FEBS Letters 352 (1994) 365–368

FEBS 14612

Peroxide-induced spectral perturbations of the 280-nm absorption band of cytochrome *c* oxidase

Randy W. Larsen*

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

Received 29 August 1994

Abstract It is now widely believed that the first two electrons transferred to the dioxygen reduction site in cytochrome *c* oxidase (CcO) are not coupled to proton translocation. The activation of the pump cycle correlates with the binding of dioxygen to the binuclear center. In order to investigate conformational changes in CcO associated with the formation of dixoygen intermediates during the catalytic cycle of CcO, the effects of hydrogen peroxide binding to CcO have been examined using UV optical absorption and second derivative techniques. Our data indicates that in the presence low concentrations of H_2O_2 (2:1 molar ratio) an initial CcO-peroxide species is formed in which the 280-nm absorption band is red shifted. This red shift occurs prior to spectral changes associated with H_2O_2 binding to cytochrome a_3. Upon addition of higher concentrations of H_2O_2 (>10 equivalents of H_2O_2 per equivalent of CcO) oxidized CcO is converted to F-state enzyme with no corresponding shift at 280 nm. It is suggested that H_2O_2 initially binds to Cu_B^{2+} resulting in a conformational change in the enzyme giving rise to a red-shifted 280 nm band. The absence of any conformational changes in F-state enzyme is consistent with the lack of bridging interactions with Cu_B^{2+} in this intermediate.

Key words: Cytochrome oxidase; Hydrogen peroxide; Proton pump; Compound C; Oxyferryl complex; Hydrogen peroxide

1. Introduction

Mammalian cytochrome *c* oxidase (CcO) catalyzes the terminal step in the respiratory chains of a wide range of aerobic organisms. As such, the enzyme plays a pivotal role in energy transduction by coupling the four electron reduction of dioxygen to the active transport of protons across the inner mitochondrial membrane [1–3]. The energy-coupling catalytic cycle of CcO utilizes four redox-active metal centers consisting of two heme A chromophores and two copper ions. The reduction of molecular oxygen takes place at a binuclear cluster containing one of the heme A chromophores (designated cytochrome a_3) and one copper ion (designated Cu_B). The other two metal centers (designated cytochrome *a* and Cu_A) have reduction potentials near that of cytochrome *c* during turnover and mediate the flow of electrons between cytochrome *c* and the binuclear center.

The redox free energy between cytochrome *c* and dioxygen is converted, in part, into a transmembrane potential gradient due to the consumption of protons (required for the formation of water from molecular oxygen) from the matrix of the mitochondrion. Concurrent with this electron flow, protons are vectorially translocated across the inner-mitochondrial membrane from the matrix to augment the the electrochemical gradient. Despite intensive research efforts spanning the last two decades, the specific nature of the pump mechanism as well as the metal center associated with proton translocation have not been identified.

There is now experimental evidence that the first two electrons transferred to the dioxygen reduction site are not coupled to proton translocation [4]. The activation of the pumping mechanism appears to begin with the binding of dioxygen to the binuclear center. Thus the binding of exogenous ligands to the binuclear center is of specific interest in proton translocation in CcO. A number of recent studies have appeared which involve the spectroscopic investigation of CcO bound with a variety of ligands including N_3^- [5–7], CN^- [5–9], CO [10–12], and more recently SCN [9]. These studies have revealed that both the cytochrome a_3 and Cu_B sites are capable of binding exogenous ligands. It has also been suggested that the initial intermediate formed during dioxygen reduction involves an O_2-bound Cu_B complex (i.e. Cu_B-O=O) [13]. The implication that Cu_B can bind exogenous ligands certainly has an impact on potential proton pump mechanisms. In fact a number of models currently exists which involve ligand binding to Cu_B [4,14,15].

2. Materials and methods

Bovine CcO was prepared as previously described [16]. The enzyme concentration is 5 mM per aa_3 in 80 mM potassium phosphate buffer (pH 7.0) with 0.08% β-D-lauryl maltoside. H_2O_2 stock solutions were prepared by diluting 30% H_2O_2 (Fisher) to 8.8 mM in 80 mM potassium phosphate buffer (pH 7.0) with 0.08% β-D-lauryl maltoside. The effects of H_2O_2 binding to oxidized CcO were examined by adding aliquots of the 8.8 mM stock H_2O_2 solution to 2 ml of 5 mM CcO in a 1-cm pathlength stirred far UV quartz optical cuvette. The cell was housed in a thermoelectrically cooled cell holder and maintained at 25°C throughout the experiment. Absorption spectra were obtained using a Milton Roy Spectronic 3000 diode array spectrophotometer (2 nm resolution). Difference spectra were obtained by mathematical subtraction of the original spectra corrected for light scattering. Second derivative spectra were obtained using Milton Roy software (quadratic least squares method with 25 point convolution).

3. Results and discussion

The goal of the current study is to investigate conformational transitions which occur during the formation of physiological intermediates associated with dioxygen reduction at the binuclear center. With this in mind we have examined the equilibrium UV absorption spectra of mammalian CcO in the presence of H_2O_2. It is now well established that the addition of H_2O_2 (K_b~6 μM [17]) to fully oxidized CcO produces optical absorption, resonance Raman, and EPR signals similar to those ob-

*Corresponding author.

SSDI 0014-5793(94)00999-6

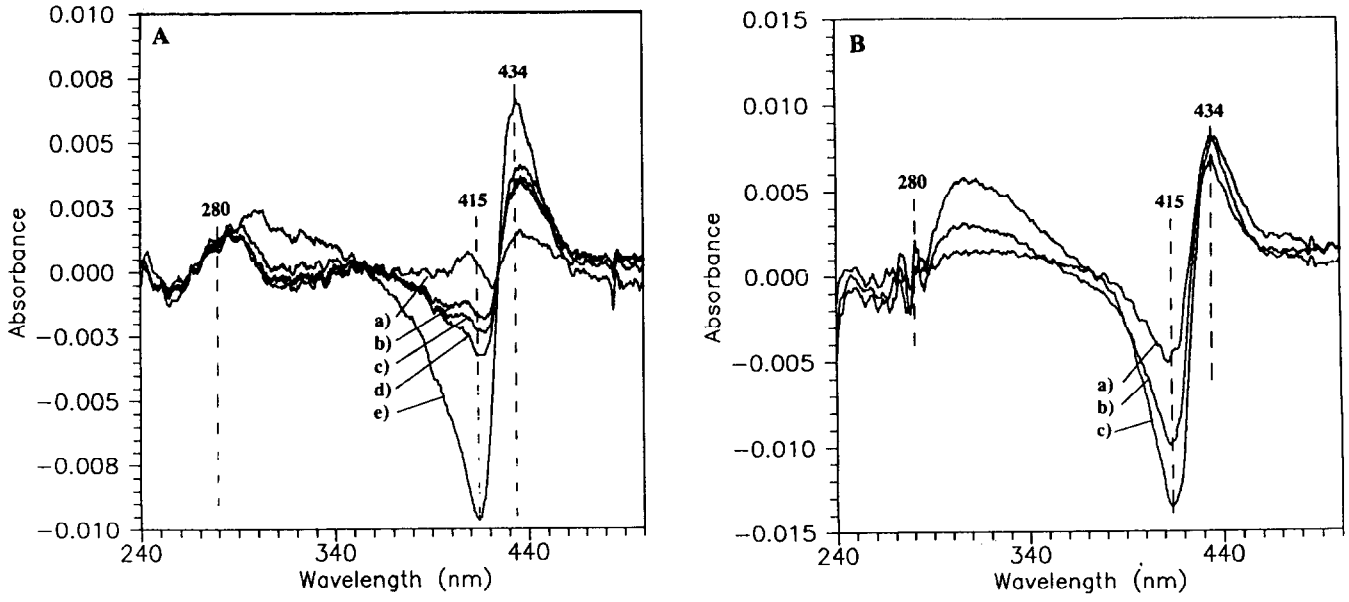

Fig. 1. (Panel A) Optical difference spectra in the near UV region (H_2O_2-oxidized CcO minus oxidized CcO) corrected for light scattering at various times after the addition of two equivalents of H_2O_2: (a) 30 s, (b) 5 min, (c) 10 min, (d) 20 min, and (e) 1.5 h. (Panel B) Optical difference spectra corrected for light scattering at various times after the addition of ten equivalents of H_2O_2: (a) 30 s, (b) 5 min, (c) 10 min, (d) 20 min, and (e) 1.5 h.

served during turnover of the fully reduced enzyme with dioxygen [17–22]. The addition of low concentrations (<10:1 H_2O_2/CcO) produces a peroxy intermediate at the binuclear center that is spectroscopically identical to the P-state (nomenclature of Wikstrom [4], Compound C in the nomenclature of Chance [22]). At higher H_2O_2 concentrations (>10:1) formation of the P-state is followed by a rapid decay to the F-state (oxyferryl cytochrome a_3). Fig. 1A displays the optical difference spectra of fully oxidized CcO (corrected for light scattering) at various times after the addition of H_2O_2 (two equivalents of H_2O_2 per equivalent of CcO). The corresponding difference spectra obtained for the visible region is displayed in Fig. 2 panel A. The spectra demonstrate that the presence of H_2O_2 induces a conformational change in CcO resulting in a red-shift of the 280nm absorption band. Interestingly the ΔA_{280nm} appears prior to shifts in the Soret band of cytochrome a_3 associated with H_2O_2 to heme a_3. On longer time scales the ΔA_{280nm} decays and gives rise to a broad increase in absorbance centered at 310nm. When CcO is treated with higher concentrations of H_2O_2 (10:1 H_2O_2/CcO) (Fig. 1, Panel B) only a broad increase in absorbance centered at 310 nm is observed. The corresponding difference spectra in the visible region is shown in Fig. 2 panel B. The ΔA_{310nm} also corresponds to a significant increase in sample light scattering indicating that some protein denaturation has

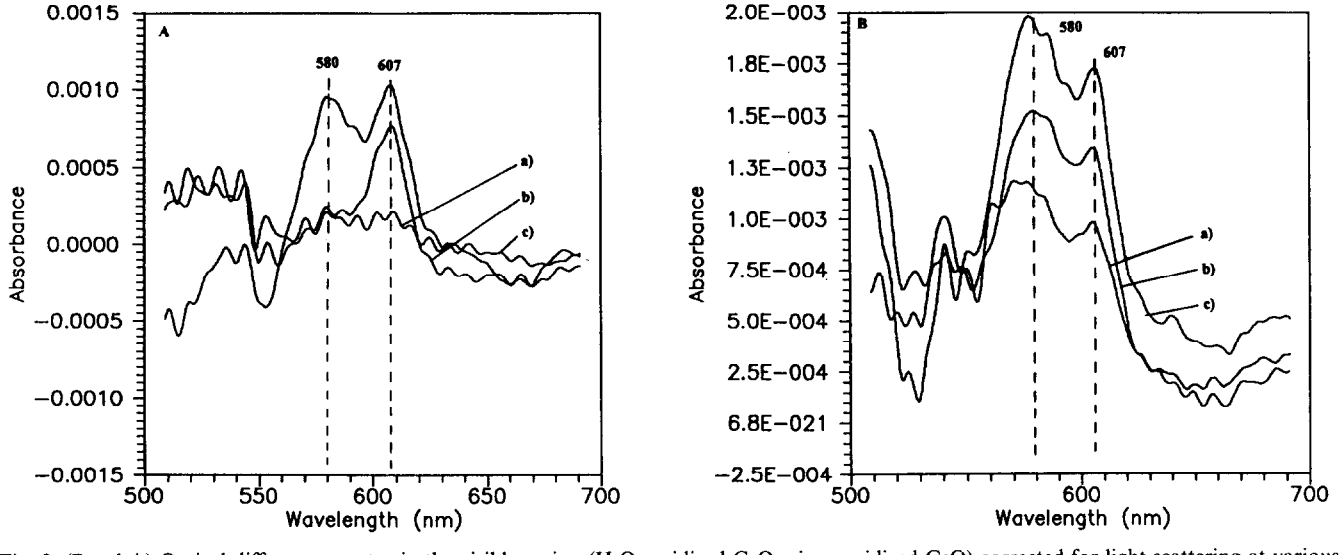

Fig. 2. (Panel A) Optical difference spectra in the visible region (H_2O_2-oxidized CcO minus oxidized CcO) corrected for light scattering at various times after the addition of two equivalents of H_2O_2: (a) 30 s, (b) 20 min, and (c) 1.5 h. (Panel B) Optical difference spectra corrected for light scattering at various times after the addition of ten equivalents of H_2O_2: (a) 30 s, (b) 20 min, and (c) 1.5 h.

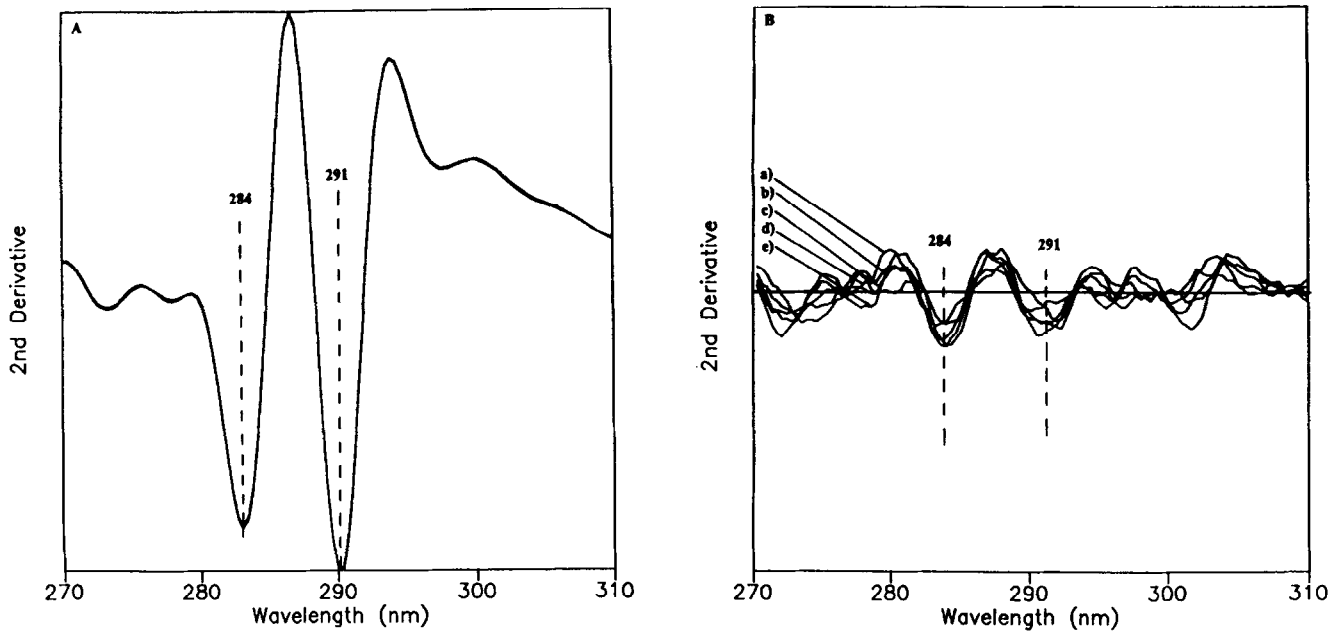

Fig. 3. Second derivative spectra obtained from the original absorption spectra of CcO (after scattering corrections) at various times after the addition of two equivalents of H_2O_2: (a) 30 s, (b) 5 min, (c) 10 min, (d) 20 min, and (e) 1.5 h. The corresponding second derivative difference spectra are displayed in Panel B.

taken place (data not shown). Analysis of the second derivative of the UV absorption spectra (Figs. 3 and 4) indicates that the ΔA_{280nm} arises primarily from perturbation of protein tyrosine and tryptophan residues. All of the observed perturbations are inhibited by incubating the oxidized with cyanide ligand prior to H_2O_2 (data not shown).

The extent to which ligand binding to the binuclear center of CcO is coupled to proton translocation is of critical importance to the elucidation of the overall energy transducing mechanism of the enzyme. The data presented here indicate that the formation of P-state enzyme results in a conformational transition in the enzyme as evident by a shift in the 280 nm absorption band. The fact that this shift is inhibited by cyanide further suggests that it is the binding of H_2O_2 to the binuclear center that induces the spectral perturbation. It is of specific interest to note that the appearance of the 280 nm band shift appears prior to the formation of significant populations of P-state enzyme (as judged by the Soret or visible band shifts versus 280 nm shift) and persists until complete formation of P-state is achieved. The absorption difference spectra of the initial com-

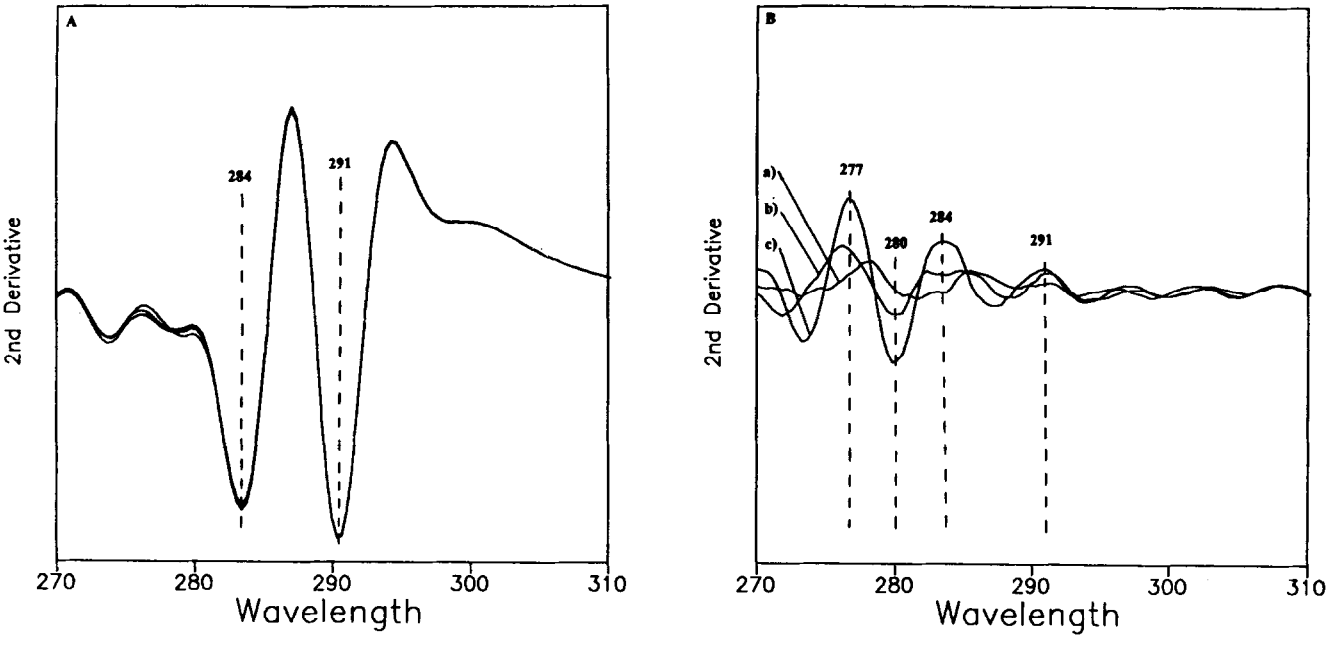

Fig. 4. Second derivative spectra obtained from the original absorption spectra of CcO (after scattering corrections) at various times after the addition of ten equivalents of H_2O_2: (a) 30 s, (b) 20 min, and (c) 1.5 h. The corresponding second derivative difference spectra are displayed in Panel B.

pound formed after the addition of two equivalents of H_2O_2 to oxidized CcO resembles absorption difference spectra of oxidized CcO in the presence of low concentrations of cyanide ligand recently reported previously Berka et al. [23]. These authors have suggested that cyanide initially binds to Cu_B^{2+} indirectly perturbing the electronic environment of cytochrome a_3. The fact that exogenous ligands can bind to Cu_B^{2+} suggests a model for the binding of H_2O_2 to the binuclear center in oxidized CcO. That is, the initial species formed involves a Cu_B^{2+}–H_2O_2 complex in which the H_2O_2 is not initially bound to cytochrome a_3 [24]. On longer time-scales H_2O_2 binds to cytochrome a_3 either through a bridging intermediate or via additional binding of H_2O_2 to cytochrome a_3. In any event it appears that ligation to Cu_B^{2+} is responsible for the shift in the 280nm absorption band.

Conformational changes triggered by ligand binding to the binuclear center are of specific interest to the proton pump mechanism of CcO. Wikstrom has presented evidence that single electron transfer to P-state followed by single electron transfer to F-state are responsible for all four protons translocated during the pump cycle giving a catalytic stoichiometry of $2H^+/e^-$ for the third and fourth electron transfer steps [4]. This implies that binding of dioxygen to the binuclear center induces a change in protein conformation that initiates the proton pump machinery of the enzyme. The implication that ligand binding to Cu_B induces a conformational change in CcO provides a mechanism for pump activation during the catalytic cycle of the enzyme. It is well known that the initial species formed during the reaction of dioxygen with a reduced binuclear center is the binding of O_2 to ferrous cytochrome a_3 followed by electron transfer from cuprous Cu_B [25–27]. The intermediate formed by the two electron reduction of dioxygen is believed to be a bridged peroxy intermediate with the peroxide bound between ferric cytochrome a_3 and Cu_B^{2+} [25–27]. Our data supports a conformational change in the enzyme at this point in the catalytic cycle which is consistent with the observed activation of the proton pump cycle. Although these results are limited, the data suggests a clear mechanistic link between ligand binding to the binuclear center and protein conformational changes during catalysis.

Acknowledgements: This work was supported by a University of Hawaii Research Council Seed Grant.

References

[1] Wikstrom, M., Kraab, K. and Saraste, M. (1981) in: Cytochrome Oxidase: A Synthesis, Academic Press, New York.
[2] Chan, S.I. and Li, P.M. (1990) Biochemistry 29, 1–12.
[3] Papa, S. (1976) Biochim. Biophys. Acta 456, 39–84.
[4] Wikstrom, M. (1988) Ann. N.Y. Acad. Sci. 550, 199–206.
[5] Yoshikawa, S. and Caughey, W.S. (1992) J. Biol. Chem. 267, 9757–9766.
[6] Li, W. and Palmer, G. (1993) Biochemistry 32, 1833–1843.
[7] Tsubaki, M. and Yoshikawa, S. (1993) Biochemistry 32, 174–182.
[8] Tsubaki, M. and Yoshikawa, S. (1993) Biochemistry 32, 164–173.
[9] Yoshikawa, S. and Caughey, W.S. (1990) J. Biol. Chem. 265, 7945–7958.
[10] Alben, J.O., Moh, P.P., Fiamingo, F.G. and Altschuld, R.A. (1981) Proc. Natl. Acad. Sci. USA 78, 234–237.
[11] Fiamingo, F.G., Altschuld, R.A., Moh, P.P. and Alben, J.O. (1982) J. Biol. Chem. 257, 1639–1650.
[12] Einarsdottir, O., Choc, M.G., Weldon, S. and Caughey, W.S. (1988) J. Biol. Chem. 263, 13641–13654.
[13] Oliveberg, M. and Malmstron, B.G. (1992) Biochemistry 31, 3560–3563.
[14] Larsen, R.W., Pan, L-P., Musser, S.M., Li, Z. and Chan, S.I. (1992) Proc. Natl. Acad. Sci. USA 89, 723–727.
[15] Mitchell, P. (1987) FEBS Lett. 222, 235–245.
[16] Hartzell and Beinert (1974) Biochim. Biophys. Acta 368, 318–338.
[17] (a) Bickar, D., Bonaventura, J. and Bonaventura, C. (1982) Biochemistry 21, 2661–2666; (b) Weng, Lichun and Baker, G.M. (1991) Biochemistry 30, 5727–5733.
[18] Gorren, A.C. F., Dekker, H. and Wever, R. (1986) Biochim. Biophys. Acta 852, 81–92.
[19] Witt, S.N. and Chan, S.I. (1987) J. Biol. Chem. 262, 1446–1448.
[20] Vygodina, T.V. and Konstantinov, A.A. (1988) Ann. NY Acad. Sci. 550, 124–138.
[21] Larsen, R.W., Li, W., Copeland, R.A., Witt, S.N., Lou, B.S., Chan, S.I. and Ondrias, M.R. (1990) Biochemistry 29, 10135–10140.
[22] Chance, B., Saronio and Leigh, J.S. (1975) Proc. Natl. Acad. Sci. USA 72, 1635–1640.
[23] Berka, V., Vygodina, T., Musatov, A., Nicholls, P. and Konstantinov, A.A. (1993) FEBS Lett. 315, 237–241.
[24] Although the Cu_B^{2+} peroxide complex is written as the protonated species, the exact protonation state of the bound peroxide is uncertain.
[25] Blair, D.F., Witt, S.N. and Chan, S.I. (1985) J. Am. Chem. Soc. 107, 7389–7399.
[26] Olivberg, M., Brzezinski, P. and Malmstrom, B.G. (1989) Biochim. Biophys. Acta 977, 322–328.
[27] Babcock, J. and Wikstrom, M. (1992) Nature 356, 301–309.