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Hydrogen Tunneling in Adenosylcobalamin-Dependent Glutamate Mutase: Evidence from Intrinsic Kinetic Isotope Effects Measured by Intramolecular Competition[†]

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ABSTRACT: Hydrogen atom transfer reactions between the substrate and coenzyme are key mechanistic features of all adenosylcobalamin-dependent enzymes. For one of these enzymes, glutamate mutase, we have investigated whether hydrogen tunneling makes a significant contribution to the mechanism by examining the temperature dependence of the deuterium kinetic isotope effect associated with the transfer of a hydrogen atom from methylaspartate to the coenzyme. To do this, we designed a novel intramolecular competition experiment that allowed us to measure the intrinsic kinetic isotope effect, even though hydrogen transfer may not be rate-determining. From the Arrhenius plot of the kinetic isotope effect, the ratio of the pre-exponential factors (A_H/A_D) was 0.17 ± 0.04 and the isotope effect on the activation energy [$\Delta E_{a(D-H)}$] was 1.94 ± 0.13 kcal/mol. The results imply that a significant degree of hydrogen tunneling occurs in glutamate mutase, even though the intrinsic kinetic isotope effects are well within the semiclassical limit and are much smaller than those measured for other AdoCbl enzymes and model reactions for which hydrogen tunneling has been implicated.

Evidence supporting quantum mechanical tunneling of hydrogen has been obtained for an increasing number of enzyme reactions (*1*−*9*). The most common, and readily interpretable, test for hydrogen tunneling involves measuring the temperature dependence of kinetic isotope effects (*9*, *10*) (KIEs),[1] although other kinetic isotope-based tests have also been used to diagnose hydrogen tunneling (*11*−*13*). Such experiments have subjected enzyme-catalyzed reactions to detailed scrutiny and required the development of improved theoretical descriptions to accurately describe the data (*1*, *2*). This, in turn, has resulted in some of the most complete descriptions of enzymatic transition states and led to an improved understanding of the important role that protein dynamics plays in catalysis.

In this report, we present data to substantiate hydrogen tunneling in the AdoCbl-dependent enzyme glutamate mutase, which catalyzes the radical-mediated rearrangement of L-glutamate to L-*threo*-3-methylaspartate (*14*−*18*). The transfer of a hydrogen atom between the substrate and coenzyme is a key step in the mechanisms of all AdoCbl-dependent enzymes (*19*, *20*). First, homolysis of the AdoCbl cobalt−carbon bond transiently generates 5′-deoxyadenosyl radical; next, this highly unstable radical immediately reacts to abstract a hydrogen atom from the substrate, thereby generating a substrate radical that undergoes subsequent steps in the mechanism.

Evidence to support hydrogen tunneling in AdoCbl reactions has been obtained from the temperature dependence of KIEs measured in AdoCbl-dependent methylmalonyl-CoA mutase (*21*) and from model studies on the thermolysis of AdoCbl and neopentyl-Cbl in protiated and deuterated ethylene glycol (*22*, *23*). In these studies, very large deuterium KIEs (ranging from ∼20 to ∼50) that exceed the semiclassical limit of ∼7 expected in the absence of tunneling were reported. However, we recently measured the intrinsic KIE for the transfer of hydrogen between methylaspartate and 5′-dA in glutamate mutase (*24*) and found it to be much smaller (KIE ∼ 4 at 10 °C), which is clearly well within the semiclassical limit. Thus, it was unclear whether hydrogen transfer in glutamate mutase also involves a significant degree of quantum tunneling, as appears to be the case in methylmalonyl-CoA mutase.

In this paper, we have determined the temperature dependence of the intrinsic deuterium KIE for the transfer of hydrogen from methylaspartate to 5′-dA, catalyzed by glutamate mutase. The KIE was found to be temperature-dependent, leading to an apparent isotope effect on the Arrhenius pre-exponential factors (A_H/A_D) of 0.17 ± 0.04 and an isotope effect on the activation energy [$\Delta E_{a(D-H)}$] of 1.94 ± 0.13 kcal/mol. These parameters are different from those measured previously for methylmalonyl-CoA mutase and the thermolysis of model alkyl cobalamins (*21*−*23*); in both of these cases, A_H/A_D was smaller and $\Delta E_{a(D-H)}$ was larger.

EXPERIMENTAL PROCEDURES

Reagents. Synthesis of d_1-methyl-(2*S*,3*S*)-methylaspartate and purification of glutamate mutase from recombinant *Escherichia coli* have been described previously (*25*, *26*). All other reagents were of the highest quality commercially available. We note that these experiments were all performed with the GlmES fusion protein in which the weakly associating S and E subunits of glutamate mutase have been genetically fused together; this simplifies kinetic analyses by removing the protein concentration dependence from the kinetics of the reaction (*25*).

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[1]Abbreviations: AdoCbl, adenosylcobalamin; 5′-dA, 5′-deoxyadenosine; KIE, kinetic isotope effect; FT-ICR-MS, Fourier transform ion cyclotron resonance mass spectrometry.

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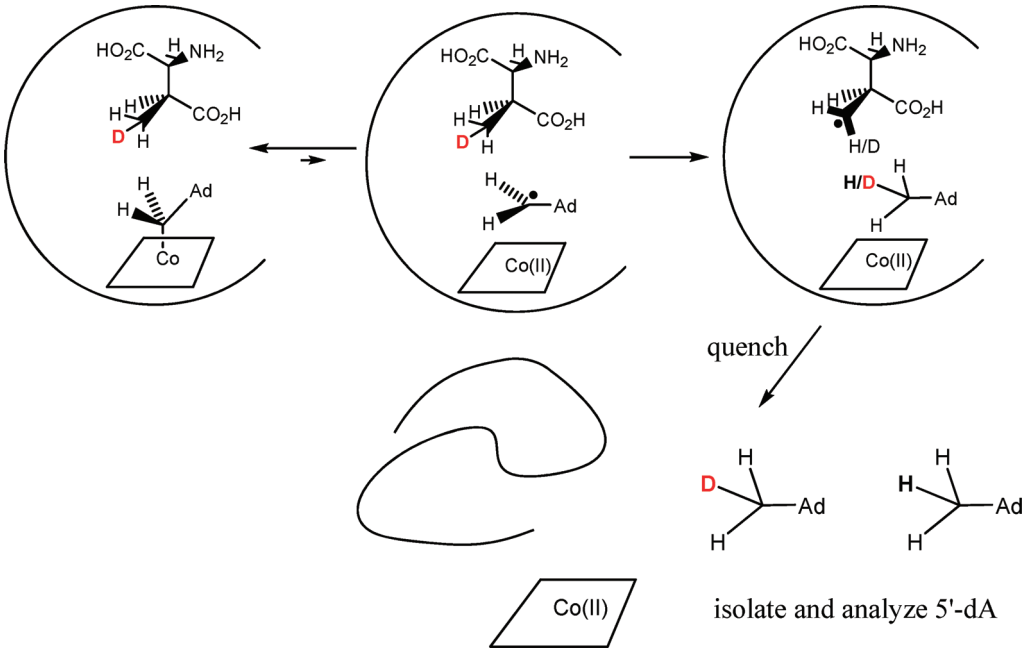

FIGURE 1: Measurement of intrinsic KIEs for the transfer of a hydrogen atom from methylaspartate to 5′-dA in glutamate mutase by intramolecular competition between deuterium and hydrogen atoms at the methyl group of the substrate.

Rapid quench experiments were performed at various temperatures between −2.5 °C (the lowest temperature accessible without needing to include cryoprotectants to prevent freezing) and 30 °C using a Hi-Tech RQF-63 apparatus; 73.5 μL of 275 μM glutamate mutase in 50 mM potassium phosphate buffer (pH 7.0) was mixed with 6.5 μL of 3.4 mM AdoCbl immediately before the experiment to reconstitute the holoenzyme. The holoenzyme mixture was reacted with 80 μL of 2.2 mM d_1-methylaspartate in 50 mM potassium phosphate buffer (pH 7.0). The reactions were quenched with 80 μL of 2.5% trifluoroacetic acid, after the mixtures had been aged for various periods of time (12−160 ms). 5′-dA was recovered and quantified by C_{18} reverse-phase HPLC as described previously (*27*). All the procedures were conducted in dim red light. Samples of labeled 5′-dA were lyophilized and stored at −20 °C prior to mass spectrometric analysis.

Mass Spectral Analysis. 5′-dA was redissolved in solvent containing 50% MeOH and 0.1% formic acid. Experiments were performed in positive ion mode at 50 μL/h with a 7 T Q-FT-ICR mass spectrometer (Bruker Daltonics, Billerica, MA) as described previously (*28*). Data were collected in narrowband mode. The masses of $^{12}C_9\,^{1}H_{13}\,^{13}C_1\,^{14}N_5\,^{16}O_3$ 5′-dA and $^{12}C_{10}\,^{1}H_{12}\,^{2}H_1\,^{14}N_5\,^{16}O_3$ 5′-dA are 252.10436 and 252.10799 Da, respectively (a mass difference of 3.6 mDa). The experimental masses of the enzymatic samples compare accurately (< 2 ppm) with the masses of the standards and the calculated theoretical masses. As an internal control, the instrument was tuned to ensure that the detected ratio of the relative abundance of [^{12}C]-5′-dA to [^{13}C]-5′-dA was as close as possible to the calculated ratio of 0.1325.

Calculation of the Intrinsic KIE. We calculated the intrinsic KIE by integrating the peak areas for [^{12}C,^{1}H]-5′-dA and [^{12}C,^{2}H]-5′-dA and directly comparing their ratio. A statistical factor of 2 was used to account for the fact that the labeled methylaspartate contains two protium atoms and only one deuterium atom in the abstractable position. Alternatively, because the natural abundance of ^{13}C is known to great precision, the intrinsic KIE was also calculated via comparison of the ratio of [^{13}C,^{1}H]-5′-dA to [^{12}C,^{2}H]-5′-dA and correction of the result for the natural abundance, n, using the following equation:

$$\mathrm{KIE} = \frac{1}{2} \times \frac{[[^{13}\mathrm{C}]\text{-}5'\text{-dA}]}{[[^{2}\mathrm{H}]\text{-}5'\text{-dA}]} \times \frac{1-n}{n}$$

The KIEs calculated by either method were the same within experimental error.

RESULTS AND DISCUSSION

To reliably diagnose hydrogen tunneling in enzymes, it is imperative to measure the intrinsic KIE for the hydrogen transfer step, undiminished by other steps in the mechanism that may be slower. Whereas for some enzymes there is a single isotopically sensitive step that is cleanly rate-determining in the mechanism, the mechanisms of AdoCbl-dependent rearrangements require two hydrogen transfer steps. Previous experiments with glutamate mutase that have employed a combination of kinetic measurements to elucidate the free energy profile of the reaction indicate that neither hydrogen transfer step is cleanly rate-determining (*18*, *27*, *29*−*34*). Pre-steady state techniques often allow the intrinsic KIE to be measured, but if a slow step precedes the isotopically sensitive step, the observed KIE will still be diminished from its intrinsic value. Results from various pre-steady state measurements using UV−visible stopped-flow spectroscopy and rapid chemical quench techniques suggest that this situation may well apply in glutamate mutase (*18*, *27*, *29*, *32*, *33*).

To overcome this problem, we developed a method to measure the intrinsic KIE in glutamate mutase that relies on intramolecular competition (*35*) between hydrogen and deuterium atoms present in the same methyl group of the substrate, methylaspartate (*24*). As illustrated in Figure 1, during the reaction, 5′-deoxyadenosyl radical, generated by homolysis of AdoCbl, is confronted with the choice of abstracting either protium or deuterium from the methyl group of the same substrate molecule. The isotopic composition of 5′-dA formed in the reaction

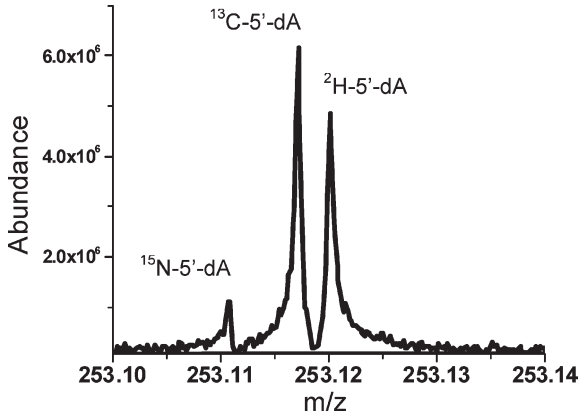

FIGURE 2: Representative FT-ICR-MS spectrum of a sample of 5′-dA obtained after reaction of glutamate mutase with [d_1-*methyl*]-methylaspartate for 44 ms at 2.5 °C. The region shown illustrates the separation of ^{15}N-, ^{13}C-, and ^{2}H-containing isotopomers of 5′-dA.

therefore reflects the intrinsic KIE. The intrinsic KIE[2] can be measured, even when the isotopically sensitive step is not rate-determining, because it is manifested though intramolecular competition between protium and deuterium atoms, which remain chemically equivalent, even in the enzyme active site, due to the rapid rotation of the methyl group.

The measurement of KIEs by intramolecular competition followed the general method that we described previously (*24*). Glutamate mutase and [d_1-*methyl*]-(2*S*,3*S*)-3-methylaspartate were reacted together at various temperatures using a rapid quench flow apparatus, as described in Experimental Procedures. The solutions were mixed in a 1:1 ratio and allowed to age for various times (10−120 ms) before the reactions were quenched and the 5′-dA formed in the reaction was recovered by reverse-phase HPLC (*27*). The deuterium content of the 5′-dA was then determined by ultra high-resolution Fourier transform ion cyclotron resonance mass spectrometry (FT-ICR-MS). The resolution of this type of mass spectrometer is sufficiently high (*36*, *37*) that the mixture of 5′-dA isotopomers, due to natural abundance ^{13}C- and ^{15}N-containing molecules, as well as ^{2}H from the substrate, is baseline resolved as shown in Figure 2. Normally, the presence of ^{13}C complicates the analysis of deuterium KIEs because the peaks due to deuterated and ^{13}C-containing molecules overlap; in this case, the ultrahigh resolution allows the ^{13}C peak to be used as an internal standard to measure the relative abundance of the peak due to ^{2}H-labeled 5′-dA.

The mechanism of the glutamate mutase-catalyzed reaction involves two hydrogen transfer reactions, one from substrate to coenzyme and the other from coenzyme to product. Therefore, measurements have to be made under pre-steady state conditions; otherwise, multiple turnovers would lead to accumulation of deuterium in 5′-dA and a diminution of the observed KIE. To ensure that this condition was met, we measured the KIE for a range of reaction times at each temperature we studied. As shown in Figure 3, the apparent KIE diminished as the reaction time was lengthened, but at times shorter than ~25 ms, it was constant within experimental error. This result is consistent with previous measurements of the rate of 5′-dA formation that show a $t_{1/2}$ of ~11 ms at 10 °C (*27*). Therefore, within this short time span, the measured KIE may be assumed to be close to the intrinsic KIE (*38*).

[2]We note that there is a contribution from a secondary KIE, due to the deuterium in the methyl group, when protium is transferred that is not present when deuterium is transferred. This may affect the magnitude of the primary KIE that is measured, but the contribution is expected to be small and not to significantly affect interpretation of the data.

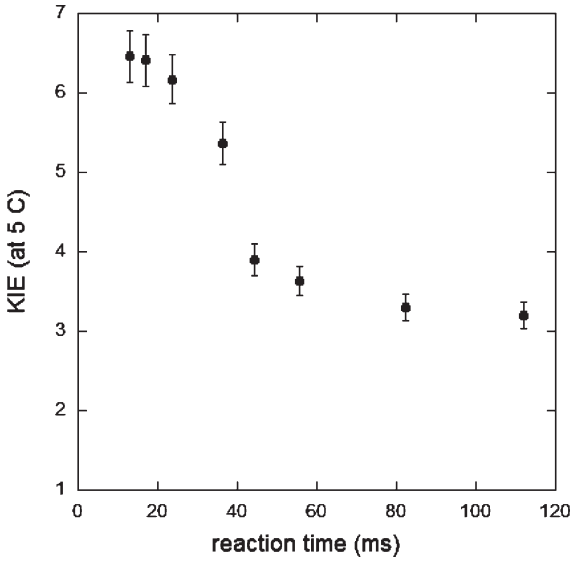

FIGURE 3: Apparent decrease in KIE as a function of reaction time due to multiple turnovers of the enzyme and equilibration of isotopes. The KIE plateaus for reaction times shorter than 25 ms, indicating that the intrinsic KIE is being measured at short reaction times. Data shown are for the reaction at 5 °C.

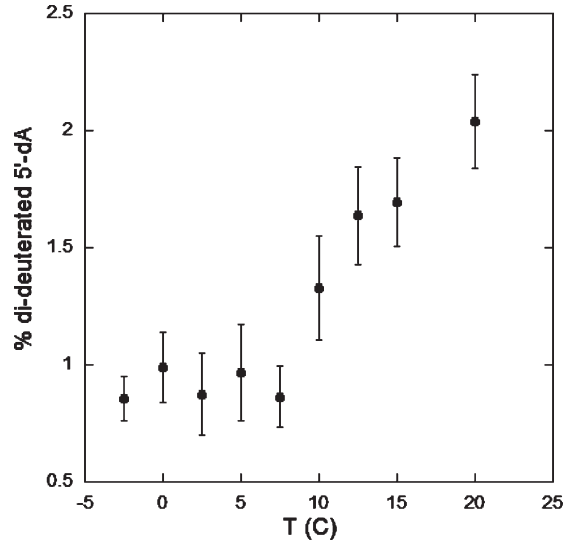

FIGURE 4: Increase in the amount of dideuterated 5′-dA formed at higher temperatures, indicative of multiple turnovers. Each point represents the average of at least three measurements made with reaction times between 12 and 24 ms.

The KIE was measured at various temperatures between − 2.5 and 30 °C. However, at temperatures above 10 °C, increasing amounts of dideuterated 5′-dA were detected, even at short reaction times, indicating that the reaction becomes too fast for the intrinsic KIE to be reliably measured (Figure 4). There is a small background amount of dideuterated 5′-dA even at lower temperatures, which is expected due to natural abundance deuterium in the sample, but which is hard to quantify accurately.

The Arrhenius plot for KIEs measured between −2.5 and 10.5 °C is shown in Figure 5. Each point represents an average of at least five independent measurements of the KIE made with reaction times between 12 and 24 ms. It is evident that at higher temperatures the KIE departs significantly from the Arrhenius

equation. This may be attributed to the contribution from multiple transits of hydrogen between the substrate and coenzyme as discussed above. However, between −2.5 and 5.0 °C, the plot is linear, and from these data, the apparent isotope effect on the pre-exponential factor (A_H/A_D) was 0.17 ± 0.04 and the isotope effect on the energy of activation (ΔE_a) 1.94 ± 0.13 kcal/mol. We note that whereas we were able to study the KIE only over a relatively narrow temperature range, it is not necessary to take measurements over a wide temperature range to reliably diagnose hydrogen tunneling. This is because the method amounts to drawing a tangent to the full temperature-dependent curve (*10*), rather than trying to extrapolate the data to infinite temperature to obtain the "true" pre-exponential factor.

The simplest interpretation of these data, in which the KIE is highly temperature dependent, is that the enzyme exhibits "moderate" tunneling, i.e., that protium is tunneling in the reaction whereas there is a reduced level of tunneling for deuterium (in contrast, temperature-independent KIEs indicate extensive tunneling of both protium and deuterium). More sophisticated analyses that incorporate "Marcus-like" or "full tunneling" models to describe the temperature dependence of KIEs in enzymes emphasize the role of protein dynamics in modulating hydrogen transfer (*1*, *9*, *39*−*41*). The physical explanation for temperature-dependent isotope effects that emerges from the models is that the substrate and coenzyme are not optimally oriented in the active site for tunneling to occur and that a thermal "gating" motion of the protein is needed to bring the reactants into optimal alignment for tunneling.

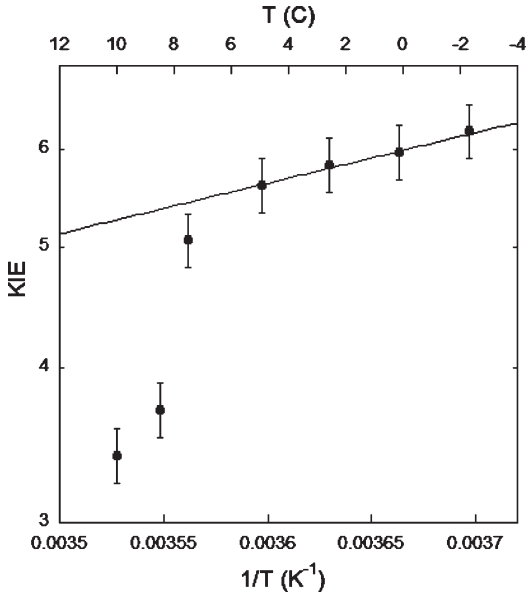

FIGURE 5: Arrhenius plot of the KIE for the formation of 5′-dA from methylaspartate and AdoCbl catalyzed by glutamate mutase over the temperature range from −2.5 to 10.0 °C. Each point represents the average of at least five measurements taken with reaction times between 12 and 24 ms. The deviation from linearity at higher temperatures may be due to multiple transits of hydrogen at higher temperatures which reduce the apparent KIE.

Hydrogen tunneling has been investigated for methylmalonyl-CoA mutase (*21*), an AdoCbl-dependent enzyme that catalyzes a carbon skeleton rearrangement very similar to that of glutamate mutase. In this case, KIEs were measured using stopped-flow spectroscopy to follow the rate of AdoCbl homolysis when the enzyme was reacted with unlabeled and deuterated methylmalonyl-CoA. Tunneling has also been investigated for the abstraction of hydrogen from ethylene glycol by adenosyl radical generated by thermolysis of AdoCbl, which mimics the reaction catalyzed by AdoCbl-dependent dioldehydratase (*22*). A similar experiment was performed using neopentylCbl (*23*) which is sufficiently reactive that neopentyl radical can be generated by thermolysis at room temperature. For comparison, the relevant parameters measured for these systems are collected together in Table 1. It is evident that the KIEs measured for these related reactions are 1 order of magnitude larger than those we determine for glutamate mutase. $\Delta E_{a(D-H)}$ is somewhat smaller for glutamate mutase, whereas A_H/A_D, which is determined least precisely in these experiments, is probably not significantly different between the different systems. Although the hydrogen atom donor is different in this case, methylaspartate as opposed to methylmalonyl-CoA or ethylene glycol, it would seem unlikely that this difference would, of itself, be responsible for substantially changing the intrinsic KIE. We also note that different experimental methods were used to measure the KIEs for each system, each of which may be subject to different types of systematic errors, but again we consider this to be an unlikely explanation for the differences in the observed KIEs.

On the basis of the similarity between the parameters listed in Table 1 for methylmalonyl-CoA mutase and the nonenzymatic systems, it was argued that there was no additional contribution from the enzyme to tunneling, i.e., that the degree of hydrogen tunneling was the same in the enzyme-catalyzed and uncatalyzed reactions (*21*−*23*). Subsequently, a computational study of methylmalonyl-CoA mutase was able to reproduce the unusually large experimental KIE through the use of a multidimensional tunneling model that included implicit protein motions in the reaction coordinate (*42*). To the best of our knowledge, similar computational studies have not been performed to examine the KIEs measured for the AdoCbl model reactions.

Our results certainly point to an influence of the protein on the transition state for hydrogen atom transfer in glutamate mutase because the intrinsic KIE is much smaller than that in either methylmalonyl-CoA mutase or the model systems. Full tunneling models predict that smaller KIEs are indicative of a better preorganized active site in which the donor−acceptor distance in the tunneling-ready state is shorter.

Although in glutamate mutase the KIE is well within the limits expected for semiclassical behavior, the temperature dependence

Table 1: Tunneling-Related Parameters Determined for AdoCbl-Dependent Enzymes and Model Systems

	k_H/k_D (0 °C)	A_H/A_D	$\Delta E_{a(D-H)}$ (kcal/mol)	ref
glutamate mutase	6.0	0.17 ± 0.04	1.94 ± 0.13	this work
methylmalonyl-CoA mutase	~56[a]	0.08 ± 0.01	3.41 ± 0.07	*21*
AdoCbl with ethylene glycol	~40[a]	0.16 ± 0.07	3.0 ± 0.3	*22*
neopentylCbl with ethylene glycol	~42[a]	0.14 ± 0.07	3.1 ± 0.3	*23*

[a]KIEs extrapolated to 0 °C to facilitate comparison.

of the KIE points to hydrogen tunneling. This observation is in accord with our previous measurements of the secondary tritium KIEs associated with the transfer of hydrogen from glutamate to 5′-dA (*43*, *44*). In these experiments, the enzyme was reacted with AdoCbl labeled with tritium at the 5′-carbon and either unlabeled or deuterated glutamate. It was found that the isotope at the primary position strongly influenced the magnitude of the secondary KIE, resulting in a breakdown in the rule of the geometric mean, i.e., that there are no isotope effects on isotope effects. It has been shown that this behavior is indicative of a transition state in which tunneling of hydrogen at the primary position is strongly coupled to the motion of the secondary hydrogen atoms (*45*, *46*), in this case at the 5′-carbon of the adenosyl radical. Furthermore, this behavior has the effect of reducing the primary kinetic isotope effect, which is "stolen" by the coupled motion of the secondary hydrogens. Similar behavior has been observed in, for example, yeast alcohol dehydrogenase (*47*, *48*), for which the primary KIEs are also well within the semiclassical range.

In conclusion, these results provide evidence of hydrogen tunneling in glutamate mutase. Together with the X-ray structure for the enzyme, they provide a starting point for computational modeling of the glutamate mutase reaction using various tunneling models. Such studies promise to provide detailed insights into the still poorly understood principles by which AdoCbl-dependent enzymes generate and control highly reactive free radical intermediates.

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