Asymmetric and Redox-specific Binding of Quinone and Quinol at Center N of the Dimeric Yeast Cytochrome bc₁ Complex

CONSEQUENCES FOR SEMIQUINONE STABILIZATION*§

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The cytochrome bc₁ complex recycles one of the two electrons from quinol (QH₂) oxidation at center P by reducing quinone (Q) at center N to semiquinone (SQ), which is bound tightly. We have analyzed the properties of SQ bound at center N of the yeast bc₁ complex. The EPR-detectable signal, which reports SQ bound in the vicinity of reduced b₄h heme, was abolished by the center N inhibitors antimycin, funiculosin, and ilicicolin H, but was unchanged by the center P inhibitors myxothiazol and stigmatellin. After correcting for the EPR-silent SQ bound close to oxidized b₄h, we calculated a midpoint redox potential (Em) of −90 mV for all bound SQ. Considering the Em values for b₄h and free Q, this result indicates that center N preferentially stabilizes SQ-b₄h³+ complexes. This favors recycling of the electron coming from center P and also implies a >2.5-fold higher affinity for QH₂ than for Q at center N, which would potentially inhibit b₄h oxidation by Q. Using pre-steady-state kinetics, we show that Q does not inhibit the initial rate of b₄h reduction by QH₂ through center N, but does decrease the extent of reduction, indicating that Q binds only when b₄h is reduced, whereas QH₂ binds when b₄h is oxidized. Kinetic modeling of these results suggests that formation of SQ at one center N in the dimer allows stabilization of SQ in the other monomer by Q reduction after intradimer electron transfer. This model allows maximum SQ-b₄h³+ formation without inhibition of Q binding by QH₂.

The cytochrome bc₁ complex couples electron transfer from QH₂ to cytochrome c to a net movement of protons across the membrane in which it is embedded. This is achieved by having two QH₂/Q-binding sites (center P and center N) in cytochrome b close to opposite sides of the membrane, as is clearly seen in crystallographic structures (1–4). The bifurcated mechanism of QH₂ oxidation at center P in the protonmotive Q cycle results in one of the electrons from the substrate being transferred to the b₄ heme and then to the b₃h heme, which is in close proximity to the center N-binding pocket. This electron is used to reduce Q, producing an SQ intermediate, which is further reduced to QH₂ after a second oxidation event at center P. For proton translocation to occur, these two sites must function in opposite directions, so protons from QH₂ oxidation at center P are released to the positive side of the membrane, whereas Q reduction at center N results in proton uptake from the negative side.

However, the b₃h heme group responsible for Q reduction at center N has a midpoint redox potential at pH 7 (Em₇) of 50–100 mV (5–7), which is close to the value of 60–90 mV for the Q pool in the membrane (8, 9). This implies that the b₃h heme should oxidize QH₂ as easily as it can reduce Q. When electrons are prevented from flowing out of cytochrome b through center N, QH₂ oxidation at center P is inhibited and results in detrimental side reactions, such as superoxide formation (10, 11). We have recently provided evidence indicating fast electron equilibration between b₄h hemes in the bc₁ complex dimer and suggested that this minimizes formation of inhibitory SQ-b₃h²⁺ complexes at center N (12). Furthermore, potentiometric studies of SQ bound at center N (13–15) have indicated that the Em of this intermediate is more positive than that of the Q pool by at least 20 mV, suggesting that formation of productive SQ-b₄h³+ complexes is favored by preferential binding of QH₂ to center N. Nevertheless, this introduces another difficulty for optimal center N function because QH₂ would prevent binding of Q to center N, especially at high QH₂/Q ratios.

We have analyzed the thermodynamic properties of bound SQ and the pre-steady-state kinetics of b₄h reduction through center N of the yeast bc₁ complex. Our results point to a mechanism in which center N sites in the dimer selectively bind QH₂ or Q depending on the redox state of the b₄h heme as well as on the occupancy of the other monomer. We discuss how this model maximizes SQ-b₄h³+ complex formation while preventing QH₂ from interfering with Q binding.

EXPERIMENTAL PROCEDURES

Materials—Decyl maltoside was obtained from Anatrace. Antimycin, myxothiazol, DBQ, and redox mediators were pur-
chased from Sigma, except for menaquinone, which was synthesized in the laboratory. Funiculosin was a gift from Novartis (Basel, Switzerland), and ilicicolin H was from the Merck sample repository. DBH$_2$ was prepared as described previously (16). All inhibitors and DBH$_2$ were quantified by UV spectroscopy (17) using previously reported extinction coefficients (18–20).

**Purification of Cytochrome bc$_1$ Complex**—Wild-type cytochrome bc$_1$ complex was isolated from Red Star cake yeast as described previously (21). Quantification of the bc$_1$ complex was performed as reported previously (22) using extinction coefficients of 17.5 mM$^{-1}$ cm$^{-1}$ at 553–539 nm for cytochrome $c_1$ (23) and 25.6 mM$^{-1}$ cm$^{-1}$ at 562–579 nm for the average absorbance of the $b_{14}$ and $b_l$ hemes in cytochrome $b$ (24). The amount of endogenous Q copurified with the bc$_1$ complex was determined as described previously (12) and varied between 0.8–1.2 molecules/bc$_1$ monomer.

**Semiaquino Redox Titration**—Purified bc$_1$ complex was diluted with 100 mM Tris and 50 mM KCl (pH 7.4) to a final concentration of 24–30 $\mu$M (based on Rieske iron-sulfur cluster concentration as measured by EPR spectroscopy; see below). Upon addition of $33 \mu$M 2,3,5,6-tetramethyl-p-phenylenediamine ($E_{m7} = +270$ mV) and $33 \mu$M phenazine ethosulfate ($E_{m7} = +55$ mV) as redox mediators, the solution was transferred into an anaerobic vessel continuously flushed with argon. The appearance of additional artificial radical signals was avoided by using only two mediator dyes, one having its $E_m$ in the range of the expected value of the Rieske iron-sulfur cluster and the other in the range of the expected $E_m$ of SQ. The sample was stirred at a constant temperature of 298 K and poised at desired potential values by adding small aliquots of SQ. EPR spectra were obtained with a Bruker ESP 300E spectrometer equipped with a double integration, and comparison with spectra from one paramagnetic species (26).

**Cytochrome b Redox Titration**—Optical potentiometric titrations were performed at 24 °C in a 3.5-ml quartz cuvette as described previously (27). The potential was measured with a platinum-Ag/AgCl (3M) microelectrode (MI-80414-6, Microelectrodes, Inc.). All values are expressed with respect to the normal hydrogen electrode. The electrode was calibrated against a pH 7 standard solution of quinhydrone ($E_m = +296$ mV). The purified bc$_1$ complex was diluted to 2 $\mu$M in 100 mM Tris (pH 7.4), 50 mM KCl, and 0.01% dodecyl maltoside. Redox equilibration between the protein and the electrode was achieved by a mixture of the following dyes (with their $E_{m7}$ values): 70 $\mu$M 2,3,5,6-tetramethyl-p-phenylenediamine (+270 mV), 25 $\mu$M 1,2-naphthoquinone (+144 mV), 25 $\mu$M phenazine methosulfate (+80 mV), 25 $\mu$M phenazine ethosulfate (+55 mV), 50 $\mu$M duroquinone (+5 mV), 25 $\mu$M menadione (−76 mV), 25 $\mu$M 2-hydroxy-1,4-naphthoquinone (−145 mV), 30 $\mu$M anthraquinone 2,6-disulfonate (−184 mV), and 30 $\mu$M anthraquinone 2-sulfonate (−225 mV).

A 10 or 100 mM solution of dithionite and ferricyanide was used for the reductive and oxidative titrations, respectively. The UV-visible spectra were recorded between 500 and 600 nm in an Amino DW-2 dual-wavelength spectrophotometer in the split beam mode. The absorbance at 562 minus 578 nm was plotted against the potential ($E_h$) of the system. The reductive and oxidative titrations were averaged, and the resulting graph was fitted in the ORIGIN 5.0 program (OriginLab Corp.) to the following $n = 1$ Nernst equation (Equation 1) with two components to obtain the redox potential for the $b_{14}$ ($E_{m(b_{14})}$) and $b_l$ ($E_{m(b_l)}$) hemes as well as the relative contribution of the $b_{14}$ heme to the total absorbance ($b$),

$$
\Delta A(562–578 \text{ nm}) = C b \left( \frac{n_f \frac{e^m(E_{m(b_{14})} - \varepsilon)}{1 + e^m(E_{m(b_{14})} - \varepsilon)}}{1 + e^m(E_{m(b_l)} - \varepsilon)} \right) + (1 - b) \left( \frac{n_f \frac{e^m(E_{m(b_l)} - \varepsilon)}{1 + e^m(E_{m(b_l)} - \varepsilon)}}{1 + e^m(E_{m(b_{14})} - \varepsilon)} \right)
$$

(Eq. 1)

where $C$ is the concentration of bc$_1$ complex monomers (in this case, 2 $\mu$M), $e$ is the added extinction coefficient of both hemes (51.2 mM$^{-1}$ cm$^{-1}$), and $p$ is the light path length (in this case, 1 cm). The temperature of the assay was maintained at 24 °C. The Nernst plots for both oxidative and reductive titrations were essentially identical, indicating full reversibility in the titration and confirming that the system was in equilibrium.

**Pre-steady-state Reduction of Cytochrome b through Center N**—Pre-steady-state reduction of cytochrome b was followed at 24 °C by stopped-flow rapid scanning spectroscopy using the
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FIGURE 1. EPR spectra of SQ at center N of the yeast bc1 complex. Data were collected at the indicated temperatures and microwave powers to reveal the EPR signature of the reduced Rieske protein together with the SQ signal (left) or to observe only the signal from SQ (right). The redox potential ($E_M$) of the system was poised approximately at the value that yielded the maximum SQ signal in the wild-type (wt) enzyme. When the bc1 complex from a yeast strain lacking Q ($\Delta Q$) was used or when antimycin, funiculosin, or ilicicolin H ($\sim 1.5$ eq/monomer) was added to the wild-type enzyme, the SQ signal was abolished.

OLIS rapid scanning monochromator as described previously (22). Reactions were started by rapid mixing of 3 $\mu$m enzyme (expressed as monomers of the bc1 complex) 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 eq of stigmatellin or myxothiazol/bc1 complex monomer and varying concentrations of DBQ against an equal volume of the same buffer (without enzyme and inhibitors) containing 48 $\mu$M DBH$_2$. For each experiment, 12–16 data sets were averaged, and the oxidized spectrum was subtracted. The time course of the absorbance change at 562 and 578 nm was extracted using software from OLIS. Using the ORIGIN program, the difference between the two wavelengths was plotted and fitted to a second- or third-order exponential, and the fitted curve was then used as the basis for an iterative smoothing procedure to decrease the noise levels of the kinetic traces. In this procedure, the difference between each data point and the corresponding value of the fitted curve at the same time point was calculated and decreased by half.

Kinetic Modeling—The DynaFit program (BioKin, Ltd.), which allows fitting to reaction mechanisms described as a series of individual reaction steps (28), was used to fit the time-dependent $b_{14}$ reduction through center N. An extinction coefficient of 36 $\text{mM}^{-1} \text{cm}^{-1}$ was used for this heme group (12, 29). Association and dissociation of DBH$_2$, DBQ, 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 $b_{14}$ hemes in the dimer through the $b_{1}$ hemes (12) was described as a single step. For $b_{14}$ reduction in the myxothiazol-inhibited enzyme, the two models used assumed that all ligands were able to bind and react with both $b_{14}$ hemes in the dimer with identical rate constants. One of the models considered only one center N to be accessible initially, with the second center N being rapidly activated by SQ formation at the first site. When stigmatellin was present, the model used to fit the reduction kinetics assumed different kinetic parameters in one of the two center N sites in the dimer from the outset. The complete DynaFit script files are available as supplemental material.

RESULTS

EPR Spectra of Semiquinone Bound at Center N—The purified yeast cytochrome bc1 complex has been reported to exhibit an EPR signal centered at $g = 2$ attributed to an SQ radical (6). However, that putative SQ signal was significantly different from SQ signals reported in other bc1 complexes (13–15) in that it had a much smaller intensity (only 5% of the Q content), yielded an $E_m$ for SQ that was $\sim 100$ mV higher than in other organisms, and was pH-independent. No evidence was provided to demonstrate that such an EPR signal came from a center N radical. As shown in Fig. 1, we have now obtained an EPR signal ($g = 2.004$ and line width = 0.9 mT) that clearly corresponds to SQ bound at center N, as judged by its sensitivity to three different center N inhibitors (antimycin, funiculosin, and ilicicolin H) and by its absence in bc1 complex lacking Q. The maximum intensity of this SQ signal at a microwave power of 0.01 milliwatt varied between 0.06 and 0.27/center N monomer, depending on the Q content of the enzyme preparation, and occurred at an $E_m$ of 50–60 mV at pH 7.4, in agreement with what has been observed in other bc1 complexes (13–15).

It was previously shown experimentally (30, 31) and confirmed theoretically (7) that the SQ bound at center N is anti-ferromagnetically coupled to the oxidized $b_{14}$ heme. Thus, to determine the true concentration and $E_m$ of SQ bound at center N, a correction needs to be made for the portion of the SQ that is EPR-silent due to this coupling. Because the reported $E_m$ of yeast cytochrome $b$ using either circular dichroism or EPR spectroscopy was found to be dependent on the type of detergent present (6), we performed a spectrophotometric redox titration of the $b$ hemes in the presence of dodecyl maltoside in the same buffer as that used for our EPR experiments.

As shown in Fig. 2A, at pH 7.4, the $b_{14}$ heme has an $E_m$ of $\sim 60$ mV. Using this value, we calculated the total percentage (relative to bc1 monomers) of SQ bound at all center N sites at
different redox potential values (\(SQtot\)) from the \(g = 2.0\) EPR signal, which reflects only SQ bound in the vicinity of \(b_H^{2+}\) heme complexes (\(SQtot^{b_H^{2+}}\)), expressed as a percentage relative to bc₁ monomers) by applying Equation 2.

\[
SQtot^b(\%) = \frac{SQtot^{b_H^{2+}}(\%)}{1 - b_H^{2+}(\%)}
\]  

(Eq. 2)

This equation implies that, for instance, when the \(b_H\) heme is half-reduced (at \(E_h = 61\) mV), if the EPR-detectable \(SQtot^{b_H^{2+}}\) amounts to 15%/bc₁ monomer, an additional 15% of the EPR-invisible SQ is expected to be bound to center N sites in which the \(b_H\) heme is oxidized. Therefore, SQ can be estimated to occupy 30% of the total center N sites, half of which have \(b_H^{2+}\) and the other half \(b_H^{3+}\). At a higher \(E_h\) of 120 mV, detecting 3% of SQ/bc₁ monomer by EPR implies that, because only 10% of center N sites have \(b_H^{2+}\), SQ is present in 30% of all center N sites, 90% of which have \(b_H^{3+}\), preventing EPR detection of SQ bound to them. An important assumption made in Equation 2 is that center N binds SQ with equal affinity irrespective of the redox state of the \(b_H\) hemes, which is supported by redox titrations in the bovine bc₁ complex that suggest a constant \(SQtot\) of 1 monomer by EPR implies that, at a higher \(E_h\) of 120 mV, detecting 3% of SQ/bc₁ monomer by EPR implies that, because only 10% of center N sites have \(b_H^{2+}\), SQ is present in 30% of all center N sites, 90% of which have \(b_H^{3+}\), preventing EPR detection of SQ bound to them. An important assumption made in Equation 2 is that center N binds SQ with equal affinity irrespective of the redox state of the \(b_H\) hemes, which is supported by redox titrations in the bovine bc₁ complex that suggest a constant \(E_m\) of the \(b_H\) heme in the presence or absence of SQ (7). As shown in Fig. 2B, the total SQ obtained using Equation 2 yielded a maximum of 40% total SQ/bc₁ monomer at \(E_h \approx 90\) mV. Because the EPR-observable SQ was zero beyond \(-150\) mV, the total SQ could not be calculated at higher \(E_h\) values, as is evident from Equation 2.

\[
SQtot^b(\%) = (C) \left( \frac{\frac{\sigma_E^{Em(SQ/Q) - E_h}}{e^{\left(e^{Em(SQ/Q) - E_h} - 1\right)}}}{1 + \frac{\sigma_E^{Em(SQ/Q) - E_h}}{e^{\left(e^{Em(SQ/Q) - E_h} - 1\right)}}} \right)
\]  

(Eq. 3)

where \(C\) corresponds to the theoretical concentration of SQ/monomer (as a percentage) that could be achieved if the \(E_m(SQ/Q)\) and \(E_m(QH2/SQ)\) values were separated enough to allow maximum accumulation. Its value was \(-35\%\) for the EPR-detectable SQ and \(-55\%\) for the total SQ calculated from Equation 2. The fitted \(E_h\) at which maximum SQ occurred, which is the mean of the two individual \(E_m\) values with their respective deviation, yielded values of 44.8 \(\pm 7.6\) mV for \(SQtot^{b_H^{2+}}\) and 86.8 \(\pm 5.3\) mV for \(SQtot\). Therefore, the peak in total bound SQ occurred when the \(b_H\) heme was 75% oxidized, implying that, at pH 7.4, SQ stabilization favors the formation of SQ\(b_H^{3+}\) complexes compared with SQ\(b_H^{2+}\) by a factor of 3. Considering that the Q pool in membranes has an \(E_m\) reported to be between 60 (6) and 90 (9, 19) mV and that this value changes by \(-60\) mV/pH unit, SQ bound at center N showed an \(E_m\) of 24–54 mV higher than unbound Q at pH 7.4. This implies that QH₂ binds to center N between 2.5- and 8.3-fold tighter than Q.

Reduction Kinetics of Heme \(b_H\) through Center N in the Presence of DBQ—When DBH₂ is added to center P-inhibited bc₁ complex, electrons equilibrate with the \(b_H\) hemes only by entry through center N (12). As shown in Fig. 3, addition of increasing

**FIGURE 2. Redox titration of cytochrome b and the center N SQ.** Reduction of cytochrome b (A) as a function of redox potential (\(E_h\)) was fitted to Equation 1. The relative \(b_H\) contribution to total absorbance (parameter \(b\) in Equation 1) was 48 \(\pm 1.2\%\). See supplemental Fig. S1 for a deconvolution of this fit and its comparison with a single Nernst component. The \(E_m\) for \(b_H\) was used to calculate the total relative concentration of oxidized \(b_H\) heme at each \(E_h\) value (dotted line) in B. The intensity of the SQ signal determined by EPR spectroscopy (○) was fitted to Equation 3 (solid line), yielding \(E_m(SQ/Q) = 73.1 \pm 7.7\) mV, \(E_m(QH2/SQ) = 16.5 \pm 7.5\) mV, and \(C = 35.1 \pm 8.3\%\). The total SQ bound at center N (○) was calculated using Equation 2. Fitting of the resulting SQ values to Equation 2 (dashed line) yielded \(E_m(SQ/Q) = 133.7 \pm 6.4\) mV, \(E_m(QH2/SQ) = 39.8 \pm 5.1\) mV, and \(C = 55.3 \pm 5.5\%\).

The Nernst equation shows that it is thermodynamically impossible to have a redox couple in which the oxidized or reduced species reaches a concentration of exactly 100%. Therefore, the denominator in Equation 2 will never reach a value of exactly zero, which would result in a mathematical indeterminacy. Also, because by definition \(SQtot^{b_H^{2+}}\) cannot be larger than the percentage of center N sites that have \(b_H^{2+}\), the \(SQtot^b\) value will always be \(<100\%\) of the total center N sites, even at high \(E_h\) values. For example, even at an \(E_h\) at which the \(b_H\) heme is 99% oxidized, \(SQtot^{b_H^{2+}}\) will be \(\leq 1\%\) simply because only 1% of all the center N sites have a reduced \(b_H\) heme that allows EPR detection of a bound SQ. Therefore, applying Equation 2 in this case would yield values of 1 or less in the numerator and 0.01 in the denominator, resulting in an \(SQtot^b\) value of \(<100\%\).

The \(E_m\) values of the QH₂/SQ and SQ/Q couples were calculated by fitting the experimental data points (SQ\(^{b_H^{2+}}\)) as well as the predicted \(SQtot^b\) concentrations to the following Nernst equation (Equation 3),

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concentrations of DBQ resulted in a progressively smaller decrease in the extent of $b_{14}$ reduction. Interestingly, this oxidation of cytochrome $b$ by DBQ was only partial, reaching a limit of $\sim 40\%$ (with myxothiazol) or $\sim 60\%$ (with stigmatellin) of the total extent observed without added DBQ. As reported previously (32), $b_{14}$ reduction by DBH₂ through center N showed biphasic kinetics when center P was inhibited with myxothiazol (Fig. 3A), whereas the presence of stigmatellin resulted in an additional re-oxidation phase that was abolished when DBQ was added (Fig. 3B).

Myxothiazol and stigmatellin affected differently the way in which DBQ decreased the extent of the two kinetic components of $b_{14}$ reduction (Fig. 4). Only the fast phase of $b_{14}$ reduction was insensitive to DBQ concentration, irrespective of the center P inhibitor present (Fig. 5). This is a surprising result, considering that the $b_{14}$ oxidation effect of DBQ shown in Fig. 3 demonstrates that DBQ can bind efficiently to the oxidized $bc₁$ complex with the same affinity as that calculated from the oxidation of the $b_{14}$ heme, a significant decrease in the rate of reduction by DBH₂ should have been expected (as simulated by the dashed lines in Fig. 5). This result indicates that DBQ does not compete with DBH₂ for binding to center N when the $b_{14}$ heme is oxidized. DBQ decreased the rate of the slower phase of $b_{14}$ reduction in the presence of stigmatellin to a value similar to that observed with myxothiazol, which was unaffected by DBQ. A $K_m$ of 2.7 μM was calculated for DBQ based on this decrease in the slow rate.

EPR Spectra of the Center N Semiquinone in the Presence of Center P Inhibitors—To determine the cause of the different $b_{14}$ reduction kinetics observed in the presence of myxothiazol and stigmatellin, we examined the properties of the EPR-detectable SQ in the absence and presence of these inhibitors (Fig. 6). The intensities and $E_m$ values of SQ in the uninhibited and myxothiazol- and stigmatellin-bound $bc₁$ complexes were very similar, within experimental error (Fig. 6A), indicating that the center P ligand had no effect on the stability of SQ at center N. The power saturation behavior of the SQ signal was also the same under the three conditions (Fig. 6B), implying that the relaxation...
properties that report the electronic environment of the unpaired electron in SQ are also independent of the center P inhibitors. These results suggest that changes in center N that are responsible for differences in $b_{14}$ reduction kinetics when stigmatellin is present are probably short-lived, as we have suggested in previous work (32), and disappear during the several minutes needed to equilibrate the enzyme to the applied $E_m$ in the EPR experiments. of one $b_{14}$ heme by equilibration with SQ and then abandoned center N. An electron would then be transferred from the remaining SQ-$b_{14}^{2+}$ complex in the dimer by intermonomeric electron transfer to yield a reduced $b_{14}$ heme with an empty center N to which DBQ or Q could bind.

A marked improvement in the fitting was obtained if the model was modified to include the condition that initial

**Kinetic Modeling of Center N**

The observations that DBQ had no effect on the initial rate of $b_{14}$ reduction and decreased only partially its extent were included in a kinetic model in which DBH$_2$ and DBQ bind only to center N when $b_{14}$ is oxidized and reduced, respectively. The kinetic model also included an intermonomeric electron equilibration between $b_{14}$ hemes in the dimer (see supplemental material for fitted parameters and details on the models). When fitting the kinetic traces obtained in the presence of myxothiazol (Fig. 7A), such a model was able to reproduce the partial decrease in the extent of $b_{14}$ reduction as well as the constant initial rate of reduction by DBH$_2$ as DBQ was varied. It was impossible to model this behavior in a model in which electron equilibration between monomers does not exist (data not shown), just as we have shown in previous work (12), in which the pre-steady-state kinetics of cytochrome $b$ reduction at different QH$_2$ and antimycin concentrations could be modeled only assuming $b_{14}$-to-$b_1$ electron transfer within the dimer. In that same work (12), we showed that this intermonomeric electron equilibration between center N sites occurs despite the thermodynamically unfavorable electron transfer from $b_{14}$ to $b_1$.

A small but fast re-oxidation phase that was not found in the experimental data was generated by the model shown in Fig. 7A. This was a consequence of allowing DBH$_2$ binding simultaneously to both center N sites in the oxidized dimer, which resulted in favoring the formation of two SQ-$b_{14}^{2+}$ complexes during the initial moments of the reaction. In this scenario, DBQ (or endogenous Q) could bind only after QH$_2$ reformed by re-oxidation...
binding of DBH₂ to the oxidized bc₁ complex occurred to only one center N in the dimer (Fig. 7B). In this mechanism, SQ formation in this initially active monomer would activate the other center N, allowing DBQ/Q to bind before DBH₂ once the b₄ heme in this second monomer receives an electron by intermonomeric electron transfer from the first b₄ heme. In this way, formation of two SQ⁻b₄²⁺ complexes in the dimer is avoided as long as DBQ or Q is present, obviating the need for b₄ re-oxidation. The fitted values for the rates of intermonomeric electron transfer and activation of the second center N after the initial SQ formation in one monomer were higher than all other rates, suggesting that these two processes were not rate-limiting.

Because endogenous QH₂ was not initially present and was expected to be formed in very small amounts by reduction of SQ in SQ⁻b₄²⁺ complexes, its rate of binding and reduction of the b₄ heme could not be accurately determined by the fitting, resulting in very low values with large deviations, especially in the model shown in Fig. 7A. Except for this value and the non-limiting rate of center N activation after the first SQ formation (Fig. 7B), all other rate values showed little deviations (see supplemental material), indicating the relevant role of each kinetic step in determining the accuracy of the fit.

Center N kinetics in the presence of stigmatellin as the center P inhibitor were considerably more complicated than those with myxothiazol. Still, we were able to fit the DBQ-independent rate of initial DBH₂ reduction together with the partial decrease in the extent of b₄ reduction by assuming that DBH₂ binds to center N only when the b₄ heme is oxidized and DBQ/Q only when the b₄ heme is reduced (Fig. 8A). However, when both center N sites in the dimer were assumed to have the same kinetic properties, the effect of increasing DBQ concentrations on the slow phase of b₄ reduction was not accurately fitted (Fig. 8B). This model was the same as that used for fitting the center N kinetics in the presence of myxothiazol as shown in Fig. 7A. If the model was modified to include the condition that initial binding of DBH₂ to the oxidized bc₁ complex occurred to only one center N in the dimer followed by a fast activation of the second monomer upon SQ formation, which improved the fitting in the presence of myxothiazol as shown in Fig. 7B, the data in the presence of stigmatellin showed an even worse fit (data not shown).

FIGURE 7. Kinetic modeling of the effect of DBQ on center N kinetics in the presence of myxothiazol. The kinetic traces obtained as described for Fig. 3A were fitted to two DynaFit models (see supplemental material for details). Both models assumed exclusive binding of QH₂ when the b₄ heme was oxidized and of Q when the heme was reduced. The model in A allowed simultaneous binding of ligands to both center N sites in the dimer, whereas the model in B allowed initial binding to only one center N, after which SQ formation transmitted a conformational change to allow binding and reaction at the other monomer. Solid curves represent the best fit to each model. Only the first 500 ms of cytochrome b reduction are shown to evidence more clearly the difference between the kinetic traces and the fitted curves.

FIGURE 8. Kinetic modeling of the effect of DBQ on center N kinetics in the presence of stigmatellin. The kinetic traces obtained as described for Fig. 3B were fitted to two DynaFit models. The first model (A) assumed equal kinetic properties for both center N sites in the dimer. The second model (B) assumed different kinetic properties in each center N site of the dimer in addition to a slow conformational change (k = 0.15 s⁻¹) that resulted in both monomers acquiring the same kinetic properties (see supplemental material for details). Both models assumed exclusive binding of QH₂ when the b₄ heme was oxidized and of Q when the heme was reduced. Solid curves represent the best fits to each model. Only the first 900 ms of cytochrome b reduction are shown to evidence more clearly the difference between the kinetic traces and the fitted curves.
On the other hand, when the model considered that one center N site exhibited different rates of SQ formation and consumption with respect to the other and also included a slow conformational change ($k = 0.15 \text{s}^{-1}$) that gradually made the second center N display the same kinetic properties as the first one (32), a much more accurate fit was obtained (Fig. 8B). This model yielded ratios of SQ-forming/SQ-consuming rates that were 1–3 orders of magnitude higher in one monomer than in the other (see supplemental material for all fitted values). These results imply that the asymmetry in SQ stabilization between monomers is preserved for at least several seconds when stigmatellin is occupying center P, in contrast with what we observed in the presence of myxothiazol, where the conformation allowing asymmetric formation of SQ in the dimer appears to be much more short-lived.

**DISCUSSION**

Previous attempts to characterize the properties of SQ bound at center N of the cytochrome bc$_1$ complex concur in two main points (13–15). First, the EPR signal originating from SQ is optimally detectable at pH values of at least 8, at which the $E_{m}$ of the Q pool falls significantly below that of the $b_{11}$ heme. Second, the $E_{m}$ of this EPR-visible SQ is higher than that of the Q pool by 20–60 mV. The only exception to this behavior seems to be the SQ signal observed in the purified yeast bc$_1$ complex (6), which appeared to be smaller in magnitude and induced significant variation in the redox properties of the $b$ hemes. Because our SQ determinations were performed using dodecyl maltoside, which yields high activities in bc$_1$ complexes from a variety of sources (21), the values we have obtained are well in agreement with the rest of the literature.

The observation concerning the increase in the SQ EPR signal mainly at high pH values can be understood by considering that only SQ bound in the vicinity of a reduced $b_{11}$ heme has paramagnetic properties. The anti-ferromagnetic coupling between the $b_{11}^{3+}$ heme and SQ bound at center N has already been experimentally demonstrated in the yeast bc$_1$ complex (30, 31). In those experiments, a strong mismatch between the reduction of the $b_{11}$ heme as measured by visible spectroscopy and the disappearance of the $g = 3.60$ EPR signal generated by $b_{11}^{3+}$ was observed (30), indicating that a paramagnetic species formed in the vicinity of the heme during the early phase of the reductive titration was eliminating the $b_{11}^{3+}$ EPR signal. Depleting the bc$_1$ complex of Q eliminated the mismatch between the $b_{11}$ reduction levels determined by visible and EPR spectroscopy (31), confirming that SQ was the species forming a diamagnetic, exchange-coupled complex with $b_{11}^{3+}$ that eliminated the EPR signals from both the heme and the Q radical.

As shown in Fig. 9, the crystal structure of the yeast bc$_1$ complex with Q bound at center N (3) reveals an orthogonal orientation and short distance (<4 Å) between the Q and heme ring with an $E_{m}$ higher than that of the $b_{11}$ heme even at high pH values, casting doubt on the identity of its source. In this work, we have demonstrated that the $g = 2.004$ signal observed by EPR in the yeast complex is similar to that in other sources in that its $E_{m}$ is lower than that of the $b_{11}$ heme and that it genuinely reflects SQ bound at center N as judged by its dependence on Q; its sensitivity to a variety of center N inhibitors (antimycin, funiculosin, and ilicicolin H); and its insensitivity to stigmatellin and myxothiazol, which bind to center P (Figs. 1, 2, and 6). Although the sensitivity of the center N SQ to antimycin was shown previously with the bovine bc$_1$ complex (13), this is the first demonstration that funiculosin and ilicicolin H eliminate the SQ signal in any species.

The reason the previous study in yeast (6) found such anomalous properties of the SQ signal is probably due to the presence of high concentrations (0.1%) of detergent combinations of Triton and deoxycholate or taurocholate, which are not optimal for bc$_1$ complex purification and activity and which also
systems that apparently allow an interaction between the magnetic fields of the unpaired electrons from both species. Interestingly, a very similar orientation and distance are present between the $b_1\text{H}$ heme and a $c$-type heme in the homologous $b_6f$ complex that occupies a position equivalent to that of Q in the $bc_1$ complex (33). Recent work has demonstrated a strong coupling between the electrons of these two hemes as revealed by their EPR spectra (34). This provides further evidence that the relative positions of the $b_1\text{H}$ heme and SQ in the $bc_1$ complex are conducive to the formation of an EPR-invisible complex.

After correcting for the amount of EPR-invisible SQ at center N, it becomes evident that most of the SQ at physiologically relevant pH values exists bound close to the oxidized $b_1\text{H}$ heme (Fig. 2). This had already been proposed to occur at pH 7.05 in simulations that tried to explain the significant increase in the $E_m$ (Fig. 2). This had already been proposed to occur at pH 7.05 in simulations that tried to explain the significant increase in the $E_m$ of a fraction of the $b_1\text{H}$ heme population (7). It is noteworthy that those simulations assumed that Q bound at center N increases the $E_m$ of the $b_1\text{H}$ heme by 68 mV (7). Although nothing was discussed in that work with respect to the binding of Q and SQ to center N, the thermodynamic consequence of such a shift in the $E_m$ of the $b_1\text{H}$ heme is that Q should be expected to bind with an affinity 14 times higher when the $b_1\text{H}$ heme is reduced than when it is oxidized. We now show that DBQ oxidizes the $b_1\text{H}$ heme with a $K_m$ of 1.4 – 12 $\mu$m (Figs. 3 – 5), which gives a relative measure of the affinity of center N for Q when the $b_1\text{H}$ heme is in the reduced state. In contrast, 100 $\mu$m DBQ was unable to compete against DBH$_2$ for binding to center N when the enzyme was oxidized. This indicates that, as can be deduced from the effects of Q on the $E_m$ of the $b_1\text{H}$ heme (7), binding of the oxidized substrate to center N is favored by one to two orders of magnitude when the $b_1\text{H}$ heme is reduced.

The second common conclusion from EPR studies of SQ at center N, that the $E_m$ of this species is higher than that of the Q pool, has been interpreted as indicative of a tighter binding of QH$_2$ than Q to this site (13–15). After correcting for the EPR-invisible SQ bound close to $b_1\text{H}^{3+}$ (Fig. 2), we also conclude that the peak of SQ occurs at an $E_m$ higher than expected for the unbound QH$_2$/Q couple. We propose that this minimizes formation of SQ-$b_1\text{H}^{2+}$ complexes, which, as we have discussed elsewhere (12), favor superoxide formation at center P due to the absence of electron acceptors in cytochrome $b$. However, a tighter binding of QH$_2$ at center N would by itself inhibit electron flow out of cytochrome $b$ by inhibiting binding of Q. This potential problem can be circumvented by assuming that QH$_2$ binds poorly to center N when the $b_1\text{H}$ heme is reduced, but tightly when the heme is oxidized. As discussed above, Q binds with an opposite redox preference, thereby avoiding competition from QH$_2$. This redox specificity in binding to center N would also ensure that QH$_2$ leaves center N whenever electrons from center P arrive at the $b_1\text{H}$ hemes.

We have already provided evidence pointing to a deficient binding of QH$_2$ to the reduced $bc_1$ complex by analyzing the kinetics of antimycin binding to center N (32). In that work, we reported that DBH$_2$ concentrations much higher than those needed for binding to center N when the enzyme is oxidized do not decrease the binding rate of antimycin when the enzyme is reduced with dithionite. It is well known that the few equivalents of Q copurified with the $bc_1$ complex are sufficient to sustain high turnover rates when steady-state activity is measured (21). Although this endogenous Q complement is diluted together with the enzyme to $\mu$m concentrations in such assays, $\mu$m concentrations of DBH$_2$ or other QH$_2$ analogs do not
appear to out-compete Q at center N. DBQ added to such assays does not activate the enzyme, but rather inhibits it by competing against DBH₂ at center P (35). These observations are consistent with our proposal that Q binds to center N without interference from QH₂ even at extraordinarily high QH₂/Q ratios. Our present results also agree with the assumption of a redox-specific binding of QH₂ and Q to center N that depends on the oxidation state of the b₃₁ heme. The immediate decrease in the extent of cytochrome b reduction through center N at concentrations of DBQ much lower than those of DBH₂ indicates a noncompetitive binding pattern between the two Q species, as confirmed by fitting to kinetic mechanisms that incorporate that assumption (Figs. 4–7). In contrast, SQ is expected to bind independently of the b₃₁ heme state. As we have discussed previously (32), this is the case with antimycin, which we consider to be an analog of SQ.

The redox-specific binding of QH₂ and Q to center N might seem to be unsupported by crystal structures in which Q is found at center N, where the b₃₁ heme is supposed to be oxidized. However, the redox state of the enzyme and Q in the crystals is not easy to determine after x-ray bombardment, and the high concentrations (up to the mM range) of both enzyme and Q in the crystals could force Q to bind to center N even when the b₃₁ heme is oxidized. Thus, crystallographic data cannot be used to support or argue against redox-specific binding of ligands at center N as measured in this work.

To prevent QH₂ from interfering with oxidation of the b₃₁ heme by Q, another condition must be met. As illustrated by the model in Fig. 10A, if only QH₂ is assumed to bind to the oxidized enzyme at center N (intermediate I), reduction of the b₃₁ heme in one monomer (step I) will result in a tightly bound SQ (intermediate II), which will prevent Q from binding to any center N with b₃₁²⁺. After QH₂ oxidation in the other monomer (step 2), only dimers with two SQ-b₃₁²⁺ complexes can be formed (intermediate III). However, if the electron in the b₃₁ heme can equilibrate with the other monomer (step 2'), QH₂ bound will be displaced as the b₃₁ heme in its proximity receives an electron (intermediate III'). Q will then be able to bind and oxidize b₃₁²⁺ in that second center N (step 3), resulting in a dimer with two SQ-b₃₁²⁺³⁻ complexes (intermediate IV), which are optimal as acceptors for electrons coming from center P.

However, even assuming that there is electron crossover within the cytochrome b dimer, the kinetic data are not explained completely unless conformational communication between center N sites is included. If QH₂ is allowed to bind simultaneously to both center N sites, Q would be able to bind and oxidize the b₃₁ heme only after three events occur: reduction of one b₃₁ heme in the dimer with formation of SQ, electron transfer to the b₃₁ heme in the other monomer, and dissociation of QH₂ bound next to this formerly oxidized heme (Fig. 10A, steps 1, 2', and 3). This implies that one electron will necessarily have to reside in the dimer during the interchange of Q for QH₂ (intermediates II' and III') before leaving the b₃₁ hemes to form a second SQ (intermediate IV). Our modeling of center N kinetics in the presence of myxothiazol suggests that this delay in Q binding and b₃₁ re-oxidation should be observable as a triphasic behavior in the presence of DBQ (see the fitted curves in Fig. 7A).

We have found that a kinetic model that assumes that one center N is initially inactive more accurately fits the center N kinetics that we have observed in the presence of myxothiazol as the center P inhibitor (Fig. 7B). As illustrated in Fig. 10B, if QH₂ is allowed to bind to only one monomer (intermediate I), SQ formation in the active site (step 1, intermediate II) activates the second center N site to bind either QH₂ (step 2') or Q (step 2''), depending on whether the electron is still residing in the original monomer (intermediate III) or has moved to the other b₃₁ heme by electron crossover (intermediate III'). Because the electron has the same probability of residing in either b₃₁ heme, the likelihood of forming a dimer with two SQ-b₃₁²⁺ (intermediate IV) or two SQ-b₃₁²⁻³⁻ (intermediate V) complexes in equilibrium with their respective single SQ forms (intermediates III and III') would depend on the relative amount of QH₂ and Q available. Both models described in Fig. 10 assume that QH₂ and Q binding depends on the redox state of the b₃₁ heme. Consequently, either of them would explain why addition of even the highest concentration of DBQ never results in a complete oxidation of cytochrome b (Fig. 3). Nevertheless, we have recently presented evidence for conformational communication between center N sites in the dimer (32), supporting the asymmetric binding model shown in Fig. 10B, which also provides a better fit to the experimental data.

The more complicated kinetic behavior at center N that we have found in the presence of stigmatellin (Figs. 3B and 8) agrees with our previous suggestion that the conformational communication that breaks the asymmetry between the two center N sites is delayed when both Rieske proteins are close to center P (32). However, this delay is not long enough to allow the observation of any difference in the SQ properties once the enzyme is in equilibrium (Fig. 6). This could also explain why Q is found at both center N sites in the dimer in the bc₁ structure even in the presence of stigmatellin (3). Nevertheless, under some conditions, this asymmetry in binding to center N might be retained at least partially and for longer periods of time, as has been reported for the yeast bc₁ complex co-crystallized with cytochrome c in the presence of stigmatellin, where Q occupancy at center N is significantly decreased in one monomer, but not completely abolished (36).

The different center N kinetics we have observed in the presence of stigmatellin support the role of long-range communication between centers P and N, as has become increasingly evident by other reports in the literature (37–39). Our present results indicate that, even in the presence of myxothiazol, which does not affect the position of the Rieske protein (40), intradimer electron transfer, together with the b₃₁ redox-dependent and asymmetric binding of QH₂ and Q, is relevant to ensure proper center N function in the bc₁ complex dimer. All of these effects serve to control SQ formation in a way that maximizes the availability of b₃₁³⁻ as acceptor for electrons coming from center P while at the same time promoting the binding of Q and the release of QH₂ at center N whenever the b₃₁ heme undergoes reduction.

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