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Role of subunit III and its lipids in the molecular mechanism of cytochrome *c* oxidase

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ABSTRACT

The terminal respiratory enzyme cytochrome *c* oxidase (C*c*O) reduces molecular oxygen to water, and pumps protons across the inner mitochondrial membrane, or the plasma membrane of bacteria. A two-subunit C*c*O harbors all the elements necessary for oxygen reduction and proton pumping. However, it rapidly undergoes turnover-induced irreversible damage, which is effectively prevented by the presence of subunit III and its tightly bound lipids. We have performed classical atomistic molecular dynamics (MD) simulations on a three-subunit C*c*O, which show the formation of water wires between the polar head groups of lipid molecules bound to subunit III and the proton uptake site Asp91 (*Bos taurus* enzyme numbering). Continuum electrostatic calculations suggest that these lipids directly influence the proton affinity of Asp91 by 1–2 p*K* units. We surmise that lipids bound to subunit III influence the rate of proton uptake through the D-pathway, and therefore play a key role in preventing turnover-induced inactivation. Atomistic MD simulations show that subunit III is rapidly hydrated in the absence of internally bound lipids, which is likely to affect the rate of O_2 diffusion into the active-site. The role of subunit III with its indigenous lipids in the molecular mechanism of C*c*O is discussed.

1. Introduction

The respiratory chains of mitochondria and many bacteria terminate in cytochrome *c* oxidase (C*c*O), which catalyzes the reduction of molecular oxygen to water, and couples the free energy of the reaction to proton pumping across the membrane [1–3]. The oxygen reduction reaction catalyzed by C*c*O is spectacular in the sense that it proceeds without any production of toxic reactive oxygen species. The electrons and protons required for oxygen reduction are supplied from the P- and N-side of the membrane, respectively, which leads to the generation of an electrical gradient across the membrane [2,3]. The concurrent translocation of protons from the N-side to the P-side further contributes to the established proton electrochemical gradient (or proton motive force — *pmf*) [1]. The *pmf* is then utilized by the ATP-synthase to generate ATP from ADP and inorganic phosphate (P_i) [2,3].

The structures of most of the intermediates of the catalytic cycle of C*c*O are known [3,4]. The thermodynamics and kinetics pertaining to elementary electron/proton transfer steps are also well understood [3,4]. Several high resolution crystal structures of C*c*O in different redox states are available [5–9], which together with site-directed mutagenesis studies have assisted in the identification of proton transfer pathways buried deep within the enzyme [10,11].

Overall, C*c*Os are classified into three subtypes: A, B, and C [12]. The three largest membrane-bound subunits (I–III) are highly conserved in the A-type mitochondrial and bacterial oxidases. Subunits I and II harbor all the necessary components that are required to catalyze oxygen reduction and proton pumping, whereas the role of subunit III, which does not contain any redox centers or known proton-conducting pathways, remains less clear. However, it is well established that subunit III provides additional stability to the enzyme while it undergoes turnover [13–15]. The catalytic activity of a two-subunit enzyme at pH = 8 is about 10–100 fold lower than when subunit III is present [15]. In contrast, at pH 6.5 and below, a two-subunit enzyme displays near wild-type activity [15]. This suggests that the presence of subunit III is critical for enzymatic activity, especially at high pH. An additional consequence of the absence of subunit III is that the enzyme rapidly undergoes turnover-induced inactivation (also called 'suicide inactivation'), by which it is irreversibly damaged [13,14]. The molecular mechanism of this turnover-induced impairment is not clear, and various possibilities such as slower rate of proton transfer via the D-pathway, and/or subsequent destabilization of the active-site, or loss of Cu_B, have been proposed [14,16]. Indeed, the N-terminal segment of subunit III houses

Abbreviations: MD, molecular dynamics; PE, phosphatidylethanolamine; PC, phosphatidylcholine; CL, cardiolipin; C*c*O, cytochrome *c* oxidase.

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histidine residues that may form a proton-buffering region, in the absence of which a slower rate of proton transfer via the D-pathway can be expected [17].

Lipids are known to play important roles in the molecular mechanism of many membrane proteins [18–20]. In the case of inner-mitochondrial membrane proteins, cardiolipins constitute the most studied and discussed lipid group [21]. It is therefore not surprising that the crystal structures of many membrane proteins have revealed tightly bound cardiolipin molecules [18,22]. Indeed, mitochondrial C*c*O is known to bind cardiolipin molecules together with other phospholipids [22,23]. The alteration of some of these lipid binding-sites destabilizes inter-subunit interactions, and also affects the catalysis [24]. Both coarse-grained and atomistic molecular dynamics (MD) simulations have assisted in highlighting the importance of cardiolipin–protein interactions in C*c*O, as well as in the cytochrome bc_1 complex [25–27].

In this study, we concentrate on the function of lipid molecules tightly bound in the V-shaped cleft formed by two clusters of transmembrane helices of subunit III [5–7,28,29] (Fig. 1). X-ray structures from different organisms show that phosphatidyl glycerol (PG), phosphatidyl ethanolamine (PE), and phosphatidyl choline (PC) lipids are found at this location [5–7,28,29]. The lipid molecules are stabilized through conserved electrostatic and hydrophobic contacts within the protein structure [22,30]. Based on X-ray structure analysis and site-directed mutagenesis studies, it has been proposed that the hydrophobic interior of subunit III forms a local reservoir for O_2, and the entrance to an O_2 diffusion pathway from the membrane into the active-site [31].

1.1. Rationale

Based on the observations that subunit III binds two lipid molecules, and any alteration of the lipid binding site results in a phenotype similar to a subunit III-deficient enzyme (suicide inactivation) [30], we hypothesize that the lipid molecules of subunit III may play an important role in channeling the protons towards Asp91, which may modulate the rate of proton transfer in the D-pathway. These viewpoints are tested here by computational methodology.

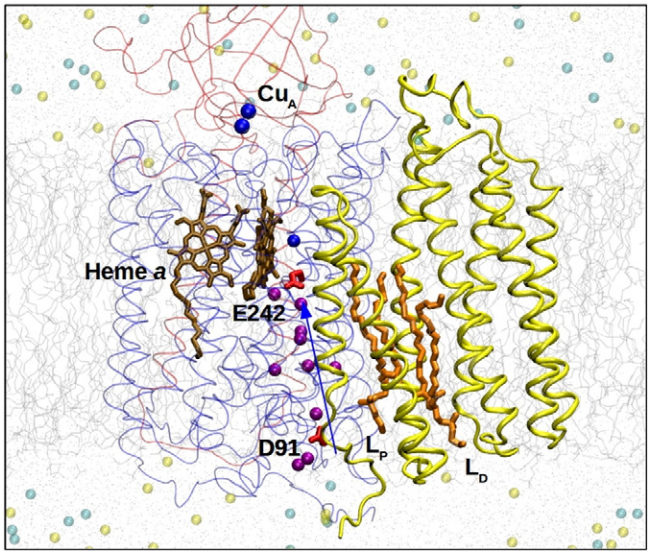

Fig. 1. Structure of C*c*O in a membrane-solvent environment. Subunits I (blue), II (red), and III (yellow/thicker) are shown as ribbons. Hemes, copper atoms, and subunit III bound lipids are shown in brown, blue, and orange, respectively. Lipids proximal (L_P) and distal (L_D) to the proton-uptake site (D91, in red) are marked. The D-pathway (blue arrow) terminates at a highly conserved residue near the active-site (E242, shown in red). Crystallographic water molecules present in the D-pathway, and Na^+ and Cl^- ions are shown as purple, blue, and yellow spheres, respectively.

2. Computational methods

2.1. MD simulations

Classical MD simulations were performed on a three-subunit C*c*O (PDB id 1V54 [5]) immersed in a hybrid lipid bilayer comprising DLiPC (1,2-dilinoleyl-sn-glycero-3-phosphatidylcholine), DLiPE (1,2-dilinoleyl-sn-glycero-3-phosphoethanolamine) and CL (4-linoleic cardiolipin) molecules (Fig. 1). The ratio of CL:DLiPC:DLiPE molecules in the system was 1:3.38:3.05, in agreement with our earlier studies [27,32]. The membrane-protein system was solvated with TIP3 water molecules, and Na^+ and Cl^- ions. The conserved lipid binding site in subunit III was modeled with or without lipids. Different types of lipids were modeled at the site; 1 CL, 2 PG or 2 PE molecules (Table 1). The model system comprised ca. 280,000 atoms. All amino acid residues were considered in their standard protonation states, except functionally critical residues for which protonation states are known from experimental data [3,32]. Glu242, Asp364 and Lys319 from subunit I were protonated, whereas Tyr244 was deprotonated (see below in this Section 2.1). 20 to 150 ns MD runs were performed on the constructed model systems. The NAMD [33] program was used to perform the simulations using the CHARMM [34] force field. The charges and parameters of metal centers were obtained from Johansson et al. [35]. In all simulations, the low-spin heme was reduced and the binuclear center was in the P_M state (ferryl heme a_3, cupric Cu_B with a fourth OH^- ligand, and Tyr244 as the neutral radical). Simulation parameters were T = 310 K for temperature, $p = 1$ atm for pressure, 1 fs for the timestep, and particle mesh Ewald (PME) used for computing electrostatic interactions [36]. Total time comprising all simulations was ~0.78 μs. Analysis of simulation trajectories was done with the help of the VMD program [37].

2.2. Continuum electrostatic calculations

The electrostatic calculations were performed on snapshots obtained from MD simulations. A total of 50 snapshots were taken from individual simulation trajectories. The MEAD package [38,39] was used to solve the linearized Poisson–Boltzmann equation. The intrinsic pK_as and site–site interaction energy data from MEAD calculations were used in the KARLSBERG [40,41] program to estimate the final pK_a. The p$K_{a,model}$ of Asp, Glu, Arg, Lys, Tyr and His was 4.5, 4.6, 12.0, 10.4, 9.6 and 6.2, respectively. The propionate groups of hemes, and the oxygenous ligand of Cu_B were also treated as titratable groups with p$K_{a,model}$ of 4.8 and 9.5, respectively.

3. Results

3.1. Proton funneling groups

We surmise that the headgroups of the lipids bound to subunit III might play an important role in channeling the protons towards Asp91 at the entrance of the D-pathway. Such information is not evident

Table 1
Simulation setup and time.

Simulation	Type of lipid modeled in su III	Time (ns)
1	No lipid (run I)	150
2	No lipid (run II)	150
3	2 PE molecules	100
4	2 PG molecules	50
5	1 CL molecule	100
6	2 PE molecules[a]	100
7	2 PE molecules/Asp91 protonated	60
8	2 PE molecules/Asp91Asn mutant	50
9	No lipid/Asp91Asn mutant	20

[a] Lipid binding site mutants; R96A in subunit I, and R221A, W58A and F86A in subunit III.