Glutamate 286 in Cytochrome aa_3 from *Rhodobacter sphaeroides* Is Involved in Proton Uptake during the Reaction of the Fully-Reduced Enzyme with Dioxygen[†]

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ABSTRACT: The reaction with dioxygen of solubilized fully-reduced wild-type and EQ(I-286) (exchange of glutamate 286 of subunit I for glutamine) mutant cytochrome *c* oxidase from *Rhodobacter sphaeroides* has been studied using the flow−flash technique in combination with optical absorption spectroscopy. Proton uptake was measured using a pH-indicator dye. In addition, internal electron-transfer reactions were studied in the absence of oxygen. Glutamate 286 is found in a proton pathway proposed to be used for pumped protons from the crystal structure of cytochrome *c* oxidase from *Paracoccus denitrificans* [Iwata et al. (1995) *Nature 376*, 660−669; E278 in *P.d.* numbering]. It is the residue closest to the oxygen-binding binuclear center that is clearly a part of the pathway. The results show that the wild-type enzyme becomes fully oxidized in a few milliseconds at pH 7.4 and displays a biphasic proton uptake from the medium. In the EQ(I-286) mutant enzyme, electron transfer after formation of the peroxy intermediate is impaired, Cu_A remains reduced, and no protons are taken up from the medium. Thus, the results suggest that E(I-286) is necessary for proton uptake after formation of the peroxy intermediate and transfer of the fourth electron to the binuclear center. The results also indicate that the proton uptake associated with formation of the ferryl intermediate controls the electron transfer from Cu_A to heme *a*.

Cytochrome aa_3 from *Rhodobacter sphaeroides* is a cytochrome *c* oxidase, and belongs to the same family of heme−copper oxidases as the extensively studied mitochondrial enzyme from bovine heart (Hosler et al., 1992). It catalyzes the reduction of dioxygen to water using electrons from cytochrome *c*. The protons needed to form water are taken up from the cytosol, and in addition, the enzyme pumps protons out of the cytosol, thus contributing to the proton gradient needed to synthesize ATP [Trumpower & Gennis, 1994; for a recent review on the structure and function of terminal oxidases, see Ferguson-Miller and Babcock (1996)].

The crystal structures of cytochrome *c* oxidase from two different sources, *Paracoccus denitrificans* (Iwata et al., 1995) and bovine heart (Tsukihara et al., 1995, 1996), have recently been solved. The enzymes contain four redox-active metal sites. Electrons from cytochrome *c* are first transferred to a dimetallic copper site, copper A (Cu_A)[1] and then consecutively to heme *a*, and to heme a_3, which is in close proximity to copper B (Cu_B), forming a bimetallic center, where oxygen is bound and reduced. The bimetallic center is located in the membrane-spanning part of the enzyme, removed from the liquid solution. Therefore, there must be pathways through which protons needed for the oxygen chemistry are transferred into the enzyme and protons to be pumped are transferred into and out from the enzyme. In the *P. denitrificans* enzyme, two proton pathways were identified, one proposed to be used for substrate protons, and the other for pumped protons. In the pathway proposed for pumped protons, glutamate 286 is the residue closest to the binuclear center that is clearly a part of the pathway (Iwata et al., 1995).

The reaction of the solubilized fully-reduced bovine enzyme with dioxygen has been extensively studied [for a review, see Babcock and Wikström (1992)]. Initially, dioxygen binds to the binuclear center at 1×10^8 M^{-1} s^{-1} (Oliveberg et al., 1989; Verkhovsky et al., 1994), forming the so-called A intermediate, a heme a_3 ferrous−oxy species (Chance et al., 1975). Then the first phase of heme oxidation at 3×10^4 s^{-1} takes place (Hill & Greenwood, 1984). During this phase, hemes *a* and a_3 are oxidized and the peroxy intermediate is produced at the binuclear center (Varotsis et al., 1993; Ogura et al., 1993; Morgan et al., 1996; Sucheta et al., 1997). This phase has been suggested to be linked to internal proton transfer within the enzyme (Hallén & Nilsson, 1992). During the next phase, Cu_B is oxidized, the ferryl intermediate is formed [see Morgan et al. (1996) and Sucheta et al. (1997)], and electrons redistribute from Cu_A to heme *a* (Hill, 1991) at 1×10^4 s^{-1}, accompanied by proton uptake from the medium (Oliveberg et al., 1991). The Cu_A-to-heme *a* electron transfer has been proposed to be controlled by the proton-uptake reaction (Hallén & Nilsson, 1992; Hallén & Brzezinski, 1994; Svensson Ek & Brzezinski, 1997). The fully-oxidized enzyme is then formed at 700 s^{-1} (at pH 7.4) (Hill, 1991), also accompanied by proton uptake from the medium (Oliveberg et al., 1991). In studies of the oxygen reaction in enzyme reconstituted in lipid vesicles, proton extrusion to the extravesicular medium is

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[1] Abbreviations: Cu_A, copper A; Cu_B, copper B; WT, wild type; *k*, rate constant; mutant-enzyme nomenclature: EQ(I-286) denotes a replacement of glutamate 286 of subunit I by glutamine.

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observed only during the ferryl-to-oxidized conversion (Nilsson et al., 1990). When instead starting with the oxidized enzyme and reversing the catalytic cycle in "energized" mitochondria, both the peroxy-to-ferryl and ferryl-to-oxidized conversions are coupled to proton translocation (Wikström, 1989). Thus, during the reaction of the solubilized, fully-reduced enzyme with O_2, protons associated with the conversion of oxygen to water are taken up and in addition, pumped protons are presumably taken up, and released. The reaction of the fully-reduced *R. sphaeroides* wild-type enzyme with oxygen displays similar kinetic components as the bovine enzyme (Ädelroth et al., 1996b).

In this work, we have investigated the single-turnover reaction with oxygen of the fully-reduced wild-type and a mutant enzyme in which glutamate 286 of subunit I has been replaced by glutamine [EQ(I-286)]. Electron-transfer reactions were investigated at a number of wavelengths in the Soret region, and at 580 and 830 nm. Proton uptake in the absence of buffer was monitored at 560 nm using the pH-indicator dye phenol red. Our results show that the EQ(I-286) mutant and wild-type enzymes display essentially the same rates and absorbance changes up to the formation of the peroxy intermediate, but the later phases of the reaction are absent in the mutant enzyme. In addition, the mutant enzyme does not take up protons from the medium during the reaction with oxygen. The results are discussed in terms of a model in which E(I-286) is involved in the proton uptake after peroxy formation that initiates the transfer of the fourth electron to the binuclear center. Furthermore, the 8×10^3 s^{-1} P to F conversion and electron redistribution from Cu_A to heme *a* are absent in the mutant enzyme, which indicates that the proton uptake to form the ferryl intermediate controls the electron transfer reaction.

MATERIALS AND METHODS

Mutagenesis, Growth of Bacteria, and Enzyme Purification. Mutagenesis was performed as described (Mitchell et al., 1995). *R. sphaeroides* wild-type and mutant strains were grown aerobically in a 20-L fermentor. The cells were harvested and the enzyme purified as described by Mitchell and Gennis (1995). After elution of the enzyme from the Ni^{2+} column, the buffer was exchanged to 0.1 M Hepes−KOH, pH 7.4, 0.1% dodecyl β-D-maltoside, glycerol was added to a final concentration of 10%, and the enzyme was frozen in liquid nitrogen, in which it was also stored until use.

Catalytic Turnover Measurements. The catalytic activities of the different enzymes were measured as the rate of oxidation of horse heart cytochrome *c*, studied optically at 550 nm. The measurements were made in 50 mM potassium phosphate buffer, pH 6.5, with 0.05% dodecyl β-D-maltoside. Horse heart cytochrome *c* (type VI, Sigma) was reduced to more than 95% by hydrogen gas using platinum black (Aldrich, WI) as a catalyst (Rosen & Pecht, 1976) and stored in liquid nitrogen until use.

Internal Electron-Transfer Reactions. The carbon monoxide-inhibited mixed-valence enzyme was prepared by incubation of the oxidized enzyme with CO (Brzezinski & Malmström, 1985). The laser and observation equipment have been described in detail elsewhere (Hallén & Brzezinski, 1994).

Flow−Flash Measurements. A 10 μM solution of the enzyme containing 5 μM PMS was transferred to an anaerobic cuvette and evacuated on a vacuum line, and air was exchanged for nitrogen, whereafter the enzyme was reduced by an anaerobic addition of ascorbate to a final concentration of 2 mM. Then, nitrogen was exchanged for carbon monoxide, and the enzyme solution was transferred anaerobically to one of the drive syringes of the flow−flash setup (a locally modified Applied Photophysics stopped-flow apparatus). The other syringe was filled with an oxygen-saturated buffer. In the measurements, the enzyme solution was diluted 1:5 with the oxygenated buffer.

Proton-Uptake Measurements. For proton-uptake measurements, the enzyme stock was diluted with 0.1 M KCl, 0.1% dodecyl β-D-maltoside, pH 7.5, and reconcentrated using Centricon-50 concentrator tubes (Amicon). The procedure was repeated until negligible amounts of buffer remained (at least a 3000-fold dilution). Phenol red at 40 μM was added to the enzyme solution, which was then placed in an anaerobic cuvette and flushed with nitrogen, and ascorbate was added anaerobically to a final concentration of 1 mM. After reduction, the pH was estimated from the absorbance of the dye, and adjusted to around 7.5 if necessary. Nitrogen was then exchanged for carbon monoxide, and the enzyme solution was transferred to the flow−flash apparatus. The enzyme was mixed in a 1:5 ratio in the flow−flash apparatus with a solution containing 0.1 M KCl, 0.05% dodecyl β-D-maltoside, 40 μM phenol red, and 1.2 mM dioxygen. Phenol red was chosen because it has a p*K* of 7.8, and an absorption maximum around 560 nm, where the redox reactions of the enzyme themselves contribute very little.

The exhaust from the flow−flash apparatus was collected in a cuvette flushed with nitrogen, and the pH of the solution was measured. To compare the buffer capacities of the different samples, the cuvette was placed in a Cary 4 spectrophotometer, and known amounts of an anaerobic solution of HCl were then added (giving each time an approximate increase in proton concentration of 5 μM), and the resulting absorbance changes were measured.

The amount of reacting enzyme was calculated from the CO dissociation absorbance change at 445 nm, using an absorption coefficient of 67 mM^{-1} cm^{-1} (Vanneste, 1966).

RESULTS

Catalytic Turnover. The EQ(I-286) mutant enzyme displays a turnover activity of about 0.2% of that of the wild-type enzyme (3 s^{-1} compared to 1500 s^{-1}).

Internal Electron Transfer in the Mixed-Valence CO-Bound Enzyme. When the fully-oxidized enzyme is incubated with carbon monoxide, a form of the enzyme called the mixed-valence state is formed. In this state, the binuclear center is reduced with CO bound to heme a_3, whereas heme *a* and Cu_A remain oxidized. After photolysis of the CO bond, the apparent redox potential of heme a_3 drops, which results in electron redistribution among the metal centers.

In the *R. sphaeroides* wild-type mixed-valence enzyme, there are three phases of electron-transfer reactions following CO photolysis. The first phase has a rate constant of 3×10^5 s^{-1} and represents electron equilibration between heme a_3 and heme *a*. It is followed by further equilibration with Cu_A with a rate constant of 3×10^4 s^{-1}(Ädelroth et al., 1995).

Table 1: Electron-Transfer Characteristics after Photolysis of CO from the Mixed-Valence CO-Bound Enzyme

	wild type		EQ(I-286)	
electron-transfer reaction	rate (s^{-1})	extent (%)	rate (s^{-1})	extent (%)
$a_3 \rightarrow a$ (at pH 7.0)	3.7×10^5	46	4.0×10^5	44
$a_3/a \rightarrow Cu_A$ (at pH 7.0)	2×10^4	<10	2×10^4	<10
H^+-coupled $a_3 \rightarrow a$ (at pH 8.8)[a]	480	~10	390	~10

[a] This reaction was studied at pH 8.8 because the absorbance change has a maximum around pH 9. The rate and amplitude of the 3.7×10^5 s^{-1} phase are independent of pH in the range 6−9.

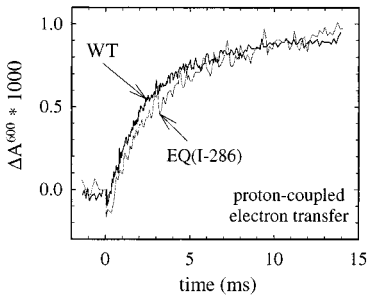

FIGURE 1: Proton-controlled electron equilibration between hemes a_3 and a in the mixed-valence wild-type and EQ(I-286) mutant enzymes in the absence of O_2, studied at 600 nm. Experimental conditions: 22 °C, 0.1 M Tris-HCl, pH 8.8, 0.1% dodecyl β-D-maltoside, 1 μM reacting enzyme, 1 mM CO. The trace shown is the average of 20 individual traces, and a laser artifact around $t = 0$ has been truncated for clarity.

The third, slowest (on the millisecond-time scale) phase displays a pH-dependent rate and amplitude and is associated with further electron equilibration between heme a_3 and heme a. This reaction has been modeled as being rate-limited by the deprotonation of a group, L, interacting with heme a_3, and in contact with the bulk phase through a proton-conducting pathway. (Hallén et al., 1994, Ädelroth et al., 1995, 1996a). All three phases are present in the EQ(I-286) mutant enzyme, and have rates and amplitudes similar to those of the wild-type enzyme (see Table 1 and Figure 1). This shows that internal electron transfer and proton transfer linked to oxidation/reduction of heme a_3 are unperturbed in the mutant enzyme.

Electron Transfer Associated with Oxygen Reduction. The *R. sphaeroides* wild-type enzyme displays at least three kinetic phases associated with electron transfer during the reaction of the fully-reduced enzyme with dioxygen (Ädelroth et al., 1996b). Based on the studies of the mitochondrial bovine enzyme, the first phase (3×10^4 s^{-1}) is interpreted in terms of oxygen binding, oxidation of both hemes, and formation of the peroxy intermediate at the binuclear center. Using absorption coefficients at 445 nm of 112 mM^{-1} and 57 mM^{-1} for reduced minus oxidized hemes a_3 and a, respectively (Vanneste, 1966), the amplitude of the 3×10^4 s^{-1} phase is consistent with approximately 70% oxidation of heme a, with the remaining fraction of the P intermediate being formed with both electrons from the binuclear center. The second phase (8×10^3 s^{-1} at pH 7.4) is associated with electron redistribution from Cu_A to heme a, oxidation of Cu_B, and formation of the ferryl state at the binuclear center. After the slowest phase (800 s^{-1} at pH 7.4), the enzyme is fully oxidized.

In the EQ(I-286) mutant enzyme, the first phase of the oxygen reaction is similar to that observed in the wild-type

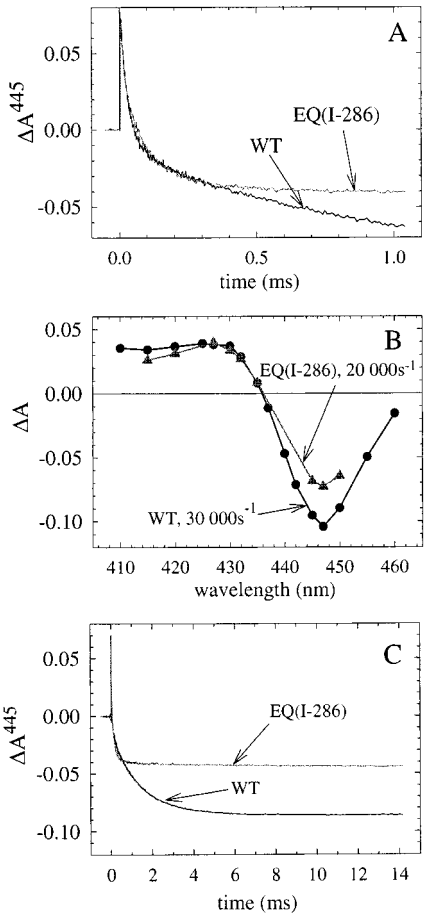

FIGURE 2: Reaction of fully-reduced WT and EQ(I-286) mutant enzyme with dioxygen. In (A), the first phase, studied at 445 nm, is shown. CO is photodissociated at $t = 0$. In (B), traces at a number of wavelengths in the Soret region were fitted to two [EQ(I-286)] or three (WT) exponentials, and the resulting spectra for the first phase are shown. In (C), the oxygen reaction on a longer time scale than in (A) and (B), studied at 445 nm, is shown. Experimental conditions: 22 °C, 0.1 M Hepes, pH 7.4, 0.05% dodecyl β-D-maltoside, 1 μM reacting enzyme, 1 mM O_2.

enzyme, with respect to both rate and the kinetic difference spectrum (Figure 2A,B), whereas the later phases are practically absent (Figure 2C). In the Soret region the 8×10^3 s^{-1} phase is mainly associated with re-reduction of heme a (Hill & Greenwood, 1984; Morgan et al., 1996; Sucheta et al., 1997), and the P to F conversion contributes very little. Therefore, the oxygen reaction was also studied at 580 nm, where the ferryl intermediate has its absorbance maximum (Wikström & Morgan, 1992). The results clearly show that the 8×10^3 s^{-1} phase is not present in the mutant enzyme, which indicates that the end-product (on this time scale) is the P intermediate (Figure 3A). Measurements were also made at 830 nm, where Cu_A has a larger relative contribution. The results show that only the 2×10^4 s^{-1} phase is seen (Figure 3B), presumably associated with oxidation of the hemes, and Cu_A remains reduced in the mutant enzyme. These data also show that the hemes contribute significantly to the absorbance at 830 nm, which is consistent with earlier observations (Hendler et al., 1994). The data obtained with the wild-type enzyme at 830 nm could be simulated using the same absorbance changes for the A to P conversion as in the EQ(I-286) mutant enzyme, and a twice as large absorbance change for the Cu_A oxidation in the 8×10^3 s^{-1} phase as for the A to P conversion at 3×10^4 s^{-1} (Figure 3C). In the fits of the EQ(I-286) data, we also had to include

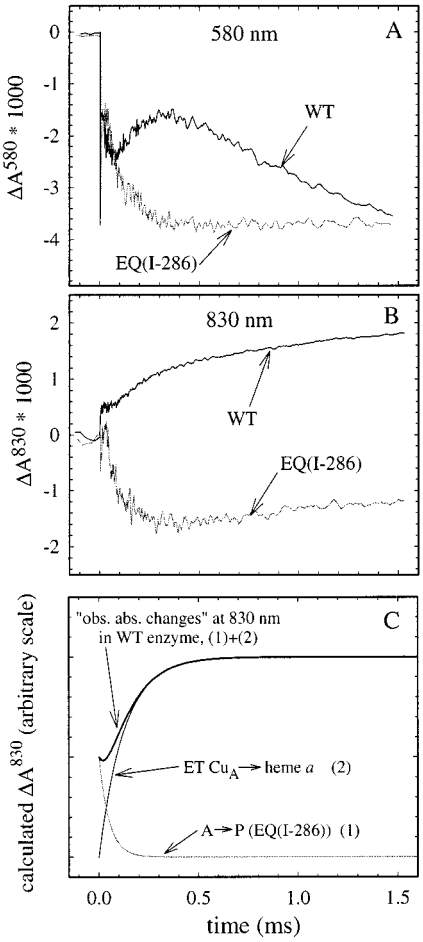

FIGURE 3: Reaction of fully-reduced WT and EQ(I-286) mutant enzyme with dioxygen, studied at 580 nm (A) and 830 nm (B). The experimental conditions were the same as in Figure 2 for the wild-type enzyme, and the absorbance changes in the mutant enzyme were scaled to give approximately the same CO dissociation absorbance changes. A laser artifact around $t = 0$ has been truncated for clarity. (C) Simulation of the absorbance changes at 830 nm associated with oxidation of the wild-type and EQ(I-286) mutant enzymes. The observed ΔA in the wild-type enzyme is composed of a sum of contributions from the A→P transition (ferrous−oxy to peroxy) (2×10^4 s^{-1}, relative $\Delta A = -1$) and a slower electron transfer (ET) from Cu_A to heme *a* (8×10^3 s^{-1}, relative $\Delta A = 2$). In the EQ(I-286) mutant enzyme, the electron transfer is impaired (because there is no H^+ uptake), and only the absorbance changes associated with the A→P transition are observed. Note that only a small lag is observed in the wild-type ΔA in (B) because two kinetic phases with different rates and amplitudes contribute.

a small component with a rate of 1000−2000 s^{-1}. Its amplitude was <10% of that associated with the first phase.

Proton Uptake during Oxygen Reduction. The *R. sphaeroides* wild-type enzyme displays proton-uptake characteristics (Figure 4) similar to those of the bovine enzyme (Oliveberg et al., 1991; Hallén & Nilsson, 1992), with two major phases of proton uptake at rates of about 7×10^3 s^{-1} and 600 s^{-1} at pH 8, associated with the P to F and the F to O conversions, respectively. The EQ(I-286) mutant enzyme displays no proton uptake at all during the reaction of the fully-reduced enzyme with dioxygen (Figure 4).

DISCUSSION

In this work, we have studied the reaction of the EQ(I-286) mutant enzyme with dioxygen using the so-called flow−flash technique. The first kinetic phase in the absor-

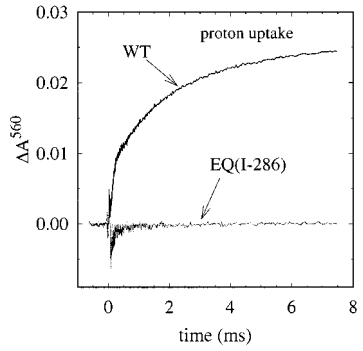

FIGURE 4: Proton uptake associated with reduction of dioxygen at pH 7.9, monitored at 560 nm using the pH-indicator phenol red. The traces shown are averages of about 20 traces, and they are the differences between the traces obtained in an unbuffered and a buffered solution. A laser artifact around $t = 0$ has been truncated for clarity. Experimental conditions: 22 °C, 0.1 M KCl (0.1 M Hepes), 0.05% dodecyl β-D-maltoside, 40 μM phenol red, 1 μM reacting enzyme, 1 mM O_2. The buffer capacities of the exhaust solutions were approximately the same for the WT and EQ(I-286) mutant enzymes.

bance changes, associated with peroxy-intermediate formation, displayed about the same rate and amplitude as the wild-type enzyme. In the wild-type enzyme, it was followed by a kinetic phase with a rate constant of 8×10^3 s^{-1} associated with oxidation of Cu_B, formation of the F intermediate, and fractional electron transfer from Cu_A to heme *a*. In the mutant enzyme the 8×10^3 s^{-1} phase was not observed, and Cu_A remained reduced (see Figure 3A,B), which strongly supports the previous suggestion that oxidation of Cu_A during the 8×10^3 s^{-1} phase is controlled by proton uptake from solution during formation of the ferryl intermediate at the binuclear center (Hallén & Nilsson, 1992; Hallén & Brzezinski, 1994; Svensson Ek & Brzezinski, 1997). Thus, in the EQ(I-286) mutant enzyme, electron transfer from Cu_A to heme *a* does not take place because the proton uptake is impaired. The coupling between proton uptake at the binuclear center and electron transfer from Cu_A to heme *a* has earlier been proposed to be of electrostatic nature (Hallén & Brzezinski, 1994). The distance between heme a_3 and Cu_A is much larger than that between heme a_3 and heme *a* (Iwata et al., 1995; Tsukihara et al., 1995, 1996), and as a consequence, addition of a positive charge at the binuclear center results in a larger increase of the apparent redox potential of heme *a* than of Cu_A. This type of interaction is supported by the observed redox interactions between the heme *a* and the binuclear center and the pH-sensitivity of the redox potential of heme *a* [e.g., see Blair et al. (1986)].

The slowest electron-transfer phase ($k \cong 800$ s^{-1}), associated with transfer of the fourth electron to the binuclear center, was also absent in the mutant enzyme. This may be either because the preceding reaction is blocked and/or because this electron transfer in the wild-type enzyme is coupled to proton uptake through E(I-286) and thus not observed in the EQ(I-286) mutant enzyme.

Internal electron-transfer reactions following photolysis of carbon monoxide from the mixed-valence enzyme in the absence of oxygen were also studied. Both the 3×10^5 s^{-1} phase, associated with electron equilibration between heme a_3 and heme *a*, and the 3×10^4 s^{-1} phase, representing further equilibration with Cu_A, were present in the EQ(I-286) mutant enzyme and had similar rates and amplitudes as in the wild-type enzyme. Since these reactions are

probably not coupled to proton transfer (Ädelroth et al., 1996a; Brzezinski, 1996), these results show that the redox potentials and/or electron-transfer pathways per se are not significantly altered in the mutant enzyme. This supports our interpretation that the disturbed electron transfer during the reaction of the fully-reduced enzyme with oxygen in the EQ(I-286) mutant enzyme is due to hindered proton uptake and not to slowing down of the actual electron transfer. In addition, the inhibition of the proton uptake is most likely responsible for the very slow turnover in the mutant enzyme. The results are consistent with studies of the dioxygen reaction of the fully-reduced cytochrome bo_3 from *E. coli* (Svensson Ek et al., 1996). That study showed that proton uptake is not observed when E(I-286) is replaced by a nonprotonatable residue, but since the fully-reduced bo_3 enzyme contains only three electrons, no effect on electron transfer was seen. The results presented here show that the lack of proton uptake prevents the reaction from proceeding, i.e., the transfer of the fourth electron to the binuclear center.

The three-dimensional *P. denitrificans* crystal structure shows that E(I-286) [E(I-278) in the *P. denitrificans* enzyme] is part of a putative proton-conducting pathway (Iwata et al., 1995). According to the suggested assignment of the pathways (Iwata et al., 1995), E(I-286) is part of the pathway used for pumped protons, through which protons are taken up to electrostatically compensate for the electrons that are transferred to the binuclear center. According to the model, protons needed to form water from oxygen are taken up through what was termed the "chemical" pathway, consisting of residues lining the transmembrane helix VIII in subunit I. The assignment of the pathways was recently questioned, based on studies of mutant enzymes in which protonatable residues in transmembrane helix VIII were modified (Hosler et al., 1996). These studies suggested that at least one of the residues in this helix is involved in proton uptake during reduction of the oxidized enzyme, which is not consistent with the model outlined by Iwata et al. (1995). In addition, while the helix VIII mutant enzymes display a greatly reduced activity, reduction of dioxygen by the fully-reduced enzymes in flow−flash experiments is essentially unaffected, with a slowing down of the final ferryl-to-oxidized transition with at most a factor of 2 (Ädelroth et al. 1997). Moreover, studies of cytochrome bo_3 from *E. coli* have shown that the helix VIII mutant enzymes react normally with oxygen up to formation of the ferryl intermediate, and take up protons to the same extent as the wild-type enzyme (Svensson et al., 1995) (the fully-reduced cytochrome bo_3 contains only three electrons per enzyme, so the end-product of the reaction should be a ferryl intermediate). This suggests that at least during the reaction of the fully-reduced enzyme with oxygen, protons used for oxygen reduction are not taken up through the helix-VIII residues.

Assuming that only two pathways are used for proton uptake, the results from this study indicate that during reaction of the fully-reduced enzyme with oxygen, all protons are taken up through the pathway containing E(I-286) (Figure 5) because residues lining helix VIII are not involved in proton uptake (see above). Therefore, most likely both types of protons are transferred through this pathway. However, the results in this work cannot be used to uniquely assign involvement of E(I-286) in transfer of pumped or oxygen-chemistry protons, because in the mutant enzyme, electron transfer could be blocked either because uptake of protons

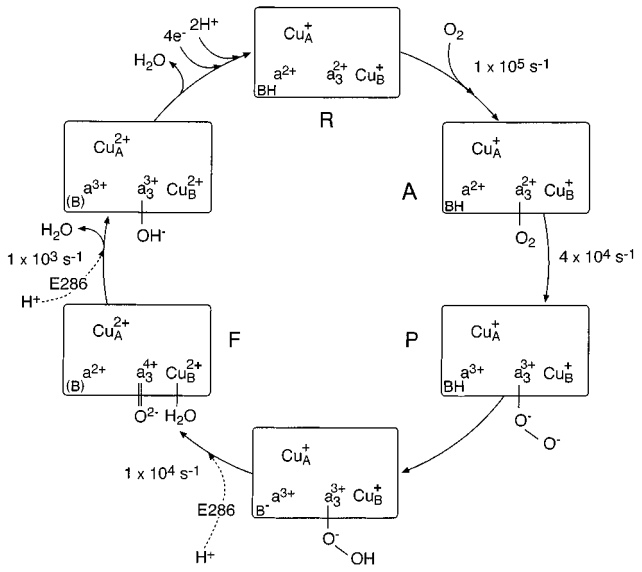

FIGURE 5: Possible reaction scheme, showing the involvement of E(I-286) in the reaction of the solubilized fully-reduced enzyme with oxygen. B is a protonatable amino acid residue previously suggested to be involved in internal proton transfer to the peroxy intermediate (Hallén & Nilsson, 1992). About two protons are taken up upon reduction of the fully-oxidized enzyme (Mitchell & Rich, 1994). One of these protons is assumed to be bound to group B. Since no proton pumping can be observed in the solubilized enzyme, it is not possible to discriminate between vectorial and substrate protons taken up during the reaction. Thus, in the scheme, the protons taken up appear to be involved in oxygen chemistry although they could instead be pumped. R, P, and F represent the fully-reduced enzyme, the peroxy, and the ferryl intermediates, respectively.

directly involved in oxygen chemistry is blocked or because uptake of pumped protons interacting electrostatically [cf. Rich (1995)] with the binuclear center is blocked.

The proton release/uptake coupled to oxidation/reduction of heme a_3 in the absence of dioxygen (in the mixed-valence enzyme) was not affected in the EQ(I-286) mutant enzyme. This indicates that the proton pathway containing the E(I-286) residue in the wild-type enzyme is not involved in proton uptake upon reduction of heme a_3.

Taken together, the results from this study and those from studies of the helix VIII mutant enzymes indicate that the proton-transfer mechanism is more complicated than that proposed by Iwata et al. (1995) and that the two pathways are most likely used for different "types of protons" during different steps of the reaction; e.g., the helix VIII pathway is used for proton uptake during reduction of the oxidized enzyme, and the pathway through E(I-286) is used for proton uptake during oxidation of the reduced enzyme.

In summary, the results presented here show that mutation of the E(I-286) residue results in inhibition of both the proton uptake after peroxy-intermediate formation, the proton-coupled electron equilibration between Cu_A and heme *a*, and the transfer of the fourth electron to the binuclear center during the reaction of the fully-reduced enzyme with oxygen (Figure 4). Moreover, the E(I-286) residue is not involved in proton release/uptake coupled to oxidation/reduction of heme a_3 in the absence of O_2 (or before O_2 binds).

NOTE ADDED IN PROOF

We have recently investigated the proton-controlled electron transfer between hemes *a* and a_3 in the absence of O_2

after flash photolysis of CO from the mixed-valence enzyme (cf. Figure 1) and the single-turnover reduction of O_2 (cf. Figure 2) also in cytochrome *c* oxidase in which amino acid residues T(I-359), K(I-362) (Ädelroth, P., Gennis, R. B., & Brzezinski, P., manuscript submitted to *Biochemistry*), and D(I-132) have been modified. Residues T(I-359) and K(I-362) are part of a proton pathway referred to as the "helix-VIII pathway" in this article. Residue D(I-132) is part of the same pathway as E(I-286). Essentially the same results were obtained with the DN(I-132) mutant enzyme as with the EQ(I-286) mutant enzyme. In the TA(I-359) and KM(I-362) mutant enzymes, the proton-controlled electron transfer in the absence of O_2 was impaired. Thus, the pathway through T(I-359)/K(I-362), but not through D(I-132)/E(I-286), is used for proton uptake upon reduction of the binuclear center (before binding of O_2). The fully-reduced TA(I-359) and KM(I-362) mutant enzymes became fully oxidized in about the same time as the wild-type enzyme (cf. Figure 2C) and displayed similar proton-uptake characteristics as the wild-type enzyme (cf. Figure 4). Taken together, the results indicate that the T(I-359)/K(I-362) pathway [but not the D(I-132)/E(I-286) pathway] is used for proton uptake upon reduction of the binuclear center. During oxidation of the fully-reduced enzyme by O_2, uptake of both substrate and pumped protons takes place through the D(I-132)/E(I-286) pathway [but not the T(I-359)/K(I-362) pathway].

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