DEER Distance Measurement Between a Spin Label and a Native FAD Semiquinone in Electron Transfer Flavoprotein

Michael A. Swanson,[1] Velavan Kathirvelu,[1] Tomas Majtan,[2] Frank E. Frerman,[2] Gareth R. Eaton,[1] and Sandra S. Eaton[1] *

[1]*Department of Chemistry and Biochemistry, University of Denver, Denver, CO 80208 and*

[2]*Department of Pediatrics, University of Colorado School of Medicine, Aurora, CO 80045*

ETF Expression and Purification

The *P. denitrificans* ETF genes[1] were subcloned into the pET-28a(+) vector at the XbaI/HindIII restriction site. The A111C mutation was introduced by site-directed mutagenesis using the Stratagene QuikChange® II XL kit according to the manufacturer's instructions. A111C ETF was expressed in *E. coli* (XL-10 gold) and the cells were broken by two passes through a French pressure cell at 1000 psi. Cell membranes were removed by centrifugation at 33,000 rpm.

The soluble supernatant from cell lysis was loaded onto a 300 mL (5 cm x 15 cm) diethylaminoethyl (DEAE) Sepharose column (Sigma-Aldrich) that had been previously equilibrated with 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 the equilibration buffer. 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. Yellow fractions were analyzed by absorption spectra and those showing the characteristic spectrum of ETF were pooled and concentrated to a volume of roughly 200 mL using an Amicon stirred ultrafiltration cell and a PM30 ultrafiltration membrane (Millipore, Billerica, MA) under N_2. The concentrated sample

was then dialyzed against 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 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 equilibration 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. Fractions showing the characteristic UV-visible spectrum of ETF were pooled and dialyzed into 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 about 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-HCl 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. ETF eluted from the column at approximately 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 into 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.[2]

DTT was removed using a HiPrep^{TM} 26/10 desalting column (GE Healthcare) at a flow rate of 10 mL/min. Immediately after elution from the column, fractions containing ETF were

combined and a 5-fold 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^{TM} 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 about 100 μM using an Amicon stirred ultrafiltration cell and a PM30 ultrafiltration membrane. The final concentration was determined using the absorbance at 436 nm (ε_{436} = 13.3 $mM^{-1}cm^{-1}$). Spin-labeled ETF was separated into 1 mL aliquots, snap frozen in liquid nitrogen and stored at -80 °C until enzymatic reduction was performed.

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 $SQ^{-\bullet}$ was carried out in stoppered cuvettes containing 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 argon 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 mutant was 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 $SQ^{-\bullet}$ was followed by monitoring the increase in absorbance at 375 nm due to formation of the anionic ETF flavin semiquinone. Spectra were recorded every 5 minutes after the addition of dehydrogenase.

When absorbance at 375 nm reached a maximum, samples (~300 μL) were immediately transferred anaerobically to 4.0 mm o.d. quartz EPR tubes 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. 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 DEER was performed.

EPR Spectroscopy of Spin-Labeled ETF Mutants

Room temperature CW spectra of spin-labeled A111C ETF in 1.0 mm glass capillaries were recorded using a Varian E109 spectrometer equipped with a TE_{102} rectangular cavity. Operating conditions were as follows; 9.23 GHz microwave frequency, 1.0 G modulation amplitude at 100 kHz modulation, a gain of 2000, 5 mW microwave power, 0.064 second time constant, 100 G sweep width, and twenty, 2 minute scans were averaged. The room temperature spectrum (Fig. S1) shows no evidence of multiple conformations.

CW EPR spectra were recorded at 100 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. Operating conditions 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.

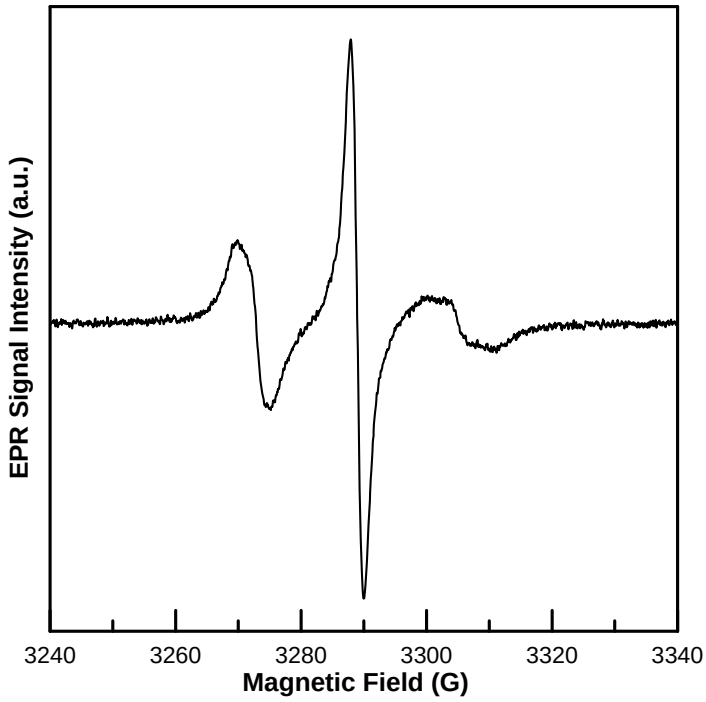


Figure S1: Room temperature continuous wave EPR spectrum of MTSL-labeled A111C ETF.

Four-pulse DEER measurements were performed using the following parameters: $\pi/2$ = 16 ns, t_1 = 124 ns, t_2 = 3200 ns, ELDOR π = 40 ns and 796 points. The pump pulse was set to 9.424 GHz which corresponds to the maximum of the echo detected CW spectrum. At this frequency the FAD $SQ^{-\bullet}$ signal overlaps with the MTSL nitroxyl signal. The observe pulse was offset by 60 MHz to higher frequency (lower resonant field) of the pump pulse (9.484 GHz). 16 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.

The distance range for DEER measurements is limited by spin echo dephasing times, T_m. Since the FAD semiquinone does not contain *tert*-butyl groups, the T_m is about 2 to 3 μs at temperatures up to about 120 K which is similar to that for nitroxyls.[3] The pulse repetition rate

for DEER experiments is determined by spin lattice relaxation. The spin lattice relaxation rates for semiquinones are somewhat slower than for nitroxyls which necessitates longer pulse repetition times, but this will not limit the general utility of DEER measurements of semiquinone-nitroxyl distances.

DEER spectra were analyzed using the program DeerAnalysis2008.[4] Data were fit to distances between 2.0 and 7.0 nm using Tikhonov regularization[5], a t_0 of 38 ns and a homogeneous 3 dimensional background correction starting at 360 ns. Tikhonov regularization gave better fits than single or double Gaussian models (see Fig 2c). The uncertainty in the distance distributions were estimated by comparing results at a regularization parameter of 10 and those obtained using regularization parameters 0.1 and 1000 (two positions away in each direction on the L curve). The average distance for the major distribution was well defined with width of approximately ± 0.1 nm. The average distance of the minor component was less well defined with values varying by approximately ± 0.2 nm. The widths at half height of both distributions (approximately ± 0.25 nm) are less well defined than the average values. These conclusions are based on 2 replicate measurements at 60 K and one at 80 K. The results for the independent data sets, analyzed with the same parameters for the Tikhonov regularization, agree within 0.05 nm for the interspin distances and within 0.1 nm for the widths of the distributions.

DeerAnalysis2008 results were compared to results obtained from the program, DEFit, which fits gaussian distributions to DEER data.[6] Two distributions were obtained using DEFit, a major component at approximately 4.3 ± 0.2 nm and a minor component at approximately 5.6 ± 0.4 nm (uncertainty values represent the dependence of the distribution average values on changing the starting position of the baseline correction). The contour plot showed that the uncertainty in the width of the longer distribution was much greater than for the 4.3 nm

distribution. The width of the major, 4.3 nm, distribution was approximately 0.5 nm ± 0.2nm. The width of the longer, 5.6 nm, distribution was 0.1 nm using the best fit to the data but the contour plot showed that widths of 1.6 or 3.0 nm were almost equally possible (within a single standard deviation). The average values and widths of the major component are consistent in both programs whereas the minor component is significantly different. Therefore, the uncertainties in the widths of the distributions calculated for the major component are proposed to be small relative to the uncertainties for the minor component.

Homology Model of *Paracoccus* ETF with Human ETF:MCAD complex

A homology model of *P. denitrificans* ETF bound to MCAD was created using the program ESyPred3D.[7] 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 the longer distance distribution (5.2 nm) was 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 (http://www.geneinfinity.org/rastop), distances between amino acids in the ETF:MCAD model and the crystal structure of *P. denitrificans* ETF were compared to estimate the change in the spin label to FAD distance when the αII domain of ETF changes conformation to facilitate electron transfer to a redox partner.

With this model it was necessary to estimate the location of the FAD, which is not included in the homology model. Three amino acids were selected in the αII domain that appeared to be in position to form hydrogen bonds with the FAD; serine 226, glutamine 263 and histidine 264 (Figure S2). In human ETF these residues are conserved (S248, Q285 and H286) and are located in the same positions relative to the FAD. Table S1 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.

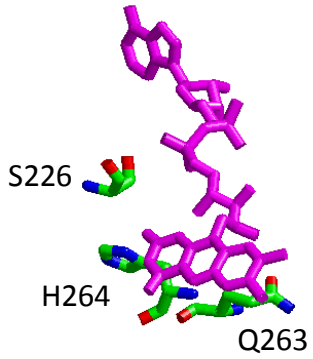


Figure S2: Amino acids in close proximity to FAD in *P. denitrificans* ETF (pdb id: 1EFP). Residues correspond to S248, Q285 and H286 in Human ETF.

Table S1: Distance, in Å, 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.

Distance Measured	***P. denitrificans* ETF (pdb: 1EFP)**	**Human ETF (pdb: 1EVP)**	**Human ETF:MCAD complex (pdb: 2A1T)**
Ser to FAD O2P	2.71	2.64	2.45
Gln to FAD O2'	2.76	2.94	2.44
His to FAD O2	2.92	2.83	3.04

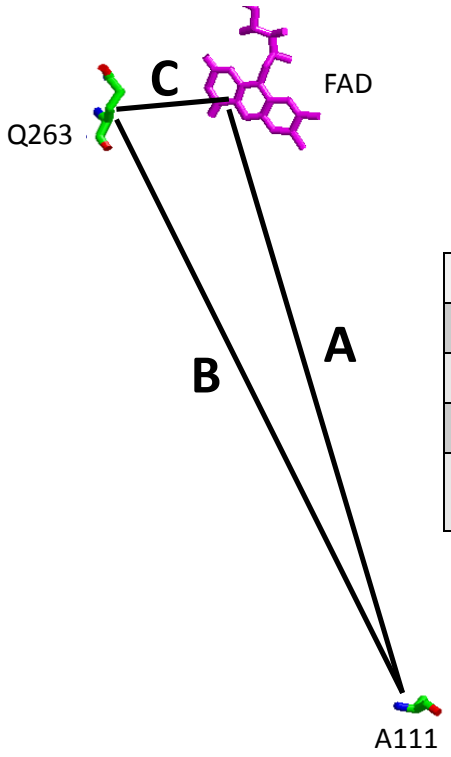
Distances between the side chains of these amino acids and the C4a position on the FAD were then measured (Table S2). The C4a position was chosen because it is thought to be a good approximation of the centroid of spin density in the FAD anionic semiquinone.[5] Glutamine is the amino acid closest to the C4a position of the FAD. It is also at the same distance from the FAD

C4a in all three ETF structures investigated (Table S2). Because of the stability with respect to the FAD, this glutamine residue was used as to estimate the FAD position in the homology model.

Table S2: Distance, in Å, between amino acid side chains and the C4a position of the FAD isoalloxazine head group in ETF crystal structures.

Amino Acid	*P. denitrificans* ETF (pdb: 1EFP)	Human ETF (pdb: 1EFV)	Human ETF:MCAD complex (pdb: 2A1T)
Ser	8.32	7.94	7.65
Gln	5.83	5.83	5.83
His	6.33	6.32	6.29

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 S3). These distances agree within 0.25 Å for the three crystal structures. The distance between Q263 and A111 in the homology model was 51.9 Å. 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 16.3 Å. The difference between the two average distances from the DEER results is roughly 9 Å. This smaller distance change may be due to ETF adopting a conformation that is an intermediate in the range of conformations predicted in the model.[9] 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.



ETF Structure	A	B	C
Human (pdb: 1EFV)	35.5	35.7	3.6
Human:MCAD (pdb: 2A1T)	57.1	57.2	3.8
P. denitrificans (pdb: 1EFP)	35.5	35.6	3.5
Homology Model of *P. denitrificans*:MCAD	NA	51.9	NA

Figure S3: Distances, in Å, between the Cβ of the spin labeling site (A111 in *Paracoccus* ETF, 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.

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