Aspartate-132 in Cytochrome *c* Oxidase from *Rhodobacter sphaeroides* Is Involved in a Two-Step Proton Transfer during Oxo-Ferryl Formation†

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ABSTRACT: The aspartate-132 in subunit I (D(I-132)) of cytochrome *c* oxidase from *Rhodobacter sphaeroides* is located on the cytoplasmic surface of the protein at the entry point of a proton-transfer pathway used for both substrate and pumped protons (D-pathway). Replacement of D(I-132) by its nonprotonatable analogue asparagine (DN(I-132)) has been shown to result in a reduced overall activity of the enzyme and impaired proton pumping. The results from this study show that during oxidation of the fully reduced enzyme the reaction was inhibited after formation of the oxo-ferryl (F) intermediate ($\tau \cong 120\ \mu$s). In contrast to the wild-type enzyme, in the mutant enzyme formation of this intermediate was *not* associated with proton uptake from solution, which is the reason the DN(I-132) enzyme does not pump protons. The proton needed to form F was presumably taken from a protonatable group in the D-pathway (e.g., E(I-286)), which indicates that in the wild-type enzyme the proton transfer during F formation takes place in two steps: proton transfer from the group in the pathway is followed by faster reprotonation from the bulk solution, through D(I-132). Unlike the wild-type enzyme, in which F formation is coupled to internal electron transfer from Cu_A to heme *a*, in the DN(I-132) enzyme this electron transfer was uncoupled from formation of the F intermediate, which presumably is due to the impaired charge-compensating proton uptake from solution. In the presence of arachidonic acid which has been shown to stimulate the turnover activity of the DN(I-132) enzyme (Fetter et al. (1996) *FEBS Lett. 393,* 155), proton uptake with a time constant of ∼2 ms was observed. However, no proton uptake associated with formation of F ($\tau \cong 120\ \mu$s) was observed, which indicates that arachidonic acid can replace the role of D(I-132), but it cannot transfer protons as fast as the Asp. The results from this study show that D(I-132) is crucial for efficient transfer of protons into the enzyme and that in the DN(I-132) mutant enzyme there is a "kinetic barrier" for proton transfer into the D-pathway.

Cytochrome *c* oxidase from *Rhodobacter sphaeroides* (cytochrome aa_3) catalyzes the reduction of oxygen by reduced cytochrome *c*, forming water and oxidized cytochrome *c*. The enzyme is an integral membrane protein which consists of three membrane-spanning subunits holding four redox-active metal sites: copper A (Cu_A)[1] bound to subunit II and heme *a*, heme a_3, and copper B (Cu_B) all bound to subunit I (for recent reviews and structural information, see refs *1*−*6*). During the catalytic cycle, electrons from cytochrome *c* are transferred consecutively to Cu_A, heme *a*, and the binuclear center consisting of heme a_3 and Cu_B, where oxygen binds and is reduced to water. The electrons from cytochrome *c* and the protons used for reducing oxygen (substrate protons) are delivered from opposite sides of the membrane resulting in a net transfer of four positive charges per O_2 reduced from the inside of the bacterial cell to the outside. In addition, during catalysis, one proton per electron is pumped through the enzyme from the inside to the outside of the membrane. As a result of these processes the free energy of the reaction between reduced cytochrome *c* and O_2 is partly conserved as a transmembrane electrochemical proton gradient. The detailed mechanism of energy conservation in this enzyme is the subject of intense investigations.

The binuclear center is situated within the membrane-spanning part of the protein. Consequently, the enzyme must provide pathways for transfer of the substrate protons to the binuclear center and for the pumped protons through the entire protein. Two proton-conducting input pathways have been proposed (*6*−*9*). One of them, called the D-pathway,

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[1] Abbreviations: R, fully reduced enzyme; A, ferrous-oxy intermediate; P, peroxy intermediate; F, oxo-ferryl intermediate ($Fe_{a_3}^{4+}{=}O^{2-}$); O, fully oxidized enzyme; Cu_A, copper A; Cu_B, copper B; WT, wild-type; substrate proton, a proton used for reduction of O_2 to water (cf. pumped proton); τ, time constant (in $\exp(-t/\tau)$); amino acid and mutant enzyme nomenclature, e.g., D(I-132) denotes aspartate-132 of subunit I, DN(I-132) denotes a replacement of aspartate-132 of subunit I by asparagine. If not otherwise indicated, the amino acid numbering is based on the *R. sphaeroides* cytochrome *c* oxidase sequence.

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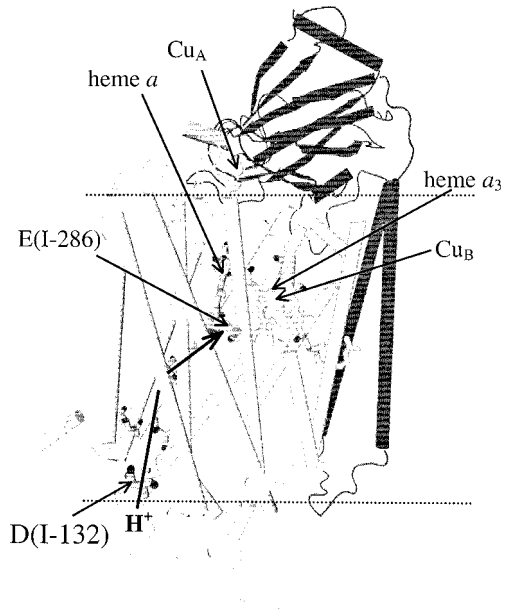

FIGURE 1: Schematic structure of subunits I and II of bovine cytochrome *c* oxidase (file 1occ.PDB from the Brookhaven Protein Data Bank (*4*)). The *R. sphaeroides* cytochrome aa_3 amino acid residue numbering is used. The binuclear center heme a_3/Cu$_B$, heme *a*, Cu$_A$, and residues D(I-132) and E(I-286) are shown. Also residues S(I-201), N(I-121), and N(I-139) in the D-pathway are shown, but not labeled. The dotted lines indicate the approximate location of the membrane surfaces. Note that the thickness of the helices (thin rods) is not to scale. The illustration was made using the Visual Molecular Dynamic Software (Theoretical Biophysics Group, Beckman Institute for Advanced Science and Technology, University of Illinois at Urbana−Champaign).

includes Asp-132 (D(I-132)) and Glu-286 (E(I-286)) of subunit I (Figure 1): the residue D(I-132) being situated at the "entrance " of the D-pathway close to the cytosolic surface of the protein and E(I-286) closer to the binuclear center, where it plays a central role in transfer of both substrate and pumped protons.

During reaction of the fully reduced (R) wild type *R. sphaeroides* enzyme with oxygen, four kinetic phases are resolved after flash-induced dissociation of CO in the presence of O_2 (*10*). Binding of oxygen to the reduced heme a_3 with a time constant of ~10 μs (at 1 mM O_2) forms the so-called ferrous-oxy intermediate (or A), followed by oxidation of hemes *a* and a_3 and formation of the so-called peroxy (P) intermediate with a time constant of ~50 μs. The oxo-ferryl (F) intermediate is then formed at the binuclear center with a time constant of 120 μs, accompanied by electron transfer from Cu$_A$ to heme *a* and proton uptake from the bulk solution. We have previously shown that the proton uptake associated with F formation controls the electron transfer from Cu$_A$ to heme *a* (*11, 12*). Finally, the oxidized enzyme (O) is formed with a time constant of 1.2 ms associated with proton uptake from the bulk solution. Previous results have shown that replacement of E(I-286) by its nonprotonatable analogue Gln results in a dramatic decrease of the enzyme activity (*13*), inhibition of the catalytic reaction after formation of the P intermediate (*12, 14, 15*), and impaired proton uptake during oxidation of the reduced enzyme (*12, 16*).

In this work we have investigated the effect of the replacement of D(I-132) by its nonprotonatable analogue Asn (DN(I-132)) on internal electron and proton transfer in the absence of oxygen and during single-turnover reaction of the fully reduced enzyme with oxygen. The turnover rate of the DN(I-132) mutant enzyme is 70 s^{-1} (electrons/s), which is ~5% of that of the wild-type enzyme, and the mutant enzyme does not pump protons (*8*). In the DN(I-135) mutant enzyme of cytochrome bo_3 from *Escherichia coli* (corresponds to D(I-132) in *R. sphaeroides*) the electron-transfer activity is reduced to 50% of that in the wild-type enzyme while proton pumping is abolished (*17*).

The results from this study show that in the reaction of the fully reduced enzyme with oxygen, the DN(I-132) mutant enzyme remained in the partly oxidized F state for >10 ms. In addition, proton uptake from solution was impaired in the mutant enzyme. The proton needed to form the F intermediate at the binuclear center was most likely extracted from the D-pathway, but it was not followed (counterbalanced) by proton uptake from solution. As a consequence, the electron transfer from Cu$_A$ to heme *a* was impaired because of the remaining, uncompensated negative charge in the D-pathway (see refs *11, 12*).

In agreement with previous studies (*18*), addition of arachidonic acid resulted in a 4−5-fold stimulation of the DN(I-132) enzyme activity. During single-turnover O_2-reduction by the DN(I-132) enzyme in the presence of arachidonic acid, the amplitude of the slowest kinetic reaction phase, associated with transfer of the fourth electron to the binuclear center, increased significantly (τ = 2 ms). In addition, we observed a proton uptake with a similar time constant and a stoichiometry of 1.5 H^+ per cytochrome aa_3 in the enzyme fraction in which the 2-ms electron-transfer phase was observed. However, no effect on proton uptake during the P → F transition (τ = 120 μs) was observed. This indicates that the fatty acid interacts with the enzyme in such a way that the $^{\bullet}COO^-$ group can replace the role of D(I-132), but it cannot shuttle protons as fast as the aspartate.

MATERIALS AND METHODS

Growth of Bacteria and Enzyme Purification. Construction of the DN(I-132) mutant enzyme has been described earlier (*8*). A six-histidine affinity tag fused to the C-terminus of subunit I was added to the mutant enzyme as described (*19*). Bacteria were grown aerobically in the dark in a 20-L fermentor. The cells were harvested and the enzyme was purified as described (*19*). After elution of the enzyme from the Ni^{2+} column with 100 mM imidazole, the buffer was immediately exchanged to 100 mM Hepes-KOH, pH 7.4, 0.1% dodecyl β-D-maltoside in which solution the purified enzyme was stored in liquid nitrogen. The cytochrome aa_3 concentration was calculated from the dithionite-reduced minus ferricyanide-oxidized difference spectrum using an absorption coefficient $\epsilon^{604} - \epsilon^{630}$ = 24 mM^{-1} cm^{-1} (*20*).

Catalytic Activity Measurement. The cytochrome *c* oxidase catalytic activity was measured in 50 mM potassium phosphate buffer, pH 6.5, 0.05% dodecyl β-D-maltoside using reduced horse-heart cytochrome *c* as an electron donor. Cytochrome *c* (type VI; Sigma) was reduced by hydrogen gas using platinum black (Aldrich, WI) as a catalyst (*21*). The turnover number was calculated from the initial oxidation rate of cytochrome *c* using ϵ^{550}(red-ox) = 21.1 mM^{-1} cm^{-1} (*22*) for cytochrome *c*. Arachidonic (5,8,11,14-eicosatetraenoic) acid from porcine liver (Sigma) or arachidonyl

alcohol (Sigma) was added as solutions in 99% ethanol to a final concentration of 0.25−2 mM (see Results and figure legends).

Electron and Proton Transfer during Reaction of the Fully Reduced Enzyme with O_2. The measurements were performed as described (*10, 12*). Briefly, the enzyme stock solution was diluted in a solution of 100 mM Hepes-KOH (pH 7.4), 0.1% dodecyl β-D-maltoside, 5 μM phenazine methosulfate (PMS) to a concentration of about 10 μM in a modified anaerobic cuvette, which was then repetitively evacuated on a vacuum line and flushed with N_2. To reduce the enzyme, sodium ascorbate at 2 mM was added to the enzyme solution which was stored at ∼4 °C until the enzyme was fully reduced, followed by replacement of N_2 by CO. The concentration of enzyme with CO bound to heme a_3 was estimated from the absorbance difference spectrum reduced •CO minus reduced using $\epsilon^{590} - \epsilon^{606} = 10$ mM^{-1} cm^{-1} (*20*). With the DN(I-132) mutant enzyme the CO complex was formed in 70−75% of the enzyme population. It should be noted that the enzyme population which did not bind CO did not participate in the reactions described in this work.

The solution of the reduced cytochrome *c* oxidase−CO complex was transferred anaerobically to one of the drive syringes of a locally modified stopped-flow apparatus (Applied Photophysics, DX-17MV). The other syringe was filled with the same buffer solution supplemented with 0.05% dodecyl β-D-maltoside, equilibrated with pure O_2 at 1 atm. The enzyme:O_2 solution mixing ratio was 1:5, giving an O_2 concentration in the experiment (after mixing) of ∼1 mM. The reaction of the fully reduced enzyme with O_2 was initiated by flash photolysis of the enzyme−CO complex about 100 ms after mixing (using a 10-ns, ∼100-mJ laser flash at 532 nm (Nd:YAG laser from Spectra Physics)). Typically, 5−20 traces were averaged.

To measure proton uptake associated with reaction of the fully reduced enzyme with O_2, buffer was exchanged for 0.1 M KCl, 0.1% dodecyl β-D-maltoside at pH 7.5−8.0 by repetitive dilution and reconcentration of the enzyme solution using Centricon-30 tubes (Amicon Inc.). Phenol red at a concentration of 40 and 5 μM PMS was added, and the pH was adjusted to about 7.8. The enzyme was reduced and equilibrated with CO as described above. If necessary, the pH was adjusted to ∼7.5, as determined from the optical absorption of the dye. The buffer capacity of the enzyme solution was determined by addition of known amounts of acid or base to the exhaust from the flow-flash apparatus, equilibrated with N_2, while monitoring the absorbance at 560 nm as described (*10*). The concentration of reacting enzyme in the kinetic experiments was calculated from the CO dissociation absorbance change at 445 nm using $\Delta\epsilon^{445} = 67$ mM^{-1} cm^{-1} (*20*).

Where applicable, arachidonic acid was added at a final concentration of 250 μM or 2 mM from a 50 mM stock solution. In the latter case an aliquot of the 50 mM stock solution was loaded into an empty anaerobic cuvette and ethanol was evaporated by a stream of nitrogen (until a small droplet remained), before the enzyme-containing sample was transferred to the same cuvette.

Measurements of Internal Electron Transfer in the Absence of O_2. The carbon monoxide mixed-valence (heme *a*/Cu_A oxidized and the binuclear center reduced) complex of cytochrome *c* oxidase was prepared by incubation of the oxidized enzyme with CO for 1−2 h as described (*23*). The laser and observation equipment have been described in detail elsewhere (*10*).

RESULTS

CO Recombination with Fully Reduced Enzyme. Figure 2A shows absorbance changes associated with flash-induced dissociation and recombination of carbon monoxide (CO) to the fully reduced wild-type and the DN(I-132) mutant enzymes. The recombination processes were monophasic and displayed the same rates in the two enzymes, which indicates that the binuclear center was intact and "homogeneous" in the CO-binding population of the DN(I-132) mutant enzyme (70−75% of the enzyme population, see Materials and Methods and Discussion).

Internal Electron Transfer. Internal electron-transfer reactions were measured following dissociation of CO from the so-called mixed-valence enzyme (Figure 2B) in which the binuclear center is reduced and heme *a* and Cu_A are oxidized (*24*). The CO ligand stabilizes the reduced state of heme a_3, thus increasing its apparent redox potential. Consequently, dissociation of CO (increase in absorbance at $t = 0$, Figure 2B) results in a fractional electron transfer from heme a_3 to heme *a*, seen as a decrease in absorbance with a time constant of ∼3 μs (Figure 2B), corresponding to ∼40% reduction of heme *a* (*24*). Both the time constant and amplitude of the 3-μs phase were about the same in the DN(I-132) as in the wild-type enzyme, which indicates that the structure around the hemes and the redox potential difference between the hemes were not affected by the mutation.

The rapid electron transfer between hemes *a* and a_3 is followed by a slower, additional reduction of heme *a* on a millisecond-time scale (Figure 2C), coupled to proton release to the bulk solution (*25*). This proton-transfer coupled electron-transfer reaction had the same time constant and extent in the DN(I-132) as in the wild-type enzyme at pH 8.8 (Figure 2C), which indicates that D(I-132) is not involved in this proton-transfer reaction. The same results were also obtained with two other D-pathway mutant enzymes: EQ-(I-286) (*12*) and SA(I-201) (*26*).

Reaction of the Fully Reduced DN(I-132) Enzyme with O_2. As described in the Introduction, after flash-induced dissociation of CO from the fully reduced wild-type *R. sphaeroides* enzyme in the presence of O_2, four kinetic phases are resolved (*10*). Binding of oxygen to the reduced heme a_3 results in an absorbance decrease at 445 nm with a time constant of ∼10 μs (formation of the A intermediate). The decrease is followed by a further decrease in absorbance at 445 nm with a time constant of ∼50 μs (Figure 3A), associated with oxidation of hemes *a* and a_3 and formation of the so-called P intermediate[2] (the molecular structure of which is under debate, see Discussion). In the wild-type enzyme, the F intermediate is then formed with a time constant of 120 μs, seen as an increase in absorbance at 580 nm (absorbance maximum for the F intermediate relative to the oxidized enzyme) (Figure 4). Formation of the F intermediate is accompanied by electron transfer from Cu_A to heme *a* (increase in absorbance at 830 nm, Figure 5) and

[2] It should be noted that at 445 nm there is contribution both from redox reactions of the hemes and also from the oxygen intermediates formed at the binuclear center.

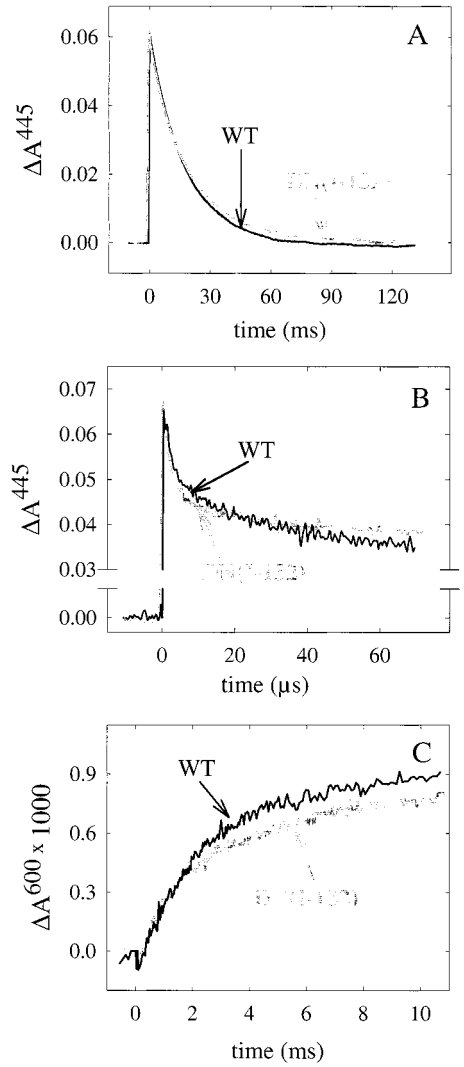

FIGURE 2: (A) Absorbance changes at 445 nm after flash photolysis of CO (at $t = 0$) from the fully reduced wild-type (WT) and DN(I-132) enzymes. (B) Flash-induced absorbance changes at 445 nm following dissociation of CO from the mixed-valence wild-type and DN(I-132) enzymes. The rapid increase in absorbance at $t = 0$ is associated with CO dissociation. The following decrease in absorbance with a time constant of ~3 μs is associated with electron transfer from heme a_3 to heme *a*. The slower decrease in absorbance is associated with CO recombination ($\tau \cong 60$ ms in the mixed-valence enzyme) and fractional electron transfer from heme *a* to Cu_A in a small fraction of the enzyme population which becomes "over-reduced" (to 3 e^-/enzyme). The difference in the kinetic traces after the 3-μs electron transfer is due to slightly different fractions of 3-electron-reduced enzyme in the different samples. (C) Flash-induced absorbance changes at 600 nm associated with proton-transfer-controlled electron transfer ($\tau \cong 2$ ms) from heme a_3 to heme *a*. Dissociation of CO is not associated with any absorbance changes at 600 nm. Conditions: (A−C) 0.5−2 μM enzyme (note that in the graphs the traces have been scaled to 1 μM enzyme), 0.1% dodecyl β-D-maltoside, 1 mM CO; (A and B) 100 mM Hepes-KOH, pH 7.4; (C) 100 mM Tris-HCl, pH 8.8. Note that the pH was 8.8 in panel C because the extent of the proton-transfer coupled electron-transfer reaction is maximal around this pH (*24*).

proton uptake from the bulk solution (Figure 6). The oxidized enzyme $(O)^3$ is formed with a time constant of 1.2 ms (Figures 3B and 4, absorbance decrease at 445 and 580 nm)

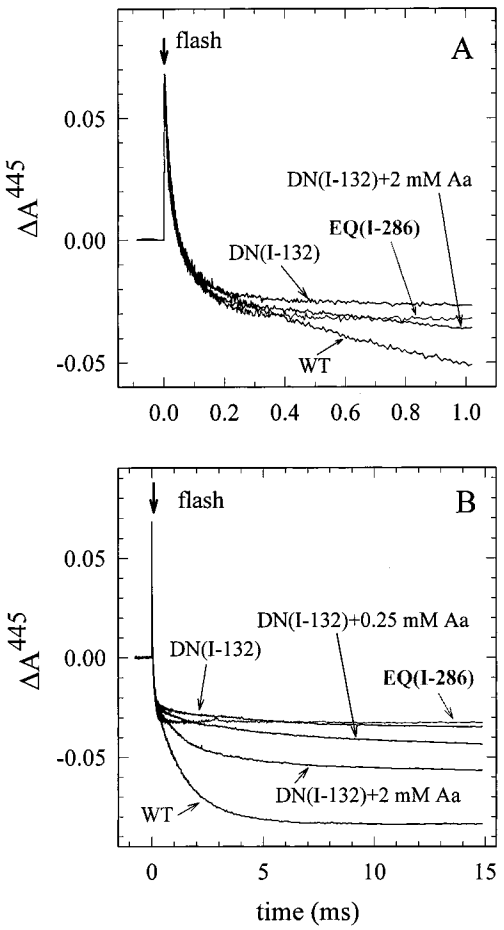

FIGURE 3: Absorbance changes at 445 nm associated with reaction of the fully reduced wild-type, DN(I-132), and EQ(I-286) mutant cytochrome aa_3 with O_2 on shorter (A) and longer (B) time scales. The effect of addition of arachidonic acid (Aa) to the DN(I-132) mutant enzyme is shown. The EQ(I-286) mutant enzyme data are from ref *12*. Experimental conditions after mixing: 0.1 M Hepes, pH 7.4, 0.1% dodecyl maltoside, 1−2 μM reacting enzyme (note that in the graph the traces have all been scaled to 1 μM enzyme), 1 mM O_2, 23 °C.

accompanied by proton uptake form the bulk solution (Figure 6).

At 445 nm, the first two phases displayed approximately the same rates and amplitudes in the DN(I-132) mutant and wild-type enzymes (Figure 3A), which indicates that the mutation did not affect O_2 reduction up to formation of the P intermediate. Furthermore, at 580 nm the 120-μs increase in absorbance was observed with the DN(I-132) mutant enzyme (Figure 4), which indicates that the F intermediate was formed. The wild-type enzyme is fully oxidized about 10 ms after initiation of the reaction. Thus, the fraction of F formed in the DN(I-132) mutant enzyme can be estimated from the absorbance difference of the DN(I-132) (at ~0.5 ms) and wild-type enzyme (at ~10 ms) in the traces shown in Figure 4. Using the absorption coefficient for the F-minus-O difference at 580 nm of 5 mM^{-1} cm^{-1} (*33*), we estimated that in the DN(I-132) mutant enzyme F was formed in almost the entire enzyme population.

With the EQ(I-286) mutant enzyme the final state was P (*12*). The absorbance level at 580 nm of the trace obtained with the EQ(I-286) enzyme, about 10 ms after the flash, i.e., at the end of the reaction, was between the levels with the wild-type (state O) and DN(I-132) (state F) enzymes (see

[3] Most likely the oxidized enzyme formed with the time constant of 1.2 ms has one or two hydroxides bound at the binuclear center (*27*–*29*). One OH^- is then (partly) released with a rate of 200 s^{-1} (*30*−*32*).

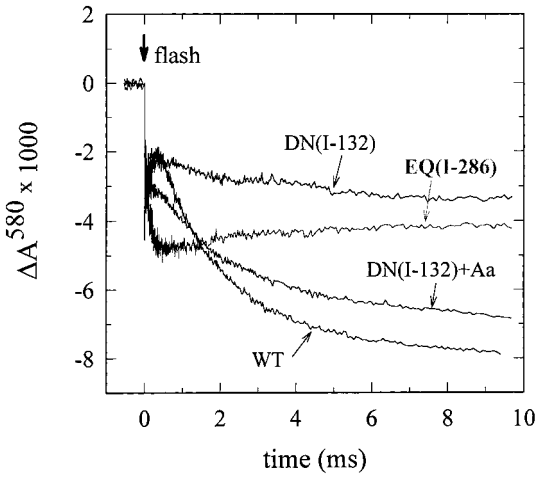

FIGURE 4: Absorbance changes at 580 nm associated with reaction of the fully reduced wild-type, DN(I-132), and EQ(I-286) mutant cytochrome aa_3 with O_2. The effect of addition of 2 mM arachidonic acid (Aa) to the DN(I-132) mutant enzyme is shown. At 580 nm the oxo-ferryl-minus-oxidized spectrum displays an absorbance maximum. The EQ(I-286) mutant enzyme data are from ref *12*. Experimental conditions and scaling were the same as in Figure 3.

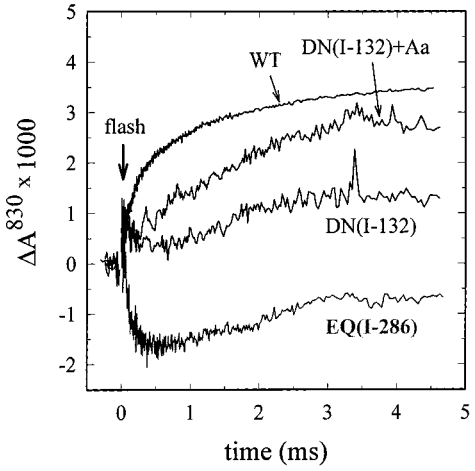

FIGURE 5: Absorbance changes at 830 nm associated with reaction of the fully reduced wild-type and DN(I-132) and EQ(I-286) mutant cytochrome aa_3 with O_2. The effect of addition of 2 mM arachidonic acid (Aa) to the DN(I-132) mutant enzyme is shown. At 830 nm the oxidized-minus-reduced spectrum of Cu_A displays an absorbance maximum. The data obtained with the EQ(I-286) mutant enzyme are from ref *12*. Experimental conditions and scaling were the same as in Figure 3.

Figure 4), which is consistent with a smaller contribution of the P intermediate (formed in EQ(I-286)) as compared to the F intermediate (formed in DN(I-132)) at 580 nm, relative to the oxidized enzyme (formed in the wild-type enzyme) (*33*).

Unlike in the wild-type enzyme, no increase in absorbance at 830 nm was observed during the first 500 μs (Figure 5) with the DN(I-132) mutant enzyme, which shows that Cu_A remained reduced on this time scale. Essentially no proton uptake from solution was observed during the first 500 μs (Figure 6).

In the DN(I-132) enzyme the slowest reaction phase, associated with the decay of the F intermediate, oxidation of Cu_A (Figure 5), and formation of the oxidized enzyme with a time constant of ~2 ms (cf. wild-type $\tau \cong 1.2$ ms), was observed although its amplitude was ≤15% of that in

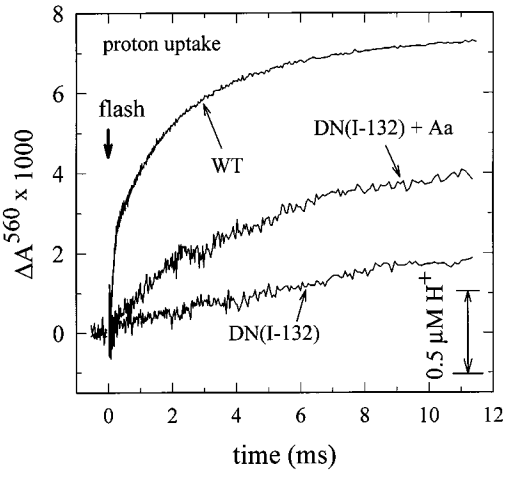

FIGURE 6: Absorbance changes at 560 nm of the pH-indicator dye phenol red, associated with proton uptake during reaction of the fully reduced enzyme with O_2. The traces shown are the differences between the traces obtained in an unbuffered (0.1 M KCl) and a buffered (0.1 M Hepes) solution at the same pH (~7.5). Experimental conditions: ~3 μM cytochrome *c* oxidase, 0.1% dodecyl maltoside, 1 mM O_2. The DN(I-132) traces were first scaled to 1 μM reacting enzyme concentration (from the CO dissociation change at 445 nm with the same sample) and then to the same buffer capacity (from a titration of the sample). The trace with the wild-type enzyme is from ref *10* and has been scaled with the DN-(I-132) enzyme traces.

the wild-type enzyme (Figure 3). In addition, a fractional proton uptake (Figure 6) with a time constant of ≥2 ms was observed.

The overall activity of the DN(I-132) mutant enzyme increased slightly with increasing buffer concentration. Consistently, at 445 nm, the fraction of the 2-ms phase increased slightly with increasing buffer concentration (not shown), which suggests that buffer can help in shuttling protons to the D-pathway, bypassing N(I-132) in the DN-(I-132) mutant enzyme.

Effect of Arachidonic Acid. It has been shown previously that addition of arachidonic acid to solubilized DN(I-132) mutant enzyme increases its activity (*18*). In agreement with this observation we observed an increase in the turnover activity by a factor of 4−5 upon addition of 250−500 μM arachidonic acid. Addition of arachidonyl alcohol, which has a −OH group instead of the $^{\bullet}COO^{-}$, did not influence the activity of the mutant enzyme.

Upon addition of arachidonic acid to the enzyme solution in the flow-flash experiments outlined above, the amplitude of the slowest reaction phase ($\tau \cong 2$ ms), monitored at 445 nm, increased significantly (Figure 3). The amplitude of the absorbance changes at 830 nm, associated with oxidation of Cu_A with the same time constant, increased to about 80% of that in the wild-type enzyme (Figure 5), and proton uptake with the same rate was observed (Figure 6). Taking into account the fraction of the mutant enzyme displaying the 2-ms reaction phase at 445 nm in the presence of arachidonic acid, a net stoichiometry of ~1.5 H^+ taken up per enzyme molecule was estimated (absorbance changes at 445 and 560 nm (proton uptake) were measured in the same sample). The extent of this proton uptake is approximately the same as the net uptake of protons during the P → F and F → O transitions in the wild-type enzyme (*10*), but in the DN(I-132) enzyme it takes place in a single kinetic phase (on the same time scale as the F → O transition).

As a control, the effect of arachidonic acid on the oxidation kinetics of the EQ(I-286) mutant enzyme was also investigated, but no effect was observed (data not shown).

DISCUSSION

In this study we have investigated the involvement of aspartate-132 of subunit I (D(I-132)) in the catalytic reaction of cytochrome *c* oxidase from *R. sphaeroides*. In the reduced form about 70−75% of the enzyme population bound CO to heme a_3, which is similar to the fraction found previously (*8*). As indicated previously (*8*), this suggests some minor change to the binuclear center in a fraction of the enzyme population. However, the CO recombination kinetics were monophasic and displayed the same time constant as with the wild-type enzyme, and internal electron transfer between hemes *a* and a_3 and oxidation of heme a_3 coupled to proton release were unaffected in the DN(I-132) enzyme. These results indicate that the redox potential difference between hemes *a* and a_3 and the structure around the hemes were unaffected by the mutation in the enzyme fraction that had bound CO. It should be noted that in the experiments discussed in this study only the CO-binding population of the enzyme participates in the investigated reactions.

Previous studies have shown that replacement of glutamate-286 by glutamine (EQ(I-286) mutant enzyme) results in inhibition of the reaction after formation of the so-called P intermediate and impairment of proton uptake during reaction of the fully reduced enzyme with O_2 (*12, 14*−*16*) (see Figure 7). Glutamate (I-286) is part of the same putative proton-transfer pathway as D(I-132) (D-pathway) (*6, 14*). As with the EQ(I-286) mutant enzyme, replacement of D(I-132) by its nonprotonatable analogue resulted in impaired proton uptake and a dramatical decrease in the extent of electron transfer from Cu_A to heme *a* during O_2 reduction. However, with the DN(I-132) mutant enzyme the reaction proceeded one step further than with the EQ(I-286) enzyme, i.e., to the F intermediate (see Figure 7). These findings are consistent with the observation made by Konstantinov et al. (*34*) who found that the F intermediate could be formed by incubation in H_2O_2 of the DN(I-132) but not the EQ(I-286) mutant enzyme.

Wikström (*35*) has shown that during turnover of the bovine cytochrome *c* oxidase, proton pumping takes place during the P → F and F → O transitions, and it is assumed that the same also applies to the *R. sphaeroides* enzyme. The P → F transition ($\tau = 120$ μs) is associated with proton uptake from solution exclusively through the D proton-transfer pathway (*12, 30*), including residues D(I-132) and E(I-286) (see Figure 1). The observation that the F intermediate is formed without uptake of protons from solution in the DN(I-132) but not in the EQ(I-286) mutant enzyme indicates that in the former the proton is taken internally from the enzyme (see Figure 7), presumably from E(I-286) or groups (e.g., water molecules) in the D-pathway located further away from the binuclear center than E(I-286). It has been suggested that E(I-286) plays a central role in alternating the accessibility of protons to different sites in the enzyme, associated with pumping of protons (*36*−*39*). In the wild-type enzyme, the proton transferred during the 120-μs phase is presumably transferred in two steps: proton transfer from, e.g., E(I-286) to the binuclear center forming F with a time constant of 120 μs, followed by a rapid (τ < 120 μs) reprotonation from solution (see Figure 7). Because D(I-132) is located at the entry point of the D-pathway, in the DN(I-132) mutant enzyme the protons extracted from the D-pathway cannot be replenished from solution as fast as in the wild-type enzyme. As indicated above, both pumped and substrate protons are transferred through the same proton-transfer pathway (D-pathway) during the P → F transition. Therefore, even though the F intermediate is formed in the DN(I-132) enzyme, it does not pump protons, presumably due to the blockage of proton uptake through D(I-132); the pump element does not deliver its proton to the output side, but rather it delivers a "pumped proton" to the binuclear center for oxygen reduction (see also ref *40*).

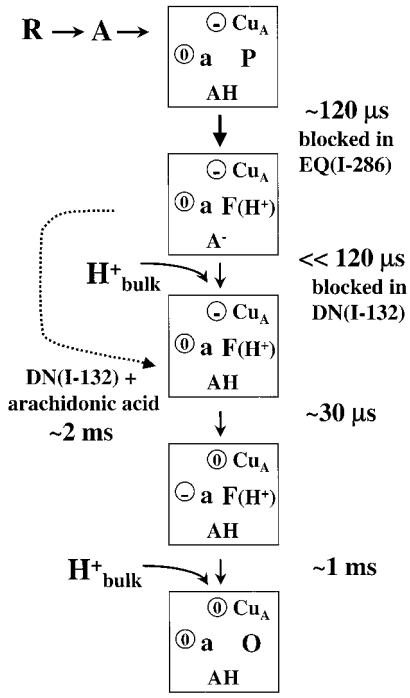

FIGURE 7: Schematic summary of the reaction of the fully reduced enzyme with O_2. Abbreviations: R, fully reduced enzyme; A, ferrous-oxy intermediate; P, peroxy intermediate; F, oxo-ferryl intermediate; O, oxidized enzyme. The redox states of Cu_A and heme *a* are denoted by "−" (reduced) or "0" (oxidized), respectively. In the wild-type enzyme the P → F transition is associated with proton uptake from solution. It is assumed that this proton is first transferred internally from a protonatable group in the enzyme (A) with a time constant of 120 μs. This proton transfer is then followed by a rapid (<120 μs) proton uptake from solution. Only if this proton is taken up from solution, then the electron is transferred from Cu_A to heme *a* with a time constant of ~30 μs (*11*). Thus, in the wild-type enzyme the apparent time constant of the transition from state P to F with reduced heme *a* is ~120 μs. With the DN(I-132) and EQ(I-286) mutant enzymes, in the major enzyme fractions, the reaction is blocked as indicted in the figure. Arachidonic acid mediates proton transfer from bulk solution to the D-pathway in the DN(I-132) mutant enzyme with a time constant of ~2 ms.

It has recently been proposed that the O−O bond is cleaved already upon formation of the P intermediate (*41*−*43*) and that the bond breaking is associated with abstraction of a hydrogen atom ($H^+ + e^-$) from Tyr(I-288) (*41*). According to this scenario, during reaction of the fully reduced enzyme with O_2, states P and F have the same structures, except that in state P a proton is missing at the binuclear center, either at $TyrO^-$ or at $Cu_B^{2+}OH^-$ (cf. *43*).

As discussed above, in the DN(I-132) mutant enzyme this proton may be transferred internally from the D-pathway without being replenished from solution. In the EQ(I-286) mutant enzyme the F intermediate cannot be formed because E(I-286) is closer to the binuclear center than D(I-132) and blockage of the D-pathway at E(I-286) blocks the proton transfer.

We have previously shown that the electron transfer from Cu_A to heme *a* with the same rate constant as the P → F transition is controlled by the proton uptake associated with this transition (*11, 12*). It was suggested that this control is of an electrostatic nature: the transfer of a positive charge toward the binuclear center increases the apparent redox potential of heme *a* more than that of Cu_A because the distance between the positive charge is smaller to heme *a* than to Cu_A. This is consistent with the results from this study since with the DN(I-132) mutant enzyme there is no proton uptake from solution (with $\tau = 120$ μs) and therefore no electron transfer from Cu_A to heme *a*.

As observed previously by Fetter et al. (*18*), addition of arachidonic acid, but not arachidonyl alcohol, resulted in an increase of the DN(I-132) mutant enzyme activity. This indicates that arachidonic acid can bind to the enzyme surface with the carboxylic group in the vicinity of N(I-132) in the DN(I-132) mutant enzyme, partly replacing the role of D(I-132). In principle, the fractional overall activity in the presence of arachidonic acid could arise from a low activity in a major fraction of the enzyme population or a high activity in a minor fraction of the enzyme population (or an intermediate case). Our results indicate that the latter alternative is correct because upon addition of arachidonic acid the fraction (but not the rate) of the 2-ms reaction increased significantly (Figures 3−6). This indicates that the effect of the arachidonic acid is specific: either it binds close to N(I-132), in which case the enzyme oxidation rate is almost the same as that of the wild-type enzyme, or it does not bind close to N(I-132), in which case there is no effect. It has been shown earlier that in the DN(I-135)[4] mutant cytochrome bo_3 from *E. coli* proton pumping and at least part of the activity could be restored by introducing carboxylates at other locations in the vicinity of N(I-135) (*44*).

The specificity of the effect of arachidonic acid is further supported by the proton-uptake stoichiometry of the DN(I-132) enzyme; in the enzyme fraction in which the 2-ms phase was restored, ~1.5 H^+/cytochrome *c* oxidase were taken up from solution, which is about the same value as in the wild-type enzyme (*10*). Thus, arachidonic acid seems to partly substitute the role of D(I-132) in providing protons to the D-pathway. However, it should be noted that with the arachidonic acid, the 2-ms phase, but *not* the 120-μs phase, was restored, which indicates that the acidic group of the arachidonic acid cannot provide protons as efficiently as does D(I-132). Therefore, it is possible that the DN(I-132) mutant enzyme does not pump protons even in the presence of arachidonic acid because the proton uptake during the P → F transition is absent.

Gutman and Nachliel have suggested that proton uptake by the proton pump bacteriorhodopsin is aided by a so-called "proton-collecting antenna", surrounding the entry point to the proton-input pathway (see, e.g., ref *45*). The antenna consists of carboxylates and histidines, forming a network which can attract protons from solution and keep them for sufficiently long times as to allow efficient transport into the pathway. In *R. sphaeroides* cytochrome *c* oxidase, D(I-132) is positioned in a cleft in a strongly negative electrostatic surface potential, surrounded by a network of six histidine residues (*46*). This arrangement hints at a similar mechanism for proton collection in cytochrome *c* oxidase as in bacteriorhodopsin (see refs *46, 47*). The results from this study indicate that aspartate-132 is a key element in such a proton-collecting antenna, constituting the "entrance" to the D-pathway.

In conclusion, even if the P → F transition is not affected in the DN(I-132) mutant enzyme, this transition is not associated with proton pumping because the proton taken internally from the D-pathway during F formation cannot be replenished from solution due to the kinetic barrier at the D-pathway entrance exhibited by the mutation. Thus, the results from this study show that in the wild-type enzyme the protonatable residue D(I-132) plays a crucial role in proton transfer into the D-pathway, shuttling protons from the bulk solution into the pathway.

[4] Corresponds to DN(I-132) in *R. sphaeroides* cytochrome aa_3.

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