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Bryce Guidry April 11, 2022

Reaction of Ethylene Glycol with Adenosylcobalamin-Dependent Ethanolamine Ammonia-Lyase

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An abstract of

a thesis submitted to the Faculty of Emory College of Arts and Sciences

of Emory University in partial fulfillment

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Bachelor of Science with Honors

Department of Physics

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Abstract

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By Bryce Guidry

Adenosylcobalamin (AdoCbl)-dependent ethanolamine ammonia lyase (EAL) is associated with gut microbe homeostasis and disease conditions in the bacteria, *Escherichia coli* and *Salmonella typhimurium*. The enzyme operates by homolytically cleaving the cobalt-carbon (Co-C) bond in AdoCbl to generate a short-lived radical state that is then used to trigger hydrogen atom transfers and radical rearrangement. When the Co-C bond is cleaved, the cobalt in cobalamin changes formal redox state from Co(III) to Co(II). Since the corresponding cobalamin states, Cbl(III) and Cbl(II), absorb different wavelengths of light, this project observed the progress of the AdoCbl-dependent EAL reaction by using optical spectroscopy. The long-term goal of this project is to characterize the reaction of the pseudo-substrate, ethylene glycol (EG), with the AdoCbl-EAL holoenzyme in the *Salmonella* system, toward understanding mechanisms for stabilizing and channeling highly-reactive radical species, and to develop a time-resolved reaction-measurement system in solution at room reaction, for kinetic studies of mechanism. The reaction of the AdoCbl-bound EAL holoenzyme with EG at different concentrations is studied in buffered aqueous solution at room temperature (295 K). Ultraviolet (UV)/visible absorbance spectroscopy was used to characterize the AdoCbl-dependent EAL reaction at room temperature. EG leads to the production of a distinct, non-native cobalt species in place of the cob(II)alamin produced by the native substrate. This unknown cobalt species has a distinct absorption feature in the range 476 nm to 525 nm and an absorption peak at 529 nm. Reaction in the presence of dithiothreitol showed that dioxygen is not involved in the reaction. The EG concentration dependence of the reaction was characterized, and a kinetic model proposed, that accounts for the first-order, exponential kinetics and concentration-dependence. Overall, visible absorption spectroscopy and time-resolution of the EG-EAL reaction in the *S. typhimurium* enzyme in solution at room temperature are developed, and the kinetic model is a foundation for future studies of the role of dynamics in EAL catalysis.

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Table of Contents

List of Figures

I. Introduction

Ethanolamine ammonia-lyase (EAL) is an adenosylcobalamin (coenzyme B_{12})-dependent enzyme capable of catalyzing the conversion of ethanolamine into acetaldehyde and ammonia, according to equation 1.[1]

$$H_3{}^+NCH_2CH_2OH \rightarrow CH_3CHO + NH_4^+ \qquad \text{(Eq. 1)}$$

EAL is found in a number of bacteria that require exogenous vitamin B_{12} for growth on ethanolamine, including *Escherichia coli* and *Salmonella typhimurium*.[1, 2] Adenosylcobalamin (AdoCbl) is a biologically active form of vitamin B_{12} and is a naturally found organometallic compound that contains a cobalt-carbon (Co-C) bond.[3] AdoCbl serves as a cofactor for enzymatic radical reactions initiated by homolysis of the Co-C bond to form a 5'-deoxyadenosyl radical.[3] AdoCbl-dependent enzymes fall into three main classes: carbons skeleton mutases, hydroxo or amino eliminases, and amino mutases.[4] EAL is an amino eliminase that plays important roles in metabolic processes associated with microbiome homeostasis and *Salmonella*- and *Escherichia coli*- induced disease conditions in the human gut.[4, 5] The cobalt carbon bond in the adenosylcobalamin (AdoCbl) cofactor is cleaved homolytically to form a short-lived 5' deoxyadenosyl radical, which is then utilized to carry out the reaction processes.[6] In AdoCbl-dependent EAL reactions, the radical state is used to carry out subreactions involving radical rearrangement and hydrogen atom transfer.[4] The EAL holoenzyme has been shown to accelerate the cleavage rate by at least 10^{10}- fold compared to the cofactor in free solution.[7]

EAL consists of a large EutB protein subunit and a small EutC protein subunit.[4] EutB consists of a $\beta_8\alpha_8$, TIM-barrel fold and its amino acid residues make the dominant contribution

to the formation of the active site for conducting the AdoCbl-dependent reactions. Figure 1 shows the structure of EAL.[2]

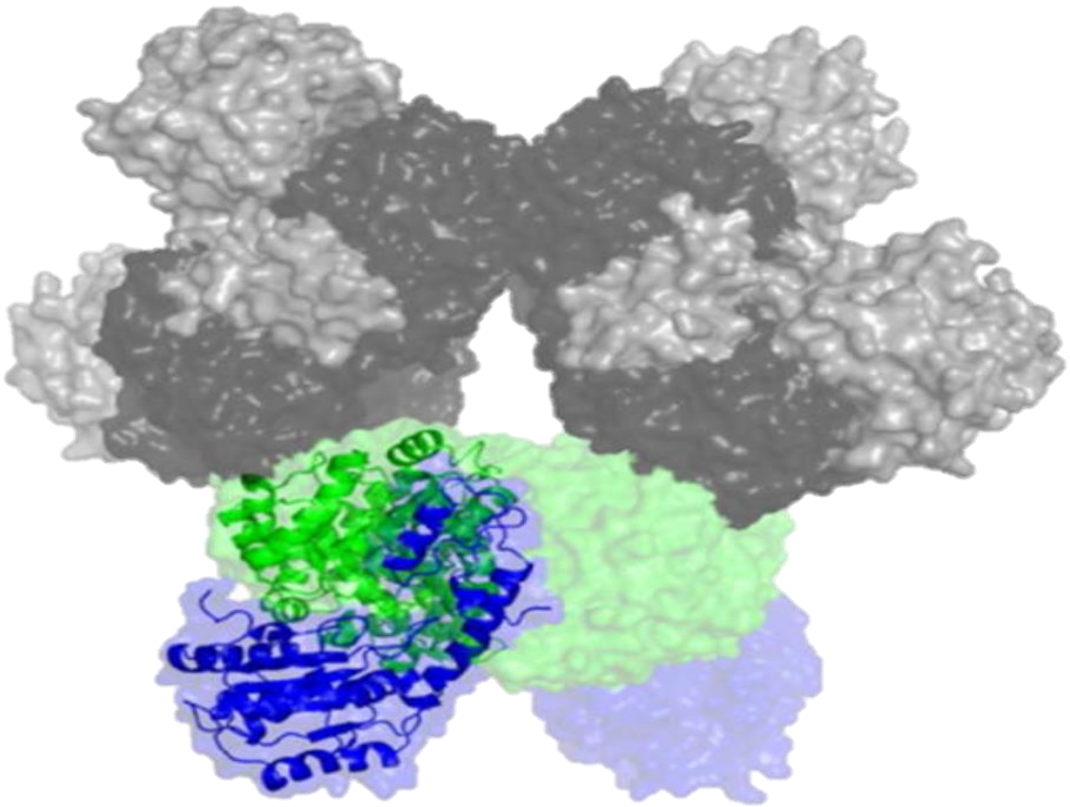


Figure 1. Structure of EAL. The colored section corresponds to one heterodimer of the EAL oligomer. For this heterodimer, The EutB subunits are in green and the EutC subunits are in blue. For the other heterodimers, the EutB subunits are dark grey while the EutC subunits are light grey.[2]

Figure 2 shows the structure of AdoCbl.[2] The notable features of AdoCbl include a cobalt atom at the center of a corrin ring. This cobalt atom is bound to a carbon atom above the corrin

ring. Cleavage of this Co-C bond occurs homolytically, allowing for the separated components to be used for hydrogen atom transfers and radical rearrangements.

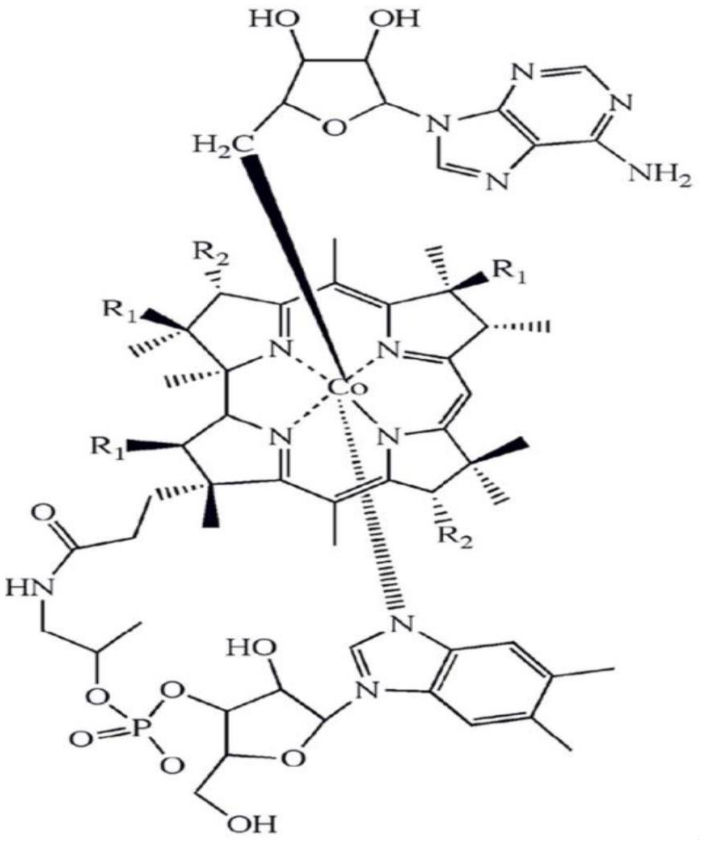


Figure 2. Structure of AdoCbl. An adenosyl moiety is at the top and bonded to a central cobalt atom. The cobalt atom is surrounded by a coring ring and a nucleotide tail trails off of the ring.[2]

Figure 3 below shows the catalytic cycle of AdoCbl-dependent EAL reactions with a substrate. The reaction begins when the bond connecting a cobalt molecule in a corrin ring to a carbon attached to the adenosyl is cleaved (step 1). This bond cleavage can be initiated by the substrate or by exposure to light. This Co-C bond is easily cleaved by light in the visible range, so AdoCbl is handled under red lights to avoid this premature bond cleavage since AdoCbl reflects light in the red range. Before and after this bond cleavage, the AdoCbl absorbs different

wavelengths of light, and the UV-visible spectrum changes. When the Co-C bond is intact, the AdoCbl absorbs light greatest in the green range (maximum absorbance wavelength λ_{max}=525 nm). When the Co-C bond is cleaved, the reacting sample absorbs light in the blue range (λ_{max}=476 nm). This feature allows the progress of the reaction to be observed using optical spectroscopy. When the Co-C bond is cleaved, a short-lived radical state is created. This radical state is used to initiate hydrogen atom transfers (steps 2, 4) and radical rearrangement (step 3) to generate the product radical, which is eventually transformed into diamagnetic products. Radical pair recombination allows the reformation of the Co-C bond before the release of the product.

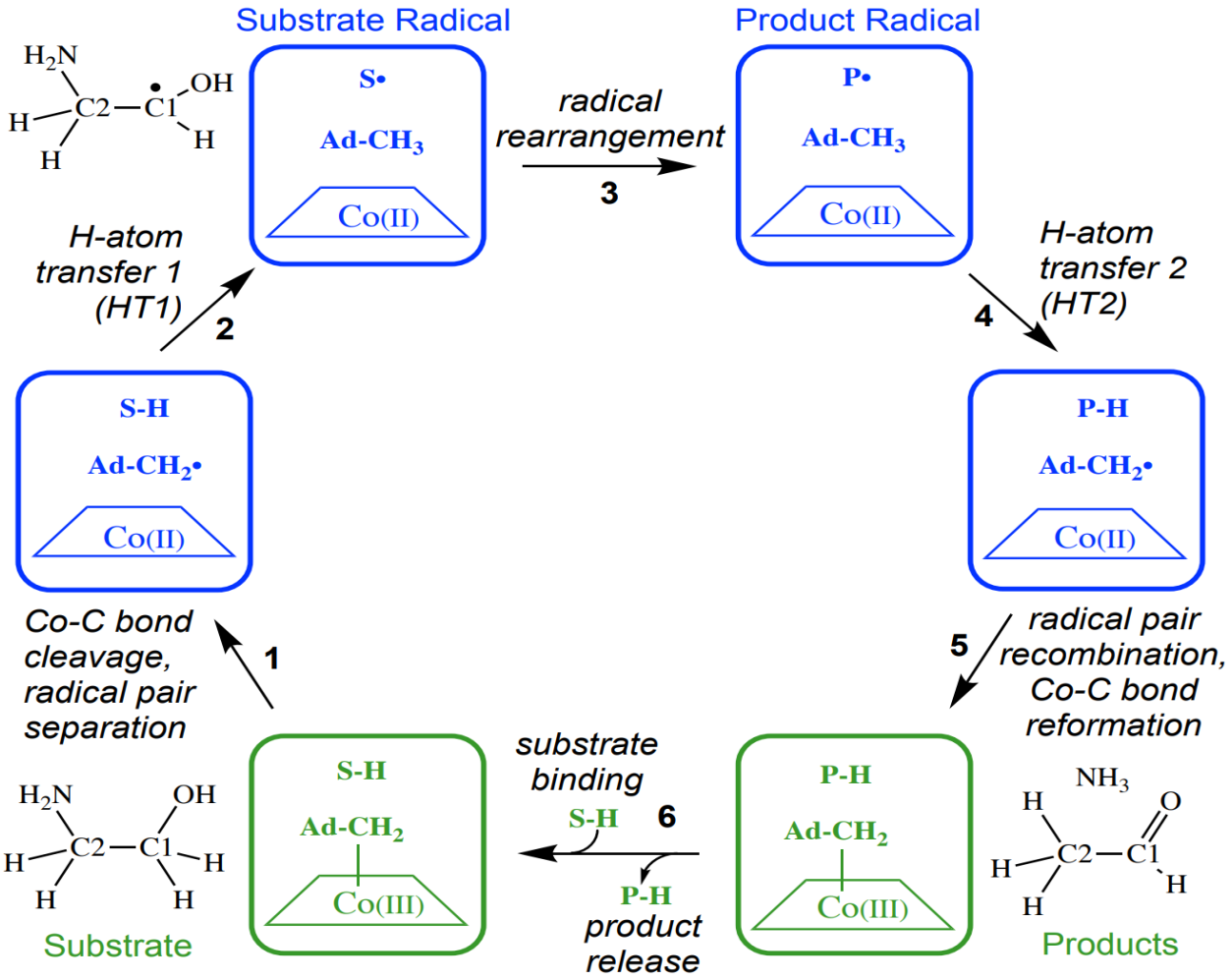


Figure 3. Catalytic cycle of AdoCbl-dependent EAL, showing canonical intermediates. The color of a step corresponds to the wavelength of light of greatest absorbance in the visible range at that step.

The native substrate for AdoCbl-dependent EAL reactions is aminoethanol (ethanolamine). Alternate substrates are molecules that are analogs of the native substrate but react by non-natural pathways. Alternate substrates and inhibitors are of great value in understanding how an enzyme works because the differences between substrates or inhibitors can expose features of the reaction mechanism not detected by study of the native substrate reaction. Our group has used time-resolved, full-spectrum EPR spectroscopy at low temperatures[8] to characterize the core reaction steps in EAL for the native substrate, aminoethanol,[9, 10, 11] and the non-native substrate, 2-aminopropanol.[5] Alternate substrates for AdoCbl-dependent reactions also include glycerol, ethylene glycol, thioglycerol, trifluoropropanediol, and isobutylene glycol.[12] This study makes use of the quasi-substate, ethylene glycol (EG).[13] EG reacts approximately 10^3-fold more slowly than aminoethanol (observed turnover rate constant of ~0.1 s^{-1}, versus ~50 s^{-1} for EAL).[13, 14] EG has the same general structure as aminoethanol, but has a hydroxyl group in place of an amine group in ethanolamine. Since EG takes longer to initiate the cleavage of the Co-C bond, it is easier to observe the progress of the reaction by using EG than with aminoethanol. However, the use of EG as a quasi-substrate inactivates the EAL after one turnover. This has been proposed to be due to the coordination of an alternate ligand to the sixth position of the Co center.[13]

Biophysical systems can be complex and sensitive to their environment. In order to better understand these elaborate systems, a variety of techniques have been developed in order to

understand the structure and mechanism of these systems. One major technique is spectroscopy, in which biological materials are understood through their interactions with electromagnetic radiation. Optical spectroscopy involving biological samples uses light to gain information about the chemical substructure of the molecules.[15] Optical spectroscopy allows for the creation of an absorption spectrum by measuring the absorbance of the samples across a range of wavelengths. Features of the absorption spectrum can be used to better understand the behavior of a molecule or what happens when a molecule reacts with another. Figure 4 below shows the absorption spectra for AdoCbl and cob(II)alamin.[5]

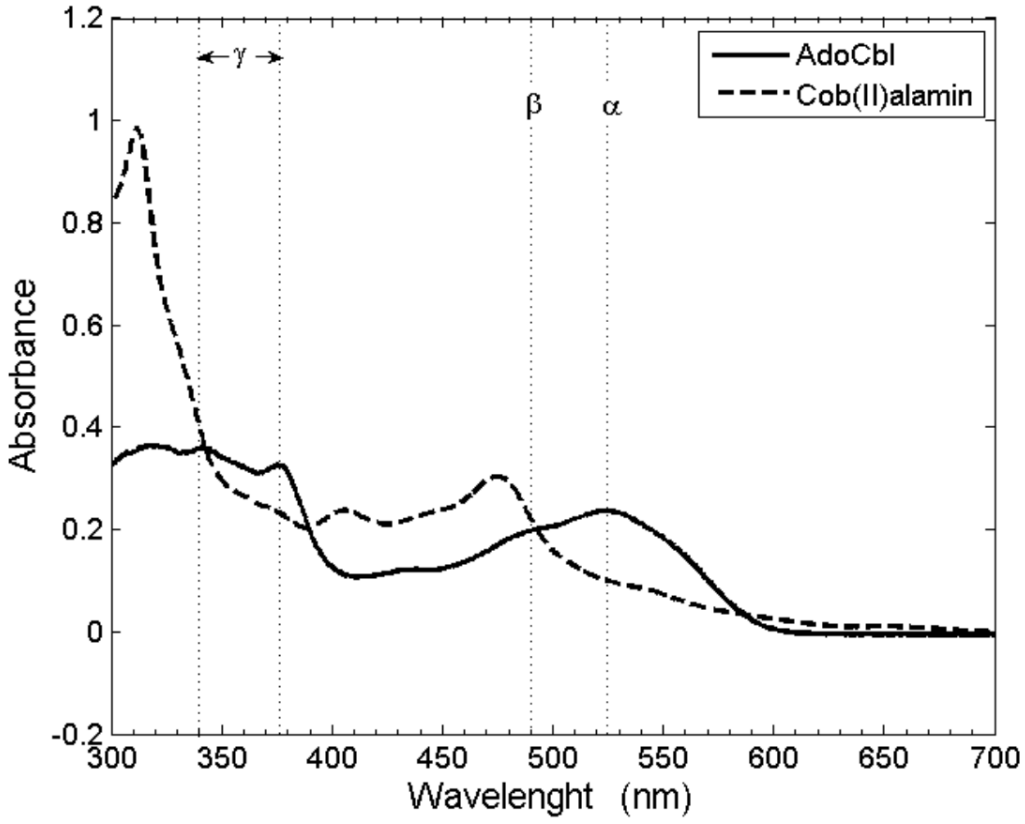


Figure 4. Absorption spectrum of AdoCbl in EAL (solid line) and cob(II)alamin (dashed line). Both samples were in solution of 10 mM phosphate buffer at pH 7.5. Spectra were recorded at room temperature. Grid lines indicate distinct features of the visible (α and β) and UV (γ) regions.[5]

The project seeks to characterize the reaction of the EAL holoenzyme with EG under different conditions, in solution at room temperature. Understanding the mechanism of the deleterious reaction will contribute to understanding the fidelity of the native reaction, with respect to the productive reaction and how the enzyme guides the highly-reactive, substrate radical species. An additional goal is to extend the technique of time-resolved measurements of first-order reactions in EAL from the low-temperature, frozen-solution conditions (T<230 K)[5, 9, 10, 11] to the room-temperature, fluid solution conditions. The original characterization of the EG reaction with EAL was performed on enzyme from a different species (*Clostridium* sp)[13], which, as currently available amino acid sequence data show, differs from the sequence of the *S. typhimurium* enzyme that is used in our studies.[2] Therefore, a reappraisal of the mechanism is required. The understanding gained from these studies has broad application for understanding how to develop drugs to treat the disease conditions associated with bacteria that make use of this EAL mechanism to utilize ethanolamine as a carbon, nitrogen, and energy source.

II. Experimental Methods

Ultraviolet-Visible Absorption Spectroscopy

Absorption spectroscopy was performed using a Shimadzu UV-1900i UV-vis spectrophotometer. The spectrophotometer compares the intensity of light passing through a sample to the intensity of a reference light beam. The ratio of the light beams (I/I_0) is the transmittance, and the determination of the transmittance allows the absorbance to be computed from

$$A = -\log(T). \quad \text{(Eq. 1)}$$

Samples for absorption spectroscopy were comprised of 0.43 ml of 10 mM KPi buffer, 0.05 ml EAL, 0.02 ml AdoCbl, and 0.05 ml EG to give a total volume of 0.55 ml in a polystyrene cuvette. The cuvettes used for the experiment could contain a total volume of 1 ml. Since the total volume of the cuvette was not used, the location that the light beam would intersect the cuvette occurred at the top of the solution, meaning that only part of the light beam would travel through the solution. To correct for this, two small spacers were added to the cuvette holder to adjust the height so that the entirety of the light beam would pass through the solution in the cuvette.

To calibrate the spectrophotometer for use in the experiment, two samples of 0.50 ml with 10 mM KPi were placed in the spectrophotometer. The auto-zero function was run to set the absorbance of the sample at 0, and the baseline function was run. The spectrophotometer was used to measure the absorbance of the sample starting from 700 nm and ending at 300 nm while recording the absorbance every 0.5 seconds. This allowed for the creation of a baseline file that could be observed to ensure that the baseline was properly calibrated since the baseline function

does not allow for visualization of the run baseline. The spectrophotometer was set to transition from the visible range to the ultraviolet range at 350 nm.

Preparation of Stock Solutions and Initial Sample Conditions

AdoCbl solutions for experimental use were prepared from 3 mM stock solution of B_{12}. Using Beer's Law,

$$A = \varepsilon C l, \quad \text{(Eq. 2)}$$

with $\varepsilon = 8700\ M^{-1}cm^{-1}$ for AdoCbl and $l = 1\ cm$ for the cuvettes used, the concentration of the prepared solution could be determined from an absorption measurement at the peak absorbance at 524.5 nm. Over 3 measurements, it was found that the prepared AdoCbl solution had an average concentration of 2.17 mM. This stock solution was diluted to 0.3 mM. This diluted stock solution was subsequently used to prepare the samples for spectroscopy. All preparation and use of AdoCbl for the experiments occurred under a dim red light due to the photosensitivity of AdoCbl to other wavelengths of visible light. EAL samples were obtained from *E. coli* modified to express the *S. typhimurium* EAL to a high degree according to methods described previously.[16] EAL stock solutions were prepared so that the concentration of EAL was 1 mg/ml.

A stock solution of 0.55 M EG for use in experiments was prepared from a larger 0.55 M EG stock solution. The 0.55 M stock solution was chosen so that the addition of 0.05 ml EG would give a concentration of 0.05 M EG in the cuvette samples. Potassium phosphate (KP_i) at a concentration of 10 mM was used as a buffer for the solution.

For experiments in which EG concentration was varied, the EG added to create 0.5-, 5-, and 50-mM samples was added from stock solutions of 5.5, 55, and 550 mM EG, respectively, thus keeping the addition volume constant. Absorption spectra were recorded for every 0.5 nm from 700 nm to 300 nm with each measurement occurring 0.5 seconds after the previous measurement.

Experiments involving dithiothreitol (DTT) were performed using 10 mM and 1 mM DTT in sample. Samples including DTT were prepared as above except the amount of buffer was lowered by the amount of DTT added, so that the total volume remained constant, at 0.55 ml. For 1 mM DTT, 5.2 microliters of DTT were added. For 10 mM DTT, 52 microliters of DTT were added.

Protocol for Spectroscopy Experiments

Experimental samples for sonication were made using 0.43 ml 10 mM KPi buffer and 0.05 ml EAL. UV/Visible spectroscopy was performed on this 0.48 ml sample to give a pre-sonication sample. The solution was then placed on ice for 3 minutes before performing sonication. Sonication was performed using a Laboratory Supplies Co. Model G112SPIT 600-volt sonicator. Sonication occurred in 3 sessions for 15 seconds each, with the solution being placed on ice for 1 minute between each sonication session. After sonication, the solution was placed back in the spectrophotometer, and a post-sonication spectrum was obtained. Figure 5 shows the effect of the sonication on the samples, which is to lower the absorption by reducing the scattering of the measuring beam by aggregated protein.

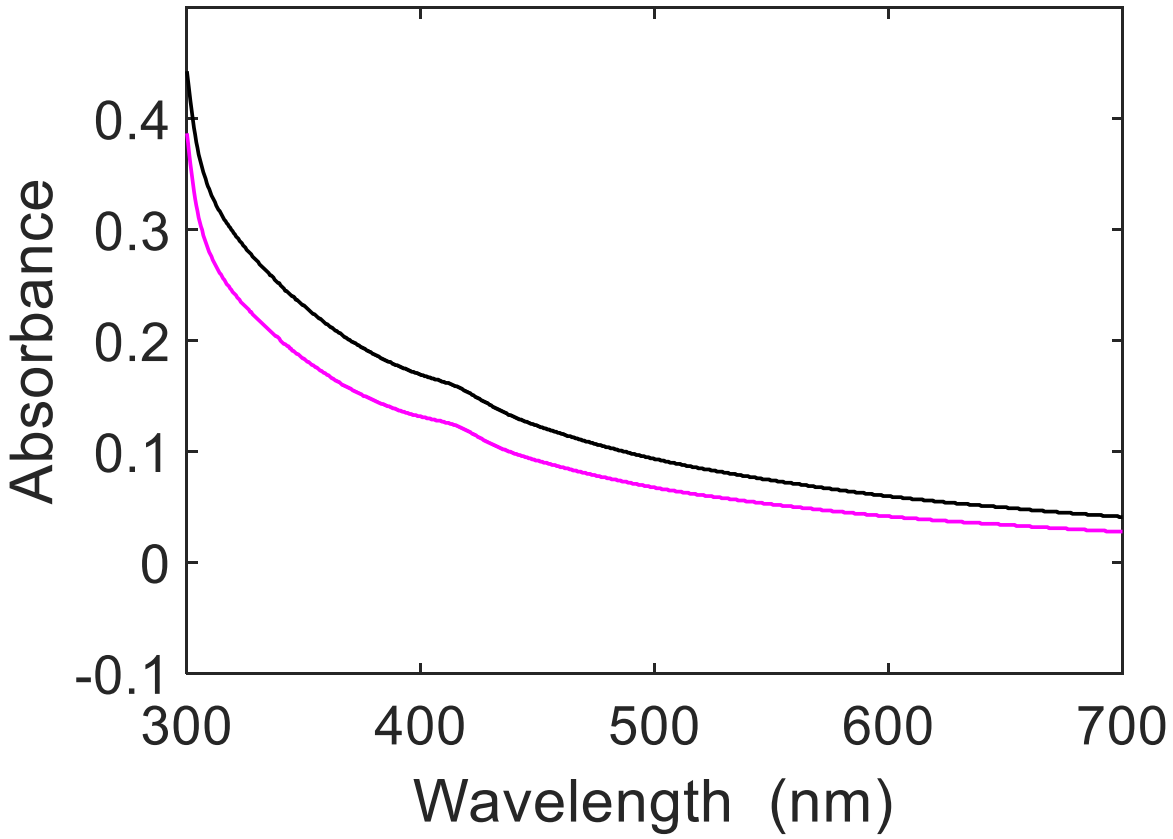


Figure 5. UV/visible absorption spectra of 0.05 ml EAL in 0.43 ml 10 mM KPi buffer before sonication (black) and after sonication (magenta).

Next, 0.02 ml AdoCbl was added to the solution under a red light to give a volume of 0.5 ml. An absorption spectrum of the sample was then taken again. Following this, 0.05 ml EG was then added to the solution to give the total volume of 0.55 ml of solution. Additional absorption spectra were taken at 1 minute, 4 minutes, 14 minutes, and 34 minutes after the addition of EG. Data for all of the absorption spectra taken were exported from the spectrophotometer software into Excel. The Excel data file was then uploaded as a workspace into MATLAB to generate plots of the data. MATLAB was then used to replot the obtained absorption spectra, along with

difference spectra obtained by taking the point-by-point difference between two spectra. This allowed for baseline corrections and for the progress of the reaction to be observed in time-dependence experiments.

Baseline Correction Analysis

The spectra collected during the experiment often featured a baseline with positive slope over the entire 300 nm to 700 nm wavelength range that complicated the interpretation of the spectra. In order to correct for this sloping baseline, a secondary baseline correction program (the primary baseline correction involved subtraction of the EAL protein-only spectrum) in MATLAB was developed to correct this. In this process, the cobalamin absorbance-free region of the spectrum, in the region 600 – 700 nm, was considered as the baseline, and was fit by using a second-order polynomial function, and the fit in this region was extrapolated to shorter wavelengths. This fitted spectrum was subtracted from the experimental spectrum. The amplitude of the polynomial function was uniformly scaled by a factor, so that the ratio of the peak long wavelength absorbance to the amplitude of minimum absorbance in the range around 400 nm obeyed the ratio from standard spectra. For AdoCbl, the wavelengths and absorbance ratio are 411 nm, 524 nm, and 0.47 (Figure 4).[5] For EG, the wavelengths and absorbance ratio are 411, 534, and 0.39, as derived from published spectra.[13] Following the completion of the experiments, it was learned that the polystyrene cuvettes used in the experiment do not properly transmit light below 320 nm. As a result, multiple plots have had the wavelength range 300 nm to 320 nm cut out of the absorption spectrum.

III. Results

Absorption Spectra of AdoCbl Samples Before and After Reaction

Figure 6 shows the absorption spectra for AdoCbl prior to the EG-induced bond cleavage and the resulting spectra 1 minute after EG addition. Comparison with Figure 4 shows that the post-EG reaction spectrum is not the same as cob(II)alamin. One minute after EG is added to the AdoCbl sample, the resulting spectrum has a peak shifted to 529 nm. Additionally, there is a new feature spanning the range between 474 nm and 525 nm in which there is a region of fairly constant slope. The secondary peak feature at 350 nm in the resulting spectrum is no longer as wide as it was in the pure AdoCbl spectrum. Also, the minimum absorption region is shifted closer to 400 nm.

The absorption spectrum of cob(II)alamin is displayed in Figure 4. Cob(II)alamin can be obtained through AdoCbl reacting with oxygen. Due to the samples used being exposed to the air during preparation, it was considered that the creation of cob(II)alamin could have been partially responsible for this resulting spectrum. However, comparison of the resulting spectrum to the spectrum for cob(II)alamin reveals some notable differences. Firstly, the cob(II)alamin spectrum begins to rise from 0 around 600 nm, similarly to the AdoCbl spectrum. However, the spectrum resulting from the reaction of EG with the AdoCbl complex does not begin to rise above the baseline until around 575 nm. In addition, the consistent downward trend of the cob(II)alamin spectrum in the range of 474 nm to 525 nm is inconsistent with the observed trends in the same region of the resulting spectrum. For these reasons, it is believed that the observed spectrum belongs to a separate cobalamin compound. This observation is consistent with the observation of the production of an XCbl from EG reactions with AdoCbl found previously.[13]

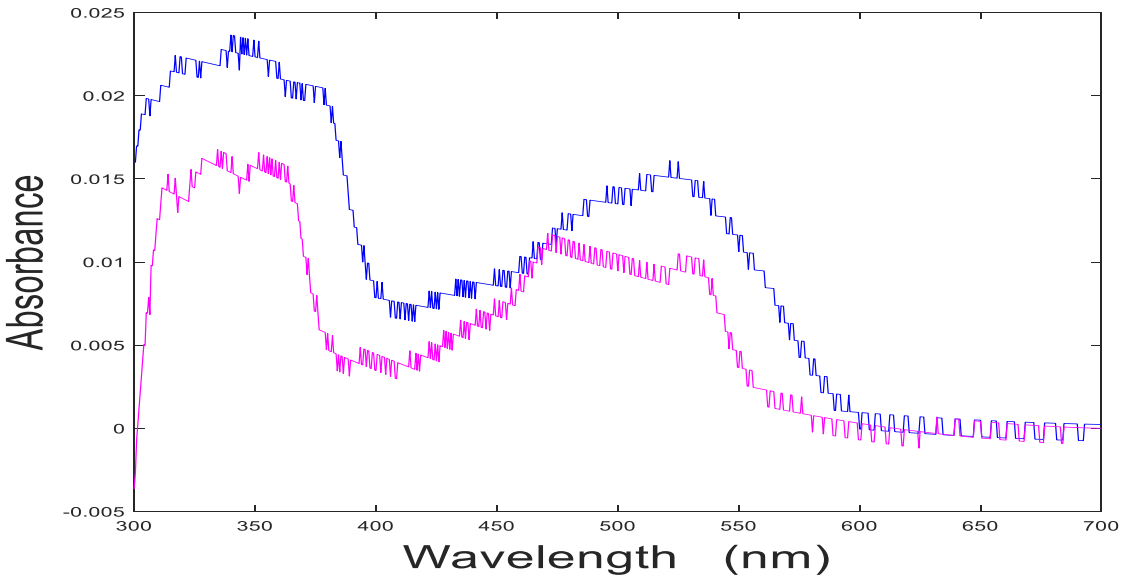


Figure 6. UV/visible absorption spectra of AdoCbl (blue) in solution and of the solution 1 minute after 0.05 ml EG was added to the solution (magenta). The AdoCbl solution is 0.43 ml 10 mM KPi, 0.05 ml EAL, and 0.02 ml AdoCbl. The EG concentration is 50 mM. The baseline correction scaling factor is -0.34 for a 411/524 ratio of 0.4734 for the AdoCbl spectrum. The baseline correction scaling factor is 0.64 for a 411/534 ratio of 0.3847 for the EG spectrum.

Absorption Spectra of AdoCbl Reaction Using DTT.

In order to ensure that the observed cobalamin spectrum following EG addition was not being influenced by the formation of an oxo-cobalamin species, dithiothreitol (DTT) was added to AdoCbl samples in order to scrub out any oxygen in the samples. DTT is easily oxidized by oxygen, so the addition of mM concentrations of DTT to samples should cause any oxygen, present at approximately 250 μM in aqueous solution at room temperature, in the samples to

react with the DTT instead of the AdoCbl, preventing the formation of cob(II)alamin. Figure 7 below displays the resulting absorption spectra for the reaction using 1 mM DTT and 10 mM DTT, 14 minutes after the addition of EG. The resulting spectra show the characteristic spectrum of the EG-generated, EAL-bound cobalamin species, obtained in the absence of DTT. Therefore, the results indicate that the formation of the EAL-bound cobalamin species formed following addition of EG is not dependent on oxygen-associated redox reactions and that the observed species is not an oxy-liganded product, such as a superoxo-cob(II)alamin or aquo/hydroxy-cob(III)alamin species.

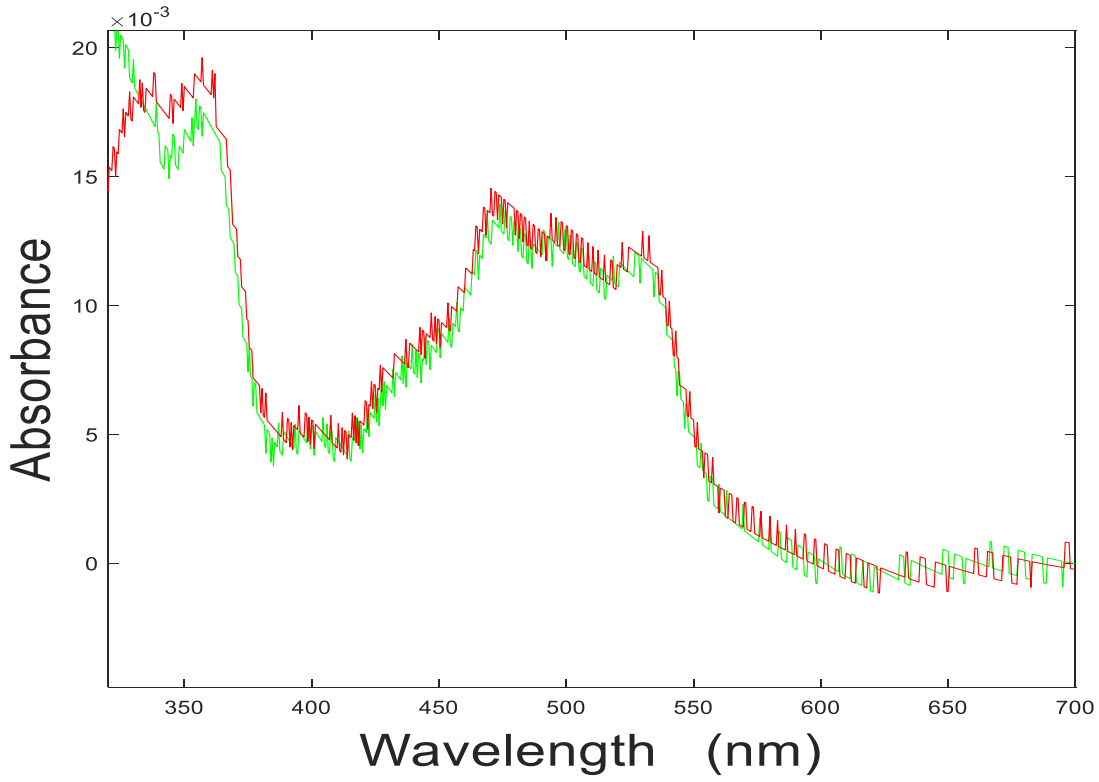


Figure 7. UV/visible absorption spectra of EAL-bound AdoCbl solution 14 minutes after 0.05 ml EG was added to the solution. The DTT concentrations are 1 mM (red) and 10 mM (green). Spectra are aligned at the baseline. The baseline correction scaling factor is 0.74 for a 411/534 ratio of 0.3948 for the 1 mM DTT spectrum. The baseline correction scaling factor is 1 for a 411/534 ratio of 0.3971 for the 10 mM DTT spectrum.

Dependence of the EAL-Bound Cobalamin Absorption Spectra on EG Concentration

In order to characterize the kinetic mechanism of the reaction of EAL-bound AdoCbl with EG, the time-dependence of the decay of the AdoCbl spectrum and rise of the EG-generated product spectrum, was measured as a function of EG concentration. Figures 8 and 9 show the absorption spectra produced when the concentration of EG was reduced from 50 mM, used for previous experiments, by factors of 100 and 10, respectively.

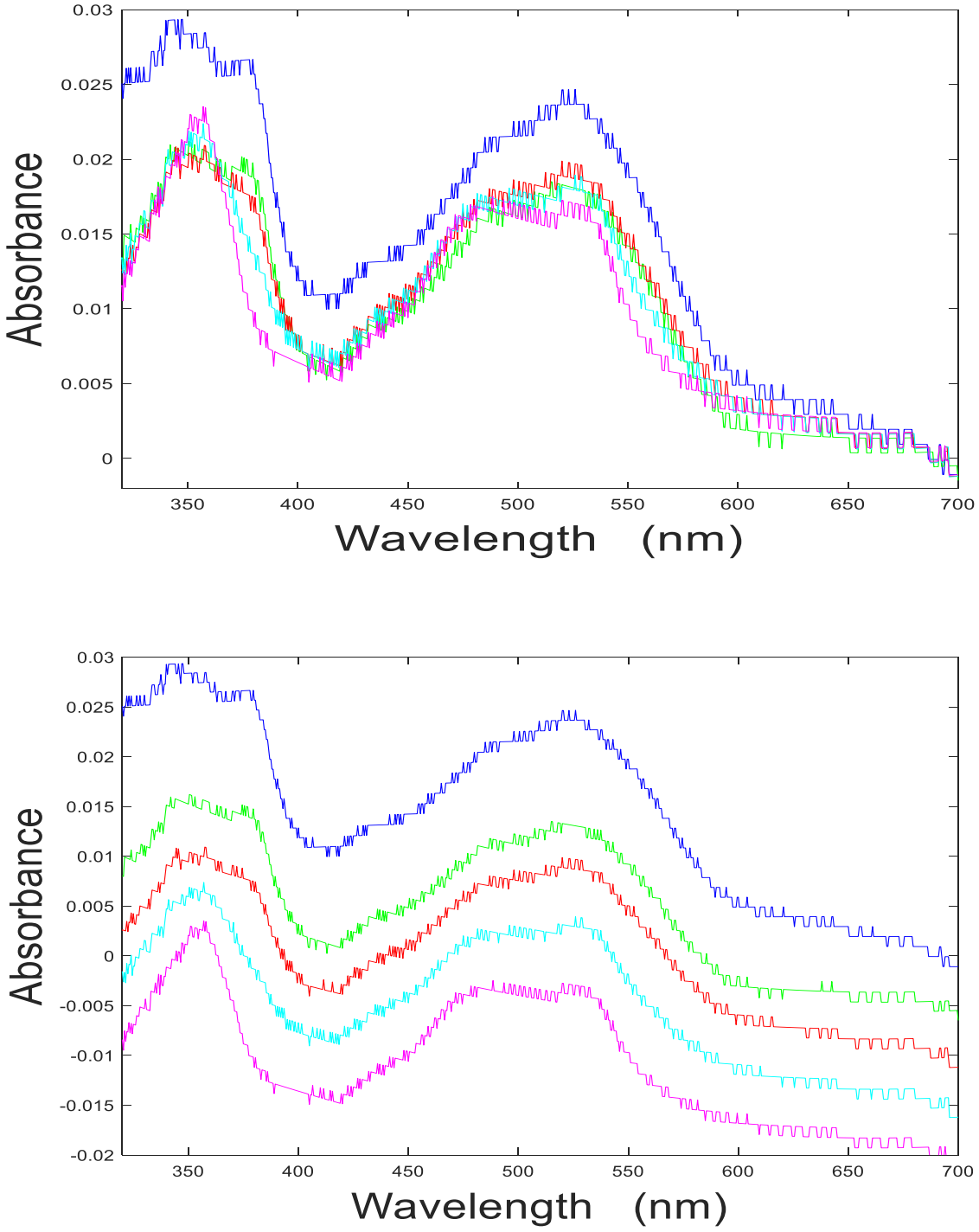
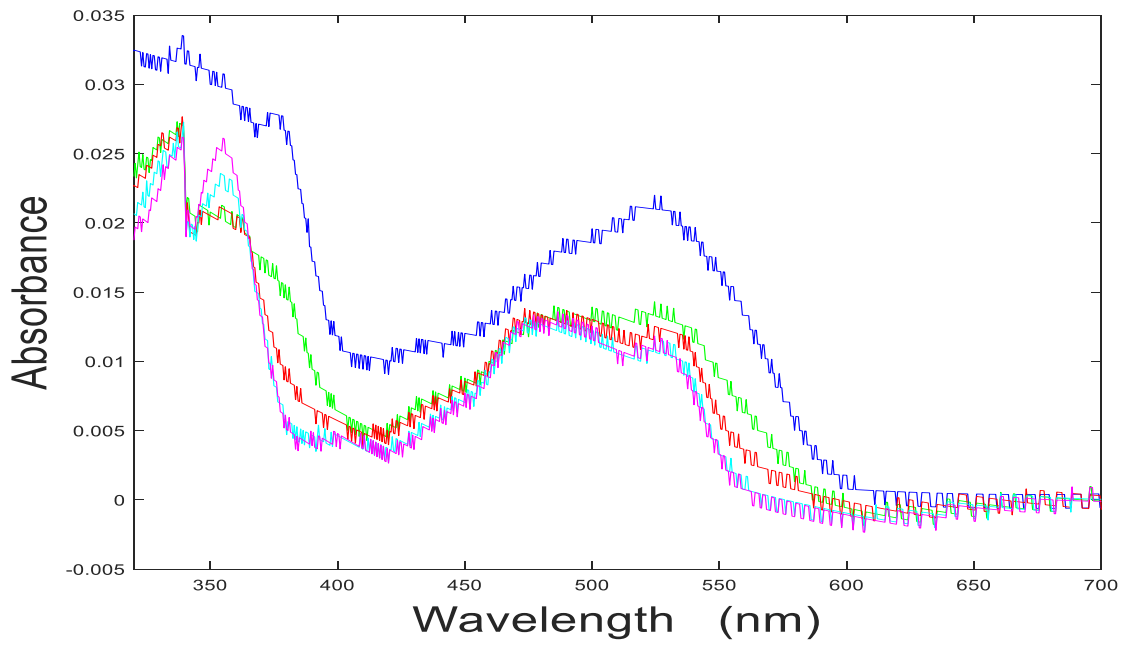


Figure 8. A) Time dependence of the UV/visible absorption spectra of cobalamin species bound to EAL before and after addition of 0.5 mM EG to the solution. Spectra were acquired at 0 time

(blue, representing the initial, pure EAL-bound AdoCbl) and at the following times after addition of EG: 1 min (green), 4 min (red), 14 min (cyan), 34 min (magenta). For the AdoCbl spectrum, the baseline correction factor is -0.01 for a 411/524 ratio of 0.4603. The remaining baseline correction factors are 0.09 for a 411/534 ratio of 0.3754 (green), 0.06 for a 411/534 ratio of 0.3686 (red), 0.07 for a 411/534 ratio of 0.3947 (cyan), and 0.07 for a 411/534 ratio of 0.3717 (magenta). B) Spectra are baseline shifted to display all absorption spectra without overlap on the same plot.



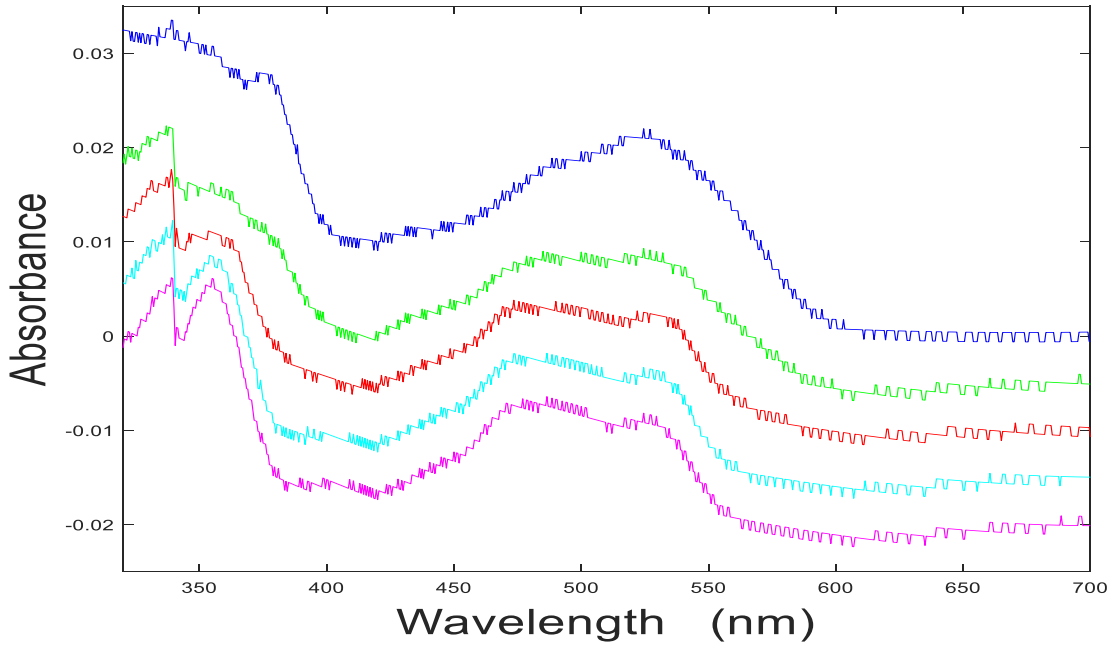


Figure 9. A) Time dependence of the UV/visible absorption spectra of cobalamin species bound to EAL before and after addition of 5.0 mM EG to the solution. Spectra were acquired at 0 time (blue, representing the initial, pure EAL-bound AdoCbl) and at the following times after addition of EG: 1 min (green), 4 min (red), 14 min (cyan), 34 min (magenta). For the AdoCbl spectrum, the baseline correction factor is 0.26 for a 411/524 ratio of 0.4721. The remaining baseline correction factors are 0.54 for a 411/534 ratio of 0.3932 (green), 0.76 for a 411/534 ratio of 0.3933 (red), 0.43 for a 411/534 ratio of 0.3804 (cyan), and 0.38 for a 411/534 ratio of 0.3947 (magenta). B) Spectra are baseline shifted to display all absorption spectra without overlap on the same plot.

Figures 8 and 9 show that, at the lowered concentration of EG of 0.5 and 5.0 mM, the decay of the AdoCbl spectrum and growth of the EG-elicited spectrum is time dependent. The decay of AdoCbl is most clear in the long-wavelength edge over 560 – 600 nm and in the region

of the ~524 nm maximum. The growth of the EG-elicited cobalamin spectrum is most clear in the growth of the ~480 nm maximum and associated low field edge (430 – 450 nm). The fall of the AdoCbl spectrum and rise of the EG-elicited cobalamin appear to occur synchronously. In addition, the rate of fall and rise is slower at 0.5 mM EG, than at 5.0 mM EG. This is shown, visually, by the essentially fully developed EG addition spectrum at 4 minutes under the 5.0 mM condition (Figure 9), whereas 34 minutes is necessary for full development of the EG addition spectrum under the 0.5 mM EG condition (Figure 8). The results show that the influence of EG on the reactions involving AdoCbl in EAL is concentration dependent.

IV. Discussion

Determination of Sonication as a Means of Reducing Sample Absorbance

One of the major issues in characterizing the EG-induced reaction of the adenosylcobalamin-dependent enzyme via UV/visible spectroscopy is that the absorbance readings can be thrown off by scattering of clusters in the solution, especially at the smaller wavelengths examined in the UV range. These experiments found that sonication provided an efficient and reproducible method to reduce the scattering of samples. The sonication process appears to be successful at breaking apart these protein clusters, which helps lower the overall absorbance of the samples.

Analysis of the Cobalamin Spectrum Following EG Addition to the EAL-AdoCbl Complex

Following the reaction of the EAL-bound AdoCbl solution with EG, a spectrum belonging to an unknown cobalamin compound is obtained (Figure 6). This spectrum is distinct from the pure AdoCbl spectrum. By visual comparison, the visible range peak of this post-reaction spectrum is located at 529 nm, which is a rightward shift from the AdoCbl spectrum with a peak at 525 nm. In addition, there is a relatively wide region with a small upward trend in absorbance between the range of 474 and 525 nm for this post-reaction spectrum. However, the AdoCbl spectrum is a downward slope in this region. Also, the AdoCbl spectrum begins to rise above the baseline around 600 nm, while the spectrum after the reaction begins to rise above the baseline around 575 nm. For these regions, the spectrum after the reaction with EG is distinct from that of AdoCbl. This post-EG reaction spectrum also does not match the absorption spectrum for cob(II)alamin. The spectrum for cob(II)alamin, as depicted in Figure 4, begins to

rise above the baseline around 600 nm and observes a less steep increase in absorbance than AdoCbl around this range. Cob(II)alamin also has a long-wavelength peak at 476 nm, and another smaller peak near 400 nm. The spectrum experiences a massive increase in absorbance around 350 nm that peaks around 320 nm. Comparison to the spectra obtained following the reaction of EG with AdoCbl in EAL reveals that the spectrum is inconsistent with that of cob(II)alamin. The observed spectrum remains along the baseline until around 575 nm, whereas the cob(II)alamin spectrum begins to rise above the baseline around 600 nm and is noticeably above the baseline by 575 nm. In addition, the recorded spectrum has a peak around 476, but this peak differs from the characteristic peak of cob(II)alamin at 476 nm at wavelengths above 476, while displaying absorption and peak features in the range between 476 nm and 530 nm that are not present in the cob(II)alamin spectrum. Finally, the peak located around 350 nm in the observed spectrum is shifted from the 320 nm peak in the cob(II)alamin spectrum while also being noticeably less in magnitude at the peak than in the cob(II)alamin spectrum. Verification that cob(II)alamin was not being produced in the samples was obtained by repeating the experiment with the addition of DTT.

Influence of DTT on the Cobalamin Spectrum Following EG Addition to the EAL-AdoCbl Complex

The two spectra obtained at DTT concentrations of 1 and 10 mM did not display a major difference in the range of interest between approximately 400 and 600 nm. The lower wavelength regions are less certain for analysis, because of the greater occurrence of scattering at shorter wavelengths and shifts in absorption when the spectrophotometer switches from the visible to the ultraviolet range that impact the continuity of the recorded spectrum. In addition,

the relatively strong absorbance and distinct peaks in the spectra of AdoCbl and cob(II)alamin, within the range of 450 to 550 nm, make this range convenient for analysis. The same general shape was observed in this region for both the 1 mM and 10 mM concentrations of DTT. Therefore, by visual comparison to the other post reaction spectra, the shape of these DTT spectra matches that of spectra without DTT in the regions of main interest. This provides further evidence that the absorption spectrum observed following the reaction with EG is not a result of cob(II)alamin formation. The removal of oxygen from the system should prevent the formation of cob(II)alamin, so these features, observed in the presence and absence of DTT, are likely to belong to another species.

Concentration-Dependence of the Kinetics of the EG-Generated Cobalamin Species in EAL

When the concentration of EG was lowered from the standard 50 mM used for previous experiments, the features of the resulting absorption spectrum were observed to change with time, over the time scale 1-34 min. For the 5 mM EG spectrum, after 1 minute, the absorption spectrum still has the general downward slope present in the AdoCbl spectrum over the range 476-525 nm. After 4 minutes, the absorption in this region has begun to develop into a feature with near constant slope, as was observed in the previous spectra after the addition of EG. The decrease in concentration appears to have lowered the rate at which the reaction occurs, influencing the resulting absorption spectra. This is further seen in the case where 0.5 mM EG was used. For the 0.5 mM EG case, the 1- and 4-min reaction spectra bear resemblance to the AdoCbl spectrum. By the time of the 14-min spectrum, the region between 476 and 525 nm has begun to develop into the previously observed feature, and by the time of the 34-min spectrum, the characteristic feature of constant slope in this region is readily observable. In addition, there

is a noticeable change that occurs in the region around 350 nm for the 0.5 mM EG case. Around 350 nm, the absorption spectrum appears fairly broad and flat in the 1-min spectrum. However, in time, the absorption spectrum begins to become narrower and develop the features of a peak. By the 34-min spectrum, the absorption spectrum around 350 nm, has developed into a sharp and narrow peak. The apparent narrowness of the peak comes from changes in the region around 400 nm. In the 1-min spectrum, there is a local minimum around 400 nm, that the absorption spectrum quickly begins to start rising again from. By the 34-min spectrum, the region of this local minimum has expanded and now remains fairly flat until around 380 nm, where the absorption once again begins to rise above this minimum. Therefore, from these observations, the conversion of AdoCbl to this cobalamin product is dependent on the concentration of the EG used.

Analysis of the Kinetics of Reaction Induced by EG in the EAL-AdoCbl Complex

Figure 10 shows the dependence of AdoCbl concentration on time after addition of EG, for EG concentrations of 0.5 mM and 5 mM. The AdoCbl concentration was measured at 575 nm, which is a wavelength at which the EG reaction product exhibits negligible absorbance. Measurement of the rise of the EG-induced AdoCbl species is not possible, owing to spectral overlap in the wavelengths below 530 nm, but it is assumed that the rise is synchronous with the decay of AdoCbl. Figure 10 shows the expected decrease in the EAL-bound AdoCbl concentration, that corresponds to the EG-induced conversion to the transformed cobalamin species. The AdoCbl reaction product is denoted as XCbl. As shown in Figure 10, the decay kinetics of AdoCbl are dependent on EG concentration. The EG concentration dependence of the kinetics is consistent with the “quasi-substrate” designation for EG.[13]

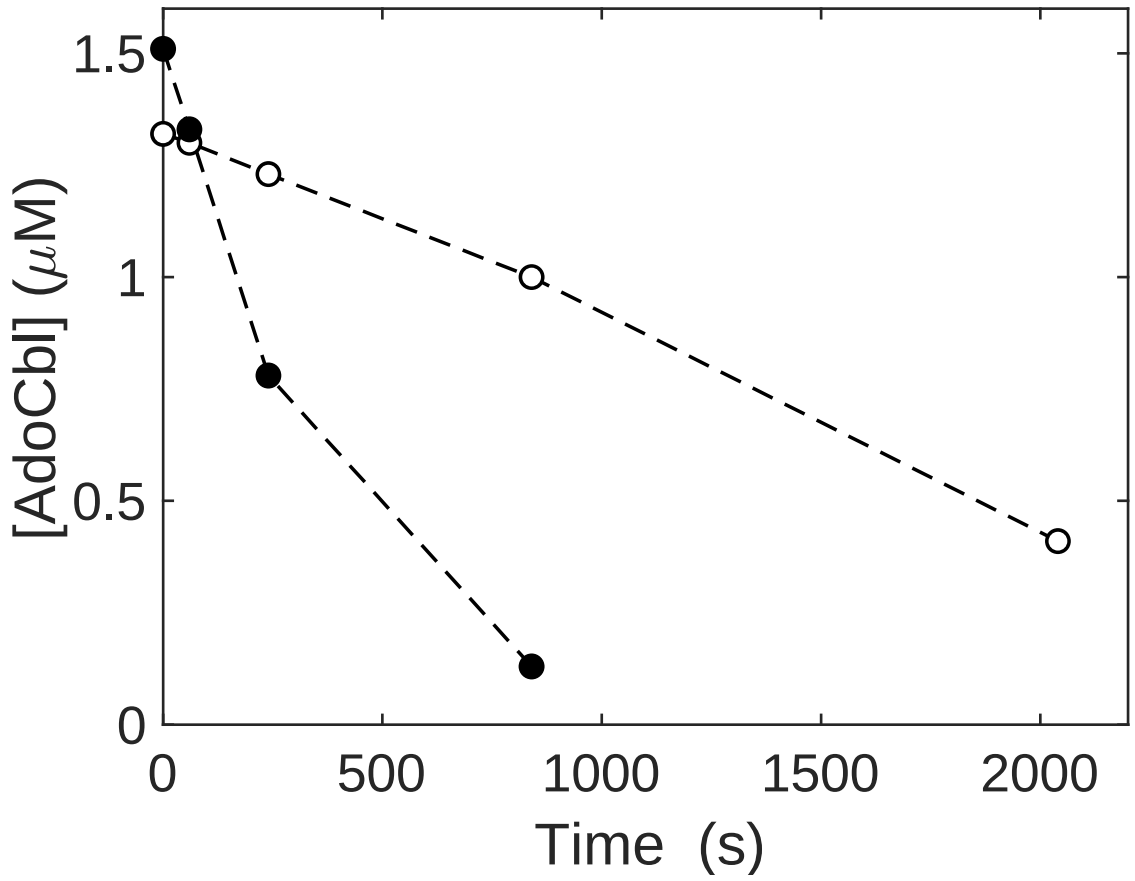


Figure 10. Dependence of AdoCbl concentration on time, following addition of EG. The curves correspond to EG concentrations of 0.5 mM (open) and 5 mM (filled). Dashed lines are drawn to guide the eye. The zero-time concentration of AdoCbl corresponds to the EG samples and was obtained by extrapolation of the $t > 0$ data.

A kinetic model for the system, that is used to account for the results presented in Figure 10, is presented in Figure 11. Unlike a true enzyme substrate, which is continuously converted to product in the steady-state enzyme reaction, EG irreversibly inactivates EAL. Therefore, the

traditional Michaelis-Menten analysis of enzyme kinetics does not directly apply. The model in Figure 11 represents the "irreversible inactivation" of EAL, although it is of the same form as the one used for derivation of the Michaelis-Menten kinetics expressions.[17] The earlier analysis of the EG reaction kinetics was based on the Michaelis-Menten formalism.[13] The first step represents the binding of EG to the EAL holoenzyme, E•AdoCbl, to form an EG-bound intermediate, E•AdoCbl•EG (the "enzyme-substrate complex"). This binding event is characterized by second-order association rate constant, k_1, and first-order dissociation rate constant, k_{-1}, and a dissociation constant, $K_{D,EG}=k_{-1}/k_1$. As in the Michaelis-Menten model, an equilibrium between EG, E•AdoCbl and E•AdoCbl•EG is assumed during the reaction ($k_2 << k_1$, k_{-1}). This appears to be justified under the assumption that the same rapid, non-rate limiting interaction of EAL with the small-molecule native substrate aminoethanol (~10^2 s^{-1}, or ~10^{-2} s time scale) holds for EG and the relatively slow reactions, over >10^2 s, that are shown in Figure 10.

$$\mathrm{EG + E{\bullet}AdoCbl} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \mathrm{E{\bullet}AdoCbl{\bullet}EG} \xrightarrow{k_2} \mathrm{E{\bullet}XCbl{\bullet}P}$$

Figure 11. Kinetic model for the decay of AdoCbl in the reaction of holo-EAL with EG. The model represents the single-turnover creation of the inactive enzyme, caused by the reaction with EG. States and rate constants are described in the text.

The modified cobalamin is identified as XCbl in Figure 11, and P represents other bound product species of the EG reaction, which may include acetaldehyde.[13] The first-order rate constant, k_2, represents the formation of the product state, E•XCbl•P by the reaction of E•AdoCbl•EG. The rate of reaction, v (units, M s^{-1}), is equivalent to the rate of AdoCbl to XCbl conversion and related to the concentration of the intermediate state as follows:

$$v = k_2[\text{E•AdoCbl•EG}]. \qquad \text{(Eq. 3)}$$

An expression for [E•AdoCbl•EG] is obtained from the equilibrium relation:

$$K_{D,EG} = \frac{[\text{E•AdoCbl}][\text{EG}]}{[\text{E•AdCbl•EG}]}. \qquad \text{(Eq. 4)}$$

Under the assumption that the concentration of free E•AdoCbl is given by the total E•AdoCbl minus the amount of enzyme in the E•AdoCbl•EG complex:

$$K_{D,EG} = \frac{([E_{tot}]-[\text{E•AdCbl•EG}])[\text{EG}]}{[\text{E•AdCbl•EG}]}. \qquad \text{(Eq. 5)}$$

After rearrangement of Eq. 5 in terms of the intermediate concentration:

$$[\text{E•AdCbl•EG}] = \frac{[E_{tot}]}{\frac{K_{D,EG}}{[EG]}+1}. \qquad \text{(Eq. 6)}$$

Substitution of Eq. 6 into Eq. 3 give an expression for the rate of XCbl formation, in terms of parameters that determine the rates of AdoCbl reaction in Figure 10:

$$v = \frac{k_2[\mathrm{E_{tot}}]}{\frac{K_{D,EG}}{[\mathrm{EG}]}+1}. \qquad \text{(Eq. 7)}$$

For the measurements performed here, the [AdoCbl] represents the number of EAL active sites that participate in the reaction in Figure 11. The [AdoCbl] is less than the total number of enzyme active sites in the sample (1.2×10^{-5} M), and the binding of AdoCbl to EAL is extremely strong ($K_{\mathrm{D,AdoCbl}}<<10^{-7}$ M). Active sites with no bound AdoCbl, that bind EG, do not contribute significantly to the equilibrium, because [EG]>>[EAL]. Therefore, the velocity or rate of AdoCbl reaction for each EG concentration, shown in Figure 11, is described by the following expression:

$$v = -\frac{d[\mathrm{AdoCbl}]}{dt} = \frac{k_2[\mathrm{AdoCbl_{tot}}]}{\frac{K_{D,EG}}{[\mathrm{EG}]}+1}. \qquad \text{(Eq. 8)}$$

The two concentrations of EG give two equations (Eq. 8) in two unknowns, which can be solved to determine $K_{\mathrm{D,EG}}$ and k_2. The following ratio expression is used to solve for $K_{\mathrm{D,EG}}$:

$$r_v = \frac{v(0.5\ \mathrm{mM\ EG})}{v(5\ \mathrm{mM\ EG})} = \frac{1+{K_{D,EG}}/{5\times10^{-3}M}}{1+{K_{D,EG}}/{5\times10^{-4}M}}. \qquad \text{(Eq. 9)}$$

The initial rates, v_i, obtained from the initial slopes of the AdoCbl reactions in Figure 10, are used in Eq. 9 to obtain a value of $K_{D,EG} = 2.2\times10^{-2}$ M, or 22 mM. This is equivalent to the value of 20 mM reported for the *Clostridium* sp EAL.[13] The K_D for EG is ~2000-fold larger (weaker binding interaction) than the K_D for aminoethanol (0.01 mM).[18] This relatively large difference in affinity, for similarly-sized molecules, may be caused by the electrostatic interactions of the protein with the positively-charged amine moiety of aminoethanol, with respect to the absence of this electrostatic interaction with the neutral hydroxyl moiety of EG. This result suggests that the electrostatic interaction with the amine nitrogen is also essential for achieving the appropriate active site positioning of the substrate, that is necessary for the next steps of reaction.

The values of v_i for 0.5 mM and 5 mM EG, that are obtained from the slopes of the data for $t\leq240$ s in Figure 10, are 3.8×10^{-10} M s^{-1} and 3.1×10^{-9} M s^{-1}, respectively. Substitution of these values, and $K_{D,EG} = 2.2\times10^{-2}$ M, into Eq. 8 for the 0.5 mM and 5 mM EG conditions, yields values for k_2 of 1.31×10^{-2} s^{-1} and 1.09×10^{-2} s^{-1}. The average is $k_2 = 1.20\times10^{-2}$ s^{-1}. The value of k_2 for EG is ~4000-fold smaller (slower reaction) than the turnover number for the native substrate, aminoethanol (52 s^{-1}).[4] This result suggests that the electrostatic interaction with the amine nitrogen is also essential for control of the reactivity of the substrate intermediate(s), in the enzyme mechanism. In the absence of the amine group, weaker interactions could result in escape of the reactive EG radical from the normal reaction channel and its reaction with the surrounding protein to form amino acid radicals, which could then react with the cob(II)alamin to form the XCbl species.[13]

Structure of the Modified AdoCbl, XCbl Product of EG Reaction in EAL

The XCbl species was originally suggested to arise from substitution of an amino acid side chain group at the axial ligand position of cobalt that was vacated by the 5'-deoxyadenosyl group following Co-C bond cleavage.[13] The most likely candidate was proposed to be the sulfur of a cysteine side chain.[13] Since this work, the amino acid sequences and high-resolution structures of the B_{12} enzymes have been obtained, including those for the *S. typhimurium* EAL.[4] In the *S. typhimurium* EAL structure, there are no cysteine sulfhydryl or methionine methylated-sulfur side chains in the vicinity of the active site, that could form an axial ligand to cobalamin, without significant structural distortion of the enzyme. Therefore, while formation of an axial ligand to cobalt in cobalamin is by an EG product or protein group is a viable explanation for the different XCbl absorbance properties, the identity of the ligand is not known. Analysis of samples of the type used for the visible absorbance experiments, by using electron paramagnetic resonance (EPR) spectroscopy (data not shown), show that the concentration of detected cob(II)alamin was ≤10% of the total cobalamin in the samples. This confirms that the dominant species observed in the visible absorbance experiments is a cob(III)alamin species.

Summary and Conclusions

The reaction of EAL with EG has been characterized at room temperature by using visible absorption spectroscopy. The results show that EG causes the formation of a novel cobalamin species, XCbl, in the EAL from *S. typhimurium*, as reported earlier for EAL isolated from *Clostridium* sp.[13] Results for anaerobic solution, created by the scrubbing of dioxygen by DTT, show that the mechanism of formation, and the final chemical constitution, of XCbl do not involve molecular oxygen (O_2). Studies of the reaction kinetics at varied EG concentrations show that the reaction to form XCbl is concentration-dependent. A kinetic model is presented for

irreversible inactivation of EAL by EG. The model differs from the traditional Michaelis-Menten model for enzyme catalysis. The model is consistent with the properties of EG concentration dependence and time-course observations of AdoCbl decay. Values of 2.2×10^{-2} M for the dissociation constant that represents equilibrium interaction of EG with EAL and 1.2×10^{-2} s^{-1} for the rate of AdoCbl reaction to form XCbl on EAL are obtained. These values differ by over three orders of magnitude, in the directions of weaker binding and slower turnover, from the corresponding values for the native aminoethanol substrate. The differences, and the destructive reaction of EG, are attributed to decreased control of the binding and reaction of EG caused by loss of electrostatic interactions with the amine (ammonium) group of the natural substrate. The amine is replaced by the charge-neutral hydroxyl group in EG. Overall, development of the time-resolved visible spectroscopy methods, and kinetics results, direct the EG–EAL system toward use in investigation of the contributions of solvent-coupled protein dynamics to enzyme catalysis, including both productive and non-productive pathways of reaction, under room temperature and fluid bulk solvent conditions. This complements the extensive low-temperature investigations of EAL enzyme catalysis, by using native and alternative substrates.[5, 8, 9, 10] Future studies will examine the temperature-dependence of the reactions, with the goal of extending investigation of the EG mechanism to the low-temperature systems used for the native reaction.

V. References.

1. Keita Akita, Naoki Hieda, Nobuyuki Baba, Satoshi Kawaguchi, Hirohisa Sakamoto, Yuka Nakanishi, Mamoru Yamanishi, Koichi Mori, Tetsuo Toraya, Purification and some properties of wild-type and N-terminal-truncated ethanolamine ammonia-lyase of *Escherichia coli*, *The Journal of Biochemistry*, Volume 147, Issue 1, January 2010, Pages 83–93, https://doi.org/10.1093/jb/mvp145

2. Bovell, A. M., and Warncke, K. (2013) Structure model of Salmonella typhimurium ethanolamine ammonia-lyase directs a rational approach to the assembly of the functional [(EutB-EutC)2]3 oligomer from isolated subunits, *Biochemistry 52*, 1419-1428.

3. Spataru, Tudor. "The Electronic Structure and Mechanism of the Adenosylcobalamin-Dependent Bio-Processes as Determined by the MCSCF Method." *Medicinal Chemistry* vol 11,8 (2021): 2161-0444.

4. Sun, L.; Warncke, K., Comparative model of EutB from coenzyme B12- dependent ethanolamine ammonia-lyase reveals a b8a8, TIM-barrel fold and radical catalytic site structural features. Proteins: Structure, Function, and Bioinformatics 2006, 64 (2), 308-319

5. Ucuncuoglu, Neslihan, and Kurt Warncke. "Protein Configurational States Guide Radical Rearrangement Catalysis in Ethanolamine Ammonia-Lyase." *Biophysical journal* vol. 114,12 (2018): 2775-2786. doi:10.1016/j.bpj.2018.03.039

6. Wang, Miao, and Kurt Warncke. "Entropic origin of cobalt-carbon bond cleavage catalysis in adenosylcobalamin-dependent ethanolamine ammonia-lyase." *Journal of the American Chemical Society* vol. 135,40 (2013): 15077-84. doi:10.1021/ja404467d

7. Finke, R.G. and B.P. Hay, Thermolysis of adenosylcobalamin: a product, kinetic, and cobalt-carbon (C5') bond dissociation energy study. Inorganic Chemistry, 1984. 23(20): p. 3041-3043.

8. Wang, M., Zhu, C., Kohne, M., and Warncke, K. (2015) Resolution and characterization of chemical steps in enzyme catalytic sequences by using low-temperature and time-resolved, full-spectrum EPR spectroscopy in fluid cryosolvent and frozen solution systems, *Meth. Enzymol. 563, Part A*, 59-94.

9. Zhu, C., and Warncke, K. (2008) Reaction of the CoII-Substrate Radical Pair Catalytic Intermediate in Coenzyme B12-Dependent Ethanolamine Ammonia-Lyase in Frozen Aqueous Solution from 190 to 217 Kelvin, Biophys. J. 95, 5890-5900.

10. Kohne, M., Zhu, C., and Warncke, K. (2017) Two dynamical regimes of the substrate radical rearrangement reaction in B12-dependent ethanolamine ammonia-lyse resolve contributions of native protein configurations and collective configurational fluctuations to catalysis, *Biochemistry 56*, 3257-3264.

11. Kohne, M., Li, W., Zhu, C., and Warncke, K. (2019) Deuterium Kinetic Isotope Effects Resolve Low-Temperature Substrate Radical Reaction Pathways and Steps in B12-Dependent Ethanolamine Ammonia-Lyase, *Biochemistry 58*, 3683-3690.

12. Bachovchin, William W. et al. “Mechanism of action of adenosylcobalamin: glycerol and other substrate analogues as substrates and inactivators for propanediol dehydratase--kinetics, stereospecificity, and mechanism.” *Biochemistry* 16 6 (1977): 1082-92 .

13. Babior, B.M., The Mechanism of Action of Ethanolamine Deaminase VI. ETHYLENE GLYCOL, A QUASI-SUBSTRATE FOR ETHANOLAMINE DEAMINASE. Journal of Biological Chemistry, 1970. 245(7): p. 1755-1766.

14. Sun, L., Groover, O. A., Canfield, J. M., and Warncke, K. (2008) Critical role of arginine 160 of the EutB protein subunit for active site structure and radical catalysis in coenzyme B12-dependent ethanolamine ammonia-lyase, *Biochemistry 47,* 5523-5535.

15. Pupeza, I., Huber, M., Trubetskov, M. *et al.* Field-resolved infrared spectroscopy of biological systems. *Nature* **577,** 52–59 (2020). https://doi.org/10.1038/s41586-019-50-7

16. Faust, L R et al. "Cloning, sequencing, and expression of the genes encoding the adenosylcobalamin-dependent ethanolamine ammonia-lyase of Salmonella typhimurium." *The Journal of biological chemistry* vol. 265,21 (1990): 12462-6.

17. Cornish-Bowden, A. "Fundamentals of Enzyme Kinetics", Butterworths, London, 1981.

18. (L. Anderson and K. Warncke, unpublished)