Published in final edited form as:

J Phys Chem B. 2017 December 14; 121(49): 11109–11118. doi:10.1021/acs.jpcb.7b09711.

Mesodomain and Protein-Associated Solvent Phases with Temperature-Tunable (200-265 K) Dynamics Surround Ethanolamine Ammonia-Lyase in Globally Polycrystalline Aqueous Solution Containing Dimethylsulfoxide

Benjamin Nforneh and Kurt Warncke*

Department of Physics, Emory University, Atlanta, Georgia 30322, United States

Abstract

Electron paramagnetic resonance (EPR) spectroscopy of the spin probe, TEMPOL, is used to resolve solvent phases that surround the ethanolamine ammonia-lyase (EAL) protein from *Salmonella typhimurium* at low-temperature (T) in frozen, globally polycrystalline aqueous solution, and to report on the T -dependence of their detectably rigid and fluid states. EAL plays a role in human gut microbiome-based disease conditions, and physical-chemical studies provide insight into protein structure and mechanism, toward potential therapeutics. Temperature dependences of the rotational correlation times (τ_c ; detection range, 10^{-11} – 10^{-7} s) and corresponding weights of TEMPOL tumbling components from 200 to 265 K in the presence of EAL, are measured in two frozen systems: (1) water-only, and (2) 1% v/v dimethylsulfoxide (DMSO). In the water-only condition, a protein-adjacent solvent component detectably fluidizes at 230 K, and melts the surrounding ice-crystalline region with increasing T , creating a bounded, relatively high-viscosity aqueous solvent domain, up to 265 K. In the EAL, 1% DMSO condition, two distinct concentric solvent phases are resolved around EAL: protein-associated domain (PAD) and mesodomain. The DMSO-aqueous mesodomain fluidizes at 200 K, followed by PAD fluidization at 210 K. The interphase-dynamical coupling is consistent with the spatial arrangement and significant contact areas of the phases, indicated by the experimentally-determined mean volume ratio, $V(\text{mesodomain}):V(\text{PAD}):V(\text{protein}) = 0.5:0.3:1.0$. The results provide a rationale for native chemical reactions of EAL at $T < 250$ K, and an advance toward precise control of solvent dynamics as a tunable parameter for quantifying the coupling between solvent and protein fluctuations and chemical reaction steps in EAL, and other enzymes.

Table of Contents Graphic

*Corresponding Author: kwarncke@physics.emory.edu.

Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website.

Supporting detailed description of EPR spectrum simulation procedures, supporting figure showing EPR spectrum from frozen aqueous solutions at 0% DMSO, no EAL, supporting tables presenting $\log \tau_c$ and W values at the different T -values for EAL, 0% DMSO, $\log \tau_c$ and W values at the different T -values for EAL, 1% v/v DMSO, $\log \tau_c$ values at the different T -values for 1% v/v DMSO, no EAL, and Arrhenius rate parameters

The authors declare no competing financial interest.

INTRODUCTION

Proteins require a minimal level of mobile hydration water, in order to execute native function.¹ The required >1 g water/g protein, corresponding to a hydration shell of thickness approximately two water molecules, arises from a dependence of the functionally-necessary protein fluctuations on solvent motions.^{1–3} Reciprocally, water dynamics influenced by protein have been distinguished from bulk water at distances of up to $20 \text{ \AA}$ from the protein surface,⁴ and computational studies suggest mechanistic features of distinct, protein-influenced water dynamics extending to $10 \text{ \AA}$.^{5–6} When water is removed by drying, protein fluctuations are lowered in frequency and suppressed in amplitude.^{7–8} Restriction of solvent water motions, by lowering temperature to solidify the solvent, also suppresses protein fluctuations and eliminates function not associated with diffusive exchange of substrates/products.^{1, 3, 9} We have shown that the protein, ethanolamine ammonia-lyase (EAL; EC 4.3.1.7),¹⁰ a bacterial enzyme that is involved in gut microbiome homeostasis,^{11–13} and in disease conditions wrought by pathogenic strains of *Salmonella* and *Escherichia coli* in humans,^{14–15} maintains the central chemical step of radical rearrangement, deep into the cryogenic temperature regime ($T \text{ 190}$), in frozen, polycrystalline aqueous solution.^{16–18} How is the enzyme able to conduct the elaborate rearrangement, and subsequent hydrogen transfer and cobalamin cofactor bond reformation reaction steps, when the bulk aqueous solvent is rigid? Insight comes from studies of the mechanism of cryopreservation, which show that frozen aqueous solutions containing relatively low concentrations of cosolvents or solutes (lower than required to form a global glass) form an interstitial “mesodomain” phase at the ice-crystallite grain boundaries.^{19–21} The mesodomain arises during freezing, as cosolvents and other solutes are excluded from the growing multiple, independently-nucleated ice-crystallite regions. The elevated cosolvent concentration lowers the melting temperature of the cosolvent/water mesodomain, and with decreasing temperature, this leads to formation of a high-viscosity fluid, and eventually, an interstitial glassy phase.²² We hypothesize that, in the presence of cosolvent, a mesodomain phase maintains fluidity and solvent fluctuations sufficient for the observed function of EAL at low temperatures. Here, we support this proposal by quantitatively describing the T -dependence, over $200\text{--}265 \text{ K}$, of the mobility of solvent phases that surround EAL in frozen $1\% \text{ v/v}$ dimethylsulfoxide (DMSO) solution, by using the nitroxide (aminoxyl) electron spin probe, TEMPOL, and continuous-wave electron paramagnetic resonance (EPR) spectroscopy.²³ The TEMPOL

mobility in frozen aqueous EAL solution without cosolvent (0% DMSO) is also determined, for comparison.

Nitroxides are stable radicals, that have been used to address the T -dependence of solvent mobility, including phase transitions and glass formation in aqueous phases.^{24–26} The rotational motion (tumbling) of the nitroxide is dependent on the medium viscosity, and is thus a proxy for solvent mobility. Tumbling leads to rotational averaging of the unpaired electron g -anisotropy and the electron- ^{14}N dipolar hyperfine interaction, and a consequent narrowing of the EPR lineshape,²³ which is quantified by the rotational correlation time (τ_c) obtained from spectral simulations. The X-band, continuous wave-EPR spin probe approach is sensitive to TEMPOL tumbling motion in the τ_c range of $\sim 10^{-11}$ (rapid limit) to 10^{-7} s (defined as the rigid limit).²⁵ The spin probe approach has been used to characterize the mesodo-main in frozen aqueous–sucrose²⁷ and aqueous–glycerol²⁶ solutions, in the absence of protein.

Here, we describe a systematic experimental and analytical strategy that leads to identification of solvent components (phases) around the protein and characterization of their T -dependent motional properties in frozen pure aqueous and 1% v/v DMSO aqueous solutions in the presence of EAL (2–20 μM) and trace TEMPOL (0.00034% w/v, 0.2 μM). The pure aqueous system is selected, to address the EAL protein effect on the surrounding solvent, in the absence of cryosolvent, through hydrogen-bonding and possible influences of surface polar amino acid side chains, which have chemical attributes of cryoprotectant molecules. DMSO is selected because it is a common cosolvent component of cryoprotectant mixtures,²⁸ with well-characterized phase behavior.^{29–30} The phase diagram of aqueous-DMSO solutions, shows water freezing point suppression at <82 % v/v, with eutectic points (53 % v/v; 210 K) and (64 % v/v; 203 K), that correspond to the presence of different DMSO hydrates.²⁹ In fluid, bulk 41% v/v DMSO/water solution at 234–248 K, EAL maintains native reactivity.^{31–33} In the present studies, DMSO is added at concentration of 1% v/v, relative to the total liquid sample volume, which leads to a polycrystalline sample upon freezing (>30% v/v DMSO is required to produce a global glass, at the mean cooling rate of 10 K/s employed here³³). The results identify two TEMPOL mobility components, with distinct T -dependences, in both 0 and 1% v/v DMSO solutions with EAL. In the 1% v/v DMSO solution, the components are assigned to a “bulk” mesodomain and a protein-associated domain (PAD), with estimated volume ratio of 1.3, and which correspond to 0.6% of the total sample volume. The results provide an unprecedented microscopic characterization of solvent structure and behavior around a protein in frozen solution, and an advance toward the precise control of solvent dynamics as a tunable parameter for quantifying the coupling between solvent and protein fluctuations and catalysis in EAL,^{18, 33} and other enzymes.

EXPERIMENTAL METHODS

Sample preparation.

All chemicals were purchased from commercial sources, including DMSO (purity, 99.9%; EMD Chemical), and deionized water was used (specific conductance, 18.2 $\text{M}\Omega\text{ cm}$; Nanopure system, Siemens). The EAL protein from *S. typhimurium* overexpressed in

Escherichia coli overexpression system and purified as described,^{34–35} with modifications.³³ The specific activity of purified EAL with aminoethanol as substrate was 20 mmol/min/mg ($T=298$ K, $P=1$ atm), as determined by using the coupled assay with alcohol dehydrogenase and NADH.³⁶ Protein samples included 10 mM potassium phosphate buffer (pH 7.5), 2–20 μ M EAL protein (20 μ M was the standard concentration), and 0.2 mM TEMPOL spin probe (4-hydroxy-TEMPO, Sigma-Aldrich) in a final volume of 0.3 ml. When present, DMSO was added to 1% v/v in the final volume of 0.3 ml. Protein and 0% DMSO solution samples were prepared aerobically, on ice in small vials, mixed, and loaded into 4 mm outer diameter EPR tubes (Wilma-LabGlass). The samples were frozen by immersion in $T=140$ K isopentane solution. This method has a characteristic cooling rate of 10 K/s.³³ Samples were transferred to liquid nitrogen for storage. Solution (no protein) samples containing 1% v/v DMSO were placed in 2mm outer diameter EPR tubes, because of their lossiness at $T>210$ K.

Continuous-wave EPR spectroscopy.

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.³³ The uncertainty in T values was ± 0.5 K.³³ In the general measurement protocol, an initial 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 TEMPOL) 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 observed for samples subjected to an additional cycle of storage and measurement. Therefore, equilibrium conditions at each T prevail. EPR acquisition parameters: Microwave frequency, 9.45 GHz; microwave power, 0.2 mW; magnetic field modulation, 0.2 mT; modulation frequency, 100 kHz; acquisition number, 4–8 spectra were averaged at each T value.

EPR Simulations.

Simulations of the CW-EPR spectra were performed by using the Chili algorithm in the program, EasySpin.³⁷ Convergence of simulations was defined by the default, local least-squares fitting criteria. Random rotational motion of the spin probe was assumed. The following established²⁷ set of parameters was used: principal values of the g tensor, $g_x = 2.0092$, $g_y = 2.0102$, $g_z = 2.0045$ (magnetic field-corrected); principal values of the ^{14}N hyperfine tensor, $A_x = 20.9$, $A_y = 19.82$, $A_z = 103.2$ MHz. The τ_c , intrinsic spectral linewidth, and, for two-component simulations, W , were varied in the simulations. Spectra were corrected by subtraction of the temperature-matched baseline sample spectrum, prior to simulation. The detailed procedure for the simulations is described in Supporting Information. Region III (210 T 265 K): Both fast- and slow-tumbling

RESULTS

Temperature dependence of the TEMPOL EPR line shape in frozen aqueous solution with EAL: 0% DMSO.

The effect of temperature on the EPR line shape of the TEMPOL spin probe at different representative temperatures from the complete addressed range of 200–265 K is shown in Figure 1A, for the EAL, 0% DMSO sample. The characteristic TEMPOL 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.²³ From 200–240 K, the spectra show the rigid-limit, powder pattern line shape, characterized qualitatively by an overall spectral width of $2A_{zz} = 7.4 \text{ mT} = 207 \text{ MHz}$, where A_{zz} is the z -component of the anisotropic hyperfine tensor. At $T = 245 \text{ K}$, narrowing of the overall spectral width and widths of individual features indicates the commencement of TEMPOL tumbling motion, which results in further line-narrowing at $T > 245 \text{ K}$.

Temperature dependence of the TEMPOL EPR line shape in frozen aqueous solution with EAL: 1% v/v DMSO.

Figure 1B shows the EPR line shape at different representative temperatures for frozen aqueous solutions of EAL protein with added 1% v/v DMSO. The rigid-limit, powder pattern line shape is observed for $T \leq 210 \text{ K}$. The overall spectral width and widths of the hyperfine features narrow at $T > 215 \text{ K}$, and this trend continues with increasing temperature. At $T \approx 250 \text{ K}$, the electron-nuclear dipolar anisotropy is well-averaged by rapid tumbling, and the overall spectral width approaches twice the value of the ^{14}N isotropic hyperfine coupling constant, $2A_{\text{iso}} = 3.4 \text{ mT} = 96 \text{ MHz}$. The comparison of the line shapes in Figure 1 shows that tumbling motion of TEMPOL in 1% v/v added DMSO is activated at $\sim 30 \text{ K}$ lower in temperature, relative to the 0% DMSO solution.

Temperature dependence of the TEMPOL rotational correlation times and normalized component weights in frozen aqueous solution with EAL: 0% DMSO.

The EPR spectra were simulated to quantify the rotational mobility in terms of the τ_c and normalized W values of TEMPOL mobility components. The temperature dependence of τ_c in the EAL, 0% DMSO condition displays two-component behavior (Figure 2A; values, Table S1), where the two components are characterized by a relatively short τ_c value (denoted as the “fast” tumbling component) and a relatively long τ_c value (denoted as “slow” tumbling component). The temperature-dependence is divided into three regions: Region I ($T < 230 \text{ K}$): The $\log \tau_c$ values lie above the tumbling detection criterion. Region II ($230 < T < 250 \text{ K}$): A fast-tumbling population is present, with $\log \tau_{c,f}$ decreasing with T , along with a rigid population. Region III ($250 < T < 265 \text{ K}$): Both fast- and slow-tumbling populations are present, with $\log \tau_{c,f}$, $\log \tau_{c,s} < -7.0$ and decreasing with T . Figure 2B shows that there is a shift in the dominant population from fast (weight, W_f) to slow (W_s). The shift reaches completion at $W_s \approx 0.8$.

Temperature dependence of the TEMPOL rotational correlation times and normalized component weights in frozen aqueous solution with EAL: 1% v/v DMSO.

The temperature dependence of τ_c for TEMPOL in the EAL, 1% v/v added DMSO condition displays two-component behavior over 200–265 K (Figure 3; values, Table S2). Three regions of tumbling behavior are observed, with boundary T values that are lower by 30–40 K, relative to the EAL, 0% DMSO condition: Region I ($T < 200$ K): The $\log \tau_c$ values lie above the tumbling detection criterion. Region II ($200 < T < 210$ K): A fast-tumbling population is present with decreasing $\log \tau_{c,f}$, along with a relatively rigid population. Region III ($210 < T < 265$ K): Both fast- and slow-tumbling populations are present, with $\log \tau_c$ values decreasing with T . Over 215–250 K, the normalized weights of the fast and slow tumbling components remain constant [mean $W_f=0.61$, $W_s=0.39 (\pm 0.01)$]. At $T > 250$ K, W_f increases slightly to a value of 0.7 at 265 K.

Temperature dependence of the TEMPOL rotational correlation times and normalized component weights in the absence of EAL: 0 and 1% v/v DMSO.

The origin of the two TEMPOL motional components in the presence of EAL was addressed by examining the motional properties of TEMPOL in solution-only samples. Frozen aqueous solutions at 0% DMSO presented a low-amplitude, single-derivative EPR line shape, with no hyperfine splitting, at all temperatures (~ 2 mT, peak-to-trough width), that was centered at $g \approx 2.006$ (Figure S1). The broad EPR signal is characteristic of multiple, strong electron spin-spin interactions, caused by aggregation of TEMPOL.^{38–39} In the frozen pure aqueous solvent system, this is expected to occur at the ice crystallite grain boundaries. In contrast, frozen 1% v/v DMSO solution yielded characteristic three-line TEMPOL EPR spectra at all temperatures. The spectra were simulated by using a single mobility component (Figure 4; values, Table S3). This is consistent with the formation of a glassy DMSO-aqueous phase in the frozen 1% v/v DMSO solution, in which the TEMPOL is dispersed. The temperature dependence of the single $\log \tau_c$ component for TEMPOL in 1% v/v DMSO matches the $\log \tau_{c,f}$ component for the +EAL condition (Figure 5). The results indicate that the W_f component in the 1% v/v DMSO, EAL condition is associated with a DMSO-aqueous mesodomain phase.

EAL protein concentration dependence of the EPR line shape in frozen 1% v/v DMSO solution.

To address the possible origin of one of the two TEMPOL motional components from a protein-associated solvent phase, the EAL concentration-dependence of W_f and W_s was determined. Experiments were performed at 1% v/v DMSO, with the concentration of the protein ranging from 2–20 μM . The series of EPR spectra obtained at $T=225$ K are shown in Figure 6. The T -value of 225 K was selected for analysis, because the lineshapes of the W_s and W_f components are the most distinct at this temperature, which optimizes the accurate detection of relative changes in weights. EPR absorption mode spectra are presented for best visualization of the full extent of the broad lineshape contributions. Upon the reduction of the protein concentration from 20 to 2 μM , W_s decreases from 0.40 to 0.09, with a compensatory increase in W_f (Table 1, Figure 7). The observed correlation of lower values

of W_s with lower EAL concentrations indicates that the slow mobility component represents a protein-associated phase.

DISCUSSION

Origin of the mobility components in the EAL, 0% DMSO condition.

Three-line TEMPOL EPR spectra are observed at all T values in 0% DMSO solution in the presence of EAL (Figure 1A). In contrast, a weak EPR signal with unresolved hyperfine features is observed in the absence of EAL (Figure S1), that indicates TEMPOL aggregation, and consequent paramagnetic broadening and quenching.^{38–39} The excellent match of the EPR spectra and two-component EPR simulations at each T value (Figure 1, Table S1) indicate that any population of this aggregate species is not significant in the presence of EAL. Therefore, we propose that the EAL protein creates a region in the immediate vicinity (1–2 solvent diameters) of the protein surface, that forms a glass-like, solid state at low- T , that, along with possible probe-protein interactions, prevents aggregation of the TEMPOL. This model is consistent with the display on the protein surface of amphiphilic, hydrogen-bonding amino acid side chains,⁴⁰ which mimic the properties of glass-promoting cosolvents^{19–21} in aqueous solution. The activation of detectable TEMPOL tumbling in the presence of EAL at 230 K is also consistent with nuclear magnetic resonance studies, which show that relatively large side chain motions on the ns timescale in hydrated globular proteins become activated over 220–250 K.^{41–42}

The origin of the three regions of T -dependence in the EAL, 0% DMSO condition (Figure 2), based on the above model, is described as follows. The protein-vicinal solvent region is rigid at $T < 230$ K (Region I). At 230 K, detectable TEMPOL mobility signals fluidization of a dominant solvent phase ($W_f=0.8$), while a smaller TEMPOL component ($W_s=0.2$) indicates a solvent phase that remains relatively solid. The dominant component is assigned to the protein-vicinal region, in line with the absence of this phase in the water-only (no EAL) system, and because the protein surface residues will disrupt the ice-crystalline hydrogen-bonding network in the vicinity of the protein surface, leading to freezing point depression. The $W_s=0.2$ component may represent TEMPOL trapped within, or on the border of, the ice crystallite region, or on the protein surface. Over 230–250 K (Region II), the mobility of the protein-vicinal, fast-motional component increases, while the minor slow-motional component remains detectably rigid. Over this same interval, W_f decreases with a compensating increase in W_s . These twin effects, and the maintenance of two-component TEMPOL mobility, suggest that TEMPOL partitions from the protein-vicinal region into a fluidizing, yet relatively viscous, aqueous solvent region, and that the volume of this aqueous region grows with increasing T . Over 255–265 K, W_s achieves a constant value of ~ 0.8 . The saturation of W_s at $T > 255$ K (Region III) may correspond to a limit on the spatial range of melting of the ice crystalline region that surrounds the protein.

Origin of the mobility components in the EAL, 1% v/v DMSO condition.

The origins of the two mobility components under the EAL, 1% v/v DMSO condition are assigned, as follows: (I) The T -dependence of $\log \tau_{c,f}$ of the W_f component in the presence of EAL matches the T -dependence of the monotonic $\log \tau_{c,f}$ for the solution-only (no EAL)

condition (Figure 5). This indicates that the W_f component for EAL, 1% v/v DMSO corresponds to an aqueous-DMSO solvent mesodomain, with bulk solvent properties. (2) The $\log \tau_{c,s}$ values in Figure 5, are distinct from the $\log \tau_{c,f}$ values, indicating that W_s originates from a different phase. The one-decade decrease in EAL oligomer concentration, from 20 to 2 μ M, reduces the total protein surface area in the sample, and therefore, reduces the volume of the protein-associated solvent. As shown in Figure 6 and Table 1, W_s decreases from 0.40 to 0.08 over the same range, with a compensating increase in W_f . Therefore, the W_s component originates from a protein-associated domain, denoted as the PAD.

Mobility transition in the EAL, 1% v/v DMSO system.

Three features of Figure 5 provide evidence for TEMPOL mobility transitions at the same T value in the mesodomain and PAD, as follows: (1) There is a discontinuity in the $\log \tau_{c,f}$ versus T dependence between 205 and 210 K: Points at $T < 210$ K do not adhere to the extrapolation of the higher- T dependence to lower T values. This discontinuity is caused by the presence of EAL, as shown by the continuous relation of $\log \tau_c$ on T for the 1% v/v DMSO solution-only (no EAL) condition over the same T -range. (2) There is an apparent discontinuity in the $\log \tau_{c,s}$ versus T dependence between 205 and 210 K. Extrapolation of the $T > 210$ K $\log \tau_{c,s}$ dependence to lower T predicts that $\log \tau_{c,s} \approx -7$ at 205 K, whereas the data at $T = 205$ K lie significantly above this value. This suggests that there is a mobility transition in the PAD. (3) Over the same T range of 205–215 K, there is a dramatic change in the fast:slow component ratio of 0.9:0.1 to 0.6:0.4. Our interpretation of the TEMPOL mobility transition in the EAL, 1% v/v DMSO system, is that the solvent in the PAD undergoes a rigid-to-mobile transition in the range $205 < T < 210$ K. This transition in the PAD influences the solvent dynamics in the mesodomain, which causes the discontinuity in $\log \tau_{c,f}$. The influence of the PAD on mesodomain is consistent with the relatively small and comparable volumes of the two phases, as estimated below, and indicates that the PAD and mesodomain abut – that is, they are not spatially isolated, independent domains in the frozen aqueous solution. A movement of $\sim 30\%$ of the total TEMPOL population from PAD to mesodomain occurs upon T decrease between 215 and 205 K. This suggests partial exclusion of TEMPOL from the PAD upon its solidification, which is facilitated by admittance into the still-fluid mesodomain.

Behavior at T -values above the mobility transition in the EAL, 1% v/v DMSO system.

In the region, $210 < T < 250$ K, the constant values of W_f , W_s and decreasing values of $\log \tau_{c,f}$ and $\log \tau_{c,s}$ indicate that the volumes of the protein hydration layer and mesodomain are constant, while the increased thermal energy promotes increased rates of rotational motion for TEMPOL in each phase. At $T > 250$ K, W_f and W_s diverge, indicating that the proportion of TEMPOL in the mesodomain increases, with a compensating loss in the protein hydration layer. This behavior may be caused by melting of the ice-crystalline region on the periphery of the mesodomain, leading to an increase in mesodomain volume.

Relative volumes of the PAD and mesodomain.

The W_s and W_f parameters are related to the relative favorability of occupancy (solvation free energy) of TEMPOL in the PAD and mesodomain, as given by the partition coefficient,

P (defined as: $P = [\text{TEMPOL}]_{\text{PAD}} / [\text{TEMPOL}]_{\text{meso}}$), and a statistical factor, which accounts for different volumes of the phases, V_{PAD} and V_{meso} . The ratio of W_s / W_f can thus be expressed as:

$$\frac{W_s}{W_f} = P \frac{V_{\text{PAD}}}{V_{\text{meso}}} \quad (1)$$

The dependence of V_{PAD} on the amount of EAL can be expressed as a scale-factor for the volume of PAD per concentration of EAL, f_{PAD} (units, $\mu\text{L}/\mu\text{M}$), multiplied by the concentration of EAL, $[\text{EAL}]$ (units, μM). Eq. 1 becomes:

$$\frac{W_s}{W_f} = P \frac{f_{\text{PAD}}[\text{EAL}]}{V_{\text{meso}}} \quad (2)$$

Figure 8 shows a linear relation between W_s / W_f and $[\text{EAL}]$ with approximately zero intercept, within the uncertainty in the weights, which is consistent with Eq. 2. The linearity indicates that the protein-solvent interaction is uniform over the one-decade range of EAL concentration.

An estimation of the relative dimensions of the PAD and mesodomain is obtained by using Figure 8. The slope of the plot, $P \frac{f_{\text{PAD}}}{V_{\text{meso}}} = 0.031$, allows estimation of V_{meso} , with the assumption of a comparable solvation interaction of TEMPOL with each phase ($P=1$), and an estimated value of f_{PAD} . An average thickness (r) of the water layer around a protein that displays dynamical properties distinct from bulk water has been identified as $\sim 10 \text{ \AA}$,⁵⁻⁶ with an upper limit of $20 \text{ \AA}$.⁴ The accessible surface area of the EAL oligomer is $1.27 \times 10^5 \text{ \AA}^2$, as determined by using ASAView.⁴³ Thus, values of $f_{\text{PAD}}(r=10 \text{ \AA}) = 2.3 \times 10^{-2}$ and $f_{\text{PAD}}(r=20 \text{ \AA}) = 4.6 \times 10^{-2} \mu\text{L}/\mu\text{M}$ are estimated. An alternative estimation is based on a minimum protein hydration level of $h=1 \text{ g H}_2\text{O/g protein}$ for functional dynamics:¹ $f_{\text{PAD}}(h=1) = 4.9 \times 10^{-2} \mu\text{L}/\mu\text{M}$. These f_{PAD} values give a range of $V_{\text{PAD}} = 0.46 - 0.98 \text{ mL}$ for $[\text{EAL}] = 20 \mu\text{M}$. By using the slope relation, $f_{\text{PAD}} / V_{\text{meso}} = 0.031$ for assumed $P=1$, the corresponding range of $V_{\text{meso}} = 0.74 - 1.58 \mu\text{L}$. Therefore, the estimated volumes of the PAD and mesodomain are comparable ($V_{\text{meso}} / V_{\text{PAD}} \approx 1.6$), for the EAL, 1% v/v DMSO condition. The combined PAD and mesodomain volume is 0.4 – 0.9 % of the total EPR sample volume.

Under standard conditions ($[\text{EAL}] = 20 \mu\text{M}$) for the 1% v/v DMSO system, the total volume of EAL oligomers, estimated by using a mean protein density of 1.35 g/cm^3 ,⁴⁴ is $2.2 \mu\text{L}$. Therefore, the estimated relative volumes of the phases, normalized to the volume of a single EAL oligomer, are $V_{\text{meso}} : V_{\text{PAD}} : V_{\text{EAL}} = 0.34 - 0.72 : 0.21 - 0.45 : 1$.

Origin of the temperature-dependence of TEMPOL tumbling mobility.

The T -dependence of τ_c over the T -range of fluidity is interpreted in terms of solution-hydrodynamic models^{44,45} to substantiate the phase assignments, and to provide

quantification of the composition and uniformity properties of the PAD and mesodomain components. The lifetime of the rotational diffusion of TEMPOL is governed by the solution viscosity, η , and the thermal energy, $k_B T$, in accordance with the Stokes-Einstein expression,

$$\tau_c = \frac{4\pi\eta a^3}{3k_B T} \quad (3)$$

where a is the effective hydrodynamic radius of TEMPOL, $\frac{4}{3}\pi a^3$ is the effective TEMPOL volume in the spherical particle limit, and k_B is Boltzmann's constant. The solution viscosity introduces the dominant T -dependence of the tumbling, and can be expressed for glass-forming solutions, in the Vogel-Tammann-Fulcher (VTF) form,⁴⁵ as:

$$\eta = \eta_0 \exp\left[\frac{B}{T - T_0}\right] \quad (4)$$

where the prefactor, η_0 , is the reference viscosity (high- T limit, $T \gg T_0$), B is a parameter characteristic of the solvent, and T_0 is related to the glass transition T -value.⁴⁵ As T exceeds T_0 , the argument of the exponential approximates the Arrhenius form, and $B \rightarrow E_a/k_B$, leading to the following approximate expression:

$$\tau_c \approx \frac{1}{A} \exp\left[\frac{E_a}{k_B T}\right] \quad (5)$$

Where $1/A = \frac{4}{3}\pi a^3 \eta_0 / k_B T$ incorporates the probe size dependence, and E_a represents a mean barrier to rotational motion of the solvent. Figure 9 shows that Arrhenius plots of $\log \tau_c$ versus $1/T$ for all conditions where $\log \tau_c \sim -7$ (tumbling detection criterion) are linear, and therefore consistent with Eq. 5 (A , E_a values, Table S4). The Arrhenius plots provide further, quantitative support for the models of EAL-associated solvent components by providing the following five insights: (1) The linear dependences indicate that the properties of the solutions are constant over the respective T -ranges, for each solvent component. Thus, the compositions of the protein-associated and mesodomain phases are uniform for $\log \tau_c \sim -7$ over the T -ranges shown in Figure 9. (2) The lower E_a values for the EAL, 1% v/v DMSO condition, relative to the EAL, 0% condition, are consistent with the fluidizing effect of DMSO cosolvent on aqueous solutions.^{29–30} (3) The near congruence of the EAL and solution-only 1% v/v DMSO relations in Figure 9 provides quantitative evidence that the mesodomain in the presence of EAL represents an aqueous-DMSO phase. (4) The comparable slopes for the EAL, 1% v/v DMSO slow and fast relations suggest that DMSO is present in both the PAD and mesodomain, when $\log \tau_c \sim -7$. (5) The higher $1/A$ value for the slow component suggests that the reference viscosity, η_0 , for the PAD is larger than for the mesodomain (assuming the same effective volume of TEMPOL in each phase).

CONCLUSIONS

The mobility parameters (correlation times, component weights) obtained from EPR spectroscopy and simulations of the TEMPOL spin probe spectra resolve solvent phases that surround the EAL protein at low- T in frozen, globally polycrystalline aqueous solution, and report on the T -dependence of their rigid and fluid states. In the qualitative model for the EAL, 0% DMSO condition, a protein-vicinal solvent component detectably fluidizes at 230 K, and melts the surrounding ice-crystalline region with increasing T , creating a bounded aqueous solvent domain with relatively high viscosity, up to 265 K. In the quantitative model for the EAL, 1% v/v DMSO condition, two distinct concentric solvent phases are resolved around EAL: PAD and mesodomain. The DMSO-enriched aqueous mesodomain phase fluidizes at low T (200 K), followed at higher T (210 K) by fluidization of the PAD. Our model for the T -dependence of mobilities in the two phases is depicted in Figure 10. The solvent rigid-to-mobile transition in the PAD at 200 K elicits a mobility change in the mesodomain, which demonstrates coupling between the solvent motions in the two phases. The interphase dynamical coupling is consistent with the spatial arrangement of the system, and significant contact area, as inferred from the estimated mean volume ratio of the concentric solvent components and EAL protein molecule, $V_{\text{meso}}:V_{\text{PAD}}:V_{\text{EAL}} = 0.5:0.3:1.0$. The results provide a rationale, and foundation for interpretation, of the reactions of EAL in frozen aqueous solutions at $T < 250$ K,^{18, 33} and represent an advance toward the precise control of solvent dynamics as a tunable parameter in quantifying the coupling between solvent and protein fluctuations, and chemical reaction steps in EAL and other enzymes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENT

We are grateful to Ms. Meghan Kohne for valuable discussions.

Funding Sources

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). Frauenfelder H; Chen G; Berendzen J; Fenimore PW; Jansson H; McMahon BH; Stroer IR; Swenson J; Young RD, A unified model of protein dynamics. *Proc. Natl. Acad. Sci* 2009, 106, 5129–5134. [PubMed: 19251640]
- (2). Jansson H; Bergman R; Swenson J, Role of solvent for the dynamics and the glass transition of proteins. *J. Phys. Chem. B* 2011, 115, 4099–4109. [PubMed: 21425816]
- (3). Vitkup D; Ringe D; Petsko GA; Karplus M, Solvent mobility and the protein ‘glass’ transition. *Nat. Struct. Biol* 2000, 7, 34–38. [PubMed: 10625424]
- (4). Ebbinghaus S; Kim SJ; Heyden M; Yu X; Heugens U; Gruebele M; Leitner DM; Havenith M, An extended dynamical hydration shell around proteins. *Proc. Natl. Acad. Sci* 2007, 104, 20749–20752. [PubMed: 18093918]
- (5). Heyden M; Tobias DJ, Spatial dependence of protein-water collective hydrogen-bond dynamics. *Phys. Rev. Lett* 2013, 111, 2181011–2181015.

- (6). Nibali VC; D'Angelo G; Paciaroni A; Tobias D, J.; Tarek, M., On the coupling between the collective dynamics of proteins and their hydration water. *J. Phys. Chem. Lett* 2014, 5, 1181–1186. [PubMed: 26274468]
- (7). Panagopoulou A; Kyritsis A; Sabater i Serra R; Gomez Ribellez JL; Shinyashiki N; Pissis P, Glass transition and dynamics in BSA-water mixtures over wide ranges of composition studied by thermal and dielectric techniques. *Biochim. Biophys. Acta* 2011, 1814, 1984–1996. [PubMed: 21798376]
- (8). Nakanishi M; Sokolov AP, Protein dynamics in a broad frequency range: Dielectric spectroscopy studies. *J. Non-Cryst. Solids* 2015, 407, 478–485.
- (9). Fenimore PW; Frauenfelder H; McMahon BH; Young RD, Bulk-solvent and hydration-shell fluctuations, similar to alpha- and beta-fluctuations in glasses, control protein motions and functions. *Proc. Natl. Acad. Sci* 2004, 101 (40), 14408–14413. [PubMed: 15448207]
- (10). Toraya T, Radical catalysis in coenzyme B12-dependent isomerization (eliminating) reactions. *Chem. Rev* 2003, 103 (6), 2095–2128. [PubMed: 12797825]
- (11). Roof DM; Roth JR, Ethanolamine utilization in *Salmonella typhimurium*. *J. Bacteriol* 1988, 170 (9), 3855–3863. [PubMed: 3045078]
- (12). Roof DM; Roth JR, Functions required for vitamin B12-dependent ethanolamine utilization in *Salmonella typhimurium*. *J. Bacteriol* 1989, 171 (6), 3316–3323. [PubMed: 2656649]
- (13). Garsin DA, Ethanolamine utilization in bacterial pathogens: Roles and regulation. *Nature reviews. Microbiology* 2010, 8 (4), 290–295. [PubMed: 20234377]
- (14). Staib L; Fuchs TM, From food to cell: Nutrient exploitation strategies of enteropathogens. *Microbiology* 2014, 160, 1020–1039. [PubMed: 24705229]
- (15). Thiennimitr P; Winter SE; Winter MG; Xavier MN; Tolstikov V; Huseby DL; Sterzenbach T; Tsolis RM; Roth JR; Bäuml AJ, Intestinal inflammation allows *Salmonella* to use ethanolamine to compete with the microbiota. *Proc. Natl. Acad. Sci* 2011, 108 (42), 17480–17485. [PubMed: 21969563]
- (16). Zhu C; Warncke K, Reaction of the CoII-substrate radical pair catalytic intermediate in coenzyme B12-dependent ethanolamine ammonia-lyase in frozen aqueous solution from 190 to 217 K. *Biophys. J* 2008, 95, 5890–5900. [PubMed: 18805934]
- (17). Zhu C; Warncke K, Kinetic isolation and characterization of the radical rearrangement step in coenzyme B12-dependent ethanolamine ammonia-Lyase. *J. Am. Chem. Soc* 2010, 132, 9610–9615. [PubMed: 20578695]
- (18). Kohne M; Zhu C; Warncke K, 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* 2017, 56, 3257–3264. [PubMed: 28548844]
- (19). Franks F, Freeze-drying of bioproducts: Putting principles into practice. *Eur J Pharm Biopharm* 1998, 45, 221–229. [PubMed: 9653626]
- (20). Goff HD; Verespej E; Jermann D, Glass transitions in frozen sucrose solutions are influenced by solute inclusions within ice crystals. *Thermochim. Acta* 2003, 399, 43–55.
- (21). Franks F, Scientific and technological aspects of aqueous glasses. *Biophys. Chem* 2003, 106, 251–261.
- (22). Mackenzie AP; Derbyshire W; Reid DS, Nonequilibrium freezing behavior of aqueous systems. *Philos T Roy Soc B* 1977, 278, 167–189.
- (23). Wertz JE; Bolton JR, *Electron Spin Resonance*. Chapman and Hall: New York, 1986.
- (24). Bhat SN; Sharma A; Bhat SV, Vitrification and glass transition of water: Insights from spin probe ESR. *Phys. Rev. Lett* 2005, 95, 2357021–2357024.
- (25). Banerjee D; Bhat SN; Bhat SV; Leporini D, ESR evidence for 2 coexisting liquid phases in deeply supercooled bulk water. *Proc. Natl. Acad. Sci* 2009, 106, 11448–11453. [PubMed: 19556546]
- (26). Banerjee D; Bhat SV, Vitrification, relaxation and free volume in glycerol-water binary liquid mixture: Spin probe ESR studies. *J. Non-Cryst. Solids* 2009, 355, 2433–2438.

- (27). Chen H; Sun L; Warncke K, Heterogeneous ordered-disordered structure of the mesodomain in frozen sucrose-water solutions revealed by multiple electron paramagnetic resonance spectroscopies. *Langmuir* 2013, 29, 4357–4365. [PubMed: 23464733]
- (28). Douzou P, Cryobiochemistry: An Introduction. Academic Press: New York, 1977.
- (29). Rasmussen DH; Mackenzie AP, Phase diagram for the system water-dimethylsulphoxide. *Nature* 1968, 220, 1315–1317. [PubMed: 5701346]
- (30). Murthy SSN, Phase behavior of the supercooled aqueous solutions of dimethyl sulfoxide, ethylene glycol, and methanol as seen by dielectric spectroscopy. *J. Phys. Chem. B* 1997, 101, 6043–6049.
- (31). Wang M; Warncke K, Kinetic and thermodynamic characterization of CoII-substrate radical pair formation in coenzyme B12-dependent ethanolamine ammonia-lyase in a cryosolvent system by using time-resolved, full-spectrum continuous-wave electron paramagnetic resonance spectroscopy. *J. Am. Chem. Soc* 2008, 130, 4846–4858. [PubMed: 18341340]
- (32). Wang M; Warncke K, Entropic origin of cobalt-carbon bond cleavage catalysis in adenosylcobalamin-dependent ethanolamine ammonia-lyase. *J. Am. Chem. Soc* 2013, 135, 15077–15084. [PubMed: 24028405]
- (33). Wang M; Zhu C; Kohne M; Warncke K, 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* 2015, 563, Part A, 59–94. [PubMed: 26478482]
- (34). Faust LP; Babior BM, Overexpression, purification, and some properties of the AdoCbl-dependent ethanolamine ammonia-lyase from *Salmonella typhimurium*. *Arch Biochem Biophys* 1992, 294 (1), 50–54. [PubMed: 1550360]
- (35). Faust LRP; Connor JA; Roof DM; Hoch JA; Babior BM, Cloning, sequencing, and expression of the genes encoding the adenosylcobalamin-dependent ethanolamine ammonia-lyase of *Salmonella typhimurium*. *J Biol Chem* 1990, 265 (21), 12462–12466. [PubMed: 2197274]
- (36). Kaplan BH; Stadtman ER, Ethanolamine deaminase a cobamide coenzyme-dependent enzyme. I. Purification, assay and properties of enzyme. *J Biol Chem* 1968, 243 (8), 1787–1793. [PubMed: 4297225]
- (37). Stoll S; Schweiger A, EasySpin, a comprehensive software package for spectral simulation and analysis in EPR. *J. Magn. Reson* 2006, 178, 42–55. [PubMed: 16188474]
- (38). Ross RT, Dipolar broadening of EPR spectra due to solute aggregation in frozen aqueous solution. *J. Chem. Phys* 1965, 42, 3919–3922.
- (39). Leigh JS; Reed GH, Electron paramagnetic resonance studies in frozen aqueous solutions. Elimination of freezing artifacts. *J. Phys. Chem* 1971, 75, 1202–1204.
- (40). Bye JW; Platts L; Falconer RJ, Biopharmaceutical liquid formulation: A review of the science of protein stability and solubility in aqueous environments. *Biotechnol. Lett* 2014, 36, 869–875. [PubMed: 24557073]
- (41). Miao Y; Yi Z; Glass DC; Hong L; Tyagi M; Baudry J; Jain N; Smith JC, Temperature-dependent dynamical transitions of different classes of amino acid residue in a globular protein. *J. Am. Chem. Soc* 2013, 134, 19576–19579.
- (42). Lewandowski JR; Halse ME; Blackledge M; Emsley L, Direct observation of hierarchical protein dynamics. *Science* 2015, 348, 578–581. [PubMed: 25931561]
- (43). Ahmad G; Gromiha M; Fawareh H; Sarai A, ASA View: Database and tool for solvent accessibility representation in proteins. *BMC Bioinformatics* 2004, 5, 1–5. [PubMed: 14706121]
- (44). Cantor CR; Schimmel PR, Biophysical Chemistry. W. H. Freeman and Company: San Francisco, 1980; Vol. 2.
- (45). Angell CA, Formation of glasses from liquids and biopolymers. *Science* 1995, 267, 1924–1935. [PubMed: 17770101]

Figure 1.

Temperature dependence of the TEMPOL EPR spectrum (black) and overlaid two-component EPR simulations (dashed red lines). (A) EAL in aqueous solution, 0% DMSO. (B) EAL in 1% v/v DMSO. EAL concentration was 20 μM . 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 2. Temperature dependence of the rotational correlation time of TEMPOL and normalized mobility component weights, for EAL, 0% DMSO. (A) Rotational correlation time. Horizontal dashed line represents upper limit of τ_c for detection of tumbling motion. (B) Normalized weight. Solid circles: fast component; open circles, slow component. Error bars represent standard deviations for three separate determinations.

Figure 3.

Temperature dependence of the rotational correlation time of the TEMPOL and normalized weight for two-component simulations for EAL, 1% v/v DMSO solution. (A) Rotational correlation time. Horizontal dashed line represents upper limit of τ_c for detection of tumbling motion. (B) Normalized weight. Solid circles: fast component; open circles, slow component. Error bars represent standard deviations for three separate determinations.

Figure 4.

Temperature dependence of the TEMPOL EPR spectrum (black) and overlaid single-component EPR simulations (dashed red lines) for 1% v/v DMSO solution in the absence of EAL. 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 5. Temperature dependence of the rotational correlation time of TEMPOL in 1% v/v DMSO solution in the absence of DMSO (single component, black circles), and comparison with values from 1% v/v DMSO solution in the presence of EAL (filled grey circles, fast component; open grey circles, slow component). Horizontal dashed line represents upper limit of τ_c for detection of tumbling motion. Error bars represent standard deviations for three separate determinations.

Figure 6.

Absorption-mode presentation of the EPR spectra for different EAL protein concentrations, and spectral deconvolutions. EAL concentrations: (A) 20 μM , (B) 10 μM , (C) 5 μM , (D) 2.5 μM . Experimental spectra, black curve; slow tumbling component, grey dashed curve; fast tumbling component, grey dotted curve. Experimental spectra were acquired at 225 K.

Figure 7.

Dependence of the normalized weights for fast and slow tumbling components on EAL protein concentration in 1% v/v DMSO at 225 K. Data are from Table 1. Fast component, W_f , solid circles; slow component, W_s , open circles.

Figure 8.

Dependence of the ratio of the slow and fast tumbling component weights on the EAL protein concentration, in the 1% v/v DMSO system, at 225 K. Error bars represent standard deviations for three separate determinations ($R^2 = 0.9974$; R , Pearson's correlation coefficient).

Figure 9.

Arrhenius plot of rotational correlation times obtained for the different conditions, and overlaid best-fit linear relations. EAL, 0% DMSO (red): fast tumbling component, filled squares; slow tumbling component, open squares. EAL, 1% v/v DMSO (black): fast tumbling component, filled circles; slow tumbling component, open circles. Solution, 1% v/v DMSO (blue): monotonic tumbling component, triangles. Horizontal dashed line represents upper limit of τ_c for detection of tumbling motion. Error bars correspond to standard deviations for three separate experiments.

Figure 10.

Model for the solvent phases that surround the EAL oligomer in frozen, 1% v/v DMSO-water solution and depiction of the temperature-dependence of the solvent mobilities. Detectably rigid (dark gray) or fluid (light grey) mesodomain and protein-associated domain (PAD) phases are reported by TEMPOL (small circles; stoichiometry, 10 per EAL oligomer), where TEMPOL is rigid ($\tau_c > 10^{-7} \text{ s}$; dark red) or tumbling ($\tau_c \leq 10^{-7} \text{ s}$; red). The depicted mesodomain, PAD, and protein sizes correspond to the experiment-based, estimated volumes of each phase, and the calculated volume of EAL.

Table 1.

Dependence of the normalized weights for fast and slow tumbling components on EAL protein concentration in 1% v/v DMSO at 225 K.^a

[EAL] (μM)	W_f	W_s
2	0.91 ± 0.01	0.09 ± 0.01
5	0.82 ± 0.01	0.18 ± 0.01
10	0.73 ± 0.01	0.27 ± 0.01
20	0.60 ± 0.01	0.40 ± 0.01

^a Mean values and standard deviations correspond to three separate experiments.