Mechanism of Inhibition of Electron Transfer by Amino Acid Replacement K362M in a Proton Channel of *Rhodobacter sphaeroides* Cytochrome *c* Oxidase[†]

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ABSTRACT: The three-dimensional structure of cytochrome *c* oxidase (COX) reveals two potential input proton channels connecting the redox core of the enzyme with the negatively charged (N-) aqueous phase. These are denoted as the K-channel (for the highly conserved lysine residue, K362 in *Rhodobacter sphaeroides* COX) and the D-channel (for the highly conserved aspartate gating the channel at the N-side, D132 in *R. sphaeroides*). In this paper, it is shown that the K362M mutant form of COX from *R. sphaeroides*, although unable to turnover with dioxygen as electron acceptor, can utilize hydrogen peroxide as an electron acceptor, with either cytochrome *c* or ferrocyanide as electron donors, with turnover that is close to that of the wild-type enzyme. The peroxidase activity is similar to that of the wild-type oxidase and is coupled to the generation of a membrane potential and to proton pumping. In contrast, no peroxidase activity is revealed in the D-channel mutants of COX, D132N, and E286Q. Reduction by dithionite of heme a_3 in the fully oxidized oxidase is severely inhibited in the K362M mutant, but not in the D132N mutant. Apparently, mutations in the D-channel arrest COX turnover by inhibiting proton uptake associated with the proton-pumping peroxidase phase of the COX catalytic cycle. In contrast, the K-channel appears to be dispensable for the peroxidase phase of the catalytic cycle, but is required for the initial reduction of the heme−copper binuclear center in the first half of the catalytic cycle.

Cytochrome *c* oxidase (COX) is a redox-driven proton pump that translocates protons electrogenically across the membrane by utilizing the free energy made available by the highly exergonic reduction of dioxygen to water. According to the general principles of energy transduction by such systems, this enzyme must be endowed with (1) redox-dependent protonatable groups in the vicinity of redox centers and (2) proton-conducting pathways, often called proton channels, that connect the buried redox-dependent ionizable groups with the inner and outer aqueous phases that are separated by the coupling membrane.

It is remarkable that the recently resolved crystal structures of COX do indeed reveal at least two potential input proton channels in subunit I of the enzyme (*18, 48*). One of these channels is formed mainly by residues in helixes VI and VIII (and internal water molecules) and leads via Y288 (*Rhodobacter sphaeroides* numbering) to one of the histidine ligands of Cu_B. The name of K-channel (for the conserved K362 residue) has been suggested in ref 21 for this putative proton-conducting pathway. The second channel, denoted as the D-channel, connects D132, at the negative (N-) surface of the membrane, to E286 at the heme−copper center (*18, 40*).

Mutations in some of the highly conserved amino acid residues in the two channels, such as E286Q and D132N in the D-channel or K362M and T359A in the K-channel, have been found to result in drastic inhibition of the enzymatic activity of COX (*2, 3, 11, 16, 17, 45−47, 50, 58*) which lends credibility to the important functional roles of these structures. A third putative proton-conducting pathway was described for bovine heart oxidase (*48*), and very recently this "pore" has been discerned in the oxidase from *Paracoccus denitrificans* (*38*).

The discussion of the specific functions of the putative proton channels in COX is closely related to the interpretation of the roles assigned to different protons participating in the energy-coupled reduction of oxygen to water. There are eight protons taken up from the N-aqueous phase during a single four-electron turnover of COX and sorting out these protons can be done according to at least two different principles.

First, an emphasis can be put on the fact that four of these protons are consumed in forming the two water molecules from the reduction of dioxygen, whereas the remaining four protons are translocated across the membrane. Conceivably, the chemistry of these processes is very different, and discrimination between the four "chemical" ("substrate", "consumed") protons and the four "pumped" protons has been a paradigm since the discovery of the proton-pumping function of COX (e.g., refs 4, 18, 31, 39, and 60). However, protonation of reduced dioxygen to form water almost certainly involves proton-donating groups in the immediate surroundings of the binuclear center rather than protons delivered directly from the bulk water phase all the way through the protein. Hence, the distinction between "chemical" and "pumped" protons at the stage of their disappearance

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from the N-aqueous phase is far from obvious. According to current thinking, both the chemical and pumped protons are first translocated within the protein to certain point(s) ("redox-linked ionizable groups") close to the oxygen-reduction site (e.g., refs 9, 18, 31, and 39). From these points, protons are directed either to the oxygen intermediates, becoming "chemical" protons, or to the exit proton channel, becoming "pumped" protons. It is not a priori clear that the initial parts of the proton trajectory within the protein should be necessarily different for chemical and pumped protons.

An alternative view is to affiliate the protons taken up from the N-phase with the partial steps of the COX catalytic cycle during which the uptake occur (*21*).

In accordance with these two approaches, there have been two alternative assignments of the functional roles of the two putative proton input pathways revealed by the structures of COX. The model of Iwata et al. (*18*) is related to the first versions of the "histidine cycle" (*31, 62*) and to the model of Rich (*39*). This model emphasizes separate delivery pathways for the protons to be consumed in the formation of water and for the protons to be translocated across the membrane. The K-channel has been assigned the role of "chemical" proton uptake pathway and the D-channel proposed to deliver "pumped" protons.

An alternative model proposes the two channels to be associated with different stages of the COX catalytic cycle, rather than with the type of protons conducted (*21, 42*). As illustrated by Figure 1, the reaction cycle of COX is comprised of two phases differing in redox chemistry and energy-coupling characteristics, and denoted as *eu-oxidase* and *peroxidase* half-reactions. As suggested in ref 21 the K-channel is functionally linked with the eu-oxidase part of the COX reaction whereas the peroxidase half-reaction is serviced by the D-channel.

To elucidate the roles of the D- and K-channels, it is important to identify the specific steps of the COX catalytic cycle that are blocked by amino acid replacements in each of these putative proton pathways. Mutations in the D-channel inhibit the F → O transition in the *R. sphaeroides* enzyme (*21*) and possibly the P → F transition as well (*3*). However, identification of the step(s) inhibited by the K362M replacement has proved more elusive. The absorption spectrum of the *R. sphaeroides* K362M oxidase in the presence of ascorbate + TMPD and oxygen shows that the enzyme is trapped in a state where heme *a* is fully reduced and heme a_3 is predominantly ferric high-spin (*21*). There are no significant contributions from the oxygen compounds (P or F) of heme a_3 (*21*), implying that the inhibition by the mutation precedes formation of compound P in the catalytic cycle (see Figure 1). Indeed, the isolated F-to-O step of the catalytic cycle proceeds about normally in the *R. sphaeroides* K362M mutant (*21*). Moreover, oxidation of the fully reduced K362M mutant of the bo_3-type quinol oxidase from *Escherichia coli* (*44*) or of the aa_3-type cytochrome *c* oxidase from *R. sphaeroides* by molecular oxygen (*3*) proceeds as in the wild-type oxidases. These data also suggest that the K362M mutant is inhibited prior to the formation of compound P.

It has been noticed previously that hydrogen peroxide oxidizes reduced heme *a* readily via heme a_3 in K362M but not in E286Q COX (Mitchell and Konstantinov, unpublished, (*21*)); apparently, electron transfer to hydrogen peroxide as electron acceptor (i.e., the peroxidase activity) in the K362M mutant of COX may not be damaged to the same extent as the overall turnover with dioxygen. These observations prompted the current work, comparing the effect of the K362M replacement with the effects of the mutations within the D-channel on the peroxidase partial reaction of COX.

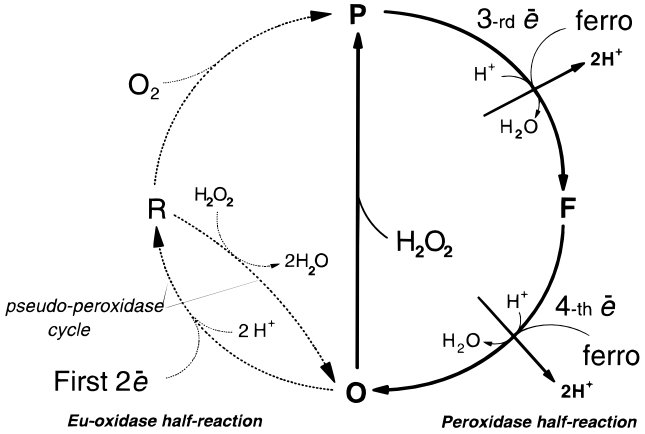

FIGURE 1: The oxidase/peroxidase cycle of cytochrome *c* oxidase. The oxidized (O), reduced (R), peroxy (P), and ferryl-oxo (F) intermediates refer primarily to the state of heme a_3. As discussed in ref 21, P may actually include two different intermediates denoted as P_0 and F_I (see the Discussion). The first (O → R → P) phase of the cycle (the eu-oxidase portion of the reaction) involves transfer of the first two electrons to the heme−copper center, and is not associated itself with proton pumping. However, these reactions generate in situ a powerful oxidant (bound peroxide) for driving proton transfer during the subsequent highly exergonic peroxidase portion of the reaction cycle. The peroxidase phase of the COX catalytic cycle (P → F → O) involves reduction of the peroxy state in two one-electron steps, back to the oxidized form by the third and fourth electrons. These steps are suggested to be coupled to transmembrane pumping of two protons each (*61*) as demonstrated recently (*55*). The K-channel is proposed to be involved in proton uptake during the eu-oxidase phase of the cycle, whereas the D-channel is presumed to be operational during the peroxidase portion of the reaction. The K362M replacement is likely to inhibit the O → R step by virtue of blocking proton uptake via the K-channel coupled to this reaction. Mutations in the D-channel suppress vectorial proton movement coupled to the F → O step (*21*) and probably that coupled to the P → F step as well (*2*).

Steady-state cytochrome *c* peroxidase activity of the mitochondrial COX has been investigated in refs 8, 25, 26, 35, and 36 and the pre-steady-state kinetics of the reduced COX oxidation by hydrogen peroxide has also been analyzed (*12*−*14*, *24*). However, under the anaerobic reducing conditions used in these studies, the cytochrome *c* peroxidase reaction is heterogeneous and involves the reaction of H_2O_2 with both the reduced and oxidized forms of heme a_3 (*25, 26, 36*). The reaction via the ferrous heme a_3 is the major pathway (*12*−*14*, *25*, *26*), but this is not the classical peroxidase mechanism and has, therefore, been denoted as pseudoperoxidase activity (*55*) (Figure 1).

More recently, it has been demonstrated to be possible to resolve the peroxidase reaction of the bovine heart COX under aerobic conditions by using a ferrocyanide/ferricyanide redox buffer as the electron donor (*22, 55, 57*). At high redox potentials of the donor, reduction of heme a_3 becomes unfavorable and is negligible. Accordingly, the reaction of COX with molecular oxygen (the oxidase activity) is attenuated to an undetectable level, whereas the peroxidase

activity is favored and proceeds by a typical peroxidase mechanism, i.e., via H_2O_2 binding to ferric heme a_3 and subsequent generation of compounds P and F (Figure 1) (*23, 53, 56, 59*). This peroxidase reaction of the bovine heart oxidase has been demonstrated to be coupled to proton pumping (*55, 57*).

This work describes the peroxidase reaction of the *R. sphaeroides* wild-type COX as well as the reaction of mutant forms with amino acid replacements in the putative proton-conducting channels. The K362M replacement, although fully inhibiting the oxidase activity of the *R. sphaeroides* COX, does not affect significantly the peroxidase activity of the enzyme. In contrast, both the peroxidase and oxidase activities are suppressed concomitantly in the D-channel mutants. These results have been reported in a preliminary form at the 1996 EBEC meeting (*54*).

MATERIALS AND METHODS

Chemicals. Hydrogen peroxide ("Suprapur") and sodium dithionite were from Merck. Asolectin (phosphatidyl choline type IIS), phosphatidyl choline from dried egg yolk type X-E, poly-L-lysine (MW 4600−6900), nigericin, valinomycin, sodium ascorbate, cytochrome *c* type VI from horse heart, ferricyanide, and ferrocyanide (potassium salts) were from Sigma. Sucrose ("chemically pure", Reachim) was recrystallized from ethanol. NTA-agarose for purification of the histidine-tagged COX from *R. sphaeroides* was purchased from Quiagen. Other chemicals were commercial products of high-purity grades from conventional sources.

The 30% H_2O_2 was diluted to stock solutions of appropriate concentrations before experiments and its concentration was checked spectrophotometrically using an extinction coefficient of 40 $M^{-1}cm^{-1}$ at 240 nm (*7*).

Cytochrome *c* was dissolved in 10 mM potassium phosphate buffer, pH 7.5, and reduced by dithionite. Excess dithionite was removed by centrifugation of the reduced cytochrome through a Sephadex G-25 column. Concentration of the reduced cytochrome *c* was determined from its absolute absorption spectrum using $\Delta\epsilon$ value of 27.7 mM^{-1} cm^{-1} at 550 nm.

Site-directed mutants were constructed on a template plasmid (pJS3) containing the *cta*D gene which codes for subunit I of cytochrome oxidase (*41*). D132N, E286Q, T359A, and K362M were constructed by an extension of a mutagenic single-stranded DNA primer on a single-stranded DNA template, using the method of Vandeyar et al. (*49*), and have been previously described (*11, 17, 28*).

Preparations. Histidine-tagged wild-type and mutant COX was isolated from membranes of *Rhodobacter spaeroides* by chromatography on NTA-agarose (Quiagen) as described (*29*). K362M COX was reconstituted in phospholipid vesicles (COV) by a cholate dialysis method essentially as described in ref *15*. Phospholipids were dispersed in 50 mM potassium phosphate buffer, pH 7.5, with 1.5% cholate, 25 mM K_2SO_4, and 2 mM $MgSO_4$ at final concentration of 40 mg/mL, bubbled with argon, and sonicated on ice (4 × 30s). After sonication, COX was added to the clear suspension to the final concentration of 3 μM and the mixture was dialyzed at pH 7.5 with a stepwise change of the buffer, as specified: first 4 h, sonication buffer but without cholate; next 4 h, 2.5 mM potassium phosphate, 50 mM K_2SO_4, 80 mM sucrose, and 2 mM $MgSO_4$; overnight, 0.25 mM potassium phosphate, 50 mM K_2SO_4, 80 mM sucrose, and 2 mM $MgSO_4$. Unless indicated otherwise, the cytochrome oxidase concentration was determined from the difference spectrum (reduced by dithionite minus oxidized by ferricyanide) using the $\Delta\epsilon_{606-630}$ value of 27 mM^{-1} cm^{-1}. This value may lead to minor underestimation of K362M concentration (ca. 10−15%) due to incomplete reduction of heme a_3 by dithionite in this mutant during the ∼10−15 min allowed for reduction of the samples (see Figure 5 of the paper); however, this is not significant for the purposes of the present work.

Spectrophotometric Measurements. Unless indicated otherwise, spectrophotometric measurements were performed with an SLM-Aminco DW 2000 split beam/dual wavelength spetrophotometer in standard 10 mm rectangular optical cells thermostated at 20 °C.

Proton Pumping. External acidification by phospholipid vesicle-reconstituted K362M-COX was monitored spectrophotometrically at 563 nm (using the absorbance at 600 nm as reference) with the pH indicator Phenol Red in a vigorously stirred optical cell with an unbuffered medium. The λ value of 563 nm was chosen rather than λ_{max} of the indicator (588 nm) to minimize interference from cytochrome *c* absorbance changes. The pH-dependent optical response of Phenol Red was calibrated by the addition of known concentrations of HCl. To evaluate the H^+/e^- ratio in the cytochrome *c* pulse experiments, the rate of electron transfer during the COV-catalyzed cytochrome *c* peroxidation was measured spectrophotometrically in a separate experiment. Cytochrome *c* oxidation was monitored by the absorbance at 550 nm minus the absorbance at 540 nm under identical conditions except the pH indicator was omitted. The rate of oxidation of cytochrome *c* was calculated using $\Delta\epsilon^{red-ox}$ (550−540) = 18.7 mM^{-1} cm^{-1} and corrected, when necessary, for direct oxidation of ferrocytochrome *c* by H_2O_2 as measured in a separate sample without COV. The background contribution due to the oxidation of 4 μM cytochrome *c* by H_2O_2 (≤2 mM) did not exceed 25%.

Data Treatment. The data were processed with the Origin 4.0 software package (Microcal) and exported into Lotus Freelance Graphic 2.1 for presentation of the figures.

RESULTS

Peroxidase Activity of the R. sphaeroides COX Mutants. Both the K362M and E286Q mutant forms of COX with amino acid replacements in the two different proton channels are virtually completely "dead" in the cytochrome oxidase assay (turnover with ferrocytochrome *c* less than 0.3% of wild-type (*11, 21*)). The oxidase and peroxidase activities of the mutants were compared using two different electron donors, ferrocyanide and ferrocytochrome *c*. Cytochrome *c* is a better substrate for COX than ferrocyanide, but there is a significant direct reaction with hydrogen peroxide, whereas ferrocyanide at pH ≤8 does not react nonenzymatically with H_2O_2 at a significant rate (Konstantinov, unpublished). Besides, the use of a ferrocyanide/ferricyanide mixture as the electron donor (see ref *22* for detailed description) allows for direct comparison of the peroxidase activities of K362M and wild-type COX under aerobic conditions. Redox interaction of ferrocyanide with COX can

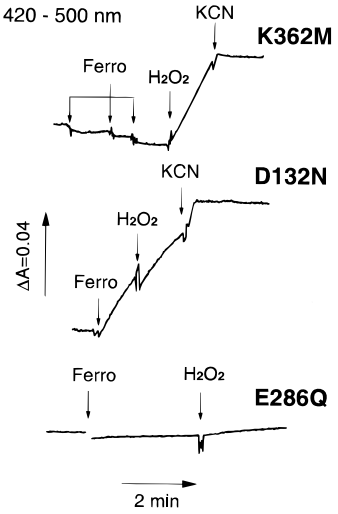

FIGURE 2: Ferrocyanide oxidation and peroxidation by the K362M, D132N, and E286Q mutants of cytochrome *c* oxidase. Ferricyanide accumulation was monitored spectrophotometrically in a dual-wavelength mode at 420 nm versus the 500 nm reference. The cell contained COX (0.5 μM) in 50 mM MOPS−KOH buffer, pH 7, with 0.5 mM EDTA and 40 μg/mL of poly-L-lysine. Additions: ferrocyanide (ferro), 0.1 mM (in case of K362M, 0.1, 0.2 and 0.5 mM); H_2O_2, 4 mM; KCN, 4 mM.

be greatly facilitated by Ca^{2+}, Mg^{2+}, or organic polycations such as poly-L-lysine, which screen the electrostatic repulsion between the ironhexacyanide anion and negatively charged surroundings of Cu_A (*33*). Therefore, all ferrocyanide-oxidation assays were performed in the presence of poly-L-lysine.

As shown in Figure 2, K362M does not oxidize ferrocyanide aerobically at any measurable rate, but ferricyanide generation can be initiated by the addition of hydrogen peroxide. Peroxidation of ferrocyanide is fully inhibited by cyanide and is not observed in the absence of poly-L-lysine (not shown, cf. ref 22). In contrast, no peroxidase reaction is observed under these conditions using the E286Q mutant form of the enzyme. In the case of D132N, there is a significant rate of ferrocyanide oxidation observed aerobically, in agreement with the residual cytochrome *c* oxidase activity (ca. 5%) exhibited by this mutant. Nevertheless, it can be seen that the addition of H_2O_2 does not induce any measurable increase in the ferrocyanide oxidation rate.

Figure 3 shows the results of analogous experiments using cytochrome *c* as the electron donor. In contrast to the experiments with ferrocyanide, some oxidation of the donor (ferrocytochrome *c*) by hydrogen peroxide can be seen in the absence of COX (e.g., traces 2 and 3). This is probably due to the microperoxidase activity of cytochrome *c* preparations (*6*). The rate of this direct reaction increases in proportion to the concentration of H_2O_2, so that special efforts are required to permit the measurements of the cytochrome *c* peroxidase activity of COX at $[H_2O_2] > 5$ mM (*65*). Nevertheless, the addition of the K362M oxidase to a mixture of ferrocytochrome *c* plus H_2O_2 greatly stimulates the oxidation of cytochrome *c*, until depletion of the electron donor (trace 1). In the absence of hydrogen peroxide, no aerobic oxidation of cytochrome *c* is observed at this concentration of K362M (e.g., trace 1). In contrast, no hydrogen peroxide-induced stimulation of cytochrome *c* oxidation is observed with the E286Q mutant (trace 3).

At 5 μM ferrocytochrome *c* and 1 mM of H_2O_2, the rate of the peroxidase reaction, after correction for the nonenzymatic oxidation of cytochrome *c*, corresponds to about 3 s^{-1}, whereas the cytochrome *c* oxidase activity under these conditions does not exceed 0.1 s^{-1}. Hence, the peroxidase reaction of the K362M oxidase, although low, is still at least 30-fold faster than the residual oxidase activity. It has to be emphasized that this peroxidase activity is far below V_{max} of the reaction and is likely to be limited by the rate of peroxide binding to heme a_3 (cf. ref 22). Indeed, in the $[H_2O_2]$ range studied (<10 mM), the rate of the peroxidase reaction with either ferrocyanide or cytochrome *c* as electron donor is proportional to H_2O_2 concentration. In case of ferrocytochrome *c*, the second-order rate constant of 2000−3000 M^{-1} s^{-1} that is observed for the peroxidase reaction of the K362M oxidase is close to the reported second-order rate constant of peroxide binding with ferric heme a_3 in beef heart COX (ca. 10^3 M^{-1} s^{-1}, which converts to 2×10^3 M^{-1} s^{-1} in terms of redox equivalents) (*14*, *59*). Experiments are in progress to determine the rate constants of hydrogen peroxide binding with the fully oxidized (ferric) COX from *R. sphaeroides*, but preliminary data indicate no drastic differences between the bacterial and mammalian enzymes in this respect (Pecoraro et al., paper in preparation). Therefore, the cytochrome *c* peroxidase activity of the K362M oxidase is likely to be limited by the rate of H_2O_2 binding to heme a_3 and there are no indications for inhibition of the intramolecular electron-transfer imposed by the mutation.

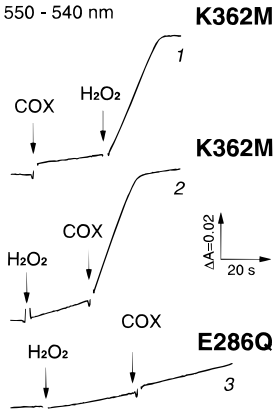

FIGURE 3: Cytochrome *c* oxidase and peroxidase activities of the K362M and E286Q mutants. Oxidation of cytochrome *c* was monitored spectrophotometrically in a dual-wavelength mode at 550 nm versus 540 nm reference. The reaction mixture contained 5 μM ferrocytochrome *c* in 50 mM MOPS−KOH buffer, pH 7.0, with 0.5 mM EDTA. Additions: COX (K362M or E286Q, as indicated), 50 nM; H_2O_2, 1 mM.

Comparison of the Peroxidase Activity of the Mutant and Wild-Type COX. The aerobic oxidation of cytochrome *c* by active (e.g. wild-type bacterial or mammalian) COX is very rapid. The apparent second-order rate constant for reaction of oxygen with reduced heme a_3 ($k \sim 10^8$ M^{-1} s^{-1}) is about 10^5 faster than for the reaction of hydrogen peroxide with ferric heme a_3. Accordingly, at H_2O_2 concentrations less than 10^{-2} M and at ambient oxygen concentration (ca. 0.25 mM), the cytochrome *c* peroxidase activity is small compared with the cytochrome *c* oxidase activity of these enzymes, as previously demonstrated for COX from beef heart (e.g., ref 37). For this reason, it is not possible to accurately measure the cytochrome *c* peroxidase activity of the wild-type enzyme

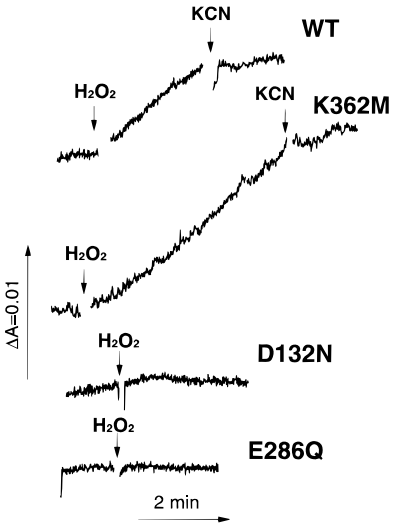

FIGURE 4: Comparison of the ferrocyanide-peroxidase activity of wild-type and mutant forms of cytochrome *c* oxidase. Conditions were as in Figure 2, but 1 mM ferricyanide was included in the reaction buffer to suppress the ferrocyanide oxidase reaction in active (wild-type, WT) or partly active (D132N) forms of COX to facilitate monitoring of the peroxidase reaction. The solution contained 0.1 mM ferrocyanide and 0.5 μM COX.

or even that of the D132N mutant (which has 5% of the normal oxidase activity) under aerobic conditions. As mentioned above (see the introduction), studying the peroxidase activity of COX under anaerobic conditions results in a complex and heterogeneous reaction. However, the ferrocyanide peroxidase activity can be easily measured with beef heart COX or with the wild-type *R. sphaeroides* enzyme under aerobic conditions without interference from the oxidase reaction when assayed at a high redox potential of the ferrocyanide/ferricyanide couple (*22*, *55*). This allows for direct comparison of the peroxidase activities of the K362M and other mutants of COX with that of the wild-type enzyme under identical conditions, albeit very far from V_{max}.

The results are shown in Figure 4. Under identical conditions, the rate of ferrocyanide peroxidation by K362M is close to that observed with the wild-type enzyme from *R. sphaeroides*. A very similar rate was obtained also for the mitochondrial beef heart COX [data not shown (*22*)]. In contrast, the mutants in the D-channel (D132N and E286Q) do not manifest any measurable peroxidase activity under these conditions. Whether D132N mutant has some residual peroxidase activity corresponding to ca. 3−5% of that in the wild-type (as implicated by the 3−5% residual oxidase activity of the mutant) cannot be inferred from the present data.

Thus, it appears that replacement of K362 by methionine, while virtually fully inhibiting the oxidase activity of COX, does not affect to any significant extent the peroxidase activity of the enzyme, whereas inhibition of the cytochrome oxidase turnover imposed by the mutations in the D-channel is accompanied by inhibition of the peroxidase activity.

Membrane Energization by the Peroxidase Reaction of K362M. The rate of peroxidase turnover of K362M at peroxide concentrations of about 1 mM, although close to that of the wild-type enzyme, is quite slow in terms of the absolute rates. Therefore, it is important to investigate whether this reaction is of functional importance. To this end, K362M was reconstituted in phospholipid vesicles

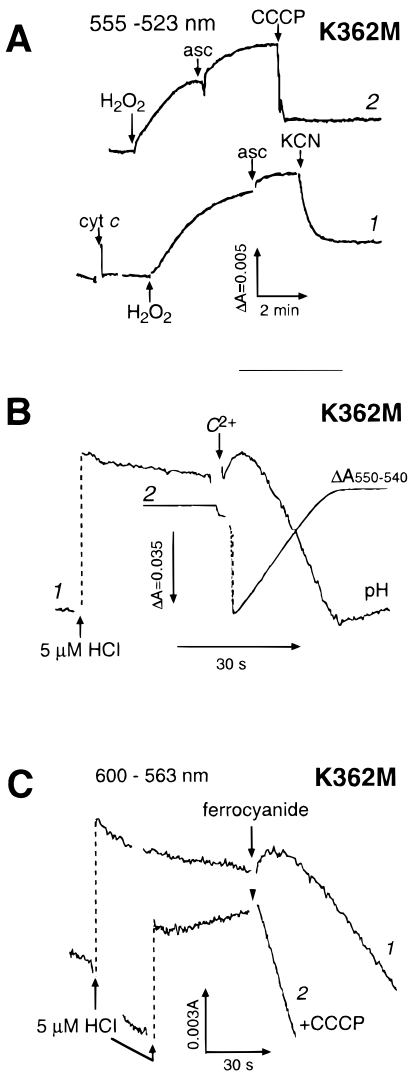

FIGURE 5: Membrane energization coupled to the peroxidase activity of liposome-reconstituted K362M mutant of COX. (A) *Membrane potential* generation has been measured spectrophotometrically with safranine as indicator by monitoring $\Delta A_{555-523}$. Cytochrome oxidase/asolectin vesicles (10 nM in COX) were in buffer containing 0.25 M sucrose, 25 mM MOPS−KOH, pH 7, 0.2 mM EDTA and also 0.5 μM nigericin and 5 μM Safranin O. Additions: ferrocytochrome *c* (cyt *c*), 2 μM (present initially in the case of trace 2); H_2O_2, 2 mM; ascorbate (asc), 5 mM; KCN, 2 mM; CCCP, 1 μM. (B, C). Proton pumping coupled to cytochrome *c*-peroxidase activity was monitored as pH changes in the medium with Phenol Red as the absorption pH indicator. Measurements were made at 600 nm minus 563 nm. Cytochrome *c* oxidase/phosphatidyl choline vesicles (0.1 μM in COX) were suspended in the unbuffered medium containing 0.25 M sucrose, 50 mM K_2SO_4, 0.1 mM EDTA and also 0.5 μM valinomycin and 2 mM H_2O_2. The pH was adjusted to 6.8 and the pH traces are started by the addition of 0.5 μL of 20 mM HCl (5 μM incremental changes in concentration) to calibrate the pH response. Proton pumping coupled to peroxidase activity of COX was initiated by the addition of 3.5 μM ferrocytochrome *c* (panel B) or 0.5 mM ferrocyanide in the presence of 0.8 μM ferricytochrome *c* (panel C). Where indicated (C, trace 2), 1 μM CCCP was present. Trace 2 in panel B shows oxidation of 3.5 μM ferrocytochrome *c* measured in a separate experiment under conditions identical to those in trace 1.

(COV), and the generation of a membrane potential, as well as proton pumping, was measured. As shown in Figure 5A, the addition of the reduced cytochrome *c* to COV under aerobic conditions does not give rise to the generation of a $\Delta\Psi$, in agreement with the full inhibition of the oxidase

activity in the mutant (note that ferrocytochrome *c* is initially present in case of trace 2). Subsequent addition of hydrogen peroxide results in generation of a transmembrane electric potential difference. This transmembrane potential is slightly stimulated by ascorbate, which regenerates the fully reduced state of cytochrome *c*, and is inhibited by the addition of either cyanide (trace 1) or the uncoupler CCCP (trace 2).

As shown in Figure 5B,C, the K362M cytochrome oxidase vesicles also pump protons coupled to the ferrocytochrome *c* peroxidase turnover. Transient acidification of the external medium followed by net proton uptake is observed using either Phenol Red as the pH indicator (Figure 5B,C) or glass electrode (data not shown). This occurs upon the addition of either ferrocytochrome *c* (Figure 5B) or of excess ferrocyanide in the presence of low concentration of ferric cytochrome *c* (Figure 5C) to the vesicles preequilibrated with K^+/valinomycin and with excess hydrogen peroxide. The acidification is abolished in the presence of the uncoupler CCCP (Figure 5C, trace 2). The latter can also be seen to accelerate the reaction (note the slopes of the final parts of traces 1 and 2 in Figure 5C). No proton pumping by K362M vesicles was observed with oxygen as electron acceptor, i.e., in the absence of hydrogen peroxide.

The experiments in Figure 5 aim primarily at a qualitative demonstration of membrane energization linked to the peroxidase activity of K362M. However, if the initial rate of the net external acidification by the vesicles is compared to that of cytochrome *c* oxidation measured under the same conditions in a parallel experiment (e.g., Figure 5B), an apparent H^+/e^- ratio higher than 1 is obtained (1.3−1.5) even without correction for the direct oxidation of cytochrome *c* by hydrogen peroxide (ca. 20% of the oxidation rate); this is comparable to results obtained with bovine COX (*55*). Simultaneous recordings of proton extrusion and cytochrome *c* oxidation in the same sample will be required for reliable quantitation (*55*) and the use of higher H_2O_2 concentrations and, hence, higher reaction rates may also be helpful (*65*). Nevertheless, the current data indicate that the peroxidase turnover of K362M, although slow at 1−2 mM of peroxide, is energy-coupled.

Inhibition of Electron Transfer to Heme a_3 in K362M. In search of the partial reactions of COX that might be blocked by the K362M mutation, we have investigated the kinetics of heme reduction by dithionite (Figure 6). At 606 nm, where heme *a* is the main contributor to the absorbance changes, rather similar traces are obtained for the wild-type and K362M as well as D132N and E286Q, showing rapid reduction of heme *a* (not shown). However, measurements in the Soret band, where both heme *a* and heme a_3 absorb to roughly the same extent, reveal drastic differences between the K362M mutant and the wild-type form of the enzyme.

Dithionite addition to the oxidized wild-type COX results in rapid, full reduction of both hemes *a* and a_3 (increase of absorbance of 444 nm to the maximal level in less than 1 min), consistent with the results reported with "rapid" forms of mammalian COX (reviewed in ref 30). In contrast, the addition of dithionite to the oxidized K362M enzyme results in rapid increase of absorbance at 444 nm to only about 50% of the final level, followed by much slower absorbance changes (Figure 6A). The difference spectrum of the slowly reduced component (Figure 6A, inset) is essentially that of the high-spin heme a_3 with a minimum at 413 nm and

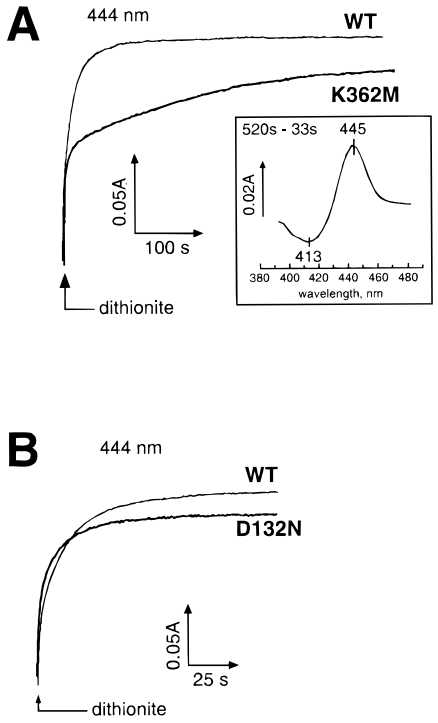

FIGURE 6: Reduction of cytochrome *c* oxidase by dithionite in the wild-type and mutant forms of the enzyme. Experiments were performed with an Applied Photophysics Bio SX-17MV sequential stopped-flow reaction analyzer in a spectra-collecting mode (diode-array configuration). The wild-type or mutant COX in 100 mM Bis-Tris-propane buffer, pH 8.0, with 0.1% dodecyl maltoside were preincubated in a syringe for 30 min in the presence of 20 μM ferricyanide and 10 μg/mL of poly-L-lysine to oxidize the enzyme and then mixed with 30 mM dithionite solution in the same buffer. The mixing volume ratio is 1:1. A total of 400 spectra were collected following the mixing for the period of 540 s in case of K362M and the wild-type control (Figure 6A) and 130 s in case of D132N/wild-type (Figure 6B). The reperesentative kinetics traces and difference spectra shown in the figure have been reconstructed from the data matrixes corresponding to each run. The enzyme concentration after mixing was 1.2 μM (wild-type), 0.82 μM (D132N), or 0.68 μM (K362M), and the traces have been normalized to a concentration of 1 μM using $\Delta A_{606-630}$ (reduced-minus-oxidized) value of 24 mM^{-1} cm^{-1} for the wild-type and D132N and 21 mM^{-1} cm^{-1} for K362M (to account for incomplete reduction of heme a_3).

maximum at 445 nm in the Soret and with low absorbance in the α-band (not shown). The rapidly reduced component has a difference spectrum characteristic of heme *a* (not shown). Therefore, it is concluded that the reduction of heme a_3 by dithionite is inhibited in K362M.

These results have been reproduced with several different preparations of the K362M oxidase. In all the experiments, the reduction of heme a_3 was impeded very significantly in K362M relative to the wild-type enzyme. The absolute rates of heme a_3 reduction varied for different K362M preparations, and the slow part of the reduction is, in fact, multiphasic. In some preparations, the reduction of a_3 in the mutant was not complete even in 2 h. Similar results are also described in the recent paper of Hosler et al. (*17*).

In contrast, mixing the D132N oxidase with dithionite shows optical changes in the Soret similar to or even slightly faster than those observed with the wild-type enzyme (Figure 6B). Curve fitting shows the presence of two phases with very similar rate constants for both the wild-type and D132N oxidases (0.62 and 0.66 s^{-1}, respectively, for the rapid phase;

0.051 and 0.059 s^{-1}, respectively, for the slow phase). It is mainly diminution of the relative amplitude of the slow phase in D132N (44% vs 61% in the wild-type) that differentiates the mutant from the wild-type oxidase. Presumably, this is associated with the altered spectroscopic characteristics and the diminished spectroscopic contribution of heme a_3 in this mutant (first noted in ref 21).

Experiments with E286Q also show rapid heme reduction by dithionite both at 606 and 444 nm (not shown). However, the major part of heme a_3 in this mutant remains in a state that does not change its absorption significantly upon addition of oxidants or reductants (manuscript in preparation) and, therefore, absorption changes of E286Q are associated almost exclusively with heme *a* oxidoreduction.

DISCUSSION

Peroxidase Activity of COX is Not Inhibited by K362M Replacement. Peroxidase activity of COX proceeding via hydrogen peroxide binding with the ferric heme a_3, and the subsequent generation of compounds P and F (bold lines in Figure 1) has been resolved recently in experiments with beef heart COX (*22*) and shown to be coupled to transmembrane translocation of protons (*55*). In this work, the peroxidase activity in the oxidase from *R. sphaeroides* has been used as a tool to locate the sites of inhibition imposed by mutations in each of the two proton pathways in subunit I of the enzyme.

The key new finding of this work is that the "dead" COX mutant K362M manifests peroxidase activity similar to the wild-type enzyme. Apparently, the step (or steps) in the COX catalytic cycle blocked by the K362M replacement is by-passed when H_2O_2 is used as the electron acceptor. In contrast, the mutants in the D-channel (D132N and E286Q) show inhibition of the peroxidase activity concomitant with the loss of normal (i.e., oxidase) catalytic function.

For a number of practical reasons, the peroxidase activity has been measured in this work under conditions far from those yielding the maximal rate of the reaction. At $[H_2O_2] < 10^{-2}$ M, the steady-state peroxidase activity of COX is proportional to peroxide concentration with either ferrocytochrome *c* or ferrocyanide as the electron donor (*22, 36*) and turnover is limited by the low second-order rate constant of H_2O_2 binding to ferric heme a_3 ($\sim 10^3$ M^{-1} s^{-1} (*14, 59*)). At the same time, the concentration of hydrogen peroxide cannot be raised beyond a few millimolar to avoid denaturation of the oxidase, nonenzymatic oxidation of cytochrome *c* by peroxide, and other undesirable reactions. Therefore, the peroxidase reaction as assayed here or in refs 22, 25, 35, 36, 37, and 55 is rather slow (<10 s^{-1}). As shown in the accompanying paper (*65*), the difficulties can be partly overcome by suppressing the microperoxidase activity of cytochrome *c* by imidazole and by using a double-mixing stopped-flow technique to avoid prolonged incubation of COX or cytochrome *c* with H_2O_2. This allows the peroxidase activity measurements to be extended to higher concentrations of H_2O_2. Under such conditions, the turnover number for cytochrome *c* peroxidase activity of the K362M oxidase has been shown to be as high as 50 s^{-1} (*65*).

Despite any limitations of the current work, three important pieces of evidence indicate that electron flow to H_2O_2 in COX is not affected by the K362M replacement to an extent comparable with inhibition of the overall oxidase activity of the mutant enzyme.

(1) Even at H_2O_2 concentrations as low as 1 mM, the peroxidase activity of K362M is at least 30-fold higher than the residual oxidase activity.

(2) Whereas the liposome-reconstituted K362M oxidase does not manifest either membrane potential generation or proton pumping with ferrocytochrome *c* as the electron donor and oxygen as the electron acceptor, the addition of hydrogen peroxide under these conditions results in both the generation of $\Delta\Psi$ and proton pumping with a H^+/e^- ratio comparable to that observed for the peroxidase reaction of the bovine (*55*).

(3) Under conditions allowing for direct comparison, the peroxidase activity of the K362M mutant is similar to that of the wild-type enzyme.

Therefore, the peroxidase part of the COX catalytic cycle is not affected significantly under the conditions examined in this work by the replacement of K362 by methionine in the K-channel.

Which Steps Are Inhibited by the Mutations in the D-Channel? Specific decoupling of proton pumping from electron transfer in the bo_3-type quinol oxidase from *E. coli* (*46*) and aa_3-type COX from *R. sphaeroides* (*11*) by nonconservative replacements of D132 (D135 in the *E. coli* enzyme) resulted in the suggestion that this residue and the II/III loop region of subunit I are part of the proton-conducting pathway involved in transmembrane translocation of pumped protons by the heme−copper oxidases. As shown here, no peroxidase activity can be observed in the D-channel mutants of the *R. sphaeroides* COX. It seems likely that mutations within the D-channel directly inhibit the proton-pumping peroxidase portion of the COX cycle (Figure 1). Suppression of intraprotein proton transfer has been demonstrated directly for the F → O transition in the D132N mutant (*21*), but whether the P → F step is inhibited to the same extent remains yet to be established. Also, whether the mutations in the D-channel might impair in addition the eu-oxidase portion of the reaction cycle (O → R) cannot be inferred from the existing data. According to the experiments examining the reduction of the enzyme by dithionite, the O → R transition is not inhibited in the D132N mutant. Results from the flow-flash studies by the Brzezinski group (*2*) indicate that the R → P transition is similar for the D132, E286Q, and wild-type. These data suggest that both the mutations in the D-channel block the reaction cycle after the formation of compound P.

Which Redox Step of the Catalytic Cycle Is Inhibited by K362M Mutation? The present finding that K362M mutation does not impair the steady-state activity of the enzyme with hydrogen peroxide as electron acceptor makes it unlikely that any of the steps within the peroxidase (P → F → O) half of the cycle is inhibited. That the cycle is arrested prior to the formation of the P state follows also from the steady-state spectra of the K362M mutant (*21*). Apparently, the severe inhibition imposed by the mutation is associated with the eu-oxidase (O → R → Oxy → P) part of the catalytic cycle (Figure 1). These steps include (1) the addition of the first two electrons to the oxidized heme-copper center, probably linked to the uptake of two protons from the N-phase (*52*); (2) transient binding of O_2 to Cu_B and its subsequent migration to the reduced heme a_3 (*51, 52*); and

(3) reduction of the bound oxygen to bound peroxide (607 nm species) (*32, 43*).

Oxidation of the fully reduced *E. coli* bo_3-type quinol oxidase with the K362M mutation proceeds with kinetics virtually the same as that of the wild-type enzyme (*44*). Similar results have been obtained recently with the K362M mutant of the *R. sphaeroides* oxidase (*3*). These data imply that neither the R-to-Oxy nor Oxy-to-P steps is impaired by the mutation. By exclusion, one can infer the O-to-R transition to be inhibited by the K362M mutation. This conclusion is now directly confirmed by the finding that the reduction of heme a_3 by dithionite is greatly impeded in the K362M oxidase. A similar observation has been described in recent papers (*17, 19*).

It is noteworthy, that the kinetics of the dithionite-induced reduction of K362M resembles that of the so-called "slow" or "resting" form of mitochondrial COX (*10, 30, 64*). Accordingly, one could suggest that K362M enzyme as isolated is simply in a "slow" form and inhibition of the reduction of heme a_3 is not a specific consequence of the mutation. However, this is not the case since K362M does not show other features typical of the "slow" form, such as poor reactivity of the oxidized enzyme with cyanide (*19*) or with hydrogen peroxide (*21* and Pecoraro et al., manuscript in preparation). Therefore, it is justified to conclude that the K362M mutation impairs the O → R transition, and it is tempting to relate the inhibition to the potential role of the K-channel in proton transfer.

Evidence suggests that electron transfer from heme *a* to heme a_3 during the O → R step is controlled by a coupled proton uptake from the N-phase (*52*). Complementary experiments have also demonstrated that the back-flow of electrons from heme a_3 to heme *a* is also controlled kinetically and thermodynamically by deprotonation of some group in the environment of heme a_3 (*1, 3*). It is reasonable to propose provisionally that the K-channel is involved in loading the protons into the enzyme concomitant with the initial two-electron reduction of the heme-copper binuclear center. One reasonable possibility is that the K-channel is required for the protonation of the heme a_3- and/or Cu_B-bound hydroxyl anions to water upon reduction of these redox centers.

Roles of the Two Putative Proton Channels of COX. As the K-channel is not required for the peroxidase portion of the COX catalytic cycle, it is likely that all the protons necessary for the P → F and F → O steps of the COX cycle can be delivered in this mutant by the D-channel. These results disagree with the concept of separate proton channels for "chemical" and "pumped" protons involved in each of these two proton pumping steps of oxygen reduction (*18, 31*), but they favor the suggestion that the two channels are functionally associated with different parts of the catalytic cycle: the K-channel being operational during the eu-oxidase half-reaction and the D-channel during the peroxidase phase of the reaction cycle (*21, 42*). At this time, the actual number of protons taken up via the K-channel during a single turnover of the enzyme cannot be specified, and might be as low as zero (*19*), but probably not more than four (*21*) (see below).

A simple provisional operative scheme would require the translocation of the first two protons in the cycle (starting from the oxidized state) via the K-channel at the O → R step (e.g., protonation of the heme a_3- and/or Cu_B-bound hydroxide anions to water) and the delivery of the last four protons associated with the P → F and F → O steps via the D-channel. The situation is least clear with the second pair of protons (third and fourth out of the total of eight taken up per turnover). As pointed out in ref 21, the COX cycle may include a step of isoelectronic spontaneous conversion of the initial peroxy intermediate (P_0) analogous to compound 0 of peroxidases (*5, 27*) to the compound I-type ferryl−oxene complex F_I (perhaps identical to the 804 cm^{-1} intermediate revealed by the resonance Raman spectroscopy (*20, 34*)). Such a step is typical for the mechanism of classical peroxidases and is likely to be involved in the peroxidase cycle of COX under the conditions of the peroxidase assay in the current work. As the P_0 → F_I transition involves heterolytic scission of the O−O bond and formation of the first H_2O molecule, it can be coupled to proton uptake from the N-phase (*21*) (two protons to form H_2O or one to form metal-bound OH^-). The insensitivity of this step to the K362M mutation may imply that the protons are delivered by the D-channel. However, in case of the peroxidase assay, the pair of protons corresponding to the uptake of the third and fourth H^+ in the oxidase catalytic cycle may be essentially delivered to the binuclear center by hydrogen peroxide itself, thus by-passing the normal conduction pathway via the input proton channel (*63*). Therefore, the insensitivity of the peroxidase activity of COX to the K362M mutation does not rule out the possibility that during the normal oxidase catalytic cycle, the K-channel is involved also in this P_0 → F_I step.

It is interesting to note in this context that the K-channel leads to the hydroxyl of tyrosine-288 (*18, 38, 48*), which could, in turn, provide a proton directly to the dioxygen at the enzyme active site and, thus, catalyze the cleavage of the O−O bond. The hydroxyl moiety of Y288 does not appear to be close enough to be an effective proton donor to the oxygen atom directly bound to the heme Fe, so the delivery of all substrate protons during normal turnover after O−O bond cleavage must come from another pathway. This could provide a simple mechanistic explanation as to why the K-channel is required only for the first portion of the catalytic cycle.

The recent discovery (Yoshikawa, personal communication; *38*) that Y288 is covalently linked to H284, which is a ligand to Cu_B, makes this a particularly attractive possibility, as it allows for direct control over the proton-donating ability of Y288 by the redox state of Cu_B. The possibility that Y288 is both an electron and a proton donor to the distal oxygen atom, hence forming a neutral tyrosine radical upon cleavage of the O−O bond, is also an attractive variant of this mechanism.

Note that in this model the protons taken up by the enzyme upon both reduction and upon heterolytic cleavage of the O−O bond are used directly in the formation of the product (water) and not to "charge" the proton pump.

The proposed limitation of Y288 to participate in proton (or hydrogen) donation only to the distal oxygen atom (Fe−O−**O**) provides a reasonable explanation for the apparently limited role of the K-channel to the initial portion of the catalytic cycle and the behavior of the K362M mutant. Several aspects of this model are subject to experimental verification, and this work is in progress.

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