Biochimica et Biophysica Acta 1837 (2014) 999–1003

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbabio

The causes of reduced proton-pumping efficiency in type B and C respiratory heme-copper oxidases, and in some mutated variants of type A☆

Virve Rauhamäki, Mårten Wikström *

Institute of Biotechnology, University of Helsinki, Viikinkaari 1, 00014 Helsinki, Finland

ARTICLE INFO

Article history:
Received 28 December 2013
Received in revised form 27 January 2014
Accepted 17 February 2014
Available online 26 February 2014

Keywords:
Heme-copper oxidase
Heme-copper oxygen reductase
Cellular respiration
Proton pumping

ABSTRACT

The heme-copper oxidases may be divided into three categories, A, B, and C, which include cytochrome *c* and quinol-oxidising enzymes. All three types are known to be proton pumps and are found in prokaryotes, whereas eukaryotes only contain A-type cytochrome *c* oxidase in their inner mitochondrial membrane. However, the bacterial B- and C-type enzymes have often been reported to pump protons with an H^+/e^- ratio of only one half of the unit stoichiometry in the A-type enzyme. We will show here that these observations are likely to be the result of difficulties with the measuring technique together with a higher sensitivity of the B- and C-type enzymes to the protonmotive force that opposes pumping. We find that under optimal conditions the H^+/e^- ratio is close to unity in all the three heme-copper oxidase subfamilies. A higher tendency for proton leak in the B- and C-type enzymes may result from less efficient gating of a proton pump mechanism that we suggest evolved before the so-called D-channel of proton transfer. There is also a discrepancy between results using whole bacterial cells vs. phospholipid vesicles inlaid with oxidase with respect to the observed proton pumping after modification of the D-channel residue asparagine-139 (*Rhodobacter sphaeroides* numbering) to aspartate in A-type cytochrome *c* oxidase. This discrepancy might also be explained by a higher sensitivity of proton pumping to protonmotive force in the mutated variant. This article is part of a Special Issue entitled: 18th European Bioenergetic Conference.

1. Introduction

"There are two kinds of truth: the truth that lights the way and the truth that warms the heart. The first of these is science, and the second is art. Neither is independent of the other or more important than the other. Without art science would be as useless as a pair of high forceps in the hands of a plumber. Without science art would become a crude mess of folklore and emotional quackery. The truth of art keeps science from becoming inhuman, and the truth of science keeps art from becoming ridiculous" (Raymond Chandler).

The respiratory heme-copper oxidases form a large superfamily of proton-pumping cytochrome *c* and quinol oxidases that may be divided into three types, A, B and C, based on the presence (type A) or absence (types B and C) of a proton-conducting D-pathway [1] (Fig. 1). The A-type cytochrome *c* oxidases from mitochondria and bacteria are known to pump 1 H^+/e^- (2 q^+/e^-, where q^+ symbolises translocated electrical charge equivalents) across the respective membrane [2]. Their quinol-oxidising counterparts (e.g. cytochrome bo_3 from *Escherichia coli*) also pump 1 H^+/e^- (and 2 q^+/e^-) although 2 H^+/e^- are released on the positively charged ***P***-side of the membrane due to oxidation of the hydrogenated electron donor [3].

With respect to the oxidases of B- and C-types there are discrepancies in the literature. Early on, the C-type cytochrome cbb_3 was reported to pump the full complement of 1 H^+/e^- in bacterial cells ([4–6], but see also [7]), as confirmed later [8]. We attributed lower stoichiometries (~0.5 H^+/e^-) reported in [9,10] to technical difficulties [8,11], and we found a unit stoichiometry also for the vesicle-reconstituted cytochrome cbb_3 albeit only at stringent experimental conditions that assured pre-reduction of the low-potential heme b_3 prior to turnover, and a very low number of turnovers in the experiment [8]. The quinol oxidase of type B from *Acidianus ambivalens* was shown to pump 1 H^+/e^- after reconstitution into proteoliposomes [12]. Subsequent work on the reconstituted B-type cytochrome ba_3 from *Thermus thermophilus* reported a stoichiometry of only 0.5 H^+/e^- [13], which has become the norm in the literature [9].

The D-pathway of proton transfer is present only in the structures of the A-type oxidases (Fig. 1), and has been generally attributed to be the conduit of all pumped protons in these enzymes [1,2,14]. A main reason for this view is the finding that mutation of conserved residues in this

☆ This article is part of a Special Issue entitled: 18th European Bioenergetic Conference.

* Corresponding author. Tel.: +358 50 467 3456.
E-mail address: marten.wikstrom@helsinki.fi (M. Wikström).

http://dx.doi.org/10.1016/j.bbabio.2014.02.020
0005-2728/

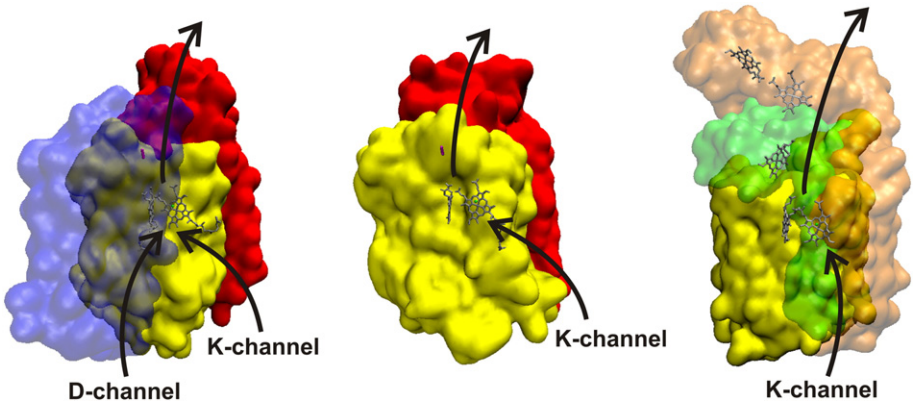

Fig. 1. Architecture of the enzymes from the three subfamilies. The structures used are, from left to right: A-type; cytochrome aa_3 from *R. sphaeroides* (1M56), B-type; cytochrome ba_3 from *T. thermophilus* (1EHK) and C-type; cytochrome cbb_3 from *Pseudomonas stutzeri* (3MK7). Subunit I (yellow) is conserved throughout the family, as is the presence of a low-spin heme (left) and the oxygen-binding heme (right) which along with Cu_B (green) are bound to this subunit. Subunit II (red) binds the electron input centre Cu_A (magenta; above the low-spin heme) in the cytochrome *c* oxidases of types A and B, but its role is taken over by three heme *c* located in two alternative subunits (green and orange) in the C-type enzyme. Black arrows depict the proton uptake (K- and D-channels) and exit pathways. The K-channel is homologous in all cytochrome *c* oxidases. Subunit III (blue) is found only in the A-type enzymes, as is the D-channel of proton transfer formed solely by residues of subunit I.

pathway, originating from mutations of the aspartate that gave its name [15], leads to loss of proton pumping observed as a lowered measured H^+/e^- ratio. In particular, mutations of conserved asparagines (e.g. N139 in aa_3 from *R. sphaeroides*, N131 in *Paracoccus denitrificans*) to aspartate have been reported to completely abolish proton pumping in reconstituted oxidase vesicles, without affecting or even enhancing the electron transfer rate [16–18]. However, also in this case there are some discrepant findings at the cell level insofar as Pfitzner et al. originally reported normal [19] and later only somewhat reduced [16] proton-pumping stoichiometries for *P. denitrificans* N131D mutant cells respiring on succinate, whilst no proton pumping was observed in the isolated N131D variant enzyme inlaid in vesicles [16,20].

2. Results

2.1. The B-type ba_3 enzyme from T. thermophilus

When scrutinizing the published experimental data on proton pumping by the reconstituted ba_3 cytochrome *c* oxidase of type B from *T. thermophilus*, we note two significant details. In the report by Kannt et al. [13] a pulse of reduced cytochrome *c* caused immediate acidification of the outside medium (see Fig. 3 in [13]) followed by slower alkalinisation. Dividing the extent of acidification by the amount of added reductant yielded an H^+/e^- ratio of less than 0.5 whereas the consumption of protons in the presence of an uncoupling agent was close to the expected 1 H^+/e^-. The former procedure assumes that all the added ferrocytochrome *c* is consumed at the peak of acidification, but the kinetics of H^+ consumption in the presence of an uncoupler shows that the reductant is oxidised much more slowly, in about 25 s. Therefore, the H^+/e^- stoichiometry was greatly underestimated for this experiment. This problem was absent in the stopped-flow experiments by the same authors (Fig. 4 in [13]), but here again the H^+/e^- stoichiometry was apparently obtained from the total extent of extravesicular acidification. The authors' parallel experiment with the reconstituted A-type cytochrome *c* oxidase from *P. denitrificans* shows a much larger amplitude of acidification, approaching 1 H^+/e^- (Fig. 4 in [13]), but there is another interesting difference: The *kinetics* of acidification (proton pumping) is linear vs. time for a much longer time span in the *P. denitrificans* case than it is for *T. thermophilus*. In fact, with *T. thermophilus* ba_3 proton ejection is highly non-linear with time, but *the initial rate is comparable to the rate of proton consumption in the presence of the uncoupler*, in which case the true H^+/e^- pumping stoichiometry would initially be unity. A very similar strong non-linearity of proton ejection in ba_3 proteoliposomes is seen in later experiments by Chang et al. [21]. Also in this case, comparison with the corresponding rate of alkalinisation in the presence of the uncoupler (where H^+/e^- is −1.0) suggests that the initial rate of proton ejection may well be compatible with an H^+/e^- ratio of 1.

Based on indirect electrometric measurements of charge translocation, Siletsky et al. [22] concluded that the proton-pumping efficiency of cytochrome ba_3 from *T. thermophilus* is only one half of that in the A-type enzymes. Recent more extensive data from our laboratory (D.A. Bloch et al., unpublished observations) suggests that the two major electrometric phases after the reaction of the reduced enzyme with O_2 may not differ significantly in amplitude, and that the conclusion of a lowered proton pumping efficiency is therefore doubtful.

von Ballmoos et al. [23] summarized their spectrophotometric and proton uptake data which they interpreted on the basis of a proton pumping stoichiometry of 0.5 H^+/e^- in *T. thermophilus* cytochrome ba_3.

We have performed oxygen pulse experiments to measure proton pumping in phospholipid vesicles inlaid with *T. thermophilus* cytochrome ba_3 obtained from the laboratory of the late James A. Fee (courtesy of Ying Chen), using methodology identical to that described for cytochrome cbb_3 [8], but with the addition of the *T. thermophilus* cytochrome *c*552 as electron donor. The choice of the setup was based on the fact that the turnover of cytochrome ba_3 is ten times slower than that for cytochrome aa_3 (~100 e^-/s versus ~1000 e^-/s) and it is therefore beneficial to use an electrochemical reduction system to remove excess oxygen. Using four independently prepared reconstitutions we found an H^+/e^- ratio of 0.85 ± 0.11 (n = 19) in the coupled vesicles (Fig. 2A), whereas the corresponding ratio was −1.0 ± 0.07 (n = 6) in the presence of the uncoupler (Fig. 2B). Now, the kinetics of proton ejection after the oxygen pulse in the cases of cytochrome aa_3 and cytochrome ba_3 are comparable (Fig. 2C). This result implies that the measuring conditions are optimal and that the amount of oxygen is small enough in comparison to the number of enzyme molecules present to allow for only a minimal number of turnovers per proteoliposome. The rise in ΔpH is thus minimised and insufficient to drive significant proton leakage in the ba_3 proteoliposomes.

We conclude from the above that the mechanistic proton-pumping stoichiometry is most probably 1 H^+/e^- also in the B-type oxidases. However, we note that our observed ratio of 0.85 H^+/e^- does not exclude a mechanistic 0.75 H^+/e^- stoichiometry where only three of the four one-electron reduction steps of the catalytic cycle would be coupled to proton pumping. Yet, we think that this possibility is unlikely,

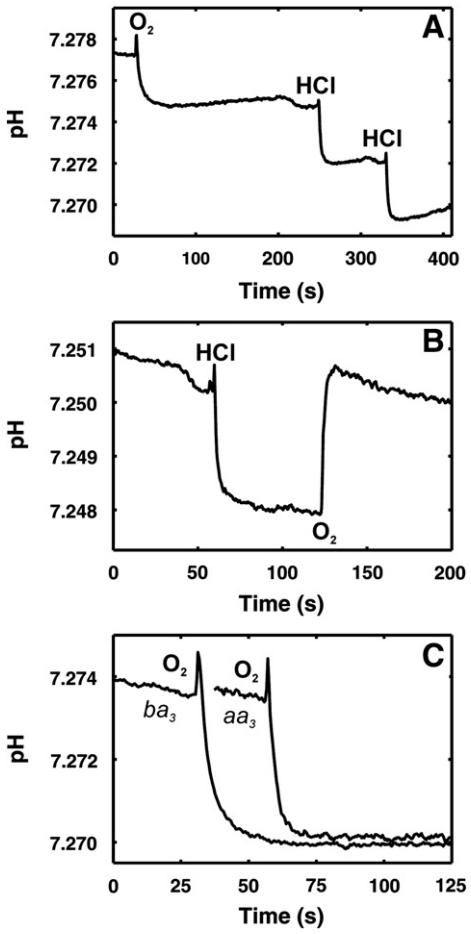

Fig. 2. Proton translocation experiments with reconstituted cytochrome ba_3 from *T. thermophilus* and aa_3 from *P. denitrificans* (see [8] for methodology). (*A*) The stoichiometry of proton-pumping was probed using proteoliposomes diluted in 1.4 ml of 100 mM KCl in the presence of 0.35 μM cytochrome ba_3. 1 μM valinomycin was used to dissipate membrane potential and 100 μM *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine (TMPD), 100 μM benzyl viologen together with 15 μM cytochrome c_{552} were used as redox mediators between the reconstituted enzyme and the gold electrode. Changes of pH were followed upon pulses of 5 μl of air-saturated water (258 μM O_2 at 25 °C, 1 atm) and of 5 μl anaerobic 1 mM HCl for calibration. The additions of O_2 and HCl thus correspond to 5.16 and 5 nmol of electron acceptor and H^+, respectively, so that an equal amplitude of the two perturbations corresponds to 0.97 H^+/e^-. (*B*) The pH responses of the reconstituted enzyme in the presence of 1 μM CCCP upon 10 μl pulses of air-saturated water and 1 mM anaerobic HCl, i.e. 10.32 nmol electron acceptor and 10 nmol H^+. (*C*) Comparison of the proton ejection kinetics between cytochrome ba_3 and cytochrome aa_3 in proteoliposomes after 6 μl pulses of air-saturated water, which corresponds to ~3 molecules of O_2 per enzyme molecule. The traces were normalised to the calibration pulses of HCl. The first 40 s of the cytochrome aa_3 trace is not shown for clarity. The response time of the pH electrode is ca. 1 s.

not least because *over*-estimation of the proton pumping efficiency is rare.

2.2. The asparagine/aspartate variant in the D-pathway

Due to the discrepancies in the results for intact cells vs. proteoliposomes for proton pumping in the N131D (*P. denitrificans* numbering) variant of the A-type cytochrome *c* oxidase (Introduction), we decided to revisit this issue. When proton translocation is probed in whole cells of *P. denitrificans* that only express wild-type cytochrome aa_3 and a quinol oxidase, an H^+/e^- ratio very close to 3.0 is obtained during succinate oxidation (Table 1), as published earlier (see e.g. [3,16]). Table 1 also shows the obtained H^+/e^- ratio for succinate oxidation in the N131D variant. We obtained a ratio close to 3.0 that decreased to 2.0 when cytochrome *c* oxidase activity is replaced by quinol oxidase upon blocking the cytochrome bc_1 complex by myxothiazol, indistinguishable

Table 1
Proton translocation stoichiometries in whole cell experiments. Values for succinate oxidation were determined by tracing pH changes upon pulses of oxygen. An H^+/e^- ratio of 3 indicates proton pumping at full efficiency by cytochrome *c* oxidase, whereas 2 H^+/e^- is explained by the quinol oxidase. AO1 is a *Paracoccus denitrificans* parent strain used in all experiments [19], and for the cases of the wild-type and the N131D mutants it is supplemented with a plasmid with the gene for subunit I [16,19] to reinstate expression of cytochrome *c* oxidase.

Strain	H^+/e^- ratio ± SD	n (number of pulses)
AO1	2.1 ± 0.28	8
AO1 + Mx	2.1 ± 0.23	5
AO1 − WT	3.0 ± 0.16	11
AO1 − WT + Mx	2.0 ± 0.18	7
AO1 − N131D[a]	3.0 ± 0.26	10
AO1 − N131D[a] + Mx	2.0 ± 0.30	5
AO1 − N131D[b]	2.9 ± 0.29	11
AO1 − N131D[b] + Mx	1.6 ± 0.12	3

Abbreviations: Mx, myxothiazol; SD, standard deviation; WT, wild-type.
[a] Mutant prepared in Helsinki.
[b] Mutant prepared in Frankfurt.

from the results with wild type enzyme. The ratio is 2.0 also in the parent strain AO1 from which genes encoding subunit I of cytochrome *c* oxidase have been deleted [19]. The DNA of the entire subunit I was sequenced to ascertain that the mutation was correct and that no second site revertants were present. Moreover, the same mutated *P. denitrificans* aa_3 variant, kindly provided to us by Prof. B. Ludwig, showed virtually identical behaviour (Table 1). There can thus be no doubt that the N131D cytochrome *c* oxidase variant pumps protons with full wild type stoichiometry under these conditions in whole cells, which agrees with the original report by Pfitzner et al. [19].

Table 2 shows corresponding data for the isolated N131D variant reconstituted into liposomes. After correction for the release of 0.5 H^+/e^- due to oxidation of ascorbate, which was the reductant of cytochrome *c*, the apparent proton pumping stoichiometry is only ~0.13, but increases to ~0.3 when the buffering capacity is increased inside the vesicles. In the presence of the uncoupler CCCP there was instead proton consumption to the extent of 0.5 H^+/e^- (not shown). This control renders strong support to the conclusion that the proton release above 0.5 H^+/e^- in the absence of the uncoupler is indeed due to proton pumping, albeit at a low efficiency. A non-zero value (~0.2) was obtained earlier also in the presence of D_2O [18] (see below). In agreement with the reports mentioned earlier (see Introduction), the observed H^+/e^- ratio for the mutant enzyme in liposomes is thus indeed very much lower than in wild type, but we do not find it to be zero.

Siletsky et al. [24] reported a lower charge translocation amplitude for the state **F** to state **O** transition in the reconstituted *R. sphaeroides* variant (N139D) corresponding to N131D in *P. denitrificans*. This result is consistent with loss of proton pumping in the reconstituted forms of this mutant, and does not contradict the data in Table 2. It seems clear that whilst full proton-pumping can clearly be demonstrated for this variant in whole cells, its isolation and reconstitution into liposomes lead to severe but not necessarily complete inhibition of proton pumping.

3. Discussion

On the basis of the evidence presented above we conclude that the nominal, mechanistic H^+/e^- ratio of proton pumping is unity not only in the A type heme-copper oxidases, but also in the oxidases of types B and C. This conclusion has immediate consequences because the B- and C-type oxidases lack the D-pathway of proton transfer that carries out all proton pumping in the A-type enzymes, but contain the structural analogue of the K-pathway [25,26] (see Fig. 1), which is therefore likely to carry out proton uptake for both O_2 reduction chemistry and proton pumping in the B- and C-type enzymes [27,28].

However, there is no doubt that the full extent of proton pumping is often difficult to accomplish experimentally with the vesicle-

Table 2
Proton translocation efficiency of the isolated reconstituted N131D variant of cytochrome *c* oxidase. Preparation of proteoliposomes and proton pumping experiments were carried out as published before [46]. A contribution of 0.5 H^+/e^- on oxidation of ascorbate was used to correct the obtained values.

Measuring conditions	H^+/e^- ratio ± SD		n (number of pulses)
	Raw data	After ascorbate correction	
100 mM HEPES pH 7.4 (inside)/100 mM KCl (outside)	0.63 ± 0.18	0.13	23
200 mM HEPES pH 7.4 (inside)/100 mM KCl (outside)	0.85 ± 0.06	0.35	11
400 mM HEPES pH 7.4 (inside)/400 mM KCl (outside)	0.76 ± 0.13	0.26	11

reconstituted B- and C-type enzymes. Based on the stringent conditions required for that described above, we suggest that the proton-pumping mechanism in these enzymes is much more sensitive to back-leakage of protons driven by protonmotive force (pmf) than in the A-type enzymes. Since the measurements of proton pumping are typically carried out in the presence of valinomycin plus K^+ (to minimise the $\Delta\psi$ term of the pmf), turnover will build up the ΔpH term instead. The stringent measuring conditions include a minimum number of turnovers, creating a minimal ΔpH term, and very strict removal of traces of O_2 in experiments started by O_2 addition. A high sensitivity of the proton pump to the build-up of ΔpH is also the most likely explanation for the strong non-linearity of net proton ejection discussed above for the experiments with reconstituted ba_3 enzyme: the more the ΔpH term increases with the number of turnovers, the higher the proton leakage, and the trace of net proton ejection bends off strongly with elapsed time. This notion is consistent with the observation that proton-pumping is more easily observable in whole cells, at least for the cbb_3-type enzyme [4,6], because the buffering capacity on the ***N***-side of the membrane (i.e. the cells' cytoplasm) is much larger than for the small-sized proteoliposomes.

In contrast, it can easily be established that the proton pump of the mitochondrial A-type cytochrome *c* oxidase shows only minimal back-leakage of protons even at a pmf as large as ~150 mV during phosphorylating State 3 of mitochondria. With TMPD + ascorbate as substrate the mean measured ATP/$2e^-$ ratio is ~0.97 [29]. Since

$$H^+/2e^- = \left(H^+/ATP\right) * (ATP/2e^-) \quad (1)$$

and $H^+/ATP = 3.67$ for intact mitochondria (see [30]), the effective $H^+/2e^-$ ratio for A-type cytochrome *c* oxidase is 3.56 which is 89% of the theoretical maximum of 4.0.[1]

There is another interesting correlation, namely between the lack of a D-pathway in the B- and C-type oxidases, their increased tendency for pmf-driven proton back-leakage as deduced here, and their very strong binding of O_2 to the reduced active site [31,32]. In contrast, the A-type oxidases bind O_2 very poorly (K_D ~ 0.3 mM), and the high physiological O_2 affinity is instead the result of efficient kinetic trapping of the ligand [33,34]. The fact that the B- and C-type oxidases invest significantly more free energy from the chemistry of O_2 reduction on binding the substrate ligand leaves correspondingly less energy for driving proton translocation. The standard free energy change of binding the ligand O_2,

$$\Delta G_o^{'} \sim -60 * \log(K_D) \quad (2)$$

where the free energy change is expressed in millivolts and K_D is the binding constant, indicates that if the binding affinity of O_2 decreases from ~0.3 mM in the A-type oxidases to the micromolar regime in the oxidases of types B and C, the loss of free energy available for proton-pumping can be as high as 180 mV.

The resulting smaller thermodynamic driving force for proton pumping may contribute to the larger sensitivity towards pmf-driven leakage. However, we put more significance on the unique presence of the D-pathway in the A-type oxidases, where it is used to transfer all pumped protons, and where the proton transfer includes redox state-dependent gating that is very important for achieving a high proton pumping efficiency at higher values of the pmf [2,35–38]. Mathematical modelling of the proton pump mechanism in A-type oxidases [39,40] has indeed shown that without such kinetic gating the proton pump will leak and will be unable to work effectively against any substantial pmf.

We consider that the orientation of the chemical reactions of cytochrome *c* oxidase with respect to the membrane, such that the electrons are taken from the ***P***-side and the protons from the ***N***-side, was an early arrangement to generate pmf in the evolution of the catalysts of cell respiration, and that proton pumping came later to enhance the energy transduction efficiency [39]. If so, it seems plausible that the K-pathway, or its structural analogue in the B- and C-type enzymes, was the original proton uptake conduit, and that a rudimentary proton-pumping mechanism evolved in this scenario using the existing K-pathway also for the pumped protons. We surmise that the D-pathway evolved later, adding kinetic gating options to the mechanism, thus increasing the energy transduction efficiency to its present 90% at the high pmf during oxidative phosphorylation in animal mitochondria. This conclusion contradicts earlier phylogenetic scenarios proposed by Brochier-Armanet et al. [41] and Han et al. [9], but are in full accordance with the ideas presented by Ducluzeau et al. [42,43].

With this view in mind it becomes less enigmatic to understand the above-mentioned apparent discrepancies in observations concerning the asparagine/aspartate variant of the D-pathway, and possibly also for other mutations in this pathway with a similar phenotype. By analogy to the observations with the B- and C-type oxidases, we suggest that proton pumping is compromised in the N131D variant especially at significant values of ΔpH (i.e. at a raised pH on the ***N***-side of the membrane), which are the conditions where the kinetic gating is required most to prevent back-leakage. In experimental conditions where the number of turnovers is small relative to the buffering power of the ***N***-phase (bacterial cytoplasm), the detrimental effect of the mutation either vanishes or is much smaller (Table 1, [19]). In proteoliposomes the decoupling effect of the mutation may be reduced by increasing the buffering power of the vesicle interior (Table 2). The non-zero H^+/e^- observed for the reconstituted N131D variant (N139D in cytochrome aa_3 from *R. sphaeroides*) when measured in heavy water [18] might have resulted from the known rise in pK_a of weak acids in D_2O [44], yielding a higher buffering capacity at an elevated pH.

Finally, it seems clear that the N131D variant pumps protons quite efficiently in whole cells, but that this is lost to a large extent, often completely, after isolation of the enzyme and reconstitution into liposomes. There may well be additional reasons for this apart from the issues of buffering power and number of turnovers addressed above. A-type cytochrome *c* oxidase easily loses subunit III due to the detergent treatment necessary during isolation, and an enzyme deficient of subunit III has been shown to be particularly sensitive to high pH [45].

[1] In addition to pumping 1 H^+/e^- from the ***N***-side of the membrane to the ***P***-side, cytochrome *c* oxidase translocates 1 e^- inwards from the ***P***-side of the membrane and 1 H^+ inwards from the ***N***-side to react with 1/4 O_2, forming 1/2 H_2O in the active site within the membrane. The latter orientation of the oxygen reduction chemistry is thermodynamically equivalent to translocation of another H^+ ion from the ***N***-side to the ***P***-side, which is why the thermodynamically relevant proton-pumping stoichiometry is 2 H^+/e^- even though only 1 H^+/e^- is released on the *P*-side.

4. Conclusion

We conclude that the A-, B- and C-types of heme-copper oxidases are all proton pumps capable of pumping 1 H^+/e^- at a low opposing protonmotive force. However, the exclusively prokaryotic B- and C-type enzymes, which lack subunit III and a D-pathway of proton transfer in subunit I, fail to pump protons at full efficiency already at low or moderate values of pmf. By contrast, proton-pumping by the A-type oxidases proceeds at high efficiency even at the high pmf encountered in phosphorylating mitochondria. We ascribe this tight coupling in the A-type enzymes to kinetic gating of proton transfer previously described as a property of the D-channel. Consequently, we suggest that the thermodynamically more efficient A-type respiratory oxidases evolved later than the oxidases of B and C types, and that this evolutionary progression included a paradoxical decrease in the strength of binding the dioxygen substrate. However, in the A-type oxidases the weaker binding of O_2 liberated free energy for driving proton translocation, and a high physiological O_2 affinity was instead ascertained by trapping the O_2 kinetically.

Acknowledgements

We thank Drs Camilla Ribacka and Anne Puustinen for the production of the N131D mutant of cytochrome *c* oxidase from *P. denitrificans*, and Professor Bernd Ludwig (Frankfurt University) for his gifts of mutated *P. denitrificans* strains. Eija Haasanen is acknowledged for skillful technical assistance. MW thanks Jonathan Hosler for valuable discussions. This work was supported by the Sigrid Jusélius Foundation, Biocentrum Helsinki, and the Academy of Finland.

References

[1] M.M. Pereira, M. Santana, M. Teixeira, A novel scenario for the evolution of heme-copper oxygen reductases, Biochim. Biophys. Acta 1505 (2001) 185–208.
[2] M. Wikström, M.I. Verkhovsky, Mechanism and energetics of proton translocation by the respiratory heme-copper oxidases, Biochim. Biophys. Acta 1767 (2007) 1200–1214.
[3] A. Puustinen, M. Finel, M. Virkki, M. Wikström, Cytochrome *o* (*bo*) is a proton pump in *Paracoccus denitrificans* and *Escherichia coli*, FEBS Lett. 249 (1989) 163–167.
[4] M. Raitio, M. Wikström, An alternative cytochrome oxidase of *Paracoccus denitrificans* functions as a proton pump, Biochim. Biophys. Acta 1186 (1994) 100–106.
[5] J.-W.L. de Gier, M. Schepper, W.N.M. Reijnders, S.J. van Dyck, D.J. Slotboom, A. Warne, M. Saraste, K. Krab, M. Finel, A.H. Stouthamer, R.J.M. van Spanning, J. van der Oost, Structural and functional analysis of aa_3-type and cbb_3-type cytochrome *c* oxidases of *Paracoccus denitrificans* reveals significant differences in proton-pump design, Mol. Microbiol. 20 (1996) 1247–1260.
[6] M. Toledo-Cuevas, B. Barquera, R.B. Gennis, M. Wikström, J.A. García-Horsman, The cbb_3-type cytochrome *c* oxidase from *Rhodobacter sphaeroides*, a proton-pumping heme-copper oxidase, Biochim. Biophys. Acta 1365 (1998) 421–434.
[7] J.-W.L. de Gier, M. Lübben, W.N.M. Reijnders, C.A. Tipker, D.-J. Slotboom, R.J.M. van Spanning, A.H. Stouthamer, J. van der Oost, The terminal oxidases of *Paracoccus denitrificans*, Mol. Microbiol. 13 (1994) 183–196.
[8] V. Rauhamäki, D.A. Bloch, M. Wikström, Mechanistic stoichiometry of proton translocation by cytochrome cbb_3, Proc. Natl. Acad. Sci. U. S. A. 109 (2012) 7286–7291.
[9] H. Han, J. Hemp, L.A. Pace, H. Ouyang, K. Ganesan, J.H. Roh, F. Daldal, S.R. Blanke, R.B. Gennis, Adaptation of aerobic respiration to low O_2 environments, Proc. Natl. Acad. Sci. U. S. A. 108 (2011) 14109–14114.
[10] R. Murali, G.G. Yildiz, F. Daldal, R.B. Gennis, Stoichiometry of proton pumping by the cbb_3-type oxygen reductase in whole cells of *Rhodobacter capsulatus* at pH 7 is about 0.5 H^+ per electron, Proc. Natl. Acad. Sci. U. S. A. 109 (2012) E2144.
[11] V. Rauhamäki, D.A. Bloch, M. Wikström, Reply to Murali et al.: Proton translocation stoichiometry of cbb_3-type cytochrome *c* oxidase, Proc. Natl. Acad. Sci. U. S. A. 109 (2012) E2145.
[12] C.M. Gomes, C. Backgren, M. Teixeira, A. Puustinen, M.L. Verkhovskaya, M. Wikström, M.I. Verkhovsky, Heme-copper oxidases with modified D- and K-pathways are yet efficient proton pumps, FEBS Lett. 497 (2001) 159–164.
[13] 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.
[14] P. Brzezinski, A.L. Johansson, Variable proton-pumping stoichiometry in structural variants of cytochrome *c* oxidase, Biochim. Biophys. Acta 1797 (2010) 710–723.
[15] J.W. Thomas, A. Puustinen, J.O. Alben, R.B. Gennis, M. Wikström, Substitution of asparagine for aspartate-135 in subunit I of the cytochrome *bo* ubiquinol oxidase of *Escherichia coli* eliminates proton-pumping activity, Biochemistry 32 (1993) 10923–10928.
[16] U. Pfitzner, K. Hoffmeier, A. Harrenga, A. Kannt, H. Michel, E. Bamberg, O.M.H. Richter, B. Ludwig, Tracing the D-pathway in reconstituted site-directed mutants of cytochrome *c* oxidase from *Paracoccus denitrificans*, Biochemistry 39 (2000) 6756–6762.
[17] A.S. Pawate, J. Morgan, A. Namslauer, D. Mills, P. Brzezinski, S. Ferguson-Miller, R.B.B. Gennis, A mutation in subunit I of cytochrome oxidase from *Rhodobacter sphaeroides* results in an increase in steady-state activity but completely eliminates proton pumping, Biochemistry 41 (2002) 13417–13423.
[18] A. Namslauer, A.S. Pawate, R.B. Gennis, P. Brzezinski, Redox-coupled proton translocation in biological systems: proton shuttling in cytochrome *c* oxidase, Proc. Natl. Acad. Sci. U. S. A. 100 (2003) 15543–15547.
[19] U. Pfitzner, A. Odenwald, T. Ostermann, L. Weingard, B. Ludwig, O.M. Richter, Cytochrome *c* oxidase (heme aa_3) from *Paracoccus denitrificans*: analysis of mutations in putative proton channels of subunit I, J. Bioenerg. Biomembr. 30 (1998) 89–97.
[20] K.L. Dürr, J. Koepke, P. Hellwig, H. Müller, H. Angerer, G. Peng, E. Olkhova, O.M. Richter, B. Ludwig, H. Michel, A D-pathway mutation decouples the *Paracoccus denitrificans* cytochrome *c* oxidase by altering the side-chain orientation of a distant conserved glutamate, J. Mol. Biol. 384 (2008) 865–877.
[21] H.Y. Chang, S.K. Choi, A.S. Vakkasoglu, Y. Chen, J. Hemp, J.A. Fee, R.B. Gennis, Exploring the proton pump and exit pathway for pumped protons in cytochrome ba_3 from *Thermus thermophilus*, Proc. Natl. Acad. Sci. U. S. A. 109 (2012) 5259–5264.
[22] S.A. Siletsky, I. Belevich, A. Jasaitis, A.A. Konstantinov, M. Wikström, T. Soulimane, M.I. Verkhovsky, Time-resolved single-turnover of ba_3 oxidase from *Thermus thermophilus*, Biochim. Biophys. Acta 1767 (2007) 1383–1392.
[23] C. von Ballmoos, P. Ädelroth, R.B. Gennis, P. Brzezinski, Proton transfer in ba_3 cytochrome *c* oxidase from *Thermus thermophilus*, Biochim. Biophys. Acta 1817 (2012) 650–657.
[24] S.A. Siletsky, A.S. Pawate, K. Weiss, R.B. Gennis, A.A. Konstantinov, Transmembrane charge separation during the ferryl-oxo -> oxidized transition in a nonpumping mutant of cytochrome *c* oxidase, J. Biol. Chem. 279 (2004) 52558–52565.
[25] 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.
[26] S. Buschmann, E. Warkentin, H. Xie, J.D. Langer, U. Ermler, H. Michel, The structure of cbb_3 cytochrome oxidase provides insights into proton pumping, Science 329 (2010) 327–330.
[27] J. Hemp, H. Han, J.H. Roh, S. Kaplan, T.J. Martinez, R.B. Gennis, Comparative genomics and site-directed mutagenesis support the existence of only one input channel for protons in the C-family (cbb_3 oxidase) of heme-copper oxygen reductases, Biochemistry 46 (2007) 9963–9972.
[28] H.Y. Chang, J. Hemp, Y. Chen, J.A. Fee, R.B. Gennis, The cytochrome ba_3 oxygen reductase from *Thermus thermophilus* uses a single input channel for proton delivery to the active site and for proton pumping, Proc. Natl. Acad. Sci. U. S. A. 106 (2009) 16169–16173.
[29] P.C. Hinkle, P/O ratios of mitochondrial oxidative phosphorylation, Biochim. Biophys. Acta 1706 (2005) 1–11.
[30] M. Wikström, G. Hummer, Stoichiometry of proton translocation by respiratory complex I and its mechanistic implications, Proc. Natl. Acad. Sci. U. S. A. 109 (2012) 4431–4436.
[31] O. Preisig, R. Zufferey, L. Thöny-Meyer, C.A. Appleby, H. Hennecke, A high-affinity cbb_3-type cytochrome oxidase terminates the symbiosis-specific respiratory chain of *Bradyrhizobium japonicum*, J. Bacteriol. 178 (1996) 1532–1538.
[32] I. Szundi, C. Funatogawa, J.A. Fee, T. Soulimane, O. Einarsdottir, CO impedes superfast O_2 binding in ba_3 cytochrome oxidase from *Thermus thermophilus*, Proc. Natl. Acad. Sci. U. S. A. 107 (2010) 21010–21015.
[33] B. Chance, C. Saronio, J.S. Leigh, Functional intermediates in reaction of cytochrome oxidase with oxygen, Proc. Natl. Acad. Sci. U. S. A. 72 (1975) 1635–1640.
[34] M.I. Verkhovsky, J.E. Morgan, A. Puustinen, M. Wikström, Kinetic trapping of oxygen in cell respiration, Nature 380 (1996) 268–270.
[35] M. Wikström, M.I. Verkhovsky, G. Hummer, Water-gated mechanism of proton translocation by cytochrome *c* oxidase, Biochim. Biophys. Acta 1604 (2003) 61–65.
[36] P.E. Siegbahn, M.R. Blomberg, Energy diagrams and mechanism for proton pumping in cytochrome *c* oxidase, Biochim. Biophys. Acta 1767 (2007) 1143–1156.
[37] V.R. Kaila, M.I. Verkhovsky, M. Wikström, Proton-coupled electron transfer in cytochrome oxidase, Chem. Rev. 110 (2010) 7062–7081.
[38] M.R. Blomberg, P.E. Siegbahn, The mechanism for proton pumping in cytochrome *c* oxidase from an electrostatic and quantum chemical perspective, Biochim. Biophys. Acta 1817 (2012) 495–505.
[39] Y.C. Kim, M. Wikström, G. Hummer, Kinetic models of redox-coupled proton pumping, Proc. Natl. Acad. Sci. U. S. A. 104 (2007) 2169–2174.
[40] Y.C. Kim, M. Wikström, G. Hummer, Kinetic gating of the proton pump in cytochrome *c* oxidase, Proc. Natl. Acad. Sci. U. S. A. 106 (2009) 13707–13712.
[41] C. Brochier-Armanet, E. Talla, S. Gribaldo, The multiple evolutionary histories of dioxygen reductases: implications for the origin and evolution of aerobic respiration, Mol. Biol. Evol. 26 (2009) 285–297.
[42] A.-L. Ducluzeau, B. Schoepp-Cothenet, F. Baymann, M.J. Russell, W. Nitschke, Free energy conversion in the LUCA: quo vadis? Biochim. Biophys. Acta (2014).
[43] A.-L. Ducluzeau, B. Schoepp-Cothenet, R. van Lis, F. Baymann, S. McGlynn, M.J. Russell, W. Nitschke, J. Roy. Soc. Interface (2014).
[44] P.K. Glasoe, F.A. Long, Use of glass electrodes to measure acidities in deuterium oxide, J. Phys. Chem. U. S. 64 (1960) 188–190.
[45] G. Gilderson, L. Salomonsson, A. Aagaard, J. Gray, P. Brzezinski, J. Hosler, Subunit III of cytochrome *c* oxidase of *Rhodobacter sphaeroides* is required to maintain rapid proton uptake through the D pathway at physiologic pH, Biochemistry 42 (2003) 7400–7409.
[46] C. Backgren, G. Hummer, M. Wikström, A. Puustinen, Proton translocation by cytochrome *c* oxidase can take place without the conserved glutamic acid in subunit I, Biochemistry 39 (2000) 7863–7867.