Supplementary Information for
An Uncharacteristically Low-Potential Flavin Governs the Energy Landscape of Electron Bifurcation

Courtney E. Wise, Anastasia E. Ledinina, David W. Mulder, Katherine J. Chou, John W. Peters, Paul W. King, and Carolyn E. Lubner

Corresponding Author: Carolyn E. Lubner
Email: Cara.Lubner@nrel.gov

This PDF file includes:
Supplementary Text
Figures S1 to S8
Tables S1 to S4
SI References

Table of Contents
Materials and Methods................................................................. S2
Figure S1 .................................................................................. S5
Figure S2 .................................................................................. S6
Figure S3 .................................................................................. S7
Figure S4 .................................................................................. S8
Figure S5 .................................................................................. S9
Figure S6 .................................................................................. S10
Figure S7 .................................................................................. S11
Figure S8 .................................................................................. S12
Table S1 .................................................................................... S13
Table S2 .................................................................................... S14
Table S3 .................................................................................... S15
Table S4 .................................................................................... S16
References .................................................................................. S17
Supplementary Materials and Methods

Plasmid Construction. The nfnL gene from *Pyrococcus furiosus* (Pf) DSM 3638, which encodes the large Nfn subunit (accession number AAL81451), was codon optimized and synthesized for heterologous expression in *Escherichia coli* by GenScript, USA in a pUC57 backbone. A Strep-II polypeptide affinity tag, with protein sequence GWSHPQFEK and corresponding sense DNA sequence 5'-GGCTGGAGCCACCAGCTTGAGAAA-3', was included at the N-terminus immediately following the methionine start codon of the synthesized nfnL construct. This gene was then subcloned into pCDFDuet™-1 (Novagen, MA, USA) using Ncol (5'-CCATGG-3') and BamHI (5'-GGATCC-3') restriction sites. The plasmid for coexpression of the NfnL and NfnS (accession number AAL81452) proteins resulted from codon-optimization of both genes for expression in *E. coli* by GenScript, USA. Each gene also encoded a C-terminal Strep-II tag with the aforementioned DNA sequence inserted just before a TAA stop codon. Both genes were subcloned from individual pUC57 vectors into a single pCDFDuet™-1 construct, using Ncol and BamHI restriction sites for nfnS and NdeI (5'-CATATG-3') and BgIII (5'-AGATCT-3') sites for nfnL. All plasmids constructed were verified by commercial sequencing (GenScript, USA).

Protein Overexpression and Purification. Heterologous expression of NfnL or NfnSL was carried out in terrific broth (TB) media using *E. coli* BL21(DE3) cells. A 1:100 inoculum from an overnight starter culture was added to 1 L volumes of sterile TB supplemented with trace metals, 0.4% glycerol, 1 mM ferric ammonium citrate (FAC), 10 μM FAD, and 125 μg·mL⁻¹ thiamine-HCl, along with 100 μg·mL⁻¹ streptomycin sulfate to select for the encoding plasmid. Cultures were incubated at 37 °C while shaking at 250 rpm until an optical density at 600 nm of ~0.6 was achieved, then additional FAC was added to a total final concentration of 2.5 mM and expression was induced via addition of 0.5 mM IPTG. Growth was continued at 37 °C and 250 rpm for ~60 minutes, after which 2 L of culture were combined with 10 mM sodium fumarate and 0.5% w/v d-glucose into one septum-sealed 2 L flask. Cultures were incubated for ~30 minutes at room temperature without shaking, then 1 mM L-cysteine was added, and cultures were sparged for ~16 hours overnight with argon gas at room temperature. Cultures were harvested anaerobically and by centrifugation for 10 minutes at 6,000 rpm, and cell pellets were washed via resuspension in anoxic 150 mM HEPES pH 8.8 with 200 mM NaCl, 5% glycerol, and 2 mM DTT followed by centrifuged, then resuspended in this buffer prior to storage in septum-sealed vials at -80 °C.

Cell lysis and protein purification were carried out in anaerobic chambers due to the O₂-lability of the Nfn cofactors. Resuspended cell pellets resulting from ~2 L of culture were thawed and combined with 75 units of Pierce Universal Nuclease, 20 mg lysozyme, and 1 tablet of Roche cOmplete EDTA-Free Protease Inhibitor Cocktail. Lysis was achieved using a Microfluidics M-110S microfluidizer, then lysate was heated at 75 °C for 1 hour and clarified by ultracentrifugation for another hour at 45,000 rpm. Clarified lysate was transferred onto a high-capacity Strept-Tactin XT column (IBA Life Sciences), which was equilibrated with 150 mM HEPES pH 8.8 with 200 mM NaCl and 5% glycerol (referred to as buffer A going forward), at a flow rate of 2 mL·min⁻¹ using an ÄKTA fast protein liquid chromatography system. Bound protein was washed with 2 column volumes (CV) of buffer A at 3 mL·min⁻¹, then eluted with 5 CV of buffer A containing 25 mM d-biotin. Eluate fractions possessing a chromophores characteristic of flavins and/or Fe/S clusters were pooled and quantified using the Bradford method. *Pf*-Nfn heterologously expressed in *E. coli* demonstrated soluble post-purification yields of 225 and 80 mg of protein per L culture for NfnSL and NfnL respectively, with purity confirmed by SDS-PAGE. As-purified proteins were found to lack full incorporation of the Fe/S and FAD cofactors required for catalysis (10 iron atoms and 2 FAD for NfnSL, and 8 iron atoms and 1 FAD per NfnL), necessitating reconstitution.

Cofactor Reconstitution and Protein Preparation. Iron-counting was carried out using previously described methodologies (1). FAD was quantified based on the flavin absorbance at 446 nm following digestion of the protein in 7.5 mM ascorbic acid and 0.1% v/v trichloroacetic acid at 65 °C for two hours and subsequent centrifugation. As-purified, both NfnL and NfnSL lacked full incorporation of the iron-sulfur (Fe/S) and FAD cofactors, which should result in 2 [4Fe-4S] clusters and 1 FAD in the former, and these plus one additional FAD and one [2Fe-2S] cluster in the latter. Following purification, reconstitution reactions containing 100 μM Nfn protein in buffer A with 0.75%
β-mercaptoethanol were incubated for 15 minutes at room temperature while stirring, then FAD was added in a 2-fold excess to the total number of flavin sites (200 µM for NfnL; 400 µM for NfnSL) and stirring continued for an additional 15 minutes. Iron was introduced by the addition of ferrous ammonium sulfate, over 5 minutes while stirring, in an 8-fold excess to the total number of Fe/S clusters (1.6 mM for L; 2.4 mM for SL). After 15 minutes, sodium sulfide was added in the same quantity and manner, and the reaction mixture was incubated for ~18 hours at 37 °C. Excess material was removed by desalting the reconstitution reaction using PD-10 desalting columns (GE Life Sciences) into buffer A and repeated washing with buffer A via ultrafiltration using a 50 mL Amicon Stirred Cell with a 10 kDa (for NfnSL) or 30 kDa (for NfnL) ultrafiltration disc until no pigment was visualized in the flow-through. Reconstituted protein was centrifuged to eliminate any precipitated material, then quantification assays were performed to assess FAD and iron-incorporation, revealing 9.9 ± 0.3 iron atoms and 1.9 ± 0.1 FAD per NfnSL (~99% Fe and ~95% FAD incorporation) and 7.5 ± 0.1 iron atoms and 1.3 ± 0.2 FAD per NfnL (~94% Fe and ~130% FAD incorporation). FeS-NfnL reconstituted only with iron and sulfur was found to contain 7.9 ± 0.3 iron atoms per NfnL (99% Fe incorporation) and retained 0.35 equivalents of FAD despite several attempts to remove any flavin by desalting. Reconstitution steps were carried out in an anaerobic chamber (Mbraun). Appropriate iron-sulfur cluster incorporation was noted to be highly dependent on reconstitution reaction pH, with optimal results achieved at pH 8.8. Both Nfn proteins and cofactors displayed stability for periods of several months when stored at 4 °C under anoxic conditions. The ability to achieve comparable reconstitution values between NfnL and NfnSL served as initial evidence that the cofactor environments in NfnL are not significantly altered in the absence of the small subunit in ways that would inhibit the incorporation of these critical prosthetic groups into the enzyme.

**Transient Absorption (TA) Spectroscopy.** The ultrafast (100 fs to 5.1 ns) TA spectrometer employed in this study used an amplified 4W Ti:sapphire laser (Libra, Coherent, 800 nm, 1 kHz, 100 fs pulse width), and Helios spectrometer (Ultrafast Systems, LLC). A fraction of the 800 nm Libra output was frequency-doubled in a beta barium borate crystal to produce the desired pump wavelength (400 nm in the data described here) for sample excitation, which was then directed into the Helios. The pump pulses were passed through a depolarizer and interrupted to 500 Hz by a synchronized chopper before reaching the sample. The pump pulse energy was 1.1 µJ per-pulse at the sample. Another fraction of the 800 nm Libra output was guided directly into the Helios for generation of the probe. Within the spectrometer, a white light continuum of wavelengths, including 340 – 800 nm, was generated using a 2 mm thick CaF₂ crystal. This beam was split into probe and reference beams. The probe beam was focused into the sample to overlap with the pump beam. The transmitted probe and reference beams were then focused into optical fibers coupled to multichannel spectrometers with complementary metal-oxide semiconductor detectors and 1 kHz detection rates. The reference signal was used to correct the probe signal for pulse-to-pulse fluctuations in the white-light continuum. The time delay between the pump and probe pulses was controlled by a motorized delay stage. For all TA measurements, 125 µM NfnL samples were prepared in an MBraun glovebox (N₂ atmosphere), sealed in a 2 mm pathlength quartz cuvette with a stir bar and stirred throughout data collection to prevent photodegradation. NfnL was poised in a partially reduced state with sub-stoichiometric amounts of NADPH so that the Fe/S clusters were reduced but the FAD was oxidized, allowing for enrichment of L-FAD SQ intermediate. Light was used to initiate formation of ASQ, as photons of sufficient energy promote L-FAD Ox to an excited state (FAD*). The highly oxidizing FAD* abstracts an electron from the nearby proximal iron-sulfur cluster to form the mechanistically relevant ASQ species which then relaxes by electron transfer to re-form L-FAD Ox. This ASQ is only observed when the iron-sulfur clusters are initially reduced. All experiments were conducted at room temperature. The change in absorbance signal (ΔA) was calculated from the intensities of sequential probe pulses with and without the pump pulse excitation. The data collection (350 pump shots per time point) was carried out four consecutive times and then averaged. The experiment was repeated at least three times. Data were corrected for spectral chirp using SurfaceXplorer (Ultrafast Systems). Fitting was performed in OriginPro using a single exponential function. Data is shown in Figure S3.
Electron Paramagnetic Resonance (EPR) Spectroscopy. EPR samples of 100 µM NfnL or NfnSL in buffer A were prepared with 1 mM NADPH or 10 mM sodium dithionite (NaDT). Following anoxic preparation in an M Braun anaerobic chamber, samples were transferred to quartz EPR tubes, frozen, and stored in liquid nitrogen. EPR spectra were collected using a Bruker ELEXSYS E500 continuous-wave X-band spectrometer and SHQ resonator at a modulation amplitude of 10 G and a microwave power of 1 mW. Cavity temperatures were maintained at 20 K via an in-cavity cryogen-free VT system from ColdEdge Technologies and Mercury iTC temperature controller from Oxford Instruments. Data was baseline-corrected using SpinCount (2), and EasySpin (3) simulations were performed in MATLAB for g-value assignment. Previous work on NfnSL has shown the reduction potentials of the [4Fe-4S] cofactors to be extremely negative (4), though overlapping signals in spectroscopic and electrochemical data did not allow for complete resolution. Notably, the absence of the [2Fe-2S] cluster and S-FAD cofactor in isolated NfnL simplified the analysis here. The EPR spectra of NfnSL and NfnL (Figure S2) revealed only partial reduction of the more positive distal cluster with NADPH ($E_m = -374$ mV at pH 8.8 (5, 6, 7, 8, 9)) as well as incomplete reduction of both clusters by sodium dithionite (NaDT; $E_m = -500$ mV at pH 10). Reduction of NfnSL with excess NaDT yielded an expectedly complex spectrum (violet) which was comprised of signals from multiple cofactors, including signals previously attributed to the NfnS [2Fe-2S]$^{1+}$ cluster (with $g = 2.047, 1.915, 1.805$ (4)) and spin interaction between this cluster and the neutral semiquinone radical of S-FAD (at $g = 2.02, 1.99$ (4)) that were absent in NfnL under the same conditions. The NaDT-reduced NfnL spectrum (red) appeared to comprise two signals arising from partial reduction of each [4Fe-4S] cluster. Isolation of the rhombic signal arising from the distal cluster was possible through reduction of NfnL with 1 mM NADPH (gold), revealing $g$-values of 2.032, 1.932, and 1.889 for this cofactor. This clarification allowed for assignment of $g = 2.035, 1.955, 1.916$ to the rhombic EPR signal deriving from reduction of the proximal cluster of NfnL, based on the NaDT-reduced spectrum along with electrochemical data for the [4Fe-4S] clusters. This information is expected to prove especially useful in future studies into the role of site differentiation for the proximal Fe/S cluster of NfnL.

Note on assignment of $E_{m,\text{ASQ/HQ}}$ from this work. SWV is a highly sensitive pulsed electrochemical technique that is able to detect species that accumulate only to trace levels (10). Detection of unstable intermediates, particularly of two electron processes, has been previously demonstrated with this methodology (11). In cases of inverted potentials, the second redox transition (SQ/HQ for flavins) may be observed in the oxidizing direction, with optimal visualization occurring when the one-electron reduced state is both thermodynamically and kinetically unstable (12). Because the one-electron reduced L-FAD ASQ of Nfn is both thermodynamically and kinetically unstable, the ASQ/HQ transition becomes detectable by SWV in the oxidizing direction.

Note on assignment of $L_{m,\text{Ox/ASQ}}$ from previous work (4). The $E_{m,\text{Ox/ASQ}}$ value for L-FAD of $-911$ mV (4) was determined from a rearrangement of Equation 7 using the $k_{ET}$ value from fitting of the ASQ intermediate decay to the Ox state in the TA experiments. The assumed parameters ($\gamma$, $\beta$, and $\lambda$) used in Equation 7 are based on averages from empirically measured enzymatic ET reactions described in SI reference (13). $R$ is the edge-to-edge distance from the PF-Nfn crystal structure (PDB 5JFC), taken as the distance from the O-atom of the L-FAD C2 to the S-atom of NfnL Cys101, which ligates the proximal [4Fe-4S]. The Ox/ASQ midpoint potential can be found by subtracting the calculated $\Delta G^\circ$ value from the $E_m$ of the proximal [4Fe-4S] acceptor. An error of ± 10 mV is estimated for the measured $E_{m,\text{Ox/ASQ}}$ value, based on the standard deviation of replicate data sets.
Figure S1. Dye-linked steady-state kinetics studies of reconstituted NfnSL (A) and NfnL (B) with the NADPH substrate reveal a decrease in activity with the isolated large subunit. Activity was assessed spectroscopically using the increase in absorbance at 600 nm, which corresponds to the reduction of the benzyl viologen dye by NADPH-reduced Nfn. Experiments were carried out in triplicate at room temperature and under anaerobic conditions. NADPH concentrations ranged from 0.25 mM to 5.0 mM while enzyme concentrations were held at 1 mg·mL$^{-1}$. Data was analyzed as described in the Methods section.
Figure S2. X-band EPR spectra of 100 µM NfnSL and NfnL reduced with 10 mM NaDT (violet and red, respectively) or 1 mM NADPH (dark blue and gold). Protein concentrations of 100 µM were incubated for ~20 minutes with the specified reductant prior to flash-freezing and storage in liquid nitrogen. Data was collected at 20 K, using a microwave power of 1 mW.
Figure S3. TA spectroscopy was performed to assess the kinetics of the L-FAD ASQ intermediate. The absorbance difference spectrum (green) at time = 1.6 ps shows ASQ absorption (360 nm) and Ox bleach (455 nm). Spectral contributions from the pump at 400 nm have been removed for clarity. As shown in the inset, kinetic traces at 360 nm (ASQ, blue) and 455 nm (Ox, teal) were fit to a single exponential (orange) to obtain the rate constant ($k_{ET}$), which was used to calculate a half-life ($t_{1/2}$) for the L-FAD ASQ of 10.8 ± 0.7 ps.
Figure S4. Representative fluorescence data showing NfnL binding to NADPH (A) and NADP⁺ (B). Following excitation of Trp and Tyr residues within the protein at 285 nm, a decrease in fluorescence emission at 345 nm (grey arrow in inset spectra) was observed which corresponded to addition of the NADPH substrate in increasing concentrations. The resulting change in protein fluorescence was fit to a Morrison (quadratic) function (Equation 8) for tight binding which yielded a $K_D$ for the interaction of NfnL and NADP(H) of 3.0 ± 0.4 µM with no appreciable difference in NfnL affinity for the oxidized versus reduced form of this substrate.
Figure S5. SWV measurements were conducted to determine the reduction potentials of the two [4Fe-4S] clusters of NfnL. The arrow indicates the direction of applied potential, which was in the reducing direction (high-to-low potential) for the data shown here. Fully reconstituted NfnL (green) showed peaks at -518 and -670 mV. When potential was applied in this direction, but not in the oxidizing direction (which is shown in Figure 3), the NfnL peak assigned to the distal cluster featured an intensity distribution similar to that observed for the concerted transfer of two electrons with free flavin, though the peak in NfnL possessed a more negative potential than that of free flavin. It was hypothesized that this altered signal distribution around the expected potential of the distal cluster and the ~50 mV more positive potential for the proximal cluster (versus previously published measurements with NfnSL (4)) derived from reduced resolution from overlapping signals between the two Fe/S clusters and the Ox/HQ potential of L-FAD. To assess this possibility, the analysis was repeated using purified NfnL that had been reconstituted with only iron and sulfur (FeS-NfnL) but which still retained 0.35 equivalents of FAD. FeS-NfnL (blue) featured signals of more similar distribution and intensity (relative to the voltammogram signals observed with NfnL) at -529 and -701 mV, which were assigned as the potentials of the distal and proximal Fe/S clusters respectively. Further analysis (Table S1) of the FAD signal contributions in the SWV of NfnL versus FeS-NfnL supported the assessment that the anomalies observed in NfnL owed to decreased resolution from the overlapping peaks of the three NfnL cofactors.
Figure S6. SWV measurements were conducted on NfnSL (dark blue) in the oxidizing direction and under the same conditions used for NfnL and FeS-NfnL (Figure 3 and Figure S5). Detection of this signal at +40 mV, attributed to the ASQ/HQ couple of L-FAD in NfnL, in NfnSL as well as isolated NfnL suggests that the $E_m^{\text{ASQ/HQ}}$ of the bifurcating flavin is largely unaltered by the absence of NfnS.
Figure S7. Oxidizing SWV scans of NfnL were measured to higher potentials (+500 mV) to screen for a signal corresponding to the previously calculated +359 mV (4) value of L-FAD $E_{m,ASQ/HQ}$, which was determined from an $E_{m,Ox/HQ}$ of -276 mV that was thought to be the average two-electron potential of the two Nfn FAD cofactors (4). Critically, no signal was observed at potentials above +40 mV.
Figure S8. Difference spectra (reduced minus oxidized) showing the optical changes occurring in free FAD (A) and NfnL L-FAD (B) from reduction during spectroelectrochemical experiments. The difference spectrum of NfnL demonstrates features characteristic of two-electron flavin reduction. The additional minor features in NfnL arise from interactions with the protein environment.
**Table S1. Determination of the FAD signal contribution to the -400 to -600 mV region in reducing SWV data.** The signal from FAD Ox/HQ overlaps with that of the distal [4Fe-4S] cluster to contribute to an increased current between -400 to -600 mV when potential is applied in the reducing direction. The approximate contribution of FAD to each signal was calculated by comparison of the peak heights from the proximal Fe/S peak and the less-negative adjacent peak in reducing-direction samples of NfnL and FeS-NfnL. From FAD counting assays, FeS-NfnL contains 0.35 FAD per NfnL and fully reconstituted NfnL contains 1.0 FAD per NfnL. The calculated FAD contribution for FeS-NfnL was found to be ~36% that of fully reconstituted NfnL, in agreement with FAD incorporation and current intensity arising from FAD.

| NfnL Sample | Fraction of signal deriving from L-FAD | FeS-NfnL L-FAD Signal/NfnL L-FAD Signal (%) |
|-------------|---------------------------------------|---------------------------------------------|
| NfnL        | 0.42 ± 0.02                           | 36 ± 2                                      |
| FeS-NfnL    | 0.15 ± 0.01                           |                                             |
Table S2. Refined reduction potentials of the NfnSL FAD cofactors. Data from SEC, TA, and SWV studies of the isolated large subunit were used to determine these values for L-FAD. The potentials for S-FAD were previously reported (4). Due to the refinement of L-FAD values, the S-FAD potentials likely require further investigation and should be regarded as tentative assignments.

| Flavin Redox Couple | L-FAD $E_m$ (mV) | S-FAD $E_m$ (mV) |
|---------------------|------------------|------------------|
| Ox/HQ               | -436             | -276 (4)         |
| Ox/SQ               | -911             | -300.5 (4)       |
| SQ/HQ               | +40              | -251.5 (4)       |
Table S3. Refined kinetic and thermodynamic parameters for Nfn electron transfer events. These values were calculated based on the experimental data from studies of the large subunit of Nfn presented here, along with previously described values for NfnS (4).

| Pathway       | Donor     | Acceptor          | $E_m$ (mV) | $\Delta G^\circ$ (eV) | R (Å) | Electronic Factor | Nuclear Factor | $k_{ET}$ (s$^{-1}$) |
|---------------|-----------|-------------------|------------|------------------------|-------|-------------------|---------------|---------------------|
| **high-potential** |           |                   |            |                        |       |                   |               |                     |
| L-FAD$_{HQ}$ | [2Fe-2S]  | +40               | +80        | -0.040                 | 13.1  | 4.5               | 1.9           | $3 \times 10^8$     |
| [2Fe-2S]     | S-FAD$_{Ox}$ | +80               | -300.5     | 0.2215                 | 9.6   | 2.9               | 3.8           | $9 \times 10^4$     |
| [2Fe-2S]     | S-FAD$_{NSQ}$ | +80               | -251.5     | 0.1725                 | 9.6   | 2.9               | 3.4           | $3 \times 10^5$     |
| **low-potential** |           |                   |            |                        |       |                   |               |                     |
| L-FAD$_{ASQ}$ | [4Fe-4S]$_{prox}$ | -911             | -701       | -0.210                 | 5.4   | 0.9               | 1.1           | $1 \times 10^{11}$ |
| [4Fe-4S]$_{dist}$ |          | -701               | -529       | -0.172                 | 9.6   | 2.9               | 1.2           | $8 \times 10^8$     |
| **short-circuits** |           |                   |            |                        |       |                   |               |                     |
| L-FAD$_{HQ}$ | [4Fe-4S]$_{prox}$ | +40               | -701       | 0.741                  | 5.4   | 0.9               | 9.2           | $9 \times 10^2$     |
| L-FAD$_{ASQ}$ | [2Fe-2S]  | -911              | +80        | -0.991                 | 13.1  | 4.5               | 0.4           | $1 \times 10^8$     |
Table S4. Comparisons of semiquinone stability constants, $K_S$, for Nfn and other electron bifurcating systems. Nfn represents the first instance in which all three redox potentials of the bifurcating flavin have been assessed empirically to allow for determination of log$K_S$ without the need for inferences from other bifurcating cofactors or assumptions regarding $E_m$ values. The log$K_S$ reported here for L-FAD highlights an emerging theme among bifurcating systems utilizing different cofactors of semiquinone instability while providing validating support for several assumptions made in calculations of this constant for other FBEB enzymes.

| System         | Bifurcating Cofactor | Log$K_S$      | Reference          |
|----------------|----------------------|---------------|--------------------|
| Pf-Nfn         | L-FAD                | -21.5 $^a$    | (4, 14, 15)        |
|                |                      | -16.1 $^b$    | This work          |
| Complex III    | Q₀ Quinone           | -14 to -16.3  | (16, 17, 18, 19)   |
| EtfAB-Bcd      | β-FAD                | -7.3 to -15.2 $^c$ | (20, 21, 22) |
| EtfABCX        | BF-FAD               | -6.5 to -15.3 $^d$ | (23) |

$^a$ Previously calculated value (14, 15) using the average Ox/HQ potential of the L-FAD and L-FAD cofactors of -276 mV (4).

$^b$ Values used in determination of this value were all experimentally derived. SEC, TA spectroscopy, and SWV were used to assess the L-FAD $E_{m,Ox/HQ}$, $E_{m,Ox/ASQ}$, and $E_{m,ASQ/HQ}$, respectively.

$^c$ Potentials used for calculation of the log$K_S$ for the EtfAB-Bcd enzyme from Acidaminococcus fermentans in a 2016 article were inferred using the $E_{m,Ox/HQ}$ for β-FAD of the Etf from Megasphaera elsdenii of -279 mV (24) along with assumed Ox/SQ and SQ/HQ couples based on potentials of acceptors of the bifurcated electrons (20). In a recent 2020 publication, the $E_{m,Ox/HQ}$ for β-FAD from Acidaminococcus fermentans was empirically determined to be -271 mV, and the single electron coupled were determined using an assumed $K_S$ value of $10^{-15}$ (22). $E_m$ values for log$K_S$ of EtfAB-Bcd from Clostridium difficile also used the $E_{m,oxid}$ of the bifurcating flavin from the M. elsdenii Etf (24) and the gap between one-electron potentials from Q₀ of the cytochrome bc₁ complex (21).

$^d$ Range was tabulated using the difference in one-electron potentials for the bifurcating quinone in complex III along with an experimentally determined Ox/HQ couple for BF-FAD, TA evidence of inverted potentials, and information regarding the acceptor $E_m$ of the bifurcated electron along the high-potential branch (23).
SI References

1. W. W. Fish, Rapid colorimetric micromethod for the quantitation of complexed iron in biological samples. *Methods Enzymol.* **158**, 357-364 (1988).
2. D. T. Petasis, M. P. Hendrich, Quantitative Interpretation of Multifrequency Multimode EPR Spectra of Metal Containing Proteins, Enzymes, and Biomimetic Complexes. *Methods Enzymol.* **563**, 171-208 (2015).
3. S. Stoll, A. Schweiger, EasySpin, a comprehensive software package for spectral simulation and analysis in EPR. *J. Magn. Reson.* **178**, 42-55 (2006).
4. C. E. Lubner, D. P. Jennings, D. W. Mulder, G. J. Schut, O. A. Zadvrony, 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, J. W. Peters, Mechanistic insights into energy conservation by flavin-based electron bifurcation. *Nat. Chem. Biol.* **13**, 655-659 (2017).
5. D. Amador-Noguez, I. A. Brasg, X. J. Feng, N. Roquet, J. D. Rabinowitz, Metabolome remodeling during the acidogenic-solventogenic transition in Clostridium acetobutylicum. *Appl. Environ. Microbiol.* **77**, 7984-7997 (2011).
6. H. Huang, S. Wang, J. Moll, R. K. Thauer, Electron bifurcation involved in the energy metabolism of the acetogenic bacterium Moorella thermoaceticola growing on glucose or H2 plus CO2. *J. Bacteriol.* **194**, 3689-3699 (2012).
7. S. K. Spaans, R. A. Weusthuis, J. van der Oost, S. W. Kengen, NADPH-generating systems in bacteria and archaea. *Front. Microbiol.* **6**, 742 (2015).
8. F. Q. Schafer, G. R. Buettner, "Redox State and Redox Environment in Biology" in *Signal Transduction by Reactive Oxygen and Nitrogen Species: Pathways and Chemical Principles*, H. J. Forman, J. Fukuto, M. Torres, Eds. (Springer Netherlands, Dordrecht, 2003), 10.1007/0-306-48412-9_1, pp. 1-14.
9. F. L. Rodkey, J. A. Donovan, Jr., Oxidation-reduction potentials of the triphosphopyridine nucleotide system. *J. Biol. Chem.* **234**, 677-680 (1959).
10. S. P. Kounaves, "Voltammetric Methods" in *Handbook of Intrumental Techniques for Analytical Chemistry*, F. Settle, Ed. (Prentice Hall, New Jersey, 1997), chap. 37, pp. 709-726.
11. M. Lovrić, Š. Komorsky-Lovrić, Theory of Square-Wave Voltammetry of Two-Electron Reduction with the Adsorption of Intermediate. *Int. J. Electrochem. Sci.* **2012**, 596268 (2012).
12. Š. Komorsky-Lovrić, M. Lovrić, Square-wave Voltammetry of Two-step Electrode Reaction. *Int. J. Electrochem. Sci.* **9**, 435-444 (2014).
13. C. C. Page, C. C. Moser, X. Chen, P. L. Dutton, Natural engineering principles of electron tunnelling in biological oxidation-reduction. *Nature* **402**, 47-52 (1999).
14. F. Baymann, B. Schoepp-Cothenet, S. Duval, M. Guiral, M. Brugna, C. Baffert, M. J. Russell, W. Nitschke, On the Natural History of Flavin-Based Electron Bifurcation. *Front. Microbiol.* **9**, 1357 (2018).
15. W. Buckel, R. K. Thauer, Flavin-Based Electron Bifurcation, A New Mechanism of Biological Energy Coupling. *Chem. Rev.* **118**, 3862-3886 (2018).
16. H. Zhang, A. Osyczka, P. L. Dutton, C. C. Moser, Exposing the complex III Qo semiquinone radical. *Biochimica et Biophysica Acta (BBA) - Bioenergetics* **1767**, 883-887 (2007).
17. H. Zhang, S. E. Chobot, A. Osyczka, C. A. Waight, P. L. Dutton, C. C. Moser, Quinone and non-quinone redox couples in Complex III. *J. Bioenerg. Biomembr.* **40**, 493-499 (2008).
18. H. Ding, C. C. Moser, C. D. Robertson, M. K. Tokito, F. Daldal, P. L. Dutton, Ubiquinone pair in the Qo site central to the primary energy conversion reactions of cytochrome bc1 complex. *Biochemistry* **34**, 15979-15996 (1995).
19. A. R. Crofts, S. Hong, C. Wilson, R. Burton, D. Victoria, C. Harrison, K. Schulten, The mechanism of ubihydroquinone oxidation at the Qo-site of the cytochrome bc1 complex. *Biochimica et Biophysica Acta (BBA) - Bioenergetics* **1827**, 1362-1377 (2013).
20. N. P. Chowdhury, K. Klomann, A. Seubert, W. Buckel, Reduction of Flavodoxin by Electron Bifurcation and Sodium Ion-dependent Reoxidation by NAD+ Catalyzed by Ferredoxin-NAD+ Reductase (Rnf). *J. Biol. Chem.* **291**, 11993-12002 (2016).
21. J. K. Demmer, N. Pal Chowdhury, T. Selmer, U. Ermler, W. Buckel, The semiquinone swing in the bifurcating electron transferring flavoprotein/butyryl-CoA dehydrogenase complex from Clostridium difficile. *Nat. Commun.* **8**, 1577 (2017).

22. J. Sucharitakul, S. Buttranon, T. Wongnate, N. P. Chowdhury, M. Prongjit, W. Buckel, P. Chaiyen, Modulations of the reduction potentials of flavin-based electron bifurcation complexes and semiquinone stabilities are key to control directional electron flow. *FEBS J.* 10.1111/febs.15343 (2020).

23. G. J. Schut, N. Mohamed-Raseek, M. Tokmina-Lukaszewska, D. W. Mulder, D. M. N. Nguyen, G. L. Lipscomb, J. P. Hобen, A. Patterson, C. E. Lubner, P. W. King, J. W. Peters, B. Bothner, A. F. Miller, M. W. W. Adams, The catalytic mechanism of electron-bifurcating electron transfer flavoproteins (ETFs) involves an intermediary complex with NAD+. *J. Biol. Chem.* **294**, 3271-3283 (2019).

24. K. Sato, Y. Nishina, K. Shiga, Interaction between NADH and electron-transferring flavoprotein from Megasphaera elsdenii. *J. Biochem.* **153**, 565-572 (2013).