A Role for Subunit III in Proton Uptake into the D Pathway and a Possible Proton Exit Pathway in *Rhodobacter sphaeroides* Cytochrome *c* Oxidase†

Denise A. Mills,‡ Zi Tan,§ Shelagh Ferguson-Miller,‡ and Jonathan Hosler*,§

Department of Biochemistry, Michigan State University, East Lansing, Michigan 48824, and Department of Biochemistry, University of Mississippi Medical Center, 2500 North State Street, Jackson, Mississippi 39216

Received January 22, 2003; Revised Manuscript Received April 22, 2003

ABSTRACT: Protons are transferred from the inner surface of cytochrome *c* oxidase to the active site by the D and K pathways, as well as from the D pathway to the outer surface by a largely undefined proton exit route. Alteration of the initial proton acceptor of the D pathway, D132, to alanine has previously been shown to greatly inhibit oxidase turnover and slow proton uptake into the D pathway. Here it is shown that the removal of subunit III restores a substantial rate of O_2 reduction to D132A. Presumably an alternative proton acceptor for the D pathway becomes active in the absence of subunit III and D132. Thus, in the absence of subunit III cytochrome oxidase shows greater flexibility in terms of proton entry into the D pathway. In the presence of $\Delta\Psi$ and ΔpH, turnover of the wild-type oxidase or D132A is slower in the absence of subunit III. Comparison of the turnover rates of subunit III-depleted wild-type oxidase to those of the zinc-inhibited wild-type oxidase containing subunit III, both reconstituted into vesicles, leads to the hypothesis that the absence of subunit III inhibits the ability of the normal proton exit pathway to take up protons from the outside in the presence of $\Delta\Psi$ and ΔpH. Thus, subunit III appears to affect the transfer of protons from both the inner and outer surfaces of cytochrome oxidase, perhaps accounting for the long-observed lower efficiency of proton pumping by the subunit III-depleted oxidase.

Protons are required by cytochrome *c* oxidase for the conversion of oxygen to water and for the production of an electrochemical gradient across the membrane that is used to drive the synthesis of ATP (*1*). The analysis of site-directed mutants (*2*−*5*) and then the crystal structures of bovine, *Paracoccus denitrificans*, and *Rhodobacter sphaeroides* cytochrome *c* oxidase (*6*−*8*) revealed two groups of residues in subunit I important for proton uptake, the D and the K pathways. The D proton-conductive pathway is composed of a series of polar residues that coordinate a hydrogen-bonded chain of water molecules between D132, located near the inner surface of subunit I, and E286, located approximately 26 Å above D132, closer to the site of O_2 binding on heme a_3 (*6*−*8*). (The inner surface of cytochrome oxidase is that facing the mitochondrial matrix or the bacterial cytoplasm.) Further proton-conductive pathways, not clearly identified, presumably transfer substrate protons from E286 to the active site where O_2 is reduced, or pumped protons from E286 to the exit pathway leading to the outer surface of the protein (*6*, *9*, *10*). Mutation of either D132 or E286 to noncarboxylic amino acid residues greatly inhibits the steady-state activity of bacterial cytochrome *c* oxidases (*4*, *11*−*14*). The addition of arachidonic acid to D132 mutants restores some O_2 reduction activity (but not proton pumping), apparently by substituting another carboxylic group in a position that allows it to transfer protons into the D pathway (*11*, *15*). Direct measurements of proton uptake into the D pathway in single turnover experiments have confirmed that D132 and E286 mutants impair the uptake of protons needed for oxidase turnover (*13*, *15*).

Zinc has become an important tool in the study of proton pathways since it has been found to inhibit the entry or exit of protons in proton pathways of the bc_1 complex (*16*), the bacterial photosynthetic reaction center (*16*, *17*), as well as in cytochrome *c* oxidases (*18*−*22*). Zinc inhibits the uptake of protons into the D pathway of *R. sphaeroides* cytochrome *c* oxidase (*21*, *22*), which is the likely explanation for the inhibition of steady-state O_2 reduction activity of the detergent-solubilized oxidase by micromolar quantities of zinc (*18*). In addition to D pathway inhibition, when the oxidase is reconstituted with phospholipids to make cytochrome oxidase vesicles (COVs),[1] zinc inhibition of a different proton-transfer pathway can be demonstrated (*18*). Catalytic turnover of COVs rapidly generates a transmembrane voltage gradient ($\Delta\Psi$) and a ΔpH of such magnitude that no net proton pumping can be measured. However, even under these "controlled" conditions a slow rate (∼15%) of

† Supported by National Institutes of Health Grants GM56824 (J.H.) and GM26916 (S.F.-M.).

* Corresponding author. Telephone: 601-984-1861. Fax: 601-984-1501. E-mail: jhosler@biochem.umsmed.edu.

‡ Michigan State University.

§ University of Mississippi Medical Center.

[1] Abbreviations: FCCP, carbonyl cyanide *p*-(trifluromethoxy)phenylhydrazone; COVs, cytochrome oxidase-containing phospholipid vesicles; Ni^{2+}-NTA, nickel nitrilotriacetic acid; RCR, respiratory control ratio; TMPD, *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine; Hepes, 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid; Taps, *N*-[tris(hydroxymethyl)methyl]-3-aminopropanesulfonic acid; LM, *n*-dodecyl β-D-maltoside; CI, confidence interval, SD, standard deviation.

10.1021/bi0341307 CCC: $25.00
Published on Web 05/29/2003

electron transfer to O_2 continues. Controlled electron transfer appears to utilize the uptake (back-flow) of protons from the outer surface of the protein, possibly by reversal of the normal exit pathway for protons (*18*). Low concentrations of zinc inhibit the controlled rate of electron transfer to O_2, apparently by inhibiting the entry of protons into the back-flow (exit) pathway (*18*). The zinc that inhibits the back-flow pathway is binding to a site on the opposite side of the protein from the entrance to the D pathway, which cannot be accessed when the oxidase is embedded in the lipid vesicles.

Subunit III of cytochrome *c* oxidase clearly affects structures within subunit I, since subunit III prevents spontaneous inactivation of the enzyme during the catalytic cycle by maintaining the structural integrity of the Cu_B center of the active site (*23*). Recent experiments indicate that subunit III also affects the entry of protons into the D pathway of subunit I; proton uptake in the absence of subunit III is 30-fold slower than that of the wild-type oxidase at pH 8, but rapid proton uptake is restored at lower pH (*24*). This decrease in the apparent pK_a of the D pathway may reflect a decrease in the pK_a of D132 since the N-terminal region of subunit III contains hydrophobic residues that partially shield D132 from bulk water [see Gilderson et al. (*24*)], and the removal of subunit III will increase the exposure of the carboxylate side chain to solvent.

Here, we have removed subunit III from the wild-type oxidase, two D pathway mutants [D132A (*4*) and E286A/I112E (*14*)], and one K pathway mutant [T359A (*3*)] in order to further explore the influence of subunit III on proton uptake from the inner surface of the protein. The removal of subunit III from D132A strongly stimulates oxidase turnover, presumably by exposing an alternative proton acceptor. Experiments with COVs containing subunit III-depleted oxidase forms show that the removal of subunit III inhibits controlled electron transfer much like zinc binding to the outer surface of the wild-type oxidase. This suggests that subunit III may also affect the activity of the back-flow proton pathway necessary for turnover in the presence of a membrane potential.

MATERIALS AND METHODS

Materials. Arachidonic acid was purchased from Sigma, and a stock solution of 50 mM was prepared in ethanol and stored at −20 °C. Asolectin (soybean phosphatidylcholine) was purchased from Associated Concentrates and recrystallized using the procedure of Sone et al. (*25*). Sodium cholate (Anatrace) was prepared as a 10% solution in water at pH 7.0, while *n*-dodecyl β-D-maltoside (Anatrace) was prepared as a 30% solution in water. Valinomycin and FCCP were from Sigma, and $ZnSO_4$ was from Columbus Chemical Industries, Inc. Horse heart cytochrome *c* was purchased from Sigma and further purified on (carboxymethyl)cellulose (*26*) when used as the substrate for COVs.

Enzyme Purification. *R. sphaeroides* cells were grown, and the wild-type and mutant cytochrome *c* oxidases were isolated using histidine affinity chromatography as described previously (*27*). Subunit III was removed from the purified oxidases using Triton X-100 as follows. The purified oxidases were exchanged into 50 mM Hepes−KOH, pH 8.0, and 200 mM KCl, and Triton X-100 was added at a ratio of 100 mg of detergent/mg of oxidase; volumes were adjusted to keep the total amount of detergent between 12% and 15%. After 30 min of gentle mixing at 4 °C, imidazole was added to a final concentration of 10 mM, and the oxidase was bound to Ni^{2+}-NTA−agarose (Qiagen) by adding 1 mL of buffer-washed agarose/milligram of oxidase and gently mixing the slurry for 1 h at 4 °C. The agarose mixture was poured into a small, disposable column and washed with 10 column volumes of 50 mM Hepes−KOH, pH 8.0, 200 mM KCl, 10 mM imidazole, and 0.1% Triton X-100 followed by 10 column volumes of 50 mM Hepes−KOH, pH 8.0, 200 mM KCl, 10 mM imidazole, and 0.1% *n*-dodecyl β-D-maltoside (LM). The oxidase was eluted with 50 mM Hepes−KOH, pH 8.0, 40 mM KCl, 100 mM histidine, and 0.1% LM and washed with 50 mM Hepes−KOH, pH 8.0, 200 mM KCl, and 0.05% LM in a 50 kD Centriprep device (Amicon) until the histidine concentration was below 1 mM. The entire procedure was repeated once in order to remove essentially all subunit III from the oxidase samples.

The rates of O_2 reduction were measured at various pH values using an oxygen electrode as described in Bratton et al. (*23*) in a reaction mixture of 25 mM Hepes−KOH, 25 mM Taps−KOH, 87.5 mM KCl, 0.5 mM EDTA, 0.6 mM TMPD, 3 mM ascorbate, 1 mg/mL phosphatidylcholine (soybean phospholipids), 0.1% LM, and 2−50 pmol of oxidase. Each reaction was initiated by the addition of 40 μM horse heart cytochrome *c*. The rates of nonenzymatic reduction of O_2 by ascorbate/TMPD at each pH were subtracted. Oxidase concentrations were determined from reduced absolute spectra using the extinction coefficients of Hosler et al. (*28*), and the turnover numbers (TN) were calculated as described in Thompson and Ferguson-Miller (*29*). Apparent pK_a values for steady-state O_2 reduction were determined by fitting the pH dependence data to a sigmoidal function that assumes a single pK_a using the equation $TN = TN_{min} + (TN_{max} - TN_{min})/(1 + 10^{pH-pK_a})$, using GraphPad Prism 3.0 (GraphPad Software). The V_{max} of steady-state O_2 reduction was determined from cytochrome *c* titrations performed in the reaction mixture listed above except that the buffer was 50 mM Hepes−KOH, pH 6.5. The data were fit to the hyperbolic Michaelis−Menten function using Microcal Origin. Zinc inhibition of steady-state O_2 reduction by the detergent-solubilized oxidases was performed in the same reaction mixture as the pH studies except the buffer was 50 mM Hepes−KOH, pH 7.4, and EDTA was omitted. The zinc inhibition constants (K_i) were determined by nonlinear least-squares fitting as described in Mills et al. (*18*) using GraphPad Prism.

Preparation of COVs and Activity Measurements. Cytochrome *c* oxidase vesicles (COVs) were prepared by mixing, to final concentrations, 20 mg/mL asolectin, 2 μM oxidase, and 3% sodium cholate in 75 mM Hepes−KOH, pH 7.4. A dialysis method was used to slowly remove the detergent (*30*). Measurements of cytochrome *c* oxidation with the COVs were made via stopped-flow spectroscopy as described previously (*18*).

RESULTS

The Removal of Subunit III Alters the Activity of D Pathway Mutants, Especially D132A. Subunit III was removed from the wild-type oxidase and D132A by washing

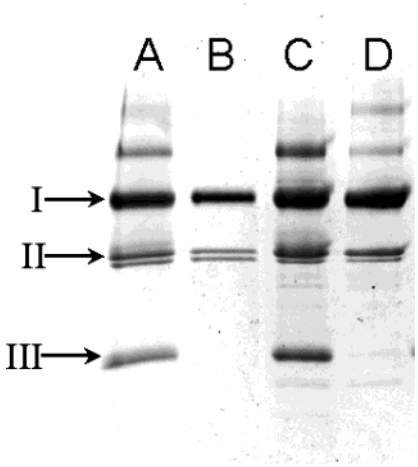

FIGURE 1: Removal of subunit III from the wild-type oxidase and D132A using Triton X-100. Oxidase samples were separated on a 14% polyacrylamide−SDS gel containing 6 M urea as described in Hosler et al. (*28*) and stained with Coomassie Blue. The locations of subunits I, II, and III are indicated by the arrows. Subunit II runs as a doublet at M_r 37 and 35 due to incomplete processing at its C-terminus (*42*). Lanes: A, wild-type oxidase (WT); B, WT III (−); C, D132A; D, D132A III (−).

Table 1: Effect of Removing Subunit III on the V_{max} Values of Steady-State O_2 Reduction (e^- s^{-1} aa_3^{-1} at pH 6.5) Catalyzed by Various Oxidase Forms

oxidase form	(+) subunit III	(−) subunit III
wild type	1905 ± 59	1578 ± 48
D132A	30 ± 0.6	669 ± 23
E286A/I112E	33 ± 0.8	5 ± 0.3
T359A	308 ± 6	202 ± 11

the purified protein complexes with Triton X-100 (Figure 1). By exchanging the enzyme back into dodecyl maltoside buffer, all of the Triton X-100 was removed from the subunit III-depleted oxidases, based on UV spectra of the final products (not shown). Using this method, the subunit III-depleted enzyme prepared from the wild-type oxidase [termed WT III (−)] generally retained 65−90% of the O_2 reduction activity of the starting material; i.e., the V_{max} of wild-type oxidase at pH 6.5 is somewhat decreased by the removal of subunit III (Table 1). In contrast, the removal of subunit III from D132A [creating D132A III (−)] *increased* its V_{max} of cytochrome *c* driven O_2 reduction more than 20-fold at pH 6.5 (Table 1). Since the strong inhibition of O_2 reduction in D132A containing subunit III is attributed to very slow proton transfer through an altered D pathway to the active site (*15*), the large increase in activity upon the removal of subunit III from D132A most likely arises from a substantial increase in the rate of proton uptake via the D pathway.

In contrast to D132A, the already low activity of E286A/I112E was almost eliminated by the removal of subunit III (Table 1). The glutamate at position 112 in transmembrane helix 2 of subunit I of this mutant occupies a position similar to that of the normal E286 in the wild-type oxidase (*14*). The loss of O_2 reduction activity upon the removal of subunit III from E286A/I112E suggests that subunit III normally helps to maintain the structure of the interior of the D pathway, well above the position of D132.

Threonine 359 of the *R. sphaeroides* oxidase is a key component of the K pathway of proton transfer to the active site (*6*), and alteration of T359 to alanine slows O_2 reduction activity without altering the structure of the active site (*3*). The removal of subunit III from T359A has no greater effect on this enzyme than the removal of subunit III from the wild-type oxidase (Table 1). This suggests that subunit III has less of an effect on the structure of the K pathway in subunit I than it has on the D pathway.

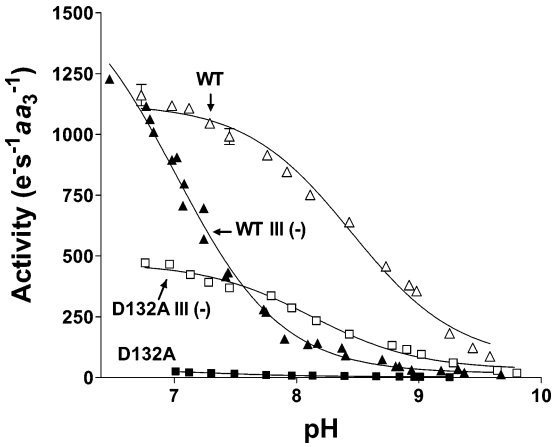

FIGURE 2: Removal of subunit III alters the pH dependence of steady-state O_2 reduction catalyzed by the detergent-solubilized forms of the wild-type oxidase (△), WT III (−) (▲), and D132A III (−) (□). The apparent pK_a values derived from nonlinear regression are shown in Table 4. The low rate of O_2 reduction catalyzed by D132A containing subunit III (■) is shown for comparison.

Effects of pH and Arachidonic Acid. Since protons are one of the substrates for cytochrome *c* oxidase, the rate of steady-state O_2 reduction by the enzyme is dependent upon solvent pH (*31*−*33*). With the wild-type *R. sphaeroides* oxidase the rate declines above pH 7, with an apparent pK_a of 8.5 (Figure 2, Table 4). In the absence of subunit III the pH dependence of O_2 reduction shifts to more acid values; the apparent pK_a of O_2 reduction by WT III (−) is 7.0 (Figure 2, Table 4). The pK_a and pH profile of WT III (−) are similar to those of the I−II oxidase, which lacks subunit III due to the deletion of its gene from the cell (*24*). Interestingly, the pH profile of subunit III-depleted D132A [D132A III (−)] is clearly different from that of WT III (−). The apparent pK_a of O_2 reduction by D132A III (−) is 8.1, and although its activity at pH 6.5 is less than that of WT III (−), at pH values above 7.6 the activity of D132A III (−) is actually greater than that of WT III (−) (Figure 2). These different pH profiles likely reflect different initial proton acceptors for the D pathways of WT III (−) and D132A III (−) (see Discussion).

The addition of certain fatty acids stimulates the O_2 reduction activity of D132A containing subunit III (*11*). The greatest stimulation occurs with long-chain, unsaturated fatty acids, particularly arachidonic acid. It is presumed that arachidonic acid restores activity by binding in such a manner that its carboxylate group serves as an alternative entry point for protons in the absence of D132 (*11*, *15*). Arachidonol has no effect on the activity of D132A, indicating that changes in O_2 reduction activity are not due to conformational responses to the binding of the acyl chain (*11*). Arachidonic acid fails to stimulate O_2 reduction by the wild-type oxidase that contains subunit III; in fact, it appears weakly inhibitory (*11*). Similarly, arachidonic acid fails to

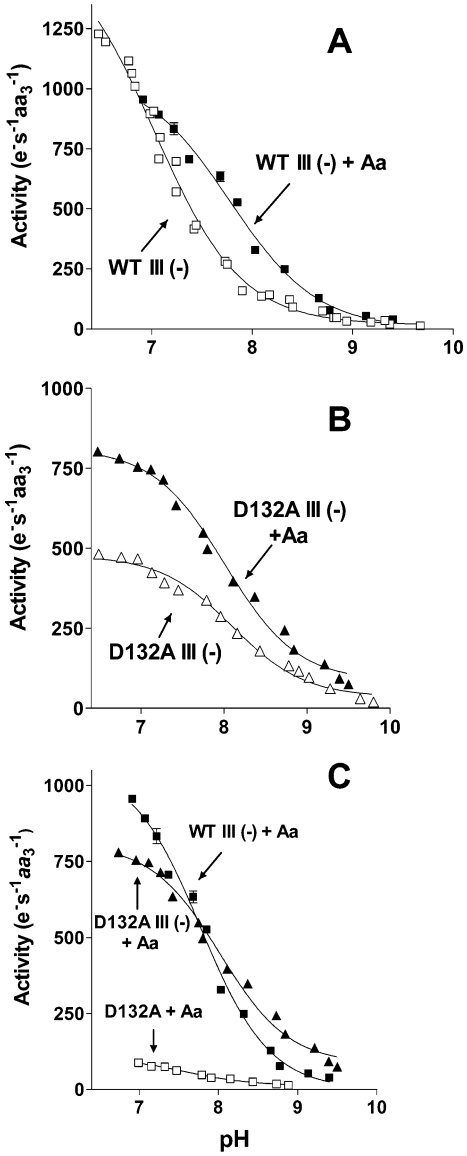

FIGURE 3: Arachidonic acid further alters the pH dependence of steady-state O_2 reduction catalyzed by WT III (−) and D132A III (−). Data sets are repeated as needed for clarity of presentation, and apparent pK_a values are shown in Table 4. (A) WT III (−) in the presence (■) and absence (□) of 250 μM arachidonic acid. (B) D132A III (−) in the presence (▲) and absence (△) of 250 μM arachidonic acid. (C) Comparison of WT III (−) (■), D132A III (−) (▲), and D132 containing subunit III (□), all in the presence of 250 μM arachidonic acid.

stimulate the activity of WT III (−) at pH 6.5, but it does stimulate enzyme activity at higher pH values (Figure 3A). In contrast, the O_2 reduction activity of D132A III (−) is stimulated at all pH values between 6.5 and 10 (Figure 3B). As with D132 containing subunit III, long-chain, unsaturated fatty acids are more effective than palmitate in stimulating the activity of D132A III (−) (Table 2). Remarkably, in the presence of arachidonic acid both the activity and the pH dependence of D132A III (−) and WT III (−) become quite similar (Figure 3C).

Inhibition by Zn^{2+}. Steady-state O_2 reduction by the detergent-solubilized wild-type oxidase is inhibited by zinc with a biphasic response (*18*). Most of the activity is inhibited with micromolar amounts of Zn^{2+}, while inhibition of the remaining activity requires at least 10 times this amount. Since zinc strongly inhibits proton uptake into the D pathway

Table 2: Long-Chain, Unsaturated Fatty Acids Increase the V_{max} of Steady-State O_2 Reduction by D132A III (−)

fatty acid[a]	V_{max} ($e^- s^{-1} aa_3^{-1}$)[b]
none	669 ± 23
palmitate (16:0)	783 ± 18
linoleate (18:2)	1089 ± 29
arachidonate (20:4)	1154 ± 33

[a] 250 μM. [b] pH 6.5.

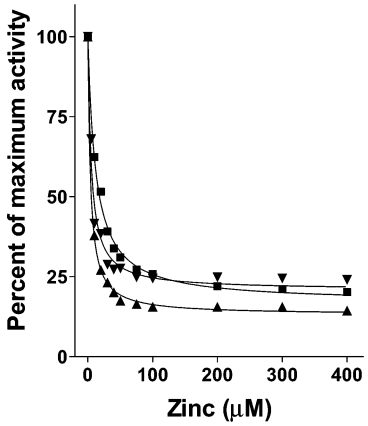

FIGURE 4: Low concentrations of zinc inhibit steady-state O_2 reduction by detergent-solubilized wild-type oxidase (■), WT III (−) (▲), and D132A III (−) (▼). Turnover of the oxidases in the presence of zinc sulfate was measured at pH 7.4, and zinc inhibition constants were determined as described in Materials and Methods. The calculated K_i values were 11.8 μM for the wild-type oxidase (95% CI from 9.6 to 14.0), 3.8 μM for WT III (−) (95% CI from 3.3 to 4.4), and 5.0 μM for D132A III (−) (95% CI from 3.1 to 6.9).

of soluble cytochrome *c* oxidase, it has been proposed that a high-affinity binding site for the metal exists near the entry point of the D pathway, possibly including D132 and two conserved histidines of subunit III (*21*, *22*). Steady-state O_2 reduction by the wild-type oxidase, WT III (−), and D132A III (−) were all inhibited by zinc with similar K_i values (4−12 μM; Figure 4), indicating that the removal of subunit III does not prevent the inhibition of the D pathway by low concentrations of zinc. In addition, neither D132 nor the histidines of subunit III are required for zinc inhibition. Steady-state O_2 reduction in the presence of arachidonic acid by both D132A III (−) and WT III (−) was also sensitive to zinc (data not shown), but the apparent K_i values were elevated since fatty acid micelles bind zinc.

The Removal of Subunit III and the Addition of Zinc Both Slow the Rate of Controlled Electron Transfer. The various oxidase forms were reconstituted with phospholipids to prepare cytochrome oxidase vesicles (COVs). The rate of cytochrome *c* oxidation was measured in stopped-flow experiments in which COVs containing the wild-type oxidase, WT III (−), D132A, or D132A III (−) were mixed with reduced cytochrome *c* (Figure 5). In the absence of ionophores and uncoupling agents, the O_2 reduction and proton pumping activities of cytochrome oxidase in COVs rapidly create a transmembrane voltage gradient ($\Delta\Psi$) and a ΔpH, both of which slow oxidase turnover. Turnover in the presence of $\Delta\Psi$ and ΔpH is termed controlled turnover. The addition of the potassium ionophore valinomycin eliminates $\Delta\Psi$, while addition of the uncoupler FCCP collapses both ΔpH and $\Delta\Psi$. The presence of these agents results in rapid "uncontrolled" catalytic turnover. As previ-

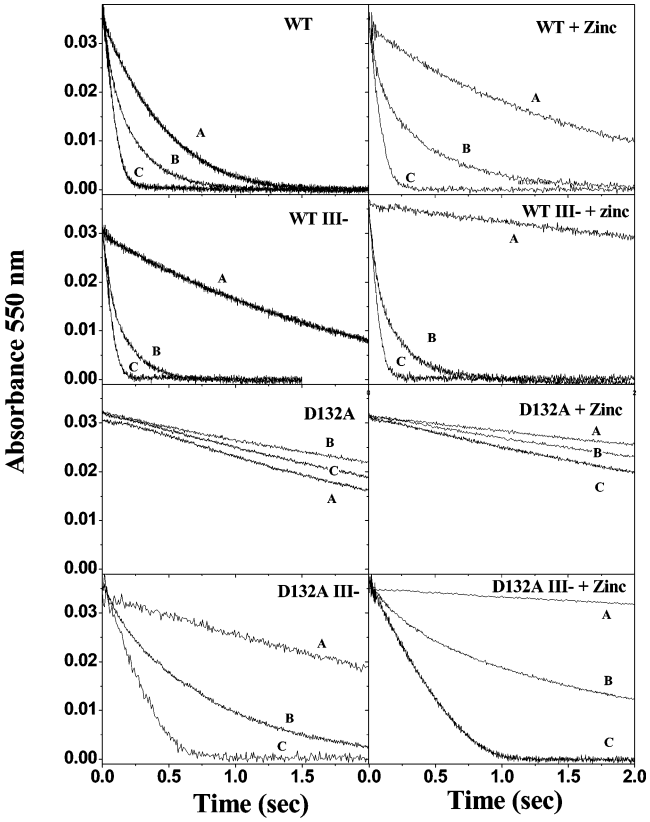

FIGURE 5: Removal of subunit III from the wild-type oxidase or from D132A decreases the rate of cytochrome *c* oxidation in the controlled state, which is further inhibited by zinc. The rate of cytochrome *c* oxidation by COVs containing different oxidase forms was measured at 550 nm using an Olis-rsm stopped-flow spectrophotometer as described in Mills et al. (*18*). Spectra: (A) no ionophores; (B) with the addition of 1.0 μM valinomycin; (C) with the addition of valinomycin plus 5.0 μM FCCP. Zinc sulfate was added to a final concentration of 100 μM as indicated.

Table 3: Effect of Zinc and Removal of Subunit III on Turnover Rates and Proton Pumping in COVs

COVs	controlled turnover rate[a]	uncontrolled turnover rate[b]	RCR[c]	proton pumping[d] (H^+/e^-)
WT	93	590	6	0.9 ± 0.2[f]
WT + Zn^{2+}[e]	16	330	21	
WT III (−)	11	390	35	0.6 ± 0.2
WT III (−) + Zn^{2+}	2	390	195	
D132A	37	18	0.5	0.0
D132A + Zn^{2+}	13	15	1.2	
D132A III (−)	10	100	10	0.0
D132A III (−) + Zn^{2+}	2	80	40	

[a] In e^- s^{-1} aa_3^{-1}; conditions were as in Figure 5. [b] Measurements were taken at pH 7.4 using subsaturating (3−5 μM) cyt c^{2+}; therefore, the rates are lower than the V_{max} values in Table 1. [c] RCR = respiratory control ratio = uncontrolled rate/controlled rate. [d] Conditions were as in Figure 6. [e] 100 μM $ZnSO_4$. [f] Standard deviation, $n = 5$.

ously shown (*18*), the addition of zinc to COVs containing the wild-type oxidase inhibits the controlled rate of turnover significantly more than the uncontrolled turnover (Figure 5). This leads to a large increase in the respiratory control ratio (RCR = uncontrolled rate/controlled rate; Table 3). In these experiments, zinc is binding to a site on the external surface of the oxidase (*18*), and the sealed vesicle prevents zinc from gaining access to its binding site(s) on the inner surface of the protein. The absence of subunit III from the wild-type oxidase has nearly the same effect as adding zinc: an 8-fold inhibition of the controlled rate of electron transfer and a large increase in the RCR (Figure 5, Table 3). Addition of zinc further inhibits the controlled rate of electron transfer by WT III (−), resulting in a very low rate of controlled electron transfer (2 e^- s^{-1} aa_3^{-1}) and an extremely high RCR of 195 (Figure 5, Table 3).

For D132A containing subunit III, turnover under both controlled and uncontrolled conditions is slow, but the controlled rate of cytochrome *c* oxidation is actually greater than the uncontrolled rate (Figure 5), as has been observed previously (*4*). In the absence of adequate rates of proton transfer through the D pathway, protons necessary for O_2 reduction are transferred to the active site from the outer surface of the protein (*4*, *11*), possibly through the normal exit pathway for pumped protons (*18*). Thus, for D132A the removal of the $\Delta\Psi$ and ΔpH that helps drive proton uptake from the outer surface slows the rate of oxidase turnover. This results in an RCR of less than 1 (Table 3). The addition of zinc to COVs containing D132A causes a 3-fold reduction in the rate of controlled turnover, and an RCR of greater than 1 is reestablished (Table 3).

The removal of subunit III from D132A slows its rate of controlled turnover approximately 4-fold (Table 3), which is qualitatively similar to the 8-fold reduction in the rate of controlled turnover of the wild-type oxidase in the absence of subunit III. In fact, the controlled rates of turnover by D132A III (−) are essentially identical to WT III (−) in both the presence and absence of zinc (Figure 5, Table 3). Therefore, the presence or absence of D132 does not affect the rate of *controlled* electron transfer when subunit III is absent. *Uncontrolled* electron transfer by D132A III (−) is not as fast as that of WT III (−) (Figure 5) with the result that the RCR values generated using D132A III (−) are not as high as those seen using WT III (−) (Table 3). When compared to D132A with subunit III, however, it is clear that the removal of subunit III from D132A increases its RCR values (Table 3).

Proton Pumping. The efficiency of proton pumping by the wild-type cytochrome *c* oxidase of *R. sphaeroides* is slightly decreased in the absence of subunit III (Figure 6, Table 3), as also occurs with mitochondrial cytochrome oxidase (*34*, *35*). In contrast, D132A III (−) shows no measurable proton pumping (Figure 6, Table 3), similar to D132A containing subunit III (*4*). Proton pumping by $W143F^{II}$, a mutant of the *R. sphaeroides* oxidase in which electron transfer to the active site is slow (*36*), is shown in order to demonstrate that the slower rates of turnover exhibited by D132A III (−) cannot explain its apparent inability to pump protons. It may be that a carboxylic amino acid at position 132 facilitates proton pumping by providing another side chain in the D pathway, in addition to E286, that can rapidly protonate the proton pump (via E286) when necessary (*37*).

DISCUSSION

Alternative Proton Acceptors in the D Pathway of Subunit I Are Recruited in the Absence of Subunit III. Recent experiments have shown that the removal of subunit III from cytochrome oxidase of *R. sphaeroides* slows the rate of proton uptake into the D pathway 30-fold at pH 8.0, but rapid

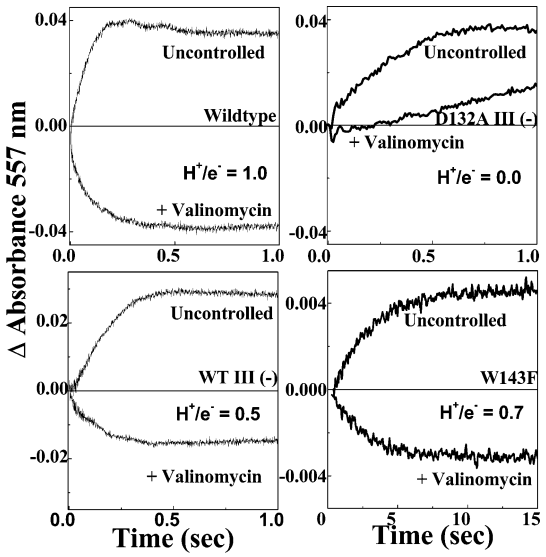

FIGURE 6: Proton pumping is observed for wild-type, WT III (−), and a low-activity mutant W143F^{II} but not for D132A III (−) COVs. Measurements were made in 50 μM Hepes−KOH, pH 7.4, 45 mM KCl, and 100 μM phenol red in an Olis-rsm stopped-flow spectrophotometer as described in Mills et al. (*18*) with 0.15 μM aa_3 and 6 μM horse heart cytochrome c^{2+}, except for W143F^{II} (*36*) which was with 0.08 μM aa_3 and 2.5 μM cyt c^{2+}. Acidification on the outside due to proton pumping is observed as a decrease in phenol red absorbance in the presence of 2 μM valinomycin (to collapse $\Delta\Psi$). Alkalinization on the outside, after the addition of valinomycin and 5 μM FCCP (collapsing $\Delta\Psi$ and ΔpH), is seen as an increase in phenol red absorbance and represents the consumed substrate protons, which have a 1:1 stoichiometry to the electrons consumed. The relative amplitudes of these two phenol red absorbance changes give the stoichiometry of proton pumping, given as H^+/e^- values. The rates of uncontrolled electron transfer, taken from the initial slope of the alkalinization profiles, are (in e^- s^{-1} aa_3^{-1}) 784 for the wild-type oxidase, 316 for WT III (−), 195 for D132A III (−), and 10 for W143F^{II}. The slow alkalinization seen for D132A III (−) in the presence of ΔpH (+valinomycin) appears to arise from the slow uptake of some substrate protons from the outer surface of this mutant oxidase rather than a nonspecific leak in the COVs.

proton uptake is restored at pH 6.5 and below (*24*). The rate of proton uptake via the D pathway appears to become the rate-limiting step for steady-state O_2 reduction in the absence of subunit III (*24*). The absence of subunit III may slow the rate of proton uptake into the D pathway by increasing the exposure of D132, the initial proton acceptor for the D pathway, to bulk water, thus decreasing its pK_a. In addition, part of a proton-collecting antenna for the D-pathway may be lost upon removal of subunit III.

When D132 of cytochrome *c* oxidase of *R. sphaeroides* is altered to alanine or asparagine, the rate of steady-state O_2 reduction and the rate of proton uptake into the D pathway are extremely inhibited (*4*, *15*). The addition of arachidonic acid stimulates the O_2 reduction activity of D132 mutants, presumably because the carboxylate of the fatty acid provides the most effective entry point into the D pathway in the absence of a carboxylic amino acid residue at position 132 (*11*). Indeed, direct measurements indicate that the rate of proton uptake into the D pathway of D132N is increased by arachidonic acid (*15*). The experiments presented here show that the removal of subunit III from D132A strongly stimulates the rate of steady-state O_2 reduction in the absence of arachidonic acid. The most likely explanation for this is that the removal of subunit III changes the conformation of the protein surface to allow the recruitment of an alternate proton acceptor for the D pathway in the absence of D132.

Table 4: Apparent pK_a Values Derived from Nonlinear Regression of pH Profiles of O_2 Reduction Activity by the Wild-Type Oxidase and D132A (Figures 2 and 3)

oxidase form	app pK_a of O_2 reduction activity (95% confidence interval)
wild type	8.5 (8.34−8.56)
WT III (−)	7.0 (6.94−7.08)
D132A	7.3 (7.10−7.48)
D132A III (−)	8.1 (7.98−8.26)
WT III (−) + Aa[a]	7.8 (7.67−7.88)
D132A III (−) + Aa	8.0 (7.93−8.12)
D132 + Aa	7.6 (7.45−7.68)

[a] 250 μM arachidonic acid.

One possible candidate for this alternative proton acceptor is His 26 of subunit I, which normally is shielded from bulk water by the carboxylate side chain of D132 and by residues in the N-terminus of subunit III (*6*). With the alteration of the D132 to alanine plus the removal of subunit III, the δ nitrogen of His 26 should be accessible to bulk water.

Proton uptake into the D pathway of the detergent-solubilized wild-type oxidase is inhibited by micromolar concentrations of Zn^{2+} (*22*). This is likely the primary cause of zinc inhibition of steady-state O_2 reduction by the detergent-solubilized oxidase. Assuming the presence of a single high-affinity binding site for zinc in the region around D132, the fact that steady-state O_2 reduction by the wild-type oxidase, WT III (−), and D132A III (−) are all inhibited by similar concentrations of Zn^{2+} suggests that the alternative proton acceptor that becomes functional in D132A III (−) is reasonably close to position 132. This would certainly be true for His 26 of subunit I. In addition, the zinc inhibition experiments indicate that neither D132 nor subunit III is required for zinc coordination. Given this, it is reasonable to propose that zinc inhibits proton uptake in the normal oxidase via electrostatic interactions (e.g., the zinc cation may strongly stabilize the deprotonated form of D132) or by conformational restraint rather than by direct interaction with D132.

If slow proton uptake into the D pathways of detergent-solubilized WT III (−) and D132A III (−) limits the rate of steady-state O_2 reduction, then the pH dependence of O_2 reduction by these subunit III-depleted oxidases may largely reflect the pK_a of the initial proton acceptor. The apparent pK_a of O_2 reduction by WT III (−), where D132 is the initial proton acceptor, is 7.0. This is considerably different from the apparent pK_a of 8.1 measured for D132A III (−), where another proton acceptor, perhaps His 26, has replaced D132.

The addition of arachidonic acid shifts the pH dependence of WT III (−) to more alkaline values (Table 4). In contrast, arachidonic acid shifts the pH dependence of D132A III (−) to slightly more acid values, and the fatty acid also stimulates the rate of O_2 reduction (Table 2). As a result of these changes, the pH profiles and activities of WT III (−) and D132A III (−) become quite similar in the presence of arachidonic acid, whereas they are considerably different in its absence (Figures 2 and 3). One explanation for these results is that the carboxylate of arachidonic acid becomes the primary entry point for D pathway protons in both of the oxidase forms lacking subunit III. The role of arachidonic

acid in the presence of subunit III is less clear. Arachidonic acid stimulates the weak O_2 reduction activity of D132A containing subunit III [Figures 2 and 3 (*11*)]. In contrast, arachidonic acid does not increase the activity of the wild-type oxidase containing subunit III at pH 6.5 (*11*) or at pH 7.5 (data not shown).

While the removal of subunit III stimulates the activity of D132A, it further inhibits the weak activity of E286A/I112E. Previous experiments have shown that the removal of subunit III from the wild-type oxidase appears to raise the apparent pK_a of E286 without slowing the rate of proton transfer between E286 and the heme a_3-Cu_B active site (*24*). Thus, subunit III influences the structure of the D pathway at more than just the entry point, although the structural changes around E286 are probably more subtle and they must occur via long-range interactions.

Proton Movement through the Exit Pathway of Cytochrome Oxidase May Be Inhibited in the Absence of Subunit III. During controlled electron transfer in COVs both a $\Delta\Psi$ and ΔpH oppose the transfer of protons through the D pathway and the K pathway to the active site and slow the turnover rate of the oxidase. Pumped protons must exit at the outer surface of the oxidase; $\Delta\Psi$ and ΔpH will also oppose proton transfer through the exit pathway and slow catalytic turnover. The components of the proton exit pathway leading from the active site to the outside of the oxidase are not yet conclusively identified (*10*, *38*).

Controlled catalytic turnover by cytochrome oxidase is even slower in the absence of subunit III, as seen by the considerable increase in the RCR (Table 3); without subunit III cytochrome oxidase turnover is more sensitive to the effects of a $\Delta\Psi$ and a ΔpH than the normal oxidase. One likely explanation for this effect comes from the observation that removing subunit III has nearly the same effect as adding zinc to COVs containing the wild-type oxidase. In COVs, zinc cannot access its interior binding sites, but in the presence of a $\Delta\Psi$ there exists a high-affinity binding site for zinc on the external surface of the protein (*18*). Electron transfer requires protons to neutralize the charge of incoming electrons (*39*, *40*) and to participate in oxygen reduction chemistry, but the $\Delta\Psi$ opposes proton transfer from the *inside* of the vesicle. However, proton uptake from the *outside* of the vesicle, via reversal of the normal exit pathway, is favored by $\Delta\Psi$ and ΔpH and may be required to allow turnover under controlled conditions. The addition of micromolar amounts of zinc appears to block this back-flow of protons and slow controlled electron transfer, leading to high RCR values (*18*; Table 3). Since the removal of subunit III also slows controlled electron transfer, it seems reasonable to propose that the transfer of protons to the active site via reversal of the proton exit pathway is inhibited by structural alterations induced by the removal of subunit III.

At the top of helix 3 of subunit III, on the outer surface of the oxidase, subunit III forms connections with subunits I and II. Lysine 103 of subunit III forms an ion pair with Asp 271 of subunit I, while Asp 271 also participates in a hydrogen bond with Arg 234 of subunit II (*6*). Disruption of these interactions by mutation of these residues weakens both the subunit I−II interface and the subunit I−III interface (*41*). The removal of subunit III may strengthen the association of Asp 271 of subunit I and Arg 234 of subunit II, alter the structure of the protein in this region of the outer surface, and constrict the proton exit (back-flow) pathway in the presence of a $\Delta\Psi$.

Clearly, the absence of subunit III does not prevent the binding of zinc on the outer surface of the oxidase. The effects of zinc and the removal of subunit III are additive, leading to an extremely high RCR value of nearly 200 for WT III (−) in the presence of zinc. The high RCR is due to the fact that controlled electron transfer is almost entirely eliminated under these conditions, while uncontrolled electron transfer is unaffected. This is not a result of high internal pH, as shown by the strong stimulation of respiration by valinomycin which will increase the internal pH. Rather, proton flow to the active site from the inside of the COVs is inhibited by $\Delta\Psi$ while proton flow via the back-leak/exit pathway is simultaneously inhibited by zinc and the absence of subunit III.

In conclusion, the removal of subunit III from cytochrome *c* oxidase apparently increases the flexibility of the enzyme in terms of the entry of protons into the D pathway. This result supports and extends the conclusion of Gilderson et al. (*24*) that subunit III plays a role in controlling the access of solvent protons to the entry of the D pathway on the inner surface of the oxidase. In addition, the removal of subunit III appears to affect the ability of the normal proton exit pathway to take up protons from the outer surface of the oxidase under controlled conditions, i.e., in the presence of $\Delta\Psi$ and ΔpH. These roles for subunit III in facilitating proton transfer in subunit I may be the reason why subunit III is required for full efficiency of proton pumping by cytochrome oxidase, as well as the reason for the high conservation of the subunit throughout evolution.

ACKNOWLEDGMENT

The authors thank Mr. Jimmy Gray and Mr. Daniel Smith for excellent technical assistance.

REFERENCES

1. Saraste, M. (1999) *Science 283*, 1488−1493.
2. Thomas, J. W., Lemieux, L. J., Alben, J. O., and Gennis, R. B. (1993) *Biochemistry 32*, 11173−11180.
3. Hosler, J. P., Shapleigh, J. P., Mitchell, D. M., Kim, Y., Pressler, M. A., Georgiou, C., Babcock, G. T., Alben, J. O., Ferguson-Miller, S., and Gennis, R. B. (1996) *Biochemistry 35*, 10776−10783.
4. Fetter, J. R., Qian, J., Shapleigh, J., Thomas, J. W., García-Horsman, A., Schmidt, E., Hosler, J., Babcock, G. T., Gennis, R. B., and Ferguson-Miller, S. (1995) *Proc. Natl. Acad. Sci. U.S.A. 92*, 1604−1608.
5. Mitchell, D. M., Fetter, J. R., Mills, D. A., Ädelroth, P., Pressler, M. A., Kim, Y., Aasa, R., Brzezinski, P., Malmström, B. G., Alben, J. O., Babcock, G. T., Ferguson-Miller, S., and Gennis, R. B. (1996) *Biochemistry 35*, 13089−13093.
6. Svensson-Ek, M., Abramson, J., Larsson, G., Törnroth, S., Brzezinski, P., and Iwata, S. (2002) *J. Mol. Biol. 321*, 329−339.
7. Iwata, S., Ostermeier, C., Ludwig, B., and Michel, H. (1995) *Nature 376*, 660−669.
8. Yoshikawa, S., Shinzawa-Itoh, K., and Tsukihara, T. (1998) *J. Bioenerg. Biomembr. 30*, 7−14.
9. Riistama, S., Hummer, G., Puustinen, A., Dyer, R. B., Woodruff, W. H., and Wikström, M. (1997) *FEBS Lett. 414*, 275−280.
10. Mills, D. A., Florens, L., Hiser, C., Qian, J., and Ferguson-Miller, S. (2000) *Biochim. Biophys. Acta 1458*, 180−187.
11. Fetter, J., Sharpe, M., Qian, J., Mills, D., Ferguson-Miller, S., and Nicholls, P. (1996) *FEBS Lett. 393*, 155−160.
12. Verkhovskaya, M. L., García-Horsman, A., Puustinen, A., Rigaud, J. L., Morgan, J. E., Verkhovsky, M. I., and Wikström, M. (1997) *Proc. Natl. Acad. Sci. U.S.A. 94*, 10128−10131.

13. Ädelroth, P., Ek, M. S., Mitchell, D. M., Gennis, R. B., and Brzezinski, P. (1997) *Biochemistry 36*, 13824−13829.
14. Aagaard, A., Gilderson, G., Mills, D. A., Ferguson-Miller, S., and Brzezinski, P. (2000) *Biochemistry 39*, 15847−15850.
15. Smirnova, I. A., Ädelroth, P., Gennis, R. B., and Brzezinski, P. (1999) *Biochemistry 38*, 6826−6833.
16. Paddock, M. L., Graige, M. S., Feher, G., and Okamura, M. Y. (1999) *Proc. Natl. Acad. Sci. U.S.A. 96*, 6183−6188.
17. Axelrod, H. L., Abresch, E. C., Paddock, M. L., Okamura, M. Y., and Feher, G. (2000) *Proc. Natl. Acad. Sci. U.S.A. 97*, 1542−1547.
18. Mills, D. A., Schmidt, B., Hiser, C., Westley, E., and Ferguson-Miller, S. (2002) *J. Biol. Chem. 277*, 14894−14901.
19. Kita, K., Kasahara, M., and Anraku, Y. (1982) *J. Biol. Chem. 257*, 7933−7935.
20. Kannt, A., Ostermann, T., Müller, H., and Ruitenberg, M. (2001) *FEBS Lett. 503*, 142−146.
21. Aagaard, A., and Brzezinski, P. (2001) *FEBS Lett. 494*, 157−160.
22. Aagaard, A., Namslauer, A., and Brzezinski, P. (2002) *Biochim. Biophys. Acta 1555*, 133−139.
23. Bratton, M. R., Pressler, M. A., and Hosler, J. P. (1999) *Biochemistry 38*, 16236−16245.
24. Gilderson, G., Salomonsson, L., Aagaard, A., Gray, J., Brzezinski, P., and Hosler, J. (2003) *Biochemistry 42*, 7400−7409.
25. Sone, N., Yoshida, M., Hirata, H., and Kagawa, Y. (1977) *J. Biochem. (Tokyo) 81*, 519−528.
26. Brautigan, D. L., Ferguson-Miller, S., and Margoliash, E. (1978) *J. Biol. Chem. 253*, 130−139.
27. Zhen, Y., Qian, J., Follmann, K., Hayward, T., Nilsson, T., Dahn, M., Hilmi, Y., Hamer, A. G., Hosler, J. P., and Ferguson-Miller, S. (1998) *Protein Expression Purif. 13*, 326−336.
28. Hosler, J. P., Fetter, J., Tecklenburg, M. M., Espe, M., Lerma, C., and Ferguson-Miller, S. (1992) *J. Biol. Chem. 267*, 24264−24272.
29. Thompson, D. A., and Ferguson-Miller, S. (1983) *Biochemistry 22*, 3178−3187.
30. Pawate, A. S., Morgan, J., Namslauer, A., Mills, D., Brzezinski, P., Ferguson-Miller, S., and Gennis, R. B. (2002) *Biochemistry 41*, 13417−13423.
31. Wilms, J., van Rijn, J. L., and Van Gelder, B. F. (1980) *Biochim. Biophys. Acta 593*, 17−23.
32. Gregory, L. C., and Ferguson-Miller, S. (1988) *Biochemistry 27*, 6307−6314.
33. Einarsdóttir, Ó., Choc, M. G., Weldon, S., and Caughey, W. S. (1988) *J. Biol. Chem. 263*, 13641−13654.
34. Thompson, D. A., Gregory, L., and Ferguson-Miller, S. (1985) *J. Inorg. Biochem. 23*, 357−364.
35. Wilson, K. S., and Prochaska, L. J. (1990) *Arch. Biochem. Biophys. 282*, 413−420.
36. Zhen, Y., Hoganson, C. W., Babcock, G. T., and Ferguson-Miller, S. (1999) *J. Biol. Chem. 274*, 38032−38041.
37. Thomas, J. W., Puustinen, A., Alben, J. O., Gennis, R. B., and Wikström, M. (1993) *Biochemistry 32*, 10923−10928.
38. Puustinen, A., and Wikström, M. (1999) *Proc. Natl. Acad. Sci. U.S.A. 96*, 35−37.
39. Mitchell, R., and Rich, P. R. (1994) *Biochim. Biophys. Acta 1186*, 19−26.
40. Verkhovsky, M. I., Morgan, J. E., and Wikström, M. (1995) *Biochemistry 34*, 7483−7491.
41. Yang, J. (2002) M.S. Thesis, Michigan State University.
42. Hiser, C., Mills, D. A., Schall, M., and Ferguson-Miller, S. (2001) *Biochemistry 40*, 1606−1615.

BI0341307