The caa_3 Terminal Oxidase of *Rhodothermus marinus* Lacking the Key Glutamate of the D-Channel Is a Proton Pump†

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ABSTRACT: The thermohalophilic bacterium *Rhodothermus marinus* expresses a caa_3-type dioxygen reductase as one of its terminal oxidases. The subunit I amino acid sequence shows the presence of all the essential residues of the D- and K-proton channels, defined in most heme-copper oxidases, with the exception of the key glutamate residue located in the middle of the membrane dielectric (E278 in *Paracoccus denitrificans*). On the basis of homology modeling studies, a tyrosine residue (Y256, *R. marinus* numbering) has been proposed to act as a functional substitute [Pereira, M. M., Santana, M., Soares, C. M., Mendes, J., Carita, J. N., Fernandes, A. S., Saraste, M., Carrondo, M. A., and Teixeira, M. (1999) *Biochim. Biophys. Acta 1413*, 1−13]. Here, *R. marinus* caa_3 oxidase was reconstituted in liposomes and shown to operate as a proton pump, translocating protons from the cytoplasmic side of the bacterial inner membrane to the periplasmatic space with a stoichiometry of $1H^+/e^-$, as in the case in heme-copper oxidases that contain the glutamate residue. Possible mechanisms of proton transfer in the D-channel with the participation of the tyrosine residue are discussed. The observation that the tyrosine residue is conserved in several other members of the heme-copper oxidase superfamily suggests a common alternative mode of action for the D-channel.

The last step of membrane-bound aerobic electron transfer chains, oxygen reduction, is performed by oxygen reductases, most of which belong to the superfamily of heme-copper oxidases (*1*−*3*). These enzymes are characterized by the presence of a low-spin heme and a binuclear center formed from a high-spin heme and a copper ion in subunit I. Most of them have been shown to operate as proton pumps (*4*): during the reduction of one dioxygen molecule to two water molecules, four protons are consumed from the cytoplasmatic or matrix space, and an additional four protons are translocated across the inner bacterial or mitochondrial membrane, contributing to an electrochemical proton gradient, which is then consumed for ATP synthesis.

Site-directed mutagenesis (*5*, *6*) together with extensive sequence analysis and structure prediction led to the proposal that the enzyme contains two proton-conducting channels, which allow the access of protons to the catalytic center. This proposal was further confirmed by the X-ray crystallographic analysis of the structure of the aa_3 oxidases from bovine heart mitochondria (*7*, *8*) and from the bacterium *Paracoccus denitrificans* (*9*, *10*). The proton pathways were named K- and D-channels according to conserved residues in these enzymes. The K-channel, named for Lys354 (K), also contains the residues Thr351 and Tyr280. The D-channel is composed of the residues Asp124 (D), Asn199, Asn113, Asn131, Tyr35, Ser134, Ser193, and Glu278 (all *P. denitrificans* numbering) (*7*, *9*, *11*−*13*). The glutamate residue, located in the middle of the membrane dielectric, has been found to be a key element in the delivery of protons for both water formation and translocation across the membrane. Most mutations of this residue result in an enzyme incapable of completing the catalytic cycle. The oxygen reaction is blocked at the P state (*11*, *12*), and consequently proton pumping is not observed (*13*−*16*).

Recently, an increasing number of terminal oxidases have been reported in which the typical residues of the channels are not conserved (*17*−*26*), thus indicating that alternative pathway(s) should exist. The recently characterized caa_3 oxidase from *R. marinus* (*27*) is particularly interesting in this context. It is the first unequivocal example of a so-called cytochrome oxidase for which the electron donor is a small, iron−sulfur protein containing a $[4Fe\text{-}4S]^{3+/2+}$ center (a high potential iron−sulfur protein, HiPIP) (*27*−*29*). The amino acid sequence of caa_3 oxidase subunit I showed that all the residues considered important in the D- and K-channels are conserved, with the exception of the glutamate residue at the hydrophobic end of the D-channel. Homology modeling studies showed that a tyrosine residue (Y-256, *R. marinus* numbering) could structurally replace the glutamate. The phenol group of this residue is very close to the spatial position of the carboxyl group of the glutamate residue in the *P. denitrificans* and bovine enzymes. This is a natural example of a single mutation in the conserved D-channel

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residue (*27*), thus providing a model to test the relevance of glutamate and alternative substitutes. In this work, we show that this oxidase is indeed a proton pump and discuss the possible role of the tyrosine residue in the functioning of the D-channel.

MATERIALS AND METHODS

Protein Purification. Bacterial growth, membrane preparation, and solubilization were done as described in ref *30*. Protein purification followed the procedure reported in ref *27*.

Protein Reconstitution and Proton Pumping Measurements. Protein reconstitution was done as described in ref *31*, with some alterations. Fifty microliters of *R. marinus* caa_3 oxidase (concentration approximately 60 μM) was diluted to a volume of 2 mL with a suspension of sonicated, preformed liposomes containing 80 mg/mL azolectin, 2% (w/v) cholic acid, and 100 mM Hepes−potassium, pH 7.4, and was mixed for 30 min at 4 °C. The detergent was removed by several additions of Bio-Beads SM-2 absorbent (Bio-Rad) followed by incubation periods. Bio-Beads were added to the mixture to a concentration of 66.5 mg/mL two times, and the mixture was stirred each time for 1 h at 4 °C. Then more absorbent was added (133 mg/mL), and the mixture was stirred for 2 h more also at 4 °C. Finally, two other additions (266 mg/mL each) were made to the mixture followed by 1 h stirring at room temperature. The proteoliposomes were separated from the Bio-Beads, diluted with 100 mM KCl, and pelleted by centrifugation at 144000*g* for 2 h to remove the external buffer. Respiratory control was determined as in ref *11*. Proton pumping measurements were performed in the apparatus described in ref *32* by the O_2 pulse method (*5*, *6*).

RESULTS AND DISCUSSION

The caa_3 oxidase from *R. marinus* reconstituted into liposomes showed a respiratory control ratio of 5. The activity of the enzyme incubated with lipids prior to removal of detergent was 314 e^-/s at 25 °C. The capability of the reconstituted caa_3 oxidase to pump protons across the liposome membrane was investigated using the O_2 pulse technique (*5*, *6*). Figure 1 shows the acidification of the external medium when caa_3 activity is initiated by the addition of air-equilibrated water. An apparent stoichiometry of 1.5 H^+/e^- was observed. Taking into account that 0.5 H^+/e^- is released upon ascorbate oxidation, a stoichiometry of proton translocation from the internal to the external aqueous phase of 1 H^+/e^- was calculated (protons for H_2O formation are consumed inside the vesicles and do not participate in fast pH changes outside). This demonstrates exactly that like most members of the heme-copper oxidase family *R. marinus* caa_3 operates as a proton pump.

The ability of *R. marinus* caa_3 to pump protons shows clearly the presence of a functional substitute of the D-channel glutamate. The input part of this channel from the aspartate residue (D124 in *P. denitrificans* and D113 in *R. marinus*) to the serine residue (S193 in *P. denitrificans* and S175 in *R. marinus*) is shown to be very similar in both enzymes (*27*). In Figure 2, we present a detailed representation of the heme a_3-Cu_B center and the amino acid residues of helix VI in the vicinity of the glutamate position from *P.*

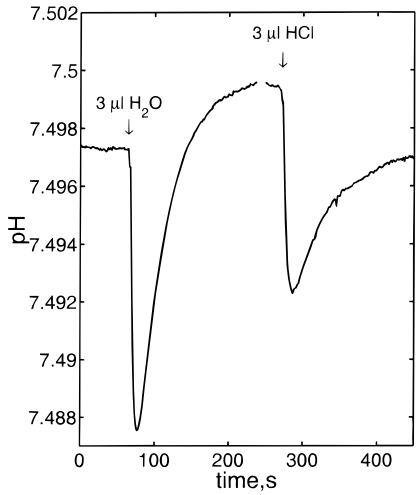

FIGURE 1: Proton translocation in caa_3 oxidase from *Rhodothermus marinus*. Changes in pH of a proteoliposome suspension containing 100 mM KCl, 5 mM potassium ascorbate, 5 μM TMPD, and 1 μM valinomycin were measured. The anaerobic proteoliposome suspension was pulsed with air-saturated water at 25 °C. The acidification of the medium was calibrated by injection of anaerobic 1 mM HCl. An apparent stoichiometry of 1.5 H^+/e^- was observed, which corresponds to 1 H^+/e^- translocated by the caa_3 oxidase across the membrane.

denitrificans oxidase (A) and the corresponding region of the previously published model of *R. marinus* enzyme (B). For the oxidases, which contain the glutamate residue, proton connectivity between the serine residue and the binuclear center has been proposed to be achieved through a network of water molecules, on the basis of the 3D structure, FTIR measurements, and theoretical calculations (*14*). The glutamate residue has been proposed to be a proton shuttle, which can take up, even if only transiently, two distinct conformations ("in" and "out"), related by a 180° rotation of the carboxylate group (*33*). Quite interestingly, in *R. marinus*, the spatial positions equivalent to the in and out conformations of the glutamate side chain are occupied by the hydroxyl groups of a tyrosine residue and a serine residue (Y256 and S257), respectively. While a small energy barrier of ~3 kcal/mol has been estimated for the movement of the glutamate residue (*33*), the phenol group of the tyrosine would be expected to present a much higher activation energy if it had to move in a similar way. In fact, taking into account the size of the tyrosine side chain and the conformational constrictions imposed by the other residues of the helix, it seems highly improbable that the tyrosine side chain could adopt different conformations.

It remains to be clarified what the actual role of the tyrosine residue and the serine residue is. They may be necessary just to maintain a well-ordered chain of water molecules in the cavity, thus enabling proton transfer without being directly involved. Alternatively, one or both might be directly involved as part of the proton wire itself, where the OH groups would function just like water molecules in a hopping mechanism. It should be mentioned that the OH group of the tyrosine may be able to adopt two opposite conformations (Mendes, J., and Soares, C. M., unpublished data), facilitating proton transfer between Ser173 of the D-channel and the binuclear center. The two alternative orientations of the hydroxyl group may be essential for the reorientation of the proton channel elements after the delivery

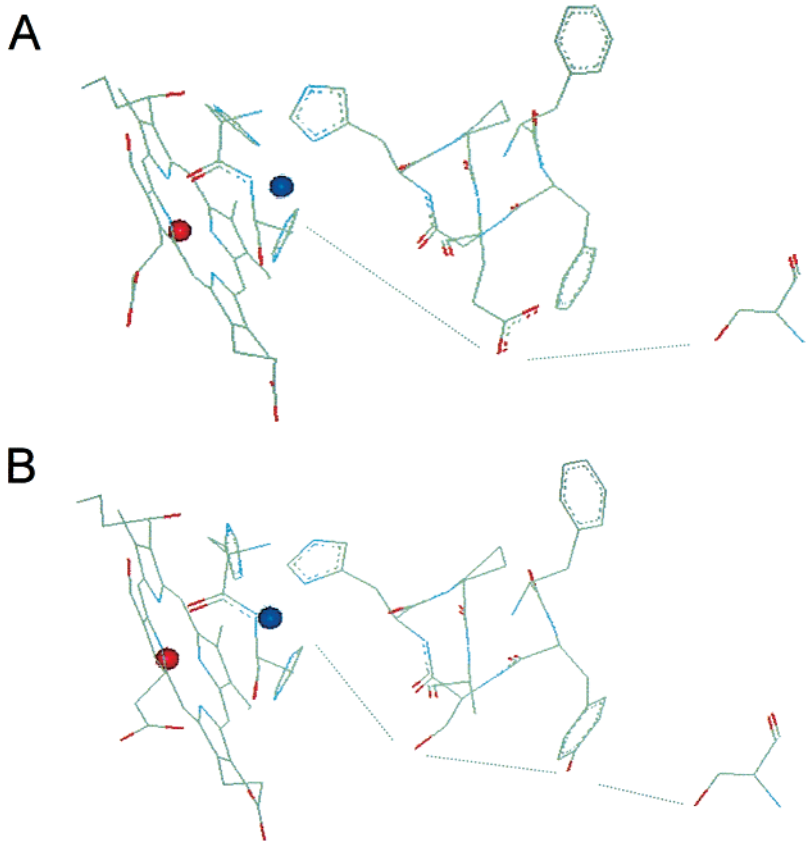

FIGURE 2: Heme a_3-Cu_B centers and amino acid residues of helix VI, FFGHPE (residues 273−278) from *P. denitrificans* aa_3 oxidase (A) (PDB code 1AR1) and FYSHPA (residues 255−260) from *R. marinus* caa_3 oxidase (B) (*27*).

of each proton, as required by the directionality of the process.

It has also been proposed that the glutamate residue is sensitive to the oxidation state of the catalytic center and is able to be deprotonated. In fact, although theoretical calculations have led to the conclusion that the glutamate remains protonated under all equilibrium conditions, the most likely situation is that it is transiently deprotonated, thus acting as an effective proton shuttle. The p*K* of a glutamate residue, in solution, is 4, but large shifts may occur, depending on the amino acid environment. For example, the essential residues Asp85, Asp86, and Glu204, present in the proton channel of bacteriorhodopsin from *Halobacterium halobium*, show increases in p*K* values of 3−4 units (*34*). A similar situation has been proposed for the glutamate in terminal oxidases, since FTIR spectra indicate that this residue is protonated at pH values as high as 9.8 (*35*). A tyrosine residue has a p*K* of 10.5 in solution, but a higher p*K* value could be expected in a hydrophobic protein environment, such as the vicinity of the glutamate residue in the D-channel. Thus, it is highly unlikely that the tyrosine residue becomes deprotonated in the case of *R. marinus* caa_3 oxidase.

Whatever the actual molecular mechanism will be, it is clear that no fully-conserved proton pathways exist in oxygen reductases of the heme-copper superfamily. While the mitochondrial-like oxidases appear to employ a carboxylate as a proton shuttle, as proposed for bacteriorhodopsin (*34*), this cannot be the case in *R. marinus*.

There are several enzymes in different species, which most probably have a similar mechanism of proton transport in the D-channel to the *R. marinus* oxidase. A protein database search already shows six other enzymes that have the similar YS motif as in *R. marinus* (Figure 3). In evolutionary terms, these oxidases have so far been found in rather phylogenetically diverse organisms: *Cyanobacteria* (*19*, *21*), *Aquificalles* (*26*), *Thermus* and *Deinococci* (20), and the *Flexibacter*, *Bacteroids*, and *Cytophaga* group (which includes

	270 285
R.marinus caa_3	FFWFYSHPAVYIMILP
A.aeolicus COXA1	IFWFYSHPVVYVQVLP
An.sp aa_3	MFWFYSHPAVYIMILP
S.sp aa_3	LFWFYSHPAVYIMILP
Sn. vulcanus aa_3	LFWFYSHPAVYLMILP
T.thermophilus caa_3	FFWFYSHPTVYVMLLP
D. radiodurans	FFWFYSHPAVYVMLLP
S.acidocaldarius aa_3	ILWFYGHPVVYYVPFP
B.japonicum cbb_3	FQWWYGHNAVGFFLTA
Ba. stearothermophilus $b(o/a)_3$	LFWYFGHPLVYFWLLP
N. pharaonis ba_3	LIWYFGHAVVYFWLMP
P. denitrificans aa_3	ILWFFGHPEVYMLILP

FIGURE 3: Alignment of subunit I amino acid sequences from oxidases containing the YS motif and only the Y residue. Sequence of the aa_3 oxidase from *P. denitrificans* was included for comparison, as an example of an enzyme containing the glutamate residue. Genus abbreviations: *R.*, *Rhodothermus*; *A.*, *Aquifex* (acc C70488); *An.*, *Anabaena* (*Anabaena* sp. strain PCC 7937, acc Z98264); *S.*, *Synechoscystis* (*Synechoscystis* sp. strain PCC 6803, acc Q06473/P73261); *Sy.*, *Synechococcus* (acc P50676); *T.*, *Thermus* (acc P98005); *D.*, *Deinococcus* (Preliminary sequence data was obtained from The Institute for Genomic Research website at http://www.tigr.org); *Su.*, *Sulfolobus* (acc P98004); *B.*, *Bradyrhizobium* (acc Q03073); *Ba.*, *Bacillus* (acc O82837); *N.*, *Natronobacterium* (acc CAA71525); and *P.*, *Paracoccus* (acc P08305). Numbering according to *P. denitrificans* sequence.

R. marinus), suggesting a parallel line of evolution. Other enzymes lacking the glutamate, such as the cbb_3 oxidases (e.g., refs *18*, *23*, *36*, and *37*) and the aa_3 oxidase from *Sulfolobus acidocaldarius* (*17*), have a tyrosine at the same sequence position, but the serine is not present. On the other hand, the bo_3 and ba_3 oxidases from *Bacillus stearothermophilus* (*38*) and *Natronobacterium pharaonis* (*24*), respectively, have a tyrosine one position earlier in the sequence. Several of these enzymes have been proposed to pump protons. The caa_3 oxidase from *Thermus thermophilus*, which has a YS motif as the *R. marinus* oxidase, shows a stoichiometry of up to $0.9H^+/e^-$ (*39*). The aa_3 oxidase from *S. acidocaldarius* (*40*) also have been shown to translocate protons. The proton pumping activity of the cbb_3 oxidases was assayed in whole cells from *P. denitrificans* (*23*, *41*) and *Rhodobacter sphaeroids* (*42*), in which the genes for other oxidases have been knocked out. The data indicate that these enzymes may also be proton pumps, but studies with purified and reconstituted proteins are still missing.

The ba_3 and bo_3 cytochrome oxidases from *T. thermophilus* and *B. stearothermophilus*, respectively, do not present any of the conserved amino acid residues of the D-channel, not even the YS motif (*20*, *38*). A stoichiometry of $0.5\ H^+/e^-$ has been determined for those oxidases (*43*, *44*), and in the case of *T. thermophilus* (*43*), it has been suggested that this pump activity is due to the functioning of a K-channel equivalent, formed by a threonine (in the place of K319, bovine numbering), a serine (in the place of T316), and a tyrosine (in the place of S255). These enzymes represent a completely different group from the mitochondrial-like oxidases, containing the glutamate residue or the YS motif.

Although the actual molecular details of the mechanism in both *R. marinus* and mitochondrial-like oxidases have yet to be fully established, a general picture emerges: the essential features are a proton channel, which allows proton conductivity through the protein from the internal to the external membrane phases, made up of a network of hydrophilic amino acid residues maintaining a chain of water molecules, and a gate element, which prevents short-circuiting. The only common elements of all heme-copper oxidases appears to be the prosthetic groups of subunit I and their ligands. Considering the prosthetic groups, only Cu_B is always conserved, since the chemical nature of the heme can vary. This further strengthens the hypothesis that the actual gating is performed by the binuclear reaction center (*45*−*47*).

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