Isotope Effects in the Transient Phases of the Reaction Catalyzed by Ethanolamine Ammonia-Lyase: Determination of the Number of Exchangeable Hydrogens in the Enzyme−Cofactor Complex†

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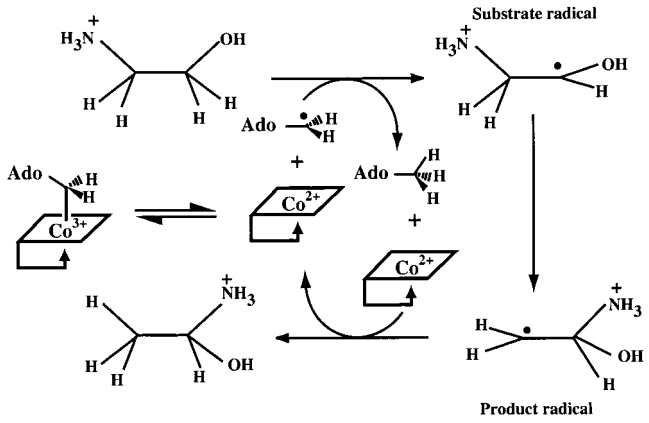
ABSTRACT: Transient phases of the reaction catalyzed by ethanolamine ammonia-lyase (EAL) from *Salmonella typhimurium* have been investigated by stopped-flow visible spectrophotometry and deuterium kinetic isotope effects. The cleavage of adenosylcobalamin (coenzyme B_{12}) to form cob(II)alamin (B_{12r}) with ethanolamine as the substrate occurred within the dead time of the instrument whenever coenzyme B_{12} was preincubated with enzyme prior to mixing with substrate. The rate was, however, slowed sufficiently to be measured with perdeutero ethanolamine as the substrate. Optical spectra indicate that, during the steady states of the reactions with ethanolamine and with *S*-2-aminopropanol as substrates, approximately 90% of the active sites contain B_{12r}. Reformation of the carbon−cobalt bond of the cofactor occurs following depletion of substrate in the reaction mixtures, and the rate constant for this process reflects k_{cat} of the respective substrates. This late phase of the reaction also exhibits ^2H isotope effects similar to those measured for the overall reaction with ^2H-labeled substrates. With unlabeled substrates, the rate of cofactor reassembly is independent of the number of substrate molecules turned over in the steady-state phase. However, with ^2H-labeled substrates, kinetic isotope effects appear in the reassembly phase, and these isotope effects are maximal after only ∼2 equiv of substrate/active site are processed. With 5′-deuterated coenzyme B_{12} and deuterated substrate, the isotope effect on reassembly is independent of the number of substrate molecules that are turned over. These results indicate that the pool of exchangeable hydrogens in the enzyme−cofactor complex is two—a finding consistent with the hydrogens in the C5′ methylene of coenzyme B_{12}.

The paradigm for action of ethanolamine ammonia-lyase (EAL;[1] EC 4.3.1.7) (Scheme 1) includes a homolysis of the carbon−cobalt bond of the cofactor to generate B_{12r} and the 5′-deoxyadenosyl radical. The 5′-deoxyadenosyl radical abstracts a hydrogen atom from the substrate to generate a substrate radical and 5′-deoxyadenosine. The substrate radical rearranges to form a product radical, and a hydrogen transfer from the 5′-methyl of the 5′-deoxyadenosine to the product radical generates the *gem*-amino alcohol and regenerates the 5′-deoxyadenosyl radical which reacts with the B_{12r} to reform coenzyme B_{12}. The resulting *gem*-amino alcohol eliminates ammonia to produce acetaldehyde (*1*, *2*).

Two salient features of the model in Scheme 1, the participation of 5′-deoxyadenosine as a hydrogen carrier and the presence of radical intermediates, have been experimentally verified. Label from tritiated substrate is transferred to the cofactor, and tritium at the 5′-position of the cofactor is transferred to product during the reaction (*3*−*5*). Radical

Scheme 1



intermediates have been observed by EPR spectroscopy during turnover with substrates (*6*−*8*). Magnetic field effects on *V*/*K* are also evidence of radical intermediates and of a radical recombination event prior to the first irreversible step in the catalytic cycle (*9*).

The magnitudes of the KIEs on transfer of ^3H from cofactor to the product radicals in EAL, however, raised doubts about the validity of the simple model shown in Scheme 1. Tritium in the 5′-position of the cofactor is discriminated against for transfer to product by a factor of ∼100 relative to protium (*5*). The deuterium isotope effect

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[1] Abbreviations: EAL, ethanolamine ammonia-lyase; coenzyme B_{12}, adenosylcobalamin; B_{12r}, cob(II)alamin; KIE, kinetic isotope effect; EPR, electron paramagnetic resonance; HEH, hydroxyethylhydrazine; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid.

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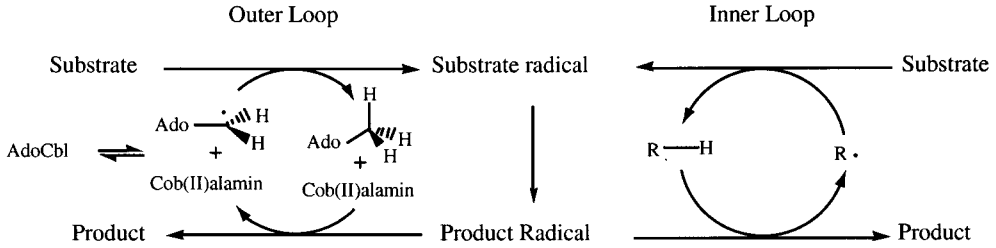
Scheme 2



in the overall reaction of EAL with perdeuterated ethanolamine is ~7 (*4*, *5*, *10*, *11*), and semiclassical theories (*12*) predict a tritium isotope effect of approximately 17. Similarly large ^{3}H KIEs have been reported for the analogous step in the reaction catalyzed by another coenzyme B_{12} dependent enzyme, dioldehydrase (*13*).

To explain the large ^{3}H KIEs, Cleland (*14*) proposed that coenzyme B_{12} does not necessarily reform at the end of each catalytic cycle whenever the enzyme is operating under conditions of V_{max}. Rather, the product radical could alternatively abstract a hydrogen atom from a group on the protein. The resulting protein radical then initiates the next catalytic cycle by hydrogen atom abstraction from the next molecule of substrate. This alternative scheme (Scheme 2) permits catalysis to proceed without reformation of coenzyme B_{12} in each cycle and allows "tuning" of the observed ^{3}H KIE such that it is more consistent with the value calculated from the deuterium isotope effect on the overall reaction. For example, to account for the deuterium isotope effect on the overall reaction, the protein radical pathway would operate in 5 out of 6 catalytic cycles. Evidence has been presented for the presence of a volatile pool of hydrogen on the enzyme that can exchange with both the substrate and the cofactor (*15*). The properties of the volatile pool are, however, not entirely consistent with the alternative pathway hypothesis. For example, although the pool should be responsible for the majority of the catalytic cycles, data from experiments designed to measure washout of ^{3}H from the pool show that ^{3}H from the pool washes-out at about the same rate as ^{3}H from the cofactor (*15*).

The deuterium KIE on the overall reaction of EAL is substantial (~7), indicating that hydrogen transfers are at least partially rate determining (*4*, *5*, *10*, *11*, *16*, *17*). The extent to which other steps might limit the rate of the overall reaction, and thereby attenuate the magnitude of the ^{2}H KIE, is not yet established. Therefore, comparisons between the ^{2}H KIE on the overall reaction and the extent of discrimination against tritium transfer, measured in the second hydrogen transfer step, may not be valid.

Cleavage of coenzyme B_{12} to give B_{12r} following addition of substrate to the preformed EAL−coenzyme B_{12} complex can be monitored by the change in the absorption spectrum of the coenzyme (*6*, *18*). In the steady state of the reaction, the active sites contain an intermediate radical and B_{12r} (*7*, *8*). The reaction catalyzed by EAL is effectively irreversible thermodynamically, and reformation of coenzyme B_{12} following depletion of substrate provides a transient kinetic phase in the latter part of the reaction. The present paper reports the results of stopped-flow visible spectrophotometry and deuterium KIE measurements which have been used to determine the contribution of hydrogen transfer steps to these two transient phases of the reaction catalyzed by EAL and the number of hydrogens in the enzyme−cofactor complex that are exchangeable with substrate and product.

EXPERIMENTAL PROCEDURES

Materials. Perdeuterated ethanolamine was from Cambridge Isotope Labs. *S*-2-Aminopropanol, ethanolamine, $LiAlD_4$, the hydrochloride salt of glycine ethyl ester and the hydrochloride salt of L-alanine methyl ester were from Aldrich. Coenyzme B_{12} was purchased from Sigma. Genes encoding the large and small subunits of EAL from *S. typhimurium* were expressed in *Escherichia coli*, and the protein was purified as previously described (*19*). Enzyme used in these studies had specific activity of ~50 IU mg^{-1} protein. Protein concentration was determined based on $\epsilon^{0.1\%}_{280nm} \approx 0.69$ (*19*).

Synthesis of [1,1-$^2H_2$]Ethanolamine. The hydrochloride salt of glycine ethyl ester was suspended in anhydrous ethyl ether and exposed to dry ammonia gas in order to neutralize the hydrochloride. The solution was dried over anhydrous $MgSO_4$, filtered, and added dropwise to a refluxing suspension of $LiAlD_4$ (1 g) in 20−30 mL of anhydrous ethyl ether. The reduction was allowed to proceed for ~2 h. Excess of the $LiAlD_4$ was quenched first with methanol, and then with water. The aqueous phase was made basic, and the suspension was extracted in a continuous extraction apparatus for ~48 h. The receiving flask contained ~20 mL of water. The contents of the receiving flask were transferred to a separatory funnel and the aqueous phase was collected. The ether phase was extracted several times with 10−20 mL aliquots of water, and the water layers were combined. The pH of the solution was adjusted to near neutral, and the solvent was removed by rotary evaporation. The purity of the material was confirmed by ^{1}H NMR.

Synthesis of [1,1-$^2H_2$]-S-2-Aminopropanol. The hydrochloride salt of L-alanine methyl ester was reduced to the deuterated alcohols using $LiAlD_4$ and purified as described above for ethanolamine. The isotopic enrichment of the product was confirmed by ^{1}H NMR.

Quantitation of Stock Solutions of Ethanolamine and S-2-Aminopropanol. Stock solutions of unlabeled and perdeuterated ethanolamine were quantitated enzymatically using the reaction of EAL coupled to NADH oxidation catalyzed by alcohol dehydrogenase. The enzymatically quantitated stock solution of unlabeled ethanolamine was used to generate the standard curves to standardize stock soltuions of *S*-2-aminopropanol using the naphthoquinone benzene sulfonate colorimetric method (*20*).

Synthesis of [5′,5′-$^2H_2$]-Coenzyme B_{12}. [5′,5′-$^2H_2$]-Coenzyme B_{12} was synthesized as previously described (*21*) with the following modifications: (1) the carboxylic acid was

Table 1: Turnover Numbers and ^2H Kinetic Isotope Effects on the Overall Reaction Catalyzed by EAL

substrate	k_{cat} (s^{-1})[a]	isotope effect[b]
ethanolamine	80 ± 2	
[1,1-$^2H_2$]ethanolamine	15.6 ± 1.4	5.1 ± 0.5
[1,1,2,2-$^2H_4$]ethanolamine	10.7 ± 0.7	7.5 ± 0.5
S-2-aminopropanol	0.27 ± 0.03	
[1,1-$^2H_2$]-*S*-2-aminopropanol	0.054 ± 0.003	5.0 ± 0.6

[a] k_{cat}s are calculated based on six active sites present in each $\alpha 6\beta 6$ oligomer of EAL (*19*). [b] Isotope effects were determined by comparing directly the rate of aldehyde production obtained with unlabeled and deuterated substrates.

esterified using trimethylsilyl-diazomethane (Aldrich); and (2) 5′-chloroadenosine was synthesized as previously described (*22*). NMR spectra of the product of each step were obtained to confirm the presence of the desired compound prior to proceeding to the following step.

Stopped-Flow Spectrophotometry. An Olis RSM-1000 rapid-scanning stopped-flow spectrophotometer was used for the measurements. Data processing was accomplished using the software package supplied with the instrument. For experiments where the effect of preincubation of EAL with cofactor was examined, EAL (~15 μM sites) and Hepes/NaOH, pH 7.5 (20 mM), were placed in one syringe, and the second syringe was filled with a solution of coenzyme B_{12} (35 μM), Hepes/NaOH, pH 7.5 (20 mM), and substrate (5 mM). The concentrations of enzyme, cofactor, and substrate were halved after mixing. For all other experiments, one syringe was filled with substrate and buffer as above. EAL, coenzyme B_{12}, and buffer were mixed and placed in the second syringe. All experiments were completed within 10−15 min of mixing of the enzyme with coenzyme B_{12}. Data from at least three repetitions were averaged. All solutions containing coenzyme B_{12} were handled in dim light.

Steady-State Kinetic Measurements. The assay mixtures contained Hepes/NaOH, pH 7.5 (50 mM), NADH (~0.15 mM), yeast alcohol dehydrogenase (45 IU), and coenzyme B_{12} (14.5 μM). Reactions were initiated by adding EAL to 0.28 μM sites in the assays with *S*-2-*aminopropanol* and to 1.4 nM in the assays with ethanolamine.

Washout of ^2H from [5′,5′-$^2H_2$]-Coenzyme B_{12}. To estimate the rate at which ^2H in coenzyme B_{12} was transferred to product, parallel assays were performed with unlabeled and deuterated coenzyme B_{12} with fixed amounts of *S*-2-aminopropanol as substrate. The assays were initated by mixing a solution of 17 μM EAL (active sites) and 33 μM coenzyme B_{12} or [5′,5′-$^2H_2$]-coenzyme B_{12} (22 mM Hepes/NaOH, pH 7.5) with an equal volume of substrate in the same buffer. The rates of coenzyme B_{12} reformation, following depletion of the *S*-2-aminopropanol, were measured at 525 nm with a Hewlett-Packard diode array spectrophotometer.

RESULTS AND DISCUSSION

Steady-State Kinetic Measurements. The k_{cat} values measured for ethanolamine and *S*-2-aminopropanol are listed in Table 1. The deuterium KIEs observed for the overall reactions with these substrates are also listed in Table 1. The values given in Table 1 are similar to those reported for these substrates with EAL from *Clostridia* sp. (*4*, *5*, *10*, *11*, *16*, *17*).

Table 2: Isotope Effects on the Rates of Formation of B_{12r}

substrate	unlabeled (s^{-1})	1,1-$^2H_2$-labeled (s^{-1})	isotope effect
ethanolamine	>300[a]	29 ± 1	>10
S-2-aminopropanol	74 ± 7	24 ± 1	3.1 ± 0.3

[a] This estimate is based on a dead-time of 3−4 ms for the stopped flow instrument.

Knowledge of the number of active sites (six) in the $\alpha_6\beta_6$ oligomer of EAL (*19*, *23*) allowed an estimate of the extent of B_{12r} and radical formation in the steady-state of the reaction. In these experiments, holo-EAL was mixed with an excess of substrate, and bleaching of the absorbance of coenzyme B_{12} at 525 nm was monitored. As an internal calibration, hydroxyethylhydrazine was used to inactivate EAL and generate a stoichiometric quantity of enzyme bound B_{12r} (*19*). In the steady-state of the reaction of *S*-2-aminopropanol, at least 90% of the available active sites contain B_{12r}. This finding is consistent with the previous results obtained with clostridial EAL with *S*-2-aminopropanol wherein the optical spectrum of a mixture of EAL and coenzyme B_{12} resembled that of B_{12r} (*18*). Furthermore, the concentration of B_{12r} obtained with [1,1-$^2H_2$]-*S*-2-aminopropanol was equivalent to that observed with the unlabeled substrate.

Isotope Effects on the Conversion of Coenzyme B_{12} to B_{12r}. Incubation of EAL (*18*) and other coenzyme B_{12}-dependent enzymes with their respective substrates (*24*, *25*) or allosteric effectors (*26*, *27*) and coenzyme B_{12} triggers homolysis of coenzyme B_{12} to generate B_{12r}. Stopped-flow spectrophotometric measurements with glutamate mutase and with methylmalonyl CoA mutase have been used to show an isotope effect on the formation of B_{12r} when deuterated substrates are used (*24*, *25*). On the other hand, experiments in which apo-EAL was mixed with a solution of B_{12} and deuterated ethanolamine, did not reveal an isotope effect on the formation of the B_{12r} (*28*). This lack of an isotope effect on the formation of B_{12r} is consistent with a dominant isotope insensitive step under the conditions of the experiment. Significantly, however, the rate of formation of B_{12r} when apo-EAL is mixed with a solution containing coenzyme B_{12} and ethanolamine is *slower* than k_{cat}. By contrast, formation of B_{12r} is completed within the dead time (~3−4 ms, k > 300 s^{-1}) of the stopped-flow instrument, whenever EAL is preincubated with coenzyme B_{12} prior to mixing with ethanolamine (*18*, *28*).

The effects of preincubation on the rates of formation of B_{12r}, when EAL is mixed with coenzyme B_{12} and substrate, or when EAL is initially mixed with coenzyme B_{12} prior to mixing with the substrate, were examined (Table 2). As in the previous studies (*18*), significant rate enhancements are observed when EAL is preincubated with coenzyme B_{12} (Table 2), indicating the presence of a slow step in the formation of holo-EAL. At present, the nature of this slow step is not known. EAL, however, binds coenzyme B_{12} in the "base-on" mode (*29*, *30*); therefore, the exchange of the dimethylbenzimidazole base of the cofactor for an enzymic side chain (such as the imidazole of a histidine) is not involved.

The deuterium KIEs on the appearance of B_{12r} were measured using [1,1-$^2H_2$]ethanolamine and [1,1-$^2H_2$]-*S*-2-

Table 3: Washout of Deuterium from Deuterated Coenzyme B_{12} during Turnover of *S*-2-Aminopropanol

[substrate]$_o$/site[a]	ratio of rates of coenzyme reformation for reactions with unlabeled and deuterated cofactor[b]
15	2.3
43	1.4
87	1.0

[a] [substrate]$_o$ is the concentration of *S*-2-aminopropanol prior to initiating the reaction with holo-EAL. [b] Uncertainties in the measurements are estimated to be ~10%.

aminopropanol. The slow formation of holo-EAL was bypassed in these experiments by preincubating EAL with coenzyme B_{12} prior to mixing with the unlabeled or deuterated substrates (Table 3). While with unlabeled ethanolamine, B_{12r} formation is complete within the dead time of the instrument, with the [1,1-$^2H_2$]ethanolamine, the rate of formation of B_{12r} is slowed to 29 s^{-1} -corresponding to a deuterium KIE of at least 10. The ^2H KIE on the formation of B_{12r} with *S*-2-aminopropanol as the substrate is ~3.

Isotope Effects on the Reformation of Coenzyme B_{12}. The first phase of the reaction, homolysis of the cofactor, is followed by a steady-state period, the duration of which depends on the initial concentration of substrate. Once the substrate has been depleted, a second transient phase appears in the optical spectrum as the absorbance due to B_{12r} disappears and that due to coenzyme B_{12} reappears. The kinetics of reformation of coenzyme B_{12} during this final phase of the reaction were examined with ethanolamine and with *S*-2-aminopropanol as substrates. In these experiments, EAL was preincubated with coenzyme B_{12} and then mixed with quantities of substrate varying from substoichiometric to an 8-fold excess over active sites. Representative examples from these experiments with varying concentrations (0.5−8 equiv/active site) of unlabeled or [1,1-$^2H_2$]-*S*-2-aminopropanol are shown in Figure 1. As expected, the rate constant for reformation of the cofactor is independent of the initial concentration of substrate (Figure 2). Furthermore, the rate constants for the reformation of the cofactor with *S*-2-aminopropanol (0.33 s^{-1}) and with ethanolamine (84 s^{-1}) are comparable to the respective k_{cat}'s for these substrates (Table 1). These findings are consistent with the idea that the rate determining steps occur after formation of B_{12r} (phase 1) and with the observation of a nearly stoichiometric amount of B_{12r} in the steady state.

The rates of reformation of coenzyme B_{12} during the final optical transient were determined with [1,1-$^2H_2$]ethanolamine and [1,1-$^2H_2$]-*S*-2-aminopropanol. As noted above with the unlabeled substrates, the rates of formation of coenzyme B_{12} are independent of amount of substrate present at time zero. With the ^2H-labeled substrates, however, the rate constants for reformation of coenzyme B_{12} decrease as the concentration of the deuterated substrate present at time zero increases, reaching minima (0.057 and 19 s^{-1} with ^2H-labeled *S*-2-aminopropanol and ^2H-labeled ethanolamine, respectively), in reactions for which ~2 equiv of substrate were present for each active site at time zero (see Figures2 and 3). Comparison of these minima to the rate of cofactor reformation measured with the unlabeled substrates indicates that with both substrates, maximum KIEs (~4.4−4.7 and 5.5−5.9 with [1,1-$^2H_2$]ethanolamine and [1,1-$^2H_2$]-*S*-2-aminopropanol, respectively) are reached after ~2 equiv of each substrate are turned over. The maximum isotope effects observed with deuterated forms of the substrates are similar to the KIEs observed on the *overall* reaction ($^Dk_{cat}$) with these same substrates (see Table 1)—a finding consistent with the match of the kinetics of coenzyme reformation with k_{cat}.

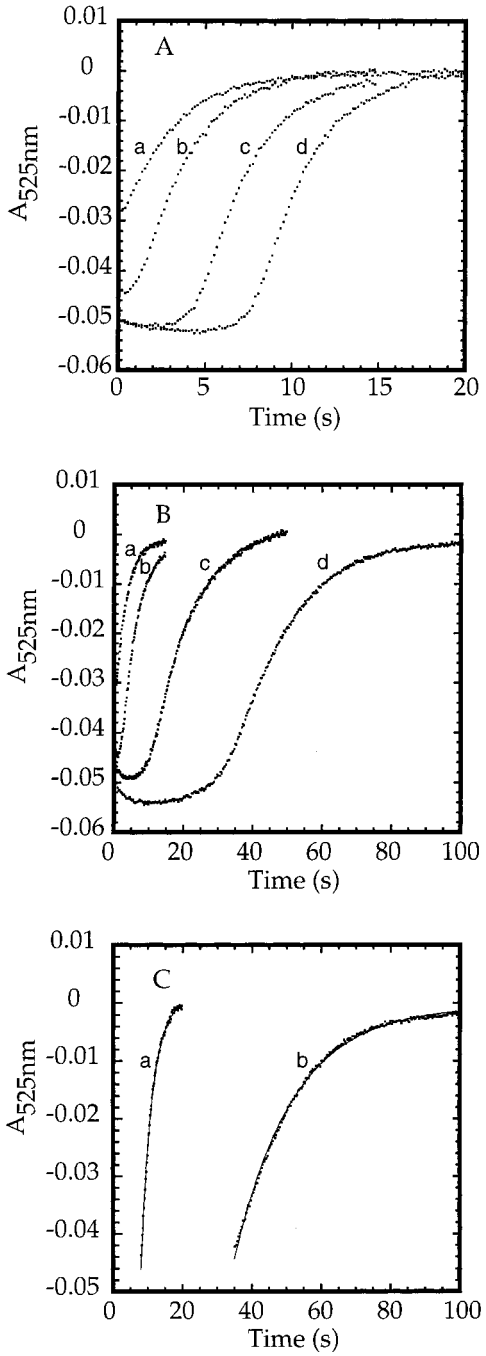


FIGURE 1: Kinetic traces showing the time course for reformation of the cofactor upon exhausting substrate. In these experiments, one syringe was filled with a solution containing EAL (~15 μM) and coenzyme B_{12} (30 μM) in 20 mM Hepes/NaOH (pH 7.4). In panel A, the second syringe contained in 20 mM Hepes/NaOH, pH 7.4, *S*-2-aminopropanol: (a) 7.5 μM; (b) 15 μM; (c) 30 μM; (d) 45 μM. In panel B, the second syringe contained in 20 mM Hepes/NaOH, pH 7.4, [1,1-$^2H_2$]-*S*-2-aminopropanol: (a) 7.5 μM; (b) 15 μM; (c) 30 μM; (d) 45 μM. The reformation of the cofactor was monited at 525 nm, and the increases in absorbance were fitted to single exponentials. Representative fits (panel C) to the reformation phases for (a) the data at 45 μM *S*-2-aminopropanol and (b) the data at 45 μM [1,1-$^2H_2$]-*S*-2-aminopropanol.

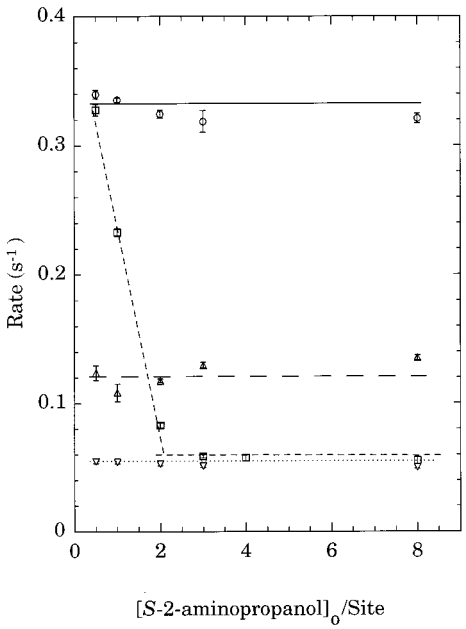


FIGURE 2: Comparison of the rate of reformation of coenzyme B_{12} as a function of the initial concentration of unlabeled or [1,1-$^2H_2$]-*S*-2-aminopropanol. These experiments were setup as described in the legend for Figure 1. (○) *S*-2-aminopropanol and coenzyme B_{12}. (□) [1,1-$^2H_2$]-*S*-2-aminopropanol and coenzyme B_{12}. (△) [5′,5′-$^2H_2$]-Coenzyme B_{12} and *S*-2-aminopropanol. (▽) [5′,5′-$^2H_2$]-Coenzyme B_{12} and [1,1-$^2H_2$]-*S*-2-aminopropanol. Substrate was varied between 0.5- and 8-fold excess over the concentration (15 μM) of active sites. Error bars represent the standard deviation for repetitive runs at each concentration. Lines are sketched through the data points.

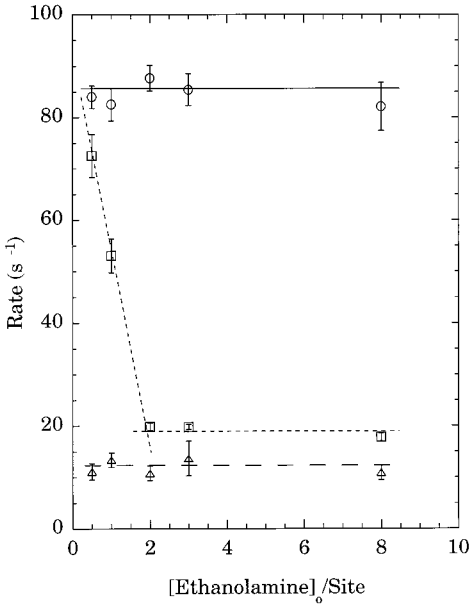


FIGURE 3: Comparison of the rate of reformation of coenzyme B_{12} as a function of the initial concentration of unlabeled or [1,1-$^2H_2$]-ethanolamine. These experiments were setup as described in the legend for Figure 1. (○) Ethanolamine and coenzyme B_{12}. (□) [1,1-$^2H_2$]-Ethanolamine and coenzyme B_{12}. (△) [1,1-$^2H_2$]-Ethanolamine [5′,5′-$^2H_2$]-coenzyme B_{12}. Substrate was varied between 0.5- and 8-fold excess over the concentration (15 μM) of active sites. Error bars represent the standard deviation for repetitive runs at each concentration. Lines are skectched through the data points.

The observation of the maximum KIEs on reformation of coenzyme B_{12} after only 2 equiv of substrate are turned over indicates that the pool of exchangeable hydrogen atoms contributed by the enzyme−cofactor complex is 2. To determine if this pool corresponds to the 5′-methylene hydrogens of coenzyme B_{12}, EAL was preincubated with [5′,5′-$^2H_2$]coenzyme B_{12} prior to mixing with deuterated substrates. If there were no other contributor to the pool of exchangeable hydrogens, with deuterated cofactor and deuterated substrate, the entire pool of exchangeable hydrogens contains deuterium at time zero. Under these conditions, the rate of reformation of coenzyme B_{12} would be at a minimum on the *first* catalytic cycle and remain so throughout additional cycles. Indeed, the rate constant for the reformation of coenzyme B_{12} is at the minimum (i.e., the maximum KIE) on the first catalytic cycle with either [1,1-$^2H_2$]-*S*-2-aminopropanol or [1,1-$^2H_2$]ethanolamine as substrates (Figures 2 and 3) and remains constant over the range of substrate concentrations examined.

Washout of ^2H from Labeled Coenzyme B_{12}. EAL was preincubated with [5′,5′-$^2H_2$]coenzyme B_{12} prior to mixing with *unlabeled S*-2-aminopropanol in the stopped flow apparatus. Under these conditions, the rate of reformation of coenzyme B_{12} exhibited a KIE of about two-thirds of the maximum for more than eight catalytic cycles (Figure 2). The washout of ^2H from the cofactor upon further turnovers was estimated by comparing the rate of coenzyme B_{12} reformation upon exhaustion of substrate for samples with either unlabeled or deuterated cofactor. Results of these measurements are summarized in Table 3. These data show that there is a substantial kinetic discrimination against transfer of ^2H from cofactor to the product radical, this discrimination is also evident in the data of Figures 2 and 3 wherein [5′,5′-$^2H_2$]coenzyme B_{12} was used with unlabeled substrates. Hence, there is a ^2H KIE on this cofactor to product radical transfer that may be more in concert with the very large ^3H KIE on this step, than with the seemingly normal ^2H KIE on k_{cat} (*5*).

Significance of Isotope Effects on Coenzyme B_{12} Homolysis. KIE's on the homolysis of coenzyme B_{12} from ^2H located in the substrates have been observed previously with methylmalonyl-CoA mutase (*25*) and with glutamate mutase (*24*). Homolysis of the cobalt−carbon bond of the cofactor, concerted or coupled to abstraction of a hydrogen atom from the substrate, has been postulated to account for the observation of these isotope effects. Concerted chemistry requires that the C−H or S−H bond of the substrate or Cys residue bind near the cofactor such that the C−Co bond is broken concomitant with the breakage of the C−H or S−H bond (*27*, *31*). There is spectroscopic and crystallographic evidence that substrates and substrate radicals bind close to (∼6−7 Å) the cofactor in the active sites of the mutases (*32*−*35*). In EAL, spectroscopic data indicate that the substrate radical is located ∼12 Å away from the cobalt of B_{12r} (*36*, *37*). Moreover, in dioldehydrase, crystallographic data show that the substrate is bound 8.4−9 Å from cobalt (*38*). These data suggest that, for EAL and dioldehydrase, the 5′-deoxyadenosyl radical must move several Å once it detaches from cobalt, to abstract a hydrogen atom from the substrate, and the distances appear to be incompatible with a concerted reaction.

Alternatively, the KIE on homolysis of the Co−C bond of coenzyme B_{12} may also derive from the existence of an equilibrium between intact and homolyzed cofactor on the enzyme, albeit one where the equilibrium constant favors intact cofactor (Scheme 1) (*24*, *39*). Addition of substrate would deplete the existing pool of 5′-deoxyadenosyl radical forcing more flux through the homolysis path. Magnetic field

effects on the EAL reaction indicate that the rate of recombination of a geminate radical pair contributes to the *V*/*K*, and this observation suggests that the 5′-deoxyadenosyl radical is a discrete participant in the catalytic pathway (*9*). Therefore, a coupled mechanism seems more likely for the homolysis step in EAL.

Size of the Pool of Exchangeable Hydrogen. The most straightforward interpretation of the results regarding the onset of the ^{2}H KIE on reformation of coenzyme B_{12} is that the only group being loaded with deuterium in experiments with unlabeled cofactor and deuterated substrates is the 5′-methylene of the adenosyl moiety of the cofactor. This interpretation is consistent with results from studies of EAL with the single turnover inactivator, hydroxyethylhydrazine, which show that there is direct hydrogen exchange between the inactivator and 5′-deoxyadenosine without an intermediate carrier (*40*). Mass spectrometry was used previously to determine that the total pool (including the substrate) of exchangeable hydrogens in methylmalonyl CoA mutase was three (*41*). Adding the single hydrogen contributed by the stubstrate to the pool of two hydrogens from the enzyme−cofactor complex in EAL gives a total pool of three hydrogens. The present findings for EAL are therefore consistent with the total pool size determined for methylmalonyl CoA mutase.

The alternative pathway hypothesis (Scheme 2) is presumed to become operational whenever the enzyme is functioning under V_{max} conditions (*14*). The present experiments were performed with limited amounts of substrate-conditions that approximate *V*/*K* conditions—where the cofactor might be expected to reform at the end of each catalytic cycle. Thus, while the present results provide no evidence of an expanded pool of exchangeable hydrogens, which is required by the alternative pathway hypothesis, they do not, alone, rule out the possibility that an amino acid side chain might participate in the hydrogen transfers when substrates are at saturation. The results are, however, inconsistent with an *obligatory* protein radical as an intermediate in every catalytic cycle. For example, an intermediate thiyl radical, such as that present in coenzyme B_{12} dependent ribonucleotide reductase (*42*), would not mediate exchange of hydrogens between substrates and the 5′-methylene group of the cofactor, which is a hallmark of EAL (*3*). Hydrogen atoms abstracted from the substrate by a putative thiyl radical would be reacquired by the product radical at the completion of each catalytic cycle. Hence, to account for the exchange of hydrogen between the substrate and the 5′-methylene group of the cofactor, any group on the protein that might mediate hydrogen transfers must contain at least two equivalent hydrogens. The latter circumstance would, however, require a cofactor/mediator pool of at least 4 hydrogens which is incompatible with the present findings.

Large KIEs are one indicator of quantum mechanical tunneling in hydrogen transfer processes (*43*). There is evidence for tunneling in the temperature dependence of the ^{2}H KIEs of EAL reported by Weisblat and Babior (*5*). The present results indicate that 5′-methylene of coenzyme B_{12} is the sole mediator of hydrogen atom exchange in EAL under *V*/*K* conditions. The possibility that this situation applies to conditions of substrate saturation as well should not be ruled out at this time.

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