Articles

Tracing the D-Pathway in Reconstituted Site-Directed Mutants of Cytochrome *c* Oxidase from *Paracoccus denitrificans*†

Ute Pfitzner,‡ Klaus Hoffmeier,‡ Axel Harrenga,§ Aimo Kannt,§ Hartmut Michel,§ Ernst Bamberg,|| Oliver-M. H. Richter,‡ and Bernd Ludwig*,‡

Molekulare Genetik, Institut für Biochemie, Biozentrum, Johann Wolfgang Goethe-Universität, Marie-Curie-Strasse 9, D-60439 Frankfurt, Germany, Abteilung Molekulare Membranbiologie, Max-Planck-Institut für Biophysik, Heinrich-Hoffmann-Strasse 7, D-60528 Frankfurt, Germany, and Abteilung Biophysikalische Chemie, Max-Planck-Institut für Biophysik, Kennedyallee 70, D-60596 Frankfurt, Germany

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ABSTRACT: Heme−copper terminal oxidases use the free energy of oxygen reduction to establish a transmembrane proton gradient. While the molecular mechanism of coupling electron transfer to proton pumping is still under debate, recent structure determinations and mutagenesis studies have provided evidence for two pathways for protons within subunit I of this class of enzymes. Here, we probe the D-pathway by mutagenesis of the cytochrome *c* oxidase of the bacterium *Paracoccus denitrificans*; amino acid replacements were selected with the rationale of interfering with the hydrophilic lining of the pathway, in particular its assumed chain of water molecules. Proton pumping was assayed in the reconstituted vesicle system by a stopped-flow spectroscopic approach, allowing a reliable assessment of proton translocation efficiency even at low turnover rates. Several mutations at positions above the cytoplasmic pathway entrance (Asn 131, Asn 199) and at the periplasmic exit region (Asp 399) led to complete inhibition of proton pumping; one of these mutants, N131D, exhibited an ideal decoupled phenotype, with a turnover comparable to that of the wild-type enzyme. Since sets of mutations in other positions along the presumed course of the pathway showed normal proton translocation stoichiometries, we conclude that the D-pathway is too wide in most areas above positions 131/199 to be disturbed by single amino acid replacements.

Heme−copper oxidases play an important role in the energy metabolism of eukaryotes and many bacteria. As terminal enzymes of the respiratory chain they catalyze the reduction of dioxygen to water. This process is coupled to proton translocation across the membrane ("proton pumping") and establishes an electrochemical gradient which drives ATP synthesis (*1*−*3*). Recent crystal structures of the aa_3-type cytochrome *c* oxidase of *Paracoccus denitrificans* (*4*, *5*) and the mitochondrial enzyme from bovine heart (*6*−*8*) provide clear evidence that both oxidases share extensive structural similarities in their catalytically relevant subunits I and II. This finding extends to bacterial quinol oxidases within this enzyme family, even though their heme compositions often vary widely (e.g., see refs *9* and *10*).

Both from the structures of the two cytochrome *c* oxidases and from a large body of mutagenesis data (for a recent compilation of several papers, see ref *11*), there is general agreement that at least two pathways for protons are distinguishable in subunit I of oxidase. The D-pathway, named after the highly conserved Asp 124[1] located at its cytoplasmic entrance (*4*), has been shown to play an essential role in proton pumping (*12*−*16*). The uptake of protons for water formation was originally assigned to the K-pathway, while the "pumped protons" were assumed to be translocated across the D-pathway (*4*). Later, a different model was proposed, taking into account the possibility that all pumped protons as well as two "substrate protons" may use the D-pathway (*17*−*19*) toward Glu 278, which may connect the D-pathway to the binuclear center (*17*, *20*, *21*). However, the precise functional assignment of both pathways is still a matter of controversy (e.g., see refs *22* and *23*).

Further amino acid positions affecting proton translocation have been defined along the D-pathway. Residues[1] corresponding to Asn 113 and Asn 131, both located close to Asp 124, have been studied in the bo_3 oxidase of *Escherichia coli* (*13*, *24*), the aa_3 oxidase of *Rhodobacter sphaeroides* (*14*), and the aa_3 oxidase of *P. denitrificans* (*15*), while Ser 193 has been analyzed in the bo_3 oxidase of *E. coli* (*25*) and the *R. sphaeroides* aa_3 oxidase (*26*). Positions Asn 199 and

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* To whom correspondence should be addressed. E-mail: ludwig@em.uni-frankfurt.de. Fax: (+49) 69 798 29244.

‡ Johann Wolfgang Goethe-Universität.

§ Abteilung Molekulare Membranbiologie, Max-Planck-Institut für Biophysik.

|| Abteilung Biophysikalische Chemie, Max-Planck-Institut für Biophysik.

[1] All amino acid positions are numbered according to the *Paracoccus* sequence.

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Thr 203 did not seem to play any significant role so far in *R. sphaeroides* (*26*) or in *P. denitrificans* (*15*).

No clear functional confirmation for the pathway of pumped protons beyond the binuclear center has been established presently. The presumed exit toward the periplasm is located within a cluster of protonatable residues, including the heme propionates, which strongly interact electrostatically (*4*, *27*, *28*). Two arginine residues (473/474[1]) in the heme vicinity have been mutated and suggested to stabilize the high-spin heme ring D propionate in its anionic form for proton translocation to occur (*29*).

In this study, specific amino acid residues presumably lining the D-pathway have been chosen for further functional investigation in the *P. denitrificans* oxidase, with the rationale of introducing mutations that might interrupt proton transfer within the pathway, such as bulky and/or hydrophobic side chains. Since previous attempts to measure the exact stoichiometry of the proton pump for the *P. denitrificans* oxidase in whole cells (*15*) were partly inconclusive, we have now used the purified mutant enzymes to assay the capacity of the pump in reconstituted vesicles on a subsecond time scale. While most of the mutations did not affect proton pumping, several replacements showed a decoupled phenotype, leading to an unequivocal identification of participating residues at the entrance region and at the periplasmic exit of the D-pathway.

MATERIALS AND METHODS

Site-Directed Mutagenesis. Amino acids were selected for mutagenesis on the basis of the crystal structure of the *Paracoccus* cytochrome *c* oxidase (*4*, *5*). Site-directed mutagenesis was performed according to the "altered sites" protocol of Promega (*30*). All mutations introduced into the *cta*DII gene encoding subunit I were confirmed by DNA sequencing.

Expression and Purification of Cytochrome c Oxidase. For homologous expression the broad host-range vector pRI (*15*), a pBBR1MCS derivative (*31*) carrying a streptomycin resistance gene (*32*), was used. A 323 bp fragment (367 bp upstream of the *cta*C gene) carrying the promoter region of the *cta* operon (*33*) was cloned into the multiple restriction site of pRI, resulting in pUP39. Mutated *cta*DII genes were subcloned into pUP39, which was then conjugated into the *Paracoccus* recipient strain AO1, lacking the aa_3- and cbb_3-type cytochrome *c* oxidase (*15*). Expression levels for cytochrome *c* oxidase mutants in AO1 were comparable to those of the wild-type enzyme.

Cells were grown on succinate medium (*34*), supplemented with 25 μg/mL streptomycin sulfate and 25 μg/mL kanamycin, at 32 °C to an $OD_{550nm} > 3.0$. Membranes isolated from these cells (*35*) were used for purifying the four-subunit oxidase complex in a single chromatographic step employing Fv fragments (*15*, *36*). Isolated oxidase complexes were characterized for their subunit composition by SDS[2] gel electrophoresis and by recording their visible redox difference spectra, including CO and CN^- derivatives, as described earlier (*15*, *30*, *35*, *37*); according to these criteria, the isolated mutants were indistinguishable from the wild type enzyme. Experimental heme *a*/protein ratios in all preparations of mutants and wild type, expressed and purified under identical conditions, varied between 10 and 16 nmol of heme *a*/mg of protein.

Determination of Enzymatic Activities of Isolated Enzymes. Cytochrome *c* oxidase activity was determined as previously described (*30*) using 20 mM Tris-HCl (pH 7.5), 20 mM KCl, 1 mM EDTA, and 0.2 g/L *n*-dodecyl β-D-maltoside. The spectrophotometric assay was performed with reduced horse heart cytochrome *c* at a concentration of 20 μM.

Proton Pumping. Proton pumping was determined either in whole cells, essentially as described previously (*15*), or with purified protein reconstituted into proteoliposomes. Purified asolectin (phosphatidylcholine, type II S, Sigma) was resuspended in 100 mM HEPES−KOH, pH 7.3, 10 mM KCl, and 2% cholate at a concentration of 40 mg/mL. The solution was stirred at 4 °C until all asolectin particles had been completely dissolved and then sonicated with a Branson sonifier at 30% duty cycle (80 W) until the solution appeared translucent. Purified oxidase, eluted from a Q-Sepharose column by 100 mM HEPES−KOH, 500 mM KCl, and 0.15 g/L dodecyl maltoside, was added to the asolectin preparation typically in a 1:8 volume ratio to yield a final protein concentration of 4 μM, and the formation of vesicles was initiated by dialysis, starting with 100 mM HEPES buffer.

For pH measurements using an electrode, the final dialysis buffer concentration was 0.5 mM HEPES (pH 7.3), 80 mM KCl, and 34 mM sucrose. For assaying proton pumping on a fast time scale, stopped-flow measurements recording absorption changes of the pH-sensitive dye phenol red photometrically were done essentially as described in ref *38*; typical concentrations, after mixing, were 0.7 μM oxidase and 18 μM horse heart cytochrome *c*, in the presence of the K^+ ionophore valinomycin. For this assay, proteoliposomes were prepared as above, but dialysis was initially against 100 mM HEPES−KOH and 10 mM KCl, while the final dialysis buffer concentration was 50 μM HEPES, 55 mM KCl, and 55 mM sucrose.

RESULTS AND DISCUSSION

For its role in pumping protons across the membrane, cytochrome *c* oxidase should provide at least one pathway for protons destined to cross the membrane dielectric. The D-pathway in subunit I, characterized from the crystal structure, mutagenesis approaches, and electrostatic calculations (see introduction and below), fulfills several criteria, such as (i) sufficient width for proton/water molecules, (ii) hydrophilic environment provided by appropriate amino acid side chains and/or water molecules within a suitable distance for establishing a hydrogen-bonding network, and (iii) a possible connection to the energy input and/or gating device.

With the two acidic landmark residues, Asp 124 and Glu 278, already investigated extensively in oxidases of different sources, we have mutated a series of residues along the presumed pathway between the cytoplasmic entrance and the exit on the periplasmic side of the *P. denitrificans* oxidase (see Figure 1). Alterations of amino acid residues were intended to change the electrostatic properties by altering the hydrophilic pathway interior and thereby impeding proton translocation. Therefore, most mutations (see Table 1) introduced a more hydrophobic and/or bulky side chain residue.

Using a standard mutagenesis protocol on the cloned *cta*DII gene coding for subunit I of the aa_3 cytochrome *c*

[2] Abbreviations: SDS, sodium dodecyl sulfate; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone.

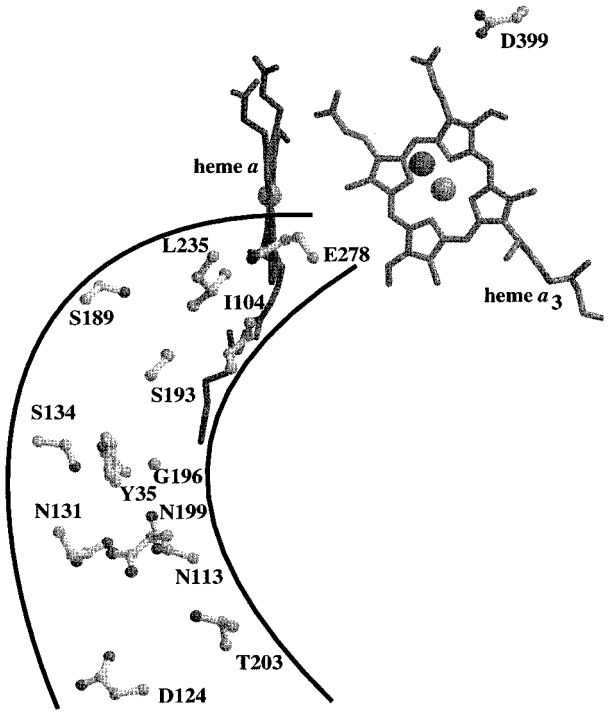

FIGURE 1: Schematic representation of amino acids presumably lining the D-pathway in subunit I of the *P. denitrificans* cytochrome *c* oxidase. Side chains of residues listed in Table 1 together with D124/E278 and subunit I redox cofactors are depicted, drawn according to the published coordinates (*5*; pdb code 1ar1) using the programs MolScript (*49*) and Raster3D (*50*, *51*).

oxidase, the gene was conjugated on a plasmid into the deletion strain AO1 (*15*) and expressed *in trans*. Strain AO1 is not only defective in producing the wild-type copy of subunit I of this oxidase but has been inactivated, in addition, in the gene coding for the alternative cytochrome *c* oxidase, *cbb*$_3$ (*15*, *39*). Therefore, any cytochrome *c* oxidase activity observed after expression of a point-mutated *cta*DII gene is strictly due to an *aa*$_3$ oxidase complex, allowing the preliminary assessment of proton pumping already in whole cells (see below).

Expression of the mutated subunit I and its assembly with the three smaller subunits were assayed by Western blotting of membranes (not shown) and purification of the oxidase complexed by an Fv fragment (see Materials and Methods); all mutant preparations showed a subunit I of normal size and were assembled into an intact four-subunit complex, as judged from SDS gel electrophoresis. Their heme compositions, relative *a* and *a*$_3$ spectral contributions, as well as specific heme/protein ratios were comparable to those of the wild-type complex. In selected cases (N131D, N199D), redox FTIR spectra were recorded to assess the structural integrity of the purified mutants (P. Hellwig, unpublished); while the former mutant showed minor changes in distinct areas of the spectrum, but no differences at the amide modes, the latter displayed full wild-type characteristics.

Periplasmic Mutations. Residue Asp 399 is located in the loop region between the transmembrane helices IX and X, near the periplasmic interface between subunits I and II of the complex. Three different replacements at this position have been studied: both the Glu and the Asn result in a moderate reduction of the turnover rate (residual activity between 40% and 60%), without any major effect on the stoichiometry of protons pumped in the reconstituted enzyme (Table 1). Only with Leu present in this position is a decoupling of both processes observed: in intact cells as well as in proteoliposomes, no proton pumping takes place, but electron transfer still persists to a certain degree. It is obvious that, with a low rate of turnover in this mutant, the pump stoichiometry determined in whole cells is not very meaningful as such (see below), but the stopped-flow spectroscopic measurement of the purified enzyme incorporated into liposomes demonstrates the complete absence of an acidification phase (see Figure 2D and, for comparison, Figure 2A illustrating a wild-type phenotype), while in the presence of CCCP the alkalinization due to water formation is clearly observable. We note, however, that this mutant phenotype is not characterized by the (idealized) situation of two completely uncoupled reactions, electron transfer and proton pumping, but should rather be described as affecting both reactions, but to varying degrees.

Asp 399 had previously been assumed to play a role at the potential exit site (*4*, *12*, *15*, *27*). However, our present data do not agree with the recent conclusion of Qian et al. (*40*), who exclude any role for this residue in the *R. sphaeroides* enzyme. While confirmed here for the *P. denitrificans* cytochrome *c* oxidase in terms of the N and E substitutions, we show that a hydrophobic residue in this

Table 1: Catalytic Activities and Proton Pump Stoichiometries of Purified *P. denitrificans* Cytochrome *c* Oxidase Mutants versus Whole Cell Pumping

		proton pumping (H^+/e^-) measured in	
strain/mutant	catalytic activity (%)[a]	whole cells[b]	reconstituted vesicles[c]
wild type	nd[d]	3.0	1.0
recombinant wt[d]	100	3.1	0.9 (1.0)
deletion mutant		2.1	
Y35F	55	3.0	0.8
Y35F/S134A	53	2.7	0.8
I104F	70	2.4	1.1
I104W	45	2.3	0.7
N131D	99	2.3	0
N131V	6	2.2	0
N131D/N113V	75	2.5	0
S134A	96	3.2	0.9 (0.9)
S189A	80	3.1	(0.8)
S189F	67	3.0	nd
S189W	47	2.9	nd
S189A/S193A	80	3.3	1.4
S193A	76	3.0	(0.7)
G196F	67	3.1	nd
G196W	70	2.6	0.8
N199D	55	2.5	0
N199D/T203V	63	2.5	0
T203V	67	3.3	(0.9)
L235F	74	2.9	nd
L235W	55	2.6	0.7
D399L	7	2.0	0
D399N	58	3.0	1.4
D399E	38	2.1	1.1

[a] Purified enzyme measured in the spectroscopic assay (see Materials and Methods). [b] Values determined for succinate oxidation; a ratio of 3 is suggestive of a $1H^+/e^-$ contribution by cytochrome *c* oxidase to the overall proton release, while a value of 2 indicates the absence of a pump at this site; see text for details. [c] Determined by stopped-flow spectroscopy; values in parentheses were determined by pH−electrode measurements. [d] Abbreviations: wt, wild type; nd, not determined.

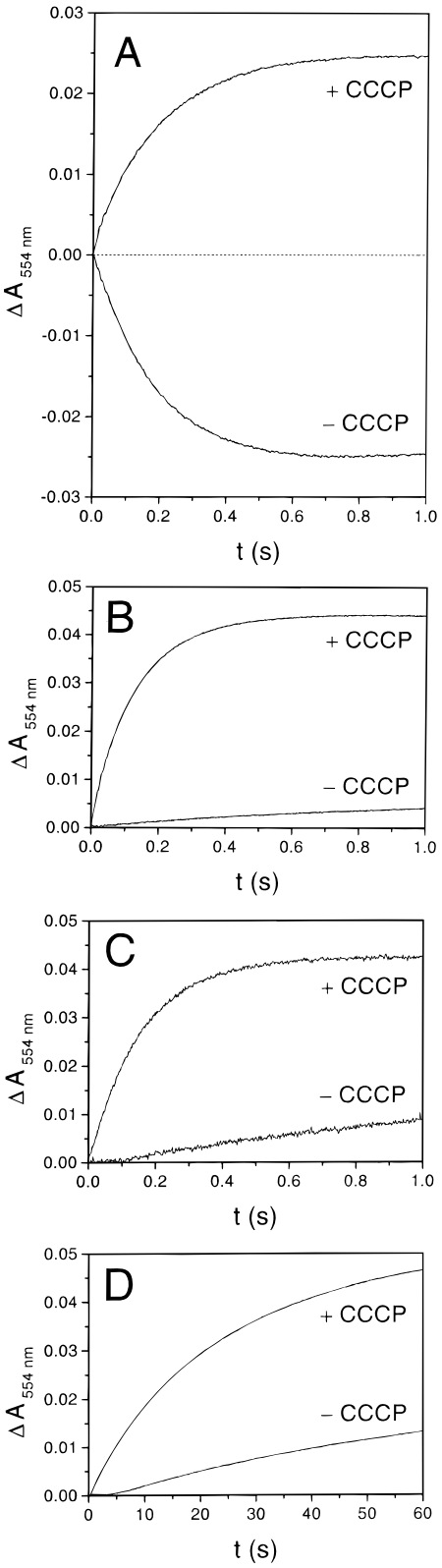

FIGURE 2: Proton translocation of reconstituted oxidase preparations measured by stopped-flow spectroscopy. Absorption changes of phenol red were recorded following a pulse of reduced cytochrome *c* in the absence or presence of CCCP, with a downward deflection indicating acidification. For further experimental details, see Materials and Methods and ref *38*. Mutants: (A) S134A, exhibiting wild-type proton translocation, see Table 1; (B) N131D; (C) N199D; (D) D399L; in this latter case, due to low catalytic turnover of this enzyme (see Table 1), absorption changes were followed for 60 s. Respiratory control ratios determined separately were between 2.7 and 4.6 for (A) and 1.7−2.7 for (B−D).

position may cause a structural change, possibly altering the H-bonding pattern around the propionate of heme a_3 (*27*). In addition, a drop in the midpoint redox potential by about 100 mV has been observed for heme a_3 in this mutant (*41*), yielding an explanation for the sharp decline of the enzymatic activity in this mutant.

The D-Pathway between Its Cytoplasmic Entrance and Residue Glu 278. Of all residues tested here, two positions in this area of the D-pathway, Asn 131 and Asn 199 (Table 1), exhibit the most interesting effects, i.e., a truely decoupled phenotype. Both are located well within the pathway above its entrance, with their amide groups almost facing each other (see Figure 1). When a negative charge is introduced individually at either of the two positions, the turnover is not affected at all (N131D) or is reduced to only 50% (N199D), but proton translocation is completely abolished. In quantitative terms, this pattern of uncoupling differs from the situation of the D399L mutation (see above) and from the N131V exchange. This latter replacement by a hydrophobic side chain also leads to a completely inactive pump but at the same time severely diminishes the turnover of the mutant enzyme to 6%. The phenotype of the N131D mutant enzyme is unique in the sense that it shows ideal uncoupling, not observed, to our knowledge, for any other cytochrome *c* oxidase mutant before; its turnover rate is virtually unaffected, yet it does not exhibit any proton translocation.

In our initial characterization of this mutation (*15*) we reported a wild-type behavior based on whole cell pumping. A later careful reappraisal of the strain identity, as well as of physiological conditions of cell growth and of inhibitor concentrations used in the whole cell pumping, consistently led to a pump ratio of close to 2 in whole cells (see Table 1 and below), fully in line with the much more direct and better controlled reconstitution assay data presented here.

Analysis of the proton pumping behavior of the N131D and N199D mutants (Figure 2B,C) does not support the interpretation [drawn for the D124N mutation in the *R. sphaeroides* oxidase (*42*)] that protons might be taken up from the external aqueous phase. As clearly shown for either case, mutants in this region of the pathway do not exhibit any fast alkalinization in the absence of the uncoupler CCCP but are characterized by a weak backflow of protons into the vesicle lumen within the relevant time scale, as a consequence of the internal consumption of protons in water formation (as seen in the corresponding trace in the presence of CCCP). Therefore, no protons, in whatever direction, are actively translocated across the membrane dielectric in any of these mutants.

From these data it is obvious that side chains in both positions belong to a proton transfer path. While previous mutations of Asn 199 in the *R. sphaeroides* aa_3 oxidase [N199A (*26*)] as well as the *Paracoccus* enzyme [N199V (*15*)] did not reveal this effect, it was shown that Asn 131 is a critical residue in the bo_3 quinol oxidase of *E. coli* (*13*); valine in this position led to a decoupled phenotype with a residual turnover rate of 22%, while N131D still retained some residual proton pump activity. The earlier conclusion (*13*) that a polar residue is sufficient at this site for effective proton pumping is not substantiated by our present study, as complete decoupling is observed for the N131D mutant in *P. denitrificans*.

How Is the Inhibition of Proton Pumping in the Asp 131/Asp 199 Mutations Rationalized Mechanistically? By introducing an acidic residue, most likely present with a negatively charged carboxylate moiety, into the pathway roughly 5 Å above Asp 124 in both mutants, it is conceivable that the hydrogen-bonding pattern in the D-pathway is altered. This should, however, affect both proton pumping *and* electron transfer, since the D-pathway provides proton access during the oxidative part of the enzyme's catalytic cycle as well. The alternative of opening the K-pathway by either mutation appears highly unlikely. The newly introduced negative charge would also have effects toward the opposite side; the possibility that it may pull in protons from the exit site and allow proton access via Glu 278 toward the active site appears to be ruled out by the results of the stopped-flow experiments as mentioned above. However, a residue which has been assumed to undergo conformational changes during the catalytic cycle is Glu 278, which appears to be protonated (*20*, *21*, *43*, *44*). It thus has an electric dipole moment along the line connecting the carboxyl oxygen atoms. The additional negative charge might influence the preferred orientation of the side chain. It has been proposed that this residue acts as a gate (*45*) or a switch (*22*, *23*), allowing proton transfer either to the binuclear center (for protons consumed in water formation) or toward the heme propionates (for pumped protons). If the additional charge favors that former orientation, then the mutant enzyme would be as active as the wild type, but proton pumping might be abolished.

As alternative explanations, one could also envisage a direct influence on the protonation behavior of the Asp 124 residue due to the additional charge introduced by either mutation or a loss of the hypothetical chain of water molecules in this region of the pathway. When two water molecules are modeled between the Asp 124 and the Asn 131/Asn 199 residues (see refs *25* and *46*), bridging the gap between the carboxylate oxygen (Asp) and the amide groups (Asn), the experimentally observed nonequivalence of three adjacent asparagine side chains (113/131/199; see Figure 1) upon replacing the Asn by either Asp or Val becomes explainable: (i) Both Asn 113 exchanges do not affect proton pumping at all (*15*), while (ii) either replacement in position 131, directly stabilizing the water structure by hydrogen bonding in the native enzyme, abolishes translocation completely (Table 1); (iii) for position 199, only the acidic residue replacement blocks the pump, while the valine exchange does not affect proton translocation (*15*); only the former mutation should interrupt the hydrogen bond in the water stabilization. This interpretation of the actual course of proton movements in this region of the pathway is in accordance with the result of the T203V mutation (Table 1); this residue is close to the Asn 113 and does not impede proton pumping upon mutation.

Either of the two latter alternatives should influence proton conductance both for protons to be pumped and for those for water formation, likewise (see above). For an explanation, one has to speculate that the availability of a "vectorial" proton has to be timed precisely for a successful translocation (coupling) event; i.e., the probability for the pump mechanism to run at idle (the uncoupled condition) is high in a D-pathway mutant.

In the further course of the D-pathway, several other, mostly hydrophilic residues were changed to hydrophobic residues with side chains of comparable size (Table I and ref *15*). Measurements of turnover and proton pumping on whole cells revealed no significant effects of these mutations, even in double mutants. Following this idea, amino acid residues Y35, S189, I104, G196, and L235 supposedly lining the pathway were replaced by rather bulky residues such as Phe and Trp (see Table 1). In all of these mutants, proton pumping was not affected, and enzymatic activity was only moderately reduced relative to the wild-type enzyme. This led to the notion that the D-pathway must be too wide in this area to link specific residues to proton translocation. In particular, the position of water molecules acting in proton transfer may not be affected by the exchange of a specific amino acid side chain, or due to its width in most parts of the pathway, alternative proton pathways may exist within the same main pathway. Indeed, a large number of water molecules can be modeled in this region of the pathway. One may speculate that the type of differential inhibition observed on turnover and/or proton pumping is dependent on whether those alternative pathways are able to act in a kinetically competent fashion in proton delivery. Nevertheless, when mutations in Ser 193 were investigated in the bo_3 quinol oxidase, the tryptophan (but not the phenylalanine) replacement mutant showed reduced proton pumping (*25*), while the alanine mutant in the *R. sphaeroides* aa_3 was fully active as a pump (*26*).

Different Experimental Approaches to Proton Pump Measurements. Proton pump determinations in whole cells at the terminal oxidase level may provide a convenient initial screening method, circumventing time-consuming purification/reconstitution steps. For the expression of mutated forms of subunit I of the aa_3-type cytochrome *c* oxidase, we have used a strain inactivated in both, the two subunit I gene copies and in the alternative cbb_3 oxidase (see Materials and Methods). As outlined elsewhere (*15*), a ratio of three protons per electron fed into the chain by succinate is indicative of full proton translocation via the cytochrome bc_1/cytochrome *c* oxidase pathway. Indeed, with an alternative control at hand (see below), we can show (see Table 1) that, for mutants with an oxidase turnover rate between ~50% and 100%, the ratio of 3 in whole cells represents a correct estimate of an oxidase fully competent in pumping, as seen from the corresponding unity ratio in the reconstituted assay. All other data sets require use of the purified enzyme reconstitution approach for reliable results, since under those conditions the flow of electrons from the Q pool will partially change from the preferred complex III/IV route to the quinol oxidase branch (*47*), leading to an inherently lower proton ratio of two protons per electron.

With respiratory control ratios for reconstituted *P. denitrificans* oxidase often very low (see legend to Figure 2), the use of a stopped-flow spectroscopic approach is superior to acidification measurements by a pH electrode (see Table 1) with its long response time. This former technique (*38*, *48*) uses phenol red for the pH determination following a pulse of reduced cytochrome *c* mixed with the proteoliposome suspension under aerobic conditions. Due to its favorable time resolution (see Figure 2), proton backflow during the relevant time interval is minimal. Measurements of vesicles with respiratory control values of as low as 2

become feasible, and its sensitivity allows a reliable assay of mutant enzyme even at low catalytic activity (see Table 1 and Figure 2).

CONCLUSION

Although the molecular mechanism of coupling electron transfer and proton translocation during turnover of cytochrome *c* oxidase and the role of its D- and K-pathway are still a matter of controversy, presently there is general agreement that all pumped protons are being translocated across the D-pathway. For the *P. denitrificans* oxidase, three new amino acid positions in subunit I have been assigned a functional role in proton translocation by mutations resulting in a decoupled phenotype. Next to the two previously well-established residues, Asp 124 at the entrance to the D-pathway and Glu 278 close to the binuclear center of the enzyme, the Asp 399 residue plays an essential role at the subunit I/II interface on the periplasmic side. Two further positions, Asn 131 and Asn 199 located above the cytoplasmic entrance, show complete loss of proton translocation as well. In either case, when the amide group is replaced by a carboxylate, the mutant enzymes show turnover between 50% and 100%; i.e., the redox cycle and water formation are not impeded by the replacements in the D-pathway. However, the pumping of all four protons during O_2 reduction is completely lost. Exchanges by hydrophobic residues (in N131V and in D399L) result in a double defect: complete decoupling and at the same time a drastic decline in the turnover rate, connected to a drop in the redox potential for heme a_3 in the case of the Asp 399 mutant enzyme.

Proton translocation is assumed to be conducted by a chain of water molecules and/or hydrophilic side chains of amino acid residues lining the pathway interior. In the course of the D-pathway between positions 124 and 278, as tested here in a set of mutants in the *P. denitrificans* cytochrome *c* oxidase, no further evidence for a direct participation of specific residues in proton pumping could be obtained. Possibly, this pathway is too wide in most parts to be disturbed, by any single side chain alteration, in its structural integrity or its electrostatic properties, even when introducing bulky residues.

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