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Prevention of leak in the proton pump of cytochrome *c* oxidase

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ABSTRACT

The cytochrome *c* oxidases (CcO), which are responsible for most O_2 consumption in biology, are also redox-linked proton pumps that effectively convert the free energy of O_2 reduction to an electrochemical proton gradient across mitochondrial and bacterial membranes. Recently, time-resolved measurements have elucidated the sequence of events in proton translocation, and shed light on the underlying molecular mechanisms. One crucial property of the proton pump mechanism has received less attention, *viz*. how proton leaks are avoided. Here, we will analyse this topic and demonstrate how the key proton-carrying residue Glu-242 (numbering according to the sequence of subunit I of bovine heart CcO) functions as a valve that has the effect of minimising back-leakage of the pumped proton.

1. Introduction

Cytochrome *c* oxidase (CcO) functions as a genuine proton pump, as distinguished from the bc_1 complex and the photosynthetic reaction centre, which operate according to the Mitchellian redox-loop principle [1,2]. Whereas several proposals have been made for the mechanism of proton pumping in CcO, less consideration has been given to the essential question of how leaks or short-circuits are prevented (but see Refs. [2–5]). The problem of short-circuits can also arise in redox-loop mechanisms, as recently discussed for the bc_1 complex [6], but is of particular concern for proton pumps. A true proton pump mechanism must, by definition, include proton transfer across the entire membrane dielectric, but paradoxically, if such continuous proton conductance is allowed, the pump will be compromised by leaks. This fact has certainly been realised, but its implications on the mechanism have only been rarely addressed.

Recent time-resolved electron and proton transfer studies of the cytochrome oxidase reaction [7–9] have stimulated a more thorough analysis of the mechanism, also in terms of how proton leaks may be prevented [2,4]. In this regard, the insightful work by Siegbahn and Blomberg [4] is particularly relevant in that it not only stresses the importance of avoiding such leaks, but also analyses the properties of the pump mechanism that are required to that effect.

A mechanistic proton pump sequence was developed on the basis of the experiments by Belevich et al. [7], where electron injection into the activated form of the oxidised enzyme was studied. This sequence starts with electron transfer from the Cu_A centre to heme *a* across ca. 1/3 of the membrane dielectric (Fig. 1). Reduction of heme *a* is suggested to raise the pK_a of a proton-loading site (PLS), the identity of which is still uncertain, but which may be the A-propionate group of heme a_3 [2,4]. Next, there is proton transfer to the PLS, initially from Glu-242[1], with subsequent reprotonation of the latter via the so-called D-pathway. The proton at the PLS raises the mid-point redox potential of heme a_3, enabling it to receive the electron from heme *a*, and resulting in further elevation of the pK_a of the PLS. The following reaction step is crucial for the mechanism, *viz*. proton transfer from the N-side of the membrane to the binuclear site to form the equivalent of water, and annihilating the charge of the electron at that site. Neutralisation of the charge in the binuclear site lowers the pK_a of the PLS, providing the driving force for proton ejection from the PLS to the P-side of the membrane to complete the reaction sequence.

It is in this last step in the sequence that one of the crucial questions concerning leakage arises, *viz*. why is the proton at the PLS not transferred backwards to the N-side of the membrane instead of being released on the P-side? This concern is of particular importance under conditions with a substantial electrochemical proton gradient across the membrane. Siegbahn and Blomberg [4] indeed identified this to be a key leakage problem of the proton pump that is not easily explained; their explanation depended on an assumed nature of the transition state of proton transfer between the PLS and the N-side of the membrane. Our recent molecular dynamics studies of the rotational isomerisation of the side chain of the residue Glu-242 may give an alternative explanation. Glu-242 may not only be a

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[1] Glu-242 is numbered on the basis of the subunit I structure of bovine heart CcO. The corresponding number is 278 in CcO from *P. denitrificans* and 286 in CcO from *Rh. sphaeroides* and the bo_3 quinol oxidase from *E. coli*.

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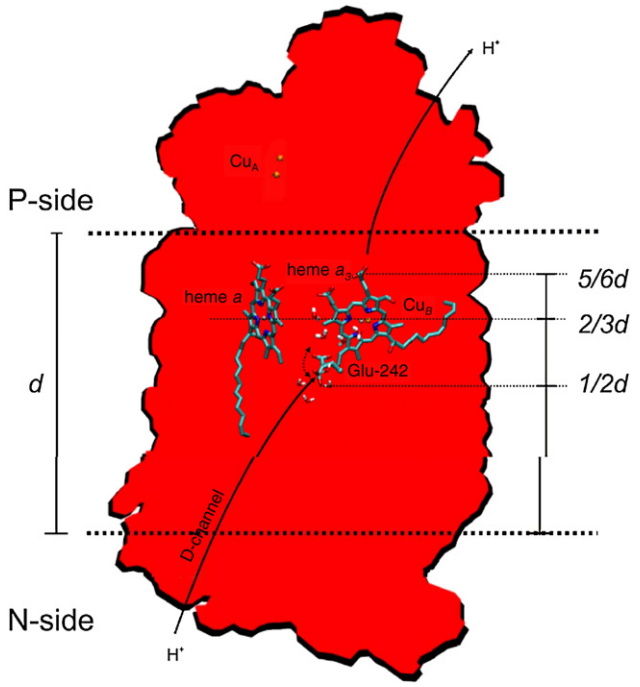

Fig. 1. The role of Glu-242 in proton transfer by cytochrome *c* oxidase. Two positions of the Glu-242 side chain are depicted, "up" and "down". In the up state the carboxylic acid either makes contact with the D-propionate of heme a_3 or with the binuclear heme a_3/Cu_B centre, both mediated by water molecules in the nonpolar cavity above Glu-242. In the down state the side chain makes contact with the N-side of the membrane via the so-called D-channel of proton transfer, again mediated by water molecules. The pumped proton is proposed to be transferred from Glu_{up} to a proton-loading site, possibly the A-propionate of heme a_3, before being released to the P-side of the membrane. Approximate relative distances across the dielectric barrier (*d*) are given for different positions along the proton transfer trajectories.

passive proton-shuttling devise, but may also function as a valve that effectively prevents proton backflux in the crucial transition considered above.

2. Dynamics and energetics of Glu-242 side chain isomerisation

The side chain of Glu-242 is directed "down" towards the D-pathway of proton transfer in all the X-ray structures, which does not allow proton transfer from this residue to the binuclear site or for proton pumping (Fig. 1). Yet, the evidence for involvement of Glu-242 in the latter events is substantial [10–13], and it has long been thought that the side chain of Glu-242 must be able to rotate to an "up" conformation towards the binuclear centre (Fig. 1 and Refs. [14–16]), where it can donate a proton either to the binuclear site or to the D-propionate of heme a_3, which is the likely transient proton acceptor for the proton to be pumped [17–19]. The latter functions are not possible without water molecules in the nonpolar cavity "above" Glu-242; evidence for such water molecules has so far been obtained only by computational methods [14,15,20–24].

We have shown by molecular dynamics simulations that the position of the protonated side chain of Glu-242 strongly depends on the hydration state [25,26]. With no water molecules added to the studied CcO structure from bovine heart mitochondria (PDB 1V54; [27]) the X-ray "down" position was favoured by a factor of ~20, but adding four water molecules to the nonpolar cavity "above" Glu-242 changed the bias towards the "up" position [25]. However, the small cavity "below" Glu-242 is also devoid of water molecules in the 1V54 structure, so that there actually does not seem to be protonic contact between the D-pathway and Glu_{down}. However, prolonged MD simulations filled this void with four water molecules [26]. Interestingly, these latter water molecules are present in the X-ray structure of the *Rh. sphaeroides* enzyme [28], which increases our confidence in the MD results. Our recent simulations with hydrated cavities both above and below Glu-242 revealed a "down/up" equilibrium constant ranging between ca. 20 and 1 for the protonated side chain, depending on the redox state of heme *a* and the binuclear site. However, the behaviour of the anionic, deprotonated side chain was drastically different. In this case Glu_{down} was favoured by a factor of at least 10^4, and the rate of "downflip" was very fast (~1 ps^{-1}), being at least in part coupled to electrostatic repulsion from the heme propionate groups. We believe that this kinetic and thermodynamic asymmetry in the positioning of the side chain may be an essential property that minimises proton leakage, as summarised below.

3. Glu-242 is a valve that prevents proton leakage

The asymmetric dynamics and energetics of the Glu-242 side chain isomerisation described above has two consequences, kinetic and thermodynamic. After proton transfer from Glu_{up} to the binuclear site, a microstate vulnerable to leakage is encountered where the anionic Glu-242 is in the up position. This is the situation described above, where the proton previously pumped into the PLS should be ejected towards the P-side of the membrane due to the lowered pK_a of the PLS, but where the anionic Glu_{up} is an alternative acceptor of the proton. If Glu-242 indeed accepts the proton in this situation, the pump will be compromised since the chemistry at the binuclear site has been completed without proton translocation across the membrane. For this reason it is essential that Glu^-_{up} will rotate to the down position at a rate that is at least two orders of magnitude faster than back-transfer of the proton from the PLS to Glu^-_{up}, in order to outcompete the latter and to secure a H^+/e^- proton translocation stoichiometry of at least 0.99. We believe that the observed very fast downflip (~1 ps^{-1}) achieves this purpose.

However, the fast downflip of the anionic side chain is not sufficient to prevent leakage, except transiently. In the steady state, where flux through the system is determined by the relatively slow release of the proton from the PLS (ca. 2.5 ms^{-1}, see Ref. [7]), thermodynamic equilibrium will be approached between the microstates prior to this rate-limiting step, among them the vulnerable state with a proton at the PLS and the Glu-242 anion in the up position. For this reason the relative steady state population of this state must be low enough to minimise the rate of backflux, which is where the down/up equilibrium constant of $>10^4$ for Glu^- becomes essential. This thermodynamic property of the side chain isomerisation ensures that the actual rate of leakage will be at least 10,000 times slower than the rate constant of back-transfer of the proton from the PLS to Glu-242.

It has not escaped our attention that the above analysis is strictly valid only for states in which the protonmotive force is relatively low across the membrane. A high protonmotive force will modulate the local pK_a values of Glu-242 and will also change the equilibrium distribution between the "up" and "down" states of Glu^-, albeit the latter effect is expected to be relatively small due to the short distance between the two states relative to the membrane dielectric. A high protonmotive force will obviously tend to enhance proton leakage of the pump mechanism, and such leakage has been reported [29,30], possibly directly affecting the "Glu-242 switch". However, careful measurements of the proton-pumping stoichiometry in actively phosphorylating mitochondria [31] provide no evidence for such leakage under conditions ("State 3"), where the protonmotive force approaches values near 170 mV (see Ref. [32]). Whether leakage occurs at higher protonmotive force is very difficult to assess experimentally (but see Refs. [29,30]).

Finally, Prutsch et al. [33] ruled out a gating function of Glu-242, presumably on the basis that this residue is absent in certain thermophilic heme-copper oxidases that nevertheless pump protons. However, such proton-pumping experiments are typically performed at zero protonmotive force where the tendency to leak is minimal. Our notion of the role of Glu-242 as a valve would suggest that these outlying oxidases might not sustain proton pumping at higher protonmotive forces.

4. Conclusion

The basic rationale for the function of Glu-242 as a valve that prevents proton backflux is based on the observation that the Glu-242 side chain is in contact with the P-side of the membrane (via the D-propionate of heme a_3) only in its "up" position, and in contact with the N-side (via the D-pathway) only in the "down" position. Hence, a key role of Glu-242 is to ensure that there will at no point in time be continuous proton conductivity across the entire dielectric, which is an essential property of the proton pump. Whilst this more general description has been obvious for some time, the asymmetric kinetic and thermodynamic properties of the side chain isomerisation in its protonated and deprotonated states completes the picture and specifically explains how backflux of the proton in the PLS is prevented, relative to its ejection towards the P-side.

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