Enzymatic Reaction Dynamics

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Is There a Dynamic Protein Contribution to the Substrate Trigger in Coenzyme B_{12}-Dependent Ethanolamine Ammonia Lyase?**

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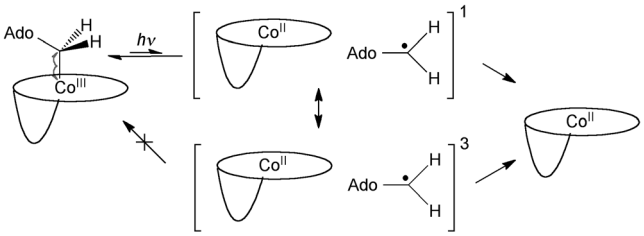
Coenzyme B_{12}, or 5′-deoxyadenosylcobalamin (AdoCbl), acts as cofactor to a number of enzymes from a range of organisms.[1,2] In all cases, the Co–C bond in the cofactor undergoes homolysis upon substrate binding, generating a singlet-born, Cbl^{II}/adenosyl radical pair (RP) and thus initiating radical-mediated catalysis. When compared to thermal homolysis of the free cofactor in solution,[3] rate increases achieved by these enzymes are in the region of 10^{11}–10^{13},[4–6] the precise origin of which is not yet fully understood. To date, the protein contribution to this catalytic power has been discussed either in terms of ground-state destabilization and a "strain" hypothesis,[7] or transition state stabilization by electrostatic factors.[8] However, there may be another contribution to consider—protein dynamics. Using a unique combination of spin-chemical and photochemical techniques we present evidence for coupling between RP reaction dynamics and protein dynamics in AdoCbl-dependent ethanolamine ammonia lyase (EAL).

The adenosyl radical has never been observed directly during turnover in an AdoCbl-dependent enzyme under ambient conditions. In EAL it is rapidly quenched by H-abstraction from the substrate to give the more stable substrate radical.[6,9,10] The Co–C bond can be photolyzed,[11] however, enabling investigation of the singlet-born geminate pair dynamics at room temperature both in the free and protein-bound cofactor. The spin-state of this RP can coherently interconvert between the singlet and the triplet sublevels (Scheme 1). If the geminate RP re-encounter, only those in the singlet state will recombine, whereas triplet pairs will separate again.[12] The extent to which the spin-states mix can be altered by the application of external magnetic fields (MFs).[13,14] For increasing MFs of moderate strength (tens to hundreds of mT) the $T_{\pm1}$ levels are gradually removed in energy and ultimately only S and T_0 interconvert. With a singlet-born RP, this process increases the relative S population and hence the probability of recombination. Such magnetic field effects (MFEs) have been observed in the rate of anaerobic, continuous wave (cw) photolysis of both free and EAL-bound AdoCbl.[15,16] Under continuous illumination the reactive adenosyl radicals are ultimately and irreversibly quenched to yield an accumulated Cbl^{II} signal, and the MFE manifests as a decrease in the apparent rate of this accumulation. The magnitude of the MFE was viscosity-dependent for unbound AdoCbl, the viscogen acting as a RP "cage". Likewise, the protein limits RP diffusion, thus enhancing the MFE over that observed in buffered water.[16] The magnetic sensitivity of homolysis is removed in EAL, however, when the Co–C bond is broken thermally by substrate binding.[6] The observation of a significant kinetic isotope effect in the pre-steady-state signal representing the conversion of $AdoCbl^{III}$ to Cbl^{II} suggests kinetic coupling of homolysis to subsequent H-abstraction from the substrate. The effect of this coupling is to rapidly quench the adenosyl radical, generating the substrate radical (which accumulates during turnover),[10] thus stabilizing against recombination of the geminate pair and removing the MFE. However, this does not preclude the possibility of MF-sensitivity in the recombination step after product release.[17]



Scheme 1. The reaction and spin dynamics of the separated Cbl^{II}/adenosyl radical pair following anaerobic photolysis of AdoCbl. During cw-photolysis, the adenosyl radicals are irreversibly quenched yielding an accumulated Cbl^{II} signal.

While the chemistry that immediately follows homolysis in the EAL-catalyzed reaction appears to favor RP dissociation, what of the protein contribution? The role of protein dynamics in enzyme function[18,19] is commonly probed by varying solvent viscosity (see, e.g. Ref. [20,21]) and assessing the extent to which this variation at the protein surface is transmitted to the active site. We therefore investigated the influence of viscosity on the cw-photolysis rate, and its MFE, of both free and EAL-bound AdoCbl at 298 K, using a specially configured MFE stopped-flow spectrophotometer.[16,22] The aim was to isolate the effect of protein dynamics on the geminate RP. Typical traces acquired at 525 nm are shown in the Supporting Information (Figure S1a and b).

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These data fit to a single exponential function, with the observed rate coefficient, k_{obs}, and MFE plot as a function of viscosity in Figure 1a and b.

Over the viscosity range studied, k_{obs} for free AdoCbl falls by 47% (no MF), and the effect is yet to saturate. On the other hand, k_{obs} for the EAL holoenzyme falls by 71% and the effect has mostly saturated, suggesting the active-site RP reaction dynamics are appreciably different from those in isotropic solution. Perhaps the most notable difference is that the magnitude of the MFE increases with viscosity in free AdoCbl (as expected), but remains constant at around 18% when protein-bound. This may tell us something about the timescales over which the viscogen is exerting influence in the protein. To observe MF-sensitivity, the geminate pair must have sufficient time: to separate for the exchange interaction $2J(r)$ to fall to less than the average hyperfine couplings of the radicals; for the spin-states to subsequently evolve; for the radicals to then reencounter.[13] These rates each depend on the radicals and their environment, but there is insufficient data available on this system to precisely determine them in full. However, a semiclassical description of spin motion in radicals,[23] does allow us to approximate the S–T mixing frequency [Eq. (1)].

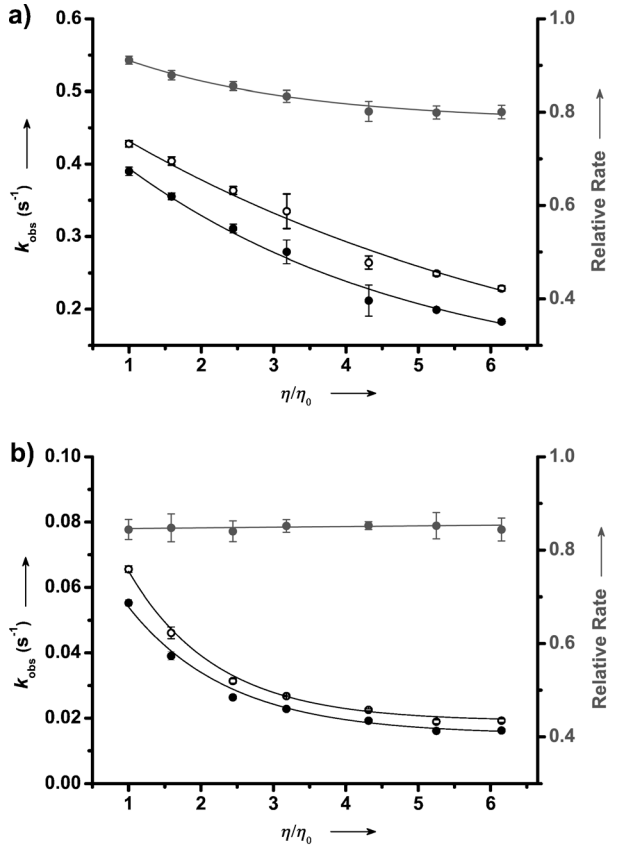


Figure 1. Observed rate coefficients, k_{obs}, for the cw-photolysis of a) AdoCbl and b) the EAL holoenzyme in the absence (empty circles) and presence (black filled circles) of an externally applied 190 mT MF, plot as a function of solvent viscosity. The MFE is also plot as a function of viscosity, and represented as a relative rate (k_{obs}[190 mT])/k_{obs}[0 mT]; gray filled circles).

$$\omega = g\beta\frac{H_1 - H_2}{\hbar} \quad (1)$$

where g is the free-electron g-value, β the Bohr magneton and H_i the average hyperfine couplings for each of the unpaired spins.[24] For the Cbl^{II}/adenosyl RP ($H_1 = 15.9$ mT and $H_2 = 2.71$ mT, respectively),[25–27] assuming zero applied field and a negligible $2J(r)$, $\omega = 2.32\ ns^{-1}$—a mixing time of 0.43 ns. In reality, the time necessary for a MFE of up to 20% to manifest will be greater than 0.43 ns when all the contributing factors are considered. It does suggest, however, that viscosity, and hence protein dynamics, is affecting the transient RP dynamics in the EAL active site on a timescale likely to be not much more than a few ns; for longer timescales one would expect some viscosity-dependence of the MFE. Furthermore, the effect of viscosity is almost certainly at the protein exterior and transmitted to the active site; if viscogen molecules access the active-site, or the adenosyl radical escapes and re-enters the active site intact (which itself is unlikely) one would likewise expect an effect on the MFE.

It has been previously reported that the majority of photogenerated radicals in this system recombine from the close pair within 10 ns without separating.[11,28–30] The quantum yield of separated radicals following photolysis of free AdoCbl is 0.20[31] to 0.24,[11] which drops to 0.08 and when bound to EAL,[32] and 0.05 in glutamate mutase.[29] To establish whether it is these close-pair dynamics that are viscosity-dependent after photolysis in the EAL holoenzyme, data were acquired over 3 ns after a 375 nm laser flash in a fs pump–probe spectrometer (see Supporting Information) in both buffered water and 30% sucrose (w/w). Selected transient difference spectra and single wavelength traces are contained in the Supporting Information (Figure S2a–d), alongside the equivalent data from unbound AdoCbl. The majority of the geminate pairs have recombined within 3 ns, and, much like in glutamate mutase, there is a marked delay in the appearance of the Cbl^{II} photoproduct when AdoCbl is EAL-bound.[29] There are also clear differences from free AdoCbl in the early-time spectra, which again resemble those in glutamate mutase where they are attributed to excited metal-to-ligand charge transfer states.[30] The transient difference spectra from the EAL holoenzyme were analyzed for principle components using single value decomposition (SVD). For each data set a single principle component was identified, the principle kinetic of which was fit to a sum of four exponentials (Figure S3). The kinetic data from the exponential fits given in Table 1 are comparable to those observed in glutamate mutase,[30] and appreciably different from free AdoCbl.[28] The value for k_1 in water corresponds to a time constant of 178 fs, which is near the instrument response function of ca. 170 fs, and may therefore explain why a sensible value for k_1 could not be extracted from the data acquired in 30% sucrose. There is a small but significant difference in the final phase, an 11% decrease in k_4 with increasing viscosity, whereas the rates preceding homolysis

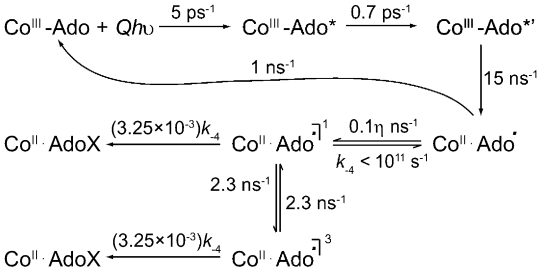
Table 1: Observed rate constants from SVD analysis of transient absorption data acquired after excitation (375 nm) of the EAL holoenzyme in both 0% and 30% sucrose (w/w, $\eta/\eta_0 = 3.18$).

	k_1 [ps^{-1}]	k_2 [ps^{-1}]	k_3 [ns^{-1}]	k_4 [ns^{-1}]
water	5.6 ± 0.5	0.66 ± 0.04	15.8 ± 0.8	1.21 ± 0.03
30% sucrose	–[a]	0.78 ± 0.12	14.0 ± 0.9	1.08 ± 0.03

[a] At the detection limit of the instrument (see main text).

(k_1–k_3) are not significantly affected. k_4 is thought to represent the kinetics of geminate pair recombination and initial radical separation.[28]

When comparing the effect of 30% sucrose in Table 1 to that in Figure 1b, there is an apparent disparity between the 11% decrease in k_4 and the 60% decrease in the cw-photolysis rate. However, neither is an intrinsic rate representing a discrete chemical step. We therefore modeled the photolysis reaction in Scheme 2 (full details in Supporting Information) in an attempt to collectively reproduce the



Scheme 2. Mechanism used to model both the transient absorption and cw-photolysis data; adapted from a published model[28] to include the separated RP reaction dynamics and spin-state mixing frequency. Rate coefficients are best estimates based on calculated and literature values.

viscosity-induced changes in the transient and cw-photolysis data. Q accounts for the difference in light intensity between the two experiments and η the influence of viscosity on the separation rate of the close RP. Using the values indicated for each step, both the transient and cw-data acquired in buffered water can be reproduced closely (Figure 2). With η set to 0.42 (i.e. a decrease in rate of 58%), the viscosity effect in each data set is also closely matched. According to this model, therefore, the initial movement (i.e. separation) of the newly formed RP in the EAL holoenzyme is slowed by increased solvent viscosity.

The influence of viscosity is therefore on the order of ps–ns, and timescales are critical in the context of protein dynamics. Different protein motions occur with different amplitudes and in different time domains[19] based on an energetic hierarchy of sub-states.[33] There are no significant differences at the overall fold level between substrate-free and substrate-bound crystal structures of EAL[34] (unlike methylmalonyl CoA mutase[35] and diol dehydratase[36]). This is consistent with the lack of viscosity-dependence of the RP dynamics that mediate magnetic sensitivity. Such separated pair dynamics in EAL occur over μs–ms,[32] which coincide with the timescales of larger domain motions in proteins. Faster motions include side-chain rotamers (ps–ns) and loop

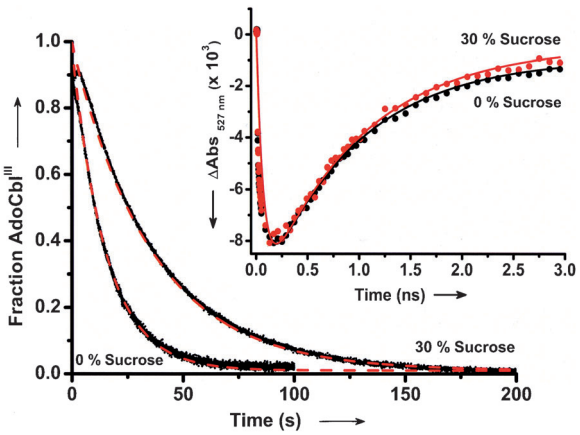
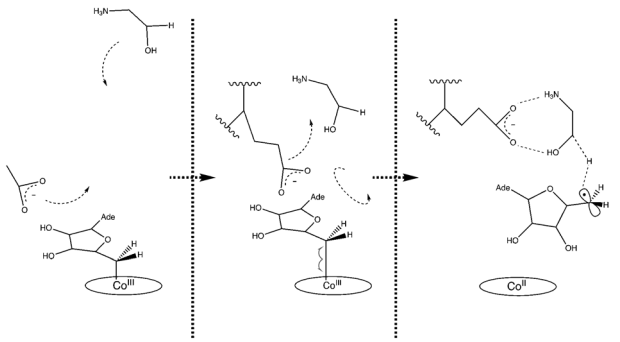


Figure 2. Normalized cw-photolysis experimental data (black lines) acquired at 525 nm in buffered water and 30% sucrose (w/w, $\eta/\eta_0 = 3.18$) alongside modeled data (red dotted lines). Inset: Scaled transient absorption data acquired at 527 nm after excitation (375 nm) of the EAL holoenzyme in buffered water (black circles) and 30% sucrose (w/w, $\eta/\eta_0 = 3.18$) (red circles) alongside modeled data (black and red lines, respectively).

motions (ns–μs), a combination of which could conceivably provide a link between the protein exterior and residues in contact with the coenzyme. The adenine ring forms at least two H-bonds with active-site residues (Serα^{247} and Gluα^{289}), and is in van der Waals contact with a further eight—one of which, Gluα^{287}, is known to be in direct contact with the ribose 2′-hydroxy group of the adenosine. Gluα^{287} is apparently mobile, being almost invisible in the electron density map in the absence of substrate, while forming two of the six hydrogen bonds with ethanolamine upon binding (spanning the OH and NH_2). It is also considered to play an intimate role in the "substrate trigger", contributing to steric strain, ribosyl rotation and stabilization of the RP state.[34]

This all raises the intriguing possibility of a dynamic contribution to the substrate trigger in EAL. Our data suggest it may be subtle, and therefore likely to happen in cohort with other factors. A subtle, cooperative effect is consistent with the conclusions of a recent study that used photolysis of the EAL ternary complex at 240 K, such that it was in a state of quasi-equilibrium with the Cbl^{II}/substrate RP (with an observed half-life for homolysis and H-abstraction of 8.3×10^2 s).[37] Under these conditions, no significant change was observed in either the AdoCbl absorption spectrum or in the reaction dynamics of the photoproducts upon substrate binding. To account for this, a model for substrate-initiated homolysis was proposed that involved a protein configuration coordinate orthogonal to the Co–C bond coordinate on a two-dimensional free energy surface. The authors speculated a diagonal path across the surface, with protein motion occurring simultaneously with, and thus guiding, the Co–C bond cleavage. To expand on this idea, the initial trigger upon substrate binding may involve the localization of the mobile Gluα^{287} (or similar mobile residue) in the substrate binding site (Scheme 3). It is feasible that such a relatively small change would be partially inhibited under the cryogenic conditions used in Ref. [37] and therefore difficult to detect in



Scheme 3. Proposed scheme of the dynamic substrate trigger for Co–C bond homolysis in EAL. Substrate binding draws a mobile residue, possibly Glu^{287}, towards the adenosine, initiating bond cleavage.

their experiments. The localization of this residue would then electrostatically initiate homolysis, perhaps also guiding bond cleavage towards the substrate. Such close control would be analogous to that observed in glutamate mutase, where an active-site glutamate is involved in "shuttling" the adenosyl radical towards the substrate, a process also likely to occur in methylmalonyl CoA mutase.[38] Homolysis is then coupled to H-abstraction, further favoring dissociation and stabilizing the RP as the Cbl^{II}/substrate radical. For AdoCbl-dependent mutases, it has even been suggested that these steps follow a concerted pathway.[8,39] A further point to consider is that protein motions in enzymatic H-transfer have been suggested to aid in barrier compression,[40] enhancing the probability of both tunneling and over-the-barrier transfer. The result would be an increase in H-transfer rate, further favoring RP dissociation in AdoCbl-dependent enzymes.

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