Hydrogen Atom Exchange between 5′-Deoxyadenosine and Hydroxyethylhydrazine during the Single Turnover Inactivation of Ethanolamine Ammonia-Lyase†

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ABSTRACT: The early steps in the single turnover inactivation of ethanolamine ammonia-lyase (EAL) from *Salmonella typhimurium* by hydroxyethylhydrazine (HEH) have been probed by rapid-mixing sampling techniques, and the destiny of deuterium atoms, present initially in HEH, has been investigated by mass spectrometry. The inactivation reaction produces acetaldehyde, the hydrazine cation radical, 5′-deoxyadenosine, and cob(II)alamin (B_{12r}) in amounts stoichiometric with active sites. Rapid-mix freeze-quench EPR spectroscopy and stopped-flow rapid-scan spectrophotometry revealed that the hydrazine cation radical and B_{12r} appeared at a rate of $\sim 3\ s^{-1}$ at 21 °C. Analysis of 5′-deoxyadenosine isolated from a reaction mixture prepared in ^2H_2O did not contain deuterium—a result which demonstrates that solvent-exchangeable sites are not involved in the hydrogen-transfer processes. In contrast, all of the 5′-deoxyadenosine, isolated from inactivation reactions with [1,1,2,2-$^2H_4$]HEH, had acquired at least one ^2H from the labeled inactivator. Significant fractions of the 5′-deoxyadenosine acquired two and three deuteriums. These results indicate that hydrogen abstraction from HEH by a radical derived from the cofactor is reversible. The distribution of 5′-deoxyadenosine with one, two, and three deuteriums incorporated and the absence of unlabeled 5′-deoxyadenosine in the product are consistent with a model in which there is direct transfer of hydrogens between the inactivator and the 5′-methyl of 5′-deoxyadenosine. These results reinforce the concept that the 5′-deoxyadenosyl radical is the species that abstracts hydrogen atoms from the substrate in EAL.

Ethanolamine ammonia-lyase (EAL;[1] EC 4.3.1.7) catalyzes the coenzyme B_{12}-dependent conversion of vicinal amino alcohols to ammonia and the corresponding oxo compound (*1*−*3*). An attractive model for the action of enzymes catalyzing coenzyme B_{12}-dependent rearrangements is that the coenzyme serves as a radical initiator (*4*, *5*). The 5′-deoxyadenosyl radical, resulting from homolytic cleavage of the cobalt−carbon bond in the cofactor, is thought to initiate the chemical transformations in the substrate directly or indirectly via hydrogen atom abstraction from the substrate molecule. The 5′-deoxyadenosyl radical has, however, eluded direct detection by spectroscopic means.

While the presence of substrate-based radicals in reactions catalyzed by EAL is firmly established (*6*−*8*), the identity of the immediate progenitor of these radicals is less certain. Experimental observations show that tritium at the 5′-position of the cofactor is discriminated against by a factor of ∼100 in transfer to product (*9*). This ^3H kinetic isotope effect is much larger than is expected from the deuterium isotope effect in the overall reaction ($^DV \approx 7$) (*9*−*12*). To rationalize these kinetic isotope effects, the presence of an alternative reaction pathway involving an enzyme-based radical has been proposed (*13*). This modified scheme removes the need to regenerate the cofactor during every turnover and thus allows "tuning" of the observed ^3H isotope effects to a value more compatible with classical predictions. Some support for the presence of such a protein radical in EAL was found in the form of a solvent exchangeable, or "volatile", pool of ^3H that is present whenever EAL is allowed to process [1-^3H]-ethanolamine (*14*). This pool of ^3H, however, washes out into the product during a chase cycle at the same rate at which ^3H in the cofactor washes out. Although the kinetics of wash out of ^3H from this volatile pool is not compatible with the modified scheme involving the alternative pathway, the identity and number of hydrogen atom abstracting species in EAL is still in question.

Incubation of EAL with the substrate analogue, HEH, leads to the complete loss of EAL activity and to the formation of 5′-deoxyadenosine, B_{12r}, the hydrazine cation radical, and acetaldehyde in amounts that are stoichiometric with respect to the available concentration of active sites (*15*). The single turnover irreversible inactivation by HEH can be used to probe early steps of EAL catalysis. For example, the fate of deuterium, present initially in the inactivator, can be examined in the absence of multiple turnovers. Thus, the location of deuterium, following the inactivation, identifies the abstracting group. This paper presents results of rapid-mix

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[1] Abbreviations: EAL, ethanolamine ammonia-lyase; coenzyme B_{12}, adenosylcobalamin; B_{12r}, cob(II)alamin; EPR, electron paramagnetic resonance; HEH, hydroxyethylhydrazine; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; GC−MS, gas chromatography−mass spectrometry.

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freeze-quench EPR spectroscopy, stopped-flow rapid-scan visible spectrophotometry, and GC−MS analysis of 5′-deoxyadenosine isolated from inactivation reactions of EAL and HEH.

EXPERIMENTAL PROCEDURES

Materials. *Salmonella typhimurium* EAL was overexpressed in *Escherichia coli* and purified as previously described (*15*). Coenzyme B_{12} and 5′-deoxyadenosine were purchased from Sigma. [1,1,2,2-$^2H_4$]Bromoethanol (98.1% ^2H) was from Cambridge Isotope Labs. Bromoethanol and ^2H_2O (99.9% ^2H) were from Aldrich. *N*-Methyl-*N*-(trimethylsilyl)trifluoroacetamide was from Alltech, and C-18 SepPak Cartridges were purchased from Millipore.

Rapid-Mix Freeze-Quench EPR Spectroscopy. The samples were prepared using an Update Instruments model 745 RAM. The experiments were performed in the two syringe mode using established protocols (*16*). One syringe contained 0.01 M Hepes/NaOH, pH 7.5, and 0.01 M HEH in a volume of 1 mL. The second syringe was filled with a 1 mL solution containing 0.17 mM EAL, 0.35 mM coenzyme B_{12}, and 0.01 M Hepes/NaOH, pH 7.5. The contents of the second syringe were typically used within 10−15 min of mixing EAL with coenzyme B_{12}. EPR spectra were acquired on a Varian E-3 X-band spectrometer. The samples were kept at 77 K during the acquisition using a standard liquid nitrogen immersion Dewar.

Analysis of [1,1,2,2-$^2H_4$] Hydroxyethylhydrazine. Unlabeled and perdeuterated HEH were synthesized by reaction of hydrazine with bromoethanol and perdeuterated bromoethanol, respectively, as described previously (*15*). The isotopic content of the perdeuterated HEH was assayed by GC−MS. Samples of perdeutero- and unlabeled-HEH were derivatized by reaction with isobutyl chloroformate prior to analysis by GC−MS. The derivatization was carried out by vortexing 0.24 mL of a 0.04 M solution of HEH or perdeutero HEH in 1 M sodium carbonate, pH 10, with 5 equiv of neat isobutyl chloroformate. The reaction mixture was extracted with 2 × 0.1 mL portions of ether. The combined organic layers were evaporated under a stream of dry N_2. The residue was dissolved in 0.5 mL of ethyl acetate and analyzed with an HP6890 GC coupled to a HP 5973 mass selective detector. The GC column was HP-5MS (30 m × 0.25 mm, 0.25 μm film thickness). The column was maintained at 140 °C for 3 min initially. The temperature was programmed to increase at a rate of 10 °C min^{-1} up to 290 °C. The chromatograms contained two peaks derived from HEH. The initial peak at 11.2 min had *m*/*z* of 276 for protio-HEH, which corresponded to molecular ion of HEH bearing two isobutyl formate groups. For perdeutero-HEH, *m*/*z* for the molecular ion of the peak at 11.2 min was at 280. A small peak at *m*/*z* 279 had 1.8 ± 0.2% of the total counts (*m*/*z* 279 + *m*/*z* 280)—a result consistent with the 98.1% isotopic enrichment of the starting perdeutero bromoethanol.

Stopped-Flow Visible Spectrophotometry. Measurements were made on an Olis RSM-1000 instrument. One syringe was filled with a mixture of EAL (18 μM), coenzyme B_{12} (37 μM) and Hepes/NaOH, pH 7.5 (0.02 M), in a total volume of 2 mL. The other was filled with unlabeled or [1,1,2,2-$^2H_4$]HEH (5 mM) and Hepes/NaOH, pH 7.5 (0.02 M), in a total volume of 2 mL. The rates of homolysis of coenzyme B_{12} were determined by fitting the decrease in absorbance at 525 nm as a function of time to a single exponential.

Incubations To Generate 5′-Deoxyadenosine. The incubation solutions contained 55 μM EAL, 0.15 mM coenzyme B_{12}, 2.5 mM unlabeled or [1,1,2,2-$^2H_4$]HEH, and 0.01 mM Hepes/NaOH, pH 7.5, in a total volume of 2 mL. Reactions were initiated by addition of EAL and allowed to proceed at room temperature for 15 min. The incubations were quenched with 0.8 mL of 1 N HCl and precipitated protein was removed by centrifugation. The supernatants were mixed with 0.8 mL of 1 N Tris base. The resulting solutions were desalted by loading onto a SepPak C-18 cartridge which had been activated by passage of ∼20 mL of methanol. The column was first washed with water, and the eluate was discarded. The column was eluted with ∼30 mL of ethanol. The ethanol was removed by rotary evaporation and the resulting residue was dissolved in 30% methanol in water. The 5′-deoxyadenosine was purified by HPLC using a C-18 column (Rainin) with a linear gradient of 30 to 100% methanol over 10 min. The peaks corresponding to 5′-deoxyadenosine from several injections were pooled, and the volume was reduced by rotary evaporation. The resulting residue was dissolved in water and the purity examined by HPLC.

Inactivation in ^2H_2O. For the reactions carried out in ^2H_2O, all components of the incubation were lyophilized and resuspended in ^2H_2O. The incubations contained 77 μM EAL, 0.23 mM coenzyme B_{12}, 5 mM HEH, and 20 mM Hepes/NaOH (pH 7.5) in a total volume of 1 mL. The reactions were quenched with 0.4 mL of 1 N HCl, protein was removed by centrifugation, and the supernatant combined with 0.4 mL of 1 N Tris base. The 5′-deoxyadenosine was purified as described above.

GC/MS Analysis of 5′-Deoxyadenosine. Purified 5′-deoxyadenosine was dissolved in pyridine, and ∼1 μg in ∼20−40 μL was combined with 100 μL of *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide and heated at 100 °C for 1 h (*17*). The resulting trimethylsilyl derivatized 5′-deoxyadenosine was injected onto the HP-5MS column for GC−MS without further purification. Standard deviations of mass peaks were estimated as the square root of the number of counts at *m*/*z*. All other conditions were as described previously (*17*).

RESULTS

Rapid-Kinetic Studies of the Inactivation of EAL by HEH. Incubation of EAL with coenzyme B_{12} and HEH leads to homolysis of coenzyme B_{12} and rapid inactivation of EAL. The rate of inactivation of EAL by HEH was too fast to be determined in the experiments where the inhibitor was manually mixed with solutions of the enzyme−cofactor complex (*15*). Stopped-flow rapid-scan visible spectrophotometry and rapid-mix freeze-quench EPR spectroscopy were used to investigate the rates of homolysis of coenzyme B_{12} and of the formation of hydrazine cation radical, respectively. In each experiment, EAL was preincubated with coenzyme B_{12} prior to mixing with HEH. Data from the stopped-flow and rapid-mix freeze-quench are shown in Figure 1. Fits of the two sets of data to single exponentials (not shown) gave slightly different time constants (2.4 ± 0.2 and 2.9± 0.2 s^{-1}

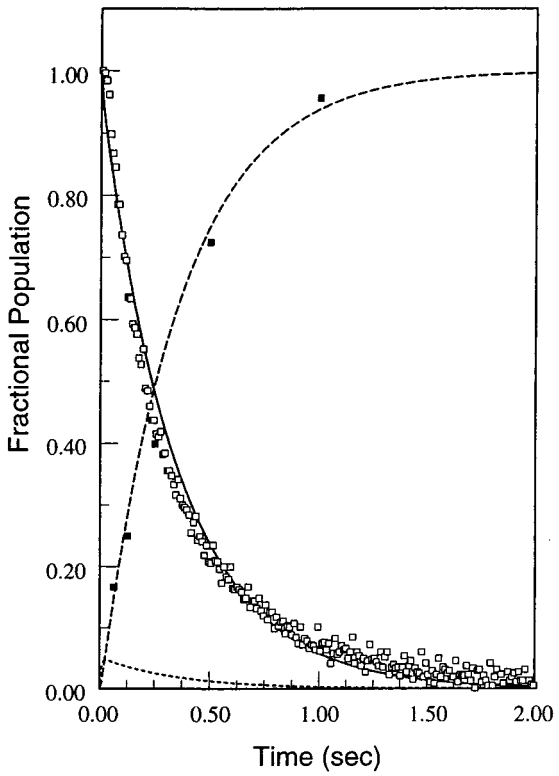


FIGURE 1: Time course of the formation of the hydrazine cation radical (solid squares) and homolysis of coenzyme B_{12} (open squares) at 21 °C. Formation of the hydrazine cation radical was monitored by rapid-mix freeze-quench EPR. Homolysis of coenzyme B_{12} was measured by following the decrease in absorbance at 525 nm using rapid scanning stopped-flow absorbance spectroscopy. Continuous curves were obtained by numerical simulation of the kinetic model of Scheme 3 using the program KINSIM and a value of 55 s^{-1} for k_7. Solid line: coenzyme B_{12}. Dashed line: hydrazine cation radical. Dotted line: HEH radical.

for the rapid-mix freeze-quench and stopped-flow, respectively). The EPR spectrum of the sample obtained at 7 ms in the freeze-quench experiments showed a weak signal at $g \approx 2$, which had an intensity slightly above background. This weak signal spanned a region about twice the width of the spectrum of the hydrazine cation radical. By 64 ms, the EPR signal of the hydrazine cation radical was prominent, and clearly the major species was absorbing near $g \approx 2$. The slight difference in the time constants obtained from rather different experimental measurements might be insignificant. However, the mechanism proposed for the inactivation involves at least two radicals (the 5′-deoxyadenosyl radical and the α-radical of HEH) which precede the hydrazine cation radical (*15*). The weak EPR signal appearing early on might contain contributions from these intermediates.

Kinetic Isotope Effect on B_{12r} Formation. The rate of homolysis of coenzyme B_{12} with [1,1,2,2-$^2H_4$]HEH was monitored by stopped-flow. A ^2H kinetic isotope effect of 4 ± 0.3 was observed.

GC−MS Analysis of 5′-Deoxyadenosine. The stoichiometry of inactivation by HEH shows that each active-site hosts a single fragmentation of the HEH radical to acetaldehyde and the hydrazine cation radical (*15*). This single turnover property provides an opportunity to follow the fate of hydrogen (deuterium) originating in HEH during this reaction. Therefore, EAL was inactivated with [1,1,2,2-$^2H_4$]HEH. The 5′-deoxyadenosine resulting from the inactivation was isolated, derivatized, and subjected to GC−MS. Mass spectra obtained with authentic 5′-deoxyadenosine and with 5′-deoxyadenosine purified from reaction mixtures of holo-EAL with unlabeled HEH were indistinguishable from the published spectrum (*17*). The mass spectrum of 5′-deoxyadenosine isolated from inactivation mixtures in ^2H_2O was also indistinguishable from the published spectrum. This result indicates that deuterium is not incorporated into 5′-deoxyadenosine from the solvent. Hence, hydrogen atom abstraction events leading to production of 5′-deoxyadenosine in the inactivation by HEH do not involve solvent exchangeable sites such as the −SH of cysteine, the −OH of serine or the $-NH_2$ of lysine. The lack of solvent participation in hydrogen exchange is consistent with the earlier report (*10*) that hydrogen transfer from carbon-1 to carbon-2 of the substrate takes place without the participation of solvent exchangeable sites.

Several ions in the mass spectrum of trimethylsilylated 5′-deoxyadenosine contain the 5′-methyl group of the 5′-deoxyribose moiety. For convenience in correcting for natural abundance of heavy isotopes, the ion at *m*/*z* 452, corresponding to loss of one methyl group from the molecular ion, was analyzed. Data obtained from authentic 5′-deoxyadenosine, and from 5′-deoxyadenosine isolated from reaction mixtures initiated with HEH and with [1,1,2,2-$^2H_4$]HEH are shown in Figure 2. The relative amounts of the various forms of 5′-deoxyadenosine, were obtained from an analysis of the peaks in the 452−458 *m*/*z* region. The mass spectrum of 5′-deoxyadenosine generated in the inactivation reaction with [1,1,2,2-$^2H_4$]HEH, contains peaks corresponding to mono (49.0 ± 0.2%)-, di (34.3 ± 0.2%)-, and tri (16.5 ± 0.2%)-deuterated 5′-deoxyadenosine. The data indicate that virtually all 5′-deoxyadenosine molecules contain at least one deuterium. The small quantity of the unlabeled 5′-deoxyadenosine (0.25 ± 0.02%) present is less than the 0.45−0.5% protium content in the 1-*pro*-*S* position (*11*, *12*) of the [1,1,2,2-$^2H_4$]-HEH. The model of the inactivation (see below) predicts an enrichment of unreacted HEH in protium.

DISCUSSION

A reaction sequence that accounts for the kinetic results as well as the finding of an incorporation of up to three deuteriums into 5′-deoxyadenosine is shown in Scheme 1.

The appearance of multiply deuterated forms of 5′-deoxyadenosine requires that the scheme include steps in which hydrogen/deuterium exchanges between the cofactor and HEH are reversible and that there be facile exchange of HEH in solution with HEH bound to the active site. The tight binding of B_{12r} in the active site is the source of the inactivation (*15*). Detection of ^3H-labeled 2-aminopropanol upon incubation of the EAL/coenzyme B_{12} complex with ^3H-labeled propionaldehyde and ammonia (*18*) showed that the hydrogen atom abstraction steps in EAL are reversible, and the reversibility of the hydrogen atom abstraction step in the inactivation by HEH is consistent with the earlier finding.

The kinetic data obtained by stopped-flow and rapid-mix freeze-quench methods provide information on the net rates through the steps in Scheme 1 while the distribution of deuterated forms of 5′-deoxyadenosine provides information

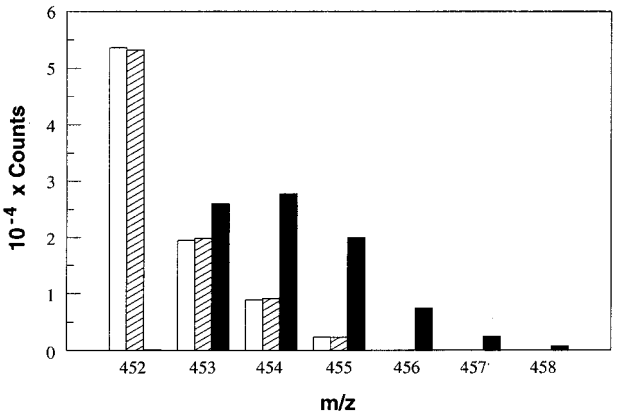
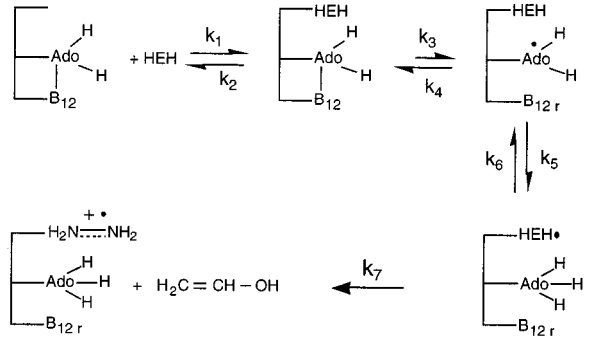


FIGURE 2: The region from *m/z* 452−458 of the mass spectrum of the trimethylsilyl derivative of 5′-deoxyadenosine. Hatched bars: authentic 5′-deoxyadenosine. Gray bars: 5′-deoxyadenosine from inactivation of EAL with HEH. Black bars: 5′-deoxyadenosine from inactivation of EAL with [1,1,2,2-^{2}H]HEH. The spectra for separate samples were normalized to the same total counts.

Scheme 1



on the partitioning of the HEH• intermediate. Introduction of ^{2}H into the scheme, however, requires some statistical corrections because of a reduction in the number of hydrogens present on the 5′-methyl group of deoxyadenosine. Scheme 2 details the pathways leading to incorporation of one, two, and three deuteriums into 5′-deoxyadenosine.

In Scheme 2, $k'_{246} = k_2k_4k_6/[k_2(k_4 + k_5) + k_3k_5]$ is a "composite rate constant"[2] (*19*, *20*) derived for the steps of Scheme 1. The statistical factor of 2/3 is included to account for the reduction in the number of hydrogens on the 5′-methyl group relative to that in Scheme 1. The HEH radical (HEH•) created by abstraction of a deuterium atom from C1 of deuterated HEH has three options—two of which can be monitored by incorporation of ^{2}H into 5′-deoxyadenosine. The third option, abstraction of a deuterium from the 5′-monodeuteriomethyl group of 5′-deoxyadenosine is a null result. Partitioning of HEH• between the inactivation route and reversal through hydrogen abstraction from the monodeutero-5′-deoxyadenosine is measured by the relative abundance of the monodeutero 5′-deoxyadenosine relative to that of the di- and trideutero products [$k_7/(2k'_{246}/3) = 0.97 \pm 0.01$, or $k_7/k'_{246} = 0.65 \pm 0.01$].

[2] The value of the composite rate constant, k'_{246} obtained from the pattern of incorporation of deuterium into 5′-deoxyadenosine differs from that corresponding to a fully protio sample by a secondary deuterium isotope effect which is appears to be ~8%. The second partitioning step has two deuteriums on the 5′-methyl group of 5′-deoxyadenosine and is therefore subject to an additional secondary deuterium isotope effect.

A second partitioning occurs following binding of the next deuterated HEH to those active sites that survived the initial round of radical production. In this second encounter, however, the HEH• has less opportunity to abstract a hydrogen because only one hydrogen remains on the dideutero-methyl group of 5′-deoxyadenosine. The ratio of inactivation to hydrogen atom abstraction in the second cycle is the ratio of the dideutero- 5′-deoxyadenosine to trideutero 5′-deoxydeoxyadenosine [$k_7/(k'_{246}/3) = 2.08 \pm 0.03$, or $k_7/k'_{246} = 0.69 \pm 0.01$]. The estimates of k_7/k'_{246} obtained from the two partitioning steps differ by ~8% probably as a result of a secondary deuterium isotope effect.[2]

The model shown in Scheme 2 predicts that HEH• formed from protio-HEH has a greater chance to undergo reversal because three hydrogens are available on the 5′-methyl group of 5′-deoxyadenosine when this radical is present. This statistical "edge" for escape of protio-HEH likely accounts for the finding of less unlabeled 5′-deoxyadenosine than expected from the isotopic purity of the [1,1,2,2-^{2}H$_4$]HEH.

Knowledge of the partitioning of HEH•, k_7/k'_{246}, obtained from the pattern of incorporation of deuterium from HEH into 5′-deoxyadenosine can be used as the basis of a more elaborate kinetic model of the stopped-flow and rapid-mix freeze-quench EPR data. The stopped-flow data measures the net rate of disappearance of coenzyme B_{12}. If one assumes that the concentration of the "elusive" 5′-deoxyadenosyl radical is evanescent, Scheme 1 can be recast using composite rate constants as Scheme 3.

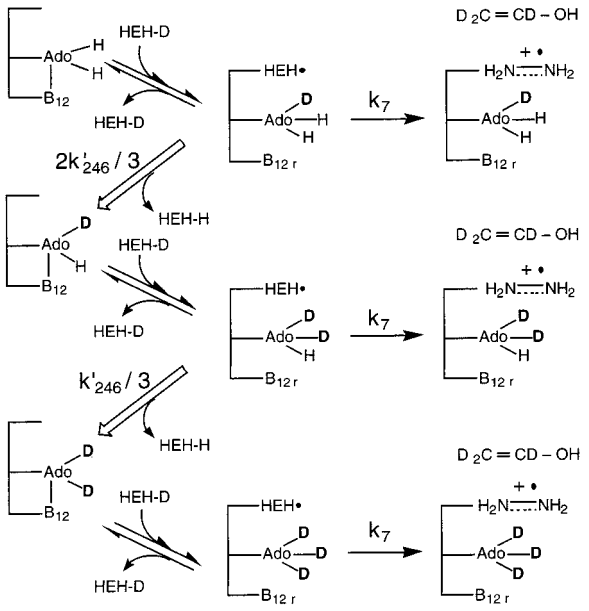
In Scheme 3, $k'_{135} = (k_1k_3k_5[\text{HEH}])/[k_2(k_4 + k_5) + k_3k_5]$. The experimental value of the rate of coenzyme B_{12} cleavage is related to the composite rate constants of Scheme 3 by eq 1:

$$2.9\ \text{s}^{-1} = \frac{k'_{135}k_7}{k'_{246} + k_7} \qquad (1)$$

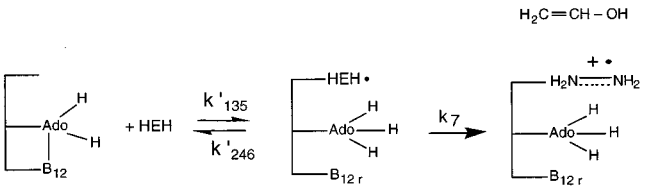
From the partitioning of HEH•, $k'_{246} \approx 1.5\ k_7$. Substitution of this result into eq 1 gives $k'_{135} = 7.2\ \text{s}^{-1}$. Knowledge of the value of k'_{135} and the relationship between k'_{246} and k_7 leaves just one variable (k_7 or k'_{246}) in simulations of the time course of the inactivation reaction using KINSIM and the model of Scheme 3 (*21*). A sample of the results of this modeling is illustrated by the continuous curves in Figure 1. The model also provides an indication of concentration/time behavior of the HEH• species. The experimental observation from the rapid freeze-quench EPR measurements that the hydrazine cation radical was the only paramagnetic species (other than B_{12r}) to achieve concentrations significantly above background provides a constraint on the kinetic model. The sample curves shown in Figure 1 were selected to be consistent with this constraint. The magnitude of k_7 ($55 \pm 20\ \text{s}^{-1}$) is, however, not well determined from the present data. The results of the simulations confirm what was apparent from the small difference in the rates of disappearance of coenzyme B_{12} and the appearance of the hydrazine cation radical and from the EPR spectra—namely, that intermediates such as HEH• do not build up to appreciable levels.

Stopped-flow measurements with [1,1,2,2-^{2}H$_4$]HEH showed that the heavy isotope, contained in HEH, influenced the rate of cleavage of coenzyme B_{12}. Kinetic isotope effects on homolysis of coenzyme B_{12}, arising from a label contained

Scheme 2



Scheme 3



in the substrate, have been observed with methylmalonyl-CoA mutase (*22*) and with glutamate mutase (*23*). Different scenarios have been proposed to account for these observations. For example, the homolysis of coenzyme B_{12} has been suggested to be either coupled to or concerted with hydrogen transfer from substrate (*22, 23*). Alternatively, it has been proposed that coenzyme B_{12} is in equilibrium with B_{12r} and 5′-deoxyadenosyl radical, albeit with an equilibrium constant that favors the intact cofactor (*23*). Hydrogen transfer from substrate to the cofactor depletes the pool of the 5′-deoxyadenosyl radical to give a substrate radical and 5′-deoxyadenosine. In the presence of deuterated substrate, the pool of the 5′-deoxyadenosyl radical is depleted more slowly leading to the observation of an isotope effect (*23*).

The observed distribution of the forms of deuterated 5′-deoxyadensoine is incompatible with any plausible scenario in which a species, other than the 5′-deoxyadenosyl radical, abstracts hydrogen atoms from HEH. Such a scheme would require that a significant fraction of 5′-deoxyadenosine be unlabeled in the inactivation with perdeutero-HEH. Hence, these results indicate that exchange of hydrogen atoms between the inactivator and the cofactor occurs via the 5′-deoxyadenosyl radical without participation of other groups in the active site. Results of the previous paper indicate that, within the active site, the hydrazine cation radical resides ∼13 Å from Co^{2+} in B_{12r} (*15*). The distance between Co^{2+} and the hydrazine cation radical is compatible with the ∼10 Å distance estimated from the magnetic interactions between Co^{2+} and the steady-state substrate radical with 2-aminopropanol (*8, 24*). Direct exchange of hydrogen/deuterium between HEH and 5′-deoxyadenosine established by the present experiments suggests that the 5′-deoxyadenosyl radical must operate between positions that are ∼10 Å apart. An implication from these findings is that separate steps in the reaction, each of which involve the 5′-deoxyadenosyl radical (i.e., the hydrogen atom abstraction events with the substrate analogue and reformation of coenzyme B_{12}), occur at locations that are separated by ∼10 Å. The means by which the 5′-deoxyadenosyl radical shuttles between these two positions provides a formidable challenge for future investigations.

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