The kinetics of reduction of the cytochrome and quinone constituents of yeast complex III by the substrate homolog Q₁H₂ have been measured under a variety of conditions.

The maximum rates of reduction of cytochromes b and c₁, and of the endogenous Q₉ by Q₁H₂ were sufficiently fast to support the V₁max for the reduction of cytochrome c by this substrate.

The absorbance at 562 nm showed an initial increase which was subsequently followed by a decrease. This decrease was synchronous with the appearance of reduced cytochrome c₁, and is interpreted as reflecting the absorbance contribution of c₁ at 562 nm under conditions where the steady state level of the b cytochromes is constant.

Prereduction of c₁ and the Fe/S cluster did not affect the initial very rapid reduction of b, but the second phase was eliminated. Antimycin abolished the very rapid rate of reduction of cytochrome b in untreated complex III and completely inhibited the reduction of cytochrome b in complex III in which c₁ and the Fe/S cluster had been prereduced. However, the reduction of the endogenous quinone was essentially unaffected by these treatments. Antimycin had no effect on the reduction of c₁. Funiculosin also suppressed the very rapid reduction of b while both myxothiazol and 5-n-undecyl-6-hydroxy-4,7-dioxobenzothiazole did not modify this phase of the reaction; no secondary decrease in absorbance was observed in the presence of any of these inhibitors.

Most of the observed kinetic changes could be reproduced by simulation of the Q-cycle; simple linear and branched schemes were unable to reproduce the data.

It is well established that electron transfer by complex III (ubiquinol cytochrome c reductase, EC 1.10.2.2) can proceed with the translocation of protons across the mitochondrial inner membrane, and there is good evidence that the stoichiometry of this process results in the appearance of two protons on the outside of the membrane for each electron transferred from the reducing substrate to cytochrome c (1).

Current models for the mechanism of this process are built upon the initial proposal of Wikstrom and Berden (2), who suggested that ubiquinol is oxidized in consecutive one-electron steps by two different redox centers. This idea was extended by Mitchell (3) as the Q-cycle and by Wikstrom and Krab (4) as the b cycle; the Q-cycle was subsequently refined by Trumpower (5). The Q-cycle proposes that proton translocation is directly coupled to redox changes in the endogenous quinone, whereas the b-cycle, in its original formulation, required one of the b cytochromes to function as a proton pump. More recently (6), the b cycle has been substantially revised; in this revision the role of Q as the proton translocator is stressed and the fundamental differences between the two competing cycles have been lessened.

Much of the experimental data which has led to these hypotheses has been obtained by following the redox changes of the cytochrome components of beef heart complex III in the absence and in the presence of a variety of inhibitors (2, 7-13), and observing the effects of manipulating the iron-sulfur center (14). The kinetic behavior of the endogenous quinone has been studied much less frequently, primarily through EPR observations of the radical anion (10, 15). In the solubilized enzyme, this species accounts for only 5% of the total endogenous Q at physiological pH values. Furthermore, despite the intense effort that has been invested in deriving the Q-cycle there seems to have been little or no evaluation of the properties of this scheme using simulation techniques.

Yeast complex III differs from the corresponding heart enzyme in a number of ways. For example, with the yeast enzyme it has not been possible to clearly demonstrate any differences in optical properties of the two b centers nor can one show any spectral shifts upon addition of the inhibitor, antimycin (16). Funiculosin has an unique effect in that it produces a large increase in the midpoint potential of the iron-sulfur cluster with little or no effect on the b hemes (16); in the heart enzyme it is the b hemes that appear to be most sensitive to funiculosin. Yeast complex III is very insensitive to 2,5-dibromo-3-methyl-6-isopropylbenzoquinone while the heart enzyme is readily inhibited by this compound. The lipid composition of the two complexes is different; in particular the yeast complex contains ergosterol and squaene whereas the heart enzyme contains cholesteral (17).

Earlier we reported our first experiments on the kinetics of reoxidation of complex III from yeast in which only the iron-sulfur center and cytochrome c₁ were reduced; these experiments led to the conclusion that the rate of electron transfer between these two redox centers is sufficiently fast as to maintain their redox equilibrium (18). We subsequently extended these experiments to enzyme in which all of the redox centers are reduced and compared the effects of using oxidants which react directly with c₁ with those that react with the endogenous quinone. In addition, the consequences of (i) removing the endogenous quinone and (ii) of adding the electron transport inhibitors antimycin, myxothiazol, and funiculosin were characterized.

As a complement to our experiments on the reoxidation of yeast complex III, we have now completed a study of the reduction of this enzyme system using Q₁H₂ as electron donor.

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1. Tsai, A.-L., Olson, J. S., and Palmer, G., (1987) J. Biol. Chem. 262, in press.
2. The abbreviations used are: Q, Q₉H₂, Q₉, and Q₁H₂ (n = 1,6,10), ubiquinone-n, its oxidized, neutral, and anionic radical forms and its hydroquinone; HPLC, high pressure liquid chromatography; UHDBT, 5-n-undecyl-6-hydroxy-4,7-dioxobenzothiazole.
donor. These experiments had the following objectives: (i) to obtain a reasonably complete set of kinetic data on both the cytochromes and endogenous quinone so that quantitative tests of possible mechanisms might be conducted, (ii) to establish whether the kinetic results could be combined with redox potentials to constrain computer simulations of plausible mechanisms, and (iii) to establish whether the clear differences in physical and chemical properties present in the yeast complex are reflected in important kinetic differences between the yeast and beef heart enzymes.

MATERIALS AND METHODS

Yeast complex III was prepared using the method described by Siedow et al. (19); a typical preparation had a coenzyme Q§ content of 1–1.5 mol/mol of c§. The yeast succinate-cytochrome c reductase was obtained as the 55% ammonium sulfate fraction after removal of complex IV (19). The pellet was resuspended in 0.1 M potassium phosphate, pH 7.4, containing 1 mM EDTA, and the residual ammonium sulfate was removed by passage through a Bio-Gel P-6 column equilibrated with the same buffer. The concentration was determined from the content of cytochrome c§.

Complex III was depleted of coenzyme Q§ by either of two methods. The first utilized repeated hexane extraction of lyophylized complex III (20), and the second required repeated fractionation with ammonium sulfate in the presence of sodium cholate (21). Reconstitution of Q§-depleted complex III was carried out essentially as described by Yu and Yu (21). When present, Q§ was first added (at a ratio of 2 mol/mol of c§) to the Q§-depleted enzyme, phospholipid (0.2–0.3 mg/ml of mg of complex III) was then added, and the reaction mixture was incubated on ice for at least 4 h before kinetic measurements were begun.

Cytochrome c (horse heart, Type IV), Q§, and antimycin were purchased from Sigma. Cardiolipin was obtained from Avanti Polar-Lipid, Inc., Ubiquinone-1 (Q§) was a generous gift from Dr. William Scott (Hoffman-La Roche), funiculosin was provided by Dr. G. Lenaz (Bologna), and myxothiazol by Dr. H. Reichenbach (Braunschweig). Stock solutions of antimycin, funiculosin, myxothiazol, and Q§ were prepared in Me2SO, and Q§ was prepared in absolute ethanol. The cardiolipin was prepared as a clear solution (10 mg/ml) in 0.5% Tween-20.

The concentrations of both Q§ and Q§ were determined spectrophotometrically using a millimolar absorbance coefficient of 12.25 cm§1 for the difference between the oxidized and reduced quinones at 275 nm.

Fast kinetic measurements were conducted on a Gibson-Durrum stopped-flow instrument maintained at 8°C. Kinetic data was collected with an OLIS model 3820 data acquisition system connected to the stopped-flow apparatus through a North Star microcomputer. The kinetic data were routinely fitted to several exponentials using standard nonlinear least-squares methods (22). In general two exponentials were required to describe the data for the absorbance changes at 553 nm. The kinetic data were routinely fitted to several exponentials using standard nonlinear least-squares methods (22). In general two exponentials were required to describe the data for the absorbance changes at 553 nm. The kinetic data were routinely fitted to several exponentials using standard nonlinear least-squares methods (22).

RESULTS

Steady-state Kinetics Studies of Complex III as a Q§H§-Cytochrome c Reductase—Although ubiquinol-6 (Q§H§) is believed to be the physiological reductant of yeast complex III, the aqueous insolubility of this quinol prompted the use of Q§H§ as a structural homolog having only a single isoprenoid unit attached to C8 of the quinonoid ring; Q§H§ is soluble in aqueous detergent solutions to a concentration of about 1 mM.

The steady-state kinetics of the rates of reduction of cytochrome c§ by Q§H§ exhibit a graphical pattern typical of "ping-pong" kinetics. The derived kinetic parameters are: Vmax (mols of c§ reduced per mol of c§) = 95 s§1; Kmax (Q§, H§) = 39 μM; Kmax (cytochrome c§) = 85 μM. These data were obtained at 8°C, the temperature used for the transient state experiments.

The Transient Kinetics of Reduction of Oxidized Yeast Complex III—When oxidized complex III was mixed with 10 eq of Q§H§ the absorbance at 562 nm increased rapidly with a rate constant of about 200 s§1 (Fig. 1). This rapid increase reached a maximum in about 10 ms; the extrapolated absorbance change for this increase was about 20% of the total to be expected. Subsequently, the absorbance at this wavelength decreased less rapidly, at a rate of about 45 s§1, finally reaching a value near that measured at the beginning of data collection. These results resemble those observed by Jin et al. (7) at 562 nm upon reaction of beef heart succinate cytochrome c reductase with succinate, and interpreted as an initial reduction and subsequent slow oxidization of cytochrome(s) b. (It should be noted that in yeast complex III it is not possible to separate the contribution of individual b centers by comparing the kinetic results obtained at 562 and 566 nm.)

The initial reduction of cytochrome b proceeded significantly faster than that of c§ (Fig. 1). Thus, when the reduction of cytochrome c§ was followed by monitoring the absorbance increase at 553 nm, the initial fast phase proceeded at a rate of about 50 s§1 and constituted 50–75% of the total absorbance change (Fig. 1); the remaining reduction of c§ proceeded at a rate of about 3 s§1. A comparable slow phase was not observed at 562 nm. In experiments with a large number of enzyme preparations, the proportion of c§ that became reduced ranged from 50–100% of that anticipated from static absorbance measurements on oxidized and reduced complex III.

The observed rate constant for the rapid increase in absorbance at 562 nm exhibited a hyperbolic dependence upon the concentration of Q§H§ (Fig. 2, left). The significant scatter in the data at this wavelength may be due to the difficulty of accurately determining the rate constants when the ratio of Q§H§ to c§ was greater than 10:1. At these higher concentrations of Q§H§, much of the initial absorbance increase occurred during the first 2–3 ms of the reaction; this is within the deadtime of the stopped-flow spectrometer that we used. The exponential fitting program was therefore presented with only a limited amount of data with which to analyze the rate and extent of the absorbance increase, and consequently some variability was to be expected.

From the double reciprocal plot of the observed rate constants versus the Q§H§ concentration, we estimated that the limiting rate for the fast phase of b reduction is 1094 s§1; the

![Fig. 1. Reaction of 10 μM fully oxidized complex III with 100 μM Q§H§ at 8°C following the absorbance changes at 562 and 563 nm. The solid lines are the fit to the kinetic data using either two (553 nm) or three (562 nm) exponentials; the experimental data are partially obscured by the fitted lines. Inset, time course of appearance of Q§H§ in a parallel experiment. The pathlength was 2 cm.](image-url)
dissociation constant for Q1H2 was calculated to be $5 \times 10^{-4}$ M (Fig. 2, left, inset).

The observed rate constant for the absorbance changes at 553 nm appeared to depend linearly on the concentration of Q1H2, although the scatter in the data precluded a definitive conclusion on this point; the slope of the concentration dependence yielded a second-order rate constant of $3 \times 10^{3}$ M$^{-1}$ s$^{-1}$. The maximum observed rate exceeded 100 s$^{-1}$ (Fig. 2, left).

The rates of the decrease in absorbance at 562 nm correlated with the rates of increase in absorbance at 553 nm over the whole concentration range examined. This correlation, taken with the faster initial rate of absorbance increase at 562 nm compared to that of 553 nm (Fig. 1), might be interpreted by assuming that one or more of the $b$ centers was reduced first and subsequently transfers electrons to the iron-sulfur cluster and cytochrome $c_1$, which are in rapid equilibrium (18).

However, we believe that this decrease in absorbance is to be attributed to the reduction of cytochrome $c_1$ that contributes a decrease in absorbance at 562 nm which is about 10% of the increase due to the reduction of the $b$ cytochromes as determined by a comparison of the optical spectra of oxidized, ascorbate-reduced, and dithionite-reduced enzyme (19). Thus, whenever the change in concentration of $c_1$ is very much larger than that of the $b$ cytochromes, the net absorbance at 562 nm will be dominated by the contribution due to $c_1$ and will exhibit a decrease. (It is most unlikely that the secondary phase represents intermolecular electron transfer between reduced and oxidized complex III, for when fully oxidized and fully reduced enzymes were mixed together in the stopped-flow instrument, several minutes were required for the oxidation of cytochrome $b$ and reduction of $c_1$ to be completed.)

**Transient Kinetics of Ascorbate-reduced Complex III**—Upon reduction of fully oxidized complex III with a large excess of Q1H2, as many as five electrons can be transferred from the reductant to the enzyme complex. This requires the consecutive reaction of the enzyme with at least 3 eq of the substrate. A simplification of the overall reaction can be achieved by taking advantage of the large potential gap between the iron-sulfur center/$c_1$ couple and the remaining redox components. Thus, treatment of the fully oxidized complex III with a small excess of ascorbate yields enzyme in which the iron-sulfur center and $c_1$ are reduced. This "two-electron reduced" enzyme only requires reaction with 2 eq of substrate to achieve complete reduction.

Upon mixing 10 eq of Q1H2 with two-electron reduced complex III, the absorbance at 562 nm increased biphasically with rate constants of 185 s$^{-1}$ and 52 s$^{-1}$ (Fig. 3); the observed extent of the absorbance changes (~19%) were comparable to the extrapolated values obtained with oxidized complex III. Furthermore, in marked contrast to experiments with fully oxidized enzyme, no subsequent decrease in absorbance was observed at this wavelength (compare Figs. 1 and 3), as was to be expected because cytochrome $c_1$ could not change oxidation state.

The apparent rate constant for the more rapid increase in absorbance was again a hyperbolic function of Q1H2 concentration (Fig. 2, right). A double reciprocal plot of the data yielded a limiting rate of 587 s$^{-1}$ for the fast phase (Fig. 2, right, inset) together with a dissociation constant for Q1H2 of $1.2 \times 10^{-4}$ M. These values are slightly different from those obtained with the fully oxidized complex, but in view of the difficulty of accurately measuring and analyzing such rapid rates, this difference may not be significant.

![Figure 2](image2.png)

**Fig. 2**. Left, concentration dependence of the fast rate of reduction of cytochrome $b$ (A) and of cytochrome $c_1$ (O). Inset, Double reciprocal plot of the data for cytochrome $b$. Right, concentration dependence of the rate constants for reduction of cytochrome $b$ in two-electron reduced complex III. Inset, double reciprocal plot of the experimental data.

![Figure 3](image3.png)

**Fig. 3** Reduction of 10 µM two-electron reduced complex III using 100 µM Q1H2 as reductant and the effect of antimycin. The solid lines represent the absorbance changes at 562 nm in the absence or presence of 3 eq of antimycin A, as indicated. O, reduction of Q0 in the absence of inhibitor; D, reduction of Q0 in the antimycin-treated complex. The pathlength was 2 cm.
When the kinetics of reduction of complex III which has been depleted of Qₐ and phospholipids was studied markedly different behavior was found with both the extent and rate of the absorbance changes at 562 and 553 nm being greatly decreased by removal of the quinone (data not shown). However, as the removal of Qₐ appears to disorganize the phospholipids (24) interpretation of this result may be complicated.

EFFECTS OF ANTIMYCIN, FUNICULOSIN, MYXOTHIAZOL, AND UHDBT ON YEAST COMPLEX III REDUCTION KINETICS

Antimycin—When 10 eq of Q₁H₂ were mixed with oxidized complex III which had been preincubated with antimycin, the absorbance at 562 nm increased biphasically at rates of 49 and 1.5 s⁻¹, reaching a final extent which was 21% of the total possible increase (Fig. 4). Thus, antimycin appeared to completely eliminate the most rapid phase of b reduction, while the second phase was converted from one of absorbance decrease (Fig. 1) to one of absorbance increase (Fig. 5).

The presence of antimycin had a much smaller effect upon the kinetics at 553 nm. The inhibitor did not change the rate constants of either the fast or slow phases of reduction from the values obtained with uninhibited enzyme and, on average, did not reduce the extent of reduction of cytochrome c₁ by more than 10% of that observed with uninhibited enzyme. These last data agree with the report of Bowyer and Trum-power (8) using beef heart enzyme, although other workers have found significant decreases in either the rate (9, 25) or amplitude (10, 25) of reduction of c₁. In particular, Degli Esposti and Lenaz (9) reported a large decrease in the rate of cytochrome c₁ reduction in the presence of antimycin. However, in experiments conducted during a visit to our laboratory, Dr. Degli Esposti did not observe any decrease in the rate or extent of the absorbance changes at 553 nm when beef heart complex III was treated with antimycin at 25 °C, thus reproducing our findings with the yeast enzyme. Low signal-to-noise ratios in the corresponding data at 562 nm prevented rate determinations at this wavelength.

Because the reduction of b and c₁ was now almost synchronous, the negative contribution of c₁ at 562 nm was obscured.

When antimycin was added to ascorbate-reduced enzyme, there was almost complete inhibition of any absorbance changes at 562 nm. No reaction could be detected in 100 ms, and several minutes were required before the extent of reduction of cytochrome b reached the levels obtained with antimycin-treated two-electron reduced enzyme (Fig. 3). Drastic inhibition of cytochrome b reduction in antimycin-inhibited, ascorbate-reduced beef heart succinate-cytochrome c reductase was first reported by Trumpower and Katki (26); it is interpreted by Q-cycle proponents as reflecting the elimination of both of two routes for the reduction of cytochrome b by Q₁H₂, an antimycin-sensitive route at center i and a route at center o which requires the presence of a functional, oxidized iron-sulfur cluster (see Fig. 8).

Funiculosin—Upon reduction of oxidized complex III by excess Q₁H₂ in the presence of funiculosin, the absorbance at 562 nm increased triphascially at rates of 79, 3.9, and 0.06 s⁻¹, respectively (Fig. 5, top left); 52% of the total possible increase was obtained, and no subsequent decrease in absorbance was observed.

Like antimycin, funiculosin did not affect either the rate or extent of the increase in absorbance at 553 nm (Fig. 5, top left, compare solid and broken lines). Again the similar rates of reduction of b and c₁ obscured the absorbance decrease at 562 nm due to reduction of c₁.

Myxothiazol—Reduction of complex III by 10 eq of Q₁H₂ in the presence of myxothiazol resulted in a rapid initial increase in absorbance at 562 nm with a rate, 192 s⁻¹, similar to that observed with uninhibited, oxidized enzyme and with two-electron reduced enzyme. The absorbance subsequently increased at a rate of 18 s⁻¹ to about 16% of the extent of dithionite-reduced enzyme (Fig. 5, top right); a third phase of decreasing absorbance was observed on occasion. Von Jagow et al. (27) have reported a myxothiazol-induced increase in reduction of b.

In agreement with observations on the beef heart enzyme by De Vries et al. (10) and Von Jagow et al. (27), the presence of myxothiazol led to a marked inhibition of the increase of absorbance at 553 nm (Fig. 6, top right); 1 min was required to obtain the maximum extent of change (56%). By contrast, less than 1 s was required for maximum reduction of uninhibited enzyme.

UHDBT—Kinetic patterns similar to those produced by myxothiazol were observed in the presence of 3 eq of UHDBT. The absorbance at 562 nm increased at a rate of 138 s⁻¹ for the initial phase, somewhat slower than that found with uninhibited enzyme (Fig. 6, bottom left). Two phases of absorbance increase followed, with rates of 6.8 and 3.2 s⁻¹, respectively, until the extent of reduction was 22%. No decrease in absorbance was observed during any of the kinetic phases.

The absorbance changes at 553 nm were strongly inhibited.
Electron Transfer in Complex III

Fig. 6. Effect of antimycin and UHDBT on the reduction of Q$_6$ by Q$_{1H2}$. Left panel, appearance of Q$_6H2$ in complex III treated with 2 eq of antimycin upon reaction with 14 eq of Q$_{1H2}$. C, control, no antimycin present; ▲, in presence of antimycin. Right panel, appearance of Q$_6H2$ in complex III treated with 3 eq of UHDBT upon reaction with 10 eq of Q$_{1H2}$. C, control, no UHDBT present; ▲, in presence of UHDBT.

by UHDBT as originally observed by Bowyer et al. (8) with the beef heart enzyme. Over 30 s were required to obtain 27% reduction, and almost no change was observed during the initial 500 ms of reaction (Fig. 6, bottom left). With both myxothiazol and UHDBT, the absence of any decrease in absorbance at 562 nm is again explained by the lack of reduction of c$_1$, together with the substantial reduction of b.

Antimycin plus UHDBT—Addition of both antimycin and UHDBT decreased the rate constants for the biphasic increase in absorbance at 562 nm to 0.81 and 0.13 s$^{-1}$. Approximately 20 s were required to achieve 16% reduction (Fig. 5, bottom right; note change in time scale). The combined action of the two inhibitors brought the absorbance changes at 562 nm into near synchrony with those at 553 nm; the latter reached a level of about 50% of the possible change in 20 s.

The Kinetics of Reduction of Q$_6$ Endogenous to Complex III—To provide direct information on the redox behavior of the endogenous Q$_6$, we have developed a rapid-quenching HPLC which allows measurement of the changes in the redox level of Q$_6$ in the millisecond range (23).

When oxidized complex III was mixed with 10 eq of Q$_{1H2}$ the amount of Q$_6H2$ increases very rapidly to an extent of about 30% within 7.5 ms, the time resolution of our observations. Further increases in the level of Q$_6H2$ were small and there was little change over the next 100 ms (Fig. 1, inset). The rate of reduction of Q$_6$ therefore, although not measured precisely, was at least as rapid as the absorbance change at 562 nm and preceded the absorbance increase at 553 nm.

After mixing ascorbate-reduced complex III with 10 eq of Q$_{1H2}$, Q$_6H2$ production rose to a value of 52% during the first 7.5 ms and averaged 54% over the next 90 ms (Fig. 3). The extent of formation of Q$_6H2$ in the uninhibited, ascorbate-reduced enzyme was significantly higher than in the reduction of fully oxidized enzyme during the first 100 ms of the reaction (34%, Fig. 1). This is to be expected from the smaller number of electrons that can be transferred from substrate to the prereduced enzyme and thus the higher effective reduction potential of the Q$_{1H2}$Q$_6$ system.

In the presence of 2 eq of antimycin, reduction of Q$_6$ occurred at a slightly slower rate than in the uninhibited complex. Reaction of complex III with 14 eq of Q$_{1H2}$ led to 22% reduction of Q$_6$ in 7.5 ms, followed by a slower increase to 46% (Fig. 6, left). In a parallel experiment with uninhibited enzyme under the same conditions, 37% of the endogenous Q$_6$ was reduced within 7.5 ms, and no further increase was observed.

When antimycin was added to two-electron reduced enzyme, the level of Q$_6H2$ rose to a value of 41% during the first 7.5 ms and stayed fairly constant for the next 90 ms (Fig. 3). Antimycin, therefore, inhibited the reduction of Q$_6$ in ascorbate-reduced complex III only slightly and certainly not enough to account for the drastic decrease in the absorbance change at 562 nm observed under the same conditions. This small decrease in the yield of Q$_6H2$ suggests that antimycin might lower the redox potentials of the Q$_6$ half-reactions; it is known that the yield of Q$_6$ is substantially decreased in the presence of antimycin (28) suggesting that the separation of the two half-reactions has been increased.

Upon mixing complex III with 10 eq of Q$_{1H2}$ in the presence of 3 eq of UHDBT (Fig. 6, right) the level of Q$_6H2$ rose to 24% in the first 7.5 ms (triangles). By 24 ms, the level of Q$_6H2$ formation increased to that observed with the uninhibited enzyme, about 45% (circles). In the experiment shown, the control values were higher than average.

**Determination of the Relative Midpoint Potential of the Q$_1$/Q$_{1H2}$ Redox Couple**—To determine the effective midpoint potential of the Q$_1$/Q$_{1H2}$ redox couple, the equilibrium levels of reduced Q$_1$ were determined in ascorbate-reduced complex III from several ratios of Q$_{1H2}$/Q$_6$.

Complex III was first prereduced with ascorbate; this eliminates electron transfer to the Fe/S and c$_1$ centers and limits reaction to the endogenous Q$_6$ and cytochromes b. The ascorbate-reduced enzyme was then incubated with one of several concentrations of Q$_{1H2}$ for 5 min, allowing the electron transfer reactions to attain equilibrium. Subsequently, the mixture was subjected to the rapid-quench HPLC protocol, mixing the preincubated enzyme with plain buffer. Rapid mixing with the quenching solvent facilitated extraction of Q$_6$ for the HPLC analysis.

The difference in midpoint potentials between the Q$_6$/Q$_{1H2}$ couple, 155 mV (29), and the Q$_1$/Q$_{1H2}$ couple, was calculated using the relationship:

$$
E_{m1} - E_{m3} = 30 \log \frac{[Q_1][Q_{1H2}]}{[Q_6][Q_{1H2}]} 
$$

(1)

where $E_{m1}$ and $E_{m3}$ are the midpoint potentials of the Q$_6$/Q$_{1H2}$ (29) and Q$_1$/Q$_{1H2}$ redox couples. For example, at a ratio of Q$_{1H2}$/Q$_6$ of 6:1, the equilibrium level of Q$_{1H2}$ was 40% while 7.2% of the total possible absorbance change at 562 nm was observed in the optical spectrum. From these two values the total amount of Q$_{1H2}$ which had been oxidized was calculated. Substituting these calculated values into Equation 1 leads to a difference in potential of ~37 mV or a value of 192 mV for the $E_{m}$ of the Q$_1$/Q$_{1H2}$ couple. The results of several experiments are summarized in Table I; these lead to an average value of 193 mV for the midpoint potential of Q$_1$ in our experiments.

**Kinetcs of Reduction of Yeast Succinate-Cytochrome c Reductase**—A common practice in studies on mitochondrial complex III is to substitute succinate-cytochrome c reductase for the ubiquinol cytochrome c oxidoreductase. This custom appears to have two origins, the ease of preparation and good

| Table I | Determination of midpoint potential of Q$_1$/Q$_{1H2}$ couple |
|---------|---------------------------------------------------------------|
| Ratio Q$_{1H2}$/Q$_6$ | % Q$_6H2$ Present | $\Delta E_{m1}$ | $E_{m1}$ (mV) |
| 1:1       | 22.7            | 32           | 187           |
| 3:1       | 28.5            | 38           | 194           |
| 6:1       | 40.2            | 37           | 192           |
| 16:1      | 49.4            | 43           | 198           |

* In millivolts.

**a** Mean = 193 millivolts.
yield of the reductase enzyme and the convenience of using succinate as electron donor. In experiments designed to determine whether this is a reliable alternative, we have compared the kinetics of reduction of yeast succinate-cytochrome c reductase with those of complex III.

The reduction of the b and c1 components of succinate-cytochrome c reductase by excess Q1H2 exhibited kinetics very similar to those obtained with complex III in both the rate and extent of the observed absorbance changes at both 553 and 562 nm; it thus appears that the kinetics of reduction of complex III are unaltered upon isolation of this enzyme from succinate-cytochrome c reductase.

By contrast, mixing succinate with succinate-cytochrome c reductase resulted in absorbance changes differing from those obtained when Q1H2 was used as reductant. The first phase of the absorbance change at 562 nm progressed at a significantly slower rate which has varied from 4–40 s⁻¹ in different preparations of succinate-cytochrome c reductase. The absorbance at 562 nm increased to 8% of the possible change in approximately 100 ms, followed by a slow decay, of about 2 s duration (Fig. 7, inset). Subsequently, a very slow increase in absorbance was observed, rising to about 35% over a 30-s period (Fig. 7). The absorbance at 553 nm increased at a rate of about 7 s⁻¹, to an extent of 33% during the initial phase, followed by a hundred-fold slower increase of another 16%. When succinate was equimolar with c1, the pattern of absorbance changes at 562 nm resembled those seen with Q1H2 but the changes were very much slower.

Reduction of Yeast Complex III by Duroquinol—For their studies on the mechanism of reaction of beef heart complex III in both succinate-cytochrome c reductase and submitochondrial particles, De Vries et al. (10) made extensive use of duroquinol (tetramethylbenzoquinol) as an alternative coenzyme Q analog. With this reductant they observed triphasic absorbance changes at 562 nm; these were interpreted as reflecting the lower midpoint potential of duroquinol (27).

Upon mixing yeast complex III with 10 eq of duroquinol, there was an initial increase in absorbance at 562 nm with a rate of about 30 s⁻¹; this increase accounted for about 18% of the total possible change. The absorbance then leveled off for about 50 ms before increasing a further 17% during the next second (data not shown). The increased extent of the absorbance change at 562 nm (35%) compared to that obtained with Q1H2 (20%) presumably reflects the lower midpoint potential of duroquinol (30).

The absorbance increase at 553 nm proceeded at an initial rate of 7.8 s⁻¹ to an extent of 72%; this was followed by an additional increase of 10% which occurred at a rate of 1.5 s⁻¹. This pattern of absorbance increase at 553 nm resembled that observed using Q1H2 as reductant (e.g. Fig. 1), but occurred at about one-fifth the rate. These absorbance changes are qualitatively similar to the data obtained by De Vries et al. (10) with the beef heart system.

Computer Simulations—The chemical equations used to describe the Q-cycle (Fig. 8) were:

\[
\begin{align*}
Q_1H_2 + Q_{c1} &\xrightleftharpoons{k_{-1}} Q_1 + Q_{c1}H_2 \\
Q_1H_2 + Q_{c1} &\xrightleftharpoons{k_{-2}} Q_1 + Q_{c1}H_2 \\
2 Q_1 &\text{ rapid } Q_1 + Q_1H_2 \\
Q_1 + Q_1H_2 &\text{ rapid } Q_1H_2 \text{redox} \\
Q_{c1} &\xrightarrow{k_3} Q_{c1}H_2 \text{redox} \\
Q_{c1} + b_{eq} &\xrightarrow{k_4} Q_{c1}H_2 + b_{eq} \\
Q_{c1}H_2 + Fe/Fe^{3+} &\xrightarrow{k_5} Q_{c1}H_2 + Fe/Fe^{3+} \\
Q_{c1}H_2 + Fe/Fe^{3+} &\xrightarrow{k_6} Q_{c1}H_2 \text{redox} + Fe/Fe^{3+} \\
Q_{c1}H_2 + Fe/Fe^{3+} &\xrightarrow{k_7} Q_{c1}H_2 + Fe/Fe^{3+} \\
Q_{c1}H_2 + b_{eq} &\xrightarrow{k_8} Q_{c1}H_2 \text{redox} + b_{eq} \\
Q_{c1}H_2 + Fe/Fe^{3+} + b_{eq} &\xrightarrow{k_9} Q_{c1}H_2 \text{redox} + Fe/Fe^{3+} + b_{eq} \\
\end{align*}
\]

Included in these equations is the step represented by \(k_a\), the reaction of \(b_{eq}\) with \(Q_1H_2\). As formulated the Q-cycle is a steady state process; this step was included as a means of initiating the reaction.

Each simulation was constrained in the following ways: (i) only forward rate constants could be varied. Reverse rate constants were deduced from the appropriate forward constant using the measured midpoint potentials for each of the redox centers used (Table II). These potentials could be varied by no more than the errors associated with original data (±20 mV). (ii) The reaction of the iron-sulfur center with \(c_1\) (\(k_{\alpha}\)) and the rate of dismutation of the semiquinone of \(Q_1\) were made sufficiently fast as not to be rate limiting. (iii) The rate of movement of \(Q_4\) and \(Q_4H_2\) between centers "i" and "o" was also made very fast and was not rate limiting.

Because most of the reactions involve intramolecular electron transfers between redox centers which are in fixed relation to one another, it was necessary to properly account for the proportion of enzyme species which had the correct electron distribution for reaction. Thus the apparent rate constant for electron transfer between a donor, \(D\), and acceptor, \(A\), was written as \(k_{\alpha}f_{DA}\), where \(f_{DA}\) and \(f_{A}\) represent the fractions of reduced donor and oxidized acceptor, respectively, and \(k_{\alpha}\) is the intrinsic rate constant when both \(f_{DA}\) and \(f_{A}\) = 1. The second-order reaction between exogenous reductant and

![Fig. 7. Kinetics of reduction of 10 µM succinate-cytochrome c reductase with 100 µM succinate. The absorbance changes were followed at 553 and 562 nm as indicated. Inset, changes observed during the early part of the reaction. The pathlength was 2 cm.](image-url)
enzyme was also weighted by the fraction of enzyme acceptor in the oxidized state. These quantities were then multiplied by the concentration of enzyme and, where appropriate, the optical spectral weights for the individual cytochromes (19). The amount of reduced Q₆ detected in the quenching experiments was taken to be the sum of the intrinsic Q₆ plus half of Q₆.

The calculated absorbance changes at 553 and 562 nm reproduced the observed data quite well, as did the calculated rapid rise and extent of formation of Q₆H₂, although the simulations did predict a subsequent slow decrease in the steady state level of Q₆H₂ which was not observed experimentally (Fig. 9).

Reproduction of the very rapid increase at 562 nm could only be obtained if the rate constant for the reduction of b₉ by Q₆H₂ (k₉) was made greater than 500 s⁻¹, a value which is comparable with the rate of the fastest phase observed with fully oxidized (Fig. 1) and two-electron reduced (Fig. 3) enzyme. Furthermore, to account for the observed rate of reduction of c₁, the rate of reduction of the iron-sulfur center by Q₆H₂ had to be set at 750 s⁻¹ while the rate of reduction of b₉ by Q₆ was required to be 1000 s⁻¹; thus both routes of reduction of the cytochromes b appear to be rapid. The rate of electron transfer between b₉ and b₁ was required to be extremely fast and was never rate limiting. (This should be contrasted with results obtained during reoxidation where slow electron transfer between the two b centers appears to prevail).¹

To simulate the reduction kinetics at other concentrations of reductant, it was necessary to make small changes in the values of a number of rate constants before a satisfactory fit to the experimental data could be obtained. In principal, no changes should be necessary, for the simulation program treated the reaction with reductant as a second-order process, and it should have been sufficient to specify that only the concentration of reductant had been changed. The required changes in rate constants were small, however, and it is possible that a single set of constants exist that provide an acceptable fit to all the concentration data. It is equally possible that the model of the Q-cycle, as implemented, lacks some feature which, while not crucial for reproducing the basic phenomena, is necessary to get a completely consistent set of simulations.

**DISCUSSION**

In assessing the significance of the kinetic data obtained during this kinetic study, we first note that the rates of the reduction of all of the redox components of complex III appear to be sufficiently rapid to support the measured turnover number of 95 s⁻¹ that we determined at the same temperature. Thus, the maximum rate of reduction of cytochrome b exceeds 500 s⁻¹ in both two-electron-reduced and fully oxidized enzyme while rates of reduction of c₁ greater than 100 s⁻¹ have been observed with high concentrations of reductant (Fig. 2). The approach to the steady state for the reduction of endogenous Q₆ appears to be essentially complete within 7.5 ms with only a modest excess of Q₆H₂, implying that rates of reduction of Q₆ greater than 200 s⁻¹ are in effect. If we assume that this endogenous Q₆ is the immediate electron donor to cytochrome b₇ then the rate of reduction of Q₆ must actually exceed 500 s⁻¹. The strongest piece of evidence in support of this assumption is provided by enzyme treated with the inhibitors, antimycin plus either myxothiazol or UHDBT, or with two-electron-enzyme treated with antimycin (i.e. the double-kill combinations). In such enzyme the reduction of b is strongly inhibited while the rate and extent of reduction of Q₆ is, at most, only slightly decreased. As we had previously shown that electron transfer between the iron-sulfur center and c₁ occurs at rates greater than 1000 s⁻¹ (18), these results make it clear that all of these redox centers can function in a catalytically competent way.

Although the responses to the inhibitors used are complicated and varied, in the main they can be rationalized using
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the Q-cycle model (Fig. 8), as has been done previously for the beef heart system (e.g. 10, 25). Two sites of action for inhibitors are invoked: antimycin blocks electron transfer between \( Q_0 \) and the high-potential \( b \) (\( b_H \)), while myxothiazol and UHDBT block electron transfer at center o. (Von Jagow et al. (27) has proposed that myxothiazol blocks electron transfer between \( Q_0 \) and the iron-sulfur cluster while UHDBT inhibits electron transfer between the iron-sulfur cluster and \( c_1 \); our data, however, do not permit such a refined assignment of the sites of action of these two reagents.) The effects of these inhibitors are then interpreted in terms of two paths for reduction of the cytochromes \( b \). The first path is the antimycin-sensitive reduction of \( b_H \) at center i. The second is the reduction of \( b_H \) linked to the reduction of the iron-sulfur cluster at center o; this latter path can be inhibited by myxothiazol, UHDBT, and prior reduction of the iron-sulfur center as is the case when two-electron reduced enzyme is used.

Thus, in the presence of antimycin, direct reduction of \( b_H \) at center i is blocked, and the very fast rate of 200 s\(^{-1}\) observed upon addition of 10 eq of \( Q_1H_2 \) to fully oxidized or two-electron-reduced enzyme is eliminated; only the subsequent processes occurring at approximately 50 s\(^{-1}\) presumably involving center o, are to be observed. Conversely, when reaction at center o is inhibited, e.g. by addition of myxothiazol or by prior reduction of the Fe/S center, the very fast rate of 200 s\(^{-1}\) is still to be observed while the rate of the second phase is substantially depressed.

Both myxothiazol and UHDBT severely inhibit the rate of reduction of \( c_1 \), while antimycin has almost no effect on this process; this pattern is just that expected from the postulated sites(s) of action of these agents (Fig. 8). The lack of inhibition of \( c_1 \) reduction observed with the yeast complex in the presence of antimycin is opposite to some published data on the heart enzyme. However, in our hands the heart and yeast enzymes behave similarly, and the observed lack of inhibition is consistent with Q-cycle predictions. As expected, when both paths for \( b \) reduction are blocked, e.g. by the addition of antimycin to two-electron-reduced enzyme or upon addition of antimycin and UHDBT together, then reduction of both \( b \) and \( c_1 \) are strongly inhibited. However, reduction of \( Q_0 \) is not strongly affected by either treatment, consistent with it being located prior to the site of action of any of the inhibitors.

The response to UHDBT is a little anomalous. Not only does it eliminate the reduction of \( c_1 \), but it also reduces the rate of the very fast phase of reduction of cytochrome \( b \) by about 30%; this decrease is likely to be a consequence of the decrease in the rate of reduction of \( Q_0 \) observed in the presence of this inhibitor. This effect of UHDBT on the kinetics of reduction of \( Q_0 \) suggests the possible existence of a second site of reaction of UHDBT. In view of the structural similarity between UHDBT and \( Q_0 \), it is quite reasonable to expect that this inhibitor might interfere with the binding of \( Q_0 \) at center i as well as center o. Zhu et al. (11) and Bowyer and Trumpower (8) have both concluded that analogs of Q might react at more than one site.

Funiculosin behaves in a unique manner for it suppresses the rate of the first, very fast phase, which implies that it, like antimycin, is a center i inhibitor. However, our potentiometric data (16) show that this reagent has a remarkable effect on the iron-sulfur cluster, raising the midpoint potential of this component from 285 to 430 mV; only a small increase in the potential of \( b_H \) is observed. This increase in midpoint potential is consistent with reoxidation studies of fully reduced enzyme, in which funiculosin was shown to completely block the oxidation of the cytochromes \( b \) and, presumably, the iron-sulfur center, while the oxidation of \( c_1 \) was unaffected. However, funiculosin clearly has no effect on the reduction of \( c_1 \) by \( Q_1H_2 \) (Fig. 5). These differences in the responses of the enzyme to reduction and reoxidation in the presence of funiculosin suggest that the modification of the iron-sulfur cluster induced by funiculosin is only manifest upon reduction of this redox center. The behavior of funiculosin is suggestive of a global action of this inhibitor with the consequences of its binding being distributed over the whole protein complex as opposed to being localized to a single redox center. A similar global consequence of inhibitor binding is also implied by the observation that antimycin markedly stabilized the beef heart enzyme complex to dissociation induced by chaotropes (31).

The complex pattern of inhibitor action just described is most difficult to interpret using any linear scheme for electron transfer in this enzyme complex, and it is one of the virtues of the Q-cycle that it provides an acceptable rationalization of the observed phenomena. Nevertheless, we felt it was useful to analyze our data in a more quantitative way and elected to use computer simulation as an additional tool to discriminate between alternative electron transfer pathways which might be applicable to this enzyme.

Initial efforts focused on linear and branched schemes, but for these cases the simulations were most unsatisfactory. Thus, for the strictly linear case, it became apparent that having \( b_H \) present as an obligatory intermediate led to very slow reduction of those centers to the oxidizing side of this component. This was also true for the branched case in which \( b_H \) was placed on the side path. Much closer fits could be obtained with \( b_H \) placed on the main path and \( b_H \) on the sidepath; however, only the general appearance of the data could be reproduced with this arrangement. Quantitative fits to the data were not satisfactory and, even then, getting the best agreement required raising the reduction potential of \( b_H \) somewhat higher than seemed reasonable.

Fitting the experimental data to the Q-cycle proved to be unexpectedly easy, and the outstanding features of the experimental results were closely approximated. In particular the simulations showed that the concentration of the \( b \) cytochromes reached a steady state within 10–15 ms thus supporting our assumption that the observed decreases in absorbance observed at 562 nm on reduction of fully oxidized enzyme reflected reduction of \( c_1 \) under conditions when the redox level of the \( b \) components was constant.

It appears that conditions which either (i) suppress the reduction of cytochrome \( c_1 \) or (ii) make the rates and extents of reduction of cytochrome \( b \) comparable in magnitude to those of \( c_1 \) will abolish the decrease in absorbance at 562 nm. The first condition can be met by the addition of myxothiazol or UHDBT to the oxidized enzyme (Fig. 5), for under these conditions reduction of \( c_1 \) is strongly inhibited (prereducing \( c_1 \) is a simple way of achieving the same result (Fig. 3)). The second condition is achieved in the presence of antimycin (Fig. 4) which eliminates the extremely rapid phase of \( b \) reduction so that the reduction of \( b \) is contingent upon oxidation of \( Q_1H_2 \) by the iron-sulfur cluster with the consequence that reduction of \( b \) and \( c_1 \) occur at similar rates.

Multiple phases in the reduction of the \( b \) cytochromes have been observed on a number of occasions, and a variety of explanations have been forthcoming (7, 10, 12). It seems probable, however, that apparent discrete oxidative phases such as those described in Fig. 1 simply reflect the combined contributions of the several heme centers to the absorbance. Chen and Zhu (32) have recently described a simple protocol for selection of reference wavelengths for elimination of the spectrophotometric contributions of the \( c \) cytochromes. They...
confirm that wavelength pairs used by earlier workers (10), (e.g. 562–575 nm) are appropriate for this purpose. Chen and Zhu (32) find that using such wavelength pairs the "oxidative" reaction of cytochrome b is eliminated but that plateaus lasting up to 20 s are clearly present. The end of the plateau appears to coincide with full reduction of c1 implying that the secondary reduction of b is a consequence of reaction at center i. Two possibilities suggest themselves: 1) reaction proceeds by a small equilibrium concentration of reductant (e.g. Q2), the reaction of which is not significant under normal conditions, and (ii) the second phase depends upon a structural change in the complex which either raises the reduction potential or alters the absorbance contributions of the b center(s).

Our experimental results point to two other complications which can arise when studying this segment of the mitochondrial electron transport chain. The first deals with the use of succinate cytochrome c reductase as an alternative source of complex III. It is clear that quite different kinetic behavior is obtained when QH2 and succinate are used as substrates with the latter typically reacting much slower than the former. Consequently, the interpretation of the kinetic changes observed when succinate is used as reductant will be complicated by the possibility of a rate-limiting step between succinate-ubiquinone reductase and complex III. Such a rate-limiting step would have the consequence of leveling the kinetic response of the b and c1 centers. The second complication introduced in the use of succinate cytochrome c reductase is the presence of a "Q" pool; direct measurement of the reduction kinetics of the endogenous QH is considerably simplified if the pool of quinone is eliminated from consideration.

The values of most of the rate constants used in the simulation are very large and are unlikely to be rate limiting. At low levels of reductant, the overall reaction is controlled by the rate at which reducing equivalents are transferred from QH2 to Qa, and the distribution of electrons within the enzyme complex is dictated principally by the electron affinities of the individual redox centers. However, as the concentration of reductant is raised, the limiting process becomes the rate at which electrons are transferred from QH2 to the iron-sulfur cluster (and directly to b7a) although thermodynamic equilibrium should still be maintained within the two b centers and between the iron-sulfur cluster and cytochrome c1. However, all the rate constants used in the simulation are significantly larger than the V_{max} of 95 s^{-1}. Thus, the observed maximum rate is not to be associated with any individual step in the cycle but rather reflects the interaction of several kinetic processes.

By setting k5 to zero, the reduction of the Fe/S center by QH2 is eliminated; this mimics the effect of studying the reduction of two-electron reduced enzyme. By so doing the kinetics of the absorbance changes at 562 nm could be well reproduced as were the overall kinetics of formation of QH2, although the calculation predicted a little less QH2 than was observed experimentally.

Attempts to fit the kinetic data obtained in the presence of inhibitors were less pleasing. The most satisfactory results were obtained with myxothiazol and UHDBT. With both of these reagents, the potentials previously reported (16) were used, and the value of k5 was made suitably small. With myxothiazol it was also necessary to reduce k3 while with UHDBT a decrease in k5 was required to obtain a fit to the optical data; as UHDBT may very well interfere with the binding site(s) for Q2, the need to change k5 was not unexpected. No satisfactory fit could be obtained to the data obtained with antimycin- or funiculosin-inhibited enzyme; this might be taken as further evidence for a global (multisite) action of these two inhibitors as proposed above.

Simulation of the reduction kinetics obtained with duroquinol as reductant could be obtained by simply lowering the midpoint potential of QH2 from 150 to 59 mV, the value for duroquinone (30), and by decreasing k5 from 750 to 50 s^{-1}.

The simulation technique also allowed us to investigate the extent of reaction between Qa and b7, a reaction that is strongly disfavored on thermodynamic grounds. This was approached by setting the value of k5 to zero, whereupon approximately 30% of b7 was observed to be reduced immediately. By contrast, no more than 1% of b7 was present in the reduced form during the simulation of Fig. 9.

It should be pointed out that we did not find it necessary to use different thermodynamic parameters for Qa and QH2. This probably reflects our assumption that the quinone and hydroquinone forms of these species were in rapid equilibrium so that the individual one-electron potentials are suitable averages of the values for the two species.

Although not shown in Fig. 9, the simulations also yield the concentration of QH radical present in the approach to steady state. In the absence of inhibitors, this proved to be quite high, almost equal to that of QH2, and with a similar time dependence. However, preliminary measurements of the concentration of radical using the rapid freeze EPR technique yielded values which were quite low, about 5% of the content of c1. De Vries et al. (13) have obtained similar values upon addition of reduced duroquinone to beef heart succinate cytochrome c reductase. An explanation for this apparent inconsistency is to be found in the suggestion (33) that ubisemiquinone (Q2-) and the ferric form of b7 form a complex which is EPR invisible, due to a magnetic interaction between the two paramagnets. The basis for this suggestion was the demonstration (19) that the loss of the EPR signal of b7 during a reductive titration occurred much more rapidly than the appearance of the characteristic absorbance at 562 nm; additional evidence in favor of this idea was provided by de la Rosa (33) who showed that this discrepancy was not present in Q-depleted complex.

In obtaining the simulation shown (Fig. 9), the rate of reaction between QH2 and Qa (represented by k5) was set to zero. Yet this step is fundamental to the Q-cycle rationalization for the stoichiometry of proton translocation, and the ability to reproduce the kinetic data without invoking this reaction could be used as evidence against the Q-cycle hypothesis. In fact, the simulations appear to be insensitive to the value of k5; thus setting this rate constant to 3 x 10^{5} M^{-1} s^{-1}, the same value as that of k3, had no effect on the predicted time course of reaction. Consequently, no significance should be attached to the value of k5 used in the calculation represented by Fig. 9.

It should be pointed out that the basic set of equations used to describe the Q-cycle (Fig. 8) should apply equally well to the Q-cycle (6). A variant of the b-cycle called the semiquinone cycle (34) which utilizes the same set of reactions but also adds an electrogenic electron transfer involving the transfer of QH between center i and center o would also satisfy our data. Thus, our results do not allow us to make any judgment on the relative merits of these three cyclic schemes. With appropriate values for the individual rate constants, the simulations should apply equally well to the variant of the Q-cycle proposed by Crofts et al. (25).

However, our data appear to be inconsistent with the local Q-cycle recently proposed by Rich (35). In this variation the direct transfer of reducing equivalents from the Q-pool to the Fe/S-b redox pair is postulated. Our demonstration that (i)
at least with the yeast enzyme, the endogenous quinone appears to be necessary for electron transfer to $b$ and $c_1$ and (ii) $Q_b$ has reached its steady-state level before there has been significant reduction of cytochrome $c_1$, are inconsistent with this postulate (the second difficulty is based on the usual assumption that the iron-sulfur center is reduced before, or simultaneously with, the low potential $b$ center).

The demonstration that the absorbance changes at 562 nm can be reproduced within the framework of a monomeric Q-cycle weakens the rationale for multiple copies of each heme center and a cubic Q-cycle as has been suggested by De Vries et al. (13) and clearly show that cyclic schemes, such as the Q-cycle, do provide a reasonably satisfying description of the kinetic data we have obtained for the reduction of yeast complex III; linear and branched schemes are either less satisfactory or implausible. At the same time, it should be recognized that the Q-cycle contains some ad hoc assertions. For example, the reduction of the iron-sulfur cluster by $Q_b$ is thermodynamically more favored than is reduction of the iron-sulfur cluster by $Q_bH_2$; the Q-cycle requires, however, that the former reaction not occur, for otherwise the $H^+/e^-$ stoichiometry would be 1, rather than the value of 2 observed experimentally (1). The simulations achieve the desired result by making $k_b$ and $k_t$ appropriately fast.

It should also be noted that we have obtained a body of kinetic data on the oxidation of fully reduced complex III with a variety of oxidants which can only be rationalized within the explicit postulates of the Q-cycle if some equally ad hoc assumptions are made about the relative physical relationships of the redox centers within the complex III. Our current efforts are directed at clarifying these difficulties.

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