Uncovering the Molecular Mode of Action of the Antimalarial Drug Atovaquone Using a Bacterial System*

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Atovaquone is an antiparasitic drug that selectively inhibits electron transport through the parasite mitochondrial cytochrome bc1 complex and collapses the mitochondrial membrane potential at concentrations far lower than those at which the mammalian system is affected. Because this molecule represents a new class of antimicrobial agents, we seek a deeper understanding of its mode of action. To that end, we employed site-directed mutagenesis of a bacterial cytochrome b, combined with biophysical and biochemical measurements. A large scale domain movement involving the iron-sulfur protein subunit is required for electron transfer from cytochrome b-bound ubiquinol to cytochrome c1, of the cytochrome bc1 complex. Here, we show that atovaquone blocks this domain movement by locking the iron-sulfur subunit in its cytochrome b-binding conformation. Based on our malaria atovaquone resistance data, a series of cytochrome b mutants was produced that were predicted to have either enhanced or reduced sensitivity to atovaquone. Mutations altering the bacterial cytochrome b at its ef loop to more closely resemble Plasmodium cytochrome bc1 increased the sensitivity of the cytochrome bc1 complex to atovaquone. A mutation within the ef loop that is associated with resistant malaria parasites renders the complex resistant to atovaquone, thereby providing direct proof that the mutation causes atovaquone resistance. This mutation resulted in a 10-fold reduction in the in vitro activity of the cytochrome bc1 complex, suggesting that it may exert a cost on efficiency of the cytochrome bc1 complex.

Malaria is one of the most intractable human afflictions in the world. Despite intensive campaigns against the parasitic disease, an estimated 300 to 500 million cases still occur each year. Plasmodium falciparum, the causative agent of the most lethal form of malaria, is responsible for over 2 million deaths annually, largely among young children and pregnant women (1, 2). Efforts to eradicate malaria have not been successful, and the situation may be worsening due, in large part, to the emergence and spread of drug-resistant parasites. The need for new antimalarial drugs is now widely recognized. Atovaquone represents a new class of drugs having a metabolic target different from extant antimalarial drugs to which resistance is widespread. Atovaquone was found to be a very effective antimalarial compound, but unsuitable for use as a single agent because of the relatively quick emergence of resistance (3–5). However, in combination with the synergistic agent proguanil, it has been effective for both therapeutic and prophylactic uses (5–7). On the other hand, once atovaquone resistance has arisen, the combination is no longer effective against malaria parasites (8, 9).

Previous studies have shown that atovaquone selectively inhibits mitochondrial electron transport in the parasite, consistent with the prevalent theory that hydroxynaphthoquinones function as ubiquinone antagonists (10, 11). Additionally, atovaquone was found to collapse the parasite mitochondrial membrane potential at nanomolar concentrations (11), and its effect on the membrane potential is enhanced by synergistic activity of proguanil (12). To derive a better molecular understanding of the mode of action of atovaquone, a series of independently derived atovaquone-resistant malaria parasites were investigated (9). All of the resistant parasite clones contained mutations in a specific, well conserved segment of the cytochrome b subunit of the cytochrome bc1 complex (ubihydroquinone-cytochrome c oxidoreductase), thus identifying a likely binding region within the ubihydroquinone oxidizing (Qo)2 site of the malaria bc1 complex (9). Similar mutations associated with atovaquone resistance have been identified in clinical isolates (13, 14), as well as in experimental rodent malaria models (15).

With mitochondrial electron transport having been validated as an important target for antimalarial drugs, it will be helpful to derive molecular details of atovaquone interactions with the cytochrome bc1 complex with the hope of exploring alternate compounds with antimalarial activity. Recent determinations of the structure of two examples of the vertebrate cytochrome bc1 complex (16, 17), followed by those of yeast (18) and the bacterium Rhodobacter capsulatus (19), have provided significant insights into the mechanisms involved in the functioning of this enzyme. Because a large body of experimental work indicates that the active centers and core subunits of the enzyme complex are well conserved (reviewed in Refs. 20–23), we have begun to explore a relatively accessible bacterial system.

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1 The abbreviations used are: Qo, ubiquinone oxidation site; Q1, ubiquinone reduction site; [Fe-2S], two iron-two sulfur (cluster).
to help uncover molecular details of the mode of action of atovaquone. The potentially key region in cytochrome b associated with atovaquone resistance exhibits a particularly high degree of similarity between the malarial and bacterial complexes. In *R. capsulatus*, the cytochrome bc₁ complex consists of only three subunits, which are amenable to site-directed mutagenesis, and its three-dimensional structure has recently been determined (19). Using this bacterial system, it has been possible to demonstrate an unprecedented large amplitude domain movement involving the [2Fe-2S] protein that accompanies electron transfer from ubiquinol-bound cytochrome b to cytochrome c₁ (24, 25) (see also Ref. 26, and references therein). In this work, we have constructed *R. capsulatus* cytochrome b substitution mutants predicted to have either enhanced or reduced sensitivity to atovaquone. Characterization of the altered cytochrome bc₁ complexes through biochemical and biophysical approaches provides new insight into the molecular mode of action of atovaquone as a potent antimarial drug.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains and Growth Conditions—Escherichia coli** strains were grown in Luria-Bertani broth, in the presence of appropriate antibiotics, and *R. capsulatus* strains in mineral/peptone/yeast extract-enriched medium (27) or in RCVB medium (28) containing 5 mM glutamate, in the presence of 10 μg/ml kanamycin. Respiratory or photosynthetic growth of *R. capsulatus* strains was at 30–35 °C in the dark under semiharmonic conditions or in an anaerobic chamber under continuous light, respectively. MT-RBC1 is a cytochrome bc₁ strain in which the chromosomal copy of the petABC operon (also called fbcFBC) has been deleted and replaced by a gene construct conferring resistance to spectinomycin (27). The strain pMTS1/MT-RBC1 corresponds to MT-RBC1 complemented in trans with the plasmid pMTS1 (29), which is a broad host-range plasmid that provides resistance to kanamycin and contains a wild-type copy of petABC.

**Molecular Genetic Techniques**—Mutations were constructed by oligonucleotide-directed mutagenesis in plasmid pPET1 (27), using either a *Phu* polymerase/DpnI strategy, as described (30), or using the "Gene Editor" T4 DNA polymerase/marker co-selection system from Promega, with minor modifications of the manufacturer’s protocol. The mutagenic oligonucleotides used were (with the changes to the parental sequences in bold italic): 5’-CGGGCGAGAGGCTTACGATCGCG-TAGA-3’ (I304M) and 5’-GCGGCGGAG-AGGCGATC-GCGCCAGAAGCAGGC-3’ (Y270C), which is a broad host-range plasmid that provides resistance to kanamycin and contains a wild-type copy of petABC.

**RESULTS**

**Properties of *R. capsulatus* with Mutated Cytochrome b**—Whereas a functional cytochrome bc₁ complex is not required for aerobic growth of *R. capsulatus* because of the presence of an alternate respiratory pathway (32), this enzyme is believed to be involved in the binding of atovaquone (9), we engineered for this study. The Met and Lys substitutions are marked below.

**Atovaquone Mode of Action**

![Atovaquone Mode of Action](http://www.jbc.org/)

**FIG. 1.** Aligned amino acid sequences from the ef loop of cytochromes b from *P. yoelii*, *R. capsulatus*, and other selected species (amino acid residues 254–273 in *P. yoelii* cytochrome b288–307 in *R. capsulatus*). The shaded boxes enclose positions that are largely conserved among the known cytochrome b sequences. Characters above the *P. yoelii* sequence show the substitutions that were found in atovaquone-resistant *P. yoelii* isolates; a rounded box surrounds the Val and Arg substitutions indicating that they occurred together in the same resistant isolate. Stars below many of the amino acid residues of the *R. capsulatus* cytochrome b mark those that are identical to the corresponding residues in *P. yoelii* cytochrome b. Characters below the *R. capsulatus* sequence denote the substitutions in the bacterial cytochrome b engineered for this study. The Met and Lys substitutions are surrounded by a box to indicate that they were engineered to occur jointly in the same cytochrome b gene product.

experiments with thermolysin were done basically according to Valkova-Valchanova et al. (25), using chromatophore membranes prepared in the presence of 17 mM EDTA, then extensively washed, dispersed in 1 mg of dodecylmaltose per mg of total proteins, and incubated for 1 h at room temperature in 50 mM Tris-HCl (pH 8.0) containing 100 mM NaCl, 5 mM CaCl₂, 2 mM of thermolysin, and 30 nmol of stigmatellin or atovaquone, when specified, in a total reaction volume of 50 μl. Aliquots were analyzed by immunoblotting with polyclonal antibodies against the iron-sulfur protein of *R. capsulatus* (25).

EPR measurements were performed on a Bruker ESP-300E spectrometer (Bruker Biosciences). Temperature control was maintained by an Oxford ESR-9 continuous flow helium Oxford model ITC4 temperature controller. The frequency was measured with a Hewlett-Packard model 5350B frequency counter. Unless otherwise noted, the operating parameters were as follows: sample temperature, 20 K; microwave frequency, 9.45 GHz; microwave power, 2 milliwatt; modulation frequency, 100 kHz; modulation amplitude, 20.243 G; and time constant, 162.34 ms. Samples were poised with the ubiquinone pool oxidized and [2Fe-2S] cluster reduced by addition of 20 mM sodium ascorbate.

Light-induced, single-turnover time-resolved kinetics were performed as described (24, 31) by using chromatophore membranes and a single wavelength spectrophotometer (Biomedical Instrumentation Group, University of Pennsylvania) in the presence of 2.5 μM valinomycin, N-ethylbenzhydrazine ethyl sulfate, N-methyl-benzhydrazine amine methyl sulfate, 2,5,6-tetramethyl-1,4-phenylenediamine, and 2-hydroxy-1,4-naphthoquinone. Transient cytochrome c re-reduction kinetics initiated by a short saturating flash (8 μs) from a xenon lamp was followed at 550 nm – 540 nm. The concentrations of antimycin A, atovaquone, myxothiazol, and stigmatellin used were 5, 10, 5, and 1 μM, respectively, and the ambient potential was poised at 100 mV.

**RESULTS**

**Properties of *R. capsulatus* with Mutated Cytochrome b**—Whereas a functional cytochrome bc₁ complex is not required for aerobic growth of *R. capsulatus* because of the presence of an alternate respiratory pathway (32), this enzyme is essential for its anoxygenic photosynthetic growth. Thus, in bacteria with a mutated cytochrome bc₁ complex, under appropriate conditions, photosynthetic growth rate reflects the functional efficiency of the cytochrome bc₁ complex. Comparing the cytochrome b sequence of *Plasmodium* and *R. capsulatus* around a highly conserved portion of the Q loop, containing the conserved "PEWY" motif (Fig. 1), which is believed to be involved in the binding of atovaquone (9), we noticed remarkable identity between these organisms, within the segment from residue 292 to 306 (bacterial numbering) all
the amino acids are identical except two, and these two represent conservative changes (Fig. 1). Because atovaquone binding affinity is likely influenced by subtle local changes, we mutated the two \textit{R. capsulatus} residues (Ile304 and Arg306) to their respective \textit{Plasmodium} counterparts (Met and Lys) to better mimic the structure of the malaria cytochrome \textit{bc}$_1$ complex in this region. We also mutated Tyr302 to cysteine (Cys302) in both the wild-type and the Met304/Lys306 backgrounds. This tyrosine corresponds to the site most frequently found altered in clinical cases of malaria atovaquone resistance (8, 14). These \textit{R. capsulatus} mutant strains were examined for their anaerobic photosynthetic growth rate as well as for the specific activity of their cytochrome \textit{bc}$_1$ complex in isolated chromatophore membranes. As shown in Table I, the photosynthetic growth rate was reduced by 13–25% of the wild-type rate in Met304/Lys306 and Cys302 strains, but was reduced by 50% in the strain bearing all three amino acid substitutions. We isolated chromatophore membranes from these strains to assess their cytochrome \textit{bc}$_1$ complex activity. The overall protein content, as well the \textit{b} and \textit{c}-type cytochrome content of these membrane preparations were similar (Table I), indicating that all strains produced and assembled comparable amounts of cytochrome \textit{bc}$_1$ complexes under the conditions used. The cytochrome \textit{bc}$_1$ complex activity was reduced only modestly in the Met304/Lys306 strain, but by about 90% in strains bearing the atovaquone resistance-associated Cys302 substitution. These observations were consistent with the greater effect of the latter substitution on photosynthetic growth of \textit{R. capsulatus}, although the degree of growth retardation was lower than its effect on enzymatic function, presumably because of the overexpression of the cytochrome \textit{bc}$_1$ complex in a trans-complemented “wild-type” strain.

\textbf{Inhibition of the Cytochrome \textit{bc}$_1$ Complex by Atovaquone and Other \textit{Q}$_0$ Site Inhibitors—}We assessed inhibition profiles of atovaquone, stigmatellin, and myxothiazol on cytochrome \textit{bc}$_1$ complex activity in chromatophore membranes prepared from the \textit{R. capsulatus} strains described above. Representative inhibition plots from the atovaquone titrations are shown in Fig. 2, and the average measured mid-point inhibition concentrations (IC$_{50}$) for the three inhibitors for each strain are given in Table II. The wild-type cytochrome \textit{bc}$_1$ complex was inhibited by atovaquone with IC$_{50}$ of about 10 nM. However, unlike stigmatellin or myxothiazol (33), \textit{R. capsulatus} growth is not inhibited by atovaquone when the drug is included in the medium, possibly because of lack of accessibility to its site of action in intact bacteria. Cytochrome \textit{bc}$_1$ complex from the strain bearing the Met304/Lys306 substitutions was inhibited by atovaquone with IC$_{50}$ of about 10 nM. This 3-fold reduction in IC$_{50}$ is consistent with our prediction that subtle changes observed in the \textit{Plasmodium} cytochrome \textit{b} are responsible for the enhanced therapeutic window for atovaquone. The substitution corresponding to one commonly seen in atovaquone-resistant human malaria parasites, Cys302, dramatically increased the IC$_{50}$ of atovaquone in the cytochrome \textit{bc}$_1$ complex. This was observed in both the wild-type and Met304/Arg306 background strains. These results now formally confirm a causal relationship between the alteration of a conserved tyrosine residue within the \textit{ef} loop and acquisition of atovaquone resistance in malaria parasites. It was interesting to note that the Cys302 substitution had much more modest effects on IC$_{50}$ for two other \textit{Q}$_0$ site inhibitors, stigmatellin and myxothiazol, increasing it by 2–20-fold compared with 100–200-fold for atovaquone (Table II). These results are consistent with the crystallo-
Effect of Inhibitors on the Ubihydroquinone Oxidation Site (Qo)-Iron-Sulfur Cluster ([2Fe-2S]) Interaction Probed by EPR Spectroscopy—The EPR spectrum of the [2Fe-2S] cluster is very sensitive to its molecular environment, and therefore, to local conditions surrounding it, including the conditions at the Qo site in cytochrome b (35). In the native enzyme, this spectrum exhibits a relatively sharp g value transition at a g = 1.80 value, which is indicative of the interactions of the reduced [2Fe-2S] cluster with ubiquinone in the Qo site (Fig. 3A) (36). Thus, to assess the effects of Y302C and I304M/R306K mutations on these interactions, the EPR spectra of appropriate wild-type and mutated enzyme complexes in chromatophores were examined in the absence and presence of inhibitors. The g signals of the cytochrome bc1 complex carrying the Met304/Lys306 substitutions is slightly shifted to lower magnetic field and broader than that of the wild-type enzyme (Fig. 3A), which could be because of subtle differences in the interactions of the [2Fe-2S] cluster with the Qo site of the mutant enzyme. In the presence of myxothiazol, stigmatellin, or atovaquone, this g signal shifts to higher magnetic field and broadens to various extents depending on the inhibitor added (Fig. 3, B–D, respectively). These spectra are similar to previously reported spectra in the case of myxothiazol and stigmatellin (35, 36), indicating that the Qo site of the cytochrome bc1 complex carrying the I304M/R306K mutations is not drastically perturbed in comparison to the wild-type enzyme. Interestingly, in the case of atovaquone and the Met304/Lys306 cytochrome bc1 complex, the g signal is sharper and shifted farther (to ~1.76) than that seen with the wild-type enzyme, suggesting that in this mutant the [2Fe-2S] cluster-atovaquone interactions are more pronounced.

When the EPR spectra of the cytochrome bc1 complexes carrying the single Y302C and the triple Y302C/I304M/R306K mutations were compared with that of the wild-type enzyme significant differences were observed (Fig. 3). The g transition broadened and shifted from 1.80 to 1.79, reflecting altered interactions between the [2Fe-2S] cluster and the Qo site and its occupants. In the presence of myxothiazol or stigmatellin, the two cytochrome bc1 complexes carrying the Y302C mutation also exhibited a broad g signal, at about the same position as those observed with atovaquone. Moreover, in this region of the spectra multiple overlapping transitions are visible in the absence of inhibitor or presence of myxothiazol (Fig. 3, A and B), suggesting some sample heterogeneity. Interestingly, in the presence of atovaquone, both of the cytochrome bc1 complexes carrying the Y302C mutation exhibited a broad g transition at a g value of 1.78, closer to that seen with the wild-type, rather than the sharper g = 1.76 signal of the Met304/Lys306 enzyme. Thus, the data clearly revealed that atovaquone has a unique and possibly stronger interaction with the [2Fe-2S] cluster of the cytochrome bc1 complex carrying the Met304/Lys306 substitutions, which is disrupted by the Y302C mutation.

Single Turnover Kinetics of the Cytochrome bc1 Complex in the Presence of Various Inhibitors—Chromatophores containing cytochrome bc1 complexes were also studied in light-initiated rapid kinetic experiments, which monitor the transfer of electrons to cytochrome c during a single turnover of the enzyme. In these experiments, the cytochrome c re-reduction kinetics are initiated by an actinic flash that excites photosynthetic reaction centers in the chromatophores, which in turn,
rapidly oxidize cytochrome c molecules, which are then re-reduced by the cytochrome bc1 complexes. The kinetics of wild-type and Met304/Lys306 mutant were virtually identical, with an initial rapid phase (because of electron transfer from the pre-reduced [2Fe-2S] cluster, completed in the dead time of the instrument (24) and only revealed in the presence of stigmatellin) and a slower phase involving re-reduction of the [2Fe-2S] cluster by ubiquihydroquinone in the Qb site and subsequent reduction of additional cytochrome c by the reduced cluster (Fig. 4). The single-turnover kinetics exhibited by the wild-type and the Met304/Lys306-substituted cytochrome bc1 complexes also responded identically to the presence of excess Qb site inhibitors. Myxothiazol blocks the slow phase of cytochrome c reduction by eliminating ubiquihydroquinone from the Qb site, whereas stigmatellin prevents both phases of the reaction by locking the [2Fe-2S] cluster at the cytochrome b position (24). Remarkably, in both the wild-type and the Met304/Lys306-substituted cytochrome bc1 complexes, atovaquone also inhibited both phases like stigmatellin (Fig. 4), indicating that it acted at the Qb site in a manner similar to stigmatellin. Stigmatellin and myxothiazol both block ubiquihydroquinone binding at the Qb site by occupying portions of the site, but stigmatellin additionally has been shown to inhibit the large amplitude movement of the [2Fe-2S] domain (24, 25) required for electron transfer to cytochrome c1.

Effect of Atovaquone on the Thermolysin Sensitivity of the Iron-Sulfur Subunit—As the domain immobilization by stigmatellin was shown to be accompanied by decreased sensitivity of the hinge segment of the Rieske [2Fe-2S] protein to cleavage by thermolysin (24, 25), we examined the thermolysin sensitivity of the cytochrome bc1 complexes in the presence and absence of atovaquone, as well as stigmatellin and antimycin A. Treatment of chromatophores with wild-type cytochrome bc1 complexes resulted in conversion of about 25% of the [2Fe-2S] protein to the 18-kDa form resulting from thermolysin cleavage (Fig. 6). Therefore, with Cys at position 302 of cytochrome b, atovaquone and stigmatellin are no longer able to stabilize the thermolysin-insensitive conformation of the Rieske [2Fe-2S] protein.

**DISCUSSION**

*R. capsulatus* has a long history as a useful system for the investigation of cyclic photosynthesis, including the function of the cytochrome bc1 complex, utilizing a combination of techniques, notably genetic and biochemical-biophysical methods (37). Employing these techniques together with the use of ubiquinone-like inhibitors, investigators identified the principal amino acid residues of cytochrome b that interact with ubiquihydroquinone/ubiquinone, i.e. the residues of the ubiquiquinone binding pockets, Qb and Qo, prior to the recent solution of the three-dimensional structure of the cytochrome bc1 complex. Because atovaquone is a ubiquinone antagonist to which *R. capsulatus*, like *Plasmodium*, is relatively sensitive, we have employed this well developed system to investigate the molecular basis of the action of the drug. The high degree of identity of the region of the *Plasmodium* cytochrome b that was previously identified as a likely atovaquone interaction site (9) with the corresponding region of the bacterial cytochrome further enhances the utility of the bacterial system for this work. Only two additional changes of the bacterial sequence (I304M and R306K) were required to achieve the complete recapitulation of the 15-residue segment (positions 258–272, reproduced in Fig. 1) of the parasite cytochrome 6 that includes all of the sites at which we found high-level resistance mutations in a previous study involving the generation of atovaquone-resistant *Plasmodium yoelii* parasites (9). This segment also includes the known site of mutations in *P. falciparum* cytochrome b (Tyr268, corresponding to Tyr302 in *R. capsulatus* numbering) that have been correlated with high-level clinical resistance (8, 14).
Trumpower and colleagues (38, 39) have recently developed the yeast cytochrome bc$_1$ complex as a model for fungal and parasite enzyme complexes, however, the yeast cytochrome b appears to be more similar to that of fungal pathogens, such as *Pneumocystis* than to that of apicomplexan parasites.

As we had hypothesized, the Met304/Lys306 double substitution increased the sensitivity of the bacterial cytochrome bc$_1$ complex to atovaquone, but not to stigmatellin or myxothiazol. This effect was modest, a 3-fold decrease in the IC$_{50}$ for atovaquone, as the wild-type enzyme complex is already relatively sensitive to the drug. Except for interactions with atovaquone, the biochemical and biophysical properties of the Met304/Lys306 cytochrome bc$_1$ complex were very similar to those of the wild-type complex. The steady state enzymatic activity in chromatophore membranes was reduced by about 20%, and the response to inhibitors in the single-turnover, flash kinetics experiments was essentially identical to that of the wild-type enzyme. The differences in the EPR spectra of the Met$_{304}$/Lys$_{306}$ versus the wild-type cytochrome bc$_1$ complex were minor, again except in the presence of atovaquone, where a significant sharpening and upfield shift of the g$_x$ signal was seen in the Met$_{304}$/Lys$_{306}$ samples. The Met$_{304}$/Lys$_{306}$ enzyme complex also behaved similarly to the wild-type complex with regard to digestion with thermolysin in the presence of stigmatellin or myxothiazol. The hinge region of the Rieske [2Fe-2S] protein of the cytochrome bc$_1$ complex is normally susceptible to cleavage by thermolysin, but in the presence of stigmatellin, the hinge segment is locked into a fully extended conformation that is resistant to cleavage. In this work, we have shown that atovaquone also protects the Rieske [2Fe-2S] protein in the wild-type and Met$_{304}$/Lys$_{306}$ complexes from proteolysis. In the case of the Cys$_{302}$-substituted complexes, however, atovaquone or stigmatellin provided...
The IC$_{50}$ is raised over 2 orders of magnitude, and the ability to immobilize the [2Fe-2S] cluster domain of the Rieske [2Fe-2S] complex, the [2Fe-2S] cluster domain of the Rieske [2Fe-2S] protein tends to be immobilized in a cytochrome $b$-binding conformation. Immobilization prevents electron transfer from the [2Fe-2S] cluster to cytochrome $b$, thus, the mechanism of inhibition by atovaquone may be 2-fold, like that of stigmatellin. First, binding of atovaquone within the Q$_o$ site probably excludes binding of the substrate ubihydroquinone. Second, binding may prevent or greatly slow electron transfer through its effect on the mobility of the cluster domain of the Rieske [2Fe-2S] protein subunit. Yet the interactions of atovaquone with the Q$_o$ site may be different from those of stigmatellin, as evidenced by the titration and EPR results. Finally, it is noted that the mutation Y302C, which is homologous to a point mutation associated with resistance to atovaquone in malaria parasites, has a strong affect on the interactions of atovaquone with the bacterial enzyme as well. The IC$_{50}$ is raised over 2 orders of magnitude, and the ability to immobilize the [2Fe-2S] cluster domain of the enzyme is lost. The distinctive features of the EPR spectrum in the presence of atovaquone are also lost. The substitution also affects the inhibition by atovaquone may be 2-fold, like that of stigmatellin. First, binding of atovaquone within the Q$_o$ site probably excludes binding of the substrate ubihydroquinone. Second, binding may prevent or greatly slow electron transfer through its effect on the mobility of the cluster domain of the Rieske [2Fe-2S] protein subunit. Yet the interactions of atovaquone with the Q$_o$ site may be different from those of stigmatellin, as evidenced by the titration and EPR results.

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**Atovaquone Mode of Action**

- The Rieske [2Fe-2S] protein tends to be immobilized in a cytochrome $b$-binding conformation. Immobilization prevents electron transfer from the [2Fe-2S] cluster to cytochrome $b$, thus, the mechanism of inhibition by atovaquone may be 2-fold, like that of stigmatellin. First, binding of atovaquone within the Q$_o$ site probably excludes binding of the substrate ubihydroquinone. Second, binding may prevent or greatly slow electron transfer through its effect on the mobility of the cluster domain of the Rieske [2Fe-2S] protein subunit. Yet the interactions of atovaquone with the Q$_o$ site may be different from those of stigmatellin, as evidenced by the titration and EPR results.

**Fig. 6. Susceptibility of wild-type, Cys$_{302}^{---}$, and Met$_{204}$/Lys$_{206}$/Cys$_{302}^{---}$ cytochrome $b_c$ complexes to proteolysis by thermolysin.** Chromatophore samples (650 µg of protein) were incubated with 2 nmol of thermolysin (T) in the absence of inhibitor and in the presence of 50 nmol of atovaquone (At) or stigmatellin (S), under the conditions described under “Experimental Procedures.” The upper panel (A) shows the results of the SDS-PAGE/Western immunoblot of the iron-sulfur protein in an aliquot of each sample. The lower panel (B) displays the fraction of the proteolytic 18-kDa fragment present in each sample as determined by densitometric analysis (see Ref. 24). WT, chromatophores from wild-type bacteria; C, chromatophores from bacteria expressing the Y302C substituted cytochrome $b$; CMK, chromatophores from bacteria expressing cytochrome $b$ with the combined Y302C, I304M, and R306K substitutions.

**Note:** The primary text contains a reference to a diagram, which is not included here. The diagram is likely provided in the original publication for illustrative purposes.

**Discussion:**

- The Rieske [2Fe-2S] protein tends to be immobilized in a cytochrome $b$-binding conformation. Immobilization prevents electron transfer from the [2Fe-2S] cluster to cytochrome $b$, thus, the mechanism of inhibition by atovaquone may be 2-fold, like that of stigmatellin. First, binding of atovaquone within the Q$_o$ site probably excludes binding of the substrate ubihydroquinone. Second, binding may prevent or greatly slow electron transfer through its effect on the mobility of the cluster domain of the Rieske [2Fe-2S] protein subunit. Yet the interactions of atovaquone with the Q$_o$ site may be different from those of stigmatellin, as evidenced by the titration and EPR results.

**Conclusion:**

- The results of the thermolysin proteolysis experiments and the single-turnover kinetics with the wild-type and Met$_{204}$/Lys$_{206}$/Cys$_{302}^{---}$ cytochrome $b_c$ complexes suggest similarities in the mechanism of inhibition of atovaquone to that of stigmatellin. Both inhibitors blocked the rapid and slow phases of electron transfer to cytochrome $c$, and both provided protection from cleavage of the Rieske [2Fe-2S] protein by thermolysin, unlike the other Q$_o$ site inhibitor myxothiazol, which has a distinctly different effect in these assays. In crystals of mammalian and yeast cytochrome $b_c$ complexes formed in the presence of stigmatellin or 5-undecyl-6-hydroxy-4,7-dioxobenzothiazole, the Rieske [2Fe-2S] protein is stabilized in a conformation with its [2Fe-2S] cluster-binding domain in contact with the ef loop region of cytochrome $b$, and its hinge region in an uncoiled, extended form (16–18). This conformation was found to be a fixed, immobilized one, which thus prevents electron transfer to cytochrome $c$. Apparently, the Rieske [2Fe-2S] protein-cytochrome $b$ contacts in the Q$_o$ site involved only two residues in the ef loop of cytochrome $b$, which contact two residues of the Rieske [2Fe-2S] subunit near its metal cluster, plus a hydrogen bond between one of its cluster ligands, His$_{356}^{---}$ (R. capsulatus numbering), and the carbonyl group of the chromone ring of stigmatellin. Atovaquone may bind in the Q$_o$ site in a manner similar to stigmatellin, with an oxygen atom from a carbonyl or hydroxyl group of the hydroxynaphthoquinone ring forming a hydrogen bond with the histidine ligand of the [2Fe-2S] cluster. In fact, Kessel et al. (39) recently constructed an energy-minimized model of atovaquone bound in the Q$_o$ site of yeast cytochrome $b_c$ complex, which predicts that the drug binds to the yeast enzyme in this fashion with a hydrogen bond between the hydroxynaphthoquinone hydroxyl oxygen and a nitrogen atom of the histidine ligand. Clearly, there are differences in the interactions of atovaquone and stigmatellin with the cytochrome $b_c$ complex, as evidenced by differences in the EPR spectra and the fact that the engineered Y302C mutation drastically increases resistance to atovaquone, but has little affect on that to stigmatellin. Most interestingly, although stigmatellin remains an inhibitor of the Cys$_{302}^{---}$-substituted complex, it no longer protects against thermolysin cleavage of the Rieske [2Fe-2S] protein. In this regard, we note that the crystallographic data show that the Tyr$_{302}$ residue participates in forming the binding site of stigmatellin (18, 34, 40). Thus, whereas stigmatellin still binds strongly to the Cys$_{302}^{---}$-substituted complex, the interaction of the [2Fe-2S] cluster domain of the Rieske [2Fe-2S] protein subunit with the bound drug or the ef loop of cytochrome $b$ appears to have been significantly weakened.

**Plasmodium spp. and R. capsulatus** are phylogenetically distant, but, as we have noted, the cytochrome $b_c$ complexes from both are strongly inhibited by atovaquone, whereas vertebrate cytochrome $b_c$ complexes are naturally resistant. The sensitivity of the malarial and bacterial enzymes to atovaquone appears to correlate with their high degree of sequence similarity in the ef loop of cytochrome $b$ (Fig. 1). When atovaquone is bound in the Q$_o$ site of these sensitive cytochrome $b_c$ complexes, the [2Fe-2S] cluster domain of the Rieske [2Fe-2S] protein tends to be immobilized in a cytochrome $b$-binding conformation. Immobilization prevents electron transfer from the [2Fe-2S] cluster to cytochrome $b$, thus, the mechanism of inhibition by atovaquone may be 2-fold, like that of stigmatellin. First, binding of atovaquone within the Q$_o$ site probably excludes binding of the substrate ubihydroquinone. Second, binding may prevent or greatly slow electron transfer through its effect on the mobility of the cluster domain of the Rieske [2Fe-2S] protein subunit. Yet the interactions of atovaquone with the Q$_o$ site may be different from those of stigmatellin, as evidenced by the titration and EPR results.

Finally, it is noted that the mutation Y302C, which is homologous to a point mutation associated with resistance to atovaquone in malaria parasites, has a strong affect on the interactions of atovaquone with the bacterial enzyme as well. The IC$_{50}$ is raised over 2 orders of magnitude, and the ability to immobilize the [2Fe-2S] cluster domain of the enzyme is lost. The distinctive features of the EPR spectrum in the presence of atovaquone are also lost. The substitution also affects the interaction of other inhibitors and the substrate ubihydroquinone with the Q$_o$ site, at the expense of decreasing the steady state activity of the cytochrome $b_c$ complex. The direct generation of these effects by site-directed mutagenesis in a defined system should dispel any doubt that the homologous substitution is the direct cause of resistance in the parasite. The mutation at the highly conserved Tyr$_{302}$ position appears to exact a cost on the efficiency of the $b_c$ complex. A homologous mutation in the mitochondrial DNA, present in the heteroplasmic state, has been associated with a multisystem disorder, decreased mitochondrial functions, and decreased Complex III enzymatic activity in a patient (41). Atovaquone-resistant malaria parasites are believed to have a growth disadvantage.
in *P. falciparum* cultures (42). Results from the present study support the speculation that atovaquone-resistant malaria parasites may have a growth disadvantage, and that withdrawal of the drug pressure may lead to replacement of the resistant parasites with a susceptible population, as has been observed for chloroquine-resistant parasites in Malawi (43).

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**Atovaquone Mode of Action**

27465
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