Electron transfer flavoprotein domain II orientation monitored using double electron-electron resonance between an enzymatically reduced, native FAD cofactor, and spin labels

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Abstract: Human electron transfer flavoprotein (ETF) is a soluble mitochondrial heterodimeric flavoprotein that links fatty acid β -oxidation to the main respiratory chain. The crystal structure of human ETF bound to medium chain acyl-CoA dehydrogenase indicates that the flavin adenine dinucleotide (FAD) domain (α II) is mobile, which permits more rapid electron transfer with donors and acceptors by providing closer access to the flavin and allows ETF to accept electrons from at least 10 different flavoprotein dehydrogenases. Sequence homology is high and low-angle X-ray scattering is identical for *Paracoccus denitrificans* (*P. denitrificans*) and human ETF. To characterize the orientations of the α II domain of *P. denitrificans* ETF, distances between enzymatically reduced FAD and spin labels in the three structural domains were measured by double electron-electron resonance (DEER) at X- and Q-bands. An FAD to spin label distance of 2.8 ± 0.15 nm for the label in the FAD-containing α II domain (A210C) agreed with estimates from the crystal structure (3.0 nm), molecular dynamics simulations (2.7 nm), and rotamer library analysis (2.8 nm). Distances between the reduced FAD and labels in α I (A43C) were between 4.0 and 4.5 ± 0.35 nm and for β III (A111C) the distance was 4.3 ± 0.15 nm. These values were intermediate between estimates from the crystal structure of *P. denitrificans* ETF and a homology model based on substrate-bound human ETF. These distances suggest that the α II domain adopts orientations in solution that are intermediate between those which are observed in the crystal structures of free ETF (closed) and ETF bound to a dehydrogenase (open).

Keywords: double electron electron resonance; FAD semiquinone; interspin distance; molecular dynamics simulation; Q-band DEER

Abbreviations: CW, continuous wave; DCPIP, 2,6-dichlorophenol-indophenol; DEER, double electron electron resonance; EPR, electron paramagnetic resonance; ETF, electron transfer flavoprotein; ETF-QO, electron transfer flavoprotein ubiquinone oxidoreductase; FAD, flavin adenine dinucleotide; MADD, multiple acyl-CoA dehydrogenation deficiency; MCAD, medium chain acyl-CoA dehydrogenase; MTSL, 1-oxyl-2,2,5,5-tetramethylpyrroline-3-methyl-methanethiosulfonate; Q, quinone; QH₂, hydroquinone/quinol; SAXS, small-angle solution X-ray scattering; SQ⁻, anionic semiquinone

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Introduction

In the mitochondrial matrix, the oxidation of fatty acids and several amino acids including lysine, leucine, valine, and isoleucine^{1–3} is coupled to the main mitochondrial respiratory chain through an electron transfer pathway involving electron transfer flavoprotein (ETF), electron transfer flavoprotein ubiquinone oxidoreductase (ETF-QO), and ubiquinone. ETF contains a flavin adenine dinucleotide (FAD) cofactor that accepts electrons from 10 flavoprotein dehydrogenases,⁴ and transfers them to ETF-QO in the inner mitochondrial membrane.^{5–7} Electrons enter ETF-QO through its [4Fe-4S]^{+2,+1} iron-sulfur cluster, are transferred to an FAD, and finally to ubiquinone.⁸ Reduced ubiquinone then transfers electrons to the main respiratory chain through the cytochrome *bc*₁ complex (complex III). Defects in human ETF or ETF-QO result in a metabolic disease known as multiple acyl-CoA dehydrogenation deficiency (MADD) or glutaric acidemia type 2. Death within the neonatal period occurs if the defects are severe.^{9,10}

ETF consists of two subunits, α and β , which combine to form three structural domains¹¹ (Fig. 1). The FAD cofactor is bound by the C-terminal region of the α -subunit (domain II). The FAD isoalloxazine ring is located in a cleft created by domains II and III. It has been postulated that for mammalian ETF to form productive electron transfer complexes with so many structurally different dehydrogenases and to transfer electrons to ETF-QO, it must be able to adopt a range of conformations.^{12,13} In addition, the rigid crystal structures of human medium chain acyl-CoA dehydrogenase and human ETF place the flavins of the two proteins 1.9–2.5 nm apart in the binary complex.^{12,13} This distance is unfavorable for electron transfer through proteins where the maximum distance is about 1.4 nm.¹⁴ The proposed mobility of the α II domain is based on the following observations. (i) The orientation of domain II in the crystal structure of human ETF bound to medium-chain acyl-CoA dehydrogenase (MCAD) is rotated relative to that in the crystal structure of free ETF.^{12,13} The electron density of domain II was not resolved until the glutamate residue at position 165 of the β subunit was replaced with an alanine. In wild-type human ETF the β E165 residue interacts with an asparagine residue (α N259) and a conserved arginine residue (α R249) that are located near the FAD. These interactions help stabilize closed orientations of domain II.¹⁵ (ii) Multiple orientations of domain II were required to model small-angle solution X-ray scattering (SAXS) of human and *P. denitrificans* ETF.⁴ The models indicated that compared with the conformation observed in the X-ray structure, domain II is rotated by 30–50° around an axis defined by domains I and III. This rotation creates an open conformation that provides access to the FAD.

Figure 1. Crystal structure of *P. denitrificans* ETF (1EFP).¹¹ The α - and β -subunits are depicted as blue and gray ribbons respectively, FAD is shown in pink and structural domains are labeled using Roman numerals. Spherical representations are used for mutated amino acids with spin labeling sites (α A43C, β A111C, and α A210C) shown in green and the glutamate residue at 162 shown in red.

The single, buried native cysteine (α C36) in *P. denitrificans* ETF makes the bacterial enzyme a more straightforward target for site-directed spin labeling than the human and pig enzymes, which contain 10 and 9 native cysteines, respectively.¹⁶ Domain motion of *P. denitrificans* ETF is expected to be similar to mammalian ETFs because of the similarity in structures and reactivity,¹⁶ and the identical SAXS data for human and *P. denitrificans* ETF.⁴ EPR experiments have shown that enzymatic reduction of the FAD in a spin-labeled ETF mutant could be performed without destroying the spin label.¹⁷ To characterize the orientations of domain II, double electron electron resonance (DEER) was used to measure distances between the enzymatically reduced FAD and 1-oxyl-2,2,5,5-tetramethylpyrroline-3-methyl-methanethiosulfonate (MTSL) spin labels at A43 in domain I, A210 in domain II, and A111 in domain III of *P. denitrificans* ETF (Fig. 1). X- and Q-band DEER measurements of enzymatically reduced, spin-labeled ETF mutants were performed to determine whether the distance distributions depend on differences in orientation selectivity at X- and Q-band. A β E162A mutation (corresponding to β E165A in human ETF) was added, which has been proposed to cause conformational changes that approximate ETF interacting with a redox partner.¹⁵

Results and Discussion

Characterization of ETF mutants

SDS-PAGE confirmed the purity of the *P. denitrificans* ETF mutants: bands near 30 and 26 kD correspond to the α -subunits (30.5 kD) and β -subunits

Figure 2. (A) CW EPR spectra of spin-labeled *P. denitrificans* ETF mutants at 270 K. The spectrum of the β A111C/E162A mutant was similar to that of the β A111C mutant.¹⁷ (B) DCPIP reduction activities of *P. denitrificans* ETF mutants, monitored by optical spectroscopy at 600 nm. Error bars represent the standard deviation among multiple trials.

(26.6 kD), respectively. Purity also was determined from the A_{270}/A_{436} ratio of 6.5 ± 0.3 , which was similar to the literature value of 5.8 for wild-type *P. denitrificans* ETF.¹⁶ Optical spectra of the oxidized (as isolated) proteins and spin-labeled mutants were similar to the wild-type spectrum (Supporting Information Fig. S1), containing two FAD peaks at 375 and 436 nm and a shoulder near 460 nm.¹⁸ An extinction coefficient (ϵ) at 436 nm of $13.6 \text{ mM}^{-1} \text{ cm}^{-1}$ was used to calculate values of $\epsilon_{375\text{nm}}$ between 12.1 and $12.2 \text{ mM}^{-1} \text{ cm}^{-1}$ in the mutants, which are similar to the literature value of $12.0 \text{ mM}^{-1} \text{ cm}^{-1}$ for wild-type ETF.¹⁶ Since the positions of the maxima in the flavin spectra are very sensitive to environment, the absence of shifts indicates that, as expected, spin labeling at α A43C, β A111C, or α A210C does not change the FAD environment.

Spin labeling sites in solvent-exposed, alpha helical regions of the protein were selected, which is expected to have negligible impact on structure,^{19,20} and permits labeling without unfolding the protein. Prolonged exposure (4°C, 16 h) of wild-type *P. denitrificans* ETF to 5-fold molar excess MTSL over protein demonstrated that there is no spin labeling of the single native cysteine residue (α C36), so this residue does not interfere with labeling at cysteines introduced by site-directed mutagenesis. The continuous wave (CW) EPR spectra of the spin-labeled ETF mutants [Fig. 2(A)] are characteristic of partially immobilized nitroxyl, confirming attachment of the spin label to the protein. Comparison of normalized integrals of the CW EPR signals with a tempol (4-

hydroxy-2,2,6,6-tetramethylpiperidino-1-oxy) standard shows that MTSL is incorporated stoichiometrically. The absence of sharp lines in the CW spectra confirms the removal of excess MTSL. Additional lines, which would be indicative of multiple spin label conformations, were not seen.²¹ CW lineshapes suggest that the motion of MTSL labels increases in the order: α A210C < α A43C < β A111C.²¹

The relative rates at which electrons are shuttled through ETF from its biological electron donor, glutaryl-CoA dehydrogenase, to the electron acceptor 2,6-dichlorophenol-indophenol (DCPIP) are shown in Figure 2(B) for spin-labeled β A111C, β A111C/E162A, α A210C, and α A43C ETF. Rates for the β A111C and α A210C spin-labeled mutants are similar to wild type ($93 \pm 3\%$ and $107 \pm 7\%$, respectively). The rate for spin-labeled α A43C ETF is lower than for wild type ($78 \pm 2\%$). A spin label at this site is not expected to interfere with the FAD or recognition loop, so the lower rate was unexpected. The rate for spin-labeled β A111C/E162A is more than twice that of the wild-type enzyme ($223 \pm 11\%$). A similar increase in activity was found for the corresponding β E165A mutant in human ETF, which was attributed to an increase in population of the electron transfer competent conformation, consistent with a concomitant 7-fold decrease in K_m .¹⁵

Enzymatic reduction of spin-labeled ETF mutants

Enzymatic reduction was performed at pH 8.0 to stabilize the FAD $\text{SQ}^{\bullet-}$. The concentration of FAD

SQ^{-•} was calculated using $\epsilon_{375} = 17 \text{ mM}^{-1} \text{ cm}^{-1}$ ¹⁶ and quantitation of the semiquinone signal in CW EPR spectra at 100 K.⁸ 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.²²

DEER measurements

DEER is a pulsed electron paramagnetic resonance (EPR) technique that is used to determine distributions of distances between unpaired electrons.^{23,24} It is commonly applied to biological systems and has recently been used to study conformational changes in the flexible protein cytochrome P450 reductase.²⁵ Four-pulse DEER measurements at X- and Q-band were performed on enzymatically reduced, spin-labeled ETF mutants. Field-swept echo-detected spectra, marked to indicate the relative locations of the pump and observe pulses used in the DEER experiments, are shown in Figure 3. To maximize the number of spins excited,²⁴ the X-band pump frequency was set to the maximum echo intensity, near $g = 2.006$. At this position the signals from the FAD SQ^{-•} and the perpendicular region of the MTSL spin label signal overlap. The observe frequency was offset by 60–65 MHz to higher frequency (lower magnetic field), where 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. Q-band DEER measurements were performed by pumping near the maximum of the FAD SQ^{-•} signal and observing at ~37 MHz higher frequency. At Q-band the pump pulse was not located on the maximum echo because that position corresponds primarily to MTSL and not FAD SQ^{-•}.

DEER signals at X- and Q-bands obtained with the same temperature, number of points, shots per point, shot repetition time, time step, and number of scans are compared in Figure 3. A signal-to-noise improvement of ~10 was calculated for Q-band relative to X-band, which is consistent with the improvement in signal-to-noise of 13 reported previously for interaction of two spin labels.^{26,27}

Time domain dipolar evolution signals at X- and Q-band, after background subtraction, are shown in Figure 4 (DEER data prior to background subtraction are shown in Supporting Information Figures S2 and S3). Larger numbers of scans were averaged at X-band to give signal-to-noise that is similar to that at Q-band. Modulation depths were slightly larger at X-band, consistent with excitation of a larger fraction of the spins by the shorter, less selective pump pulses. Dipolar evolution signals were fit to a single Gaussian, two Gaussians or by Tikhonov regularization using the program DeerAnalysis2009.²⁸ As shown in Supporting Information Fig-

Figure 3. Comparison of X- and Q-band DEER measurements. Positions of the pump (solid arrows) and observe (dashed arrows) pulses are shown on the field-swept echo-detected spectra for enzymatically reduced, spin-labeled α A43C ETF (top). Contributions from MTSL³⁹ (short dashes) and FAD SQ^{-•40} (long dashes) to the field-swept echo-detected spectra were simulated using a locally written program⁴¹ and are shown, with arbitrary y-axis scales. Time domain DEER dipolar evolution signals for enzymatically reduced, spin-labeled β A111C ETF (95 μ M MTSL, ~45 μ M FAD SQ^{-•}) were recorded at 80 K, averaging 23 scans overnight, followed by a second degree polynomial background subtraction (bottom). X- and Q-band data are shown in gray and black, respectively. Signal-to-noise at Q-band is enhanced by ~10 times relative to X-band. The sample within the active volume of the resonators was ~40 μ L for X-band and ~5 μ L for Q-band.

ure S3, fits to the dipolar evolution curves obtained by Tikhonov regularization were marginally better than for one or two Gaussians. Therefore results are reported for single Gaussians for all samples except α A43C at Q-band for which data were fit with two Gaussians (Table I).

FAD-nitroxyl distance distributions obtained by DEER at X-band and Q-band are similar (Table I, Fig. 5), except for α A43C. The EPR signal extends over about 75 G at X-band and about 90 G at Q-band (Fig. 3). The smaller available power at Q-band (1 W) than at X-band (1 kW), required longer 90° pulses at Q-band than at X-band. The longer Q-band pulses excite a smaller segment of the spectrum, which combined with the larger spectral extent, makes the excitation substantially more selective than at X-band. This can cause orientation selection, where only certain spin label conformations relative to the interspin axis are excited and

Figure 4. X- and Q-band dipolar evolution signals for enzymatically reduced, spin-labeled ETF mutants after background subtraction (gray). Fits to a single Gaussian are shown as dashed black lines, except for α A43C at Q-band which was fit with the sum of two Gaussians.

observed in the DEER measurement.²⁹ The Pake patterns (Supporting Information Fig. S4) for α A43C, β A111C, β A111C/E162A show negligible evi-

dence of orientation selection at X- or Q-band. For α A210C, which has shorter interspin distances, the largest dipolar couplings are undersampled at Q-band. The small impact of orientation selection is attributed to many conformations of the spin label such that, for example, a pulse along the magnetic z -axis of the nitroxyl excites spins that have many different orientations of the interspin vector relative to the external magnetic field. The similarity between the distance distributions for β A111C and β A111C/E162A indicates that the additional mutation had little impact on the solution conformations.

Modeling of MTSL bound to ETF

Modeling was performed to relate the interspin distances to the structure of the protein. Three approaches were taken to model the conformations of MTSL bound to ETF based on the crystal structure of *P. denitrificans* ETF.¹¹ The C4a atom of the FAD was used as the centroid of spin density for the semiquinone.³⁰ (i) The native amino acids were replaced by a cysteine to which the MTSL was added. Energy minimization was performed allowing only the MTSL and cysteine residue to vary. (ii) Molecular dynamics (MD) simulations with 10 ns runs were performed to model the effect that MTSL motion would have on the interspin distance distributions. Mutated cysteine residues and MTSL labels were allowed to move while the rest of the protein structure was fixed. (iii) *In silico* spin labeling experiments also were performed using the MMM2009 software.³¹ Stable MTSL conformations at the three labeling positions were calculated using a rotamer library (at 175 K) contained in the program.³¹ A second spin density was added to the C4a position of the FAD, and distance distributions between the labels and FAD were calculated. Because it is less computationally intensive, rotamer library analysis is an attractive alternative to MD simulations for modeling interspin distance distributions. The average interspin distances calculated using these procedures are summarized in Table II.

For α A210C the distance obtained by DEER (2.8 ± 0.15 nm) is within 0.2 nm of the values obtained

Table I. Average Nitroxyl to FAD Distances (nm) and Distribution Widths at X- and Q-Band^{a,b}

ETF Mutant	X-Band		Q-Band	
	Avg Distance	Width	Avg Distance	Width
α A210C	2.7 ± 0.03	0.3 ± 0.02	2.8 ± 0.03	0.3 ± 0.02
β A111C	4.3 ± 0.02	0.3 ± 0.04	4.3 ± 0.02	0.3 ± 0.03
β A111C/E162A	4.3 ± 0.02	0.5 ± 0.02	4.3 ± 0.01	0.3 ± 0.01
α A43C	4.3 ± 0.02	0.7 ± 0.02	$4.0, 4.5^c \pm 0.02$	0.7 ± 0.1

^a Distances and full-width at half-height distribution widths for a single Gaussian, in nm. Values cited in the text are $\pm$ half width at half height.

^b Uncertainties were estimated by shifting t_0 by ± 10 ns and changing the start and end point for the fitting of the background.

^c Fit with two Gaussians. If a single broad Gaussian is used the average distance is 4.4 nm.

Figure 5. Left: Calculated interspin distances, measured between MTSL oxygens and the C4a position of the FAD (inset), based on the crystal structure of *P. denitrificans* ETF (1EFP).¹¹ Cysteine mutations, the addition of MTSL spin labels to the crystal structure and the energy minimization of MTSL conformations were made using Discovery Suite software (Accelrys). Right: distance distributions obtained from DEER data by fitting to a single Gaussian, expect for α A43C at Q-band, which was fit with the sum of two Gaussians. The y-axis are probabilities. Results from X- and Q-band measurements are shown in gray and black, respectively.

by the three methods to model the conformations of the spin label attached to the crystal structure of *P. denitrificans* ETF (2.7–3.0 nm). Since the α A210C label is in the same subunit as the FAD, the spin-label to FAD distance is independent of the orientation of domain II relative to domains I and III. For β A111C the distance obtained by DEER (4.3 ± 0.15 nm) is significantly longer than the distances obtained by the three modeling methods (3.8–4.1 nm). For α A43C the interspin distance obtained by DEER ($4.0\text{--}4.5 \pm 0.35$ nm) is again significantly longer than the distances found by the modeling methods (3.3–3.9 nm). The discrepancies for α A43C and β A111C, which are in domains I and III, respectively, suggested that the orientations of the FAD-containing domain II in solution are different than in the crystal structure.

Homology modeling of ETF bound to MCAD

A homology model of *P. denitrificans* ETF bound to MCAD was created using the program ESyPred3D.³² The crystal structure of human ETF in the electron transfer active ETF:MCAD complex (2A1T)¹³ was used as the template. MTSL molecules were added to the model and the conformations of the labels were energy minimized. Since FAD is not present in the ETF:MCAD structure, glutamate 263 in the α II domain was used to approximate the centroid of FAD SQ[•] spin density.¹⁷ Based on the structure of *P. denitrificans* ETF¹¹ the distance from spin label at β A111C to FAD C4a is the same as β A111C to α Q263. For the two other labeling sites the distance

to α Q263 is within 0.2 nm of the distance to FAD C4a. MD simulations were performed to model the range of conformations adopted by spin labels at α A43C and β A111C. Distances between MTSL labels and α Q263 in the ETF:MCAD 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 α II domain of ETF changes conformation to facilitate electron transfer to a redox partner (Fig. 6). For the conformations found by MD, the change in interspin distance on going from a closed

Table II. Comparison of Average Distances(nm)^a

Label Site	Spin Label			
	DEER ^b	Energy Minimization ^c	MD Simulation ^d	Rotamer Analysis ^e
α A210C	2.8	3.0	2.7	2.8
β A111C	4.3	4.1	3.9	3.8
α A43C	4.0–4.5	3.9	3.3	3.4

^a The C4a atom of the FAD is used as the centroid of the spin density. Distances were calculated to the nitroxyl oxygen.

^b DEER values are averages of X- and Q-band results.

^c Local energy minimization of spin label and mutated cysteine attached to the crystal structure of *P. denitrificans* ETF (1EFP).¹¹

^d Average values from MD simulations performed on MTSL labels attached to the crystal structure of *P. denitrificans* ETF (1EFP).

^e Average values from rotamer library analysis performed on MTSL labels³¹ attached to the crystal structure of *P. denitrificans* ETF (1EFP).

Figure 6. Distances between spin label sites and the approximate FAD location in (A) the crystal structure of *P. denitrificans* ETF (1EFP) and (B) the homology model of *P. denitrificans* ETF based on the structure of the human ETF:MCAD complex (2A1T). Distances are measured between MTSL (black ball and stick) oxygen atoms and the β carbon of α Q263 (gray ball and stick).

to open conformation is 1.1 nm for the label at the β A111C site and 0.6 nm for label at the α A43C site.

Comparison of distance distributions

The distance distributions determined by DEER are overlaid in Figure 7 on the distance distributions calculated by MD based on the crystal structure and homology model of ETF and by rotamer library analysis based on the crystal structure of ETF. For α A210C (domain II) the DEER distance distribution agrees well with the results of the MD simulations and reasonably well with the results from the rotamer analysis. For this label the width of the DEER distribution is attributed to conformations of the MTSL spin label as opposed to ETF domain motion.

For α A43C (domain I) the MD simulations and rotamer analysis indicate a wide range of conformations of the spin label (Fig. 7), which is consistent with a wide range of interspin distances found by DEER. The modeling of the spin label conformations was based on fixed conformations of the protein backbone. Since α A43C is near the end of a small alpha helix, protein flexibility may also contribute to the wide distance distribution at X-band and two distance distributions at Q-band. These factors make it difficult to determine, based on this mutant, the range of orientations of domain II. The distances found by DEER are intermediate between the values predicted for free (closed) ETF and ETF bound to MCAD.

The distance distributions found by MD and rotamer analysis are narrower for β A111C than for α A43C, which indicates fewer conformational options for the label at this location. The distance distribution found by DEER is again intermediate between the distances predicted for the free or bound ETF. The wider distribution of distances found by DEER than by MD or rotamer analysis for β A111C suggests that some of the width of the DEER distance

distribution may be due to variations in the orientation of domain II relative to domains I and III.

The DEER data demonstrate that the conformations of domain II in solution are different from that in the X-ray structures of human and *P. denitrificans* ETF.^{11,12} This result is consistent the SAXS data on human and *P. denitrificans* ETF, which showed rotation of domain II by 30–50°. This rotation provides access of the dehydrogenases to the FAD, to facilitate electron transfer. Determination of the range of conformations in solution will require data from additional mutations at multiple locations on the three domains.

Figure 7. Molecular dynamics (MD) simulations of MTSL spin label to FAD distances based on the crystal structure of *P. denitrificans* ETF (red histograms) and the homology model of *P. denitrificans* ETF based on the structure of human ETF:MCAD complex (green histograms). MTSL rotamer library analysis gave distance distributions shown as blue lines.³¹ Distance distributions calculated from Q-band DEER data are shown as black lines.

Conclusions

DEER measurements, at X- and Q-band, of the distance between the enzymatically reduced FAD and MTSL spin labels in the three structural domains of *P. denitrificans* ETF are similar, indicating very little orientation selection. Measurements at Q-band gave a signal to noise enhancement of approximately 10 fold relative to X-band. Interspin distances obtained from DEER measurements were 4.3 ± 0.35 , 2.8 ± 0.15 , and 4.3 ± 0.15 nm for labels in domain I (α A43C), II (α A210C), and III (β A111C), respectively. For the α A210C site the distance obtained by DEER is in good agreement with interspin distances calculated from the crystal structure and distributions calculated from MD simulations and rotamer library analysis. Because α A210C and the FAD are located on the same subunit, a change in the orientation of this subunit will not change the FAD-nitroxyl interspin distance. For both β A111C and α A43C the distances obtained by DEER are longer than expected for free ETF and are shifted toward distances expected for ETF bound to an electron transfer partner. These results are consistent with the α II domain adopting orientations in solution that deviate from the crystal structure of free ETF towards the active, substrate-bound orientation.

Experimental Procedures

Preparation of ETF mutants

Site-directed mutagenesis of the *P. denitrificans* ETF genes were conducted using the QuikChange® II XL site-directed mutagenesis kit (Stratagene).³³ The two genes were previously subcloned into the pET-28a(+) expression vector at the XbaI/HindIII restriction site. Mutated plasmids were transformed into BL21-Gold(DE3)® Ultracompetent *E. coli* cells. Plasmid DNA was isolated using the QIAprep Spin Miniprep Kit (QIAGEN). Levels of expression by transformed cells were determined by western blots of cells from single colonies. Sequencing, to confirm mutations, was performed at the University of Colorado Denver Cancer Center DNA Sequencing and Analysis Core. Single colonies expressing high levels of ETF were used to create cell stocks that were stored at -80°C . Expression, purification, and spin labeling of ETF mutants were done as described previously.¹⁷

Expression and purification of *P. denitrificans* glutaryl CoA dehydrogenase

The glutaryl CoA dehydrogenase that was used to enzymatically reduce ETF was isolated from *P. denitrificans* (ATCC 13543) grown at 30°C in media containing 2% glutaric acid as the carbon source.³⁴ Cells were harvested late during the exponential phase and then broken using two passes through a French

pressure cell at 1000 psi. Cell membranes were removed by centrifugation at $100,000g$ for 90 min. Glutaryl CoA dehydrogenase was purified by the method of Husain and Steenkamp³⁴ except that the first DEAE-cellulose column was not used and the purification started with the DEAE-Sepharose column. Glutaryl-CoA dehydrogenase-containing fractions were identified by enzyme assay and UV-visible absorption spectroscopy. These fractions were pooled and dialyzed against 10 mM potassium phosphate, pH 7 containing 10% glycerol. Purified glutaryl-CoA dehydrogenase was stored at -80°C .

Enzyme assay

The activities of wild type and spin-labeled ETF mutants were measured in a coupled reaction containing 0.1M potassium phosphate buffer, pH 7.5, 0.4 μM *P. denitrificans* glutaryl-CoA dehydrogenase, 10 μM glutaryl CoA and 57 μM 2,6-dichlorophenol-indophenol (DCPIP) at 25°C . DCPIP reduction was initiated by the addition of ETF, giving a final concentration of ~ 10 nM. Reduction of DCPIP was monitored by the decrease of absorbance at 600 nm (DCPIP, $\epsilon = 20 \text{ mM}^{-1} \text{ cm}^{-1}$). Baselines were measured for 40 seconds before the addition of ETF. Kinetic data were recorded for an additional 140 seconds after the addition of ETF. Activities of the spin-labeled mutants, expressed as turnover numbers (min^{-1}), are reported as percent of wild-type ETF.

Enzymatic reduction of spin-labeled ETF mutants

The flavin cofactor of spin-labeled ETF mutants was reduced with glutaryl-CoA under anaerobic conditions at pH 8.0 in the presence of a catalytic concentration of *Paracoccus* glutaryl-CoA dehydrogenase.¹⁷ FAD $\text{SQ}^{\bullet-}$ formation was followed by monitoring the absorbance at 375 nm, which increases as the semiquinone is formed, but decreases upon formation of the hydroquinone. When the absorbance at 375 nm reached a maximum ($\sim 50\%$ yield of FAD $\text{SQ}^{\bullet-}$), samples 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 1.6 mm o.d. quartz EPR tubes using a gastight syringe inside a glove bag under constant N_2 flow. Samples were rapidly frozen in liquid nitrogen immediately after transfer and the vapor space was subjected to five cycles of alternate evacuation and purging with helium gas. Rapid freezing is expected to trap the protein in conformations sampled at temperatures between ambient and the freezing point of the solution. Samples were then flame sealed and stored in liquid nitrogen until spectroscopic measurements were performed.

Continuous wave EPR spectroscopy

CW EPR spectra were recorded using the parameters reported in the initial study of the β A111C mutant.¹⁷

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 observe pulse of $\pi/2 = 16$ ns. Inter pulse delay times were optimized for each sample, and varied between 124 to 130 ns and 2300 to 3200 ns for τ_1 and τ_2 , respectively. ELDOR pulse lengths varied from 38 to 40 ns and were offset by 60–65 MHz higher frequency from the observe pulses. Data were recorded in 4 or 8 ns steps with 1024 shots at each time point. The repetition time (1.5–3.6 ms) was set to about 1.2 times the T_1 relaxation time of the samples, which has been shown to be an acceptable compromise between incomplete return to equilibrium and an increased number of averages per unit time.³⁵ Multiple scans were averaged using eight step phase cycling. For data sets in which proton modulation was observed (Supporting Information Fig. S2), the sharp peak in the Fourier transformed data corresponding to the modulation frequency was manually removed, followed by reverse Fourier transformation before the DEER analysis was performed. DEER measurements on a standard sample with 2.8 nm interspin distance (provided by Dr. Jeschke, ETH Zurich) with the same instrumental parameters was used to determine the zero time (t_0) of ~ 50 ns.

Q-band four-pulse DEER measurements at 80 K were performed on a Bruker ELEXSYS E580 spectrometer equipped with a Super Q-FT bridge, an E580-400U ELDOR unit, an EN 5107D2 Q-band resonator and an Oxford CF 935 cryostat. Pulse lengths were optimized for each sample and ranged from 28 to 38 ns for the $\pi/2$ observe pulse and 60–66 ns for the ELDOR π pump pulse. The ELDOR pulse was offset by 37 MHz higher frequency than the observe pulse. The delay time between the $\pi/2$ and π observe pulses (τ_1) was optimized for each sample to give maximum echo and ranged from 120 to 136 ns. The delay between the first and second π observe pulses (τ_2) was 3400 ns for all mutants except α A210C, where it was 1650 ns. A shorter τ_2 was used for α A210C because of the shorter interspin distance. Data were recorded in 8 ns steps using 1024 shots at each time point. At each point, 1024 shots were done using a shot repetition time (1.0–2.4 ms) that was 1.2 times the T_1 relaxation time. Multiple scans were averaged using eight step phase cycling.

DEER data were analyzed using the program DeerAnalysis2009.²⁸ Background correction, using a

second degree polynomial, was used to separate the long distance intermolecular interactions from the intramolecular interaction of interest. Fourier transformation of the DEER traces in Figure 4 gives characteristic Pake pattern (Supporting Information Fig. S4). The shape of the Pake pattern is dependent on the function that was used for the background subtraction and the interval selected to define the background, so it was used as a measure of the quality of the background subtraction. Data were fit to distances between 2.0 and 8.0 nm using one or two Gaussians or Tikhonov regularization.³⁶ The uncertainties in the distance distributions were estimated by comparing results obtained with different fitting models. The widths of the distributions at half height are less well defined than the average values.

MD simulations of spin label conformations

Residues α A43, α A210, and β A111 were replaced with cysteine residues in the crystal structure of *P. denitrificans* ETF (1EFP)¹¹ using the biopolymer function of the Discovery Studio program (Accelrys). Cysteine residues were then modified by adding a MTSL spin label. MD simulations were performed using Discovery Studio 2.5 (Accelrys). The structure of ETF with attached spin labels was prepared for simulations by protonation at pH 8 and atoms were typed using CHARMM.³⁷ The positions of all atoms except those of the cysteine residue and the MTSL substituent were constrained. Energies of the MTSL labels were minimized individually using the smart minimizer algorithm with 200 maximum steps, an RMS gradient of 0.1 kcal/mol $\text{\AA}$, an energy change tolerance of 0.0 kcal/mol and a dielectric constant of 1 (vacuum). The motion of each MTSL label was analyzed using the Standard Dynamics Cascade protocol, which consists of energy minimization, heating, equilibrium, and production stages.³⁸ Energy minimization was done in two parts, first using the steepest descent algorithm with 500 steps with a RMS gradient of 0.1 kcal/mol $\text{\AA}$, followed by the conjugate gradient algorithm with 500 steps with a RMS gradient of 0.0001 kcal/mol $\text{\AA}$. Next the temperature was increased from 50 to 600 K in 1 ns, which was followed by a 1 ns equilibration at 600 K. Finally a 10 ns MD production run at 300 K was performed using 2 fs steps and 320 trajectories were saved. MD simulations were carried out using the distance-dependent dielectrics implicit solvent model, with a dielectric constant of 80, as well as the SHAKE constraint, which fixes all bonds to hydrogen atoms. Nitroxyl-FAD distances (between the oxygen of the MTSL and the C4a position of the FAD) from the MD production runs were monitored using the Analyze Trajectories protocol. Distances were sorted from smallest to largest and were rounded to two significant figures. Histograms of the distance distributions were constructed by adding

the number of trajectories with nitroxyl-FAD distances in 0.025 nm steps.

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