Photochemical and ligand-exchange properties of the cyanide complex of fully reduced cytochrome *c* oxidase

Bruce C. HILL* and Stephen MARMOR

Department of Chemistry and Biochemistry, Concordia University, Montreal, P.Q. H3G 1M8, Canada

Cytochrome oxidase, in its fully reduced state, forms a complex with CN having a K_d of 230 μM with a stoichiometry of 1 CN molecule per cytochrome oxidase. We do not detect a second CN-binding site as seen by i.r. spectroscopy [Yoshikawa & Caughey (1990) *J. Biol. Chem.* **265**, 7945–7958]. The ferrocyanide a_3 -CN complex, like the analogous ferrocyanide a_3 -CO complex, is photosensitive but with a 15-fold lower quantum yield for photolysis. Analysis of the recombination kinetics after CN photolysis establishes a simple bimolecular binding constant of 235 $\text{M}^{-1}\cdot\text{s}^{-1}$, in agreement with the value obtained from stopped-flow studies [Antonini, Brunori, Greenwood, Malmström & Rotillo (1971) *Eur. J. Biochem.* **23**, 396–400]. A rate of 0.07 s^{-1} for the first-order dissociation of CN from cytochrome a_3 is found by the rate of exchange of CO with ferrocyanide a_3 -CN, and is consistent with the value calculated from the equilibrium binding constant and the CN on rate. However, O_2 is able to oxidize the fully reduced CN compound at a rate well in excess of the CN off rate. The product of this oxidation reaction is a partially reduced CN complex. This implies that O_2 either promotes CN dissociation or is able to oxidize the CN-bound enzyme directly. These results are discussed in the context of the structure and dynamics of the ligand-binding site of cytochrome oxidase.

INTRODUCTION

Cytochrome *c* oxidase catalyses the transfer of reducing equivalents from cytochrome *c* to molecular oxygen. It has a ligand-binding site which includes one of the haem centres (cytochrome a_3) and one of the copper centres (Cu_B). Cytochrome a_3 and Cu_B are physically close, magnetically coupled and may even share a common inner-sphere ligand (Naqui *et al.*, 1986). These structural features are recognized as important determinants of the functional properties of this binuclear site. It has been demonstrated that each of these metals may simultaneously bind an exogenous ligand. A complex with CN bound to ferricytochrome a_3 and NO bound to oxidized Cu_B has been characterized (Hill *et al.*, 1983). It has also been proposed that Cu_B serves in binding O_2 during the reaction cycle (e.g. Chance & Leigh, 1977). Thus a role for Cu_B in O_2 activation and reduction of O_2 , as well as ligand binding, has been postulated.

Cytochrome oxidase forms stable complexes with well-known haem ligands, such as CO and CN^- . These ligands derive their inhibitory properties by binding at the binuclear cytochrome a_3 - Cu_B centre and thereby prevent binding of the substrate O_2 . Cytochrome oxidase forms a variety of complexes with CN (van Buuren *et al.*, 1972a). CN binds to the fully oxidized enzyme to form a low-spin complex with ferricytochrome a_3 in which the magnetic coupling between cytochrome a_3 and Cu_B is maintained (Thomson *et al.*, 1982). This complex is non-reactive with O_2 , and three reducing equivalents may be added to yield a partially reduced species in which cytochrome a , Cu_A and Cu_B are reduced (Johnson *et al.*, 1981). CN binding greatly stabilizes ferricytochrome a_3 and the reduction of this centre only occurs very slowly in the presence of excess reductant at the rate at which CN dissociates (van Buuren *et al.*, 1972b).

CN also forms a complex with cytochrome oxidase in the fully reduced state in which CN is bound to ferrocyanide a_3 . CN binding to ferrohaemoproteins has also been reported for horseradish peroxidase (HRP), very weakly for ferromyoglobin and

only transiently for ferrohaemoglobin (Phelps *et al.*, 1971; Yoshikawa *et al.*, 1985). I.r. spectroscopy is able to detect two sites on the reduced enzyme for CN binding, one on cytochrome a_3 and another on Cu_B (Yoshikawa & Caughey, 1990). However, previous binding studies have indicated a stoichiometry of 1 for CN binding (Antonini *et al.*, 1971; van Buuren *et al.*, 1972a). We have studied the complex formed between CN and fully reduced cytochrome oxidase. We have compared its photochemical properties with the CO complex of the oxidase and with the CO and CN complexes formed with ferroHRP. We have measured the binding kinetics by following recombination after flash photolysis and the dissociation kinetics by measuring the rate of CN replacement by CO. These results indicate that the oxidase forms a kinetically and thermodynamically stable complex in its fully reduced state with CN. However, when the fully reduced cytochrome oxidase-CN complex reacts with O_2 , the rate is not limited by the dissociation of CN. The product of the reaction with O_2 is a partially reduced CN complex in which cytochrome a_3 is oxidized and CN bound. Thus O_2 must either act to promote CN dissociation or be able to oxidize directly the reduced oxidase-CN complex. We suggest that O_2 is able to oxidize the binuclear centre directly, generating CN-bound oxidized cytochrome a_3 .

EXPERIMENTAL

Cytochrome oxidase was extracted from mitochondrial membranes of bovine heart muscle (Yonetani, 1961) by following a modified version of the procedure of Kuboyama *et al.* (1972). $(\text{NH}_4)_2\text{SO}_4$ fractionation of the enzyme was performed completely in sodium cholate-containing buffer and the final pellet was suspended in 0.05 M-sodium phosphate buffer containing 1 mg of lauryl (dodecyl) maltoside/ml. Sodium cholate was from Fluka, and lauryl maltoside was from Boehringer-Mannheim.

The fully reduced complex of cytochrome oxidase with CN was prepared in the following way. Cytochrome oxidase was

Abbreviations used: HRP, horseradish peroxidase; TMPD, tetramethyl-*NNN'*-*p*-phenylenediamine.

*To whom correspondence and reprint requests should be addressed at: Division of Biochemistry, Department of Biological Sciences, University of Calgary, Calgary, Alberta T2N 1N4, Canada.

diluted from a stock sample to a concentration of 3–5 μM . The sample was deoxygenated by exchange with pre-purified N_2 . Sodium ascorbate and tetramethyl-*NNN'*-*p*-phenylenediamine (TMPD) were added to the oxidase solution to give concentrations of 1.2 mM and 0.75 μM respectively. This solution was allowed to incubate for 2 h to obtain complete reduction. KCN was added to the reduced enzyme from a freshly prepared 2 M stock solution. The stock KCN solution was prepared in deoxygenated N_2 -equilibrated buffer containing 5 mM-sodium dithionite. In titration experiments, small volumes of CN were sequentially added and the absorbance spectrum was measured after each addition. The concentration of sodium dithionite in the oxidase solution was never more than 15 μM and thus would not significantly affect the O_2 reaction, but was necessary for anaerobic CN transfer. Ligand-exchange experiments were done by adding a small volume of buffer containing either CO or O_2 , mixing and then monitoring the change in absorbance as a function of time. The mixing time in these experiments was about 3 s.

Flash-photolysis experiments were done using a Phase-R flash-pumped dye laser which delivered 0.5–0.7 J flashes of 350 ns duration. Absorbance measurements were made using the light from a 75 W Xe arc lamp situated at 90° to the photolysis pulse. The observation beam was focused on the sample so as to pass through the centre of the volume irradiated by the photolysis flash. The change in light transmission through the sample was measured as a change in voltage of a Photodyne silicon photodiode connected via a 100 k Ω termination to a Nicolet digital oscilloscope. The data were transferred in digital form to an AT&T 6300 WGS computer into the Asystant+ software package for conversion into absorbance, analysis and plotting.

Stopped-flow experiments were done on a Hi-Tech SF-51 stopped-flow spectrophotometer. The digital data were transferred to the Asystant+ software package and fitted to exponential decay curves to determine first-order rate constants.

RESULTS

Fig. 1 shows an absorbance difference spectrum between the CN complex of fully reduced cytochrome oxidase and the unliganded fully reduced enzyme. The peak and trough wavelength positions and the difference absorption coefficients for this species are shown as an inset to Fig. 1. The spectrum of the CN complex of fully reduced cytochrome oxidase is similar, in the visible region, to the complexes that fully reduced oxidase forms with NO and CO, but differs in the Soret region. The absorption coefficients of the difference absorbance bands in the visible region for the CN complex are nearly of equal intensity to those in the Soret, whereas the Soret/visible intensity ratios are about 5:1 for the CO and NO complexes.

Formation of the CN complex with fully reduced cytochrome oxidase can be monitored spectrophotometrically. A simple titration curve is obtained which conforms to an apparent stoichiometry of 1 CN molecule per oxidase with an equilibrium dissociation constant of 230 μM (Fig. 2). These results are in reasonable agreement with those obtained by previous workers who report the same stoichiometry, but a K_d of 100 μM (van Buuren *et al.*, 1972a) and 555 μM (Antonini *et al.*, 1971). We do not attribute any significance to these differences in binding constants, but assume they represent small differences in experimental conditions.

The photosensitivity of the ferrocyanochrome a_3 -CN complex relative to the ferrocyanochrome a_3 -CO complex is measured using a laser pulse technique (Brunori & Giacometti, 1981). Fig. 3 shows the efficiency of photolysis plotted as a function of flash

intensity obtained for the two ferrocyanochrome a_3 species. The relative slopes of the two plots are a measure of the relative quantum yields. Since the quantum yield is close to one for the CO complex, then we calculate a quantum yield of 0.065 for the CN complex. Keilin & Hartree (1955) reported that the complex of reduced HRP with CN was photosensitive with a quantum yield of 0.80. We have measured the quantum yield of the ferroHRP-CN complex relative to ferroHRP-CO and ferromyoglobin-CO. These results and those for the oxidase are summarized in Table 1. We find a significantly lower value for the quantum yield for photolysis of ferroHRP-CN complex than originally reported by Keilin & Hartree (1955), although we obtain similar values to those of Brunori & Giacometti (1981) for the photolysis quantum yield of HRP-CO. Although we do not know the origin of this discrepancy, it should be pointed out that the data were obtained by different experimental approaches. Furthermore, the general result is the same, namely that the

Fig. 1. Difference spectrum between the CN complex of fully reduced cytochrome oxidase and the fully reduced ligand-free species [i.e. $(a^{2+}a_3^{2+}\text{-CN}) - (a^{2+}a_3^{2+})$]

The absorption coefficients shown in the inset are for the peak and trough positions and are based on the enzyme concentration as determined using a $\Delta\epsilon(605\text{--}630)$ (reduced–oxidized) of 27 $\text{mm}^{-1} \text{cm}^{-1}$. The concentration of oxidase was 4.55 μM in 0.2 M-sodium phosphate buffer, pH 7.4, with 1 mg of lauryl maltoside/ml and 5 mM-CN. All other conditions are as described in the Experimental section.

Fig. 2. Hill plot of equilibrium CN binding to fully reduced cytochrome oxidase

The y-axis is a function of the fractional absorbance (i.e. $\alpha = A_x/A_\infty$, where A_x is the absorbance at a particular cyanide concentration and A_∞ is the absorbance at infinitely high cyanide). A is measured as the difference in absorbance at 589.5 nm–609.5 nm in the difference spectrum between reduced CN-bound oxidase and reduced unliganded oxidase. The buffer conditions and enzyme concentration were the same as those described in Fig. 1.

Fig. 3. Quantum yield for photolysis of the CN complex of fully reduced cytochrome oxidase relative to the CO complex of the reduced enzyme

Fe-X₀ is the concentration of liganded species before the flash and Fe-X₁ the concentration of liganded species immediately after the flash, where the ligand X is either CO or CN. These values were corrected for variation in the flash energy and absorbance at the photolysis wavelength. The concentration of the enzyme was 5 μM, CO 1 mM and CN 5 mM, and the buffer conditions were the same as those described for Fig. 1.

Fig. 4. Comparison of the static and kinetic difference spectra in the Soret region for the CN complex of fully reduced cytochrome oxidase

The static spectrum is shown in the solid line. The kinetic spectrum is a plot of the absorbance change induced by laser photolysis as a function of wavelength (●). For this comparison the observed absorbance values have been normalized to 1 since complete photolysis is not obtained for the CN complex. The enzyme concentration was 4.55 μM and CN 5 mM, and the buffer conditions were the same as those in Fig. 1.

ferroHRP-CN complex is significantly more photolabile than the ferrocycytochrome a₃-CN species.

Fig. 4 shows the wavelength-dependence of the absorbance changes produced by the photolysis of ferrocycytochrome a₃-CN compared with the equilibrium difference spectrum generated by the addition of CN to the fully reduced enzyme. The close agreement between the static and kinetic difference spectra seen in Fig. 4 suggests that the process of CN photolysis does not yield a state of the haem or protein that differs from the unliganded oxidase produced in the thermal dissociation of CN. In addition we have determined the kinetics of CN binding by monitoring

Table 1. Photolysis quantum yields for some ferrohaem-ligand complexes

Species	Relative quantum yield	Reference
Reduced myoglobin-CO	1	Sawicki & Gibson (1979)
Reduced aa ₃ -CO	1	Relative to myoglobin-CO, this paper
Reduced aa ₃ -CO	0.065	This paper
Reduced HRP-CO	0.65	This paper
	0.70	Brunori & Giacometti (1981)
Reduced HRP-CN	0.30	This paper
	0.80	Kelin & Hartree (1955)

Fig. 5. Dependence of the rate of CN binding to fully reduced cytochrome oxidase on CN concentration

Rates of CN binding were obtained from recombination time courses after laser photolysis. The bimolecular rate, obtained from the slope, is 235 M⁻¹·s⁻¹ and the dissociation rate constant (i.e. k_{off}) is 0.05 s⁻¹, obtained from the y-intercept. The enzyme concentration was 4.55 μM and the buffer conditions were the same as those outlined in Fig. 1.

the re-formation of the complex after laser photolysis. The time-course of rebinding follows a single exponential rate law from which we obtain a pseudo-first-order rate constant (k_{obs}). The pseudo-first-order rate is reported for a number of CN concentrations, and a typical plot of k_{obs} as a function of ligand concentration is shown in Fig. 5. The plot is well fitted by a straight line, the slope of which is 235 M⁻¹·s⁻¹, the second-order binding rate constant. This value is slightly higher than the value measured previously for CN combination to reduced cytochrome oxidase in stopped-flow experiments (van Buuren *et al.*, 1972a; Antonini *et al.*, 1971), but identical with the combination rate constant we have measured by stopped-flow (B. C. Hill & J. D. Doran, unpublished work). The intercept on the y-axis is a measure of the dissociation rate constant and is 0.05 s⁻¹, in good agreement with the estimate of 0.1–0.2 s⁻¹ reported by Antonini *et al.* (1971).

We have also measured the cyanide-dissociation rate by following the rate of exchange of CO. A typical time course for ligand exchange with CO is shown in Fig. 6. This process follows a single-exponential time course consistent with the model that these two ligands react competitively, but independently, at the same binding site. The bimolecular binding constant for CO with reduced cytochrome oxidase is 8 × 10⁴ M⁻¹·s⁻¹, giving an expected pseudo-first-order rate of 4 s⁻¹ for the binding of CO to unliganded ferrocycytochrome a₃. The observed first-order rate for CO exchange is 0.07 s⁻¹, and is taken as the CN-dissociation rate constant.

Fig. 6. Time course of CN displacement from ferrocyanochrome a_3 by CO

At time zero, CO was added to commence ligand exchange. The continuous line on the graph is a single exponential fit to the data points. The concentration of enzyme was $3.65 \mu\text{M}$, CN 7 mM , and CO $50 \mu\text{M}$, and the buffer conditions were the same as those described in Fig. 1.

Fig. 7. Time course for the reaction of the reduced cytochrome oxidase-CN complex with O_2

The absorbance change is recorded at 445 nm as a function of time on a scale in (a) to 20 ms and in (b) to 50 s . After mixing, the concentrations of reactants were; cytochrome oxidase, $4.2 \mu\text{M}$; KCN, 6 mM ; O_2 , $135 \mu\text{M}$; ascorbate, 0.6 mM ; TMPD, $0.375 \mu\text{M}$; and sodium dithionite, $5 \mu\text{M}$. The temperature was 20°C and the buffer was 0.2 M -sodium phosphate at $\text{pH } 7.4$ with 1 mg of lauryl maltoside/ml. In (a) the trace begins with a sudden increase in absorbance as new reactants enter the observation cell because observation is triggered prior to the flow being stopped. (b) records the reaction from 10 ms to 50 s .

We have assessed the reactivity of the CN complex of reduced cytochrome oxidase with O_2 . Fig. 7 shows the course of this reaction, monitored at 445 nm , on two time scales. Fig. 7(a) shows the reaction to 20 ms and Fig. 7(b) to 50 s . There is a very fast reaction which corresponds to haem oxidation and is virtually complete in 5 ms . We can fit this process to a single exponential decay with a first-order rate of 500 s^{-1} , but this is very near the limits of the resolution of the stopped-flow method and is only an approximation. A large portion of this reaction is lost in the dead time (i.e. 1 ms) of the mixing apparatus. Fig. 7(b) shows a very slow reaction on a time scale of seconds which is consistent with further haem oxidation.

Fig. 8 shows Soret difference spectra recorded at different times after the addition of O_2 to the CN complex of fully reduced cytochrome oxidase. Spectrum a is the difference between the 'immediate' product, taken 3 s after mixing and recorded in 2.5 s , with fully reduced oxidase as reference. Spectrum b is after

Fig. 8. Difference spectra in the Soret region during the reaction of cytochrome oxidase-CN with O_2

The concentration of cytochrome oxidase was $4.40 \mu\text{M}$ in the sample and the reference after the addition of all the reagents. The enzyme was brought to full reduction by incubation under N_2 with 1.2 mM -sodium ascorbate and $0.75 \mu\text{M}$ -TMPD. KCN was added to the sample to a concentration of 6.2 mM , including $7 \mu\text{M}$ -sodium dithionite. O_2 was then added to a level of $78 \mu\text{M}$ to the sample cuvette. The first spectrum was recorded 3 s after O_2 addition. The spectrometer scanning at 40 nm/s required 2.5 s to record the full spectrum. Therefore spectrum a is the difference between the product of the very fast process and fully reduced enzyme in the reference cuvette. Spectrum b is the difference spectrum after the slow reaction seen in Fig. 7.

50 s with the fully reduced spectrum as reference. The magnitude of the trough at 445 nm represents the extent of haem oxidation in the two phases of the reaction. The absorption coefficient at 445 nm for the difference between fully oxidized CN-bound cytochrome oxidase and fully reduced cytochrome oxidase is $150 \text{ mm}^{-1} \cdot \text{cm}^{-1}$. After 3 s , 49% of this change has occurred and 76% occurs after 50 s . We suggest that these changes represent the complete oxidation of ferrocyanochrome a_3 -CN in the initial fast phase and 50% oxidation of cytochrome a in the slower phase. Spectrum a in Fig. 8 is close to that for $(a^{2+}a_3^{3+}\text{-CN}) - (a^{2+}a_3^{2+})$ (see, e.g., van Buuren *et al.*, 1972a,b). However, in the spectrum of van Buuren *et al.* (1972a,b) cytochrome a_3 changes both spin and oxidation state, whereas in our spectrum only the oxidation state of cytochrome a_3 is changed. This spectral similarity reflects the small effect of CN binding, and presumably spin state, on the Soret spectrum of ferrocyanochrome a_3 .

In the experiment shown in Fig. 8 the oxidase was originally reduced with an excess of ascorbate (1.2 mM) and a catalytic amount of TMPD ($0.75 \mu\text{M}$). The reaction product of Fig. 8 gives rise to the conventional mixed-valence CN complex, with cytochrome a fully reduced, on a time scale of hours, which is consistent with the original reduction kinetics. In addition, neither of the species shown in Fig. 8 is reactive with CO on a ms or s time-scale, but the CO complex of the oxidase begins to form only after 30 min . This result indicates that CO reacts at the rate of dissociation of CN from oxidized cytochrome a_3 and supports our conclusion that cytochrome a_3 is the initial site of oxidation in the reaction of O_2 with the CN complex. Thus we assign the initial fast reaction of O_2 with the reduced cytochrome oxidase-CN complex to the oxidation of ferrocyanochrome a_3 -CN. O_2 reacts with ferrocyanochrome a_3 -CN at a rate that is about 1000 times faster than the measured dissociation rate of CN.

Scheme 1. Reactivity of ferrocyanide a_3 -CN with CO and O_2

DISCUSSION

Scheme 1 shows some features of the reactions studied in this work. CN forms an apparently simple complex with reduced cytochrome oxidase in which it is bound to the Fe atom of cytochrome a_3 . However, its electronic spectrum is different from the better known CO and NO complexes of cytochrome oxidase. In addition, Storey (1970) has reported that the ferrocyanide a_3 -CN complex formed in intact mitochondrial membranes is sensitive to O_2 . We have confirmed this finding using the isolated detergent-solubilized enzyme. The cytochrome oxidase-CO complex reacts with O_2 slowly and essentially at the CO-dissociation rate (Gibson & Greenwood, 1963; Antonini *et al.*, 1987). In contrast, O_2 is able to oxidize the ferrocyanide a_3 -CN complex without the need for CN to dissociate or, alternatively, O_2 is able to promote the rate of CN dissociation. As shown in Scheme 1, we favour the direct oxidation of ferrocyanide a_3 -CN by O_2 and present two possible mechanisms, which are discussed below.

The product of the reaction with O_2 after 5 ms has cytochrome a_3 oxidized and CN bound. However, we have not directly assessed the redox status of Cu_B in the kinetically generated partially reduced CN complex. But, if Cu_B has remained reduced, as is found in the partially reduced CN complex produced by equilibrium titration (Johnson *et al.*, 1981), then this would imply a one-electron reduction of O_2 to superoxide. However, the addition of one electron to dioxygen is thermodynamically unfavourable. The drive for this reaction could be found in the relative stabilities of the fully reduced and partially reduced CN complexes. The K_d for the fully reduced complex is about $2.5 \times 10^{-4} \text{ M}$, and for the partially reduced CN complex a K_d of $1 \times 10^{-7} \text{ M}$ has been reported (van Buuren *et al.*, 1972a). This

corresponds to a free-energy drive in terms of reduction potential of -0.2 V , but is still short of the redox potential for one-electron reduction of O_2 at -0.33 V (Wood, 1988). The two-electron reduction of oxygen is thermodynamically favourable, and therefore we suggest that the initial product is a CN complex with Cu_B oxidized. There is some evidence for this species as a kinetic intermediate in the reduction of the oxidized CN-bound oxidase (Nicholls & Hildebrandt, 1978).

Further, we suggest that the initial rapid two-electron oxidation is followed at longer times by electron redistribution from cytochrome a and Cu_A to re-reduce Cu_B . This accounts for the oxidation we observe for cytochrome a on a time scale of 10–50 s. As shown in titration experiments, Cu_B is the most oxidizing centre in this complex and at equilibrium would tend to oxidize cytochrome a and Cu_A . Thus we would predict that, if this reaction were examined by e.p.r. spectroscopy, the initial product would be e.p.r.-silent with spin coupling between oxidized Cu_B and cytochrome a_3 , and that, on a time scale of 50 s, signals from cytochrome a and Cu_A would appear to one-half their values in the oxidized enzyme.

The mechanism for this reaction is hard to reconcile with what is known for the oxygen reaction of reduced unliganded cytochrome oxidase. When oxygen attacks reduced cytochrome oxidase, a complex is formed initially between ferrocyanide a_3 and O_2 , and then four reducing equivalents are transferred, generating two water molecules and the oxidized enzyme. This process takes place in 2–3 ms at room temperature and is generally postulated not to involve any ligand-exchange steps after the initial complex-formation (Hill *et al.*, 1986). One would expect that, once an O_2 molecule gains access to its oxidative site, complete oxidation would occur since it is kinetically efficient and energetically downhill. Thus it is surprising that the presence

of CN does not protect cytochrome a_3 from oxidation but does manage to inhibit complete oxidation of the enzyme. We suggest that CN must remain bound during this process, as depicted in the two postulated intermediates shown in Scheme 1. One way to reconcile such a finding is to allow oxygen to oxidize the enzyme via Cu_B and for cyanide to remain bound at cytochrome a_3 . In such a mechanism one (or two) electrons could be extracted via Cu_B -oxidizing cytochrome a_3 (and Cu_B) and generating the very stable ferricytochrome a_3 -CN complex. Alternatively, O_2 could still attack at cytochrome a_3 but with CN remaining bound, which would imply an expansion of the inner-sphere ligation shell for cytochrome a_3 .

Spectroscopic and equilibrium and kinetic binding data suggest that cytochrome oxidase in the fully reduced state binds CN in a simple complex stabilized by a ferrocyanide a_3 -CN bond. Our results agree with those reported previously (van Buuren *et al.*, 1972a,b; Antonini *et al.*, 1971). However, Yoshikawa & Caughey (1990) have suggested that the oxidase binds two equivalents of CN and that there is independent ligation to Cu_B . These authors do not report the relative affinity of CN at these two sites nor the degree of their occupancy, and their experiments are all conducted at very high CN concentrations. If the second CN-binding site has a much lower affinity than the site we and others have observed, then it might not be detected in our work. In addition, if the second binding site for CN is Cu_B , then it might be difficult to detect spectrophotometrically since this centre does not give rise to a well-defined electronic spectrum in the visible region.

The Fe-CN bond in the ferrocyanide a_3 -CN complex is photolabile, a property it shares with other ferrohaemoprotein-ligand complexes. However, the quantum yield is much reduced for CN-bound reduced cytochrome oxidase species, implying geminate recombination in this system or a much reduced efficiency for the initial photolytic step. In either case such a finding differentiates the CN complex from the ferrocyanide a_3 -CO species. It should be possible to determine the limiting factor in the quantum yield of these species by the use of ultrashort laser pulses to photolyse the haem-ligand complexes.

We thank Dr. T. W. Swaddle and Dr. H. Doine of the Department of Chemistry at the University of Calgary for the use of the Hi-Tech

stopped-flow spectrometer. We are grateful to Dr. A. M. English and other members of the Enzyme Research Group, Department of Chemistry and Biochemistry at Concordia University, for valuable discussions. This work was supported by an operating grant from the Natural Sciences and Engineering Research Council (Canada) to B. C. H.

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