Published in final edited form as:

Free Radic Res. 2018 March ; 52(3): 307–318. doi:10.1080/10715762.2017.1412433.

Electron spin-labeling of the EutC subunit in B₁₂-dependent ethanolamine ammonia-lyase reveals dynamics and a two-state conformational equilibrium in the N-terminal, signal-sequence-associated domain

Benjamin Nforneh^a, Adonis M. Bovell^a, and Kurt Warncke^{a,*}

^aDepartment of Physics, Emory University, Atlanta, GA 30322

Abstract

The B₁₂ (adenosylcobalamin) -dependent ethanolamine ammonia-lyase (EAL) is a product of the ethanolamine utilization (eut) gene cluster, that is involved in human gut microbiome homeostasis and in disease conditions caused by pathogenic strains of *Salmonella* and *Escherichia coli*. Toward elucidation of the molecular basis of EAL catalysis, and its intracellular trafficking and targeting to the Eut biocompartment (BMC), we have applied electron spin-labeling and electron paramagnetic resonance spectroscopy to wild type (wt) EAL from *S. typhimurium*, by using the sulfhydryl-specific, 4-maleimido-TEMPO (4MT) spin label. One cysteine residue per active site displays exceptional reactivity with 4MT. This site is identified as β C37 on the EutC subunit, by using 4MT-labeling of site-specific cysteine-to-alanine mutants, enzyme kinetics, and accessible surface area calculations. EPR spectra of 4MT-labeled wt EAL are collected over 200–265 K in frozen, polycrystalline water-only and 1% v/v DMSO solvents. EPR simulations reveal two mobility components for each condition. Detectable spin probe reorientational motion of the two components occurs at 215 and 225 K with 1% v/v DMSO, relative to the water-only condition, consistent with formation of an aqueous-DMSO solvent mesodomain around EAL. Parallel trends in fast- and slow- reorientational correlation times and interconversion of the two populations with increasing temperature, indicate 4MT labeling of a single site (β C37). A two-state model is proposed, in which the fast and slow motional populations represent EAL-bound and free conformations of the EutC N-terminal domain. The approximately equal proportion of each state may represent a balance between EutC and EAL protein stability and efficient targeting to the BMC.

*Corresponding author: Kurt Warncke, Department of Physics, N201 MSC, 400 Dowman Drive, Emory University, Atlanta, GA 30322-2430, kwarncke@physics.emory.edu.

Associated content

Supplementary information

The Supplementary Information is available free of charge on the Taylor & Francis Publications website at DOI: 10.1080/#####
The supplementary information showing tables presenting the primers used in the development of the EAL single mutant proteins, and log τ_c and W values at the different T -values for the EAL, 0% DMSO and EAL, 1% v/v DMSO systems, and enthalpy and entropy values obtained from van't Hoff analysis of the mobility component weights in the 1% v/v DMSO system.

Disclosure statement

The authors declare no competing financial interest.

Keywords

electron paramagnetic resonance; spin labeling; protein conformation; signal sequence; *Salmonella*

Introduction

The B₁₂ (adenosylcobalamin) -dependent ethanolamine ammonia-lyase (EAL; EC 4.3.1.7), [1] is a bacterial enzyme that is involved in gut microbiome homeostasis,[2–4] and in disease conditions caused by pathogenic strains of *Salmonella* and *Escherichia coli*, in humans.[5, 6] The EAL oligomer is composed of the EutB (49.4 kDa, *S. typhimurium*; α -subunit) and EutC (32.0 kDa, *S. typhimurium*; β -subunit) proteins (one active site per EutBEutC) in a [(EutBEutC)₂]₃ stoichiometry. [7–9] EAL catalyzes the conversion of ethanolamine to acetaldehyde and ammonia, and is thought to function under pathogenic conditions with other proteins of the ethanolamine utilization pathway (products of the *eut* operon), in the Eut biomicrocompartment (BMC), a sub-cellular organelle.[10, 11] Elucidation of the molecular basis of EAL catalysis, and the intracellular trafficking and targeting of EAL to the biomicrocompartment, is a direction for development of therapeutics. Toward this, we have used low-temperature ($T=193\text{--}250\text{ K}$), full-spectrum, time-resolved electron paramagnetic resonance (EPR) spectroscopy[12] in frozen, globally-polycrystalline aqueous solutions to characterize kinetics and approximately $>1\text{ \AA}$ require a fluid state of the surrounding solvent. [15–17] The origin of the low- T reactivity of EAL is therefore hypothesized to originate from a solvent “mesodomain” in the microscopic region that surrounds the EAL protein. In cryopreservation processes, the protective “mesodomain” is created by the exclusion of cosolvents from the growing ice crystallite regions during sample freezing.[18–20] For the cosolvent, dimethylsulfoxide (DMSO),[21] this condition is met for proportions of $<30\%$ v/v.[22, 23] The increased concentration of cosolvent at the interstitial, ice crystallite grain boundaries in the relatively low-volume mesodomain, leads to depression of the liquid/solid transition T , and formation of supercooled fluid and eventual glassy solid phases with decreasing T , in the mesodomain.[18–20] Here, we address the T -dependence of the solvent liquid-solid state around EAL from *S. typhimurium* over $200\text{--}265\text{ K}$, from the vantage of an electron spin label on the protein surface, by using spin label EPR spectroscopy in frozen, polycrystalline aqueous water-only and 1% v/v DMSO solutions.

The technique of site-directed spin-labeling (SDSL) of proteins involves replacement of a selected amino acid with cysteine (Cys, C) by using protein mutagenesis techniques, followed by covalent modification of the substituted Cys side chain by a sulfhydryl-specific molecule that carries an electron spin ($S=1/2$), the spin label.[24–26] Development and classical applications of SDSL were performed in proteins with native Cys-devoid or Cys-sparse backgrounds.[27, 28] In contrast, spin labelling studies of wild-type (wt) proteins (WTSL) are typically fraught with multiple Cys labeling sites, which give a heterogeneous distribution of label, and consequent poor spectral resolution of individual sites. In order to achieve site-specificity in WTSL, one Cys site must be exceptionally accessible to reaction with the spin label, relative to other, partially-accessible and buried Cys sites. The

subsequent identification of the spin-labeled site by incremental mutagenesis to replace the Cys sites with an unreactive amino acid, must incur minimal perturbations of the protein fold, that might alter the accessibility of sites, relative to the native state.

Here, we describe the application of WTSL to wt EAL from *S. typhimurium*, by using the sulfhydryl-specific, 4-maleimido-TEMPO spin label, at a stoichiometry of 1 spin label per active site. EAL presents a challenge to EPR spin-labeling approaches, because there are 16 Cys residues in each EutBEutC unit (16 per active site; *S. typhimurium*). [9] As required for successful application of WTSL, we find that one cysteine residue per EutBEutC displays exceptional reactivity with the 4MT spin label, while other cysteines are effectively buried and inaccessible. EAL structure-based Cys solvent accessibility predictions [29, 30] guide site-directed Cys to alanine (Ala, A) mutations, and room-*T* EPR studies, that locate the labeled residue in the N-terminal segment of the EutC subunit (β C37). The N-terminal region of EutC includes a signal sequence, that is involved in intracellular targeting of EAL to the Eut BMC. [31][32] The *T*-dependence of the spin label motion in wt EAL over 200–265 K in frozen, polycrystalline water-only and 1% v/v DMSO solvents reveals two mobility components at the labeled site (quantified by τ_c and normalized weight, W , values). The results are interpreted in terms of a two-state model for bound and free association of the EutC N-terminal domain with EAL, and indicate that the persistence of catalysis reactions of EAL [12–14] and the two-state equilibrium deep into the cryogenic-*T* regime, are promoted by low-*T* fluidization of the local solvent region around the EAL protein.

Material and Methods

Protein preparation

All chemicals were purchased from commercial sources, including DMSO (purity, 99.9%; EMD Chemical), 4-maleimido-TEMPO (4MT, Sigma-Aldrich). Deionized water was used to prepare aqueous solutions (specific conductance, 18.2 M Ω cm; Nanopure system, Siemens). The wt EAL protein was overexpressed in *Escherichia coli* and purified as described, [7, 33] with modifications. [12] Enzyme activity of purified EAL with aminoethanol as substrate was determined at *T*=298 K and *P*= 1 atm by using the coupled assay with alcohol dehydrogenase and NADH. [34]

EPR sample preparation

Frozen, polycrystalline EPR samples for low-*T* studies were prepared under aerobic conditions, on ice. A proportion of 1:1 4MT/active sites was added to 20 μ M EAL (active site concentration; 120 μ M) in 10 mM potassium phosphate buffer (pH 7.5). At 1:1 4MT/active sites, all added 4MT reacts with protein Cys sites (there is no free-solution 4MT EPR spectrum in the samples). Therefore, a procedure to separate unreacted 4MT from the reaction mixture was not necessary. The buffered sample pH is 3 units lower than the *pK_a* of the lysine side chain, and therefore, reaction of 4MT with the deprotonated form of the ϵ -amino group was considered negligible. This assumption is supported by the results of the Cys-to-Ala mutagenesis studies, described below. The reaction of 4MT with EAL Cys sites was rapid (<60 s), on the time scale of the addition of 4MT to the EAL protein samples, mixing, and loading into EPR tubes. EAL was allowed to react with 4MT for 5 min, prior to

cryotrapping. The final volume of each sample was 0.3 ml. When present, DMSO was added to 1% v/v in a final volume of 0.3 ml. Samples were loaded into 4 mm outer diameter EPR tubes (Wilmad-LabGlass) and frozen by plunging into $T=140$ K isopentane solution. This method has a characteristic cooling rate of 10 K/s.[12] Samples were transferred to liquid nitrogen for storage. Room temperature, bulk liquid EPR samples were prepared in 2 mm outer diameter EPR tubes (Wilmad-LabGlass).

Site-directed mutagenesis

Ala was selected for replacement of Cys residues in EAL, following the Ala scanning approach, because Ala displays side-chain space-filling and maintenance protein secondary structure, which minimizes alteration of the native protein fold.[35] The pBR322 plasmid including the EutB and EutC genes of wt EAL from *S. typhimurium* (created by L. P. Faust and B. M. Babior [33]; gift of C. B. Grissom, University of Utah) was isolated from single colony growth of wt EAL by using the Plasmid Mini kit (QIAGEN, Hilden, DE). Primers containing the desired mutations (Table S1) were used for mutational polymerase chain reaction (PCR) with the QuikChange II XL Site-Directed Mutagenesis Kit, according to the manufacturer's instructions (Stratagene, La Jolla, CA, US). The parental EAL plasmid was digested with *DpnI*, and the mutant plasmid was transformed into XL10 Gold competent cells (Agilent Genomics, Santa Clara, CA, US). Single colonies of the mutant bacteria were grown, and the plasmid extracted for sequencing (Cogenics, UK). Mutation was considered successful when 100% of the desired sequence identity within the EAL reading frame was obtained. Absence of significant protein structural rearrangements was evidenced by successful purification, according to the protocol for wt EAL, without insolubility (improper folding), and by enzyme activity equivalent to wt EAL (Table 1).

Continuous-wave EPR simulations

Continuous wave (CW) -EPR experiments were performed by using a Bruker E500 ElexSys EPR spectrometer and ER4123SHQE X-band cavity resonator. A Bruker ER4131VT temperature controller and cooling system, based on nitrogen gas flow through a coil immersed in liquid nitrogen, was used to establish T values of 200–265 K. The Bruker ER4131VT temperature readout was calibrated by using a 19180 4-wire RTD probe and Oxford Instruments ITC503 unit, as described.[12] The uncertainty in T values was ± 0.5 K. [12] For the low- T measurement protocol, the first EPR spectrum was obtained at $T=200$ K. The controller was then set to the next higher T -value, and the system was allowed to equilibrate for 5 min at the new T -value. At the end of the 5 min period, the cavity was retuned, and the spectrum was acquired at the new T -value. This procedure was repeated for the entire T range. A baseline sample (all components, but without 4MT) was subjected to the same measurement protocol. Accessory, control experiments were carried out in the direction of decreasing T , starting from 265 K. No hysteresis in correlation times or component amplitudes was observed. No hysteresis was also not observed for samples subjected to an additional cycle of storage and measurement. Therefore, equilibrium conditions are established at each T value. **EPR acquisition parameters, 200–265 K:** Microwave frequency, 9.45 GHz; microwave power, 0.2 mW; magnetic field modulation, 0.2 mT; modulation frequency, 100 kHz; acquisition number, 4–16 spectra at each T value. **EPR**

acquisition parameters, 295 K: Same as for 200–265 K, except microwave frequency, 9.36 GHz; acquisition number, 64 or 100 spectra.

Continuous-wave EPR simulations

Simulations of the CW-EPR spectra were performed by using the program, EasySpin.[36] Convergence of simulations was defined by the default, least-squares fitting criteria. The following established[37] set of parameters was used: principal values of the g tensor, $g_x = 2.0120$, $g_y = 2.0130$, $g_z = 2.0073$; principal values of the ^{14}N hyperfine tensor, $A_x = 20.9$, $A_y = 19.82$, $A_z = 103.2$ MHz. A simple model of random rotational motion about the three Cartesian axes was assumed. Spectra were corrected by subtraction of a baseline sample spectrum (EAL, no 4MT), collected at the same temperature, prior to simulation. Simulations of the experimental spectra required two components, and were performed by variation of the correlation times and normalized weights for the slow-motional ($\tau_{c,s}$, W_s) and fast-motional ($\tau_{c,f}$, W_f) components, and the corresponding intrinsic line widths (Gaussian line width, 0.02 mT full width at half maximum). The two-component simulation procedure was initiated by using the best-fit parameters from an preliminary one-component simulation as input values for the fast-motional component. This was followed by three iterations of fixing τ_c , W , and intrinsic line width of one component, while the other component's parameters were varied, and a fifth iteration with simultaneous variation of τ_c , W , and intrinsic line width of both components. This derivative-mode spectrum simulation procedure provides rapid convergence to the correct region of the parameter space. The following final steps in the two-component simulations were performed to assure that the slow-motional, broader-line component was correctly represented, particularly for spectra in which the slow component was present in small proportion. The best-fit parameters from the two-component, derivative-mode simulations were used as starting parameters for an absorption-mode EPR simulation of the integrated experimental derivative EPR spectrum. In the first iteration, the slow component correlation time, $\tau_{c,f}$, was fixed, and all other adjustable parameters were varied. A scan of $\tau_{c,s}$ about the above fixed value was performed at up to six increments of ± 0.1 of the value of $\log(\tau_{c,s})$, and the $\tau_{c,s}$ value that gave the best match to the slow component was identified. This final $\tau_{c,s}$ was then fixed, and $\log(\tau_{c,f})$ was varied by ± 0.1 , to obtain the final $\tau_{c,f}$ value.

Criteria for detection of spin label motion

The characteristic 4MT spectrum arises from interaction of the unpaired electron spin ($S=1/2$) with the nitroxide ^{14}N nuclear spin ($I=1$), which produces three dominant spectral features that correspond to electron spin-spin transitions ($m_s = \pm 1/2$) among $m_I=0, \pm 1$ energy levels created by the electron-nuclear hyperfine interaction.[38] The time-dependent reorientation of the spin label leads to averaging of the unpaired electron- ^{14}N dipolar hyperfine interaction in the nitroxide spin label, and a consequent narrowing of the EPR line shape,[38] which is quantified by the τ_c value obtained from spectral simulations. The X-band, CW-EPR spin probe approach is sensitive to 4MT reorientational motion in the τ_c range of $\sim 10^{-11}$ (rapid reorientation limit) to 10^{-7} s (defined as the rigid limit).[39] Thus, the inverse bandwidth of mobility detection is defined as $10^{-11} \tau_c 10^{-7}$ s.

Results

Accessibility of cysteines in wt EAL

The solvent accessibility of Cys residues in wt EAL was estimated, in order to efficiently identify the most probable sites of 4MT spin label attachment, among the 16 Cys residues per active site (16 per EutBEutC).[9] The accessibility of cysteines in the *S. typhimurium* wt EAL structure[9] was assessed by using the Accessible Surface Area web server (ASAview), [29] which uses the algorithm, Define Secondary Structure of Proteins (DSSP),[30] to calculate the relative surface area (*RSA*) of the residue (area accessible to solvent in the protein, scaled by the total accessible surface area). The standard criterion of *RSA* > 0.2 [40–43] was applied to identify accessible cysteine sites. The *RSA* values for each cysteine residue in wt EAL (Table 1), predict that 1 site is accessible (β C260), and 13 are inaccessible (buried). The β C260 side chain lies within the buried active site region of wt EAL, where it makes van der Waals contact with the adenosylcobalamin cofactor in the holoenzyme.[9] The accessible area calculated for β C260 corresponds to the active site cavity, which the EAL structure shows is accessible by the water- equivalent sphere used for calculation in DSSP,[30] but not to bulky molecules, such as 4MT. Therefore, we conclude that β C260 is not a primary site of 4MT reaction. The two residues without *RSA* values in Table 1, β C37 and β C46, are part of the N-terminal sequence of EutC, that is not present in the X-ray crystallographic structure of *E. coli* EAL (deletion up to residue 44 was necessary to achieve conditions conducive to EAL crystallization),[8, 44] or in the *S. typhimurium* EAL model.[9] We thus provisionally consider β C37 and β C46 to be accessible to reaction with 4MT.

Identification of the 4MT labeling site in wt EAL

To experimentally identify the primary labeling site in wt EAL, site-directed mutagenesis was used to substitute Ala for the two candidate accessible Cys residues, β C37 and β C46. [35] Steady-state enzyme activity parameters for the β C37A and β C46A EAL were measured, as an assessment of native protein structure in the mutants. Table 2 shows that β C37A and β C46A EAL have k_{cat} and K_m values that are the same, within uncertainty, as for wt EAL. The absence of an effect on enzyme activity parameters is consistent with an accessible, protein surface location of β C37 and β C46, and a significant distance from the active site. The zero enzyme activity of the β C260A mutation is consistent with the buried, active site location of β C260.

The EAL single-site mutants, β C37A and β C46A were analyzed by using 4MT spin labeling and EPR spectroscopy, performed at 295 K. The product of the reaction of wt EAL with 4MT, added at a stoichiometry of one 4MT per active site, shows a line shape characteristic of a relatively mobile species (Figure 1). If a mutated cysteine site corresponds to a 4MT attachment site, then the EPR spectrum of the mutant protein should be altered significantly, and present a broader line shape, characteristic of a low-mobility species. This is because, deprived of the accessible cysteine site by mutation to alanine, 4MT would react with one or more of the less accessible sites, which are more deeply buried. The sterically-restricted spin label motion at the buried site would display a broadened EPR line shape. Figure 1 shows that the EPR line shape of β C46A EAL is comparable to wt EAL, which suggests that β C46

is not the site of 4MT reaction. In contrast, the EPR line shape of β C37A EAL is significantly altered, relative to wt EAL, suggesting that β C37 is the primary site of 4MT reaction. These conclusions are quantified by simulations of the EPR spectra. As shown by the overlaid simulated spectra (Figure 1) and parameter values (Table 2), the wt EAL spectrum is well-fit by a two-component simulation, with relatively short (fast motion) τ_c values for both components. As demonstrated by the low- T results in following sections, these two mobility components correspond to a single reaction site on wt EAL. The β C46A mutant spectrum is also a two-component simulation, with τ_c and W values comparable to wt EAL. In contrast, the simulation of the spectrum from β C37A EAL requires a dominant slow-motional component, consistent with 4MT reaction at one or more lower-accessibility, buried sites. The minor faster-motion component in the β C37A EAL spectrum may arise from increased probability of reaction at β C46, in the absence of β C37, or an enhancement of Cys accessibility in a sub-population of EAL, owing to destabilization of the fold by 4MT reaction at one or more buried sites. In summary, based on predictions of the RSA values, enzyme activity parameters, and EPR line shape analysis of 4MT- labeled wt and mutant proteins, we conclude that β C37A is accessible, and that it is the primary site of 4MT reaction in wt EAL.

Temperature dependence of the 4MT-labeled EAL EPR line shape in frozen aqueous solution, in the absence of DMSO

The effect of temperature on the EPR line shape of the 4MT spin label in wt EAL at different representative T -values from the complete addressed range of 200–265 K is shown for the 0% DMSO sample in Figure 2A. From 200–240 K, the spectra show the rigid-limit, powder pattern line shape. At $T > 240$ K, narrowing of the overall spectral line width and individual features indicates the commencement of detectable ($\log \tau_c \sim -7.0$) 4MT motion, which results in further line narrowing as T is increased.

Temperature dependence of the 4MT-labeled EAL EPR line shape in frozen aqueous solution with 1% v/v DMSO

Figure 2B shows the EPR line shape of frozen aqueous solutions of 4MT-labeled wt EAL with added 1% v/v DMSO at representative T -values. The rigid-limit, powder pattern line shape is observed for $T \sim 230$ K. The overall line width and widths of the hyperfine features narrow at $T > 230$ K, and this trend continues with increasing temperature. Comparing the line shapes in Figure 2 shows that tumbling motion of 4MT in 1% v/v added DMSO is at 20–30 K lower in temperature, relative to the 0% DMSO sample.

Temperature dependence of the 4MT-labeled EAL rotational correlation time and normalized component weights in frozen aqueous solution, in the absence of DMSO

Simulations of the EPR spectra were performed in order to quantify the mobility of 4MT on EAL, in terms of the τ_c and W parameters. The temperature dependence of τ_c in EAL, 0% DMSO displays two-component behavior (Figure 3A; values, Table S2), and is divided into three regions: Region I ($200 < T < 230$ K): The $\log \tau_c$ values lie above the tumbling detection criterion. Region II ($230 < T < 240$ K): A fast-tumbling population is present, with $\log \tau_{c,f}$ decreasing with T , along with a rigid population. Region III ($240 < T < 265$ K): Both fast- and

slow-tumbling populations are present, with $\log \tau_{c,f}$, $\log \tau_{c,s} < -7.0$, and decreasing with T . Figure 3B shows that there is trend of decreasing dominant slow population component (W_s) with a compensating increase in fast population component (W_f) in region **III**.

Temperature dependence of the 4MT-labeled EAL rotational correlation time and normalized component weights in frozen aqueous solution with 1% v/v DMSO

The temperature dependence of τ_c for 4MT in EAL with 1% v/v added DMSO displays two-component behavior over the full T range (Figure 4; values, Table S3). Three regions of motional behavior are observed, as for the 0% DMSO condition, but the boundary T values are lower by 20–30 K. The dependence of $\log \tau_c$ on T is divided into three regions (Figure 4A): Region **I** ($200 < T < 210$ K): The $\log \tau_c$ values lie above the motion detection criterion. Region **II** ($215 < T < 225$ K): A fast-tumbling population is present with decreasing $\log \tau_{c,f}$, along with a relatively rigid population. Region **III** ($225 < T < 265$ K): Both fast- and slow-tumbling populations are present, with $\log \tau_c$ values that decrease that there is a trend of decreasing in W_s with increasing T , with a compensating increase in W_f .

Discussion

Nature of the primary β C37 spin-labeling site in wt EAL

The study of proteins that contain multiple cysteines by the technique of electron spin-labeling is challenged by the loss of single-site resolution. Buried cysteine residues have small individual probabilities for SL attachment, relative to exposed, surface cysteine sites, but the summed probabilities of multiple buried sites can combine to collectively recruit spin label. Multiple buried sites create a background, lower-mobility spin label line shape in EPR spectra, that can interfere with spectral resolution of the desired spin label site. The challenge is overcome in spin-labeling studies of wt EAL from *S. typhimurium* by the exceptionally high reactivity of a single residue, β C37. The high reactivity of β C37 is attributed to its location in the N-terminal polypeptide segment of the EutC subunit.[9] Amino acids 1–19 of the N-terminus of EutC have been identified as a signal sequence for intracellular targeting of EAL to the Eut BMC.[31][32] Signal sequences require flexible, or disordered regions for protein-target recognition and mooring. A partially unbound or unstructured state of the N-terminus of EutC is consistent with rapid trypsinization of *E. coli* EutC in the EAL oligomer,[44] the requirement for N-terminal deletion up to residue 44 in *E. coli*, to eliminate insolubility and achieve crystallization of EAL,[8] and the calculated high protein Instability Index of 51.4 for *S. typhimurium* EutC (Instability Index > 40 connotes high instability; the index for EutB is 27.0). [45] The high reactivity of β C37 with 4MT in wt EAL is consistent with these previous results, and suggests that β C37 lies in a dynamic region, which dramatically enhances the accessibility of its side chain-SH function to 4MT.

The β C37 spin label site provides a probe of equilibrium states and conformational fluctuations of the EutC N-terminus. As described previously, β C37 is not present in the *S. typhimurium* EAL model.[9] Therefore, preliminary modeling studies were performed to delimit the possible β C37 backbone C_α position, in the context of the folded [EutCEutB]₂ homodimer unit in the EAL oligomer. In the fully-extended conformation of the N-terminal

polypeptide, β C37 is positioned 32 Å from the first modeled residue in the *S. typhimurium* EAL structure, β A47. Figure 6 depicts this upper-limit range of 32 Å for β C37 displacement from β A47. The β C37 residue is associated principally with the surface of the EutB subunit. Electron spin-spin distance measurements, performed by using β C37–4MT interactions with SDSL-generated sites, as well as interactions of β C37–4MT with the intrinsic paramagnets in EAL [$S = 1/2$ Co(II) in the cobalamin cofactor, substrate radical], [46] in concert with computational approaches, [47][48][49] will be used to refine the location of the β C37 side chain in EAL.

Two-state model for 4MT mobility in EAL in frozen solution in the absence and presence of DMSO (0 and 1% v/v DMSO)

In the frozen aqueous system in the absence of DMSO, 4MT displays two mobility components at all T values (Figure 3, Table S2). The $\log \tau_c$ values of both the relatively fast-motional and slow-motional components are present in the detection window at 240 K. Both $\log \tau_{c,s}$ and $\log \tau_{c,f}$ subsequently display a continuous, uniformly-decreasing dependence on T , in concert with compensatory changes in W_s and W_f . This suggests that the $\log \tau_{c,s}$ and $\log \tau_{c,f}$ values represent spin label motion within the same microscopic regions, over the full T -range. Figure 4 shows that $\log \tau_{c,s}$, $\log \tau_{c,f}$ and W_s , W_f for the 1% v/v DMSO condition display the same trends as for 0% DMSO: The $\log \tau_{c,f}$ value enters the detection window at 215 K, and $\log \tau_{c,s}$ enters at 225 K, followed by a continuous, uniformly-decreasing dependence of each parameter on increasing T , with compensatory changes in W_s and W_f . Therefore, we propose a two-state model for both the water-only and aqueous-DMSO systems, with states, $S_{s,w}$, $S_{f,w}$ (subscript “w” denotes the water only, 0% DMSO condition), and $S_{s,d}$, $S_{f,d}$ (subscript “d” denotes the 1% v/v DMSO condition), that correspond to the slow- and fast-motional components of the 4MT label at the β C37 site.

The uniformly lower values of $\log \tau_{c,s}$ and $\log \tau_{c,f}$ for the 1% DMSO condition relative to 0% DMSO, are consistent with lowering of the T -value of the liquid-solid transition, and the lowering of the liquid-state viscosity, in bulk aqueous solutions that contain high proportions (40–50 % v/v) of DMSO.[22, 23] Therefore, the results indicate the presence of an aqueous-DMSO mesodomain, that surrounds the EAL protein, within the bulk frozen polycrystalline solution.

Solvent- and temperature-dependence of the populations in the two-state system

The normalized weights, W_s , W_f that correspond to the states, S_s , S_f , show that the fast-motional component is present at low proportion at the lowest T -values for motion detection: $W_f = 0.14$ in 0% DMSO and $W_f = 0.13$ in 1% DMSO (Figures 2, 3). Over 245–255 K for 0% DMSO, there is an abrupt, partial conversion of $S_{s,w}$ to $S_{f,w}$, leading to $W_f = 0.33$ –0.34, followed by a gradual rise to $W_f = 0.40$ at 265 K. In stark contrast, the W_s , W_f versus T relation for 1% v/v DMSO is continuous, with a uniform trend to increasing W_f values at higher T values, ending with $W_f = 0.40$ at 265 K. The abrupt W_s , W_f interconversion in 0% DMSO suggests the effect of a change in solvent phase, that alters the solvent environment around the β C37 site. The smooth T -dependence of W_s , W_f in the 1% v/v DMSO system arises because the aqueous-DMSO mesodomain around the protein is in a fluid state over the entire T -range. We attribute the abrupt change in W_s , W_f over 235–245 K in 0% DMSO to

melting of an ice-crystalline domain in the vicinity of the protein. This protein-vicinal ice-crystalline domain is not present in the 1% v/v DMSO system.

Equilibrium between the states, S_s and S_f

The smooth T -dependence of the W_s , W_f values for the 1% DMSO condition over a broad T -range affords van't Hoff analysis of the equilibrium between $S_{s,d}$ and $S_{f,d}$, to determine the thermodynamic parameters.[50] The equilibrium constant (defined for the direction of $S_{s,d} \rightarrow S_{f,d}$) is described as:

$$K = \frac{W_{f,d}}{W_{s,d}} \quad \text{Eq. 1}$$

The Gibbs free energy (ΔG) and corresponding enthalpy (ΔH) and entropy (ΔS) terms are introduced through the equilibrium expressions, $\Delta G = -RT \ln K$ and $\Delta G = \Delta H - T \Delta S$, where R is the gas constant, leading to:

$$\ln K = -\frac{\Delta H}{RT} + \frac{\Delta S}{R} \quad \text{Eq. 2}$$

The ΔH and ΔS values, obtained from the slope and intercept of the linear fit of data in the van't Hoff plot in Figure 6, are 2.4 kcal/mol and 8.4 cal/mol/K, respectively. Therefore, $S_{f,d}$ has both a higher (more positive) enthalpy and higher entropy, relative to $S_{s,d}$. This corresponds qualitatively to the higher relative mobility of the spin label in $S_{f,d}$, which suggests both “looser” interactions with the site region (larger, more positive ΔH) and increased motional freedom (larger, more positive ΔS). The overall free energy difference between $S_{s,d}$ and $S_{f,d}$ is relatively small over the observed T -range, an formation of $S_{f,d}$: ΔG varies from 0.8 to 0.2 kcal/mol from 210 to 265 K.

The W_s and W_f values for the 0 and 1% DMSO conditions become coincident at $T = 245$ K, above the abrupt mobility transition in the 0% DMSO sample (Table S2, S3). Therefore, DMSO does not influence the equilibrium free energy difference between the states: $K_d = K_w$, for $T = 245$ K. In contrast, the difference in mobility between the two fast-motional states, $S_{f,d}$, $S_{f,w}$, and difference between the two slow-motional states, $S_{s,d}$, $S_{s,w}$, quantified as $(\log \tau_{c,f,d} - \log \tau_{c,f,w})$ and $(\log \tau_{c,s,d} - \log \tau_{c,s,w})$, is approximately maintained, across the full T -range of detectable SL mobility. Therefore, DMSO influences (enhances) the mobility of SL in both S_s and S_f , which is consistent with the effect of DMSO on the dynamical property of solvent viscosity. Solvent viscosity does not influence the thermodynamic relations between states at equilibrium.

Model for the origin and temperature-dependence of the two-state, S_s , S_f system for EAL in 0 and 1% DMSO solution

The model proposed for the origin of the two mobility states of the 4MT spin label at the $\beta C37$ site and the T -dependence of the mobilities is presented in Figure 7. The model in Figure 7 applies to both the 0% and 1% DMSO conditions, but the detailed, microscopic

effects of solvent composition on the T -dependence of spin label mobility differ for the two solvent systems. The model incorporates the following prior literature results, that inform on the context of the $\beta C37$ site in the N-terminal domain of the EutC subunit: (1) $\beta C37$ is located in the disordered N-terminal region identified by X-ray crystallographic studies. [8] (2) Rapid trypsinization of the N-terminal domain of EutC indicates a disordered region.[44] (3) The N-terminal region of EutC is part of the signal sequence that targets EAL to the biomicrocompartment,[31, 32] which implies a functional requirement for a dissociated state. A dissociated state of the N-terminal region is also suggested by the calculated relatively high Instability Index [45] of the N-terminal region of EutC. The explicit model in Figure 7 also incorporates the proposition, that lowered temperature, over the wide range of 265→200 K sampled here, will reveal the corresponding bound state of the N-terminal domain, because this state is expected to be relatively restricted, and thus, favored by lowered temperature. The explicit model in Figure 7 is one of several possible interpretations that could be based on consideration of side chain rotamer states, static protein environments local to the spin label, possible effects of delocalized protein structure changes, and possible direct effects of protein dynamics. For example, an alternative interpretation is that the two spin label mobility states arise from two different rotamer states, within a static protein environment. The hypothesis, that is represented by the explicit model in Figure 7, is consistent with the following findings, presented here: (1) the exceptional 4MT- reactivity of the $\beta C37$ site, and (2) the positive sign of the equilibrium entropy change for conversion of S_s to S_f (the sign of ΔS is not linked to spin label mobility). We will further test the hypothesis and explicit model, by probing for consistent behavior of the temperature-dependent spin label dynamics at genetically-engineered Cys at other sites in the N-terminal domain.

In the model, depicted in Figure 7, the two spin label mobility states, S_s and S_f , correspond to two conformations of the N-terminal EutC sequence. In S_s , the N-terminal domain is relatively strongly-associated, or “bound,” at the protein surface, and in S_f , the domain is relatively loosely-associated with the protein, or “free.” The conformation of the N-terminal domain dictates the motional freedom of the spin label at the $\beta C37$ site. In the bound state, S_s , the spin label experiences a relatively constrained environment, which leads to lower mobility, and in the free state, S_f , the spin label experiences an environment that allows higher mobility. The W_f and W_s spin label mobility populations directly represent the equilibrium thermodynamics of the bound and free N-terminal sequence conformations. The kinetics of S_s - S_f interconversion will be addressed in future work, by monitoring the relaxation of the W_f and W_s populations following T -step perturbation.[12]

The results, interpreted in terms of the model in Figure 7, indicate that the EutC N-terminus-bound state is favored over the range of low T values examined. The N-terminus-free state has higher relative entropy, and thus its population grows with increasing T , to a solvent- independent value of 0.4 at 265 K. At 295 K, significant populations of each state are present (Figure 1, Table 2). This may represent the physiological situation, and a trade-off between EutC and EAL protein stability (N-terminus-bound state), and an efficient stochastic search for the BMC inside the bacterial cell, which requires molecular recognition at the BMC target (N-terminus-free state).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK) of the National Institutes of Health (NIH) (grant R01 DK054514). The Bruker E500 EPR spectrometer was funded by the National Center for Research Resources of the NIH (grant RR17767) and by Emory University.

References

1. Toraya T (2003) Radical Catalysis in Coenzyme B12-Dependent Isomerization (Eliminating) Reactions. *Chem. Rev* 103, 2095–2127. [PubMed: 12797825]
2. Roof DM and Roth JR (1988) Ethanolamine utilization in salmonella-typhimurium. *J Bacteriol* 170 (9), 3855–3863. [PubMed: 3045078]
3. Roof DM and Roth JR (1989) Functions required for vitamin-B12-dependent ethanolamine utilization in salmonella-typhimurium. *J Bacteriol* 171 (6), 3316–3323. [PubMed: 2656649]
4. Garsin DA (2010) Ethanolamine utilization in bacterial pathogens: roles and regulation. *Nat. Rev. Microbiol* 8 (4), 290–5. [PubMed: 20234377]
5. Staib L and Fuchs TM (2014) From food to cell: nutrient exploitation strategies of enteropathogens. *Microbiology* 160, 1020–1039. [PubMed: 24705229]
6. Thiennimitr P et al. (2011) Intestinal inflammation allows Salmonella to use ethanolamine to compete with the microbiota. *Proc. Natl. Acad. Sci* 108 (42), 17480–17485. [PubMed: 21969563]
7. Faust LP and Babior BM (1992) Overexpression, purification, and some properties of the AdoCbl-dependent ethanolamine ammonia-lyase from salmonella typhimurium. *Arch. Biochem & Biophys* 294 (1), 50–54. [PubMed: 1550360]
8. Shibata N et al. (2010) Crystal structures of ethanolamine ammonia-lyase complexed with coenzyme B-12 analogs and substrates. *J. Biol. Chem* (285), 26484–26493. [PubMed: 20519496]
9. Bovell A and Warncke K (2013) Structure model of salmonella typhimurium ethanolamine ammonia-Lyase directs a rational approach to the assembly of the functional [(EutB-EutC)₂]₃ oligomer from isolated subunits. *Biochemistry* 52 (8), 1419–1428. [PubMed: 23374068]
10. Tanaka S et al. (2010) Structure and mechanisms of a protein-based organelle in Escherichia coli. *Science* 327, 81–84. [PubMed: 20044574]
11. Bobik TA et al. (2015) Bacterial microcompartments: widespread prokaryotic organelles for isolation and optimization of metabolic pathways. *Molec. Microbiol* 98, 193–207. [PubMed: 26148529]
12. Wang M et al. (2015) Resolution and characterization of chemical steps in enzyme catalytic sequences by using low-temperature and time-resolved, full-spectrum EPR spectroscopy in fluid cryosolvent and frozen solution systems. *Meth. Enzymol* 563, Part A, 59–94. [PubMed: 26478482]
13. Zhu C and Warncke K (2008) Reaction of the Co-II-substrate radical pair catalytic intermediate in coenzyme B-12-dependent ethanolamine ammonia-lyase in frozen aqueous solution from 190 to 217 K. *Biophys. J* 95, 5890–5900. [PubMed: 18805934]
14. Kohne M et al. (2017) Two dynamical regimes of the substrate radical rearrangement reaction in B12-dependent ethanolamine ammonia-lyase resolve contributions of native protein configurations and collective configurational fluctuations to catalysis. *Biochemistry* 56, 3257–3264. [PubMed: 28548844]
15. Fenimore PW et al. (2004) Bulk-solvent and hydration-shell fluctuations, similar to alpha- and beta-fluctuations in glasses, control protein motions and functions. *Proceedings of the National Academy of Sciences of the United States of America* 101 (40), 14408–14413. [PubMed: 15448207]
16. Frauenfelder H et al. (2009) A unified model of protein dynamics. *Proc. Natl. Acad. Sci* 106, 5129–5134. [PubMed: 19251640]

17. Vitkup D et al. (2000) Solvent mobility and the protein 'glass' transition. *Nat. Struct. Biol* 7, 34–38. [PubMed: 10625424]
18. Franks F (1998) Freeze-drying of bioproducts: putting principles into practice. *Eur. J. Pharm. Biopharm* 45, 221–229. [PubMed: 9653626]
19. Goff HD et al. (2003) Glass transitions in frozen sucrose solutions are influenced by solute inclusions within ice crystals. *Thermochim. Acta* 399, 43–55.
20. Franks F (2003) Scientific and technological aspects of aqueous glasses. *Biophys. Chem* 106, 251–261.
21. Douzou P (1977) *Cryobiochemistry: An Introduction*, Academic Press.
22. Rasmussen DH and Mackenzie AP (1968) Phase diagram for the system water-dimethylsulphoxide. *Nature* 220, 1315–1317. [PubMed: 5701346]
23. Murthy SSN (1997) Phase behavior of the supercooled aqueous solutions of dimethyl sulfoxide, ethylene glycol, and methanol as seen by dielectric spectroscopy. *J. Phys. Chem. B* 101, 6043–6049.
24. Bordignon E and Steinhoff H-J (2007) *Membrane Protein Structure and Dynamics Studied by Site-Directed Spin-Labeling ESR In ESR Spectroscopy in Membrane Biophysics; Biological Magnetic Resonance*, Springer.
25. Jeschke G (2013) Conformational dynamics and distribution of nitroxide spin labels. *Prog. Nucl. Magn. Reson. Spectrosc* 72, 42–60. [PubMed: 23731861]
26. Altenbach C et al. (2015) Exploring structure, dynamics, and topology of nitroxide spin-labeled proteins using continuous wave electron paramagnetic resonance spectroscopy. *Meth. Enzymol* 564, 59–100. [PubMed: 26477248]
27. Todd AP et al. (1989) Site-directed mutagenesis of colicin E1 provides specific attachment sites for spin labels whose spectra are sensitive to local conformation. *Proteins* 6, 294–305. [PubMed: 2560193]
28. Mchaurab HS et al. (1996) Motion of spin-labeled side chains in T4 lysozyme. Correlation with protein structure and dynamics. *Biochemistry* 35, 7692–7704. [PubMed: 8672470]
29. Ahmad S et al. (2004) ASAView: database and tool for solvent accessibility representation in proteins. *BMC Bioinformatics* 5, 51. [PubMed: 15119964]
30. Kabsch W and Sander C (1983) Dictionary of protein secondary structure: pattern recognition of hydrogen-bonded and geometrical features. *Biopolymers* 22, 2577–2637. [PubMed: 6667333]
31. Fan C et al. (2010) Short N-terminal sequences package proteins into bacterial microcompartments. *Proc. Natl. Acad. Sci* 107, 7509–7514. [PubMed: 20308536]
32. Choudhary S et al. (2012) Engineered protein nano-compartments for targeted enzyme localization. *PLoS ONE* 7, e33342. [PubMed: 22428024]
33. Faust LRP et al. (1990) Cloning, sequencing, and expression of the genes encoding the adenosylcobalamin-dependent ethanolamine ammonia-Lyase of salmonella-typhimurium. *J. Biol. Chem* 265 (21), 12462–12466. [PubMed: 2197274]
34. Kaplan BH and Stadtman ER (1968) Ethanolamine deaminase a cobamide coenzyme-dependent enzyme .I. purification assay and properties of enzyme. *J. Biol. Chem. Rev* (243), 1787–1793.
35. Morrison KL and Weiss GA (2001) Combinatorial alanine-scanning. *Curr. Opin. Chem. Biol* 5, 302–307. [PubMed: 11479122]
36. Stoll S and Schweiger A (2006) EasySpin, a comprehensive software package for spectral simulation and analysis in EPR. *J. Magn. Reson.* 178, 42–55. [PubMed: 16188474]
37. Chen H et al. (2013) Heterogeneous ordered-disordered structure of the mesodomain in frozen sucrose-water solutions revealed by multiple electron paramagnetic resonance spectroscopies. *Langmuir* 29, 4357–4365. [PubMed: 23464733]
38. Wertz JE and Bolton JR (1986) *Electron Spin Resonance*, Chapman and Hall.
39. Banerjee D et al. (2009) ESR evidence for 2 coexisting liquid phases in deeply supercooled bulk water. *Proc. Natl. Acad. Sci* 106, 11448–11453. [PubMed: 19556546]
40. Carugo O (2000) Predicting residue solvent accessibility from protein sequence by considering the sequence environment. *Protein Eng.* 13, 607–609. [PubMed: 11054454]

41. Chen H and Zhou H-X (2005) Prediction of solvent accessibility and sites of deleterious mutations from protein sequence. *Nucleic Acids Res.* 33, 3193–3199. [PubMed: 15937195]
42. Zhang H et al. (2009) On the relation between residue flexibility and local solvent accessibility in proteins. *Proteins* 76, 617–636. [PubMed: 19274736]
43. Wu W et al. (2017) Accurate prediction of protein relative solvent accessibility using a balanced model. *BioData Min.* 10, 1. [PubMed: 28127402]
44. Akita K et al. (2010) Purification and some properties of wild-type and N-terminal-truncated ethanolamine ammonia-lyase of *Escherichia coli*. *J. Biochem* 147, 83–93. [PubMed: 19762342]
45. Guruprasad K et al. (1990) Correlation between stability of a protein and its dipeptide composition - a novel approach for predicting in vivo stability of a protein from its primary sequence. *Protein Eng.* 4, 155–161. [PubMed: 2075190]
46. Canfield JM and Warncke K (2002) Geometry of reactant centers in the Co-II substrate radical pair state of coenzyme B-12-dependent ethanolamine deaminase determined by using orientation-selection-ESEEM spectroscopy. *J. Phys. Chem. B* 106, 8831–8841.
47. Polyhach Y et al. (2011) Rotamer libraries of spin labelled cysteines for protein studies. *Physical Chemistry Chemical Physics* 13, 2356–2366. [PubMed: 21116569]
48. Hatmal MM et al. (2012) Computer modeling of nitroxide spin labels on proteins. *Biopolymers* 97, 35–44. [PubMed: 21792846]
49. Hagelueken G et al. (2012) MtsslWizzard: In silico spin-labelling and generation of distance distributions in PyMOL. *Appl. Magn. Reson.* 42, 377–391. [PubMed: 22448103]
50. Cornish-Bowden A (1981) *Fundamentals of Enzyme Kinetics*, Butterworths and Company, Ltd.

Figure 1.

EPR spectra of 4MT spin-labeled wild-type and mutant EAL, acquired at 295 K. The spectra are normalized to the central peak-to-trough amplitude, to illustrate the differences in line shape. The normalized spin counts for each spectrum, which represent the double-integral of the presented absorption-derivative spectra, are: 1.0 (WT), 0.9 (β C46A), 0.6 (β C37A).

Figure 2.

Temperature dependence of the 4MT-labeled EAL EPR spectrum (black) and overlaid EPR simulations (dashed red lines). (A) EAL in frozen aqueous solution, 0% DMSO. (B) EAL in frozen aqueous solution containing 1% v/v DMSO. The spectra are normalized to the central peak-to-trough amplitude. Alignment along the magnetic field axis corresponds to the microwave frequency at 200 K.

Figure 3.

Temperature dependence of the rotational correlation time of 4MT-label on wt EAL and normalized mobility component weights, for the water-only, 0% DMSO condition. (A) Rotational correlation time. (B) Normalized weights. Solid circles: fast component; open circles, slow component. Error bars represent standard deviations for three separate determinations.

Figure 4.

Temperature dependence of the rotational correlation time of the 4MT on wt EAL and normalized mobility component weights, for the 1% v/v DMSO condition. (A) Rotational correlation time. (B) Normalized weights. Solid circles: fast component; open circles, slow component. Error bars represent standard deviations for three separate determinations.

Figure 5.

Structure showing possible positions of EutC residue, $\beta C37$, in the *S. typhimurium* EAL model. [9] The cartoon structure of the EutB (green) and EutC (blue), $[EutBEutC]_2$ dimer is shown in two orientations, A and B, related by a 90° rotation about the horizontal. The position of the EutC N-terminus at $\beta A47$ is shown as a red sphere in each EutC subunit. The upper-limit range of possible positions of $\beta C37$, assuming a fully-extended (32 Å) polypeptide chain, are shown as a red surface. The protein was modeled by using PyMOL (Schrödinger).

Figure 6. Temperature-dependence of the relation of the ratio of fast- and slow-motional component weights in the form of the van't Hoff plot. Solid line is the best-fit linear relation ($R^2 = 0.9113$; R is Pearson's correlation coefficient).

Figure 7.

Depiction of the two-state model proposed for the origin of the temperature-dependence of the mobility of 4MT spin label in *S. typhimurium* EAL, based on the context of the β C37 label site in the N-terminal domain of the EutC subunit (black). The 4MT spin label site (hexagon) has two mobility states, W_s (cyan) and W_f (red). The S_s and S_f states correspond to protein-“bound” (left, blue) and relatively loosely-associated, or “free” (right, dark red) conformations of the N-terminal sequence of EutC, respectively. The equilibrium between the two conformations is governed by equilibrium constant, K (K_d , 1% v/v DMSO; K_w , water-only). The grey halo represents the aqueous-DMSO mesodomain around EAL, that maintains the liquid state at cryogenic temperatures, or the side-chain fluidized region vicinal to the protein in the absence of DMSO. Blue hatched regions represent the polycrystalline water-ice domains in the surrounding frozen aqueous solution.

Table 1.Relative solvent accessibility (*RSA*) of cysteine residues in wt EAL.

Residue	<i>RSA</i> ^a
αC71	0.140
αC129	0.031
αC159	0
αC223	0.007
αC249	0
αC283	0.130
αC361	0
αC363	0
αC364	0
αC388	0.007
βC37 ^b	--
βC46 ^b	--
βC81	0
βC158	0.100
βC242	0
βC260	0.440

^aSolvent accessibility calculated by DSSP.[30]^bThese residues are absent in the X-ray crystallographic structure of *E. coli* EAL,[8] and in the *S. typhimurium* EAL model.[9]

Table 2.Steady-state enzyme kinetic and EPR simulation parameters for wild-type and mutant EAL.^a

EAL	$k_{\text{cat}}(\text{s}^{-1})$	$K_M(\mu\text{M})$	$\text{Log}[\tau_c, \text{s (s)}]$	$\text{Log}[\tau_c, \text{f (s)}]$	W_s	W_f	RMSD^b
WT	21.8±7.1	31.4±5.7	−8.37	−9.18	0.32	0.68	0.01913
βC37A	22.1±1.1	33.2	−8.17	−9.01	0.71	0.29	0.3441
βC46A	21.7±2.9	35.8±4.5	−8.25	−9.26	0.59	0.41	0.01956
βC260A	0	--	--	--	--	--	--

^aSteady-state enzyme kinetics parameters: catalytic constant, k_{cat} ; Michaelis constant, K_M . Simulation parameters: reorientational correlation time, τ_c ; normalized component weight, W .

^bRMSD, root mean square deviation of fit of simulated EPR spectrum to experimental EPR spectrum.