Breaking the Q-cycle: finding new ways to study Qo through thermodynamic manipulations

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Abstract Thirty years ago, Peter Mitchell won the Nobel Prize for proposing how electrical and proton gradients across bioenergetic membranes were the energy coupling intermediate between photosynthetic and respiratory electron transfer and cellular activities that include ATP production. A high point of his thinking was the development of the Q-cycle model that advanced our understanding of cytochrome bc_1. While the principle tenets of his Q-cycle still hold true today, Mitchell did not explain the specific mechanism that allows the Qo site to perform this Q-cycle efficiently without undue energy loss. Though much speculation on Qo site mode of molecular action and regulation has been introduced over the 30 years after Mitchell collected his Prize, no single mechanism has been universally accepted. The mystery behind the Qo site mechanism remains unsolved due to elusive kinetic intermediates during Qo site electron transfer that have not been detected spectroscopically. Therefore, to reveal the Qo mechanism, we must look beyond traditional steady-state experimental approaches by changing cytochrome bc_1 thermodynamics and promoting otherwise transient Qo site redox states.

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Introduction

In 1976, Peter Mitchell developed the Q-cycle theory, which explained how the proton coupled electron transfer of cytochrome bc_1 was driven by the ubiquinone cofactors (Mitchell 1975, 1976). According to Mitchell's proposal, which also incorporated the previous observation of "oxidant-induced reduction of cytochrome b" by Chance (Chance et al. 1970) and a suggested role for semiquinone by Wikstrom and Berden (Wikstrom and Berden 1972), a reduced quinone molecule at the interface between the high and low potential chains of cytochrome bc_1 (bound at the Qo site) initiates a bifurcated electron transfer reaction upon its oxidation. One electron is accepted by the iron–sulfur cluster (FeS), and follows the c-chain via cytochrome c_1 to external c-type cytochromes (Fig. 1). The other electron passes through two b-type hemes and reduces a quinone molecule at the Qi site via a relatively stable semiquinone intermediate. This unique electron split sets into motion a quasi-equilibrium state of cytochrome bc_1 that balances the redox potentials of the high potential c-chain and low potential b-chain evenly on either side of the redox potential of the quinone pool. This quasi-equilibrium behavior shows that cytochrome bc_1 catalysis is rapidly and readily reversible. Collapse of this quasi-equilibrium into complete equilibrium, in which all chains and pools reach the same redox potential, takes place on a tens of seconds timescale and can be assisted by the short-circuiting action of redox mediator dyes.

Under normal forward electron transfer conditions, the efficiency of cytochrome bc_1 turnover is high, and
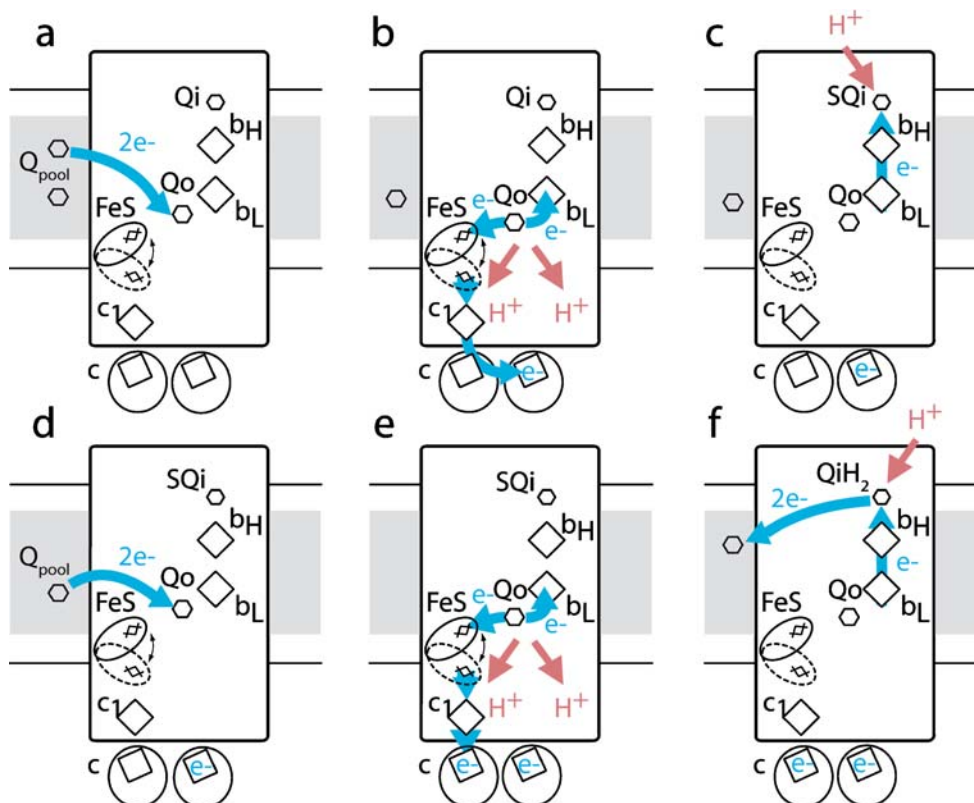
Fig. 1 Schematic representation of the Q-cycle as we think of it today in cytochrome bc_1 (represented here as a monomer for simplicity). Each panel represents a step in the electron and/or proton transfer process of one complete Q-cycle. **a** One reduced quinone molecule is delivered to the Qo site from the Q-pool. **b** The Qo site becomes oxidized, transferring two protons across the membrane and distributing two electrons to the high and low potential chains of cytochrome bc_1. **c** A proton and electron are delivered to the Qi site, forming a stable semiquinone intermediate. **d** The quinone pool delivers another reduced quinone molecule to the Qo site to restart cytochrome bc_1 turnover. **e** The Qo site turns over again, delivering a second set of electrons to the high potential c-chain and the low potential b-chain, as well as two more protons across the membrane. **f** Upon receiving an additional proton and an electron from heme b_H, the Qi semiquinone becomes fully reduced, and delivers a reduced quinol back to the quinone pool. The net result of this cycle is oxidation of one quinone, reduction of two cytochromes c, two electrogenic transmembrane electron transfers, uptake of two protons from one membrane face and delivery of 4 protons to the other

deleterious short circuit or bypass reactions involving either Qo or heme b_L are minimal (Boveris 1984). We define short circuit reactions as any electron transfer reaction between two cytochrome bc_1 cofactors that results in an unproductive loss of energy (Osyczka et al. 2004). Alternatively, bypass reactions result when the electron transfer processes at the Qo site are intercepted by an extraneous reagent, such as oxygen, which steals the electron from cytochrome bc1 and initiates harmful side reactions that produce reactive oxygen species (Cape et al. 2007; Sun and Trumpower 2003). In order for the Qo site to be efficient, these reactions must be suppressed, even though the driving forces for these reactions are highly favorable. Understanding Qo site engineering means understanding how electron transfer is regulated such that productive electron-transfer steps overwhelm unproductive steps.

The Q-cycle scheme developed in 1976 provides a successful general model for electron and proton flow

during cytochrome bc_1 turnover; however, the structural and chemical factors that promote and control this unusual bifurcated electron-transfer reaction at Qo remain controversial. Crystal structures have failed to convincingly resolve oxidized or reduced quinone in the Qo site (Gao et al. 2003; Iwata et al. 1998; Lange and Hunte 2002); in fact, the Qo site appears to be large enough to potentially accommodate two sequential positions for a moving ubiquinone headgroup (Crofts et al. 1999, 2006), or conceivably bind multiple ubiquinones at the same time (Bartoschek et al. 2001; Ding et al. 1992, 1995). Without the direction of structural information on Qo binding, a range of mechanistic models has emerged with no real consensus as to which one is the most successful. Moreover, the work of Osyczka et. al. prompted revision of all contemporary Q-cycle models in order to accommodate suppression of unwanted short-circuits in a reversible, energy-coupling mechanism of the kind evident in cytochrome bc_1 (Osyczka et al. 2004, 2005). However, it was

made clear that models that are truly concerted two-electron transfer reactions (Osyczka et al. 2004), with no detectable semiquinone intermediate state on more than a femto-seconds timescale, were not prone to the same kind of short circuit possibilities as sequential models which included a semiquinone intermediate state (Hong et al. 1999; Snyder et al. 2000). Therefore, all sequential mechanisms that exploit the properties of a semiquinone intermediate (Crofts et al. 2006, 2008; Osyczka et al. 2005, 2006; Rich 2004), must be modified to include effective gating mechanisms, sensitive to different combinations of redox states of the FeS, Qo and heme b_L redox partners, to promote productive catalysis and prevent unproductive short-circuits.

Double gated models

While there are numerous ways that reversible Q-cycle models can be modified to impede short circuits, Osyczka et al. made it clear that a minimum of two gates or barriers are mandatory to regulate Qo redox states. One model has been amplified by Peter Rich, who introduced a double, "logic gated" electron transfer model for Qo site electron transfer (Rich 2004). In short, this model suggests that reduced quinone is forbidden from binding in the Qo site if FeS and heme b_L are not both oxidized, and oxidized quinone is forbidden from binding if both of its redox partners are not reduced. This can, in principle, be achieved by regulation of oxidized and reduced quinone binding by manipulation of the conformations of Qo hydrogen bonding partners by the redox and protonation states of heme b_L and FeS (see also Osyczka et al. 2004, 2005).

Crofts et al. has modified his sequential mechanism (Crofts et al. 2000) to introduce more redox-state sensitive gating to avoid short circuits (Crofts et al. 2006, 2008). They propose that when a semiquinone intermediate is formed, it can move closer to oxidized heme b_L and participate in productive electron transfer. However, when heme b_L is reduced, there is a coulombic repulsion which keeps the semiquinone away from reduced heme b_L towards the FeS end of the site, and inhibits the unproductive, and energetically favorable, short-circuit reduction of semiquinone by heme b_L. While in principle coulombic interactions could provide a redox-state sensitive gate to inhibit a short-circuit reaction, more than one gate is needed. The coloumbic push of semiquinone from reduced heme b_L moves it closer to FeS, which when oxidized, can accept an electron from the semiquinone in another type of short circuit. A second gate for this type of model requires the FeS and semiquinone to overcome their coulombic *attraction* and enter some sort of conformation to prevent energetically favorable, but wasteful electron transfer. In addition, the site must be designed to overcome a coulombic *repulsion* between semiquinone and reduced FeS. This would allow a favorable interaction between semiquinone and reduced FeS, fostering productive and rapid reverse reactions in which oxidized quinone is doubly reduced by FeS and heme b_L.

Qo site mechanism thermodynamics

The thermodynamics of concerted and sequential mechanisms are distinctly different, which presents an opportunity to eliminate models and resolve Qo mechanism. The *top* panel of Fig. 2 illustrates when the energetic landscape for a concerted mechanism at Qo has an activation barrier that depends on both FeS *and* heme b_L redox midpoint potentials. In this mechanism, the activation barrier for forming the semiquinone intermediate (purple curve) is significantly higher; normally semiquinone formation will be minimal, but not impossible. In contrast, the sequential mechanism will have a reduced quinone oxidation rate that depends on *either* the midpoint potential of the FeS *or* the heme b_L. Experimentally, changing the midpoint potential of the FeS often changes the rate; however, changing the heme b_L midpoint potential has proven more difficult and the changes that have been made are modest, so the heme b_L midpoint potential dependence of the rate is still unclear.

The bottom panel of Fig. 2 illustrates the energetic picture of one of the short-circuit reactions. The rapid reversibility of the reactions at the Qo site means that semiquinone will be reformed by reverse electron transfer on roughly the same timescale as it is formed in the normal, forward reaction. But the short-circuit reduction of reoxidized FeS is highly favorable and energetically disastrous. Thus, if cytochome bc_1 is operating by a sequential mechanism, redox-state activated gates must be present to avoid unproductive short-circuit reactions.

Uncovering a semiquinone intermediate at the Qo site would be a critical step in distinguishing a sequential from a concerted mechanism. Numerous laboratories have chased a semiquinone intermediate at Qo experimentally after Mitchell included such a state in his Q-cycle, despite a lacking in experimental kinetic resolution. In 1979, Takamiya et al. failed to detect a cytochrome bc_1 semiquinone signal by electron paramagnetic resonance (EPR) spectroscopy in the presence of antimycin (Takamiya and Dutton 1979), an inhibitor of the semiquinone at Qi (Ohnishi and Trumpower 1980), indicating that the mechanism at Qo avoided the generation of an observable semiquinone redox state. A few years later, de Vries et al. claimed to detect the semiquinone at Qo by EPR spectroscopy (de Vries et al. 1981), but subsequent work by Rich's laboratory revealed the signal was really due to stable radicals of other quinones in the respiratory complex (Junemann et al. 1998).
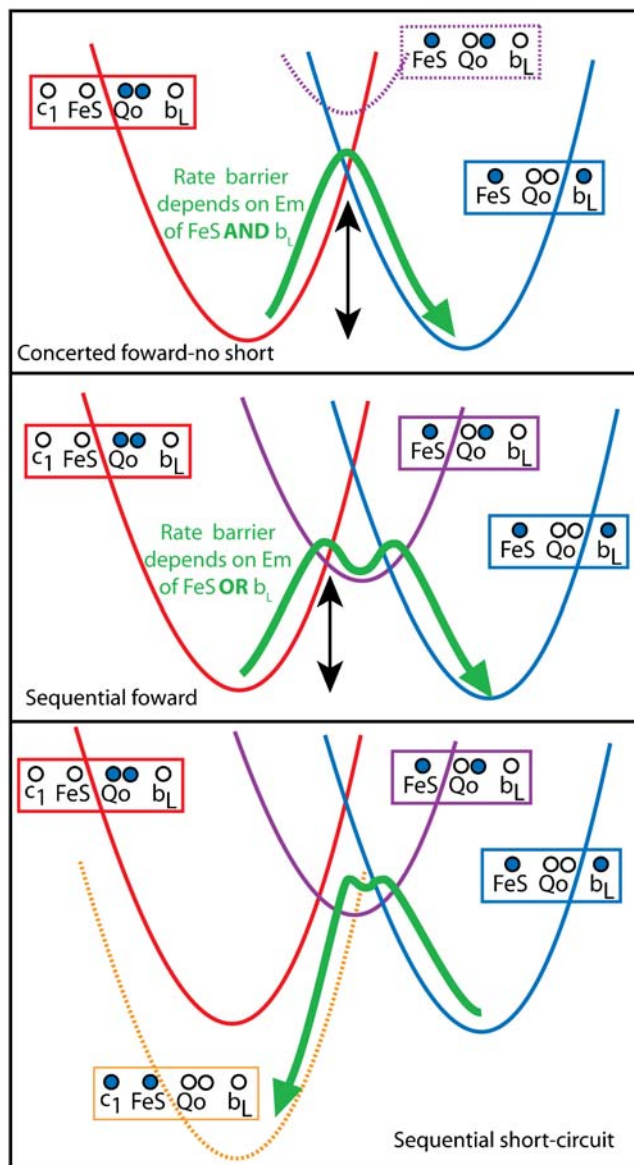
Fig. 2 Electron transfer reaction energy surfaces for Qo site operation by concerted and sequential mechanisms. Marcus-like parabolic potential surfaces are shown for each redox state. In a concerted reaction (*top*), electron transfer to form a semiquinone state (*purple*) takes more thermal energy than forming the two-electron transition state. In a sequential mechanism (*middle*), electron transfer to form a semiquinone intermediate state takes less energy than the simultaneous two electron transfers, but leaves cytochrome bc_1 potentially exposed to highly exergonic short-circuit reactions such as the one illustrated in the *bottom panel*

The field reached a stalemate for a long period before the hunt for the semiquinone returned vigorously. In 2007, two different laboratories revealed candidates for the elusive semiquinone by two different experimental approaches. Cape et al. used freeze-quench experiments (Cape et al. 2007) to produce a stigmatellin-sensitive signal that was also oxygen sensitive. They proposed that the semiquinone formed at the Qo site is consumed more rapidly than it is

formed, either by heme b_L reduction or superoxide formation. Using a completely different approach that used light-activated electron transfer, Zhang et al. also reported the appearance of a stigmatellin-sensitive semiquinone EPR signal at Qo (Zhang et al. 2007). Zhang's mutagenesis strategy to effectively knockout heme b_H and limit cytochrome bc_1 to single turnovers pushed the thermodynamics of the Qo site sufficiently to favor semiquinone production.

Even though an EPR signature attributed to a Qo semiquinone has been experimentally isolated and characterized, it is not yet clear if this is a legitimate, transient mechanistic intermediate, or a side reaction forced into existence by experimental design. Therefore, it is still too soon to reject the possibility that the normal Qo site mechanism is concerted, with the transfer of both electrons occurring in a very short interval so that there is no time for an oxidized quinone to relax into any intermediate state.

Breaking the Q-cycle: turning questions into answers

Much has been revealed about the Q-cycle and cytochrome bc_1 since Mitchell first developed his model 30 years ago; however, though elegant experiments have been completed, the data continues to fall short of telling the whole story of Qo electron transfer. Instead, we have relied on extrapolation and educated guesswork to fill in the missing pieces. Experiments aiming to generate information on the Qo site mechanism seem to yield more controversy than progress. To break this cycle of generating more questions than answers, we must design new experiments that will account for Qo redox states that could not be considered previously. As shown by current work studying a semiquinone at Qo, transient redox states will always be formed fleetingly in miniscule amounts unless larger scale manipulations of
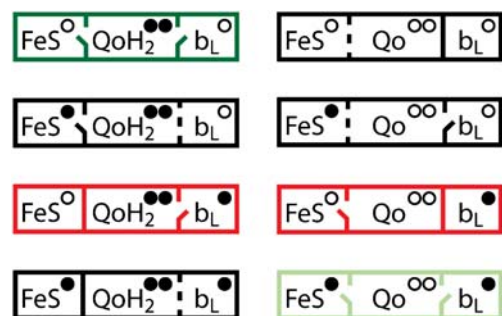

Fig. 3 Eight possible redox state combinations for the Qo site and its electron transfer partners, FeS and heme b_L. Equilibrium states are shown in *black*, productive transient intermediates are shown in *green*, and non-physiological quasi-equilibrium states are shown in *red*. Solid lines indicate closed gates, and *solid lines* with a break in the middle indicate open gates for electron transfer. *Dashed lines* indicate that a gating mechanism is optional for this electron transfer
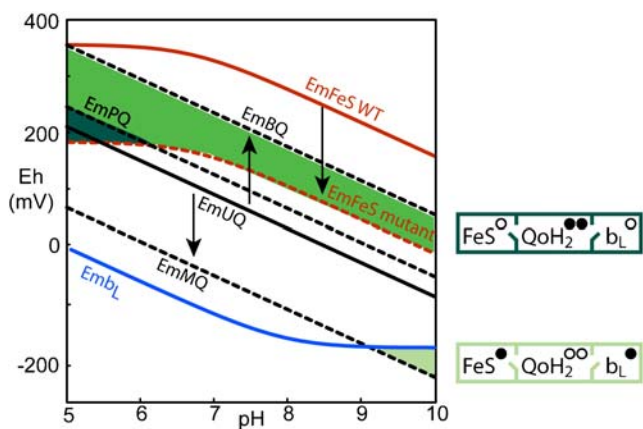
Fig. 4 Manipulation of the midpoint potentials of cytochrome bc_1 cofactors allows us to create, at equilibrium, two different intermediate states of the Qo site. The *darker green* regions of the graph represent the midpoint potential and pH combinations that would promote a reduced quinone redox state, where Qo is poised to transfer electrons to FeS and heme b_L. Shown in *light green* is the fully oxidized Qo redox state, where FeS and heme b_L are both fully reduced

cytochrome bc_1 thermodynamics are applied to systematically raise and lower the natural energetic barrier favoring or disfavoring semiquinone formation.

Though cofactor knockouts have proven to be successful in isolating a measurable Qo semiquinone signature, we are not limited to such severe measures to selectively access transient Qo site redox states. Instead, we can use information gained from classical Em/pH plots (see previous paper by Zhang et al.) to modify the redox midpoint potentials of cytochrome bc_1 cofactors and tip the thermodynamics of electron transfer more in favor of normally difficult to observe non-equilibrium states. As shown in Fig. 3, there are eight possible combinations of redox states for Qo and its redox partners, heme b_L and

FeS. Half of these states are equilibrium states readily achieved by simple redox poising (shown in black), and half are non-equilibrium states that to-date remain unstable and out of reach for convenient characterization by experiment (shown in red and green). The green states are particularly important since they are the enzyme-substrate and enzyme-product states immediate to physiologically productive quinone oxidation- and reduction-coupled energy transduction at the Qo site, while the states shown in red are subject to short-circuit reactions that are high in driving force and physiologically unproductive. Assuming for a moment a sequential-gated Qo site mechanism in this model, the gates controlling semiquinone activity must be open so that catalysis can occur in the green states, while the gates close in the red states in order to protect cytochrome bc_1 from short circuiting (Osyczka et al. 2004; Rich 2004).

While the red short-circuit states may stay out of experimental reach for some time to come, we have recognized an experimental opportunity to access and stabilize the green enzyme-substrate and enzyme-product intermediates that are key to any proposed Qo site mechanism of action. Figure 4 illustrates how the two key green states can be thermodynamically stabilized using a combination of the following thermodynamic manipulations: 1) replacing ubiquinone with other quinones and analogues that have either a higher redox midpoint potential, such as plastoquinone (PQ) and benzoquinones (BQ), or a lower redox potential, such as menaquinone (MQ); 2) using mutants to lower the midpoint potential of FeS or raise the midpoint potential of heme b_L; and 3) exploiting the differential Em/pH relationships, as described by Zhang et al. in this issue.

For example, by using a FeS mutant with the lowest midpoint potential currently available in combination with
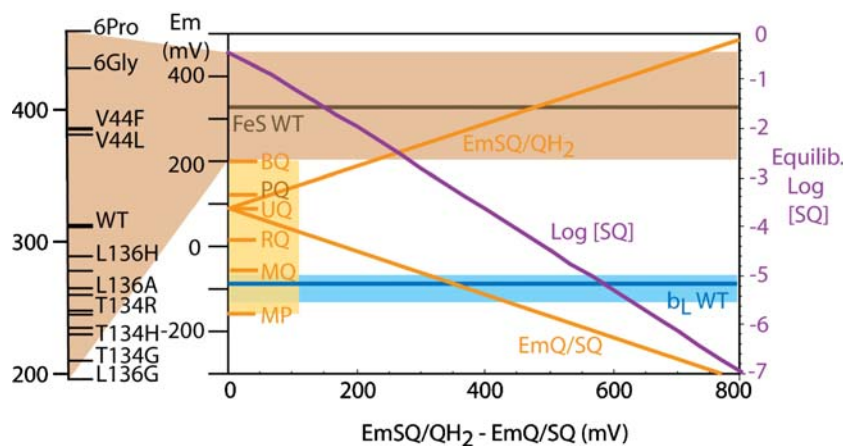

Fig. 5 The range of midpoint potentials of FeS that are available via characterized mutants are illustrated in *brown*. So far, heme b_L mutations show a more limited range of midpoint potentials, as shown in *blue*. We also show here the midpoint potentials for several quinone molecules, including ubiquinone (*UQ*), menaquinone (*MQ*), plasto-

quinone (*PQ*), rhodoquinone (*RQ*), benzoquinone (*BQ*), and methanophenazine (*MP*). As demonstrated in *purple*, depending on the split of the two electron transfer couples for the quinone, the maximum amount of semiquinone generated on an equilibrium timescale will vary

substituting PQ for the native ubiquinone (UQ) at Qo, the normally unstable reactant state of reduced quinone and oxidized FeS and heme b_L will become thermodynamically stable and experimentally accessible at the lowest pH values sustainable by cytochrome bc$_1$ (darkest green area of Fig. 4). Substituting the native UQ in the Qo site with the higher potential BQ creates this same redox state, and it is more thermodynamically stable over a much wider pH range (medium green area of Fig. 4). Stabilizing the other catalytically active state, where Qo is oxidized and both FeS and heme b_L are reduced, requires the opposite strategy (light green of Fig. 4). Attaining thermodynamic stability for this state requires replacing the native UQ with quinones that have intrinsically *lower* midpoint potential values, such as MQ, and *raising* the pH; mutants that *raise* heme b_L midpoint potential can assist the formation of this redox state.

While mutational changes to FeS midpoint potential have created a wide thermodynamic range for exploration, as shown in Fig. 5 (Darrouzet et al. 2002; Guergova-Kuras et al. 2000; Davidson et al. 1992; Merbitz-Zahradnik et al. 2003), a similar adjustment of the midpoint of heme b_L has proven more challenging (Liebl et al. 1992). There may be some hope from preliminary electrostatic calculations that suggest glutamate and arginine residues outside of the PEWY loop that could potentially lower heme b_L midpoint potential (Gunner MR (2007) personal communication). Another approach could change heme b_L ligands to allow CO ligation or a methionine, which successfully raised the midpoint potential of the heme in cytochrome b_{562} by 180 mV (Barker et al. 1996).

Once equilibrium is attained, these two states can be followed by traditional EPR or FTIR approaches, since they would no longer be constrained by a quasi-equilibrium timescale. However, the most important use of this stabilization strategy may be the potential accessibility of these new equilibria to X-ray crystallography techniques. One explanation for the absence of bound quinone in the Qo site is that quinone is only bound in the unstable, catalytically active states, a hypothesis that could be confirmed once these states have been stabilized.

While low midpoint potential FeS mutants are potentially useful in stabilizing the physiologically active oxidized and reduced quinone catalytic states for leisurely study at equilibrium, high midpoint potential FeS mutants are potentially useful for facilitating the creation of pseudo-equilibrium Qo semiquinone states using the light activation method of Zhang et al. (Zhang et al. 2007). Figure 5 shows that as the midpoint potential split between the oxidizing and reducing couple of the two quinone electron transfers gets larger, and the equilibrium concentration of semiquinone at Qo becomes extremely small, the quinone redox couples approach and eventually surpass the midpoint potential

values of FeS and heme b_L. By using a mutant of FeS with the highest possible midpoint potential (and therefore, the greatest oxidizing power), it becomes easier to strip one electron off the reduced quinone at Qo and leave the other stranded on the semiquinone when the b-chain is fully reduced. Similarly, the use of higher potential quinones instead of native UQ should also facilitate the quasi-equilibrium formation of semiquinone at Qo. Instead of the pH 9 conditions required for semiquinone formation with native UQ, high midpoint potential FeS mutants and/or quinone substitutes at lower pH values can be used to verify that the semiquinone formed is relevant to the Qo catalytic cycle, and also to detail its engineering in the physiological mechanism.

Conclusion

Assigning a Qo site mechanism remains an elusive goal despite the experimental progress we have made since Peter Mitchell's Q-cycle proposal in 1976. In order to differentiate between the mechanisms currently proposed, we need to push the boundaries of cytochrome bc$_1$ thermodynamics and generate redox states that are, at present, too short-lived to be studied spectroscopically. The basic tools to perform this thermodynamic push are largely available and are ready to be applied.

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