Ilicicolin H is an antibiotic isolated from the “imperfect” fungus Cylindrocladium iliciola strain MFC-870. Ilicicolin inhibits mitochondrial respiration by inhibiting the cytochrome bc\(_1\) complex. In order to identify the site of ilicicolin action within the bc\(_1\) complex we have characterized the effects of ilicicolin on the cytochrome bc\(_1\) complex of Saccharomyces cerevisiae. Ilicicolin inhibits ubiquinol-cytochrome c reductase activity of the yeast bc\(_1\) complex with an IC\(_{50}\) of 3–5 \(\mu\)M, while 200–250 \(\mu\)M ilicicolin was required to obtain comparable inhibition of the bovine bc\(_1\) complex. Ilicicolin blocks oxidation-reduction of cytochrome b through center N of the bc\(_1\) complex and promotes oxidant-induced reduction of cytochrome b but has no effect on oxidation of ubiquinol through center P. These results indicate that ilicicolin binds to the Qn site of the bc\(_1\) complex. Ilicicolin induces a blue shift in the absorption spectrum of ferro-cytochrome b, and titration of the spectral shift indicates binding of one inhibitor molecule per Qn site. The effects of ilicicolin on electron transfer reactions in the bc\(_1\) complex are similar to those of antimycin, another inhibitor that binds to the Qn site of the bc\(_1\) complex. However, because the two inhibitors have different effects on the absorption spectrum of cytochrome b, they differ in their mode of binding to the Qn site.

Electron transfer through the cytochrome bc\(_1\) complex occurs by the protonotive Q cycle mechanism in which ubiquinol is oxidized at one center, referred to as center P, and ubiquinone is re-reduced at a second center, referred to as center N (1). Numerous inhibitors that act specifically at center P or center N within the bc\(_1\) complex have been discovered and characterized (2). The so-called Qp inhibitors block oxidation of ubiquinol at center P and prevent reduction of the high potential redox centers of the bc\(_1\) complex, cytochrome c\(_1\), and the Rieske protein. The Qp inhibitors include stigmatellin, hydroxyquinones, and methoxyacrylates such as myxothiazol and methoxyacrylate (MOA)-stilbene. These inhibitors are generally thought to mimic intermediate states during ubiquinol oxidation. The bc\(_1\) complexes from chicken, bovine, and yeast mitochondria have been crystallized with stigmatellin bound (3–5), and the yeast enzyme has also been crystallized with a hydroxyquinone anion bound (6). Consequently, a significant amount of detailed structural information is available regarding the binding of these Qp inhibitors.

The Qn inhibitors block reduction of ubiquinone by cytochrome b\(_{1}\) at center N and also block reduction of cytochrome b by ubiquinol that can occur by reversal of this reaction. At present there are two antibiotics that inhibit at the Qn site of the bc\(_1\) complex. Antimycin is a natural product of various species of Streptomyces and is one of the most extensively studied inhibitors of the bc\(_1\) complex (7). Antimycin binds with high affinity (K\(_{D}\) = 3.2 \(\times\) 10\(^{-10}\)) close to heme b\(_1\) of cytochrome b\(_1\) and causes a red shift in the \(\alpha\) band of the optical bc\(_1\) spectrum of the ferro-cytochrome b\(_1\). The crystal structure of the chicken bc\(_1\) complex with antimycin bound shows that the antimycin binding site is near the high potential (b\(_{1}\)) heme of cytochrome b\(_1\) and surrounded by adjacent residues from helices A, D, and E (3). The aromatic ring of the inhibitor is in close proximity to the heme, as expected from the quenching of the inhibitor fluorescence when bound to the bc\(_1\) complex (7, 8).

Funiculosin, an antibiotic isolated from Penicillium funiculosum Thom, also inhibits at the Qn site. However, funiculosin is no longer available and thus little is known about the structural details of its binding, although it is generally assumed to bind in a manner similar to antimycin (2).

Ilicicolin H is one of several ilicicolin antibiotics isolated from the “imperfect” fungus Cylindrocladium iliciola strain MFC-870 (9). Ilicicolin was cytotoxic against HeLa cells but had only limited antibacterial activity. Subsequent studies showed that it was also toxic to various yeast, and characterization of ilicicolin H effects on mitochondria suggested that the inhibitor acts at the level of the cytochrome bc\(_1\) complex (1). In the experiments reported below we characterize the effects of ilicicolin H on the purified yeast bc\(_1\) complex. We show that ilicicolin H is a Qn site inhibitor and compare its effects to those of antimycin, another inhibitor of the bc\(_1\) complex that also acts at the Qn site of the enzyme.

**Experimental Procedures**

Materials—Dodecylmaltoside was obtained from Roche Applied Science. DEAE-Biogel and ammonium persulfate were obtained from Bio-Rad Laboratories. Antimycin, diisopropylfluorophosphate (DFP), phenylmethylsulfonyl fluoride, menadione, and dithionite were purchased from Sigma Chemical Co. Stigmatellin was purchased from Fluka Biochemicals. Ilicicolin H was obtained from the Merck sample repository.

Purification of bc\(_1\) Complexes—Yeast cytochrome bc\(_1\) complex was isolated from yeast mitochondrial membranes as described previously (10, 11). The bovine bc\(_1\) complex was prepared from beef heart mitochondrial membranes using the same protocol for the yeast bc\(_1\) complex, except that the membranes were extracted with 1.2 g of dodecyl maltoside per gram of protein, compared with 0.8 g of dodecyl maltoside per gram of protein used to extract the yeast enzyme.

**Determination of Inhibitor Concentrations**—Inhibitors were diluted in ethanol and the concentrations were determined from optical spectra obtained in an Aminco DW2aTM UV/Visible spectrophotometer with the OLIS (On-Line Instrument Systems Inc. Bogart, GA) DW2 conversion and OLIS Software. The spectrum, after subtracting the ethanol background, was used to calculate the absorbance at the desired wavelength.

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† To whom correspondence should be addressed: Dept. of Biochemistry, Dartmouth Medical School, 7200 Vail, Hanover, NH 03755. Tel.: 603-650-1621; E-mail: Trumpower@Dartmouth.edu.

‡ J. Onishi, submitted for publication.
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ground, was recorded from 230 to 400 nm. To accurately determine the concentration for each inhibitor, the absorbance was measured at concentrations that yielded 0.1–0.15 absorbance units after diluting the sodium dithionite-reduced minus ascorbate-reduced enzyme using an extinction coefficient of 25 mM⁻¹ cm⁻¹ at 450 nm; for antimycin, 4.8 mM⁻¹ cm⁻¹ at 320 nm; for stigmatellin, 65.5 mM⁻¹ cm⁻¹ at 267 nm; for illicicolin H, 23.2 mM⁻¹ cm⁻¹ at 248 nm and 5.3 mM⁻¹ cm⁻¹ at 349 nm (2, 9). All of the inhibitor dilutions were prepared daily, and the concentrations were determined before use.

Determination of bc₁ Complex Concentrations—For each inhibitor titration, the bc₁ complex was prediluted to ~3 μM in assay buffer minus cytochrome c, and the concentration determined by difference spectra recorded in the Aminco DW2A™ spectrophotometer. The cytochrome c concentration was determined from the difference spectrum of the ascorbate-reduced versus ferricyanide-oxidized enzyme, using an extinction coefficient of 17.5 mM⁻¹ cm⁻¹ at 553–539 nm (12). Cytochrome b concentration was determined from the difference spectrum of the sodium dithionite-reduced minus ascorbate-reduced enzyme using an extinction coefficient of 25 mM⁻¹ cm⁻¹ at 563–578 nm (13). The activity of this stock solution of enzyme was stable for a week at 4°C.

Ubiquinol-Cytochrome c Reductase Assays with 2.5 nM bc₁ Complex—Ubiquinol-cytochrome c reductase activities of the purified bc₁ complexes were assayed at room temperature in assay buffer containing 50 mM potassium phosphate, pH 7.0, 0.25% w/v Tween-20, 1 mM sodium azide, 0.2 mM EDTA, 0.01% Tween-20. After diluting in assay buffer, the enzyme was diluted daily to 10% activity for the inhibitor titration. This was taken as 100% activity for the inhibitor titration.

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Ilicicolin Inhibition of the Cytochrome bc₁ Complex

Fig. 1. Structures of antimycin, funiculosin, and ilicicolin H. Antimycin is a 3-FASA linked via an amide bond to an alkyl- and acyl-substituted dilacon ring. Funiculosin is an N-methyl-substituted 4-hydroxy-2-pyridone ring with a hydrophobic side chain in position 1 and a tetrahydrocyclodopentane ring in position 3. Ilicicolin H consists of a 5-(4-hydroxyphenyl)-α-pyridone with a bicyclic decalin system.

The titration curves in Fig. 2b show the inhibition of the ubiquinol-cytochrome c oxidoreductase assay using 50 nM enzyme concentration. Under these conditions one equivalent of antimycin per enzyme inhibits ~99% of the catalytic activity, and the same result is obtained with ilicicolin H. From these results one can estimate that the $K_i$ of ilicicolin H is between $2.5 \times 10^{-9}$ M and $5 \times 10^{-8}$ M for the isolated yeast bc₁ complex, while that for antimycin is <2.5 $\times 10^{-9}$ M.

There is a significant hysteresis in the titration curves for both inhibitors in the assay with the higher enzyme concentration. In addition, at low ratios of inhibitor per enzyme both inhibitors cause the ubiquinol-cytochrome c reductase activity to increase by 10–20% above the activity in the absence of inhibitor. This rate enhancement is consistently seen in cytochrome c reductase assays when the enzyme concentration is high, as in Fig. 2b, and also occurs but is less evident when the enzyme concentration is low. Although the hysteresis can be attributed to electron transfer between the two monomers in the dimeric bc₁ complex, the rate enhancement is more difficult to explain. The rate enhancement is not due to formation of superoxide anion, because it could not be eliminated by including superoxide dismutase in the assay (results not shown). An explanation for the rate enhancement is discussed below.

We also compared the inhibition of the bovine bc₁ complex by ilicicolin H and antimycin. In the standard catalytic assay with 2.5 nM bc₁ complex antimycin inhibits the bovine bc₁ complex with stoichiometric affinity as shown in Fig. 3a. With ilicicolin, however, the IC₅₀ for the bovine enzyme is ~200 nM (Fig. 3b). From this comparison it is clear that ilicicolin H is a much more potent inhibitor of the yeast bc₁ complex than of the bovine enzyme.

Effect of Ilicicolin H on the Presteady State Reduction of Cytochrome b—In order to determine whether ilicicolin H is a Qn or Qp site inhibitor we characterized the effect of the inhibitor on the presteady state reduction of cytochromes b and c₁. When the bc₁ complex is reduced by menadiol in the absence of inhibitor, cytochrome b is reduced in a triphasic manner, consisting of a relatively rapid partial reduction, partial reoxidation, and re-reduction (11). With 50 μM menadiol the triphasic reduction of cytochrome b is faster, so that the reoxidation phase appears as a lag between the two reduction phases, as shown in Fig. 4a.

Antimycin, a Qn site inhibitor, blocks reoxidation of cytochrome b and the re-reduction of b through center N and the triphasic reduction of cytochrome b becomes biphasic, as shown in Fig. 4b. The effects of antimycin on the triphasic reduction have been described in detail elsewhere (11). Antimycin also slows the rate of cytochrome c₁ reduction through center P and the rate matches the slow phase of cytochrome b reduction. As discussed elsewhere, the linkage between the rate of c₁ reduc-
tion and the slow phase of b reduction is indicative of a concerted mechanism of ubiquinol oxidation (22).

Ilicicolin H has the same effects on the presteady state reduction of the bc1 complex as antimycin, changing the triphasic reduction of cytochrome b into a biphasic reaction, and also slowing down cytochrome c1 reduction (Fig. 4). This suggests that ilicicolin H is also a Qn site inhibitor.

Effect of Ilicicolin H on the Presteady State Reduction of the bc1 Complex through Center N—To confirm that ilicicolin H inhibits at the Qn site and to determine the stoichiometry of binding, we titrated ilicicolin into the bc1 complex in the presence of stigmatellin to block reduction through center P and then followed reduction of cytochrome b through center N. As a control we performed the same experiment with antimycin. As can be seen in Fig. 5 both antimycin and ilicicolin H inhibit the reduction of cytochrome b through center N with a stoichiometry of 0.8–1.0 equivalent per bc1 complex. This shows conclusively that ilicicolin H binds to the Qn site of the bc1 complex.

The effects of ilicicolin in the presteady state reduction assays show that ilicicolin H acts at the Qn site of the enzyme. To ensure that ilicicolin did not also affect the Qp site we added 2 equivalents of ilicicolin H per bc1 complex in the presence of antimycin and followed the reduction of the enzyme by menadion. When added together, ilicicolin and antimycin had the same effects on the presteady state reduction of the enzyme as when either inhibitor was added alone. Cytochrome b reduction was biphasic, and cytochrome c1 reduction occurred at a slower rate than when no inhibitor is present (results not shown). If ilicicolin inhibited reduction of the enzyme through the Qp site in addition to its effects at the Qn site, there would have been no reduction of either cytochrome b or cytochrome c1. From this result we conclude that ilicicolin, like antimycin, is specific for the Qn site.

Oxidant-induced Reduction of Cytochrome b in the Presence of Ilicicolin H—If the cytochrome bc1 complex is partially reduced and ubiquinol is present, oxidation of cytochrome c1 causes reduction of cytochrome b through center P (23). In the absence of inhibitors this oxidant-induced reduction of cytochrome b is transient, as the b is rapidly re-oxidized through center N. Antimycin blocks the reoxidation of the b through center N, trapping electrons in the b hemes, and thus promotes the oxidant-induced reduction of cytochrome b. The traces in Fig. 6a show the oxidant-induced reduction of cytochrome b in the presence of antimycin as cytochrome c1 is oxidized by the addition of cytochrome c plus cytochrome c oxidase.

Ilicicolin H also promotes oxidant-induced reduction of cytochrome b coincident with cytochrome c1 oxidation, as shown in Fig. 6b. We also observed a similar oxidant-induced reduction
of cytochrome $b$ when ilicicolin was added to respiring mitochondrial membranes (results not shown). This result further confirms that ilicicolin acts at the Qn site, like antimycin, and not at the Qp site.

**Effects of Ilicicolin H on the Absorption Spectrum of Cytochrome $b$**—When antimycin binds to the $bc_1$ complex, it causes a shift to longer wavelengths in the visible absorption spectrum of cytochrome $b$ in the reduced enzyme. This red shift is evident in the $a$ and $y$ peaks of cytochrome $b$ (24). The resulting difference spectrum shows a peak at 565 nm and a trough at 559 nm as shown in Fig 7a. By titrating antimycin and measuring this spectral shift it is possible to demonstrate that the inhibitor binds with a stoichiometry of one per enzyme.

Ilicicolin H causes a shift toward shorter wavelengths in the spectrum of reduced cytochrome $b$. As shown in Fig. 7b this blue shift results in a difference spectrum with a peak at 559 nm and a trough at 563 nm. Titration of the spectral shift demonstrates that ilicicolin also binds with a stoichiometry of one per $bc_1$ complex.

The effects of ilicicolin H on the presteady state reduction of
cytochrome \( b \) suggest that ilicicolin \( H \) binds at the Qn site, as does antimycin. However, the two inhibitors apparently differ in their mode of binding to the Qn site, since they have different effects on the absorption spectrum of cytochrome \( b \). From the concentrations of inhibitors required to inhibit the \( bc_1 \) complex (Fig. 2), we estimated that antimycin binds to the Qn site of the yeast enzyme \( \sim 10 \times \) more tightly than ilicicolin. If the binding sites for the two inhibitors are identical or overlapping, it should be possible to displace ilicicolin with antimycin.

As shown in Fig. 8, \( a \) and \( b \), when antimycin is added to the \( bc_1 \) complex after ilicicolin the blue shift that is induced by ilicicolin is eliminated and replaced by a red shift, indicative of antimycin binding. This result suggests that the more tightly binding antimycin has displaced ilicicolin from the Qn binding site. As a control we performed a similar experiment with stigmatellin, which binds at the Qp site of the \( bc_1 \) complex. Stigmatellin did not eliminate the blue shift induced by ilicicolin (Fig. 8c).

**DISCUSSION**

Ilicicolin is an antibiotic that inhibits the cytochrome \( bc_1 \) complex in a manner similar to antimycin. Ilicicolin blocks oxidation-reduction of cytochrome \( b \) through center N of the \( bc_1 \) complex and promotes oxidant-induced reduction of cytochrome \( b \). These results indicate that ilicicolin binds to the Qn site of the \( bc_1 \) complex, as does antimycin, and both inhibitors bind with a stoichiometry of one inhibitor per \( bc_1 \) complex.

Although antimycin and ilicicolin both bind to the Qn site, which is near the \( b_H \) heme, the two inhibitors have different effects on the absorption spectrum of cytochrome \( b \). When antimycin binds to the reduced \( bc_1 \) complex it has a bathochromic effect, causing a red shift of 1 nm in the \( \alpha \) band of the cytochrome \( b \) spectrum. When ilicicolin \( H \) binds to the yeast \( bc_1 \) complex it has a hypsochromic effect, causing a blue shift of about 1 nm in the \( \alpha \) band of the cytochrome \( b \) spectrum. Funiculosin, another Qn inhibitor, also induces a blue shift in the cytochrome \( b \) spectrum upon binding to the reduced \( bc_1 \) complex (2, 24, 25). These different effects on the absorption spectrum indicate different effects on the electronic environment of the \( b_H \) heme.

Howell and Robertson (25) pointed out that the \( \pi-\pi^* \) transitions of the heme result in movement of electrons to the periphery of the heme macrocycle and, along with the heme iron \( d \) orbitals, contribute to the \( \alpha \) band in the absorption spectrum. Inhibitors can alter the environment near the heme macrocycle. Increasing the electron withdrawing character of the environment around the heme results in a lower energy requirement to excite the electron to a higher orbital, which translates into a red shift in the \( \alpha \) band in the optical spectrum. Antimycin apparently has such an effect on the \( b_H \) heme environment.

Inhibitor binding could also result in an increase in the electron donating character of the heme environment, thus producing a blue shift in the \( \alpha \) band of the spectrum. Funiculosin and ilicicolin \( H \) affect the heme environment in this manner. This may be due to location of the electron-donating pyridine ring in these ligands proximal to the \( b_H \) heme.

Ilicicolin differs markedly from antimycin in efficacy of inhibi-
hibitation of yeast and bovine bc₁ complexes. Whereas antimycin inhibits the enzyme from these species with similar efficacy, ilicicolin inhibits the yeast enzyme at much lower concentrations than required to inhibit the bovine enzyme. In ubiquinol-cytochrome c reductase assays with 2.5 nM enzyme in the assay, 3–5 nM ilicicolin was required to inhibit the yeast bc₁ complex by 50%, while 200–250 nM was required to inhibit the bovine enzyme to the same extent. This difference obviously reflects subtle structural differences in the Qₖ pocket in the yeast and bovine enzymes. The amino acid residues that are likely responsible for this difference are probably among those amino acids that confer resistance to funiculosin but do not confer cross-resistance to antimycin (26).

A more exact understanding of the structural basis for the species specificity must await a crystal structure of the bc₁ complex with ilicicolin bound.

We noted a significant hysteresis in the titration curves for inhibition of ubiquinol-cytochrome c reductase activity by both antimycin and ilicicolin. We also noted that both inhibitors cause the ubiquinol-cytochrome c reductase activity to increase by 10–20% when substoichiometric amounts of inhibitor are added to the enzyme. One would expect hysteresis in the inhibitor titration curves if electrons can cross from one monomer to the other in the dimeric enzyme, in which case binding of inhibitor to only one monomer would not inhibit the enzyme. This would not, however, account for the increase in enzyme activity that is observed with 0.1–0.2 equivalents of inhibitor per enzyme.

We suspected that the increased activity might result from formation of superoxide anion induced by low amounts of the inhibitors. We reasoned that superoxide anion might mediate electron transfer from the bc₁ complex to cytochrome c faster that cytochrome c could bind and dissociate from the enzyme. However, we were unable to eliminate the rate enhancement in the inhibitor titration curves by including superoxide dismutase in the assays, thus excluding this explanation.

We previously showed that inhibitory analogs of ubiquinol bind in an anticooperative manner to the Qₖ site in the bc₁ complex and suggested that the bc₁ complex operates by an alternating sites mechanism (15). An alternative explanation for the increased activity is that antimycin and ilicicolin disrupt the negative cooperativity between the two monomers in the dimeric enzyme so that ubiquinol can be oxidized at both Qₖ sites in the dimer simultaneously. If electrons can cross from one monomer to the other in the dimeric enzyme, disruption of negative cooperativity by binding of inhibitor to the Qₖ site would lead to activity enhancement and hysteresis in inhibitor titration curves as is seen with antimycin and ilicicolin.

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