The Respiratory Substrate Rhodoquinol Induces Q-cycle Bypass Reactions in the Yeast Cytochrome bc₁ Complex

MECHANISTIC AND PHYSIOLOGICAL IMPLICATIONS

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Jonathan L. Cape‡, Jeff R. Strahan§, Michael J. Leneus§, Brook A. Yuknis§, Trieu T. Le§, Jennifer N. Shepherd§, Michael K. Bowman*, and David M. Kramer†

From the ‡Institute of Biological Chemistry, Washington State University, Pullman, Washington 99164-6340, §Department of Chemistry, Gonzaga University, Spokane, Washington 99258-0005, and †Structural Biology and Microimaging, Battelle Northwest Laboratory, Richland, Washington 99352-0999

The mitochondrial cytochrome bc₁ complex catalyzes the transfer of electrons from ubiquinol to cyt c while generating a proton motive force for ATP synthesis via the “Q-cycle” mechanism. Under certain conditions electron flow through the Q-cycle is blocked at the level of a reactive intermediate in the quinol oxidase site of the enzyme, resulting in “bypass reactions,” some of which lead to superoxide production. Using analogs of the respiratory substrates ubiquinol-3 and rhodoquinol-3, we show that the relative rates of Q-cycle bypass reactions in the Saccharomyces cerevisiae cyt bc₁ complex are highly dependent by a factor of up to 100-fold on the properties of the substrate quinol. Our results suggest that the rate of Q-cycle bypass reactions is dependent on the steady state concentration of reactive intermediates produced at the quinol oxidase site of the enzyme. We conclude that normal operation of the Q-cycle requires a fairly narrow window of redox potentials with respect to the quinol substrate to allow normal turnover of the complex while preventing potentially damaging bypass reactions.

The cyt bc₁ complex (EC 1.10.2.2) functions as the ubiquinol:cytochrome c oxidoreductase in the electron transport chains of mitochondria and bacteria, where it couples the energy released from the oxidation of ubiquinol (UQH₂) by high potential electron carriers (e.g. cyt c) to establish a proton motive force used to drive ATP synthesis (1, 2). Although the cyt bc₁ complex usually conserves energy across the membrane, deleterious side reactions can occur that bypass normal electron flow in the enzyme, robbing the cell of energy and potentially producing reactive oxygen species (3–7). To deal with this problem, the cyt bc₁ complex has apparently developed strict control mechanisms to prevent side reactions of ubiquinone/ubiquinol (UQ (1)/UQH₂; see Fig. 1) reaction intermediates (3, 6, 8–10).³

The turnover mechanism of the cyt bc₁ complex is best described by the modified Q-cycle (11, 12). The key feature of this mechanism is the bifurcated oxidation of UQH₂ at the quinol oxidase (Qo) site of the enzyme that sends one UQH₂ electron to a high potential chain (composed of the Rieske 2Fe2S cluster and cyt c₁), whereas the other electron reduces a low potential chain (cyt b₁ and cyt b₂) and is eventually recycled back into the Q pool through UQ reduction at a quinone reductase (Qr) site on the opposite side of the membrane. In most models UQH₂ oxidation proceeds by the initial reduction of the Rieske 2Fe2S cluster (13–15), which produces an unstable semiquinone (SQ) intermediate localized to the Qo site (SQₒ), which then reduces the low potential chain and UQ at the Qr site, leaving a labile UQ in the Qₒ pocket.

Certain conditions such as mutation (16–18), imposition of a high proton motive force (17, 19, 20), or inhibitor treatment (3–5, 7) can interrupt electron flow from SQₒ to the low potential chain and can lead to a large fraction of reactions that bypass the normal Q-cycle. Most Q-cycle bypass reactions are thought to result from the buildup of a highly reactive SQₒ species, although some are suggested to occur via reduced cyt b₁ (18); some of these reactions include (3, 4, 7) 1) double reduction of the high potential chain by SQₒ; 2) re-oxidation of reduced cyt b₁ by SQₒ, Qₒ, or O₂; 3) oxidation of SQₒ by O₂; and 4) direct reduction of cyt c by a labile SQₒ that leaves the Qₒ site. Although each of these reactions is thermodynamically unfavorable in comparison to the Q-cycle, they are typically not detected during uninhibited turnover of the complex, leading to the hypothesis that the cyt bc₁ complex has evolved a mechanism to steer its reactive intermediate toward productive, rather than harmful, reactions.

Several variants of the Q-cycle have been proposed to explain the prevention of bypass reactions, most of which impart strict constraints on the chemical and thermodynamic properties of the substrate QH₂ and its proposed initial oxidation product, SQₒ (6, 8, 11–14, 21–26). Previous studies (27–29) have suggested a link between these thermodynamic properties and the overall turnover rate of the bc₁ complex, but it is unclear how the intrinsic redox properties of the QH₂ substrate and the influence of the protein environment on these properties work together to enforce normal electron flow in the Q-cycle while also preventing bypass reactions. We suggest that both these factors tailor QH₂ oxidation in the Qₒ site to minimize the concentration of reactive intermediates that can participate in these bypass reactions, which can be achieved through the use of a substrate whose oxidation results in a less reducing redox poise in the Qₒ site (i.e. by preventing the accumulation of either SQₒ or reduced cyt b₁).

³ D. M. Kramer, J. L. Cape, I. Forquer, and M. K. Bowman, manuscript in preparation.
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In this work we use synthetic analogues of the quinone substrates UQ and rhodoquine (RQ) (2), see Fig. 1) to test boundary conditions on how the intrinsic thermodynamic properties of the substrate and its associated SQ stability influence the rate of Q-cycle bypass reactions. RQ is used in certain eukaryotes (30–36) and prokaryotes (37, 38) to shuttle electrons from the NADH dehydrogenase to a fumarate reductase (31, 36, 39–41). This alternative electron transport chain is used during anoxia to maintain cellular redox balance by using fumarate as a terminal acceptor instead of oxygen. Although RQ is chemically similar to UQ (a OCH3 substituent on the ring is replaced by NH2), the electron donating nature of its amino group gives it substantially more reducing power than its counterpart UQ (42–44), thus offering the opportunity to probe the efficiency of the Q-cycle under conditions where the relative stability of SQ species are modulated in both the Qo and Q, sites.

In certain organisms (nematodes (45), parasitic helminths (35, 46), mussels and oysters (35), and the photosynthetic bacterium Rhodospirillum rubrum (34)), RQ is co-localized in the mitochondrial or plasma membrane with UQ (32, 34, 47). This occurs even under aerobic conditions where biosynthesis of RQ is down-regulated but not entirely absent, providing the potential for the interaction of RQ or RQH2 with the cyt bc complex. The use of the synthetic substrates ubiquinone-3 (UQ3) and rhodoquine-3 (RQ3), where n = 3 isoquinone units (instead of 9 or 10 in the natural substrates), allows us to probe the Q-cycle and bypass reactions as well as to measure the relevant redox potentials of the electrochemical couples involved in these reactions. From these results we predict the potential impact due to bypass of the Q-cycle in organisms that contain a mixed UQ/RQ pool (32, 37, 48) and test the relationship between the rates of bypass reactions and the thermodynamic properties of these two substrates.

**EXPERIMENTAL PROCEDURES**

Chemicals for RQ3 Synthesis—All reactions were performed under a positive pressure of Ar with minimal exposure to light. Diethyl ether, ethyl acetate, hexanes, methanol, and tetrahydrofuran were purchased from Fisher (ACS-certified grade). Tetrahydrofuran was distilled from sodium benzenophenone ketyl, and methanol was dried over 3-Å molecular sieves. Mesyl chloride, sodium azide, sodium sulfate, triethylamine, sodium benzophenone ketyl, and methanol was dried over 3-Å molecular sieves. Mesyl chloride, sodium azide, sodium sulfate, triethylamine, sodium benzophenone ketyl, and methanol was dried over 3-Å molecular sieves. Mesyl chloride (0.046 ml, 0.59 mmol) was dissolved in tetrahydrofuran (5 ml) and cooled to –20 °C before treating with triethylamine (0.082 ml, 0.59 mmol). The solution immediately turned dark purple, and the reaction was quenched with H2O (5 ml). The mixture was extracted three times with diethyl ether (Et2O, 15 ml total). The organic layers were combined and rinsed with brine (10 ml). The organic layers were dried over Na2SO4 and concentrated in vacuo to yield compound 4, a yellow solid (162 mg, 73% yield). 31H NMR (300 MHz, CDCl3): δ 8.05 (m, 2H), 4.89 (t, 1H, J = 6.9 Hz), 4.20 (s, 3H), 3.47 (s, 3H), 3.19 (d, 2H, J = 7.1 Hz), 1.97 (s, 3H), 1.83 (m, 8H), 1.62 (m, 12H); 13C NMR (75 MHz, CDCl3): 182.6, 182.4, 149.0, 142.3, 139.5, 138.2, 135.3, 132.6, 131.3, 124.3, 123.8, 118.2, 61.5, 40.4, 39.7, 29.7, 26.7, 26.4, 25.7, 25.5, 17.6, 16.3, 16.0, 12.0; IR (thin film on NaCl): 2924, 2853, 1670, 1654, 1615, 1484, 1371, 1262, 1176, 790 cm–1. High resolution mass spectrum calculated for C23H34O3Na (M+ + Na) was 473.1968; found was 473.1919.

2-Azido-5-farnesyl-3-methoxy-6-methyl-1,4-benzoquinone (5)—The mesylate 4 (200 mg, 0.44 mmol) was dissolved in methanol (MeOH, 3.7 ml) and treated with sodium azide (NaN3, 58 mg, 0.89 mmol). The solution turned from a reddish-orange color to a reddish-brown color after 5 min. The reaction was allowed to proceed for 1.5 h before quenching with H2O (5 ml). The mixture was extracted three times with ethyl acetate (15 ml total), and the organic layers were washed with brine (10 ml), dried over Na2SO4, and concentrated in vacuo. Flash chromatography (9.1 hexanesethyl acetate) afforded 5, a reddish-orange solid (58 mg, 33% yield). Unreacted mesylate was recovered during chromatography. 31H NMR (300 MHz, CDCl3): δ 8.05 (m, 2H), 4.91 (t, 1H, J = 6.8 Hz), 4.07 (s, 3H), 3.19 (d, 2H, J = 7.0 Hz), 2.06 (s, 3H), 1.94 (m, 8H), 1.62 (m, 12H); 13C NMR (75 MHz, CDCl3): 183.8, 181.7, 144.8, 142.7, 139.1, 137.9, 135.2, 132.1, 126.9, 124.3, 123.8, 118.5, 61.4, 39.7, 39.6, 26.8, 26.4, 25.7, 25.3, 17.6, 16.3, 16.0, 12.1; IR (thin film on NaCl): 2920, 2346, 2141, 1654, 1602, 1429, 1219, 1146 cm–1; high resolution mass spectrum calculated for C23H34N3O3Na (M+ + Na+) was 420.2258; found was 420.2244.

2-Amino-5-farnesyl-3-methoxy-6-methyl-1,4-benzoquinone (RQ3) 7—Compound 5 (50 mg, 0.13 mmol) was dissolved in tetrahydrofuran (1 ml), and triphenylphosphine (33 mg, 0.13 mmol) was added. The solution immediately turned dark purple, and the reaction was allowed to proceed for 2 h. The reaction was quenched with H2O (2 ml), and a standard ethyl acetate/brine work-up was performed to provide 6. Compound 6 was hydrolyzed by slow gravity elution on SiO2 gel (9.1 hexaneethyl acetate) to provide pure 7 (27 mg, 64% for 2 steps). 31H NMR (300 MHz, CDCl3): δ 8.67 (m, 15H, 5.06 (m, 2H), 4.99 (t, 1H, J = 6.8 Hz), 3.76 (s, 3H), 3.17 (d, 2H, J = 7.0 Hz), 2.06 (s, 3H), 1.94 (m, 8H), 1.62 (m, 12H); 13C NMR (300 MHz, CDCl3): δ 5.06 (m, 2H), 4.99 (t, 1H, J = 6.8 Hz), 4.68 (broad s, 2H), 3.86 (s, 3H), 3.18 (d, 2H, J = 7.0 Hz), 2.03 (m, 8H), 1.96 (s, 3H), 1.67 (m, 12H); 13C NMR (75 MHz, CDCl3): δ 185.1, 180.8, 143.8, 137.2, 136.8, 135.8, 135.1, 134.7, 131.2, 124.3, 123.8, 119.3, 60.1, 39.7 (2C), 26.7, 26.5, 25.7, 25.3, 17.6, 16.3, 16.0, 11.6; IR (thin film on NaCl): 3485, 3362, 2918, 2849, 1652, 1606, 1468, 1440, 1375, 1302, 1206 cm–1; high resolution mass spectrum calculated for C23H34N3O3Na (M+ + Na+) was 372.2533; found was 372.2519.

Purification of the Cyt bc Complex—Yeast (Saccharomycyes cerevisiae) cyt bc complex was isolated from store-bought bakers’ yeast using the protocol of Ljungdahl et al. (49) with modifications (4). The concentration of the cyt bc complex was determined by the ferricyanide-ascorbate-dithionite absorbance difference spectra using published values of the extinction coefficients (49).

Steady State Turnover Measurements—Steady state turnover was measured after the reduction of cyt c using an extinction coefficient of 17 mM–1 cm–1 for the 550–542 nm difference absorption (3, 4). Just before measurement, synthetic substrate, QH2 (UQH2 or RQH2), was mixed in a cuvette containing 50 μM cyt c and 1 mM NaN3 in reaction buffer (50 mM MOPS, 50 mM Tricine, and 100 mM KCl at pH 7.0 before treatment with triethylamine (0.082 ml, 0.59 mmol).
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SCHEMЕ 1. Synthesis of RQ₃. Reaction conditions: a, mesyl chloride, triethylamine, tetrahydrofuran tetrahydrofuran, -20 °C; b, NaN₃, MeOH; c, triphenylphosphine, tetrahydrofuran; d, 8.2 hexanes/ethyl acetate, SiO₂.

In previous work (3, 4) we used only MOPS as a buffer but have now included Tricine to ensure a high buffering capacity in the pH range 8.0 and above; this additional component did not cause any measurable changes in the activity of the complex. Cyt bc₁ (1–3 nM) was added and mixed in the cuvette after collection of 20 s of background cyt c reduction. Turnover numbers were calculated by subtracting the background cyt c reduction rate from the initial rate observed after the addition of cyt bc₁. Measurement of Q-cycle bypass reactions under partially inhibited conditions was performed by the addition of 30 μM antimycin-A (AA) to the cuvette before Q₃H₂ addition and mixing. Cyt bc₁ (20–40 nM) was used for bypass reaction measurements involving UQ₃H₂ due to the very low rate of this reaction (1–5 s⁻¹). All experiments were repeated once with the addition of stigmatellin as a control, which completely abolished activity in all cases.

Measurement of Super oxid eProduction—Under partially inhibited conditions, i.e. conditions where AA blocks reduction of Q at the Q₁ site, the intermediate SQ₂ can reduce oxygen to superoxide, which then reduces cyt c directly in a diffusion-limited reaction (4). Thus, the rate of superoxide production from the Q₁ site can be determined by taking the difference in rates of AA-resistant cyt c reduction with and without added manganese superoxide dismutase (SOD). In most cases the addition of SOD inhibits the rate of cyt c reduction by 25–50%; the residual non-SOD-inhibitable cyt c reduction is caused by other Q-cycle bypass reactions such as the direct reduction of cyt c by SQ₂, or double reduction of the high potential chain (4). A typical superoxide production assay measuring cyt c reduction was performed by the addition of 300 units of manganese SOD to the steady state assay mixture described above.

The Extent of Cyt b Reduction in the Steady State—Measurement of the reduction state of both cyt b hemes during steady state turnover was performed by mixing 30 μM Q₃H₂ into a cuvette containing 265 nM cyt bc₁ and 5 μM cyt c. The low concentration of cyt c was used to slow depletion of the substrate through normal turnover and background cyt c reduction, which allowed us to observe steady state turnover of the complex over a timescale of minutes. The reaction was followed using the 561-nm minus 578-nm wavelength pair using an extinction coefficient of 26 mm⁻¹ cm⁻¹ for total cyt b. Saturating concentrations of AA and stigmatellin were added as needed. Sodium azide was omitted from the assay medium to allow re-oxidation of cyt c by the cyt c oxidase, which is a trace impurity in our preparations of cyt bc₁ complex. The reduction state of the cyt c pool was measured optically to verify that the complex had actually reached the steady state. Re-oxidation of reduced cyt c after an initial spike in the concentration (up to 300 nM) brought the steady state reduced cyt c concentration to ~60 nM. Over this time, the extent of cyt b reduction was nearly constant after the initial increase in reduction, suggesting rapid establishment of steady state conditions in the low potential chain.

Cyclic Voltammetry and Determination of Redox Potentials—Voltammetric measurements of the Q/Q₃H₂ and Q/Q’ couples for UQ₁ and RQ₂ were performed in aqueous solution under an Ar atmosphere at 23 °C using a BAS C50-W potentiostat with an Ag/AgCl reference electrode, glassy carbon working electrode, and Pt wire auxiliary electrode. The working electrode was washed with dilute H₂SO₄, repeatedly rinsed with distilled water, and then polished with alumina before all measurements. Determination of E(Q/Q’₃) was performed in unbuffered solution (100 mM KCl) as described in Shim and Park (50) at pH > 5.0. Aqueous E(Q/Q’₃) values were determined at several pH values above pH 5.0 to ensure that the E₀ and ΔE values were independent of pH and, thus, reflect a pure electron transfer process. The two-electron Q/QH₂ potential was measured in 50 mM phosphate, 100 mM KCl buffer at pH 7.0. E₀ values for each couple were taken as the average of the cathodic and anodic peaks at half height. All potentials given in the text are referenced to the standard hydrogen electrode.

We used experimentally determined values for the Q/Q’₃ and Q/QH₂ couples of UQ₁ and RQ₂ in aqueous solution to estimate reasonable values of the aqueous stability constant, Kₑ, and Eₐ values for the various redox couples contributing to Kₑ according to Equation (1) (51–53),

\[
Kₑ = \frac{[QH]²/[Q][QH₂]}{\exp(2F[E(Q/QH₂) - E(Q/Q’₃) + 0.059(pH - pKₑ)])/RT} \quad \text{(Eq. 1)}
\]

where pKₑ is the pKₐ for deprotonation of QH². The pKₑ for UQ₁ was previously determined to be 5.6 in aqueous solution using pulse radiolysis (54). We used a calibrated computational technique using a combination of density functional theory calculations and thermodynamic cycles to estimate the pKₐ of RQ₂H₂; the details of these calculations are to be presented in a forthcoming manuscript. This pKₐ was found to be similar to that of UQH², 5.0–5.6.

RESULTS

Synthesis of RQ₃ and UQ₃—A novel and efficient synthesis of RQ₃ was developed. The farnesylated analogue of demethylubiquinone (3), a known biosynthetic precursor of UQ (55–57), was prepared from fumagatin using an improved method that utilized farnesytrimethyl stannane and BF₃-O(CH₂CH₂)₂ followed by oxidation with Ag₂O (Scheme 1) (58–60). Compound 3 was converted to the mesylate 4 using mesyl chloride and triethylamine in a 77% yield (38). The mesylate was then treated with NaN₃ to generate the azide 5 via a nucleophilic addition–elimination reaction (62). This reaction produced a 33% yield of azide; however, unreacted starting material could be recovered. Reduction of the azide with triphenylphosphine formed an iminophosphorane 6, which was not readily hydrolyzed during an aqueous work-up as previously reported (63, 64). However, treatment of 6 with dilute acid or slow elution through a silica gel column provided pure RQ₂ (7) in a 64% yield (two steps). UQ₁ was prepared using published procedures (55).

Thermodynamic Characterization of UQ₁ and RQ₂—Cyclic voltammetry of UQ₁ in unbuffered aqueous solution (50) at pH > 5 exhibited

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Quasi-reversible cathodic and anodic peaks (ΔE = 190 mV). The lack of complete reversibility likely reflects adsorption of UQ₃ to the electrode surface. Rigorous cleaning of the electrode surface did not affect the reversibility of these peaks. The Eₒ value obtained from this measurement was −23 mV (see TABLE ONE), which is consistent with a previous report of Eₒ = −30 mV for the UQ (Q/Q') couple in 80% aqueous ethanol (65). The (Q/Q') couple for RQ₃ also exhibited quasi-reversible voltammetric peaks (ΔE = 109 mV), although to a much lesser extent than UQ₃, with Eₒ = −170 mV.

As an additional assurance that the Eₒ values measured here are reasonable estimates of the actual equilibrium potentials, we compared the (Q/QH₂) couple of RQ₃ with the previously published polarographic value. Our result (TABLE ONE), Eₒ = −45 mV, is in reasonable agreement with the previously published value of −65 mV (44). Measurement of the Eₒ for the UQ₃ (Q/QH₂) couple was not possible due to complete irreversibility of the cathodic peak, resulting in the absence of an anodic peak under these conditions.

Normal Turnover and Q-cycle Bypass Reactions Induced by UQ₃H₂ and RQ₃H₂—TABLE TWO summarizes the results from steady state turnover of the cyt bc₁ complex driven by UQ₃H₂ and RQ₃H₂ under uninhibited (Fig. 2 and TABLE TWO, row 1) and AA-inhibited (TABLE TWO, row 2) conditions. Cyt c reduction observed under AA-inhibited conditions results from partial turnover of the complex and one or more reactions that bypass the Q-cycle and directly reduce cyt c. Using UQ₃H₂ as a substrate, the addition of 30 μM AA decreased the rate of cyt bc₁ turnover from 143 to 3 s⁻¹, consistent with previous results (3, 4, 7). On the other hand, the use of RQ₃H₂ exhibited an uninhibited turnover rate substantially lower than UQ₃H₂, 76 s⁻¹, and exhibited a relative insensitivity to AA treatment, 88 s⁻¹.

We verified that AA did indeed bind to the Q site of the complex under these conditions by observing the red-shifted absorbance spectrum of cyt b₆₆ induced by AA binding in the presence of RQ₃ (66) (data not shown). Further titration of RQ₃ into the AA-treated complex did not reverse the red-shifted spectrum of cyt b₆₆, showing that RQ₃ does not displace AA during the assay.

A large degree of curvature in the kinetic traces with RQ₃H₂ was observed, suggesting that the true initial rate of cyt c reduction was obscured during the manual mixing time. We thus repeated the AA-induced bypass reaction experiments using stopped flow. Indeed, as shown in TABLE ONE, the rate of UQ₃H₂ AA-resistant turnover was 2.3 s⁻¹, consistent with steady state measurements, whereas the rate of RQ₃H₂ AA-resistant turnover was very high, at 207 s⁻¹, a difference in bypass rates of more than 100-fold. This rate might be limited by the 0.2-s time resolution of our spectrophotometer.

Both RQ₃H₂ and UQ₃H₂ exhibited similar apparent Km values with the bc₁ complex, 10 and 8 μM for UQ₃H₂ and RQ₃H₂, respectively. These Km values are similar to those measured previously for the isolated yeast bc₁ complex by various groups (7, 67–69).

Partial Inhibition of the Q-cycle by RQ₃H₂/RQ₃₆—The curvature in the steady state kinetic traces described above suggested some form of product inhibition (Fig. 2). We first tested for inhibition of Q reduction at the Q site induced by RQ₃ or UQ₃. Typically, efficient oxidation of cyt b by Q at the Q site results in only a small accumulation of reduced cyt b in the low potential chain under steady state conditions, whereas blockage of cyt b reoxidation at the Q site with inhibitors results in a substantial accumulation (30–50%) of reduced cyt b (10). The data shown in Fig. 3 confirm these observations in our system using UQ₃H₂ as substrate. Approximately 10% of total cyt b was reduced under uninhibited conditions, whereas 52% went reduced in the presence of AA. In contrast, when RQ₃H₂ was used (Fig. 3), 77% of cyt b went reduced under uninhibited conditions, suggesting that the re-oxidation of cyt b by RQ₃ was slowed with respect to its reduction. The addition of AA in the presence of RQ₃H₂ resulted in eventual reduction of nearly all (92%)

### TABLE ONE

| Quinone midpot (mV) | Ubiquinone | Rhodoquinone |
|---------------------|------------|--------------|
| E(Q/QH₂)            | +110 mV    | +45 (−65) mV|
| E(Q'/Q')            | −23 (−30) mV | −170 mV     |
| E(Q'/QH')           | −105 mV    | −252 mV      |
| ΔE[Q'/QH'] − QH'/QH₂ | −430 mV    | −414 mV      |
| Log Kₒ             | −7.22      | −7.00        |

*a* E(Q'/Q') for ubiquinone in 80% EtOH:20% H₂O, reported in Bauscher and Manele (65) and references therein.

### TABLE TWO

| Quinol donor | Ubiquinol | Rhodoquinol |
|-------------|-----------|-------------|
| Vₘₐₓ uninhibited (s⁻¹) | 143 | 76 |
| Vₘₐₓ + AA (s⁻¹) | 3 | 88 |
| Vₘₐₓ⁺ (s⁻¹) | 1.5 | 44 |
| Vₘₐₓ + AA + SOD (s⁻¹) | 2.0 | 59 |
| Vₘₐₓ⁺⁺ (s⁻¹) | 1.0 | 29 |
| Fraction SO⁺ | 0.66 | 0.66 |
| Vₘₐₓ + AA pre-steady state (s⁻¹) | 2.3 | 207 |
| Kₘ (μM) | 10 (7) | 5 |

*a* Vₘₐₓ⁺⁺ = 0.5 (Vₘₐₓ + AA) due to the fact that the first electron reduces the high potential chain in all cases; therefore, accounts for half of the overall rate.

**Figure 2.** Representative kinetic assays of cyt bc₁ with either UQ₃H₂ or RQ₃H₂. Assays were performed as described under “Experimental Procedures.” Both traces shown here were collected using either 70 μM UQ₃H₂ (open circles) or 30 μM RQ₃H₂ (filled squares) with 4 nM cyt bc₁ and 50 μM cyt c. The background rate of cyt c reduction by the substrate alone is subtracted for simplicity. Ubiquinol always reached the full extent of cyt c reduction, whereas RQ₃H₂ did not (see Normal Turnover and Q-cycle Bypass Reactions Induced by UQ₃H₂ and RQ₃H₂).
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**FIGURE 3.** Steady state measurement of cyt b reduction in the presence of UQ$_3$H$_2$ and RQ$_3$H$_2$. Measurement of the total cyt b reduction state under steady state conditions in the cyt bc$_1$ complex is described under “Experimental Procedures.” Data were determined in triplicate, and results were consistent between two different preparations of the cyt bc$_1$ complex. Error bars represent the S.D. of the measurement. Assays were performed using 265 nM bc$_1$ complex (yielding 530 nM total cyt b heme) using either 30 μM RQ$_3$H$_2$ or UQ$_3$H$_2$ with 5 μM cyt c used as an acceptor to the complex in all assays. AA (30 μM) was added as needed, and sodium azide was omitted to allow re-oxidation of cyt c by residual cyt c oxidase. Cyt b reduction was followed using the 561 − 578 nm wavelength pair. Inset, time-based absorbance traces during the steady state measurement for RQ$_3$H$_2$ + AA (A), RQ$_3$H$_2$ with no AA (B), UQ$_3$H$_2$ + AA (C), and UQ$_3$H$_2$ with no AA (D). The slow autooxidation of cyt b shown in the inset for traces A, B, and C is due to substrate depletion after several minutes through background cyt c reduction and Q$_1$ autooxidation.

of cyt b. The statistically significant overall lower accumulation of cyt b during RQ$_3$H$_2$ oxidation in the absence of AA probably indicates that reduction of RQ$_3$ at the Q$_1$ site occurs, albeit at a lower rate than with UQ.$_3$.

In addition to functioning as a Q$_1$ site inhibitor, Fig. 4 demonstrates that RQ$_3$ also interferes with UQ$_3$H$_2$ binding to the Q$_1$ site. The addition of up to 20 μM RQ$_3$ both increased the apparent $K_m$ for UQ$_3$H$_2$ from 8.5 to 13.7 μM, and lowered the apparent $V_{max}$ from 98 to 84 s$^{-1}$, for UQ$_3$H$_2$ oxidation. These results are consistent with RQ$_3$ acting as a weak mixed competitive inhibitor of the bc$_1$ complex.

**Superoxide Production Resulting from Q-cycle Bypass Dramatically Increases Using RQ$_3$H$_2$ as a Substrate**—The SOD sensitivity of AA-resistant cyt c reduction was used to estimate the fraction of bypass reactions resulting in superoxide production. The initial rate of AA-resistant UQ$_3$H$_2$-induced turnover resulting in superoxide production was 1.0 s$^{-1}$, consistent with previous measurements (3, 4, 7), whereas the same measurements performed with RQ$_3$H$_2$ resulted in an astonishing superoxide production rate of 29.5 s$^{-1}$. The ～30-fold increase in rates for RQ$_3$H$_2$-induced superoxide production relative to UQ$_3$H$_2$ (see TABLE TWO, row 5) correlates well with the overall increased rate of bypass reactions when this substrate is used (see TABLE TWO, row 3), which also increases by a factor of ～30, suggesting that about 66% of all QH$_2$ oxidations result in the reduction of oxygen to superoxide (3, 4, 7) under partially inhibited conditions regardless of the substrate used (TABLE TWO, row 6).

We observed complex (or strongly multi-phasic) cyt c reduction kinetics, probably reflecting a progressive onset of product inhibition (see “Partial Inhibition of the Q-cycle by RQ$_3$H$_2$”). Because the curvature was apparent even at short reaction times, we suggest that our measured rates of RQ$_3$H$_2$-induced superoxide production represent a lower limit. Unfortunately, even with steady state kinetics using stopped-flow, it is not presently possible to distinguish between the first two non-inhibited turnovers (which result in cyt $b_2$ and $b_1$ reduction) and the subsequent true bypass reactions.

**FIGURE 4.** Mixed competitive inhibition of the cyt bc$_1$ complex by RQ$_3$. UQ$_3$H$_2$ was titrated from 2.5 to 60 μM with 2 nM cyt bc$_1$ complex in the presence of 0 μM (filled squares), 10 μM (open circles), and 20 μM (open triangles) RQ$_3$. Measurements were also performed using decyl-UQ$_3$H$_2$, a synthetic UQ$_3$ analogue, with similar results (not shown). All activity was demonstrated to be stigmatellin-sensitive (data not shown). Inset, replot of extrema in double reciprocal form showing the decrease in $V_{max}$, and increase in $K_m$ upon increasing [RQ] from 0 μM (filled squares) to 20 μM (open circles).

**DISCUSSION**

The Thermodynamic Properties of RQ and UQ and Predictions for Reactivity—Our electrochemical data (TABLE ONE) show that, as expected, the redox potential of the E(QH$^+$/QH$_2$) for RQH$_2$ is substantially more negative than the same couple for UQ$_3$H$_2$ (by nearly 200 mV). We predict that the low potential of the RQH$^+$/RQH$_2$ couple will result in increased concentrations of rhodosemiquinone (RSQ) in the Q$_1$ site due to an increase in the equilibrium constant for RSQ formation by over 3 orders of magnitude, thus increasing the rate of bypass reactions. Similarly, the aqueous values of E(RQH$^+$/RQH$_2$) and E(RQH$_2$/RSQ$^+$) are ～150 mV lower than the corresponding UQ species, likely decreasing the equilibrium constant for reduction of RQ at the Q$_1$ site by more than 2 orders of magnitude. We, thus, predict that the slow oxidation of the low potential chain during steady state turnover will lead to an AA-like inhibition of the complex, again having the effect of increasing the rate of bypass reactions. Consistent with these predictions, we observe that the rates of bypass reactions and superoxide production are drastically increased (more than 100-fold, see TABLE TWO) when RQ$_3$H$_2$ is used as a substrate instead of UQ$_3$H$_2$.

The SQ stability constant, $K_s$, is often used alone as a qualitative predictor of SQ reactivity and steady state concentration for many Q$_1$ site models (6, 8, 11, 70). Despite the clear differences between these substrates in our estimates for the one-electron couples, E(QH$^+$/QH$_2$) and E(Q/Q$^+$), the estimated semiquinone stability constants (TABLE ONE) for RSQ and USQ differ by only a factor of 2, with RSQ ($K_s = 1.0 \times 10^{-5}$) slightly more stable than USQ ($K_s = 5.3 \times 10^{-6}$). The semiquinone stability constant by itself is, thus, an incomplete predictor of reactivity and SQ concentration and must be used in conjunction with the overall two-electron QH$_2$/QH$_2$ couple to derive the one electron couples, so that meaningful predictions can be made.

It is important to note that the in situ potentials of the QH$_2$/Q$_1$ species in the Q$_1$ and Q$_3$ sites are likely to differ substantially from the aqueous potentials measured and estimated here due to the unique electrostatic environment of the protein and dielectric effects (71–74). Interestingly, the two Q binding sites in the complex likely exhibit disparate effects on the stability of the SQ species; the Q$_1$ site has been shown to stabilize the SQ (74, 75), whereas some models suggest the Q$_3$ site greatly destabilizes the SQ (11–13, 70). Nevertheless, it is reasonable to assume that the
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changes in the redox potentials of the RQ species in the Qₒ pocket will parallel those of the UQ species so that the differences measured in solution will persist in the Qₒ pocket.

RQ/RQH₂ Alters the Redox Poise of the bc₁ Complex to Favor Bypass Reactions—Turnover of the cyt bc₁ complex using RQ₃H₂ as a substrate exhibited three features distinguishing it from that with UQ₃H₂ (Fig. 2); 1) upon manual mixing of substrate into reaction buffer (with a mixing time of ~2 s) the initial rate of uninhibited turnover (76 s⁻¹) was about half that with UQ₃H₂ (143 s⁻¹), 2) the kinetic trace exhibited a high degree of curvature as the reaction proceeded, lowering the steady state rate over time, and 3) unlike UQ₃H₂ oxidation, the addition of AA had little effect on the initial rate of turnover.

We suggest that all of these observations can be explained by product inhibition at both the Qₒ and Q_s sites. This interpretation is supported by the "backup" of reducing equivalents in the low potential chain (Fig. 3) when RQ₃H₂ is used as a substrate and the mixed inhibition observed from RQ₃ using UQ₃H₂ as a substrate (see Fig. 4).

The Q_s site inhibition by RQ₃/RQ₃H₂ can be explained by two possible, non-exclusive models, both of which are consistent with the predictions based on solution thermodynamics given in the previous section. First, using a pure thermodynamic argument, RQ₃H₂ is a considerably stronger reductant (~150 mV) than UQ₃H₂, allowing for the establishment of a more reducing cyt b redox poise. Likewise, the lower redox potential of the RQ/RQ⁺ couple should make it a poor substrate for the Q_s site, effectively "trapping" electrons in the cyt b chain. Second, using a kinetics argument, RQ₃H₂, RQ, or even RSQ could act as inhibitors of or slow substrates for the Q_s site. The simplest interpretation is that both the kinetic and thermodynamic properties of RQ/RQH₂ result in a more reduced cyt b chain, leading to a buildup of reactive intermediates in the Q_s site, possibly an SQ, which increases the rate of superoxide production and other bypass reactions (TABLE TWO). These data offer an explanation for previous findings that RQ₃/RQ₃H₂ inhibition by the cyt bc₁ complex in R. rubrum chromatophores is insensitive to AA (34) (i.e. it is likely that the bc₁ complex is already partially inhibited by RQ).

The Physiological Impact of a Mixed Q Pool in Q-producing Organisms—The results discussed above beg the question, How do organisms that use both RQ/RQH₂ and UQ/UQH₂ in their quinone pools prevent deleterious side reactions at the cyt bc₁ complex? For example, Euglena gracilis possesses both RQ/RQH₂ and UQ/UQH₂ simultaneously (although RQ synthesis is down-regulated under aerobic conditions, residual RQ persists for extended periods, with a ratio of UQ:RQ approaching 3:1 under fully aerobic conditions) (32). Simultaneous accumulation of RQ and UQ has also been observed in certain nema-
todes (45), parasitic helminths (35, 46), mussels and oysters (35), and the photosynthetic bacterium R. rubrum (34). The presence of the two quinones is apparently physiologically necessary for rapid transitions between aerobic and anaerobic respiration in these organisms. Our model system, S. cerevisiae, does not synthesize RQ, but we suggest that if the cyt bc₁ complex in these RQ-containing organisms is similar to that in yeast, then co-mingling of RQ and UQ in cyt bc₁-accessible compartments should result in production of large amounts of super-
oxide and other unwanted bypass reactions.

We consider three possible mechanisms by which this superoxide production could be prevented in these organisms. First, the cyt bc₁ complexes from RQ-containing organisms might have different sub-
strate specificities. Sequence alignments of cyt b proteins between organisms known to contain mixed RQ/UQ pools (Mytilus edulis, R. rubrum) and UQ-only species (chicken, yeast) did not reveal any discernable trends in conserved residues known to be involved in Qₒ and Q_s site binding (not shown), making this possibility seem unlikely. Nevertheless, we are currently testing this possibility directly by characterizing the cyt bc₁ complexes from several RQ-containing organisms.

Another possibility is that the mitochondrial inner membrane or bacterial plasma membranes are compartmentalized either into separate membrane systems or domains (76, 77) that contain separate electron transfer chains. A third possibility is that the cyt bc₁ complex and/or fumarate reductase are under regulatory control to prevent the co-ex-
istence of RQH₂ and an active cyt bc₁ complex in the same bioenergetic membrane.

Implications for the Function (and Dysfunction) of the Cyt bc₁ Complexes—This work shows that the propensity of the bc₁ complex to participate in Q-cycle bypass reactions can be greatly modified by changing the thermodynamic properties of the substrates Q and QH₂, particularly those that affect the steady state concentration of reactive intermediates in the Q_s site. In principle either SQ or cyt b could be responsible for the reduction of oxygen during bypass reactions, although it has been argued that cyt b is not sufficiently reducing to act as an effective O₂ reductant (4). Our data show that the total amount of reduced cyt b does accumulate to a larger extent with RQ₃H₂ than with UQ₃H₂, but in most Q-cycle models the accumulation of reduced low potential chain components would also imply an increase in the concent-
ration of SQ. Alternatively, RSQ might be very labile relative to USQ in the Q_s site, thus allowing it to more readily escape and collide with O₂.

In principle, both thermodynamic and kinetic factors will influence the amount of reactive intermediates formed in the steady state, i.e. how much of the intermediate is formed, how fast it is formed, and how fast it is consumed. All of these factors will be influenced by the redox and binding properties of SQ₃/QH₂ and Q/SQ couples, which in turn are determined by the structural properties of the substrate and the binding site.

We propose that the Q-cycle requires a fairly narrow window of substrate thermodynamic (redox and binding) properties to ensure a low steady state concentration of reactive intermediates while allowing for rapid Q-cycle turnover. UQ/UQH₂ seems to be particularly well suited to this task in comparison to RQ/RQH₂. Intriguingly, this “matching” of the quinone/quinol redox properties with those of the cyt bc₁ complex appears to be well conserved; menaquinol-oxidizing cyt bc-type complexes have electron carriers with redox potentials about 150 mV lower than those in UQ₃H₂-oxidizing complexes, perfectly matching the 150-mV lower potential of the substrate Q/QH₂ couple (61, 78–80).

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The Respiratory Substrate Rhodoquinol Induces Q-cycle Bypass Reactions in the Yeast Cytochrome $bc_1$ Complex: MECHANISTIC AND PHYSIOLOGICAL IMPLICATIONS

Jonathan L. Cape, Jeff R. Strahan, Michael J. Lenaeus, Brook A. Yuknis, Trieu T. Le, Jennifer N. Shepherd, Michael K. Bowman and David M. Kramer

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