Proton Translocation by Cytochrome *c* Oxidase Can Take Place without the Conserved Glutamic Acid in Subunit I[†]

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ABSTRACT: A glutamic acid residue in subunit I of the heme−copper oxidases is highly conserved and has been directly implicated in the O_2 reduction and proton-pumping mechanisms of these respiratory enzymes. Its mutation to residues other than aspartic acid dramatically inhibits activity, and proton translocation is lost. However, this glutamic acid is replaced by a nonacidic residue in some structurally distant members of the heme−copper oxidases, which have a tyrosine residue in the vicinity. Here, using cytochrome *c* oxidase from *Paracoccus denitrificans*, we show that replacement of the glutamic acid and a conserved glycine nearby lowers the catalytic activity to <0.1% of the wild-type value. But if, in addition, a phenylalanine that lies close in the structure is changed to tyrosine, the activity rises more than 100-fold and proton translocation is restored. Molecular dynamics simulations suggest that the tyrosine can support a transient array of water molecules that may be essential for proton transfer in the heme−copper oxidases. Surprisingly, the glutamic acid is thus not indispensable, which puts important constraints on the catalytic mechanism of these enzymes.

The structurally and functionally related heme−copper oxidases catalyze the respiratory reduction of O_2 to water. This is coupled to proton translocation across the mitochondrial or bacterial membrane, by which energy is conserved for the synthesis of ATP (*1*). The mechanism of proton translocation in this class of enzymes has been intensively studied, and much work has been done to identify proton-transfer pathways to the bimetallic O_2 reduction site, which lies within the membrane part of the protein structure (*2*, *3*). Site-directed mutagenesis combined with measurements of proton translocation in cytochrome bo_3 from *Escherichia coli* (*4*, *5*) and cytochrome aa_3 from *Rhodobacter sphaeroides* (*6*) identified an aspartic acid (Asp-124 in cytochrome aa_3 from *Paracoccus denitrificans*)[1] as the entry site of pumped protons on the inside of the membrane. The crystal structures (*2*, *3*, *7*) of two cytochrome *c* oxidases confirmed that a proton-conducting pathway (the D-channel, Figure 1) leads from this aspartic acid toward a well-conserved glutamic acid residue (Glu-278) in the middle of the membrane domain of subunit I. Several water molecules in this pathway were predicted by computational techniques (*8*, *9*), and many of them have been identified in the refined crystal structures (*7*, *10*). The D-pathway is probably connected to Glu-278 via bound water molecules (*2*, *8*, *9*). However, proton transfer beyond this point is not clear from the crystal structures, but statistical−mechanical calculations have suggested that water molecules may, at least transiently, connect the glutamic acid further toward the bimetallic heme a_3−Cu_B site (*8*).

Glu-278 is one of the best conserved residues among the heme−copper oxidases, and mutations at this locus have been shown to block the oxygen reaction and the uptake of protons, as well as proton translocation (*11*−*13*), suggesting that this residue plays an important role in the catalytic mechanism and that it is a key residue for proton translocation. FTIR data provided evidence for connectivity between Glu-278 and Cu_B, possibly through a hydrogen-bonded water array (*14*), although the crystal structures do not reveal any bound water molecules in the vicinity of the bimetallic center (*2*, *3*, *7*, *10*). Proton conduction from Glu-278 forward might involve conformational isomerization of the side chain of this amino acid (*8*, *9*, *15*), and therefore Glu-278 may have a proton-shuttling role rather than being a mere proton conductor.

Despite the obvious importance of Glu-278, some distantly related heme−copper oxidases lack this residue but are nevertheless able to pump protons. This suggests that these enzymes have either alternative proton-transfer pathways or perhaps even a different proton translocating mechanism (*16*−*19*). Cytochrome caa_3 from *Rhodothermus marinus*, for example, contains all of the other residues known to be involved in the D-pathway but lacks the key glutamic acid. Comparison between the amino acid sequences in transmembrane helix VI in subunit I of the heme−copper oxidases shows that, in many enzymes that lack the glutamic acid, the fourth residue upstream from this locus (usually a phenylalanine) has been changed to a tyrosine (*20*) (Figure

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[1] If not otherwise specified, all amino acid numbering refers to subunit I of the aa_3-type cytochrome *c* oxidase from *P. denitrificans*.

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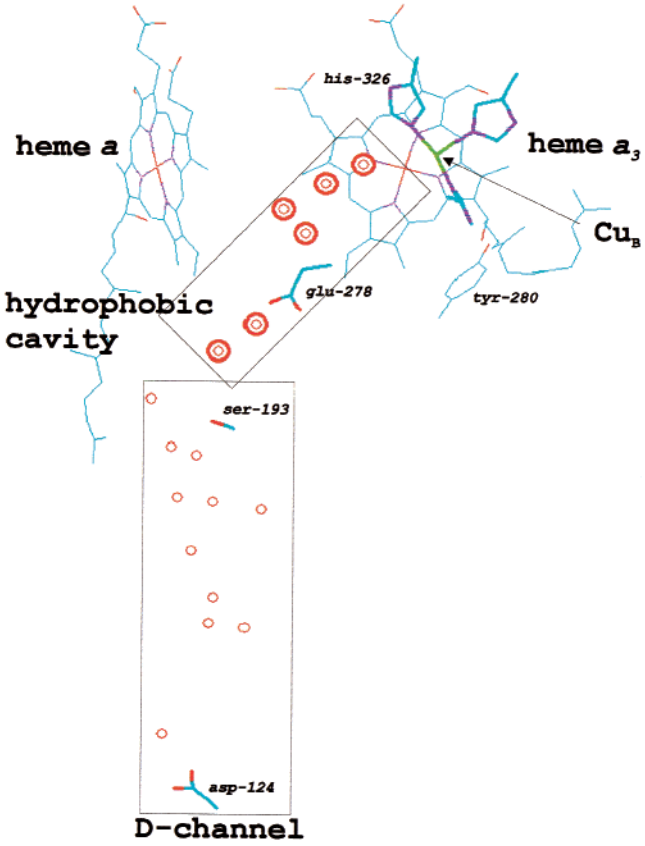

FIGURE 1: The D-channel and glutamic acid 278. Schematic view of the cytochrome *c* oxidase structure along the plane of the membrane, showing the bimetallic heme a_3−Cu_B center and the low-spin heme *a*. The D-channel transfers protons from Asp-124 on the inside, ending at Ser-193 in the middle of the membrane. A hydrophobic cavity leads from this point toward the bimetallic center and contains the residue Glu-278. Predicted water molecules along this path (see the text) are shown as red circles; those in the hydrophobic cavity are labeled by double circles.

2). Structural modeling suggests that the side chain of such a tyrosine residue may be close to the position of the carboxyl group of the glutamic acid in the main group of heme−copper oxidases (*20*). The fact that this tyrosine is the only protonatable amino acid residue near the Glu-278 site in these distant members of the enzyme family has been taken to suggest that the OH group of the tyrosine might take the role of the carboxyl group of the glutamic acid in proton transfer (*20*). However, given the large differences in properties between these side chains, full functional equivalence would be surprising a priori.

In cytochrome caa_3 from *Rh. marinus* there is an alanine in the Glu-278 locus. In addition, the highly conserved Gly-275 is changed to serine and Phe-274 to tyrosine (Figure 2). We set out to mimic this structural theme in the mitochondrial-type cytochrome *c* oxidase from *P. denitrificans* to find out whether the glutamic acid in the latter is indispensable for function.

MATERIALS AND METHODS

Site-directed mutagenesis, bacterial growth conditions, and purification of cytochrome aa_3 enzymes from *P. denitrificans* were as described previously (*21*). On the basis of the methodology developed by Rigaud et al. (*22*), reconstitution of the *Paracoccus* cytochrome aa_3 into proteoliposomes was

Paracoccus denitrificans aa$_3$	W	F	F	G	H	P	E	V	Y
Bovine heart *aa*$_3$	W	F	F	G	H	P	E	V	Y
Rhodobacter sphaeroides aa$_3$	W	F	F	G	H	P	E	V	Y
Escherichia coli bo$_3$	W	A	W	G	H	P	E	V	Y
Rhodothermus marinus caa$_3$	W	F	Y	S	H	P	A	V	Y
Thermus thermophilus caa$_3$	W	F	Y	S	H	P	T	V	Y
Rhodobacter sphaeroides cbb$_3$	W	W	Y	G	H	N	A	V	G
Paracoccus denitrificans cbb$_3$	W	W	Y	G	H	N	A	V	G

FIGURE 2: Amino acid sequence alignment for the region around Glu-278 in transmembrane helix VI of subunit I in three types of heme−copper oxidases. The one-letter code for amino acids is used. The fully conserved histidine shown enlarged in blue in the center is one of the three ligands of Cu_B. Residues Glu-278 and Tyr-274 are shown in red. The boxes indicate the three loci that were mutated in this work in the *Paracoccus* aa_3 enzyme.

carried out as described for bovine heart cytochrome *c* oxidase (*23*), omitting the extra sucrose gradient purification step. Isolated cytochrome aa_3 was added to a final concentration of 0.5 μM. Respiratory control ratios were between 3 and 9. Proton translocation was determined by the O_2 pulse method (*24*) under constant argon flow. Proteoliposomes were added to anaerobic medium containing 100 mM KCl, 15 μM horse heart cytochrome *c*, 10 μM *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine (TMPD), and 5 mM potassium ascorbate. One micromolar valinomycin was added, and the pH was kept in the range 7−7.4. Oxygen was added as a small volume of air-saturated water (0.258 mmol of O_2/L at 25 °C and 1 atm) and anaerobic HCl to calibrate the observed proton ejection.

RESULTS AND DISCUSSION

If, in the *P. denitrificans* enzyme, Glu-278 is replaced by alanine, the activity is reduced to ca. 1%, while the replacement of Gly-275 with serine decreased the activity to 3%. In combination, these two mutations yielded an additive effect, and only <0.1% of the activity remained (Table 1). Clearly, the residual activity of both single mutant enzymes depends crucially on the functionality of the other amino acid. Quite strikingly, however, when Phe-274 is replaced by a tyrosine in the background of these two mutations, the activity is raised by more than 2 orders of magnitude and reaches ca. 10% of the wild-type activity. Moreover, this raised O_2 consumption activity in the triple mutant enzyme is coupled to proton translocation with almost the same efficiency as in the wild type (Table 1) (see Note Added in Proof).

Table 1 also shows that the mutation of Phe-274 to tyrosine alone, as well as in combination with the Glu-278-Ala mutation, yielded ca. 10% activity as well as proton translocation, i.e., a result similar to that of the triple mutant described above. Thus, the mutation of Phe-274 to tyrosine has two significant effects: On the one hand, it lowers the activity to about 10% of the wild-type enzyme with retained proton translocation efficiency, but on the other, it completely nullifies the strongly additive inhibitory effect of the mutations at Gly-275 and Glu-278.

These data confirm that the glutamic acid is important as such, but surprisingly, it is not indispensable for either the

Table 1: Enzymatic Activity and Proton Translocation Efficiency of Wild-Type and Mutant Cytochrome aa_3 Enzymes from *P. denitrificans*[a]

enzyme	activity		proton translocation, H^+/e^-
	% of wt	$e^- s^{-1} aa_3^{-1}$	
wild type	100	1150	0.7−1
E278A	1	12.2	0
G275S	3	32.3	0
G275S/E278A	<0.1	<1.2	ND
F274Y/G275S/E278A	9	103	0.6−0.7
F274Y/G275S	7	86	0.6
F274Y	11	124	0.6
F274Y/E278A	9	99.2	0.5−0.9
F274Y/G275S/E278T	3	39.4	0.4−0.6

[a] Oxygen consumption was measured using a Clark-type oxygen electrode. The reaction medium was 50 mM phosphate buffer, pH 6.5, supplemented with 0.05% DM, 2.9 mM ascorbate, 34 μM horse heart cytochrome *c*, 0.6 mM TMPD, and 1.1 mg/mL asolectin. The wild-type activity range was 1150 (±187 SD) $e^- s^{-1}$ (cytochrome $aa_3)^{-1}$. The H^+/e^- ratios measured in proteoliposomes have been corrected for the 0.5 H^+/e^- released on oxidation of ascorbate. ND, not determined.

O_2 reduction or the proton translocation activities of the heme−copper oxidases. Therefore, the glutamic acid is unlikely to play a direct role in the proton-pumping mechanism as is the case, for example, with the Schiff base in bacteriorhodopsin (bR; *25*). Interestingly, the aspartic acid residue in bR (Asp-96), which reprotonates the Schiff base, is substituted by a tyrosine in the structurally related sensory rhodopsin I (sRI; *26*). This substitution leads to a slow rate of sRI photocycling, which is still coupled to proton translocation (*27*). The slow rate might be necessary to mediate transmission of light signals to the flagellar motor. In bR, the Asp-96 residue is located in a hydrophobic cavity approximately 10 Å from the Schiff base, and water molecules are needed for proton transfer between the two. The general structural features of this cavity are similar to the cavity between the Glu-278 and the bimetallic center in the heme−copper oxidases (Figure 1), although the two enzymes are otherwise entirely different (*28*).

An initial model of the triple mutant structure was constructed on the basis of the 2.3 Å crystallographic structure of bovine cytochrome *c* oxidase (access code 2OCC; *10*). After energy minimization, a 19 amino acid region of approximately 15 Å diameter around Tyr-274 was subjected to molecular dynamics (MD) simulation using the AMBER force field and program (*29*, *30*), with the bimetallic center fully reduced. Also included in the MD simulations were six of the water molecules placed originally in the 2.8 Å bovine structure (*3*) on the basis of statistical−mechanical calculations (Figure 1; *8*). Modeling of this triple mutant structure shows that the stress induced by the serine side chain can be alleviated by a small change in the backbone dihedral angles, and the other two mutations (Glu → Ala and Phe → Tyr) can be accommodated easily. The Glu → Ala mutation produces a hole toward the Ser-193 side chain at the end of the D-channel.

One picosecond equilibration at 150 K was followed by 100 ps of MD at 300 K. Within 3 ps at 300 K, the four water molecules in the cavity between His-326 (a ligand of Cu_B in the bimetallic center) and Tyr-274 rearranged to form a hydrogen-bonded chain connecting Nϵ of His-326 to the OH group of Tyr-274, as shown in Figure 3. Moreover, two water molecules, which were initially placed between the end of the D-pathway and the glutamic acid (*8*), now connect the Ser-157 to the tyrosine, thus establishing a protonic link all the way between the copper-bound histidine and the D-pathway. One water molecule moved by 2.3 Å from a position near Ile-66 (Met-99 in the *Paracoccus* enzyme) to a position within hydrogen-bonding distance of the carbonyl oxygen of Ser-275. A second water molecule positioned originally near Leu-381 (Met-416 in the *Paracoccus* enzyme) and the vinyl group of heme a_3 moved about 6 Å toward the OH group of Tyr-274. The formed water chain persists for the duration of the simulation (100 ps) and is anchored by the Nϵ of His-326, the OH group of Tyr-274, and the carbonyl oxygen of Ser-275. During the 100 ps simulation, fluctuations in the water chain led to occasional breaks of hydrogen bonds, but the overall structure remained stable. It should be stressed that, lacking an "anchor", no such water chain was formed in control simulations with the wild-type enzyme where the glutamic acid was positioned according to the crystal structure (*10*).

The OH group of the Ser-275 side chain does not interact directly with the water chain, which explains why the Gly-275-Ser mutation does not affect the activity in Phe-274-Tyr mutant enzymes. The reduction of activity in the Gly-275-Ser single mutant enzyme can be understood from direct interactions of the isomerized side chain of Glu-278 (*8*, *15*) with the OH group of Ser-275, which does not participate in proton transfer toward the active site. The rate of proton delivery by isomerization of the Glu-278 side chain would thus be reduced, lacking a transfer path beyond Ser-275. The small residual activity of the Glu-278-Ala single mutant enzyme can be understood from the very infrequent formation of a long water chain between the D-channel and the active site, which would have to pass through the hole left by the Glu-278-Ala mutation. Such a water chain will be perturbed further by a Gly-275-Ser mutation, practically eliminating activity in the double mutant enzyme (Gly-275-Ser *plus* Glu-278-Ala), as observed. Inhibition of activity to ca. 10% of wild type by the Phe-274-Tyr mutation, without changing Glu-278 (Table 1), can be understood by formation of a hydrogen bond between the OH of the tyrosine and the carboxyl of glutamic acid, which would prevent side-chain isomerization of the latter.

The OH group of Tyr-274 is about 10 Å away from the nearest proton acceptor on the exit side so that proton transfer beyond Tyr-274 appears virtually impossible without recruitment of water molecules. We conclude that a transient water chain can indeed establish a functional proton-transfer connection between the D-channel and the bimetallic site (Nϵ of His-326) in the triple mutant. Since the water chain bypasses the Glu-278 position, this explains why all Phe-274-Tyr mutants show activity independent of the analyzed mutations at the glycine and glutamic acid sites, and it provides further evidence for our proposal of water-mediated proton transfer beyond Glu-278 also in the wild-type enzyme (*8*). The formation of the water chain requires the transient presence of water molecules in the cavity. These may be produced by the O_2 reduction reaction at the bimetallic site in preceding enzyme turnovers. The product of oxygen reduction—water—is thus possibly recycled as "wiring" for proton transfer into the bimetallic center before diffusing out

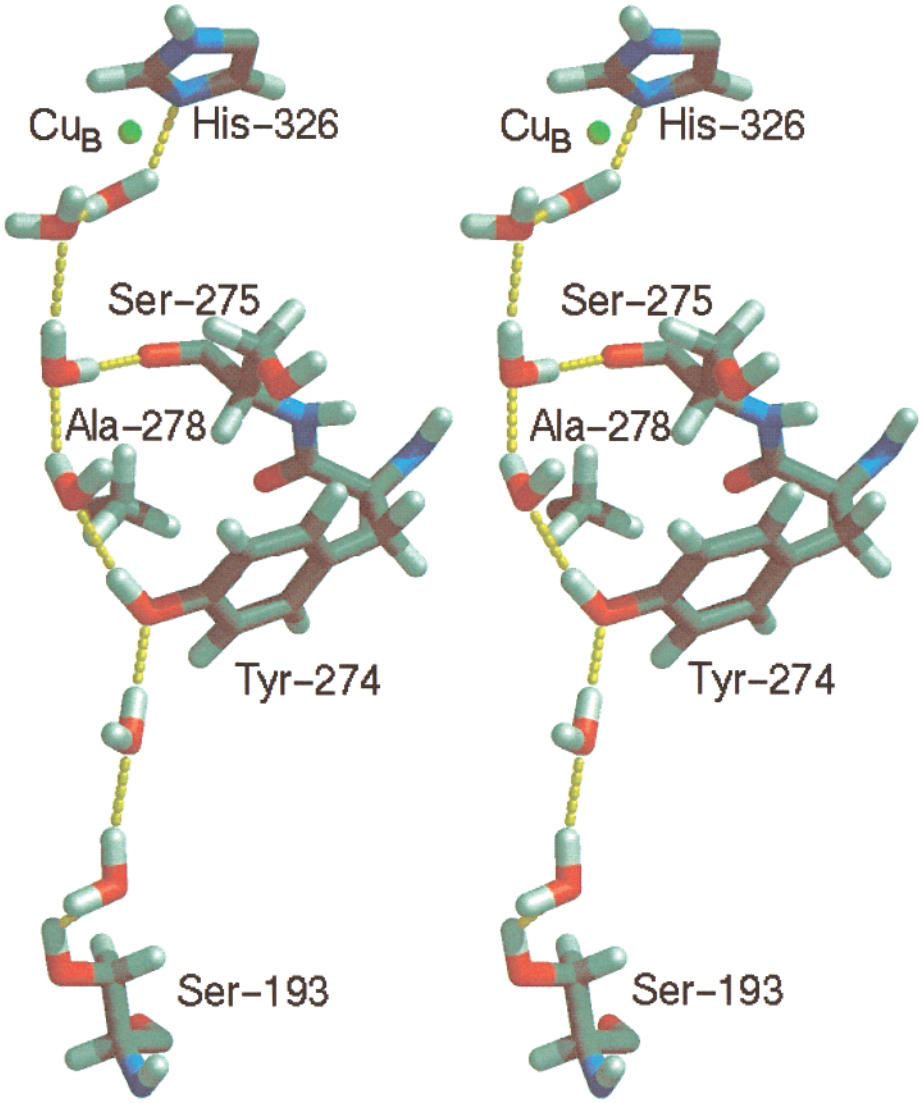

FIGURE 3: Stereoview of the hydrogen-bonded water chain connecting the active site (Nϵ of His-326) to the D-channel residue Ser-193 via Tyr-274, formed after 3 ps of MD simulation at room temperature. Note that although the simulations were done with the structure of the bovine enzyme (*10*), the amino acid numbering in the figure refers to the *Paracoccus* enzyme. Oxygen, nitrogen, carbon, and hydrogen atoms are shown in red, blue, gray, and white, respectively.

into the aqueous medium. As the MD simulations show, when there is a sufficient number of water molecules in the cavity, they can rapidly (within a few picoseconds) reorganize to form a hydrogen-bonded chain. Proton transfer is thus limited by the dynamics of production and removal of water molecules within the cavity.

These considerations also shed light on the specific role of the glutamic acid. Due to its acidic property, it divides the proton conduction path between the D-channel and the bimetallic site into two short water chains that may form independently of one another and, therefore, with higher probability (Figure 1). In contrast, with tyrosine in the cavity, proton transfer depends on the smaller probability of forming a single six-membered water chain between the end of the D-channel and the bimetallic site (Figure 3).

Finally, we suggest that the glutamic acid may have a proton-shuttling function between two short water chains, functioning as a true proton donor and acceptor. In contrast, the tyrosine will probably not donate protons owing to its much higher pK_a value. Instead, its function may be to stabilize a single long water chain, which, when formed, facilitates fast transfer of a proton by a Grotthuss-type mechanism from aspartic acid 124 in the beginning of the D-pathway to the bimetallic site.

NOTE ADDED IN PROOF

More detailed experiments have revealed that the proton translocating efficiency of the triple mutant enzyme is as high as it is in the wild-type enzyme.

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