A new era for electron bifurcation

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Electron bifurcation, or the coupling of exergonic and endergonic oxidation-reduction reactions, was discovered by Peter Mitchell and provides an elegant mechanism to rationalize and understand the logic that underpins the Q cycle of the respiratory chain. Thought to be a unique reaction of respiratory complex III for nearly 40 years, about a decade ago Wolfgang Buckel and Rudolf Thauer discovered that flavin-based electron bifurcation is also an important component of anaerobic microbial metabolism. Their discovery spawned a surge of research activity, providing a basis to understand flavin-based bifurcation, forging fundamental parallels with Mitchell's Q cycle and leading to the proposal of metal-based bifurcating enzymes. New insights into the mechanism of electron bifurcation provide a foundation to establish the unifying principles and essential elements of this fascinating biochemical phenomenon.

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In the simplest terms, electron bifurcation effectively couples exergonic and endergonic oxidation-reduction reactions in an overall thermodynamically spontaneous process [1••,2]. Based on Marcus theory, rates of biological electron transfer reactions are governed by the difference in reduction potentials of the donors and acceptors, their mutual distance, and the nature of the physical medium between them, in particular its response to the change in charge – the reorganization energy [3]. Electron transfer reactions are fastest when the thermodynamic driving force matches the reorganization energy and when the separation distance between donor and acceptor is short. Electrons can flow 'uphill,' but that comes with a kinetic penalty. Interestingly, when the thermodynamic driving force exceeds the reorganization energy, the reactions slow down; this is known as the 'inverted regime' for electron transfer. Electron bifurcation exploits these characteristic properties of electron transfer to direct electrons down spatially separated and energetically distinct landscapes to low and high potential cofactors. Most importantly, the electron flow along the two branches is highly concerted.

Harnessing the free energy of electrochemical potential

Aerobic respiratory chains in biology couple the oxidation of substrates with the reduction of oxygen to generate a transmembrane proton motive force (Figure 1). They are

found in the mitochondria of eukaryotes [4], as well as in diverse lineages of Bacteria and Archaea. Transmembrane electron transfer is a primary mechanism underpinning energy conservation in living systems. The hallmark of electron transfer chain function, and the essence of Mitchell's 'chemiosmotic hypothesis', is that harnessing the free energy of electron transfer reactions down an electrochemical potential gradient drives proton translocation across a membrane [5]. The transmembrane proton gradient essentially stores free energy in an entropic form (a concentration gradient) as well as in an enthalpic form (if the membrane has a nonzero resting voltage). The redox chains thus convert the electrochemical potential energy of low potential substrates, such as NADH and $FADH_2$, to potential energy in the form of the chemiosmotic potential. In biological energy transduction, the chemiosmotic potential is used to drive ATP synthesis. At the center is an elegant dance of electrons and protons that is of paramount importance to life in general and is driven by electrochemical potential energy.

In addition to the chemiosmotic hypothesis, Mitchell is credited with the discovery of 'electron bifurcation' in his elucidation of the 'Q cycle' that links oxidized quinone (Q) to reduced quinol (QH_2) in complex III [6,7] (Figure 2). Complex III is an electron transfer-driven proton pump of the aerobic respiratory chain, oxidizing QH_2 equivalents produced by respiratory complexes I and II and reducing cytochrome c, which provides the link to complex IV (cytochrome c oxidase) and oxygen reduction. Mitchell found that complex III functions rather differently from the other respiratory complexes: a fraction of the electron flux and electrochemical potential is diverted to catalyze a

seemingly illogical reaction coupling the oxidation of QH_2 near the exterior of the membrane to the reduction of Q near the interior of the membrane. The reaction, however, rings of sheer brilliance when considering the mobility of Q/QH_2 in the membrane, and its ability to generate a proton gradient through this process.
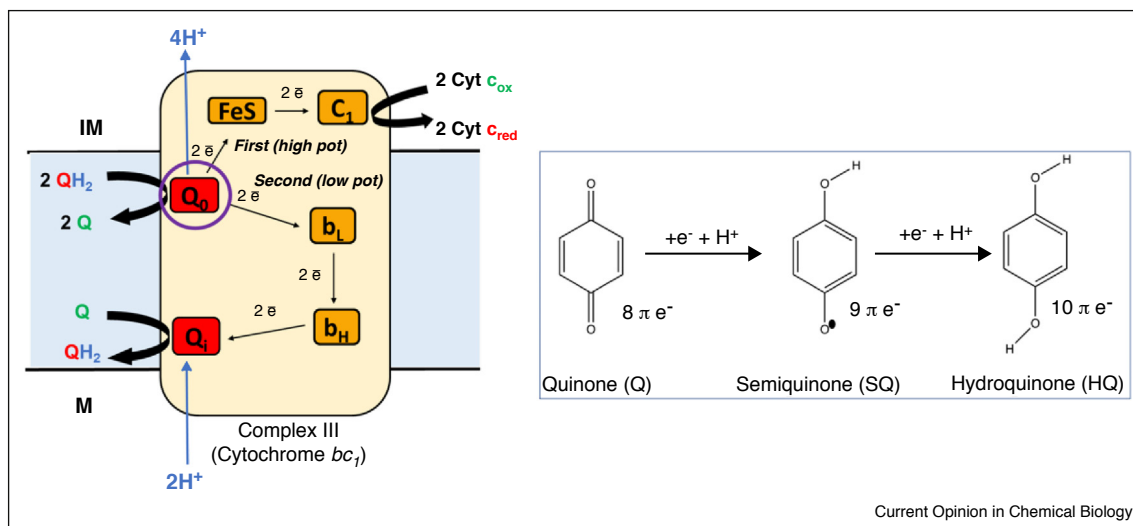
Q cycle and the birth of electron bifurcation

An additional puzzling element of the 'Q cycle' reaction is reconciling the thermodynamics of its two-electron chemistry. Complex III-dependent QH_2 oxidation leads to the simultaneous reduction of both cytochrome c (downhill thermodynamically) and the reduction of a b-type cytochrome (uphill thermodynamically), along two different spatially separated redox pathways (Figure 2). 'Electron bifurcation' was subsequently proposed to reconcile the two paths within the thermodynamics of the 'Q cycle,' and the requirement that all spontaneous reactions at constant pressure require negative values for the Gibbs free energy. Two half reactions were proposed in which the initial one involves the exergonic electron transfer of a single electron to an iron-sulfur cluster, which in turn transfers an electron to cytochrome c. In the second half reaction, the other electron traverses an endergonic path to the reduction of cytochrome b and then Q within complex III. Diffusion of the quinol through the membrane and subsequent oxidation on the outside of the membrane by complex III completes this elegant cycle of redox-driven proton pumping. A key to the mechanism of electron bifurcation is that the exergonic transfer to the iron-sulfur cluster is the production of an energetic intermediate species on Q with a sufficiently low potential to drive the reduction of cytochrome b. The overall energy

Figure 1
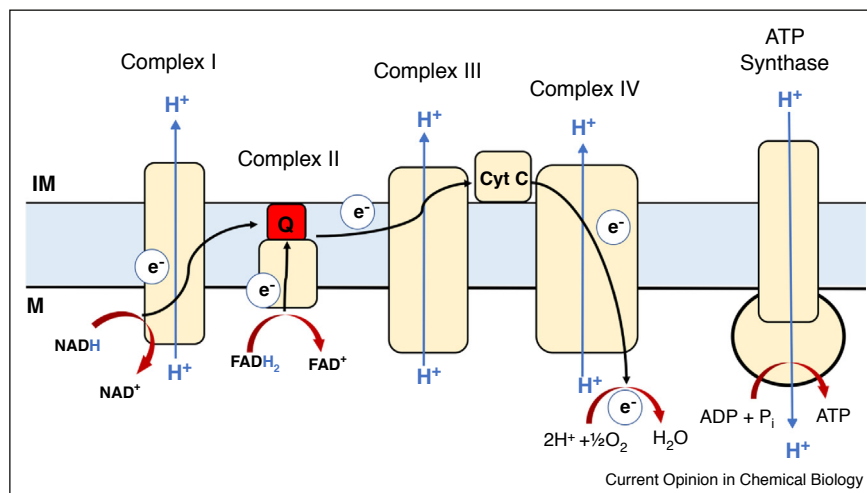
The electron transport chain illustrating the electron transfer from the unifying currency of reducing equivalents in biology NADH and $FADH_2$ through three proton translocating respiratory complexes, I, III, and IV from the mitochondrial matrix (M) into the intermembrane space (IM) resulting in the eventual reduction of oxygen to water and the generation of proton motive force that is harnessed to drive ATP synthesis through an ATPase.

Figure 2

Electron transfer in respiratory complex III and the 'Q cycle' illustrating the transfer of electrons from QH_2 simultaneously to an FeS cluster and a b-type cytochrome in an electron bifurcation reaction. The electron transferred to the FeS cluster is on the path to the eventual reduction of cytochrome c (the substrate for complex IV). The electron transferred to cytochrome b will be coupled with an additional electron from the subsequent round of the Q cycle to reduce Q to QH_2. The link between the Q cycle and the generation of proton motif force is the oxidation of QH_2 and the release of protons in the intermembrane space (IM) and the reduction of Q and consumption of protons in the matrix (M). On the right are the structures of the Q, SQ, and QH_2 states.

landscape is thermodynamically favorable ($\Delta G < 0$) since the potential difference in the exergonic half-reaction (reduction of FeS and cytochrome c) is larger than the potential difference of the endergonic half-reaction.

Electron bifurcation thus harnesses the free energy of exergonic electron transfer to drive endergonic electron transfer. Q-based electron bifurcation occurs when QH_2 is oxidized by a single electron through an exergonic electron transfer generating a highly energized semiquinone (SQ) intermediate. The SQ intermediate has a low enough reduction potential to favorably drive the endergonic half reaction [8]. The key to high fidelity electron bifurcation is a robust mechanism to direct electron flow along the 'orthogonal' pathways, so that electrons are sent down the two pathways in a specific sequence, which is presumably controlled by a combination of distance and free energy effects dictated by electron tunneling and Marcus theory constraints [9]. This electronic choreography is accomplished in complex III through a large scale conformational change that significantly increases the distance of the iron-sulfur cluster acceptor on the exergonic path following electron transfer, making the kinetics for the transfer of a second electron down this pathway much less favorable [10,11]. Electron bifurcation in complex III is an evolutionary innovation that increases the efficiency in the production of a proton motive force by the electron transport chain, and its presence increases the amount of chemiosmotic potential generated in the electron

transport chain on the order of 20% without any net change in the overall electron flow.

Flavin-based electron bifurcation

For nearly forty years, electron bifurcation as described by Mitchell was presumed to be unique to complex III. About a decade ago, however, bifurcation re-emerged in the context of anaerobic metabolism [12–14]. Enzymatic activities were described that involved the coupling of the exergonic reduction of NAD^+ to an endergonic reaction that allowed the reduction of the low potential electron carrier ferredoxin (Fd) [15]. In anaerobic metabolism, where free energy cannot be squandered without dire consequences to cell viability, electron bifurcation provides a high-fidelity mechanism to promote efficient energy conservation [1••,16]. The discovery of electron bifurcation associated with anaerobic metabolism involved the oxidation of NADH coupled to the simultaneous reduction of crotonyl-CoA and Fd in a butyric acid-forming bacterium [13]. The enzyme responsible for this reaction is a member of an electron transferring flavoprotein family (ETF) of enzymes that characteristically possess only flavin redox cofactors, pointing to the fact that electron bifurcation is flavin-based for this class of enzymes [17].

Studies of flavin-based electron bifurcation are emerging rapidly; parallels and new features are being discovered, which compare with a single version of Q-based bifurcation [1••]. The best characterized electron bifurcating enzyme is the dimeric NADH-dependent reduced

ferredoxin NADP$^+$ oxidoreductase (Nfn) [18,19$^{\bullet\bullet}$]. This enzyme has been structurally characterized in multiple states from two different microbial sources (*Thermotoga maritima* and *Pyrococcus furiosus*). The dimer consists of a large subunit with one flavin and two [4Fe-4S] clusters, and a small subunit with an additional flavin and a [2Fe-2S] cluster (Figure 3). These five redox cofactors are arranged linearly, with one of the flavins (the bifurcating flavin) located in the large subunit at the site of NADPH oxidation. Exergonic and endergonic electron transfer paths connect the bifurcating flavin with NAD$^+$ and ferredoxin (Fd) reduction sites. The exergonic path contains a [2Fe-2S] cluster about 14 Å from the bifurcating flavin on a pathway to a second flavin located another 9 Å away, at the site of NAD$^+$ reduction. Interestingly, the [2Fe-2S] cluster has a non-canonical coordinating ligand set of three Cys and one Asp, with an unusual reduction potential of +80 mV, estimated by EPR potentiometric titrations [20]. The endergonic branch has two [4Fe-4S] clusters located in the large subunit that connects the bifurcating flavin with the Fd reduction site. The proximal [4Fe-4S] cluster is located less than 8 Å from the bifurcating flavin and, by analogy with the bifurcating flavin proximal [2Fe-2S] cluster, is coordinated with three Cys ligands and an acidic Glu side chain. The distal cluster, which presumably transfers electrons directly to Fd, is approximately 10 Å further removed from the bifurcating flavin.

A combination of experimental techniques has been employed to provide insights into the mechanism of Nfn-catalyzed electron bifurcation and to understand the requirements for electron bifurcation [19$^{\bullet\bullet}$]. Nfn is an excellent model system for probing bifurcating electron transfer due, in part, to the available structures and to accessible interrogation through a variety of biophysical approaches. Electrochemical and spectroscopic methods have been used to estimate the reduction potential of the five redox cofactors, revealing surprising results. The [4Fe-4S] clusters have dramatically different reduction potentials compared to the [2Fe-2S] cluster of the exergonic branch (−718 mV and −513 mV compared to the estimated potential of +80 mV versus the standard hydrogen electrode). This arrangement sets up an unusual energy landscape and indicates that the bifurcating flavin site is likely unique in nature in its ability to generate the driving force needed to reduce the −718 mV cluster at a reasonable rate [21$^{\bullet}$].

Free energy landscape

Key mechanistic insights into Nfn electron bifurcation have been provided through spectroelectrochemistry and transient absorption spectroscopy [19$^{\bullet\bullet}$]. The SQ state of the bifurcating flavin cannot be observed in spectroelectrochemical titrations, suggesting that it is very short-lived. The SQ state, however, can be observed using ultrafast transient absorption spectroscopy of a semi-reduced Nfn that is generated by reduction with NADPH in the absence of NAD$^+$ or Fd as electron acceptors which characteristically exists with two electrons residing on the FeS clusters. Laser excitation generates strongly oxidizing flavin sites which are immediately reduced to SQ

Figure 3
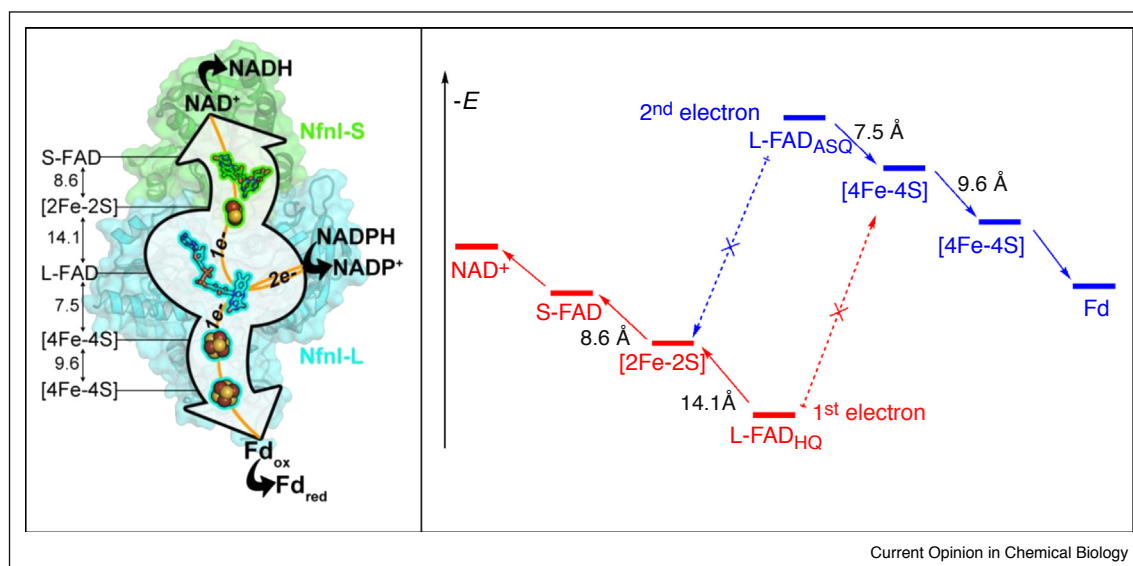
Left: Nfn structure showing cofactor arrangements along pathways and distances between cofactors. Right: Redox potential (E) landscape for bifurcating Nfn. After bifurcation at L-FAD, the first electron flows uphill (red relay) eventually to NAD$^+$, and the second electron flows downhill (blue relay), on a different physical pathway, to ferredoxin (Fd).

flavins. Rates of electron transfer back to the FeS clusters can be measured and, conveniently, the S-flavins and L-flavins can be distinguished because they exist in neutral and anionic SQ states, respectively, that have different optical signatures. Electron transfer from the SQ state of the bifurcating flavin to the [4Fe-4S] cluster occurs at a very fast rate (ps) which, when accounting for the reduction potential of the proximal [4Fe-4S], indicates that the reduction potential of SQ to oxidized flavin must be $\sim-911\,mV$, so a HQ to SQ reduction potential would be on the order of $+359\,mV$. This establishes a surprising energy landscape (Figure 3), with the bifurcating flavin having strongly crossed potentials. Although this is not a requirement for bifurcation [21•], the potential crossing rationalizes a mechanism to generate the driving force needed to satisfy the energetics required of the endergonic branch, and the overall energy landscape and is in line with the principles of Q-based bifurcation observed in complex III.

## Controlling electron flow down the exergonic path

Maintaining the 1:1 electron fidelity of bifurcation (moving electrons with an equal flux down the two paths) in complex III is associated with a large conformational change that increases the distance between the outer Q oxidation site and the Rieske FeS site on the exergonic branch [22•]. Increasing the distance

presumably lowers the electron transfer rate on the exergonic path, in favor of the second electron moving to cytochrome b on the endergonic path. Although this kind of mechanism has been proposed for other members of the bifurcating electron transferring flavoprotein family of enzymes [17,23•,24••] and suggested to potentially be employed in Nfn, the structural and mass spectrometry results are not consistent with conformational changes on the scale anticipated to support such a mechanism [25]. As an alternative, it has been suggested that the electron transfer is electrochemically controlled and the very large difference between the very low potential bifurcating flavin SQ ($-911\,mV$) and the iron-sulfur cluster on the exergonic branch ($+80\,mV$) places the electron transfer in the Marcus inverted regime: this inverted effect coupled with the nearly 7 Å shorter distance to the acceptor along the endergonic path is predicted to send the second electron down the endergonic path. Exploiting both distances and driving forces of electron transfer reactions between redox cofactors shows how macromolecular machines can exploit the intrinsic biophysical chemistry of electron transfer to direct high value multi-electron redox processes.

New frontiers

The nature of Q-based bifurcation revealed decades ago, coupled with the rapidly appearing insights into flavin-

Figure 4
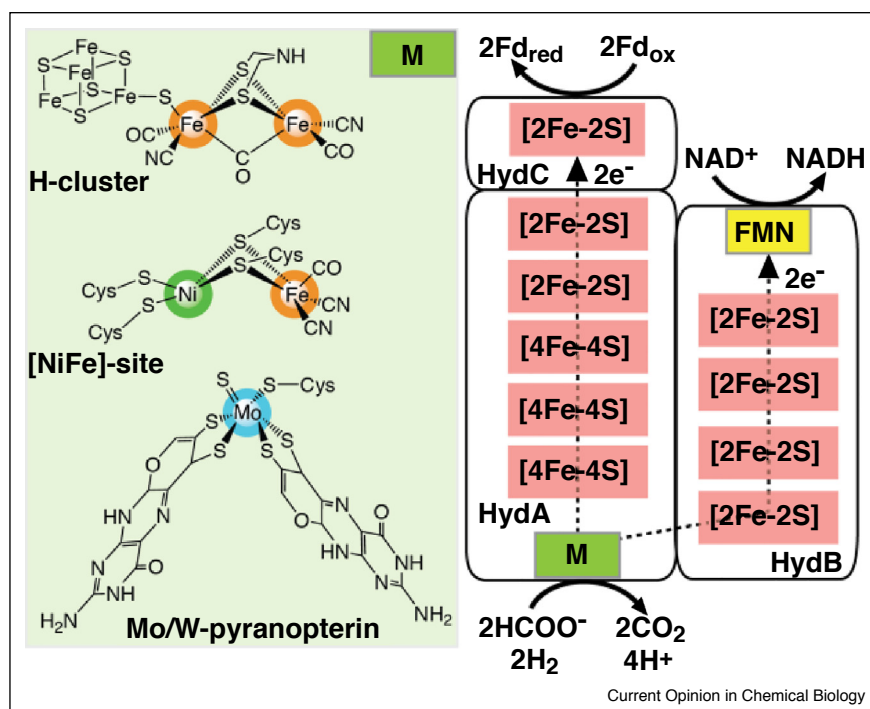
The HydABC scaffold of proposed metal-site bifurcating enzymes (*right*) where the M site is the H cluster, the [NiFe]-site or the W/Mo-pyranopterin catalytic site (*left*) of the [FeFe]-hydrogenase, [NiFe]-hydrogenase or formate dehydrogenase, respectively.

based bifurcation, establish the core requirements for electron bifurcation in proteins. The major requirements are 1) a redox site that can perform one-electron and two-electron chemistry where one redox state is inherently less stable than the other, and 2) an effective mechanism to control the 1:1 bifurcation fidelity to affect equal fluxes along exergonic and endergonic pathways. Now that flavin-based bifurcation has been demonstrated, several other bifurcating reactions and associated enzymes have been identified [26] and the structures of these enzymes are emerging [23•,24••,27,28•]. It will be interesting to see if mechanistic features different than those observed for Nfn modulate this unique and elegant electron transfer catalysis. Interestingly, it has been observed that a common multiple redox cofactor protein architecture, termed HydABC, is found to be associated with a canonical Nfn bifurcating architecture as well as bifurcating tungsten-containing formate dehydrogenase [29], [FeFe]-hydrogenase [14], and [NiFe]-hydrogenase sites [30••] (Figure 4 [30••]). The implication of these observations is that these metal-containing active sites may serve as the site of bifurcation. The role of protons in electron bifurcation is unclear, but a proton-coupled electron transfer process is suspected [31–35]. The growing relevance of electron bifurcation points to a very high level of control that proteins can impose on redox energetics and electron transfer kinetics, pointing to conceivably game-changing paradigms to be exploited in future bioinspired energy production and utilization technologies.

Conflict of interest statement
Nothing declared.

Acknowledgements

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