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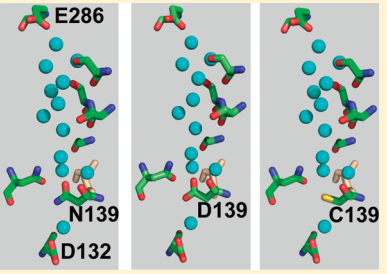
Alternative Initial Proton Acceptors for the D Pathway of *Rhodobacter sphaeroides* Cytochrome *c* Oxidase

Lakshman Varanasi and Jonathan Hosler*

Department of Biochemistry, University of Mississippi Medical Center, Jackson, Mississippi 39216, United States

ABSTRACT: To characterize protein structures that control proton uptake, we assayed forms of cytochrome *c* oxidase (C*c*O) containing a carboxyl or a thiol group in line with the initial, internal waters of the D pathway for proton transfer in the presence and absence of subunit III. Subunit III provides approximately half of the protein surrounding the entry region of the D pathway. The N139D/D132N mutant contains a carboxyl group 6 Å within the D pathway and lacks the normal, surface-exposed proton acceptor, Asp-132. With subunit III, the steady-state activity of this mutant is slow, but once subunit III is removed, its activity is the same as that of wild-type C*c*O lacking subunit III (~1800 H^+/s). Thus, a carboxyl group ~25% within the pathway enhances proton uptake even though the carboxyl has no direct contact with bulk solvent. Protons from solvent apparently move to internal Asp-139 through a short file of waters, normally blocked by subunit III. Cys-139 also supports rapid steady-state proton uptake, demonstrating that an anion other than a carboxyl can attract and transfer protons into the D pathway. When both Asp-132 and Asp/Cys-139 are present, the removal of subunit III increases C*c*O activity to rates greater than that of normal C*c*O because of simultaneous proton uptake by two initial acceptors. The results show how the environment of the initial proton acceptor for the D pathway in these C*c*O forms dictates the pH range of C*c*O activity, with implications for the function of Asp-132, the normal proton acceptor.



The aa_3-type cytochrome *c* oxidase complex (C*c*O) is the final member of the electron transfer chain of oxidative phosphorylation in the inner membrane of mitochondria and in the cytoplasmic membrane of many bacteria. To generate a voltage gradient across these membranes, C*c*O transfers electrons from its outer (positive) surface and protons from its inner (negative) surface to a buried heme−Cu active site where the charges combine as O_2 is reduced to water.[1−3] As a proton pump, C*c*O also uses the energy of O_2 reduction to transfer protons completely through the protein, across the membrane.[3−6] The four-subunit aa_3-type C*c*O of the bacterium *Rhodobacter sphaeroides* is one of the popular, mutable models of the catalytic core of mitochondrial C*c*O.[7,8]

C*c*O takes up the protons necessary for catalysis via two long pathways, the D and K paths, leading from the inner surface of the complex to the buried active site (Figure 1).[2,4,9,10] Protons are introduced into the D pathway by Asp-132 of subunit I (*R. sphaeroides* numbering) and transferred 26 Å to Glu-286, near the active site, through a series of hydrogen-bonded water molecules.[11−14] From Glu-286, protons branch to O_2 reduction intermediates in the active site or to the site of proton pumping.[15−17] The K pathway begins far from the entry region of the D pathway, at Glu-101 of subunit II.[18] The K pathway transfers protons primarily via protein side chains to the cross-linked Tyr-288/His-284 group of the active site.[6,11,12]

Aspartate 132 is defined as the initial proton acceptor of the D pathway based upon its absolute requirement for D pathway activity and its partial exposure on the surface of the protein. Alteration of Asp-132 to alanine, histidine, or asparagine essentially eliminates proton uptake,[19,20] indicating that Asp-132 provides the sole point of entry into the D pathway of normal C*c*O. Only one of the carboxyl oxygens of Asp-132 is exposed at the bottom of a narrow cavity some 6−10 Å deep.[21] The protein partner of subunit I in the membrane, subunit III, provides approximately half of the protein residues that form the cavity surrounding Asp-132.[21−24]

Subunit III can be removed from *R. sphaeroides* C*c*O using detergent, leaving an active C*c*O form composed of subunits I and II.[25] The removal of subunit III results in a sizable acid shift (>1 pH unit) of the pH profile of the uptake of protons into the D pathway.[20,26] The shift apparently reflects a decrease in the affinity of Asp-132 for protons[20,26] consistent with a greater exposure of Asp-132 to solvent water and increased stability of the carboxyl anion.

The removal of subunit III also restores significant rates of proton uptake (~400 H^+/s) to D pathways inactivated by the alteration of Asp-132 to alanine, histidine, or asparagine.[20,25,27] The absence of subunit III allows protons from solvent to access components of the D pathway beyond residue 132, such as the crystal water seen between Asp-132 and Asn-139 in wild-type C*c*O structures.[14,17,20,28] Ergo, subunit III normally provides a structural barrier that prevents protons from solvent from bypassing Asp-132.

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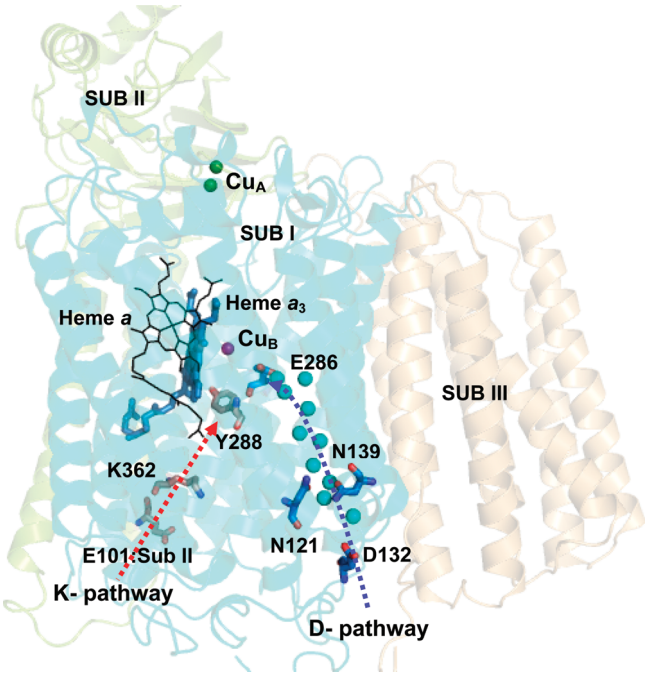

Figure 1. D and K pathways of the aa_3-type cytochrome *c* oxidase of *R. sphaeroides*. Subunits I−III are shadowed in cyan, green, and tan, respectively. The waters and selected residues of the D pathway are shown along the blue arrow and selected residues of the K pathway along the red arrow.

Even following the removal of subunit III, steady-state proton uptake by the D pathways of D132A III (−) and D132H III (−) is considerably slower (~400 s^{-1}) than that of WT III (−) (~1800 s^{-1}). The addition of arachidonic acid to D132A III (−) stimulates the rate of proton uptake, restoring up to 60% of normal TN_{max}, as the carboxyl of the fatty acid takes over as the initial proton acceptor.[20,25] This demonstrates the utility of an anionic proton acceptor at the entrance of the pathway even though full activity is not restored and the precise location of the fatty acid carboxyl group remains unknown.

Work in *Paracoccus denitrificans*[29] and *R. sphaeroides*[30] has shown that mutant C*c*Os with a substitution of aspartic acid for an asparagine that lies in the D pathway 6 Å above Asp-132 (Asn-131 in *Paracoccus* and Asn-139 in *Rhodobacter*) retain high rates of O_2 reduction activity. Consistent with this, the N139D mutant of *R. sphaeroides* exhibits rapid proton uptake in single-turnover experiments.[30,31] In a study of proton pumping, Asp-132 of N139D was altered to asparagine, creating N139D/D132N.[32] Of particular interest for this study of proton uptake is the finding that N139D/D132N III (+) shows greater activity (~250 s^{-1}) than D132A, -H, or -N III (+) (~40 s^{-1}).[20,25,32,33] This suggests that Asp-139, even though buried, facilitates the entry of solvent protons into the D pathway.

On the basis of previous results,[20,25] we first hypothesized that the access of solvent protons to Asp-139 of N139D/D132N is restricted by subunit III. In fact, we find that without subunit III, buried Asp-139 supports as much C*c*O activity as surface-exposed Asp-132 of WT III (−). We propose that Asp-139 becomes the initial proton acceptor for the D pathway in the absence of Asp-132 and subunit III. A short file of waters provides the likely pathway for the transfer of protons between Asp-139 and solvent. The general utility of an anionic proton acceptor is demonstrated by examination of C*c*O forms with cysteine at position 139. Further experiments show that the environment of the initial acceptor can control the pH range of C*c*O activity, that multiple acceptors can increase rates of proton uptake to rates greater than that of normal C*c*O, and that proton uptake by the D pathway is inherently more limiting to C*c*O activity than proton uptake by the K pathway.

MATERIALS AND METHODS

Materials. Ni^{2+}-NTA agarose was obtained from Qiagen. Arachidonic acid, methyl arachidonate (mAa), horse heart cytochrome *c*, CCCP, valinomycin, and soybean phospholipids (asolectin) were from Sigma. Stock solutions (50 mM) of arachidonic acid and mAa were prepared in ethanol and stored at −20 °C. Soybean phospholipid (asolectin, Sigma type II-S) was recrystallized using the procedure of Sone et al.[34] *N*-Dodecyl β-D-maltoside was from Anatrace. All other chemicals were ACS grade.

Construction of N139C/D132N and N139D/D132A. A pUC18 construct with an ~2 kb *Hin*dIII−*Eco*RI fragment containing *R. sphaeroides coxI*, the gene for subunit I of C*c*O, with the N139C mutation, was obtained from R. B. Gennis (University of Illinois, Urbana, IL). Quik-Change mutagenesis (Stratagene) was used to insert the D132N mutation, and the presence of the N139C and D132N mutations, and the absence of other alterations, was verified by DNA sequencing. The altered *Eco*RI−*Hin*dIII fragment was then excised from the pUC plasmid and moved into broad host range vector pRK415[35] to create pJG273. This plasmid was conjugated into *R. sphaeroides* JS100, a strain lacking the wild-type gene for subunit I,[36] for the expression of N139C/D132N. The same procedure was used to create N139D/D132A, starting from the *Hin*dIII−*Eco*RI fragment of *coxI* containing the N139D mutation. The *R. sphaeroides* C*c*O mutants N139D,[30] N139C, and N139D/D132N[32] were obtained from the Gennis laboratory.

Isolation of C*c*O, Removal of Subunit III, Preparation of COVs, and Measurements of Activity. Bacterial growth and oxidase purification by Ni-affinity chromatography were performed as described by Zhen et al.[37] The removal of subunit III was performed as described by Mills et al.[25] Measurements of the rates of O_2 reduction (consumption) by purified, detergent-solubilized C*c*O were taken using an oxygen electrode as described previously[25] in assay mixtures containing 25 mM MES-KOH (pH 6.5) adjusted to an *I* of 50 mM with KCl, 0.3 mM TMPD, 3.0 mM ascorbate, 0.1% dodecyl maltoside (DDM), 1000 units of catalase, 1 mg/mL soybean phospholipids, 5−25 pmol of C*c*O and horse cytochrome *c*, and other additions as noted. For determinations of C*c*O activity at different pH values, the buffer consisted of 25 mM MES-KOH from pH 6.2 to 6.8, 25 mM Hepes-KOH from pH 6.8 to 8.2, and 25 mM CHES-KOH from pH 8.2 to 9.2. Each buffer was adjusted to a final ionic strength of 50 mM using KCl. The actual pH was measured in the O_2 electrode chamber before and after each activity measurement. The rates of nonenzymatic reduction of O_2 by ascorbate/TMPD at each pH were subtracted. 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 $TN = TN_{min} + (TN_{max} - TN_{min})/(1 + 10^{pH-pK_a})$ in GraphPad Prism 5.0.

Cytochrome *c* oxidase was reconstituted into phospholipid vesicles (COVs) as previously described.[30] Controlled steady-state activities of the COVs were measured using the O_2

Table 1. Comparison of the TN_{max} Values at pH 6.5 of Wild-Type CcO and Asn-139 Mutants in the Presence and Absence of Subunit III

CcO form	III (+)	III (−)
WT	2062[a] ± 24[b]	1835 ± 48
D132N	43 ± 2	383 ± 11
N139D/D132N	328 ± 7	1799 ± 75
N139C/D132N	251 ± 4	1587 ± 23
N139D	2187[c] ± 81	2505 ± 24
N139C	1781[c] ± 36	2256 ± 36
N139D/D132A	1017 ± 37	ND

[a] e^- or H^+ consumed per second. [b] Standard error of the fit to the Michaelis−Menten function. [c] The activities of N139D and -C III (+) are in good agreement with the relative activities published for the corresponding mutants of *P. denitrificans* CcO[41] and with the absolute activities published previously for these *R. sphaeroides* CcO forms.[42]

electrode in a reaction mixture containing 25 mM Hepes (pH 7.4), 24 mM KCl, ascorbate, TMPD, horse cytochrome *c*, and catalase as described above. Uncontrolled (uncoupled) activities were measured in the same reaction mixture with 5 μM CCCP and 3 μM valinomycin.

RESULTS

Effect of Removing Subunit III on the Steady-State Proton Uptake Activity of D132N and the Asn-139 Mutants. If one begins with normal CcO, the alteration of Asp-132, the initial proton acceptor of the D pathway, to alanine, histidine, or asparagine (D132A, -H, or -N, respectively) nearly eliminates proton uptake by this pathway.[19,20] Slow O_2 reduction activity continues (~40 e^-/s), as substrate protons normally supplied by the D pathway are slowly taken up by another route, apparently from the opposite side of the complex.[25,33,38] The removal of subunit III from D132A and D132H restores steady-state CcO activity to rates 20−25% of that of normal CcO by increasing the rate of D pathway proton uptake to ~400 H^+/s.[20,25] (All proton uptake rates presented here are those for substrate protons.) D132N exhibits the same behavior as D132A and -H: removing subunit III increases its low activity to a TN_{max} of ~400 s^{-1}.[27] (Table 1).

If one starts with D132N, the further alteration of Asn-139 to aspartic acid creates N139D/D132N,[32] thereby placing a carboxyl group within the D pathway, 6 Å above the normal position of Asp-132 (Figures 1 and 2). Computer modeling of the Asn-139 → Asp substitution in the structure of the subunit I−II form of wild-type *R. sphaeroides* CcO (Protein Data Bank entry 2GSM[39]), using Swiss PDB Viewer DeepView,[40] shows that the carboxyl of Asp-139 occupies nearly the same position as the amide group of Asn-139 in normal CcO. The group has no exposure to bulk solvent. One carboxyl O of Asp-139 is 2.8 Å from the water above (O−O, center−center), while the other lies 2.9 Å from the water below, which is the water normally seen between Asp-132 and Asn-139 (Figure 2). While a structure of the N139D/D132N double mutant is not yet available, a high-resolution structure of N139D of *R. sphaeroides* (J. Zhu and R. Gennis, unpublished observations) agrees with the modeled position of Asp-139. In addition, the same position for the equivalent of Asp-139 is seen in a structure of N131D of *P. denitrificans*, although the water normally present above Asn-139

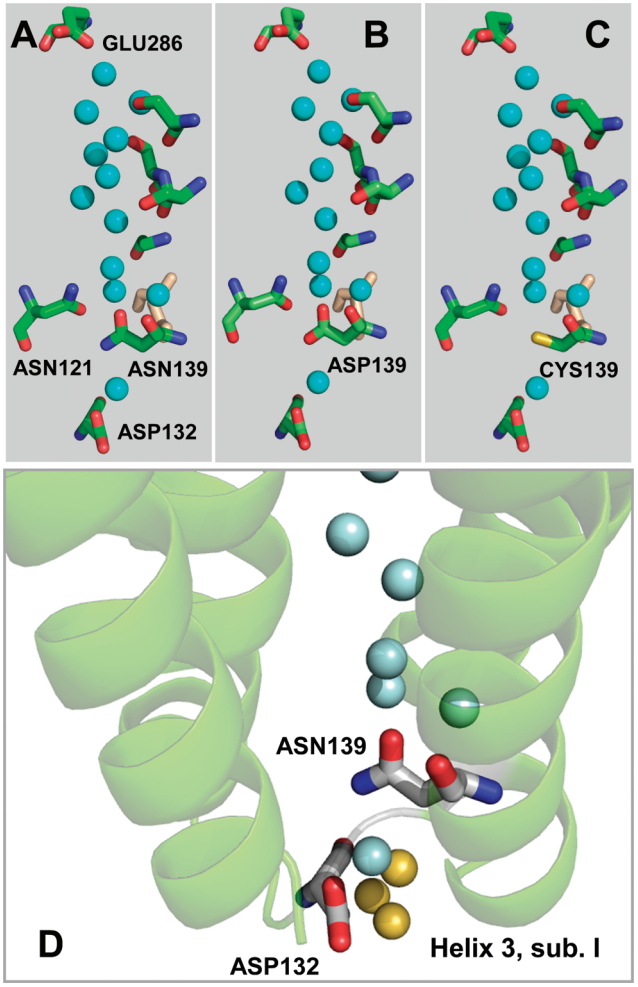

Figure 2. Normal D pathway and variants containing aspartic acid or cysteine at position 139. The normal D pathway (A) is that of the structure of the subunit I−II CcO of *R. sphaeroides* at 2.0 Å resolution (Protein Data Bank entry 2GSM[39]). The structures shown for N139D (B) and N139C (C) were modeled from the 2GSM structure using Swiss PDB Viewer DeepView.[40] It is assumed that the alteration of Asp-132 to Asn in N139D/D132N and N139C/D132N does not significantly alter the position of Asp-139 or Cys-139. Therefore, models of these CcO forms are not shown. In panel D, three waters (gold, 2.6 Å apart) have been added to the 2GSM structure using DeepView to show one possibility of a water file sufficient to bypass Asp-132 or Asn-132 and transfer protons from solvent to the first crystal water (blue) shown between Asp-132 and Asn-139 in panel A. In the model of panel D, the two lower waters are both exposed to solvent. Both make steric clashes with Pro-136 and Met-133 of subunit I, which would be relieved if the lower region of helix 3 of subunit I moved less than 1 Å. Such flexibility seems likely in the absence of subunit III.

is not present in the structure of N131D.[41] The carboxyl of Asp-139 appears well placed to transfer protons, consistent with published results showing high activity for N131D of *P. denitrificans*[29,41] and N139D of *R. sphaeroides*,[30,42] and the observation of rapid proton uptake in single-turnover experiments.[30,31] Prior to the removal of subunit III, the N139D/D132N double mutant exhibits low activity with respect to normal CcO (Table 1 and ref 32) because of the absence of Asp-132. However, with the removal of subunit III, the O_2 reduction rate of N139D/D132N increases to ~1800 s^{-1}, the same as that of WT III (−) (Table 1).

This shows that Asp-139 of N139D/D132N and Asp-132 of wild-type C*c*O are equally efficient at supporting steady-state proton uptake, as long as subunit III is removed to allow protons from the solvent to access Asp-139.

With subunit III in place, the activity of N139D/D132N is low (~300 s^{-1}) but significantly greater than that of D132N III (+) (~40 s^{-1}) (Table 1 and ref 32). This suggests that Asp-139 obtains protons from solvent even in the presence of subunit III, albeit at a much slower rate.

Similar to N139D, the mutant N139C is highly active [41,42] (Table 1), indicating that a cysteine at position 139 also permits rapid proton flow through the D pathway. We created the N139C/D132N double mutant to determine if Cys-139, like Asp-139, is capable of functioning as the sole initial proton gate for the D pathway, i.e., without Asp-132. As for Asp-139, computer modeling indicates that the thiol of a cysteine at position 139 is well placed to transfer protons from water to water given the longer hydrogen bonds of a thiol (Figure 2). The thiol of Cys-139 is 3.5 Å from the "upstream" water and 3.4 Å from the "downstream" water. This is confirmed by the nearly identical position of the thiol group in a high-resolution structure of *R. sphaeroides* N139C (J. Zhu and R. Gennis, unpublished observations). Similar to that of N139D/D132N, the activity of N139C/D132N is more rapid (~250 s^{-1}) than that of D132N even with subunit III present (~40 s^{-1}) (Table 1), indicating that Cys-139 stimulates D pathway proton uptake. Upon the removal of subunit III, the activity of the double mutant increases to ~1600 s^{-1}, indicating enhanced access of solvent protons to Cys-139. The TN_{max} of N139C/D132N III (−) is only slightly lower than that of WT III (−) (Table 1), showing that Cys-139 functions as an efficient initial proton gate in the absence of Asp-132.

The structures of C*c*O indicate that the most probable pathway for the transfer of protons from solvent to Asp-139 or Cys-139 is a short series of hydrogen-bonded waters within the normal D pathway channel (Figure 2). Subunit III appears to inhibit the formation of this water chain. If this is so, then creating more space for waters to enter this channel should enhance proton uptake even in the presence of subunit III. To test this, Asn-132 of N139D/D132N and N139C/D132N was altered to alanine, which replaces the amide group at the mouth of the channel with the smaller methyl group. Indeed, N139D/D132A has significantly greater activity with subunit III present (1017 s^{-1}) than N139D/D132N does (328 s^{-1}) (Table 1). Thus, creating more space at the mouth of the D channel increases the rate of transfer of protons to Asp-139, most probably by making it more possible for waters to enter and form a hydrogen-bonded path for the transfer of protons from solvent. A similar result was obtained using N139C/D132A III (+) (data not shown).

In the N139D mutant, two carboxylic groups are present in the initial region of the D pathway, Asp-132 and Asp-139. The situation is similar in N139C, except that the smaller thiol group is present at position 139. With subunit III present, the steady-state activities of both mutants are roughly similar to those of our preparations of wild-type C*c*O; N139D is slightly, but consistently, more active than normal C*c*O, while N139C is slightly less active (Table 1). The removal of subunit III stimulates the steady-state O_2 reduction activity of both mutant C*c*Os to rates greater than that of normal C*c*O, at least up to pH 8 (Table 1 and Figure 4). The result requires the conclusion that the altered D pathways of N139D III (−) and N139C III (−) take up substrate protons more rapidly than wild-type C*c*O that contains subunit III.

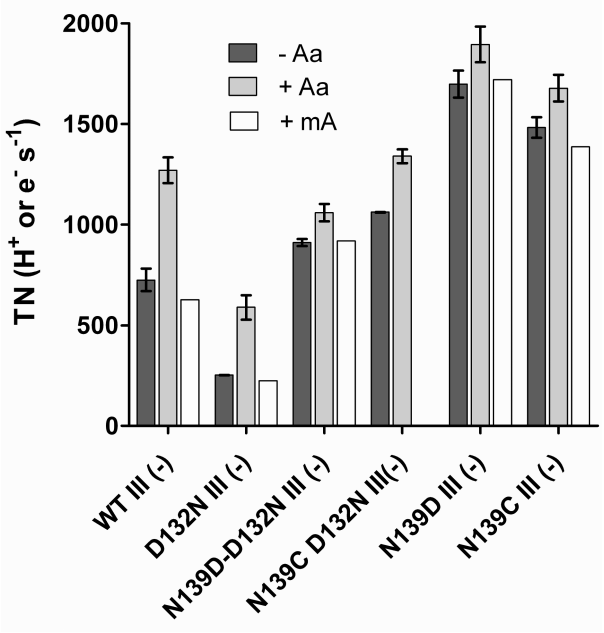

Figure 3. Effect of 250 μM arachidonic acid or 250 μM methyl arachidonate on the steady-state activities of subunit III-depleted wild-type C*c*O, D132N, and four Asn-139 mutants. Activities were measured at pH 7.5 as described in Materials and Methods. The error is the standard deviation.

Arachidonic Acid Enhances the D Pathway Activity of the Asn-139 Mutants. The addition of long chain, unsaturated fatty acids, notably, arachidonic acid (Aa), restores activity to C*c*O forms lacking Asp-132,[33] especially in the absence of subunit III.[20,25,27] Arachidonic acid also enhances the activity of WT III (−), which contains Asp-132, but if subunit III is present Aa has no effect.[25] Single-turnover experiments confirm that Aa increases the activity of these C*c*O forms by increasing the rate of uptake of protons into their D pathways.[20] Methyl arachidonate has no effect[33,43] (Figure 3), indicating that the carboxyl group is required for the stimulation of proton uptake. Apparently, Aa binds on the surface of C*c*O in such a way that its carboxyl group transfers protons into the D pathway in the absence of Asp-132, or in the presence of Asp-132, it provides an additional entry point for protons. The ability of the fatty acid carboxyl to transfer protons into the D pathway is enhanced by removing subunit III. Arachidonic acid can also stimulate the uptake of protons by the K pathway of *R. sphaeroides* C*c*O, but only when the surface-exposed proton acceptor of the K pathway, Glu-101 of subunit II, is replaced with a residue that cannot be protonated.[43] Therefore, the K pathways of the C*c*O forms presented here will not be affected by Aa. Figure 3 shows that Aa enhances the activity of D132N III (−), as it does for D132A III (−).[25] The addition of Aa also stimulates the activities of the double mutants containing Asp-139 or Cys-139 as the initial proton gate, similar to the effect of Aa on WT III (−) (Figure 3). Methyl arachidonate has no effect. On the basis of the background work described above, we conclude that Aa increases the rate of uptake of protons into the D pathways of these C*c*Os by restoring an initial proton gate in D132N III (−) and by providing an additional proton entry point in N139D/D132N III (−) and N139C/D132N III (−). The data also indicate that the uptake of protons into the D pathway is a rate-limiting process for steady-state O_2 reduction

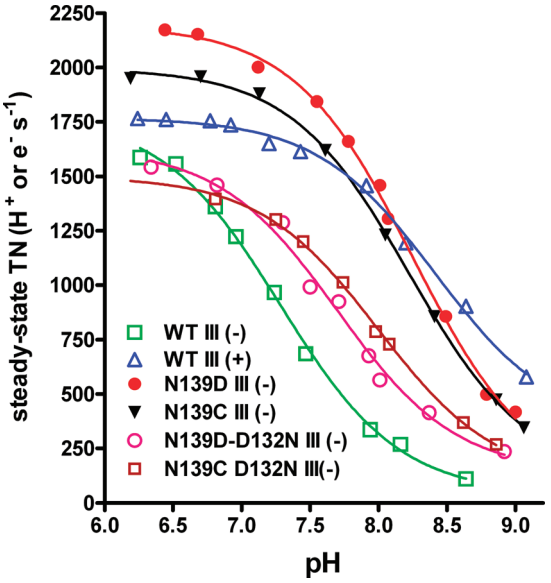

Figure 4. pH dependence of the steady-state activity by wild-type C*c*O and subunit III-depleted C*c*O forms. Activity measurements and fits to the Henderson−Hasselbalch equation are as described in Materials and Methods.

by these C*c*O forms, as suggested by previous studies.[20,25,44] Interestingly, Aa also stimulates the activities of N139C III (−) and N139D III (−) (Figure 3), even though these C*c*O forms already take up substrate protons at a rate greater than that of normal C*c*O.

Estimating the Functional pK_a Values of the Initial Proton Acceptors of the Asn-139 Mutants. In comparison to normal C*c*O, subunit III-depleted C*c*O exhibits significantly slower uptake of protons into the D pathway at pH values greater than ~6.5[21,26] (Figure 4). Analyses of single-turnover experiments, during which only D pathway protons are taken up, support the argument that the shift in the pH dependence of proton uptake occurs with a decrease in the apparent pK_a of Asp-132 to ~7.[20,26] This is consistent with the structure of C*c*O, because the removal of subunit III will increase the exposure of Asp-132 to solvent water and thereby confer greater stability to the anionic carboxyl. When the uptake of protons into the D pathway has been made rate-limiting, the pH dependence of steady-state O_2 reduction can be used to estimate the pK_a of the initial proton acceptor.[20] For example, the pH dependence of steady-state O_2 reduction by WT III (−) yields a pK_a of 7.0−7.3 (Figure 4, Table 2, and refs 21 and 25), similar to that obtained from the pH dependence of proton uptake during a single turnover.[20,26] The pK_a values obtained for Asp-132 and other initial proton gates from steady-state activity data are not "true" pK_a values because they are not derived from direct, equilibrium measurements of the protonation state of these residues. Rather, these are "apparent" or "functional" pK_a values that characterize the group(s) whose protonation and deprotonation control the uptake of protons into the D pathway during continuous catalytic turnover.

For N139D/D132N III (−), a pK_a of 7.7 is obtained from the pH dependence of steady-state activity, ~0.5 pH unit higher than that of WT III (−) (Figure 4 and Table 2). Similar to that of WT III (−), this value should reflect the functional pK_a of Asp-139 (sans subunit III) if the rate of C*c*O activity is limited by the rate at which protons enter the D pathway via Asp-139. This assumption is supported by the close similarity of N139D/D132N III (−) to WT III (−). For both C*c*O forms, an altered D pathway leads to O_2 reduction activity that is slower than that of WT III (+) over a wide pH range (Figure 4). Furthermore, the activity of both N139D/D132N III (−) and WT III (−) is further enhanced by arachidonic acid (Figure 3), indicating that the transfer of solvent protons into the D pathway limits the rate of O_2 reduction, as discussed above. The same arguments hold true for N139C/D132N III (−), where a functional pK_a of 8.0 is assigned to Cys-139 (Figure 4 and Table 2).

Table 2. Functional pK_a Values of Steady-State Activity of Wild-Type C*c*O and the Asn-139 Mutants

C*c*O form	pK_a of steady-state TN[a]	initial proton acceptor
WT III (+)	8.4 (8.3−8.5)[b]	Asp-132
WT III (−)	7.3 (7.2−7.4)[b]	Asp-132
N139D/D132N III (−)	7.7 (7.5−7.9)[b]	Asp-139
N139C/D132N III (−)	8.0 (7.9−8.1)[b]	Cys-139
N139D III (−)	8.3 (8.2−8.4)[b]	Asp-132 and Asp-139
N139C III (−)	8.2 (8.2−8.3)[b]	Asp-132 and Cys-139

[a] TN is the turnover number as e^- or H^+ consumed per second. [b] The 95% confidence interval.

For N139D III (−) and N139C III (−), the pH dependencies show their activities to be greater than that of normal C*c*O containing subunit III, up to approximately pH 8 (Figure 4). The further stimulation of their O_2 reduction activities by Aa (Figure 3) suggests that the uptake of protons into the D pathway of these C*c*O forms may still be a limiting factor for active site chemistry when Aa is not present, regardless of the fact that their O_2 reduction activity is greater than normal. The pH dependencies of steady-state O_2 reduction activity yield functional pK_a values of 8.2 for N139C III (−) and 8.3 for N139D III (−). With at least two potential proton acceptors in each of these mutants, Asp-132 and Asp-139 in N139D III (−) and Asp-132 and Cys-139 in N139C III (−), the assignment of functional pK_a values to a specific residue is not possible.

Activity of the Subunit III-Depleted Asn-139 Mutants in Lipid Vesicles. Once purified C*c*O is incorporated into phospholipid vesicles (COVs), the consumption of protons from the interior of the COVs raises the pH near the entry region of the D pathway. At the same time, charge separation generates a membrane potential, positive outside. Both conditions slow the uptake of protons from the COV interior via the D and K pathways and lower the O_2 reduction activity of C*c*O. Under controlled conditions, the rate of controlled O_2 reduction by WT III (−) in COVs is slow, ~15 s^{-1} (Table 3). In contrast, the controlled activities of the subunit III-depleted Asp-139 mutants are 2−4-fold higher than that of WT III (−) (Table 3). The high RCR values (Table 3) indicate that the increased activity cannot be ascribed to the leakage of protons through the vesicle membrane. The controlled activities of the subunit III-depleted Asp-139 mutants in COVs increase in the same order as the functional pK_a values obtained for these C*c*O forms in detergent solution (Table 2). This indicates that controlled C*c*O activity by these forms is determined by the activity of the D pathway that, in turn, is controlled by the proton affinities of the initial proton acceptors of the different D pathways. The controlled activity of D132N III (−), which lacks an initial proton acceptor, is even lower than that of WT III (−).

Table 3. Controlled and Uncoupled Activity of COVs Containing Wild-Type C*c*O and the Subunit III-Depleted D Pathway Mutants

COVs	controlled TN[a]	uncoupled TN[b]	RCR[c]
WT III (−)	15.5 ± 3.5	647 ± 107	42
D132N III (−)	10.2 ± 0.9	72 ± 8	7.2
N139D/D132N III (−)	31 ± 5	730 ± 31	23.4
N139C/D132N III (−)	37 ± 5	405 ± 12	10.9
N139D III (−)	54 ± 1	1315 ± 56	25
N139C III (−)	55 ± 3	1092 ± 14	20

[a] The error is the standard deviation; TN is the turnover number as e^- or H^+ consumed per second. [b] In the presence of 3 μM valinomycin and 5 μM CCCP. [c] Controlled TN/uncoupled TN.

The activities of the uncontrolled COVs of the Asp-139 mutants at pH 7.4 (Table 3), are similar to those of the detergent-solubilized forms (Figure 4) indicating that the lipid environment does not alter the characteristics of the mutant C*c*Os.

DISCUSSION

In the Absence of Subunit III and Asp-132, Asp-139 within the D Pathway Becomes the Initial Anionic Proton Acceptor. The normal initial proton acceptor of the D pathway is the carboxyl group of Asp-132 of subunit I.[2] When Asp-132 is altered to asparagine, alanine, or histidine, the uptake of protons by the D pathway is essentially eliminated.[19,20] However, ~20% of the normal rate of steady-state proton uptake is restored by removing subunit III,[20,21,25] apparently because this modification creates space for a short chain of waters to transfer protons from solvent to the first crystal water of the D pathway, immediately below Asn-139 (Figure 1). Because the amide side chain of Asn-139 of normal C*c*O is thought to adopt a conformation that allows the formation of a continuous chain of waters between Asp-132 and Glu-286 during proton transfer,[13] the D pathways for proton transfer in D132N, -A, and -H III (−) likely consist of a chain of waters all the way from solvent to Glu-286. The relatively low efficiency of proton uptake measured for D132A and -H III (−) (~400 s^{-1} [20]) may reflect the lack of a protein group for attracting and transferring solvent protons into the D pathways.

The N139D/D132N mutant also lacks Asp-132 near the surface, but it contains a carboxyl group at position 139, ~6 Å farther into the D pathway, in the interior of subunit I (Figure 1). Similar to D132N, -A, and -H, the removal of subunit III increases the activity of N139D/D132N, but to a much greater extent (Table 1 and Figure 4). In fact, the TN_{max} value of subunit III-depleted N139D/D132N is similar to that of WT III (−), which retains Asp-132 as the initial proton acceptor of the D pathway.

It seems most likely that Asp-139 plays a direct role in proton transfer, rather than moving aside, as proposed for Asn-139 of normal C*c*O,[13] or by causing a structural disruption that allows the removal of subunit III to create a continuous pathway of water from solvent to Glu-286. The reasons for this conclusion, which also apply to Cys-139, are as follows. (1) The modeled and actual structures show that Asp-139 does not distort C*c*O structure and that its carboxyl is positioned to participate in proton transfer (Figure 2; see Results). (The actual structures are, in fact, obtained in the absence of subunit III.) (2) The structures show no obvious pathways bypassing Asp-139. (3) If the functional D pathway of N139D/D132N III (−) did not include Asp-139, i.e., if it consisted solely of water from solvent to Glu-286, its characteristics should be similar to those described above for D132N, -A, and -H III (−). However, the D pathway of N139D/D132N III (−) is at least 4-fold more active than those of D132A, -H, and -N III (−) [steady-state proton uptake of 1800 s^{-1} vs ~400 s^{-1} (Table 1)]. (4) The functional pK_a value of proton uptake facilitated by Asp-139 [7.7 (Table 2)] is consistent with an internal carboxyl group and considerably different from the high pK_a measured for the uptake of protons by D132A and -H III (−) (>10, ref 20). We conclude that Asp-139 participates directly in proton transfer.

A separate consideration is whether Asp-139 of N139D/D132N III (−) protonates and deprotonates during proton transfer. Given its position, the carboxyl of Asp-139 could remain neutral by participating in proton transfer as a Grotthus-type element,[10,45] that is by acquiring a proton from a hydronium "below" its side chain while simultaneously releasing a proton to the water "above". However, the results are more consistent with Asp-139 cycling between its protonated and deprotonated forms. The higher rate of proton uptake supported by Asp-139 is consistent with the presence of an anion at position 139 that attracts and "traps" protons in the same manner as Asp-132. In contrast, uncharged Asp-139 would not be expected to enhance the rate of proton uptake in comparison to those of D132A, -H, and -N III (−). In addition, we observe a further increase in the functional pK_a of proton uptake for N139D III (−) because of electrostatic interaction between Asp-139 and Asp-132 (see below). Obviously, this requires that Asp-139 can ionize. Finally, FTIR analysis suggests that the equivalent of Asp-139 in a mutant of the aa_3-type C*c*O of *P. denitrificans* is deprotonated at pH 7.[41] From the sum of these results, we conclude that Asp-139 can function as an efficient initial proton acceptor in lieu of Asp-132, once subunit III is removed, and that Asp-139 is ionized during proton transfer.

Cysteine as an Initial Proton Acceptor for the D Pathway. Cysteine also provides a side chain capable of protonation and deprotonation in the physiologic pH range. Like Asp-139, the C*c*O structures show that Cys-139 is well positioned to transfer protons (see Results). The high activity of N139C C*c*O[41,42] (Table 1) shows that Cys-139 does not interrupt the flow of protons to the site of O_2 reduction. N139C/D132N was created to place a thiol group at position 139 in the absence of Asp-132 (Figure 2). As for N139D/D132N, the removal of subunit III from N139C/D132N increases its TN_{max} to a value similar to that of WT III (−) (Table 1). Cys-139 of N139C/D132N III (−) participates directly in proton transfer, and it appears to ionize, based on evidence similar to that discussed above for Asp-139. We conclude that Cys-139 functions as the initial proton acceptor for the D pathway of N139C/D132N III (−). To the best of our knowledge, such a role for cysteine is not found in naturally occurring proton transfer pathways. The success of Cys-139 verifies the general utility of anionic groups as initial proton acceptors.

Transfer of Protons from Solvent to Asp- or Cys-139. Neither Asp-139 nor Cys-139 is exposed to bulk solvent, even in the absence of subunit III. The most likely pathway for the transfer of protons from solvent to Asp- or Cys-139 is a short series of waters within the initial region of the normal D pathway channel. Because protons move rapidly along short chains of hydrogen-bound waters,[10] a water file would allow facile transfer of protons from solvent to Asp- or Cys-139. Moreover,

simulations show that carboxylate side chains organize short water files that facilitate the transfer of protons to the carboxyl group.[46] With subunit III removed, a slight outward movement (~1 Å) of the lower region of helix 3 of subunit I would create space sufficient for the formation of a water file leading from bulk solvent to the water molecule normally observed between the carboxyl group of Asp-132 and the amide side chain of Asn-139[14,17,28] (Figure 2). The lower region of helix 3 of subunit I participates directly in the subunit I−III interface, so the removal of subunit III should allow increased flexibility. Indeed, a molecular dynamics simulation of an *R. sphaeroides* C*c*O form lacking subunit III shows the spontaneous movement of three solvent water molecules into the initial region of the D pathway.[13] Experimentally, the replacement of the amide group at position 132 with the smaller methyl group increases the rate of steady-state transfer of protons to Asp-139 3-fold even when subunit III is present [N139D/D132A III (+) vs N139D/D132N III (+) (Table 1)]. This result strongly supports the proposal of a water file to position residue 139 within the existing D pathway channel (Figure 2).

The finding that Asp-139 and Cys-139 facilitate rapid steady-state proton uptake demonstrates that protein groups that accelerate proton uptake need not be in direct contact with bulk solvent. This has relevance for Asp-132 of normal C*c*O. With subunit III present, Asp-132 has limited accessibility to solvent water because it is located at the bottom of a narrow cavity some 6−10 Å deep.[22,24,28] As such, it seems likely that the protonation of anionic Asp-132 involves the transfer of protons from solvent via a short series of hydrogen-bonded waters.

Multiple Proton Acceptors Enhance Steady-State Proton Uptake. The activities of the subunit III-depleted C*c*O forms containing Asp-132 and Asp-139 or Cys139 are actually greater than that of normal C*c*O containing subunit III, and this higher activity is sustained at lower pH (Figure 4). Electrostatic interactions cannot account for the greater activity of these C*c*O forms at low pH because the functional pK_a values show that Asp-132, Asp-139, and Cys-139 will lose charge. It could be argued that the Asp-132 and Asp-139 pair or the Asp-132 and Cys-139 pair provide tandem side chains for the storage of two protons at the entry of the D pathway, which could accelerate proton uptake and hence activity. However, this arrangement is also present in N139D and -C III (+), and therefore, it does not explain the increased steady-state activity of these forms upon the removal of subunit III.

A reasonable explanation for the higher than normal activities of N139D and -C III (−) arises from our findings that Asp-132, Asp-139, and Cys-139 can all function, independently, as efficient proton entry points. Therefore, upon the removal of subunit III from N139D and -C, two pathways deliver protons to Asp- and Cys-139, respectively, for transfer into the rest of the D pathway. In one path, protons flow from solvent to Asp-132 and onto Asp or -Cys-139 via one intervening water.[14] In a second path, protons from solvent bypass Asp-132 and flow to Asp- or Cys-139 by the water file shown in Figure 2. The delivery of solvent protons to Asp- or Cys-139 by two pathways accelerates proton uptake and C*c*O activity. The creation of two proton entry points in N139D III (−) appears similar to the stimulation of the activity of WT III (−) by arachidonic acid.[20,25] In both cases, the addition of a second carboxyl group increases the rate of proton uptake.

The situation is further complicated by the observation that the addition of arachidonic acid (but not its methylated form) to N139D III (−) and N139C III (−) further increases their rates of steady-state proton uptake. The result raises the possibility that three proton acceptors, Asp-132, Asp- or Cys-139, and the carboxyl of Aa, can simultaneously deliver protons into the D pathway in the absence of subunit III.

The Normal K Pathway Exceeds the Proton Uptake Requirements of Wild-Type C*c*O. During each catalytic cycle, the D pathway takes up two (possibly three) of the four substrate protons required for O_2 reduction and the K pathway takes up the remaining one or two.[2] While the D and K pathways converge near the active site, their proton entry points on the inner surface of C*c*O are far apart (Figure 1). There is no experimental or structural evidence that the alteration of residues in the initial regions of either pathway affects the other. The greater than normal O_2 reduction rates of N139D and -C III (−) require greater than normal rates of proton uptake by both their D and K pathways. Because the K pathways in these mutant C*c*Os are not modified, it follows that the K pathway of normal C*c*O imposes no limitation on the rate of O_2 reduction. The same argument holds true for C*c*O in lipid vesicles, where the uptake of protons into both the K and D pathways is inhibited by a transmembrane voltage gradient and high pH in the interior of the vesicles. Thus, across the range of possible pH values and voltage gradient conditions encountered by wild-type C*c*O, it appears that the proton uptake capability of the K pathway exceeds the requirements for normal activity. In contrast, the uptake of protons into the D pathway may be a rate-limiting process.

The Initial Proton Acceptor of the D Pathway Controls C*c*O Activity. The ability of C*c*O to function at physiological pH is considerably compromised by the removal of subunit III. For example, the activity of WT III (−) is only 20% of that of normal C*c*O at pH 8, while at pH 6.5, the activities of the two forms are similar (Figure 4). The primary source of this difference appears to be a decline in the functional pK_a of Asp-132 because of the greater exposure of Asp-132 to solvent in the absence of subunit III.[20]

With this study, we demonstrate that even in the absence of subunit III the pH range of C*c*O activity can be extended to match that of normal C*c*O by manipulating the environment and the identity of the initial proton acceptor of the D pathway. In N139D/D132N III (−), the initial aspartate is moved to a position inside the D pathway that eliminates its exposure to bulk solvent. This change increases the proton affinity of the pathway approximately 3-fold in comparison to that of WT III (−) (Table 2 and Figure 4) where the carboxyl of Asp-132 is partially exposed to solvent. Hence, shielding the initial proton acceptor of the D pathway from bulk solvent can extend the pH range of the enzyme without lowering its overall activity. This strategy appears to be used by normal C*c*O, in which a ring of residues from subunits I and III shields the carboxyl of Asp-132 from bulk solvent.

The pH dependence of C*c*O activity is extended another 0.3 pH unit with the introduction of Cys-139 as the initial proton acceptor, presumably because of the greater inherent proton affinity of the thiol group for protons. In fact, a greater pH shift might be expected with a thiol as the proton acceptor because continuum electrostatics calculations predict a higher pK_a for Cys-139 in the resting enzyme.[41] However, the functional pK_a value measured here reflects the situation during continuous turnover where the apparent proton affinity of the initial acceptor is also influenced by the rate at which protons are withdrawn to the active site. This may prevent large increases in the functional pK_a value.

When Asp-132 is present along with either Asp-139 or Cys-139, the pH profiles become similar to that of normal CcO containing subunit III (Table 2 and Figure 4). Electrostatic interaction between the deprotonated forms of Asp-132 and Asp-139 or Cys-139 should increase the proton affinities of these groups, providing the likely explanation for the retention of activity at higher pH.

CONCLUSIONS

The examination of CcO mutants with an aspartate or a cysteine inside the D pathway for proton transfer reveals new information about the process of proton uptake.

(1) In the absence of subunit III, protons from solvent water gain access to Asp-139 in the interior of the D pathway such that Asp-139 becomes as efficient an initial proton acceptor for steady-state activity as surface-exposed Asp-132. Solvent protons are likely transferred to Asp-139 through a short file of waters. In normal CcO, subunit III provides a barrier for the influx of solvent water and the formation of an alternative proton pathway that can bypass Asp-132.

(2) A protein group that accelerates the uptake of protons from solvent need not be in direct contact with bulk solvent. In these experiments, both a carboxylate group and a thiolate group accelerate the uptake of protons from an internal position. It is likely that their anionic nature is the key to their function in that the negative charge will both attract protons and facilitate the formation of the hydrogen-bound water file from solvent.[46]

(3) Proton uptake can be driven to higher rates by placing multiple anionic proton acceptors in the initial region of the D pathway.

(4) The uptake of protons into the K pathway is not rate-limiting for CcO activity at physiologic pH.

(5) The pH range of CcO activity can be manipulated by altering the proton affinity of the initial proton acceptor for the D pathway. The experiments here show that this can be achieved by restricting the access of the acceptor to bulk solvent, by changing the chemical identity of the acceptor, and by the electrostatic interaction of the acceptor with another group. Normal CcO appears to use the first of these strategies to maintain a high functional pK_a for Asp-132.

AUTHOR INFORMATION

Corresponding Author

*Phone: (601) 984-1861. Fax: (601) 984-1501. E-mail: jhosler@umc.edu.

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ABBREVIATIONS

Aa, arachidonic acid; CcO, cytochrome *c* oxidase; COV, cytochrome *c* oxidase vesicle; CCCP, carbonyl cyanide 3-chlorophenylhydrazone; Ni-NTA, Ni^{2+}-nitriloacetic acid agarose; RCR, respiratory control ratio; TMPD, *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine; TN, turnover number as e^- or H^+ consumed per second; WT III (+), wild-type CcO; WT III (−), wild-type CcO lacking subunit III.

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