Activation volumes for intramolecular electron transfer in *Escherichia coli* cytochrome bo_3

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Abstract **In this report we describe the activation volumes associated with the heme–heme electron transfer (ET) and CO rebinding to the binuclear center subsequent to photolysis of the CO-mixed-valence derivative of *Escherichia coli* cytochrome bo_3 (Cbo). The activation volumes associated with the heme–heme ET ($k = 1.2\times10^5$ s^{-1}), and CO rebinding ($k = 57$ s^{-1}) are found to be +27.4 ml/mol and −2.6 ml/mol, respectively. The activation volume associated with the rebinding of CO is consistent with previous Cu X-ray absorption studies of Cbo where a structural change was observed at the Cu_B site (loss of a histidine ligand) due to a change in the redox state of the binuclear center. In addition, the volume of activation for the heme–heme ET was found to be quite distinct from the activation volumes obtained for heme–heme ET in bovine heart Cytochrome *c* oxidase. Differences in mechanisms/pathways for heme *b*/heme o_3 and heme *a*/heme a_3 ET are suggested based on the associated activation volumes and previously obtained Marcus parameters. © 2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.**

Key words: High pressure; Cytochrome bo_3; Cytochrome *c* oxidase; Electron transfer; Mixed valence; Activation volume

1. Introduction

Heme copper oxidases comprise a diverse class of respiratory proteins found in most aerobic organisms [1–3]. These enzymes range in molecular weight and subunit composition, and yet, have several characteristic features that are found throughout the class. The majority of heme copper oxidases contain at least three subunits (SU I, SU II, and SU III) where SU I contains the bulk of the redox active metal centers. These enzymes contain two heme chromophores and at least one copper ion. One of the two hemes is a six coordinate, low-spin heme and catalyzes the transfer of electrons to a dioxygen reduction site consisting of the second heme (five coordinate/high-spin) and a Cu_B ion. Heme copper oxidases from higher organisms contain an additional binuclear copper cluster that catalyzes the ET from cytochrome *c* to the low-spin heme chromophore. The common functionality of these proteins is the four electron reduction of dioxygen to water. In addition, it has been demonstrated that most enzymes in this class are energy transducing, i.e. redox free energy is coupled to active proton translocation against a membrane potential.

The eukaryotic bacteria, *Escherichia coli*, can express two different terminal oxidases depending upon growth conditions. At low concentrations of oxygen cytochrome *bd* is the terminal oxidase, while at higher concentrations of oxygen, cytochrome bo_3 (Cbo) is predominant [4]. Cbo is a four subunit enzyme that catalyzes the four electron reduction of dioxygen to water while actively transporting protons across the periplasmic membrane of the cell [5]. Of particular interest is the high degree of sequence similarity between Cbo and bovine heart cytochrome *c* oxidase (CcO) and the fact that both enzymes have similar crystal structures [4,6,7]. Cbo contains a six coordinate low-spin heme *b* and a binuclear center consisting of a five coordinate high-spin heme o_3 and a copper ion (Cu_B). All of the redox metal centers are located within the largest subunit of the enzyme (SU I).

Previous studies have shown that Cbo can be prepared as a mixed-valence derivative in which CO is bound to ferrous heme o_3 (i.e. Fe^{2+}/Cu^{1+}) while heme *b* remains in the Fe^{+3} form. Photolysis of CO from heme o_3 results in rapid ET from heme o_3 to heme *b* with a rate constant of $\sim3\times10^5$ s^{-1} [8,9]. CcOs can also form CO-mixed-valence derivatives [10–13]. The rate constant for intramolecular ET between heme a_3 and heme *a* for these enzymes is $\sim2\times10^5$ s^{-1} [10,11]. CcOs also contain a low potential mixed-valence Cu_A center which catalyzes ET from the substrate cytochrome *c* to the low-spin cytochrome *a*. In the mixed-valence derivatives of these enzymes the observed ET from cytochrome *a* to Cu_A occurs with a rate constant of $\sim6\times10^4$ s^{-1} [10,11].

In a recent study from our laboratory, the activation volumes related to intramolecular ET processes for the mixed-valence form of the bovine heart CcO were examined [14]. The observed activation volume for ET between cytochrome *a* and cytochrome a_3 was found to be +41 ml/mol. On longer timescales, subsequent ET from cytochrome *a* to Cu_A was observed with an activation volume of +28 ml/mol. It was suggested that the activation volume associated with the ET between cytochrome *a* and cytochrome a_3 is due primarily to structural changes at the cytochrome a_3 site subsequent to a change in redox state. Additionally, the activation volume related to the ET between Cu_A and cytochrome *a* was attributed to structural changes localized at the Cu_A site. Thus, cytochrome *a* does not appear to regulate the ET processes within the bovine heart CcO.

Our laboratory has also utilized photoacoustic calorimetry

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as a tool to probe conformational dynamics associated with ligand dissociation from the fully reduced Cbo and fully reduced bovine heart CcO ($Fe^{+2}_{b/a}$ $Fe^{+2}_{o3/a3}/Cu^{+1}$) [15,16]. The volume change associated with CO dissociation from the binuclear center was found to be −5.1 ml/mol for the fully reduced Cbo. In contrast, two phases were resolved in the photodissociation of CO from the fully reduced bovine heart CcO on time-scales of <50 ns and $\sim$1.5 μs with volume changes of +13.7 ml/mol and −6.8 ml/mol, respectively. Additionally, the activation volumes for the fully reduced forms of Cbo and the bovine heart CcO were found to be +13.3 ml/mol, and −9.0 ml/mol, respectively [unpublished data]. These results clearly demonstrate conformational dynamics associated with ligand binding to heme copper oxidases and that these structural changes are distinct between heme copper oxidases from different species.

In this study, the pressure dependence of the ET between cytochrome *b* and cytochrome o_3 from the mixed-valence form of Cbo was examined to obtain the associated activation volume. For Cbo the rate constant for ET between cytochrome *b* and cytochrome o_3 and the CO rebinding rate constant are $\sim 1.2\times10^5$ s^{-1} and $\sim 8.7\times10$ s^{-1}, respectively [8,9]. The associated activation volumes for CO rebinding to the binuclear center and the ET of cytochrome *b*/cytochrome o_3 are −2.6 ml/mol and +27.4 ml/mol, respectively. Taken together, these results demonstrate clear differences in the control of ET and CO binding among members of the heme/copper oxidase family. In addition, these differences may suggest distinct mechanisms for modulating the energy transduction processes among enzymes within this class of protein.

2. Materials and methods

Cbo was purified from *E. coli* strain GO105/pJRHISA [17]. A histidine tag on subunit II of the enzyme extends it by seven amino acids, which allows purification in one step. The enzyme is stored as a stock solution ($\sim$150 μM) in 100 mM HEPES buffer containing 0.1% β-D-lauryl maltoside. Samples were prepared by diluting the stock solution to $\sim$10 μM in 50 mM HEPES buffer containing 0.05% β-D-lauryl maltoside (pH $\sim$7.5). Samples were placed in a 1-cm path length quartz cuvette, sealed with a septum cap and purged with Ar for 30 min, followed by an additional purge with CO for 20 min. Samples were then transferred to a high-pressure bomb (with an initial CO atmosphere) and sealed.

Variable pressure transient absorption spectroscopy was performed as follows. Briefly, the arc of a 150 W Xe arc lamp is passed through the sample housed in a high-pressure bomb (equipped with quartz optical windows). The light emerging from the sample is then focused onto the entrance slit of a Spex 1680B 1/4M double monochromator and detected using a Hamamatsu R928 PMT coupled to a 500 MHz pre-amplifier/amplifier system of our own design. The signal is then digitized using a Techtronix RTD710A 200 MHz transient digitizer coupled to an IBM-based PC. The photochemistry is initiated by a pulse from a frequency doubled Nd:YAG laser (Continuum SureLite I, 532 nm, 7 ns pulse width, 3 mJ/pulse) passing perpendicular to the probe spot. The pressure is regulated using a high-pressure piston, giving pressures up to 3 kbar. The resulting data was first smoothed using a five point binomial algorithm in Grams386™ and fit using either one or two exponential decay schemes with SigmaPlot™ software.

Pressure experiments were performed by bringing the sample up to the desired pressure and then allowing the sample to equilibrate for $\sim$20 min prior to the photolysis experiments. Each trace is the average of 50 laser pulses. At the end of each set of experiments the pressure was released (i.e. back to ambient) and the kinetics re-measured to insure integrity of the sample. In all experiments the kinetics observed after pressure release were identical to those obtained prior to pressurization.

3. Results and discussion

The pressure dependence of CO rebinding to cytochrome o_3 and ET between cytochrome *b* and cytochrome o_3 for the CO-mixed-valence form of Cbo was monitored by transient absorption changes at 436 nm on a 25 ms and 200 μs time-scale, respectively (Fig. 1, top panel). The data obtained on a 25 ms time-scale could be fit to a single exponential decay at ambient pressure (Fig. 1, bottom panel) with a rate constant of 57 ± 1 s^{-1} (25°C, 1 atm), consistent with CO rebinding to the heme o_3 site [9]. The data obtained on a 200 μs time-scale shows a rapid increase in absorption due to the formation of the five coordinate cytochrome o_3 species followed by an additional monophasic absorption decrease that has been previously attributed to intramolecular ET between cytochrome o_3 and cytochrome *b* [9]. The rate constant for the absorption decay was found to be $(1.2\pm0.6)\times10^5$ s^{-1} at ambient pressure (Fig. 2). The corresponding plots of ln (k_{obs}) vs. pressure for data obtained on a 25 ms (circles) and 200 μs (triangles) time-scale are shown in Fig. 2. Fits of the data to:

$$\ln(k_{obs}) = -\Delta V^{\ddagger}/RT \quad (1)$$

give activation volumes of −2.6 ml/mol and +27.4 ml/mol for the long and short time-scale transients, respectively.

3.1. CO rebinding

Preliminary studies from our laboratory on the pressure dependence of CO rebinding to fully reduced Cbo (Fe^{+2} Fe^{+2}/Cu^{+1}) from *E. coli* give an activation volume of −9.0 ml/mol (unpublished results) while CO rebinding to the mixed-valence Cbo derivative (Fe^{+3} Fe^{+2}/Cu^{+1}) has an activation volume of −2.6 ml/mol. From a variety of spectroscopic studies of the fully reduced and mixed-valence derivatives of heme/copper oxidases, a reaction mechanism for the photolysis and rebinding of the CO ligand to cytochrome x_3 (where *x* is either *a* or *o*) has been proposed (Fig. 3). Subsequent to photolysis of CO from cytochrome a_3, the CO ligand binds to the Cu_B center within a few picoseconds. The CO ligand dissociates from Cu_B with a rate constant of 4.7×10^5 s^{-1}. In CcO from bovine heart the overall rate constant for CO rebinding to cytochrome a_3 is 2×10^3 s^{-1} [18]. The same reaction mechanism is observed for the fully reduced Cbo, however, the dissociation rate of CO from Cu_B is 1.4×10^3 s^{-1}, i.e. two orders of magnitude slower than what is observed for the bovine heart CcO [19]. Additionally, the association rate constant of the Cu_B–CO complex is 7×10^5 M^{-1} s^{-1} and the rebinding rate constant of CO to the cytochrome o_3 is 190 s^{-1} [19,20]. Thus, the CO does not dissociate from Cu_B on the time-scale of heme–heme ET in the CO-mixed-valence derivative of Cbo. Recent EXAFS studies have suggested that upon CO binding to the heme o_3 the coordination geometry of $Cu_B(1+)$ is significantly altered [21,22]. The EXAFS results are consistent with the loss of one of the Cu_B histidine ligands and a weakly coordinated water molecule as well as the transfer of a Cl^- from cytochrome o_3 to Cu_B. In the absence of intramolecular ET the activation volumes for the binding of CO to the binuclear center can be analyzed by considering two processes. The first process, CO binding to Cu_B, would contribute activation volumes associated with bond formation between the CO and Cu_B, dissociation of the weakly bound histidine ligand, and dissociation of a bound water molecule.

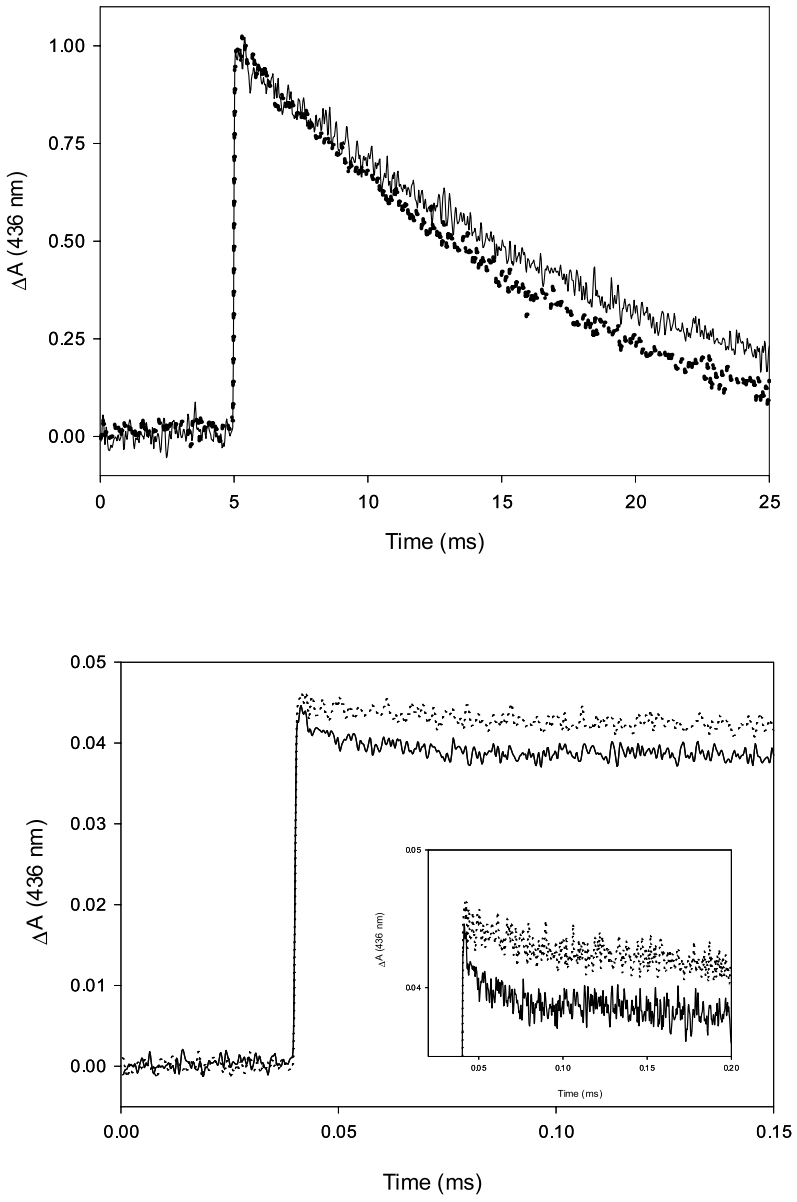

Fig. 1. Transient absorption data obtained at 436 nm for the photolysis of the CO-mixed-valence derivative of Cbo_3 as a function of pressure. Top panel: Traces obtained on a 25 ms at ambient pressure (solid trace) and 1.72 kbar (dotted trace). Bottom panel: Traces obtained on a 200 μs at ambient pressure (solid trace) and 1.72 kbar (dotted trace). Inset: Expansion of the 200 μs traces.

The subsequent transfer of CO from Cu_B to cytochrome o_3 involves Cu_B–CO bond cleavage, Fe_{o3}–Cl^- bond cleavage, Cu_B–Cl^- formation and Fe_{o3}–CO bond formation as well as a high-spin to low-spin transition at the cytochrome o_3 site. Typically, bond formation reactions contribute, on average, −15 ml/mol while bond cleavage contributes ∼5 ml/mol to the activation volume [23]. In addition, high-spin to low-spin transitions for transition metals typically contribute −10 ml/mol to the activation volume change. Using these values the activation volume for CO binding to Cu_B would be on the order of −5 ml/mol (assuming no significant protein conformational changes), while the subsequent transfer of the CO to cytochrome o_3 would have an activation volume of roughly −30 ml/mol. The observed activation volume is ∼−9 ml/mol suggesting that the rate limiting step to CO binding is the initial binding of CO to Cu_B.

In the case of CO rebinding to the CO-mixed-valence derivative an additional electron transfer step needs to be considered. Thus, the starting state for CO rebinding to the photolysed CO-mixed-valence form is the Cu_B^{2+} ion with three histidine ligands and one water molecule. Electron transfer between cytochrome o_3 and Cu_B prior to CO binding (Fe_{o3}^{2+} $Cu_B^{2+}0 \leftrightarrow Fe_{o3}^{3+}$ Cu_B^{1+}) results in the lengthening of one of the histidine–Cu_B bonds which should give a small (∼2–3 ml/mol) activation volume plus an additional small volume change due to the change in the overall charge on the Cu_B center. From the pressure dependence of charge redistribution in bacterial photosynthetic reaction centers an activation volume of −9 to −10 ml/mol is expected for the charge transfer between cytochrome o_3 and Cu_B. This would give an overall activation volume for CO binding to Cu_B of ∼−10 to −11 ml/mol (i.e. more negative activation volume than for CO binding to Cu_B in the fully reduced enzyme). The fact that the observed activation volume is much more positive suggests

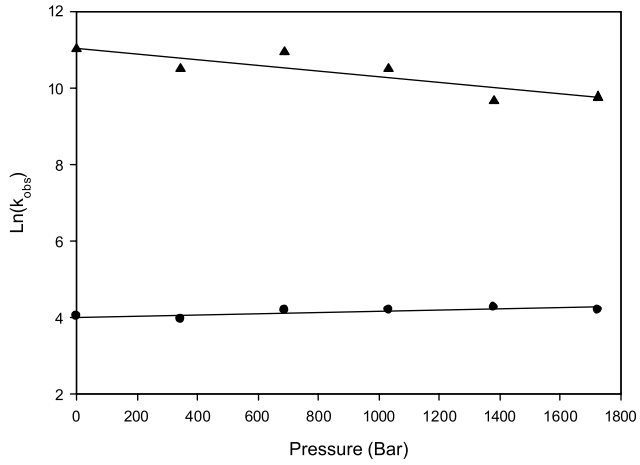

Fig. 2. Plot of ln (k_{obs}) versus pressure for the changes in absorption at 436 nm subsequent to photolysis. Triangles: 200 μs data; circles: 25 ms data.

an additional conformational process may be taking place and/or proton uptake accompanying the ET reaction ($\Delta V^{\ddagger}$ for the protonation of an acid residue would be on the order of +20 ml/mol). In fact, preliminary photothermal beam deflection evidence from our laboratory suggests proton uptake on a time-scale much faster than the CO rebinding rate (J. Miksovska and R. Larsen, unpublished results).

3.2. Electron transfer

The fast phase data can be considered within the context of semi-classical Marcus theory. The rate of intramolecular ET in Cbo can be described in the non-adiabatic limit as:

$$k = (2\pi/h)|T_{DA}^2|FC \quad (2)$$

where T_{DA} is an electronic coupling term and FC is the Franck–Condon weighted density of states [24]. The FC term is also related to the reaction free energy and the reorganizational energy by:

$$FC = (\beta/4\pi\lambda)^{1/2} \exp(-\beta(\lambda + \Delta G)^2/4\lambda) \quad (3)$$

The corresponding electronic coupling term can be expressed as:

$$T_{DA} = (H_{DA} - S_{DA}H_{DD})/(1 - |S_{DA}|^2) \quad (4)$$

where $H_{DA} = -V_A < \iota_A|\iota_D >$, $H_{DD} = -V_A < \iota_D|\iota_D >$ and $S_{DA} = < \iota_D|\iota_A >$. The relationship between the activation volume and parameters related to the intramolecular ET are described by:

$$\Delta V_{ET}^{\ddagger} = \Delta V_{FC}^{\ddagger} + \Delta V_{EC}^{\ddagger} \quad (5)$$

where $\Delta V_{FC}^{\ddagger}$ is the activation volume associated with the FC factor and the $\Delta V_{EC}^{\ddagger}$ term relates to volume changes associated with the electron coupling [25]. The $\Delta V_{FC}^{\ddagger}$ term represents the difference between the average volume of the system at the crossing point and the average volume of the system at equilibrium. The term, $\Delta V_{EC}^{\ddagger}$, is the activation volume related to the electronic coupling factor and contributes to the overall activation volume when changes in pressure cause structural changes to the coupling orbitals. Thus, the pressure effect of the coupling term is dominated by the through space gaps. Assuming that the contribution of $\Delta V_{EC}^{\ddagger}$ is negligible (which is likely to be the case for most proteins), Eq. 5 can be reduced to:

$$\Delta V = \Delta V_{FC}^{\ddagger} = ((\lambda + \Delta G)/2\lambda)\Delta V \quad (6)$$

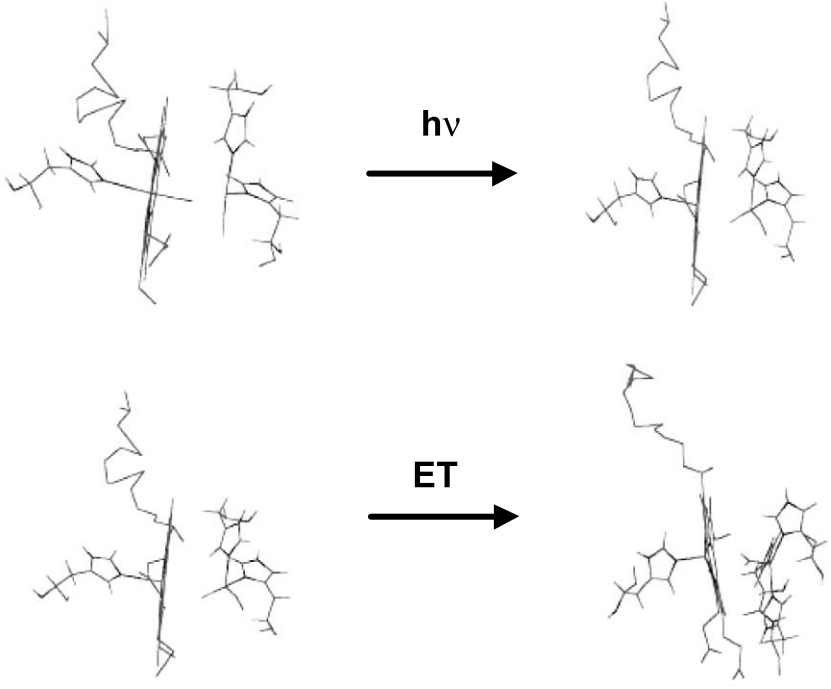

Fig. 3. Diagram showing the changes in bonding at the binuclear center of Cbo subsequent to CO binding and electron transfer. Top: Transfer of CO from heme o_3 to Cu_B subsequent to photolysis of the fully reduced binuclear center. Bottom: Changes at Cu_B subsequent to a change in redox state ($Cu_B(2+)/Cu_B(1+)$).

where ΔV is the total reaction volume.

In a recent study from our laboratory the reorganizational energy (λ) associated with the intramolecular ET between cytochrome *b*/cytochrome o_3 in Cbo from *E. coli* was determined to be 1.3 eV using $\Delta G^\circ = -22$ meV [26]. Thus, using Eq. 6 ΔV would have a value of 55.7 ml/mol and is equal to the volume changes associated with Fe_b^{+3} to Fe_b^{+2} and Fe_{o3}^{+2} to Fe_{o3}^{+3}. A previous high-pressure study of intramolecular ET in CcO [14] indicated that the volume changes associated with Fe_a^{+3} to Fe_a^{+2}, Fe_{a3}^{+2} to Fe_{a3}^{+3}, and Cu_A^{+2} to Cu_A^{+1} transitions are -106.5 ml/mol, 193.5 ml/mol, and -48.5 ml/mol respectively. An appreciable amount of the overall reaction volume for the ET between Cu_A and cytochrome *a* was attributed to the pressure dependence of the Franck–Condon factor. It was concluded that a majority of the structural changes occurring during intramolecular ET are localized at cytochrome a_3 and Cu_A. Assuming that, like in CcO, most of the structural changes due to the ET between cytochrome *b* and cytochrome o_3 occur at the binuclear center, the Fe_b^{+3} to Fe_b^{+2} volume change should be similar to the Fe_a^{+3} to Fe_a^{+2} volume change for bovine heart CcO (-106.5 ml/mol). The volume change for the oxidation of the binuclear center can then be estimated by:

$$\Delta V = \Delta V^{b(3+)\rightarrow b(2+)} + \Delta V^{o3(2+)\rightarrow o3(3+)} \quad (7)$$

then:

$$\Delta V^{o3(2+)\rightarrow o3(3+)} = \Delta V - \Delta V^{b(3+)\rightarrow b(2+)}. \quad (8)$$

This analysis gives $\Delta V^{o3(2+)\rightarrow o3(3+)} \sim 162$ ml/mol (assuming $\Delta V^{b(3+)\rightarrow b(2+)} \sim \Delta V^{a(3+)\rightarrow a(2+)}$) which is smaller than that estimated for the bovine enzyme ($\Delta V^{a3(2+)\rightarrow a3(3+)} \sim 194$ ml/mol) by roughly 30 ml/mol. This is also consistent with ligation state changes occurring at the binuclear center subsequent to intramolecular ET.

Together these results suggest significant mechanistic differences among enzymes within the heme/copper oxidase class. It is now apparent that the Cu_B site is quite labile to ligand substitution and both redox state changes and direct ligand transfer from cytochrome o_3 to Cu_B can trigger changes in the Cu_B ligand sphere. The activation volumes also demonstrate that the activation barriers for intramolecular ET are also influenced by Cu_B ligation state changes. It is interesting to note, however, that the heme/heme ET rates are quite similar between bacterial and mammalian enzymes ($\sim 2\times 10^5$ s^{-1}) despite the fact that no Cu_B ligation state changes have been observed accompanying redox state changes and/or CO binding. Thus, the role of these conformational changes in regulating intramolecular ET within the heme/copper oxidase family is unclear.

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