We have determined the kinetics of ilicicolin binding and dissociation at center N of the yeast bc1 complex and its effect on the reduction of cytochrome b with center P blocked. The addition of ilicicolin to the oxidized complex resulted in a non-linear inhibition of the extent of cytochrome b reduction by quinol with a shift of the reduced bH heme spectrum, indicating electron transfer between monomers. The possibility of a fast exchange of ilicicolin between center N sites was excluded in two ways. First, kinetic modeling showed that fast movement of an inhibitor between monomers would result in a linear inhibition of the extent of cytochrome b reduction through center N. Second, we determined a very slow dissociation rate for ilicicolin (k = 1.2 × 10^{-3} s^{-1}) as calculated from its displacement by antimycin. Ilicicolin binding to the reduced bc1 complex occurred in a single phase (k_{on} = 1.5–1.7 × 10^6 M^{-1} s^{-1}) except in the presence of stigmatellin, where a second slower binding phase comprising ~50% of the spectral change was observed. This second kinetic event was weakly dependent on ilicicolin concentration, which suggests that binding of ilicicolin to one center N in the dimer transmits a slow (k = 2–3 s^{-1}) conformational change that allows binding of the inhibitor in the other monomer. These results, together with the evidence for intermonomeric electron transfer, provide further support for a dimeric model of regulatory interactions between center P and center N sites in the bc1 complex.

The cytochrome bc1 complex is a multisubunit enzyme that generates an electrochemical gradient across the inner mitochondrial or bacterial membrane by transferring electrons from QH2 to cytochrome c. Structurally, the bc1 complex is a dimer of 9–11 subunits, with redox groups in cytochrome b, the Rieske iron-sulfur protein, and cytochrome c1 (1–4). The binding sites for QH2 and Q, termed center P (or Qo site) and center N (or Q1 site), are present at opposite sites of each cytochrome b subunit, close to the b3 and b4 hemes, respectively (Fig. 1). The two b3 hemes in the dimer are, depending on the organism, within 13–14 Å of each other, a distance that should theoretically allow electron transfer rates of at least 10^4 s^{-1} between the two redox groups (5). The Rieske protein interacts with both monomers by traversing the membrane in a tilted angle from the vicinity of center N of one monomer to center P of the other monomer, where its movable extrinsic domain shuttles one electron at a time from QH2 to cytochrome c1.

We have previously provided experimental evidence of the functional importance of the dimeric structure of the bc1 complex (reviewed in Ref. 6). Our results have indicated that only one center P in the dimer is able to oxidize QH2 when both center N sites are occupied by the tightly bound inhibitor antimycin but that electrons are able to reduce both b4 hemes, implying electron crossover at the level of the b4 hemes (7). We obtained more direct evidence for intermonomeric electron equilibration by blocking both center P sites and titrating cytochrome b reduction by QH2 through center N with antimycin (8). In these experiments, non-linear inhibition by antimycin was obtained, and electrons were observed reaching the b4 heme where antimycin had been bound before the addition of QH2. Assuming that antimycin did not dissociate from center N during the time scale of the experiments (<1 s), we interpreted these results as proof of electron equilibration between center N sites via the b4 heme. However, determination of dissociation constants for center N ligands is required to definitively discard alternative models that attempt to explain non-linear titration curves in terms of fast exchange of inhibitor molecules between center N sites (9, 10).

In the present work, we have analyzed the binding and dissociation kinetics of ilicicolin, a center N inhibitor with a lower affinity than antimycin but that binds almost stoichiometrically to the yeast bc1 complex in the μM range (11). We show that the non-linear inhibition of cytochrome b reduction and the b4 heme spectral shift induced by ilicicolin cannot be attributed to a fast exchange of the inhibitor between center N sites but instead reveals intermonomeric electron equilibration. Furthermore, we show that binding of ilicicolin is also sensitive to the center P occupants in a manner that indicates a dimeric regulation of the bc1 complex based on the position of the extrinsic domain of the Rieske protein. These results support a dimeric mechanism of half-of-the-sites regulation of the bc1 complex that involves non-rate-limiting electron movement between cytochrome b subunits (12).
**EXPERIMENTAL PROCEDURES**

**Materials**—Dodecylmaltoside was obtained from Anatrace. DEAE-Bio Gel A was from Bio-Rad Laboratories. Stigmatellin, myxothiazol, antimycin, and decylubiquinone (2,3-dimethoxy-5-methyl-6-decyl-1,4-benzoquinone) were purchased from Sigma. Ilicicolin was obtained from the Merck sample repository. DBH2 was prepared from decylubiquinone as described previously (13) and quantified by UV spectroscopy using an extinction coefficient of 36 mM$^{-1}$ cm$^{-1}$ at 579 nm (14). Inhibitors were dissolved in ethanol and quantified by UV spectroscopy (15) using extinction coefficients of 4.8 mM$^{-1}$ cm$^{-1}$ at 320 nm for antimycin, 65.5 mM$^{-1}$ cm$^{-1}$ at 267 nm for stigmatellin, 10.5 mM$^{-1}$ cm$^{-1}$ at 313 nm for myxothiazol, and 23.2 mM$^{-1}$ cm$^{-1}$ at 248 nm for ilicicolin (11, 16).

**Purification of Cytochrome bc$_1$ Complex**—Wild-type cytochrome bc$_1$ complex was isolated from Red Star cake yeast as described previously (17), except that dodecylmaltoside concentration was increased to 0.05% in the elution buffers and the volume of DEAE-Bio Gel A was reduced to 25 ml to increase the yield of active enzyme. Quantification of the bc$_1$ complex was performed as described before (18) using extinction coefficients of 17.5 mM$^{-1}$ cm$^{-1}$ at 553–539 nm for cytochrome c$_1$ (19) and 25.6 mM$^{-1}$ cm$^{-1}$ at 563–579 nm for the average absorbance of the b$_{54}$ and b$_{59}$ hemes in cytochrome b (20).

**Inhibition of the Pre-steady State Reduction of the b$_{59}$ Heme and Determination of Spectral Shift by Ilicicolin**—Pre-steady state reduction of cytochrome b was followed at room temperature by stopped flow rapid scanning spectroscopy using the OLIS rapid scanning monochromator as described before (18). For these experiments, 3 μM bc$_1$ complex was incubated with 3.6 μM stigmatellin and the indicated concentration of ilicicolin for 5 min in assay buffer containing 50 mM potassium phosphate, pH 7.0, 2 mM azide, 0.2 mM EDTA, and 0.05% Tween 20. The reaction was started by rapid mixing against an equal volume of the same buffer containing 30 μM DBH$_2$. For each experiment, 8–10 data sets were averaged after subtracting the oxidized spectrum. The time course of absorbance changes at 563–579 nm were extracted using software from OLIS and exported to the Origin 5.0 program (OriginLab Corp.).

Spectra from each kinetic trace collected between 0.2 and 0.3 s were averaged using the OLIS software and exported to Origin. To determine spectral shift displacements induced by ilicicolin bound before the addition of DBH$_2$, the average spectrum collected in the absence of ilicicolin was normalized to create a reference spectrum that was subtracted from those collected in the presence of different ilicicolin concentrations. The normalization procedure has been described previously (8) and involves converting to zero the absorbance of each spectrum at two reference wavelengths and then equalizing the maximum absorbance for the b$_{59}$ heme in the reference spectrum to that of each spectrum collected with ilicicolin. In the present work, we decided to use the isosbestic points at 516 and 569 nm as reference wavelengths instead of 539 and 579 nm, which are minimum absorbance values for cytochrome c$_1$ and b, respectively, resulting in more symmetrical ilicicolin-induced spectral shifts than those reported before in the presence of antimycin (8).

**Kinetic Modeling**—Cytochrome b reduction was simulated using the Dynafit program (Biokin Ltd.), which allows the generation of time-dependent data according to different reaction mechanisms described as a series of kinetic steps (21). The script files describing the mechanisms used for simulations are provided as supplemental data. In all models, an extinction coefficient of 36 mM$^{-1}$ cm$^{-1}$ was assumed for b$_{59}$ reduction based on a 70% contribution of this heme to the total absorbance of cytochrome b (22).

All models assumed equilibration of the oxidized dimer (E) with ilicicolin (I) to form three possible complexes (E$I$, I$E$, and I$I$). In the model that allowed intermonomer electron transfer, association (k$_{on}$) and dissociation rate constants (k$_{off}$) of $0.15 \times 10^6$ M$^{-1}$ s$^{-1}$ and $1 \times 10^{-3}$ s$^{-1}$, respectively, were used for ilicicolin based on experimental values we report in this work. QH$_2$ binding and oxidation at center N resulted in reduc-
Ilicicin Inhibition at Center N of the Dimeric bc₁ Complex

A.

FIGURE 2. Inhibition of the pre-steady state reduction of cytochrome b (cyt b) through center N by ilicicolin. A shows the reduction traces of 1.5 μM stigmatellin-inhibited cytochrome bc₁ complex by 15 μM DBH₂ preincubated in the presence of increasing ilicicolin concentrations (from 0 to 1.2 ilicicolin/BC₁, monomer in 0.085 intervals). Solid curves correspond to the simulated kinetics at each ilicicolin concentration assuming intermonomeric electron transfer between center N sites at a rate of 500 s⁻¹. The maximum extent of reduction at each ilicicolin concentration is shown in B (solid circles) taken from the spectra collected at 0.25 s (inset). The solid curve represents the expected extent of cytochrome b reduction assuming that dimers with only one ilicicolin bound contribute to the total absorbance as much as dimers with no ilicicolin. Enzyme-inhibitor complexes were calculated using the equilibration model described in the supplemental data assuming association and dissociation rates for ilicicolin of 4 × 10⁴ M⁻¹ s⁻¹, respectively.

Results

Non-linear Inhibition of Cytochrome b Reduction by Ilicicolin—The pre-steady state reduction of the stigmatellin-bound bc₁ complex by DBH₂ at different ilicicolin concentrations is shown in Fig. 2. Cytochrome b reduction kinetics were compared with a model that assumed non-rate-limiting intermonomeric electron equilibration (see supplemental data for details), which was able to reproduce the different increase in inhibition at low and high ilicicolin concentrations (Fig. 2A, solid curves). This non-linear inhibition in the extent of cytochrome b reduction (Fig. 2B, circles) was simulated by assuming that inhibition of one center N site per dimer by ilicicolin still allowed both monomers to be reduced to the same extent as dimers with no inhibitor (Fig. 2B, solid curve). The lack of contribution of center P to cytochrome b reduction in the presence of stigmatellin during the time scale of the assay was indicated by the lack of cytochrome c₁ reduction (Fig. 2B, inset).

Unimpeded electron equilibration of both cytochrome b subunits through only one center N site per dimer is consistent with fast electron transfer between monomers. However, an alternative model (9) that is still invoked to explain non-linear inhibition curves in the bc₁ complex (10) proposes the existence of fast intermonomeric movement of tightly bound inhibitors
Ilicicolin Inhibition at Center N of the Dimeric bc₁ Complex

between center N sites. As shown by the simulation in Fig. 3, such a mechanism would result in linear inhibition of cytochrome b reduction by a center N inhibitor. Interestingly, the same kinetic pattern was obtained irrespectively of the value assigned to the rate of the hypothetical intermonomeric inhibitor exchange (not shown), as long as both center N sites are assumed to be simultaneously active. Because movement of an inhibitor between monomers would still result in the same fraction of inhibited center N sites, linear inhibition curves would always be expected in such a mechanism. A variant of this model in which the dissociation rate \((k_{\text{off}})\) for the center N inhibitor was increased while maintaining the \(k_{\text{off}}/k_{\text{on}}\) ratio constant also yielded a linear decrease in the extent of cytochrome b reduction together with a loss of stoichiometric binding (see supplemental Fig. S1).

**Reduction of the b₁ Heme in Ilicicolin-blocked Center N Sites**—Ilicicolin bound at center N induces a blue shift in the absorbance maximum of the reduced b₁ heme (11). However, if substoichiometric concentrations of ilicicolin are bound to the oxidized and center P-blocked bc₁ complex before the addition of DBH₂, no spectral shift is expected to occur unless electrons can equilibrate into the b₁ heme of the ilicicolin-bound center N from the uninhibited monomer. As shown in Fig. 4, such a shift was observed within the first 300 ms after the addition of DBH₂ (Fig. 4A). The amplitude of the ilicicolin-induced shift corresponded to the expected proportion of dimers with one inhibitor bound per dimer (Fig. 4B). This observation indicates that electrons entering the dimer through the uninhibited center N can reach the b₁ heme in the opposite monomer in a few hundred ms, unless ilicicolin is assumed to move within this time scale from an oxidized to a reduced center N site. This last possibility is in conflict with the kinetic analysis of Figs. 2 and 3 (see above) and also requires the doubtful assumption that the stable SQ formed in the vicinity of the reduced b₁ heme can dissociate rapidly to allow ilicicolin binding (8).

**Slow Dissociation of Ilicicolin from Center N**—To examine the possibility of fast ilicicolin dissociation from center N and re-equilibration in the dimer, the dissociation rate of this inhibitor was determined by measuring the rate of its displacement by antimycin, which binds more tightly to center N and generates a distinct red shift in the spectrum of reduced b₁ heme (16). Antimycin binds to available center N sites within a few hundred ms when added at μM concentrations (12). As shown in Fig. 5A, when ilicicolin was already bound to center N, the anti-
Ilicicolin Inhibition at Center N of the Dimeric bc$_1$ Complex

mycin-induced red shift occurred simultaneously with the disappearance of the blue shift caused by bound ilicicolin. The time scale of the displacement by antimycin was in the order of tens of minutes (Fig. 5B), yielding a dissociation rate of ($k_{off} = 1.2 \times 10^{-3}$ s$^{-1}$) for ilicicolin. This value was unmodified by the presence of center P inhibitors during the displacement assay or by varying the concentrations of ilicicolin and antimycin (data not shown), indicating that antimycin and ilicicolin are competing only for center N and not for some unspecific hydrophobic site in the enzyme or in the detergent micelles to which the inhibitors might need to bind before gaining access to cen-

ter N. These results conclusively exclude the possibility of fast movement of ilicicolin between center N sites because this would require a fast dissociation rate from center N that should have resulted in a much faster replacement by antimycin than was experimentally observed.

Binding Kinetics of Ilicicolin to Center N—The time course of ilicicolin binding to the vicinity of the b$_{4}$ heme showed monophasic kinetics in the presence of myxothiazol (Fig. 6A) or in the absence of center P inhibitors (not shown). In contrast, stigmatellin induced a change in the kinetics of ilicicolin binding that resulted in two kinetic phases, each comprising approximately half of the spectral shift amplitude (Fig. 6B). The first phase had a rate similar to that observed when center P was unoccupied or bound with myxothiazol. As shown in Fig. 7A, this fast phase was dependent on ilicicolin concentration, indicating that it was limited by the diffusion of the inhibitor to center N. However, the second slower phase observed only in the presence of stigmatellin was very weakly dependent on the concentra-
tion of ilicicolin, suggesting that a relatively slow change in conformation at half of the center N sites was limiting binding of the inhibitor.

The fast phase of ilicicolin binding to the reduced bc$_1$ complex was insensitive to the presence of DBH$_2$ (Fig. 7B), whereas the slow phase observed in the presence of stigmatellin disappeared at the lowest concentrations of DBH$_2$ assayed, rendering the kinetics of ilicicolin binding monophasic (data not shown). As we have shown previ-
ously for antimycin binding (8), these results suggest a very weak affinity for DBH$_2$ in the reduced complex that prevents competition with binding of center N inhibitors. However, transient binding of DBH$_2$ before mixing with the inhibitor was apparently able to trigger the conformational change that allowed all center N sites to bind ligands in a single phase.

**DISCUSSION**

Ilicicolin is an antibiotic that exerts an inhibitory effect at center N of the bc$_1$ complex in a manner that differs in certain respects from that of antimycin (11). The most notable differ-
Illicicolin Inhibition at Center N of the Dimeric bc₁ Complex

FIGURE 7. Illicicolin binding rates at different inhibitor or DBH₁ concentrations. In A, the rates of illicicolin binding obtained in the absence of center P inhibitor (squares) with myxothiazol (triangles), as well as the initial rate in the presence of stigmatellin (solid circles), were fitted to a linear regression (solid lines) to obtain the binding rate constant for illicicolin ($k_{on}$). The slower illicicolin binding rates obtained with stigmatellin at center P (open circles) showed a very weak concentration dependence, as shown by the very low apparent $k_{on}$ value obtained by a linear fit (dashed line). B shows the binding rate of 60 μM illicicolin to center N in the presence of different DBH₁ concentrations and in the absence of center P inhibitors.

ence is the opposite direction of the spectral shift of the $b_H$ heme absorbance induced by illicicolin with respect to antimycin, which indicates a different effect on the electronic environment of the heme macromolecule. Another difference is the affinity of these two ligands, with antimycin ($K_{d} \sim 10$ ps, according to Ref. 16) binding much tighter to most bc₁ complexes than illicicolin, which inhibits the activity of the yeast and bovine bc₁ complexes with an IC₅₀ of 3–12 and 200–250 nM, respectively (11, 23) and very poorly in the case of the bacterial enzyme (24). The mutations that confer resistance to both inhibitors are also different (23, 24), indicating that several of the residues that interact with each of these molecules are not the same, and that might possibly contribute to the weaker binding of illicicolin. Nevertheless, the fact that both inhibitors displace the stable SQ formed at center N in the presence of antimycin displaces illicicolin (Fig. 5) indicates that their binding sites are overlapping.

In a previous work, we showed that incubation with increasing concentrations of antimycin resulted in cytochrome $b$ reduction kinetics through center N that could not be fitted to a model of fast inhibitor movement (8). In the present work, we have shown that the non-linear inhibition pattern in the extent of cytochrome $b$ reduction upon titration with illicicolin, which we also observed previously with antimycin, cannot be reproduced by assuming fast exchange of inhibitors (Figs. 2 and 3). Careful examination of the original work where such a model was first proposed (9) reveals that non-linear curves are expected only if active sites in the dimer are assumed to function in an alternating fashion so that a single movable inhibitor molecule always shifts to the site that is already inactive and therefore does not inhibit the overall reaction. Our kinetic modeling shows that if the two sites in the dimer are assumed to be active from the outset, fast or slow inhibitor movement becomes irrelevant in terms of the fraction of inhibited sites in the population of enzyme (Fig. 3).

Other authors have claimed that non-linear titration curves can be generated if the inhibitor blocks a non-rate-limiting step in the reaction (10). That argument is not applicable to our present results because the inhibitor used directly blocks the only reaction measured, that is, cytochrome $b$ reduction by QH₂ at center N. Therefore, the only mechanism that can explain the kinetic pattern of inhibition by center N inhibitors is intermonomeric electron transfer (Fig. 2).

As we have discussed elsewhere (6), electron equilibration from one center N to the other in the dimer is expected to occur in <20 ms based on the distance between heme groups and electron tunneling calculations. Recently, it has been argued that $b_L$ to $b_H$ electron transfer does not exist based on the linear inhibition by myxothiazol of cytochrome $b$ reduction through center P, and poorly characterized interference effects were invoked in an attempt to justify how two heme groups at such a close distance from each other do not share electrons across the dimer interface (10). However, it is difficult to conceive a logical reason for natural selection to conserve a close distance between the $b_H$ hemes across the phylogenetic scale without taking advantage of the beneficial effects that electron equilibration would have in maintaining cytochrome $b$ maximally oxidized (8). Furthermore, we have already explained why myxothiazol inhibition is in fact expected to be linear based on the reported half-of-the-sites activity of the center $P$ sites in the dimer (6), rendering such titration curves irrelevant in terms of proving or disproving intermonomeric electron transfer.

We previously reported that one antimycin molecule bound to oxidized dimers was able to generate a red shift in the reduced $b_H$ heme absorbance upon the addition of QH₂ (8), indicating that electrons could rapidly equilibrate from the uninhibited center N to the heme in the blocked monomer. The reasonable assumption was made that antimycin could not dissociate from the oxidized center N to bind to a reduced site. However, we did not experimentally determine the dissociation rate for antimycin at that time. In the present work, we show that illicicolin also induces a spectral shift in those dimers where one inhibitor was bound before the addition of QH₂ (Fig. 4). More importantly, we experimentally demonstrate that this effect is not due to a fast dissociation of illicicolin from center N (Fig. 5). A very slow dissociation rate was also reported (although no numerical value was provided) for funiculosin (26), another center N inhibitor with an affinity similar to that of illicicolin (24). Thus, illicicolin can still be defined as a tightly bound or pseudo-irreversible inhibitor in the case of the yeast
Ilicicolin Inhibition at Center N of the Dimeric bc₁ Complex

bc₁ complex, mainly due to its very slow dissociation rate from center N, which was found to be in the range of tens of minutes.

The values we have obtained for the ilicicolin association (Kₐ₁ = 1.5–1.7 × 10⁸ M⁻¹ s⁻¹, Fig. 7A) and dissociation rate constants (Kₐ₋₁ = 1.2 × 10⁻³ s⁻¹, Fig. 5) allow us to calculate a Kₑ of 7–8 nm, which is within the range of Kₑ values reported before from activity assays using yeast mitochondrial membranes or isolated enzyme (11). There is consequently an ~1000-fold difference in the Kₑ of antimycin and ilicicolin. Because the kₐ value we have previously reported for antimycin (12) is only five times higher than the present value for ilicicolin, we conclude that most of the difference in affinity between the two inhibitors is attributable to the dissociation rate, which can thus be estimated to be 200 times lower for antimycin or close to 6 × 10⁻¹ s⁻¹. This means that antimycin dissociates from center N in a time scale of days, and not in ms, as implied by models that have proposed fast movement of inhibitors between center N sites within (or even between) dimers (9). Consequently, our previous and present results regarding the spectral shift induced by antimycin or ilicicolin bound to the oxidized enzyme provide direct evidence of intermonomeric electron equilibration between cytochrome b subunits.

Electron movement between monomers in the bc₁ complex is especially relevant under physiological conditions in which the mitochondrial transmembrane potential favors electron occupancy at the b₄ hemes by decreasing the effective potential difference with respect to the b₄ hemes (27). As discussed before (8, 28), intradimeric electron equilibration would also aid in maintaining forward electron flow at higher QH₂/Q ratios that might exist under pathological conditions, such as ischemia (29). Dimeric functioning of the bc₁ complex under these conditions would minimize inhibition of cytochrome b oxidation and subsequent electron leakage to oxygen at center P caused by an excess of QH₂ binding at center N.

Further evidence for the functional relevance of the dimeric structure of the bc₁ complex comes from the effect that the position of the Rieske protein has on half of the center N sites as evidenced by the biphasic binding of ilicicolin in the presence of stigmatellin (Fig. 6). We have previously discussed similar results with antimycin to propose a model in which the simultaneous location of the two Rieske protein peripheral domains close to the center P sites in the dimer delays or transiently impedes SQ stability at one center N (12). This proposed mechanism was based on the concentration independence of the second phase of antimycin binding, which we interpreted as reflecting a conformational change that was transmitted from one center N site to the other upon initial binding of antimycin to one monomer. Interestingly, the rate of the concentration-independent binding event we have now obtained with ilicicolin (Fig. 7A) is identical to that we determined with antimycin (12), although the diffusion-limited initial phase is different by a factor of ~5. This supports our interpretation that binding of any tight ligand to the second center N site is limited by the same relatively slow conformational change. The poor binding of DBH₂ to center N in the reduced enzyme as evidenced by the lack of its effect on antimycin (12) and ilicicolin (Fig. 7B) binding rates is consistent with other kinetic results that suggest that center N binds QH₂ preferentially when the b₄ heme is oxidized and Q when the heme is reduced, favoring the formation of SQ-b₄⁺ complexes that maintain cytochrome b favorably poised to accept electrons from center P (25). Therefore, we conclude that the binding properties of tight inhibitors at center N resemble those of the stabilized SQ, evidencing the regulatory interactions between center P and center N sites that impede simultaneous activity of the four QH₂/Q binding sites in the bc₁ complex dimer to promote optimal electron flow (6).

REFERENCES