Mechanistic Importance of Redox Potentials and Conformational Flexibility in Electron-Transferring Flavoproteins

Michael Anthony Swanson
University of Denver

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Mechanistic Importance of Redox Potentials and Conformational Flexibility in Electron-Transferring Flavoproteins

Abstract
The mitochondrial matrix flavoproteins electron transfer flavoprotein (ETF) and electron transfer flavoprotein-ubiquinone oxidoreductase (ETF-QO) are responsible for linking fatty acid β-oxidation with the main mitochondrial respiratory chain. Electrons derived from flavoprotein dehydrogenases are transferred sequentially through ETF and ETF-QO to ubiquinone and then into the respiratory chain via complex III. In this study, the effects of changes in ETF-QO redox potentials on its activity and the conformational flexibility of ETF were investigated.

ETF-QO contains one [4Fe-4S]^{2+}_{2},^{1+}_{1} and one flavin adenine dinucleotide (FAD). In the porcine protein, threonine 367 is hydrogen bonded to N1 and O2 of the flavin ring of the FAD. The analogous site in Rhodobacter sphaeroides ETF-QO is asparagine 338. Mutations N338T and N338A were introduced into the R. sphaeroides protein by site-directed mutagenesis to determine the impact of hydrogen bonding at this site on redox potentials and activity. FAD redox potentials were measured by potentiometric titration probed by electron paramagnetic resonance (EPR) spectroscopy. The N338T and N338A mutations lowered the midpoint potentials, which resulted in a decrease in the quinone reductase activity and negligible impact on disproportionation of ETF_{1e} catalyzed by ETF-QO. These observations indicate that the FAD is involved in electron transfer to ubiquinone, but not in electron transfer from ETF to ETF-QO. Therefore it is proposed that the iron-sulfur cluster is the immediate acceptor from ETF.

It has been proposed that the αII domain of ETF is mobile, allowing promiscuous interactions with structurally different partners. Double electron-electron resonance (DEER) was used to measure the distance between spin labels at various sites and an enzymatically reduced FAD cofactor in Paracoccus denitrificans ETF. Two or three interspin distance distributions were observed for spin-labels in the αI (A43C) and βIII (A111C) domains, but only one is observed for a label in the βII (A210C) domain. This suggests that the αII domain adopts several stable conformations which may correspond to a closed/inactive conformation and an open/active conformation. An additional mutation, E162A, was introduced to increase the mobility of the αII domain. The E162A mutation doubled the activity compared to wild-type and caused the distance distributions to become wider. The DEER method has the potential to characterize conformational changes in ETF that occur when it interacts with various redox partners.

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Mechanistic Importance of Redox Potentials and Conformational Flexibility in Electron-Transferring Flavoproteins

A Dissertation
Presented to
The Faculty of Natural Sciences and Mathematics
University of Denver

In Partial Fulfillment
of the Requirements for the Degree
Doctor of Philosophy

by
Michael A. Swanson
June 2010
Advisor: Dr. Sandra S. Eaton
ABSTRACT

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ETF-QO contains one \([4\text{Fe}-4\text{S}]^{2+}\) and one flavin adenine dinucleotide (FAD). In the porcine protein, threonine 367 is hydrogen bonded to N1 and O2 of the flavin ring of the FAD. The analogous site in *Rhodobacter sphaeroides* ETF-QO is asparagine 338. Mutations N338T and N338A were introduced into the *R. sphaeroides* protein by site-directed mutagenesis to determine the impact of hydrogen bonding at this site on redox potentials and activity. FAD redox potentials were measured by potentiometric titration probed by electron paramagnetic resonance (EPR) spectroscopy. The N338T and N338A mutations lowered the midpoint potentials, which resulted in a decrease in the quinone reductase activity and negligible impact on disproportionation of ETF\(_{1e^-}\) catalyzed by ETF-QO. These observations indicate that the FAD is involved in electron transfer to...
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I would like to thank Professors Gareth and Sandra Eaton for allowing me the privilege of pursuing my doctorate in their lab. They have been great mentors and it has been an honor to learn under them. I take great pride in my time spent in the lab and I hope that one day I can use what I have learned to make them as proud of me.

The work described in this dissertation was in collaboration with Professor Frank Frereman at the University of Colorado, Denver (Anshutz medical campus). His support throughout this process has been vital and is greatly appreciated.

Dr. Velavan Kathirvelu deserves special thanks for his help with DEER measurements and analysis of ETF mutants. Without his help, these measurements would have taken much longer. Other individuals who I would like to acknowledge for their contributions to this work include: Dr. Robert Usselman for his help with the potentiometry of ETF-QO mutants, Dr. Tomas Majtan (University of Colorado, Denver) for performing mutagenesis on the *P. denitrificans* ETF genes, and Professor David Britt and Dr. Stefan Stoll (University of California, Davis) for the use of their spectrometer to obtain the initial Q-band DEER measurements of ETF mutants.

I would also like to thank Dr. Bowler for his tutelage in the brief time I was a member of his lab. Consultation with him before his departure led me to join the Eaton lab.
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CONTENTS

Chapter 1: Introduction
  1.1: Mitochondrial Electron Transport .................................................................1
  1.2: Protein Structure ............................................................................................5

Chapter 2: ETF-QO Background
  2.1: ETF-QO Crystal Structure .............................................................................7
  2.2: Effects of Mutation on the [4Fe-4S]^{2+,1+} of ETF-QO ....................................8
  2.3: Selection of Mutation Sites in This Study ...................................................11
  2.4: Determining Midpoint Potentials Using EPR Spectroscopy .......................14
  2.5: Relaxation Enhancement Distance Measurements in Proteins ...............18

Chapter 3: Experimental Methods for ETF-QO Mutation Investigation
  3.1: Preparation of ETF-QO Mutants .................................................................20
  3.2: Expression and Purification of ETF-QO Mutants .......................................28
  3.3: Iron and FAD Quantitation ..........................................................................32
  3.4: Potentiometric Titration ...............................................................................33
  3.5: Spectrophotometric Titration .......................................................................37
  3.6: Enzyme Activity Assays ...............................................................................38
  3.7: Pulsed Electron Spin Echo (ESE) Experiments ...........................................41

Chapter 4: ETF-QO Mutation Results
  4.1: Characterization of ETF-QO Mutants .........................................................45
  4.2: Potentiometric Titration ...............................................................................53
  4.3: Spectrophotometric Titration .......................................................................60
  4.4: Quinone Reductase Activity ........................................................................64
  4.5: Disproportionation of ETF Semiquinone ....................................................67

Chapter 5: Mechanistic Conclusions from ETF-QO Study .................................71

Chapter 6: ETF Background
  6.1: Electron Transfer Flavoprotein ....................................................................73
  6.2: Evidence of αII Domain Motion in ETF ....................................................77
  6.3: Observation of Domain II Conformational Change ....................................79
  6.4: Double Electron-Electron Resonance (DEER) ..........................................81
  6.5: Selection of ETF Mutation Sites .................................................................86
Chapter 7: Experimental Methods for DEER Measurements on ETF

7.1: Preparation of ETF Mutants .................................................................88
7.2: Purification and Spin Labeling of ETF Mutants.................................88
7.3: Expression and Purification of Glutaryl-CoA Dehydrogenase ...........92
7.4: ETF Activity Assay .............................................................................94
7.5: Enzymatic Reduction of Spin-Labeled ETF Mutants .......................94
7.6: EPR Spectroscopy of Spin-Labeled ETF Mutants .............................95
7.7: Modeling of ETF Structures ...............................................................99

Chapter 8: Results of DEER Distance Measurements of ETF

8.1: Characterization of ETF Mutants .......................................................100
8.2: Spin Labeling of ETF Mutants ............................................................102
8.3: ETF Activity Assays ..........................................................................104
8.4: Enzymatic Reduction of Spin-Labeled ETF Mutants .......................106
8.5: DEER Measurements of Spin-Labeled ETF Mutants .......................110
8.6: Modeling of ETF .............................................................................126

Chapter 9: Summary of DEER Distance Measurements of ETF

9.1: Conclusions ......................................................................................137
9.2: Future Work .....................................................................................141

References ..............................................................................................144

Appendices

Appendix 1: Derivation of the Nernst Equation ......................................155
Appendix 2: Media Recipes .......................................................................157
Appendix 3: Plasmid DNA Purification ....................................................159
Appendix 4: Bradford Protein Assay Instructions .....................................160
LIST OF TABLES

2.1 - Quinone reductase and ETF disproportionation activities for ETF-QO mutants in the vicinity of [4Fe-4S]^{2+},^{1+} compared to the wild-type enzyme ....................10

3.1 – ETF-QO mutagenic primers ........................................................................................................21

3.2 - PCR temperature cycling parameters used in ETF-QO mutagenesis.........................21

4.1 – EPR g-values and linewidths for [4Fe-4S]^- and FAD SQ^- ............................................49

4.2 - Midpoint potentials for wild-type and mutant ETF-QOs..................................................56

4.3 - Quinone reductase activities for N338A, N338T and wild-type ETF-QO.................66

8.1 - DCPIP reduction rates for spin-labeled ETF mutants .........................................................105

8.2 - Optimization of the enzymatic reduction of ETF............................................................108

8.3 - Distances between amino acid side chains and groups on the FAD as measured in the crystal structures of human ETF, human ETF:MCAD complex and \textit{P. denitrificans} ETF. ..........................................................................................................................131

8.4 – Distances between amino acid side chains and the C4a position of the FAD in ETF crystal structures..............................................................................................................131

9.1 – Comparison of distances calculated from DEER measurements and crystal structures ..............................................................................................................140
LIST OF FIGURES

1.1 – Location of ETF-QO in the mitochondrion ......................................................... 2
1.2 – ETF and ETF-QO connect β-oxidation with the respiratory chain ................... 4
1.3 – Number of structures in the Protein Data Bank .................................................. 6
2.1 – Crystal structure of porcine ETF-QO .................................................................. 8
2.2 – Hydrogen bonding interactions with the [4Fe-4S]^{2+,1+} cluster ......................... 9
2.3 – Potentiometric titration curves of ETF-QO [4Fe-4S]^{2+,1+} site mutants ............ 10
2.4 – Oxidation states of flavin cofactors ................................................................ 12
2.5 – Hydrogen bonding interactions with the FAD in ETF-QO ................................. 14
2.6 – Zeeman energy level splitting ......................................................................... 17
2.7 – Oxidation states of the [4Fe-4S]^{2+,1+} and FAD in ETF-QO ......................... 18
3.1 – DNA sequence of \textit{R. sphaeroides} ETF-QO ................................................. 24
3.2 – Potentiometric titration experimental set-up ...................................................... 34
3.3 – Inversion recovery pulse sequence .................................................................. 42
4.1 – Absorption spectra of N338A, N338T and wild-type ETF-QO ...................... 45
4.2 – Standard curve used to determine iron concentration in ETF-QO ................... 46
4.3 – CW EPR spectra of FAD SQ^- in N338A, N338T and wild-type ETF-QO .......... 47
4.4 – CW EPR spectra of [4Fe-4S]^+ in N338A, N338T and wild-type ETF-QO .......... 48
4.5 – Temperature dependence of T_1 rates for [4Fe-4S]^+ in ETF-QO ..................... 50
4.6 – Inversion recovery of FAD SQ^- in N338A and Y501F/T525A ETF-QO .......... 52
4.7 – CW EPR spectra of the potentiometric titration of N338T ETF-QO ....................54
4.8 – FAD SQ− potentiometric titration curves at 100 K ...........................................55
4.9 – FAD SQ− potentiometric titration curves at 293 K ...........................................56
4.10 – Comparison of wild-type potentiometric titration curves at 100 and 293 K ......58
4.11 – Comparison of N338T potentiometric titration curves at 100 and 293 K ..........58
4.12 – Comparison of N338A potentiometric titration curves at 100 and 293 K ...........59
4.13 – [4Fe-4S]+ potentiometric titration curves..........................................................60
4.14 – UV-VIS spectra of anaerobic reductive titration of wild-type ETF-QO ..........61
4.15 – UV-VIS spectra of anaerobic reductive titration of N338A ETF-QO ...............62
4.16 – UV-VIS spectra of anaerobic reductive titration of N338T ETF-QO ...............63
4.17 – Change in ε430 during reductive titration of N338A, N338T and wild-type ETF-QO ...............................................................64
4.18 – Time dependence of quinone reductase activity .............................................65
4.19 – Relative quinone reductase activities of ETF-QO mutants...............................67
4.20 – Disproportionation of ETF1e− by wild-type ETF-QO ......................................68
4.21 – Time dependence of ETF1e− disproportionation assay ....................................68
4.22 – Relative ETF1e− disproportionation activities of ETF-QO mutants ...............69
6.1 – Crystal structure of human ETF.........................................................................75
6.2 – Conformational changes in ETF during complex formation ............................79
6.3 – MTSL spin-labeling reaction .............................................................................80
6.4 – DEER pulse sequence ....................................................................................83
6.5 – Crystal structure of P. denitrificans ETF with spin label locations ..................87
8.1 – SDS-PAGE of purified P. denitrificans A111C ETF ..................................100
8.2 – UV-VIS spectra of ETF mutants compared with wild-type ........................................102
8.3 – CW EPR spectra of spin-labeled ETF mutants............................................................103
8.4 – ETF DCPIP reduction activity assay raw data..............................................................104
8.5 – Relative DCPIP reduction rates of spin-labeled ETF mutants......................................106
8.6 – Visible spectra of wild-type ETF during enzymatic reduction....................................107
8.7 – Visible spectra of A111C+E162A ETF during enzymatic reduction .........................109
8.8 – CW EPR spectra of oxidized and reduced spin-labeled A111C ETF .........................110
8.9 – Field-swept echo-detected EPR spectra at X- and Q-band........................................111
8.10 – X-band DEER data for reduced, spin-labeled ETF mutants ....................................113
8.11 – X-band dipolar evolution signals ..........................................................................114
8.12 – X-band DEER frequency domain data .................................................................115
8.13 – Distance distributions calculated from X-band DEER data ....................................117
8.14 – L curve obtained by Tikonov regularization of A111C X-band data and error estimation ......................................................................................................................118
8.15 – Q-band DEER data for reduced, spin-labeled ETF mutants ....................................119
8.16 – Q-band dipolar evolution signals ..........................................................................121
8.17 – Q-band frequency domain data .............................................................................122
8.18 – Distance distributions calculated from Q-band data ................................................124
8.19 – Comparison of X- and Q-band distance distributions ............................................126
8.20 – Crystal structure and MTSL-FAD interspin distances ............................................128
8.21 – Homology model of *P. denitrificans* ETF bound to MCAD ....................................129
8.22 – Amino acids in close proximity to FAD in *P. denitrificans* ETF .........................130
8.23 – Differences between crystal structure and homology model distances ..........132
8.24 – A111 Cβ-FAD distances in crystal structures and homology model ..........134
8.25 – A210 Cβ-FAD distances in crystal structures and homology model ..........135
8.26 – A43 Cβ-FAD distances in crystal structures and homology model ..........136
**LIST OF ABBREVIATIONS**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>APS</td>
<td>ammonium persulfate</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine-5’-triphosphate</td>
</tr>
<tr>
<td>β−ME</td>
<td>β-mercaptoethanol</td>
</tr>
<tr>
<td>Co-A</td>
<td>coenzyme A</td>
</tr>
<tr>
<td>CW</td>
<td>continuous wave</td>
</tr>
<tr>
<td>DDM</td>
<td>n-dodecyl-β-D-maltopyranoside</td>
</tr>
<tr>
<td>DEER</td>
<td>double electron-electron resonance</td>
</tr>
<tr>
<td>DEAE</td>
<td>diethylaminoethyl</td>
</tr>
<tr>
<td>DCPIP</td>
<td>2,6-dichlorophenol-indophenol</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
</tr>
<tr>
<td>PELDOR</td>
<td>pulsed electron double resonance</td>
</tr>
<tr>
<td>EPR</td>
<td>electron paramagnetic resonance</td>
</tr>
<tr>
<td>ETF</td>
<td>electron transfer flavoprotein</td>
</tr>
<tr>
<td>ETF-QO</td>
<td>electron transfer flavoprotein ubiquinone oxidoreductase</td>
</tr>
<tr>
<td>FAD</td>
<td>flavin adenine dinucleotide</td>
</tr>
<tr>
<td>FMN</td>
<td>flavin mononucleotide</td>
</tr>
<tr>
<td>GA2</td>
<td>glutaric acidemia type II</td>
</tr>
<tr>
<td>HA</td>
<td>hydroxyapatite</td>
</tr>
</tbody>
</table>
IPTG isopropyl β-D-1-thiogalactopyranoside
IR infrared
LB lysogeny broth
MADD multiple acyl-CoA dehydrogenase deficiency
MCAD medium chain acyl-CoA dehydrogenase
MTSL 1-oxyl-2,2,5,5-tetramethylpyrroline-3-methyl-methanethiosulfonate
NMR nuclear magnetic resonance
NZY+ broth containing enzymatic digest of casein and yeast extract
OD optical density
PAGE polyacrylamide gel electrophoresis
PBS phosphate buffered saline
PCR polymerase chain reaction
PMSF phenylmethanesulphonylfluoride
PVDF polyvinylidene fluoride
Q quinone
Q1 coenzyme Q1
QH2 hydroquinone/quinol
RCF relative centrifugal force
redox oxidation-reduction
rpm revolutions per minute
SAXS small-angle X-ray scattering
SDS sodium dodecyl sulfate
SHE  standard hydrogen electrode
SQ¯•  anionic semiquinone
TEMED  tetramethylethylenediamine
tempol  4-hydroxy-2,2,6,6-tetramethylpiperidino-1-oxy
TLCK  tosyl-L-lysine chloromethylketone
UQ  ubiquinone
W3A1  *Methylophilus methylotrophus*
Chapter 1. Introduction

1.1 Mitochondrial Electron Transport

The transfer of electrons is of extreme biochemical importance. Living organisms derive the free energy they need to do work from electron transfer [Nelson and Cox 2005 pg507; Voet and Voet 1995 pg434]. In mitochondria the transfer of electrons derived from the oxidation of nutrients is coupled to the synthesis of adenosine-5’-triphosphate (ATP) in a process called oxidative phosphorylation. Electron transfer flavoprotein–ubiquinone oxidoreductase (ETF-QO or ETFDH) is responsible for linking electrons derived from fatty acid β oxidation and the oxidation of some amino acids to the main mitochondrial respiratory chain [Ruzicka and Beinert 1977]. ETF-QO is a monotopic membrane protein located on the inner mitochondria membrane facing into the mitochondrial matrix (Figure 1.1) and has two oxidation-reduction (redox) active cofactors, one \([4\text{Fe}-4\text{S}]^{2+}\) cluster and one flavin adenine dinucleotide (FAD). ETF-QO oxidizes electron transfer flavoprotein (ETF), a soluble matrix protein responsible for oxidizing acyl-coenzyme A (CoA) dehydrogenases, sarcosine dehydrogenase and dimethylglycine dehydrogenase [Ghisla and Thorpe 2004; Toogood et al. 2007], and reduces ubiquinone (UQ, Coenzyme Q\(_{10}\)). UQ then transfers the electrons to the cytochrome bc\(_1\) complex, also known as complex III. Electrons are transferred from complex III to complex IV by cytochrome c, where they are used to reduce O\(_2\) to H\(_2\)O.
Complexes III and IV (along with complex I) create a proton gradient by pumping $\text{H}^+$ out of the mitochondrial matrix and into the inner membrane space (Figure 1.2). This proton gradient is used by complex V (ATP Synthase) to generate ATP [Mitchell 1961]. For every pair of electrons transferred through ETF and ETF-QO, 2 molecules of ATP are formed [Voet and Voet 1995 p669].

**Figure 1.1** Crystal structure of porcine ETF-QO (protein data bank: 2GMH). The approximate position and depth in the mitochondrial inner membrane is shown (inner membrane represented by the blue rectangle). On the right a cartoon depiction of a mitochondrion is shown. The arrow indicates the location of ETF-QO on the matrix side of the inner membrane of mitochondria. ETF-QO’s electron donor, ETF, is a soluble protein located in the matrix.

Defects in ETF-QO, or its electron donor ETF, result in a metabolic disease known as multiple acyl-CoA dehydrogenase deficiency (MADD) or glutaric acidemia type 2 (GA2). The severity of this autosomal recessive disease depends upon the particular mutation and can range from a milder, late-onset form usually developed in the teenage years (type III) to more severe forms that cause death within the neonatal period.
(types I and II) [Goodman et al. 2002; Beresford et al. 2006]. Clinical studies have shown that there is a correlation between the phenotype and genotype [Olsen et al. 2003; Schiff et al. 2006]. The most severe forms of the disease are caused by mutations that disrupt FAD binding, cause ETF or ETF-QO to be rapidly degraded or delete large portions of the ETF or ETF-QO genes. Late-onset GA2 appears to be caused by point mutations where a stable but less active form of ETF or ETF-QO is expressed. It is important to better understand the electron-transfer properties of these enzymes because of their important role in linking fatty acid β oxidation with mitochondrial respiration. This electron transfer pathway is biologically crucial but only a few sentences are devoted to it in chapters on fatty acid metabolism in biochemistry texts [Nelson and Cox p699; Voet and Voet p669]. A more complete understanding of the electron transfer mechanisms of ETF and ETF-QO could lead to a better understanding of the disease GA2 and of electron transfer mechanisms in metabolic pathways in general.
Figure 1.2   ETF and ETF-QO connect fatty acid β-oxidation with the mitochondrial electron transport chain. Black arrows indicate the direction of electron transfer. Grey arrows are steps in the fatty acid β-oxidation pathway. Electrons derived from the oxidation of acyl-CoA molecules are transferred to ETF (highlighted in blue) via at least 10 different dehydrogenases. Electrons are then transferred from ETF to ETF-QO (highlighted in yellow). ETF-QO then transfers the electrons to ubiquinone which finally transfers them to complex III [Nelson and Cox].
1.2 Protein Structure

A central dogma in the field of protein chemistry is that the function of a protein is determined by its three-dimensional structure and that structure is determined by the sequence of amino acids [Crick 1958; Crick 1970; Morange 2008]. The vast majority of atomic resolution structures of proteins are determined by X-ray crystallography and high-resolution NMR spectroscopy. There are roughly 55,000 structures in the protein data base determined using X-ray crystallography and another 8,250 structures determined by NMR spectroscopy (RCSB Protein Data Bank, http://www.rcsb.org/pdb, accessed on 4/12/10). These methods are important tools for the study of proteins but both have serious limitations. NMR spectroscopy can provide valuable structural information, including information on mobile regions, but there are size limitations on the proteins studied. Size limitations are caused by the proton transverse relaxation rate increasing, the tumbling rate decreasing and the complexity of the spectra increasing with protein size [Markus et al. 1994; Liu et al. 2009]. X-ray crystallography is the most widely utilized technique for obtaining protein structures but interpretation requires the assumption that the conformation of the protein in the crystal resembles the biologically active conformation. This is not a valid assumption in some instances, such as in the case of arrestin [Hanson et al. 2007]. It has also been shown that the high concentrations of stabilizing solutes used to generate protein crystals can lead to altered conformations that are significantly different than conformations adopted under physiological conditions [Kim et al. 2008].
Three dimensional structures are an important way to gain information about proteins and a powerful way of representing them, but these examples illustrate the importance of collecting supporting experimental evidence before making mechanistic conclusions based on structural information. This fact is of increasing importance as the total number of available protein structures increases every year (Figure 1.3).

![Graph showing the number of protein structures added to the Protein Data Bank from 1999 to 2009. Blue bars represent the number of structures added in a given year and red bars represent the total number of structures in the data bank.](http://www.rcsb.org/pdb/statistics/contentGrowthChart.do?content=total&seqid=100, accessed on 9/16/09).

**Figure 1.3** Total number of structures added to the Protein Data Bank from 1999 to 2009. Blue bars represent the number of structures added in a given year and red bars represent the total number of structures in the data bank. The plot was taken from the RCSB Protein Data Bank website (http://www.rcsb.org/pdb/statistics/contentGrowthChart.do?content=total&seqid=100, accessed on 9/16/09).
Chapter 2. ETF-QO Background

2.1 ETF-QO Crystal Structure

The locations of the redox centers in the crystal structure of porcine ETF-QO provide insights into the possible mechanism of electron transfer in this enzyme [Zhang et al. 2006]. The UQ molecule is closer to the FAD than to the $[4\text{Fe}-4\text{S}]^{2+,1+}$ cluster (9.9 Å as opposed to 18.8 Å, closest approach) and the iron-sulfur cluster is closer to the surface of the protein than the 7,8-dimethylisoalloxazine ring of FAD [Zhang et al. 2006] (Figure 2.1). The relative locations of the cofactors suggest that the $[4\text{Fe}-4\text{S}]^{2+,1+}$ cluster is responsible for accepting electrons from ETF and that the FAD is responsible for reducing UQ. The closest approach between the $[4\text{Fe}-4\text{S}]^{2+,1+}$ cluster and the FAD is 11.5 Å which is consistent with electron transfer between the two cofactors [Page et al. 1999]. There is 98% sequence identity between porcine and human ETF-QO and 67% sequence identity between human and *Rhodobacter sphaeroides* ETF-QO (N. J. Watmough, F. E. Frerman, and J. N. Butt, 2008, unpublished results). This high sequence similarity predicts that the tertiary structures are closely related [Chothia and Lesk 1986] and that the mechanism of electron transfer is likely similar in all three enzymes. Although the crystal structure of porcine ETF-QO suggests the pathway by which electrons are transferred through the enzyme, experimental evidence is needed to validate this hypothesis.
Figure 2.1  Crystal structure of porcine ETF-QO (PDB id: 2GMH) highlighting the positions of the three redox centers, \([4Fe-4S]^{2+,1+}\) cluster (red and yellow), FAD (pink), and ubiquinone (blue). On the right the ribbon structure has been removed for clarity and distances of closest approach of the three redox centers are shown. (Representation was made using the program RasTop 2.2, http://www.geneinfinity.org/rastop).

2.2  Effects of Mutations on the \([4Fe-4S]^{2+,1+}\) of ETF-QO

Recently the mutations Y501F, T525A, and Y501F/T525A were introduced into \textit{R. sphaeroides} ETF-QO [Usselman et al. 2008]. These residues were chosen for mutation because the same residues (although numbered differently) form hydrogen bonds with the cysteine S\(_\gamma\) atoms that are ligated to the \([4Fe-4S]^{2+,1+}\) cluster of porcine ETF-QO (Figure 2.2). The mutations eliminate these hydrogen bonds and thus change the redox potential of the \([4Fe-4S]^{2+,1+}\) cluster, which in turn affects the activity of the enzyme. The
[4Fe-4S]^{2+,1+} midpoint potentials were lowered by about 100 mV for either single mutation and by about 165 mV for the double mutant (Figure 2.3). The lower redox potentials of these mutants decreased the quinone reductase activity and the rates of disproportionation of ETF_{1e} compared to the wild-type enzyme as shown in table 2.1 [Usselman et al. 2008]. Both single mutations had similar impacts on activities. These results demonstrate that reduction of [4Fe-4S]^{2+,1+} is required for proper enzyme function. As expected these mutations caused no change in FAD midpoint potentials.

**Figure 2.2** Relative locations of the iron-sulfur cluster and the residues that were mutated based on the crystal structure of porcine ETF-QO (PDB id: 2GMH). Residues Y533 and T558 are in position to form hydrogen bonds with the Sγ atoms in C559 and C556, respectively. These residues correspond to Y501 and T525 in the *R. sphaeroides* enzyme. (Representation was made using the program Insight II 2000, Biosym Technologies Inc., San Diego, CA, USA).
Figure 2.3  Potentiometric titration curves are shown of the iron-sulfur cluster for \textit{R. sphaeroides} (○) native, (□) Y501F, (◊) T525A, and (×) Y501F/T525A ETF-QOs. The oxidation-reduction midpoint potentials of the cluster were determined by the increase in the X-band electron paramagnetic resonance signal at 15 K ([4Fe-4S]$^{±}$) and fit using a single Nernst curve with \( n=1 \) [Usselman et al. 2008].

Table 2.1  Quinone reductase and ETF disproportionation activities for ETF-QO mutants in the vicinity of [4Fe-4S]$^{2+,1+}$ compared to the wild-type enzyme [Usselman et al. 2008]. Relative percent of wild-type activity is listed for each mutant in parenthesis.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Quinone Reductase Activity (s$^{-1}$)</th>
<th>ETF Disproportionation Activity (s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild Type</td>
<td>24.2 (100%)</td>
<td>8.3 (100%)</td>
</tr>
<tr>
<td>Y501F</td>
<td>8.9 (37%)</td>
<td>4.1 (51%)</td>
</tr>
<tr>
<td>T525A</td>
<td>8.5 (35%)</td>
<td>5.1 (62%)</td>
</tr>
<tr>
<td>Y501F/T525A</td>
<td>1.8 (8%)</td>
<td>0.66 (8%)</td>
</tr>
</tbody>
</table>
2.3 Selection of Mutation Sites in This Study

Proteins containing a flavin cofactor, FAD, FMN (flavin mononucleotide) or riboflavin (vitamin B2), are known as flavoproteins and are biochemically diverse (Figure 2.4). Flavoproteins are found in both prokaryotic and eukaryotic cells and are responsible for a wide range of enzymatic reactions including electron transfer, dehydrogenation, hydration, signal transduction, deoxyribonucleic acid (DNA) repair and photosynthesis [Massey 2000]. Flavin cofactors can accept one or two electrons and thus exist in three oxidation states; oxidized, one electron reduced semiquinone (SQ), and two electron reduced hydroquinone (QH$_2$) (Figure 2.4). Flavoproteins can participate in such a diverse range of chemical reactions because the flavin cofactors are highly influenced by the environment in their binding sites and their interactions with the apoprotein. Computer simulations have shown that the electron density of the 7,8-dimethylisoalloxazine head group of a flavin cofactor is altered by hydrogen bonding interactions with neighboring amino acids [Zheng and Ornstein 1996; Weber et al. 2001; Bhattacharyya et al. 2007]. These hydrogen bonding interactions modulate the redox potentials of the flavin by stabilizing or destabilizing certain oxidation states of the flavin. In other words, the specific interactions between the enzyme and flavin cofactor lead to unique redox potentials for that enzyme, which can be quite different than the redox potentials of the flavin cofactor free in solution [Nelson and Cox pg515]. Studies on various flavoprotein variants show that changing or eliminating hydrogen bonding interactions at the N1, N5 and C4O positions of the flavin cofactor changes the redox potential of these enzymes [Dwyer et al. 1999; Yang and Swenson 2007; Salazar et al. 1997; Xu et al. 1999]. The changes in flavin redox potentials are also
accompanied by a decrease in biological activity suggesting that the flavin cofactors are finely tuned by hydrogen bonds.

![Figure 2.4](image)

**Figure 2.4** Oxidation states of flavin cofactors. In ETF-QO the singly reduced FAD adopts the anionic semiquinone form. The numbering scheme of the isoalloxazine ring system is shown in red on the oxidized structure. Flavin cofactors; riboflavin, FMN and FAD all contain the same 7,8-dimethylisoalloxazine head group and ribityl chain (R). Structures of the three X groups on the ribityl chain are shown in the box.
In the study reported in this dissertation, mutations were introduced to modulate the FAD redox potentials and investigate the role of the FAD for *R. sphaeroides* ETF-QO function. The X-ray crystal structure of porcine ETF-QO [Zhang et al. 2006] shows that the location of threonine 367 is suitable for formation of hydrogen bonds with the N1 and C2O of the FAD. In *R. sphaeroides* ETF-QO asparagine 338 is at the position equivalent to threonine 367 of the porcine enzyme. Therefore, it is proposed that this residue is in position to form hydrogen bonds with N1 and C2O of the FAD (Figure 2.5). All other hydrogen bonding interactions with the FAD occur through the protein backbone and therefore cannot be changed by mutagenesis. Studies on model flavoenzyme systems done by Rotello and colleagues suggest that hydrogen bonding of C2O is important in stabilizing the anionic semiquinone form of the flavin [Breinlinger et al. 1995; Legrand et al. 2003]. In ETF-QO the FAD is reduced to the anionic SQ so the environment of C2O is important. The mutations N338A and N338T were introduced to eliminate or lower the energy of the hydrogen bond interaction with the FAD, respectively. The impact of these changes on FAD redox potentials and enzyme activity was determined. Results from this study and the previous mutagenesis near the [4Fe-4S]^{2+;1+} cluster are combined to give a description of the roles of the FAD and [4Fe-4S]^{2+;1+} cluster in ETF-QO electron transfer.
2.4 Determining Midpoint Potentials Using Electron Paramagnetic Resonance

Redox reactions occur when electrons are transferred from one chemical species to another. The equation for a generic redox reaction where species A gains electrons from species B is shown below:

\[ A_{ox} + B_{red} \rightleftharpoons A_{red} + B_{ox} \]  

(1)

This equation can be divided into two half reactions, also known as redox couples, for the reduction of species A and the oxidation of species B:

\[ A_{ox} + e^- \rightleftharpoons A_{red} \]  

(2)

\[ B_{red} \rightleftharpoons B_{ox} + e^- \]  

(3)

The forward reaction in equation 1 will be spontaneous if species A has a higher redox potential than species B. The relationship between the redox potential (electromotive
force) and the state of oxidation or reduction of a redox couple is given by the Nernst
equation [Nernst 1888; Nernst 1889]:

\[ E_h = E_{mx} - \frac{RT}{nF} \ln \left( \frac{[A_{red}]}{[A_{ox}]} \right) \]  

(4)

Where \( R \) is the gas constant, \( T \) is the temperature, \( n \) is the number of electrons transferred
per molecule, \( F \) is the electrical charge of 1 mol of electrons (faraday), \( A_{red} \) and \( A_{ox} \) are the
concentrations of A in the reduced and oxidized forms respectively, \( E_h \) is the redox
potential of the redox pair with respect to the standard Hydrogen electrode, and \( E_{mx} \) is the
midpoint potential of the couple where \( A_{ox} = A_{red} \) at pH \( x \). A derivation of the Nernst
equation is given in Appendix 1.

Experimentally the midpoint potentials of biological redox molecules are
determined by varying the electrode potential in a reaction vessel and monitoring the
corresponding changes in the ratio of concentrations of oxidized or reduced forms
[Dutton 1978]. The value of \( E_{mx} \) in the Nernst equation (equation 4) is then varied to fit
the data and determine the midpoint potential. The redox state of the biological molecule,
at various \( E_h \) values, is measured by a physical technique suitable for the given system.
Most commonly visible absorbance spectroscopy is used to determine midpoint potentials
of proteins [Dutton 1971; Stotz et al. 1938; Sato-Watanabe 1995]. Although there are
redox dependent changes in the absorption spectrum of FAD, the absorption spectrum
cannot be used to monitor the FAD redox state in ETF-QO because the spectrum of the
[4Fe-4S]\(^{2+,1+}\) overlaps with the spectrum of the FAD. Other techniques commonly used in
redox potentiometry are infrared (IR) spectroscopy [Hamacher et al. 1996], fluorescence [Krieger et al. 1995], NMR [Chang and Swenson 1999], and electron paramagnetic resonance (EPR) spectroscopy [Caldeira et al. 1996; Cooley et al. 2004; Creevey et al. 2008].

In their oxidized forms the [4Fe-4S]^{2+,-1+} cluster and the FAD of ETF-QO are diamagnetic but, upon reduction, both cofactors become paramagnetic [Beckmann and Frerman 1985]. Electrons possess a quantized property called spin which has been described in classical terms as the electron rotating around an axis through its center. This property has a corresponding magnetic moment associated with it, symbolized by the spin quantum number $m_s$, which can have one of two possible values, $m_s = +1/2$ or $m_s = -1/2$ for a single electron. When molecules containing unpaired electrons are placed in a magnetic field the electron’s magnetic moment can align in two ways, parallel ($m_s = -1/2$) or anti-parallel ($m_s = +1/2$), to the external magnetic field. The two spin states were degenerate in the absence of the external magnetic field, but now have different energy levels. This process is known as the Zeeman effect and it is what makes EPR spectroscopy possible (Figure 2.6). Electrons move from the lower energy level to the higher energy level by the absorption of energy. Continuous wave (CW) EPR spectrometers use constant frequency radiation, most commonly microwaves at roughly 10 GHz (X Band), and the magnetic field is swept.
Figure 2.6  Representation of the Zeeman effect. The degenerate energy levels of the two electron spin states are split when an external magnetic field is applied (solid lines). The difference in energy between the two states (dashed line) is proportional to the external magnetic field and is given by the equation listed above where $g_e$ is the electron’s $g$-factor, $\beta$ is the Bohr magneton, and $B_0$ is the magnetic field strength.

EPR was used to monitor the redox states of the two cofactors in this study (Figure 2.7). The use of EPR spectroscopy to measure the state of reduction of a protein during a potentiometric titration was pioneered by Dutton and co-workers [Wilson et al. 1970]. This method has been used to determine the redox potentials of many cofactors including $[4Fe-4S]^{2+,1+}$ clusters [Hinckley and Frey 2006; Fritz et al. 2002] and FAD [Parschat et al. 2001]. In ETF-QO, the $[4Fe-4S]^{2+,1+}$ can exist in two redox states, the oxidized form: $[4Fe-4S]^{2+}$, and the EPR detectable reduced form: $[4Fe-4S]^{1+}$. The increase in $[4Fe-4S]^{1+}$ EPR signal as the electrochemical potential is lowered can be fit using the Nernst equation to find the midpoint potential of the couple.
The FAD is slightly more complicated because it has three redox states; Q (oxidized), SQ\textsuperscript{−}, and QH\textsubscript{2}, where only the SQ\textsuperscript{−} form is detectable by EPR. When going from high to low potential the FAD SQ\textsuperscript{−} EPR signal grows in and then disappears. These data are fit to the sum of two Nernst equations to give midpoint potentials of both couples: reduction of Q to SQ\textsuperscript{−} and SQ\textsuperscript{−} to QH\textsubscript{2}. Stankovich and coworkers used analogous methods to determine redox potentials of the [4Fe-4S]\textsuperscript{2+:1+} and FAD in human ETF-QO [Paulsen et al. 1992].

\[
[4Fe4S]^{2+} \overset{e^-}{\Rightarrow} [4Fe4S]^{1+} \quad FAD_{ox} \overset{e^-}{\Rightarrow} FAD_{SQ} \quad \overset{e^-}{\Rightarrow} FAD_{QH2}
\]

**Figure 2.7** Oxidation states of the iron-sulfur cluster and FAD of ETF-QO. Red indicates EPR active states.

### 2.5 Relaxation Enhancement Inter-spin Distance Measurements in Proteins

Mutations may change the three-dimensional structure of proteins. Before any conclusions can be made from activity data for the ETF-QO mutants, the structural integrity of the mutants needs to be confirmed to ensure that any changes observed are due to the measured modulation of FAD potentials rather than an unexpected disruption in the protein structure. The distance between the two cofactors in ETF-QO mutants were compared to the distance found in wild-type ETF-QO to ensure that the mutations were not deforming the three-dimensional structure. One way to determine interspin distances
between two paramagnetic centers is by monitoring the effect of a rapidly relaxing paramagnetic metal center on the electron spin relaxation rate of a more slowly relaxing center [Eaton and Eaton 2000 pp. 347-381]. In ETF-QO, \([4\text{Fe}-4\text{S}]^{1+}\) has a faster spin-lattice relaxation rate \((1/T_1)\) than the FAD SQ\(^{-}\). Therefore the iron-sulfur cluster enhances the relaxation of the FAD and the degree of enhancement can be used to determine a distance between the two [Fielding et al. 2008]. Similar methodology was used previously in the study of spin-labeled metmyoglobin to measure interspin distances between heme iron (Fe(III)) and nitroxyl spin labels [Zhou et al. 2000].
3.1 Preparation of ETF-QO Mutants

3.1.1 Site-directed Mutagenesis of R. sphaeroides ETF-QO gene

The R. sphaeroides ETF-QO gene was cloned into the pET21a expression vector (containing the lac operon and an ampicillin resistance gene) as previously described [Usselman et al. 2008]. Site-directed mutagenesis was carried out using the Stratagene QuikChange® II XL site-directed mutagenesis kit according to the manufacturer’s instructions. Mutagenic primers were designed and obtained from Integrated DNA Technologies, Inc. (San Diego, CA). Sequences of the mutagenic primers used are listed in Table 3.1. The following were added to a 100 μL thin walled Eppendorf tube: 5 μL 10X reaction buffer (included in QuikChange® II XL kit), 1 μL ETF-QO plasmid (~20 ng/μL), 1 μL forward mutagenic primer (~125 ng/μL), 1 μL reverse mutagenic primer (~125 ng/μL), 1 μL dNTP mix, 3 μL QuikSolution™, and 38 μL ddH2O. Next 1 μL of PfuUltra™ High Fidelity DNA polymerase (2.5 U/μL) was added to the 50 μL reaction mixture and Polymerase Chain Reaction (PCR) temperature cycling was done using a Thermo Hybaid JMBS 0.2G temperature cycler. Temperature cycling parameters used in ETF-QO mutagenesis can be found in Table 3.2. Denaturation (melting) of the double stranded plasmid DNA was done at 95 °C, annealing of the mutagenic primers with the single stranded plasmid DNA was done at 60 °C, and extension of the DNA
chain by PfuUltra™ was done at 68 °C. In this procedure, 18 cycles of denuauration/annealing/extension were used. After PCR, parental (non-mutated) DNA was digested by the addition of Dpn I restriction enzyme. 1 μL of Dpn I (10 U/μL) was added to the PCR reaction mixture followed by incubation at 37 °C for 1 hour.

Table 3.1  ETF-QO mutagenic primer sequences and melting points.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’ to 3’)</th>
<th>Tm (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>N338A</td>
<td>CGCGCATCAAGGGCGCGCACAATGCC</td>
<td>70.5</td>
</tr>
<tr>
<td>forward</td>
<td></td>
<td></td>
</tr>
<tr>
<td>N338A</td>
<td>GCATGGCATATTGTGCACGCACCCTTGATGCACG</td>
<td>71.8</td>
</tr>
<tr>
<td>reverse</td>
<td></td>
<td></td>
</tr>
<tr>
<td>N338T</td>
<td>CCGCGCATCAAGGGCAACCACCAAATGCC</td>
<td>69.6</td>
</tr>
<tr>
<td>forward</td>
<td></td>
<td></td>
</tr>
<tr>
<td>N338T</td>
<td>CCGAGAGCATGGCATATTGTGGGTGGCCTTGATGCACG</td>
<td>72.8</td>
</tr>
<tr>
<td>reverse</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 3.2  PCR temperature cycling parameters used in ETF-QO mutagenesis

<table>
<thead>
<tr>
<th>Segment</th>
<th>Cycles</th>
<th>Temperature (°C)</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>95</td>
<td>1 min</td>
</tr>
<tr>
<td>2</td>
<td>18</td>
<td>95</td>
<td>50 s</td>
</tr>
<tr>
<td></td>
<td></td>
<td>60</td>
<td>50 s</td>
</tr>
<tr>
<td></td>
<td></td>
<td>68</td>
<td>7.5 min</td>
</tr>
<tr>
<td>3</td>
<td>1</td>
<td>68</td>
<td>7 min</td>
</tr>
</tbody>
</table>
The digest was then transformed into XL 10-Gold® Ultracompetent cells (Stratagene). In a prechilled 14-mL BD Falcon polypropylene round-bottom tube, 2 μL of Stratagene β–ME (β-Mercaptoethanol) mix was added to 45 μL of XL 10-Gold® cells. The β–ME/cell mixture was swirled and incubated on ice for 10 minutes with additional swirling every 2 minutes. Next 2 μL of the mutagenesis digest was added to the β–ME/cell mixture and the tube was incubated on ice for 30 minutes. The tube was then heat-pulsed at 42 °C for 30 seconds and placed back on ice for 2 minutes. Finally 0.5 mL of preheated (42 °C) NZY+ (NZ amine, yeast extract) broth was added to the tube and the mixture was incubated at 37 °C for 1 hour with shaking at 225 rpm. On two lysogeny broth (LB) plates containing 50 μg/mL ampicillin, 25 and 150 μL of the transformation mixture was plated. Plates were incubated overnight (~18 hours) at 37 °C producing numerous single colonies. Composition of broths used in this work can be found in Appendix 2.

3.1.2 Mutant Plasmid DNA Purification and Sequencing

Three single colonies were used to inoculate three flasks containing 10 mL of LB broth with 100 μg/mL ampicillin. The flasks were incubated at 37 °C overnight (~16 hours) and were then harvested by centrifugation. Mutant ETF-QO plasmid DNA was purified from the cells using the QIAprep Spin Miniprep Kit (QIAGEN) following the manufactures instructions (Appendix 3). This procedure yielded 50 μL of approximately 100 ng/μL plasmid DNA per flask.

Sequencing of the isolated plasmid DNA was done to confirm that the correct mutations had been generated. Sequencing was done at the University of Colorado.
Denver Cancer Center DNA Sequencing and Analysis Core following the instructions listed on the website (http://loki.uchsc.edu/, accessed on 4/12/10). The *R. sphaeroides* ETF-QO gene is roughly 1.7 kb long (551 amino acids), which is longer than can be reliably sequenced using the T7 and T7ter sequencing primers that flank the gene on the pET21a vector. Sequencing primers were designed, using Primer3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi, accessed on 4/12/10) [Rozen and Skaletsky 2000], and ordered from Integrated DNA Technologies, Inc (Coralville, IA). The sequencing primers were as follows: left primer (3’-ATCTATCATGCGAGAACAATCA-5’), right primer (3’-CGAAGAACGAGAAGTTGAACAG-5’). Full sequences were obtained when the sequencing primers were used in combination with the T7 and T7ter sequencing primers. Samples submitted for sequencing contained roughly 1250 ng of plasmid DNA and 10 pmoles of the sequencing primers. The DNA sequence of *R. sphaeroides* ETF-QO with the locations of the synthesized sequencing primers is shown in Figure 3.1.

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23
Figure 3.1  DNA sequence of *R. sphaeroides* ETF-QO. Start (ATG) and stop (TAA) codons are highlighted in bold. Locations of the synthesized sequencing primers are also highlighted in bold with the arrows indicating sequencing direction. The N338 codon that was mutated (AAC) is shown in red.

3.1.3 Transformation of Mutant Plasmids into Competent Cells

Mutated plasmids were transformed into *E. coli* C43 (DE3) cells after sequencing confirmed that the correct mutation had been introduced and that no unwanted mutations were present. The C43 strain of *E. coli* was used because it has been shown to allow high levels of membrane protein expression [Miroux and Walker 1996]. The transformation was done on a 50 μL aliquot of C43 cells using 1 μL of mutated plasmid DNA
(~100 ng/μL). The plasmid DNA was added to C43 cells and then the mixture was incubated on ice for 20 minutes, heat pulsed in a 42 °C water bath for 2 minutes and placed back on ice for 3 minutes. LB media (400 μL) was then added and the mixture was incubated at 37 °C for 15 minutes with shaking at 225 rpm. Next 2 LB agar plates, containing 50 μg/mL ampicillin, were inoculated with 50 μL of the transformation mixture and were incubated at 37 °C for 18 hours.

Single colonies from the LB agar plates were used to make cultures for frozen cell stocks. Cultures consisted of 10 mL LB media with 100 μg/mL ampicillin and were inoculated with single colonies (4 N338A colonies and 6 N338T colonies were used). They were then incubated at 37 °C for 16 hours with shaking at 250 rpm. Cell stocks were made by combining 600 μL of the culture with 600 μL of 2X freezing medium (in 1 liter of distilled water: 12.6 g K_2HPO_4, 0.9 g sodium citrate, 0.18 g MgSO_4, 1.8 g (NH_4)_2PO_4, 3.6 g KH_2PO_4, and 88 g glycerol) [Schleif and Wensink 1981]. This mixture was frozen on dry ice and then stored at -80 °C.

3.1.4 Confirmation of ETF-QO Expression

Western blots were used to confirm ETF-QO (MW = 60 kDa) expression from transformed C43 cells and to select the highest expressing colonies. N338A and N338T cell stocks were used to inoculate flasks containing 10 mL LB media, 100 μg/mL ampicillin, ~2 μM riboflavin, and 40 μM FeCl_3. Flasks were shaken at 250 rpm at 30 °C until the OD_600nm reached between 0.6 and 1. Isopropyl β-D-1 thiogalactopyranoside (IPTG) was then added to each flask to induce ETF-QO expression (final IPTG concentration of 0.5 mM). Flasks were incubated at 30 °C and 250 rpm for 16 hours.
After incubation, the optical density (light scattering) at 600 nm was determined and 1 mL of each of the cultures was centrifuged at a relative centrifugal force (RCF) of 4500 x g (note: all RCF values in this dissertation are reported as the average acceleration and are expressed as multiples of earth’s gravity), discarding the supernatant. The cell pellets were then washed in 1 mL of 10 mM Tris-HCl, pH 8.0, centrifuged at 4500 x g, washed with 1 mL of distilled H2O, and again centrifuged at 4500 x g. Cell pellets were resuspended in distilled water, 300 μL for the culture with the lowest OD600 and the remainder scaled to give equal cell concentration. Laemmli sample buffer (Bio-Rad), 60 μL, was added to 60 μL of the cell suspensions and these mixtures were placed in a boiling water bath for 5 minutes, on ice for 10 minutes, and back in the boiling water bath for 5 minutes more. Samples were then centrifuged at 18,000 x g for 2 minutes and were then loaded (5 - 20 μL) onto 10 % SDS-PAGE (sodium dodecyl sulfate – polyacrylamide gel electrophoresis) polyacrylamide gels.

Gels contained a stacking gel (4% acrylamide) on top of a resolving gel (10% acrylamide) and were formed using the following ingredients; resolving gel: 3.3 mL distilled H2O, 3 mL Protogel (National Diagnostics), 2.25 mL 1.5 mM Tris-HCl pH 8.9, 78 μL 10 % SDS, 450 μL 14 mg/mL ammonium persulfate (APS) and 3 μL TEMED (tetramethylthelylenediamine), and stacking gel: 1.125 mL distilled H2O, 325 μL Protogel, 625 μL 0.5 M Tris-HCl pH 6.8, 25 μL 10 % SDS, 300 μL APS (14 mg/mL), and 1 μL TEMED. SDS-PAGE gels were run at a constant current of 16 mA with a molecular weight standard. Current was stopped when the blue sample buffer reached the end of the gel which was usually around 1.5 hour.
Protein was transferred electrophoretically from the SDS-PAGE gels to polyvinylidene fluoride (PVDF) membranes using the western blot procedure pioneered by Towbin and coworkers [Towbin et al. 1979]. SDS-PAGE gels were soaked in Towbin buffer (48 mM Tris-HCl, 38 mM glycine, 1.3 mM SDS and 20 % methanol) for 5 minutes. Immobilon-PTM blotting membranes (Millipore) were cut to size and were soaked in methanol for 30 seconds, rinsed with distilled H₂O and then soaked in Towbin buffer for 5 minutes. SDS-PAGE gels and blotting membranes were placed together in the middle of 6 sheets of gel blotting paper soaked in Towbin buffer. Transfer to the blotting membrane was done at 120 mA for 1 hour using a semi-dry electroblotter (Continental Lab Products). After transfer, the blotting membrane was placed in 100 mL PBS, pH 7.0 containing 0.2 % (volume) Tween-20 and 5 % (weight) non-fat milk powder and was incubated for 2 hours and was then rinsed with distilled H₂O.

Binding of the primary antibody was carried out using a 1:5000 dilution of antibody in PBS, pH 7.0 containing 0.2 % Tween-20 and 3 % bovine serum albumin (BSA). Primary antibody was raised in rabbits against R. sphaeroides ETF-QO (polyclonal). The blotting membrane was incubated in the 1:5000 antibody solution for 1 hour. Following incubation the membrane was washed twice with PBS, pH 7.0 containing 0.2 % Tween-20 for 20 minutes and twice with PBS, pH 7.0 without Tween-20 for 10 minutes. The secondary antibody (horse radish peroxidase-conjugated goat anti-rabbit IgG) was then bound to the membrane by incubation for 1 hour in a 1:5000 dilution of the secondary antibody in PBS, pH 7.0 containing 0.2 % Tween-20 and 5 % non-fat milk powder. Again the membrane was washed twice with PBS, pH 7.0
containing 0.2 % Tween-20 for 20 minutes and twice with PBS, pH 7.0 without Tween-20 for 10 minutes after incubation.

Development of the membrane was done using the Amersham™ ECL Plus Western Blotting Detection System (GE Healthcare). In a darkroom the membrane was exposed on X-ray X-OMAT Blue film (Kodak) using a FBXC 810 Autoradiography Cassette (FisherBiotech). Exposure times varied from 5 seconds to 5 minutes depending on the strength of the signal. Film was developed using a Konica Minolta SRX-101A tabletop X-ray film processor.

3.2 Expression and Purification of ETF-QO Mutants

3.2.1 Growth of C43 E. coli Cells and Expression of ETF-QO

A starting culture of 25 mL LB media with 100 μg/mL ampicillin was inoculated using frozen cell stocks that gave the highest levels of mutated ETF-QO expression. The starting culture was shaken at 250 rpm overnight (16 hours) at 37 °C. This starting culture was used to inoculate 12 flasks containing 700 mL of LB media with 100 μg/mL ampicillin, 2 μM riboflavin, and 40 μM FeCl₃. Inoculation was done by adding approximately 2 mL of the starting culture to each of the 12 flasks. After inoculation the flasks were incubated at 30 °C and 250 rpm until the optical density (OD) at 600 nm reached between 0.6 and 1 (approximately 5 hours). ETF-QO expression was induced by the addition of IPTG to a final concentration of 0.5 mM in each flask. After the addition of IPTG, incubation was continued at 30 °C and 250 rpm until stationary phase was reached (OD₆₀₀ ~8).
Cells were harvested by centrifugation at a RCF of 4,500 x g, 4 °C for 10 minutes in a JLA 10.500 rotor on a Beckman Avanti J-25 centrifuge. Cells were washed once with phosphate buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄, pH 7.4) and centrifuged again at 4,500 x g, 4 °C for 10 minutes and then weighed. Approximately 5 g of cells were obtained per liter of culture.

3.2.2 Cell Lysis

Harvested cells were resuspended in lysis buffer (20 mM Tris, 50 mM NaCl, 10% glycerol, 0.1 mM Dithiothreitol (DTT), pH 7.4) at 4 times buffer volume to cell weight and were homogenized. The following protease inhibitors were also present in the lysis buffer: phenylmethanesulphonylfluoride (PMSF, 1 mM), pepstatin A (1 μM), tosyl-L-lysine chloromethylketone (TLCK, 50 μM), aprotinin (0.3 μM), and leupeptin (1 μM). Approximately 10 mg of DNase I was added to the homogenized solution and the cells were broken by two passes through a French pressure cell at 1000 psi. The broken cell suspension was placed in a Ti45 rotor and spun at 100,000 x g for 90 minutes at 4 °C in a Beckman L8-70M ultracentrifuge. The supernatant was discarded leaving a green pellet containing membrane bound ETF-QO.

3.2.3 Cell Membrane Detergent Extraction

The cell membrane pellet was resuspended and homogenized in 30 mL of lysis buffer (20 mM Tris, 50 mM NaCl, 10% glycerol, 0.1 mM DTT, pH 7.4) containing protease inhibitors as before (1 mM PMSF, 1 μM pepstatin A, 50 μM TLCK, 0.3 μM aprotinin, and 1 μM leupeptin). The total membrane bound protein concentration of the
homogenized suspension was determined using the Bradford method [Bradford 1976] and Bio-Rad protein assay dye reagent (Bio-Rad cat# 500-0006). The complete Bio-Rad Bradford procedure can be found in Appendix 4. About 1.2 g of membrane bound protein was found for all cell growths. The sample was diluted with lysis buffer (approximately 30 mL) to give a total protein concentration of 20 mg/mL.

The detergent, n-Dodecyl-β-D-Maltopyranoside (DDM, Antrate cat# D130S), was used to extract and solublize ETF-QO. A solution of approximately 3 g of DDM in 60 mL of lysis buffer containing protease inhibitors was prepared. On ice and in a 4 °C refrigerator the DDM solution was added to the homogenized membrane sample in 1 mL aliquots with constant stirring. When all the DDM solution was added the final volume of the homogenized membrane sample was 120 mL with a protein concentration of approximately 10 mg/mL and a detergent to protein ratio of 2.5:1. This solution was kept on ice in a 4 °C refrigerator with constant stirring for 1 hour.

Centrifugation at 100,000 x g, 4 °C, for 1 hour was used to separate the detergent solublized ETF-QO and the cellular membrane component (type Ti45 rotor on a Beckman L8-70M ultracentrifuge).

3.2.4 Purification of Protein Extract

Supernatant from the DDM cell membrane extract was loaded at 2 mL/min onto a 70 mL (2.5 cm x 14 cm) Q Sepharose Fast Flow column (GE Healthcare). The column had been previously equilibrated with 1 L of 20 mM Tris, pH 7.4, 150 mM NaCl, 0.1 mM DTT, 0.02% Triton X-100. The cell membrane extract formed a large greenish-brown band on the top third of the column. The column was then washed with 20 mM Tris,
pH 7.4, 150 mM NaCl, 0.1 mM DTT, 0.02% Triton X-100 at a flow rate of approximately 0.8 mL/min for 20 hours (approximately 1 L total volume). After washing, a small, yellowish-green band containing ETF-QO remained at the top of the column. ETF-QO was eluted with a 1 L linear gradient (225 to 600 mM NaCl) at a flow rate of 0.7 mL/min (starting buffer: 500 mL of 20 mM Tris, pH 7.4, 225 mM NaCl, 0.1 mM DTT, 0.02% Triton X-100 and ending buffer: 500 mL of 20 mM Tris, pH7.4, 600 mM NaCl, 0.1 mM DTT, 0.02% Triton X-100). Using a Teledyne Isco Foxy 200 automatic fraction collector, 10 mL fractions were collected. ETF-QO eluted from the column near the end of the gradient and was located by its characteristic visible absorbance (Simadzu UV-2401 PC). Those fractions showing the characteristic spectrum of ETF-QO were combined and dialyzed. Concentrated ETF-QO was dialyzed to eliminate the high salt concentration as well as to exchange the buffer. Purified ETF-QO was dialyzed against three changes of 1 L, 20 mM Hepes(K+), pH 7.4.

After dialysis Bio-Beads® (SM-2 Adsorbent, 20-50 mesh) were used to remove detergent from the sample. ETF-QO was added to roughly 4 g of Bio-Beads®, that had been previously equilibrated with 20 mM Hepes(K+), pH 7.4, and the mixture was stored in a 4 °C refrigerator overnight (16 hours) with occasional swirling. ETF-QO was removed from the Bio-Beads® by vacuum filtration.

Purified enzyme was concentrated using an Amicon stirred ultrafiltration cell and a PM30 ultrafiltration membrane (Millipore, Billerica, MA) under N₂ at a pressure of approximately 12 psi. The final concentration was determined using the absorbance at 430 nm ($\varepsilon_{430} = 24 \text{ mM}^{-1}\text{cm}^{-1}$) on a Simadzu UV-2401 PC spectrometer. Aliquots of 1 mL
were snap frozen in liquid nitrogen and stored at -80 °C until experiments could be carried out.

3.3 Iron and FAD Quantitation

The iron content of the two mutants and of wild type ETF-QO was determined spectrophotometrically at 535 nm on a Shimadzu UV-2401 PC spectrophotometer by the method of Wu and coworkers [Wu et al. 2002]. First, 60 μL of concentrated HCl was added to 200 μL of protein sample and the mixture was heated at 100 °C for 15 minutes. Centrifugation at 18,000 x g for 5 minutes was done to remove precipitate. Next 100 μL of supernatant was mixed with 1.3 mL of 0.5 mM Tris-HCl (pH 8.5), 100 μL of freshly prepared 5% sodium ascorbate solution and 400 μL of 0.1% bathophenanthroline disulfonate solution. The resulting mixture was incubated at room temperature for 1 hour. Standard curves were made following the same procedure except varying concentrations (~ 300 – 50 μM) of FeCl₃ were substituted for the protein in the first step. Iron concentration was quantitated by comparing absorbance at 535 nm of the protein samples with a calibration curve made using the FeCl₃ solutions.

To quantitate the amount of FAD present in the purified enzyme, SDS was used to denature ETF-QO and cause the release of FAD. 50 μL of 10% (w/v) SDS was added to 200 μL protein solution giving a final SDS concentration of 2%. The mixture was incubated at room temperature for 15 minutes and FAD concentration was determined by the optical absorption at 450 nm using ε₄₅₀ = 11.3 mM⁻¹ cm⁻¹ as the extinction coefficient
of the free FAD [Siegel 1978]. Optical measurements were again done on a Shimadzu UV-2401 PC spectrophotometer.

### 3.4 Potentiometric Titration

Potentiometric titrations of ETF-QO were carried out in a glass vessel under continuous N₂ (g) flow at 4°C using dithionite as reductant. Figure 3.2 shows the experimental set-up used in the titrations. Enzyme concentration ranged from 40 to 100 µM in 20 mM Hepes(K⁺), 20% glycerol (v/v), pH 7.4. The vessel was made anaerobic by 10 cycles of evacuation and purging with helium gas. Samples of 5 to 6 mL were poised by the addition of 10 mM sodium dithionite stock (in 0.1 M sodium pyrophosphate, pH 8.0) and were allowed to equilibrate under constant stirring. Equilibrium was judged to have occurred when the potential changed less than 2 mV in 5 minutes. The solution potential was monitored using a Ag/AgCl platinum ORP electrode (Orion 9678BNWP, Thermo Scientific) filled with 4M KCl and a pH/mV meter (Fisher Scientific Accumet Basic). The ORP electrode was calibrated using Orion 967901 ORP standard. Experimental values were converted to a Standard Hydrogen Electrode (SHE) scale by addition of 200 mV. Electrodes used to measure \( E_h \) do not achieve sufficient contact with the redox centers in most proteins so small molecule redox mediators are required for potential measurement [Dutton 1978]. The following mediator dyes were added at a final concentration of 25 µM to facilitate measurement of the potential; 2,3,5,6-tetramethyl-p-phenylenediamine (+260 mV), 2,6-dichlorophenol-indophenol (+217 mV), phenazine methosulphate (+80 mV), methylene blue (+11 mV), pyocyanine (-34 mV), indigo carmine (-125 mV), and
9,10-anthraquinone 2,6-disulphonic acid (-185 mV). For samples measured at 100 K, poised enzyme aliquots of roughly 300 μL were transferred anaerobically to 4.0 mm OD quartz EPR tubes and were immediately frozen in liquid nitrogen. The tubes were flame sealed and stored at -80 °C. Samples measured at 293 K were collected by placing 1.0 mm ID capillary tubes into the poised solution and quickly sealing the ends with Critoseal® (Oxford Labware, St. Louis, MO). The filled capillary tubes were then immediately placed in a 4.0 mm EPR tube that was flushed with N₂ (g) to keep the sample anaerobic.

**Figure 3.2** Schematic diagram of the reaction vessel used in the potentiometric titrations of ETF-QO. The total volume was approximately 10 mL. Typically 5 mL of protein solution was used. The vessel was made anaerobic and was under continuous N₂ (g) flow during the titrations. Samples measured at 100 K were transferred to EPR tubes using N₂ (g) flow and were immediately frozen in liquid nitrogen and flame sealed. Samples measured at 293 K were transferred to capillary tubes. The minimum volume in the vessel was about 1 mL corresponding to the smallest amount of sample that could contact the ORP electrode to give a reading.
3.4.1 CW EPR Spectroscopy

CW EPR spectra of the [4Fe-4S]$^+$ were recorded at 15 K and 9.35-9.42 GHz on a locally constructed spectrometer [Quine et al. 1987] equipped with a GaAsFET amplifier, Bruker split-ring resonator and Oxford CF 935 cryostat. The operating conditions were: 4.0 G modulation amplitude at 100 kHz modulation frequency, and microwave power of 50 $\mu$W. Spectra were recorded with sweep widths between 1500 and 2000 G averaging five, 2 minute scans. Resonator background spectra for 20 mM Tris-HCl at pH 7.4 containing 20% glycerol were recorded under identical conditions and subtracted from spectra of the iron-sulfur cluster.

CW EPR spectra of the FAD SQ$^{••}$ at 9.23 GHz were measured using a Varian E109 spectrometer equipped with a GaAsFET amplifier, a TE$_{102}$ rectangular cavity, and a Varian liquid-nitrogen-cooled gas flow system. Spectra were recorded at 100 K and at 293 K to determine what effect the freezing of the samples had on the FAD redox potentials. CW EPR spectra of FAD SQ$^{••}$ at 100 K were recorded in 4.0 mm EPR tubes using the following operating conditions: 2.0 G modulation amplitude at 100 kHz modulation, a gain of 2000, a microwave power of 10 $\mu$W, 0.25 s time constant, 200 G sweep width, and averaging ten, 2 minute scans. Spectra at 293 K were recorded in 1.0 mm ID capillaries using the following operating conditions: 2.0 G modulation amplitude at 100 kHz modulation, a gain of 8000, a microwave power of 0.5 mW, a time constant of 0.25 seconds, 200 G sweep width, and averaging five, 2 minute scans. A smaller diameter tube had to be used for measurements at 293 K because of the high microwave energy loss associated with liquid water in the resonator. FAD SQ$^{••}$ concentration was calculated by double integration of the EPR signal and comparison
with the double integral for the signal from 0.5 and 0.8 mM tempol (4-hydroxy-2,2,6,6-tetramethylpiperidinooxy) standards. EPR spectra for tempol standards at 293 K were recorded using the following conditions: 2.0 G modulation amplitude at 100 kHz, a gain of 250, a microwave power of 0.5 mW, a time constant of 64 ms, 200 G sweep width and averaging 2, 4 minute scans. Because different instrument settings were used, the EPR signal integrals obtained from ETF-QO samples and tempol standards were normalized for comparison. Integrals were normalized by dividing the second integral of the EPR signal by the product of gain, modulation amplitude, number of scans and the square root of microwave power. FAD SQ⁻ EPR spectra were recorded at higher temperatures compared to the spectra of [4Fe-4S]⁺ because relaxation rates for the FAD SQ⁻ are so slow at lower temperatures that it is difficult to obtain spectra that are not power-saturated. Also the relaxation rate of the [4Fe-4S]⁺ signal is so fast at temperatures above about 60 K that it is not detected and overlap with the FAD SQ⁻ signal is not a problem.

### 3.4.2 Midpoint Potential Calculation

Relative concentrations of the paramagnetic [4Fe-4S]¹⁺ that are needed for the calculations of the midpoint potentials were based on peak-to-peak signal amplitudes [Usselman et al. 2008]. Double integration was not used because at 15 K the signals for [4Fe-4S]¹⁺ and FAD SQ⁻ overlap. For the [4Fe-4S]¹⁺ titration curves the signal at $g_x$ was selected for the calculations, because it is relatively strong and does not overlap with the resonator background signal that was seen at lower field, overlapping with $g_z$. The $g_y$ signal was not used because of the close proximity of the FAD SQ⁻ signal. Relative
concentration of reduced [4Fe-4S]$^{1+}$ was estimated by normalizing EPR data to the peak with the highest amplitude. Midpoint potentials of the [4Fe-4S]$^{2+}$,$^{1+}$ cluster ($E_m$) were calculated by nonlinear least squares fitting to plots of relative $g_z$ EPR signal amplitude versus $E$ (mV) using the Nernst equation [Usselman et al. 2008; Fielding et al. 2008]. The Mathcad software package (PTC, Needham, MA) was used to fit the data by manually changing the midpoint potential ($E_m$) to satisfy a least squares error constraint. The scatter in the data points determines the uncertainty in $E_m$, which is estimated at about ±5 mV. The first ($E_{m1}$) and second ($E_{m2}$) reduction potentials for the FAD were calculated by nonlinear least squares fitting to plots of [SQ$^-\cdot$] versus $E$ (mV) using the Nernst equation. The solver function in Excel was used to vary the $E_{m1}$ and $E_{m2}$ values until a minimum sum of squares error between the data points and Nernst equation was found. The average of $E_{m1}$ and $E_{m2}$ can be determined with an uncertainty of about ±5 mV. The maximum percentage of SQ$^-\cdot$ formed at 293 K are: 75% for wild type, 39% for N338T, and 41% for N338A ETF-QO. These values are higher than those measured at 100 K, which are: 53%, 32% and 35% for wild type, N338T, and N338A respectively. The best-fit separation between the two potentials, $E_{m1} - E_{m2}$, is strongly dependent on the concentration of SQ$^-\cdot$ observed at the peak of the titration curve, so the uncertainties in $E_{m1}$ and $E_{m2}$ are estimated as ±15 mV.

3.5 Spectrophotometric Titration

Reduction of ETF-QO was carried out either enzymatically using octanoyl-CoA as the electron donor or chemically with Na$_2$S$_2$O$_4$ (sodium dithionite) [Beckmann and Frerman 1985; Simkovic et al. 2002; Johnson et al. 1987]. Enzymatic reductive titrations
of ETF-QO were performed in stoppered cuvettes containing 800 μL of 20 μM ETF-QO in 20 mM Hepes(K⁺) at pH 7.4, 8 mM CHAPS, 0.5 μM human medium chain acyl-CoA dehydrogenase (MCAD), 1.5 μM human ETF, and 0.4 mM protocatechuate. Reaction mixtures were made anaerobic by 10 cycles of alternate evacuation and purging with argon. Residual O₂ was removed by addition of protocatechuate dioxygenase to a final concentration of 0.13 μM [Patil and Ballou 2000]. The dioxygenase was a gift from Dr. David Ballou (University of Michigan) or purchased from Sigma-Aldrich. ETF-QO was then reduced by titrating in 0.5 μL aliquots of 4.3 mM octanoyl-CoA. Absorption spectra following each addition were measured after the optical spectrum had been stable for 5 minutes.

Reduction with dithionite was performed on 400 μL samples of 20 μM ETF-QO in 10 mM Hepes(K⁺), pH 7.4, 8 mM CHAPS, and 0.4 mM protocatechuate. Alternate evacuation and purging with argon was again used to make the reaction mixtures anaerobic and residual O₂ was removed by addition of protocatechuate dioxygenase (0.13 μM final concentration). For complete reduction of ETF-QO, an excess of Na₂S₂O₄ (5 μL of 5 mM in anaerobic 0.1 M sodium pyrophosphate at pH 8.0) was added to the cuvette and the absorption spectrum was recorded.

3.6 Enzyme Activity Assays

ETF-QO activity was assayed by two methods to determine the effects of the mutations on the electron transferring properties of ETF-QO. First a quinone reductase activity assay was performed to determine how the specific mutations effect ETF-QO in
its biological role of electron transfer from ETF to ubiquinone. Equation 5 shows the 
flow of electrons in the assay.

\[
e^{-} \quad e^{-} \quad e^{-} \quad e^{-} \quad e^{-} \\
\text{octanoylCoA} \rightleftharpoons \text{MCAD} \rightleftharpoons \text{ETF} \rightleftharpoons \text{ETFQO} \rightleftharpoons Q \_1 \tag{5}
\]

In the quinone reductase assay, MCAD is reduced by octanoyl-CoA and subsequently 
transfers electrons to ETF which in turn transfers electrons to ETF-QO (present in 
catalytic amounts). ETF-QO reduces coenzyme Q\(_1\). The second assay monitors the 
ETF-QO catalyzed disproportionation of ETF semiquinone (Equation 6). Rates from this 
assay can be used to determine the effects of mutations on the electron transfer between 
ETF-QO and its biological electron donor ETF.

\[
2\text{ETF}_{1e^-} \xleftrightarrow{\text{ETFQO}} \text{ETF}_{\text{ox}} + \text{ETF}_{2e^-} \tag{6}
\]

### 3.6.1 Quinone Reductase Activity Assay

ETF-QO quinone reductase activity was measured spectrophotometrically in a 
coupled reaction containing 10 mM Hepes(K\(^+\)) buffer, pH 7.4, 2 µM human MCAD, 
2 µM porcine ETF, 100 µM octanoyl CoA, 1 mM DDM, and 60 µM Q\(_1\) (coenzyme Q\(_1\), 
Sigma Chemical Co.) at 25°C [Ramsay et al. 1987]. Q\(_1\) reduction was initiated by the 
addition of ETF-QO, giving a final concentration of about 8 nM. Reduction of Q\(_1\) was 
monitored by the decrease of absorbance at 275nm (Q\(_1\), Δε = 7.4 mM\(^{-1}\) cm\(^{-1}\)) as a function 
of time. Absorbance versus time data were plotted and slopes were calculated for the
initial, linear regions. Activities were determined by dividing the absolute value of these slopes by the product of the extinction coefficient of ETF-QO ($\epsilon_{275} = 7.5$ mM$^{-1}$ cm$^{-1}$) and the working concentration of ETF-QO in the assay.

### 3.6.2 Disproportionation of ETF$_{1e}$

Disproportionation of ETF$_{1e}$ catalyzed by ETF-QO was assayed under anaerobic conditions as described by Beckmann and Frerman [Beckmann and Frerman 1985]. For the assay 0.8 mL reaction mixtures were prepared, in sealed cuvettes, containing $10 \mu$M ETF in 10 mM Tris-HCl, pH 7.5 with 0.4 mM protocatechuate. Cuvettes were made anaerobic by 10 cycles of alternate evacuation and purging with argon gas. Residual oxygen was removed by the addition of protocatechuate dioxygenase (0.13 $\mu$M final concentration). The absorption spectrum of oxidized ETF was recorded, and then ETF was quantitatively reduced to the semiquinone with 0.5 mM sodium dithionite in anaerobic 0.1 M sodium pyrophosphate at pH 8.0 [Husain and Steenkamp 1983]. Reactions were initiated by the addition of 2.5 to 5 $\mu$L of 0.8 $\mu$M ETF-QO and the decrease in absorbance at 370 nm was monitored with respect to time. The concentration of the oxidized form of ETF was calculated using the equation:

$$[ETF]_{ox} = \frac{Abs_{370nm} - (\epsilon_{sq} \times [ETF]_{Total})}{\epsilon_{ox} - 2\epsilon_{sq} + \epsilon_{hq}}$$

where $\epsilon_{ox} = 11.7$ mM$^{-1}$cm$^{-1}$, $\epsilon_{sq} = 18.1$ mM$^{-1}$cm$^{-1}$ and $\epsilon_{hq} = 5.1$ mM$^{-1}$cm$^{-1}$ [Beckmann and Frerman 1985]. A plot of $[ETF]_{ox}$ versus time was made for the initial...
linear portion of the disproportionation data and a slope was calculated by a linear fit function. This slope was then divided by the total concentration of ETF-QO added in the assay to give the turnover number with the units, s\(^{-1}\). \(K_m\) of \(R.\ sphaeroides\) ETF-QO for human ETF is 25 \(\mu\)M so it was impractical to use saturating concentrations of ETF in these assays. However, the assay conditions are satisfactory for comparative purposes because under non-saturating conditions, the velocity is proportional to the second order rate constant [Fersht 1988].

3.7 Pulsed Electron Spin Echo (ESE) Experiments

3.7.1 Temperature Dependence of \([4Fe-4S]^+\) Spin-lattice Relaxation

Electron spin-lattice relaxation times (\(T_1\)) of the \([4Fe-4S]^+\) were determined by inversion recovery at temperatures between 8 and 14 K and by contributions to the CW line shape at temperatures from 25 to 40 K. Three-pulse inversion recovery measurements were performed on a Bruker E580 spectrometer with a split-ring resonator and Oxford CF 935 cryostat using a \(\pi - T_{\text{var}} - \pi / 2 - \tau - \pi - \tau - \text{echo}\) sequence with pulse lengths of 80, 40 and 80 ns (Figure 3.3). Values of \(\tau\) and \(T_{\text{var}}\) were 370 ns and 120 ns respectively. \(T_1\) relaxation times were determined by fitting the recovery curves to a sum of two exponentials using the program MULTIFIT [Provencher 1976]. The short component was attributed to spectral diffusion processes and the long component was assigned as the \(T_1\) relaxation time. CW EPR spectroscopy was performed on a Bruker E580 spectrometer with split-ring resonator and Oxford CF 935 cryostat at 25, 30, 35 and 40 K using a sweep width of 1000 G at 3380 G center field, 4 G modulation amplitude, 80° modulation phase and a receiver gain of 70. The microwave power was 0.5 mW (26 dB) for the
measurements at 25 and 30 K and 2 mW (20 dB) for the measurements at 35 and 40 K. 

\( T_1 \) values were determined by fitting the CW spectra using the locally written program SATMON [Rakowsky et al. 1998] assuming that the spin-spin relaxation time (\( T_2 \)) was equal to \( T_1 \) and the inhomogeneous broadening was independent of temperature.

**Figure 3.3** Electron Spin Echo (ESE) inversion recovery pulse sequence. A Hahn echo (\( \pi/2 - \pi \)) pulse sequence is performed after an initial \( \pi \) pulse to give an inverted echo (A). The time between the initial \( \pi \) pulse and Hahn echo sequence (\( T_{var} \)) is stepped allowing the magnetization to return to equilibrium (B). An inversion recovery curve is the measurement of echo amplitude as a function of \( T_{var} \). This curve is fit to determine the spin-lattice (\( T_1 \)) relaxation time.
The temperature dependence of \([4\text{Fe-4S}]^+\) \(T_1\) values was analyzed by fitting with the model shown in equation 8.

\[
\frac{1}{T_1} = A_{\text{dir}} T + A_{\text{Ram}} \left( \frac{T}{\Theta_D} \right)^9 J_b \left( \frac{\Theta_D}{T} \right) + A_{\text{Orb}} \left[ \frac{\Delta_{\text{Orb}}^3}{\left( \frac{\Delta_{\text{Orb}}}{T} \right)^{-1}} \right]
\]  

(8)

In the model, \(T\) is the temperature in Kelvin, \(\Theta_D\) is the Debye temperature, and \(\Delta_{\text{Orb}}\) is the Orbach energy (energy separation, in K, between ground and excited state for the Orbach process). \(A_{\text{dir}}, A_{\text{Ram}},\) and \(A_{\text{Orb}}\) are the coefficients for the contributions from the direct process, the Raman process, and the Orbach process respectively. \(J_b\) is the transport integral defined by equation 9.

\[
J_b \left( \frac{\Theta_D}{T} \right) = \int_0^{\Theta_D/T} x^9 \frac{e^x}{(e^x - 1)^2} dx
\]  

(9)

3.7.2 Relaxation Enhancement Distance Measurements

Enhanced relaxation of the FAD SQ•• signal due to interaction with rapidly relaxing \([4\text{Fe-4S}]^+\) was measured using inversion recovery EPR analogous to the procedure for the \([4\text{Fe-4S}]^+\), with pulse lengths of 80, 40 and 80 ns. Measurements were performed on samples prepared in the potentiometric titration, selected for the maximum FAD SQ•• signal intensity. Values of \(\tau\) and \(T_{\text{var}}\) were 370 ns and 120 ns respectively. The attenuation of the pulses was adjusted to give the maximum echo intensity and the resonator was over coupled (\(Q \sim 100\)). Recovery curves were recorded at temperatures between 30 and 60 K for the FAD SQ•• signal located at \(g = 2.005\).
Enhancement of the relaxation rates for FAD SQ$^-$ was modeled using the locally written program MENOSR, which utilizes a modified version of the Bloembergen equation [Eaton and Eaton 2000 pp. 347-381; Rakowsky et al. 1995]. The inversion recovery curves for the FAD SQ$^-$ are comprised of contributions from radicals with neighboring diamagnetic [4Fe-4S]$^{2+}$ and paramagnetic [4Fe-4S]$^+$, but only the paramagnetic form of the iron-sulfur cluster enhances the FAD relaxation. In the MENOSR program the distance between the two centers and the fraction of iron-sulfur cluster in the diamagnetic state are both varied to give the best fit to the data [Fielding et al. 2008].

The double mutation Y501F/T525A [Usselman et al. 2008], which deletes two hydrogen bonding interactions with the [4Fe-4S]$^{2+}$, was used to measure FAD SQ$^-$ relaxation in the absence of enhancement. The [4Fe-4S]$^+$ midpoint potential for Y501F/T525A ETF-QO is sufficiently low that at -15 mV all of the iron-sulfur cluster is in the diamagnetic +2 oxidation state and does not interact with the FAD SQ$^-$. In the previous study of ETF-QO by Fielding et al. [Fielding et al. 2008] the FAD SQ$^-$ in ETF was used to estimate the relaxation rate in the absence of relaxation enhancement. The relaxation rates that were measured for the SQ$^-$ in reduced Y510F/T524A were in good agreement with values obtained previously for the SQ$^-$ in ETF [Usselman et al. 2008; Fielding et al. 2008].
Chapter 4. ETF-QO Mutation Results

4.1 Characterization of ETF-QO Mutants

The optical spectrum of oxidized (as isolated) N338A ETF-QO is shown in Figure 4.1 (blue trace). It is made up of overlapping contributions from the $[4\text{Fe-4S}]^{2+\text{-}1+}$ and the FAD. The FAD contributes the dominant peaks at 380 and 430 nm, which are similar for the wild type and two mutants (Figure 4.1). The absorbance at $\lambda > 500$ nm is primarily due to the $[4\text{Fe-4S}]^{2+\text{-}1+}$. These clusters typically have broad optical spectra from about 350 to 800 nm with maxima between 400 and 420 nm [Rousset et al. 2008; Crack et al. 2009] caused by S to Fe charge transfer [Maes et al. 2000].

![Figure 4.1](image)

Figure 4.1 Absorption spectra of N338A (---), N338T (—) and wild-type (—) ETF-QO. It was assumed that the extinction coefficient of 24 mM$^{-1}$cm$^{-1}$ at 430 nm was unchanged by mutation.
Quantitation of the redox cofactors in wild-type ETF-QO and the two mutants was done to demonstrate the incorporation of the $[4\text{Fe-4S}]^{2+,1+}$ and the FAD in the correct 1:1 ratio which corresponds to a 4:1 molar ratio of iron to FAD. Iron/FAD ratios were found to be 4.0:1 for wild type, 3.6:1 for N338T and 4.1:1 for N338A (uncertainties were estimated as ± 0.3 based on standard deviations of multiple measurements using the method described in section 3.3). These approximately 4:1 iron:FAD ratios confirm that the mutations did not have an effect on FAD incorporation. A standard curve for absorbance of bathophenanthroline disulfonate as a function of FeCl$_3$ concentrations used in the iron quantitation is shown in Figure 4.2 [Wu et al. 2002].

![Figure 4.2](image)

**Figure 4.2** Example of standard curve used to calculate iron concentration of ETF-QO samples.
The \([4\text{Fe-4S}]^{2+,1+}\) cluster and the FAD are diamagnetic in the oxidized as-isolated enzyme, but can be reduced to EPR-detectable forms. The continuous wave (CW) EPR spectra of the FAD SQ” at 100 K in the two mutants are indistinguishable from those of wild-type protein (Figure 4.3). The FAD signal was studied at 100 K and 293 K because the relaxation rate of the \([4\text{Fe-4S}]^+\) is so fast at these temperatures that it does not contribute to the CW signal [Fielding et al. 2008].

![Figure 4.3](image)

**Figure 4.3** X-band CW EPR spectra of *R. sphaeroides* ETF-QO FAD semiquinone. Spectra were recorded at 100 K, 9.23 GHz with 0.01 mW power. A similar lineshape, but with lower signal-to-noise, was observed at 293 K. Anaerobic samples contained 20 mM Hepes and 20% glycerol (v/v) at pH 7.4. Spectra are shown for samples with maximum semiquinone signals, from the series prepared in the potentiometric titrations. The corresponding potentials were: wild type (-10 mV), N338A (-75 mV), and N338T (-35 mV).
The FAD $\mathrm{SQ}^-$ signal becomes severely power-saturated at the lower temperatures and higher microwave powers that were used to monitor the $[4\mathrm{Fe}-4\mathrm{S}]^+$ signal [Fielding et al. 2008]. Figure 4.4 shows the CW spectra of the $[4\mathrm{Fe}-4\mathrm{S}]^+$ in the two mutants and wild-type ETF-QO at 15 K. As expected, the $[4\mathrm{Fe}-4\mathrm{S}]^+$ linewidths and $g$-values are not changed by mutation near the FAD (Table 4.1).

![Figure 4.4](image)

**Figure 4.4** X-band CW EPR spectra of *R. sphaeroides* ETF-QO $[4\mathrm{Fe}-4\mathrm{S}]^+$. Spectra were recorded at 15 K, 9.35-9.42 GHz with 0.05 mW power. The saturated FAD semiquinone signal was deleted to aid in comparison (dashed lines). Samples were anaerobic and contained 20 mM Hepes and 20% glycerol (v/v) at pH 7.4.
Table 4.1  \( g \)-values\(^{a,b}\) and linewidths\(^{c}\) (G) for \([4Fe-4S]^{+}\) at 15 K and FAD SQ\(^{-}\) at 100 K.

<table>
<thead>
<tr>
<th>Sample</th>
<th>([4Fe4S]^{+})</th>
<th>([4Fe4S]^{+})</th>
<th>([4Fe4S]^{+})</th>
<th>SQ(^{-})</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( g_z )</td>
<td>( g_y )</td>
<td>( g_x )</td>
<td>( g )</td>
</tr>
<tr>
<td></td>
<td>(linewidth)</td>
<td>(linewidth)</td>
<td>(linewidth)</td>
<td></td>
</tr>
<tr>
<td>Wild Type</td>
<td>2.089 (22)</td>
<td>1.934 (15)</td>
<td>1.875 (29)</td>
<td>2.0036</td>
</tr>
<tr>
<td>N338T</td>
<td>2.084 (22.5)</td>
<td>1.930 (17)</td>
<td>1.870 (30.5)</td>
<td>2.0037</td>
</tr>
<tr>
<td>N338A</td>
<td>2.084 (22.5)</td>
<td>1.930 (17.5)</td>
<td>1.870 (30.5)</td>
<td>2.0039</td>
</tr>
</tbody>
</table>

\(^{a}\)The uncertainty of the iron-sulfur cluster \( g \)-values was \( \pm 0.003 \).  
\(^{b}\)The uncertainty of the semiquinone \( g \)-values was \( \pm 0.0005 \).  
\(^{c}\)Peak-to-peak first derivative linewidths were obtained by computer simulation.  
Uncertainty of the linewidths was \( \pm 2 \) G.

The temperature dependence of the spin lattice relaxation rates, \( 1/T_1 \), for \([4Fe-4S]^{+}\) at the \( g_y \) turning point in the spectrum was measured for the two mutants. Relaxation rates are the same, within experimental uncertainty, as in the wild-type protein (Figure 4.5). Analysis of the temperature dependence of the relaxation in terms of contributions from Raman and Orbach process [Fielding et al. 2008; Eaton and Eaton 2000 pp. 29-154] showed that the energy of the low-lying excited state, \( \Delta E = 210 \pm 5 \) K (146 cm\(^{-1}\)), is unchanged by the mutations.
Figure 4.5 Temperature dependence of spin-lattice relaxation rates for [4Fe-4S]$^+$ in *R. sphaeroides* ETF-QO. The circles are the average values for wild type, N338T and N338A mutants. The error bars reflect the standard deviations for the three proteins. The fit line is the sum of contributions from the Raman and Orbach processes. The Debye temperature was fixed at 100 K and the energy of the low-lying excited state was $\Delta E = 210$ K (146 cm$^{-1}$).

Interaction between the rapidly-relaxing [4Fe-4S]$^+$ and the more slowly relaxing FAD $\text{SQ}^{-}$ enhances the electron spin relaxation rate for the $\text{SQ}^{-}$, which can be used to find the point dipole distance between the two centers [Fielding et al. 2008; Eaton and Eaton 2000 pp. 347-381]. This method was used to determine a distance of 18.4 Å between the center of the iron sulfur cluster and the weighted average position of the spin densities of the FAD in *R. sphaeroides* ETF-QO [Usselman et al. 2008; Fielding et al. 2008]. The inversion recovery curves for the FAD $\text{SQ}^{-}$ signal in the two mutants were modeled using the program MENOSR [Rakowsky et al. 1995].
inversion recovery curves for wild type and N338T were very similar to that for N338A (Figure 4.6). For the N338T mutation the calculated distance between centers was 17.7 ± 0.9 Å and the fraction of the cluster in the non-interacting, diamagnetic form was 28 ± 10%. Inversion recovery data for the N338A mutant were in good agreement, with a calculated distance of 17.4 ± 0.9 Å and 30 ± 10% non-interacting [4Fe-4S]^{2+} cluster (Figure 4.6). The stated errors are standard deviations for results at temperatures between 31 and 50 K. Also shown in Figure 4.6 is the inversion recovery curve for the SQ•− in the double mutant, Y501F/T525A, which lacks relaxation enhancement because very little of the iron-sulfur cluster is reduced at the potential that gives the maximum concentration of SQ•−. The distances in the mutants are slightly smaller, although within error, than the value of 18.4 Å previously calculated for wild-type ETF-QO. One explanation for this difference could be that the mutants alter the spin density of the FAD SQ•− so that the point dipole is slightly closer to the cluster. The similarities in lineshapes of the FAD SQ•− signals (Figure 4.3) indicate that any changes in spin density distributions are too small to have large impacts on major hyperfine couplings.
Figure 4.6  Inversion recovery curves for semiquinone in the reduced N338A (upper) and Y501F/T525A (lower) samples of R. sphaeroides ETF-QO at 50 K. The samples were produced by reductive titration to the potentials that give the maximum FAD SQ$^-$ signal: N338A (-75 mV) and Y501F/T525A (-10 mV). The curves are the sums of contributions from semiquinone with neighboring diamagnetic [4Fe-4S]$^{2+}$ and paramagnetic [4Fe-4S]$^+$. The difference between the two curves reflects the impact of the paramagnetic [4Fe-4S]$^+$: N338A (70-80%) and Y501F/T525A (0%). The dashed line is a simulated curve calculated with MENOSR. Simulated curves for the N338A and N338T mutants give an average inter-spin distance of 17.6 ± 0.9 Å.

The similarity in CW EPR line shapes and g-values, the similarity in optical spectra, the fact that no observed change in the [4Fe-4S]$^+$ relaxation times was seen, the observed 4:1 iron to FAD ratio, and the similarity in the distances between the iron-sulfur cluster and the FAD SQ$^-$ radical all suggest that the mutations did not cause major structural changes to the enzyme.
4.2 Potentiometric Titration

CW EPR signals of FAD SQ\(^{-}\) and [4Fe-4S]\(^{+}\) were used to monitor the dithionite titrations. Figure 4.7 shows EPR spectra taken at various potentials during a titration of N338T ETF-QO. In the beginning of the titration the majority of the [4Fe-4S] clusters and the FADs are in oxidized, diamagnetic forms and the majority of the signal seen is due to paramagnetic materials in the resonator (Figure 4.7 top trace). As the potential is lowered the signals for the FAD SQ\(^{-}\) and [4Fe-4S]\(^{+}\) increase in intensity with the FAD SQ\(^{-}\) reaching a maximum and then decreasing and the [4Fe-4S]\(^{+}\) reaching a maximum and then remaining approximately constant. Wild type and N338A ETF-QO both exhibited similar behavior.
Figure 4.7  Potentiometric titration of N338T ETF-QO as followed by CW EPR spectroscopy at 15 K. As the potential is varied from high to low, the FAD SQ$^-$ signal increases to a maximum near -35 mV, and then decreases back to nearly zero. The [4Fe-4S]$^+$ signal increases to a maximum and remains constant as the potential is decreased. N338A and wild-type ETF-QO show similar behavior but the potentials that produce the maximum FAD SQ$^-$ signals vary. At 15 K the SQ$^-$ signal is power-saturated to differing degrees, so the redox potential for the SQ$^-$ signal cannot be calculated from these data.
The FAD potentiometric titration curves for wild type, N338T and N338A mutants of *R. sphaeroides* ETF-QO are shown in Figure 4.8 (titration at 277 K and spectra recorded at 100 K) and Figure 4.9 (titration at 293 K and spectra recorded at 293 K). The solid lines are least squares fits of the Nernst equation to the data. Calculated midpoint potentials are shown in Table 4.2.

**Figure 4.8** Potentiometric titration curves of the FAD semiquinone EPR signals recorded at 100 K for *R. sphaeroides* wild type (●), N338T (+), and N338A (♦) ETF-QOs. Solid lines are least squares fits of the Nernst equation to the data.
Figure 4.9  Potentiometric titration curves of the FAD semiquinone EPR signals recorded at 293 K for *R. sphaeroides* wild type (●, ▲), N338T (+), and N338A (♦) ETF-QOs. Solid lines are least squares fits of the Nernst equation to the data.

Table 4.2  Midpoint potentials and enzymatic titration $\Delta \varepsilon_{430}$ values for wild-type and mutant ETF-QOs. $[4\text{Fe}4\text{S}]^{2+,1+}$ midpoints were measured at 15 K. FAD midpoint potentials were measured at 293 K. Values at 100 K are given in parentheses.

<table>
<thead>
<tr>
<th>Sample</th>
<th>$[4\text{Fe}4\text{S}]^{2+,1+}$ $E_m$ (mV)</th>
<th>$\text{Q/SQ}^-/$ $E_{m1}$ (mV)</th>
<th>$\text{SQ}^-/$QH$<em>2$ $E</em>{m2}$ (mV)</th>
<th>$\text{Q/QH}<em>2$ $(E</em>{m1}+E_{m2})/2$ (mV)</th>
<th>Reductive Titration $\Delta \varepsilon_{430}$ (M$^{-1}$cm$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>wild type</td>
<td>+37</td>
<td>+47</td>
<td>-30</td>
<td>+8 ( +9 )</td>
<td>12790</td>
</tr>
<tr>
<td>N338T</td>
<td>+48</td>
<td>-11</td>
<td>-19</td>
<td>-15 (-31 )</td>
<td>10560</td>
</tr>
<tr>
<td>N338A</td>
<td>+42</td>
<td>-37</td>
<td>-49</td>
<td>-43 (-78 )</td>
<td>9490</td>
</tr>
</tbody>
</table>
The midpoint potentials calculated based on FAD SQ⁻ spectra recorded at 293 K differed significantly from those calculated from spectra at 100 K. These differences are illustrated in Figures 4.10, 4.11 and 4.12 for wild type, N338T and N338T, respectively. The averages of $E_{m1}$ and $E_{m2}$ determined at 100 K were 16 to 45 mV more negative than at 293 K (Table 4.2). Differences in reduction potentials determined from EPR spectra at ambient and cryogenic temperatures have been reported previously for other proteins including *Saccharomyces cerevisiae* flavocytochrome $b_2$ and *E. coli* sulfite reductase [Tegoni et al. 1998; Coves et al. 1997]. In the remainder of this dissertation emphasis is placed on FAD potentials determined by measurements at 293 K. At 293 K the N338T and N338A mutations lower $E_{m1}$, the midpoint potential of the quinone/semiquinone (Q/SQ⁻) couple from +47 mV for wild type to -11 mV and -37 mV, respectively. Mutations shift the $E_{m2}$, the midpoint potential of the semiquinone/quinol (SQ⁻/QH₂) couple, to -19 and -49 mV for N338T and N338A compared to -30 mV for wild-type ETF-QO.
Figure 4.10  Comparison of potentiometric titration curves for *R. sphaeroides* wild-type ETF-QO at 100 and 293 K. Values for the maximum fraction of FAD SQ• observed are higher for spectra recorded at 293 K (○) than for spectra recorded at 100 K (●). Calculated values of the two FAD reduction potentials, $E_{m1}$ and $E_{m2}$, also differ.

Figure 4.11  Comparison of potentiometric titration curves for *R. sphaeroides* N338T ETF-QO at 100 and 293 K. Values for the maximum fraction of FAD SQ• observed are higher for spectra recorded at 293 K (□) than for spectra recorded at 100 K (■). Calculated values of the two FAD reduction potentials, $E_{m1}$ and $E_{m2}$, also differ.
Figure 4.12   Comparison of potentiometric titration curves for *R. sphaeroides* N338A ETF-QO at 100 and 293 K. Values for the maximum fraction of FAD SQ•• observed are higher for spectra recorded at 293 K (∧) than for spectra recorded at 100 K (∗). Calculated values of the two FAD reduction potentials, $E_{m1}$ and $E_{m2}$, also differ.

Figure 4.13 shows the $[4\text{Fe}-4\text{S}]^{2+,1+}$ potentiometric titration curves for the two mutants and wild-type ETF-QO, calculated from EPR data at 15 K. The low temperature was used because the EPR signal becomes too broad to quantitate at higher temperatures. The $E_m$ of the $[4\text{Fe}-4\text{S}]^{2+,1+}$ in the N338T (+48 mV) and N338A (+42 mV) mutants are within experimental uncertainty of the wild-type enzyme (+37 mV). These results show that mutations in the vicinity of the FAD do not impact the redox potentials of the $[4\text{Fe}-4\text{S}]^{2+,1+}$. This is consistent with the observation that the $[4\text{Fe}-4\text{S}]^{2+,1+}$ site mutations did not impact the redox potentials of the FAD [Usselman et al. 2008].
4.3 Spectrophotometric Titration

ETF-QO can be reduced either enzymatically using octanoyl-CoA as the electron donor or chemically with dithionite. The extent of reduction can be monitored by changes in the optical spectra at 430 nm [Ruzicka and Beiner 1977; Usselman et al. 2008; Beckmann and Frerman 1985; Paulsen et al. 1992]. Absorption spectra for wild-type, N338A and N338T ETF-QO (Figures 4.14, 4.15 and 4.16) are shown for oxidized enzyme (trace 1), samples reduced with 0.5:1, and 1:1 mole ratios of octanoyl-CoA to ETF-QO (traces 2 and 3 respectively), and with excess dithionite (trace 4). For wild-type ETF-QO these ratios represent roughly 1 and 2 electron reduced ETF-QO because each
mole of octanoyl–CoA can transfer two electrons to the enzyme. Figure 4.17 is a plot of the change in $\epsilon_{430}$ as a function of octanoyl-CoA added, for wild-type and mutant ETF-QOs. The mutant proteins have higher limiting values for $\epsilon_{430}$ than wild type because octanoyl CoA is not a strong enough reductant to fully reduce the FAD in the two mutants, which have lower $E_{m1}$ compared to wild-type ETF-QO.

![UV-VIS spectra of anaerobic reductive titration of wild-type ETF-QO.](image)

**Figure 4.14** UV-VIS spectra of anaerobic reductive titration of wild-type ETF-QO. The oxidized sample (spectrum 1) was titrated with octanoyl-CoA. After each addition spectra were recorded every 5-10 minutes until less than 0.001 a.u. change per minute in absorbance was observed. Spectra 2 and 3 correspond to mole ratios of 0.5:1 and 1:1 octanoyl-CoA:ETF-QO, respectively, and spectrum 4 is the dithionite reduced enzyme.
Figure 4.15  UV-VIS spectra of anaerobic reductive titration of N338A ETF-QO. The oxidized sample (spectrum 1) was titrated with octanoyl-CoA. After each addition spectra were recorded every 5-10 minutes until less than 0.001 a.u. change per minute in absorbance was observed. Spectra 2 and 3 correspond to mole ratios of 0.5:1 and 1:1 octanoyl-CoA:ETF-QO, respectively, and spectrum 4 is the dithionite reduced enzyme.
Figure 4.16  UV-Vis spectra of anaerobic reductive titration of N338T ETF-QO. The oxidized sample (spectrum 1) was titrated with octanoyl-CoA. After each addition spectra were recorded every 5-10 minutes until less than 0.001 a.u. change per minute in absorbance was observed. Spectra 2 and 3 correspond to mole ratios of 0.5:1 and 1:1 octanoyl-CoA:ETF-QO, respectively, and spectrum 4 is the dithionite reduced enzyme.
4.4 Quinone Reductase Activity

The quinone reductase activity assay measures the rate at which electrons are shuttled through the enzyme from its biological electron donor, ETF, to its biological electron acceptor, ubiquinone (equation 5, chapter 3). Absorbance (275 nm) versus time data were used to calculate rates of quinone reduction (Figure 4.18). The slopes of the initial, linear regions were divided by the product of the extinction coefficient of ETF-QO ($\varepsilon_{275} = 7.5$ mM$^{-1}$ cm$^{-1}$) and the working concentration of ETF-QO in the assay. Rates from 6 trials for N338A and wild type ETF-QO and 5 trials for the N338T mutant were averaged and the standard deviations were used as an estimate of the experimental error (Table 4.3). The activity of wild type ETF-QO (12.6 s$^{-1}$) was significantly lower than the
value of 24.2 s$^{-1}$ calculated in the previous study [Usselman et al. 2008]. This difference was attributed to the fact that MCAD and ETF stocks were from different preparations in the two studies. It was therefore necessary to determine mutant activities relative to the corresponding wild type values so that results from the two studies could be compared.

Figure 4.18  The time dependence of reduction of Q$_1$ in the quinone reductase activity assay for N338A (---), N338T (---) and wild-type (—) ETF-QOs. ETF-QO quinone reductase activity was measured at 25°C in a coupled reaction containing 10 mM Hepes(K$^+$) buffer, pH 7.4, 2 µM human MCAD, 2 µM porcine ETF, 100 µM octanoyl-CoA, 60 µM Q$_1$ and 1 mM DDM. Q$_1$ reduction was initiated by the addition of ETF-QO to a final concentration of approximately 8 nM.
Table 4.3 Quinone reductase activities (s⁻¹) for trials of wild-type, N338T and N338A ETF-QO. Average values and standard deviations are shown at the bottom.

<table>
<thead>
<tr>
<th>Trial</th>
<th>Wild Type</th>
<th>N338T</th>
<th>N338A</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>11.48</td>
<td>5.27</td>
<td>6.67</td>
</tr>
<tr>
<td>2</td>
<td>11.07</td>
<td>5.81</td>
<td>5.48</td>
</tr>
<tr>
<td>3</td>
<td>11.53</td>
<td>5.35</td>
<td>4.56</td>
</tr>
<tr>
<td>4</td>
<td>14.37</td>
<td>9.10</td>
<td>3.45</td>
</tr>
<tr>
<td>5</td>
<td>13.55</td>
<td>10.73</td>
<td>3.02</td>
</tr>
<tr>
<td>6</td>
<td>13.47</td>
<td></td>
<td>3.25</td>
</tr>
<tr>
<td>Average</td>
<td>12.6</td>
<td>7.3</td>
<td>4.4</td>
</tr>
<tr>
<td>Standard Deviation</td>
<td>1.4</td>
<td>2.5</td>
<td>1.4</td>
</tr>
</tbody>
</table>

The relative quinone reductase activities for the N338T, N338A, Y501F, T525A and Y501F/T525A mutated ETF-QOs are shown in Figure 4.19. These values are reported as percents of wild type activities to facilitate comparisons. Mutations in the vicinity of the [4Fe-4S]²⁺,⁺ : Y501F, T525A, and Y501F/T525A, lowered the relative activity to 37%, 35% and 7% of wild type respectively. Mutations in the vicinity of the FAD: N338T and N338A lowered the relative activity to 58% and 35%, respectively. The differences can be explained by the fact that N338A mutation removes the hydrogen bonding interaction with the FAD, whereas the N338T mutation only weakens the interaction. More generally it is observed that mutations at both centers decrease the quinone reductase activity. It can be concluded that both the [4Fe-4S]²⁺,⁺ and the FAD are required for full biological function of the enzyme. It can also be concluded that the redox potentials of the two centers are carefully tuned in the wild-type enzyme and that small changes in potentials of one of the centers, such as in the N338T mutant, can cause a substantial decline in the enzyme’s activity.
Figure 4.19  Relative quinone reductase activities of ETF-QO mutants. Activities for the FAD site mutations, N338T (red) and N338A (blue), are shown as percents of wild-type ETF-QO. Also shown are the relative activities of previous [4Fe-4S]^{2+,1+} site mutations, Y501F (orange), T525A (purple) and the double mutant Y501F/T525A (green) [Usselman et al. 2008].

4.5 Disproportionation of ETF Semiquinone

In the disproportionation assay, ETF-QO catalyzes the disproportionation of ETF_1e^-, where ETF_1e^- serves as both an electron acceptor and donor. Absorbance spectra for ETF_OX, ETF_1e^-, and the equilibrium of the two reached after disproportionation are shown in Figure 4.20. Activities were calculated (Equation 7 in chapter 3) by monitoring the formation of ETF_OX (absorbance at 370 nm) with respect to time (Figure 4.21). Rate constants were measured for the disproportionation of human ETF_1e^- by mutant R. sphaeroides ETF-QOs and scaled to that for the wild-type enzyme (Figure 4.22).
Figure 4.20  Disproportionation of ETF$_{1e}$ by wild-type ETF-QO. Oxidized ETF (-----), at a concentration of 10 μM in 10 mM Tris-HCl, pH 7.5, was reduced quantitatively to ETF$_{1e}$ (-----) by titration with dithionite. Disproportionation was initiated by the addition of ETF-QO to a final concentration of 5 nM. The decrease in absorbance at 370 nm was monitored with respect to time until an equilibrium had been reached (- - - -).

Figure 4.21  Time dependence of the concentration of oxidized ETF in the disproportionation assays of wild-type (○), N338T (■) and N338A (◊) ETF-QOs. Solid lines are least squares fits to the data.
Figure 4.22  Relative ETF$_{1e}$ disproportionation activities of ETF-QO mutants. Activities for the FAD site mutations, N338T (red) and N338A (blue), are shown as percents of wild-type ETF-QO. Also shown are the relative activities of previous [4Fe-4S]$^{2+,-1+}$ site mutations, Y501F (orange), T525A (purple) and the double mutant Y501F/T525A (green) [Usselman et al. 2008].

The activity, expressed as an average turnover number for wild-type ETF-QO was 8 s$^{-1}$, which is considerably lower with this heterologous coupled system than the 81.4 s$^{-1}$ for the human ETF and ETF-QO homolog system [Simkovic et al. 2002]. The value of 8 s$^{-1}$ is similar to the value of 8.3 s$^{-1}$ from previous work [Usselman et al. 2008]. The turnover numbers found previously for the Y501F, T525A, and Y501F/T525A mutants near the iron-sulfur cluster decreased to 4.1 s$^{-1}$, 5.1 s$^{-1}$, and 0.66 s$^{-1}$, respectively. These disproportionation rates correspond to roughly 51%, 64% and 8% of wild type activity.
It is clear that mutations that remove a hydrogen bonding interaction to the cysteine $\gamma$ ligands of the $[4\text{Fe-4S}]^{2+},^{1+}$ cluster reduce the rate at which ETF-QO can catalyze the disproportionation of ETF. This is not the case for the N338T and N338A mutations where a slight increase in disproportionation activity was seen. Relative to wild type, the N338A and N338T mutants had about 110% activity with rates of 8.8 and 8.9 s$^{-1}$, respectively. The fact that changing the redox potential of the FAD has no negative effect on catalysis of the disproportionation of ETF $\text{SQ}^{-}$ suggests that the FAD does not directly interact with the ETF. The increase in disproportionation rate in the N338A and N338T mutants could be caused by the lowering of the FAD $\text{Q}/\text{SQ}^{-}$ redox potential. Lowering the $\text{Q}/\text{SQ}^{-}$ redox potential makes the FAD harder to reduce. The point in the reductive titration curve where $\Delta \varepsilon$ corresponds to the same extent of FAD reduction for the wild-type and mutant proteins is at a lower potential for the mutants. At this potential there is a higher concentration of the $[4\text{Fe-4S}]^{+}$ form of the cluster, which may enhance interaction with ETF.
Chapter 5. Mechanistic Conclusions from ETF-QO Study

The asparagine residue at position 338 of *R. sphaeroides* ETF-QO is postulated to interact with the FAD through hydrogen bonding interactions. Replacement of the asparagine by threonine lowered $E_{m1}$ by 58 mV and raised $E_{m2}$ by 11 mV. The alanine mutation lowered $E_{m1}$ by 84 mV and $E_{m2}$ by 19 mV compared to wild type. It is proposed that the hydrogen bonding interaction of the asparagine is completely removed by the alanine mutation but only weakened by the threonine mutation. No change in the midpoint potential of the $[4\text{Fe}-4\text{S}]^{2+,1+}$ was detected in these mutants. No evidence of the mutations causing changes in the structure of the enzyme was observed based on the visible and EPR spectra. Quinone reductase activity was lowered by both mutations demonstrating that reduction of the FAD is required for proper enzyme function. Rates of disproportionation of ETF$_{1e-}$ were not lowered by the N338 mutations near the FAD, suggesting that FAD does not interact directly with ETF [Swanson et al. 2008].

The previous finding that the ubiquinone reductase and disproportionation activities decreased when the midpoint potential of the $[4\text{Fe}-4\text{S}]^{+}$ decreased demonstrates that reduction of the cluster is required for activity. Based on this evidence and the crystal structure of porcine ETF-QO [Zhang et al. 2006] it was suggested that electrons from ETF enter ETF-QO through the $[4\text{Fe}-4\text{S}]^{2+,1+}$ [Usselman et al. 2008]. The mutations in this study shifted the redox potentials of the FAD while leaving the redox potential of the
[4Fe-4S]^{2+,1+} unchanged. The shifted FAD redox potentials cause a decrease in the ubiquinone reductase activity, but no decrease in the disproportionation activity. The lower ubiquinone reductase activity suggests that the FAD is required to shuttle electrons through the protein and reduce UQ and is consistent with crystallographic data [Zhang et al. 2006]. The slight increase in the disproportionation activity suggests that the FAD is not directly involved in the electron transfer between ETF and ETF-QO. These results combined with the previous results for the mutations near the [4Fe-4S]^{2+,1+} support the proposal that electrons are accepted from ETF through the [4Fe-4S]^{2+,1+}, and are then transferred to FAD which reduces UQ as shown in the scheme below.

\[ \text{ETF} \xrightarrow{e} \text{ETF-QO} [4\text{Fe-4S}]^{2+} \xrightarrow{e} \text{ETF-QO (FAD)} \xrightarrow{e} \text{ubiquinone} \quad (10) \]

Biologically the FAD in ETF-QO is reduced to SQ^- and not QH_2, but UQ is reduced by two electrons to UQH_2 by ETF-QO. A possible function of the [4Fe-4S]^{2+,1+} could be as a reservoir for the second electron that is needed to fully reduce UQ.
Chapter 6. ETF Background

6.1 Electron Transfer Flavoprotein

Electron transferring flavoproteins are found in all kingdoms of life. They are soluble heterodimeric proteins that use FAD to transfer electrons in diverse metabolic pathways. ETFs are divided into three groups based on sequence homology and function [Toogood et al. 2007]. Group I ETFs (sometimes referred to as “housekeeping”) are typically found in mammalian cells, but are also present in a few bacteria such as Paracoccus denitrificans. These ETFs accept electrons from dehydrogenases and transfer them to ETF-QO. Group II is comprised of the FixA and FixB proteins found in nitrogen-fixing and diazotrophic bacteria. Based on structure, FixA and FixB correspond to the β and α-subunits of group I ETFs respectively [Tsai and Saier 1995]. These ETFs transfer electrons that are used to reduce N₂ from the atmosphere to NH₃ by nitrogenase. Nitrogen fixation makes the biosynthesis of amino acids and nucleotides possible in higher organisms, thus is an essential process for life. The proteins YaaQ and YaaR make up group III ETFs. These proteins are evolutionarily distant from those in the other two groups and have evolved in Escherichia coli to deliver electrons to CaiA, which is thought to convert crotonobetaine to γ-butyrobetaine [Tsai and Saier 1995]. In the remainder of this dissertation the term ETF will be used to describe enzymes from group I.
Mammalian ETFs are located in the mitochondrial matrix. They use a single equivalent of FAD to shuttle electrons between flavoprotein dehydrogenases and the membrane-bound ETF-QO [Toogood et al. 2007; Zhang et al. 2006]. Human ETF accepts electrons from ten mitochondrial matrix flavoprotein dehydrogenases [Chohan et al. 2001] that play critical roles in fatty acid β-oxidation and the oxidation of several amino acids including lysine, valine, and isoleucine [Al-Walid and Vockley 1995; Binzak et al. 1998; Dwyer et al. 2000]. The critical physiological role of human ETF is demonstrated by the finding that mutations in ETF or ETF-QO result in the metabolic disease multiple acyl-CoA dehydrogenase deficiency [Olsen et al. 2003]. The severity of the disease depends on the location of the mutation in the protein, with the most severe mutations causing death in infancy [Curcoy et al. 2003].

Like all group I ETFs, the human enzyme is made up of two subunits, denoted α and β, which combine to form three structural domains (Figure 6.1). Domain I is formed from the N-terminal region of the α-subunit, domain II from the C-terminal region of the α-subunit (IIα) and a small C-terminal region of the β-subunit (IIβ) and domain III from the majority of the β-subunit. These three domains form a T-shaped structure where domain II rests in a shallow bowl formed by domains I and III [Chohan et al. 2001]. The FAD cofactor is bound exclusively by the C-terminal portion of the α-subunit (domain IIα). It is in an orientation where the isoalloxazine ring is located in a cleft created by domains II and III. A highly conserved region of FAD enzymes (β₁αβ₂) is also contained in domain II [Toogood et al. 2007]. This β₁αβ₂ region binds the FAD’s adenosine pyrophosphoryl moiety. Domain III contains a buried adenosine monophosphate (AMP)
molecule that plays a structural role and is required for renaturation of guanidine-denatured ETF [Roberts et al. 1996; Griffin et al. 1997]. Also located in domain III is a recognition loop (DLRLNEPRYA[S/T]LPNIMKAKKK) that is responsible for partner binding and is highly conserved in group I ETFs [Toogood et al. 2007]. Binding occurs due to hydrophobic residues in the loop, most importantly the highly conserved leucine residue at position 195 (human), interacting with hydrophobic pockets on partner proteins. The spatial separation between the recognition loop and the FAD domain allows ETF to interact with specific, structurally distinct, partners [Toogood et al. 2004].

Figure 6.1  Crystal structure of human ETF (PDB id: 1EFV). The α subunit is shown in light blue and the β subunit is shown in grey with the three structural domains labeled using roman numerals. FAD (pink) is located in domain II and the structural AMP (yellow) is located in domain III. The recognition loop in domain III is depicted using red ribbon.
Under anaerobic heterotrophic growth conditions, the bacterium *P. denitrificans* synthesizes an electron-transport system similar to the eukaryotic mitochondrial respiratory system [Bedzyk et al. 1993]. *P. denitrificans* expresses glutaryl-CoA dehydrogenase, ETF and ETF-QO as well as other electron-transport system proteins that are very similar to the eukaryotic mitochondrial electron transport chain [Watmough et al. 1992]. Because *P. denitrificans* resembles a mitochondrion much more closely than any other known bacterium, it was suggested as a possible ancestor of mitochondria [John and Whatley 1975]. *Paracoccus* ETF contains three domains and has a structure that is nearly identical to the human enzyme. The major difference in the two occurs in residues β90-96 which form an α-helix in human ETF but are a random loop in the bacterial enzyme. The structural similarity is not surprising considering the fact that there is 54% similarity in the sequences of human and *P. denitrificans* ETF [Chohan et al. 2001]. Like mammalian ETFs, the *Paracoccus* enzyme has the same highly resolved, blue-shifted visible absorption spectrum, from the FAD chromophore. Similarities in the spectra indicate that the local environments and hydrogen bonding interactions of the FAD cofactors are similar [Watmough et al. 1992]. Because of the similar environments, the two FAD redox couples are essentially identical in *Paracoccus* and porcine ETF. The two enzymes have enough similarity that *P. denitrificans* ETF can function as an electron acceptor for porcine butyryl-CoA and octanoyl-CoA dehydrogenases [Watmough et al. 1992].
6.2 Evidence of αII Domain Motion in ETF

It has been postulated that in order for mammalian ETF to accept electrons from at least 10 structurally different dehydrogenases and to transfer the electron to ETF-QO, it must be able to adopt a range of conformations. The concept of conformational flexibility is important for understanding a wide variety of electron transfer proteins [Leys et al. 2003]. Electron transfer proteins obtain optimal conformations with maximum electronic coupling by conformational sampling, which allows for efficient communication with structurally distinct partners [Toogood et al. 2007]. A striking feature of all known ETF structures is the diversity of conformations of the αII domain [Toogood et al. 2007]. Multiple conformations of the αII domain were required to model results obtained by small-angle solution X-ray scattering (SAXS) of human and *P. denitrificans* ETF [Chohan et al. 2001]. SAXS results indicate that the group II ETF from *Methylophilus methylotrophus* (W3A1) had an almost identical solvent envelope as human and *Paracoccus* ETF even though domain II in W3A1 adopts a different conformation in the crystal structure. Domains I and III fit well when the solvent envelope of W3A1 ETF was superimposed onto the crystal structure, which indicates that these domains have little motion relative to each other in solution. However, the solvent envelope greatly exceeded the size of domain II indicating that this domain undergoes a change in conformation. To fit the solvent envelope it was proposed that the αII domain rotates by 30 to 50° relative to an axis defined by domains I and III. Modeling suggests that in solution domain II samples a range of discrete, transient states [Toogood et al. 2007]. There is no evidence of a large change in conformation as a result
of reducing ETF because the solvent envelopes of oxidized and reduced W3A1 ETF are approximately identical [Chohan et al. 2001].

In the crystal structure of human ETF bound to medium-chain acyl-CoA dehydrogenase (MCAD) the flavin binding domain was barely visible in the electron density [Toogood et al. 2004]. The size of the disordered region was consistent with the envelope of structures observed by SAXS. When the glutamate residue at position 165 of the β subunit in human ETF was replaced with an alanine, the crystal structure of the resulting complex with MCAD showed a vast increase in the density of the FAD domain (about 70% occupancy). Eβ165A ETF also has a higher electron transfer rate and lower $K_m$ when compared to wild-type enzyme [Toogood et al. 2005]. In wild-type human ETF the Eβ165 residue interacts with an asparagine residue (Nα259) and a conserved arginine residue (Rα249) that are located near the FAD. These interactions help stabilize domain II in the inactive conformation [Toogood et al. 2005]. Rα249 forms a salt bridge with the E212 residue of MCAD upon ETF:MACD complex formation. Removing the interaction between Eβ165 and Rα249 by mutation leads to a shift in equilibrium from inactive conformations toward the active ones (Figure 6.2). Eβ165 is a highly conserved residue in all group I ETFs. The corresponding residue in the Paracoccus enzyme is Eβ162.
Figure 6.2  Cartoon representation of conformational changes in ETF free in solution (A) and complexed with a partner (B). In ETF, Eβ165 is represented as a red arrow and Rα249 as a blue arrow. MCAD is used as an example partner with only the surface close to ETF domain II shown (green line) and residue E212 represented as a red arrow. When ETF is free in solution the equilibrium is shifted towards the inactive conformation. Complex formation shifts the equilibrium towards the active conformation through interactions with Rα249 (residue E212 in MCAD).

6.3 Observation of Domain II Conformational Change

In this study site-directed mutagenesis of *P. denitrificans* ETF was done to introduce cysteine residues at desired positions. This was followed by spin-labeling using the cysteine-specific, nitroxyl spin label MTSL (1-oxyl-2,2,5,5-tetramethylpyrroline-3-methyl-methanethiosulfonate) [Hubbell et al. 2003]. Figure 6.3 shows the reaction of a spin label with the thiol group of a cysteine residue. FAD, in the various mutants, was then enzymatically reduced to the anionic semiquinone form so that it would have an
EPR signal. Interspin distances between the reduced FAD and spin labels at various sites were obtained by DEER measurements. A separate mutation, Eβ162A, was added to change the stability of the FAD domain conformations. It was proposed that this change in stability would lead to conformational changes that approximate ETF interacting with a redox partner [Toogood et al. 2005].

![MTSL] + [Protein] → [Spin-Labeled Protein]

**Figure 6.3** Reaction of MTSL with the thiol group of a cysteine residue.

ETF from *P. denitrificans* was used in this study because it contains only one native cysteine whereas human and pig ETF contain 10 and 9 native cysteines, respectively [Watmough et al. 1992]. Deeply buried cysteines do not react with MTSL (unless denaturing agents are used). Other cysteines need to be removed by mutagenesis before site-directed spin labeling can be performed. The single, buried native cysteine in ETF from *P. denitrificans* makes it a more feasible target for site-directed spin labeling.
than the mammalian proteins. Conformational results for Paracoccus ETF are relevant to mammalian systems because of the similarity in structures, potentials and reactivity [Watmough et al. 1992].

6.4 Double Electron-Electron Resonance (DEER)

X-ray crystallography is the most utilized technique for determining protein structures, but protein conformation in the crystal is not always biologically relevant [Hanson et al. 2007; Kim et al. 2008]. Crystallography also requires a uniform conformation throughout the crystal and does not give information on dynamics. NMR spectroscopy can give information on the dynamics of regions in a protein-protein complex formation such as in the case of GroEL-GroES complex [Fiaux et al. 2002] and the 48-kDa IIA\textsuperscript{Mannose} -HPr complex [Williams et al. 2005]. Because of their size, these proteins require $^{13}$C and $^{15}$N labeling techniques [Kay et al. 1990; Riek et al. 1999; Liu et al. 2009] which increases the cost and complexity of the experiments. Another technique commonly used to obtain conformational information of proteins is distance measurements by Förster or fluorescence resonance energy transfer (FRET) [Heyduk 2002; Hillisch et al. 2001]. Using FRET, distances between an artificially introduced probe molecule and a native fluorophore (such as a tryptophan residue) can be made assuming that the emission spectrum of one overlaps with the absorbance spectrum of the other. This limits the number of proteins that can be studied using this technique as some proteins do not contain native fluorophores near the region of interest. In such cases it is necessary to selectively label the protein with a distinct donor and acceptor molecule at two sites or use a probe that can transfer energy to itself. Covalently labeling proteins
with distinct donor and acceptor probes requires more complex approaches such as introducing nonsense codons into the gene coding for the fluorescence probes [Heyduk 2002]. Fluorescein- and rhodamine-conjugated molecules are commonly used as site specific donor-acceptor pairs [Matyus 1992; Lamichhane et al. 2010]. Recently fluorescent proteins, such as CFP (cyan fluorescent protein) and YFP (yellow fluorescent protein), have been used as donor-acceptor probes to measure conformational changes and complex formation in proteins [Giepmans et al. 2006].

A complementary method of determining structures of proteins is site-directed spin labeling (SDSL) probed by pulsed EPR spectroscopy [Berliner et al. 2000]. Double electron-electron resonance (DEER), also known as pulsed electron double resonance (PELDOR), experiments can provide information on the distribution of distances between two paramagnetic sites in a protein [Milov et al. 1981; Jeschke et al. 2000]. A major advantage of DEER is that the experiments do not require samples with a unique distance like in crystallography. Another advantage of DEER is that generally it does not require any isotopic labeling (although sometimes deuteration is used to remove strong proton modulation). Probes used in DEER measurements are also smaller than probes used in FRET so theoretically they have less of an effect on protein structure and give more accurate information of the backbone position. DEER has proven to be an effective way to study the conformational changes induced by protein-protein interactions [Banham et al. 2006].

A DEER measurement is made by exciting one spin population with microwave radiation and observing the effect on the two-pulse spin echo amplitude of a second spin population. A change in the echo amplitude occurs because the excitation of one spin
population affects the local magnetic field of the other population. The timing of excitation is varied which causes a modulation of the observed echo [Fajer et al. 2007]. Figure 6.4 shows the pulse sequence of a 4-pulse DEER measurement.

\[ \frac{\pi}{2} \quad \pi \quad \pi \]

\[ \tau_1 \quad \tau_1 \quad \tau_2 \quad \tau_2 \]

\[ v_{\text{observe}} \]

\[ v_{\text{pump}} \]

**Figure 6.4** Four-pulse DEER pulse sequence. At the observer frequency \( (v_{\text{observe}}) \) a \( \frac{\pi}{2} \) pulse is followed by a \( \pi \) pulse to give a Hahn echo (dashed green) which is then refocused with another \( \pi \) pulse. At a second frequency \( (v_{\text{pump}}) \), another group of spins is excited by a \( \pi \) pulse at a time \( t \) (red arrow) after the first observer \( \pi \) pulse. Time \( t \) is sequentially stepped while \( \tau_1, \tau_2 \) and \( \tau_2 \) (blue arrows) are constant. Stepping the pump pulse causes a modulation in the amplitude of the refocused echo (solid green). This modulation is measured by recording the echo intensity in a constant gate (g).
Dipole-dipole coupling between two spins is the basis for distance determination in DEER measurements. The electron-electron coupling, $\omega_{ee}$, between two unpaired spins can be expressed as the sum of the dipolar coupling ($\omega_{dd}$) and exchange coupling ($J$):

$$\omega_{ee} = \omega_{dd}(3\cos^2\theta - 1) + J \quad (11)$$

where $\theta$ is the angle between the interspin vector and the external magnetic field. Dipolar coupling is proportional to the inverse cube of the distance between the two spins ($r$) as shown by the equation:

$$\omega_{dd} = \frac{g_A g_B \beta^2 \mu_0}{4\pi h} \frac{1}{r^3} \quad (12)$$

where $g_A$ and $g_B$ are the g values of the two electrons, $\beta$ is the Bohr magneton, $\mu_0$ is the permeability of free space, $h$ is the Planck constant. DEER can access a range of interspin distances from 1.5 to 8 nm [Berliner et al. 2000, Chpt. 11]. At short interspin distances electron-electron coupling becomes large, therefore the lower limit for DEER measurements is the point at which excitation bandwidth is less than the electron-electron coupling. Theoretically, the upper limit of measurable interspin distance is determined by the lifetime of the transverse magnetization of electron spins (spin-spin relaxation time) and intermolecular spin-spin interactions.

In most cases DEER measurements are made between two artificial spin labels, such as MTSL, covalently bonded to cysteine residues that have been added in desired
locations by site-directed mutagenesis [Hubbell et al. 2003]. A limitation to distance measurements between two spin labels is that both spin labels can adopt multiple conformations, which can make the interpretation of the DEER data challenging. Also adding two non-native cysteines to a protein can have a greater effect on its structure and biological activity than a single mutation. Utilizing a tightly bound, natural paramagnetic cofactor in place of a spin label can lower the uncertainty that arises from the rotational freedom of a spin label. Previously DEER has been used to determine a distance of 26 Å between flavin radicals in augmenter liver regeneration (ALR) dimers [Kay et al. 2006]. It has also been used to study complex formation in *E. coli* ribonucleotide reductase by measuring the distance between a tyrosyl radical on the R2 subunit and a radical formed by the inhibitor 2’-azido-2’-deoxyuridine-5’-diphosphate in the active site of the R1 subunit [Bennati et al. 2005]. The work of Borovykh et al. on the photosynthetic reaction center of *Rhodobacter sphaeroides* is the only known example of DEER measurements between a spin label and a tightly bound, natural organic cofactor; the photochemically generated anionic semiquinone of QA [Borovykh et al. 2006]. In the present study DEER was used to measure the distance between a spin label and an enzymatically reduced flavin adenine dinucleotide (FAD) cofactor in electron transfer flavoprotein (ETF) from *Paracoccus denitrificans*. Enzymatic formation of the anionic semiquinone makes the method applicable to a larger number of proteins than the photochemical radical generation used by Borovykh et al.

DEER measurements are most commonly performed at X-band (~9 GHz) because of the commercial availability of X-band EPR instrumentation. It was recently reported that DEER measurements at Q-band (34 GHz) give a 13-fold signal enhancement
compared to measurements at X-band [Ghimire et al. 2009]. X- and Q-band DEER measurements of enzymatically reduced, spin-labeled ETF mutants were performed to compare the results obtained at the two frequencies and to determine if a signal enhancement could be achieved at Q-band for the ETF samples.

6.5 Selection of ETF Mutation Sites

Spin labeling sites in solvent-exposed, alpha helical regions of the protein were selected. Sites in alpha helical regions were chosen because it has been shown that site-directed spin labeling on alpha-helical regions of a protein has a negligible impact on structure [Hubbell et al. 1998; Altenbach et al. 2001]. Sites located in relatively solvent exposed regions were chosen because they can be labeled without unfolding the protein, which can cause the loss of the FAD cofactor in ETF.

Conformational changes in ETF are purposed to involve motion of domain II relative to domains I and III. Sites in all three domains were selected to characterize distances relative to the FAD, whose position is well-known from the crystal structure. Spin labeling sites are shown in Figure 6.5: A43 and G82 in domain I, A210 in domain II, and A111 in domain III. Positions A43C and A111C were chosen because nitroxyl-FAD distance distributions obtained at these sites are predicted to change relative to protein conformation. Specifically, when going from the inactive conformation to the active one, interspin distances are expected to become longer. As a control the site A210C was chosen. Located in domain II with the FAD, the nitroxyl-FAD distance will remain constant during the proposed conformational changes. As another control the G82C site was chosen. During the proposed conformational changes, nitroxyl-nitroxyl distances
between labels at G82C and A111C will not change but distances between label at G82C and A210C will. Distances between the FAD semiquinone and the 3 nitroxyl NO groups are in the range of 27 to 41 Å (from the crystal structure), which are well within the range for DEER measurements [Berliner et al. 2000, Chpt. 11].

**Figure 6.5** Crystal structure of *P. denitrificans* ETF (PDB id: 1EFP). The α subunit is shown in light blue and the β subunit is shown in grey. Proposed sites for spin labels are shown in green. Addition of the MTSL spin labels to the crystal structure was made using Insight II (Accelrys) software. Graphic representation and estimation of inter-spin distances were made using the program RasTop 2.2 (http://www.geneinfinity.org/rastop/, accessed on 4/12/10).
Chapter 7. Experimental Methods for DEER Measurements on ETF

7.1 Preparation of ETF Mutants

The *P. denitrificans* ETF genes [Bedzyk et al. 1993] were subcloned into the pET-28a(+) expression vector at the XbaI/HindIII restriction site. Site-directed mutagenesis was carried out using the Stratagene QuikChange® II XL site-directed mutagenesis kit according to the manufacturer’s instructions. Mutated plasmids were transformed into XL 10-Gold® Ultracompetent E. coli cells (detailed procedure can be found in chapter 3, page 20 of this dissertation). DNA sequencing to confirm mutations, production of glycerol cell stocks for long term storage at -80 °C and western blots confirming expression were done in similar fashion as in the work on ETF-QO in Chapter 3 of this dissertation, except the cell stocks were made using XL 10-Gold® cells.

7.2 Purification and Spin Labeling of ETF Mutants

7.2.1 Cell Growth and Expression of ETF

Starting cultures of 25 mL LB media, containing 30 μg/mL kanamycin monosulfate, were inoculated using frozen cell stocks and were shaken at 250 rpm overnight (16 hours) at 37 °C. Large-scale growths were started by the addition of approximately 2 mL starter culture to each of 12 flasks containing 700 mL of TYP media with 30 μg/mL kanamycin and 2 μM riboflavin. Flasks were incubated at 30 °C and
250 rpm until the optical density at 600 nm (light scattering) reached between 0.6 and 1 (approximately 5 hours). ETF expression was induced by the addition of IPTG to a final concentration of 0.5 mM in each flask. After the addition of IPTG incubation continued at 30 °C and 250 rpm until stationary phase was reached (OD₆₀₀ ~8).

Cells were harvested by centrifugation at a RCF of 7,900 x g, 4 °C for 10 minutes in a JLA 10.500 rotor on a Beckman Avanti J-25 centrifuge. Harvested cells were washed once with phosphate buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄, pH 7.4) solution and centrifuged again at 7,900 x g, 4 °C for 10 minutes and then weighed and frozen at -80 °C. The 8.4 L cultures yield approximately 80 grams of cells.

7.2.2 Cell Lysis

Harvested cells were subjected to 3 freeze-thaw cycles between -80 °C and room temperature. After thawing for the third time cells were suspended in buffer containing 10 mM potassium phosphate, pH 6.8 with 10% ethylene glycol, 0.1 mM ethylenediaminetetraacetic acid (EDTA) and 0.1 mM DTT at 3 times buffer volume to cell weight and were homogenized. The following protease inhibitors were added to the cell suspension: phenylmethanesulphonylfluoride (PMSF, 1 mM), pepstatin A (1 μM), tosyl-L-lysine chloromethylketone (TLCK, 50 μM), aprotinin (0.3 μM), and leupeptin (1 μM). Approximately 10 mg of DNase I was added to the homogenized solution and the cells were broken by two passes through a French pressure cell at 1000 psi. The broken cell suspension was placed in a Beckman Ti45 rotor and spun at 100,000 x g for 90 minutes at 4 °C in a Beckman L8-70M ultracentrifuge.
The cell membrane pellet was discarded and the supernatant containing ETF was retained for purification.

### 7.2.3 ETF Purification

Supernatant from cell lysis was loaded at 2 mL/min onto a 300 mL (5 cm x 15 cm) diethylaminoethyl (DEAE) Sepharose column (Sigma-Aldrich) that had been previously equilibrated with approximately 3 L of 10 mM potassium phosphate, pH 6.8 with 10% ethylene glycol, 0.1 mM EDTA and 0.1 mM DTT. The column was then washed with approximately 600 mL of 10 mM potassium phosphate, pH 6.8 with 10% ethylene glycol, 0.1 mM EDTA, 0.1 mM DTT, and protease inhibitors. Protein was eluted from the DEAE Sepharose column by a 2400 mL linear gradient of 10 to 150 mM NaCl in the same potassium phosphate buffer as above. The gradient was run at a flow rate of 1.66 mL/min and 10 minute fractions were collected using a Teledyne Isco Foxy 200 automatic fraction collector. Fractions showing the characteristic visible spectrum of ETF (Simadzu UV-2401 PC) were pooled and concentrated to a volume of approximately 200 mL using an Amicon stirred ultrafiltration cell and a PM30 ultrafiltration membrane (Millipore, Billerica, MA) under N₂ at a pressure of approximately 12 psi. The concentrated sample was then dialyzed against two, 2 L volumes of 10 mM potassium phosphate, pH 6.8 with 10% ethylene glycol and 0.1 mM DTT.

Next the protein was loaded onto a 250 mL (5 cm x 12.5 cm) Q Sepharose (GE Healthcare) column that had been previously equilibrated with approximately 2.5 L of 10 mM potassium phosphate, pH 6.8 with 10% ethylene glycol and 0.1 mM DTT. The column was then washed with 600 mL of the same buffer. Protein was eluted from
the Q Sepharose column using a 2 L linear gradient of 10 to 150 mM potassium phosphate, pH 6.8 with 10% ethylene glycol and 0.1 mM DTT. The gradient was run at a flow rate of 1.38 mL/min and 10 minute fractions were collected. Fractions showing the characteristic UV-visible spectrum of ETF were pooled and dialyzed against three, 2 L volumes of 20 mM BisTris/Cl pH 6.5, containing 10% ethylene glycol and 0.1 mM DTT.

In the final step of purification, the protein was loaded onto an 80 mL (2.5 cm x 16 cm) ω-aminooctyl-agarose column (Sigma-Aldrich) that had been previously equilibrated with approximately 1 L of 20 mM BisTris/Cl pH 6.5, with 10% ethylene glycol and 0.1 mM DTT and the column was then washed with about 400 mL of the BisTris/Cl buffer. ETF was eluted from the ω-aminooctyl-agarose column using a 1 L linear gradient of 10 to 200 mM KCl in 20 mM BisTris/Cl pH 6.5, containing 10% ethylene glycol and 0.1 mM DTT. The gradient was run at a flow rate of 0.69 mL/min and 10 minute fractions were collected. ETF eluted from the column at 50 – 60 mM KCl. Fractions containing ETF were pooled and concentrated using an Amicon stirred ultrafiltration cell and a PM30 ultrafiltration membrane. ETF was then dialyzed against three, 1 L volumes of 10 mM potassium phosphate, pH 7 with 10% ethylene glycol and 0.1 mM DTT. ETF purity was confirmed by determining the ratio of the absorbance at 270 nm to the absorbance at 436 nm and comparing it to the literature value of 5.8 for pure *P. denitrificans* ETF [Watmough et al. 1992]. SDS-PAGE gels were performed on purified ETF mutants to confirm that both subunits were present. The procedure for SDS-PAGE gels was the same used on page 26 of this dissertation.
7.2.4 Spin-Labeling of ETF

DTT, which was added to prevent the formation of intermolecular disulfide bonds between mutated cysteine residues, can react with the sulfhydryl group of cysteine specific spin labels. It is therefore necessary to remove DTT before spin-labeling reactions are performed. To remove DTT, purified ETF was separated into 10 mL fractions and the fractions were chromatographed on a HiPrep™ 26/10 desalting column using an ÄKTA FPLC system under the control of UNICORN 3.21 software (GE Healthcare). The system was run using 10 mM potassium phosphate, pH 7 with 10% ethylene glycol at a flow rate of 10 mL/min and 5 mL fractions were collected after the addition of ETF to the column. Immediately after exit from the column, fractions containing ETF were combined and a 5 times molar excess of the spin label MTSL was added. Spin labeling was done at 4 °C overnight with gentle shaking. Excess MTSL was removed the following morning by another pass through the HiPrep™ desalting column using identical operating conditions as in the removal of DTT except 10 % glycerol was used in place of 10 % ethylene glycol in the buffer. Spin-labeled ETF was then pooled and concentrated to a concentration of approximately 100 μM using an Amicon stirred ultrafiltration cell and a PM30 ultrafiltration membrane. Final concentration was determined using the absorbance at 436 nm (ε_{436} = 13.6 mM^{-1}cm^{-1}) on a Simadzu UV-2401 PC spectrophotometer. Spin-labeled ETF was separated into 1 mL aliquots, snap frozen in liquid nitrogen and stored at -80 °C until experiments were performed.
7.3 Expression and Purification of *P. denitrificans* Glutaryl Co-A Dehydrogenase

*Paracoccus denitrificans* (ATCC 13543) was grown in mineral salts medium (Appendix 2) containing 2% glutaric acid as the carbon source at 30 °C [Husain and Steenkamp 1985]. Cells were harvested late during the exponential phase and then broken using the procedures detailed in sections 7.2.1 and 7.2.2. Supernatant from cell lysis was loaded onto a 300 mL (5 cm x 15 cm) DEAE Sepharose column (Sigma-Aldrich) that had been previously equilibrated with 10 mM potassium phosphate, pH 6.5 with 10% ethylene glycol, 0.1 mM EDTA and 0.1 mM PMSF. The column was then washed with approximately 600 mL of the equilibration buffer containing 50 mM NaCl. Protein was eluted from the DEAE Sepharose column by a 2400 mL linear gradient of 50 to 400 mM NaCl in the same potassium phosphate buffer as above. Fractions were tested for glutaryl-CoA dehydrogenase activity using 10 μM glutaryl-CoA, 1.33 mM phenazine methosulfate and 57 μM 2,6-dichlorophenol-indophenol (DCPIP) as the terminal electron acceptor in 100 mM potassium phosphate at pH 7.5. Active fractions were pooled, concentrated by ultrafiltration (Millipore YM100 membrane) and dialyzed against 10 mM potassium phosphate, pH 6.5 with 10% ethylene glycol. Next the protein was loaded onto a 100 mL (2.5 cm x 20 cm) hydroxyapatite (HA) column that had been previously equilibrated with 10 mM potassium phosphate, pH 6.5 with 10% ethylene glycol. The HA column was washed with 500 mL of 50 mM potassium phosphate, pH 6.5 with 10% ethylene glycol. Protein was eluted from the HA column using a 1300 mL linear gradient of 50 to 200 mM potassium phosphate, pH 6.5 with 10% ethylene glycol. Fractions showing the characteristic UV-visible spectrum of glutaryl-CoA dehydrogenase were pooled and
dialyzed against 10 mM potassium phosphate, pH 7 containing 10 % glycerol. Purified glutaryl-CoA dehydrogenase was stored at -80 °C.

7.4 ETF Activity Assay

The activities of spin-labeled ETF mutants were measured relative to wild-type in a coupled reaction containing 0.1 M potassium phosphate buffer, pH 7.5, 0.4 µM *P. denitrificans* glutaryl-CoA dehydrogenase, 10 µM glutaryl CoA and 57 µM DCPIP at 25°C. DCPIP reduction was initiated by the addition of ETF, giving a final concentration of approximately 10 nM. Reduction of DCPIP was monitored by the decrease of absorbance at 600nm (DCPIP, $\varepsilon = 20 \text{ mM}^{-1} \text{ cm}^{-1}$) as a function of time. Baselines were measured for 40 seconds before the addition of ETF. Absorbance versus time data was recorded for an additional 140 seconds after the addition of ETF and slopes were calculated for these linear regions. Activities were determined by dividing the absolute value of these slopes by the product of the extinction coefficient of DCPIP ($\varepsilon_{600} = 20 \text{ mM}^{-1} \text{ cm}^{-1}$) and the working concentration of ETF in the assay (approximately 10 nM).

7.5 Enzymatic Reduction of Spin-Labeled ETF Mutants

Spin-labeled ETF mutant aliquots were thawed and dialyzed into 20 mM tris-HCl pH 8.0, with 10 % glycerol. A pH of 8.0 was used to stabilize the formation of anionic FAD semiquinone. Reduction to FAD SQ$^-$ was carried out in stoppered cuvettes containing between 350 and 800 µL of approximately 100 µM ETF, 20 mM glucose and 800 µM glutaryl-CoA. These mixtures were then made anaerobic by 10 cycles of
evacuation and purging with helium gas. Residual oxygen was removed by addition of 15 units of glucose oxidase and 90 units of catalase; reaction mixtures were preincubated for 10 minutes at room temperature. The absorption spectra of the fully oxidized spin-labeled ETF mutants were then recorded. To initiate the reduction of ETF, glutaryl-CoA dehydrogenase was added to the cuvette at a final concentration of about 5 nM. Reduction to FAD SQ$^-$ was followed by monitoring the increase in absorbance at 375 nm due to formation of FAD SQ$^-$. After the addition of dehydrogenase, spectra were recorded every 5 minutes on a Thermo Scientific Evolution 300 or a Varian Cary 300 spectrometer.

When absorbance at 375 nm reached a maximum, samples (~300 μL) were immediately transferred anaerobically to 4.0 mm o.d. quartz EPR tubes (X-band measurements) using nitrogen gas and a Teflon transfer line. To remove oxygen, nitrogen gas was allowed to flow into the empty EPR tube for several minutes before the transfer. For Q-band measurements, reduced ETF samples were transferred to 1.0 mm o.d. quartz EPR tubes using a gastight syringe inside a glove bag under constant N$_2$ flow. Samples were frozen in liquid nitrogen immediately after transfer and were subjected to 5 cycles of alternate evacuation and purging with helium gas. Samples were then flame sealed and stored in liquid nitrogen until spectroscopic measurements were performed.

7.6 EPR Spectroscopy of Spin-Labeled ETF Mutants

7.6.1 Continuous Wave EPR Spectroscopy

CW EPR spectra were used to confirm the incorporation of the MTSL spin label into single cysteine ETF mutants. Spectra were recorded at 100 K and 293 K using a
Varian E109 spectrometer equipped with a GaAsFET amplifier, a TE\(_{102}\) rectangular cavity and a Varian liquid-nitrogen-cooled gas flow system. Spectra of ETF samples in 1.0 mm ID capillaries at 293 K were recorded using the following operating conditions; 9.23 GHz microwave frequency, 1.0 G modulation amplitude at 100 kHz modulation, a gain of 2000, 0.5 mW microwave power, 0.064 second time constant, 100 G sweep width, and 20 scans of 2 minutes were averaged. Spectra recorded at 100 K used ETF samples of approximately 300 μL in 4.0 mm EPR tubes that had been evacuated and flame sealed. Operating conditions at 100 K were as follows; 9.23 GHz microwave frequency, 1.0 G modulation amplitude at 100 kHz modulation, a gain of 2000, 0.2 mW microwave power, 0.064 second time constant, 200 G sweep width, and five, 2 minute scans were averaged. MTSL concentration was calculated by double integration of the EPR spectra and compared to the double integral from EPR spectra of 0.5 and 0.8 mM tempol standards recorded under the same conditions.

7.6.2 DEER Measurements of Reduced Spin-Labeled ETF Mutants

X-band four-pulse DEER measurements at 60 and 80 K were performed on a Bruker ELEXSYS E580 spectrometer equipped with a Oxford CF 935 cryostat using an observer pulse of \(\pi/2 = 16\) ns. Delay times, \(\tau_1\) and \(\tau_2\), and the ELDOR \(\pi\) pulse were optimized for each sample. Delay times varied between 124 to 130 ns and 2300 to 3200 ns for \(\tau_1\) and \(\tau_2\) respectively. ELDOR pulse lengths varied from 38 to 40 ns. The pump pulse was set to 9.424 GHz which corresponds to the maximum of the echo detected field swept spectrum. At this frequency the FAD SQ^- signal overlaps with the MTSL nitroxide signal. The observer pulse was off-set by 60 MHz down field of the
pump pulse (9.484 GHz). Data was recorded in 4 or 8 ns steps with 1024 shots at each time point. The repetition rate was set to 1.2 times the T₁ relaxation time of the samples. Multiple scans were averaged using 8 step phase correction. There was strong proton modulation in the raw data. The sharp peak in the Fourier transformed data corresponding to the proton modulation was manually removed, followed by reverse Fourier transformation before the DEER analysis was performed. DEER measurement of a 2.8 nm inter-spin distance standard sample (provided by Dr. Jeschke, ETH Zurich) using the same parameters was used to determine the zero time (t₀) of approximately 40 ns.

Q-band four-pulse DEER measurements at 60 and 80 K were performed on a Bruker ELEXSYS E580 spectrometer equipped with a Super Q-FT bridge, an E580-400U ELDOR unit, EN 5107D2 Q-band resonator and an Oxford CF 935 cryostat. Pulse times were optimized for each sample and ranged from 40 to 44 ns for the π/2 probe pulse and 60 to 72 ns for the ELDOR π pulse. Delay times were set at 200 ns and 3000 ns for τ₁ and τ₂ respectively. The observer frequency was set to 33.960 GHz which corresponds to the maximum of the echo detected field swept spectrum. The pump pulse was off-set by 50 MHz up field of the pump pulse (33.910 GHz) and t was varied from 40 to 3000 ns in 8 ns steps. At each step point, 100 shots were done using a shot retention time of 1 ms. Multiple scans were averaged using 8 step phase correction.

DEER spectra were analyzed using the program DeerAnalysis2008 developed by Jeschke [Jeschke 2006]. To separate the long distance intermolecular interactions from the intramolecular interaction of interest, a background correction using a three-dimensional homogeneous exponential decay model (equation 13) was used.
\[ B(t) = e^{-kt^{d/3}} \]  

(13)

In the model \( d \) is the dimensionality of the distribution, which was set to 3 and \( k \) is an expression of the spin density. The \( t_0 \) and the beginning and ending points of the background subtraction were varied for each sample to give the lowest sum of squares error.

Data were fit to distances between 2.0 and 7.0 nm using Tikhonov regularization, which gave better fits than using single or double Gaussian models [Chiang et al. 2005]. In Tikhonov regularization, optimum distance distributions \( P(r) \) are found by minimizing the sum of the functions \( \rho(\alpha) \) and \( \eta(\alpha) \), where \( \rho(\alpha) \) is the mean square deviation between the simulated and experimental time domain data and \( \eta(\alpha) \) is the square norm of the second derivative of \( P(r) \) multiplied by the regularization parameter \( \alpha \). This procedure is done for a predetermined set of \( \alpha \) values. The set of \( \alpha \) values used in fitting the experimental DEER data were: 0.001, 0.01, 0.1, 1, 10, 100, 1000, 10000, and 100000. The function \( \rho(\alpha) \) represents the error associated with the fit of the data for a given \( P(r) \) and \( \eta(\alpha) \) is a measure of smoothness of \( P(r) \). The goal of Tikhonov regularization is to find the optimum \( \alpha \) value where a compromise between \( \rho(\alpha) \) and \( \eta(\alpha) \) is reached. The optimum \( \alpha \) is determined by using a plot of log \( \eta(\alpha) \) versus log \( \rho(\alpha) \), which is known as the L curve because of its shape. Small \( \alpha \) values are responsible for the left side of the plot where the slope is steep and negative. As \( \alpha \) is increased in this region, the value of \( \eta(\alpha) \) decreases rapidly while the value of \( \rho(\alpha) \) slightly increases. Large \( \alpha \) values are responsible for the right side of the plot where the slope is much smaller. For increasing
values in this region, the value of $\eta(\alpha)$ decreases slightly but the value of $\rho(\alpha)$ increases rapidly. For data with good signal-to-noise, the optimum $\alpha$ is located at the point of maximum curvature or corner of the “L” [Chiang et al. 2005]. At this position, changes in both $\eta(\alpha)$ and $\rho(\alpha)$ are small with respect to $\alpha$, which is a good compromise between undersmoothing and oversmoothing of the distance distribution function $P(r)$.

7.7 Modeling of ETF Structures

Crystal structures of both human and *P. denitrificans* ETF have been reported, presumably in the closed conformation (pdb id’s: 1EFV and 1EFP respectively). The structure of human ETF in an open conformation is also available as the ETF:MCAD complex (pdb id: 2A1T). Currently there is no crystal structure of *P. denitrificans* in an open conformation, so it was necessary to create a model to interpret the results from DEER experiments. A homology model of *P. denitrificans* ETF was created using the program ESyPred3D [Lambert et al. 2002]. The crystal structure of human ETF in the electron transfer active conformation from the ETF:MCAD complex was used as a template. Using the program RasTop, distances between amino acids in the homology model and in the crystal structure of *P. denitrificans* ETF were compared to estimate the change in the spin label to FAD distance when the $\alpha$II domain of ETF changes conformation to facilitate electron transfer to a redox partner.
Chapter 8. Results of DEER Distance Measurements of ETF

8.1 Characterization of ETF Mutants

*Paracoccus denitrificans* ETF purity was measured by calculating the ratio of the absorbance at 270 nm to the absorbance at 436 nm. The ratios obtained for ETF mutants were all approximately 6.5 ± 0.3, which was similar to the literature value of 5.8 for pure *P. denitrificans* ETF [Watmough et al. 1992]. SDS-PAGE was used to confirm the purity of the ETF mutants. Figure 8.1 shows the results of the SDS-PAGE of the βA111C mutant. Two bands, one near 26 kD and the other near 30 kD, are seen. These correspond to the α- (30.5 kD) and β- (26.6 kD) subunits of ETF. SDS-PAGE of other ETF mutants gave similar results.

![SDS-PAGE of purified P. denitrificans A111C+E162A ETF. Lane one is molecular weight standards (Bio-Rad Precision Plus Dual Color) with the masses in kilodaltons. Lane 2 is approximately 5 μg of purified A111C+E162A ETF. The gel confirms the presence of both the α- and β-subunits (30.5 and 26.6 kD respectively). SDS-polyacrylamide gel electrophoresis of the other mutants gave similar results.](image)

Figure 8.1 SDS-PAGE of purified *P. denitrificans* A111C+E162A ETF. Lane one is molecular weight standards (Bio-Rad Precision Plus Dual Color) with the masses in kilodaltons. Lane 2 is approximately 5 μg of purified A111C+E162A ETF. The gel confirms the presence of both the α- and β-subunits (30.5 and 26.6 kD respectively). SDS-polyacrylamide gel electrophoresis of the other mutants gave similar results.
Figure 8.2A shows the optical spectrum of oxidized (as isolated) wild-type *P. denitrificans* ETF. The spectrum contains two peaks at 375 and 436 nm and a shoulder near 450 nm. These optical bands are due to FAD $\pi \rightarrow \pi^*$ transitions, as well as a mixing of $n \rightarrow \pi^*$ for the band at 375 nm [Auer and Frereman 1980]. Similar spectra are seen in the A111C (Figure 8.2B), A210C (Figure 8.2C) and A43C mutants. An extinction coefficient (ε) at 436 nm of 13.6 mM$^{-1}$cm$^{-1}$ was used to calculate values of $\varepsilon_{375nm}$ between 12.1 and 12.2 mM$^{-1}$cm$^{-1}$ in the single cysteine mutants. These values are similar to the literature value of 12.0 mM$^{-1}$cm$^{-1}$ for wild-type ETF [Watmough et al. 1992]. As expected the spin label mutation sites do not change the FAD environment. However, the maximum absorbance peak in the A111C+E162A mutant is shifted from 436 nm to 431 nm (Figure 8.2D). The relative absorbance at 375 nm in the A111C+E162A mutant is also lower (~10.9 mM$^{-1}$cm$^{-1}$) compared to the other mutants and wild-type ETF. This suggests that the FAD environment is altered by the E162A mutation presumably through changes in solvent exposure. The $\pi \rightarrow \pi^*$ transition that gives rise to the band at 436 nm responds to changes in solvation by shifts in position [Auer and Frereman 1980]. At pH 8, the absorbance at 500 nm is also much higher for the A111C+E162A mutant suggesting the possibility of the protein denaturing. No denaturing is believed to have taken place because there was no noticeable precipitate and the absorbance at 350 nm did not increase. A color change from yellow to orange was seen when the A111C+E162A mutant was dialyzed against 20 mM Tris, pH 8, indicating that the optical spectrum of the A111C+E162A mutant is be pH dependent.
Figure 8.2  UV-visible spectra of oxidized wild-type (A), A111C (B), A210C (C), and A111C+E162A (D) *P. denitrificans* ETF. Spectra are normalized to an extinction coefficient of 13.6 mM$^{-1}$cm$^{-1}$ for the absorption peak at 436 nm. The A43C mutant gave similar results as in B and C. Spectra at pH 7 (−−−) and pH 8 (-----) are shown for the A111C+E162A mutant (D). No pH-dependent spectral changes are observed for the other mutants.

8.2 Spin Labeling of ETF Mutants

Wild-type *P. denitrificans* ETF contains a single native cysteine residue (C36 of the α-subunit). Prolonged exposure to 5-fold excess MTSL has demonstrated that there is no spin labeling of the native αC36 residue. This residue does not interfere with labeling at cysteines introduced by site-directed mutagenesis.

ETF single cysteine mutants showed characteristic partially immobilized nitroxyl CW EPR spectra after exposure to 5 fold excess MTSL confirming attachment of the spin
label to the protein (Figure 8.3). The absence of sharp lines in the CW spectra confirms that the removal of excess MTSL after the spin labeling reaction was successful. Comparison of normalized integrals of the CW EPR signal of spin-labeled ETF mutants with tempol samples of known concentrations shows that the spin label is incorporated stoichiometrically into the enzyme. Multiple $g_x$ and $g_z$ lines would be expected if multiple conformations of the MTSL spin label were present in the singly labeled ETF mutants [Columbus et al. 2001]. Room temperature CW EPR spectra show no evidence of multiple spin label conformations (Figure 8.3).

**Figure 8.3** CW EPR spectra of spin-labeled *P. denitrificans* ETF mutants at 275 K. Spectra were recorded using 5 mW power, 1 G modulation amplitude, a gain of 2000 and a sweep width of 100 G. The spectrum of the A111C+E162A mutant was recorded on a Bruker EMX spectrometer at a frequency of 9.426 GHz which gave an increased signal to noise ratio compared to the Varian E109 spectrometer that was used to record spectra of the other mutants.
8.3 ETF Activity Assays

The activity assay of spin-labeled ETF mutants measures the rate at which electrons are shuttled through ETF from its biological electron donor, glutaryl-CoA dehydrogenase, to the electron acceptor, DCPIP. Absorbance (600 nm) versus time data were used to calculate rates of DCPIP reduction (Figure 8.4). The slopes of the initial, linear regions were divided by the product of the extinction coefficient of DCPIP ($\varepsilon_{600} = 20 \text{ mM}^{-1} \text{ cm}^{-1}$) and the working concentration of ETF in the assay. Rates from 5 trials were averaged and the standard deviations were used as an estimate of the experimental error (Table 8.1).

![Figure 8.4](image)

**Figure 8.4** The time dependence of DCPIP reduction in the activity assays of spin-labeled A111C and A111C+E162A ETF mutants compared with wild-type enzyme. Linear fits of the data are shown in red. The slopes obtained for the A210C and A43C mutants were similar to wild type.
Table 8.1  DCPIP reduction rate values (min\(^{-1}\)) for trials of spin-labeled A111C, A111C+E162A, A210C and A43C ETF mutants as well as the wild-type enzyme. Average values and standard deviations are shown at the bottom.

<table>
<thead>
<tr>
<th>Trial</th>
<th>Wild Type</th>
<th>A111C</th>
<th>A111C+E162A</th>
<th>A210C</th>
<th>A43C</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>38.1</td>
<td>36.5</td>
<td>95.1</td>
<td>39.6</td>
<td>30.3</td>
</tr>
<tr>
<td>2</td>
<td>39.6</td>
<td>37.1</td>
<td>86.7</td>
<td>43.2</td>
<td>32.1</td>
</tr>
<tr>
<td>3</td>
<td>40.2</td>
<td>37.4</td>
<td>86.7</td>
<td>47.7</td>
<td>31.5</td>
</tr>
<tr>
<td>4</td>
<td>43.8</td>
<td>39.3</td>
<td>88.5</td>
<td>43.5</td>
<td>31.5</td>
</tr>
<tr>
<td>5</td>
<td>41.4</td>
<td>38.7</td>
<td>95.7</td>
<td>44.4</td>
<td>33.0</td>
</tr>
<tr>
<td>Average</td>
<td>40.6</td>
<td>37.8</td>
<td>90.5</td>
<td>43.7</td>
<td>31.7</td>
</tr>
<tr>
<td>Standard Deviation</td>
<td>2.1</td>
<td>1.1</td>
<td>4.5</td>
<td>2.9</td>
<td>1.0</td>
</tr>
</tbody>
</table>

Relative rates of DCPIP reduction, reported as percents of wild type, for spin-labeled A111C, A111C+E162A, A210C and A43C ETF are shown in Figure 8.5. The A111C and A210C spin-labeled mutants have DCPIP reduction rates similar to wild type (93 ± 3% and 107 ± 7%, respectively). Spin-labeled A43C ETF has a rate that is slightly lower than wild type (78 ± 2%). A spin label at this site is not expected to interfere with the FAD or recognition loop so the lower rate was surprising. The DCPIP reduction rate of the spin-labeled A111C+E162A mutant is more than two times that of the wild-type enzyme (223 ± 11%). The increased rate observed in the A111C+E162A mutant could be due to an increased interaction with glutaryl-CoA dehydrogenase or an increased ability to transfer electrons to DCPIP.
Figure 8.5  Relative DCPIP reduction rates of spin-labeled ETF mutants shown as percents of wild type. Error bars represent the standard deviation of five trials.

8.4  Enzymatic Reduction of Spin-Labeled ETF Mutants

Spin-labeled ETF mutants were enzymatically reduced under anaerobic conditions at pH 8.0 to form FAD SQ$^-$ using a coupled reaction with glutaryl-CoA and glutaryl-CoA dehydrogenase [Swanson et al. 2009]. Formation of FAD SQ$^-$ causes an increase in absorbance at 375 nm and a decrease in absorbance at 436 nm (Figure 8.6). FAD SQ$^-$ formation was followed by monitoring the increase in absorbance at 375 nm. The absorbance at 375 nm can be used to measure FAD SQ$^-$ because it increases as the semiquinone is formed but decreases upon formation of the hydroquinone. The absorbance at 436 nm is not a good measure of FAD SQ$^-$ formation because it continues to decrease as FAD QH$_2$ is formed. For 100% formation of FAD SQ$^-$, $\varepsilon_{375}$ is
17 mM$^{-1}$cm$^{-1}$ [Watmough et al. 1992]. This value was used to calculate the expected change in absorbance at 375 nm for 100% conversion to FAD SQ$^{-}$ before the enzymatic reduction was initiated. Using the change in absorbance and comparing it to the expected change, the percentage of FAD SQ$^{-}$ was calculated. Between 50 and 60% of the ETF flavin was reduced to FAD SQ$^{-}$ before a decrease in absorbance at 375 nm indicated disproportionation by the dehydrogenase/enoyl-CoA complex [Gorelick et al. 1985]. Optimal conditions for the enzymatic reduction of ETF were found by varying the pH, CoA concentration and the dehydrogenase used (Table 8.2).

![Figure 8.6](image)

**Figure 8.6** Time dependence of the visible spectra during enzymatic reduction of wild-type *P. denitrificans* ETF. Upon formation of FAD SQ$^{-}$, the absorbance at 375 nm increases and the absorbance at 436 nm decreases (arrows). Spectra were recorded of oxidized ETF (black) and for 20 (red), 45 (blue), 60 (green), 70 (magenta) and 110 minutes (gray) after the addition of dehydrogenase. Similar results were obtained for the A111C, A210C and A43C ETF mutants.
Table 8.2  Optimization of the enzymatic reduction of ETF. Reduction of ETF was performed using medium chain acyl-CoA dehydrogenase (MCAD) or glutaryl-CoA dehydrogenase (GDH). Butyryl CoA was used as the substrate for MCAD and glutaryl CoA was used as the substrate for GDH.

<table>
<thead>
<tr>
<th>Sample</th>
<th>[ETF] (μM)</th>
<th>[CoA] (μM)</th>
<th>[DH] (nM)</th>
<th>pH</th>
<th>Max SQ%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild Type</td>
<td>10</td>
<td>100</td>
<td>MCAD, 20</td>
<td>8.0</td>
<td>64</td>
</tr>
<tr>
<td>Wild Type</td>
<td>10</td>
<td>100</td>
<td>GDH, 5</td>
<td>8.0</td>
<td>70</td>
</tr>
<tr>
<td>A111C+MTSL</td>
<td>80</td>
<td>100</td>
<td>GDH, 20</td>
<td>7.5</td>
<td>41</td>
</tr>
<tr>
<td>A111C+MTSL</td>
<td>80</td>
<td>420</td>
<td>MCAD, 15</td>
<td>7.5</td>
<td>22</td>
</tr>
<tr>
<td>Wild Type</td>
<td>80</td>
<td>420</td>
<td>MCAD, 90</td>
<td>7.5</td>
<td>22</td>
</tr>
<tr>
<td>Wild Type</td>
<td>73</td>
<td>420</td>
<td>GDH, 5</td>
<td>8.0</td>
<td>55</td>
</tr>
<tr>
<td>Wild Type</td>
<td>78</td>
<td>800</td>
<td>GDH, 15</td>
<td>8.0</td>
<td>58</td>
</tr>
<tr>
<td>Wild Type</td>
<td>67</td>
<td>800</td>
<td>GDH, 10</td>
<td>8.5</td>
<td>50</td>
</tr>
<tr>
<td>A111C+MTSL</td>
<td>107</td>
<td>800</td>
<td>GDH, 5</td>
<td>8.0</td>
<td>51</td>
</tr>
</tbody>
</table>

Unlike the single cysteine mutants and wild type, an increase in absorbance at both 375 and 436 nm was seen during the enzymatic reduction of spin-labeled A111C+E162A ETF (Figure 8.7). The E162A mutation eliminates the negative charge of one glutamate residue. The change in the enzymatic reduction of ETF by replacing the negatively charged glutamate residue at position 162 of the α-subunit with an uncharged alanine suggests that this negative charge plays a role in the FAD environment.
Figure 8.7  Visible spectra of the enzymatic reduction of A111C+E162A ETF. Unlike the other mutants both peaks (375 and 436 nm) increased upon the addition of glutaryl Co-A dehydrogenase. Spectra were recorded for oxidized (black) A111C+E162A ETF and at 2 (red), 5 (blue), 10 (green), 16 (magenta) and 22 minutes (gray) after the addition of dehydrogenase.

CW EPR spectra at 100 K were used to confirm the formation of FAD SQ$. The difference between CW EPR spectra before and after enzymatic reduction of A111C ETF matches the spectrum of FAD SQ$ from unlabeled, reduced wild-type ETF [Figure 8.8]. Normalized integrals for EPR spectra of oxidized and reduced spin-labeled A111C ETF were compared to the normalized integral of an EPR spectrum of a tempol standard of known concentration. Comparison of these integrals showed that approximately 50 % of the FAD in spin-labeled A111C ETF was reduced to the semiquinone during the enzymatic reduction. This agreed with the value of 51 %, calculated by using the change in absorbance at 375 nm.
Figure 8.8  CW EPR spectra of as-isolated (black) and enzymatically reduced (red) spin-labeled A111C ETF. The difference between the two spectra is due to the formation of FAD SQ$^\cdot$ (green). The spectrum of FAD SQ$^\cdot$ in reduced wild-type ETF is shown for comparison (blue). Spectra were recorded at 100 K using 0.2 mW power, 1 G modulation amplitude, a gain of 2000 and a sweep width of 200 G.

8.5  DEER Measurements of Reduced, Spin-Labeled ETF Mutants

Four-pulse DEER measurements were performed on enzymatically reduced, spin-labeled ETF mutants at X- and Q-band. Field-swept echo-detected spectra at X- and Q-band, with the relative locations of the excitation and observing pulses used in the DEER experiments are shown in Figure 8.9. In DEER measurements the excitation frequency is usually set to correspond with the maximum echo in the field-swept spectrum to excite the largest number of spins [Jeschke et al. 2000; Fajer et al. 2007].
The X-band excitation frequency was set to the maximum echo intensity near \( g = 2.006 \). At this position the signals from the FAD SQ\(^-\) (\( g_{iso} = 2.0036 \) [Swanson et al. 2008]) and the perpendicular region of the MTSL spin label overlap. The X-band observe frequency was offset by approximately 60 MHz higher frequency (down field) which is close to \( g = 2.018 \). At this position there is no contribution from the FAD SQ\(^-\). Thus, the effects on the MTSL spin label of exciting the FAD SQ\(^-\) spins are seen with pulses at these locations.

![Field-swept echo-detected EPR spectra of enzymatically reduced spin-labeled A43C ETF at X- and Q-band. The approximate location of the pump and probe frequencies used in the DEER measurements are shown.](image)

**Figure 8.9** Field-swept echo-detected EPR spectra of enzymatically reduced spin-labeled A43C ETF at X- and Q-band. The approximate location of the pump and probe frequencies used in the DEER measurements are shown.

Initial Q-band DEER measurements were performed by pumping at the maximum echo in the field swept spectrum (\( g = 2.010 \)) and observing at approximately 50 MHz lower frequency (\( g = 2.006 \)). Pulses at these locations, unlike at X-band, excite the MTSL
and observe the effects on the MTSL and FAD SQ\(^{-}\). No modulations were seen in the DEER data with pulses at these positions in the spectrum. The locations of the excitation and observing pulses were then reversed so that the FAD SQ\(^{-}\) (and MTSL) were excited and only the MTSL was observed, as was the case in X-band measurements (Figure 8.9 right panel). Modulations were observed in the DEER data with pulses at these positions.

### 8.5.1 X-Band DEER Data

DEER data were analyzed using the DeerAnalysis2008 program [Jeschke et al. 2006]. Figure 8.10 shows X-band DEER traces at 60 K and the background corrections. For the A111C, A43C and A111C+E162A mutants, a three dimensional homogeneous background model was used. A 2\(^{nd}\) order polynomial background model was used for the A210C mutant because the three dimensional homogeneous model did not fit the data. The different background signal in the A210C data might have been due to the fact that the concentration of this sample was higher than for the other mutants.
Figure 8.10  X-band DEER data (black) and fitted background (red) for enzymatically reduced, spin-labeled ETF mutants. Vertical lines indicate the starting (---) and ending (-----) points for the background calculation. Background decays were fit to a three-dimensional homogenous spin distribution model (A111C, A43C and A111C+E162A) or a second degree polynomial function (A210C).

X-band DEER traces after background subtraction are shown in Figure 8.11. Data from the first mutant investigated (A111C) were fit using a single Gaussian model or Tikhonov regularization [Chiang et al. 2005]. A better fit was obtained using Tikhonov regularization than with a single Gaussian distribution centered at 4.3 nm (Figure 8.11 upper left panel). Therefore, Tikhonov regularization was used to fit data for all subsequent mutants.
Figure 8.11  X-band dipolar evolution signals for enzymatically reduced, spin-labeled ETF mutants obtained from the DEER data in Figure 8.10 by background subtraction. Tikhonov regularization fitting is shown in red. Single Gaussian fit of the A111C mutant is shown in yellow.

Fourier transformation of the X-band DEER traces in Figure 8.11 gives frequency domain dipolar evolution spectra having the characteristic Pake pattern (Figure 8.12). The shape of the frequency domain spectrum is dependent on the interval chosen in the raw data for background subtraction, therefore the shape of the frequency domain spectrum can be used as a measure of the quality of the background subtraction. The traces in Figure 8.12 do not have positive spikes (part of the background attributed to biradical) or holes (part of the biradical signal attributed to background) suggesting that the background subtractions used were appropriate [Jeschke 2006].
Figure 8.12  X-band DEER frequency domain data for enzymatically reduced, spin-labeled ETF mutants by Fourier transformation of the data in Figure 8.11. Tikhonov regularization fitting is shown in red.

Interspin distance distributions obtained by Tikonov regularization fitting of the X-band DEER data are shown in Figure 8.13 (where $P(r)$ is the probability). Two or three distributions of FAD-nitroxyl distances are found for spin-labels located in domains I and III of ETF (the A43C and A111C sites, respectively). The A43C mutant gave two distributions of distances: a major component centered at $4.3 \pm 0.05$ nm with a width of $0.34 \pm 0.15$ nm and a minor component centered at $3.8 \pm 0.05$ nm with a width of $0.23 \pm 0.1$ nm (Figure 8.13 upper right panel). Three distributions of distances were seen for the A111C mutant: a major component centered at $4.2 \pm 0.1$ nm, a minor component
centered at 5.1 ± 0.2 nm and a very small component centered at 3.7 nm (Figure 8.13 upper left panel). The two larger components of the A111C distance distribution had widths of approximately 0.3 ± 0.25 nm at half height and the 3.7 nm component had a width of 0.16 nm. Error estimates were not made for the 3.7 nm component because of its significantly smaller magnitude. The A111C+E162A mutant gave three distributions that were similar to the A111C mutant: a major component centered at 4.3 ± 0.05 nm with a width of 0.47 ± 0.15 nm and a minor component centered at 5.1 ± 0.1 nm with a width of 0.36 ± 0.1 nm and another minor component centered at 3.6 ± 0.1 nm with a width of 0.32 ± 0.15 nm (Figure 8.13 lower right panel). These components had larger widths than for the A111C mutant which could be caused by the E162A mutation making domain II of ETF less rigid. It is unclear whether or not the relative amplitude of the short component observed in the A111C+E162A mutant is due to a higher population because of the E162A mutation or the increased signal to noise ratio in the data.
Figure 8.13  Distance distributions calculated from X-band DEER data shown in Figure 8.12.

The spin label in domain II (A210C) shows only one significant component in the calculated distance distribution (Figure 8.13 lower left panel). The distribution is centered at 2.8 ± 0.02 nm and has a width of 0.27 ± 0.075 nm. Only one component is expected at this spin labeling site because it is located on the same subunit as the FAD so a conformational change of this subunit should not cause a change in the FAD-nitroxyl interspin distance. The width of the distribution at the A210C site is slightly smaller than the distribution widths of the other spin-labeling sites major components. The location of this label suggests that the width of this distribution may be due primarily to motion of the MTSL spin label as opposed to ETF domain motion.
Uncertainties in the distance distribution values are estimates based on variation with fitting parameters. The uncertainties were estimated by comparing results at the optimum regularization parameter (located at the bend in L curve) and those obtained using regularization parameters two positions away in each direction on the L curve. Figure 8.14 shows uncertainty estimation of the A111C mutant data as an example.

Figure 8.14  L curve obtained by Tikonov regularization of the A111C X-band data (Figure 8.10 upper left panel). The optimum position in the L curve is shown in red which corresponds to a regularization parameter of 10. Grey circles show regularization parameters two positions away (0.1 and 1000) that were used to estimate the error in the distance distributions. Distances distributions calculated at regularization parameters 10 (—), 0.1 (—) and 1000 (—) are shown in the right panel.

For all mutants, the average distances for the distributions were well defined (variations of approximately ± 0.1 nm or less for the major contributions). The average distances in the A111C mutant were less well defined than the other mutants. This is most likely due to the lower signal to noise ratio in the A111C data. For all mutants, the widths at half height of the distributions are less well defined than the average values.
8.5.2 Q-Band DEER Data

Initial Q-band four-pulse DEER measurements were performed in the laboratory of Professor David R. Britt at the University of California Davis. Q-band DEER data were analyzed using the same method as for the X-band data. Figure 8.15 shows Q-band DEER traces at 80 K and the background corrections. Q-band data were fit using a three dimensional homogeneous background model (A111C+E162A) or a 2nd order polynomial background model (A111C and A43C). Initial Q-band measurements appeared to give similar signal to noise ratios as in the X-band data.

Figure 8.15 Q-band DEER data (black) and fitted background (red) for enzymatically reduced, spin-labeled ETF mutants. Vertical lines indicate the starting (---) and ending (——) points for the background calculation.
Q-band DEER traces after background subtraction are shown in Figure 8.16. Data were fit using Tikhonov regularization [Chiang et al. 2005]. The modulation depths obtained from Q-band measurements were smaller than the modulation depths at X-band. This indicates that the fraction of FAD SQ⁻ spins excited at Q-band is less than the fraction excited at X-band.

At Q-band the g anisotropy in the FAD SQ⁻ signal is resolved. Because of this increased resolution, pumping at a g value of 2.006 excites a smaller fraction of the FAD orientations compared to X-band. Thus, DEER measurements at Q-band have greater dependence on the field positions selected for the excitation and observe pulses than X-band measurements. Another reason why a smaller fraction of the FAD SQ⁻ spins are excited at Q-band is because 90° pulses are longer at Q-band due to the use of a 1 W source (compared to 1 kW at X-band). Longer pulses excite a smaller bandwidth of frequencies and are therefore more selective.
Figure 8.16  Q-band dipolar evolution signals for enzymatically reduced, spin-labeled ETF mutants obtained from the DEER data in Figure 8.15 by background subtraction. Tikhonov regularization fitting is shown in red.

Characteristic Pake patterns are obtained by Fourier transformation of the Q-band DEER traces in Figure 8.16 (Figure 8.17). Like the X-band data, the traces of the Q-band data in Figure 8.17 do not have positive spikes or holes suggesting that the background subtractions used were appropriate [Jeschke 2006].
Figure 8.17 Q-band DEER frequency domain data for enzymatically reduced, spin-labeled ETF mutants by Fourier transformation of the data in Figure 8.16. Tikhonov regularization fitting is shown in red.

Figure 8.18 shows interspin distance distributions obtained by Tikhonov regularization fitting of the Q-band DEER data. Similar to X-band data, spin labels at the A111C and A43C sites show multiple distributions of FAD-nitroxyl distances. The A43C mutant gave two distributions of distances: a major component centered at $4.3 \pm 0.05$ nm with a width of $0.3 \pm 0.15$ nm and a minor component centered at $3.6 \pm 0.05$ nm with a width of $0.2 \pm 0.1$ nm (Figure 8.18 left panel). A major component centered at
4.2 ± 0.05 nm with a width of 0.58 ± 0.25 nm and a minor component centered at 5.3 ± 0.4 nm with a width of approximately 0.4 ± 0.15 nm was found for the A111C+E162A mutant (Figure 8.18 left panel). The A111C mutant gave three distributions of distances: a major component centered at 4.2 ± 0.05 nm with a width of 0.4 ± 0.2 nm, a minor component centered at 5.3 ± 0.1 nm with a width of 0.4 ± 0.15 nm and another minor component centered at 3.7 ± 0.1 nm with a width of 0.2 ± 0.1 nm (Figure 8.18 left panel). Q-band DEER measurements for the A210C mutant was done using the initial pulse settings, where the maximum echo near $g = 2.010$ was excited, and therefore, did not give usable data.
8.5.3 Comparison of X- and Q-Band DEER Data

A comparison of the distance distributions obtained at X-and Q-band for the A43C, A111C and A111C+E162A mutants are shown in Figure 8.19. Average values and widths in the distance distributions from the A43C and A111C mutant are similar at both frequencies. There is a slight difference of the average value of the minor component of the A43C mutant (0.2 nm) which can be attributed to poorer
signal-to-noise (less averaging) at Q-band. There is also a slight difference (0.2 nm) in the average value of the longer distance minor component in the A111C mutant, which can be attributed to poorer signal to noise of the X-band data. The average values of the major component of the A111C+E162A mutant agree well at X- and Q-band (within 0.1 nm). At X-band, minor components of the A111C+E162A distance distribution are seen at 3.6 and 5.1 nm. At Q-band, only one minor component is seen at 5.3 nm. The smaller relative amplitude of the 5.3 nm component in the Q-band data might be caused by the shorter interval of $t$ values used in the Q-band measurements (~2.2 $\mu$s compared to ~2.9 $\mu$s at X-band). Measuring longer distances require longer $t$ values. The absence of the minor component around 3.6 nm might be the result of poorer signal-to-noise (less averaging) of the Q-band data.
Figure 8.19  Comparison of distance distributions calculated from X-band (black) and Q-band (green) EPR data.

8.6 Modeling of ETF

8.6.1 MTSL-FAD Interspin Distances

DEER results were compared with the crystal structure in the closed conformation (pdb id: 1efp). Using Insight II software (Accelrys), alanine residues at positions 43 and 210 of the α-subunit and 111 of the β-subunit were replaced with cysteines and MTSL labels were attached to the $S_\gamma$ of the cysteines (Figure 8.20). Distances between the C4a
of the FAD and the N-O group of MTSL labels were then measured using the program RasTop 2.2 (http://www.geneinfinity.org/rastop/, accessed on 4/12/10). The C4a position was chosen because it is thought to be a good approximation of the centroid of spin density in the FAD anionic semiquinone [Legrand et al. 2003].

A nitroxyl to FAD distance of approximately 4.1 nm was found for the spin-label at position A111C. This distance is in agreement with the center of the major distance distribution found in the DEER measurements (X-band: 4.2 nm). The distance of 2.76 nm, calculated for the spin label at position A210C also agrees with the results from DEER (X-band: 2.8 nm). The calculated nitroxyl-FAD distance for the MTSL at the A43C position was 3.14 nm. This distance is much shorter than the center of the major distance distribution found in the DEER measurements (X-band: 4.3 nm). The calculated distance is also shorter than the center of the minor distribution (X-band: 3.8 nm). One possible reason for the underestimation of the distance could be due to the conformation of the MTSL spin label in the model. At this position, the cysteine side chain is approximately perpendicular to the interspin vector. Therefore, calculated distances from the spin label at A43C are more dependent on Cβ-Sγ bond rotation than the A111C or A210C sites.
8.6.2 Homology Model of *Paracoccus* ETF with Human ETF:MCAD

A homology model of *P. denitrificans* ETF bound to MCAD, shown in Figure 8.21, was created using the program ESyPred3D [Lambert et al. 2002]. The crystal structure of human ETF in the electron transfer active conformation from the ETF:MCAD complex (pdb id: 2A1T) was used as a template. This was done to determine if multiple distributions were due to a fraction of the ETF adopting a conformation similar to that when it is bound to a redox partner. Using the program RasTop 2.2
distances between amino acids in the ETF:MCAD model and the crystal structure of \textit{P. denitrificans} ETF were compared to estimate the change in the spin label to FAD distance when the \(\alpha\)II domain of ETF changes conformation to facilitate electron transfer to a redox partner.

\textbf{Figure 8.21} Homology model of \textit{P. denitrificans} ETF bound to MCAD. The crystal structure of human ETF from the ETF:MCAD complex (pdb id: 2A1T) that was used in the homology model is shown on the left. Blue ribbons represent the \(\alpha\)-subunit in human ETF and the FAD is shown in pink. FAD is absent in the homology model.

For this comparison it was necessary to estimate the location of the FAD, which is not included in the crystal structure on which the homology model is based. Three amino acids were selected in the \(\alpha\)II domain that appeared to be in position to form hydrogen bonds with the FAD; serine 226, glutamine 263 and histidine 264 (Figure 8.22).
In human ETF these residues are conserved (S248, Q285 and H286) and are located in the same positions relative to the FAD. Table 8.3 shows the distance between side chains of these amino acids and potential hydrogen bonding partners on the FAD in crystal structures of human and *P. denitrificans* ETF. The distances in the *P. denitrificans* enzyme are similar to those in the human enzyme and there is only a small change in these distances when the human enzyme is bound to MCAD (pdb id: 2A1T), which indicates that binding to MCAD causes little change in the orientation of the FAD in the protein.

![Figure 8.22](image)

**Figure 8.22** Amino acids in close proximity to FAD in *P. denitrificans* ETF (pdb id: 1EFP). Residues correspond to S248, Q285 and H286 in Human ETF.
Table 8.3  Distance, in nm, between amino acid side chains and groups on the FAD as measured in the crystal structures of human ETF, human ETF:MCAD complex and *P. denitrificans* ETF.

<table>
<thead>
<tr>
<th>Distance Measured</th>
<th>P. denitrificans ETF (pdb: 1EFP)</th>
<th>Human ETF (pdb: 1EVP)</th>
<th>Human ETF:MCAD complex (pdb: 2A1T)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ser to FAD O2P</td>
<td>0.27</td>
<td>0.26</td>
<td>0.25</td>
</tr>
<tr>
<td>Gln to FAD O2*</td>
<td>0.28</td>
<td>0.29</td>
<td>0.24</td>
</tr>
<tr>
<td>His to FAD O2</td>
<td>0.29</td>
<td>0.28</td>
<td>0.30</td>
</tr>
</tbody>
</table>

Distances between the side chains of these amino acids and the C4a position on the FAD were then measured (Table 8.4). Glutamine is the amino acid closest to the C4a position of the FAD. It is also at the same distance from the FAD C4a in all three ETF structures investigated (Table 8.4). Because of the stability with respect to the FAD, this glutamine residue was used to estimate the FAD position in the homology model.

Table 8.4  Distance, in nm, between amino acid side chains and the C4a position of the FAD isoalloxazine head group in ETF crystal structures.

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>P. denitrificans ETF (pdb: 1EFP)</th>
<th>Human ETF (pdb: 1EFV)</th>
<th>Human ETF:MCAD complex (pdb: 2A1T)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ser</td>
<td>0.83</td>
<td>0.79</td>
<td>0.77</td>
</tr>
<tr>
<td>Gln</td>
<td>0.58</td>
<td>0.58</td>
<td>0.58</td>
</tr>
<tr>
<td>His</td>
<td>0.63</td>
<td>0.63</td>
<td>0.63</td>
</tr>
</tbody>
</table>
Figure 8.23 shows how the distances between the spin-label sites and the FAD are expected to change when ETF forms a complex with MCAD. When the conformation of ETF changes from the closed to open form, a change in interspin distance of approximately 0.9 nm is expected at the A43C site. The same conformational change is expected to give a difference in interspin distances of approximately 1.6 nm at the A111C site. When the spin-labeling site is on the same domain as the FAD, as is the case with the A210C site, the model predicts minimal change (< 0.1 nm) in interspin distance upon conformational change.

![Crystal Structure](image1)

**Figure 8.23** Distances between spin label sites and the approximate FAD location in the crystal structure (1EFP) and the homology model. Distances, in nm, are measured between β carbons of residues at spin label sites and glutamine 263.
Distances between the Cβ atom on the A111 residue of the β subunit (K114 in human) and the FAD C4a or the Q263 Cβ of the α subunit (Q285 in human) were measured in the three crystal structures (distances A and B in Figure 8.24). These distances agree within 0.02 nm for the three crystal structures. The distance between Q263 and A111 in the homology model was 5.19 nm. The change in distance between the Cβ atoms of A111 and Q263 in the crystal structure (unbound conformation) and the homology model (MCAD bound conformation) is 1.63 nm. The difference between the two average distances from the DEER results is approximately 0.9 nm in the A111C mutant and 0.8 nm in the A111C+E162A mutant. This smaller distance change may be due to ETF adopting a conformation that is intermediate in the range of conformations predicted in the model [Chohan et al. 2001]. Modeling does not predict the shortest distance in the A111C+E162A distance distribution (3.6 nm). This distance may be due to the protein adopting a third conformation. The difference values measured from A111 and the FAD are expected to be similar to the difference between FAD-nitroxyl distance in the bound and unbound conformations because the structure of domain III, where the spin label is located, does not appear to change during αII domain movement.
Figure 8.24 Distances, in nm, between the Cβ of the A111 spin labeling site (corresponding to K114 in Human ETF) and either the C4a position on the FAD or the Cβ of the glutamine residue close to the FAD head (Q263 in Paracoccus, Q285 in Human). Distances A and C could not be measured in the homology model because of the absence of the FAD molecule.

Next, the A210 site of the α subunit (K232 in human) in the three crystal structures was analyzed in an analogous way. Distances between the Cβ atom on the A210 residue and the FAD C4a or the Q263 Cβ of the α subunit (Q285 in human) were calculated (distances A and B in Figure 8.25). The distance between Q263 and A210 in the homology model was calculated and compared to distances obtained from the crystal structures. Modeling predicts that there is no change in the orientation of the A210 residue with respect to the FAD as ETF changes from the closed to the open
conformation. Distances calculated for both the open and closed conformations of ETFs were similar (within 0.1 nm). This agrees with experimental data, where only one distribution is given by DEER measurements (bottom left panel, Figure 8.13).

**Figure 8.25** Distances, in nm, between the Cβ of the A210 spin labeling site (corresponding to K232 in Human ETF) and either the C4a position on the FAD or the Cβ of the glutamine residue close to the FAD head (Q263 in Paracoccus, Q285 in Human). Distance A could not be measured in the homology model because of the absence of the FAD molecule.

<table>
<thead>
<tr>
<th>ETF Structure</th>
<th>A</th>
<th>B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human (pdb: 1EFV)</td>
<td>2.34</td>
<td>2.58</td>
</tr>
<tr>
<td>Human:MCAD (pdb: 2A1T)</td>
<td>2.40</td>
<td>2.65</td>
</tr>
<tr>
<td>P. denitrificans (pdb: 1EFP)</td>
<td>2.32</td>
<td>2.55</td>
</tr>
<tr>
<td>Homology Model of P. denitrificans:MCAD</td>
<td>NA</td>
<td>2.62</td>
</tr>
</tbody>
</table>

Finally, distances between the Cβ and the FAD C4a or Q263 Cβ were measured with respect to the A43 site of the α subunit (K62 in human) in the three crystal structures (Figure 8.26). The homology model predicts the difference in interspin distance associated with the conformational change between the unbound and MCAD-bound conformation of Paracoccus ETF to be approximately 0.9 nm. A difference between the two average distances of 0.5 and 0.7 nm was found in the DEER measurements at X- and Q-band respectively. As for the A111C site, the model
predicts a larger interspin distance change than is seen in the data. ETF adopting a conformation that is an intermediate in the range of conformations predicted in the model could explain the smaller observed difference. Unlike the A111C mutant, the longer of the two average distances in the A43C mutant has a higher population. If these distances represent two ETF conformations, it is unclear why the A43C mutant has a higher percentage in the long distance conformation compared with the A111C mutant. Because of the amplitude of the difference in distances in the DEER data (approximately 0.5 nm) and the fact that the location of the A43C residue is approximately perpendicular to the interspin vector, two conformations of the spin label at this site can’t be ruled out.

**Figure 8.26** Distances, in nm, between the Cβ of the A43 spin labeling site (corresponding to K62 in Human ETF) and either the C4a position on the FAD or the Cβ of the glutamine residue close to the FAD head (Q263 in *Paracoccus*, Q285 in Human). Distance A could not be measured in the homology model because of the absence of the FAD molecule.
Chapter 9. Summary of DEER Distance Measurements of ETF

9.1 Conclusions

Human ETF, located in the mitochondrial matrix, is responsible for transferring electrons derived from fatty acid β-oxidation and the oxidation of several amino acids to the main respiratory chain via ETF-QO. It is postulated that multiple conformations of the αII domain, containing FAD, allow ETF to interact with so many structurally different partners. The goal of this work was to characterize the motion of domain II using DEER measurements of the distance between the enzymatically reduced FAD cofactor, located in domain II, and spin labels at various sites.

The spin label MTSL was attached to single cysteine mutations that were introduced into ETF by site-directed mutagenesis. Spin labeling sites were placed in each structural domain of ETF (A43C, domain I; A210C, domain II; A111C, domain III). Incorporation of the MTSL spin label was shown to be stoichiometric by CW EPR spectroscopy. A coupled reaction with glutaryl-CoA and glutaryl-CoA dehydrogenase was used to enzymatically reduce ETF. Formation of FAD SQ− was confirmed by the difference in CW EPR spectra of as-isolated and reduced spin-labeled ETF mutants, and demonstrated that the FAD cofactor could be reduced without destruction of the spin
label. Using visible absorption, it was determined that the maximum amount of FAD $\text{SQ}^-$ was produced at pH 8 before the onset of disproportionation.

Four-pulse DEER measurements were initially performed, at X-band, on the enzymatically reduced, spin-labeled A111C mutant. The excitation pulse was set to the maximum echo in the field swept spectrum ($g = 2.006$) and the observe pulse was set approximately 60 MHz down field ($g = 2.0018$). Two distributions of distances were found: a major component centered at 4.2 nm and a minor component centered at 5.1 nm. A third component at 3.7 nm was also seen but, because of the relative amplitude of this component in the distance distribution, the significance of this distance was questioned. Widths of both distributions were approximately 0.3 nm at half height. Comparing these results with the crystal structure of $P. \text{denitrificans}$ suggested that the contribution at 4.2 nm was due to an ETF conformation similar to the crystal structure. The longer contribution at 5.1 nm was purposed to be due to an ETF conformation that was intermediate between the substrate-free and substrate-bound forms [Swanson et al. 2009].

X-band DEER measurements were then performed on enzymatically reduced ETF with spin labels at the other two mutation sites. The A43C mutant gave two distributions of distances: a major component centered at 4.3 nm with a width of 0.34 nm and a minor component centered at 3.8 nm with a width of 0.23 nm. As expected, only one component was found in the calculated distance distribution of the spin-labeled A210C mutant. The distribution is centered at 2.8 nm and has a width of 0.27 nm. Because this spin labeling site is located on the same subunit as the FAD, conformational changes of this subunit relative to the other subunits should not cause a change in the FAD-nitroxyl interspin distance.
In addition to the three single cysteine mutations, a second mutation, E162A, was introduced into A111C ETF. DEER measurements of enzymatically reduced, spin-labeled A111C+E162A ETF showed three distributions: a major component centered at 4.3 nm with a width of 0.47 nm, a minor component centered at 5.1 nm with a width of 0.36 nm and a second minor component centered at 3.6 nm with a width of 0.32 nm. These components were also seen in the data from the A111C mutant, but the component at 3.6 nm was much more prominent in the A111C+E162A mutant. It was expected that adding the E162A mutation would favor the open conformation and cause the population of the 5.2 nm component in the distance distribution of A111C to increase relative to the 4.2 nm component. This increase was not seen. However, the widths of the components were larger in the A111C+E162A mutant suggesting that the E162A mutation causes domain II of ETF to be less rigid, perhaps by destabilization of the 4.3 nm conformation. It is also possible that the larger relative amplitude of the component at 3.6 nm observed in the A111C+E162A mutant is due to a higher population of a third protein conformation caused by the E162A mutation.

Distance distributions for all mutants were compared to the expected distances based on the crystal structure of *P. denitrificans* ETF and a homology model of *P. denitrificans* ETF bound to MCAD. Average values of the major components in distance distributions from the results of DEER measurements at the A111C and A210C sites agreed with distances obtained from the crystal structure, with MTSL labels added (Table 9.1). For the A43C mutant, the nitroxyl to FAD distance in the crystal structure was approximately 3.1 nm which was closer to the minor component (3.8 nm) than to the major component (4.3 nm) seen in the DEER measurements.
Table 9.1  Average values of the major and minor components in the distance distributions calculated from DEER measurements of ETF mutants compared to interspin distances in the crystal structure. Also shown are differences between the short and long contributions to the distance distributions and the differences in interspin distances calculated from the crystal structure and the homology model. All values are in nm.

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Major Component Distance</th>
<th>Crystal Structure Distance</th>
<th>Minor Component/s Distance/s</th>
<th>Δ, Between Components</th>
<th>Δ, Model – Crystal Structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>A111C</td>
<td>4.2</td>
<td>4.07</td>
<td>5.1</td>
<td>0.9</td>
<td>1.6</td>
</tr>
<tr>
<td>A111C+ E162A</td>
<td>4.3</td>
<td>4.07</td>
<td>5.1, 3.6</td>
<td>0.8</td>
<td>1.6</td>
</tr>
<tr>
<td>A210C</td>
<td>2.8</td>
<td>2.76</td>
<td>NA</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>A43C</td>
<td>4.3</td>
<td>3.14</td>
<td>3.8</td>
<td>0.5</td>
<td>0.9</td>
</tr>
</tbody>
</table>

Interspin distance changes predicted by the homology model were approximately twice as large as the differences observed between the contributions to the distance distributions calculated from DEER measurements of the A111C and A43C mutation sites (Table 9.1). Therefore, it is proposed that ETF adopts a conformation that is intermediate between the substrate-free and substrate-bound conformations. The two contributions in the distance distribution of the A111C mutant are not the result of multiple conformations of the spin label. Multiple spin label conformations were ruled out because the maximum increase in interspin distance caused by varying the dihedral angles of the spin label is ~0.5 nm at this site. Multiple spin label conformations could not be ruled out for the A43C mutant because the cysteine side chain at this site is in an approximately perpendicular orientation with respect to the interspin vector. In this orientation, spin label rotation will lead to much greater interspin distance differences between spin label conformations.
DEER measurements were repeated at Q-band for the A43C, A111C and A111C+E162A mutants. When the excitation pulse was set to the maximum in the field swept echo-detected spectrum and the observe pulse was offset 50 MHz up-field, no dipolar interactions between the FAD SQ\textsuperscript{−} and the MTSL spin label could be detected in the DEER measurement. Modulations, indicating dipolar interaction of two spins, were observed in the DEER data when the locations of the excitation and observing pulses were reversed. With pulses located at these positions in the spectrum, the FAD SQ\textsuperscript{−} (and MTSL) were excited and only the MTSL is observed, analogous with X-band measurements. Similar distance distributions were obtained at X- and Q-bands.

9.2 Future Work

Future work will be required to determine the validity of the distance distributions calculated from the DEER measurements and to confirm that the multiple contributions in the distance distributions are indeed multiple conformations of ETF and not of the label. The amplitude of a minor component of the distance distribution, at 3.6 nm, was found to be larger in the A111C+E162A mutant compared to the A111C mutant. A111C was the first ETF mutant investigated and the signal to noise ratio of the data collected was less than ideal. More signal averaging is needed for X-band DEER measurements of the A111C mutant to determine if the lower amplitude of the 3.7 nm component in the A111C mutant is because of the lack of the E162A mutation or because of poor signal to noise. The E162A mutation should also be added to the A43C mutant to determine if a third contribution, corresponding to the 3.6 nm contribution in A111C+E162A, is observed. This should also be done to verify that broadening of the components in the
distance distribution observed in A111C+E162A ETF was in fact due to the E162A mutation.

Q-band DEER measurements should be made on the remaining A210C mutant. Initial attempts at collecting Q-band DEER data for this mutant were unsuccessful. Optimization of the Q-band DEER procedure for this system is also required because of the orientation dependence. Optimization will be done by determining the observe pulse frequency that gives the strongest dipolar signal while keeping the excitation pulse frequency fixed. Once optimal conditions are found, repeat measurements of the A111C+E162A and A43C mutants will also be done to obtain higher signal to noise.

The A210C mutation site is located in the \( \alpha \text{II} \) domain so the interspin distance between a spin label at this site and the FAD SQ\(^{-}\) does not change with respect to domain II motion. To characterize the motion of the \( \alpha \text{II} \) domain at least one more mutation site is needed to triangulate the position of the FAD SQ\(^{-}\).

The two average distances in the distribution obtained from the A43C mutant can be explained by ETF adopting a conformation that is intermediate between the conformations observed in the crystal structures of free ETF and MCAD bound ETF. What was unexpected was the relative abundance of each average distance, which suggests that a higher amount of the A43C mutant is in the intermediate conformation. This is opposite of the result from the A111C mutation site where the major contribution to the distance distribution was attributed to the substrate-free conformation of ETF. Additional spin labeling sites in domain I and domain III of ETF are needed to check the impact of spin label conformation and verify the results from the A43C and A111C mutants.
Using data from new spin labeling sites along with the data in this dissertation, a model can be made of ETF in the open conformation. This method has the potential to characterize conformational changes in ETF that occur when it interacts with various redox partners.
References


Griffin, K. J., Dwyer, T. M., Manning, M. C., Meyer, J. D., Carpenter, J. F. and Frerman, F. E. (1997) \( \alpha \)T244M mutation affects the redox, kinetic, and in vitro folding properties of paracoccus denitrificans electron transfer flavoprotein, Biochemistry 36, 4194-4202.


Appendix 1: Derivation of Nernst Equation

A generic redox reaction where species $B_{\text{red}}$ donates electrons to species $A_{\text{ox}}$ is described by the following expression:

$$A_{\text{ox}} + B_{\text{red}} \rightleftharpoons A_{\text{red}} + B_{\text{ox}} \quad (A1.1)$$

Assuming the concentration of species $B_{\text{red}}$ is much greater than species $A_{\text{ox}}$ so the change in $B_{\text{red}}$ can be neglected, the free energy associated with the reduction of species $A$ is:

$$\Delta G = \Delta G^\circ + RT \ln \left( \frac{[A_{\text{red}}]}{[A_{\text{ox}}]} \right) \quad (A1.2)$$

Under reversible conditions free energy is equal to:

$$\Delta G = -w' = -w_{\text{el}} \quad (A1.3)$$

where $w'$ is the non-pressure-volume work, which in this case is the electrical work, $w_{\text{el}}$. The electrical work is defined as the work required to transfer $n$ moles of electrons through an electrical potential difference $E$, according to the equation:

$$w_{\text{el}} = nFE \quad (A1.4)$$

where $F$ is the electrical charge of 1 mol of electrons (faraday). Substituting equation A1.4 into equation A1.3 gives:

$$\Delta G = -nFE \quad (A1.5)$$
Combining equations A1.2 and A1.5 yields:

\[-nFE = \Delta G^\circ + RT \ln \left( \frac{[\text{red}]}{[\text{ox}]} \right) \]  \hspace{1cm} (A1.6)

Rearranging A1.6 to solve for the potential gives:

\[E = \frac{-\Delta G^\circ}{nF} - \frac{RT}{nF} \ln \left( \frac{[\text{red}]}{[\text{ox}]} \right) \]  \hspace{1cm} (A1.7)

Equation A1.5 can be rearranged to give an expression for the standard redox potential of the couple (midpoint potential):

\[E^\circ = \frac{-\Delta G^\circ}{nF} \]  \hspace{1cm} (A1.8)

This equation can then be substituted into equation A1.7 giving the familiar form of the Nernst equation:

\[E = E^\circ - \frac{RT}{nF} \ln \left( \frac{[\text{red}]}{[\text{ox}]} \right) \]  \hspace{1cm} (A1.9)
Appendix 2: Media Recipes

All media were autoclaved at 250 °F for 20 minutes.

**NZY**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Casein hydrolysate</td>
<td>10 g / L</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>5 g / L</td>
</tr>
<tr>
<td>NaCl</td>
<td>5 g / L</td>
</tr>
</tbody>
</table>

Adjust pH to 7.5. The following filter sterilized supplements were added prior to use: 12.5 mL of 1 M MgCl₂, 12.5 mL of 1 M MgSO₄, 20 mL of 20% (w/v) glucose.

**LB**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tryptone</td>
<td>10 g / L</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>10 g / L</td>
</tr>
<tr>
<td>NaCl</td>
<td>5 g / L</td>
</tr>
</tbody>
</table>

Adjust pH to 7.4

**LB Agar**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tryptone</td>
<td>10 g / L</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>10 g / L</td>
</tr>
<tr>
<td>NaCl</td>
<td>5 g / L</td>
</tr>
<tr>
<td>agar</td>
<td>20 g / L</td>
</tr>
</tbody>
</table>

Adjust pH to 7. Cool in 40 °C water bath after autoclaving before adding antibiotic. Use ~50 mL of LB agar per plate. Dry in 37 °C incubator over night.

**TYP**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tryptone</td>
<td>16 g / L</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>16 g / L</td>
</tr>
<tr>
<td>HCl</td>
<td>5 g / L</td>
</tr>
<tr>
<td>K₂HPO₄</td>
<td>2.5 g / L</td>
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</table>

Adjust pH to 6.8
Mineral Salts Medium

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
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</thead>
<tbody>
<tr>
<td>Glutaric acid</td>
<td>20 g/L</td>
</tr>
<tr>
<td>K$_2$HPO$_4$</td>
<td>50 mM</td>
</tr>
<tr>
<td>NH$_4$Cl</td>
<td>75 mM</td>
</tr>
<tr>
<td>Na$_2$SO$_4$</td>
<td>11.5 mM</td>
</tr>
<tr>
<td>MgCl$_2$</td>
<td>1.25 mM</td>
</tr>
<tr>
<td>Citric acid</td>
<td>1 mM</td>
</tr>
<tr>
<td>Succinic acid</td>
<td>10 g/L</td>
</tr>
<tr>
<td>Salt Stock</td>
<td>1 mL/L</td>
</tr>
</tbody>
</table>

Adjust pH to 6.2. After medium is autoclaved add 1 mL 50 mM Biotin per liter of media.

Salt Stock for Mineral Salts Medium

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>CaCl$_2$</td>
<td>0.1 M</td>
</tr>
<tr>
<td>FeCl$_3$</td>
<td>90 mM</td>
</tr>
<tr>
<td>MnCl$_2$</td>
<td>50 mM</td>
</tr>
<tr>
<td>ZnCl$_2$</td>
<td>25 mM</td>
</tr>
<tr>
<td>CoCl$_2$</td>
<td>10 mM</td>
</tr>
<tr>
<td>CuCl$_2$</td>
<td>5 mM</td>
</tr>
<tr>
<td>H$_3$BO$_3$</td>
<td>5 mM</td>
</tr>
<tr>
<td>Na$_2$MoO$_4$</td>
<td>10 mM</td>
</tr>
</tbody>
</table>

Dissolve in 50% HCl.
Appendix 3: Plasmid DNA purification using QIAprep spin miniprep kit

Procedure:

1. Cells were resuspended in 250 μl Buffer P1 and transferred to a microcentrifuge tube.
2. Added 250 μl Buffer P2 and mixed thoroughly by inversion.
3. Added 350 μl Buffer N3 and mixed immediately by inversion.
4. Centrifuged for 10 min at 13,000 rpm in a microcentrifuge.
5. Applied the supernatants from step 4 to the QIAprep spin column.
6. Centrifuged for 60 seconds. Flow-through was discarded.
7. Washed QIAprep spin column with 0.5 ml Buffer PB, centrifuged for 60 seconds, and discarded the flow-through.
8. Washed QIAprep spin column with 0.75 ml Buffer PE and centrifuged for 60 seconds.
9. Discarded the flow-through, and centrifuged for an additional 60 seconds to remove residual wash buffer.
10. Placed the QIAprep column in a 1.5 ml microcentrifuge tube. DNA was eluted by adding 50 μl of water to the center of each spin column, waiting 1 minute, and then centrifuging for 1 minute.

Appendix 4: Bio-Rad Bradford Method Protein Assay

Standard Sample

1. Prepared five dilutions of a BSA (bovine serum albumin) standard by adding 2, 4, 6, 8 and 10 μL of 1 mg/mL BSA to 800 μL of dd H₂O in disposable cuvettes.
2. Added 200 μL of dye reagent concentrate to each cuvette and mixed by inversion.
3. Incubated cuvettes at room temperature for 5 minutes.

ETF-QO Sample

1. Prepared five dilutions of membrane suspension by adding 2, 4, 6, 8 and 10 μL of a 30 X dilution of the resuspended membrane fraction to 800 μL of dd H₂O in disposable cuvettes.
2. Added 200 μL of dye reagent concentrate to each cuvette and mixed by inversion.
3. Incubated cuvettes at room temperature for 5 minutes.
5. Compared slope of the membrane suspension plot versus the slope of the BSA standard to determine the concentration of protein in the membrane suspension.