Volume Changes Associated with CO Photodissociation from Fully Reduced Cytochrome *bo*₃ from *Escherichia coli*

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Received April 6, 1998

Terminal oxidases represent a unique class of heme proteins capable of transducing redox free energy into a transmembrane electrochemical gradient that is subsequently used to drive the synthesis of ATP.[1,2] These enzyme share a similar structural motif consisting of at least three core subunits and both redox active heme and copper centers. What is particularly intriguing is the considerable sequence homology among heme/copper oxidases from a wide array of aerobic organisms.[2,3] The recent X-ray structures of cytochrome *c* oxidase from bovine heart muscle[4] and from the aerobic bacterium *Paracoccus denitrificans*[5] as well as mutagenesis results with cytochrome bo_3 from *Escherichia coli*[6,7] and *Rhodobacter sphaeroides*,[8−11] coupled with data from a multitude of biophysical techniques[2,12] has shed considerable light into the mechanism of enzyme function. It is clear that in some manner events at the heme/copper binuclear center are coupled to the proton pump mechanism.[13−16] Thus, the relationship between ligand binding to the binuclear dioxygen reduction site and conformational changes within the enzyme that could be associated with proton uptake, either for active proton transport or for dioxygen reduction, is of considerable interest.

Time-resolved optical and vibrational spectroscopies have been employed to probe the ligand binding reactions of both the cytochrome *c* oxidases (CcO) and quinol oxidases, such as cytochrome bo_3 (CbO).[12,17−20] What has emerged from these studies is a mechanism (Figure 1) in which CO, photodissociated from heme a_3/o_3, binds to the nearby Cu_B site with a $t_{1/2} < 1$ ps, followed by dissociation and diffusion of CO into the bulk solvent. For bovine CcO, the half-time for the dissociation of CO from Cu_B is on the order of 1 μs, but for CbO, the rate of dissociation appears to be considerably slower.[19] Furthermore, the bimolecular rate constant for the formation of the Cu_B−CO complex is about 100-fold slower for CbO than for the bovine oxidase.[19] It is clear that CO must bind to Cu_B as a necessary step prior to binding to the heme Fe, but the details of the kinetics of CO binding vary significantly among the different heme/copper oxidases.

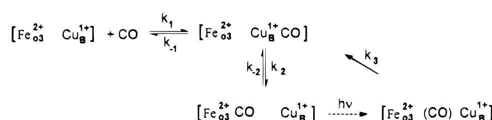

Figure 1. Mechanism of CO binding to heme/copper terminal oxidases.

In the present study we have employed photoacoustic calorimetry (PAC) to probe volume changes associated with CO binding to fully reduced cytochrome bo_3 from *E. coli*.[21] Photoacoustic calorimetry is proving to be powerful technique for determining the magnitude and time scale of conformational changes as well as reaction thermodynamics in proteins and enzymes. For example, PAC has been previously employed to investigate conformational changes associated with ligand photolysis from hemoglobin, myoglobin, and cytochrome P450 as well as the catalytic cycle of rhodopsin and bacteriorhodopsin.[22−25] The physical principle behind PAC is that photoexcited molecules dissipate excess energy via vibrational relaxation to the ground state accompanied by thermal heating of the surrounding solvent. For solvents such as water, this causes rapid volume expansion resulting in an acoustic wave that can be detected with a sensitive piezoelectric crystal-based microphone. In addition, volume changes in the system of interest resulting from a photoinitiated reaction also contribute to the acoustic wave. The resulting acoustic signal can be written as

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(21) Purified Cbo was purified from *E. coli* strain GO105/pJRHISA. The enzyme contains a histidine tag extending subunit II by seven amino acids and is used for single-step purification. The enzyme is stored as stock solutions (∼150 μM) in 100 mM HEPES buffer containing 0.1% lauryl maltoside. PAC signals were obtained by placing a 1 × 1 cm quartz optical cuvette containing the samples on the center of our photoacoustic detector. The acoustic detector is based upon a Transducer Products PZT-2H piezoelectric crystal (2 mm in diameter) that is silvered on each end. The crystal is housed in an aluminum case such that one side of the crystal makes contact with the top of the cylinder. The crystal is held in place by a steel spring which also serves as the signal carrier to a BNC connector. Sound waves generated by pulsed laser photolysis (Continium SureLite II frequency doubled Nd: YAG laser, 7 ns, ∼80 mJ/pulse) of the sample result in compression of the crystal and generation of a voltage between the two sides that is amplified and recorded using a 200-MHz transient digitizer (Tektronix RTD710A). The trigger for the digitizer is supplied by a photodiode sensing the laser pulse. This ensures correct timing of the data collection.
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S0002-7863(98)01143-3 CCC: $15.00 © 1998 American Chemical Society
Published on Web 08/18/1998

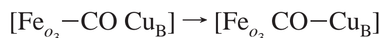
$$\phi E_{h\nu} = (S/S_{ref})E_{h\nu} = Q + \{\Delta V_{con}/F(T)\} \quad \text{(I)}$$

where $F(T) = (\beta/C_p\rho)$ (β is the coefficient of thermal expansion of the solvent (K^{-1}), C_p is the heat capacity (cal/(g-K)), ρ is the density (g/mL)), Q is the amount of heat released to the solvent subsequent to photolysis, and $E_{h\nu}$ is the energy of the excitation photon.[22,23] A plot of the normalized photoacoustic amplitude versus $1/F(T)$ gives a straight line with a slope equal to ΔV_{con} associated with a change in conformation and an intercept equal to the heat evolved (Q). Subtracting Q from $E_{h\nu}$ gives ΔH for the reaction.

Figure 2 displays an overlay of the acoustics waves generated by a reference compound (bromocresol purple) that degrades the absorbed photon into heat only (i.e., no conformational change) and for the photolysis of fully reduced CO-bound CbO at 24° C (left panel). The fact that the two waves overlap in time indicates that the process that gives rise to volume/enthalpy changes after photolysis occurs within the response time of the instrument (~50 ns). A plot of $(S_{samp}/S_{cal})E_{h\nu}$ vs $(C_p\rho/\beta)$ (where S_{samp} is the amplitude of the CO−CbO signal (mV) and S_{cal} is the amplitude of the reference signal (mV)) is shown in Figure 2, right. The slope of the line corresponds to a volume change of -5.1 ± 0.9 mL/mol for CbO upon photodissociation of CO. The intercept of the line is 59.5 (at $1/F(T) = 0$) and corresponds to the amount of energy released to the solvent as heat. Thus, $\Delta H = E_{h\nu}$ (amount of energy absorbed) $- Q$. However, at 532 nm, heme *b* and heme o_3 make roughly equal contributions to the total absorbance. Thus, the acoustic wave for CO−CbO is a combination of an acoustic wave derived from heme *b*, originating from vibrational relaxation of the heme excited-state only, and heme o_3, originating from vibrational relaxation as well as CO bond cleavage and protein conformational change. With this in mind the acoustic signal for CO photodissociation can be written as[26]

$$(S/S_{cal})E_{h\nu} = [Q^{\text{Heme } b}(A_{\text{Heme } b}/A_{TOT}) + Q^{\text{Heme } o_3}(A_{\text{Heme } o_3}/A_{TOT})] + \Delta V_{con}{}^{\text{Heme } o_3}/(C_p\rho/\beta) \quad \text{(II)}$$

where $A_{\text{Heme } b/o_3}$ is the absorbance of heme b/o_3 at the excitation wavelength, A_{TOT} is the total absorbance at the excitation wavelength, and ΔV_{CON} is the volume change due to conformational changes. It is assumed that heme *b* does not make a significant contribution to ΔV_{CON}. The value of Q for each heme is then one-half the value of Q obtained from the plot in Figure 3 (since $A_{\text{Heme } o_3}/A_{TOT} = A_{\text{Heme } b}/A_{TOT} = 0.5$). The thermodynamic parameter ΔH for the photodissociation of CO from Cbo is then equal to $E_{h\nu} - Q^{\text{Heme } o_3}$. Using $E_{h\nu} = 53.73$ kcal/mol at 532 nm, a ΔH of 22.7 ± 6.8 kcal/mol is obtained for the photodissociation reaction. This value is very near that obtained for the dissociation of CO from $Cr(CO)_6$ ($\Delta H = 27$ kcal/mol),[27] CO−myoglobin ($\Delta H = 17.3$ kcal/mol),[23] and CO−hemoglobin ($\Delta H = 18.0$ kcal/mol)[22] as well as the thermal dissociation of CO from chelated heme ($\Delta H = 17.5$ kcal/mol).[28] However, the ΔH value obtained for the photolysis of CO−CbO actually includes the ΔH for the thermal association of CO to Cu_B. The acoustic waves of the CO−CbO overlap in time with those obtained from the reference compound, indicating that the process giving rise to ΔV occurs within ~50 ns and that no volume changes are observed between ~50 ns and ~5 μs, the longest time period detectable by piezoelectric-based detectors. Within this time, CO does not dissociate from Cu_B.[19] Thus, the reaction measured by the photoacoustic method is



$$[Fe_{o_3}{-}CO\ Cu_B] \rightarrow [Fe_{o_3}\ CO{-}Cu_B]$$

Assuming the contribution to ΔH from heme−CO bond cleavage to be ~18 kcal/mol (average value for heme model compounds), then the corresponding ΔH for CO binding to Cu_B is roughly 5 kcal/mol. This value is considerably higher than would be expected for thermal association of typical metal carbonyl complexes,[27] suggesting that a more endothermic process accompanies CO binding.

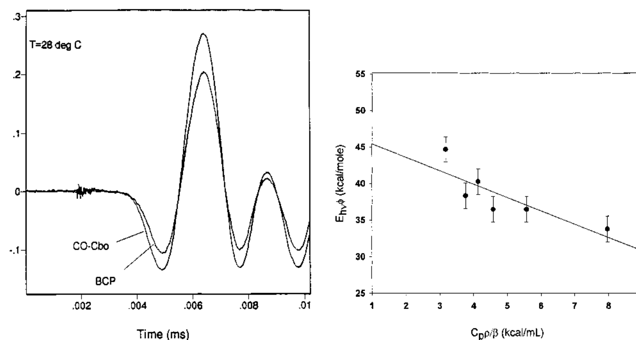


Figure 2. (left) Overlay of the photoacoustic waves for bromocresyl purple (1 mM) and CO−Cbo (75 μM) at 24 °C. (right) Plot of $E_{h\nu}\phi$ (kcal/mol) vs $C_p\rho/\beta$ (kcal/mL) for CO−Cbo. Temperature range is 15−26 °C.

Volume changes associated with this reaction include Cu_B−CO bond formation, Fe−CO bond breaking, low-spin to high-spin transition (heme o_3) and possibly ligand dissociation from Cu_B or some other alteration in the protein. Bond formation should contribute a substantial negative ΔV due to the fact that the bound ligand−metal complex is smaller than the corresponding contact pair.[29−31] As a corollary, bond breaking should contribute a significant positive ΔV. Thus, the dissociation of CO from heme o_3 and the corresponding binding of CO to Cu_B would have offsetting contributions to the overall ΔV (considering only bond formation/breaking processes). However, heme Fe low-spin to high-spin transitions have ΔV values near +10 mL/mol.[31]

The fact that the overall $\Delta V < 0$ suggests that another process must contribute a significant negative ΔV to the overall reaction. It is possible that a local protein structural change accompanies CO binding to Cu_B, resulting in an overall volume contraction. This is the case, for example, with myoglobin. Photolysis of CO from sperm whale myoglobin results in the formation of an intermediate species with an overall volume decrease (−9 mL/mol) and is believed to be due, in part, to the breaking of a salt bridge between Arg-45 and the heme propionate group. The accompanying solvation of the charged residues gives rise to electrostriction and an overall volume decrease.[22] Recent FTIR data[13−15] have clearly demonstrated protein changes in CbO, in particular at glutamate-286, that accompany the photolytic transfer of CO from the heme Fe to Cu_B. Hence, both spectroscopic data and the results of the current work indicate a structural change in the protein associated with the transfer of CO from the heme Fe to Cu_B. It is not known whether this structural change is unique to CbO, and possibly related to the differences in the kinetics of CO recombination in comparison with the bovine oxidase, or whether this phenomenon is typical of the heme/copper oxidases. Such a protein conformational change could "gate" ligand access to the binuclear center or be related to the proton pumping mechanism. Further experimentation will be required to make this determination.

Acknowledgment. The authors acknowledge an American Heart Association Grant-in-Aid (R.W.L.) and the Department of Energy (DEFG-02-87ER13716, to R.B.G.) for support of this work.

JA981143C

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