



Understanding the cytochrome *bc* complexes by what they don't do. The Q-cycle at 30

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The cytochrome (cyt) *bc*₁, *b*_{6f} and related complexes are central components of the respiratory and photosynthetic electron transport chains. These complexes carry out an extraordinary sequence of electron and proton transfer reactions that conserve redox energy in the form of a trans-membrane proton motive force for use in synthesizing ATP and other processes. Thirty years ago, Peter Mitchell proposed a general turnover mechanism for these complexes, which he called the Q-cycle. Since that time, many opposing schemes have challenged the Q-cycle but, with the accumulation of large amounts of biochemical, kinetic, thermodynamic and high-resolution structural data, the Q-cycle has triumphed as the accepted model, although some of the intermediate steps are poorly understood and still controversial. One of the major research questions concerning the cyt *bc*₁ and *b*_{6f} complexes is how these enzymes suppress deleterious and dissipative side reactions. In particular, most Q-cycle models involve reactive semiquinone radical intermediates that can reduce O₂ to superoxide and lead to cellular oxidative stress. Current models to explain the avoidance of side reactions involve unprecedented or unusual enzyme mechanisms, the testing of which will involve new theoretical and experimental approaches.

What are the cytochrome *bc* complexes and why are they important for plants?

The cytochrome (cyt) *bc*₁, *b*_{6f}, and related complexes are essential components of energy transduction in mitochondria, chloroplasts and many bacteria. They are sometimes referred to collectively as cyt *bc* complexes but, given that the truly conserved functional components are the 'Rieske'-type 2Fe2S center and the *b*-type cytochromes, a better name might be Rieske/cytochrome *b* complexes, as suggested by Michael Schultz *et al.* [1]. However, for historical continuity, we will use the term cyt *bc* complexes in this review.

High-resolution crystal structures for some cyt *bc* complexes have been solved (Box 1), shedding new light on their function. These complexes catalyze the transfer of

electrons between a hydroquinone (QH₂) reductant (see Glossary) and a soluble electron transfer protein carrier. The specific QH₂ and soluble electron carriers used to drive the complexes vary between different species and organelles: ubiquinol and cyt *c* are found in the mitochondrial inner membrane, plastoquinol and plastocyanin are found in the chloroplast thylakoid membrane, and menaquinol and a variety of soluble or membrane-associated electron carriers are found in many bacteria. The energy liberated during electron transfer is used to

Glossary

Q-cycle: general scheme originally proposed by Peter Mitchell for the proton translocation mechanism of the cyt *bc* complexes. This hypothesis proposes that some of the electrons derived from QH₂ oxidation in the cyt *bc* complex are recycled back into the Q-pool via a Q-reductase site on the enzyme, thereby increasing the proton translocation stoichiometry relative to a simple linear electron shuttle mechanism (see Box 2 for details). This type of reaction is called bifurcated electron transfer and its details are the subject of contentious debate. Box 2

Q, SQ and QH₂: the three accessible redox states of quinone species – quinone (Q), semiquinone (SQ) and quinol (QH₂) – each differing by one electron. Both the QH₂ and SQ species are capable of assuming different protonation states at physiological pH. It is not clear which of these species participate in the Q-cycle. We use the vague term SQ, which does not discriminate between protonation states.

SQ_o: a putative SQ species that is formed in the Q_o site of the cyt *bc* complex from QH₂ oxidation by the Rieske 2Fe2S cluster. Although certain models of the Q-cycle invoke the existence of this intermediate species, its thermodynamic stability is still open to debate. However, other models deny the existence of this intermediate altogether during normal Q-cycle turnover, favoring a concerted oxidation of QH₂ to Q in the Q_o site.

pmf: the proton motive force that results from the vectorial transfer of protons across the energy conserving membrane by various electron transport proteins. This force is partitioned into both osmotic (ΔpH) and electric field (ΔΨ) components that are energetically interchangeable for most purposes [4]. The proton motive force both activates and drives the synthesis of ATP at the CF₁CF₀-ATP synthase. [4]

Q_o and Q_i sites: the quinol oxidase (Q_o) and quinone reductase (Q_i) sites housed in the cyt *b* subunit of the *bc* complex. The Q_o site catalyzes the transfer of electrons from QH₂ to the Rieske 2Fe2S cluster and cyt *b_L*, and the Q_i site catalyzes the reduction of quinone by cyt *b_H*. In the *b*_{6f} complex, the Q_i site contains an additional heme cofactor, tentatively named heme *c_i* or *x*, whose function is presently unknown.

***n*- and *p*-sides:** the negatively and positively charged sides of the energetic membrane. QH₂ oxidation and proton release from the Q_o site occurs on the *p*-side of the membrane, and quinone reduction with associated proton uptake occurs on the *n*-side of the membrane.

Low- and high-potential chains: the cyt *bc* complex contains two separate redox chains. The high-potential chain transfers electrons from the QH₂ at the Q_o site to a soluble protein electron carrier via the Rieske 2Fe2S cluster and cyt *c_i* (in mitochondria) or cyt *f* (in the chloroplast). The low-potential chain links the Q_o site to the Q_i site via two *b*-type hemes, cyt *b_L* and cyt *b_H*, named for their respective redox potentials.

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Box 1. Comparative structures of the bovine cytochrome bc_1 and *Chlamydomonas reinhardtii* cytochrome b_6f complexes

The *cyt bc* complexes all share a common core structure of three catalytic subunits: *cyt b*, the Rieske iron sulfur protein (ISP), and either *cyt c₁* in the bc_1 complex or *cyt f* in the b_6f complex, which are arranged in an integral membrane-bound dimeric complex (Figure 1). Most eukaryotic complexes can contain up to 11 subunits; the b_6f complex also contains an additional major component, subunit IV, which is evolutionarily derived from the N-terminal section of the main *cyt b* subunit in the bc_1 complex. *Cyt b* is an integral membrane subunit that contains two *b*-type cytochromes, b_L and b_H (depicted in cyan in Figure 1), that span the membrane. The *cyt b* subunit also contains two quinol and quinone binding sites, termed the Q_o (quinol oxidase) and Q_i (quinone reductase) sites, respectively, which are linked to one another by the *cyt b* hemes, forming one branch, called the low-potential chain, of two redox pathways within the complex. A second redox branch in the complex, the high-potential chain, begins at the Q_o site with the Rieske ISP, containing a 2Fe2S cluster (orange) that forms one 'wall' of the site, and extends down to a third essential subunit, *cyt c₁* (red), which contains a single *c*-type heme. In the *cyt b_{6f}* complex, *cyt c₁* is replaced by the *cyt f* subunit, which likewise contains a single *f*-type heme (red). The *cyt b_{6f}* complex also contains an additional

heme cofactor not found in the bc_1 complexes, tentatively called heme c_i (purple) because of its unusual covalent linkage to the conserved residue Cys35 on *cyt b*, which has been identified recently in the newly elucidated structures of the cyanobacterial and chloroplast *cyt b_{6f}* complexes.

The identification of heme c_i in the recently reported structures of the *Mastigocladus laminosus* [12] and *Chlamydomonas reinhardtii* [11] cytochrome b_6f complexes raise several fundamental issues about the divergent structures of the *cyt bc* and *bf* complexes and the function of their respective low-potential chains. It was previously proposed that heme c_i might play a role in cyclic electron transfer around photosystem I and the b_6f complex by functioning as the long sought after ferredoxin-plastoquinone oxidoreductase [12,13]. However, caution must be exercised in assigning this role to heme c_i because of previous reports of kinetic limitations in the reduction of *cyt b* by NADPH/ferredoxin, and mutational work on alternative Fd:PQ oxidoreductases that indicate that the b_6f complex might not be a component of the physiological cyclic electron transport pathway [13]. In addition, the functional significance of heme c_i as a five coordinate high-spin heme, which typically bind substrate ligands, has yet to be fully explained.

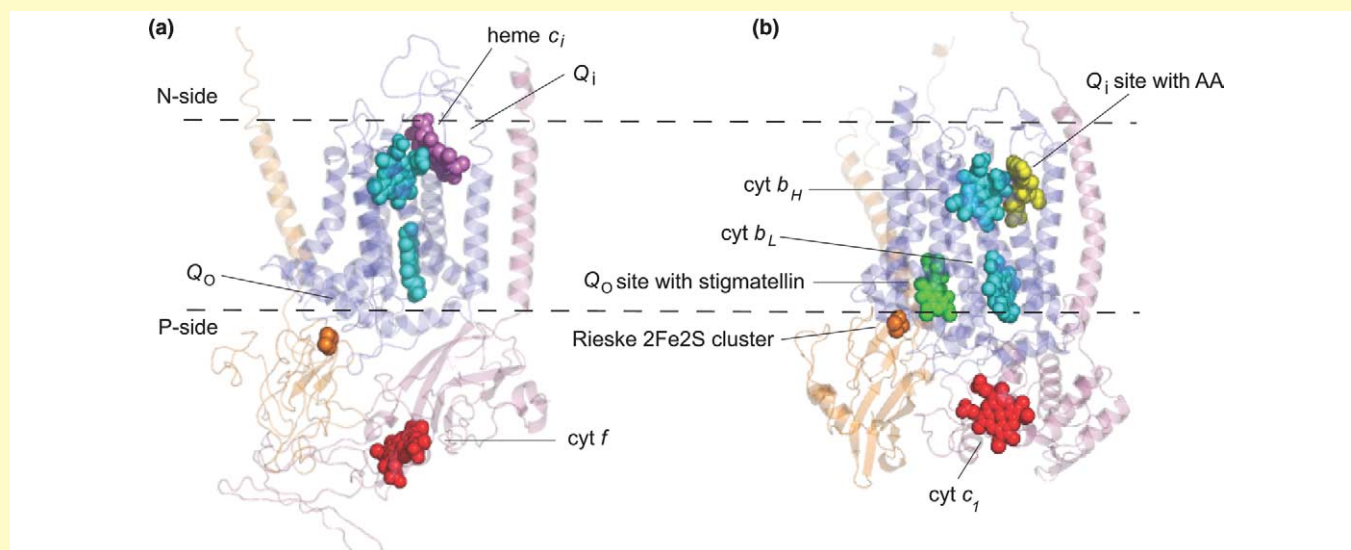


Figure 1. The *Chlamydomonas reinhardtii* cytochrome b_6f (a) and *Bos taurus* bc_1 (b) complexes. The broken lines running through the membrane-spanning portions of the complexes represents either the chloroplast thylakoid membrane or mitochondrial inner membranes, with the p-side representing either the thylakoid lumen or intermembrane space, and the n-side representing the chloroplast stroma or mitochondrial matrix. Color scheme for protein subunits: the Rieske ISP (orange); *cyt b* (with subunit IV in the b_6f structure) (slate); *cyt f* and *cyt c₁* (red). Color scheme for cofactors and inhibitors: *cyt b_L* and *cyt b_H* (cyan); *cyt f* and *cyt c₁* (red); the Rieske 2Fe2S cluster (orange); heme c_i (purple); stigmatellin (in *cyt bc₁* structure) (green); antimycin-A (AA, yellow).

translocate protons across the membrane (e.g. the thylakoid membrane in chloroplasts or the mitochondrial inner membrane), resulting in the formation of an electrochemical proton gradient termed the proton motive force (*pmf*) [2,3]. The *pmf* in turn drives the synthesis of ATP [4].

It has been suggested that the *bc* complexes also play multiple roles beyond energy transduction, particularly in plant chloroplasts where the b_6f complex has been implicated in regulating the balance of excitation energy delivery between photosystems I and II, in initiating cyclic electron transfer around photosystem I [5], and even in regulating gene expression [6]. In this review, we will concentrate on the energy transduction functions of these complexes; for a recent review on the regulatory roles of these complexes see [6].

Thirty years ago Peter Mitchell [7] proposed the Q-cycle within the context of his celebrated chemiosmotic theory to explain how electron flow through energy-conserving membranes such as the mitochondria and the chloroplast could be coupled to proton translocation. Today, we view the Q-cycle as a mechanistic 'roadmap' that describes the intricate sequence of reactions that allow the *bc* complexes to function in energy transduction. The important structural and mechanistic features of these complexes that allow them to perform the Q-cycle are still open to debate.

Two roles for the Q-cycle

The complex 'ballet' of electron and proton transfer reactions in the Q-cycle (Box 2) can be considered from

Box 2. Working model for the uninhibited Q-cycle

This version of the Q-cycle is broadly based on models presented in the literature (see text) and is described graphically below (Figure 1a). For illustration, we begin the cycle with binding QH₂ to the Q_o site, with both the high- and low-potential chains fully oxidized, although the cycle can also start from other states. QH₂ binding in the site probably involves hydrogen bonds between the QH₂ head group and at least two polar Q_o site residues (His161 on the Rieske iron sulfur protein (ISP) and Glu272 on the cyt *b* subunit, both using the *Saccharomyces cerevisiae* numbering system). At this point, electron transfer through the complex bifurcates, or forks, with one electron from QH₂ flowing downhill in energy along the high-potential chain, and the second QH₂ electron entering the low-potential chain, retaining its energy. The electron carried on the 2Fe2S cluster is eventually transferred to cyt *f* (or cyt *c*₁ in the *bc*₁ complex) through a large domain movement in the entire Rieske ISP headgroup [2,53] that pivots nearly 60° to carry the 2Fe2S cluster into reasonable electron transfer proximity so that it can reduce cyt *f* or *c*₁. A mobile plastocyanin eventually oxidizes cyt *f* to provide reducing equivalents to PSI, while, at some point, the Rieske 'resets' its position proximal to cyt *b* in anticipation of a second turnover cycle. At nearly the same time, the electron carried on cyt *b*_L equilibrates along the low-potential chain (and a quinone at the Q_i site of the *bc*₁ complex, forming a stable SQ_i intermediate in this complex). The cycle is then fully reset by the exchange of the Q for another QH₂ in the Q_o site, after which a second turnover reduces another plastocyanin and both electrons built up on the low-potential chain fully reduce Q to a QH₂ in the Q_i site, thus recycling half of all the QH₂ electrons back into the Q-pool. Proton translocation is provided by the

release and uptake of protons at the Q_o and Q_i sites, which occurs as a natural consequence of oxidizing or reducing QH₂ and Q on opposite sides of the membrane.

We are purposefully vague about the exact sequence and timing for some of the reactions described above because current experimental data does not allow us to piece them all together definitively in the correct order. Certain constraints on these events are obvious, such as the need for the bifurcated reaction to occur before the Rieske domain movement, and the resetting of the Rieske ISP back at the Q_o site for a second bifurcated reaction to occur. However, it is not clear if the low-potential chain is reduced before Rieske domain movement, or whether it is the domain movement itself that forces the intermediate SQ_o, with no other acceptor present, to reduce the low-potential chain. Indeed, a thermodynamically favored bypass of the Q-cycle is possible by the Rieske oxidizing QH₂, transferring its electron to cyt *f* or cyt *c*₁, then returning back to the Q_o site to oxidize the SQ_o it left there, with all this happening before SQ_o has a chance to reduce the low-potential chain [see bypass reaction (i) in text]. Alternatively, another major bypass reaction of the Q-cycle involves the reduction of oxygen by SQ intermediates generated at the Q_o site (Figure 1b). But Q-cycle bypass reactions are a rare event under optimal conditions, which suggests a control mechanism in the cyt *bc* complexes to prevent the wasteful (and sometimes harmful) dissipation of energy. Somehow, the cyt *bc* complexes choreograph these events so that exactly half the electrons from QH₂ end up on the low-potential chain; possible mechanisms for enforcement of the bifurcated reaction are described in the text.

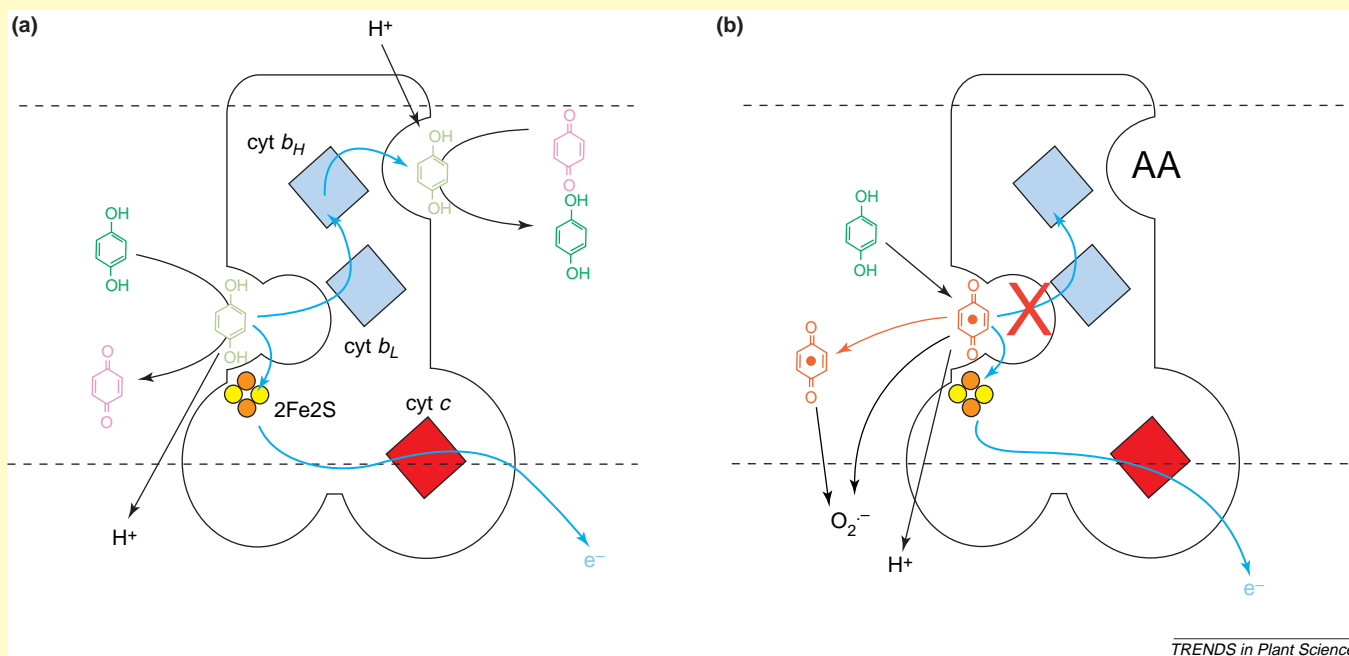


Figure 1. Schematic illustration of electron flow through (a) the uninhibited Q-cycle and (b) the antimycin A-induced Q-cycle bypass reactions. See Box 2 text for details.

two points of view: as enhancing storage of redox energy as *pmf* or as suppressing harmful side reactions.

Energy storage

The cyt *bc*₁ complexes enhance energy storage in the form of *pmf* via the Q-cycle mechanism, a process that sometimes appears complex for students (and professors alike), but can be easily understood by counting the protons released and electrons transferred through the complex at the quinol oxidase (Q_o) site in what is known as the

'bifurcated reaction'. At the Q_o site, QH₂ is oxidized by two electrons, one electron follows the high-potential chain (the Rieske 2Fe2S cluster, and cyt *f* or *c*₁), and the other electron follows the low-potential chain (cyt *b*_L and cyt *b*_H), and the two QH₂ protons are released into the *p*-side aqueous phase (Boxes 1 and 2). Only one of the two QH₂ electrons is passed to the mobile carrier (plastocyanin or cyt *c*) per QH₂ oxidized, yet two protons are released to the *p*-side of the membrane. The remaining electron from QH₂ is recycled through the low-potential chain and re-enters

the Q pool by reduction of Q at the Q_i site, picking up an additional proton along the way from the *n*-side of the membrane. The recycling of electrons back into the Q-pool increases the proton pumping stoichiometry of the *bc* complex from 1H⁺/e⁻ transferred if both QH₂ electrons followed the high-potential chain to 2H⁺/e⁻ when the Q_i site recycles the second electron back into the Q-pool.

The overall structure, and probably the mechanism, of the Q_o site appears to be well conserved over the cyt *bc*₁, *b₆f* and menaquinol-oxidizing *bc*-type complexes [8,9]. By contrast, there are significant evolutionary deviations in the components that handle electron transfer after the initial bifurcated reaction. Whereas the cyt *bc*₁ complexes possess cyt *c*₁, with a *c*-type heme as an electron acceptor to the 2Fe2S cluster of the Rieske iron sulfur protein (ISP), this function is supplied by a variety of other hemes in evolutionarily diverse species, including cyt *f* in the cyt *b₆f* complex from cyanobacteria and chloroplasts, and a diheme cyt in the menaquinol-oxidizing *Heliobacillus* [9]. These substitutions of the terminal high-potential chain carrier on the *bc* complexes probably represent simple variations on a common theme and not differences in function.

The Q_i site exhibits radical differences between the cyt *bc*₁ and *b₆f* complexes. The Q_i site semiquinone (SQ) species in cyt *bc*₁ is stabilized by the protein, allowing sequential transfer of two electrons from the low-potential chain without generating a highly reactive SQ intermediate [3]. By contrast, earlier work suggested that the Q_i site of the cyt *b₆f* complex did not stabilize its SQ intermediate to the same extent, and that two electrons accumulated before the near simultaneous reduction of plastoquinone [10]. A potential structural basis for this difference was revealed in the high-resolution structures of the cyt *b₆f* complex, which showed the existence of a previously unknown 'extra' heme, termed either heme *c_i* or heme *x* [11,12], with unusual properties [11,13]. We prefer the term heme *c_i* because it better describes its properties. Jean Alric *et al.* [60] have recently characterized the redox properties of heme *c_i*, and suggested that it operates as a redox carrier between the *b* cytochromes and the Q_i site. Various other functions have also been proposed, including a role in 'gating' the flow of two electrons to the Q_i site Q to prevent accumulation of reactive SQ intermediates (F. Bayman, personal communication) or as a redox wire, allowing ferredoxin or other electron carriers to reduce the Q pool via the Q_i site, for example, in cyclic electron transport in chloroplasts [13].

'Taming' of potentially harmful side reactions

With the widespread acceptance of the energy-storing function of the Q-cycle, the field is now considering a second function of these enzymes: how they prevent deleterious side reactions of their reactive radical intermediates [14–17].

Any time a QH₂ is oxidized by one electron, a SQ will be formed. This situation would be expected in the Q_o site if the Rieske 2Fe2S cluster simply removes one electron from QH₂, as in many Q-cycle models (Box 2). We term this SQ intermediate SQ_o. In solution, such SQ species are highly reactive and readily reduce O₂ to superoxide [18].

Superoxide production within a cell can lead to a cascade of reactions, causing lipid peroxidation and protein damage [19,20] or inappropriate cellular signaling [21], even at low yields. These oxidative processes are causative factors for various diseases and stresses, some ageing-related, in animals [19,22] and in plants [23,24].

Superoxide is produced by the *bc* complexes as a result of one of several side reactions that bypass the Q-cycle under some conditions [14,15,17,25]. These bypass reactions are energetically favored compared with the Q-cycle because energy storage in the form of *pmf* conserves energy whereas the bypass reactions release this energy in the form of heat (and other processes), much like a steam valve. Apparently, the reactive intermediates in the Q_o site can have several fates besides the Q-cycle, including: (i) reduction of the high-potential chain by both QH₂ electrons, either by direct oxidation of SQ_o or by relaying an electron from the reduced low-potential chain; (ii) escape of SQ_o from the Q_o site, leading either to disproportionation (2SQ → Q + QH₂) or other reductive processes; and (iii) reaction of oxygen with SQ_o to produce superoxide. All these reactions decrease energy storage by decreasing proton translocation. The third reaction also produces toxic superoxide and is the most active [26]; we will focus on the superoxide-producing bypass route in this review.

Under optimal conditions, normal electron flow in the Q-cycle is able to out-compete these bypass reactions kinetically through some unknown mechanism(s) to control the reactive intermediates of the Q_o site. The rates and yield of the bypass reactions are greatly increased upon slowing down the 'normal' Q-cycle reactions, for example, by exposure to certain inhibitors [14,15]; by mutations in the *bc* complex [27,28] or by buildup of a large *pmf* [29,30]. Some of these conditions, particularly high *pmf* or the accumulation of mitochondrial mutations with cellular ageing, are expected *in vivo*, suggesting that Q-cycle bypass reactions are physiologically relevant.

General mechanisms to avoid Q-cycle bypass reactions

Here, we ask how the *bc* complexes prevent Q-cycle bypass reactions. We particularly focus on the chemistry occurring at the Q_o site because this is where the reductant to O₂ is likely to reside [14,15,31]. Each protection strategy puts certain constraints on the chemistry and energetics of the Q_o site reactions and illustrates a proposed or potential fundamental mechanism of enzyme catalysis.

One of the major problems in the field is that the intermediates at the Q_o site have not been physically observed or characterized, making it difficult to test the various proposed Q_o site models. The resulting lack of experimental constraints has led to the proliferation of many Q_o site mechanistic models. It is not possible in this review to introduce each of these models, so we have attempted to group them into broad classes of strategies. We hope this exercise will clarify this sometimes-bewildering field and provide predictions so that experimental tests for each strategy can be devised.

How we describe Q-cycle models

A full description of the Q-cycle models would involve thermodynamic and kinetic arguments, involving at least some mathematics. In this review, we have attempted to use real world analogies to make the concepts accessible to a wider audience but there are limitations as to how far these analogies can be taken. For example, although some properties of the enzyme can be described using a flowing water analogy (Figure 2), the microscopic behavior of flowing electrons is different from water. Readers should consult the references for a more complete (accurate) description of these models.

Strategy I: controlling SQ_o stability

Stabilized SQ models

Most enzymes are thought to work by stabilizing activated intermediates, so it is natural to propose that the Q_o site might also stabilize (i.e. lower the free energy of) its intermediates [32,33], including the SQ_o intermediate. Indeed, this strategy can prevent bypass reactions by making the reaction of SQ_o with O₂, or other oxidants or reductants, energetically uphill processes. An analogy for this view is given in Figure 1, going from QH₂ to Q with the SQ_o intermediate in an energetic 'valley'. It would be 'easy' (energetically downhill) to get to SQ_o, going down into the valley, but 'difficult' (energetically uphill) for SQ_o to make superoxide and Q, thus lowering the overall yield of superoxide production when the Q-cycle is blocked or slowed.

In thermodynamic terms, such stabilization requires tighter binding of SQ_o than Q and QH₂. Support for this idea originates from the strong, specific binding of the Q_o site inhibitor stigmatellin, which is hypothesized to bind

in a similar manner to the SQ_o intermediate [32–34]. Good precedents exist for SQ stabilization in many redox enzymes, including the Q_i site of the cyt *bc*₁ complexes [34], the Q_B sites of photosystem II and bacterial reaction centers [35], and the Q binding sites of the cyt *bo*₃ oxidase [36]. In these examples, the SQ is rendered so stable that it can be readily observed by electron paramagnetic resonance over extended times, even under aerobic conditions.

No SQ_o species have been observed by electron paramagnetic resonance in the cyt *bc* complexes [37], which is a crucial point that must be rationalized in these models. Some suggest that the characteristic SQ_o signal is 'smeared out' by a strong interaction with the reduced Rieske 2Fe2S center [37].

Stabilized SQ models predict that additional energy must be added to the system to bring SQ_o out of the energetic valley in Figure 1 and to allow it to drive the reduction of cyt *b*_L and the rest of the low-potential chain. SQ_o is formed before the activated intermediate of the catalytic cycle (i.e. before the top of the thermal energy barrier), therefore, the main energy barrier in the Q-cycle involves activating SQ_o to reach the top of the barrier. This prediction appears to be at odds with kinetic observations that demonstrate [38,39] that the energy barrier involves the formation of SQ_o, or some other QH₂ oxidation product, and with our observation that the energy barrier for Q formation is the same as for superoxide production [16,39].

Destabilized SQ models

Alternatively, the Q_o site might destabilize the intermediate SQ_o or simply maintain its solution instability. This is illustrated in Figure 1, with SQ_o going from QH₂ to Q with

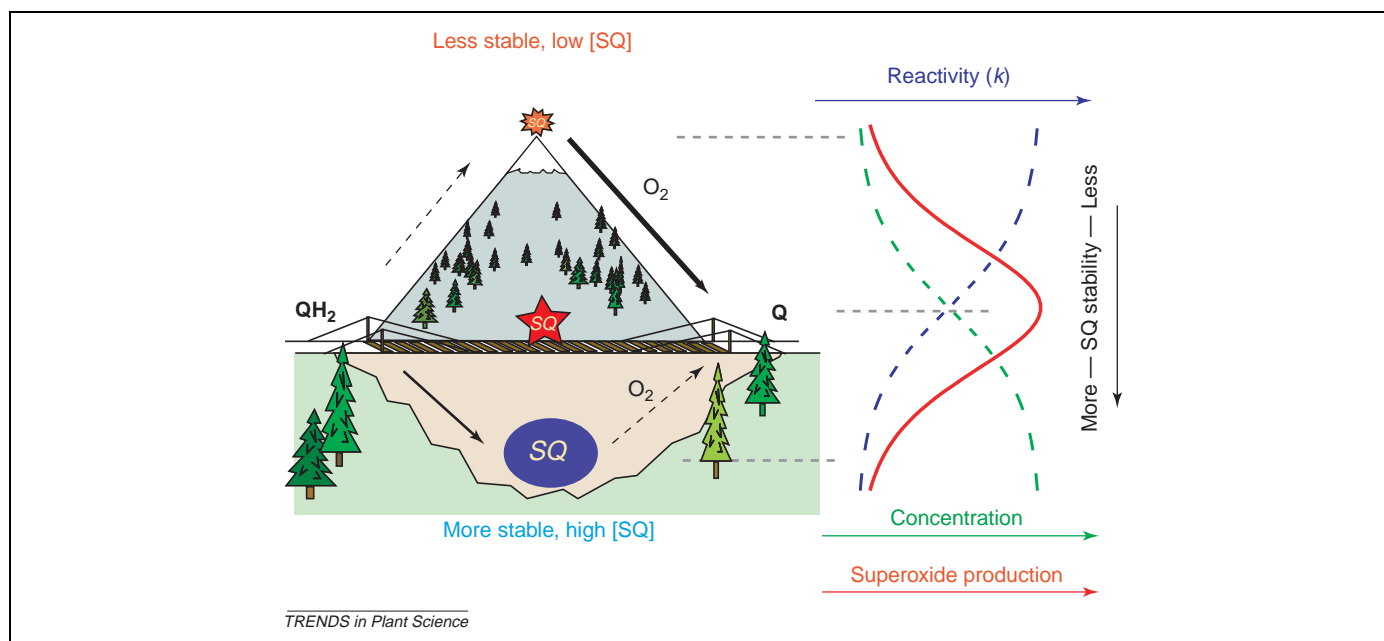


Figure 1. Thermodynamically controlled models for minimizing Q-cycle bypass reactions can be described by analogy with a mountain, bridge and valley landscape. In this analogy, the altitude of the SQ in the mountain landscape corresponds to its thermodynamic stability, and the 'size' of the SQ corresponds to its relative concentration at equilibrium with Q-pool and acceptors. To the right of this landscape is a graph plotting the relative stability of the SQ species versus its equilibrium concentration (green), its reactivity (i.e. rate constant for reaction with oxygen) (blue), and the overall rate of superoxide production expected (red). The overall rate of superoxide production is the product of the reactivity and [SQ] according to a second order rate law where [O₂] is considered constant. The lowest rate of superoxide production is expected with either a very stable, or very unstable SQ, whereas the highest rate of superoxide production occurs with a moderately stable SQ. Thus, one way that the cyt *bc* complexes can prevent superoxide production, and other bypass reactions, is by either stabilizing or destabilizing the intermediate SQ.

the intermediate SQ at, or near, the 'summit'. At first glance, this might appear counterintuitive because destabilizing the SQ only makes it more reactive to oxygen, as amply demonstrated by kinetic assays of SQ reactivity in solution [18]. However, as the reactivity of SQ towards O_2 is increased, it becomes more difficult to form (i.e. the equilibrium constant for its formation becomes more unfavorable), and thus its steady-state concentration will decrease dramatically. The overall effect will be a decrease in the reaction with oxygen owing to the low concentration of SQ.

The greatest rate of superoxide production would be expected from a significant concentration of a reasonably reactive SQ, rather than from a small concentration of highly reactive SQ or a high concentration of unreactive SQ. This is illustrated in Figure 1 as going from QH_2 to Q via the 'bridge'. With increasing SQ stability, the concentration [SQ] will increase until most of the Q species are in the SQ form. At the same time, the SQ reactivity (i.e. second order rate constant for reduction of O_2) will decrease. The rate of superoxide production depends on [SQ] and its reactivity, and both of these factors vary with the SQ stability in an opposing manner. These conflicting trends produce a bell-shaped curve with a maximum rate of superoxide production occurring when SQ is moderately stable.

A good example of this effect is the formation of an NAD^\cdot radical from either NAD^+ or $NADH$, and its reaction with oxygen. NAD^\cdot , an extremely unstable species [40], should react vigorously with oxygen, but the overall rate would be extremely low because of the low concentrations of NAD^\cdot that would be formed from either $NADH$ oxidation or NAD^+ reduction. Hence, $NADH$ is restricted to almost always being a two-electron donor and does not typically react with oxygen.

Destabilizing the SQ intermediate also limits the overall rate of the productive Q-cycle reactions, which initially appears to be harmful to the cell. However, we must remember that the *bc* complexes have evolved to optimize energy conservation, but not necessarily for speed.

The destabilized SQ model predicts that SQ_0 formation would be a major energy-requiring step in the catalytic cycle, placing the formal SQ_0 intermediate towards the top of the energetic barrier of the Q-cycle [41]; this feature might be used to test these models. Superoxide production and *cyt b_L* reduction will then occur as downhill reactions from the SQ_0 intermediate, with their relative yields determined by kinetic competition (see also strategy II below).

There is something provocative about the destabilized SQ models: in contrast to the textbook view of enzymes, the *cyt bc* complexes might de-stabilize the activated intermediate for the reaction it catalyzes to suppress the unwanted bypass reactions.

Strategy II: kinetic steering

An enzyme can also prevent harmful side reactions by kinetic 'steering' of the reactive intermediates towards the reduction of *cyt b* and away from deleterious side reactions in the same way that a 'water-park' is designed to deliver

its riders to the bottom in safety. This can be achieved in several ways, as described below.

Engineered reaction probabilities

It is possible to favor one reaction over the other by simply changing their respective reaction probabilities (or activation entropies), as illustrated by the flowing water analogy in Figure 2a. Water flows out via two holes near the bottom of a container. Let us say that flow through hole 'Q' represents the turnover of the Q-cycle, whereas flow through hole 'S' represents production of superoxide (or

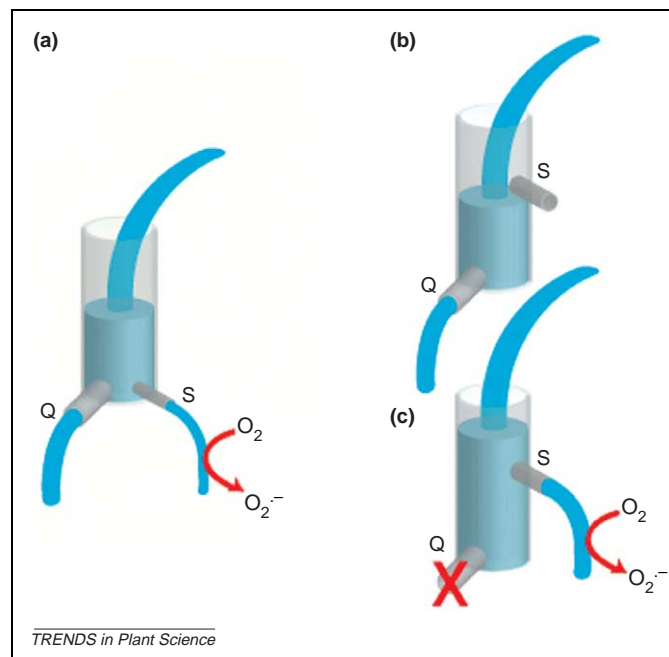


Figure 2. Kinetically steered Q-cycle models are well described by analogy with water flowing into and out of a bucket with pipes leading out of the bottom. In the 'engineered reaction probability' form of these models, efflux of water out of the bucket is controlled by both the level of water in the bucket and the (relative) diameter of the two pipes allowing the water to escape out of the bottom (a). The level of water in the bucket corresponds to the 'amount' (i.e. thermodynamic properties) of the SQ intermediate, and the sizes of the two efflux pipes describe consumption of the SQ intermediate by the productive Q-cycle reactions (Q) and the bypass reactions or superoxide production (S). The reaction can be steered towards Q by increasing the size of the Q hole relative to the size of S, this makes the probability of water flowing out of the Q hole greater than that flowing out of the S hole. The overall level of the water can remain constant under steady-state conditions because the probability of water efflux from the sum of both the Q and S holes should always equal unity, unless we turn the faucet on full blast and knock the system out of the steady state (an obvious drawback of this analogy). In this model, a constant, but small, rate of efflux out of the S hole can occur as long as there is water in the bucket. However, the 'non-equilibrium cascade' models (b) and (c) suggest that the bypass reactions are prevented by a cascade of energetically downhill steps that remove the SQ before it can participate in other, possibly energetically unfavorable, bypass reactions. The cascade of downhill reactions is represented here by positioning the Q efflux pipe lower in the container than the S efflux pipe. Note that the relative diameters of these efflux pipes are the same in this analogy because a change in entropic driving force is not necessary for operating this type of kinetic steering. Under conditions where Q is unblocked (b), no (or little) flux to bypass reactions will be observed out of S because the level of water in the bucket is too low to reach S. When Q is blocked (c), the water level in the bucket will rise (corresponding to a buildup of SQ_0) until it reaches S, and then flows out of the bucket through S to participate in Q-cycle bypass reactions. This model shows that efflux through Q and S can be controlled enthalpically by keeping the steady state [SQ] low. Obviously, these analogies can only be taken so far. If Q is blocked, the water level will rise, eventually reaching S. The transition between Q and S flow in the enzyme would be more sudden because the energy differences between Q-cycle and superoxide production pathways are much smaller than those in the water analogy, and can be bridged by local fluctuations in energy (i.e. heat). (One could stretch the analogy by shaking the cylinder to add waves that would simulate these fluctuations.)

other bypass reactions). Simply making 'Q' larger than 'S' will increase the yield of the Q-cycle relative to superoxide production. Although the overall energetic properties, or driving force, of the two reactions can remain roughly equal, the reaction with the large 'hole' will be entropically favored.

A familiar example of this strategy is how photons are delivered to the photosynthetic reaction center by chlorophyll-containing antenna complexes [42]. After light absorption by the antenna, some of the energy within the antenna complex is 'lost' through fluorescence or heat, and the rest of this energy arrives at the reaction center to perform useful photochemistry. The photosynthetic apparatus has evolved to optimize the rate of energy delivery to the reaction center and subsequent electron transfer to compete effectively with energy loss from the antenna. This strategy is realized by tuning the distances, orientations and relative energetics of the reacting species.

Increasing the Q-cycle yield can be achieved either by increasing the rate constants for reactions leading to the Q-cycle or by decreasing the rate constants for the side reactions, or both (Figure 2a). Indeed, these tactics are implicit in many existing models. These differences in rates would be reflected in the activation entropy of turnover for either the Q-cycle or bypass reactions; one should observe a decrease in reaction probability as the complex is inhibited (i.e. the number of possible configurations that can consume SQ_o will decrease), as suggested by recent experimental evidence [39].

One problematic feature of pure 'engineered reaction probability' models is that they predict a substantial rate of superoxide production even under uninhibited conditions. Instead, what is generally observed is an unobservable, or low rate and yield of superoxide production in uninhibited complexes [15,25]. Thus, if it occurs, engineered reaction probability steering probably functions along side other strategies, for example, stabilization or destabilization of SQ or gating (see below).

Non-equilibrium cascades

An interesting situation, which we term a non-equilibrium cascade, can occur when the reaction resulting in superoxide production has to pass through a slightly higher (enthalpic) energy barrier than it does through the Q-cycle. (This could result from a complex potential energy surface for the reaction, or from the requirement for additional discrete steps from a common intermediate, to form the activated intermediate for the bypass reaction.) In these models, the SQ is rapidly removed by the Q-cycle, and the intermediate for the bypass reactions does not have a chance to accumulate [3,43]. However, when that Q-cycle cascade is blocked, the reactive intermediate (probably SQ_o) is not removed, and builds up until it approaches equilibrium with QH_2 , accelerating the bypass reactions.

In such models, the production of superoxide can be much less (possibly zero) under normal Q-cycle operation than when the Q-cycle is blocked, as has been observed [15,25].

A possible analogy can be seen in Figure 2b, where water flows into a vessel with two holes, one at a lower elevation, representing the Q-cycle (Q) and one at a higher elevation, representing superoxide production or other bypass reactions (S). The water level is prevented from reaching S, hence no bypass flow, unless Q is blocked. When efflux of water through hole Q is blocked (Figure 2c), water begins to accumulate in the container (the SQ is approaching equilibrium with the Q-pool), and will eventually reach S, thus leading to bypass reactions.

In this type of kinetic steering, the enzyme 'tunes' the energetics for consumption of the SQ by each competing decay pathway to make the Q-cycle much faster than the others. Suggestions for the mechanism by which the *bc* complex kinetically 'steers' the SQ intermediate towards the productive Q-cycle reactions include: the formation of hydrogen bonded intermediates [44,45], the modulation of E_m of the Rieske 2Fe2S cluster when it docks to the Q_o site [46,47], the movement of the SQ between different binding niches in the Q_o pocket [14,48], the mediation of fast electron transfer from SQ to *cyt* b_L via a second Q species [49], and the allowance of other secondary reactions, for example, electron transfer between monomers, that prevent the buildup of reduced intermediates [25,50].

Protection of the SQ in the Q_o site

A special case of kinetic steering can be seen in models where superoxide production is avoided simply by preventing any reactive SQ_o from encountering O_2 . For instance, the protein surrounding the Q_o site could form a good barrier against oxygen diffusion to SQ_o , while also preventing SQ_o from escaping into solution. In this case, the Q_o site acts as a protective 'reaction vessel' for the 'dangerous' reactions. This mechanism could work in conjunction with either thermodynamically or kinetically tuned models (see above), but could also stand alone as a simple way to prevent the worst of the bypass reactions. The prevention of O_2 diffusion could either be kinetic or thermodynamic, although both lead to the same experimental prediction, that SQ_o would need to escape from the Q_o site to react with O_2 . Structural changes (e.g. caused by mutation or in the substrate QH_2) that increase the propensity of SQ_o to leave the Q_o site would increase superoxide production, possibly leading to oxidative stress or disease. Artificial changes that specifically increase leakage should have the same effect, potentially providing a test of this model.

Strategy III: gated models

Modern automobiles usually have a mechanism called an interlock that requires that the brake be applied when starting the engine of a car. This mechanism is a foolproof way of preventing an unsafe situation such as starting the car with the transmission in gear. In a similar way, internal combustion engines have valves that open to allow fuel and air in and exhaust out but that seal to contain the explosion.

Some hypothesize that the *bc* complex is an efficient nano-machine, complete with 'interlock' or valve mechanisms [2,17,51] that prevent the formation of the reactive SQ_o except when the enzyme is in a state that can

process it efficiently. For instance, the Q_o site might be disabled (unable to bind QH_2) under conditions where the SQ_o would accumulate, for example, when the cyt b hemes or the Q_i site are reduced [17,25,52]. Alternatively, oxidation of bound QH_2 to SQ might be prevented by a similar interlock mechanism.

Could the cyt bc complex be such a nano-machine? The interlock mechanisms described above would probably require allosteric or electrostatic changes within the Q_o site that respond to redox changes in the cyt b hemes or the binding of substrate (or inhibitors) in the Q_i site. These gates would need to sense the 'readiness' of the low-potential chain to consume SQ_o .

The soluble headgroup of the Rieske ISP undergoes a domain movement between the Q_o site and cyt c_1/f during catalysis and it has been suggested that this movement acts as a conformational gate to regulate the reactions of QH_2 and SQ at the Q_o site [51]. The ISP movements are the only obvious large-scale conformational changes yet defined, so it is an obvious choice. The conformational gate could, in principle, be controlled from the Q_i site or low-potential chain. Indeed, the orientation of the ISP soluble domain (the part that protrudes into the aqueous phase, see Box 2) has been observed to change with inhibitor or substrate occupancy of the Q_o [2,53] or Q_i site [54] as well as the overall redox state of the complex [55], but it is neither clear whether these changes are relevant to the functional enzyme or if they have sufficient thermodynamic driving force to accomplish their proposed function, nor how these conformational changes are transmitted through the quaternary structure of the complex.

More subtle gating modes have been proposed, involving electrostatic polarization of water or amino acid side chains, dictated by the reduction states of the Rieske ISP and cyt b_L , in the Q_o site, which gate the progression of QH_2 to SQ , and finally to Q [17]. Deprotonation of SQ_o by the conformationally flexible glutamate residue (Glu272 on cyt b , using the *Saccharomyces cerevisiae* numbering system) has also been suggested to gate the further reactions of SQ_o [3], although mutation of the homologous residue appears to have variable effects depending upon species [56]. Overall, a major problem with these models is that there are, as yet, no well-defined structural mechanisms that account for these gating processes. No one has suggested how the interlock or valves work at a molecular level. Testing such models will require new structural or computational techniques that allow us to resolve clearly defined mechanisms.

Strategy IV: the avoidance of SQ_o

Perhaps most provocative of the models to explain the avoidance of superoxide production is one in which the SQ does not form at all, and QH_2 is oxidized by a 'truly' concerted double electron transfer from QH_2 to both the Rieske 2Fe2S cluster and cyt b_L [25,57]. In theory, the oxidation of QH_2 to Q in the Q_o site does not have to occur by sequential single-electron oxidation steps but could instead result from a simultaneous, concerted oxidation of QH_2 in a single step. The double concerted electron transfer step for the QH_2 to Q oxidation step can only occur when both electron transport chains are ready to

each receive an electron because SQ formation is simply not possible in this model.

Of course, the superoxide production that is observed under partially inhibited conditions can only be explained by invoking a new reaction for superoxide production that is completely different from the concerted electron transfer reaction of the Q -cycle. The physical details of this model have yet to be elaborated in the theoretical literature, mostly because of the complicated coupling between the simultaneously transferring charges. As it stands, the current interpretation of this mechanism requires 'two events taking place without time for significant atomic rearrangement on the scale of femto-seconds' [25]. The QH_2 oxidation reaction would probably require the coupled transfer of up to four particles – two electrons and two protons – because QH_2^{2+} is a physically unrealistic oxidation product. (It is conceivable that one, or even two, of the QH_2 protons are released before the rate-limiting electron transfer reactions, although the energy required to form Q^{2-} would be enormous). The most believable concerted reactions would be either $QH_2 \leftrightarrow Q + 2e^- + 2H^+$ or $QH^- \leftrightarrow Q + 2e^- + H^+$. Such complex multi-particle concerted reactions would involve unprecedented chemistry.

Theoretical considerations [58] lead to predictions and tests of this double concerted model: (i) a stronger deuterium kinetic isotope effect for normal turnover relative to the bypass reactions; (ii) substantially different reaction coordinates, and thus activation energies, for Q -cycle and bypass reactions; and (iii) the E_m of both the Rieske 2Fe2S cluster and the cyt b_L would affect the rate and activation energy of normal turnover.

Conclusion: the radical hypotheses

In our view, each of the mechanistic categories presented above involves aspects that are radical or unprecedented:

- (i) A complex that can either stabilize the intermediate SQ_o , rendering it inert and invisible through some unknown mechanism, or that can use the unprecedented tactic of destabilizing its reactive intermediates.
- (ii) A kinetic 'water-park' that tunes reaction activation enthalpies or entropies to route 'water' (electron) flow into productive channels.
- (iii) A nano-machine that gates the electron and proton transfer reactions of SQ_o according to its recognition of the different redox and/or conformational states of the complex.
- (iv) An extraordinary, and unprecedented, double concerted oxidation of QH_2 that simultaneously distributes two electrons and at least one proton between at least three different acceptors.

The bc complexes are widely spread through the kingdoms of life, and yet have strong structural and functional similarities (with the interesting exceptions noted above), suggesting that the Q -cycle makes use of some novel mechanisms that were discovered only once. But how do we discriminate among these mechanistic strategies given that we have not yet observed the intermediate species? In other words, how can we test for something we cannot see?

Of course, the entire field might be turned on its head with the observation of a reaction intermediate at the Q_o site (e.g. SQ_o), but, in the meantime, we are restricted to indirect methods for characterizing the intermediates.

Our view is that, rather than introduce new models, the broad mechanistic classes introduced above should be put to experimental tests. In our opinion, the simplest, or most testable, model (in accordance with William of Occam^{*}) is that there is nothing exotic about the chemistry of QH_2 in the Q_o site, and that the reactive intermediates in the Q_o site for the Q-cycle and superoxide production are similar, as suggested by recent studies on QH_2 solution [16,39,59]. This simple model tells us that the avoidance of bypass reactions in the cyt *bc* complexes is probably because the concentration of SQ_o species is minimized. Whatever the form of the actual mechanism by which *bc* complexes achieve this goal (see above), such a model will represent a 'radical' deviation from our standard view of enzymes.

The situation with the *bc* complex field is, in this respect, not that different from that of many other enzymes: structures are solved and the broad, descriptive outlines of the mechanism appear to be established, but moving forward leads us to the interface between biology, chemistry and physics. The questions being posed by the cyt *bc*₁ complexes are at the same fundamental levels as those posed by other such 'well-characterized' systems. Even though we agree with the Q-cycle with respect to it being a 'roadmap', the interesting mechanistic aspects are still far from understood.

Acknowledgements

We are grateful to Isaac Forquer, Les Dutton, Anthony Crofts, Florian Muller, Bernard Trumpower, Edward Berry, Tomoko Ohnishi, Arieh Warshel, Giovanni Finazzi, Francesca Zito, Fabrice Rappaport, Wolfgang Nitschke, Artur Osyczka and Marilyn Gunner for stimulating discussions. Work from our laboratories was supported by National Institutes of Health, GM61904 (M.K.B.) and US Department of Energy DE-FG02-04ER15559 (D.M.K.). Part of this work was performed at the WR Wiley Environmental Molecular Sciences Laboratory, a national scientific user facility sponsored by the Department of Energy's Office of Biological and Environmental Research and located at Pacific Northwest National Laboratory.

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* William of Occam (sometimes spelled Ockham) was perhaps the most influential philosopher of the 14th century. He advocated a principle of parsimony called Occam's Razor, which is revered today as a central principle of the scientific method because it cuts through to the central issues of a problem. The principle states that one should not increase, beyond what is necessary, the number of entities required to explain anything. In other words, one should first test the simplest (less exotic) explanation for something, before adding complexity.

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