Electron Bifurcation

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Electron bifurcation is the recently-recognized third mechanism of biological energy conservation. It simultaneously couples exergonic and endergonic oxidation-reduction reactions to circumvent thermodynamic barriers and minimize free energy loss. Little is known about the details of how electron bifurcating enzymes function, but specifics are beginning to emerge for several bifurcating enzymes. To date, those characterized contain a collection of redox cofactors including flavins and iron-sulfur clusters. Here we discuss the current understanding of bifurcating enzymes and the mechanistic features required to reversibly partition multiple electrons from a single redox site into exergonic and endergonic electron transfer paths.

The unifying feature of biological energy conservation mechanisms is the conversion of electrochemical potential to chemical bond energy. During substrate-level phosphorylation, metabolism of organic compounds is accompanied by formation of phosphorylated high-energy intermediates that can undergo phosphoryl transfer reactions with ADP to form ATP. Similarly, both organic and inorganic electron donors serve as substrates for electron transport chain complexes in electron transport phosphorylation. This process couples exergonic electron transfer from a more negative to a more positive electrochemical potential, to ion pumping and formation of a chemiosmotic potential. This in turn drives production of ATP by the ATPase. For decades it was generally accepted that substrate level phosphorylation and electron transport phosphorylation were the two fundamental biological energy conservation mechanisms. However, a third mode of energy conservation, referred to as electron bifurcation, has been recently recognized.

A conceptual framework

Electron bifurcation involves coupling exergonic and endergonic electron transfer reactions to limit energy loss. A redox reaction $A \rightarrow B$ with a significant positive free energy change at the concentrations of A and B in the cell does not occur spontaneously. In contrast, the reaction $A \rightarrow C$ may have a significantly negative free energy change and occur spontaneously at cellular concentrations of A and C. In biology, significant negative free energy represents energy wasted as heat unless it is harnessed through energy-conserving mechanisms. Electron bifurcation in its simplest

terms is a mechanism through which the reactions $A \rightarrow B$ and $A \rightarrow C$ are coupled ($2A \rightarrow B + C$) to generate a net exergonic reaction with minimal negative free energy change and maximal energy conservation.

The thermodynamics of electron transfer is dictated by the relative reduction potentials of the redox partners. Electron transfers that occur from a more negative to a more positive potential are favorable. Reduction potentials are sensitive to concentrations of reactants and products according to the Nernst equation. In complex electron transfer pathways having multiple stepwise electron transfer events, individual intermediate electron transfer reactions from more positive to more negative potentials can occur readily if the overall sequence of reactions is exergonic and thermodynamically favorable [1]. Alternately by coupling reactions via electron bifurcation, reactions that at face value appear to be thermodynamically unfavorable can be made to occur spontaneously. In short, a redox reaction from a more positive to a more negative reduction potential can occur spontaneously as long as the net change in electrochemical potential of the coupled reactions is still from more negative to more positive (**Figure 1**). Note that some types of bifurcating enzymes actually catalyze a “confurcating” reaction, where the oxidation of low and high potential donors are coupled to simultaneously reduce an acceptor of intermediate potential, although they are still referred to as bifurcating enzymes (**Figure 1**).

Electron bifurcation in biology was first proposed based on quinones some forty years ago by Peter Mitchell to explain the so-called Q-cycle catalyzed by Complex III of the

aerobic respiratory chain [2]. In this cycle, reducing equivalents produced by oxidation of quinol ($E_0' \sim +100$ mV) are fed into two distinct redox chains directed to opposite sides of the membrane. The first electron reduces a Rieske FeS cluster ($E_0' \sim +300$ mV) at the starting-point of a thermodynamically favorable, higher potential chain. The second electron reduces a low potential b-type heme ($E_0' \sim -100$ mV) [3]. In comparison, flavin-based electron bifurcation occurs at much more negative potentials, typically in the -300 to -500 mV range, than does quinone-based bifurcation. Flavin-based bifurcation can therefore be exploited by anaerobic microorganisms to generate low potential, high energy compounds such as hydrogen gas and reduced ferredoxin. Hence, as pointed out by Thauer [4], flavin-based bifurcation is probably as important for energy coupling in anaerobic metabolism as the quinone-based system is in aerobic metabolism. Indeed, it has been speculated that electron bifurcation may have played a key role in origin of life [5].

The discovery of flavin-based bifurcating enzymes

Flavin-based electron bifurcation was discovered as a result of observations by Wolfgang Buckel and Rolf Thauer in 2008. They showed that metabolism in some anaerobic microorganisms couples oxidation of NAD(P)H, with a standard reduction potential of -320 mV (at pH 7, E_0'), to reduction of the low potential electron carrier ferredoxin, a small iron and sulfur-containing protein with E_0' typically near -500 mV. This seemingly thermodynamically-unfavorable reaction occurs without additional energy input because it is intimately coupled to simultaneous reduction of the more

positive electron acceptor crotonyl CoA ($E_0' = -30$ mV) by NAD(P)H [6,7]. Shortly thereafter, it was shown that an unusual type of [FeFe]-hydrogenase couples reduction of protons to generate hydrogen gas (H^+/H_2 , $E_0' = -420$ mV) to endergonic oxidation of NADH with concomitant exergonic oxidation of reduced ferredoxin [8]. Hence, as shown in **Figure 2**, conventional hydrogenases cannot spontaneously evolve H_2 from NADH, but bifurcating hydrogenases evolve H_2 simultaneously from NADH and reduced ferredoxin. Importantly, the increased energy yields of these electron bifurcating reactions within a microbial cell can significantly affect yields of ATP and reduced metabolic end products. For example, fermentation from glucose to acetate can yield 4 ATP and 4 H_2 when a bifurcating hydrogenase is present to balance electron flow. However, without bifurcation, the yield of ATP and H_2 is lower since carbon products more reduced than acetate, such as alcohols or hydroxyacids, must also be generated to reoxidize the NADH produced by fermentation (see **Figure 2**).

Balancing the pyridine nucleotide pool

Other bifurcating enzymes play important roles in the efficiency of microbial metabolism by balancing electron flow associated with the pyridine nucleotide pool (NAD(P)H), see **Figure 4**. For example, NADH-dependent reduced ferredoxin:NADP⁺ oxidoreductase (Nfn) provides a means to control the relative amounts of NADH and NADPH, quantities thought to be key in regulating the balance of catabolism and anabolism in some organisms. These enzymes couple oxidation of NADH or NADPH with oxidation of reduced ferredoxin to drive reduction of NAD⁺ or NADP⁺ against unfavorable, non-

equilibrium ratios of NADH and NADPH. Another example is the Fix system present in some nitrogen-fixing microorganisms. Fix is hypothesized to couple NADH oxidation to reduction of quinone ($E_0' \sim 0$ mV) and ferredoxin ($E_0' \sim -500$ mV). The latter can then function as an electron donor for ammonia production by nitrogenase. In this case, the bacteria sacrifice the proton-motive force generated by the activity of Complex I of the aerobic respiratory chain, which oxidizes NADH and reduces quinone, to generate reduced ferredoxin to drive nitrogen fixation, thereby balancing the ratios of ATP and reducing equivalents for optimal nitrogen fixation. In energetic terms, transfer of two electrons from NADH (-320 mV) to ferredoxin (-500 mV), which is what Fix is hypothesized to accomplish, requires a driving force comparable to that released by the hydrolysis of one ATP (30.5 kJ/mol). Additional examples of bifurcation include H_2 - and formate-dependent reduction of heterodisulfides coupled to ferredoxin reduction in methanogenesis [9], formate dehydrogenases that couple oxidation of NADPH and reduced ferredoxin to drive formate production [10] and lactate dehydrogenase that couples exergonic electron flow from reduced ferredoxin to NAD to the endergonic reduction of NAD by lactate [11] (**Figure 1**).

Flavins in bifurcation

The bifurcating enzyme complexes have multiple electron transfer centers including both iron-sulfur (FeS) clusters and flavins, and a flavin is proposed to be the site of electron bifurcation [6,12]. The most common flavins are flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD), wherein the redox chemistry is associated with

the isoalloxazine (flavin) ring (**Figure 3**). The 2-electron E_0' of free flavins is slightly higher than that of the pyridine nucleotides, making flavins common pairwise electron acceptors from NADH and NADPH. However, the larger ring system of the flavin makes the one-electron reduced states (semiquinones, SQs) more accessible. Thus the flavin cofactors have redox properties intermediate between those of the single-electron transferring FeS clusters and the two-electron pyridine nucleotides. The reduction potentials of flavins can be tuned by interactions with the protein to allow flavoenzymes to interact with a wide variety of donors and acceptors involved in a multitude of metabolic processes.

Conventionally, successive reductions of a molecule become more difficult as a result of accumulation of negative charge. However, for free flavin, the two one-electron reduction potentials, i.e. $E_{Ox/SQ}^{0'}$ for reduction of oxidized flavin (Ox) to SQ and $E_{SQ/HQ}^{0'}$ for reduction of SQ to hydroquinone (HQ), are crossed. $E_{SQ/HQ}^{0'} > E_{Ox/SQ}^{0'}$, and the SQ is thermodynamically unstable (**see Figure 3**) [13]. This is a counter-intuitive situation in which it is easier to reduce the cofactor by a second electron than by the first, and the flavin appears to undergo a concerted two-electron reduction at the average of $E_{SQ/HQ}^{0'}$ and $E_{Ox/SQ}^{0'}$. Thus, flavins can accept electrons as a pair from pyridine nucleotides, bypassing the SQ state. However for the resulting doubly-reduced HQ state, removal of a first electron is less favorable than the second oxidation. Thus a stronger oxidant is needed, and the SQ intermediate that results is a much stronger reductant with a lower $E_{Ox/SQ}^{0'}$ than the HQ [5]. This is sometimes referred to as oxidation-triggered reduction because exergonic electron transfer to an acceptor with an E_0' above that of NAD(P)H

pays for endergonic electron transfer to a second acceptor with an E_0' below that of NADH. As we describe below, in addition to the thermodynamic constraints, this type of activity requires gating to prevent the second electron from also following the exergonic path [14].

Analogous thermodynamics can explain reversible bifurcating systems, wherein an oxidized pyridine nucleotide gets reduced but only one of the two reductants has a reduction potential more negative than that of NAD(P). The excess driving force available from the low E_0' redox reaction is conserved by accepting the other electron from a less reducing source. The strongly reducing donor produces SQ which, due to its proposed high $E^{0'}_{\text{SQ/HQ}}$, can be reduced by even a modest electron donor to the HQ capable of reducing a pyridine nucleotide. This suggests that regardless of the direction in which they operate, we can expect bifurcating enzymes to possess at least three redox cofactors, one of which will serve as the transducer between 2-electron and 1-electron reactions at a branch point. More complicated systems may be needed to explain how bifurcating enzymes orchestrate catalysis of 4-electron reactions involving the simultaneous oxidation of two different 2-electron donors and the reduction of two molecules of a 2-electron acceptor.

Gating electron flow

As described above, a cofactor with the appropriate thermodynamics for bifurcation is necessary but not sufficient. Additionally, bifurcation requires mechanisms that prevent

both electrons from traveling down the exergonic one of the two electron transfer pathways from a bifurcating site, i.e. the paths must be insulated from one another. In essence, electron transfer from the bifurcating site must be strictly gated. In quinone-based bifurcation in Complex III (and related cytochrome *bc* or *bf* complexes) gating of electron transfer is proposed to involve conformational and/or redox based mechanisms [15-18]. In the conformational gating model, a second electron is prevented from entering the thermodynamically favorable, higher potential chain by a pivoting motion of a Rieske-containing subunit that carries the FeS cluster to a distance too great for direct reduction by the SQ [19]. The gating motions of the Rieske subunit appear to be coupled to the redox state of the quinone [15-18].

The recent X-ray structure of the bifurcating flavoenzyme Nfn from *Thermotoga maritima* suggests that an analogous conformational gating of electron transfer may also play a key role in flavin-based bifurcation. Nfn is composed of two subunits, NfnA and NfnB, with a proposed bifurcating flavin site (within NfnB) positioned between two redox chains, see **Figure 4**. The chain in NfnA starts with a [2Fe-2S] cluster with Cys₃Asp coordination while that in NfnB commences with a [4Fe-4S] cluster with Cys₃Glu ligation (NfnB). The reduction potential of the [2Fe-2S] cluster was determined from EPR redox titration of the NfnI of *Pyrococcus furiosus* to be + 80 mV, but the E_0' values of the two [4Fe-4S] clusters in NfnB have yet to be established [20,21]. These E_0' s are expected to be significantly lower than -400 mV to couple to the reduction of ferredoxin ($E_m \sim -500$ mV) [22,23]. In the X-ray crystal structure of *T. maritima* Nfn, the [2Fe-2S] cluster is at the NfnA/NfnB interface, and at a distance of ~ 15 Å from the

proposed bifurcating flavin site (edge-to-edge), whereas the proximal [4Fe-4S] cluster is ~ 7 Å from the bifurcating b-FAD in the NfnB subunit. The difference in cofactor distances alone would contribute a $\sim 10^4$ s⁻¹ difference in electron transfer rate from the bifurcating b-FAD in favor of the [4Fe-4S] cluster [1].

One hypothesis for mechanistic control of electron transfer in Nfn is that the two subunits undergo a redox state-dependent, conformational change that alters the distance between the bifurcating b-FAD and the [2Fe-2S] cluster [16], analogous to Rieske FeS cluster domain movements in cytochrome *bc* Complex III. In essence, as shown in **Figure 4**, in one conformation, the redox centers are close enough to one another for fast electron transfer but, after electron transfer to form a-FAD SQ, NfnA shifts relative to NfnB to both promote transfer from b-FADH• to oxidized ferredoxin and to block additional electron transfer to a-FADH•. Movement effectively increasing the separation between [2Fe-2S] and b-FAD could prevent such electron transfer. In combination with the instability of SQ, this would virtually guarantee reduction of the less thermodynamically favorable, lower potential [4Fe-4S] cluster chain, i.e. separation of the two electrons. At present, this mechanism is a hypothesis, and we look forward to experimental results that will clarify details of this process.

Conclusions

It is yet to be determined how prevalent flavin-based bifurcating enzymes are and studies of these systems are still in their infancy. However, we know already that they

represent a unique mechanism of balancing electron flow in metabolism and of limiting free energy loss. We know bifurcating enzymes are complex, multi-subunit proteins with several redox cofactors including flavins and FeS clusters. Hypotheses to explain how these cofactors work as an ensemble to perform bifurcation and gate the resulting electron flow to the endergonic half reaction are just emerging. It is nonetheless clear that as the unifying features of the mechanisms of electron bifurcation are elucidated, fundamentally new chemistry will be revealed.

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Figure Legends

Figure 1. Bifurcating enzymes. The schemes depict electron transfer in the two types of bifurcating enzyme. *Left:* a two-electron donor (D) of intermediate reduction potential simultaneously provides electrons to electron acceptors with more negative (A_1) and more positive (A_2) potentials. *Right:* a two-electron acceptor (A) of intermediate reduction potential simultaneously accepts electrons from electron donors with more negative (D_1) and more positive (D_2) potentials. Seven types of reaction currently known to be catalyzed by bifurcating flavoenzymes are listed. Details on the enzymes can be found in [6,11,12].

Figure 2. Comparison of the reactions catalyzed by H_2 -producing bifurcating and non-bifurcating [FeFe]-hydrogenases, and their impacts on metabolic electron flow and energy conservation. *Upper:* Conventional hydrogenase spontaneously evolves H_2 from the low potential donor reduced ferredoxin (Fd) but oxidation of higher potential NADH coupled to proton reduction is an endergonic reaction. The bifurcating hydrogenase catalyzes ferredoxin and NADH oxidation simultaneously to produce H_2 . *Lower:* Glucose oxidation generates both NADH and reduced Fd and energy is conserved by ATP production via substrate level phosphorylation (SLP). Conventional hydrogenase oxidizes only reduced Fd, and NADH oxidation must be coupled to the generation of reduced carbon products (from pyruvate) that would otherwise be used for SLP. The bifurcating hydrogenase oxidizes both reduced Fd and NADH to generate the maximum amount of H_2 and ATP.

Figure 3. Properties of flavin that facilitate electron bifurcation. Destabilization of the semiquinone state of a flavin site in which the individual 1-electron potentials are crossed (below), compared with the conventional case of sequential electron transfer (above). These illustrative examples employ the two 1-electron potentials of *Azotobacter chroococcum* flavodoxin of $E^{0'}_{\text{SQ/HQ}} = -520 \text{ mV}$ and $E^{0'}_{\text{Ox/SQ}} = -110 \text{ mV}$ [24]. However, for the crossed potentials they are $E^{0'}_{\text{SQ/HQ}} = -110 \text{ mV}$ and $E^{0'}_{\text{Ox/SQ}} = -520 \text{ mV}$. By definition, these are the potentials at which $[\text{HQ}]=[\text{SQ}]$ and $[\text{SQ}]=[\text{Ox}]$, respectively (green circles). In the case of crossed potentials, the population of SQ is magnified by 100-fold to make it visible. Reduction potentials are reported relative to NHE. The anionic semiquinone structure is depicted although the neutral form may occur if it is protonated. R = ribitylphosphate for FMN, and R = ribose adenosine diphosphate for FAD.

Figure 4. Hypothetical mechanism of electron transfer in NADH-dependent reduced ferredoxin:NADP⁺ oxidoreductase (Nfn). This model is adapted from [16]. Electrons added to Nfn are indicated and counted in green, those leaving are in red. Beginning in the top left, upon binding of NADPH two electrons are proposed to be transferred (red arrows) to the b-FAD [16] yielding the HQ state of that flavin. This flavin is proposed to donate a single electron to each of two paths: exergonic transfer to the a-FAD at more positive potential via a Fe_2S_2 cluster in NfnA, and endergonic transfer to ferredoxin at more negative potential via two Fe_4S_4 clusters in NfnB. This would yield a-FAD SQ and reduced ferredoxin. A conformational change, here depicted as movement

of NfnA that separates the Fe_2S_2 from the b-FAD, is proposed to sever electron transfer between the two FADs. Dissociation of reduced ferredoxin and its replacement by an oxidized ferredoxin bearing an oxidized Fe_4S_4 cluster is depicted as associated with return of NfnA to its electron transfer competent position. Upon dissociation of NADP and binding of a second NADPH, b-FAD can be re-reduced, which once again would distribute one electron to each path producing a second reduced ferredoxin and providing a second electron to a-FAD. Doubly-reduced a-FAD would then be able to reduce NAD to NADH. Elements of this model are inspired by knowledge of ferredoxin NADP reductase, dihydroorotate dehydrogenase B [25], glutamate synthase [22] and butyryl-CoA dehydrogenase/electron transfer flavoprotein [14], but are currently hypothetical. The model nonetheless provides examples of gating needed to ensure that only one of each pair of electrons from b-FAD passes to NADH, and these will need to be tested experimentally.

Figure 1.

NADH-dependent acyl-CoA and ferredoxin reduction

H₂ or formate-dependent reduction of heterodisulfides and ferredoxin

NADPH- and ferredoxin-dependent formate production

NADH- and ferredoxin-dependent NADP reduction

NAD(P)H- and ferredoxin-dependent H₂ production

NADH-dependent quinone and ferredoxin reduction

Ferredoxin-dependent reduction of NAD by lactate

Figure 2.

Figure 3.

Figure 4.

Graphical Abstract