Arginine 391 in Subunit I of the Cytochrome bd Quinol Oxidase from Escherichia coli Stabilizes the Reduced Form of the Hemes and Is Essential for Quinol Oxidase Activity*

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The cytochrome bd quinol oxidase is one of two respiratory oxidases in Escherichia coli. It oxidizes dihydrobioquinoi or dihydromenaquinol while reducing dioxygen to water. The bd-type oxidases have only been found in prokaryotes and have been implicated in the survival of some bacteria, including pathogens, under conditions of low aeration. With a high affinity for dioxygen, cytochrome bd not only couples respiration to the generation of a proton motive force but also scavenges O2. In the current work, the role of a highly conserved arginine residue is explored by site-directed mutagenesis. Four mutations were made: R391A, R391K, R391M, and R391Q. All of the mutations except R391K result in enzyme lacking ubiquinol oxidase activity. Oxidase activity using the artificial reductant N,N,N′,N′-tetramethyl-p-phenylenediamine in place of ubiquinol was, however, unimpaired by the mutations, indicating that the catalytic center where O2 is reduced is intact. UV-visible spectra of each of the mutant oxidases show no perturbations to any of the three heme components (heme b558, heme b595, and heme d). However, spectrophotometric titrations of the R391A mutant reveal that the midpoint potentials of all of the heme components are substantially lower compared with the wild type enzyme. Since Arg391 is close to Met393, one of the axial ligands to heme b595, it is to be expected that the R391A mutation might destabilize the reduced form of heme b595. The fact that the midpoint potentials of heme d and heme b595 are also significantly lowered in the R391A mutant is consistent with these hemes being physically close together on the periplasmic side of the membrane.

The cytochrome bd respiratory oxidase has a very high affinity for O2 (in nm range) and is found in many prokaryotes (1–4). The bd-type oxidases are being increasingly recognized as physiologically significant. Frequently, these respiratory oxidases are induced under conditions of very low aeration, either to generate a proton motive force by reducing O2 to water or by scavenging O2 to protect the cell (5, 6). For example, cytochrome bd was recently found in what were previously identified as strict anaerobes, Bacteroides fragilis (7) and Desulfovibrio gigas (8), which allowed these organisms to grow aerobically with very low concentrations of O2. Mutations in the enzyme have been implicated in reduced virulence in some pathogens (9, 10). Although cytochrome bd catalyzes the two-electron oxidation of either ubiquinol or menaquinol and the reduction of O2 to water, cytochrome bd is completely unrelated to the proton-pumping heme/copper superfamily of respiratory oxidases. Indeed, the bd-type oxidases do not pump protons, although they do generate a proton motive force (11, 12). It appears that quinol oxidation occurs near the periplasmic surface (13–15) but that the protons used to form water are taken from the bacterial cytoplasm. Hence, the reaction is coupled to transmembrane charge separation and the generation of a membrane potential.

The best characterized bd-type oxidase is from E. coli (1, 2). The enzyme is a heterodimer with one copy each of two subunits (CydA and CydB). There are three heme prosthetic groups but no copper. Heme b595 is ligated to His19 in CydA (20, 21) (Fig. 1), and heme d is both high spin hemes that appear to form a binuclear center where O2 is reduced to water (19). It is known that O2 binds to ferrous heme d and, in the absence of additional reducing equivalents, forms a stable heme d Fe2⁺−O2 adduct. The axial ligand of heme b595 has been identified as His19 in CydA (20, 21) (Fig. 1), but the nature and location of the axial ligand to heme d are not known.

In the current work, the role of Arg391 in the CydA subunit of the Escherichia coli oxidase is investigated. This arginine is very highly conserved and is only two residues away from Met393, one of the axial ligands to heme b595 (Fig. 1). It was expected, therefore, that this residue might play either a structural or functional role related to heme b595 or the putative quinol binding site that is proposed to be adjacent to this heme (1, 22). Four site-directed mutants were made: R391K, R391A, R391Q, and R391M. The R391K mutant oxidase is partially active as a quinol oxidase (25% of wild type activity), but the other three unchanged amino acid substitutions all result in eliminating quinol oxidase activity. The optical spectrum of each of the enzymes is normal, indicating that the hemes are properly assembled and there is no major conformational change. All of the mutants are active using an alternative artificial substrate TMPD,1 which has previously been characterized as utilizing a different site from that of ubiquinol (23). This indicates that the O2 binding site and catalytic function

1 The abbreviations used are: TMPD, N,N,N′,N′-tetramethyl-p-phenylenediamine; FTIR, Fourier transform infrared; HPLC, high pressure liquid chromatography; HNE, normal hydrogen electrode.
are not perturbed by the Arg391 mutants. The major effect of the R391A mutation (and presumably the R391Q and R391M substitutions) is to decrease the electrochemical midpoint potential of heme b₅₅₈, which is sufficient to explain the lack of quinol oxidase activity. The positive charge on Arg391 appears to stabilize the reduced form of heme b₅₅₈ in the E. coli oxidase by about 210 mV (versus 130 mV). Substitution by another amino acid residue, other than lysine, makes it much harder to reduce heme b₅₅₈ and inactivates the enzyme. Remarkably, the R391A mutant also results in a lower potential for both heme b₅₉₅ and heme d. If this is due simply to an electrostatic effect, all of the hemes must be physically close to Arg391. A local conformational change due to the R391A mutation cannot be ruled out as the cause of the lower midpoint potentials of hemes b₅₉₅ and d. However, this is unlikely, since the optical and EPR spectra appear unperturbed, and the TMPD oxidase activity is unimpaired.

MATERIALS AND METHODS

Strains and Plasmids—E. coli strain GO105 (cydAB::kan, cyo, recA), which lacks both cytochrome bᵩ₃ and cytochrome bᵩ₁ quinol oxidases (17), was used as the host strain for expressing both the wild type and mutant cytochrome bᵩ₁ from a plasmid. To obtain wild type cytochrome bᵩ₁, plasmid pTK1 (24) was introduced into the strain. Mutants of cytochrome bᵩ₁ were expressed using pTK1 plasmid as well. Phagemid pZG1 was used as the template for site-directed mutagenesis. This phagemid, prepared by Dr. Tamma Zuberi, was generated by ligating the cydA gene into phagemid pT7T3 19u using two unique restriction sites HindIII and SalI.

Site-directed Mutagenesis, Expression, and Complementation Test of the Mutant Cytochrome bᵩ₁—Plasmid pZG1 (see above) was used as template. The oligonucleotide primers were synthesized by the University of Illinois Biotechnology Center and purified using oligonucleotide purification cartridges with melting temperature around 80 °C based on the Stratagene formula. The mutagenesis was performed as described (24). Once the mutant sequences were confirmed from DNA sequencing (CBI Inc.), the mutant plasmids were digested by restriction endonuclease SalI and HindIII. The digestions were then subjected to agarose gel electrophoresis. The bands containing DNA of cydA were cut from the gel, and DNA was extracted using “GenElute minus EtBr” columns (Sigma) and ligated to vector pTK1 that had been pretreated with the same restriction endonucleases. The resulting mutant cydAB operon was again confirmed from DNA sequencing (CBI Inc.). Complementation was tested as described previously (24).

Cell Growth and Protein Sample Preparation—Large scale cell growth of strains that grow aerobically (i.e. expressing wild type or R391K mutant) was carried out in 24 2-liter flasks shaking at 230 rpm 37 °C using an Innova 4330 incubator shaker (New Brunswick Scientific). Strains expressing the inactive mutants (e.g. R391A) could not be grown aerobically and were, therefore, grown in a 20-liter fermenter anaerobically at the Fermentation Facility at the University of Illinois using LB containing 100 μg/ml Amp, 50 μg/ml Kan, and 0.3% glucose, pH 7, at 37 °C.

The mutant cytochrome bᵩ₁ oxidases were purified as described previously (24). The pooled fractions were concentrated using an Amicon concentrator with a 50-kDa molecular weight cut-off filter and then dialyzed three times against 50 mM sodium phosphate buffer, pH 7.9, containing 5 mM EDTA, 0.05% N-lauroylsarcosine. Both wild type and mutant cytochrome bᵩ₁ samples were then examined, using the same dialysis buffer for appropriate dilution unless specified otherwise. All of these assays were carried out as described in Ref. 24.

UV-visible Spectroscopic Measurements—All of the absorbance spectra in the UV-visible region were obtained with a DW2000 spectrophotometer (Aminco) or UV-2101PC spectrophotometer (Shimadzu) using a 1-cm path length cuvette. The absorbance spectra for midpoint potential measurements were obtained using an ultrathin layer spectrophotometer (Shimadzu).

FIG. 1. Scheme of subunit I (CydA) of the cytochrome bᵩ₁ quinol oxidase from E. coli. The filled circles represent highly conserved residues, and the arrows point to the three known heme axial ligands and to the Arg391 residue. The model has nine transmembrane helices, and the large “Q loop” has been implicated in quinol oxidation.
TABLE I

Characteristics of the Arg<sup>391</sup> mutants of subunit I of E. coli cytochrome bd

<table>
<thead>
<tr>
<th>Sample name</th>
<th>Complementation test</th>
<th>UQH&lt;sub&gt;1&lt;/sub&gt;-oxidase activity</th>
<th>TMPD-oxidase activity</th>
<th>Heme b&lt;sub&gt;1&lt;/sub&gt; b&lt;sub&gt;d&lt;/b&gt; ratio</th>
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<tbody>
<tr>
<td>Wild type</td>
<td>Yes</td>
<td>100</td>
<td>100</td>
<td>2–2.5</td>
</tr>
<tr>
<td>R391A</td>
<td>No</td>
<td>&lt;0.1</td>
<td>170</td>
<td>2.85</td>
</tr>
<tr>
<td>R391K</td>
<td>Yes</td>
<td>25</td>
<td>140</td>
<td>2.15</td>
</tr>
<tr>
<td>R391M</td>
<td>No</td>
<td>&lt;0.5</td>
<td>100</td>
<td>3.16</td>
</tr>
<tr>
<td>R391Q</td>
<td>No</td>
<td>&lt;0.1</td>
<td>100</td>
<td>2.15</td>
</tr>
</tbody>
</table>

**EPR Spectroscopic Measurements**—X-band (9.09-GHz) EPR spectra were acquired on a Varian E-122 spectrometer with a modulation amplitude of 5 gauss at 100 kHz at 9 K. Samples were prepared as frozen glasses in 3-mm inner diameter tubes using an Air Product Helitran cryostat. The magnetic field was calibrated with a Varian NMR gauss meter, and the microwave frequency was determined by an EIP frequency meter (San Jose, CA). Spectra were recorded with ferriyanide-oxidized samples of the R391A mutant oxidase (30 μM) at 9 K and a power of 1 milliwatt.

**Difference Spectroscopy Showing Binding of CO**—The R391A mutant oxidase (2.3 μM) was made anaerobic by purging with argon gas for 1 h and was then reduced by adding fresh dithionite solution. The absorption spectrum of the reduced enzyme was recorded and taken as a baseline. The sample was then purged with CO gas flow for 5 min, following which the absorbance spectrum was taken. The difference spectrum was generated by subtracting the spectrum of reduced enzyme from the one after binding CO.

**Redox Titrations of Wild Type Cytochrome bd and the R391A Mutant**—Both wild type and the Arg<sup>391</sup> mutant oxidases were prepared as described above. The redox titration was carried out using the ultrathin layer spectroelectrochemical cell for UV-visible spectroscopy as previously described (25). The gold grid working electrode was chemically modified by 2 mM cysteamine solution as reported before (26). In order to separate the redox reaction, 15 different mediators were added, as reported in Ref. 26, to a total concentration of 40 μM each. At this concentration and with the path length below 10 μm, no spectral contribution from the mediators in the visible region could be detected in the control experiment with samples lacking the protein. As a supporting electrolyte, 100 mM KCl was added. Approximately 25–30 μl of the protein sample was used to fill the spectroelectrochemical cell. Absorbance changes were monitored at multiple wavelengths using a UYPC-2101 spectrophotometer (Shimadzu). The equilibration time was less than 15 min under the conditions described above. Redox titrations were performed by stepwise setting the potentials and recording the spectra after sufficient equilibrations. All measurements were obtained at 10 °C and were repeated at least twice. For the wild type enzyme, absorbance changes at 429, 560, and 628 nm were used to generate the heme titration curves. For the R391A mutant, due to the dilute concentration, absorbance changes at 628 nm were too small to generate an accurate curve. The midpoint potentials (E<sub>m</sub> values) were obtained by interactive fitting to a Nernst equation using the program offered by Origin (Microcal).

**FTIR Measurements**—Purified wild type enzyme (grown anaerobically) and the R391A mutant (20 μM) were concentrated using a microconcentrator (Amicon, 50-kDa cut-off filter) by centrifugation using 50 mM sodium phosphate buffer, pH 8.3, 5 mM EDTA, 0.05% lauryl sarcosine. FTIR and visible difference spectra as a function of the applied potential were obtained simultaneously from the same sample with a setup combining an IR beam from the interferometer (modified IFS 25; Bruker, Germany) for the 4000 to 1000 cm<sup>-1</sup> range and a dispersive spectrometer for the 400–900-nm range. Electrochemically induced difference spectra were recorded and processed as previously described in Ref. 25. In all experiments, the protein solution was first equilibrated at the initial potential of the electrode, and single beam spectra in the IR range were recorded. Then a potential step toward the final potential was applied, and the single beam spectrum of this state was again recorded after equilibration. Subsequently, difference spectra, as presented in this work, were calculated from the two single beam spectra with the initial single beam spectrum taken as the reference. The equilibration at the applied potential generally took less than 5–6 min in the potential range from −0.3 to 0.7 V. Typically, 5 × 128 scans were co-added, and 10–20 spectra were averaged. No smoothing or deconvolution procedures were applied. The noise level in the difference spectra was estimated to be around 30–50 × 10<sup>−5</sup> absorbance units in the spectral range under consideration except for the region of the strongly absorbing water bending and protein amide I modes at ~1650 cm<sup>−1</sup>, where the noise was slightly higher.

**HPLC Analysis of Quinone Content**—Membranes from different growth conditions were isolated as previously described (27), and small aliquots were homogenized using a buffer containing 25 mM Tris-HCl, 1 mM EDTA (disodium salt), pH 7.5. Either this membrane suspension or, alternatively, purified protein was prepared to contain 3–6 nmol of cytochrome bd in 500 μl. Quinone was extracted by vortex-mixing 500 μl of the sample with 3 ml of methanol/petroleum ether (1/ν<sub>2</sub> ratio 3:2) and centrifuged to collect the supernatant. The vortex and centrifuge step were repeated three times to ensure complete extraction of quinone. Supernatant containing extracted quinone was dried under nitrogen gas flow and quinone was redissolved in 100 μl of ethanol just prior to HPLC injection. A BIO-CAD Sprint<sup>TM</sup> (PerSeptive Biosystems) HPLC and Microsorb-MV 100A HPLC column (Varian Analytical Instruments) were used. The running phase was 100% ethanol, and eluted material was monitored at 278 nm. The peak area was calculated, and the amount of quinone bound to the sample was obtained by comparing with the standard peak, in which the amount of UQ-10 added was known. The extraction efficiency was also taken into account.
FIG. 4. Redox difference spectra of the wild type and R391A mutant oxidases. A, two redox “cuts” for the wild type enzyme. The data show that none of the hemes is oxidized as the potential is raised from −342 to 0 mV (NHE). Each of the hemes has a midpoint potential significantly above 0 mV (NHE). B shows the corresponding data for the R391A mutant. In this case, a substantial amount of heme b (560 nm) and heme d (630 nm) is oxidized between −342 and 0 mV (NHE).
confirming that the active site for O₂ binding (heme b₅₉₅/heme d binuclear center) is essentially unchanged by the mutation.

**FTIR Oxidized Minus Reduced Difference Spectroscopy**—The oxidized minus reduced (−0.3 to +0.7 V) FTIR difference spectrum (1800 to 1200 cm⁻¹) of the wild type, R391K, and R391A mutant oxidases were recorded and compared. Fig. 3 compares the difference spectra of the wild type and R391A oxidases. The amplitudes have been normalized based on spectra measured in the visible spectral range. The spectra of both mutants are very similar to that of the wild type enzyme, consistent with the conclusion that the mutations do not result in any large conformational changes. The bands previously assigned (30) to menaquinone (1264 cm⁻¹) and ubiquinone (1295 cm⁻¹) are both present in the spectra of the mutants (arrows in Fig. 3). Small variations indicate differences in quinone ratio in accordance with Ref. 30. The quinones were extracted from the isolated wild type and R391A oxidases and analyzed by HPLC chromatography. The results were consistent with the FTIR
spectroscopy and indicated that both ubiquinone and menaquinone co-purify with the enzymes (data not shown).

Potentiometric Titrations of the Wild Type and R391A Oxidases—The electrochemical properties of the oxidase are greatly perturbed by the R391A mutation. This can be seen in Fig. 4, which shows the UV-visible difference spectra for the enzymes at different redox cuts at pH 8.3. For the wild type oxidase (Fig. 4A), the enzyme is fully reduced at $/H_11002 -342$ mV (NHE) and at 0 mV (NHE). The enzyme is fully oxidized at $/H_11001 +558$ mV (NHE), as shown by the difference spectrum. The R391A mutant behaves very differently. As shown in Fig. 4B, there is considerable oxidation of the enzyme at 0 mV (NHE). It appears that about 70% of heme $b_{558}$ and probably all of heme $b_{595}$ are oxidized at 0 mV. The status of heme $b_{595}$ is not clear from these spectra.

Full electrochemical titrations were performed and monitored at 429, 560, and 628 nm. Figs. 5 and 6 show representative plots of absorbance versus solution potential for the wild type (Fig. 5) and the R391A mutant (Fig. 6). The electrochemical properties of the wild type enzyme are similar to those reported previously (31): heme $b_{558}$, $/H_11001 +60$ to $+80$ mV; heme $b_{595}$, $+220$ to $+240$ mV; heme $d$, $+250$ to $+260$ mV. For the R391A mutant oxidase, the midpoint potentials of heme $b_{558}$ and heme $b_{595}$ are shifted downward dramatically: heme $b_{558}$, $-132$ to $-121$ mV; heme $b_{595}$, $+20$ to $+37$ mV. The midpoint potential of heme $d$ was not determined because the sample concentration was insufficient to obtain a sufficient absorption at 628 nm. However, the spectra in Fig. 4 clearly show that about 70% of heme $d$ and probably all of heme $b_{558}$ are oxidized at 0 mV. The status of heme $b_{595}$ is not clear from these spectra.

An electrochemical redox titration was also performed on the R391K mutant oxidase. The midpoint potentials of the three hemes were found to be essentially the same as those of the wild type enzyme (data not shown), which is consistent with the observation that this mutant is partially active and able to complement the aerobic growth of oxidase-deficient E. coli strain GO105.

Kinetics of Reduction of the Enzyme by Ubiquinol under Anaerobic Conditions—Under anaerobic conditions, the wild type oxidase can be reduced upon the addition of the substrate, ubiquinol-1, plus dithiothreitol to keep the substrate fully reduced. The reduction of the enzyme (about 1 pmol) was monitored at 560 nm and was carried out under aerobic conditions. The conditions are given under “Results.” The wild type oxidase is reduced within a few seconds after adding the substrate, presumably after the oxygen has been depleted. The mutant oxidase is reduced at least 80-fold more slowly.

Fig. 7. Kinetics of anaerobic reduction of the “as isolated” wild type oxidase (solid line) and the R391A mutant (dashed line) by ubiquinol-1 in the presence of dithiothreitol. The absorbance changes were normalized to the same protein concentrations. The progress of the reduction of the enzymes was monitored at 560 nm (A, formation of ferrous heme $b$ from ferric heme $b$), 628 nm (B, formation of ferrous heme $d$ from ferric heme $d$), and 650 nm (C, conversion of oxyferrous heme $d$ (O$_2$ adduct) to ferrous heme $d$). The data show that the R391A mutation has little effect on the kinetics of these reactions. Note that the rates of reaction are much slower than turnover (see “Results”) and that the rate of reaction of the enzyme population with ferrous heme $d$-O$_2$ complex is significantly faster than reduction of the fully oxidized enzyme, although still slower than turnover.

Fig. 8. Kinetics of reduction by menadiol of cytochrome bd (wild type (WT), solid line) and the R391A mutant (dashed line). The reduction of the enzyme (about 1 pmol) was monitored at 560 nm and was carried out under aerobic conditions. The conditions are given under “Results.” The wild type oxidase is reduced within a few seconds after adding the substrate, presumably after the oxygen has been depleted. The mutant oxidase is reduced at least 80-fold more slowly.
duced. The rate of this reaction is, however, very slow and multiphasic, with the fastest rate constant being about 0.3 s\(^{-1}\). This is much slower than turnover, which is on the order of 1000 s\(^{-1}\). Hence, the slow reduction of the enzyme by ubiquinol-1 under anaerobic conditions must represent a reaction unrelated to catalytic function. Under the same conditions, the Arg\(^{391}\) mutants are all reduced in a similar manner. The shifted midpoint potential of heme \(b_{558}\) does not impede the reaction, which is driven by the presence of dithiothreitol, which has a midpoint potential of \(-330\) mV (NHE). The dithiothreitol itself does not reduce the enzyme. Representative data are shown in Fig. 7.

More informative is the reaction of menadiol with the oxidase (Fig. 8). The wild type enzyme in the presence of menadiol under aerobic conditions rapidly turns over and depletes the oxygen in solution and is then fully reduced in a matter of a few seconds. In contrast, the R391A mutant oxidase is not substantially reduced by menadiol under the same conditions. These data are consistent with the large drop of the midpoint potential of heme \(b_{558}\) to a point where it is not significantly reduced by menadiol.

**Kinetics of Oxidation of the Fully Reduced Enzyme by \(O_2\)**

The fact that the Arg\(^{391}\) mutants all have TMPD oxidase activity implies that the oxygen reactive site is not perturbed greatly, if at all, by these mutations. This was confirmed also by adding \(O_2\) to dithionite-reduced enzyme and monitoring the oxidation of the hemes optically. For both the wild type and mutants, the reaction is rapid and goes to completion (not shown). Rates were not determined using rapid mixing, but qualitatively, the kinetic traces appear identical.

**DISCUSSION**

Arg\(^{391}\), although not totally conserved, is present in the vast majority of sequences of CydA. Since it is only two residues away from Met\(^{396}\), which has been shown to be an axial ligand to heme \(b_{558}\) (17), it is reasonable to speculate that Arg\(^{391}\) might be important in determining the properties of this heme component of the enzyme. The current work confirms this speculation. Whereas the R391K mutant retains substantial catalytic function, substitution by a neutral residue (Ala, Met, or Gln) eliminates quinol oxidase activity. UV-visible, EPR, and FTIR spectroscopies show no significant alteration of the enzyme structure of these inactive mutants. The mutant enzymes all retain TMPD oxidase activity, and the reduced enzymes are rapidly and fully reoxidized by \(O_2\), which suggests that the mutants do not displace the heme \(b_{558}\)/heme \(d\) binuclear site.

One major effect of the R391A mutation is the large shift of the electrochemical midpoint potential of heme \(b_{558}\) from about +80 to \(-132\) mV (NHE). This is a shift in the direction one would predict from removing a positive charge in the immediate vicinity of heme \(b_{558}\). Indeed, the midpoint potential of heme \(b_{558}\) in the R391K mutant, which retains the positive charge, is identical to that of the wild type oxidase. The low midpoint potential of heme \(b_{558}\) in the R391A mutant would be expected to make it more difficult for the quinol substrates to reduce heme \(b_{558}\), which is clearly observed with menadiol as the substrate (Fig. 8). As expected from the electrochemical studies, the R391K mutant oxidase reacts with menadion in the same way as does the wild type oxidase. The unusually slow reduction of the wild type enzyme by ubiquinol-1 under anaerobic conditions (Fig. 7) is not relevant to turnover and is not understood. The data are not informative about the effects of the Arg\(^{391}\) mutants on the enzyme, and further studies are required to clarify this reaction.

Surprisingly and significantly, the R391A mutation also results in substantially lower midpoint potentials of heme \(d\) (Fig. 4) and heme \(b_{558}\) (Fig. 6). It might be argued that the large drop of the midpoint potential of heme \(b_{558}\) caused by the R391A mutation slows down the rate of internal electron transfer to heme \(d\) and heme \(b_{558}\) and, therefore, results in the appearance of these hemes also having abnormally low midpoint potentials (i.e. they do not readily reduce). However, this cannot be the case, since one of the mediators present in the solution is TMPD, and it is clear that hemes \(d\) and \(b_{558}\) readily react with TMPD. Possibly, Arg\(^{391}\) may be critically placed to have an electrostatic influence on all three of the heme components. It is known that all three hemes are located on the periplasmic side of the membrane (15), and the heme \(b_{558}/\text{heme }d\) dinuclear center must be close to heme \(b_{558}\) to facilitate electron transfer from the bound quinol to each heme center (32).

Previous work has strongly suggested that the quinone binding site is near heme \(b_{558}\) (1, 13, 14). A quinone-like inhibitor, decyl-aurachin D, results in shifting the midpoint potential of heme \(b_{558}\) to much higher values and also eliminates quinol oxidase activity while not inhibiting TMPD oxidase activity. Hence, it is possible that the Arg\(^{391}\) mutants may also be perturbing the quinol binding site (similar to decyl-aurachin D) and that this may be the primary cause for the loss of quinol oxidase activity. Another possibility is that the Arg\(^{391}\) mutations might destabilize the semiquinone intermediate, presumably required for function (33). The FTIR difference spectrum of the R391A mutant shows that both ubiquinone and menaquinone remain bound to the enzyme, and HPLC analysis shows quinone co-purifying with the enzyme. Hence, the mutations probably do not prevent quinol binding per se. A determination of whether the R391A mutant influences the orientation of the bound quinol or the stability of the semiquinone will require further experimentation.

The R391A mutant is reduced anaerobically by ubiquinol-1 at a rate similar to the wild type oxidase. However, the reduction of the oxidase by reduced ubiquinol is very slow (\(k < 0.3\) s\(^{-1}\)) even with the wild type enzyme, and this process cannot be directly related to the reactions occurring during turnover (1000 s\(^{-1}\)). In the kinetic mechanism proposed by Jüinemann et al. (32), it is predicted that the species that reacts rapidly with quinol is actually the transiently formed one-electron reduced form of the enzyme with ferrous heme \(b_{558}\). If this is correct, then perhaps the reaction of ubiquinol with the fully oxidized enzyme would be much slower, as observed here. However, the R391A mutant dramatically slows down the reduction of cytochrome \(bd\) by an alternate substrate, menadiol, a soluble analogue of menaquinone (Fig. 8). This is consistent with the lack of quinol oxidase activity by the enzyme. Further studies are required to clarify whether the R391A mutant perturbs quinol binding to the enzyme in addition to influencing the heme electrochemical properties.

The simplest conclusion is that the substitution of Arg\(^{391}\) by a neutral residue abolishes quinol oxidation activity primarily due to a large shift in the midpoint potential of heme \(b_{558}\). There are variants of cytochrome \(bd\) in which Arg\(^{391}\) is not conserved, mostly in thermophilic microorganisms and in *Bacillales*. In one of the sequences from *B. subtilis*, for example, the Arg\(^{391}\) position is occupied by asparagine. Presumably, the reduced form of heme \(b_{558}\) is stabilized by the interaction with a positively charged residue at another position but still close to the heme. Alternatively, some of these organisms may utilize substrates with considerably lower midpoint potentials.

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