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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

Miao Wang², Chen Zhu³, Meghan M. Kohne¹, and Kurt Warncke^{1,*}

¹Department of Physics, Emory University, Atlanta, GA 30322

Abstract

Approaches to the resolution and characterization of individual chemical steps in enzyme catalytic sequences, by using temperatures in the cryogenic range of 190–250 K, and kinetics measured by time-resolved, full-spectrum electron paramagnetic resonance (EPR) spectroscopy in fluid cryosolvent and frozen solution systems, are described. The preparation and performance of the adenosylcobalamin-dependent ethanolamine ammonia-lyase enzyme from *Salmonella typhimurium* in the two systems exemplifies the biochemical and spectroscopic methods. General advantages of low-temperature studies are: (1) Slowing of reaction steps, so that measurements can be made by using straightforward *T*-step kinetic methods and commercial instrumentation, (2) resolution of individual reaction steps, so that first-order kinetic analysis can be applied, and (3) accumulation of intermediates that are not detectable at room temperatures. The broad temperature range from room temperature to 190 K encompasses three regimes: (1) Temperature-independent mean free energy surface (corresponding to native behavior), (2) the narrow temperature region of a glass-like transition in the protein, over which the free energy surface changes, revealing dependence of the native reaction on collective protein/solvent motions, and (3) the temperature range below the glass transition region, for which persistent reaction corresponds to non-native, alternative reaction pathways, in the vicinity of the native configurational envelope. Representative outcomes of low-temperature kinetics studies are portrayed on Eyring and free energy surface (landscape) plots, and guidelines for interpretations are presented.

Keywords

Adenosylcobalamin; vitamin B12; cryosolvent; enzyme; EPR; free energy surface; glass transition; protein dynamics; reaction kinetics; time-resolved

* Corresponding author: Kurt Warncke, Department of Physics, N201 Mathematics and Science Center, 400 Dowman Drive, Emory University, Atlanta, GA 30322-2430, Phone: 404-727-2974, Fax: 404-727-0873, kwarncke@physics.emory.edu.

²Present Address: Wilmad-LabGlass/SP Industries, 1172 NW Boulevard, Vineland, NJ 08360

³Present Address: BBVA Compass, 1718 Peachtree Street NE, Suite 900, Atlanta, GA 30309

1. INTRODUCTION

Catalysis of complex chemical transformations by enzymes is conducted in a sequence of events, that distribute the formidable barrier for coincident bond-breaking and bond-making among relatively low-barrier steps. Characterization of enzyme catalysis thus requires an understanding of the mechanisms of the individual chemical steps. Reaction kinetics, or the time-dependence of populations of reactant states, are a principal source of mechanistic information (Cornish-Bowden, 1981; Moore & Pearson, 1981). At room temperature, however, steady-state or transient enzyme reaction kinetics are often dominated by a single step, corresponding to the highest barrier, which limits insight. Kinetic resolution may be further thwarted by short time scales (ns to ms) for the lower-barrier steps, and low levels of accumulation of the corresponding intermediates. In particular, steps with a large activation entropy are likely to be relatively fast, and therefore latent, at room temperatures. Here, we describe two experimental approaches for resolving the kinetics of individual, chemical steps in enzyme reactions involving paramagnetic intermediates, by using fluid cryosolvent and frozen solution systems. The approaches are distinguished by temperature variation from cryogenic to ambient (190 – 295 K), and the use of time-resolved, full-spectrum continuous-wave (CW) electron paramagnetic resonance (EPR) spectroscopy (Wang & Warncke, 2008, 2013; Zhu & Warncke, 2008, 2010). Possible outcomes of low-temperature transient enzyme kinetic studies, and guidelines for interpretations, are also presented.

We developed and applied the cryo-kinetic approaches to dissect the sequence of radical reactions in the adenosylcobalamin (AdoCbl, coenzyme B₁₂) -dependent enzyme (Banerjee, 1999; Brown, 2005; Toraya, 2003), ethanolamine ammonia-lyase (EAL; EC 4.3.1.7) (Bandarian & Reed, 1999; Frey, 2010) from *S. typhimurium*. EAL catalyzes the deamination of aminoethanol and (*R*)- and (*S*)-2-aminopropanol through the multi-step reaction cycle depicted in Figure 1. The central adiabatic reaction of radical rearrangement (step 3) is surrounded by chemical sub-steps, that include: Cobalt-carbon (Co-C) bond cleavage leading to radical pair formation (step 1), substrate radical-forming and product radical-quenching hydrogen atom transfer reactions (steps 2 and 4, respectively), and Co-C bond reformation and return to the diamagnetic state (step 5). Following Co-C bond cleavage, the low-spin Co²⁺ (d⁷; electron spin, *S*=1/2) in cobalamin interacts with the radical (*S*=1/2) to form a radical pair, which is detected by EPR spectroscopy (Gerfen, 1999). The Co²⁺-substrate radical pair state can be formed and cryotrapped in EAL by using 2-aminopropanol (Babior, Moss, Orme-Johnson, & Beinert, 1974) and aminoethanol (Bender, Poyner, & Reed, 2008; Warncke, Schmidt, & Ke, 1999). Figure 2 shows the EPR spectrum of the Co²⁺-aminopropanol substrate radical pair state. Simulations of the radical pair EPR spectra yield the magnetic dipolar interaction coupling constant, *D* (and, *E*, in the case of rhombic dipolar interaction symmetry), from which the electron-electron dipolar distances, *R*_{ee}, and the isotropic exchange coupling constant, *J*, are obtained (Gerfen, 1999). The cryotrapped Co²⁺-substrate radical pair states in EAL display *R*_{ee} values of 9–11 Å, and magnitudes of the isotropic exchange interaction, *|J|*=200–300 MHz (Bender et al., 2008; Canfield & Warncke, 2002; S. C. Ke, 2003). The simulation-derived *R*_{ee} values and radical-cobalamin geometries are consistent with high-resolution X-ray crystallographic and modeled structures of EAL (Bovell & Warncke, 2013; Shibata et al., 2010). The substrate radical component of the

radical pair EPR spectrum has been monitored in the ensemble kinetics studies, and step 1 (Section 2) and step 3 (Section 3) of the reaction cycle (Figure 1) are the foci in this chapter.

2. LOW-TEMPERATURE FLUID CRYOSOLVENT SYSTEM

2.1 Background

Two general experimental approaches have been used to temporally resolve the progress of reactions in ensembles of enzymes in fluid solution samples, when starting from a synchronized initial state. One approach is to decrease the detection time constant, by using rapid-mixing with stopped-flow (Chance, 1951; Gomezhens & Perezbendito, 1991), rapid-flow-quench (Barman & Travers, 1985), flash photolysis (Porter, 1950), or pressure jump (Jacob et al., 1999), coupled with different time-resolved spectroscopies, for example, ultraviolet (UV) -visible, infrared, resonance Raman, EPR, and nuclear magnetic resonance (NMR) (Cantor & Schimmel, 1980). The second approach is to slow the reaction, through manipulation of the Boltzmann thermal contribution, by lowering the temperature. This family of “cryoenzymology” methods (Fink, 1977; Travers & Barman, 1995) has been used to study a large selection of enzymes in the sub-273 K temperature range (Douzou, Sireix, & Travers, 1970; Feig, Ammons, & Uhlenbeck, 1998; Mustafi, Hofer, Huang, Palzkill, & Makinen, 2004; Travers, Bertrand, Roseau, & Vanthoai, 1978). The cryoenzymology systems are in the fluid (or supercooled fluid) state, and therefore, reaction can be initiated by mixing components (*e.g.*, enzyme and substrate). The ratio of the mixing and instrument deadtime (τ_{dead}) to the observed reaction time constant ($\tau_{\text{obs}}=k_{\text{obs}}^{-1}$) provides a quantification of the low temperature advantage. For example, in EAL, rapid-mixing/stopped-flow experiments show that the room temperature ratio, $\tau_{\text{obs}}/\tau_{\text{dead}}$, for the rise of the 2-aminopropanol substrate radical (monitored optically by using the cobalamin Co^{3+} to Co^{2+} absorbance change) is 14 ms/3.5 ms=4 (Bandarian & Reed, 2000). For the cryosolvent system described below (Wang & Warncke, 2008), at 240 K, $\tau_{\text{obs}}/\tau_{\text{dead}}=900\text{ s}/30\text{ s}=30$, and at 234 K, the ratio is 120 (Wang & Warncke, 2013). Thus, improvements in resolution of >10-fold are realized in the EAL cryosolvent system. Further, because the low-temperature reaction in EAL is slow ($\tau_{\text{obs}}>10^3\text{ s}$), relative to spectrum acquisition sweep times (typically, 24 s), the entire CW EPR spectrum can be collected as a function of time, during the reaction. This “time-resolved, full-spectrum EPR” is valuable for simultaneously characterizing kinetics and structure of the paramagnetic reactant state(s). The cryosolvent is also nominally optically transparent, unlike for frozen, polycrystalline solution. Thus, excitatory and probe ultraviolet (UV)-visible light are transmitted through the sample, allowing optical experiments (Wesley D. Robertson, Wang, & Warncke, 2011). To realize these advantageous features, care must be taken in the preparation and characterization of the protein in the low-temperature cryosolvent system.

2.2 Selection of the cryosolvent

The goal of cryosolvent use is the depression of the freezing point of water, which allows addition and mixing of the enzyme with substrate and other solutes at sub-273 K temperatures, and also thwarts protein aggregation or denaturation, that arises from polycrystalline water (ice) formation. Cryosolvent selection criteria are based on the maintenance of the physical properties of the native aqueous solvent at low temperature, and

on the absence of components that perturb the enzyme. To achieve these criteria, several organic solvents can be used, owing to their high miscibility with water, low viscosity and relatively high dielectric constants (Douzou, 1977). Among them are ethylene glycol (Bicknell & Waley, 1985; Tesi, Travers, & Barman, 1990; Travers et al., 1978), methanol (Bicknell & Waley, 1985; Feig et al., 1998), propanediol (Fahy, Lilley, Linsdell, Douglas, & Meryman, 1990; Mehl, 1993), dimethyl sulfoxide (DMSO) (Douzou et al., 1970; Mustafi et al., 2004), and their ternary or higher-order mixtures (Bragger, Dunn, & Daniel, 2000). For the EAL enzyme, methanol and ethylene glycol react with, and inactivate, the holoenzyme (Babior, 1970; Babior & Krouwer, 1979). Therefore, we chose a binary mixture of DMSO and water as the cryosolvent system. Here, we describe the formation and properties of the 41% (volume/volume, v/v) DMSO/water system (corresponding to 1 mol DMSO/7 mol water).

2.3 Physical properties of the cryosolvent

The density (ρ), viscosity (η) and dielectric constant (ϵ) of water are three physical properties, whose values for aqueous solutions define the conditions essential for native protein structure and function. For pure water solution at 293 K, $\rho=0.998$ g/ml, $\eta=1.0020$ cP and $\epsilon=80.4$ (Douzou, 1977). Values of the physical constants for 41% (v/v) DMSO/water over the approximately 200–300 K range of inquiry are derived from empirical relationships, that are constructed from available data for DMSO/water binary mixtures (Douzou, 1977; Gaylord, 2015; Schichman & Amey, 1971). The temperature-dependence of ρ (g/cm³) can be fitted by using the following linear relation:

$$\rho = 1.247 - 6.48 \times 10^{-4}T \quad (1)$$

where temperature, T , is in units of K. At 240 K, which is in the range for the EAL studies, the density of 41% (v/v) DMSO/water is 1.09 g/cm³.

The dependence of η (cP) on temperature can be fit by using an Arrhenius-type plot, as follows:

$$\log \eta = -5.3 + 1600/T \quad (2)$$

At 240 K, the viscosity of 41% (v/v) DMSO/water cryosolvent is 23 cP. Although this value is significantly higher than that of pure water at 293 K, we find that EAL catalysis is independent of viscosity for $\eta < 40$ cP. In general, the thermally-slowed internal reactions and bound-state equilibria for enzymes will be less sensitive to η than the encounter rate (on-rate) for the protein-substrate interaction.

The relation between ϵ and temperature can also be expressed as a linear relation, as follows:

$$\epsilon = 195.1 - 0.4T \quad (3)$$

Thus, at 240 K, the value of e for 41% (v/v) DMSO/water cryosolvent is calculated to be 99.

The effective pH in the cryosolvent is critical for maintaining the native protonation states of protein and solutes. Organic solvents alter acid-base equilibria (Douzou, 1977). The dependence of pH on temperature for aqueous-organic mixtures has been determined (Maurel, Huibonhoa, & Douzou, 1975; Travers, Douzou, Pederson, & Gunsalus, 1975). Under low ionic strength conditions, which are characteristic of EAL samples (typically, 10 mM buffer, no added salt), the dependence of pH on inverse temperature is linear over 277 – 293 K. Therefore, the pH at $T < 273$ K is estimated by extrapolation of pH *versus* T^{-1} dependences, measured for $T > 273$ K by using a glass electrode. Although the standard buffer for EAL studies is potassium phosphate (pH=7.5), cacodylic acid/potassium cacodylate (dimethylarsinic acid) buffer displays a relatively weak dependence of pH on temperature and relative insensitivity to the presence of cryosolvent. Thus, potassium cacodylate buffer was selected for EAL cryosolvent studies. The measured pH-temperature dependence of 5.3 mM potassium cacodylate buffer (used in the preparation of EAL in the cryosolvent), poised at pH=7.0 prior to addition of DMSO to 41% (v/v), was fit by the function:

$$\text{pH} = 3.98 + 1010/T \quad (4)$$

Catalytic activity of EAL has a broad optimum range, from 6.6 – 8.2 (Babior, 1982), which is an advantage for low-temperature studies, because it provides a wide latitude for pH variations.

2.4 Cryosolvent phase under cooling and determination of critical cooling rate

DMSO/water mixtures of 30% and 80% (v/v) have freezing points of 254 K and 235 K, respectively, and bracket a region of supercooled fluid behavior for $30 < \% \text{ (v/v)} < 80$ (Douzou, 1977). An important parameter for achieving the supercooled fluid state is the critical cooling rate, R_c , which is defined as the minimum rate of cooling, $\Delta T / \Delta t$, required to avoid formation of a crystalline phase. Values of R_c are sensitive to DMSO/water % (v/v), and decrease non-linearly with the amount of added DMSO cosolvent: 43% ($R_c = 5$ °C/min), 41% ($R_c = 12$ °C/min), and 39% ($R_c = 150$ °C/min) (Baudot, Alger, & Boutron, 2000).

The cooling system was based on the liquid nitrogen (LN₂) flow temperature control apparatus for standard EPR resonant cavities. The cooling system was used to assess the critical cooling rate for the protein and solute-containing DMSO/water samples, and to monitor the state of the cryosolvent during protein sample preparation. The standard variable temperature (VT) dewar insert (Suprasil; Wilmad/LabGlass) allowed visual inspection of the samples throughout the cooling process. Temperature regulation and read-out were performed by using a 1/16, Deutsches Institut für Normung (DIN) -standard, autotune, proportional-integral-derivative (PID) temperature/process controller (CN77000, Omega Engineering), which controls a coil heater, based on the set-temperature and the feedback voltage from an ultra-thin T-band thermocouple (5STRC-TT-T-30-36, Omega Engineering), that was inserted inside the dewar insert. Dry nitrogen gas (N₂, thermal mediator) flowed through a heat-exchange coil (stainless steel), immersed in LN₂, and into the insert through a

transfer dewar. The system hosts samples contained in 4 or 5 mm outer diameter (o. d.) EPR tubes, and has a temperature accuracy of ± 1.5 K in the range from 200 K to 300 K.

2.5 Preparation of EAL protein in the low-temperature cryosolvent

The procedure described here was developed for EAL, in a cryosolvent of 41% (v/v) DMSO/water (Wang & Warncke, 2008). However, the principles of coupled incremental additions of cryosolvent and decreases in temperature, to avoid dielectric and pH “shock,” and thus, to maintain protein integrity and activity, are general. Assembly of the EAL holoenzyme is first performed at 295 K, by adding a molar excess of the AdoCbl cofactor, relative to EAL active sites (typically, 2:1), in buffered aqueous solution (10 mM potassium cacodylate). Small volumes (less than 15% of the volume of the holoenzyme-containing solution) of 70% (v/v) DMSO/water solvent are then added, with continuous slow mixing, in four stages marked by temperature decreases over the range from 273 to 240 K, to achieve a final 41% (v/v) DMSO/water solution of holoenzyme. The sample can be used immediately for kinetics measurements, or stored at low temperature for future use. All procedures, as depicted in Figure 3, are performed in the flow cryostat, described in Section 2.4. In the case of EAL, all operations are also performed under a dim red light, to prevent photolysis of the cofactor. Visual inspection of the sample in the cryostat assures that the system maintains the nominally transparent, fluid phase upon cooling. A detailed, step-by-step protocol for the preparation of protein EPR samples in DMSO/water cryosolvent is presented in the following section. This protocol is based on the method developed for EAL, but it illuminates the general aspects, that can be adjusted and optimized for preparation of any macromolecule sample.

2.6 Procedure for preparation of protein in low-temperature 41% (v/v) DMSO/water cryosolvent

The desired amount of protein is prepared in 100 μ L of 10 mM potassium cacodylate buffer (pH 7.2; determined at 25 $^{\circ}$ C). The 100 μ L solution yields a total of at least five samples in 2 mm outer diameter (o. d.) EPR tubes (P/N 712-SQ-250 M; Wilmad/Lab Glass). The preparation of an individual 2 mm o. d. EPR tube sample is described below. Standard small borosilicate glass test tubes are used (12 $\times$ 75 mm disposable culture tube, P/N 14-961-26; Fisher Scientific).

The starting solutions are as follows: *Tube A*: Prepare 100 μ L protein in 10 mM potassium cacodylate buffer (pH 7.2). EAL is typically present at a concentration of 480 μ M active sites, corresponding to 40 mg/ml, at this stage. *Tube B*: Mix 140 μ L of DMSO (grade, 99.5%) with 60 μ L 10 mM potassium cacodylate buffer (pH 7.2). *Tube C*: Prepare desired amount of substrate (in water), and dilute with same volume of 99.5% DMSO.

The step-by-step procedure is as follows:

- Cut a 5 mm o. d. NMR tube (P/N 710-SQ-250 M; Wilmad/Lab Glass) to 10.2 cm length (keep the sealed end intact).
- Cut a 2 mm o. d. EPR tube to 16.0 cm length (both ends open). Mark two black lines on the outer shell, one at 4.0 cm (from the cut end, this will be the liquid

level), and one at 16.0 cm (also from the cut end; this line indicates the position of the teflon insert/adaptor on the top of the EPR cavity stack, when the tube is later mounted for EPR measurements). Insert one open end into a clean disposable syringe.

- Turn on the cryostat controller, and set temperature to 1 °C. • Prepare isopentane (2-methylbutane) freezing solution at –60 °C (213 K). A standard, small (~25 cm height) dewar flask is filled with isopentane (~75% level), and an empty, thick-walled glass test tube is inserted into the isopentane solution. LN₂ is poured into the tube, intermittently, between boil-off of the LN₂, until the solution reaches the desired temperature (measured with a thermocouple inserted into the isopentane solution). All operations with the isopentane freezing solution are performed in a fume hood.
- Use a long-stem glass transfer pipette (P/N 803A; Wilmad/LabGlass) to slowly transfer the solution in tube A to the 5 mm o. d. NMR tube.
- Insert the 5 mm o.d. NMR tube (loaded with tube A solution) into the cryostat. Wait ~2 min to reach thermal equilibrium.
- Use a long-stem glass pipette to transfer 15 µL of the DMSO/water solvent in tube B into the 5 mm o. d. NMR tube. Wait 3 min to reach thermal equilibrium.
- Set the cryostat to –8 °C, and wait 2 min. If solution freezing occurs (indicated by the solution becoming opaque), insert a rod in the 5 mm o. d. NMR tube, and stir until clear. A 1 mm-diameter, teflon-coated-steel stir rod can be used.
- Use the long-stem glass pipette to transfer another 30 µL DMSO/water solvent in tube B into the 5 mm o. d. NMR tube.
- Set the cryostat to –16 °C, and wait 2 min. If solution freezing occurs, stir with the rod.
- Use the long-stem glass pipette to transfer another 30 µL DMSO/water solvent in the tube B into the 5 mm o. d. NMR tube.
- Set the cryostat to –24 °C, and wait 2 min. If solution freezing occurs, stir with the rod.
- Use the long-stem glass pipette to transfer another 30 µL DMSO/water solvent in the tube B into the 5 mm o. d. NMR tube.
- Set the cryostat to –34 °C, and wait 2 min. If solution freezing occurs, stir with the rod.
- Use the long-stem glass pipette to transfer a final 50 µL DMSO/water solvent in the tube B into the 5 mm o. d. NMR tube.
- Set the cryostat to –43 °C, and wait 2 min. If solution freezing occurs, stir with the rod.
- Use a separate, clean long-stem glass pipette to slowly transfer the substrate in the tube C into the 5 mm o. d. NMR tube. Stir with the stir rod for 30 seconds.

- Prepare melted wax in a 1.5 mL “Eppendorf” microcentrifuge tube: Place a 5 mm section of “wax wire” (Rey Industries; similar blue-colored wax wire is available from other vendors) into the tube, and melt the wax into a liquid by using a hot plate. Set a timer to count down from 90 s.
- As the wax melting timer counts down, insert the 2 mm o. d. tube into the 5 mm o. d. NMR tube, and use a syringe on top to carefully raise the solution level to the 4.0 cm black line, on the 2 mm o. d. tube.
- Quickly immerse the 2 mm o. d. tube into the cold isopentane. The 5 mm o. d. NMR tube can be frozen by immersion in LN₂, if future preparation of additional 2 mm o. d. tube samples is desired.
- When the timer alarm sounds, quickly pull out the 2 mm o. d. tube from the isopentane solution, and push it into the wax to make a seal. This step must be completed within 15 s.
- Quickly immerse the 2 mm o.d. tube back into the cold isopentane. Wait for 1 min.
- Remove the syringe and seal the other end with the wax, as in above step.
- The sample in the 2 mm o. d. tube is ready for EPR experiments. If desired, it can be solidified by immersion in the isopentane freezing solution, and used at a later time.

2.7 Protein structural integrity in the low-*T* fluid cryosolvent system

The EPR spectroscopic properties of the protein in the cryosolvent system can be compared with the properties under native conditions in aqueous solution, to assess structural perturbations by the cryosolvent. In the case of a radical pair, the EPR simulation-derived *D*, *E*, and *J* parameters characterize the interaction between the unpaired electron spins (Wertz & Bolton, 1986). The *J* value reports primarily on through-bond, or electron spin delocalization-mediated, coupling, and *D* is directly proportional to the inter-electron spin distance, as R_{ee}^{-3} . The CW-EPR spectrum of the Co²⁺-substrate radical pair, that accumulates during steady-state turnover in aqueous solution at 277 K (Babior et al., 1974), is essentially identical to the spectrum recorded for the radical pair prepared by mixing of substrate with ternary complex in the cryosolvent system (Wang & Warncke, 2008), as shown in Figure 2. Thus, the values of $R=11$ Å and $J=300$ MHz are comparable, to within the standard deviation of the measurements, indicating that the native EAL protein structure is maintained in the cryosolvent. The *R* and *J* values are also in agreement with those obtained for the (*S*)-2-aminopropanol-generated Co²⁺-substrate radical pair in EAL by other groups (Bandarian & Reed, 2002; Boas, Hicks, Pilbrow, & Smith, 1978). High-resolution EPR spectroscopies of hyperfine interactions of the substrate radical (Canfield & Warncke, 2002; LoBrutto et al., 2001; Sun, Groover, Canfield, & Warncke, 2008; Warncke & Utada, 2001), both internal and with surrounding protein nuclei, may detect more subtle differences (< 1 Å) between native aqueous and cryosolvent conditions. Superhyperfine interactions of Co²⁺ in cobalamin are also sensitive detectors of structure (S.-C. Ke, Torrent, Museav, Morokuma, & Warncke, 1999; Torrent, Musaev, Morokuma, Ke, & Warncke, 1999).

2.8 Time-resolved, full-spectrum measurements of reaction kinetics in the low-temperature fluid cryosolvent system

The X-band EPR measurements were performed on a Bruker ELEXSYS E500 EPR spectrometer, by using a Bruker ER4123SHQE X-band cavity resonator, and Bruker ER4131VT system for temperature control. A nitrogen flow cooling system was used, with a 15 cm heating coil installed in a transfer dewar (length ~1 m, P/N 1100104, Bruker), approximately 60 cm from the EPR cavity dewar insert. The accuracy of the temperature value is an important consideration for the experiment. The temperature gradient over the height (long-axis) of the upright sample, was measured to be ± 0.2 K, by using an ultra-thin, T-band thermocouple probe. The temperature readout of the Bruker ER4131VT controller unit was calibrated by using a model 19180 4-wire RTD probe (± 0.3 K accuracy in the 230 K to 273 K region; factory-calibrated), interfaced with the temperature controller (ITC503, Oxford Instruments). Thus, the total temperature uncertainty of the EPR measurements is estimated to be ± 0.4 K.

The fluid state of the protein samples leads to microwave absorption and consequent reduction in the cavity quality factor, Q . Therefore, the sample volume was minimized by using the 2 mm o. d. quartz EPR tubes. The tubes are also stable under the large changes in temperature in the experiment.

Prior to reaction initiation, (*S*)-2-aminopropanol substrate in 41% (v:v) DMSO/water cryosolvent, was added, and mixed with the solution of holoenzyme, at 230 K. The ternary complex is incubated for 2–3 min at 230 K, which allows ample time for substrate to bind to the active site. The reaction of the EAL ternary complex is initiated by a temperature-step (T -step), caused by a change from a starting, low-temperature set point (typically, 230 K), to the desired higher temperature (~ 250 K) for kinetics measurement. The deadtime of the experiment is determined by the time intervals for the T -step and re-balancing of the microwave bridge at the higher set temperature. The PID parameters of the temperature controller, along with the heater power and N_2 gas flow rate of the Bruker ER4131VT temperature controller, are adjusted to minimize the following factors: (1) Rise time from 230 K to the set higher temperature, (2) overshoot of the set temperature, and (3) the settling time. A set of PID parameters is obtained, which guarantee that the temperature rising and settling process, from the low- to high-temperature set point requires <10 s, with an overshoot of ~ 1.5 K. Re-balancing of the microwave bridge at the high- T set point, by using the auto-calibration (“auto-tune” mode) of Bruker instrument control software, requires ~ 10 s. Therefore, the optimal, total deadtime of the instrument is 30 s. Figure 4 shows the monotonic rise of the substrate radical component of the EPR line shape as a function of time after T -step from the low- to high-temperature set point.

2.9 Measurement of equilibria in the low-temperature fluid cryosolvent system

The temperature dependence of equilibria among catalytic states can be measured in the cryosolvent system (Wang & Warncke, 2008). A method was developed to measure the EPR amplitudes for samples in the solid state at a common, low-temperature value (120 K). Measurement of the EPR spectral amplitude at 120 K provides a uniform standard for all incubation temperature values, obviating interference with quantitation from T -dependent

line shape effects, and allows larger sample volume [4 mm o. d. EPR tube (P/N 707-SQ-250 M, Wilmad/LabGlass), relative to the 2 mm o. d. tube], for increased signal-to-noise ratio (*SNR*).

The EPR hardware used in this experiment is the same as that described for the time-resolved, full-spectrum CW EPR experiments. The sample, containing ternary complex and a 100-fold excess of substrate relative to active sites, is initially adjusted to the incubation temperature, T_{inc} , in the ER4123SHQE X-band resonator, and then incubated to achieve a constant amplitude of the Co^{2+} -substrate radical pair EPR signal (time generally $>3\tau_{obs}$). In EAL, the time scale of the substrate-to-product radical reaction (Figure 1, step 3) after radical pair formation is >10 -fold longer than the radical pair formation time. Therefore, the long-time amplitude represents well the equilibrium level of Co^{2+} -substrate radical pair formation. The temperature of the sample is then step-decreased to 120 K, in a time interval that is significantly shorter than the equilibration time (15 s, $\ll \tau_{obs}$). Therefore, the amplitude of the EPR spectrum acquired at 120 K faithfully represents the radical pair population at T_{inc} . The equilibrium peak-to-trough EPR amplitude (measured as the difference in amplitude between the lowest field peak and highest field trough of the substrate radical lineshape component; see Figure 2) is denoted as $A_{pt}(t=\infty, T=T_{inc})$. The sample temperature is then stepped to the next temperature incubation set point, and the incubation and measurement process is repeated. In order to determine if the observed changes in the EPR amplitude of the Co^{2+} -substrate radical pair are reversible, a nonlinear temperature perturbation sequence is applied: 238 $\rightarrow$ 242, 242 $\rightarrow$ 240, 240 $\rightarrow$ 246, 246 $\rightarrow$ 244, 244 $\rightarrow$ 248 K. A linear amplitude *versus* temperature relation indicates a reversible equilibrium. The $A_{pt}(\infty, T_{inc})$ values can be normalized, by using a reference state, for which the sites are saturated. In the case of EAL, after completion of the incubation series, the sample temperature is raised stepwise to 273 K, to avoid sudden pH and e changes, held for 5 min, and then step-decreased to 120 K. This leads to the formation of the Co^{2+} -substrate radical pair in $>90\%$ of the functional EAL active sites, as found previously (Hollaway et al., 1978). The fraction of EAL active sites occupied by the Co^{2+} -substrate radical pair, v_{inc} , was computed for each incubation temperature, by using the following expression:

$$v_{inc} = \frac{A_{pt}(\infty, T_{inc})}{A_{pt}(\infty, 273 \text{ K})} \quad (5)$$

For EAL, the value of v increased with increasing temperature from 0.39 (standard deviation, ± 0.07) to 0.61 (± 0.04) over 238–248 K (Wang & Warncke, 2008). Therefore, the ternary complex and the Co^{2+} -substrate radical pair have comparable stabilities over the temperature range, with a trend towards a more stable Co^{2+} -substrate radical pair state as temperature increases.

2.10 Optical properties and measurements in the low-temperature fluid cryosolvent system

The supercooled DMSO/water cryosolvent system is nominally optically transparent. This allows flash-lamp or laser excitation of samples, and measurements of component concentrations and kinetics of reaction, by using ultraviolet (UV)/visible absorption spectroscopy. For example, we studied the pulsed-laser photolysis of the AdoCbl cofactor Co-C bond in the ternary and inhibitor-bound complexes of EAL in 50% (v/v) DMSO/water at 240 K, and the subsequent msms recombination kinetics, by using time-resolved optical absorption spectroscopy (Wesley D. Robertson et al., 2011), performed on a home-built transient UV-visible spectrophotometer (Wesley. D. Robertson & Warncke, 2009).

2.11 Experimental results, models, and analyses: EAL in the cryosolvent system

The 41% (v/v) DMSO/water system was used to measure the temperature-dependence of the kinetics of formation of the Co^{2+} -substrate radical pair, starting from the ternary complex (Wang & Warncke, 2013). The first-order kinetics of substrate radical formation were interpreted in terms of a linear, two-step, three-state kinetic model (Moore & Pearson, 1981) (representing the first three states in Figure 1). For the particular case of EAL over 234–248 K, the first-order rate constant was shown to represent the forward step of Co-C bond cleavage (step 1 in Figure 1) (Wang & Warncke, 2013). In other systems, multiple kinetic phases would require interpretation by using different models and approximations (Moore & Pearson, 1981), in order to resolve rate constants for single steps.

The Eyring formalism was used to analyze the temperature-dependence of the first-order rate constant, by using the following equation:

$$\ln(k/T) = \ln\left[\frac{k_B}{h}\right] + \frac{\Delta S^\ddagger}{R} - \frac{\Delta H^\ddagger}{R} \frac{1}{T} \quad (6)$$

where k_B is Boltzmann's constant, h is Planck's constant, and $\Delta H^\ddagger$ and $\Delta S^\ddagger$ are the activation enthalpy and entropy, respectively (Cornish-Bowden, 1981). The values for $\Delta H^\ddagger$ and $\Delta S^\ddagger$ for Co-C bond cleavage were obtained from the slope ($-\Delta H^\ddagger/R$) and y -intercept $[\ln(k_B/h) + \Delta S^\ddagger/R]$ of the plot of $\ln(k/T)$ versus T^{-1} . The $\Delta H^\ddagger$ values for enzyme (Wang & Warncke, 2013) and solution (B. P. Hay & Finke, 1986) Co-C bond cleavage were comparable (32 kcal/mol), but the $\Delta S^\ddagger$ value for the enzyme was 61 cal/mol/K, compared to 7 cal/mol/K for solution. The findings are consistent with significant entropy contributions to the two-step (corresponding to coupled steps 1 and 2, Figure 1) formation of the Co^{2+} -thiyl radical pair in ribonucleotide triphosphate reductase (Licht, Lawrence, & Stubbe, 1999) and Co^{2+} -substrate radical pair in methylmalonyl-CoA mutase (Chowdhury & Banerjee, 2000) at room temperature, suggesting that significant entropy contributions to radical pair formation are general for AdoCbl-dependent enzymes. Our first resolution of the Co-C bond cleavage step in a B_{12} enzyme, and fundamental insights into enzyme mechanism (described in section 4), were achieved by virtue of the low-temperature, cryosolvent system. In particular, the Co-C bond cleavage step is latent at room temperature (step 2 in Figure 1 is

rate-determining), but is rendered rate-limiting, and therefore accessible to measurement, at low temperature.

The 41% (v/v) DMSO/water system was used to address the equilibrium between the ternary complex and the Co^{2+} -substrate radical pair state (Wang & Warncke, 2008). For the general equilibrium, $A \xrightleftharpoons{K} B$, the equilibrium constant, K , at a particular T_{inc} value is related to the concentrations of species A and B (here, the ternary complex and Co^{2+} -substrate radical pair, respectively), and to the ν_{inc} parameter obtained above, as follows:

$$K_{\text{inc}} = \frac{[B]}{[A]} = \frac{1}{\frac{1}{\nu_{\text{inc}}} - 1} \quad (7)$$

The relation of K_{inc} to the equilibrium Gibbs free energy (G), is given by the van't Hoff relation (Cornish-Bowden, 1981):

$$K_{\text{inc}} = \exp \left[\frac{-\Delta G}{RT_{\text{inc}}} \right] \quad (8)$$

By relating G with the equilibrium enthalpy (H) and entropy (S) by using the Gibbs equation, $G = H - TS$, and taking the natural logarithm of both sides of Eq. 8, the following expression is obtained for $T = T_{\text{inc}}$:

$$\ln K_{\text{inc}} = \frac{\Delta S}{R} - \frac{\Delta H}{R} \frac{1}{T_{\text{inc}}} \quad (9)$$

where R is the gas constant. Thus, a plot of $\ln K_{\text{inc}}$ versus T_{inc}^{-1} yields the values of H and S from the slope ($-H/R$) and y-intercept ($-S/R$). A relatively large $S = 45$ cal/mol/K was found to be associated with formation of the Co^{2+} -substrate radical pair state. This is consistent with the relatively large $S^\ddagger$ for Co-C bond cleavage, and suggests that the radical pair intermediate is a state of relatively high entropy.

3. LOW-TEMPERATURE FROZEN SOLUTION SYSTEM

3.1 Background

Three general types of methods have been developed to resolve reaction steps and intermediate states in metalloproteins in solid state samples at cryogenic temperatures. The first method is radiolytic cryoreduction, followed by annealing, in which a sample containing a redox-poised metal center in the protein at equilibrium, is subsequently frozen, and then reduced by using γ -irradiation, at 77 K (Blumenfeld, Davydov, Magonov, & Vilu, 1974; Davydov & Hoffman, 2011; Davydov et al., 2002). The reduced metal center is no longer at equilibrium, and is thermally activated by graded-annealing to relax through protein conformational changes, electron transfer, or chemical reaction. This technique has

been primarily applied to heme and non-heme iron proteins. The second method is the low temperature photodissociation of protein-bound metal-ligand complexes in frozen solutions. The prototype is optically-monitored migration and rebinding of carbon monoxide (CO) or dioxygen (O₂) to the heme iron in myoglobin after photolysis of the carboxy- or oxy-heme state in frozen solutions at temperatures from <10 to 270 K (Austin, Beeson, Eisenstein, Frauenfelder, & Gunsalus, 1975; Frauenfelder et al., 2009). This technique has been extended to other heme proteins and metalloproteins. The third method is to prepare and cryotrap a kinetically unstable enzyme intermediate state, which can be subsequently promoted to relax by annealing (Lukoyanov, Barney, Dean, Seefeldt, & Hoffman, 2007; Zhu & Warncke, 2008, 2010). Here, we consider the third method.

The Co²⁺-substrate radical pair state, formed by using the two EAL substrates, 2-aminopropanol or aminoethanol, accumulates as the only detectable paramagnetic intermediate during steady-state turnover of EAL at room temperature (Babior et al., 1974; Bandarian & Reed, 2002; Bender et al., 2008). The kinetically unstable substrate radical intermediate state formed from the aminoethanol substrate can be cryotrapped (Warncke et al., 1999), and is stabilized by storage at 77 K. The forward reaction of the substrate radical ensemble can be synchronously initiated at low temperature by a *T*-step, and the time-evolution of the reaction is monitored by time-resolved, full-spectrum EPR spectroscopy (Zhu & Warncke, 2008, 2010). The methods have allowed the study of the radical rearrangement and second hydrogen atom transfer (Figure 1, steps 3 and 4) for natural isotopic abundance and 1,1,2,2-²H₄-labeled aminoethanol substrate over the exceptional range of 173–235 K in the bulk frozen solid state (173–187 K range; H. Chen and K. Warncke, unpublished).

3.2 Trapping Protocols

The Co²⁺-substrate radical pair samples were prepared by first accumulating the intermediate during steady-state turnover at 277 K, and then rapidly lowering the temperature to trap the radical pair (Zhu & Warncke, 2008). The standard procedure for manual cryotrapping of steady-state intermediates in EAL (Warncke et al., 1999; Zhu & Warncke, 2008) starts with manual mixing, on ice, of the prepared EAL-AdoCbl holoenzyme solution (~0.27 ml; in a 12×75 mm disposable culture tube, P/N 14-961-26; Fisher Scientific) with substrate (~0.03 ml of 0.1–1.0 M stock; added with an automatic pipette, under vortex mixing; elapsed time, ~5 s). The sample is then removed from the test tube with a long-stem glass transfer pipette (9 inch Pasteur Pipet, P/N 13-678-6B; Fisher Scientific), and discharged into the middle region of a 4 mm o. d. EPR tube. The open, top end of the EPR tube is held firmly between thumb and index finger, and two high-to-low height, sweeping “whip” motions of the tube move the solution contents to the bottom, and the tube is then plunged into LN₂-chilled isopentane (*T* = 133 K) to trap the Co²⁺-substrate radical pair state. The total elapsed time from substrate mixing to isopentane immersion by this procedure is ~15 s. The procedure can be performed in a cold room, or at room temperature, by keeping all components on ice. Samples are stored in LN₂, prior to use.

The cooling rate during trapping is relevant to conservation of the accumulated paramagnetic state, because, at some temperature during the trapping, diffusive exchange of substrate/

product with the active site ceases. If the cooling rate is much greater than the rate of substrate radical reaction (step 3, Figure 1), then the total accumulated population is trapped. If the cooling rate and reaction rate are comparable, a proportion of the steady-state level of Co^{2+} -substrate radical pair will decay. In the case of EAL, the decay is to the EPR-inactive, diamagnetic product state. The loss of radical intermediate during cooling is partially mitigated by the slowing of the reaction, itself, with decreasing temperature.

Cooling rate is also an adjustable parameter, that can be used to dissect mechanism. For studies of the effect of cooling rate on sample properties, “slow” and “fast” cooling protocols were developed. A sample was prepared for slow cooling by cryotrapping, as described above for the standard sample. The sample temperature was then raised to 223 K (in the protein glass transition region, described below), and systematically stepped to lower temperature at a rate of 1 K per 15 s until $T=214$ K was reached, after which the sample was immersed in LN_2 -chilled isopentane ($T=133$ K).

A “fast” cooling rate sample was prepared, by using a home-built cooling apparatus (design available upon request). A 2 mm o. d. EPR tube, cut to be open at both ends, was mounted in the inner volume of a modified LN_2 dewar, with a section of length ~ 2 cm protruding from the bottom of the dewar. A disposable syringe (35 ml), with ram in the compressed position, was fitted snugly (air-tight seal) over the top end of the EPR tube, and then fitted over the support in the dewar, and sealed (Great Stuff, Dow Chemical Co.). LN_2 was then added to the dewar. A small fan, mounted below the dewar, maintained the tip of the lower protruding portion of the tube near room temperature. The holoenzyme and substrate were mixed in a micro Eppendorf tube, and the tube was immediately raised under the LN_2 dewar, to insert the EPR tube end in the enzyme solution. The syringe ram was moved out, to draw the sample into the tube. This action flash-freezes the rising sample in the tube. The tip of the EPR tube that is outside of the dewar is broken-off, and the 2 mm o. d. tube, containing the now-frozen sample, is removed from the dewar, and placed in a 4 mm o. d. EPR tube for storage in LN_2 , prior to use.

Both the medium and fast cooling rates through the transition temperature range were determined by placing a thermocouple, which was attached to an oscilloscope, into a mock sample. The mock sample was then flash-frozen in isopentane at 133 K. The temperature *versus* time profile on the oscilloscope is the “cooling curve.” The fast cooling rate was determined in the same way, except that an ultra-thin thermocouple was partially inserted into the mock sample capillary tube, through a small opening in the capillary wall (produced by using the edge of a grinding wheel). The following values were obtained for the different cooling methods, and correspond to the traverse time of the 214–219 K interval around the protein glass transition: Slow, 0.07 K/s; standard, 10 K/s; fast, 400 K/s. The cooling rates differ by over 3 decades.

3.3 Time-resolved, full-spectrum EPR measurements

EPR measurements were made by using the Bruker E500 EPR spectrometer. EPR samples were held at a staging temperature of 160 K or 180 K in the microwave cavity, under control of the ER4131VT cryostat system, and the microwave bridge was tuned. T -steps from 160 K or 180 K to the decay measurement temperatures between 190 and 230 K were initiated by

changing the ER4131VT temperature set-point. The temperature at the sample was calibrated, as described above. Different cryostat/controller systems were used, depending upon the experiment run time, which, in general, was the time required for the sample to decay to 10% of the initial radical pair amplitude. For measurements over the lower temperature range, 190–207 K, the ER4131VT cryostat/controller system was used with the ER4121VT-1011 evaporator/transfer line (including 26 L LN₂ bath; TR276, Air Liquide) and ER4121VT-1013 heater/thermocouple. This system provided relatively long run times for an evaporator configuration (8 h, N₂ gas flow rate-dependent), which were necessary for relatively long sample decays. For measurements at $T \geq 210$ K, the standard ER4131VT components were used (~8 L LN₂ bath; KGW Isotherm), which afforded run times of 6 hours. A third system utilized the vent gas from a 250 L LN₂ storage dewar to drive N₂ gas flow (N₂ flow rate regulated by using a ball flow gauge; King Instrument Co.) through a coil immersed in a LN₂ bath in a ~4 L dewar. This system allows effectively unlimited run time, under the requirement of periodic replenishment of the LN₂ in the bath. For each system, the uncertainty in the temperature value was approximately ± 0.5 K.

Once the sample temperature stabilized at the set point, the pre-set auto-tune/auto-scan mode of the spectrometer was triggered, and the sample was auto-tuned at the high-temperature set point, followed immediately by repeated, continuous spectrum acquisition. The time from initiation of the temperature step to the start of acquisition of the first spectrum was 30–60 s. The first EPR spectrum was collected upon stabilization at the set point. The acquisition time of an individual spectrum was typically 24 s for the sweep across the radical component of the EPR spectrum (record length, 1024 points; dwell time, 23 ms/point), with a time constant of 2.56 ms, but could be as short as 6 s (with a corresponding shorter time constant) for more rapid decays at higher temperatures. Figure 5 shows a representative time-resolved, full-spectrum EPR decay time series, that captures the full Co²⁺-substrate radical pair EPR spectrum.

3.4 Experimental results, models, and analyses: EAL in the frozen solution system

The temperature-dependence of the decay kinetics of the Co²⁺-substrate radical pair state are monotonic (monoexponential) from 230 to 220 K (Zhu & Warncke, 2008, 2010) (M. Kohne and K. Warncke, unpublished). The interval from 219 to 214 K marks a discontinuous transition. At $T < 214$ K, the decay kinetics diverge, and are well-fit by a biexponential decay function. The upper, high-temperature limit of the measurements is dictated by comparable lifetimes for the decay ($k^{-1} = 25$ s at 230 K) and the experimental deadtime of ~30 s. The rate constants were assigned to the radical rearrangement reaction (step 3, Figure 1), based on substrate ¹H/²H kinetic isotope effects (Zhu & Warncke, 2010). If possible, it is valuable to make a connection between the low- and room-temperature regimes, toward supporting the biological relevance of the low-temperature results. In the case of EAL, the k_{cat} for steady-state turnover and the room-temperature rate constant for decay of the steady-state (corresponding to the accumulated Co²⁺-substrate radical pair state) following substrate depletion are comparable (Bandarian & Reed, 2000; Hollaway et al., 1978), and values of k_{cat} , measured at 277 and 295 K, adhere to the linear extrapolation of the monotonic $\ln k$ versus T^{-1} data for 220–230 K (C. Zhu, M. Kohne, K. Warncke, unpublished). This is consistent with rate-determination by the radical rearrangement reaction from room- to low-

temperature, and with the partial substrate $^{14}\text{N}/^{15}\text{N}$ steady-state isotope effect, that is assigned to the substrate C-N bond cleavage sub-step (Frey, 2010; Poyner, Anderson, Bandarian, Cleland, & Reed, 2006).

The presence of two kinetic components at $T < 220$ K could correspond to a two-step sequential reaction (for EAL, steps 3 and 4 in Figure 1), or to two parallel, first-order reactions (for example, two different microscopic paths for step 3 in Figure 1) (Moore & Pearson, 1981). In the case of the substrate radical reaction in EAL, simulations of the biexponential decay kinetics, by using the two-step model (implemented by using MATLAB; MathWorks, Natick MA), predicted the accumulation of the intermediate state, to $\sim 50\%$ of total states. However, the accumulation of a product radical was not detected by EPR, which is consistent with the high calculated energy of product radical intermediates (Semialjac & Schwartz, 2003; Wetmore, Smith, Bennet, & Radom, 2002), relative to substrate radical. Therefore, a model of two parallel first-order reactions was adopted (Zhu & Warncke, 2008).

In the case of parallel first-order reactions, additional information about the system can be obtained by manipulating the populations. An enriched subset of states can be prepared by executing a partial decay. For example, if there are two states, 1 and 2, of equal initial amplitude, with lifetimes that are related as $\tau_1 = \tau_2/10$, then after a time, $t = 3\tau_1$, state 1 decays to a normalized amplitude of $\exp(-t/\tau_1) = 0.05$, while state 2 decays to $\exp(-0.3\tau_1/\tau_2) = 0.74$. The sample is therefore enriched in component 2, by a factor of 15, with a modest loss of signal amplitude. Longer decay times increase the enrichment of the slower decay component. For EAL, partial decays are performed over periods of up to 16 h, by incubation in a -80°C (193 K) freezer, and result in $>95\%$ enrichment in the slow component. The enriched sample is an advantage toward accurate determination of the slow component decay kinetics at higher temperatures, for which the fast and slow decay rate constants, when both components are present, differ by <3 -fold. Distinguishing k values by simulation of decay data with reasonable *SNR* and incomplete decay is generally not reliable when the difference is <3 -fold.

The enrichment can also be used to resolve structural differences between the states, by acquiring EPR spectra of the slow-decay component, in isolation. This can be followed by difference spectroscopic analysis, by subtracting the appropriately-scaled, enriched slow-decay sample spectrum from the time-zero sample, which includes both the slow- and fast-decay components, to reveal the spectrum of the fast-decay component. This approach, applied by using CW-EPR spectroscopy, does not reveal spectral differences between the two-components in EAL, which suggests that the origin of the two components lies in the protein structure, rather than in a significant difference in substrate radical structure, or distance from Co^{2+} .

The normalized amplitudes of the fast and slow phases were found to be independent of cooling rate. These rates correspond to traverse times over the approximate temperature-range of the protein glass-like transition ($214\text{--}219$ K; $T = 5$ K) of 13 ms to 71 s. The cooling rate-independence of the amplitudes suggests that the two components do not arise from an equilibration process, that is truncated by the decreasing temperature.

4. ANALYSIS AND INTERPRETATIONS: GENERAL CASES AND GUIDELINES

The deep low-temperature regime is uncharted for the majority of enzymes. To illustrate the possible outcomes and guide interpretations of low-temperature experiments, four general cases of rate-temperature dependence are considered for an enzyme reaction step, and compared with results from EAL studies from the 190–250 K range. In practice, more than one of these behaviors may occur over the experimental temperature range. The Eyring plot of $\ln(k/T)$ versus T^{-1} (or, alternatively, the Arrhenius plot of $\ln(k)$ versus T^{-1}) is the starting point for analysis. Figure 6 shows the four general types of behavior on the Eyring plot, and Figure 7 displays corresponding free energy surfaces (or landscapes), which portray the free energy of the system as a function of a reaction (chemical) coordinate (RC), and a configuration coordinate (CC), that represents the protein and associated solvent configurations that define the trajectories of the reaction (Kamerlin & Warshel, 2011; Stillinger, 1995). In Figure 6A, the linear relation indicates that the same rate-determining step and mechanism are maintained over the examined temperature range. The temperature dependence of the reaction arises from the change in the Boltzmann population of states, over an invariant mean free energy surface, as depicted in Figure 7A.

Figure 6B depicts the effect of a change in rate-determining step for the overall reaction. As for case A, the observed temperature-dependence arises from the change in the Boltzmann population distribution. The gradual, continuous change in slope suggests that the mean free energy surface is invariant with temperature. The crossing of two curves, that represent different steps in a reaction sequence, on the $\ln(k/T)$ versus T^{-1} plot, requires differences in both the $H^\ddagger$ and $S^\ddagger$ components of the reaction. Owing to the enthalpy-entropy compensation phenomenon (Lumry & Rajender, 1970; Qian & Hopfield, 1996), $H^\ddagger$ and $S^\ddagger$ are expected to vary in tandem for different reaction steps (*e.g.*, an increase in $H^\ddagger$ will be accompanied by an increase in $S^\ddagger$, and *vice versa*) and the corresponding $\ln(k/T)$ versus T^{-1} curves will display different slopes and intercepts (see Eq. 6). This increases the probability of observing curve-crossing, even if $G^\ddagger$ values for different steps are comparable.

In Figure 6B, step 1 in a two-step reaction sequence is represented as having higher $H^\ddagger$ (steeper slope) and $S^\ddagger$ (higher y-intercept value), relative to step 2. This leads to rate-determination by step 1 in the low temperature limit, and by step 2, at higher temperature. This is the behavior proposed for the first two steps in the catalytic cycle of EAL (Figure 1), based on the low-temperature kinetics of Co^{2+} -substrate radical pair formation in the DMSO/water cryosolvent system (Wang & Warncke, 2013). The revelation of high- $S^\ddagger$ reaction steps is a specific, predicted outcome of low- T kinetic studies. Therefore, the proposed “configurational catalysis” model for the high- $S^\ddagger$ Co-C bond cleavage reaction step in EAL is described in more detail. The total configurational entropy, S_{conf} , of the protein can be expressed as follows (Karplus, Ichiye, & Pettitt, 1987):

$$S_{conf} = \sum_{i=1}^N p_i S_i^I - k_B \sum_{i=1}^N p_i \ln p_i \quad (10)$$

The first term on the right-hand side of Eq. 9 represents an individual protein configuration, i , with Boltzmann weighting factor p_i and entropy S_i^I , and the second term represents the number of distinct protein configurations. The difference between S_{conf} at the transition state and at the equilibrium reactant state contributes to $S^\ddagger$. On the free energy surface, an increase of S_i^I deepens the channel along a particular reaction path, but enthalpy-entropy compensation (Lumry & Rajender, 1970; Sturtevant, 1977) limits large contributions from this term to $G^\ddagger$. Therefore, the dominant contribution of S_{conf} to the activation entropy for Co-C bond cleavage was proposed to arise from a change in protein configurational entropy (Wang & Warncke, 2013). The increase of the configurational entropy with progress along the reaction coordinate can be represented by an expansion of the configurational coordinate, transverse to the reaction path, on the free energy surface. In Figure 7B, this is represented as a wider transition state, or “saddle,” region for step 1, relative to step 2.

The large absolute value of $S^\ddagger$, and the significant difference between $S^\ddagger$ for Co-C bond cleavage in the protein (61 ± 6 cal/mol/K) and in solution (6.8 ± 1.0 cal/mol/K) (B. P. Hay & Finke, 1986), led to the proposal of a “configurational catalysis” mechanism for this step in EAL (Wang & Warncke, 2013). The contribution of multiple paths through configuration space, and the associated $S^\ddagger > 0$, to the free energy barriers of the *chemical steps* of reactions in solution (Leffler & Grunwald, 1989) and in enzymes (Fan, Cembran, Ma, & Gao, 2013; Kamerlin & Warshel, 2011) have been considered, theoretically. There have also been proposals for roles of protein conformational dynamics in steps that bracket (precede, follow) the chemical steps in enzyme reactions (Boehr, McElheny, Dyson, & Wright, 2006; Henzler-Wildman, Lei, et al., 2007; Henzler-Wildman, Thai, et al., 2007; Whitford, Onuchic, & Wolynes, 2008), including setting the stage for reaction coordinate-specific vibrations (S. Hay, Johannissen, Sutcliffe, & Scrutton, 2010; Nunez, Antoniou, Schramm, & Schwartz, 2004; Pudney et al., 2013), as summarized by (Benkovic, Hammes, & Hammes-Schiffer, 2008; Bohr et al., 2006; Klinman, 2013). Some of these proposals have been criticized, on the basis that they are not true catalytic effects (*i.e.*, relative to a solution reference reaction), or that they are not rigorously defined (*i.e.*, in terms of an energy surface description) (Kamerlin & Warshel, 2011; Olsson, Parson, & Warshel, 2006). The configurational catalysis model for Co-C bond cleavage in EAL represents an enzyme-catalytic effect (referenced to AdoCbl cleavage in solution) (B. P. Hay & Finke, 1986), with an articulated origin in terms of a free energy surface (Figure 7B) (Wang & Warncke, 2013).

Figure 6C represents the case of a *temperature-dependent* mean free energy surface. The abrupt change in slope occurs, because of a glass-like transition in the protein, which arises from a change in the free energy surface itself, over a narrow temperature range of a few Kelvin (Goldstein, 1969; Stillinger, 1995). The change in the mean free energy surface is associated with a change in the protein “dynamics,” or rate of fluctuations among

configurational states, that occurs at a glass transition temperature (Angell, 1995), T'_g (the prime denotes that the value is dependent on cooling rate (Chen, Sun, & Warncke, 2013; Franks, 2003)). The glass transition in solutions effectively quenches the relatively large spatial-scale, collective motions of solvent molecules, which are termed α -fluctuations (Angell, 1995). Studies of the motions of proteins, at different hydration levels and on different spatial and temporal scales, have led to the proposal of a “protein glass transition” (at a temperature specified here as $T'_{g,p}$), that is coupled to solvent dynamics, in the range from 160–220 K (Jansson, Bergman, & Swenson, 2011; Panagopoulou, Kyritsis, Shinyashiki, & Pissis, 2012; Ringe & Petsko, 2003). Care must be taken to distinguish the role of the bandwidth of the measurement technique in interpretations of the presence of a glass transition (Khodadadi et al., 2008). In detailed studies of the gas-binding function of the protein, myoglobin, the effective quenching of protein α -fluctuations at $T'_{g,p}$ was related to a change in the rates of ingress/egress of CO between protein and solution (Fenimore, Frauenfelder, McMahon, & Young, 2004). The persistence of CO migration through the myoglobin protein interior at T -values below $T'_{g,p}$ was proposed to be mediated by dynamics associated with localized displacements of small numbers of atoms, or b -fluctuations (Fenimore et al., 2004; Frauenfelder et al., 2009). A reaction step in an enzyme, that is dependent upon collective motions of the protein atoms among protein configurations (α -fluctuations), will experience an abrupt change in rate constant with lowering temperature, near the effective $T'_{g,p}$. Figure 7C depicts the fate of the surface in Figure 7A, for the case of $T < T'_{g,p}$. The glass-like transition effectively blocks the progress along configurations characteristic of the native path (for example, the path shown in Figure 7A). The observed continuation of the reaction at $T < T'_{g,p}$ in Figure 6C is depicted in Figure 7C as an alternative path through the configuration space (higher $H^\ddagger$), that follows a series of smaller displacements that are linked by b -like fluctuations (higher $S^\ddagger$). The emergence of multiple states in the transition region, that are longitudinal to the reaction trajectory in Figure 7C, are a second possible origin of a large $S^\ddagger$ (relative to a transverse distribution of states, described above) as represented in Figure 7B, step 1 (Kamerlin & Warshel, 2011).

Figure 7D shows a case comparable to case C, but for which there are multiple, parallel paths for reaction. The two-path model in case D represents the situation for the low-temperature substrate radical rearrangement reaction in EAL, below the kinetic divergence temperature range of ~219 K (Zhu & Warncke, 2008).

5. CONCLUSIONS

Low-temperature, EPR-based kinetic and structural methods allow a broad canvassing of the free energy surface for the enzyme reaction sequence, with the reward of comprehensive, and more detailed, insights into the microscopic mechanisms of enzyme catalysis. This chapter has described two systems, fluid cryosolvent and frozen solution, and associated EPR measurement and analysis approaches, that allow study of enzyme reactions deep into the low-temperature regime. The general advantages of low-temperature studies are: (1) Slowing of reaction steps, so that kinetics can be measured by using straightforward T -step, time-resolved, full-spectrum CWEPR spectroscopy, performed with commercial

instrumentation, (2) resolution of individual reaction steps by using different reaction-initiation methods, so that first-order kinetic analysis can be applied, and (3) accumulation of intermediates in the catalytic sequence, that may be short-lived, or present at undetectable concentration, at physiological temperature. The particular advantages of low-temperature studies, that may be realized by the extended range of reaction rate–temperature dependence, are as follows: (1) Reaction steps in the catalytic cycle, that have different $H^\ddagger$ and $S^\ddagger$, may display curve-crossing on the Eyring plot, thus revealing additional steps for analysis, beyond a single, dominant step that may be rate-determining at room temperature. This case corresponds to an invariant mean free energy surface. (2) Studies that extend into the region of approximately $T < 220$ K may encounter a glass-like transition in the protein, which signifies the effective quenching of collective atom motions, or α -fluctuations, that convey the protein among configurational states. Dependence of the reaction step on collective atom motions can be identified by an abrupt change in reaction rate constant, at $T'_{g,p}$, which arises from a temperature-dependent change in the mean free energy surface. The protein configurational states involved in the fluctuations can be addressed by using high-resolution structural EPR and other spectroscopic approaches, and microscopic origins at the level of single or clusters of amino acid residues can be probed by site-directed mutagenesis (Dantsker, Samuni, Friedman, & Agmon, 2005; Samuni, Dantsker, Roche, & Friedman, 2007). The advantages (1) and (2) provide direct information about the native mechanism.

In the regime of $T < T'_{g,p}$, the reacting system may follow a non-native pathway over the free energy surface. Nevertheless, the system samples microscopic states in the vicinity of the native path, and reaction progress may depend on alternative fluctuations (for example, combinations of smaller scale atom movements, or β -type fluctuations), that involve incremental steps within the native reaction configuration coordinate envelope. This information about redundant, non-native pathways is of keen interest for discerning origins of the native mechanism, and for informing *de novo* biological catalyst design.

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Figure 1.

Minimal mechanism for the catalytic cycle of ethanolamine ammonia-lyase and structures of substrate, substrate radical, and products. The forward direction of reaction is indicated by arrows, and steps are numbered. The 5'-deoxyadenosyl b-axial ligand is represented as Ad-CH₂- in the intact coenzyme, and as Ad-CH₂• (5'-deoxyadenosyl radical) or Ad-CH₃ (5'-deoxyadenosine) following cobalt-carbon bond cleavage. The cobalt ion and its formal oxidation states are depicted.

Figure 2.

X-band CW-EPR spectra of the cryotrapped Co^{2+} -substrate radical pair formed by using (*S*)-2-aminopropanol substrate. The free electron resonance position at $g=2.0$ is shown by the arrow. *Solid line*: Co^{2+} -substrate radical pair formed at $T=242$ K in the 41% (v/v) DMSO/water cryosolvent system. *Dashed line*: Co^{2+} -substrate radical pair formed at $T=277$ K during steady-state turnover in buffered aqueous solution (10 mM potassium phosphate, pH 7.5). In each case, the samples were cryotrapped (isopentane, 133 K), and spectra were obtained at $T=120$ K. *Conditions*: Microwave frequency, 9.44 GHz; microwave power, 10 dB (20 mW); magnetic field modulation, 10 G peak-peak; modulation frequency, 100 kHz; field sweep rate, 1.5 G s^{-1} ; time constant, 200 ms. A baseline has been subtracted from each spectrum.

Figure 3. Minimal cryosolvent sample preparation scheme. The image shows the 2 mm o. d. EPR tube, guide marks, protein in cryosolvent (lower right), blue-wax seals at each end, and penny to illustrate scale.

Figure 4.

Rise of the EPR spectrum of the 1-²H-labeled (*S*)-2-aminopropanol substrate radical component of the Co²⁺-substrate radical pair state in EAL in the 41% (v/v) DMSO/water cryosolvent system at *T*=242 K, following *T*-step initiation of reaction. The free electron resonance position at *g*=2.0 is shown by the arrow. The full extents of the magnetic field sweep and time course are 560 Gauss and 6.43×10^3 s, respectively, and spectrum sweep interval is 15 s. The first peak and second trough are positioned at 3284 and 3415 Gauss, respectively. *Conditions*: Microwave frequency, 9.36 GHz; microwave power, 10 dB (20 mW); magnetic field modulation, 12 Gauss; modulation frequency, 100 kHz; scan rate: 53 Gauss s⁻¹; time constant, 164 ms. The *t*=0 baseline spectrum has been subtracted from each spectrum.

Figure 5.

Decay of the EPR spectrum of the $^2\text{H}_4$ -aminoethanol-generated Co^{2+} -substrate radical pair state in EAL at $T=207$ K, following after T -step reaction initiation. The free electron resonance position at $g=2.0$ is shown by the arrow. Selected spectra from the decay series are shown. *Conditions:* microwave frequency, 9.34 GHz; microwave power, 10 dB (20 mW); magnetic field modulation, 1.0 mT; modulation frequency, 100 kHz; scan rate, 6.52 mT s^{-1} ; time constant, 2.56 ms. A baseline spectrum has been subtracted from each spectrum.

Figure 6.

Eyring, or $\ln(k/T)$ versus inverse T , plots for four representative dependences of first-order reaction rate constants on temperature, that may be encountered over the 190 K to room temperature range. (A) Single-step reaction, with T -independent $H^\ddagger$ and $S^\ddagger$. (B) Two-step reaction, with T -independent $H^\ddagger$ and $S^\ddagger$ for both step 1 and step 2, under the conditions, $\Delta H_1^\ddagger > \Delta H_2^\ddagger$ and $\Delta S_1^\ddagger > \Delta S_2^\ddagger$. Extrapolated individual dependencies are shown by dashed lines. (C) Single-step reaction, with T -dependent $H^\ddagger$ and $S^\ddagger$, as shown by the discontinuity, marked by the dashed line. (D) Single-step reaction (high- T regime), with T -dependent $H^\ddagger$ and $S^\ddagger$, as shown by the discontinuity, marked by the dashed line. The reaction diverges into two kinetic components, with different $H^\ddagger$ and $S^\ddagger$.

Figure 7.

Free energy surface (landscape) depictions of reactions, that correspond to the four Eyring plots in Figure 6. RC, reaction (chemical) coordinate; CC, configurational coordinate. The reaction pathways are depicted by white dotted lines, which represent the central, or most-probable, region of the flow of population over the landscape. Panels B and A/D depict free energy surfaces that correspond to step 1 (for 234–248 K) and step 3 (190–295 K), respectively, in EAL. (A) Single-step reaction, with T -independent free energy surface. (B) Two-step reaction, with T -independent free energy surface for both step 1 (rear to middle minimum) and step 2 (middle minimum to front). The conditions, $\Delta H_1^\ddagger > \Delta H_2^\ddagger$ and $\Delta S_1^\ddagger > \Delta S_2^\ddagger$ are represented by a relatively higher barrier, and broader transition region transverse to the reaction coordinate, for step 1 relative to step 2. (C) Single-step reaction, below the protein glass-like transition temperature ($T < T'_{g,p}$). Relative to the reference free energy surface for $T > T'_{g,p}$ in panel A, barriers to collective atom motions cause the reaction to follow a trajectory involving smaller, incremental configuration changes. (D) Single-step reaction, below the protein glass-like transition temperature ($T < T'_{g,p}$). Comparable to panel C, but for the specific case of two paths.