Glutamate-89 in Subunit II of Cytochrome bo_3 from *Escherichia coli* Is Required for the Function of the Heme−Copper Oxidase[†]

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ABSTRACT: Recent electrostatics calculations on the cytochrome *c* oxidase from *Paracoccus denitrificans* revealed an unexpected coupling between the redox state of the heme−copper center and the state of protonation of a glutamic acid ($E78^{II}$) that is 25 Å away in subunit II of the oxidase. Examination of more than 300 sequences of the homologous subunit in other heme−copper oxidases shows that this residue is virtually totally conserved and is in a cluster of very highly conserved residues at the "negative" end (bacterial cytoplasm or mitochondrial matrix) of the second transmembrane helix. The functional importance of several residues in this cluster ($E89^{II}$, $W93^{II}$, $T94^{II}$, and $P96^{II}$) was examined by site-directed mutagenesis of the corresponding region of the cytochrome bo_3 quinol oxidase from *Escherichia coli* (where $E89^{II}$ is the equivalent of residue $E78^{II}$ of the *P. denitrificans* oxidase). Substitution of $E89^{II}$ with either alanine or glutamine resulted in reducing the rate of turnover to about 43 or 10% of the wild-type value, respectively, whereas E89D has only about 60% of the activity of the control oxidase. The quinol oxidase activity of the W93V mutant is also reduced to about 30% of that of the wild-type oxidase. Spectroscopic studies with the purified E89A and E89Q mutants indicate no perturbation of the heme−copper center. The data suggest that $E89^{II}$ (*E. coli* numbering) is critical for the function of the heme copper oxidases. The proximity to K362 suggests that this glutamic acid residue may regulate proton entry or transit through the K-channel. This hypothesis is supported by the finding that the degree of oxidation of the low-spin heme *b* is greater in the steady state using hydrogen peroxide as an oxidant in place of dioxygen for the E89Q mutant. Thus, it appears that the inhibition resulting from the $E89^{II}$ mutation is due to a block in the reduction of the heme−copper binuclear center, expected for K-channel mutants.

Cytochrome bo_3 from *Escherichia coli* is a member of the heme−copper oxidase superfamily (*1*). Whereas most of the known members of this superfamily function as cytochrome *c* oxidases, cytochrome bo_3 is part of a subgroup that catalyzes the two-electron oxidation of membrane-bound quinol. Cytochrome bo_3 is also a proton pump, as are the cytochrome *c* oxidases (*2*, *3*). The *E. coli* oxidase has one copy each of four subunits (*4*, *5*). Subunits I−III are homologues of the three mitochondrially encoded subunits of the eukaryotic cytochrome *c* oxidases, and are common to most members of the superfamily. Subunit IV is unique to cytochrome bo_3, and its function is not known (*6*). The structures of two different cytochrome *c* oxidases are known: that from bovine heart mitochondria (*7*−*9*) and that from the bacterium *Paracoccus denitrificans* (*10*, *11*). These two structures exhibit remarkable similarities, which clearly provide a guide to features that are common to the superfamily of enzymes. The enzyme from *P. denitrificans* contains four subunits (*10*), while the bovine heart oxidase has 13 different subunits (*7*). However, it has been shown clearly in the case of the oxidase from *P. denitrificans* that subunits I and II are sufficient for cytochrome *c* oxidase function, including proton pumping (*12*). The X-ray structure of the two-subunit form of the oxidase from *P. denitrificans* has recently been reported (*11*).

Subunit I is the most highly conserved among the superfamily and is the defining feature of the superfamily (*1*, *13*). This subunit contains the heme−copper center, where dioxygen is activated and reduced to water, as well as a low-

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spin heme component that serves as a conduit for electrons to the heme−copper center (*13*). The heme−copper center is deeply buried within the protein, and at least one pathway is required to deliver protons from the bacterial cytoplasm (mitochondrial matrix) that are needed for the chemical conversion of dioxygen to water. A proton-conducting channel(s) is also needed for those protons that are pumped across the membrane. These channels are located within subunit I (*7*, *9*, *11*). Both X-ray structures (*7*, *11*) show the so-called K-channel, providing a path for proton translocation to the heme−copper center from the aqueous phase (bacterial cytoplasm or mitochondrial matrix). The central residue in this channel is a lysine ($K354^I$ in *P. denitrificans* and $K319^I$ in the bovine heart oxidase). A substantial accumulation of evidence has demonstrated that this lysine is essential for function. The function of the K-channel appears to be to deliver protons that are required to stabilize the reduced form of the heme−copper center, prior to the interaction with dioxygen (*14*−*18*). There is substantial evidence that the protons required after the binding of dioxygen for both chemistry and for the proton pump are delivered via a second input channel called the D-channel (*19*−*24*). This has been structurally defined in the *P. denitrificans* oxidase leading from $D124^I$, near the cytoplasmic surface, to $E278^I$, near the heme−copper center (*10*, *11*), and is not well-defined past this point. Support for the functional roles of the K-channel and D-channel has been provided by the study of site-directed mutants, and much of this work has been with cytochrome bo_3 (*20*, *25*−*29*). The critical lysine in subunit I of cytochrome bo_3 is $K362^I$, and the two key residues in the D-channel are $D135^I$ and $E286^I$.

The mechanism by which the redox chemistry at the heme−copper center is coupled to the proton pump is not known, though different models have been proposed (*9*, *10*, *30*−*32*). Recently, Kannt et al. (*33*) used the coordinates of the oxidase from *P. denitrificans* to perform electrostatic calculations to predict which protonatable residues would be influenced by changing the redox status of the metal centers. The most surprising result was the relatively strong coupling between the heme−copper center and an otherwise obscure glutamic acid residue ($E78^{II}$) in subunit II that is located 25 Å away. Kannt et al. (*33*) also point out that a significant coupling would also be expected between $E78^{II}$ and the lysine in the K-channel ($K354^I$ in *P. denitrificans*), which is separated from $E78^{II}$ by about 10 Å.

Subunit II in most of the heme−copper oxidases is anchored to the membrane by two transmembrane helices that are in direct contact with subunit I (*7*, *10*, *11*). The glutamic acid $E78^{II}$ is part of a cluster of very highly conserved amino acids in the second transmembrane span, located near the bacterial cytoplasm. The equivalent residue in the bovine oxidase is $E62^{II}$, and in cytochrome bo_3, it is $E89^{II}$. In the work presented here, the functional importance of this residue, as well as others in the conserved cluster, is examined by site-directed mutagenesis of cytochrome bo_3. The data indicate that a negative charge is important at this position, possibly to facilitate either the entry of protons into the K-channel or the transit of protons through this channel.

MATERIALS AND METHODS

Materials. The restriction enzymes *Kpn*I, *Sal*I, *Hin*dIII, and *Msp*I were purchased from Bethesda Research Laboratories. The restriction enzyme *Hha*I, T4 DNA ligase, and T4 DNA kinase were from New England Biolabs. T4 DNA polymerase, exonuclease III, and sequenase kits were obtained from U.S. Biochemistry Corp. Nucleotides dATP, dGTP, dCTP, dTTP, and 5 Me-dCTP were from Pharmacia LKB Biotechnology, Inc. ATP and phenylmethanesulfonyl fluoride (PMSF) were from Sigma. Oligonucleotides used for mutation or sequencing were obtained from the Biotechnology Center at the University of Illinois (Urbana, IL). Ubiquinone-1 was a gift from Hoffman-LaRoche AG (Basel, Switzerland).

Mutagenesis. Single-stranded M13 DNA was prepared following the procedures of Messing (*34*), and the mutagenesis was performed as described previously (*35*) with some modifications (J. Ma et al., manuscript submitted for publication). Once desired mutations were identified on M13 phage by single-stranded DNA sequencing, the fragments containing the mutations were cloned back into the *cyo* operon in plasmid pMC31 (*36*). After subcloning, all mutations were confirmed by double-stranded DNA sequencing. GL101 (*36*) was used to propagate both wild-type and mutant plasmids.

Complementation Analysis of the Mutant Plasmids. Genetic complementation was conducted using the procedure previously described (*36*). The host strain used for complementation analysis was either RG129 (*37*) or GO105 (*38*) which cannot grow aerobically on nonfermentable substrates because of mutations in both the chromosomal *cyo* and *cyd* operons. Overnight aerobic growth on succinate−lactate minimal plates at 37 °C was the criterion used to determine the presence of a functional cytochrome bo_3.

Cell Growth and Membrane Preparation. For functional mutant oxidases (i.e., supporting aerobic growth), strain GO105 (*38*) was used as the host strain for further characterization. The protocol for growing the cells has been described (J. Ma et al., manuscript submitted for publication). After the cell density was increased by about 70 Klett units (red filter), the cells were harvested by centrifugation and then washed with 10 mM Tris-HCl (pH 8.0). The washed cells from a 1 L culture were resuspended in 20 mL of buffer containing 10 mM Tris-HCl, 0.5 mM phenylmethanesulfonyl fluoride, 0.5 μg/mL leupeptin, 5 mM $MgSO_4$, and 4 μg/mL DNAse. The cell suspension was passed through a French pressure cell at 1200 psi twice, and cell debris and most of the outer membrane were removed by centrifugation at 9000*g* for 30 min, followed by a second centrifugation at 25000*g* for 30 min. The supernatant was centrifuged at 160000*g* in a 60 Ti rotor for 1.5 h. The red colored cytoplasmic membranes were collected and homogenized in 2 mL of ice-cold 50 mM potassium phosphate buffer (pH 7.5) with 0.5 μg/mL leupeptin for subsequent characterization.

For the two noncomplementing mutants, $E89^{II}A$ and $E89^{II}Q$, a strain containing the chromosomal *cyd* operon was used as the host strain. The strain that was used was a derivative of RG129 (*37*). Growth and preparation of membranes were performed as described above, and the membranes were used to purify the $E89^{II}A$ and $E89^{II}Q$ oxidase mutants.

Protein Purification. The wild-type and mutant cytochrome bo_3 oxidases were solubilized and purified to homogeneity according to the published protocol (*39*).

Oxidase Activity Assay. Ubiquinol-1 oxidase activity at 37 °C was measured with a YSI (model 53) oxygen electrode

(Yellow Springs Instrument Co., Yellow Springs, OH) and a temperature-controlled 1.8 mL electrode chamber (Gibson). The buffer for this assay of membrane samples was 50 mM Tris and 1 mM EDTA (pH 7.4) containing 2 mM dithioerythritol and 150 mM ubiquinone-1. For assays with the purified oxidase, the buffer contained 50 mM HEPES, 1 mM EDTA, 0.01% dodecyl maltoside, 2 mM dithioerythritol, and 150 μM ubiquinol-1 (pH 7.4). The concentration of O_2 in the air-saturated buffers at this temperature was assumed to be 250 μM. The reaction was initiated by injecting 5 μL of appropriately diluted membrane preparations or pure enzymes. The background drift prior to the addition of oxidases was subtracted.

UV−Visible Steady-State Spectroscopy. The rapid-mixing experiments were performed using and Applied Photophysics (Leatherhead, U.K.) SX-18MV stopped-flow apparatus. The mixing ratio for the experiments was 1:1. For all reactions, one syringe contained 2 mM air-oxidized cytochrome bo_3 E89Q mutant protein in 50 mM HEPES buffer (pH 7.5), 0.01% dodecyl maltoside, 1 mM Q_1H_2, and 10 mM DTT. The protein sample was equilibrated for at least 30 min at room temperature prior to loading into the syringe where it became fully reduced (FR). The second syringe contained either air-saturated buffer or was supplemented with 2 M H_2O_2. Analyses were performed using Applied Photophysics Origin software (Microcal Software, Inc.).

Proton Pumping Assay. Proton pumping was assessed as previously described (*3*, *20*).

Electrostatics Calculations. The electrostatic potential contours were calculated using the program GRASP (*40*) and the structure of the bovine oxidase (*7*), entry 1occ from the Brookhaven Protein Data Bank. The dielectric constant inside the protein was set to 4, and the ionic strength was set to 0. Similar results were obtained using the coordinates of the oxidase from *P. denitrificans* (*10*).

Miscellaneous. The protein concentration was determined using the BCA method (Pierce Chemical Co.). Optical spectroscopy was carried out on a DW2000 UV-Vis spectrometer (SLM Instruments, Inc.). FTIR difference spectroscopy was performed as previously described (*20*, *41*). Analysis of the heme composition of purified mutant cytochrome bo_3 oxidases was performed as previously described (*42*).

RESULTS

Sequence alignmnets from more than 300 sequences of subunit II from heme−copper oxidases showed that some residues in the region corresponding to **E**^{89}AVV**WT**V**P**^{96} in cytochrome bo_3 are very highly conserved (indicated in bold). This region is at the cytoplasmic end of the second transmembrane span of subunit II, and several of these residues are at or close to the interface with subunit I. All the subunit II sequences examined have the equivalent of proline-96 in the *E. coli* oxidase, and most have tryptophan-93. Essentially, all the sequences have the equivalent of E89^{II}, though in some sequences this residue is displaced by one position in relation to W93^{II}. What appears constant is the seven-residue spacing (six intervening residues) between the glutamate and proline. For example, in the *E. coli* quinol oxidase, the sequence is **E**^{89}AVV**WT**V**P**^{96}, whereas in the quinol oxidase from *Bacillus subtilis*, the equivalent sequence is **E**^{95}VV**WT**VI**P**^{102}. The *E. coli* sequence has one more residue between the E and W, but one less separating the W and P in the sequence. In the cytochrome *c* oxidase from *P. denitrificans*, the sequence is **E**^{78}VI**WT**LV**P**^{85}, and in the oxidase from bovine heart mitochondria, it is **E**^{62}TI**WT**IL**P**^{69}. In the X-ray structures of both the *P. denitrificans* (*10*, *11*) and the bovine oxidases (*7*), the carboxyl of this glutamate in subunit II is about 4 Å from a serine hydroxyl from subunit I (S357^{I} in *P. denitrificans* and S322^{I} in the bovine oxidase). This serine is very highly conserved among the heme−copper oxidases as either serine or asparagine (e.g., N365^{I} in subunit I of the *E. coli* oxidase).

Table 1: Properties of the Mutants in Subunit II of Cytochrome bo_3

	complementation	relative oxidase specific activity[a]	relative level of expression[b]	K_M (UQ-1) (μM)
wild type	yes	100	100	38
E89A	no	43	25	31
E89Q	no	10	28	37
E89D	yes	59	78	29
W93V	yes	30	70	47
T94A	yes	99	96	41
P96A	yes	100	94	81

[a] The specific activity of the wild-type oxidase was taken to be 100%. The ubiquinol-1 oxidase activity was evaluated per mole of CO-binding heme o_3. Both the E89A and E89Q mutants were purified, and the specific activities of these mutants are given relative to that of a preparation of the purified wild-type oxidase. [b] Determined by the relative amount of CO-binding heme o_3 in the membrane samples, using an extinction coefficient of 135 mM^{-1} cm^{-1}.

To examine the importance of residues in the transmembrane span of subunit II, a set of site-directed mutants were made in cytochrome bo_3: E89^{II} (to Q, A, or D), W93^{II} (to V), T94^{II} (to A), and P96^{II} (to A). The results are summarized in Table 1. Most dramatic are the effects of replacing E89^{II} with glutamine. This mutant cannot complement an oxidase-deficient strain, i.e., support aerobic growth in a strain lacking other oxidases. To further examine this "dead" mutant, it was expressed in a strain that can grow aerobically due to the presence of the alternate oxidase, cytochrome *bd*. It was observed spectroscopically that the mutant oxidase was present in the membranes of these strains, albeit at a relatively low level. The dead oxidase was then solubilized and purified using a standard protocol (*39*). In addition, the W93V mutant and wild-type oxidase were also purified and examined along with the E89^{II} mutants. The purified enzymes were similar to the wild type as determined by SDS−PAGE analysis. Immunoblotting demonstrated that there was no contamination of these preparations by cytochrome *bd*, so any quinol oxidase activity catalyzed by these preparations must be due to the cytochrome bo_3 mutants.

The purified mutants were examined spectroscopically. Figure 1A shows that all of the enzymes (wild type, E89A, E89Q, and W93V) form the expected CO adduct, as measured by the dithionite-reduced plus CO-minus-dithionite-reduced difference spectrum. The characteristic peak at 416 nm and trough at 430 nm are observed in all cases. The dithionite-reduced-minus-air-oxidized difference spectra, recorded at 77 K, are shown in Figure 1B. The split α-band is apparent in all cases, as expected. The relative heights of the two peaks are reversed in the E89A and E89Q mutants. This might be due to a perturbation sensed by the low-spin heme, which is responsible for the spectroscopic features in

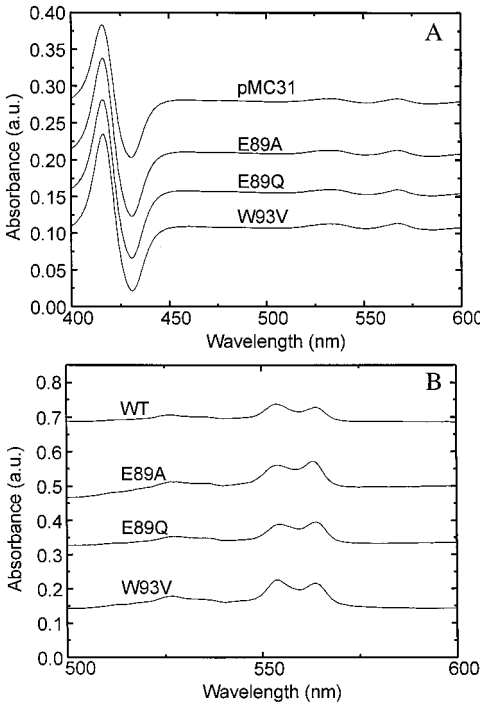

FIGURE 1: Optical spectra of the purified oxidases. (A) Dithionite-reduced plus CO-minus-dithionite-reduced difference spectra. Spectra were recorded at room temperature with a protein concentration of 0.2 mg/mL. (B) Dithionite-reduced-minus-air-oxidized difference spectra recorded at 77 K with a protein concentration of 2 mg/mL.

this region, or it might also be due to a change in the occupancy of the low-spin heme binding site by heme *o* versus heme *b* (*43*). For this reason, the hemes were extracted from each enzyme and analyzed by HPLC. The data indicated no differences in the ratio of heme *b* to heme *o* when comparing the wild type with any of the mutant oxidases. It was discovered during the course of this work that the use of plasmid pMC31 resulted in a heme ratio that favored heme O more than observed with other vectors, but this was consistent in all the enzymes examined in this work. Hence, the spectroscopic differences observed in Figure 1B could be due to a subtle change in the low-spin heme component of the oxidase.

Figure 2 shows the reduced-minus-oxidized spectra recorded from 600 to 800 nm at 77 K. The spectra of all of the enzymes exhibit a peak around 756 nm which is a charge-transfer band (*44*) between the porphyrin and iron of the high-spin ferrous heme component of the heme−copper center (*45*). The "conformation" band is sensitive to the structure of the heme site, and the results, therefore, suggest that there is no signifcant change in the environment of the high-spin heme due to any of these mutants. This is further confirmed by using low-temperature FTIR difference spectroscopy of the CO adduct of the reduced enzymes. This technique has been used previously with success to detect perturbations due to mutations at the heme−copper center (*28*, *46*, *47*). The CO adduct of the high-spin heme o_3 is made and then cooled to about 20 K. The FTIR spectrum of this sample includes the stretching band of CO bound to the heme Fe (1959 cm^{-1}). Upon photolysis at this low temperature, the CO moves from the heme Fe to the nearby Cu_B, and the FTIR spectrum now contains a feature due to CO bound to Cu. The difference spectra of the wild-type and

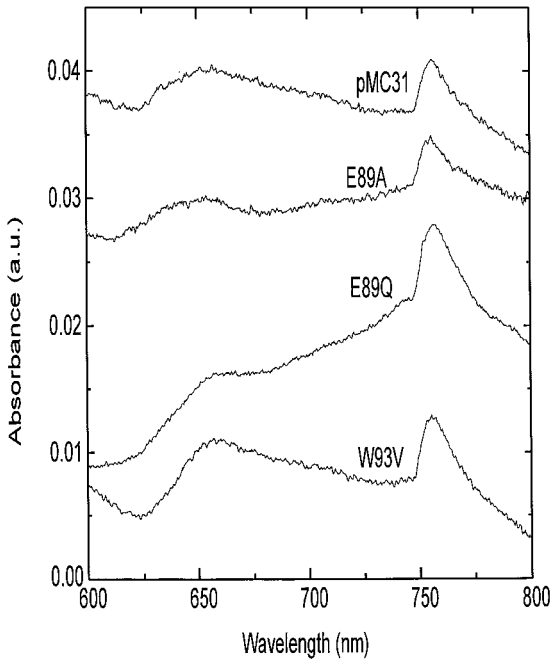

FIGURE 2: Dithionite-reduced-minus-air oxidized difference optical specta in the near-infrared region of the purified oxidases. Spectra were recorded at 77 K with a protein concentration of 2 mg/mL.

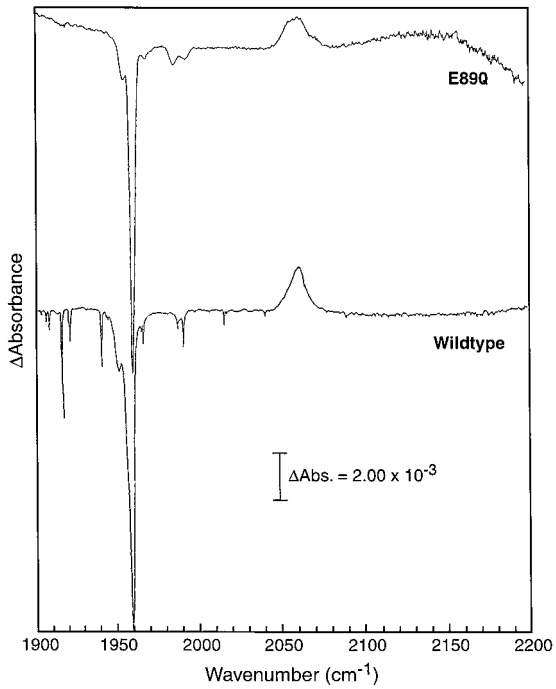

FIGURE 3: "Light-minus-dark" FTIR absorbance difference spectra of the wild-type and E89Q mutant cytochrome bo_3. Spectra were recorded at 20 K with a path length of 27 μm. The absorption band at 1959 cm^{-1} is the Fe−CO vibrational mode, and the 2063 cm^{-1} band is the Cu−CO vibrational mode.

E89Q mutant oxidases are shown in Figure 3. The Fe−CO band remains as a very narrow feature in the mutant (1959 cm^{-1}), indicating a very ordered and well-defined heme−copper center. The Cu−CO band shows evidence of heterogeneity that is also evident in the wild-type enzyme, and is not significantly perturbed. There is no indication of a perturbation of the active site due to the E89Q mutation, which has only about 9% of the turnover of the wild-type oxidase.

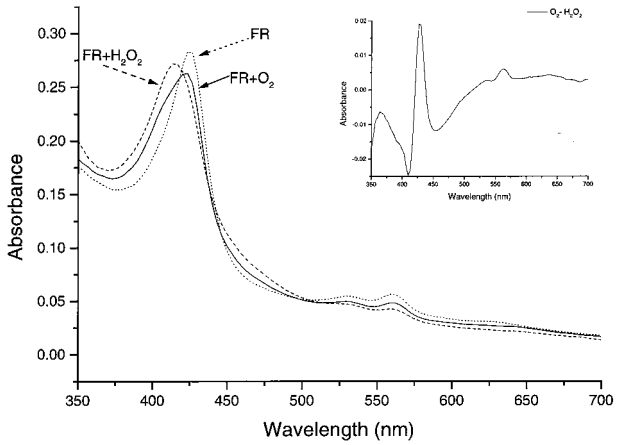

FIGURE 4: Steady-state spectra of the E89Q mutant after the addition of either O_2 or H_2O_2 to the fully reduced (FR) enzyme. Shown are the spectrum of the fully reduced cytochrome bo_3 mutant (FR) in the presence of excess ubiquinol-1, the spectrum in the steady state after mixing the fully reduced enzyme with 2 M H_2O_2, and the spectrum in the steady state after mixing the fully reduced enzyme with air-saturated buffer. The difference spectrum of the steady state obtained in the presence of O_2 minus the steady state in the presence of H_2O_2 is shown in the inset. The difference is attributed to the more complete oxidation of heme *b* by H_2O_2.

Table 2: Proton Pumping by Cytochrome bo_3 Mutants

	H^+/e^-		
	pH 6.0	pH 7.0	pH 8.0
wild type	1.6−2.0	1.3−1.6	1−1.3
T94A	1.5−2.0	1.3−1.8	1.0
P96A	1.8−2.0	1.7	−
S299A	1.6−2.0	−	−

The ubiquinol-1 oxidase activity of the mutants was examined, in the membrane or, where feasible, after purification. The specific activity was determined relative to the amount of CO-binding heme o_3 in each preparation. The data (Table 1) show virtually no effect due to either the T94A or P96A mutant. The specific activity of the W93V mutant is only 30% of that of the wild type, whereas the E89A and E89Q mutants are only 43% and 10% as active as the wild type. Even the conservative replacement of E89D causes a drop in the rate of turnover to about 60% of the control.

Since these mutants are located on the cytoplasmic side of the membrane, it is not expected that they would be directly involved in the interation with ubiquinol, which appears to be on the periplasmic side of the membrane (*48*). This is confirmed by the fact that the K_m for ubiquinol-1 is essentially unchanged due to these mutations (Table 1), with the possible exception of P96A, where the small increase (about double) in the K_m is close to the limits of accuracy of the assay. Ubiquinol-1 was also used to reduce each of the mutants. In all cases, both the low-spin and high-spin heme components were fully reduced by ubiquinol-1, consistent with the fact that all of the mutants have measurable quinol oxidase activity.

Figure 4 compares the spectra of the fully reduced (FR) enzyme, the fully reduced enzyme mixed with air-saturated buffer (FR+O_2), and the fully reduced enzyme mixed with 2 M H_2O_2 (FR+H_2O_2). The inset shows the difference in the absorbance of the two steady states. Heme *b* is fully reduced in the FR+O_2 spectrum, whereas the addition of 2 M H_2O_2 oxidizes heme *b*. In other words, in the presence of H_2O_2, but not O_2, a significant amount of heme *b* becomes oxidized under steady-state turnover conditions. This shows that the oxidation of the low-spin heme *b* by H_2O_2 is faster than the oxidation by O_2, indicating that the peroxidase activity of the enzyme is higher than its oxidase activity. This is consistent with the behavior of K-channel mutants in the cytochrome *c* oxidase from *Rhodobacter sphaeroides* (*15*). This behavior is also exhibited by the K362M mutant of cytochrome bo_3 (not shown).

Finally, the proton pumping stoichiometry and pH dependence were examined for those mutants with sufficient activity, i.e., excluding W93V, E89A, and E89Q. These data are shown in Table 2. Not surprisingly, neither the T94A nor the P96A mutant has any effect on the proton pumping of the oxidase.

The evident conclusion of this study is that $E89^{II}$ is an important residue, possibly for controlling the proton input to the K-channel. The input pathway to the K-channel lysine has been proposed to involve a serine residue in both the bovine heart oxidase ($S255^I$) and the *P. denitrificans* oxidase ($S291^I$). This serine is hydrogen bonded to the K-channel lysine ($K319^I$ and $K354^I$, respectively) through an intervening water molecule, and appears to be a component of a hydrogen bond network connecting the lysine to the bulk solvent (*7*, *10*). Mutation of the equivalent serine in the *E. coli* oxidase ($S299^I$) to alanine in cytochrome bo_3 has no effect, however. The enzyme is fully active, and the proton pumping stoichiometry is similar to that of the wild type (Table 2).

It was reported (*33*) that electrostatics calculations using the coordinates of the *P. denitrificans* oxidase show a coupling between the $E78^{II}$ residue and $K354^I$ in the K-channel. This is also indicated by calculations using the coordinates of the bovine oxidase, summarized in Figure 5. The calculations show the difference in electrostatic potential around $K319^I$ (bovine) upon removal of the negative charge of $E62^{II}$ (bovine). These residues are the bovine equivalents to $K354^I$ and $E78^{II}$ in the *P. denitrificans* oxidase. As seen in Figure 5, the charge of $E62^{II}$ influences the electrostatic potential around the lysine. The "mutation" of $E62^{II}$ results in a less negative electrostatic potential at $K319^I$. Although these calculations have limited quantitative value, they do indicate that changing the charge at the position occupied by the glutamate in subunit II will have a significant influence on the electrostatic field sensed by the lysine in the K-channel.

CONCLUSIONS

The primary conclusion of this work is that $E89^{II}$ is important for the proper function of cytochrome bo_3. In view of the fact that this residue is virtually completely conserved in all the heme−copper oxidases that have a "traditional" subunit II, and that the electrostatics calculations (*33*) show this residue to be remarkably strongly coupled to the redox state of the heme−copper metals despite being 25 Å away, the mutagenesis results suggest interesting possibilities. The most intriguing is that $E89^{II}$ controls the entry of protons into the K-channel. The fact that the E89Q mutant apparently has more rapid peroxidase activity than oxidase activity, as judged by the steady-state oxidation of heme *b*, is consistent with the phenotype expected of a block in the K-channel

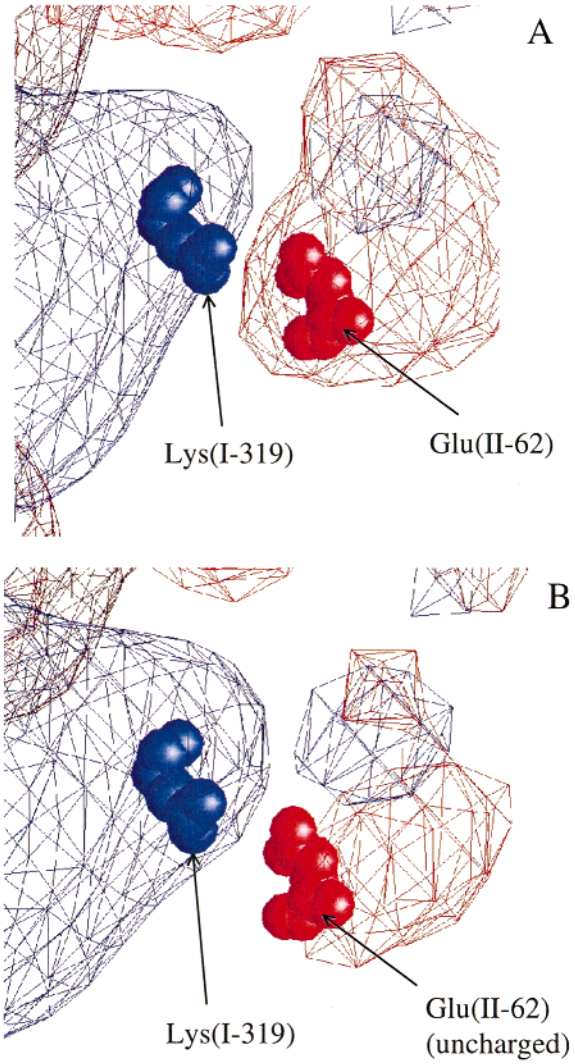

FIGURE 5: Electrostatic potential contours around $E62^{II}$ and $K319^{I}$ in the bovine heart mitochondrial oxidase using the program GRASP (*40*). The ionic strength was set at 0, and the dielectric constant inside the protein was set at 4. Potentials of −3 kBT and 12 kBT are marked by red and blue, respectively. The different values were chosen because they give a clearer view of the effect of the removal of the charge of the glutamate. In panel A, the charge of $E62^{II}$ was set at −1, whereas in panel B, the charge was set to 0.

(*15*). This could be an indirect effect through electrostatic coupling. The protonation status of $E89^{II}$ appears to be regulated by the redox state of the heme−copper center (*33*), and the net charge at $E89^{II}$ could, in turn, influence the electrostatic field around $K362^{I}$ in the K-channel. This could alter the rate of proton transit by influencing the transient protonation or change in conformation, each of which appears to be necessary for conveying protons to the heme−copper center.

A more direct role for $E89^{II}$ could also be postulated. The possibility should be considered that the actual site for proton entry into the K-channel is via $E89^{II}$, and not via the route suggested by the two X-ray structures (*7*, *10*, *11*), which proceeds through subunit I via K308 (*E. coli*) at the protein surface (*49*) and $S299^{I}$ (*E. coli*). The X-ray structures of neither the bovine (*7*) nor the *P. denitrificans* oxidases (*10*, *11*) show hydrogen bond connectivity linking $E89^{II}$ and $K362^{I}$ in the K-channel. These residues are separated by about 10 Å. However, a conformational shift of the side chain of K362 must be postulated to convey protons in the channel as it is presently postulated anyway. It is noted that the K-channel appears to be required only for the initial portion of the catalytic cycle, up to the formation of intermediate P (*14*−*18*). Therefore, this channel need not be "open" to proton influx during the entire catalytic cycle. The fact that the S299A mutant oxidase is fully functional cannot be taken as definitive evidence that the currently postulated input pathway to $K362^{I}$ (*49*) is incorrect. The equivalent mutant in the *P. denitrificans* oxidase (S291A) also has no effect (*50*). However, the serine could be functionally replaced by a water molecule in these alanine-substituted mutants. A series of serine residues in the D-channel can also be mutated without dramatic consequences (*51*). The current data, however, do point to the possibility that $E89^{II}$ plays a role analagous to that of $D135^{I}$ (in cytochrome bo_3) at the entrance to the D-channel. Certainly, the next necessary step is to determine whether mutations of the equivalent glutamate in other heme−copper oxidases (e.g., from *P. denitrificans* or *Rhodobacter sphaeroides*) indicate an importance of this residue beyond cytochrome bo_3. If so, it can be determined whether the phenotype is similar to those of other mutants that impede proton flux through the K-channel (*14*−*16*), or whether the explanation is more mundane, perhaps relating to the detailed structure of the interface between subunits I and II. Such experiments are in progress.

REFERENCES

1. Garcia-Horsman, J. A., Barquera, B., Rumbley, J., Ma, J., and Gennis, R. B. (1994) *J. Bacteriol. 176*, 5587−5600.
2. Puustinen, A., Finel, M., Haltia, T., Gennis, R. B., and Wikström, M. (1991) *Biochemistry 30*, 3936−3942.
3. Verkhovskaya, M., Verkhovsky, M., and Wikström, M. (1992) *J. Biol. Chem. 267*, 14559−14562.
4. Rumbley, J. N., Nickels, E. F., and Gennis, R. B. (1997) *Biochim. Biophys. Acta 1340*, 131−142.
5. Saiki, K., Mogi, T., Tsubaki, M., Hori, H., and Anraku, Y. (1997) *J. Biol. Chem. 272*, 14721−14726.
6. Saiki, K., Nakamura, H., Mogi, T., and Anraku, Y. (1996) *J. Biol. Chem. 271*, 15336−15340.
7. Tsukihara, T., Aoyama, H., Yamashita, E., Takashi, T., Yamaguichi, H., Shinzawa-Itoh, K., Nakashima, R., Yaono, R., and Yoshikawa, S. (1996) *Science 272*, 1136−1144.
8. Tsukihara, T., Aoyama, H., Yamashita, E., Tomizaki, T., Yamaguchi, H., Shinzawa-Itoh, K., Nakashima, T., Yaono, R., and Yoshikawa, S. (1995) *Science 269*, 1069−1074.
9. Yoshikawa, S., Shinzawa-Itoh, K., Nakashima, R., Yaono, R., Yamashita, E., Inoue, N., Yao, M., Fei, M. J., Libeu, C. P., Mizushima, T., Yamaguchi, H., Tomizaki, T., and Tsukihara, T. (1998) *Science 280*, 1723−1729.
10. Iwata, S., Ostermeier, C., Ludwig, B., and Michel, H. (1995) *Nature 376*, 660−669.
11. Ostermeier, C., Harrenga, A., Ermler, U., and Michel, H. (1997) *Proc. Natl. Acad. Sci. U.S.A. 94*, 10547−10553.
12. Hendler, R. W., Pardhasaradhi, K., Reynafarje, B., and Ludwig, B. (1991) *Biophys. J. 60*, 415−423.
13. Ferguson-Miller, S., and Babcock, G. T. (1996) *Chem. Rev. 7*, 2889−2907.
14. Konstantinov, A. A., Siletsky, S., Mitchell, D., Kaulen, A., and Gennis, R. B. (1997) *Proc. Natl. Acad. Sci. U.S.A. 94*, 9085−9090.
15. Zaslavsky, D., and Gennis, R. B. (1998) *Biochemistry 37*, 3062−3067.
16. Vygodina, T. V., Pecoraro, C., Mitchell, D., Gennis, R., and Konstantinov, A. A. (1998) *Biochemistry 37*, 3053−3061.
17. Ädelroth, P., Gennis, R. B., and Brzezinski, P. (1998) *Biochemistry 37*, 3062−3067.

18. Jünemann, S., Meunier, B., Gennis, R. B., and Rich, P. R. (1997) *Biochemistry 36*, 14456−14464.
19. Ädelroth, P., Ek, M. S., Mitchell, D. M., Gennis, R. B., and Brzezinski, P. (1997) *Biochemistry 36*, 13824−13829.
20. Thomas, J. W., Puustinen, A., Alben, J. O., Gennis, R. B., and Wikström, M. (1993) *Biochemistry 32*, 10923−10928.
21. Verkhovskaya, M. L., Garcia-Horsman, A., Puustinen, A., Rigaud, J.-L., Morgan, J. E., Verkhovsky, M. I., and Wikström, M. (1997) *Proc. Natl. Acad. Sci. U.S.A. 94*, 10128−10131.
22. Watmough, N. J., Katsonouri, A., Little, R. H., Osborne, J. P., Furlong-Nickels, E., Gennis, R. B., Brittain, T., and Greenwood, C. (1997) *Biochemistry 36*, 13736−13742.
23. Fetter, J. R., Qian, J., Shapleigh, J., Thomas, J. W., Garcia-Horsman, A., Schmidt, E., Hosler, J., Babcock, G. T., Gennis, R. B., and Ferguson-Miller, S. (1995) *Proc. Natl. Acad. Sci. U.S.A. 92*, 1604−1608.
24. Smirnova, I. A., Ädelroth, P., Gennis, R. B., and Brzezinski, P. (1999) *Biochemistry 38*, 6826−6833.
25. Svensson, M., Hallen, S., Thomas, J. W., Lemieux, L., Gennis, R. B., and Nilsson, T. (1995) *Biochemistry 34*, 5252−5258.
26. Svensson-Ek, M., Thomas, J. W., Gennis, R. B., Nilsson, T., and Brzezinski, P. (1996) *Biochemistry 35*, 13673−13680.
27. Thomas, J. W., Lemieux, L. J., Alben, J. O., and Gennis, R. B. (1993) *Biochemistry 32*, 11173−11180.
28. Thomas, J. W., Calhoun, M. W., Lemieux, L. J., Puustinen, A., Wikström, M., Alben, J. O., and Gennis, R. B. (1994) *Biochemistry 33*, 13013−13021.
29. Garcia-Horsman, J. A., Puustinen, A., Gennis, R. B., and Wikström, M. (1995) *Biochemistry 34*, 4428−4433.
30. Morgan, J. E., Verkhovsky, M. I., and Wikström, M. (1994) *J. Bioenerg. Biomembr. 26*, 599−608.
31. Rich, P. R., Jünemann, S., and Meunier, B. (1997) *J. Bioenerg. Biomembr. 30*, 131−137.
32. Michel, H. (1998) *Proc. Natl. Acad. Sci. U.S.A. 95*, 12819−12824.
33. Kannt, A., Lancaster, C. R. D., and Michel, H. (1998) *Biophys. J. 74*, 708−721.
34. Yanisch-Perron, C., Vieira, J., and Messing, J. (1985) *Gene 33*, 103−119.
35. Vandeyar, M. A., Weiner, M. P., Hutton, C. J., and Batt, C. A. (1988) *Gene 65*, 129−133.
36. Lemieux, L. J., Calhoun, M. W., Thomas, J. W., Ingledew, W. J., and Gennis, R. B. (1992) *J. Biol. Chem. 267*, 2105−2113.
37. Au, D. C.-T., and Gennis, R. B. (1987) *J. Bacteriol. 169*, 3237−3242.
38. Calhoun, M. W., Lemieux, L. J., Thomas, J. W., Hill, J. J., Goswitz, V. C., Alben, J. O., and Gennis, R. B. (1993) *Biochemistry 32*, 13254−13261.
39. Minghetti, K. C., Goswitz, V. C., Gabriel, N. E., Hill, J. J., Barassi, C., Georgiou, C. D., Chan, S. I., and Gennis, R. B. (1992) *Biochemistry 31*, 6917−6924.
40. Nicholls, A., Sharp, K. A., and B., H. (1991) *Proteins: Struct., Funct., Genet. 11*, 281−296.
41. Calhoun, M. W., Thomas, J. W., Hill, J. J., Hosler, J. P., Shapleigh, J. P., Tecklenburg, M. M. J., Ferguson-Miller, S., Babcock, G. T., Alben, J. O., and Gennis, R. B. (1993) *Biochemistry 32*, 10905−10911.
42. Puustinen, A., and Wikström, M. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 6122−6126.
43. Puustinen, A., Morgan, J. E., Verkhovsky, M., Thomas, J. W., Gennis, R. B., and Wikström, M. (1992) *Biochemistry 31*, 10363−10369.
44. Chavez, M. D., Courtney, S. H., Chance, M. R., Kiula, D., Nocek, J., Hoffman, B. M., Friedman, J. M., and Ondrias, M. R. (1990) *Biochemistry 29*, 4844.
45. Ingledew, W. J., Bacon, M., and Rich, P. R. (1992) *FEBS Lett. 305*, 167−170.
46. Calhoun, M. W., Hill, J. J., Lemieux, L. J., Ingledew, W. J., Alben, J. O., and Gennis, R. B. (1993) *Biochemistry 32*, 11524−11529.
47. Mitchell, D. M., Shapleigh, J. P., Archer, A. M., Alben, J. O., and Gennis, R. B. (1996) *Biochemistry 35*, 9446−9450.
48. Tsatsos, P. H., Reynolds, K., Nickels, E. F., He, D.-Y., Yu, C.-A., and Gennis, R. B. (1998) *Biochemistry 37*, 9884.
49. Villani, G., Capitanio, N., Bizzoca, A., Palese, L. L., Carlino, V., Tattoli, M., Glaser, L., Danchin, A., and Papa, S. (1999) *Biochemistry 38*, 2287−2294.
50. Pfitzner, U., Odenwald, A., Ostermann, T., Weingard, L., Ludwig, B., and Richter, O.-M. H. (1998) *J. Bioenerg. Biomembr. 30*, 89−93.
51. Mitchell, D. M., Fetter, J. R., Mills, D. A., Ädelroth, P., Pressler, M. A., Kim, Y., Aasa, R., Brzezinski, P., Malmström, B. G., Alben, J. O., Babcock, G. T., Ferguson-Miller, S., and Gennis, R. B. (1996) *Biochemistry 35*, 13089−13093.

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