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Biochimica et Biophysica Acta 1757 (2006) 1047–1051
BIOCHIMICA ET BIOPHYSICA ACTA
BBA
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Review

Towards the mechanism of proton pumping by the haem-copper oxidases

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Received 5 December 2005; received in revised form 28 January 2006; accepted 30 January 2006
Available online 24 February 2006

Abstract

The haem-copper oxidases comprise a large family of enzymes that is widespread among aerobic organisms. These remarkable membrane-bound proteins catalyse the respiratory reduction of dioxygen to water, and conserve free energy from this reaction by operating as proton pumps. The mechanism of redox-dependent proton translocation has been elusive despite the availability of high resolution crystal structures from several oxidases. Here, we discuss some recent as well as some older results that may shed light on this mechanism. We conclude that proton-pumping is initiated by vectorial proton transfer from a conserved glutamic acid (Glu242 in the bovine enzyme) to a proton acceptor above the haem groups, and that this primary event is mechanistically coupled to electron transfer from haem *a* to the binuclear haem a_3/Cu_B centre. Subsequently, Glu242 is reprotonated from the negatively charged side of the membrane. Next this proton is transferred to the binuclear site to complete the chemistry, Glu242 is reprotonated once more, and the "prepumped" proton is ejected on the opposite side of the membrane. The different kinetics of electron-coupled proton transfer in different steps of the catalytic cycle may be related to differences in the driving force due to different E_m values of the electron acceptor in the binuclear site.

Keywords: Electron transfer; Proton transfer; Energy transduction; Cell respiration

1. Introduction

Dioxygen reduction in the catalytic cycle of cytochrome *c* oxidase is fairly well understood (Fig. 1). Since O_2 reduction to water requires four electrons, and only one electron is transferred at the time to the binuclear haem a_3/Cu_B site, intermediate states of that site are formed in the cycle, and may be monitored by time-resolved spectroscopic techniques [1,2]. O_2 binding to the reduced binuclear site is followed by essentially a single step of internal four-electron reduction of the bound O_2 molecule, which generates the P_M intermediate: The O–O bond is broken and the two oxygen atoms are bound, one to the haem, which forms a ferryl species, and the other to Cu_B as an OH^- ligand. Two of the required electrons derive from haem a_3 and the third from Cu_B. The fourth electron equivalent presumably derives from a conserved tyrosine residue in the site forming a neutral tyrosyl radical [3]. The P_M state thus contains four oxidising equivalents and is analogous to the S4 state of the water-oxidising manganese cluster of Photosystem II. In each subsequent reaction step of the cycle, one electron is transferred to the binuclear site, accompanied by net uptake of a "substrate proton". Each of these four reaction steps is also coupled to pumping of one proton across the membrane [4].

Also shown in Fig. 1 is the alternative path taken when the fully reduced enzyme reacts with O_2, i.e., when apart from the binuclear site also the Cu_A and haem *a* centres are reduced prior to the reaction. In this much studied case, the A→P transition is accompanied by transfer of an electron from haem *a* to the binuclear site, in which case the product is the P_R state. The structure of P_R is otherwise similar to P_M, but the fourth electron required for O–O bond scission has been donated from haem *a*, rather than from the tyrosine [5]. Net protonation of the P_R state ensues next to form the F state, and this reaction has been shown to be linked to proton translocation [6,7]. It is of particular interest that this sequence of events is the only case known thus far where electron transfer into the binuclear site (i.e., during A→P_R) is kinetically distinguishable from net proton uptake and proton pumping (during P_R→F).

Abbreviations: $\Delta\Psi$, membrane potential; $E_{m,7}$, midpoint redox potential at pH 7; eT, electron transfer; N-side, negatively charged side of the membrane; pmf, protonmotive force or electrochemical proton gradient; P-side, positively charged side of the membrane; WT, wild type

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doi:10.1016/j.bbabio.2006.01.010

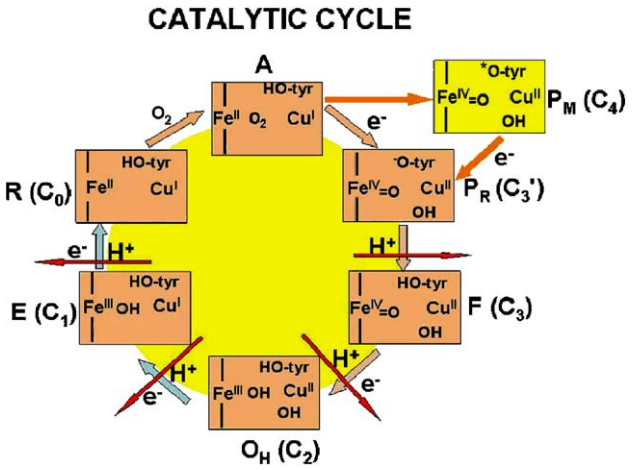

Fig. 1. Catalytic cycle of cytochrome c oxidase. The rectangles depict the binuclear centre with haem a_3, Cu_B and a conserved tyrosine residue in different states of the cycle. Pink arrows show the sequence when the fully reduced oxidase reacts with O_2. Blue arrows show the "reductive" phase. The subscript in the C state nomenclature describes the number of oxidising equivalents in the centre. Red arrows depict reactions that include proton pumping events. As shown in this paper, also the reaction $A \rightarrow P_R$ should be included in this category. H^+ describes net uptake of a proton to form the equivalent of water.

The other issue to be addressed here is the "reductive phase" of the catalytic cycle (O→R, Fig. 1), which has been shown to be coupled to proton translocation provided that it occurs immediately after the oxidative phase [4,8], but where the driving force for proton-pumping appears to be far too small.

2. Metastable catalytic states of the binuclear site

The cycle described above applies only for continuous turnover. Reduction of the oxidised enzyme (as isolated) from the O state to the R state is not linked to proton pumping. Therefore, a different O state (called O_H, or O~) was postulated to be an immediate metastable product of the oxidative phase of the cycle [4,8], which relaxes to O in the absence of an electron source. The structural and thermodynamic difference between these two states is not yet understood, but may well be related to the fact that reduction of state O to state R appears to be thermodynamically insufficient to drive proton translocation based on available E_m data: anaerobic redox titrations have shown that the midpoint redox potentials of haem a_3 and Cu_B are both <0.4 V [9]. Since the E_m of the donor, cytochrome *c*, is 0.26 V, the driving force is <140 mV, which is insufficient to drive translocation of two charge equivalents against a protonmotive force higher than 70 mV. Although some help may be obtained from high ratios of the relative occupancy of the redox couples involved ([O]/[E] and [E]/[R]), these ratios would have to attain such extremes to yield sufficiently high acceptor potentials that kinetic performance (e.g., the rate of O_2 binding) would be compromised. The apparently very low driving force for the redox reactions in the reductive phase contrasts to the much higher force in the oxidative phase, where the E_m values of the tyrosine radical and ferryl haem electron acceptors are expected to be in the 0.8 V regime ([10,11]; see below).

The above survey suggests that the E_m of reduction of the metastable O_H state might be considerably higher than the E_m of O. This possibility may be tested by a different independent approach. The sum of the free energies of the partial reactions in the catalytic cycle must be equal to the overall free energy for the reduction of O_2 (E_m ~0.8 V per electron) by cytochrome *c* (E_m ~0.26 V), i.e., ~2.2 eV. The $E_{m,7}$ values for the P_M/F and F/O redox couples may be estimated from the results of redox titrations in mitochondria at high protonmotive force ([12]; see Table 1). The effect of protonmotive force (pmf) may be corrected for, using the current knowledge that each of the two transitions is coupled to translocation of two electrical charge equivalents across the membrane [4], and this indeed yields $E_{m,7}$ values near 0.8 V for these redox couples at zero pmf (Table 1). It should be noted here that our previously estimated $E_{m,7}$ values for these redox couples were too high [13] because at that time it was assumed that each of these reaction steps would be linked to pumping of two protons in addition to the translocation of one charge due to electron transfer and uptake of the substrate proton. The driving forces for the O/E and E/R reactions are obtained here by assuming $E_{m,7}$=0.4 V for both couples (see above). The remaining reactions in the cycle, i.e., O_2 binding to the R state, and conversion of R into P_M, have free energies of ca. 0 and −5 kcal/mol (~0.22 eV), respectively [14,15]. The overall driving force obtained in this way is ca. 1.6 eV (Table 1). Although conservative energies have been used in this estimate, this is still 0.6 eV short of the driving force for the entire cycle (2.2 eV), which supports the contention that the driving forces during the reductive phase in the active enzyme may be considerably higher than those deduced from the E_m values measured in anaerobic redox titrations.

3. The $O_H \rightarrow E$ reaction step

Preliminary data has shown that photochemical electron injection into the enzyme in the O_H state results, first, in fast reduction of Cu_A, followed by reduction of heme *a* by Cu_A (τ ~10 μs for the enzyme from *P. denitrificans*). The latter electron transfer is accompanied by the first phase of $\Delta\Psi$ generation with the same kinetics (cf. [4]). Then follows two

Table 1
Measured $E_{m,7}$ values and driving forces in the catalytic cycle

	E_m(obs) [mV]	$E_{m,7}$(obs) [mV]	$E_{m,7}$(pmf=0) [mV]	$-\Delta G$ [eV]
$P_M \rightarrow F$	375 (pH 7.7)	417	817	0.56
$F \rightarrow O$	350 (pH 7.2)	362	762	0.50
$O \rightarrow E$			400	0.14
$E \rightarrow R$			400	0.14
$R \rightarrow A$				0
$A \rightarrow P_M$				0.22
Sum of driving forces				1.56

The observed E_m values (E_m(obs)) are from redox titrations in intact mitochondria at a high pmf [12]. They are corrected to pH=7 by assuming that one net proton is taken up on reduction [29]. The correction to pmf=0 assumes that each reaction is associated with translocation of two electrical charges across the membrane [4,8], and that the pmf is 200 mV. The driving force ($-\Delta G$) is obtained from the difference in redox potential between cytochrome *c* (0.26 V) and the $E_{m,7}$ (pmf=0) of the acceptor redox couple (see text).