Suicide Inactivation of Cytochrome *c* Oxidase: Catalytic Turnover in the Absence of Subunit III Alters the Active Site†

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ABSTRACT: The catalytic core of cytochrome *c* oxidase is composed of three subunits: I, II, and III. Subunit III is a highly hydrophobic membrane protein that contains no redox centers; its role in cytochrome oxidase function is not obvious. Here, subunit III has been removed from the three-subunit mitochondrial-like oxidase of *Rhodobacter sphaeroides* by detergent washing. The resulting two-subunit oxidase, subunit III (−), is highly active. Ligand-binding analyses and resonance Raman spectroscopy show that its heme a_3−Cu_B active site is normal. However, subunit III (−) spontaneously and irreversibly inactivates during O_2 reduction. At pH 7.5, its catalytic lifetime is only 2% that of the normal oxidase. This suicide inactivation event primarily alters the active site. Its ability to form specific O_2 reduction intermediates is lost, and CO binding experiments suggest that the access of O_2 to reduced heme a_3 is inhibited. Reduced heme *a* accumulates in response to a decrease in the redox potential of heme a_3; electron transfer between the hemes is inhibited. Ligand-binding experiments and resonance Raman analysis show that increased flexibility in the structure of the active site accompanies inactivation. Cu_B is partially lost. It is proposed that suicide inactivation results from the dissociation of a ligand of Cu_B and that subunit III functions to prevent suicide inactivation by maintaining the structural integrity of the Cu_B center via long-range interactions.

As the terminal member of the respiratory electron-transfer chain of mitochondria and many aerobic bacteria, cytochrome *c* oxidase catalyzes the four-electron reduction of oxygen to water. The resulting redox energy drives a transmembrane proton pump (*1*, *2*). While mammalian oxidase is a large complex of 13 different subunits, only the three largest form the "catalytic core" of the enzyme (*3*, *4*). Subunits I and II contain all of the redox-active centers of the oxidase. Four soluble cytochromes *c* bind sequentially to subunit II to donate electrons to Cu_A (*5*, *6*). Electrons are then transferred to the six-coordinate heme *a* site in subunit I and then to the heme a_3−Cu_B O_2 reduction site, also in subunit I. The O_2 reduction mechanism has recently been proposed to involve a radical form of a tyrosine residue that is cross-linked to one of the histidine ligands of Cu_B (*7*, *8*). The proton-pumping mechanism is yet to be solved, but it has been hypothesized to involve a histidine ligand of Cu_B (*9*).

The aa_3-type cytochrome *c* oxidases of the non-sulfur purple bacteria *Paracoccus denitrificans* and *Rhodobacter sphaeroides* are highly similar to cytochrome oxidase of mitochondria (*10*−*14*). Indeed, the structure of the *P. denitrificans* enzyme is nearly identical to the three-subunit core of the bovine oxidase (*15*−*17*).

Subunit III is a large hydrophobic protein that binds to the transmembrane face of subunit I opposite the transmembrane helices of subunit II (*15*, *16*). Subunit III contains no metal centers and thus plays no direct role in electron transfer. However, subunit III is clearly a member of the catalytic core of cytochrome oxidase along with subunits I and II (*3*, *4*). The gene for subunit III is retained in the mitochondrial genome along with those for subunits I and II (*18*), and the primary structure of subunit III is highly conserved [∼71% similarity between *Rb. sphaeroides* and humans (*12*)]. Subunit III can be removed from the enzyme using polyoxyethylene detergents (*19*−*25*), indicating relatively weak binding to subunit I. Subunit III-depleted oxidase preparations generally exhibit decreased proton-pumping efficiency, and the reported rates of O_2 reduction by these preparations varies considerably (*20*−*23*, *26*, *27*). Subunit III has been suggested to play a role in proton-pumping efficiency (*27*, *28*), proton channeling (*20*, *27*, *29*), oxidase assembly (*23*), and O_2 channeling (*30*). The molecular mechanisms for these proposed roles remain to be elucidated.

During O_2 reduction by cytochrome oxidase, highly reactive intermediates are produced at the active site. Some of these reactive intermediates, including the heme a_3 oxyferryl and the proposed tyrosine radical, may accumulate to high levels depending upon the rate of electron flow into heme a_3−Cu_B active site (*7*, *31*). Inappropriate chemistry by such intermediates could be detrimental to the enzyme. For example, prostaglandin H synthase (*32*) and linoleate

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diol synthase (*33*) both cleave dioxygen and generate a heme oxyferryl and a tyrosine radical during their catalytic cycle. Both of these enzymes rapidly inactivate during catalysis. In contrast, cytochrome oxidase reduces O_2 for extended periods with little loss of activity (*26* and this report). Working with the mitochondrial-like cytochrome *c* oxidase of *P. denitrificans*, Haltia et al. (*26*) showed that oxidase forms that lack subunit III rapidly lose O_2 reduction activity during turnover. Thus, a potential role for subunit III is to protect the active site of cytochrome oxidase from oxidative damage during turnover.

Here, we show that turnover-induced loss of activity (suicide inactivation) also occurs in the mitochondrial-like cytochrome oxidase of *Rb. sphaeroides* if subunit III is absent. The inactive oxidase has been examined to determine the cause of suicide inactivation and to initiate an investigation of how subunit III prevents inactivation of the normal oxidase. Inactivated subunit III (−) shows major changes in the access of CO to heme a_3 and disruption of the Cu_B center. Suicide inactivation also leads to a decrease in the redox potential of heme a_3 that inhibits electron transfer from heme *a*. The results indicate that subunit III functions to maintain active conformations of the heme a_3-Cu_B site during turnover.

EXPERIMENTAL PROCEDURES

Materials. L-α-Phosphatidylcholine (asolectin, Sigma type II-S) and horse heart cytochrome *c* (Sigma, type VI) were dissolved in 2% sodium cholate and 50 mM KH_2PO_4, pH 7.2, respectively, and stored at −80 °C. *N*-Dodecyl-*β*-D-maltoside was purchased from Anatrace. All other reagents were of the highest grade available.

Bacterial Growth and Oxidase Purification. Rb. sphaeroides strains YZ300 (*34*) and ΔCOXIII (see below) were grown in Sistrom's media A (*35*) containing 1.0 μg/mL tetracycline, 50 μg/mL streptomycin, and 50 μg/mL spectinomycin. Cultures of 500 mL were shaken vigorously at 32 °C in baffled flasks and harvested in late exponential phase (OD_{660} = 1.0−1.2). Cytoplasmic membranes were prepared as in Hosler et al. (*13*), and the oxidases were purified on Ni^{2+}-NTA[1] agarose (Qiagen) as in Zhen et al. (*34*).

Preparation of Subunit III (−). Subunit III was removed from the purified three-subunit oxidase by stirring the oxidase in 50 mM KH_2PO_4, pH 7.2, 1 mM EDTA, and 4% Triton X-100 for 1 h at 0 °C. The product was repurified on Ni^{2+}-NTA agarose as before, with the exception that detergent exchange was performed on the column by washing with 5 vol of 10 mM Tris-HCl, pH 8.0, 40 mM KCl, and 10 mM imidazole running buffer containing 0.5% Triton X-100, followed by 10 vol of the running buffer containing 0.1% dodecyl maltoside. Triton washing and purification was repeated. The resulting two-subunit oxidase was termed subunit III (−).

Activity Assays. Oxygen reduction assays were performed at 25 °C in a mixture of 5 pmol of oxidase (unless otherwise noted), 25 mM Bis-Tris-HCl, pH 6.5, 87.5 mM KCl, 0.1% dodecyl maltoside, 3 mM ascorbate, 0.6 mM TMPD, and 1 mg/mL phosphatidylcholine (PC). The reactions were initiated by the addition of 1−100 μM horse-heart cytochrome *c*. Oxygen consumption rates were measured using a Hansatech DW1 oxygen electrode unit, a CB1-D3 control box and Minirec software (Hansatech) on a PC. The data were imported into Origin 5.0 (Microcal) for subtraction of the slow rate of nonenzymatic reduction of O_2 by ascorbate and calculation of initial slopes. Turnover numbers (TN) were calculated as described in Thompson and Ferguson-Miller (*36*). V_{max} values were obtained by fitting plots of TN vs [cytochrome *c*] to a hyperbolic function.

Estimation of the Rate of Suicide Inactivation: $CC_{60\%}$. Origin 5.0 was used to isolate the point on individual O_2 consumption traces where the tangent was equal to 60% of the initial rate. The amount of oxygen consumed (nmol) to this point was calculated and divided by the amount of oxidase (nmol) used in the reaction. This gave the average number of catalytic cycles ($1O_2 \rightarrow 2H_2O$) performed by each oxidase monomer to the point where the entire population reduced O_2 at 60% of the initial rate. This value, termed $CC_{60\%}$, is inversely proportional to the actual rate of suicide inactivation. For example, if the rate of suicide inactivation is fast, fewer catalytic cycles are required to decrease the overall activity to 60% of the initial activity than if the rate of suicide inactivation is slow. In addition to being a simple method for comparing rates of oxidase inactivation, the $CC_{60\%}$ value is independent of time (unlike a $t_{1/2}$) and the amount of oxidase used in the assay. Therefore, the $CC_{60\%}$ value allows comparison of inactivation experiments performed under different conditions, such as varying pH. The value of 60% of the initial rate was chosen empirically: it can be accurately determined at all of the pH values tested.

Preparation of Suicide-Inactivated Subunit III (−) for Spectroscopic Analyses. Two milliliter solutions of 1 μM oxidase were vigorously stirred in 10 mL beakers in 25 mM Bis-Tris-HCl, pH 7.5, 87.5 mM KCl, 50 mM ascorbate, 0.6 mM TMPD, 0.1% dodecyl maltoside, 130 units superoxide dismutase, and 140 units catalase. Subunit III (−) samples with 20−75% of their original activity were prepared by using 1−5 μM cytochrome *c* and reaction times of 5−25 min. The reactions were terminated by slowing the input of O_2 by stopping the stir motor and by adding KH_2PO_4, pH 7.2, to 200 mM in order to inhibit the binding of cytochrome *c*. The samples were concentrated using a 30K Ultrafree centrifugal filter (Millipore) and chromatographed on a 50 × 1 cm Sephadex G-50 column in a running buffer of 200 mM KH_2PO_4, pH 7.2, and 0.1% dodecyl maltoside in order to separate subunit III (−) from cytochrome *c*.

Optical Spectroscopy and Ligand-Binding Reactions. Optical spectra were recorded at 25 °C using a Hitachi-U3000 UV−VIS spectrophotometer. Oxidase samples were reduced using 3 mM ascorbate, 0.6 mM TMPD, or solid sodium dithionite in 50 mM KH_2PO_4, pH 7.2, 1 mM EDTA, 0.1% dodecyl maltoside, and the concentrations determined using the extinction values of Vanneste (*37*). To produce the carbon-monoxy form, 0.5 mL of CO was bubbled through the reduced oxidase sample. The extent of CO binding by active subunit III (−) was calculated from the amplitude of the 430−447 nm signal in reduced + CO minus reduced

[1] Abbreviations: NTA, nitrilotriacetic acid; TMPD, *N*,*N*,*N*′,*N*′-tetramethyl-1,4-phenylenediamine dihydrochloride; PC, phosphatidylcholine; TN, turnover number; $CC_{60\%}$, number of catalytic cycles to 60% of the initial O_2 reduction activity (catalytic cycle = $1O_2 \rightarrow 2H_2O$); **P**, "peroxy" O_2 reduction intermediate; **F**, "oxyferryl" O_2 reduction intermediate; SOD, superoxide dismutase.

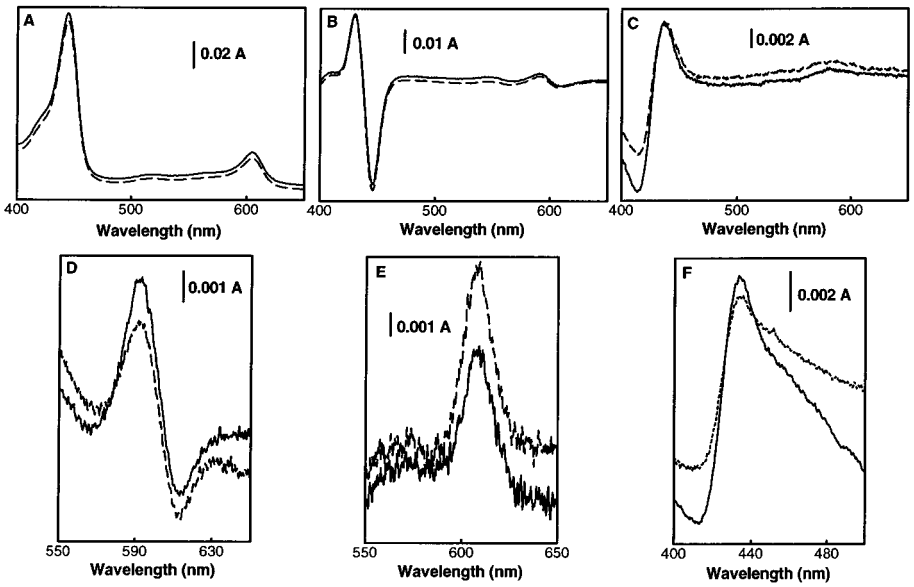

FIGURE 1: Spectroscopic comparison of subunit III (−) (- - -) and the normal three-subunit oxidase (—). (A) Absolute spectra of the two oxidase forms reduced with dithionite. The difference spectra shown in panels B−F were obtained as described in Experimental Procedures. (B) CO binding to reduced heme a_3. (C) Formation of O_2 reduction intermediate **F**. (D) CN^- binding to reduced heme a_3. (E) Formation of O_2 reduction intermediate **P**. (F) CN^- binding to oxidized heme a_3. The concentration of the oxidases was 0.7−1.1 μM.

difference spectra. The signal exhibited by the normal, three-subunit oxidase was considered to be 100%. In determining the extent of CO binding by the *inactive* form of subunit III (−), the signal produced by *active* subunit III (−) was considered to be 100%. These conventions were followed for the other four ligand-binding reactions explained below. Note that for these *relative* measurements of ligand binding extinction coefficients are not used. For cyanide binding to reduced oxidase, the samples were reduced as above but at pH 6.5, followed by the addition of KCN to 40 mM (*38*). The extent of CN^- binding was calculated from the 590−613 nm signal of the reduced + CN^- minus reduced difference spectrum. For CN^- binding to the oxidized enzymes, the oxidases were oxidized with 10 μM $KMnO_4$ in 50 mM KH_2PO_4, pH 7.2, and 0.1% dodecyl maltoside followed by the addition of KCN to 5 mM. The extent of CN^- binding was calculated from the 435−415 nm signal in oxidized + CN^- minus oxidized difference spectra. Generation of the "peroxy" O_2 reduction intermediate (**P**) was accomplished by bubbling 0.5 mL of CO into a $KMnO_4$ oxidized sample of the oxidase in 50 mM KH_2PO_4, pH 7.2, 1 mM EDTA, and 0.1% dodecyl maltoside. The amount of **P** produced was determined from the 607−650 nm signal of oxidized + CO/O_2 minus oxidized difference spectra. Generation of the "oxyferryl" O_2 reduction intermediate (**F**) was accomplished by adding 2 mM H_2O_2 to the oxidized enzymes at pH 7.2. The amount of **F** produced was determined from the 435−415 nm signal of oxidized + H_2O_2 minus oxidized difference spectra.

In oxidase samples in which heme a_3 was incompletely reduced, the extent of heme a_3 reduction was estimated from the ratio of the amplitudes of the Soret band and the α band of absolute reduced spectra. The Soret/α value is 5.3 when hemes *a* and a_3 are completely reduced and 2.9 when only heme *a* is reduced (*37*). Thus, the fraction of heme a_3 reduced is given by $(x - 2.9)/2.4$, where x is the measured Soret/α value and 2.4 is the difference between the estimated Soret/α values of $a^{2+}a_3^{2+}$ and $a^{2+}a_3^{3+}$ (from ref *37*). This method is reasonably accurate since absorption by oxidized heme a_3 at the wavelengths of the reduced Soret and α peaks is slight.

Other Assays. Heme A determinations and resonance Raman spectroscopy were performed as described earlier (*13*). Metal analysis of 1 mL samples of 25 μM oxidase were performed by inductively coupled plasma atomic emission spectroscopy at the Chemical Analysis Laboratory of the University of Georgia.

RESULTS

Preparation and Characterization of Subunit III (−). To create subunit III (−), subunit III was removed from the three-subunit aa_3-type oxidase of *Rb. sphaeroides* using Triton-X-100, as described in Experimental Procedures. By gel analysis, less than 5% of the original amount of subunit III remained in subunit III (−) after two rounds of Triton washing and repurification. The V_{max} of the initial oxygen reduction activity of subunit III (−) was 2400 e^-/s (assayed at pH 6.5 in the presence of 1 mg/mL PC). This was only slightly lower than that of the normal, three-subunit oxidase (V_{max} = 2600 e^-/s), indicating that the removal of subunit III with Triton had little effect on oxidase activity.

The absolute spectrum of subunit III (−) was identical to that of the three-subunit oxidase (Figure 1A), indicating that the removal of subunit III did not perturb the environments of the heme centers. A series of ligand-binding experiments showed that the heme a_3-Cu_B active site of subunit III (−) was only slightly altered by the removal of subunit III. Carbon monoxide binds tightly to reduced heme a_3 (*38*). Subunit III (−) bound normal levels of CO with either ascorbate/TMPD or sodium dithionite as the reductant (Figure 1B). Cyanide binds tightly to oxidized heme a_3 and with less affinity to the reduced heme (*38*). Oxidized heme a_3 of subunit III (−) bound ∼75% of the normal amount of cyanide (Figure 1F), while reduced heme a_3 bound as much CN^- as the normal enzyme (Figure 1D). Bubbling CO through an aerobic solution of resting cytochrome oxidase

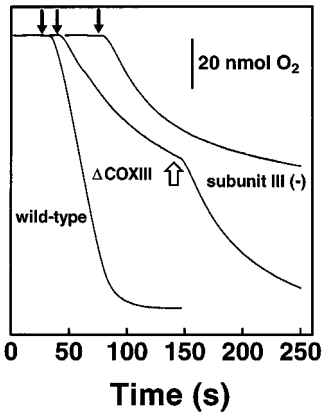

FIGURE 2: O_2 reduction activity of the normal, three-subunit oxidase, ΔCOXIII, and subunit III (−). Wild-type (three-subunit) oxidase (10 pmol), subunit III (−) (10 pmol), and ΔCOXIII (15 pmol) were assayed for O_2 reduction activity (O_2 uptake) as described in the Experimental Procedures, but using 25 mM Bis-Tris-HCl, pH 7.5. Reactions were initiated by the addition of 80 μM horse heart cytochrome *c* (solid arrow). Fifteen picomoles of fresh ΔCOXIII was added at the point indicated by the open arrow. 100% O_2 (120 nmols total) was present at the beginning of the experiment, whereas the cessation of O_2 uptake by the wild-type oxidase marks the point at which all O_2 was consumed.

effects a two-electron reduction of heme a_3 and Cu_B; residual O_2 is then reduced to produce the "peroxy" intermediate, **P** (*39*−*42*). Subunit III (−) produced the same amount of **P** by this method as the normal oxidase (Figure 1E). Oxidized heme a_3 in normal cytochrome oxidase binds hydrogen peroxide to transiently form **P**; the oxidation of a second molecule of H_2O_2 rapidly yields the three-electron reduced "oxyferryl" intermediate, **F** (*38*, *42*−*44*). This reaction proceeded to 75% of normal in subunit III (−) (Figure 1C). Presumably, the initial binding of H_2O_2 to oxidized heme a_3 was inhibited to the same extent (∼25%) as CN^- binding to oxidized subunit III (−). Thus, the reduced active site of subunit III (−) appeared identical to the normal oxidase, while the oxidized heme a_3−Cu_B center showed evidence of a slight alteration.

Direct expression of an oxidase that assembles in vivo without subunit III was achieved by reintroducing an expression vector that lacks *coxIII* into *Rb. sphaeroides* strain YZ200, a strain in which the *coxII/III* operon has been deleted (*34*). The resulting oxidase was termed ΔCOXIII; details of its construction and characteristics will be published elsewhere. ΔCOXIII showed the same pattern of active-site alterations as subunit III (−) with suicide inactivation (described below). Experiments using ΔCOXIII are presented, as necessary, in this report to support the results obtained with subunit III (−).

Suicide Inactivation of Subunit III (−) and ΔCOXIII. During steady-state O_2 reduction, an obvious difference appeared in the three forms of cytochrome oxidase: both subunit III (−) and ΔCOXIII rapidly lost activity, while the three-subunit oxidase containing subunit III did not (Figure 2). For example, at pH 7.5 the lifetime of subunit III (−) was 2% of that of the normal oxidase containing subunit III (Table 1). The loss of O_2 reduction activity occurred only during turnover, and no condition was found that reversed the loss of activity, except for the addition of fresh enzyme (Figure 2). Thus, the loss of activity can be accurately termed turnover-induced inactivation or suicide inactivation. The rate

Table 1: Effects of Phosphatidylcholine, Catalase and Superoxide Dismutase on the Rate of Suicide Inactivation

oxidase and additions	$CC_{60\%}$[a]
wild-type	>500 000[b]
wild-type + PC[c]	>850 000[b]
subunit III (−)	11 000
subunit III (−) + PC[c]	20 000
ΔCOXIII	11 000
ΔCOXIII + PC[c]	21 000
subunit III (−)[d]	27 000
subunit III (−) plus catalase[e]	29 000
subunit III (−) plus SOD [f]	26 000

[a] $CC_{60\%}$ is the number of catalytic cycles per oxidase molecule required to reach 60% of the initial activity, as explained in the Experimental Procedures. A catalytic cycle is defined as $1O_2 \rightarrow 2H_2O$, or one-fourth of the turnover number. The $CC_{60\%}$ value is inversely proportional to the rate of suicide inactivation. The $CC_{60\%}$ values presented are rounded to the nearest one thousand. O_2 reduction activity was measured as in Experimental Procedures, but at pH 7.5. [b] Extended turnover was achieved by adding catalase plus H_2O_2 each time the enzyme depleted the O_2 in the cell (see *26*). These values are underestimates of the actual $CC_{60\%}$. [c] 1 mg/mL PC. [d] Control for the catalase and SOD experiments; 1 mg/mL PC was included in these experiments. [e] 5000 units. [f] 1500 units.

of suicide inactivation was independent of oxidase concentration (data not shown). The phenomenon can be easily overlooked, however, if higher concentrations of oxidase are used in the O_2 electrode assay since all of the O_2 in the reaction cell will be consumed before significant amounts of inactive oxidase accumulate.

The O_2 consumption tracings of subunit III (−) and ΔCOXIII (Figure 2) fit well to single-exponential decay kinetics, after the slow, nonenzymatic reduction of O_2 by ascorbate was subtracted (fits not shown). This indicates that a spontaneous event inactivated subunit III (−) or ΔCOXIII, i.e., each monomer in the oxidase populations was either fully active or inactive. Since the time resolution of the O_2 electrode is 100−500 times slower than a single catalytic turnover, the kinetic analysis cannot distinguish between a single event leading to inactivation or a closely spaced series of events.

Suicide Inactivation Is Not Caused by Residual Triton-X-100, Thermal Instability, the Lack of Phospholipid, or by the Accumulation of Superoxide or H_2O_2. Triton-X-100 is well-known to slow the rate of O_2 reduction by cytochrome oxidase when it is present in significant amounts (*25*). It is possible that a small amount of Triton remained bound to subunit III (−), even though none could be detected by UV spectroscopy. Residual Triton, however, could not have been the cause of suicide inactivation since ΔCOXIII inactivated without exposure to Triton (Figure 2). Moreover, the normal oxidase containing subunit III did not suicide inactivate when assayed in the presence of 0.1% Triton-X-100 (data not shown).

The resting forms of subunit III (−) and ΔCOXIII showed no greater thermal instability than the wild-type, three-subunit oxidase. All three could be incubated for 70 min at 25 °C without any change in their initial O_2 reduction activity or in their rates of suicide inactivation (data not shown). In another experiment, inactivated subunit III (−) was repurified on Ni^{2+}-NTA agarose and examined by SDS−PAGE. Both subunits I and II were equally present in active and inactive subunit III (−) (gel not shown), indicating that subunit dissociation does not occur with suicide inactivation.

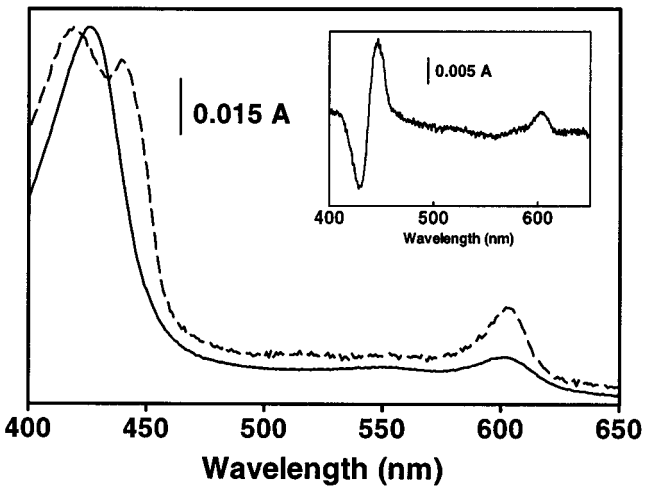

FIGURE 3: Absolute visible spectra of the resting forms of active (—) and inactive (- - -) subunit III (−). The active oxidase was equilibrated in 50 mM KH_2PO_4, pH 7.5, and 0.1% dodecyl maltoside. The spectrum of the inactive oxidase was obtained immediately after chromatography on Sephadex G-50 (see Experimental Procedures). The inset shows the difference of these two spectra, inactive minus active.

The addition of 1 mg/mL PC enhanced the initial O_2 reduction activity of the wild-type oxidase, as well as subunit III (−) and ΔCOXIII, by ~150%. The omission of exogenous phospholipid doubled the rate of suicide inactivation by subunit III (−) (or halved the $CC_{60\%}$ value; Table 1), but it did not alter the characteristics of the inactivated oxidase (described below). Thus, the mechanism of suicide inactivation in the presence or absence of additional phospholipid appeared to be the same. Increasing the concentration of PC to 2 mg/mL did not further increase the initial activity or further slow the rate of suicide inactivation.

It was considered that subunit III (−) and ΔCOXIII might release H_2O_2 or superoxide during turnover, which could then attack the oxidase to cause the loss of activity. The addition of catalase after extensive turnover of subunit III (−) showed that H_2O_2 was not released (data not shown). Moreover, the addition of catalase or superoxide dismutase (SOD) had no effect on the rate of suicide inactivation (Table 1). [Catalase and SOD were included in the large-scale inactivation reactions (see Experimental Procedures) in order to eliminate the accumulation of reduced O_2 species generated by the direct reduction of O_2 by ascorbate.]

Suicide Inactivation Causes a Decrease in the Redox Potential of Heme a_3. Both hemes *a* and a_3 of *active* subunit III (−) were fully oxidized in the resting form (Figure 3). Following the inactivation and repurification of subunit III (−), its spectrum showed two distinct peaks in the Soret that correspond to oxidized heme a_3 (418 nm) and reduced heme *a* (441 nm) (Figure 3). The inactive minus active difference spectrum (Figure 3, inset) has peaks at 605 and 444 nm and a trough at 426 nm. This is a spectrum of heme *a* only (*37*, *45*), indicating that suicide inactivation altered the oxidase such that electrons accumulated on heme *a* while heme a_3 remained oxidized. In preparations of subunit III (−) that were inactivated to various extents, the accumulation of reduced heme *a* was found to be proportional to the amount of inactivated oxidase, indicating that the transfer of electrons from heme *a* to heme a_3 was lost with suicide inactivation. The loss of interheme electron transfer could occur for three reasons. The redox potential of heme *a* could increase, that of heme a_3 could decrease, or the pathway of electron flow between the two hemes could be sufficiently altered to prevent electron transfer. These possibilities were examined as follows.

Table 2: Reduction of Heme a_3 of Inactive Subunit III (−) in the Presence of Ascorbate/TMPD or Sodium Dithionite

extent of inactivation of the subunit III (−) sample (% of original O_2 reduction activity remaining)	extent of heme a_3 reduction in the inactive fraction of subunit III (−) in the presence of exogenous reductant (%)[a,b]	
	ascorbate/TMPD[c] (E = 94 mV)[e]	sodium dithionite[d] (E = <−500 mV)[e]
19	43	59
21	57	58
24	51	57
25	53	59
54	57	52
62	42	47
	mean: 50 ± 7	mean: 55 ± 5

[a] The extent of reduction of heme a_3 in fully active subunit III (−) is 100%. [b] After the extent of total heme a_3 reduction was calculated from the Soret/α value of absolute reduced spectra as described in the Experimental Procedures, the contribution of the residual active fraction of subunit III (−) was subtracted by assuming that all of its heme a_3 was reduced in the presence of these reductants. [c] 3.0 mM ascorbate + 0.6 mM TMPD. [d] Several grains of solid dithionite were added. [e] Redox potentials were measured directly using a Corning redox electrode.

Horse heart cytochrome *c* (E_m = 230 mV when bound; *46*) completely oxidized heme *a* of the inactive oxidase by reverse electron transfer through Cu_A (data not shown). Thus, the midpoint potential of heme *a* after suicide inactivation could not be significantly greater than its normal value [195−270 mV (*47*−*49*)].

The extent to which heme a_3 of inactive subunit III (−) was reduced in the presence of exogenous reductants was determined from the Soret/α value of absolute reduced spectra (see Experimental Procedures). Pyridine hemochrome analysis (not shown) showed that both hemes were still present in the inactivated oxidase. Thus, a Soret/α value lower than the maximum value of 5.3 could not be due to loss of heme A. Since heme a_3 was completely oxidized in resting, inactive subunit III (−), any increase in the Soret/α value above 2.9 (*37*) was attributed to the reduction of heme a_3. Significant amounts of heme a_3 in inactive subunit III (−) could be reduced by heme *a* when either ascorbate/TMPD (E = 94 mV) or sodium dithionite (E < −500 mV) were added to rereduce heme *a* (Table 2). This indicated that the pathway of electron transfer between hemes *a* and a_3 was not inhibited by suicide inactivation. Since the potential of heme *a* was not increased (see above), it follows that the loss of normal interheme electron transfer with suicide inactivation is due to a decrease in the potential of heme a_3.

The full reduction of heme a_3, however, could not be achieved. Of the total heme a_3 in the *inactive* fraction of subunit III (−), 50−55% was reduced in the presence of ascorbate/TMPD or sodium dithionite (Table 2). Thus, two populations of heme a_3 were generated by suicide inactivation. One-half had a lowered redox potential, but could be reduced by dithionite, while the other half had a much lower potential and could not be reduced. These two forms were observed in subunit III (−) preparations inactivated to

Table 3: Ligand Binding and the Formation of O_2 Reduction Intermediates by Suicide-Inactivated Subunit III (−)

ligand[a] (O_2 reduction intermediate)	redox state of subunit III (−)	extent of ligand binding (%)[b]
CO	reduced (dithionite)	48 ± 2
CO	reduced (ascorbate/TMPD)	36 ± 4
CN^-	oxidized ($KMnO_4$)	91 ± 4
CN^-	reduced (dithionite)	0
CO/O_2 (formation of **P**)	oxidized ($KMnO_4$)	0
H_2O_2 (formation of **F**)	oxidized ($KMnO_4$)	0

[a] Ligand binding reactions were performed as in Figure 1. [b] The amount of ligand binding to *active* subunit III (−) is 100%. The data were obtained from three inactivation experiments with an average final activity of 24 ± 2% of the initial O_2 reduction rate, except for the CO binding data, which was obtained from the six experiments of Table 2. Ligand binding due to the remaining active oxidase was subtracted and the percent binding was recalculated using the formula [(% total ligand binding) minus (% residual oxidase activity)]/% inactive oxidase.

different extents (Table 2) and the rough 1:1 stoichiometry was not affected by the length of time between oxidase inactivation and spectral analysis. Therefore, the generation of these two forms appears to be a direct result of the inactivation process.

Suicide Inactivation Leads to Significant Alteration of the Cu_B Center and Flexibility in the Active Site. The five ligand-binding reactions used to characterize the active form of subunit III (−) (Figure 1) were also used to elucidate alterations in the active site due to suicide inactivation (Table 3). Note that the results presented below focus on *inactive* subunit III (−) only. The amount of ligand binding by residual *active* subunit III (−) in the inactivated samples has been subtracted.

In contrast to active subunit III (−), the reduced form of suicide-inactivated subunit III (−) bound CO very slowly (Figure 4, inset). The rate of CO binding was not further affected by lowering the concentration of CO below 1 mM. This indicates that CO binding was limited by a slow step prior to the actual binding event, such as a structural rearrangement in the active site. This suggests that the inactive heme a_3−Cu_B center is not a static structure, but rather one that is capable of rearranging with time. The total amount of CO bound (Table 3) was limited by the extent of heme a_3 reduction (Table 2).

The oxidized form of suicide-inactivated subunit III (−) bound almost as much CN^- as active subunit III (−) (Table 3). However, while active subunit III (−) bound CN^- at a single rate, the kinetics of CN^- binding to the inactive oxidase were distinctly biphasic (Figure 4). Approximately one-half of the inactive oxidase bound CN^- at the same rate as the active subunit III (−), while the other half bound the ligand slowly (Figure 4).

The addition of 1 mM CO to the oxidized, aerated form of inactive subunit III (−) led to the reduction of heme *a* rather than the production of **P** (Table 3). Two minutes after the introduction of CO, an oxidized + CO *minus* oxidized difference spectrum (not shown) was obtained that was similar to the heme *a* spectrum shown in the inset of Figure 3. Rather than trapping the electrons in the high-potential **P** intermediate, the heme a_3−Cu_B centers that were reduced

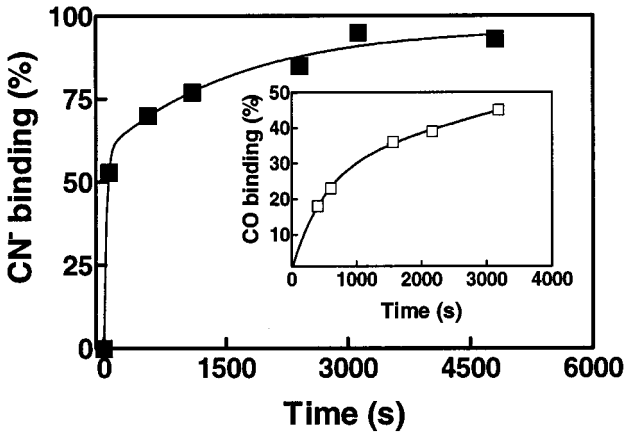

FIGURE 4: The kinetics of CN^- and CO binding to inactive subunit III (−). Subunit III (−) was inactivated to 25% of its original activity, repurified, and reacted with CO (reduced enzyme) or KCN (oxidized enzyme), and the extent of ligand binding (see Experimental Procedures) was measured as a function of time. The amount of CO or CN^- bound by subunit III (−) prior to inactivation is 100%. In both data sets, the amount of ligand bound by the remaining 25% of *active* subunit III (−) has been subtracted. Each data set was fit to a two-phase exponential association equation using Prism 2.01 (GraphPad). Estimated first-order rate constants for CN^- binding are 3.4×10^{-2} s^{-1} (60% of the total) and 6.1×10^{-4} s^{-1} (40%) while those for CO binding are 1.9×10^{-3} s^{-1} (8%) and 3.4×10^{-5} s^{-1} (92%).

by CO [$E_m = -0.5$ V at pH 7.2 (*41*)] transferred electrons to heme *a* (and possibly Cu_A). This was another indication of the low-potential nature of heme a_3 following suicide inactivation. The amount of heme *a* reduced by reverse electron transfer from CO corresponded to approximately 25% of the heme *a* present in the inactive fraction of subunit III (−). Reduction of Cu_A was not assessed.

Suicide inactivation completely eliminated any measurable reaction with two ligands. First, cyanide failed to bind to the *reduced* form of inactivated subunit III (−) (Table 3) even when KCN concentrations up to 80 mM were employed. The loss of cyanide binding to reduced subunit III (−) was directly proportional to the extent of oxidase inactivation (data not shown). Second, H_2O_2 failed to react with the oxidized form of suicide inactivated subunit III (−) (Table 3). Given the results presented above, it seems unlikely that the structural changes caused by inactivation could have completely prevented these ligands from approaching heme a_3. Cyanide binding to reduced oxidase and the reaction of H_2O_2 with the oxidized enzyme are similar in that their binding is very weak in the absence of Cu_B (*50*). These results, therefore, indicated that the Cu_B center was altered by the suicide inactivation reaction.

Several lines of evidence indicated that Cu_B itself was partially lost following suicide inactivation. Metal analysis of a sample of inactivated subunit III (−) gave a Cu/Fe value of 1.2, while the normal oxidase gave the expected Cu/Fe value of 1.5. Since the reverse electron-transfer experiment presented above argues for the retention of Cu_A in the inactivated oxidase, the loss of copper was expected to be from the Cu_B center. This was confirmed by obtaining the EPR spectrum of inactivated subunit III (−) (not presented), which shows an increased signal for high-spin heme a_3. Heme a_3 is normally EPR silent due to spin coupling with Cu_B; the loss of the latter reveals the high-spin heme signal (*51*). The ability of CO to donate two electrons to at least

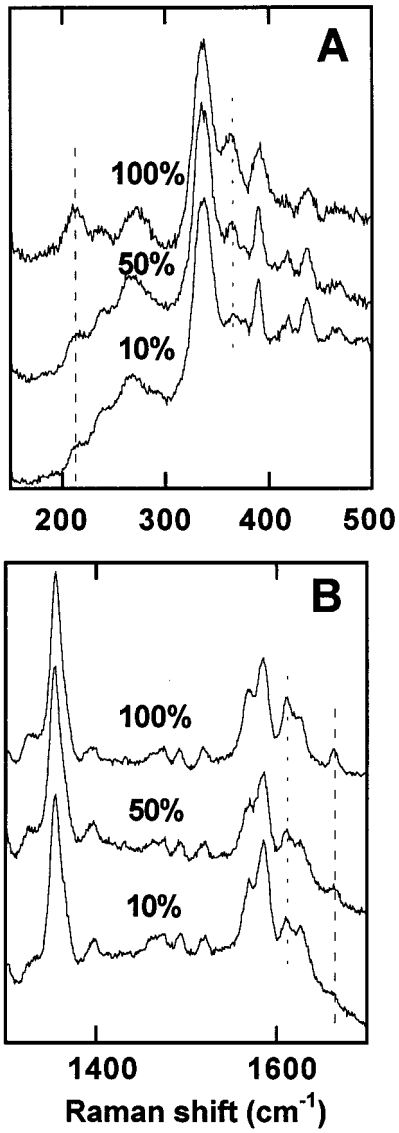

FIGURE 5: Resonance Raman analysis of the environments of hemes *a* and a_3 upon suicide inactivation. Resonance Raman spectra of dithionite reduced samples of subunit III (−) were obtained as in Hosler et al. (*13*). The subunit III (−) samples were 100% active (no prior turnover), 50% active, or 10% active. The Fe−N_{his} stretch at 214 cm^{-1} and the ring bending mode at 365 cm^{-1}, both specific for heme a_3, are marked in panel A. The formyl stretching modes specific for heme *a* (1612 cm^{-1}) and heme a_3 (1662 cm^{-1}) are marked in panel B.

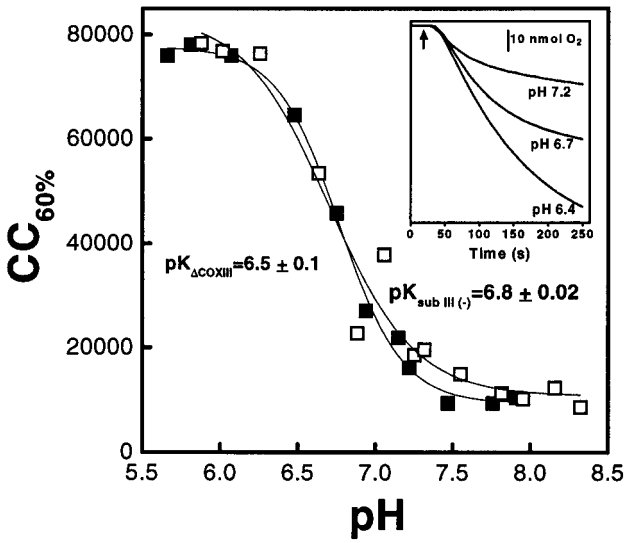

FIGURE 6: The effect of pH on the rate of suicide inactivation of subunit III (−) (■) and ΔCOXIII (□). $CC_{60\%}$ values (inversely proportional to the rate of suicide inactivation) were obtained as described in Experimental Procedures. The O_2 reduction assay buffer was adjusted to different pH values using 25 mM Bis-Tris-HCl or HEPES-HCl. The points were fit to the sigmoidal function of Origin 5.0 to determine apparent p*K* values. The inset shows O_2 consumption traces of 10 pmol subunit III (−) samples assayed at three pH values.

25% of the heme a_3−Cu_B centers (see above) argues for the retention of Cu_B in a significant portion of the inactive oxidase. Therefore, the loss of Cu_B is not required for suicide inactivation; rather, it appears to be a secondary event.

Resonance Raman Analysis Indicates Movement of Heme a_3 in Inactivated Subunit III (−). Resonance Raman spectroscopy is capable of sampling the protein environment of each heme of cytochrome oxidase (*52*, *53*). The spectra of active subunit III (−) (Figure 5) were identical to that of the normal, three-subunit oxidase (*13*). Signals specific for the interaction of heme a_3 with the apoprotein include the Fe−N_{his} stretch at 214 cm^{-1}, a ring-bending mode at 365 cm^{-1}, and the formyl stretch at 1662 cm^{-1}. These signals diminished as suicide inactivation progressed (Figure 5). In contrast, two signals specific for heme *a*, the formyl and vinyl stretches at 1612 and 1624 cm^{-1}, respectively, were less affected by oxidase inactivation (Figure 5B). This suggests that the heme a_3−Cu_B center was most affected by the inactivation process. Turnover of the normal, three-subunit oxidase under the same conditions that decrease the activity of subunit III (−) by 90% had no effect on the resonance Raman spectrum of the oxidase (not shown).

The progressive loss of the 214 cm^{-1} signal with suicide inactivation is unlikely to reflect the actual loss of the bond between His419 and heme a_3 (*15*, *16*, *54*). In fact, the position of the heme a_3 peak in the optical spectrum of the inactive oxidase (Figure 3) is strong evidence that heme a_3 retains its five-coordinate, high-spin configuration (*45*). The simultaneous loss of all three of the heme a_3 signals is consistent with the ligand-binding experiments that indicate considerable flexibility in the inactive heme a_3−Cu_B pocket. If heme a_3 moves through a variety of slightly different orientations, the resonance Raman signals that reflect its interactions with the surrounding protein will broaden and diminish. This same type of spectrum, showing the loss of these three heme a_3 modes, but retention of the two heme *a* modes, has been obtained for numerous mutant oxidases with alterations of residues in and around the active site (*54*, *55*).

An Apparent Deprotonation Event Increases the Probability of Suicide Inactivation. Increasing the solvent pH accelerated the rate of suicide inactivation by subunit III (−) (Figure 6, inset) and ΔCOXIII. Plots of $CC_{60\%}$ (inversely proportional to the rate of suicide inactivation) vs pH were fit to sigmoidal curves that gave apparent p*K* values of 6.5 and 6.8 (Figure 6). Full deprotonation of this unidentified group(s) did not immediately render subunit III (−) inactive, since subunit III (−) (and ΔCOXIII) underwent several thousand catalytic cycles at high pH before inactivating (Figure 6). Preincubation of subunit III (−) at pH 8.3 or pH 6.5 prior to turnover did not alter the rate of suicide inactivation at pH 7.0 (data not shown). These data indicate

that deprotonation of a group(s) during turnover increased the probability that any given oxidase molecule would convert to the inactive form during each catalytic cycle.

DISCUSSION

The removal of subunit III from the mitochondrial-like oxidase of *Rb. sphaeroides* has little effect on the V_{max} of O_2 reduction or on the structure of the active site prior to turnover. Active subunit III (−) reacts with CO and CN^- and forms the **P** and **F** intermediates in the same manner as the normal oxidase, and the environments of hemes *a* and a_3 appear unaltered. There is no discernible effect on the stability of the protein in the absence of catalytic turnover. These results are consistent with crystal structures and FTIR data that show no large differences in the resting conformations of the mitochondrial-like oxidase of *P. denitrificans* upon the removal of subunit III (*56*, *57*).

However, the removal of subunit III from cytochrome oxidase is not benign. In its absence, the enzyme becomes highly susceptible to spontaneous inactivation during O_2 reduction at physiologic pH values. For example, the catalytic lifetime of subunit III (−) is only ∼2% of the normal, three-subunit oxidase at pH 7.5. Irreversible inactivation only occurs during catalytic turnover; thus, the process may be termed suicide inactivation. ("Suicide inactivation" is often used to describe enzyme inhibition by a "suicide inhibitor", generally an artificial substrate. In this study, suicide inactivation describes the spontaneous inactivation of cytochrome oxidase during turnover with normal substrates.) Our results clearly show that oxidase inactivation is not caused by Triton-X-100, by the accumulation of superoxide or H_2O_2, by simple thermal denaturation of the two-subunit oxidases, or by loss of the lipid environment. Suicide inactivation of cytochrome oxidase occurs in two-subunit forms of the mitochondrial-like oxidase of *P. denitrificans* (*26*) in addition to *Rb. sphaeroides* subunit III (−) and ΔCOXIII. The phenomenon is not restricted to bacterial oxidases, since subunit III-depleted forms of mitochondrial cytochrome oxidase rapidly lose activity upon catalytic turnover (L. Prochaska, and S. Ferguson-Miller; personal communications). Thus, it appears that one reason for the high conservation of subunit III from bacterial to mammalian species is its ability to prevent rapid inactivation of the enzyme.

Suicide Inactivation Alters the Active Site. The functional alterations associated with suicide inactivation appear to be localized to the heme a_3−Cu_B active site. The ability of soluble cytochrome *c* to completely oxidize reduced heme *a* of the resting, inactive oxidase demonstrates that heme *a*, Cu_A, and the cytochrome *c* binding site of the inactive oxidase are intact and functional in electron transfer. This result is consistent with the gel analysis of repurified inactive subunit III (−), which shows that suicide inactivation does not alter the association of subunits I and II. In addition, resonance Raman analysis shows that the environment of heme *a* is not significantly altered. In contrast, structural changes at the heme a_3−Cu_B active site result in 10^6 times slower CO binding, a large decrease in the redox potential of heme a_3, dramatic alterations in CN^- binding, the inability of the active site to form the **P** and **F** O_2 reduction intermediates, movement of heme a_3, and partial loss of Cu_B.

Suicide Inactivation and the Histidine−Tyrosine Ligand of Cu_B. Recent structural information reveals a covalent bond between His284, one of the three histidine ligands of Cu_B, and Tyr288 (*Rb. sphaeroides* numbering) (*56*, *58*, *59*). Spectroscopic experiments suggest that this Cu-His-Tyr group forms a tyrosine radical to facilitate rapid cleavage of the O−O bond after the binding of O_2 (*7*, *8*). Three key characteristics of the heme a_3−Cu_B center of suicide inactivated subunit III (−) are remarkably similar to those of oxidase mutants with alterations of His284 or Tyr288. Both the inactive oxidase and these mutants contain a significant fraction of heme a_3 that cannot be reduced in the presence of dithionite, both show very slow CO binding, and both exhibit at least partial loss of Cu_B (*54*, *55*, *60*−*62*; Hosler and Ferguson-Miller, unpublished results). Significantly, these particular characteristics are not present in other mutants of the *Rb. sphaeroides* oxidase that disturb the environment of the active site (*54*, *63*). For example, the amount of CO binding is decreased in mutants of His333 and His334, the other two ligands of Cu_B, but the *rate* of CO binding is not slowed (*54*; Hosler and Ferguson-Miller, unpublished results). The similarity of the His284 mutants and the inactivated oxidase strongly suggests that suicide inactivation involves the release of His284 from Cu_B, perhaps due to the formation of a cross-link between the Tyr288 radical and another protein group. The dissociation of the His284−Tyr288 group from Cu_B may be the primary event that inactivates the oxidase, since (1) resultant structural changes should slow the binding of O_2, similar to CO and (2) Tyr288 would be unable to participate in the formation and/or stabilization of O_2 reduction intermediates, as recently proposed (*7*).

Suicide Inactivation Inhibits Interheme Electron Transfer. Suicide inactivation inhibits normal electron transfer between hemes *a* and a_3, resulting in the accumulation of electrons on heme *a*. This is due to a decrease in the redox potential of heme a_3. Cytochrome oxidase normally neutralizes the introduction of negative charge (electrons) to the buried active site by the uptake and binding of an equal number of protons (*50*, *64*−*66*). Loss of charge neutralization is capable of decreasing the redox potential of heme a_3 by more than 1 V (*50*). Thus, the lowered potential of heme a_3 upon suicide inactivation may result from decreased proton binding capability in or near the active site. However, the ability of ascorbate/TMPD to drive the reduction of at least one-half of heme a_3 following inactivation suggests that the inhibition of interheme electron transfer, by itself, would not completely inhibit oxidase activity.

The complete loss of CN^- binding to the dithionite-reduced form of inactive subunit III (−) is consistent with the loss of proton-binding sites. Cyanide enters the active site as HCN, but binds to heme a_3 as CN^-; a binding site is required for the proton that must dissociate. An intact Cu_B center is required for this reaction (*50*), and analysis of the electrostatics of the oxidase implicates a putative hydroxyl group on Cu_B as the proton acceptor (*65*, *66*).

Secondary Alterations Follow the Primary Inactivation Event. Three consistent characteristics of inactivated subunit III (−) are more difficult to understand. First, Cu_B is lost in some of the inactive oxidase, but not all. Second, the reduction experiments reveal the existence of two populations of heme a_3 in the inactivated oxidase. One is low potential,

but can be reduced by dithionite, while the other cannot be reduced. A further loss of proton-binding sites necessary for charge neutralization is likely to have occurred in the very low-potential form (see above). Third, approximately one-half of the oxidized form of the inactive oxidase binds CN^- at the same rate as the active oxidase, while the other half binds the ligand much more slowly. The partial loss of Cu_B, the further loss of proton-binding capacity, and the appearance of slow CN^- binding reveal one or more structural changes in the active site that must be secondary to the primary inactivation event. One way to explain why these secondary changes occur in approximately one-half of the inactive oxidase is to propose that (1) subunit III (−) exists as a dimer, regardless of its activity, and (2) an active monomer is capable of protecting its partner from secondary structural changes, but an inactive monomer is not. For example, the first monomer of the dimer to inactivate would not release Cu_B, but the second would. In experiments not presented here, it appears that ΔCOXIII, as isolated, is a dimer. Thus, the absence of subunit III may promote self-association of the two-subunit oxidases in dodecyl maltoside.

pH Dependence of Suicide Inactivation. Increasing the pH of the solvent accelerates the rate of suicide inactivation by increasing the probability that any given oxidase molecule will inactivate during the catalytic cycle. The data are consistent with the deprotonation of a single, unidentified protein group with a p*K* around 6.7. Deprotonation of this group may disrupt the network of hydrogen bonds that surrounds the heme a_3−Cu_B center (*67*) or increase the lifetime of a reactive intermediate. The pH effect is only seen in the absence of subunit III; high pH does not induce suicide inactivation in the normal oxidase.

Role of Subunit III. The results presented here indicate that the primary target of suicide inactivation is the Cu_B center, and the major determinant of the probability of suicide inactivation is the presence or absence of subunit III. Since subunit III makes no direct contact with the ligands of Cu_B or groups in the outer shell of the Cu_B center (*15*, *16*, *58*), it must be concluded that subunit III participates in long-range interactions critical for preserving the normal ligand environment of Cu_B during the catalytic cycle. The analysis of site-directed mutants should reveal which regions of subunit III are required for this protective function.

We have proposed that suicide inactivation of subunit III (−) and ΔCOXIII could result from the formation of a cross-link between Tyr288 and a nearby group that leads to the dissociation of His284 from Cu_B. Suicide inactivation of prostaglandin H synthase is also proposed to result from destructive cross-linking by a tyrosine radical in its active site (*32*). For cytochrome oxidase, the absence of subunit III may allow greater flexibility in the active site, thus increasing the mobility of the tyrosine radical. In this light, it is interesting to note that the only members of the heme-Cu family of terminal oxidases that clearly lack subunit III are the cbb_3-type oxidases that do not contain a residue corresponding to Tyr288 (*68*), and thus cannot utilize an O_2 reduction mechanism involving a tyrosine radical linked to Cu_B.

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