Slow Proton Transfer through the Pathways for Pumped Protons in Cytochrome *c* Oxidase Induces Suicide Inactivation of the Enzyme†

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ABSTRACT: In the absence of subunit III the aa_3-type cytochrome *c* oxidase exhibits a shortened catalytic life span (total number of turnovers) due to an increased probability of undergoing irreversible inactivation during steady-state turnover. Inactivation results from structural alteration of the heme a_3−Cu_B active site in subunit I [Hosler (2004) *Biochim. Biophys. Acta 1655*, 332−339]. The absence of subunit III also dramatically slows proton uptake to the active site via the D proton pathway, as well as inhibiting the proton backflow/exit pathway that connects the active site/proton pump with the outer surface of the oxidase complex. Here we demonstrate that these phenomena are linked: slow proton delivery to the active site through these pathways induces suicide inactivation, thus shortening the catalytic life span of the enzyme. Mutations that inhibit the D pathway, but not the K pathway, increase the probability of suicide inactivation. Strong inhibition of the D pathway allows suicide inactivation to occur even in the presence of subunit III. Arachidonic acid, which stimulates proton uptake by the D pathway, retards suicide inactivation. Steady-state turnover in the presence of $\Delta\Psi$ and ΔpH, which inhibits proton uptake from the inner surface of the protein, enhances suicide inactivation. Simultaneous inhibition of proton uptake from both sides of the protein by a double mutation affecting the D pathway and the proton backflow/exit pathway greatly shortens the catalytic life span of the oxidase even in the presence of subunit III. Thus, maintenance of rapid proton transfer through the D pathway and the backflow/exit pathway is one mechanism by which subunit III normally functions to prevent suicide inactivation of cytochrome *c* oxidase. The experiments suggest that increased lifetimes of the heme a_3 oxoferryl intermediates as well as the anionic form of Glu286 of the D pathway cause suicide inactivation in the active site.

As the terminal member of the respiratory electron transfer chain in the inner membrane of mitochondria and the cytoplasmic membrane of many aerobic bacteria, cytochrome *c* oxidase reduces O_2 to H_2O. The enzyme uses some of the energy of this redox reaction to pump protons through the protein, across its host membrane. The α-proteobacterium *Rhodobacter sphaeroides* synthesizes a heme A-containing oxidase with high genetic and structural similarity to the subunits of the catalytic core (I, II, and III) of the mammalian oxidase (*1*−*3*).

In one possible catalytic mechanism for steady-state O_2 reduction by cytochrome *c* oxidase, O_2 binds to heme a_3 following the reduction of heme a_3 and Cu_B by electrons from cytochrome *c* via Cu_A in subunit II and heme *a* in subunit I (Figure 1, intermediate 3). In a rapid electronic rearrangement, bound O_2 is reduced by four electrons, two from heme a_3, one from Cu_B, and one from a covalently linked histidine−tyrosine group in the active site that forms a radical (*4*−*7*). This chemistry breaks the O═O bond and leaves an oxoferryl species on heme a_3 (a_3^{4+}═O^{2-}) plus a hydroxyl group on Cu_B (Figure 1, intermediate 4). The rapid kinetics of O_2 reduction avoid the accumulation and possible release of superoxide and hydrogen peroxide, but the active site must deal with the persistence of the histidine−tyrosine radical and the oxoferryl species until two more electrons and two more protons are delivered (Figure 1, intermediate 1). These protons and electrons rereduce the radical and regenerate heme a_3^{3+}.

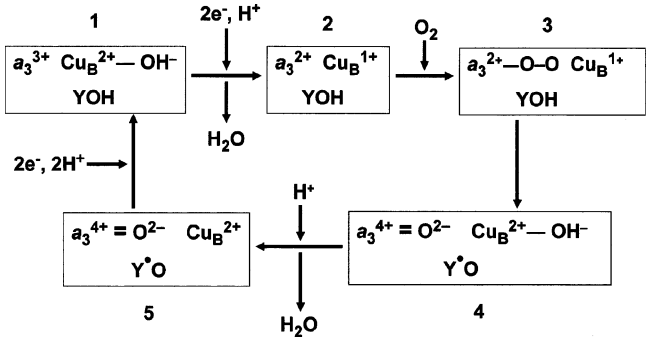

FIGURE 1: A scheme for steady-state O_2 reduction at the active site of cytochrome *c* oxidase. Y = Y288 (*R. sphaeroides* numbering).

The protons required for the synthesis of water (shown in Figure 1), as well as those that are pumped through the protein (not shown), are taken up from the inner surface of the protein (facing the mitochondrial matrix or bacterial

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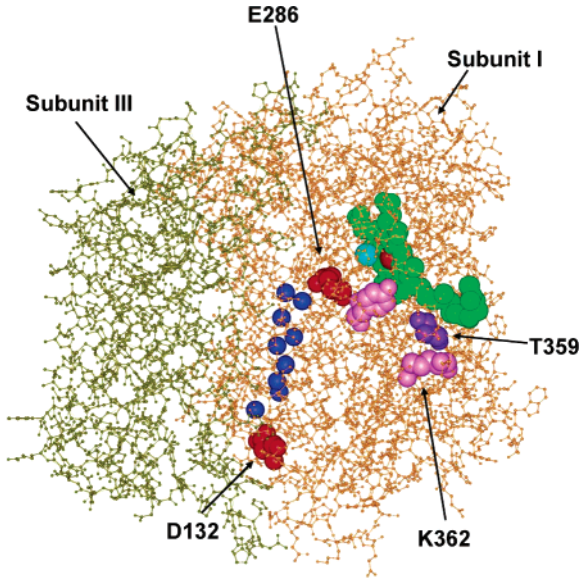

FIGURE 2: Separation of the D and K pathways in subunit I and the proximity of the D pathway to subunit III. The D pathway in subunit I (brown) is shown as a series of bound waters (blue) between surface-exposed D132 and E286 in the interior of the protein. Other waters, not resolved in the crystal structure, connect E286 with the heme a_3 (green)−Cu_B (cyan) O_2 reduction site. The K pathway in subunit I is shown from K362 to T359 to Y288 (pink), near the active site. Heme *a* of subunit I is not shown, and neither are subunits II and IV. The structure is that of the aa_3-type cytochrome *c* oxidase of *R. sphaeroides* (*3*).

cytoplasm) and transferred ~30 Å through two proton pathways to the buried heme a_3−Cu_B active site. The first of these is the K pathway, named for a conserved lysine (Figure 2). 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 prior to 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*, *12*). In the D pathway, a series of ordered water molecules form a hydrogen-bonded proton-conductive pathway between D132 and E286, located ~26 Å above D132. From E286 each proton is transferred either to the heme a_3−Cu_B active site, apparently through a shorter series of waters, or to the site of proton pumping, presumably near the heme a_3−Cu_B active site (*4*, *13*−*15*). Another pathway transfers protons from the site of pumping to the outer surface of the oxidase, but the components of this proton exit pathway are yet to be identified (*16*−*18*). If proton transfer through the D pathway is severely inhibited, protons for O_2 reduction are transferred to the active site from the outer surface of the protein, probably by reversal of the normal exit pathway for pumped protons (*19*, *20*). This is termed the proton backflow pathway.

Mutations that alter key residues in the D and K pathways, such as D132 and E286 of the D pathway and K362 and T359 of the K pathway, have strong effects on the rate of proton transfer (*8*, *21*−*24*). These same mutations either slow or eliminate steady-state O_2 reduction, depending upon the extent to which the mutation inhibits proton transfer (*8*, *20*, *22*, *24*−*27*).

The role of subunit III in cytochrome oxidase function is becoming more clear. This integral membrane protein binds to subunit I, is highly conserved, and is clearly a member of the catalytic core of cytochrome oxidase. However, it does not bind metal and thus cannot be assigned a direct role in electron transfer. One critical function of subunit III is its ability to prevent the oxidase from undergoing spontaneous inactivation during turnover (suicide inactivation) (*28*, *29*) caused by structural changes at the active site in subunit I (*29*). This function is likely to be of physiological significance since (1) the catalytic life span of cytochrome *c* oxidase (total number of catalytic cycles) is 100−1000-fold less in the absence of subunit III and (2) suicide inactivation also occurs in mitochondrial cytochrome *c* oxidase lacking subunit III (Ferguson-Miller, personal communication).

The oxidase structures show contacts between subunit III and the D pathway of subunit I but not the K pathway (*3*, *30*−*32*). Consistent with this, we have found that subunit III strongly affects the pH optimum for proton uptake by the D pathway but has little effect on the K pathway for protons (*33*). In the presence of subunit III, the rate of proton uptake into the D pathway at pH 8.0 exceeds 10000 s^{-1}, but in the absence of subunit III the rate declines to 350 s^{-1}. Rapid proton uptake is restored to the subunit III-depleted oxidase at low pH (~10000 s^{-1} at pH 5.5). The effective pK_a value for proton uptake by the D pathway is ~1.8 pH units more acid than normal in the absence of subunit III (*33*). The probability of suicide inactivation by subunit III-depleted cytochrome oxidase increases with pH, with an apparent pK_a value similar to that of proton uptake by the D pathway of the subunit III-depleted oxidase (*29*). Since high pH both enhances suicide inactivation and slows proton uptake through the D pathway, we hypothesized that other factors that slow proton uptake through the D pathway should increase the probability of suicide inactivation and shorten the catalytic life span of cytochrome *c* oxidase. The experiments presented here support this hypothesis. The data suggest that the reactive heme a_3 oxoferryl O_2 reduction intermediate, whose lifetime is increased by slow proton delivery, is involved in the suicide inactivation process. Subunit III protects the active site from suicide inactivation, in part, by contributing structures necessary for rapid proton transfer through those pathways in subunit I that are used for both the transfer of protons to the active site and the transfer of pumped protons.

EXPERIMENTAL PROCEDURES

Materials. L-α-Phosphatidylcholine (asolectin, Sigma type II-S) was recrystallized using the procedure of Sone et al. (*34*) and prepared as a 40 mg/mL solution in 2% sodium cholate (Anatrace). Horse heart cytochrome *c* (Sigma, type VI) was dissolved in 50 mM KH_2PO_4, pH 7.2. Arachidonic acid and *n*-dodecyl β-D-maltoside were purchased and prepared as in Mills et al. (*20*). Valinomycin and CCCP[1] were from Sigma. All other reagents were of the highest grade available.

[1] Abbreviations: Aa, arachidonic acid; CCCP, carbonyl cyanide 3-chlorophenylhydrazone; CC, catalytic cycle; COVs, cytochrome oxidase-containing phospholipid vesicles; Hepes, 4-(2-hydroxyethyl)-piperazine-1-ethanesulfonic acid; TMPD, *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine; SD, standard deviation; TN, turnover number; WT, wild-type.

Bacterial growth, oxidase purification, and the removal of subunit III were as in Zhen et al. (*35*) and Mills et al. (*20*). To create a version of T359A containing a six-histidine tag, the QuikChange mutagenesis system (Qiagen) was used to introduce the mutation into pJS3, a plasmid containing the gene for subunit I (*36*). A 1.3 kbp *Eco*RI−*Bgl*II fragment containing the mutation was excised and moved into pAH1H32, a pUC-based plasmid containing all of the oxidase genes (except the gene for subunit IV), including the codons for a six-histidine tag at the C-terminus of subunit I (*37*). A 7.5 kbp *Eco*RI−*Hin*dIII fragment, containing all of the oxidase genes, was excised from pAH1H32 and moved into the broad host range vector pRK415 (*38*). The resulting plasmid was conjugated into *R. sphaeroides* JS100, a strain lacking the wild-type gene for subunit I (*1*), for the expression of T359A. Similar methods were used to create D132A with a six-histidine tag at the C-terminus of subunit I (Qian and Ferguson-Miller, unpublished results). The catalytic properties of the histidine-tagged versions of T359A and D132A appeared to be identical to the original untagged versions of these mutants. Mutant oxidases E286D (*22*), E286A-I112E (*39*), R481K (*40*), and M263L (*41*, *42*) are as previously described. The construction of the double mutant D132A-R481K will be described elsewhere (Mills and Ferguson-Miller, to be published).

Activity Assays. Steady-state O_2 reduction was measured at 25 °C in a 1.7 mL water-jacketed chamber (Gilson) using a Clark-type O_2 electrode (Yellow Springs Instruments) and a YSI 5300A biological oxygen monitor. The analogue output from the YSI monitor was ported to an 18-bit analogue to digital converter in a Hansatech Oxygraph unit and from there to a PC for data analysis (see below). For experiments using detergent-solubilized oxidase forms, the reaction mixture included 25 mM KH_2PO_4 or 25 mM Hepes−KOH (pH as noted), 0.1% dodecyl maltoside, 1 mg/mL phosphatidylcholine, 3 mM ascorbate, 0.3 mM TMPD, 40 μM horse cytochrome *c* (except where noted), and 1000 units of catalase. KCl was added to adjust the ionic strength to 100 mM. The reactions contained 4−50 pmol of oxidase depending upon the activity of the oxidase form. Steady-state oxygen reduction by cytochrome oxidase vesicles [COVs; prepared as in Mills et al. (*20*)] was measured as above in a reaction mixture containing 25 mM Hepes, pH 7.4, and 24 mM KCl, plus ascorbate, TMPD, horse cytochrome *c*, and catalase as above. Turnover numbers (TN) were calculated as described in Thompson and Ferguson-Miller (*43*).

Measurements of Suicide Inactivation. Suicide inactivation progresses exponentially with time and linearly with the number of catalytic cycles (CC: $1O_2 \rightarrow 2H_2O$). The parameter chosen to measure suicide inactivation of cytochrome oxidase is the number of catalytic cycles per oxidase molecule required for the population of oxidase monomers to reach 50% of the initial rate of O_2 reduction activity. This number is termed the CC_{50}. The CC_{50} is also the average catalytic life span of the oxidase (see Results).

Three methods were employed to obtain the CC_{50} values. Control experiments using WT III (−) and D132A III (−) showed that each of these methods yielded nearly identical CC_{50} values. For oxidase populations that rapidly lost O_2 reduction activity, either as the detergent-solubilized oxidase or as COVs, exponentially decaying O_2 reduction curves [such as those shown in Bratton et al. (*29*)] were obtained within 200 nmol of O_2 (approximately one-half of the total amount of O_2 in the reaction cell). In this region there is no loss of activity due to low O_2 concentrations. After capturing the data on a PC, as described above, each curve was corrected for the rate of nonenzymatic O_2 reduction by ascorbate plus TMPD, and the corrected curve was fit to a single exponential using Prism 4 (GraphPad Software). The R^2 parameter for goodness of fit was ≥ 0.9991 for every exponential fit. The fit itself was differentiated to obtain the turnover number (TN) at 150 time points. At each time point the number of catalytic cycles was determined by dividing the nanomoles of O_2 consumed (after correction for the amount of O_2 consumed by ascorbate plus TMPD alone) by the total nanomoles of oxidase present. The linear plot of TN vs catalytic cycles yielded the CC_{50} by calculating the CC value at one-half the initial TN, given by the *y*-intercept.

For oxidase populations in which the loss of O_2 activity was slow, the decay curve obtained within 200 nmol of O_2 was too shallow for an accurate exponential fit. In these cases, a series of experiments with longer periods of catalytic turnover were performed in order to create the data for a linear plot of TN vs catalytic cycles, and as above, the CC_{50} was determined from this plot. Extended catalytic turnover was accomplished by periodically replenishing the O_2 in the reaction cell by blowing humidified O_2 over the surface of the stirred reaction mixture. Particularly long experiments also required small additions of ascorbate. The final TN was determined from the slope of the O_2 reduction curve at the end of the experiment after correction for the nonenzymatic O_2 reduction rate. The number of catalytic cycles to the point at which the final TN was measured was estimated by summing the total nanomoles of O_2 consumed throughout the experiment, subtracting the amount of nonenzymatic O_2 consumption, and dividing the remainder by the total nanomoles of oxidase present.

Similar experiments were used to measure the CC_{50} for COVs that showed a slow loss of O_2 reduction activity. In these experiments, catalytic turnover of the COVs took place under controlled conditions, i.e., in the presence of $\Delta\Psi$ and ΔpH. At the end of various amounts of controlled turnover, the remaining activity of the oxidase population was determined after adding detergent (0.1% dodecyl maltoside) and 1 mg/mL phosphatidylcholine. The addition of these reagents eliminated $\Delta\Psi$ and ΔpH, thus maximizing the remaining O_2 reduction activity.

RESULTS

Subunit III can be quantitatively removed from the aa_3-type cytochrome *c* oxidase of *R. sphaeroides* using Triton X-100 to create a form termed WT III (−) (*29*). While the initial O_2 reduction activity of detergent-solubilized WT III (−) at pH 6.5 is similar to the enzyme containing subunit III, the catalytic life span of WT III (−) is at least 300-fold less than the normal oxidase at the physiologic pH of 7.4 [Figure 6 (*44*)]. [Catalytic life span is the number of catalytic cycles (CCs) between the birth and death of the enzyme.] The shorter life span of the wild-type enzyme in the absence of subunit III is due to its increased tendency to irreversibly lose activity, or suicide inactivate, due to some malfunction during the catalytic cycle (*29*). Inactivation is caused by a

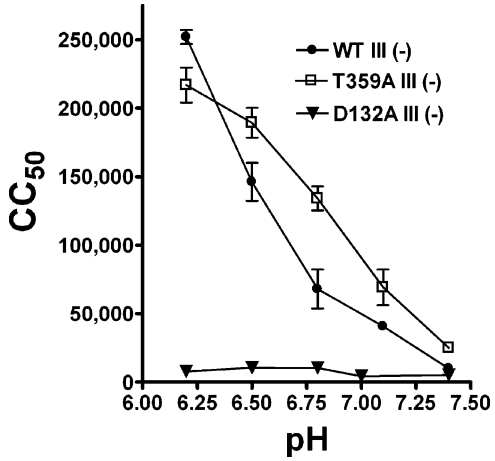

FIGURE 3: Effect of D pathway inhibition, K pathway inhibition, and pH on the probability of suicide inactivation in the absence of subunit III. Average catalytic life spans (as CC_{50} values) of detergent-solubilized WT III (−), T359A III (−), and D132A III (−) were measured at pH values between 6.2 and 7.4. Decreases in the CC_{50} value indicate a greater probability of inactivation during any given catalytic cycle. Error bars show the SD.

structural change at the active site that targets the Cu_B center, but the details of the inactivation chemistry are yet to be defined (*29*, *44*).

In this study, the tendency of different oxidase forms to suicide inactivate under a variety of experimental conditions is compared by measuring their average catalytic life spans. The average catalytic life span of cytochrome oxidase is given by the CC_{50} (see Experimental Procedures), which has several definitions and uses. First, the CC_{50} is the number of catalytic cycles ($1CC = 1O_2 \rightarrow 2H_2O$) per oxidase molecule that an oxidase population has performed at the point where the entire oxidase population exhibits one-half of its initial O_2 reduction activity (see Experimental Procedures). At the point of the CC_{50}, one-half of the oxidase molecules in the total population have inactivated and one-half are still fully active. The single exponential decay in the rate of O_2 reduction exhibited by a suicide-inactivating oxidase population (*29*) indicates a balanced distribution of lifetimes in the oxidase population. Thus, the CC_{50} is the average catalytic life span of each oxidase molecule in the population in terms of catalytic cycles. Although the CC_{50} is derived from time-based measurements of O_2 reduction activity (as described in Experimental Procedures), the CC_{50} is a number of catalytic cycles, not a measure of time. The CC_{50} can also be viewed as a measure of probability. The smaller the CC_{50}, the greater the probability that any given oxidase molecule in that oxidase population will inactivate on each turnover.

Slow Proton Transfer through the D Pathway but Not the K Pathway Induces Suicide Inactivation. The CC_{50} or average catalytic life span of detergent-solubilized WT III (−) decreases dramatically with pH [Figure 3 (*29*, *44*)], suggesting that a reduced rate of proton delivery to the active site sets up the conditions for the suicide inactivation event. A 25-fold decrease in the CC_{50} occurs as the pH changes from 6.2 to 7.4, from an average catalytic lifetime of ~250000 CCs to one of ~9000 CCs (Figure 3). Over this same pH range, the rate of proton uptake into the D pathway of WT III (−) shows an approximate 20-fold decrease, from ~10000 to ~500 s^{-1} (*33*). Thus, the probability of suicide inactivation increases as the rate of proton uptake by the D pathway decreases.

Table 1: Initial O_2 Reduction Activities (i.e., Prior to Suicide Inactivation) of the Oxidase Forms Used in the Experiments Presented in This Study

oxidase form	assay conditions	initial TN (s^{-1}) at pH 7.4
WT III (+)	uncontrolled[a]	992 ± 46
WT III (−)	uncontrolled	549 ± 6
WT III (−)	uncontrolled plus Aa[b]	1136 ± 65
WT III (−)	controlled[c]	20.3 ± 2.3
D132A III (+)	uncontrolled	11 ± 3.3
D132A III (−)	uncontrolled	322 ± 40
D132A III (−)	uncontrolled plus Aa	722 ± 42
D132A III (−)	controlled	22 ± 1
T359A III (−)	uncontrolled	114 ± 6
T359A III (−)	controlled	7.5 ± 1.9
E286D III (−)	uncontrolled	140 ± 35
E286D III (−)	uncontrolled plus Aa	269 ± 46
E286A-I112E III (−)	uncontrolled	2.3 ± 0.5
E286A-I112E III (+)	uncontrolled	20.3 ± 0.4
D132A-R481K III (+)	uncoupled[d]	7.7 ± 0.4
D132A-R481K III (+)	controlled	11.5 ± 0.4

[a] Measured as the detergent-solubilized oxidase; no $\Delta\Psi$ or ΔpH. [b] 250 μM arachidonic acid. [c] Measured in COVs in the presence of $\Delta\Psi$ plus ΔpH. [d] Measured in COVs with valinomycin and CCCP to collapse $\Delta\Psi$ and ΔpH.

The proximity of the D pathway to subunit III (Figure 2) suggests that the relationship between proton uptake and suicide inactivation may be specific for the D pathway. If suicide inactivation follows from slow proton uptake through the D pathway, but not the K pathway, then a mutation that inhibits the D pathway should lower the CC_{50}, while a mutation that inhibits the K pathway should not. Initially, two well-characterized subunit I mutations were chosen to test this. Alteration of D132, the initial proton acceptor of the D pathway, strongly inhibits proton uptake by this pathway (*21*), while alteration of T359 to alanine inhibits proton transfer through the K pathway, with little effect on the D pathway (*8*). In the first set of experiments, subunit III was removed from both D132A and T359A [creating D132A III (−) and T359A III (−)] so that the effect of the mutations could be compared to the WT III (−), the wild-type oxidase lacking subunit III.

The average catalytic life span (CC_{50}) of D132A III (−) is 2-fold to 50-fold less than that of WT III (−), depending upon pH, and the CC_{50} of D132A III (−) is independent of external pH from pH 6.2 to pH 7.4 (Figures 3 and 4). Over this same pH range, direct measurements show that the rate of proton uptake by the D pathway of D132A III (−) remains constant and 20-fold slower than wild type, at 500 s^{-1} (Ädelroth et al., unpublished results, and see ref *20*). Thus, the catalytic life spans of both WT III (−) and D132A III (−) are proportional to their rates of D pathway proton uptake between pH 6.2 and pH 7.4.

Alteration of T359 of the K pathway to alanine has been demonstrated to strongly impair the proton transfer activity of this pathway (*8*), leading to a 70% reduction in the rate of steady-state O_2 reduction (*8*, *27*). The steady-state activity of T359A III (−) was 20% that of WT III (−) (Table 1), indicating that, as expected, the mutation also inhibits proton transfer in the absence of subunit III. This inhibition of the K pathway did not decrease the CC_{50} of T359A III (−) below that exhibited by WT III (−) (Figure 3). Thus, slowing proton

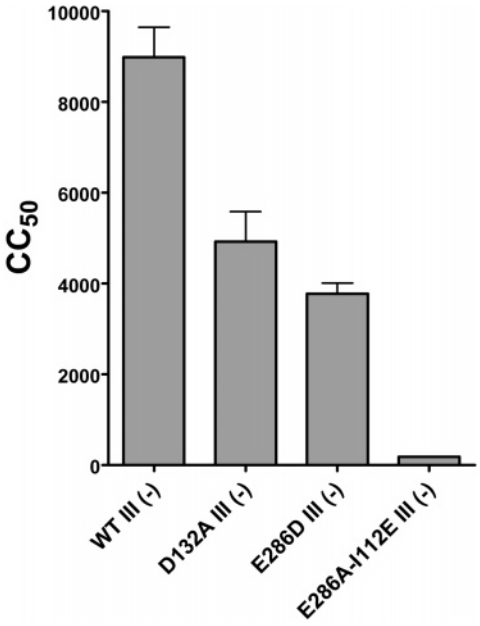

FIGURE 4: Various D pathway alterations induce suicide inactivation and shorten the catalytic life span of cytochrome *c* oxidase. Average catalytic life spans (as CC_{50} values) were measured for detergent-solubilized oxidase forms at pH 7.4. The actual CC_{50} values, with errors as SD, are as follows: WT III (−), 8981 ± 660; D132A III (−), 4922 ± 662; E286D III (−), 3774 ± 236; E286A-I112E III (−), 105 ± 53.

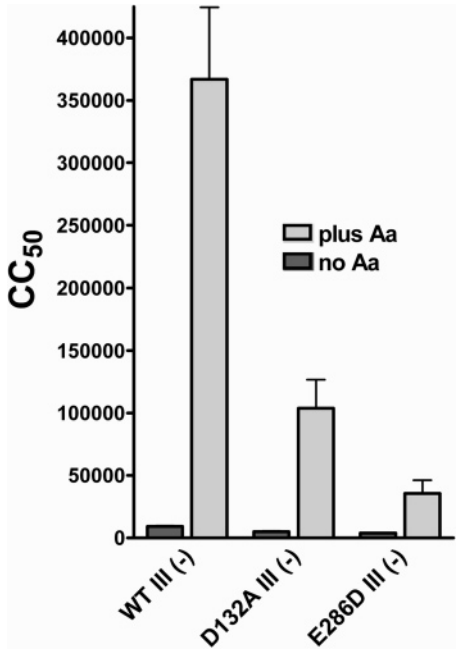

FIGURE 5: Stimulation of the rate of proton uptake into the D pathway by arachidonic acid slows suicide inactivation. CC_{50} values (average catalytic life spans) were measured at pH 7.4 using detergent-solubilized oxidase forms, with and without the addition of 250 μM arachidonic acid (Aa). The measured CC_{50} values without arachidonic acid are those given in the legend of Figure 4. The CC_{50} values obtained in the presence of arachidonic acid are as follows: WT III (−), 366844 ± 57470; D132A III (−), 103913 ± 22732; E286D III (−), 35567 ± 10446.

uptake through the K pathway does not increase the probability of suicide inactivation.

Other mutations in the D pathway that slow proton transfer to the active site also increase the probability of suicide inactivation in the absence of subunit III. In the form of E286D containing subunit III, the rate of proton uptake has been shown to be 2-fold slower than the normal enzyme (*22*), consistent with a 50% reduction in its O_2 reduction activity (*22*, *26*). E286D III (−) exhibits a 75% decrease in its O_2 reduction activity, compared to WT III (−) (Table 1), indicating that proton transfer through D pathway of the subunit III-depleted mutant is inhibited to a similar extent as in E286D III (+). The CC_{50} or catalytic life span of detergent-solubilized E286D III (−) was half that of WT III (−), or about the same as D132A III (−) (Figure 4), indicating that the mutation enhanced suicide inactivation. The double mutant E286A-I112E relocates the internal glutamate from helix VI to helix II and shifts the carboxylate side chain an estimated 2 Å from its normal position (*23*, *39*). The rate of proton transfer to the active site in E286A-I112E III (+) is 100−400-fold slower than in the wild-type oxidase, depending upon external pH, leading to a slower rate of O_2 reduction (*23*). At pH 7.4, a TN of 20 s^{-1} was measured for E286A-I112E III (+), or 2% of the activity of WT III (+) (Table 1). Following the removal of subunit III, the activity of E286A-I112E III (−) was 0.4% that of the subunit III-depleted wild-type oxidase (Table 1). Therefore, like E286D, the subunit III-depleted form of E286A-I112E shows a similar level of inhibition of proton transfer as the form that contains subunit III. Consistent with its very slow rate of proton transfer to the active site, detergent-solubilized E286A-I112E III (−) inactivates with high probability and exhibits a very short average catalytic life span of approximately 100 catalytic cycles (Figure 4).

Enhancement of D Pathway Activity by Arachidonic Acid Slows Suicide Inactivation. A unique characteristic of the D pathway is the ability of long-chain, unsaturated fatty acids, particularly arachidonic acid, to bind at or near the entrance of the pathway and enhance the rate of proton transfer (*20*, *21*, *45*) (see Discussion). If suicide inactivation is enhanced by slow proton uptake into the D pathway, then the addition of arachidonic acid should delay suicide inactivation by increasing the rate of proton uptake. This is precisely the case: arachidonic acid increases the average catalytic life span of detergent-solubilized WT III (−) approximately 40-fold, that of D132A III (−) approximately 20-fold, and that of E286D III (−) by 10-fold (Figure 5). The fact that the life spans of all of these oxidase forms are not increased to the same level by arachidonic acid (Figure 5) indicates that each D pathway mutation continues to impose an underlying limitation.

Extreme Inhibition of Proton Uptake through the D Pathway Leads to Suicide Inactivation in the Presence of Subunit III. The rates of D pathway proton uptake exhibited by E286A-I112E III (+) and D132A III (+) are very slow. A rate of approximately 6 s^{-1} has been reported for E286A-I112E III (+) at pH 7.5 (*23*), while a rate of 5 s^{-1} has been measured for proton uptake by D132A III (+) at pH 7.4 (Ädelroth et al., unpublished results). [In a seeming paradox, proton uptake into the D pathway of D132A is much slower in the *presence* of subunit III than in its absence (*20*). This appears to be due to a strong contribution by the N-terminal region of subunit III to the environment of D132 (*20*).] The rate of proton uptake exhibited by D132A is similar to that exhibited by the related mutant D132N (*21*). These slow rates of proton uptake by the D pathway lead to equally slow

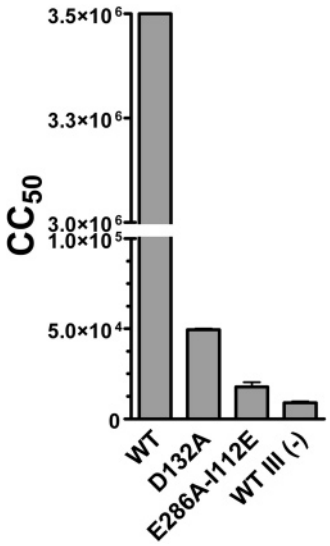

FIGURE 6: Suicide inactivation in the presence of subunit III. CC_{50} values, obtained at pH 7.4, for two detergent-solubilized D pathway mutants containing normal amounts of subunit III are compared to WT III (+) and WT III (−). The CC_{50} values are as follows: WT III (+), 3.5×10^6 (from a representative experiment); D132A III (+), 49436 ± 714; E286A-I112E III (+), 17825 ± 2544; WT III (−), 8981 ± 660. A break is included in the *Y* axis to allow the high CC_{50} of WT III (+) to be included.

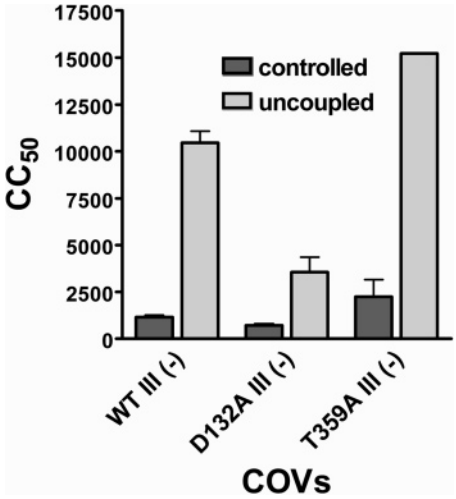

FIGURE 7: Catalytic turnover under controlled conditions (+ΔΨ, ΔpH) stimulates suicide inactivation. CC_{50} values were measured at pH 7.4 for three oxidase forms in cytochrome oxidase vesicles (COVs) under controlled and uncoupled conditions. For the latter, 5 μM valinomycin and 7 μM CCCP were added to collapse ΔΨ and ΔpH. The CC_{50} values under controlled conditions were as follows: WT III (−), 1136 ± 108; D132A III (−), 716 ± 79; T359A III (−), 2227 ± 917. Those under uncoupled conditions were as follows: WT III (−), 10449 ± 621; D132A III (−), 3537 ± 804; T359A III (−), 15210.

steady-state rates of O_2 reduction by D132A III (+) and E286A-I112E III (+) (ref *20* and Table 1). E286A-I112E and D132A suicide inactivate even when they contain stoichiometric amounts of subunit III (protein gels not shown). D132A III (+) had an average catalytic life span of approximately 50000 CCs at pH 7.4, while E286A-I112E III (+) showed an average life span of ∼18000 CCs (Figure 6). These catalytic life spans are longer than those measured for the subunit III-depleted forms of these mutants, but they are 70−200-fold shorter than the catalytic life span of the wild-type oxidase that contains subunit III (Figure 6).

In contrast to the D pathway mutants containing subunit III, the subunit III-containing form of the K pathway mutant T359A exhibits a catalytic life span of $(3-4) \times 10^6$ CCs (data not shown), similar to WT III (+) (Figure 6). This is consistent with the finding that the rate of proton uptake through the D pathway of T359A III (+) is similar to that of WT III (+) (*8*).

Inhibition of Proton Uptake by ΔΨ and ΔpH Further Accelerates the Rate of Suicide Inactivation. Once cytochrome oxidase is reconstituted into a phospholipid vesicle, catalytic turnover generates a transmembrane voltage gradient (positive outside) that electrostatically opposes proton uptake from the inside of the vesicle through both the D and K pathways. Catalytic turnover under such "controlled" conditions increases the probability of suicide inactivation such that the catalytic life span of WT III (−) is decreased approximately 10-fold, from ∼10000 CCs to ∼1000 CCs, by the transmembrane voltage gradient (Figure 7). On the basis of the above, this increase in suicide inactivation is due to the inhibition of proton uptake through the D pathway by the ΔΨ and ΔpH. A similar pattern is seen for D132A III (−) (Figure 7), although the inhibition imposed by the D132A mutation itself further increases suicide inactivation under both controlled and uncoupled conditions. Once T359A III (−) is incorporated into COVs, this mutant oxidase, also, shows a decreased catalytic life span in the presence of a membrane potential (Figure 7). Unlike D132A III (−), however, the catalytic life span of T359A III (−) is no less than that of WT III (−). This is consistent with the earlier conclusion that inhibition of the K pathway fails to enhance suicide inactivation.

Proton Uptake from the Outer Surface of the Oxidase Complex Helps To Slow Suicide Inactivation and Extend the Life Span of the Enzyme. During controlled turnover in COVs (i.e., in the presence of ΔΨ and ΔpH) a considerable portion of proton flow to the active site comes from the outer surface of cytochrome oxidase, perhaps by reverse proton flow through the normal exit pathway for pumped protons (*19*, *46*). The importance of this pathway becomes particularly evident using mutants that inhibit proton transfer through the D pathway, such as D132A (see Discussion). The double mutant D132A-R481K alters both D132 plus an arginine that may form part of the proton exit pathway (*17*, *46*). The catalytic life span of D132A-R481K, which contains stoichiometric amounts of subunit III, is 10-fold shorter than that of WT III (−) during uncontrolled or uncoupled turnover (Figure 8). Such a high probability of suicide inactivation, even in the presence of subunit III, likely occurs because proton flow from the outer surface of the protein is inhibited by the R481K mutation at the same time that proton flow from the inner surface is inhibited by the D132A mutation (see Discussion). Note that the catalytic life span of D132A-R481K III (+) is actually longer during controlled turnover than it is during turnover in the presence of uncouplers (Figure 8). This indicates that the driving force of the transmembrane potential (negative inside) is able to at least partially overcome the inhibition of reverse proton flow imposed by the R481K mutation.

Is Slow Electron Input into the Active Site the Actual Cause of Suicide Inactivation? The experiments presented to this point support the hypothesis that slow proton transfer to the

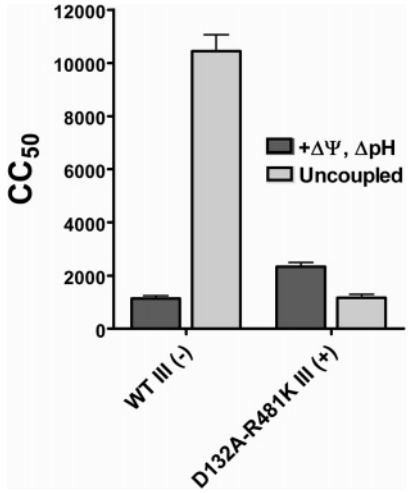

FIGURE 8: Simultaneous inhibition of the D proton pathway and the backflow/exit proton pathway enhances suicide inactivation in the presence of subunit III. The CC_{50} values of COVs containing D132A-R481K III (+) were 2337 ± 158 (controlled turnover) and 1162 ± 136 (uncoupled turnover). The CC_{50} values for WT III (−) COVs are presented in the legend of Figure 7.

active site increases the probability that cytochrome *c* oxidase will suicide inactivate during its catalytic cycle. Since electron input into the active site of the enzyme is controlled by the rate of proton uptake (*47*−*49*), it could be argued that the direct cause of suicide inactivation is actually slow electron input into the active site, brought about by slow proton uptake. This hypothesis was examined in three ways.

First, the ability of the subunit II mutant M263L to induce suicide inactivation was examined. The M263L mutation inhibits electron transfer from cytochrome *c* to Cu_A, setting up conditions where electron input into the active site of M263L is over 30-fold slower than the intrinsic rate of proton uptake via the D pathway (*42*, *48*). Catalytic turnover of M263L, containing subunit III, does not result in suicide inactivation (data not shown). In the absence of subunit III, M263L does inactivate, but no faster than WT III (−) (data not shown). These experiments argue against slow electron input as the primary trigger for suicide inactivation.

Second, the tendency of the wild-type oxidase, containing subunit III, to suicide inactivate at very slow turnover rates was examined. This is a important control experiment because the mutant oxidase forms shown above to inactivate in the presence of subunit III, D132A-R481K III (+), D132A III (+), and E286A-I112E III (+), exhibit very slow initial turnover rates due to slow proton delivery to the active site (*21*, *23*, *25*, *27*, *39*) (Table 1). Slowing the turnover rate of the wild-type oxidase, with subunit III, to ∼10 s^{-1} (by adjusting the concentration of cytochrome *c*) did not induce the enzyme to suicide inactivate (data not shown).

Finally, an experiment was conducted to slow the rate of electron input into the active site of detergent-solubilized WT III (−) by progressively limiting the amount of cytochrome *c*, thus slowing electron input via Cu_A and heme *a*. Figure 9 shows that the average catalytic life span of WT III (−) during uncontrolled, steady-state turnover is the nearly the same at a TN of 21 s^{-1} as it is at a TN of 825 s^{-1}. Taken alone, these two points suggest that slow electron input into the active site in the absence of subunit III does not induce suicide inactivation. However, the catalytic life span of WT

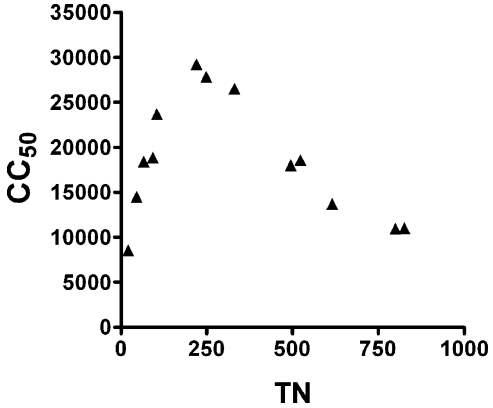

FIGURE 9: Effect of the rate of electron delivery to the active site on the rate of suicide inactivation. The average catalytic life span (CC_{50}) of detergent-solubilized WT III (−) at pH 7.2 was measured at different rates of initial turnover (O_2 reduction), achieved by varying the concentration of horse cytochrome *c* in the reaction mixture.

III (−) did vary with TN in that it increased 3-fold as the TN declined to 250 s^{-1}, below which the life span decreased once again (Figure 9). Since the rate of proton uptake by WT III (−) under these conditions is <500 s^{-1} (*33*), it may be that at the turnover rate of 250 s^{-1} the enzyme is "tuned"; i.e., the rate of delivery of substrate electrons and substrate protons to the active site has been optimized. If such electron−proton tuning helps to resist suicide inactivation, it follows that slow electron delivery to the active site may sometimes play a role in the chemistry of suicide inactivation (see Discussion).

DISCUSSION

Suicide Inactivation and the D Pathway. In the absence of subunit III, cytochrome *c* oxidase exhibits a tendency to suicide inactivate during any given catalytic cycle (*28*), due to irreversible structural alterations at the active site of the enzyme (*29*, *44*). The probability of the inactivation event increases substantially at pH values above neutral (*44*). The experiments presented above explain the pH effect: the probability of suicide inactivation increases as the rate of proton delivery to the heme a_3−Cu_B O_2 reduction site decreases. For protons taken up from the inner surface of the oxidase complex, the effect is specific for the D proton pathway. Mutations that disrupt the D pathway increase the probability of suicide inactivation while mutational inhibition of the K pathway does not. We have previously shown that the removal of subunit III strongly inhibits proton uptake by the D pathway (and not the K pathway) at pH values above neutral (*20*, *33*). Together with this previous finding, the experiments presented here demonstrate that one way that subunit III protects the oxidase from suicide inactivation is to maintain rapid proton transfer through the D pathway to the active site.

Long-chain, unsaturated fatty acids, particularly arachidonic acid, stimulate the slow O_2 reduction activity of D132 mutants by increasing proton uptake into the D pathway, apparently by replacing the carboxylate function of the absent D132 with the carboxylate group of the fatty acid (*21*, *45*, *46*). More recent experiments indicate that the removal of subunit III allows for a more productive binding of the fatty

acid in terms of its ability to direct protons into the D pathway (*20*). In keeping with its function of enhancing proton uptake into the D pathway, arachidonic acid strongly retards suicide inactivation by WT III (−) and two subunit III-depleted oxidase mutants with structural alterations in the D pathway, D132A III (−) and E286 III (−) (Figure 5).

An informative negative result was obtained when we attempted to use zinc to induce suicide inactivation. The addition of micromolar quantities of Zn^{2+} strongly inhibits steady-state O_2 reduction by detergent-solubilized cytochrome oxidase (*50*). Single-turnover experiments show that zinc inhibits proton uptake into the D pathway (*51*). However, the addition of zinc at a variety of pH values did not increase suicide inactivation by WT III (−) (data not shown) even though it did inhibit steady-state O_2 reduction, as expected. From this we propose that the predominant effect of zinc during steady-state turnover is *not* inhibition of proton uptake by the D pathway; it may be that zinc slows steady-state O_2 reduction by the detergent-solubilized oxidase by inhibiting proton uptake through the K pathway. This finding need not be viewed as inconsistent with the inhibitory effect of zinc on the D pathway in the single-turnover experiments (*51*). In the latter, Zn^{2+} has extensive time to equilibrate with all of its binding sites on the oxidase surface prior to the initiation of the single-turnover reduction of O_2. It is entirely possible that proton and Zn^{2+} compete for a site near the entrance to the D pathway where the k_{on} for proton is greater than that for Zn^{2+}. If this is so, Zn^{2+} will have little effect on proton uptake into the D pathway during steady-state turnover even though the metal may have a large effect once Zn^{2+} and proton have sufficient time to reach equilibrium for binding prior to the single-turnover experiment.

The Role of the Proton Backflow/Exit Pathway. Considerable evidence now exists for a proton backflow pathway leading from the outer surface of the oxidase complex to the active site (*19*, *20*, *46*). This pathway, which may function by reversal of the normal exit pathway for pumped protons, allows at least a slow delivery of protons to the active site from the outer surface of the oxidase under conditions where proton delivery from the inner surface is compromised. For example, a membrane potential (negative inside) stimulates the slow O_2 reduction activity of D132A III (+), apparently by driving the influx of protons from the outer surface (*25*, *45*). In other experiments, the slow rate of controlled O_2 reduction (plus $\Delta\Psi$ and ΔpH) by COVs containing the wild-type oxidase is further inhibited by the addition of zinc, because the zinc cation binds to the outer surface of the oxidase and blocks the entry of protons (*19*). (In the latter experiment the lipid bilayer of the COVs prevents access of zinc to its binding sites on the inner surface of the oxidase complex.) Recently, we have found that the removal of subunit III also inhibits proton transfer from the outer surface of the oxidase, similar to but additive to the effect of zinc (*20*).

Of particular interest with regard to the backflow of protons is the D132A-R481K double mutant containing subunit III. This mutant oxidase exhibits a 10-fold shorter catalytic life span than WT III (−) during uncontrolled turnover (Figure 8) even though it contains normal amounts of subunit III. This phenomenon may be explained by strong induction of suicide inactivation upon simultaneous structural

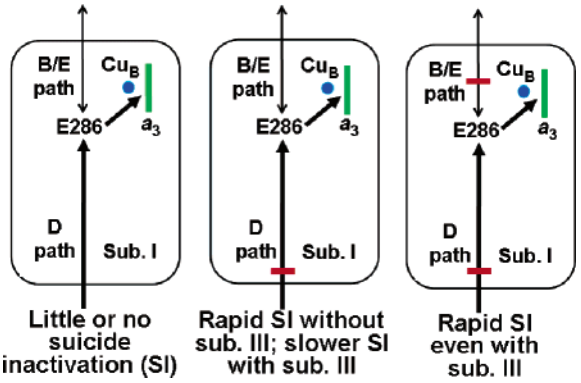

FIGURE 10: Relationship between the D pathway and the backflow/exit pathway to suicide inactivation of cytochrome *c* oxidase. The cartoon shows subunit I only, with the two metal centers of the active site. The D pathway (D) leads to E286 from the inner surface the oxidase complex while a backflow/exit pathway (B/E) is proposed to lead to E286 from the outer surface of the oxidase complex. The involvement of E286 in the B/E pathway is purely hypothetical. A short arrow depicts the pathway for protons leading from E286 to the active site. The red blocks represent the substantial but incomplete inhibition of proton flow through the pathways as a result of site-directed mutations, the removal of subunit III, or, for the D pathway only, the presence of $\Delta\Psi$ and ΔpH.

inhibition of both the D-pathway and the proton backflow pathway by the amino acid alterations. The D132A mutation strongly retards proton uptake by the D pathway, especially in the presence of subunit III (*20*). Arg481 normally interacts with the propionate groups of hemes *a* and a_3 (*3*, *40*), one or both of which may be participants in the proton backflow/exit pathway (*16*, *52*, *53*). When proton uptake from the inner surface of R481K (the single mutant) is inhibited by $\Delta\Psi$ and ΔpH (i.e., by turnover in COVs), its rate of controlled turnover is ~25% that of the wild-type oxidase (Mills and Ferguson-Miller, unpublished results). This indicates restricted delivery of protons to the active site of R481K from the outer surface of the oxidase complex via the proton backflow pathway, an effect that only becomes evident when proton uptake from the inner surface of the oxidase complex is inhibited. Thus, the rapid inactivation of D132A-R481K III (+) occurs because proton transfer from both sides of the oxidase complex is inhibited in the double mutant, with the result that the active site is starved for protons.

An alternative explanation for the rapid inactivation of D132A-R481K is that the R481K mutation distorts the structure of the active site in such a manner that suicide inactivation becomes more likely. However, the CC_{50} of detergent-solubilized R481K is approximately 3.5×10^6; i.e., with uninhibited proton uptake through the D pathway, the catalytic life span of R481K is the same as the detergent-solubilized wild-type oxidase containing subunit III. Thus, the R481K mutation does not appear to introduce an active site alteration that predisposes the enzyme to suicide inactivate.

A scheme for the roles of the D pathway and the backflow/exit (B/E) pathway in preventing or inducing suicide inactivation is presented in Figure 10. The D pathway is viewed as primary, since, as yet, we have no definitive evidence that the restriction of proton transfer through the B/E pathway in the absence of D pathway inhibition induces suicide inactivation. However, once the D pathway is inhibited by a membrane potential (or by mutation), proton

uptake through the B/E pathway helps to slow suicide inactivation. Inhibition of both pathways leads to rapid suicide inactivation even in the presence of subunit III, which, in addition to its other effects, is likely to directly stabilize the structure of the active site (see below).

Our results suggest an important physiological role for the backflow pathway for protons from the outer surface of the oxidase complex. Under conditions of a high membrane potential, where proton transfer from the inner surface is strongly inhibited, the transfer of protons through the backflow pathway will help to prevent the rapid inactivation of cytochrome oxidase. Since suicide inactivation is irreversible, this would prevent the cell from having to synthesize large amounts of the cytochrome oxidase complex following transient periods of low ADP availability. The ability of subunit III to enhance the function of the proton backflow pathway (*20*) becomes a second way in which this membrane partner of subunit I helps to prevent the onset of suicide inactivation at the active site.

The Mechanism of Suicide Inactivation. The finding that slow proton transfer through the D pathway, but not the K pathway, enhances suicide inactivation provides important clues about the mechanism of inactivation. The inactivation event itself presents as an irreversible alteration in the structure of the active site (*29*), which culminates in the release of Cu_B (Hosler, unpublished results). Since the D pathway is required for the uptake of the protons that carry the O_2 reduction chemistry past the oxoferryl intermediates (Figure 1, intermediates 4 and 5) (*4*), it follows that slow proton delivery by the D pathway will increase the lifetimes of these intermediates in the catalytic cycle during steady-state turnover. The oxoferryl (Fe^{4+}═O) group is capable of nucleophilic attack on a nearby target, leading to irreversible oxygenation or hydroxylation. An extended lifetime for the oxoferryl may allow its reaction with the protein scaffold of subunit I in the same manner that the oxoferryl intermediate of cytochrome P450 monooxygenases reacts with substrate molecules (*54*, *55*).

Slow proton uptake through the D pathway will also increase the lifetime of deprotonated E286. A series of bound water molecules, unresolved in current crystal structures, has been proposed to direct protons from E286 to the heme a_3−Cu_B O_2 reduction site, a distance of ∼10 Å (*56*). Spectroscopic studies reveal the existence of a through-bond, polarizable connection between E286 and the Cu_B center, via water (*56*, *57*). Anionic E286 may polarize the waters between it and the Cu_B center, bringing a partial negative charge on a water oxygen close to a histidine ligand of Cu_B. Electrostatic stabilization by this partial negative charge could promote the occasional dissociation and protonation of the histidine from Cu_B as the initial step in suicide inactivation. Nucleophilic attack of the dissociated histidine by the oxoferryl group on heme a_3 could make the dissociation of the histidine permanent, thus weakening the interaction of Cu_B with the protein and allowing loss of the metal. We have previously noted that the structural features of the active site of the suicide-inactivated oxidase are remarkably similar to those of oxidase mutants containing alterations of the Cu_B ligand H284 or its covalently attached neighbor, Y288 (*29*). Thus, we propose the dissociation of H284 from Cu_B as a central component of suicide inactivation chemistry. This proposal is strengthened by theoretical studies indicating that the Y288-H284 Cu_B ligand is the most likely of the three Cu_B ligands to dissociate (*58*).

The catalytic cycle of cytochrome oxidase may include the formation of a radical form of Y288, close to Cu_B (*5*, *7*, *59*) (Figure 1). The ability of an amino acid radical to initiate destructive chemistry makes this an attractive candidate for the species that leads to inactivating structural changes in the active site. During steady-state turnover, the reduction of heme *a* may be limited by the supply of electrons from cytochrome *c*, and this should extend the lifetime of the Y288 radical. The finding that the probability of suicide inactivation by WT III (−) is increasing as electron delivery slows (Figure 9) leaves open the possibility that the Y288 radical may augment suicide inactivation at slow turnover rates in the absence of subunit III. In the presence of subunit III, however, slow turnover of the wild-type oxidase does not lead to suicide inactivation (see Results).

Suicide Inactivation and Proton Pumping. Suicide inactivation results from decreased rates of proton transfer through the D pathway and the backflow/exit pathway. Together, these two pathways provide the route for the transfer of pumped protons to and from the site of proton pumping, presumably near the heme a_3−Cu_B O_2 reduction site (*4*). Consistent with this, oxidase forms that suicide inactivate either fail to pump protons or pump with decreased efficiency. *R. sphaeroides* D132A III (+), D132A III (−), and D132A-R481K III (+) all fail to pump protons (*20*, *25*) (Mills and Ferguson-Miller, unpublished results). *R. sphaeroides* WT III (−) and E286A-I112E III (+) pump with lower efficiency than the wild-type oxidase containing subunit III (*20*, *39*). Consistent with our finding that inhibition of the K pathway does not induce suicide inactivation, the K pathway mutant T359A III (+) pumps with normal efficiency (*25*).

The Role of Subunit III. We have previously shown that the removal of subunit III from cytochrome *c* oxidase (1) induces suicide inactivation at the active site (*29*), (2) slows proton uptake into the D pathway at physiologic pH (*33*), and (3) inhibits the backflow of protons to the active site (*20*). The experiments presented here link these phenomena by demonstrating that slow proton transfer to the active site induces suicide inactivation and that both the D pathway and the backflow pathway for protons play a role in this process. Thus, it can be argued that the ability of subunit III to prevent suicide inactivation in the normal oxidase is due to its ability to maintain effective rates of proton flow through these two pathways in subunit I. The ability of subunit III to prevent suicide inactivation can be overcome, however, by severely limiting proton flow to the active site.

Does subunit III protect the active site from suicide inactivation by any mechanism in addition to its ability to facilitate proton transfer in subunit I? The answer appears to be yes, based on the following observations. D132A suicide inactivates both in the presence and in the absence of subunit III. However, the average catalytic life span of D132A III (−) is 10 times less than that of D132A III (+) (Figures 4 and 6) even though the rate of proton delivery to the active site is some 40-fold faster for D132A III (−) than for D132A III (+) (Ädelroth et al., unpublished results, and ref *20*). Comparison of these two forms of D132A, therefore, suggests that subunit III protects the active site by some mechanism in addition to the maintenance of proton delivery.

It seems likely that the binding of subunit III to subunit I exerts structural influence that also helps to protect the active site from the chemistry of suicide inactivation.

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