Hydrogen Bonding to the Substrate Is Not Required for Rieske Iron-Sulfur Protein Docking to the Quinol Oxidation Site of Complex III*

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Complex III or the cytochrome (cyt) bc1 complex constitutes an integral part of the respiratory chain of most aerobic organisms and is a part of the photosynthetic apparatus of anoxygenic purple bacteria. The function of cyt bc1 is to couple the reaction of electron transfer from ubiquinol to cytochrome c to proton pumping across the membrane. Mechanistically, the electron transfer reaction requires docking of its Rieske iron-sulfur protein (ISP) subunit to the quinol oxidation site (Qₚ) of the complex. Formation of an H-bond between the ISP and the bound substrate was proposed to mediate the docking. Here we show that the binding of oxazolidinedione-type inhibitors famoxadone, jg144, and fenamidone induces docking of the ISP to the Qₚ site in the absence of the H-bond formation both in mitochondrial and bacterial cyt bc1 complexes, demonstrating that ISP docking is independent of the proposed direct ISP-inhibitor interaction. The binding of oxazolidinedione-type inhibitors to cyt bc1 of different species reveals a toxophore that appears to interact optimally with residues in the Qₚ site. The effect of modifications or additions to the toxophore on the binding to cyt bc1 from different species could not be predicted from structure-based sequence alignments, as demonstrated by the altered binding mode of famoxadone to bacterial cyt bc1.

The ubiquinol-cytochrome c oxidoreductase, also known as complex III of mitochondrial respiratory chain or cytochrome (cyt)3 bc1 complex, catalyzes the reaction of electron transfer from ubiquinol (QH₂) to cyt c and concomitantly transports protons across the inner membrane of mitochondria or the plasma membrane of photosynthetic purple bacteria, contributing to a cross-membrane potential important for cellular function (1, 2). Although catalyzing the same enzymatic reaction, cyt bc1 complexes isolated from different organisms have very different subunit compositions. Prokaryotic bc1 complexes often consist of 3–4 subunits, whereas mitochondrial enzymes have 10–11 different subunits (1). Nevertheless, only three subunits are essential for the ET function (Fig. 1A): cyt b, cyt c₁, and the Rieske iron-sulfur protein (ISP) (3). The cyt b subunit contains two b-type hemes, b₁ and b₄, for the low and high potential hemes, respectively, and is entirely embedded in the membrane with eight transmembrane (TM) helices (4). The cyt c₁ subunit, anchored to the membrane by a single TM helix, has a c-type heme covalently attached to its active domain that is located in the intermembrane space of mitochondria or periplasm in bacteria. The ISP subunit features an integrated 2Fe-25 cluster in its extrinsic domain (ISP-ED) that is on the same side as the cyt c₁ subunit and also anchored to the membrane by a helix.

The ET-coupled proton translocation function, as modeled by the Q-cycle mechanism (Fig. 1B), requires two active sites: a QH₂ oxidation (Qₚ) site and a ubiquinone (Q) reduction (Qₐ) site (5–7), which were shown to exist by crystal structures of mitochondrial and bacterial bc1 complexes in complexes with various bc1-specific inhibitors (4, 8–12). A characteristic feature of the Q-cycle mechanism is the bifurcated ET pathway at the Qₚ site, in which the two electrons of the substrate QH₂ take two separate routes (Fig. 1B): one takes a high potential route going from ISP to cyt c₁ and to substrate cyt c, and the other follows the low potential route traveling to the b₁ and b₄ hemes sequentially, ending in ubiquinone/ubisemiquinone bound at the Qₐ site. Strikingly, structural studies of bovine mitochondrial cyt bc₁ (Bbc₁) in complex with various respiratory inhibitors revealed the inhibitor–dependent conformational switch of ISP-ED. This observation not only suggested a mechanism for the QH₂ oxidation at the Qₚ site but also offered a
means for inhibitor classification (13–15). Thus, inhibitors that immobilize the ISP-ED to the Q_p site are called Pf-type inhibitors, and those that mobilize it are termed Pm-type inhibitors (13).

High resolution structural studies of cyt bc_1 have so far failed to show the binding of substrate QH_2 at the Q_p site. Studies of cyt bc_1 in the presence of inhibitors that are substrate QH_2 homologs such as stigmatellin (4, 11–13, 16, 17), 3-Undecyl-2-hydroxydioxobenzothiazol (13), 5-n-heptyl-6-hydroxy-4,7-dioxobenzothiazole (18), and 2-nonyl-4-hydroxyquinoline N-oxide (13) revealed conformational immobilization or docking of the ISP-ED to the Q_p site and a hydrogen bond (H-bond, 2.8–

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**FIGURE 1.** Structure and mechanism of cyt bc_1 complex. A, ribbon representation of the structure of dimeric cyt bc_1 in complex with famoxadone from photosynthetic bacterium *R. sphaeroides*. The cyt b subunit is shown in green, the cyt c_1 is in blue, and ISP is in yellow. Heme groups are shown as the ball and stick models with carbon in black, nitrogen in blue, and oxygen in red; they are labeled in black as b_H, b_L, and c_1, respectively. The iron-sulfur cluster (FeS) of ISP is shown as spheres with Fe in orange-brown and S in green. The two parallel horizontal lines represent the cytoplasm membrane bilayer. The bound famoxadone (Fam) at the Q_p site is shown as the CPK model with carbon in orange, oxygen in red, and nitrogen in blue. One detergent molecule (β-OG) bound at cyt c_1 is drawn as a brown/red ball and stick model. A bound strontium ion (Sr^{2+}) bound to cyt c_1 is also shown together with its amino acid ligands Asp^{6}, Glu^{14}, and Glu^{129} in the stick models. B, Q-cycle mechanism. The cyt bc_1 consists of two reaction sites: Q_p and Q_N sites. The Q_p site is near the P side, where the two electrons of QH_2 diverge. The first electron goes to the high potential chain via the ISP and cyt c_1, ending in cyt c_2 (cyt c in mitochondrial cyt bc_1), which can be interrupted by stigmatellin. The second electron enters the low potential chain via hemes b_L and b_H, end in substrate Q or radical Q' bound at the Q_N site. The low potential path can be inhibited by myxothiazol at the Q_p site and antimycin at the Q_N site.
Binding of cyt bc₁ by Oxazolidinedione-type Inhibitors

3.4 Å depending on the structure) between the histidine ligand to the 2Fe-2S cluster of ISP (His¹⁴¹, bovine sequence) and oxygen moieties of the inhibitor, leading to the proposal that docking of the ISP-ED to the Q₉ site is likely the consequence of this H-bond (11, 19, 20). Despite evidence that suggests the docking of the ISP-ED to the Q₉ site may be independent of the H-bond (21), systematic study is lacking.

The dire consequence of obstructing cellular respiration has made cyt bc₁ one of the most frequent targets of antibiotics. However, allelopathic inhibition has been observed as a strategy employed by many organisms to gain survival advantage (22). Consequently, toxin-producing organisms are intrinsically resistant to their own toxin. Although many mechanisms may be at work to render this intrinsic resistance, changes in target structures by amino acid substitutions are believed to contribute significantly to the phenomenon, leading to amino acid sequence diversification at the site of inhibition across organisms. Inhibitors of bc₁ have been used not only for the purpose of disease control but also in studies of the mechanisms of bc₁ function, inhibition, and resistance (13, 14, 23–28).

Famoxadone (5-methyl-5-(4-phenoxy-phenyl)-3-phenylamino-2,4-oxazolidinedione) (Fig. 2A) is an oxazolidinedione-type Q₉ site inhibitor (29). It has reportedly displayed various IC₅₀ values for submitochondrial particles from different species (30). However, evidence is mounting that some fungal species are intrinsically more resistant (30, 31), and field applications of famoxadone for fungal disease control have led to rapid development of resistance that renders the inhibitor ineffective (32–34). How inhibitors such as famoxadone react to fluidity in its binding environment has not been fully explored experimentally, let alone understood.

It is known that the ET between the ISP and cyt c₁ requires movement of the ISP-ED, which could be controlled, as we proposed earlier (14), by the bimodal conformation switch of the ISP-ED. Thus, the question regarding the involvement of the H-bond in this control process needs to be addressed. In the present study, we used three oxazolidinedione-type inhibitors to form complexes with either mitochondrial or bacterial cyt bc₁ or both and showed that all three inhibitors can induce docking of the ISP-ED at the Q₉ site without forming a direct H-bond with the ISP, which is consistent with the notion that the ISP conformation switch does not require a direct H-bond. We established the structural basis for the toxophore of oxazolidinedione-type inhibitors and showed the effect of alterations to the toxophore on inhibitor binding to the cyt bc₁, both structurally and biochemically. These observations have strong implications concerning the development of drugs that are designed to target a broad spectrum of pathogens.

Results

Differential Inhibition of Mitochondrial and Bacterial cyt bc₁ Complexes by Famoxadone—Previously, we reported that binding of famoxadone to Btbc₁ induces docking of the ISP to the Q₉ site (21). Unlike other Q₉ site inhibitors such as stigmatellin and 3-Undecyl-2-hydroxydioxobenzothiazol, famoxadone does not make a direct H-bond with the ISP-ED because the shortest distance between His¹⁴¹ of the ISP and the phenylamino moiety of famoxadone is 6.1 Å (Fig. 3A). However, whether the fixation of the ISP-ED by famoxadone binding can be interpreted as part of the Q-cycle mechanism remains unclear. Thus, to understand this phenomenon, one would have to investigate the effect of famoxadone on cyt bc₁ of other organisms, which are evolutionarily remote from mammals or yeast, such as bacteria. Doubts that the capture of the ISP-ED induced by famoxadone binding is indeed part of a conserved mechanism were initially cast by the observation that the experimentally determined IC₅₀ values of 1.4 and 418 nM of isolated cyt bc₁ from photosynthetic bacterium Rhodobacter sphaeroides (Rsb₁) and mitochondrial cyt bc₁ (Btbc₁), respectively, differ by a factor of nearly 300 (Fig. 4A). Further differences were noticed in the shapes of the inhibition curves for Rsb₁ and Btbc₁, suggesting that Rsb₁ might exhibit a different set of binding interactions with famoxadone.

Paradoxically, the difference in IC₅₀ values is not supported by the apparent conservation in the Q₉ site, because all residues in contact with famoxadone in Btbc₁ are conserved, except for two residues: Phe²⁷⁶ and Ala³⁰⁶ (Pro³⁰⁶ and Phe³⁰¹ in Rsb₁, Fig. 5). The closest distances between these two Btbc₁ residues and bound famoxadone are 4.53 and 3.72 Å, respectively (Fig. 3A). Judging by the alignment between Btbc₁ (PDB code 1L0L) and Rsb₁ (PDB code 2FYN) structures, it seems unlikely that these two amino acid substitutions would result in a significant change in the binding of famoxadone, because modeling exercises indicate residue Phe³⁰¹ in Rsb₁ appears to be in a good position to replace the function of Phe²⁷⁶ in Btbc₁.

Distinct Conformation of Famoxadone as the Q₉ Site Occupant of Rsb₁—To answer questions about the binding mode of the inhibitor famoxadone and its local and global effect, we determined the crystal structure of Rsb₁ in complex with famoxadone (Fig. 1A, Table 1). There are four copies of cyt bc₁ in the Rsb₁ crystal per asymmetric unit, each binding one mol-

FIGURE 2. Chemical structures of famoxadone (A), jj144 (B), and fenamidone (C).
Binding of cyt bc₁ by Oxazolidinedione-type Inhibitors
Binding of cyt bc1 by Oxazolidinedione-type Inhibitors

eucleotide and inhibitors. Even though no non-crystallographic symmetry (NCS) restraints were applied to the inhibitor during refinement, the four famoxadone molecules superpose within 0.198 Å root mean square (RMS) deviation, allowing our analysis to focus on just one molecule.

The structure of the Rsbc1-famoxadone complex reveals that famoxadone binds in the same location as in Btbc1 (Fig. 3B). Like Btbc1, the chiral environment of the Qp site of Rsbc1 selects the S(-)-isomer of famoxadone for binding. Most importantly for mechanistic implications, the ISP-ED is found docking in the Qp position, as evidenced by the strong peak of the anomalous difference Fourier density at the location of the 2Fe-2S cluster of ISP. At the same time, residue His152 (ligand to the 2Fe-2S cluster in Rsbc1 and equivalent of His141 of Btbc1) has a distance of 5.7 Å to the phenylamino group of famoxadone. This result confirms that recruitment of the ISP to the Qp site does not depend on the H-bond formation between the ISP-ED and the inhibitor.

As in the Btbc1-famoxadone structure, the inhibitor bound to Rsbc1 is capable of forming an H-bond between atom O6 and the backbone amide nitrogen atom of Glu239 over a distance of 2.69 Å and between atom N1 and the conserved water molecule (W1) (Fig. 3B). Unexpectedly, the terminal phenoxy group adopts a position essentially opposite to that found in Btbc1-famoxadone, which is quantifiable by a change in torsion angle (C12-C11-O14-C15) from −116° to +145° (Fig. 6A). However, this difference in positions of the phenoxy group is consistent with the observation that the residues lining the entrance to the Qp pocket are quite variable between cyt b subunits of different organisms. The adaptation of the terminal phenoxy group to a different environment appears to be based on both exclusion and attraction. The exclusion is caused by the substitution of Ala277 of Rsbc1 (Fig. 5), which would not allow the phenoxy group to be in the same position as in Btbc1. Being flexible to swing around, the aromatic phenoxy group of famoxadone finds stabilization by aromatic attractions from Phe166, Phe337, and Phe144 (Figs. 3B and 5A), which correspond to Ile150, Ala295, and Phe284 in the bovine sequence.

Famoxadone has been noted to prefer aromatic environments (21), which is demonstrated most convincingly by the 13-fold increase in IC50 value for the F129L mutation in yeast (30). Thus, the combined effect of the naturally occurring aromatic residues Phe166 and Phe337 in the cyt b subunit of Rsbc1 (as opposed to the respective residues Leu150 and Ala295 in Btbc1) might cause the stabilization of famoxadone in the present conformation and explain the difference in IC50 values compared with Btbc1 (Fig. 4A).

Mapping the interactions between Qp site residues and bound famoxadone, it became immediately apparent that Rsbc1 uses an overlapping but non-identical set of residues from Btbc1 for famoxadone binding (Fig. 5). Most notably, Rsbc1 uses Phe166, Met336, and Phe144 to engage famoxadone, whereas the equivalent residues Leu150, Leu295, and Ala295 in Btbc1 remain uninvolved with the inhibitor. Conversely, not all interactions observed in Btbc1 are also found in the Rsbc1-famoxadone complex, as seen in residues Phe91, Tyr95, Phe276, Ala277, and Ile298 in Btbc1 (Phe105, Tyr109, Pro300, Phe301, and Ile140 in Rsbc1) (Fig. 5). Thus, these structural observations qualitatively explain the differences in IC50 values for famoxadone binding to Btbc1 and Rsbc1, which could not be predicted based on sequence alignment.

Oxazolidinedione-type Inhibitors Induce Docking of the ISP-ED to the Qp Site without Direct H-bonding to the ISP and Inhibitor-bound Btbc1. Structures Reveal a Conserved Core-binding Motif—Superposition of the cyt b subunits of Rsbc1 and Btbc1 complexed with famoxadone shows a good structural alignment of the oxazolidinedione core of famoxadone and its two directly connected aromatic rings (Fig. 6A), which raises the question as to whether the difference in binding affinity of famoxadone between Rsbc1 and Btbc1 is entirely due to the difference in interaction with the terminal phenoxy group. To evaluate the amount of binding energy contributed by various portions of famoxadone other than the phenoxy group, we measured the binding of two additional oxazolidinedione-type compounds, 5-methyl-5-(4,6-difluorophenyl)-3-phenylamino-2,4-oxazolidinedione (jg144) and 5-methyl-2-(methylsulfanyl)-5-phenyl-3-(phenylamino)-3,5-dihydro-4H-imidazol-4-one (fenamidone), to both Btbc1 and Rsbc1 (Fig. 4, B and C). The chemical structures of jg144 and fenamidone closely resemble that of famoxadone except that they do not have the conformationally flexible terminal phenoxy group (Fig. 2, B and C). Although jg144 replaces the phenoxy group and the hydrogen atom H9 of famoxadone each with fluorine, fenamidone features a substituted imidazole ring in place of the oxazolidinedione ring.

The IC50 values for jg144 inhibition were determined to be 1.1 and 1.2 nM, respectively, for Rsbc1 and Btbc1 (Fig. 4B). Similarly, the IC50 values for fenamidone were 3.5 and 8.3 nM, respectively, for Rsbc1 and Btbc1 (Fig. 4C). More importantly, the profiles of the jg144 and fenamidone inhibition curves are very similar regardless of whether they bind to Rsbc1 or Btbc1, indicating that similar interactions are employed for their binding.

We determined and refined the structure for the complex Btbc1-fenamidone at 2.65 Å resolutions (Table 1) and reanalyzed the structure of Btbc1-jg144 (PDB code 2FYU) at 2.25 Å resolution. In both cases, their respective ISP-EDs are docked at the Qp site, as indicated by the high anomalous peak heights of the 2Fe-2S clusters (Table 2). The arrest of the ISP-ED is a
characteristic trait of $P_r$-type inhibitors (13), and here the observed shortest distances between His$^{141}$ of the ISP and the bound inhibitors are 6.34 and 6.74 Å, respectively, for jg144 and fenamidone (Fig. 3, C and D, Table 2). Thus, no direct H-bond is formed between the ISP and the inhibitors.

The structures of both $Btbc_1$ jg144 and $Btbc_1$ fenamidone showed no significant differences in side chain rotamers from $Btbc_1$ famoxadone (Fig. 6B). This in turn suggests that the terminal phenoxy group of famoxadone is capable of sampling the environment around the entrance of the QP site without perturbing any side chains in the Q$_{b}$ site. The consistency of interactions and the measurements showing little difference in IC$_{50}$ values between $Btbc_1$ and $Rsbc_1$ for jg144 and fenamidone firmly establish the structural basis for the three-ring unit as the toxophore of the oxazolidinone-type inhibitors (29).

**Discussion**

**Mechanistic Implications Concerning the Bifurcated Electron Transfer at the Q$_{p}$ Site**—Quinol oxidation at the Q$_{p}$ site requires strict, consecutive bifurcated ET steps that are subject to a high degree of control, an idea that has received support over the years by a substantial amount of experimental evidence and features a bimodal ISP-ED conformation switch at its core (14, 15). Although most structural data on this switch were first obtained from $Btbc_1$, structural information of several avian mitochondrial cyt $bc_1$ ($Ggbc_1$) complexes recently made public in the Protein Data Bank (PDB codes 3L74 and 3L73) is in perfect agreement with the former. However, the conformational switch of the ISP-ED in bacterial $bc_1$ has been difficult to observe crystallographically. This is because all structures of bacterial cyt $bc_1$ complexes obtained so far were crystallized with bound stigmatellin, and it has been difficult to crystallize them in a different $P_r$-type inhibitor such as famoxadone. Conceivably, crystallizing bacterial cyt $bc_1$ bound with a $P_m$-type inhibitor would be even more difficult. Prior to this work, complexes of bacterial $bc_1$ were crystallized only in the presence of the $P_r$-type inhibitor stigmatellin with the ISP-ED immobilized at the Q$_{p}$ position (4, 12, 17). In this work, we show for the first time that another $P_r$-type inhibitor, namely famoxadone, is able to fix the conformation of ISP-ED without providing a direct H-bond to the ISP (13, 21).

The question becomes whether the presence or absence of the H-bond can support the required bimodal conformation switch of the ISP-ED.
The bifurcated ET at the Qₚ site begins with the binding of substrate QH₂, which also triggers the docking of ISP-ED. An H-bond forms between a hydroxyl group of QH₂ and a histidine of the ISP-ED. After deprotonation, the QH₂ transfers its first electron to the 2Fe-2S cluster of the ISP, which is an energetically favorable process. The second electron of QH₂, however, has to travel to the Qₐ site via the hemes, b₇ and b₅, eventually reducing a ubiquinone (Q) or ubisemiquinone (Q₁) molecule (Fig. 1B). It is important to emphasize (i) that to avoid short circuit reactions, the 2Fe-2S cluster cannot be allowed to leave the Qₚ site and return oxidized until the product has been completely removed and/or replaced by QH₂ and (ii) that although the ISP-ED remains at the Qₚ site, the H-bond should always exist between the reduced ISP and the product ubiquinone (Q) after the completion of the two-electron transfer reaction (Fig. 7). In other words, the H-bond between the substrate and the ISP cannot control the conformation switch of the ISP-ED.

### TABLE 1

Statistics on the quality of diffraction data sets of cyt bc₁, crystals and structural models

|       | Mtbₐ, fen | Rsbc₁, fam | Rsbc₁, stg/ant |
|-------|-----------|------------|---------------|
| Diffraction data |                     |             |               |
| Space group | I₄₁₂₂ | P₁ | P₁ |
| Cell parameters | 154.0, 154.0, 592.7 | 120.8, 128.3, 128.3 | 118.9, 126.9, 127.9 |
| a, b, c (Å) | 90, 90, 90 | 63.9, 88.6, 63.4 | 64.7, 87.7, 61.8 |
| Resolution (Å) (outer shell) | 50–2.5 (2.59–2.50) | 31.9–2.95 (3.06–2.95) | 37.8–3.00 (3.10–3.00) |
| Rmerge | 0.091 (0.442) | 0.102 (0.549) | 0.175 (0.730) |
| Completeness (%) | 98.5 (97.8) | 96.5 (98.1) | 93.6 (81.1) |
| <T> × aₚ | 13.8 (2.0) | 5.6 (1.2) | 5.0 (1.4) |
| No. unique obs. | 120,848 (11,829) | 122,242 (11,289) | 110,064 (9,558) |
| Refinement |                     |             |               |
| Resolution (Å) | 34.3–2.65 (2.75–2.65) | 29.7–2.97 (3.04–2.97) | 37.7–3.00 (3.07–3.00) |
| Rfree | 0.269 (0.343) | 0.258 (0.337) | 0.243 (0.374) |
| Rwork | 0.228 (0.338) | 0.215 (0.362) | 0.211 (0.329) |
| No. atoms | 16,913 | 27,918 | 27,938 |
| No. residues | 2,079 | 3,484 | 3,476 |
| No. ligand atoms | 548 | 962 | 1022 |
| No. solvent molecules | 50 | 4 | 0 |
| RMS deviations |                     |             |               |
| Bond length | 0.005 | 0.014 | 0.036 |
| Bond angles | 0.75 | 1.33 | 1.13 |
| Ramachandran plot | Favored (%) | 96 | 97 | 95 |
| Outliers (%) | 0.34 | 0.2 | 0.12 |

*a The statistics for the highest resolution shell are shown in parentheses.
In this work, we present evidence from the famoxadone-bound Rsbc1 structure and two Btbc1 structures with bound oxazolidinedione-type inhibitors, jg144 and fenamidone, showing that docking of the ISP-ED to the Qp site, both in mitochondrial and bacterial cyt bc1, does not require a direct H-bond between the ISP and the inhibitor. This lends strong support for the hypothesis that electron bifurcation at the Qp site is achieved through a controlled bimodal conformational change of the ISP-ED (14, 15).

Crystal Contacts Do Not Significantly Influence the Conformation of ISP-ED—The head domain of ISP in Rsbc1 is involved in crystal-packing contacts, whereas that of Btbc1 is not. This observation provides a good opportunity to qualitatively assess how much influence crystal contacts have on the conformation of the intrinsically moving domain. For the purpose of an unbiased comparison, we also determined the structure of the doubly inhibited (stigmatellin and antimycin) ternary complex of Rsbc1 (Table 1). Antimycin is a QN site inhibitor and is not.
known to influence ISP-ED conformation; thus, its presence is of no consequence for this analysis. Stigmatellin inhibited Rsbc$_1$ has been structurally characterized before (4). However, the newly determined structure that we are using as a reference was obtained from the same batch of protein under the same conditions, and importantly, the crystals features the same space group $P1$, the same number of molecules per asymmetric unit (two dimers) and has the same cell dimensions and crystal packing. To quantify how much the ISP-ED moves in response to a change in the Qp site occupant, we superposed only the C$_{N}$subunits only between the structures with bound antimycin and famoxadone. The red vectors in the ISP trace (yellow trace) of the stigmatellin-inhibited Rsbc$_1$ represent the connection to the C$_a$ atoms of the famoxadone-inhibited complex. The blue vectors represent the equivalent repositioning of the C$_a$ atoms of the ISP-ED in Btbc$_1$. This vector diagram illustrates qualitatively that the amount of change (vector length) is approximately the same in both sets. However, the direction in which the ISP-ED is displaced is indicated. The RMS deviation of stigmatellin vs. famoxadone$^d$ (vector length) is approximately the same in both sets. How- ever, the direction in which the ISP-ED is displaced.

**TABLE 2**
Normalized anomalous peak heights for iron atoms in crystals of bovine mitochondrial bc$_1$ in complex with oxazolidinedione inhibitors

| Bound inhibitor       | cyt bc$_1$ complex Distance to ISP $b_1$ $b_2$ $c_1$ ISP Inhibitor class |
|-----------------------|-------------------------------|-----------------|---------|---------|---------------------|---------|
| Stigmatellin$^a$      | Btbc$_1$                      | 3.03            | 1.0     | 0.99    | 0.84               | 2.20    |
| Famoxadone$^a$        | Btbc$_1$                      | 5.07            | 1.0     | 0.85    | 0.64               | 1.02    |
| Fenamidone            | Btbc$_1$                      | 3.24            | 1.0     | 0.91    | 0.78               | 1.30    |
| JG144                 | Btbc$_1$                      | 6.34            | 1.0     | 0.80    | 0.78               | 1.30    |
| Azoxystrobin$^c$      | Btbc$_1$                      | 6.74            | 1.0     | 0.91    | 0.78               | 1.30    |
| Stigmatellin$^a$      | Rsbc$_1$                      | 2.14            | 1.0     | 0.85    | 0.95               | 0.36    |
| Famoxadone$^d$        | Rsbc$_1$                      | 5.73            | 1.0     | 0.91    | 0.95               | 0.36    |

$^a$ The peaks are normalized against anomalous signals of their respective $b_1$ heme iron.
$^b$ Anomalous signals were calculated using diffraction data in the range of 2.0–5.0 Å.
$^c$ These numbers were taken from Esser et al. (13) for comparison.
$^d$ These two inhibitors fix ISP-ED to the Q$_p$ site allowing a true state of equilibration in solution.

The angular displacement of the ISP-EDs between Btbc$_1$ and Rsbc$_1$ when bound with different inhibitors can also be visualized using vectors (Fig. 8A), which is calculated based on superposition of cyt $b$ subunits only between the structures with bound antimycin and famoxadone. The red vectors in the ISP trace (yellow trace) of the stigmatellin-inhibited Rsbc$_1$ represent the connection to the C$_a$ atoms of the famoxadone-inhibited complex. The blue vectors represent the equivalent repositioning of the C$_a$ atoms of the ISP-ED in Btbc$_1$. This vector diagram illustrates qualitatively that the amount of change (vector length) is approximately the same in both sets. However, the direction in which the ISP-ED is displaced is indicated. The RMS deviation of stigmatellin vs. famoxadone$^d$ (vector length) is approximately the same in both sets. However, the direction in which the ISP-ED is displaced is indicated.

**TABLE 3**
RMS deviation values of C$_{N}$ traces between equivalent subunits in stigmatellin versus famoxadone inhibited cyt bc$_1$ after superimposing cyt $b$ subunit only

| Structures                | RMS deviation of stigmatellin vs. famoxadone |
|---------------------------|-----------------------------------------------|
|                           | cyt $b$ | ISP-ED          | cyt $c_1$          |
| Rsbc$_1$ (residue range, no. residue) | 0.40 (3–430, 428) | 2.55 (49–187, 139) | 0.60 (1–222, 222) |
| Btbc$_1$ (residue range, no. residue) | 0.40 (10–378, 369) | 2.86 (73–196, 124) | 0.93 (1–197, 179) |
Binding of cyt bc\(_1\) by Oxazolidinedione-type Inhibitors
Binding of cyt bc1 by Oxazolidinedione-type Inhibitors

versus Btbc1, is characterized by an angle of 58° (represented by half-transparent triangles). It is conceivable that, as a consequence of packing forces in Rsbc1 crystals, the ISP-ED undergoes an angular adjustment but importantly maintains the proper distance from the Qp site of cyt b.

Implications Concerning the Design and Use of Broad Spectrum Antifungal Agents—Despite considerable pressure that must exist to maintain the intricate function of cyt b and its active sites, several residues, individually or in groups, may have changed in the course of evolution. These changes are seen as the root cause of intrinsic drug resistance by target site mutations. By determining the structure of Rsbc1 in complex with famoxadone and comparing it with that of Btbc1, we uncovered the structural basis for the effects of sequence polymorphisms on the binding of inhibitors to cyt bc1 of different organisms, which were manifested by varied efficacies of inhibitors against cyt bc1 complexes. This structural observation is consistent with measurements of IC50 values, indicating that famoxadone inhibits Rsbc1 more strongly than Btbc1 (Fig. 4A); it agrees also with the binding energy of −12.0 kcal/mol and −12.8 kcal/mol, respectively, estimated by molecular docking experiments for mitochondrial and bacterial cyt bc1 complexes. The conformation of famoxadone in the binding pocket shows sensitivity to the environment and is characterized by a tendency to optimize Ar-Ar interactions (21).

Because of the absence of an apo Rsbc1 structure, the only comparisons that can be made are between complexes of Rsbc1 with famoxadone and stigmatellin (Fig. 8B) and the equivalent pair of Btbc1 complexes (Fig. 8C). The comparison made it immediately clear that the accommodation of famoxadone in the Qp pocket requires adjustments of torsion angles in both the inhibitors and cyt b residues. Direct steric interactions force Phe299 (Phe274 in Btbc1) to adopt two distinct conformations depending on the binding of either stigmatellin or famoxadone in both Rsbc1 and Btbc1. Similarly pronounced are changes in the positions of Glu295 (Glu271 in Btbc1) in response to the inhibitors. In the Rsbc1-famoxadone structure, Glu295 returns to the solvent channel or to the “native” position as observed in Btbc1 structures because of the loss of a hydrogen bond to the inhibitor (8).

From the Rsbc1 structure, Phe166, Met336, and Phe337 (Leu150, Leu294, and Ala295 in Btbc1) appear to play a major role in repositioning the terminal phenoxy moiety of famoxadone. Although this is convincing based on qualitative energy considerations, the situation seems subtler in the recently published Ggbc1 structure with famoxadone (35), showing the same moiety in an orientation nearly identical to the one found in Rsbc1 but in contrast to that in Btbc1. Despite the higher sequence identity between Ggbc1 and Btbc1 (Fig. 5), residue Phe151 in the avian Gg cyt b is also a phenylalanine, as in Rsbc1 (Btbc1, Phe166). However, residue Phe337 of Rs cyt b coincides with the non-aromatic Ala296 in Gg cyt b, implying that a single change in the amino acid composition (Leu294Phe) at the entrance to the Qp pocket is sufficient to stabilize an alternate conformation of famoxadone to the one that was first found in Btbc1. This observation underscores the importance of Ar-Ar interaction in inhibitor binding.

The fact that famoxadone is able to adapt to the different microenvironments of the target sites of a wide range of organisms suggests two important principles for designs of broad spectrum inhibitors: one is the flexibility to adopt different conformations as needed, and the other is the ability to fully engage in Ar-Ar interactions. A single pair of Ar-Ar interactions could provide as much as 1.3 kcal/mol of free energy (36), which can be exploited either for the enhancement of specificity or for the evasion of resistance and is also consistent with previous observations that the phenoxyphenyl group of famoxadone is most tolerant to variations (29).

Experimental Procedures

Expression of Δ-sub IV Rsbc1 —The purification procedure for Rsbc1, published earlier (37), was used with minor changes to obtain protein for crystallization. Briefly, chromatophore membranes were prepared from cells harboring the plasmid (pRKDJfbcFBc1) coding for Δ-sub IV wild type Rsbc1 protein, by disrupting cells with a French press followed by differential centrifugations. To purify His6-tagged Rsbc1, the chromatophore suspensions were adjusted to a cyt b concentration of 25 μM with 50 mM Tris-Cl (pH 8.0 at 4 °C), containing 20% glycerol, 1 mM MgSO4, and 1 mM PMSF. Dodecyl-d-maltopyranoside (DDM) solution (10% w/v) was added dropwise to a final detergent to protein ratio of 0.57 (mg/mg). After centrifugation, the supernatant was loaded on a nickel-nitrilotriacetic acid column, which was washed with 6 column volumes of buffer A (50 mM Tris-HCl, pH 8.0, at 4 °C, 200 mM NaCl, 0.01% DDM), 6 column volumes of buffer A in the presence of 5 mM histidine, and 4 column volumes of buffer B (50 mM Tris-HCl, pH 8.0, at 4 °C, 200 mM NaCl, 0.5% β-OG) in the presence of 5 mM histidine. The desired protein fractions were eluted with buffer B containing 200 mM histidine and concentrated with Centriprep-50 to a final concentration of 300 μM.

Crystallization of Btbc1 in Complex with Inhibitors—The final concentration of purified Btbc1 complex was adjusted to 20 mg/ml in a solution containing 50 mM MOPS buffer at pH 7.2, 20 mM ammonium acetate, 20% (w/v) glycerol, and 0.16% sucrose monopurate. This solution was further incubated with a 5× molar excess amount of the desired inhibitor and set up for crystallization as described in previous publications (8, 21). Crystals were treated with a cryoprotectant in 5% increments until the final glycerol concentration reached 42% and then cryo-cooled in liquid propane.

Figure 8. Stereoscopic pairs showing the changes in subunits of cyt bc1 upon binding of different inhibitors. A, effects of inhibitor binding on the conformation of ISP-ED. The tracing of the Cα atoms of the ISP-ED from Rsbc1, in complex with stigmatellin is drawn in yellow. A red vector marks the distance to the same Cα atom of Rsbc1, in complex with famoxadone. The blue vectors represent changes in Cα positions between famoxadone- and stigmatellin-bound Btbc1. The dotted lines connect residues that have no equivalent in Btbc1. B, superposition of the cyt b subunits of Rsbc1-famoxadone and Rsbc1-stigmatellin. The famoxadone bound structure is shown with carbon atoms in yellow, whereas that with bound stigmatellin is given with carbon atoms in gray. In both structures, nitrogen atoms are colored blue, and oxygen is red. Residues that undergo large conformational changes with different inhibitors bound are labeled. C, superposition of the cyt b subunits of Btbc1-famoxadone and Btbc1-stigmatellin.
Binding of cyt bc₁ by Oxazolidinedione-type Inhibitors

Crystallization of Famoxadone-inhibited Rsbc₁—A solution of Δ-sub IV Rsbc₁ (57 mg/ml) in a buffer containing 0.5% β-OG was treated overnight with a 3-fold molar excess of famoxadone (Chem. Service Inc.). The solution was diluted 4-fold with buffer consisting of 50 mM Tris-HCl, pH 8.0, 0.3% β-OG, 200 mM histidine, 150 mM NaCl, 10 mM sodium ascorbate, and 10% glycerol. The resulting solution of 14 mg/ml Rsbc₁ in 0.35% β-OG was augmented with sucrose monophosphate (0.06%, ~0.5 critical micelle concentration) and 10 mM strontium nitrate. PEG 400, added to a final concentration of 7%, served as the precipitant. The solution was allowed to stand overnight at 4 °C. Any precipitate was centrifuged off, and 5 µl of the supernatant was used in sitting drop vapor diffusion crystallization trays over 1 ml of 100 mM Tris- HCl, pH 8.0, 20% glycerol, 600 mM NaCl, 26% PEG 400, and 5 mM NaN₃. After several weeks, small red crystals appeared which froze cleanly in liquid propane without requiring additional cryoprotectants.

Diffraction Data Collection, Structure Determination, and Refinement—Crystals of the tetragonal Btbc₁-fenamidone and the triclinic famoxadone-inhibited Rsbc₁ were stable when cryo-cooled to 100 K, permitting several hours of data collection at the SER-CAT Beamline (ID22) of the Advanced Photon Source, Argonne National Lab. Raw diffraction frames were processed with the program package HKL2000 (38). A diffraction data set for the Btbc₁ crystal was phased with coordinates of the 11-subunit apo structure of Btbc₁ (PDB code 1NTM) (23). The program REFMAC (39) was employed for initial rigid body refinement followed by iterative maximum likelihood and TLS (Translation, Libration, and Screw tensor) refinement. In between REFMAC runs, sigma A (40) weighted 2Fo – Fc and Fc – Fo Fourier maps were calculated and used to identify and build bound ligand and solvent molecules manually with the program Coot (41). The data set for the Rsbc₁ was phased with molecular replacement using an edited version of the Rsbc₁ dimer (PDB code 2QYI) in MolRep (CCP4) (42), producing a solution with two dimers/unit cell. Care was taken in the subsequent refinement to allow the head domain of the ISP to adjust to a position possibly different from that in stigmatellin-inhibited complexes. Rigid body refinement, simulated annealing, positional, and atomic displacement (ADP, TLS) refinement were carried out with phenix.refine 1.8–1512 (43). 4-fold NCS restraints were applied throughout the refinement. However, no NCS restraints were imposed on the four famoxadone molecules during refinement, yet they are superposed within 0.2 Å RMS deviation (average from mean structure), allowing our analysis to focus on just one molecule. Manual adjustments of the coordinates were carried out in O (44) and Coot 0.6. As the last step, famoxadone was fitted into the difference density. Final refinement statistics are given in Table 2.

Measurement of IC₅₀ Values for bc₁ Inhibitors—The activities of Mtbnc₁ and Rsbc₁ were assayed following the reduction of substrate cyt c. The bc₁ preparation was diluted to a final concentration of 0.1 and 1 µM, respectively, for Btbc₁ and Rsbc₁, in a buffer containing 50 mM Tris- HCl, pH 8.0, 0.01% β- DDM, and 200 mM NaCl. To a 2-ml assay mixture containing 100 mM phosphate buffer, pH 7.4, 0.3 mM EDTA, and 80 µM cyt c, QoC₁₀BrH₂ was added to a final concentration of 25 µM, and the solution was split evenly into two cuvettes. To one cuvette, 5 µl of diluted bc₁ solution was added, and we immediately began recording the cyt c reduction at 550-nm wavelengths for 100 s in a two-beam Shimadzu UV-2250 PC spectrophotometer at room temperature. The amount of cyt c reduced at a given period of time was calculated using a millimolar extinction coefficient of 18.5 mM⁻¹ cm⁻¹. To measure the effect of bc₁ inhibitors, bc₁ was preincubated with various concentrations of inhibitors for 5 min on ice prior to the measurement of bc₁ activity. The IC₅₀ value for each inhibitor was calculated by a least squares procedure fitting the equation (Y = Aₘ₉ + (Aₘ₉ − A₅₉)/(1 + 10⁶[-logIC₅₀])) implemented in a commercial package Prism, where A₅₉ and A₉₉ are maximal and minimal activities, respectively.

Author Contributions—D. X. conceived and coordinated this study and wrote most of this paper. L. E. wrote significant parts of this paper, prepared Figs. 1 and 8, and crystallized and solved the structures of RS-fam and RS-stg/an. F. Z. expressed and purified R. sphaeroides bc₁ needed in this study and performed the assays in Fig. 4. Y. Z., Y. X., and Z. Q. contributed to the crystallization and structure determination of Btbc₁-fenamidone complex. W.-K. T. advised on the interpretation of the kinetics of the binding studies. C.-A. Y. contributed to the discussion and interpretation of the effect of inhibitors in bc₁.

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