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The pathway of CO binding to cytochrome *c* oxidase
Can the gateway be closed?

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Abstract Addition of cyanide to the CO complex of cytochrome oxidase reduces the apparent photosensitivity of the Fe-CO bond. This effect is not seen with azide, or when cyanide is added to ferromyoglobin-CO. It is proposed that cyanide binds to Cu_B, and restricts the passage of CO out of the protein. This restriction favors geminate recombination of CO and ferrocytochrome a_3, thereby lowering the apparent quantum yield for CO photolysis. The apparent K_d of cyanide for Cu_B is 15.4 mM. These data support a direct role for Cu_B in ligand binding by cytochrome *c* oxidase.

Key words: Cytochrome *c* oxidase; Ligand binding; Binuclear center; Cytochrome a_3-Cu_B; Cyanide; Carbon monoxide

1. Introduction

Cytochrome *c* oxidase catalyses electron transfer from cytochrome *c* to molecular O_2. It reduces O_2 to water without releasing any of the potentially dangerous, partially-reduced states of O_2. In addition, the enzyme conserves energy from the redox process in the form of a transmembrane proton gradient that can be used to drive other energy requiring processes [1]. The oxidase has four metal centers; two haem A groups and two copper centers, that are involved in its functional cycle. One haem A, cytochrome a_3 and one Cu atom; Cu_B, sit close to one another and form a binuclear center. The binuclear center is the site where the substrate O_2 and inhibitory ligands, such as CO and CN, bind.

CO forms a stable complex with fully reduced cytochrome oxidase and binds as an inner sphere ligand to cytochrome a_3. The CO-cytochrome a_3 bond is photodissociable with an apparent, μs quantum yield near one. Furthermore, it has been known for some time that photodissociation of CO from cytochrome a_3 is irreversible at low temperature (e.g. < 100 K) [2]. FTIR[1] spectroscopy at ultra low temperature (e.g. 20 K) reveals that CO, photodissociated from cytochrome a_3, migrates and binds to reduced Cu_B [3]. The binding of CO to Cu_B accounts for the high apparent quantum yield for CO photolysis at room temperature and its irreversibility at low temperature.

Woodruff and his colleagues [4–7] have applied time resolved spectroscopy to demonstrate some further features of this CO transfer reaction. CO moves from the Fe of cytochrome a_3 to Cu_B in less than 1 ps, and then CO dissociates from Cu_B into solution with a half-time of 1 μs [4,5,7]. Such remarkable speed and efficiency implies that CO has little freedom for other modes of motion. Other work has shown that CO binding from solution shows saturation kinetics at CO concentrations above 1 mM indicative of a pre-equilibrium binding site for CO [6]. Woodruff [8] has developed a ligand-shuttle model in which binding to Cu_B is required in the association and dissociation of ligands to and from cytochrome a_3.

Cu_B has been invoked previously as a binding site for ligands other than CO. In a mixed-ligand complex, NO is suggested to bind to oxidized Cu_B while CN binds oxidized cytochrome a_3 [9]. FTIR studies suggest that cyanide is able to ligate to Cu_B in fully reduced enzyme, with either CN [10] (but cf. [11]) or CO bound to cytochrome a_3 [12]. In addition optical studies suggest transient binding of CN to Cu_B in the oxidized enzyme [13]. There has also been evidence presented in support of transient association of O_2 with Cu_B in the oxidation of reduced cytochrome oxidase [14–16].

In this work I have shown that addition of cyanide to fully reduced, CO-bound cytochrome oxidase drastically reduces the apparent quantum yield for CO photolysis from cytochrome oxidase. This effect is proposed to be mediated by ligation of CN to reduced Cu_B. The binding of CN to Cu_B blocks the first step in the pathway of CO out of the binding pocket and thereby promotes immediate or geminate recombination of CO and cytochrome a_3. This work supports the suggestion that Cu_B is an obligate binding site for ligands to the binuclear center of cytochrome oxidase.

2. Materials and methods

Cytochrome oxidase was purified from fresh bovine heart mitochondria according to the procedure outlined by Kuboyama et al. [17]. The purified enzyme was precipitated with ammonium sulfate and resuspended in 25 mM sodium phosphate pH 7.4 with 1 mg per ml lauryl maltoside. Ammonium sulphate, sodium phosphate, potassium cyanide and sodium dithionite were all from BDH (Montreal, Que.). Lauryl maltoside was from Boehringer-Mannheim (Laval, Que.). CO was from Canox Limited (Missassauga, Ont.).

Cytochrome oxidase samples were prepared for photolysis in the following manner. A 3 ml sample of oxidized, resting cytochrome *c* oxidase, in 0.2 M sodium phosphate, pH 7.4, with 1 mg/ml lauryl maltoside, was made anaerobic by incubation under a stream of prepurified N_2 gas for 15–20 min. The enzyme sample was reduced by addition of 5 mM ascorbate plus 200 μM TMPD. The sample was left for 1 h under these conditions to insure that full reduction was reached. The N_2 atmosphere was replaced with CO and the sample allowed to sit for a further 15 min, prior to being transferred under a slight positive pressure of CO to a small volume cuvette so that the sample completely filled the available volume. At this point single wavelength and multiwavelength transient data were collected. A stock solution of KCN was made at a concentration of 2–4 M in 200 mM sodium phosphate, pH 7.4, that had been previously equilibrated with N_2. Small volumes of cyanide were added to the sample to give final concentrations ranging

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Abbreviations: FTIR, Fourier transform infrared; TMPD, *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine.

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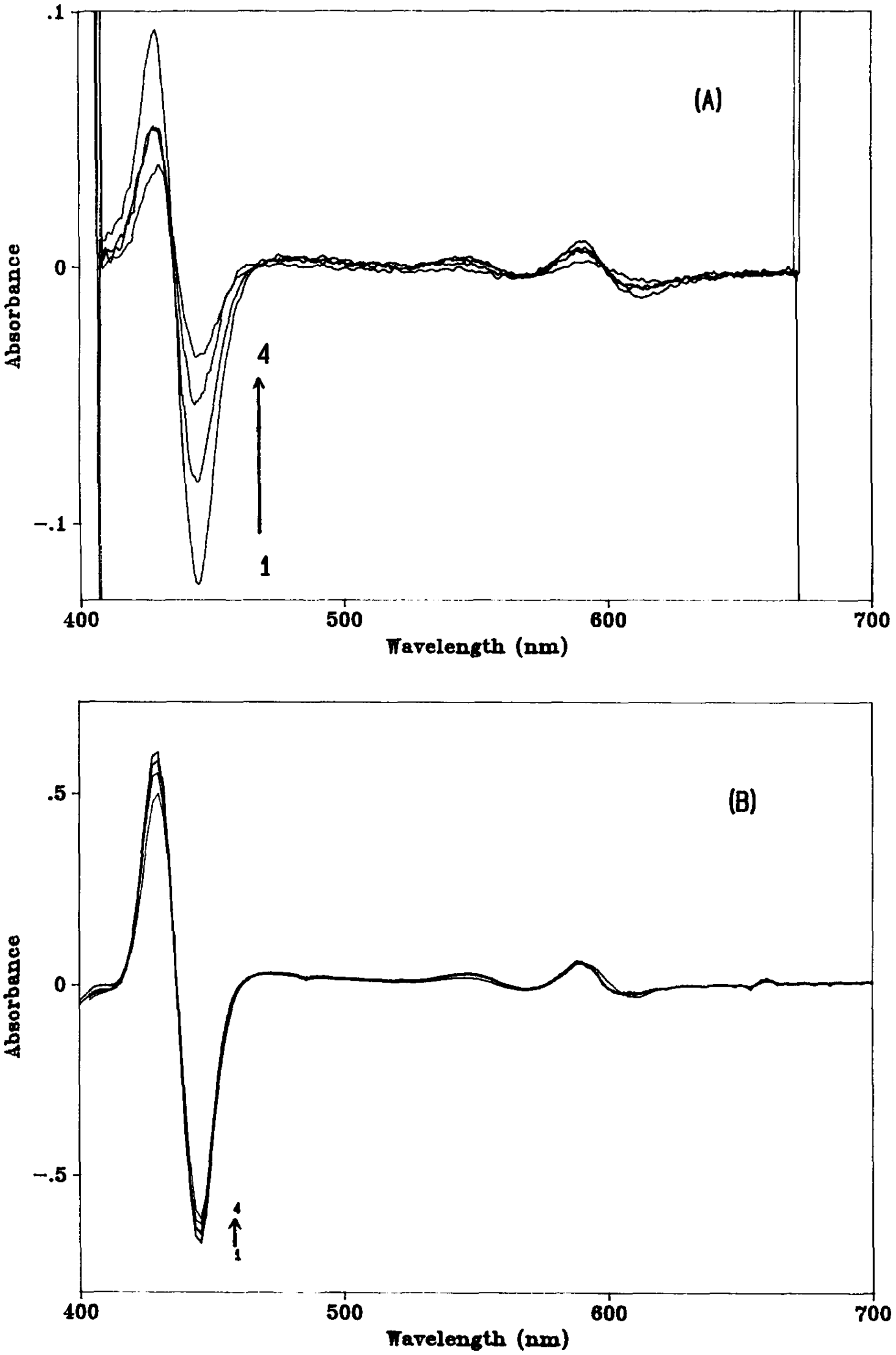


Fig. 1. The effect of cyanide on the CO complex of cytochrome *c* oxidase. (A) Kinetic difference spectra for unphotolysed-photolysed samples of the CO complex of cytochrome oxidase. Spectra 1–4 correspond to 0, 9.18, 18.2, and 36.3 mM KCN, respectively, all in the presence of 1 mM CO. The concentration of cytochrome oxidase was 9.9 μM. (B) Static difference spectra for the CO complex of cytochrome oxidase in the absence and presence of KCN. The conditions of spectra 1–4 are the same as in panel A.

from about 4 to 40 mM. At each addition of cyanide 5 μl of 1 M sodium dithionite was added to the stock cyanide to insure the transfer was anaerobic. The final concentration of dithionite ranged from 5 to 50 μM. All manipulations and measurements were carried out at 20°C.

Static spectra were obtained on a Hewlett-Packard diode array spectrophotometer (Model HP8452A). Kinetic difference spectra were obtained using a gated optical multichannel analyzer from Princeton Instruments. The 2nd harmonic output from a Q-switched, Nd:YAG laser (Lumonics, HY-400) at 532 nm with 50 mJ per 8 ns pulse, was used for photolysis. The time between the photolysis pulse and the detector gate pulse (0.27 μs), and the pulse width (0.18 μs) were controlled by a Princeton Instruments PG-10 timer. Kinetic spectra are averages of 128 single shots taken at a frequency of 2 per second. Static and kinetic spectra were plotted using the Spectracalc software package.

Single wavelength time courses were measured using the kinetic spectrometer described previously [18], except the liquid dye laser was replaced by the Nd:YAG laser. The time courses are averages of 64 single shots taken at 2 per second. The samples did not show any changes with regard to their spectral or kinetic properties over the time of data collection. Exponential analysis of the time courses was done by Asystant software using a fourth order Runge-Kutta method.

3. Results

Time-resolved optical difference spectra between unphotolysed and photolysed CO-bound oxidase show peaks at 430 and 590 nm, and troughs at 445 and 610 nm characteristic of CO photolysis from ferrocytochrome a_3 (see Fig. 1A). These features are retained when cyanide is added to the CO complex, but the intensity declines as the cyanide concentration increases. Panel B shows corresponding static, CO difference spectra taken under the same solution conditions. The intensity of the static Soret transition declines by 15% and the visible band shifts to longer wavelength at the highest cyanide concentration used in Fig. 1B. However, the main spectral features of the CO complex are still evident indicating that KCN addition has not destroyed the CO complex, but altered its form. It is concluded that cyanide, which can bind to ferrocytochrome a_3 to form a photosensitive species, is not displacing CO. This conclusion is in keeping with the relative binding constants of CO and CN for ferrocytochrome a_3, which is 1,000 to 1 in favor of CO [19].

As seen in Fig. 2 (a) CO recombination, on the µs–ms timescale, is a single exponential process the rate of which is CO concentration dependent, at least up to 1 mM CO (cf. [6]). The inset shows a representative plot of the residual differences between the kinetic data obtained here and a single exponential process with a pseudo-first-order rate of 40 s^{-1}. The process with a lifetime of 1 µs observed in multi-wavelength kinetic studies (e.g. [20]) is not observed here by single wavelength kinetics due to its small intensity. Addition of KCN does not alter the observed kinetic parameters, but does diminish the transient intensity, in Fig. 2 traces (b) by 80% for 46.4 mM KCN. As above, this observation supports the idea that cyanide has not displaced CO from its binding site, because different kinetics would be anticipated for cyanide recombination.

There is concern that the high concentrations of cyanide used in this study might lead to irreversible changes in cytochrome oxidase. Indeed exposure of the oxidase to high cyanide for prolonged periods has been used to deplete copper from the enzyme [21]. In an attempt to ascertain if the cyanide effect seen here is reversible a cyanide-exposed sample was flushed for a second time with CO. Since the KCN added would largely be in the form of HCN it should be possible to deplete much of the cyanide in this manner. The signal observed originally in the absence of cyanide is partially restored when the CO,CN-oxidase sample is flushed a second time with CO (see Fig. 2). This result indicates that the interaction of cyanide with the cytochrome c oxidase–CO complex is reversible.

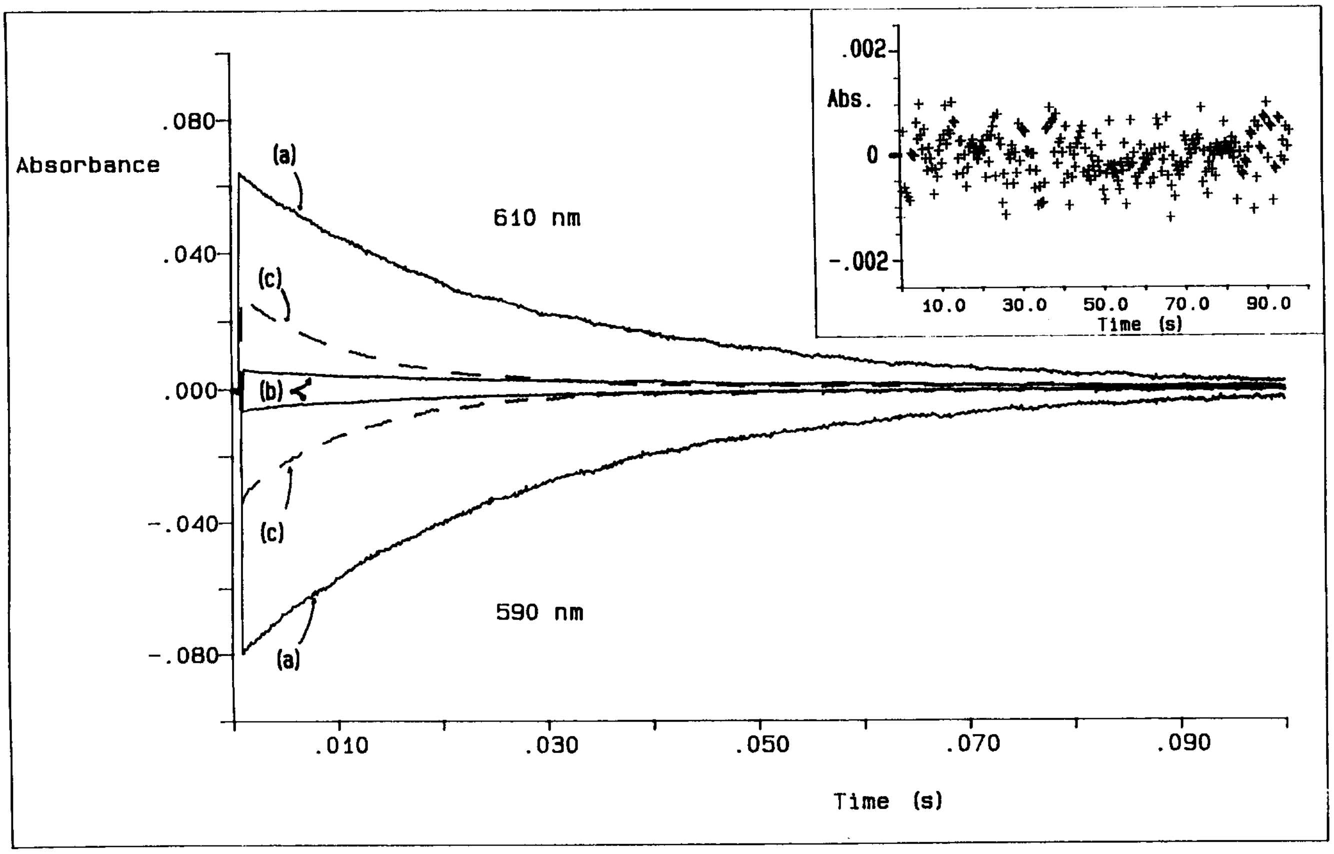


Fig. 2. Kinetics of CO recombination following photolysis of the cytochrome oxidase–CO complex. The traces showing increased absorbance upon photolysis were measured at 610 nm and those with decreased absorbance upon photolysis at 590 nm. The concentration of cytochrome oxidase was 24.5 µM and CO was 1 mM. Traces (a) were in the absence of cyanide, traces (b) plus 46.4 mM KCN, and traces (c) the same sample purged with CO for 15 min. The inset shows a plot of the residual difference between the data of trace (a) at 610 nm and a single exponential process with an first order rate of 40 s^{-1}.

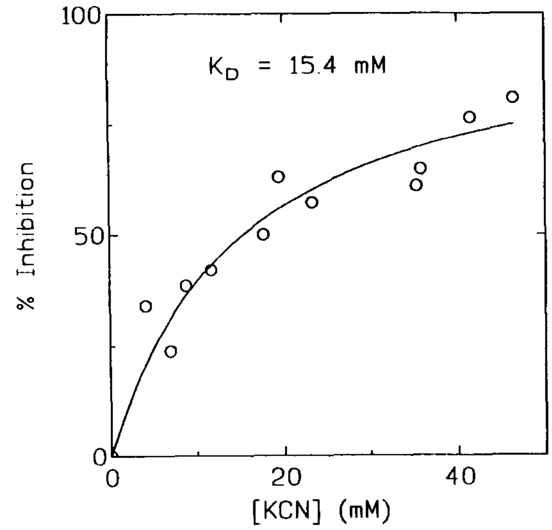


Fig. 3. Dissociation constant of cyanide binding to Cu_B. The % of the CO-photolysis transient lost in the presence of cyanide is plotted against KCN concentration. The smooth line is a non-linear least squares fit of the data to a single-site binding isotherm.

In contrast to the effect of cyanide on the CO complex of cytochrome oxidase, the addition of NaN_3 had no effect on either the spectral intensity or the kinetics of the cytochrome oxidase-CO photolysis reaction. Moreover, the addition of cyanide had no effect on the photolysis reaction of ferromyoglobin-CO. These control experiments indicate the specific nature of the interaction of cyanide with the binuclear center of cytochrome oxidase and are consistent with the view that cyanide is binding at a site nearby, but distinct from the CO site.

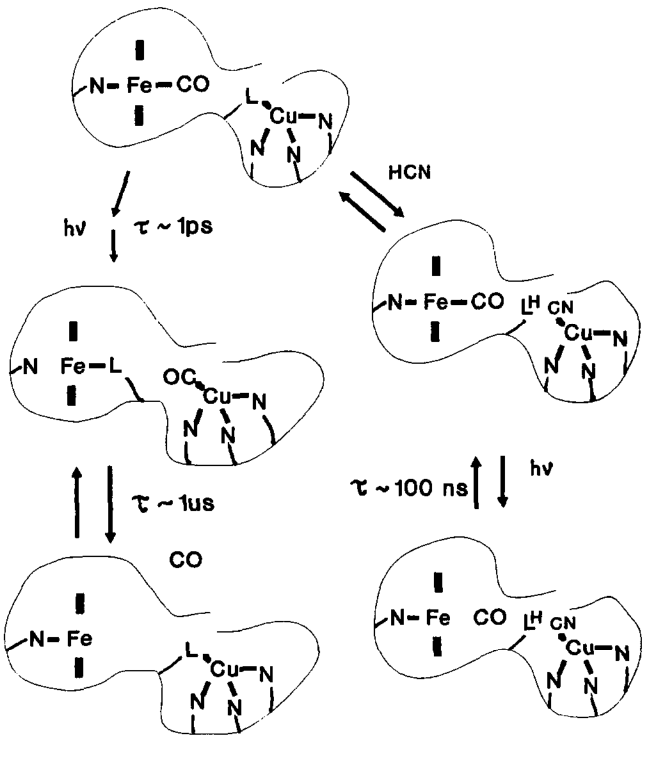
The loss in intensity of the spectral signal upon CO photolysis is plotted as a function of KCN concentration in Fig. 3. The smooth line through the data is a single site binding curve with a K_d of 15.4 mM. This binding constant is relatively weak for hemoprotein-ligand complexes, but is consistent with suggestions of binding constants for the transient complexes of CO [6] and O_2 [16] with Cu_B.

4. Discussion

Scheme 1 is based on the ligand-shuttle proposal of Woodruff [8] for the dynamics of CO binding with cytochrome *c* oxidase. This scheme starts with the conventional ferrocytochrome a_3-CO adduct of cytochrome oxidase. Along the left side some features are shown of the species seen during CO photolysis in the absence of cyanide. CO moves to Cu_B and a ligand, L, from Cu_B moves to cytochrome a_3. The overall lifetime of this process is less than 1 ps, and may involve direct transfer of CO from Fe_{a3} to Cu_B [7]. The Fe bond to imidazole is also shown to be broken by photolysis as suggested by Woodruff [8]. CO dissociation into solution occurs with a lifetime of about 1 μs. Along the right side a model for the structure of the mixed-ligand complex of CO and CN with the binuclear center of cytochrome oxidase is shown. Cyanide is shown bound to Cu_B taking up the position of the ligand L, that is proposed to undergo ligand exchange when CO is transferred to Cu_B. When cyanide is bound to Cu_B, this pathway for CO is blocked. In the photoproduct of the mixed-ligand species only the Fe-CO bond is shown to be photolysed. Geminate recombination of CO with ferrocytochrome a_3 is suggested to occur with a lifetime less than 100 ns.

In keeping with the principle of electroneutrality in ligand binding by cytochrome oxidase (e.g. [22]) cyanide binding to Cu_B is shown to be accompanied by proton uptake. It is proposed that this proton uptake takes place to ligand L and this may be an intimate feature of the subsequent change in the photolabilty of the cytochrome a_3-CO adduct. This model implies that pH studies of this complex will be informative and these are underway at present. The cyanide complex of ferrocytochrome a_3 is also known to have a low quantum yield for photolysis [19] and this could also be moderated by coupled proton binding to L, or by a second CN binding to Cu_B.

Cytochrome oxidase has an apparent quantum yield for photolysis of CO that is near one. The same is true for the complex of CO with ferromyoglobin, but for a different reason. In the case of cytochrome oxidase the Fe-CO adduct is efficiently photolysed and geminate recombination is precluded by the efficient transfer of CO to Cu_B. In contrast, the near unity value for the photolysis of myoglobin-CO reflects the lack of any substantial barrier between recently photolysed CO, and CO molecules in the bulk solvent [23]. The results reported here for cytochrome oxidase imply that CO dissociation proceeds through Cu_B and that there is no other easy way for CO to escape to the solvent phase. Presumably, this reactivity reflects the arrangement of the protein pocket around the binuclear center and that this arrangement defines a very precise pathway for ligands into and out of the active site of cytochrome oxidase. Ligand binding must involve at least transient interaction with



Scheme 1. CO and CN binding to the binuclear center of cytochrome oxidase. The left side shows the fate of CO following photolysis in the absence of cyanide. The right side proposes how CN might bind to Cu_B and effect the subsequent photolysis of CO from cytochrome a_3.

Cu_B and if this Cu_B site is occupied access to cytochrome a_3 is blocked.

This scheme predicts an unusual photoproduct on the ns or less timescale for photolysis of CO from cytochrome oxidase in the presence of KCN. The detection system used here is limited to a time resolution of 0.14 μs, but ns and ps studies are being pursued. In addition, work at high CO pressures to raise the CO solution concentration to the 10 mM range show that a second molecule of CO can, presumably, be bound at the binuclear center [6]. This scheme predicts that such a species would have a much reduced apparent quantum yield relative to the conventional complex with one CO molecule bound at the binuclear center.

References

[1] Babcock, G.T. and Wikström, M. (1992) Nature 356, 301–309.

[2] Chance, B., Schoener, B. and Yonetani, T. (1965) in: Oxidases and Related Redox Systems (King, T.E., Mason, H.S. and Morrison, M. Eds.) Vol. 2, pp. 609–614, John Wiley & Sons, New York.

[3] Alben, J., Moh, P.P., Fiamingo, F.G. and Altschuld, R.A. (1981) Proc. Natl. Acad. Sci. USA 78, 234–237.

[4] Dyer, R.B., Einarsdóttir, Ó., Killough, P.M., López-Garriga, J.J. and Woodruff, W.H. (1989) J. Am. Chem. Soc. 111, 7657–7659.

[5] Dyer, R.B., Peterson, K.A., Stoutland, P.O. and Woodruff, W.H. (1991) J. Am. Chem. Soc. 113, 6276–6277.

[6] Einarsdóttir, Ó., Dyer, R.B., Lemon, D.D., Killough, P.M., Hubig, S.M., Atherton, S.J., López-Garriga, J.J., Palmer, G. and Woodruff, W.H. (1993) Biochemistry 32, 12013–12024.

[7] Dyer, R.B., Peterson, K.A., Stoutland, P.O. and Woodruff, W.H. (1994) Biochemistry 33, 500–507.

[8] Woodruff, W.H. (1993) J. Bioenerg. Biomembr. 25, 177–188.

[9] Hill, B.C., Brittain, T., Eglinton, D.G., Gadsby, P.M., Greenwood, C., Nicholls, P., Peterson, J., Thomson, A.J. and Woon, T.C. (1983) Biochem. J. 215, 57–66.

[10] Tsubaki, M. and Yoshikawa, S. (1993) Biochemistry 32, 164–173, (see Correction, Biochemistry 32, 926).

[11] Caughey, W.S., Dong, A., Sampath, V., Yoshikawa, S. and Zhao, X.-J. (1993) J. Bioenerg. Biomembr. 25, 81–91.

[12] Yoshikawa, S. and Caughey, W.S. (1990) J. Biol. Chem. 265, 7945–7958.

[13] Berka, V., Vygodina, T., Musatov, A., Nicholls, P. and Konstantinov, A.A. (1993) FEBS Lett. 315, 237–241.

[14] Blackmore, R.S., Greenwood, C. and Gibson, Q.H. (1991) J. Biol. Chem. 266, 19245–19249.

[15] Oliveberg, M. and Malmström, B.G. (1992) Biochemistry 31, 3560–3563.

[16] Verkhovshy, M.I., Morgan, J.E. and Wikström, M. (1994) Biochemistry 33, 3079–3086

[17] Kuboyama, M., Yong, F.C. and King, T.E. (1972) J. Biol. Chem. 247, 6357–6383.

[18] Hill, B.C. (1991) J. Biol. Chem. 266, 2219–2226.

[19] Hill, B.C. and Marmor, S. (1991) Biochem. J. 279, 355–360.

[20] Georgiadis, K.E., Jhon, N.-I. and Einarsdóttir, O. (1994) Biochemistry 33, 9245–9256.

[21] Weintraub, S.T. and Wharton, D.C. (1981) J. Biol. Chem. 256, 1669–1676.

[22] Mitchell, R. and Rich, P.R. (1994) Biochim. Biophys. Acta 1186, 19–26.

[23] Henry, E.R., Sommer, J.H., Hofrichter, J. and Eaton, W.A. (1983) J. Mol. Biol. 166, 443–451.