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Minireview

The Q-cycle – a personal perspective

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Abstract

In this Minireview, I provide an overview of the developments over the period 1970 to 1990 that led to the current view of the Q-cycle mechanism of the cytochrome bc_1 complex. The perspective is necessarily personal, and places some emphasis on research on the complex in the photosynthetic bacteria, where the kinetics could be studied *in situ* and with better time resolution than in mitochondria. Peter Mitchell's original Q-cycle underwent several early revisions. The version of the Q-cycle currently accepted in most labs owed much to a perceptive critique by Peter Garland, who proposed a modified Q-cycle that allowed the complex to act independently. This was among several variants discussed by Mitchell in a seminal review from 1976. Six years later, despite significant advances in both mitochondrial and bacterial work, discrimination between the half-dozen or so variants that remained in active contention had proved elusive, and the kinetic data from both mitochondrial and photosynthetic systems was refractory. This was the basis of my own opposition to the Q-cycle. While trying to explain this opposition to an undergraduate student in the lab I was led to a re-evaluation of the kinetic data in the light of the substantial advances in our understanding of the biochemistry and thermodynamic properties of the complex. From this it became apparent that one version of the Q-cycle could account with satisfactory economy for the data from the photosynthetic bacteria, and for most results from work with mitochondrial complexes. The resulting model was highly constrained, and, since it incorporated Garland's suggestions for an independent mechanism, was called the modified Q-cycle. The modified Q-cycle has stood the test of time well, and the recent structural information has both confirmed the general mechanism, and allowed extension to a more detailed understanding of the molecular architecture, and the relation between structure and function.

Abbreviations: BAL – British Anti Lewisite, 2,3-dimercaptopropanol; chromatophores – sealed vesicles produced by mechanical disruption of the invaginated cell membranes from photosynthetic bacteria; Cyt – cytochrome; Cyt b_H – high-potential heme of cytochrome *b* (also known as Cyt b_K or Cyt b_{561}); Cyt b_L – low-potential heme of cytochrome *b* (also known as Cyt b_T); Cyt bc_1 (also referred simply as bc_1) complex – ubiquinol:cytochrome *c* oxidoreductase (EC 1.10.2.2); DBMIB – 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone; E_h – ambient redox potential; E_m – midpoint redox potential; ISP – (Rieske) iron–sulfur protein; P-phase, N-phase – aqueous phases in which the proton gradient is positive or negative, respectively; P^+/P – oxidized and reduced forms of the reaction center primary donor; Q – quinone, oxidized form of ubiquinone; Q_i-site – quinone reducing site of bc_1 complex; Q_o-site – quinol oxidizing site of bc_1 complex; QH_2 – quinol, reduced form of ubiquinone (ubihydroquinone); *Rb.* – *Rhodobacter*; RC – photosynthetic reaction center; *Rps.* – *Rhodopseudomonas*; SQ – semiquinone form of ubiquinone; UHDBT – 5-undecyl-6-hydroxy-4,7-dioxobenzothiazol; UHNQ – 2-undecyl-3-hydroxy-1,4-naphthoquinone

Introduction

The general concept of Peter Mitchell's Q-cycle mechanism was, as revealed by the great man himself, '... invented at about 3 A.M. on Tuesday May 20, 1975 ...' (Mitchell 1990). At that time, Peter Mitchell (1920–1992, Figure 1) was still embroiled in the great controversy about his chemiosmotic mechanism

Figure 1. Peter Mitchell c. 1943 (adapted from Mitchell 1981). A young Peter Mitchell in the Department of Biochemistry at Cambridge. *Left to right*: Joan Keilin, Jim Danielli, Peter Mitchell and Mary Danielli. The ideas of David Keilin on the cytochromes and Jim Danielli on the lipid bilayer were seminal in the development of Mitchell's views on chemiosmosis and vectorial metabolism.

(see Harold (2001) for a historical review, and Crofts (1993) for a brief biography). His ideas had received wide recognition (cf. Paul Boyer et al. 1977, and the Nobel Prize in Chemistry, 1978), and there was some measure of agreement, after 14 years of argument, that the mechanism of coupling between electron transfer and phosphorylation was through the coupled proton pumping activity of the electron transfer chain and the ATP synthase, mediated by the electrochemical proton gradient. However, as attention turned to mechanism, the central span of the mitochondrial chain was proving particularly troublesome, as discussed in greater detail below. With Mitchell's proposal of the Q-cycle several odd experimental observations on the kinetic behavior of the *b* and *c*-type cytochrome (Cyt) intermediates of the mitochondrial electron transfer chain were neatly explained. In addition, his mechanism allowed for the energy transduction function of the central span of the chain in the context of the protonmotive activity required by the chemiosmotic hypothesis (Mitchell 1961, 1966, 1968, and see Jagendorf, 2002, for a historical minireview). The energy coupling behavior was first shown by the 'cross-over point' in this span, discussed at length by Britton Chance and Ron Williams (1956). This was the name given to a kinetic behavior of Cyt *b* and Cyt *c* that was observed when the coupled state of the mitochondrial membrane was changed. Electrons piled up in Cyt *b*, and disappeared from Cyt *c*, as if the coupling mechanism exerted a back-pressure on the electron transfer chain at this point. The cytochrome *b* observed was later called Cyt b_{561}, or Cyt b_K, where the K recognized the rediscovery of the cytochromes by David Keilin (Mitchell 1978; Ferguson 2001; see also papers by D.S. Bendall and W.A. Cramer, this issue), who first identified cytochromes *a*, *b* and *c* in cells and tissues, using a hand spectroscope. In the schemes shown in this review, Cyt b_K is referred to as Cyt b_H, where the H stands for *h*igh redox potential. Chance and colleagues (Chance et al. 1970) later demonstrated that two *b*-cytochrome components, which had previously been identified in redox titrations by Les Dutton and Dave Wilson (1976, and Dutton et al. 1970), participated in the respiratory chain. They showed that the equilibrium constant between the two hemes was strongly dependent on the coupled state. The lower potential, longer wavelength, component was identified as uniquely involved in energy transduction and called Cyt b_T (where the T stands for transducing), and is equivalent to Cyt b_L (*l*ow redox potential) in the schemes shown in this review. At the time, this response to the coupled state was taken as evidence for a chemical high-energy intermediate. The other kinetic effects that the Q-cycle explained were those related to the phenomenon of 'oxidant-induced reduction of cytochrome *b*.' Observations in several labs (nicely reviewed by Bill Slater 1981) had shown that on addition of oxygen to mitochondrial suspensions, the *c*-type cytochromes became oxidized, as expected from the linear electron transfer schemes current at the time, but, unexpectedly, the *b*-type cytochromes became more reduced. This behavior was strongly enhanced in the presence of antimycin (Wikström and Berden 1972) (see Figures 2 and 3 for group photographs from two meetings some 29 years apart, with some of the actors in the Q-cycle drama, including Brit Chance, hardly changed!).

Early variants of the Q-cycle theme

Mårten Wikström and Jan Berden (1972) had suggested a neat explanation for the oxidant-induced reduction effect. They pointed out that oxidation of ubihydroquinone (quinol or QH_2) likely occurred via an intermediate semiquinone, opening the possibility that the two electrons released might be transferred to two different acceptor chains. In the scheme they suggested (similar to that in Scheme 1 of Figure 4),

Figure 2. A section of the audience on the occasion of a lecture delivered by Robin Hill on Joseph Priestley and the discovery of photosynthesis, at the Second International Congress on Photosynthesis Research, Stresa, 1972. *Front row*: Hans Heldt, Tony Crofts, Paula Crofts, Wolfgang Junge; *second row*: Herrick Baltscheffsky, Mrs. Stacy French, Stacy French; *third row*: second from left, Britton Chance; *fourth row*: (from right) Barbara Cogdell, Richard Cogdell, Hillary Evans; *fifth row*: (second from left) Richard Gregory, Alison Telfer, Mike Evans, and back row, middle, with glasses) Yaroslav de Kouchkovsky.

electron donation from QH_2 to Cyt *b* was pulled over by consumption of the intermediate semiquinone in the reduction of Cyt *c*. Since this latter reaction would occur in response to the oxidation of Cyt *c* by O_2 through the terminal oxidase, the result would be an oxidant-induced reduction of Cyt *b*. In their original scheme, the first electron was transferred to Cyt *b*, and then to Cyt c_1, through an antimycin-sensitive step, then Cyt *c*, and cytochrome *c* oxidase as final acceptor. The second electron transfer chain, in which Cyt *c* was the immediate acceptor, was connected in a somewhat nebulous manner to the same terminal oxidase.

Mitchell's Q-cycle followed the Wikström–Berden scheme in assignment of electron acceptors, but proposed that the *b*-cytochromes were re-oxidized by ubiquinone (quinone, or Q) at a second catalytic site on the other side of the membrane, and that the high potential chain (then thought to consist of Cyt c_1, Cyt *c* and cytochrome oxidase) accepted the second electron (Scheme 2, Figure 5). Mitchell called the two quinone processing sites the Q_o-site (the quinol oxidizing site) and the Q_i-site (the quinone reducing site); the subscripts denoted the reactions in which protons came ***o***ut or went ***i***n to the cycle. Because reduction of quinone required two electrons, Mitchell invoked an odd process for his acceptor site, in which one electron came from the *b*-cytochromes, and one from the dehydrogenases. Even to those involved in the field, this Q-cycle idea appeared at first sight somewhat absurd – the mechanism had two sites, one apparently catalyzing the reverse reaction of the other. However, closer inspection showed that, in the context of a bifurcated oxidation of QH_2 in which the protons released appeared on the Cyt *c* side of the membrane, and a vectorial arrangement of the reactions so that the electrons traveled across the membrane to reduce quinone (and take up protons) on the other side, the new scheme provided a pretty mechanism for the proton-pumping activity. It retained from the Wikström–Berden scheme the useful explanation for oxidant-induced reduction of Cyt *b*.

The Q-cycle also helped to solve a major problem for the chemiosmotic hypothesis. To see this problem we need to recall the general mechanism that Mitchell had proposed for the proton-pumping activity of electron transfer chains: the proton-pumping loops of Figure 6. Each loop consisted of an electrogenic arm in which a membrane-embedded catalytic center transferred an electron across the membrane between a donor catalytic site at which a H-carrier was oxidized (releasing a H^+), and an acceptor site at which a second carrier was reduced (taking up a H^+ from the opposite phase). Shuttling of H-equivalents between neighboring electrogenic spans provided the neutral arm of the proton-pumping loop, and release or uptake of protons on oxidation or reduction of the H-carrier gave a stoichiometry of $1H^+/e^-$ for the overall loop activity.

So, what was the problem? For each loop, a H-carrier was needed, and there were not enough H-carriers to go around. The 'cross-over' experiments of Chance and Williams had identified a coupling site in the middle span, between Cyt *b* and Cyt c_1. For this span, only ubiquinone appeared as a candidate with suitable redox properties, yet two carriers were needed for the loops, one each for the donor and acceptor sites. By recycling the Q, the Q-cycle could use the same H-carrier for each site. The Q-cycle scheme also provided a neat explanation for the effect of antimycin, and identified the site of action as an inhibition of the reaction by which the *b*-type cytochromes reduce Q (or semiquinone, SQ) at the Q_i-site. Also, by allowing the Cyt *b* chain to span the membrane, the differential response of Cyt b_K and Cyt b_T to the coupled state could be explained in chemiosmotic terms by the

Figure 3. The group photo from the (most oddly named) 'Et: pcet: HAT: pt: 40 years of Tunneling in Biology' Meeting, organized by Les Dutton, University Pennsylvania, May 2001. Actors in the Q-cycle story, shown in bold, can be readily identified. Participants from the bioenergetics community include (*front row, from left*:) Rudi Marcus (1), Bill Rutherford (2), Rick Debus (3), Shelagh Ferguson-Miller (4), **Britton Chance** (5), Chris Moser (6), Cecilia Tommos (7), Toshi Kakitani (8), Graham Palmer (9), (*second/third row*) Peter Brzezinski (10), **Mårten Wikström** (11), Dennis Rousseau (12), Takashi Yonetani (13), Woody Woodruff (14), 'Yoshi' Yoshikawa (15), Paul Mathis (16), Tomoko Ohnishi (17), Bridgette Barry (18), Dan Nocera (19), Gary Brudvig (20), **Peter Rich** (21), Josh Wand (22), **Les Dutton** (23), **Colin Wraight** (24), Bob Cukier (25), Joshua Jortner (26), Harry Gray (27), Dave Britt (28), Charlie Yocum (29), Wilfred van der Donk (30), (*back row, left*) Frazer Armstrong (31), Bob Gennis (32), Jim Barber (33), Bruce Diner (34) and **Tony Crofts** (35).

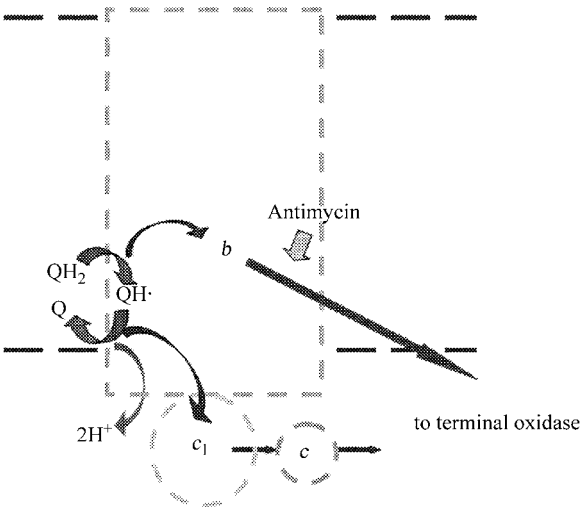


Figure 4. The bifurcated reaction. Adapted from the model proposed by Wikström and Berden (1972) so as to follow the lay-out for later Q-cycle schemes shown here.

difference in electrical potential seen by the hemes placed at different distances across the membrane.

The first exposure of the Q-cycle hypothesis to public debate was at the 1975 Fasano meeting organized by the Italian bioenergetics community. Among many interesting contributions, several turned out to be of importance in defining the debate. Paul Wood (see Crofts et al. 1975) had pointed out that, given the redox chemistry of the quinone system, the alternative sequence for the bifurcated reaction, in which the first electron from QH_2 was donated to the high potential chain, was a more natural fit with the known chemistry of quinone systems. Peter Garland (Garland et al. 1975) made three important points, all reflecting a criticism of the odd reaction Mitchell had proposed at the Q_i-site. This required that one electron to reduce the quinone should come from the dehydrogenases, and implied a close coupling between complexes. Garland pointed out (i) that the isolated Cyt bc_1 complex was

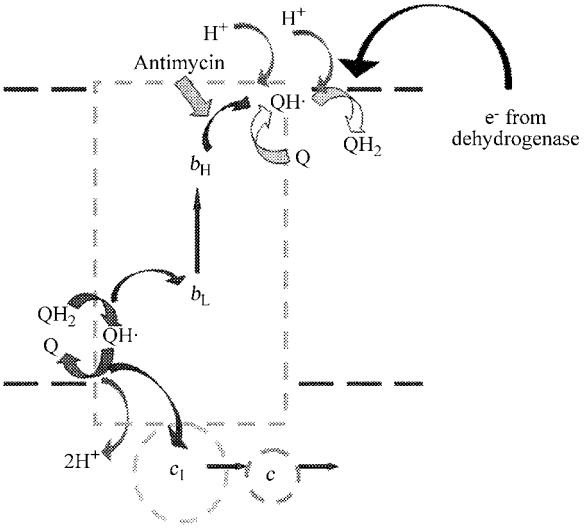


Figure 5. Mitchell's original Q-cycle (Mitchell 1975a). The Q-cycle later favored by Mitchell and many others was similar, but with the first electron transfer reaction from QH_2 to the high potential chain, leaving semiquinone as the reductant for Cyt b_L. The cyclic nature of the reaction can be appreciated by noting that the Q consumed and the QH_2 generated at the Q_i-site can be provided by (in the case of Q) or can replace (in the case of QH_2) equivalents involved in oxidation of QH_2 at the Q_o-site, by simple diffusion in the membrane phase.

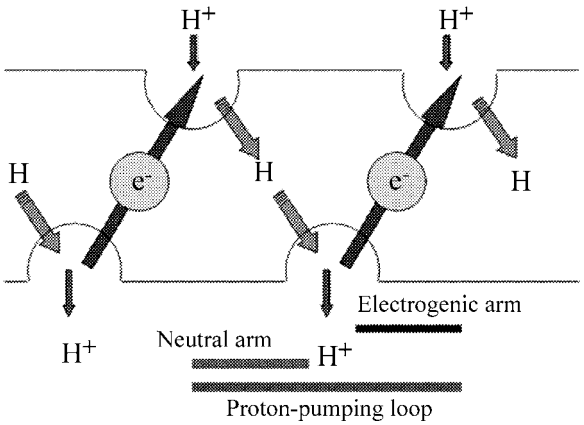


Figure 6. Mitchell's proton pumping loops (Mitchell 1961, 1966).

able to act independently (Leung and Hinkle 1975), (ii) that reversal of the complex did not require succinate dehydrogenase, and (iii) that antimycin did not inhibit the dehydrogenases. These observations indicated that the system operated through a self-contained mechanism. Garland suggested that a self-contained Q-cycle could be achieved if the oxidizing site turned over twice for a complete reaction, delivering two electrons through the *b*-cytochrome chain to the Q_i-site to generate one QH_2, with a net yield of 1 QH_2 oxidized. He called this a *modified Q-cycle*.

A third set of observations that were later to figure prominently in discussion related to the mismatch between the kinetics of reduction of Cyt *b* and Cyt *c*. The kinetics, especially in the presence of antimycin, appeared to be contrary to the expectations of a Q-cycle. In a simple interpretation, the bifurcated reaction provided electrons in equal stoichiometry to two electron transfer chains. In the isolated complex, Cyt c_1 was the terminal acceptor of the high potential chain. In the presence of antimycin, Cyt b_H was the terminal acceptor of the low potential chain. From this, one might have expected that electrons would arrive at these terminal acceptors with similar rates, and in equal stoichiometry. Tsu King (King et al. 1975) reported briefly on the pre-steady state kinetics of the isolated complex, in which a faster reduction of Cyt *b* than of Cyt *c* was observed, both in the presence and absence of antimycin.

Les Dutton (see Figure 7) and Baz Jackson (Dutton and Jackson 1972a, b; Jackson and Dutton 1972), and our lab (Crofts et al. 1972, 1974; Evans and Crofts 1974), had developed protocols for detailed kinetic measurements of the activity of the Cyt bc_1 complex *in situ* in 'chromatophores.' These were the sealed vesicles produced from the invaginated cell membrane by mechanical disruption of cells of the photosynthetic bacteria *Rhodopseudomonas* (later renamed *Rhodobacter*) *sphaeroides* and *Rb. capsulatus*. In these bacteria, the Cyt bc_1 complex was oxidized and reduced by the photochemical reaction center (RC), making it possible to activate the enzyme rapidly with a flash of light. As I discussed at the 1975 Fasano meeting, this work showed that the kinetics of

Figure 7. Left to right: Jerry Babcock, Tony Crofts and Les Dutton, on an outing from Helsinki to Tallin, Estonia, after the 7th European Bioenergetics Conference (EBEC) Meeting, 1992.

cytochrome changes could not readily be explained by the Q-cycle (Crofts et al. 1975). The rates and stoichiometries of electron transfer to the *b*- and *c*-type cytochromes, particularly in the presence of antimycin, did not show the matching expected from a simple interpretation of the bifurcated reaction.

A more detailed exploration of the Q-cycle theme came in an important review by Mitchell (Mitchell 1976) in which he discussed a number of different versions of the Q-cycle that could account for the general behavior of the mitochondrial electron transfer chain, based on the limited data available at that time. One of these was the version now adopted by most labs working in the field as the modified Q-cycle, which borrowed from Garland his ideas for a stand-alone mechanism, and from Wood the sequence of the partial reactions of QH_2 oxidation. Several other of the variants discussed in this review had, at one time or another, strong support from different labs in the field. The longest lived of these was the SQ-cycle, another mechanism that allowed the complex to operate in stand-alone mode. This was achieved by having the quinol oxidizing site catalyze two different reactions. The first was as in a conventional Q-cycle; the second used the semiquinone (SQ, hence the name) generated on oxidation of the second QH_2, as a donor to the Q_i-site, where it could contribute the second electron needed to complete the reduction of quinone. This mechanism was strongly supported by Pierre and Anne Joliot until quite recently to account for the kinetics of electron transfer and electrogenic events associated with turnover of the Cyt b_6f complex in algae (Joliot and Joliot 1994).

Because the experimental evidence available at the time did not allow distinction between these different hypotheses, in this review Peter Mitchell regarded the Q-cycle hypotheses in broad terms as covering all these possibilities, but later continued to favor a version of his original Q-cycle (updated to include Wood's sequence for the Q_o-site reactions), in which the reduction of Q at the Q_i-site was shared with a dehydrogenase. This was the version (with minor modifications) favored and discussed in most subsequent publications from other labs (cf. Slater 1981; Trumpower 1981).

Gathering of evidence

Over the 7 years from its inception in 1975, the Q-cycle and its variants were a topic for heated discussion and extensive experiment. Many workers provided experimental support for the main features of the Q-cycle [see Slater (1981) for a comprehensive discussion], but the difficulties arising from the kinetic anomalies identified in the chromatophore system were not reconciled, and similar difficulties also became better appreciated in the context of the mitochondrial chain. A major contribution came from the extensive kinetic studies by Simon de Vries, working with Jan Berden in Bill Slater's group, on the isolated beef heart mitochondrial complex, in which optical and EPR (Electron Paramagnetic Resonance) techniques were used to look at the pre-steady state kinetics of the cytochromes (by stopped-flow kinetics), and the semiquinone and Rieske *i*ron *s*ulfur *p*rotein (ISP) (by using a rapid mix/flow/freeze-quench approach and EPR), as discussed further below. The semiquinone had previously been characterized as a Q_i-site-linked species from its sensitivity to antimycin (Yu et al. 1978; De Vries et al. 1980, 1982; Ohnishi and Trumpower 1980), and its thermodynamic properties suggested a stable intermediate in the reduction of quinone at the site (De Vries et al. 1982; Robertson et al. 1984).

In the chromatophore world, studies of the kinetics of turnover of the Cyt bc_1 complex advanced rapidly. An advantage of using the chromatophore system (and other photosynthetic systems) was the ability to measure on a rapid time scale the electrical events associated with transfer of charge across the membrane. This was based on absorbance changes associated with the light-harvesting pigments. The late Jan Amesz (1934–2001) had noted that a strong absorbance change seen in *Rhodobacter sphaeroides*, which had a very low quantum requirement, was due to a small red shift of the carotenoid and bacteriochlorophyll pigments (Vredenberg et al. 1965; Vredenberg and Amesz 1967). Meg Baltscheffsky (1969) studied a similar change for carotenoids in chromatophores from *Rhodospirillum rubrum*, and showed that it was linked to the 'high energy state.' Wolfgang Junge and Horst Witt (1968), on the basis of the sensitivity to ionophores, suggested that the similar '515 nm change' observed following flash activation of chloroplasts was an electrochromic response of the accessory pigments (the so-called 'membrane voltmeter' (Figure 8 shows data for the changes seen in chloroplasts and chromatophores, taken from a nice review by Junge and Jackson (1982)). Baz Jackson and I (Jackson and Crofts 1969) demonstrated that the absorbance change of the carotenoid pigments in

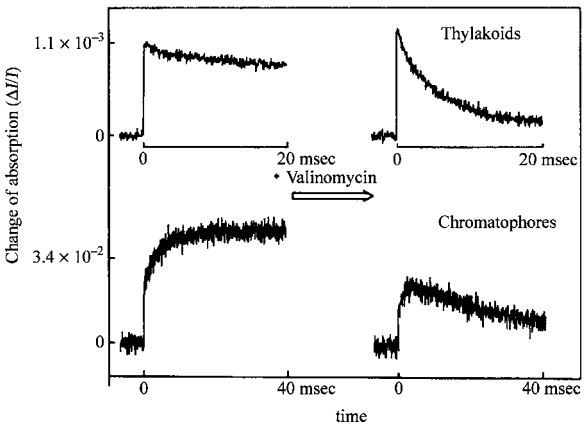


Figure 8. The kinetics of the 515 nm and carotenoid electrochromic changes in the presence and absence of valinomycin. The top pair of traces shows the effect of valinomycin in accelerating the decay of the electrochromic change following flash illumination of a thylakoid suspension (chloroplasts salt-treated so as to release the stromal contents). The bottom pair shows similar experiments with chromatophores. Note the two distinct phases of the rise kinetics. Adapted from Junge and Jackson (1982).

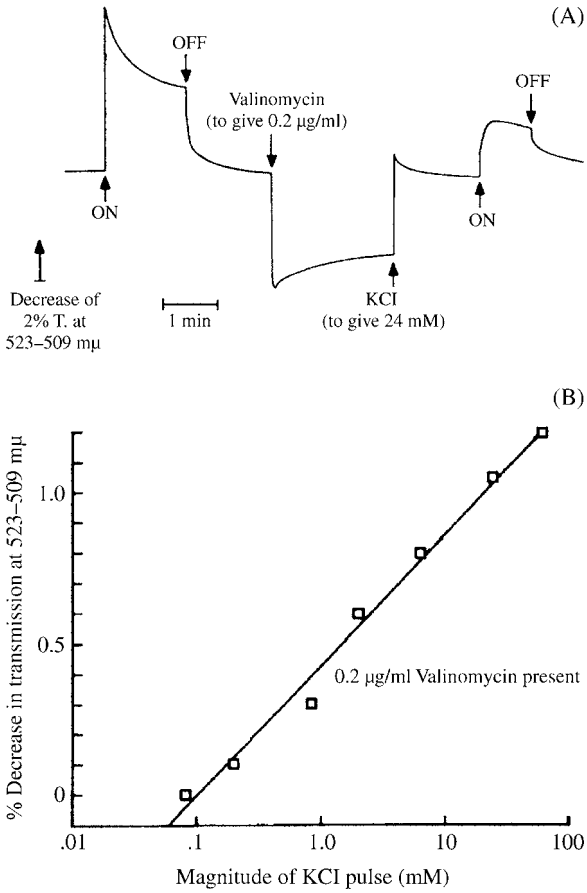


Figure 9. Calibration of the electrochromic change. (A) Kinetic traces showing the absorbance change due to the carotenoid electrochromic response on continuous illumination (on and off arrows), followed by addition of valinomycin, then KCl. (B) The absorbance change due to the electrochromic response of the carotenoids to the membrane potential was calibrated, and shown to be linear, by using K^+ gradients in the presence of valinomycin to generate known changes in $\Delta\psi$ (adapted from Jackson and Crofts 1969). Identical experiments to that shown in (A) were performed to the point of addition of KCl. At this point, the K^+-gradient had equilibrated, so that addition of KCl at different concentrations generated a membrane potential positive with respect to the inside of the chromatophores. Since the $[K^+]_{in}$ was constant between experiments, the change in membrane potential was proportional to $\log_{10}[K^+]_{added}$, from the Nernst equation: $\Delta\psi_{in\text{-}out} = (2.303RT/F)\log_{10}([K^+]_{added}/[K^+]_{in})$.

chromatophores from *Rb. sphaeroides* was proportional to the membrane potential, by calibrating the change induced when a potential was generated across the membrane by K^+ gradients operating through valinomycin (Figure 9). From the linearity of absorbance change with potential established in this work, the carotenoid change could be used to quantify the electrogenic events of the photosynthetic chain. In collaboration with Les Dutton (Jackson and Crofts 1971; Jackson and Dutton 1972, 1973), we went on to show that the kinetics of the carotenoid change following a single flash showed distinct kinetic phases. The fast components ($t_{1/2} < 50\,\mu s$, the fast phase) were well separated from a slower component – the slow phase. The fast components were observed whenever the photochemical reactions were occurring; the slow component only when the Cyt bc_1 complex was functional.

Les Dutton was an early advocate of the Q-cycle (cf. Dutton and Prince 1978; Van den Berg et al. 1979). Following earlier studies by Baz Jackson, Richard Cogdell and Roger Prince in my lab (Crofts et al. 1971a, b; Cogdell et al. 1972, 1973; Cogdell and Crofts 1974), the extensive work with Roger Prince and Katie Petty (Petty et al. 1979; Prince et al. 1982) led to a better characterization of the kinetics of electron transfer, the electrogenic processes, and the H^+-uptake reactions. The authors recognized that the kinetic matching of the electrochromic carotenoid change, and the re-reduction of the oxidized *c*-type cytochromes after flash activation through the reaction center, both measured in the absence of inhibitors, and the re-oxidation kinetics for Cyt b_H, inferred from the difference kinetics with and without antimycin, were as expected from the Q-cycle. However, the kinetic mismatch in the presence of antimycin remained intransigent. In retrospect, an understanding of these kinetic features in the context of the Q-cycle was hampered by a paucity of more biochemical infor-

mation. It was not at first appreciated that the bacterial complexes contained the full complement of redox centers found in the better-characterized mitochondrial complexes. On the other hand, the mitochondrial world could ignore these kinetic difficulties and proceeded earlier to acceptance of the Q-cycle.

An important development over this period was the characterization of the role of the Rieske ISP in the Cyt bc_1 complex. Advances in this area came from several directions. The first was the characterization of inhibitors of the Cyt bc_1 complex and the discovery of new inhibitors, leading to recognition that there were two distinct sites for binding, later to be recognized as the Q_i- and Q_o-sites of the Q-cycle. Antimycin had long been recognized as an inhibitor of Cyt *b* oxidation (Slater 1973), and in the context of the Q-cycle, this could be explained by a binding at the Q_i-site. However, the only inhibitory treatment that appeared to block the Q_o-site was incubation with British Anti Lewisite (BAL, 2,3-dimercaptopropanol), a rather nonspecific sulfhydryl reagent. BAL-treatment had been shown by Deul and Thorn (1962) to block a site different from that affected by antimycin. Although overall electron transfer was inhibited in the presence of either inhibitory treatment alone, the *b*-cytochromes could be reduced by addition of substrate. However, on addition of antimycin to BAL-treated preparations, this reduction of Cyt *b* was lost, suggesting two different inhibitor binding sites catalyzing two different pathways for Cyt *b* reduction by QH_2. Later work by Bill Slater and Simon de Vries (1980) showed that BAL-treatment destroyed the [2Fe–2S] cluster of the ISP.

This brings up an additional important line of evidence, which had come from Bernie Trumpower's work on reconstitution of activity in isolated Cyt bc_1 complex from which the ISP had been extracted. This showed that extraction of the ISP had an effect similar to that of BAL-treatment, indicating that there was at least a structural requirement for ISP for function of the BAL-sensitive site. Trumpower (1981) interpreted the result as showing a functional requirement, and as strong evidence for a Q-cycle mechanism similar to the one favored by Mitchell, but with the [2Fe–2S] center of the ISP as the immediate acceptor for the first electron from QH_2. This view was much strengthened by identification of the BAL target.

Further progress came from parallel work following the introduction of 5-undecyl-6-hydroxy-4,7-dioxobenzothiazol (UHDBT) (Roberts et al. 1978), which was shown by John Bowyer and I (Bowyer and Crofts 1978) to block electron transfer between QH_2 and Cyt *c*. Interestingly, after addition of UHDBT, the amplitude of Cyt *c* oxidation following a single saturating flash was almost double that seen in the absence of inhibitor, indicating that an invisible donor could rapidly (in $<150\,\mu s$) transfer an electron to Cyt *c* in the absence of the inhibitor (Bowyer and Crofts 1978; Bowyer et al. 1979). A similar effect of DBMIB (Trebst et al. 1970) on Cyt *f* kinetics had been observed independently by Koike et al. (1978). We suggested that this donor might be the ISP, and in subsequent work in collaboration with Dutton's group (Bowyer et al. 1981), we were able to demonstrate that this was indeed the case. An important additional finding from this latter study was that UHDBT induced a shift in the E_m of ISP, interpreted as a preferential binding of the inhibitor by the reduced form of the ISP, as now seen in the structures (Zhang et al. 1998). Trumpower and colleagues (Trumpower and Haggerty 1980; Bowyer and Trumpower 1981; Bowyer 1982; Edwards et al. 1982) later went on to characterize similar effects of UHDBT in the mitochondrial system. Also, 2-undecyl-3-hydroxy-1,4-naphthoquinone (UHNQ) was shown to behave similarly (Matsuura et al. 1983).

In addition to UHDBT, the early 1980s saw the introduction of the first of a new class of inhibitors acting at the Q_o-site, the fungal antibiotic myxothiazol (Thierbach and Reichenbach 1981). Steve Meinhardt (Meinhardt and Crofts 1982a) found that myxothiazol completely blocked the re-reduction of Cyt *c* through the Q_o-site, but did not lead to the increased amplitude of Cyt c_t oxidation after a single flash induced by UHDBT addition. Myxothiazol in excess could reverse this effect of UHDBT. We suggested that both inhibitors must share a common binding domain, displacing QH_2 from the site. However, in contrast to UHDBT, myxothiazol did not interact with the ISP. From this, and the shift in the absorbance spectrum of Cyt b_L seen on addition of myxothiazol, we could postulate that in the common binding site, myxothiazol must bind closer to heme b_L and UHDBT closer to ISP, a prediction nicely confirmed by the recent structures (Kim et al. 1998; Zhang et al. 1998; Crofts et al. 1999a, b). Later work from Gebhard von Jagow's group, with Thomas Link and Uli Brandt (Brandt et al. 1988; von Jagow and Link 1988), confirmed and extended this work. Work from this same lab also led to characterization of the inhibition induced by stigmatellin, another fungal antibiotic, shown to represent a third class of inhibitor with properties more similar to

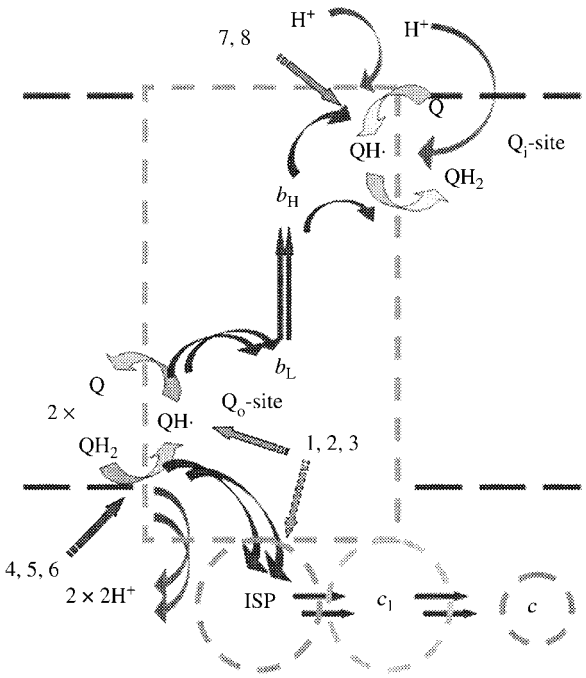


Figure 10. The modified Q-cycle. The experiments from the Crofts lab in the early 1980s provided severe constraints that limited the types of plausible Q-cycle model. This version is essentially the same as that proposed by Crofts et al. (1982a, b), and reviewed by Crofts (1985). Sites of action of inhibitors are shown as follows: 1, 2, 3 (light arrows): UHDBT, UHNQ and stigmatellin. These displace quinone species from the Q_o-site, and also interact directly through a H-bond with the ISP in its reduced form. The effect of this is to block electron transfer through the site, and also to prevent the reduced ISP from accessing Cyt c_1 (as represented by the dotted arrow). 4, 5, 6 (darker arrow): Myxothiazol, other MOA-type inhibitors, famoxadone. These also displace quinone species from the Q_o-site, but bind at the end of the quinone-binding pocket closer to heme b_L, and do not interact with the ISP. 7, 8 (intermediate arrow): Antimycin, and HQNO (and similar) displace quinone species from the Q_i-site.

UHDBT than to myxothiazol. (Sites of action of inhibitors, and a brief explanation, are shown in relation to the modified Q-cycle of Figure 10.)

A third important area of advance came from the refinement of kinetic studies in the chromatophore system. The protocols developed owed much to the use of redox potentiometry, introduced in Dutton's group in the context of work on the chromatophore system (Dutton and Jackson 1972a, b), and in collaboration with Dave Wilson and C.P. Lee for the mitochondrial chain (Dutton et al. 1970; Dutton and Wilson 1976). This work established values for the midpoint potentials of the redox centers of the photosynthetic and respiratory chains, and allowed for calculation of the thermodynamic parameters controlling the partial processes involved. As later developments were to show, important pieces of the thermodynamic jigsaw were E_m values for the two *b*-type hemes of the Cyt bc_1 complexes (see discussion of Cyt b_T above), and for the ISP (Prince and Dutton 1976). In addition, by using redox poising coupled to flash-activated kinetic spectrophotometry in the chromatophore systems, the redox dependence of partial reactions in the photosynthetic chain could be assayed, allowing the measurement of kinetic parameters as a function of substrate concentration (Crofts et al. 1972; Dutton and Jackson 1972a, b; Jackson and Dutton 1972). A critical role was later to emerge from identification in this early work of an electron donor that seemed to determine many features of the turnover. It was observed that on reduction over the E_h range 200–100 mV, a number of kinetic events increased dramatically in rate. This led to the recognition that an electron donor (initially called Z by Evans and Crofts (1974)) needed for rapid reduction of Cyt b_H, rapid re-reduction of Cyt c_t, the antimycin-sensitive H^+-uptake, and a rapid slow-phase of the carotenoid change, had the properties expected of a quinone, but had a different apparent E_m from that of the quinone pool ($\sim$140 mV compared to 90 mV).

Kinetic anomalies

By late 1981, there was neither consensus on whether a Q-cycle operated, nor any agreement among those favoring a Q-cycle about which variant was best. The controversy was encapsulated in several places. Interesting variants that allowed the Cyt bc_1 complex to function independently had been introduced by Malviya et al. (1980) and (for the Cyt b_6f complex) by Velthuys (1982). A volume to honor Peter Mitchell, edited by Vladimir Skulachev and Peter Hinkle (1981), contained articles by Slater (1981), Bowyer and Trumpower (1981), King (1981), and Konstantinov et al. (1981) showing a preponderance of support for a Q-cycle mechanism, though with several different variants. A meeting on 'The Function of Quinones in Energy Conserving Systems' was organized by Trumpower in the fall of 1980, and the updated book of the proceedings, published in 1982, contains papers favoring several different Q-cycle models, and some fairly baroque alternative schemes. These include my own, which tried to justify by other means the features well explained by the Q-cycle, in order to accommodate the apparently

intractable kinetic problems that seemed against it. Also around this time, Colin Wraight wrote a nice review (1982) in which he referred to the state of research as a Benghazi gallop, and illustrated the confusion by schemes for Q-cycle and linear pathways that could have served as choreography for the Whirling Dervishes. In this same volume, William (Bill) Cramer and Crofts (1982) reviewed the photosynthetic arena, and discussed two different Q-cycle variants for the chloroplast and chromatophore complexes, the latter model being the modified Q-cycle that my lab had recently adopted (Crofts and Meinhardt 1982; Crofts et al. 1982b). The chloroplast Q-cycle discussed was an interesting variant suggested by Bruno Velthuys (1982) in which the *b*-heme chain provided a parallel pathway for electron transfer between the Q_o- and Q_i-sites. A somewhat later contribution by Peter Rich (1984) provided a serious look at the underlying physico-chemical constraints, and careful review of several variants of the Q-cycle still considered to be in serious contention. These included the SQ- and b-cycles that he, and Mårten Wikström (Wikström et al. 1981), were enamored of at the time. Although space considerations preclude detailed discussion of further developments in the Cyt b_6f field (see G. Hauska, this issue), the early contributions of John Whitmarsh (Selak and Whitmarsh 1982; Jones and Whitmarsh 1988), and David Crowther and Geoffery Hind (1982) deserve recognition.

Although the question of the kinetic anomalies looked hopelessly confused, the groundwork for a resolution in the chromatophore system was already laid. The careful work of John Bowyer in quantifying the components of the photosynthetic chain showed that the stoichiometric ratio of reaction center (RC) to Cyt bc_1 complex in *Rb. sphaeroides* was close to 2 (Bowyer et al. 1979, 1981; Crofts et al. 1982a). Bowyer had also demonstrated that Cyt b_L was a player in the chromatophore chain, and could be rapidly reduced on a second flash in antimycin inhibited chromatophores (Bowyer et al. 1981; Crofts et al. 1982a). Paul Wood had identified Cyt c_1 as a component of the high potential chain through biochemical work (Wood 1980) and we had demonstrated its involvement kinetically (Crofts et al. 1982a; Meinhardt and Crofts 1982b). Together with the work showing involvement of the ISP, these contributions demonstrated that the bacterial complex had the same redox centers as the mitochondrial complex.

Why did the kinetics appear to be in contradiction to the Q-cycle? In steady-state experiments, the kinetic behavior of the complex seemed consistent with the Q-cycle (cf. Trumpower 1981; Bowyer 1982). The difficulties arose from measurement of the pre-steady-state kinetics of the complex. Several different approaches had been used, either with the isolated complex or the complex *in situ*, all showing basically the same problem; when Cyt c_1 and Cyt b_H were oxidized, and QH_2 was available to reduce them, the kinetics of reduction of the cytochromes did not match, as exemplified in the early work using photosynthetic bacteria (cf. Crofts et al. 1975). An example will explain the difficulties. In Figure 11 (adapted from Bowyer 1979) the kinetics of cytochrome changes measured in the absence (top) and presence (bottom) of antimycin are shown. The suspending medium was poised at an ambient redox potential of E_h 100 mV, so that the high potential chain was reduced, the low potential chain oxidized, and the Q pool ~30% reduced. The traces are normalized to the same concentration scale. Interpretation of these traces in the context of existing Q-cycle schemes appeared problematic for the following reasons:

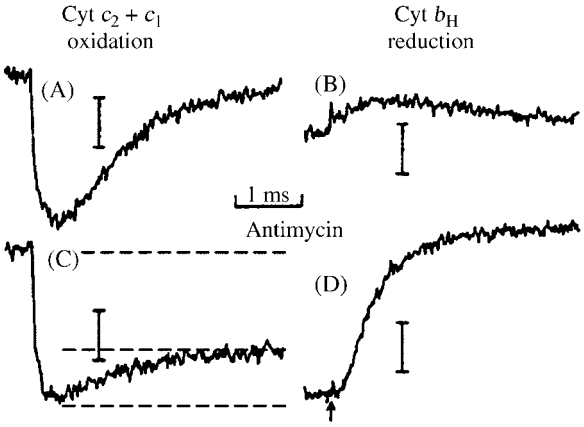


Figure 11. The kinetics of cytochrome changes, measured in chromatophores form *Rb. sphaeroides*, showing the mismatched kinetics of Cyt b_H and Cyt c_t. The traces show the changes in redox state of the cytochromes on excitation with a single saturating flash of short (~5 μs) duration. Cyt c_1 plus c_2 (Cyt c_t, left, measured at 551–542 nm), and for Cyt b_H (right, measured at 561–569 nm), are shown in the absence (top) or presence (bottom) of antimycin at 10 μM (the traces are adapted from Figure 7.5 of Bowyer 1979).

(1) Following a saturating flash, the Cyt c_1 plus Cyt c_2 (Cyt c_t) was rapidly oxidized (Figure 11, traces (A) and (C)). Resolution on a more rapid time scale (not shown) indicated a rapid phase (<10 μs) and a slower phase (~150 μs), later shown to represent the oxidation of Cyt c_2 by RC,

then of Cyt c_1 by Cyt c_2 (Meinhardt and Crofts 1982b). This oxidation of the high potential chain generated a system similar to that used as the starting point in experiments with the isolated mitochondrial complex (the fully oxidized complex), except that QH_2 was available immediately in the partly reduced quinone pool of the chromatophore membrane.

(2) In the absence of antimycin, re-reduction of Cyt c_t occurred with $t_{1/2} \sim 2$ ms and then was completed after ~10 ms. The complete reduction indicated that all the oxidizing equivalents from the RC had been consumed (trace A). Over this time scale, Cyt b_H was transiently reduced and oxidized (trace B). The kinetics observed here are those of the 'oxidant-induced reduction' mentioned above, and the general behavior is that expected in a Q-cycle.

(3) In the presence of antimycin, the kinetics of reduction of Cyt b_H were more rapid ($t_{1/2} \sim 0.65$ ms), and the extent after one flash was nearly maximal (trace D) – compatible with reduction of Cyt b_H in almost all of the Cyt bc_1 complexes functionally connected to the photochemical reactions. The increased amplitude and rate of reduction reflected the enhancement of 'oxidant-induced reduction of Cyt b_H' on addition of antimycin previously seen in mitochondrial work. However, there was no corresponding kinetics for Cyt c_t reduction (trace C). The traces show a truncated re-reduction kinetics of Cyt c_t so that a substantial fraction (~65%) remained oxidized at 10 ms after the flash.

(4) Inspection of the classical Q-cycle scheme (Scheme 2, Figure 5) shows that (at $E_h \sim 100$ mV, where Cyt b_H is almost completely oxidized) after photoactivation with two equivalents of oxidant, both chains should contain two equivalents of acceptor. In a simple analysis, there appears to be no reason why the Q_o-site should not turnover twice to fill both pools, even in the presence of antimycin. Why was this not seen in the reduction of Cyt c_t (trace C)? Why was no reduction of Cyt b_L seen under these conditions after one flash (not shown here, but readily demonstrated)? (Bowyer et al. 1981).

(5) In the bifurcated reaction of the Q-cycle, electrons should be delivered simultaneously to the high and low potential chains. In a simple interpretation, in the presence of antimycin, the electrons might be expected to reach the terminal acceptors (Cyt b_H and Cyt c_1) with the same kinetics, and in equal stoichiometry. Clearly, they did not [traces (C) and (D)].

(6) In any Q-cycle mechanism, the rate of oxidation of QH_2 could not be slower than the electron delivery through the bifurcated reaction to Cyt b_H, as revealed in the reduction kinetics in the presence of antimycin (trace D). If the bifurcated reaction occurred with a relatively unstable semiquinone, as required to match the potentials of the two chains, the two electron transfers would be effectively concerted. From this, the rate of re-reduction of Cyt c_t in the absence of antimycin should reflect the rate of QH_2 oxidation. However, the rate observed was slower by a factor of ~3 (trace A) than Cyt b_H reduction in the presence of antimycin (trace D).

These apparent kinetic anomalies were the basis of my own reluctance to accept that the Q-cycle provided an adequate mechanistic account.

Similar anomalies were apparent in work on the mitochondrial complexes. Following the early contribution from Tsu King's group, in which Chan-An Yu was a participant (King et al. 1975), detailed measurements of the kinetics of the cytochromes, semiquinone and ISP were initiated by Simon De Vries, working with Jan Berden in Bill Slater's lab (De Vries et al. 1979, 1982, 1983). The approach was to use rapid mixing of a quinol substrate with the fully oxidized complex, and to measure the kinetics of reduction of the redox centers. For the cytochromes, a conventional stopped flow apparatus was used. For the EPR detectable components (the reduced ISP, semiquinone, the oxidized cytochromes), a rapid-mix/freeze-quench method was used. The kinetics using this latter approach could be correlated with the cytochrome changes by measuring the latter using reflectance spectrophotometry. This extensive study represents a *tour de force* that still ranks as the most complete set of data available. This work, the earlier report from King et al. (1975), and similar studies in other labs (cf. Tsai et al. 1983), showed several important features.

(1) In the kinetics measured in the absence of inhibitors, Cyt *b* was reduced with multiple phases, which were variable depending on starting conditions, but included a rapid reductive phase. The Cyt *b* reduction kinetics seen in the presence of myxothiazol or after BAL-treatment showed the same rapid reductive phase as seen in the absence of inhibitor.

(2) In the presence of antimycin, the rate was slower, and the kinetics simpler. When wavelengths appropriate for Cyt b_H were used, the kinetics were fairly monotonic.
(3) In the absence or presence of antimycin, the kinetics of Cyt c_1 reduction were slower than those of Cyt *b*, and of lower amplitude. In particular, the kinetics in the presence of antimycin were much slower than the Cyt *b* reduction in the absence of antimycin.
(4) In the absence of antimycin, the Q_i-site semiquinone appeared with kinetics similar to those for reduction of Cyt *b*. Neither the rapid reduction of Cyt *b*, nor the appearance of semiquinone, was inhibited by BAL-treatment or myxothiazol addition.
(5) On reduction of the fully oxidized complex, the signal of the reduced ISP appeared with kinetics similar to those of Cyt c_1 reduction.

Interpretation of these results was again difficult in the context of the simple Q-cycle scheme. Some of the difficulties, in particular the failure to match the kinetics of reduction of Cyt c_1 and Cyt b_H in the presence of antimycin, were similar to those observed in the chromatophore system. Other aspects, in particular the multiphasic kinetics of Cyt *b* reduction in the absence of inhibitors, were peculiar to the experimental approach. For this latter case, the fact that similar rapid kinetics were observed in the presence of myxothiazol, or after BAL-treatment, demonstrated that reduction of Cyt *b* through the antimycin-sensitive site was more rapid than that through the BAL/myxothiazol-sensitive site (Slater 1981; De Vries et al. 1983). This antimycin sensitivity showed that the most rapid phase of the multiphasic kinetics was a Q_i-site function. However, the results left open the question of the rate of oxidation of QH_2 at the Q_o-site in the absence of antimycin. Was the rate equal to or faster than that observed through the reduction of Cyt *b* in the presence of antimycin? In either case, the slow and/or incomplete reduction of Cyt c_1 (or Cyt c_t in the chromatophore system) was clearly contrary to expectations from a simple interpretation of the Q-cycle mechanism.

The complexities were compounded by an apparent proliferation of redox species in the Cyt bc_1 complex. Redox titrations had identified at least three Cyt *b* species (Dutton and Jackson 1972a, b; Dutton and Wilson 1976). The low potential component, Cyt b_L (also known as Cyt *b*-566, or Cyt b_T) had two peaks, and some labs reported that these appeared to behave independently, suggesting a total of four *b*-heme components. The ISP also showed several forms, depending on the state of the quinone pool. De Vries et al. (1983) had proposed an ingenious but elaborate double Q-cycle to accommodate these different species.

The modified Q-cycle

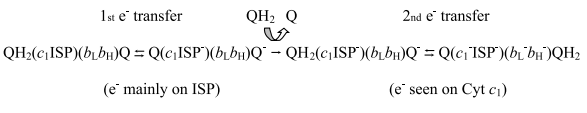
The process by which I changed my point of view with respect to the Q-cycle was an epiphany of sorts, but also somewhat salutary. A bright young undergraduate student, Kevin Jones, was doing a research project in the lab involving measurements of the kinetics of the Cyt bc_1 complex. Sometime around mid October 1981, he asked me if I could once again explain why the kinetics we were observing were inconsistent with the Q-cycle. With a sigh, I turned to the blackboard, drew up the standard Q-cycle scheme, and started saying 'Well, Kevin – it's pretty simple. We expect the electrons in these two chains to end up in Cyt b_H and Cyt c_t, ...' at which point I suddenly realized that things were not that simple. I was reiterating ideas ingrained from an earlier period, before we had all the new information about stoichiometry and thermodynamic properties of components, and the involvement of Cyt b_L, Cyt c_1, and the Rieske ISP. To my shame, I had neither thought through the implications in terms of the equilibrium constants involved, nor taken account of the stoichiometric consequences. What stopped me in mid flow was the realization that three new factors had to be included:

(1) The stoichiometry determined for our chromatophores prepared from *Rb. sphaeroides* Ga strain was always close to 2RC:1 Cyt c_2:1Cyt bc_1 (Crofts et al. 1982). As a consequence, two oxidizing equivalents were generated for each Cyt bc_1 complex following a saturating flash. The kinetics of Cyt c_t changes showed that all Cyt *c* oxidized after the flash was re-reduced within 10 ms if the Q-pool was initially partly reduced (Figure 10). To account for this in a Q-cycle, the Q_o-site would have to turnover twice.
(2) From the E_m values of Cyt c_1, ISP, Cyt c_2, and the RC donor, P, the equilibrium constants determining distribution of electrons and oxidizing equivalents (electron 'holes') in the high potential chain could be calculated. On generation of 2 P^+ by a flash, the holes would end up in the lower potential components. Because E_m(ISP) (~300 mV)

was higher than E_m(Cyt c_1) (~270 mV), the latter would be preferentially oxidized as holes were introduced into the initially reduced high potential chain. Conversely, ISP would be preferentially reduced as electrons entered the oxidized chain as the Q_o-site turned over.

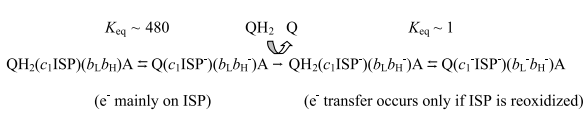
(3) The equilibrium constants for the bifurcated reaction at the Q_o-site could also be calculated. In the presence of antimycin, it was obvious that the first quinol oxidized would experience a different value for K_{eq} than the second quinol. This is because oxidation of the first quinol would consume the most favorable acceptors in each of the two chains – Cyt b_H in the low potential chain and ISP in the high potential chain – leaving the less favorable acceptors, Cyt b_L and Cyt c_1, for the second QH_2.

How did these affect the discussion? The two turnovers of the Q_o-site raised the question of how the electrons introduced in the first and second turnover distributed themselves – provoking consideration of the second and third points above. Because the equilibrium constant favored reduction of ISP over that of Cyt c_1, the *first* electron from QH_2 arriving in the high potential chain (after oxidation by 2 P^+) would go mainly to ISP, and therefore *would not be seen* in the Cyt c_t kinetics. The kinetics of reduction seen in the absence of antimycin must therefore reflect predominantly the electron arriving from the *second* turnover of the Q_o-site. On the other hand, the rate of reduction of Cyt b_H in the presence of antimycin must reflect the first QH_2 oxidized. This would explain why the reduction of Cyt b_H was faster than the re-reduction of Cyt c_t [see simplified reaction Equation (1) below].



(1)

What about the small amplitude of Cyt c_t reduction in the presence of antimycin? For the first electron, going to Cyt b_H and ISP, the equilibrium constant was favorable (K_{eq} ~ 480) Calculation of K_{eq} for the oxidation of the second QH_2, using $E_m(b_L) \sim -90$ mV and E_m (Cyt c_1) ~ 270 mV gave a value of ~1. Since the quinone pool was only 30% reduced at the E_h of 100 mV in the experiment of Figure 11, and, after the first turnover had reached completion, the ISP was 75% reduced, further reduction would be severely limited by the low equilibrium constant. In effect, the Q_o-site was restricted to one turnover in the presence of antimycin under these conditions. The fractional reduction of Cyt c_t seen in the presence of antimycin was therefore just what was expected from the distribution of the one electron in the high potential chain [see reaction Equation (2)]!



(2)

A frantic few days of calculation and a refresher course on the Q-cycle literature followed, during which it became apparent that the values we had measured were perfectly consistent with a Q-cycle, but only one in which the mechanism was highly constrained. The resulting mechanism is shown as Scheme 4 (Figure 10), and I outlined the main points in a letter to Peter Mitchell (copied to many colleagues in the field) in early November 1981. The mechanism (Crofts and Meinhardt 1982; Crofts et al. 1982b) was similar to the one proposed by Garland et al. (1975), and also to one of the options Mitchell had discussed in his 1976 review. The scheme also incorporated the suggestion of Wood about the sequence of the semiquinone half-reactions. Since the resulting mechanism was substantially different from the models being considered in other labs, I borrowed from Garland the name modified Q-cycle. The new mechanism explained with satisfactory parsimony all the things that had previously appeared contradictory, and seemed also to account quite well, with modification of equilibrium constants to take account of differences in E_m values and the different quinol donors used, for the anomalies observed in the kinetic work on mitochondrial complexes.

Over the next few years, we were able to demonstrate the main features of the hypothesis (reviewed in Crofts 1985). Important among these were:

(a) The availability of two acceptors in each chain in the presence of antimycin, and the effect of the small equilibrium constant for oxidation of the second QH_2. These first two features could be demonstrated by measurement of the dependence of the extent of Cyt b_H and Cyt b_L reduction in the presence of antimycin on flash number and on ambient potential (Meinhardt and Crofts 1983), and the amplitude of Cyt c_1 and Cyt c_2 oxidation in the presence of myxothiazol and/or UHDBT (or UHNQ) (Meinhardt and Crofts 1982a, b; Meinhardt 1984). We showed that the amplitudes

could be well fit by computational modeling of the distribution of electrons in the high and low potential chains to be expected from the equilibrium constants calculated from the measured E_m values.

(b) The double turnover of the Q_o-site, implicit in the Cyt c_t re-reduction kinetics in the absence of antimycin, could be confirmed by measurement of the kinetics and amplitude of the slow phase of the carotenoid change (Crofts et al. 1983; Glaser and Crofts 1984). According to the mechanism, the slow phase must reflect the electrogenic processes accompanying electron transfer from the Q_o-site across the membrane to the Q_i-site. In the absence of antimycin, the slow phase should represent the electric work done moving two charges (from the two turnovers of the Q_o-site) across the full width of the membrane. In the presence of antimycin, with the turnover constrained to oxidation of 1 QH_2, the change should represent the transfer of one charge, and also the location of heme b_H in the low dielectric phase. Our results, based on comparison of the kinetics of the slow phase with those of Cyt b_H, measured under different conditions of inhibition and redox poise, showed unambiguously that the full electrogenic process in the uninhibited complex was due to two successive turnovers of the Q_o-site. Furthermore, the same rate-limiting step was observed in the presence or absence of antimycin.

(c) The successive turnovers raised the question of the rates of exchange of Q and QH_2 at the Q_o-site. This was answered by our recognition of the second-order nature of the kinetics of QH_2 oxidation at low [QH_2], and the saturation of rate at higher [QH_2] – the site behaved as expected for an enzyme whose substrate was bound from the lipid phase (Baccarini-Melandri et al. 1982; Crofts et al. 1983; Snozzi and Crofts 1984, 1985; Venturoli et al. 1986, 1988). We were able to explain the characteristics of the component Z in terms of the preferential binding of QH_2 to form the enzyme–substrate (ES-) complex (Crofts and Wang 1989).

(d) We extended our understanding of the electrogenic processes by identifying partial processes associated with reduction of Cyt b_H through the Q_i-site, and demonstrated that heme b_H was located so that the electrogenic events associated with reduction and oxidation of this center each contributed about half of the full span (Glaser and Crofts 1984, 1987; Glaser et al. 1984).

(e) We later demonstrated that the rate limiting step under conditions of substrate saturation was the oxidation of QH_2 bound in the ES-complex. This partial reaction was the location of the highest activation barrier (Crofts and Wang 1989).

(f) The paradox of the multiple *b*-type cytochromes was resolved. As previously shown from extensive biochemical work, and now emphasized by the structures (Xia et al. 1997; Zhang et al. 1998), the protein contains only two heme *b* centers. Full-spectrum redox titration and kinetic resolution of the native complex in *Rb. sphaeroides* had showed that both peaks of Cyt b_L behaved the same, and likely represented a single component (Meinhardt and Crofts 1983). The third component with $E_{m.7} \sim 150$ mV, seen in redox titration of complexes from bacteria and mitochondria performed *in situ*, or on the isolated complex, continues to be of interest. The component was lost in the presence of antimycin, suggesting a link to occupancy of the Q_i-site (Meinhardt and Crofts 1984). Several different mechanisms have been proposed in which the potential of heme b_H is modified by differential interaction with different bound species of the Q/SQ/QH_2 system at the Q_i-site, but no consensus has been reached as to how this is affected (Salerno et al. 1989; Rich et al. 1990; Crofts et al. 1995).

(g) Using the electrochromic carotenoid change, and careful correction of the cytochrome and RC absorbance changes, we measured the changes in poise of the reactants of the chain as the system approached the coupled steady-state on continuous illumination or with a series of saturating flashes (Chen 1989; Chen and Crofts 1990; Crofts et al. 1990). The results showed that in the steady-state, the electron transfer reactions were in quasi-equilibrium with the proton gradient, but only if the reactions of the modified Q-cycle provided the mechanism.

Members of the Crofts group during this period can be seen in Figure 12.

Recent developments

The development of the modified Q-cycle is an ongoing work, now greatly stimulated by the availability of structures. Insights from the structural studies have been reviewed extensively elsewhere (Crofts and

Figure 12. Members of the Crofts lab in the early 1980s. *Top* (*left to right*): John Bowyer and Steve Meinhardt. *Middle* (*left to right*): Mario Snozzi, Elzbieta Glaser, and Tony Crofts. *Bottom* (*left to right*) (circa 1987): Zhenggan Wang, Mike Kulikowski, Shinichi Taoka (front), Yue Chen (back), Steve Van Doren, Kathe Andrews, Tony Crofts, Jim Fenton, Dave Kramer, Omar Adawi, Chris Yerkes, and Howie Robinson. My thanks to John Bowyer and Steve Meinhardt, who provided photos in the top two rows.

Berry 1998; Kim et al. 1998; Berry et al. 2001; Darrouzet et al. 2001; Hunte 2001). However, it is interesting to note that the structures have provoked a revival of the issue of anomalous kinetics. To understand why this should be, we need to note that the structures introduced a big surprise: the extrinsic head of the ISP was found in several different positions in different crystals (Zhang et al. 1998), including eight different configurations in seven native structures [one from Iwata et al. (1998) with two positions in the same crystal!]. Consideration of the distance dependence of electron transfer (Moser et al. 1995) made it obvious that no single position could allow the electron transfer rates from QH_2 and to Cyt c_1 observed experimentally (Zhang et al. 1998; Crofts et al. 1999b). This led to the conclusion that the ISP head must move during catalysis. Effectively, the extrinsic head acts like a second substrate at the Q_o-site. After formation of an ES-complex with QH_2 in the Q_o-site of the Cyt *b* subunit, and transfer of a H from QH_2, the extrinsic

head moves ~23 Å through a rotational displacement to deliver an electron to Cyt c_1, and release H^+, at a separate catalytic interface on Cyt c_1.

The movement of the ISP as a prerequisite for catalysis has now been demonstrated through both biophysical and mutagenesis approaches from several labs (reviewed in Darrouzet et al. 2001). This dynamic role of the ISP introduced questions about how the observed kinetics might be affected by the movement. In addition to the electron transfer reactions, factors involved are the restricted diffusion imposed by the tethering N-terminal 'tail,' and on and off rate constants for the different binding processes. This has raised the possibility of an alternative explanation for the kinetic disparity between the reduction rates of the *b*- and *c*-type cytochromes discussed above – that the movement is limiting (cf. Hansen et al. 2000). This reminds me of a remark that Peter Mitchell made to me, *apropos* the kinetic anomalies, at a Gordon Conference around 1981 – 'Don't you think the electron might be getting hung up on the Rieske?' – a prescient observation, though I never knew whether Peter was thinking of a kinetic or a thermodynamic hang-up. Several labs have recently adopted the idea that the Cyt c_1 reduction kinetics are slower than those of Cyt b_H because the movement of the reduced ISP from its site of reduction close to the Q_o-site to its site of oxidation close to Cyt c_1 is the rate limiting step (Hansen et al. 2000; Yu et al. 2002).

I believe these authors have failed to take account of the simpler explanation for the apparent kinetic anomalies provided by the modified Q-cycle mechanism. In our explication of the modified Q-cycle, the arguments were based on electron distribution determined by the equilibrium constants calculated from the E_m values. In formulating these arguments, several implicit assumptions were made. The kinetic behavior was well explained without invoking any special modifications to equilibrium constants by interactions between centers, or between different sites. It was assumed that equilibration of the ISP and of Cyt b_L with their reaction partners was much faster than the limiting oxidation of QH_2. In line with this, our earlier results had shown that the electron transfer from the reduced ISP to Cyt c_1 was very rapid ($<10\,\mu s$), that electron transfer from heme b_L to b_H was rapid ($<100\,\mu s$), and that the oxidation of bound QH_2 was rate limiting ($\sim 750\,\mu s$) (Crofts and Wang 1989). This later reaction was also the partial process with the highest activation barrier. The kinetic anomalies were therefore accounted for in terms of thermodynamic distribution, not kinetic limitation. Our own more recent experiments have shown unambiguously that in the chromatophore system the movement of the ISP is not the rate limiting step, either kinetically, or in terms of activation barriers (Hong et al. 1999; Crofts et al. 2003). These conclusions have been confirmed by work from Frank Millett's lab, in which the same reactions have been explored on a rapid time scale through photoactivation of the isolated enzymes from both mitochondria and *Rb. sphaeroides* using bound ruthenium complexes (Engstrom et al. 2002). From our results and analysis of the Cyt bc_1 complex *in situ* in chromatophores (Crofts et al. 2003), it would seem likely that the thermodynamic (distribution) factors also could provide an adequate explanation for the kinetic effects seen in the pre-steady state work with mitochondrial complexes. A precise accounting is made difficult by uncertainties in the thermodynamic parameters, both in the E_m values for the redox centers in mitochondria from different species and those arising from use of artificial donor quinones with E_m values that differ markedly from the native ubiquinone. More detailed studies under well-controlled conditions from the labs working on the mitochondrial complexes are clearly needed to answer these questions.

The modified Q-cycle accounts quite neatly for the data, and continues to provide a simple basis for further exploration. The structures provide a firm basis for detailed understanding at a molecular level, and research in this direction has been a major theme of much recent work (reviewed in Crofts 2004a, b). Other directions that look interesting are questions arising from the dimeric nature of the complex. The modified Q-cycle was formulated in the context of a monomeric mechanism, and independent sites, but there are some tantalizing indications that things might be more complicated, and suggestions, and even structural evidence, that some interaction between Q_o- and Q_i-sites might play a role (Iwata et al. 1998; Hunte et al. 2002; Lange and Hunte 2002; Trumpower 2002). It will be interesting to see if, in addition to their connection through the *b*-heme chain (Figure 10), the two Q-sites communicate across the membrane through allosteric interaction, or if the two monomers communicate across the dimer interface. In both cases, complications might be expected from coulombic interactions (to modify local potentials), as has been seen between hemes b_H and b_L (Shinkarev et al. 2001). In addition to simple electron transfer, there is the possibility of allosteric interactions within or between monomers (Trumpower 2002). Distinguishing

between the possibilities will present a serious experimental challenge, complicated further by the inevitable heterogeneity of nanoscale vesicular systems (Crofts et al. 1998).

Finally, the recognition that the Cyt bc_1 complex is a major contributor to the production of reactive oxygen species (ROS) has placed work on the complex at center stage in this important medical area (Skulachev 1996; Muller 2000, Chen et al. 2003). The ROS are thought to play a major role in cellular aging through damage to both DNA and protein. The most sensitive target is mitochondrial DNA, for which the repair mechanism in animals is less sophisticated than that in the nucleus. Since the mitochondrial DNA encodes several subunits of the respiratory chain, including Cyt *b*, mutagenesis may progressively increase ROS effects and exacerbate their destructive effects over an individual life span. It seems clear that the semiquinone generated at the Q_o-site acts as a donor in production of the 1-electron reduced superoxide that is a progenitor of ROS. The rate of generation of superoxide in the presence of antimycin (which maximizes the rate by inhibiting removal of the semiquinone) is about 80% of that required to account for all the ROS production in the cell. However, the physiological rate is likely considerably less. It is determined by the inhibitory effect of the back-pressure from the proton gradient, which is itself under metabolic control. Nevertheless, production of superoxide at the Q_o-site is clearly of importance, and may also contribute to the damage in some mitochondrial myopathies (Fisher and Meunier 2001). Presumably, the mechanism of the site must reflect a design by evolution that has minimized this harmful side-reaction, and it will be interesting to see how this has been achieved. Studies of mutant strains to see which residue changes lead to excess production will be a powerful tool for exploration of this problem.

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