

Regulatory Interactions between Ubiquinol Oxidation and Ubiquinone Reduction Sites in the Dimeric Cytochrome bc_1 Complex^{*[5]}

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We have obtained evidence for conformational communication between ubiquinol oxidation (center P) and ubiquinone reduction (center N) sites of the yeast bc_1 complex dimer by analyzing antimycin binding and heme b_H reduction at center N in the presence of different center P inhibitors. When stigmatellin was occupying center P, concentration-dependent binding of antimycin occurred only to half of the center N sites. The remaining half of the bc_1 complex bound antimycin with a slower rate that was independent of inhibitor concentration, indicating that a slow conformational change needed to occur before half of the enzyme could bind antimycin. In contrast, under conditions where the Rieske protein was not fixed proximal to heme b_L at center P, all center N sites bound antimycin with fast and concentration-dependent kinetics. Additionally, the extent of fast cytochrome b reduction by menaquinol through center N in the presence of stigmatellin was approximately half of that observed when myxothiazol was bound at center P. The reduction kinetics of the b_H heme by decylubiquinol in the presence of stigmatellin or myxothiazol were also consistent with a model in which fixation of the Rieske protein close to heme b_L in both monomers allows rapid binding of ligands only to one center N. Decylubiquinol at high concentrations was able to abolish the biphasic binding of antimycin in the presence of stigmatellin but did not slow down antimycin binding rates. These results are discussed in terms of half-of-the-sites activity of the dimeric bc_1 complex.

The cytochrome bc_1 complex is a dimer of 9–11 subunits in mitochondria (1–3) and 3–4 in bacteria (4) that catalyzes electron transfer from quinol (QH_2)² to cytochrome c coupled to proton movement across the membrane as described by the proton motive Q cycle model (5, 6). The Rieske iron-sulfur protein of each monomer transports one electron from the QH_2 oxidation site (center P) in cytochrome b to cytochrome c_1 . This

electron shuttling by the Rieske protein requires a considerable movement of its extrinsic domain (7–9). The other electron from QH_2 oxidation is transferred to the center N site in cytochrome b through the b_L and b_H hemes, where it reduces Q to form a stable, tightly bound semiquinone (SQ) (10).

When center N is blocked by inhibitors such as antimycin, center P starts to use oxygen as an electron acceptor when cytochrome b becomes reduced after a few turnovers, resulting in the formation of reactive oxygen species (11). A similar situation could potentially arise when the Q pool is highly reduced, which would slow down the capacity of center N to reoxidize the b hemes by binding and reducing Q. We have recently provided evidence for mechanisms that maximize the availability of electron acceptors in cytochrome b in order to prevent reduction of oxygen at center P. We have found that only one center P in the dimer is active when both center N sites are occupied by antimycin (12) and that electrons coming from that QH_2 oxidation site can equilibrate to any of the two b_H hemes in the dimer by means of rapid b_L - b_L electron transfer (13).

According to these previous findings, there would be four b hemes available to accept electrons from the one active center P site in the dimer. Under uninhibited conditions, electron equilibration between the cytochrome b subunits would favor the formation of SQ with oxidized b_H heme at each center N, adding two more electron acceptors per active center P. Our kinetic analysis also suggested that both center P sites can be active simultaneously when only one center N is inhibited by antimycin (12), hinting at possible conformational communication between center P and center N in the dimer. Antimycin is known to increase the susceptibility of the extrinsic domain of the Rieske protein to proteolysis (14), suggesting that occupancy at center N affects its mobility. It has also been found recently that inhibitor binding, as well as mutations at center N, modify the interaction of the Rieske extrinsic domain with center P occupants (15). However, the role of center P-center N conformational communication in regulating the half-of-the-sites-reactivity of the dimeric bc_1 complex has not been explored.

In the present work, we have demonstrated that occupancy of both center P sites by inhibitors that have opposite effects on the mobility of the extrinsic domain of the Rieske protein results in different kinetics of antimycin binding and QH_2 oxidation at center N. Our results support a model in which interaction of both Rieske proteins with center P ligands largely inactivates one center N in the dimer, implying conformational

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² The abbreviations used are: QH_2 , quinol; MQ, menaquinone; MQH₂, menaquinol (2,3-dimethyl-1,4-naphthoquinol); Q, quinone; SQ, semiquinone; DBH₂, decylubiquinol (2,3-dimethoxy-5-methyl-6-decyl-1,4-benzoquinol).

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communication not only between center P and center N but also between center N sites. We discuss these findings as part of a mechanism that allows only one center P in the dimer to be active at a time.

EXPERIMENTAL PROCEDURES

Materials—Dodecylmaltoside was obtained from Roche Applied Science. DEAE-Bio-Gel was obtained from Bio-Rad Laboratories. Stigmatellin was from Fluka. Antimycin, myxothiazol, diisopropyl fluorophosphate, horse heart cytochrome *c*, decylubiquinone, sodium ascorbate, sodium dithionite, and sodium borohydride were purchased from Sigma. Menaquinone (MQ) was synthesized in the laboratory. DBH_2 and MQH_2 were prepared as described before (16, 17). Antimycin, myxothiazol, stigmatellin, and DBH_2 were quantified by UV spectroscopy (18) using reported extinction coefficients (19, 20). MQH_2 was quantified by determining the amount of cytochrome *c* reduced by 50 nM isolated bc_1 complex, assuming a ratio of 2 cytochromes *c* reduced/ MQH_2 oxidized.

Purification of Cytochrome bc_1 Complex—Wild-type bc_1 complex was isolated from Red Star cake yeast as described previously (16, 21). Quantification of the bc_1 complex was performed as reported before (17), using extinction coefficients of $17.5 \text{ mM}^{-1} \text{ cm}^{-1}$ at 553–539 for cytochrome c_1 (22) and $25.6 \text{ mM}^{-1} \text{ cm}^{-1}$ at 562–579 for the average absorbance of the b_H and b_L hemes in cytochrome *b* (23). The amount of endogenous Q copurified with the bc_1 complex was determined as described before (13) and determined to be 1.0–1.2 molecules/ bc_1 monomer.

Kinetics of Antimycin Binding to the bc_1 Complex—The appearance of the red shift of the reduced spectrum of the b_H heme upon antimycin binding was followed at 20 °C by stopped flow rapid scanning spectroscopy using the OLIS Rapid Scanning Monochromator as previously reported (13). Purified yeast bc_1 complex (5–6 μM) in assay buffer containing 50 mM phosphate, pH 7.0, 1 mM sodium azide, 0.2 mM EDTA, 0.05% Tween 20, and, where indicated, 1.2 equivalents/ bc_1 complex monomer of stigmatellin or myxothiazol and varying concentrations of DBH_2 were reduced with a few grains of solid dithionite and mixed rapidly against an equal volume of the same buffer (without enzyme or center P inhibitors) containing different concentrations of antimycin. For each experiment, eight to ten data sets were averaged and the reduced spectrum was subtracted. The wavelength maximum and minimum for the antimycin-induced red shift (565 and 559 nm, respectively) were extracted using software from OLIS. The difference between the two wavelengths was plotted and fitted to a first or second order exponential equation using the Origin 5.0 (OriginLab Corp.) program.

Pre-steady State Reduction of bc_1 Complex—Pre-steady state reduction of cytochrome *b* was followed at 20 °C by stopped flow rapid scanning spectroscopy using the OLIS Rapid Scanning Monochromator as described before (17). Reactions were started by rapid mixing of 3 μM enzyme (expressed as monomers of bc_1 complex) in the same assay buffer used for the antimycin binding experiments containing 3.6 μM stigmatellin or myxothiazol against an equal volume of the same buffer (without enzyme and inhibitors) containing different concen-

trations of MQH_2 or DBH_2 . For each experiment, eight to ten data sets were averaged and the oxidized spectrum was subtracted. The time course of absorbance change at 562 and 578 nm was extracted using software from OLIS. The difference between the two wavelengths was plotted and fitted to a second or third order exponential equation using the Origin program.

Kinetic Modeling—The Dynafit program (Biokin, Ltd.) calculates and solves a system of differential equations that correspond to the time-dependent change in concentration for each species involved in a given reaction mechanism, including substrates and products as well as any other ligands (24). The mechanism is described as a series of individual reaction steps. A family of kinetic traces where one or more ligand concentrations are changed can be fitted globally to one or more kinetic models. An extinction coefficient can be assigned to some of those species that contribute to observed absorbance changes.

Two models were used to fit the cytochrome *b* reduction kinetics by DBH_2 . For simplicity, only b_H was assumed to undergo reduction, and an extinction coefficient of $36 \text{ mM}^{-1} \text{ cm}^{-1}$ was used for this heme group (12, 13). In addition, association and dissociation of DBH_2 , decylubiquinone, QH_2 , and Q were included together with the corresponding electron transfer reactions into single steps, thereby reducing the number of intermediate species. SQ species formed from partial reduction or oxidation of DBH_2 or endogenous Q were assumed not to dissociate from the enzyme. Electron equilibration between the two b_H hemes in the dimer through the b_L hemes (13) was described as a single step. One model assumed that all ligands were able to bind and react with both b_H hemes in the dimer with identical rate constants, whereas the second model considered only one center N to be accessible initially, with the second center N being slowly and irreversibly activated by reaction at the first center N. The complete Dynafit script files are available as supplemental data.

RESULTS

Binding of Antimycin to Center N in the Presence or Absence of Center P Inhibitors—The dithionite-reduced bc_1 complex bound antimycin at the vicinity of the b_H heme with the kinetics shown in Fig. 1. The absorbance red shift induced by antimycin binding when center P was unoccupied or when myxothiazol was occupying center P occurred with monophasic kinetics (Fig. 1A). In contrast, when stigmatellin was present at center N, only half the center N sites were occupied with a rate similar to that observed in the vacant or myxothiazol-inhibited center P (Fig. 1B). The remaining half of center N sites bound antimycin with markedly slower kinetics. The contribution of the fast and slow phase to the total absorbance red shift between different antimycin concentrations and enzyme preparations varied between 45 and 55%, averaging 50% overall. The calculated second order rate constants for the binding of antimycin with or without myxothiazol were practically identical to each other and to the fast binding of antimycin to the stigmatellin-bound enzyme (Fig. 2).

Interestingly, the slow binding phase appeared to be largely independent of the concentration of antimycin (Fig. 2B), suggesting that a process slower than the diffusion of antimycin to center N was delaying binding of this inhibitor to half of the bc_1

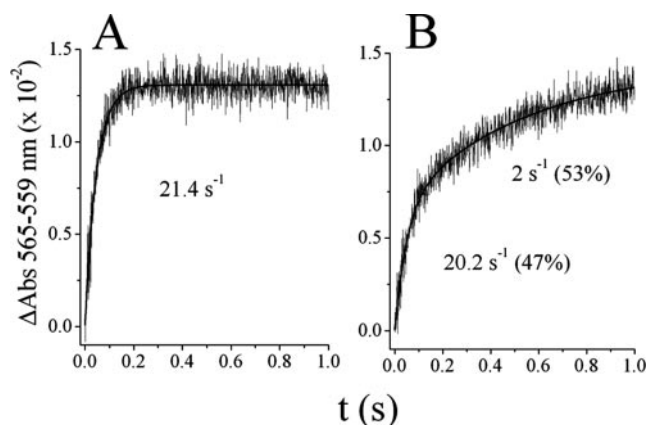


FIGURE 1. **Antimycin binding kinetics in the presence of center P inhibitors.** The time course of the reduced b_H spectral red shift induced by antimycin ($20 \mu\text{M}$) binding to the dithionite-reduced bc_1 complex ($2.5 \mu\text{M}$ final) was determined in the presence of $3 \mu\text{M}$ myxothiazol (A) or stigmatellin (B). The indicated binding rates were obtained by fitting the kinetic traces to a one-exponential (A) or two-exponential (B) function. The relative amplitude of each kinetic phase is indicated in parentheses in panel B.

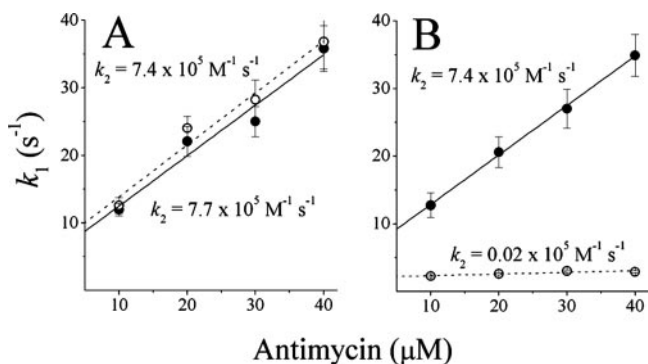


FIGURE 2. **Concentration dependence of antimycin binding rates.** The rates of appearance of the antimycin-induced red shift with no center P inhibitors (A, open circles), in the presence of myxothiazol (A, solid circles) or stigmatellin (B, solid circles, fast phase; open circles, slow phase) were plotted as a function of antimycin concentration. Each family of points was fitted to a straight line with the value of the slope corresponding to the second order rate constant of antimycin binding to center N. Center P inhibitors and bc_1 complex concentrations were as in Fig. 1.

complex monomers. Because only one equivalent of antimycin/ bc_1 complex monomer was still sufficient to achieve maximal red shift even in the presence of stigmatellin (data not shown), the slow antimycin binding to half of the center N sites was not accompanied by a significant loss in overall affinity toward the inhibitor. In these experiments, center P inhibitors were added at an almost stoichiometric ratio (1.2 equivalents/ bc_1 complex monomer), and given that the same red shift kinetics were observed with higher (2 or 3 equivalents) concentrations of stigmatellin or myxothiazol (data not shown), the observed differences in antimycin binding cannot be attributed to unspecific binding of the center P inhibitors at center N.

Reduction of b_H Heme by MQH₂ in the Presence of Myxothiazol or Stigmatellin—To determine whether the center P inhibitor-dependent binding pattern of antimycin was relevant to the interaction of substrate at center N, the low redox potential substrate MQH₂ ($E_{m7} = -70$ mV) was used to reduce cytochrome *b* through center N (Fig. 3). In the presence of myxothiazol, MQH₂ reduced $\sim 90\%$ of the b_H hemes in a fast single phase, whereas stigmatellin bound at center P only

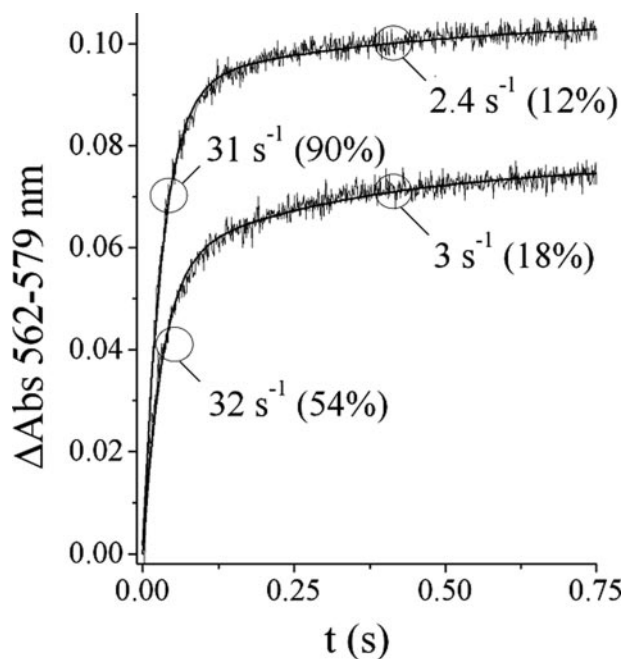


FIGURE 3. **Cytochrome *b* reduction by MQH₂ in the presence of center P inhibitors.** Cytochrome bc_1 complex ($1.5 \mu\text{M}$ final) preincubated with 1.2 equivalents of myxothiazol (upper trace) or stigmatellin (lower trace) was reduced with 16 equivalents of MQH ($24 \mu\text{M}$ final). Rate constants were calculated from fitting to a two-exponential function. The relative amount of b_H reduced is indicated in parentheses.

allowed slightly over half of the b_H hemes to undergo rapid reduction. In both cases a slower subsequent reduction was observed that resulted in some b_L heme reduction when myxothiazol was present. In the stigmatellin-inhibited bc_1 complex, less than half of the remaining oxidized b_H heme was reduced during this slower phase. These results are consistent with the observed antimycin binding kinetics (Fig. 1) and indicate that half of the monomers are not able to bind MQH₂ rapidly when stigmatellin is occupying center P. The fast reduction through center N had the same rate regardless of the center P inhibitor added, suggesting that the affinity for MQH₂ binding and the stability of menaquinone (formed after the one electron oxidation of MQH₂) are the same in all those center N sites that are able to react with the substrate (half of the total in the presence of stigmatellin or all of them with myxothiazol).

Reduction of b_H Heme by DBH₂ in the Presence of Myxothiazol or Stigmatellin—Because decylubiquinone has the same benzoquinone ring structure and redox potential ($E_{m7} = 90$ mV) as the natural Q substrate of the bc_1 complex, DBH₂ was used to compare the effect of myxothiazol and stigmatellin on center N reduction kinetics. As shown in Fig. 4A, $\sim 50\%$ of the b_H hemes were reduced by DBH₂ in the presence of myxothiazol, mostly in a single fast phase.

In contrast, a more complicated kinetic pattern was evident when stigmatellin was bound at center P (Fig. 4B). In this case, $\sim 26\%$ of the b_H hemes were reduced with a rate similar to that of the fast phase of reduction observed with myxothiazol. An additional 24% was reduced with slower kinetics, followed by an even lower reoxidation rate that involved $\sim 6\%$ of the total b_H hemes. Once again, these results suggest that only half of the center N sites that react rapidly in the presence of myxothiazol

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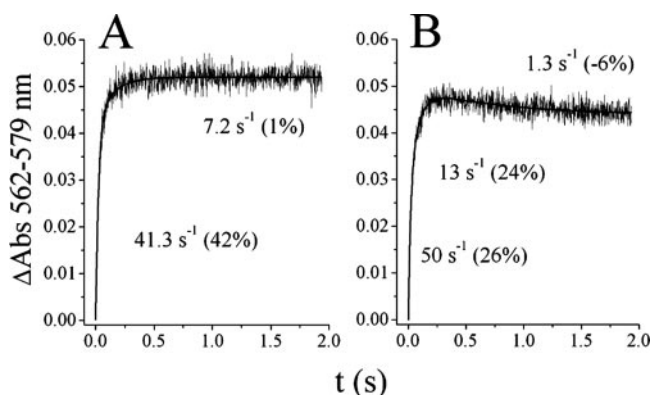


FIGURE 4. Cytochrome b reduction by DBH_2 in the presence of center P inhibitors. Cytochrome bc_1 complex ($1.5 \mu\text{M}$) preincubated with 1.2 equivalents of myxothiazol (A) or stigmatellin/ bc_1 monomer (B) was reduced with 16 equivalents of MQH_2 ($24 \mu\text{M}$). Rate constants were calculated from fitting to a two-exponential (A) or three-exponential (B) function. The relative amount of b_H reduced is indicated in parentheses.

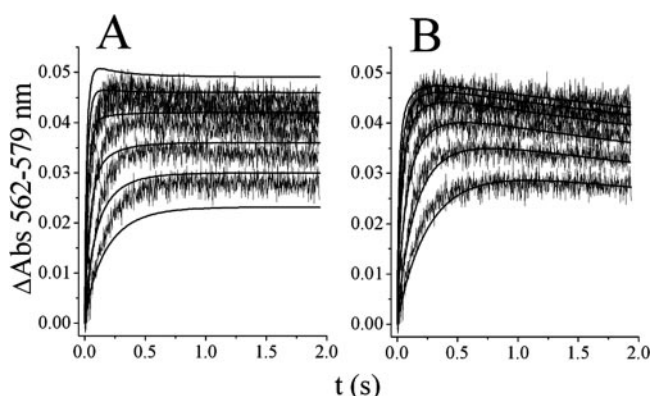


FIGURE 5. Kinetic modeling of cytochrome b reduction by DBH_2 in the presence of center P inhibitors. Cytochrome bc_1 complex ($1.5 \mu\text{M}$) preincubated with 1.2 equivalents of stigmatellin (B) was reduced with 1, 2, 4, 8, 12, and 16 equivalents of DBH_2/bc_1 monomer. Traces were fitted to a model that assumed that both center N sites were equally active (A) or that one center N was inactive until formation of SQ at the active site induced a slow conformational change to activate the second center N site (B). Solid lines correspond to the fitted curves. See supplemental data for details on the kinetic models and fitted values for rate constants.

are able to undergo rapid reduction when stigmatellin is bound at center P. A subsequent slow equilibration of electrons occurs once slower conformational changes allow the other half of the center N sites to bind the ~ 1 equivalent of endogenous Q/monomer that is present in our purified bc_1 complex preparations.

To further test this possibility, we fitted the center N reduction kinetics at different DBH_2 concentrations in the presence of stigmatellin to two different models of electron equilibration within the cytochrome b dimer (Fig. 5; see "Experimental Procedures" and supplemental data for detailed descriptions). When both center N sites were assumed to bind and react simultaneously with DBH_2 and Q to form the corresponding SQ, a poor fitting to the experimentally measured rates was obtained (Fig. 5A). This model could not accurately account for the different extent of b_H reduction at the various DBH_2 concentrations and predicted a very weak reoxidation only at the highest substrate concentration. In contrast, good fitting was obtained using a model that assumed that center N in one monomer could not bind and react with any ligands until a slow

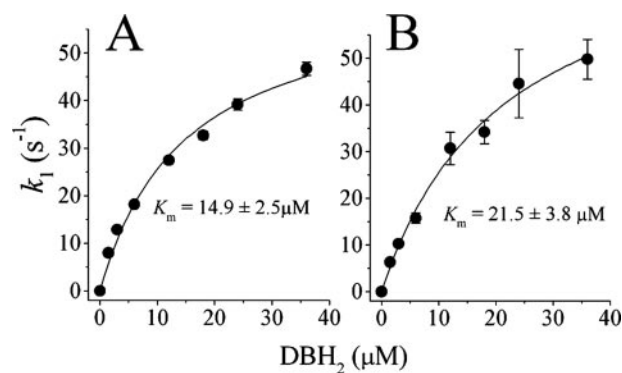


FIGURE 6. Kinetic parameters of cytochrome b reduction by DBH_2 in the presence of center P inhibitors. The rates of the first phase of b_H reduction in the presence of 1.2 equivalents/ bc_1 monomer of myxothiazol (A) or stigmatellin (B) in an experiment similar to that shown in Fig. 5 are plotted as a function of DBH_2 concentration. Data points were fitted to a hyperbolic function to obtain the indicated K_m values for reduction by DBH_2 .

irreversible conformational change occurred upon SQ formation in the active monomer (Fig. 5B).

The fitted value for the rate of the intermonomeric conformational change (0.15 s^{-1}) was one order of magnitude lower than the slow antimycin binding observed in the presence of stigmatellin (Figs. 1B and 2B), suggesting that the SQ formed from DBH_2 oxidation is less efficient than antimycin in eliciting the activation of center N in the second monomer. Interestingly, cytochrome b reduction kinetics in the presence of myxothiazol could be fitted equally well to both models (see supplemental data). This occurred because the value for the rate of intermonomeric conformational change in the second model increased to very high, non-rate-limiting values, rendering this mechanism essentially equivalent to the first model in which both center N sites in the dimer are assumed to react simultaneously.

Further proof that the active center N in the stigmatellin-inhibited bc_1 dimer has the same kinetic properties as the center N sites in the myxothiazol-bound complex was obtained by calculating the affinities for DBH_2 from the fast cytochrome b reduction kinetics in the presence of both inhibitors. As shown in Fig. 6, the K_m values for DBH_2 are similar irrespective of the center P inhibitor and the small difference can be attributed to the increased uncertainty in the rate of the fast b_H reduction with stigmatellin due to the appearance of the reoxidation phase at the highest DBH_2 concentrations.

Effect of DBH_2 on Antimycin Binding to the Center P Inhibited bc_1 Complex—Antimycin binding rates were also determined after adding DBH_2 to the dithionite-reduced bc_1 complex bound with myxothiazol or stigmatellin (Fig. 6). Surprisingly, DBH_2 did not decrease the single rate of antimycin binding in the presence of myxothiazol even at concentrations close to its solubility limit and one order of magnitude higher than its K_m value in the oxidized bc_1 complex (0.15 mM), even though antimycin was used at a low concentration of 2 equivalents/ bc_1 monomer (Fig. 7A). Likewise, in the presence of stigmatellin, the rate of the fast binding of antimycin to half of the center N sites was insensitive to DBH_2 (Fig. 7B). However, DBH_2 at the concentration shown did eliminate the second slow phase of antimycin binding, resulting in a single fast phase (Fig. 7B) sim-

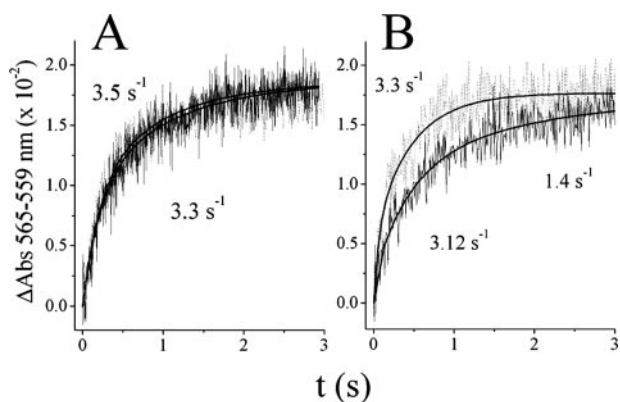


FIGURE 7. Effect of DBH₂ on the kinetics of antimycin binding to center N. The time course of the ferro- b_H spectral red shift induced by 6 μM antimycin binding to 3 μM dithionite-reduced bc_1 complex preincubated with (dotted traces) or without (solid traces) 150 μM DBH₂ was determined in the presence of 3.6 μM myxothiazol (A) or stigmatellin (B). The indicated binding rates were obtained by fitting the kinetic traces to a first order function (after correcting for the change in free inhibitor concentration), with an additional phase in the case of the enzyme incubated with stigmatellin but without DBH₂ (solid trace in panel B) accounting for $\sim 50\%$ of the total red shift. Solid lines correspond to the fitted curves.

ilar to the kinetic pattern observed in the presence of myxothiazol. The disappearance of the slow phase was dependent on DBH₂ concentration, with a K_m similar to that obtained in the oxidized bc_1 complex (data not shown).

This result could suggest that DBH₂ does bind to the active center N in the reduced stigmatellin-bound enzyme, where it is able to trigger the slow conformational change that allows fast binding of antimycin to the second monomer. In this scenario, the lack of decrease in the observed fast binding rate of antimycin to the active center N sites would be due to binding of antimycin to a different form of the enzyme than that to which DBH₂ binds. In this case, however, saturation with DBH₂ would be expected to maintain the enzyme in a conformation different from that to which antimycin binds, resulting in a decreased apparent binding rate for the inhibitor. Therefore, it seems more likely that the reduced bc_1 complex has a very low affinity toward DBH₂ and that during the 1–2 min between the addition of DBH₂ to the reduced enzyme and the moment of rapid mixing with antimycin the substrate was able to transiently bind to the active center N site in the dimer to promote the intermonomeric conformational change that activates the other center N. The high dissociation rate for the bc_1 -DBH₂ complex, however, would prevent significant competition against antimycin, as evidenced by the lack of effect on the inhibitor binding rate to the active center N sites.

DISCUSSION

We have previously shown that only one center P in the dimeric yeast bc_1 complex is active when both center N sites are occupied by antimycin (12) and that electrons coming from that QH₂ oxidation site can equilibrate to either of the two b_H hemes in the dimer by means of non-rate-limiting ($>10^3 \text{ s}^{-1}$) electron transfer between cytochrome b subunits (13). These previous results imply that four b hemes are available to accept electrons from the active center P site in the dimer and that, as our kinetic modeling suggested (13), the formation of the SQ- b_H^{3+} complex at center N is favored even at high QH₂/Q ratios.

Superoxide formation by the bc_1 complex is observed mainly under conditions in which cytochrome b is reduced and center N is inhibited. We have therefore proposed that a mechanism in which electrons coming from only one center P equilibrate rapidly between two cytochrome b monomers that are maintained largely oxidized by SQ formation at both center N sites minimizes superoxide formation (12, 13).

Our present results provide further insight as to how center P-center N communication maintains half-of-the-sites activity in the dimer. We observed that binding of stigmatellin, but not of myxothiazol, to both center P sites produces an asymmetric binding of ligands to the center N sites in the dimer. This was clearly the case for antimycin and MQH₂ (see Figs. 1B and 3), where only half the b_H hemes presented a red shift or under-reduction in a fast phase. The main difference in the effects of stigmatellin and myxothiazol binding to the bc_1 complex is that stigmatellin fixes the position of the extrinsic domain of the Rieske protein close to cytochrome b , completely impeding its movement toward cytochrome c_1 (2, 3). In contrast, myxothiazol allows the free movement of the Rieske protein, as evidenced by the high degree of disorder in the extrinsic domain of the Rieske seen in bc_1 structures with this inhibitor (and in the native enzyme without center P ligands), which indicates multiple conformations (25). Therefore, the fixation of both Rieske domains close to cytochrome b appears to be the signal that initially inactivates one center N in the dimer.

Having the two Rieske proteins fixed close to cytochrome b resembles the situation in which QH₂ oxidation at both center P sites can occur simultaneously. Such a symmetrical configuration at center P can exist only when one center N site is active, as evidenced by our results showing fast binding of antimycin to only one center N/dimer (see Fig. 1B). The fact that antimycin binds slowly to the remaining half of center N sites in a concentration-independent manner (see Fig. 2) can be explained by assuming that a slow conformational change caused by binding to the active center N is transmitted to the inactive site. Our kinetic modeling of cytochrome b reduction kinetics by DBH₂ suggests that not even the endogenous Q that is copurified with the bc_1 complex binds to the inactive center N in the dimer until the active site in one monomer reacts and a slow conformational change occurs. This would explain the partial and slow reoxidation of cytochrome b that is observed only in the presence of stigmatellin (see Fig. 4). These results suggest that both center N sites in the dimer communicate conformational changes to each other and that they are able to sense the position of the extrinsic domain of the Rieske protein at both center P sites and react to it in an asymmetrical manner.

Crystal structures of the bc_1 complex dimer reveal a potential transmission route for conformational communication between center N sites in the dimer, comprised of the amino-terminal region of each cytochrome b subunit, also known as the α -a helix (26). In the yeast bc_1 complex (Fig. 8), the ring of Tyr-16 and the backbone oxygen of Ser-15 are in contact with the conserved His-202, which is critical for Q binding (3) and participates in the interaction with antimycin as revealed by the structure of the bovine bc_1 complex (26). Mutations at the nearby Ile-17 are known to induce resistance to some center N inhibitors such as HQNO (27). The α -a helix extends toward cyto-

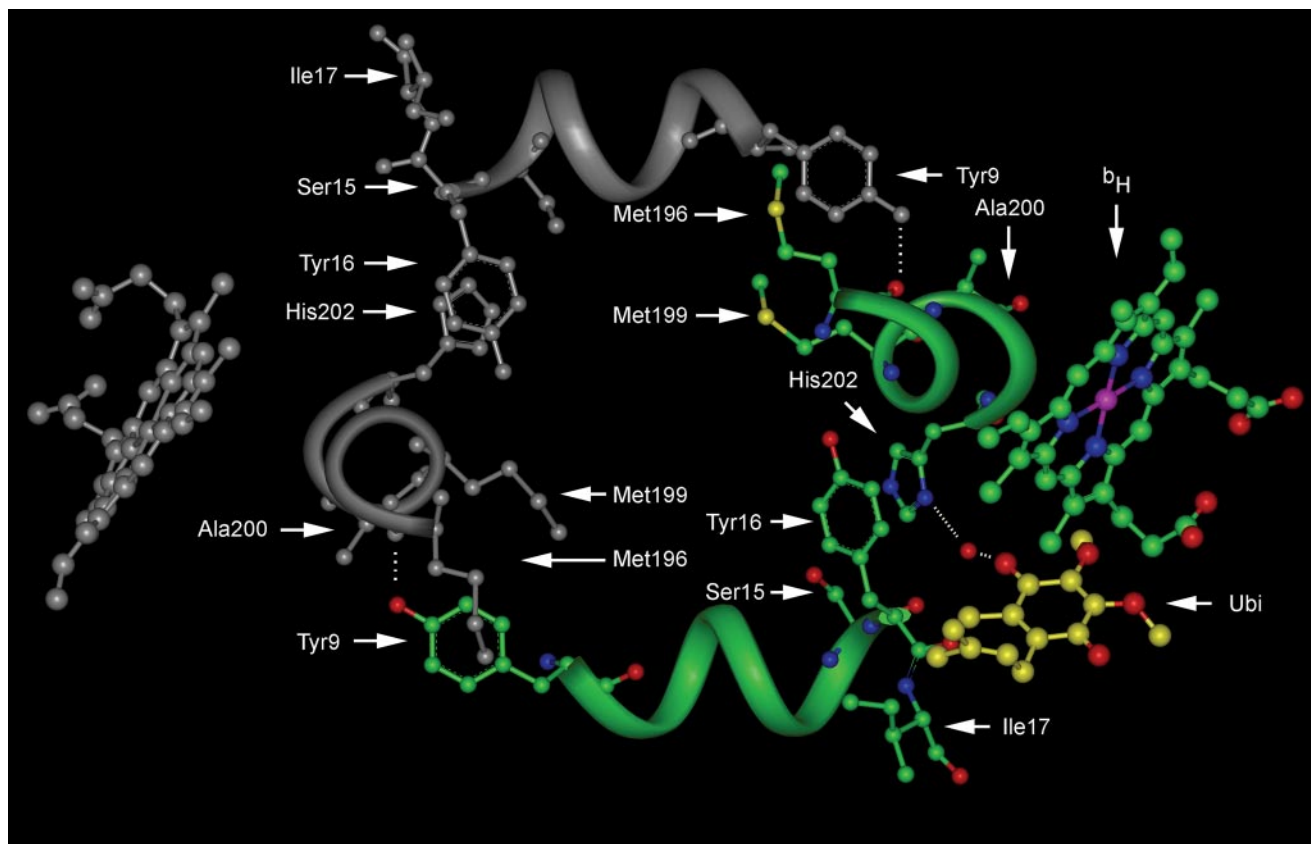


FIGURE 8. **Close-up view of the center N pockets in the yeast cytochrome bc_1 dimer.** The figure shows the b_H hemes and amino acid residues proposed to be involved in conformational communication between center N sites as discussed under "Discussion," which are colored in one monomer and gray in the other. Portions of the α -a helices are shown as ribbons traversing horizontally from one center N to the other. Also shown are ubiquinone (*Ubi*) and the water-mediated hydrogen bond to His-202 in one monomer. The structure is excerpted from the stigmatellin-bound bc_1 complex (Protein Data Bank code 1EZV, Ref. 3).

chrome b of the other monomer, where Tyr-9 forms a hydrogen bond to the backbone oxygen of Met-196 of the opposite cytochrome b and establishes contacts with other atoms of that same residue, as well as of Met-199 and Ala-200, very close to His-202. Although there is poor residue conservation in this α -a helix between different organisms, the overall structure and interaction pattern of this helix are maintained. For example, Asp-31 in *Rhodobacter capsulatus* (4) and Leu-21 in bovine (26) interact with the critical center N His in a similar way as Tyr-16 does in yeast. In the bacterial enzyme, Leu-23 from one cytochrome b monomer contacts Trp-214 and Thr-218, close to the essential His-217, resembling the intermonomeric interactions of Tyr-9 with Met-196 and Met-199 in yeast. Interestingly, most atomic resolution structures available show poor order and multiple conformations in the α -a helix, consistent with a role in conformational communication (26).

Less clear from available crystal structures is the precise pathway that allows center N to be affected by the position of the Rieske protein. However, it is evident that the CD and EF loops and helices that cover center P undergo conformational changes as the extrinsic domain of the Rieske protein moves to and from center P (2, 25, 28). These changes could be transmitted to center N through the transmembrane helices D and E of cytochrome b , where the critical residues for antimycin and Q binding (His-202, Lys-228, and Asp-229) reside. Interestingly, subtle shifts in the EPR spectrum of the b_L heme when antimycin is bound to the bacterial

enzyme or when His-202 (His-217 in *R. capsulatus*) is mutated suggest a change in rigidity in helix D (15).

Even though no changes are evident in any of these helices when comparing the bovine stigmatellin-bound bc_1 structure with or without antimycin (26), or antimycin-bound bc_1 structures with different center P inhibitors (2), our results indicate that the conformational changes that prevent binding to one center N are transient. In the presence of stigmatellin, antimycin does eventually bind to both monomers, and QH_2/Q also appear to be able to equilibrate with the b_H heme in the previously inaccessible center N within a few seconds (see Figs. 1B and 5B). When the enzyme is incubated in the presence of inhibitors for days for crystallization, the dimer has a long time period to relax to a configuration in which both center N sites are available. Further proof for communication between center P and center N comes from the enhanced mobility of the extrinsic domain of the Rieske protein upon antimycin binding to center N as evidenced by the increased susceptibility of the Rieske protein to proteolysis (14) and by the loss of orientation dependence of the iron-sulfur cluster EPR signal (15).

Our observation that DBH_2 does not compete with antimycin for center N binding in the fully reduced enzyme (see Fig. 7) suggests that QH_2 binds with poor affinity to center N when the b_H heme is in the reduced state. The K_m values for DBH_2 binding to the oxidized bc_1 complex (15–20 μM), as determined from b_H reduction at center N (Fig. 6), allow the prediction that a significant

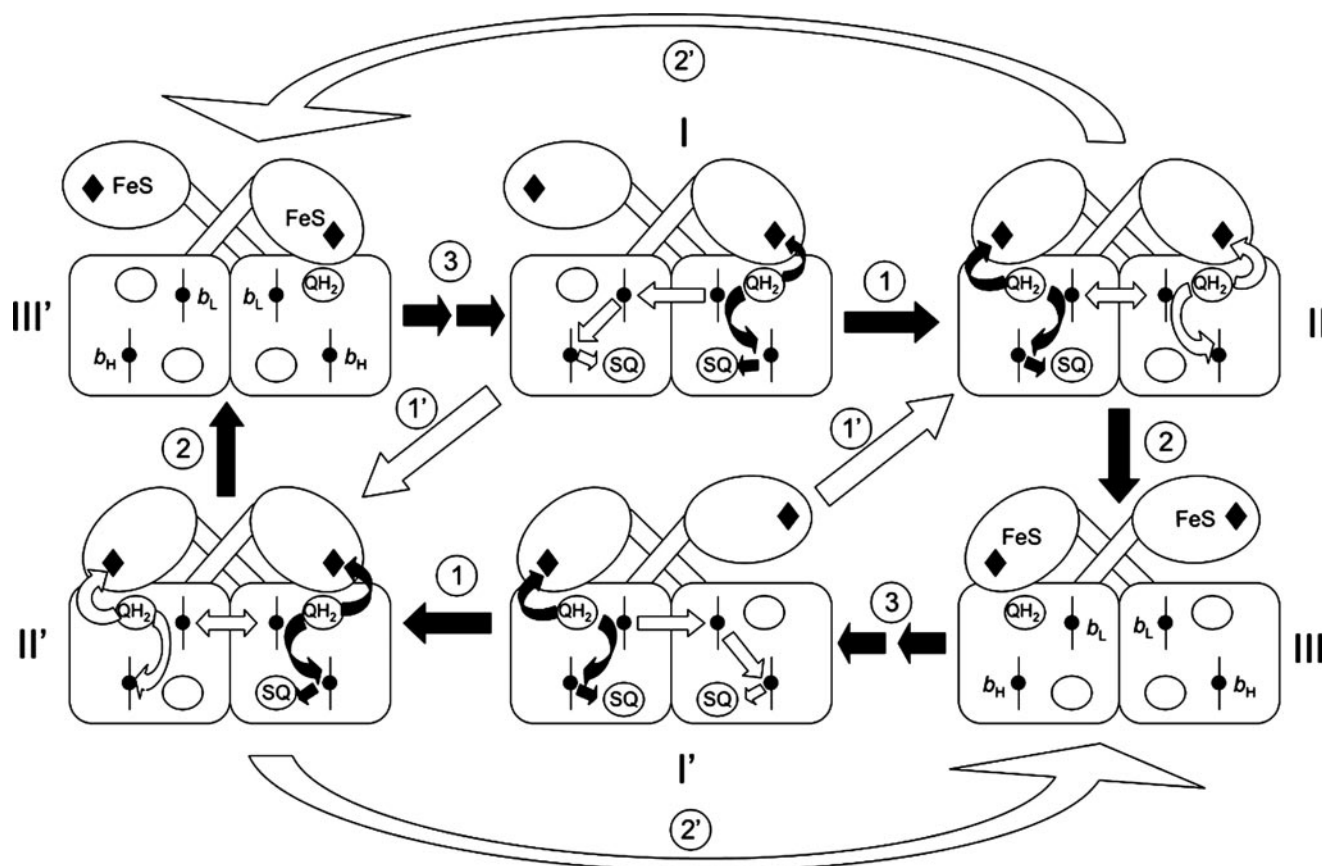


FIGURE 9. **Model of half-of-the-sites activity in the dimeric bc_1 complex.** Cytochrome b subunits are shown as rectangles; the Rieske proteins are depicted as ovals (extra-membrane domain) attached to an α helix (cylinders). Potentially active center P sites are shown as small ovals containing quinol (QH_2) close to the b_L hemes. Center N sites are the small ovals next to the b_H hemes. For simplicity, only occupancy by semiquinone (SQ) is shown at center N, while quinol and quinone binding and release steps have been omitted. Arrows within each dimer represent possible electron transfer steps. Black arrows between dimers represent conformational changes induced by electron transfer steps within a monomer (black arrows inside dimers). White arrows between dimers correspond to conformational changes generated by electron transfer steps between monomers (white arrows between monomers). Double black arrows in step 3 represent the various binding and electron transfer events involved in formation of semiquinone at both center N sites by means of electron equilibration of the b_H hemes with a partially reduced quinone pool. Roman numerals refer to intermediate states, and circled numbers refer to reaction steps as discussed in the text. See under "Discussion" for a full discussion of the model.

slowing of antimycin binding to center N should have occurred upon addition of $150 \mu M$ by DBH_2 , which did not occur.

This result has two important implications. First, because antimycin binds effectively to center N when the enzyme is oxidized (13) or reduced (see Fig. 1), this inhibitor appears to be an analog not of QH_2 , as previously proposed (15), but of tightly bound SQ, which is expected to be stabilized at center N when the b_H heme is oxidized (after its formation by the b_H^{2+} to Q electron transfer) and also when it is reduced (by reduction of b_H by QH_2 , as in Figs. 3–5). The high concentration of DBH_2 added to the reduced and stigmatellin-inhibited bc_1 complex was able to bind transiently to the active center N and transmit the conformational change needed to open the second center N site after a few minutes (see Fig. 7B). The fact that the one equivalent of endogenous Q copurified with the bc_1 complex was not able to induce the same conformational change to activate the second monomer during the few minutes elapsed after addition of stigmatellin (see Figs. 3 and 4) also suggests that the affinity of center N for Q when b_H is oxidized is low compared with when the enzyme is reduced. Therefore, the only physiological center N ligand that shares the tight and b_H redox-independent binding pattern shown by antimycin is SQ.

Second, binding of Q, but not of QH_2 , to center N sites containing b_H^{2+} would logically result in a preferential formation of $SQ-b_H^{3+}$ complexes at center N. Even when the Q pool was in a highly reduced state, the few Q molecules present would still be able to readily bind to and accept electrons from center N sites with b_H^{2+} without competition from QH_2 , which would bind only when b_H was oxidized. This would maximize cytochrome b oxidation through center N throughout a wide range of redox states of the Q pool. Because superoxide formation occurs only when the b hemes are not available to accept the second electron from QH_2 oxidation at center P, electron leakage to oxygen would also be minimized.

Based on our previous findings (12, 13) and the present results, we propose a mechanism for the half-of-the-sites activity in the bc_1 complex (29). In this model (Fig. 9), formation of SQ at both center N sites in the dimer induces one of the center N sites to transmit a change through the D or E helices of cytochrome b that alters the CD-EF surface regions where the Rieske protein docks, preventing interaction of the Rieske His-161 with the substrate and impeding oxidation at that center P (I and I' intermediates in Fig. 9). This configuration can be fixed by having two antimycin molecules/dimer, with only one center

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P able to oxidize QH_2 (12). Catalysis at the active center P (*step 1* in Fig. 9) reduces one of the two b_H hemes in the dimer (b_L to b_L electron transfer would allow either b_H heme to be equally likely to be reduced), causing one of the two SQ molecules stabilized at center N to accept an electron and vacate center N as QH_2 (*II* and *II'* intermediates in Fig. 9).

With only one SQ present in the dimer, the extrinsic domain of the Rieske protein in the previously inactive center P would then be able to dock to the surface of cytochrome *b* to oxidize QH_2 , and, at least transiently, both center P sites would have the Rieske protein available to interact with the center P substrate (also shown in the *II* and *II'* intermediates in Fig. 9). This configuration of the dimer would be similar to the condition in which two stigmatellin molecules fix the extrinsic domain of both Rieske proteins proximal to cytochrome *b* at center P and only one center N is able to bind ligands rapidly (see Figs. 1*B* and 3*B*). If antimycin binds to only one monomer and some short time interval is required for the activation of the second center N, this dimer conformation would allow simultaneous steady-state activity at both center P sites, as we have proposed to explain the increased activity that is observed after incubation of the bc_1 complex with low antimycin concentrations (12). In the absence of inhibitors, the state with only one SQ/dimer and both Rieske proteins able to oxidize QH_2 (*II* and *II'*) would be short lived, because the single SQ at the asymmetrical center N would quickly be reduced to QH_2 by an electron from one of the two active center P sites (*step 2* in Fig. 9).

The absence of SQ from both center N sites (*III* and *III'* intermediates) implies a regained symmetry that can reactivate the dormant center N and again inactivate one center P in the dimer by changing the cytochrome *b* CD-EF docking surface in one monomer. This symmetric center N configuration without SQ at any site corresponds to the situation where both center P sites are vacant or occupied by myxothiazol, in which ligands can bind and react at both center N sites (see Figs. 1*A* and 3*A*). The two vacant and active center N sites can rapidly equilibrate again with the partially reduced Q pool (*step 3*) to reform the initial double SQ configuration in which only one center P is active (intermediates *I* and *I'*).

Because of rapid b_L - b_L transfer, the electron coming from the active center P would have the same probability of reducing either of the two SQ molecules in the dimer. Whenever the electron crosses over to reduce SQ in the opposite monomer (*step 1'*), SQ will remain in the originally active monomer. During the transient symmetric P/asymmetric N state (*II* and *II'*), the electron from one of the two active center P sites can also cross over to reduce SQ in the other monomer (*step 2'*). Thus, electron transfer between cytochrome *b* subunits allows center P in each monomer to alternate randomly between the active and inactive states. This results in an alternating site mechanism (29) in which each center P turns over the same number of times on average over long periods of time.

This model implies that the bc_1 complex dimer will exist predominantly in a conformation that allows up to six redox acceptors (four *b* hemes and two bound SQ molecules) for each center P that oxidizes QH_2 . A very similar dimeric structure of this enzyme exists in bacteria, yeast, and vertebrates (1–4). We

have also observed that only half of the center P sites are active when antimycin is occupying both center N sites in the dimer in the bc_1 complexes from *Paracoccus denitrificans* and bovine mitochondria,³ as we have reported for the yeast enzyme (12). Therefore, the dimeric architecture of the bc_1 complex, with its b_L - b_L electron transfer that allows electron transfer between the two monomers, its high tendency to maintain cytochrome *b* oxidized by forming SQ at center N, and its half-of-the-sites reactivity, seems to have been selected very early and retained during evolution to minimize the formation of damaging reactive oxygen species while maintaining a maximal turnover rate over a broad range of redox potentials of the Q pool.

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