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Relocation of an internal proton donor in cytochrome *c* oxidase results in an altered pK_a and a non-integer pumping stoichiometry

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Abstract

Cytochrome *c* oxidase from *Rhodobacter sphaeroides* has two proton-input pathways leading from the protein surface towards the catalytic site, located within the membrane-spanning part of the enzyme. One of these pathways, the D-pathway, contains a highly conserved Glu residue [E(I-286)], which plays an important role in proton transfer through the pathway. In a recent study, we showed that a mutant enzyme in which E(I-286) was re-located to the opposite side of the D-pathway [EA(I-286)/IE(I-112) double mutant enzyme] was able to pump protons, although with a stoichiometry that was lower than that of the wild-type enzyme ($\sim$0.6 H^+/e^-) (Aagaard et al. (2000) Biochemistry 39, 15847–15850). These results showed that the residue must not necessarily be located at a specific place in the amino-acid sequence, but rather at a specific location in space. In this study, we have investigated the effect of moving E(I-286) on the kinetics of specific reaction steps of the catalytic cycle in the pH range 6–11. Our results show that during the reaction of the four-electron reduced enzyme with O_2, the rates of the two first transitions (up to formation of the 'peroxy' intermediate, P_r) are the same for the double mutant as for the wild-type enzyme, but formation of the oxo-ferryl (F) and fully oxidized (O) states, associated with proton uptake from the bulk solution, are slowed by factors of $\sim$30 and $\sim$400, respectively. Thus, in spite of the dramatically reduced transition rates, the proton-pumping stoichiometry is reduced only by $\sim$40%. The apparent pK_a values in the pH-dependencies of the rates of the $P_R \rightarrow F$ and $F \rightarrow O$ transitions were >3 and $\sim$2 units lower than those of the corresponding transitions in the wild-type enzyme, respectively. The relation between the modified pK_as, the transition rates between oxygen intermediates and the pumping stoichiometry is discussed[1].

Keywords: Proton transfer; Electron transfer; Gating; Respiration; Kinetics; Rhodobacter sphaeroides

Abbreviations: WT: wild type; Cu_A: copper A; Cu_B: copper B; Binuclear center, heme a_3 and Cu_B; Catalytic site: the binuclear center and redox-active/protonatable groups in its immediate vicinity; *N*-side: negative side of the membrane; *P*-side: positive side of the membrane; R: fully reduced binuclear center; A: ferrous-oxy intermediate; P_R: the peroxy intermediate formed at the binuclear center upon reaction of the fully reduced (R) cytochrome *c* oxidase with O_2; F: oxo-ferryl intermediate; O: fully-oxidized binuclear center; Amino-acid residue and mutant-enzyme nomenclature, E(I-286): a glutamate of subunit I at position 286; EA(I-286): replacement of E(I-286) by alanine.

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[1] If not otherwise indicated, amino-acid residues are numbered according to the *R. sphaeroides* cytochrome aa_3 sequence.

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1. Introduction

Cytochrome *c* oxidases belong to the so-called heme-copper oxidase superfamily. These enzymes are integral membrane proteins that catalyze the four-electron reduction of dioxygen to water (for review see [1–3]). Four protons are consumed in this reaction, which takes place at a heme-copper binuclear center. These protons are taken up specifically from one side of the membrane (*N*-side, proton-input side), contributing to maintaining an electrochemical proton gradient across the membrane. In addition, the enzyme is a proton pump [4] that pumps one proton per electron [5] across the membrane from the *N*- to the *P*-side.

Cytochrome *c* oxidase from *Rhodobacter sphaeroides* contains four redox-active metal sites. Electrons from a water-soluble cytochrome *c* are transferred consecutively to the primary electron acceptor, Cu_A, the intermediate electron acceptor, heme *a*, and finally to the terminal acceptor, the heme a_3-Cu_B binuclear center, where O_2 binds and is reduced to water. The binuclear center is located about two-thirds of the membrane thickness from the proton-input side of the membrane. Thus, the enzyme needs pathways to transfer protons from the bulk solution to the binuclear center as well as a controlled pathway used for the transfer of the pumped protons. Two proton-transfer pathways have been suggested on the basis of site-directed mutagenesis [6] and the crystal structures of cytochrome *c* oxidases from various species ([7–9], see also [10]). They are named the D-pathway and the K-pathway after the key residues D(I-132) and K(I-362), respectively, in the two pathways. The D-pathway consists of a number of polar residues and structurally ordered water molecules connected via hydrogen bonds, leading up to a highly conserved glutamate [E(I-286)], which has been shown to be essential for the transfer of both substrate and pumped protons [11–13]. This residue has been proposed to be able to adopt different conformations, where a switching between these conformations during turnover enables the glutamate to deliver protons to different sites in the enzyme [14–16]. In recent years, several different terminal oxidases that lack the residue corresponding to E(I-286) have been isolated. Nevertheless, many of them have been shown to translocate protons (for review see [17]). One of these enzymes is the cytochrome aa_3-type quinol oxidase from the archaeon *Acidanus ambivalens.* This enzyme not only lacks E(I-286), but also most residues defining the K-pathway and all residues defining the D-pathway [18]. Still, the transfer of protons and electrons during the reaction of the fully reduced *A. ambivalens* enzyme with O_2 has been shown to be essentially the same as in the quinol oxidase from *Escherichia coli* [19,20], which contains E(I-286) and the other conserved residues of the proton pathways. Also, recently, the enzyme from *A. ambivalens* was shown to pump protons with a stoichiometry of about one proton per electron [21]. On the basis of a modeled structure of this enzyme, a number of protonatable residues have been suggested to constitute an alternative D-pathway [21]. One of these residues, E(I-80) (*A. ambivalens* numbering) is located at helix II pointing towards the position occupied by E(I-286) in the mitochondrial-like oxidases. Keeping this observation in mind and with the intention of further investigating the role of E(I-286), we constructed the double mutant EA(I-286)/IE(I-112) of cytochrome *c* oxidase from *R. sphaeroides* [22]. In this mutant enzyme the glutamic acid, E(I-286), was basically moved from helix VI to helix II, keeping the protonatable group close to its original position in space (see Fig. 1). We have previously shown that this mutant enzyme has more than 10-fold higher activity than the EA(I-286) single mutant and that it pumps protons, but with a stoichiometry of $\sim$0.6 protons per electron [22].

In this work, we have investigated the separate steps in the oxidative part of the reaction cycle of the EA(I-286)/IE(I-112) mutant enzyme as a function of pH, using the so-called flow-flash technique. The results are discussed in terms of possible proton pumping mechanisms of respiratory oxidases.

2. Materials and methods

2.1. Enzyme purification and characterization

Expression and purification of the His-tagged *Rhodobacter sphaeroides* cytochrome *c* oxidase

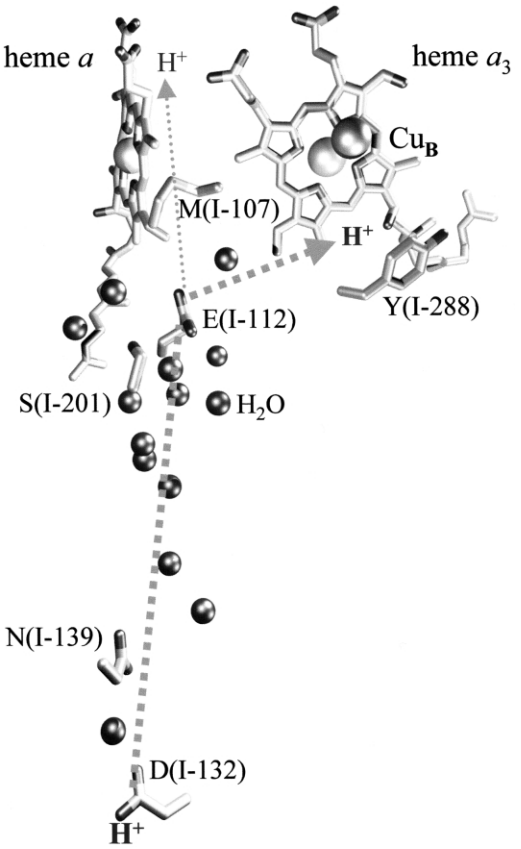

Fig. 1. A view of the proton conducting D-pathway of the double mutant enzyme EA(I-112)/IE(I-112), where E(I-286) in the wild-type enzyme has been 'moved' to the opposite side of the D-pathway. Both protons used in water formation ('substrate protons') and 'pumped protons' are transferred from the N-side of the membrane towards the upper part of the D-pathway. At a possible branching point in the vicinity of the (I-286) site, the protons are either transferred towards the P-side to be pumped or towards the binuclear center for water formation (see also [15,16]). The illustration was made using the Visual Molecular Dynamic Software [38].

were performed as described [23], with the exception that in the last step the enzyme was eluated from the Ni-affinity column with imidazole rather than with Histidine. The enzyme was repeatedly concentrated and diluted in 100 mM Hepes-KOH (pH 7.4), 0.1% L-dodecyl-β-D-maltoside (LM) using Centriprep tubes (Millipore) until the imidazole concentration was smaller than 10 μM. The enzyme was then rapidly frozen and stored in liquid nitrogen until use. The steady-state activity measurements were performed as described [22].

The catalytic activity of the single mutant enzyme EA(I-286) was less than 5 electrons/s at pH 6.5, i.e. $<0.5\%$ of that of the wild-type enzyme. Introduction of a Glu at position I-112 in the EA(I-286) mutant enzyme [EA(I-286)/IE(I-112) double-mutant enzyme] resulted in an increase in the activity to about 60 electrons/s at pH 6.5. The static absorbance spectra (between 400 and 700 nm) of the oxidized, fully reduced and CO-ligated enzymes were not affected by the mutations.

2.2. Sample preparation

The enzyme was diluted to ~15 μM in 100 mM Hepes-KOH (pH 7.8), 0.1% LM. For proton-uptake measurements the buffer was exchanged to 100 mM KCl, 0.1% LM by repeatedly diluting and re-concentrating the enzyme solution using Centriprep tubes (Millipore), followed by an adjustment of the pH to 6.8. The enzyme solution was transferred to a modified anaerobic cuvette and the electron mediator phenazine methosulfate (PMS) was added to a concentration of 0.7 μM. After exchange of air by nitrogen, the enzyme was reduced by adding 2 mM ascorbate. The carbon monoxide adduct was formed by exchanging N_2 for CO. The mixed-valence enzyme was prepared by diluting the enzyme in 100 mM Tris–KOH, pH 8.5, 0.1% LM and then incubation of the enzyme in CO for ~1 h at room temperature.

2.3. Flow-flash experiments

The experimental set-up has been described in detail earlier [24]. Measurements of absorbance changes associated with reaction of the fully reduced enzyme with oxygen were performed as described [11,25]. Briefly, the fully reduced enzyme-CO complex was mixed with an O_2-saturated solution in a stopped-flow/flow-flash apparatus (Applied Photophysics Ltd.). Approximately 100 ms after mixing, the CO ligand was flashed off using a ~100 mJ, ~5 ns laser flash at 532 nm, which allows O_2 to bind and initiate the reaction. Electron-transfers and transition

between oxygen intermediates were recorded at five different wavelengths (445, 580, 590, 605 and 830 nm). In the experiments in which the pH-dependence of the enzyme kinetics were investigated, the enzyme solution was kept in 100 mM KCl, 0.1% LM at pH ~7. It was then mixed in the stopped-flow apparatus with an oxygen-saturated solution of 100 mM Bis-Tris propane (the buffer has two pK_as of 6.8 and 9.0), 1 mM EDTA, 0.1% LM at various pH in the range 6–9.5. This procedure was also used in measurements with the wild-type *R. sphaeroides* enzyme and was shown to give identical results as compared to incubating the enzyme at an appropriate pH prior to the measurement (Katsonouri et al., unpublished data).

2.4. Proton-uptake measurements

The proton-uptake measurements were performed as described [26] with the difference that the pH was 6.8 instead of 7.8 and the phenol red concentration was 160 μM instead of 40 μM.

2.5. Measurements of internal electron transfer

Internal electron transfer in the absence of O_2 was measured after flash photolysis of CO from the mixed-valence enzyme as described [24].

2.6. Potentiometric titration of heme a

Chemical redox titrations were performed as described in [27], with the exception that DAD (2,3,5,6-Tetramethyl phenylenedianine) was exchanged for Quinhydrone. Briefly, a solution of oxidized enzyme was supplemented with 5 mM KCN, which binds at the binuclear center and stabilizes it in the oxidized state (see [28]). The redox mediators PMS, Quinhydrone and Ferrocene were added at a concentration of 10 μM of each mediator. After addition of these mediators the reduction potential (E_h) was approximately +250 mV, giving the maximum absorbance difference A(605 nm)−A(620 nm), i.e. heme *a* was fully reduced. To gradually oxidize heme *a*, aliquots of potassium ferricyanide were added and at each E_h value, the A(605 nm)−A(620 nm) absorbance was determined.

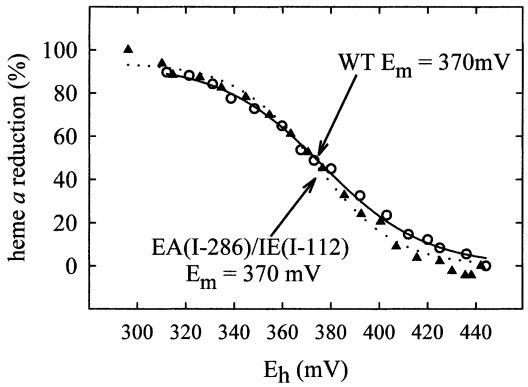

Fig. 2. Redox titration of heme *a* (see Section 2). The heme *a* absorbance level was determined from the absorbance at 605 nm minus that at 620 nm. The 100% reduction level is defined as the level obtained at E_h=250 mV (obtained after addition of all redox mediators), where E_h is the redox potential measured vs. a standard hydrogen electrode. The solid and dotted lines are fits of the wild-type (○, WT) and EA(I-286)/IE(I-112) (▲) enzyme data, respectively, with standard redox titration equations. In both cases, a value of 370 mV was obtained for the midpoint potential. Conditions: ~2 μM enzyme, 100 mM phosphate buffer, pH 7.4, 0.1% dodecyl-β-D-maltoside, 5 mM KCN, T=24 °C.

3. Results

3.1. Redox and co-binding properties of the mutant enzyme

The kinetics of CO recombination after photolysis is a powerful tool to study the protein dynamics around the binuclear center (see [29]). After flashing off CO from the fully reduced EA(I-286) and EA(I-286)/IE(I-112) mutant enzymes, CO rebound to the binuclear center with the same rate as to the wild-type enzyme (k=35 s^{-1}, data not shown), which indicates that the structure around the binuclear center is intact in the two mutant enzymes.

The midpoint potential of heme *a* in the oxidized enzyme in the presence of KCN was the same in the EA(I-286)/IE(I-112) as in the wild-type enzyme (Fig. 2, E_m=370 mV, see also [28]).

3.2. Electron transfer in the mixed-valence enzyme after flash photolysis of CO

After flashing off CO from the reduced binuclear center, the apparent redox potential of heme

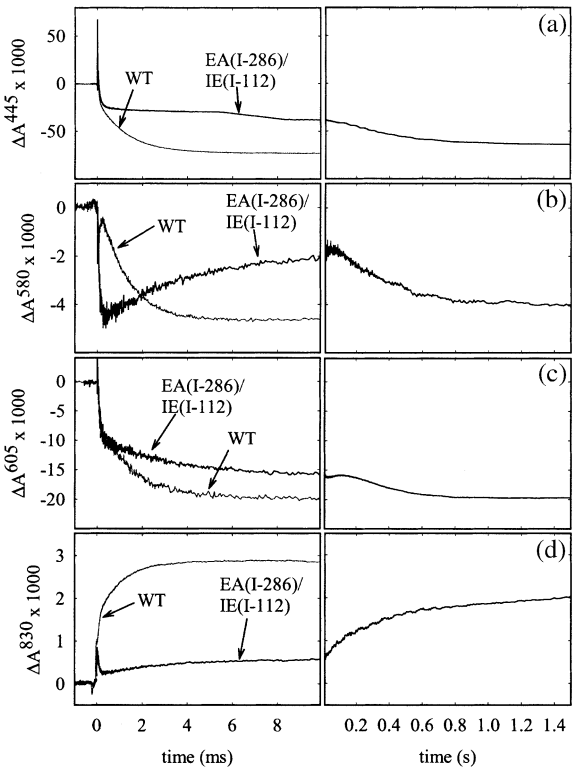

Fig. 3. Absorbance changes at 445 nm (a), 580 nm (b), 605 nm (c) and 830 nm (d), associated with the reaction of the fully reduced wild-type and EA(I-286)/IE(I-112) mutant enzyme with dioxygen. The traces have been scaled to 1 μM reacting enzyme. Experimental conditions after mixing: 0.1 M Hepes-KOH, pH 7.4, 0.1% dodecyl maltoside, 1–2 μM reacting enzyme, 1 mM O_2, $T=22$ °C.

a_3 decreases, which in the mixed-valence enzyme results in electron transfer from heme a_3 to heme a with a time constant of ~3 μs. The electron then equilibrates with Cu_A with a time constant of ~40 μs. These rapid electron transfers are followed by a slower oxidation of heme a_3 ($\tau \cong 2$ ms at pH 8.5), coupled to proton release to the bulk solution (for a more detailed description of these events, see [30]). Both the rate end extent of electron transfers between the redox centers were the same in the double-mutant as in the wild-type enzyme (data not shown), which indicates that the mutations did not affect the intrinsic electron-transfer rates between the redox centers. Thus, any effects on these rates seen during reaction of the reduced enzyme with O_2 (see below) are due to a modification proton-transfer reactions coupled to the electron transfer.

3.3. Reaction of the fully reduced enzyme with O_2

In this experiment, the fully reduced CO-bound enzyme is rapidly mixed with an O_2-saturated buffer solution. Approximately 100 ms after mixing, the CO ligand is flashed off with a short laser pulse, which allows O_2 to bind to the reduced binuclear center. Absorbance changes associated with the electron and proton transfers during the reaction of the fully reduced enzyme with oxygen are shown in Fig. 3. After flash-photolysis of CO, seen as a rapid increase of the absorbance at 445 nm (Fig. 3a), oxygen binds to the reduced binuclear center with a rate constant of 10^5 s^{-1} (at 1 mM O_2, [28]) forming compound A. This reaction is seen as a decrease in absorbance that follows immediately after the CO-dissociation change at 445 nm (Fig. 3a). Then the P_R intermediate is formed, with a rate constant of $\sim 1.4 \cdot 10^4$ s^{-1} [25]. This event is associated with oxidation of both hemes and is seen as a decrease in the absorbance at 445, 580 and 605 nm (Fig. 3a-c). Up to this point, both the EA(I-286)/IE(I-112) and the EA(I-286) mutant enzymes displayed the same behavior as the wild-type enzyme. In the EA(I-286) mutant enzyme, no additional phases could be observed after P_R formation (Fig. 4), a behavior

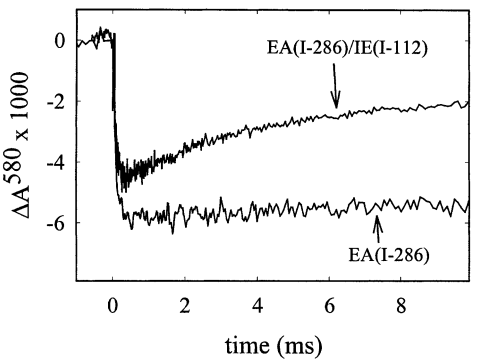

Fig. 4. Absorbance changes at 580 nm, associated with the reaction of the fully reduced EA(I-286) and EA(I-286)/IE(I-112) mutant enzymes with dioxygen. Conditions were the same as in Fig. 3.

that has previously been reported for the EQ(I-286) mutant enzyme [11].

In the wild-type enzyme, the next intermediate (F) is formed with a rate constant of $8{\cdot}10^3$ s^{-1} [25], accompanied by electron transfer from Cu_A to heme *a*. This intermediate has an absorbance peak at 580 nm in the difference spectrum with the oxidized enzyme (see increase in absorbance in the time interval ~0–200 μs in Fig. 3b), but the process can also be followed at 605 nm (contribution mainly from heme *a*) and 830 nm (contribution mainly from Cu_A). In the double mutant enzyme, the rate of F formation was slowed to 250 s^{-1} at pH 7.5, i.e. by a factor of 30 (see increase in absorbance in Fig. 3b). In addition, a smaller fraction of Cu_A was oxidized in this step (20% compared to 80% in the wild-type enzyme). Instead, this phase was followed by additional electron transfer from Cu_A to heme *a* with a rate constant of 50 ± 20 s^{-1} (this phase is best seen at 605 nm). In the wild-type, enzyme formation of F is associated with the uptake of approximately one proton per enzyme molecule from the bulk solution [11]. In the double-mutant enzyme we observed an uptake of approximately 0.6 protons with the same rate as formation of the F intermediate (Fig. 5).

In the last step, the fully oxidized enzyme is formed, with a rate of 800 s^{-1} in the wild-type enzyme [25]. Both hemes and Cu_A are oxidized and a proton is taken up from the bulk solution. In the double mutant enzyme this rate was slowed by a factor of ~400 to ~2 s^{-1}.

It was difficult to determine the number of protons taken up on the time scale of the F→O transition in the EA(I-286)/IE(I-112) mutant enzyme, due to a slow drift in the pH of the buffer-free solution. To minimize this drift, the proton-uptake measurements were performed at a pH of 6.8, i.e. lower than that used previously [25] because the transition rates between the intermediates increase at lower pH. By subtracting the drift from the observed signals, the number of protons taken up during the F→O transition was estimated to be ~0.6. The smaller total number protons taken up at this pH is consistent with results from studies of the bovine enzyme [31].

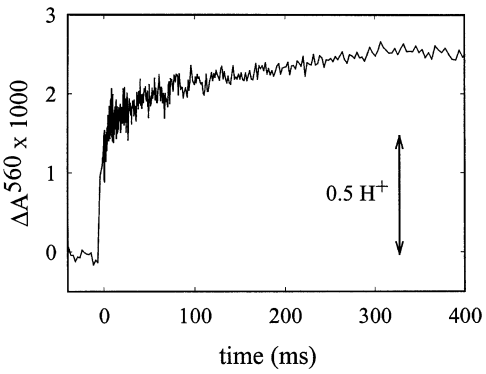

Fig. 5. Absorbance changes of the pH-indicator dye phenol red, associated with proton uptake during the reaction of fully reduced EA(I-286)/IE(I-112) double mutant enzyme with dioxygen. The trace shown is the difference between the traces obtained at 560 nm with an unbuffered (KCl) and a buffered (Bis–Tris Propane) solution at the same pH. A slow pH-drift was fitted to a straight line and subtracted from the trace for clarity. The number of protons taken up were determined by adding known amounts of protons to the reaction mixture and measuring the corresponding absorbance changes at 560 nm. Experimental conditions after mixing: 0.1 M KCl or 0.1 M Bis–Tris–KOH, pH 6.8, 0.1% dodecyl maltoside, 160 μM phenol red, 3 μM reacting enzyme, 1 mM O_2.

3.4. pH-dependence of the F and O formation rates

The kinetics of the reaction between the fully reduced double mutant enzyme and O_2 were measured at eight different pH values. As for the *R. sphaeroides* wild-type enzyme (Katsonouri et al., unpublished) and the bovine cytochrome *c* oxidase [31], the first two phases were independent of pH. However, the $P_R \rightarrow F$ and F→O rates increased with decreasing pH, as shown in Fig. 6. For comparison, data obtained with the wild-type enzyme are also shown in the same figure (note the different scales on the ordinates). Both phases of the double-mutant enzyme titrate with a considerably lower apparent[2] pK_a than the corresponding phases observed with the wild-type enzyme. The

[2] The pK_as are referred to as 'apparent' pK_as because they are determined from the pH dependence of the *kinetics* and they may therefore not be the true equilibrium values of a specific group. However, we assume that a change in the apparent pK_a upon mutation of a specific group reflects changes in the pK_as of that group (or groups in the vicinity).

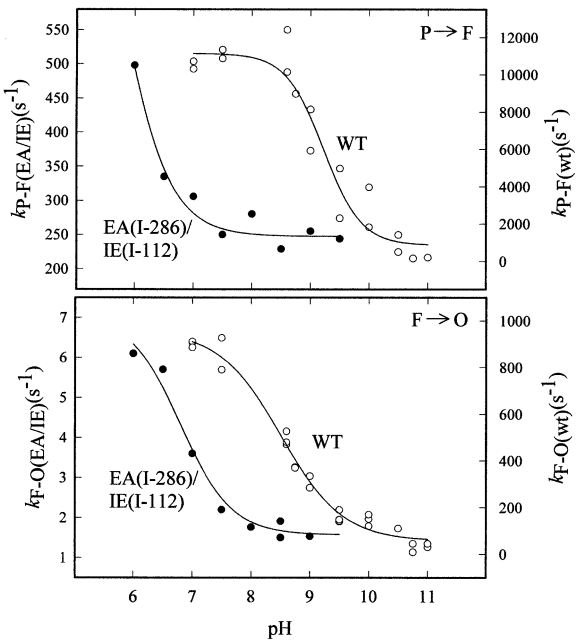

Fig. 6. pH dependence of the rates of the $P_R \rightarrow F$ and $F \rightarrow O$ transitions in the wild-type and EA(I-286)/IE(I-112) mutant enzymes. The solid lines are fits with standard titration curves of a single protonatable group. The data with the wild-type enzyme are from (Katsonouri et al., submitted for publication). Experimental conditions after mixing: 83 mM Bis-Tris propane, pH 6–9.5, 17 mM KCl, 0.1% dodecyl maltoside, 3 μM reacting enzyme, 1 mM O_2.

pK_a value for the $P_R \rightarrow F$ transition could not be determined, because the rate did not level out at the lowest pH values at which measurements were possible. However, a limiting value of <6.5 could be determined, i.e. >3 units lower than that measured with the wild-type enzyme (~ 9). For the $F \rightarrow O$ transition an apparent pK_a value of 6.7 was determined, which is about two units lower than that measured with the wild-type enzyme (~ 8.5).

4. Discussion

We have studied the details of the reaction of the fully reduced EA(I-286)/IE(I-112) double mutant enzyme with dioxygen, using the flow-flash technique. In contrast to mutant forms of cytochrome *c* oxidase, in which E(I-286) has been replaced by non-protonatable groups, [e.g. EA(I-286) and EQ(I-286) mutant enzymes], this double mutant enzyme has the ability to completely reduce oxygen to water (see also [32]). All the kinetic steps associated with the reaction of the fully reduced enzyme and oxygen that were observed with the wild-type enzyme were also observed with this mutant enzyme. The first two intermediates (A and P_R) were formed with the same rates as in the wild-type enzyme, which was also found for the EA(I-286) single-mutant enzyme and previously for the EQ(I-286) mutant enzyme [11]. This observation is consistent with the fact that there is no proton uptake from the bulk solution associated with the formation of these intermediates (see [33]).

The subsequent steps, $P_R \rightarrow F$ and $F \rightarrow O$, which are coupled to proton uptake from the bulk solution through the D-pathway were dramatically slowed in the double-mutant enzyme. In the wild-type enzyme the $P_R \rightarrow F$ transition at the binuclear center is accompanied by several events, which occur simultaneously with a rate constant of $1.4 \cdot 10^4$ s^{-1} at pH 7.5. Formation of the F intermediate itself requires proton transfer to the binuclear center. This proton is initially transferred from an internal proton donor (presumably E(I-286)) [34]. The internal donor is then rapidly reprotonated from the bulk solution, which restores the charge in the D-pathway and results in an increase in the redox potential of heme *a*. As a consequence, the electron in the Cu_A/heme *a* equilibrium is transferred towards heme *a*. Since the intrinsic electron-transfer rate is faster than the proton uptake, the electron is transferred with the same observed rate as the $P_R \rightarrow F$ transition [34,35]. In the EA(I-286)/IE(I-112) double mutant enzyme, F formation was slowed by a factor of ~ 30 ($k \cong 250$ s^{-1}), which presumably is an effect of a slowed proton transfer, because the intrinsic rates of the electron-transfer reactions between the redox centers were not affected by the mutations. All events during the $P_R \rightarrow F$ transition were slowed because of the protonic control of the electron-transfer reactions (see above). In addition, a smaller fraction proton uptake and electron transfer from Cu_A to heme *a* was observed during the $P_R \rightarrow F$ transition. A second phase of

electron transfer from Cu_A to heme *a* was observed with a rate of approximately 50 s^{-1}. This event is probably coupled to further proton uptake from the bulk solution, although it was difficult to resolve this phase from that associated with the following F→O transition (see Section 3).

The pH-dependence of the observed rate of F formation displayed an apparent pK_a of <6.5 (Fig. 6), i.e. much lower than that observed with the wild-type enzyme. The change in the apparent pK_a value most likely is a reflection of the replacement of E(I-286) by E(I-112) in the double-mutant enzyme (see also footnote 2). The low apparent pK_a indicates that E(I-112) is not protonated above pH ~7. Consequently, in this pH range, the proton used to form F must be transferred from the bulk solution and not internally from the Glu. This explains in part why the P_R→F transition rate is slowed dramatically in the double mutant enzyme.

Assuming that E(I-286) is the proton donor to the catalytic site during F formation in the wild-type enzyme, the observed P_R→F rate should depend on the protonation state of the Glu. Consequently, to compare the rates of these transitions in the wild-type and EA(I-286)/IE(I-112) enzymes, one must take into account the different pK_as and consider the maximum, limiting rates at low pH. Even though we were not able to do measurements below pH 6 to determine the saturation level with the EA(I-286)/IE(I-112) enzyme, the data in Fig. 6a could only be fitted with titration curves that saturate at values lower than 10^4 s^{-1} (maximum value observed with the wild-type enzyme). This indicates that also the intrinsic proton-transfer rate from E(I-112) to the binuclear center is slowed, which is consistent with a longer distance between the proton donor (E(I-112)) and acceptor (catalytic site) and/or a less optimal connectivity through the water chain in the double-mutant enzyme between the donor and acceptor.

In the next step, formation of the fully oxidized state is associated with the transfer of the fourth electron to the binuclear center from the Cu_A-heme *a* equilibrium. In the wild-type enzyme, the transition is associated with the uptake of one proton per enzyme molecule from the bulk solution. At pH 7.5, the rate of this transition was ~400 times slower in the EA(I-286)/IE(I-112) mutant than in the wild-type enzyme ($k_{EA/IE}$=2 s^{-1}, k_{wt}=800 s^{-1} at pH 7.5) and at low pH the rate saturated at ~6 s^{-1} (see Fig. 6), i.e. at a value that is more than 100 times slower than that observed with the wild-type enzyme. Since the saturation rate should reflect the proton-transfer rate through the pathway with fully protonated protonatable group(s), the result of the mutations is apparently that the proton-conducting pathway is less efficient (see above).

As indicated in Section 1, the D-proton-transfer pathway containing E(I-286) is used for transfer of both substrate and pumped protons during enzyme turnover [11,13]. This dual role of the D-pathway indicates that there must be a branching point in the pathway at which protons are directed along different trajectories. One possible branching site has been suggested to be E(I-286). However, even though the residue is most likely closely associated with the pumping machinery and it is important for an efficient function of the D-pathway, E(I-286) alone is not the pumping element of cytochrome *c* oxidase. In this context it is noteworthy that despite the dramatic changes in the rates of the P_R→F and F→O transitions (in which proton pumping takes place) due to the relocation of E(I-286), the enzyme still pumps on average ~0.6 protons per electron. This observation shows that the proton-pumping machinery is robust and coupling is maintained even if the rates of the transitions at which proton pumping takes place are slowed by orders of magnitude.

Previous studies with the *Paracoccus denitrificans* cytochrome *c* oxidase showed that introduction of an Asp residue at either of the sites N(I-131) or N(I-199) (*P.d.* enzyme numbering), lining the D-pathway resulted in mutant enzymes with almost full overall activity, but in which proton pumping was uncoupled from the O_2-reduction reaction [36]. Also in the *R. sphaeroides* enzyme, introduction of an acidic residue near E(I-286) resulted in uncoupling of proton pumping from oxygen reduction and a decrease in the apparent pK_a of the F→O transition to <6 [37], which suggests that an alteration of the electrostatic environment of E(I-286), leads to an altera-

tion of the pumping stoichiometry of cytochrome *c* oxidase.

A non-integer pumping stoichiometry may simply be due to competing proton-transfer reactions from the D-pathway around E(I-286) to the binuclear center ('substrate protons') and towards the proton output side ('pumped protons'), respectively (see Fig. 1). The net driving force for the transfer of substrate protons to the catalytic site of cytochrome *c* oxidase is very high. On the other hand, in the 'pumping pathway' the pK_as of the proton donors and acceptors must be fine-tuned, so that the driving force in each step is adjusted to yield large enough forward rates compared to the competing proton-transfer rates to the binuclear center, but small enough to prevent loss off energy into heat. Thus, even though both the substrate and pumped protons are transferred through the same (D) pathway, an alteration of the pK_a of one protonatable group in the pathway is likely to result in larger effects on the effective proton-transfer rates for the pumped than for the substrate protons. Consequently, the probability that a substrate proton is transferred to the catalytic site before a pumped proton is transferred to the acceptor site in the output pathway increases, which results in a non-integer pumping stoichiometry that is smaller in the mutant than in the wild-type enzyme.

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