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The caa_3 terminal oxidase of the thermohalophilic bacterium *Rhodothermus marinus*: a HiPIP:oxygen oxidoreductase lacking the key glutamate of the D-channel

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Abstract

The respiratory chain of the thermohalophilic bacterium *Rhodothermus marinus* contains a novel complex III and a high potential iron-sulfur protein (HiPIP) as the main electron shuttle (Pereira et al., Biochemistry 38 (1999) 1268–1275 and 1276–1283). In this paper, one of the terminal oxidases expressed in this bacterium is extensively characterised. It is a caa_3-type oxidase, isolated with four subunits (apparent molecular masses of 42, 19 and 15 kDa and a C-haem containing subunit of 35 kDa), which has haems of the A_s type. This oxidase is capable of using TMPD and horse heart cytochrome *c* as substrates, but has a higher turnover with HiPIP, being the first example of a HiPIP:oxygen oxidoreductase. The oxidase has unusually low reduction potentials of 260 (haem C), 255 (haem A) and 180 mV (haem A_3). Subunit I of *R. marinus* caa_3 oxidase has an overall significant homology with the subunits I of the COX type oxidases, namely the metal binding sites and most residues considered to be functionally important for proton uptake and pumping (K- and D-channels). However, a major difference is present: the putative essential glutamate (E278 in *Paraccocus denitrificans*) of the D-channel is missing in the *R. marinus* oxidase. Homology modelling of the *R. marinus* oxidase shows that the phenol group of a tyrosine residue may occupy a similar spatial position as the glutamate carboxyl, in relation to the binuclear centre. Moreover, sequence comparisons reveal that several enzymes lacking that glutamate have a conserved substitution pattern in helix VI: -YSHPXV- instead of -XGHPEV-. These observations are discussed in terms of the mechanisms for proton uptake and it is suggested that, in these enzymes, tyrosine may play the role of the glutamate in the proton channel. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Terminal oxidase; High potential iron-sulfur protein (HiPIP); *Rhodothermus marinus*; Thermophile; Proton pathway

1. Introduction

Rhodothermus marinus is a thermohalophilic Gram-negative bacterium, isolated from shallow marine hot springs off the coast of Iceland (strain DSM 4253) [1] and from hydrothermal areas on the beach Praia da Ribeira Quente (PRQ) of the island of São Miguel, Azores (strain PRQ) [2]. It is a strict aerobe and halophile, which grows optimally at 65°C and at

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1–2% sodium chloride, classified as a new genus of the rather heterogeneous group of *Flexibacter*, *Bacteroids* and *Cytophaga* species (FBC-group) [3].

A preliminary analysis of its membrane-bound electron transport chain has suggested some unique characteristics [4]. Most remarkable is the finding of the first membrane-bound HiPIP[1] [4] which was shown to be the electron carrier between this bacterium unique complex III and the caa_3 terminal oxidase [5,6].

Cytochrome *c*:oxygen oxidoreductase (EC 1.9.3.1) is the terminal electron transfer complex of respiratory chains, using O_2 as terminal electron acceptor. This complex catalyses the transfer of electrons from cytochrome *c* to molecular oxygen, producing water with consumption of protons and simultaneous translocation of additional protons across the membrane. Almost all terminal oxidases belong to the same family of proteins, the Haem-Copper oxidases Superfamily [7–9]. Exceptions are the *bd* type oxidase described to be expressed in *Escherichia coli* under low oxygen tension [10] and which is apparently the only oxidase present in *Haemophilus influenzae* [11], the so called alternative oxidase in plants [12,13] and the newly described oxidase from the sulphate reducer *Desulfovibrio gigas* [14, 15].

The members of the haem-copper superfamily (including the mitochondrial enzyme) have their larger subunit, subunit I, very similar. All have as their characteristic feature (what gives this family name) the unique bimetallic centre consisting of a high spin haem and a copper ion, Cu_B. Another haem, low-spin, is also present in all subunits I. The homology of terminal oxidases can also be extended to the other fundamental subunit for the catalysis, subunit II, which in the case of cytochrome *c* oxidases contains the binuclear copper centre, Cu_A, while in the case of quinol oxidases this centre is absent. Exceptions to this subunit II homology are the aa_3 quinol oxidases from *Sulfolobus acidocaldarius* (SOXABCD) [16] and *Acidianus ambivalens* [17] and all cbb_3 oxidases [18–23]. Subunit II, in some cases, can also present a longer C-terminus with a binding site for a C type haem.

A great impulse on the functional aspects of terminal oxidases was achieved after the structures of the aa_3 oxidases from bovine heart mitochondria [24] and from *Paracoccus denitrificans* [25,26] were determined to atomic resolution. Both structures show that the active site is buried within the protein and near the opposite side of the membrane from where the pumped protons and those necessary for the water formation are taken up. To facilitate the protons' access to the reaction centre three pathways were proposed based on the crystal structures and on site directed mutagenesis studies. These pathways are named D-, K- and H-channel according to those conserved residues in each channel [27–31]. The K-channel besides the conserved lysine has a tyrosine. All mutational substitutions of those residues result in the elimination of oxidase activity ([27–30] and references therein). D91 and E278 (*P. denitrificans* numbering) are the conserved residues of the D-channel. Site directed mutations of the glutamate block all the reactions occurring in the oxygen cycle after the formation of the P-state ([27–30] and references therein). It is proposed that this channel is necessary to provide protons for the complete reduction of oxygen and protons to be translocated ([28] and references therein, [32]). However, the essential residues of the D-channel are not conserved in all oxidases [16–18,21,33,34], suggesting either alternative proton pathways or functional substitutions. The H-channel (first named E-channel) was proposed to be essential for the bovine enzyme [35], although there are no mutagenesis studies that can corroborate this assumption. With different residues this putative H-channel is also present in the bacterial enzymes; however site-directed mutations of the residues of the channel had no effect on the oxidase activity ([27] and references therein).

As *R. marinus* has an HiPIP as the main electron carrier in the respiratory chain, we extensively studied the terminal oxidases from this bacterium. In this paper, we report the characterisation of one of the terminal oxidases of *R. marinus*, a caa_3 oxidase which acts as a HiPIP oxidase. The amino acid sequence of subunit I shows important differences in the residues considered to be essential for proton uptake.

2. Materials and methods

2.1. Protein purification

Bacterial growth, membrane preparation and sol-

ubilisation were done as described in Pereira et al. [6]. The first part of the purification procedure was adapted from Warne et al. [36]. All chromatographic steps were done on Pharmacia HiLoad or LKB-HPLC systems, at 4°C. The purification steps were monitored by activity measurements (TMPD oxidase), visible and EPR spectroscopies as well as SDS-PAGE. The detergent solubilised extract was applied to a Fast Flow DEAE column, using as buffer 20 mM Tris-HCl pH 8, 0.1% DM and eluted in a linear gradient of 0–50% 1 M NaCl. The fraction containing TMPD oxidase activity, which also contains virtually all haem centres, as determined by visible spectroscopy, was then applied to a Chelating Sepharose Fast Flow column saturated with Cu^{2+} and equilibrated with 20 mM Tris-HCl pH 8, 0.1% DM. Two linear gradients of 0–10% and 10–100% 125 mM imidazole were applied. The *caa*$_3$ oxidase eluted in the second gradient was applied to a Q-Sepharose column and eluted in 20 mM Tris-HCl pH 8, 0.1% DM with a linear gradient of 0–50% 1 M NaCl. After this step, the fraction containing the *caa*$_3$-type oxidase was pure as judged by gel electrophoresis and visible spectroscopy. To check for possible proteolytic degradation, a purification was also performed by the same procedure, but using in all buffers protease inhibitors (sodium EDTA and phenylmethylsulfonylfluoride (PMSF)); SDS-PAGE of the *caa*$_3$ fractions after each step showed a constant electrophoretic pattern, identical to that of the enzyme purified in the absence of these inhibitors.

2.2. Haem extraction and HPLC analysis

Non-covalently bound haems were extracted according to [37]. Haem composition was analysed on a System Gold, Beckman chromatograph with a Deltapak C18 (3.9×150 mm, Waters) reverse phase HPLC column. Standard haems were obtained from myoglobin and membrane extracts of *E. coli* (B and O haems), *A. ambivalens* (A_s haems) and *P. denitrificans* (A haems).

2.3. Protein and haem determination

Protein concentrations were determined using the modified MicroBiuret method for membrane proteins [38]. Haem contents were determined by pyridine haemochrome using molar absorptivities of $\varepsilon_{r-o,550-535} = 23.97\ mM^{-1}\ cm^{-1}$ for haem C, and $\varepsilon_{r-o,peak-620} = 25.02\ mM^{-1}\ cm^{-1}$ for haem A [39]. Although *R. marinus* is shown to have haems of the A_s type, the spectral properties of cytochromes containing haems A and A_s were reported to be identical [37] and so the last value for molar absorptivity is used.

2.4. Metal determination

Iron and copper were analysed by atomic absorption on a graphite chamber, at the Laboratório de Análises, Instituto Superior Técnico, Lisbon.

2.5. Electrophoresis

Tricine-SDS-PAGE was carried out as described by Schägger and von Jagow [40] with 10% T, 3% C. Haem staining procedure followed Goodhew et al. [41].

2.6. Spectroscopic techniques

Electronic spectra were obtained on a Beckman DU-70 or on an OLIS DW2 spectrophotometers, at room and liquid nitrogen temperatures. Extinction coefficients of $\varepsilon_{592-605} = 3.5\ mM^{-1}\ cm^{-1}$ (cytochrome a_3, α-band), and $\varepsilon_{429-445} = 91\ mM^{-1}\ cm^{-1}$ (cytochrome a_3, Soret-band) [42] were used for CO difference spectra. EPR spectra were measured and analysed as in Pereira et al. [6].

2.7. Catalytic activity assays

TMPD oxidase activity was measured by monitoring the change in absorbance of TMPD at 560 nm at 65°C ($\varepsilon_{560} = 12\,100\ M^{-1}\ cm^{-1}$). Cytochrome *c* oxidase activity was determined following the change in absorbance of cytochrome *c* at 550 nm, at 65°C, ($\varepsilon_{550} = 28\,000\ M^{-1}\ cm^{-1}$). Horse heart cytochrome *c* was pre-reduced by the addition of solid sodium dithionite followed by passage over a Sephadex G-10 size exclusion column. Oxygen consumption was measured polarographically at 35 and at 40°C, with a Clark-type oxygen electrode, YSI Model 5300, Yellow Springs. All assays were carried out using 20 mM Tris-HCl pH 8, 0.1% DM as buffer.

2.8. Redox titrations

Anaerobic potentiometric titrations were performed and analysed as in Pereira et al. [6].

2.9. Homology modelling

The structure of subunit I of the terminal oxidase from *R. marinus* was modelled based on the structures of subunits I of cytochrome *c* oxidase from *P. denitrificans* [26] (PDB code 1AR1; resolution 2.7 Å) and cytochrome *c* oxidase from bovine heart [35] (PDB code1OCC; resolution 2.8 Å). The sequences and structures of these subunits are very conserved. The sequence alignment with the sequence of *R. marinus* oxidase of unknown structure is also good. The program Modeller (release 3) [43] was used to derive the structure of this terminal oxidase. This program models protein 3D structure by satisfaction of spatial restraints, which are derived from the 3D structure of the known proteins and alignments of their sequences with the sequence of unknown structure. Therefore, the quality of the final model will depend on the sequence and structural homology between the known and unknown proteins and on the correct alignment between these. An initial alignment between the sequences from *P. denitrificans* and bovine heart was performed, and this alignment, together with the X-ray structures, was used by Modeller to derive a 3D alignment and a new sequence alignment based on this superposition. This alignment (Fig. 1) was then used to align the sequence of *R. marinus*. The alignment has a rather good identity (38.4 and 39.7% with the bovine and *P. denitrificans* enzymes, respectively). However, the last 71 residues of the sequence from *R. marinus* did not show any homology with the other two structures. Therefore these residues were excluded from the sequence alignment and from the final model.

Several models were derived by Modeller. We selected the one with the lowest value of the objective function.

2.10. Side-chain modelling

Modeller is able to position side-chains in the homology derived models. However, despite the quality of the main-chain coordinates that can be obtained, side-chain conformations can be less precise (J. Mendes et al., unpublished results). Therefore, we chose to use another method to position side-chains in the final model. To predict the side-chain conformations we used the Self Consistent Mean Field Theory (SCMFT) method [44], with some improvements in the optimisation procedure and interaction energy function [45]. Interaction energies used in the SCMFT procedure were calculated using molecular flexibility [46], a method that improves predictions substantially. Since we were more interested in the zone close to the reaction centre, we selected residues whose Cα atoms were at a distance from the copper atom lower than or equal to 25 Å. All residues within this radius (around 230 residues) were selected for prediction, while all others were kept rigid in the conformations predicted by Modeller. The final model was refined using restrained minimisation with the GROMOS 87 package [47] and the force field with polar and aromatic hydrogen atoms [48].

3. Results

3.1. Isolation and biochemical characterisation of caa_3-type oxidase

A caa_3-type oxidase was isolated from *R. marinus*. The purified enzyme shows clearly three bands in tricine-SDS-PAG, corresponding to subunits with apparent molecular masses of 42, 35 and 19 kDa (Fig. 2). A minor intensity band is observed with an apparent mass of 15 kDa (not shown). Haem staining reveals the presence of a C-type haem centre in the 35 kDa subunit. The gene cluster encoding this oxidase has been sequenced (our own unpublished data), showing the presence of four subunits with deduced molecular masses of 62, 36, 26 and 13 kDa and of a haem C binding motif in the 36 kDa subunit. As observed for other oxidases, subunits I and III have anomalous migration, which has been attributed to a high hydrophobicity [49,50]. Exactly the same electrophoretic pattern was obtained with the enzyme sample purified in the presence of protease inhibitors, indicating the anomalous apparent low molecular mass of subunit I is not due to proteolytic degradation. The assignment of the bands at 42 and 19 kDa to subunits I and III was confirmed

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1OCC        M-F-INRWLFSTNHKDIGTLYLLFGAWAGMVGTALS----LLIRAELGQP
1AR1        -GFFT-RWFMSTNHKDIGILYLFTAGIVGLISVCFT----VYMRMELQHP
MOD         MATQT-VAAHKTAQPEIHYLNHAKGLKSWLLTLDHKRIGILYLISVVFFF
                      .* : :*  *    .    ::    .    : :   :

1OCC        GT-LL----------------GDDQIYNVVVTAHAFVMIFFMVMPIMIGG
1AR1        GVQYMCLEGARLIADASAECTPNGHLWNVMITYHGVLMMFFVVIPALFGG
MOD         IVGGILAVLIRLELLQPGQTLMTADTYNHIFTLHGAIMIFLFLIPAVPAV
             .  :                      . :*:.* *.  :*:*:.::* : .

1OCC        FGNWLVPLMIGAPDMAFPRMNNMSFWLLPPSFLLLLASSMVE----A-GA
1AR1        FGNYFMPLHIGAPDMAFPRLNNLSYWMYVCGVALGVASLLAPGGNDQMGS
MOD         LGNFALPIMIGAKDVAFPRLNLASWYIFWLGALTMLVGIVTS------GL
            :**: :*: *** *:****:*  *::: .    :.. :.       *

1OCC        GTGWTVYPPLAGNLAHAGASVDLTIFSLHLAGVSSILGAINFITTIINMK
1AR1        GVGWVLYPPLST-T-EAGYSMDLAIFAVHVSGASSILGAINIITTFLNMR
MOD         DTGWTFYTPYSTMTSSGVTWVALGVFIL---GFSSILTGLNFIVTVHKMR
            ..**..*.* :   . .   : * :* :  .* **** .:*:*.*. :*:

1OCC        PPAMSQYQTPLFVWSVMITAVLLLLSLPVLAAGITMLLTDRNLNTTFFDP
1AR1        APGMTLFKVPLFAWSVFITAWLILLSLPVLAGAITMLLMDRNFGTQFFDP
MOD         APGLTWSRLPLFVWGLYATSIVQILATPVLGITVLLLALERIMKIGIFDP
            .*.:: ' : ***.*.:  *: : :*: ***.  : :*  :* :   :***

1OCC        AGGGDPILYQHLFWFFGHPEVYILILPGFGMISHIVTYYSGKKEPFGYMG
1AR1        AGGGDPVLYQHILWFFGHPEVYIIILPGFGIISHVISTFAKK-PIFGYLP
MOD         ALGGDPILFQHFFWFYSHPAVYIMILPAFGVISELIGTFSRK-GIFGYKF
            * ****:*:**:.**:.**.***:***.**:**.::  :: *   ***

1OCC        MVWAMMSIGFLGFIVWAHHMFTVGMDVDTRAYFTSATMIIAIPTGVKVFS
1AR1        MVLAMAAIGILGFVVWAHHMYTAGMSLTQQAYFMLATMTIAVPTGIKVFS
MOD         VALSSVAIAFLGFLVWGHHMFVSGQSATAATVFSLLTFLIGVPTGVKVLN
            :. :  :*.:***:**.***:. * .    : *   *: *.:***:**:.

1OCC        WLATLHGGNIKWSPAMMWALGFIFLFTVGGLTGIVLANSSLDIVLHDTYY
1AR1        WIATMWGGSIEFKTPMLWAFGFLFLFTVGGVTGVVLSQAPLDRVYHDTYY
MOD         WVASLYRGSIWLRTPLLYALAFLFVFPIGGFTGIALGTLGLDVPLHDTYF
            *:*::  *.*   .:::*:.*:*:*.:*.**:.*.   **   ****:

1OCC        VVAHFHYVLSMGAVFAIMGGFVHWFPLFSGYTLNDTWAKIHFAIMFVGVN
1AR1        VVAHFHYVMSLGAVFGIFAGVYYWIGKMSGRQYPEWAGQLHFWMMFIGSN
MOD         VVAHFHYVMVSGGLLAFLGGLHYWWPKMFGRLYNEKLAQIAALLIFVGFN
            ********:  *.::.::.*. :*   :*    :  .::    ::*:* *

1OCC        MTFFPQHFLGLSGMPRRYSDYPDAYTMWNTISSMGSFISLTAVMLMVFII
1AR1        LIFFPQHFLGRQGMPRRYIDYPVEFAYWNNISSIGAYISFASFLFFIGIV
MOD         VTFFPQFILGTQGMPRRYFDYVPEFTTLHQLSTVGSWILGLGLLLVAVCL
            : ****.:** .****** **   ::: :*::*::*     ..::.   :

1OCC        WEAFASK-REV-LT-V--DLTT--TNLEWLNGCPPPYHTFEEPTYVNLK
1AR1        FYTLFA-GKRVNV-PNYWNE--HADTLEWTLPSPPPEHTFET-------
MOD         LHSLL-----------------------------------------
              ::
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Fig. 1. Sequence alignment of subunits I used as an input to Modeller. Sequences are: 1OCC, cytochrome *c* oxidase from bovine heart (PDB code); 1AR1, cytochrome *c* oxidase from *P. denitrificans* (PDB code); and MOD, *R. marinus* caa_3 oxidase.

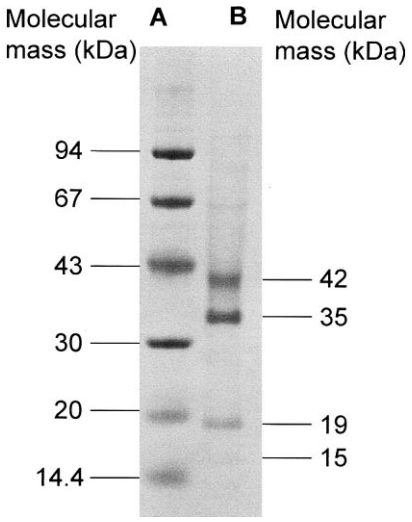

Fig. 2. Tricine-SDS-PAG. (A) Molecular mass standards (Pharmacia-Biotech, low molecular weight calibration kit). (B) Subunit composition of the purified *R. marinus* cytochrome caa_3 oxidase.

by amino acid analysis after electroblotting of these bands (data not shown).

The pyridine haemochrome difference spectrum shows α-peaks at 550 and 587 nm indicating, respectively, the presence of C- and A-type haems, in a 1:2 stoichiometry. These haems are of the A_s type as deduced by HPLC analysis. Atomic absorption metal analysis gave an iron to copper ratio of 1.2:1; this value, within the experimental error, is consistent with the presence of three copper ions plus three haems, being fully supported by the visible and EPR spectroscopic data (see below).

The *R. marinus* caa_3 oxidase is able to use TMPD and horse heart cytochrome *c* as substrates with molecular activities (mol substrate/mol haem A per min) of 1100 and 182 min^{-1}, respectively, at 65°C. TMPD oxidase activity is maximal at 70°C (1155 min^{-1}), increasing ca. 4 times from 35 to 70°C. Activity assays carried out polarographically at 40°C indicate an oxidation rate of 340 mol O_2/mol haem A per min, using TMPD as substrate and 74 mol O_2/mol haem A per min, using horse heart cytochrome *c* as substrate. The enzyme catalyses the complete reduction of O_2 to water, as polarographic studies show

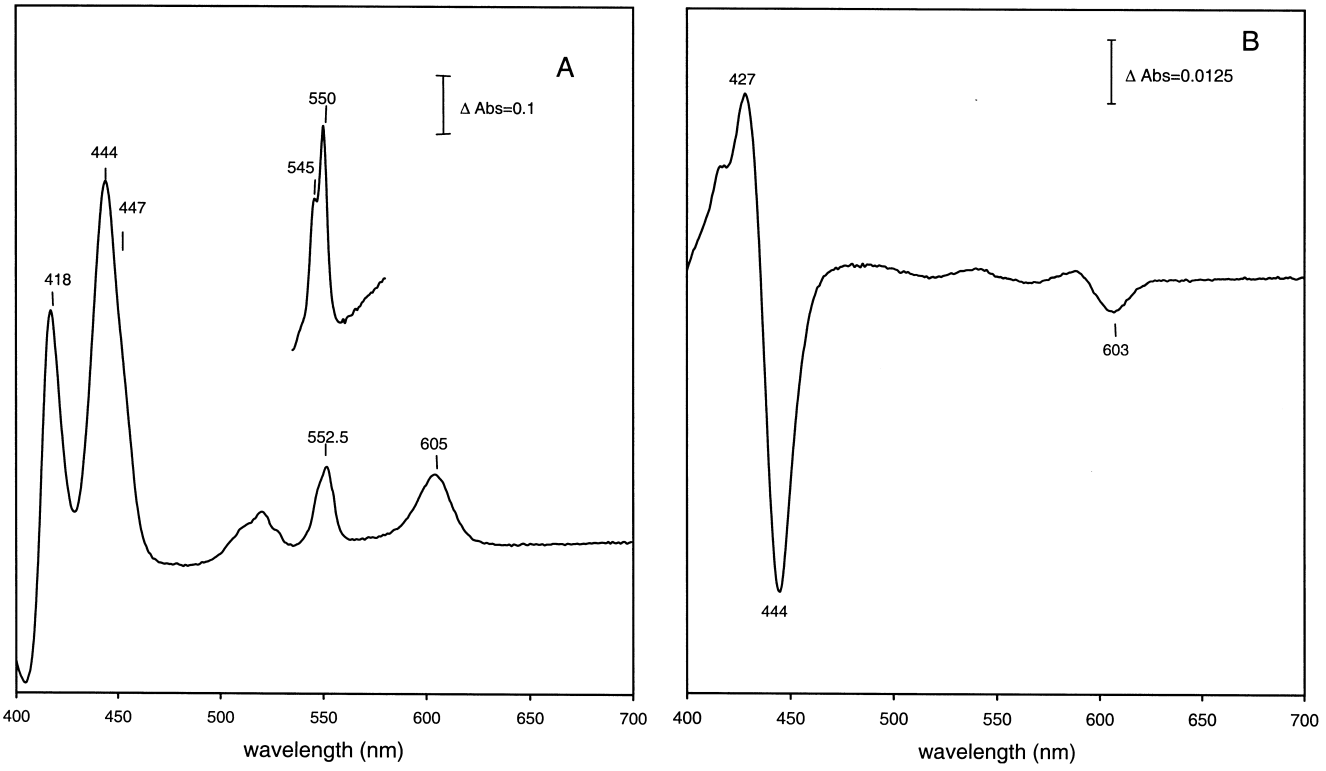

Fig. 3. (A) Dithionite reduced minus oxidised visible spectra of *R. marinus* caa_3 oxidase, at room and at liquid nitrogen (insert) temperatures. (B) Dithionite-reduced+CO minus dithionite-reduced visible spectra of *R. marinus* caa_3 oxidase, at room temperature.

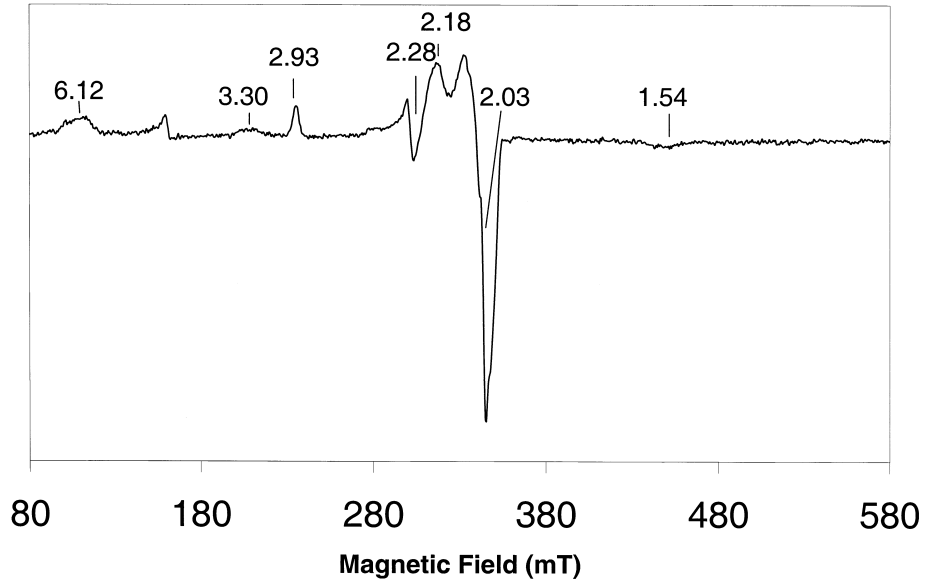

Fig. 4. EPR spectra of *R. marinus caa*$_3$ oxidase at 10 K. Microwave frequency, 9.64 GHz; microwave power, 0.74 mW; modulation amplitude, 0.9 mT.

that O_2 consumption is the same in the presence and in the absence of catalase.

3.2. Spectroscopic and redox characterisation

The redox visible spectrum of the purified enzyme (Fig. 3A) presents α- and Soret bands characteristic of cytochromes *a* and *c* (Table 1) and is similar to redox spectra of *caa*$_3$-type oxidases [49,51–58]. The CO difference spectrum of the *caa*$_3$-type oxidase (Fig. 3B, Table 1) shows features characteristic of cytochrome a_3. A stoichiometry of one cytochrome *a* per cytochrome a_3 was obtained. Interestingly, the α-band of cytochrome *c* is split, even at room temperature; at liquid nitrogen (Fig. 3A, insertion), a split of 5 nm (167 cm^{-1}) is observed, with the high-energy peak at 545 nm (18 348 cm^{-1}), and a lower intensity transition at 550 nm (18 181 cm^{-1}). This split is well within the range found for ferrous porphyrins [57] and has been observed in several haem proteins, including the cytochrome *c* oxidase from *T. thermophilus* [51]. Recently it was suggested that it arises from an *xy* asymmetry at the haem centre, imposed by the protein polypeptide chain [57].

The EPR spectrum (Fig. 4) of the isolated oxidase indicates the presence of a very small amount of high-spin ferric haem centre, with g_{max} at ~6 and of two low-spin haem centres with g_{max} at 3.30 and $g_{max,med,min}$ = 2.93, 2.28, 1.54 (Table 1). Theoretical simulations yield a 1:1 ratio for each low-spin haem species. By analogy to the *B. subtilis* [54] and *T. thermophilus* [51] oxidases, these resonances are assigned to the low-spin haem centres C and A, re-

Table 1
Visible and EPR data and respective reduction potentials (pH 7) for each cytochrome component of *R. marinus caa*$_3$ oxidase

Soret, nm	α, nm	CO spectra, troughs (peak), nm	EPR $g_{max,med,min}$	E', mV
418 (*c*)	552.5 (*c*)		3.30, (2.05, 1.00)[b] (*c*)	260 (*c*)
444 (a_3)	605 (a+a_3)	444 (428), 603	2.93, 2.28, 1.54 (*a*)	180 (a_3)
447[a] (*a*)				255 (*a*)

Cytochrome type is indicated between brackets.
[a]Shoulder.
[b]Values between brackets were used for the simulations.

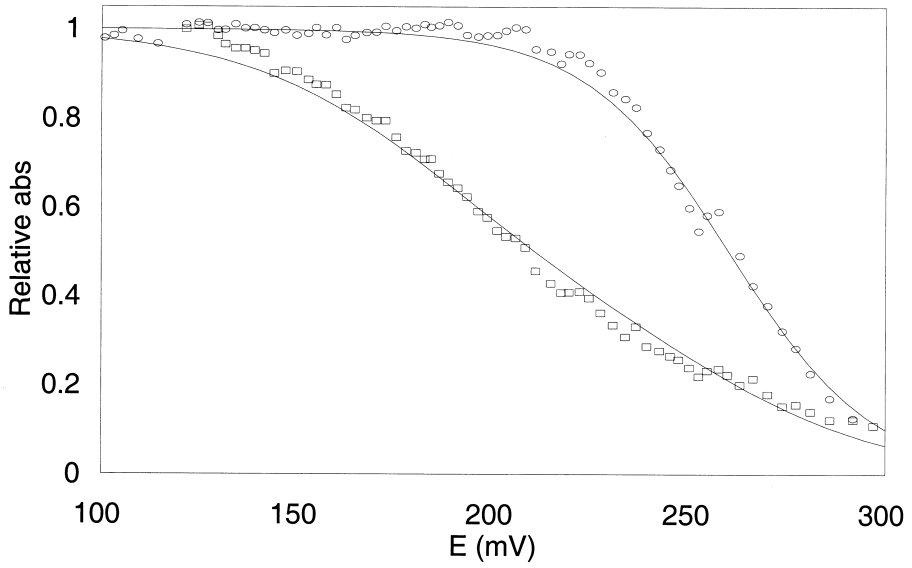

Fig. 5. *R. marinus caa*$_3$ oxidase visible titration. The change in absorbance at 418 nm (cytochrome *c*, circles) is described by a single Nernst equation with $n=1$ and $E_0=260$ mV. The change in absorbance at 444 nm (cytochrome *aa*$_3$, squares) is described by two Nernst equations with $n=1$ and $E_0=180$ (60%) and $E_0=255$ (40%).

spectively. In the $g\sim2$ region, resonances typical of the binuclear Cu_A centre are observed with $g_{max}=2.18$, $g_{med}=2.03$ and $g_{min}=2.00$, which integrate in a 1:1 stoichiometry with each of the low spin haem species. Altogether, the pyridine haemochrome, CO-redox spectra, metal analysis and EPR quantification confirm the presence of three haem centres and three copper ions per molecule of enzyme.

A visible redox titration of the *caa*$_3$-type oxidase (Fig. 5) was carried out at pH 7, to obtain a general description of the reductive redox profile of the enzyme. The Soret band due to cytochrome *aa*$_3$ is asymmetric, with a maximum at 444 nm and a shoulder at 447 nm (Fig. 3A). By analogy with the mitochondrial oxidase, these features are assigned to cytochromes *a*$_3$ and *a*, respectively [58]. The data obtained following the absorbance changes at 444 nm can be described by two Nernst equations, with midpoint redox potentials of 180 and 255 mV, and relative contributions of 0.6 and 0.4, respectively. Since cytochrome *a*$_3$ has its maximum at 444 nm and a slightly higher absorptivity than cytochrome *a* at this wavelength, the first transition is attributed to cytochrome *a*$_3$ and the second to cytochrome *a* (Table 1). In spite of the lower spectral resolution at the α region, the data obtained at 605 nm can be described by the same Nernst equations, but with different relative proportions, i.e. 0.3 for the lowest potential and 0.7 for the highest. This is in perfect agreement with the relative absorptivity coefficients assigned to these cytochromes at that wavelength [58]. For cytochrome *c*, the data obtained for both the Soret and α-bands show a single redox transition with a midpoint redox potential of 260 mV. Similar reduction potentials associated with the cytochrome *aa*$_3$ were observed in the titration of the membrane extract, following the shoulder at 444 nm (data not shown).

3.3. Analysis of the modelled structure

The gene cluster encoding *R. marinus caa*$_3$ oxidase was sequenced (our unpublished data). The deduced

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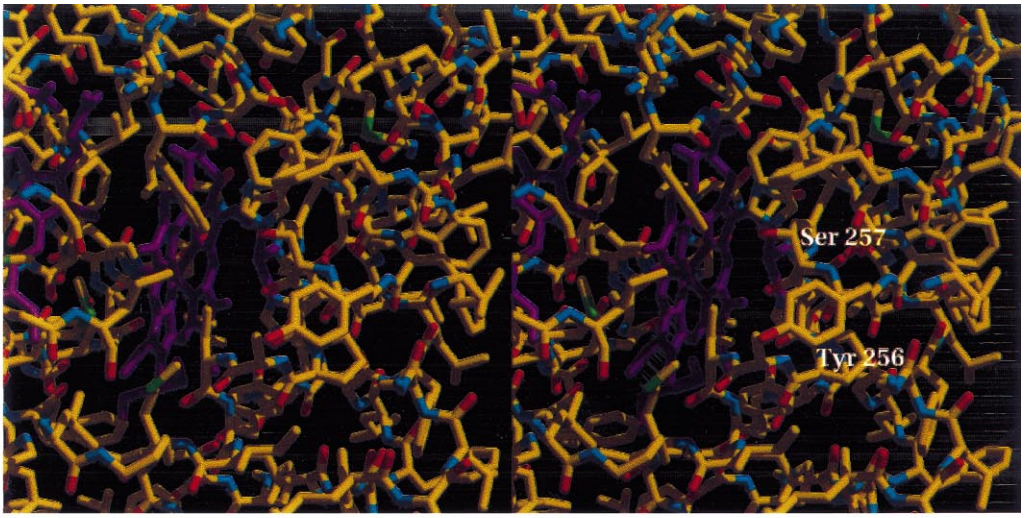
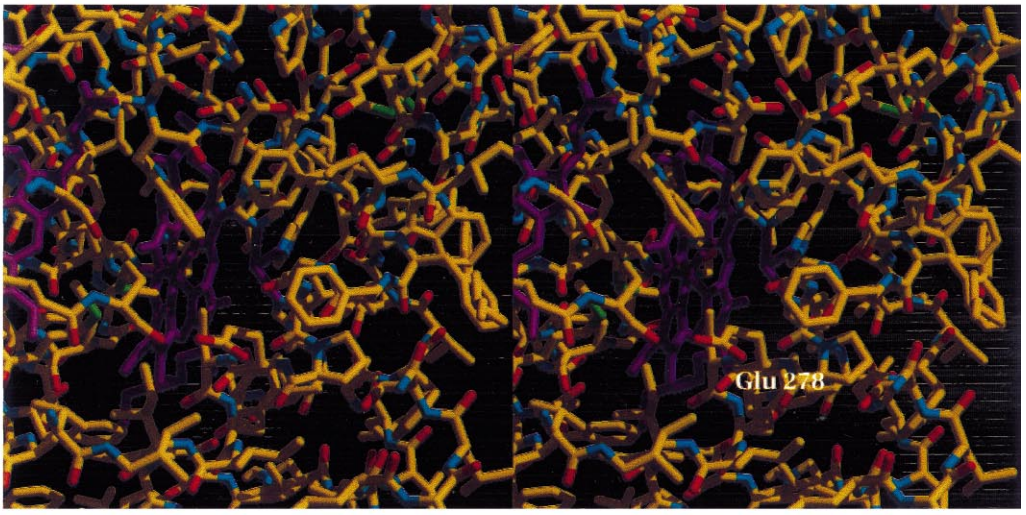
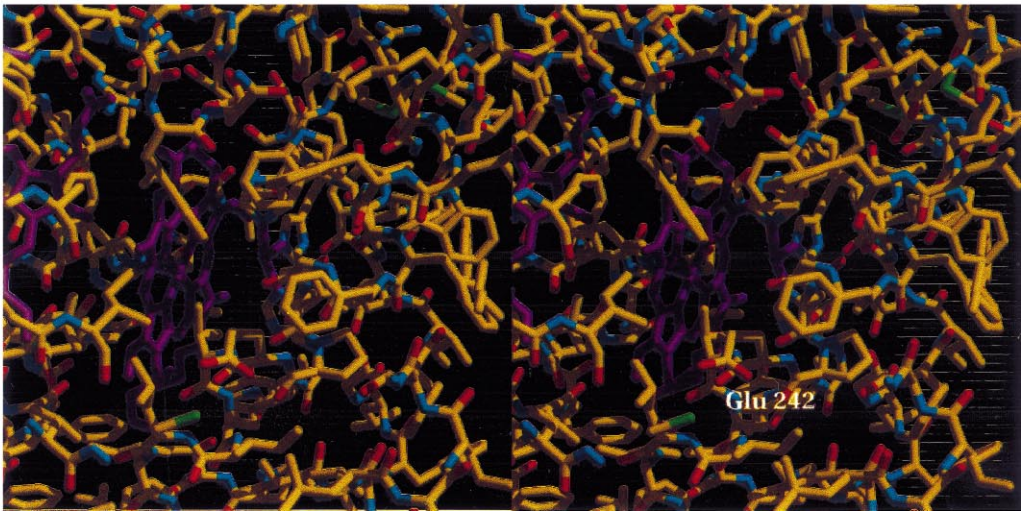
Fig. 6. Stereo view (produced using XtalView 3.2 [84] and Raster3D 2.3 [85]) of the zone near the copper centre in the different terminal oxidases. The heme groups and the copper atom are coloured in magenta. (A) *caa*$_3$ terminal oxidase from *Rhodothermus marinus*. The positions of Tyr-256 and Ser-257 are labelled. (B) Cytochrome *c* oxidase from *Paracoccus denitrificans*. The position of Glu-278 is labelled. (C) Cytochrome *c* oxidase from bovine heart. The position of Glu-242 is labelled.



[A]



[B]



[C]

amino acid sequence of subunit I shows a significant homology with the equivalent subunits of the haem-copper oxidases superfamily. All the metal binding residues are conserved, namely the six histidines necessary for haem and Cu_B binding, as well as the tyrosine (Y280, *P. denitrificans* numbering), which is covalently bound to a Cu_B binding histidine. With one major exception, the postulated important residues of the K- and D-channels are also conserved. The exception is the absence of the glutamate residue (E278 in *P. denitrificans*). Sequence analysis and the modelled structure of the protein show the absence of any acidic residues in the place or vicinity of the missing glutamate. In this vicinity in the modelled structure (Fig. 6) it is noteworthy the presence of a tyrosine residue, Tyr-256 (a Phe in *P. denitrifcans* and in the bovine structures) and a serine residue, Ser-257 (a glycine in both *P. denitrifcans* and bovine structures). The tyrosine residue is the only titratable residue in the zone around the equivalent position of the glutamate. The phenol group of this tyrosine residue is indeed very close to the spatial position occupied by the carboxyl group of the glutamate in the *P. denitrificans* and bovine aa_3 oxidases (Fig. 6). Moreover, analysis of the sequence alignment (Fig. 7) suggests that the presence of these residues is a typical pattern common to several oxidases lacking the glutamate residue: the oxidases from cyanobacteria (*Synechoscystis* sp. strain PCC 6803 [33], *Synechococus vulcanus* [34] and *Anabaena* sp. strain PCC 7937 (accession number Z98264)), *Thermus thermophilus* caa_3 oxidase [59] and one of *Aquifex aeolicus* oxidases [60].

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                    270                 288
R.marinus_caa3      FFWFYSHPAVYIMILPAFG
A.aeolicus_COXA1    IFWFYSHPVVYVQVLPVFG
An.sp_aa3           MFWFYSHPAVYIMILPFFG
S.sp_aa3            LFWFYSHPAVYIMILPFFG
T.thermophilus_caa3 FFWFYSHPTVYVMLLPYLG
B.subtillis_ caa3   LFWIFGHPEVYILILPAFG
P.denitrificans_aa3 ILWFFGHPEVYMLILPGFG
E.coli_bo3          LIWAWGHPEVYILILPVFG
bovine_aa3          LFWFFGHPEVYILILPGFG
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Fig. 7. Alignment of *R. marinus* caa_3 oxidase subunit I with other subunit I from higher homologous (first four sequences) and from some representative (four last sequences) oxidases. The YS motive common to the first five oxidases and possibly involved in the proton pathway is shaded. In the last four sequences, the key glutamate is shaded. *R.*, *Rhodothermus*; *A.*, *Aquifex* (accession number C70488); *An.*, *Anabaena* (*Anabaena* sp. strain PCC 7937, accession number Z98264); *S.*, *Synechoscystis* (*Synechoscystis* sp. strain PCC 6803, accession number Q06473/P73261); *T.*, *Thermus* (accession number P98005); *B.*, *Bacillus* (accession number P24010); *P.*, *Paracoccus* (accession number P08305); *E.*, *Escherichia* (accession number P18400); bovine (accession number P00396). Numbering according to *P. denitrificans* sequence.

4. Discussion

R. marinus respiratory chain contains complexes I and II analogues [5] and a novel cytochrom*e bc* complex: a multihaemic menadiol:HiPIP oxidoreductase [6]. In this paper, we characterised one of the two distinct terminal oxidases known to be expressed in this bacterium, *a* caa_3-oxidase functioning as a HiPIP:oxygen oxidoreductase. As the other oxidase from *R. marinus* is a cbb_3 type oxidase (our unpublished data), both oxidases expressed by this bacterium contain an extra redox site. It is not clear as yet whether this feature is physiologically relevant in relation to oxygen utilisation or to higher energetic needs, i.e. if it provides an increased efficiency of the terminal oxidases. *R. marinus* thrives in microaerophilic environments [61], and hence efficient oxygen reductases may be needed to support its bioenergetics. However, while the cbb_3 type oxidases have been reported to have a higher oxygen affinity than the other cytochrome or quinol oxidases, caa_3 oxidases do not appear to differ significantly from these enzymes [62].

R. marinus caa_3 oxidase shows at the same time quite distinct properties as well as several features common to the large superfamily of haem-copper oxidases. It is isolated as a four subunits complex as most bacterial oxidases and, interestingly, contains haems of the A_s type, which so far have been reported to be present only in *T. thermophilus* and in archaeal species [37]. These haems have a hydroxyethylgeranylgeranyl group instead of a farnesyl as its side chain in position 2 [37]. The enzyme has clearly a higher activity using HiPIP as the electron donor, in comparison with horse cytochrome *c* or *R. marinus* membrane bound cytochrome *c*, with which the enzyme has a turnover ca four times and ten times lower, respectively, than with the HiPIP [5].

R. marinus oxidase has equilibrium reduction potentials lower than those generally determined for aerobic respiratory chains, namely for the terminal oxidases (e.g. [63]) and in particular for the caa_3

oxidase from *T. thermophilus* [51]. Although the high-spin haem, at the catalytic site, has an equilibrium redox potential lower than cytochrome *a*, as observed for the mitochondrial enzyme [63,64], the cytochrome *c* has an equilibrium potential identical, within experimental error, to that of cytochrome *a* and, most striking, to that of its electron donor, HiPIP [4,5]. It should be stressed that similar reduction potentials were obtained by titrating the full membrane extract (our unpublished data). These data indicate a considerably lower driving force for the electron transfer reactions from HiPIP to the caa_3 oxidase, via the haem C. However, the reduction potentials were determined under equilibrium, and only at one pH value; preliminary data on *R. marinus* membrane extracts show that several haem centres have a strong pH dependence of their reduction potential (our unpublished data). Moreover, functionally, the actual reduction potential of each centre may be strongly altered from the equilibrium value. For example, for the terminal oxidases, not only homotropic and heterotropic interactions have been shown to be important (e.g. [63–65]), but also the reduction potential of the active site, upon binding of the substrate and during the catalytic cycle becomes undetermined. Furthermore, for the other centres, functioning only as electron transfer agents, a strong deviation from the equilibrium behaviour may occur, depending on the redox state of the other centres, as well demonstrated for the tetrahaemic cytochromes from the photosynthetic reaction centre or from sulfate reducing bacteria [66,67] and as proposed to be a key feature of the coupling of proton and electron transfer in terminal oxidases [28,29,68].

The amino acid sequence of *R. marinus* subunit I shows the absence of the glutamate residue (E278 in *P. denitrificans*), which is proposed, based on structural and site directed mutagenesis analysis, to be a key element of the D-channel. The D-channel delivers protons to complete the reduction of O_2 to water after the formation of the peroxy state, as well as protons to be translocated ([27–30] and references therein). *R. marinus* caa_3 oxidase works as a true terminal oxidase, i.e. performs the complete reduction of molecular oxygen to water. Thus in this oxidase the D-channel has to be operative, as well. The homology derived model shows the presence of the phenol group of a tyrosine residue (Y256) in the spatial location of the carboxyl group of the *P. denitrificans* aa_3 oxidase glutamate (E278) (Fig. 6). Although the pK_a of tyrosine in solution is much higher than that of glutamate (pK_a of 10.1, versus 4.3), this pK_a can change within the protein core [69–72]. In addition, the pK_a of some residues in redox proteins can change substantially with the redox state [73–78]. Being in a special environment, near the reaction centre, the tyrosine pK_a could be different from the solution value and sensitive to redox changes. In such a case, this residue could work as a key element for delivering protons to the binuclear site. On the other hand, this tyrosine may not change its normal protonated state if it would function just as a mediator in a proton wire. Continuum electrostatics pK_a calculations on the *P. denitrificans* structure have suggested [79,80] that the glutamate (E278) may be always protonated in the operational conditions of the protein, serving as a proton conductor in the D-channel proton wire. Interestingly, the position of a serine (S257), halfway between the tyrosine and the binuclear centre, may also suggest the involvement of that residue in the proton channel, either to the binuclear centre or linking the proton pathway to the cytochrome a_3 propionates. Sequence comparisons corroborate these hypotheses, since several enzymes lacking the glutamate have in the same place the pattern YSHP (Fig. 7). It is worth noting that the glutamate residue is also absent in the cbb_3 oxidases [18,21], in SoxABCD oxidase from *Sulfolobus acidocaldarius* [16] and one of *Aquifex aeolicus* oxidases [60], in both cases a tyrosine residue is observed as in the *R. marinus* enzyme. These observations can also be extended to *Natronobacterium pharaonis* ba_3 oxidase [81] that has a tyrosine one residue before. The oxidase from *A. ambivalens* [17], the ba_3 oxidase from *T. thermophilus* [82] and the other oxidase from *Aquifex aeolicus* [60] remain as examples of terminal oxidases that do not present any of the *P. denitrificans* or *R. marinus* oxidases characteristics. For the *T. thermophilus* ba_3 oxidase is has been recently shown that it indeed pumps protons [83]. Thus it seems clear that terminal oxidases may adopt different residues to be constituents of their possible proton channels and so the glutamate residue may be considered as a characteristic of a restricted group of terminal oxidases and not as a general key element in proton uptake.

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