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Pathways of Proton Transfer in Cytochrome *c* Oxidase

Peter Brzezinski[1] and Pia Ädelroth[1]

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During the last few years our knowledge of the structure and function of heme copper oxidases has greatly profited from the use of site-directed mutagenesis in combination with biophysical techniques. This, together with the recently-determined crystal structures of cytochrome *c* oxidase, has now made it possible to design experiments aimed at targeting specific pump mechanisms. Here, we summarize results from our recent kinetic studies of electron and proton-transfer reactions in wild-type and mutant forms of cytochrome *c* oxidase from *Rhodobacter sphaeroides*. These studies have made it possible to identify amino acid residues involved in proton transfer during specific reaction steps and provide a basis for discussion of mechanisms of electron and proton transfer in terminal oxidases. The results indicate that the pathway through K(I-362)/T(I-359), but not through D(I-132)/E(I-286), is used for proton transfer to a protonatable group interacting electrostatically with heme a_3, i.e., upon reduction of the binuclear center. The pathway through D(I-132)/E(I-286) is used for uptake of pumped and substrate protons during the pumping steps during O_2 reduction.

KEY WORDS: Electron transfer; flow-flash; proton pumping; cytochrome aa_3; flash photolysis.

INTRODUCTION[2]

Cytochrome *c* oxidase is a membrane-bound protein complex which catalyzes the one-electron oxidation of cytochrome *c* to the four-electron reduction of dioxygen to water. Part of the released energy is conserved by pumping ~1 H^+ across the membrane per electron transferred in the opposite direction. Cytochrome aa_3 from *Rhodobacter sphaeroides* consists of three protein subunits which hold four redox-active metal centers involved in the catalytic reaction; copper A (Cu_A), the electron-input site, heme *a*, the intermediate electron acceptor, and the binuclear center consisting of heme a_3 and copper B (Cu_B), where dioxygen is bound and reduced to water. As most of the energy is released at the binuclear center, recently, most of the attention has been focused on this center and its vicinity in search for the pump element (for a recent review, see Ferguson-Miller and Babcock, 1996).

The crystal structures of cytochrome *c* oxidase from *Paracoccus denitrificans* (Iwata *et al.*, 1995) and bovine heart (Tsukihara *et al.*, 1995, 1996) have recently been determined to atomic resolution. They show that the binuclear center is in the membrane-spanning part of the protein, removed from the protein surface on the proton-input side by about 30 Å. Consequently, the protein must provide pathways for transfer of substrate protons to the binuclear center (used for reduction of O_2) and for pumped protons through the protein, via the "pump element." Such proton-transfer pathway(s) may be provided by a so-called "proton wire" (Nagle and Morowitz, 1978), a protein-spanning chain of protonatable amino acid residues and/or bound

[1] Department of Biochemistry and Biophysics, Göteborg University and Chalmers University of Technology, Medicinaregatan 9C, S-413 90 Göteborg, Sweden.

[2] Abbreviations and definitions: R, fully-reduced enzyme; A, ferrous-oxy intermediate; P, peroxy intermediate; F, ferryl intermediate; O, fully-oxidized enzyme; τ, time constant ($\exp(-t/\tau)$); ET, electron transfer; PT, proton transfer; WT, wild type; Cu_A, copper A; Cu_B, copper B; *substrate proton*, a proton used for reduction of O_2 to water (cf. *pumped proton*); mutant-enzyme nomenclature: EQ(I-286) denotes a replacement of glutamate-286 of subunit I by glutamine; D- and K-pathways, proposed proton-transfer pathways through D(I-132)/E(I-286) and K(I-362)/T(I-359), respectively. **If not otherwise indicated, amino-acid residues are numbered according to the *R. sphaeroides* sequence.**

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water molecules, as has been proposed for a number of biological systems such as, e.g., bacteriorhodopsin (Lanyi, 1993), photosynthetic reaction centers (Baciou and Michel, 1995), F_1F_o ATP synthase (Schulten and Schulten, 1985; Akeson and Deamer, 1991), and the cytochrome b_6f complex of oxygenic photosynthetic membranes (Martinez *et al.*, 1996).

PROTON-TRANSFER PATHWAYS

In the *P. denitrificans* structure model, two proton-input pathways in subunit I have been proposed. One pathway includes lysine 362 [K(I-362)] and threonine 359 [T(I-359)] and the other aspartate 132 [D(I-132)] and glutamate 286 [E(I-286)], denoted K- and D-pathways, respectively. Iwata *et al.* (1995) suggested that the K- and D-pathways may be used for substrate and pumped protons, respectively. These pathways are also found in the bovine-enzyme model (Tsukihara *et al.*, 1996) (Fig. 1), but the connection between E(I-286) [E(I-242) in the bovine enzyme] and the binuclear center was less obvious. Possible proton-exit pathways leading from the vicinity of the binuclear center to the protein surface have been discussed on the basis of the structural information, but so far no obvious candidates have been identified experimentally (see Qian *et al.*, 1997; Ferguson-Miller and Babcock, 1996).

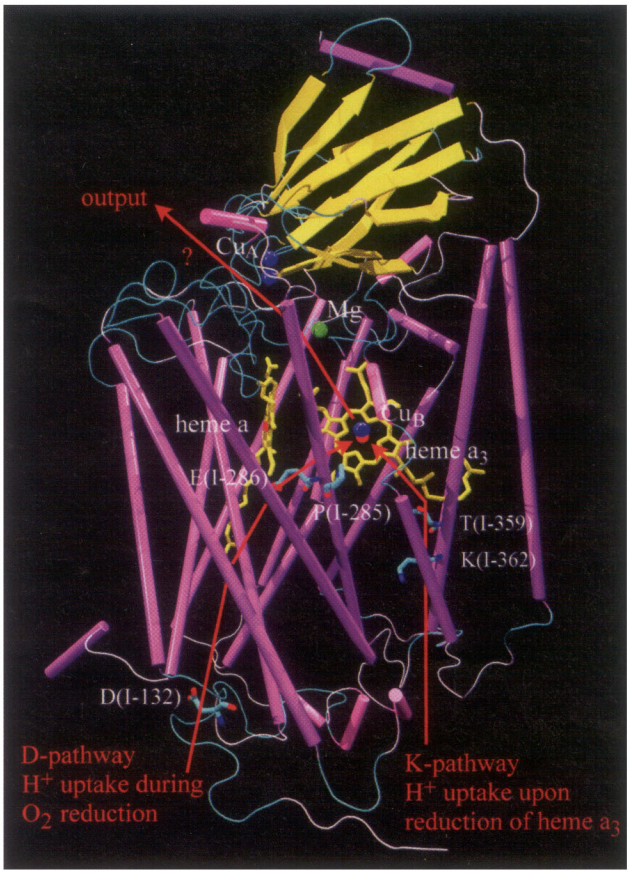

Fig. 1. Structure of cytochrome *c* oxidase. Two proton-transfer pathways through D(I-132)/E(I-286) (D-pathway) and K(I-362)/T(I-359) (K-pathway), respectively, are indicated (see text). In addition, residue P(I-285) in the D-pathway is shown. It is likely to provide an appropriate structure for binding of solvent molecules used for proton conductance (Iwata *et al.*, 1995). Thus, mutation of this residue is expected to disrupt the D-pathway "water chain" and impair proton transfer (see text). Enzymatic activities of the mutant enzymes were (in percent of wild type): DN(I-132), <5 (Fetter *et. al.*, 1995); EQ(I-286), <5 (Mitchell *et al.*, 1995); KM(I-362), <2; TA(I-359), ~30 (Hosler *et al.*, 1996). Coordinates are from Tsukihara *et al.* (1996). The illustration was made using the Visual Molecular Dynamic Software (Theoretical Biophysics Group, Beckman Institute for Advanced Science and Technology, University of Illinois at Urbana-Champaign, USA).

KINETICS OF ELECTRON AND PROTON TRANSFER IN THE WILD-TYPE ENZYME

Reaction of the Fully-Reduced Enzyme with O_2

The kinetics of electron and proton transfer during reaction of the fully-reduced enzyme with O_2 were investigated using the so-called flow-flash technique (Gibson and Greenwood, 1963). The fully-reduced enzyme with CO bound at heme a_3 is mixed rapidly with an O_2-containing buffer. The CO ligand is flashed off using a short laser flash, which allows O_2 to bind. This reaction in the bovine enzyme has been investigated in detail by several research groups using various spectroscopic techniques (for review see, e.g., Ferguson-Miller and Babcock, 1996; Hill, 1993; Babcock and Wikström, 1992).

A schematic outline of the reaction in the wild-type *R. sphaeroides* enzyme is shown in Fig. 2. Typical traces of absorbance changes at 445 and 605 nm associated with reaction of the fully-reduced (R) wild-type enzyme with O_2 are shown in Fig. 3. First, O_2 binds to heme a_3 forming the ferrous-oxy intermediate (A) ($\tau_{R\rightarrow A} \cong 8$ μs at 1 mM O_2) [absorbance decrease at 445 nm ($\Delta A^{445}<0$) and an initial lag at 605 nm]. This is followed by oxidation of hemes *a* and a_3, and formation of the peroxy intermediate (P) at the binuclear center ($\tau_{A\rightarrow P}\cong 50$ μs) (ΔA^{445}, $\Delta A^{605}<0$). Copper B is oxidized and the ferryl intermediate (F) is formed ($\tau_{P\rightarrow F}\cong 120$ μs at pH 7.4) without additional electron

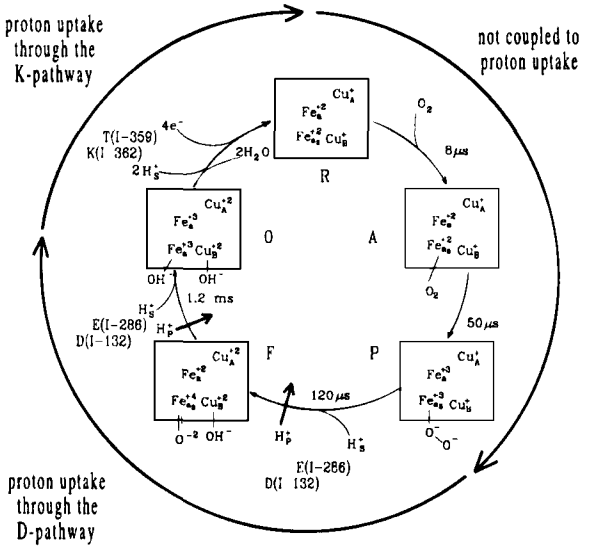

Fig. 2. A simplified reaction scheme showing the sequence of reactions during oxidation of the fully-reduced cytochrome *c* oxidase. It is largely based on results with the bovine enzyme (see text), but time constants are from experiments with the *R. sphaeroides* enzyme (P. Ädelroth, M. Svensson Ek, and P. Brzezinski, unpublished). The involvement of residues D(I-132), E(I-286), K(I-362), and T(I-359) in proton uptake is indicated. R, A, P, F, and O represent the fully reduced, ferrous-oxy, peroxy, ferryl, and fully-oxidized states, respectively. The structures of the partly-reduced oxygen intermediates illustrate only the reduction state and their actual structures may differ from those shown in the figure. The hydroxide in F may be released earlier than during O → R, i.e., the O state may bind less than two hydroxide ions. H_s^+ and H_p^+ represent substrate and pumped protons, respectively. Where indicated, the number of protons taken up is rounded off to integer numbers (cf. text). The bold arrows represent steps in which protons are pumped during turnover (P → F and F → O, Wikström, 1989). The number of pumped protons *in the flow-flash experiment* is still under debate (see text).

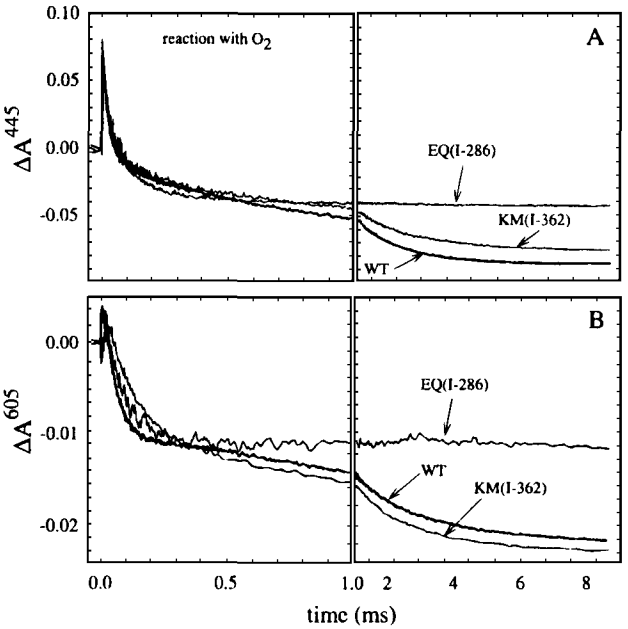

Fig. 3. Absorbance changes at 445 nm (A) and 605 nm (B), associated with electron-transfer reactions during oxidation of the wild-type (WT), KM(I-362), and EQ(I-286) enzymes. The fully-reduced enzyme–CO complex was mixed with an O_2-saturated buffer solution. After about 100 ms CO was photodissociated (at $t=0$). In the WT enzyme four kinetic phases were observed (see text). Absorbance changes observed with the DN(I-132) and TA(I-359) enzymes were about the same as those observed with the EQ(I-286) and KM(I-362) enzymes, respectively (see Ädelroth *et al.*, 1997, 1998). The concentration of reactive enzyme, ~1 μM, was calculated from the CO-dissociation change at 445 nm. Experimental conditions: 22°C, 0.1 M Hepes, pH 7.4, 0.05% dodecyl-β-D maltoside, 1 mM O_2 (for experimental details see Ädelroth *et al.*, 1997, 1998).

transfer to the binuclear center. At the same time there is fractional electron transfer from Cu_A to heme *a* (ΔA^{445}, $\Delta A^{605}>0$), seen as a "plateau" at 605 nm around 300 μs after the flash (Fig. 3B). Finally, the fourth electron is transferred to the binuclear center and the fully oxidized state (O) is formed ($\tau_{F\rightarrow O}\cong 1.2$ ms at pH 7.4) (ΔA^{445}, $\Delta A^{605}<0$). Figure 4 shows absorbance changes associated with proton uptake from the bulk solution during O_2 reduction. In the solubilized enzyme a net uptake of 0.75 ± 0.15 H^+ per enzyme was observed during each of the P → F and F → O transitions, respectively (P. Ädelroth, M. Svensson-Ek, and P. Brzezinski, unpublished), similar to that observed in the bovine enzyme (Oliveberg *et al.*, 1991). Also a slower component with a time constant of ~5 ms was observed, possibly associated with protonation or release of a hydroxide bound at the binuclear center (see below).

Proton Uptake upon Reduction

A net of ~1.8 protons are taken up during oxidation of the solubilized *R. sphaeroides* enzyme (including the slower ~5 ms phase, see above). Since a net of 4 protons are consumed during O_2 reduction to water, the oxidized enzyme must pick up ~2.2 protons upon reduction, assuming that there are no changes in the protonation state after the 5-ms phase. Using the same approach, about the same number was obtained with the bovine enzyme (Oliveberg *et al.* 1991; Mitchell and Rich, 1994).

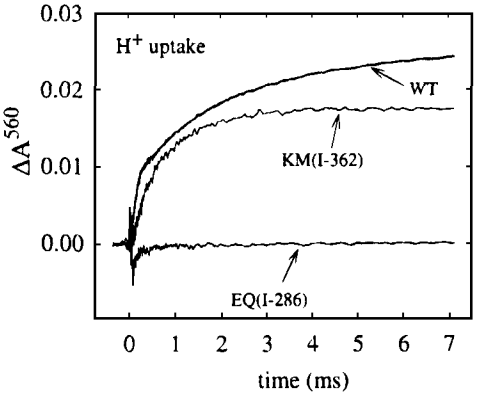

Fig. 4. Absorbance changes at 560 nm of the dye phenol red, showing proton uptake during oxidation of the fully-reduced wild-type (WT), KM(I-362), and EQ(I-286) enzymes. Absorbance changes observed with the DN(I-132) and TA(I-359) enzymes were about the same as those observed with the EQ(I-286) and KM(I-362) enzymes, respectively (see Ädelroth *et al.*, 1997, 1998). The traces shown are the differences between the traces obtained in an unbuffered (with 0.1 M KCl) and a buffered solution (0.1 M Hepes) pH 7.5–8.0. Other conditions were the same as in Fig. 3.

INTRAMOLECULAR ELECTRON TRANSFER IN THE ABSENCE OF O_2

Internal electron-transfer reactions in cytochrome *c* oxidase in the absence of O_2 were studied following flash photolysis of the mixed-valence CO complex. In this complex, reduced heme a_3/Cu_B are stabilized by the binding of CO whereas heme *a* and Cu_A are oxidized. After flash photolysis of CO, electrons are first transferred from heme a_3 to *a* ($\tau \cong 3$ μs), which results in a ~40% reduction of heme *a*, followed by electron transfer from the heme a_3/heme *a* equilibrium to Cu_A ($\tau \cong 30$ μs) (Fig. 5AC). The time scale of these electron-transfer reactions is shorter than that of proton transfer (Ädelroth *et al.*, 1995; Brzezinski, 1996) and therefore during these electron transfers the enzyme is in the same protonation state as with a reduced binuclear center.

Proton-Coupled Electron Transfer

The rapid electron equilibria described above are followed by an additional, slower electron transfer from heme a_3 to *a* ($\tau \cong 3$ ms at pH 9) (Fig. 5B) with a pH-dependent time constant and amplitude. It is coupled to proton release to the medium of ~0.7 H^+/e^- at pH 9.5. (Ädelroth *et al.*, 1996). This reaction was modeled in terms of electrostatic interactions between

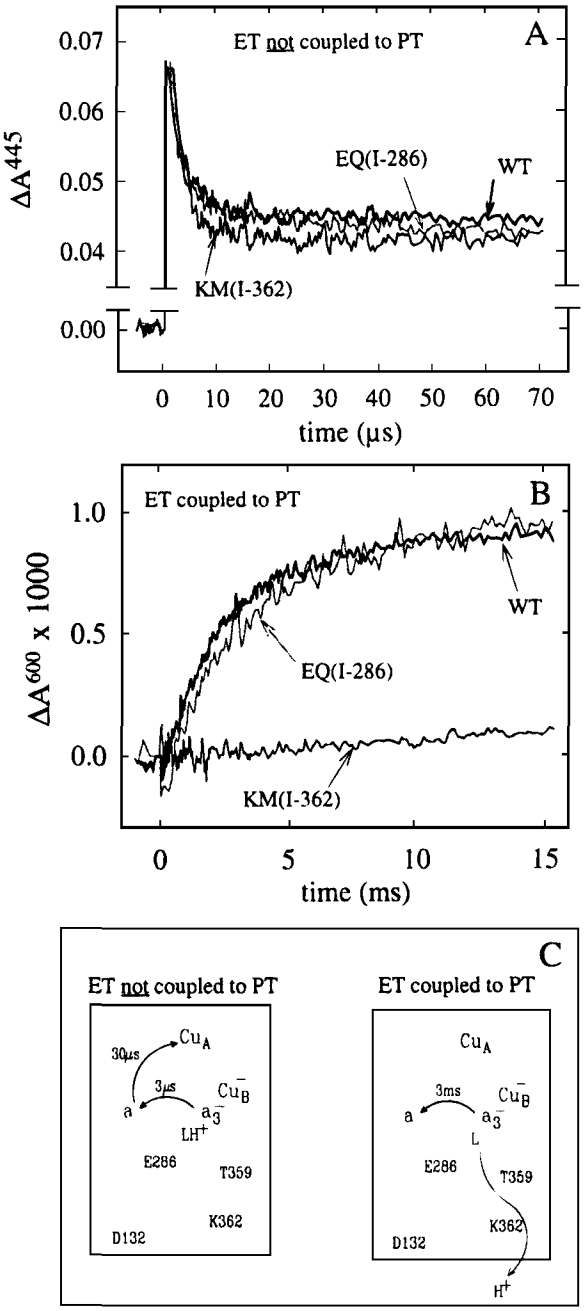

Fig. 5. (A, B) Absorbance changes following flash photolysis of CO from mixed-valence wild-type (WT), KM(I-362), and EQ(I-286) enzymes in the absence of O_2. In (A) the kinetic phases at 445 nm with time constants of 3 and 30 μs (after CO dissociation at $t=0$) are associated with electron transfer from heme a_3 to *a* and from hemes a/a_3 to Cu_A, respectively (there is also a contribution from CO recombination with a rate of about 50 s^{-1}). In (B), absorbance changes at 600 nm [note the different wavelength as compared to (A)] associated with proton-controlled electron transfer from heme a_3 to *a* are shown. Absorbance changes observed with the DN(I-132) and TA(I-359) enzymes were about the same as those observed with the EQ(I-286) and KM(I-362) enzymes, respectively

heme a_3 and a protonatable group(s), *L*, (Fig. 5C) that changes its pK_a from 10.3 to 9.1 upon oxidation of heme a_3 (interaction energy ~70 meV). The group *L* was proposed to be in contact with the bulk solution through a proton-transfer pathway, which determines the rate of proton release and, indirectly also of the electron transfer from heme a_3 to *a* (Ädelroth *et al.*, 1996; Hallén *et al.*, 1994). Thus, investigation of electron transfer after flash photolysis of CO from the partly-reduced enzyme provides information both about rates and driving forces of electron transfers and, in addition, about proton-transfer reactions coupled to oxidation/reduction of heme a_3. *Thus, this is an ideal experimental system for investigation of mechanisms of coupling between electron and proton transfer in cytochrome c oxidase using site-directed mutagenesis because it is possible to independently probe effects of a mutation on electron and proton transfer, respectively.*

ELECTRON AND PROTON TRANSFER IN PROTON-PATHWAY MUTANT ENZYMES

To identify amino acid residues and proton-transfer pathways involved in specific steps of the cytochrome *c* oxidase reaction cycle, we have investigated electron and proton-transfer reactions in mutant enzymes in which amino-acid residues in the D- and K-pathways (see Fig. 1), respectively, have been modified (Mitchell *et al.*, 1995; Fetter *et al.*, 1995; Hosler *et al.*, 1996). Mutations of these residues result in reduced overall enzymatic activities (see legend of Fig. 1). In addition, we discuss results from studies of mutants of cytochrome bo_3 from *E. coli* in which residues around E(I-286) in the D-pathway have been modified (Svensson-Ek *et al.*, 1996). These mutations are expected to alter the structure of water molecules in the proton wire through the D-pathway (Hofacker and Schulten, 1997; Riistama *et al.*, 1997).

(see **Ädelroth** *et al.*, 1997a, b). (C) Schematic model showing the reactions after CO dissociation from the mixed-valence enzyme. Note that in the 3-μs reaction ~40% heme a_3 is oxidized. Proton release from *L* is coupled to *additional* oxidation of heme a_3. Conditions: 0.1 M Hepes, pH 7.4 [in (A)]/0.1 M Tris-HCl, pH 8.8 [in(B)], 0.1% β-D-dodecyl maltoside, 1 μM reacting enzyme, 1 mM CO, 22°C (for experimental details, see Ädelroth *et al.*, 1997, 1998).

K-Pathway Mutant Enzymes

Figures 3 and 4 show absorbance changes associated with electron and proton transfer, respectively, during the single turnover reaction of the fully-reduced KM(I-362) enzyme with O_2. The same results were obtained also with the TA(I-359) enzyme (not shown). The results show that the mutant enzymes become essentially fully oxidized within the same time as the wild-type enzyme (Ädelroth *et al.*, 1998), with a slowing of the individual rates of at most a factor of 3. In addition, the net proton uptake during the P → F and F → O transitions is about the same in the mutant as in the wild-type enzymes, which indicates that the K-pathway is not involved in proton uptake during oxidation of the fully-reduced enzyme, i.e., the decreased (impaired) activity of the K-pathway mutant enzymes is not due to blocked "electron output" during O_2 reduction. Similar results were also obtained in studies of the reaction with O_2 of the fully-reduced KM(I-362) and TA(I-359) of cytochrome bo_3 from *E. coli* (Svensson *et al.*, 1995). However, since this enzyme accommodates only three redox-active metal sites, the reaction could not be studied beyond formation of the F intermediate.

While no significant effects were observed on the oxidation rates of the KM(I-362) and TA(I-359) enzymes, the reduction rate of heme a_3 in oxidized KM(I-362) was at least an order of magnitude slower that that of the wild-type enzyme. A slow reduction rate of the binuclear centre was also observed by other investigators (Hosler *et al.* 1996; Jünemann *et al.*, 1997; Konstantinov *et al.*, 1997).

In the mixed-valence KM(I-362) and TA(I-359) enzymes, electron transfer between heme a_3, heme *a* and Cu_A in the absence of O_2 was not significantly affected, whereas the slower, proton-coupled electron transfer was impaired (Fig. 5B) (Ädelroth *et al.*, 1998). This shows that the mutations do not affect internal electron transfer *per se* but impair proton transfer coupled to oxidation/reduction of heme a_3. In addition, the results indicate that the proton pathway suggested to limit the rate of this proton transfer is the K-pathway (see also below and Fig. 5C).

In conclusion, the results indicate that the K-pathway is used for proton uptake during reduction of heme a_3 (or the binuclear center). Once the enzyme is fully reduced [which in the KM(I-362) enzyme requires long ($>10^3$ s) incubation time with a reductant], oxidation of the enzyme does not involve proton transfer through the K-pathway.

D-Pathway Mutant Enzymes

In contrast to the K-pathway mutant enzymes, the EQ(I-286) enzyme did not become fully oxidized in the flow-flash experiment (Fig. 3); the 120 μs and 1.2 ms phases were not observed and the enzyme was "trapped" at the level of the P intermediate with Cu_A remaining reduced (Ädelroth *et al.*, 1997). In addition, proton uptake from the medium was impaired (Fig. 4). Similar results were also obtained with the DN(I-132) enzyme (Smirnova, Ädelroth, Gennis, and Brzezinski, unpublished), which shows that the D-pathway is involved in proton uptake after formation of the P intermediate. In addition, these experimental results support the proposal that D(I-132) and E(I-286) are part of the same pathway (Iwata *et al.*, 1995).

Konstantinov *et al.* (1997) found recently that electrogenic events associated with proton pumping during the F → O transition were impaired in the EQ(I-286) enzyme reconstituted in lipid vesicles, which is consistent with our results. Also in cytochrome bo_3 from *E. coli* proton uptake concomitant with the P → F transition was impaired when E(I-286) was modified (Svensson Ek *et. al.*, 1996). In addition, mutations of P(I-285) (see Fig. 1) and W(I-280), in the vicinity of E(I-286), also resulted in impaired proton uptake. As suggested previously (Svensson Ek *et al.*, 1996), mutation of P(I-285) and most likely of W(I-280) result in alterations of the hydrogen-bonding pattern in the D-pathway. Theoretical calculations show that the D-pathway can accommodate about ten ordered water molecules (Hofacker and Schulten, 1997; Riistama *et al.*, 1997), which form a hydrogen-bonded "proton wire" (Nagle and Morowitz, 1978). Other calculations show that proton transfer through such a wire is a semicollective process where the proton within the pathway is present as an $O_2H_5^+$ ion (i.e., the proton is shared by two water molecules) and the excess positive charge orients a large number of water molecules in the pathway (Pomès and Roux, 1997; see also review by Gutman and Nachliel, 1990). Thus any modification of the protein structure around the pathway is expected to impair proton transfer.

In the EQ(I-286) and DN(I-132) enzymes neither internal electron transfer nor the slower proton-coupled electron transfer were affected in the mixed-valence enzyme in the absence of O_2 (Ädelroth *et al.*, 1997a). This shows that the mutations do not affect internal electron transfer and that proton uptake/release directly coupled to reduction/oxidation of heme a_3 (binuclear center) does not take place through the D-pathway. Thus, taken together the above described results indicate that the D-pathway is involved in proton uptake exclusively during the P → F and F → O transitions.

MECHANISTIC IMPLICATIONS

Electron and Proton Transfer in the Absence of Oxygen

The two rapid internal electron-transfer reactions in the mixed-valence enzyme in the absence of O_2 (~3 and ~30 μs, not coupled to PT) were not significantly affected in the mutant enzymes, which indicates that the mutations do not affect electron-transfer pathways or redox potentials, and any effects on electron transfer in the mutant enzymes were due to altered proton-transfer rates, linked to the electron transfer. Oxidation of heme a_3 in the absence of O_2, coupled to proton release was impaired in the K-pathway but not in the D-pathway mutant enzymes. This indicates that protonation of the group *L*, interacting with heme a_3, takes place through the K-pathway and not through the D-pathway. Possible candidates for *L* were discussed based on the measured interaction energy of *L* with heme a_3 (Ädelroth *et al.*, 1996) and the crystal structure of cytochrome *c* oxidase. With the additional information from the mutant-enzyme studies it is now possible to rule out E(I-286) and presumably also K(I-362) [because it is further away from heme a_3 than T(I-359)], and limit the number of candidates to those that are located closer to heme a_3 than T(I-359): Y(I-288), T(I-352), the histidine ligands of Cu_B, and the propionate side chains of heme a_3 (Ädelroth *et al.*, 1996). The residue Y(I-288) is a particularly interesting candidate because in the bovine-enzyme structure it has recently been found to be covalently bound to H(I-284), a Cu_B ligand (S. Yoshikawa, personal communication), which is an unusual structure and may suggest a specific role for Y(I-288) in proton pumping [Y(I-244) and H(I-240), respectively, in the bovine-enzyme sequence]. It is also conceivable that *L* is a water molecule which releases a proton upon oxidation of heme a_3 and binds to oxidized heme a_3 as a hydroxide ion.

Proton Uptake upon Reduction and after Oxidation of the Binuclear Center—the K-Pathway

In the flow-flash experiment, in wild-type enzyme, *after* oxidation of the fully-reduced enzyme,

proton uptake with a time constant of about 5 ms was observed (Fig. 4) without any significant further absorbance changes of the enzyme. This component was much slower or smaller in the KM(I-362) enzyme. Since two OH^- are formed during O_2 reduction (Han *et al.*, 1990; Varotsis *et al.*, 1993), one OH^- may be released or pick up a proton through the K-pathway after the enzyme is oxidized (~5 ms phase) and one OH^- may remain bound at the binuclear center in the oxidized enzyme (Lanne *et al.*, 1979; Wikström 1988; Han *et al.*, 1990; Varotsis *et al.*, 1993; Fann *et al.*, 1995). Upon reduction of the oxidized binuclear center at least one proton is taken up through the K-pathway, presumably to the bound hydroxide (cf. discussion of *L* above). This conclusion is in line with results of Jünemann *et al.* (1997) who found that in the KM(I-362) mutant enzyme one, but not two, electrons can rapidly enter the binuclear center, which indicates that during reduction at least one proton is taken up through the K-pathway (Jünemann *et al.*, 1997 interpreted their results in a different way).

The ~5-ms proton uptake after oxidation may appear not to be catalytically relevant because the turnover rate of the wild-type enzyme is of the order of $10^3\ s^{-1}$ (pH 7.4). However, during turnover, the proton uptake would most likely be accelerated by reduction of the binuclear center during the next reaction cycle.

Proton Uptake during Reduction of O_2—the D-Pathway

The results indicate that all protons taken up during oxidation of the fully-reduced enzyme are transferred through the D-pathway. Assuming that there are only two proton-input pathways (see Introduction), at least two of these protons must be substrate protons because about two protons are "preloaded" in the fully-reduced enzyme and four protons are needed to reduce O_2 to water. The number of pumped protons transferred through the D-pathway during oxidation of the fully-reduced enzyme is uncertain because inconsistent numbers of protons pumped in the flow-flash experiment have been obtained. Results from measurements of pH-sensitive dye absorbance changes associated with proton release out from enzyme reconstituted in lipid vesicles indicate that 1–2 protons are released to the output side (pumped) with a time constant of ~1 ms (Oliveberg *et al.*, 1991) whereas based on results from measurements of electrogenic events in enzyme reconstituted in lipid membranes Verkhovsky *et al.* (1997) suggested that the number is 4. We leave this question open and conclude that at least 1–2 pumped protons are transferred through the D-pathway to the pump element.

As discussed above, the transfer of substrate protons through the D-pathway occurs only during the two last reaction steps (P → F and F → O). Since only these two steps are coupled to proton pumping (Wikström, 1989), the use of the same pathway for both pumped protons and substrate protons *during the pumping steps* may be a way for the enzyme to prevent substrate protons from being transferred to the binuclear center before pumped protons are taken up.

The results summarized in this work indicate somewhat different roles for the proton pathways from those proposed earlier (Iwata *et al.*, 1995). The earlier assignment of the D-pathway was based on results from studies of site-directed mutants of D(I-132) [or D(I-135) in cytochrome bo_3 from *E. coli*], which lost their ability to pump protons while retaining some of their O_2-reduction ability (Thomas *et al.*, 1993; Fetter *et al.*, 1995). However, recent results indicate that the DN(I-132) enzyme reconstituted in a membrane can pick up protons from the *output side* (Fetter *et al.*, 1996), and therefore the interpretation of the earlier results must be revised.

Proton-Transfer-Controlled Electron Transfer

During oxidation of cytochrome *c* oxidase the intermediate states build up to detectable concentrations because the reaction gradually decelerates as it proceeds, which is most likely a consequence of the proton-pumping function of the enzyme; a tight coupling between proton pumping and the exergonic electron transfers to partly-reduced oxygen intermediates, which drive the pump, can be maintained if the electron-transfer rates (and the associated uptake of substrate protons) are controlled by the uptake of pumped protons (Babcock *et al.*, 1996; Ferguson-Miller and Babcock, 1996; Babcock and Wikström, 1992).

A way by which proton transfer may control electron transfer to the binuclear center is evident from the lack of the 120 μs phase (ET from Cu_A to heme *a* during the P → F transition) and proton uptake in the EQ(I-286) enzyme (Ädelroth *et al.*, 1997). In this step, proton uptake, the P → F transition, and electron transfer from Cu_A to heme *a* all display the same time constant (120 μs) even though the intrinsic electron-transfer rate from Cu_A to heme *a* is faster ($\tau \cong 30$ μs).

During the P → F transition the charge of the binuclear center increases by +1 (see Fig. 2), and it has been suggested that this increase in positive charge may increase the apparent redox potential of heme *a* (which is much closer to the binuclear center than Cu_A is), which "opens the gate" for electron transfer between Cu_A and heme *a* (Hallén and Brzezinski, 1994; Svensson Ek and Brzezinski, 1996; Ädelroth *et al.*, 1997). Thus, in the EQ(I-286) enzyme electron transfer from Cu_A to heme *a* is impaired, because proton uptake is impaired and the charge of the binuclear center does not change. Direct electron transfer from Cu_A to heme a_3 is presumably much slower than that through heme *a* (Brzezinski, 1996; see also Regan *et al.*, 1998).

SUMMARY

From the studies described above it is evident that modification of the structure or components of a proton pathway may have dramatic effects on the proton-transfer rates. This is consistent with theory and studies of model complexes, which indicate that an increase in the distance between components of a "proton wire" by 1–2 Å can dramatically diminish the proton-transfer rate (Gutman and Nachliel, 1990). This also suggests that the pump can operate without any major structural changes and the movement of, e.g., a single amino acid residue can control the accessibility of protons to the two sides of the membrane.

The K-pathway, but not the D-pathway, is used for proton uptake/release due to reduction/oxidation (electrostatic) interactions of a group *L* (which may be H_2O/OH^-) with the binuclear center. During O_2 reduction, uptake of two of the substrate protons during the pumping steps, as well as of the pumped protons, take place through the D-pathway. Thus, the results are consistent with a model in which the K-pathway is used for proton transfer coupled to reduction of the binuclear center, and proton transfer through the D-pathway is strictly linked with protonation/deprotonation of the "pump element" and substrate protons are transferred through this pathway only during the pumping steps (P → F and F → O).

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REFERENCES

Ädelroth, P., Brzezinski, P., and Malmström, B. G. (1995). *Biochemistry* **34**, 2844–2849.
Ädelroth, P., Sigurdson, H., Hallén, S., and Brzezinski P. (1996). *Proc. Natl. Acad. Sci. USA* **93**, 12292–12297.
Ädelroth, P., Svensson Ek, M., Mitchell, D.M, Gennis, R. B., and Brzezinski, P. (1997). *Biochemistry* **36**, 13824–13829.
Ädelroth, P., Gennis, R. B., and Brzezinski, P. (1998). *Biochemistry* **37**, 2470–2476.
Akeson, M., and Deamer, D. W. (1991). *Biophys. J.* **60**, 101–109.
Babcock, G. T., and Wikström, M. (1992). *Nature* **356**, 301–309.
Babcock, G. T., Floris, R., Nilsson, T., Pressler, M., Varotsis, C., and Vollenbroek, E. (1996). *Inorg. Chim. Acta* **243**, 345–353.
Baciou, L., and Michel, H. (1995). *Biochemistry* **34**, 7967–7972.
Brzezinski, P. (1996). *Biochemistry* **35**, 5611–5615.
Fann, Y. C., Ahmed, I., Blackburn, N. J., Boswell, J. S., Verkhovskaya, M. L., Hoffman, B.M., and Wikström, M. (1995). *Biochemistry* **34**, 10245–10255.
Ferguson-Miller, S., and Babcock, G. T. (1996). *Chem. Rev.* **96**, 2889–2907.
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.
Fetter, J. R., Sharpe, M., Qian, J., Mills, D., Ferguson-Miller, S., and Nicholls, P. (1996). *FEBS Lett.* **393**, 155–160.
Gibson, Q. H., and Greenwood, C. (1963). *Biochem. J.* **86**, 541–554.
Gutman, M., and Nachliel, E. (1990). *Biochim. Biophys. Acta* **1015**, 391–414.
Hallén, S., and Brzezinski, P. (1994). *Biochim. Biophys. Acta* **1184**, 207–218.
Hallén, S., Brzezinski, P., and Malmström, B. G. (1994). *Biochemistry* **33**, 1467–1472.
Han, S, Ching, Y. C., and Rousseau, D. L. (1990). *Nature* **348**, 89–90.
Hill, B.C. (1993). *J. Bioenerg. Biomembr.* **25**, 115–120.
Hofacker, I., and Schulten, K. (1998). *Proteins* **30**, 100–107.
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.
Iwata, S., Ostermeier, C., Ludwig, B., and Michel, H. (1995). *Nature* **376**, 660–669.
Jünemann, S., Meunier, B., Gennis, R. B., and Rich, P. R. (1997). *Biochemistry* **36**, 14456–14464.

Konstantinov, A. A., Siletsky, S., Mitchell, D., Kaulen, A., and Gennis, R. B. (1997). *Proc. Natl. Acad. Sci. USA* **94**, 9085–9090.

Lanne, B., Malmström, B.G., and Vänngård, T. (1979). *Biochim. Biophys. Acta* **545**, 205–214.

Lanyi, J.K. (1993). *Biochim. Biophys. Acta* **1183**, 241–261.

Martinez, S.E., Huang, D., Ponomarev, M., Cramer, W.A., and Smith, J.L. (1996). *Protein Sci.* **5**, 1081–1092.

Mitchell, D.M., Aasa, R., Ädelroth, P., Brzezinski, P., Gennis, R.B., and Malmström, B.G. (1995). *FEBS Lett.* **374**, 371–374.

Mitchell, R., and Rich, P. R. (1994). *Biochim. Biophys. Acta* **1186**, 19–26.

Nagle, J.F., and Morowitz, H.J.(1978). *Proc. Natl. Acad. Sci. USA* **75**, 298–302.

Oliveberg M., Hallén, S., and Nilsson, T. (1991). *Biochemistry* **30**, 436–440.

Pomès, R., and Roux, B. (1997). *Biophys. J.* **71**, 19–39.

Qian, J., Shi, W., Pressler, M., Hoganson, C., Mills, D., Babcock, G.T., and Ferguson-Miller, S. (1997). *Biochemistry* **36**, 2539–2543.

Regan, J. J., Ramirez, B. E., Winkler, J. R., Gray, H. B., and Malmstrom, B. G. (1998). *J. Bioenergetics and Biomembranes* **30**, 35–40.

Riistama, S., Hummer, G., Puustinen, A., Dyer, B.R., Woodruff, W.H., and Wikström, M. (1997). *FEBS Lett.* **414**, 275–280.

Schulten, Z., and Schulten, K. (1985). *Eur. Biophys J.* **11**, 149–155.

Svensson M., Hallén, S., Thomas, J. W., Lemieux, L. J., Gennis, R. B., and Nilsson, T. (1995). *Biochemistry* **34**, 5252–5258.

Svensson Ek, M., and Brzezinski, P. (1997). *Biochemistry* **36**, 5425–5431.

Svensson Ek, M., Thomas, J.W, Gennis, R.B, Nilsson, T., and Brzezinski, P. (1996). *Biochemistry* **35**, 13673–13680.

Thomas, J.W., Puustinen, A., Alben, J.O., Gennis, R.B., and Wikström, M. (1993). *Biochemistry* **32**, 10923–10928.

Tsukihara, T., Aoyama, H., Yamashita, E., Tomizaki, T., Yamaguchi, H., Shinzava-Itoh, K., Nakashima, R., Yaono, R., and Yoshikawa, S. (1995). *Science* **269**, 1069–1074.

Tsukihara, T., Aoyama, H., Yamashita, E., Tomizaki, T., Yamaguchi, H., Shinzava-Itoh, K., Nakashima, R., Yaono, R., and Yoshikawa, S. (1996). *Science* **272**, 1136–1144.

Varotsis, C., Zhang, Y., Appelman, E.H., and Babcock, G.T. (1993). *Proc. Natl. Acad. Sci. USA* **90**, 237–241.

Verkhovsky, M. I., Morgan, J. E., Verkhovskaya, M. L., and Wikström, M. (1997). *Biochim. Biophys. Acta* **1318**, 6–10.

Wikström, M. (1988). *Chemi. Scr.* **28A**, 71–74.

Wikström, M. (1989). *Nature* **338**, 776–778.