Kinetics of Electron and Proton Transfer during the Reaction of Wild Type and Helix VI Mutants of Cytochrome bo_3 with Oxygen†

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ABSTRACT: Site-directed mutagenesis was used to investigate the mechanism of electron and proton transfer in the ubiquinol oxidase, cytochrome bo_3, from *Escherichia coli*. The reaction between the fully reduced form of the enzyme and dioxygen was studied using the flow−flash method. After rapid mixing of CO-bound enzyme with an O_2-containing solution, CO was photodissociated, and the subsequent electron- and proton-transfer reactions were measured spectrophotometrically, the latter using a pH-indicator dye. In the wild-type, pure bo_3 enzyme, without bound quinones, we observed a single kinetic phase with a rate constant of about 2.4×10^4 s^{-1}, associated with formation of the ferryl oxygen intermediate, followed by proton uptake from solution with a rate constant of about 1.2×10^4 s^{-1}. Enzyme in which heme *o* instead of heme *b* was incorporated into the low-spin site displayed a slower ferryl formation with a rate constant of about 3.6×10^3 s^{-1}. Upon replacement of the acidic residue glutamate 286 in helix VI of subunit I with a nonprotonatable residue, electron transfer was slightly accelerated, and proton uptake was impaired. Mutations of other residues in the vicinity of E286 also resulted in a dramatic decrease of proton uptake, suggesting that the environment of this residue is important for efficient proton transfer. In the closely related cytochrome aa_3 from *P. denitrificans*, the corresponding residue (E278) has been suggested to be part of a proton-transfer pathway [Iwata, S., Ostermeier, C., Ludwig, B., & Michel, H. (1995) *Nature 376*, 660−669]. The results are discussed in terms of a model for electron−proton coupling during dioxygen reduction.

Cytochrome bo_3[1] (ubiquinol:O_2 oxidoreductase) is one of the membrane-bound terminal oxidases of *Escherichia coli*. It catalyzes the four-electron reduction of dioxygen to water with ubiquinol as a reductant, and couples this redox reaction to vectorial proton transport across the membrane (Puustinen et al., 1989, 1991). The enzyme contains three redox-active metal sites: one low-spin heme *b* accepting electrons from the reducing substrate and a heme−copper binuclear center (o_3−Cu_B) where dioxygen is bound and reduced to water [for a review, see Hosler et al. (1993)]. The mechanism of electron-transfer-driven proton pumping is not known, but recently much attention has been focused on the binuclear center as the coupling site [see, e.g., Babcock and Wikström (1992) and Morgan et al. (1994)].

High-resolution three-dimensional structures of both the mitochondrial and *P. denitrificans* bacterial cytochrome *c* oxidases (cytochromes aa_3), structurally and functionally similar to the bo_3 enzyme (Chepuri et al., 1990; Hosler et al., 1993), have recently been determined. According to these structures, the binuclear center is located in the transmembrane part of the protein near the periplasm, removed from the cytoplasm from where protons are taken up. Proton uptake associated both with reduction of dioxygen to water (scalar protons) and with proton pumping (vectorial protons) requires, therefore, efficient transport of protons through the protein. Consequently, the protein must have at least one proton-transfer pathway. As found in several other biological systems, such pathways may consist of a chain of protonatable amino acid residues and water molecules [see, e.g., Gutman and Nachliel (1990) and Okamura and Feher (1992)]. The *P. denitrificans* (Iwata et al., 1995) and bovine (Tsukihara et al., 1996) enzyme structures suggest at least two such proton-transfer pathways leading from the protein surface on the cytoplasmic side to the binuclear center. Some of the protonatable amino acid residues included in the pathways, proposed to be involved in vectorial proton transfer to the binuclear center, are found along helix VI, located near the binuclear center. One of these residues is glutamate 286[2] (Iwata et al., 1995). Earlier studies have shown that mutation of this residue to alanine

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[1] The enzyme has two heme sites. As suggested by Puustinen et al. (1992), in analogy with the mitochondrial cytochrome aa_3, the heme of the binuclear center where oxygen reacts is denoted with a subscript "3". The heme of the second, low-spin site does not bind ligands and is denoted without an index. In many preparations, a fraction of these sites is occupied by heme *o*, instead of heme *b*; i.e., the enzyme is a cytochrome oo_3. Throughout this paper, we will refer to this site as the low-spin site and, where applicable, specify which heme is found in the site.

[2] *E. coli* numbering of amino acid residues is used throughout this work. To get the *P. denitrificans* numbering, subtract eight from these numbers.

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(E286A) results in an inactive enzyme (Thomas et al., 1994) whereas a mutation to glutamine (E286Q) results in an active enzyme (Thomas et al., 1993). The E286Q mutant cytochrome aa_3 from *Rhodobacter sphaeroides* is inactive (Mitchell et al., 1995).

In this work, we use the flow−flash technique to investigate the effects of mutation of this residue as well as tryptophan 280 and proline 285, in the vicinity of E286, on the kinetics of electron and proton transfer during the reaction of the fully-reduced enzyme with dioxygen. The flow−flash technique has previously been used to investigate the reaction between different oxidases and dioxygen. Carbon monoxide is bound to the enzyme which is reduced to different degrees. After rapid mixing with a dioxygen-containing solution, CO is photodissociated, and the following oxidation of the enzyme is studied time-resolved using various spectroscopic techniques. The most extensive studies have been performed on the mitochondrial cytochrome aa_3, and many of the different intermediates during reduction of dioxygen to water have been identified [for a review, see Babcock and Wikström (1992)]. Also a number of studies of oxygen reduction catalyzed by the *E. coli* cytochrome bo_3 enzyme have been done, but the results have not been conclusive. Some studies show that there is only one kinetic phase with a rate constant of about 3×10^4 s^{-1} in the absorbance changes [e.g., see Orii et al. (1995) and Puustinen et al. (1996)] whereas other studies show two kinetic phases with rate constants of about 3×10^3 s^{-1} and 3×10^4 s^{-1}, respectively (Wang et al., 1995; Svensson & Nilsson, 1993). This discrepancy has been suggested to be due to an enzyme population, with heme *o* instead of heme *b* incorporated in the low-spin site, resulting in a fractional population of cytochrome oo_3 (Puustinen et al., 1992). The faster and slower components have been suggested to originate from reactions in cytochromes bo_3 and oo_3, respectively (Verkhovsky et al., 1994, 1996).

In both the mitochondrial and the *E. coli* oxidases, it has been shown that during oxygen reduction protons are taken up with a rate of about 1×10^4 s^{-1} (Hallén & Nilsson, 1992; Hallén et al., 1993). This proton uptake was suggested to be associated with reprotonation of an internal group involved in proton delivery to an oxygen intermediate formed at the binuclear center during earlier steps of the reaction.

In the present study, we have investigated the reaction between the fully reduced enzyme and dioxygen in a number of wild-type and mutant ubiquinol oxidases. The different preparations contained different fractions of hemes *b* and *o* in the low-spin site, and these fractions were determined in each preparation. Also in this study we observed two kinetic phases, and the results show that the fraction of the 3×10^4 s^{-1} phase correlates well with the fraction of cytochrome bo_3; i.e., the rapid and slow kinetic phases are associated with cytochromes bo_3 and oo_3, respectively (Verkhovsky et al., 1994, 1996). Recently, Puustinen et al. (1996) reported a single kinetic phase in cytochrome bo_3 without bound quinone.

Effects of mutations of glutamate 286, tryptophan 280, proline 285, and tyrosine 288 were investigated. Both E286 and W280 have been suggested to be part of the pathway proposed for pumped protons and were replaced by the nonprotonatable residues alanine and phenylalanine, respectively. Replacement of P285 is expected to alter the hydrogen-bonding structure of helix VI near E286. In addition, it has been suggested that the carbonyl oxygen atom of P285 may bind solvent molecules involved in proton conduction (Iwata et al., 1995). Y288 has been proposed to form a hydrogen bond with one of the Cu_B−histidine ligands.

Mutant enzymes were purified, and both electron- and proton-transfer reactions associated with oxygen reduction were investigated. The results show that upon replacement of E286 by alanine, electron transfer is slightly accelerated whereas the 1.2×10^4 s^{-1} proton uptake is impaired. Mutation of either W280 or P285 has no effect on electron transfer, but also results in an impaired proton uptake.

MATERIALS AND METHODS

Materials. Decylubiquinone, ubiquinone-10 (synthetic), *N*-octyl glucoside, and the pH indicator cresol red were purchased from Sigma. Other chemicals were of the purest grade available. The pK_a of cresol red was determined by spectrophotometric titration under the same conditions as those used in the present work and was found to be 8.3.

Mutagenesis, Growth of Bacteria, and Enzyme Purification. Site-directed mutagenesis and cloning were performed as described by Thomas et al. (1994). Bacteria were grown aerobically in a 20-L fermentor (Chemap, Switzerland) in TB-medium (380 g of yeast extract and 180 g of tryptone), Minimal A [15 g of $(NH_4)_2SO_4$, 7.5 g of sodium citrate, 225 g of K_2HPO_4, and 15 g of KH_2PO_4], pH 7.5, and 1% (v/v) lactate. They were harvested in the late-exponential phase of growth. Membranes (Svensson & Nilsson, 1993) and the enzyme were isolated as described (Minghetti et al., 1992). In two enzyme preparations from the *E. coli* strain GO105 with a pJRhis plasmid incorporated, in the following called GO105, a histidine-tagged protein purification protocol was used; a number of histidine residues were inserted at the C-terminal of subunit II. In these preparations, instead of an anion-exchange column a Ni^{2+}-chelate affinity column was used to specifically bind the histidine-modified enzyme. Membranes were solubilized with Triton X-100 and octyl glucoside to a final concentration of 1% of each in 50 mM potassium phosphate buffer, pH 8.1. After ultracentrifugation, the supernatant was loaded on a Ni^{2+} column equilibrated with 50 mM potassium phosphate, 0.1% Triton X-100, and 25 mM imidazole at pH 8.1. The bound enzyme was eluted from the column with a 25−300 mM imidazole gradient. Imidazole was removed by dialysis against 10 mM Tris-HCl, 0.1% *N*-dodecyl sarcosinate, pH 7.5, and/or successive concentration/dilution several times. Enzyme concentrations were determined from the pyridine hemochrome spectra (Berry & Trumpover, 1987), using the absorption coefficient $\Delta\Delta\epsilon = \Delta\epsilon^{553}_{\text{red -ox}} - \Delta\epsilon^{535}_{\text{red -ox}} = 24$ mM^{-1} cm^{-1} (Puustinen et al., 1991). The stock solution of the enzyme was stored in liquid nitrogen. Optical spectra were recorded on a Schimadzu UV-3000 or a Cary 4 (Varian) spectrophotometer.

Steady-State Activity Measurements. The steady-state turnover rates (catalytic activities) were determined spectrophotometrically by measuring the consumption rate of the substrate decylubiquinol at 278 nm on a Cary 4 (Varian) spectrophotometer in a buffer composed of 0.1 M Hepes,[3] 1 mM EDTA, and 0.1% sodium *N*-dodecyl sarcosinate at pH 7.5 and 25 °C. The decylubiquinone was reduced with sodium borhydride as described (Rieske, 1967; Svensson et al., 1995). The enzyme concentration was 0.5 nM and

$\sim$1 μM for the wild-type and mutant enzymes, respectively, and the ubiquinol concentration was varied in the range 4−65 μM.

Quinone Analysis. If quinol is present in a flow−flash experiment, electrons in excess of those found at the redox sites may take part and interfere with the studied reactions. Therefore, all enzyme preparations were checked for bound quinol (summarized in Table 2).

Ubiquinones were extracted from approximately 3 nmol of purified enzyme with 6:4 methanol/light petroleum ether. The samples were supplemented with 3 nmol of ubiquinone-10 as a standard. The aqueous phase was extracted a second time with light petroleum ether, and the organic phases were combined. The solvent was evaporated under a stream of nitrogen and the residue resuspended in 1 mL of ethanol (Kröger & Dadak, 1969). The amount of ubiquinone-8 present was determined by HPLC using an Microsorb-MV column (Rainin, 4.6 × 250 mm) with 4:3:3 ethanol/methanol/acetonitrile as the mobile phase at a flow rate of 0.8 mL/min (Sato-Watanabe et al., 1994). The quantity of ubiquinone-8 was determined through comparison of the 13 min peak area at 278 nm with the ubiquinone-10 standard which elutes at 25 min.

Heme Analysis. The low-spin site can hold either heme *b* or heme *o*. The fraction of enzymes with the different hemes incorporated in this site varies in different preparations and in different mutant forms. Consequently, the heme *b*-to-heme *o* ratio must be determined in each enzyme preparation. The fraction of cytochrome bo_3 was determined from this ratio (Table 2).

Hemes were extracted from approximately 5 nmol of purified enzyme with 9:1:1 acetone/HCl/water solution. The hemes were then extracted into diethyl ether which was washed twice with water. The organic phase containing the hemes was dried under a stream of nitrogen. The residue was dissolved in 500 mL of a 13:17:70 water/acetic acid/95% ethanol solution. The heme composition was determined by HPLC using a Microsorb-MV column (Rainin, 4.6 × 250 mm) with a flow rate of 1.0 mL/min. The mobile phase consisted of 7:17:70 water/acetic acid/95% ethanol mixture. The ratio of the 7 min heme *b* peak to the 14 min hemo *o* peak at 402 nm was equated with the heme ratio (Puustinen & Wikström, 1991).

Flow−Flash Experiments. Flow−flash experiments were carried out as described (Svensson et al., 1995). Briefly, the enzyme was solubilized in 0.1% *N*-dodecyl sarcosinate containing 0.1 M Hepes−KOH at pH 7.5 and 1 mM EDTA to a concentration of 10 μM. To prepare the CO-bound fully reduced enzyme, it was first incubated under an N_2 atmosphere for about 10 h with 2 mM sodium ascorbate and 5 μM phenazine methosulfate as a mediator. Then N_2 was replaced with CO (to a final concentration of about 1 mM). The solution was transferred anaerobically to a 500-μL syringe of a mixing device (RX1000, Applied Photophysics). The other syringe (2.5 mL) was filled with an O_2-saturated detergent/buffer solution with the same composition as described above. The mixing ratio was 1:5 (enzyme/O_2 buffer).

[3] Abbreviations: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; CHES, 2-(cyclohexylamino)ethanesulfonic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid.

Proton Uptake Measurements. To remove buffer from the enzyme solution, samples were washed several times with a solution containing 0.1 M KCl and 0.1% *N*-dodecyl sarcosinate at pH 7−7.5 using Centricon-50 tubes (Amicon Inc.). After washing, the enzyme was diluted to 30 μM, and the pH was adjusted to about 8.6 so that the final pH of the reduced enzyme/CO solution after addition of sodium ascorbate was 8.3. The solution was transferred anaerobically to the mixing device (see above). The other syringe was filled with an O_2-saturated solution with the same composition as described above and 30 μM cresol red at pH 8.3. After each experiment, the dye/enzyme solution was collected, and the pH was measured using a FTPH-2 microflow (LASAR Research Inc., Los Angeles, CA) or a 5 mm pH electrode (Radiometer). All samples without buffer were kept under a N_2 atmosphere to avoid exposure to air CO_2. To calibrate the dye absorbance changes at 580 nm to changes in proton concentration, absorbance changes were measured after injecting small volumes (15 μL) of a 0.500 mM H_2SO_4 solution. Typically, about five additions were made, and the average $\Delta A^{580}/\Delta H^+$ was calculated.

RESULTS

Figure 1A shows absorbance changes following flash photolysis of CO from the fully reduced enzyme immediately after mixing of the sample with an oxygen-saturated buffer solution (within $\sim$100 ms). The mixing ratio was 1:5 which gives an oxygen concentration of about 1 mM in the experiment. To eliminate the effect of variations in the photoactive enzyme−CO concentrations after mixing, all traces in Figure 1A were normalized to the CO dissociation absorbance changes, i.e., the absorbance changes immediately after the flash. This normalization was particularly important for the Y288F mutant enzyme because it was not 100% reduced prior to O_2 addition.

In the wild-type enzyme, two rapid kinetic phases with apparent rate constants of $(2.4 \pm 0.3) \times 10^4$ s^{-1} and $(3.6 \pm 0.4) \times 10^3$ s^{-1} were observed [cf. Svensson and Nilsson (1993)]. As determined from the optical absorption spectrum after completion of the reaction (not shown), the final state is a ferryl intermediate [Puustinen et al. (1996); see Scheme 1], consistent with the presence of three electrons in the fully reduced enzyme in the absence of bound quinol (see also Discussion).

Three different wild-type strains with different heme *b*:heme *o* ratios were investigated (GO103, RG145, and GO105 with a pJRhis plasmid) (Table 2, Figure 1). Assuming that all hemes are bound at the active sites of the enzyme (except for the GO103 enzyme, see below), the fraction of the fast phase amplitude correlates well with the cytochrome bo_3 fraction (Table 2, Figure 2). Therefore, as suggested by Verkhovsky et al. (1994), the rapid and slow phases probably originate from reactions in cytochromes bo_3 and oo_3, respectively. In the GO105 enzyme, following the 3×10^4 s^{-1} decay, we observed a slight increase in absorbance. This effect is most likely due to re-reduction of heme *b* by the bound quinol in a fraction of enzyme molecules (see Table 1) as observed previously by Puustinen et al. (1996).

The rates of both phases were essentially pH-independent in the range $\sim$5.5−10; the rate of the faster phase was $\sim$20% slower at pH 10 than at pH 5.5 (Figure 3).

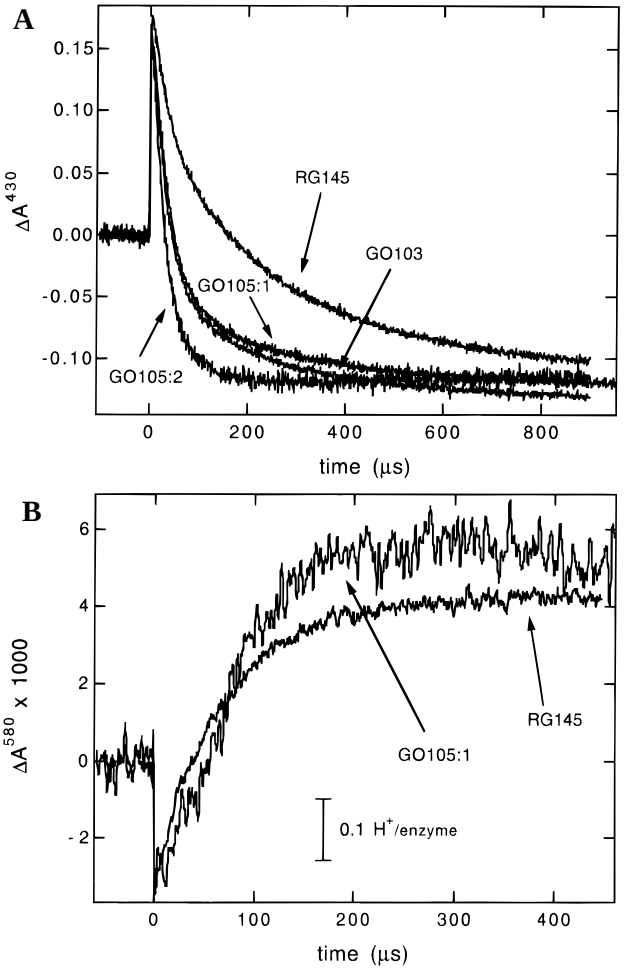
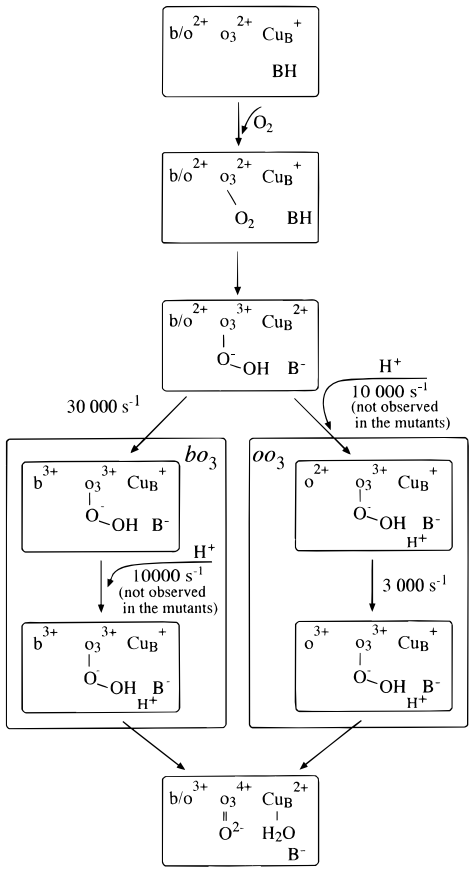


FIGURE 1: (A) Absorbance changes after flash photolysis of CO from fully reduced wild-type ubiquinol oxidases in the presence of oxygen. The different strains and preparations contained different ratios of cytochromes bo_3 and oo_3 (see Table 1). Experimental conditions after mixing: 1.7 μM enzyme, 0.1 M Hepes−KOH, 0.1% sodium *N*-dodecylsarcosinate, pH 7.5, and 1 mM oxygen. A flash artifact has been truncated. (B) Time courses of proton uptake during oxygen reduction by wild-type enzymes. The reactions were monitored at 580 nm with the pH indicator cresol red, with and without buffer. The traces shown are differences obtained by subtracting the time courses obtained in the presence of buffer from those without buffer. Experimental conditions after mixing: 5 μM enzyme, 0.1 M Hepes−KOH or 0.1 M KCl, 30 μM Cresol red, 0.1% sodium *N*-dodecylsarcosinate, and 1 mM oxygen. A flash artifact has been truncated.

The enzyme purified from the GO103 strain contained heme *b* in excess of what could be ascribed to cytochrome bo_3; the heme *b*:heme *o* ratio was 1.4, i.e., larger than 1. In addition, a significant fraction of heme *b* remained reduced after reaction of the fully-reduced enzyme with oxygen. This phenomenon was not observed in any of the other wild-type or mutant enzymes and indicates that there is excess heme *b* in this preparation, presumably unspecifically bound to the enzyme. This heme is not expected to bind CO/O_2 and does not contribute to the absorbance changes observed in the flow−flash experiments. However, it introduces an error in the cytochrome determination (overestimation of the cytochrome bo_3 fraction, see Discussion).

Proton uptake during reduction of dioxygen was studied using the pH-indicator cresol red. Buffer was excluded in these experiments, and the pH was measured using a pH electrode placed immediately after the measuring cell. Absorbance changes were monitored at 580 nm, where the dye displays the maximum pH-induced changes. To eliminate possible contributions from absorbance changes of the

Scheme 1: Proposed Mechanism for Electron Transfer and Proton Uptake during Oxygen Reduction in the Ubiquinol Oxidase[a]



[a] The low-spin site can be occupied by either heme *b* or heme *o*. After binding of O_2 to the fully reduced enzyme, heme o_3 and Cu_B are oxidized, and the peroxy intermediate is formed. It is stabilized by an internal proton transfer from a protonatable group (BH) in the enzyme. There is no net proton uptake from solution during this reaction. Electron transfers from hemes *b* and *o* to the binuclear center have rates of $\sim 3 \times 10^4$ s^{-1} and $\sim 3 \times 10^3$ s^{-1}, respectively, and proton uptake from solution has a rate of $\sim 1 \times 10^4$ s^{-1}. Therefore, in cytochrome bo_3, electron transfer is followed by proton uptake whereas in cytochrome oo_3 proton uptake precedes electron transfer. This proton may be associated with reprotonation of the internal group B^-, or it may be taken up by another group interacting electrostatically with the binuclear center. It is blocked in the E286A, W280F, P285G, and Y288F mutant enzymes which makes the transfer of a fourth electron to the binuclear center unfavorable and may be the reason why these mutant enzymes have reduced activities.

Table 1: Percentage of the First-Phase Amplitude, Percentage of Cytochrome bo_3 of the Total Content of Cytochromes bo_3 and oo_3, and the Quinone Content

strain	fraction fast phase (% of A_{tot})	fraction bo_3 (%)	fraction bound quinone (%)[a]
RG145	36	25	10−15
GO105:1	79	86	55
GO105:2	100	ND[b]	68
GO103	75	84	<5
W280F	43	46	0
P285G	29	29	<5
E286A	79	46	0
Y288F	58	64	6

[a] 100% corresponds to 1 bound quinone/enzyme molecule. [b] Not determined.

Table 2: Steady-State Parameters for the Ubiquinol Oxidase Activity, Measured with Decylubiquinol (Q_2) as a Substrate[d]

strain	k_{cat}[b] (electrons·s^{-1})	activity (% of wild-type)[c]
RG145	510 ± 30	
GO103	450 ± 5	
GO105:1[a]	620 ± 30	
GO105:2[a]	500 ± 20	
W280F	30 ± 2	6
P285G	150 ± 5	29
E286A	31 ± 1	6
Y288F	1.4 ± 0.4	0.3

[a] Two different batches of the GO105 enzyme were used. [b] Standard deviation based on 12 measurements. [c] Percent of the mean value of the activities for the different wild-type enzymes. [d] The K_m values were found to be ≤2 μM for both wild-type and mutant enzymes except for Y288F which has a K_m of 21 μM.

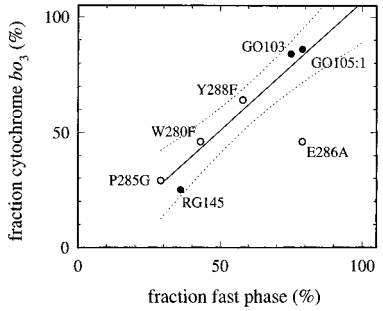


FIGURE 2: Fraction of cytochrome bo_3 (Table 1) as a function of the fraction of fast phase during oxygen reduction (see Figure 1A). (●) represent wild-type enzymes, and (○) represent mutant enzymes. The dashed lines show a 99% confidence interval.

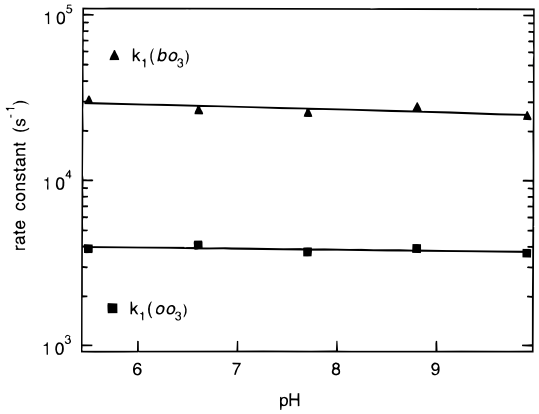


FIGURE 3: pH dependence of the rates of the two phases observed during oxygen reduction (see Figure 1A). Experimental conditions after mixing: 1.7 μM enzyme; the buffers used were 100 mM MES (pH 5.5 and 6.6), 100 mM Hepes (pH 7.7), and 50 mM CHES (pH 8.8, 9.9).

enzyme at this wavelength, the same measurements were repeated with buffer present (100 mM Tris-HCl at pH 8.3), and these absorbance changes were then subtracted from those measured without buffer (Figure 1B). After the subtraction, a kinetic phase with an apparent rate constant of (1.2 ± 0.2 × 10^4 s^{-1} was observed in the wild-type strains corresponding to 0.7 ± 0.1 H^+/enzyme molecule (Figure 1B).

All investigated mutant oxidases (E286A, W280F, P285G, and Y288F) displayed reduced overall turnover activities (Table 1). As in the wild-type enzyme, two kinetic phases with rate constants of 2.4 × 10^4 and 3.6 × 10^3 s^{-1} were found in the oxygen reaction of the mutant enzymes (Figures 4A and 5A). In all studied mutant oxidases, except E286A,

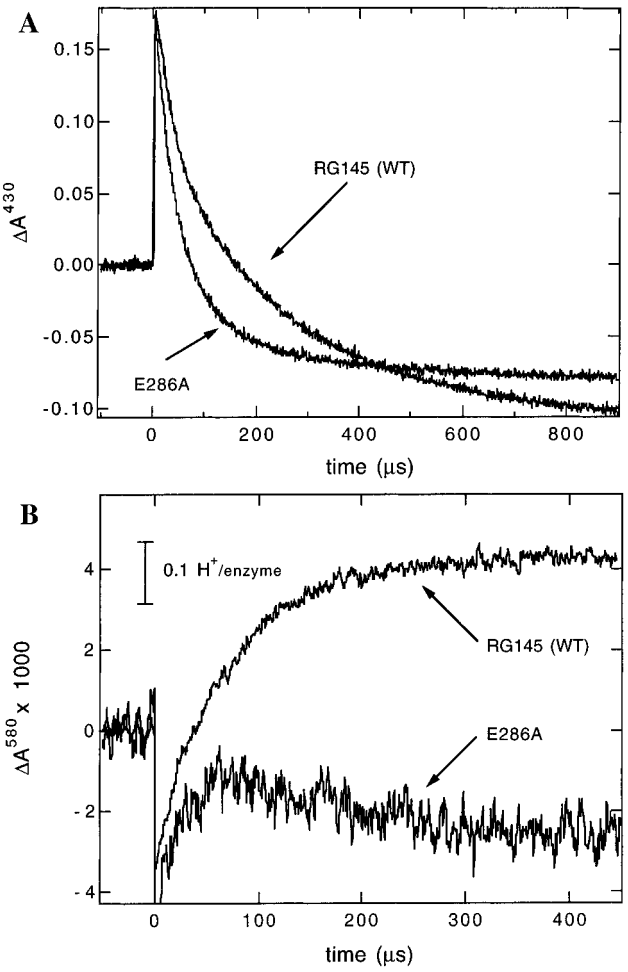


FIGURE 4: Absorbance changes associated with electron transfer (A) and proton uptake (B) after flash photolysis of CO from the fully reduced E286A mutant and RG145 wild-type enzymes in the presence of oxygen. Conditions are the same as those in Figure 1.

the fractions of the rapid phases correlate well with the fractions of cytochrome bo_3 (see Table 2 and Figure 2).

In the E286A mutant oxidase, the fraction of the rapid phase was larger than that predicted from the fraction of heme bo_3, and the rate constant of the slower phase was increased to 6.1 × 10^3 s^{-1}. This suggests that exchange of glutamate 286 with alanine results in an increased electron-transfer rate in the cytochrome oo_3 population (see Discussion). This is also supported by a comparison of the electron-transfer kinetics in the E286A and wild-type enzymes at other wavelengths (400−450 nm) where E286A displays on average a faster rate than wild-type enzyme (Figure 2).

Taking into account the different absorption coefficients of hemes *b* and *o* and the different cytochrome bo_3/oo_3 ratios, in all mutant forms, except in the Y288F mutant oxidase, the total absorbance change amplitude at 430 nm after 2 ms was ≥80% of that found in the wild-type strains, which suggests about the same degree of oxidation of the mutant as of the wild-type enzymes. However, it is difficult to determine the end product of the different mutant forms. The fully reduced-minus-oxidized spectrum of the E286A enzyme was the same as that of the wild-type enzyme. In the Y288 mutant enzyme, the absorption spectra of the different enzyme forms are altered, which may be a consequence of the strong interaction of Y288 with one of the Cu_B histidine ligands (Iwata et al., 1995; see also Discussion). For example, in the reduced-CO-minus-reduced spectrum, the

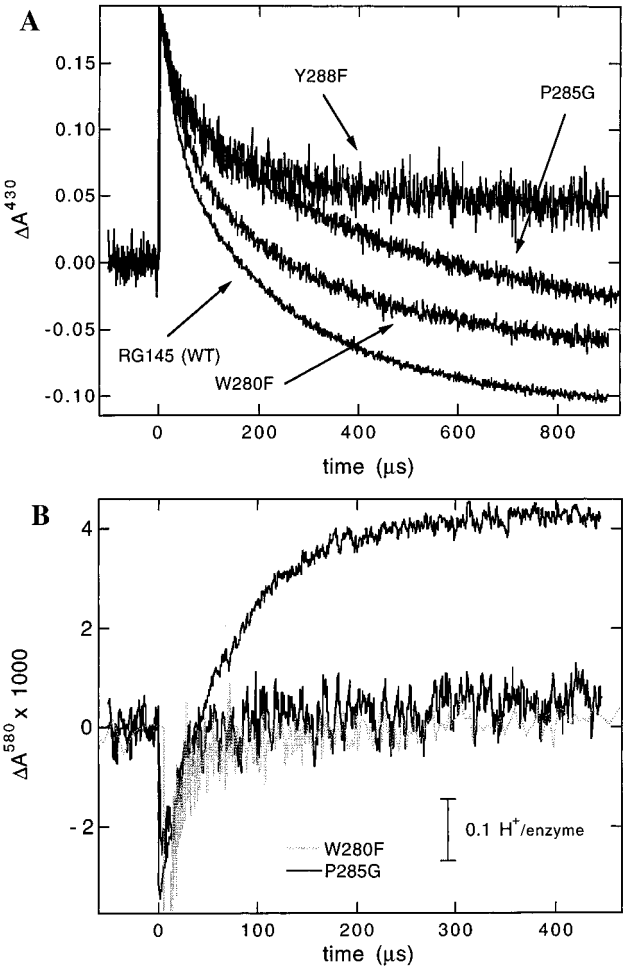


FIGURE 5: Absorbance changes associated with electron transfer (A) and proton uptake (B) after flash photolysis of CO from the fully reduced W280F, P285G, Y288F mutant, and RG145 wild-type enzymes in the presence of oxygen; the proton uptake trace for Y288F is not shown but looks like the P285G trace. Conditions are the same as those in Figure 1.

peak at 430 nm, was smaller in the mutant enzyme. It is therefore difficult to directly compare absorbance changes of this mutant with those of the wild-type enzyme.

Figures 4B and 5B show dye absorbance changes associated with proton uptake by the mutant enzymes. All traces were normalized to the enzyme concentration. All helix VI-mutant enzymes displayed a dramatically decreased extent of 1×10^4 s^{-1} proton uptake.

DISCUSSION

In this work, we have investigated the reaction between the fully reduced wild-type and mutant forms of cytochrome bo_3 and dioxygen using the so-called flow−flash technique. Both electron transfer and proton uptake were studied. The reaction stops at the ferryl intermediate (see Scheme 1) because only three electrons are available in the enzyme. Therefore, dioxygen cannot be fully reduced to water. Recently, it has been suggested that there may be an amino acid radical involved in the oxygen reaction of ubiquinol oxidase (Watmough et al., 1994; Wang et al., 1995). In this study, we have not considered this possibility because a ferryl intermediate appears to be formed at the end of the reaction after about 2 ms (see also below).

In samples containing a mixture of enzymes with both heme *b* and heme *o*, two kinetic phases were observed with rate constants of 2.4×10^4 s^{-1} and 3.6×10^3 s^{-1}. Their relative amplitude ratios depended on the relative contents of heme *b* and heme *o* in the different strains and preparations. Assuming that all heme is bound at the active sites of the enzyme, the fractions of the faster of the two kinetic phases correlate well with the fraction of enzymes with heme *b* at the low-potential site (see Figure 2). Consequently, we ascribe the rapid and slow phases to the oxygen reactions in the subpopulations of cytochromes bo_3 and oo_3, respectively (Verkhovsky et al., 1994). This model is used as a basis for the following discussion.

The GO103 strain displayed two kinetic phases during the reaction between the fully reduced enzyme and dioxygen, consistent with a fraction of cytochrome oo_3 in the preparation. In an earlier study (Svensson et al., 1995), the GO103 strain was assumed to produce pure cytochrome bo_3, based on the heme content determination. However, as pointed out under Results, after oxidation of the fully-reduced GO103 enzyme a large fraction of heme *b* remained reduced. This heme is presumably unspecifically bound to the enzyme and leads to an overestimation of the cytochrome bo_3 content. Also, Wang et al. (1995) found a large fraction of reduced heme *b* after completion of the reaction between the fully-reduced enzyme with dioxygen. However, they interpreted these results in terms of a radical involved in oxygen reduction.

The reaction between fully reduced mitochondrial cytochrome *c* oxidase and dioxygen has been characterized extensively, and detailed models for the reaction sequence have been proposed [reviewed in Babcock and Wikström (1992) and Einarsdóttir (1995)]. In this enzyme, four kinetic phases have been identified in the Soret band at pH 7. An absorbance change with a rate constant of $\sim 10^5$ s^{-1} (at ~1 mM O_2, corresponding to a second-order rate constant of $\sim 10^8$ M^{-1} s^{-1}), associated with binding of O_2 (Oliveberg et al., 1989; Verkhovsky et al., 1994), is followed by oxidation of heme *a* and formation of the peroxy intermediate with a rate constant of $\sim 3 \times 10^4$ s^{-1}. The rate of this phase is independent of O_2 concentration above ~300 μM O_2 (below this O_2 concentration, it is presumably limited by O_2 binding). It is then followed by stabilization of the peroxy intermediate by proton uptake and electron transfer from Cu_A to heme *a* with a rate constant of $\sim 1 \times 10^4$ s^{-1}, followed by oxidation of Cu_B forming the ferryl intermediate. Finally, heme *a* is oxidized with a rate constant of $\sim 10^3$ s^{-1}, and water is formed at the binuclear center.

As discussed above, in the pure cytochrome bo_3, in the absence of bound quinol, only one major kinetic phase with a rate constant of 3×10^4 s^{-1} was observed. Assuming a similar reaction sequence in the ubiquinol as in the mitochondrial oxidase and that the products formed after the 3×10^4 s^{-1} reaction phase are the same, the difference in the number of kinetic phases can be explained as follows. In contrast to the bovine enzyme, the rates of the kinetic phases in both cytochrome bo_3 and cytochrome oo_3 decrease with decreasing O_2 concentration below 1 mM O_2 (Svensson & Nilsson, 1993), which shows that they are at least partly rate-limited by O_2 binding. In cytochrome *c* oxidase, the first states formed during reduction of dioxygen are at equilibrium, and the observed reaction rate constant is a combination of several rate constants of the individual steps (Varotsis & Babcock, 1995; Verkhovsky et al., 1994). Thus, the initial $\sim 10^5$ s^{-1} phase is probably not observed in cytochrome bo_3

because of a slower dioxygen binding (Puustinen et al., 1996) or a different equilibrium constant for dioxygen binding resulting in a smaller population of heme $o_3^{2+}-O_2$. The second, 3×10^4 s^{-1} phase most likely corresponds to the same reaction in both enzymes. Absorbance changes associated with the third (10^4 s^{-1}) phase in cytochrome *c* oxidase in the Soret band are presumably mainly associated with electron transfer from Cu_A to heme *a* and are therefore not expected to be observed in the ubiquinol oxidase. During this phase, electrons are also redistributed within the binuclear center during formation of the ferryl intermediate, but these reactions are not expected to contribute significantly to absorbance changes at 430 nm in the ubiquinol oxidase. The reaction corresponding to the fourth and slowest (10^3 s^{-1}) phase, observed in cytochrome *c* oxidase, is not observed in the ubiquinol oxidase because there are only three electrons in the fully-reduced enzyme.

In the E286A mutant oxidase, the fraction of the faster phase was ~80% even though the cytochrome bo_3 content was ~50%; i.e., the fast-phase amplitude was larger than that predicted from the cytochrome bo_3 content (see Table 1 and Figure 2). This suggests that in this mutant oxidase in the oo_3 population, electron transfer from heme *o* to o_3 is accelerated. Qualitatively, this may be explained in terms of electrostatic interactions between E286 and heme o_3 (stronger than with the low-spin heme). Upon replacement of the negatively charged E286 by a neutral residue, the driving force for the heme *b*/*o*-to-heme o_3 transfer could increase, resulting in an acceleration of the reaction. This effect may be more pronounced in cytochrome oo_3 than in cytochrome bo_3 because in the latter the reaction is rapid and may be close to saturation, limited by other reactions associated with the oxygen chemistry.

As in the mitochondrial cytochrome *c* oxidase (Oliveberg et al., 1989), the rate of the 3×10^4 s^{-1} phase in the ubiquinol oxidase was essentially pH-independent (Figure 3). This is consistent with a model in which the first proton, needed to stabilize the peroxy intermediate, is present in the enzyme prior to CO photolysis, bound to a group in the vicinity of the binuclear center (Hallén & Nilsson, 1992). This proton is transferred internally during peroxy formation and is accompanied by the transfer of the third electron to the binuclear center from heme *a* with a rate of about 3×10^4 s^{-1}. The slower proton uptake *from solution* with a rate of about 1×10^4 s^{-1} is associated with reprotonation of the internal group.

Assuming the same mechanism in the ubiquinol oxidase, the lack of retardation of the 3×10^4 s^{-1} electron transfer in the E286A mutant enzyme indicates that E286 is not involved in transfer of the first proton to the binuclear center, needed to stabilize the peroxy intermediate. However, this residue appears to be involved in proton uptake that follows peroxy formation and is necessary for the following electron transfer. This interpretation is supported by results from studies of the reaction of fully-reduced cytochrome aa_3 from *Rhodobacter sphaeroides* with dioxygen, which show that the slowest phase with a rate of about 10^3 s^{-1} (at 445 nm, pH 7, 20 °C), associated with transfer of the fourth electron to the binuclear center, is not observed in the E286Q mutant enzyme (P. Ädelroth, D. M. Mitchell, R. B. Gennis, and P. Brzezinski, unpublished results). Taken together, the results from the ubiquinol oxidase and bacterial cytochrome aa_3 indicate that E286 is involved in proton uptake that must precede transfer of the fourth electron. The ferryl intermediate with OH^- instead of H_2O bound to Cu_B can be formed when this proton uptake is blocked, but the following reactions are impaired.

Also in the W280F and P285G mutant oxidases electron transfer during the reaction of the fully-reduced enzyme and dioxygen was not affected, whereas proton uptake was impaired. The structure of *P. denitrificans* cytochrome aa_3 shows that W280, P285, and E286 may be part of a proton-transfer pathway leading from the surface in the direction toward the binuclear center, suggested to be used for pumped protons (Iwata et al., 1995). The absence of proton uptake in these mutant enzymes suggests that an intact pathway structure is necessary for efficient proton transfer. The effect of the P285G mutation is presumably indirect because a modification alters the hydrogen-bonding pattern of helix VI. In addition, it has been suggested that the carbonyl oxygen atom may bind solvent molecules involved in proton conduction (Iwata et al., 1995). Thus, mutation of this residue may interrupt the proton pathway.

As shown in Table 2, the turnover activities of W280, P285, and E286 mutant oxidases are much smaller than that of the wild-type enzyme. This lower activity is most likely due to blocking of proton uptake that is necessary for transfer of the fourth electron to the binuclear center during turnover. The Y288F mutant enzyme displayed a lower overall activity and also a disruption of oxygen reduction in the flow−flash experiment. As pointed out under Results, this effect may be due to structural changes in the binuclear center because this residue may form a hydrogen bond with one of the Cu_B ligands (H284).

Our research groups have shown previously that in the ubiquinol oxidase there is no effect on either electron or proton transfer in mutant oxidase in which protonatable residues in helix VIII have been modified (Svensson et al., 1995). In *P. denitrificans* cytochrome aa_3, this helix lines a second proton-transfer pathway proposed to be involved in transfer of scalar protons to the binuclear center. Results from experiments on the *Rb. sphaeroides* cytochrome aa_3 show that when T359 or K362 of helix VIII are replaced with nonprotonatable residues the 10^3 s^{-1} phase, associated with the transfer of the fourth electron to the binuclear center, was slowed down (P. Ädelroth, D. M. Mitchell, R. B. Gennis, and P. Brzezinski, unpublished results). However, this effect could not alone account for the reduced activities of these mutant enzymes. Therefore, most likely in the helix VIII mutant enzymes the initial preloading of protons during reduction of the enzyme is blocked.

In conclusion, the results from this work, together with results from the helix VIII mutant enzymes and those from *Rb. sphaeroides* cytochrome aa_3, suggest that E286 is needed to take up a proton after formation of the peroxy intermediate. This proton may not necessarily directly participate in dioxygen reduction but may instead interact electrostatically and stabilize negative charges in the binuclear center, as has been discussed previously by, for example, Rich (1995) (electroneutrality principle), Morgan et al. (1994) (histidine cycle), and Ädelroth et al. (1996) and Brzezinski (1996) (electrostatic interactions between heme a_3 and a protonatable group). The proton uptake through E286 is required for the transfer of the fourth electron to the binuclear center.

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