Inhibitory Analogs of Ubiquinol Act Anti-cooperatively on the Yeast Cytochrome bc1 Complex

EVIDENCE FOR AN ALTERNATING, HALF-OF-THE-SITES MECHANISM OF UBQUINOLOXIDATION*

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The cytochrome bc1 complex is a dimeric enzyme that links electron transfer from ubiquinol to cytochrome c by a proton motive 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 bc1 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 bc1 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 ferrocyanochrome 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 bc1 complex, we propose that the yeast cytochrome bc1 complex oxidizes ubiquinol by an alternating, half-of-the-sites mechanism.

Electron transfer through the cytochrome bc1 complex occurs by the proton motive 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. Crystal structures of the bovine (2, 3), chicken (4), and yeast (5) bc1 complexes have revealed that the mitochondrial cytochrome bc1 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 bc1 complex by acting specifically at center P or center N. The so-called Qn inhibitors block oxidation of ubiquinol at center P and prevent reduction of the high potential redox centers of the bc1 complex. Stigmatellin, hydroxyquinones, and methoxyacrylates such as myxothiazol and MOA3 stilbene, all act at center P (6). The Qn inhibitors block re-reduction of ubiquinone by cytochrome b at center N and block re-reduction of cytochrome b that otherwise can occur by reversal of this reaction. Antimycin, one of the most extensively studied inhibitors of the bc1 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 bc1 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 ferrocyanochrome 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 bc1 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 bc1 Complexes—Yeast cytochrome bc1 complexes were isolated from Red Star cake yeast as described previously (8, 9). The Δcoq2 yeast mutant was obtained from Dr. C. Clarke (UCLA). The wild-type yeast strain, W303a, and the Δcoq2 yeast mutant were grown in 1% yeast extract, 2% peptone, 2% dextrose medium and harvested by centrifugation.

Reduction of Decylubiquinone—The ubiquinol analog, decylubiquinol (DBH2), was used as substrate in the ubiquinol-cytochrome c reductase

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assays and was obtained by reducing the quinone as described by Trumpower 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 μM EDTA. The mixture was reduced by slowly adding solid dithionite and then solid sodium cyanide. The reduced quinone was extracted by adding 1 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 Amino DW2 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, 66.5 μM cm⁻¹ at 300 nm; for myxothiazol, 10.5 μM cm⁻¹ at 313 nm; for antimycin, 4.8 μM cm⁻¹ at 320 nm (6); and for MOA stilbene, 26.5 μM cm⁻¹ at 300 nm (11). All of the inhibitor dilutions were prepared daily, and the concentrations were determined from a calibration curve that was stored.

**Ubiquinol-Cytochrome c Reductase Assays with 2.5 nM bc₁ Complex**—Ubiquinol-cytochrome c reductase activities of the purified bc₁ 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 μM cytochrome c. Cytochrome bc₁ 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 μM DBH₂ (final concentration), and reduction of cytochrome c was monitored from 550–539 nm with the Aminco DW2a™ spectrophotometer in the dual wavelength mode. The extinction coefficient used to calculate cytochrome c reduction was 21.5 μM cm⁻¹ at 550–539 nm (12).

For each inhibitor titration, the bc₁ complex was pre-diluted in assay buffer minus cytochrome c and the concentration determined by difference spectra recorded in the Amino DW2a™ spectrophotometer. The cytochrome c₁ concentration was determined from the difference spectrum of the ascorbate reduced versus ferricyanide-oxidized enzyme, using an extinction coefficient of 17.5 μM cm⁻¹ at 553–548 nm (13). Cytochrome bc₁ complex concentration was determined from the difference spectrum of the sodium dithionite reduced versus ferricyanide-oxidized enzyme, using an extinction coefficient of 25 μM cm⁻¹ at 563–578 nm (13). This pre-diluted enzyme was considered the stock solution, and the concentration was usually 3 μM cytochrome c₁. The activity of this stock solution of enzyme was stable for a week at 4 °C.

After determining the bc₁ complex concentration, the enzyme was diluted to the 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 μM cytochrome c and 0.5 mM KCN. The activity of the bc₁ 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 V₀. At the end of each titration the activity of the bc₁ complex without inhibitor was again determined to check the stability of the enzyme during the experiment. The non-enzymatic reduction of cytochrome c by DBH₂ was subtracted from each activity trace. Because the enzyme was preincubated with inhibitors in the assay buffer containing cytochrome c₁, we could not correct for the non-catalytic rate of cytochrome c reduction by DBH₂ 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 30 nM bc₁ Complex**—Ubiquinol-cytochrome c reductase activities using the higher enzyme concentration were assayed 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 μM bc₁ 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 μM menaquinol. The bc₁ 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 prior to starting the reaction. An oxidized spectrum was obtained by mixing the oxidized bc₁ 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 these three-dimensional data set composed of wavelength, absorbance, and time, the time course and amplitude change for cytochrome c 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 μM bc₁ complex was incubated for 15 min with 8 μM antimycin in assay buffer, pH 7.0, and 30 μM DBH₂ to determine the oxygen level. A base line was obtained by reducing the quinol with 0.5 mM potassium cyanide and 100 μM DBH₂ 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 of wavelength, absorbance, and time, the time course and amplitude change for cytochrome c reduction was calculated from the absorbance increase at 550 nm, using an extinction coefficient of 18.5 μM⁻¹ 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 μM bc₁ 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 μM menaquinol. The bc₁ 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 prior to starting the reaction. An oxidized spectrum was obtained by mixing the oxidized bc₁ 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 these 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.

**Measurement of the Red Shift in the Cytochrome b Spectrum**—The bc₁ complex was diluted to an approximate concentration of 3 μM in assay buffer, and the exact concentration was determined as described above. A base line was obtained by reducing the bc₁ complex with dithionite in both sample and reference cuvettes in the Amino DW2a™ 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 bc₁ Complex with Antimycin**—In the experiments reported below we show that some inhibitors of the bc₁ 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
catalytic assay using 2.5 nM bc1 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 μM in this buffer, it remained stable for 1 week at 4°C. A similar result was reported previously for the bovine bc1 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 bc1 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 μM bc1 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 bc1 complex concentration, the stoichiometry of antimycin per bc1 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 μM bc1 complex, it is clear that the stoichiometry for inhibition of the yeast bc1 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 bc1 Complex with Stigmatellin**—Fig. 2A shows the inhibition of ubiquinol-cytochrome c reductase activity by stigmatellin in a catalytic assay using 2.5 nM bc1 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 bc1 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 Kₜ 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 bc1 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 bc1 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 bc1 complex is present at 1.5 μM (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 bc1 complex. Furthermore, the lack of displacement to values greater than 0.5 eq per cytochrome c₁ 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 bc1 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 bc1 complex is...
partially reduced with DBH2. This reduces the iron-sulfur protein, cytochrome c1, 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 c1, and the Rieske protein. The inset in Fig. 3 shows the redox status of the cytochromes in partially reduced bc1 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 bc1 Complex with MOA Stilbene**—MOA stilbene is a member of the methoxyacrylate class of inhibitors that includes myxothiazol, stroblurin, 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 bc1 complex with MOA stilbene is shown in Fig. 4A. Under these conditions, using 2.5 nM bc1 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 bc1 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 bc1 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 bc1 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 μM bc1 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 bc1 monomer.

**Titration of Inhibitors into bc1 Complex Lacking Endogenous Ubiquinone**—Inhibition of the dimeric bc1 complex by 0.5 eq of inhibitor per bc1 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 bc1 complex, we repeated these experiments with bc1 complex from the acq2 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 bc1 monomer. These results establish that ubiquinone is not responsible for the anti-cooperative binding of these two Qp inhibitors in the yeast bc1 complex.

**Measurement of the Stoichiometry of MOA Stilbene Binding from the Red Shift in the Cytochrome b Spectrum**—Methoxyacyrlates cause a red shift in the α band of the reduced cyto-
Anti-cooperative Inhibition of the Cytochrome bc\textsubscript{1} Complex

Fig. 4. Titration of the bc\textsubscript{1} complex with MOA stilbene. The figure shows titrations of bc\textsubscript{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\textsubscript{1} complex in the assay. The activity without inhibitor was 184 s\textsuperscript{-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\textsubscript{1} complex. The activity of the enzyme without inhibitor was 126 s\textsuperscript{-1}. C shows the titration of the pre-steady state cytochrome b reduction with 1.5 \mu 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\textsubscript{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 M bc\textsubscript{1} complex in the presence of excess antimycin. Each point in the titration represents the average of 5 reactions. B shows a MOA stilbene titration of the pre-steady state reduction of cytochrome b, using 1.15 \mu M bc\textsubscript{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.

The biphasic titration of the red shift at 2.8 \mu M enzyme that the \(K_I\) value for the low affinity MOA stilbene site must fall between these two concentrations.

Titration of the bc\textsubscript{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\textsubscript{1} complex. When ubiquinol-cytochrome c reductase activity of the bc\textsubscript{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\textsubscript{1} monomer (Fig. 7A). When the inhibitor is titrated in a cytochrome c reductase assay, using 50 nM bc\textsubscript{1} complex, some of the data points fall below the theoretical curve for a titer of one inhibitor per bc\textsubscript{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 M yeast bc\textsubscript{1} complex in the assay, the titer for full inhibition is one myxothiazol per bc\textsubscript{1} monomer (Fig. 7C).

We also measured myxothiazol binding to the bc\textsubscript{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\textsubscript{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\textsubscript{1} complex, we investigated the interaction of several inhibitors that act on the ubiquinol oxidation site with the isolated yeast bc\textsubscript{1} complex. We found that stigmatellin fully inhibits the enzyme at 0.5 eq per bc\textsubscript{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\textsubscript{1} complex in two
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 site of the dimeric enzyme. Rather, the binding affinity for the second inhibitor is decreased sufficiently. The binding affinity is not a constant binding to 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 $K_d$ for the high affinity site from these titration curves, the curvilinear and linear titration curves obtained with 2.5 and 50 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 $\mu$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 $\mu$M. Together these results suggest that the $K_d$ value of the second site for stigmatellin is $-1-1.5$ $\mu$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 nM) or a high (1.5 $\mu$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.

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,
MOA stilbene also exhibited anti-cooperative, half-of-the-sites binding to the $b_c$ 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 $b_c$ complex is 2.5 nM. The reported $K_s$ value for MOA stilbene is 14 nM (19), and a similar value was reported for an independently measured $K_d$ = 19 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 nM or 1.5 $\mu$M $b_c$ complex revealed the half-of-the-sites titer most clearly. The titer of 0.5 eq of inhibitor per $b_c$ monomer was confirmed by following the red shift in the cytochrome $b$ spectrum induced by MOA stilbene binding to the reduced $b_c$ complex. This binding-dependent parameter was measured at enzyme concentrations well above the reported $K_s$ value of the inhibitor and is independent of electron transfer.

Myxothiazol was not an anti-cooperative inhibitor for the yeast $b_c$ 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 $b_c$ 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 $b_c$ complex reported a titer for myxothiazol of 1.6 molecules of inhibitor per $b_c$ 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.

Electron transfer between the $b_c$ 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 $b_c$ hemes, because access to the $b_c$ 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 $b_c$ 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).

![Fig. 8. Titration of the red shift in the cytochrome b spectrum with myxothiazol. Cytochrome $b_c$ complex (2.4 $\mu$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 $b_c$ complex. The dashed line shows fitted curves, assuming high affinity binding of one inhibitor molecule per cytochrome $b_c$ complex monomer and binding of a second inhibitor molecule at a low affinity site.](image)

when we examined the antimycin titration curves with different enzyme preparations that varied in activity from 140 to 240 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 an electron crossover between the two monomers. The proximity of the $b_c$ hemes in the crystal structures of the $b_c$ complex (2–5) would allow for electron transfer from the $b_c$ heme in one monomer to the other. Another mechanism for inter-monomer electron transfer is that superoxide anion produced by aberrant electron transfer is that superoxide anion produced by aberrant 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).
ing is that the side chain of stigmatellin or MOA stillbene 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-ligated bc1 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 stillbene 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 bc1 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 bc1 complex the flexible linker between the extrinsic domain and the transmembrane helix extends and the extrinsic domain of the iron-sulfur protein rotates ~57°. Simultaneously, there is movement of up to 2.3 Å, mainly in the α-cd1 and α-cd2 helices and the α-ε-δ linker, in cytochrome b (25). The crystal structures of the bc1 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 Kp value for ubiquinol and the Kc value for stigmatellin (28). At present the only crystal structure of the yeast bc1 complex is with stigmatellin bound (5). When structures of the yeast bc1 complex in the native state and with MOA stillbene 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 stillbene 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 bc1 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 E0 redox/H+ 250 mV and the ubiquinone pool was expected to be fully oxidized, it was found that one molecule of ubiquinol per bc1 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 bc1 in inhibitor titrations using Rhodobacter sphaeroides chromatophores, although they interpreted their results as indicating that the bc1 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 bc1 complex.

We had suggested previously that the yeast bc1 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 bc1 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.2

REFERENCES


2 C. Hunte, personal communication.