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

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

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The cytochrome bc1 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 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 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 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 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 fluoroephosphatase, phenylmethysulfonyl 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 (DBH), was used as substrate in the ubiquinol-cytochrome c reductase

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Determination of Inhibitor Concentrations—Each of the inhibitors was diluted in ethanol, and the concentration was determined by optical spectroscopy in an Aminco DW2TM 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{ m } \text{M}^{-1} \text{ cm}^{-1} at 300 nm; for myxothiazol, 10.5 \text{ m } \text{M}^{-1} \text{ cm}^{-1} at 313 nm; for antimycin, 4.8 \text{ m } \text{M}^{-1} \text{ cm}^{-1} at 320 nm (6); and for MOA stilbene, 26.5 \text{ m } \text{M}^{-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 bc Complex-Assays with 2.5 nm bc Complex**—Ubiquinol-cytochrome c reductase activities of the purified bc 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, and 0.01% Tween 20, and 50 \text{ m } \text{M} cytochrome c. Cytochrome bc 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 \text{ nM} DBH$_2$ (final concentration), and reduction of cytochrome c was monitored at 550–539 nm with the Aminco DW2aTM spectrophotometer in the dual wavelength mode. The extinction coefficient used to calculate cytochrome c reduction was 21.5 \text{ m } \text{M}^{-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 DW2aTM spectrophotometer. The cytochrome c$_1$ concentration was determined from the difference spectrum of the ascorbate reduced versus ferricyanide-oxidized enzyme, using an extinction coefficient of 17.5 \text{ m } \text{M}^{-1} \text{ cm}^{-1} at 553–548 nm (13). Cytochrome bc$_1$ concentration was determined from the difference spectrum of the sodium dithionite reduced versus ferricyanide-oxidized enzyme, using an extinction coefficient of 25 \text{ m } \text{M}^{-1} \text{ cm}^{-1} at 563–578 nm (13). This pre-diluted enzyme was considered the stock solution, and the concentration was usually 3 \text{ m } \text{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 to second time, to 53 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 \text{ m } \text{M} cytochrome c and 0.5 \text{ 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$_c$. 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$_2$ 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$_2$ 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 bc 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 \text{ m } \text{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 \text{ m } \text{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 c reduction at 550 nm was extracted using the OLIS software.

**Oxidant-induced Reduction of Cytochrome b**—Prior to mixing to initiate the oxidant-induced reduction, 3 \text{ m } \text{M} bc$_1$ complex was incubated for 15 min with 8 \text{ m } \text{M} antimycin in assay buffer, pH 7.0, and 30 \text{ \text{mM} } DBH$_2$ to pre-oxidize the bc$_1$ complex. The bc$_1$ complex was added to the bc$_1$ complex after the incubation with DBH$_2$ 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 \text{ m } \text{M} cytochrome c oxidase and 30 \text{ m } \text{M} cytochrome c. A spectrum of the oxidized mixture of cytochrome c plus cytochrome c oxidase was obtained by mixing with equal amounts 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 \text{ m } \text{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 DW2aTM 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**

**Titrations of the bc$_1$ Complex with Antixygen**—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

**Anti-cooperative Inhibition of the Cytochrome bc Complex**
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, 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 Kᵣ 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...
Anti-cooperative Inhibition of the Cytochrome bc₁ Complex

The redox status of the cytochromes in partially reduced bc₁ complex and the increment in cytochrome dimeric nature for this hysteresis is discussed below. 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, strobilurin, and oudemansin (4, 5). 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. 6A and B, suggests that the Kᵢ 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.

FIG. 2. Titration of the bc₁ complex with stigmatellin. A shows a stigmatellin titration of the ubiquinol-cytochrome c reductase activity with 2.5 nM bc₁ complex in the assay. The activity with inhibitor was 147 s⁻¹ and was used as 100% activity for the plot. B shows a titration of 50 nM yeast bc₁ complex and the cytochrome c reductase assay. The activity of the bc₁ complex without inhibitor was 130 s⁻¹. For each inhibitor concentration an average of four assays was used. C shows the titration of 1.5 μM yeast bc₁ 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 bc₁ 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 bc₁ complex.

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 Δcoq2 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—Methoxyacrylates cause a red shift in the α band of the reduced cyto-
the biphasic titration of the red shift at 2.8 \mu M enzyme that the $K_v$ value for the low affinity MOA stilbene site must fall between these two concentrations.

Titrations 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. 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
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 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 $\sim 1-1.5$ $\mu$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. 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,
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 nM. The reported $K_d$ 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 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 structural matrices 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 deltaog2 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).
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-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 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 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-e-f 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 K_m value for ubiquinol and the K_i 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 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 should provide insight into the structural basis for the anti-cooperative, half-of-the-sites reactivity toward ubiquinol and inhibitory analogs. In experiments with Rhodobacter capsulatus chromatophores in which the redox poise was clamped at $E_{m} = \sim 250$ 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

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2 C. Hunte, personal communication.
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

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