

Inhibition of the Yeast Cytochrome bc_1 Complex by Ilicicolin H, a Novel Inhibitor That Acts at the Qn Site of the bc_1 Complex*

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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 nM, while 200–250 nM 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 protonmotive 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.

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The Qn inhibitors block reduction of ubiquinone by cytochrome b_H 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 ($K_d = 3.2 \times 10^{-11}$) close to heme b_H of cytochrome *b* and causes a red shift in the α band of the optical spectrum of the ferro-cytochrome *b*. The crystal structure of the chicken bc_1 complex with antimycin bound shows that the antimycin binding site is near the high potential (b_H) heme of cytochrome *b* 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 Biochemica. 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 DW2a™ UV/visible spectrophotometer with the OLIS (On-Line Instrument Systems Inc. Bogart, GA) DW2 conversion and OLIS Software. The spectrum, after subtracting the ethanol back-

¹ J. Onishi, submitted for publication.

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 stock solutions of the inhibitors. To minimize random dilution errors, each dilution was performed 5 or 6 times, and the diluted solutions were combined. The extinction coefficients used to calculate the concentrations of the stock solutions were, for antimycin, $4.8 \text{ mM}^{-1} \text{ cm}^{-1}$ at 320 nm; for stigmatellin, $65.5 \text{ mM}^{-1} \text{ cm}^{-1}$ at 267 nm; for ilicicolin H $23.2 \text{ mM}^{-1} \text{ cm}^{-1}$ at 248 nm and $5.3 \text{ mM}^{-1} \text{ cm}^{-1}$ at 349 nm (2, 9). All of the inhibitor dilutions were prepared daily, and the concentrations were determined before use.

Determination of bc_1 Complex Concentrations—For each inhibitor titration, the bc_1 complex was prediluted to $\sim 3 \mu\text{M}$ in assay buffer minus cytochrome c , and the concentration determined by difference spectra recorded in the Aminco DW2aTM spectrophotometer. The cytochrome c_1 concentration was determined from the difference spectrum of the ascorbate-reduced *versus* ferricyanide-oxidized enzyme, using an extinction coefficient of $17.5 \text{ mM}^{-1} \text{ cm}^{-1}$ 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 $25 \text{ mM}^{-1} \text{ cm}^{-1}$ 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 bc_1 Complex—Ubiquinol-cytochrome c reductase activities of the purified bc_1 complexes were assayed at room temperature in assay buffer containing 50 mM potassium phosphate, pH 7.0, 250 mM sucrose, 1 mM sodium azide, 0.2 mM EDTA, 0.01% Tween-20. After determining the bc_1 complex concentration, the enzyme was diluted daily a second time with the same buffer to 90 nM and incubated on ice for 30 min prior to the activity measurements. To initiate the assay an aliquot of the enzyme was diluted to a final concentration of 2.5 nM in assay buffer containing 40 μM cytochrome c and 0.5 mM potassium cyanide. The reaction was started by adding the ubiquinol analog, decyl-ubiquinol (DBH₂),² to a final concentration of 50 μM and reduction of cytochrome c was monitored at 550–539 nm with the Aminco DW2aTM spectrophotometer in the dual wavelength mode. The extinction coefficient used to calculate cytochrome c reduction was $21.5 \text{ mM}^{-1} \text{ cm}^{-1}$ at 550–539 nm (14).

The activity of the bc_1 complex without inhibitor and after stirring 2 min in the assay buffer was determined at the beginning of each titration. This was taken as 100% activity for the inhibitor titration. Under these conditions, and after 2 min stirring, the activity of the enzyme was always between 180 and 200 s⁻¹. At the end of each titration the activity of the bc_1 complex without inhibitor was again determined to check the stability of the enzyme during the experiment. Since the enzyme was preincubated with inhibitors in the assay buffer containing cytochrome c we could not correct for the non-enzymatic rate of cytochrome c reduction by DBH₂ at the beginning of each measurement (see Ref. 15). Instead, the non-enzymatic rate of reduction of cytochrome c by DBH₂ was measured in the absence of enzyme and subtracted from each activity trace.

Ubiquinol-Cytochrome c Reductase Assays with 50 nM bc_1 Complex—Ubiquinol-cytochrome c reductase activities using higher enzyme concentrations were measured at room temperature by stopped flow rapid scanning spectroscopy, using an OLIS-Rapid Scanning Monochromator, which is described below.

Reactions were started by mixing 100 nM bc_1 complex in assay buffer with 0.5 mM potassium cyanide and 100 μM DBH₂ against an equal volume of 100 μM cytochrome c in assay buffer. The inhibitors were incubated with the bc_1 complex for 2 min before mixing into the stopped flow chamber. The non-enzymatic rate of reduction of cytochrome c by DBH₂ was obtained by mixing equal volumes of 100 μM cytochrome c in assay buffer against 100 μM DBH₂ in assay buffer. The reaction was followed for 2 s. For each inhibitor concentration, four data sets were averaged, and the non-enzymatic rate was subtracted from each scan. From the three-dimensional data set comprised of wavelength, absorbance, and time, the time course of cytochrome c reduction was extracted using the OLIS software. The rate of cytochrome c reduction was calculated from the absorbance increase at 550 nm, using an extinction coefficient of $18.5 \text{ mM}^{-1} \text{ cm}^{-1}$ (16).

Reduction of Decyl-ubiquinone—The ubiquinol analog, decyl-ubiquinol (DBH₂) used as substrate in the ubiquinol-cytochrome c reductase assays was obtained by reducing the quinone as described previously (15). The concentration of DBH₂ was determined by the amount of

cytochrome c reduced in a catalytic assay, allowing the reaction to proceed to completion.

Presteady State Reduction of Cytochrome b —Presteady state reduction of cytochrome b was followed at room temperature by stopped flow rapid scanning spectroscopy using the OLIS Rapid Scanning Monochromator (17). The spectrophotometer was equipped with a 1200 lines/nm grating blazed at 500 nm. This produced a 75-nm spectrum, centered at 550 nm, with a resolution of 0.4 nm. The dead time of the instrument was 2 ms, and the end of this period was chosen as time 0. Data was collected at 1000 scans/s.

Reactions were started by rapidly mixing 3 μM bc_1 complex in assay buffer containing 50 mM potassium phosphate, pH 6.0, 250 mM sucrose, 1 mM sodium azide, 0.2 mM EDTA, and 0.01% Tween-20 with an equal volume of the same buffer containing 50 μM menadiol. A fresh solution of menadiol substrate was prepared from menadione before each experiment as described previously (17). The bc_1 complex was diluted shortly before each titration. The exact bc_1 concentration was determined by difference spectra recorded in the Aminco DW2aTM spectrophotometer as indicated above.

To determine the titer for inhibition of cytochrome b reduction with antimycin or ilicicolin H, the inhibitors were incubated with the enzyme 2 min before starting the reaction. The enzyme was premixed with a 2-fold excess of stigmatellin to block the reaction through center P. A spectrum of the oxidized bc_1 complex was obtained by mixing the oxidized bc_1 complex with assay buffer and averaging the data sets to a single scan. For each inhibitor concentration, three data sets were averaged, and the oxidized spectrum was subtracted from each scan. From the three-dimensional data set comprised of wavelength, absorbance and time, the time course and amplitude change for cytochrome b reduction at 563 nm was extracted using the OLIS software.

Oxidant-induced Reduction of Cytochrome b —The oxidant-induced reduction of cytochrome b was followed at room temperature by stopped flow rapid scanning spectroscopy using the OLIS-Rapid Scanning Monochromator. Prior to mixing to initiate the oxidant-induced reduction, 3 μM bc_1 complex was incubated for 15 min with 8 μM antimycin or ilicicolin H in assay buffer, pH 7.0, without Na₂S₃, and 30 μM DBH₂ to partially reduce the enzyme. A spectrum of the mixture of cytochrome c plus cytochrome c oxidase was obtained by mixing with an equal volume of assay buffer, and the data set was averaged to one scan. Oxidant-induced reduction reactions were started by mixing the partially reduced enzyme against an equal volume of buffer containing 6 μM cytochrome c oxidase and 30 μM cytochrome c . For each inhibitor, three data sets were averaged, and the spectrum of cytochrome c plus cytochrome c oxidase was subtracted from each scan. From the data sets the amplitude change for cytochrome b reduction was obtained as described above.

Measurement of the Absorbance Shift in the Cytochrome b Spectrum—The bc_1 complex was diluted to $\sim 3 \mu\text{M}$ in assay buffer, pH 7.0, and the concentration was determined as described above. A baseline was obtained by reducing the bc_1 complex with dithionite in both sample and reference cuvettes in the Aminco DW2aTM spectrophotometer. Increasing amounts of antimycin were added to the sample cuvette and an equal amount of ethanol to the reference cuvette. After allowing the inhibitor to equilibrate with the enzyme in the cuvette, a difference spectrum was recorded for each concentration of inhibitor added. For each inhibitor concentration the absorbance difference at 565–558 nm was determined. The same procedure was used to measure the blue shift induced by ilicicolin H binding, except that the absorbance difference at 559–563 nm was determined. To demonstrate that the ilicicolin-induced blue shift is eliminated by antimycin and not by stigmatellin, the inhibitors were added at 4.5 μM to the dithionite-reduced bc_1 complex, and optical spectra were recorded until no further change in the spectrum was observed.

RESULTS

Comparison of Ilicicolin H to Antimycin and Funiculosin—The structures of antimycin, funiculosin, and ilicicolin H are shown in Fig. 1. The first two are known Qn inhibitors. Antimycin consists of a 3-formamidosalicylic acid (3-FASA) linked via an amide bond to an alkyl- and acyl-substituted dilactone ring. It has been known for some time that the 3-FASA is the segment of the inhibitor closest to heme b_H (18, 19). Funiculosin consists of an *N*-methyl-substituted 4-hydroxy-2-pyridone ring with a hydrophobic side chain in position 1 and a tetrahydrocyclopentane ring in position 3.

Ilicicolin H is a 5-(4-hydroxyphenyl)- α -pyridone with a bicy-

² The abbreviations used are: DBH₂, decylubiquinol; 3-FASA, 3-formamidosalicylic acid.

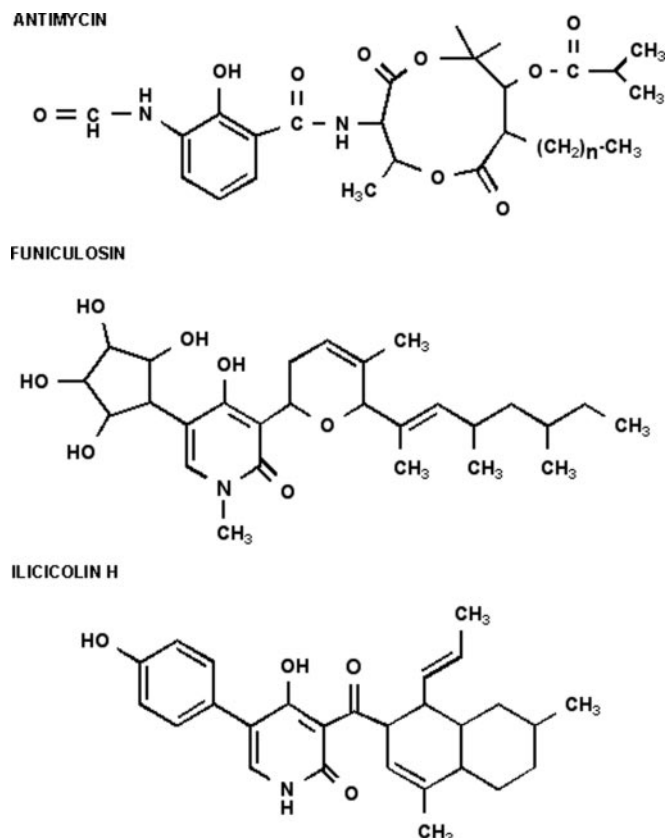


FIG. 1. Structures of antimycin, funiculosin, and ilicicolin H. Antimycin is a 3-FASA linked via an amide bond to an alkyl- and acyl-substituted dilactone ring. Funiculosin is an *N*-methyl-substituted 4-hydroxy-2-pyridone ring with a hydrophobic side chain in position 1 and a tetrahydrocyclopropentane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

clic decalin system. The 5-(4-hydroxyphenyl)- α -pyridone is the chromophore of tenellin, produced by the insect-pathogenic fungus *Beumeria bassiana*. Attached to this is a bicyclic decalin system (20, 21). Although the structures of the three antibiotics are clearly different, the structure of ilicicolin H resembles that of antimycin in containing a phenol ring and resembles that of funiculosin because of the pyridone ring system. The similar effects of ilicicolin H and funiculosin on the cytochrome *b* optical spectrum, shown below, point to a more prominent role of the pyridone ring system in ilicicolin binding.

Inhibition of the Yeast and Bovine Cytochrome bc_1 Complexes by Ilicicolin H—The results in Fig. 2 compare the inhibition of the yeast cytochrome bc_1 complex by ilicicolin H and antimycin. The titration curves in Fig. 2a show the inhibition of ubiquinol-cytochrome *c* oxidoreductase activity by antimycin or ilicicolin H in a catalytic assay using 2.5 nM enzyme concentration. Antimycin inhibits ~92% of the enzyme activity with a titer very close to one inhibitor per bc_1 complex. Ilicicolin H binds less tightly, and concentrations significantly greater than one inhibitor per bc_1 complex are required to fully inhibit the enzyme under these assay conditions.

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 2.5×10^{-9} M and 5×10^{-8} M for the isolated yeast bc_1 complex while that for antimycin is $<2.5 \times 10^{-9}$ M.

There is a significant hysteresis in the titration curves for

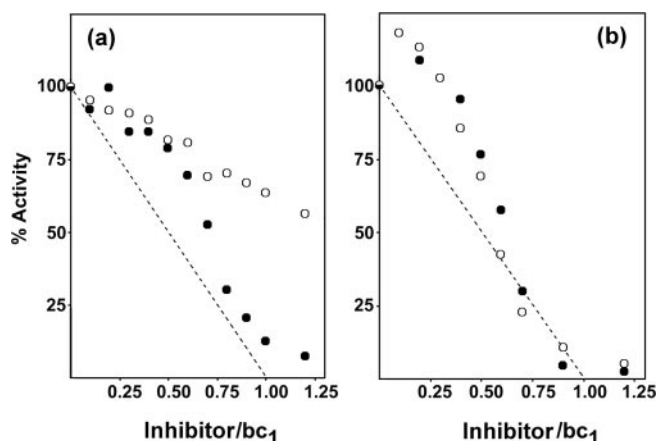


FIG. 2. Inhibition of ubiquinol-cytochrome *c* reductase activity of yeast cytochrome bc_1 complex by ilicicolin H and antimycin. The activities in the presence of ilicicolin are indicated by open circles and those in the presence of antimycin are indicated by solid circles. In panel a the concentration of cytochrome bc_1 complex in the assay was 2.5 nM, and the activity of the enzyme in the absence of inhibitor was 140 s^{-1} . In panel b the concentration of cytochrome bc_1 complex in the assay was 50 nM, and the activity of the enzyme in the absence of inhibitor was 160 s^{-1} . The dashed lines show theoretical titration curves for stoichiometric binding of one inhibitor per bc_1 complex monomer.

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 bc_1 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 bc_1 complex by ilicicolin H and antimycin. In the standard catalytic assay with 2.5 nM bc_1 complex antimycin inhibits the bovine bc_1 complex with stoichiometric affinity as shown in Fig. 3a. With ilicicolin, however, the IC_{50} 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 bc_1 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 c_1 . When the bc_1 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 c_1 reduction through center P and the rate matches the slow phase of cytochrome *b* reduction. As discussed elsewhere, the linkage between the rate of c_1 reduc-

FIG. 3. **Inhibition of ubiquinol-cytochrome c reductase activity of bovine cytochrome bc_1 complex by antimycin and ilicicolin.** Inhibition of the bovine enzyme by antimycin is shown in panel *a* and by ilicicolin is shown in panel *b*. The enzyme concentration in both assays was 2.5 nM. The dashed line in panel *a* shows the theoretical titration curve for stoichiometric binding of one inhibitor per bc_1 complex monomer. Activities in the absence of inhibitors were 414 s^{-1} and 350 s^{-1} in panels *a* and *b*, respectively.

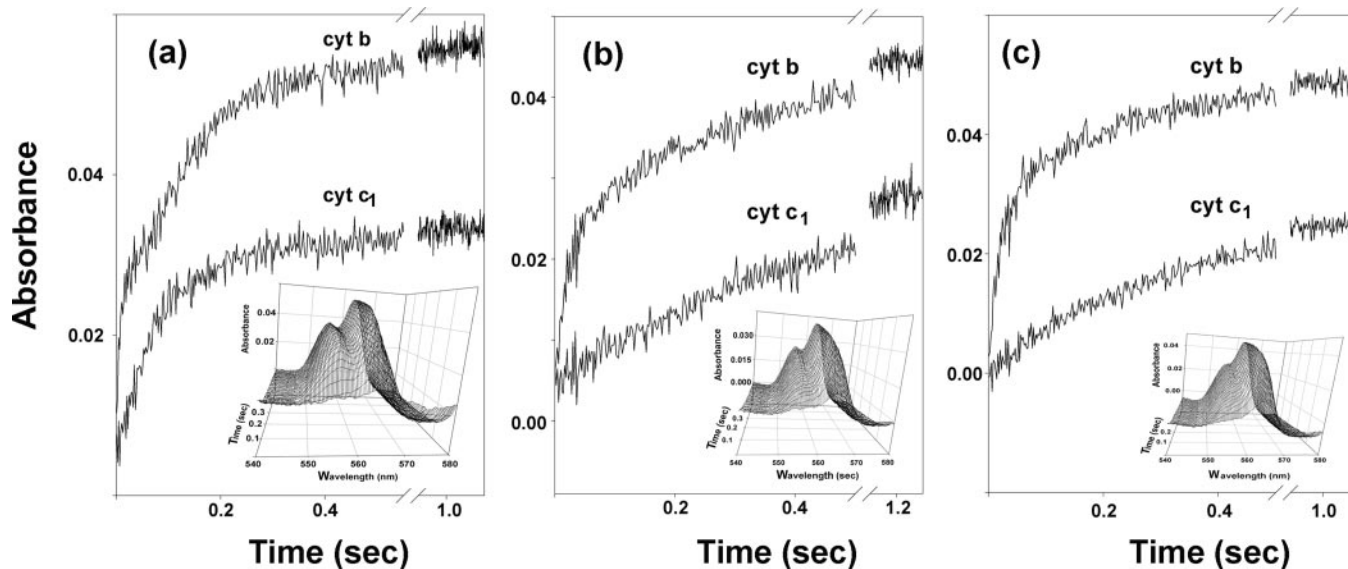
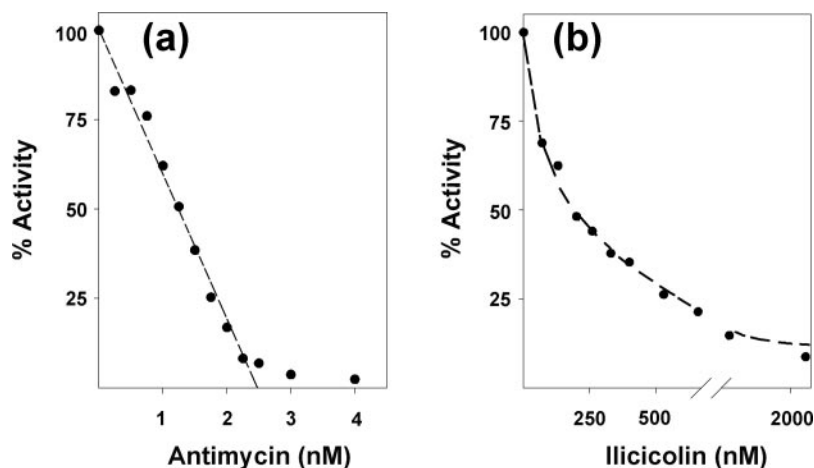


FIG. 4. **Effect of antimycin and ilicicolin on presteady state reduction of cytochrome b and cytochrome c_1 .** The tracings show reduction of the bc_1 complex by 50 μM menadiol in the absence of inhibitors (panel *a*), in the presence of a 2 equivalents of antimycin (panel *b*), and in the presence of 2 equivalents of ilicicolin (panel *c*). The insets show three-dimensional profiles of the reaction, in which optical spectra were collected at 2-ms intervals.

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 bc_1 complex as antimycin, changing the triphasic reduction of cytochrome b into a biphasic reaction, and also slowing down cytochrome c_1 reduction (Fig. 4c). This suggests that ilicicolin H is also a Qn site inhibitor.

Effect of Ilicicolin H on the Presteady State Reduction of the bc_1 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 bc_1 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 bc_1 complex. This shows conclusively that ilicicolin H binds to the Qn site of the bc_1 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 bc_1 complex in the presence of antimycin and followed the reduction of the enzyme by menadiol. 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 c_1 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 c_1 . 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 bc_1 complex is partially reduced and ubiquinol is present, oxidation of cytochrome c_1 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 c_1 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 c_1 oxidation, as shown in Fig. 6b. We also observed a similar oxidant-induced reduction

FIG. 5. Inhibition of presteady state reduction of cytochrome b through center N by antimycin and ilicicilin. The amount of inhibitor required to block reduction of cytochrome b through center N was determined by examining the presteady state reduction of cytochrome b . Two equivalents ($6 \mu\text{M}$) of stigmatellin were added to the bc_1 complex to block reduction through center P , prior to reducing the enzyme with $50 \mu\text{M}$ menadiol. The titration in *panel a* was performed with antimycin, and the concentration of enzyme was $3.0 \mu\text{M}$, while that in *panel b* was performed with ilicicilin, and the concentration of enzyme was $3.4 \mu\text{M}$. The dashed lines show theoretical titration curves for stoichiometric binding of one inhibitor per bc_1 complex monomer.

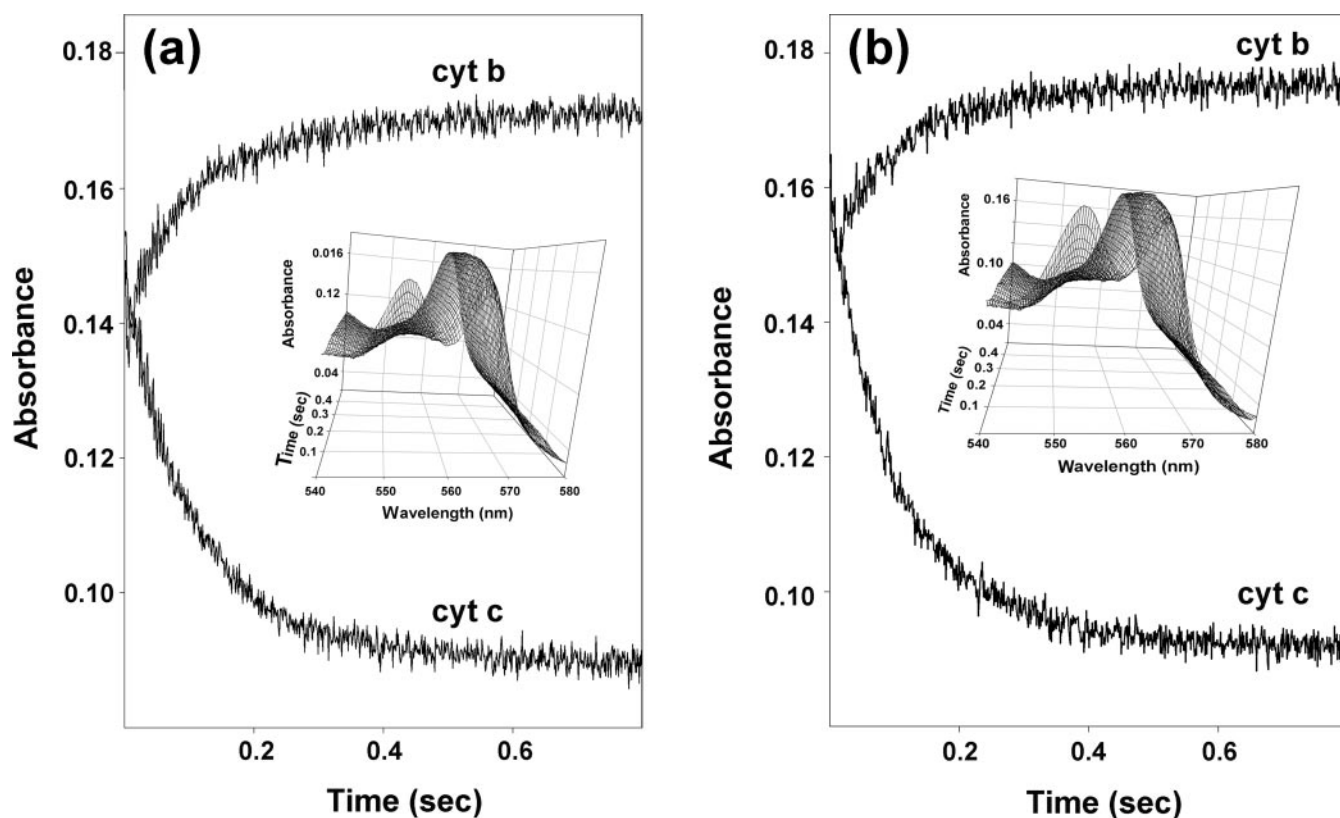
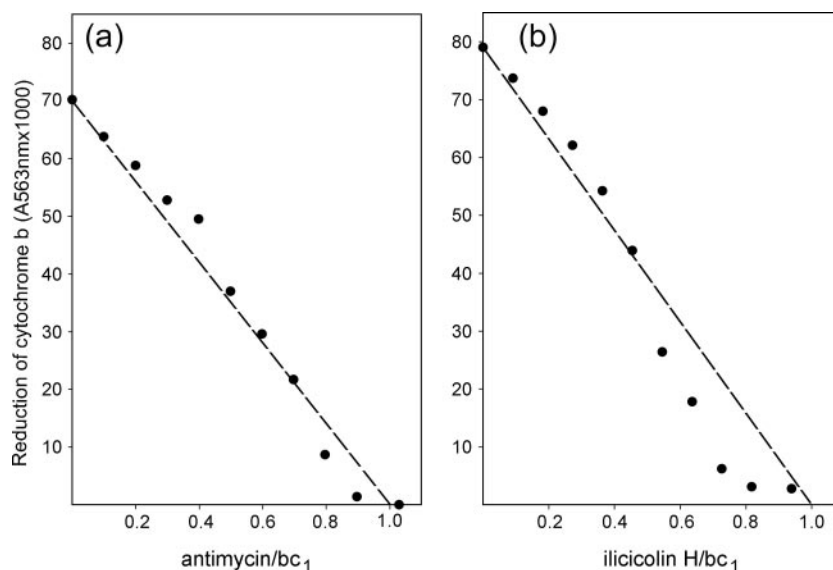


FIG. 6. Oxidant-induced reduction of cytochrome b in the presence of antimycin or ilicicilin. The cytochrome bc_1 complex was partially reduced by decyl-ubiquinol and incubated for 1 min with 2 equivalents of antimycin or ilicicilin. The partially reduced enzyme was then mixed in the stopped-flow spectrophotometer with 10 equivalents of cytochrome c and 2 equivalents of cytochrome c oxidase, and optical spectra were recorded at 2-ms intervals during the ensuing reaction. *Panel a* shows the reaction in the presence of antimycin, and *panel b* shows the reaction in the presence of ilicicilin. The tracings show reduction of cytochrome b and oxidation of cytochrome c . The inset shows a three-dimensional profile of the reaction.

of cytochrome b when ilicicilin was added to respiring mitochondrial membranes (results not shown). This result further confirms that ilicicilin acts at the Q_n site, like antimycin, and not at the Q_p site.

Effects of Ilicicilin 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 α and γ 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.

Ilicicilin 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 ilicicilin also binds with a stoichiometry of one per bc_1 complex.

The effects of ilicicilin H on the presteady state reduction of

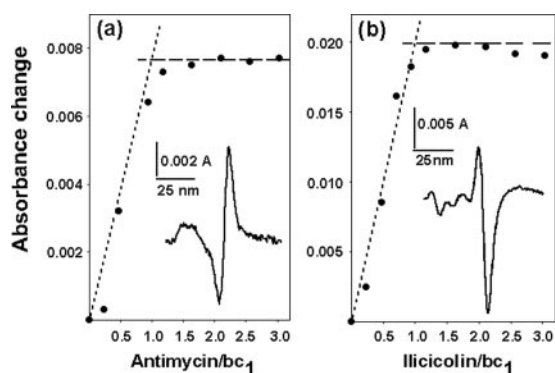


FIG. 7. Determination of the binding stoichiometries of antimycin and ilicicolin by titration of the shift in the optical spectrum of cytochrome b . The absorbance change was measured from the difference spectra as shown in the insets and plotted against inhibitor concentration. The dashed lines show theoretical titration curves for stoichiometric binding of one inhibitor per bc_1 complex monomer. The insets show the red shift induced in the difference spectrum of ferrocytochrome b by antimycin binding and the blue shift induced in the difference spectrum of ferrocytochrome b by ilicicolin binding.

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. 8*c*).

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 α 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 α 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 α band in the absorption spectrum. Inhibitors can alter the environment near the heme macro-

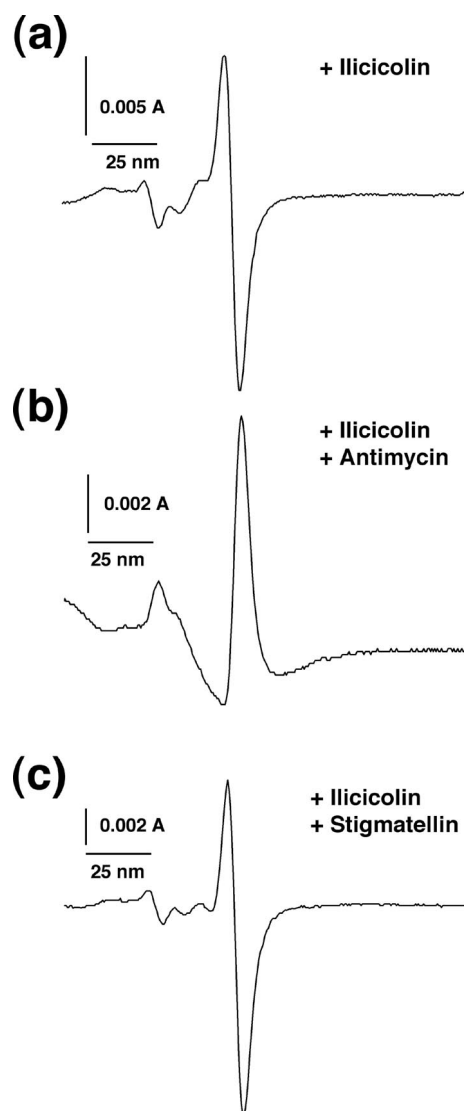


FIG. 8. Elimination of the ilicicolin-induced blue shift in the optical spectrum of cytochrome b by binding of antimycin. The spectrum in *a* is a difference spectrum of ferrocytochrome b plus ilicicolin versus ferrocytochrome b . The spectrum in *b* is a difference spectrum of ferrocytochrome b plus ilicicolin and antimycin versus ferrocytochrome b . The spectrum in *c* is a difference spectrum of ferrocytochrome b plus ilicicolin and stigmatellin versus ferrocytochrome b . To obtain the spectra in *b* and *c* ilicicolin was first added to the dithionite-reduced bc_1 complex, and a difference spectrum of the bc_1 complex plus ilicicolin versus bc_1 complex was recorded to demonstrate the blue shift induced by ilicicolin as in *a*. Antimycin or stigmatellin was then added to the ilicicolin-treated enzyme, and another difference spectrum of the bc_1 complex plus ilicicolin and antimycin or of the bc_1 complex plus ilicicolin and stigmatellin versus bc_1 complex was recorded. The concentration of cytochrome bc_1 complex was $3\ \mu\text{M}$, and the inhibitors were added at $4.5\ \mu\text{M}$.

cycle. 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 α 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 α 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 pyridone ring in these ligands proximal to the b_H heme.

Ilicicolin differs markedly from antimycin in efficacy of inhi-

bition of yeast and bovine bc_1 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 bc_1 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 Qn 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 bc_1 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 bc_1 complex to cytochrome c faster than 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 Qp site in the bc_1 complex and suggested that the bc_1 complex operates by an alternating sites mechanism (15). An alternative explanation for the increased activity is that antimycin and ilicicolin dis-

rupt the negative cooperativity between the two monomers in the dimeric enzyme so that ubiquinol can be oxidized at both Qp 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 Qn site would lead to activity enhancement and hysteresis in inhibitor titration curves as is seen with antimycin and ilicicolin.

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