

PARTITIONING OF REACTIVE OXYGEN SPECIES VIA THE RE-OXIDATION OF
ELECTRON TRANSFER FLAVOPROTEIN

by

Chase Kennor Austvold

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NOMENCLATURE

Reactive Oxygen Species (ROS). Superoxide anion ($\text{O}_2^{\bullet-}$). Hydrogen peroxide (H_2O_2).
Electron Transfer Flavoprotein (ETF). Flavin Adenine Dinucleotide (FAD). Medium
Chain-Acyl CoA Dehydrogenase (MCAD). Magnetic field (M_f).

ABSTRACT

The biology of Reactive Oxygen Species are poorly understood. Within a healthy cell, Reactive Oxygen Species behave as signaling molecules, although overproduction leads to oxidative damage. In order to understand when the overproduction of Reactive Oxygen Species takes place, or leads to oxidative damage, the elementary step of quantification becomes necessary. Electron Transfer Flavoprotein is a known Reactive Oxygen Species producing enzyme and was studied. Electron Transfer Flavoprotein is a key-player within the production of energy within the eukaryotic mitochondria. The redox nature of Electron Transfer Flavoprotein's catalytic cofactor, flavin adenine dinucleotide produced two types of ROS; the superoxide anion ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2). Electron Transfer Flavoprotein produced roughly five-fold more $O_2^{\bullet-}$ compared to H_2O_2 as the enzyme became oxidized. It has been put forward that the production of these two Reactive Oxygen Species is dictated by the formation of a radical pair between the flavin adenine dinucleotide of Electron Transfer Flavoprotein and molecular oxygen. Two types of radical pairs can be formed, either in a triplet or singlet state, and the rate in which these states occur can be influenced by a static magnetic field. Therefore, the effect of a magnetic field on these products was also studied. Upon the suppression of magnetic field strength, the production of H_2O_2 decreased and a proportional increase of $O_2^{\bullet-}$ was observed.

BACKGROUND

Introduction to Reactive Oxygen Species (ROS)

The involvement of Reactive Oxygen Species (ROS) in biological systems has been of great interest in recent years. Elevated levels of ROS result in oxidative damage and “normal levels” result in regulated signaling pathways. To begin to understand what is “elevated” or “normal”, quantification of ROS is the first stepping-stone to defining the role of ROS in biology. Since ROS are not well understood, selecting a specific system which has significant biological impact would be useful in beginning to characterize the line of elevated vs. normal, or in other words, defining a phenotypic boundary that can solidify an understanding of when ROS begin to cause damage. To begin, the specific system of choice will be elucidated after an introductory prelude that explains ROS biology and delineations of important ROS producers within the cell.

Reactive Oxygen Species (ROS) in Biological Systems

Reactive Oxygen Species (ROS) are inherent products of aerobic metabolism (1). ROS all are small molecules that contain an oxygen atom and are generally derived from molecular oxygen (O_2). ROS include hydrogen peroxide (H_2O_2), hydroxyl radicals ($OH^\cdot$), and the superoxide anion (O_2^-) (1). Each of these oxygen-containing species have inherent chemical differences that coincide with their reactivity or role within biological systems (1).

In terms of current research, ROS are most closely associated with oxidative stress, which indicates elevated levels of ROS that result in damage to lipids, proteins, and DNA within the cell (2). Oxidative stress has been linked to a host of pathological consequences such as the onset of several diseases (3). Diseases of particular interest with ROS implications are atherosclerosis, inflammation, onset of cancer, and neurodegenerative diseases such as Alzheimer's and Parkinson's (4). Although much of the research revolves around defining the specifics of Reactive Oxygen Species' role in these diseases, under normal levels, ROS serve as signaling molecules to regulate biological and physiological processes (1).

ROS Signaling Regulated production of Reactive Oxygen Species has been demonstrated as an important redox signaling mechanism (5). Initially, it may be hard to comprehend how these potentially toxic molecules can also play a regulatory role. From an evolutionary perspective, since through the course of time cells have been able to cope with the toxicity of ROS, it would make logical sense that a slight divergence in levels could produce signaling phenomena (6). Cells utilize ROS as signal transduction mechanisms that allow for adaptation to a changing oxidative environment (7). Since ROS are toxic, cells produce proteins that are responsible for scavenging ROS (8,9). This fluid interplay between producers and scavengers are how ROS levels result in a signal.

Production of ROS Reactive Oxygen Species are produced through the interaction of redox active macromolecules with molecular oxygen (10). Most of the

oxidative metabolic pathways occur within the mitochondria, and all of these pathways involve redox intermediates that can interact with O_2 to produce ROS (10), therefore, mitochondria are a vital source of ROS production within most eukaryotic cells (11).

β -Oxidation

Mitochondrial β -oxidation is a major catabolic pathway that involves the degradation of saturated fatty acids and has been shown as a major producer of ROS (12,13). The process of β -oxidation begins with a thiol ester bond becoming formed between the fatty acid and the thiol group of coenzyme A (14). Once initiated, fatty acids are degraded by successive cycles of carbon-carbon bond cleavage that yields two-carbon units in the form of acetyl-CoA (14). To accomplish this, a repeating pattern of four reactions occurs. The first step is an oxidation step of the beta carbon of the fatty acid that leads to the formation of a double bond, the second is a hydration reaction across the newly formed double bond, the third and fourth steps are successive oxidation and cleavage by an enzyme, respectively.

The first reaction of this cycle is catalyzed by one of 11 acyl-CoA dehydrogenase enzymes that accept electrons via a Flavin Adenine Dinucleotide (FAD) cofactor (15). Each of these 11 dehydrogenases reduce a single protein, electron transfer flavoprotein (ETF), which goes on to reduce a membrane bound protein whose respective electrons are transferred to the ubiquinone pool of cellular respiration (15). Electron transfer flavoprotein acts as an electron-funnel, which indicates an imperative role that acts as a “pinch-point” within β -oxidation. Given its catalytic activity as an electron acceptor,

ETF also contains a redox active FAD cofactor that can interact with O₂ as a potential production site of ROS.

Electron Transfer Flavoprotein

Mammalian electron transfer flavoprotein (ETF) is a heterodimeric protein located in the mitochondria (16). In terms of catalytic role, ETF serves as the functional electron shuttle between primary flavoprotein dehydrogenases that are involved in mitochondrial fatty acid and amino acid catabolism and the inter-membrane bound electron flavoprotein ubiquinone oxidoreductase (ETF-QO) (16). Specifically, ETF is reduced by 11 unique mitochondrial flavoprotein dehydrogenases before transferring those respective electrons to the ubiquinone pool (15). ETF conducts the transfer of electrons through a single equivalent of flavin adenine dinucleotide (FAD), as made apparent by the protein's name (15). In terms of biological relevance, ETF's implications are being investigated on two fronts; first of which is understanding the mechanism and subsequent reduction/oxidation with ETF's respective dehydrogenase partners (17), and secondly, defining the partitioning of superoxide and hydrogen peroxide that is generated by ETF (18).

Multiple acyl-CoA dehydrogenase deficiency (MADD), also known as glutaric acidemia Type II, is an autosomal recessive disorder that is the result of either defective ETF or ETF-QO (19). On a metabolic level, this rare condition results in abnormal fatty acid, amino acid, and choline breakdown within its patients (19). The inability to oxidize fatty acids becomes imperatively important within the mammalian cardiac system, which

is the upstream process before the synthesis of ketone bodies, an essential alternative energy source for the heart (15). Within a chemical scale, MADD also results in irregular excretion of glutaric, lactic, ethylmalonic, and other organic acids (19).

Phenotypically, MADD can result in a wide range of outcomes. In extreme cases, early infant death from either metabolic acidosis or stress-induced hypertrophic cardiomyopathy and lipid accumulation in the heart is a likely outcome (15). Milder cases can be treated with riboflavin therapy and manifests itself in adolescence or early adulthood as muscle weakness associated with intracellular lipid accumulation becomes apparent (20). These cases are linked to different point mutations that result in a single amino acid substitution in close proximity to electron transferring cofactors (20), or involved in protein—protein interaction interface of ETF and acyl-CoA dehydrogenases (U). Therefore, understanding the structural implications of the electron transfer mechanism within the mitochondria and the respective protein—protein interaction interfaces have become the primary area of research relevant to ETF.

The second front of research involving ETF involves the partitioning of reactive oxygen species and hydrogen peroxide by ETF. Reactive oxygen species production by mitochondrial enzymes, including ETF, play a fundamental role in cellular signaling and the progression of dysfunctional states (18). Upon interacting with any of its partners, ETF's semiquinone FAD can potentially produce ROS or H_2O_2 as a byproduct, and of concern is the relative distribution of these molecules between healthy individuals and ones with an irregular system (21). Although ROS and H_2O_2 serve as signaling

molecules, disrupting this controlled system can lead to health complications including, of particular research interest, the onset of neurodegenerative diseases.

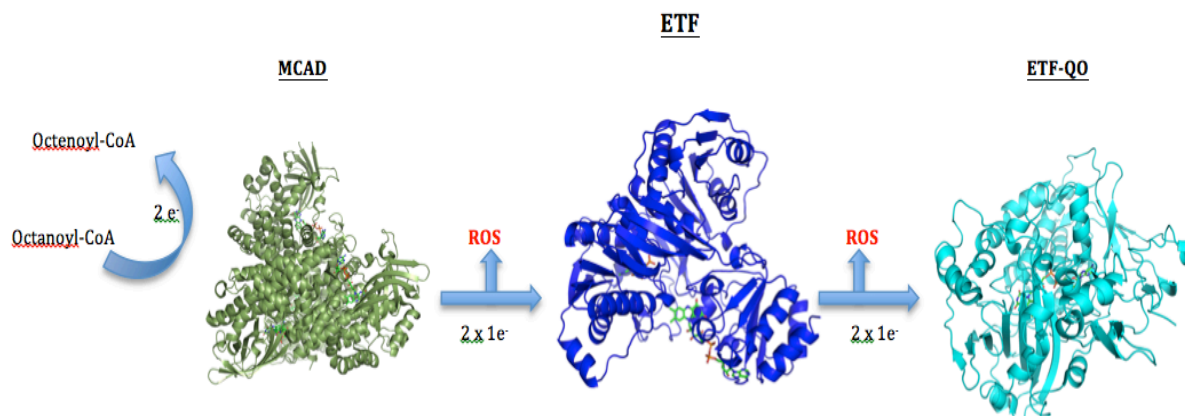


Figure 1. Catalytic activity of ETF. ETF's FAD cofactor is reduced to its semiquinone state (FADH*, one electron reduction) by a mitochondrial protein, in this example medium chain acyl-CoA dehydrogenase (MCAD) is shown. The completely reduced MCAD reduced two equivalents of ETF. ETF then transfer's its newly acquired electron to the membrane bound electron transfer protein-quinone oxidoreductase (ETF:QO) where it is used in other metabolic processes (1). During this electron shuttling, ETF's FADH* can react with oxygen or water to produce hydrogen peroxide or ROS. It has been hypothesized that the relative conformations of the FAD cofactor influence the partitioning of H₂O₂:ROS, and these signaling molecules can impact the cellular signaling/energetics of an organism (3)

Structure

ETF is a 63-kDA (570 amino acid residues) $\alpha\beta$ heterodimeric protein located in the mitochondria (22). Although ETF is a heterodimer, it contains a pseudo two fold axis of symmetry that brings about structural specificity and can be understood in evolutionary terms (16). As stated in the background section, ETF is a ubiquitous electron carrier that interacts with a host of dehydrogenases directly involved in a host of metabolic processes. These reducing partners exhibit residue-specific diversity when interacting with ETF and possess unique structural implications upon successive redox

chemistry (22). For practical applications as why to study ETF, a detailed structural understanding of ETF and its redox partners is essential.

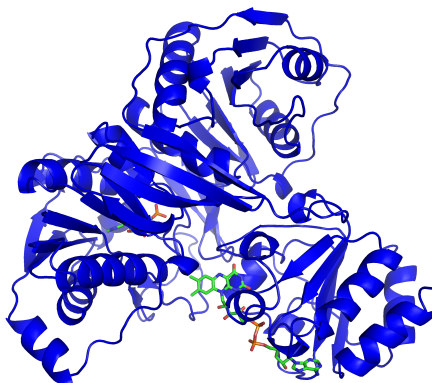


Figure 2. Depiction of electron transfer protein (ETF). Backbone is indicated in a blue cartoon image with cofactors FAD and AMP colored by atom. The way ETF is orientated will be referred to as “Initial View” in future images as a point of reference. PDB ID: 1EFV.

Three-Dimensional Structure As stated above, ETF is a 570 amino acid heterodimeric protein, with 315 residues belonging to the larger α -subunit and 255 amino acid residues make up the smaller β subunit, see Figure 3 (16). In terms of secondary structure elements, ETF can qualitatively be characterized as an α/β protein consisting of 18 α -helices 28 main β -sheets that are inter-mixed, or somewhat evenly distributed (16). Among these secondary elements, the α and β -subunits of ETF share many of similar length and orientation. Specifically, the two subunits share 11 similar β -sheets along with 5 α -helices that are strikingly similar structurally and in terms of positioning but share little sequence similarity (16).

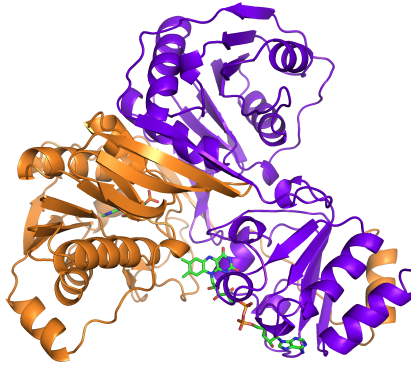


Figure 3. Initial view of the two subunits of ETF, a $\alpha\beta$ heterodimer. The α -subunit is colored in purpleblue and β -subunit is colored in orange.

The two subunits of ETF form three separate domains (16). Domain I is the N-terminal portion of α subunit. Domain II is made up of portions from both α and β subunits, which consists the C-terminal portions of the α subunit along with a small C-terminal portion of the β subunit. Domain III makes up the vast majority of the β subunit of ETF (16).

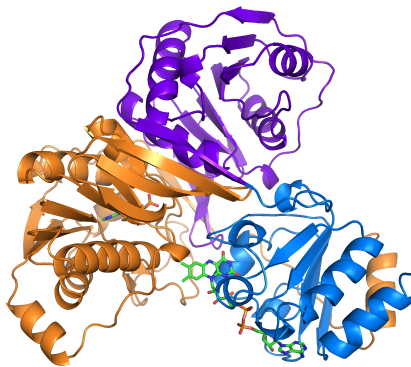


Figure 4. Initial view of the three domains of ETF. Domain I is indicated in purple blue. Domain II, colored in skyblue, consists of portions of the α -subunit and β -subunit. Domain III is colored in orange and consists solely of β -subunit contribution.

Domain I's core composed of seven-stranded parallel β -sheet with solvent exposed α -helices (16). These seven strands are numbered 1-7 in Figure 5. Domain I also contains a three-stranded anti-parallel β sheet (referred to as β -sheets 1'-3' in Figure 5) that shares a fourth β -strand (4') with Domain III (16). This interacting anti-parallel β sheet with domain I's pseudo-symmetric partner, domain III, form tight interactions that contribute to the stability of the quaternary structure of ETF. These interactions also results in a shallow bowl in which domain II is located (16).

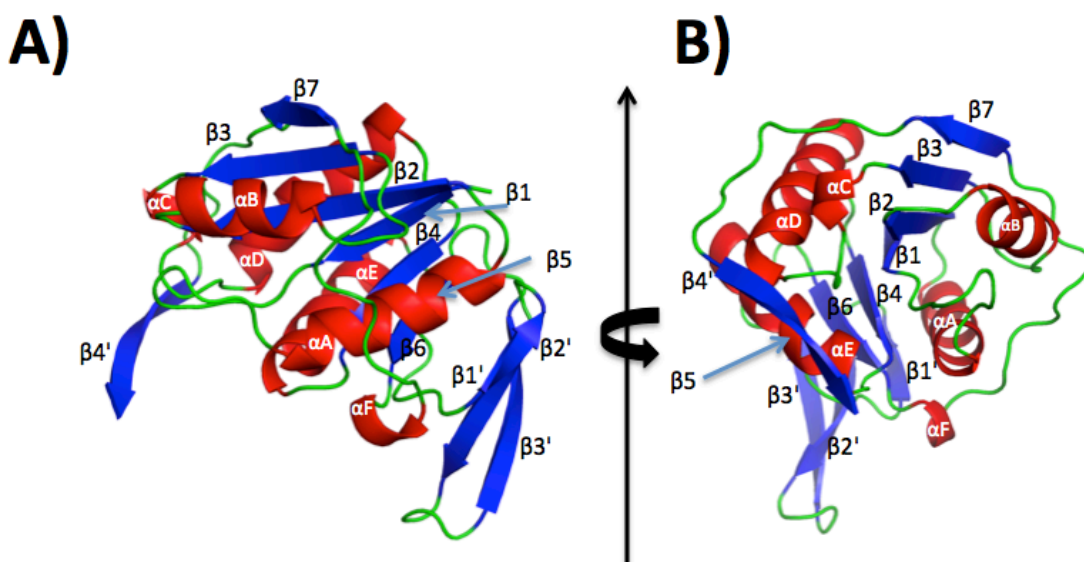


Figure 5. Two views of domain I of ETF. (A) View of domain I Initial View. (B) Same domain after a 90 degrees rotation about y-axis.

The fold of domain I belongs to a superfamily of proteins called Adenine Nucleotide A Hydrolase-Like (AANH) according the Structural Classification of Proteins (SCOP) database. This superfamily is sub-divided into three families, and domain I is a member of the ETFP family (SCOP). This family solely consists of homologous proteins that are referred to as electron transfer flavoproteins and are found across all domains of

life (15). According to SCOP, proteins of this fold are α/β heterodimers of homologous subunits, with sharing strands for quaternary structure stability (indicated with asterisks in Figures 5 and 7).

Domain II is composed of two parts, domain II α and domain II β . Domain II α is a five-stranded parallel β sheet connected by four α -helices that connect these five strands through right-handed helical crossovers (16). The C-terminal of the α subunit therefore contains a dinucleotide-binding motif, which is expected in FAD-binding proteins. In contrast to a traditional Rossmann fold that binds NADH, this dinucleotide-binding domain consists of a five, instead of six, parallel β -strands (β 1-5) interspersed by α -helices that lie on both sides of the five-stranded β -sheet (23). Respective stereo images of this fold can be seen in Figure 6.

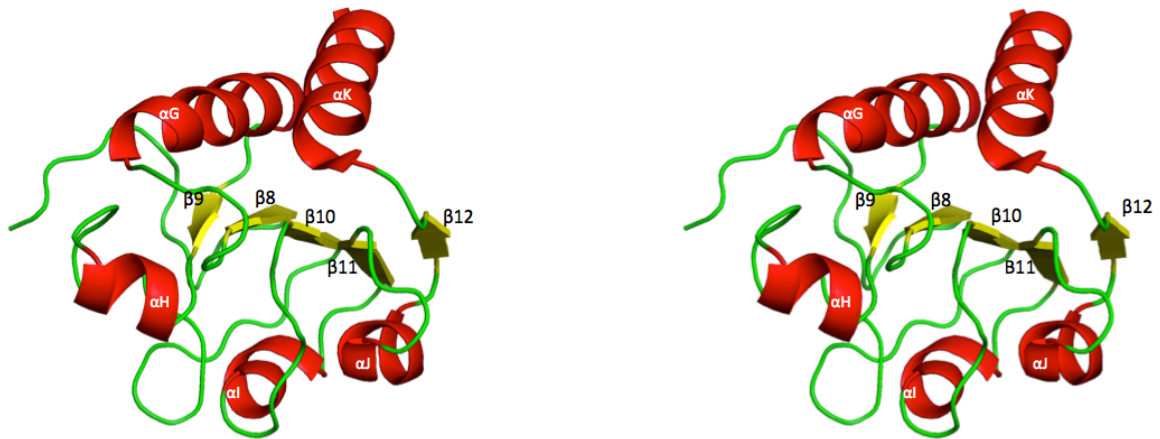


Figure 6. Stereo image of ETF's domain II or FAD-binding domain. β -sheets are colored in yellow and labeled 8-12 and α -helices G-K. This domain resembles a Rossmann fold, although one less β -strand is observed.

Domain II α makes up the C-terminal portion of the α -subunit and is separated by domain I through a hinge-like region (16). This hinge region is likely to behave as an

intrinsically disordered region with can bring about specificity through ETF's interactions with certain dehydrogenases in the mitochondria (24).

Similar to that of domain I, domain III (see Figure 7) is composed of a seven-stranded parallel β -sheet at its core, flanked by solvent exposed α -helices (16). It also shares β -sheets with the anti-parallel sheets of domain I, its pseudo-symmetric partner, to stabilize the quaternary structure of ETF (16). Domain III's fold family is described in the section on domain I.

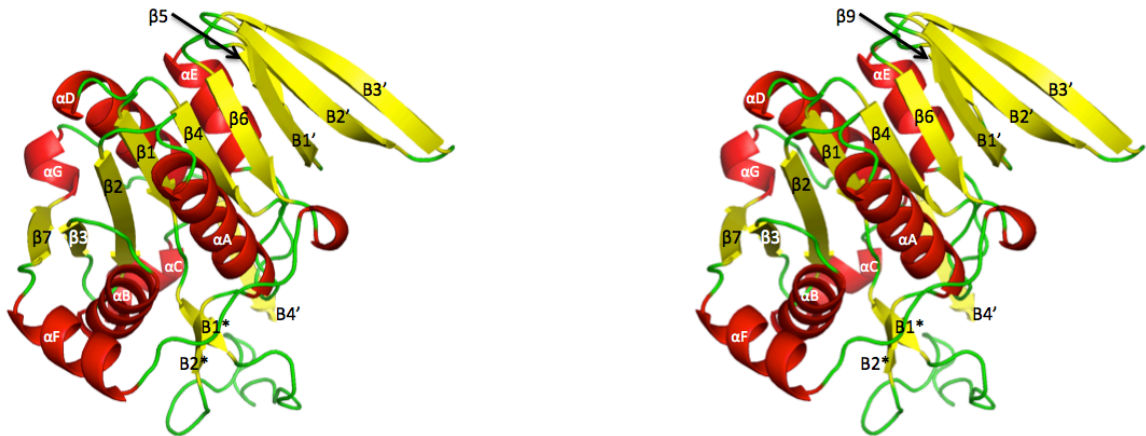


Figure 7. Stereo image of Domain III of ETF. β -sheets are indicated in yellow and full α -helices in red. A seven-stranded parallel β -sheet (1-7) makes up the core of the fold with flanking α -helices (A-G). The fold also has flanking anti-parallel β -sheets (labeled 1'-3' and 4') that share a β -sheet with domain I making a four-stranded anti-parallel β -sheet that stabilizes the quaternary structure of ETF. Unique to domain III are the two small β -strands indicated with an asterisk that come after $\beta 1$.

Symmetry Implications Domains I/III have been called pseudo-symmetric (16).

Their respective folds are identical, with the exception of a few loops. A topographical analysis of these domains can be seen in Figure 8. Interestingly, the sequence similarity between domains I/III are very low (13.6% identity) (16). This likely can be categorized into a gene duplication event followed by the truncation of the β -subunits

FAD domain, providing genetic economy in conjunction with extended reaction partners of ETF (16).

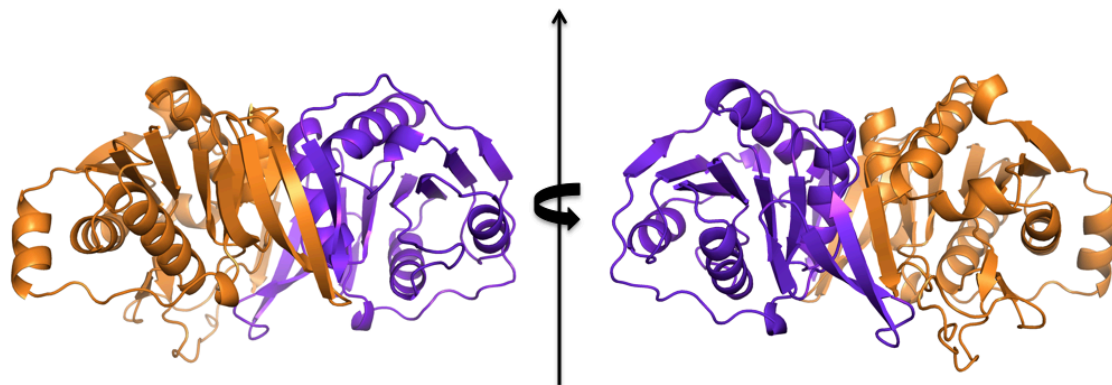


Figure 8. Image reflecting the pseudo-symmetry of domains I and II in electron transfer protein. 180 degree rotation.

Sequence

Within the sequence of ETF, the conserved residues are either involved in the coordination of the FAD cofactor (i.e. influence catalytic activity) or involved in protein-protein interactions. With that being said, the entirety of conserved residues of ETF are found on the surface or are solvent exposed to some extent. This can be seen in Figure 9. As one can see, almost the entirety of residues around the isoalloxazine ring are conserved, the catalytic portion of the FAD cofactor. Secondly, with respect to the image, the most prevalent interaction sites for ETF are within the β subunit in close proximity to ETF's catalytic center, the isoalloxazine ring, which also can be observed in Figure 9.

FAD Coordinating Residues In contrast to traditional dinucleotide-binding motifs, the FAD cofactor of ETF is bound in an elongated conformation of the second $\beta\alpha\beta$ unit

rather than the first (23), and with that, certain sequential characteristics are seen. ETF has been categorized to have sequence similarity with one other protein, pyruvate oxidase (23). Since it was characterized first, the pyruvate oxidase (PO) sequential family was developed to include itself and ETF (23).

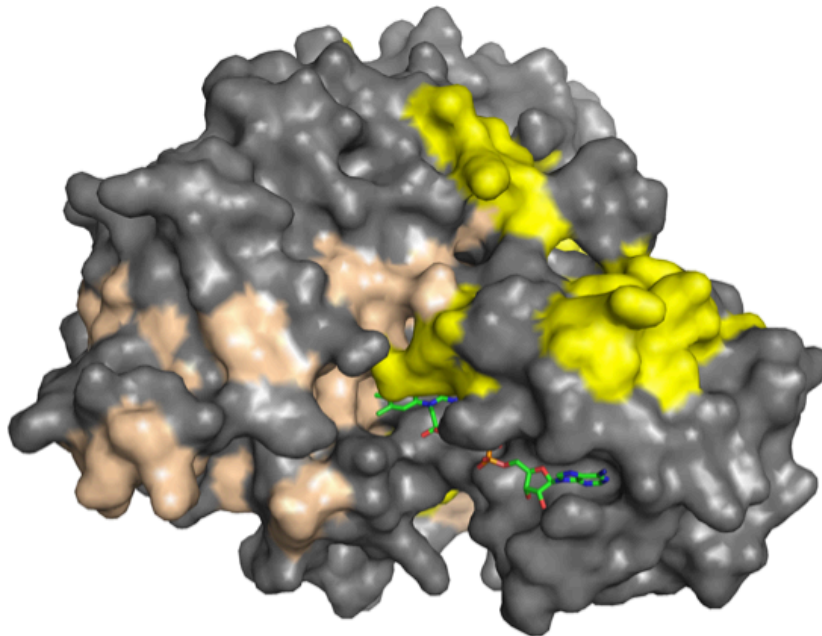


Figure 9. Conserved surface residues of ETF. Almost all conserved residues of ETF are on the surface either involved in FAD coordination or recognition of interactions partners. Yellow residues are conserved alpha-subunit residues and wheat colored residues are located in the beta-subunit.

The major difference between the cofactor coordination of a Rossmann fold and the PO family, is the absence of the conserved motif, GxGxxG (23). The absence of this motif is replaced by internal hydrogen-bonding within the FAD molecule. One such conserved sequence within the PO family is the motif KxLxxLxxxL(x)6SR(x)6V (23). This sequence is located in α H and β 9 of domain II (see Figure 6 for reference). The

highly conserved Thr and Ser are within hydrogen bonding distances of the OP1 and OP2 atoms of the pyrophosphate moiety, see Figure 12 (16,23). Another conserved motif is VGxS and is part of β 10 of domain II and stabilized the negative charge of the FAD cofactor (23).

Recognition Loop ETF's recognition loop is highly conserved in terms of structural/chemical composition (see Figure 10) (25). This loop extends from the β -subunit's externally exposed α -helix (helixF in Figure 7). The role of this loop is in the recognition of ETF's interaction partners, as the name implies. The sequence displays a host of complimentary stabilizing potential when undergoing protein—protein interactions of ETF (25). These different chemical components of the recognition loop and their importance can be seen “Electron Transfer Mechanisms” of this section.

GINKPRYASLRG I KQA	(M. <u>methylo</u> trophus)
GLNSPRYP T LPN I MKS	(C. <u>tepidum</u>)
GLNSPRYP T LPN I MKS	(R. <u>sphaeroides</u>)
RLNEPRYASLPN I MKA	(P. <u>dentrificans</u>)
RLNQPRYASLPN I MKA	(A. <u>thaliana</u>)
RLNTPRYVSLPK L MKA	(S. <u>cerevisiae</u>)
GLNKPRYASLRG I KQA	(C. <u>elegans</u>)
RLNEPRYATLPN I MKA	(H. <u>sapiens</u>)

Figure 10. Sequence of ETF's recognition loop across domains of life. The highly conserved residue Leu¹⁹⁴ is colored in green and influences the kinetic activity of ETF the greatest in mutagenic studies. Residues involved in hydrophobic interactions are colored in red and residues that stabilize the recognition loop are colored in blue.

Ligand Interactions

FAD ETF's domains II α and II β are responsible for coordinating the flavin adenine dinucleotide (FAD) cofactor (16). Domain II α resembles a flavodoxin (SCOP), although the FAD coordination is distinct between the two classes. 16 residues from the α -subunit and two residues from the β -subunit help to stabilize FAD in ETF (16). Of these residues of interest with respect to the FAD cofactor, they can be split into two categories; (1) first, aiding in maintaining the FAD cofactor in ETF, and (2) secondly (and albeit more interesting) are the residues that influence the chemistry of the FAD cofactor, and thus, having the most influence on ETF's catalytic activity.

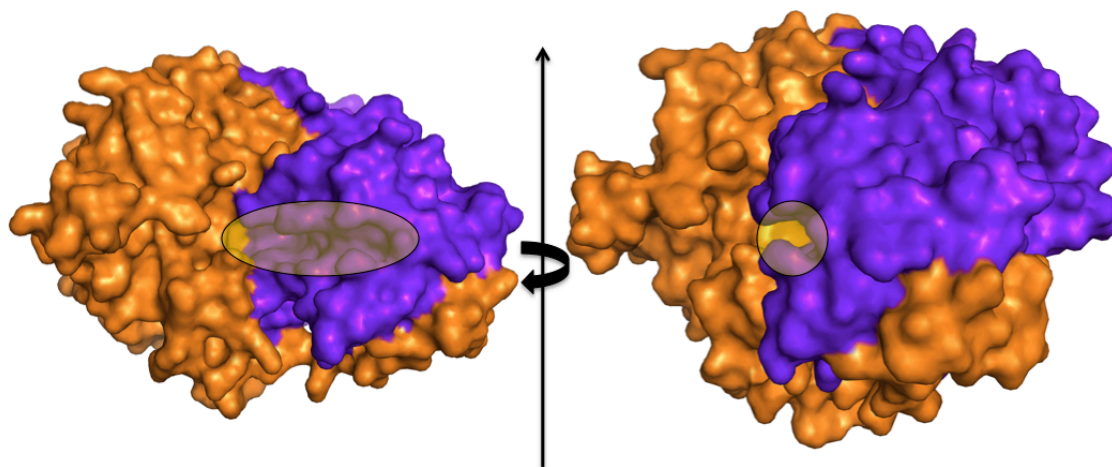


Figure 11. Surface view of the FAD binding pocket of ETF. Domains I/II are in purple blue and domain III in orange. A stereo image of the depth of the binding pocket is supplied in the supplementary information.

(1) Most of the residues that coordinate FAD in ETF are involved in hydrogen bonding with the cofactor (16). These residues stabilize the general location of FAD to fit within the topological binding pocket of ETF. The hydrogen bonding of these residues are either given by the residues R groups or from the amide backbone. In terms

of conservation, most of these residues are not highly conserved, although their basic chemical properties are. In reference to Figure 9, you can see the residues that likely coordinate the phosphate and adenine moieties are not highly conserved, although the residues surrounding the isoalloxazine ring (the catalytic center) are indeed highly conserved. These residues also can be seen in Figure 13, where they are indicated in blue.

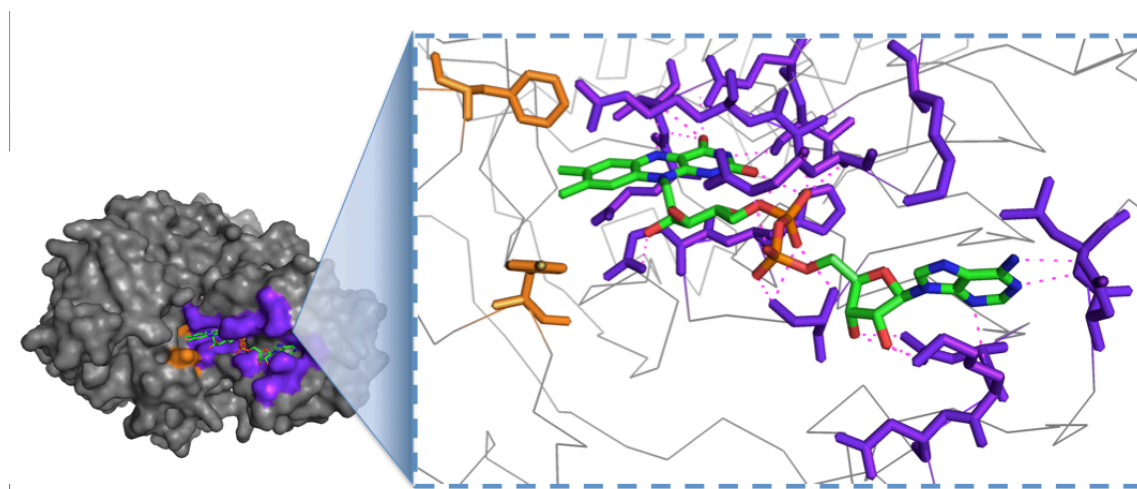


Figure 12. Zoomed in view of FAD coordination in ETF. Purple residues indicate coordination from domain II α and orange indicates contributions from domain II β .

(2) The residues that have been shown to impact the catalytic activity of ETF and are responsible for the coordination of FAD are indicated in yellow in Figure 13. These residues are highly conserved (25), and have been shown to impact the midpoint potential of FAD (18). These residues are in close proximity to the isoalloxazine ring of FAD and their electronic contributions can influence the semiquinone state of ETF (26). Of particular interest, α R249 has been shown to stabilize the anionic semiquinone state of the FAD cofactor in ETF along with the electron rich electron donors in which ETF

interacts with (16). Its positive charge accommodates the stability of the negatively charged catalytic site of ETF.

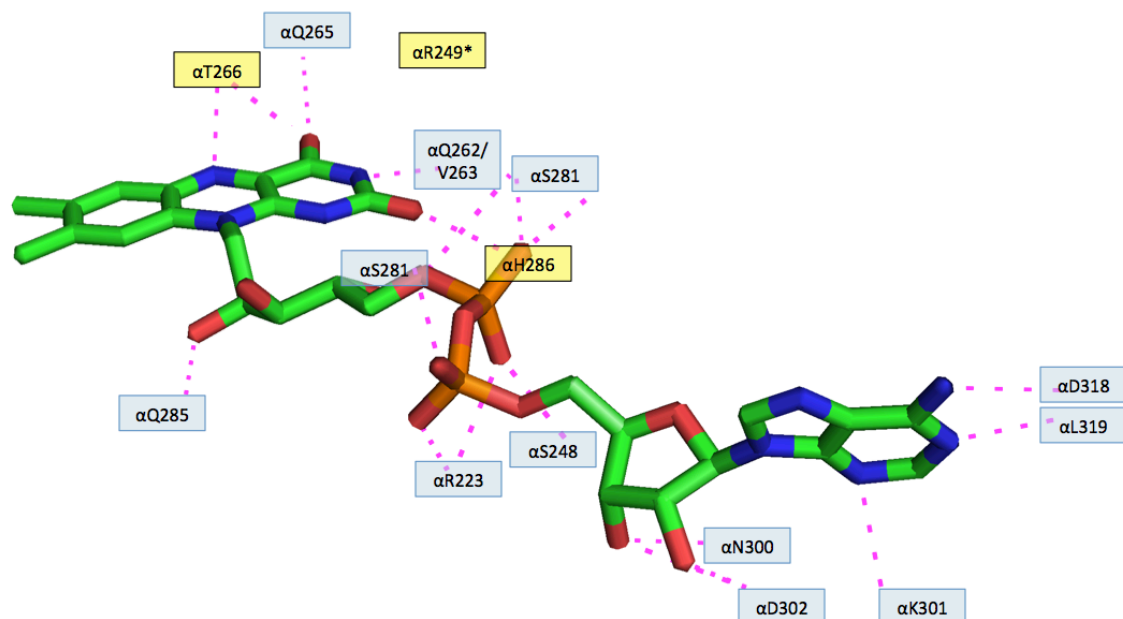


Figure 13. Coordination of the FAD cofactor in ETF. Blue residues are non-conserved residues that are involved in the coordination of FAD. Yellow residues, on the other hand, are considered electronically active and can impact the midpoint potential (i.e. catalytic activity) of the isoalloxazine ring of ETF, and as expected, are highly conserved. R249* residue is indicated with an asterisk due to the absence of direct H-bonding with FAD, although the positive charge stabilized the anionic semiquinone state of FAD.

AMP Little is known about the role of AMP's internal presence in ETF (16). It is neither solvent exposed (16), shown to influence the midpoint potential of FAD (21), or be immediately required to assemble the heterodimer (18). Although the role is not clearly defined, one can hypothesize a regulatory role (depletion of energy stimulating β -oxidation) or more likely, stabilizing the tertiary structure the β -subunit (16). The coordinating residues can be seen in Figure 14.

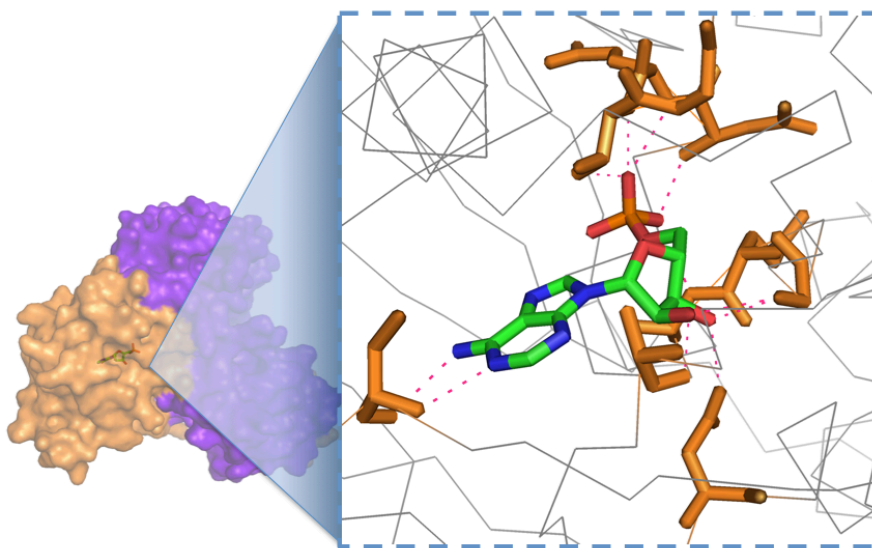


Figure 14. Zoomed in view of AMP coordination in ETF. TV_orange residues are the sole contributors for AMP stability and from domain III.

Electron Transfer Mechanism

Within eukaryotic mitochondrial matrix, ETF links the activity of a host of dehydrogenase enzymes and to the respiratory chain through successive redox chemistry (13). Therefore ETF plays a synonymous role to that of cytochrome-c in the intermembrane space of the mitochondria (27). ETF interacts with three groups of proteins involved in different catabolism of carbon containing compounds (17). Interestingly, each one of these groups share limited structural and sequential similarity making them evolutionarily distinct (17). Given that, ETF's interactions with a wide range of structurally distinct proteins implies that ETF possesses a certain degree of promiscuity, but also, in terms of regulatory impacts, it must possess a required degree of specificity (17). This interplay between diverse interacting partners and specific interactions separates ETF from other mitochondrial electron transporters, such as cytochrome c (27).

ETF's promiscuity with respect to its interaction partners comes from two factors; first of which is a one electron transfer through its FAD cofactor, second, is its highly conserved recognition loop (23). This recognition loop contains several factors that provide stability (17) when ETF undergoes electron transfer and will be discussed in the following case study of the ETF:MCAD complex.

Case Study—MCAD: Dehydrogenase Enzymes The crystal structure of ETF in complex with medium chain acyl-CoA dehydrogenase (MCAD) can provide essential insight on how ETF interacts with various proteins while undergoing its redox-dependent catalytic activity. ETF possesses a dual-mode interaction when transferring accepting electrons from MCAD; first, ETF uses its recognition loop (see “Sequence” section) to act as a static anchor within the ETF:MCAD interface, and secondly, utilizes the induced highly mobile redox active FAD domain to accept electrons from MCAD (17). In principle, this type of reaction contradicts the traditional thought process of “lock and key” protein interactions that are stabilized upon binding. In the case of ETF, the static anchor provides the mobility of domain II to sample various conformations of the FAD cofactor (17). This “sampling” of conformations is the basis for the interactions of ETF with structurally diverse proteins, i.e specificity on the surface provides enough stability for the FAD-containing domain to sample many conformations until a favorable electron transfer conformation is obtained. In terms of entropic penalty, ETF's catalytic activity is minimal upon interacting with its partners (17).

The initial understanding of the ETF:MCAD complex came from the crystal structure of the two proteins in complex with one another. In the crystal structure,

domain II (FAD-containing domain) of ETF lacks any significant electron density, indicating a highly transient or mobile state (17). Figure 15 represents a docking model obtained using Pymol of this complex.

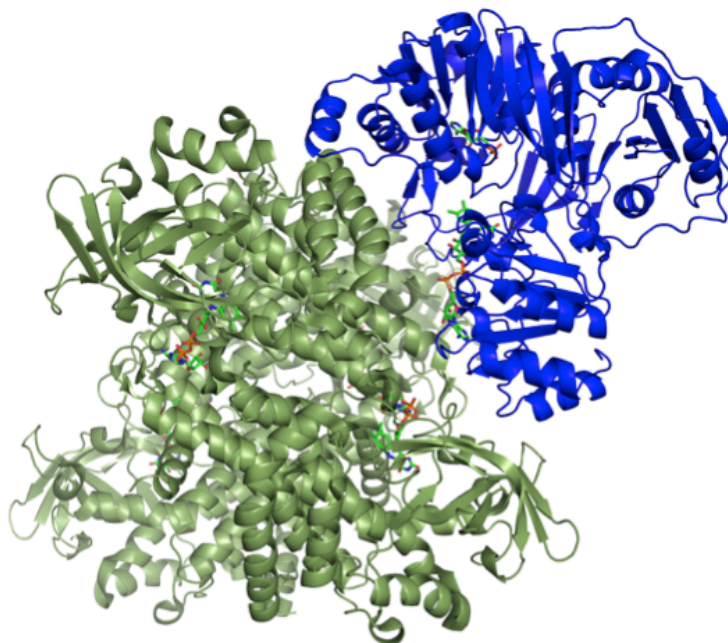


Figure 15. Docking model of ETF:MCAD. ETF is colored in blue and MCAD in smudge. For how this model was constructed, refer to Figure S6.

The interaction of ETF:MCAD surface is rather small, only utilizing 4.3% (ETF) and 3.2% (MCAD) of the complex's total surface area (17). Although small, this interaction area is highly complementary. The central relevance as to why this reaction takes place is through the ETF recognition loop (see "Sequence" section) (17). Three factors contribute to this highly specific, yet somewhat flexible interaction (due to small surface area interaction). The first of which is three hydrogen bonds formed from six respective residues from ETF and MCAD (Figure 16 Panel B). The second is the formation of a hydrophobic path on both ETF and MCAD that align during complex

formation (Figure 16 Panel C). Finally, the last stabilizing force of ETF:MCAD static anchor is the alignment of helix dipoles from helix C of MCAD and helix β F of ETF (Figure 16 Panel D) (17).

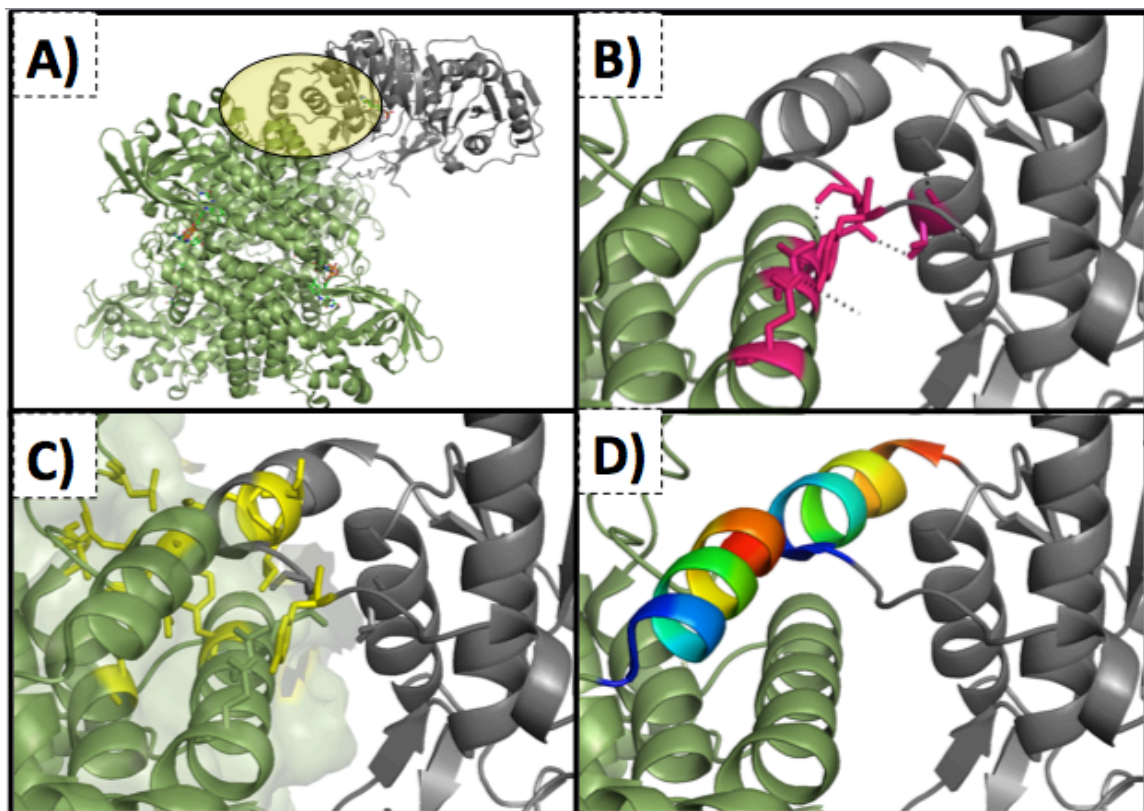


Figure 16. Stabilizing interactions of the static anchor of ETF:MCAD complex. (A) Zoomed out view of PDB ID 1T9G with static anchor region highlighted. (B) H-bonding of ThrMCAD26 - Ala β 193, GluMCAD34 - Tyr β 192, and GluMCAD22 - Thr β 77. (C) Hydrophobic residues in proximity of recognition region. (D) Aligned Helix dipoles from MCAD and ETF.

The interaction of ETF:MCAD at the recognition loop can be regarded as a “ball-and-socket” type of motion (17). Where the anchor is MCAD and the ETF moves above the static anchor somewhat freely. Although, this freedom of motion of ETF cannot simply explain the electronic transfer of the ETF:MCAD complex. When the static anchor of the complex is formed through ETF’s recognition loop, the closest respective

FADMCAD is 35 Angstroms away, which is clearly incompatible with electron transfer through space in which the transferring components need to be within the accepted 14 Angstroms (21). Therefore, a hinge region on ETF would make sense to accommodate this lack of space. This hinge region is made up of the stretches of amino acids connecting domain I and domain III to domains II α / β respectively (17). The hinge region contains conserved proline residues at the β 230/231 (23). These proline residues provide structural stability with the formation of sp² hybridized atoms within the amide backbone, promoting extended conformations of the hinge region (see Figure 17).

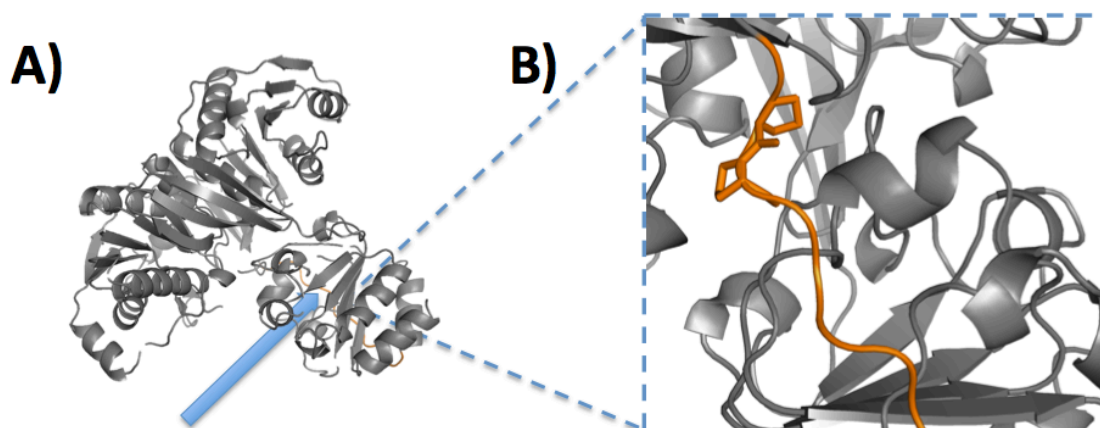


Figure 17. Indicates conserved proline residues within β -subunit's connector arm that contributes to domain II of ETF. (A) Depicts a gray scale image of ETF along with domain II's β -subunit's contribution orange color. (B) Zoomed in view after a 180 degrees rotation about y-axis.

Although the extended conformation capability of ETF allows extension of the FAD domain, it does not solely allow for specificity of ETF and its electron transfer partners. Two residues in particular, ArgETF249 from the FAD-coordinating domain of ETF and a Glu212 from one of the MCAD monomers are involved in increased specificity of electronic transfer. ArgETF249 and Glu212 undergo an electrostatic

interaction to bring the FAD cofactors into close proximity, thus promoting electron transfer of MCAD to ETF (see Figure 18) (17).

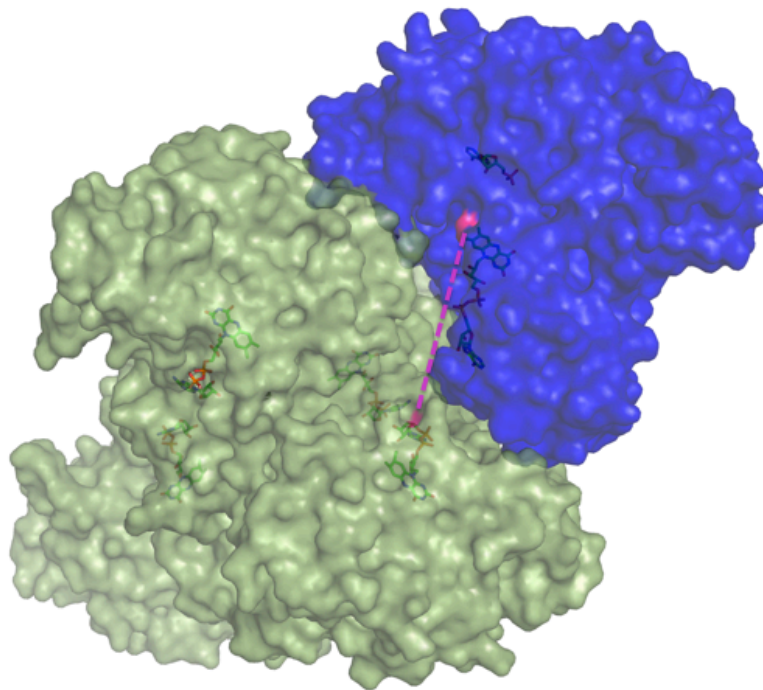


Figure 18. Indicates the two residues (in pink) from ETF and MCAD that undergo an electrostatic interaction to bring FAD cofactors into close proximity to undergo electron transfer. Arg²⁴⁹ of ETF and Glu²¹² of MCAD.

Overall, the ball of the socket is made up by domains I and III of ETF, the non-FAD coordinating domains. From there the static ETF domains can sample various accommodations in compliment with the hinge region to obtain acceptable electron transfer distances and conformations to permit fast electron transfer between ETF and MCAD (17, 22, 24). The recognition loop of ETF is highly conserved and other proteins interact with ETF in a similar manner.

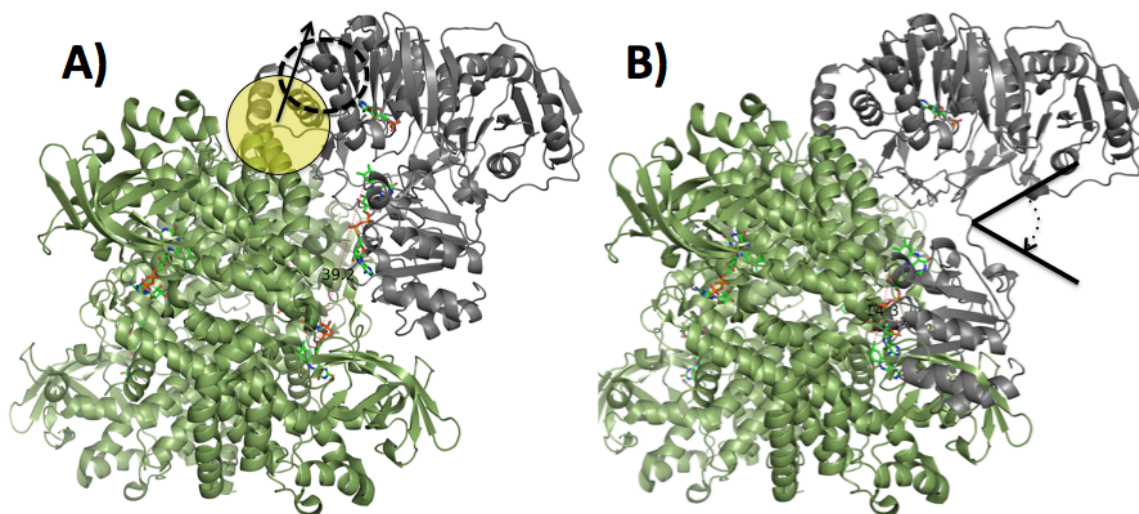


Figure 19. Model of how ETF is reduced by MCAD with “dual-mode” of mechanistic action with the distances of FAD electron transfer cofactors indicated. (A) Shows ETF docking to MCAD with the recognition loop acting as a static anchor permitting multiple conformations acting as a ball-and-socket joint. (B) Shows the hinge region of ETF permitting allowable electron transfer distance to occur of 14 Ångströms.

Regulation

Not much research has gone into detail pertaining the regulation of ETF. This could be because ETF could be regulated on so many levels due to its interactions with a host of enzymes within the mitochondria. Another way to view this is that if ETF is down regulated, many systems would be impaired, thus, down regulation of ETF would be detrimental to an organism. Although little research has been conducted, two types of modification to ETF has influenced its catalytic activity (28, 29).

The first potential area of regulation of ETF can come from bulky covalent substitutions (29). The C8 portion of the FAD cofactor is exposed to solvent in order to be able to undergo its catalytic activity. With that being true, this carbon is susceptible to the attachment of covalent substitutions (29). In particular, a CoA moiety has been shown to interact at this position (29), and disrupt ETF’s catalytic activity. It then can be

rationalized that if β -oxidation is over-active, a CoA moiety may react with ETF and down-regulating its activity to reduce levels of β -oxidation.

The second area of regulating ETF is through post-translational modifications, specifically, methylation. Within mammalian mitochondria, protein methylation is a fairly uncommon post-transcriptional modification, although within the mitochondria, methyltransferases and their substrates have been shown to regulate a host of cellular processes/products (28). Methyltransferase-like protein 20 (METTL20) has been shown the potential to regulate the activity of ETF (28). This protein achieves this regulation by methylating lysine residues 199 and 202 of the β -subunit of ETF (28). These residues are within the recognition-loop region of ETF, thus methylating these residues impairs recognition loop association, impairing ETF's catalytic activity (28). Interestingly, METTL20 shows remarkable specificity towards this region with no methylation being observed in the α -subunit (28). Therefore if METTL20 is up-regulated, the catalytic activity of ETF would be impaired.

Theory

Flavoenzymes Activating O₂

Reactive Oxygen Species (ROS) are produced when molecular oxygen (O₂) interacts with redox active molecules. O₂'s molecular orbital (MO) diagram contains two unpaired electrons in antibonding orbitals with parallel spins, or in other words, O₂'s ground state is triplet in nature. O₂ can form an ROS, O₂^{•-}, by undergoing a one-electron reduction step with a radical redox intermediate. One such intermediate is electron transfer flavoprotein (ETF). In general, flavoenzymes have been shown to be a major source of biological ROS (30). When mixed with O₂, reduced flavins immediately become oxidized due to the accumulation of more reactive species present (31).

Radical Pairs O₂ can accept one electron at a time to form the superoxide anion. Reduced flavoenzymes can activate molecular oxygen in this manner by forming a radical pair intermediate between the flavin semiquinone (FADH[•]) and superoxide anion. This transient stabilization of the two radicals can either release O₂^{•-} or continue to stabilize the radical pair to eventually release H₂O₂ (32).

Mechanistically, radical pairs of flavoenzymes are formed in a two-step manner (see Figure 20). First, the reaction is initialized via a charge transfer process of the reduced enzyme (E-Fl_{red}) that allows the newly formed negative semiquinone (E-FlH[•]) to undergo an electron transfer to O₂. Second, the now neutral semiquinone state of flavin within the enzyme (E-Fl_{sq}H[•]) forms a radical pair with the superoxide anion, denoted FADH[•]:O₂^{•-} (33). Once the radical pair is formed, it is important to note that the spin-

correlated radical pair is in the triplet state. This is because once the electron transfer is initiated, there is no spin-inversion upon the pairs formation, yielding a triplet state (33).

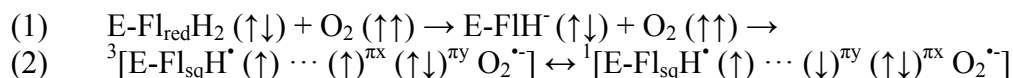


Figure 20. Two-step scheme, proposing how a radical pair is formed between a flavoenzyme and molecular oxygen.

Redox Switch—Influencing Singlet-Triplet Intersystem Crossing Once a spin-correlated radical pair is formed between a flavin cofactor and molecular oxygen, hyperfine interactions allow for interconversion between the initial triplet state of the pair and a singlet state (34). When radical pairs of this nature are formed (i.e. knowledge of spin-states and orientation), a relative ratio of recombinant products correlating with spin multiplicity can be determined (34, 35). External magnetic fields (R_f) impact electronic motion, therefore, can also dictate yields (singlet vs. triplet) of recombinant radical pair products (36). Implementing a magnetic field to flavin-containing systems has shown to tune the equilibrium of intersystem crossing (k_{isc}) between singlet and triplet states, redistributing the relative ratio of products (33, 37, 38).

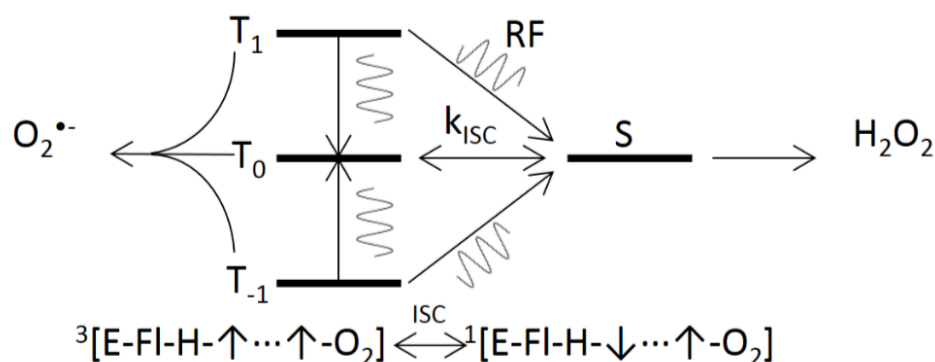


Figure 21. Energy levels of spin-correlated radical pairs. The intersystem crossing rate (k_{isc}) can be modulated by a magnetic field.

Singlet-triplet mixing of radical pairs dictate ROS products (see Figure 21).

Either the application or suppression of a magnetic field should modulate the relative distribution of ROS products formed via a radical pair of a flavoenzyme and molecular oxygen. Impacting ROS production as a function of a magnetic field as a potential switch could have substantial biological effect. Further understanding this redox system, or “redox switch”, could elucidate fundamental knowledge in illustrating the biology of Reactive Oxygen Species.

Scope of Thesis

The overall focus of this project is to quantify Reactive Oxygen Species within a known ROS-producing system. This system of choice is the reduction and re-oxidation of Electron transfer flavoprotein (ETF). ETF is a central component to cellular respiration, in which it is an electron funnel that relays electrons to the ubiquinone pool that are generated through the first step of fatty acid β -oxidation (18). Knowledge of how this important metabolic system produces ROS, and any insight on relative rates or ways one can influence the production of specific ROS products will aid in the limited understanding of ROS' role in biology. To address these issues, this project contained two aims that will contribute to the understanding of ROS biology, in terms of both production and potential therapeutic insights.

Aim 1: Define the ROS Produced by the Re-oxidation of ETF

Since the reduction ETF is a one-electron process, we have hypothesized that the semiquinone state of the FAD cofactor will produce more $O_2^{\bullet-}$ relative to the amount of H_2O_2 produced. Experimentally, this aim will be addressed by setting up a system of completely reduced ETF, followed by simultaneous assays that quantify specific ROS. Utilizing an assay that specifically reacts with H_2O_2 and one with $O_2^{\bullet-}$, investigation of the relative rates at which ETF produces ROS was performed.

Aim 2: Understand How the Implementation of a Magnetic Field Influences Products

It has been shown that a magnetic field can impact electronic motion and dictate the distribution of singlet-triplet states of radical pairs (33, 36, 37, 38). Therefore, if the

implementation of a magnetic field impacts the intersystem crossing rates (k_{isc}) of singlet and triplet states, we can relatively change the ratio of specific ROS produced. Again using the same reduced protein, we performed the same ROS-specific experiments at two different magnetic fields. The fields of choice were 50 μ T (micro Tesla) and 20 nT. Low magnetic fields have been shown to suppress the production of ROS in cancer cells (39), hence, further defining of a magnetic field can impact ROS productions could potentially have therapeutic benefits.

METHODS

Purification of ETF

The growth and purification of ETF was completed by adopting a procedure used at University of Colorado—School of Medicine (40). Modifications were made to optimize protein yield.

Growth

A scraping of frozen ETF cells were seeded in 50 mL of Terrific Broth (TB). 50 μ L of ampicillin stock (100 mg/mL) was also added to the seed culture and was allowed to grow overnight in a shaking incubator set to 37° C.

The following day, 5 mL of seed culture was inoculated to 1 liter of autoclaved TB media (6 liters per growth). The bacteria were allowed to grow throughout the day in the presence of 100 μ g/mL ampicillin until the OD₆₀₀ was between 0.6 and 1.0. After the culture reached the respective OD₆₀₀, the cultures were induced with 25 mL of saturated riboflavin in Ultrapure H₂O, 1 mL of 0.5 M IPTG, and 500 μ L of 100mg/mL ampicillin. Cultures were grown over night after induction in a shaking incubator set to 37° C.

Harvest/Lysis

Flasks were removed from incubators and placed on ice. Cultures were centrifuged in 400 mL centrifuge bottles and spun at 6000 rpm for 20 minutes. After all of cultures were spun down, pellets were combined and weighed. Cell pellets were snap

frozen in liquid nitrogen and stored in -80° C freezer until lysis and purification was desired.

Cell pellet was thawed on ice the day of lysis. Pellet was resuspended in 3-5 volumes of 10 mM KH_2PO_4 , pH=7.5, 10% glycerol. A lysis cocktail containing 0.5 M PMSF, 0.5 M EDTA, 100 mM DTT, 50 mM FAD, 25 mM TLCK, and 10 mM leupeptin was added to suspended pellet in a 1:1000 dilution. With a small spatula, lysozyme and DNase were added (~0.5 g) and allowed to stand for 10 minutes.

Enzymatic lysate was spun at 20,000 rpm for 20 minutes and supernatants were pooled. The supernatants were then spun in an ultracentrifuge at 30,000 for 60 minutes, and again, supernatants were pooled.

Purification

All column development was performed at 4° C. After centrifugation, supernatants were loaded onto a DEAE fast flow column equipped with a flow adaptor and equilibrated with 10 mM KH_2PO_4 , pH=7.5, 10% glycerol. Using a Biorad Econopump, a flow rate of 3 mL/min was ran and fractions were collected immediately. ETF did not stick to the DEAE column, impurities did.

Yellow fractions (indicative of flavin presence) were concentrated using an EMD Millipore centrifuge concentrator (10,000 MWCO) by spinning fractions at 6000 rpm for 5 minutes and repeating the process until less than 100 mL remained.

Concentrated fractions were then loaded on to an SP sepharose column equilibrated with 5 mM KH_2PO_4 , pH=7.5, 10% glycerol. A gradient of 50-200 mM KCl was ran to elute ETF off column. The migration of ETF was monitored by the migration

of a dark yellow band down the column. Once approaching the bottom of the column, fractions were collected, and again were then concentrated down to less than 100 mL in the same manner as described earlier.

To remove unwanted salt, the concentrated protein was dialyzed against 10 mM KH_2PO_4 , pH=7.5, 10% glycerol overnight. After dialysis, samples were snap frozen in liquid nitrogen and stored in -80°C freezer.

Determination of Flavin Loading

An imperative step in setting up the partitioning assays was the calculation of flavin that was present or coordinated within the flavoproteins. This percentage of flavin absorbance vs. protein concentration is referred to as the flavin loading percentage. To accomplish this a total protein concentration was obtained using a Nano Drop device. Next, the absorbance at 450nm from the same sample was recorded. Using Beer's law ($A = \epsilon bc$) the concentration of flavin cofactor was calculated (see Table 1). All concentrations of either [ETF] or [MCAD] in "Methods" section indicated the concentration of flavin bound in each respective enzyme.

Electron Transfer Flavoprotein (ETF)	97% Flavin Loading
Medium Chain Acyl-CoA Dehydrogenase	33% Flavin Loading

Table 1. Shows the percentage of flavin adenine dinucleotide bound to protein.

FAD Reduction

The reduction and re-oxidation of a free flavin in solution was performed. The smallest flake of flavin was suspended in 1 mL PBS and transferred into an anaerobic 1

mL Starna quartz cuvette. 10 μ L of 100 mM DTT was also added to the cuvette and was sealed with silicon septum. Solution was purged of O₂ by undergoing 20 cycles of Argon purging using an anaerobic Schlenk line equipped with an O₂ scrubber.

After the solution was made anaerobic, a UV-Vis scan was taken of the solution from 300-600nm using a Cary 300. The cuvette solution was then transferred to an ice bath within a 250 mL glass beaker. The solution was exposed to a 250 W Halogen work lamp from a distance of 10 cm. UV-Vis scans were performed every 5 minutes in the same manner as earlier described.

Once the A₄₅₀ ceased to decrease in a five-minute interval, the sample was deemed completely reduced. The cap and septum of the anaerobic cuvette was removed and oxygen was exposed to the reduced flavin. UV-Vis scans were performed every 30 seconds to monitor the increased A₄₅₀, which corresponded with the flavin becoming re-oxidized.

Enzymatic Reduction

Preparation of Protein

Both ETF and MCAD were removed from -80° C freezer and allowed to thaw on ice. Once thawed, a 24 μ M solution of ETF was prepared by diluting stock solution in 10 mM Tris Buffer, pH = 7.5, to a final volume of 800 μ L. The ETF solution was then transferred into an anaerobic cuvette and purged with Argon gas for 20 cycles. During each cycle, a small spatula was used to lightly tap the solution to ensure the entirety of O₂ was removed from solution.

MCAD solution was prepared by first adding 50 μL of 2 mM Octanoyl-CoA to the protein solution. Octanoyl-CoA was the chosen electron source for this reaction due to MCAD's high affinity for this length of fatty acid. This solution was then diluted to obtain a final volume of 0.1 μM MCAD with the same Tris buffer used to prepare ETF. After dilutions were made, the MCAD solution was transferred to a 1.5 mL crimp vial and purged with Argon gas for 20 cycles in the same manner as ETF. Once reduction reaction was initiated the final ratio of ETF:MCAD was 20 μM ETF to 0.02 μM MCAD.

Monitoring Reduction

An Agilent Cary 300 was used to monitor the reduction spectra of ETF. The settings were as followed, a range of 350-600 nm, a scan rate of 600 nm/min, and a baseline subtraction of a 10 mM Tris Buffer, pH = 7.5. The Cary 300 was also equipped with a cuvette temperature control accessory that held the protein samples at a constant temperature of 20° C (see Appendix A1).

After ensuring an O_2 free environment of both ETF and MCAD, an initial oxidized scan of ETF was obtained (Figure 28). After oxidized scan, a 500 μL Hamilton syringe was made anaerobic through ten purges of Argon gas. After syringe purging, the Hamilton syringe remained in an upright orientation to ensure heavier Argon gas remained in syringe and MCAD solution in crimp vial was punctured in the same upright orientation. This technique ensured anaerobic nature of transferring solutions and can be affirmed by the same technique being applied to the O_2 sensitive FAD solution (see Figure 27).

200 μ L of MCAD solution was transferred into ETF anaerobic cuvette using the technique described above. Visible spectra were obtained in regular increments until the A_{450} remained unchanged. After many trials, this time point was found to be 15 minutes after the addition of MCAD and Octanoyl-CoA were added to the oxidized ETF solution.

Imposing Magnetic Field

Two incubators equipped with an electrical system were used to impose various magnetic fields on samples. A constant magnetic field was obtained by using a quantified electrical current that correlates with the intensity of the magnetic field. Both the current input and a sensory reading of the magnetic field of the incubators was monitored continuously (see Appendix A2). The two magnetic fields employed were 50 μ T, which corresponds to the magnetic field of the earth, and a suppressed 20 nT. The incubators were held at 20° C during the re-oxidation of ETF and while conducting the partitioning assays to quantify ROS of samples.

Partitioning Assays

To quantify both ROS species of interest, two assays were used to quantify H_2O_2 and $O_2^{\bullet -}$. Both assays resulted in a fluorescent product that indicates the presence of a specific ROS within experiments. Assays quantified the specific ROS produced upon the re-oxidation of the FAD cofactor in ETF. To perform assays, ETF was enzymatically reduced (described above) in an anaerobic environment, and molecular oxygen was re-introduced to the system by the addition of oxygenated buffers containing the reagents

necessary to perform ROS-specific assays in a simultaneous manner. Both H_2O_2 assay (Amplex Red) and $\text{O}_2^{\bullet-}$ assay (DHE) were performed using the same reduced protein and in the presence of varying magnetic fields.

Amplex Red Assay

Amplex Red is a fluorescent probe that is used to quantify and detect H_2O_2 (41). In the presence of enzyme Horseradish Peroxidase, Amplex Red reacts with H_2O_2 in a 1 to 1 fashion to produce a small molecule called resorufin (see Figure 22). Resorufin is quantified using its fluorescent signature of an excitation wavelength of 554 nm and emission of 593 nm (41).

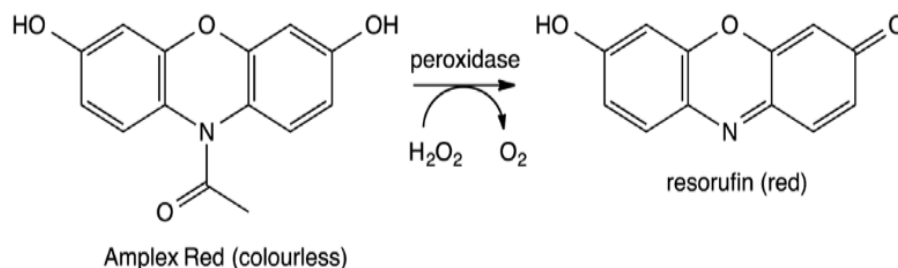


Figure 22. Mechanism of Amplex Red Assay. In conjunction with enzyme Horseradish Peroxidase (HRP), a colorless Amplex Red (AR), reacts in a 1:1 fashion with H_2O_2 to produce a fluorescent molecule resorufin.

10 mM Amplex Red stocks were prepared by dissolving the salt in DMSO and distributing the solution into 10 μL aliquots. Horseradish peroxidase (HRP) stock was 20 activity units per mL (U/mL). Both Amplex Red and HRP stock solutions were stored in -20°C freezer.

The day of performing experiments, both Amplex Red and HRP stock solutions were thawed on ice. Once thawed, 10 μL Amplex Red stock and 40 μL HRP stock were

combined and diluted to a final volume of 1 mL using 10 mM Tris Buffer, pH = 7.5. This resulted in a “reaction buffer” that contained 100 μ M Amplex Red and 0.8 U/mL HRP.

250 μ L of the above reaction buffer was transferred into four 1.5 mL crimp vials and sealed. To each of these vials, 250 μ L of solution was added, yielding final concentrations of 50 μ M Amplex Red and 0.4 U/mL HRP. Two of these vials contained the reduced ETF solution and two would serve as blanks containing anaerobic Tris buffer. The addition of either ETF or Tris Buffer was performed using a Hamilton syringe and conducted within each a respective incubator with a constant magnetic field of 50 μ T or 20 nT.

Solutions were allowed to re-oxidize for 10 minutes before being loaded on to a black plate reader tray. 10 minutes was chosen due to a cease in A_{560} increase that was performed in control experiments. Fluorescent readings were performed in triplicate and experimental value was obtained after the subtraction of absorbance of blank solution. A sample of what an experimental output produces can be seen in Appendix B1 and a standard curve was obtained with a quantifiable range of up to 10 μ M using an H_2O_2 solution in Tris Buffer (Figure 23).

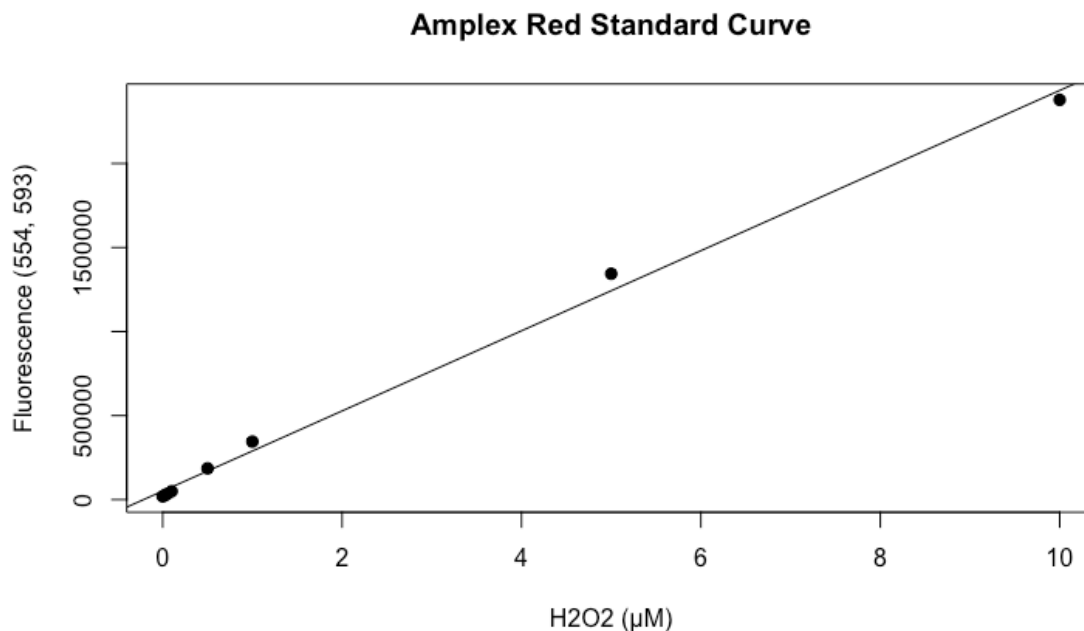


Figure 23. Amplex Red Standard curve used to quantify H₂O₂. A linear equation of $y = 238,432x + 50,799$ with an $R^2 = 0.99$ was used to quantify H₂O₂ of experiments.

Dihydroethidium Assay

Dihydroethidium (DHE) is a fluorescent probe used to quantify the production of O₂^{•-} (41). DHE reacts specifically with the superoxide anion to produce a hydroxylated product, 2-hydroxyethidium (2-OH-E⁺ or DHEO) (Figure 24) (42). Although a specific product is produced, other non-specific oxidative products are observed in these assays that have overlapping fluorescent signals (42). Traditionally, this issue has been addressed by employing HPLC-based techniques to separate these products based on a fluorescent signal coming off of the column and isolated peaks used to allow for quantification (42). Although this technique is regularly utilized, there seems to be a lot of issues getting substantial peak separation. Also, even once the peaks are separated adequately, further testing needs to be done to identify the particular product that is

producing that respective peak due to identical fluorescent signatures. To address this, we developed a Mass Spectrometric technique that allows for separation and identification of $O_2^{\bullet-}$ -specific products to allow for quantification.

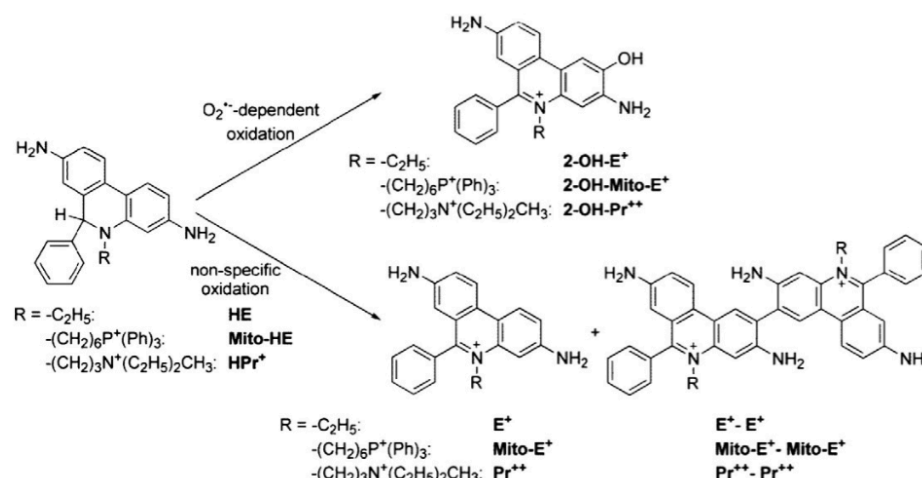


Figure 24. Oxidative products of DHE. DHE has a m/z value of 316, the $O_2^{\bullet-}$ specific product, 2-OH-E⁺ or DHEO, has a corresponding m/z value of 330, and the dominant non-specific product ethidium, has a m/z value of 314. Corresponding chromatography peaks were separated and could be extracted on the basis of size to allow for specific quantification.

Experimental Set-Up All experimental steps were performed with lights off in the lab to eliminate interaction with light-sensitive DHE. 10 mM DHE stock solution was distributed to create 5 μ L aliquots and stored in -20° C freezer. On the day of experiments, an aliquot was removed and wrapped in tin foil to eliminate interaction with light. Once covered, aliquot was allowed to thaw on ice. The thawed DHE solution was diluted using 10 mM Tris Buffer, 100 μ M DTPA, pH = 7.5 to a final volume of 1 mL to create a reaction buffer containing 50 μ M DHE. 250 μ L of this reaction buffer was added to both the controls and reduced ETF solution in the same manner as described above in “Amplex Red” section. After re-oxidation occurred for 10 minutes, samples were

transferred into a 1.5 mL Eppendorf tube and flash frozen in liquid nitrogen. Samples were stored in -80° C freezer until MS quantification took place.

Sample Preparation When quantification means were available, DHE frozen samples were thawed on ice in a sealed cooler. Once thawed, 500 µL of DHE samples were mixed with 1 mL of cold acetone and stored in -80° C freezer for two hours to precipitate unwanted protein. Samples were spun at 6000 rpm and supernatant was transferred into snap-close vials for MS-analysis.

MS Method Prepared samples were analyzed via liquid chromatography mass spectrometry (LCMS) using an Agilent 6538 quadrupole time of flight (Q-TOF) mass spectrometer in positive mode coupled with an Agilent 1290 ultra high-performance liquid chromatography (UHPLC) system. Chromatographic separation was achieved with a 1.8µm x 100mm Agilent Eclipse Rapid Resolution High Definition (RRHD) C-18 reverse-phase column.

A 20-minute method was developed with mobile phase A consisting of HPLC grade water with 0.1% formic acid as a counter ion and mobile phase B consisting of HPLC grade acetonitrile with 0.1% formic acid. Mobile phase B was increase from 5% to 95% using a linear gradient from 2 minutes to 18 minutes. 95% B was held for 1.5 minutes followed by a return to the original solvent concentrations. An injection volume of 15 µL was used along with a column compartment temperature of 30° C.

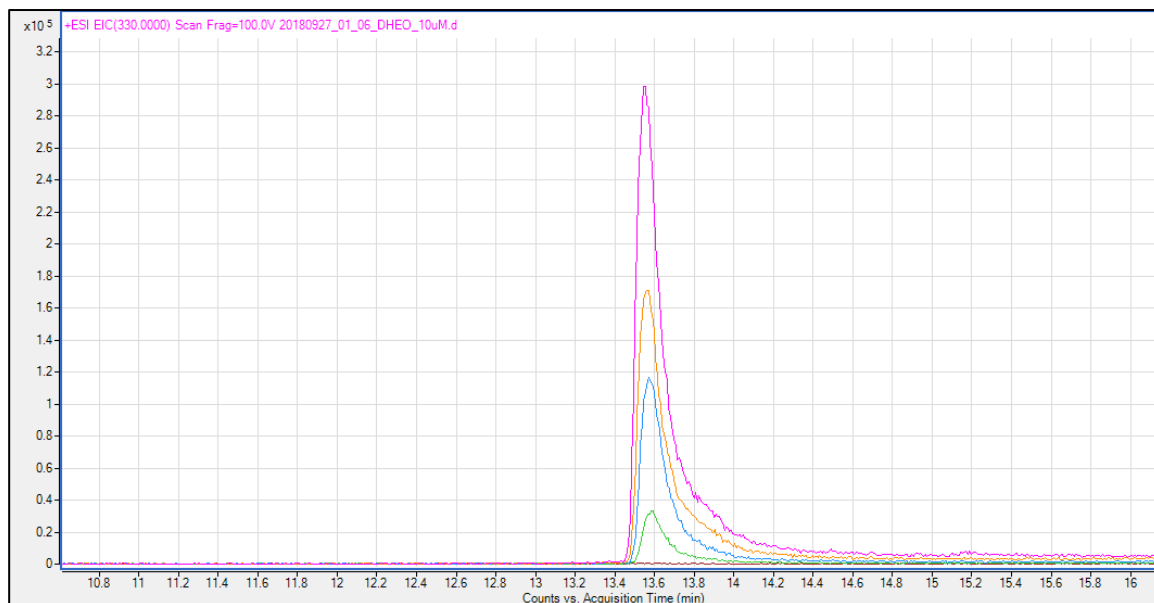


Figure 25. Extracted chromatograms that were used to create DHE standard curve.

Using the described mass spectrometry method, DHE and DHEO standards were analyzed (Figure 25). From this data, peak retention time and verification were determined using MassHunter. Extracted ion chromatograms of m/z values 314 and 330 were analyzed corresponding to DHE and DHEO, respectively. To further validate identification, ultraviolet-visible spectroscopy (UV-Vis) data was collected using a diode array detector (DAD). The wavelength of 485 was used to verify DHEO retention time. After compound verification, DHEO standards were integrated using MassHunter and a standard curve was created (Figure 26). Standards of $0.1\mu\text{M}$, $1\mu\text{M}$, $2.5\mu\text{M}$, $5\mu\text{M}$ and $10\mu\text{M}$ were used. Experimental samples were analyzed immediately after the standards were ran. Samples were then integrated and concentrations were determined using the standard curve.

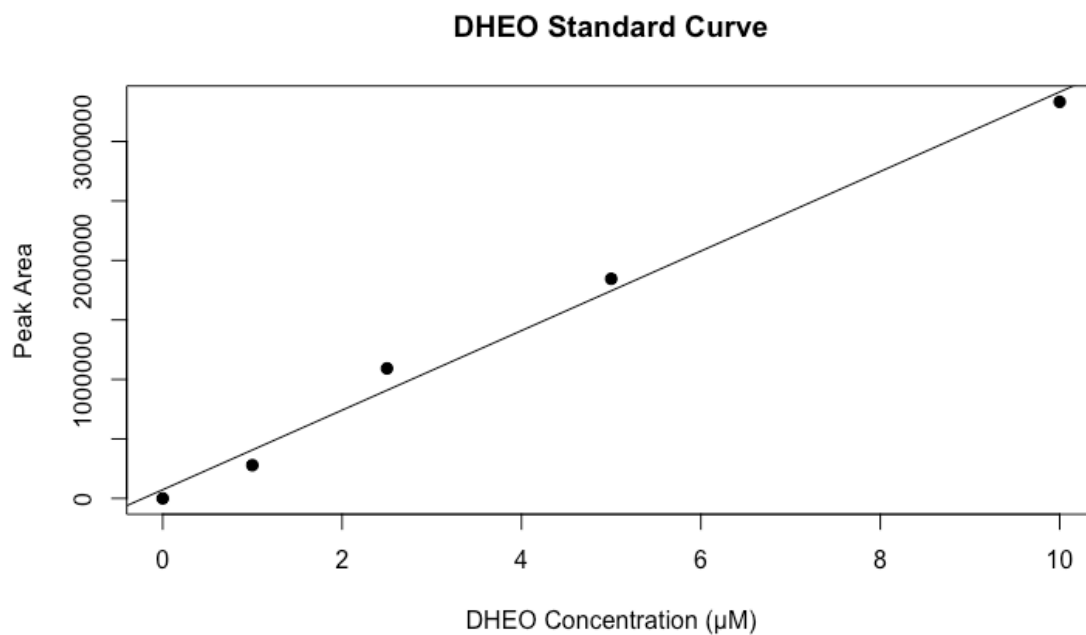


Figure 26. Standard Curve used for quantification of $\text{O}_2^{\bullet-}$. A linear equation of $y = 334,408x + 72,751$ with an $R^2 = 0.989$ was used to quantify the production of $\text{O}_2^{\bullet-}$ produced during the re-oxidation of ETF.

RESULTS

Flavin Adenine Dinucleotide Reduction

As stated earlier, reduced flavins interact readily with molecular oxygen. The identification of how long both the reduction and re-oxidation of a flavin takes was imperative to determining the experimental set-up of the reduction of ETF.

FAD_{ox} has a maximum absorbance at $\lambda = 450$ nm. The reduction of FAD_{ox} was monitored as described in Methods section and can be seen in Figure 27, Panel A. The complete reduction was observed at time points 15 and 20 min. The reaction was monitored for two hours with no change in A_{450} . Once anticipated complete reduction was achieved and maintained, re-oxidation of FAD was observed (Figure 27, Panel B). The re-oxidation of the FAD occurred rapidly, with the first time point being 30 seconds and almost complete re-oxidation was observed. The sensitivity to molecular oxygen demonstrates that the techniques used imposed and held an anaerobic environment during the reduction experiments.

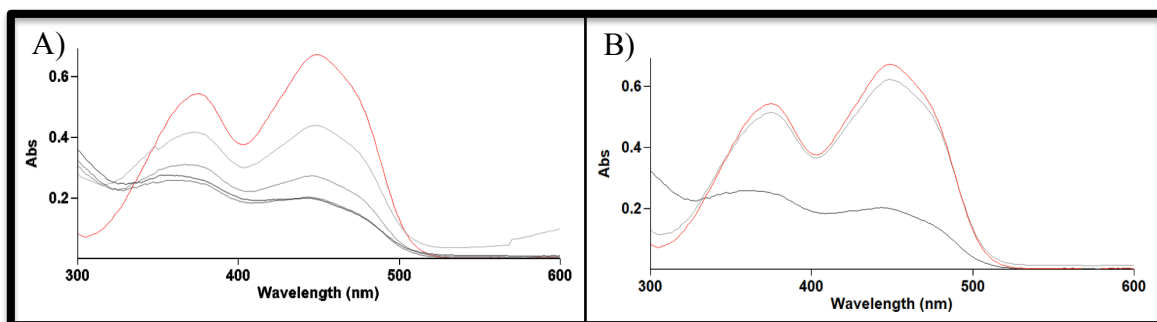


Figure 27. Monitoring the reduction and re-oxidation of flavin adenine dinucleotide (FAD). (A) Indicates the reduction of FAD. Initially, oxidized FAD (red) was photoreduced in the presence of electron donor DTT. The reduction was monitored by a decrease in absorbance at 450 nm and spectrum taken at 5, 10, 15, and 20 minutes. (B) Shows the re-oxidation of FAD. FAD_{reduced} is in black and two spectra were obtained at a time interval of 30 sec (gray) and two minutes (red).

Enzymatic Reduction

Enzymatic reduction assays were carried out in a 1000:1 ratio between Electron transfer flavoprotein (ETF) and Medium Chain Acyl-CoA Dehydrogenase (MCAD), respectively. This ratio was chosen to allow the complete reduction/re-oxidation of ETF and the ROS products produced to be attributed to the re-oxidation of the FAD cofactor present in ETF.

Complete enzymatic reduction of ETF was achieved in a time frame of 15 minutes. The reduction was monitored by the loss of absorbance at $\lambda = 450$ nm, which coincides with the λ_{max} of the oxidized FAD cofactor (Figure 28). Figure 28 shows one of the enzymatic reducing spectra, others can be seen in Appendix B2.

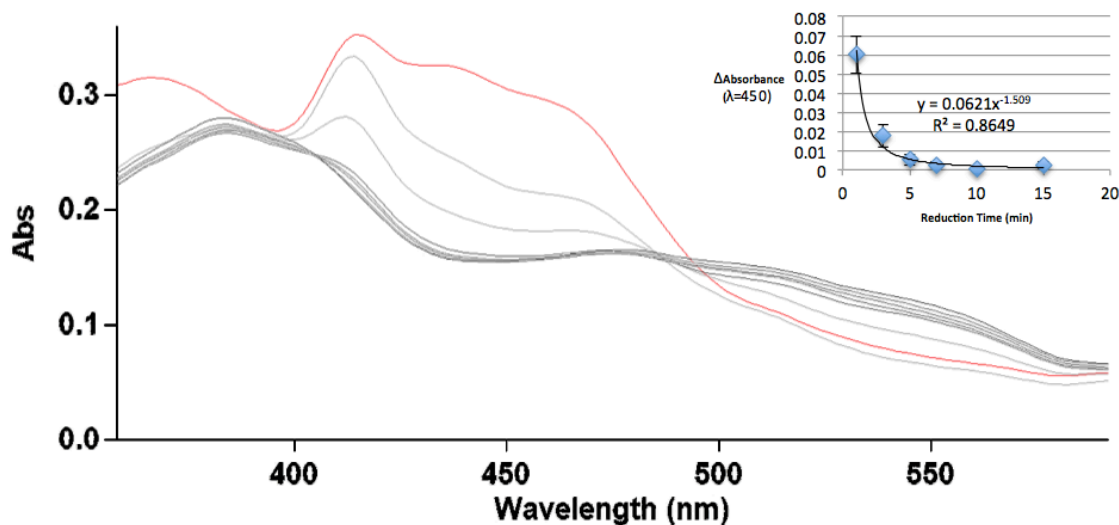


Figure 28. Enzymatic reduction spectra of ETF. Oxidized ETF (red) loses its absorbance around 450 nm, corresponding with the reduction of the FAD cofactor. Time points shown are 1 min, 3 min, 5 min, 7 min, 10 min, and 15 minutes in increasing grayscale saturation. The insert indicates the change in Absorbance at $\lambda = 450$ nm as time progresses.

Partitioning Assays

Amplex Red

Amplex Red results can be seen on Table 2 and a graphical representation can be seen on Figure 30. The results reflect the amount of H_2O_2 produced within the reaction (full list of raw data can be found in Appendix B3).

	H ₂ O ₂ (μM)	Average	St Dev
20 nT	1.85	2.14	0.26
	2.24		
	2.33		
50 μT	2.14	2.37	0.22
	2.45		
	2.50		
	p-value=0.289		

Table 2. Amplex Red results.

Dihydroethidium Assay

DHE-superoxide assay was successfully carried out using a novel MS approach. Our results demonstrate that DHE was successfully separated from the $O_2^{\bullet-}$ - specific product, DHEO. Separation of these species can be seen on Figure 29. The method was even able to see separation the Total Ion Chromatogram (TIC) (Appendix B4). The extracted ion peaks were used and a m/z value affirmed either the $O_2^{\bullet-}$ -specific product, non-oxidized DHE or the presence of non-specific products.

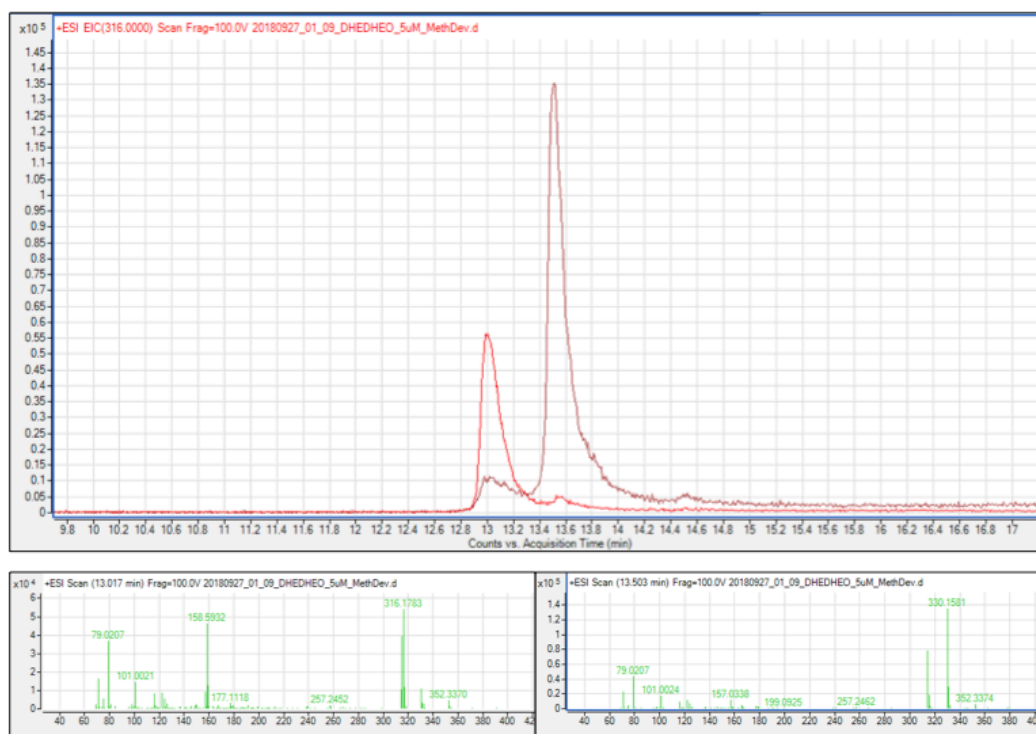


Figure 29. Extraction ion chromatograms and corresponding m/z values.

Dihydroethidium results can be seen on Table 3 and a graphical representation on Figure 30, at the end of this section.. The results show the amount of $O_2^{\bullet-}$ produced during the re-oxidation of Electron transfer flavoprotein. The data was performed in

triplicate and showed a 9.76% increase in $O_2^{\bullet-}$ production with the decrease in magnetic field strength.

	O ₂ ⁻ (uM)	Average	St Dev
20 nT	9.49	10.57	1.36
	10.13		
	12.1		
50 uT	8.85	9.63	0.76
	9.68		
	10.38		
	p-value=0.35		

Table 3. DHE Results.

Statistical Analysis

To address Aim 2, the differences between H_2O_2 produced (Table 2) versus the amount of $O_2^{\bullet-}$ produced (Table 3), a p-value of 2.3×10^{-9} was obtained. This shows that there is substantial significant difference between the type of ROS produced by the re-oxidation of ETF.

To address Aim 2, when comparing the two different magnetic fields, the p-values of the H_2O_2 and $O_2^{\bullet-}$ triplicate experiments were 0.289 and 0.35, respectively. Both of these values indicate a non-significant difference between the two fields.

Both the quantification of the superoxide anion and hydrogen peroxide are conducted independently in terms of experiment and are mechanistically independent. Therefore, since the experiments are independent $P(A \text{ and } B) = P(A) \times P(B)$ and can yield a modified p-value. The p-value of both independent events is 0.10, a trend towards significance. Although this is still statistically insignificant, simple modifications such as an increase in protein concentration may lead to an increased distribution of ROS. Also,

further MS experiments need to be prepped and ran to assure the authenticity of quantification.

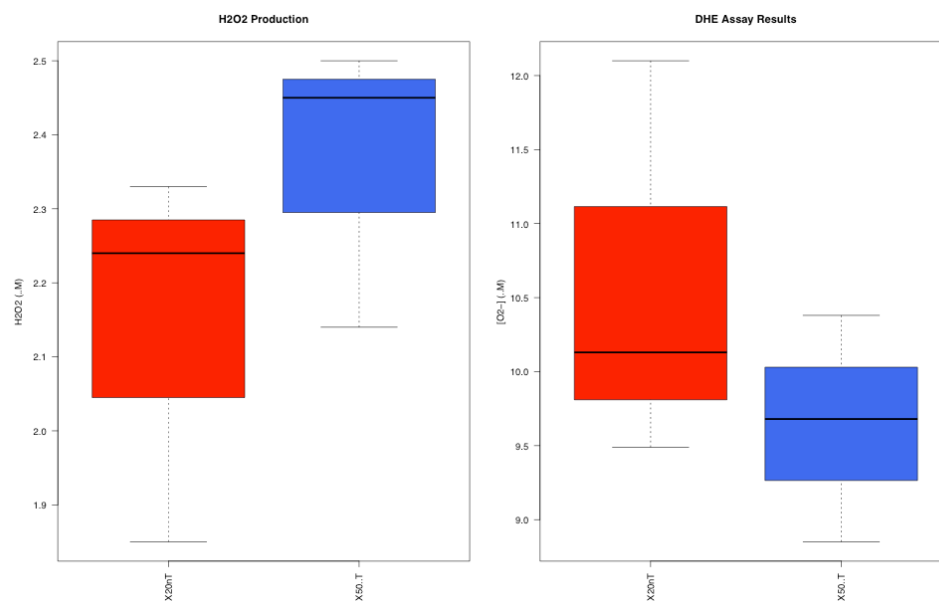


Figure 30. Quantification of partitioning assay results. (left) A decrease in H₂O₂ was seen with a decrease in magnetic field strength with respect to Earth's 50 μT. (right) Conversely, an increase in production of the superoxide anion was observed with the decreasing magnetic field.

DISCUSSION

Defining the role of Reactive Oxygen Species (ROS) in biological systems lacks definitive boundaries. Under “normal” conditions ROS act as regulatory signaling molecules, but the overproduction of these same molecules can lead to oxidative damage and a host of physiological and pathological outcomes. To begin to understand the phenotypic boundary that dictates either positive or negative biological outcomes, quantification of these species becomes the elementary step. To address this, an experimental approach has been put forward and results obtained that begin to address this elementary step.

Electron transfer flavoprotein (ETF) was successfully reduced and the ROS species produced upon the re-oxidation of the system was quantified. ETF was chosen due to its imperative role in a metabolic pathway that feeds electrons to cellular respiration. From an experimental standpoint, ETF was successfully reduced by one of its biological partners, and affirmative ROS products were quantified upon its re-oxidation monitored a visible absorbance at $\lambda = 450 \text{ nm}$.

Aim 1 of this research aimed at defining the ROS produced by ETF. The re-oxidation of ETF produced approximately five-fold more $\text{O}_2^{\bullet -}$ than H_2O_2 , and demonstrated statistical significance. These findings compare with other results (18, 31) and make sense from a theoretical perspective. The ground triplet-state of molecular oxygen interacts favorably with the semiquinone radical of the flavin cofactor, resulting in a transient reaction that releases $\text{O}_2^{\bullet -}$.

Aim 2 sought to understand the role of a magnetic field in potentially dictating ROS products. Although shown to be statistically insignificant, the relative ratios of hydrogen peroxide and the superoxide anion changed in a proportional manner (roughly 9%). Other research (39) and the use of theoretical models (44) support the relative distributions from the results obtained through the re-oxidation of the ETF cofactor. Using the basis of magnetoreception theory that radical pairs may dictate the ability of birds to utilize the earth's magnetic field as a compass (43), theoretical simulations have been performed that aim at quantifying ROS products that are dictated by singlet-triplet mixing of radical pairs (44). Using spin dynamics calculations to determine singlet and triplet yields, relative distributions of H_2O_2 and $\text{O}_2^{\cdot-}$ have been predicted. The yields change as a function of the strength of an external magnetic field (Figure 31) (44).

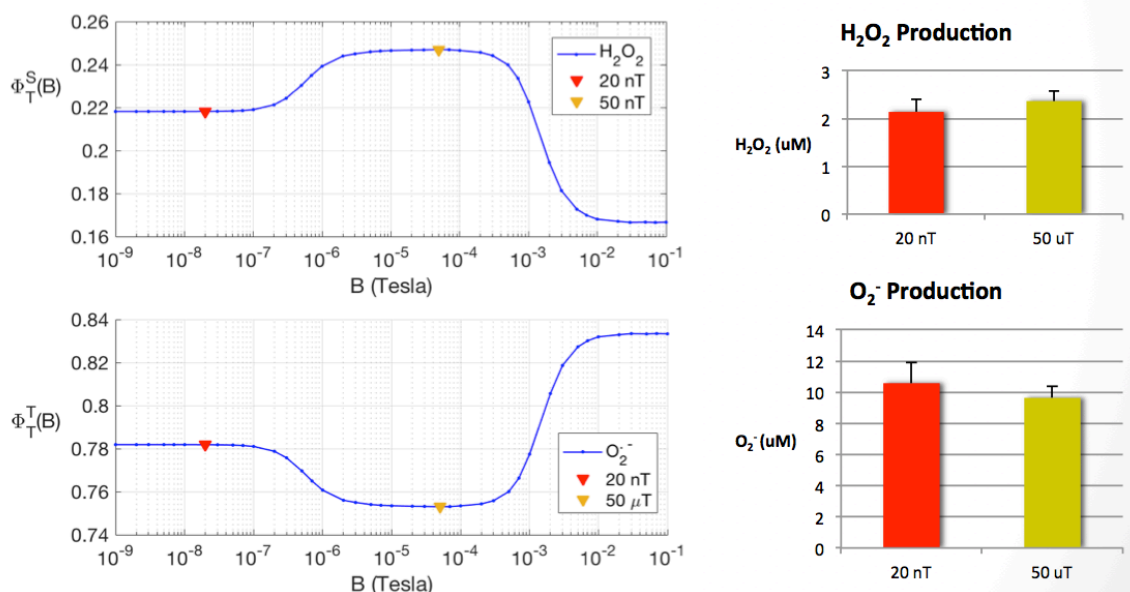


Figure 31. Singlet-triplet yields of flavin radical with molecular oxygen as a function of an external magnetic field. (Top) Singlet mixing (ϕ_S^T) results in the production of H_2O_2 , and changes in the same manner as the obtained results. (Bottom) Triplet mixing (ϕ_T^T) results in the release of $\text{O}_2^{\cdot-}$ and coincides with results.

The results obtained from the ETF re-oxidation assays saw a decrease in H_2O_2 production with a decrease in magnetic field by 9.6%. Conversely, the same reduced protein increased the production of $\text{O}_2^{\bullet-}$ by 9.8% with a decreasing magnetic field. The results of the simulations seem to correlate with the behavior of ROS produced via the re-oxidation of ETF.

One of the major limitations of studying ROS biology, is the fact these species are not only highly transient, but that they are produced by many different systems. ETF re-oxidation produces ROS, although this small subset of data gives us only a glimpse of the behavior of these molecules in biological systems. To address this major limitation, first, a refined approach must be taken to divulge some sort of statistical significance before applying these experimental approaches to other flavoprotein systems. From there, more general characteristics of ROS productions at a flavin cofactor of an enzyme may be made.

Overall, a known ROS-producing system was characterized and studied. The re-oxidation of the flavin adenine dinucleotide cofactor bound in ETF produced predictable ratios of the ROS, H_2O_2 and $\text{O}_2^{\bullet-}$. The implementation of a magnetic field appeared to impact the ratios of these ROS, although further investigation needs to be conducted to establish statistical significance. These assays sought out to contribute some understanding of the poorly defined nature of ROS by starting at the beginning; the elementary step of quantification.

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APPENDICES

APPENDIX A: PICTURES OF EXPERIMENTAL EQUIPMENT

PICTURES OF EXPERIMENTAL EQUIPMENT

1. Cary 300 Temperature Control



2. Incubator—Imposing a Constant Magnetic Field



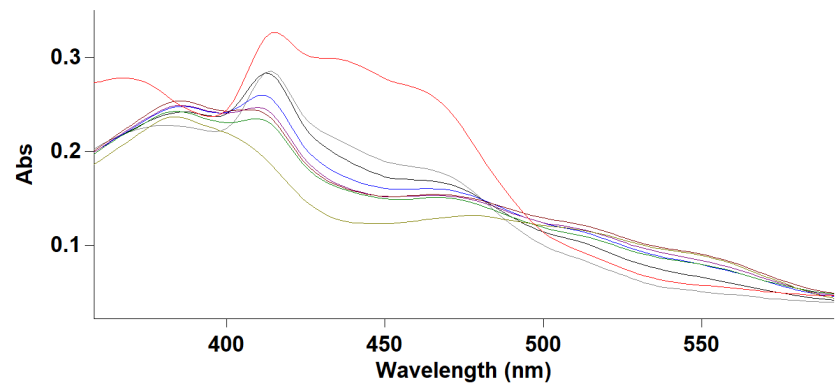
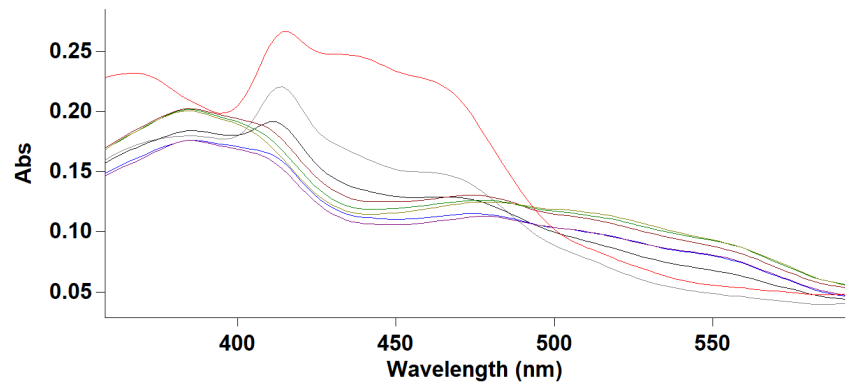
APPENDIX B: DATA

DATA

1. Plate Reader Data

16-May	1	2	3	4	5	6	7	8	9	10	11	12	
A	122502	243851	246460	194598	372577	499428	1262366	1951526	10	10	10	7	554,593
B	118846	242119	244428	232514	267391	500072	1273070	1951952	7	8	10	8	554,593
C	120341	238235	242015	229349	374716	505183	1165250	1950917	7	8	8	9	554,593
D	9	8	9	8	12	8	9	8	7	13	11	8	554,593
E	11	10	9	7	10	9	9	13	11	13	9	10	554,593
F	34402	45514	57063	75187	396748	391325	1738744	2195763	8	9	6	10	554,593
G	33671	44461	54091	67584	380487	421630	1727431	2134499	6	9	7	9	554,593
H	35807	47354	56441	67153	390550	425335	1695953	2462190	8	9	8	7	554,593

2. Magnetic Field Spectra



3. Amplex Red Data

	10-Oct	11-Oct	12-Oct	H2O2 (uM)
501	1.094219736	1.052899779	1.07003674	1.072385418
502	1.397371158	1.183630972	1.015679523	1.198893884
503	1.300653156	1.213580112	1.237238849	1.250490706
201	0.932297958	0.944733369	0.892252997	0.923094775
202	0.97149292	1.168324722	1.217059791	1.118959144
203	1.161765199	1.19665565	1.140253825	1.166224892

4. Total Ion Chromatogram

