Abstract:

Flavin/Protein interactions that Differently Shape the Reactivities of the Two Cofactors in Bifurcating Electron Transfer Flavoproteins

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Electron bifurcation provides ancient bacterial lineages with enhanced energy efficiency, which we would like to harness. The two flavins in bifurcating electron transfer flavoproteins (Bf-ETF) have contrasting reactivities and play complementary roles, despite being chemically identical. Cutting-edge infrared spectroscopic techniques are proposed (1) to understand how hydrogen bonding between protein and flavin shape reactivity of the latter (2) to determine whether a conserved nearby cysteine interacts with the bifurcating flavin and/or the conserved arginine that is also nearby and (3) to test the effects of protein conformational change on the flavins. We propose that redistribution of hydrogen bonds triggered by flavin oxidation state change in turn initiate a protein conformation change that orchestrates electron transfer. Experimental work will occur at the FU and be closely linked to computational studies in the group of Maria Andrea Mroginski (TU), with proteins to be provided by our collaborator Anne-Frances Miller (University of Kentucky and Einstein Visiting Fellow).

Extended description version of the project:

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1. Overall goal of the project

The two flavins in Bf-ETFs are chemically identical flavin adenine dinucleotides (FADs), so their distinct activities must be created by the different interactions each derives from its different protein environment. These interactions are predominantly hydrogen bonding (H-bonding) interactions, and moreover are expected to change in the course of enzyme turnover because the protonation states of the flavins change depending on their oxidation states. Thus, we want to understand how the two flavin-binding sites of the Bf-ETF protein differ with respect to the H-bonds they make with their flavins. Infrared spectroscopy (IR) is perfectly suited to the task because it directly detects the protonation states and H-bonding functionalities. We will apply in-situ electrochemical FTIR difference spectroscopy as well as exploiting the fact that the flavin is a chromophore and can be converted from oxidized (OX) to anionic semiquinone (ASQ) by illumination.[1] Thus, the H-bonds to the flavin will be affected but not the others in the protein. For example, we will use IR spectroscopy to test for H-bonding between a Cys thiolate anion and an Arg cation, which are conserved near the bifurcating flavin. Finally, cyano derivatives of the flavins will be used as vibrational Stark effect probes to scrutinize the prediction of large changes in the environments of the two flavins induced by a conformational change, and by binding of partner proteins. Thus we will learn how the protein shapes the environments and thereby the reactivities of the two flavins, to enable them to play different roles.

2. State of the art

Bifurcating Electron Transfer Flavoproteins (Bf-ETF) exploit the remarkable versatility of the flavin cofactor regarding its ability to conduct either pairwise or sequential electron transfer. One of the two flavins, the 'electron transfer' flavin (ET-flavin) transfers electrons one at a time thanks to a stable anionic semiquinone (ASQ) one-electron reduced state, whereas the other flavin (the so-called bifurcating flavin or Bf-flavin), accepts electrons as a pair from NADH and is thermodynamically very *un*stable in the semiquinone (SQ) state. Thus the Bf-flavin is an all-or-none site, whereas the ET-flavin is a one-after-the-other site. They work together to consume a pair of medium-strength electrons from NADH to produce one super-reducing electron able to drive essential but demanding reactions such as nitrogen fixation or production of H_2. The laws of thermodynamics are satisfied by exergonic transfer of the other electron to a partner enzyme, thereby paying for the production of reduced ferredoxin (or reduced flavodoxin) that carries the super-reducing electron. This is nature's molecular voltage converter, that enables cheap abundant fuels (NADH) to drive demanding chemistry (nitrogen fixation).

Flavin-based bifurcation was recognized by Buckel and Thauer comparatively recently, so the essential principles are still emerging.[2] A protein conformational change is proposed to play a key role in preventing unproductive electron transfer between the two flavins,[3] and destabilization of the SQ states of the Bf-flavin is believed to be crucial in enforcing strong coupling between the two electron transfers from that site to two different acceptors.[4] Anne-Frances Miller´s group has characterized the redox reactivity of the two flavins in the Bf-ETF from the nitrogen-fixing bacterium *Rhodopseudomonas palustris*, including measurement of the reduction midpoint potentials (E°s). They demonstrated thermodynamic suppression of the SQ of the Bf-flavin[5] and tested experimentally and computationally which protein site harbours which flavin function,[6] confirming the structure-based model.[2] Moreover the Miller group can now produce Bf-ETF protein bearing mutations

in the different flavin sites, and with either one of the flavins missing, to permit uncomplicated observation of the other one. Thus, the stage is set for spectroscopic observation of the interactions between specific flavins and specific amino acid side chains.

3. Specific aims and how they may be reached:

WP1 Objective: to understand how H-bonding between protein and flavin shape flavin reactivity. We propose to use IR spectroscopy of Bf-ETF containing either the Bf-flavin or the ET-flavin (one at a time) to observe H-bonds between the protein and the flavin. Electrochemically-induced IR difference spectroscopy will be performed on monolayers of wild-type Bf-ETF and its variants using SEIRAS (surface-enhanced infrared absorption spectroscopy).[7] By manipulating the oxidation state of the flavin, we will learn which H-bonds are coupled to flavin oxidation state, and therefore can conversely affect the reduction midpoint potential of the flavin. In particular, the ASQ state is stable for the ET-flavin, but unstable for the Bf-flavin. Therefore we anticipate that some interaction(s) in the ET site will be stronger upon formation of ASQ. For example, literature suggests a H-bond between the flavin N5 and a nearby Thr (sometimes Ser, in other ETFs). Therefore we propose comparison with a mutant in which the Thr is converted to Ala. We will also try mutating the Thr to Cys, which will change the H-bonding strength. These weak but crucial interactions will be recorded via the acute sensitivity of IR spectroscopy. Time-resolved IR experiments after pulsed excitation will be performed using our new quantum cascade laser system. [2]

WP2 Objective: to determine whether a conserved nearby Cys interacts with the Bf-flavin and/or the conserved Arg that is also nearby. We will use IR instrumentation optimized for observation of H-bonds involving Cys side chains, to test for H-bonds formed by a Cys residue near the Bf-flavin that is close enough to bond with that flavin, albeit at a different position than in the light- oxygen- or voltage-sensitive (LOV) domains. [3] Because this Cys is conserved in Bf-ETFs associated with nitrogen fixation we propose a regulatory role for it. The structure suggests that the Cys H-bonds with an Arg side chain that is also conserved near the Bf-flavin, so we wonder if anionic Cys neutralizes the usual positive charge of the Arg and thereby neutralizes its ability to stabilize an ASQ state of Bf-flavin. This is mechanistically crucial because bifurcating activity is believed to require that the ASQ state be *de*stabilized.

WP3 Objective: to test changes in the local electrostatics. FAD can be prepared with the 8-methyl group replaced by a cyano group. This has been incorporated into a Bf-ETF and supports electron transfer (albeit at altered rates and at altered E°s). Of particular note, this flavin has not been perturbed with respect to the H-bonding functionalities on the uracyl ring, but now posesses a highly informative IR probe with a C≡N vibration whose frequency strongly absorbs in an otherwise empty spectral region and an enhanced dipole moment due to the coupling to the isoalloxazine ring making it an excellent VSE (vibrational Stark effect) probe. The 8-methyl group of the Bf-flavin is believed to protrude into the binding site of the electron-accepting ferredoxin or flavodoxin.[2] If this is true, the CN of 8-cyanoflavin should respond strongly to binding of these proteins. The Miller group has overexpressed the appropriate flavodoxin. The 8-methyl of the ET-flavin is believed to contact another protein domain when the Bf-ETF is in the conformation that favours bifurcation, but is proposed to be exposed to solvent in another conformation proposed to occur at another point in the catalytic cycle.[3] Thus we will use IR spectroscopy to test for large-scale protein binding/dynamic events proposed to occur as part of the catalytic cycle.

(1) Kottke et al (2016) J. Phys Chem B **121**:335-350; (2) Chowdhury et al (2014) *J. Biol. Chem.* **289**, 5145-5157; (3) Demmer et al (2017) *Nat. Commun.* **8**, 1577; (4) Nitschke & Russell (2012) *Bioessays* **34**, 106-109; (5) Duan et al (2018) *J. Biol. Chem.* **293**, 4688-4701; (6) Mohamed-Raseek et al (2019) *Chemical Sci.* **10**, 7762-7772. (7) Ataka & Heberle (2007) *Anal. Bioanal. Chem. 388*, 47-54. (8) Schultz et al. (2018) *Spectrochim Acta A* **188**, 666-674. (9) Ataka et al. (2003) *Biophys. J.* **84,** 466-474.