The influence of subunit III of cytochrome c oxidase on the D pathway, the proton exit pathway and mechanism-based inactivation in subunit I

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Abstract

Although subunit III of cytochrome c oxidase is part of the catalytic core of the enzyme, its function has remained enigmatic. Comparison of the wild-type oxidase and forms lacking subunit III shows that the presence of subunit III maintains rapid proton uptake into the D pathway at the pH of the bacterial cytoplasm or mitochondrial matrix, apparently by contributing to the protein environment of D132, the initial proton acceptor of the D pathway. Subunit III also appears to contribute to the conformation of the normal proton exit pathway, allowing this pathway to take up protons from the outer surface of the oxidase in the presence of \(D_{W}\) and \(D_{pH}\). Subunit III prevents turnover-induced inactivation of the oxidase (suicide inactivation) and the subsequent loss of Cu B from the active site. This function of subunit III appears partly related to its ability to maintain rapid proton flow to the active site, thereby shortening the lifetime of reactive \(O_2\) reduction intermediates. Analysis of proton pumping by subunit III-depleted oxidase forms leads to the proposal that the trapping of two protons in the D pathway, one on E286 and one on D132, is required for efficient proton pumping.

Keywords: Cytochrome oxidase; Proton pathway; Proton pumping; Suicide inactivation

Subunits I, II, and III of cytochrome c oxidase are encoded in the mitochondrial genome of eukaryotes and are evolutionarily conserved from bacteria to humans. These three subunits comprise the catalytic core of the mitochondrial oxidase and essentially all of the \(aa_3\)-type bacterial cytochrome c oxidases [1–4]. Subunit III is as highly conserved as subunit I (45% vs. 47% identity between the \(\alpha\)-proteobacter \(Rhodobacter sphaeroides\) and human subunits III and I, respectively) and considerably more conserved than subunit II (29% identity between subunit II of \(R. sphaeroides\) and humans). Subunit III contains no metal centers and therefore has no direct role in electron transfer. Subunit III has been implicated in proton pumping by cytochrome oxidase beginning with reports that labeling a DCCD-reactive glutamate in subunit III affected proton pumping more than electron transfer [5]. The removal of subunit III from bacterial and mitochondrial oxidase decreases the efficiency of proton pumping in reconstituted systems [6,7]. The reasons for this have remained unclear, as has the molecular mechanism of the pump itself.

Protons required for the synthesis of water, as well as those that are pumped through the protein, are taken up from the inner surface of the protein (facing the mitochondrial matrix or bacterial cytoplasm) and transferred \(\sim 30\) Å through two independent pathways to the vicinity of the buried heme \(a_3–Cu_B\) active site. The first of these is the K pathway, named for a conserved lysine (Fig. 1). The K pathway is responsible for the uptake of one or two protons during the ‘reductive’ phase of the catalytic cycle, when heme \(a_3\) and Cu B are being reduced before the binding of \(O_2\) [8–11]. The second is the D pathway, named for D132, the conserved aspartic acid residue that serves as the initial proton acceptor. The D pathway transfers the remaining ‘substrate’ protons to the active site for \(O_2\) reduction, plus all of the pumped protons [11–14]. In the D pathway, a series of ordered water molecules, plus an asparagine residue (N139), forms a hydrogen-bonded proton-conductive pathway between D132 on the inner surface of the oxidase and E286, located \(\sim 26\) Å above D132 [4]. Protons are apparently transferred from E286 to the heme \(a_3–Cu_B\) center, a distance of 10–12 Å, through another series of waters. Protons from E286 are also transferred to a site involved in proton pumping, which may be at or near the heme \(a_3–Cu_B\) active site [15–19]. Pumps protons must be transferred from the site of pumping
to the outer surface of the oxidase. The components of the proton exit pathway leading from E286 to the outside of the oxidase are yet to be identified. A hydrogen-bonded network of arginines, histidines, and carboxylic groups above hemes $a$ and $a_3$ are possible components [20,21]. If proton transfer through the D pathway is strongly inhibited, the protons required for continued low rates of O$_2$ reduction appear to be supplied from the outer surface of the protein, probably by reversal of the normal exit pathway for pumped protons [22,23]. Our current evidence indicates that subunit III strongly influences the function of both the D pathway and the exit pathway for protons [24,25]. Subunit III also acts to prevent the rapid demise of the enzyme by protecting the active site from deleterious chemistry that leads to the loss of Cu$B$ [26]. Here we present evidence that slowed proton transfer to the active site through the D pathway accelerates the rate of turnover-induced (suicide) inactivation and we present a model to explain the apparent requirement of D132 for proton pumping.

1. Cytochrome oxidase suicide inactivates in the absence of subunit III, with the loss of Cu$B$

Haltia et al. [27] prepared two active forms of the mitochondrial-like cytochrome $c$ oxidase of Paracoccus denitrificans that lacked subunit III. In one form, subunit III was removed from wild-type oxidase using the detergent Triton X-100 (Triton was subsequently removed) while the other form was purified from cells lacking the gene for subunit III [28]. The absence of subunit III caused little or no difference in the thermal stability of the subunit I–II component of the resting oxidase. However, both of the subunit III-depleted oxidase forms rapidly lost activity during catalytic turnover while the oxidase containing subunit III did not [27]. We have prepared equivalent subunit III-depleted forms of the $aa_3$-type cytochrome $c$ oxidase of R. sphaeroides [26,29]. The oxidase in which subunit III is removed using Triton is termed WT III ($-$) while the oxidase that is synthesized in the absence of subunit III is termed I–II oxidase. The initial activities of WT III ($-$) and I–II oxidase are generally similar to the normal oxidase ($V_{max} = 1600–1800$ s$^{-1}$), but both forms spontaneously and irreversibly inactivate (suicide inactivate) during steady state turnover [26]. It might be expected that the isolated I–II oxidase would show low activity due to turnover-induced inactivation of the enzyme in vivo, especially since the I–II oxidase does appear to inactivate during turnover in purified cytoplasmic membranes when near-saturating amounts of horse heart cytochrome $c$ are used as substrate (data not shown). However, cells expressing the I–II oxidase also contain two additional terminal oxidases. These are a $cbb_3$-type cytochrome $c$ oxidase [30,31,35], present in high amounts, and a $bd$-type quinol oxidase [31,32]. In the presence of these two alternative terminal oxidases, the flux of electron flow through the $aa_3$-type oxidase in vivo may be limited, leading to little accumulation of the inactivated enzyme in cells grown only to mid-log phase.

Suicide inactivation of enzymes is classically induced by the addition of a suicide substrate, often a compound that is oxidized during catalysis to a radical species that goes on to bind to amino acid residues in the active site. In contrast, suicide inactivation of cytochrome oxidase is the spontaneous loss of activity during normal turnover, more analogous to the inactivation of prostaglandin H synthase and other
O2-activating enzymes [33,34]. Suicide inactivation of cytochrome oxidase follows single exponential kinetics when followed as the loss of O2 reduction activity with increasing catalytic cycles (CC = O2 → 2H2O) [26]. The rate of suicide inactivation is inversely proportional to the CC50 value, which is the number of catalytic cycles required for O2 reduction activity to drop to half the original rate (i.e., the half-life of the oxidase in terms of catalytic cycles). At pH 7.5, the wild-type oxidase shows a CC50 value >3 × 106, while under identical conditions WT III (−) and the I–II oxidase each exhibit a CC50 of approximately 3000. Therefore, at pH 7.5, the catalytic lifetime of the subunit III-depleted enzymes is less than 0.1% that of the wild-type oxidase that contains subunit III. Reconstitution of WT III (−) or the I–II oxidase into phosphatidylethanolamine vesicles has no protective effect and the elimination of exogenous reactive oxygen species (by adding catalase and superoxide dismutase) also does nothing to slow the rate of inactivation [26]. Detailed spectroscopic analysis of inactivated WT III (−) and I–II oxidase indicated structural alterations at the heme α3–CuB active site [26]. In particular, loss of CuB appeared to be the primary result of suicide inactivation.

Three histidines, H333, H334, and H284, serve as the ligands of CuB [4,36–39]. The crystal structures of cytochrome oxidase show a covalent bond between the imidazole ring of H284 and the phenyl ring of Y288 [39–41]. The Babcock group was quick to suggest a role for a Y288 radical in O2 reduction chemistry [42] and to provide direct evidence for formation of a Y288 radical during formation of the oxyferryl intermediate P [43]. We have determined, via EPR spectroscopy and metal analysis, that CuB is completely lost from the oxidase as a result of suicide inactivation. The EPR spectrum of the inactivated oxidase is very similar to that of the ΔCuB oxidase that assembles in cells lacking Cox11p, a copper chaperone [44] that appears to function in the insertion of CuB [45]. The EPR spectra of ΔCuB [45] and the inactivated oxidase both show a strong signal arising from high-spin heme α3; heme α3 is normally EPR silent due to its spin interaction with CuB [46]. Unpublished EPR spectra of H284 and Y288 mutants, obtained in the Babcock laboratory (~1990), show equivalent amounts of high spin heme α3, consistent with significant loss of CuB from these mutants. (Metal analysis of Y288F has also indicated loss of copper [47].) The H284 and Y288 mutant oxidases also exhibit another hallmark of the inactivated oxidase, namely extremely slow binding of CO to reduced heme α3 [26,37]. From these results, we propose that the loss of CuB following suicide inactivation may be due to dissociation of His-284 from the metal as a result of structural alteration of the H284–Y288 dimer. Proshlyakov et al. [43] have argued that the bond between H284 and Y288 is necessary to stabilize the orientation of Y288 with respect to oxyferryl intermediate P. Subunit III may also play a role in stabilizing active site conformers during O2 reduction, particularly if ligand rearrangements occur. For instance, the absence of subunit III may allow increased motion of H284 and Y288, thereby increasing the probability of oxygenation or further cross-linking of the side chains of these residues in the P state, where a radical form of Y288 is proposed to exist [42,43]. This could result in the dissociation of H284 and the release of CuB.

The rate of suicide inactivation by WT III (−) or the I–II oxidase is strongly influenced by the bulk pH [26]. At pH 6.2, the CC50 is 4 × 105, but this decreases to 1 × 103 at pH 8, indicating that suicide inactivation is 400 times faster at the higher pH (Fig. 2). One explanation for this pH dependence is that a slower rate of proton transfer to the active site accelerates the rate of suicide inactivation. In support of this, conditions that slow proton uptake into the D pathway (mutation of D132 or the presence of ΔΨ) also speed suicide inactivation (data not shown). These conditions also slow electron transfer, but slowing electron transfer by means other than inhibition of the D pathway does not accelerate suicide inactivation. Thus, an increased probability of suicide inactivation correlates with increased lifetimes of deprotonated, reactive intermediates during the catalytic cycle, such as the H284–Y288 radical and the deprotonated oxyferryl intermediate, P. Subunit III may prevent suicide inactivation by maintaining rapid proton transfer to the active site (see below), thus limiting the lifetime of these reactive intermediates. As suggested above, subunit III may also function as a structural buffer to decrease the probability of destructive chemistry at the active site, such as protein cross-links or oxygenation reactions. In this regard, it seems significant that two other oxygen-activating enzymes that also produce heme oxy-

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Fig. 2. The pH dependence of the rate of suicide inactivation by WT III (−). The CC50 is the average half-life of the oxidase complex in terms of catalytic cycles. O2 consumption profiles at different pH values were obtained as in Bratton et al. [26] but using Hepes–KOH and Taps–KOH as buffers. The CC50 values were extracted from single exponential fits of the O2 consumption curves after subtracting the rate of the nonenzymatic reduction of O2 by ascorbate. The fit to the data was performed by nonlinear regression using the equation CC50 = CC50min + (CC50max − CC50min)/(1 + 10(pH − pKα)). The fit predicts a pKα of 5.2 with a R2 value of 0.9911. The identity of the group(s) being titrated is yet unknown.
ferryl and protein radical intermediates during their catalytic cycles, prostaglandin H synthase and linoleate diol synthase, exhibit rapid suicide inactivation during normal turnover [33,34].

2. Subunit III and proton transfer through the D pathway

The pH dependence of steady state O$_2$ reduction catalyzed by WT III (—) and the I–II oxidase is considerably different from that of oxidase containing subunit III. In the absence of subunit III, the initial steady state activity of the subunit III-depleted enzyme (before suicide inhibition has set in) declines sharply with increasing pH, yielding an apparent pK$_a$ of 7.0 (Fig. 3). In contrast, normal cytochrome oxidase containing subunit III is considerably more active at higher pH and the apparent pK$_a$ of steady state O$_2$ reduction is 8.4. Since the structures of cytochrome oxidase show close connections between subunit III and the D pathway in subunit I (Figs. 4 and 7), the increased sensitivity of WT III (—) and I–II oxidase to pH could be due to changes in the rate of proton transfer through the D pathway. Subunit III clearly contributes to the environment of D132, the apparent initial proton acceptor for the D pathway, which is not completely surface-exposed (Fig. 4). Rather, D132 is situated at the bottom of a shallow depression formed by a ring of residues, one half of which is composed of four hydrophobic residues from the N-terminal tail of subunit III. These residues appear to partially shield D132 from the aqueous phase, which may have the effect of raising the pK$_a$ (proton affinity) of the initial proton acceptor. The N-terminal region of subunit III also contains histidine residues that may form part of a proton-collecting antenna for the D pathway that speeds proton uptake at higher pH [48].

![Fig. 3. The pH dependence of steady state O$_2$ reduction (before suicide inactivation) by cytochrome oxidase in the presence and absence of subunit III [WT III (—)]. Oxidase activity was measured and nonlinear regression performed as in Mills et al. [25].](image1)

![Fig. 4. The D132 pocket in *R. sphaeroides* cytochrome c oxidase [4]. Subunit III is yellow, subunit I light blue. Subunit III residues surrounding D132 include Ile-11 (magenta), Leu-12 (orange), Pro-13 (green), and Pro-14 (pink). In blue, clockwise from the right, are His-10 (III), His-7 (III), and His-549 (I).](image2)

![Fig. 5. Proton uptake into the D pathway during the single turnover reduction of O$_2$ by the fully reduced wild-type and I–II oxidase at pH 8.0 (from Gilderson et al. [24]). In the wild-type oxidase, one ‘substrate’ proton is taken up during the P$_R$ → F transition and one during the F → O transition. In the absence of subunit III, the slow uptake of two protons is observed. In this experiment, the uptake of ‘pumped’ protons is not observed. R = reduced; P$_R$ = oxyferryl intermediate; F = oxyferryl intermediate; O = oxidized.](image3)
capacity for rapid proton uptake. In the final step of the single-turnover reduction of O$_2$, a heme $a_3$ oxyferryl (F) is converted to the oxidized form (O); this $F \rightarrow O$ transition requires the transfer of a proton from the bulk solution to the active site through the D pathway [50,51]. For the I–II oxidase, the rate and the apparent pK$_a$ of the $F \rightarrow O$ transition (7.0) closely match the rate and apparent pK$_a$ of steady state O$_2$ reduction [24]. Therefore, in the absence of subunit III, the rate of steady state O$_2$ reduction at pH 7.0 and above appears to reflect the rate at which protons are transferred through the D pathway to the active site. In contrast, the rate of proton transfer through the K pathway does not appear affected by the absence of subunit III [24,25]. These results indicate another important role for subunit III, i.e., maintaining rapid proton uptake into the D pathway, particularly at the pH values typical of the bacterial cytoplasm and the mitochondrial matrix (pH 7.5). Whether this is due to the ability of subunit III residues to raise the pK$_a$ of D132 or to the presence of key components of a proton antenna in this region of subunit III, or both, is under investigation.

Alteration of the initial proton acceptor of the D pathway, D132, to alanine has previously been shown to greatly inhibit oxidase turnover [22,52]. One way to test whether subunit III influences the immediate environment of D132 is to remove subunit III from D132A [creating D132A III (−)] and observe the effect on oxidase activity. Remarkably, we found that the removal of subunit III from D132A (via Triton extraction) increased its $V_{\text{max}}$ of steady state O$_2$ reduction more than 20-fold at pH 6.5, from ~30 to ~670 s$^{-1}$ [25]. This substantial increase in the rate of O$_2$ reduction must be due an increase in the rate of proton transfer through the D pathway. [This has now been verified by direct measurement (Adelroth, unpublished).] Apparently, recruitment of an alternative proton acceptor for the D pathway occurs with the removal of subunit III, partially replacing the function of the deleted D132. One possible candidate is H26 of subunit I, which normally lies behind D132 and forms a hydrogen bond with the more buried carboxylate oxygen of D132. The pH dependence of O$_2$ reduction by D132A III (−) is considerably different from that of WT III (−), consistent with a different proton acceptor [25].

Subunit III contains seven transmembrane helices that are split into two domains; a bundle of five helices (3–7) is separated from a helix 1–2 pair by a deep V-shaped cleft or groove (Fig 6). The front of the cleft is open to the lipid bilayer, while the back wall is formed by helices 3, 4, and 5 of subunit I. A remarkable feature of subunit III is that it contains a large number of conserved residues that tightly bind phospholipid in highly specific orientations. This is true for the bovine oxidase as well as the two bacterial enzymes [2,4,53]. Six phosphatidylethanolamines (PE) are resolved in the crystal structure of the R. sphaeroides oxidase [4]. Five of these PEs are coordinated by residues of subunit III, and two are located within the cleft (Fig. 6). One of the fatty acids of the lipid positioned at the back of the subunit III cleft (termed PE1) forms three connections with the proton conductive pathway between D132 and E286 of the D pathway. This part of the D pathway is composed of a series of residues that coordinate a hydrogen-bonded chain of waters [4]. Asn-139 on helix 3 of subunit I,
3. Subunit III and the proton back-leak/exit pathway

Pumped protons exit through the outer surface of the oxidase complex, but the pathway leading from the site of proton pumping to the surface has yet to be defined (Fig. 1) [21,55]. Once cytochrome oxidase has been reconstituted into phospholipids vesicles (COVs), catalytic turnover of the COVs rapidly generates a transmembrane voltage gradient (ΔΨ) and a ΔpH of sufficient magnitude that the flow of pumped protons from the inner surface to the outer surface of the oxidase is inhibited. However, even under these ‘controlled’ conditions, a slow rate (~15%) of electron transfer to O₂ continues [25,55]. Electron transfer requires protons to neutralize the charge of incoming electrons [56,57], and to participate in oxygen reduction chemistry, but the ΔΨ opposes proton transfer from the inside of the vesicle. However, proton uptake from the outside of the vesicle, apparently by reversal of the normal exit pathway (Fig. 1) [23], is favored by ΔΨ and ΔpH. Such proton uptake through the back-leak/exit pathway appears to be an important contributor to continued turnover under controlled conditions [23].

When added on the outside of COVs, low concentrations of zinc strongly inhibit the controlled rate of electron transfer to O₂, apparently by binding to a site that becomes available in the presence of ΔΨ and inhibiting the entry of protons into the back-leak/exit pathway [23]. We have found that the removal of subunit III also slows controlled electron transfer, to a similar extent as (and additive to) the addition of zinc [25]. The best explanation for this result is that the removal of subunit III, like the addition of zinc, alters the conformation of the normal proton exit pathway and inhibits its ability to take up protons from the outer surface of the oxidase in the presence of ΔΨ and ΔpH.

4. Subunit III and proton pumping

Proton pumping by mitochondrial cytochrome oxidase is less efficient (i.e., has a lower H⁺/e⁻) in the absence of subunit III [6,7], although the reasons for this have never been clearly defined. Proton pumping by the R. sphaeroides oxidase is also less efficient in the absence of subunit III. The H⁺/e⁻ value of the wild-type oxidase averages 0.9 ± 0.2 in the presence of subunit III and 0.6 ± 0.2 in its absence [25]. Under the conditions of the proton pumping experiments (pH 7.4, no ΔΨ'), the rate of proton uptake into the D pathway of the normal oxidase is at least 10,000 s⁻¹ while the rate of proton uptake into the D pathway of WT III (−) is approximately 600 s⁻¹ [24,25]. Thus, slowing the proton uptake capability of the normal D pathway more than 16-fold may inhibit proton pumping, but only slightly. Under the same conditions, no proton pumping could be detected for D132A III (−), although the rate of proton uptake into the D pathway of D132A III (−) at pH 7.4 is the same as that of WT III (−) (approximately 600 s⁻¹ [24,25]; Ådelroth, unpublished). Thus, the presence of D132 appears required for proton pumping for some reason in addition to maintaining a sufficient rate of proton uptake. Considerable evidence indicates that E286 of the D pathway functions as an internal reservoir for a proton [12,48,50,58]. We propose that D132 facilitates proton pumping by functioning as another reservoir for a proton within the D pathway. In this paradigm, D132 and E286 ‘trap’ two protons within the D pathway for those reaction steps that require the transfer of two protons through E286 in rapid succession, e.g., one to a high pKₐ O₂ reduction intermediate and a second to the proton pump. In the mutant oxidase D132A III (−), the alternative initial proton acceptor is not an efficient trap for a second proton within the D pathway and the rate of proton uptake from the bulk is not sufficient to deliver a proton to the proton pump during the short period in which the pump is in its input configuration. Thus, D132A III (−) has considerable O₂ reduction activity but it fails to pump protons. In the normal oxidase, the pKₐ (proton affinity) of D132 is set by its protein environment to be high enough to capture protons from the bulk solution and high enough to retard the exit of protons from the D pathway, but low enough that E286 easily competes for the proton on D132. This proposal is consistent with a previous explanation that the D135N mutation of cytochrome bo₃ of E. coli eliminates measurable proton pumping by inhibiting proton transfer and allowing the proton pump to switch into its output configuration while still unprotonated [59].

5. Conclusions

Subunit III has several roles in the function of cytochrome oxidase sufficient to explain its high level of conservation. The presence of subunit III maintains rapid proton uptake into the D pathway at the pH of the bacterial cytoplasm or mitochondrial matrix (~7.5) [24]. In part, this may be accomplished by tuning the pKₐ of D132 to a value ~7.5 or greater. The presence of subunit III appears to contribute to the control of back-leak of protons from the...
outer surface of subunit I, when the transmembrane voltage gradient attains a magnitude sufficient to inhibit proton uptake from the inner surface [25]. By extrapolation, it seems likely that subunit III is necessary to maintain the exit pathway for pumped protons in the physiologic situation, i.e., in the presence of a significant $\Delta \Psi$. Subunit III clearly prevents turnover-induced inactivation of the oxidase [26], which is especially rapid at physiologic pH values and in the presence of $\Delta \Psi$. This function of subunit III appears related to its ability to maintain proton flow to the active site, but it also appears that subunit III exerts some structural influence that more directly protects the active site from destructive chemistry during the O$_2$ reduction cycle. Any or all of the connections between subunit III and subunit I (Fig. 1) could contribute to optimum proton pumping efficiency. One of the goals of our current work is to elucidate how each of the areas of contact between subunit III and the pathways for pumped protons in subunit I affects the efficiency of pumping. This may provide further insight into the mechan-ism of the pump.

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References


