# Two distinct quinone-modulated modes of antimycin-sensitive cytochrome *b* reduction in the cytochrome $bc_1$ complex

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Reduction of cytochrome *b*-560 (analogous to cyt *b*-562 of mitochondria) via an antimycin-sensitive route has been revealed in chromatophores of the photosynthetic bacterium, *Rhodopseudomonas sphaeroides* Ga. Indeed, the results suggest that two reductive mechanisms can be operative. One is consistent with the idea that the quinol generated at the reaction center $Q_B$ site enters the Q pool and, via the $Q_c$ site, equilibrates with cytochrome *b*-560. The other reductive mode circumvents redox equilibrium with the pool; we consider that this could result from a direct encounter of the reaction center with the $bc_1$ complex perhaps involving a direct $Q_B$-$Q_c$ site interaction. This latter reaction is suppressed by occupancy of the $Q_c$ site, not only by antimycin but by ubiquinol and ubiquinone.

*Ubiquinol-cytochrome* $c_2$ *oxidoreductase* *Reaction center* *Ubiquinone* *Quinone pool* *Cytochrome* b *Antimycin* *Myxothiazol*

## 1. INTRODUCTION

In his Q-cycle model for the operation of ubiquinol-cytochrome $c_2$ oxidoreductase ($bc_1$ complex), Mitchell [1,2] proposed that there are two quinol/quinone sites on the protein that catalyze redox reactions between the two-electron quinone pool sequestered in the membrane and the one-electron *b*-cytochromes in the protein. The sites were designated $Q_{in}$ and $Q_{out}$ according to their postulated relative locations on the protein with respect to the membrane surfaces.

Experimental evidence supports the existence of two distinct catalytic sites for quinol/quinone on the $bc_1$ complex of mitochondrial and photosynthetic systems (review, [3]). One site is recognized as catalyzing cyt *b* reduction concurrent with (via Rieske FeS) cyt $c_1$ + $c_2$ reduction [4–12]. In this mechanism it is thought that a quinol occupying the site is oxidized by the Rieske FeS center [10,11] to a semiquinone which is able to reduce cyt *b*. The antibiotic myxothiazol displays a high affinity for this site and strongly inhibits its reaction [14–18]. The site has been called $Q_z$ [19–23] and has the characteristics expected of Mitchell's $Q_{out}$ site.

The existence of another quinone binding site associated with the $bc_1$ complex has been revealed as the EPR signal of a stable semiquinone [24–26]. This species is commonly called $Q_c$ and with the arguments below can be circumstantially identified

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*Abbreviations:* $bc_1$ complex, the ubiquinol-cytochrome $c_2$ oxidoreductase; $Q_A$, the reaction center primary acceptor quinone; $Q_B$, the reaction center secondary acceptor quinone; $Q_c$, $Q_{in}$, the antimycin-sensitive quinone of the cyt $bc_1$ complex; $Q_z$, $Q_{out}$, the myxothiazol-sensitive quinone of the cyt $bc_1$ complex; cyt, cytochrome; Q, quinone; $Q^{\cdot -}$, semiquinone; $QH_2$, quinol

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as $Q_{in}$. The EPR signal of the semiquinone is specifically abolished by the antibiotic antimycin [24–26]. The working proposal that the effect of antimycin represents an inactivation of the $Q_c$ site and that $Q_c$ can serve as an oxidant of cyt *b* [24–26] rationalizes the well-known inhibition by antimycin of cyt *b* oxidation [8,27,28]. The proposal can also explain the demonstration of cyt *b* reduction that does not involve the myxothiazol-sensitive, $Q_z$-mediated mechanism [14–18]. This mode of cyt *b* reduction is antimycin-sensitive and thus displays this antibiotic in the less familiar role as an inhibitor of the reduction of cyt *b*; it also implicates $Q_c$, under these conditions, as the reductant of cyt *b*.

These reactions involving the $Q_z$ and $Q_c$ sites have also been clearly demonstrated in a light-activatable hybrid system comprising photochemical reaction centers from *Rhodopseudomonas sphaeroides* R26, $bc_1$ complex from beef heart and horse heart cyt *c* [29,30]. Single-turnover flash activation promotes both the myxothiazol-sensitive ($Q_z$-mediated) and the antimycin-sensitive ($Q_c$-mediated) routes to cyt *b*. However, in the native photosynthetic bacterial chromatophore membrane, while the $Q_z$-mediated route is evident, the $Q_c$-mediated, antimycin-sensitive route to cyt *b* reduction has not been observed.

The work presented here demonstrates the antimycin-sensitive $Q_c$-mediated route to cyt *b* reduction in chromatophores of *Rps. sphaeroides*. Two reductive mechanisms are revealed. One involves redox equilibrium of cyt *b* with the quinone pool. The other may involve a direct interaction of the reaction center and $bc_1$ complex. Physiological considerations are discussed.

## 2. MATERIALS AND METHODS

Chromatophores were prepared from *Rps. sphaeroides* Ga [31]. Redox potentiometry and dual-wavelength spectrophotometry after flash activation were performed using methods and equipment described in [32,33]. Reaction center and cyt $c_1 + c_2$ concentrations in chromatophore suspensions were determined using previously reported extinction coefficients [31]. Partial extraction and reconstitution of ubiquinone-10 was performed in isooctane and the quinone content determined as in [34]. Treatment of submitochondrial particles and chromatophores with organic solvents is an effective way to remove selectively ubiquinone without disrupting electron transfer pathways. Isooctane extraction lowers the content of functional $bc_1$ complex and reaction center by less than 10%. The functional cyt $c_2$ content, however, may be diminished by as much as 40–50% after two to three cycles of extraction. In this work a single cycle of extraction was sufficient to lower quinone content by 85–95% while retaining 80% of the original cyt $c_2$.

Myxothiazol was a generous gift of Dr W. Trowitzsch, Gesellschaft für Biotechnologische Forschung, FRG. All solvents used for quinone extraction and determination were HPLC grade.

## 3. RESULTS

Cyt *b* reduction via the antimycin sensitive route has not been observed in chromatophores, possibly due to a thermodynamically unfavorable path from the Q pool ($E_{m7}$ = 90 mV; $E_m$/pH = −60 mV/pH unit [34]) to cyt *b*-560 (see later; $E_{m7}$ = 50 mV; $pK_{red}$ = 7.5 [36]) at pH 7.0. Strategies may be devised, however, which alter the operating potential of the Q pool or which exploit differential $E_m$/pH relationships and render the route more favorable. For example, a natural suppression of the route may relate simply to the Q pool's redox buffering capacity. The redox potential of the pool according to the Nernst equation is governed by the $[Q_p]/[Q_pH_2]$ ratio. Thus, the effect of decreasing $[Q_p]$ by extraction while maintaining the $QH_2$ supply constant is to lower the effective redox potential of the pool. Alternatively, raising the ambient pH increases the midpoint potential difference ($\Delta E_m$) between $Q_p$ and cyt *b*-560. At pH values of 6, 7, 8, 9 and 10, for example, the difference in $E_m$ values is +40, +40, +10, −50 and −110 mV, respectively. Each of these protocols was used in an attempt to observe cyt *b* reduction in myxothiazol treated chromatophores.

Fig.1 shows kinetics of flash-induced cyt *b* reduction in lyophilized (traces a–d) and in lyophilized and solvent extracted (traces e–h) chromatophores poised at pH 7.0 and an $E_h$ sufficient to render the quinone pool completely oxidized prior to activation. Traces a and b demon-

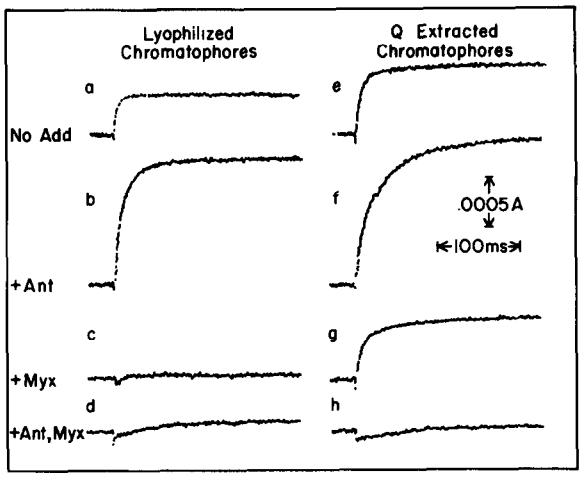


Fig.1. Flash-activated cyt *b* reduction kinetics in lyophilized (a–d) and quinone-extracted (e–h) chromatophores. Chromatophores were lyophilized and extracted as outlined in section 2 and were suspended to final reaction center concentrations of 0.14 $\mu$M (left column) or 0.16 $\mu$M (right column) in 20 mM Mops, 100 mM KCl, 1 mM $MgCl_2$; pH 7.0. Valinomycin (1 $\mu$M) and CCCP (0.2 $\mu$M) were added in addition to the redox mediators 2,3,4,5-tetramethylphenylenediamine (2.5 $\mu$M), phenazine methosulfate (1 $\mu$M) and phenazine ethosulfate (1 $\mu$M). The ambient redox potential was adjusted to 230 ± 10 mV. Myxothiazol and antimycin were each added where indicated to 5 $\mu$M and 10 $\mu$M, respectively. The optical change of cyt *b* was measured as the difference between 561 and 540 nm.

strate the familiar stimulation of cyt *b* reduction by antimycin in chromatophores containing a full complement of quinone (41 per reaction center). In the presence of myxothiazol, however, no cyt *b* is reduced (trace c), independent of the presence of antimycin (trace d) indicating that all cyt *b* reduction occurs by the $Q_z$-mediated route. In contrast, extracted chromatophores, in which there were 7 quinones per reaction center, display strikingly different patterns of cyt *b* reduction. These chromatophores exhibit, in the presence of myxothiazol, a significant amount of cyt *b* reduction after the flash (trace g; cf. trace c). This reduction is independent of the presence of redox-mediating dyes (not shown), and as trace h demonstrates, it is inhibited by antimycin.

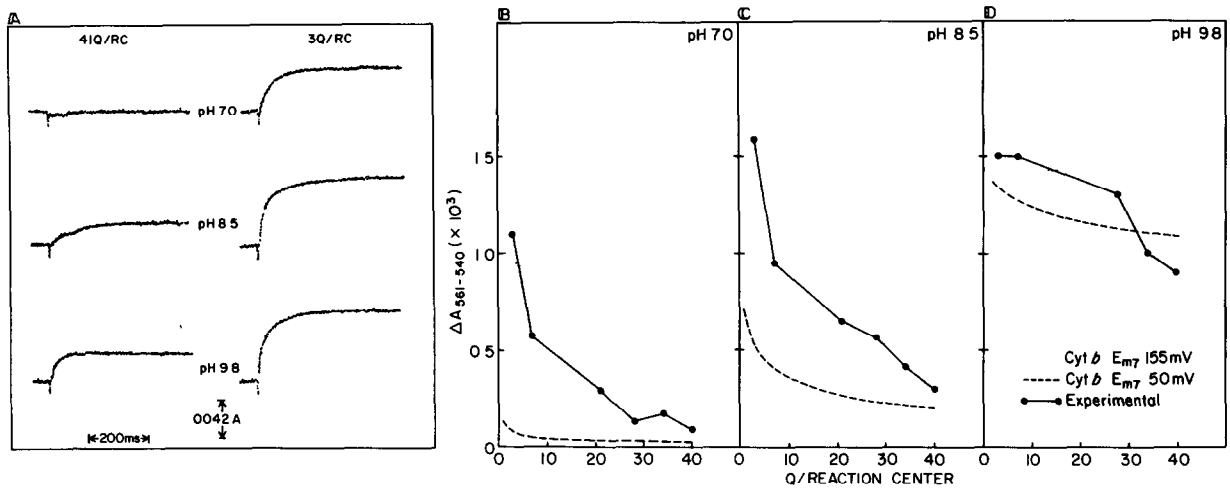


Fig.2. Cyt *b* reduction *vs* pH and quinone pool size. Conditions for kinetic traces were the same as those in fig.1 with the following exceptions: Mops was substituted by Tricine (pH 8.5) or glycine (pH 9.8). Experiments were performed at 240 ± 10 mV at pH 7.0, 190 ± 10 mV at pH 8.5 and 160 ± 10 mV at pH 9.8. Each experimental point (●---●) in panels B–D was calculated from the absorbance difference at the wavelength pair 561–540 nm, 50 ms following delivery of a single flash. A myxothiazol plus antimycin control trace was subtracted at each quinone concentration. Simulations of quinone pool-cyt *b*-560 redox equilibria (B–D) were performed by calculating the $E_h$ of a variable pool ($E_{m7}$, $E_{m8.5}$, $E_{m9}$ = 90, 0, −78 mV, respectively) with one reduced quinone and by assuming contact with either the low potential cyt *b*-560 ($E_{m7}$, $E_{m8.5}$, $E_{m9.8}$ = 50, 20, 20 mV, respectively; – – –) or the high potential cyt *b*-560 ($E_{m7}$, $E_{m8.5}$, $E_{m9.8}$ = 155, 120, 120 mV, respectively; ···).

Fig.2A shows the effect of pH on the extent of antimycin-sensitive cyt *b* reduction. As predicted, this route to cyt *b* emerges as the pH of the chromatophore suspension is raised. At pH 8.5 and 9.8 even unextracted chromatophores display some antimycin-sensitive cyt *b* reduction. Also, as expected with quinone-extracted chromatophores, the route is revealed at lower pH values; for example, fig.2A shows that with 3 quinones per reaction center, cyt *b* reduction is nearly maximal between pH 7.0 and 8.5.

Fig.2B–D shows these results in detail, presenting the yield of antimycin-sensitive cyt *b* reduction as a function of the size of the quinone pool at 3 pH values. The data are compared to theoretical lines which assume a simple redox equilibrium between the ubiquinone pool and either the low potential cyt *b*-560 or the high potential cyt *b*-560 ($E_{m7}$ = 155 mV [36]; p$K_{red}$ = 7.5 [37]) detectable in dark titrations of chromatophores. Neither of the theoretical treatments conforms to the experimental data with the exception of low potential cyt *b*-560 reduction at maximum values of quinone content. These results are not due to any artifact of the quinone extraction procedure since reconstitution of the full quinone complement restores cyt *b* reduction behavior typical of unextracted chromatophores.

Fig.3 shows, under optimized conditions of high pH and low quinone pool size, kinetics (A) and spectra (B and C) of the events that accompany cyt *b* reduction via either the myxothiazol-sensitive or the antimycin-sensitive route. Fig.3A demonstrates that in the presence of myxothiazol, cyt *b* reduction occurs on a time scale that is independent of that of the redox reactions of cyt $c_1 + c_2$. In contrast, in the presence of antimycin, myxothiazol-sensitive cyt *b* reduction occurs concomitant with the partial reduction of cyt $c_1 + c_2$, consistent with expectations concerning the $Q_z$-mediated route to cyt *b* [8,38].

In fig.3B are spectra obtained from experimental traces at various wavelengths, each relative to 540 nm. Fig.3C is composed of two resolved spectra which correspond to the routes to cyt *b* reduction. The top spectrum shows absorbance changes when cyt *b* reduction proceeds via the antimycin-sensitive route and confirms that cyt *b* is reduced with no significant accompanying cyt *c* reduction. The bottom spectrum shows the absorbance

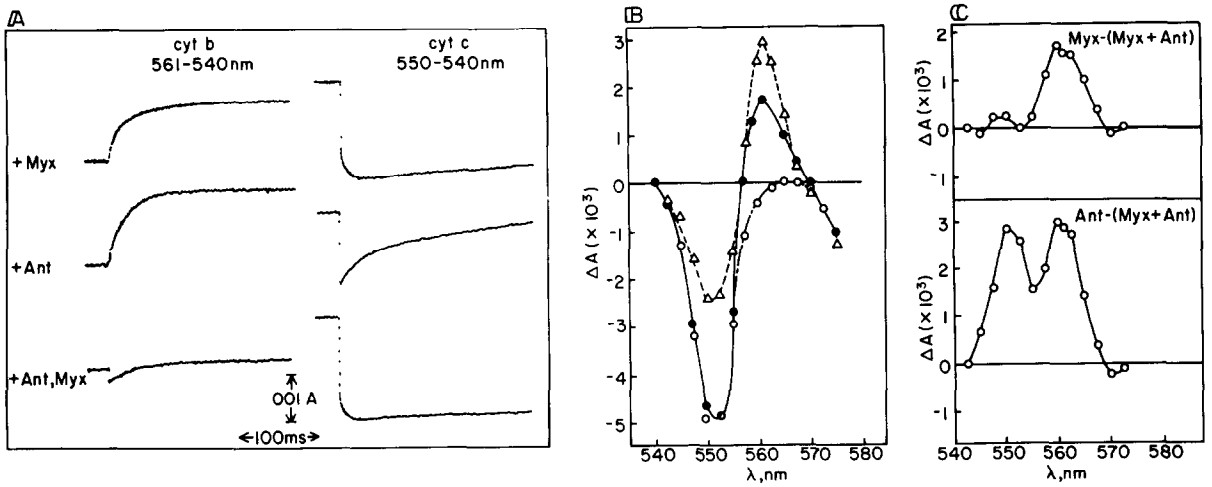


Fig.3. Flash-activated optical changes of cyt *b* (measured at 561–540 nm) and cytochromes *c* (550–540 nm) at pH 9.0. Chromatophores were suspended in 20 mM glycine, 100 mM KCl, 1 mM $MgCl_2$, pH 9.0, to a final reaction center concentration of 0.18 μM and experiments were carried out at 200 ± 10 mV. All other conditions were identical to those of fig.1. (Panel A) Kinetic traces of cyt *b* and cyt *c* absorbance changes in the presence of myxothiazol (5 μM) or antimycin (10 μM). (Panel B) Spectra of optical changes with myxothiazol (●——●), antimycin (Δ---Δ) or myxothiazol plus antimycin (○---○). Changes at each wavelength are plotted *vs* a reference wavelength of 540 nm. (Panel C) Corrected spectra in the presence of myxothiazol (upper) or antimycin (lower). Spectra obtained with myxothiazol plus antimycin were subtracted from those obtained with each inhibitor separately.

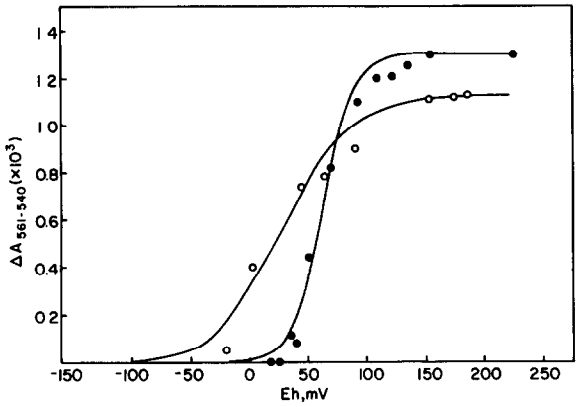


Fig.4. Redox titrations of cyt *b* reduction at pH 9.0 via the antimycin-sensitive (●—●) or myxothiazol-sensitive (○—○) routes. Conditions were as described in fig.1 and section 2 with the following exceptions: in addition to the redox mediators used previously, pyocyanine (2 μM) and 2-hydroxy-1,4-naphthoquinone (2 μM) were added. Each point represents the absorbance change at 50 ms following a single saturating flash. The ambient redox potential was adjusted by addition of minute amounts of sodium dithionite or potassium ferricyanide.

changes occurring during cyt *b* reduction via the myxothiazol-sensitive route; here extensive cyt *c* reduction accompanies cyt *b* reduction. These spectra are identical to those obtained from unextracted, myxothiazol- or antimycin-treated chromatophores at high pH (not shown) and from the reaction center $bc_1$ complex hybrid system (in preparation). The reduced-minus-oxidized spectrum in each case has a maximum at 560 nm consistent with cyt *b*-560.

Finally, we have measured the dependency of flash-induced cyt *b* reduction on the redox potential poise of the chromatophore suspension. This has been done for the myxothiazol-sensitive route

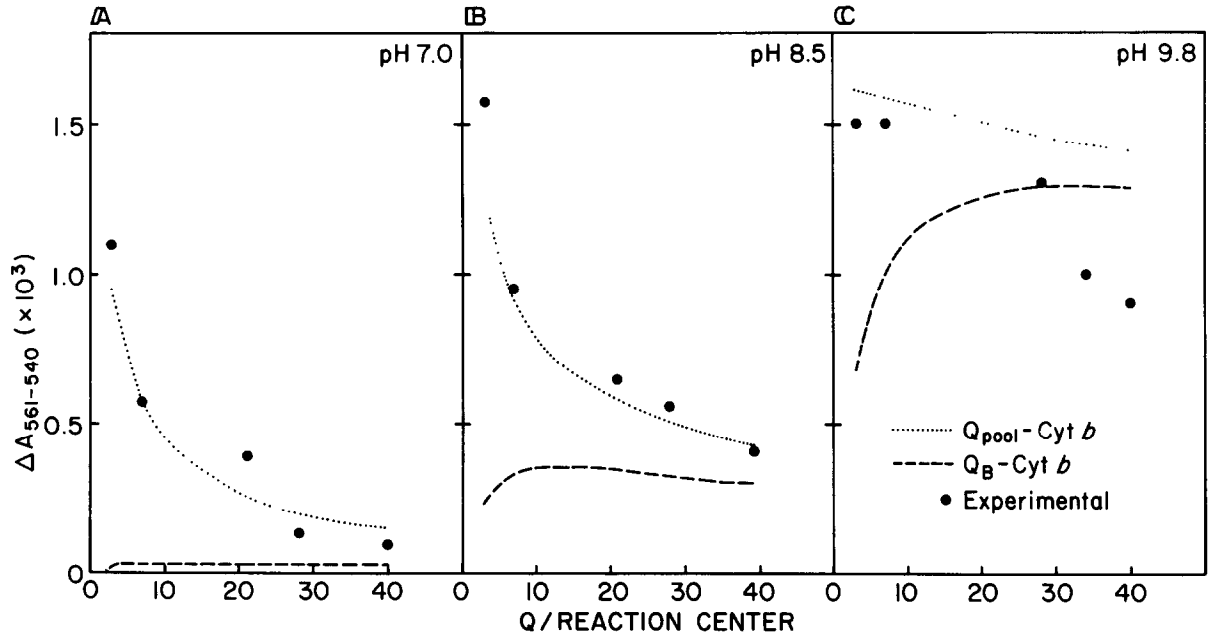


Fig.5. Theoretical behavior of two simultaneous modes of antimycin-sensitive cyt *b*-560 reduction *vs* [Q]. The experimental concentrations of redox components and those used for simulation were: [RC] = 0.14 μM; [$bc_1$ complex] = 0.09 μM; [$Q_BH_2$] after a flash = 0.07 μM. For simulation, $E_m$ $Q_BH_2/Q_B^{\cdot-}$ = −150 mV; $E_m$ Q pool at pH 7, 8.5, 9.8 = 90, 0, −78 mV, respectively; $E_m$ cyt *b*-560 at pH 7, 8.5, 9.8 = 40, 20, 20 mV, respectively. A $K_d$ of the $Q_c$ site for Q of 0.45 μM provided the best fit to the experimental data. It was assumed that the $Q_B$–cyt *b*-560 mode proceeds only in the absence of a site occupant. Simulation of total cyt *b*-560 reduction via either the sum of the $Q_P$–cyt *b*-560 and $Q_B$–cyt *b*-560 modes (···) or of the $Q_P$–cyt *b*-560 mode (– – –) viewed alone are shown.

[29] and is again shown in fig.4 for comparison with the antimycin-sensitive route. The failure of myxothiazol-sensitive cyt *b*-560 reduction as the redox potential is lowered follows the familiar course of the $n = 1$ Nernst curve of cyt *b*-560 ($E_{m9} = 20$ mV); the failure in this case results simply from the fact that the cytochrome is already reduced prior to activation. In contrast, the failure of cyt *b* reduction via the antimycin-sensitive route describes an $n = 2$ curve with an $E_{m9}$ value of 62 mV.

## 4. DISCUSSION

Cyt *b*-560 is reduced in flash-activated chromatophores via two independent routes distinguished by their sensitivities to either antimycin or myxothiazol and defined by their characteristic difference spectra. The familiar myxothiazol-sensitive pathway is part of an oxidant-induced reduction sequence involving electron transfer by $Q_z$ to both the high and low potential ends of the $bc_1$ complex. The other, described here in detail, is an antimycin-sensitive route presumably involving the $Q_c$ site. This route may be revealed by increasing the redox potential difference between the Q pool and cyt *b*-560. However, a simple thermodynamic contact between these components is insufficient to describe the higher than predicted levels of cyt *b* reduction observed after quinone extraction. To explain these results, we propose that antimycin-sensitive cyt *b* reduction may proceed by two discrete, simultaneously operating mechanisms; one, dominant at high $[Q_P]$ and directly $Q_P$ mediated ($Q_P$–cyt *b*-560), the other dependent upon circumvention of $Q_P$ and upon $Q_B$ as the ultimate reductant ($Q_B$–cyt *b*-560). The $Q_P$–cyt *b*-560 mode operates by exchange of reducing equivalents between the Q pool and cyt *b*-560, presumably involves the $Q_c$ site and relies for effective electron transfer on the redox potential of $Q_P$. The $Q_B$–cyt *b*-560 mode is expressed in inverse proportion to the size of $Q_P$, requires an unoccupied $Q_c$ site and implies collision and complex formation between the reaction center (RC) and the $bc_1$ complex.

There are a number of assumptions implicit in the idea of the $Q_B$–cyt *b*-560 mechanism: (1) The reducing power of $Q_B$ is translated directly to cyt *b*-560 without loss due to interaction with $Q_P$; (2) a quinol at the $Q_B$ site is the reductant of cyt *b*-560. This is based on the observation of binary oscillations of antimycin-sensitive cyt *b* reduction in a dark-adapted hybrid system (unpublished); (3) the operating $E_m$ of $Q_BH_2/Q_B^{\cdot -}$ is approx. $-150$ mV. This value is derived from the operating $E_m$ of $Q_A/Q_A^{\cdot -}$ [39] and the potential difference calculated from the equilibrium constant for electron exchange between $Q_A$ and $Q_B$ [40]; (4) cyt *b*-560 reduction by this mode will not proceed if the $Q_c$ site is occupied (i.e., by antimycin, Q, $Q^{\cdot -}$, or $QH_2$); (5) normally, $[Q_P]$ is higher than the $K_d$ of $Q_c$ for quinone. Extraction brings $[Q_P]$ within the range of $K_d$ and, in an oxidized pool, leaves $Q_c$ sites unoccupied for time periods which permit productive RC/$bc_1$ interactions; (6) the $K_d$ for quinol is 100-fold lower than that of quinone [26]. Prereduction of a single free quinone per $bc_1$ complex is therefore sufficient to populate $Q_c$ sites and to block cyt *b* reduction at any $[Q_P]$. A simulation based on these assumptions and compared to experimental data is shown in fig.5. In each panel the dotted line describes concerted cyt *b* reduction by the two postulated mechanisms based on a $K_d$ for Q binding to $Q_c$ of 0.45 $\mu$M. $K_d$ was derived from redox component concentrations and by assuming that any $bc_1$ complex exhibiting cyt *b* reduction above that expected from a pure $Q_P$–cyt *b*-560 mechanism lacked an occupied $Q_c$ site. The lower, dashed line in each panel is an isolated view of the $Q_P$–cyt *b*-560 reduction mode. Other mechanistic treatments fail to provide the combination of a strong reductant and a reliance on $[Q_P]$ and therefore a less reasonable fit to the experimental data.

Antimycin-sensitive cyt *b* reduction failing at an $E_m$ of 88 mV has been noted in the RC/$bc_1$ hybrid system in the absence of cyt *c* [29]. A non-Q pool mechanism involving $Q_B$ and a special quinone comparable to $Q_c$ was postulated to explain the data. Authors in [30] have also alluded to $Q_B$ as a possible reductant of cyt *b*-560 in the hybrid system. Q pool dependent and independent modes of inter-complex electron transfer have been postulated to explain the onset and resolution of anomalous kinetics and inhibitor sensitivities after removal and subsequent reconstitution of quinone in mixtures of complex I and III [41,42].

Redox potential dependent failure of antimycin-

sensitive cyt *b* reduction provides some insight into the proposed mechanisms. The idea that occupancy of the $Q_c$ site prevents $Q_B$ interaction with cyt *b*-560 is easily fitted into a mechanism which fails at redox potentials higher than the $E_m$ of cyt *b*-560. The $E_m$ of failure would correspond well to the half-maximal $[QH_2]$ at $Q_c$ (see fig.4 and [26]). $Q_P$–cyt *b*-560 may fail for the same reason if a ligand at $Q_c$ insulates cyt *b*-560 from $Q_P$.

The physiological implication of a route to cyt *b* suppressed by $[Q_P]$ is that the size of $Q_P$ and the properties of $Q_c$ combine to bias the direction of $bc_1$ complex electron flow. As such, $Q_c$ may act as the rectifier of the Q cycle.

## ACKNOWLEDGEMENT

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