Fatty acids stimulate activity and restore respiratory control in a proton channel mutant of cytochrome *c* oxidase

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Abstract (1) Removal of a carboxyl at residue 132 of subunit I of *Rhodobacter sphaeroides* cytochrome *c* oxidase significantly inhibits electron transfer and makes proton pumping undetectable [Fetter et al. (1995) Proc. Natl. Acad. Sci. USA 92, 1604–1608]. When reconstituted into phospholipid vesicles (COV), wild-type oxidase shows respiratory control that is partially released by either valinomycin or nigericin and fully released by the two ionophores combined. Under the same conditions, the D132A mutant COV show anomalous ionophore responses, including inhibition by valinomycin or by CCCP. Nevertheless, oxidase activity results in development of a similar membrane potential in COV containing either wild-type or D132A oxidase, and the ionophore responses of the membrane potential are similar for both enzymes. (2) Long chain fatty acids such as arachidonic acid, but not fatty alcohols, stimulate steady-state electron transfer activity 3–7-fold, with either detergent-solubilized (purified) D132A oxidase or the reconstituted form. The effect is specific for this mutant and is not seen with wild-type or other mutants of similar overall activity. Arachidonate-treated D132A COV show normal ionophore responses to valinomycin and nigericin and full release of respiration in presence of both ionophores or of CCCP. Thus, arachidonate and some other fatty acids abolish the ionophore anomalies seen when the D132A enzyme is reconstituted in their absence. (3) Fatty acid addition does not restore proton pumping, likely because fatty acids also induce proton permeability and some degree of uncoupling. A model of D132A function is presented and possible roles for the fatty acids in 'chemical rescue' of the mutant are discussed.

Key words: Respiratory control; Cytochrome *c* oxidase; Proton channel mutant; Fatty acid

1. Introduction

The structures of cytochrome *c* oxidases both from a eukaryote, beef heart [1,2], and from a prokaryote, *Paracoccus* [3], have been determined to 2.8 Å resolution by X-ray crystal analysis. In addition to defining the positions of the electron acceptors and donors, the resulting structures show possible channels for the movement of both scalar protons to the oxygen-reactive binuclear center and pumped protons across the membrane [3]. At the entry of the proposed 'pumped proton' channel there is an evolutionarily conserved aspartate residue, numbered as D132 in *Rhodobacter sphaeroides*, mutation of which to remove the carboxyl moiety by substitution with either an alanine (D132A) or an asparagine (D132N) strongly diminishes oxygen reduction activity and abolishes proton pumping. D132 is a substantial distance from the oxygen-reactive site and mutation of the residue does not perturb the spectral properties of the metal centers [4]. Similar effects are seen when the corresponding residue in *Escherichia coli* cytochrome bo_3 is mutated [5,6]).

Another effect of the D132N/A mutations is that the reconstituted enzymes show anomalous responses to uncouplers and ionophores [4]. Valinomycin and CCCP, both singly and in combination, inhibit instead of stimulating turnover in reconstituted enzyme. The ionophores do not inhibit the purified enzyme directly and the oxygen reduction activity shows a similar decrease with increasing pH as wild-type, indicating that the inhibition is likely caused by changes in membrane potential ($\Delta\Psi$) and/or pH gradient (ΔpH) [4]. An understanding of these responses might clarify the pumping process itself. The present paper will show that (i) a normal membrane potential is formed by COV containing mutant enzyme and (ii) the defect of the D132A/N mutations can partially be overcome by addition of free fatty acids. The fatty acids also rectify the anomolous responses to uncouplers and ionophores, but repair of proton pumping has not been demonstrated.

2. Materials and methods

2.1. Methods

COV were reconstituted using wild-type and D132A-mutated *Rhodobacter sphaeroides* cytochrome *c* oxidases as described previously [4,7]. Mutant enzymes were also obtained as described previously [4] and both mutant and wild-type enzymes were isolated by the methods of Hosler et al. [7].

Spectrophotometry was performed using either a Perkin-Elmer Lambda 6 or a Beckman DU-7HS spectrophotometer. Membrane potentials were determined using the carbocyanine dye method. Fluorescence intensity was measured using a Perkin Elmer LS-50 fluorescence spectrophotometer with an excitation wavelength of 622 nm, and emission wavelength of 688 nm (10 nm slits), using 0.45 μM $diSC_{(3)}$-5 as the membrane potential probe, a technique described previously for reconstituted beef heart enzyme [8]. Reconstituted vesicles, containing either wild-type enzyme or D132A mutant, were added to 3 ml 10 mM K^+ HEPES buffer, pH 7.4, with 20 mM K_2SO_4 and 50 mM sucrose at 30°C. Cytochrome *c* was added at a concentration of 1.1 μM. As detailed in the legend to Fig. 1, nigericin was used to abolish ΔpH and valinomycin to release the membrane potential, returning fluorescence to its control level.

Activities and respiratory controls were measured polarographically with appropriate closed thermostatted chambers containing Clark-type oxygen electrodes. Steady-state turnover was measured with horse heart cytochrome *c*, ascorbate and TMPD as substrates as described by Hosler et al. [7]. Proton pumping in wild-type, mutant, and beef heart-containing COV was estimated according to the method of Fetter et al. [4].

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2.2. Materials

Fatty acids, fatty alcohols and arachidonate analogues were purchased from Sigma Chemical Co. Arachidonic acid was also purchased from Cayman Chemical. Samples from the two companies gave similar results. All were made up as 250 mM stock solutions in ethanol and stored at −20°C. Horse heart cytochrome *c*, purified as in [9], valinomycin, nigericin and CCCP were from Sigma. Asolectin used in vesicle reconstitution (as 'type VI phosphatidylcholine') was also a Sigma product.

3. Results

3.1. Turnover and respiratory control in COV reconstituted with the D132A mutant, wild-type, and beef heart oxidases

As described previously [4], the D132A mutant enzyme reconstituted into COV has a relatively low turnover that is slowed even more by valinomycin or by CCCP. Subsequent complete abolition of pH gradients by nigericin fails to restore the original low level of activity (see Fig. 3). This suggested the possibility that abnormal membrane potentials might be generated by the mutant enzyme, and experiments were carried out to examine membrane potential generation by the mutant enzyme.

3.2. Determination of membrane potentials in reconstituted D132A and reconstituted wild-type COV

As shown in Fig. 1, the time courses and extent of membrane potential-dependent quenching of $diSC_{(3)}$-5 fluorescence are similar for reconstituted COV containing wild-type enzyme as for those containing the D132A mutant. Beef heart cytochrome *c* oxidase also gave similar responses (not shown). Addition of oxidized cytochrome *c* decreases measured fluorescence by optical quenching. Ascorbate and TMPD addition initiates steady-state turnover and a build-up of membrane potential (decreased fluorescence). Nigericin abolishes ΔpH, with a concomitant small further increase in membrane potential. Valinomycin, which inhibits D132A activity, abolishes the membrane potential and the fluorescence returns to the value seen before addition of reductant. Thus a membrane

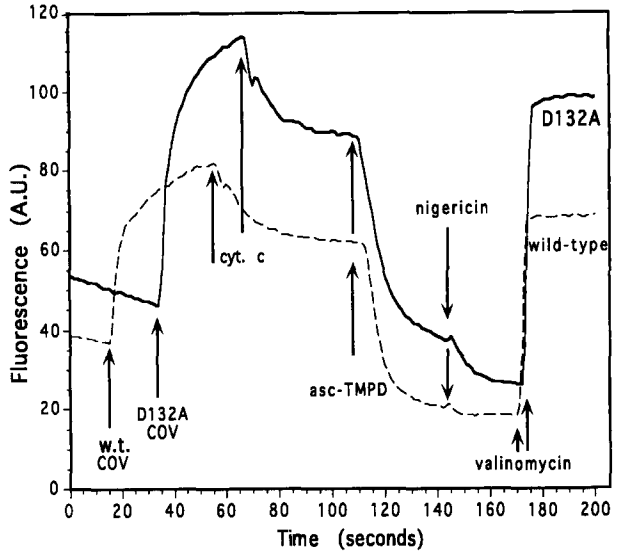

Fig. 1. Reconstituted $Asp^{132}Ala$ mutant enzyme shows a normal membrane potential. 1.1 μM oxidized cytochrome *c* was added as the substrate, causing an initial decrease in fluorescence unrelated to turnover. To generate steady-state turnover, 2.8 mM ascorbate and 1.1 mM TMPD were added. This leads to a decrease in the fluorescence of the probe, indicating the formation of a membrane potential. Nigericin was added to 0.5 μM to release the pH gradient. Valinomycin was then added to 0.67 μM which releases the membrane potential and causes an increase in fluorescence.

Table 1
Fatty acid stimulation of D132A mutant activity increases with longer alkyl chains, while fatty alcohols have no effect

Fatty acid or fatty alcohol added	% activity		
	Acid or alcohol concentration		
	5 μM	50 μM	250 μM
To D132A mutant oxidase			
Acetate	90	80	70
Propionate	80	70	70
Propanol	90	90	90
Octanoate	90	100	120
Octanol	100	80	80
Laurate	120	120	150
Lauryl alcohol	110	100	70
Palmitate	140	210	150
Hexadecanol	120	90	80
Oleate ($18:1^{\Delta 9}$)	120	160	140
Linoleate ($18:2^{\Delta 9,12}$)	190	260	310
Linolenate ($18:3^{\Delta 9,12,15}$)	130	170	150
Arachidonate (20:Δ5,8,11,14)	180	300	410
Arachidonoyl alcohol	100	90	100
Docosahexanoate (22:Δ4,7,10,13,16,19)	260	380	330
Docosahexanol	90	90	130
To wild-type oxidase			
Arachidonate	100	90	60
Palmitate	110	110	100
Hexadecanol	100	100	100
Oleate	100	100	70

Activity assayed polarographically as described in Section 2 with 13 nM $Asp^{132}Ala$ mutant or wild-type enzyme in a medium containing 30 μM cytochrome *c*, 1.1 mM TMPD, and 2.8 mM ascorbate in 50 mM potassium phosphate, pH 6.5 at 25°C. All fatty acids or alcohols were dissolved in ethanol. Consecutive additions were made to a final concentration of 5, 50, and 250 μM to the enzyme during turnover. The numbers in the table are in percentages where 100% represents a turnover of 1200 s^{-1} for the wild-type enzyme and 50 s^{-1} for the mutant enzyme.

potential equivalent to that produced by wild-type and beef heart enzyme is also produced by the reconstituted D132A mutant enzyme. The anomalous ionophore responses therefore do not reflect anomalies of membrane potential formation by the mutant enzyme.

3.3. Stimulation of Asp^{132} mutants by free fatty acids

Fig. 2 shows the polarographically measured activities of purified, detergent-solubilized D132A mutant enzyme with cytochrome *c* and ascorbate plus TMPD, and the effects of fatty acid addition to such systems. Arachidonic acid stimulated the activity of the mutant enzyme significantly, but not that of wild-type. With increasing fatty acid concentration, a maximal turnover of nearly 200 s^{-1} was attained, which compares with 1200 s^{-1} obtained for wild-type enzyme under these conditions in the presence or absence of fatty acids. The induced cytochrome *c* oxidase activity is blocked by cyanide (trace A) or by azide (trace B) indicating that the fatty acid is affecting the oxidase and not inducing a secondary reaction. Controls without oxidase showed no significant changes in the background rate of autoxidation induced by fatty acids. Another oxidase mutant at the same position, D132N, showed a similar stimulation by fatty acids (not shown).

Is the fatty acid carboxyl group or the hydrophobic tail more important for stimulation? Table 1 lists results that show that no stimulation occurred with propionate or acetate, indicating that more than an acidic group was required. Medium chain fatty acids (octanoic and lauric) showed some stimulation, but long chain unsaturated fatty acids (C16–22) had the greatest effects, while alcohols of all sizes were ineffective. Stimulation requires both a sizeable hydrophobic tail and an acidic head group. Flurbiprofin and indomethacin, analogues of arachidonic acid, stimulated Asp^{132}Ala activity 40 and 100%, respectively, emphazing the requirement of a large hydrophobic group and a carboxyl moiety (data not shown).

Similar experiments with arachidonate were performed with various wild-type, beef heart, and mutant oxidases (Table 2). Detergent-solubilized oxidases are commonly stimulated by phospholipid mixtures and the effects of 'asolectin' phospholipids were therefore also tested. No significant stimulation by arachidonate occurs with wild-type (cf. Table 1), beef heart or most mutant enzymes. T359A was slightly stimulated but a similar effect was seen with the alcohol. F391Q showed only activation by the fatty alcohol and asolectin, while M262L showed slight stimulation by both fatty acid and alcohol, but no mutant showed the stimulation seen with D132N. There was no correlation between phospholipid and fatty acid effects, indicating a unique fatty acid role in activating D132 mutants.

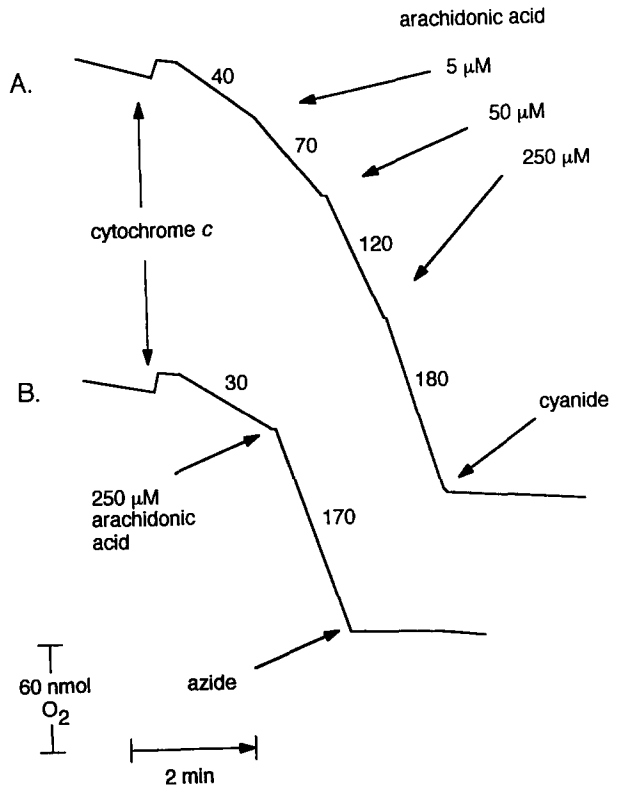

Fig. 2. Arachidonic acid stimulation of the Asp^{132}Ala mutant enzyme. Enzyme activity was assayed as indicated in Section 2 and Table 1. (A) Stimulation of enzyme activity by multiple additions of arachidonic acid and inhibition by 2 mM cyanide. (B) Effect of 250 μM arachidonic acid with subsequent inhibition by 9 mM azide. Numbers beside traces are activities in electron/aa_3 per s.

3.4. Restoration by fatty acids of respiratory control in D132A COV, but no demonstrable restoration of proton pumping

If fatty acids can partially restore enzyme turnover (Fig. 2) what are their effects upon the anomalous ionophore responses of COV [4]? The behaviour of COV towards fatty acids either added to the enzyme being reconstituted or to the reaction mixture before addition of valinomycin and nigericin is shown in Fig. 3. The inhibitory effects of valinomycin and nigericin are prevented by fatty acid. Although this effect may be complicated by a dual fatty acid role of uncou-

Table 2
Stimulation of activity by arachidonic acid is unique to the D132A mutant enzyme

Oxidase	Initial activity	% stimulation of activity with addition of		
	(s^{-1})	Arachidonic acid	Arachidonyl alcohol	Asolectin
Wild-type	1400	0	0	20
Beef heart	450	0	0	10
Asp^{132}Ala	50	300	0	0
Thr^{359}Ala	350	20	30	30
Asp^{412}Asn	370	0	0	40
Phe^{391}Gln	30	0	70	70
Lys^{362}Met	20	0	0	0
Met^{262}Leu(II)	130	40	20	0
His^{259}Asn(II)	10	0	0	0

Mutant enzymes were tested by the protocol in Table 1 for stimulation by arachidonic acid or arachidonoyl alcohol. They were also tested with 2 mg of asolectin phospholipid (cholate solubilized). Two mutations are in subunit II (II, noted in parentheses), while the others are all in subunit I.

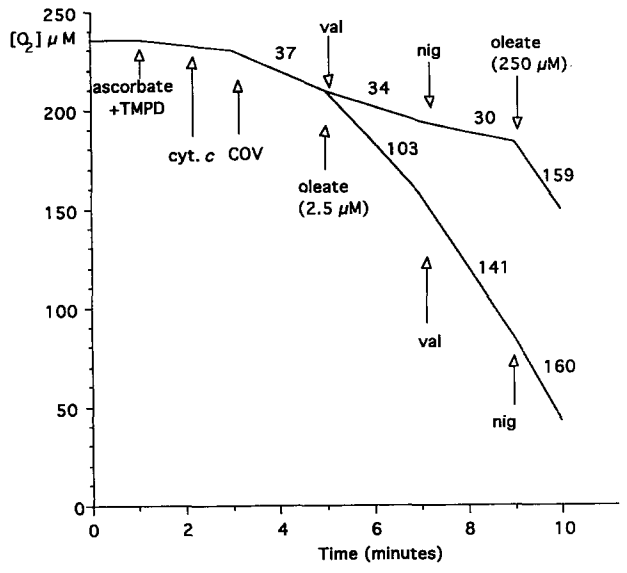

Fig. 3. Effects of oleic acid upon respiratory control and activity of cytochrome *c* oxidase proteoliposomes. Polarographic experiments in sucrose-KCl-10 mM Hepes buffer pH 7.4, at 30°C, in 4 ml total volume. Additions of 6 mM Na ascorbate, 0.6 mM TMPD, 6 μM cytochrome *c* (horse heart) and 20 μl COV (approx. 3.5 nM cytochrome aa_3 final) as indicated. Valinomycin (10 nM) and nigericin (1 μM) were subsequently added after (lower trace) or before (upper trace) addition of fatty acid. The lower trace shows the effect of adding 2.5 μM oleate to controlled state COV. Under these conditions a very small amount of added fatty acid was sufficient to induce increased turnover and conventional ionophore responses. The upper trace shows the effect of adding a higher amount of oleate (250 μM) to uncontrolled COV. Numbers on the traces indicate turnover in electrons/s per aa_3.

pling and stimulation, it is clear that the COV now behave more like those made with wild-type enzyme.

Experiments were then carried out to determine whether proton pumping by mutant COV might also be restored by fatty acids. Arachidonate in the concentration range required for stimulation of vesicles, 50–250 μM, caused some loss of respiratory control in wild-type COV and lowering of H^+/e^- ratios. Under these conditions, no restoration of proton pumping was detected with D132A COV.

4. Discussion

4.1. Effects of Ionophores and membrane potentials in D132A COV

Why are D132N and D132A mutant COV inhibited by valinomycin and CCCP ('reverse respiratory control')? This inhibition occurs despite the abolition of membrane potential and ΔpH by the ionophores and, unlike the case with inhibition by low levels of valinomycin [10], cannot be accounted for by an increase in ΔpH. In steady state, cytochrome oxidase takes up protons from the interior to generate membrane potential and ΔpH. Reconstituted D132A cytochrome *c* oxidase generates a normal membrane potential under steady-state conditions, indicating that the enzyme is inserted correctly in the membrane and at least the scalar protons are taken up normally from the vesicle interior via a 'substrate proton' pathway. However, if turnover is limited by blockage of proton uptake via a separate 'pumping' pathway involving D132, it is possible that this block could be partially overcome through reversal of the normal exit route. The existence of a gradient would make it easier for protons to be pulled in from the outside of the vesicles (Fig. 4C), while removal of membrane potential would decrease the rate of H^+ uptake and slow enzyme turnover, as is observed (Fig. 4B).

4.2. Fatty acid stimulation of activity and restoration of respiratory control

Fatty acids restore significant electron transfer activity in the D132N/A mutant enzymes. No stimulation is observed for other mutants with equally low activity, or wild-type enzyme, although a small stimulation can sometimes be detected with normal or BSA-treated samples of beef heart enzyme [11–13]. The D132N/A mutants are not markedly stimulated by phospholipids; therefore fatty acid stimulation must be of a different nature.

Mutation of D132 to remove the carboxyl moiety may create a defect that can be remedied by insertion of the fatty acid in the enzyme so that its carboxyl is in an appropriate position to replace the missing functional group. A less likely alternative is that the mutation has induced loss of an intrinsic fatty acid that has a functional role (cf. [13]). If the fatty acid is complementing the loss of the D132 carboxyl, or interacting at another site to improve proton access, this resembles the chemical rescue phenomenon observe with carboxylate mutants of other proton transferring membrane proteins. In mutated bacteriorhodopsin [14,15] and bacterial photosynthetic reaction center [16] activity can be restored by weak acid anions such as azide. These water soluble acids are required at much higher concentrations (approaching molar), as opposed to micromolar for the fatty acid effects. The most effective of these soluble weak acids cannot be used with cytochrome oxidase because they are inhibitors.

The model in Fig. 4A illustrates how protons could be taken up and released in the wild-type oxidase to accomplish oxygen reduction and proton pumping in a manner consistent with mutagenesis results and theoretical considerations suggesting separate pumping and substrate proton pathways [3,17]. The mutation of D132 is depicted in Fig. 4B in the absence of a membrane potential. Uptake of protons from the matrix through the normal proton channel is blocked, but some activity is permitted due to proton back leak through the exit path. Proton backleak would be required to provide protons at the active site for charge compensation to enable turnover. Establishing a membrane potential (Fig. 4C) would increase the rate of influx of protons from outside the vesicles through the exit route, accounting for the higher activity of the mutant in the absence of valinomycin or uncoupler. In Fig. 4D, fatty acids act to repair the loss of a carboxyl in the pumping pathway, facilitating proton uptake through the correct route and instigating higher activity with normal respiratory control. If this is the case, restoration of proton pumping would also be expected, along with electron transfer activity and the ionophore response. The latter two effects are seen, but so far we have been unable to show restoration of pumping. This is technically difficult because of the uncoupling effect fatty acids. It is also possible that the lack of pumping is due to the carboxyl of the fatty acid having a different pK_a than the aspartate it is replacing, resulting in facilitation of both influx and efflux of protons in the normal influx route.

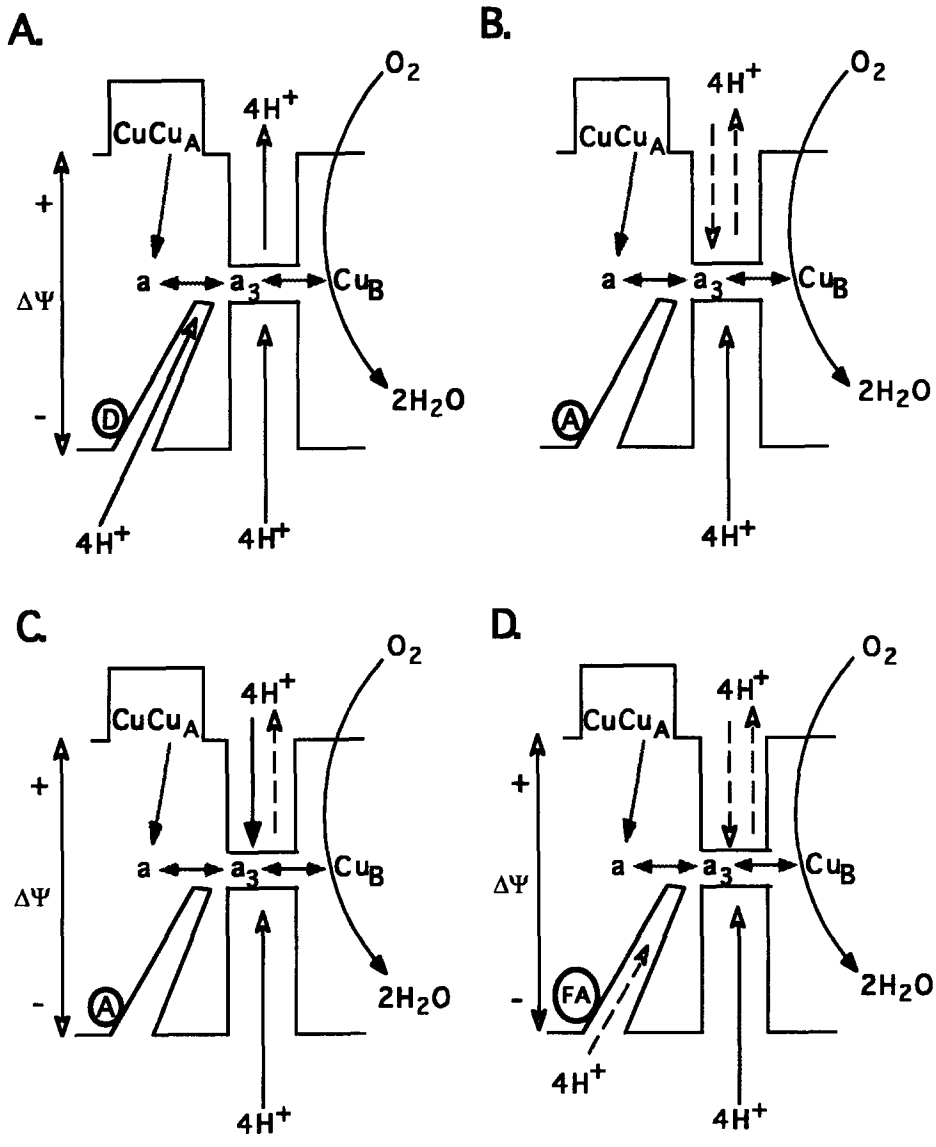

Fig. 4. Model of proton pathways in wild-type and D132 mutant cytochrome oxidases: effect of membrane potential and of fatty acid addition. (A) Wild-type with an aspartate residue at position 132. (B) D132A mutant with no membrane potential but some activity due to reversibility of the normal exit pathway. (C) D132A mutant with a negative internal membrane potential giving higher activity due to facillitation of the entry of protons through the exit path (reverse respiratory control). (D) D132A mutant with fatty acid replacing the lost carboxyl at the proton entry site and facillitating proton entry through the normal uptake pathway (higher activity with normal respiratory control).

An alternative model for the fatty acid effect is that the fatty acid binds in or near the proton exit channel and further facilitates reverse proton uptake, lessening the need for the membrane potential but still producing an uncoupled activity. This second model does not explain the reestablishment of respiratory control or the lack of effect of fatty acids on wild-type or other mutants.

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