Rapid Electron Transfer between Monomers when the Cytochrome bc₁ Complex Dimer Is Reduced through Center N*

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We have obtained evidence for electron transfer between cytochrome b subunits of the yeast bc₁ complex dimer by analyzing pre-steady state reduction of cytochrome b in the presence of center P inhibitors. The kinetics and extent of cytochrome b reduction by quinol in the presence of variable concentrations of antimycin decreased non-linearly and could only be fitted to a model in which electrons entering through one center N can equilibrate between the two cytochrome b subunits of the bc₁ complex dimer. The b₃₄ heme absorbance in a bc₁ complex inhibited at center P and preincubated with substoichiometric concentrations of antimycin showed a red shift upon the addition of substrate, which indicates that electrons from the uninhibited center N in one monomer are able to reach the b₃₄ heme at the antimycin-blocked site in the other. The extent of cytochrome b reduction by variable concentrations of menaquinol could only be fitted to a kinetic model that assumes electron equilibration between center N sites in the dimer. Kinetic simulations showed that non-rate-limiting electron equilibration between the two b₃₄ hemes in the dimer through the two b₃₄ hemes is possible upon reduction through one center N despite the thermodynamically unfavorable b₃₄ to b₃₄ electron transfer step. We propose that electron transfer between cytochrome b subunits minimizes the formation of semiquinone-ferrocytochrome b₃₄ complexes at center N and favors ubiquinol oxidation at center P by increasing the amount of oxidized cytochrome b.

According to the protonmotive Q¹ cycle (1, 2), the cytochrome bc₁ complex is able to move charges across the membrane in which it is embedded by using one of the two electrons obtained from quinol oxidation at center P to reduce quinone bound at center N. A key feature of this cycle is that the semiquinone formed at center N after one center P turnover needs to be stabilized until the second electron from another QH₂ oxidation at center P can arrive. A stable SQ at center N has been detected by EPR spectroscopy in the bc₁ complex from different sources (3–6), and this stabilization helps to explain why superoxide formation at center N is virtually non-existent when center P is blocked with stigmatellin (7, 8). However, considering that reactions at center N are fully reversible, stabilizing SQ at center N should also favor the one electron transfer from QH₂ to heme b₃₄. When heme b₃₄ is reduced, center P catalysis is slowed down, as has been observed when antimycin blocks cytochrome b reoxidation or when there is no Q available to oxidize heme b₃₄ (9). Therefore, a mechanism should exist that avoids b₃₄ reduction through center N without destabilizing SQ at that site.

Crystal structures of the bc₁ complexes from various sources (10–13) show a dimeric structure in which the b₃₄ hemes of each monomer are close to each other with an edge-to-edge distance ranging from 10.4 to 11.2 Å (Fig. 1). According to electron tunneling calculations, such distance should allow electron transfer at a rate in the order of 10⁻⁶ to 10⁻⁷ s⁻¹ (14). Before this structural information was available, kinetic and spectroscopic evidence was used to propose direct electron equilibration between the two center N sites of the dimer (15, 16). However, such equilibration was assumed to occur by direct electron transfer between the Q molecules bound at the two center N sites and not by electron communication between the b₃₄ hemes. Crystal structures have since shown such transfer to be impossible due to distance constraints.

We have recently provided evidence that two b₃₄ hemes per bc₁ dimer can be reduced through one center P when both center N sites are blocked with antimycin and that the stimulation of steady state catalysis by low concentrations of antimycin requires both monomers to use only one center N for Q reduction (17). However, rapid mobility of an antimycin molecule between the two N sites of the dimer has also been proposed to account for non-linear inhibition of steady state activity (18). In the present work, we have analyzed the pre-steady state kinetics of reduction through center N and conclude that electron equilibration between cytochrome b subunits through the b₃₄ hemes of the dimeric bc₁ complex is the only model consistent with the experimental data. We also suggest that a possible function of this intermonomer electron communication is to use the stabilization of SQ at center N to maintain the b₃₄ hemes in the oxidized state, ensuring a maximal rate of QH₂ oxidation at center P.

EXPERIMENTAL PROCEDURES

Materials—Dodecylmaltoside was obtained from Roche Applied Science. DEAEBio-Gel was obtained from Bio-Rad Laboratories. Stigmatellin was from Fluka. Antimycin, myxothiazol, diisopropyl fluorophosphate, phenylmethylsulfonyl fluoride, horse heart cytochrome c, decylubiquinone, sodium ascorbate, sodium dithionite, and sodium borohydride were purchased from Sigma. MQ was synthesized in the laboratory. DBH₂ and MQH₂ were prepared as described before (19, 20). Antimycin, myxothiazol, stigmatellin, and DBH₂ were quantified by UV spectroscopy (21) by using reported extinction coefficients (22, 23). MQH₂ was quantified by determining the amount of oxidized cytochrome c.
reduced by 50 nM bc1 complex, assuming two cytochrome c molecules reduced per MQH2 oxidized.

**Purification of Cytochrome bc1 Complex—**Cytochrome bc1 complex was isolated from Red Star cake yeast as described previously (19, 24). Quantification of the bc1 complex was performed as reported before (20) by using extinction coefficients of 17.5 mM cm⁻¹ at 553–539 for cytochrome c (25) and 25.6 mM cm⁻¹ at 563–579 for the average absorbance of the bc1 and bc1 heme in cytochrome b (26).

Quantification of Endogenous Ubiquinone in Purified bc1 Complex—A modification of the procedure reported by Kroger (27) was used. Briefly, 100 μl of a 30–80 μM solution of purified bc1 complex was mixed with 2.4 ml of a mixture of 60% methanol: 40% cyclohexane in a 10-ml glass tube with screw cap. The upper phase containing cyclohexane was collected, and the lower layer was re-extracted with 1 ml of methanol. UV spectra of these samples were recorded in the range of 240–340 nm by using an Aminco DW-2 dual wavelength spectrophotometer. The reduced spectra were subtracted from the oxidized spectra, and Q content was calculated from these difference spectra by using an Aminco DW-2 dual wavelength spectrophotometer. The reduced spectra were subtracted from the oxidized spectra of these samples were recorded in the range of 240–340 nm by using an Aminco DW-2 dual wavelength spectrophotometer. The reduced spectra were subtracted from the oxidized spectra, and Q content was calculated from these difference spectra by using an extinction coefficient (26, 29). Since the mixing chamber in the OLIS-rapid scanning monochromator has a path length of 2 cm, full bc1 reduction of the bc1 complex present in the reaction (1.5 μM) would result in a Δ absorbance value of 563–579 of ~0.198, whereas full bc1 and bc1 reduction should yield Δ absorbance ~ 0.154. Dynafit also allows modeling of an equilibration phase in which two or more ligands interact before dilution and the addition of other reactants. A simplified explanation of the models used in fitting and simulating data is provided below. The complete script files are provided as Supplemental Data.

For fitting of pre-steady state kinetics of cytochrome bc1 reduction by QH2 through center N in the presence of variable antimycin concentrations, two models were used. One of the models assumed that the dimeric oxidized enzyme (E) bound tightly one or two inhibitor (I) molecules in a random, non-cooperative manner before the addition of substrate to form three possible complexes ([E], I.E, and I.E.I). Then, binding of QH2 was allowed to form five possible productive complexes ([E.QH2], [E.QH2], [E.QH2], [E.QH2], and [E.QH2].I). Electron transfer reactions were assumed to occur for each of these complexes as shown in Fig. 2, Scheme 1, in which reactions for only two of all possible complexes ([E.QH2], and [E.QH2].I) are depicted. This model assumed that an electron in the bc1 heme of one monomer (EβH or βHE) could be transferred to the bc1 heme in the other monomer reversibly in a single step (with k5 = k3), which would include intermediate reactions occurring through the bc1 hemes not explicitly considered in this particular model. The result of this intradimer equilibration is that both bc1 hemes can be reduced through only one center N in the dimer. This is illustrated in Scheme 1, in which both bc1 hemes in QH2.E.I and I.E.QH2 can be reduced to yield QbHEbH.I and IbHEbH.Q. In this model, SQ was considered to be a tightly bound ligand, not dissociating from E.

The second model omitted the intermonomer electron transfer step and included instead the movement of I from one monomer to the other (18), as shown in Fig. 2, Scheme 2. In this model, QH2 (E2 = 90 mV) is not able to reduce heme b1 (E2 = −30 mV, see Ref. 30) significantly to form Q, which would then be able to leave center N. Thus, SQ had to be assumed to dissociate, allowing the inhibitor to move from the oxidized to the reduced bc1 monomer. This made it possible for QH2 to bind and reduce the second bc1 in the dimer, as illustrated in Fig. 2, Scheme 2, in which both the EbcHbHbc2 and the EbcHbHbc2 can only be formed after SQ dissociates from SQ.bHebH.E and I.EbHEbH.Q. This dissociation was considered to be irreversible due to the reactivity of the highly reducing SQ molecule outside center N. Movement of I between monomers was described by a rate constant (k3a) different from the dissociation rate constant that would result in the formation of free I (k3a).

Reduction of cytochrome b by MQH2 through center N in the absence of antimycin was also fitted to two different models. One of them was a...
modified version of the mechanism explained in Fig. 2, Scheme 1 in which equilibration of one molecule of endogenous Q per bc₁ complex monomer was allowed before dilution and mixing with MQH₂. For simplicity, no distinction was made between the monomers, as was done in the models described above. Electron transfer between monomers was implicitly included by making ligands in either site exchange electrons with either one of the b₅₅₆ hemes. This is illustrated in Fig. 2, Scheme 3, in which some of the possible reactions included for E.MQH₂.Q are shown. The same sequence of possible reactions would apply for E.MQH₂ and E.MQH₂.MQH₂ with additional binding and dissociation of ligands from any of the two center N sites of the dimer. Only the semiquinones SQ and MSQ were assumed not to dissociate during dissociation of ligands from any of the two center N sites of the dimer.

Only the semiquinones SQ and MSQ were assumed not to dissociate during dissociation of ligands from any of the two center N sites of the dimer. This is reasonable considering that the reactions involving these two ligands will be mostly unidirectional, with MQH₂ being formed in low concentrations from the two-electron reduction of only 1 eq of endogenous Q per monomer. For yeast enzyme for at least 2 min with 6 μM M. stigmatellin and different concentrations of antimony in assay buffer before rapid mixing against an equal volume of buffer containing 24 μM MQH₂ by using the OLIS rapid scanning monochromator. The kinetic traces showed no further change in cytochrome b absorbance measured at 568–579 nm after 1.2 s of collection. Therefore, the 800 spectra collected between 1.2 and 2.0 s were averaged by using the OLIS software. The absorbance values for

\[ \Delta E_m = 59.2 \text{mVlog}_{\text{red}_{10}} \frac{[\text{red}_{10}]}{[\text{red}_{1}]} \]  

(Eq. 1) Using a difference in redox potential (ΔE₉₅) between the two b hemes in yeast of -150 mV (30), an equilibrium constant (Kₑ₉₅) of 0.0029 for the electron transfer from b₅₅₆ to b₅₅₆ is obtained. However, to calculate the ratio of [red₉₅]/[red₅₉₅] when only one electron is moving between the two hemes, the following applies:

\[ \frac{[\text{red}_{1}]}{[\text{red}_{9}]} = \frac{[\text{ox}_{1}]}{[\text{ox}_{9}]} \]  

(Eq. 3) As a result of these assumptions, the Nernst equation can be expressed as:

\[ \Delta E_m = 59.2 \text{mVlog}_{\text{red}_{10}} \frac{[\text{red}_{1}]}{[\text{red}_{9}]} \]  

(Eq. 4) Rearranging, we obtain:

\[ \frac{[\text{red}_{1}]}{[\text{red}_{9}]} = 10^\frac{\Delta E_m}{59.2 \text{mV}} \]  

(Eq. 5) For ΔEₙ₉₅ = -150 mV, the [red₉₅]/[red₅₉₅] ratio when one electron is equilibrating within a cytochrome b monomer is 0.054. Therefore, a ratio of k₆₅₆/k₅₉₅ = 0.054 was also used for those reactions in Fig. 2, Scheme 4 describing electron transfer between b₅₅₆ and b₅₅₅. Since k₅₅₅ was chosen to be 10⁷ s⁻¹, k₅₉₅ was set to 5400 s⁻¹. It is noteworthy that the ratio of these unimolecular rate constants is not the same as the value obtained for the formal Kₑ₉₅ from Equation 2 (0.0029) but is equal to Kₑ₊ₙ. This comes from the fact that the k₆₅₆/k₅₉₅ ratio was used to express the [red₉₅]/[red₅₉₅] ratio as deduced from Equations 3-5 and not the complete ratio of [ox₅₉₅]/[red₉₅]/[redₙ₉₅]/[oxₙ₉₅], which would require bimolecular constants (in units of M⁻¹s⁻¹) to be used.

**Determination of the Antimycin-induced Red Shift of Heme b₅₅₆ Absorbance**—Red shifts induced by antimony upon reduction with substrate after inhibitor addition were determined after incubating 3 μM yeast enzyme for at least 2 min with 6 μM stigmatellin and different concentrations of antimony in assay buffer before rapid mixing against an equal volume of buffer containing 24 μM MQH₂ by using the OLIS rapid scanning monochromator. The kinetic traces showed no further change in cytochrome b absorbance measured at 568–579 nm after 1.2 s of collection. Therefore, the 800 spectra collected between 1.2 and 2.0 s were averaged by using the OLIS software. The absorbance values for

\[ \Delta E_m = 59.2 \text{mVlog}_{\text{red}_{10}} \frac{[\text{red}_{1}]}{[\text{red}_{9}]} \]  

(Eq. 1)

\[ Kₑ₉₅ = \frac{[\text{ox₉₅}][\text{red₉₅}]}{[\text{red₅₉₅}][\text{ox₅₉₅}]} = 10^\frac{\Delta E_m}{59.2 \text{mV}} \]  

(Eq. 2)
The reduction kinetics of cytochrome b through center N in the presence of variable antimycin concentration. The reduction kinetics of 1.5 μM cytochrome b by 24 μM DBH₂₃ in the presence of increasing antimycin concentrations (from 0 to 1 antimycin/bc₁ complex monomer in 0.1 intervals) was fitted to a model that assumes intermonomer electron transfer (A) or movement of antimycin between monomers (B). Solid lines represent fitted curves at each inhibitor ratio. Center P was inhibited by incubating the bc₁ complex with 6 μM stigmatellin before rapid mixing. See “Experimental Procedures” (descriptions of Fig. 2, Schemes 1 and 2) and Supplemental Data for details of the kinetic models and fitted values for rate constants.

**RESULTS**

**Titration of the Pre-steady State Reduction of Cytochrome b through Center N with Antimycin**—When center P was blocked, increasing concentrations of antimycin at intervals of 0.1 mol of bc₁ monomer modified the kinetics of cytochrome b reduction by DBH₂ as shown in Fig. 3. A good fit to the experimental data (Fig. 3A) was obtained by using a model that allowed electrons to move between monomers as described in Fig. 2, Scheme 1, and under “Experimental Procedures.” A fitted value of 21.1 s⁻¹ was obtained for the intradimer electron transfer rate (k₂ in Fig. 2, Scheme 1, see Supplemental Data for fitted values of all other rate constants). In contrast, fitting to a model assuming fast antimycin movement between monomers and no intradimer electron transfer yielded sharply biphasic kinetics and a larger extent of reduction than experimentally observed, especially at low antimycin concentrations (Fig. 3B). This was a consequence of the SQ dissociation step (fitted value of k₃SQ = 3.4 s⁻¹) included in the model, which was needed to allow movement of an inhibitor molecule from an oxidized to a reduced monomer (Fig. 2, Scheme 2). According to this model, the excess of QH₂ present (16 mol of DBH₂/monomer) should result in full b₃ reduction in both monomers (ΔAbs → 0.1) when little or no inhibitor is present, which was clearly not the case. Furthermore, the fitted value for the rate of intermonomer movement of antimycin (k₁ = 1.9 × 10⁸ s⁻¹) had a standard error several orders of magnitude higher than the fitted value. This means that any value for this constant could be used with little effect on the overall kinetics because movement of antimycin from one monomer to the other would be limited by the rate of SQ dissociation (k₃SQ). Modifying the model by allowing SQ dissociation to be reversible by including an association rate constant (k₃₅SQ) with a separate irreversible step describing SQ autoxidation to Q outside center N did not improve the fitting (data not shown).

When the total extent of cytochrome b reduction was plotted as a function of antimycin concentration, non-linear titration curves were obtained (Fig. 4). This occurred independently of the substrate (DBH₂ or MQH₂) or center P inhibitor (stigmatellin or myxothiazol) used. Titrating center N with another inhibitor such as illicolin (31) yielded the same results (not shown). These non-linear titration curves could be fitted by assuming that the same extent of reduction could be obtained when either one or both center N sites per dimer were not bound by antimycin (Fig. 4, solid lines). This implies that both
$b_{1\text{H}}$ hemes in a dimer can equilibrate with the substrate through only one center N, as assumed in the model used for fitting in Fig. 3A. In contrast, if each $b_{1\text{H}}$ heme is assumed to be reducible only through its adjacent center N in the same monomer, linear inhibition would be expected (Fig. 4, dashed lines). Linear binding of antimycin to center N was observed by measuring the red shift induced in the spectrum of dithionite-reduced $b_{1\text{H}}$ upon binding of the inhibitor (not shown), as has been reported in other $bc_1$ complexes (32). This eliminates the possibility that non-linearity in the extent of cytochrome $b$ reduction was due to poor accessibility of antimycin to center N at low inhibitor concentrations due to the presence of detergent.

Reduction of $b_{1\text{H}}$ Heme in the Antimycin-blocked Center N—Hydrophobic tightly bound inhibitors similar to antimycin dissociate very slowly from their binding sites, with dissociation rates in the order of at least minutes (33, 34). This means that antimycin bound at a particular center N will not dissociate within the 1–2 s needed to reduce cytochrome b with QH$_2$ or MQH$_2$, thus preventing $b_{1\text{H}}$ reduction at that center N whenever center P sites are also blocked. Even if a higher dissociation rate for antimycin were to exist, the inhibitor would not be able to bind to a center N with a reduced $b_{1\text{H}}$ until the highly stable SQ dissociated from the site, which is unlikely (especially when DBH$_2$ is used as a substrate) in view of the poor fitting obtained with such a model (Fig. 3B). Consequently, if substoichiometric concentrations of antimycin are used, only those $b_{1\text{H}}$ hemes located at non-inhibited center N sites should undergo reduction if electron crossover from the other monomer is not possible. Simultaneously, the $b_{1\text{H}}$ hemes at antimycin-bound center N sites should remain oxidized, and no antimycin-induced red shift should be observable if there is no electron crossover from the other monomer.

The results in Fig. 5A show that substoichiometric concentrations of antimycin bound to the stigmatellin inhibited $bc_1$ complex before the addition of MQH$_2$ were able to induce a red shift in the $b_{1\text{H}}$ heme absorbance. The asymmetry in the red shift spectra (larger absolute absorbance of the peak than the trough) was due to the normalization procedure needed to generate a reference spectrum for each of the spectra obtained with antimycin (see “Experimental Procedures”). A decrease in the width of the red shift peak was observed at some of the high antimycin concentrations ($0.8bc_1$ monomer, for example, in Fig. 5A). This was likely due to a decreased signal to noise ratio at those concentrations in which there was little overall reduction (and thus decreased magnitude of the red shift). The amplitude of the red shift closely followed the theoretically predicted change in the relative concentration of dimers with a free and a blocked center N (Fig. 5B). This indicates that the electrons reaching the $b_{1\text{H}}$ hemes adjacent to antimycin were coming from the uninhibited center N in the other monomer. Similar results were obtained with DBH$_2$ as reducing substrate, which produces a more stable SQ after reduction of a $b_{1\text{H}}$ heme. The amplitude of the red shifts obtained with MQH$_2$ was larger than with DBH$_2$ because of the lower extent of reduction obtained with the latter substrate (Fig. 5B).

Cytochrome b Reduction Kinetics through Center N with Variable MQH$_2$ Concentrations—Since MQH$_2$ has a very low redox potential ($E_m = -70\text{ mV}$) relative to the $b_{1\text{H}}$ heme ($E_m = 120\text{ mV}$, see Ref. 30), reduction with at least 1 eq of MQH$_2$ per center N should result in almost full $b_{1\text{H}}$ reduction. Surprisingly, as shown in Fig. 6, full $b_{1\text{H}}$ reduction (corresponding to $\Delta A_{\text{Abs}} = 0.108$) in the presence of myxothiazol as center P inhibitor required at least 4 eq of MQH$_2$. Since $-1$ eq of endogenous Q was copurified with the yeast $bc_1$ complex, oxidation of cytochrome b by Q should account for the lack of full $b_{1\text{H}}$ reduction. There are two possibilities for electrons to be enzymatically transferred from MQH$_2$ to Q through center N. If center N sites in a dimer are assumed to be electronically insulated from each other, Q should only take electrons by binding to those center N sites in which MQH$_2$ has already been oxidized by cytochrome b and MQ has dissociated. This would require a reduction phase that would reduce $b_{1\text{H}}$ and $b_{1\text{L}}$ to completely oxidize MQH$_2$ to MQ followed by a reoxidation phase. On the other hand, if electrons can equilibrate rapidly between the center N sites of the dimer, electrons should be transferred rapidly to Q without accumulating in cytochrome b following the route $\text{MQH}_2 \rightarrow b_{1\text{H}} \rightarrow b_{1\text{L}} \rightarrow b_{1\text{L}} \rightarrow Q$.

As shown on Fig. 6A, fitting to a model that assumes electron communication between $b_{1\text{H}}$ hemes in a dimer (Fig. 2, Scheme 3) closely followed the difference in the extent of cytochrome b reduction with variable concentrations of MQH$_2$. The experimentally determined amount of $-1$ endogenous Q per monomer was used for the fitting to this model. In contrast, fitting the experimental data to a model in which each center N functions independently (Fig. 2, Scheme 4) resulted in poor fitting (Fig. 6B), mainly due to the inherent prediction of this model that full $b_{1\text{H}}$ (and $b_{1\text{L}}$) reduction should occur even at the lowest concentration of MQH$_2$. Even the kinetic traces with higher MQH$_2$ concentrations were poorly fitted because the reduction of $b_{1\text{L}}$ was minimized to bring down the extent of...
Electron Transfer between Monomers in bc₁ Complex Dimer

**DISCUSSION**

The close proximity of the b₃ hemes observed in crystal structures of the bc₁ complex dimer (10–13) has been taken in itself as evidence of intermonomer electron transfer and discussed in terms of its effect on different models of QH₂ oxidation at center P (14). The distance between b₃ heme edges (10–11 Å) is bridged by three pairs of aromatic residues (Phe-180, Tyr-184, and Phe-188 in yeast). Aromatic residues are conserved at these three positions in most cytochrome b₃ complexes (24), and the few exceptions have at least 2 aromatic residues at the corresponding positions (35). This suggests that intermonomer electron transfer at the level of the b₃ hemes is of importance in the overall function of the bc₁ complex. Recently, it was reported that replacing one of these pairs of aromatic residues in the bacterial bc₁ complex (equivalent to Phe-180) decreased slightly the steady state activity and increased the production of superoxide at center P severalfold, suggesting that this mutation interfered with electron transfer between monomers (36). However, the short b₃ to b₃ distance theoretically could support fast electron transfer even without considering participation of these aromatic residues in intermonomer electron transfer (14).

Electron communication between center N sites has been included in previous dimeric models of electron transfer within the bc₁ complex based on the EPR detection of no more than 1 SQ per dimer (15) and the kinetics of Q reduction by duroquinol when center P was inhibited (16). However, it was proposed that such intradimer transfer occurred between two Q molecules while bound at the center N sites and not between the b₃ hemes (15, 16). Crystal structures of the bc₁ complex show a distance of over 26 Å between the Q molecules in the dimer and of 27 Å between b₃ hemes, without any discernible electron pathway formed by protein atoms in most of this space. Such distances could support electron transfer rates of only 10⁻⁷ s⁻¹ (37), which are much lower than the fitted value of 2.1 10⁻⁴ s⁻¹ we have obtained for the complete b₁H to b₁L electron transfer (Fig. 3A).

Therefore, electron equilibration must occur following the route b₁H (monomer A) → b₁H (monomer A) → b₁H (monomer B) → b₁L (monomer B). Considering that the difference in redox potential between the b₁H and b₁L hemes results in a 95% b₁H:5% b₁L reduction ratio at equilibrium as explained under “Experimental Procedures,” it might seem that the energetically unfavorable b₁H → b₁H reaction would make the electron equilibration between monomers too slow to account for the present results. This is not the case, however, according to the simulated kinetics shown in Fig. 7. An electron residing in one of the b₁H hemes of a cytochrome b₃ dimer will equilibrate within 50 ms with the other b₁L heme via the b₁L hemes, even if b₁H to b₁L reduction of the traces corresponding to 1 or 2 eq of MQH₂. If this model was modified to allow for dissociation of SQ and MSQ species (violating the assumption of highly stabilized semiquinones), a better fit was obtained (data not shown). However, the resulting fitted K_d values for MSQ and SQ were 0.3 and 6 μM, respectively, implying that an extremely highly unstable SQ at center P would be needed to explain the kinetic data when no electron crossover between monomers is allowed.

**Fig. 5.** Antimycin induced red shift of the b₁L heme absorbance upon reduction through center N. MQH₂ or DBH₂ (24 μM) was used to reduce 1.5 μM bc₁ complex preincubated with 6 μM stigmatellin and the relative concentrations of antimycin to bc₁ complex indicated. A shows the red shift induced by the indicated concentrations of antimycin expressed as inhibitor/bc₁ complex monomer ratio after reduction with MQH₂. The amplitudes of the absorbance changes between 567 and 559 nm after reduction with MQH₂ (solid circles) or DBH₂ (open circles) are shown in B. Solid lines represent the relative change in the concentration of dimer with one center N bound with antimycin normalized to the observed red shift.

**Fig. 6.** Kinetics of cytochrome b reduction through center N. Cytochrome bc₁ complex (1.5 μM) preincubated with 6 μM myxothiazol was reduced with the indicated equivalents of MQH₂. Solid lines represent the best fit of the kinetic data to a model that assumed rapid equilibration between center N sites in the dimer (A) or to a model that did not include communication between monomers (B). See “Experimental Procedures” (descriptions of Fig. 2, Schemes 3 and 4) and Supplemental Data for details of the kinetic models and fitted values for rate constants.
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Electron transfer is assumed to be the same order of magnitude as the slow \( b_{11} \) to \( b_1 \) transfer. The simulation in Fig. 7A represents the lower limit of the actual electron transfer rate between \( b_{11} \) and \( b_1 \), which is in the energetically favorable direction (\( b_1 \) to \( b_{11} \)) probably occurs within a few microseconds.

Even if the \( b_{11} \) to \( b_1 \) transfer is assumed to be one order of magnitude slower (Fig. 7B), the electron equilibrates between the four redox centers in the dimeric cytochrome \( b \) within the same time scale. These very conservative estimates demonstrate that intermonomer electron communication could occur within the time scale of the observed cytochrome \( b \) reduction kinetics, which under our conditions was in the order of hundreds of milliseconds. These simulations are also consistent with the value obtained from the fitting shown in Fig. 3A for the complete electron transfer from one \( b_{11} \) to the other \((21 \ s^{-1})\), which would allow electron equilibration from one monomer to the other in \( \sim 0.1 \) s. This shows that, even assuming slower than expected electron transfer rates, the preferential residence of the electron in the \( b_{11} \) heme due to its 150 mV higher \( E_m \) relative to \( b_1 \) (30) does not preclude rapid equilibration of the center \( N \) ligands through \( b_1 \) to \( b_{11} \) transfer.

Further evidence for \( b_1 \) to \( b_{11} \) electron communication in the dimer comes from the red shift observed when the oxidized bc₁ complex was preincubated with substoichiometric concentrations of antimycin before reduction with MQH₂ or DBH₂ (Fig. 5). These results indicate that an oxidized \( b_1 \) heme with antimycin bound in one center \( N \) can be reduced by oxidation of substrate occurring at center \( N \) in the opposite monomer, with the resulting shift in the reduced \( b_1 \) spectrum caused by the close proximity of the inhibitor. Since antimycin blocks \( Q \) binding to the center \( N \), where it is bound, the \( b_1 \) heme close to the inhibitor would not be able to receive an electron through the route MQH₂ (bound to monomer \( A \)) \( \rightarrow \) MQH₁ (bound to monomer \( B \)) \( \rightarrow \) MQH₂ (bound to monomer \( B \)), as proposed elsewhere (15, 16). Therefore, the only electron transfer pathway that can explain the results of Fig. 5 is MQH₂ (monomer \( A \)) \( \rightarrow \) MQH₁ (monomer \( A \)) \( \rightarrow \) MQH₂ (monomer \( B \)) \( \rightarrow \) MQH₁ (monomer \( B \), with antimycin bound in close proximity).

The pre-steady state reduction of cytochrome \( b \) and its inhibition by antimycin could only be fitted assuming electron transfer between cytochrome \( b \) subunits (Figs. 3 and 4). The proposal that an antimycin molecule can move rapidly between center \( N \) sites in a dimer (18) was also considered but resulted in poor fitting of the experimental data (Fig. 3B). This was expected since this model was used originally to explain the non-linear inhibition curves of steady state activities at center \( P \) and not of pre-steady state kinetics through center \( N \). One of the assumptions of this model is that non-linear titration curves are observed only when the inhibitor binds to a site different from that at which the rate-limiting step occurs (18). This condition is clearly not fulfilled in the present experiments, in which antimycin binds to the only site of the reaction. In addition, we have previously reported that an antimycin-movement model cannot explain even the non-linear inhibition of steady state activity (17). Fast movement of antimycin between center \( N \) sites is also inconsistent with the crystal structures of the bc₁ complex, which show that there is no continuous cavity in the protein that could allow antimycin to move from one center \( N \) to the other without the inhibitor having to dissociate into the lipid/detergent phase.

Therefore, any intradimer movement of the inhibitor, which has been proposed to occur with a rate of \( \sim 5 \times 10^5 \) \( \text{m}^{-1} \text{s}^{-1} \) (18), could not occur faster than the dissociation of antimycin out of center \( N \). The \( K_p \) for antimycin in the isolated bc₁ complex is in the \( 10^{-10} \text{ to } 10^{-11} \) \( \mu \) range (38). We have determined the association rate constant for antimycin binding \((k_{11})\) to be \( \sim 5 \times 10^5 \) \( \text{m}^{-1} \text{s}^{-1} \), which is in the same order of magnitude as reported for other tightly bound hydrophobic inhibitors such as stigmatellin and myxothiazol (33, 34). This value, together with the \( K_p \) of \( 10^{-10} \text{ to } 10^{-11} \) \( \mu \) (38), results in a very slow dissociation rate of \( \sim 10^{-5} \text{s}^{-1} \) for antimycin in the isolated bc₁ complex. An antimycin-movement model would also require SQ to dissociate from center \( N \) in order for antimycin to leave the oxidized monomer (Fig. 2, Scheme 2), which is in conflict with the known stabilization of SQ at center \( N \) (3–6) and the lack of superoxide anion formation when center \( P \) is inhibited by stigmatellin (7, 8). Even assuming that all of these requirements, which are in conflict with what is known about center \( N \) structure and binding properties, do exist, the antimycin-movement model still resulted in poor fitting of the experimentally observed reduction kinetics (Fig. 3B).

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2 R. Covian and B. L. Trumpower, unpublished results.
The evidence against fast intradimer movement of antimycin also indicates that the red shifts observed in Fig. 5 can only be explained in terms of $b_1$ to $b_1$ electron transfer in the dimer. Given the poor fitting obtained when SQ was assumed to dissociate from center N (Fig. 3B), reduction of $b_1$ by DBH$_2$ in a monomer would form a tightly bound SQ at that center N. This SQ would prevent any “rapidly moving” antimycin molecule from dissociating from an oxidized center N to bind in proximity to the DBH$_2$-reduced $b_1$ heme within the 2-s time scale of the experiment. Considering the very slow dissociation rate for antimycin, even the red shifts observed after MQH$_2$ reduction forms a less stable MSQ can be interpreted as evidence of electrons reaching those $b_1$ hemes in close contact with the tightly bound inhibitor via the $b_1$ hemes in the dimer.

In contrast to the frequently observed non-linear inhibition of steady state activities (Ref. 18 and see Ref. 38 for a review), inhibition of cytochrome b reduction through center N as a function of antimycin concentration has been reported on only a few occasions and only by our laboratory (19, 31, 39). Non-linear titration curves were observed in these reports, although there was no attempt to explain this kinetic pattern. In Fig. 4, we show that this non-linearity can be explained by using the same intermonomer electron transfer model used for fitting the kinetic traces shown in Fig. 3. Cytochrome $b$ kinetics without center N inhibitors, especially at low, but super-stoichiometric, concentrations of MQH$_2$ that result in incomplete $b_1$ reduction, could be fitted only to a model that includes electron crossover in the dimer (Fig. 6). In contrast, a model that treated each center N site in the dimer as electronically insulated from the other predicted a higher extent of $b_1$ reduction.

Recently, we found that only half of the center P sites in the antimycin inhibited bc$_1$ complex were catalytically active even under conditions in which an excess of cytochrome $c$ was available as an oxidant (17). Still, two $b_1$ hemes per dimer were found to undergo reduction. This suggested that a second turnover through the only active center P in the dimer reduced the $b_1$ heme in the adjacent monomer by $b_1$ to $b_1$ transfer. The non-linearity in the inhibition of steady state activity by antimycin, which includes a slight activation at low inhibitor concentrations, was also modeled assuming electron crossover between monomers. Our present center N kinetic results complement those obtained at center P to show that intermonomer electron transfer via the $b_1$ hemes is the only mechanism that can account for all of the experimental data.

Previous results from our laboratory have demonstrated that reduction of cytochrome $b$ inhibits QH$_2$ oxidation at center P (9), either by electrostatic or by conformational effects (40, 41). This is the case when cytochrome $b$ reoxidation is prevented by using center N inhibitors and also when Q is absent (9). Electron equilibration between the center N sites of the bc$_1$ complex dimer has been proposed to prevent the accumulation of reduced b heme (14) because of the effect of stabilizing SQ at center N, which favors oxidation of QH$_2$. Formation of a stable complex of reduced $b_1$ heme with SQ at center N is analogous to formation of an antimycin:$b_1^{2+}$ complex after oxidation of one QH$_2$ at center P when center N is inhibited.

We investigated the consequences of electron communication between cytochrome $b$ subunits in the formation of inhibitory SQ:$b_1^{2+}$ complexes at center N by using kinetic modeling, assuming that center P sites were inactive. For these simulations, we used modified versions of the models used for the fitting shown in Fig. 6, with no MQH$_2$ present and a ratio of 20 QH$_2$ + Q molecules per bc$_1$ complex monomer, as found in yeast mitochondria (42). Rate constants were taken from the fitted values obtained from Fig. 6A (see Supplemental Data for full scripts). As shown in Fig. 8, the relative proportion of SQ:$b_1^{2+}$ at center N at QH$_2$/Q ratios of 0.5 and 8 varied between 15 and 50%, assuming no electron equilibration between center N sites in the dimer (solid lines). In contrast, if electron crossover between monomers was allowed, no more than 23% of SQ:$b_1^{2+}$ at center N at QH$_2$/Q ratios of 0.5 and 8 varied between 15 and 50%, assuming no electron equilibration between center N sites in the dimer (solid lines). In contrast, if electron crossover between monomers was allowed, no more than 23% of SQ:$b_1^{2+}$ at center N at QH$_2$/Q ratios of 0.5 and 8 varied between 15 and 50%, assuming no electron equilibration between center N sites in the dimer (solid lines). In contrast, if electron crossover between monomers was allowed, no more than 23% of SQ:$b_1^{2+}$ at center N at QH$_2$/Q ratios of 0.5 and 8 varied between 15 and 50%, assuming no electron equilibration between center N sites in the dimer (solid lines). In contrast, if electron crossover between monomers was allowed, no more than 23% of SQ:$b_1^{2+}$ at center N at QH$_2$/Q ratios of 0.5 and 8 varied between 15 and 50%, assuming no electron equilibration between center N sites in the dimer (solid lines). In contrast, if electron crossover between monomers was allowed, no more than 23% of SQ:$b_1^{2+}$ at center N at QH$_2$/Q ratios of 0.5 and 8 varied between 15 and 50%, assuming no electron equilibration between center N sites in the dimer (solid lines).
Electron transfer between monomers could have additional functions, such as controlling an alternating reaction mechanism between center P sites in the dimer (17, 44). For example, the formation and interconversion of SQ: b$_{11}^{3+}$ to SQ: b$_{11}^{2+}$ by electron crossover between monomers could have effects on center P catalysis, either by electrostatic effects in the b$_{11}$ heme of the same monomer or by conformational changes transmitted through the Rieske iron-sulfur protein, which traverses from center N in one monomer to center P in the other. Evidence for such communication between center N and P of multisubunit enzyme but also is an essential part of its energy conserving mechanism.

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