The Entry Point of the K-Proton-Transfer Pathway in Cytochrome *c* Oxidase†

Magnus Brändén,‡ Farol Tomson,§ Robert B. Gennis,§ and Peter Brzezinski*,‡

Department of Biochemistry and Biophysics, The Arrhenius Laboratories for Natural Sciences, Stockholm University, SE-106 91 Stockholm, Sweden, and School of Chemical Sciences, University of Illinois, Urbana, Illinois 1801

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ABSTRACT: Cytochrome *c* oxidase is a redox-driven proton pump. The enzyme has two proton input pathways, leading from the solution on the N-side to the binuclear center. One of these pathways, the K-pathway, is used for proton uptake upon reduction of the binuclear center. It is also important for local charge compensation during reaction of the fully reduced enzyme with O_2. Two different locations have been proposed to constitute the entry point of the K-pathway: near S(I-299) or near E(II-101), respectively, in the *Rhodobacter sphaeroides* enzyme. The experiments discussed in this study are aimed at identifying the location of the entry point. The kinetics and extent of flash-induced proton release coupled to oxidation of heme a_3 ($\tau \cong 2$ ms at pH 8.8 in the wild-type enzyme) in the absence of O_2 were investigated in the ED(II-101), SD(I-299), and KM(I-362) mutant enzymes, i.e., at the two proposed entry points and in the middle of the pathway, respectively. This reaction was completely blocked in KM(I-362), while it was slowed by factors of 25 and 40 in the ED(II-101) and SD(I-299) mutant enzymes, respectively. During reaction of the fully reduced enzyme with O_2, electron transfer from heme *a* to the catalytic site (during P_R-formation) was blocked in the KM(I-362) and SD(I-299)/SG(I-299) but not in the ED(II-101)/EA(II-101) mutant enzymes. The results are interpreted as follows: Residue K(I-362) is involved in both proton transfer and charge compensation (in different reaction steps). The impaired proton release in the S(I-299) mutant enzymes is an indirect effect due to an altered environment of K(I-362). E(II-101), on the other hand, is likely to be part of the K-pathway since mutation of this residue results in impaired proton release but does not affect the P_R formation kinetics; i.e., the properties of K(I-362) are not altered. Consequently, we conclude that the entry point of the K-pathway is located near E(II-101).

Cytochrome *c* oxidase is a membrane-bound protein that catalyzes the reduction of oxygen to water, concomitant with the oxidation of cytochrome *c*. Four protons are needed to form water, and they are taken up by the enzyme from the N-side[1] of the membrane. Part of the energy released during oxygen reduction drives the translocation of protons across the membrane from the N-side to the P-side. The electrochemical gradient across the membrane, maintained by both the redox reaction and the translocation of protons, is used for the production of ATP catalyzed by ATPase (for review see refs *1*−*3*). Cytochrome *c* oxidase from *Rhodobacter sphaeroides* contains four redox-active metal centers. The diatomic copper center, Cu_A, located close to the surface on the P-side, is reduced by the water-soluble cytochrome *c*. The electron is then transferred via heme *a* to the binuclear center, consisting of a monatomic copper center, Cu_B, and heme a_3, where the reduction of oxygen takes place. When the binuclear center is reduced, oxygen binds to heme a_3, followed by formation of the so-called "peroxy" (P) intermediate (for review see refs *2* and *4*). At this stage the O−O bond is broken, which results in formation of a ferryl group at heme a_3 and a hydroxide at Cu_B (*5*−*9*). Four electrons are needed to break the O−O bond. Three of them are provided by the binuclear center where $Fe_{a_3}{}^{2+}$ is oxidized to $Fe_{a_3}{}^{4+}$ and $Cu_B{}^{+}$ is oxidized to $Cu_B{}^{2+}$. If the enzyme is initially reduced only by two electrons (binuclear center reduced, heme *a* and Cu_A oxidized, mixed-valence enzyme), the fourth electron is presumably taken from Y(I-288) (the intermediate is called P_M). A proton is also needed to break the O−O bond, and since no protons are taken up from the bulk solution upon formation of P_M, it must be taken internally from the enzyme (*10*), presumably from Y(I-288), which forms a neutral radical, Y•(I-288) (*11*, *12*). If all four redox centers are reduced initially, an intermediate called P_R is formed transiently (*9*). The P_R intermediate has presumably the same structure as that of P_M, but the neutral radical, Y•(I-288), is immediately rereduced by heme *a* to

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* Corresponding author. Fax: (+46)-8-153679. Phone: (+46)-8-163280. E-mail: peterb@dbb.su.se.

‡ Stockholm University.

§ University of Illinois.

[1] Abbreviations: WT, wild type; Cu_A, copper A; Cu_B, copper B; τ, time constant [$\exp(-t/\tau)$]; binuclear center, heme a_3 and Cu_B; catalytic site, the binuclear center and redox-active/protonatable groups in its immediate vicinity; N-side, negative side of the membrane; P-side, positive side of the membrane; P_M and P_R, the peroxy intermediate formed at the binuclear center upon reaction of the two-electron reduced (mixed-valence, "M") and fully reduced ("R") cytochrome *c* oxidase, respectively, with O_2 (in P_R there is one more electron at the catalytic site as compared to P_M); F, ferryl intermediate; O, fully oxidized enzyme. Mutant enzyme nomenclature: E(II-101), glutamate of subunit II at position 101; ED(II-101), replacement of E(II-101) by aspartic acid. If not otherwise indicated, amino acid residues are numbered according to the *R. sphaeroides* cytochrome *c* oxidase sequence.

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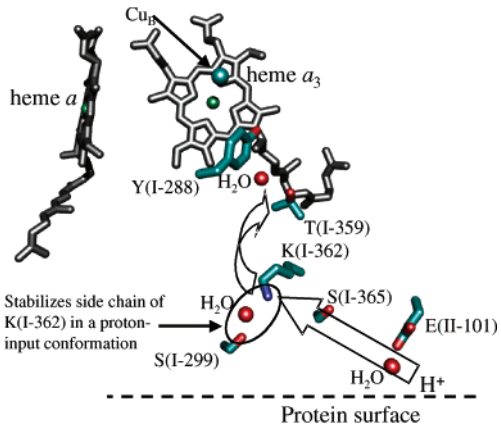

FIGURE 1: The K-pathway in cytochrome *c* oxidase from *R. sphaeroides* (*26*). The results from this work suggest that a proton is taken up from the bulk solution through E(II-101) and is likely to be transferred via S(I-365) to K(I-362). The side chain of K(I-362) moves up toward the binuclear center, and the proton is transferred to T(I-359). Next, the proton is transferred via a water molecule to the catalytic site. S(I-299) is hydrogen bonded via a water molecule to K(I-362). Mutation of S(I-299) results in an alteration of the environment of K(I-362) (see text).

produce a tyrosinate, Y^-(I-288). Thus, the P_R intermediate contains one more negative charge at the catalytic site than P_M. Formation of P_R is impaired if the highly conserved lysine [K(I-362)] is removed or its environment is modified, which was explained in terms of a movement of the positively charged side chain of K(I-362) up toward the catalytic site in order to compensate for the extra charge introduced there (*13*).

In the last two reaction steps, i.e., formation of intermediates F and O, the last electron and two protons are transferred to the catalytic site to complete the reduction of O_2 to water. The P_R to F and the F to O transitions are coupled to proton pumping across the membrane (*14*, *15*). These protons, as well as the substrate protons taken up in these two reaction steps, are transferred through the D-pathway (*16*−*18*).

During reduction, the enzyme picks up two protons from the bulk solution. At least one of these protons is transferred through the K-pathway [named after K(I-362) in the middle of the pathway] (*17*−*21*).

On the basis of an analysis of the three-dimensional structures of cytochrome *c* oxidase, electrostatic calculations, and site-directed mutagenesis (*22*−*26*), two possible entry points of the K-pathway have been proposed, namely, S(I-299) and E(II-101), both of which are conserved in oxidases from a wide range of organisms. As shown in Figure 1, S(I-299) is hydrogen bonded via a water molecule to K(I-362) (*26*). The glutamate E(II-101) is located in subunit II, and a proton could be transferred via S(I-365) to K(I-362). There is no direct connectivity between the side chain of K(I-362) and the upper part of the K-pathway. Therefore, the side chain of K(I-362) has to move up toward the binuclear center in order to transfer a proton to the next protonatable group, T(I-359). Next, the proton can be transferred toward Y(I-288) at the catalytic site.

The aim of this study was to investigate which of the two amino acid residues, S(I-299) or E(II-101) (or both), that is located at the entry point of the K-pathway. The problem was addressed by investigating the effect of mutations at positions I-362, I-299, and II-101, respectively, using two different experimental approaches. (i) Proton uptake, coupled to the reduction of the binuclear center, was investigated after flash photolysis of CO from the two-electron reduced, mixed-valence enzyme in the absence of oxygen. (ii) The flow-flash technique was used to investigate the rates and extents of formation and decay of the individual intermediates during reaction of the fully reduced enzyme with oxygen.

MATERIALS AND METHODS

Mutagenesis, Growth of Bacteria, and Enzyme Purification. Mutants of cytochrome *c* oxidase from *R. sphaeroides* at the S(I-299) position were designed using site-directed mutagenesis and have been characterized as described (*13*). The ED(II-101)/EA(II-101) strains were prepared essentially as described in ref *27*. The *R. sphaeroides* bacteria were grown anaerobically, at 30 °C, in shake incubators. The enzyme, with a histidine tag attached to subunit I, was purified using Ni^{2+}-NTA affinity chromatography (*28*). After elution, the buffer was exchanged for 100 mM HEPES−KOH at pH 7.4, containing 0.1% dodecyl β-D-maltoside, and then the enzyme was concentrated to ∼40 μM.

Catalytic Turnover Rates of the Mutant Enzymes. Cytochrome *c* was reduced by H_2 using platinum black (Aldrich) as a catalyst. The reduced cytochrome *c* was mixed with oxidized cytochrome *c* oxidase in a 50 mM phosphate buffer at pH 6.5, containing 0.1% dodecyl β-D-maltoside. The catalytic turnover rate was determined from the initial rate of oxidation of cytochrome *c*, measured at 550 nm. The turnover rates of the five mutant enzymes KM(I-362), SD-(I-299)/SG(I-299), and ED(II-101)/EA(II-101) were 0.3%, 40/100%, and 10/7%, respectively, of that of the wild-type enzyme.

Preparation of Mixed-Valence and Fully Reduced Enzymes. (A) Mixed-Valence Enzyme. The buffer of the stock solution of the oxidized enzyme was exchanged to 100 mM Bis-Tris-propane at pH 8.8, containing 0.1% dodecyl β-D-maltoside, on a PD-10 column (Pharmacia) and diluted to ∼5 μM. When proton release/uptake was measured, the buffer was exchanged to 100 mM KCl, 40 μM cresol purple at pH 8.8, and 0.1% dodecyl β-D-maltoside. The enzyme was then transferred to an anaerobically sealed cuvette, and the atmosphere was exchanged first to nitrogen and then to carbon monoxide. Carbon monoxide is a two-electron reductant and reacts with the enzyme to yield the mixed-valence state (*29*) in which Cu_A/heme *a* are oxidized and heme a_3/Cu_B are reduced with CO bound to heme a_3. Occasionally, in a fraction of the enzyme population heme *a* became reduced (three-electron reduced), in which case anaerobic ferricyanide was added until the two-electron reduced state was obtained again, as determined from the optical absorption spectra.

(B) Fully Reduced Enzyme. The stock solution of the enzyme was diluted to ∼15 μM and then transferred to an anaerobically sealed cuvette. After exchange of air for N_2, the enzyme was reduced by adding 2 mM sodium ascorbate and 1 μM PMS (phenazine methosulfate) as a mediator, followed by exchange of N_2 for CO.

Flash Photolysis of the Mixed-Valence−CO Complex in the Absence of Oxygen. The CO ligand was dissociated from the reduced binuclear center with a 5 ns laser flash (for a description of the experimental setup, see ref *13*). Optical

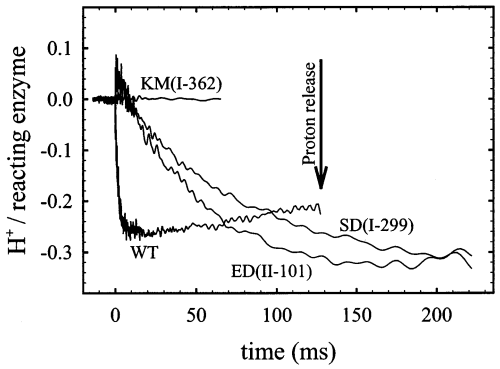

FIGURE 2: Absorbance changes of the dye cresol purple at 560 nm associated with pH changes of the bulk solution after flash photolysis of the mixed-valence enzyme−CO complex in absence of O_2. The figure shows the absorbance difference between a buffer-free and a buffered solution (to subtract any contribution arising from absorbance changes of the hemes). The concentration of the reacting enzyme was determined in each experiment from the CO-dissociation absorbance change at 445 nm. The ordinate scale shows the number of protons per enzyme molecule. Typical absorbance changes of the pH dye were $\sim 10^{-3}$. To obtain a relation between the observed absorbance changes and the number of protons released/taken up, the buffer-free solution was titrated by several consecutive additions of a 5 mM HCl solution. Experimental conditions: ~ 5 μM reacting enzyme, 40 μM cresol purple, pH 8.8, 0.1% dodecyl β-D-maltoside, 1 mM CO, and 22 °C. In addition, 0.1 M KCl or 0.1 M Bis-Tris-propane was present in the unbuffered and buffered samples, respectively.

absorption spectroscopy was used to monitor the reaction following the dissociation of CO, as described in refs *13* and *30*).

Flow-Flash Measurements of the Fully Reduced Enzyme with Oxygen. The fully reduced CO-bound enzyme was mixed with an oxygen-saturated buffer at a ratio of 1:5. The CO ligand was dissociated with a 5 ns laser flash, and the oxidation of the enzyme was followed using optical absorption spectroscopy, with a time resolution of 100 ns, using a modified custom-built combined stopped-flow/flash photolysis apparatus (LKS.60, FF.60, from Applied Photophysics, Surrey, U.K.), as described in detail in ref *13*. The cuvette path length was 1 cm.

RESULTS

Proton Transfer after Flash Photolysis of CO from the Two-Electron Reduced Enzyme in the Absence of Oxygen. The reduced state of the binuclear center is stabilized by CO, keeping heme *a* and Cu_A oxidized. When CO is flashed off, the apparent redox potential of heme a_3 decreases, which results in an equilibration of the electron between heme a_3 and heme *a* ($\tau_{WT} \cong 3$ μs). This electron transfer is followed by a slower equilibration between heme *a*/heme a_3 and Cu_A ($\tau_{WT} \cong 30$ μs). The oxidation of heme a_3 is coupled to the deprotonation of a group close to the binuclear center and the release of a proton through the K-pathway ($\tau_{WT} \cong 2$ ms at pH 8.8) (*31*, *32*). On a longer time scale, the electron in the heme *a*/Cu_A equilibrium is transferred back to heme a_3, and the proton is taken up, driven by the rebinding of the CO ligand to heme a_3. Figure 2 shows absorbance changes at 560 nm of the dye cresol purple associated with proton release from the enzyme. The proton release in the SD(I-299) and ED(II-101) mutant enzymes was slowed by factors of 40 and 25, respectively, compared to that of the wild-type enzyme. In the KM(I-362) mutant enzyme the proton release was blocked. The increase in absorbance seen in the trace measured with the wild-type enzyme in Figure 2 is due to CO recombination (i.e., proton uptake associated with the recombination reaction). It is not seen with the SD(I-299) nor with the ED(II-101) enzymes because in these mutant enzymes the CO recombination reaction is slowed dramatically (*32*).

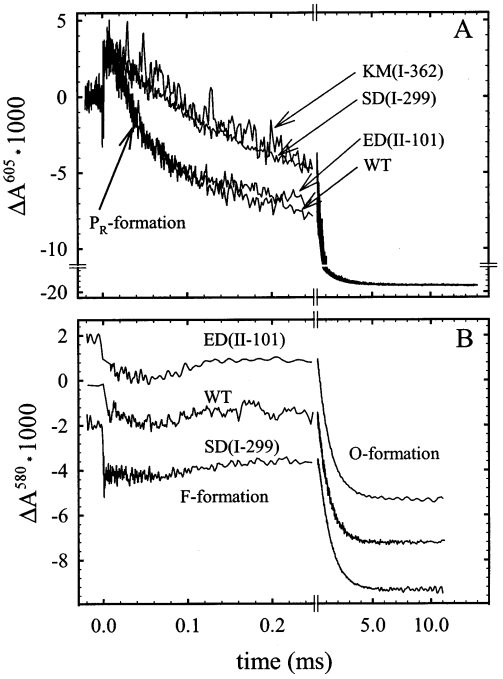

FIGURE 3: Absorbance changes after flash photolysis of CO from the fully reduced enzyme in the presence of O_2. (A) At 605 nm the redox state of heme *a* is monitored. The initial, unresolved increase in absorbance is associated with formation of intermediate A. In the wild-type enzyme this is followed by formation of P_R (oxidation of heme *a*) seen as a decrease in absorbance ($\tau \cong 60$ μs). The ED/EA(II-101) mutant enzymes display the same time constants of all transitions as those observed with the wild-type enzyme. In the SD/SG(I-299) and KM(I-362) mutant enzymes the oxidation of heme *a* is slowed and takes place with the same rate as that of F-formation ($\tau \cong 130$ μs). (B) At 580 nm the formation of the F- and O-intermediates is seen as an increase followed by a decrease in the absorbance, respectively. The mutant enzymes display the same rate constants as that of the wild-type enzyme in these two steps. Experimental conditions: 0.1 M HEPES−KOH, pH 7.4, 0.1% dodecyl β-D-maltoside, ~ 2 μM reacting enzyme (all traces have been normalized to 1 μM reacting enzyme), 1 mM O_2, and 22 °C.

Reaction of the Fully Reduced Enzyme with Oxygen. We have previously shown that electron transfer to the binuclear center during formation of the P_R-intermediate is impaired when K(I-362) or S(I-299) is modified (*13*). These results were interpreted in terms of a movement of the K(I-362) side chain to compensate for the transfer of the additional charge from heme *a* to the binuclear center (see Discussion). To test whether also mutations of E(II-101) display any effects on the P_R-formation rate, we investigated the reaction of the fully reduced enzyme with oxygen in the ED(II-101) and EA(II-101) mutant enzymes. The fully reduced enzyme with CO bound to heme a_3 was mixed with an O_2-saturated buffer solution. About 100 ms after mixing, the CO ligand was flashed off, allowing O_2 to bind to heme a_3. Figure 3A shows absorbance changes at 605 nm where heme *a* has a significant contribution. In the wild-type enzyme, oxidation of heme *a* during P_R-formation displays a time constant of

~60 μs (*10*, *33*), while in the KM(I-362) and SD(I-299)/SG(I-299) mutant enzymes, it is slowed significantly and occurs concomitant with the next phase, i.e., formation of the ferryl (F) intermediate, which is formed with a time constant of ~130 μs, both in the wild-type and in these mutant enzymes. In the ED(II-101)/EA(II-101) mutant enzymes, oxidation of heme *a* was not affected, and formation of P_R was observed with a time constant of 60 μs. The F- and O-intermediates were formed with time constants of ~130 μs and 1.1 ms, respectively, i.e., the same as with the wild-type enzyme [see increase ($P_R \rightarrow F$) and decrease ($F \rightarrow O$) in absorbance, respectively, at 580 nm; Figure 3B].

DISCUSSION

Two conserved amino acid residues, S(I-299) and E(II-101), have been proposed to constitute the proton-entry point of the K-pathway. Both residues are located near the N-side surface (see Figure 1), which excludes that they transfer protons sequentially to K(I-362). S(I-299) is located in subunit I where it is hydrogen bonded, via a water molecule, to K(I-362). The other proposed entry point, E(II-101), is located in subunit II, ~12 Å from K(I-362). Residue S(I-365) is situated between residues E(II-101) and K(I-362), and although not resolved in the crystal structure, water molecules between the three residues could ensure proton conductivity from E(II-101) to K(I-362). To determine which one of the two residues, E(II-101) or S(I-299), that is the entry point or if there could be two parallel pathways, we investigated mutant enzymes in which the two amino acid residues were modified.

One method that has been used to study the kinetics of proton release/uptake, coupled to oxidation/reduction of the binuclear center, is to flash off CO from the mixed-valence enzyme under anaerobic conditions and monitor the proton release (using pH-sensitive dyes in the bulk solution) that follows the oxidation of heme a_3. Oxidation of heme a_3 results in a lowering of the pK_a of a protonatable group close to the binuclear center, presumably a water molecule (*32*). Thus, in a pH range between the pK_as of the water molecule with oxidized and reduced heme a_3, respectively, the electron transfer is followed by proton release to the bulk solution. The deprotonation of this group is presumably coupled to further oxidation of heme a_3 and/or binding of OH^- to the oxidized heme a_3, which can be measured as a spectral change at 598 nm. Earlier measurements showed that this spectral change was not observed with the KM(I-362) nor with the TA(I-359) mutant enzymes (*17*). By measuring the changes in pH of the bulk solution using the dye cresol purple, we show here that also the proton release was blocked in the KM(I-362) mutant enzyme (see Figure 2). Similarly, the proton release was slowed by factors of 40 and 25 with the SD(I-299) and ED(II-101) mutant enzymes, respectively, compared to the rate observed with the wild-type enzyme ($\tau_{WT} \cong 2$ ms) at pH 8.8. These results are consistent with measurements of the reduction kinetics of the fully oxidized enzyme, which show that the reduction rate of the binuclear center was slowed in the SD(I-299) and ED(II-101) mutant enzymes (Tomson et al., unpublished results).

Since similar effects on the proton-transfer rates were observed when either of the two residues, E(II-101) or S(I-299), was mutated, the results suggest at first that both S(I-299) and E(II-101) are part of the K-pathway. However, on the basis of the results from this and other studies we argue below that E(II-101) is a more likely component of the K-pathway.

As indicated above, a sequential proton transfer through E(II-101) and S(I-299) can be excluded from an analysis of the enzyme structure. Assuming that the effects of the mutations at either site are local and do not effect the other site, two parallel input sites can be excluded on the basis of the following arguments. In the wild-type enzyme, the proton-release rate, k_{WT}, is ~500 s^{-1} ($\tau \cong 2$ ms at pH 8.8). If there were two parallel pathways, the measured rate with the wild-type enzyme would be a sum of the rates through E(II-101) (k_{E101}) and through S(I-299) (k_{S299}), i.e.

$$k_{WT} = k_{E101} + k_{S299} \quad (1)$$

In the ED(II-101) mutant enzyme the rate was slowed by a factor of 25, i.e., the transfer rate through the S(I-299) pathway, k_{S299}, would be ≤ 20 s^{-1}, while in the SD(I-299) mutant enzyme it was slowed by a factor of 40, i.e., $k_{E101} \leq 12$ s^{-1}, which is incompatible with eq 1. Thus, we conclude that only one of the two pathways is used for proton transfer.

To shed further light on the identity of the proton-entry point into the K-pathway, we discuss the effect of the mutations on the P_R-formation rates. During P_R-formation an electron is transferred from heme *a* to the catalytic site. To compensate for the extra negative charge introduced in the catalytic site, the positively charged side chain of K(I-362) was proposed to move toward the binuclear center (*13*). As shown previously, the formation of P_R was impaired in the SD(I-299), SG(I-299) (*13*), and KM(I-362) (*17*) mutant enzymes but, as shown in this study, not in the ED(II-101) or EA(II-101) mutant enzymes. In the KM(I-362) mutant enzyme, the side chain is not charged, and therefore electron transfer from heme *a* to the binuclear center does not occur (see ref *13*). In the SD(I-299) or SG(I-299) mutant enzymes the hydrogen bonds between the residue at I-299, the water molecule, and K(I-362) are altered (see Figure 1), which is likely to affect the charge and mobility of the K(I-362) side chain, so that it can no longer act as a charge compensator. It is worth noting that the neutral substitution, SG(I-299), has the same effect as introducing a polar/charged amino acid residue [SD(I-299)], suggesting that the substituted residue in itself does not affect the P_R-formation through electrostatic interactions. Neither the conserved substitution, ED(II-101), nor a substitution where the polar/negatively charged side chain was removed, EA(II-101), had any effect on the P_R-formation rates. These results indicate that the properties of K(I-362) are not altered upon mutation of E(II-101). Therefore, the slowed proton-release/uptake rate in the ED(II-101)/EA(II-101) enzymes is most likely due to the modification of E(II-101) itself and not due to secondary effects. The different effects of mutating E(II-101) and S(I-299), respectively, are consistent with the longer distance between K(I-362) and E(II-101) (~12 Å) than between K(I-362) and S(I-299) (4.7 Å).

Since the electron transfer from heme *a* to heme a_3 during P_R-formation is impaired in the SD(I-299) mutant enzyme, presumably due to an alteration of the K(I-362) environment, it is likely that the slower proton release in the SD(I-299) mutant enzyme is due to an indirect effect on K(I-362). In

other words, S(I-299) in itself does not take part in the proton transfer, and the slowed proton-release rate in the SD(I-299) mutant enzyme is due to an alteration in the ability of K(I-362) to transfer protons. Furthermore, as discussed above, if S(I-299) was a kinetically competent input site of the K-pathway, then a proton release with the same rate as that observed with the wild-type enzyme would be expected upon introduction of the conserved substitution in the ED(II-101) mutant enzyme, because in this mutant enzyme the side chain of K(I-362) should be unaffected and the proton could be released via S(I-299).

On the basis of the discussion above we conclude that only one of the two sites, S(I-299) or E(II-101), is the entry point of the K-pathway. Mutation of either E(II-101) or S(I-299) impairs proton release. However, while mutation of S(I-299) also alters the mobility and/or charge of K(I-362), mutation of E(II-101) has no indirect effect on K(I-362). Therefore, E(II-101) is the most likely candidate for the entry point to the K-pathway.

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