Oxygen Reaction and Proton Uptake in Helix VIII Mutants of Cytochrome bo_3[†]

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ABSTRACT: The oxygen reaction of wild-type and helix VIII mutants of cytochrome bo_3 from *Escherichia coli*, and the associated proton uptake during this reaction, has been studied using flash photolysis of the CO complex of the reduced protein after rapid mixing with oxygen. We have focused on mutations in the transmembrane helix VIII where protonatable residues have been exchanged, and mainly on the inactive mutants (i.e., T352A, T359A, and K362L, -M, and -Q). The kinetics for electron transfer during oxidation for the mutants are similar to the wild-type; two rate constants of 3.2×10^4 and 3.4×10^3 s^{-1} (at 1 mM oxygen) are detected. Proton uptake is observed for wild-type as well as for the mutant enzymes, but the mutations within helix VIII have affected the rate of proton uptake; it is significantly accelerated in the mutants. These results show that none of the protonatable residues in helix VIII are required in the reaction between the fully reduced cytochrome bo_3 and oxygen. We have also studied electron redistribution after photolysis of CO from the mixed-valence compound; we found three kinetic components for wild-type and the mutants T352A and T359A, but for K362M only the first and third components are observed, with amplitudes that are lower than those for the corresponding components in the wild-type enzyme, suggesting that the characteristics of internal electron transfer in the K362M mutant are different from those of the wild-type enzyme.

The final step in the respiratory chain of *Escherichia coli* grown aerobically is the oxidation of ubiquinol by molecular oxygen, catalyzed by cytochrome bo_3 [we use the nomenclature suggested by Puustinen et al. (1992)], the dominating terminal oxidase under this condition. This enzyme belongs to the superfamily of heme−copper oxidases that includes both quinol oxidases and cytochrome *c* oxidases, such as the mitochondrial cytochrome aa_3. In addition to catalyzing the transfer of electrons from their substrate to oxygen, the heme−copper oxidases couple this transfer to vectorial proton translocation across a membrane, generating a proton electrochemical gradient. The function and structure of the heme−copper oxidase superfamily have been reviewed recently by Hosler et al. (1993).

The active site for binding and reduction of dioxygen in cytochrome bo_3 contains two metals, one iron in cytochrome o_3 (the high-spin heme) and one copper atom termed Cu_B. The third redox-active metal site, cytochrome *b* (the low-spin heme), serves as the primary acceptor of electrons from the reducing substrate. In wild-type bacteria, the chemical structures of the two cytochrome components *b* and o_3 are heme B (iron protoporphyrin IX) and heme O, respectively (Puustinen & Wikström, 1991). On overexpression, however, it appears that some heme O is incorporated in the cytochrome *b* site (Puustinen et al., 1992) with the consequence that enzyme isolated from overproducing strains is a mixture of two species. The first species is the normal cytochrome bo_3 that contains one molecule of heme B and one molecule of heme O, whereas the second species contains two molecules of heme O. Following Puustinen et al. (1992), we will refer to the latter species as cytochrome oo_3. Although recent work (Puustinen et al., 1992; Morgan et al., 1993) indicates that the two species may differ with respect to redox properties, their enzymatic activity is similar.

Identification of the metal ligands using site-directed mutagenesis (Lemieux et al., 1992; Minagawa et al., 1992; Shapleigh et al., 1992; Calhoun et al., 1993a,b,c) in combination with topological information has allowed the formulation of a structural model for the catalytic core of the heme−copper oxidases (Figure 1). The metals are bound by histidine residues in the transmembrane α-helices II, VI, VII, and X of the largest subunit, subunit I. As seen in Figure 1, the positions of the histidine ligands suggest that the oxygen-binding site is located within the hydrophobic, transmembrane part of the protein near the periplasm. It is known, however, that the protons consumed on reduction of oxygen to water are taken up from the cytosolic side of the membrane (Wikström, 1988). This position of the active site requires that its access to protons and water molecules from the bulk medium and the cytosolic side is provided by the protein. In addition to providing access for protons required for the oxygen chemistry, the protein must also allow a passage for protons that are translocated from the cytoplasm to the periplasm.

Earlier work on proton uptake in the oxygen reduction in cytochrome aa_3 has suggested that at least two groups close

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to the bimetallic site are involved in proton delivery (Hallén & Nilsson, 1992). One protonatable group is proposed to be in near proximity to the bimetallic site. A second, at least, is proposed to mediate proton transfer from the bulk medium into the site. A proton pathway leading to cytochrome a_3/Cu_B has also been suggested earlier (Konstantinov et al., 1986; Wikström, 1988; Hallén et al., 1994). The transmembrane helix VIII has been noted to contain highly conserved polar residues that fall along one face of the helix. These residues could be important components of a pathway providing a conduit for protons from the cytoplasm to the binuclear center. It has been shown that some of the polar residues within this helix are necessary for activity. Mutations in the region change the spectroscopic properties of the metal centers (Thomas et al., 1993). From these results, it has been suggested that residues in helix VIII are located in the immediate vicinity of the binuclear center. They are therefore potential components of a proton-conducting channel from the cytoplasmic surface to the heme−copper center where oxygen is reduced to water (Hallén & Nilsson, 1992; Babcock & Wikström, 1992).

In this paper, we test the hypothesis that polar side chains in helix VIII are involved in proton uptake during the oxidative part of the reaction. We have used the flow-flash method to investigate the kinetics for electron transfer and to observe proton uptake for different mutants of cytochrome bo_3. The kinetics for electron transfer during oxidation are similar to the wild-type. Proton uptake is observed, but the mutations within helix VIII have affected the rate of proton uptake; it is significantly accelerated for the mutants. We have also investigated electron redistribution after photodissociation of CO from the CO mixed-valence complexes. We found that the reverse electron transfer from the cytochrome o_3 to the low-spin cytochrome is affected in the mutants K362M and, to a smaller extent, T352A. The partially active mutant T359A is unaffected.

From these results, we suggest that none of the polar residues in helix VIII are necessary for the proton uptake that accompanies oxygen reduction in the flow-flash experiment. Impaired proton uptake in other parts of the catalytic reaction is, however, still a possible reason for the loss of activity in some of the mutants.

MATERIALS AND METHODS

Materials. D_2O (99.9%), the pH indicator cresol red (Na^+-salt), and decylubiquinone were purchased from Sigma. The pK_a value for cresol red was determined to be 8.3 by spectrophotometric titration under the conditions used in the present work.

Mutagenesis. Site-directed mutagenesis and cloning were performed as described previously (Thomas et al., 1993).

Cell Growth and Enzyme Isolation. Cells were grown aerobically in a 150-L or a 20-L fermentor (Chemap, Switzerland) and were harvested as in Georgiou (1988). Membranes were isolated as in Svensson and Nilsson (1993), and enzyme isolation was as in Minghetti et al. (1992). The concentration of enzyme in stock solutions was estimated using the pyridine hemochrome method as described by Puustinen et al. (1991), with the extinction coefficient given by these authors [$\Delta\Delta\epsilon$(red−ox, 553−535) = 24 mM^{-1} cm^{-1}]. Optical spectra were recorded on a Shimadzu UV-3000 or a Cary 4 spectrophotometer.

Measurement of Steady-State Activities. Decylubiquinone was reduced with sodium borohydride as described by Rieske (1967). The activities were measured both in H_2O and in D_2O for the mutants that do not complement *in vivo* (Thomas et al., 1993). The reaction medium was 0.1 M Hepes,[1] 1 mM EDTA,[1] and 0.1% sodium *N*-dodecyl sarcosinate at pH* 7.5 [pH-meter reading; see Schowen (1978)] containing 55 μM decylubiquinol. Reactions were initiated by the addition of enzyme and were followed at 278 nm on a Shimadzu UV-3000 spectrophotometer.

Flow-Flash Experiments. The CO complex of the fully reduced enzyme was prepared as by Svensson and Nilsson (1993) using ascorbate (2 mM) as reductant and phenazine methosulfate (5 μM) as mediator. The buffer was 0.1 M Hepes−KOH, pH 7.5, and 0.05% sodium *N*-dodecyl sarcosinate. Rapid mixing and flash photolysis with monitoring of the chromophores in the enzyme were done as described by Hallén and Nilsson (1992), except that the monitoring light passed through a 430 nm interference filter placed before the sample, and that a Nicolet Model 490 oscilloscope was used to collect and digitize the signals. Proton uptake experiments were done as in Hallén et al. (1993). To minimize the buffer capacity in the proton uptake experiments, the enzymes were dialyzed against 3 × 100 volumes of 0.1 M KCl, and 0.05% sodium *N*-dodecyl sarcosinate adjusted to pH 8. This was also the medium used in the proton uptake measurements. In some of the experiments, we attempted to reduce the buffer capacity of the sample by lipid removal following Robinson and Wiginton (1985). The following procedure was used. The protein was kept at room temperature in the presence of 1 M NaCl and then centrifuged to remove a whitish precipitate. The supernatant was desalted on a G-25 column equilibrated with 0.1 M KCl and 0.1% sodium *N*-dodecyl sarconsinate, pH 8, eluted, and concentrated to the desired concentration. There was, however, no difference in the buffer capacity between the two procedures used.

Electron Redistribution after Photodissociation of CO from the CO Mixed-Valence Complexes. The CO mixed-valence complexes of the wild-type and mutant enzymes were prepared as described by Morgan et al. (1993). The buffer used was the same as in the flow-flash experiments. To take into account the possibility that the yield of the CO mixed-valence complex could be different with different mutant enzymes, the absorbance increase at 565−650 nm on incubation with CO was used as a relative measure of the amount of the CO mixed-valence complex formed. Flash photolysis and data collection were as in the flow-flash experiments. The reaction was monitored in the α band using an 560 nm interference filter (bandwidth 5 nm) placed before the sample in the monitoring beam. The monitoring wavelength was fine adjusted using the double monochromator placed after the sample, and a suitable wavelength was determined in each experiment as described below.

RESULTS

Activity Measurements. The mutants made in the predicted helix VIII are K362M, -L, and -Q; T359A and -S; P358A;

[1] Abbreviations: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid.

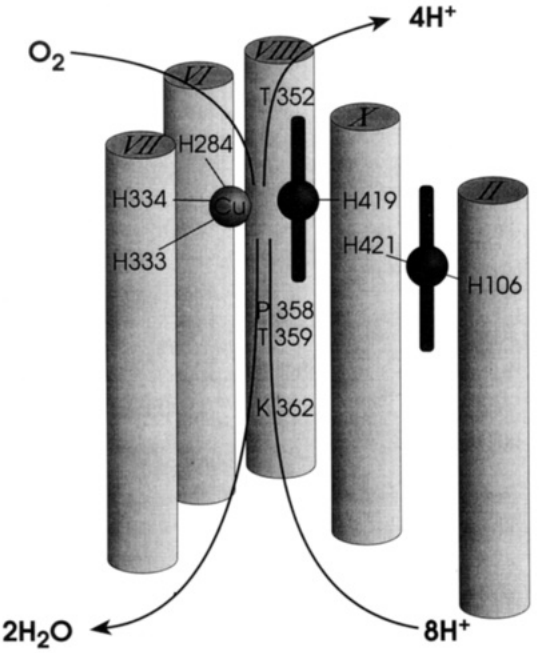

FIGURE 1: Schematic model of the catalytic core. The vertical positions of the histidine ligands and the mutated residues are shown.

Table 1: Activities of Purified Protein for Wild-Type and Helix VIII Mutants in H_2O and D_2O

strain	turnover in H_2O (electrons/s)	activity relative to wild-type (%)	isotope effect (H_2O/D_2O)
wild-type (RG145)	530	100	1.8
T352A	95	18	1.6
T359A	80	15	1.7
K362L	1	0.2	1.6
K362M	2	0.4	2.5

M353A; and T352S, -N, and -A. The approximate position of the mutated residues on the helix is shown in Figure 1. When tested for activity *in vivo* (i.e., for the ability to support aerobic growth as the sole oxidase), the K362M, -L, and -Q; T359A; and T352A derivatives have been found to be inactive (Thomas et al., 1993). As a starting point for the kinetic characterization, we have measured the *in vitro* ubiquinol oxidase activity of enzymes isolated from the strains carrying the inactivating mutations. The results are shown in Table 1. The inactive mutants fall into two categories. The two threonine-to-alanine mutants show considerable residual activity, whereas the mutants in the 362 position are virtually inactive. Since it has been suggested earlier that the inactive mutants may be deficient in proton transfer (Thomas et al., 1993), we have also compared the deuterium isotope effect on the steady-state activity of the mutants to that of the wild-type enzyme in an attempt to detect an increased barrier to proton transfer in the steady-state. However, a steady-state isotope effect of about 2 is found in the wild-type as well as in the mutant enzymes.

Reaction of Reduced Enzymes with Oxygen. In the bovine cytochrome aa_3, the last two steps in the reaction of the fully reduced protein with oxygen appear limited by proton uptake from the medium (Hallén & Nilsson, 1992). Although it has been shown that cytochromes aa_3 and bo_3 differ with respect to the pH and deuterium isotope sensitivities of the oxygen reaction, rapid proton uptake is observed also in

Table 2: Observed Rate Constants in the Oxygen Reaction of Fully Reduced Wild-Type and Helix VIII Mutant Proteins

strain	first phase ($k_1 \times 10^{-3}$, s^{-1})	second phase ($k_2 \times 10^{-3}$, s^{-1})	fraction of first phase (%)	A_{tot}[a]
GO103 (batch 1)	27	3.9	71	1.79
GO103 (batch 2)[b]	23	3.3	66	1.88
RG145 (batch 1)	22	3.3	28	1.73
RG145 (batch 2)	32	4.2	29	1.60
RG145 (batch 3)[c]	24	3.5	29	1.71
T352A	15	2.5	18	1.58
T352N	21	3.2	11	1.51
T352S	27	3.0	27	1.34
M353A	20	3.2	20	1.35
P358A	15	2.8	26	1.44
T359A	26	2.9	35	1.55
T359S	22	3.1	34	1.52
K362L	23	3.0	30	1.53
K362M	23	2.7	31	1.39
K362Q	13	3.1	22	1.39

[a] The amplitude is given as relative to the amplitude of the CO photodissociation to adjust for variations in enzyme concentrations. [b] This batch contains less than 5% of the oo_3 species, as determined by the pyridine−hemochrome method and from the reduced *minus* oxidized spectra (Puustinen et al., 1992). [c] In addition to ascorbate, decylubiquinol was added in this experiment.

cytochrome bo_3 (Hallén et al., 1993). We have therefore investigated the oxygen reaction in the helix VIII mutants using the flow-flash method. In earlier flow-flash studies on the wild-type cytochrome bo_3 (Svensson & Nilsson, 1993), two rapid kinetic phases with apparent rate constants of 3.2×10^4 s^{-1} and 3.4×10^3 s^{-1} (at 1 mM oxygen) were resolved. Table 2 compares the kinetic parameters found for the mutants with those of the wild-type enzyme. Two kinetic components were also found in all the mutants, and the rates seen in the active, partially active, and inactive mutants are all similar to the wild-type rates. The relative contributions of the first and second phase to the total amplitude vary, however, among different mutant enzymes as well as between wild-type enzymes prepared from strains RG145 and GO103. This result is probably a consequence of variation in the oo_3/bo_3 ratio, and is discussed in detail below.

It has been reported that cytochrome bo_3 contains bound ubiquinone as isolated (Orii et al., 1994). It would therefore be possible that a variable quinone content could give rise to the variable contributions of the first and second phase to the total amplitude. Addition of excess ubiquinol to the enzyme in a flow-flash experiment did, however, not change the relative contributions of the two phases (Table 2).

Kinetics of Proton Uptake in the Oxygen Reaction. We have also investigated the kinetics of proton uptake during the oxygen reaction in the helix VIII mutants using the pH indicator method. Figure 2 shows representative traces obtained by comparing the time course at 580 nm in the presence of the pH indicator cresol red, and in the presence and absence of buffer as described earlier (Hallén et al., 1993). Proton uptake is detected for the wild-type enzyme as well as for all the mutant enzymes. In Figure 2, the proton uptake traces for the partially active mutants T352A and T359A and for one of the almost inactive K362 mutants are shown. The apparent rate constants obtained from these time courses and from similar experiments with the other mutant enzymes are summarized in Table 3 together with the parameters found for the wild-type enzyme in a parallel

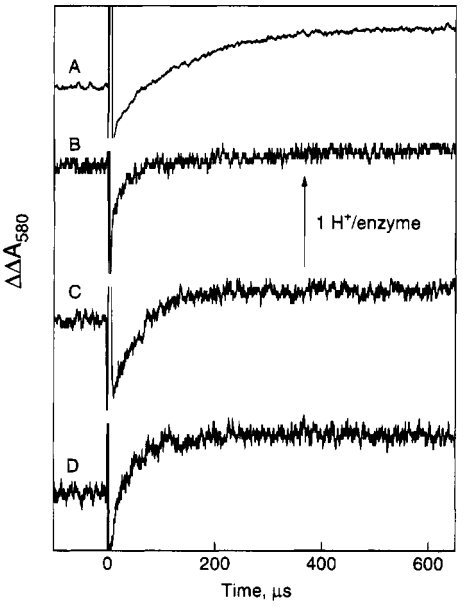

FIGURE 2: Time courses of proton uptake during oxygen reduction by wild-type enzyme (A), the partially active mutants T352A (B) and T359A (C), and the inactive mutant K362L (D). The reactions were monitored at 580 nm with cresol red as the indicator, and the traces shown are differences obtained by subtracting the time course obtained in the presence of buffer from those obtained without buffer. These traces were averages of 20−40 individual transients. The concentration of each sample was determined from its reduced *minus* oxidized difference spectrum before the addition of CO, giving the following concentrations after mixing: wild-type enzyme, 5.2 μM; T352A, 2.8 μM; T359A, 2.8 μM; K362L, 4.4 μM. Stoichiometries were calculated using the enzyme concentrations given above, and the absorbance changes produced by the addition of known amounts of acid to the flow cell exhaust after adjustment to the proper pH. Since the stepwise decrease in absorbance immediately after the laser pulse seen in all traces is probably an artifact caused by slightly different concentrations of the enzyme in buffered and unbuffered medium, the absorbance immediately after the laser pulse was taken as the starting absorbance in the stoichiometry calculation.

Table 3: Observed Proton Uptake Rate in the Oxygen Reaction of the Fully Reduced Wild-Type and Helix VIII Mutant Proteins

strain	proton uptake rate ($k \times 10^{-3}$, s^{-1})	strain	proton uptake rate ($k \times 10^{-3}$, s^{-1})
wild-type (RG145)	10	T359A	19
T352A	30	T359S	21
T352S	24	K362L	22
T352N	14	K362M	17
M353A	18	K362Q	20
P358A	24		

experiment. The rate constant seen for proton uptake is significantly higher for the mutant enzymes than for the wild-type protein, with the effect that the proton uptake appears synchronous with the first phase of the oxygen reaction. The higher proton uptake rate is, however, observed in all mutants and does not correlate with the effect of the mutation on the activity. It appears, therefore, that neither the redox chemistry nor the proton uptake associated with the oxygen reaction has been impaired by any of the mutations in helix VIII.

Internal Electron Transfer after Photodissociation of CO from the CO Mixed-Valence Compounds. The results obtained in the flow-flash experiments do not explain the loss of activity in K362M, -L, and -Q and the two threonine-to-alanine mutants. In order to study the effect of these mutations on other parts of the catalytic cycle, we have investigated electron transfer between the low-spin cytochrome and the oxygen-binding site in cytochrome bo_3 as suggested by the recent work of Morgan et al. (1993). These authors demonstrated that electron transfer from the oxygen-binding site to the low-spin cytochrome takes place after the photodissociation of CO from cytochrome o_3 in the mixed-valence compound of cytochrome bo_3. Several kinetic components were detected by Morgan et al. (1993), the fastest three of which could be attributed to electron transfer from cytochrome o_3 to the low-spin cytochrome. In the present investigation, we have chosen to focus on the three fastest reactions, and have limited our study to the first 0.2 ms after CO photodissociation.

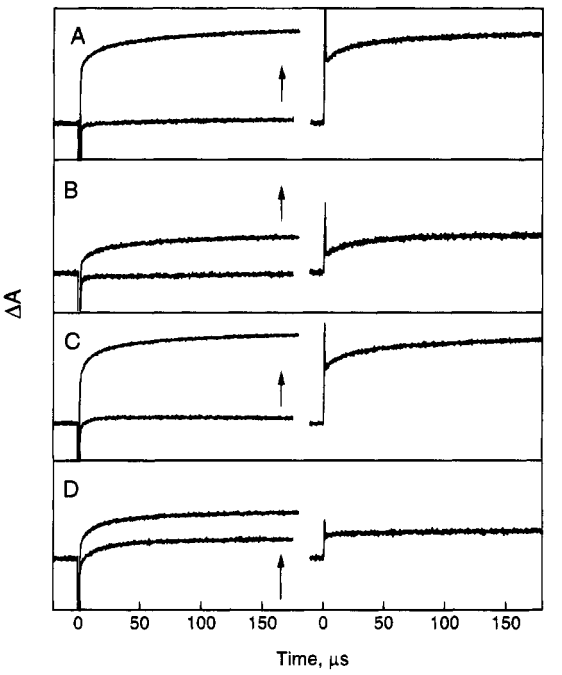

FIGURE 3: Electron redistribution after photodissociation of CO from the CO mixed-valence compound of wild-type enzyme and mutants T352A, T359A, and K362M measured in the α band. For each derivative, a wavelength was chosen that minimized the absorbance change after photodissociation of CO from the corresponding fully reduced CO complex. The traces to the left were obtained from the CO mixed-valence samples (top traces) and the fully reduced CO complexes (bottom traces). The traces to the right are the differences obtained by subtracting the fully reduced traces from the mixed-valence traces. (A) Wild-type enzyme (562 nm); (B) T352A (567 nm); (C) T359A (562nm); (D) K362M (565 nm). Enzyme concentrations were 15−20 μM. To facilitate comparison, the traces have been scaled according to the relative concentrations of the mixed-valence species in the samples as estimated from the increase in absorbance at the wavelength pair 565−650 nm on incubation with CO. Each trace is the average of 1000 individual transients. The arrows represent an absorbance increase of $\Delta A = 0.005$.

In order to isolate the absorbance changes due to interheme electron transfer from those due to CO dissociation and recombination, a wavelength was chosen where the absorbance changes obtained on photolyzing a sample of the fully reduced CO complex were minimized. The latter reaction was found to be close to isosbestic at wavelengths around 560 nm as described by Brown et al. (1994). In Figure 3, time courses obtained at these wavelengths with samples of fully reduced and mixed-valence complexes of wild-type enzyme, T352A, T359A, and K362M are compared. Since

Table 4: Kinetic Parameters for Electron Redistribution after Photodissociation of CO from the CO Mixed-Valence Compounds Obtained by Curve-Fitting to the Scaled Difference Traces in Figure 3 (Traces to the Right)[a]

derivative	first phase (unresolved) amplitude	second phase		third phase	
		$k \times 10^{-3}$ (s^{-1})	amplitude	$k \times 10^{-3}$ (s^{-1})	amplitude
wild-type (RG145)	0.0082	96	0.0023	13	0.0024
T352A	0.0023	89	0.0011	23	0.0020
T359A	0.0076	80	0.0021	9	0.0027
K362M	0.0033	ND[b]	ND	11	0.0006

[a] Amplitudes are given in absorbance units. [b] Not detected.

we were not able to find a wavelength without observable absorbance changes in the fully reduced samples in all cases (in particular with K362M), the kinetic analyses were carried out on difference traces obtained by subtracting the time courses obtained with the fully reduced samples from those obtained with the mixed-valence enzymes. The kinetic parameters found from curve-fitting to these difference traces are summarized in Table 4.

With the wild-type enzyme, we find, in good agreement with Morgan et al. (1993), three kinetic components. The first component could not be resolved and is seen only as a stepwise absorbance increase immediately after photolysis. This reaction is clearly more than 90% complete already after the initial spike caused by scattered laser light. With a duration of about 4 μs for the spike, one can estimate a lower limit of 6×10^5 s^{-1} (a lifetime of less than 2 μs). For the second and third phases, rate constants of 1×10^5 and 1.4×10^4 s^{-1} are obtained with the wild-type enzyme, in reasonable agreement with the results of Morgan et al. (1993) and Brown et al. (1994). With the mutant T359A, very similar results are obtained (trace C). Mutant T352A (trace B) displays a qualitatively similar behavior, but here the total amplitude is smaller and the proportion of the slowest phase has increased somewhat at the expense of the initial absorbance jump. With the mutant K362M, it was particularly difficult to find a wavelength without contributions from the fully reduced CO complex. The traces shown (D) were taken at a wavelength where the CO recombination at about 50 s^{-1} was almost undetectable in a sample of the fully reduced CO complex of the K362M enzyme. However, there are still some fast reactions seen after CO photolysis in the reduced sample, whose nature is not presently understood (Figure 3). The difference trace obtained with the mutant K362M clearly deviates from the behavior seen with the wild-type enzyme. The overall amplitude is only about 30% of that seen in the wild-type enzyme, and most of the absorbance change takes place in the very rapid component. Curve-fitting to the difference trace showed that the slow component accounts for the absorbance change after the initial jump, and that the intermediate component is completely absent (Table 4).

DISCUSSION

The present results, together with earlier investigation of the activities of the helix VIII mutants *in vivo* and in isolated membranes (Thomas et al., 1993), show that these mutant proteins form three distinct categories. The first category contains the mutants T359S; P358A; M353A; and T352S and -N, which are active in the complementation assay. The second group is formed by the two threonine-to-alanine mutants T359A and T352A, which are inactive in the complementation assay, and show a reduction of their specific activity *in vitro* to about 10−20%. Third, the mutations tested in the 362 position, K362M, -Q, and -L, are inactive in complementation and have *in vitro* activities less than 1% of that of the wild-type. Solvent deuterium isotope effects on the steady-state activity of cytochrome bo_3 have not been reported earlier, but the approximately 2-fold reduction of activity seen here (Table 1) is similar to the effect seen with the bovine cytochrome *c* oxidase (Wilms et al., 1981; Thörnström et al., 1984). The isotope effect on the residual activity of the mutants inactive in the complementation assay was similar to that of the wild-type enzyme, which suggests that the reason for the reduced activity is not an increased kinetic barrier for rate-limiting proton transfer in the mutants.

In the time course of the reaction between reduced enzyme and oxygen, only minor variations of the apparent rate constants were observed (Table 2). However, the contribution of the first phase to the total amplitude varies among the mutants. We have observed earlier (Svensson & Nilsson, 1993) that the enzyme isolated from strain RG145, which is a mixture of the bo_3 and oo_3 species, has a smaller contribution of the first phase than enzyme isolated from the nonoverproducing strain GO103 (which has been reported to contain only the bo_3 species). It is therefore likely that the variable relative contribution of the first phase is due to variable heme content, i.e., that the bo_3/oo_3 ratio is different for the different mutant enzymes. In this case, one also expects a corresponding variability of the combined amplitudes of the first and second phases, since the differential extinction coefficient for low-spin cytochrome *o* oxidation at 430 nm is only about half of that for cytochrome *b* oxidation (Morgan et al., 1993). It can be seen in Table 2 that the largest total amplitude, as well as the largest relative contribution from the first phase, is found in the GO103 enzyme. This result supports the notion that it is the bo_3/oo_3 ratio that is the main determinant of the relative contributions of the first and second phases. A variation in the heme content among the mutant enzymes is in accordance with the observation by Puustinen et al. (1992) that a very high proportion of the oo_3 species is present in a particular mutant enzyme (i.e., E286Q).

It has recently been suggested by Verkhovsky et al. (1994) that the effect of heme content on the relative contributions of the first and second phases is due to a lower intrinsic rate for intramolecular electron transfer in the oo_3 species than in the bo_3 species. The substantial contribution from the second phase with enzyme isolated from strain GO103 (see Table 2) shows, however, that at least part of the amplitude of the second phase is due to a reaction constitutive to oxygen reduction, and cannot be attributed solely to slow oxidation of cytochrome *o* in the low-spin site. On the other hand, if there were no oxidation of low-spin cytochrome *o* in the

slow phase, its amplitude would not depend on the heme content. The results presented in Table 2 demonstrate, however, that the amplitude of the second phase is substantially larger in wild-type enzyme isolated from the RG145 strain, and in the mutant enzymes, than in wild-type enzyme from the GO103 strain. These considerations suggest a scenario in which cytochrome *b* oxidizes in the first phase, and low-spin cytochrome *o* in the second phase, as suggested by Verkhovsky et al. (1994), but that a reaction subsequent to the oxidation of low-spin cytochrome also contributes to the second phase.

Very recently, another flow-flash study of the oxygen reaction in cytochrome bo_3 was published by Orii et al. (1994). These authors report only one kinetic component in contrast to the two components found by us with wild-type enzyme prepared from the overproducing RG145 strain as well as the nonoverproducing GO103 strain. However, the absence of a clear isosbestic point in the kinetic difference spectra presented by Orii et al. (1994) suggest the presence of additional processes also in this experiment, but their relative contribution is smaller than that found by us for the second phase. Also, the kinetic scheme used by these authors in describing their results appears incomplete, including only reversible binding of oxygen, with the consequence that the rate equation obtained from the scheme cannot describe reactions beyond oxygen binding to the reduced enzyme.

Since the enzyme prepared from the GO103 strain in the present study was found to contain the pure bo_3 species, it is not likely that the different results are due to a variation in the heme content. Other possible explanations include a variation in the amount of bound ubiquinone (Orii et al., 1994), and the different reductants used to prepare the fully reduced enzyme. In order to address the former possibility, we performed a flow-flash experiment in the presence of excess decylubiquinol, but we were not able to detect any effect on the relative contribution of the two phases (Table 2). Clearly, understanding the nature of the second kinetic phase requires further studies.

Our observation of proton uptake during the oxygen reaction in all mutants suggests that none of the residues mutated are required for this particular proton uptake reaction. However, the fully reduced enzyme contains only three electrons, and the expected end product in its reaction with oxygen is a ferryl species (Svensson & Nilsson, 1993). The present experiments do therefore not allow the detection of a counterpart to the proton uptake observed in connection to the transfer of the fourth electron to the oxygen-binding site in the bovine cytochrome *c* oxidase (Oliveberg et al., 1991). Impaired proton uptake during the conversion of the ferryl species to the oxidized enzyme thus remains a viable explanation for the effect of the mutations in helix VIII on the catalytic activity. The acceleration of proton uptake observed in the mutant enzymes is probably due to structural effects since it is seen in both active and inactive mutants. Structural effects are in agreement with the perturbed FTIR spectra (Thomas et al., 1993), suggesting that these residues are in the vicinity of the active site.

In order to further characterize electron transfer between the low-spin cytochrome and the oxygen-binding site in the inactive mutants, we have studied electron redistribution after photolysis of CO from the mixed-valence compound as described by Morgan et al. (1993). These authors found three kinetic components that all displayed the spectral characteristics of electron transfer from cytochrome o_3 to the low-spin cytochrome. They suggested, therefore, that the different rates observed reflect the rates of relaxations of the enzyme into a state where the electron transfer is faster or energetically more favorable, rather than different intrinsic rates for internal electron transfer. In the current picture of the structure of the heme−copper oxidases, both the cytochrome at the oxygen-binding site and the low-spin cytochrome are coordinated by histidine residues located in the transmembrane helix X. It has been pointed out by Woodruff (1993) that this arrangement provides a σ-bonded pathway for electron transfer between the two heme groups. Consequently, a change in the intrinsic rate for interheme electron transfer would require changes in heme coordination. It appears, therefore, more likely that the different kinetic components reflect transitions into states where the electron transfer is thermodynamically more favorable. For the corresponding reaction in the bovine cytochrome *c* oxidase, two kinetic components with similar spectral characteristics have been observed, and in this case the slower component is probably limited by proton transfer (Hallén et al., 1994).

The similarity of the results obtained in the experiment of electron redistribution after photolysis of CO from the mixed-valence compound with the mutant T359A and with the wild-type enzyme suggests that neither the overall thermodynamics of the reaction nor the rates of the suggested secondary relaxations have been affected. Also in the mutant T352A, all three kinetic components found in the wild-type enzyme are present, but here the extent of the reverse electron transfer is less than in the wild-type enzyme. This suggests that the T352A enzyme is capable of relaxing into states more favorable for electron transfer similarly to the wild-type enzyme, but that the relative reduction potentials of the low-spin cytochrome and the oxygen-binding site are less favorable for reverse electron transfer. Alternatively, the decreased total extent of electron transfer in this mutant is due to an increased content of the oo_3 species as was found for the E286Q mutant by Morgan et al. (1993). Another possibility is that the extent of electron transfer is diminished by overreduction of the low-spin cytochrome or underreduction of Cu_B in the preparation of the mixed-valence compounds. Optical spectra taken before and after the experiments suggest, however, that overreduction of the low-spin cytochrome has not taken place. On the other hand, we cannot exclude that the redox state of Cu_B is different in the different samples.

With the K362M enzyme, only the first and third components are observed, with amplitudes that are lower than those for the corresponding components in wild-type enzyme. Given that neither the optical spectrum nor the light-induced FTIR difference spectrum of the CO adduct of the mutant K362M is different from those seen in the wild-type enzyme (Thomas et al., 1993), it is highly unlikely that the arrangement of the heme groups is different. One would therefore expect a similar intrinsic rate for electron transfer between the two hemes. A more likely explanation for our observations is that the secondary relaxations have been affected by the mutation. Although the nature of these relaxations is presently unknown, it is interesting to note that a similar (albeit slower) subsequent component in reverse electron transfer in the bovine cytochrome *c* oxidase has been shown to involve proton transfer (Hallén et al., 1994).

In conclusion, we have demonstrated that none of the protonatable residues in helix VIII are required in the reaction between the fully reduced cytochrome bo_3 and oxygen. The impaired catalytic activities observed in the mutants T352A, T359A, and K362M are therefore probably due to effects of the mutations on catalytically important reactions that are not included in the flow-flash experiment. These include the transfer of the fourth electron to the oxygen-binding site and electron input to the enzyme, both of which have been shown to be associated with proton uptake in the bovine cytochrome *c* oxidase (Oliveberg et al., 1991; Mitchell et al., 1992). The results obtained in the reverse electron transfer experiment with K362M are consistent with an effect of the mutation on internal electron transfer.

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