Substitution of Lysine-362 in a Putative Proton-Conducting Channel in the Cytochrome *c* Oxidase from *Rhodobacter sphaeroides* Blocks Turnover with O_2 but Not with H_2O_2

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ABSTRACT: The recently reported X-ray structures of cytochrome oxidase reveal structures that are likely proton-conducting channels. One of these channels, leading from the negative aqueous surface to the heme a_3/Cu_B bimetallic center, contains a lysine as a central element. Previous work has shown that this lysine (K362 in the oxidase from *Rhodobacter sphaeroides*) is essential for cytochrome *c* oxidase activity. The data presented demonstrate that the K362M mutant is impeded in the reduction of the heme a_3/Cu_B bimetallic center, probably by interfering with the intramolecular movement of protons. The reduction of the heme−copper center is required prior to the reaction with dioxygen to form the so-called peroxy intermediate (compound P). This block can be by-passed to some extent by the addition of H_2O_2, which can react with the enzyme without prereduction of the heme−copper center and can then be reduced to water using electrons from cytochrome *c*. Hence, the K362M mutant, though lacking oxidase activity, exhibits cytochrome *c* peroxidase activity. Rapid mixing techniques have been used to determine the kinetics of this peroxidase activity at concentrations of H_2O_2 up to 0.5 M. The K_m for peroxide is about 50 mM and the V_{max} is 50 electrons s^{-1}, which is considerably slower than the turnover that can be obtained for the oxidase activity of the wild-type enzyme (1200 s^{-1}). The turnover of the mutant oxidase with H_2O_2 appears to be limited by the rate of reaction of the enzyme with peroxide to form compound P, rather than the rate of reduction of compound P to water by cytochrome *c*. The data require a reexamination of the proposed roles of the putative proton-conducting channels.

Cytochrome *c* oxidase from *Rhodobacter sphaeroides* is a member of the superfamily of heme−copper respiratory oxidases that includes the eukaryotic mitochondrial cytochrome *c* oxidase as well as most prokaryotic (*5*−*7*). All of the oxidases in this superfamily share the same structural features of the catalytic site at which the reduction of dioxygen is catalyzed, namely, a bimetallic center consisting of a high-spin heme (heme a_3 in the case of the *R. sphaeroides* oxidase) and a copper atom (Cu_B). The initial binding of O_2 requires the prereduction of the heme−copper center by two electrons, which is coupled to the uptake of two protons by the enzyme (*19*, *20*, *33*). The reduced binuclear center (R) reacts with O_2 to form "compound P" (*22*, *27*), the structure of which is still being debated (*6*, *14*). One-electron reduction of compound P yields the oxoferryl complex (compound F) of heme a_3 which is structurally related to compound II of peroxidases (i.e., heme $Fe^{4+}{=}O^{2-}$) (*4*, *19*, *20*, *33*, *34*). Finally, the one-electron reduction of compound F completes the catalytic cycle and returns the enzyme to the oxidized state (Ox) (*4*, *23*, *34*). Recent studies of the kinetics of the oxidation of the fully reduced oxidase from bovine heart mitochondria have explored the details of this fundamental scheme (*3*, *4*, *14*, *22*, *27*).

The heme−copper oxidases are of particular interest because these membrane-bound enzymes conserve the considerable free energy, made available by the reduction of dioxygen to water, in the form of a transmembrane proton electrochemical gradient ($\Delta\mu H^+$). This is done by two means. First, since the electrons and protons used for the formation of H_2O from O_2 originate from different sides of the coupling membrane, the chemical reaction itself causes electric charge separation and hence the generation of a membrane potential. Second, the heme−copper oxidases are proton pumps, using the driving force of the chemical reaction to actively transport protons from the electronegative to the electropositive side of the membrane (1 H^+/e^-).

The recently reported high-resolution structures of the cytochrome oxidases from both bovine heart mitochondria (*30*, *31*) and from *Paracoccus denitrificans* (*12*, *25*) provide the basis for understanding the catalytic and pumping mechanisms and the roles of specific amino acids. In both crystal structures, lysine-362 (*R. sphaeroides* numbering; K354 in *P. denitrificans* and K319 in the bovine heart oxidase) is observed to be a central component in what appears to be a channel leading from the bulk aqueous phase on the electronegative side (bacterial cytoplasm) to the heme−copper center (*12*, *25*, *30*). It has been proposed (*12*) that this channel, here referred to as the K-channel, functions to deliver protons used for the conversion of O_2 to H_2O, so-called "chemical" protons. Mutants in which K362 has been substituted would be expected to be inactive, and this has been previously demonstrated to be the case both for

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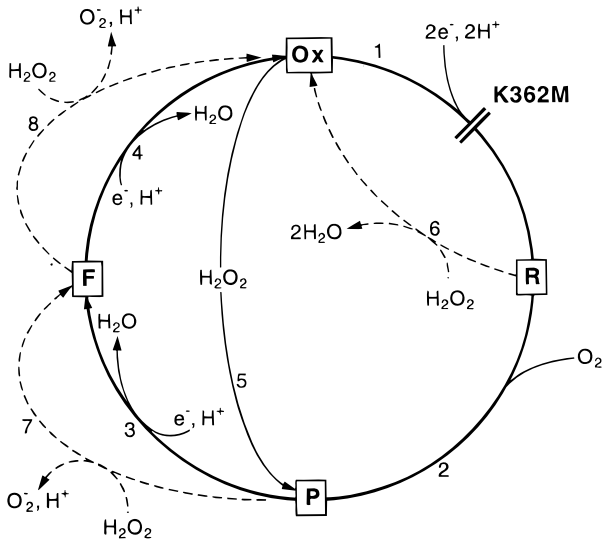

FIGURE 1: Interconvertion of different intermediates of the cytochrome oxidase catalytic cycle in the presence of O_2, H_2O_2, and reducing equivalents. Reaction 1 is a two-electron reduction of the heme−copper binuclear center, which is obligatory for O_2 to bind. The reaction is accompanied by the uptake of two protons. The addition of O_2 results in compound P formation (reaction 2). The single-electron reduction of compound P results in the formation of compound F, the reaction being coupled to the uptake of a proton. The addition of the fourth electron completes the catalytic cycle, regenerating the oxidized form of the enzyme (reaction 4). In the presence of H_2O_2 compound P can be formed in the reaction of the oxidized enzyme with 1 equiv of H_2O_2 (reaction 5), forming a bypass around reaction 1, which is blocked by the K362M mutation. Reaction 6 represents the oxidation of the binuclear center by H_2O_2, which makes possible the catalytic cycle route 1−6 for the peroxidase reaction of the wild-type cytochrome oxidase, but not with the K362M mutant. Reactions 7 and 8 represent secondary reactions of compounds P and F with hydrogen peroxide, which produce superoxide radicals.

the *R. sphaeroides* oxidase (*1*, *11*, *13*) and for the cytochrome bo_3 quinol oxidase from *E. coli* (*28*, *29*).

Recent models of the mechanism of proton pumping propose that the protons taken up by the enzyme prior to the formation of compound P are used to "prime" the pump (*12*, *21*, *26*). That is, these protons are not "chemical" protons but are, rather, use to preload the pump mechanism. If the K-channel were required strictly for "chemical" protons, this would predict that the K362M mutant would be blocked in the catalytic cycle after the formation of compound P but not prior to this intermediate. To examine the extent of a possible block following the formation of compound P, the cytochrome *c* peroxidase activity of the K326M oxidase was examined. Previous work has established that H_2O_2 can serve as an alternate electron acceptor from the bovine cytochrome oxidase (*2*, *16*, *17*, *24*). H_2O_2 reacts directly with the oxidized bovine enzyme to yield the "peroxy" intermediate, compound P (*40*). The reduction by cytochrome *c* of compound P, first to the oxoferryl state and then to the fully oxidized form of the enzyme is coincident with the "peroxidase" portion of the catalytic cycle with oxygen (Figure 1) (*15*, *35*, *37*). The work of Vygodina et al. (*37*) demonstrates that the K362M oxidase does have cytochrome *c* peroxidase activity, and the current work establishes a maximum turnover rate (V_{max}) of 50 s^{-1}. The primary inhibition in the oxidase cycle due to the K362M mutant is prior to the formation of compound P.

MATERIALS AND METHODS

The K362M mutant of the *R. sphaeroides* cytochrome oxidase was engineered with a histidine tag at the carboxyl terminus of subunit I and was isolated by affinity chromatography as described previously (*18*). The enzyme concentration was determined using $\Delta\epsilon_{605-700} = 40$ mM^{-1} cm^{-1} for the spectrum of the fully reduced enzyme. All reagents used were analytical grade. The concentration of hydrogen peroxide was determined by using $\epsilon_{240} = 40$ M^{-1} cm^{-1}.

The absorption spectra of the oxidase were recorded using an SLM-Aminco DW2000 spectrophotometer. The stopped-flow experiments were performed using an Applied Photophysics SM17X with either a photomultiplier or photodiode array detection. The diode array was used to collect oxidase spectra during steady-state peroxidase turnover. The rate of cytochrome *c* oxidation was measured using either a Shimadzu UV-2101PC spectrometer, for slow rates, or the photomultiplier detector of the stopped-flow machine.

The cytochrome *c* peroxidase activity was measured in the presence of imidazole (10−100 mM) in order to reduce to negligible levels the background due to the nonenzymatic oxidation of cytochrome *c* by H_2O_2. The background was reasoned to be due to a small proportion of high-spin cytochrome *c* in the population. The addition of imidazole to the assay was found to effectively eliminate this problem without any deleterious effects on the enzymatic assay. The concentration of oxidase used was between 40 and 100 nM for the steady-state peroxidase assays.

RESULTS

Aerobic Oxidase Activity of the K362M Mutant. Consistent with published data, the purified K362M mutant enzyme is almost totally inactive as a cytochrome *c* oxidase (*11*). The activity under the conditions examined does not exceed 5 min^{-1}, which is 20 000-fold less than that of the wild-type oxidase. The spectrum of the enzyme under steady-state turnover conditions shows that heme *a* is reduced (peaks at 445 and 605 nm), but that heme a_3 remains in the ferric form, as judged by the absorption maximum near 416 nm (*15*); also see trace b in Figure 2A. This suggests that the most severely blocked step in the catalytic cycle is not the breakdown of compound P but the reduction of the heme−copper binuclear center (step 1, Figure 1). Otherwise, the oxidized form of the heme−copper center would not accumulate under steady-state conditions.

The inhibition in the rate of reduction of the fully oxidized K362M mutant has been reported (*11*). To measure any effects by the mutation specifically on the rate of reduction of heme a_3, the K362M mutant enzyme was first reduced aerobically by a mixture of ascorbate and ruthenium hexamine, thus reducing heme *a* (see Figure 2A, trace b), followed by the addition of dithionite, which eliminates O_2 from the solution and permits the reduction of heme a_3. The deaeration of the solution by dithionite results in absorption changes in the visible spectrum of the enzyme (Figure 3). The difference spectrum (Figure 3A) has a minimum at 413 nm and maximum at 446 nm, indicating that it is the reduction of heme a_3 which is being monitored (ferrous-minus-ferric). These optical changes are biphasic, with a fast phase occurring with a half-time of about 40 s and a slow phase taking tens of minutes to develop (Figure 3B).

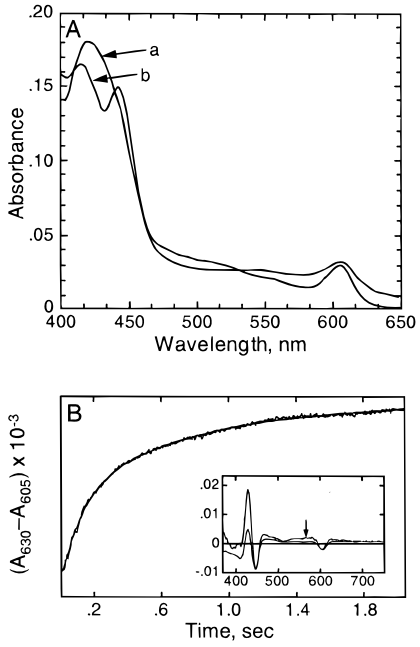

FIGURE 2: Reduction of the K362M mutant (panel A) and the oxidation of heme *a* by H_2O_2 (panel B). Heme *a* of the K362M cytochrome oxidase was partly prereduced by incubation of the enzyme with 10 mM ascorbate, which reduces the enzyme slowly (trace a in panel A). The addition of ruthenium hexamine results in further reduction of heme *a* (trace b). The solution (in 50 mM phosphate buffer, pH 7) was mixed in a 1:1 ratio with 400 mM H_2O_2. The reaction was followed by means of diode array spectrometer with data point collection each 2.5 ms. Subsequent analysis reveals two components of the response, the spectra of which are shown in the insert to panel B. The rate of oxidation of heme *a* was monitored by the absorbance difference 630 nm − 605 nm and is shown in panel B.

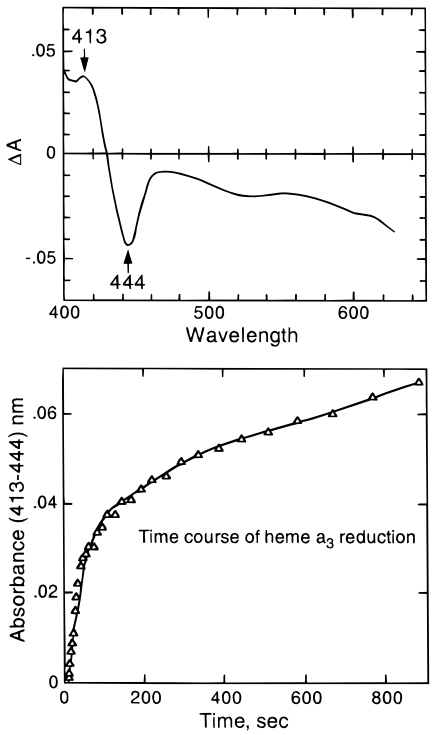

FIGURE 3: Reduction of heme a_3 in the K362M mutant under anaerobic conditions. The starting conditions are as described in the legend to Figure 2. The oxidase with heme *a* prereduced by ascorbate was mixed with dithionite-containing solution in a stopped-flow apparatus to deaerate the sample and, thus, permit reduction of heme a_3. The response is biphasic. The spectrum represents the fast component of the response, and that of the slow component is essentially the same. This corresponds to the reduction heme a_3. The time course of the reduction of heme a_3 is given in panel B.

The extent of the slow phase varies with the enzyme preparation, and in some cases is negligible. The so-called "fast" phase (0.025 s^{-1}) of the reduction of heme a_3 is very slow in comparison to that expected with the wild-type oxidase, and this is a direct measure of the inhibition due to the K362M mutation of the observed electron-transfer rate between reduced heme *a* and the oxidized heme−copper center. Since the reduction of the heme−copper center requires the uptake of two protons (step 1 in Figure 1), perhaps the K362M mutation impedes the reduction of the metals by preventing the coupled proton uptake required to stabilize the reduced heme−copper center (*33*).

Steady-State Peroxidase Activity of the K362M Mutant. As shown schematically in Figure 1 (step 5), compound P can be formed directly upon the binding of H_2O_2 to the fully oxidized enzyme (Ox) without preliminary reduction of the heme−copper center (*40*) and can then be reduced to regenerate the oxidized form of the enzyme, resulting in peroxidase activity (*2*, *24*). Analagous procedures have been used with cytochrome P-450, and this is referred to as a "peroxide shunt".

Indeed, the mutant does exhibit peroxidase activity (steps 5 → 3 → 4 in Figure 1) (also see ref 37). At low concentrations of H_2O_2, the rate of the cytochrome *c* peroxidase reaction increases linearly with H_2O_2 concentration with a slope of about 1000 M^{-1} sec^{-1} (quantifying cytochrome *c* oxidation), corresponding to a rate constant of 500 M^{-1} sec^{-1} for the binding of H_2O_2 to the enzyme, since two electrons are required to reduce peroxide to water. Note that the reactions of compound P and compound F with excess H_2O_2 (steps 7 and 8) are very slow compared to the reaction with cytochrome *c* (steps 3 and 4) (*36*, *38*), so it is justified to assume 2 equiv of cytochrome *c* oxidized per mole of H_2O_2 in the cytochrome *c* peroxidase reaction.

If the cytochrome *c* peroxidase turnover were to approach the rate of the oxidase activity of the wild-type oxidase, this rate would require a concentration of about 10 M H_2O_2 (K_m of H_2O_2 of about 1 M. To experimentally measure the rate of the cytochrome *c* peroxidase activity at high levels of H_2O_2, it is necessary to use rapid mixing to avoid complications due to possible deleterious reactions of peroxide with the enzyme. Typical traces of cytochrome *c* oxidation monitored at 550 nm are shown in Figure 4. It is evident that the reaction rate saturates, since there is little increase of the rate as the peroxide concentration is increased from 100 to 500 mM. The concentration dependence of the turnover of cytochrome *c* as a function of H_2O_2 concentration is shown in Figure 5. The peroxidase activity of the K362M mutant exhibits classical Michaelis−Menten kinetics, but saturates at much lower concentrations than expected, with a K_m for H_2O_2 of 50 mM and a V_{max} of 50 s^{-1} (electron turnover, equivalent to 25 s^{-1} peroxide turnover).

It is appropriate to compare the rate of the peroxidase reaction of the K362M mutant with the oxidase activity, but not the peroxidase activity, of the wild-type enzyme. The cytochrome *c* peroxidase activity of the wild-type enzyme can proceed, in principle, by either of two mechanisms

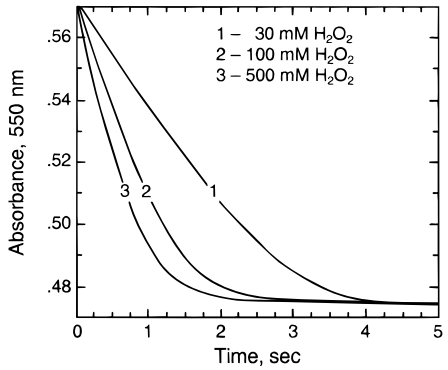

FIGURE 4: Saturation of the cytochrome *c* peroxidase activity at high concentrations of H_2O_2. A stopped-flow machine with sequential mixing was used for the peroxidase activity assay. First, a solution of 40 μM ferrocytochrome *c* and 200 mM imidazole in 50 mM phosphate buffer, pH 7, was mixed with an equal volume of 500 nM K362M. The resulting mixture was mixed in a 1:1 ratio with solutions of H_2O_2 ranging from 6 mM to 1 M. The resulting final concentrations of H_2O_2 are shown.

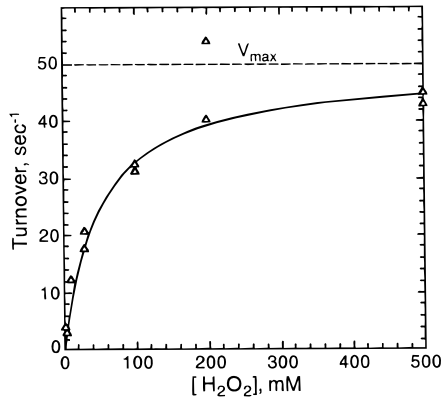

FIGURE 5: Dependence of the cytochrome *c* peroxidase activity of the K362M mutant on the concentration of H_2O_2. The number of cytochrome *c* molecules oxidized in 1 s by a molecule of K362M is given as a function of the concentration of H_2O_2. The reaction behaves in accordance with Michaelis−Menten kinetics with a K_m for H_2O_2 of 50 mM and V_{max} of 50 s^{-1} electrons.

(Figure 1). Peroxide can interact with the reduced binuclear center (route 1−6 in Figure 1) or by first binding to the oxidized binuclear center (route 5 → 3 → 4 in Figure 1). The favored pathway in the wild-type enzyme is through the reduced binuclear center (route 1−6), because the rate of reaction of peroxide with the reduced binuclear center is very fast (data not shown). The cytochrome *c* peroxidase reaction of the wild-type enzyme under anaerobic conditions is even faster than the oxidase reaction (data not shown). However, this pathway does not proceed through compound P and does not share steps 3 and 4 (Figure 1) with the dioxygen reduction mechanism. Hence, the peroxidase activity of the wild-type enzyme does not proceed by a route that corresponds to the mechanism that must be utilized by the K362M mutant (route 5 → 3 → 4).

The 20-fold difference between the V_{max} for the oxidase activity of the wild-type oxidase and the peroxidase activity of the K362M mutant can, in principle, be due to either of two reasons. First, the rate of binding of H_2O_2 to the enzyme (step 5 in Figure 1) might saturate and be rate-limiting in the entire range of H_2O_2 concentrations studied. Second, the rate of binding of H_2O_2 might not saturate, so that the rate-limitation of the peroxidase cycle at high concentrations of H_2O_2 would arise from an effect of the K362M mutation on the rate of reduction of compound P (steps 3 and 4 in Figure 1).

Oxidation of Reduced Heme a by H_2O_2 in the K362M Mutant. The addition of ascorbate to the K362M oxidase results in the partial reduction of heme *a* whereas heme a_3 remains oxidized (Figure 2A). That reduction of heme *a* is incomplete is demonstrated by the further addition of ruthenium hexamine, which reduces the magnitude of the peak at 445 nm (Figure 2A). Since the midpoint potential of Cu_A is lower than that of heme *a* and the two centers are in rapid equilibrium, under these conditions it is expected that most of the Cu_A is oxidized. Hence, a substantial fraction of the oxidase is one-electron-reduced under these circumstances. The addition of H_2O_2 is expected to form compound P transiently and then compound F, since the binding of H_2O_2 permits electron transfer from heme *a* to the heme−copper center, as shown below (Ox, P, and F refer to the status of the heme a_3/Cu_B center).

$$H_2O_2 + \text{heme } a^{2+}\cdot\text{Ox} \xrightarrow{\text{step 5a}} [\text{heme } a^{2+}\cdot\text{P}] \xrightarrow{\text{step 3a}} \text{heme } a^{3+}\cdot\text{F}$$

These reactions are the equivalent of steps 5 and 3 in Figure 1, except that heme *a* has been prereduced prior to the addition of peroxide. Hence, this experiment essentially measures the rate of reaction of the partially reduced enzyme with H_2O_2 under conditions where only a single turnover of H_2O_2 will be observed.

The course of the reaction was followed by using a stopped-flow diode array spectrophotometer to record the spectrum of the enzyme. Spectroscopic analysis with the SVD procedure using the software provided by Applied Photophysics revealed biphasic kinetics, and the spectroscopic changes associated with each phase are similar, indicating in both phases the simultaneous oxidation of heme *a* and the formation of compound F at the heme−copper center (Figure 2B). At 200 mM H_2O_2 ($4K_m$ for the cytochrome *c* peroxidase activity), the rapid phase has a calculated pseudo-first-order rate constant of 40 s^{-1} and represents about 60% of the total change observed. The slower phase accounts for the remaining 40% and has a pseudo-first-order rate constant of 1.3 s^{-1}. These results suggest that there are two populations of enzyme and that the turnover with H_2O_2 is due to only about 60% of the total enzyme present. Considering that there are two molecules of cytochrome *c* oxidized per H_2O_2 in the cytochrome *c* peroxidase reaction, the observed V_{max} can be reasonably accounted for by this 60% of the enzyme population: 40 $s^{-1} \times 0.6 \times 2 = 48$ s^{-1}.

The obligatory intermediate, [heme a^{2+}·P], is not observed during the experiment, i.e., there is no delay between the binding of H_2O_2 and the oxidation of heme *a*. This demonstrates that the electron transfer from heme *a* and reduction of compound P (step 3a, above) are at least several-fold faster than the formation of compound P (step 5a, above) and, subsequently, must be substantially faster than the V_{max} of the cytochrome *c* peroxidase reaction (50 s^{-1}). These data strongly suggest that it is the rate of formation of compound P (step 5) that is rate-limiting for the overall reaction (steps 5 → 4 → 3).

DISCUSSION

The value of examining the peroxidase activity of cytochrome oxidase to study those steps in common with the oxidase catalytic cycle has been long recognized (*2*, *24*, *40*). This is particularly important given that proton pumping is associated with this portion of the oxidase catalylic cycle (*32*, *39*). Orii and his colleagues used strictly anaerobic conditions to study the cytochrome *c* peroxidase activity of the bovine heart oxidase (*16*, *17*, *24*). Unfortunately, there are two possible mechanisms for cytochrome *c* peroxidase activity (*17*). In addition to the reaction initiated by the interaction of peroxide with the oxidized enzyme (Steps 5 → 6 → 7, Figure 1), the reduced enzyme can be oxidized by peroxide (Steps 1−6, Figure 1) (*8*, *9*, *10*). Since the reduction of the wild-type oxidase by cytochrome *c* is relatively rapid compared to the rate of interaction with peroxide, this is a severe complication (*17*). In the K362M oxidase, this reaction does not take place because the heme−copper center is not reduced by cytochrome *c*.

The current data and those of Vygodina (*37*) demonstrate that the K362M mutant is clearly defective in the reduction of the heme−copper center prior to the binding of dioxygen. This is reasonably interpreted as being due to a defect caused by the mutation in the delivery of protons to the vicinity of the binuclear center that must accompany reduction of the metals. Other explanations are also feasible, such as the K362M mutation causing a change in the local dielectic (*13*) or a shift in the p*K* of another critical residue (H. Rottenberg, personal communication). In any event, the K-channel is very unlikely to be utilized for the delivery of all of the "chemical" protons as described in the model of Iwata et al. (*12*).

The cytochrome *c* peroxidase activity of the K362M oxidase has a V_{max} of 50 s^{-1}, which, although substantial, is much lower than the oxidase activity of the wild-type enzyme. The peroxidase activity of the K362M mutant is limited by the rate of binding of peroxide, which does not exceed 25 s^{-1}, even at saturating concentrations. For this reason, the maximal rate of reduction of compounds P and F in the K362M mutant cannot be obtained from the steady-state peroxidase activity measurements, although a minimal rate of 50 s^{-1} is established. Therefore, significant inhibition during multiple turnover of the P-to-F and F-to-Ox steps due to the K362M mutation cannot be entirely excluded. Single-turnover experiments suggest that the K362M mutant is not inhibited in these steps, particularly F-to-Ox (*1*, *15*). However, single-turnover experiments do not require that the enzyme "recharge" in preparation for the next turnover, so any defect due to the mutation in any such "recharging" step would not be detected. Hence, it is important to evaluate the results of the K362M mutation under multiple-turnover conditions and at the maximal possible turnover rates, as initiated in the current work.

A plausible explanation of the current data is that the K-channel and D-channel are utilized at different steps in the catalytic cycle (*15*, *37*). It is important to recognize, however, that it has yet to be demonstrated that mutations in the K-channel do not substantially inhibit reaction steps following formation of compound P during multiple turnover conditions and, similarly, that mutations in the D-channel have effects that are strictly limited to steps following the formation of compound P. Because these questions are still open, the existing data can be easily accommodated by models quite different from that described above. For example, the model of Iwata et al. (*12*) can be rationalized with the existing data by switching the roles of the K-channel and the D-channel and by asserting that pumped protons can be used at a slow rate for oxygen chemistry when the delivery of chemical protons is blocked. Additional experimental data are clearly required to distinguish the many possibilities.

The data presented in the current work, however, show that the cytochrome *c* peroxidase reaction catalyzed by the K362M mutant should allow the steps in the oxidase cycle following the formation of compound P to be examined in more detail and without complicating side reactions, to provide further information about the coupling of these steps to proton pumping.

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