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Review Article

Electrochemistry of flavin-based electron bifurcation: ‘Current’ past and ‘potential’ futures

Syed Muhammad Saad Imran[a], Seth A. Wiley[a] and Carolyn E. Lubner

Abstract
Flavin-based electron bifurcation (FBEB) was discovered as a significant process of microbial energy conservation less than two decades ago. Since then, several classes of enzymes engaging in FBEB have been identified, all of which utilize a flavin cofactor that accepts two electrons and then transfers one along an exergonic (high-potential) pathway and the other along an endergonic (low-potential) pathway. We describe the critical role of electrochemical techniques, especially protein-film voltammetry and spectroelectrochemistry, in determining the mechanism and energetic landscape of FBEB in a characteristic enzyme. A prospectus of future directions involving currently unutilized electrochemical techniques is discussed with regards to the salient open questions in the field of FBEB.

Addresses
Biosciences Center, National Renewable Energy Laboratory, Golden, CO 80401, USA

Corresponding author: Lubner, Carolyn E. (Cara.Lubner@nrel.gov)
[a] Contributed equally.

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Introduction

Electron bifurcation is an efficient mechanism of energy conservation where a median-energy electron pair is split into different energy levels, akin to an electronic fulcrum (Figure 1a): one electron is raised to a higher energy level while the other's energy is decreased to a lower level [1–3]. Relative to the median-energy electron pair, the two bifurcated electrons have comparable potential gaps, leaving very little or no energy wasted in the process (Figure 1b). Of the two known types of biological electron bifurcation, flavin-based electron bifurcation (FBEB) was discovered more recently [4] and is the focus of this review, in contrast to the more established quinone-based electron bifurcation (QBEB) [2] involved in generating proton gradients. FBEB generally utilizes a more negative energetic landscape than QBEB (Figure 1c), which presents unique experimental challenges.

FBEB is an energetically significant, evolutionarily ancient [5], and widely utilized [6] process in many microorganisms, allowing for the generation of high-energy cellular reductants by coupling exergonic reactions, such as NAD^+ reduction, to endergonic reactions, such as ferredoxin reduction. These electron bifurcation reactions allow microbes to generate low potential reducing equivalents without expending excess energy. Conversely, electron confurcation, the reverse process of electron bifurcation, can be utilized to off-load excess reducing equivalents within the cell under highly reducing conditions. There have been several FBEB enzyme families discovered to date, most notably: NADH-dependent ferredoxin:$NADP^+$ oxidoreductase (Nfn) [7,8], Electron transferring flavoprotein (Etf) [4,9,10], the newly-designated Bfu family [11,12], and heterodisulfide reductase (Hdr) [13,14] (Figure 2). These structurally and functionally diverse enzymes employ FBEB to couple oxidation of several median-energy electron donors to reductions of low- and high-potential acceptors (Figure 2).

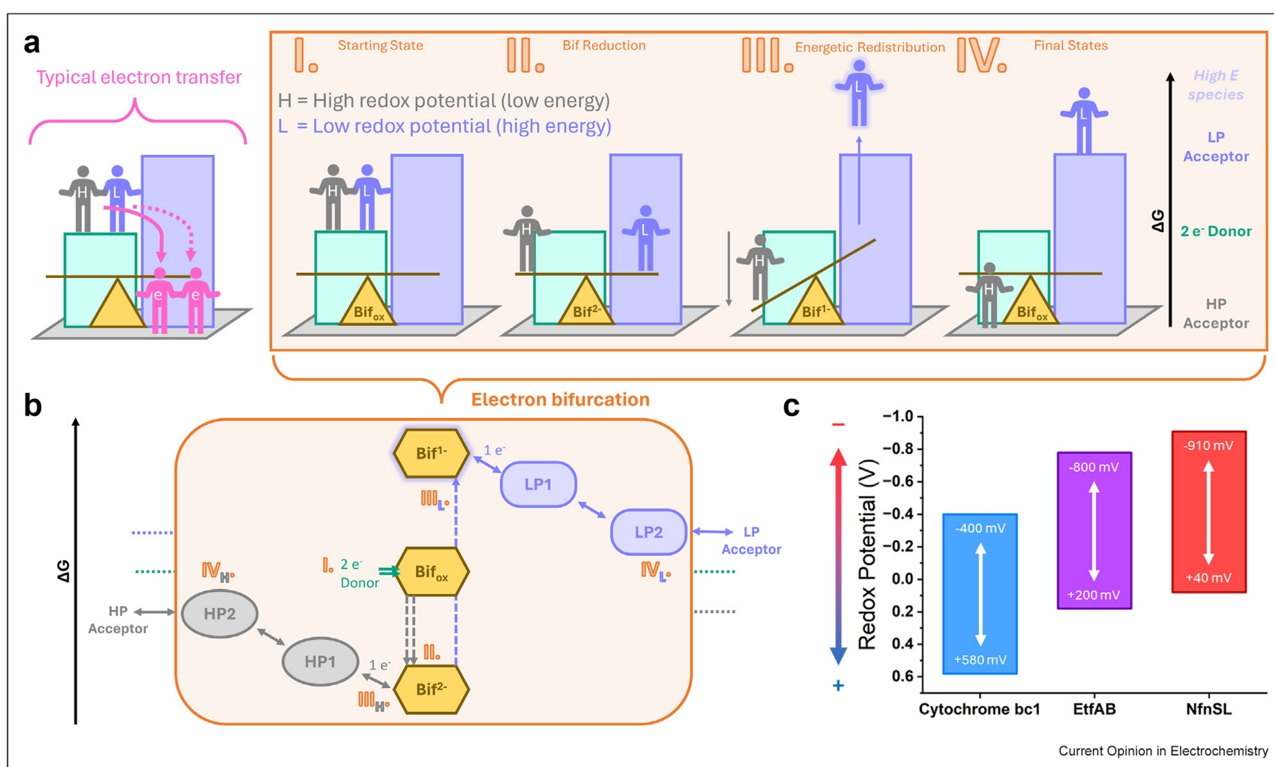
As the name suggests, FBEB relies on a flavin cofactor, such as flavin adenine dinucleotide (FAD) or flavin mononucleotide (FMN) for electron bifurcation [3]. Flavins can exist in three redox states: fully oxidized (Ox), singly-reduced semiquinone (SQ), and fully reduced hydroquinone (HQ) [15]. This makes them an ideal candidate for accepting an electron pair and then transferring it as two single electrons. Moreover, the bifurcating flavin has been shown to exhibit a specific arrangement of its one- and two-electron couples, termed ‘crossed potentials’ [2,16]. Many flavins that do not perform FBEB have a standard reduction potential arrangement: the first (Ox→SQ) reduction has a less negative potential than the second (SQ→HQ) reduction. However, in bifurcating flavins, the first reduction

Abbreviations

ASQ	anionic semiquinone
CD	circular dichroism
EPR	electron paramagnetic resonance
FAD	flavin adenine dinucleotide
Fd	ferredoxin
FMN	flavin mononucleotide
HQ	hydroquinone
KIE	kinetic isotope effect
MCD	magnetic circular dichroism
NAD(H)	nicotinamide adenine dinucleotide (hydride)
NADP(H)	nicotinamide adenine dinucleotide phosphate (hydride)
NSQ	neutral semiquinone
PCET	proton coupled electron transfer
PFC	protein film chronoamperometry
PF-CV	protein film cyclic voltammetry
PF-rFTV	protein film ramped Fourier transform voltammetry
PF-SWV	protein film square wave voltammetry
PFV	protein film voltammetry
SQ	semiquinone
SWV	square wave voltammetry
UV-Vis	ultraviolet-visible spectroscopy
XAS	X-ray absorption spectroscopy

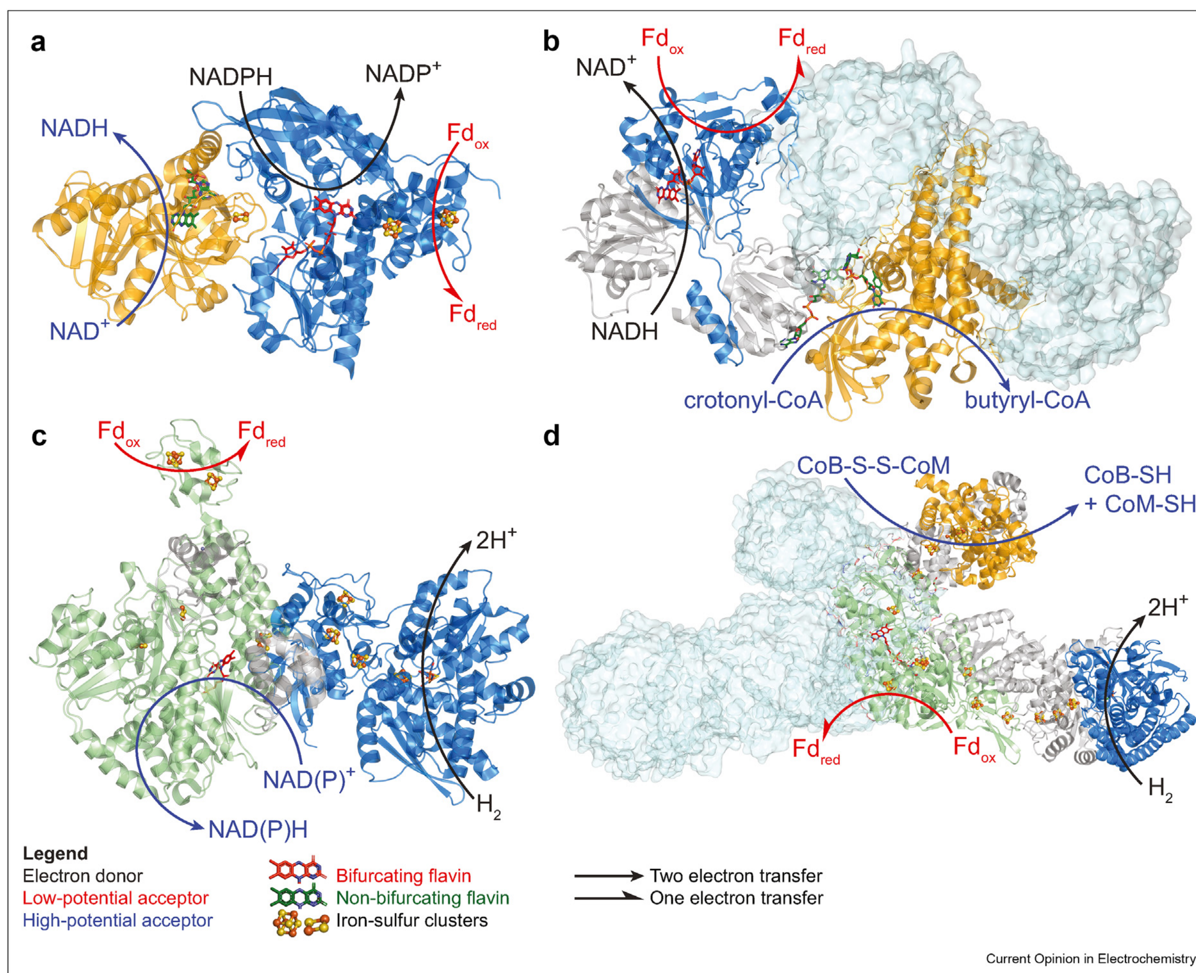
has a more negative potential than the second — in other words, the two potentials become ‘crossed’ or inverted, which appears critical to the energy conservation property of FBEB. In addition, FBEB enzymes typically contain other cofactors, most commonly additional FAD or FMN and iron-sulfur clusters (especially [2Fe−2S]

Figure 1



Generalized representation of electron bifurcation and its overall energetic landscape. Panel **a** shows a cartoon representation of both typical electron transfer (pink) and generalized electron bifurcation (orange box), highlighting the unique energetic coupling key to the bifurcation mechanism. “Typical” electron transfer moves an electron or electron pair from one higher energy state to a lower state (pink arrows). Electron bifurcation starts with an external 2-electron reductant with a median midpoint potential (I), which then reduces the site of electron bifurcation, represented as a yellow fulcrum, in a concerted manner (II). Electrons are bifurcated into high- and low-potential pathways (III), represented here simply as an energetic redistribution, condensing a complex multistep mechanism where the favorable transfer of one electron results in an unfavorable short-lived intermediate state. The electrons ultimately reach their respective high- and low-potential electron acceptors, where the overall energetic change is conserved between the electron pair (IV). Graph **b** depicts the overall bifurcation energy changes represented in panel **a**. The energy cost required to produce the low-potential acceptor is coupled to the energy redistributed from the high-potential acceptor, represented in III. Graph **c** shows some representative electron bifurcating enzymes involved in QBEB (cytochrome bc_1) and FBEB (EtfAB and NfnSL), and the large redox potential ranges spanned by their bifurcation mechanisms (refs. [7,33,38]).

Figure 2



Structural and functional diversity of the FBEB enzyme families discovered to date. **(a)** NfnSL from *Pyrococcus furiosus* (PDB ID: 5JCA, ref. [7]), **(b)** EtfAB/Bcd from *Clostridium difficile* (PDB ID: 5OL2, ref. [9]), **(c)** HydABC from *Thermoanaerobacter kivui* (PDB ID: 8A6T, ref. [12]), representative of the broader Bfu family (ref. [11]), and **(d)** HdrABC-MvhAGD from *Methanothermococcus thermolithotrophicus* (PDB ID: 5ODH, ref. [13]). The arrows and colored subunits indicate where the given reaction (likely) takes place, and subunits involved only in electron transfer between subunits are colored gray. Cyan surfaces in (b) and (d) represent the identical half of the dimeric enzyme structure not shown in cartoon representation.

and [4Fe−4S] clusters) [7,9], which facilitate electron transfer between donors and acceptors. In some cases, multimeric FBEB enzymes contain additional domains such as [NiFe]- and [FeFe]-hydrogenase, formate dehydrogenase, and heterodisulfide reductase units among others [11−13] (Figure 2). Most of the FBEB enzyme families also utilize large scale conformational movements that are thought to facilitate bifurcation by enabling specific electron transfer reactions, with Nfn being a notable exception [17,18].

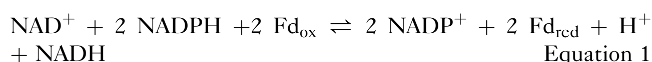
Commonly, it is microbes living at thermodynamic limits [19], where there are no electrons to spare, that utilize the highly efficient process of electron bifurcation. This makes its incorporation in biotechnology an attractive target, as electron bifurcation can confer many benefits: improving metabolic efficiency in currently used industrial organisms [20], increasing the pool of low-potential electron donors for carbon-fixation to enhance biofuel production, and decreasing reliance on inefficient metabolic pathways. Studying and understanding the mechanisms of electron bifurcation and confurcation is imperative for its more widespread adoption in biotechnological applications.

As with other redox active enzymes, electrochemical techniques have played a vital role in elucidating the mechanism of FBEB. Given that only a fraction of the range of electrochemical techniques available have been utilized to study FBEB, there is immense potential for unearthing more insights through electrochemistry. This review provides an overview of the electrochemical techniques that have been employed to date to probe FBEB and highlights others that should be considered in

future studies, to generate knowledge of phenomena such as electrophysical cofactor parameters, overall electron transfer kinetics, coupling between redox processes, and isolation and accumulation of mechanistic intermediates.

Overall mechanism of FBEB in Nfn

NADH-dependent ferredoxin:$NADP^+$ oxidoreductase (Nfn) is a prototypical FBEB enzyme. Compared to the other types of FBEB enzymes with multiple subunits and several cofactors, Nfn is a structurally less complex system to understand in the context of FBEB (Figure 2a). Consequently, Nfn is biochemically [21,22], biophysically [7,23], and electrochemically [7,23] the best characterized FBEB system. Nfn is a heterodimer of two subunits, NfnS and NfnL [7] (also termed NfnA and NfnB, respectively [8]), which each hold various cofactors comprising the high- and low-potential pathways (Figure 3a). Electron bifurcation and transfer along the low-potential pathway occurs within NfnL, while NfnS houses the high-potential pathway. In the bifurcating direction, the median-energy electron pair donor is NADPH, the high-potential acceptor is NAD^+, and the low-potential acceptor is oxidized ferredoxin (Fd). NADPH binds to NfnL at the oxidized bifurcating FAD (L-FAD), to which it transfers two electrons. The fully reduced L-FAD sends its first electron along the high-potential pathway to the [2Fe−2S] cluster of NfnS. This transforms L-FAD into the high-energy anionic semiquinone (ASQ) species with a highly negative reduction potential. The ASQ is very reactive and rapidly reduces the [4Fe−4S] cluster in NfnL proximal to it, which regenerates the oxidized L-FAD. The proximal cluster then transfers the electron to the second (distal) [4Fe−4S] cluster, which in turn reduces Fd, the low-potential acceptor. In the high-potential pathway, the reduced [2Fe−2S] cluster transfers its electron to the non-bifurcating FAD bound to NfnS (S-FAD), which then forms a neutral semiquinone (NSQ). After a second round of electron bifurcation and transfer along the high-potential pathway, S-FAD becomes fully reduced, and can then transfer a hydride to the NAD^+ bound to NfnS, generating NADH. The overall, reversible reaction in the bifurcating direction is:



$$NAD^+ + 2\ NADPH + 2\ Fd_{ox} \rightleftharpoons 2\ NADP^+ + 2\ Fd_{red} + H^+ + NADH \qquad \text{Equation 1}$$

These mechanistic details were informed by the integration of biophysical and electrochemical techniques which resulted in an overall thermodynamic landscape of electron bifurcation in Nfn. In particular, elucidation of the reduction potentials for each Nfn cofactor was pivotal to the construction of this landscape. Much of the electrochemical studies of Nfn were performed with the enzyme from *Pyrococcus furiosus* (*Pf* Nfn).

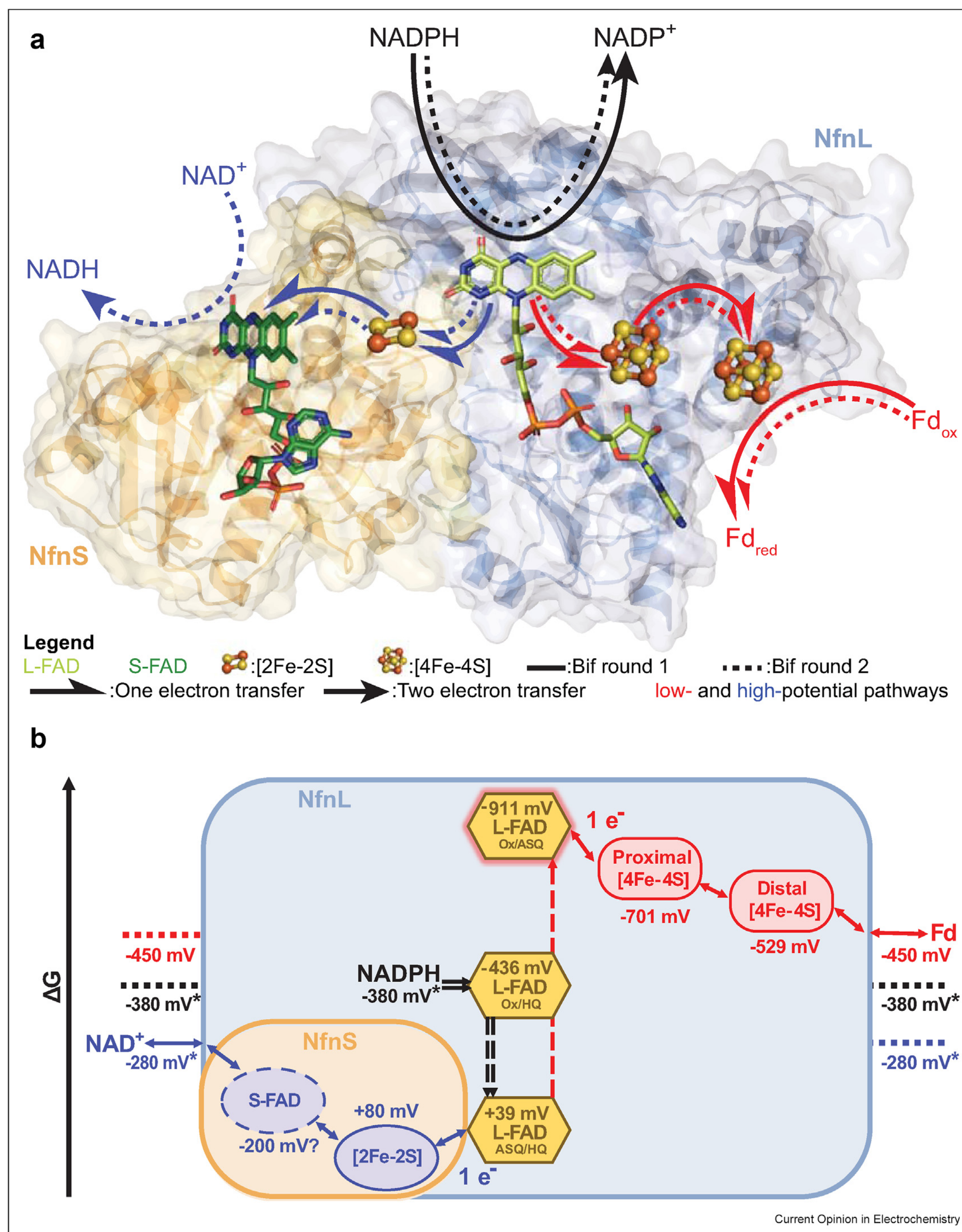
Electrochemistry of FBEB through the lens of Nfn

Early electrochemical studies were conducted with *Pf* Nfn natively expressed in *P. furiosus*. Protein film voltammetry (PFV) techniques, in particular square wave voltammetry (SWV), were utilized to probe the reduction potentials for the various cofactors [7]. Utilizing this highly-sensitive technique resulted in distinct values for the one-electron redox couples of the two [4Fe−4S] clusters, for transitions between their +1 and + 2 oxidation states. However, the two flavins gave overlapping peaks and thus an assignment could only be made for the average two-electron couple of L-FAD and S-FAD. Dye-based spectroelectrochemical redox titrations [24] were also conducted, but those too did not resolve the two-electron reduction potentials of the two FAD molecules.

Because L-FAD and the two [4Fe−4S] clusters are embedded deep enough within the structure of NfnL, it was possible to study recombinantly produced, isolated NfnL which yielded biophysical parameters without potential interference and spectral overlap from NfnS cofactors [23]. For the two [4Fe−4S] clusters, protein film SWV was utilized to determine their midpoint reduction potentials. Peak broadening and shifting was observed, indicating overlap between the clusters and L-FAD. Therefore, NfnL with minimal FAD loading was also studied. This protein gave sharper and more homogenous signals in square-wave voltammograms, centered around more accurate values of midpoint reduction potentials at −701 mV and −529 mV for the proximal and distal clusters, respectively. Therefore, SWV was suitable for NfnL with minimal L-FAD loading because the protein retained structural integrity − especially around the two clusters − even in the absence of much of L-FAD. Spectroelectrochemical titrations [25,26] of NfnL were also carried out to accurately determine the two-electron reduction potential of the bifurcating L-FAD, bypassing the limitations of SWV. This technique was feasible because FAD is the prominent chromophore around 400−500 nm in the optical spectrum of NfnL, with little interference from the [4Fe−4S] clusters [27]. The absorbance in this region also significantly changes in response to FAD reduction due to the applied potential. Due to the crossed potentials of the L-FAD, applied potential sweeps between 0 and −600 mV (in both reducing and oxidizing directions) yielded the two-electron reduction potential, $E_{OX/HQ}$, of −436 ± 8 mV at pH 8.8, calculated by fitting the absorbance at 450 nm to a Nernst function [23].

L-FAD ASQ is reactive and transient, and therefore unsuitable for being probed through equilibrium electrochemical methods. To overcome these limitations of electrochemistry, ultrafast transient absorption

Figure 3



Electron transfer and energetic landscape of FBEB in Nfn. (a) Overview of electron transfer between *Pf* Nfn cofactors (not shown to scale with protein structure, PDB ID: 5JCA, ref. [7]) during FBEB. Electron bifurcation (Bif) transfers one electron along each pathway, but NAD^+ is a two-electron acceptor. Therefore, the process – initiated by NADPH-driven reduction of L-FAD – repeats before NAD^+ is reduced to NADH in the second round of Bif (Equation (1)). **(b)** The energetic landscape of FBEB in *Pf* Nfn. Reduction potential of S-FAD is an estimate based on the average two-electron reduction potentials of the two FADs. *: NAD^+ and $NADP^+$ reduction potentials are estimates under physiological conditions (ref. [1]) and can vary significantly.

spectroscopy [28] was used to study its lifetime and rate of electron transfer, which also indirectly yielded its reduction potential. Briefly, L-FAD was photoreduced using a femtosecond-pulsed laser at 400 nm, generating an FAD excited state which rapidly abstracts a nearby electron to form ASQ [29,30]. The kinetics of the decay

of ASQ to regenerate the oxidized L-FAD was measured and yielded an extremely fast rate of electron transfer, k_{ET}, of 6.4–6.8 × 10^{10} s^{-1} (half-life of 10.2–10.8 ps) [7,23]. The Moser-Dutton formula [31–33] was then used to calculate ΔG°, the difference in free energies of the electron donor (ASQ) and acceptor (proximal [4Fe–4S] cluster):

$$\log k_{ET} = 13 - \frac{\beta(R-3.6)}{2.303} - \frac{\gamma(\Delta G^\circ + \lambda)^2}{\lambda}$$

Equation 2

where the parameters γ, β, and λ are based on averages from measured enzymatic electron transfer reactions, and R is the edge-to-edge distance between the bifurcating FAD and the proximal [4Fe–4S] cluster calculated from the *Pf* Nfn crystal structure (PDB: 5JFC [7]). The reduction potential of ASQ was calculated by subtracting the reduction potential term of the proximal cluster (−701 mV, *vide supra*) from ΔG°, to give $E_{OX/ASQ}$ = −911 ± 10 mV. This also enables the calculation of $E_{ASQ/HQ}$ = +39 mV through the following equation:

$$E_{OX/HQ} = \tfrac{1}{2}\ E_{OX/ASQ} + \tfrac{1}{2}\ E_{ASQ/HQ}$$

Equation 3

Therefore, through electrochemical approaches paired with ultrafast spectroscopy, all three reduction potentials for the bifurcating L-FAD were empirically determined, which show how crossed potentials underpin the Nfn FBEB mechanism.

The aforementioned experiments were critical in establishing the energetic landscape of electron bifurcation (Figure 3b). The first electron transfer, from L-FAD HQ to the [2Fe–2S] cluster of NfnS, is then exergonic by ∼40 mV (E_m values of +39 mV and +80 mV ([34], *vide infra*), respectively). This event triggers the formation of L-FAD ASQ, and the subsequent electron transfer events to the proximal and distal [4Fe–4S] clusters and Fd are exergonic (E_m values of −911, −701, −529, and ∼−450 mV, respectively). While the energetics of the non-bifurcating S-FAD are being investigated, the high-potential pathway involves some endergonic steps after the first electron transfer in order to reduce NAD^+.

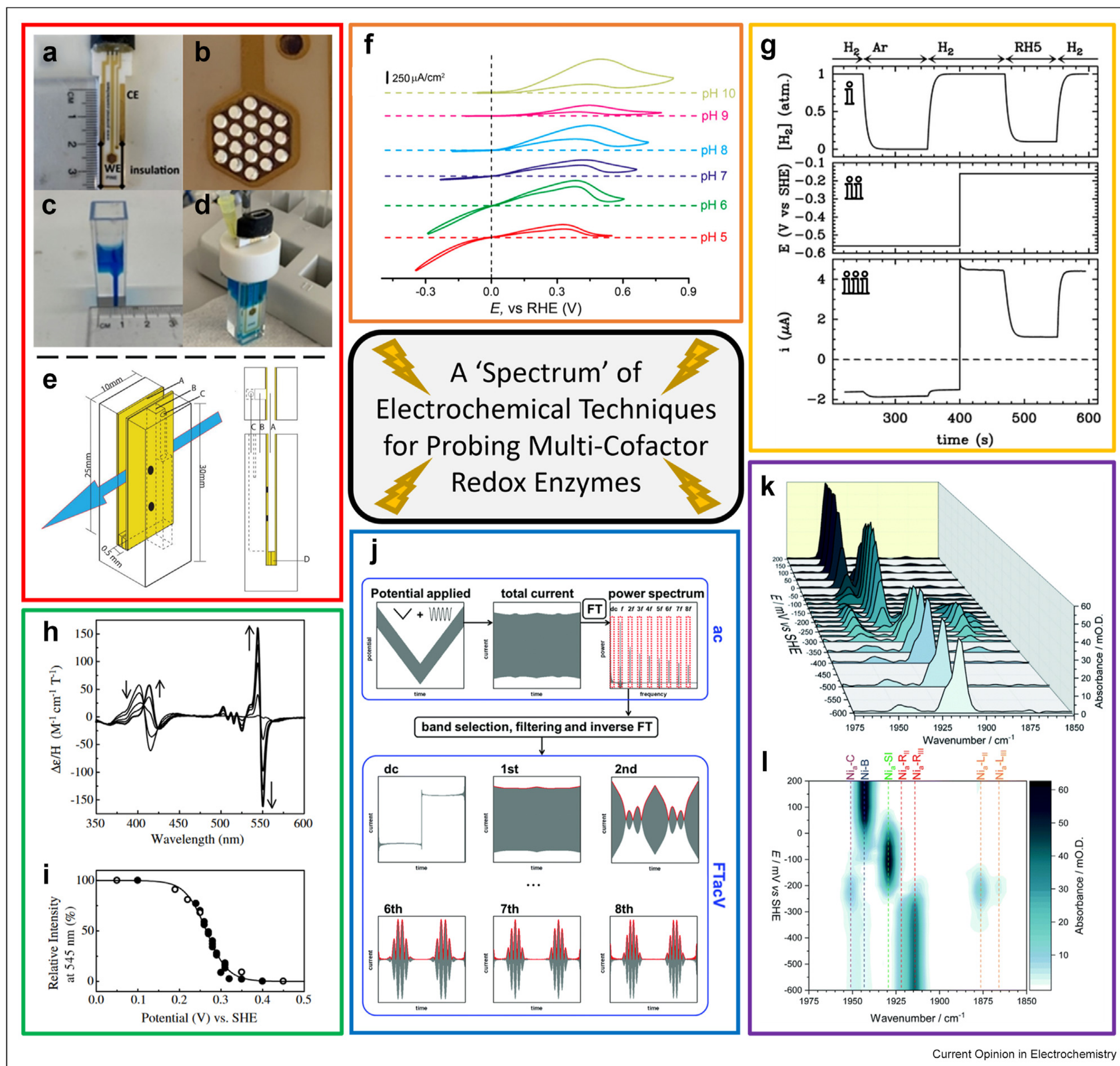
It should be noted that redox titration-coupled electron paramagnetic resonance (EPR) is a common technique used to assign reduction potentials to redox-active cofactors with unpaired electrons. In fact, it was used to determine the reduction potential of *Pf* NfnS [2Fe–2S] cluster (+80 mV) [34] when *Pf* Nfn was first discovered and mischaracterized as a sulfide dehydrogenase. However, given the highly negative reduction potentials involved for NfnL, not all cofactors could be characterized this way due to the difficulties associated with chemically reaching low potentials < −500 mV, and spectral interference from reductant [7,35] and mediator signals even if such low potentials were achieved. Moreover, due to the crossed potentials of L-FAD, a direct two-electron reduction (or oxidation) takes place between its Ox and HQ forms – both of which are EPR silent – in response to redox or electrochemical titrations. During equilibration of applied potentials, neither form converts to the EPR-active ASQ form, which renders EPR-coupled titrations ineffective as a means of determining any of the three reduction potentials of L-FAD. These factors emphasize the advantages of direct electrochemical techniques like protein-film SWV and spectroelectrochemistry, and why they were necessary in the determination of most of the midpoint reduction potentials for the Nfn cofactors.

Although there has been recent progress in structural determinations of a variety of FBEB enzymes [7,9,12,13,36,37] and many reports regarding their biochemical [10,21,22,38–40] and physiological activities [19,41,42], most systems do not have defined energetic landscapes yet. Mechanistic descriptions analogous to Nfn are still needed for many FBEB enzymes, particularly for the emerging Bfu family [11,12,36,37,43] which presents unique challenges for deconvoluting and assigning spectroscopic signals to discrete redox centers. Collectively, this knowledge will propel our understanding of the requisite properties and atomic level determinants for enabling FBEB. For example, are bifurcating electron transfer events controlled by similar thermodynamic considerations or is there flexibility across the FBEB enzymes in how they promote and control energy flux? In the following section, we describe the potential of current and upcoming electrochemical methods for deciphering the mysteries of FBEB.

Methods applicable to electron bifurcation

While some electrochemical techniques have been successfully applied to FBEB, at least for the Nfn enzyme, there is far more potential for incorporating other electrochemical applications to investigate open questions remaining in the field. One area deserving of further investigation involves using electrochemistry to probe electron transfer kinetics and the catalytic preferences within the bifurcation reaction to tease out the roles that kinetics and thermodynamics play in facilitating electron bifurcation. Building upon the current understanding of mechanisms and pathways, electrochemistry is a direct way to measure the impact of electron-transfer facilitated by the bifurcating site, and how the pathways are thermodynamically tuned for electron transfer. Despite the current lack of diversity of electrochemical methodology employed to unweave the mechanism of electron bifurcation, there are several methods used in the greater redox enzyme field that can be applied and adapted to study electron bifurcation in finer detail (Figure 4).

Figure 4



Various electrochemical and spectroelectrochemical methods used for studying multi-cofactor redox enzymes. **a-e** show two spectroelectrochemical cell setups, with **a-d** depicting the Pine Labs honeycomb cell (ref. [26], © 2022 The Authors. Published by Wiley-VCH GmbH), and **e** showing a 3D-printed insert for a standard 1 × 1 cm quartz cuvette (from Ref. [25]). **f** shows pH-dependent rotating disk cyclic voltammetry experiments performed on the [FeFe]-hydrogenase from *Clostridium reinhardtii* (*Cr*HydA1) (Reprinted with permission from Ref. [48]. Copyright 2019 American Chemical Society.) **g** shows a representative example of rotating disk protein film chronoamperometry on *Cr*HydA1, where **gi** shows the gas composition, **gii** shows the applied potential, and **giii** shows the resulting catalytic current from the conditions above (Reproduced from Ref. [44] with permission from the Royal Society of Chemistry.) **h** & **i** show an MCD-monitored *in situ* potentiometric titration of cytochrome *c* from horse heart, where **h** depicts room temperature MCD spectra and **i** shows the resulting redox curve of **h** at 545 nm (ref. [55]). **j** shows the iterative increase in signal sensitivity over noise of the FTacV method (Reproduced from Ref. [66] with permission from the Royal Society of Chemistry.) **k** & **l** show the 3D IR-spectroelectrochemical potential titration of a single crystal of Hyd1 [NiFe]-hydrogenase from *Escherichia coli* (Reproduced from Ref. [56] with permission from the Royal Society of Chemistry.).

A new frontier in the field of FBEB would be studying the catalytic processes of electron bifurcation via electrochemical methods. One method used extensively to study H_2 oxidation and competitive inhibition with hydrogenase enzymes is protein film chronoamperometry (PFC) and may be particularly useful for studying electron bifurcation [44–46]. PFC could be utilized to study bulk electron bifurcation and

confurcation kinetics under various substrate conditions [45,46], and to compare site-directed variants' activity to one another. With the PFC method, real-time rates for the oxidation or reduction of substrates can be measured directly by an electrode rather than with the addition of common mediator dyes, which often interact at non-specific sites within enzymes, as the readout. Protein film cyclic voltammetry (PF-CV), while currently underused in this field, has great potential [45–50] and offers a complimentary method for validating highly sensitive PF-SWV, with the added benefit of determining catalytic potentials. A major issue when applying these methods to bifurcating enzymes is that pyridine nucleotides (NAD(P)(H)), involved in three out of four FBEB enzyme families discovered to date (Figure 2), may give rise to interfering electrochemical signals through direct interaction with electrode surfaces [51,52]. Since FBEB reactions are tuned to specific stoichiometric conditions with these substrates, direct electrode interactions could potentially alter the ratios of reduced and oxidized cofactors and thus affect catalytic activity in unforeseen ways. This drawback severely limits the ability of electrochemical methods to deconvolute the mechanism of bifurcation, and necessitates advances in electrode design and development. Numerous issues plague current bioelectrochemical setups, including mass transfer, protein and substrate adsorption, and difficulty in teasing apart multiple electrochemical traces, which are compounded by factors intrinsic to FBEB: several cofactors involved, multiple oxidations states of flavin(s), conformational dynamics, and at least three substrates and products. Some of these issues could be addressed by working with individual subunits of FBEB enzymes. However, the aforementioned methodological issues require significant development in multichannel electrochemical setups in order to study catalysis in whole systems that are as complex as electron bifurcation.

To understand the effects of redox potential on spectroscopically-active species, combined electrochemical methods are necessary for studying FBEB. These include the simple combination of potentiostatic electrochemistry, including bulk electrolysis, with various spectroscopies such as EPR [53], conventional or magnetic circular dichroism (CD or MCD) [47,54,55], crystallographic microspectroscopy [56,57][b], X-ray absorption spectroscopy (XAS) [58,59], and possibly even infrared spectroscopy [49,60]. These simple, yet effective potentiostatic combination methods can help resolve intermediate states or favored interstate conditions as a function of the redox environment in contrast to the use of chemical reductants, which are often used in lieu of long equilibration times present in potentiostatic experiments [38,61]. Despite the utility, a caveat is that potentiostatic methods may be, depending on the spectroscopy, highly time and resource intensive, requiring high concentrations of active enzyme or specialized instrumental setups to facilitate the proper combined electrochemical method. This is especially germane if native or heterologous protein expression is not sufficiently developed. Yet another important route of further study is assessing the effect of temperature on the electrochemical characteristics [62] of bifurcating enzymes, especially those from hyperthermophilic organisms such as *P. furiosus* [63,64], as this may have large impacts on the understanding of the conditions and mechanisms operational *in vivo*. Temperature, however, has broad effects on all species within a sample, leading to highly convoluted results that require dedicated attention to overcome. Electrode and electrochemical cell design and development to utilize materials that can withstand the high temperatures that some FBEB systems naturally operate under (>65 °C) may additionally be needed to probe physiologically relevant regimes.

Further musing on the applicability of electrochemistry in breaking down the mechanisms across FBEB systems, there are several mechanistically significant proton-coupled electron transfer (PCET) events (for example hydride transfer and intermediate formation) [7,8,23]. Therefore, both potentiometric and SWV methods should also be used in conjunction with changes in buffer pH (or pD in D_2O for kinetic isotope effect (KIE) studies), to probe the effects of proton-dependence on kinetics and midpoint potentials. This will enable a better understanding of the role of protonated states (and PCET) in the bifurcation mechanism, such as with hydride transfers between pyridine nucleotides and flavins, provided further methodologies are developed to avoid the aforementioned issues, such as mass transfer and specificity. Experiments probing the effects of pH on redox potentials and subsequent intermediate-state populations have been used to great success in many hydrogenase systems [48,49,60], and while proton availability has more direct importance within the hydrogenase mechanism, proton effects on the bifurcation reaction may still have considerable mechanistic influence (see Equation (1)), especially with the more recently discovered complex, multimeric systems thought to utilize electron bifurcation [9,11–14,36,43]. Using newly developed bioelectrochemical techniques, such as protein film ramped Fourier transformed alternating current voltammetry (PF-rFTacV) [65,66], increased sensitivity and signal-to-noise can be applied to pH-sensitive cofactors as well for teasing apart the large, multimeric complexes facilitating a push to the boundaries of the current bifurcation field.

Conformational dynamics, which are central to many FBEB enzyme mechanisms, present a further confounding variable in the application of electrochemical techniques to the study of bifurcation. In

[b] This content is a preprint and has not been peer-reviewed.

general, the effects of conformational dynamics on reduction potentials are challenging to probe and not well understood. The electrode surface may not only inhibit or constrain protein adsorption, but it may also affect domain motion leading to non-physiologically relevant signals. However, an advantage could exist in looking at a rigid system in order to probe single pathway or non-productive electron transfer events, which may provide insights into how and why some electron transfer events are favored or disfavored. Given that the dynamics are an important feature to the FBEB mechanism in many systems, developing new electrode or immobilization strategies which allow for flexibility in the protein environment while still facilitating efficient electron transfer with the electrode will be required. Redox active hydrogels [67] may constitute a potential solution, depending on the desired mechanistic questions to be explored. Another option would be to investigate subunits individually and build complexity to examine how electrochemical and spectroscopic signals respond in different populations of subunit components. Crosslinking to favor specific conformations could also be used and may prove helpful in assigning electrochemical signals to discrete cofactors. Iterative processes like these may ultimately allow for a better understanding of the impact of conformational dynamics on cofactor tuning and their contributions to complex enzymatic mechanisms.

Furthermore, to confirm much of the observed electrochemical characteristics of electron bifurcation, models and simulations of the observations are integral to fully understanding the complete mechanism on a fundamental level. With the increasing accessibility and power of computer modeling, applying analytical simulations to electrochemical data provides context to observed phenomena and a path to understanding the physical parameters dictating redox events [68]. For protein electrochemistry, the modeling program QSoas allows for deconvolution of complex and overlapping electrochemical signals to pull apart multicomponent redox processes [69,70]. Employing models and simulations will be essential to the study of simple FBEB systems as well as the large, multimeric bifurcating complexes due to their colossal size and numerous redox-active cofactors, particularly to integrate signals from individual subunits (*vide supra*). Moreover, electrochemical data has been utilized to build theoretical models describing a universal landscape of electron bifurcation [71], and the expanded use and incorporation of electrochemical models and simulations will likely be pivotal in advancing the theoretical descriptions of complex biological phenomena.

Future outlook

While the importance of electrochemical experiments in teasing apart key physical characteristics of cofactors in the Nfn mechanism has been demonstrated, the broad range of techniques available in the electrochemical toolkit have yet to be used to their full potential in the field of FBEB as a whole. The inherent complexity found in even the simplest of bifurcation systems necessitates a strong mechanistic foundation, of which electrochemistry has yielded many details (for Nfn), especially in combination with other biochemical and biophysical methods (*vide supra*). Furthermore, as mentioned previously, elucidation of the larger, more complex multimeric systems [11,12,36,42,43] with their abundance of redox-active components is challenging due to the overlapping spectroscopic and electrochemical signals (EPR, UV-Vis, voltammograms etc.), for which combined electrochemical methods may be critical for deconvolution. Once the redox characteristics of bifurcating enzymes are fully defined, novel insights regarding the atomic and molecular determinants of FBEB will likely become clear. For example, is FBEB facilitated by similar energetic landscapes across systems or are unique features utilized to bifurcate electrons at drastically different potentials? Answers to this question will drive future endeavors to apply this knowledge to target bifurcation pathways for industrial or bioengineering purposes [72] as well as to build synthetic bifurcation and energy-conserving devices [73]. Holistically, electrochemistry is necessary to understand the kinetics and thermodynamics of electron transfer within the electron bifurcation process and its expanded implementation is encouraged. We hope this opinion on the current state of electrochemistry applied to FBEB enzymes encourages broader usage of more diverse electrochemical experiments in studying the complex mechanisms found in Nature.

Author contributions

Author contributions: S.M.S.I.: Conceptualization, Writing – Original Draft, Writing – Review and Editing, Visualization; S.A.W.: Conceptualization, Writing – Original Draft, Writing – Review and Editing, Visualization; C.E.L.: Conceptualization, Writing – Review and Editing, Visualization, Funding acquisition.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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