Heme-copper oxidases with modified D- and K-pathways are yet efficient proton pumps

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Received 2 January 2001; revised 12 March 2001; accepted 17 April 2001

First published online 4 May 2001

Edited by Richard Cogdell

Abstract The cytochrome aa_3-type quinol oxidase from the archaeon *Acidianus ambivalens* and the ba_3-type cytochrome *c* oxidase from *Thermus thermophilus* are divergent members of the heme-copper oxidase superfamily of enzymes. In particular they lack most of the key residues involved in the proposed proton transfer pathways. The pumping capability of the *A. ambivalens* enzyme was investigated and found to occur with the same efficiency as the canonical enzymes. This is the first demonstration of pumping of 1 H^+/electron in a heme-copper oxidase that lacks most residues of the K- and D-channels. Also, the structure of the ba_3 oxidase from *T. thermophilus* was simulated by mutating Phe274 to threonine and Glu278 to isoleucine in the D-pathway of the *Paracoccus denitrificans* cytochrome *c* oxidase. This modification resulted in full efficiency of proton translocation albeit with a substantially lowered turnover. Together, these findings show that multiple structural solutions for efficient proton conduction arose during evolution of the respiratory oxidases, and that very few residues remain invariant among these enzymes to function in a common proton-pumping mechanism.

Key words: Quinol oxidase; Cytochrome *c* oxidase; Proton transfer; Archaeon

1. Introduction

The heme-copper oxidases are the terminal elements of the membrane-bound respiratory chains [1–3] that catalyze the four-electron reduction of dioxygen to water, and concomitantly pump protons across the membrane. Thus, they need pathways for proton transfer that may be described as proton wires [4], i.e. protein-spanning chains of hydrophilic residues and water molecules. Key residues of two such proton pathways were first identified functionally by site-directed mutagenesis data on the bo_3-type quinol oxidase from *Escherichia coli* ([5–8]) and were corroborated by the crystal structures of the cytochrome *c* oxidases from *Paracoccus denitrificans* [9], and bovine heart mitochondria [10], and the quinol oxidase from *E. coli* [35]. The D-pathway is composed of a series of polar residues and water molecules that connect the input side to a glutamic acid residue (Glu278 in *Paracoccus*) in the middle of the membrane, that was shown to be essential for proton translocation [11]. The K-pathway is named after a conserved lysine residue (Lys354 in *Paracoccus*) and seems to lead directly to the binuclear center (see [8,9,12–18]).

The thermoacidophilic archaeon *Acidianus ambivalens* expresses an aa_3-type quinol oxidase as its only terminal oxidase [17,19,20]. The electron donor is caldariella quinone (CQ), the predominant benzo-thiophene quinone of this organism. This oxidase has unique features in that it diverges significantly from most other members of the heme copper superfamily on the basis of the primary structure [21]. Strikingly, most residues that are necessary for proton conduction in the canonical oxidases are absent. On the other hand, the heme reduction potentials are pH-dependent [20], as they are in the more common oxidases. In addition, reduction of heme a_3 is associated with a transient change of the hydrogen bonding network around the formyl group of this heme, which might have significance for the proton translocation mechanism [17]. Hence, this enzyme is an excellent model system for inquiring whether proton pumping can be achieved by an oxidase without the previously described characteristic proton transfer pathways.

The ba_3-type cytochrome *c* oxidase from *Thermus thermophilus* is likewise very different from the common heme-copper oxidases in the design of the so-called D- and K-channels. The X-ray structure of this enzyme was recently solved [22], and while its main features were found to be similar to those of the common group of heme-copper oxidases, there are dramatic differences with respect to the two proton transfer pathways, as had been expected from the primary structure. Kannt et al. [23] found a H^+/e^- ratio of only 0.5 for the reconstituted *T. thermophilus* enzyme, which led to the suggestion that proton translocation might occur by a mechanism that differs from that of the main group of heme-copper oxidases.

Here we demonstrate that: (i) the *A. ambivalens* oxidase, despite lacking typical D- and K-channels is a canonical proton pump with an efficiency of 1.0 H^+/e^-; and (ii) a double mutant of cytochrome *c* oxidase from *P. denitrificans* that

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Abbreviations: CQ, caldariella quinone; CCCP, carbonylcyanide *p*-chloromethoxy phenylhydrazone; TMPD, *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine

0014-5793/01/$20.00
PII: S0014-5793(01)02431-0

simulates the structure of the *T. thermophilus* oxidase D-channel, also translocates protons with full efficiency.

2. Materials and methods

2.1. Protein purification

Cells of *A. ambivalens* were grown and the membrane fraction was prepared as previously described [24]. All chromatographic steps were carried out on a Pharmacia HiLoad™ system, at 4°C. The membrane fraction was solubilized with dodecyl maltoside (DM), in the proportion of 2 g detergent per g of protein, and the resulting suspension was centrifuged at 138 000×*g* for 6 h at 4°C. The green supernatant, corresponding to the solubilized membranes, was applied to a 330-ml DEAE–Sepharose Fast flow column. After elution at 10 ml min^{-1} with 330 ml of low ionic strength buffer (20 mM potassium phosphate pH 6.5, 0.1% DM) a gradient from 0 to 80% of high ionic strength buffer (20 mM potassium phosphate pH 6.5, 0.1% DM) was applied at the same flow. The oxidase fraction, which eluted at 250 mM NaCl, was applied directly to a 70-ml home-packed HTP Micro-beads column, at 1 ml min^{-1}, which had been previously equilibrated with 20 mM potassium phosphate pH 6.5, 0.1% DM. An elution gradient of six column volumes of 500 mM potassium phosphate pH 6.5, 1 M NaCl, 0.1% DM was applied and the oxidase fraction eluted at around 400 mM potassium phosphate. This fraction was concentrated by ultrafiltration over a 10-kDa cut-off membrane and applied to a 330-ml gel filtration Superdex S-200 column, equilibrated and eluted with 20 mM potassium phosphate, pH 6.5, 200 mM NaCl, 0.1% DM at 1.6 ml min^{-1}. The purified enzyme was divided in aliquots and stored at −70°C.

2.2. Reconstitution of the A. ambivalens enzyme into liposomes

CQ was purified as described in [25], and reconstitution was performed as described in [11], with the following modifications. Preformed liposomes containing CQ were prepared by mixing 150 μl of 1 mM CQ with 16 mg asolectin and chloroform (∼2 ml). The solvent was evaporated and the asolectin/CQ mixture was solubilized in 200 mM HEPES–KOH, pH 7.4/55 mM octyl glucoside by brief ultrasonic treatment. Purified aa_3 quinol oxidase (10 μM) was added to a final concentration of 0.2 μM, and the mixture stirred for 10 min. From this point on, the described procedure was followed. The reconstituted oxidase was reduced by dithiothreitol (2 mM) and ubiquinone-1 (0.25 mM).

2.3. P. denitrificans aa_3 mutant

Mutagenesis, isolation of the mutant enzyme, and its reconstitution into liposomes were performed as described earlier [26].

2.4. Proton translocation measurements

Proton-pumping measurements were performed using the O_2 pulse method [27]. An all-glass vessel was used under constant flow of argon. To initiate the reaction, a small accurate pulse of O_2 was added as 1–10 μl of pure water equilibrated under air at 25° (0.258 mM O_2 at 1 atm) into the final volume of 1.2 ml. The response of the pH electrode was converted to H^+ release (or consumption) using an anaerobic HCl standard. The pH range was 7.0–7.5. Stirring by a magnetic disk assured mixing in ca. 0.2 s; the pH electrode response time was ca. 1 s. Under such conditions the added O_2 is typically consumed in a few seconds.

3. Results and discussion

The *A. ambivalens* quinol oxidase was co-reconstituted with CQ into artificial proteoliposomes. The rate of oxygen consumption using DTT and ubiquinone-1 as the electron source increased up to 2-fold following addition of valinomycin plus uncoupler. This respiratory control is lower than that observed for the reconstituted quinol oxidase from *E. coli* [11], which is consistent with the faster rates of relaxation of the H^+ transients observed here (Fig. 1A). However, an average ratio of 1.75 H^+/e^- was observed in the proton translocation experiments, despite the higher membrane permeability for

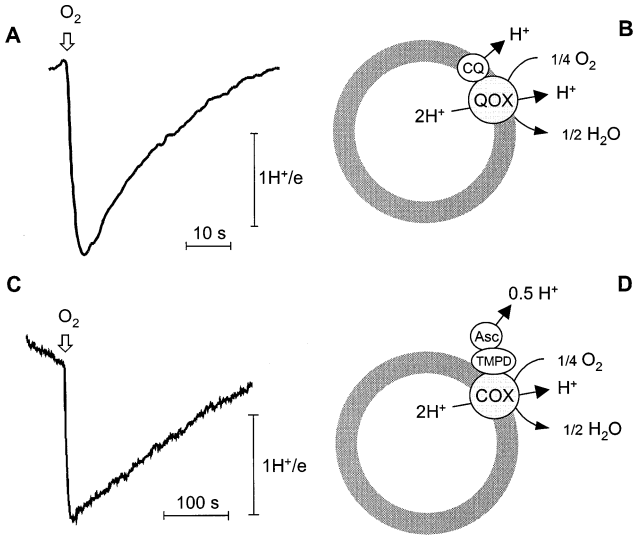

Fig. 1. Proton translocation in reconstituted proteoliposomes containing *A. ambivalens* aa_3 quinol oxidase: proton translocations experiments (A) were done at 25°C in proteoliposomes containing CQ and the oxidase, incubated anaerobically in a medium containing 200 mM KCl, 2 mM DTT, 0.25 mM ubiquinone-1 and 1 μM valinomycin. Note that oxidation of the hydroquinone by the oxidase results in the scalar release of 1 H^+/e^- to the outside of the membrane, whereas the second released proton is due to proton translocation across the membrane (B). In the presence of 1 μM carbonylcyanide *p*-chloromethoxy phenylhydrazone (CCCP) no proton ejection or consumption upon addition of O_2 was observed. *P. denitrificans* cytochrome *c* oxidase mutant Phe274Thr/Glu278Ile: Proton translocation for the reconstituted mutant (C) was measured at 25°C as described in Section 2. Anaerobic 100 mM KCl medium, proteoliposomes (ca. 0.1 μM cytochrome aa_3), 10 μM *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine (TMPD), and 5 mM potassium ascorbate were supplemented with 15 μM horse-heart cytochrome *c*, and 1 μM valinomycin (all final concentrations). The arrow marked O_2 indicates the addition of 1 μl of air-saturated water. Note that 0.5 H^+/e^- is released due to oxidation of ascorbate. D: Shows schematically the function of the reconstituted cytochrome *c* oxidase system (cf. B).

protons, which indicates that the majority of the reconstituted oxidase molecules has an adequate orientation. The oxidation of hydroquinone by the enzyme results in the scalar release of 1 H^+/e^- to the outside of the membrane, whereas the second proton is vectorially translocated from the inside to the outside (cf. [11]; Fig. 1B). Hence, the *A. ambivalens* quinol oxidase is a true proton pump that pumps 1 H^+/e^- as do the canonical members of the heme-copper superfamily. Clearly, this means that since the canonical D- and K-pathways are missing, this enzyme must possess alternative pathways of proton transfer to be able to conduct protons from the input side of the membrane to the binuclear site, as well as across the membrane.

The *A. ambivalens* and *T. thermophilus* enzymes have quite a low amino acid identity relative to the canonical oxidases, and only very few residues are strictly conserved among all oxidases [21,22,28,29]. Striking differences are noticeable with respect to the proposed proton-conducting D- and K-pathways. Although most usually conserved residues are absent from the K-channel of the *A. ambivalens* enzyme, possible functional substitutions were anticipated on the basis of sequence alignments and the analysis of a 3D homology model ([30]; Figs. 2–4 and Table 1); these substitutions were also

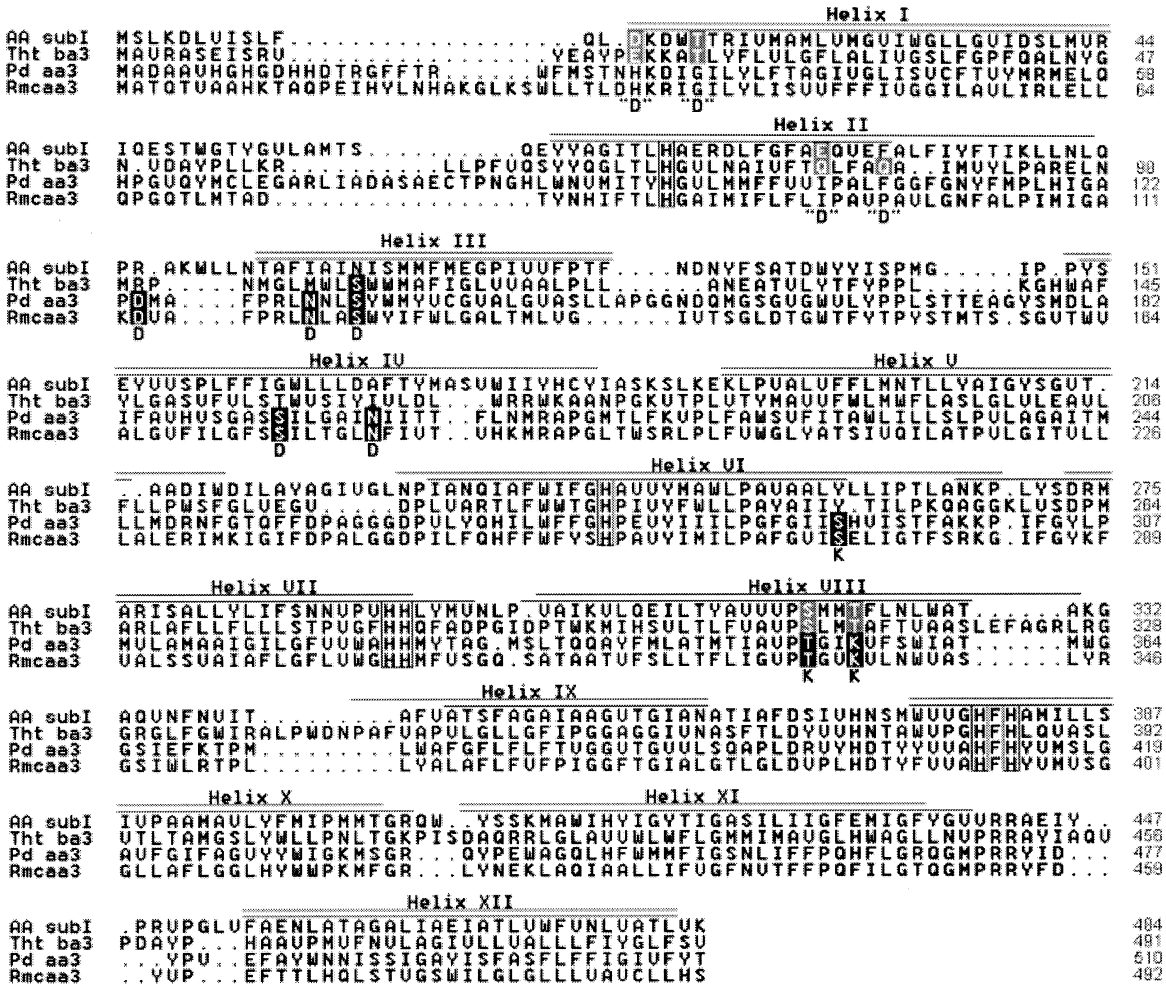

Fig. 2. Structure-oriented sequence alignment of terminal oxidases subunit I. Alignment of subunit I sequences from *A. ambivalens* (*Aa* aa_3), *T. thermophilus* (*Tt* ba_3), *P. denitrificans* (*Pd* aa_3) and *R. marinus* (*Rm* caa_3). Gray-shaded boxes represent the pseudo D- and K-channels (marked as 'D' and 'K') proposed for the *A. ambivalens* aa_3, whereas black boxes represent residues involved in the canonical D- and K-channels (marked as D and K). Helices are indicated as defined for *Tt* ba_3 (upper trace) and for *Pd* aa_3 (lower trace). Heme ligands are boxed.

proposed for the *T. thermophilus* ba_3 oxidase [22,23]. In contrast, none of the residues that form the D-channel in the canonical oxidases are conserved in the *A. ambivalens* or *T. thermophilus* ba_3 enzymes. In the latter, Glu278 is substituted by an isoleucine and Phe274 by a threonine (Fig. 3, Table 1). These two amino acid replacements were done in the cytochrome *c* oxidase from *P. denitrificans* to mimic this situation. Although the turnover of this double mutant was only ca. 2% of the wild-type enzyme, proton translocation was observed at almost normal efficiency (Fig. 1C).

As a first conclusion from this work, we can therefore confirm that full proton-pumping efficiency can be attained in the absence of the glutamic acid (Glu278 in *Paracoccus* cytochrome *c* oxidase) that has been described as one of the key elements of this pathway. Earlier evidence for this view was given by work on the the caa_3 oxidases from *T. thermophilus* [31] and *Rhodothermus marinus* [32], both of which also pump 1 H^+/e^-. However, although these enzymes lack the glutamic acid, all other residues of the D-channel are conserved in contrast to the case for the ba_3 from *T. thermophilus* and the aa_3 from *A. ambivalens*. In the caa_3-type enzymes above (as well as in some other heme-copper oxidases), a tyrosine substituting for Phe274 in *Paracoccus* was proposed to functionally replace the glutamic acid [2,26,32]. However, this tyrosine is not present in the *A. ambivalens* and *T. thermophilus* ba_3 oxidases. The latter enzyme has a threonine in this site, which is apparently sufficient to support normal proton-translocating efficiency, as suggested by the present work (Fig. 1C). Our data show that the earlier conclusions that the *T. thermophilus* ba_3 enzyme pumps only 0.5 H^+/e^- [23] have to be regarded with caution.

On the other hand, the *A. ambivalens* enzyme has a phenylalanine in the Phe274 (*Paracoccus*) site, like most of the common oxidases, and therefore the question remains as to how the lack of the glutamic acid has been compensated for in this case. Detailed analysis of the 3D homology model for this enzyme suggests several amino acids that may participate in an alternative pseudo D-channel, including Asp14, Thr18, Glu80, and Tyr89 ([30]; Figs. 2 and 3 and Table 1). Some of these residues were recently suggested to have such a function also on the basis of the crystal structure of the *T. thermophilus* ba_3 oxidase [22]. However, Glu80 is unique for the *A. ambivalens* enzyme and is of particular interest here. It is located in helix II, and faces the hydrophobic domain close to

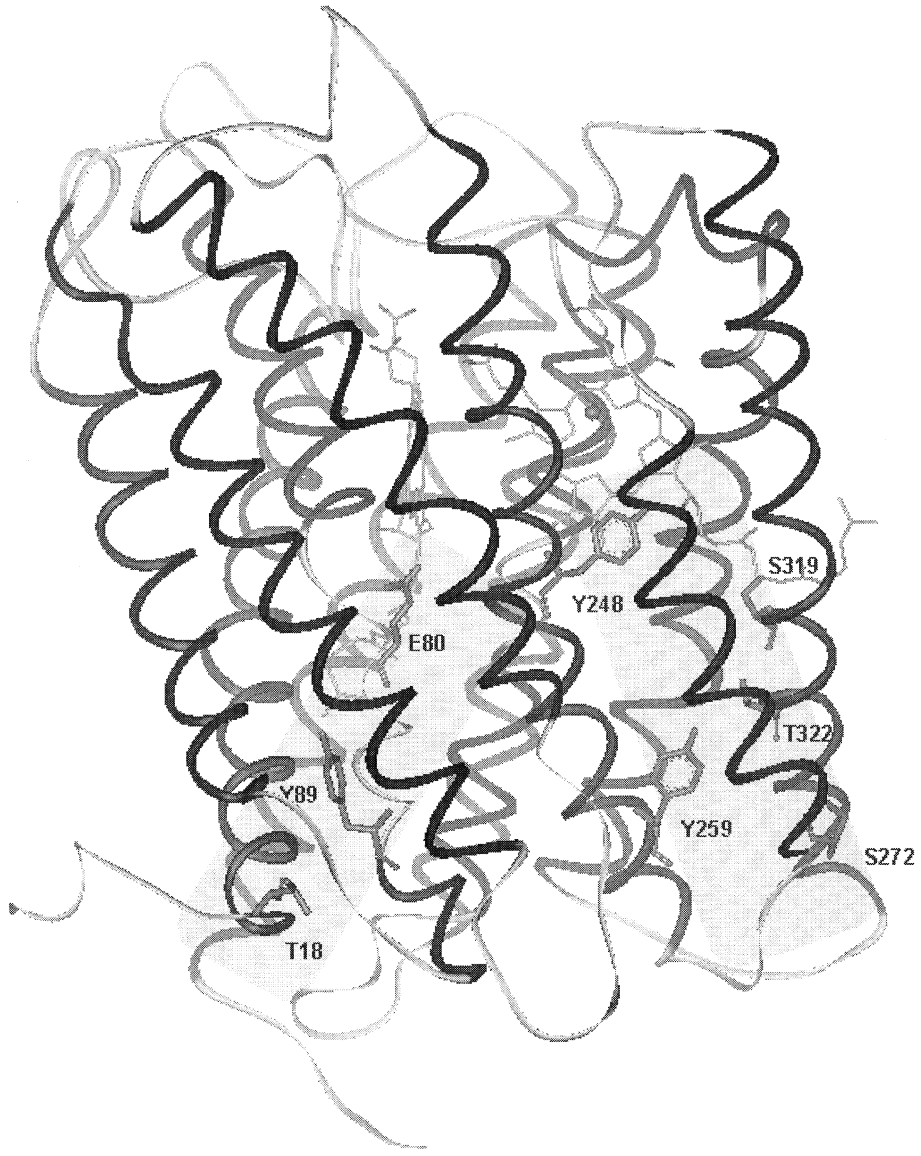

Fig. 3. Structural model of the *A. ambivalens* cytochrome aa_3 highlighting two putative proton channels. Gray boxes highlight residues involved in the pseudo K-channel (right) and in the pseudo D-channel (left). The pseudo K-channel leads directly to the binuclear site (Tyr248), while the pseudo D-channel leads to Glu80, at the bottom of an hydrophobic cavity; from Glu80 onwards the proton connectivity has to be assured by water molecules.

the position of Glu278 in *Paracoccus* (Fig. 4). In the *T. thermophilus* ba_3 oxidase this position is occupied by a glutamine; the equivalent site is Ile104 in *Paracoccus*, Met116 in the bo_3-type quinol oxidase from *E. coli*, and Met71 in the mitochondrial enzyme. An arginine or lysine substitution at this locus was previously shown to interfere with the glutamic acid in the *E. coli* enzyme (Glu286; [33]). Furthermore, substitution of the isoleucine in this position to glutamic acid in the *Rho-*

Table 1
Comparison of residues proposed to be involved in subunit I proton pathways

	Pd aa_3	*Rm* caa_3	*Tt* ba_3	*Aa* aa_3
'K-pathway'	Tyr280[a], Thr351, Lys354	Tyr258[a], Thr329, Lys332	Tyr237[a], Ser309, Thr312	Tyr248[a], Ser319, Thr322
'D-pathway'	Tyr35, Asn113, Asp124, Ser134, Ser193, Asn199	Tyr41, Asn102, Asp113, Ser123, Ser175, Asn181	Gln82, Ser155, Gln86, Ser109, Thr21, Tyr91	Thr18, Tyr89
	Glu278	Tyr256 (274)	Thr156 (274)	Glu80 (104)
References	[9]	[2,33]	[22]	This work

[a]Tyrosine covalently bound to a Cu_B histidine ligand. Glu278 (*Pd*) and its functional substitutes are highlighted (the equivalent numbering in *Pd* is indicated in parentheses).

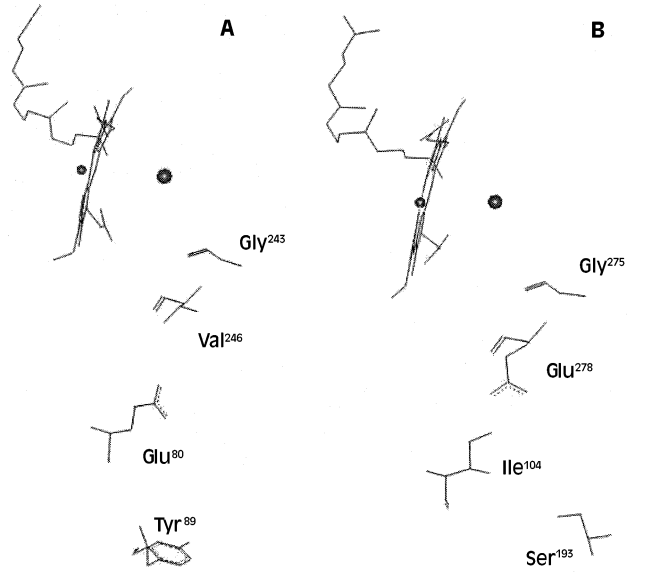

Fig. 4. Comparison between the predicted position of Glu80 and the position of Glu278 in *A. ambivalens* and *P. denitrificans* aa_3 oxidases, in relation to the heme a_3-Cu_B center. A: *A. ambivalens* quinol oxidase model. B: *P. denitrificans* cytochrome oxidase (PDB code 1AR1). In both oxidases, a series of hydrophilic residues leads from the cytoplasmatic side of the membrane to Glu80 (*Aa*) or Glu278 (*Pd*), at the bottom of a hydrophobic cavity connecting to the binuclear center. The *Pd* residues equivalent to those of *A. ambivalens* are shown.

dobacter sphaeroides aa_3 enzyme was recently shown to confer proton-translocating activity to a mutant enzyme where the glutamic acid (Glu278, *Paracoccus*) had been changed to alanine (P. Brzezinski et al., personal communication). Hence, Glu80 is a very good candidate for functionally replacing Glu278 (*Paracoccus*), and thus establishing an alternative D-pathway in the *A. ambivalens* enzyme. Since it is somewhat further away from the binuclear center than Glu278 (*Paracoccus*), proton conductivity towards the binuclear center might have to be assured by a longer chain of water molecules, a situation reminiscent to that proposed in *R. marinus* caa_3 [2] and in the *P. denitrificans* D-pathway mutant enzymes [26]. Quite remarkably, the pseudo D-channels in the *A. ambivalens*, *R. marinus*, and probably the *T. thermophilus* ba_3 oxidases, are as efficient as the D-channel in the canonical oxidases, and they also appear kinetically competent: proton uptake from the bulk solution during the state P to state F transition of the binuclear site in the *A. ambivalens* oxidase at room temperature occurs with a rate constant of $\sim 10^4$ s^{-1}, which is identical to that of the more common oxidases [34].

In conclusion, it is clear from this work that proton transfer pathways may differ considerably in structural detail but may yet support the oxygen reduction chemistry as well as efficient proton-translocating activity in the large family of heme-copper oxidases. This suggests that multiple structural solutions for these pathways have been encountered during the evolution of these enzymes. Probably these solutions have been optimized for the growth conditions of the organism, since the alternative proton transfer pathways are mostly found in heme-copper oxidases of microorganisms that thrive under unusual conditions.

Acknowledgements: A. Kannt and H. Michel are acknowledged for the structural model of the *A. ambivalens* oxidase. This work was supported by Grants PRAXIS XXI BIO37/96 to M.T., EMBO ASTF 9415 to C.M.G., and by grants from the Academy of Finland, Biocentrum Helsinki and the Sigrid Juselius Foundation to M.W. P. Brzezinski is acknowledged for communicating data prior to publication.

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