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Review

Proton pathways, ligand binding and dynamics of the catalytic site in haem-copper oxygen reductases: a comparison between the three families

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Abstract

Haem-copper oxygen reductases are the widest spread enzymes involved in aerobic respiratory chains, in Eukarya, Bacteria and Archaea. However, both the catalytic mechanism for oxygen reduction and its coupling to proton translocation remain to be fully understood. In this article we analyse the experimental data gathered in recent years for haem-copper reductases presenting features distinct from the mitochondrial-like enzymes. These features further support the classification of several families of haem-copper oxygen reductases based on their proton pathways and previously proposed by us [Biochim. Biophys. Acta 1505 (2001) 185], and allow to identify the minimal essential elements for these enzymes.

Keywords: Cytochrome oxidase; Proton channel; Binuclear site

Haem-copper oxygen reductases play a key role in aerobic respiratory chains, reducing the ultimate electron acceptor, dioxygen, to water. This reaction is associated with charge separation, both due to the chemical reaction and to the pumping of protons across the mitochondrial (in eukaryotes) or periplasmic (in prokaryotes) membranes, contributing to the establishment of the membrane potential, which allows the synthesis of ATP. These enzymes are able to use cytochromes, quinols, high-potential iron–sulfur proteins (HiPIPs) [1] and probably blue-copper proteins [2] as electron donors, being characterised for having in their subunit I a haem-copper binuclear centre and a low-spin haem.

Particular members of the haem-copper oxygen reductases superfamily have been extensively studied, namely the mitochondrial enzyme and its close evolutionary related oxygen reductases from purple bacteria. In more recent years, enzymes from phylogenetically distant prokaryotes began to be deeply studied, revealing novel features that challenged several assumptions established by the study of the mitochondrial-type enzymes. In this short review, we will summarise what has been learnt by the study of these enzymes, based on specific examples for which more data is available, and focusing on (i) types of haem-copper oxygen reductases; (ii) intraprotein proton channels; (iii) properties of the haem-copper binuclear centre. These last properties correlate with the differences observed for intraprotein proton channels and thus further support the classification proposed by us [3].

1. Types of haem-copper oxygen reductases: proton pathways

While the reasons for the choice to study the mitochondrial-type enzymes are obvious, it does not allow to identify general structural and functional elements indispensable for the mechanism of these enzymes, i.e., general conclusions based on a very restricted group of highly similar enzymes are highly biased. A good sampling comprising haem-copper oxygen reductases from phylogenetically distant organisms allows the recognition of common features, which being kept conserved should be functionally and/or structurally important. Surprisingly, among all haem-copper oxygen reductases so far sequenced, in the catalytic subunit I besides the six histidyl ligands of the prosthetic groups, only three amino acid residues are strictly conserved (Vall-279, Trp-272 and

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AgrI-474) (*Paracoccus* (*P.*) *denitrificans* numbering) [3]. These residues were considered to be critical for oxygen diffusion, proton pumping and electron transfer. This surprising observation strongly suggests that, besides the obvious conservation of the catalytic site, it is also the redox groups that are determinant for proton pumping.

We were able to classify the haem-copper oxygen reductases into families according to their proton channels, which correlated with the overall amino acid similarities [3]. The members of this superfamily were grouped into three families (A, B and C), one of which comprises two subfamilies (A1 and A2) (Table 1). It is important to notice that the distribution of several types of oxygen reductases does not correlate with extreme life conditions, although the most studied examples of non-canonical enzymes are from thermophilic organisms; similar enzymes are found in mesophiles from both the Bacteria and Archaea domains. As we will show in the next sections, several other properties (such as specific characteristics of the catalytic centre) of the enzymes from each family correlate well with this general classification.

1.1. Type A family

This family includes the enzymes more similar to the mitochondrial one. The A1 subfamily is constituted of oxygen reductases having the D- and K-channels first established for the mitochondrial-like enzymes. Besides AspI 124 (D), the D-channel is composed by hydrophilic amino acid residues ending at a glutamyl, GluI-278. The K-channel includes the residues LysI-354 (K), ThrI-351, SerI-291 and TyrI-280 (e.g., Refs. [4–7]) (Table 1). This last tyrosyl is covalently bound to one of the histidyl ligands of Cu_B (HisI-276), and has been proposed to play an important role in the catalytic cycle, namely in the heterolytic splitting of the O_2 molecule [8–10]. The members of the subfamily A2 have all the residues of the D- and K-channels, with the exception of the helix VI glutamyl (GluI-278) at the hydrophilic end of the D-channel (Table 1). A homology model performed for the *Rhodothermus* (*R.*) *marinus* caa_3 oxygen reductase suggested that a tyrosine residue, in a position equivalent to PheI-274, i.e., one helix turn below the glutamate residue in helix VI, whose hydroxyl group occupies the spatial place of the carboxyl group of the glutamyl, and a consecutive seryl participate in proton transfer [1,11]. This so-called YS motif is the fingerprint of the members of type A2 subfamily (Table 1) present in a quite diverse type of organisms, both mesophilic and thermophilic [3].

Table 1
Classification of haem-copper oxygen reductases on the basis of the proton channels on subunit I [3]

	Channels					
	"D"[a]		"K"[b]			
Oxygen reductase type	E/Y	Others	K/T	T/S	S/Y	Y
A1	E	+	K	T	S	Y
A2	Y	+	K	T	S	Y
B	–	–	T	S	Y	Y
C	Y	–	–	S	Y	–

[a] GluI-278, TyrI-256 (*R. marinus*), Others: AspI-124, AsnI-199, AsnI-113, AsnI-131, TyrI-35, SerI-134, SerI-193.

[b] LysI-354, ThrI-351, SerI-291 and TyrI-280. This tyrosyl is covalently bound to one of the histidyl ligands of Cu_B (HisI-276).

1.2. Type B family

The residues forming the D- and K-channels in type A enzymes are not conserved in the haem-copper oxygen reductases of the B type. However, a K-channel homologue with a threonine, a serine and a tyrosine residue replacing LysI-354, ThrI-351 and SerI-291, respectively (Table 1), could be operative. These enzymes also contain the tyrosyl covalently bound to the histidyl coordinating Cu_B (TyrI-280). The crystallographic structure of the ba_3 oxygen reductase from *Thermus* (*T.*) *thermophilus* [12] suggests that apart from the alternative K-channel, there are two other possible proton channels, but their functionality remains to be established. Furthermore, inspection of the sequence alignment of the known type B oxygen reductases shows that none of the amino acid residues (or equivalent ones), constituent of those putative channels is common to all of them [3]. For the aa_3 oxygen reductase from *Acidianus* (*A.*) *ambivalens* a pseudo D-channel was suggested on the basis of structural models [13]. Interestingly, the hydrophilic part of this channel ends at a glutamate residue (GluI-80, *A. ambivalens* numbering), located at helix II rather than in helix VI as in the type A1 enzymes; this proposal was corroborated by a double mutant in *Rhodobacter* (*Rh.*) *sphaeroides* aa_3 oxygen reductase mimicking the *A. ambivalens* enzyme, which is competent in proton pumping [14].

1.3. Type C family

The cbb_3 oxygen reductases are members of the type C family. These reductases apparently have only part of the alternative K-channel conserved, with a seryl and tyrosyl in the place of the *P. denitrificans* ThrI-351 and SerI-291 (Table 1), and do not have an equivalent to TyrI-280, the tyrosyl covalently bound to a copper histidyl ligand. None of the canonical residues of the D-channel is present; there is a tyrosine residue in all cbb_3 oxygen reductases, with the possible exception of the one from *Helicobacter pylori*, in the same sequence position as the tyrosyl of the YS motif of the type A2 reductases that, as for these reductases, may play a role in the proton pathway [3].

A particularly important conclusion from the comparison of the 3D structures and models of these several enzymes, as well as of their primary sequences, is that many oxygen reductases do not have any protonatable residues in between their surface facing the inner membrane side and the catalytic centre [15]. Thus, the intraprotein proton conduc-

tion has to be assured by chains of water molecules, through a mechanism possibly related to that of, for example, the gramicidin channels (e.g., Ref. [16]). This raises two important issues: which is the gating mechanism/element in these several enzymes? Are all these enzymes proton pumps? The answer to the second question has already been obtained: oxygen reductases from the three families have been shown to translocate protons, albeit with apparent different stoichiometries. For several type A1 oxygen reductases, including the quinol bo_3 oxidase from *Escherichia* (*E.*) *coli*, the stoichiometry is close to 1 H^+/e [17–19] (Table 2); type A2 enzymes also pump protons, with the same stoichiometry [20,21], emphasising the functional substitution of the glutamate (GluI-278) residue by the YS motif. For type B oxygen reductases, proton pumping has been demonstrated for the *T. thermophilus* ba_3 [22] and *Bacillus* (*B.*) *stearothermophilus* $b(o)a_3$ cytochrome oxidases [23], and for the *A. ambivalens* aa_3 quinol oxidase [24]. While for the first two enzymes, a stoichiometry of ca. 0.5 H^+/e was measured, for the *A. ambivalens* enzyme a ratio close to 1 was obtained (Table 2). Proton pumping by the type C (cbb_3) oxygen reductases has also been demonstrated, using either whole cells [25,26], or the purified *Bradyrhizobium japonicum* cbb_3 oxygen reductase reconstituted in artificial liposomes [27] (Table 2). A proposed coupling mechanism for haem-copper oxygen reductases is based on the electoneutrality principle [28]. If not all oxygen pump protons with a stoichiometry of $H^+/e = 1$, is this principle still obeyed? Or do the different stoichiometries reflect different coupling mechanisms? Due to the structural similarities of haem-copper oxygen reductases, the existence of such a common mechanism would seem more plausible. The pumping of protons is intimately associated with the coupling between the redox and chemical processes during the catalytic cycle, and the protonic affinities of amino acid residues participating in the uptake and release of protons. The extent of this heterotropic interaction (redox-Bohr effect) [29,30], together with the intrinsic proton affinity (pK_a) of relevant amino acid residues and/or the protein redox groups, with the fine-tuning of the kinetics of proton transfer, and with possible structural modifications along the catalytic cycle (even if localised), will have a determinant effect on the proton stoichiometry, thus explaining the different values obtained.

It is also relevant the observation that a much higher conservation of the K-channels is observed, which raises the question of whether there will be distinct channels for the so-called chemical protons and pumped protons. In this context, the D-channel mutants of *P. denitrificans* aa_3 reductase, which exhibit an almost unaltered turnover, but have a completely uncoupled behaviour, i.e., do not pump protons [31], raise the possibility that indeed the channels may deliver functionally distinct protons.

2. Catalytic site characteristics

As mentioned before, a high-spin haem and a copper ion, named Cu_B, compose the catalytic site of haem-copper oxygen reductases. With the exception of the members of

Table 2
Characteristics of members of the haem-copper oxygen reductase families and subfamilies inferred by different techniques

	Oxygen reductases family or subfamily						
	A1		A2		B		C
	B. taurus aa_3	*R. sphaeroides* aa_3	*R. marinus* caa_3	*T. thermophilus* caa_3	*A. ambivalens* aa_3	*T. thermophilus* ba_3	cbb_3
Proton pumping H^+/e	1 [57]	1 [58]	1 [20]	1[21]	~ 1 [24]	0.5 [22]	0.5–1 [25–27]
CO-FTIR, haem	α form	α (β) form		β(α) form	γ, unassigned	γ unassigned	β form
Cu_B	α form	α form		α form	unassigned	unassigned	α form
Binuclear site	close [40]	close [41]	nd	open [43,44]	++ close	++ close [43,45]	++ open [46,47]
Flash photolysis							
$FeCu(CO) \xrightarrow{k_1} Fe(CO)Cu$ (s^{-1})	0.027	0.01	0.1	34.1	1.2	0.8	nd
$Fe(CO)Cu \xrightarrow{k_2} FeCu(CO)$ (s^{-1})	1030	750	450	50	1.9	8	nd
K_{21} (k_2/k_1)	3.8×10^4	7.5×10^4	4.5×10^3	1.4	1.6	10	nd
k_{off} (s^{-1})	7.9×10^5	nd	2.2×10^4	2×10^4	1.4×10^4	nd	nd
k_{on} (M^{-1} s^{-1})	6.8×10^7	nd	5.4×10^6	8×10^7	$\sim \times 10^9$	nd	nd
K_{CO}^{-1} (mM)	11 [59]	16 [51]	4 [50]	0.25 [44,60]	$\sim 1.3 \times 10^{-2}$ [51]	$<10^{-1}$ [52]	nd
NO reductase activity							
V_{max} (min^{-1})	inactive	nd	nd	32 ± 8	nd	3 ± 0.7	100 ± 9
K_M (μM NO)	inactive [53]	nd	nd	nd [54]	nd	40 [54]	12 ± 2.5 [55]
Fast kinetics							
R → A ($\times 10^{-3}$ s^{-1})	100	120	120	nd	270	nd	nd
A → Pr ($\times 10^{-3}$ s^{-1})	39	18	28	nd	nd	nd	nd
Pr → F ($\times 10^{-3}$ s^{-1})	14	7.4	4.2	nd	3.7	nd	nd
F → O ($\times 10^{-3}$ s^{-1})	1.0 [61]	0.75 [61]	0.4 [50]	nd	nd [56]	nd	nd

(nd—not determined).

the type C family, the high-spin haem is of type A, O or their derivatives, thus always containing a hydroxylethylfarnesyl or a hydroxylethylgeranylgeranyl substitution. Type A haems, which are not exclusive or unique within each oxygen reductases family, possess another substitution, a formyl group.

Resonance Raman studies of examples from the three haem-copper families (A, B and C) and from the two subfamilies (A1 and A2) have revealed that the haem at the binuclear centre is high-spin and six-coordinated in the oxidised state; in the fully reduced state the haem iron is out of the ring plane, reflecting the loss or the weakening of the bond to the sixth ligand (e.g. [32–36]). For the ba_3 oxygen reductases from *T. thermophilus* and *Natronobacterium pharaonis* [36], as well for the Sox ABCD reductase from *Sulfolobus acidocaldarius* [35], an equilibrium between two positions, in the plane and out of the plane, for the haem iron atom has been observed. This distinct observation for members of the B type family has been attributed to a possible difference in the hydrogen network in these oxygen reductases, close to the binuclear site [36]; they may also be due to slightly different architectures of the catalytic sites (see next section), which nevertheless are not detected with the available resolution of the crystal structure. A major point that remains to be clarified is which is the nature of the haem sixth ligand.

2.1. Interaction with ligands

The interaction of haem-copper oxygen reductases with different ligands, such as CO, NO and O_2 has been a fruitful tool in the characterisation of the catalytic binuclear site. FTIR studies of the C—O stretching mode (ν_{C-O}) showed different conformations of the active site. These conformations, named α, β and γ [37], have been attributed to changes in the distance between the iron atom of the high-spin haem and Cu_B [38,39]. According to the same authors, the α and γ forms reflect constricted pockets (more for γ than for α) that do not allow CO to coordinate to the haem iron or Cu_B without strong distal polar or steric interactions between CO and the other metal site of the catalytic centre. The β form reflects a more ‘‘open’’ binuclear site, where the bound CO is not influenced by the presence of the second metal ion. In type A1 oxygen reductases the α form is the major/only form for CO bound to the haem and to Cu_B (e.g. [40–42]), whereas for *T. thermophilus* caa_3 reductase (a type A2 enzyme), the major forms observed are the β form CO bound to the haem and the α form CO bound to Cu_B [43,44] (Table 2). *T. thermophilus* ba_3 reductase is a type B enzyme, and the major peaks observed for the C—O stretching modes bound to the haem were assigned to the γ form, and to a second form representing an even more constricted pocket by Cu_B[43,45] (Table 2). The C—O stretching modes bound to Cu_B could not be assigned either to α or β forms; similar values for those stretching modes have also been measured for the aa_3 oxygen reductase from *A. ambivalens*, also a B type reductase (M. Lübben, personal communication). For the type C enzymes, such as cbb_3 reductases from *Rh. capsulatus* and *Pseudomonas* (*Ps.*) *stutzeri* the β and α forms have been assigned to the CO bound to the haem and to Cu_B [46–48] (Table 2). Thus, the available FTIR data of the CO-bond form suggests different characteristics for the binuclear site of each oxygen reductases family as summarised in Table 2.

It is not clear at this stage whether these different conformations of the binuclear site, indirectly observed through the binding of CO, correlate with the distribution of five- and six-coordinated forms of the high-spin haem. Furthermore, although CO is an inhibitor of haem-copper oxygen reductases, its electronic characteristics are quite distinct from those of O_2, either in the free or bound forms; also the respective preferential orientations in relation to the haem plane are different, which may result in distinct H-bonding and/or electrostatic interactions with the amino acid residues surrounding the binuclear site [49]. Thus, caution should be taken when extrapolating the behaviour with CO to the physiological substrate, O_2.

Thermodynamic and kinetic parameters, obtained by flash-photolysis, for CO binding to the catalytic centre also reflect the differences between the binuclear centre of type A and B families of oxygen reductases (Table 2). The dissociation constant for CO (K_{CO}^{-1}) in type A enzymes is two to three orders of magnitude higher than for type B enzymes (e.g., Ref. [50]). It is also observed that for the former family of reductases the binding of CO to Cu_B is endergonic, while the binding to the high-spin haem is exergonic, thus the equilibrium is displaced towards the binding of the ligand to the haem. Oppositely, an exergonic binding of CO to Cu_B is observed in the case of the aa_3 and ba_3 oxygen reductases from *A. ambivalens* [51] and *T. thermophilus* [52], respectively. These reductases are typical members of the type B family. No data is available for type C oxygen reductases.

2.2. Reaction with NO

Due to the structural similarities between the haem-copper oxygen and the bacterial NO reductases, which contain in their subunit I a binuclear site constituted by a high-spin haem and an iron ion (instead of Cu_B), NO reductase activity was tested for several haem-copper oxygen reductases. Type A1 oxygen reductases seem to be unable to reduce NO [53], but the opposite was observed for types A2, B and C oxygen reductases. The caa_3 enzyme from *T. thermophilus*, a type A2 member, reduces NO with a V_{max} of 32 ± 8 min^{-1} [54]. The ba_3 reductase from the same organism, a type B enzyme, shows a V_{max} of 3 ± 0.7 min^{-1} for NO reduction and a K_M of 40 μM NO [54], whereas the type C cbb_3 oxygen reductase from *Ps. stutzeri* has an activity with a V_{max} of 100 ± 9 min^{-1} and a K_M of 12 ± 2.5 μM NO [55] (Table 2). It should also be mentioned that in any case, the NO

reductases activities are very slow. Although the available data is still limited to allow general conclusions, it appears that the enzymes having a less "close" site exhibit a higher turnover towards NO, as it would be expected due to the necessary binding of two NO molecules.

2.3. Kinetics

Most kinetic analyses have been mainly performed for the mitochondrial and related oxygen reductases, similarly to what happened with other studies. With the few data available for other types of oxygen reductases, such as *R. marinus* caa_3 (type A2) [50], *A. ambivalens* aa_3 [56] and *T. thermophilus* ba_3 [52] (type B), it can be observed that the rates for the formation of the different catalytic intermediates have the same order of magnitude (Table 2). Taking into account that the above mentioned organisms are thermophilic, and the data were obtained at 20–25 °C, it can be expected that the values obtained would be higher if determined at the optimum temperatures for each of those reductases. Further, these data clearly show that the overall kinetics is not limited by the distinct characteristics of the proton channels; for example, the P to F transition, which is associated with proton uptake, has essentially the same rate for types A1, A2 and B oxygen reductases.

3. Summary

Our previous classification of haem-copper oxygen reductases into three major families was based on the analyses of the proton pathways and the amino acid sequences [3]. The properties of the catalytic centres of those reductases now discussed, further sustain our classification. But these different properties do not seem to influence the efficiency of the haem-copper oxygen reductases, in the sense that the enzymes so far studied from the several families have similar kinetic rates and turnover numbers. Thus it is clear that along evolution, slightly different solutions to the same functions have been established, but it should be stressed that the number of non-canonical enzymes studied is still very small. Regarding the pumping stoichiometry, it is an issue that clearly needs more examples before any general conclusion may be reached. Thus if questions concerning the minimal functional elements, namely the catalytic intervenients, and the trigger and gating mechanism/element are to be solved, studies of more examples of oxygen reductases from the other families must be performed. Nevertheless, all data so far gathered, and putting together those for type A1, mitochondrial-like enzymes, and for type A2 and B enzymes, show that only three main features are conserved: the overall helical structure of subunit I (the ideal structure for an ion channel); the catalytic site composition; and the presence of a low-spin six-coordinated haem. As the only common possible coupling elements are the metal centres: the redox-chemical events along the catalytic cycle, by inducing rearrangements of the hydrogen bonding networks (including arrays of water molecules), and changes of protonic affinities of amino acid residues and/or of the redox centres, may simultaneously trigger the proton pumping and assure directionality of the process. Finally, in the overall process, the relative rates of each step, namely of the chemical reaction and of intraprotein transfer, may be decisive to the overall energetics of the process, i.e., the number of protons pumped, an issue that has been scarcely studied.

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References

[1] M.M. Pereira, M. Santana, C.M. Soares, J. Mendes, J.N. Carita, A.S. Fernandes, M. Saraste, M.A. Carrondo, M. Teixeira, The caa_3 terminal oxidase of the thermohalophilic bacterium *Rhodothermus marinus*: a HiPIP:oxygen oxidoreductase lacking the key glutamate of the D-channel, Biochim. Biophys. Acta 1413 (1999) 1–13.

[2] M. Lubbern, S. Arnaud, J. Castresana, A. Warne, S.P. Albracht, M. Saraste, A second terminal oxidase in *Sulfolobus acidocaldarius*, Eur. J. Biochem. 224 (1994) 151–159.

[3] M.M. Pereira, M. Santana, M. Teixeira, A novel scenario for the evolution of haem-copper oxygen reductases, Biochim. Biophys. Acta 1505 (2001) 185–208.

[4] H. Michel, J. Behr, A. Harrenga, A. Kannt, Cytochrome *c* oxidase: structure and spectroscopy, Annu. Rev. Biophys. Biomol. Struct. 27 (1998) 329–356.

[5] R.B. Gennis, Multiple proton-conducting pathways in cytochrome oxidase and a proposed role for the active-site tyrosine, Biochim. Biophys. Acta 1365 (1998) 241–248.

[6] T. Tsukihara, H. Aoyama, E. Yamashita, T. Tomizaki, H. Yamaguchi, K. Shinzawa-Itoh, R. Nakashima, R. Yaono, S. Yoshikawa, Structures of metal sites of oxidized bovine heart cytochrome *c* oxidase at 2.8 Å, Science 269 (1995) 1069–1074.

[7] S. Iwata, C. Ostermeier, B. Ludwig, H. Michel, Structure at 2.8 Å resolution of cytochrome *c* oxidase from *Paracoccus denitrificans*, Nature 376 (1995) 660–669.

[8] M.R. Blomberg, P.E. Siegbahn, G.T. Babcock, M. Wikstrom, O—O bond splitting mechanism in cytochrome oxidase, J. Inorg. Biochem. 80 (2000) 261–269.

[9] G. Buse, T. Soulimane, M. Dewor, H.E. Meyer, M. Bluggel, Evidence for a copper-coordinated histidine–tyrosine cross-link in the active site of cytochrome oxidase, Protein Sci. 8 (1999) 985–990.

[10] D.A. Proshlyakov, M.A. Pressler, G.T. Babcock, Dioxygen and bond cleavage by cleavage by mixed-valence cytochrome *c* oxidase, Proc. Natl. Acad. Sci. U. S. A. 95 (1998) 8020–8025.

[11] M. Santana, M.M. Pereira, N.P. Elias, C.M. Soares, M. Teixeira, Gene cluster of *Rhodothermus marinus* high-potential iron–sulfur protein: oxygen oxidoreductase, a *caa*(3)-type oxidase belonging to the superfamily of heme-copper oxidase, J. Bacteriol. 183 (2001) 687–699.

[12] T. Soulimane, G. Buse, G.P. Bourenkov, H.D. Bartunik, R. Huber, M.E. Than, Structure and mechanism of the aberrant *ba*(3)-cytochrome *c* oxidase from *Thermus thermophilus*, EMBO J. 19 (2000) 1766–1776.
[13] C.M. Gomes, PhD dissertation, Instituto de Tecnologia Química e Biológica, Universidad Nova de Lisboa, 1999.
[14] A. Aagaard, G. Gilderson, D.A. Mills, S. Ferguson-Miller, P. Brzezinski, Redesign of the proton-pumping machinery of cytochrome *c* oxidase: proton pumping does not require Glu(I-286), Biochemistry 39 (2000) 15847–15850.
[15] M.M. Pereira, C.M. Gomes, M. Teixeira, Plasticity of proton pathways in haem-copper oxygen reductases, FEBS Lett. 522 (2002) 14–18.
[16] R. Pomes, B. Roux, Structure and dynamics of a proton wire: a theoretical study of H^+ translocation along the single-file water chain in the gramicidin A channel, Biophys. J. 71 (1996) 19–39.
[17] G. Antonini, F. Malatesta, P. Sarti, M. Brunori, Proton pumping by cytochrome oxidase as studied by time-resolved stop-flow spectrophotometry, Proc. Natl. Acad. Sci. U. S. A. 90 (1993) 5949–5953.
[18] N. Capitanio, G. Capitanio, E. De Nitto, S. Papa, Vectorial redox nature of Bohr effects in bovine heart cytochrome *c* oxidase, FEBS Lett. 414 (1997) 414–418.
[19] M.L. Verkhovskaya, A. Garcia-Horsman, A. Puustinen, J.L. Rigaud, J.E. Morgan, M.I. Verkhovsky, M. Wikstrom, Glutamic acid 286 in subunit I of cytochrome bo_3 is involved in proton translocation, Proc. Natl. Acad. Sci. U. S. A. 94 (1997) 10128–10131.
[20] M.M. Pereira, M.L. Verkhovskaya, M. Teixeira, M.I. Verkhovsky, The *caa*(3) terminal oxidase of *Rhodothermus marinus* lacking the key glutamate of the D-channel is a proton pump, Biochemistry 39 (2000) 6336–6340.
[21] K. Honnami, T. Oshima, Purification and characterization of cytochrome *c* oxidase from *Thermus thermophilus* HB8, Biochemistry 23 (1984) 454–460.
[22] A. Kannt, T. Soulimane, G. Buse, A. Becker, E. Bamberg, H. Michel, Electrical current generation and proton pumping catalyzed by the ba_3-type cytochrome *c* oxidase from *Thermus thermophilus*, FEBS Lett. 434 (1998) 17–22.
[23] K. Nikaido, J. Sakamoto, S. Noguchi, N. Sone, Over-expression of cbaAB genes of *Bacillus stearothermophilus* produces a two-subunit SoxB-type cytochrome *c* oxidase with proton pumping activity, Biochim. Biophys. Acta 1456 (2000) 35–44.
[24] C.M. Gomes, C. Backgren, M. Teixeira, A. Puustinen, M.L. Verkhovskaya, M. Wikstrom, M.I. Verkhovsky, Heme-copper oxidase with modified D- and K-pathways are yet efficient proton pumps, FEBS Lett. 497 (2001) 159–164.
[25] J.W. de Gier, M. Lubben, W.N. Reijnders, C.A. Tipker, D.J. Slotboom, van Spanning, R.J. van Spanning, A.H. Stouthamer, J. van der Oost, The terminal oxidases of *Paracoccus denitrificans*, Mol. Microbiol. 13 (1994) 183–196.
[26] M. Toledo-Cuevas, B. Barquera, R.B. Gennis, M. Wikstrom, J.A. Garcia-Horsman, The cbb_3-type cytochrome *c* oxidase from *Rhodobacter sphaeroides*, a proton-pumping heme-copper oxidase, Biochim. Biophys. Acta 1365 (1998) 421–434.
[27] E. Arslan, A. Kannt, L. Thony-Meyer, H. Hennecke, The symbiotically essential *cbb*(3)-type oxidase of *Bradyrhizobium japonicum* is a proton pump, FEBS Lett. 470 (2000) 7–10.
[28] P.R. Rich, Towards an understanding of the chemistry of oxygen reduction and proton translocation in the iron–copper respiratory oxidases, Aust. J. Plant Physiol. 22 (1995) 479–486.
[29] A.V. Xavier, A mechano-chemical model for energy transduction in cytochrome *c* oxidase: the work of a Maxwell's god, FEBS Lett. 532 (2002) 261–266.
[30] S. Papa, F. Guerrieri, G. Izzo, Redox Bohr-effects in the cytochrome system of mitochondria, FEBS Lett. 105 (1979) 213–216.
[31] U. Pfitzner, K. Hoffmeier, A. Harrenga, A. Kannt, H. Michel, E. Bamberg, O.M. Richter, B. Ludwig, Tracing the D-pathway in reconstituted site-directed mutants of cytochrome *c* oxidase from *Paracoccus denitrificans*, Biochemistry 39 (2000) 6756–6762.
[32] W.A. Oertling, K.K. Surerus, O. Einarsdottir, J.A. Fee, R.B. Dyer, W.H. Woodruff, Spectroscopic characterization of cytochrome ba_3, a terminal oxidase from *Thermus thermophilus*: comparison a3/CuB site to that of bovine cytochrome aa_3, Biochemistry 33 (1994) 3128–3141.
[33] G.E. Heibel, P. Hildebrandt, B. Ludwig, P. Steinrucke, T. Soulimane, G. Buse, Comparative resonance Raman study of cytochrome *c* oxidase from beef heart and *Paracoccus denitrificans*, Biochemistry 32 (1993) 10866–10877.
[34] S. Gerscher, P. Hildebrandt, T. Soulimane, G. Buse, Resonance Raman spectroscopic study of the caa_3 oxidase from *Thermus thermophilus*, Biospectroscopy 4 (1998) 365–377.
[35] S. Gerscher, S. Dopner, P. Hildebrandt, M. Gleissner, G. Schafer, Resonance Raman spectroscopy of the integral quinol oxidase complex of *Sulfolobus acidocaldarius*, Biochemistry 35 (1996) 12796–12803.
[36] S. Gerscher, P. Hildebrandt, G. Buse, T. Soulimane, The active site structure of ba_3 oxidase from *Thermus thermophilus* studied by resonance Raman spectroscopy, Biospectroscopy 5 (1999) S53–S63.
[37] J.O. Alben, P.P. Moh, F.G. Fiamingo, R.A. Altschuld, Cytochrome oxidase (a_3) heme and copper observed by low-temperature Fourier transform infrared spectroscopy of the CO complex, Proc. Natl. Acad. Sci. U. S. A. 78 (1981) 234–237.
[38] E. Pinakoulaki, U. Pfitzner, B. Ludwig, C. Varotsis, The role of the cross-link His–Tyr in the functional properties of the binuclear center in cytochrome *c* oxidase, J. Biol. Chem. 277 (2002) 13563–13568.
[39] T.K. Das, F.L. Tomson, R.B. Gennis, M. Gordon, D.L. Rousseau, pH-dependent structure changes at the heme-c cytochrome *c* oxidase, Biophys. J. 80 (2001) 2039–2045.
[40] J.O. Alben, R.A. Altschuld, F.G. Fiamingo, P.P. Moh, in: C. Ho (Ed.), Transport Electron and Oxygen Utilization, Elsevier, Amsterdam, 1982, pp. 205–208.
[41] J. Wang, S. Takahashi, J.P. Hosler, D.M. Mitchell, S. Ferguson-Miller, R.B. Gennis, D.L. Rousseau, Two conformations of the catalytic site in the aa_3-type cytochrome *c* oxidase from *Rhodobacter sphaeroides*, Biochemistry 34 (1995) 9819–9825.
[42] M. Tsubaki, T. Mogi, Y. Anraku, H. Hori, Heme-copper binuclear center of the cytochrome *bo* complex of *Escherichia coli*: WPR and Fourier transform infrared spectroscopic studies, Biochemistry 32 (1993) 6065–6072.
[43] O. Einarsdottir, P.M. Killough, J.A. Fee, W.H. Woodruff, An infrared study of the binding and photodissociation of carbon monoxide in cytochrome ba_3 from *Thermus thermophilus*, J. Biol. Chem. 264 (1989) 2405–2408.
[44] E. Pinakoulaki, T. Soulimane, C. Varotsis, Fourier transform infrared (FTIR) and step-scan time-resolved FTIR spectroscopies reveal a unique active site in cytochrome caa_3 oxidase from *Thermus thermophilus*, J. Biol. Chem. 277 (2002) 32867–32874.
[45] K. Koutsoupakis, S. Stavrakis, E. Pinakoulaki, T. Soulimane, C. Varotsis, Observation of the equilibrium CuB–CO complex and functional implications of the transient heme a3 propionates in cytochrome ba_3–CO from *Thermus thermophilus*. Fourier transform infrared (FTIR) and time-resolved step-scan FTIR studies, J. Biol. Chem. 277 (2002) 32860–32866.
[46] S. Stavrakis, K. Koutsoupakis, E. Pinakoulaki, A. Urbani, M. Saraste, C. Varotsis, Decay of the transient Cu(B)–CO complex is accompanied by formation of the heme Fe–CO complex of cytochrome *cbb*(3)–CO at ambient temperature: evidence from time-resolved Fourier transform infrared spectroscopy, J. Am. Chem. Soc. 124 (2002) 3814–3815.
[47] J. Wang, K.A. Gray, F. Daldal, D.L. Rousseau, The cbb_3-type cytochrome oxidase from *Rhodobacter capsulatus* contains a unique site, J. Am. Chem. Soc. 117 (1995) 9363–9364.
[48] J.A. Garcia-Horsman, E. Berry, J.P. Shapleigh, J.O. Alben,

R.B. Gennis, A novel cytochrome *c* oxidase from *Rhodobacter sphaeroides* lacks Cu_A, Biochemistry 33 (1994) 3113–3119.
[49] R. Jain, M.K. Chan, Mechanisms of ligand discrimination by heme proteins, J. Biol. Inorg. Chem. 8 (2003) 1–11.
[50] H. Sigurdson, A. Namslauer, M.M. Pereira, M. Teixeira, P. Brzezinski, Ligand binding and the catalytic reaction of cytochrome *caa*(3) from thermophilic bacterium *Rhodothermus marinus*, Biochemistry 40 (2001) 10578–10585.
[51] A. Aagaard, G. Gilderson, C.M. Gomes, M. Teixeira, P. Brzezinski, Dynamics of the binuclear center of the quinol oxidase from *Acidianus ambivalens*, Biochemistry 38 (1999) 10032–10041.
[52] A. Giuffre, E. Forte, G. Antonini, E. D'Itri, M. Brunori, T. Soulimane, G. Buse, Kinetic properties of ba_3 oxidase from *Thermus thermophilus*: effect of temperature, Biochemistry 38 (1999) 1057–1065.
[53] G. Stubauer, A. Giuffre, M. Brunori, P. Sarti, Cytochrome *c* oxidase does not catalyze the anaerobic reduction of NO, Biochem. Biophys. Res. Commun. 245 (1998) 459–465.
[54] A. Giuffre, G. Stubauer, P. Sarti, M. Brunori, W.G. Zumft, G. Buse, T. Soulimane, The heme-copper oxidases of *Thermus thermophilus* catalyze the reduction of nitric oxide: evolutionary implications, Proc. Natl. Acad. Sci. U. S. A. 96 (1999) 14718–14723.
[55] E. Forte, A. Urbani, M. Saraste, P. Sarti, M. Brunori, A. Giuffre, The cytochrome cbb_3 from *Pseudomonas stutzeri* displays nitric oxide reductase activity, Eur. J. Biochem. 268 (2001) 6486–6491.
[56] G. Gilderson, A. Aagaard, C.M. Gomes, P. Adelroth, M. Teixeira, P. Brzezinski, Kinetics of electron and proton transfer during O(2) reduction in cytochrome aa_3 from *A. ambivalens*: an enzyme lacking Glu(I-286), Biochim. Biophys. Acta 1503 (2001) 261–270.
[57] K. Krab, M. Wikstrom, Proton-translocating cytochrome *c* oxidase in artificial phospholipid vesicles, Biochim. Biophys. Acta 504 (1978) 200–214.
[58] J.R. Fetter, J. Qian, J. Shapleigh, J.W. Thomas, A. Garcia-Horsman, E Schmidt, J. Hosler, G.T. Babcock, R.B. Gennis, S. Ferguson-Miller, Possible proton relay pathways in cytochrome *c* oxidase, Proc. Natl. Acad. Sci. U. S. A. 92 (1995) 1604–1608.
[59] O. Einarsdottir, R.B. Dyer, D.D. Lemon, P.M. Killough, S.M. Hubig, S.J. Atherton, J.J. Lopez-Garriga, G. Palmer, W.H. Woodruff, Photodissociation and recombination of carbonmonoxy cytochrome oxidase: dynamics from picoseconds to kiloseconds, Biochemistry 32 (1993) 12013–12024.
[60] W.H. Woodruff, Coordination dynamics of heme-copper oxidases. The shuttle and the control and coupling of translocation, J. Bioenerg. Biomembranes 25 (1993) 177–188.
[61] P. Adelroth, M. Ek, P. Brzezinski, Factors determining electron-transfer rates in cytochrome *c* oxidase: investigation of the oxygen reaction in *R. sphaeroides* enzymes, Biochim. Biophys. Acta 1367 (1998) 107–117.