The Magnetic Field Dependent Step in B_{12} Ethanolamine Ammonia Lyase Is Radical-Pair Recombination

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The rate of chemical and biological reactions with radical-pair (RP) intermediates can be altered by magnetic fields (B ≈ 10−3000 G) that perturb intersystem crossing (ISC) rates between the singlet and triplet spin states.[1] Recently, we reported that the steady-state kinetic parameter V_{max}/K_m for the B_{12} dependent enzyme ethanolamine ammonia lyase (EAL) from *Salmonella typhimurium* is decreased by 25% at 1000 G with unlabeled ethanolamine and 60% at 1500 G with deuterated ethanolamine (1,1,2,2-tetradeuterioethanolamine).[2] V_{max} is unaffected by an applied magnetic field in this range. This is the first example of a magnetic field dependent enzyme with known RP intermediates.[2]

Although the observation of magnetic field dependent steady-state kinetic parameters suggests the existence of a spin-correlated RP in an ES intermediate, it does not indicate the identity of the magnetic field dependent step(s). In this report, we have used rapid-scanning stopped-flow spectrophotometry as a function of magnetic field to demonstrate that one of the magnetic field dependent steps is nonproductive recombination of enzyme-bound 5′-deoxyadenosyl radical (5′-AdoCH$_2$•) and cob(II)alamin (Cbl^{II}).[3]

EAL catalyzes the conversion of ethanolamine to acetaldehyde and ammonia by promoting a 1,2-migration of the amine group to produce a hydrolytically-unstable carbinolamine (Figure 1).[4] All of the B_{12} enzymes that promote a 1,2-shift require adenosylcob(III)alamin (coenzyme B_{12}; $AdoCbl^{III}$) as an exogenous cofactor.[5] The purported role of $AdoCbl^{III}$ in these enzymes is to serve as a transient radical source to initiate catalysis. After the enzyme binds substrate and cofactor, homolysis of the C−Co bond in $AdoCbl^{III}$ produces enzyme-bound 5′-AdoCH$_2$• and Cbl^{II} (Figure 1).

Fast kinetic measurements were carried out with a rapid scanning stopped-flow spectrophotometer outfitted with an electromagnet surrounding the reaction chamber.[6−8] One complete absorbance spectrum was collected each millisecond to provide a spectral data file of absorbance vs wavelength and time. Global kinetic analysis and single-value decomposition were applied to each data file using the implementation of Matheson.[9] Various kinetic models were tested to find the best fit of the experimental data. In each case, the data were fitted best by a single-exponential model with offset.

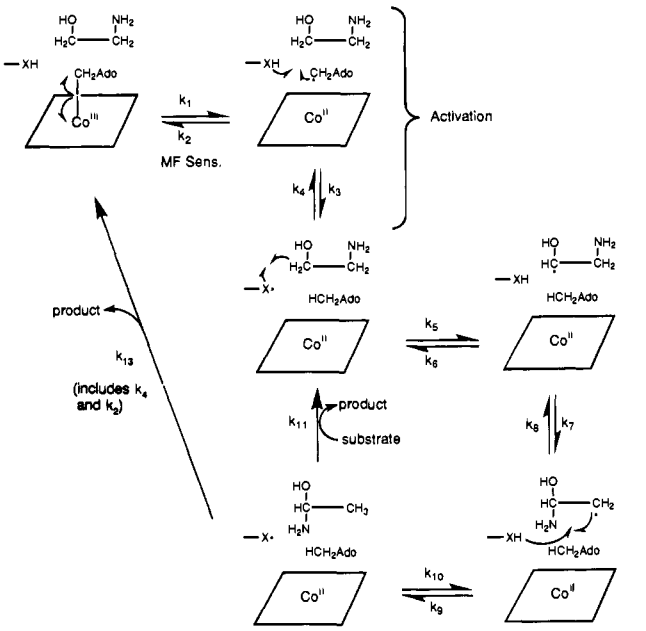

Figure 1. Mechanism of ethanolamine ammonia lyase. Homolysis of the C−Co bond produces the 5′-deoxyadenosyl radical and cob(II)alamin in the singlet spin state. The 5′-deoxyadenosyl radical abstracts H• from a group in the enzyme active site to generate the radical carrier, X•.[16] The protein radical, X•, abstracts H• from the substrate to generate the substrate radical. Migration of the amine group produces the carbinolamine radical that abstracts H• from XH to produce the hydrolytically-unstable carbinolamine and regenerate X•. Subsequent catalytic cycles can occur without re-forming the C−Co bond of adenosylcob(III)alamin. Implicit in step k_{13} are k_4 (XH formation from X•) and k_2 (C−Co bond re-formation), in addition to product dissociation. In this scheme, nonproductive recombination of the C−Co bond, k_2, is the MF-sensitive step.

Figure 2A shows a series of absorbance spectra that were obtained shortly after flow stopped. The kinetic trace of absorbance at 471 nm (ascribed to Cbl^{II}) is shown in Figure 2B. The disappearance and appearance of species A and B is first-order (Figure 2B, inset). Because the data set contains information on the time-dependent absorbance at all wavelengths, it is possible to calculate the absorbance spectra for species A and B that give rise to their respective kinetic traces.

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(3) A preliminary report of this work was presented at the 208th National Meeting of the American Chemical Society, Washington, DC, August 1994.

(4) For a thorough discussion of the kinetic mechanism of EAL from *Clostridium* sp., see: (a) Babior, B. M. In *B_{12}*; Dolphin, D., Ed.; Wiley: New York, 1991; Vol. 2, Chapter 10. (b) Kaplan, B. H.; Stadtman, E. R. *J. Biol. Chem.* **1968**, *243*, 1787, (c) 1794. (d) Babior, B. M.; Carty, T. J.; Abeles, R. H. *J. Biol. Chem.* **1974**, *249*, 1689. (e) Babior, B. M. *Acc. Chem. Res.* **1975**, *8*, 376. (f) Abeles, R. H.; Dolphin, D. *Acc. Chem. Res.* **1976**, *9*, 114. (g) Babior, B. M.; Krouwer, J. S. *Crit. Rev. Biochem.* **1979**, *6*, 35.

(5) For reviews on the mechanism of B_{12} enzymes, see: (a) Schneider, Z.; Stroinski, A. *Comprehensive B_{12}*; de Gruyter, Berlin, 1987. (b) Pratt, J. M. *Chem. Soc. Rev.* **1985**, *14*, 121. (c) Diziol, P.; Haas, H.; Retey, J.; Graves, S. W.; Babior, B. M. *Eur. J. Biochem.* **1980**, *106*, 211.

(6) Stopped-flow kinetic studies were carried out with an OLIS, Inc., RSM-1000 rapid-scanning spectrophotometer and an OLIS, Inc., USA-SF stopped-flow mixing device with 3 ms dead-time. The stopped-flow spectrophotometer was modified in order to place the thermostated reaction cuvette (1.7 cm optical path) in the air gap of a GMW Associates electromagnet (7.5 cm cylindrical poles; Model 5403). Absorbance spectra were recorded from 390 to 600 nm at a sampling rate of 1 kHz. The double-subtractive monochromator and rotating slit wheel design of the OLIS RSM-1000 ensures that the sample is dark 97% of the time. The sample is illuminated with spectrally-dispersed light. Irradiation is insufficient to photodissociate more than 0.01% of the cofactor during the 5−10 s measurement.

(7) The gene for ethanolamine ammonia lyase from *S. typhimurium* was cloned and expressed in *Escherichia coli* and purified as described (Faust, L. P.; Babior, B. M. *Arch. Biochem. Biophys.* **1992**, *294*, 50). Immediately before use, EAL was dialyzed against buffer T (100 mM Hepes, pH 7.45; 10 mM KCl; 5 mM dithiothreitol; 10 mM urea; and 10% v/v glycerol). The enzyme was concentrated to a total activity of 500 units/mL (unit = 1 μmol min^{-1} mg^{-1}), with a specific activity of 24−35 units/mg depending upon the preparation. The protein was soluble to 21 mg/mL.

(8) Solutions of EAL (24−35 units/mg in buffer T) and 24 μM $AdoCbl^{III}$/60 mM ethanolamine (unlabeled or deuterated in buffer T) were sparged with a gentle stream of hydrated Ar for 1 h. The solutions were transferred anaerobically to separate syringes. The syringe bath and reaction cell were thermostated at 24.0 °C. Absorbance vs time and wavelength data were collected for up to 10 s after mixing.

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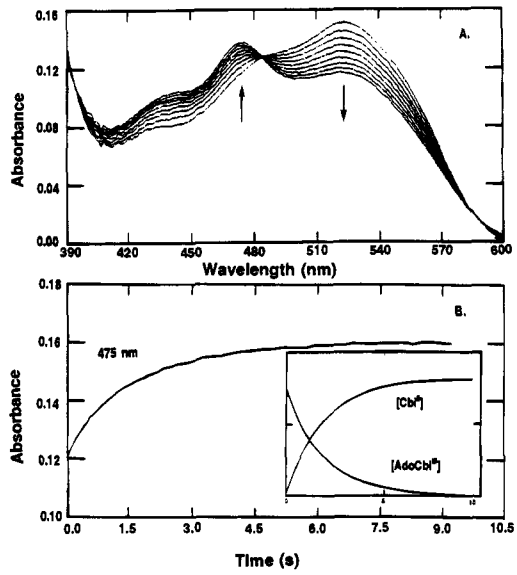

Figure 2. Stopped-flow kinetics of ethanolamine ammonia lyase from *S. typhimurium*. (A) Absorbance vs wavelength after flow has stopped. Arrows at 471 and 525 nm indicate absorbance maxima for Cbl^{II} and $AdoCbl^{III}$ formation, respectively. Each spectrum was collected in 1 ms. The top spectrum was acquired 100 ms after flow stopped, with each depicted spectrum taken at 100 ms intervals. Each data file contains 10 000 spectra. Contents of syringes prior to mixing (anaerobic, 24 °C): syringe 1, EAL, 24–35 units/mL in buffer T; syringe 2, 24 μM $AdoCbl^{III}$ and 60 mM ethanolamine in buffer T (100 mM Hepes, pH 7.45, 10 mM KCl, 10 mM urea, 10% v/v glycerol). (B) Kinetic trace at 471 nm. Inset shows the calculated rate of disappearance of $AdoCbl^{III}$ and the calculated rate of appearance of Cbl^{II} (note: axis on inset is arbitrary concentration scale and not absorbance).

From an examination of these calculated absorbance spectra, and a comparison with published absorbance spectra,[5a,10] species A and B can be assigned to $AdoCbl^{III}$ and Cbl^{II}, respectively. The spectrophotometrically-observed change can now be ascribed to the net conversion of $AdoCbl^{III}$ to Cbl^{II}.[11] The observed rate is the net rate for the appearance of Cbl^{II}, including Cbl^{II} formation (via k_1), as well as Cbl^{II} disappearance (via k_2 and k_3). Under single-turnover conditions, k_{13} can be ignored. Placing EAL and $AdoCbl^{III}$ in separate syringes slows the rate of Cbl^{II} appearance to $0.70 \pm 0.03\ s^{-1}$, as compared to the faster turnover rate of $\geq 300\ s^{-1}$ that is observed for *Clostridium* sp. EAL when enzyme and cofactor are in the same syringe prior to mixing with substrate.[11,12]

Figure 3 illustrates the rate of Cbl^{II} appearance vs magnetic field (MF). A 17% decrease in the net rate of Cbl^{II} appearance is observed at 500 G. The data shown were obtained within 1 h to achieve maximum precision of measurement. Triple repetition of the entire rate vs MF curve gave identical results (one additional data set is shown in the supplementary material). The biphasic curve that exhibits a minimum at 500 G is remarkably similar to the MFE curve for the photolysis of adenosylcob(III)alamin.[13] Further confidence in the small but significant magnetic field effect on the rate is obtained from replicate measurements that were obtained by alternating between 0 and 500 G (supplementary material).

No additional change in the rate of Cbl^{II} appearance is observed with perdeuterated ethanolamine as substrate. The absence of an isotope effect is in contrast to the large kinetic isotope effect that is observed on the steady-state kinetic parameters: $^{D}V_{max} = 6.8 \pm 0.2$ and $^{D}(V_{max}/K_m) = 5.4 \pm 0.4$.[3] This strongly suggests that the event being monitored by stopped-flow kinetics is the net rate for the conversion AdoCbl $\rightarrow$ 5′-$AdoCH_2^{\bullet}$ + Cbl^{II} and does not involve $H^{\bullet}$ abstraction from ethanolamine. In the steady-state kinetic parameters reported previously, the MF-induced decrease in V_{max}/K_m was enhanced to 60% by deuteration, and the maximum effect was shifted from 1000 G to 1500 G.[2] In those experiments, multiple turnovers produce a heterogeneous population of 5′-deuterated $AdoCbl^{II}$ because of the large isotope effect on washout of the deuterium.[14] Deuteration of the 5′-CH_2 group in the cofactor will decrease the commitment to catalysis for ethanolamine and perhaps alter the spin state of 5′-$AdoCH_2^{\bullet}$ by changing the small degree of hyperfine coupling from the methylene hydrogens. In the current experiment, no difference in the rate vs magnetic field dependence curves with unlabeled and deuterated substrate (or resulting isotopic distribution of cofactor) is observed because catalysis is limited to one turnover where only Cbl^{II} formation is being monitored. EAL is the first example of an enzyme that undergoes magnetic field dependent ISC. The stopped-flow kinetic studies reported herein identify one of the magnetic field dependent steps as nonproductive recombination of {5′-$AdoCH_2^{\bullet}$ Cbl^{III}} in the enzyme active site. This result suggests that other B_{12} dependent enzymes that utilize $AdoCbl^{III}$ to initiate radical chemistry during catalysis may also exhibit magnetic field dependent chemistry. The possibility of magnetic spin dependent chemistry in enzymes with radical intermediates has been considered before,[2b,15] but $AdoCbl^{III}$ dependent enzymes that rely on C–Co bond homolysis to generate a RP may be the best candidates for further investigation.

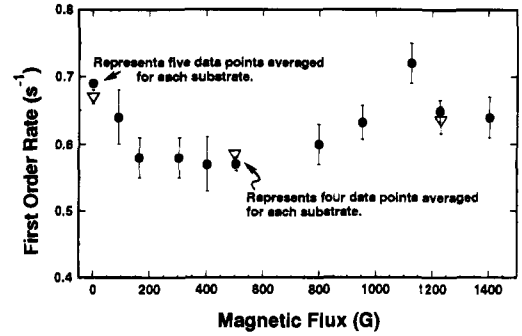

Figure 3. Magnetic field dependence of the first-order rate of Cbl^{II} appearance (see Figure 2 for reaction conditions). The observed rate includes any conformational change that is necessary to accommodate $AdoCbl^{III}$ binding. The rate of $AdoCbl^{III}$ disappearance and Cbl^{II} appearance was fitted to the integrated first-order rate equation. Symbols are as follows: unlabeled ethanolamine (▽); deuterated ethanolamine (●). Identical rates are observed with unlabeled and deuterated ethanolamine (see supplementary material for additional replicates).

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Supplementary Material Available: Replicate of rate vs magnetic field (cf. Figure 3) (2 pages). This material is contained in many libraries on microfiche, immediately follows this article in the microfilm version of the journal, and can be ordered from the ACS; see any current masthead page for ordering information.

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