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On the nature of organic and inorganic centers that bifurcate electrons, coupling exergonic and endergonic oxidation-reduction reactions

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Bifurcating electrons to couple endergonic and exergonic electron-transfer reactions has been shown to have a key role in energy conserving redox enzymes. Bifurcating enzymes require a redox center that is capable of directing electron transport along two spatially separate pathways. Research into the nature of electron bifurcating sites indicates that one of the keys is the formation of a low potential oxidation state to satisfy the energetics required of the endergonic half reaction, indicating that any redox center (organic or inorganic) that can exist in multiple oxidation states with sufficiently separated redox potentials should be capable of electron bifurcation. In this *Feature Article*, we explore a paradigm for bifurcating electrons down independent high and low potential pathways, and describe redox cofactors that have been demonstrated or implicated in driving this unique biochemistry.

Introduction

Almost half a century ago, Peter Mitchell introduced the principle of electron bifurcation (EB) to explain the function of respiratory Complex III to describe how quinol (QH_2) oxidation in the mitochondrial inner membrane space was coupled to quinone (Q) reduction in the lumen, producing an overall thermodynamically favorable sequence of multi-electron transfer reactions. The mechanism described in Mitchell's Q cycle indicated that the coupling of exergonic and endergonic reactions could occur by a phenomenon he referred to as EB, reasoning that the free energy change of an exergonic electron transfer reaction could be captured to drive an endergonic electron transfer reaction. Additional experimental support was subsequently presented to support this elegant mechanism for generating chemiosmotic potential. Yet, for more than thirty years, EB was thought to be a feature unique to respiratory Complex III.

In the late 2000s, Wolfgang Buckel and Rolf Thauer discovered that bifurcation was also involved in certain aspects of anaerobic metabolism in microorganisms. Subsequently, EB was implicated in a variety of reactions in anaerobic metabolism, many of which involved the oxidation of NADH and the reduction of the redox protein ferredoxin, coupled to exergonic reactions such as the reduction of unsaturated organics, quinones, or disulfides. EB reactions are predominantly associated with anaerobic metabolism, but the EB enzyme called FixABCX is found in some nitrogen-fixing microorganisms including aerobes. It seems clear that EB in aerobic metabolism during nitrogen-fixing growth is critical for the optimization of metabolic efficiency and redox homeostasis.

The EB enzymes that have been characterized biochemically are all complex iron-sulfur flavoproteins containing numerous [4Fe-4S] and [2Fe-2S] clusters, and at least one flavin site. It was presumed that a flavin site was the most likely site of EB since an implied requirement of bifurcating redox sites is that they coordinate both one and two electron transfer reactions. The first structurally characterized bifurcating enzyme was the NADH-dependent ferredoxin (Fd) $NADP^+$ oxidoreductase abbreviated Nfn. This protein has an architecture with a central and presumably bifurcating flavin that is intimately coupled to two separate electron-transfer pathways that couple the reversible two-electron oxidation of NADPH to the reduction of NAD^+ and Fd. Results from spectroscopic and electrochemical studies support a developing paradigm for flavin-based bifurcation that mirrors that of the quinone-based bifurcation in complex III: the first one-electron oxidation of flavin produces an energetic, short lived semiquinone species that drives the half reaction leading to a highly reducing product species (Fd).

In Nfn, the two flavin cofactors were determined to serve (separately) as (1) the bifurcating site (mentioned above) as well as the site for reversible NADPH oxidation and (2) the site for reversible NAD^+ reduction. Interestingly, the bifurcating hydrogenases, which couple reversible NADH and reduced Fd oxidation to proton reduction, appear not to follow this paradigm. Instead, the bifurcating hydrogenases have only a

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single flavin site that appears to interact only with NAD(H). By analogy with Nfn, the lack of a flavin bifurcating site prompted us to examine whether or not other redox centers can bifurcate electrons in a manner analogous to quinone and flavin bifurcation. The paradigm for quinones and flavins that perform EB requires: 1) a redox center that can support more than two oxidation states, participate in more than one one-electron redox transitions along spatially separated paths and 2) a large energetic separation of the reduction potentials of the two electron transfer active states. We propose that the hydrogenase catalytic site, a unique iron-sulfur center known as the H cluster, can serve as a bifurcating site, since the H cluster should be able to satisfy the two above requirements for EB. The broader implication of this proposed function of the H cluster is that other metal-containing cofactors in biology, including those based on nickel, molybdenum and tungsten, could act as bifurcating redox centers.

In this Feature Article, we discuss the history of the discovery of quinone- and flavin-based EB, and the associated mechanistic studies that established the bifurcating charge transfer paradigm. The Article provides compelling bioinformatics support that EB may extend well beyond quinone and flavin cofactors and may be driven by other metal centers.

Mitchell and the Q cycle

EB, in the context of coupling endergonic and exergonic reactions, was a concept introduced by Peter Mitchell to explain the activity of respiratory complex III (the cytochrome bc_1 complex) in the electron transport chain.[3] The ingenious mechanism of proton translocation catalyzed by Complex III, termed the Q cycle, harnesses the reducing power of electrons from the mobile electron donor quinone in its reduced state, quinol (QH_2) in a unique manner. Complex III oxidizes QH_2 and sends one electron to the mobile electron carrier cytochrome c (cytc) and the other, oddly enough, is energized (its reduction potential lowered). It then travels along a separate pathway to reduce a second oxidized quinone via a cytochrome b cofactor. This bifurcation of electrons drives electrons on two very different thermodynamic landscapes. The redox pathway directed toward cyt c reduction is the first step: it involves transfer of a single electron uphill ~330 mV from the 570 mV potential Q_o species (dihydroquinone(HQ)/semiquinone(SQ) redox couple) to the 242 mV potential cyt c_1 species.[4,x] The semiquinone thus produced is strongly reducing, with a negative reduction potential (-390 mV) capable of reducing of a second Q (first reduction potential 20-70 mV, second reduction potential 260-290 mV at the Q_i site).[4] Interestingly, the Q_o donor and Q_i acceptor are located on the outside and inside of the membrane, respectively, such that oxidation of QH_2 at the Q_O site releases protons in the inter-membrane space and the reduction of Q to QH_2 on the inside results in the net translocation of 4 protons per Q cycle. The key to the Q cycle is the bifurcation of electrons along the endergonic (cytochrome c) and exergonic (cytochrome b) pathways. In Mitchell's 1975 study of the Q cycle, he wrote "The type of two-equivalent redox reaction in which the two electrons transferred are each in equilibrium with separate specific centers that are at different redox potentials may be somewhat unfamiliar inasmuch as this specific type of reaction may proceed reversibly when the stability constant of the intermediary is either greater or smaller than unity, depending on the sequence of electron transfer".[5] The latter is in reference to the energetic nature of the SQ intermediate that creates the driving force for the reduction of Q by QH_2.

Conceptually, that idea that the SQ→Q redox couple would have a lower potential than the HQ→SQ redox couple is counter-intuitive. Typically we think about the transfer of more than one electron into a single redox site as requiring increasingly negative reduction potentials for each successive step, based on simple electrostatic charging arguments (neglecting charge neutralization by coupled proton transfer). The subsequent (sequential) oxidation of a doubly reduced redox species presumably occurs through steps in which the two electron reduced state would be more reducing (would have a more negative reduction potential). This is not the case for the Q cycle, as the HQ→SQ couple has a significantly more positive reduction potential that the SQ→Q couple. Thus, the first and second potentials are "crossed". The crossed potentials are produced through an intermediate state that is very unstable and short lived relative to states with one more or one less electron. Complex III transfers an electron from QH_2 at the Q_o site to a [2Fe-2S] cluster in a reaction that is counter to an electrochemical potential gradient (i.e. is endergonic). The resulting SQ state at Q_o is a strongly reducing unstable intermediate, sufficiently reducing to transfer an electron to the Q_i site, eventually producing QH_2. The release of protons in the inter-membrane space couples to proton capture from the matrix to generate the chemiosmotic potential without net translocation of Q molecules. These events are all possible by the unique function at the Q_O site, which Mitchell termed "EB."

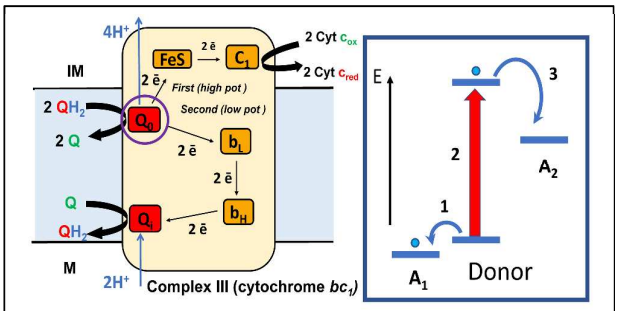

Fig 1. Schematic diagram showing the electron flux in Complex III. The first electron (step 1) leaves the HQ (donor) and flows to cytochrome *c* (A_1) via the iron-sulfur cluster and cytochrome c_1, causing the SQ state to be energized (step 2). The second (more reducing) electron flows (step 3) to the cytochrome b_L (A_2). The FeS cluster may not compete for the second electron because the time scale for its delivery of the first electron to cytochrome c_1 and consequent return to proximity of the Q_O site. M indicates the matrix (inside or i) and IM indicates the intermembrane space between the inner and outer mitochondrial membranes (the outside, o).

Electron bifurcation in the Q-cycle of Complex III

The cytochrome bc_1 complex oxidizes ubihydroquinone, reduces cytochrome c, and contributes to the transmembrane proton gradient that powers ATP synthase (Complex V) (Fig 1). The bc_1 enzyme complex (Complex III) lies in the inner mitochondrial membrane and in photosynthetic membranes. A puzzle surrounding the Q cycle of Complex III was that adding oxygen to mitochondrial suspensions was found to produce (the expected) oxidation of cytochrome c, but surprisingly also led to the reduction of the b-type cytochromes.[6] Indeed, Wikstrom and Berden suggested in 1972 that the dihydroquinone (QH_2) likely parsed its two electrons to two separate redox chains, involving the quinone, semiquinone and hydroquinone species. Interestingly, the first redox step would be triggered by cytochrome *c* oxidization (by cytochrome *c* oxidase, complex IV). Why must the oxidized cytochrome b_L await the production of oxidized cytochrome c by complex IV, its diffusion to complex III, and reduction by QH_2 via cytochrome c_1, *prior* to cytochrome b_L reduction?

In Complex III, the quinone in the Q_o site is about 12 Å from the cytochrome b_L but only about 7 Å from the FeS cluster (when the somewhat mobile FeS cofactor is in its proximal position), the shuttle that leads to cyt c_1.[4] Upon reduction, the FeS cluster moves about 16 Å to its distal position, delivering the electron to cytochrome c_1, which then passes the electron to cytochrome *c*. The second electron transfer from the energized semiquinone to the 12 Å distant cytochrome b_L is apparently not in competition with the more than 20 Å distant cytochrome c_1 electron acceptor, because of the prohibitive cost for tunneling such a long distance in a single step. As well, reduction of the mobile FeS shuttle by the energized Q_o electron is apparently not accessible (based on the energy gap law, the fact that the FeS cluster may still be reduced, or the possibility that the FeS cluster may be out of position to receive a second electron), so cytochrome b_L is reduced by the energized Q_o electron. [6-8]

How do quinones bifurcate electrons?

A puzzle surrounding EB in Complex III is why the first electron to leave the quinone that reduces the FeS cluster, cytochrome c_1, and cytochrome *c* exits at a different (higher) reduction potential than the second electron, and how this redox level crossing, in concert with the three-dimensional structure and dynamics of cytochrome bc_1, produce EB.

The question of the redox potential shift in the Q/SQ/HQ redox couples is addressed in an approximate way by examining the trend in the HOMO energies of the three quinone species. The structures of the three species are shown schematically in Fig. 2. Note that one electron oxidation of HQ changes the hybridization of an oxygen atom from sp^3 to p, thus destabilizing the semiquinone species. A simple Hückel calculation of pi-electron HOMO energies demonstrates that this energizes the second redox active electron. Hence the mechanism of how electron flow is controlled in EB-quinones is at least qualitatively understood.

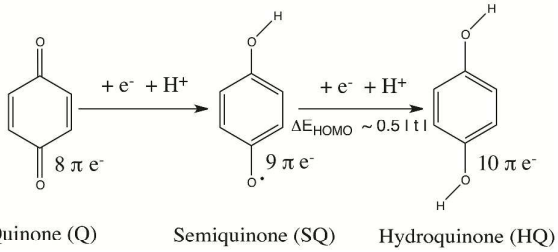

Fig 2. The quinone (oxidized), semiquinone (singly reduced), and hydroquinone (doubly reduced) states are indicated. Not that the first electron leaves the hydroquinone causes a hybridization change in the remaining open shell oxygen atom, form sp^3 to p. This causes a concomitant destabilization of the HOMO energy by about 0.5|t|, where t is the Hückel interaction energy between nearest-neighbor carbon p-orbitals. This hybridization change leads to a destabilization of the SQ HOMO, thus energizing the second redox.

Buckel, Thauer, and the discovery of flavin-based bifurcation

The concept of EB as described by Mitchell in the Q cycle was thought to be an isolated paradigm for energy transduction for more than thirty years, until the observations in 2008 that EB is pervasive in biology[9]. Many anaerobes require reduced low potential electron carriers for H^+, CO, and CO_2 reduction as well as for N_2 fixation. Reduced ferredoxin (Fd) is produced during metabolism by heterotrophic anaerobes through the oxidation of pyruvate by pyruvate ferredoxin oxidoreductase, but the reduction of Fd (E_m ~ -450mV) via NADH oxidation (E_m -320 mV) is an endergonic process. For many decades, it remained a mystery as to how Fd reduction was accomplished as part of several fundamental metabolic pathways, especially under typical cellular conditions where [NAD]/[NADH] >1. It was also clear that in many, if not all cases, that the NADH-dependent reduction of Fd was independent of membrane potential or ion gradients. The breakthrough came in 2008, when Buckel and coworkers proposed that Fd reduction by NADH could be catalyzed by a single cytoplasmic enzyme but that that the reaction requires another substrate that, in essence, drives the reaction. Specifically, they proposed that in butyric acid-forming clostridia such as *C. kluyveri*, the reduction of Fd by NADH is coupled to the reduction of crotonyl-CoA (E_m – 10 mV) by NADH in an overall thermodynamically favorable reaction, and that the reaction is catalyzed by a cytoplasmic electron-transfer flavoprotein (ETF) [10]. ETFs in eukaryotes catalyze the oxidation of fatty acids and some amino acids coupled to the transfer of electrons to the quinone pool and the generation of chemismotic potential and ATP through oxidative phosphorylation in mitochondria. The distinction between the reactions carried out by these enzymes and the reaction proposed by Buckel is the harnessing of the fatty acid

oxidation free energy to drive the endergonic reduction of Fd, indicating a clearly more complex mechanism. Since the enzyme complex purified and characterized by Buckel and Thauer contained only flavin-based redox cofactors, it was concluded that bifurcation must occur at a flavin-cofactor, i.e., "flavin-based EB."

Since this discovery, other enzymes have been found to catalyze bifurcation reactions and several were shown to be flavin-based bifurcators [11-20]. In addition to the butyryl-CoA dehydrogenase described above, there are now other ETF enzymes that have been found to bifurcate, including the related caffeyl-CoA reductase coupling the oxidation of NADH to the simultaneous reduction of caffeyl-CoA and Fd [11], and a lactate dehydrogenase that couples the oxidation of lactate and Fd to the reduction of NAD [21]. Very recently, it was shown that another ETF enzyme, termed the FixABCX complex, can couple the oxidation of NADH to the coupled reduction of quinone and Fd or flavodoxin (Fld). The name Fix was coined due to its association with nitrogen fixation in the purple sulfur bacteria *Rhodobacter capsulatus*. FixABCX was purified from nitrogen fixing *Azotobacter vinelandii* and shown to couple quinone reduction to the reduction of Fld via NADH oxidation [14]. All of the above reactions couple the oxidation of NADH, an intermediate reduction potential electron carrier, to the reduction of either low potential electron carriers (Fd or Fld) and more positive potential organic carbonyl or olefenic reductions reactions. In the case of the butyryl-CoA dehydrogenase, caffeyl-CoA reductase, and lactate dehydrogenase, these bifurcating reactions are presumably serving the role of increasing the metabolic efficiency. The implicated role of the Fix system in *A. vinelandii,* which is an obligate aerobe, is somewhat different than those involved in anaerobic metabolism. Fix can be thought of as diverting the flow of electrons in the electron transport chain by bypassing Complex I, where half of the electrons from NADH are used to reduce ferredoxin and the other half are delivered back to the electron transport chain at Complex III, as quinol. During nitrogen fixation, the Fix reaction helps balance not only the pools of electron carriers, but the chemiosmotic potential and ATP production.

In 2008, Thauer also suggested [22] that coupling exergonic oxidation of the coenzyme M (CoM) – coenzyme B (CoB) heterodisulfide to the reduction of Fd could be invoked to explain the observations from Ralph Wolfe's lab in the late 1970's that the reduction of methyl-coenzyme M to methane by H_2, and the reduction of CO_2 by H_2, were somehow coupled without the involvement of processes occurring in the membrane. This observation led him to suggest later that methanogenesis is a cyclic process [23]. Results from biochemical experiments of Thauer and coworkers in 2011 and of Leigh and coworkers in 2010 found that CoM-CoB oxidation was indeed coupled to Fd reduction in a cyclic manner, prompting Thauer's proposal to name the cycle that balances the energetics for hydrogenotrophic methanogenesis the "Wolfe cycle" [24].

The central bifurcating enzyme in hydrogenotrophic methanogenesis is the heterodisulfide reductase-hydrogenase (MvhADG/HdrABC) complex that consists of a non-F_{420}-reducing [NiFe]-hydrogenase and a heterodisulfide reductase [13]. The complex catalyzes the coupled reduction of CoM-S-S-CoB and Fd with 2 H_2 via an EB mechanism. The recently solved structure of this MvhADG/HdrABC complex show the elegant mechanism of this enzyme in which the central EB-FAD is connected to the three redox sites by strings of [FeS] clusters[25]. The HdrABC forms the core of this bifurcating enzyme class. Members are widespread in the archaeal and bacterial domain, such as the heterodisulfide reductase F_{420} dehydrogenase complex in some *Methansosarcina* species and heterodisulfide reductase NADH dehydrogenase of sulfate reducing bacteria [20, 26].

How do flavins bifurcate electrons?

At present, the most extensively studied flavin-based bifurcating enzyme is the NADH-dependent reduced ferredoxin $NADP^+$ oxidoreductase (Nfn) [2, 19, 27-30]. This is another enzyme that has been demonstrated to catalyze an EB reaction that plays a fundamental role in redox homeostasis. It is directly involved in partitioning pools of redox equivalents between NADH, NADPH and reduced Fd and plays a pivotal in balancing anabolism and catabolism in anaerobic metabolism.[30] A combination of structural, spectroscopic, and electrochemical probes have defined the basic physical nature of bifurcation in Nfn[2]. The structural characterization of Nfn from *Thermotoga maritima* [15] and the homologous NfnI from *Pyrococcus furiosus* [2] (Pf NfnI) showed that the dimeric Nfn exists with a central bifurcating flavin located within the large subunit that is in close proximity, and within electron-transfer

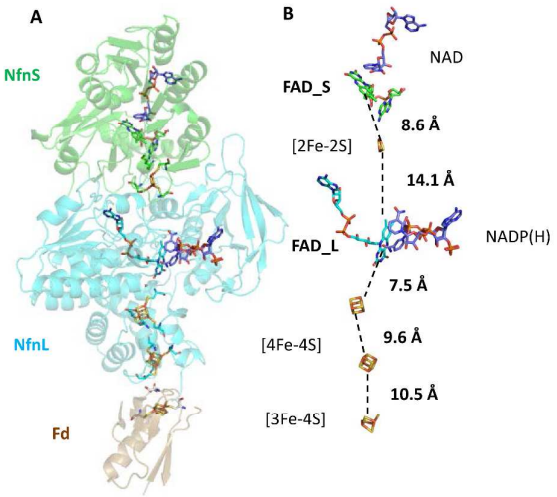

Fig. 3. (A) Pf NfnI as a heterodimer of small subunit (NfnI-S, green), and large subunit (NfnI-L, cyan) and computationally docked ferredoxin (Fd, brown). NfnI-S contains one FAD (S-FAD) and a [2Fe-2S] cluster. NfnI-L contains one FAD (L-FAD) that is the site of EB, and two [4Fe-4S] clusters. (B) Distances between NfnI cofactors are given in Å. S-FAD and L-FAD bind NADH and NADPH, respectively. Fd binds near the distal [4Fe-4S] cluster of NfnI-L.

distance, of a large subunit [4Fe-4S] clusters and a [2Fe-2S] cluster of the small subunit (Fig. 3). The distal redox cofactors are the second [4Fe-4S] cluster of the large subunit and the flavin located in the small subunit. These cofactors serve as the sites for reversible Fd and NAD^+ reduction, respectively. The structural studies provided a framework for a general mechanistic hypothesis concerning the flow of electrons during EB. NADPH binding and reduction of the central bifurcating site leads to flavin two EB from a hydroquinone state. The bifurcating flavin's oxidation first to the semiquionone and then to the two-electron oxidized product allows the electron to be bifurcated to effect the eventual reduction of NAD^+ coupled to the reduction of Fd. The two-electron reaction is not complete without a second round of NADPH-induced reduction of the bifurcating center, leading to one fully reduced NADH and two reduced Fds in the following overall reaction:

$$2NADPH + NAD^+ + 2Fd_{ox} < -- > 2NADP^+ + NADH + 2Fd_{red}$$

The structures of NfnI serves a hypothetical framework to design experiments that elucidated a detailed redox mechanism. Perhaps the most important questions surrounding the flavin-based EB mechanism are 1) what are the properties of a bifurcating flavin that results in the low potential to drive Fd reduction and 2) how are electrons prevented from always traveling down the more favorable electrochemical potential gradient to reduce NAD^+? On discovering EB it was suggested[31] that the crossed potentials of the flavin bifurcating site involved an obligatory two-electron reaction that could be important in EB and in generating a landscape that is thermodynamically favorable to drive the endergonic half reaction. A set of complementary spectroscopic experiments revealed that this was indeed the case and that, coupled with the results of square wave voltammetry, a detailed mechanism could be proposed [2]. Transient absorption spectroscopy (TAS) was used to probe the semiquinone state of the bifurcating flavin and to determine the rate of electron transfer from the semiquinone to the proximal [4Fe-4S] cluster acceptor. In this experiment the pump laser excitation induces electron transfer to the flavin sites and generates the reducing semiquinone state. The oxidation of the flavin semiquinone can be monitored as a function of time and the electron transfer rates from the flavin to the FeS clusters can be determined. The bifurcating flavin can be differentiated from the proximal NAD^+-reducing flavin because they exist as anionic and neutral semiquinone states, respectively, which have significantly different optical signatures. These studies indicated that the rate of electron transfer from the anionic semiquinone bifurcating flavin to the proximal [4Fe-4S] cluster is on the order of 10 ps, indicating highly crossed flavin potentials and a very short lived and highly reducing semiquinone state.

The next set of experiments, involving the characterization of the FeS clusters, provided significant insights into the overall electron flow. The results of square wave voltammetry (SWV) experiments provided evidence for the assignment of the

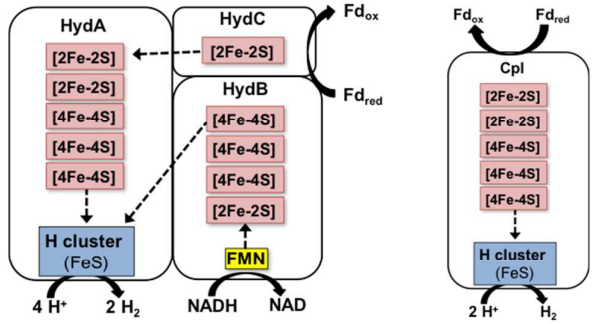

Fig. 4 Models of EB-hydrogenase of *T. maritima* (*left)* and the non-EB-hydrogenase of *C. pasteurianum.*

redox potentials of two of the three FeS clusters [2]. The reduction potential of the [2Fe-2S] cluster in the high potential branch was previously reported from the EPR characterization at ~ +80mV,.[32] The reduction potentials of the [4Fe-4S] clusters in the low potential branch determined by the SWV experiments were somewhat surprising, since they were estimated at ~-513 and at -718mV. The presence of the very low potential cluster was also supported by EPR data, indicating that maximal signal intensities could not be achieved without the addition of the low potential electron donor, Ti(III) citrate (~-0.8 V). Spin coupling analysis allowed the tentative assignment of the low potential cluster as the [4Fe-4S] cluster closest to the bifurcating flavin.

The ability to measure rates of electron transfer from the bifurcating flavin, combined with the ability to estimate the reduction potentials of the FeS clusters, allows estimation of the reduction potentials for the two redox couples of the bifurcating flavin during catalysis. A fast electron-transfer rate from the bifurcating flavin to the -718mV [4Fe-4S] cluster at a distance of only 7.5 Å means that the reduction potentials of the HQ/ASQ and ASQ/OX are calculated to be highly crossed at +359mV and -911mV respectively (although these specific deduced value are sensitive to the chosen non-adiabatic electron transfer rate parameters used).

The very low reduction potential (-911mV) of the ASQ/OX couple answers one of the key questions of how the Fd reduction is accomplished. The second main question is how electron transfer is used to direct electrons down this path to the reduction of Fd and not down the path to reduce NAD^+. For Complex III, the solution was a large scale conformational change that increased the distance of donors and acceptors causing the relative rate of electron transfer faster for the reduction pathway of Q than for the reduction pathway of cytochrome c. In the case of Nfn, the results of hydrogen-deuterium exchange experiments found only subtle conformational changes that are presumably insufficient for this manner of conformational control [27]. With the large potential differences observed in the two possible electron acceptors of the bifurcating flavin, the [2Fe-2S] cluster at +80mV and the [4Fe-4S] cluster at -718mV, an alternative mechanism for control can be proposed. The difference of nearly 1V in the reduction potential between the ASQ/OX (-911mV) and the [2Fe-2S] (+80mV) could place this transfer in

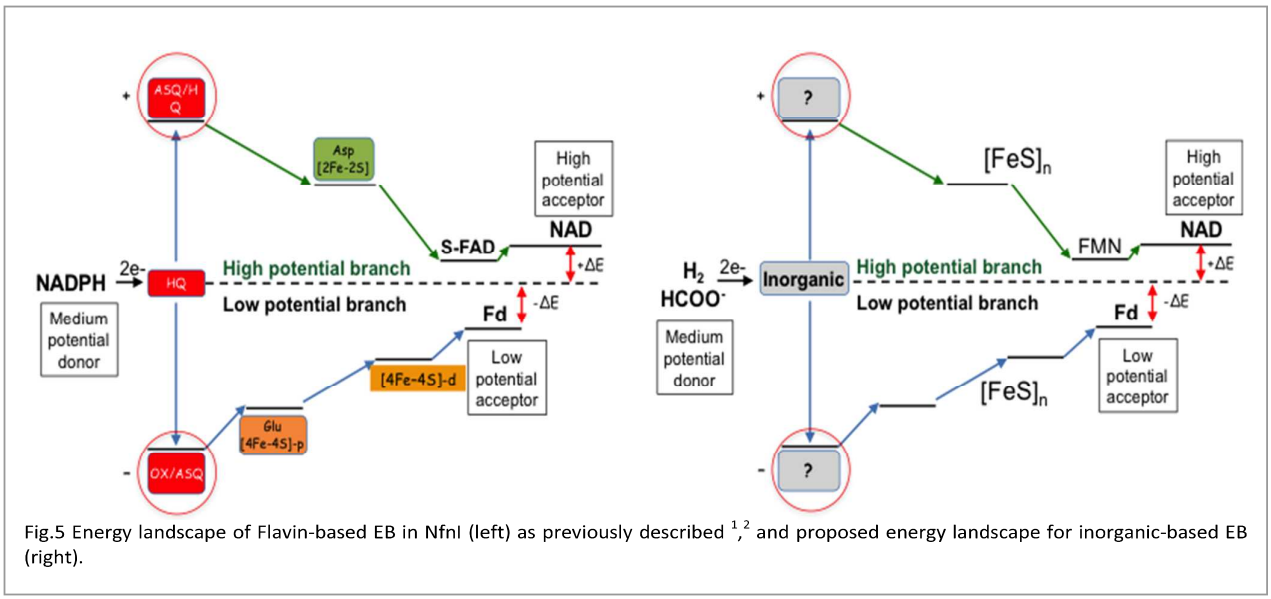

Fig.5 Energy landscape of Flavin-based EB in NfnI (left) as previously described [1,2] and proposed energy landscape for inorganic-based EB (right).

the Marcus inverted region and thus make it slower than the transfer to the -718mV [4Fe-4S] cluster. Marcus inversion provides an elegant scheme to control electron transfer and to effectively direct electrons down the low potential path. Separation distance clearly also work in favor of transferring the second electron in the direction of Fd.

The conclusions from the NfnI studies [2] can be extrapolated to suggest that bifurcating redox cofactors: 1) exist with at least three oxidation states, and the reduction potential shifts among these states must be characterized by a difference that enables the driving of two energetically separate half reactions, 2) favor two electron chemistry and extracting a single electron results in the generation of an energized intermediate with a lower potential than the reduction potential of the electron pair reaction, and 3) possess architectures capable of extracting the first electron producing a strongly reducing second donor, which uses key electron transfer control parameters (distance and potential difference) to direct the second reaction in the required direction. We propose that the principles that we established in Nfn also apply to bifurcating metal centers, which are described next.

Can metal centers function as electron bifurcating sites?

One bifurcating enzyme that is distinctly different from the group of flavin-based EB-enzymes described above is the H_2-evolving hydrogenase from *Thermotoga maritima* [15]. This protein catalyzes the reduction of protons (E_m – 420 mV) to H_2 by coupling it to the exergonic oxidation of low potential reduced Fd (E_m ~ - 450 mV) to the endergonic oxidation of high potential NADH (E_m -320mV) in an overall $4e^-$ reaction:

$2Fd_{red} + NADH + 3\ H^+ \leftrightarrow 2H_2 + 2Fd_{ox} + NAD$

In contrast to Nfn, where the intermediate potential reaction, the reversible oxidation of NADPH, occurs at a bifurcating flavin site, the intermediate potential reaction of the hydrogenase, reversible hydrogen oxidation, occurs at a novel iron sulfur cluster known as the H cluster. We therefore hypothesize that the site of EB in the EB-hydrogenases is not flavin, but is rather the H_2-evolving H cluster. In support of this hypothesis, only one flavin-binding motif is present in the amino acid sequence of the *T. maritima* enzyme, and this is assumed to be the NADH binding site (in the HydB subunit). A structure of the *T. maritima* enzyme is not available, but a model with the proposed pathways of electron flow are shown in **Fig. 4**, together with that of the structurally-characterized *C. pasteurianum* enzyme that does not bifurcate and simply oxidizes reduced Fd and evolves H_2. In the *T. maritima* enzyme, electrons flow to the proposed EB-H cluster from two strings of FeS clusters, similar to the strings of FeS clusters seen in EB-MvhADG/HdrABC, one fed by reduced Fd and one fed by NADH. Hence the thermodynamic landscape of the EB-hydrogenase is analogous to that of EB by EB-Nfn and EB-MvhADG/HdrABC, except that an inorganic metal cluster rather than flavin is the site of EB (**Fig. 5**). The 6Fe-H cluster catalyzes the $2e^-$ reduction of two protons to H_2 gas and in principle could accommodate even more electrons as bifurcation is nominally a $4e^-$ reduction reaction per catalytic turnover. Accommodating 4 electrons may not be necessary, given the two 'strings' of multiple FeS clusters that are proposed to direct electrons into the catalytic site.

The H cluster has the requisite features of serving as a bifurcating site, analogous to the function of the bifurcating flavin in Nfn described above, where the key to bifurcation is a low potential one-electron intermediate. Accordingly, the properties of the H clusters of EB and non-EB hydrogenases are not identical. For example, the oxidized H cluster of the non-EB *C. pasteurianum* enzyme exhibits a unique and characteristic EPR signal but this is not evident with the EB-hydrogenase of *T. maritima.* It is possible that the EB-H cluster operates in a different redox potential range that than

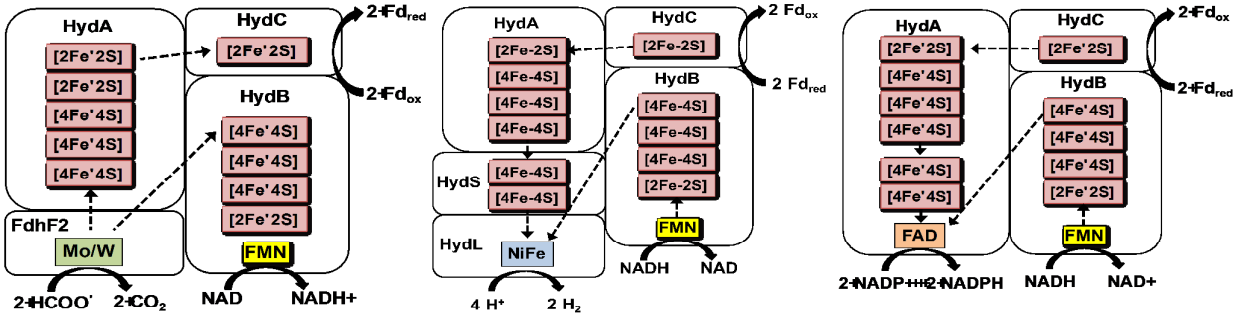

Fig. 6. Stabilization of inorganic (Mo/W and NiFe) and organic (FAD) EB-catalytic sites by the HydABC architecture. See text for details.

observed for the non-EB-H cluster. Other differences might include polarity and charge (dielectric) of their protein environments and availability and affinity for protons (pH and pKa). Adding another layer of complexity to the analysis of EB-hydrogenases is the recent finding that the gene cluster encoding the *T. maritima* enzyme also encodes enzymes related to protein kinases and phosphatases, and that two of the subunits (HydA and B) of the purified enzyme are phosphorylated[33]. Whether this post-translational modification has any role in bifurcation remain to be seen.

In support of the HydB and HydC subunits of the *T. maritima* enzyme facilitating bifurcation by providing distinct pathways of electron flow for the high potential and low potential donors (**Fig. 4**), bioinformatics analysis show that such a system is not unique to the H_2-evolving EB-hydrogenase. The anaerobe *Clostridium acidurici* does not metabolize hydrogen gas, and its genome does not encode any conventional hydrogenase. However, Thauer and coworkers showed that it contains a HydABC-like protein complex that catalyzes the formate-dependent reduction of Fd and NAD [17]. Encoded by *hylCBA-fdhF2,* this enzyme resembles *T. maritima* EB-hydrogenase in that it contains homologs of HydABC (designated HylCBA) except that it lacks the H-cluster domain. The fourth gene (*fdhF2*) encodes a Mo/W-pyranopterin binding site and this is proposed to catalyze formate oxidation in this formate dehydrogenase complex (FDH). The midpotential acceptor/donor for this enzyme is therefore the formate/CO_2 redox couple, which has same potential (E_o' = -420 mV) as the hydrogen electrode, and formate oxidation is catalyzed by the Mo/W-pyranopterin (it is not clear which metal the enzyme uses). Like the H cluster, Mo/W sites catalyze a $2e^-$ reaction (IV/V and V/VI redox states) and this is the proposed site of bifurcation in the EB-FDH. Hence, we postulate that HydABC forms a framework to provide two electron pathways, one for the high potential carrier, NAD(H), and one for the low potential carrier, Fd, to and from the bifurcating site, which is the H-cluster in the EB [FeFe] hydrogenase and Mo/W-pterin in EB-FDH. Our model of the enzyme and the proposed thermodynamic landscape for bifurcated electron flow are shown is **Figs. 5 and 6,** respectively**.**

In further support of the H cluster and Mo/W as the sites of EB in the EB-hydrogenases and EB-FDHs, the genomes of some thermophilic halophiles harbor a 5-gene cluster that also encodes homologs of HydABC [34]. In this case, HydA is truncated and lacks the H cluster domain, while the other two genes in the cluster encode homologs of the large (L) and small (S) subunits of NiFe-hydrogenases. The HydL subunit contains the catalytic NiFe-site and the HydS subunit contains two (rather than the typical three) iron-sulfur clusters. Hence, as shown in **Fig. 6**, this HydABCLS enzyme is proposed to be a homolog of *T. maritima* EB-hydrogenase and to catalyze exactly the same reaction, the NADH- and Fd-dependent production of H_2, except that the H cluster is replaced by a NiFe-hydrogenase. Like the H cluster and Mo/W sites, the NiFe-site catalyzes a $2e^-$ reaction and presumably can also generate a destabilized one-electron intermediate, stabilized in part by the HydABC framework.

Additional evidence for the unique properties of the HydABC system comes from the genomes of some thermophilic archaea [35]. These also contain homologs of *T. maritima* HydABC except that the H cluster domain in HydA is replaced by the equivalent of the NfnL subunit that contains the bifurcating flavin and two iron-sulfur clusters (**Fig. 6**). We therefore propose that this Hyd-Nfn enzyme catalyzes the same overall reaction as conventional Nfn. Clearly, the HydABC architecture has special features that facilitate EB in a manner yet to be elucidated and we hypothesize that this structure provides two distinct pathways of multiple FeS clusters for electron transfer to flow to and from both inorganic (FeFe, W/Mo and NiFe) and organic (flavin) EB-sites.

A unified perspective on electron bifurcation by organic and inorganic centers

Modern electron transfer was framed more than 60 years ago. Its key constraints on electron transfer kinetics are the so-called energy gap law and the exponential sensitivity of electron tunneling to donor-acceptor distance. It is very unusual for the thermodynamics of chemical reactions to dictate their kinetics, but this is exactly the case for electron-transfer reactions. Electron transfer reactions are special because the reaction coordinate is defined by a large number of small nuclear librations, motions that respond to the charge flow from donor to acceptor cofactors.[36] As such, a protein's response to charge transfer is somewhat generic, determined by its effective dielectric environment. As a consequence, the

free energy for activation is linked to the Gibbs free energy of electron transfer, as this determines the offset of the two potential energy surfaces, defined by the elegant Marcus relation: $G^{\ddagger} = (\Delta G + \lambda)^2/4\lambda$, where $G^{\ddagger}$ is the activation free energy, ΔG is the Gibbs free energy for reaction, and λ is the reaction free energy (larger in more polar media, smaller in less polar media).[36] Importantly, there is a value of -$\Delta G = \lambda$ that maximizes the rates (the case of activationless ET); rates with $-\Delta G < \lambda$ are slower ("normal" regime) and rates with $-\Delta G > \lambda$ are also slowed ("inverted" regime). This energy gap law, or Marcus relation, interconnect the kinetics and thermodynamics of electron transfer.

More than 40 years ago, the influence of the protein medium on electron tunneling was described for biological electron transfer.[37] The protein must allow the redox equivalents to transfer from donor and acceptor sites, but must not allow high-energy redox equivalents to wander off randomly, dissipating energy or producing chemical damage. The redox active centers must sequester charge in "traps" that are much deeper than thermal energies, to sequester the charge.[38,39] How does biology achieve both charge sequestration *and* the delivery to "the right place at the right time"? The answer is that Nature has employed the physics of electron tunneling, producing rates that drop approximately exponentially with donor-acceptor distances.

The central dogma of biological electron transfer theory – kinetic and thermodynamic linkage of rates, and an electron tunneling mechanism for transport – set the stage for understanding EB reactions. Directionality is assured by *both* the energy gap law and the positions of the redox cofactors in the proteins. The control of EB has come into quantitative focus very recently,[1] but it is not a new concept by any means. Consider, for example, photoinduced charge separation in the bacterial photosynthetic apparatus.[40] Why does the photoexcited special pair transfer its electron to the bacterial chlorophyll monomer and pheophytin, rather than short-circuiting to fill the "hole" on the oxidized cytochrome that conveys electrons to the special pair? Filling the hole on the cytochrome is further "downhill" thermodynamically than the physiologically productive reaction. The resolution is two-fold. First, the energetics for reduction of the oxidized cytochrome is likely inverted: its activation free energy is much higher than the activation free energy for the charge flow in the physiologically productive directions. Second, the distance from the special pair to the productive acceptors is shorter than to the cytochrome. Our recent theoretical studies[1] on the EB flavin (L-FAD) in Nfn indicates that precisely these same two directional control factors are responsible for sending the first and second electrons to depart the EB site down their separate pathways, directing one toward NAD^+ and one toward oxidized ferredoxin. The same factors underpin the productive function of mitochondrial complex III bifurcation from the Q_O quinone site, as described above.

Summary and Perspective

Once thought to be an isolated phenomenon associated with the Q cycle of respiration, EB, as it was coined by Mitchell, is now known to be much more pervasive in biology. The paramount importance of efficiency in metabolism provides tremendous selective pressure for maximizing the free energy that is directed toward cellular function, rather than dissipate the energy as heat. In EB, Nature has exploited some of the most novel characteristics of multi-state redox centers, harnessing the low potential driving force of crossed potentials to make the low potential half reaction possible, triggered by a high potential first step. Exploiting many of the key control elements that have come to be understood in the context of modern electron transfer theory, suites of biological redox centers in sophisticated architectures balance potential differences and distances to stoichiometrically bifurcate electrons in an elegantly controlled manner. Significant progress has been made to unravel some of the mysteries concerning the molcular mechanisms of EB enzymes. A fundamental understanding of the mechanisms' impeccable fidelity and its elegant control imposed by EB enzymes provide important principles for the design of redox catalysts for energy production. This emerging understanding has even wider significance, as we are now finding that Nature employs inorganic as well as organic cofactors to carry out electron bifurcation.

Conflicts of interest

There are no conflicts to declare.

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Notes and references

1. P. Zhang, J. L. Yuly, C. E. Lubner, D. W. Mulder, P. W. King, J. W. Peters and D. N. Beratan, *Acc Chem Res*, 2017, **50**, 2410-2417.
2. C. E. Lubner, D. P. Jennings, D. W. Mulder, G. J. Schut, O. A. Zadvornyy, J. P. Hoben, M. Tokmina-Lukaszewska, L. Berry, D. M. Nguyen, G. L. Lipscomb, B. Bothner, A. K. Jones, A. F. Miller, P. W. King, M. W. W. Adams and J. W. Peters, *Nat Chem Biol*, 2017, **13**, 655-659.
3. P. Mitchell, *J. Theor. Biol.*, 1976, **62**, 327-367.
4. C. C. Moser, T. A. Farid, S. E. Chobot and P. L. Dutton, *Biochim Biophys Acta*, 2006, **1757**, 1096-1109.
5. P. Mitchell, *FEBS Lett.*, 1975, **59**, 137-139.
6. A. R. Crofts, *Photosynth Res*, 2004, **80**, 223-243.
7. A. R. Crofts, S. W. Rose, R. L. Burton, A. V. Desai, P. J. A. Kenis and S. A. Dikanov, *J Phys Chem B*, 2017, **121**, 3701-3717.
8. A. Osyczka, C. C. Moser, F. Daldal and P. L. Dutton, *Nature*, 2004, **427**, 607-612.
9. G. Herrmann, E. Jayamani, G. Mai and W. Buckel, *J Bacteriol*, 2008, **190**, 784-791.
10. F. Li, J. Hinderberger, H. Seedorf, J. Zhang, W. Buckel and R. K. Thauer, *J Bacteriol*, 2008, **190**, 843-850.
11. J. Bertsch, A. Parthasarathy, W. Buckel and V. Muller, *J Biol Chem*, 2013, **288**, 11304-11311.
12. N. P. Chowdhury, J. Kahnt and W. Buckel, *FEBS J.*, 2015, **282**, 3149-3160.

13. A. K. Kaster, J. Moll, K. Parey and R. K. Thauer, *Proc Natl Acad Sci U S A*, 2011, **108**, 2981-2986.
14. R. N. Ledbetter, A. M. Garcia Costas, C. E. Lubner, D. E. Mulder, M. Tokmina-Lukaszewska, J. H. Artz, A. Patterson, T. S. Magnuson, Z. J. Jay, H. D. Duan, J. Miller, M. H. Plunkett, J. P. Hoben, B. M. Barney, R. P. Carlson, A.-F. Miller, B. Bothner, P. W. King, J. W. Peters and L. C. Seefeldt, *Biochemistry*, 2017, **56**, 4177-4190.
15. G. J. Schut and M. W. Adams, *J Bacteriol*, 2009, **191**, 4451-4457.
16. S. Wang, H. Huang, J. Kahnt, A. P. Mueller, M. Kopke and R. K. Thauer, *J Bacteriol*, 2013, **195**, 4373-4386.
17. S. Wang, H. Huang, J. Kahnt and R. K. Thauer, *Appl Environ Microbiol*, 2013, **79**, 6176-6179.
18. S. Wang, H. Huang, J. Kahnt and R. K. Thauer, *J Bacteriol*, 2013, **195**, 1267-1275.
19. S. Wang, H. Huang, J. Moll and R. K. Thauer, *J Bacteriol*, 2010, **192**, 5115-5123.
20. Z. Yan, M. Wang and J. G. Ferry, *MBio*, 2017, **8**.
21. M. C. Weghoff, J. Bertsch and V. Muller, *Environ Microbiol*, 2015, **17**, 670-677.
22. R. K. Thauer, A. K. Kaster, H. Seedorf, W. Buckel and R. Hedderich, *Nat Rev Microbiol*, 2008, **6**, 579-591.
23. P. E. Rouviere and R. S. Wolfe, *J Biol Chem*, 1988, **263**, 7913-7916.
24. R. K. Thauer, *Proc Natl Acad Sci U S A*, 2012, **109**, 15084-15085.
25. T. Wagner, J. Koch, U. Ermler and S. Shima, *Science*, 2017, **357**, 699-703.
26. A. R. Ramos, F. Grein, G. P. Oliveira, S. S. Venceslau, K. L. Keller, J. D. Wall and I. A. Pereira, *Environ Microbiol*, 2015, **17**, 2288-2305.
27. L. Berry, S. Poudel, M. Tokmina-Lukaszewska, D. R. Colman, D. M. N. Nguyen, G. J. Schut, M. W. W. Adams, J. W. Peters, E. S. Boyd and B. Bothner, *Biochim Biophys Acta*, 2018, **1862**, 9-17.
28. J. K. Demmer, H. Huang, S. Wang, U. Demmer, R. K. Thauer and U. Ermler, *J Biol Chem*, 2015, **290**, 21985-21995.
29. H. Huang, S. Wang, J. Moll and R. K. Thauer, *J Bacteriol*, 2012, **194**, 3689-3699.
30. D. M. N. Nguyen, G. J. Schut, O. A. Zadvornyy, M. Tokmina-Lukaszewska, S. Poudel, G. L. Lipscomb, L. A. Adams, J. T. Dinsmore, W. J. Nixon, E. S. Boyd, B. Bothner, J. W. Peters and M. W. W. Adams, *J Biol Chem*, 2017, **292**, 14603-14616.
31. W. Nitschke and M. J. Russell, *Bioessays*, 2012, **34**, 106-109.
32. W. R. Hagen, P. J. Silva, M. A. Amorim, P. L. Hagedoorn, H. Wassink, H. Haaker and F. T. Robb, *J Biol Inorg Chem*, 2000, **5**, 527-534.
33. S. Poudel, M. Tokmina-Lukaszewska, D. R. Colman, M. Refai, G. J. Schut, P. W. King, P. C. Maness, M. W. Adams, J. W. Peters, B. Bothner and E. S. Boyd, *Biochim Biophys Acta*, 2016, **1860**, 1910-1921.
34. A. Antunes, I. Alam, V. B. Bajic and U. Stingl, *J Bacteriol*, 2011, **193**, 4553-4554.
35. A. V. Mardanov, N. V. Ravin, V. A. Svetlitchnyi, A. V. Beletsky, M. L. Miroshnichenko, E. A. Bonch-Osmolovskaya and K. G. Skryabin, *Appl Environ Microbiol*, 2009, **75**, 4580-4588.
36. R. A. Marcus and N. Sutin, *Biochimica Et Biophysica Acta*, 1985, **811**, 265-322.
37. J. J. Hopfield, *Proc. Natl. Acad. Sci. U. S. A.*, 1974, **71**, 3640-3644.
38. D. N. Beratan, C. R. Liu, A. Migliore, N. F. Polizzi, S. S. Skourtis, P. Zhang and Y. Q. Zhang, *Accounts of Chemical Research*, 2015, **48**, 474-481.
39. D. N. Beratan, S. S. Skourtis, I. A. Balabin, A. Balaeff, S. Keinan, R. Venkatramani and D. Q. Xiao, *Acc. Chem. Res.*, 2009, **42**, 1669-1678.
40. R. E. Blankenship, *Molecular Mechanisms of Photosynthesis*, Wiley, 2014.