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Continuous Wave Photolysis Magnetic Field Effect Investigations with Free and Protein-Bound Alkylcobalamins

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Abstract: The activation of the Co−C bond in adenosylcobalamin-dependent enzymes generates a singlet-born Co^{II}−adenosyl radical pair. Two of the salient questions regarding this process are: (1) What is the origin of the considerable homolysis rate enhancement achieved by this class of enzyme? (2) Are the reaction dynamics of the resultant radical pair sensitive to the application of external magnetic fields? Here, we present continuous wave photolysis magnetic field effect (MFE) data that reveal the ethanolamine ammonia lyase (EAL) active site to be an ideal microreactor in which to observe enhanced magnetic field sensitivity in the adenosylcobalamin radical pair. The observed field dependence is in excellent agreement with that calculated from published hyperfine couplings for the constituent radicals, and the magnitude of the MFE (<18%) is almost identical to that observed in a solvent containing 67% glycerol. Similar augmentation is not observed, however, in the equivalent experiments with EAL-bound methylcobalamin, where all field sensitivity observed in the free cofactor is washed out completely. Parallels are drawn between the latter case and the loss of field sensitivity in the EAL holoenzyme upon substrate binding (Jones et al. *J. Am. Chem. Soc.* **2007**, *129*, 15718−15727). Both are attributed to the rapid removal of the alkyl radical immediately after homolysis, such that there is inadequate radical pair recombination for the observation of field effects. Taken together, these results support the notion that rapid radical quenching, through the coupling of homolysis and hydrogen abstraction steps, and subsequent radical pair stabilization make a contribution to the observed rate acceleration of Co−C bond homolysis in adenosylcobalamin-dependent enzymes.

Introduction

Adenosylcobalamin (AdoCbl, coenzyme B_{12}) is an active form of vitamin B_{12}, which acts as cofactor to a number of enzymes that employ closely controlled radical chemistry as a catalytic tool.[1−3] It is a diamagnetic, six-coordinate Co^{III} complex with an unusual covalent linkage to the C5′ of an equatorial 5′-deoxyadenosyl group. In all AdoCbl-dependent enzymes, substrate binding prompts rapid homolysis of this Co−C bond, generating a Co^{II}−adenosyl radical pair and thus initiating radical catalysis. When compared to thermal homolysis of the free cofactor in solution,[4] rate increases achieved by these enzymes are estimated to be in the region of 10^{11}−10^{13} (e.g., ref 5), yet the precise origin of such vast enhancements remains elusive.[6]

Biological radical reactions, in systems as varied as the photosynthetic reaction center,[7,8] enzymes,[9−11] and the avian magnetic compass,[12] have been probed by exploiting the unique features of the radical pair mechanism (RPM).[13−16] When pairs of radicals are created simultaneously, the unpaired spins are correlated depending on the multiplicity of the radical precursors. In AdoCbl-dependent enzyme activation, the Co^{II}−adenosyl RP (both spin 1/2) is singlet-born, and because each radical has different magnetic properties, the partially separated pair has the potential to undergo coherent spin-state interconversion between this singlet state (S) and the near degenerate triplet states (T_0, $T_{\pm1}$). The direct relevance of this process to AdoCbl-dependent enzymes was initially addressed by Chagovetz and Grissom, when, in 1993,[17] they reported a magnetically induced reduction in the Co^{II} quantum yield during continuous

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(1) Brown, K. L. *Chem. Rev.* **2005**, *105*, 2075–2149.
(2) Banerjee, R. *Chem. Rev.* **2003**, *103*, 2083–2094.
(3) Toraya, T. *Chem. Rev.* **2003**, *103*, 2095–2127.
(4) Hay, B. P.; Finke, R. G. *J. Am. Chem. Soc.* **1987**, *109*, 8012–8018.
(5) Jones, A. R.; Hay, S.; Woodward, J. R.; Scrutton, N. S. *J. Am. Chem. Soc.* **2007**, *129*, 15718–15727.
(6) Brown, K. L. *Dalton Trans.* **2006**, 1123–1133.
(7) Blankenship, R. E.; Schaafsma, T. J.; Parson, W. W. *Biochim. Biophys. Acta* **1977**, 461.
(8) Boxer, S. G.; Chidsey, E. D.; Roelofs, M. G. *Annu. Rev. Phys. Chem.* **1983**, *34*, 389–417.
(9) Taraban, M. B.; Leshina, T. V.; Anderson, M. A.; Grissom, C. B. *J. Am. Chem. Soc.* **1997**, *119*, 5768–5769.
(10) Jones, A. R.; Scrutton, N. S.; Woodward, J. R. *J. Am. Chem. Soc.* **2006**, *128*, 8408–8409.
(11) Buchachenko, A. L.; Kouznetsov, D. A.; Orlova, M. A.; Markarian, A. A. *Proc. Natl. Acad. Sci. U.S.A.* **2005**, *102*, 10793–10796.
(12) Rodgers, C. T.; Hore, P. J. *Proc. Natl. Acad. Sci. U.S.A.* **2009**, *106*, 353–360.
(13) Steiner, U. E.; Ulrich, T. *Chem. Rev.* **1989**, *89*, 51–147.
(14) Grissom, C. B. *Chem. Rev.* **1995**, *95*, 3–24.
(15) Woodward, J. R. *Prog. React. Kinet. Mech.* **2002**, *27*, 165–207.
(16) Woodward, J. R.; Foster, T. J.; Jones, A. R.; Salaoru, A. T.; Scrutton, N. S. *Biochem. Soc. Trans.* **2009**, *37*, 358–362.
(17) Chagovetz, A. M.; Grissom, C. B. *J. Am. Chem. Soc.* **1993**, *115*, 12152–12157.

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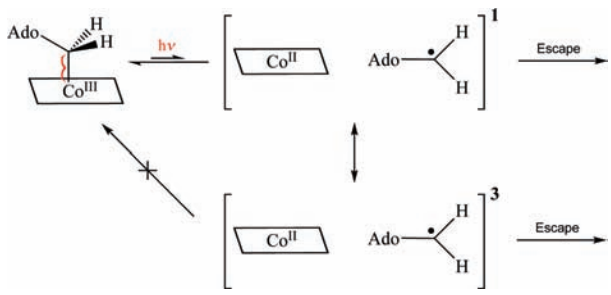
Scheme 1. Singlet−Triplet Spin-State Interconversion of the Diffusing Co^{II}−Adenosyl RP after Photolysis of AdoCbl in Solution[a]



[a] RP recombination is energetically highly unlikely from the triplet spin-state.

wave (cw) photolysis of free AdoCbl in solution. Much like enzyme activation, Co−C photolysis generates a singlet-born RP. Application of an external magnetic field (MF) to the system alters the energy of the $T_{\pm 1}$ sublevels relative to S and T_0, thus decreasing the mixing efficiency to these levels from S. The population of singlet RPs is therefore increased in relation to when no MF is applied, as is the RP recombination probability, hence the reduction in Co^{II} quantum yield. The photoinduced spin dynamics are represented in Scheme 1.

The same group reported magnetic field and isotope effects in the steady-state parameters V_{max}/K_m for AdoCbl-dependent ethanolamine ammonia lyase (EAL) with both protiated and perdeuterated ethanolamine.[18] Although reversible coenzyme activation (Scheme 2) was considered the likely position of MF sensitivity, it was subsequent presteady-state studies that claimed to pinpoint this step as being so.[19] However, we recently demonstrated the specific stopped-flow procedure employed in ref 19 to be insensitive to the magnetically induced changes reported, and that even when the methodology was modified accordingly, no magnetic field effect (MFE) was observed.[5] Yet the cw-photolysis MFE[17] for free AdoCbl was successfully reproduced, a result that prompted us to consider reasons for the removal of field-sensitivity in the presence of enzyme and bound substrate. A hypothesis of RP stabilization (against recombination) by radical separation and quenching was suggested and discussed as making a possible contribution to the homolysis rate enhancement achieved by AdoCbl enzymes.

Reported here are cw-photolysis MFE studies with both free and enzyme-bound AdoCbl and MeCbl in the absence of substrate. The intention has been to not only further explore rapid radical removal as a contributing factor to catalysis in AdoCbl-dependent enzymes, but also to confirm (or otherwise) the existence of MF sensitivity in the EAL holoenzyme.

Materials and Methods

The plasmid, pET-SEAL, encoding the small (32.0 kDa) and large (49.1 kDa) subunits of EAL from *Salmonella typhimurium* was overexpressed in *Escherichia coli* and purified essentially as described previously.[20] AdoCbl (coenzyme B_{12}, ≥98% Sigma), MeCbl (methylcobalamin, Sigma), TEMPO (free radical, sublimed, ≥99% Aldrich), and glucose oxidase (from *Aspergillus niger*, Sigma) were all used as purchased with no further purification. The triple-coated neodymium−iron−boron (NeFeB) permanent magnets were purchased from e-Magnetics UK.

cw-Photolysis MFE: Instrumentation and General Method. The construction and testing of the magnetic field effect stopped-flow spectrophotometer (MFESFS) employed in these studies have been described in full elsewhere.[10] Whereas in conventional single wavelength stopped-flow investigations the light is monochromated presample, the MFESFS was reconfigured for cw-photolysis MFE studies by monochromating postsample (Figure S1, Supporting Information). Thus, the sample is exposed to the entire emission spectrum of the 150 W Xe arc lamp, which acts as both photolysis and probe light source, and the transmitted light is then monochromated to 525 nm before passing to a photomultiplier tube. To restrict photodegradation of the protein during acquisition in the experiments involving EAL-bound cofactors, a < 400 nm cutoff filter was placed between the arc lamp and sample cell.

Static MFs of ≤80 mT were generated by passing a current of up to ∼40 A through a pair of coils built in-house from hollow copper wiring wound on an iron−alloy core machined from a decommissioned EPR magnet. These were kept cool by continuously passing water (plus antifreeze) at −5 °C through the central bore of the copper (a separate thermostatic water bath controlled the temperature of the reaction cell). The MF at the cell position was calibrated to both the current passing through the coils and the MF at the outside edge of the coils immediately prior to the data acquisition process. MFs of >80 mT were generated by placing pairs of NeFeB permanent magnets in the unoccupied light-guide ports during acquisition.

For each cw-photolysis (light intensity = 140 ± 1 μmol s^{-1} m^{-2}) experiment conducted under safe-light at 25 °C: absorbance measurements were acquired over a linear timebase at 525 nm; the first 4−5 stopped-flow shots were discarded; the homogeneous MF was applied to the sample position throughout the data acquisition period; each experiment was comprised of 5−6 field-on/field-off data acquisition pairs (the order of which was randomized) so that the MF dependence could be quantified where appropriate as relative rates.

cw-Photolysis MFE: Unbound AdoCbl and MeCbl. AdoCbl (26 μM) and MeCbl (10−40 μM) samples were prepared in degassed (bubbling $N_{2(g)}$ with continuous stirring for ∼1 h) 20 mM Hepes/67% w/w glycerol pH 7.5 containing 1 mM TEMPO. Excess oxygen was scavenged by glucose oxidase (∼13 units mL^{-1}) and glucose (10 mM) prior to loading into the MFESFS under safe-light.

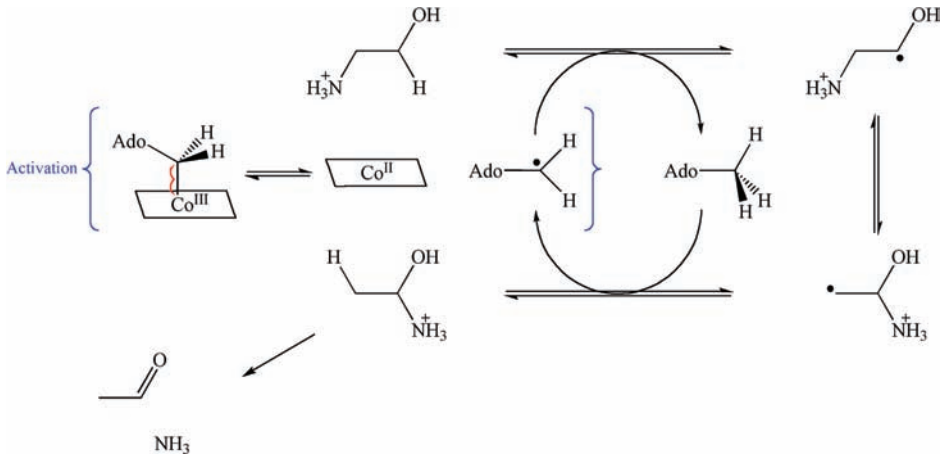
cw-Photolysis MFE: EAL-Bound AdoCbl and MeCbl. All samples were prepared in aqueous 20 mM Hepes/NaOH pH 7.5 saturated with air (oxygen concentration ≈ 0.23 mM[21]). EAL (∼5−26 μM active sites) was preincubated with AdoCbl or MeCbl (10 μM) and loaded into the MFESFS under safe-light. Some samples were prepared anaerobic, where appropriate, as for the free cofactor experiments. Photodiode array (PDA) spectra were acquired in each case, and the MF dependencies were established between 0 and 190 mT from single wavelength studies. To probe the protective effect of the enzyme, aerobic cw-photolysis data were acquired in the MFESFS as above for AdoCbl and MeCbl (10 μM), with an increasing concentration of EAL added for each repetition.

Results

Past cw-photolysis studies with naturally occurring B_{12} compounds have been conducted under anaerobic conditions and in the presence of TEMPO, to selectively scavenge the alkyl radical and allow an accumulation of the Co^{II} radical.[22,23] This strategy was also employed here with the cw-photolysis MFE of free AdoCbl in viscous solvent, and the resulting traces

(18) Harkins, T. T.; Grissom, C. B. *Science* **1994**, *263*, 958–960.
(19) Harkins, T. T.; Grissom, C. B. *J. Am. Chem. Soc.* **1995**, *117*, 566–567.
(20) Bandarian, V.; Reed, G. H. *Biochemistry* **1999**, *38*, 12394–12402.
(21) Yomo, T.; Urabe, I.; Okada, H. *Anal. Biochem.* **1989**, *179*, 124–126.
(22) Chen, E.; Chance, M. R. *Biochemistry* **1993**, *32*, 1480–1487.
(23) Natarajan, E.; Grissom, C. B. *Photochem. Photobiol.* **1996**, *64*, 286–295.

Scheme 2. Proposed Reaction Cycle of the EAL-Catalyzed Conversion of Ethanolamine to Acetaldehyde and Ammonia[a]



[a] Upon substrate binding, the enzyme causes rapid Co−C homolysis in the cofactor to generate a Co^{II}−adenosyl radical pair and thus initiate radical catalysis.

acquired at 525 nm in the absence and presence of a 190 mT MF are in Figure S2a of the Supporting Information. A large MFE is apparent in the rate of the initial downward phase, representing Co^{II} accumulation, and when the relative rate coefficient is calculated for each MF investigated, a saturating MF dependence is evident (Supporting Information Figure S2b). We are currently preparing a comprehensive experimental and theoretical account of viscosity-dependent MFEs in free B_{12} RP systems, and a full treatment of the various considerations specific to them will be contained therein.[24] The pertinent information to this study, however, is the perhaps unsurprising observation that the magnitude of the MFE increases in each case with increasing solvent viscosity, a result of a longer cage lifetime and hence both the potential for a longer separation time for enhanced spin-state mixing and an improved probability of RP reencounter.

In the experiments with EAL-bound AdoCbl (for a full description, see the Materials and Methods), neither an anaerobic environment nor the addition of TEMPO is necessary; the protein protects the radicals from side reactions with exogenous species such as oxygen (Figure 1a),[25−28] and the alkyl radical is quenched in the absence of TEMPO, presumably by hydrogen abstraction from the protein cage. As an increasing proportion of AdoCbl is bound to the protein, the absorbance increase at 525 nm, indicative of the efficient reaction between the Co^{II} radical and molecular oxygen to 5′-peroxyadenosylcobalamin (which then slowly decomposes to hydroxycobalamin), is replaced by an absorbance decrease as Co^{II} becomes protected by the active site. The relative extinction coefficients of the intermediates are also shown in Figure 1.[28,29] Figure 1b shows the kinetic trace and corresponding single exponential fit from the cw-photolysis of EAL-bound AdoCbl. The absorbance change at 525 nm corresponds to the loss of the $AdoCbl^{III}$ $\alpha\beta$-band on Co−C homolysis (see PDA spectra in the Supporting Information Figure S3). Although close-pair recombination is highly favored,[29,30] a fraction (RP quantum yield after 10^{-7} s in EAL $\approx 0.08 \pm 0.01$)[26] of the adenosyl radicals diffuse away from the Co^{II} with the possibility of then being removed. The observed accumulation of Co^{II} is a result of the repeated photolysis of this bond and gradual removal of the majority of alkyl radicals. As a consequence, continuous wave, as opposed to transient, photolysis experiments have enabled us to enhance the signal and detect potentially subtle magnetic perturbations.

Figure 1c represents the change in the cw-photolysis signal at 525 nm induced by the application of a 190 mT MF. The difference trace reveals that the MFE is mostly kinetic in origin, with single exponential fits of both data sets giving a relative rate[10] of 0.82 ± 0.01 (i.e., an 18% reduction in cw-photolysis rate in the presence of 190 mT). A slight quantum yield effect is also apparent in Figure 1a and c, which does imply competing reaction pathways. Although the origins of this effect are not known, alternative photodegradation processes are possible and kinetically feasible. The data in Figure 1c closely resemble those acquired under anaerobic conditions (see Supporting Information Figure S4), supporting the idea of a protein protective effect, which in turn confirms that these MFEs are a result of the protein-bound AdoCbl RP reaction dynamics.

Relative rates were calculated for all fields investigated, resulting in the MF-dependence plot in Figure 2. There is a clear saturation of the MFE (characteristic of the energetic removal of $T_{\pm 1}$ spin-states) at a relative rate and MF of around 0.82 and of 110 mT, respectively, and the overall dependence is almost identical to that of free AdoCbl in a 67% solution of glycerol (see Supporting Information Figure S2b).[24]

The observed MF dependence in Figure 2 is in excellent agreement with that predicted by the Lorentzian line shape, which was produced from the calculated $B_{1/2}$ value using the method described by Woodward and Vink,[31] and scaled vertically to fit the data. The $B_{1/2}$ value is the field strength at half saturation, and experimental values are commonly

(24) Jones, A. R.; Woodward, J. R.; Scrutton, N. S., in preparation.
(25) Rétey, J. *Angew. Chem., Int. Ed. Engl.* **1990**, *29*, 355–361.
(26) Robertson, W. D.; Warncke, K. *Biochemistry* **2009**, *48*, 140–147.
(27) Hogenkamp, H. P. C. *Biochemistry* **1966**, *5*, 417–422.
(28) Schwartz, P. A.; Frey, P. A. *Biochemistry* **2007**, *46*, 7284.
(29) Walker, L. A.; Shiang, J. J.; Anderson, N. A.; Pullen, S. H.; Sension, R. J. *J. Am. Chem. Soc.* **1998**, *120*, 7286–7292.
(30) Chen, E.; Chance, M. R. *J. Biol. Chem.* **1990**, *265*, 12987–12994.
(31) Woodward, J. R.; Vink, C. B. *Phys. Chem. Chem. Phys.* **2007**, *9*, 6272–6278.

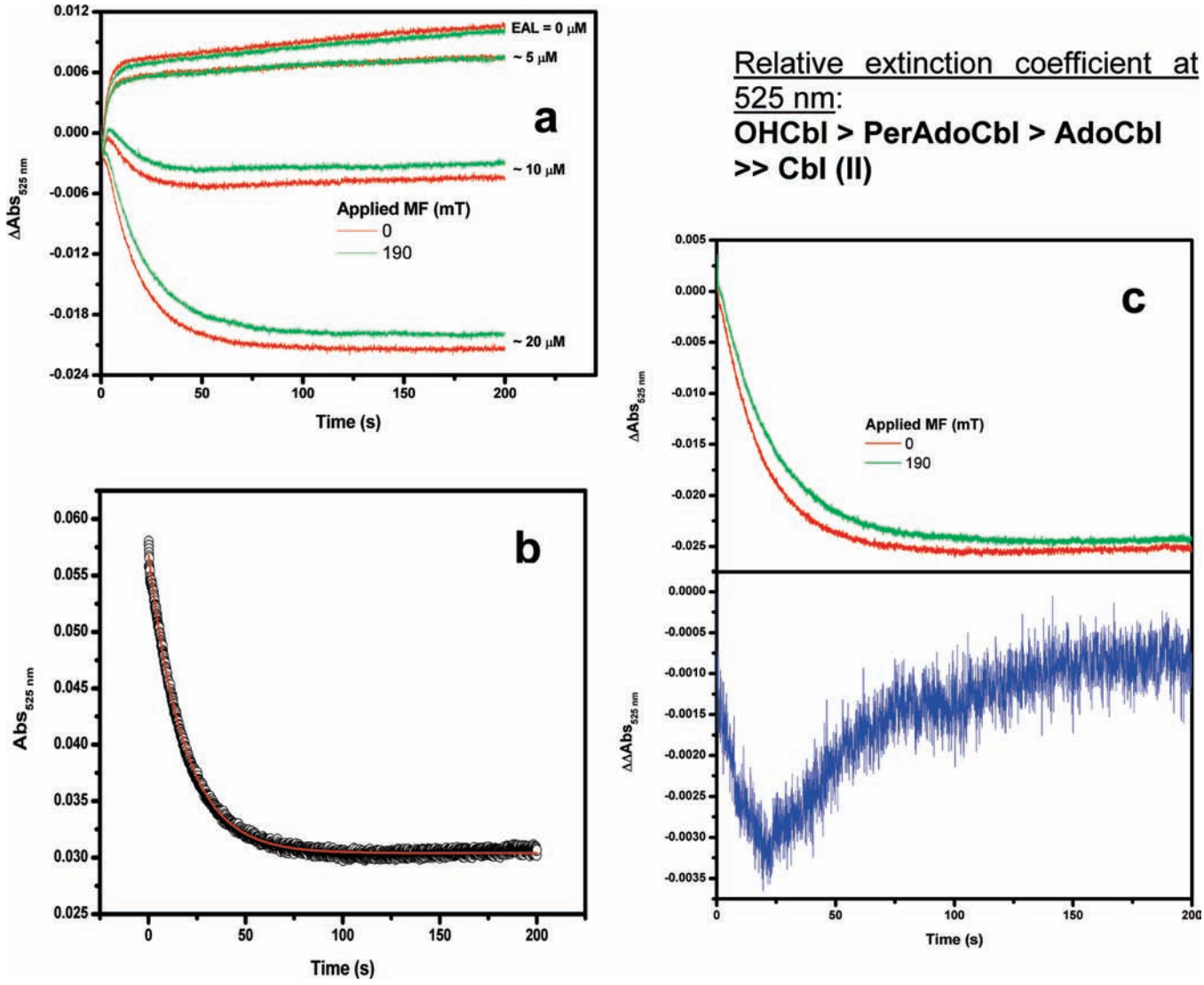


Figure 1. (a) The aerobic cw-photolysis of 10 μM AdoCbl in the presence and absence of a 190 mT MF titrating EAL apoenzyme (~0−20 μM active sites). The relative extinction coefficients at 525 nm of the various cobalamin species implicated are listed: OHCbl, hydroxycobalamin; PerAdoCbl, 5′-peroxyadenosylcobalamin; AdoCbl, 5′-adenosylcobalamin; Cbl(II), cob(II)alamin. (b) Data (○) and single exponential fit (red line) representing the aerobic cw-photolysis of the EAL holoenzyme (~26 μM active sites, 10 μM AdoCbl), acquired at 525 nm, 0 mT, and 25 °C. (c) Overlaid traces acquired at 0 and 190 mT, and corresponding difference trace (blue line); conditions as for (b). A clear magnetically induced change in photolysis rate is evident.

compared to theoretical values calculated using eq 1[32] from the average hyperfine couplings (eq 2)[33] of the radicals in the pair.

$$B_{1/2} = \frac{2(a_1^2 + a_2^2)}{(a_1 + a_2)} \quad (1)$$

where

$$a_i = \sqrt{\sum_j I_{ij}(I_{ij} + 1)a_{ij}^2} \quad (2)$$

Average hyperfine couplings of 2.71[34] and 15.9 mT[35,36] were calculated from published values for the adenosyl and $^{59}Co^{II}$ radicals, respectively, to give a $B_{1/2}$ of 28 mT.

The anaerobic cw-photolysis of free MeCbl in the presence of TEMPO and 67% glycerol results in a reduced cw-photolysis MFE when compared to AdoCbl (Supporting Information Figure S5a),[24] because the relatively small methyl radical will have an increased likelihood of cage escape. However, the relative distribution of initial MeCbl photoproducts is also wavelength-dependent. At an excitation wavelength of 400 nm, ~25% undergo homolysis directly, with the remaining ~75% forming a metastable state, whereas at 520 nm only the latter is observed.[37,38] In both instances, the metastable state either decays to the ground state or dissociates into radicals. These differing initial product distributions are pertinent because the cw-photolysis experiments with EAL-bound MeCbl were conducted with a < 400 nm cutoff filter, which was not in place for most of the free MeCbl experiments. However, when the

(32) Weller, A.; Nolting, F.; Staerk, H. *Chem. Phys. Lett.* **1983**, *96*, 24–27.
(33) Schulten, K.; Bittl, R. *J. Chem. Phys.* **1986**, *84*, 5155–5161.
(34) Bussandri, A. P.; Kiarie, C. W.; Van Willigen, H. *Res. Chem. Intermed.* **2002**, *28*, 697–710.
(35) Babior, B. M.; Moss, T. H.; Orme-Johnson, W. H.; Beinert, H. *J. Biol. Chem.* **1974**, *249*, 4537–4544.
(36) Baumgarten, M.; Lubitz, W.; Winscom, C. J. *Chem. Phys. Lett.* **1987**, *133*, 102–108.
(37) Walker, L. A.; Jarrett, J. T.; Anderson, N. A.; Pullen, S. H.; Matthews, R. G.; Sension, R. J. *J. Am. Chem. Soc.* **1998**, *120*, 3597–3603.
(38) Shiang, J. J.; Walker, L. A.; Anderson, N. A.; Cole, A. G.; Sension, R. J. *J. Phys. Chem. B* **1999**, *103*, 10532–10539.

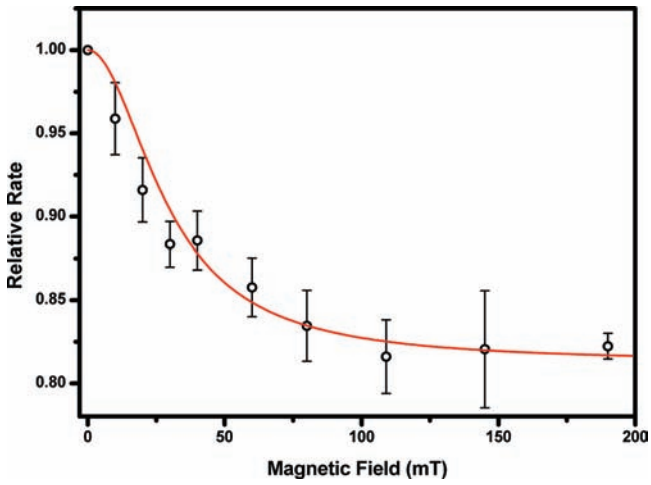


Figure 2. Magnetic field dependence (○) of the aerobic cw-photolysis relative rate of the EAL holoezyme. The data points are the mean of 5−7 acquisitions, and the error bars represent the standard deviation. The observed relative rate coefficient, extracted from the data in Figure 2b, decreases with increasing field strength until saturation at a relative rate of ~0.82. A Lorentzian line shape (red line) predicting the MF dependence was produced from the calculated $B_{1/2}$ value (see main text). The predicted and experimental dependencies are in good agreement.

cw-photolysis of free MeCbl is compared with and without this filter, the extent and kinetics are clearly affected (Figure S5b), but the magnitude of the MFE is not (Figure S5a). The aerobic cw-photolysis of EAL-bound MeCbl again reveals at least a partial protective effect of the enzyme against the side reaction with molecular oxygen (Supporting Information Figure S6); however, all magnetic sensitivity of the system in this case has been removed. The negative absorbance change at 525 nm (Figure 3a) representing the loss of $MeCbl^{III}$ is more complex than for EAL-bound AdoCbl, and the product spectrum from PDA data acquisition (see Supporting Information Figure S7) closely resembles that of aquocobalamin, with the appearance of a more prominent γ-band at ~350 nm characteristic of a weak axial ligand at the sixth position.[37]

Alongside faster quenching kinetics, and a much reduced amplitude than the equivalent studies with EAL-bound AdoCbl, these spectral features suggest more numerous and rapid RP quenching processes in the cw-photolysis of EAL-bound MeCbl. Although the traces acquired at 525 nm fit reasonably well to a double exponential function, and one might reasonably speculate as to their physical origin, they are, however, not precisely known. The lack of field dependence is therefore presented by difference spectra in Figure 3b.

Discussion

The original observation of MF sensitivity in AdoCbl-dependent EAL was a significant one,[18,19] as was the proposition that it is facilitated by the RPM. It represented the first experimental evidence, for a wild-type system with natural substrate, of a biological mechanism sensitive to the application of externally applied MF, and remains the only one with no obvious evolutionary advantage for such sensitivity. Under certain conditions, *Salmonella typhimurium* will utilize ethanolamine as its source of carbon and nitrogen, and EAL initiates the breakdown of this molecule.[39,40] The notion that a critical physiological process such as this may in some way be impaired by exposure to external fields raised the spectre of human pathology by environmental or occupational MF exposure.[41,42] Concerns were heightened by the observation of the so-called low field effect in chemical RP systems.[43] Here, MF as weak as ~50 μT,[44] which would otherwise make no thermodynamic impression on most biological systems, have the potential to influence the course or kinetics of (bio)chemical reactions by the removal of zero field, sublevel degeneracies.

It was also significant, therefore, that in a previous study[5] we were unable to reproduce the presteady-state MFE that claimed to identify the magnetically sensitive step as initial Co−C homolysis on substrate binding.[19] Although the MFE of free AdoCbl photolysis has been reproduced, the former result seriously called into question the existence of magnetic sensitivity when the cofactor is bound to the EAL apoenzyme. However, the observation of up to ~18% magnetically induced change in the cw-photolysis rate of EAL-bound AdoCbl reported here reveals the enzyme active site to be an ideal microreactor in which to observe MFEs in this RP system. When the MF dependence in Figure 2 is compared to that for free AdoCbl photolysis in viscous solvent (Figure S2b), the EAL active site clearly acts as a cage in which the RP are free to separate, but that increases the probability of RP reencounter (by limiting the probability of cage escape) and in doing so enhances the MF sensitivity. This is to be expected when one considers the protective effect of the apoenzyme against the radical side-reaction with molecular oxygen (Figure 1a), and the concept of negative catalysis.[25] It is clearly in the interest of the enzyme to not only keep foreign species out of the active site (although it is unlikely that EAL developed to specifically keep out oxygen), but also to retain the highly reactive primary carbon radical that is so intimately involved in catalysis.

In solution, a newly created "close" RP must diffuse apart sufficiently within a solvent cage for the electron exchange interaction to become small enough for S−T spin-state mixing to become energetically feasible. The fact that both the corrin ring (and therefore the Co^{II}) and adenosine are bound to the protein, alongside the protective surroundings restricting access to the active site by species such as oxygen and water, renders such conventional diffusive processes unlikely. The necessary freedom the RP requires to separate and reencounter to exhibit the observed MF dependence in Figure 2 appears to run contrary to the controlled environment of an enzyme active site. The idea that RPs can be immobilized within an enzyme, with a significant and constant exchange interaction such that spin mixing cannot occur, has been discussed previously.[45] *J*-resonance (the level-crossing of the S and T_{-1} sublevels) was suggested as a mechanism that might overcome this; yet it would not result in the field dependence observed in Figure 2. One might reasonably consider macromolecular dynamics[46] (Figure 4a), however, as a means by which large molecules such as proteins facilitate the requisite RP separation. Indeed, protein motion upon substrate binding in AdoCbl-dependent enzymes

(39) Chang, G. W.; Chang, J. T. *Nature* **1975**, *254*, 150–151.
(40) Roof, D. M.; Roth, J. R. *J. Bacteriol.* **1989**, *171*, 3316–3323.
(41) Ahlbom, A.; Cardis, E.; Green, A.; Linet, M.; Savitz, D.; Swerdlow, A. *Environ. Health Perspect.* **2001**, *109*, 911–933.
(42) Crumpton, M. J. *Philos. Trans. R. Soc. London, Ser. B* **2005**, *360*, 1223–1230.
(43) Timmel, C. R.; Henbest, K. B. *Philos. Trans. R. Soc. London, Ser. A* **2004**, *362*, 2573–2589.
(44) Maeda, K.; Henbest, K. B.; Cintolesi, F.; Kuprov, I.; Rodgers, C. T.; Liddell, P. A.; Gust, D.; Timmel, C. R.; Hore, P. J. *Nature* **2008**, *453*, 387–390.
(45) Brocklehurst, B.; McLauchlan, K. A. *Int. J. Radiat. Biol.* **1996**, *69*, 3–24.
(46) Henzler-Wildman, K.; Kern, D. *Nature* **2007**, *450*, 964–972.

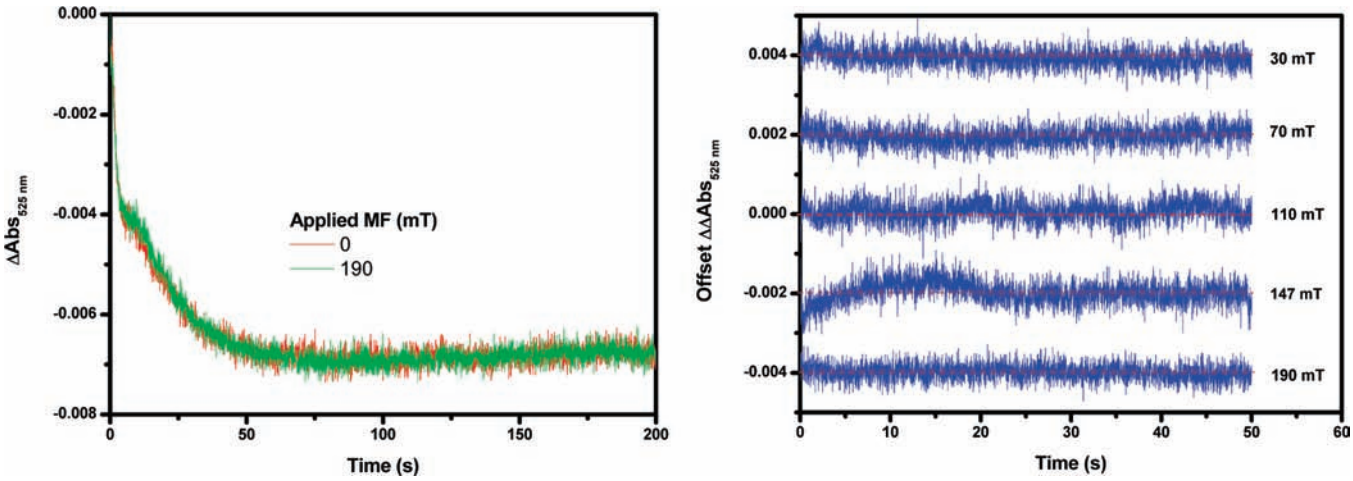


Figure 3. (a) Overlaid absorbance traces acquired at 525 nm for the aerobic cw-photolysis of MeCbl (10 μM) bound to EAL (~26 μM), in the presence and absence of an applied 190 mT magnetic field. (b) Difference spectra calculated from the cw-photolysis data acquired for EAL-bound MeCbl in the presence and absence of a range of magnetic fields. The original traces all passed through zero on the *y*-axis, but all except 110 mT are offset here for clarity. In sharp contrast to EAL-bound AdoCbl, no magnetic sensitivity is evident.

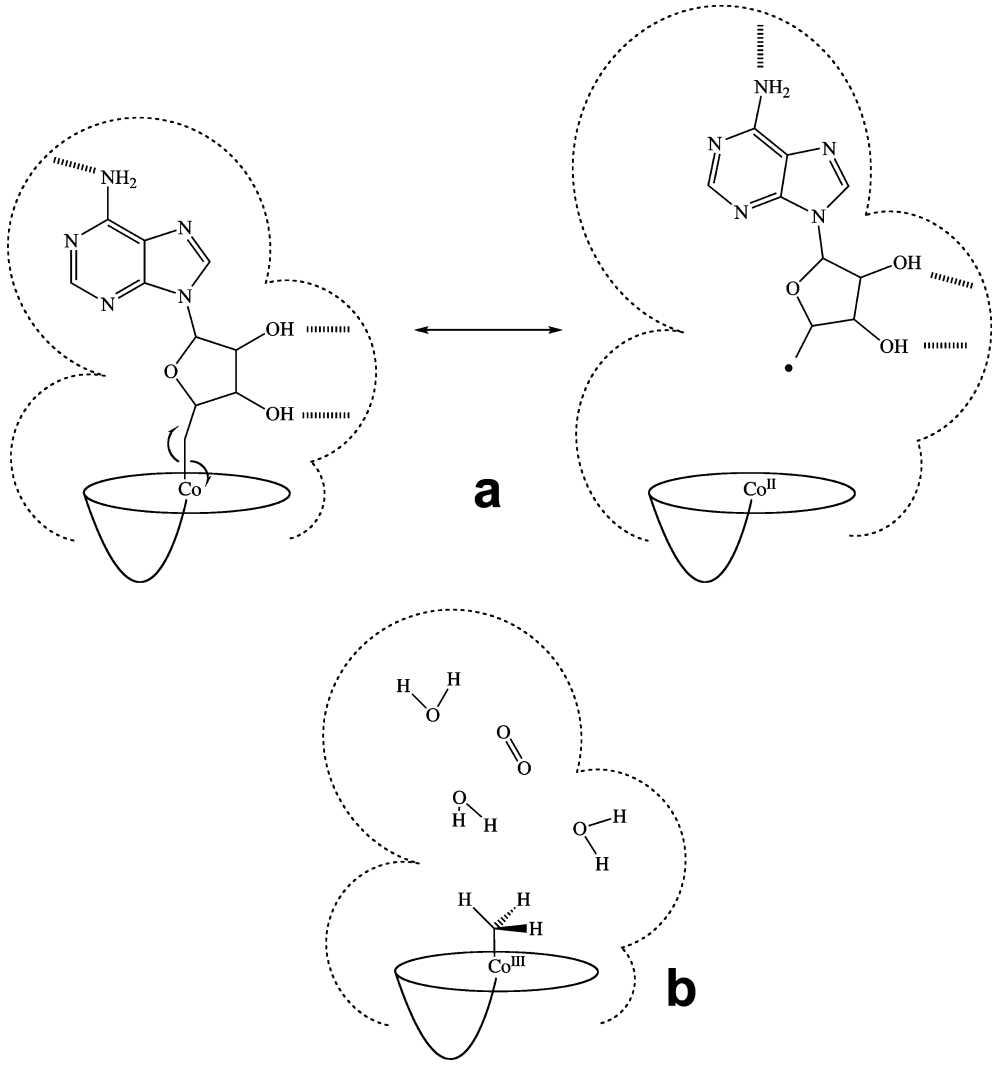


Figure 4. (a) A diagrammatical representation of the idea that protein "breathing" may facilitate RP separation and reencounter in the protected environment of the EAL active site. Possible hydrogen bonds of the adenine binding pocket are shown as dashed connections. (b) The smaller methyl in the adenine binding pocket may allow exogenous species such as water and oxygen access to the RP.

is considered an influential factor in the separation of the RP during catalysis. Further study is necessary to explore this idea.

In ref 5, we reasoned that the removal of the MFE was most likely a result of changes made to the RP reaction dynamics on

substrate binding, and the observations in Figures 1 and 2 confirm that assertion. We went on to discuss coupling of the Co−C homolysis to H-abstraction from substrate,[3,47,48] indicated by the large apparent kinetic isotope effect in the signal representing the conversion of AdoCbl to Cbl^{II},[5,49] and the implications this may have for limiting the extent of RP recombination necessary for spin-selective reactivity and hence the observation of MFEs. In other words, does rapid removal of the adenosyl radical by H-abstraction from the substrate and subsequent RP separation remove magnetic sensitivity of the RP, and does this reduction in recombination probability contribute to the homolysis rate enhancement achieved by EAL and other AdoCbl-dependent enzymes?

To assess the plausibility of this idea in B_{12} systems, we turn our focus to Figure 3. One might expect a similar augmentation of the MFE magnitude as a consequence of the active-site cage effect with EAL-bound MeCbl; yet all magnetic sensitivity is removed. The size, geometry, and reactivity of the methyl radical mean those that separate enough to overcome the electron exchange interaction, and thus enable S−T interconversion, are likely to be removed very rapidly, with little or no back reaction. In fact, the subnanosecond recombination that occurs readily in AdoCbl photolysis has not been observed in the equivalent investigations with MeCbl.[37,50] Excess energy from photolysis might plausibly propel the smaller methyl radical away from the cobalt,[51,52] rendering this process unlikely. Further, the methyl radical is almost planar; the unpaired spin therefore has access to the face of the molecule that lies opposite to the Co^{II}, thus increasing the probability of chemical quenching when compared to the relatively localized unpaired spin of the adenosyl radical,[50] especially if the quenching process involves H-abstraction from an active-site residue that necessarily exists within the RP "cage". This process is analogous to the early stages of catalysis in EAL and similar enzymes, where rapid radical quenching is instead mediated by ribosyl rotation about the *N*-glycosidic bond so that the adenosyl radical is optimally positioned to abstract a hydrogen from the substrate. The PDA spectra in Figure S7 implicate further radical quenching avenues are possible in EAL-bound MeCbl cw-photolysis. A weak equatorial ligand is evident, which suggests that water molecules might have access to the Co^{II} radical (Figure 4b), a feature that may also mean the methyl radical is able to escape the active-site cage completely.

The primary photochemistry of MeCbl, however, does exhibit some unusual behavior. Depending on the excitation wavelength,[38] varying extents of the initially excited MeCbl form a metastable photoproduct with a spectrum similar to that of cob(III)alamin compounds.[37] This is now thought to be a Co^{III}−methyl anion metal to ligand charge transfer (MLCT) complex,[53] which forms in competition with prompt homolysis, and then either decays to the ground state (~86%) or dissociates into radicals (~14%).[38] Although the initial formation of this state is independent of factors such as solvent dielectric (ε), its lifetime does appear to be influenced by the cofactor's environment, being more than twice as long in ethylene glycol ($t_{1/2}$ = 2.4 ns, ε = 37 at 25 °C) than in water ($t_{1/2}$ = 1.0 ns, ε = 78.5 at 25 °C).[51] Recent time-dependent DFT calculations also invoke this MLCT state, suggesting that it is the lowest excited singlet (S_1), and all photolysis, be it direct or via the metastable state, is thought to be mediated by the repulsive $^3(\sigma_{Co-C} \rightarrow \sigma^*_{Co-C})$ triplet state.[54,55] However, the phase of the MF-dependence plot for free MeCbl in viscous solvent (Figure S5a) is characteristic of a singlet-born RP, and the multiplicity of the radical precursors, in this case the excited MeCbl immediately prior to dissociation, must also be a singlet. This fact, however, does not preclude triplet-born pairs altogether, as long as a majority of singlet-born pairs are generated under the excitation conditions for these experiments. This would be possible if prompt homolysis and that which occurs via the MLCT state have different RP precursor multiplicities.

The data in Figures S5a and S5b show that the presence of a < 400 nm cutoff filter affects the extent and kinetics of MeCbl cw-photolysis, perhaps as a consequence of differing levels of the MLCT complex that are formed. However, the magnitude of the MFE is unaffected. The relative proportion of singlet and triplet-born RPs must therefore remain unchanged when wavelengths <400 nm are excluded (if, indeed, both are present). The implications of this for assigning spin multiplicities to the RP precursors, however, require further investigation by single-wavelength excitation studies. When considering the influence of cofactor environment on the lifetime of the metastable intermediate, perhaps most relevant to this study is the change in polarity between solution and enzyme. The difference in ε between a 67% glycerol/water solution (~55 at 25 °C) and that estimated for the interior of a folded protein (<4)[56,57] is significant, and one might therefore expect the lifetime of the MeCbl MLCT state to be extended accordingly when photolyzed within the EAL active site. However, the lifetime of this metastable state should not have a deleterious effect on the magnetically induced changes observed in the cw-photolysis signal. The MLCT complex is not radical in nature, and heterolytic cleavage is not observed in steady-state photolysis measurements.[37] The accumulated (not transient) signal observed here requires the gradual removal of a species bound to the Co after repeated photolysis; the obvious candidate for this is the methyl radical. The cw-photolysis signal, therefore, most likely represents the RP reaction dynamics. Furthermore, such a metastable state is also observed during AdoCbl photolysis when bound to glutamate mutase,[58] where none is evident in the free coenzyme under different solution conditions,[59] and is therefore likely in the magnetically sensitive photolysis of the EAL holenzyme.

X-ray crystallographic studies give some indication of how AdoCbl-dependent enzymes achieve the optimal relative positioning of the adenosyl radical and substrate. Adenine-binding

(47) Padmakuma, R.; Padmakuma, R.; Banerjee, R. *Biochemistry* **1997**, *36*, 3717–3718.
(48) Marsh, E. N. G.; Ballou, D. P. *Biochemistry* **1998**, *37*, 11864–11872.
(49) Bandarian, V.; Reed, G. H. *Biochemistry* **2000**, *39*, 12069–12075.
(50) Lott, W. B.; Chagovetz, A. M.; Grissom, C. B. *J. Am. Chem. Soc.* **1995**, *117*, 12194–12201.
(51) Sension, R. J.; Harris, D. A.; Cole, A. G. *J. Phys. Chem. B* **2005**, *109*, 21954–21962.
(52) Stickrath, A. B.; Carroll, E. C.; Dai, X.; Harris, D. A.; Rury, A.; Smith, B.; Tang, K.-C.; Wert, J.; Sension, R. J. *J. Phys. Chem. A* **2009**, *113*, 8513–8522.
(53) Cole, A. G.; Yoder, L. M.; Shiang, J. J.; Anderson, N. A.; Walker, L. A.; Banaszak Holl, M. M.; Sension, R. J. *J. Am. Chem. Soc.* **2002**, *124*, 434–441.
(54) Jaworska, M.; Lodowski, P.; Andruniow, T.; Kozlowski, P. M. *J. Phys. Chem. B* **2007**, *111*, 2419–2422.
(55) Lodowski, P.; Jaworska, M.; Andruniow, T.; Kumar, M.; Kozlowski, P. M. *J. Phys. Chem. B* **2009**, *113*, 6898–6909.
(56) Gilson, M. K.; Honig, B. H. *Biopolymers* **1986**, *25*, 2097–2119.
(57) Johnson, E. T.; Parson, W. W. *Biochemistry* **2002**, *41*, 6483–6494.
(58) Sension, R. J.; Harris, D. A.; Stickrath, A.; Cole, A. G.; Fox, C. C.; Marsh, E. N. G. *J. Phys. Chem. B* **2005**, *109*, 18146–18152.
(59) Yoder, L. M.; Cole, A. G.; Walker, L. A.; Sension, R. J. *J. Phys. Chem. B* **2001**, *105*, 12180–12188.

pockets have been identified from the crystal structures of methylmalonyl-CoA mutase,[60] diol dehydratase,[61] and glutamate mutase,[62] and although the structure of EAL is not yet solved, a similar pocket is implied from a model of the active-site containing EutB (49.1 kDa, α) subunit, alongside an aperture leading to the substrate binding site.[63] The "adenine-attracting" effect[3] upon substrate binding is considered by many as a major contributing factor to weakening the Co−C bond,[64] and it appears that subsequent ribosyl rotation about the *N*-glycosidic bond toward the substrate[3,65] alongside coupled radical quenching may go some way to completing the picture. Substrate binding in methylmalonyl−CoA mutase causes the adenosyl to move away from the Co^{II}, placing the C5′ sufficiently close to the substrate for hydrogen abstraction.[66] A similar "radical shuttling" is proposed in glutamate mutase, where a superposition of two adenosyl conformers in the crystal structure shifts the C5′ from above the Co^{II} toward the substrate binding site.[62] The existence of this pocket helps explain the more rapid quenching of the methyl radical in our current photolysis studies. The hydrogen-bonding network of the adenine-binding pocket, which anchors the adenine ring during ribosyl rotation, will further restrict the cage escape of the adenosyl, but not methyl, radical, and the latter will far from fill this space (Figure 4a and b). This may allow species such as water and oxygen access to the RP, and aside from their interactions with Co^{II}, methyl radicals are also known to efficiently react with molecular oxygen.[67]

Studies on the single turnover inactivation of EAL by perdeuterated hydroxyethylhydrazine indicated that, although kinetically coupled to homolysis, hydrogen abstraction is at least partially reversible,[68] a scenario also invoked by observed ^{15}N isotope effects in V_{max}/K_m.[69] Indeed, the opportunity for back H-transfer is there since Bender et al. identified the alkyl radical intermediate that accumulates during turnover by EPR (trapped by rapid-mix freeze−quench methods) as the more stable substrate radical,[70] not the product radical as previously reported.[71] The relative stability of the substrate over the adenosyl radical, however, does intimate that back hydrogen transfer is still less favored than the enzyme-mediated forward reaction, and the potential for subsequent RP recombination is likely restricted by the physical distancing of the adenosyl−Co^{II} RP during turnover.[72−74] We expect spin-correlation to be conserved during any reversible H-transfer steps, and an appreciable extent of back reaction from the Co^{II}−substrate RP to the intact AdoCbl would plausibly result in magnetic sensitivity of the system. This is supported by EPR data[70] that indicate the Co^{II}−substrate RP separation is adequate (∼8.7 Å) for the electron exchange interaction to have dropped sufficiently (−5.3 mT) to allow the average hyperfine couplings to facilitate spin-state interconversion.

If reversible H-transfer from and to the substrate is a necessary condition for this interconversion to take place, the same hydrogen will not necessarily be reabstracted from the adenosine methyl group. The effect would be to expose the unpaired electron spin to a new distribution of nuclear spins in the secondary adenosyl radical, and an altered precession frequency of this spin would result, analogous to that encountered as a consequence of degenerate electron exchange in photoinduced electron transfer reactions.[75−77] However, such an occurrence normally serves to modify the shape of the MF dependence and tends not to remove it completely. It does appear, therefore, that inadequate back reaction is responsible for MFE removal and that the dissociated state is reached rapidly.

In summary, we have demonstrated that the EAL active site is a suitable environment in which to observe enhanced MFEs in B_{12} RP reactions, but that when the rate of radical removal is sufficient, magnetic sensitivity is removed. These data also support an assertion we made in ref 5, based on the observation of a large kinetic isotope effect in the homolysis signal. We speculated that if the kinetic coupling of Co−C homolysis to H/^{2}H from the substrate, and subsequent RP stabilization, are sufficient to remove MFEs in EAL, such influences are likely to make at least a minor contribution to the rate enhancement affected by this class of enzyme. Any contribution would, of course, work in synergy with other influences from the protein upon substrate binding, which in turn may influence the magnetic sensitivity of the initial Co−C in a manner not related to RP stabilization. One such possibility would be a change in the nature of coordination between the lower axial ligand and the cobalt, such that incoherent spin relaxation processes compete with the coherent spin-state interconversion. However, the excellent agreement between the observed MF dependence in Figure 2 and the Lorentzian line shape calculated from the hyperfine couplings of the radicals in the pair suggests a minimal influence of relaxation during the RP reaction dynamics following photolysis. To account for a complete removal of MF sensitivity on substrate binding, the increase in relaxation rate would have to be significant.

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

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(60) Mancia, F.; Evans, P. R. *Structure* **1998**, *6*, 711–720.
(61) Masuda, J.; Shibata, N.; Morimoto, Y.; Toraya, T.; Yasuoka, N. *Structure* **2000**, *8*, 775–788.
(62) Gruber, K.; Reitzer, R.; Kratky, C. *Angew. Chem., Int. Ed.* **2001**, *40*, 3377–3380.
(63) Li, S.; Kurt, W. *Proteins: Struct., Funct., Bioinf.* **2006**, *64*, 308–319.
(64) Sharma, P. K.; Chu, Z. T.; Olsson, M. H. M.; Warshel, A. *Proc. Natl. Acad. Sci. U.S.A.* **2007**, *104*, 9661–9666.
(65) Banerjee, R. *Biochemistry* **2001**, *40*, 6191–6198.
(66) Mancia, F.; Smith, G. A.; Evans, P. R. *Biochemistry* **1999**, *38*, 7999–8005.
(67) Martin, R. B.; Noyes, W. A. *J. Am. Chem. Soc.* **2002**, *75*, 4183–4185.
(68) Bandarian, V.; Poyner, R. R.; Reed, G. H. *Biochemistry* **1999**, *38*, 12403–12407.
(69) Poyner, R. R.; Anderson, M. A.; Bandarian, V.; Cleland, W. W.; Reed, G. H. *J. Am. Chem. Soc.* **2006**, *128*, 7120–7121.
(70) Bender, G.; Poyner, R. R.; Reed, G. H. *Biochemistry* **2008**, *47*, 11360–11366.
(71) Warncke, K.; Schmidt, J. C.; Ke, S.-C. *J. Am. Chem. Soc.* **1999**, *121*, 10522–10528.
(72) LoBrutto, R.; Bandarian, V.; Magnusson, O. T.; Chen, X.; Schramm, V. L.; Reed, G. H. *Biochemistry* **2001**, *40*, 9–14.
(73) Canfield, J. M.; Warncke, K. *J. Phys. Chem. B* **2002**, *106*, 8831–8841.
(74) Canfield, J. M.; Warncke, K. *J. Phys. Chem. B* **2005**, *109*, 3053–3064.
(75) Knapp, E.-W.; Schulten, K. *J. Chem. Phys.* **1979**, *71*, 1878–1883.
(76) Staerk, H.; Treichel, R.; Weller, A. *Chem. Phys. Lett.* **1983**, *96*, 28–30.
(77) Batchelor, S. N.; Kay, C. W. M.; McLauchlan, K. A.; Shkrob, I. A. *J. Phys. Chem.* **1993**, *97*, 13250–13258.