Reaction Dynamics

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Protein Motions Are Coupled to the Reaction Chemistry in Coenzyme B_{12}-Dependent Ethanolamine Ammonia Lyase**

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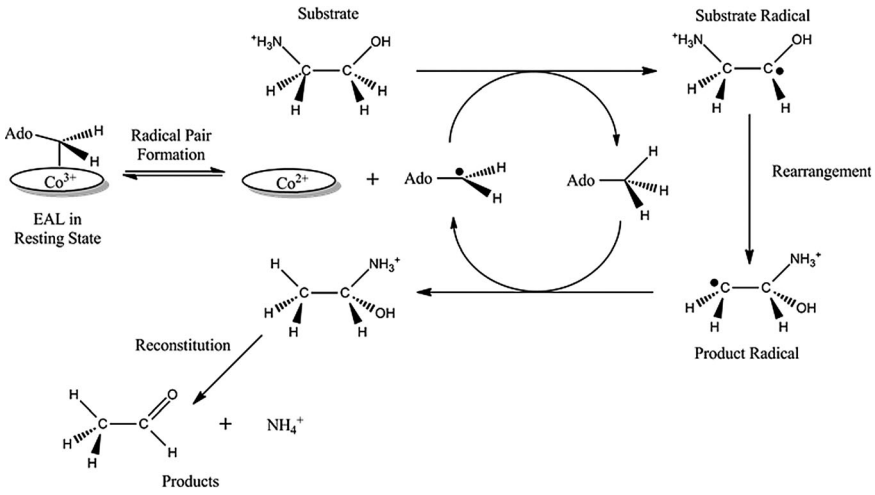
Coenzyme B_{12} (5′-deoxyadenosylcobalamin, AdoCbl)-dependent enzymes facilitate radical rearrangement reactions in a variety of organisms.[1,2] Upon substrate binding, the unique covalent Co–C bond at the center of AdoCbl is broken homolytically, yielding a singlet-born 5′-deoxyadenosyl and cob(II)alamin radical pair.[1,2] Remarkably, thermal homolysis rates achieved by these enzymes are about 10^{12} times greater than that of the free cofactor in solution.[3–6] Recent data indirectly suggest protein motions may be coupled to the reaction chemistry,[7] a hotly debated issue in modern enzymology.[8,9] Herein, from studies using both laser flash photolysis and stopped-flow infrared (IR) techniques, we present the first direct evidence for a protein motion that correlates with coupled Co–C bond homolysis and H abstraction from the substrate in ethanolamine ammonia lyase (EAL) from *Salmonella enteric*a.



Scheme 1. Current reaction mechanism for EAL adapted from Ref. [11]. Substrate binding initiates homolysis, with the 5′-deoxyadenosyl radical generating the substrate radical by H abstraction, followed by rearrangement to the product radical, which then dissociates to the acetaldehyde and ammonia.

EAL is in many ways typical of AdoCbl-dependent enzymes that catalyze isomerization and elimination reactions.[7] After Co–C homolysis, the adenosyl radical abstracts hydrogen from ethanolamine, followed by radical rearrangement and dissociation to products (Scheme 1).[10,11] In theory, the only steps resolvable by UV/Vis spectroscopy are the interconversion of cob(III)alamin and cob(II)alamin at the beginning and end of turnover. However, much like methylmalonyl-CoA mutase[3] and glutamate mutase,[4] homolysis of the Co–C bond and H abstraction from the substrate are kinetically coupled in EAL, such that kinetic isotope effects are observable in the signal representing homolysis.[5,11] This coupling has been shown to remove magnetic field-sensitivity from the Co–C homolysis in EAL, by limiting the extent of geminate recombination, and thus favoring the dissociated state.[5,12] EPR spectroscopy has also shown the substrate radical to accumulate during turnover and that it is separated from the Co^{II} by 8.7 Å,[13] further stabilizing against geminate recombination. Although these factors no doubt contribute to the catalytic power of EAL, there remains little evidence for an explicit role for the protein. Unlike methylmalonyl-CoA mutase,[14] diol dehydratase[15] and ornithine 4,5-aminomutase,[16,17] there is yet no evidence of any large-scale motions associated with substrate binding in EAL.[18] Instead, Robertson et al. have proposed a more subtle, cooperative effect from the protein,[19] and, in support of this, we have recently published evidence that implicates a dynamic contribution from an active-site residue.[7]

EAL exhibits three broad amide absorptions in the mid-IR spectrum (Figure 1a, solid line) with the amide I band (1625–1675 cm^{-1}) representing the most intense signal. AdoCbl also contributes to the signal in this region (Figure 1a, dashed line), with a broad band centered around

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Supporting information for this article, including experimental details, is available on the WWW under http://dx.doi.org/10.1002/anie.201202502.

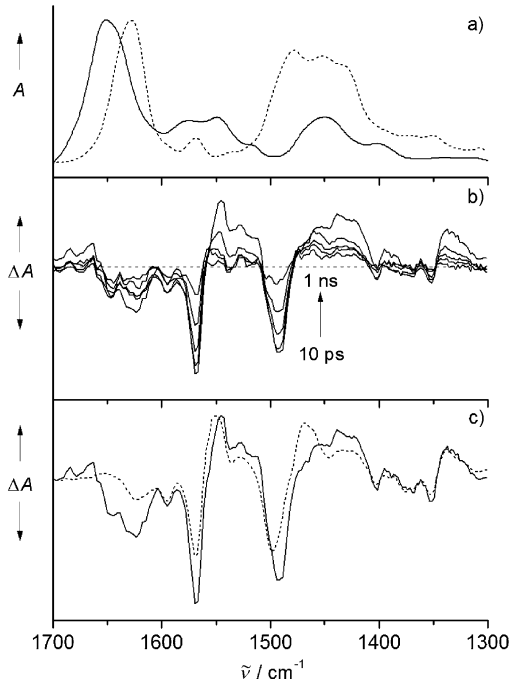

Figure 1. a) Ground-state FTIR spectra of EAL holoenzyme (solid line) and AdoCbl (dashed line), normalized to peak absorbance. b) Selected TRIR difference spectra from EAL holoenzyme. c) Overlaid 10 ps TRIR difference spectra from the EAL holoenzyme (solid line) and AdoCbl (dashed line). The EAL spectrum varies in the region around 1450 cm^{-1}, a bleach at 1650 cm^{-1}, and a transient at 1661 cm^{-1} (A = absorbance and $\tilde{\nu}$ = wavenumber).

1625 cm^{-1} (likely owing to amide groups of the corrin ring). AdoCbl also absorbs strongly in the fingerprint region (1300–1500 cm^{-1}), where many vibrational modes overlap.[20] IR spectroscopy has been used to good effect in AdoCbl-dependent systems previously; however, without time resolution.[21,22] In an attempt to directly assess the influence of protein motions on the reaction chemistry in EAL, we have employed two dynamic IR methods. A stopped-flow sample handling unit coupled to a Fourier transform IR spectrometer (SF-FTIR) allowed spectral changes to be monitored following rapid mixing of reactants.[23,24] The ultrafast time-resolved IR (TRIR) spectrometer, on the other hand, employs a pump–probe setup and enabled spectral measurements with femtoseconds time resolution after photoexcitation of the sample.[25] TRIR has been previously employed to probe cofactor–protein interactions in systems such as bacteriorhodopsin.[26,27] As will become evident, such interactions are subtle during photolysis of EAL-bound AdoCbl. In this case, therefore, the TRIR data serve to illustrate the distinction between Co–C homolysis by light and by substrate binding. Overall signals in the spectral window measured by TRIR are very different to those measured by SF-FTIR experiments. We conclude that substrate binding, therefore, has a considerably stronger impact on the protein, which appears to move in conjunction with the reaction chemistry.

Comparing TRIR measurements of free and EAL-bound AdoCbl provided the opportunity to determine whether there is interaction between the protein and cofactor following photoexcitation and Co–C bond photolysis. Our previous TRIR data from free AdoCbl showed a sub-picosecond appearance of signal, which then decays over about 2 ns.[20] Kinetic analysis of these data fit to the sum of four exponentials, in close agreement with similar UV/Vis kinetic measurements.[28] Selected TRIR difference spectra acquired between 1 ps–2 ns from the EAL holoenzyme (Figure 1b) are compared to data from free AdoCbl in Figure 1c. Of particular note are the appearance of a ground-state bleach at about 1650 cm^{-1} and a transient signal at about 1661 cm^{-1} in the EAL measurements, both of which coincide with the protein amide I band and decay with similar kinetics to the signal from the cofactor. There are also differences around 1425–1475 cm^{-1}, which coincide with the amide II bands of deuterated proteins.[29] The lower concentration of the protein sample (about 200 μM) compared to the free cofactor (about 8 mM) meant that we had to average more spectra to acquire good quality data, and therefore collected fewer data points. Consequently, the principle kinetics from single value decomposition analysis only fit to the sum of two exponentials as opposed to the four exponential fit to the AdoCbl data (see Figure S1 and Table S1 in the Supporting Information). Although protein binding is known to affect the kinetics of AdoCbl photolysis,[7,30,31] the most marked effect is on the excited state processes, which have not been fully resolved here. However, values for k_3 and k_4—which represent radical pair formation and dynamics—are of the same order across the available TRIR and UV/Vis data sets (see Table S1 in the Supporting Information).

Our SF-FTIR spectrometer measures spectra every 72 ms, and therefore acquisition of a full data set of the pre-steady states for ethanolamine or 2-aminopropanol (slow substrate) was not possible. Therefore, initial measurements focused on ethylene glycol (EG), a quasi-substrate that initiates homolysis and H abstraction to give cob(II)alamin and the substrate radical (EG$^{\bullet}$), and produces acetaldehyde as product (P). However, it renders EAL inactive after a single turnover by coordination of a different ligand (X) to the 6th position of the Co center [Eq. (1)].[32]

$$(\mathrm{Co^{III}}\text{-Ado}) + \mathrm{EG} \xrightarrow{\text{slow}} (\mathrm{Co^{II}} \cdot \mathrm{AdoH}) \cdot \mathrm{EG}^{\bullet} [\rightarrow (\mathrm{Co^{III}}\text{-X} \cdot \mathrm{Ado}) + \mathrm{P}] \quad (1)$$

UV/Vis absorbance changes were initially acquired by stopped flow at 525 nm and show the conversion of cob(III)alamin to cob(II)alamin over 15 s (see Figure S2 in the Supporting Information). Reversion to cob(III)alamin (the part of Equation (1) in squared brackets) is not observed in this time. Normalized difference spectra from similar SF-FTIR experiments were calculated by subtracting the first clean spectrum—which is dominated by the intact holoenzyme and free substrate—from the subsequent time points (Figure 2a). The most striking spectral features are at 1650 and 1661 cm^{-1}, where the signals evolve at similar rates. An example trace at 1661 cm^{-1} illustrated in Figure 2b was fit to the sum of two exponentials, with rate constants $k_1 = (1.15 \pm 0.18)$ s^{-1} and $k_2 = (0.27 \pm 0.04)$ s^{-1}. Interestingly, these peaks correspond closely in position to the additional signals observed in the TRIR data from photolysis of the EAL

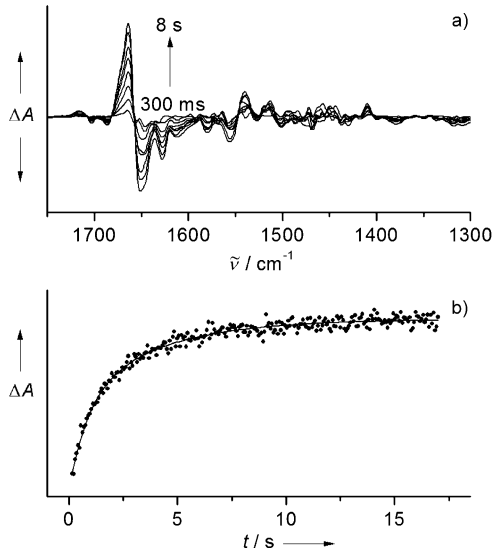

Figure 2. a) Selected SF-FTIR difference spectra of 150 μM EAL versus 800 mM ethylene glycol. The major signal has peaks at 1661 and 1650 cm^{-1}. b) Example SF-FTIR trace at 1661 cm^{-1} for 150 μM EAL versus 800 mM ethylene glycol, fit to the sum of two exponentials.

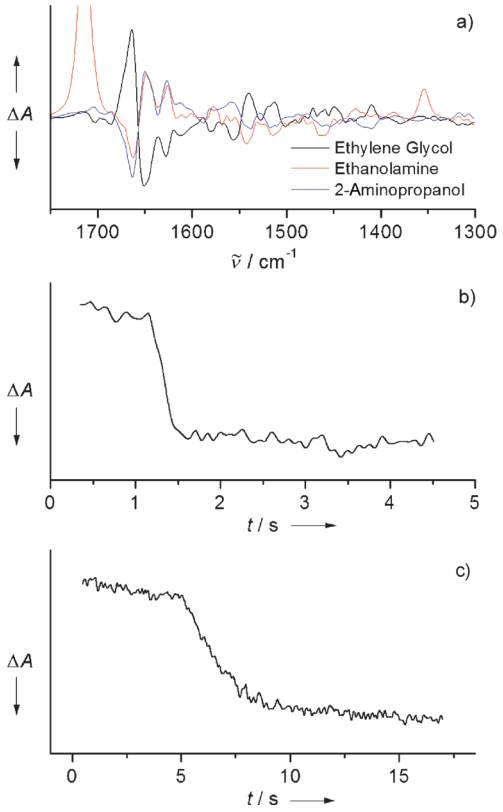

Figure 3. a) Overlaid, final time point SF-FTIR difference spectra relative to the 144 ms spectrum for ethylene glycol (black) and ethanolamine (red), and relative to the 482 ms spectrum for 2-aminopropanol (blue). b, c) Example SF-FTIR traces at 1661 cm^{-1} for 150 μM EAL versus b) 10 mM ethanolamine and c) 400 μM 2-aminopropanol. These traces are comparable with the equivalent UV/Vis stopped-flow traces (Figures S4 and S5).

holoenzyme (Figure S3). EAL and AdoCbl both have amide bands in this region, and therefore both the SF-FTIR and TRIR signals could either represent structural changes within the cofactor or the protein, or interactions between the two. Other notable features include bleaches at 1625 and 1555 cm^{-1}, and a transient at 1540 cm^{-1}.

Although full measurement of pre-steady state data with ethanolamine and 2-aminopropanol (the part of Equation (2) set in square brackets) was not possible, there remained the possibility of capturing signal changes at the end of turnover.

$$[(Co^{III}\text{-Ado}) + S \xrightarrow{fast}](Co^{II} \cdot AdoH) \cdot S^{\bullet} \xrightarrow{slow} (Co^{III}\text{-Ado}) + P \qquad (2)$$

Absorbance changes at 525 nm after rapid mixing of EAL with excess substrate (S) show a period of steady state turnover followed by a relatively sharp absorbance change for ethanolamine (about 300 ms), with a more gradual (about 10 s) change for 2-aminopropanol (Figures S4 and S5, respectively). These correspond to chemical changes between cob(II)alamin/substrate radical (S•) pair that accumulates during turnover,[13] and the recombination of the Co–C bond upon substrate exhaustion [Eq. (2)].[5,11] SF-FTIR difference spectra were again calculated by subtracting the first clean spectrum (144 ms for ethanolamine and 482 ms for 2-aminopropanol) from spectra at subsequent points in time. In contrast to ethylene glycol, these early spectra are dominated by the cob(II)alamin/substrate radical (S•) pair that accumulates during turnover. The most intense signals that evolve after the exhaustion of substrate are found at the same frequencies as those from the EG experiment, but are inverted (Figure 3a).

Much like the UV/Vis data in Figure S4, the SF-FTIR difference spectra with ethanolamine as substrate (Figure S6) show a period of steady-state turnover followed by the evolution signals over 300 ms (Figure 3b and Figure S7). These difference spectra also included two additional signal increases at 1715 and 1354 cm^{-1} that had linear evolution profiles (Figure 3a and Figure S6), and are due to the formation of acetaldehyde (Figure S8).[33] SF-FTIR spectroscopy of EAL versus 2-aminopropanol yielded similar difference spectra to ethanolamine (Figure 3a and Figure S9). They differ in that the signal changes appear over about 10 s (Figure 3c and Figure S10) similar to that observed in the UV/Vis region, and the propanal (product) peaks are not visible because of the lower substrate concentration (400 μM, compared to 10 mM ethanolamine). The signal inversion compared to EG is perhaps unsurprising, as the difference spectra in each case are calculated with reference to different early time spectra—the intact holoenzyme for EG, and cob(II)alamin/substrate radical pair for ethanolamine and 2-aminopropanol. Therefore, inversion at 1625, 1650, and 1661 cm^{-1} represent, at least in part, the equivalent but opposing processes, that is, Co–C bond homolysis and recombination. The signals at 1555 to 1540 cm^{-1} are similarly inverted.

To assign the major SF-FTIR signal changes at 1650 and 1661 cm^{-1} to either the protein or cofactor, we isotopically labeled the EAL apoenzyme with ^{15}N, which results in a detectable red shift of 2 cm^{-1} of the amide I band.[34] EAL

was then incubated with non-labeled AdoCbl shortly before data acquisition. Since the EAL-ethanolamine SF-FTIR experiment yields a large acetaldehyde signal at 1715 cm^{-1}, this was used as an internal control that should not shift between isotope experiments. SF-FTIR data for nonlabeled and ^{15}N-labeled EAL yielded comparable difference spectra (Figure 4a). As expected, the acetaldehyde product peak has

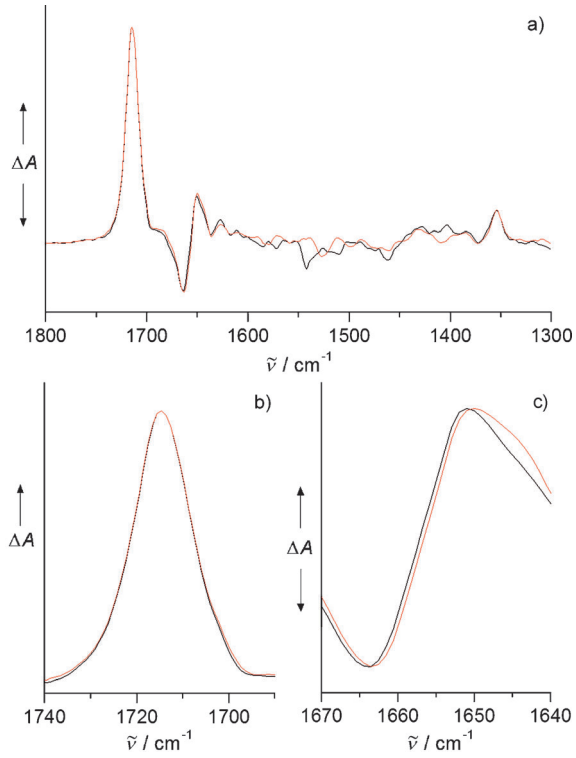

Figure 4. Superposition of normalized SF-FTIR difference spectra of nonlabeled (black line) and ^{15}N-labeled EAL (red line). a) Measured after turnover of 10 mM ethanolamine. b, c) Spectra 1.6 s post-mixing showing: b) no shift in the acetaldehyde product peak at 1715 cm^{-1} and c) a 2 cm^{-1} red-shift in the peaks at 1650 and 1661 cm^{-1} from ^{15}N-labeled EAL. The signal variation in (a) between 1400–1600 cm^{-1} may be owing to noise, but can also be attributed in part to peak shifts that result from potentially different extents of deuteration.

not shifted (Figure 4b). However, the 1650 and 1661 cm^{-1} signals from ^{15}N-labeled EAL are red-shifted by 2 cm^{-1} (Figure 4c and Figure S11), which suggests that they are at least in part reporting on changes in the protein. As the amide II band can vary significantly depending on the extent of protein deuteration, the signal at 1555–1540 cm^{-1} was not similarly analyzed.

In conclusion, both TRIR and SF-FTIR data from studies with EAL show a signal bleach at 1650 cm^{-1} and transient at 1661 cm^{-1} (amide I region) that correlates with the reaction chemistry. It is possible that the AdoCbl spectrum shifts on binding to EAL, and that both sets of signal changes in this region result from the coenzyme. However, the red-shift in the SF-FTIR data after isotopic labeling of the protein strongly suggests that much of the signal results from a structural change in the protein scaffold. The about 3 cm^{-1} resolution of TRIR prevented a similar ^{15}N-labeling experiment.[25] However, the coincidence of these TRIR signals with those from SF-FTIR spectroscopy (Figure S3) is certainly compelling, and may indicate a measurable impact on the EAL protein from Co–C bond photolysis. Such an interaction is consistent with the viscosity dependence of the recombination kinetics after photolysis in EAL, recently attributed to a mobile active-site residue (likely Glu^{287})[18] interacting with the adenosyl radical.[7] Whether the TRIR peaks at 1650 and 1661 cm^{-1} are from the protein or otherwise, they are a relatively minor component of the signal, the majority of which resembles the predicted changes in the cofactor that accompany homolysis (calculated using DFT).[20] The fact that such cofactor signals are not clearly evident above the noise in the SF-FTIR spectra suggest that these data are dominated by signals representing changes in the protein following substrate binding or turnover. The signal bleach at 1650 cm^{-1} represents a change of approximately 1% of the overall IR absorbance for the holoenzyme at this wavenumber, which corresponds to about 7 ± 2 peptide bonds in the EAL heterodimer that binds each cofactor (see the Supporting Information). These changes accompany the reaction chemistry, and appear to be more substantial than those observed in the EAL crystal structure from substrate binding alone,[18] where the chemistry was arrested by using a AdoCbl derivative that does not undergo homolysis. The implications of directly monitoring the protein structure by IR methods during the EAL-catalyzed reaction are wide ranging, and suggest that such techniques provide a powerful resource for answering long-standing questions regarding AdoCbl-dependent enzymes, and potentially other B_{12}-dependent processes.

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