Role of the Pathway through K(I-362) in Proton Transfer in Cytochrome *c* Oxidase from *R. sphaeroides*†

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ABSTRACT: In this study we have combined the use of site-directed mutants with time-resolved optical absorption spectroscopy to investigate the role of the protonatable subunit-I residues lysine-362 (K(I-362)) and threonine-359 (T(I-359)) in cytochrome *c* oxidase from *Rhodobacter sphaeroides* in electron and proton transfer. These residues have been proposed to be part of a proton-transfer pathway in cytochrome oxidases from *Paracoccus denitrificans* and bovine heart. Mutation of K(I-362) and T(I-359) to methionine and alanine, respectively, results in reduction of the overall turnover activities to <2% and ∼35%, respectively, of those in the wild-type enzyme. The results show that in the absence of dioxygen, electron transfer between hemes a_3 and *a* with a time constant of ∼3 μs, not coupled to protonation reactions, is not affected in the mutant enzymes. However, the slower electron transfer between hemes a_3 and *a*, coupled to proton release with a time constant of ∼3 ms (at pH 9.0) is impaired in the KM(I-362) and TA(I-359) mutant enzymes. This is consistent with the slow reduction rate of heme a_3 in the oxidized KM(I-362) enzyme because in the wild-type enzyme reduction of heme a_3 is coupled to proton uptake. On the other hand, when reacting with O_2, both the wild-type and mutant fully reduced enzymes become oxidized in ∼5 ms, and proton uptake on this time scale is not affected. Hence, the results indicate that the KM(I-362) mutant enzyme is inactive because the proton-transfer pathway through K(I-362) and T(I-359) is involved in proton uptake during reduction of the oxidized binuclear center. Proton uptake during oxidation of the fully reduced enzyme takes place through a different pathway [through E(I-286) (Ädelroth, P., et al. (1997) *Biochemistry 36*, 13824−13829)].

Cytochrome *c* oxidase catalyzes oxidation of cytochrome *c* by dioxygen and conserves part of the energy of this reaction by creating an electrochemical proton gradient across a membrane (for a recent review, see *1*). During turnover, electrons are first transferred from cytochrome *c* to Cu_A[1] and then to heme *a* and the binuclear center heme a_3/Cu_B, where O_2 is reduced to water. In the bovine enzyme, protons used for reduction of dioxygen to water (substrate protons) as well as the pumped protons are taken up from the same side of the membrane (*2*). Consequently, both types of protons contribute to the maintenance of the electrochemical proton gradient

$$4\text{cyt } c^{2+} + O_2 + 4H^+_{S1} + 4H^+_{P1} \rightarrow 4\text{cyt } c^{3+} + 2H_2O + 4H^+_{P2} \quad (1)$$

where indexes "1" and "2" indicate the proton input and output sides of the membrane, respectively, and indexes "P" and "S" indicate the pumped and substrate protons, respectively. It has been suggested that the pumped and substrate protons are transferred through different pathways (e.g., *3*, *4*).

The three-dimensional structures of cytochrome *c* oxidase from *Paracoccus denitrificans* (*4*) and bovine heart (*5*, *6*) have been determined to atomic resolution. In the *P. denitrificans* structure two proton-input pathways in subunit I were identified. One pathway includes the protonatable residues aspartate 132 (D(I-132))/glutamate 286 (E(I-286)), and the other includes lysine 362(K(I-362))/threonine 359 (T(I-359)), here denoted D- and K-pathways, respectively. On the basis of studies of site-directed mutants of D(I-132) (D(I-135) in cytochrome bo_3 from *Escherichia coli*), which lost their ability to pump protons while retaining some of their electron-transfer ability (*7*, *8*), the D-pathway was suggested to be used for pumped protons. On the basis of a proposal of Hosler et al. (*3*), the K-pathway was suggested to be used for substrate protons. The K- and D-pathways were also found in the bovine enzyme (*6*), but the connection between E(I-286) (E(I-242) in the bovine enzyme) and the binuclear center was less obvious.

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[1] Abbreviations and definitions: R, fully reduced enzyme; A, ferrous-oxy intermediate; P, peroxy intermediate; F, ferryl intermediate; O, fully oxidized enzyme; ET, electron transfer; PT, proton transfer; Cu_A, copper A; Cu_B, copper B; WT, wild-type; *substrate proton*, a proton used for reduction of O_2 to water (cf. *pumped proton*); τ, time constant (in $\exp(-t/\tau)$); p*K* is used for pK_a; amino acid and mutant-enzyme nomenclature: e.g. K(I-362) denotes lysine-362 of subunit I, KM(I-362) denotes a replacement of lysine-362 of subunit I by methionine. If not otherwise indicated, the amino acid numbering is based on the *R. sphaeroides* cytochrome *c* oxidase sequence.

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In this study we have investigated internal electron-transfer reactions and proton-transfer coupled electron transfer in the absence of O_2 in mutant enzymes in which the residues lysine (I-362) and threonine (I-359) were replaced by methionine (KM(I-362)) and alanine (TA(I-359)), respectively. In addition, we have used the so-called flow-flash technique to investigate the kinetics of electron and proton transfer during single-turnover oxidation of the fully reduced wild-type and mutant enzymes.

A preliminary account of part of this work has been presented (*9*).

MATERIALS AND METHODS

Mutagenesis, Growth of Bacteria, and Enzyme Purification. Mutagenesis was performed as described (*10*). Bacteria were grown aerobically in a 20-l fermentor. The cells were harvested, and the histidine-tagged enzyme was purified as described (*11*). After elution of the enzyme from the Ni^{2+}-column, the buffer was exchanged to 0.1 M Hepes-KOH, pH 7.4, 0.1% β-D-dodecyl maltoside. The enzyme was stored in liquid nitrogen until use.

Overall Turnover Activities of the Mutant Enzymes. The overall turnover activities of the KM(I-362) and TA(I-359) mutant enzymes were determined from the oxidation rates of reduced cytochrome *c* as described in (*11*).

Preparation of the Mixed-Valence Enzyme. The carbon monoxide mixed-valence complex of cytochrome *c* oxidase was prepared by incubation of the oxidized enzyme with CO (*12*). The reduction level of the enzyme was determined from the optical absorption spectrum. The laser and observation equipment have been described in detail elsewhere (*13*).

Preparation of the Fully-Reduced CO Complex of Cytochrome c Oxidase. A 10 μM solution of the enzyme containing 5 μM PMS was evacuated on a vacuum line and repetitively flushed with nitrogen. To prepare the fully reduced CO complex of the enzyme, ascorbate was added anaerobically to a final concentration of 2 mM, nitrogen was exchanged for carbon monoxide, and the solution was stored for ~3 h at 20 °C or ~10 h at 4 °C.

Flow-Flash Measurements. The enzyme solution was transferred anaerobically to one of the drive syringes of a locally modified stopped-flow apparatus (Applied Photophysics). The other syringe was filled with an oxygen-saturated buffer (~1.2 mM O_2). In the measurements, the enzyme solution was diluted 1:5 with the oxygenated buffer. About 100 ms after mixing, CO was flashed off using a 10-ns, ~100-mJ laser flash at 532 nm (Nd:YAG laser from Spectra Physics) and the reaction between the fully reduced enzyme and O_2 was studied at various wavelengths. The amount of reacting enzyme was calculated from the CO-dissociation absorbance change at 445 nm, using an absorption coefficient of 67 $mM^{-1}cm^{-1}$ (*14*).

The output signal from the photomultiplier was fed into a current-to-voltage converter (with a bandwidth of 3 MHz) and a preamplifier with a variable RC filter (Tektronix, model AM 502) and was recorded using a digital transient recorder (Nicolet, model 490). The photomultiplier was protected from the laser light using various interference filters. The cuvette path length was 1.00 cm. Typically, 6×10^4 data points were collected, and the data set was then reduced to 500−1000 points by averaging over a progressively increasing number of points (logarithmic time scale). Time constants (τ in $\exp(-t/\tau)$) were determined using a computer program based on a nonlinear fitting algorithm (simultaneous fitting at many wavelengths) written in Matlab (Math Works) by M. Karpefors in this laboratory.

Proton-Uptake Measurements. For proton-uptake measurements, the enzyme stock was repetitively diluted with 0.1 M KCl, 0.1% β-D-dodecyl maltoside, pH 7.5, and reconcentrated using centricon-50 concentrator tubes (Amicon) until negligible amounts of buffer remained (at least a 3000-fold dilution). Alternatively, the buffer in the sample was removed chromatographically. Phenol red at 40 μM was added to the enzyme solution, and the fully reduced-CO complex of the enzyme was prepared as described above, except the final concentration of ascorbate was 1 mM. After reduction, the pH was estimated from the absorption spectrum of the dye and, if necessary, adjusted to 7.5. The exhaust from the flow-flash apparatus was collected in a cuvette flushed with nitrogen, and the pH of the solution was determined. The buffer capacity of the sample relates the observed absorbance changes (change in pH) to the number of protons added or removed from the sample. It was determined in each sample by additions of specific amounts of an anaerobic solution of HCl (giving each time an approximate increase in proton concentration of 5 μM), and the corresponding absorbance changes were recorded.

RESULTS

The turnover activities of the KM(I-362) and TA(I-359) mutant enzymes were found to be <2% and ~35%, respectively, of that of the wild-type enzyme.

As described in the Materials and Methods section, the fully reduced enzyme with CO bound to heme a_3 was prepared by incubation of the oxidized enzyme in the presence of ascorbate and CO. The reduction rate of heme a_3 in the KM(I-362) mutant enzyme was much slower than that of the wild-type enzyme (see below and *10*, *15*, *16*). After incubation in ascorbate and CO for about 10 h, >50% of the enzyme was reduced and formed a complex with CO. Only this fraction contributed to the absorbance changes after flash photolysis of CO. The fully reduced CO enzyme was mixed rapidly with an oxygen-containing solution in a stopped-flow apparatus. About 100 ms after mixing, CO was flashed off and absorbance changes associated with oxidation of the enzyme were followed in time.

Figure 1 shows absorbance changes at 445 and 605 nm after flash photolysis of CO from the fully reduced wild-type, KM(I-362) and TA(I-359) mutant enzymes in the presence of ~1 mM O_2. The initial increase in absorbance at $t = 0$ is associated with dissociation of CO. In the wild-type enzyme the following reaction with O_2 is manifested in four kinetic components associated with binding of O_2 to reduced heme a_3 (intermediate A) ($\tau_1 \cong 8$ μs), formation of the peroxy (P) intermediate ($\tau_2 \cong 52$ μs), formation of the ferryl (F) intermediate ($\tau_3 \cong 125$ μs at pH 7.4), and oxidation of the enzyme ($\tau_4 \cong 1.2$ ms at pH 7.4) (O), respectively. It is seen in Figure 1 that the mutant enzymes become essentially fully oxidized within the same time as the wild-type enzyme (i.e., within about 5 ms). In the mutant enzymes the individual rate constants were affected by at most a factor of 3 (see Figure 1B and Discussion).

Figure 2 shows absorbance changes of the pH-sensitive dye phenol red at 560 nm, associated with proton uptake

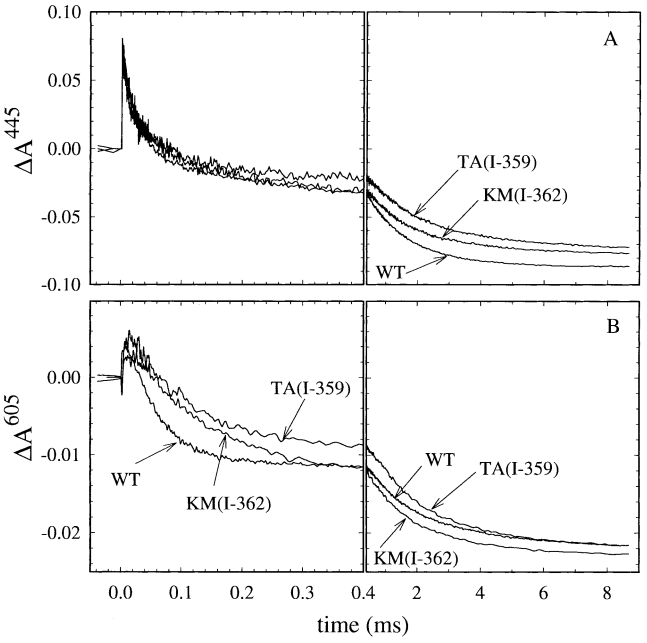

FIGURE 1: Absorbance changes associated with the reaction of fully reduced wild-type, KM(I-362) and TA(I-359) cytochrome *c* oxidase with dioxygen. The fully reduced enzyme−CO complex was mixed with an O_2-saturated buffer solution. After about 100 ms, CO was photodissociated (at $t = 0$) and the reaction was studied at 445 nm (A) and 605 nm (B). The concentration of reacting enzyme, ∼1 μM, was calculated from the CO-dissociation change at 445 nm (see Materials and Methods). Experimental conditions: 22 °C, 0.1 M Hepes, pH 7.4, 0.05% dodecyl-β-D-maltoside, 1 mM O_2.

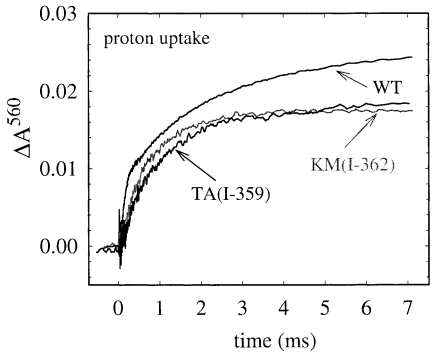

FIGURE 2: Absorbance changes at 560 nm of the pH-indicator dye phenol red (40 μM) associated with proton uptake during reaction of the fully reduced wild-type, TA(I-359) and KM(I-362) mutant enzymes with dioxygen. The traces shown are the differences between the traces obtained in an unbuffered (with 0.1 M KCl, no buffer, pH 7.4) and a buffered solution (0.1 M Hepes, pH 7.4). The buffer capacities of the solutions were about the same for the wild-type and KM(I-362) mutant enzymes, but the capacity was not determined for the TA(I-359) enzyme (see Materials and Methods). Other conditions were the same as in Figure 1.

during oxidation of the fully reduced wild-type and mutant enzymes. In both the wild-type and mutant enzymes, two kinetic phases were observed, associated with the P → F and F → O transitions (see above). The net proton uptake after 5 ms was 1.2−1.6 H^+/enzyme, with approximately equal contributions from the two kinetic phases. In the wild-type enzyme also a slower proton-uptake component with a time constant of about 5 ms was observed (Figure 2) *after* oxidation of the enzyme (cf. Figure 1). This component was much slower or smaller in the KM(I-362) mutant enzyme (see Discussion).

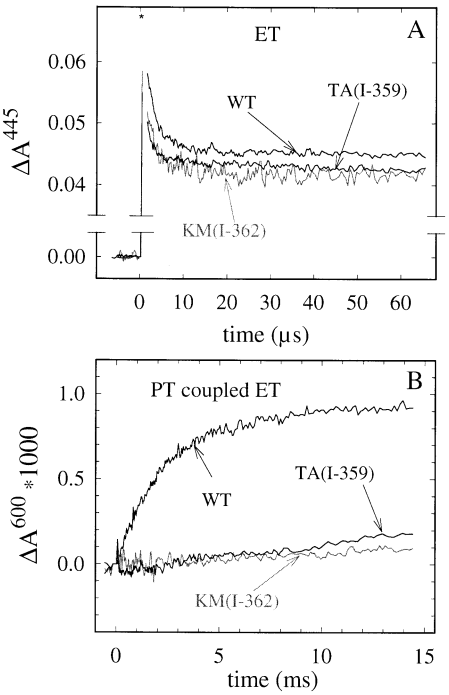

FIGURE 3: Normalized absorbance changes following flash photolysis of CO from mixed-valence wild-type and KM(I-362)/TA-(I-359) mutant enzymes in the absence of O_2. In (A) the initial increase in absorbance at $t = 0$ at 445 nm is associated with dissociation of CO. The following decrease in absorbance with time constants of 2−3 μs is associated with electron transfer (ET) from heme a_3 to *a*. The slower absorbance decrease with a time constant of ∼60 ms is associated with CO recombination. A laser artifact has been truncated. The absorbance immediately after the flash (marked with "*") has been calculated in each trace from a nonlinear fit of the data to a sum of exponential functions, extrapolated to $t = 0$. In (B) the absorbance changes at 600 nm (note the different wavelength than in (A)) in the wild-type enzyme is associated with proton-transfer (PT) controlled electron transfer from heme a_3 to *a*. Conditions: 0.1 M Hepes, pH 7.4 (in (A))/0.1 M Tris-HCl, pH 8.8 (in (B)), 0.1% β-D-dodecyl maltoside, 1 μM reacting enzyme, 1 mM CO, 22 °C.

Intramolecular Electron and Proton Transfer in the Absence of O_2. Binding of CO to heme a_3 stabilizes the reduced state. In addition, the p*K*s of protonatable groups interacting electrostatically with heme a_3 are higher in the presence of reduced heme a_3 than in the presence of oxidized heme a_3 (*17*, *18*). In other words, the protonated states of these groups are stabilized when heme a_3 is reduced (cf. redox Bohr effect, i.e., a link between the redox potential of a redox center and the p*K* of a protonatable group).

After flash photolysis of CO from the mixed-valence-CO complex, electrons were first transferred from heme a_3 to heme *a* with a time constant of ∼3 μs, which resulted in ∼40% reduction of heme *a* (Figure 3A) (*19*). The time scale of this electron transfer is shorter than that of proton transfer (*18*), and therefore during this electron transfer the enzyme was in the same protonation state as with a reduced binuclear center. In the mutant enzymes, no significant effects were observed on the 3 μs electron transfer (Figure 3A), which shows that the mutations did not affect the electron transfer between hemes *a* and a_3.

The rapid fractional electron transfer from heme a_3 to heme *a* was followed by additional, slower oxidation of heme a_3 with a pH-dependent time constant (∼3 ms at pH 9) and amplitude, coupled to proton release to the medium with a

maximum of ~0.7 H^+/e^- (at pH 9.5) (*17*). This reaction was modeled in terms of electrostatic interactions between heme a_3 and a protonatable group(s), L (interaction energy ~70 meV, assuming a single protonatable group), that changes its p*K* from 10.3 to 9.1 upon oxidation of heme a_3 (*17*). On the basis of the pH dependence of the kinetics of proton release, the protonatable group was proposed to be in contact with the bulk solution through a proton-transfer pathway, which determines the rate of proton release and, indirectly, also of the electron transfer from heme a_3 to *a*. In the mutant enzymes, the proton-transfer coupled electron transfer was impaired (on the time scale of the measurements, Figure 3B), which indicates that the protons released/taken up during oxidation/reduction of heme a_3 are transferred through the K-pathway (see Discussion).

DISCUSSION

We have investigated internal electron-transfer and proton-transfer coupled electron-transfer reactions in the absence of O_2 and the reaction of the fully reduced enzyme with O_2 in wild-type and KM(I-362)/TA(I-359) mutants of cytochrome *c* oxidase.

Reaction of the Fully-Reduced Enzyme with O_2. The fully reduced mutant enzymes became oxidized and displayed proton uptake from the bulk medium in about the same time as the wild-type enzyme, which indicates that the K-pathway is not involved in proton uptake during oxidation of the enzyme by O_2 (cf. also below). In the mutant enzymes, the rate constants of the kinetic phases associated with formation of the P- and F-intermediates were slowed by at most a factor of 3, which may be a consequence of a change of the water structure in the vicinity of the binuclear center. It should be noted that even if the formation of the P- and/or F-intermediate is slowed by a factor of 3, this does not affect the overall oxidation rates of the mutant enzymes. Consequently, this effect is not the reason for the decreased turnover activity of the KM(I-362) and TA(I-359) mutant enzymes.

In the wild-type enzyme also a slower proton-uptake component with a time constant of about 5 ms was observed (see Figure 2), i.e., slower than oxidation of the enzyme (cf. Figure 1). This component was not observed in the KM(I-362) mutant enzyme on the time scale of the experiment. During O_2 reduction, two hydroxide ions are formed at the binuclear center (cf. Figure 4). One of these is released (as H_2O or OH^-) and in the oxidized enzyme one hydroxide is most likely bound at the binuclear center (*20*−*24*). Thus, one possibility is that the observed 5-ms proton uptake is associated with release of one hydroxide ion or proton uptake to the OH^- through the K-pathway. Since the turnover rate of the wild-type enzyme is on the order of 10^3 s^{-1} at pH 7.4, this proton uptake/hydroxide-ion release may appear not to be catalytically relevant. However, during turnover it may be accelerated by reduction of the binuclear center during the next reaction cycle. In other words, during turnover this proton uptake/hydroxide-ion release takes place in the reductive part of the second (and the following) cycle(s).[2]

The reaction of the three-electron reduced enzyme with O_2 has been studied earlier in the KM(I-362) and TA(I-359) mutants of the ubiquinol oxidase cytochrome bo_3 (*25*). Essentially no differences were observed in the reactions of the wild-type and mutant enzymes. Since this enzyme accommodates only three redox-active metal sites, the reaction could not be studied beyond formation of the F-intermediate. Consequently, the question was raised whether during turnover the reaction in the mutant enzymes was blocked at the F → O transition or at electron input to the binuclear center (or both). The present results with the *Rhodobacter sphaeroides* enzyme can now resolve this question.

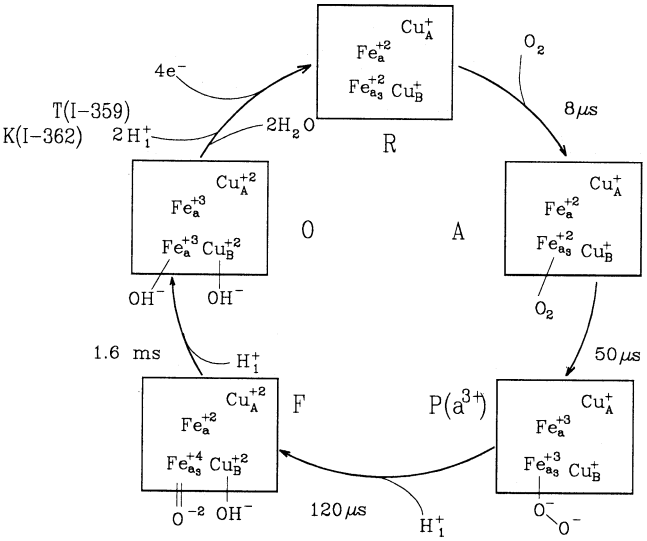

FIGURE 4: Simplified reaction scheme, based on the data shown in Figures 1−3 and described in the text. The involvement of K(I-362) and T(I-359) in proton uptake in cytochrome *c* oxidase is indicated. R, A, P(a^{3+}), F, and O represent the fully reduced, ferrous-oxy, peroxy, ferryl, and fully oxidized states, respectively. The state P can be formed either with electrons from hemes a/a_3 (i.e. heme *a* becomes oxidized P(a^{3+})) or from heme a_3/Cu_B (in the two-electron reduced enzyme) (see *34*, *40*). The results from this work show that the *upper limit* for proton uptake through the K-pathway is 2.5 H^+ (rounded off to 2). Protons released to side "2" (see eq 1) are not shown in the figure. Proton uptake during the P(a^{3+}) → F and F → O transitions most likely takes place through the D-pathway (*29*). One of the two hydroxide ions (see states F and O) may be protonated or released without reduction of the binuclear center. The time constants were determined from experiments with the wild-type enzyme.

Our research group has found earlier that during oxidation of the fully reduced bovine cytochrome *c* oxidase reconstituted in lipid vesicles, 1−2 protons are released to side "2" (cf. eq 1) (*26*). As shown in Figure 2 the solubilized wild-type enzyme picks up a *net* of ~1.5 protons during full oxidation (during <5 ms). Hence, assuming that 1−2 protons are released to side "2" also in the *R. sphaeroides* enzyme, during oxidation of the fully reduced enzyme, 2.5−3.5 protons are taken up from side "1". Recent studies of the electrogenic events associated specifically with the F → O transition in the KM(I-362) mutant enzyme reconstituted in lipid vesicles showed that there is no effect on the electrogenicity associated with this reaction step (*15*). Since during the F → O transition protons are pumped across the membrane (*27*), these results together with those from the

[2] It should be noted that even if we assume that this proton uptake (or hydroxide-ion release) is accelerated by reduction of the binuclear center it takes place spontaneously also when the binuclear center is oxidized. Thus, this proton uptake is not included in the ~1.5 protons taken up upon reduction of the binuclear center in the fully oxidized enzyme.

present study indicate that *during oxidation of the fully reduced enzyme*, the K-pathway is not involved in uptake of substrate or pumped protons because the net proton uptake is about the same in the mutant as in the wild-type enzyme. In other words, the K-pathway is not involved in proton-transfer reactions during (coupled to) "electron output" from the fully reduced enzyme to dioxygen.

On the other hand, we have recently shown that replacement of Glu (I-286) by its nonprotonatable analogue Gln results in inhibition of proton uptake during reaction of the fully reduced enzyme with O_2 in both ubiquinol (from *E. coli*) (*28*) and cytochrome *c* oxidases (from *R. sphaeroides*) (*29*, *30*). Consequently, the reaction stops after formation of the P-intermediate. This indicates that the D-pathway is used for proton uptake only during reaction of the fully reduced enzyme with O_2 (Figures 4 and 5B). The inhibition of the reaction at the level of the P-intermediate in the EQ-(I-286) enzyme has since been demonstrated also by a number of other research groups (*15*, *31*, *32*).

Internal Electron- and Proton-Transfer Reactions. In the partly reduced KM(I-362) and TA(I-359) mutant enzymes, the intrinsic electron-transfers rates between heme a_3 and heme *a*, were the same as in the wild-type enzyme (Figure 3A). However, the slower, proton-transfer-coupled electron transfer was impaired in the KM(I-362) and TA(I-359) mutant enzymes. We found earlier the DN(I-132) and EQ-(I-286) mutant enzymes displayed essentially the same proton-transfer coupled electron-transfer characteristics as the wild-type enzyme (*29*). Thus, taken together, the results show that the pathway earlier predicted to be involved in the proton uptake/release coupled to reduction/oxidation of heme a_3 (*17*) in the absence of O_2 is the K-pathway and not the D-pathway. In addition, the results suggest that in the KM(I-362) mutant enzyme, reduction of heme a_3 is dramatically slowed not because electron transfer to heme a_3 is impaired but because proton uptake through the K-pathway, coupled to reduction of heme a_3, is impaired. The upper limit for the extent of this uptake is $\sim$2.5 H^+. Since one hydroxide is bound at the binuclear center in the fully oxidized enzyme (see above), most likely one of these protons is used to protonate the hydroxide ion (alternatively, OH^- is released).

Which Protonatable Group(s) Interacts with the Binuclear Center? We have previously presented a model for the proton-transfer coupled electron transfer between hemes *a* and a_3 and assumed that a protonatable group L (see above) interacts electrostatically with heme a_3 with an interaction energy of 70 meV (*17*). On the basis of this assumption, the distance between heme a_3 and L was estimated to be in the range 5−10 Å (*17*), and from the three-dimensional structure of bovine heart cytochrome *c* oxidase (*6*) a number of possible candidates for L were proposed (Figure 5A): T(I-359), Y(I-288), T(I-352), the histidine ligands of Cu_B, and the propionate side chains of heme a_3 (*17*, *30*). It is also possible that L is a water molecule that releases a proton upon oxidation of heme a_3 and binds to oxidized heme a_3 as a hydroxide ion. Since the proton-transfer coupled electron transfer was impaired in both the TA(I-359) and KM(I-362) enzymes and K(I-362) is further away from the binuclear center than T(I-359), it is unlikely that L is K(I-362). The residue Y(I-288) is a particularly interesting candidate. In the bovine enzyme, it was recently found to

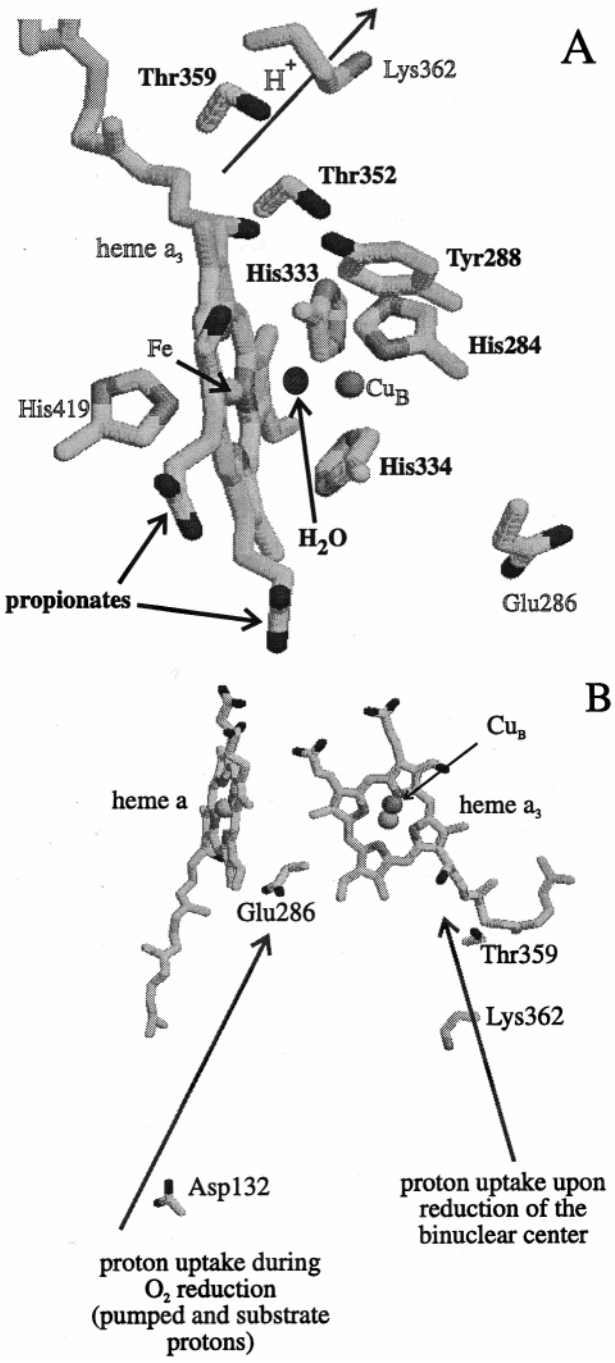

FIGURE 5: (A) Possible candidates for L, the protonatable group-(s) interacting electrostatically with heme a_3 (and Cu_B) (marked with bold text). The positions of Glu(I-286), Lys(I-362), and His-(I-419) are also indicated. All amino acid residues shown are found in subunit I. Upon oxidation/reduction of heme a_3, protons are released/taken up through the K-pathway (see also (B)). In the figure proton release upon oxidation of heme a_3 is indicated (cf. Figure 3B). (B) Role of the D and K proton-transfer pathways. Results from the experiments described in this work indicate that the K-pathway is used for proton uptake/release upon reduction/oxidation of the binuclear center. Results described by Ädelroth et al. (*29*) indicate that the D-pathway is used for proton uptake only during oxygen reduction. Amino acid residues are numbered according to the *R. sphaeroides* sequence. Coordinates are from the bovine heart enzyme structure (*6*; file 1occ.PDB from the Brookhaven Protein Databank). In the amino acid residues and heme groups, atoms marked in black and dark gray are oxygens and nitrogens, respectively. The figures were prepared using the programs Rasmol and Canvas.

be covalently bound to H(I-284) (*R. sphaeroides* sequence numbering), a Cu_B ligand (S. Yoshikawa, personal communication). This unusual structure may hint at a specific role for the Y(I-288)−H(I-284) pair in electron transfer and/or proton pumping. A similar structural arrangement was also found in the two-subunit form of the *P. denitrificans* enzyme (*35*).

Does Heme a Interact with Protonatable Groups Near the Binuclear Center? We have assumed that L interacts only with heme a_3. However, since deprotonation of L is coupled to electron transfer from heme a_3 to heme *a*, the only conclusion that can be drawn from the experimental data is that L interacts more strongly with heme a_3 than with heme *a* (*17*, *18*). This situation is similar to that found in the photosynthetic reaction centers in which the secondary quinone Q_B (cf. heme a_3) interacts more strongly than the primary quinone Q_A (cf. heme *a*) with protonatable groups, and electron transfer from Q_A^- to Q_B results in fractional proton uptake (see *18*). Also the observation that the extent of proton uptake upon reduction of the binuclear center is larger ($\sim$2 H^+) than upon reduction of heme *a* ($\sim$0.5 H^+) in the bovine cytochrome *c* oxidase (*33*) indicates that the binuclear center interacts more strongly than heme *a* with protonatable groups in the protein.

It has been observed that the redox potential of heme *a* displays a significantly smaller pH dependence when the binuclear center is reduced than when it is oxidized (*36*, *37*). This difference may also be explained by the stronger interactions of protonatable groups near the binuclear center with heme a_3/Cu_B than with heme *a*. When the binuclear center is reduced, the protonatable groups interacting with this center are essentially fully protonated and there is no additional proton uptake upon reduction of heme *a*. However, when the binuclear center is oxidized, the groups are not protonated and reduction of heme *a* results in a fractional proton uptake by these groups. This interpretation implies that the binuclear center and heme *a* interact with the same protonatable groups. In addition, considering the results from the present work, this implies that the fractional proton uptake upon reduction of heme *a* takes place through the K-pathway.

Recently, Rich (*38*) proposed a model in which E(I-286) is (fractionally) protonated upon reduction of heme *a*. During electron transfer from heme *a* to heme a_3, the proton is then transferred from E(I-286) to a protonatable group closer to the binuclear center. As discussed above, this is a conceivable scenario even though, presumably, groups other than E(I-286) are involved because in the EQ(I-286) mutant enzyme the proton-transfer coupled electron transfer between hemes *a* and a_3 was not affected (*29*).

Proton Uptake during Reduction of the Oxidized Enzyme. Since a net of $\sim$1.5 protons are taken up during oxidation of the *R. sphaeroides* enzyme and a total of four protons are consumed during O_2 reduction to water, the oxidized enzyme must pick up 2.5 protons upon reduction (in the solubilized enzyme, only the net proton uptake is observed and pumped protons do not contribute). These results are similar to those obtained with the bovine enzyme (*26*), which picks up about 2.4 protons upon reduction of the enzyme, about 2 of which are associated with reduction of the binuclear center (*33*; see also *17*). As found previously (*10*, *15*, *16*), the reduction rate of the oxidized KM(I-362) mutant enzyme was much slower than that of the wild-type enzyme, which in light of the results presented here, is most likely due to impaired proton transfer through the K-pathway (see also below and Figures 4 and 5).

CONCLUSIONS

Investigation of the proton-transfer coupled electron transfer after flash photolysis of CO from the partly reduced enzyme shows that the K-pathway (and not the D-pathway) is involved in proton uptake/release upon reduction/oxidation of heme a_3 and presumably also Cu_B. Since the fully reduced KM(I-362) and TA(I-359) enzymes became fully oxidized when reacting with O_2 on the same time scale as the wild-type enzyme, the results indicate that the K-pathway is not used during oxidation of the fully reduced enzyme. Thus, the turnover activity of the KM(I-362) mutant enzyme is impaired (and presumably that of TA(I-359) reduced to $\sim$35%) because proton uptake during reduction of the binuclear center is blocked.

The opposite situation was observed in the EQ(I-286) mutant enzyme in which the proton-transfer coupled electron transfer after flash photolysis of CO from the partly reduced enzyme was the same as in the wild-type enzyme. During reaction of the fully reduced enzyme with O_2, the reaction stopped after formation of the P intermediate and proton uptake was impaired (*29*). This means that after cytochrome *c* oxidase is reduced (and the protons associated with reduction are taken up through the K-pathway) during oxidation, all protons (pumped and substrate) are taken up through the second, D-pathway (Figures 4 and 5).

In future studies it will be interesting to investigate the kinetics of electron and proton transfer also in other oxidases with different structures of the proton pathways, for example, the quinol oxidase from the thermoacidophilic archaeon *Sulfolobus acidocaldarius*, which lacks the D-pathway (*39*).

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