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Thermodynamics of carbon monoxide photodissociation from the fully reduced cytochrome aa_3 oxidase from *Rb. sphaeroides*

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Abstract

Photodissociation of the fully reduced carbonmonoxy bound cytochrome aa_3 from *Rb. sphaeroides* results in ultrafast ligand transfer between heme a_3 and Cu_B, which is followed by thermal dissociation from Cu_B on longer time scales. We have utilized photoacoustic calorimetry to obtain a detailed thermodynamic description of the mechanism of ligand photodissociation and transfer between heme a_3 and Cu_B. Subsequent to ligand photodissociation an additional process, which has not been characterized previously, was observed with the lifetime of 485 ns at 18 °C and is coupled to a volume expansion of 3.3 ml mol^{-1}. From the temperature dependence, an activation barrier of 4 kcal mol^{-1} was determined. We attribute the observed 500 ns process to changes in Cu_B ligation subsequent to ligand translocation. In a photoacoustic study on CO photodissociation from bovine heart aa_3 oxidase, no volume changes were observed on the ns timescale, indicating that a different mechanism may control ligand dissociation and binding within the binuclear center of the bacterial and bovine enzymes.

Keywords: Photoacoustic calorimetry; Cytochrome c oxidase; CO photolysis; Respiratory protein

1. Introduction

Heme-copper oxidases represent a unique class of metalloenzymes that catalyze the four electron reduction of dioxygen to water [1–3]. The free energy released during this process is utilized to actively transport protons against a potential gradient. These enzymes require at least three core subunits to maintain catalytic competency. The largest subunit (SUI) contains the catalytic site consisting of a five-coordinate and high spin heme (designated heme x_3 where $x=a$, b or o depending upon the organism) and a three-coordinate Cu center 4.6 Å away (designated Cu_B). This subunit also contains an additional heme group that is six-coordinate and low-spin and is responsible for shuttling electrons to the binuclear center (this heme is designated heme x). An additional binuclear copper cluster (designated Cu_A) is present in all cytochrome c oxidase and is located in SUII.

The reduction of dioxygen to water has been extensively investigated using both optical and vibrational methods [4–11]. Starting from the fully oxidized enzyme at least three intermediates localized at the binuclear center have been identified. The catalytic cycle for cytochrome c oxidases (CcO) is initiated by the sequential transfer of two electrons from cytochrome c to the binuclear site via the low potential metal centers (heme a and Cu_A). At this point, dioxygen binds to heme a_3 ultimately forming an intermediate in which dioxygen has been reduced to the equivalent of an oxyferryl with a protein radical (P-state) (presumably a tyrosine radical). The next two electrons transferred from cytochrome c to the binuclear site reduce the P-intermediate to an oxyferryl species in which the tyrosine radical has been reduced (F-state) and then to a heme-bound hydroxy species (or water depending upon the protonation state). This catalytic reaction results in the consumption of four electrons on the cytosolic side of the inner-mitochondrial membrane and the consumption of four protons on the matrix side of the membrane.

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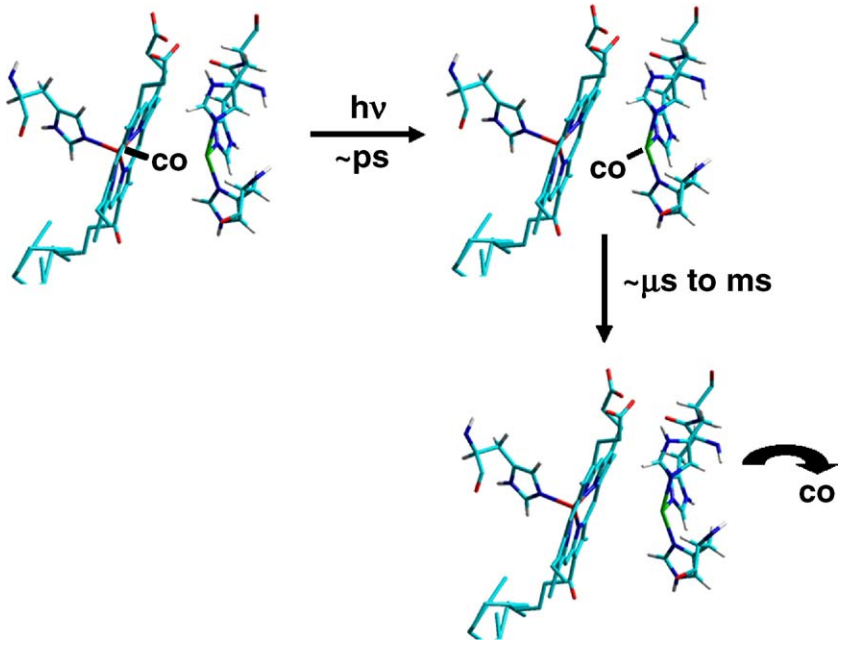
Studies using site directed mutants as well 3D structural models have also revealed two proton translocating channels that direct protons to the binuclear center [1–3]. The so-called K-channel begins with Lys319 and proceeds through Thr316, Thr309, Ser255, Tyr244 and ends at the binuclear center (bovine heart numbering). The D-channel has an entryway on the cytoplasmic side formed by Asp91 and continues through Asn98 and comes into close proximity of the binuclear center through Glu242. It is believed that the K-channel is active during the first phase of the reaction cycle in which the enzyme accepts two electrons and takes up two protons whereas the D-channel conducts protons to the binuclear center as well as to the periplasm during the reduction of various dioxygen intermediates.

The binding of dioxygen to the binuclear center represents the initial step in the catalytic cycle. Carbon monoxide provides an extremely useful probe for examining the mechanistic details of ligand binding to a wide range of heme proteins due to the high quantum yield for photolysis and the reversibility of the reaction. In the case of heme/copper oxidases, CO has been used to examine ligand binding to the fully reduced form of the enzyme as well as several mixed valence states [12–20]. These studies have revealed that, subsequent to photolysis of CO from heme x_3 CO binds transiently to Cu_B within <1 ps and thermally dissociates from Cu_B with a rate constant that is dependent on the nature of the enzyme (see Scheme 1). Cytochrome *c* oxidase from bovine heart muscle exhibits a thermal dissociation rate constant of $\sim 7\times 10^5$ s^{-1} [13], cytochrome bo_3 from *E. coli* of ~ 500 s^{-1} [15] and cytochrome ba_3 from *Thermus thermophylus* of ~ 35 s^{-1} [19].

These results are quite striking from two perspectives. First, the thermal release of CO from the Cu_B site does not result in significant rebinding to heme x_3 despite the fact that the two metal centers are only 4.6 Å apart. Second, the thermally activated off-rates for CO dissociation from Cu_B vary dramatically between organisms despite the fact that the Cu_B site appears to maintain nearly identical conformations between heme/copper oxidases of very diverse organisms. The fact that the thermally dissociated CO (dissociated from the Cu_B site) does not rebind directly with the nearby heme x_3 suggests that the barrier for ligand escape is much lower than the barrier for recombination. It has been suggested that transient protein fluctuations modulate these barriers in order to facilitate CO migration to the bulk solution [19]. The nature of the diverse CO off rates from the Cu_B of heme/copper oxidase from various organisms is not clear but may also involve transient localized conformational changes within the binuclear center subsequent to CO binding to the Cu center.

It has been previously shown that CO photo-dissociation from the binuclear center of the fully reduced CcO from bovine heart, CcO from *Paracoccus denitrificans* and cytochrome bo_3 from *E. coli* also results in perturbations to Glu (I-286) suggesting a conformational linkage between the binuclear center and the D-channel [14,16]. These perturbations are not due to changes in the protonation state. Additional perturbations to the Glu (II-89) residue have also been observed subsequent to CO photolysis from fully reduced cytochrome bo_3. It has been suggested that electrostatic coupling exists between this residue and the Lys residue making up the entrance of the K-channel [15]. Time-resolved step scan FTIR studies have suggested that the perturbations to Glu (I-286) occur faster than ~5 μs [14,15]. Interestingly, similar perturbations to Glu (I-286) are not observed in CcO from *R. sphaeroides* upon ligand transfer to Cu_B. Instead, perturbations are observed in the vicinity of Glu (I-286) upon reduction of the enzyme [14,18].

We have previously examined the thermodynamics of CO photodissociation from the fully reduced forms of both bovine heart CcO and cytochrome bo_3 from *E. coli* as well as the mixed



Scheme 1.

valence form of cytochrome bo_3 using photoacoustic calorimetry (PAC) and it is now clear that significant mechanistic differences exist in the CO photodissociation processes in heme/copper oxidases [21–23]. Here, we continue our efforts to obtain thermodynamic profiles for ligand binding processes in heme/copper oxidases by utilizing PAC to obtain molar volume and enthalpy profiles for CO photodissociation from the fully reduced form of cytochrome *c* oxidase from *Rhodobacter sphaeroides*.

2. Materials and methods

Cytochrome *c* oxidase was purified from *Rb. sphaeroides* according to Mitchell and Gennis, 1995 [24]. A 6-histidine tag on the C-terminus of subunit I allows purification in one step using Ni^{2+}–NTA affinity chromatography. The enzyme was stored as a stock solution (~150 μM) in 100 mM HEPES buffer containing 0.1% DM (pH 7.5). Samples were prepared by dilution the stock solution to ~10 μM in 50 mM HEPES buffer containing 0.05% DM (pH 7.5). The fully reduced CO bound of RbCcO was prepared by de-aerating the sample with Ar for 20 min in a septum sealed 1-cm quartz cuvette. The sample was then purged with CO for 10 min followed by the addition of sodium dithionite. Formation of the CO-RbCcO was verified by UV/VIS spectra obtained using a Shimadzu UV-2401 PC spectrophotometer. Fe(III) (4-sulphonatophenyl) Porphine (Fe4SP) (Porphyrin Products Inc.) in 50 mM HEPES buffer, 0.05% DM (pH 7.5) was used as a calorimetric reference compound for the PAC measurements. Absorbance of the sample and the reference at the excitation wavelength (532 nm) were adjusted to be 0.3.

The instrumentation and application of PAC to study the electron transfer reactions and ligand binding in proteins has been reviewed elsewhere [25–28]. PAC measurements were performed by placing a 1×1 cm quartz cuvette containing 2 mL of a sample in a temperature controlled sample holder housing a Panametric V103 transducer. Contact between the cuvette and the detector was facilitated with a thin layer of vacuum grease. Photo-dissociation of CO was achieved with a 532 nm laser pulse (Continuum Minilite I frequency double Q-switched Nd:YAG laser, 6 ns pulse, <80 μJ). The acoustic signal was amplified with an ultrasonic preamplifier (Panametrics) and recorded using an NI 5102 Oscilloscope (15 MHz) controlled by VirtualBench software (National Instrument). The PAC data were analyzed using the multiple temperature method in which sample and calorimetric reference acoustic traces are obtained as a function of temperature. The ratio of the amplitudes of the acoustic signals are then plotted versus $1/(\beta/C_p\rho)$ according to Eq. (1).

$$(S/R)E_{h\nu} = \phi E_{h\nu} = \Phi[Q + (\Delta V_{con}/(\beta/C_p\rho)], \quad (1)$$

where Φ is the quantum yield, $E_h\nu$ is the energy of the photon, Q is the heat released to the solvent, β is the coefficient of thermal expansion of the solvent (K^{-1}), C_p is the heat capacity (cal g^{-1} K^{-1}), ρ is the density (g mL^{-1}) and ΔV_{con} represents conformational/electrostriction contributions to the solution volume change. A plot of $\phi E_{h\nu}$ versus $(C_p\rho/\beta)$ gives a straight line with a slope equal to ΦV_{con} and an intercept equal to the released heat (ΦQ). Subtracting (ΦQ) from $E_{h\nu}$ gives ΔH for processes occurring faster than the time resolution of the instrument (<50 ns). The ΦQ values for subsequent kinetic processes represent $-\Delta H$ for that step (i.e., heat released). Since the acoustic transducer is sensitive to the amplitude of the acoustic waves as well as to their temporal profile, individual contributions to ΔV_{con} and Q arising from longer time kinetic events can be resolved. The observed time dependent acoustic signal $E(t)_{obs}$ is produced by the convolution of an instrument response function $T(t)$ (the reference acoustic wave) with a time dependent function of the decay processes, $H(t)$:

$$E(t)_{obs} = H(t)*T(t) \quad (2)$$

where

$$H(t) = \phi_1\exp(-\tau/t_1) + [\phi_2 k_1/(k_2 - k_1)][\exp(-\tau/t_1) - \exp(\tau/t_2)]. \quad (3)$$

$T(t)$ can be independently determined from the reference compound. The deconvolution of the signal involves estimation of the lifetime (τ_I) and preexponentional factor (ϕ_i) parameters for $H(t)$ and a convolution of estimated $H(t)$ function with the $T(t)$ function. The parameters are varied until the estimated $E(t)$ fits the $E(t)_{obs}$ based on residual and χ^2. We use a simplex parameter estimation software developed in our laboratory. Processes occurring faster than roughly 50 ns cannot be resolved in time but the integrated enthalpy and volume changes can be quantified from the amplitude of the acoustic wave.

3. Results and discussion

Fig. 1 displays an overlay of the acoustic waves for the photolysis of CO from fully reduced RbCcO as a function of temperature while an overlay the acoustic waves for CO RbCcO photolysis and the calorimetric reference Fe4SP at 20 °C are shown in Fig. 2. The observed shift in the phase of the RbCcO acoustic wave relative to the reference wave indicates a process (or processes) occurring on a time scale between ~50 ns and ~15 μs. The kinetic volume and enthalpy changes taking place faster than roughly 50 ns cannot be resolved, but their magnitudes may be determined. From deconvolution of the acoustic traces (see Materials and methods), two events were resolved subsequent to CO photodissociation, a fast phase (τ<50 ns) and the slow phase (τ=485 ns). We did not observe any kinetics on the μs time scale, which would correspond to the CO release from Cu_B in bovine heart CcO suggesting that these kinetics may be too slow to be resolved by our instrumentation.

Extraction of the molar volume and enthalpy changes for these phases was accomplished using Eq. (1) (the $\phi E_{h\nu}$ vs. $C_p\rho/\beta$ plot is shown in Fig. 3). The fast phase (which includes vibrational relaxation of the excited state of heme *a* and heme a_3, Fe–CO bond cleavage, Cu_B–CO bond formation, the low-spin to high-spin transition associated with heme a_3, and conformation changes within the protein) does not exhibit any significant volume changes and the amount of heat (Q) released to the solution corresponds to 38 kcal mol^{-1}. However, RbCcO contains two hemes that can absorb the incident photon. Photons absorbed by heme *a* will simply degrade the absorbed photon energy to heat and deposit this energy into the solution within the laser pulse while photons absorbed by heme a_3 initiate the photolysis reaction. The amount of absorbed energy by each heme depends on their relative absorbance at the

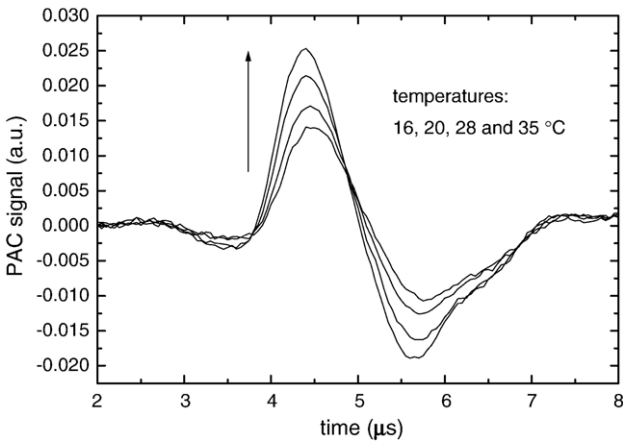


Fig. 1. Temperature dependence of the acoustic waves for fully reduced CO Rb CcO. Conditions: ~15 μM Rb CcO in 50 mM HEPES, 0.05% dodecyl maltoside, pH 7.5. The absorbance of reference and sample at 532 nm were 0.3.

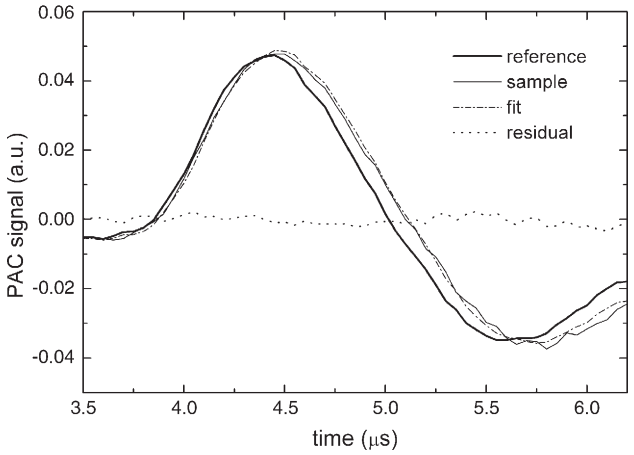


Fig. 2. Example of a double exponential fit of the acoustic waves for CO–Rb CcO at 20 °C. Shown are an overlay of the photoacoustic waves for the reference, CO–Rb CcO, best-fit sample acoustic wave using a double exponential decay scheme and the residuals are displayed. Conditions are as given in Fig. 1.

excitation wavelength. As a first approximation, we may consider that both hemes contribute equally to the total absorbance at 532 nm (excitation wavelength). Thus, the heat deposited due to the photolysis event at heme a_3 and the corresponding ΔH, can be expressed as:

$$Q_{a3} = 0.5Q_{\text{total}} = 38.3\ kcalmol^{-1}/2 = 19.2\ kcalmol^{-1}$$

$$\Delta H = E_{h\nu} - Q_{a3} = 53.7 - 19.2 = 34.5\ kcalmol^{-1}.$$

We have previously examined the thermodynamics of CO photodissociation from the fully reduced forms of both bovine heart CcO and cytochrome bo_3 from *E. coli* (Cbo) as well as the mixed valence form of cytochrome bo_3 using PAC [21–23]. The PAC results for CO-cytochrome bo_3/aa_3 photolysis give ΔH of +22.7±6.8 kcal mol^{-1}/+39±2 kcal mol^{-1} and an accompanying volume changes of −5.1±0.9 mL mol^{-1}/+7±1 mL mol^{-1} occurring in <∼50 ns [21]. In the case of CO CcO from bovine heart an additional phase has been resolved from deconvolution of the PAC signal with a lifetime of 1.7 μs (20 °C) and $\Delta H/\Delta V$ of −17±3 kcal mol^{-1}/+7±1 mL mol^{-1}. The two processes observed for CO CcO correspond to the transfer of CO form heme a_3 to Cu_B and the subsequent thermal dissociation of CO from Cu_B (∼2 μs). The volume changes associated with CO photo-dissociation from heme o_3/a_3 and binding to Cu_B include CO–Fe bond cleavage, CO–Cu_B bond formation and a low spin to high spin transition at heme o_3/a_3. These changes are expected to contribute ∼+10 mL mol^{-1} to the overall volume changes occurring <1 ps (time scale of the CO binding to Cu_B). For Cbo CO photo-dissociation from the fully reduced enzyme results in a negative volume change of −5.1 mL mol^{-1} occurring faster than 50 ns suggesting that protein conformational changes accompany the ligand transfer between heme o_3 and Cu_B (possibly conformational changes involving Glu (I-286)). Since the thermal dissociation from the Cu_B center occurs on a timescale outside the range of the PAC instrument (∼2 ms for the thermal dissociation while the long time limit for the PAC instrument is ∼15 μs), the thermodynamics of this process could not be determined. In the case of CO photolysis from CcO a volume increase is observed suggesting that the volume change associated with the heme a_3 low-spin to high-spin transition dominates the overall volume change upon CO photolysis. If a conformational change occurs resulting in a volume decrease (as suggested for the CO–Cbo system) then it is sufficiently small in CcO as to make a minimal contribution to the overall change in volume. The longer time volume expansion may be attributed to the relaxation of the protein upon ligand release from the Cu_B site since the time scale is similar to that of the Fe–His relaxation observed in resonance Raman and transient absorption experiments.

The observed enthalpy change for CO photolysis from RbCcO is close to that previously determined for CO photodissociation from Cbo heme o_3 (23 kcal mol^{-1}) [6] and bovine heart CcO heme a_3 (∼39 kcal mol^{-1}) (analysis of Q_{a3} from [22]). Previous studies have suggested that the enthalpy associated with Fe–CO bond cleavage is on the

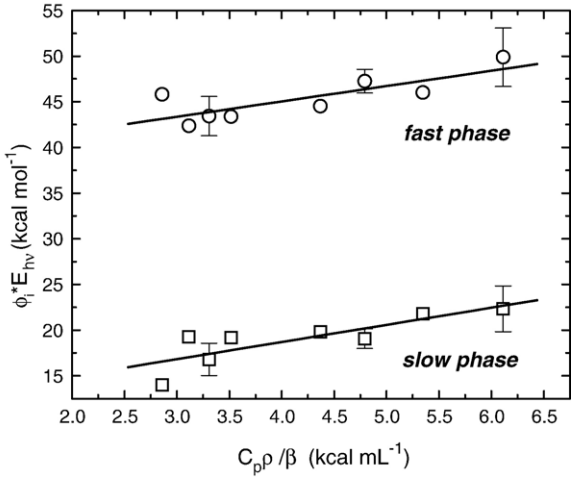


Fig. 3. Plot of ϕ_i $E_{h\nu}$ (kcal/mol) versus $C_p\rho/\beta$ (kcal/mL) for CO photodissociation from Rb CcO.

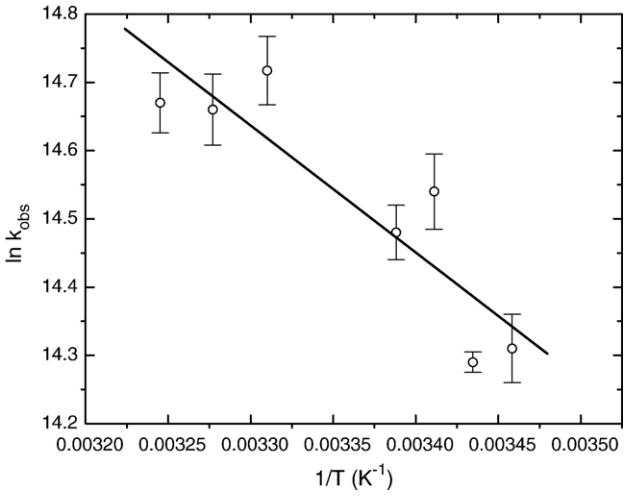


Fig. 4. Arrhenuis plot for the 500 ns phase subsequent to CO photodissociation.

Table 1
Summary of volume enthalpy changes for CO photolysis from the fully reduced forms of various heme/Cu oxidases

Species	τ_1	ΔV_1 (mL mol^{-1})	ΔH_1 (kcal mol^{-1})	τ_2	ΔV_2 (mL mol^{-1})	ΔH_1 (kcal mol^{-1})	Reference
RbCcO	<50 ns	0	35	~500 ns	+3	0	This work
Cyt. bo_3	<50 ns	−5±1	23±7	–	–	–	[21]
Bovine CcO	<50 ns	+7±1	36±5	1.5 μs	+7±1	+34±5	[22]

order of 17–20 kcal mol^{-1} while the binding of CO to the Cu_B site is estimated to be on the order of −2 kcal mol^{-1} [29,30]. Thus, of the observed 35 kcal mol^{-1}, ~15 kcal mol^{-1} can be attributed to ligand dissociation from the heme a_3 iron and subsequent CO–Cu bond formation. The origin of remaining ~20 kcal mol^{-1} is not clear. One possibility is that binding of the CO to the Cu_B site results in local structural reorganization of the Cu_B coordination sphere and/or perturbations near the heme a_3 site subsequent to CO photodissociation. In fact, previous time-resolved FTIR studies have shown significant shifts in vibrational frequencies attributed to Cu–His bonding upon the transfer of CO from heme a_3 to Cu_B in bovine heart CcO (Cu–His ν_{38},1535 cm^{-1} observed for the CO–Cu_B complex but not observed in the CO–Fe_{a3} species) occurring within the first 5 μs subsequent to photolysis [14]. Similar perturbations were observed in the FTIR spectrum for *Rb. sphaeroides* enzyme upon CO binding to Cu_B. Since this spectrum was obtained 40 ms subsequent to photolysis the lifetime of the perturbation could not be determined.

The ~500 ns phase of CO RbCcO photolysis is characterized by an expansion of ~3 ml mol^{-1} and a negligible change in enthalpy. Interestingly, this phase has not been observed in time resolved absorbance measurements indicating that the changes corresponding to this event do not lead to perturbations of the heme electronic structure. From the Arrhenius plot, the activation energy for this phase is found to be 4.1±0.9 kcal mol^{-1} (Fig. 4). The absence of any significant enthalpy change indicates that the volume expansion is mainly entropy driven. Although the precise origin of this phase is unclear it is likely that this phase involves either a relaxation at the heme a_3 center subsequent to CO photolysis or a relaxation following CO binding to Cu_B or both. Previous time resolved resonance Raman studies have shown that, subsequent to CO photolysis from fully reduced bovine heart CcO an Fe–His relaxation occurs with the time constant of ~1 μs (as evident by a relaxation of ν_{Fe-His} from ~222 cm^{-1} to ~215 cm^{-1} with 215 cm^{-1} being the equilibrium value) and that no other structural changes are observed at the heme a_3 site [31]. Thus, it is unlikely that the

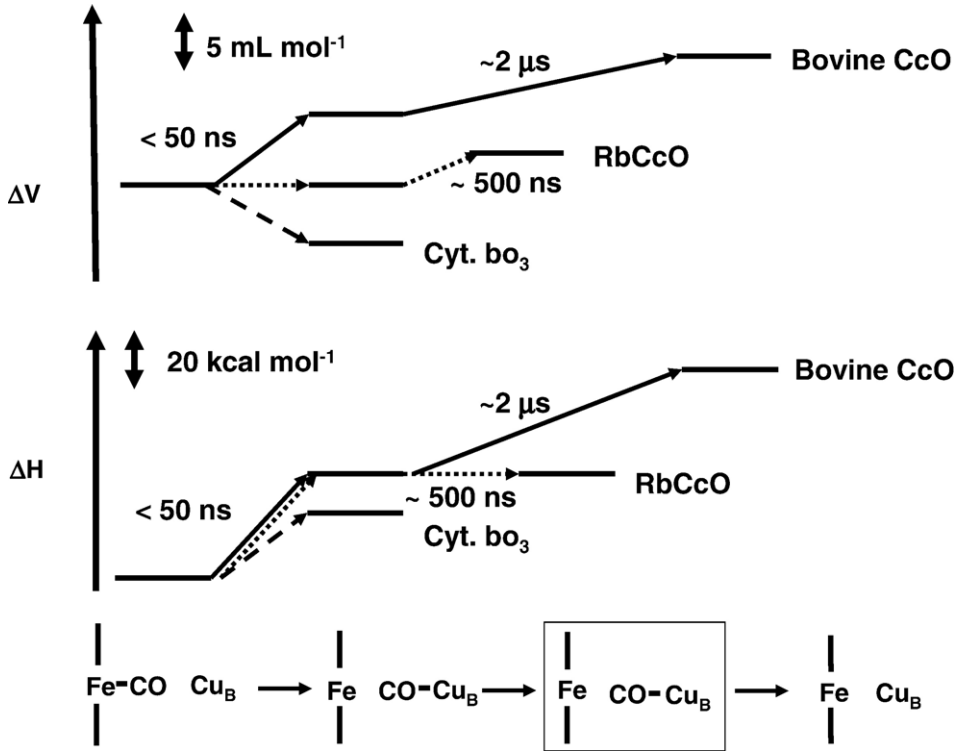


Fig. 5. Thermodynamic profiles for the photolysis of CO from the fully reduced CO bound forms of bovine heart CcO (solid lines), *E. coli* cytochrome bo_3 (dashed lines) and Rb CcO (dotted lines). The 'boxed' species represents the 500-ns transient, only observed for CORbCcO photolysis.

500 ns phase observed in the PAC experiments are due to heme a_3 relaxation.

As already mentioned, upon dissociation from heme a_3 CO binds transiently to Cu_B^{1+} prior to diffusion out of the active site. Interestingly, thermal dissociation of CO from Cu_B does not result in CO rebinding to heme a_3 which might be expected due to the close proximity of the two metal centers (4.6 Å). Thus, CO binding to Cu_B must trigger a conformational rearrangement at the Cu_B/heme a_3 site that significantly increases the energy barrier for heme a_3 ligand binding while lowering the barrier for CO diffusion out of the active site pocket. With this in mind it is important to point out that previous EXAFS studies of cytochrome bo_3 have suggested that CO dissociation from heme o_3 and subsequent binding to Cu_B leads to an increase of the bond length between the copper and two histidine ligands [32]. The fact that the 500 ns process only involves a change in volume (i.e., no enthalpic component) is consistent with this type of rearrangement rather than Cu_B–His bond dissociation. At this point it is not clear how such a rearrangement would affect the barrier for CO recombination to heme a_3.

Examination of Table 1 reveals that the <50 ns processes in bovine heart CcO and the combined <50 ns and ~500 ns events in RbCcO are similar with ΔH ~35 kcal mol^{-1} and ΔV ~+5 mL mol^{-1} suggesting that conformational perturbations to the Cu_B site upon CO transfer from heme a_3 are similar for the two enzymes but occur on different timescales. This is in clear contrast to the quinol oxidase from *E. coli* which exhibits distinct dynamics upon CO binding to Cu_B. Since both bovine heart CcO and RbCcO display similar thermodynamics subsequent to CO transfer to Cu_B it is unlikely that these dynamics involve Glu (I-286) perturbations suggesting that this event must take place on a time scale between ~50 ns and ~2 μs at which time thermal dissociation of CO from the Cu_B site takes place in the bovine enzyme (Fig. 5).

Overall, these results demonstrate that, although the family of heme/copper oxidases shares structural similarities in their active sites, the local dynamics associated with ligand migration are quite distinct. It is likely that these variations in ligand binding dynamics are responsible for the dramatically different rates for CO thermal dissociation from Cu_B and subsequent migration to the solvent.

Acknowledgements

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