Tritium Partitioning and Isotope Effects in Adenosylcobalamin-Dependent Glutamate Mutase†

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ABSTRACT: Tritiated adenosylcobalamin, labeled at the exchangeable position, has been used to investigate the partitioning of tritium between substrate and product in the reaction catalyzed by glutamate mutase. The isotope partitions between glutamate and methylaspartate in nearly 1:1 ratio, regardless of the direction in which the overall reaction is proceeding. This is consistent with a free-energy profile in which the interconversion of the intermediate glutamyl and methylaspartyl radicals is rapid relative to the transfer of tritium from 5′-deoxyadenosine to either substrate or product. Initial velocity measurements have been used to measure the tritium isotope effects for the transfer of tritium from adenosylcobalamin to product in each direction. The isotope effect is 21 for the formation of glutamate and 19 for the formation of methylasparate. The large magnitude of these isotope effects makes it likely that the rate-determining step may be altered by the substitution of tritium for hydrogen in the reaction. The results of these experiments are compared with previous isotope effect measurements made on other adenosylcobalamin-dependent enzymes.

Adenosylcobalamin (AdoCbl, coenzyme B_{12})-dependent[1] glutamate mutase (EC 5.4.99.1) catalyzes the reversible interconversion of L-glutamate to L-*threo*-3-methylaspartate as the first step in the fermentation of L-glutamate by various species of Clostridia (*1*−*4*). It is one of a group of AdoCbl-dependent isomerases that catalyze unusual isomerizations in which a hydrogen atom on one carbon atom is interchanged with an electron-withdrawing group on an adjacent carbon (*5*−*8*). These rearrangements proceed through a mechanism involving free-radical intermediates that are generated by homolysis of AdoCbl. The B_{12}-dependent isomerases are themselves one group in a larger, newly recognized class of enzymes that use carbon-based free radicals to catalyze a variety of chemical transformations on otherwise unreactive substrates (*9*, *10*).

Glutamate mutase provides a simple system with which to study the phenomenon of radical-mediated enzymatic catalysis: both the substrates are small, stable molecules, the reaction is freely reversible, and the enzyme requires no cofactors other than AdoCbl (*3*). The crystal structure of the enzyme complexed with substrate and coenzyme analogues has recently been determined at 1.4 Å resolution (*11*). In the past few years, there have been a number of mechanistic studies on this enzyme and our current knowledge of the reaction mechanism, illustrated in Figure 1, may be summarized as follows.

The reaction is initiated by glutamate (or methylaspartate) binding to the enzyme (**I** or **VII**) which triggers homolysis of the Co−C bond of AdoCbl, resulting in the formation of 5′-deoxyadenosyl radical (**II** or **VI**). This species is very short-lived and immediately reacts to abstract a hydrogen atom from the substrate to form 5′-dA and the C-4 glutamyl radical (**III**), in a process that probably involves a high degree of hydrogen tunneling (*12*, *13*). EPR measurements indicate that the glutamyl radical, which appears to be the most stable radical species in the reaction pathway, is separated from the cobalt atom by about 6.6 Å (*14*). This distance is in good agreement with that predicted from the crystal structure (*11*). The next step in the reaction, the interconversion of glutamyl and methylaspartyl radicals, is most unusual as it formally involves the 1,2-migration of an sp^3-hybridized carbon, which is highly unfavorable. Recently, we have demonstrated that this step occurs through a fragmentation and recombination mechanism in which glycyl radical and acrylate (**IV**) are formed as intermediates (*15*). The final steps in the reaction are the reverse of the initial steps. A hydrogen is transferred from 5′-dA to the methylaspartyl radical to form methylaspartate and regenerate the 5′-deoxyadenosyl radical (**VI**), which immediately recombines with cob(II)alamin to form AdoCbl (**VII**). Finally, methylaspartate is released from the active site. Product release, in either direction, appears to be rapid (*16*).

In this paper we report results of tritium partitioning experiments on glutamate mutase and the measurement of tritium isotope effects for the transfer of tritium from 5′-dA to glutamate and methylaspartate. The partitioning of hydrogen isotopes from an enzyme-bound intermediate between products and substrates provides an elegant way of investigating the relative heights of energetic barriers in an enzyme-catalyzed reaction. Notably, this approach was used by Knowles and co-workers to elucidate the free-energy profile of triosephosphate isomerase. More recently, tritium partitioning experiments have been used to investigate the free-energy profile of AdoCbl dependent methylmalonyl-CoA mutase (*17*).

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[1] Abbreviations: AdoCbl, adenosylcobalamin; Cbl(II), cob(II)alamin; 5′-dA, 5′-deoxyadenosine; TFA, trifluoroacetic acid.

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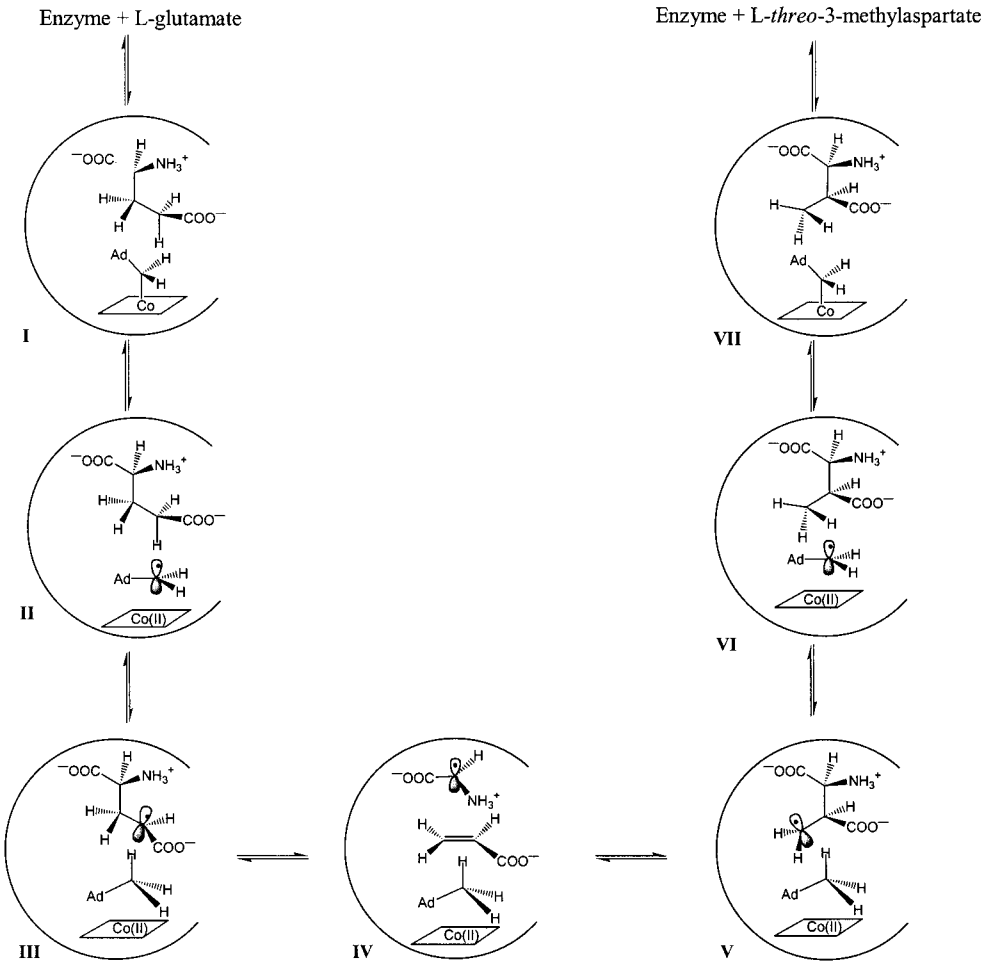

FIGURE 1: Mechanism for the reversible rearrangement of L-glutamate to L-*threo*-3-methylaspartate catalyzed by glutamate mutase.

MATERIALS AND METHODS

Materials. The purification of the glutamate mutase fusion protein, GlmES, from a recombinant *Escherichia coli* strain has been described previously (*4*). AdoCbl, 5′-dA, L-glutamate, D,L-*threo*-3-methylasparate, leucine, and dansyl chloride were purchased from the Sigma Chemical Company. The sources of other materials have been described previously (*3*, *4*, *12*) or were purchased from commercial suppliers. [5′-^{3}H]AdoCbl was prepared enzymatically by using GlmES protein to exchange tritium into the coenzyme from ^{3}H-labeled glutamate in a slight modification of the procedure described previously (*18*). Racemic *threo*-3-methylaspartate was used in these experiments as the D-isomer has previously been shown to be neither a substrate nor an inhibitor of the enzyme (*4*).

Rapid Quench Kinetic Experiments. Tritium partitioning experiments were performed at 10 °C with a Hi-Tech Scientific (U.K.) RQF-63 rapid mixing apparatus. The temperature of the mixing chamber was maintained at 10 °C using a circulating water bath. The enzyme solution contained 100 μM GlmES in 50 mM potassium phosphate buffer, pH 7.0. Immediately before the experiment, radiolabeled 5′-[^{3}H]-AdoCbl, specific activity of 42 000 dpm/nmol, was added to a final concentration of 120 μM. The effective concentration of holoenzyme after mixing was 45 μM. The substrate solution contained either 20 mM L-glutamate or 4 mM L-*threo*-3-methylaspartate (final concentrations 10 and 2 mM, respectively) dissolved in the same buffer as the enzyme and also containing 500 μM leucine and 100 μM L-tryptophan as internal standards. Reactions were started by automated mixing of 80 μL of enzyme solution with 80 μL of substrate solution. The solution was allowed to age for various times (30−250 ms) before being quenched with a further 80 μL of 5% trifluoroacetic acid. A total of 25 μL of the solution was taken from each sample and stored in the dark at −20 °C prior to HPLC analysis of AdoCbl and 5′-dA. The remainder of the sample was centrifuged for 30 min in order to remove precipitated protein. The supernatant was removed and stored at −20 °C prior to derivatization with dansyl chloride and HPLC analysis for glutamate and methylaspartate content.

Derivatization and HPLC Analysis of Glutamate and Methylaspartate. The volume of quenched reaction mixture was reduced to 50 μL using a Speedvac apparatus, and then 100 μL of 0.5 M $NaHCO_3$ was added to raise the pH above 8.5. Fifty microliters of a 20 mg/mL solution of dansyl chloride in acetone was added and the sample was incubated at 45 °C for 1 h to derivatize the substrate, product, and internal standard. Dansylated glutamate, methylaspartate, and leucine were separated by HPLC using a 25 cm C_{18} reversed-phase column (Alltech Alltima C18 5 μm). The column was preequilibrated in 92.5% solvent A: 25 mM potassium phosphate buffer containing 7% acetonitrile and 3% methanol; and 7.5% solvent B: 70% acetonitrile, 30% methanol.

A total of 200 μL of sample was injected onto the column, and the derivatized amino acids were eluted with an ascending gradient of solvent B as follows: 0 to 8.5 min, 7.5 to 50% B; 8.5 to 18.5 min, 50 to 80% B; 18.5 to 20 min, 80% B; 20 to 20.5 min, 80 to 100% B; 20.5 to 25.5 min, 100% B; 25.5 to 26 min, 100 to 7.5% B; 26 to 36 min, 7.5% B. The flow rate was 1.4 mL/min, and compounds were detected by monitoring absorbance at 330 nm. Dansyl-glutamate and dansyl-methylaspartate peaks were collected and tritium content determined by scintillation counting. A blank run was performed prior to each sample injection to remove any contamination from the compounds left from previous sample analysis.

Analysis of 5′-dA and AdoCbl. 5′-dA and AdoCbl were separated by HPLC using a 25 cm C_{18} reversed-phase column as described previously (*16*). L-Tryptophan was included as an internal standard. The amount of 5′-dA in the sample was calculated from standard curves obtained by chromatography of known amounts of 5′-dA.

RESULTS

These experiments used the engineered fusion protein of glutamate mutase, GlmES, that we have used in previous mechanistic investigations (*4*, *12*, *15*, *16*, *19*). In this construct, the S subunit is fused to the C-terminus of the E-subunit by an 11 amino acid flexible linker. This protein is better suited to kinetic analysis because complications due to concentration-dependent dissociation of the subunits are avoided (*4*).

Experimental Design. In designing a partitioning experiment, it is important to ensure that the transfer of tritium from the coenzyme to either the substrate or product of the reaction is effectively irreversible. If this is not the case, and the tritium can make multiple passages through the enzyme, then the distribution of tritium will tend to reflect the relative concentrations of the reactants in the mixture. Previously, we have measured the tritium isotope effect for the transfer of tritium from AdoCbl to methylaspartate (*18*). In this case, it was possible to make the reaction effectively irreversible by coupling it to the formation of mesaconate through the action of methylaspartase. However, this experimental set up did not allow us to measure the reaction in the reverse direction nor was the possibility that tritium might be partitioning back to glutamate addressed.

Previous tritium isotope effect measurements on AdoCbl-dependent enzymes have followed the time course for the complete transfer of tritium from AdoCbl to the product. Under these conditions, the specific activity of the coenzyme decreases exponentially with time, and the rate constant for tritium transfer can readily be calculated. Comparison of this number with the turnover number for the enzyme yields the apparent isotope effect. However, if the tritium transfer is slow (large isotope effect), and the reaction is reversible (as is the case with glutamate mutase), then the reaction may approach equilibrium before all the isotope is transferred. To overcome this problem, we have taken a different approach to determining the kinetics of tritium transfer by measuring the initial rates at which tritium appears in either substrate or product.

Our experiments employed relatively high concentrations of enzyme and coenzyme to avoid possible complications due to the dissociation of AdoCbl from the protein (*18*). The effective concentration of holo-enzyme (after mixing) was 45 μM, which is over 20-fold higher than the K_d of 2 μM for AdoCbl. Furthermore, previous studies on the wild-type two subunit enzyme indicate that coenzyme exchange is very slow relative to turnover, $k_{off} = 0.01\ s^{-1}$ (*18*), and coenzyme exchange in GlmES appears to be similarly slow. Therefore, in the rapid time scale of the present experiments, exchange of coenzyme is unlikely to contribute to the kinetics of tritium transfer.

To ensure that the reaction did not approach equilibrium during the experiments, high concentrations of substrates and short reaction times were used. The holoenzyme was reacted with either 10 mM L-glutamate or 2 mM L-*threo*-3-methylaspartate (final concentrations). These substrate concentrations are 17 x K_m and 14 x K_m, respectively, so that the enzyme was effectively saturated with substrate. The reaction between the substrate and holoenzyme was initiated by automated mixing in a rapid quenched flow apparatus, and the solution was allowed to age for various times between 37 and 260 ms before quenching with 5% TFA solution. On this time scale, less than 0.5% of the glutamate and 2.5% of the methylaspartate were consumed during the reaction, ensuring that the reaction remained far from equilibrium and that the reverse reaction was negligible.

The quenched solutions were divided into two portions; one portion was used to determine the concentration and tritium content of AdoCbl and 5′-dA, and the other to determine the concentration and tritium content of glutamate and methylaspartate, as described in Materials and Methods. To facilitate comparison of the data, we have expressed the concentrations of tritiated products in micromolar units, based on the specific activity of the starting tritiated AdoCbl.

Loss of tritium from 5′-dA. The concentration of 5′-dA present in the reaction mixture at each time point was determined by measuring the peak area of the 5′-dA peak in the HPLC chromatogram, from which the concentration could be obtained by comparison with standard curves, as described previously (*16*). The tritium content of the recovered 5′-dA was determined by scintillation counting. From tritium counting, the concentration of 5′-dA could be determined independently based on the known specific activity of the AdoCbl starting material. As shown in Figures 2A and 3A, the two methods for determining the initial concentration of 5′-dA yielded results that were in good agreement with each other. As expected, on the basis of previous kinetic experiments (*16*) the concentration of 5′-dA was constant throughout the time course of the experiment, and the steady-state concentrations of 5′-dA were different, depending upon which substrate was used (Figures 2A and 3A).

Tritium loss from the coenzyme was linear with time (there may be a short initial lag phase, although this is difficult to discern from the data). The rate at which tritium was lost was similar regardless of the substrate and approximated the rate at which tritium appeared in glutamate and methylaspartate. During the course of the experiment, less than 20% of the tritium was transferred from 5′-dA to substrate or product, thus the reaction did not deviate significantly from pseudo-first-order conditions.

Appearance of Tritium in Substrate and Product. The time courses for tritium partitioning between substrate and product

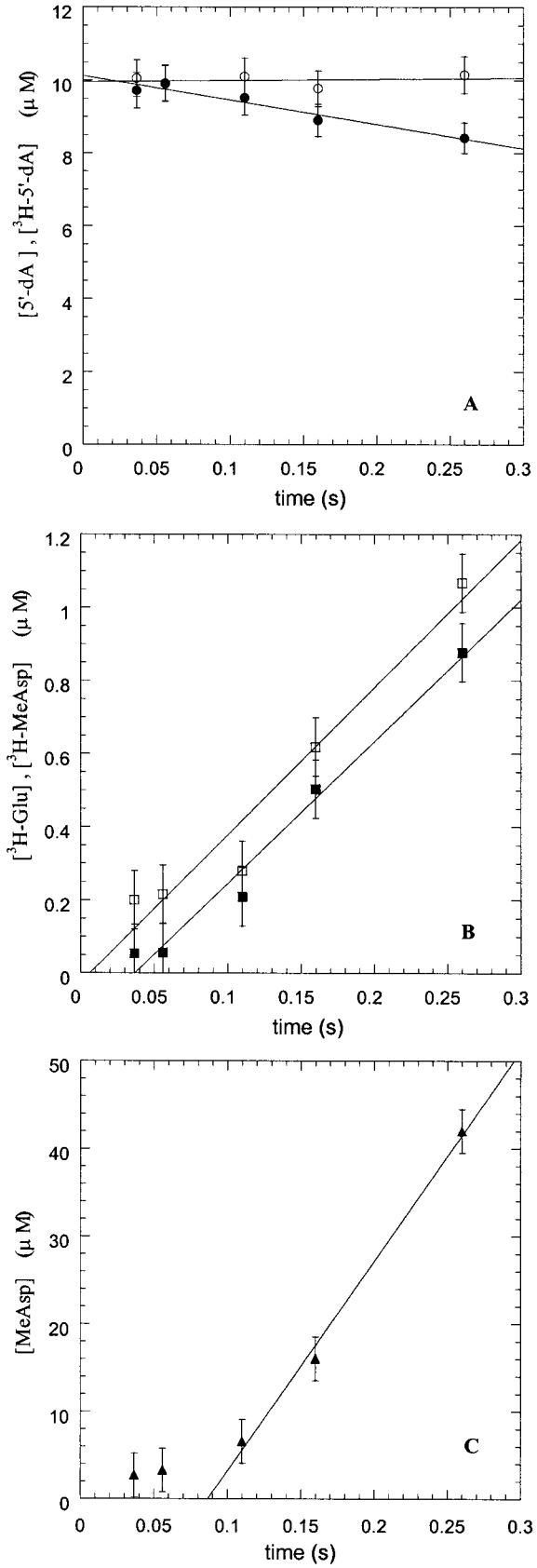

FIGURE 2: Kinetics of tritium transfer and product formation when glutamate mutase was reacted with 10 mM L-glutamate. (A) Variation of 5′-dA concentration (○) and reduction of specific activity of 5′-dA (●) as a function of time. (B) Formation of tritiated glutamate (□) and methylaspartate (■) as a function of time. (C) Formation of product methylaspartate (▲). Experimental details are given in the text.

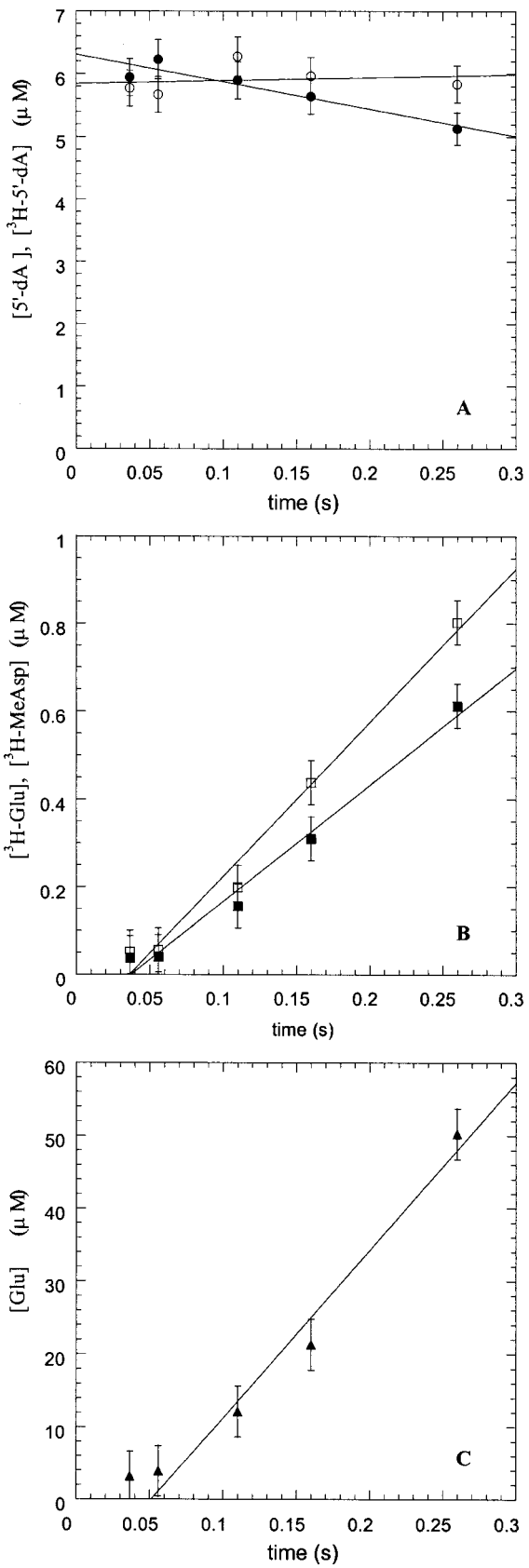

FIGURE 3: Kinetics of tritium transfer and product formation when glutamate mutase was reacted with 2 mM L-*threo*-3-methylaspartate. (A) Variation of 5′-dA concentration (○) and reduction of specific activity of 5′-dA (●) as a function of time. (B) Formation of tritiated glutamate (□) and 3-methylaspartate (■) as a function of time. (C) Formation of product glutamate (▲). Experimental details are given in the text.

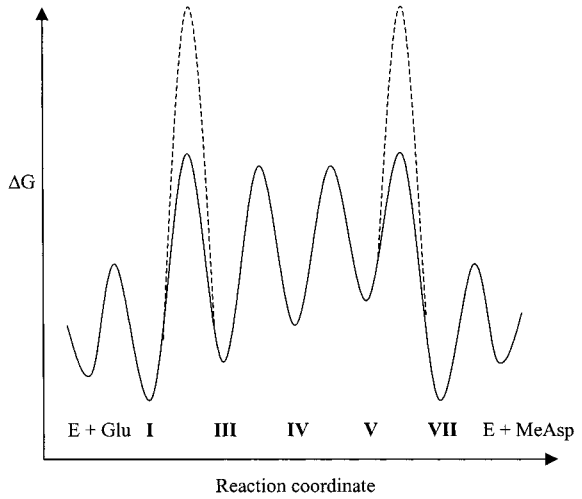

FIGURE 4: Qualitative free energy profile for the reaction catalyzed by glutamate mutase in which the tritium isotope effects are indicated by dashed lines. The intermediates represented by Roman numerals correspond to the chemical species illustrated in Figure 1.

when the enzyme was reacted with either glutamate or methylaspartate are shown in Figures 2B and 3B, respectively. It is evident that with either substrate there is an initial lag phase before the velocity of tritium transfer becomes linear with time. The origin of this lag phase is not certain, but is probably associated with the approach toward the steady state. When glutamate is the substrate, the velocities with which tritium appears in the substrate and product are, within error, equal, and the partition coefficient, v_{Glu}/v_{MeAsp}, equals 1.1 ± 0.15. When methylaspartate is the substrate, the velocity at which tritium appears in glutamate is slightly greater than that with which tritium appears in methylaspartate. However, the partition coefficient, $v_{Glu}/v_{MeAsp} = 1.3 \pm 0.15$, is not significantly different from the results obtained with glutamate. Overall, these results indicate that the barriers for the transfer of tritium between 5′-dA and either methylaspartyl radical or glutamyl radical are of very similar energy. Furthermore, since the partitioning ratios are independent of the direction in which the reaction is proceeding, the interconversion of methylaspartyl and glutamyl radicals must be rapid compared with tritium transfer.

Velocities of Product Formation. The velocity of product formation under the conditions of the partitioning experiments was determined by integrating the areas of either the dansyl-glutamate or dansyl-methylaspartate product peaks in the HPLC chromatogram. The concentration of product in the reaction mixture was then calculated by comparison with standard curves (Figures 2C and 3C). With each substrate an initial lag phase was observed before the reaction became linear. With 10 mM glutamate as substrate the reaction velocity was 229 ± 25 μM/s, whereas with 2 mM methylasparate as substrate the reaction velocity was 238 ± 30 μM/s. Assuming an effective holoenzyme concentration of 45 μM, the turnover numbers with glutamate and methylaspartate were 5.3 ± 0.5 and 5.1 ± 0.5 s^{-1} respectively. These numbers are in good agreement with k_{cat} values for GlmES previously determined from coupled spectroscopic assays: $k_{cat} = 5.8$ s^{-1} with glutamate as substrate and $k_{cat} = 5.8$ s^{-1} with methylaspartate as substrate (*4*).

The velocities of the various reactions discussed above, calculated from the data shown in Figures 2 and 3, are summarized in Table 1.

DISCUSSION

Tritium Isotope Effects. Previous measurements of tritium isotope effects in AdoCbl-dependent isomerases have calculated the isotope effects from the ratio of two apparent rate constants k_{cat} and k_T (k_T is the apparent first order rate constant for tritium transfer). This method carries the disadvantage of having to know accurately the concentration of enzyme active sites, which may be difficult to determine. This quantity is used to calculate the rate constant for (unlabeled) hydrogen transfer from the coenzyme, which is assumed to equal k_{cat}. The active-site concentration is usually assumed to equal the concentration of tritiated AdoCbl in the reaction, assuming that all the AdoCbl is bound when the enzyme is in excess, but this may not be so. Furthermore, exchange between bound and unbound coenzyme can contribute to the kinetics of tritium transfer (*18*), which could potentially give rise to spuriously large isotope effects.

From the data collected in Table 1, the tritium isotope effects can be calculated very easily by direct comparison of the initial velocities of formation of tritiated and nontritiated products. This method has the advantage that there is no need to know the enzyme concentration; it is only necessary to ensure that the specific activity of the tritiated coenzyme does not change significantly during the time course of the experiment. We were able to demonstrate that this requirement was met by isolating 5′-dA from the reaction and determining its specific activity.

To calculate the tritium isotope effect for the transfer of isotope from 5′-dA, a statistical factor must be introduced to account for the fact that the tritium atom is only one of three equivalent hydrogens in the methyl group. Initially, Abeles and co-workers used a statistical factor of 2 in calculating the tritium isotope effect in dioldehydrase (*20*). Subsequent measurements on other enzymes have also used a statistical factor of 2 (*17*, *18*, *21*). However, it has been pointed out[2] that this is incorrect because the factor of 2 only accounts for the *intra*molecular competition between one tritium and two hydrogen atoms in the methyl group. In fact, the statistical factor should be 3 because tritium is present only in trace amounts in 5′-dA, and therefore, the major flux of reactants through the enzyme will encounter completely unlabeled 5′-dA. The calculation of the statistical factor is dealt with in detail in the appendix to this article. To facilitate comparisons between previous isotope effect measurements and the present work, the corrected tritium isotope effects for the various enzymes are summarized in Table 2.

From the data presented in Table 1, the tritium isotope effect for the transfer of hydrogen from 5′-dA to methylaspartyl radical is 19, and that for the transfer of hydrogen from 5′-dA to glutamyl radical is 21. These isotope effects are larger (although not greatly so) than that determined previously in our laboratory for the transfer of tritium from 5′-dA to methylaspartyl radical (*18*) (Table 2). There are

[2] We thank Prof. Rowena Matthews and Prof. Duilio Arigoni for bringing this fact to our attention.

Table 1: Velocities of Tritium Transfer between 5′-dA and Product and Substrate and Velocities of Product Formation[a]

substrate	reaction measured	velocity of reaction (μM/s)
L-glutamate	loss of tritium from 5′-dA	7.5 ± 0.9
L-glutamate	appearance of tritium in Glu	4.4 ± 0.6
L-glutamate	appearance of tritium in MeAsp	4.1 ± 0.3
L-glutamate	formation of MeAsp	238 ± 30
L-*threo*−3-methylaspartate	loss of tritium from 5′-dA	5.3 ± 0.2
L-*threo*−3-methylaspartate	appearance of tritium in Glu	3.7 ± 0.2
L-*threo*−3-methylaspartate	appearance of tritium in MeAsp	2.8 ± 0.1
L-*threo*−3-methylaspartate	formation of Glu	229 ± 25

[a] Values calculated from data shown in Figures 2 and 3.

Table 2: Tritium Isotope Effect for the Transfer of Tritium from 5′-Deoxyadenosine to Product Measured for Various AdoCbl-Dependent Enzymes[a]

enzyme	transfer reaction examined	tritium isotope effect	ref
diol dehydrase	5′-dA to acetaldehyde	83*	*20*
ethanolamine ammonia lyase	5′-dA to 1,1-aminoethanol	107*	*21*
glutamate mutase	5′-dA to 3-methylaspartate	12*	*18*
methylmalonyl-CoA mutase	5′-dA to succinyl-CoA	3.3*	*17*
glutamate mutase	5′-dA to glutamate	21	this work
glutamate mutase	5′-dA to 3-methylaspartate	19	this work

[a] The values marked by an asterisk (*) differ from those originally published in that they have been corrected by dividing by a statistical factor of 3, rather than a statistical factor of 2 that was used in the cited reference.

several reasons why the results may differ. First, the current measurements were conducted on an engineered fusion protein, GlmES, which we have used in almost all of our mechanistic studies. The initial tritium isotope measurements on glutamate mutase were conducted using the wild-type two-subunit enzyme (MutE + MutS) with an excess of MutS protein to drive formation of the holoenzyme (*18*). These two enzymes have similar, but not identical, kinetic properties, including slightly different V_{max} deuterium isotope effects (*4*), so it would not be surprising if the tritium isotope effects also differed slightly. Second, the two isotope effect measurements were made using different experimental approaches, as discussed above, and these differences may have introduced some systematic error into one or the other measurement.

As Table 2 illustrates, an extremely wide range of values for tritium isotope effects have been measured for AdoCbl-dependent isomerases. Even after the corrected statistical factor has been applied, the isotope effects for ethanolamine ammonia lyase and diol dehydrase are remarkably large. These very large isotope effects have been rationalized by invoking the existence of a second pool of hydrogen atoms on the protein that can equilibrate with the coenzyme (*22*). However, recent kinetic studies on ethanolamine ammonia lyase demonstrated that only two hydrogen atoms in the coenzyme:enzyme complex are exchangeable (*23*). This result makes it very unlikely that an intermediate protein radical, akin to that formed in ribonucleotide reductase, is formed in this enzyme.

It is more likely that the anomalously large tritium isotope effects are manifestations of hydrogen atom tunneling between the substrate and coenzyme. Pre-steady-state stopped-flow spectroscopic studies have examined the intrinsic deuterium isotope effects for the transfer of hydrogen from substrate to coenzyme. Very large deuterium isotope effects have been measured for glutamate mutase, $k_H/k_D = 28-35$, (*12*), methylmalonyl-CoA mutase, $k_H/k_D = 35-50$, (*13*) and a large isotope effect, >10, is estimated for ethanolamine ammonia lyase (*23*). Tunneling is most firmly established for methylmalonyl-CoA mutase where the temperature dependence of the deuterium kinetic isotope effect has been investigated (*13*).

Partition Ratios and Free-Energy Profile. Isotope effect measurements when combined with partitioning analysis can provide much useful information on the free-energy profile of enzyme-catalyzed reactions. Leadlay and co-workers have previously examined tritium-partitioning from AdoCbl in methylmalonyl-CoA mutase (*17*). Their data also indicated that interconversion of product and substrate radicals was fast compared with tritium transfer, although in this enzyme, transfer of tritium from 5′-dA to succinyl-CoA radical is favored by about 3:1 over transfer to methylmalonyl-CoA radical which reflects the overall equilibrium for the reaction that lies 20:1 in favor of succinyl-CoA. Furthermore, the tritium isotope effect they measured was very small, indicating that a slow, isotopically insensitive step contributed to the overall rate of reaction. Since the partitioning analysis indicated that interconversion of substrate and product radicals was fast, it is most likely that product release is significantly rate determining for this enzyme.

In the glutamate mutase reaction, tritium at the exchangeable position of the coenzyme partitions in an almost 1:1 ratio between substrate and product. This indicates that the energetic barriers for the transfer of tritium from 5′-dA to glutamyl radical and methylaspartyl radicals are of equal height. Furthermore, the partitioning ratio is essentially independent of the direction in which the reaction is proceeding, implying that the interconversion of glutamyl and methylaspartyl radicals is fast relative to tritium transfer. In contrast to methylmalonyl-CoA mutase though, the tritium isotope effects are quite large, suggesting that hydrogen transfer steps may be fully rate determining in this enzyme. Furthermore, pre-steady-state measurements on glutamate mutase found no evidence that slow product release, in either direction, contributes to the kinetics of the reaction (*16*). However, as discussed below, we think that this is unlikely

to be the case because the intrinsic tritium isotope effects may be large enough to alter the rate-determining step in the reaction.

Several previous kinetic studies point to the likelihood that both hydrogen transfer from substrate to coenzyme and the rearrangement of substrate radical to product radical contribute to the overall rate of the reaction in glutamate mutase (*12*, *15*, *16*). In particular, measurements of the deuterium isotope effects for the transfer of hydrogen between substrate and coenzyme indicate that an isotopically insensitive step is partly rate determining. Thus, the steady-state deuterium isotope effects on V_{max} are 3.9 and 6.3 determined with 4-d_2-L-glutamate and d_3-methyl-L-*threo*-3-methylaspartate respectively (*4*). Whereas the corresponding isotope effects for the transfer of hydrogen from substrate to coenzyme measured by pre-steady state techniques are 28 and 35, respectively (*12*), and are probably close to their intrinsic values. Comparison of these two sets of measurements indicates that in the overall catalytic cycle the intrinsic deuterium isotope effects are suppressed 5−7-fold by isotopically insensitive steps in the mechanism. One would therefore expect the tritium isotope effects to be suppressed by a similar factor.

The intrinsic tritium isotope effects may also be calculated from the pre-steady-state deuterium isotope effects using the Swain-Schaad relationship (*24*), $1.44 \log(k^H/k^D) = \log(k^H/k^T)$. This relationship predicts the intrinsic tritium isotope effects to be in the range of 120−160, although, if tunneling plays a significant role, they are expected to be even larger than this (*25*). This would place the tritium isotope effects for glutamate mutase in the range of those measured for dioldehydrase and ethanolamine ammonia lyase, and they would certainly be much larger than that expected classically. Since tritium substitution most likely slows the rate of hydrogen transfer by over 100-fold it is very likely that the rate-determining step in the reaction is altered.

APPENDIX

Statistical Factors in Kinetic Isotope Experiments. There has been some confusion in the B_{12} literature regarding the method used to calculate the statistical factor necessary to correct tritium kinetic isotope effects arising from transfer of tritium from a methyl group. Therefore, a derivation of the statistical factor is given below. Although illustrated for a tritium isotope effect, the argument may be generalized for any competitive isotope experiment in which both *intra*molecular and *inter*molecular competition effects are possible.

Consider a substrate molecule, *S*, that possesses *n* chemically equivalent reactive atoms or groups. (In the present case, this would be the reactive hydrogen atoms in the methyl group of 5′-deoxyadenosine, so $n = 3$.) The probability, Φ, of any given atom undergoing reaction is simply $1/n$. If some fraction of these molecules contains a single tritium atom at the reactive position, the specific activity, A^S, of the substrate is defined by eq 1. Similarly, the specific activity of the product molecules to which tritium is transferred in the reaction, A^P, is defined by eq 2 (where the superscripts H and T refer to the protiated and tritiated molecules, respectively.)

$$A^S = \frac{[S^T]}{[S^T] + [S^H]} \quad (1)$$

$$A^P = \frac{[P^T]}{[P^T] + [P^H]} \quad (2)$$

For a reaction proceeding under initial velocity conditions, so that A^S does not change significantly, then the experimentally measured kinetic isotope effect, KIE_{obs}, is simply the ratio of the specific activities of the substrate and product molecules (eq 3).

$$KIE_{obs} = \frac{A^S}{A^P} \quad (3)$$

Furthermore, A^P is related to the rates, ν^T and ν^H, at which the tritiated and protiated substrates react by eq 4

$$A^P = \frac{\nu^T}{\nu^T + \nu^H} \quad (4)$$

Under first-order conditions, ν^T is simply given by the apparent rate constant for tritium transfer, k^T, multiplied by the concentration of tritiated substrate molecules, multiplied by Φ (eq 5). Similarly, ν^H is given by the apparent rate constant for protium transfer, k^H, multiplied by the concentration of protiated substrate molecules, but must also include a term to account for protium undergoing reaction in the tritiated molecules (eq 6)

$$\nu^T = k^T[S^T]\Phi \quad (5)$$

$$\nu^H = k^H[S^H] + k^H[S^T](1 - \Phi) \quad (6)$$

Substitution of eqs 5 and 6 into eq 4 yields the expression for A^P in terms of rate constants and substrate concentrations (eq **7**).

$$A^P = \frac{k^T[S^T]\Phi}{k^T[S^T]\Phi + k^H[S^H] + k^H[S^T](1 - \Phi)} \quad (7)$$

Further substitution of eqs 1 and 7 into eq 3 yields eq 8, which is the general solution relating the observed kinetic isotope effect to the specific activity of the substrate and the rate constants for tritium and hydrogen transfer.

$$KIE_{obs} = \frac{[S^T]}{[S^T] + [S^H]} \cdot \frac{k^T[S^T]\Phi + k^H[S^H] + k^H[S^T](1 - \Phi)}{k^T[S^T]\Phi} \quad (8)$$

Two limiting cases are now considered.

Isotope Present as Tracer. Most experiments involve tritium as a tracer label so that $[S^T] \gg [S^H]$, in which case eq 8 simplifies to give eq 9, for which $\Phi = 1/3$ in the present case.

$$KIE_{obs} = \frac{k^H}{k^T\Phi} \quad (9)$$

All Molecules Isotopically Labeled. If carrier-free substrate is employed (more commonly encountered for stable isotopes), then $[S^H] = 0$ and eq 8 simplifies to give eq 10, in

which the $k^T\Phi$ term in the numerator may be neglected if $k^H \gg k^T$.

$$KIE_{obs} = \frac{k^T\Phi + k^H(1-\Phi)}{k^T\Phi} \approx \frac{k^H(1-\Phi)}{k^T\Phi} \quad (10)$$

Equation 10 describes the case where only *intra*-molecular competition occurs and is the basis for Abeles' use of a statistical factor of 2 in his original isotope measurements on diol dehydrase *(20)*.

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