The role of the D- and K-pathways of proton transfer in the function of the haem–copper oxidases

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Abstract

The X-ray structures of several haem–copper oxidases now at hand have given important constraints on how these enzymes function. Yet, dynamic data are required to elucidate the mechanisms of electron and proton transfer, the activation of O₂ and its reduction to water, as well as the still enigmatic mechanism by which these enzymes couple the redox reaction to proton translocation. Here, some recent observations will be briefly reviewed with special emphasis on the functioning of the so-called D- and K-pathways of proton transfer. It turns out that only one of the eight protons taken up by the enzyme during its catalytic cycle is transferred via the K-pathway. The D-pathway is probably responsible for the transfer of all other protons, including the four that are pumped across the membrane. The unique K-pathway proton may be specifically required to aid O–O bond scission by the haem–copper oxidases.

1. Introduction

Since comprehensive reviews on the structure and function of the haem–copper oxidases are available (see, e.g., [1,2]), only a brief general account is given here. Fig. 1 shows the overall catalytic scheme. Electron input occurs from cytochrome c on the outside of the membrane into the bimetallic Cuₐ centre, which donates electrons – one at the time – to the low-spin haem a (Feₐ). Feₐ, in turn, donates electrons to the haem a₃–copper centre (Feₐ₃/Cuₐ), which serves to bind and activate O₂ and to reduce it to water. For the reduction of O₂ there is – overall – uptake of eight protons from the opposite side of the membrane: four ‘substrate’ protons to complete the chemistry, and four to be translocated across the dielectric (proton-pumping). This picture is fairly well agreed upon, but it pertains only to the average functioning of the enzyme under multturnover, level flow, conditions. Following the pioneering work by Gibson and Greenwood [3] and Chance et al. [4], the recent years have seen an increasing amount of time-resolved studies of different parts of the enzyme’s catalytic cycle using spectroscopic and electrometric techniques. Combined with specific mutagenesis of bacterial haem–copper oxidases, as directed by the available X-ray structures [5–8], these studies have revealed several important aspects of the function, but they have also opened up new questions.

Site-directed mutagenesis experiments [9–12] and the crystal structures [5–8] have together revealed two proton transfer pathways that lead from the N-side of the membrane towards the haem–copper site (Fig. 2). It seems clear from these results (reviewed in Ref. [15]) that the D-pathway is used for the transfer of all protons to be pumped across the
membrane, as well as for uptake of ‘substrate’ protons during the oxidative phase of the catalytic cycle, and that the K-pathway is used at least for some of the proton uptake accompanying reduction of the binuclear site. Until recently, it was not clear whether the K-pathway is used for uptake of both or only one of the two protons taken up in this latter process. It is of crucial importance to determine precisely the role of these pathways in the different reactions of the catalytic cycle.

2. Protons and oxidoreduction of haem a

Recently, the idea [16,17] has been revived that reduction of haem a is linked to uptake of protons from the N-side of the membrane, to be later pumped across to the P-side [18,19]. However, titrations of CO-inhibited cytochrome c oxidase have failed to reveal redox-linked protonic coupling to an extent higher than, depending on pH, 0.2–0.4 H⁺ per haem a/Cu₄ pair [20,21]. Moreover, this weak protonic coupling could be attributed solely to the P-side of the membrane-embedded enzyme [20,21]. This stands in contrast to the claim that oxidation of the haem a/Cu₄ pair is coupled to release of ca. 0.75 protons to the P-side, and that reduction of this pair would cause uptake of the same number of protons from the N-side [22].

We reduced Paracoccus denitrificans cytochrome c oxidase with hexammine ruthenium (plus dithionite),
and compared the kinetics of haem $a$ reduction, as measured spectrophotometrically, in wild-type and in mutant enzymes where either the D (Asp124Asn, Glu278Gln or Glu278Ser) or the K pathway (Lys354Met) is blocked. In none of these four mutants was there any significant difference in the rate or extent of haem $a$ reduction (now shown, but see Fig. 4). We conclude, therefore, that the reduction of haem $a$ is unlikely to be linked to proton uptake via either of these pathways.

3. Protons and reduction of the binuclear centre

Ruitenberg et al. [23] recently reported that photo-injection of one electron into oxidised cytochrome $c$ oxidase from $P$. denitrificans results in fast generation of $\Delta \Psi$ due to electron transfer to Fe$_a$ (cf. [24]). These authors attributed a subsequent slower phase of $\Delta \Psi$ formation to proton uptake via the K-pathway to a site near the binuclear centre, linked to the prior reduction of Fe$_a$. As discussed above, however, it is improbable that proton uptake via either the K- or the D-pathway accompanies reduction of Fe$_a$. It seems more likely that it is the subsequent electron transfer from Fe$_a$ to the haem–copper centre that is coupled to proton uptake, and that this is responsible for the secondary electrometric phase observed in [23]. Fig. 3 (left panel) confirms these results, showing two distinct electrometric phases on flash-induced one-electron reduction of the $P$. denitrificans enzyme. Fig. 3 (right panel) shows the corresponding spectral change at 1 ms, with clear partial reduction of the binuclear site in addition to the reduction of Fe$_a$. This is better seen in Fig. 3 (inset), where the reduced minus oxidised spectrum of haem $a$ has been subtracted.

Fig. 4 shows the kinetics of haem reduction by hexammineruthenium in the $P$. denitrificans enzyme as measured spectrophotometrically. In this type of experiment, with excess reductant, the redox centres
of the enzyme are reduced essentially in an order that is determined by their midpoint potentials. The initial fast reduction of Feα is not resolved in the plots of Fig. 4 (but see above), and corresponds to the initial fast absorption change of ca. 0.4. This is followed by slower reduction of haem α3. The latter process is indeed decelerated in mutant enzymes where either the D (D124N, Fig. 4; or E278Q, not shown) or the K (K354M) pathway is blocked (cf. [26,27]). This substantiates the conclusion [19,23,28,29] that both pathways are used in the reductive phase, but in contrast to [19,23] we assign all proton uptake to be linked to reduction of the binuclear site rather than to reduction of Feα. This interpretation is also better in line with the observation that the secondary electrometric phase associated with input of the first electron (cf. above). Together with our present results this allows for an interesting conclusion: In agreement with [23] we conclude that proton uptake associated with the first electron transfer to the enzyme takes place via the K-pathway. However, we ascribe this proton uptake to be coupled to electron transfer to the binuclear site, and not to Feα. This first electron may be distributed between CuB and haem α3 if proton uptake via the K pathway is possible, and when there is a strong driving force (as in the experiment of Fig. 4). The second electron transfer into the site is linked to proton uptake via the D-pathway. Thus, a block of the K-pathway strongly retards electron transfer into Feα3, whereas a block in the D-pathway allows input of the first electron into the site at a virtually normal rate and allows it to equilibrate between Feα3 and CuB, but uptake of the second electron is retarded. Jünemann et al. [26] found evidence for reduction of CuB in the K-pathway mutant enzyme, and Ruitenberg et al. [23] found a residual slow (0.9 ms) electrometric phase with low relative amplitude in this mutant. Thus the first electron can probably be transferred into CuB even when the K-pathway is inhibited (cf. below).

While H+ transfer by the D-pathway has been relatively well substantiated, the K-pathway has alternatively been proposed to transfer OH− ions [30], or to be a ‘dielectric well’ that charge compensates electron input into the binuclear site [27,28]. The finding that a block in the K-pathway strongly inhibits a major electrogenic phase associated with input of the first electron [23] does not distinguish between these possibilities, nor does the deuterium isotope effect on this phase [23]. Input of the first electron might therefore not be associated with any net proton uptake, but with reorganisation of charges in the K-pathway, which must occur perpendicular to the plane of the membrane to produce the

Fig. 4. Kinetics of reduction of the haem groups in the wild-type (WT) cytochrome aα3 from P. denitrificans and in two mutant enzymes. Stopped flow measurements at 445 minus 470 nm with 25 μM ruthenium[II]hexamine and 10 mM sodium dithionite in one syringe and enzyme in 100 mM Tricine buffer (pH 8) and 0.1% dodecylmaltoside in the other syringe. Mixing ratio 1:1. Room temperature. The traces are normalised to the 7.2 μM wild-type enzyme concentration (before mixing). Note that the first fast phase due to reduction of Feα (amplitude ca. 0.4) is time-resolved in this experiment. Though not shown, this phase has the same rate and extent in wild-type and mutant enzymes.
electrometric response. If so, input of the second electron must be associated with uptake of two protons via the D-pathway. However, the observation by Ådelroth and Brzezinski [31] that net proton release coupled to oxidation of the binuclear site is inhibited in the K354M mutant enzyme is more consistent with the notion that the K-pathway is a true H$^+$ transfer device.

Ruitenberg et al. [23] found a 0.6/0.4 ratio for the amplitudes of the fast/slow electrometric phases on one-electron reduction, and this is confirmed in Fig. 3 (left panel). The fast phase corresponds to reduction of Fe$_a$ via Cu$_A$, which occurs across a dielectric fraction $d$ of the enzyme, where $d$ is the dielectric depth of Fe$_a$ (as well as of Fe$_{a3}$ and Cu$_B$) from the $P$-side of the membrane (see Fig. 1), whereas the slow phase corresponds to proton uptake from the $N$-side into the binuclear site. Since the slow phase probably corresponds to uptake of one proton (per electron), the authors in [23] concluded from the relative amplitudes that $d = 0.6$, which is almost twice the value of 0.32 estimated earlier [32] and in poor accord with the crystal structure [33]. However, the value of 0.6 for $d$ is probably extensively overestimated because the proton uptake is more likely the result of electron transfer to the Fe$_{a3}$-Cu$_B$ site (see above). Since this electron transfer is only partial, due to the similar redox potentials of Fe$_a$ and Cu$_B$ [24], the proton uptake coupled to it would be partial as well, in the same proportion. From data such as those in Fig. 3B we estimate that about 44% of the electron injected into Fe$_a$ is subsequently transferred to the binuclear site, which is in good agreement with the reported 20 mV redox potential difference between Cu$_B$ and Fe$_a$ [26]. From this, a value for $d$ just under 0.4 may be calculated, in reasonable agreement with the previous estimate [32] as well as with the crystal structure [33].
4. Conclusions

The fate of the two protons taken up on reduction of the binuclear site [34] is not known, but we presently favour the scenario shown in Fig. 5. The oxidised binuclear site (state O) may have a water ligand on Fe43 and an OH ligand on Cu8 [19,33,35], probably hydrogen-bonded to one another. In fact, it may be more accurate to state that the binuclear site metals share one OH ligand. The first electron preferentially reduces CuB since Fe43 has a lower mid-point redox potential at this stage. However, in order for CuB to accept the electron, the OH ligand must receive a proton. This can only occur from the aquo ligand next to it because the D-pathway does not connect to the binuclear site (vide infra). Next, the uptake of the first electron is charge-compensated by uptake of a proton via the K-pathway, possibly to the hydroxyethyl group of the farnesyl side chain of Fe43 at the end of this pathway (Ref. 36; Fig. 2). CuB, but not Fe43, may be reduced without this proton, as reported by Jönemann et al. [26]. Thus the proton uptake via the K-pathway is a prerequisite for the equilibration of the first electron with Fe43. The slow electrometric phase of low amplitude found by Ruitenberg et al. on injecting the first electron in the K354M mutant enzyme [23] may be due to some rearrangement of charges within the blocked K-pathway.

The formed cuprous CuB prefers trigonal geometry and releases the aquo ligand towards the hydrophobic cavity that leads towards Glu-278 and the D-pathway (Fig. 2). This water molecule establishes proton conduction from Glu-278 to the binuclear centre, i.e., it ‘opens’ the D-pathway (cf. [37]).

In the half-reduced state E, and with an operative K-pathway, the electron on CuB can equilibrate with Fe43, with corresponding exchange of the OH ligand. An EPR signal, which should be distorted by the nearby high spin ferrous haem iron, should be created from the fraction of the site in this state where CuB is cupric (Fig. 5), and this prediction can be tested experimentally.

The second electron taken up by the site is coupled to uptake of a second proton via the now active D-pathway, as first proposed by Michel [19]. However, we find it difficult to ascribe this proton uptake to be coupled to reduction of haem of (see above). Instead, we suggest that it protonates the OH ligand of CuB (Fig. 5). It is possible that protons transferred via the D-pathway can access the Fe43 site only by way of the oxygenous ligand on CuB. The K-pathway cannot be engaged further at this stage until the proton at the hydroxyethyl farnesy1 group has been consumed. Interestingly, the observations in Fig. 4 mean that the two pathways do not equilibrate protonically with one another via the binuclear site during the reductive phase.

We conclude that neither the original idea of K/D pathway segregation by transfer of ‘chemical’ and ‘pumped’ protons, nor that based on the reductive and oxidative phases of the catalytic cycle holds. Only one out of the eight protons taken up in the catalytic cycle (Fig. 1) is transferred via the K-pathway. Hence, the K-pathway proton must presumably have a very special role in the mechanism – a role that cannot be fulfilled by proton uptake via the D-pathway. Blomberg et al. [36] recently suggested such a role for this proton: it was proposed to be associated to the OH group of the hydroxyethyl farnesyl side chain of haem of, and H-bonded to the nearby conserved tyrosine residue (Fig. 2). Quantum chemical calculations of the O–O bond scission event in the binuclear site indicated the necessity of such an additional proton in order to promote hydrogen transfer from the tyrosine to the iron-bound dioxygen [36]. In its absence, the activation energy for O–O bond scission was found to be far too high for this process to take place. According to this mechanism, an additional proton must be present near the tyrosine side chain, which resides at the end of the K pathway, in order to make O2 reduction possible. Our present findings indeed suggest that the D-pathway cannot provide an alternative access to this site during reduction of the binuclear centre, and this is also apparent from the X-ray structures.

References


