

# Inhibitory Analogs of Ubiquinol Act Anti-cooperatively on the Yeast Cytochrome $bc_1$ Complex

EVIDENCE FOR AN ALTERNATING, HALF-OF-THE-SITES MECHANISM OF UBIQUINOL OXIDATION\*

Received for publication, September 20, 2001, and in revised form, November 5, 2001  
Published, JBC Papers in Press, November 7, 2001, DOI 10.1074/jbc.M109097200

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The cytochrome  $bc_1$  complex is a dimeric enzyme that links electron transfer from ubiquinol to cytochrome  $c$  by a protonmotive Q cycle mechanism in which ubiquinol is oxidized at one center in the enzyme, referred to as center P, and ubiquinone is re-reduced at a second center, referred to as center N. To understand better the mechanism of ubiquinol oxidation, we have examined the interaction of several inhibitory analogs of ubiquinol with the yeast cytochrome  $bc_1$  complex. Stigmatellin and methoxyacrylate stilbene, two inhibitors that block ubiquinol oxidation at center P, inhibit the yeast enzyme with a stoichiometry of 0.5 per  $bc_1$  complex, indicating that one molecule of inhibitor is sufficient to fully inhibit the dimeric enzyme. This stoichiometry was obtained when the inhibitors were titrated in cytochrome  $c$  reductase assays and in reactions of quinol with enzyme in which the inhibitors block pre-steady state reduction of cytochrome  $b$ . As an independent measure of inhibitor binding, we titrated the red shift in the optical spectrum of ferrocytochrome  $b$  with methoxyacrylate stilbene and thus confirmed the results of the inhibition of activity titrations. The titration curves also indicate that the binding is anti-cooperative, in that a second molecule of inhibitor binds with much lower affinity to a dimer in which an inhibitor molecule is already bound. Because these inhibitors bind to the ubiquinol oxidation site in the  $bc_1$  complex, we propose that the yeast cytochrome  $bc_1$  complex oxidizes ubiquinol by an alternating, half-of-the-sites mechanism.

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). Crystal structures of the bovine (2, 3), chicken (4), and yeast (5) cytochrome  $bc_1$  complexes have revealed that the mitochondrial cytochrome  $bc_1$  complex is a symmetrical dimer. The role of the dimeric structure in the Q cycle mechanism is not fully understood. It is not known whether each monomer operates independently or whether there is electron transfer between the two monomers.

There are numerous inhibitors that block electron transfer within the  $bc_1$  complex by acting specifically at center P or

center N. 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. Stigmatellin, hydroxyquinones, and methoxyacrylates such as myxothiazol and MOA<sup>1</sup> stilbene, all act at center P (6). The Qn inhibitors block reduction of ubiquinone by cytochrome  $b$  at center N and block reduction of cytochrome  $b$  that otherwise can occur by reversal of this reaction. Antimycin, one of the most extensively studied inhibitors of the  $bc_1$  complex, acts at center N (6, 7).

In the experiments reported here, we show that some of the inhibitors that block ubiquinol oxidation at center P inhibit the yeast enzyme with a stoichiometry of 0.5 per  $bc_1$  complex, indicating that one molecule of inhibitor is sufficient to fully inhibit the dimeric enzyme. The titration curves also indicate that the binding is anti-cooperative, in that a second molecule of inhibitor binds with markedly lower affinity to the dimer in which an inhibitor molecule is already bound. As an independent measure of inhibitor binding, we titrated the red shift in the optical spectrum of ferrocytochrome  $b$  with MOA stilbene and found that the inhibitor binds to the dimeric enzyme at two sites with two very different affinities, consistent with a model in which a second molecule of inhibitor does not bind to an enzyme dimer until all of the dimers are occupied by one inhibitor.

To test the possible involvement of ubiquinone in the anti-cooperative behavior of the inhibitors, we titrated stigmatellin and MOA stilbene in a yeast mutant that lacks ubiquinone. The titer for the two inhibitors in the mutant was also 0.5 inhibitor per enzyme monomer, indicating that ubiquinone is not responsible for the anti-cooperative interactions in the dimeric enzyme. These results are discussed in the context of the crystal structures of the  $bc_1$  complex and the implications for the mechanism of ubiquinol oxidation.

## EXPERIMENTAL PROCEDURES

**Materials**—Dodecylmaltoside was obtained from Roche Molecular Biochemicals. DEAE-Bio-Gel was obtained from Bio-Rad. Yeast extract and peptone were from Difco. Antimycin, myxothiazol, diisopropyl fluorophosphate, phenylmethylsulfonyl fluoride, menaquinone, horse heart cytochrome  $c$ , and decylubiquinone were purchased from Sigma. Stigmatellin was purchased from Fluka. MOA stilbene was obtained from Dr. U. Brandt (University of Frankfurt).

**Purification of  $bc_1$  Complexes**—Yeast cytochrome  $bc_1$  complexes were isolated from Red Star cake yeast as described previously (8, 9). The  $\Delta coq2$  yeast mutant was obtained from Dr. C. Clarke (UCLA). The wild-type yeast strain, W303a, and the  $\Delta coq2$  yeast mutant were grown in 1% yeast extract, 2% peptone, 2% dextrose medium and harvested by centrifugation.

**Reduction of Decylubiquinone**—The ubiquinol analog, decylubiquinol (DBH<sub>2</sub>), was used as substrate in the ubiquinol-cytochrome  $c$  reductase

\* This work was supported by National Institutes of Health Grant GM 20379 and a postdoctoral fellowship from the American Heart Association, New England affiliate (to E. G.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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<sup>1</sup> The abbreviations used are: MOA, methoxyacrylate; DBH<sub>2</sub>, decyl ubiquinol.

assays and was obtained by reducing the quinone as described by Trumppower and Edwards (10). One-hundred mg of decylubiquinone were dissolved in 5 ml of ethanol and then mixed with 10 ml of a buffer containing 25 mM potassium phosphate, pH 7.2, and 25  $\mu$ M EDTA. The mixture was reduced by slowly adding solid sodium dithionite and then solid sodium borohydride. The reduced mixture was extracted with 16 ml of cyclohexane and the fractions washed with buffer. The cyclohexane was evaporated with a stream of argon and the reduced quinol was dissolved in 2 ml of ethanol containing 10 mM HCl.

**Determination of Inhibitor Concentrations**—Each of the inhibitors was diluted in ethanol, and the concentration was determined from optical spectra obtained in an Aminco DW2a<sup>TM</sup> UV-visible spectrophotometer with the OLIS DW2 conversion and OLIS Software. The difference spectrum, after subtracting the ethanol background, was recorded from 250 to 400 nm. To determine accurately 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 are as follows: for stigmatellin, 65.5  $\text{mm}^{-1} \text{cm}^{-1}$  at 300 nm; for myxothiazol, 10.5  $\text{mm}^{-1} \text{cm}^{-1}$  at 313 nm; for antimycin, 4.8  $\text{mm}^{-1} \text{cm}^{-1}$  at 320 nm (6); and for MOA stilbene, 26.5  $\text{mm}^{-1} \text{cm}^{-1}$  at 300 nm (11). All of the inhibitor dilutions were prepared daily, and the concentrations were determined before a titration was started.

**Ubiquinol-Cytochrome  $c$  Reductase Assays with 2.5 nM  $bc_1$  Complex**—Ubiquinol-cytochrome  $c$  reductase activities of the purified  $bc_1$  complex were assayed at room temperature in an 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, and 50  $\mu$ M cytochrome  $c$ . Cytochrome  $bc_1$  complex was added to a final concentration of 2.5 nM and allowed to equilibrate with inhibitor by stirring for 2 min in the cuvette. Potassium cyanide was added to a final concentration of 0.5 mM. The reaction was started by adding 50  $\mu$ M DBH<sub>2</sub> (final concentration), and reduction of cytochrome  $c$  was monitored at 550–539 nm with the Aminco DW2a<sup>TM</sup> 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 (12).

For each inhibitor titration, the  $bc_1$  complex was pre-diluted in assay buffer minus cytochrome  $c$  and the concentration determined by difference spectra recorded in the Aminco DW2a<sup>TM</sup> 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 553–548 nm (13). Cytochrome  $b$  concentration was determined from the difference spectrum of the sodium dithionite reduced *versus* ferricyanide-oxidized enzyme, using an extinction coefficient of 25  $\text{mm}^{-1} \text{cm}^{-1}$  at 563–578 nm (13). This pre-diluted enzyme was considered the stock solution, and the concentration was usually 3  $\mu$ M cytochrome  $c_1$ . The activity of this stock solution of enzyme was stable for a week at 4 °C.

After determining the  $bc_1$  complex concentration, the enzyme was diluted daily a second time, to 33 nM, and incubated on ice for 30 min prior to the activity measurements. To initiate the assay an aliquot of the 33 nM dilution was diluted to a final concentration of 2.5 nM in assay buffer containing 50  $\mu$ M cytochrome  $c$  and 0.5 mM KCN. 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, or  $V_0$ . 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. The non-enzymatic reduction of cytochrome  $c$  by DBH<sub>2</sub> was subtracted from each activity trace. Because the enzyme was preincubated with inhibitors in the assay buffer containing cytochrome  $c$ , we could not correct for the non-catalytic rate of cytochrome  $c$  reduction by DBH<sub>2</sub> at the beginning of each measurement. However, we found that this rate was less than 1% of the catalytic rate; therefore, an average of two non-catalytic rates of cytochrome  $c$  reduction was subtracted from the catalytic rate.

**Ubiquinol-Cytochrome  $c$  Reductase Assays with 50 nM  $bc_1$  Complex**—Ubiquinol-cytochrome  $c$  reductase activities using the higher enzyme concentration were assayed at room temperature by stopped flow rapid scanning spectroscopy, using an OLIS-Rapid Scanning Monochromator (On-Line Instrument Systems Inc. Bogart, GA) equipped with a 1200 lines/mm grating blazed at 500 nm. This produced a spectrum of 75 nm width, 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 were chosen as time 0. Data were collected at 1000 scans/s.

Reactions were started by mixing 100 nM  $bc_1$  complex in assay buffer

with 0.5 mM potassium cyanide and 100  $\mu$ M DBH<sub>2</sub> against an equal volume of 100  $\mu$ M cytochrome  $c$  in assay buffer. The inhibitors were added to the  $bc_1$  complex and incubated with the enzyme for 2 min before mixing into the stopped flow chamber. The non-enzymatic rate of reduction of cytochrome  $c$  by DBH<sub>2</sub> was obtained by mixing equal volumes of 100  $\mu$ M cytochrome  $c$  in assay buffer against 100  $\mu$ M DBH<sub>2</sub> 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 composed 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  $\text{mm}^{-1} \text{cm}^{-1}$  (14).

**Pre-steady State Reduction of Cytochrome  $b$** —Pre-steady state reduction of cytochrome  $b$  was followed at room temperature by stopped flow rapid scanning spectroscopy using the OLIS Rapid Scanning Monochromator. The rationale for this pre-steady state kinetics method was discussed previously (15).

Reactions were started by rapid mixing of 3  $\mu$ M  $bc_1$  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 against an equal volume of the same buffer containing 50  $\mu$ M menaquinol. The  $bc_1$  complex was diluted shortly before each titration, and the exact concentration was determined as described above. A fresh solution of menaquinol substrate was prepared before every experiment as described previously (15). The inhibitors were incubated with the enzyme 2 min before starting the reaction. An oxidized spectrum was obtained by mixing the oxidized  $bc_1$  complex against 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 composed of wavelength, absorbance, and time, the time course and amplitude change for cytochrome  $b$  reduction at 563 nm was extracted using the OLIS software.

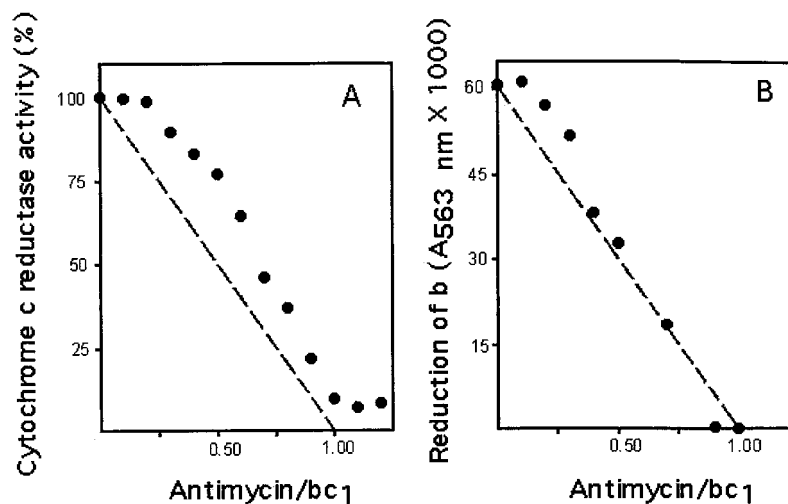
**Oxidant-induced Reduction of Cytochrome  $b$** —Prior to mixing to initiate the oxidant-induced reduction, 3  $\mu$ M  $bc_1$  complex was incubated for 15 min with 8  $\mu$ M antimycin in assay buffer, pH 7.0, and 30  $\mu$ M DBH<sub>2</sub> to partially reduce the enzyme. Varying amounts of stigmatellin were added to the  $bc_1$  complex after the incubation with DBH<sub>2</sub> and incubated for 2 min prior to mixing with the oxidase and cytochrome  $c$ . Oxidant-induced reduction reactions were started by mixing the partially reduced enzyme against an equal volume of buffer containing 6  $\mu$ M cytochrome  $c$  oxidase and 30  $\mu$ M cytochrome  $c$ . A spectrum of the oxidized mixture of cytochrome  $c$  plus cytochrome  $c$  oxidase was obtained by mixing with an equal amount of buffer and averaging the data set to one scan. The oxidant-induced reduction of cytochrome  $b$  was followed at room temperature by stopped flow rapid scanning spectroscopy using the OLIS-Rapid Scanning Monochromator. For each inhibitor concentration, three data sets were averaged, and the oxidized spectrum was subtracted from each scan. From the data sets the amplitude change for cytochrome  $b$  reduction was obtained as described above.

**Measurement of the Red Shift in the Cytochrome  $b$  Spectrum**—The  $bc_1$  complex was diluted to an approximate concentration of 3  $\mu$ M in assay buffer, and the exact concentration was determined as described above. A base line was obtained by reducing the  $bc_1$  complex with dithionite in both sample and reference cuvettes in the Aminco DW2a<sup>TM</sup> spectrophotometer. Increasing amounts of MOA stilbene or myxothiazol 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 for 2 min, a difference spectrum was recorded for each concentration of inhibitor added. A 2-fold excess of inhibitor was added at the end of the titration to establish the maximum change of the red shift. For each inhibitor concentration the absorbance difference at 568–560 nm, for MOA stilbene, or at 564–559 nm, for myxothiazol, was measured.

## RESULTS

**Titration of the  $bc_1$  Complex with Antimycin**—In the experiments reported below we show that some inhibitors of the  $bc_1$  complex that block ubiquinol oxidation at center P fully inhibit the enzyme with a stoichiometry of 0.5 inhibitor per enzyme monomer. As a control for these experiments, we performed a set of inhibitor titrations with antimycin, which inhibits the enzyme at center N. The results in Fig. 1A show the inhibition of ubiquinol-cytochrome  $c$  reductase activity by antimycin in a

FIG. 1. Titration of the  $bc_1$  complex with antimycin. A shows an antimycin titration of the ubiquinol-cytochrome  $c$  reductase activity with 2.5 nM  $bc_1$  complex in the assay. Activities are expressed as percentage of the activity without inhibitor and plotted versus the ratio of antimycin per  $bc_1$  complex. The activity of the enzyme without inhibitor was 184  $s^{-1}$ . B shows a titration of the pre-steady state reduction of cytochrome  $b$  with 1.5  $\mu M$  enzyme in the assay. The  $bc_1$  complex was pre-mixed with 2 eq of stigmatellin to block reduction of cytochrome  $b$  through center P and then reduced with 50  $\mu M$  menaquinol. Reduction of cytochrome  $b$  was followed at 563 nm. The dashed lines show the linear fitting to 1 eq of inhibitor per  $bc_1$  complex.



catalytic assay using 2.5 nM  $bc_1$  complex. The dashed line shows the fitting of a linear titration curve with an intercept of one inhibitor per enzyme. Antimycin fully inhibits the enzyme at a titer of one inhibitor per enzyme, although there is a significant hysteresis in the titration curve at low antimycin concentrations. An explanation for the hysteresis is discussed below.

In establishing optimal conditions for the ubiquinol-cytochrome  $c$  reductase assays, we found that a buffer containing 0.01% Tween 20 and 250 mM sucrose was essential to obtain consistently turnover numbers greater than 100  $s^{-1}$ . In addition, when the yeast enzyme was diluted to 3  $\mu M$  in this buffer, it remained stable for 1 week at 4  $^{\circ}C$ . A similar result was reported previously for the bovine  $bc_1$  complex (14).

We also found that it was necessary to incubate the enzyme with inhibitor for 2 min in the assay buffer before beginning the reaction, to obtain maximum inhibition, particularly in cytochrome  $c$  reductase assays using low (2.5 nM) concentrations of  $bc_1$  complex. Lack of equilibration of inhibitor with enzyme may account for the higher inhibitor stoichiometries observed in some studies, as discussed below.

To establish that the titer for inhibition by antimycin is independent of enzyme concentration, we also performed a titration using 1.5  $\mu M$   $bc_1$  complex, following the pre-steady state reduction of cytochrome  $b$ . In this assay a stoichiometric excess of stigmatellin is included to block reduction of cytochrome  $b$  through center P, and the reduction of cytochrome  $b$  through center N is inhibited by varying amounts of antimycin. As shown in Fig. 1B, at this high  $bc_1$  complex concentration, the stoichiometry of antimycin per  $bc_1$  complex is also 1:1. In this assay also there was a slight hysteresis in the titration curve at low antimycin concentrations. From titrating the inhibitor with 2.5 nM or 1.5  $\mu M$   $bc_1$  complex, it is clear that the stoichiometry for inhibition of the yeast  $bc_1$  complex by antimycin is one inhibitor per enzyme monomer. This agrees with previous results for titration of the yeast enzyme with this inhibitor (16).

**Titration of the  $bc_1$  Complex with Stigmatellin**—Fig. 2A shows the inhibition of ubiquinol-cytochrome  $c$  reductase activity by stigmatellin in a catalytic assay using 2.5 nM  $bc_1$  complex. The dotted line shows a theoretical linear titration curve with a slope of 2, which would correspond to a titer of 0.5 eq of inhibitor per enzyme monomer. At low inhibitor concentrations, the data points in the titration fall on the theoretical titration curve and extrapolate to a titer of 0.5.

Because these results were unexpected, we repeated this titration with different preparations of enzyme and made measurements in triplicate each time. We also took special care

to determine accurately the concentrations of inhibitor and  $bc_1$  complex for each experiment as described under "Experimental Procedures." With 14 preparations of enzyme, which differed in activity from 140 to 240  $s^{-1}$ , which we attribute to different degrees of delipidation during the ion-exchange chromatography in the presence of detergent, we found that there were slight variations in the degree of linearity of the titration curves, but the data consistently indicated a titer that ranged from 0.45 to 0.55 eq of inhibitor per enzyme monomer.

The deviation from linearity of the titration curve in Fig. 2A would be expected if the  $K_d$  value of the inhibitor is comparable with or greater than the enzyme concentration in the assay, because a portion of the inhibitor would not be bound to the enzyme. To test this possibility, we repeated the titration with stigmatellin, using 50 nM  $bc_1$  complex in a ubiquinol-cytochrome  $c$  reductase assay. At this high enzyme concentration, reduction of cytochrome  $c$  occurs so rapidly that the reaction must be followed in a stopped flow spectrophotometer, and data points are collected over a 2-s interval. As shown in Fig. 2B, at the higher enzyme concentration the experimental points fit the theoretical linear titration curve very well, and at 0.5 eq of stigmatellin per  $bc_1$  monomer more than 95% of the enzyme is inhibited.

We also examined the amount of stigmatellin required for inhibition of cytochrome  $b$  reduction in a pre-steady state assay in which the  $bc_1$  complex is present at 1.5  $\mu M$  (Fig. 2C). In this assay a stoichiometric excess of antimycin is included to block reduction of cytochrome  $b$  through center N, and the reduction of cytochrome  $b$  through center P is inhibited by varying amounts of stigmatellin. In this assay also 0.5 eq of stigmatellin fully inhibit the enzyme.

The results from the titrations of cytochrome  $c$  reductase activity and pre-steady state reduction of cytochrome  $b$  indicate that one molecule of stigmatellin fully inhibits the dimeric yeast  $bc_1$  complex. Furthermore, the lack of displacement to values greater than 0.5 eq per cytochrome  $c_1$  in the linear titration curves indicates that the inhibitor binds in an anti-cooperative manner, *i.e.* a second molecule of inhibitor does not bind to a dimer to which one molecule of inhibitor is already bound.

**Titration of the Oxidant-induced Reduction of Cytochrome  $b$  with Stigmatellin**—As a further measure of the stoichiometry of stigmatellin interaction with the  $bc_1$  complex, we examined the amount of stigmatellin required to inhibit the oxidant-induced reduction of cytochrome  $b$ . Binding of stigmatellin depends on the redox state of the Rieske iron-sulfur protein (6, 17), and in this reaction the antimycin-inhibited  $bc_1$  complex is

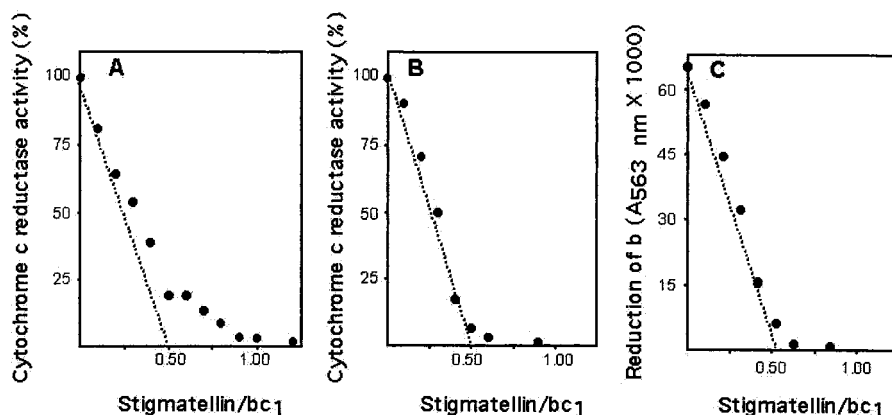


FIG. 2. **Titration of the  $bc_1$  complex with stigmatellin.** A shows a stigmatellin titration of the ubiquinol-cytochrome  $c$  reductase activity with 2.5 nM  $bc_1$  complex in the assay. The activity without inhibitor was  $147 \text{ s}^{-1}$  and was used as 100% activity for the plot. B shows a titration of 50 nM yeast  $bc_1$  complex in the cytochrome  $c$  reductase assay. The activity of the  $bc_1$  complex without inhibitor was  $130 \text{ s}^{-1}$ . For each inhibitor concentration an average of four assays was used. C shows the titration of 1.5  $\mu\text{M}$  yeast  $bc_1$  complex with stigmatellin in an assay that measures pre-steady-state reduction of cytochrome  $b$ . The enzyme was pre-mixed with 2 eq of antimycin to block reduction of  $b$  through center N. Reduction of cytochrome  $b$  was followed at 563 nm and is plotted against the ratio of stigmatellin per  $bc_1$  complex. Each data point is the average of three pre-steady state reactions in the stopped flow spectrophotometer. The dashed lines show the linear fitting to 0.5 eq of inhibitor per  $bc_1$  complex.

partially reduced with  $\text{DBH}_2$ . This reduces the iron-sulfur protein, cytochrome  $c_1$ , and a portion of the high potential cytochrome  $b$  and the quinone pool. Subsequent addition of cytochrome  $c$  plus cytochrome  $c$  oxidase then elicits additional reduction of cytochrome  $b$  concomitant with oxidation of cytochrome  $c_1$  and the Rieske protein. The inset in Fig. 3 shows the redox status of the cytochromes in partially reduced  $bc_1$  complex and the increment in cytochrome  $b$  reduction that results from the oxidation of the high potential redox components.

As seen from the titration results in Fig. 3, 0.5 eq of stigmatellin fully inhibits the oxidant-induced reduction of cytochrome  $b$ . This result is the same as that obtained from the titrations of cytochrome  $c$  reductase activity and pre-steady state reduction of cytochrome  $b$ , but in this case there is a pronounced hysteresis in the titration curve. A possible explanation for this hysteresis is discussed below.

**Titration of the  $bc_1$  Complex with MOA Stilbene**—MOA stilbene is a member of the methoxyacrylate class of inhibitors that includes myxothiazol, strobilurin, and oudemansin (6). These inhibitors block ubiquinol oxidation at center P, but they differ from stigmatellin in that they prevent reduction of the Rieske iron-sulfur cluster (17), whereas stigmatellin allows reduction of the cluster and locks the Rieske protein in the reduced conformation, proximal to cytochrome  $b$  (4, 5).

A representative titration of the ubiquinol-cytochrome  $c$  reductase activity of the  $bc_1$  complex with MOA stilbene is shown in Fig. 4A. Under these conditions, using 2.5 nM  $bc_1$  complex in the standard catalytic assay, the binding of the inhibitor is not sufficiently tight to extrapolate a stoichiometry of binding directly from the titration curve. However, if 50 nM  $bc_1$  complex is used for the cytochrome  $c$  reductase assay, the data from the inhibitor titration fits well to a linear curve corresponding to 0.5 molecules of inhibitor per  $bc_1$  monomer, as shown in Fig. 4B.

The difference in the titration curves in Fig. 4, A and B, suggests that the  $K_i$  of MOA stilbene for the  $bc_1$  complex is in the range of the 2.5 nM enzyme concentration used in the standard catalytic assay. Further evidence to this effect was obtained by titrating the pre-steady state reduction of cytochrome  $b$ , using 1.5  $\mu\text{M}$   $bc_1$  complex in the assay. Varying amounts of MOA stilbene were used to inhibit cytochrome  $b$  reduction through center P, while blocking reduction through center N with an excess of antimycin. As seen in Fig. 4C, there is a slight hysteresis in the titration curve at low inhibitor concentrations, but the reduction of cytochrome  $b$  is fully inhibited at 0.5 eq of MOA stilbene per  $bc_1$  monomer.

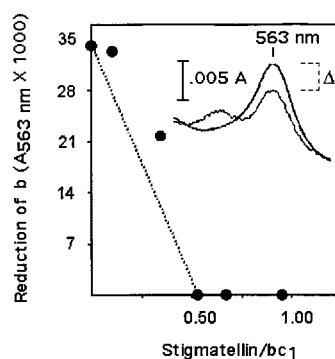


FIG. 3. **Titration of oxidant-induced reduction of cytochrome  $b$  with stigmatellin.** The  $bc_1$  complex was pre-mixed with 2 eq of antimycin and 30  $\mu\text{M}$   $\text{DBH}_2$ . Oxidant-induced reduction was initiated by mixing the partially reduced enzyme at a final concentration of 1.5  $\mu\text{M}$   $bc_1$  complex with 3  $\mu\text{M}$  cytochrome  $c$  oxidase + 15  $\mu\text{M}$  cytochrome  $c$  in the stopped flow spectrophotometer. Reduction of cytochrome  $b$  was measured at 563 nm 3 s after the addition of oxidase and is plotted against the ratio of stigmatellin to  $bc_1$  complex. The inset shows spectra from 540–580 nm extracted from the kinetic data after the partial reduction with 30  $\mu\text{M}$   $\text{DBH}_2$  and after the oxidant-induced reduction. Each of the data points in the titration is an average of three reactions in the stopped flow spectrophotometer. The dashed line shows a fitted curve with an intercept of 0.5 eq of inhibitor per enzyme.

**Titration of Inhibitors into  $bc_1$  Complex Lacking Endogenous Ubiquinone**—Inhibition of the dimeric  $bc_1$  complex by 0.5 eq of inhibitor per  $bc_1$  monomer and the anti-cooperative nature of the inhibition indicate that binding of the inhibitor in one monomer prevents oxidation of ubiquinol or binding of a second molecule of inhibitor at the second ubiquinol oxidation site in the dimer. To test whether this behavior is dependent on the endogenous ubiquinone in the  $bc_1$  complex, we repeated these experiments with  $bc_1$  complex from the  $\Delta\text{coq2}$  yeast mutant that lacks endogenous quinone (18). The titration curves in Fig. 5 show inhibition of the pre-steady state reduction of cytochrome  $b$  by stigmatellin (Fig. 5A) and MOA stilbene (Fig. 5B). With both inhibitors the reduction of cytochrome  $b$  is completely blocked by 0.5 eq of inhibitor per  $bc_1$  monomer. These results establish that ubiquinone is not responsible for the anti-cooperative binding of these two Qp inhibitors in the yeast  $bc_1$  complex.

**Measurement of the Stoichiometry of MOA Stilbene Binding from the Red Shift in the Cytochrome  $b$  Spectrum**—Methoxyacrylates cause a red shift in the  $\alpha$  band of the reduced cyto-

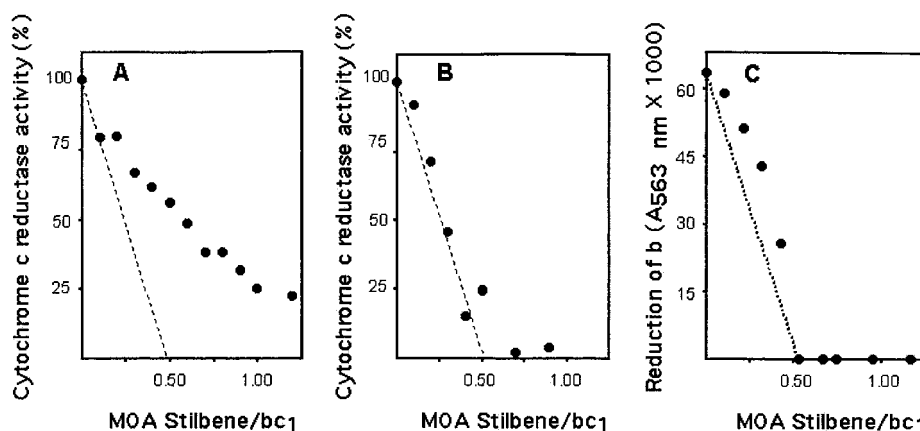


FIG. 4. **Titration of the  $bc_1$  complex with MOA stilbene.** The figure shows titrations of  $bc_1$  complex with MOA stilbene, using the same methods to determine the inhibitor stoichiometries as used with stigmatellin in Fig. 2. A shows an inhibitor titration of the ubiquinol-cytochrome  $c$  reductase activity with 2.5 nM  $bc_1$  complex in the assay. The activity without inhibitor was  $164 \text{ s}^{-1}$  and was used as 100% of the activity for constructing the plot. B shows a cytochrome  $c$  reductase assay titration with 50 nM yeast  $bc_1$  complex. The activity of the enzyme without inhibitor was  $126 \text{ s}^{-1}$ . C shows the titration of the pre-steady state cytochrome  $b$  reduction with  $1.5 \mu\text{M}$  enzyme. The enzyme was pre-mixed with 2 eq of antimycin to block reduction through center N. Each data point is the average of three reactions in the stopped flow spectrophotometer. The dashed line shows a fitted curve with an intercept of 0.5 eq of inhibitor per enzyme.

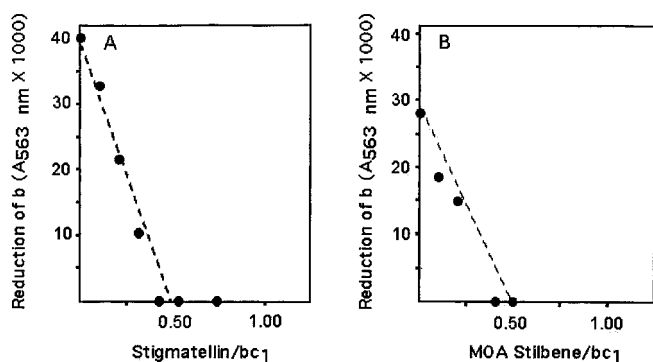


FIG. 5. **Titration of the pre-steady state reduction of cytochrome  $b$  in  $bc_1$  complex from a mutant lacking ubiquinone.** A shows a stigmatellin titration of the pre-steady state reduction of cytochrome  $b$ , using  $1.4 \mu\text{M}$   $bc_1$  complex in the presence of excess antimycin. Each point in the titration represents the average of 3 reactions. B shows a MOA stilbene titration of the pre-steady state reduction of cytochrome  $b$ , using  $1.15 \mu\text{M}$   $bc_1$  complex in the presence of excess antimycin. The dotted lines show fitted curves with an intercept of 0.5 eq of inhibitor per enzyme.

chrome  $b$  when they bind to the  $bc_1$  complex (6, 19). We used this characteristic to obtain a titer for binding of MOA stilbene to the  $bc_1$  complex that does not depend on inhibition of electron transfer within the enzyme. The results of such a titration with  $bc_1$  complex from a wild-type yeast strain are shown in Fig. 6A. The inset shows the difference spectrum that results from the red shift and the increment in absorbance at 568 nm versus 560 nm that was used to quantitate binding of the inhibitor. As increasing amounts of MOA stilbene were added to the enzyme, the absorbance increased in a linear manner until 0.5 eq of inhibitor per  $bc_1$  monomer was bound.

A similar result was obtained when the red shift was titrated in the  $\Delta coq2$  mutant (Fig. 6B), although the titration curve is less linear, suggesting that the inhibitor binds somewhat less tightly in the mutant. With  $bc_1$  complexes from both the wild-type strain and the  $\Delta coq2$  mutant, a second line can be fitted where the absorbance signal increases only slightly, attributable to lower affinity binding in the second monomer. It is not possible to determine accurately the binding constant for the low affinity site from these data, because of the anti-cooperative nature of the interaction between the high and low affinity sites. However, one can estimate from the linear nature of the pre-steady state titration curves at  $1.5 \mu\text{M}$  enzyme (Fig. 4C) and

the biphasic titration of the red shift at  $2.8 \mu\text{M}$  enzyme that the  $K_d$  value for the low affinity MOA stilbene site must fall between these two concentrations.

**Titration of the  $bc_1$  Complex with Myxothiazol**—Myxothiazol is a methoxyacrylate that blocks ubiquinol oxidation at center P in a manner like MOA stilbene. The two inhibitors differ, however, in the manner in which they inhibit the yeast  $bc_1$  complex. When ubiquinol-cytochrome  $c$  reductase activity of the  $bc_1$  complex is titrated with myxothiazol, the experimental points fit very well to a theoretical titration curve with a stoichiometry of one inhibitor per  $bc_1$  monomer (Fig. 7A). When the inhibitor is titrated in a cytochrome  $c$  reductase assay, using 50 nM  $bc_1$  complex, some of the data points fall below the theoretical curve for a titer of one inhibitor per  $bc_1$  monomer (Fig. 7B). However, this result was difficult to reproduce, and when myxothiazol is used to inhibit pre-steady state reduction of cytochrome  $b$ , using  $1.5 \mu\text{M}$  yeast  $bc_1$  complex in the assay, the titer for full inhibition is one myxothiazol per  $bc_1$  monomer (Fig. 7C).

We also measured myxothiazol binding to the  $bc_1$  complex by titrating the red shift in the optical spectrum of ferrocytochrome  $b$ . As can be seen in Fig. 8, 1 eq of myxothiazol per  $bc_1$  monomer is required to saturate the shift in the optical spectrum, confirming the results obtained by titrating the inhibitor against electron transfer activities. At higher amounts of myxothiazol there is an additional increment in the optical spectrum beyond a titer of one inhibitor per binding site. This might indicate double occupancy of the myxothiazol binding site or nonspecific binding of the inhibitor at another site on the enzyme.

## DISCUSSION

To understand better the mechanism of ubiquinol oxidation by the cytochrome  $bc_1$  complex, we investigated the interaction of several inhibitors that act on the ubiquinol oxidation site with the isolated yeast  $bc_1$  complex. We found that stigmatellin fully inhibits the enzyme at 0.5 eq per  $bc_1$  monomer; in other words occupancy of half of the inhibitor-binding sites in the dimer fully inhibits the enzyme. This behavior was not noticed when stigmatellin was initially tested in isolated mitochondria of the yeast *Saccharomyces cerevisiae* (20), but this difference can readily be attributed to difficulties in achieving complete equilibration of the inhibitor with the enzyme. The present study is the first to report the titration of the isolated yeast enzyme with stigmatellin. We have shown that one molecule of stigmatellin fully inhibits the dimeric yeast  $bc_1$  complex in two

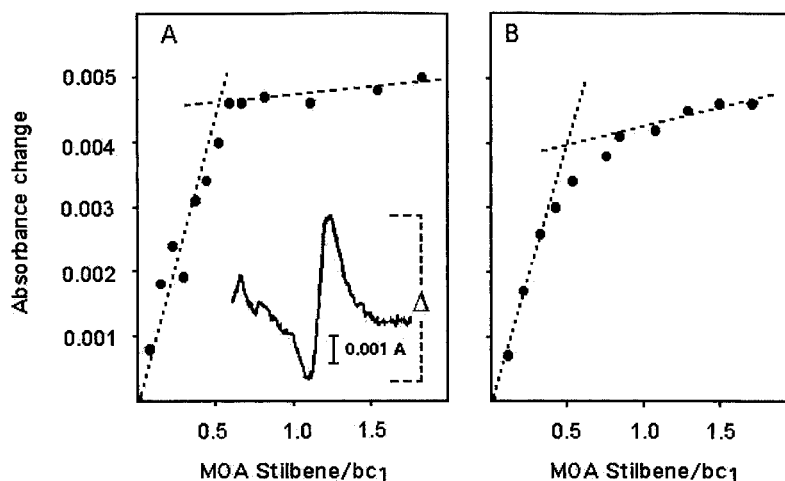


FIG. 6. Titration of the red shift in the cytochrome  $b$  spectrum with MOA stilbene. *A*,  $2.8 \mu\text{M}$   $bc_1$  complex from the wild-type yeast strain, W303a, was reduced with dithionite and titrated with increasing amounts of MOA stilbene. The inset shows the difference spectrum resulting from the red shift in the optical spectrum of the reduced  $b$  upon binding of MOA stilbene and the method used to measure the absorbance increment at 568–560 nm due to the red shift. *B*,  $2.8 \mu\text{M}$   $bc_1$  complex from the  $\Delta\text{coq}2$  mutant was titrated with increasing amounts of MOA stilbene. The absorbance change at each inhibitor concentration was measured as in *A* and plotted against the ratio of inhibitor per  $bc_1$  complex. The dashed lines show fitted curves, assuming high affinity binding of one inhibitor molecule per dimer and binding of a second inhibitor molecule at a low affinity site.

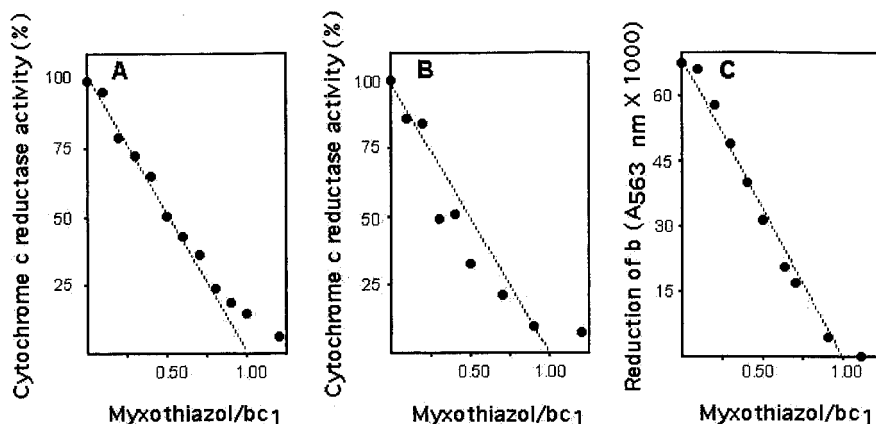


FIG. 7. Titration of the  $bc_1$  complex with myxothiazol. The figure shows titrations of  $bc_1$  complex with myxothiazol, using the same methods to determine the inhibitor stoichiometries as used with stigmatellin and MOA stilbene. *A* shows an inhibitor titration of the ubiquinol-cytochrome  $c$  reductase activity with  $2.5 \text{ nM}$  enzyme. The activity without inhibitor was  $156 \text{ s}^{-1}$ . *B* shows a cytochrome  $c$  reductase assay titration with  $50 \text{ nM}$  yeast  $bc_1$  complex. The activity of the  $bc_1$  complex without inhibitor was  $149 \text{ s}^{-1}$ . Each point in the titration represents the average of four reactions. *C* shows the myxothiazol titration of the pre-steady state reduction of cytochrome  $b$  with  $1.5 \mu\text{M}$  enzyme pre-mixed with 2 eq of antimycin. Each point is the average of three reactions. The dashed lines in the three panels show fitted curves with an intercept of 1 eq of inhibitor per enzyme monomer.

cytochrome  $c$  reductase assays with significantly different concentrations of enzyme and in two pre-steady state assays in which cytochrome  $b$  was reduced through center P.

The extrapolated intercepts of the titration curves also indicate that the binding of stigmatellin is anti-cooperative. Inhibitor binding in one monomer interferes with inhibitor binding to the second monomer. The anti-cooperative binding does not preclude binding of inhibitor to the second monomer. Rather, the binding affinity for the second inhibitor is decreased sufficiently that inhibitor does not bind at the second site in the dimeric enzyme until half of the sites in all of the dimers are occupied with inhibitor. Although the binding of stigmatellin is too tight to accurately determine a  $K_d$  value for the high affinity site from these titration curves, the curvilinear and linear titration curves obtained with  $2.5$  and  $50 \text{ nM}$  enzyme, respectively, are consistent with a  $K_d$  for stigmatellin between these two concentrations. Stigmatellin is seen in both halves of the dimer in the yeast enzyme (5), which is crystallized at a concentration of  $1 \mu\text{M}$  in the presence of a slight excess of stigmatellin, whereas the pre-steady state titration curve (Fig. 2C) is nearly linear at an enzyme concentration of  $1.5 \mu\text{M}$ . Together

these results suggest that the  $K_d$  value of the second site for stigmatellin is  $\sim 1\text{--}1.5 \mu\text{M}$ .

The anti-cooperative, half-of-the-sites inhibitor binding appears to be exclusive to center P inhibitors. In control titrations with antimycin, which inhibits electron transfer at center N, we found that this inhibitor acted with a stoichiometry of one per enzyme monomer, using a low ( $2.5 \text{ nM}$ ) or a high ( $1.5 \mu\text{M}$ ) enzyme concentration. However, in titrating the  $bc_1$  complex with antimycin, we consistently observed a significant lag, or hysteresis, in the titration curves at low antimycin concentrations. This was especially pronounced in the cytochrome  $c$  reductase assays but was also observed, although to a lesser extent, in the pre-steady state reduction of cytochrome  $b$ . A survey of the literature shows that this effect is observed in most antimycin titrations, if data points are reported for low antimycin concentrations.

Hysteresis in an inhibitor titration curve, appearing as a lag in the titration curve at low inhibitor concentrations, indicates that inhibitor is binding without inhibiting the enzyme activity. Binding of the inhibitor to a sub-population of enzyme that is inactive would result in such a titration curve. However,

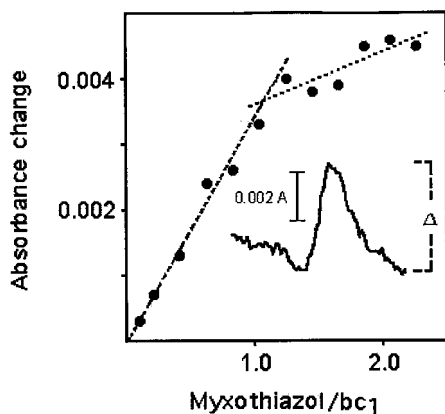


FIG. 8. **Titration of the red shift in the cytochrome  $b$  spectrum with myxothiazol.** Cytochrome  $bc_1$  complex ( $2.4 \mu\text{M}$ ) was reduced with dithionite and titrated with increasing amounts of myxothiazol. The inset shows the difference spectrum resulting from the red shift in the optical spectrum of the reduced  $b$  upon binding of myxothiazol and the method used to measure the absorbance increment at 564–559 nm due to the red shift. The absorbance change at each inhibitor concentration was measured and plotted against the ratio of inhibitor per  $bc_1$  complex. The dashed line shows fitted curves, assuming high affinity binding of one inhibitor molecule per cytochrome  $bc_1$  complex monomer and binding of a second inhibitor molecule at a low affinity site.

when we examined the antimycin titration curves with different enzyme preparations that varied in activity from 140 to 240  $\text{s}^{-1}$ , we found that the more active preparations showed the most pronounced hysteresis. The opposite would be expected if binding to a sub-population of inactive enzyme were occurring.

An alternative explanation for the hysteresis is that there is electron crossover between the two monomers. The proximity of the  $b_L$  hemes in the crystal structures of the  $bc_1$  complex (2–5) would allow for electron transfer from the  $b_L$  heme in one monomer to the other. Another mechanism for inter-monomer electron transfer is that superoxide anion produced by aberrant reactivity of a low potential semiquinone anion at center P could act as a mediator, carrying an electron out of one monomer to reduce the  $b_L$  heme of the other monomer. The generation of superoxide radicals by the  $bc_1$  complex has been reported when antimycin is bound to the enzyme (21–22).

Electron transfer between the  $b_L$  hemes, either directly or mediated by superoxide anion, could account for the hysteresis that is observed in the inhibitor titration curves in the cytochrome  $c$  reductase assays or the oxidant-induced reduction of cytochrome  $b$ , because in these reactions electrons are entering the  $b$  hemes via center P. Either of these mechanisms is also consistent with the observation that the hysteresis in the antimycin titration curve is less pronounced during the pre-steady state reduction of cytochrome  $b$  in which electrons enter the  $b$  hemes via center N. Under these conditions there would likely be less electron crossover between the  $b_L$  hemes, because access to the  $b_L$  hemes is limited due to inhibition of center P with an excess of stigmatellin, and less superoxide anion would be formed by the relatively stable semiquinone at center N. Additional experimentation is in progress to test these possible mechanisms.

To test whether the anti-cooperative, half-of-the-sites binding is exclusive to stigmatellin, we investigated the interaction of two additional Qp site inhibitors, MOA stilbene and myxothiazol, with the yeast  $bc_1$  complex. These methoxyacrylates differ from stigmatellin in their mechanism of inhibition in that they inhibit electron flow from ubiquinol to the iron-sulfur protein. Stigmatellin allows iron-sulfur protein reduction and traps the reduced iron-sulfur protein in a position proximal to cytochrome  $b$ , thus preventing its oxidation by cytochrome  $c_1$  (4, 23).

MOA stilbene also exhibited anti-cooperative, half-of-the-sites binding to the  $bc_1$  complex, but this was less obvious than it was with stigmatellin. This difference can be attributed to a lower affinity of MOA stilbene for the yeast enzyme. In the standard cytochrome  $c$  reductase assay the titer of 0.5 MOA stilbene per enzyme monomer was not as obvious as it was with stigmatellin, due to the curvilinear nature of the titration curve. In this assay the concentration of  $bc_1$  complex is  $2.5 \text{ nM}$ . The reported  $K_i$  value for MOA stilbene is  $14 \text{ nM}$  (19), and a similar value was reported for an independently measured  $K_d = 19 \text{ nM}$  (11). Although these values were obtained with the bovine enzyme, they are consistent with the results we obtained, in which the assays with  $50 \text{ nM}$  or  $1.5 \mu\text{M}$   $bc_1$  complex revealed the half-of-the-sites titer most clearly. The titer of 0.5 eq of inhibitor per  $bc_1$  monomer was confirmed by following the red shift in the cytochrome  $b$  spectrum induced by MOA stilbene binding to the reduced  $bc_1$  complex. This binding-dependent parameter was measured at enzyme concentrations well above the reported  $K_d$  value of the inhibitor and is independent of electron transfer.

Myxothiazol was not an anti-cooperative inhibitor for the yeast  $bc_1$  complex, even at high enzyme concentrations. By titrating cytochrome  $c$  reductase assays, pre-steady state reduction of cytochrome  $b$ , and the red shift in the optical spectrum of the reduced  $bc_1$  complex, we found a stoichiometry for myxothiazol of one inhibitor per enzyme monomer. The titer of one inhibitor per enzyme monomer and lack of anti-cooperativity with myxothiazol was somewhat surprising, because myxothiazol is a methoxyacrylate, like MOA stilbene. The difference in mode of binding of these two structurally related inhibitors implies that very subtle differences in ligand-protein interaction can have profound effects on the binding behavior. Previous titrations with the yeast  $bc_1$  complex reported a titer for myxothiazol of 1.6 molecules of inhibitor per  $bc_1$  complex, extrapolated from the amounts required for 50% inhibition (16). Based on our experience with these inhibitors, we attribute the higher titer in these earlier experiments to incomplete equilibration of the inhibitor with the enzyme.

Anti-cooperative binding in a dimeric enzyme requires that a structural interaction must be transmitted from the ligand-binding site in one monomer to the other. Because the crystal structures of the mitochondrial  $bc_1$  complex show that ubiquinone occupies a cleft that spans the dimer (3–5), we tested whether the anti-cooperative binding of stigmatellin or MOA stilbene was dependent on the presence of endogenous ubiquinone. We found that anti-cooperative binding of stigmatellin and MOA stilbene was retained in a yeast mutant completely devoid of endogenous ubiquinone.

Although the anti-cooperative binding was retained in the ubiquinone-deficient mutant, the titration of the red shift in the cytochrome  $b$  spectrum indicated subtle differences in the titration curves in the mutant compared with the wild-type  $bc_1$  complex, as if binding of the methoxyacrylate to the ubiquinone-deficient mutant was not as profoundly anti-cooperative as in the wild-type strain. Because the anti-cooperative nature of the binding was retained, but slightly diminished, in the  $bc_1$  complex from the  $\Delta\text{coq2}$  mutant, this subtle difference is most likely due to structural changes in the enzyme resulting from lack of ubiquinone during enzyme assembly, and not due to transmission of a structural change within the dimer by ubiquinone. We have found that the  $bc_1$  complex isolated from the ubiquinone-deficient mutant is only about 25% as active as the enzyme from wild-type yeast (results not shown), and others have found that the respiratory enzyme complexes are thermolabile in ubiquinone-deficient strains (24).

Another possible explanation for the anti-cooperative bind-

ing is that the side chain of stigmatellin or MOA stilbene in one monomer extends into the other monomer and inhibits binding of a second molecule of inhibitor by interfering with entry of the side chain into the free monomer. Similarly, one might envision an inhibitor in one monomer might block access of the ubiquinol side chain to the second monomer. We think this explanation can be ruled out by two observations. The crystal structure of the stigmatellin-liganded  $bc_1$  complex (4, 5) shows no direct interaction between stigmatellin molecules, which are 29 Å apart at the closest point in the symmetrical dimer. Also, the half-of-the-sites inhibition was observed in the pre-steady state reduction of cytochrome  $b$  by menaquinol, a substrate that has no side chain, which precludes the possibility of contact between this substrate in one monomer and stigmatellin in the other.

It seems most likely that the anti-cooperative binding of stigmatellin and MOA stilbene involves transmission of a subtle structural change from the center P of one monomer to the other via an interaction between the iron-sulfur protein and cytochrome  $b$ . The iron-sulfur protein extends its cluster-containing domain to form the ubiquinol oxidation site in one monomer, while its transmembrane helix abuts the cytochrome  $b$  helices in the other monomer. In the available crystal structures of the  $bc_1$  complex, there are multiple van der Waals contacts in the abutting regions of these two proteins that could transmit such a change across the dimer. When stigmatellin binds to the  $bc_1$  complex the flexible linker between the extrinsic domain and the transmembrane helix extends and the extrinsic domain of the iron-sulfur protein rotates  $\sim 57^\circ$ . Simultaneously, there is movement of up to 2.3 Å, mainly in the  $\alpha$ -cd1 and  $\alpha$ -cd2 helices and the  $\alpha$ E- $\alpha$ F linker, in cytochrome  $b$  (25).

The crystal structures of the  $bc_1$  complexes with stigmatellin bound have shown the inhibitor bridging the imidazole ring of His-181 and a carboxyl oxygen of Glu-272 (4, 5). If ubiquinol must similarly bridge these two residues to allow a concerted (26) or thermodynamically linked (27) oxidation mechanism, it is easy to envision how small changes in the distance or relative orientation of these two residues could impact significantly on substrate or inhibitor binding. It has been shown already that changes in the structure of the ubiquinol oxidation site inferred from changes to the length of the flexible linker region can have profound effects on the  $K_m$  value for ubiquinol and the  $K_i$  value for stigmatellin (28). At present the only crystal structure of the yeast  $bc_1$  complex is with stigmatellin bound (5). When structures of the yeast  $bc_1$  complex in the native state and with MOA stilbene and myxothiazol bound are obtained, these should provide insight into the structural basis for the anti-cooperative, half-of-the-sites reactivity of this dimeric enzyme.

Stigmatellin and the methoxyacrylate part of MOA stilbene are structurally related to ubiquinol, and it is generally thought that their binding mimics a transition state in ubiquinol oxidation (27). This leads us to propose that ubiquinol binding is likewise anti-cooperative, and that ubiquinol oxidation alternates between the two monomers, with only half-of-the sites reactive at any one time.

There have been two previous reports in the literature that could be interpreted as indicating that the  $bc_1$  complex exhibits half-of-the sites reactivity toward ubiquinol or inhibitory analogs. In experiments with *Rhodobacter capsulatus* chromatophores in which the redox poise was clamped at  $E_h \sim 250$  mV and the ubiquinone pool was expected to be fully oxidized, it was found that one molecule of ubiquinol per  $bc_1$  dimer remained reduced for an interval as long as several minutes (29). Subsequent flash activation resulted in oxidation of this

ubiquinol on the first flash. Although this result was interpreted as indicating a dimeric Q cycle mechanism, it is also consistent with a half-of-the sites mechanism for ubiquinol oxidation of the type we propose.

Also, Fernandez-Velasco and Crofts (30) found a stoichiometry of 0.33–0.4 mol of stigmatellin per mol of cytochrome  $b_H$  in inhibitor titrations using *Rhodobacter sphaeroides* chromatophores, although they interpreted their results as indicating that the  $bc_1$  complex is dimeric and forms ternary complexes in chromatophores. In these experiments the stoichiometry for stigmatellin was not altered by the redox state of the quinone pool. This result agrees with our finding that anti-cooperative binding of stigmatellin is not altered by the absence of quinone in the  $bc_1$  complex.

We had suggested previously that the yeast  $bc_1$  complex exhibits half-of-the-sites reactivity toward cytochrome  $c$  (31), and our current work suggests that a similar mechanism applies to oxidation of ubiquinol. Interestingly, a recent crystal structure of the yeast  $bc_1$  complex co-crystallized with cytochrome  $c$  shows only one molecule of cytochrome  $c$  bound to the dimeric enzyme, and ubiquinone is present in only one-half of the dimer.<sup>2</sup>

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<sup>2</sup> C. Hunte, personal communication.