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AUTHOR(S) R. Brian Dyer, CLS-4
Kristen A. Peterson, CLS-4
Page O. Stoutland, INC-4
William H. Woodruff, INC-4

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Time-resolved infrared studies of the dynamics of ligand binding to cytochrome *c* oxidase.

R. Brian Dyer*, Kristen A. Peterson, Page O. Stoutland and William H. Woodruff*

Photochemistry and Photophysics Group (CLS-4, Mail Stop J567)
Isotope and Structural Chemistry Group (INC-4, Mail Stop C345)
University of California, Los Alamos National Laboratory, Los Alamos, NM 87545

ABSTRACT

Time-resolved infrared spectroscopy (TRIRS) has been employed to study the reactions of small molecules with the cytochrome a3-Cu_B site of cytochrome *c* oxidase (CcO). All phases of these reactions have been investigated, from ultrafast phenomena (hundreds of femtoseconds) to relatively slow processes (milliseconds). The ligation dynamics immediately following photodissociation have been studied using a TRIR technique with time resolution of less than 1 ps. The rate of photoinitiated transfer of CO from Fe_{a3}^{2+} to Cu_B^+ was measured directly by monitoring the development of the transient Cu_B^+-CO absorption. The development of a stationary Cu_B^+-CO spectrum which is constant until the CO dissociates from Cu_B^+ occurs in less than 1 ps, indicating that the photoinitiated transfer of CO is remarkably fast. This unprecedented ligand transfer rate has profound implications with regard to the structure and dynamics of the cytochrome a3-Cu_B site, the functional architecture of the protein and coordination dynamics in general. The photodissociation and recombination of CN^- has also been studied using a real-time TRIR technique. The CN^- recombination rate of 430 s^{-1} is consistent with a recombination pathway similar to the one we have previously proposed for CO. We suggest the rate determining step for CN^- recombination is the thermal dissociation of the Fe_{a3}^{2+}-L bond.

1. INTRODUCTION

Cytochrome *c* Oxidase (CcO or ferrocytochrome *c*: dioxygen oxidoreductase, EC 1.9.3.1) is a complex transmembrane protein which catalyzes the rapid reduction of dioxygen to water by cytochrome *c*, a key reaction in aerobic energy production in all eukaryotes and many prokaryotes. The considerable energy produced in this reaction (nearly 0.5 V or 40 kJ/mole) is conserved by the enzyme as a transmembrane proton gradient which it produces via uptake of protons in the course of the reduction of O_2 to H_2O and by active translocation of protons in a redox-linked proton pumping process.[1] A detailed understanding of the molecular basis of these functions, including the relevant structures and mechanisms, is generally lacking despite the enormous effort directed towards this end.[2] The dioxygen reduction site is accepted to be the bimetallic cytochrome a3-Cu_B center. The ligation reactions of small molecules such as CO and CN^- with the cytochrome a3-Cu_B site exemplify the mechanisms available to O_2, potentially revealing the molecular details of the enzyme function in the activation of O_2, in effecting its reduction to water and perhaps in conserving the energy of the redox reaction.[3] Infrared spectroscopy is uniquely suited as a probe for these processes, particularly the behavior of Cu_B^+, which generally is not observable by other spectroscopies.[4,5] Using time-resolved infrared (TRIR) spectroscopy and kinetics measurements we have recently shown that coordination to Cu_B^+ is an obligatory mechanistic step for CO entering the a3 heme site and departing the protein after photodissociation.[3,6,7] The early time dynamics of the CO transfer from cytochrome a3 to Cu_B^+ were not observed on the timescale of these measurements (10^{-7} s). Here we report a picosecond TRIR study of the ligation dynamics immediately following photodissociation.

We have also explored the fate of photodissociated ligands on longer timescales in real-time TRIR experiments. A unique feature of CcO is that geminate recombination of CO is not observed on any timescale. Real time TRIR measurements have revealed that the protein erects a long lived (milliseconds) barrier to CO recombination with Fe_{a3}^{2+}.[3,6] We have attributed the formation of this barrier to the binding of an endogenous ligand L to Fe_{a3}^{2+}, triggered by the transfer of CO to Cu_B^+.[3] The rate of recombination of photodissociated CO is therefore determined by the rate of thermal breaking of the Fe_{a3}^{2+}-L bond. We have initiated real-time TRIR studies with ligands other than CO to test the generality of these mechanisms. Cyanide is another ligand with accessible infrared absorptions and which binds to CcO, acting as a potent inhibitor of respiration. Here we report the use of CN^- as an infrared probe of the photodissociation and recombination dynamics of ligands bound to Fe_{a3}^{2+}.

2. MATERIALS AND METHODS

Beef heart cytochrome *c* oxidase was isolated and the CO derivative and IR samples were prepared as described previously.[6,7] The transient IR signals were optimized by exchanging the solvent with D_2O to minimize background absorbance, concentrating the enzyme as much as possible (approximately 1 mM) and adjusting the pathlength of the cell to 200 μm to give a reasonable transmission (10%) of the IR probe beam. The cyanide complex was prepared by addition of sufficient 0.5 M KCN in D_2O solution at pD 7.8 to the fully reduced enzyme solution (1 mM) to give an excess concentration (20 mM) of cyanide. Visible-Soret spectra of the infrared samples were obtained directly in the infrared cells before and after TRIR measurements, as a test of complex formation and integrity.

Real-time TRIR measurements (10^{-8} to 10^{-1} s) were obtained using a pulsed, Q-switched Nd:YAG laser pump, a cw infrared diode laser probe and a fast InSb detector, as previously described.[7] Ultrafast TRIR measurements were obtained by means of a pump-probe experiment, shown schematically in Figure 1, in which the time resolution is accomplished by optical delay. The visible pump (photodissociation) pulse (1.7 ps, 595 nm, 30 μJ) is generated by a dye laser synchronously pumped by a frequency doubled, mode-locked Nd:YAG laser (Coherent Antares 76) then amplified in a three stage dye amplifier pumped at 30 Hz and 532 nm by a Nd:YAG regenerative amplifier (Quantel RGA60). The infrared probe pulse is created by generating the difference frequency between the 532 nm pulses and the amplified dye pulse in a $LiIO_3$ crystal. Tuning the dye laser yields infrared pulses (1.7 ps, 100 nJ) tunable from 1800 to 2500 cm^{-1}. The infrared light is split into "sample" and "reference" pulses. The time of arrival of the pump pulse is adjusted using a computer controlled optical delay line (Klinger). It is then made colinear with the infrared "sample" pulse using a dichroic mirror and both are focused to a 100 μm spot on the sample. The infrared "sample" and "reference" intensities are then detected with matched InSb detectors and a dual channel boxcar integrator and ratioed to correct for shot-to-shot amplitude fluctuations. The adverse effects of long term laser drift are minimized by synchronously chopping the pump at half the repetition rate (15 Hz) to yield alternating "light" and "dark" signals from which ΔA is determined at each optical delay. Before and after each protein measurement, the zero-of-time and the instrument temporal response (dependent solely on the pump/probe cross-correlation width) are determined by substituting for the sample a Si wafer in which the pump causes an instantaneous decrease in transmission of the probe.[8] The temporal response varies with laser conditions but generally is well described by a $sech^2$ function of FWHM = 3.5 ps.

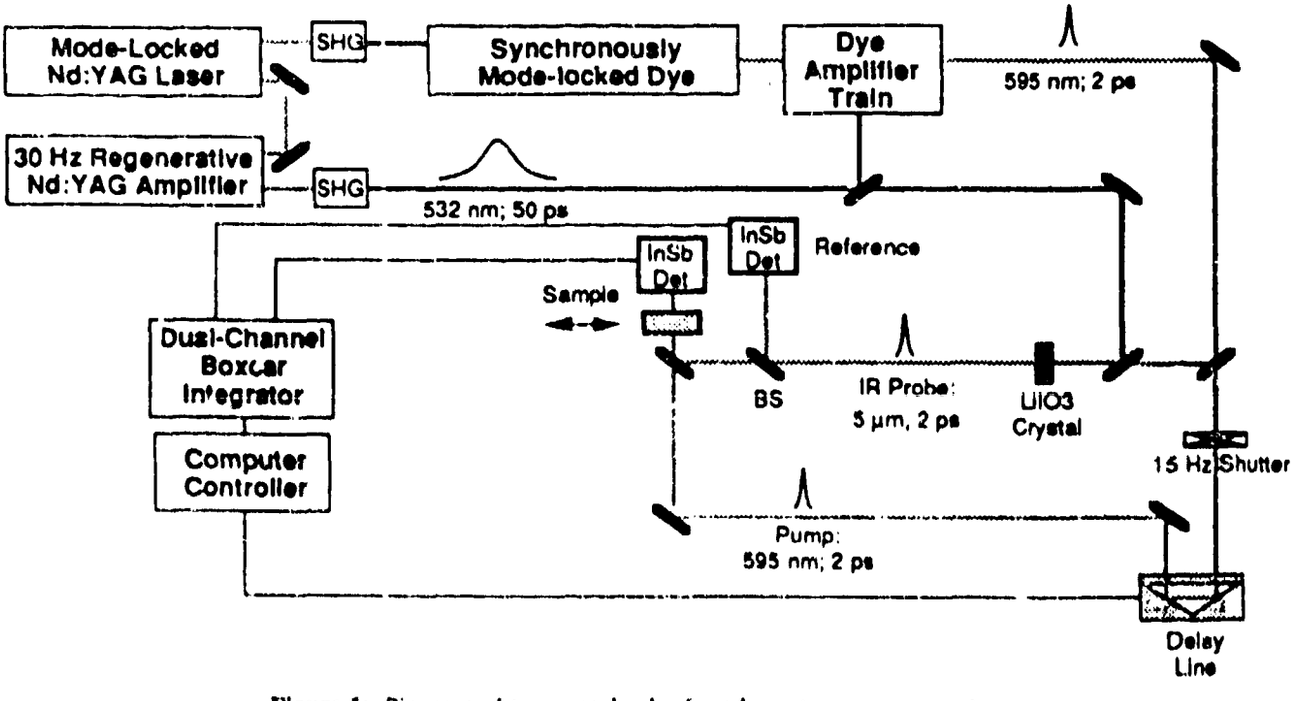

Figure 1: Picosecond time resolved infrared spectroscopy apparatus.

3. RESULTS AND DISCUSSION

We have directly observed the photodissociation of CO from cytochrome a3 by means of the picosecond TRIR spectrum. The time-resolved bleaching of the Fe_{a3}^{2+}-CO absorption at 1963 cm^{-1} is shown in Figure 2. The instantaneous instrument response, determined before and after the protein measurement using a Si wafer, is also shown with its intensity scaled to that of the protein measurement. Given the measured zero-of-time and instrument response together with the excellent signal-to-noise ratio of the TRIR data it is possible to resolve an absorption transient having a rise of 1 ps or greater. In this case however, the development of the bleach is indistinguishable from the instrument response function. Accordingly, the Fe_{a3}^{2+}-CO bleach must occur in less than 1 ps. This observation is consistent with recent UV-Vis measurements we have made on CcO, which indicate that CO photodissociation occurs in less than 150 fs, probably on the timescale of one vibrational period of the Fe-CO stretch (520 cm^{-1}, 64 fs).[9] Ultrafast photodissociation of CO has also been observed for other heme proteins, including hemoglobin and myoglobin.[10,11] The Fe_{a3}^{2+}-CO infrared transient was recorded for 100 ps following the pump pulse with no observable decrease in the bleach which could be attributed to geminate recombination, indicating that the barrier to recombination must form rapidly. The absence of rapid geminate recombination of CO is typical for heme proteins and has been attributed to electronic (spin) barriers[10] and frictional forces due to iron displacement opposing rebinding.[11] In CcO another barrier to recombination is also possible, namely the kinetic trapping of CO resulting from rapid binding to Cu_B^+. The formation of this barrier depends on a rate of transfer of the CO to Cu_B^+ which is much faster than the intrinsic heme-CO recombination rate.

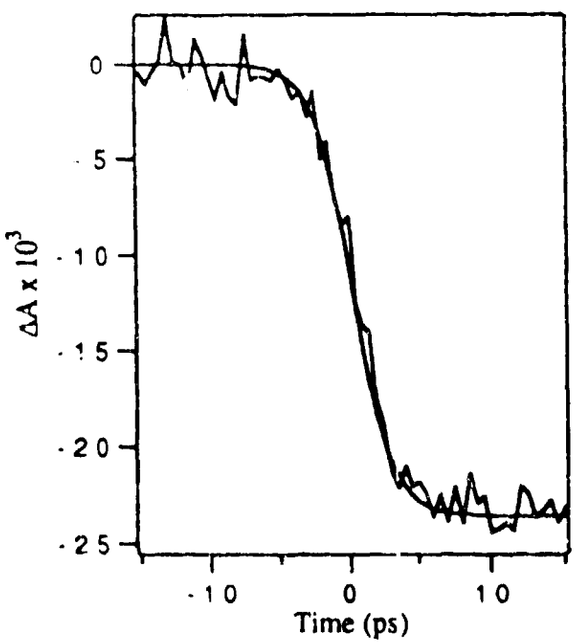

Figure 2: Time-resolved infrared transient for photodissociated carbonmonoxy CcO obtained at the peak of the Fe_{a3}^{2+}-CO absorption at 1963 cm^{-1}. The smooth trace is the instrument response function scaled to fit the ΔA of the CcO transient.

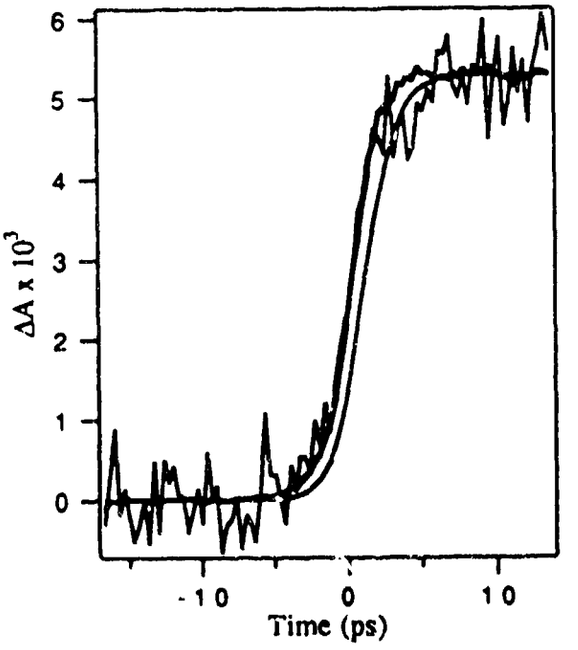

Figure 3: Time-resolved infrared transient for photodissociated carbonmonoxy CcO obtained at the peak of the Cu_B^+-CO absorption at 2051 cm^{-1}. The smooth traces are the experimentally determined instrument response function ($sech^2$, FWHM = 3.2 ps) and that function convoluted with a 1 ps exponential.

The events immediately following the photodissociation of CO from cytochrome a3 were probed in additional picosecond TRIR experiments. The rapid formation of the Cu_B^+-CO complex was observed by monitoring the appearance of an infrared transient at 2061 cm^{-1} (Figure 3), the peak of the Cu_B^+-CO absorption. The experimentally determined instrument (Si) response, which is best fit with a $sech^2$ function of FWHM = 3.2 ps, is also shown. A calculated curve which represents the convolution of the 3.2 ps $sech^2$ instrument response function with a 1 ps exponential function

describing the appearance of the Cu_B^+-CO absorption is also plotted in Figure 3. The failure of this convolution to fit the data clearly indicates that the rise of the IR transient is less than 1 ps.

The magnitude of the transient at early times is determined both by the dynamics of the CO transfer and the dynamics of evolution of the Cu_B^+-CO absorption. At later times the complex is fully formed and the absorption spectrum is completely developed consequently a *static* absorption intensity is obtained. The magnitude of the static Cu_B^+-CO absorption has been previously determined in two separate experiments. At sufficiently low temperature (< 180 K), the photodissociated CO remains on Cu_B^+ long enough to obtain a static FTIR spectrum, which was first accomplished by Alben *et al* using mitochondrial preparations[4] and later by Einarsdóttir *et al* using the purified enzyme.[6] At room temperature however, the transient is short lived ($t_{1/2}$ = 1.5 μs) and a real-time TRIR experiment with 10^{-7} s resolution was performed to observe the Cu_B^+-CO absorption before the complex begins to decay.[7] The low temperature and the room temperature measurements yield an extinction coefficient of 0.64 mM^{-1} cm^{-1} at 2061 cm^{-1}, a factor of 7 less than the Fe_{a3}^{2+} CO extinction coefficient at 1963 cm^{-1} (4.58 mM^{-1} cm^{-1}). Correlation of vibrational frequencies with absorption intensities for CO bound to heme and copper proteins shows that the relative intensities of the Fe_{a3}^{2+}-CO and Cu_B^+-CO absorbances below 180 K and at room temperature represent quantitative transfer of CO upon photodissociation. In the picosecond TRIR experiments, the observed ratio $\Delta A_{Cu\text{-}CO}/\Delta A_{Fe\text{-}CO}$ is equal to the *static* ratio when care is taken to perform the two ps TRIR measurements under the same optical conditions (beam overlap, fraction of photodissociation, etc.). This suggests that complete development of the static spectrum of Cu_B^+-CO occurs on this timescale.

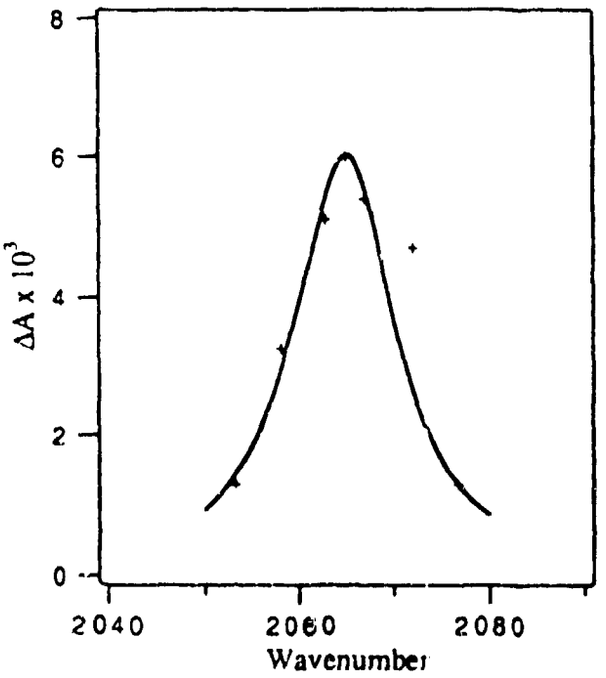

Figure 4: Five picosecond time-resolved infrared spectrum of photodissociated carbonmonoxy CcO in the Cu_B^+-CO region. The solid curve represents the sum of two lorentzians having the following band parameters: ν = 2060 cm^{-1}, Δν = 12 cm^{-1}, ε_m = 0.07 mM^{-1} cm^{-1} and ν = 2065 cm^{-1}, Δν = 12 cm^{-1}, ε_m = 0.23 mM^{-1} cm^{-1}.

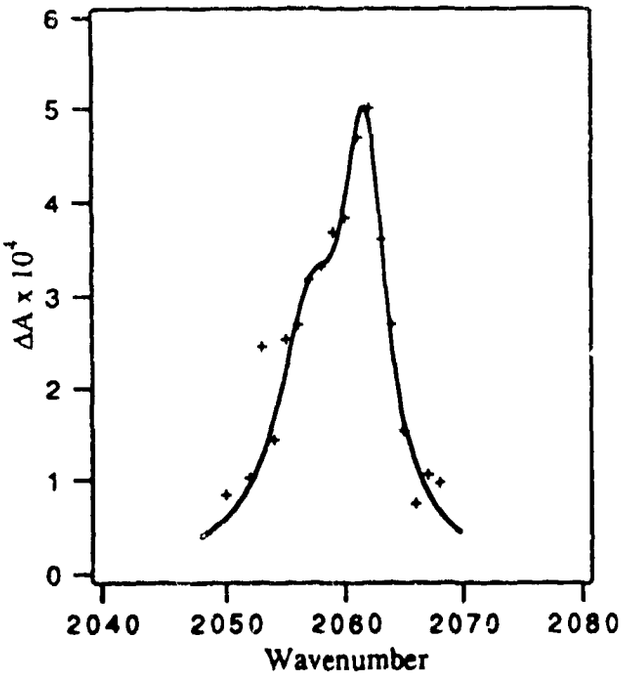

Figure 5: One hundred nanosecond time-resolved infrared spectrum of photodissociated carbonmonoxy CcO in the Cu_B^+-CO region. The solid curve represents the sum of two lorentzians having the following band parameters: ν = 2057 cm^{-1}, Δν = 6 cm^{-1}, ε_m = 0.09 mM^{-1} cm^{-1} and ν = 2062 cm^{-1}, Δν = 4.5 cm^{-1}, ε_m = 0.17 mM^{-1} cm^{-1}.

Further evidence for this interpretation is found by comparing the 5 ps TRIR spectrum shown in Figure 4 with the 100 ns TRIR spectrum shown in Figure 5. These spectra were generated in a point by point manner by obtaining transients at various probe frequencies within the Cu_B^+-CO absorption band and measuring the magnitude of the ΔA at

the appropriate delay. In the case of the 5 ps spectrum, if an earlier delay is chosen, such that the transient rise is incomplete, the spectrum obtained is essentially identical except that the intensity is slightly diminished. These spectra were simulated (solid lines) using the sum of two lorentzian lineshapes corresponding to the two components of the Cu_B^+-CO absorption seen in the high resolution low temperature FTIR spectra of the kinetically trapped Cu_B^+-CO complex.[4,6] Two distinct peaks are observed at low temperatures, a major one near 2061 cm^{-1} and a minor one near 2043 cm^{-1}, which have been suggested to arise from discrete conformers of CcO.[12] The frequency of the minor peak increases linearly with temperature in the range (4 to 180 K) observable in this experiment (Figure 6). If the data are extrapolated to room temperature, this minor component is expected to shift to near 2055 cm^{-1} and appear as an incompletely resolved shoulder. This is exactly what is observed in the room temperature transient spectra (Figures 4 and 5). The wavelengths of the individual lorentzian components used to simulate each spectrum were fixed according to the values predicted from extrapolation of the low temperature FTIR values to room temperature, while the intensities and bandwidths were varied to give the most reasonable fit to the data. Within experimental error, the parameters used to simulate the 100 ns and 5 ps spectra are the same, except that the peak widths are a factor of two greater in the case of the 5 ps spectrum. The broadening is an experimental artifact inherent to this particular picosecond TRIR technique. The effective resolution for the 5 ps spectrum is 10 cm^{-1}, determined by the transform limited width of the probe pulse, compared to 4 cm^{-1} for the 100 ns spectrum, determined by the bandpass of the monochrometer used to filter the multimode output of the IR diode probe laser. It is clear from these observations that the Cu_B^+-CO spectrum is fully developed within the 1 ps time resolution of this experiment and persists for microseconds, until the CO dissociates. We conclude that the CO is quantitatively transferred and the equilibrium Cu_B^+-CO conformation is obtained in 1 ps or less.

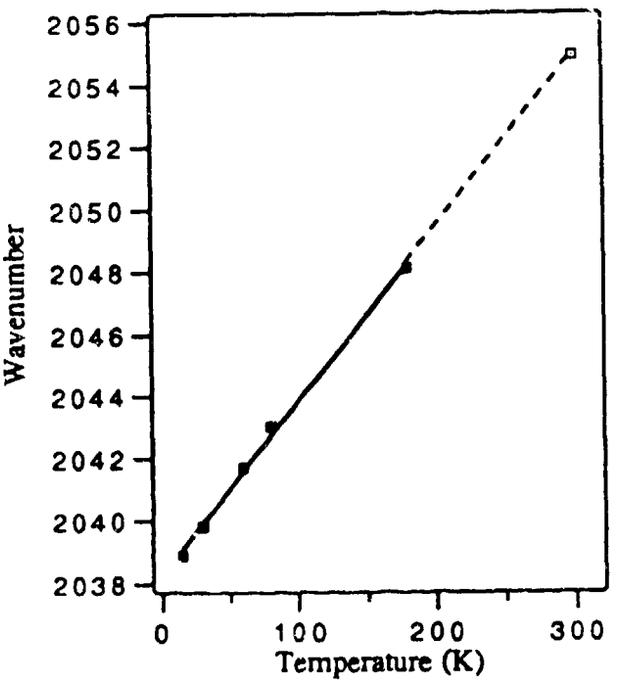

Figure 6: Dependence of the frequency of the minor Cu_B^+-CO IR peak on temperature (data from reference 12).

In order to understand the remarkable rate of transfer of CO from Fe_{a3}^{2+} to Cu_B^+ we employ a heuristic model which describes the four processes that must occur to produce the observed Cu_B^+-CO transient absorption (we emphasize that these processes need be neither separate nor sequential). These are: 1) CO dissociation from Fe_{a3}^{2+}, 2) translation and rotation of CO into position to bind to Cu_B^+, 3) formation of the Cu_B^+-CO bond and 4) evolution of the new Cu_B^+-CO absorption.

The rates of processes 1 and 4 can be predicted from direct spectroscopic measurements. The photodissociation occurs in less than 150 fs as discussed previously. Process 4, the evolution of the static lineshape and extinction coefficient, occurs on the timescale of the vibrational dephasing.[13] A lower limit for the rate of evolution of the new Cu_B^+-CO absorption can be estimated from the static Cu_B^+-CO absorption linewidth by assuming that it is homogeneous. This linewidth is 6 cm^{-1}, yielding a lower limit for development of the new stationary spectrum of 900 fs. The time necessary to form the new stationary spectrum therefore represents a significant contribution to the observed rise-time of the Cu_B^+-CO absorption.

The rate of process 2 can be estimated on the basis of the following arguments. Cu_B^+ is generally believed to be within 5 Å of the iron of cytochrome a_3.[14,15] We have measured the orientation of the CO bound to Fe_{a3}^{2+} as 21° from the heme normal compared to an angle of 51° when it is bound to Cu_B^+.[16] Molecular modeling suggests that CO will experience significantly unfavorable van derWaals contact with the heme if the CO vector of Cu_B^+-CO points towards the heme plane. The more favorable conformation has the CO vector directed away from the heme plane. The CO must

therefore move approximately 4 Å to bind to Cu_B^+, and rotate 30°. The terminal velocities of separation of photofragments are typically 10 Å/ps.[17] The energetics of CO photodissociation from HbCO have been estimated to deposit 9.4 kcal/mole of excess energy in the Fe and CO, resulting in an initial recoil velocity of ~14 Å/ps for the CO.[18] For CcO, the energy of the dissociative state ($^3T_1$ or $^5T_2$) and the Fe_{a3}-CO dissociation energy are expected to be very similar to the HbCO values, yielding a similar recoil velocity of the CO. Accordingly, in free flight, the ejected CO could move 4 Å within 300 fs. It is highly improbable, however, that the CO is able to move this far without colliding with other atoms in the heme pocket. In the case of Hb, the crystal structure indicates that the CO can move no more than 1 Å in free flight[19,20] and it has been concluded that collisions rapidly (~300 fs) render the motion of the CO diffusive.[18] Although direct evidence from crystallographic data is unavailable for CcO, the unusually narrow infrared linewidth for CO bound to Fe_{a3}^{2+} (half that in HbCO[21]) suggests a rigid, confined heme pocket.[21,22] If this restrictive heme environment results in rapid collisional thermalization of the motion of the photodissociated CO and renders its motion purely diffusive, it would move from its starting point at a rate of only 1.1 Å/ps. Clearly this picture is not consistent with the observed risetime of the Cu_B^+-CO transient which requires the motion of the CO from the Fe_{a3}^{2+} to Cu_B^+ to be closer to free flight at supersonic velocity than purely diffusive at a thermalized velocity. The photodissociation of CO is likely to produce CO in high J states as is observed for other metal carbonyl complexes.[23] As a result, the rotation of the CO is expected to be rapid and concurrent with translation to its new position.

The CO transfer includes one other process, namely Cu_B^+-CO bond formation and the associated intramolecular vibrational energy transfer. Although the CO likely suffers numerous collisions with surrounding atoms upon photodissociation, the rapid rate of CO transfer suggests that it is not completely translationally cooled. Hence it arrives at the Cu_B^+ with considerable excess energy which must be dissipated along with the energy of the Cu_B^+-CO bond formation. It is difficult to estimate the rates of such processes since the magnitude of the excess energy and the structure and vibrational coupling of the Cu_B^+-CO center are not known. The observed rise of the Cu_B^+-CO absorption requires that these processes be very rapid, at most hundreds of femtoseconds.

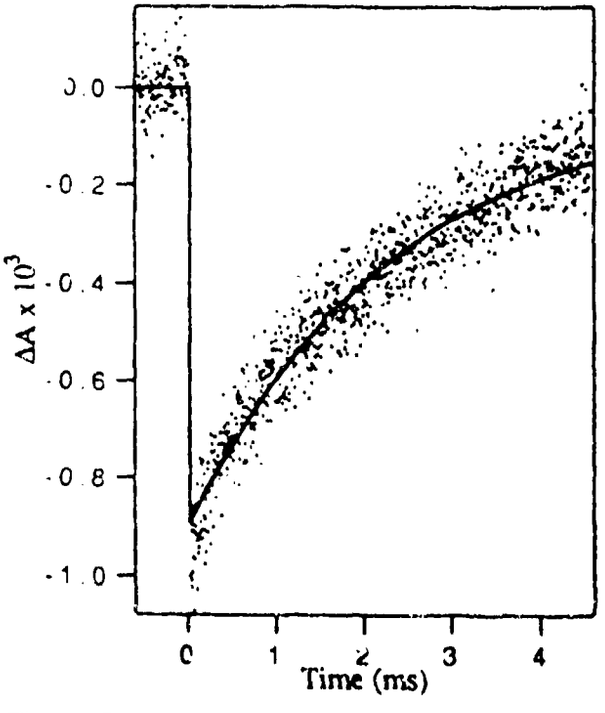

Figure 7: Time-resolved infrared transient for photodissociated, fully reduced cyanide CcO obtained near the peak of the Fe_{a3}^{2+}-CN absorption at 2059 cm^{-1} with 1 mM enzyme and a 50 μm pathlength.

Once the CO is on Cu_B^+, it remains there for 1.5 μs, then dissociates into solution. It does not return to Fe_{a3}^{2+} for milliseconds, despite the lack of intrinsic heme barriers to CO recombination on this timescale. Typical dynamics of five-coordinate hemes yield geminate CO rebinding rates on the nanosecond timescale.[10,11] Clearly, the protein has erected a substantial barrier to heme-CO rebinding subsequent to the photoinitiated transfer of CO to Cu_B^+, which we have suggested is the binding of L to Fe_{a3}^{2+} triggered by the transfer of CO to Cu_B^+.[3] The generality of these mechanisms can be tested using other small ligands which bind to Fe_{a3}^{2+}. Recently, Yoshikawa and Caughey have reported convincing infrared evidence for the binding of cyanide to the cytochrome a_3 and Cu_B metal centers of CcO.[25] They found that for the fully reduced enzyme and high CN^- concentrations, a CN^- is bound to each metal. Infrared peaks at 2058 cm^{-1} (ε = 0.22 mM^{-1} cm^{-1}) and 2092 cm^{-1} (ε = 0.15 mM^{-1} cm^{-1}) were assigned to Fe_{a3}^{2+}-CN and Cu_B^+-CN absorptions respectively. We have observed the photodissociation of CN^- from Fe_{a3}^{2+} in a real-time TRIR experiment. Figure 7 shows the transient IR bleach observed by pumping at 532 nm (7 ns pulse) and monitoring at 2059 cm^{-1}. The magnitude of the bleach ($\Delta A = 1 \times 10^{-3}$) is consistent with 100% photodissociation of the CN^- with no rapid geminate recombination. The rise of the bleach is faster than the instrument response of 30 ns. This is the first report of the dynamics of photo

dissociation of CN^- from a heme. This result emphasizes the generality of the photolabile nature of axial ligands bound to ferrous, six-coordinate hemes. The recovery of the bleach is fit well by a single exponential having $\tau = 430\ s^{-1}$. This rate is comparable to the saturation value of $700\ s^{-1}$ for the recombination of photodissociated CO, suggesting that the rate determining step for recombination is similar. The CO recombination pathway requires the formation of the Cu_B^+-CO complex in a rapid preequilibrium step, followed by the relatively slow transfer of CO to Fe_{a3}^{2+} with the loss of L by Fe_{a3}^{2+} as the rate determining step. We suggest that the CN^- on Cu_B^+ displaces L and when the CN^- on Fe_{a3}^{2+} is photodissociated, L is transferred to Fe_{a3}^{2+}. Consequently, the rate of recombination of the CN^- is also determined by the thermal rate of the Fe_{a3}^{2+}-L bond dissociation.

4. CONCLUSIONS

The remarkably fast rate of CO transfer from Fe_{a3}^{2+} to Cu_B^+ provides new insight into the structure of CcO. The results suggest that an unhindered pathway or channel is required to expedite ligand transfer from one metal center to the other. The heme pocket must be constructed in such a manner that it restricts the motion of bound CO but facilitates rapid transfer of the photodissociated CO between metal centers. The rapidity of this transfer does not allow for any barriers to CO rotation nor for any ligand reorganization at Cu_B^+. It is possible that nuclear tunneling is a factor in this extremely fast reaction.[24] In any case, the protein and metal centers must be elegantly designed to facilitate ligand transfer from Fe_{a3} to Cu_B. This feature of the protein is significant to the role of Cu_B as a ligand shuttle to Fe_{a3} in the functional dynamics of the protein. This reaction is also of interest from a fundamental standpoint in understanding the elementary steps involved in coordination and atom transfer dynamics, in the same sense that chlorophyll reaction centers have become a paradigm for understanding ultrafast electron transfer. The real-time TRIR results for the fully reduced CN^- complex provide insight to another feature of the protein, the general mechanism by which it controls the binding of exogenous ligands. These results, together with previous measurements for CO suggest that the rate determining step for ligand binding to Fe_{a3}^{2+} is the thermal breaking of the Fe_{a3}^{2+}-L bond.

5. ACKNOWLEDGEMENTS

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