Supporting Information

The catalytic mechanism of electron bifurcating electron transfer flavoproteins (ETFs) involves an intermediary complex with NAD^+

Gerrit J. Schut[1], Nishya Mohamed-Raseek[2], Monika Tokmina-Lukaszewska[3], David W. Mulder[4], Diep M. N. Nguyen[1], Gina L. Lipscomb[1], John P. Hoben[2], Angela Patterson[3], Carolyn E. Lubner[4], Paul W. King[4], John W. Peters[5], Brian Bothner[3], Anne-Frances Miller[2] and Michael W. W. Adams[1]*

[1]Department of Biochemistry and Molecular Biology, University of Georgia, Athens, GA 30602,

[2]Department of Chemistry, University of Kentucky, Lexington, KY 40506

[3]Department of Chemistry and Biochemistry, Montana State University, Bozeman, MT 59717

[4]Biosciences Center, National Renewable Energy Laboratory, Golden, CO 80401

[5]Institute of Biological Chemistry, Washington State University, Pullman WA 99163

Supplemental Figures S1 – S13

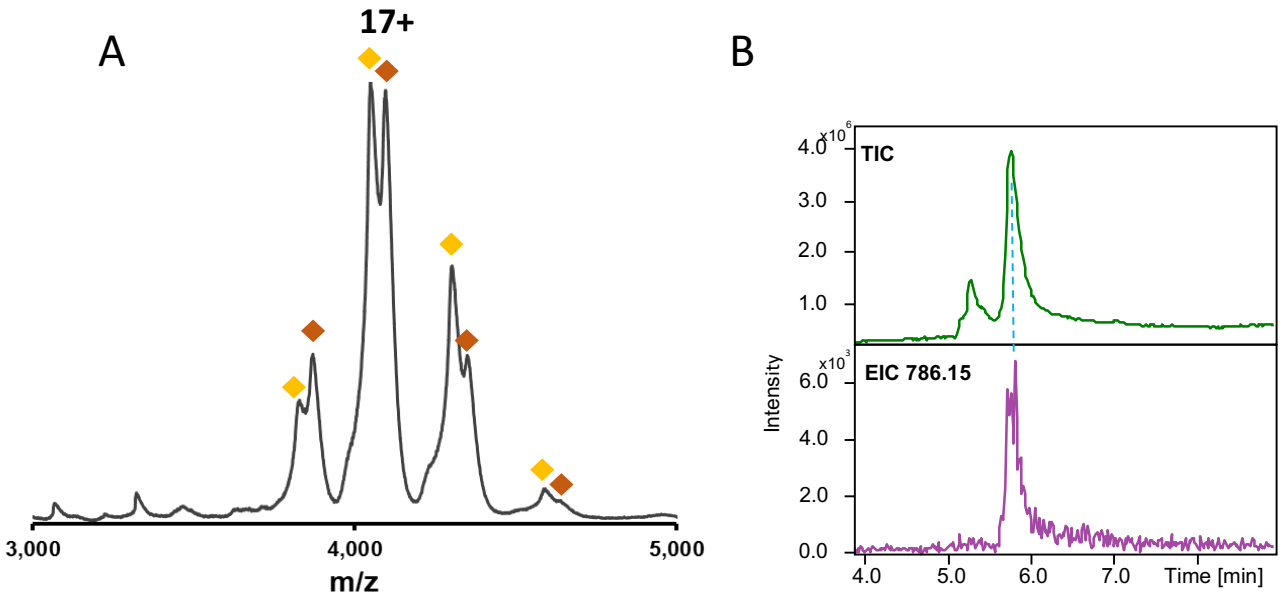
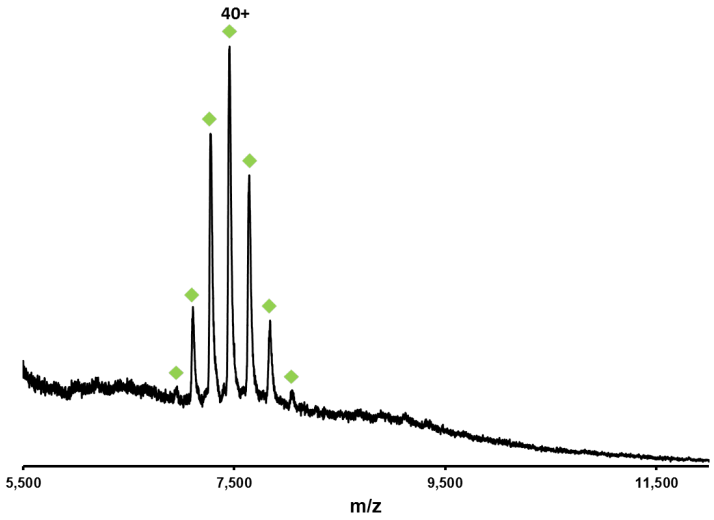
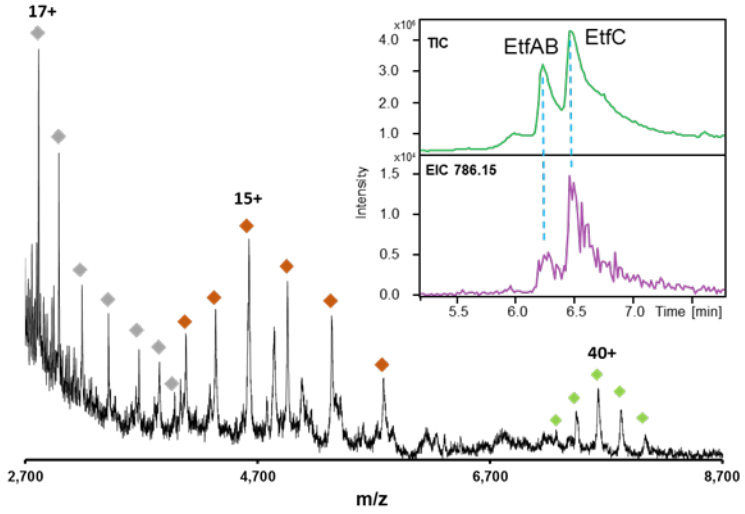


Figure S1. A. *Pae* EtfAB in the gas phase. The as purified EtfAB complex in the gas phase is a mixture of the AB dimer with one or two FAD molecules (approximate ratio of 50:50). Yellow (complex with one FAD) and brown (complex containing two FADs) diamonds denote charge state envelopes centered around a charge of 17+. **B.** total ion current (TIC) of *Pae* EtfAB complex separation on reverse phase column; bottom panel represents extracted ion chromatogram (EIC) of the molecular ion of FAD (proton adduct).

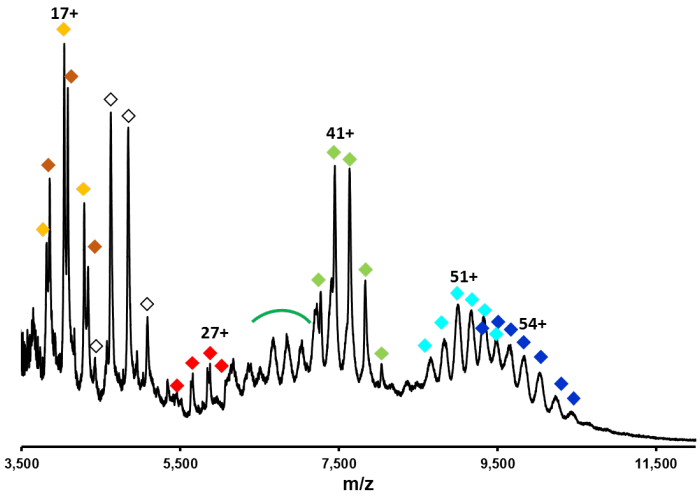
A


40+
5,500
7,500
9,500
11,500
m/z

B


17+
15+
40+
TIC
EtfAB
EtfC
EIC 786.15
Intensity
Time [min]
2,700
4,700
6,700
8,700
m/z

C


17+
41+
51+
54+
27+
3,500
5,500
7,500
9,500
11,500
m/z

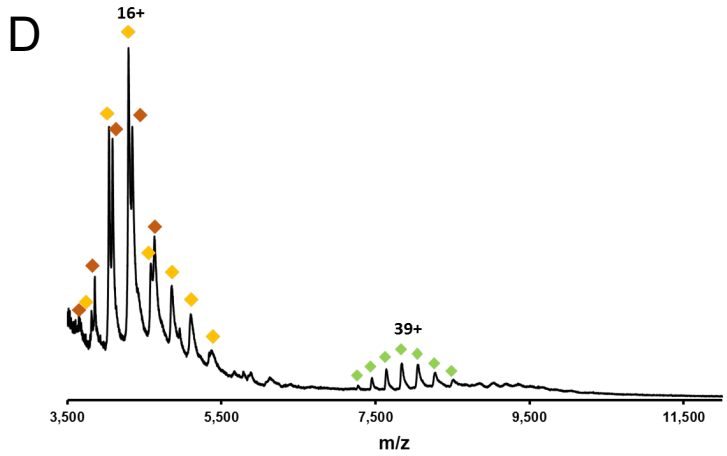


Figure S2. Native mass spectra of the *Pae* EtfABCX in the gas phase recorded at different energies. **A.** Spectrum of the intact EtfABCX complex (dimer) recorded at low collision energy (trap collision energy set to 10V) and low capillary voltage (1.7kV) and minimum back pressure (0.02Bar). **B-D.** Spectra of the partially dissociated EtfABCX complex recorded at variable conditions: **B**, trap 20V, capillary 1.7V, back pressure 0.02Bar; **C**, trap 20V, capillary 1.7V, back pressure 0.2Bar; **D**, trap 60V, capillary 1.7V, back pressure 0.2Bar. Differently color-coded diamonds correspond to the charge state distribution of the following *Pae* Etf complex species: green, intact EtfABCX complex dimer; brown, EtfAB dimer with 2 FAD (full occupancy); grey, EtfC with 1FAD (full occupancy); yellow, EtfAB dimer with 1 FAD; red, EtfABCX (monomer); blue and cyan, EtfABCX tetramer and trimer, respectively; white, unknown sub-complex. The green arch denotes several forms of incomplete EtfABCX. The predominant charge state is indicated above each envelope. **B Inset.** The TIC of the *Pae* EtfABCX complex separation on a reverse phase column while the bottom panel represents the EIC of the molecular ion of FAD (proton adduct).

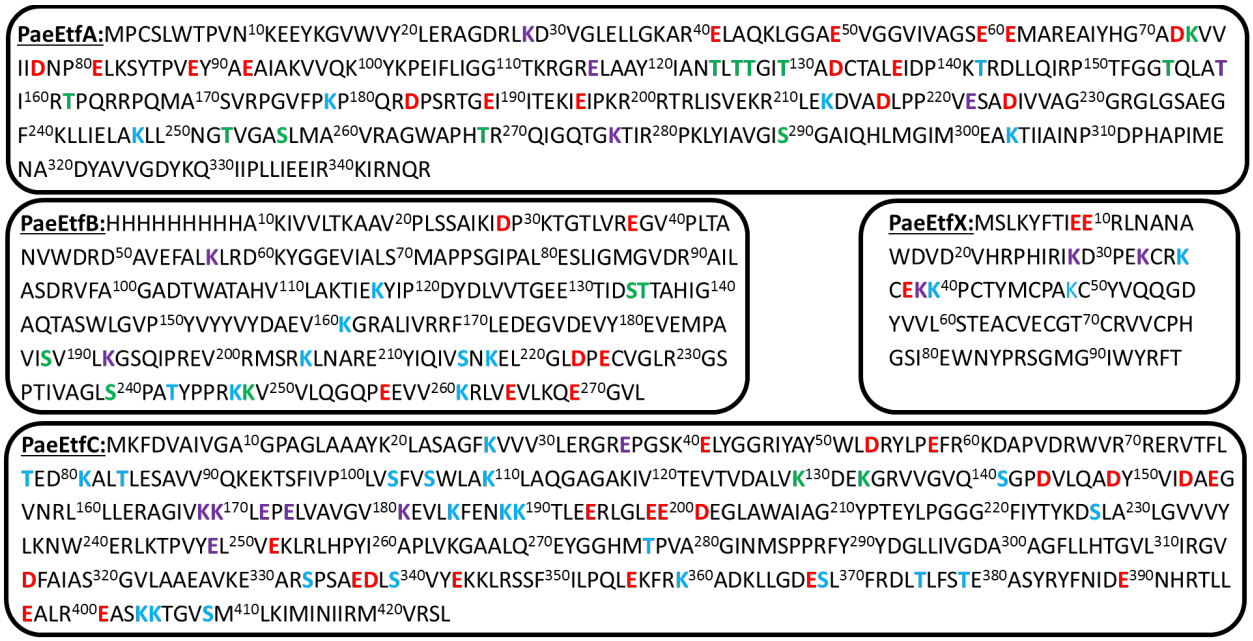


Figure S3. Complete data on surface labeling of intact *Pae* EtfABCX complex. Blue (DnsCl) and red (GEE) colored residues denote DnsCl and GEE moiety incorporated in to complex structure during the shortest exposure to labeling reagent, 5 minutes and 3 minutes respectively. Green colored residues represent DnsCl/GEE labels incorporated only in the latest time points (10/30 minutes) and residues highlighted in purple disagree with the model

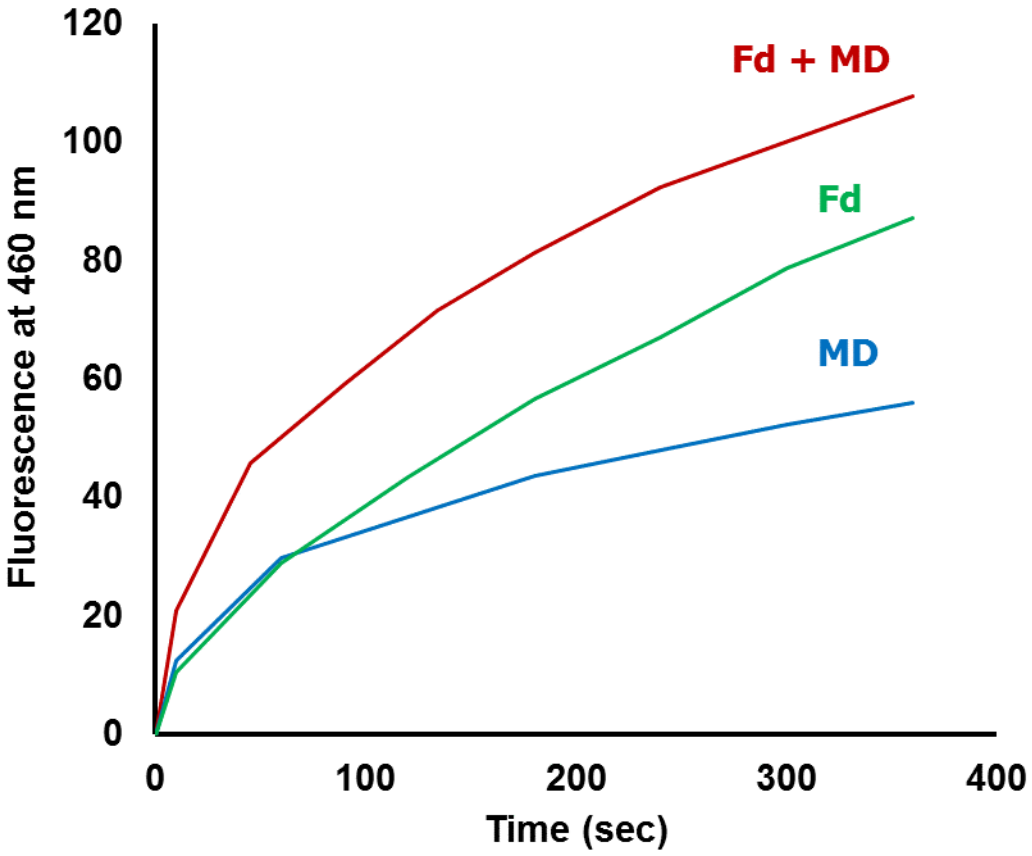
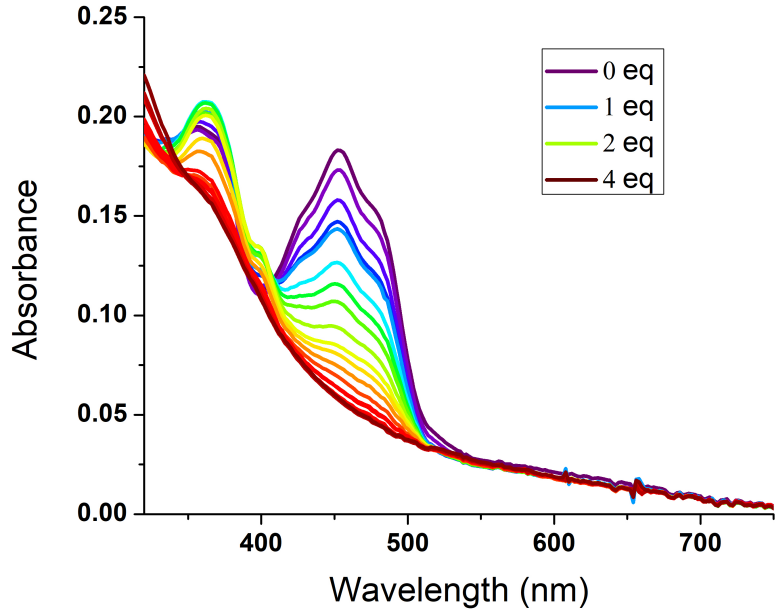
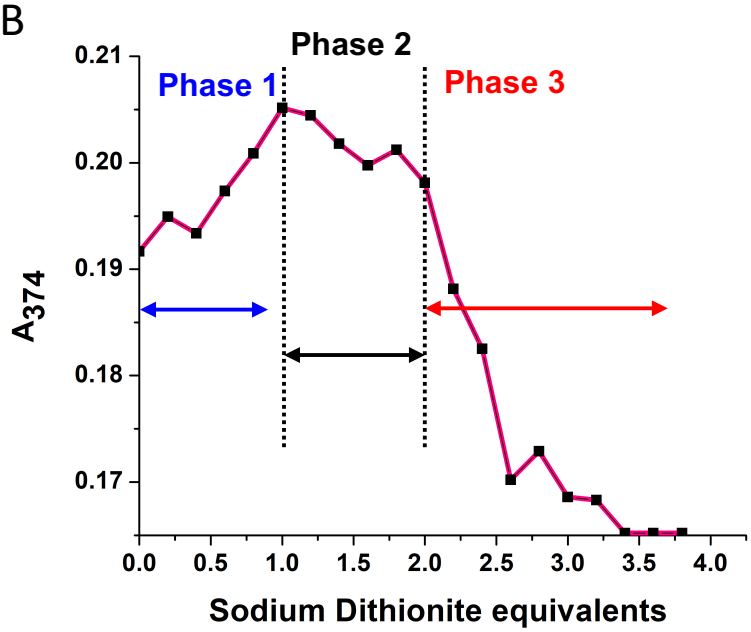


Figure S4. Electron bifurcation by the EtfABCX complex of *P. aerophilum* under anaerobic conditions. Formation of NADH from ferredoxin and menadione reduced with Ti-citrate was followed by the fluorescence of NADH (excitation 340 nm/emission 460 nm). The abbreviations are: MD, menadione only reduced with Ti-citrate; Fd, *P. furiosus* ferredoxin only reduced with Ti-citrate; Fd + MD, menadione and *P. furiosus* ferredoxin reduced with Ti-citrate.

A


0.25
0.20
0.15
0.10
0.05
0.00
Absorbance
400
500
600
700
Wavelength (nm)
0 eq
1 eq
2 eq
4 eq

B


Phase 1
Phase 2
Phase 3
0.21
0.20
0.19
0.18
0.17
A374
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
Sodium Dithionite equivalents

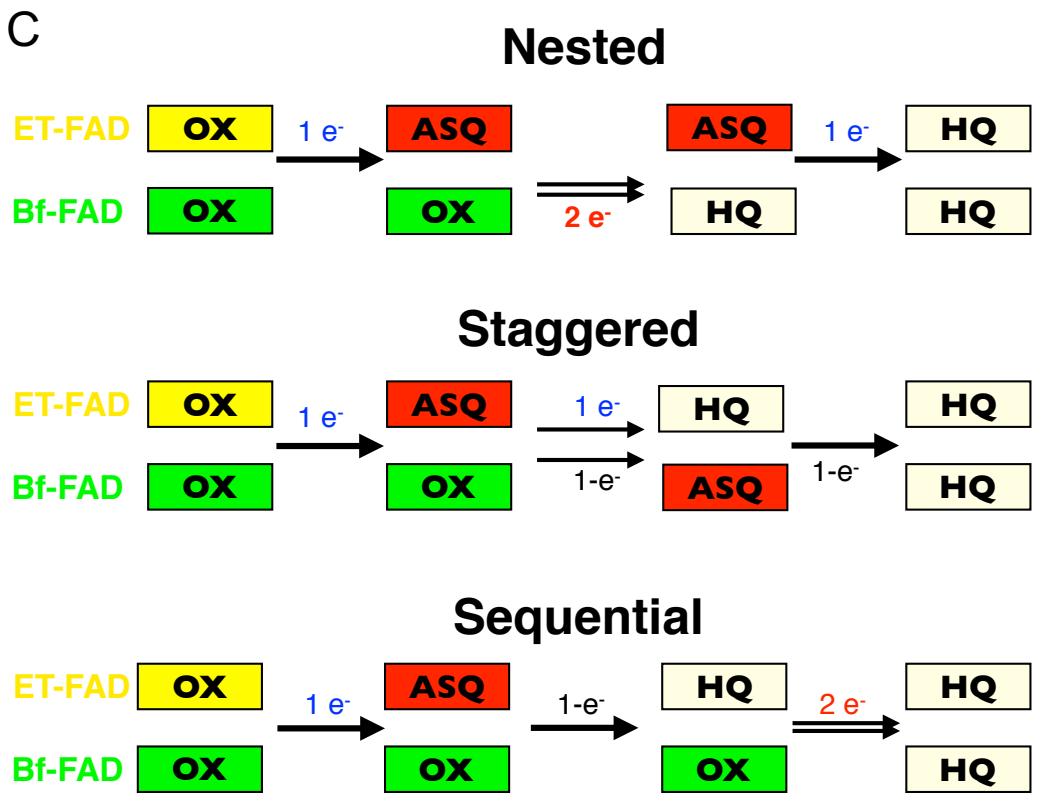


Figure S5. A. Spectra resulting from stepwise reduction of 10 μM EtfAB with sodium dithionite. **B.** Stepwise reduction of EtfAB showing initial formation of ASQ followed by its conversion to HQ and then reduction of remaining OX to HQ by absorbance at 374 nm at each step in the titration. Straight lines connect the measured values to guide the eye. Three phases are seen characterized by increasing 374 nm (blue arrow) then gradual loss of 374 nm (black arrow) and then steep decrease in 374 nm as well as 454 nm (red arrow). **C.** Possible sequential reduction events of each FAD cofactor in EtfAB.

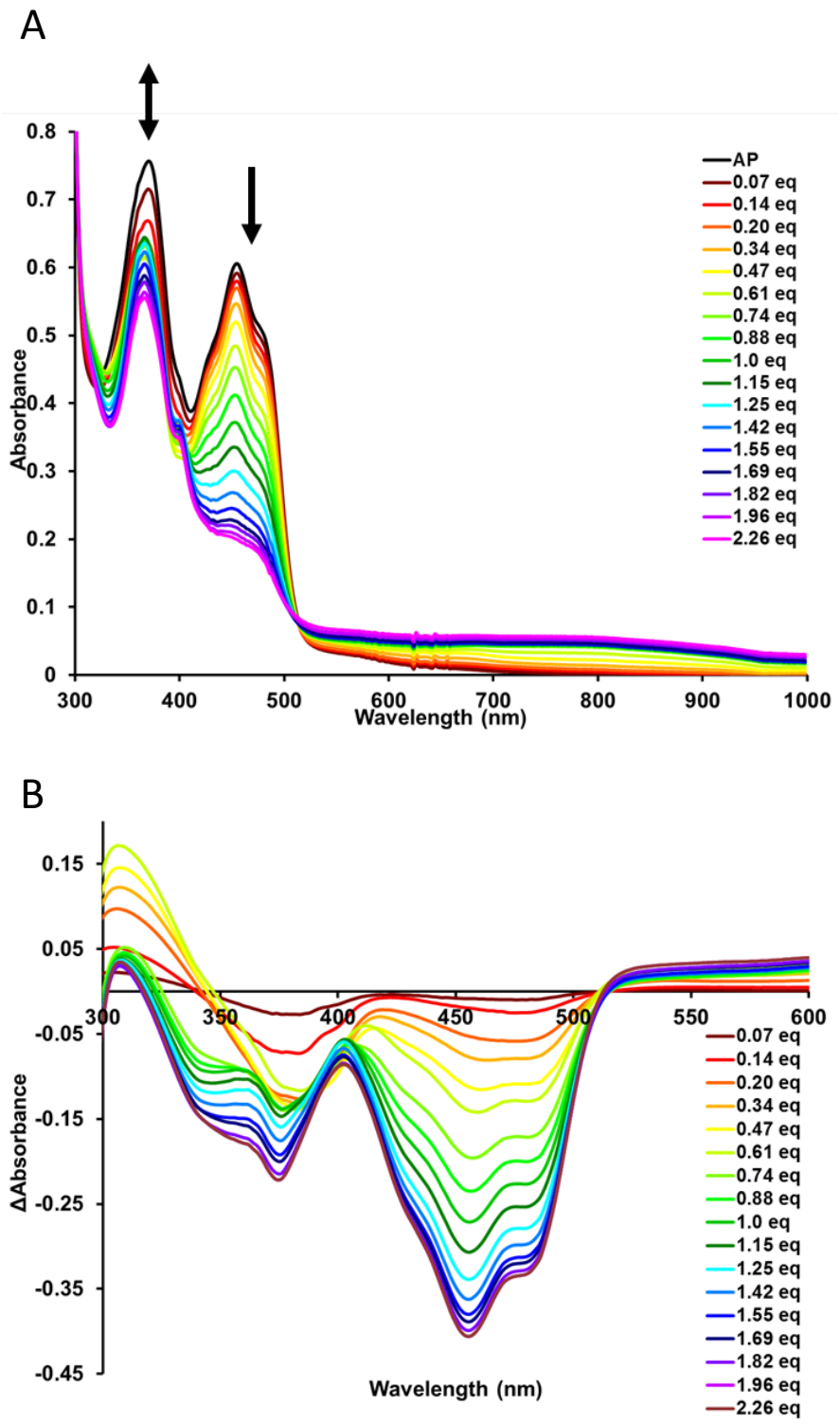
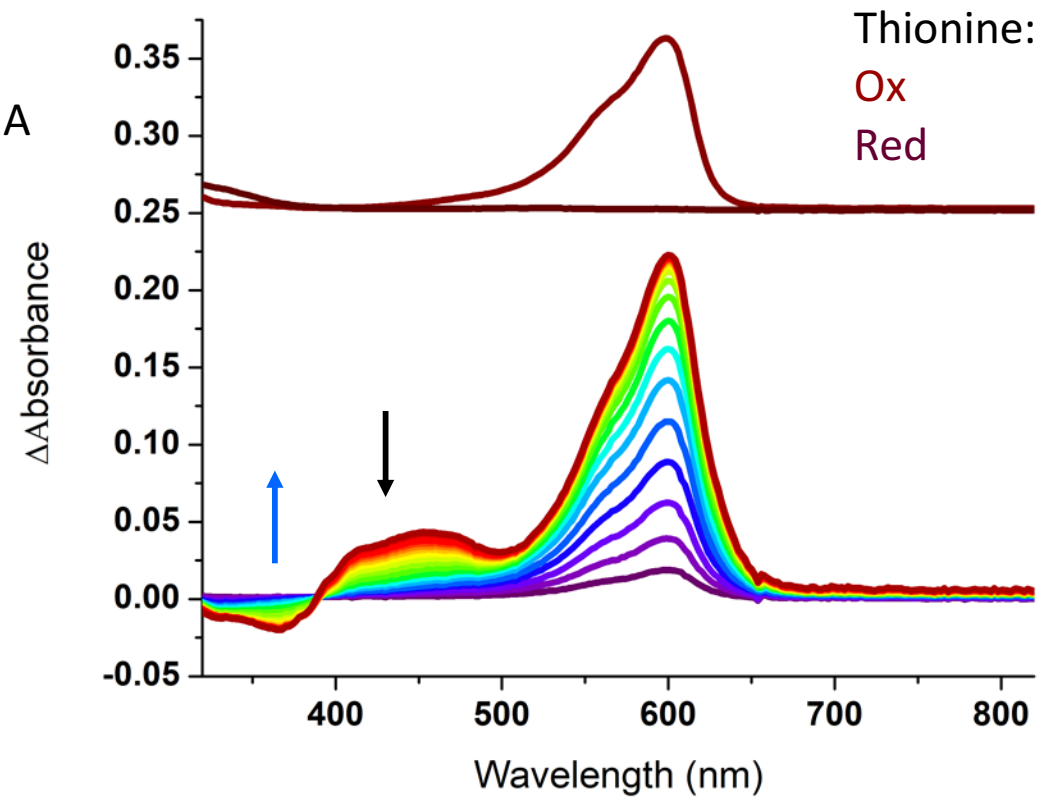
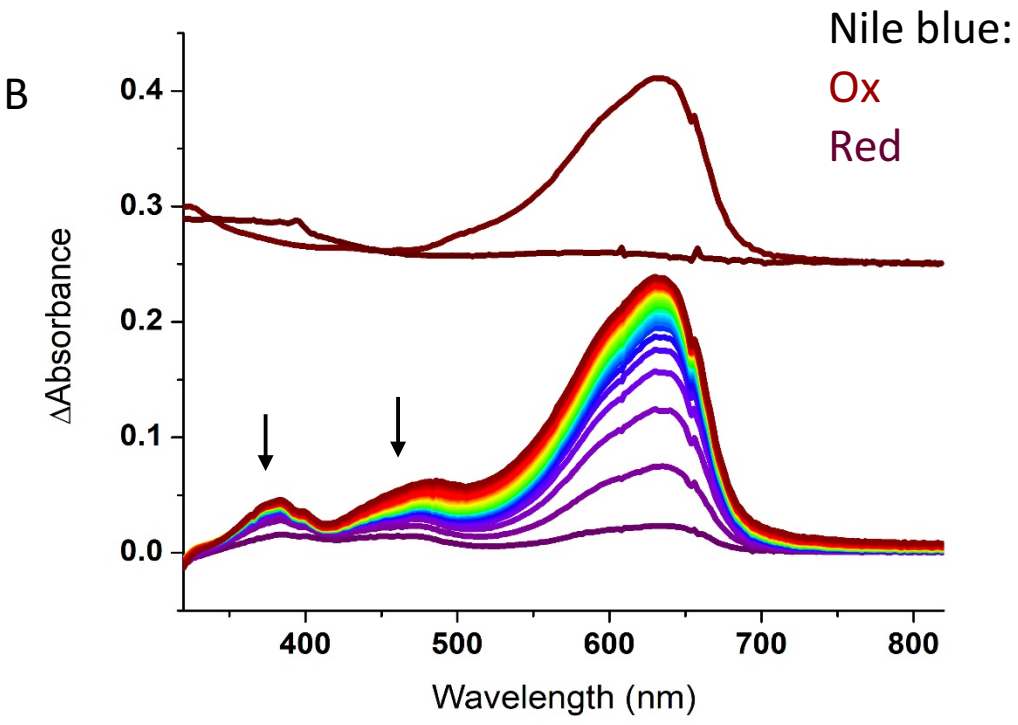


Figure S6. **A.** Anaerobic titration of *Pae* EtfAB (74 μM) as purified, with sequential aliquots of Ti-citrate. In the as purified state the ET-FAD is partly in the ASQ state as evident by the absorbance at 374 nm which represents signal from both ASQ and OX flavin. Arrows from left to right indicate reduction OX flavin absorbance. **B.** Difference spectrum relative to starting spectrum.

A


Thionine:
Ox
Red
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
-0.05
ΔAbsorbance
400
500
600
700
800
Wavelength (nm)

B


Nile blue:
Ox
Red
0.4
0.3
0.2
0.1
0.0
ΔAbsorbance
400
500
600
700
800
Wavelength (nm)

C

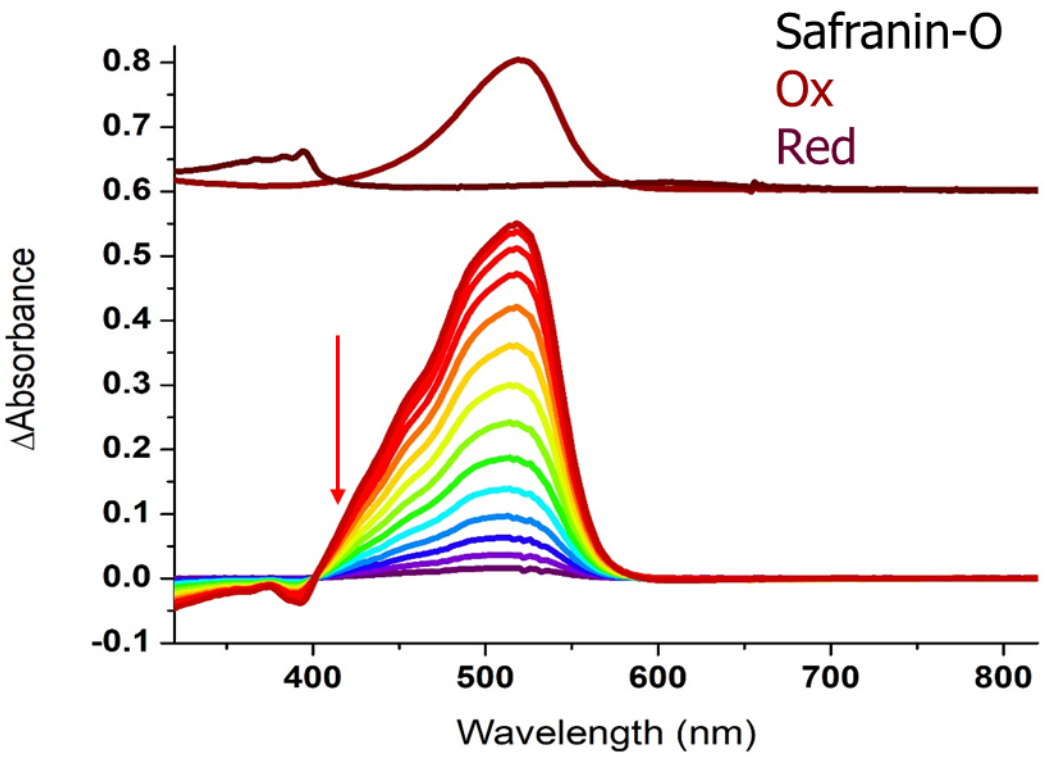
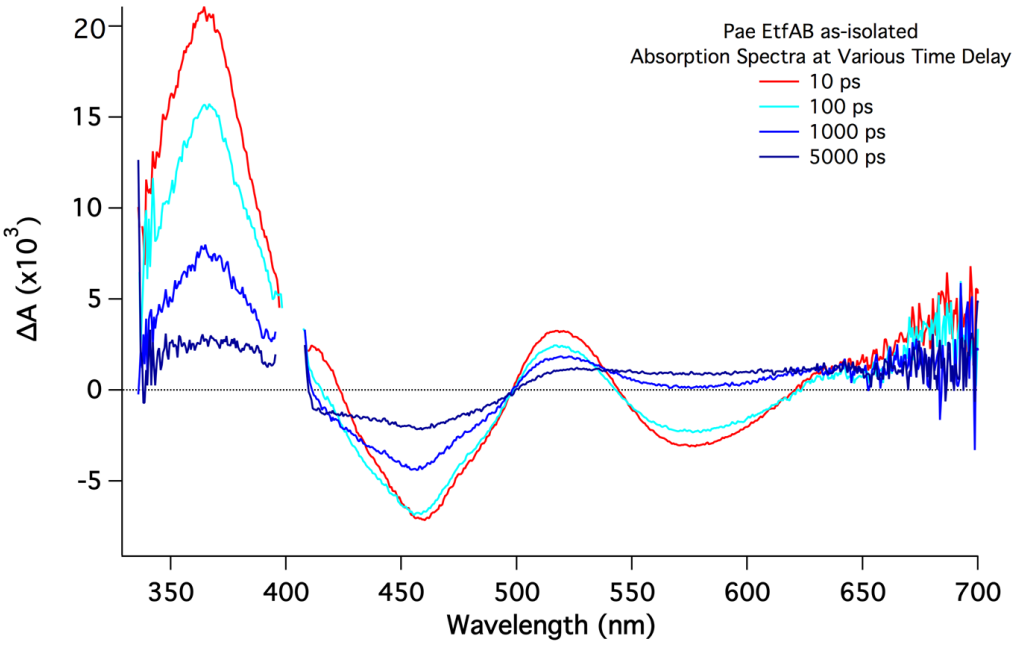


Figure S7. A. Formation of ASQ (blue arrow) at the expense of OX (black arrow) in the course of reduction in the presence of thionine. **B** Conversion of ASQ to HQ (black arrows) in phase 2 in the presence of Nile blue. **C.** Two-electron reduction of lower-E° FAD from OX to HQ (red arrow) in the presence of safranin-O.

A



B

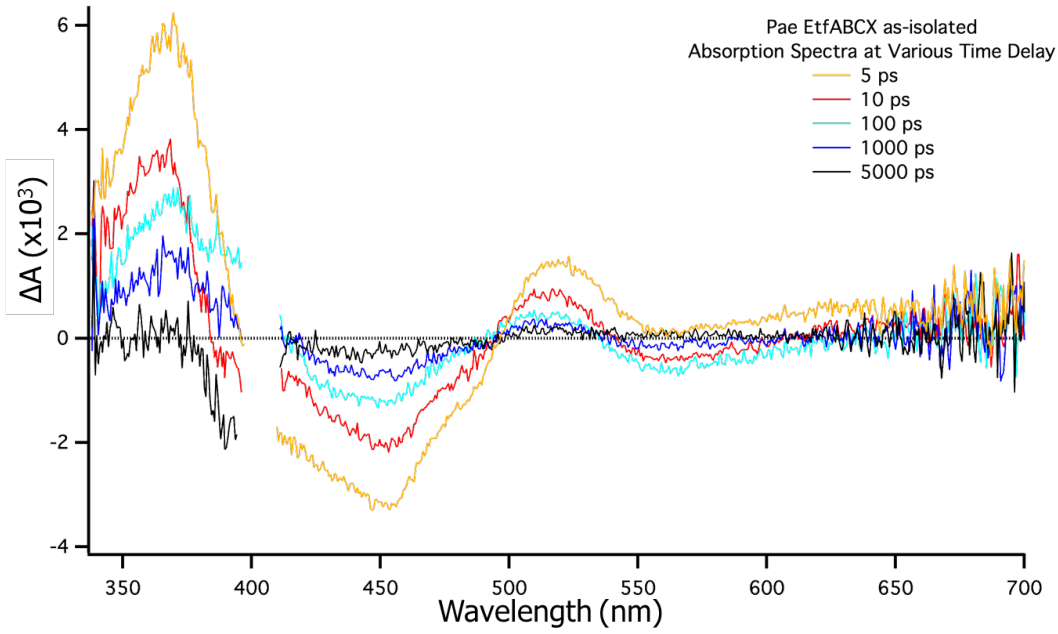
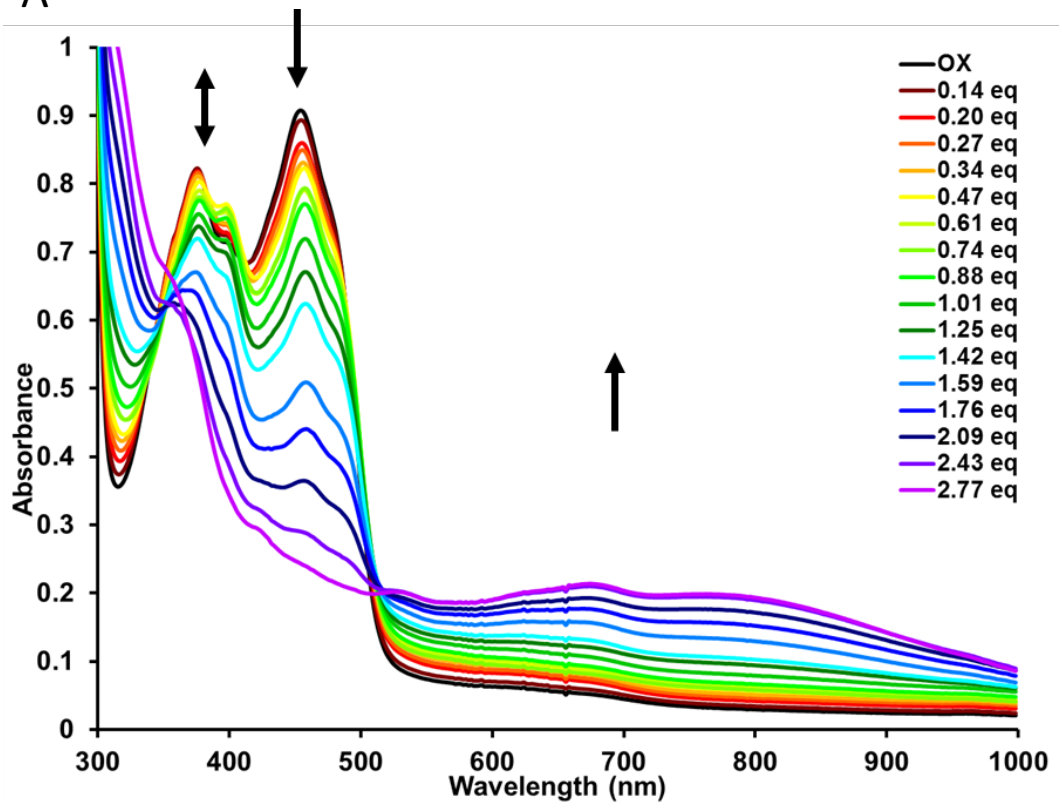
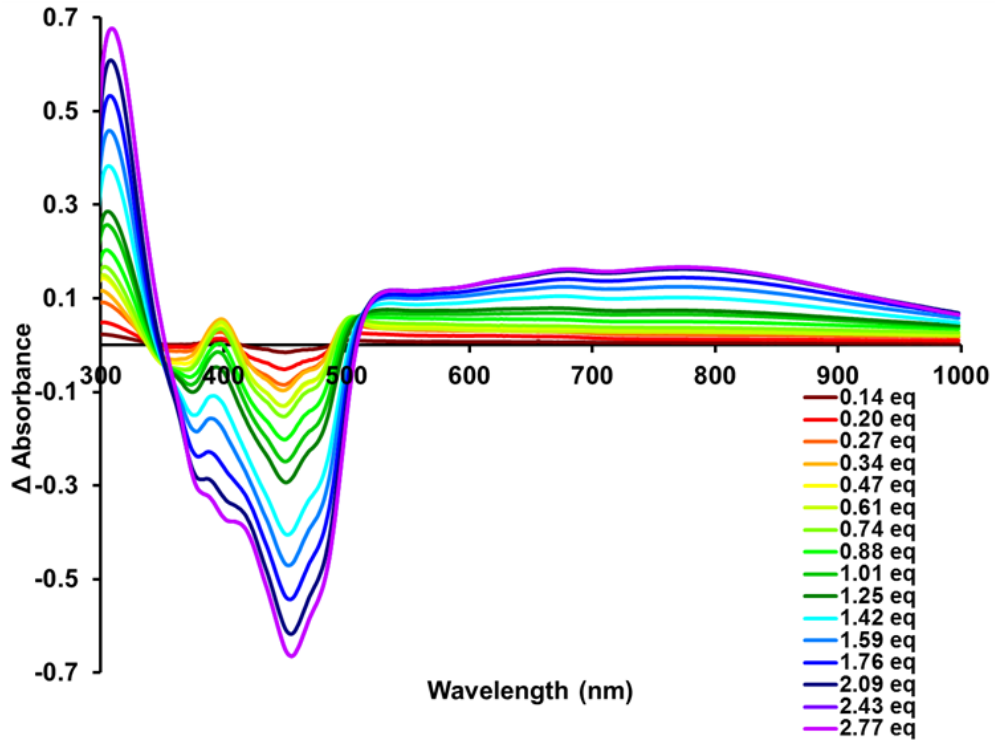


Figure S8. Absorbance spectra of *Pae* EtfAB **A** and EtfABCX **B** at various time points in the TAS experiment. At early times (10 ps), a strong ASQ absorption at 374 nm is formed concurrent with a bleaching of oxidized flavin at 454 nm. A stimulated emission is observed ~ 570 nm (with a lifetime of < 2 ps), although the exact energy levels/states causing it are unknown.

A


Absorbance
Wavelength (nm)
OX
0.14 eq
0.20 eq
0.27 eq
0.34 eq
0.47 eq
0.61 eq
0.74 eq
0.88 eq
1.01 eq
1.25 eq
1.42 eq
1.59 eq
1.76 eq
2.09 eq
2.43 eq
2.77 eq

B


Δ Absorbance
Wavelength (nm)
0.14 eq
0.20 eq
0.27 eq
0.34 eq
0.47 eq
0.61 eq
0.74 eq
0.88 eq
1.01 eq
1.25 eq
1.42 eq
1.59 eq
1.76 eq
2.09 eq
2.43 eq
2.77 eq

C

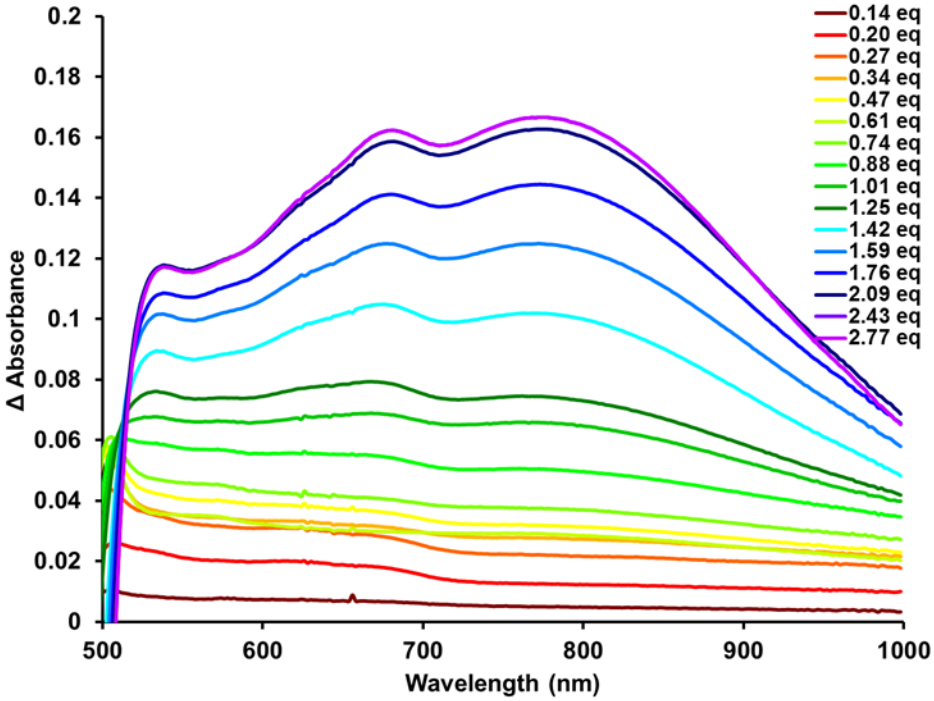


Figure S9. **A.** Anaerobic titration of *Pae* EtfAB (74 μM) with NADH. Arrows from left to right indicate formation and disappearance of aionic semiquinone, disappearance of oxidized flavin absorbance and the formation of a broad charge transfer complex. **B.** Differential spectrum relative to starting spectrum. **C.** Differential spectrum relative to starting spectrum, zoom in of the 600-1000 nm region.

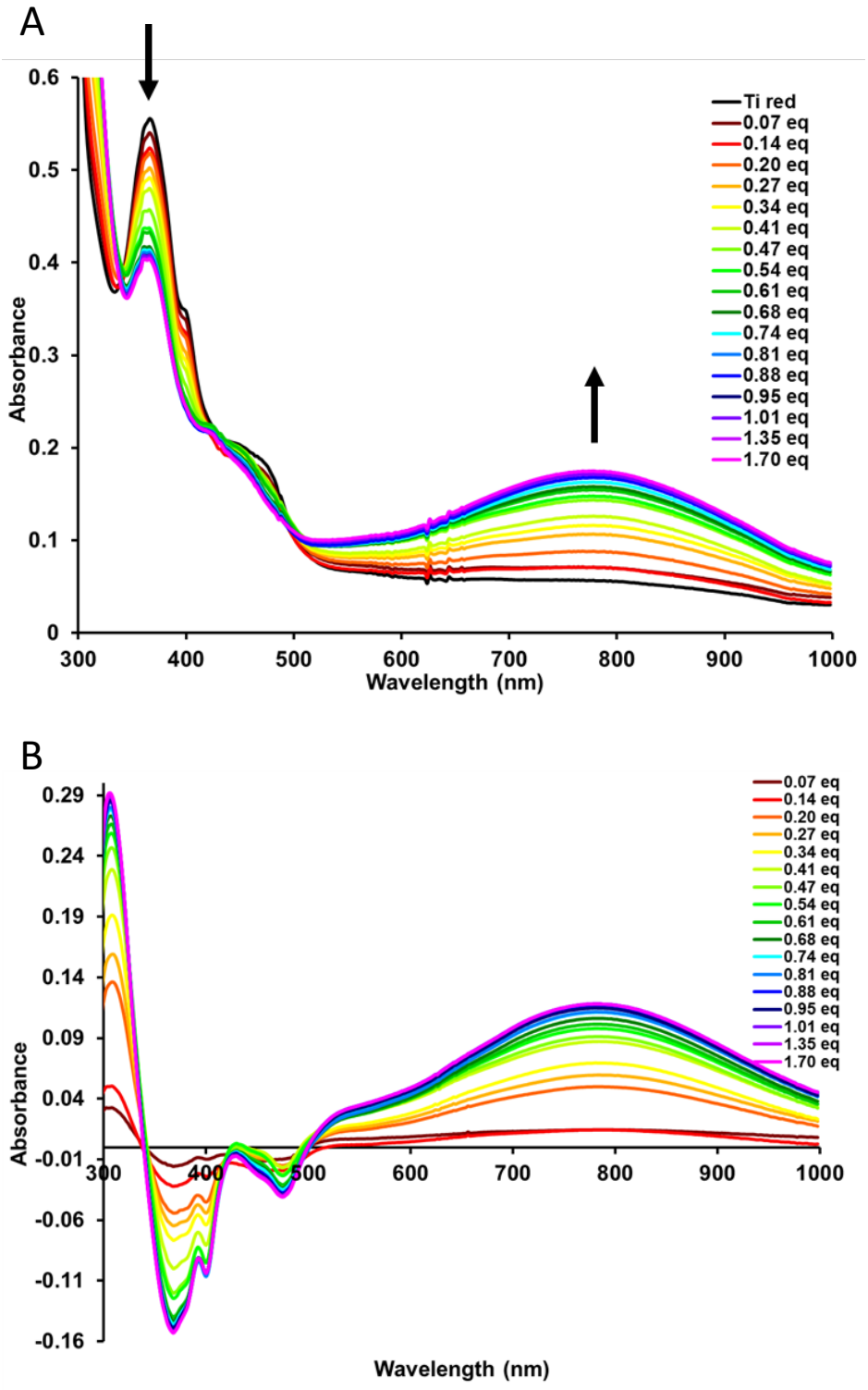
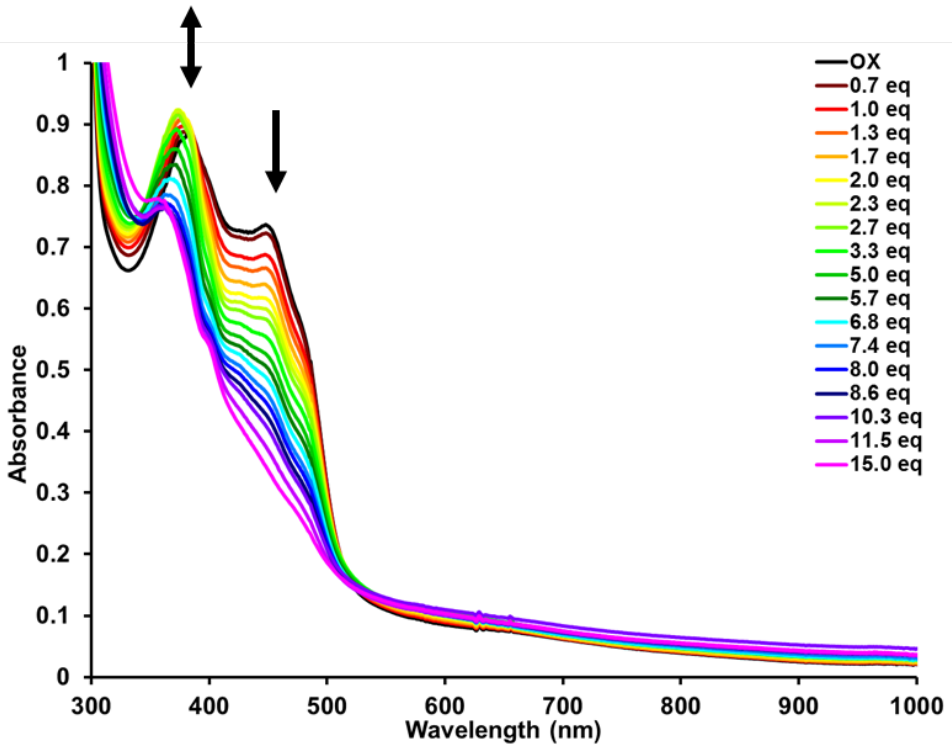
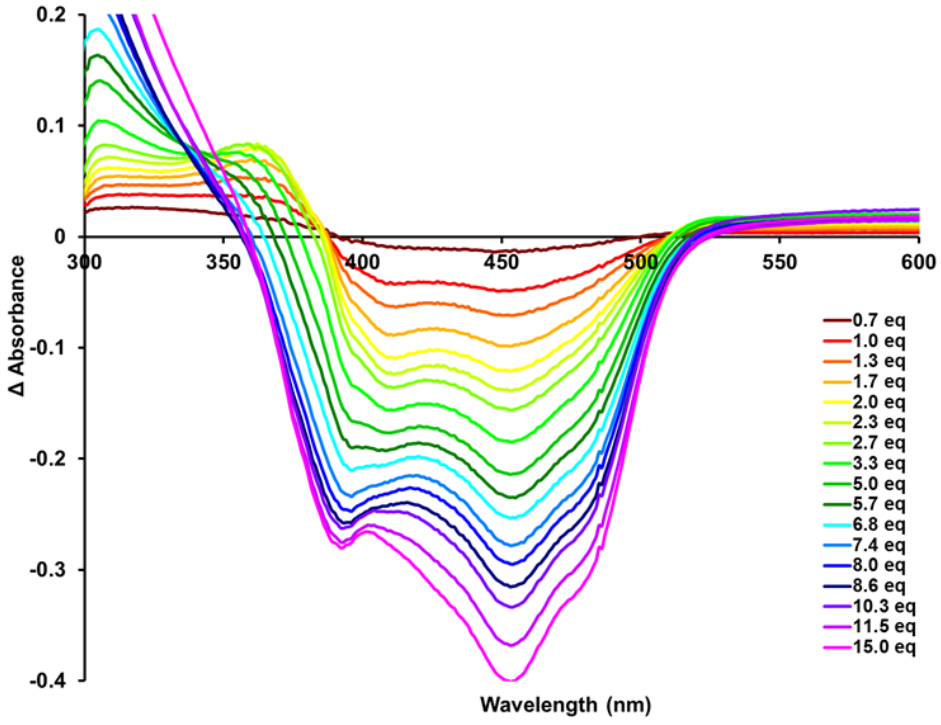


Figure S10. **A.** Anaerobic titration with NAD^+ of *Pae* EtfAB (74 μM) reduced excess Ti-citrate. Arrows from left to right indicate disappearance of anionic semiquinone and the formation of a broad charge transfer complex. **B.** Difference spectrum relative to starting spectrum.

A


Absorbance
Wavelength (nm)
OX
0.7 eq
1.0 eq
1.3 eq
1.7 eq
2.0 eq
2.3 eq
2.7 eq
3.3 eq
5.0 eq
5.7 eq
6.8 eq
7.4 eq
8.0 eq
8.6 eq
10.3 eq
11.5 eq
15.0 eq

B


Δ Absorbance
Wavelength (nm)
0.7 eq
1.0 eq
1.3 eq
1.7 eq
2.0 eq
2.3 eq
2.7 eq
3.3 eq
5.0 eq
5.7 eq
6.8 eq
7.4 eq
8.0 eq
8.6 eq
10.3 eq
11.5 eq
15.0 eq

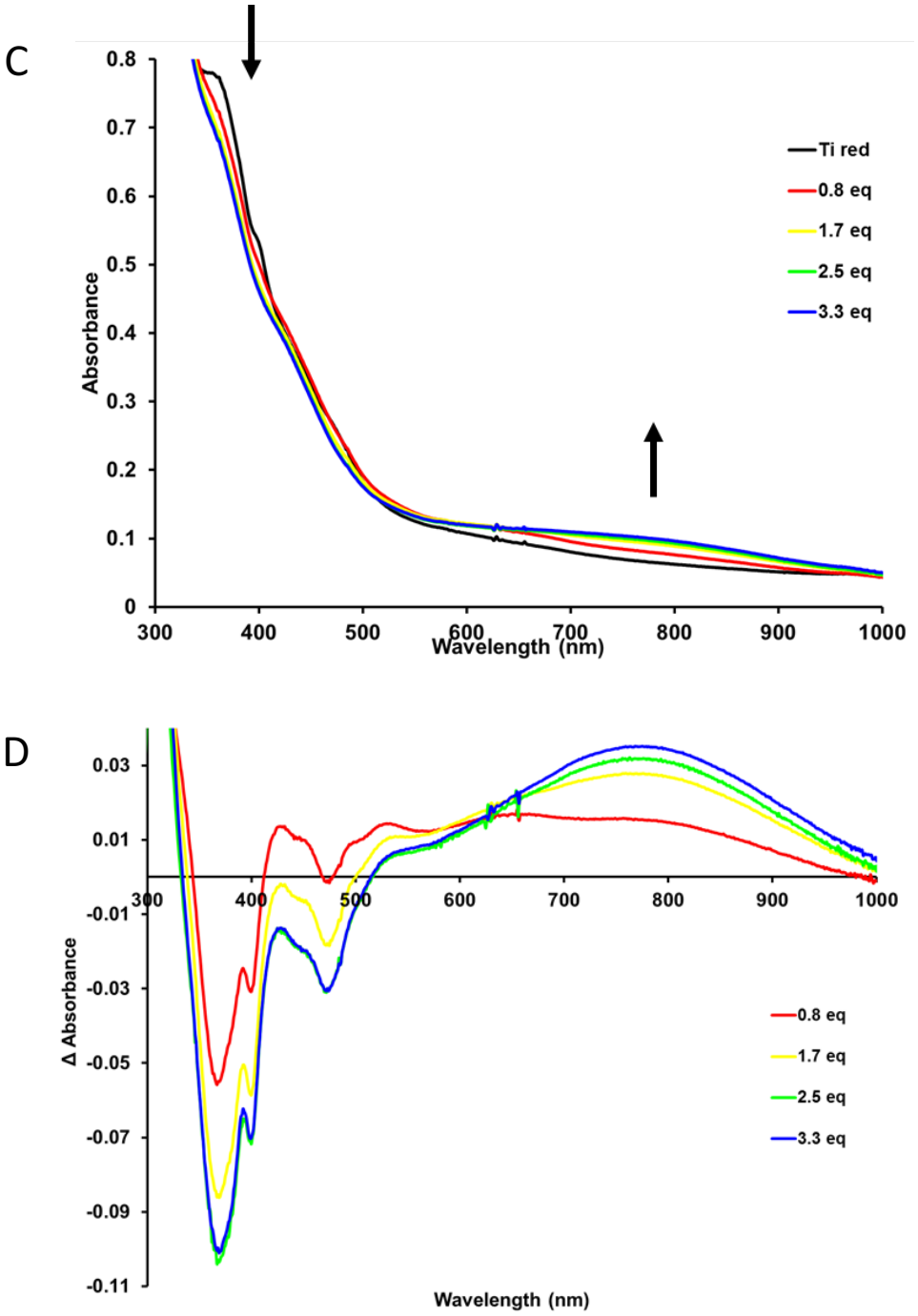
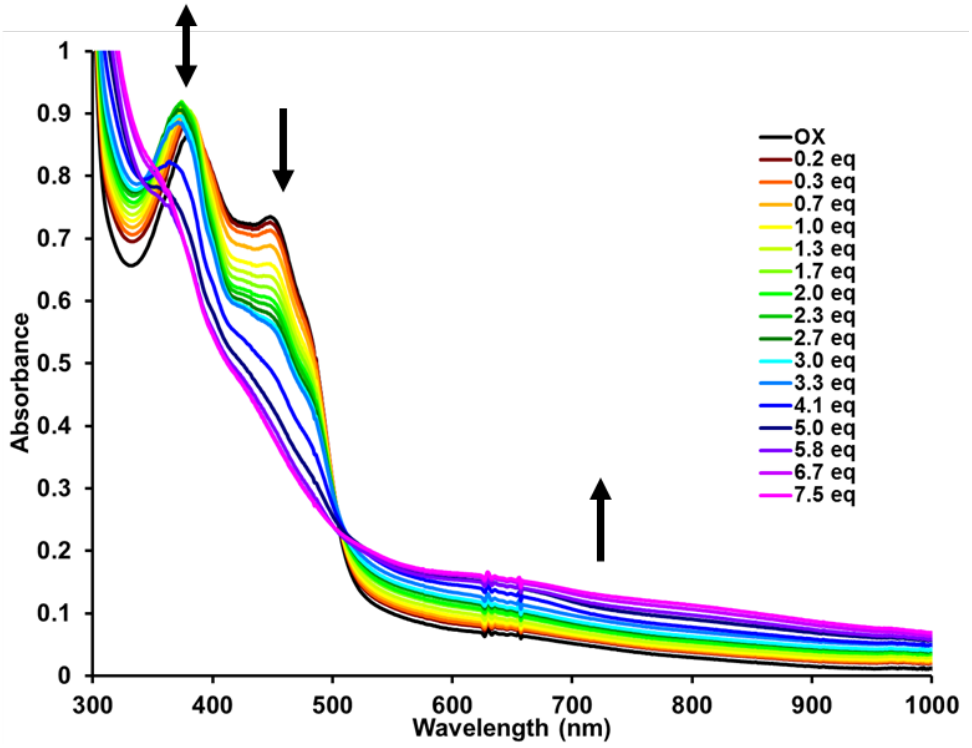
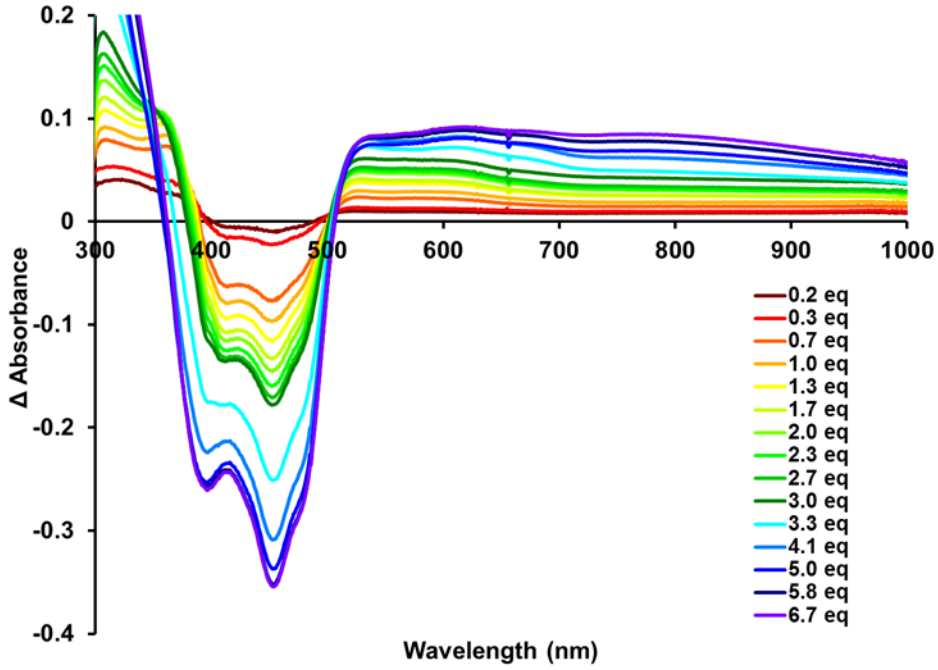


Figure S11. A. Anaerobic titration of *Pae* EtfABCX (15 μM) with Ti-citrate. Arrows from left to right indicate formation and disappearance of anionic semiquinone and disappearance of oxidized flavin absorbance. **B.** Difference spectrum of A relative to starting spectrum. **C.** Anaerobic titration with NAD^+ of *Pae* EtfABCX (15 μM) reduced excess Ti-citrate. Arrows from left to right indicate disappearance of anionic semiquinone and the formation of a broad charge transfer complex. **D.** Difference spectrum of C relative to starting spectrum.

A


Absorbance
Wavelength (nm)
OX
0.2 eq
0.3 eq
0.7 eq
1.0 eq
1.3 eq
1.7 eq
2.0 eq
2.3 eq
2.7 eq
3.0 eq
3.3 eq
4.1 eq
5.0 eq
5.8 eq
6.7 eq
7.5 eq

B


Δ Absorbance
Wavelength (nm)
0.2 eq
0.3 eq
0.7 eq
1.0 eq
1.3 eq
1.7 eq
2.0 eq
2.3 eq
2.7 eq
3.0 eq
3.3 eq
4.1 eq
5.0 eq
5.8 eq
6.7 eq

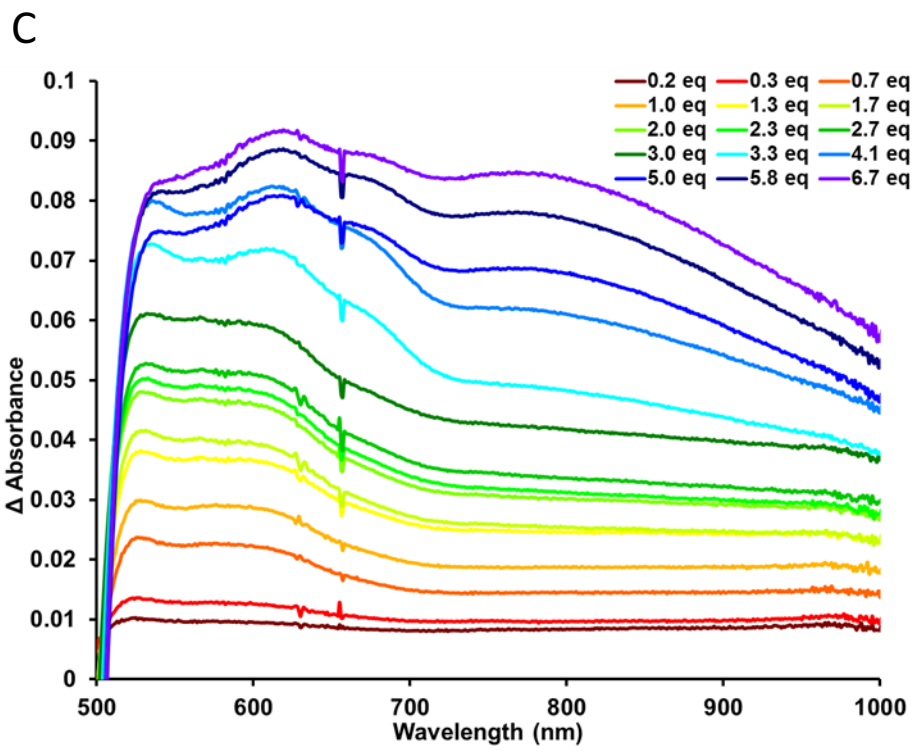


Figure S12. **A.** Anaerobic titration of *Pae* EtfABCX (15 μM) with NADH. Arrows from left to right indicate formation and disappearance of anionic semiquinone , disappearance of oxidized flavin absorbance and the formation of a charge transfer complex. **B.** Difference spectrum of A relative to starting spectrum. **C.** Difference spectrum of A relative to starting spectrum zoomed in to illustrate the formation of NSQ signal mixed with the charge transfer complex signal.

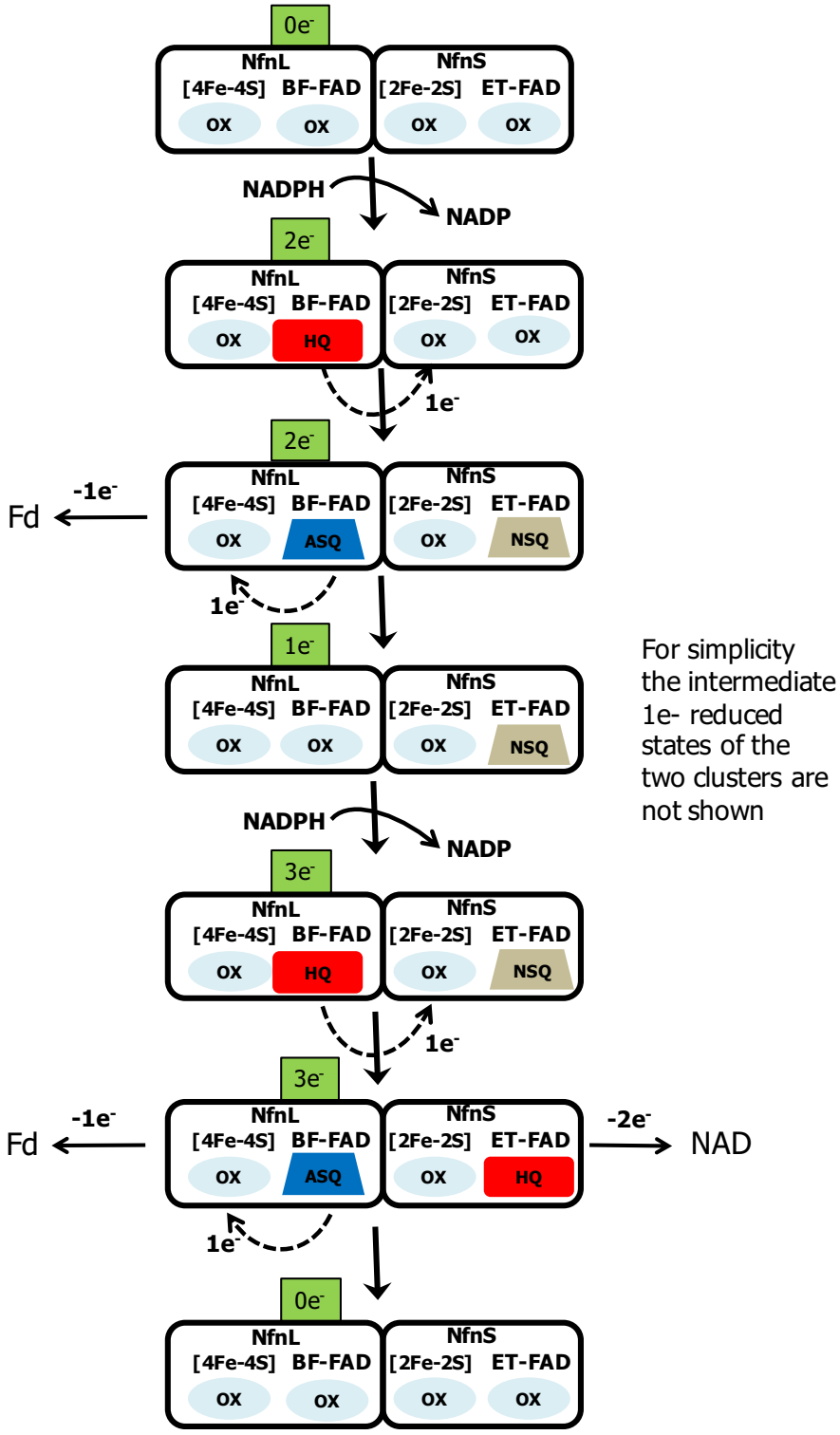


Figure S13. Mechanism of NfnI (in the same format as for EtfABCX in Figure 6). Note that this depicts the full 4 e- reaction pathway utilizing two NADPH, whereas two turns of the cycle are required in Figure 6 for NfnI to utilize two NADH.