Oxidation of Ubiquinol by Cytochrome bo_3 from *Escherichia coli*: Kinetics of Electron and Proton Transfer†

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ABSTRACT: In this study we have used the so-called flow−flash technique to investigate electron and proton transfer during the reaction between cytochrome bo_3 with bound ubiquinol (QH_2) and dioxygen. The results are compared to those from the well-characterized mitochondrial cytochrome aa_3. Qualitatively, the same type of absorbance changes associated with electron transfer were observed in both enzymes whereas the protonation reactions were markedly different. In the bacterial QH_2-bound enzyme, three kinetic phases with time constants of ∼45 μs, ∼700 μs, and ∼4 ms associated with electron-transfer reactions were observed. The first phase is attributed to oxidation of hemes *b* and o_3 and formation of the "peroxy" intermediate. The second and third phases were not observed after addition of the herbicide HQNO, which displaces QH_2 from its binding site. They are attributed to electron transfer from QH_2 to heme *b* and from heme *b* to the binuclear center, respectively. In both enzymes, the initial electron transfer was followed by a slower uptake of 0.9 ± 0.3 proton per enzyme molecule ($\tau \cong 90$ μs), previously attributed to protonation of a group near the binuclear center. Only in the bacterial enzyme, the second electron-transfer reaction was accompanied by a net release of 1.1 ± 0.3 H^+, which is attributed to proton release during oxidation of QH_2. It was followed by a slower uptake of 1.2 ± 0.4 H^+ during transfer of the fourth electron to the binuclear center. The two slowest protonation reactions were not observed in the presence of HQNO.

Cytochrome bo_3[1] (ubiquinol:O_2 oxidoreductase) is a component of the respiratory chain of *Escherichia coli* grown under aerobic conditions. It is a membrane-bound integral protein complex which catalyzes oxidation of ubiquinol (QH_2) by molecular oxygen. Part of the energy released in this reaction is conserved by the enzyme through vectorial pumping of protons across the membrane (Puustinen et al., 1989, 1991). The enzyme consists of four protein subunits which accommodate three redox-active metal sites, all bound to subunit I: a low-spin heme *b* and a heme−copper binuclear center (heme o_3−Cu_B) [for a review see Hosler et al. (1993)]. Electrons from QH_2, which binds to subunit II (Welter et al., 1994), are first transferred to heme *b* and then to the binuclear center, where dioxygen is bound and reduced to water [for a review, see Babock and Wikström (1992) and Ferguson-Miller and Babcock (1996)].

The reaction between the fully reduced enzyme and O_2 has been investigated using the so-called flow−flash technique [developed by Gibson and Grenwood (1963)]; the fully reduced enzyme with carbon monoxide bound to heme o_3 is mixed with an O_2-containing solution. Oxidation of the enzyme is slow ($\sim 10^{-1}$ s^{-1}) because the reaction is limited by the CO-off rate. Immediately after mixing, CO is photodissociated and the reaction between the fully reduced enzyme and O_2 is studied using various spectroscopic techniques. This method, in combination with optical absorption spectroscopy, has been used by our and several other research groups to investigate electron-transfer reactions in cytochrome bo_3 (Svensson & Nilsson, 1993; Orii et al., 1995; Puustinen et al., 1996; Svensson et al., 1995,1996). In addition, our research group has also used this technique for studies of proton uptake and release both by cytochrome aa_3 [e.g., Hallén and Nilsson (1992)] and cytochrome bo_3 (Hallén et al., 1993; Svensson et al., 1995, 1996). These reactions were investigated by monitoring absorbance changes of pH-sensitive dyes present in the solution. In cytochrome bo_3, oxidation of hemes *b* and o_3 and formation of the "peroxy" intermediate observed as a single kinetic phase (at 430 nm) with a time constant of about 45 μs is followed by proton uptake from solution with a time constant of about 90 μs (Svensson Ek et al., 1996), accompanied by formation of the "ferryl" intermediate (Puustinen et al., 1996; Svensson Ek et al., 1996). This proton-uptake reaction has been attributed to protonation of an internal protonatable group near the binuclear center (Hallén & Nilsson, 1992; Svensson Ek et al., 1996).

In most studies of the reaction between the fully reduced cytochrome bo_3 and O_2 using the flow−flash technique, only part of the reaction has been investigated because there are only three electrons in the enzyme and the final state after completion of the reaction is the ferryl intermediate (Puustinen et al., 1996, Svensson Ek et al., 1996). Recently, Puustinen et al. (1996) investigated the reaction between the fully reduced cytochrome bo_3 with bound ubiquinol (QH_2) and O_2. They observed multiphasic kinetics indicating re-reduction of heme *b* by QH_2 following the initial heme *b* oxidation. In addition, this experimental system makes it also possible to investigate reactions associated with transfer of the fourth electron to the binuclear center from heme *b*. In this work, we have studied the kinetics of both electron and proton transfer during reaction of the QH_2-containing

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[1] Nomenclature suggested by Puustinen et al. (1992).

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fully reduced cytochrome bo_3 and O_2. Our results show that after the initial ($\tau \cong 45$ μs)[2] oxidation of hemes *b* and o_3 and proton uptake ($\tau \cong 90$ μs) follows proton release and electron transfer from QH_2 to heme *b*. The electron is then transferred from heme *b* to the binuclear center associated with proton uptake.

MATERIALS AND METHODS

Materials. Decylubiquinone, *N*-octyl glucoside, HQNO, and the pH indicators cresol red and phenol red were purchased from Sigma. Other chemicals were of the purest grade available.

Bacteria were grown aerobically in a 20 L fermentor in LB-medium supplemented with 0.1 M potassium phosphate buffer (pH 7.5), 1 g of $(NH_4)_2SO_4$, 0.5 g of sodium citrate, 2.5 mg of $CuSO_4 \times 5H_2O$ per liter of medium, and 1% (w/v) lactate. They were harvested in the mid-exponential phase of growth. Membranes were isolated as described (Svensson & Nilsson, 1993), solubilized with Triton X-100 and octyl glucoside to a final concentration of 1% of each or 1% dodecyl maltoside in 50 mM potassium phosphate buffer, and ultracentrifuged. The supernatant was loaded on a Ni^{2+}−chelate affinity column (Ni^{2+}−NTA−agarose, Qiagen) to specifically bind the histidine-modified enzyme and equilibrated with 50 mM K-phosphate, 0.1% Triton X-100 and 25 mM imidazole at pH 8.1. Alternatively, when dodecyl maltoside was used, the protocol of Puustinen et al. (1996) was followed. A gradient from 25 to 300 mM imidazole was used to elute the bound enzyme. Imidazole was removed by dialysis against 50 mM potassium phosphate, 0.025% *N*-dodecyl sarcosinate, pH 8.3 and/or successive concentration/dilution several times. Enzyme concentrations were determined from reduced-minus-oxidized spectra using the absorption coefficient $\Delta\Delta\epsilon = \Delta\epsilon^{560}_{red-ox} - \Delta\epsilon^{580}_{red-ox} = 24$ mM^{-1} cm^{-1} (Puustinen et al., 1991). The stock solution of the enzyme was stored in liquid nitrogen. The maximum turnover rate of the enzyme was ~500 s^{-1}. The fraction-bound quinone varied between different preparations in both preparation methods. The results shown in this work were obtained with the Triton/octyl glucoside preparation with a fraction bound quinone of 0.7 per enzyme molecule.

Flow−Flash Experimental Setup and Data Analysis. The flow−flash experiments were performed using a locally modified stopped-flow apparatus (Applied Photophysics, DX-17MV). The syringes had total volumes of 500 and 2500 μL, giving a mixing ratio of 1:5. The smaller syringe was filled with the enzyme solution and the larger with the O_2-saturated solution (see below). The output signal from the photomultiplier was fed into a 3 MHz current-to-voltage converter, a preamplifier with a variable RC filter (Tektronix, model AM 502), and was recorded using a digital transient recorder (Nicolet, model 490). The stop-syringe switch was connected to a delay circuit which triggered a Nd:YAG laser (Spectra Physics) about 100 ms after mixing. The photomultiplier was protected from the laser light using various interference filters, depending on wavelength. The cuvette path length was 1 cm. Time constants [$\exp(-t/\tau)$] were determined using a nonlinear fitting algorithm using the Igor software (Wavemetrics, Inc) or a global non-linear fitting program written in Matlab (MathWorks, Inc.). Typically, 60 000 data points were collected with a sampling time of 500 ns. The data set was then reduced to about 1000 points with a logarithmic time scale.

Studies of Electron-Transfer Reactions. The enzyme, solubilized in 0.1% *N*-dodecyl sarcosinate, 0.1 M Hepes-KOH (pH 7.5), and 1 mM EDTA, was first incubated under a N_2 atmosphere for about 10 h in 2 mM sodium ascorbate and 5 μM phenazine methosulfate as a mediator. After formation of the fully-reduced enzyme, as confirmed from an optical absorption spectrum, N_2 was replaced with CO to a final concentration of about 1 mM (10^5 Pa). The enzyme solution was transferred anaerobically to the smaller syringe (500 μL) of the stopped-flow apparatus. The larger syringe (2500 μL) was filled with a detergent−buffer solution (same as used for solubilization of the enzyme), equilibrated with pure O_2 at 10^5 Pa. After mixing, the enzyme and O_2 concentrations were 0.3−1.5 μM (see figure legends) and ~1 mM, respectively. Where applicable, the endogenous QH_2 was displaced by addition of HQNO (70 μM) to the enzyme solution followed by incubation for about 30−60 min.

Proton-Uptake Measurements. Buffer was removed from the enzyme solution by washing several times with 0.1 M KCl and 0.1% *N*-dodecyl sarcosinate at pH 7−7.5 using centricon-50 tubes (Amicon Inc.). The pH was adjusted to about 8.6, and the enzyme was reduced as described above. The pH decreased slightly upon addition of sodium ascorbate so that the final pH of the carboxy fully reduced enzyme solution was 8.3. The smaller syringe of the stopped-flow apparatus was filled with the enzyme solution and the larger syringe with an O_2-saturated buffer-free solution (see above), supplemented with 36 μM cresol red at pH 8.3. Care was exercised to avoid exposure to air CO_2 during handling of the buffer-free samples. After each experiment, the output solution was collected and its pH was measured. About five additions of 15.0 μL of 0.500 mM H_2SO_4 to the collected enzyme−dye solution (~2.5 mL) were made, and absorbance changes at 580 nm were monitored using a Cary 4 (Varian) spectrophotometer. The average ratio of changes in proton concentration and in absorbance ($\Delta H^+/\Delta A^{580}$) was determined. The degree of proton uptake/release in the kinetic experiments was determined from this ratio. The pK_as of cresol red and phenol red were determined by spectrophotometric titration under the same conditions as those used in this study and were found to be 8.3 and 7.8, respectively. The above described procedure is for cytochrome bo_3. Proton uptake in the bovine enzyme was measured in the same way, except that phenol red was used instead of cresol red (see figure legends).

RESULTS

A detergent solution of fully reduced carboxycytochrome bo_3 was mixed with a dioxygen-containing solution in a stopped-flow apparatus. About 100 ms after mixing, CO was photodissociated with a 10 ns laser flash and the reaction between the fully reduced enzyme and O_2 was studied time resolved at different wavelengths in the range 410−440 nm. Figure 1A shows absorbance changes at 430 nm. A rapid unresolved ($\tau < 0.5$ μs) increase in absorbance associated

[2] Abbreviations: τ, time constant [defined as $A \exp(-t/\tau)$]; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; HQNO, 2-*n*-heptyl-4-hydroxyquinolone *N*-oxide.

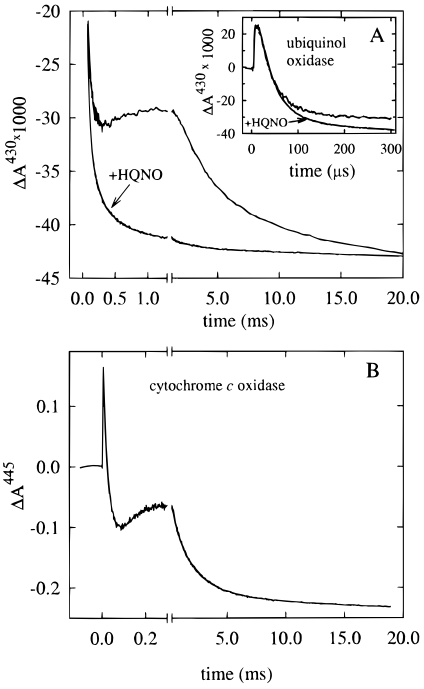

FIGURE 1: Absorbance changes following flash photolysis of the fully reduced CO complex of cytochrome bo_3 (A, at 430 nm) and of bovine cytochrome *c* oxidase (B, at 445 nm) in the presence of oxygen. The lower trace in panel A shows an experiment done in the presence of HQNO (70 μM), which displaces QH_2 from its binding site. It has been moved down by 0.01 absorbance unit for clarity. The inset in panel A shows the time courses of the first electron-transfer phases on an expanded time scale. Triton X-100 and octyl glucoside were used in the preparation of the enzyme used in this experiment. The enzyme prepared with dodecyl maltoside gave qualitatively the same results; relative amplitudes of the kinetic phases were different depending on the QH_2 content. Experimental conditions after mixing: (A) 0.3 μM cytochrome bo_3, 1 mM oxygen, 0.1 M Hepes-KOH, 0.1% *N*-dodecyl sarcosinate, pH 7.5; (B) ~2.5 μM cytochrome *c* oxidase, 1 mM oxygen, 0.1 M Hepes-KOH, 0.1% dodecyl maltoside, pH 7.4. A flash artifact at $t = 0$ has been truncated.

with dissociation of CO was followed by three kinetic phases with time constants of 45 ± 5 μs, 700 ± 200 μs, and 4 ± 1 ms in the following referred to as the first, second, and third phases, respectively. These time constants were determined from a global fit to data collected at nine different wavelengths (errors are SD determined from fits at the different wavelengths) using the following model:

$$A \xrightarrow{45\,\mu s} B \xrightarrow{90\,\mu s} C \xrightarrow{700\,\mu s} D \xrightarrow{4\,ms} E \qquad (1)$$

where the states A−E are defined in the Discussion. Step B → C was included in the model, but it does not contribute to the absorbance changes because it is associated with a protonation reaction (see below). Figure 2B shows kinetic difference spectra of the three kinetic phases. They were determined from the global fit and show absorbance changes corresponding to the reactions steps in eq 1 at the different wavelengths. It is seen that the spectra are consistent with heme redox reactions. The first phase was associated with oxidation of hemes *b* and o_3 and formation of the peroxy intermediate (Svensson Ek et al., 1996). The spectra of the second and third phases are consistent with reduction and oxidation of heme, respectively. The difference spectrum of the absorbance changes associated with CO dissociation were fitted with a static reduced-minus-(CO-reduced) spectrum (Figure 2A), and the concentration active enzyme was calculated from this fit to be 0.3 μM. Due to a laser artifact, the CO-dissociation amplitude was determined by extrapolating the fitted curve to $t = 0$. The deviation between the static and the kinetic spectra is most likely due to the presence of a fast phase associated with O_2 binding (Oliveberg et al., 1989; Verkhovsky et al., 1996), not included in the fit.

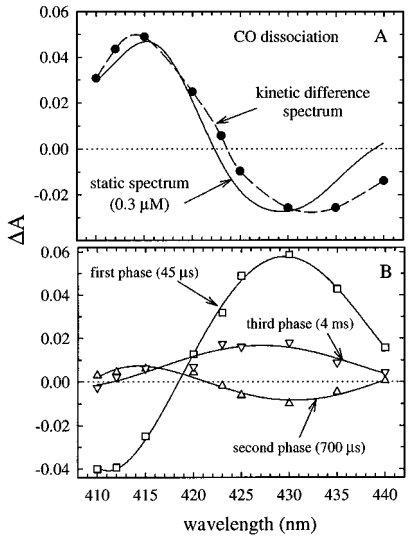

FIGURE 2: Kinetic difference spectra of the CO dissociation (A) and the three phases of electron transfer (B) in cytochrome bo_3. The CO dissociation spectrum (● in panel A) is compared with a static reduced/CO-*minus*-reduced spectrum of cytochrome bo_3 from the strain RG145, normalized to 0.3 μM cytochrome bo_3 (solid line). The dashed line in panel A and solid lines in panel B are drawn through the data points for clarity. Experimental conditions: 0.1 M Hepes-KOH, 0.1 *N*-dodecyl sarcosinate, pH 7.5.

Figure 1B shows the same experiment as that described above but with the mitochondrial bovine cytochrome aa_3 at 445 nm. The O_2 reduction reaction in this enzyme has been characterized in detail previously [reviewed by Babcock and Wikström (1992) and Ferguson-Miller and Babcock (1996)], and therefore these results are helpful for the interpretation of the cytochrome bo_3 data. It is seen that similar absorbance changes are observed as in cytochrome bo_3 at 430 nm. In the bovine enzyme, the first phase[3] ($\tau \cong 30$ μs) is also associated with oxidation of the hemes and formation of the peroxy intermediate. The second phase, observed as an increase in absorbance with a time constant of ~100 μs, is due to partial re-reduction of heme *a* (cf. heme *b* in ubiquinol oxidase) by Cu_A and formation of the ferryl intermediate. The slowest decrease in absorbance with a time constant of ~1 ms is due to oxidation of heme *a* and formation of the fully oxidized enzyme.

In analogy with the reactions observed in the mitochondrial enzyme, the second and third phases observed in cytochrome bo_3 are probably due to re-reduction of heme *b* (see Discussion) and electron transfer from heme *b* to the binuclear center, respectively. In the ubiquinol oxidase, the fourth electron is donated by QH_2 instead of Cu_A (Puustinen et al., 1996).

After addition of HQNO, which displaces ubiquinone from its binding site (Meunier et al., 1995), the first phase was

[3] There is also a more rapid absorbance change associated with binding of O_2, but it is not discussed here.

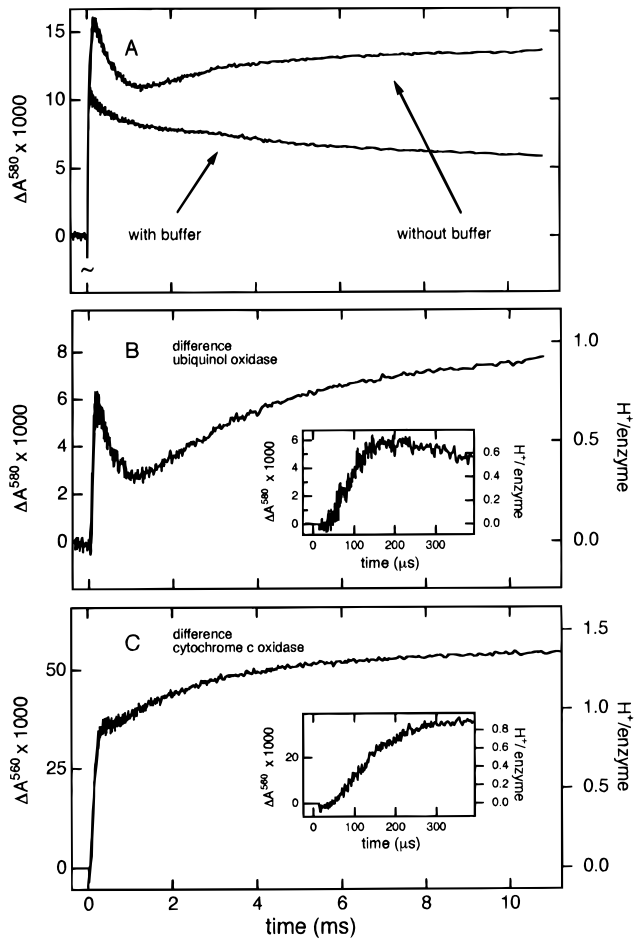

FIGURE 3: (A) Absorbance changes associated with proton uptake/release without (lower trace) and with (upper trace) buffer during the reaction between cytochrome bo_3 and oxygen and the difference between the two traces (B). The trace in panel C shows the difference trace (without minus with buffer) of the same reaction in bovine cytochrome *c* oxidase. The insets in panels B and C show the time courses of the first proton uptake phase on an expanded time scale. The reactions were monitored at 580 nm (cytochrome bo_3) and 560 nm (cytochrome *c* oxidase) where the dyes cresol red and phenol red, respectively, display maximum absorbance changes associated with pH changes. Experimental conditions after mixing: (A and B) 1.5 μM cytochrome bo_3, 0.1 M Tris-HCl or 0.1 M KCl, 30 μM cresol red, 0.1% *N*-dodecyl sarcosinate, pH ~8.0, and 1 mM O_2. (C) 2.5 μM cytochrome *c* oxidase, 0.1 M Hepes-KOH or 0.1 M KCl, 40 μM phenol red, 0.1% dodecyl maltoside, pH 7.7 and 1 mM O_2.

unaffected whereas the second and third phases were not observed and were replaced by a small decrease (~10%) in absorbance at 430 nm with a time constant of ~300 μs (Figure 1A, lower trace), presumably associated with a small fraction (~10%) cytochrome oo_3 present in the preparation (see Discussion).

Figure 3A (upper trace) shows absorbance changes at 580 nm measured during the reaction of fully reduced cytochrome bo_3 with O_2 as described above, but without buffer and in the presence of the pH-sensitive dye cresol red in the solution. At this wavelength, changes in the dye absorbance have maximum contribution whereas the contribution from redox changes in the enzyme are small. Consequently, the observed absorbance changes are mainly associated with proton uptake and release. To eliminate the small contributions from heme absorbance changes, the same measurement was also repeated after addition of 100 mM buffer to the dye-containing solution (Figure 3A, lower trace) and the

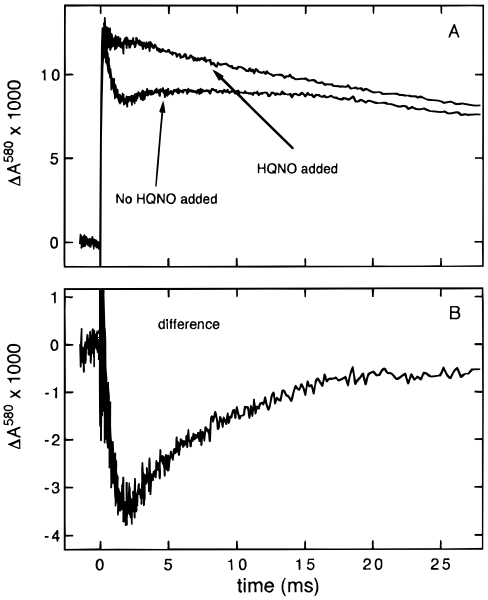

FIGURE 4: Time courses of proton uptake and release without buffer in the presence and absence of HQNO (A) and the difference between these traces (B). Only the two slowest phases ($\tau \cong 550$ μs, 3.3 ms) are observed in the difference trace. The 90 μs proton uptake is not affected by HQNO. The concentrations of cytochrome bo_3 and of HQNO were ~1 and 70 μM, respectively. Other conditions were the same as in Figure 3.

difference before and after addition of buffer was calculated (Figure 3B). After dissociation of CO, we observed a proton-uptake reaction with a time constant of 90 ± 10 μs followed by a slower proton release with a time constant of 550 ± 100 μs and proton uptake with a time constant of 3.3 ± 0.3 ms (errors are SD determined from a least-square fit, data is the average of 40 traces). The first proton-uptake phase is about 2 times slower than the first electron-transfer phase, i.e., proton uptake follows electron transfer. The second and third phases of proton transfer have about the same time constants as the corresponding phases during electron transfer, measured in the Soret region (see note below).

Figure 3C shows protonation reactions during reaction of the fully reduced mitochondrial cytochrome aa_3 with O_2. In this enzyme, two kinetic phases are observed with time constants of ~100 μs and ~1 ms, both associated with proton uptake. The intermediate proton-release reaction observed with cytochrome bo_3 is not observed with the aa_3 enzyme.

After addition of HQNO to the cytochrome bo_3 solution, the first phase was unaffected whereas the two slowest phases were not observed (Figure 4A); only the two slowest phases were found in the difference trace before-minus-after addition of HQNO (Figure 4B).

An EPR spectrum of the end product (~2 s after CO photolysis) of the reaction of the fully-reduced QH_2-containing ubiquinol oxidase with O_2 at pH 7.5 (not shown) indicates that a semiquinone radical is present in the sample. The radical signal was not observed in the fully-oxidized enzyme, and in the fully-reduced enzyme the signal was ~10% of that found in the end product. It is difficult to give an accurate value for the radical content because it is difficult to estimate the degree of CO dissociation in the EPR tube, but a limiting value is ≥15%. The lower limit is similar to that found by Ingledew et al. (1995).

The time constants of the two slower electron- and proton-transfer phases (only observed with bound QH_2) varied in

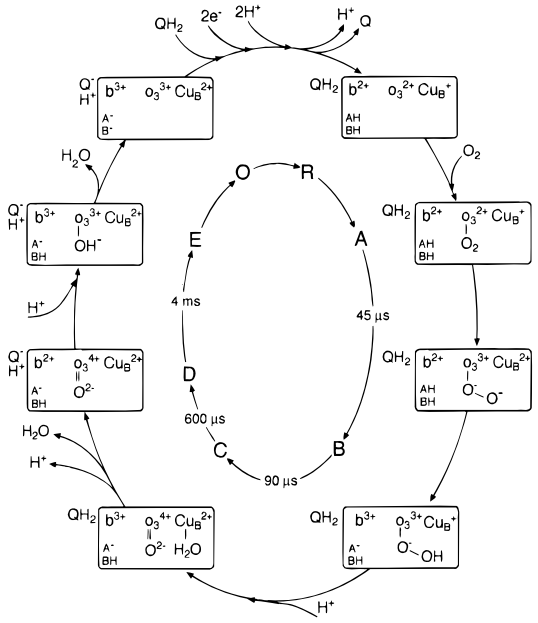

FIGURE 5: Proposed mechanism for the reaction of the fully reduced cytochrome bo_3 and dioxygen. A−E indicate intermediates which are referred to in the text. O and R are the oxidized and fully reduced enzyme, respectively. For simplicity, it is indicated in the model that the fifth electron stays on Q^-. The experimental data do not exclude that it may equilibrate with the other redox centers. In addition to the quinol protons, upon reduction, two protons are assumed to be picked up by the protonatable groups A and B, in analogy with the mitochondrial cytochrome *c* oxidase (Mitchell & Rich, 1994). These protons are used during oxygen reduction. The A → B transition is divided in two steps although it is observed as one single kinetic phase [cf. Svensson Ek et al. (1996)]. The proton near Q^- in states D, E, and O may be bound to Q^- or to groups interacting with the semiquinone (see also text). In the model, it is indicated that it is released during the O-to-R transition but during turnover it may be released earlier.

different enzyme preparations. This is presumably because of a variation of the QH_2 binding mode or site [cf. Sato-Watanabe et al. (1994) and Meunier et al. (1995)].

DISCUSSION

We have investigated the reaction between the fully reduced cytochrome bo_3 with bound quinol (QH_2) and dioxygen using the flow−flash methodology. Both electron- and proton-transfer reactions were studied.

Three kinetic phases associated with electron-transfer reactions were observed. The first phase was associated with oxidation of hemes *b* and o_3 and formation of the peroxy intermediate (Svensson Ek et al., 1996; Figure 5). The second and third electron-transfer phases were only observed in enzyme containing bound quinol and are therefore attributed to electron-transfer reactions associated with quinol oxidation (Puustinen et al., 1996).

During oxygen reduction in the structurally and functionally closely related, Cu_A-containing bovine cytochrome *c* oxidase, qualitatively the same type of absorbance changes were observed at 445 nm as at 430 nm in cytochrome bo_3 (compare Figure 1, panels A and B), but with different time constants. In the bovine enzyme, after the initial O_2 binding, the decrease in absorbance with a time constant of about 30 μs is associated with peroxy-intermediate formation. The following increase in absorbance is mainly associated with fractional electron transfer from Cu_A to heme *a* with a time constant of ∼100 μs (and ferryl-intermediate formation). The slowest decrease in absorbance with a time constant of ∼1 ms is due to electron transfer from heme *a* to the binuclear center and decay of the ferryl intermediate.

A comparison of the kinetic traces in the bovine and the bacterial enzymes, together with the difference spectra in Figure 2B, suggest that in the ubiquinol oxidase during the second phase (absorbance increase at 430 nm) an electron from the bound quinol is transferred to heme *b* with a time constant of ∼700 μs, followed by electron transfer from heme *b* to the binuclear center with a time constant of ∼4 ms. This is also indicated from the kinetic difference spectra of the second and third phases which are consistent with reduction and oxidation of heme *b*, respectively (Morgan et al., 1993). On the basis of the heme *b* spectrum presented by Morgan et al. (1993), the fraction of reduced heme *b* in enzymes containing QH_2 was estimated to be about 60%. The third phase probably also contains contribution from decay of the ferryl intermediate when the fourth electron arrives to the binuclear center.

When measured with the herbicide HQNO at 430 nm, we observed a small fraction (∼10%) slow ($\tau \cong 300$ μs) absorbance decay following the main 45 μs phase. The slower decay was presumably due to a ∼10% fraction cytochrome oo_3, i.e., oxidase with heme *o* instead of heme *b* incorporated in the low-spin site (Puustinen et al., 1992; Verkhovsky et al., 1994; Svensson Ek et al., 1996).

While the absorbance changes associated with electron-transfer reactions were qualitatively similar in the QH_2-containing ubiquinol and in the mitochondrial oxidases, the protonation reactions displayed different behaviors in these enzymes. In the mitochondrial enzyme, two kinetic phases were observed associated with proton uptake with time constants of ∼100 μs and ∼1 ms, respectively, i.e., the same time constants as those of the second and third electron-transfer phases, respectively [see also Hallén and Nilsson (1992)]. The first proton uptake has been suggested to be associated with protonation of a protonatable group in the vicinity of the binuclear center (Hallén et al., 1993; Svensson Ek et al., 1996). The slower proton uptake is associated with transfer of the fourth electron to the binuclear center. In cytochrome bo_3 the first proton-uptake reaction with a time constant of ∼90 μs has been observed earlier in QH_2-free enzyme (Hallén et al., 1993; Svensson Ek et al., 1996), and, as in the bovine enzyme, it has been proposed to be associated with protonation of an internal group. The proton release with a time constant of ∼550 μs was not observed after addition of HQNO. It is attributed to proton release during oxidation of QH_2. It is not observed in cytochrome aa_3 because in this enzyme, in the corresponding reaction, Cu_A donates the electron to heme *a*. As in cytochrome aa_3 the slowest proton uptake is likely to be associated with transfer of the fourth electron to the binuclear center from the low-spin heme.

To calculate the stoichiometry of proton uptake/release, we fitted the data with the following model:

$$\mathrm{A} \xrightarrow{45\,\mu s} \mathrm{B} \xrightarrow[+\chi_1 H^+]{90\,\mu s} \mathrm{C} \xrightarrow[-\chi_2 H^+]{550\,\mu s} \mathrm{D} \xrightarrow[+\chi_3 H^+]{3.3\,ms} \mathrm{E}$$

where the states A−E are defined in Figure 5 and χ_1, χ_2, and χ_3 are the fractions proton uptake (+) or release (−) in the different reaction steps. These parameters were calculated from a fit to the data in Figure 3. Since the two slowest

Table 1: Time Constants and Extents of Electron- and Proton-Transfer Reactions during Reaction Between Fully Reduced Cytochrome bo_3 with Bound Ubiquinol and Dioxygen[a]

	electron transfer	proton transfer	
reaction	time constant (μs)	time constant (μs)	number of protons
oxidation of hemes *b* and o_3 R → P	45 ± 5		
H^+ uptake P → F		90 ± 10	0.9 ± 0.3
H^+ release, reduction of heme *b* $b^{3+}QH_2 \rightarrow b^{2+}Q^- + 2H^+$	700 ± 200	550 ± 100	−1.1 ± 0.3
H^+ uptake, oxidation of heme *b* $b^{2+}F \rightarrow b^{3+}O$	4000 ± 1000	3300 ± 300	1.2 ± 0.4

[a] R, P, F, and O are the fully reduced enzyme, the peroxy and ferryl intermediates, and the fully oxidized enzyme, respectively.

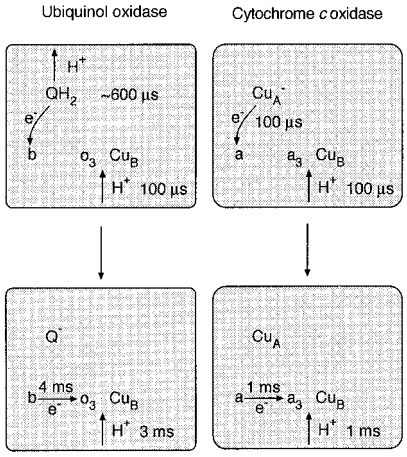

FIGURE 6: Comparison between cytochrome bo_3 and bovine cytochrome *c* oxidase showing similarities and differences in proton uptake and release. Initially, in both enzymes the peroxy intermediate is formed (not shown in the scheme), followed by proton uptake with a time constant of about 100 μs. In cytochrome *c* oxidase, this is accompanied by electron transfer from Cu_A to heme *a* with the same time constant (~100 μs), whereas in ubiquinol oxidase the re-reduction of heme *b* by QH_2 and the associated proton release is slower ($\tau \cong 600$ μs). Different time constants are observed for the re-reduction of heme *a*/heme *b* because in cytochrome *c* oxidase the reaction is limited by the 100 μs proton uptake whereas in the ubiquinol oxidase it is limited by proton release during oxidation of QH_2. The fourth electron is transferred from heme *a*/heme *b* to heme a_3/heme o_3 with time constants of about 1 and 4 ms, respectively.

reactions take place only in cytochrome bo_3 with bound QH_2, the two latter parameters must be divided by the fraction QH_2 (i.e., 0.7). The following values were obtained: $\chi_1 = 0.9 \pm 0.3$, $\chi_2 = 1.1 \pm 0.3$, $\chi_3 = 1.2 \pm 0.4$. Thus, electron transfer from QH_2 to heme *b* is accompanied by release of about 1.1 protons to the medium (Figure 3, Table 1).

There are several possible explanations for an observed net release of less than two protons associated with oxidation of the quinol; an equilibrium is probably established between QH_2 and heme *b* resulting only in a fractional electron transfer (heme $b^{3+} + QH_2 \leftrightarrow Q^- + 2H^+ +$ heme b^{2+}) (cf. above). Since the pK_a of the enzyme-bound semiquinone is 7−8 (Ingledew et al., 1995), a fraction of the semiquinone formed may be protonated. In addition, the unprotonated semiquinone formed during electron transfer may interact with nearby protonatable residues resulting in a fractional proton uptake [cf. interactions between heme a_3 and protonatable groups, Ädelroth et al. (1996)]. Similar effects have been observed in bacterial photosynthetic reaction centers where formation of the secondary semiquinone (Q_B^-) is associated with fractional proton uptake of up to 1 H^+/reaction center, depending on pH (McPherson et al., 1988; Maróti & Wraight, 1988). This proton uptake is due to electrostatic interactions between Q_B^- and nearby protonatable groups. Upon reduction of quinone, the pK_as of these groups increase and they become protonated to different degrees depending on pH and the interaction energy.

The slowest, third-phase electron transfer is accompanied by uptake of about 1.2 protons. As discussed above, this phase is associated with oxidation of heme *b* and reduction of the ferryl intermediate at the binuclear center forming water and oxidized heme o_3/Cu_B. Also, in bovine (Hallén & Nilsson, 1992) and *Rhodobacter sphaeroides* (P. Ädelroth, M. Svensson Ek, and P. Brzezinski, unpublished results), cytochrome *c* oxidases transfer of the fourth electron to the binuclear center is accompanied by uptake of 1−2 protons. However, in the ubiquinol oxidase this electron transfer may be accompanied by re-reduction of heme *b* either by Q^-H^+ or by Q^-, which may result in concomitant fractional proton release (cf. above).

CONCLUSION

The results from this work are summarized in Figures 5 and 6. Both in ubiquinol oxidase and in bovine cytochrome *c* oxidase during the reaction of the fully reduced enzymes with O_2 the peroxy intermediate is formed with time constants of ~45 and ~30 μs, respectively. In both enzymes, it is followed by proton uptake with a time constant of ~90 μs. In the bovine enzyme, re-reduction of heme *a* by Cu_A has the same time constant as that of the proton uptake. The *intrinsic* electron-transfer time constant from Cu_A to heme *a* is ~50 μs [see Brzezinski (1996)], but the reaction rate is limited by the 90 μs proton uptake (Hallén & Nilsson, 1992; Hallén & Brzezinski, 1994), i.e., the electron transfer is controlled by H^+ uptake at the binuclear center. In the QH_2-containing ubiquinol oxidase, re-reduction of heme *b* is much slower (~700 μs). This is presumably because this reaction *in itself* is slower since it is limited by the rate of proton release during QH_2 oxidation. In both enzymes, transfer of the fourth electron to the binuclear center from heme *b*/heme *a* is associated with proton uptake.

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