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

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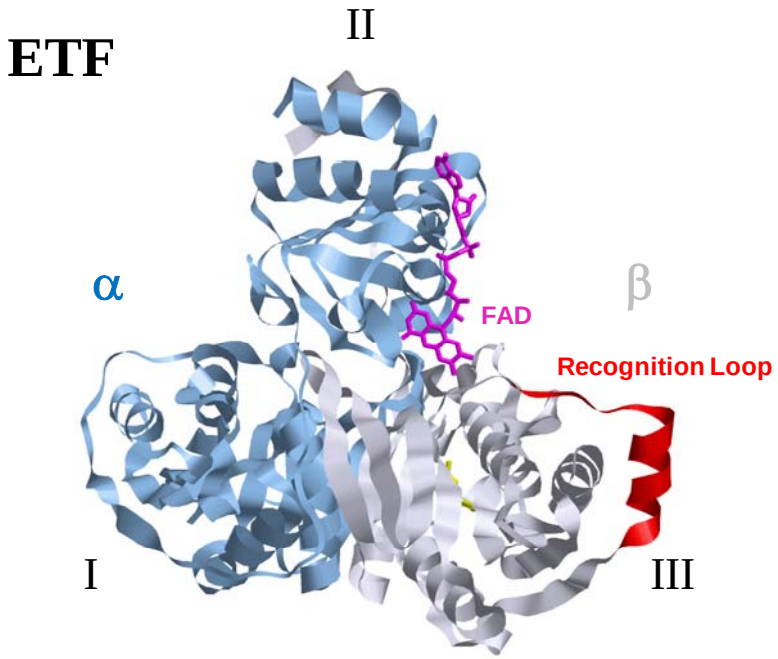
Outline

- Electron Transfer Flavoprotein
- Designing the DEER Experiments
- X-band vs. Q-band
- Distance Distributions
- Interpreting Distances from DEER

Electron Transfer Flavoprotein (ETF)

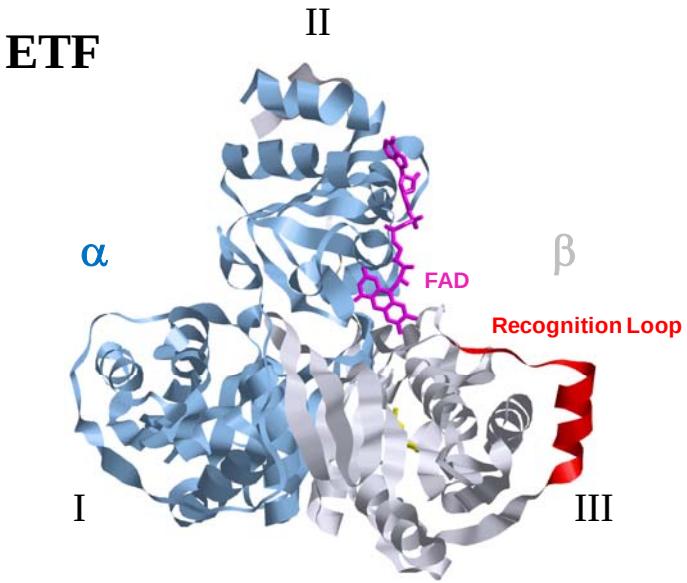
- Mitochondrial fatty acid oxidation is the major source of energy for heart, kidney and type I (slow twitch) skeletal muscle fibers.
- The first oxidative step is catalyzed by the acyl-CoA dehydrogenases.
- The electron transfer pathway is

  Dehydrogenase → ETF → ETF-QO →Ubiquinone → complex III of main respiratory chain
- Defects in ETF or ETF-QO result in the metabolic disease multiple acyl-CoA dehydrogenation deficiency.



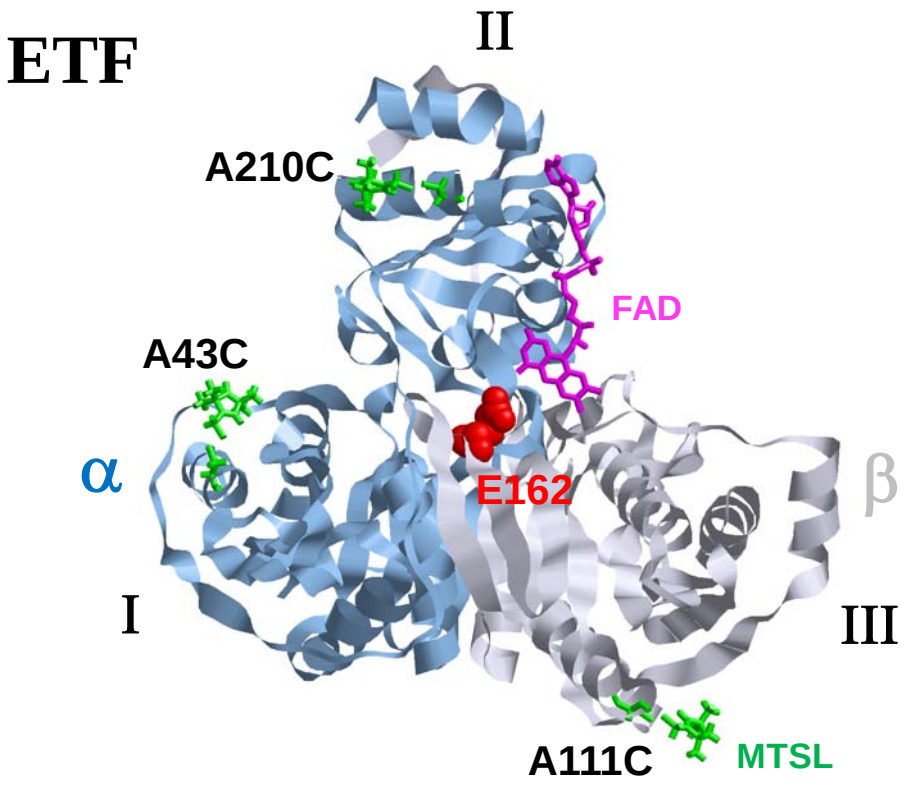
- Mammalian ETF is located in the mitochondrial matrix.
- Reactivity is very similar for mammalian and bacterial ETF.
- It is a soluble heterodimer.
- It contains one FAD, which accepts electrons from at least 10 different flavoprotein dehydrogenases and transfers the electron to ETF-QO.
- In the crystal structures of mammalian and bacterial ETF the FAD is located in a crevice between domains II and III.

Conformational Sampling by Domain II



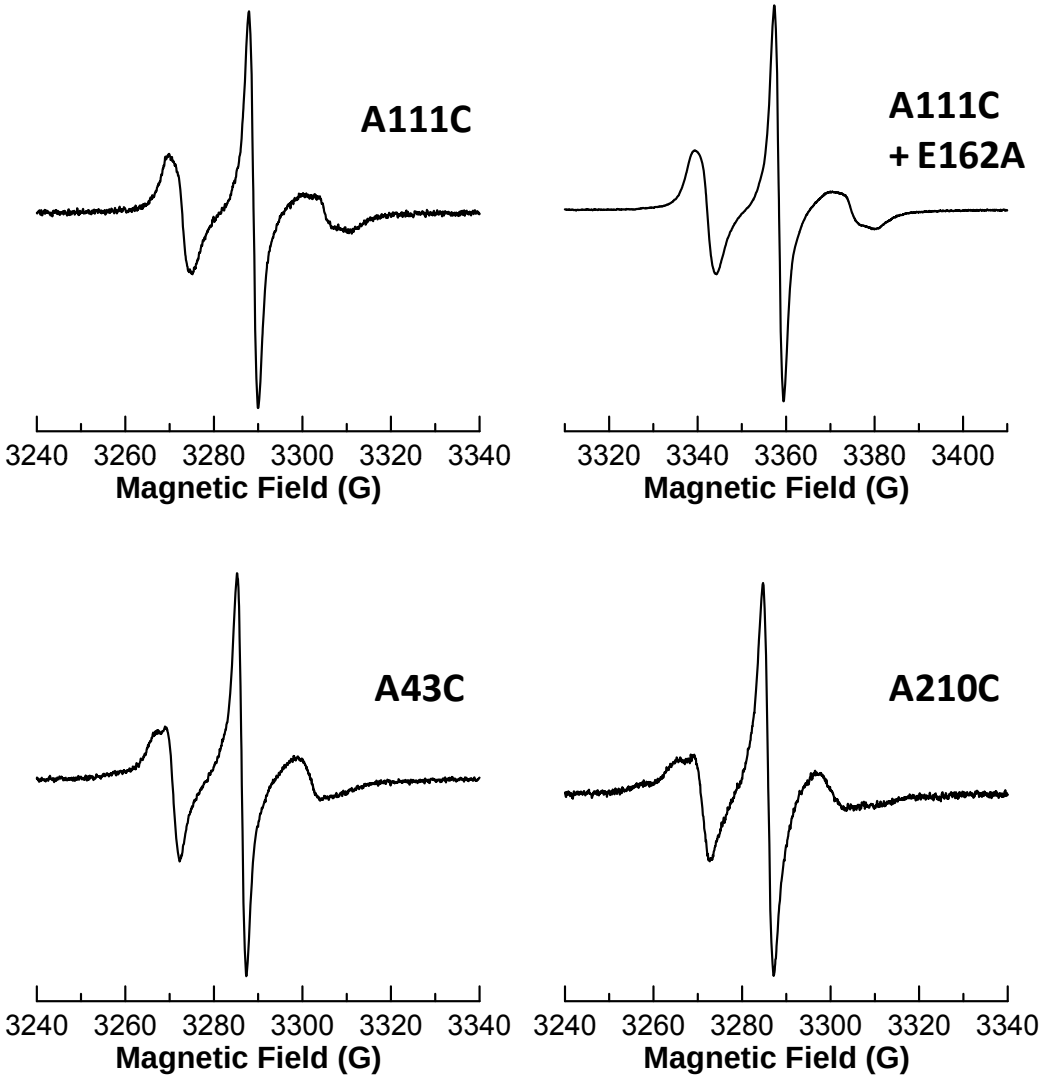
In structural studies of human and bacterial ETF, free and bound to electron transfer partners, the folds within the 3 domains are similar, but the orientation of domain II relative to domains I and III varies dramatically.

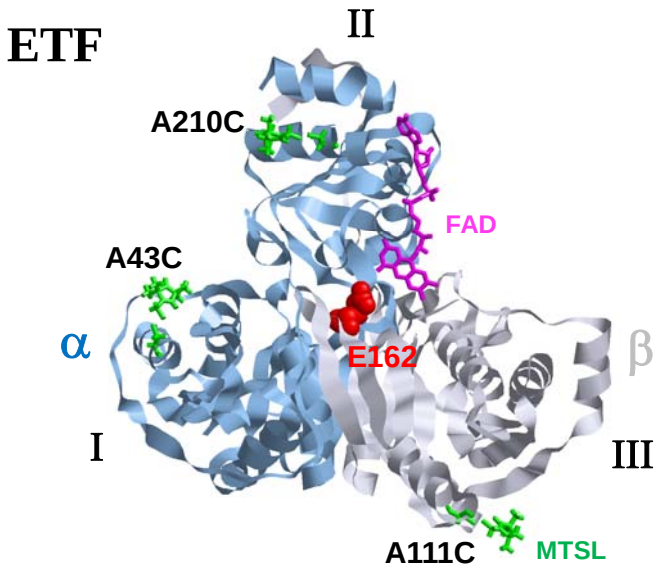
- In crystal structures of complexes of ETF with dehydrogenases, domain II is highly disordered.
- Multiple conformations of domain II were required to model small-angle solution X-ray scattering (SAXS) of human and *P. denitrificans* ETF.
- It was proposed that upon binding to a redox partner domain II rotates by 30 to 50^{o} relative to an axis defined by domains I and III.
- ETF may need to adopt a variety of conformations to accept electrons from so many different dehydrogenases.



- MTSL label sites were added to *P. denitrificans* ETF: domain I – A43C, domain II – A210C, domain III – A111C.
- Additional mutation, E162A, is proposed to stabilize the open conformation.

CW Spectra at 270 K, as isolated

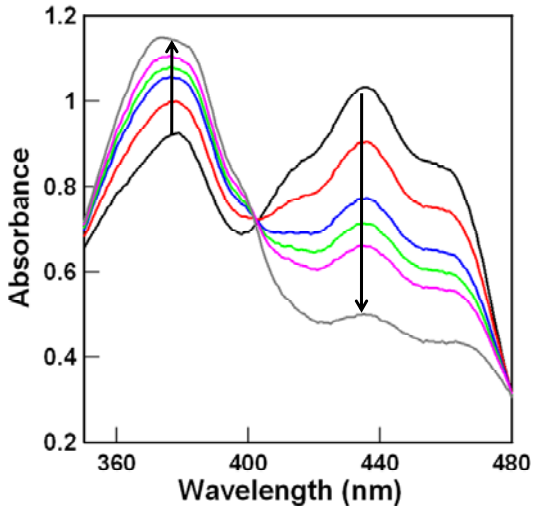




Spectra show substantial immobilization.
Double integrals show full labeling.

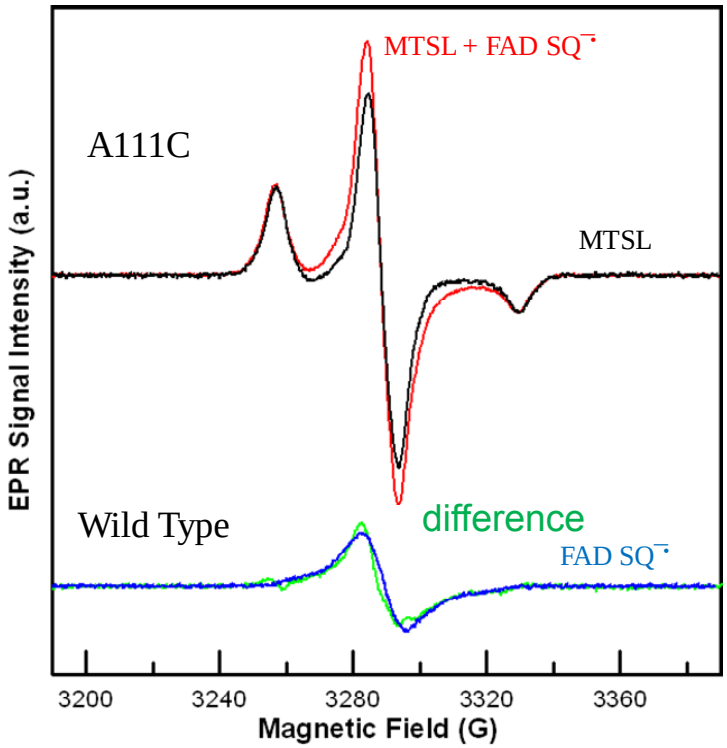
Reduction of FAD to semiquinone

Reduction conditions
- pH = 8, anaerobic
- ETF (~100 μM)
- glutaryl CoA (~800 μM)
- glutaryl-CoA dehydrogenase (~5 nM)



~50–60% of ETF was reduced to FAD $SQ^{-\bullet}$ before a decrease in absorbance at 375 nm indicated disproportionation.

Spectra @ 100K



Double integral indicated ~ 50% reduction to FAD $SQ^{-\bullet}$.

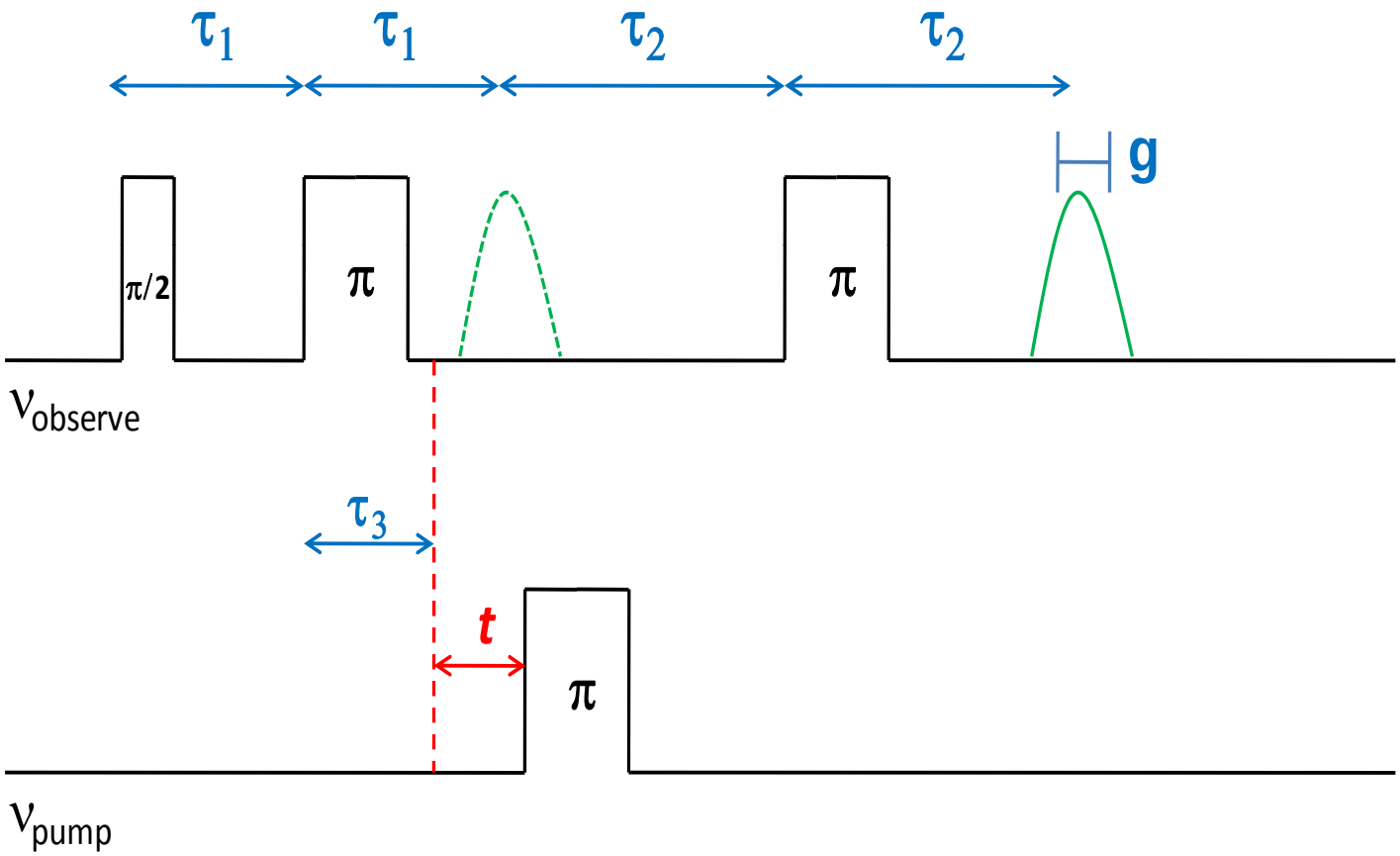
Methods to Determine Distances between Slowly Relaxing Spins

Methods based on continuous wave (CW) EPR - distances up to about 20 Å

- Intensity of forbidden transition at 'half-field'
- Simulation of lineshape changes
- Fourier analysis to determine broadening function

Pulsed EPR double resonance (DEER) – distances up to about 60 Å, and distribution width

Four-pulse DEER

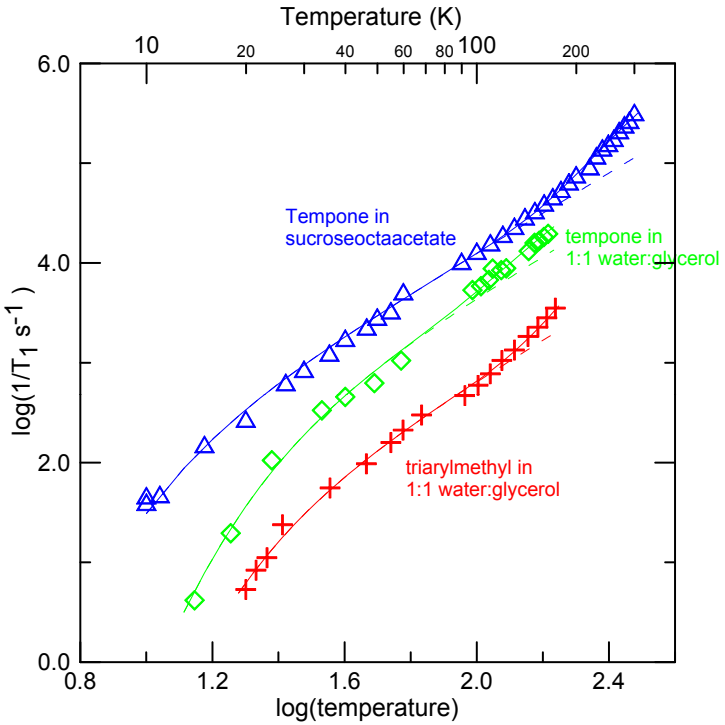


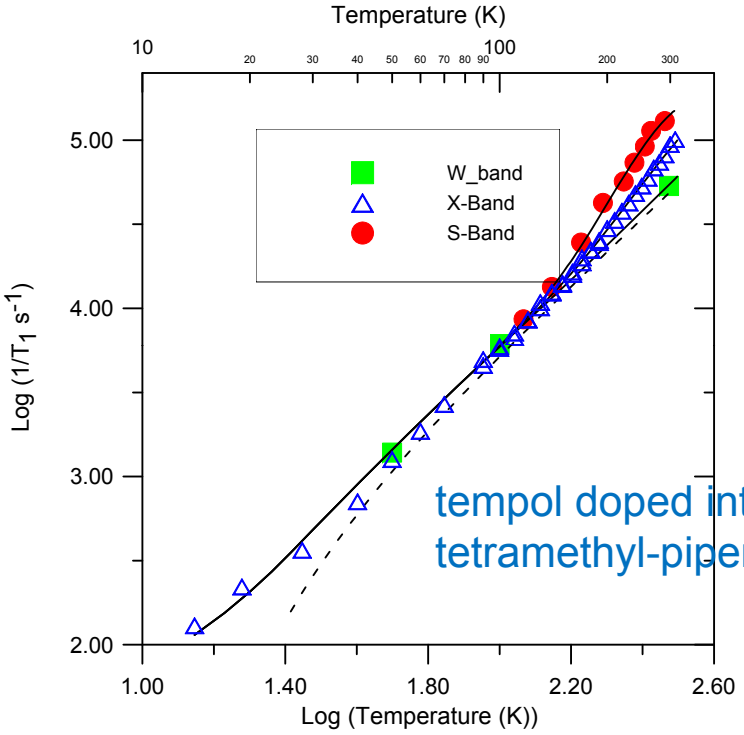
Selection of Temperature for DEER Measurements

- EPR signal is proportional to the difference in Boltzmann populations of spin energy levels, which depends approximately linearly on 1/temperature.

- However, as temperature decreases T_1 gets longer, which means that the pulse repetition rate must be slower.

Temperature Dependence of Nitroxyl T_1

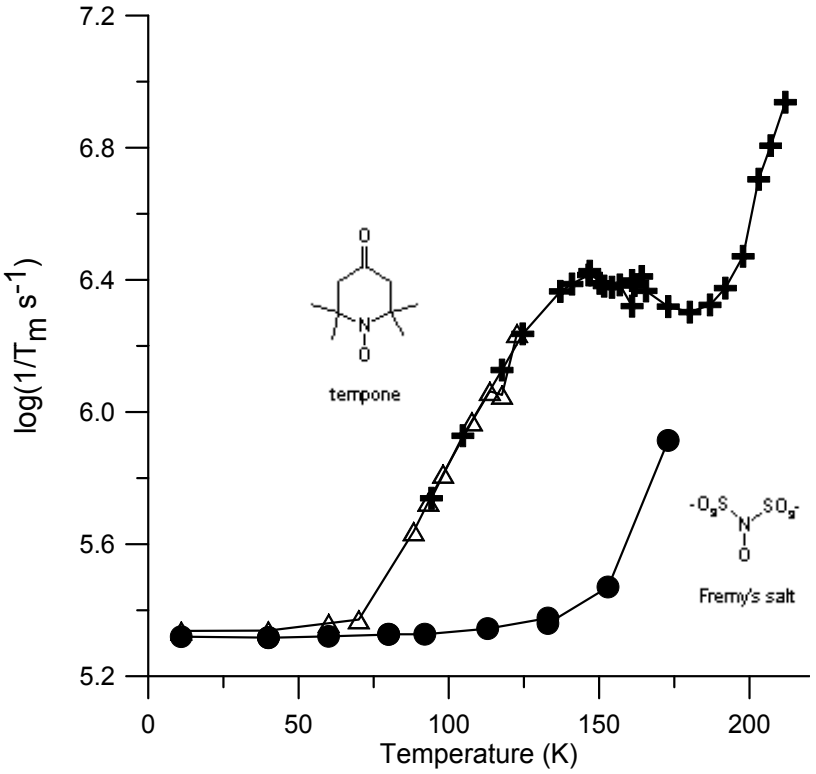




tempol doped into 4-hydroxy-tetramethyl-piperidinol

- T_1 has T^n dependence with n > 2, so if increase T by factor of 2 can increase rep rate by more than a factor of 4.
- Signal intensity varies as ~ T^{-1}, so if increase T by factor of 2, lose a factor of 2 in S/N.
- Faster rep time more than compensates for loss in Boltzmann population.
- T_1 at about 100 K is not frequency dependent.

Temperature Dependence of Nitroxyl T_m

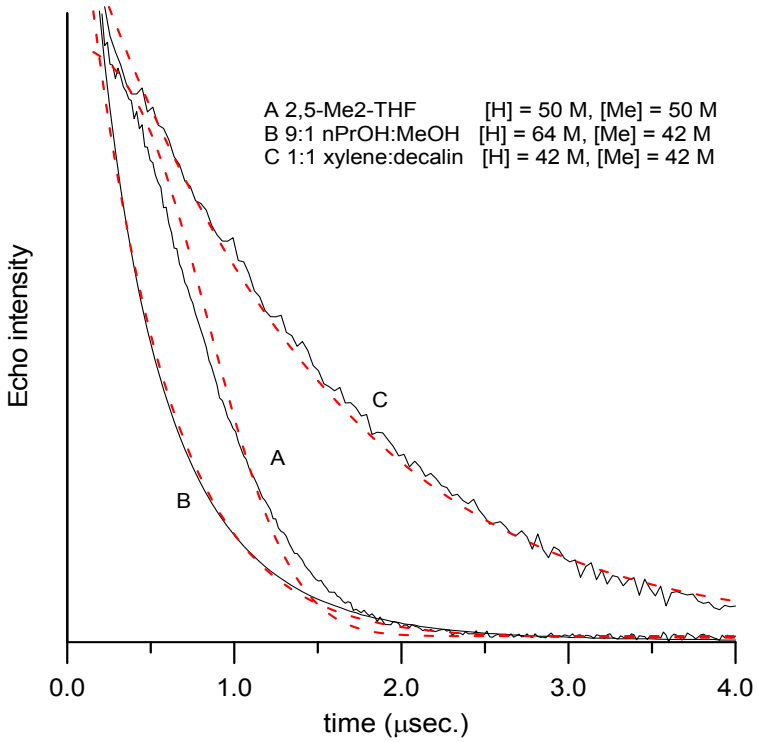


- Below about 60 K dephasing is dominated by spin diffusion of solvent nuclei.
- Above about 60 K rotation of nitroxyl methyl groups at rates comparable to the anisotropy of electron proton couplings dominates dephasing.

When consider T_1, Boltzmann populations, and T_m, optimum temperature for DEER with nitroxyls typically is about 60 K.

Why is T_m for spin labels on proteins sometimes shorter than in H_2O:glycerol?

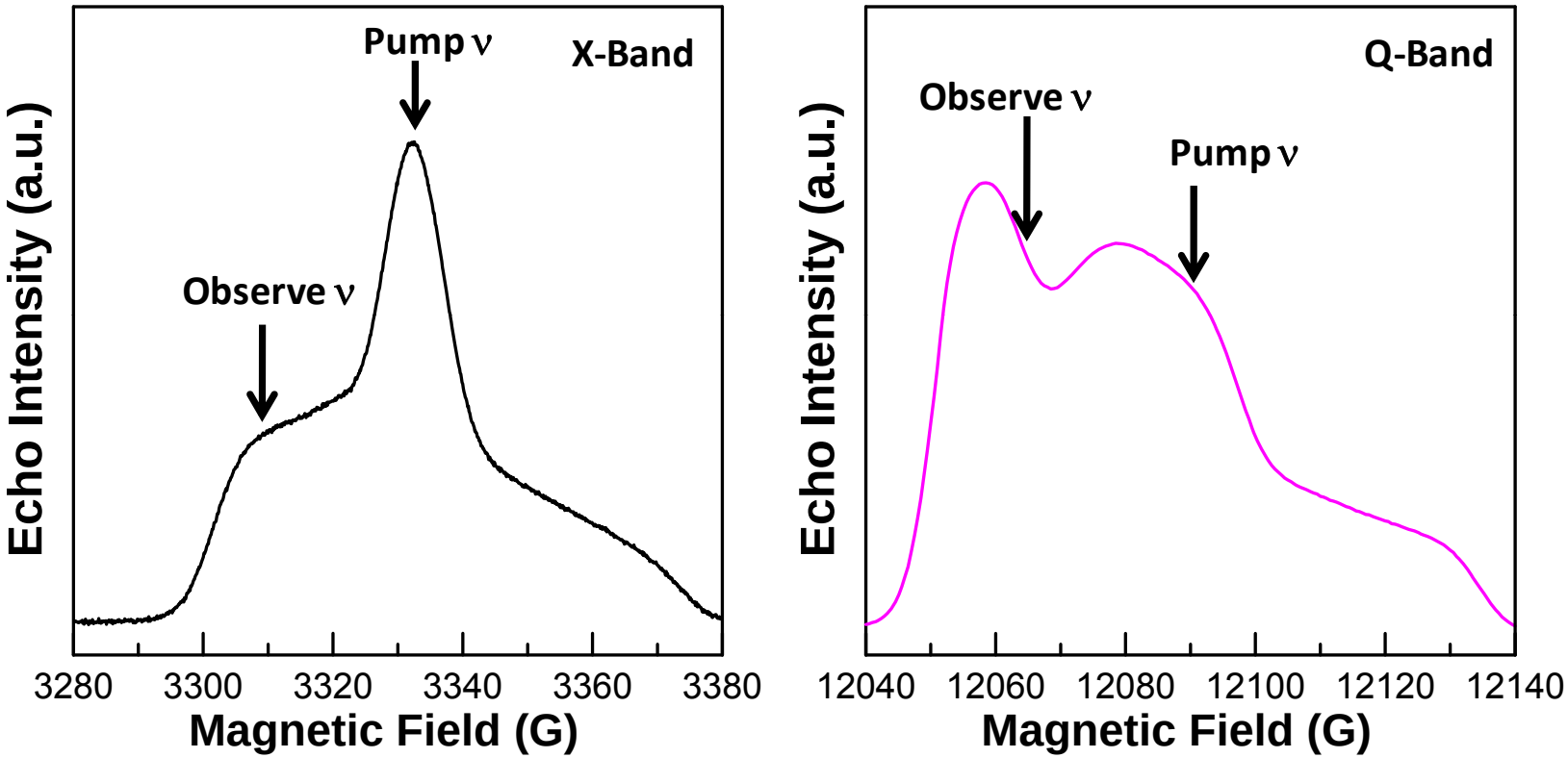
- Locally high concentrations of spins due to solvent freezing can shorten T_m.
- Methyl groups in the vicinity of the spin label can shorten T_m.



T_M of Spin Labeled HCA II at 40 K and Comparison with Information Concerning Probe Location

residue		L79C	W97C	C206	F176C	W245C	W16C
location		buried	buried	buried	at/near surface	surface/ intermed	surface
Probe mobility, CW, RT		rigid	rigid	rigid	mobile	rigid/ mobile	mobile
number of CH_3 in 5 to 10 Å shell		24	15	11	13	6	7
T_M [μs] in H_2O/glycerol		1.6	2.7	3.8	3.7	4.7	4.3

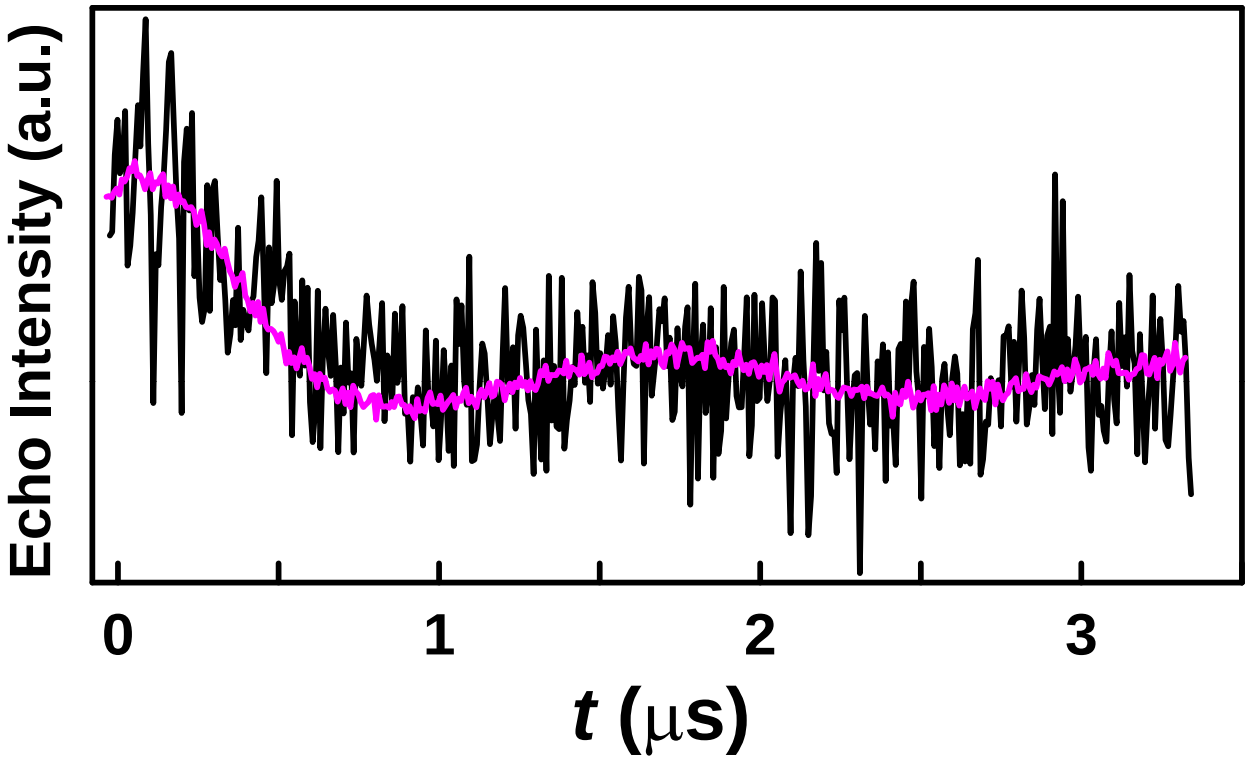
DEER Setup



Pump: semiquinone signal

Observe: nitroxyl low-field line

X-band/Q-band Comparison



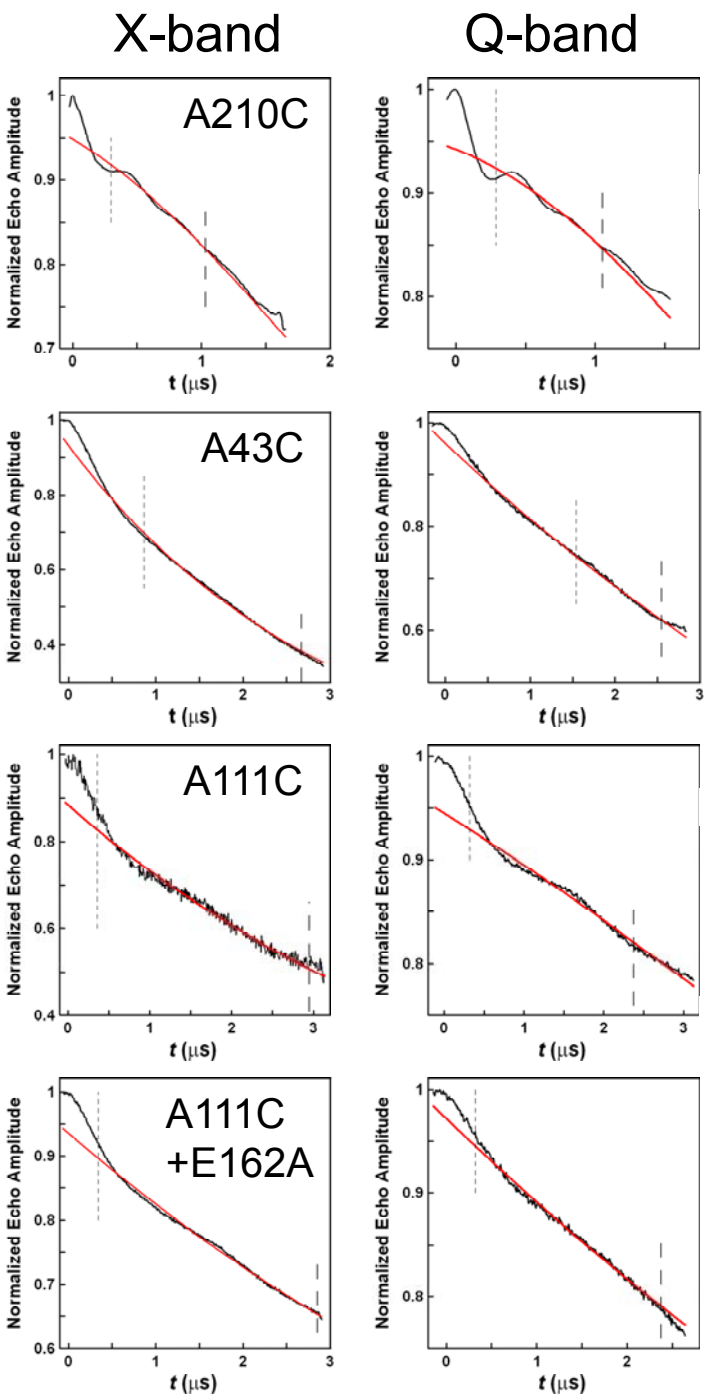
80 K
23 scans

Sample Tubes (o.d.):

X-Band – 4.0 mm

Q-Band – 1.0 mm

Signal-to-noise is ~10x better
at Q-band than at X-band.

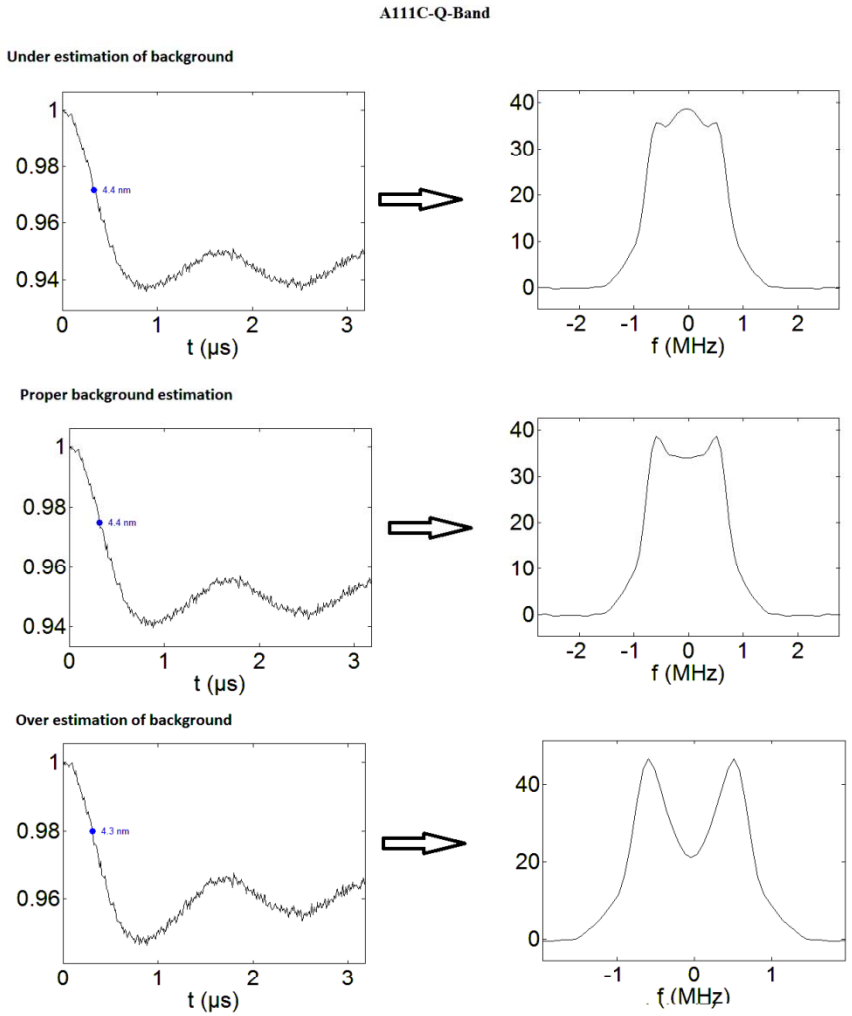


DEER experimental data

"background" subtraction corrects for random intermolecular spin-spin interactions

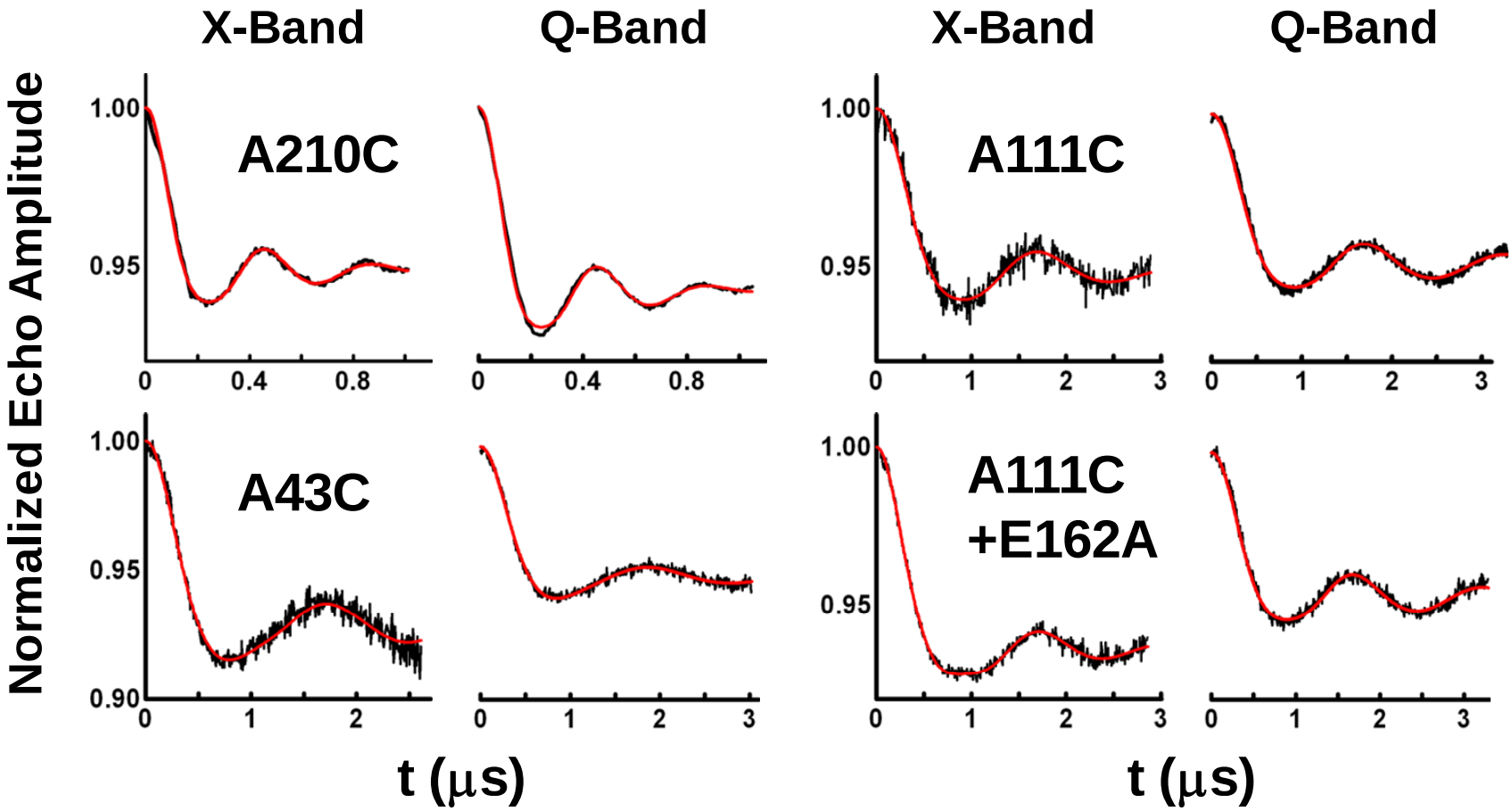
Data analysis performed with DEER2009
www.epr.ethz.ch/software/index
By Gunnar Jeschke

How much background to subtract?



The background subtraction impacts the shape of the Pake pattern that is obtained by Fourier transformation of the dipolar evolution signal.

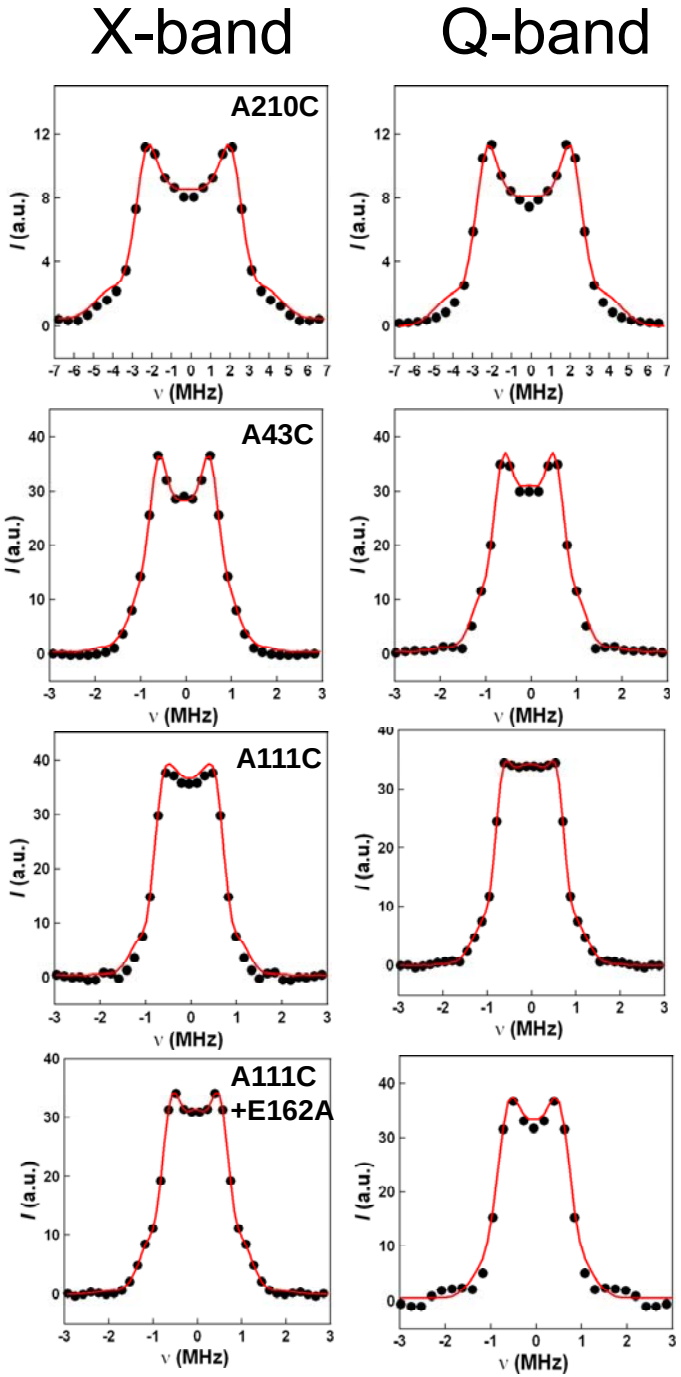
X-band and Q-band Dipolar Evolution Signals



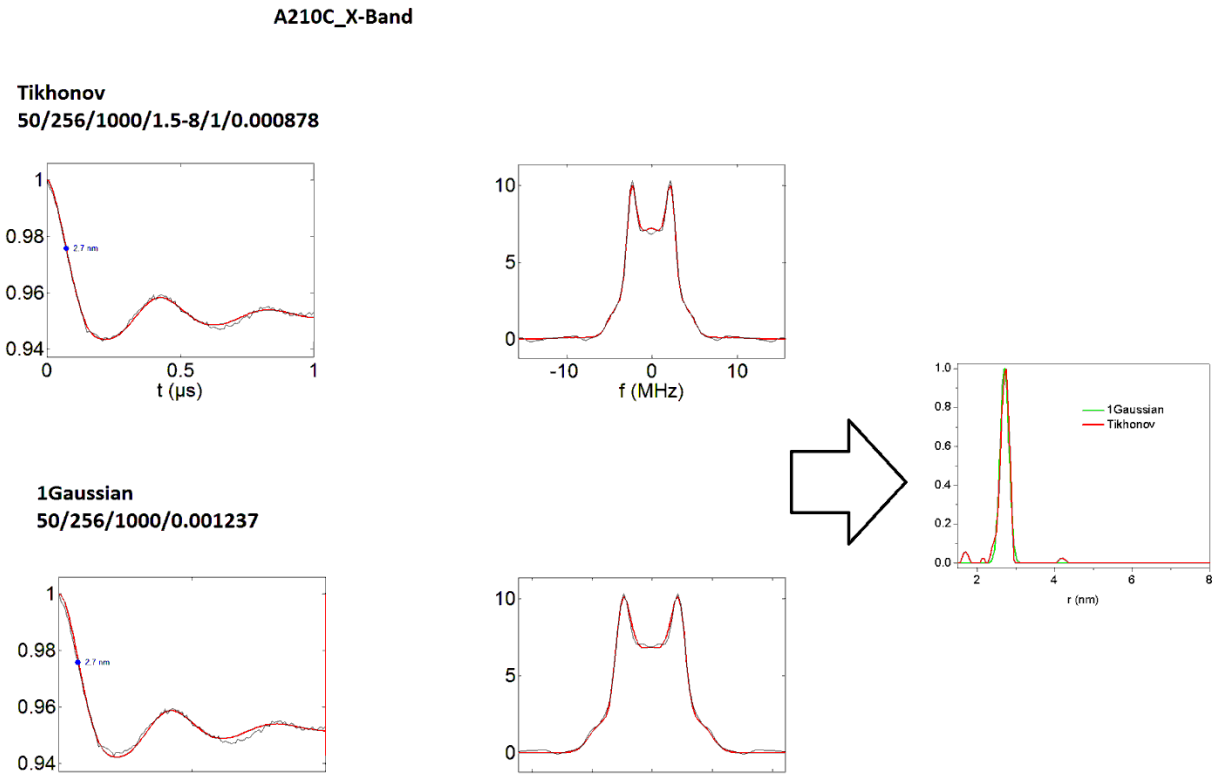
Pake patterns for spin-labeled ETF mutants

Black circles: FT of dipolar evolution

Red line: Modeling of distance distribution using Tikhonov regularization



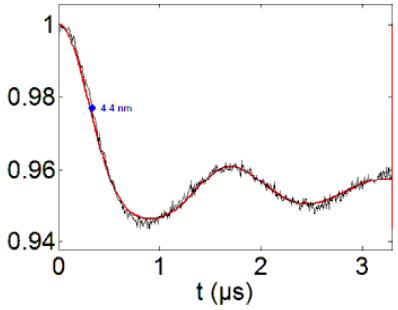
Small changes in fitting parameters can have large impact on smaller components of distance distribution

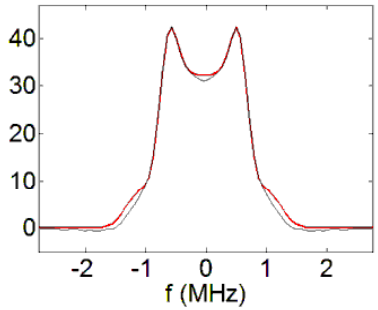


Another Comparison

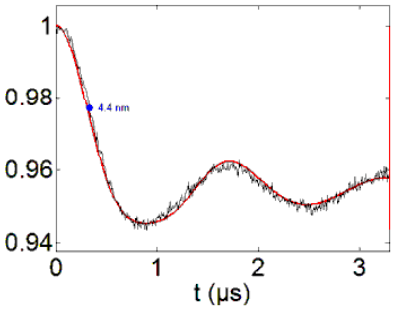
A111C_Q-band

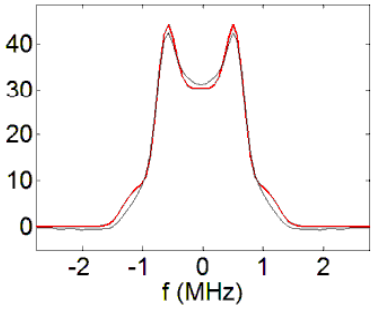
Tikhonov
65/1048/3288/1.5-8/10/0.001319

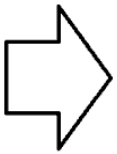


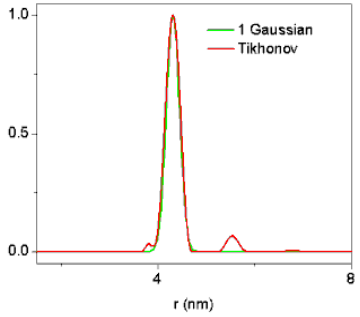


1Gaussian
65/1048/3288/0.00145

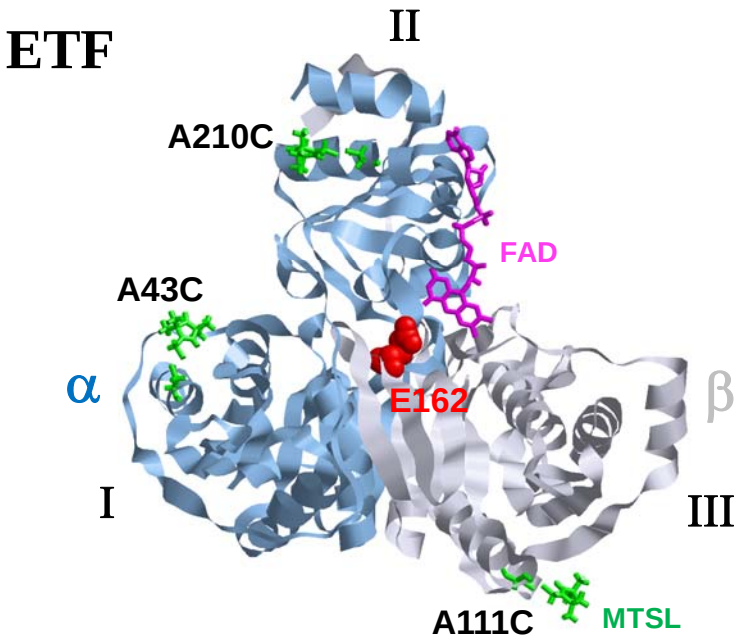




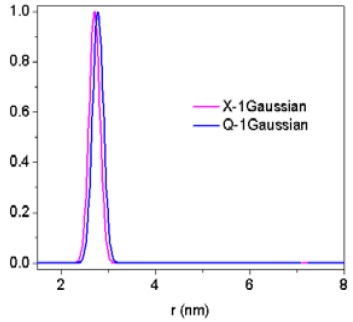




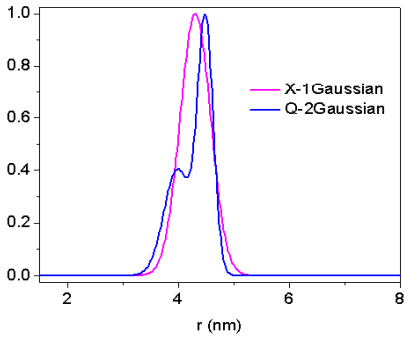
DEER Distance Distributions



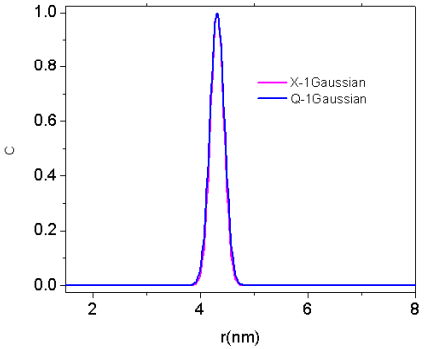
A210C



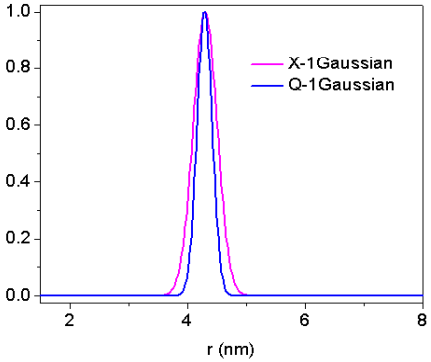
A43C



A111C



A111C+E162G



X-Band (—)
60 K

Q-Band (—)
80 K

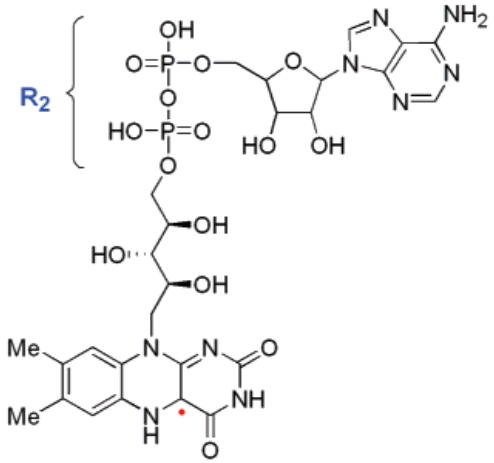
Summary of Interspin Distances

Distances and distribution widths for the major components, in nm.

ETF Mutant	X-Band		Q-Band	
	Avg Distance	Width	Avg Distance	Width
A210C	2.7	0.3	2.8	0.4
A111C	4.3	0.5	4.3	0.5
A111C+ E162A	4.3	0.5	4.3	0.3
A43C	4.4	0.7	4.4	0.7

Interpretation of the DEER distances

- Distances obtained by DEER are between paramagnetic centers
  - For nitroxyls the unpaired electron is primarily on the N-O bond
  - For FAD semiquinone the centroid of spin density is near C4a.



- Need to relate the spin-spin distances to protein structure

Model conformations of the linkage to the spin label

- Use library of nitroxyl rotamers – Polyhach and Jeschke. (2009) MMM Version 2009: http://www.epr.ethz/software/index
- Use Discovery Studio to do molecular dynamics (MD)

Molecular dynamics (MD) simulations of the motion of MTSL labels were performed using Discovery Studio (Accelrys).

- MTSL labels were added to the crystal structure of *P. denitrificans* ETF and the homology model of *P. denitrificans* ETF based on the crystal structure of human ETF:MCAD complex.
- Positions of all atoms except those in the MTSL and cysteine residues were restrained.
- MD simulations consisted of three stages:
  - Heating: 1 ns, 50 – 600 K
  - Equilibrium: 1 ns, 600 K
  - Production: 10 ns, 300 K
- Distance dependent implicit solvent model was used (ε = 80) along with SHAKE constraint.

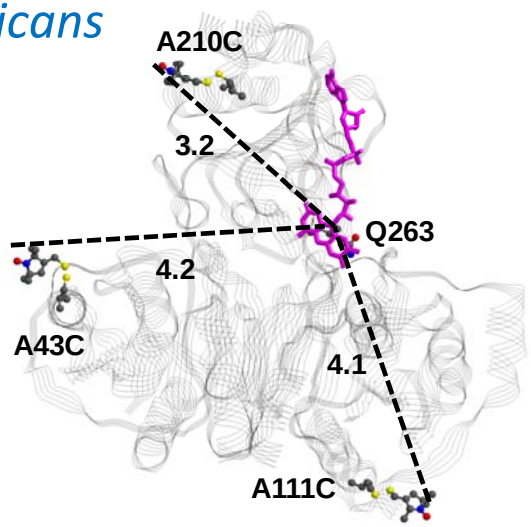
Homology Modeling of ETF:MCAD structure

- X-ray structures are known for *P. denitrificans* and human ETF.
- Spin labelling studies used P. *denitrificans* because it has a single buried native cysteine.
- The crystal structure is known for human ETF:MCAD complex.
- A homology model of *P. denitrificans* ETF was created using the program ESyPred3D.
- FAD was not present in the structure of the human ETF:MCAD complex, so Q263 was used as a approximation of the FAD position.
- Nitroxyl oxygen to β-carbon distances were measured in the model and the crystal structure of ETF to determine the approximate change in interspin distance associated with ETF going from the closed to open conformation.

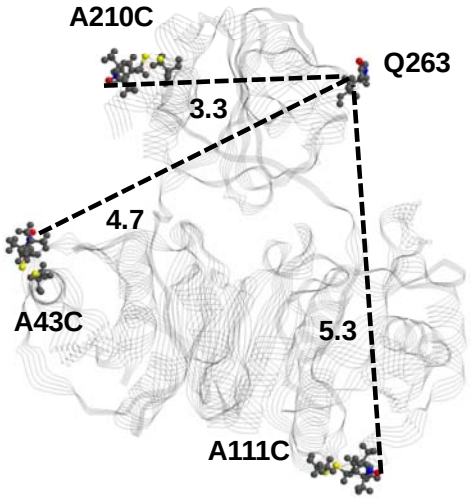
Crystal Structure of ETF and Homology Model of ETF bound to MCAD

ETF

P. denitrificans



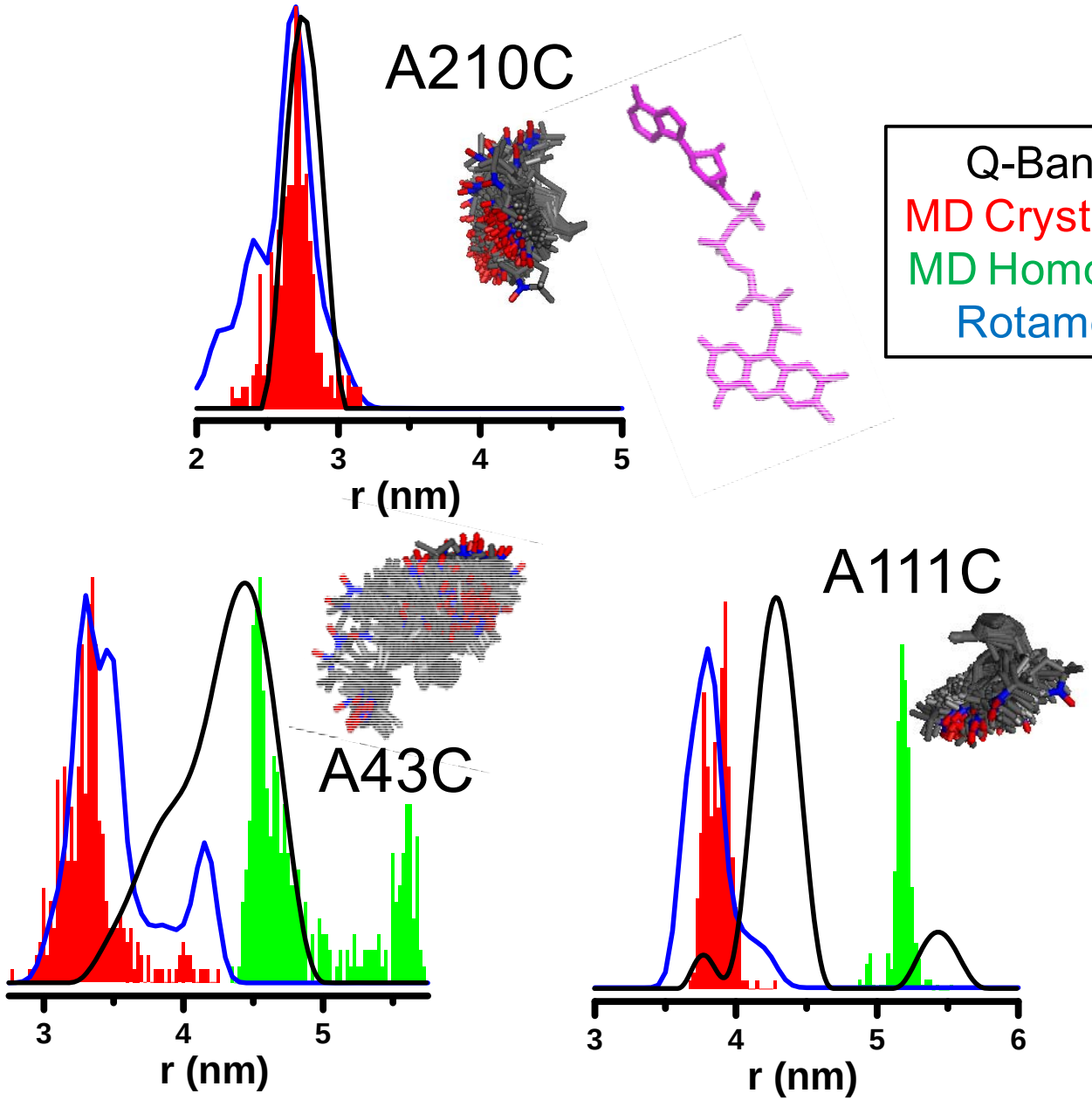
ETF:MCAD



Based on crystal structure for human ETF:MCAD complex.

M. A. Swanson et al., *J. Amer. Chem. Soc*. **131**, 15978-15979 (2009).

Molecular Dynamics Simulations



Summary

- Distributions of distances between the FAD $SQ^{-\bullet}$ and MTSL labels in the three structural domains of ETF were determined.
- One narrow component was found for the A210C site, indicating that the structure of the FAD domain (II) is well defined.
- Average distances for the major components of the distributions obtained from the A43C and A111C sites are intermediate between distances predicted for free and bound ETF.
- The E162A mutation has little impact on the distance distribution.