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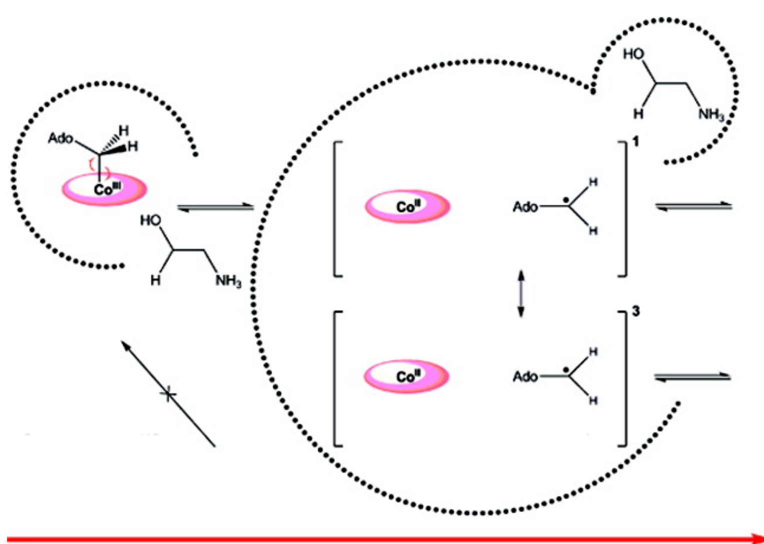

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Magnetic Field Effect Studies Indicate Reduced Geminate Recombination of the Radical Pair in Substrate-Bound Adenosylcobalamin-Dependent Ethanolamine Ammonia Lyase

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Abstract: The apparent conflict between literature evidence for (i) radical pair (RP) stabilization in adenosylcobalamin (AdoCbl)-dependent enzymes and (ii) the manifestation of magnetic field sensitivity due to appreciable geminate recombination of the RP has been reconciled by pre-steady-state magnetic field effect (MFE) investigations with ethanolamine ammonia lyase (EAL). We have shown previous stopped-flow MFE studies to be insensitive to magnetically induced changes in the net forward rate of C−Co homolytic bond cleavage. Subsequently, we observed a magnetic-dependence in the continuous-wave C−Co photolysis of free AdoCbl in 75% glycerol but have not done so in the thermal homolysis of this bond in the enzyme-bound cofactor in the presence of substrate. Consequently, in the enzyme-bound state, the RP generated upon homolysis appears to be stabilized against the extent of geminate recombination required to observe an MFE. These findings have strong implications for the mechanism of RP stabilization and the unprecedented catalytic power of this important class of cobalamin-dependent enzymes.

Introduction

The radical pair mechanism (RPM) describes the reaction dynamics of spin correlated radical pairs (SCRP) in solution.[1] Coherent spin-state mixing, in the most part between the near degenerate singlet and triplet states in two spin $^1/_2$ radicals, can result in spin-selective reactivity. As this process is magnetic in origin, the rate at which it occurs is sensitive to the application of external magnetic fields (MF).[2] A consequence is that relatively weak MF can alter the ratio of reaction through singlet and triplet reaction channels, which may give rise to different reaction products or produce products at different rates. Magnetic field effects (MFE), therefore, may be observed for magnetic flux densities that give rise to Zeeman energies that are small compared to the average thermal energy of molecules, k_BT. This is pertinent when one considers the potential interaction between environmentally sourced MF and biological systems containing RP reaction intermediates.[3−5] Moreover, the prediction,[6] and subsequent observation,[7−13] of the low field effect (LFE) in chemical systems has further raised the profile of biological MFE.

Enzyme reaction systems are an example of potential carriers of biological field sensitivity. The first MFE to be reported in an enzyme was in the steady-state parameters V_{max}/K_m of ethanolamine ammonia lyase (EAL) from the bacterium *Salmonella typhimurium*.[14] This was a significant finding, not least because it introduced the possibility of MFE in *human* enzyme systems containing RP intermediates. Subsequently, the same group have carried out a number of studies on a range of horseradish peroxidase (HRP) catalyzed reactions,[15−17] one of which reported a unique LFE.[15] However, the current authors were unable to reproduce any field effect described in ref 15 and recently published a substantial reappraisal of this work.[18] The work described here includes another reassessment of previous MFE reported in an enzyme, this time in stopped-flow (pre-steady-state) studies with EAL.[19]

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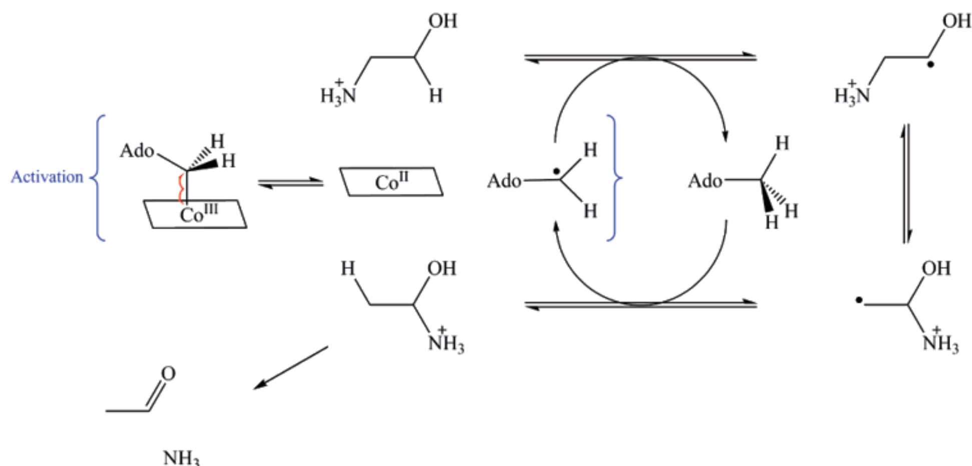

Figure 1. Proposed mechanistic scheme for the ethanolamine ammonia lyase (EAL) catalyzed conversion of 2-aminoethanol (2AE) to acetaldehyde and ammonia, based on ref 25. The cobalt(III) containing species is a simplified representation of coenzyme B_{12} (adenosylcobalamin, AdoCbl) that acts as a radical source and that promotes the 1,2-rearrangement of the amine group. The proposed radical pair comprises the Cbl^{II} and 5′-deoxyadenosyl radicals (see main text and Figure 2). *S*-2-aminopropanol (2AP) is an alternative substrate.

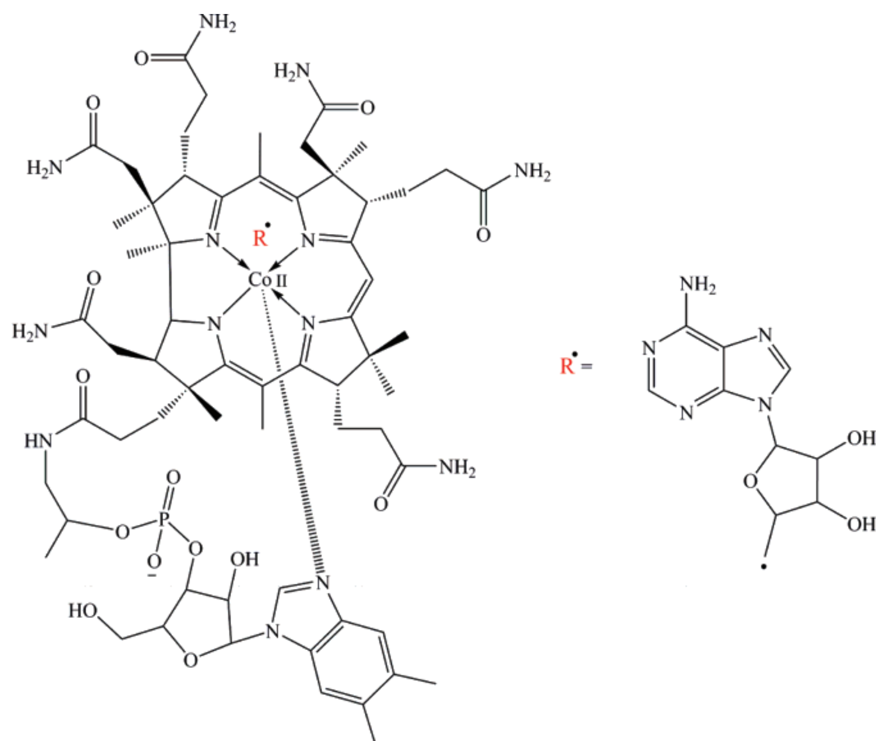

Figure 2. Structures of the proposed radical pair in the AdoCbl-dependent ethanolamine ammonia lyase catalyzed reaction represented in Figure 1. C−Co bond homolysis generates a low-spin Co^{II}, ligated equatorially by four nitrogens of a corrin ring and with an axial 5,6-dimethylbenzimidazole, and a 5′-deoxyadenosyl radical, both of spin $^1/_2$. Based on structures in ref 22.

EAL is a coenzyme B_{12} (adenosylcobalamin, AdoCbl)[20−23] dependent enzyme that catalyzes the conversion of 2-aminoethanol (2AE) to acetaldehyde and ammonia. Figure 1 represents the proposed catalytic cycle.[24] The original stopped-flow MFE studies[19] were used to pinpoint the magnetically sensitive step as the reversible C−Co bond homolysis in AdoCbl activation on substrate binding, with the proposed RP comprising a low spin, cobalt (Co^{II}) containing radical (Cbl^{II}), and a 5′-deoxyadenosyl radical (see Figure 2 for structures). The 5′-deoxyadenosyl radical abstracts hydrogen from the substrate to form the substrate radical, which then undergoes a 1,2-rearrangement of the amine group to the product radical before abstracting hydrogen back from the adenosine and dissociating into products. Significant kinetic isotope effects (KIE) have been reported on the rate of bond homolysis with [1,1,2,2-$^2H_4$]-2AE ($^2H_4$-2AE), [1,1-$^2H_4$]-2AE, and [1,1-$^2H_4$]-*S*-2-aminopropanol (2AP) as substrates,[25] and an enhanced MFE was observed in V_{max}/K_m with $^2H_4$-2AE.[14]

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There are a range of publications on the dynamic spin chemistry of AdoCbl, including: MFE in AdoCbl photolysis in solution;[26] calculated and modeled MFE in AdoCbl-dependent enzymes;[27,28] CIDNP[29] and CIDEP[30,31] on AdoCbl, model compounds, and analogues. The MFE in V_{max}/K_m has been reproduced in collaboration with the original authors,[32] and a heavy atom effect was also observed in these parameters for EAL.[33] New stopped-flow data will now be presented for EAL that challenge the relevance of the original stopped-flow methodology to studying MFE and offer a new protocol that in turn questions the established wisdom on the proposed magnetically sensitive step in this system. These results have wider implications for the catalytic power of AdoCbl-dependent enzymes in terms of stabilization of the RP against geminate recombination relative to free coenzyme.

Experimental Procedures

Materials. The plasmid, pET-SEAL, encoding the small (32.0 kDa) and large (49.1 kDa) subunits of EAL from *Salmonella typhimurium* (kindly donated by Prof. George Reed), was expressed in *Escherichia coli* and purified as described previously.[34] AdoCbl (coenzyme B_{12}, >98% Sigma), 2AE (ethanolamine, >98%, Sigma), 2AP (*S*-(+)-2-amino-1-propanol, >98%, Aldrich), and $^2H_4$-2AE (D_4-ethanolamine, >98%, Cambridge Isotopes) were all used as purchased.

Stopped-Flow Spectrophotometry. Two mixing regimes were employed for stopped-flow studies with EAL. In the first instance, an EAL *apoenzyme* solution (protein only) is loaded into one drive syringe, with a solution mixture of AdoCbl and substrate in the second. Alternatively, EAL apoenzyme and AdoCbl cofactor are incubated as the *holoenzyme* in the same drive syringe prior to rapid mixing with a solution of just the substrate. For a full justification of the different protocols, refer to the Results and Discussion section. Construction and testing of the dedicated magnetic field effect stopped-flow spectrophotometer (MFESFS) in use throughout these studies is detailed in the supporting information of ref 18. EAL begins to denature within approximately 2 h of thawing, even when kept on ice. Care was therefore taken to prepare solutions and carry out experiments well within this time. No benefit was apparent when utilizing degassed (see Supporting Information) reagent solutions. All comparisons made are with the previous EAL stopped-flow MFE study.[19]

1.1. Apoenzyme Pre-Steady-State Kinetic and MFE Studies. The apoenzyme mixing regime is the protocol adopted by the original MFE stopped-flow study, and therefore, this reproduction adhered to the same conditions wherever possible and appropriate. The buffer solution used previously contained: 100 mM Hepes/NaOH, pH 7.45; 10 mM KCl; 10 mM urea; 5 mM dithiothreitol (DTT); and 10% glycerol; (designated buffer "*B4*"). However, the enzyme purification process employed[34] is based on the limited solubility of the EAL protein near neutral pH in buffers also containing glycerol. Consequently, a second buffer solution ("*B3*") was also prepared which has the same composition as *B4*, but excluding glycerol. All solutions were prepared separately in both *B4* and *B3*, with identical MFESFS investigations carried out in each case. All concentrations quoted are post-mixing: EAL ~10 μM active-sites; AdoCbl 20 μM, determined spectrophotometrically at 525 nm using ϵ = 8.0 mM^{-1}cm^{-1}; and 2AE 30 mM. An AdoCbl concentration of 20 μM was used in this case, compared to 12 μM previously, because the concentration ratio (~1.7) corresponds to the ratio between reaction cell optical pathlengths in this (10 mm) and the original study (17 mm). Under safe-light, reagent solutions were passed through a 0.45 μm filter on loading into the drive syringes: syringe 1, EAL apoenzyme; syringe 2, AdoCbl and 2AE.

All experiments were conducted under safe-light at 25 °C. Absorbance measurements were acquired over a linear time-base using a single wavelength of 525 nm[25] (which corresponds to the most significant absorbance change on C−Co homolysis, see Supporting Information) and a photomultiplier tube (PMT). The homogeneous MF (30, 50, and 80 mT) was applied to the sample position throughout the data acquisition period and generated by mounting pairs of rare-earth permanent magnets in opposite, unoccupied light-guide ports in the cell block. The first 5−6 shots were discarded on every occasion. Each experiment comprised 6−7 field-on/field-off data acquisition pairs (the order of which was randomized) and was repeated several times for every MF data point.

1.2. AdoCbl Binding Studies. To assess the rate of cofactor binding to the apoenzyme under the same conditions as above, but in the absence of an applied MF, EAL was rapidly mixed with varying concentrations of AdoCbl and absorbance measurements acquired at 555 nm (see Supporting Information). Reagent solutions were prepared in *B3*: EAL ~13 μM active-sites; and AdoCbl 50, 75, 100, 125, and 150 μM. A similar experiment was then carried out with 2AE alongside AdoCbl (i.e., apoenzyme mixing) and absorbance measurements acquired at 525 nm. Reagent solutions were prepared in *B3*: EAL ~13 μM active-sites; AdoCbl 20, 37.5, 50, 75, 100, and 125 μM; and 2AE 30 mM.

2.1. Holoenzyme Pre-Steady-State Kinetic and MFE Studies. Previous stopped-flow experiments with EAL[25] have successfully used a simpler buffer solution than described above: 20 mM Hepes/NaOH, pH 7.5 ("*B20*"). Reagent solutions were therefore prepared in *B20*: EAL 10−13 μM active-sites (consistent for each substrate); AdoCbl 20 μM; and substrate (2AE, $^2H_4$-2AE, or 2AP) 2.5 mM. The general experimental procedure was conducted as for the apoenzyme mixing regime, with the thermostatic water bath at 5 or 25 °C depending on requirements, and syringes loaded as follows: syringe 1, EAL holoenzyme; syringe 2, substrate. Absorbance measurements were acquired over linear and split (an equal number of acquisition points distributed over two unequal time periods) time bases at 525 nm. A Helmholtz pair mounted upon the cell block of the MFESFS and a pulsed power supply (both constructed in-house) were used to generate an adjustable, homogeneous MF (10 and 30 mT) at the sample position throughout data acquisition. Due to the restrictions of this apparatus, rare-earth permanent magnets were mounted as above for the 50 and 80 mT field points. Each experiment comprised 6−7 field-on/field-off data acquisition pairs as above and was repeated several times for every MF data point.

2.2. Holoenzyme Post-Steady-State MFE Studies. After the substrate has been exhausted by the enzyme turnover, a second transient may be observed. This has been attributed to RP recombination of the C−Co bond in AdoCbl[25] and was also monitored for MF sensitivity. Reagent solutions were prepared in *B20*: EAL ~13 μM active-sites; AdoCbl 20 μM; and 2AE 2.5 mM. The general experimental procedure was conducted as before, with absorbance measurements acquired at 25 °C over a linear time-base at 525 nm. A magnetic field of 80 mT was generated with rare-earth permanent magnets as above. This field was applied to alternate shots for the entire 10 s data acquisition, which, under the conditions, was sufficient time to ensure exposure of the full steady-state turnover and post-steady-state transient.

3. MFE Studies on the Anaerobic Continuous-Wave Photolysis of AdoCbl. The relevance of MFE to AdoCbl-dependent enzymes was based on the observation of field sensitivity in the net quantum yield of C−Co photolysis in the free cofactor.[26] We have reinvestigated the

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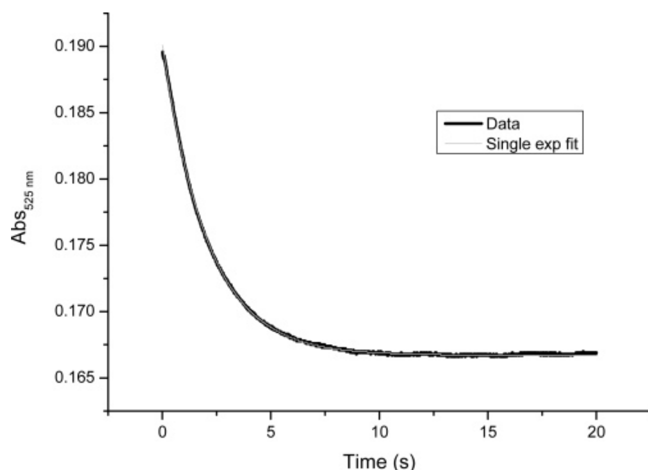

Figure 3. Averaged absorbance trace (and corresponding first-order fit) recorded at 525 nm for EAL apoenzyme (*B3*, ~10 μM active-sites) rapidly mixed with AdoCbl (*B3*, 20 μM) and 2AE (*B3*, 30 mM). In this case, k_{obs} = 0.48 s^{-1}.

anaerobic continuous-wave (cw) photolysis studies at 80 mT in a viscous solvent; 20 mM Hepes pH 7.5, 75% glycerol was degassed by bubbling nitrogen gas with stirring for 1 h, and 200 μM of free AdoCbl was prepared in this buffer solution and loaded into both drive syringes of the MFESFS. The instrument was configured in such a way as to expose the contents of the reaction cell to the entire emission spectrum of the Xe arc-lamp and to monochromate the light transmitted from the cell to 525 nm before reaching the PMT. The light source, therefore, served both to photolyze the sample and to monitor its absorbance over time. Experiments were conducted at 25 °C under safe-light, and field-on/field-off pairs were acquired over a linear time-base for 500 s. The 80 mT MF was generated using rare-earth permanent magnets as outlined above.

Results and Discussion

The rate of C−Co bond homolysis is so rapid when holoenzyme is mixed with 2AE that it is complete within the dead-time (T_D) of typical commercial stopped-flow spectrophotometers, and to date has never been observed using this method. Bandarian and Reed[25] estimated the first-order rate coefficient at >300 s^{-1}, based on an instrument T_D of 3−4 ms (as did Hollaway et al. for EAL from *Clostridium sp.*[35]). Consequently, Harkins and Grissom in ref 19 slowed the rate of homolysis by using the apoenzyme mixing regime described above, allowing them to investigate the magnetic sensitivity by comparing observed first-order rate coefficients (k_{obs}).

1.1. Apoenzyme Pre-Steady-State Kinetic and MFE Studies. Figure 3 shows the absorbance change at 525 nm for EAL apoenzyme rapidly mixed with AdoCbl and 2AE, and the corresponding single-exponential fit. The rate has been slowed substantially from >300 s^{-1}, to a typical first-order $k_{obs} \approx 0.5$ s^{-1}. The presence of glycerol in the buffer solution significantly reduced the observed amplitude (by a factor of ~0.55, see Supporting Information), presumably as a consequence of the limited solubility of the apoenzyme under these conditions.

In ref 19, Harkins and Grissom reported a MF dependence in the absolute first-order k_{obs} for the appearance of Cbl^{II} with both 2AE and $^2H_4$-2AE as substrate. Neither a magnetic isotope effect nor a kinetic isotope effect was observed. A decrease in the net rate of Cbl^{II} appearance was reported with increasing MF to a minimum at 50 mT (a fall of 17%). Further increase in the MF resulted in k_{obs} gradually returning to its zero field value, and hence, a biphasic curve was obtained, characteristic of combined Zeeman and Δg mechanisms.[2] The field response is consistent with a singlet born RP and a decreased efficiency of spin-state mixing to the $T_{\pm 1}$ sublevels on application of MF. The resulting increase in population of the singlet state would augment RP recombination and lead to an overall decrease in the net, forward reaction rate. Larger applied fields may result in enhanced $S \leftrightarrow T_0$ spin-state mixing through the Δg mechanism, reducing the singlet state population and hence the probability of RP recombination.

The present study has attempted to recreate the results of Harkins and Grissom[19] using conditions and methods similar to the original (refer to the Experimental Procedures). However, the MF dependence will be analyzed here in terms of relative, as opposed to absolute, rate coefficients.[15,18] Recording each kinetic trace with MF applied, with a corresponding trace with no applied MF, not only allows for accurate comparison of data from multiple experiments as mean relative rate coefficients, but should also overcome any systematic instrumental error within single experiments. The relative rate coefficient at 0 mT applied MF is set at 1.

Figure 4a shows the MF dependence of the relative k_{obs}, and it is clear that the original MFE from the apoenzyme mixing regime has not been reproduced. An MFE was neither observed in *B3* nor *B4* buffer solutions, and therefore, data from the former (no glycerol present) has been presented here because of the improved quality of data acquired. The kinetic trace from each shot was fit with a single-exponential function using the instrument software (Applied Photophysics Ltd SX.18MV Kinetic Spectrometer workstation Software 4.52) and relative rate coefficients calculated for every field-on/field-off acquisition pair by dividing the field-on by the corresponding field-off coefficient. The mean and standard deviation were then calculated across all experiments for individual data points, which were then plot against MF. Figure 4b further illustrates the lack of an observable MFE by overlaying averaged traces for the experiments carried out at 50 mT, which corresponds to the most significant data point from ref 19. No equivalent field-on/field-off traces were published from the original study for comparison; therefore, a simulated trace of the same amplitude is included to account for the magnetically induced changes a 17% reduction in k_{obs} (as reported) would have on the shape of the 50 mT trace from the current study. These traces appear quite different, yet the 0 and 50 mT traces superimpose almost exactly. The mean values of k_{obs} for 0 and 50 mT are 0.484 ± 0.026 and 0.483 ± 0.027 s^{-1}, respectively.

1.2. AdoCbl Binding. To reconcile this discrepancy, one must consider the method used in slowing the kinetics to achieve an observable spectral change. In placing the apoenzyme and AdoCbl cofactor in separate syringes, an additional slow step has been introduced that alters the rate-limiting chemistry being monitored. This is likely to be generation of the active holoenzyme through AdoCbl binding to the apoenzyme with possible accompanying and/or subsequent conformational changes.[35] Figure 5 displays the results of cofactor binding studies, which involved rapid mixing in the MFESFS of the apoenzyme with varying AdoCbl concentrations in the absence and presence of 2AE alongside the cofactor. In the absence of

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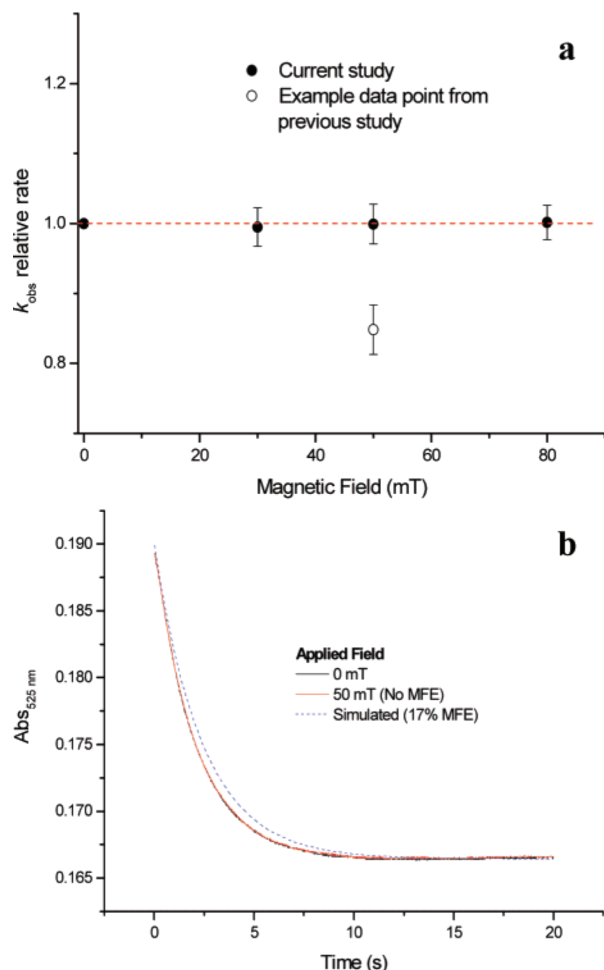

Figure 4. (a) Magnetic field dependence of the first-order observed rate coefficient, k_{obs}, expressed in terms of relative rates. (●) data from the current study, (○) example data point from ref 19, calculated as k_{obs} relative rate from published rate coefficients. 50 mT corresponds to the most significant field point from the original study. (b) Overlaid, averaged absorbance traces for 0 mT (—) and 50 mT (red —) applied field recorded at 525 nm for EAL apoenzyme mixed with AdoCbl and 2AE. Conditions as for Figure 3. (---) is a simulated trace with the same amplitude change but 17% slower first-order apparent rate coefficient than the 50 mT data point (see text).

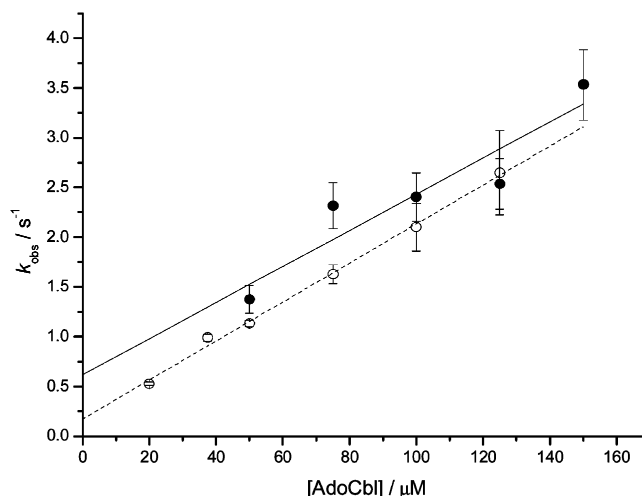

Figure 5. Dependence of first-order k_{obs} on AdoCbl concentration. (●) EAL apoenzyme (*B3*, ~13 μM active sites) against AdoCbl (*B3*: 50, 75, 100, 125, and 150 μM), absorbance measurements recorded at 555 nm. (○) EAL (*B3*, ~13 μM active sites) against AdoCbl (*B3*: 20, 37.5, 50, 75, 100, and 125 μM) and 2AE (*B3*, 30 mM), absorbance measurements recorded at 525 nm. (—) and (---) are corresponding linear regression fits.

substrate, mixing of apoenzyme with AdoCbl results in a small absorbance change at 555 nm (see Supporting Information), potentially due to lengthening of the C−Co bond in the cofactor upon binding. For both investigations, the trace resulting from each shot was fit with a single-exponential function on the instrument software (as above), with the mean and standard deviation then calculated and plot as a function of AdoCbl concentration.

The concentration dependences in Figure 5 are indistinguishable within error, strongly suggesting that AdoCbl binding is rate-limiting when the apoenzyme mixing regime is employed and not C−Co bond homolysis. Both data-sets were fit to:

$$k_{obs} = k_f[\text{AdoCbl}] + k_r \quad (1)$$

where k_f is the apparent second-order rate coefficient for the forward reaction, [AdoCbl] is the cofactor concentration, and k_r is the apparent first-order rate coefficient for the reverse reaction. Figures extracted for k_f in the absence and presence of 2AE are $(1.80 \pm 0.4) \times 10$ and $(1.96 \pm 0.06) \times 10^4$ $M^{-1}s^{-1}$, respectively, and for k_r are 0.6 ± 0.4 and 0.17 ± 0.04 s^{-1}, respectively. These values correspond between data sets within error which, alongside the linear dependence of k_{obs} with [AdoCbl] in the presence of substrate, support the above notion that the apparent rate measured using this mixing regime is largely or wholly limited by AdoCbl binding steps preceding C−Co homolysis.

Consequently, even if the net rate of rapid, reversible C−Co homolysis is MF dependent, the rate-limiting step of AdoCbl binding (and any conformational changes) may be sufficiently slow to render this protocol insensitive to any magnetically induced changes. Hollaway et al. carried out a range of apoenzyme and holoenzyme transient kinetic experiments[35] on EAL from *Clostridium sp.* and, in light of their results, concluded that it was essential to conduct holoenzyme rather than apoenzyme mixing when investigating the catalytic steps in EAL catalyzed reactions; otherwise, the initial and comparatively slow formation of the holoenzyme will impact the results. This may go some way to explain the differences in MF dependencies between the steady-state[14] and stopped-flow[19] studies carried out by the original authors, including the size and position of the rate minima.

2.1. Holoenzyme Pre-Steady-State Kinetic and MFE Studies. Although to date C−Co homolysis initiated by the binding of 2AE has never been observed by stopped-flow spectrophotometery, the 20 μL cell of the MFESFS has a functioning T_D of 1.5−2 ms, a marked improvement on the 3−4 ms used previously to estimate the single turnover rate coefficient of >300 s^{-1}.[25,35] Pre-steady-state kinetic experiments were therefore conducted with EAL holoenzyme and both 2AE and $^2H_4$-2AE in an attempt to improve on this estimate and to see whether a viable alternative to apoenzyme mixing can be developed for MFE studies. Figure 6a is the first reported example of this elusive transient for EAL holoenzyme with its natural substrate (2AE) at 25 °C.

If the averaged trace in Figure 6a is fit to a single-exponential fitting function, as for the apoenzyme investigations, a first-order rate coefficient of ~1100 s^{-1} is estimated. How-

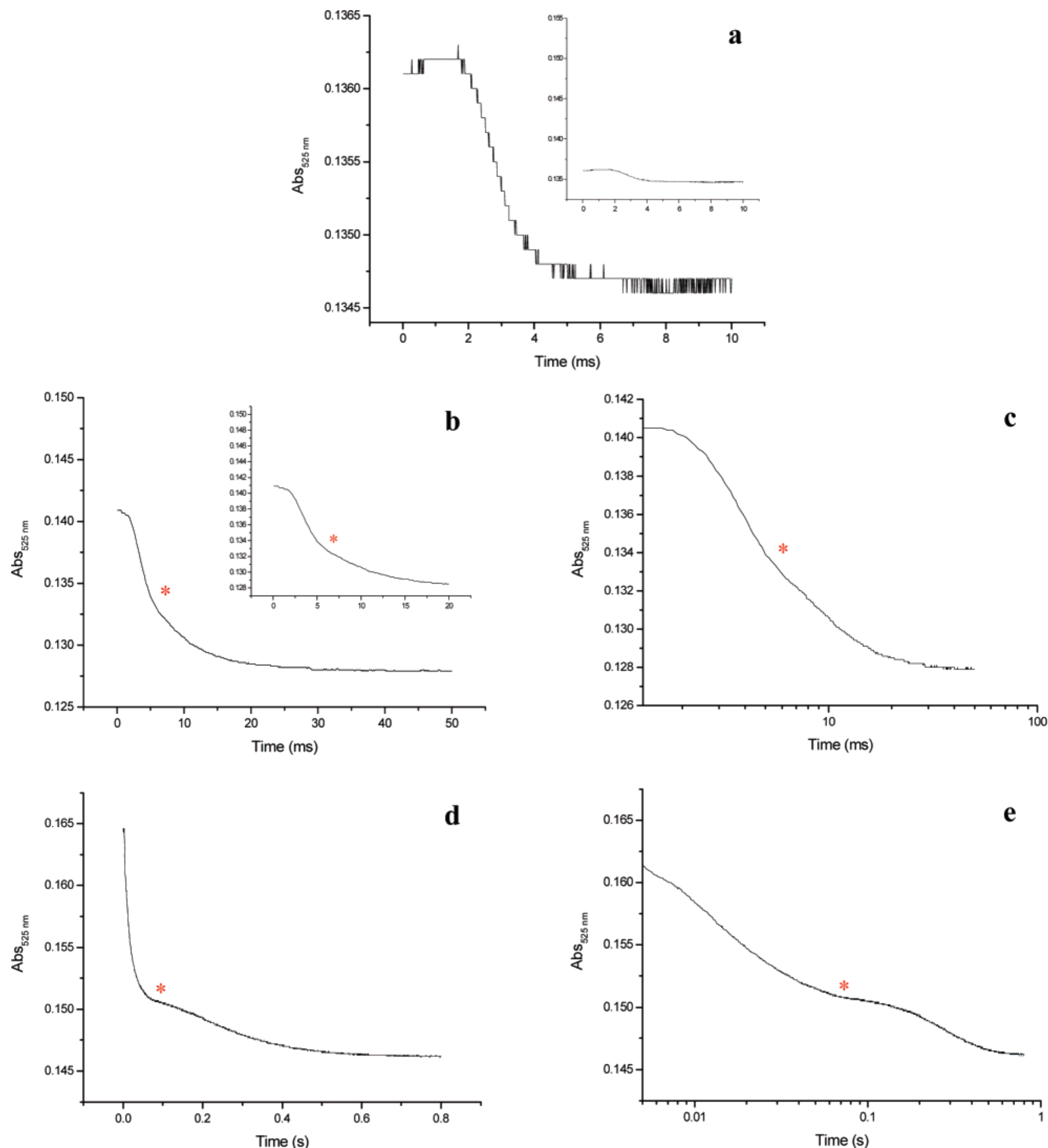

Figure 6. Averaged kinetic traces recorded at 525 nm from rapid mixing of EAL holoenzyme (*B20*, EAL ~13 μM, AdoCbl 20 μM) and 2AE (*B20*, 2.5 mM) at (a) 25 °C (insert shows trace on the scale of the expected amplitude) and (b,c) 5 °C. (d,e) Equivalent traces from mixing of EAL holoenzyme and $^2H_4$-2AE at 5 °C, reagent concentrations and conditions as in (b,c). Red "*" identifies an unusual feature of the transient that appears to be consistent between substrates and exaggerated in $^2H_4$-2AE, and the insert in (b) serves to highlight this. Variations in absorbance magnitude are attributed to fluctuation in photomultiplier voltage.

ever, several factors may call into question the relevance of such a figure. First, the insert in Figure 6a illustrates the amplitude of this transient on the scale of that observed for the apoenzyme. The vast majority of the signal disappears within the instrument T_D, and therefore the kinetic fit will not be a reliable representation of the true kinetics. Second, our investigations at 5 °C with both 2AE and $^2H_4$-2AE (Figures 6b,c and 6d,e, respectively) suggest a more complicated story than has been previously assumed. Although homolysis is expected to be a first-order process, there are at least two distinct phases (with the transition highlighted with a red "*" in Figure 6) that appear to be common to both the protiated and deuterated substrates. In fact, the multiphase nature of these transients closely resembles those observed for protiated and deuterated glutamate when rapidly mixed with the holoenzyme of AdoCbl-dependent glutamate mutase.[36]

It appears, therefore, that first-order kinetics is an oversimplified approach, and a more detailed analysis is required.

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However, such a full investigation is outside the remit of the current study and must be left to future consideration. A number of pertinent observations can be made at this point based on the data in Figure 6, which support assertions laid out in part I of this section. It is well established that the reduction in absorbance at around 525 nm does in some way represent the conversion of $AdoCbl^{III}$ to Cbl^{II} on bond homolysis.[37] Consequently, Figure 6b and c indicate a substantial KIE in this step that was not observed in ref 19. The original authors explained this absence by stating that it was conversion of $AdoCbl^{III}$ to Cbl^{II} and the 5′-deoxyadenosyl radical that was responsible for the spectral change and that this step does not involve H-abstraction; therefore, no KIE would be expected. Experimental evidence of a significant KIE in C−Co homolysis from this study (and previously[25]) strongly contests this notion and suggests that, like methylmalonyl-CoA mutase,[38] glutamate mutase,[36] and diol dehydratase,[20] C−Co bond homolysis is kinetically coupled to subsequent H/^{2}H-abstraction from the substrate. Instead, the lack of a KIE in the original stopped-flow MFE study is most likely indicative of the transient being rate-limited by generation of the active holoenzyme. If the apoenzyme mixing regime is insensitive to a net rate change of approximately an order of magnitude, it is unlikely to be able to detect an MFE of 17−25% (the latter being the maximum MFE in V_{max}/K_m[14]). The multiphase features are also not resolved in the apoenzyme kinetic transients, adding further support to this case. To accurately establish the magnitude of the KIE, further study is required.

The original stopped-flow study by Harkins and Grissom[19] was used to pinpoint the magnetically sensitive step in this EAL catalyzed reaction as reversible C−Co bond homolysis, but it has been established that the experimental protocol adopted is not sensitive to changes in this step. To confirm the presence of an MFE at this point in the reaction cycle, the kinetic transient *must* be rate-limited by homolysis. This can only be achieved by employing the holoenzyme mixing regime for pre-steady-state MFE studies. Unfortunately, due to the rate of homolysis with 2AE as substrate, there is still an insufficient proportion of the transient observable at either 25 or 5 °C for adequate MFE analysis to be possible. Therefore, an alternative means of slowing the net rate of this step has been identified. An enhanced MFE was observed in V_{max}/K_m with $^2H_4$-2AE,[14] and a substantial KIE has been identified for this substrate resulting in the vast majority of the transient being observable. Investigations have therefore been conducted in the MFESFS, with both $^2H_4$-2AE and 2AP (the "lesser" substrate of EAL that also yields sufficient signal) at 5 °C, to reassess the identity of the magnetically sensitive step.

The pre-steady-state, averaged kinetic traces yielded for 2AP at 25 and 5 °C are shown in the Supporting Information. At 25 °C, the transient closely resembles that for 2AE at 5 °C in Figure 6b, and like this trace, there is insufficient signal to conduct reliable MFE analysis. A further similarity is that both sets of data presented appear to represent more than one phase, and in fact, the 2AP transients accurately fit two exponentials. Again, this is a new development for EAL, and some basic kinetic analysis is also presented in the Supporting Information. In terms of MFE studies, although there is adequate signal for both $^2H_4$-2AE (Figure 6d,e) and 2AP when data is acquired at 5 °C, there is insufficient evidence to meaningfully assign microscopic rate coefficients to specific chemical steps. MFE analysis shall therefore be presented as difference spectra for each MF data point investigated, alongside examples of overlaid spectra at 50 mT for each substrate (Figure 7). The difference spectra illustrated in Figure 7a and c were calculated by subtracting the kinetic trace, averaged across experiments from a single field point for data acquired with no applied MF, from the corresponding trace averaged for data acquired with MF applied. They are offset in Figure 7 for clarity. This is an established method for reporting MFE[12] and should reveal any magnetically induced changes in the kinetic transients yielded. No MFE is evident for either substrate. The lack of any MFE is further supported by the examples of overlaid traces (Figure 7b and d) for the respective 50 mT experiments. The transients are plotted with a log time base in these instances as it better resolves each phase and is more sensitive to changes in amplitude than the equivalent plot with a linear time base. As a consequence, this method of presenting data should not only identify magnetic perturbations in net rate but also in reaction yield manifest as a difference in absorbance change. Again, no effect is observed. An additional, slow pre-steady-state phase has also been identified for 2AP. The amplitude change is relatively small in comparison with what appear to be the proceeding two phases; however, the same range of MF points were investigated with the same negative result (for details, refer to the Supporting Information).

These results represent a significant shift from the received wisdom on the position of MF sensitivity in the reaction cycle of EAL. The MFESFS has been thoroughly tested[18] with a chemical system well established to exhibit an MFE[9,10] and has generated reproducible data of a high quality. If an appreciable field effect did exist in reversible C−Co homolysis in EAL, one would expect the holoenzyme pre-steady-state kinetic investigations in this instrument to identify it. Nevertheless, prior to the studies on EAL, Chagovetz and Grissom observed a 2-fold decrease in the net quantum yield of the cw-photolysis of free AdoCbl in a buffered solution of 75% glycerol at 80 mT.[26] Figure 8 shows the results from our reinvestigation of this system, which strongly suggest an MFE does exist in the reversible C−Co photolysis in the free cofactor. Not only does this represent a positive MFE observed in the MFESFS for a biologically relevant system, but it also reveals a discrepancy between the magnetic sensitivity of the photolysis in free AdoCbl in a viscous solvent and the thermal homolysis in the enzyme-bound cofactor in the presence of substrate. It is now left for us to speculate on biochemical reasons for this difference.

It is probable that after activation (i.e., homolysis) the *enzyme* fosters conditions that stabilize the RP, be it thermodynamically or kinetically, against the extent of geminate recombination required for an MFE to manifest. The highly reactive 5′-deoxyadenosyl radical (a primary carbon radical) of the proposed pair, although inferred in the majority of mechanistic schemes put forward for AdoCbl-dependent enzymes, has never been observed in any direct way spectroscopically. In many cases, it is now considered a high-energy, fleeting intermediate, with homolysis kinetically coupled to subsequent H-abstraction from the substrate.[20,36,38] The large KIE reported here and previously[25] for EAL provides experimental evidence for such a coupling

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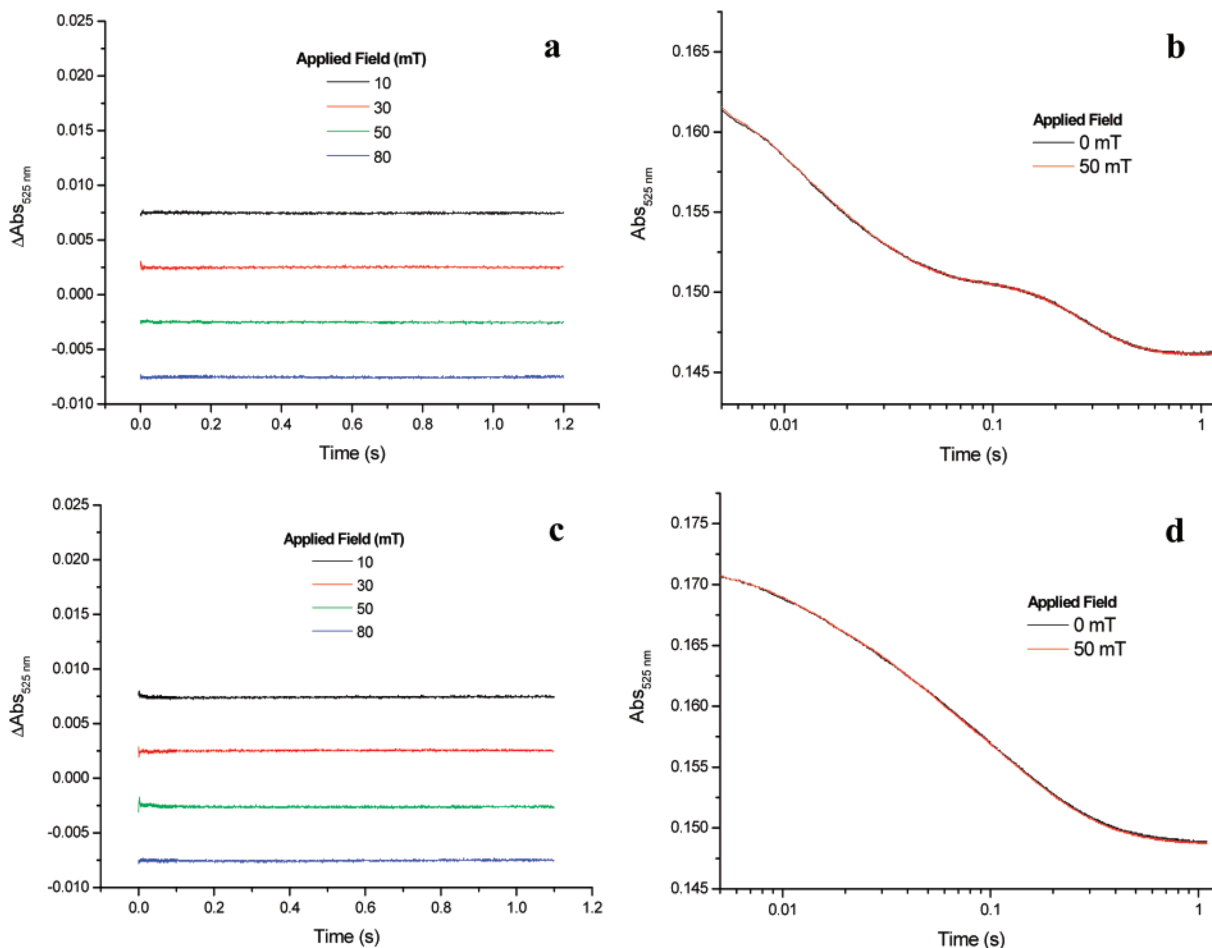

Figure 7. Magnetic field dependencies of averaged kinetic traces recorded at 525 nm for the rapid mixing of EAL holoenzyme (*B20*, EAL ~13 μM, AdoCbl 20 μM) and $^2H_4$-2AE (*B20*, 2.5 mM) presented as (a) difference spectra ("field-off" trace subtracted from "field-on" trace) for each field value, offset for clarity, and (b) example overlaid trace from 50 mT experiment. Equivalent field dependencies for EAL holoenzyme (*B20*, EAL ~10 μM, AdoCbl 20 μM) and 2AP (*B20*, 2.5 mM), (c) difference spectra, and (d) 50 mT overlaid traces. Nowhere is a magnetic field dependence evident.

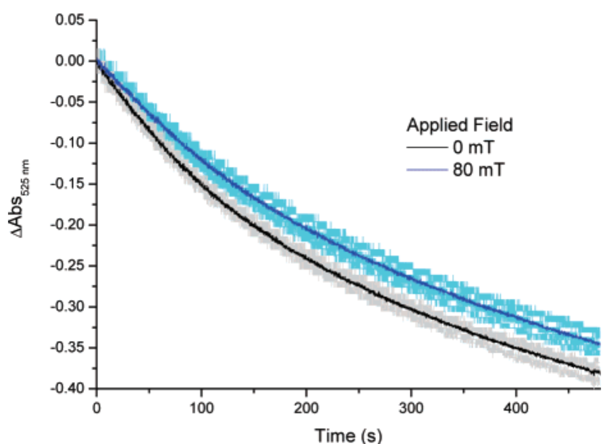

Figure 8. Anaerobic continuous-wave photolysis of AdoCbl (200 μM, 20 mM Hepes pH 7.5, 75% glycerol) monitored at 525 nm at 25 °C. A magnetic field effect is observed in the extent of photolysis with time on the application of an 80 mT field (top trace). Averaged traces are presented in bold, with the full range of experimentally observed transients indicated in lighter shades.

of steps that, it has been argued,[20] may be in part responsible for the >90% dissociation reported of the C−Co bond with 2AP as substrate.[25] This may result in "trapping", and hence stabilization, of the RP in the dissociated state as Cbl^{II} and substrate radicals. One must be careful, however, of over interpreting the KIE observed in the current stopped-flow studies. Very similar results were obtained by Marsh and Ballou in 1998 for glutamate mutase,[36] but subsequent deuterium isotope effect studies by the same group using HPLC and electrospray mass spectrometry to analyze the 5′-deoxyadenosine produced from rapid chemical quench studies[39] have resulted in reinterpretation of the original data. The original KIE, elucidated from the second phase of the pre-steady-state transient representing C−Co homolysis from stopped-flow investigations, was found to be much smaller than previously thought (from ~28 to 2.4 ± 0.4). Alongside a large, inverse *equilibrium* isotope effect, this indicates that homolysis is more rate-determining than originally postulated.

For a similar AdoCbl-dependent enzyme, ribonucleoside triphosphate reductase, concerted homolysis and thiyl radical formation was proposed,[40] and although hydrogen is known to be abstracted from a protein residue in this case, the concerted mechanism is now considered less likely.[41] Analysis of electron spin−echo envelope modulation (ESEEM) data[42] has indicated that the 5′-deoxyadenosyl radical in EAL directly abstracts hydrogen from the substrate, and electron nuclear double

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(41) Chen, D.; Abend, A.; Stubbe, J.; Frey, P. A. *Biochemistry* **2003**, *42*, 4578.
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resonance (ENDOR),[24] EPR,[43] and electron-spin echo electron paramagnetic resonance (ESE-EPR)[44] studies have estimated the Co^{II} and substrate radical separation to be 10−12 Å, rendering a direct concerted reaction for EAL also unlikely. However, a relatively large separation such as this may serve to additionally stabilize against appreciable geminate recombination. Ke et al. compared X-band ESEEM spectra of enzyme-bound and free Cbl^{II}.[45] They identified a 14% increase in the isotropic hyperfine coupling of the axial 5,6-dimethylbenzimidazole ^{14}N in the bound case, indicating increased delocalization of the unpaired spin to the axial ligand, which would result in a decrease in the RP recombination probability.

RP stabilization by separation apparently continues through the rearrangement step. Further ESE-EPR and ESEEM analysis from Ke and Warncke[46] suggests that the C2 of the 2AE-derived product radical is further from Co^{II} than the C1 of the 2AP-derived substrate radical. Related X-band EPR[47] investigations reported accumulation of the product radical (through identifying unpaired spin density almost exclusively on C2) in the steady state with 2AE as substrate, indicating that, after H-abstraction, 1,2-rearrangement to the product occurs rapidly and irreversibly. More-recent work[48] has identified the product radical in question as the 1-aminoethanol-2-yl radical from 1,2-rearrangement as opposed to the ethanal-2-yl radical produced on direct ammonia elimination, with this mechanism also supported by ab initio calculations.[49] There is a rather different picture with 2AP, however, where the substrate radical accumulates and 1,2-rearrangement is thought to be rate limiting.[43] Warncke has suggested that the C5′ of the 5′-deoxyadenosine remains in close proximity of the substrate/product radicals species during rearrangement.[50]

Single turnover inactivation of EAL by hydroxyethylhydrazine (HEH) has been used to investigate early steps in the catalytic cycle of EAL, in particular the fate of ^2H originally on the inactivator molecule (i.e., $^2H_4$-HEH).[51] Gas chromatography mass spectra (GCMS) of the 5′-deoxyadenosine resulting from inactivation show peaks corresponding to mono-, di-, and tri-deuterated species. It seems therefore, that although H/^{2}H-abstraction from the substrate by the 5′-deoxyadenosyl radical is coupled to homolysis, it is also reversible, with accompanying exchange of HEH between the active site and solution. This analysis is consistent with the recent deuterium isotope studies for glutamate mutase[39] mentioned previously. Here, Cheng and Marsh attribute the multiphase nature of the pre-steady-state transients yielded from a previous study,[36] which closely resemble those acquired for 2AE and $^2H_4$-2AE at 5 °C in Figure 6, to sequential incorporation of H/^{2}H atoms into 5′-deoxyadenosine.

All of the factors outlined above can contribute to the acceleration of homolysis in the enzyme-bound state, which will in some way reflect the stability of the resultant RP(s). Yet, they do not comprehensively account for the enormous catalytic power of AdoCbl-dependent enzymes. The first-order rate coefficient for thermal C−Co bond homolysis for free AdoCbl and $AdoCbi^+OH^-$ ([adenosylcobinamide]$^+OH^-$) aqueous solution has been measured at 10^{-9} and 10^{-11} s^{-1}, respectively,[52] which would imply a rate increase of 10^{11}−10^{13} when bound to EAL alongside 2AE. There are currently two competing theories to explain such a substantial weakening of the C−Co bond: (i) mechanicochemical strain and (ii) electrostatic effects. The former has been around for some time now and claims that upon substrate binding, upward distortion of the corrin ring in AdoCbl (see Figure 2) results in sufficient steric repulsion between itself and the 5′-deoxyadenosyl substituent to achieve necessary bond weakening.[23] Very recent computational studies[53] have developed the idea of electrostatic catalysis to challenge this theory, postulating that the enzyme stabilizes the transition-state (as opposed to reactant state destabilization) by attaching a very polar 5′-deoxyadenosyl group to the leaving carbon, which becomes increasingly stable, through interactions with polar residues in the active-site, as the bond length increases. It is inappropriate for us to discuss the relative merits of each theory. However, be it through C−Co bond destabilization or RP stabilization, there is a strong preference for the dissociated state, an idea further supported by the removal of an MFE in the enzyme-bound state.

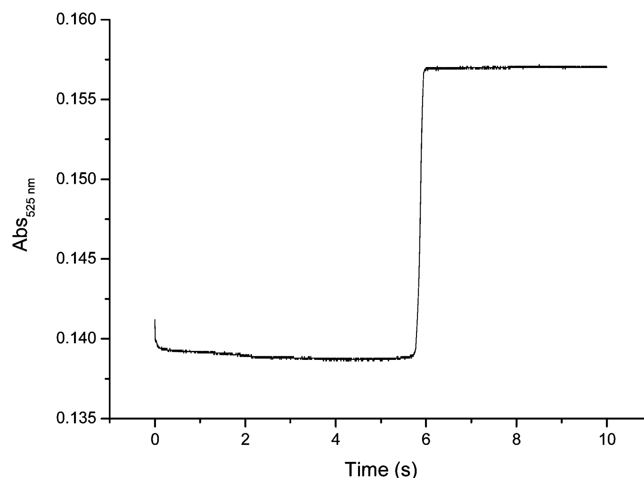

Figure 9. Averaged kinetic trace recorded at 525 nm for rapid mixing of EAL holoenzyme (*B20*, EAL ~13 μM, AdoCbl 20 μM) and 2AE (*B20*, 2.5 mM) at 25 °C. After a period of steady-state turnover, a second transient occurs.

2.2. Holoenzyme Post-Steady-State Transient. If, when conducting stopped-flow investigations with EAL holoenzyme and 2AE at 25 °C, data is acquired at 525 nm for up to 10 s after rapid mixing, a second transient is evident at ~ 6 s (see Figure 9). The spectral change here is in the opposite sense (i.e., an increase in absorbance at 525 nm) and has a comparable amplitude to the pre-steady-state transient (when, of course, the entire pre-steady-state signal is observable as in 2AE apoenzyme mixing at 25 °C and $^2H_4$-2AE/2AP holoenzyme mixing at 5 °C). It also transpires after a period of apparent steady-state turnover. These features indicate that when the substrate has been exhausted by enzyme turnover, the Cbl^{II} and 5′-deoxyadenosyl RP undergoes geminate recombination to reform AdoCbl, and that the increase in absorbance at this wavelength represents

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(53) Sharma, P. K.; Chu, Z. T.; Olsson, M. H. M.; Warshel, A. *PNAS* **2007**, *104*, 9661.

the conversion $Cbl^{II} \rightarrow Cbl^{III}$ (see Figure 1). Such a feature has been reported previously with 2AP as substrate[25] and has in fact been observed in the current study for all substrates and mixing regimes described above (at a range of points in time depending on substrate and conditions). Stabilization by the enzyme of the RP *against* geminate recombination has been offered as a potential explanation for the lack of observable MFE in *pre*-steady-state kinetic transients. However, is it possible that the *post*-steady-state recombination of the RP, which appears to occur readily, is the origin of the field sensitivity manifest in V_{max}/K_m?[14]

MFE investigations were conducted for this transient at 80 mT to test the feasibility of probing magnetic sensitivity at this point in the reaction cycle. Unfortunately, the position and slope of this transient is not consistent enough, shot-to-shot, for reliable MFE analysis to be made, even after normalizing the traces in terms of their *x* position. This may be due to a number of factors, not least the fact that the signal occurs after the steady-state turnover. Consequently, we can make no definitive statements about MFE (or lack thereof) at this point in the reaction cycle. Despite this apparent setback, it remains the most likely position of field sensitivity in the reaction cycle of EAL, and we shall therefore pursue this line of enquiry alongside new steady-state MFE investigations. This said, the fact that the transient is relatively sharp does suggest a tight binding affinity for 2AE, and magnetically induced changes at this point would therefore be unlikely to manifest in V_{max}/K_m. A tight 2AE binding affinity may also limit the precision with which K_m can be measured. Typically, the steady-state kinetics of EAL are monitored in a coupled assay using alcohol dehydrogenase and NADH,[14] and the few μM 2AE expected is likely to result in a small absorbance change ($\Delta\epsilon_{340\ nm}$ of NADH = 0.00622 $\mu M^{-1}\ cm^{-1}$) when using a standard optical path length. We will approach this problem in a significantly different way in our reinvestigations, which should overcome these issues with the conventional, potentially error-prone, steady-state assay method.

Concluding Remarks

We have demonstrated that, when it is rate-limiting in pre-steady-state kinetic studies with EAL, the net forward rate of C−Co bond homolysis is insensitive to the application of external magnetic fields in the presence of substrate. In doing so, we have not only identified what appears to be a substantial deuterium KIE (which could imply quantum mechanical tunneling during hydrogen transfer)[54] but also a higher level of complexity than previously assumed in the pre-steady-state transients. The implications are that homolysis is kinetically coupled to subsequent hydrogen abstraction from the substrate and, alongside subsequent conformational and chemical changes, the RP is stabilized by the enzyme in the dissociated state. The apparent discrepancy between the magnetic sensitivity of geminate recombination in free AdoCbl and the enzyme-bound cofactor in the presence of substrate reflects the extent of the homolysis back reaction in both cases. This, in turn, has implications for the role of RP stabilization in the massive increase in the rate of thermal C−Co homolysis observed in AdoCbl-dependent enzymes.

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Supporting Information Available: Additional spectra and experimental details. This material is available free of charge via the Internet at http://pubs.acs.org.

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