a better position to evaluate signal-to-noise problems if highly smoothed spectra are not presented. Any uncertainty arising from a high noise level in any one averaged spectrum for a given experiment is assuaged by the fact that similar weak bands are always seen in repeated experiments and by observation of the shifts expected on isotopic substitution with $^{13}$C and $^{15}$N (Table III). Furthermore, the bands are so very narrow that it is possible to determine $\nu_{CN}$ values within ±1 cm$^{-1}$ despite the low signal-to-noise ratio.

Substitution of D$_2$O for H$_2$O in the medium improved the signal-to-noise ratio and permitted a more effective examination of the region near 2090 cm$^{-1}$. The typical results of Fig. 11, which include the spectrum of CcO(IV)CN, are all obtained with an enzyme preparation which contains no measured Cu$^+$. The fully oxidized enzyme cyanide spectrum has only a band at 2151 cm$^{-1}$ (Fig. 11A). Spectral changes that occur as the degree of reduction increases are shown in Fig. 11. With one-electron reduction, level(III), the spectrum develops bands at 2165, 2151, 2131, and 2093 cm$^{-1}$ (Fig. 11B). By level (II) (Fig. 11C), the bands at 2165 and 2151 cm$^{-1}$ have disappeared, the band at 2131 cm$^{-1}$ is slightly intensified, and the band at 2093 cm$^{-1}$ is greatly intensified. The addition of
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Fig. 5. Infrared spectra of a solution of fully reduced cytochrome c oxidase cyanide with and without exposure to carbon monoxide. The spectra were measured in the same infrared cell used for the visible-Soret measurements of Fig. 4. Upper panel, no CO. Lower panel, CO saturated. The reference cell contained water.

Fig. 6. Effects of isotopic substitution on the infrared spectra of cyanide bound to fully reduced cytochrome c oxidase. The solution contains 9.98 mM oxidase (O) in 10 mM sodium phosphate buffer, pH 7.4. Upper panel, 30 mM total cyanide as \(^{15}\)CN. Lower panel, 26 mM total cyanide as \(^{13}\)CN. The reference cell contained water. Each spectrum represents the average of 10 accumulated single scans.

Fig. 7. Infrared spectrum of fully reduced cytochrome c oxidase cyanide in D\(_2\)O solution. The solution contains 1.85 mM oxidase (O) and 19.4 mM cyanide in 46 mM potassium phosphate buffer, pH 7.4, in D\(_2\)O. This enzyme preparation was washed with EDTA, and the infrared spectrum of CcO(IV)CN showed no contaminant copper, CuCN. The spectrum represents the average of 25 accumulated single scans. The band near 2050 cm\(^{-1}\) can be deconvoluted into two Gaussian bands. The band parameters of the deconvoluted bands are as follows: \(v = 2058\) cm\(^{-1}\), \(\Delta v = 9\) cm\(^{-1}\), and \(c = 0.22\) mM\(^{-1}\) cm\(^{-1}\); \(v = 2042\) cm\(^{-1}\), \(\Delta v = 18\) cm\(^{-1}\), and \(c = 0.29\) mM\(^{-1}\) cm\(^{-1}\). The reference cell contained the enzyme at the same concentration as in the sample cell but no cyanide.

Fig. 8. Infrared spectrum of fully reduced cytochrome c oxidase cyanide in the C-O stretch region after exposure to carbon monoxide. The enzyme and cyanide concentrations are 2.6 and 19.2 mM, respectively. The reference cell contained the same solution without cyanide. The spectrum is the average of six single scans. The C-O stretch band can be deconvoluted into four Gaussian bands. The band parameters of these bands are as follows: \(v = 1968.5\) cm\(^{-1}\), \(\Delta v = 3.5\) cm\(^{-1}\), \(c = 0.14\) mM\(^{-1}\) cm\(^{-1}\); \(v = 1963.3\) cm\(^{-1}\), \(\Delta v = 4.3\) cm\(^{-1}\), \(c = 4.58\) mM\(^{-1}\) cm\(^{-1}\); \(v = 1960.0\) cm\(^{-1}\), \(\Delta v = 3.0\) cm\(^{-1}\), \(c = 0.72\) mM\(^{-1}\) cm\(^{-1}\); \(v = 1955.0\) cm\(^{-1}\), \(\Delta v = 3.5\) cm\(^{-1}\), \(c = 0.20\) mM\(^{-1}\) cm\(^{-1}\). This lower tracing represents the observed spectrum minus the sum of the four bands from deconvolution.

A third one-electron equivalent to give CcO(I)CN has little effect on the infrared spectrum (Fig. 11D) compared with CcO(II)CN. When only one-half an electron equivalent is added to CcO(IV)CN, the spectrum (not shown) contains no band at 2093 cm\(^{-1}\), a band at 2131 cm\(^{-1}\) of about one-half the intensity of the 2131 cm\(^{-1}\) band in the CcO(III)CN spectrum, a 2151 cm\(^{-1}\) band nearly as strong as in CcO(III)CN, and a much weaker 2165 cm\(^{-1}\) band. Upon exposure of CcO(IV) to cyanide for long periods, e.g. 40 h, the 2165 cm\(^{-1}\) band sometimes developed concomitantly with a weak band at 2131 cm\(^{-1}\), phenomena which can be ascribed to partial reduction upon standing. These results suggest that the fully oxidized...
enzyme only binds cyanide at the 2151 cm⁻¹ center, CcO(III) binds cyanide at the 2131 and 2165 cm⁻¹ centers, as well as at the 2151 cm⁻¹ center, while CcO(I) and (II) have only two bands at 2131 and 2093 cm⁻¹ (Tables II and IV). The weak band at 2093 cm⁻¹ at overall level (III) indicates the presence of CcO(II)CN as a minor component and the less than maximal intensity of the 2131 cm⁻¹ band (75% of the maximum) suggests that a small amount of unred CcO(IV)CN as a second minor component at overall level (III). The development of the 2165 cm⁻¹ band accompanies intensification at 2131 cm⁻¹ upon reduction of the (IV) level. A reciprocal intensity relationship between the 2093 and 2151 cm⁻¹ bands is evident in Fig. 11, A–C. Also, the 2165 cm⁻¹ band was absent when the intensity of the 2093 cm⁻¹ band was maximal. No minor component is detected at either (I) or (II) overall level.

Partial reductions were also carried out under aerobic conditions in solutions of CcO(IV)CN by treatment with ascorbate. When a solution of 1.85 mM CcO(IV) and 23 mM cyanide in 43 mM potassium phosphate D₂O buffer, pH 7.4, open to the air was made 4.3 mM in ascorbate and closed to the atmosphere, visible-Soret spectral changes were complete in 40 min. (Fig. 12). The final visible-Soret spectra varied from a spectrum analogous to CcO(II)CN as shown in Fig. 12B to a spectrum similar to the CcO(III)CN spectrum of Fig. 9. The product obtained is denoted as CcO(MV)CN where MV represents "mixed valence." The infrared spectrum has bands with ¹⁵C¹N at 2155, 2132, and 2093 cm⁻¹ (Fig. 12A) similar to an infrared spectrum that is similar to the level (III) spectrum of Fig. 11B. Thus, ascorbate may reduce the heme iron to a greater extent than the copper component compared with the NADH/PMS reduction system.

Infrared parameters obtained at each redox level in D₂O are given in Table II. The 2151 and 2151 cm⁻¹ bands are narrower and the ε values higher in fact than the values given in Table II due to the lower than optimal resolution used (3–3.5 cm⁻¹ at 2150 cm⁻¹) to improve the signal-to-noise ratio. The resolution was high enough for measurements of Δνₐ for the 2093, 2088, and 2042 cm⁻¹ bands of CcO(IV)CN in Fig. 9 in contrast to an infrared spectrum that is similar to the level (III) spectrum of Fig. 11B. The spectral variations probably result from different amounts of residual ascorbate present following reduction of variable amounts of O₂. The visible-Soret spectrum of CcO(MV)CN CN in Fig. 12 is nearly the same as that of CcO(ICN) in Fig. 9 in contrast to an infrared spectrum that is similar to the level (III) spectrum of Fig. 11B. Thus, ascorbate may reduce the heme iron to a greater extent than the copper component compared with the NADH/PMS reduction system.

Infrared parameters obtained at each redox level in D₂O are given in Table II. The 2151 and 2151 cm⁻¹ bands are narrower and the ε values higher in fact than the values given in Table II due to the lower than optimal resolution used (3–3.5 cm⁻¹ at 2150 cm⁻¹) to improve the signal-to-noise ratio. The resolution was high enough for measurements of Δνₐ for the 2093, 2088, and 2042 cm⁻¹ bands because of the low background absorption due to D₂O in the lower wavenumber region. A measurement at a resolution of 2 cm⁻¹ at 2150 cm⁻¹ gave a Δνₐ value of 5 cm⁻¹ for the 2151 cm⁻¹ band.

**DISCUSSION**

**Preparation of Cyanide Complexes of Cytochrome c Oxidase at Different Redox Levels**—The method of CcO isolation from bovine heart developed in this laboratory consistently and rapidly provides good yields of enzyme in the fully oxidized (Iₐₐ) form with high purity and activity (21). Furthermore, this preparation remains soluble at the high concentrations required for the measurement of weak infrared bands. The visible-Soret spectra for CcO(IV)CN and CcO(II)CN of Table I and Figs. 1 and 4 agree closely with spectra reported by others (23–25, 27, 28); the spectra of the I, II, and III cyanides in Fig. 9 are similar to those reported by Johnson et al. (29). However, the plots of absorbance changes at 430 and 600 nm in Fig. 13 are distinctly non-linear whereas Johnson et al. reported linear plots. The fairly large increase in absorbance from 3e⁻ to 4e⁻/oxidase in Fig. 13 is due to reduction of Fe⁺CN to Fe⁺⁺CN.

Nonintrinsic copper (Cuₐₐ) exhibits a C–N band at 2093 cm⁻¹ and apparently at 2125 cm⁻¹ as well (Fig. 9). Exposure of the enzyme to extraneous copper resulted in retention of the metal ion, i.e. the oxidase acted as an effective chelator. Although usual protein fractionation procedures, including crystallization, do not effectively remove Cuₐₐ, the treatment of a Cuₐₐ containing preparation with EDTA under the mild conditions described above proved effective in Cuₐₐ removal as demonstrated by the loss of Cuₐₐ-CN infrared bands. The unusually high Cu/Fe ratios given in earlier reports (30–32)
**Measurement of C-N Stretch Bands for Cyanide Bound to Cytochrome c Oxidase**—The bands of Tables II and III were established by the reproducibility of their observation and by the expected isotopic shifts. Bands due to HCN can be differentiated from bands due to metal-bound CN by use of either $^{15}$C$^{14}$N$^-$ or D$_2$O. The $^{12}$C/$^{13}$C isotope shift is about 10 cm$^{-1}$ greater for metal-bound cyanide than protonated cyanide (22). Metal-cyanide bands are little affected by D$_2$O exchange for H$_2$O whereas the v$_{CN}$ for DCN (1887 cm$^{-1}$) is far removed from the value for HCN (2093 cm$^{-1}$) (22). The use of D$_2$O also significantly enhances the signal-to-noise ratio due to the greater energy passage through D$_2$O than H$_2$O in the C-N stretch region.

**Metal and Oxidation State Assignments of C-N Stretch Bands**—The minimum functional unit of Cc0 is conventionally viewed as containing two heme irons and two copper atoms with only two metal centers potentially able to bind ligands, one iron and one copper (3, 8, 21). Although much evidence supports ligand binding to heme a$_3$, the evidence for binding of an external ligand to a copper center under physiologically relevant conditions has been weak and indirect. The possibility that cyanide can bind to Zn, Mg, and Cu$_{ex}$ (the copper in excess of Fe is represented as Cu$_{ex}$) as well as to Fe$_{a3}$ and Cu$_{a1}$ must be considered.

The fully reduced enzyme in D$_2$O exhibits three metal cyanide ($^{15}$C$^{14}$N$^-$) bands at 2093, 2058, and 2042 cm$^{-1}$ (Table II). The disappearance of the 2058 cm$^{-1}$ band observed when the solution in either H$_2$O or D$_2$O is exposed to CO with concomitant appearance of the heme carbonyl in infrared and visible-Soret spectra demonstrates that the 2058 cm$^{-1}$ band is due to Fe$^{2+}$CN. A v$_{CN}$ value of 2058 cm$^{-1}$ is consistent with other Fe$^{2+}$ cyanides and is much lower than is found for oxidized heme protein cyanides (22). The 2093 cm$^{-1}$ band observed in D$_2$O is at a frequency appropriate for cuprous cyanide (26). For example, reported v$_{CN}$, and $\delta_{PC}$ values for the Cu(CN)$_2^-$ ion (2094 cm$^{-1}$ and $\sim$13 cm$^{-1}$) are similar (26). However, a simple cuprous cyanide ion is not expected to be the source of the 2093 cm$^{-1}$ band since higher concentrations of cyanide and higher pH are required for displacement of copper from the Cc0 (34). Furthermore, the changes in visible-Soret spectra that accompany copper removal by cyanide have not been observed here. The 2093 cm$^{-1}$ band is unaffected by exposure to CO and no Cu$^{+}$CO band (expected near 2060 cm$^{-1}$) appears.

The 2131 cm$^{-1}$ bands of the partially reduced oxidases (I),
Cyanide Binding to Fe and Cu Sites in Cytochrome c Oxidase

FIG. 11. Infrared spectra of fully oxidized and partially reduced species of cytochrome c oxidase cyanide in D2O. The oxidase for the partially reduced enzyme cyanides is 2.1 mM, cyanide 17.4 mM, and phenazine methosulfate 17 μM in 30 mM potassium phosphate buffer, pH 7.4. The electron equivalents of NADH added/two hemes are the same as in Fig. 9. For CcO(IV)CN, the oxidase is 2.15 mM and cyanide 24.8 mM. The reference cell contained fully oxidized enzyme at the same concentration as in the sample cell but with no cyanide present. The same enzyme preparation (EDTA-treated) was used for all measurements. Each spectrum represents the average of 25 accumulated single scans.

TABLE IV

<table>
<thead>
<tr>
<th>Redox level</th>
<th>( \nu ) (cm(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>IV</td>
<td>2165 2151 2131 2093 2058 2042</td>
</tr>
<tr>
<td>III</td>
<td>+ + + + + + + +</td>
</tr>
<tr>
<td>II</td>
<td>+ + + + + + + +</td>
</tr>
<tr>
<td>I</td>
<td>+ + + + + + + +</td>
</tr>
<tr>
<td>O</td>
<td>+ + + + + + + +</td>
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</table>

(II), and (III) can be assigned to Fe\(^{3+}\)CN on the basis of the similarity in \( \nu_{CN} \) values for Fe\(^{3+}\)CN bands of hemin cyanides and other oxidized heme protein cyanides (Table II) (22). CcO(III)CN exhibits a second band at 2131 cm\(^{-1}\) with an intensity comparable to the 2151 cm\(^{-1}\) band. A somewhat variable band appears at 2165 cm\(^{-1}\) in addition to the 2151 cm\(^{-1}\) band. The \( \nu_{CN} \) values of these bands strongly suggest that both bands are due to cyanide bound to copper. The Cu\(^{2+}\)CN of lactase, ascorbate oxidase, and Cu(II) Leu-Leu-Leu cyanides exhibit \( \nu_{CN} \) bands near 2168 cm\(^{-1}\) (35). The

FIG. 12. Visible-Soret and infrared spectra of cytochrome c oxidase partially reduced with ascorbate in D2O with cyanide present. The oxidase is 1.85 mM, sodium ascorbate 4.3 mM, and 23.2 mM cyanide in 42.6 mM potassium phosphate buffer, pH 7.4. A, infrared spectrum. The reference cell contained fully oxidized enzyme at the same concentration as in the sample cell. The spectrum is the average of 30 accumulated single scans. B, visible-Soret spectrum. The same cell was used for both visible-Soret and infrared measurements.

FIG. 13. Changes in absorbance at \( \alpha \) and Soret band maxima upon progressive reduction of cytochrome c oxidase in the presence of cyanide. From spectra in Figs. 1, 4, and 9 absorbance values are normalized to the same oxidase concentrations (2.2 mM). Values for the fully oxidized resting oxidase (IVR) are without added ligand. Values of no electrons/two hemes are for the fully oxidized enzyme exposed to \( ^{13} \)C\(^{14}\)N\(^{-}\) for 20 h.
1,10-phenanthroline complexes of the type \([\text{Cu(phen)}_2(\text{CN})_X]\) where \(X = \text{Cl}, \text{Br}, \text{I}, \text{NO}_2\), and \(\text{ClO}_4\) in Nujol mulls exhibit C-N stretch bands within the range 2136–2149 cm\(^{-1}\) (36). The redox level-dependent interconversion between the 2151 and the 2093 cm\(^{-1}\) band, which can be assigned to cuprous cyanide as discussed above, is consistent with a cupric cyanide assignment for the 2151 cm\(^{-1}\) band. The 2165 cm\(^{-1}\) band was completely absent when the 2093 cm\(^{-1}\) band was at maximum intensity. This reciprocal intensity relationship suggests that the 2165 cm\(^{-1}\) band is also due to \(\text{Cu}^{+}\)CN. Thus, two different ligand binding environments of cyanide bound to \(\text{Cu}^{+}\) appear possible. The 2151 cm\(^{-1}\) band is found in both \(\text{CcO(III)}\) and \(\text{CcO(IV)}\) but, the 2165 cm\(^{-1}\) band is only in \(\text{CcO(III)}\). Although the 2165 cm\(^{-1}\) band sometimes appeared in \(\text{CcO(IV)}\), in that case a weak 2131 cm\(^{-1}\) band was always associated with the 2165 cm\(^{-1}\) band, indicating a small amount of \(\text{CeO(III)}\) was present. The 2151 cm\(^{-1}\) band in \(\text{CcO(III)}\) seems to be interconvertible with the 2166 cm\(^{-1}\) band. However, the intensity at 2151 cm\(^{-1}\) does not decrease as much as the intensity at 2166 cm\(^{-1}\) increases which suggests that the 2165 cm\(^{-1}\) band has appreciable absorbance at 2151 cm\(^{-1}\). Furthermore, the extinction coefficient of the 2165 cm\(^{-1}\) band at the peak position must be higher than that of the 2151 cm\(^{-1}\) band.

The cyanide complex prepared from the resting Cytochrome c oxidase exhibits only one C–N stretch band at 2151 cm\(^{-1}\) (Figs. 2 and 4). The 2151 cm\(^{-1}\) bands for the \(\text{CcO(III)}\) and \(\text{CcO(IV)}\) cyanides are of comparable intensity and width as well as frequency (Table II). As discussed above, a frequency of 2151 cm\(^{-1}\) is fully consistent with a CN\(^-\) bound as a terminal ligand to \(\text{Cu}^{+}\) (38). However, this band appears inconsistent with either the classical view that cyanide is bound only to \(\text{FeL}^{3+}\) in \(\text{CcO(IV)}\) (37) or the recent suggestions that the N atom of \(\text{FeL}^{3+}\) bonded to \(\text{Cu}^{+}\) to form a \(\mu\)-cyano complex, \(\text{FeL}^{3+}\)–C–N–\(\text{Cu}^{+}\) (38). The band at 2131 cm\(^{-1}\), a value of \(\nu_{\text{CN}}\) expected for a typical terminal cyanide ligand bound to \(\text{FeL}^{3+}\) in an iron porphyrin or other heme proteins (22), is found in \(\text{CcO(III)}\) in addition to the 2151 cm\(^{-1}\) band. If the 2151 cm\(^{-1}\) band were due to \(\text{FeL}^{3+}\)CN, the insensitivity of its parameters to the absence (as in CeO(IV)CN) or the presence (as in CeO(III)CN) of a second cyanide ligand to \(\text{FeL}^{3+}\), as shown by a band at 2131 cm\(^{-1}\), is not expected. For example, replacement of the pyridine of pyridine cyanohemins by a second cyanide ligand typically reduces \(\nu_{\text{CN}}\) by about 14 cm\(^{-1}\) (Table V) (22). The 2151 cm\(^{-1}\) band is also not likely to represent a bridging ligand between \(\text{FeL}^{3+}\) and \(\text{Cu}^{+}\). The \(\nu_{\text{CN}}\) value for a cyanide ligand bound via carbon as a terminal ligand is expected to increase upon formation of a bond between the distal nitrogen atom and a second metal atom (39, 40). Strong bonding of the N-atom of \(\text{Fe}^{3+}\)CN to \(\text{Cu}^{+}\) is expected to result in a greater blue shift than is indicated by a \(\nu_{\text{CN}}\) value of 2150 cm\(^{-1}\). To be sure a \(\nu_{\text{CN}}\) value of 2150 cm\(^{-1}\) has been reported for a \(\text{Fe}^{3+}\) porphyrin–\(\text{Cu}^{+}\) cyanide complex in which the presence of \(\text{Fe}^{3+}\)–C–N–\(\text{Cu}^{+}\) bonding was suggested (41). However, the structure has not yet been established by a crystal structure and the magnetic coupling between the \(\text{Fe}^{3+}\) and \(\text{Cu}^{+}\) paramagnetic centers was weak so the structure may not in fact contain a \(\mu\)-cyano ligand with strong bonds to both metals. Perhaps more convincing evidence against the 2151 cm\(^{-1}\) representing a bridging cyanide is the observation that band intensity decreases as a result of bridging between two metals (39), whereas the intensities of the 2151 and 2131 cm\(^{-1}\) bands in CeO(III)CN are similar. It is possible, but not very likely, for an undetected bridging cyanide to be present as well as a terminal cyanide at \(\text{Cu}^{+}\).

The overall independence of the 2151 and 2131 cm\(^{-1}\) bands for the oxidase (IV) (I) (II) and (I) cyanides provides strong evidence that neither band represents a bridging ligand with strong bonds to both \(\text{FeL}^{3+}\) and \(\text{CuL}^{2+}\). The classical view that cyanide is bound only to \(\text{FeL}^{3+}\) in (IV) is based on the visible-Soret spectral change (and possibly on an over confidence in the ligand binding ability of \(\text{FeL}^{3+}\)). As discussed below, the modification of the strong magnetic coupling between \(\text{FeL}^{3+}\) and \(\text{CuL}^{2+}\) in \(\text{CcO(IV)}\) by cyanide, as well as the visible-Soret spectral change, can be induced by CN binding to \(\text{CuL}^{2+}\) without formation of a \(\mu\)-cyano linkage.

\(\text{Cu}^{+}\) cyanides are typically difficult to prepare because of the reduction of \(\text{Cu}^{2+}\) to \(\text{Cu}^{+}\) by cyanide (42, 43). An interesting property of the \(\text{Cu}^{2+}\) and \(\text{Cu}^{+}\) centers in the oxidase is their resistance to reduction by CN\(^-\). The ferromagnetic coupling observed between \(\text{CuX}^{2+}\) and \(\text{FeL}^{3+}\) in \(\text{CcO(IV)}\) (37) demonstrates the resistance of \(\text{CuX}^{2+}\) to reduction by cyanide. The loss of absorbance near 2151 cm\(^{-1}\) and the gain of absorbance at 2093 cm\(^{-1}\) upon reduction beyond the (III) level is therefore explained by conversion of \(\text{CuX}^{2+}\) to \(\text{CuX}^{+}\)CN. On the other hand, the 2093 and 2125 cm\(^{-1}\) bands due to \(\text{CuX}^{+}\)CN do not necessarily indicate the presence of cuprous \(\text{CuX}^{+}\).

The 2042 cm\(^{-1}\) band has a frequency similar to that of ferrocyanide (22). However, as stated above this band is most likely to be due to CN\(^-\) bound to an intrinsic metal other than \(\text{FeL}^{3+}\) and \(\text{CuL}^{2+}\). Thus, only \(\text{CuL}^{2+}\) and \(\text{ZnL}^{2+}\) are likely candidates for the binding site, since Mg is expected to have a low affinity for cyanide. The band position suggests \(\text{CuL}^{2+}\) is more likely but the possibility of \(\text{ZnL}^{2+}\) cannot be excluded. It is also of interest that this intrinsic metal is accessible only in the fully reduced state.

Other possibilities of cyanide binding to \(\text{CUX}\) and Zn should be considered. The fact that a 2151 cm\(^{-1}\) band is also found in \(\text{CcO(IV)}\) CN where the binding of cyanide has been shown to modify the magnetic coupling between \(\text{FeL}^{3+}\) and \(\text{CuL}^{2+}\) (38), supports the assignment of the 2151 cm\(^{-1}\) band to \(\text{CuL}^{2+}\)CN rather than \(\text{CuX}^{2+}\)CN. The reciprocal intensity relationship between the 2151 and 2093 cm\(^{-1}\) bands indicate that the 2093 cm\(^{-1}\) band is also unlikely to be due to \(\text{CuX}^{+}\)CN. It is possible for a zinc cyanide to have a frequency of 2151 cm\(^{-1}\); a \(\nu_{\text{CN}}\) value of 2149 cm\(^{-1}\) is reported for the \(\text{Zn(CN)}^{2+}\) anion (44, 45). However, the 2151 cm\(^{-1}\) band is highly sensitive to the overall redox level of oxidase and Zn\(^+\) will not undergo reduction under these conditions. The \(\nu_{\text{CN}}\) values and binding sites for cyanide, assigned as above, are summarized in Tables IV–VI.

Our recent investigation of infrared spectra of cyanide...
bound to heme and hemeproteins (22) provides a basis for concluding that all the cyanide species of CeO involve the commonly found metal cyanide structure (M–C≡N) with the CN− anion as ligand. The rarely seen HCN metal complex (46), where nitrile-like bonding between metal and nitrogen occurs (i.e. M–NCH), is in no case consistent with the infrared data obtained for oxidase cyanides. The changes in pH that accompany cyanide inhibition have been interpreted as evidence that HCN is the form of cyanide bound to metal (47), a view currently widely accepted (48-50). However, no heme cyanides or hemeprotein cyanides for which infrared data is available exhibit nitrile-like HCN bonding; C–N infrared spectra indicate each cyanide has the commonly found metal cyanide structure (22). Proof of M-CN bonding comes from the magnitude of the 13C isotope shifts and the minor effects of exchange of H2O by D2O on "cN for the 2152, 2131, 2093, and 2058 cm−1 bands for oxidase cyanides, based on criteria discussed earlier (22). The effect of pH changes on cyanide inhibition (47) indicates that the protein contains a functional group which controls the reactivity of the enzyme with cyanide. The band positions and binding sites assigned as discussed above are given in Tables IV and V.

Ligand Binding and Electron Distribution—The infrared and visible-Soret spectra demonstrate an interdependence between ligand binding and the electron distribution among metal centers. The ligand infrared spectra show the oxidation state of the liganded metal whereas the visible-Soret spectra reflect heme oxidation and spin states with only a small contribution from copper complexes. The ligand-binding sites and estimated electron distributions among centers based upon our spectral findings are summarized schematically in Fig. 14. Oxidase species become increasingly reduced on descending from top to bottom. Unliganded species are in the center with carbonyls on the left and cyanides on the right. The fully oxidized species, (IV), at top center normally receives electrons donated by cytochrome c*+ on the outside of the inner mitochondrial membrane as designated by a small solid arrow to FeL and CuL*, which are the first centers to receive electrons from cytochrome c*+ (3, 8). FeL and CuL* are shown to exchange electrons but do not couple magnetically. Passage of electrons from the FeL–CuL pair to the Fe2+, CuL pair is represented by an open double-ended arrow. The FeL and CuL sites are at the dioxygen reaction site near the inner surface of the membrane. In IVa, FeL*+ and CuL* experience antiferromagnetic coupling via an unidentified ligand, X, (possibly S or Cl) and may be within 5 Å of each other (51, 52, 58).

The unliganded CeO(IVa) forms no detectable carbonyl species. Neither FeL*+ nor CuL*+ is expected to bind CO. However, as shown at the top left of Fig. 14, upon exposure of CeO(IVa) to CO, CeO(III)CO slowly forms (53). The unliganded CeO(IVa) contains FeL*+ as a low spin species and FeL*+ as high spin (54, 55). Cyanide binding occurs at CuL*+. The changes in visible-Soret spectra that accompany cyanide binding, although small (Fig. 11, Table I), are consistent with conversion of FeL*+ from high spin to low spin, a widely observed spin state change. In Fig. 14 open ligand access to CuL*+ is denoted by a dashed line below CuL*+ whereas ligand access to FeL*+ is represented as blocked by a double line below FeL*+.

The one-electron reduction of CeO(IVa) to unliganded CeO(III) is shown by the large solid arrow at the upper center of Fig. 14. The reaction of CeO(III) with cyanide is on the right and with carbon monoxide on the left. The changes in visible-Soret spectra that accompany the addition of one reducing equivalent to CeO(IVa) are consistent with FeL*+ and FeL*+ each becoming one-fourth reduced, i.e. for both the FeL*+ and FeL*+ of unliganded CeO(III) approximately one-fourth are FeL*+ and three-fourths are FeL*+ as reported earlier (19). For this reason, both FeL*+ and FeL*+ are represented as FeL*+ in Fig. 14. CuL*+ and CuL*+ are each represented as CuL*+ since no direct evidence of their states of reduction is available. CO binding occurs only at FeL*+. With FeL*+ reduced, FeL*+, CuL*+ and CuL*+ must each be oxidized in CeO(III)CO. The binding of CO to FeL*+ forces an electron redistribution from unliganded CeO(III) to make FeL*+, CuL*+, and CuL*+ completely oxidized. Since no CO binding to CuL*+ is observed, an alternative electron distribution to make all the CuL*+ sites reduced with CO bound, and all the CuL*+, FeL*+, and FeL*+ sites oxidized does not occur. Cyanide binds to CeO(III) at both FeL*+ and CuL*+ with all metal oxidized. The changes in visible-Soret spectra upon CN-binding (Fig. 13) indicate that FeL*+ becomes one-half reduced and one-half oxidized. Thus, CO and CN− induce different electron distributions among the metal centers. The greater stability of the cyanide species is demonstrated by the conversion of CeO(III)CO to CeO(III)CN by addition of cyanide, whereas exposure of CeO(III)CN to CO is without effect. The broken lines beneath both FeL*+ and CuL*+ in unliganded CeO(III) of Fig. 14 indicate both metals are accessible to ligands. The double lines under CuL*+ in CeO(III)CO are to show that CO does not bind to CuL*+.

When two electrons are added to CeO(IVa), the unliganded CeO(II) obtained exhibits a visible-Soret spectrum consistent with equal amounts of oxidized and reduced species in both FeL*+ and FeL*+ (19). After exposure to CO, the infrared spectrum shows CO is only bound to FeL*+ in CeO(II)CO, and the visible-Soret spectrum indicates about one-third of FeL*+ is reduced. Addition of cyanide to CeO(II) develops a band at 2131 cm−1 (FeL*+CN) but no 2151 cm−1 band (CuL*+CN). A band at 2093 cm−1 consistent with CuL*+CN was shown to be present in CeO(II)CN prepared in D2O medium. CeO(II)CN also exhibited a visible-Soret spectrum indicative of FeL*+ about 80% reduced. Cyanide displaces the CO of CeO(II)CO to produce CeO(II)CN.

The addition of three electrons to CeO(IVa) yields CeO(I) which in the unliganded form has FeL*+ and FeL*+ each about 80% reduced based upon the visible-Soret spectrum (19). Binding of CO to FeL*+ causes FeL*+ to become about one-third oxidized. Cyanide binds to FeL*+ thereby forcing FeL*+, CuL*+, and CuL*+ each to be reduced.

In fully reduced CeO(0), all metals are in the lower oxidation state. Infrared spectra show that CO binds only to FeL*+, with both CO and CN− present CO binds to FeL*+ and CN− to CuL*+, and without CO present CN− binds to both FeL*+ and CuL*+.

Structures Inferred from CN and CO Infrared Spectra— The ligand infrared spectra indicate the O2 reduction site has structures that have not been recognized previously. The FeL*+ and CuL*+ binding sites are independent of each other in the sense that changes at one site have little effect on the infrared spectrum of the other site. Furthermore, no major changes in protein conformation appear to occur between redox states 0 and III for these liganded species. The band widths of the FeL*+CO and FeL*+CN bands are extraordinarily narrow compared with other hemeprotein carbonyls and cyanides (20–22). The narrow band widths demonstrate that the mobility of the groups adjacent to the ligand must be restricted so there is little diversity in the surfaces presented to the ligand by these groups (56) and, in each case, the ligand must be well isolated from the external aqueous medium. Thus, the reduction of O2 to water occurs at a site that is remote from the aqueous medium and relatively immobile, at
least when ligands are bound to heme iron. Isolation of the
site may explain why no incompletely reduced O₂ species are
released during the enzymic reaction.

The O₂ reduction by the oxidase involves hydrogen (H⁺ or
H²) transfers to the dioxygen substrate as well as electron
transfers from cytochrome c⁰⁻ (Fig. 15). In order for hydrogen
transfers to occur at a site of restricted mobility isolated from
the external aqueous medium, hydrogen donor groups must
be positioned immediately adjacent to the bound dioxygen. In
analog, previous studies supported Structure I for the heme
α₅ ligand site with CO bound (19). Bending and/or tilting of
the CO bonding to heme iron is expected because the sub-
strate, O₂, prefers a bent-end-on stoichiometry as found in
oxyhemoglobin and oxymyoglobin (20, 57). The structure for
the cyanides liganded to FeL³⁺ and FeL⁵⁺ should be similar to
Structure I as shown in Structure II. The unusually high
affinity of CcO(0) for CN⁻ compared with several other re-
duced hemeproteins (22, 25) may result from hydrogen bond-
ing from an adjacent proton donor to the CN⁻ ligand whose
negative charge cannot be neutralized by the dipositive metal
ion since the number of positive charges on metal equals the
number of negative charges on porphyrin nitrogens.

The structure at the CuL-binding site is less clear. The
possibility must be considered that CuL is in fact the Cu
Cyanide Binding to Fe and Cu Sites in Cytochrome c Oxidase

FIG. 15. Basic steps required in the reduction of dioxygen to water by cytochrome c oxidase.

associated with the heme of cytochrome a which is the heme that receives electrons most directly from cytochrome c and is not the Cu nearest to heme a. Perhaps the strongest evidence in support of CuL being the Cu most closely associated with heme a is the observed effect of cyanide binding to CcO(IV) on the spin-state of heme a, as discussed above. It is much less likely that the binding of cyanide to Cu a could so markedly affect the spin-state of heme a. Therefore, we must ask how, in a stereochemical sense with respect to FeL, CN- bind to CuL. For example, does CN- bind to CuL, oriented toward FeL, as in Structure III, or away from FeL, as in Structure IV (all ligands other than cyanide are omitted in Structures III and IV). EXAFS data indicate the FeL to CuL distance should be about 5 Å or less (51, 58). The ligand infrared spectra reveal an independence between the FeL and CuL ligand-binding sites in that changes at FeL do not affect the infrared bands for a ligand at CuL and, conversely, changes at CuL do not affect bands for a ligand at FeL. It is unlikely that CN- can bind to CuL at a site directly pointing toward FeL without perturbing the spectrum for the ligand bound to FeL. Therefore, we conclude that Structure IV is much more likely than Structure III.

Binding of CN- to CcO(IV) can be represented by the conversion of Structure V to Structure VI in which the binding of CN- to CuL weakens CuL-X bonding to result in stronger (shorter) FeL-X bonding. The binding of CN- to CuL is assumed to alter the bonding of a bridging ligand X (e.g. S or Cl) between CuL and FeL (58) such as to effect a change from high spin FeL3+ to low spin FeL3+.

Reaction Mechanism for Catalysis of the Reduction of Dioxygen to Water—The chemistry of Reaction 1 necessarily involves a number of steps: O2 binding, electron transfers to oxygen, hydrogen transfers to oxygen, cleavage of the O-O bond, and release of products (Fig. 15) (20, 57). O2 is expected to bind to five coordinate FeL as in proposed Structure VII to give Structure VIII (20). The bent-end-on dioxygen ligand is expected to acquire electron density on the distal oxygen atom which promotes H-bonding with R2YH and/or R2ZG to stabilize the structure.

The first two-electron reduction of dioxygen can occur if one additional electron is made available to the FeL-CuL pair to form Structure IX from Structure VIII (20, 21, 59). The infrared evidence that CN- binds to CuL as well as FeL may be considered experimental support for the possibility of this reduction resulting in a p-peroxo intermediate with a FeL3+-O-O-CuL2+ linkage. However, as discussed earlier, the hydroperoxide (Fe-O-O-H) of Structure X may also be rapidly produced (20). The conversion of an oxyheme to a hemin
peroxide with a low energy of activation requires structures of the appropriate stereochemistry to permit facile electron transfer onto the dioxygen ligand (59, 60). And, unless a μ-peroxo linkage between Fe" and Cu" is formed, facile proton (or hydrogen atom) transfer to the distal oxygen atom is also required. Since the infrared data indicate that a ligand site may not be readily available on the FeL side of CuL, formation of a peroxo bridge may not represent a low energy path. The electron and hydrogen transfers involved for the conversion of Structure IX to Structure X will require very little structural movement, an advantage for the reduction of the energy barrier to electron-hydrogen transfers. Electron transfer across networks such as FeO=O...H-Y-Cu' of Structure IX has not been demonstrated. However, a crystal structure of a compound with a Cu"-C≡N...H-N-Cu" linkage has been reported (61). Since this Cu" dimer exhibits antiferromagnetic exchange interaction (62), the linkage may well also provide a pathway for electron transfer. The transfer of two electrons and three hydrogens to the peroxide will result in the splitting of the O-O bond and the formation of two water molecules (Fig. 15). Stereochemical optimization of these transfers must be achieved by the same active site structure used to bind O2 and convert it to the peroxide.

Oxidation Linked Deprotonation at Cu"—The oxidation of Cu" to Cu"", as must occur on going from CeO(II)CO to CeO(III)CO, causes little change in the interactions of CO with its immediate environment, as implied by stable CO bond infrared parameters (19, 20). The lack of change in these interactions can result at least in part from the maintenance of an essentially constant effective charge at CuL by loss of a proton from a ligand (e.g. L1) upon oxidation of Cu" to Cu"" and by structures at CuL which minimize the extent of change in ligand stereochemistry. The reduction of Cu"" in Structure VIII to give Structure IX is shown to be accompanied by a pickup of a proton on L1. The subsequent oxidation of Cu" to Cu"" to give Structure X results in the loss of the proton from L1. If the proton uptake on L1 results in withdrawal of protons from the inside of the mitochondrial membrane and the deprotonations of L1H+ causes proton transfer to the outside of the membrane, this process can contribute to the coupling of electron transfers from cytochrome c" to O2 to proton pumping from the inside to the outside of the inner mitochonrdial membrane (63, 64). The complete reduction of O2 to water requires four electrons and, by this process, two changes in the oxidation state of CuL. Since one proton can
to the pumping of waters in the plant. Support for these theories comes from the observations of plant growth in the presence of water and from the observations of plant growth in the absence of water.

**Conclusion**

The results of this study provide evidence for the importance of water in the growth of plants. Further research is needed to understand the mechanisms by which water influences plant growth and to develop strategies for optimizing plant growth in different conditions.
Infrared evidence of cyanide binding to iron and copper sites in bovine heart cytochrome c oxidase. Implications regarding oxygen reduction.

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