Activation volumes for intramolecular electron transfer in bovine heart cytochrome *c* oxidase

Randy W. Larsen*

Department of Chemistry, University of Hawaii at Manoa, 2545 The Mall, Honolulu, HI 96822, USA

Received 2 September 1999; received in revised form 2 November 1999

Abstract The present work examines the activation volumes associated with intramolecular electron transfer (ET) within the CO-mixed-valence form of bovine heart cytochrome *c* oxidase (CcO). Activation volumes for intramolecular ET between cytochrome a_3 and cytochrome *a* ($k = (6.7 \pm 0.9) \times 10^5$ s^{-1} at ambient pressure) and between cytochrome *a* and Cu_A ($k = (5.9 \pm 1.7) \times 10^4$ s^{-1}) are found to be $+41 \pm 5$ ml/mol and $+28 \pm 4$ ml/mol, respectively. Examination of the crystal structures of both the fully oxidized and fully reduced forms of bovine heart CcO suggest that the activation volume for the ET between cytochrome a_3 and cytochrome *a* arises from structural changes localized at cytochrome a_3 upon heme reduction. Similarly, the activation volume for the ET between cytochrome *a* and Cu_A is primarily due to structural changes localized at Cu_A upon reduction of this site. Reduction/oxidation of cytochrome *a* does not appear to make any significant contribution to the activation volume. Overall, these results suggest conformational regulation of ET by both Cu_A and cytochrome a_3 but not cytochrome *a*.

Key words: High pressure; Cytochrome oxidase; Electron transfer; Activation volume

1. Introduction

Cytochrome *c* oxidase (CcO) represents a unique class of biomolecules that is capable of converting redox free energy into usable potential energy that is stored across a membrane barrier [1,2]. Thus, CcO operates as a molecular machine in a fashion similar to that of other protein-based transducers such as rhodopsin and bacteriorhodopsin. Physiologically, CcO catalyzes the terminal step in the respiratory chain of a wide range of aerobic organisms including fungi, eukaryotic and prokaryotic bacteria, plants and mammals. The preponderance of CcO containing mitochondria in critical mammalian tissue such as heart, liver and brain exemplifies the enzyme's importance in maintaining health and well being of higher organisms.

The recent crystal structures of both bovine CcO as well as CcO isolated from the aerobic bacteria *Paracoccus denitrificans* coupled with site-directed mutagenesis studies with CcO isolated from *Rhodobacter sphaeroides* and the quinol oxidase from *Escherichia coli* (cytochrome bo_3) have shed considerable light onto the mechanism of redox-linked proton translocation [3–8]. It is now apparent that two proton conduction channels (referred to as K- and D-channels) connect the inner membrane surface to the binuclear center of both the *P. denitrificans* and bovine enzymes. The K-channel begins with a highly conserved lysine (K362 in *R. sphaeroides*) and ultimately connects to tyrosine 288. The D-channel begins at an aspartate residue (D132 in *R. sphaeroides*) and ends at glutamate 286. It is proposed that several water molecules then connect E286 to the binuclear center. The specific role of these two channels in proton transport has not been fully elucidated, but it is suggested that the K-channel is responsible for proton input to the binuclear center upon reduction while the D-channel may serve as a conduit for both substrate protons as well as pumped protons [9,10].

Conformational dynamics are required to complete these channels, consistent with previous models which suggest that the enzyme complex must undergo an ordered sequence of conformational transitions to complete its catalytic cycle [2]. The binding of dioxygen to the two electron-reduced binuclear center appears to induce a conformational change in the enzyme that 'activates' the proton pump cycle [2,11,12]. Additional conformational changes are then required to modulate ('gate') both electron and proton transfer as well as to physically transport the protons through the enzyme [13]. Such conformational changes are required to insure kinetic favorability of proton translocation and must be linked to a change in oxidation state of one or more of the four redox active metal sites contained within the enzyme complex. It must be pointed out that these conformational changes are likely to occur transiently during the catalytic cycle of the enzyme and in some cases, it may represent the rate limiting step in the overall reaction. Previous studies of CcO involving high pressure optical spectroscopy of steady state enzyme turnover have revealed large volume changes associated with conversion between various intermediates and redox states of the enzyme [14–16]. For example, full reduction of the enzyme results in a volume decrease of 95 ml/mol while reduction of both cytochrome *a* and Cu_A in the CO mixed-valence form of the enzyme results in a volume decrease of 155 ml/mol [16].

It is clear that elucidation of the molecular basis of energy transduction requires the determination of the magnitudes and time scales of conformational changes as well as the thermodynamics associated with substrate binding and electron transfer (ET) within CcO. With this in mind, the goal of the present work is to identify the magnitudes and time scales of volume changes and enthalpies associated with ET between various metal centers within the enzyme. CcO can be prepared in a state in which the binuclear center contains two electrons with CO bound to the ferrous heme (so called mixed-valence forms). The presence of CO at the binuclear center shifts the reduction potential of the low-spin heme such that electron redistribution does not occur. Upon photolysis, the reduction

*Corresponding author. Fax: (1) 808-956-5908.
E-mail: rlarsen@gold.chem.hawaii.edu

PII: S0014-5793(99)01517-3

potentials of the high-spin and low-spin hemes become nearly equivalent and subsequent intramolecular ET between the hemes (and possibly Cu_B) occurs. In CcO, ET between cytochrome a_3 to cytochrome a occurs with a rate constant of roughly 10^5 s^{-1} with subsequent ET between cytochrome a and Cu_A occurring with a rate constant in the order of 10^4 s^{-1} [17–20]. Further ET takes place between the heme groups on a slower time scale (roughly 800 s^{-1}) and this ET has been found to be coupled to proton uptake into the enzyme [19,20].

Determination of activation volumes associated with intramolecular ET in CcO provides important information concerning the conformational control of the ET. By utilizing the recent crystal structures of both the fully oxidized and fully reduced forms of the enzyme, information concerning the molecular origins of the activation volumes can be obtained providing clues to ET modulation within the enzyme complex.

2. Materials and methods

Bovine CcO was purified as described previously [21]. Samples were prepared by diluting the CcO stock solution (~250 μM) to ~20 μM CcO in 100 mM HEPES buffer/0.1% lauryl maltoside (pH 7.5) in a 0.5 cm path length quartz container (total volume 0.5 ml). The container was then sealed with a septum cap. The sample was then purged with Ar for 30 min (the sample was placed on ice during purging), followed by an additional purge with CO for 20 min. The sample was then transferred to a high pressure bomb (with an initial CO atmosphere) and sealed.

Pressure dependent 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 monochrometer 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 Tecktronix 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 (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 were first smoothed using a five point binominal algorithm in Grams386 and fit using either one or two exponential decay schemes with SigmaPlot software.

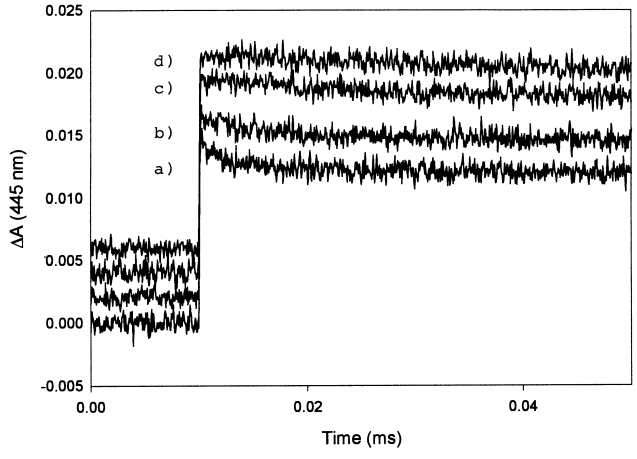

Fig. 1. Transient absorption data obtained at 445 nm for photolysis of the CO mixed-valence derivative of CcO on a 50 μs time scale as a function of pressure. Trace (a) ambient, (b) 0.69 kbar, (c) 1.38 kbar and (d) 2.07 kbar.

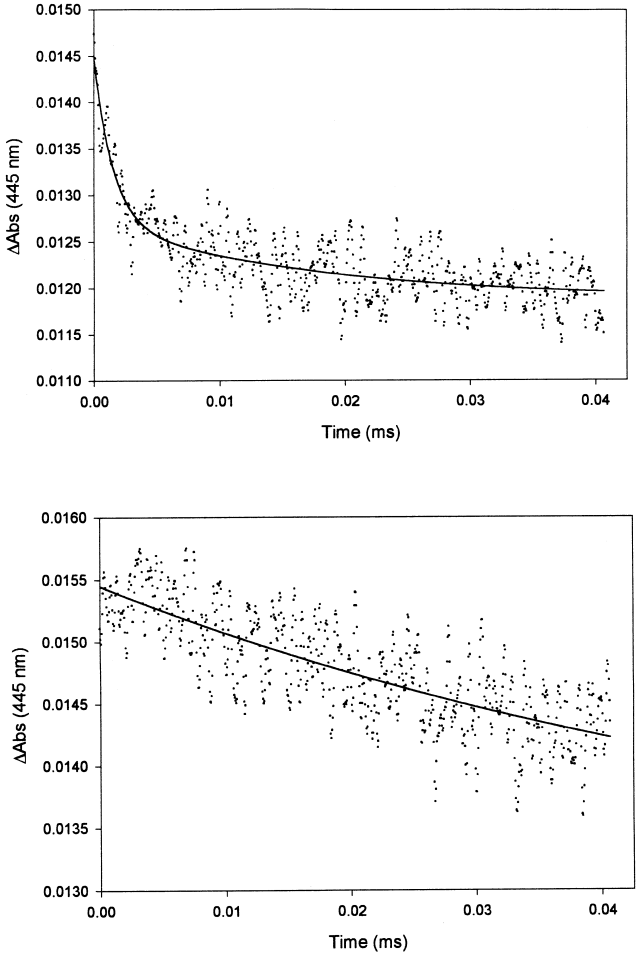

Fig. 2. Double exponential fit to the data obtained at ambient pressure (top panel) and single exponential fit to the data obtained at 2.07 kbar (bottom panel). Rate constants are found to be 6.7×10^5 s^{-1} and 5.9×10^4 s^{-1} for the fast and slow phases, respectively, at ambient pressure.

3. Results and discussion

3.1. Activation volume for ET

Previous studies have shown that photolysis of the CO mixed-valence derivative of CcO results in electron redistribution between cytochrome a_3, cytochrome a and Cu_A. Three distinct kinetic phases have been resolved corresponding to ET between cytochrome a_3 and cytochrome a (not coupled to proton uptake, $\tau\sim3$ μs), between cytochrome a and Cu_A (not coupled to proton uptake, $\tau\sim35$ μs) and between cytochrome a_3 and cytochrome a that is coupled to proton uptake at the binuclear center ($\tau\sim1$ ms) [17–20]. Recently, thermodynamic parameters associated with the various ET reactions have been determined. Specifically, the reorganization energies have been determined for ET between cytochrome a_3/cytochrome a ($\lambda=0.8$ eV) and between cytochrome a/Cu_A ($\lambda=0.2$ eV [17,19].

Fig. 1 displays pressure dependent transient absorption data obtained at 445 nm on a 50 μs time scale from ambient to 2 kbar for the CO mixed-valence derivative of CcO. The data could be fit to two exponential processes at ambient pressure (Fig. 2). The rate constants from the two processes are found to be $(6.7\pm0.9)\times10^5$ s^{-1} and $(5.9\pm1.7)\times10^4$ s^{-1}.

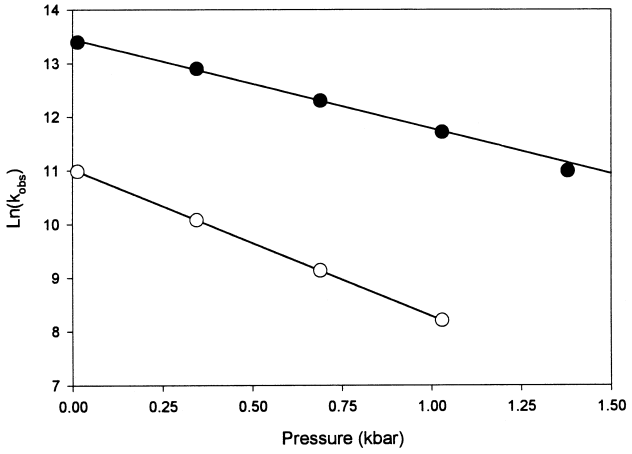

Fig. 3. Plot of $\ln(k_{obs})$ versus pressure for the decay at 445 nm subsequent to photolysis. Open circles: fast phase data. Filled circles: slow phase data.

These processes can be assigned to intramolecular ET between cytochrome *a* and cytochrome a_3 (fast phase) and between cytochrome *a* and Cu_A (slow phase). The corresponding plots of $\ln(k_{obs})$ vs. pressure for both the fast and slow phases are shown in Fig. 3. Fits of the data in Fig. 3 to

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

give activation volumes of $+41 \pm 5$ ml/mol and $+28 \pm 4$ ml/mol for the fast and slow phases, respectively (see Fig. 3) [22].

The rate of intramolecular ET in CcO can be described in the non-adiabatic limit as:

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

where T_{DA} is a term describing the electronic coupling between the donor and acceptor and FC is the Franck–Condon weighted density of states [23]. The FC term is related to both the reaction free energy and the overall reorganization energy:

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

The corresponding T_{DA} term can be written as:

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

where $H_{DA} = -V_A\langle\Psi_A^*|\Psi_D\rangle$, $H_{DD} = -V_A\langle\Psi_D^*|\Psi_D\rangle$ and $S_{DA} = \langle\Psi_D|\Psi_A\rangle$. The correlation between the activation volume and parameters associated with intramolecular ET are then described as follows:

$$\Delta V^{+}_{ET} = \Delta V^{+}_{FC} + \Delta V^{+}_{EC} \quad (5)$$

where ΔV^{+}_{FC} is related to the pressure dependence of the FC factor and ΔV^{+}_{EC} is related to the pressure dependence of the electronic coupling term [24]. If the pressure dependence of the electronic coupling is small, then, the FC term dominates the activation volume. On the other hand, if the FC term is small, then, the pressure dependence of the electronic coupling dominates. It has previously been shown that if the electronic coupling contribution is small, then:

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

where ΔV is the overall reaction volume [24]. Whether ΔV^{+}_{EC} is small for the ET reactions in mixed-valence CcO can be determined by calculating the total volume change for the reduction of cytochrome *a* and Cu_A assuming a small ΔV^{+}_{EC} (i.e. using Eq. 5) and then comparing this value to that previously determined for reduction of these two metal centers under equilibrium conditions [16]. Using reorganization energies (λ) and reaction driving forces (ΔG) previously determined, the values for the total volume changes are 87 ml/mol and 58 ml/mol for cytochrome *a* to Cu_A and cytochrome a_3 to cytochrome *a* ET reactions, respectively (using Eq. 5, $\Delta V = 2\lambda\Delta V^{+}_{FC}/(\lambda+\Delta G)$ and Cu_A to cytochrome *a*: $\Delta G° = -12$ meV, $\lambda = 0.4$ eV; cytochrome a_3 to cytochrome *a*: $\Delta G° = -40$ meV, $\lambda = 0.76$ eV) [17,19]. Using these values along with equilibrium volume change data from Kornblatt et al., the volume change for reduction of cytochrome *a* would be in the order of -155 ml/mol while that for the reduction of Cu_A would be in the order of -97 ml/mol[1] [16]. Thus, the total volume change for the first two electrons input into CcO would be in the order of -252 ml/mol. However, Kornblatt et al. have shown that the total volume change for the first two electrons input into CcO is -155 ml/mol [16]. This suggests that the FC term is not the dominant contribution to ΔV^{+}. This is in contrast to theoretical studies on Ru-modified cytochrome *c*, which indicate relatively small values for ΔV^{+}_{EC} (i.e. in the order of several ml/mol) [24].

The analysis presented above suggests that both reorganization and electronic coupling factors contribute to the intramolecular ET activation volumes. These results are consistent with a recent description of electron tunneling pathways in CcO as well as the crystal structures of the fully oxidized and fully reduced forms of the enzyme [3,4,5,25]. According to Regan et al., three nearly equivalent pathways exist for ET between cytochrome *a* and cytochrome a_3 with all three pathways starting/ending at the heme Fe (cytochrome *a* or cytochrome a_3) and involving the heme ligands His-378 (cytochrome *a*) and His-376 (cytochrome a_3) [25]. Upon reduction of the enzyme, the most significant change in the cytochrome *a*/cytochrome a_3 region is a decrease in the Fe-His bond length of cytochrome a_3 by roughly 0.2 Å (Fig. 4). Since this residue is directly involved in all three tunneling pathways, this change in bond length would make a contribution to ΔV^{+}_{EC}. This structural change would also make a contribution to ΔV^{+}_{FC} since the His is an inner-sphere ligand to the heme iron of cytochrome a_3. Interestingly, little change is observed in the ligation of the heme iron of cytochrome *a*, indicating that the magnitude of ΔV^{+} for the ET reaction between cytochrome *a* and cytochrome a_3 is primarily due to structural changes at cytochrome a_3.

Reduction of the enzyme also causes apparent structural changes to the Cu_A site (Fig. 4). Specifically, the Cu-Cu distance increases by roughly 0.2 Å upon reduction. This results in the lengthening of both Cu-S (from bridging Cys-200 and Cys-196) and Cu-N (from coordinated His-204 and His-161)

[1] The volume changes for the reduction of cytochrome *a* and Cu_A were calculated as follows. The volume changes calculated from Eq. 5 correspond to $\Delta V_I = 87$ ml/mol $= \Delta V_{a3^r\rightarrow a3^o} + \Delta V_{a^o\rightarrow a^r}$ and $\Delta V_{II} = 58$ ml/mol $= \Delta V_{a^r\rightarrow a^o} + \Delta V_{CuA^o\rightarrow CuA^r}$ for the fast and slow phases, respectively. From [16], the volume change for the two electron reduction of the mixed-valence enzyme is $\Delta V_{III} = -155$ ml/mol $= \Delta V_{a^o\rightarrow a^r} + \Delta V_{CuA^o\rightarrow CuA^r}$. Noting that $\Delta V_{a^o\rightarrow a^r} = -\Delta V_{a^r\rightarrow a^o}$, $\Delta V_{II} = -\Delta V_{a^o\rightarrow a^r} + \Delta V_{CuA^o\rightarrow CuA^r}$. Adding ΔV_{II} and ΔV_{III} gives $\Delta V_{CuA^o\rightarrow CuA^r} = -97$ ml/mol. Substituting this value into ΔV_{II} gives $\Delta V_{a^o\rightarrow a^r} = -155$ ml/mol.

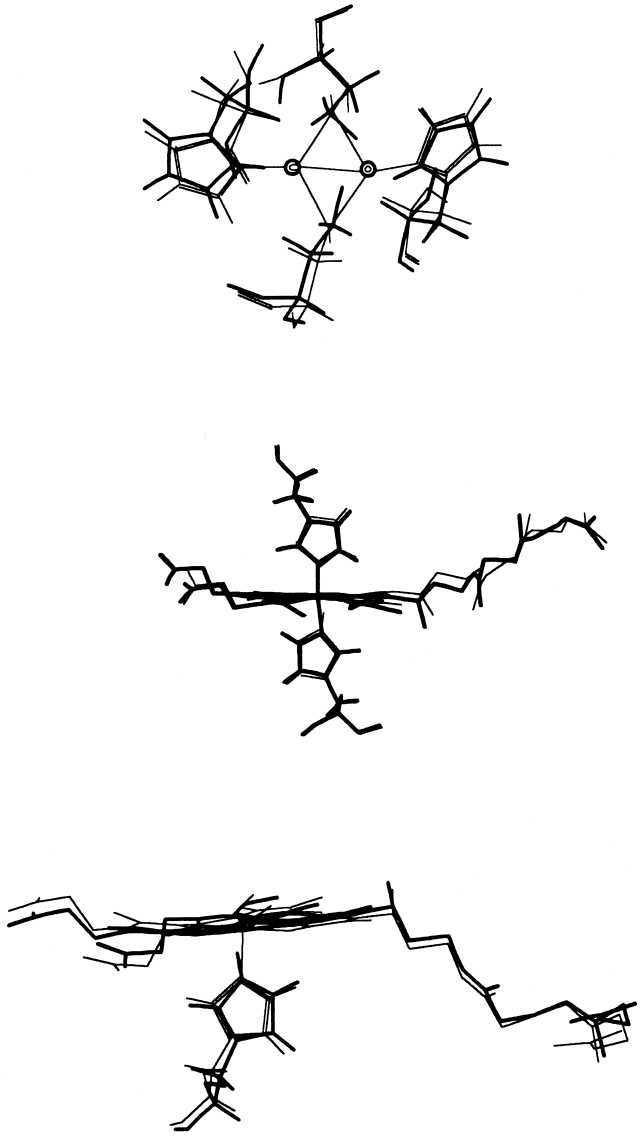

Fig. 4. Structural changes to the Cu_A site (top), cytochrome *a* site (middle) and cytochrome a_3 site (bottom). Bold face structures are of the reduced form. Structures were obtained from [4] (PBD10CC) for the oxidized enzyme and [5] (PBD10CR) for the fully reduced form.

bond lengths. The calculated electron tunneling pathway between Cu_A and cytochrome *a* involves His-204 due to connectivity with the heme propionate of cytochrome *a*. Thus it is apparent that ΔV^{+} for the ET reaction between cytochrome *a* and Cu_A presumably arises from both ΔV^{+}_{FC} and ΔV^{+}_{EC} associated with the Cu_A site and little or no contribution from cytochrome *a*. It should be pointed out that the structural differences in the metal centers between the oxidized and reduced forms of the enzyme are less than the S.D. reported for the two structures and must be viewed in that context. However, the activation volume data are consistent with these changes. Overall, the data presented here suggest that both cytochrome a_3 and Cu_A can gate ET rates via structural changes involving metal site ligands while cytochrome *a* appears to simply shuttle electrons between Cu_A and cytochrome a_3 without the ability to gate the ET.

Acknowledgements: The author would like to acknowledge the American Heart Association (HIGS-11-97) and the National Science Foundation (MCB9904713) for support of this work.

References

[1] Michel, H., Behr, J., Harrenga, A. and Kannt, A. (1998) Ann. Rev. Biophys. Biomol. Struct. 27, 329–356.
[2] Musser, S.M., Stowell, M.H.B. and Chan, S.I. (1995) in: Adavnces in Enzymology and Related Areas of Molecular Biology (Meister, A., Ed.), Vol. 71, Wiley and Sons.
[3] Iwata, S., Ostermeier, C., Ludwig, B. and Michel, H. (1995) Nature 376, 660–669.
[4] Tsukihara, T., Aoyama, H., Yamashita, E., Tomizaki, T., Yamaguchi, H., Shinzawa-Itoh, K., Nakashima, R., Yaono, R. and Yoshikawa, S. (1995) Science 269, 1069–1074.
[5] Yoshikawa, S., Shinzawa-Itoh, K., Nakashima, R., Yaono, R., Yamashita, E., Inoue, N., Yao, M., Fei, M.J., Libeu, C.P., Mizushima, T., Yamaguchi, H., Tomizaki, T. and Tsukihara, T. (1998) Science 280, 1723–1729.
[6] Fetter, J.R., Qian, J., Shapleigh, J., Thomas, J.W., Garcia-Horsman, A., Schmidt, E., Hosler, J., Babcock, G.T., Gennis, R.B. and Ferguson-Miller, S. (1995) Proc. Natl. Acad. Sci. USA 92, 1604–1608.
[7] Hosler, J.P., Shapleigh, J.P., Mitchell, D.M., Kim, Y., Pressler, M.A., Georgiou, C., Babcock, G.T., Alben, J.O., Ferguson-Miller, S. and Gennis, R.B. (1996) Biochemistry 35, 10776–10783.
[8] Pfitzner, U., Odenwald, A., Ostermann, T., Weingard, L., Ludwig, B. and Richter, O.H. (1998) J. Bioenerg. Biomembr. 30, 89–97.
[9] Mills, D.A. and Ferguson-Miller, S. (1998) Biochim. Biophys. Acta 1365, 46–52.
[10] Gennis, R.B. (1998) Nature 280, 1712–1713.
[11] Larsen, R.W. (1994) FEBS Lett. 352, 365–368.
[12] Niu, S.L. and Larsen, R.W. (1998) J. Biochem. Mol. Biol. Biophys. 1, 287–293.
[13] Musser, S.M. and Chan, S.I. (1995) Biophys. J. 68, 2543–2555.
[14] Kornblatt, J.A., Hoa, G.H.B. and Heremans, K. (1988) Biochemistry 27, 5122–5128.
[15] Kornblatt, J.A. and Hoa, G.H.B. (1990) Biochemistry 29, 9370–9376.
[16] Kornbaltt, J.A., Kornblatt, M.J., Rajotte, I., Hoa, G.H.B. and Kahn, P.C. (1998) Biophys. J. 75, 435–444.
[17] Brzezinski, P. (1996) Biochemistry 35, 5611–5615.
[18] Hallen, S., Brzezinski, P. and Malmstrom, B.G. (1994) Biochemistry 33, 1467–1472.
[19] Adelroth, P., Brzezinski, P. and Malmstrom, B.G. (1995) Biochemistry 34, 2844–2849.
[20] Morgan, J.E., Li, P.M., Jang, D.J., El-Sayed, M.A. and Chan, S.I. (1989) Biochemistry 28, 6975–6983.
[21] Niu, S.-N. (1997) Dissertation, University of Hawaii at Manoa, HI.
[22] van Eldik, R., Asano and le Noble, W.J. (1989) Chem. Rev. 89, 549.
[23] Marcus, R.A. and Sutin, N. (1985) Biochim. Biophys. Acta 811, 265–322.
[24] Miyashita, O. and Go, N. (1999) J. Phys. Chem. B 103, 562–571.
[25] Regan, J.J., Ramirez, B.E., Winkler, J.R., Gray, H.B. and Malmstrom, B.J. (1998) J. Bioenerg. Biomembr. 30, 35–39.