Isotope Effects for Deuterium Transfer between Substrate and Coenzyme in Adenosylcobalamin-Dependent Glutamate Mutase†

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ABSTRACT: A key step in the mechanism of all adenosylcobalamin-dependent enzymes is the abstraction of a hydrogen atom from the substrate by a 5′-deoxyadenosyl radical generated by homolytic fission of the coenzyme cobalt−carbon bond. We have investigated the isotope effects associated with this process for glutamate mutase reacting with deuterated glutamate. The kinetics of deuterium incorporation into 5′-deoxyadenosine (5′-dA) during the reaction were followed by rapid chemical quench, using HPLC and electrospray mass spectrometry to analyze the 5′-dA formed. The kinetics of 5′-dA formation are biphasic, comprising a rapid phase $k_{app} = 37 \pm 3\ s^{-1}$ and a slower phase $k_{app} = 0.9 \pm 0.4\ s^{-1}$. The mass spectral data clearly show that the faster phase is associated with the formation of monodeuterated 5′-dA whereas the slower phase is associated with the incorporation of a second and then a third deuterium into 5′-dA. This observation implies that a large inverse equilibrium secondary isotope effect is associated with the formation of 5′-dA from adenosylcobalamin. The primary deuterium kinetic isotope effects on *V* and *V*/*K* for the formation of 5′-dA were determined from time-based and competition experiments. $^{D}V = 2.4 \pm 0.4$ whereas $^{D}(V/K) = 10 \pm 0.4$, implying that an isotopically insensitive step is partially rate-determining. The additional data provided by these experiments cause us to revise our interpretation of earlier UV−visible stopped-flow kinetic measurements of AdoCbl homolysis obtained with deuterated substrates.

Adenosylcobalamin (coenzyme B_{12}, AdoCbl)[1] serves as the coenzyme for a group of enzymes that catalyze unusual rearrangement or elimination reactions (*1*−*6*). The coenzyme serves as a masked form of a 5′-deoxyadenosyl radical that is generated through homolytic fission of the AdoCbl cobalt−carbon bond. The carbon skeleton rearrangement of L-glutamate to L-*threo*-3-methylaspartate catalyzed by glutamate mutase (Figure 1) provides an attractive model reaction to investigate the principles by which enzymes generate reactive radical intermediates and harness them for catalysis of chemically difficult reactions (*7*, *8*). Our laboratory has studied this enzyme using a variety of spectroscopic and kinetic measurements, combined with mutagenesis of active site residues, to gain insight into the reaction mechanism and the role of the protein in catalyzing the reaction (*9*−*17*).

Previously, we have used UV−visible stopped-flow spectroscopy to investigate the kinetics of cobalt−carbon bond homolysis when the enzyme was reacted with either unlabeled substrates or substrates deuterated in the abstractable position (*17*). Our results demonstrated that two key steps in the reaction, homolysis of the AdoCbl cobalt−carbon bond and hydrogen atom transfer from the substrate to the resulting 5′-deoxyadenosyl radical, are kinetically coupled together. The results imply that the adenosyl radical can only exist fleetingly, as a high-energy intermediate in the reaction. Similar results have been obtained for other AdoCbl-dependent enzymes for which this phenomenon has been investigated (*18*−*20*).

Most recently, we have used rapid chemical quench techniques to measure the α-secondary tritium isotope effect associated with the formation of 5′-deoxyadenosine from 5′-[^{3}H]-labeled AdoCbl (*13*). We found that formation of 5′-dA is accompanied by a large inverse secondary kinetic isotope effect of 0.76 and an equilibrium isotope effect of 0.72. These results suggest both a late transition state for the overall reaction and that the 5′-C−H bonds are significantly stiffer in 5′-dA than they are in AdoCbl.

The large secondary tritium isotope effects on 5′-dA formation have prompted us to reinvestigate the very large primary isotope effect associated with 5′-dA formation we observed previously when glutamate mutase was reacted with deuterated glutamate (*17*). Whereas previously we measured the primary deuterium isotope effect on 5′-dA formation indirectly by following the kinetics of Cbl(II) formation, in the experiments described here we have followed the kinetics of 5′-dA formation (Figure 1) directly, using rapid chemical quench techniques. This has allowed us to analyze the deuterium content of 5′-dA as a function time by electrospray mass spectrometry. Although the kinetics of 5′-dA formation and Cbl(II) formation measured by the two different techniques are consistent with each other, the additional information provided by the mass spectral analysis leads us to reinterpret our original data; in particular, it appears that the primary kinetic isotope effect on 5′-dA formation is much smaller than we originally thought.

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[1] Abbreviations: AdoCbl, adenosylcobalamin; Cbl(II), cob(II)alamin; 5′-dA, 5′-deoxyadenosine; d_1-5′-dA, [5′-$^{2}H_1$]-5′-deoxyadenosine; d_2-5′-dA, [5′-$^{2}H_2$]-5′-deoxyadenosine; d_3-5′-dA, [5′-$^{2}H_3$]-5′-deoxyadenosine; TFA, trifluoroacetic acid.

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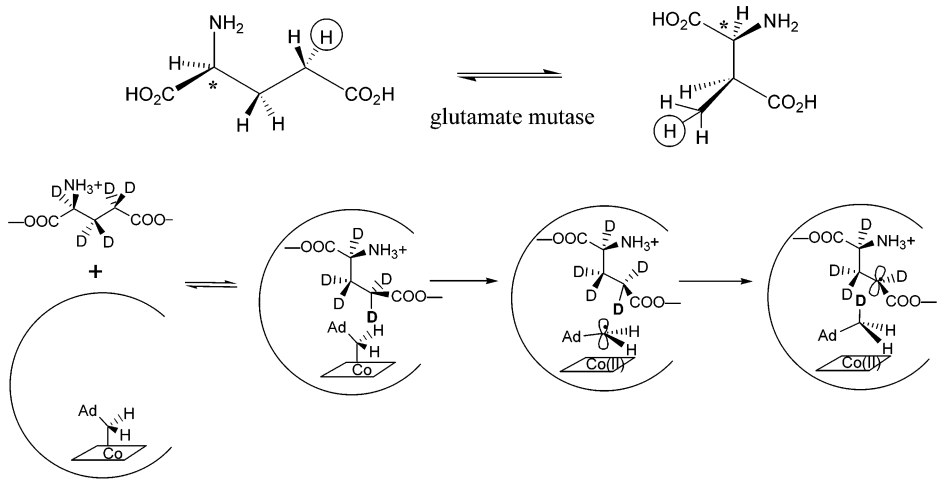

FIGURE 1: Top: Isomerization reaction catalyzed by glutamate mutase. The migrating hydrogen is circled. Bottom: Scheme illustrating steps in the mechanism leading to the formation of deuterated 5′-dA from AdoCbl and deuterated glutamate.

EXPERIMENTAL PROCEDURES

Materials. The purification from recombinant *Escherichia coli* of the engineered single subunit glutamate mutase protein, GlmES, which has been used in this and previous mechanistic studies, has been described previously (*21*). D,L-[2,4,4-$^2H_3$]Glutamic acid and D,L-[2,4,4,3,3-$^2H_5$]glutamic acid were purchased from Cambridge Isotope Laboratories Inc. Deuterated glutamate was only available in racemic form, and therefore in these experiments racemic protiated glutamate was used for comparative purposes. However, control experiments established that D-glutamate is neither a substrate nor an inhibitor of the enzyme, and in all of the experiments described here concentrations refer only to the active L-isomer. AdoCbl was purchased from Sigma Chemical Co.

Rapid Quench Flow. Experiments were performed at 10 °C using a HiTech RQF-63 apparatus (*22*). Eighty microliters of a solution containing 120 μM glutamate mutase, 100 μM AdoCbl, and 20 μM L-tryptophan, as an internal standard, in 50 mM potassium phosphate buffer, pH 7.0, was rapidly mixed with an equal volume of 20 mM glutamate in the same buffer. The solution was allowed to age for various times (10−1800 ms) before being quenched with a further 80 μL of 5% TFA. 5′-dA, AdoCbl, and L-tryptophan were separated by HPLC using a 25 cm C_{18} reverse-phase column (Spherisorb S5 ODS2). The column was preequilibrated in 0.1% TFA and eluted with a 20 mL linear gradient of 0−45% acetonitrile containing 0.1% TFA. 5′-dA eluted at ≈10%, L-tryptophan at 40%, and AdoCbl at 45% acetonitrile. The amount of 5′-dA in the sample was calculated from standard curves obtained by chromatography of known amounts of 5′-dA.

Mass Spectroscopy. Samples collected after HPLC were concentrated to dryness by freeze-drying and redissolved in 15 μL of a solution of water−acetonitrile−formic acid, ratio 1:1:0.003. The samples were analyzed by time-of-flight electrospray mass spectrometry (LCT Micromass). The samples were introduced into the spectrometer through an in-line HPLC pump, in a carrier solvent of 90% methanol/10% water at 0.1 mL/min. The desolvation temperature was 150 °C. The spectral data were analyzed using the MassLynx 4.0 software suite.

Data Fitting. In general, plots of kinetic data were generated, and curve fitting was performed using the KaleidaGraph program (Abelbeck Software). Data describing the kinetics of multiple deuterium incorporation into 5′-dA were globally fitted using the program Matlab 6.0 (Mathworks Software) assuming a model in which A → B → C → D for the concentrations of *A*, *B*, *C*, and *D* as a function of time are described by the equations:

$$A(t) = A_0 e^{-k_1 t}$$

$$B(t) = A_0 \frac{k_1}{k_1 - k_2}(e^{-k_2 t} - e^{-k_1 t})$$

$$C(t) = A_0 k_1 k_2 \left(\frac{e^{-k_1 t}}{(k_1 - k_2)(k_1 - k_3)} - \frac{e^{-k_2 t}}{(k_1 - k_2)(k_2 - k_3)} + \frac{e^{-k_3 t}}{(k_1 - k_3)(k_2 - k_3)} \right)$$

$$D(t) = A_0 \left(1 + \frac{k_2 k_3 e^{-k_1 t}}{(k_1 - k_2)(k_1 - k_3)} + \frac{k_1 k_3 e^{-k_2 t}}{(k_1 - k_2)(k_2 - k_3)} - \frac{k_1 k_2 e^{-k_3 t}}{(k_1 - k_3)(k_2 - k_3)} \right)$$

RESULTS

We have previously used UV−visible stopped-flow spectroscopy to measure the kinetics of AdoCbl homolysis and Cbl(II) formation when glutamate mutase was reacted with either glutamate or methylaspartate or their deuterated analogues (*17*). The protiated substrates reacted rapidly to cause homolysis of the coenzyme in a single step, which was well fitted by a single apparent first-order rate constant. However, the deuterated substrates reacted in a more complicated fashion: homolysis was clearly biphasic and required two exponential functions to adequately fit the data. Subsequently, we used rapid chemical quench techniques to investigate the kinetics of 5′-dA formation when glutamate mutase was reacted with unlabeled glutamate (*22*). In this case 5′-dA formation appeared to be a simple reaction that was well described by a single apparent first-order rate

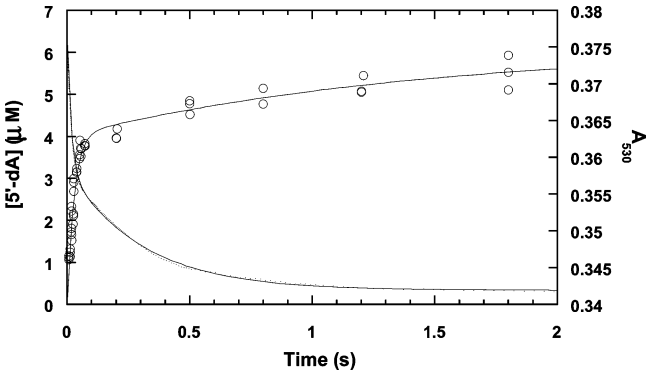

FIGURE 2: Time course for the formation of 5′-dA when hologlutamate mutase was reacted with 10 mM d_5-L-glutamate. For comparison, the stopped-flow trace following the disappearance of AdoCbl at 530 nm when the enzyme was reacted with d_5-L-glutamate under similar conditions is also shown (data from ref *17*). Both reactions follow a similar biphasic time course.

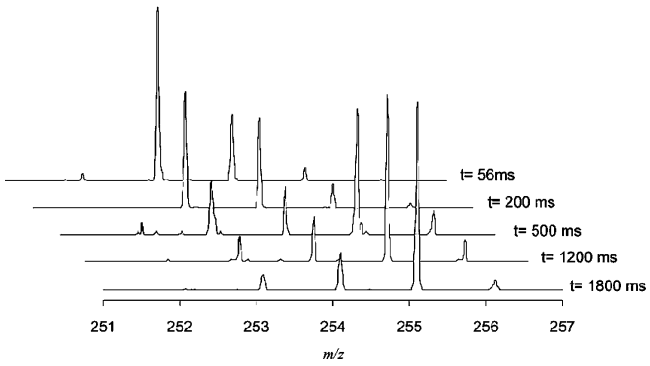

FIGURE 3: Representative mass spectra of deuterated 5′-dA samples recovered from the enzyme after various reaction times illustrating the incorporation of successive deuterium atoms into the coenzyme. The M_r of the initially formed $[d_1$-5′-dA + H$]^+$ ion is 253.

constant. We were therefore interested in knowing whether the biphasic kinetics we observed for AdoCbl homolysis when the enzyme was reacted with deuterated substrates was also reflected in the kinetics of 5′-dA formation.

Kinetics of 5′-dA Formation. We examined the kinetics of 5′-dA formation when hologlutamate mutase (final concentration after mixing = 45 μM) was reacted with a saturating concentration (10 mM) of L-glutamate at 10 °C in 50 mM potassium phosphate buffer, pH 7.0. These are very similar to the conditions that we used in the previous kinetic studies of the enzyme described above (*17*). Under these conditions substrate binding is rapid, and the rate of 5′-dA formation is independent of substrate concentration (*17*, *22*). The reaction was allowed to proceed for various times between 10 and 1800 ms before quenching with TFA. The products of the reaction were separated by reverse-phase HPLC, and the peak corresponding to 5′-dA was collected for subsequent analysis by electrospray mass spectrometry. The amount of 5′-dA formed was determined from the HPLC peak area by reference to a standard curve.

The time course for the formation of deuterated 5′-dA was clearly biphasic, as shown in Figure 2, and mirrors the time course for the disappearance of AdoCbl, which we measured previously by stopped flow. The data shown are from one experiment in which all of the measurements were made with the same batch of enzyme. Similar data were obtained from experiments on another batch of enzyme. The formation of 5′-dA was well fitted by a double exponential function, yielding apparent rate constants $k_{\text{fast(5′−dA)}} = 37 \pm 3\ \text{s}^{-1}$ and $k_{\text{slow(5′−dA)}} = 0.9 \pm 0.3\ \text{s}^{-1}$ for the faster and slower phases, respectively. These rate constants are slower than those measured previously by stopped flow for the formation of Cbl(II) from AdoCbl when the enzyme was reacted with deuterated glutamate ($k_{\text{fast(CblII)}} = 64\ \text{s}^{-1}$ and $k_{\text{slow(CblII)}} = 3.4\ \text{s}^{-1}$). The difference between $k_{\text{fast(5′−dA)}}$ and $k_{\text{fast(CblII)}} = 64\ \text{s}^{-1}$ may be explained in part by experimental error and the fact that the data were obtained by different experimental methods. It is also consistent with the fact that 5′-dA is formed after Cbl(II) in the mechanism, and therefore the apparent rate constant $k_{\text{fast(5′−dA)}}$ must be slower or equal to $k_{\text{fast(CblII)}}$, even though these two steps are kinetically coupled.

Deuterium Content of 5′-dA. We next analyzed the recovered 5′-dA by electrospray mass spectrometry to determine the deuterium content of the samples (Figure 3). Because this technique was not as sensitive as HPLC for detecting 5′-dA, samples for mass spectral analysis at short reaction times (20 ms or less) were made by pooling the products of several reactions made at the same time point. Under the conditions of the experiment 5′-dA was readily detected as its singly protonated ion, and deconvolution of the spectral peaks allowed the number of deuterium atoms in the molecules to be determined. As expected, at early times only a single species of $M_r = 253$ was detected, corresponding to the $[M + 1]^+$ ion of monodeuterated 5′-dA (d_1-5′-dA). However, as the reaction progressed, a species of $M_r = 254$, corresponding to dideuterated 5′-dA (d_2-5′-dA), accumulated at the expense of the monodeuterated material, and at longer times this, in turn, was converted to trideuterated 5′-dA (d_3-5′-dA) ($M_r = 255$), as shown in Figure 3. The average deuterium content of the 5′-dA increased from 1 atom per molecule at the start of the reaction to about 2.9 atoms per molecule after 1.8 s.

The formation of 5′-dA containing more than one deuterium must arise from multiple hydrogen abstraction reactions. As illustrated in Figure 4, this could occur if the glutamyl radical either rearranges to produce methylapartate or partitions back to form d_4-glutamate, which, after diffusion from the enzyme, would allow another molecule of deuterated substrate to react with the enzyme. Indeed, given that k_{cat} for the turnover of deuterated glutamate is 1.4 s^{-1} [determined from measurements made in the steady state (*21*)], on average two to three turnovers are expected to occur during the 2 s time course of the experiment. This observation suggests that the slow increase in the concentration of 5′-dA on the enzyme is associated with an *equilibrium* secondary isotope effect (rather than a kinetic one) in which the presence of deuterium at C-5′ shifts the equilibrium between the enzyme−AdoCbl−substrate and enzyme−Cbl(II)−5′-dA−substrate−radical complexes in favor of the latter.

The concentrations of d_1-5′-dA, d_2-5′-dA, and d_3-5′-dA at each time point were calculated by multiplying the total amount of 5′-dA, as determined by HPLC, by its relative abundance, as determined by mass spectrometry. Figure 5 shows how the concentrations of each species vary as a function of time. The data in Figure 5 could be fitted reasonably well to a kinetic model involving three sequential first-order reactions in which monodeuterated 5′-dA is

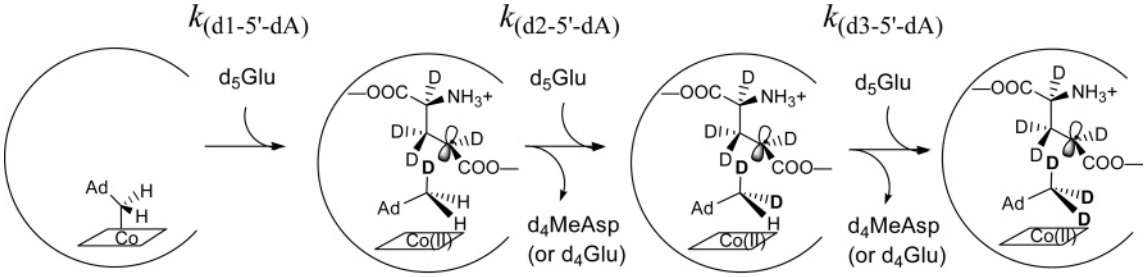

FIGURE 4: Kinetic scheme for the formation of multiply deuterated 5′-dA molecules. The initial reaction of d_5-glutamate with the enzyme results in formation of d_1-5′-dA. Subsequently, partitioning of the glutamyl radical, either forward to methylaspartate or backward to glutamate, followed by diffusion of substrate or product from the enzyme will allow another molecule of deuterated substrate to react to form dideuterated and then trideuterated 5′-dA.

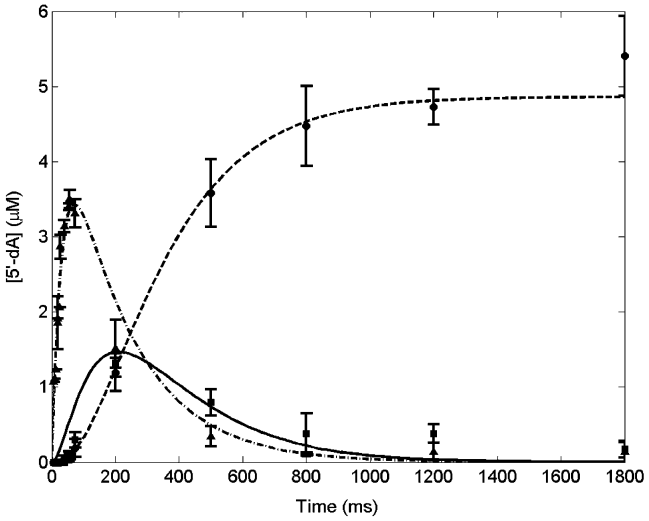

FIGURE 5: Deconvolution of the data in Figure 2 to show the time course for formation and decay of deuterated 5′-dA species: (▲) d_1-5′-dA; (■) d_2-5′-dA; (●) d_3-5′-dA. The data were fitted assuming the kinetic model illustrated in Figure 4. The reasons for the possible small deviations of the data from the fit at longer times are discussed in the text.

initially generated by reaction of the coenzyme with deuterated substrate and subsequently converted into dideuterated and trideuterated 5′-dA by exchange reactions, as shown in Figure 4. Global fitting of the data yielded the following values for the apparent first-order rate constants: $k_{d_1-5'-dA} = 31 \pm 2\ s^{-1}$ for the formation of d_1-5′-dA; $k_{d_2-5'-dA} = 4.9 \pm 0.4\ s^{-1}$ for the conversion of d_1-5′-dA to d_2-5′-dA, and $k_{d_3-5'-dA} = 6.9 \pm 0.6\ s^{-1}$ for the conversion of d_2-5′-dA to d_3-5′-dA.

However, it is also evident from Figure 5 that not all of the 5′-dA becomes trideuterated, even at longer times, as it should do if the kinetic model was strictly correct. There are several reasons why not all the 5′-dA may become fully deuterated. First, the deuterated glutamate contains a small amount, approximately 2%, of protium at exchangeable positions. Second, some small fraction of enzyme may become inactivated during the reaction leaving partially deuterated 5′-dA molecules that are unable to participate further. Third, although we tried to correct for background noise and possible low levels of contamination from previously analyzed samples, there may be some systematic error associated with quantifying the very small amounts of 5′-dA by mass spectrometry that leads to an overestimation of the amount of mono- and dideuterated samples. Whereas a better fit to the data could be obtained by introducing into the kinetic model a pathway for the return of protium to the cofactor, this did not significantly change the values for the rate constants reported above.

The deuterium kinetic isotope effect on the formation of 5′-dA, ^{D}V, can be calculated by dividing the rate constant for the formation of 5′-dA measured for the enzyme reacting with unlabeled glutamate, $k_{5'-dA}$, by the rate constant for the formation of monodeuterated 5′-dA, $k_{d_1-5'-dA}$, calculated from the data in Figure 5. Previously, we measured $k_{5'-dA}$ to be $73 \pm 8\ s^{-1}$ (*22*), a measurement that has been reconfirmed during the course of these experiments. From these data $^{D}V = 2.4 \pm 0.4$. This is much smaller than the apparent deuterium isotope effect on homolysis and hydrogen transfer of ~28 that we calculated previously from stopped-flow spectroscopic measurements. The possible reasons for this discrepancy are discussed below.

V/K Isotope Effect on 5′-dA Formation. Given the unexpectedly small value of ^{D}V, we decided to measure the deuterium isotope effect on the formation of 5′-dA directly by a competition experiment. Such experiments generally provide a more accurate way to measure isotope effects because both deuterated and unlabeled substrates are reacting under identical conditions. We note also that competition experiments measure the isotope effect on V_{max}/K_m [$^{D}(V/K)$] rather than ^{D}V.

Hologlutamate mutase (final concentration after mixing = 45 μM) was reacted with a mixture of 7.5 mM 2,4,4-d_3-L-glutamate and 2.5 mM *proteo*-L-glutamate (final concentrations after mixing) at 10 °C in 50 mM potassium phosphate buffer, pH 7.0. At various times ranging between 35 and 1000 ms the reaction was quenched with TFA, and the 5′-dA produced was recovered and quantified by reverse-phase HPLC, as described above. The deuterium content of the 5′-dA was then determined by mass spectral analysis.

The time course for the formation of 5′-dA when the enzyme was reacted with the mixture of glutamates is shown in Figure 6A and resembles that for the enzyme reacting with completely unlabeled glutamate. The relative amounts of 5′-dA, d_1-5′-dA, and d_2-5′-dA formed between 35 and 100 ms are shown in Figure 6B. During this time the relative amounts of 5′-dA and d_1-5′-dA are roughly constant with time, and the amount of d_2-5′-dA is less than 1%. At shorter times there was insufficient material to accurately quantify the relative amounts of each species by mass spectrometry, whereas at longer times the amounts of di- and trideuterated material became significant. $^{D}(V/K)$ for 5′-dA formation, calculated from the average of all of the data shown in Figure 6B, is 10 ± 0.4. $^{D}(V/K)$ is thus significantly larger than ^{D}V

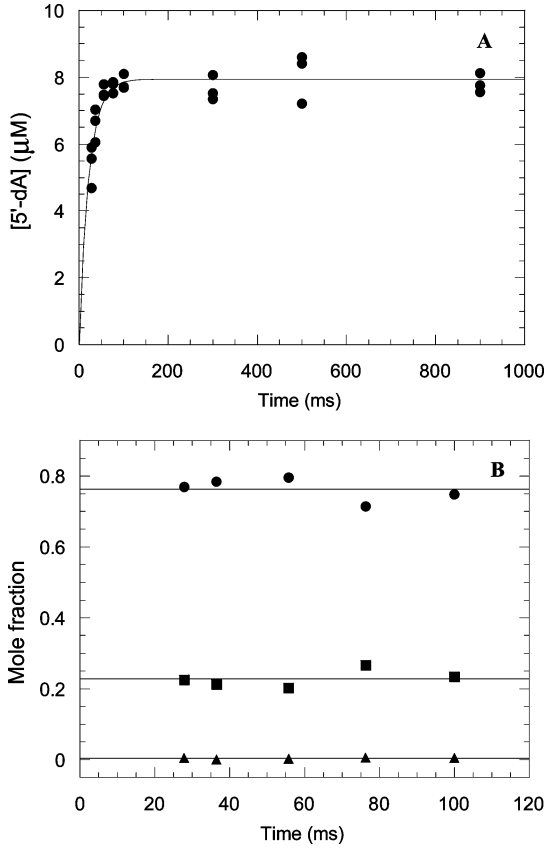

FIGURE 6: Measurement of $^{D}(V/K)$ kinetic isotope effect on 5′-dA formation. (A) Time course for the formation of 5′-dA when hologlutamate mutase was reacted with a mixture of 2.5 mM L-glutamate and 7.5 mM d_3-L-glutamate. (B) Relative amounts (mole fraction) of 5′-dA (●), d_1-5′dA (■), and d_2-5′dA (▲) determined by mass spectrometry at times between 20 and 100 ms, when multiply deuterated molecules comprise less than 1% of total.

but still considerably smaller than we had assumed on the basis of our previous data.

DISCUSSION

These experiments have provided insight into some unusual kinetic behavior that we uncovered several years ago when we examined kinetics of Cbl(II) formation when glutamate mutase was reacted with its substrates (*17*). We observed biphasic kinetics when the enzyme was reacted with deuterated substrates, which we interpreted as arising from negative cooperativity between the two active sites of the homodimeric enzyme. This explanation appeared to be reasonable because the two phases of the reaction had approximately equal amplitudes and we had previously observed weakly cooperative binding of the monomeric S subunit to the dimeric E subunit of the wild-type enzyme (*23*). Also, the stopped-flow traces obtained when the enzyme was reacted with protiated substrates provided some evidence of a very rapid reaction that was almost complete within the dead time of the spectrometer, implying that the protiated substrates were also reacting in a biphasic manner. However, it was not possible to measure either the rate or the amplitude of this phase because the reaction was occurring so rapidly. We therefore calculated the apparent deuterium kinetic isotope effects on Cbl(II) formation by comparing the rate constant for the reaction with protiated glutamate with that for the *slower* phase of the reaction with deuterated glutamate. This calculation yielded a value of ~28 for the isotope effect.

A further reason for our confidence in our original interpretation of the stopped-flow data was that similarly large deuterium isotope effects, of up to 50, on AdoCbl homolysis have been measured for the closely related methylmalonyl-CoA mutase reacting with deuterated methylmalonyl-CoA (*24*). Furthermore, very large kinetic isotope effects are also implicated in the mechanisms of AdoCbl-dependent diol dehydrase and ethanolamine ammonia lyase (*18*, *25*, *26*). More generally, large kinetic isotope effects are seen in enzymes such as lipoxygenase that catalyze reactions involving hydrogen atom abstraction from carbon (*27*, *28*).

The data we have presented here clearly show that the biphasic nature of the reaction with deuterated glutamate arises from the initial formation of monodeuterated 5′-dA, followed by the exchange of a second and then a third deuterium into the coenzyme in subsequent turnovers. If we recalculate the deuterium isotope effects from our original spectroscopic data, $^{D}V = 1.5$ for the enzyme reacting with deuterated glutamate, a value that is in reasonable agreement with the ^{D}V isotope effect of 2.4 calculated from the data we present here. It should also be noted that both ^{D}V and $^{D}(V/K)$ measurements will include a small, presumably normal, contribution from the secondary isotope effect associated with the presence of deuterium at the C-4 *pro-R* position of glutamate.

Comparison of the ^{D}V and $^{D}(V/K)$ isotope effects is also informative. The fact that ^{D}V is much smaller than $^{D}(V/K)$ implies that an isotopically insensitive step is kinetically significant in the mechanism of 5′-dA formation. We have previously undertaken pre-steady-state measurements of product formation on the enzyme, which rule out substrate binding and product release as rate-determining steps (*22*). We expect, therefore, that cobalt−carbon bond homolysis is most likely partially rate-determining and is responsible for suppressing the ^{D}V isotope effect, although another event such as possible conformational change in the protein cannot be ruled out. It is also likely that the intrinsic isotope effect on hydrogen transfer between substrate and coenzyme is significantly larger than the observed $^{D}(V/K)$ effect of 10 that we have measured. Indeed, it may be as large as the deuterium isotope effects of 30−50 measured for the radical enzymes discussed above.

Perhaps the most surprising observation to arise from these experiments is the large secondary equilibrium isotope effect on homolysis of AdoCbl and formation of 5′-dA that appears to be associated with incorporation of deuterium atoms into the coenzyme. Assuming that the amplitude of the slower phase of the reaction represents perturbation of the equilibrium between AdoCbl and 5′-dA and Cbl(II) caused by incorporation of two deuterium atoms at the 5′-position of AdoCbl and that this perturbation is the product of two identical secondary isotope effects, we estimate that the secondary equilibrium deuterium isotope effect is about 0.85 per deuterium atom. This implies that the 5′-hydrogens of 5′-dA make stiffer bonds to carbon (C−H bending modes occur at higher frequencies) than do the 5′-hydrogens of AdoCbl and is consistent with the equilibrium secondary tritium isotope effect of 0.72 that we measured previously (*13*).

Given that the 5′-carbon does not change its hybridization state in going from AdoCbl to 5′-dA, one might expect the equilibrium isotope effect to be close to unity. Indeed, fractionation factors for methylene hydrogens tend to be slightly larger than those for methyl hydrogens (*29*), so that a small normal equilibrium isotope effect might even have been predicted. One explanation is that the cobalt atom changes the vibrational modes of the methylene carbon bonded to it, resulting in much smaller fractionation factors. (To our knowledge fractionation factors for methylene groups bonded to transition metals have not been determined.) Another, more mechanistically interesting, interpretation of the isotope effect is that the enzyme distorts the geometry of the 5′-carbon so that it becomes more "sp^2-like" in character in the enzyme−substrate complex, thereby activating the coenzyme toward homolysis. Further theoretical and model studies should prove to be informative in helping to distinguish between these possibilities and to interpret better the data we have presented here.

In conclusion, we have investigated the kinetics of deuterium transfer from glutamate to 5′-dA catalyzed by glutamate mutase, and using mass spectrometry we have been able to observe the formation of multiply deuterated 5′-dA molecules. These measurements have caused us to reinterpret stopped-flow kinetic data on Cbl(II) formation obtained previously. We have found that the isotope effect on AdoCbl homolysis and 5′-dA formation is much smaller than we previously thought. The biphasic kinetics we observe with deuterated substrates appear to be the result of a large inverse equilibrium isotope effect that favors 5′-dA formation in multiply deuterated coenzyme molecules. Taken together, our data suggest that a step prior to hydrogen transfer, most likely cobalt−carbon bond homolysis, is significantly more rate-determining in the enzyme mechanism than we previously thought.

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