Tryptophan-136 in Subunit II of Cytochrome bo_3 from *Escherichia coli* May Participate in the Binding of Ubiquinol†

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ABSTRACT: In the cytochrome *c* oxidases, the role of subunit II is to provide the electron entry site into the enzyme. This subunit contains both the binding site for the substrate, cytochrome *c*, and the Cu_A redox center, which is initially reduced by cytochrome *c*. Cytochrome bo_3 and other quinol oxidases that are members of the heme−copper oxidase superfamily have a homologous subunit II, but the Cu_A site is absent, as is the docking site for cytochrome *c*. Speculation that subunit II in the quinol oxidases may also be important as an electron entry site is supported by the demonstration several years ago that a photoreactive substrate analogue, azido-Q, covalently labeled subunit II in cytochrome bo_3. In the current work, a sequence alignment of subunit II of heme−copper quinol oxidases is used as a guide to select conserved residues that might be important for the binding of ubiquinol to cytochrome bo_3. Results are presented for point mutants in 24 different residue positions in subunit II. The membrane-bound enzymes were examined by optical spectroscopy and by determining the activity of ubiquinol-1 oxidase. In each case, the K_m for ubiquinol-1 was determined as a measure of possible perturbation to a quinol binding site. The only mutant that had a noticeably altered K_m for ubiquinol-1 was W136A, in which the K_m was about sixfold increased. Thus, W136 may be at or close to a substrate (ubiquinol)-binding site in cytochrome bo_3. In the cytochrome *c* oxidases, the equivalent tryptophan (W121 in *Paracoccus denitrificans*) has been identified as the "electron entry site".

Cytochrome bo_3 from *Escherichia coli* is a member of the heme−copper oxidase superfamily that includes the cytochrome *c* oxidases (*1*). These enzymes all catalyze the 4-electron reduction of dioxygen to water and are proton pumps, generating a protonmotive force (*2*). Cytochrome bo_3, however, does not utilize cytochrome *c* as a substrate but instead catalyzes the 2-electron oxidation of ubiquinol-8 in the cytoplasmic membrane of *E. coli*. The enzyme contains one copy each of four subunits (*3*). Subunits I, II, and III are homologues to those found in other heme−copper oxidases, whereas subunit IV is unrelated to the subunits of the cytochrome *c* oxidases (*4*). Because the structure of the cytochrome *c* oxidases from bovine heart mitochondria (*5, 6*) and from the bacterium *Paracoccus denitrificans* (*7, 8*) have been determined by X-ray crystallography, a reasonable model of the structure of subunits I, II, and III of cytochrome bo_3 can be made (*9*). Subunit I, which has the highest sequence homologies among the heme−copper oxidases, contains both the active site (a heme−copper bimetallic center) and a low-spin heme component whose function is to transfer electrons to the heme−copper center (*1, 2*). The proton pump machinery is also contained within subunit I. In the cytochrome *c* oxidases, one role of subunit II is clearly to provide electrons to subunit I. The binding site for cytochrome *c* is located on the surface of a large hydrophilic COOH-terminal domain of subunit II (*10, 11*), which is anchored to the membrane by two NH_2-terminal transmembrane helices. Also contained within subunit II is the Cu_A redox center, which accepts electrons from cytochrome *c* and passes them along to the low-spin heme within subunit I (*2, 6, 7*).

Subunit II of cytochrome bo_3 shares the same general structure as that of the cytchrome *c* oxidases (*12*−*14*) but lacks both the binding site of cytochrome *c* and the Cu_A center. The structure of the COOH-terminal hydrophilic domain has been determined by X-ray crystallography (*14*), and is clearly closely related to the homologous domains of the cytochrome *c* oxidases (*6, 7*). Indeed, by site-directed mutagenesis, the Cu_A site has been restored to the subunit II domain and to cytochrome bo_3 (*15*). We therefore postulate that the role of subunit II in the quinol oxidases may be analogous to that of the equivalent subunit in the cytochrome *c* oxidases, i.e., providing electrons to the metal centers within subunit I. However, whereas cytochrome *c* is water-soluble and binds to subunit II from the aqueous phase, ubiquinol-8 is hydrophobic and is confined to the membrane. Clearly, if subunit II of cytochrome bo_3 does contain a ubiquinol-binding site, it cannot be a simple modification of the analogous binding site for cytochrome *c*.

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Subunit II has two transmembrane helices, both in contact with subunit I, and the hydrophilic domain sits on the top of subunit I (on the periplasmic surface for cytochrome bo_3) and forms a cap over the heme centers that is essentially at the membrane interface (*6*, *7*). Support for a role for subunit II in the binding of ubiquinol in cytochrome bo_3 is provided by the use of a photoreactive substrate analogue, azido-Q (*16*, *17*). Upon photolysis in the presence of purified cytochrome bo_3, azido-Q is covalently linked to subunit II (*16*). To further localize the binding site for ubiquinol within subunit II, we tried two approaches. In one, we used the covalent attachment of azido-Q, presumably at a ubiquinol-binding site, to identify a peptide likely to participate in ubiquinol binding. By mass spectroscopy, a trypsin fragment consisting of residues 165−178 of Subunit II was determined to be linked to azido-Q (*17*). In the second, parallel approach, we used site-directed mutagenesis, directed at selected residues within subunit II, to determine which residues are functionally important and might be involved in ubiquinol binding. Here, we report the results of the mutagenesis study. Of the 24 different sites within subunit II that we altered by mutagenesis, only 1 (residue W136) appeared to be functionally important and was thus potentially a candidate for direct involvement in the binding of ubiquinol.

MATERIALS AND METHODS

Materials. The restriction enzymes *Kpn*I, *Sal*I, *Hind*III, and *Msp*I were purchased from Bethesda Research Laboratories (BRL) . 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. Biochemicals. The nucleotides dATP, dGTP, dCTP, dTTP, and 5′Me-dCTP were from Pharmacia LKB Biotechnology. ATP and phenylmethylsulfonyl fluoride (PMSF) were from Sigma. Oligonucleotides used for mutation or sequencing were obtained from the Biotechnology Center at the University of Illinois. Ubiquinone-1 was a gift from Hoffmann-LaRoche AG (Basel, Switzerland).

Mutagenesis. Single-stranded M13 DNA was prepared according to the procedures of Messing and colleagues (*18*). Mutagenesis was performed as described (*19*), with the following modifications. The mutagenic primer (∼75 pmol) was 5′-phosphorylated with 1 unit of T4 nucleotide kinase in a 30-μL reaction containing 100 mM Tris-HCl, 10 mM $MgCl_2$, 7 mM dithiothreitol, and 1 mM ATP, at pH 8.0. After incubation at 37 °C for 30 min, the reaction was stopped by heating to 70 °C for 10 min. We then mixed 2 μL of 0.1 μg/μL M13 DNA template with 2 μL of the above reaction mixture, added 5 μL of H_2O and 1 μL of 10× React 2 (BRL) buffer (500 mM Tris-HCl, 100 mM $MgCl_2$, and 500 mM NaCl, pH 8.0) and incubated the mixture at 65 °C for 5 min. After the mixture cooled at room temperature for 15 min, the second DNA strand was synthesized by adding to the same tube 8 μL of 10× React 1 (BRL) buffer (500 mM Tris-HCl and 100 mM $MgCl_2$, pH 8.0), 19 μL of mixed dNTPs solution (1.33 mM each of dATP, dGTP, dTTP, and 5-methylated-dCTP, plus 2.67 mM ATP), 1 μL of T4 DNA ligase, and 5 units of T4 DNA polymerase. Water was added to the mixture to bring the total volume to 100 μL. After incubation for 90 min at 37 °C, the extension and ligation reactions were terminated by incubation at 70 °C for 10 min. The parent DNA template was nicked and digested with 1 μL each of *Msp*I and *Hha*I for 45 min, followed by 1 μL of exonuclease III at 37 °C for 45 min. The mixture was heated to 70 °C for 10 min, and then transformed into strain Jmr$^-$, which allows methylated DNA to be propagated.

Once the desired mutations were identified on M13 phage by single-stranded DNA sequencing, the fragments containing the mutations were cloned back into *cyo* operon in plasmid pMC31 (*20*). After subcloning, all mutations were confirmed by double-stranded DNA sequencing. GL101 (*20*) was used to propagate both wild-type and mutant plasmids.

Complementation Analysis of the Mutant Plasmids. Genetic complementation was conducted with the procedure previously described (*20*). The host strain used for complementation analysis was either RG129 (*21*) or GO105 (*22*), which cannot grow aerobically on nonfermentable substrates because of mutations in both the *cyo* and *cyd* operons of the chromosomes. 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. Strain GO105 (*22*) was used as the host strain for further characterization. Typically, a single colony harboring a mutant plasmid or the wild type was inoculated into 10 mL of liquid LB supplemented with ampicillin (100 μg/mL) and kanamycin (50 μg/mL). After overnight aerobic growth, the 10-mL culture was used to inoculate 1 L of LB with 0.3% D,L-lactate, 200 μM $CuSO_4$, and the antibiotics mentioned above. The cells were grown at 37 °C in 2-L baffled flasks with vigorous shaking. After the cell density was increased by ∼70 Klett units (determined with a 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 PMSF, leupeptin 0.5 μg/mL, 5 mM $MgSO_4$, and DNAse 4 μg/mL. The cell suspension was passed twice through a French pressure cell at 1200 psi (8.28 MPa), 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 25 000*g* for 30 min. The supernatant was centrifuged at 160 000*g* in a 60-Ti rotor for 1.5 h. The red cytoplasmic membranes were collected and homologized in 2 mL of ice-cold 50 mM potassium phosphate buffer (pH 7.5) with leupeptin (0.5 μg/mL) for subsequent characterization.

Oxidase Activity Assay. Ubiquinol-1 oxidase activity at 37 °C was measured with a model 53 YSI oxygen electrode (Yellow Springs Instrument Co., Yellow Springs, OH) and a temperature-controlled 1.8-mL electrode chamber (Gibson). The buffer for this assay on membrane samples was 50 mM Tris and 1 mM EDTA, pH 7.4, containing 2 mM dithioerythritol and 150 μM ubiquinone-1. For assays with the purified oxidase, the buffer contained 50 mM Hepes, 1 mM EDTA, 0.01% dodecyl maltoside, 2 mM dithiothreitol, 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 from the results.

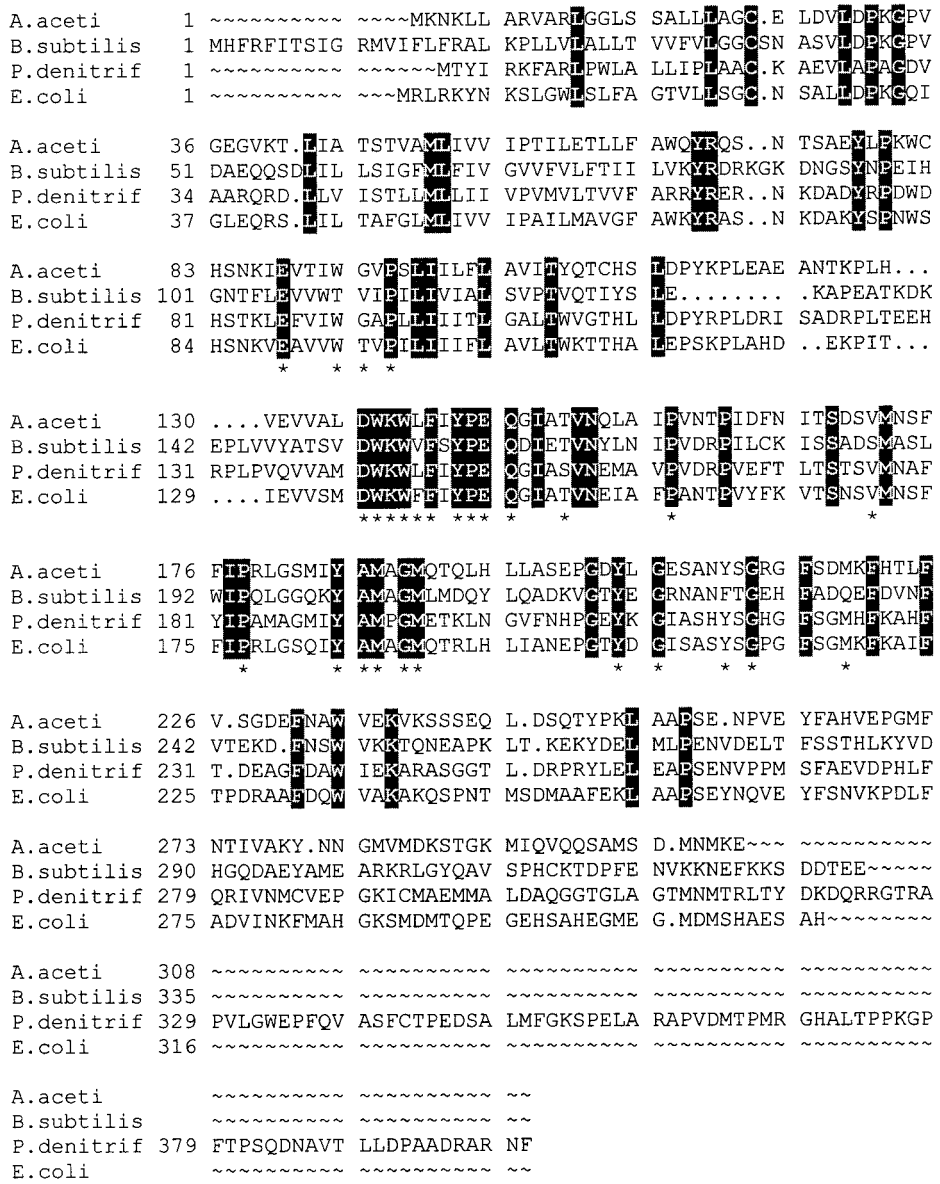

FIGURE 1: Sequence alignment of subunit II of the bacterial quinol oxidases. Residues that are totally conserved are boxed. The sequences are from the quinol oxidases from *E. coli* (*4*), *Acetobacter aceti* (*35*), *Bacillus subtilis* (*36*), and *P. denitrificans* (*37*).

Protein Purification: Wild type and W136A mutants were purified according to the published procedure of Minghetti et al. (*23*).

Proton Pumping Assay. Proton pumping was measured as previously described (*24*, *25*).

Miscellaneous. Protein concentration was determined with the bicinchoninic acid method (Pierce Chemical Co.). Optical spectroscopy was carried with a DW2000 UV−Vis spectrometer (SLM Instruments). The heme composition of purified mutant cytochrome bo_3 oxidases was analyzed as previously described (*26*).

RESULTS

Site-directed mutagenesis was used to screen a large set of residues within subunit II of cytochrome bo_3 for structural or functional importance. Figure 1 shows a sequence alignment of four ubiquinol oxidases within the heme−copper superfamily. Excluded from this list are the quinol oxidases from the archaebacteria, which have unique properties and are grouped in a separate phylogenetic category (*27*−*29*). As Figure 1 shows, a number of residues are conserved within the quinol oxidases; some of these are also highly conserved in the cytochrome *c* oxidases (not shown). Although residues common to the quinol oxidases are scattered throughout the sequences, three clusters are apparent, roughly E89 to I99, D135 to Q145, and Y184 to M189 (*E. coli* numbering). By comparison with the structures of the cytochrome *c* oxidases (*5*, *7*, *8*), we determined the first cluster (E89 to I99) to be located near the cytoplasmic side of the membrane within the second transmembrane helix. Therefore, those residues are not likely to be involved in the binding of ubiquinol, which is expected to be oxidized at the periplasmic surface of the membrane. Protons from the oxidation of ubiquinol are released into the bacterial periplasm (*9*, *30*), and the metal centers are located toward this side of the membrane (*6*, *7*). Therefore, we selected the targets for mutagenesis from residues within the periplasmic COOH-terminal domain, i.e., encompassing the other two clusters of conserved residues: D135 to Q145 and Y184 to M189 (Figure 1). The selected residues do not

Table 1: Summary of Properties of Site-Directed Mutants of Subunit II of Cytochrome bo_3

mutant	complementation	relative oxidase specific activity[a], %	relative expression[b], %	K_M, μM
WT	yes	100	100	38
D135N	yes	118	90	56
W136A	yes	72	99	242
K137M	yes	95	93	44
W138V	yes	63	52	45
F139A	yes	72	88	39
F140A	yes	95	106	59
Y142F	yes	95	83	48
Y142V	yes	63	56	31
P143A	yes	105	85	86
E144Q	yes	120	64	67
Q145A	yes	97	84	73
T149A	yes	95	104	58
P156A	yes	97	83	37
V170A	yes	109	64	66
P177A	yes	118	76	81
Y184F	yes	94	67	34
Y184V	yes	106	69	43
A185V	yes	102	103	59
M186A	yes	104	108	75
G188A	yes	103	86	52
M189A	yes	117	93	54
Y203A	yes	98	101	63
Y203F	yes	109	115	31
G205A	yes	110	79	65
Y210F	yes	100	115	61
G212A	yes	76	81	45
M218A	yes	88	90	59

[a] The specific activity of the wild-type oxidase from the parent plasmid was taken as 100%. Ubiquinol-1 oxidase activity was expressed per mole of CO-binding heme o_3. All assays were performed with membrane preparations. [b] Determined by the relative CO-binding units in membrane samples, using an absorptivity of 135 $mM^{-1}cm^{-1}$.

include all of those that are conserved in the quinol oxidases in this region but are spread through the region from ~130 to 230. The residues were also selected without regard for their positions in the structure of the hydrophilic domain of subunit II of cytochrome bo_3 (*14*) to provide a potentially wider range of phenotypes. In addition, several of the residues selected are not fully conserved.

Table 1 summarizes the properties of the mutants. All of the mutants are expressed to approximately the same amount and are functional, both by genetic complementation and by biochemical assay. Since a subtle perturbation to the ubiquinol binding site might not be apparent in a single measurement of catalytic velocity, we determined the dependence of activity on the concentration of ubiquinol-1. The data in all cases could be interpreted in terms of a K_m for ubiquinol-1. As shown in Table 1, only the W136A mutation had a K_m significantly different from that of the wild-type control. The W136A mutation had a sixfold increase of the K_m of the oxidase for ubiquinol-1, considerably more than the fluctuations in K_m values observed for the other mutant oxidases.

The W136A oxidase was solubilized and purified for further analysis. Spectroscopic studies, including the reduced-minus-oxidized spectrum and the CO-reduced-minus-reduced spectrum, showed no difference from the results for the wild-type oxidase. There is thus no indication of any perturbation to either the low-spin heme or the heme−copper binuclear center. The dependence of the ubiquinol-1 oxidase activity

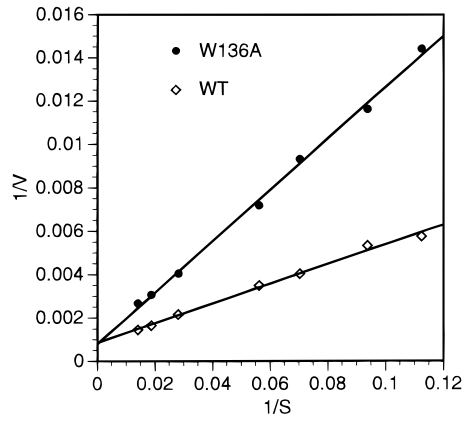

FIGURE 2: A Lineweaver−Burk plot showing the dependence of the steady-state kinetics of the ubiquinol-1 oxidase activity on the concentration of ubiquinol-1 for the purified wild type (WT) and W136A mutant of cytochrome bo_3. The data were analyzed to obtain the K_m values, which are threefold higher for the W136A mutant. The K_m values in Table 1 were obtained by using membrane-bound enzymes, in which case there is a sixfold increase due to the W136A mutation.

Table 2: Proton Pumping Stoichiometry of Selected Mutants of Cytochrome bo_3

mutant	H^+/e^- pH 6.0	H^+/e^- pH 7.0	H^+/e^- pH 8.0
WT	1.6−2	1.3−1.6	1−1.3
W136A	1.7−1.9	1.5−1.8	1
W138V	1.7−1.85	1.3−1.7	0.9
Y142F	1.5−2	1.4−1.5	1−1.3

was also determined by using the purified wild-type and W136A mutant oxidases. Each has an identical maximal turnover number (V_{max}), ~1200 electrons per second, but the K_m for ubiquinol-1 is threefold higher for the W136A mutant (Figure 2). The reason the influence of the mutation is less after purification (threefold increase in K_m) than in the membrane-bound form (sixfold increase) is not known, but both increases in K_m were consistent, as determined with several independent preparations of the enzyme.

Although no other mutants showed a significant change in the K_m for quinol, two mutations displayed a lower specific activity than that of the wild type: W138V and Y142V (but not Y142F) (Table 1). In each case, the strains harboring these mutant oxidases grew slowly, and the specific quinol oxidase activities were ~60% of that of the wild type. However, the K_m for ubiquinol-1 was not altered in either case. As with W136A, neither W138V nor Y142F showed any spectroscopic perturbations.

Finally, proton pumping assays (at pH 6.0, 7.0, and 8.0) were performed with cells containing the W136A mutant and with cells containing W138V or Y142F (Table 2). No difference from wild-type behavior was observed. These mutations have no influence on the proton pumping stoichiometry of cytochrome bo_3 or on its pH-dependence (*25*).

DISCUSSION

Our motivation in this study was to obtain information about the location of the ubiquinol-binding site(s) of cytochrome bo_3, given the finding that a photoreactive ubiquinol analogue, azido-Q, covalently labels subunit II (*16*). Presumably, some of the residues making up the ubiquinol-

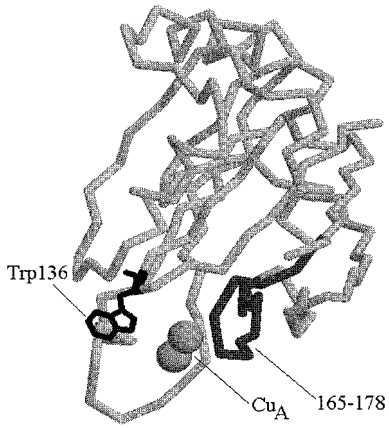

FIGURE 3: Structure of the hydrophilic COOH-terminal domain of subunit II of cytochrome bo_3 from *E. coli* (*14*). The location of tryptophan-136 is indicated along with the peptide that has been implicated in the binding site for ubiquinol (*17*). Also shown is the reconstructed site where the bimetallic Cu_A site is located. This redox site is not present in wild-type cytochrome bo_3 but is present in the related cytochrome *c* oxidases.

binding site would be common to the set of related quinol oxidases. The sequence alignment of subunit II of the ubiquinol oxidases indicates a number of conserved residues, some of which are also common to the much larger set of the related cytochrome *c* oxidases. Residue C25 is one of the residues conserved in the set of four quinol oxidases. We previously showed (*31*) that in the *E. coli* oxidase the posttranslational processing results in cleavage of the protein C25 and covalent addition of lipids to this residue, anchoring the modified NH_2-terminus of the subunit to the membrane. However, this NH_2-terminal modification is not important for the interaction of the enzyme with ubiquinol (*31*).

Of the 27 mutants examined in the current study, only the W136A mutation resulted in a significant, albeit modest, effect on the K_m of ubiquinol-1. We conclude that W136 is probably located at or very close to a ubiquinol-binding site in cytochrome bo_3. Concurrent with this work, we also determined that the azido-Q photolabel covalently binds to peptide residues 165−178 in subunit II, implicating some portion of this peptide in the binding of ubiquinol (*17*). Figure 3 shows a model of the structure of cytochrome bo_3, highlighting both this peptide as well as W136. Clearly, these are close to each other, which suggests a ubiquinol-binding site near the interface between subunits I and II. Since quinol is oxidized by the metal centers in subunit I, and since the binding site must be accessed from the lipid bilayer, this location for the binding site is plausible. Some evidence suggests that cytochrome bo_3 contains two sites for binding ubiquinol (*32*, *33*): One site has high affinity and appears not to readily exchange with free ubuqinol (*33*, *34*), whereas the second site has lower affinity and appears to be a traditional substrate-binding site. Presumbly, W136A perturbs the low-affinity site, which would be expected to result in an altered K_m for the substrate. A model of this site has been generated by molecular modeling, docking a ubiquinone close to both W136 and the peptide 165−178 (*17*). Four of the seven residues that were predicted to be within 5 Å, but further than 3 Å, from the docked quinone were coincidentally altered by mutagenesis in the current work: D135, V170, Y210, and M218. The mutations at these sites (Table 1) did not alter the K_m for ubiquinol-1, however, which suggests they are not directly involved in or required for the binding of ubiquinol, presumably because they are too far away. Additional sites will be targeted for mutagenesis to further examine the model.

The current data are also consistent with recent studies from the group of Anraku (Sato-Watanabe et al., unpublished) in which mutants resistant to quinol analogues were isolated and mapped. Given that these mutations were all within the hydrophilic domain of subunit II, the work independently suggests a quinol-binding site in the region near W136 and the Cu_A domain.

Of particular interest, the residue equivalent to W136 in the cytochrome *c* oxidase from *P. denitrificans*, W121, has been shown to be functionally important in mediating electron transfer from cytochrome *c* to Cu_A (*11*). W121 is located within the binding domain for cytochrome *c* on the surface of subunit II. A significant role for W136 in the substrate-binding site of cytochrome bo_3 would represent an interesting functional evolution from the cytochrome *c* oxidases to the quinol oxidases.

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