Redesign of the Proton-Pumping Machinery of Cytochrome *c* Oxidase: Proton Pumping Does Not Require Glu(I-286)

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ABSTRACT: One of the putative proton-transfer pathways leading from solution toward the binuclear center in many cytochrome *c* oxidases is the D-pathway, so-called because it starts with a highly conserved aspartate [D(I-132)] residue. Another highly conserved amino acid residue in this pathway, glutamate-(I-286), has been indicated to play a central role in the proton-pumping machinery of mitochondrial-type enzymes, a role that requires a movement of the side chain between two distinct positions. In the present work we have relocated the glutamate to the opposite side of the proton-transfer pathway by constructing the double mutant EA(I-286)/IE(I-112). This places the side chain in about the same position in space as in the original enzyme, but does not allow for the same type of movement. The results show that the introduction of the second-site mutation, IE(I-112), in the EA(I-286) mutant enzyme results in an increase of the enzyme activity by a factor of >10. In addition, the double mutant enzyme pumps ∼0.4 proton per electron. This observation restricts the number of possible mechanisms for the operation of the redox-driven proton pump. The proton-pumping machinery evidently does require the presence of a protonatable/polar residue at a specific location in space, presumably to stabilize an intact water chain. However, this residue does not necessarily have to be at a strictly conserved location in the amino acid sequence. In addition, the results indicate that E(I-286) is not the "proton gate" of cytochrome *c* oxidase controlling the flow of pumped protons from one to the other side of the membrane.

The terminal component of the respiratory chain in many archea, prokaryotes, and eukaryotes is a cytochrome *c* or quinol oxidase, which catalyzes the four-electron reduction of oxygen to water and couples this reaction to pumping of protons across the membrane [for review, see (*1*)]. The heme−copper oxidase superfamily is defined by the presence of a protein subunit (subunit I) with conserved histidine residues that ligate a copper ion (Cu_B) and two heme groups. One of the hemes is coordinated by two histidines; i.e., it has a total of six ligands (low-spin heme). The other heme has an open coordination site, and together with copper B it forms a heme−copper binuclear center, where oxygen binds and is reduced to water. In addition to these three redox-active metal sites, cytochrome *c* oxidases have a fourth center, Cu_A, composed of two copper ions. During catalysis, electrons from water-soluble cytochrome *c* are first transferred to Cu_A from where they are transferred intramolecularly to the low-spin heme and then to the binuclear center.

The process of reduction of dioxygen to water proceeds by the build-up and decay of partly reduced oxygen intermediates [see (*1, 2*)], and several of the transitions between these intermediates are coupled to proton pumping (*3, 4*). However, the molecular mechanism of proton pumping is still not understood, and the involvement of specific protein elements in the pumping machinery is still debated (*3, 5*).

During the past few years, insights about the proton-pumping mechanism have been gained from analyses of the three-dimensional structures of several oxidases (*6*−*8*) and the use of site-directed mutagenesis in combination with biophysical techniques (*9*).

One basic feature of a redox-driven bioenergetic device, which transfers protons against a gradient, is regulation of the intraprotein proton-transfer rates providing alternating access of the proton to the two sides of the membrane, termed gating. One of the amino acid residues that have been suggested to be involved in gating the proton flow though cytochrome *c* oxidase is glutamate(I-286) [E(I-286)],[1] a highly conserved amino acid residue in the mitochondrial-type oxidases, located in the so-called D-proton-transfer pathway, used for transfer of both substrate and pumped protons during enzyme turnover (*10*−*12*). The dual role of the D-pathway suggests that during turnover the side chain of E(I-286) has to move between well-defined distinct positions directing the pumped and substrate protons along

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[1] If not otherwise indicated, the amino acid numbering is based on the *Rhodobacter sphaeroides* cytochrome *c* oxidase sequence.

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different trajectories. In addition, an analysis of the three-dimensional structure of cytochrome *c* oxidase indicates that an adequate proton-transfer connectivity through the D-pathway can only be achieved if the side chain of the glutamate moves. The dynamic nature of the E(I-286) side chain is also evident from numerous experimental studies (*13*−*19*). In addition, Fourier transform infrared studies showed a connection between changes in the environment of the side chain of E(I-286) and redox changes or ligand binding at the binuclear center (*14*, *15*, *20*).

In recent years a number of oxidases that lack E(I-286) have been isolated and shown to be capable of translocating protons across a biological membrane (*21*−*24*), although with variable stoichiometries. These findings have resulted in speculations that these enzymes may utilize a different proton-pumping mechanism which does not require the involvement of the glutamate [see, e.g., (*8*)].

To test the involvement of E(I-286) in proton pumping in the mitochondrial-like oxidases, in this work we moved the glutamate E(I-286) side chain to the opposite side of the D-pathway, which does not allow for the same dynamics as in the wild-type enzyme.

MATERIALS AND METHODS

Site-Directed Mutagenesis. The mutant was constructed with the Quick-Change site-directed mutagenesis kit (Stratagene) on pJS3(X6H) (see *25*). The mutation was verified by DNA sequencing.

Expression and Purification of the Mutant Protein. The mutant pRKpAH1H32 plasmid was mobilized into the *Rhodobacter sphaeroides* strain JS100 (*26*) by biparental conjugation using *Escherichia coli* strain S-17-1 as the donor (*27*). Growing of *R. sphaeroides* and purification of the mutant enzyme were performed as described (*28*).

Measurements of the Catalytic Activity. Horse heart cytochrome *c* (type VI, Sigma) was reduced (>95%) by hydrogen gas using platinum black (Aldrich, WI) as a catalyst. The catalytic activity was determined from the initial change in absorbance at 550 nm upon mixing the reduced cytochrome *c* with cytochrome *c* oxidase. The experiments were performed in 50 mM potassium phosphate buffer at pH 6.5, with 0.1% dodecyl β-D-maltoside.

Measurements of Proton Pumping. The cytochrome *c* oxidase-containing vesicles were prepared as described (*29*). The respiratory control of the vesicles with the wild-type enzyme was typically 6−10, while smaller values (2−4) were obtained with the double mutant enzyme. Measurements were made in 50 μM Hepes−KOH, 45 mM KCl, 44 mM sucrose, 1 mM EDTA, 100 μM phenol red, pH 7.4. Proton pumping was measured upon mixing (using a stopped-flow apparatus from Applied Photophysics) reduced cytochrome *c* and the cytochrome *c* oxidase-containing vesicles in the presence of 10 μM valinomycin. The pH changes were determined from absorbance changes of the dye phenol red at 559.5 nm, which is an isosbestic point for cytochrome *c* oxidation. Control measurements were made without phenol red to confirm the wavelength of the isosbestic point.

RESULTS AND DISCUSSION

To test whether or not E(I-286) is involved in the proton-pumping machinery of the mitochondrial-like enzymes, we

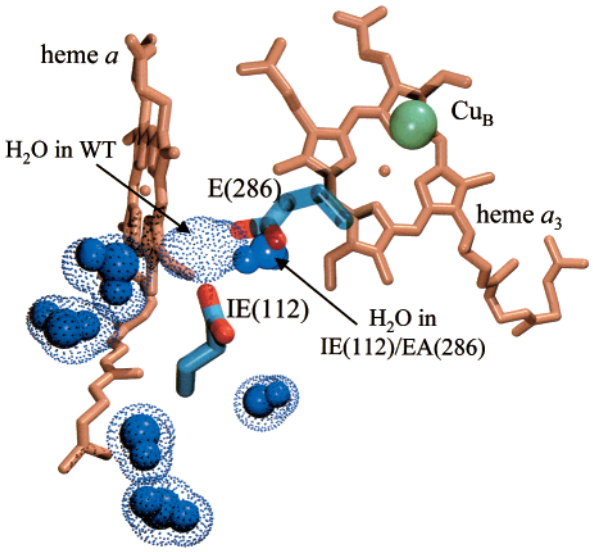

FIGURE 1: Overview of the *R. sphaeroides* cytochrome *c* oxidase structure (Svensson Ek et al., unpublished data) around the heme groups and Glu(I-286). The double mutant [IE(I-112)/EA(I-286)] enzyme structure was modeled on the basis of an energy-minimization calculation performed using the program INSIGHT II (Molecular Simulations Incorporated). The position of E(I-286) in the wild-type enzyme is shown. The locations of water molecules in the wild-type (WT) and double mutant enzymes are shown as dotted blue surfaces and solid blue spheres, respectively. In the IE(I-112)/EA(I-286) double mutant enzyme, one of the water molecules moves into a location occupied by the E(I-286) carboxyl group in the wild-type enzyme. The locations of the other water molecules are not affected by the mutations. Note that in the double mutant enzyme E(I-286) is replaced by an alanine. The illustration was prepared using the Visual Molecular Dynamic Software (*32*).

constructed a double mutant of the *R. sphaeroides* enzyme in which E(I-286) (located on helix VI) of the D-pathway was replaced by an alanine [EA(I-286)] and a glutamate was introduced on the other side of the D-pathway at the location of I(I-112) [IE(I-112), on helix II], slightly "below" the original position of E(I-286) (see Figure 1). Assuming that the overall structure of the double mutant enzyme does not change, the position of the E(I-112) side chain was estimated by an energy-minimization calculation applied to the structure of the *R. sphaeroides* enzyme (Svensson Ek et al., unpublished) at a radius of ~15 Å around the residue. As shown in Figure 1, in the redesigned enzyme the protonatable group of the glutamate is kept at about the same position in space as that of the original E(I-286), the carboxyl group of E(I-112) being about 2 Å from that of the original E(I-286). However, the E(I-112) residue cannot move along the same trajectory as E(I-286) because the amino acid residue is fixed on helix II instead of helix VI. In addition, the through-bond connection between E(I-286) and the binuclear center is lost in the mutant enzyme because of the location of E(I-112) at a different helix. Thus, the results from this study indicate that such a connection is not important for proton pumping (see the introduction).

The turnover activity of the EA(I-286) mutant enzyme was <5 e^-/s, i.e., <0.5% of that of the wild-type enzyme. Introduction of the second-site mutation [double mutant EA-(I-286)/IE(I-112)] resulted in an increase of the turnover activity by a factor of >10 to about 60 e^-/s (at pH 6.5), i.e., ~5% of that of the wild-type enzyme. The lower overall activity of the EA(I-286)/IE(I-112) mutant enzyme as

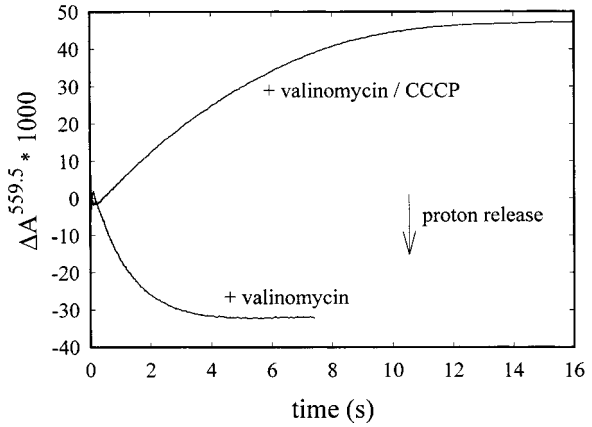

FIGURE 2: Absorbance changes of the dye phenol red at 559.5 nm, associated with proton pumping by the EA(I-286)/IE(I-112) double mutant cytochrome *c* oxidase from *R. sphaeroides* (0.25 μM), reconstituted in phospholipid vesicles. The lower trace shows the acidification upon mixing the vesicles with reduced horse heart cytochrome *c* (8 μM) in the presence of 10 μM valinomycin. The upper trace shows the alkalization caused by the uptake of four protons for the oxygen reduction in the same experiment as above, but in the presence of 50 μM CCCP. The traces have been corrected for a slow back-leak of protons through the vesicles. Conditions: 50 μM Hepes−KOH, 45 mM KCl, 44 mM sucrose, 1 mM EDTA, 100 μM phenol red, pH 7.4, $T = 22$ °C.

compared to that of the wild-type enzyme is most likely a consequence of a weaker proton connectivity through the D-pathway due to disruption of the hydrogen-bonded water chain (*16*, *30*).

As discussed in the introduction, in the wild-type enzyme an efficient proton connectivity through the D-pathway may require a transient movement of the E(I-286) side chain to reach a putative chain of water molecules forming a proton wire that connects to the binuclear center. In the structural model of the wild-type *R. sphaeroides* cytochrome *c* oxidase, a water molecule is hydrogen-bonded to E(I-286) (Svensson Ek et al., unpublished data). In the energy-minimized structure of the EA(I-286)/IE(I-112) double mutant enzyme, this water molecule moves "up" the D-pathway to a location previously occupied by the side chain of E(I-286). In this model structure, the hydrogen-bonding connectivity between the D-pathway and the binuclear center is likely to be weaker because the water molecule occupying the (I-286) site cannot play the same role in proton transfer as E(I-286) (see above). In addition, the different environment of E(I-112) than that of E(I-286) most likely leads to a shift of the pK_a of the side chain; i.e., the driving force for the proton transfer is altered.

To investigate the proton-pumping activity of the double mutant enzyme, it was incorporated into phospholipid vesicles. Proton release to the bulk solution was measured by monitoring absorbance changes at 559.5 nm of the pH dye phenol red. Figure 2 shows absorbance changes upon mixing the vesicle solution with a solution of reduced cytochrome *c* in a stopped-flow apparatus in the presence of valinomycin and valinomycin/CCCP, respectively. As shown in the figure the EA(I-286)/IE(I-112) mutant enzyme pumped protons with a stoichiometry of 0.4 ± 0.1 H^+/e^- (as compared to ~ 1 H^+/e^- for the wild-type enzyme), which demonstrates that the mutant enzyme has an intact proton-pumping machinery.

In a very recent study Backgren et al. (*31*) found that the turnover activity and proton-pumping efficiency of the EA-(I-278)/GS(I-275) (*Paracoccus denitrificans* enzyme amino acid residue numbering) double mutant of the *P. denitrificans* cytochrome *c* oxidase can be dramatically increased by placing a tyrosine residue near the (I-278) position [E(I-278) of the *P. denitrificans* enzyme corresponds to E(I-286) of the *R. sphaeroides* enzyme], which also indicates that proton translocation can take place without the conserved glutamic acid residue.

Most likely, efficient proton pumping requires a fine-tuning of the driving force (pK_a of the proton-donating group) and dynamics of the proton transfer through the D-pathway allowing an accurate timing of the events associated with proton pumping and the oxygen−reduction chemistry. This requirement is supported by results from studies of the EC-(I-286) mutant enzyme of the *E. coli* enzyme, in which the oxygen−reduction chemistry was functional but uncoupled from proton pumping (*13*). Thus, the lower pumping stoichiometry in the double mutant enzyme is most likely due to the altered proton connectivity through the D-pathway and an altered dynamics and/or pK_a of E(I-112) as compared to E(I-286). Earlier results have shown that when the E(I-286) side chain was locked onto a stable position in the D-pathway, proton transfer through the D-pathway was blocked, which also indicates the necessity of dynamics of the side chain for efficient proton transfer (*16*). Results pointing to the same direction showed that the relatively large kinetic deuterium-isotope effect of ~ 7 observed for the proton transfer through the D-pathway (over a distance of ~ 30 Å) was associated with a single protonatable site (*17*, *18*), identified as E(I-286), that could adopt different conformations.

The results from this work indicate that the presence of a protonatable/polar residue at a specific location in space in the D-pathway is required to stabilize an intact water chain. However, since this residue can be located on either side of the D-pathway, i.e., not at a strictly conserved location in the amino acid sequence, it is unlikely that E(I-286) is the pumping element ("gate") of cytochrome *c* oxidase controlling the accessibility of protons to the two sides of the membrane. Furthermore, the results show that the absence of a glutamate in the nonmitochondrial-like oxidases does not exclude a common proton-pumping mechanism for all respiratory oxidases. In addition, since the D-pathway is alone responsible for the transfer of both pumped and substrate protons during oxygen reduction, the pumping element is presumably located closer to the binuclear center than E(I-286), presumably in close association with the binuclear center itself.

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