

Evidence for Coupled Motion and Hydrogen Tunneling of the Reaction Catalyzed by Glutamate Mutase[†]

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ABSTRACT: Glutamate mutase is one of a group of adenosylcobalamin-dependent enzymes that catalyze unusual isomerizations that proceed through organic radical intermediates generated by homolytic fission of the coenzyme's unique cobalt–carbon bond. These enzymes are part of a larger family of enzymes that catalyze radical chemistry in which a key step is the abstraction of a hydrogen atom from an otherwise inert substrate. To gain insight into the mechanism of hydrogen transfer, we previously used pre-steady-state, rapid-quench techniques to measure the α -secondary tritium kinetic and equilibrium isotope effects associated with the formation of 5'-deoxyadenosine when glutamate mutase was reacted with [5'-³H]-adenosylcobalamin and L-glutamate. We showed that both the kinetic and equilibrium isotope effects are large and inverse, 0.76 and 0.72, respectively. We have now repeated these measurements using glutamate deuterated in the position of hydrogen abstraction. The effect of introducing a *primary* deuterium kinetic isotope effect on the hydrogen transfer step is to *reduce* the magnitude of the *secondary* kinetic isotope effect to a value close to unity, 1.05 ± 0.08 , whereas the equilibrium isotope effect is unchanged. The significant reduction in the secondary kinetic isotope effect is consistent with motions of the 5'-hydrogen atoms being coupled in the transition state to the motion of the hydrogen undergoing transfer, in a reaction that involves a large degree of quantum tunneling.

Glutamate mutase is one of a group of adenosylcobalamin (AdoCbl,¹ coenzyme B₁₂) dependent enzymes that catalyze unusual carbon skeleton isomerizations. These rearrangements formally involve a 1,2-hydrogen atom migration and proceed through a mechanism involving carbon-based free radical intermediates (1–6). The initial steps of these reactions involve homolysis of the reactive cobalt–carbon bond of the coenzyme to form cob(II)alamin and 5'-deoxyadenosyl radical. The adenosyl radical then abstracts the migrating hydrogen from the substrate to form 5-deoxyadenosine and substrate radical (or protein radical in the case of AdoCbl-dependent ribonucleotide reductase). These steps have been studied in some detail for several B₁₂ enzymes, and in each case homolysis and hydrogen abstraction are found to be kinetically coupled (7–10), as evidenced by the appearance of a kinetic isotope effect on cobalt–carbon bond homolysis when the enzymes are reacted with deuterated substrates.

This observation implies that adenosyl radical can only be present in very low concentrations as a high-energy intermediate that does not accumulate on the enzyme. Arguments have been advanced for a formally concerted mechanism for homolysis and hydrogen abstraction (11),

although this is now considered to be less likely (12). In many cases these deuterium isotope effects are unusually large, in the range of 40–30, and the most likely explanation for this is that hydrogen transfer involves a large degree of quantum tunneling (13, 14).

We have recently described rapid-quench experiments (15) to measure the α -secondary tritium isotope effects associated with the formation of 5'-deoxyadenosine under pre-steady-state conditions, as illustrated in Figure 1. AdoCbl, tritium-labeled in the 5'-position, was used to measure both the secondary equilibrium and kinetic isotope effects on the formation of 5'-dA. The measurements were made at very short time intervals between 10 and 100 ms so that loss of tritium from the 5'-position was minimal. Both the kinetic and equilibrium isotope effects were found to be large and inverse, $k_H/k_T = 0.76 \pm 0.02$ and $K_H/K_T = 0.72 \pm 0.04$. These results indicate that the 5'-C–H bonds become significantly stiffer in going from AdoCbl to 5'-dA, even though the 5'-carbon remains formally sp³ hybridized, and are consistent with the hydrogen transfer, as opposed to cobalt–carbon bond cleavage, being the slower step. Classically, the large inverse kinetic isotope effect would be interpreted as indicating a late transition state. However, if quantum tunneling is occurring in the hydrogen transfer step, this conclusion cannot be drawn (16).

To investigate whether quantum tunneling is likely to be important in the mechanism of glutamate mutase, we have repeated the α -secondary tritium isotope effect measurements for the formation of 5'-dA using deuterated glutamate to trigger homolysis of AdoCbl. This introduces a primary deuterium isotope effect at the 5'-carbon, which should not

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¹ Abbreviations: AdoCbl, adenosylcobalamin; 5'-dA, 5'-deoxyadenosine; ^{H,H,D}5'-dA, 5'-deoxyadenosine specifically containing protium and deuterium in the 5'-position; ^{H,D,T}5'-dA, 5'-deoxyadenosine specifically containing tritium and deuterium in the 5'-position.

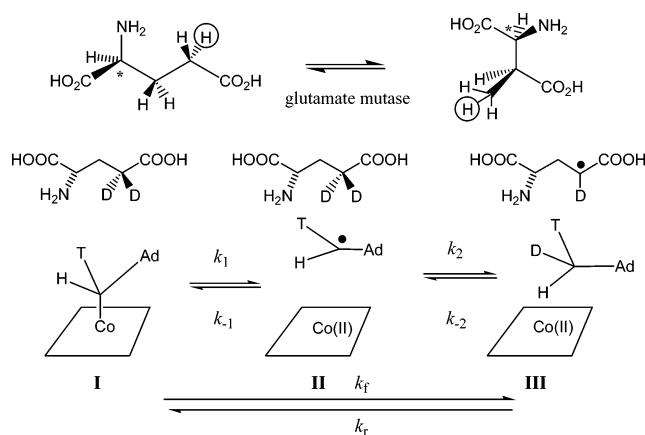


FIGURE 1: Top: Isomerization reaction catalyzed by glutamate mutase, with the migrating hydrogen atom circled. Bottom: Secondary isotope effects associated with the homolysis of AdoCbl and the formation of 5'-dA when holo-glutamate mutase is reacted with deuterated L-glutamate.

change the secondary isotope effect if this arises purely semiclassically (16). However, we observe that the secondary kinetic isotope effect is significantly reduced in magnitude, which implies that the motions of the primary and secondary hydrogen atoms are coupled together in the transition state and that the reaction involves a significant degree of quantum tunneling.

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 (17). L-[G-³H]Glutamate, 250 Ci/mol, was purchased from Amersham Pharmacia Biotech and [8-¹⁴C]adenosine, 50 Ci/mol, was purchased from American Radiochemicals. [2,3,3,4,4-²H₅]-L-Glutamic acid was purchased from Cambridge Isotope Laboratories, Inc., and AdoCbl was purchased from Sigma Chemical Co.

Synthesis of [5'-³H]-AdoCbl and [8-¹⁴C]-AdoCbl. [5'-³H]-AdoCbl was synthesized enzymically by exchange of tritium from L-[G-³H]glutamate to unlabeled AdoCbl and purified by reverse-phase HPLC as described previously (18). [8-¹⁴C]-Adenosylcobalamin was synthesized using established protocols described by Brown et al. (19) from [8-¹⁴C]adenosine that was purchased from Amersham; the radiolabeled material was first converted to 5'-chloroadenosine that was subsequently used to alkylate cob(I)alamin generated by *in situ* reduction of hydroxocobalamin with zinc. The resulting [8-¹⁴C]-AdoCbl was purified by reverse-phase HPLC.

Rapid-Quench Flow. Experiments were performed at 10 °C using a HiTech RQF-63 apparatus. Eighty microliters of a solution containing 100 μ M glutamate mutase, 120 μ M [5'-³H,8-¹⁴C]-AdoCbl (specific activity of each isotope \sim 3500 dpm/nmol), and 20 μ M 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 L-glutamate in the same buffer. After various times reactions were quenched with 80 μ L of 5% trifluoroacetic acid (TFA). 5'-dA was recovered by reverse-phase HPLC (20), and the ¹⁴C:³H ratio of the 5'-dA was determined by dual label scintillation counting. Isotope effects were calculated by

comparing the ratio of ¹⁴C:³H in the recovered 5'-dA with that of the starting AdoCbl. The progress of the reaction with time was followed by monitoring the ¹⁴C content of the 5'-dA; the radioactivity of individual samples was normalized by reference to the tryptophan internal standard.

RESULTS

These experiments were performed under the same conditions as our previously published measurement of secondary isotope effects associated with the formation of 5'-dA using unlabeled glutamate as substrate (15). Hologlutamate mutase was reconstituted with ¹⁴C,³H-labeled AdoCbl and reacted with a saturating concentration (10 mM after mixing) of [2H₅]-L-glutamate at 10 °C. Under these conditions substrate binding is rapid, and the rate of 5'-dA formation is independent of substrate concentration (7, 20). The reaction was allowed to proceed for various times between 7 and 80 ms, after which time the concentration of 5'-dA had essentially reached internal equilibrium on the enzyme. Previous work from our laboratory (21) has demonstrated that at times shorter than 80 ms exchange of hydrogen between 5'-dA and substrate, as evidenced by the formation of doubly deuterated 5'-dA, is minimal.

The time course for formation of ^{H,H,D}5'-dA and ^{H,D,T}5'-dA (the superscripts denote which hydrogen isotopes are present at the 5'-methyl group) was described by single exponential functions, as shown in Figure 2A. The data shown are from one experiment and represent the average of at least three independent measurements at each time point that were all made with the same batch of enzyme. Similar data were obtained from experiments on another batch of enzyme. The apparent first-order rate constants for the formation of ^{H,H,D}5'-dA and ^{H,D,T}5'-dA were determined as $k_{\text{H,H,D}5'-\text{dA}} = 40 \pm 8 \text{ s}^{-1}$ and $k_{\text{H,D,T}5'-\text{dA}} = 60 \pm 10 \text{ s}^{-1}$, respectively.

Previously, when we measured the apparent rate constant for the formation of 5'-dA with protium in the primary position, we found $k_{5'-\text{dA}} = 53 \pm 5 \text{ s}^{-1}$ (for nontritiated 5'-dA) (15). Therefore, we might have expected to see a larger primary isotope effect on the apparent rate constant with deuterium in the primary position, as in other published experiments we have concluded that $k_{\text{H}}/k_{\text{D}}$ for the reaction is about 2 (21). In this case, comparing $k_{5'-\text{dA}}$ with $k_{\text{H,H,D}5'-\text{dA}}$ (no influence of tritium in the secondary position) gives a smaller primary isotope effect of about 1.5. However, this study was not designed to measure primary isotope effects, and so this number must be treated with caution. The lower precision with which $k_{\text{H,H,D}5'-\text{dA}}$ can be measured leads to a large uncertainty in the primary KIE. The two sets of experiments were conducted at different times with different batches of enzyme, and small variations in experimental conditions may lead to significant systematic error. Furthermore, for our determination of the primary deuterium isotope effect (21) we used HPLC (rather than radioactivity) to quantify the amount of 5'-dA produced in the reaction, and this difference in experimental design could also introduce some systematic error.

Lastly, we note that we have recently measured the intrinsic primary deuterium isotope effect for transfer of hydrogen from methyl aspartate to 5'-dA by an internal competition experiment; the isotope effect is about 4 (M.

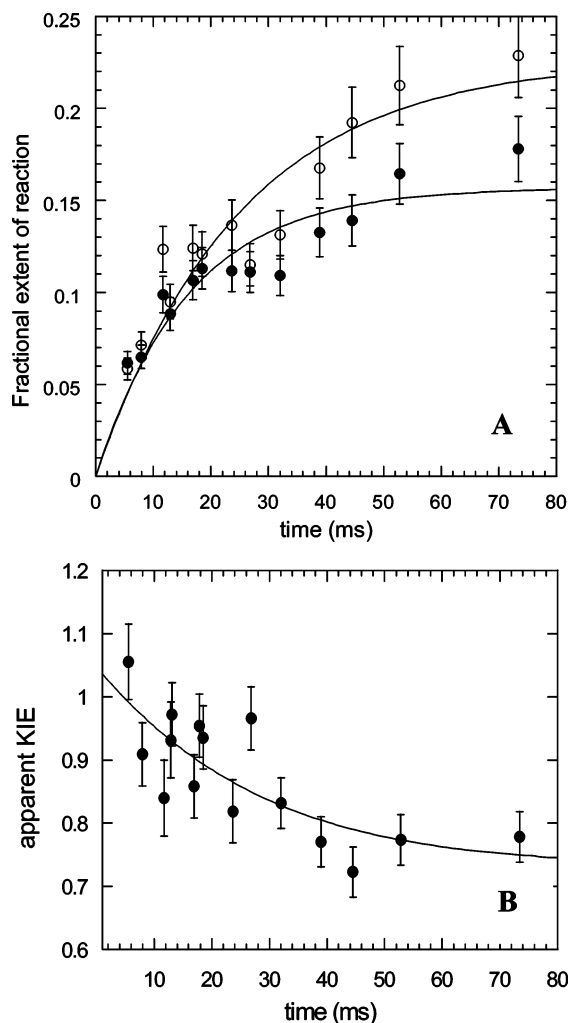


FIGURE 2: (A) Kinetics of 5'-dA formation (approach to internal equilibrium) for holo-glutamate mutase reacting with [2H₅]-L-glutamate (10 mM). Key: (●) formation of [5'-H]-dA, as determined by ¹⁴C counts; (○) formation of [5'-³H]-dA, as determined by tritium counts. Each time point represents an average of three independent measurements. (B) Variation of the apparent α -secondary tritium kinetic isotope effect on 5'-dA formation as a function of time, showing the fit of the data to eq 2.

Yoon, K. Hakansson, and E. N. G. Marsh, unpublished data). Thus we are quite confident that the primary deuterium isotope effects are indeed much smaller than those we originally measured by stopped-flow spectroscopy (7), for reasons we have discussed previously (21).

It is also important to note that it is the relative magnitudes of $k_{H,H,D}5'-dA$ and $k_{H,D,T}5'-dA$, rather than the absolute values, that are important in determining the secondary isotope effects, as we discuss below. Also, as we have discussed previously (7, 15), because the reaction is reversible, the apparent rate constant for the formation of 5'-dA is a function of both the forward and reverse rate constants associated with the equilibrium between the E•AdoCbl•S and E•5'-dA•Cbl(II)•S• complexes (species I and III in Figure 1), such that $k_{5'-dA} = k_f + k_r$.

The apparent secondary isotope effect, KIE_{app} , was calculated by comparing the ratio of ³H to ¹⁴C counts in 5'-dA with that of the starting AdoCbl. The isotope effect is plotted as a function of time in Figure 2B. Much of the error present in the measurement of the apparent rate constants for $k_{H,H,D}5'-dA$, and $k_{H,D,T}5'-dA$ formation cancels out in the calculation

of KIE_{app} . Thus although there is some scatter in the data, it is clear that at shorter times the apparent isotope effect is diminished in magnitude and tends toward unity, whereas at longer times it tends toward a value of ~ 0.7 . This behavior is in contrast to that observed when the experiment was conducted with unlabeled glutamate and the apparent isotope effect was essentially invariant with time (15).

Calculation of Secondary Isotope Effects. As we have discussed previously, because these measurements are made under single turnover conditions, they do not rely on competition between labeled and unlabeled molecules for the isotope effect to be manifested (15). Instead, tritiated and unlabeled AdoCbl molecules are reacting in parallel reactions under identical conditions, and therefore the ratio of $^{H,H,D}5'-dA$ to $^{H,D,T}5'-dA$, as a function of time is described by eq 1 (in which the subscripts attached to the rate constants denote the hydrogen isotopes present at the secondary position).

$$\frac{^{H,H,D}5'-dA_t}{^{H,D,T}5'-dA_t} = \frac{^H[AdoCbl]_0 k_{f(H)}(k_{f(T)} + k_{r(T)})(1 - e^{-(k_{f(H)} + k_{r(H)})t})}{^T[AdoCbl]_0 k_{f(T)}(k_{f(H)} + k_{r(H)})(1 - e^{-(k_{f(T)} + k_{r(T)})t})} \quad (1)$$

Equation 1 can be rewritten to describe how the apparent kinetic isotope effect, KIE_{app} , varies as a function of time by rearranging it and replacing the terms $(k_f + k_r)$ by the corresponding apparent rate constants for the formation of $^{H,H,D}5'-dA$ and $^{H,D,T}5'-dA$, $k_{H,H,D}5'-dA$ and $k_{H,D,T}5'-dA$, respectively; this gives eq 2.

$$(KIE_{app})_t = \frac{^{H,H,D}5'-dA_t}{^{H,D,T}5'-dA_t} \frac{^T[AdoCbl]_0}{^H[AdoCbl]_0} = \left(\frac{k_{f(H)}}{k_{f(T)}} \right) \frac{k_{H,D,T}5'-dA (1 - e^{-(k_{H,H,D}5'-dA)t})}{k_{H,H,D}5'-dA (1 - e^{-(k_{H,D,T}5'-dA)t})} \quad (2)$$

The values for these apparent rate constants can be determined from the data in Figure 2A, as discussed above. Extrapolation of the data to $t = 0$ gives the secondary kinetic isotope effect, $k_{f(H)}/k_{f(T)}$, on 5'-dA formation, whereas extrapolation to $t = \infty$ gives the secondary equilibrium isotope effect, K_H/K_T , on 5'-dA formation.

To determine $k_{f(H)}/k_{f(T)}$, the data in Figure 2B were fitted to eq 2, using the values for $k_{H,H,D}5'-dA$ and $k_{H,D,T}5'-dA$ determined from the data in Figure 2A and allowing $k_{f(H)}/k_{f(T)}$ to float. Similarly good fits could be obtained if the initial values for $k_{H,H,D}5'-dA$ and $k_{H,D,T}5'-dA$ were also allowed to vary during the fitting process. Although the values for $k_{H,H,D}5'-dA$ and $k_{H,D,T}5'-dA$ are not determined with any precision by fitting to eq 2, the value of $k_{f(H)}/k_{f(T)}$ is not very sensitive to these parameters. The value for the secondary kinetic isotope effect at the 5'-carbon when deuterium is in the primary position, $k_{f(H)}/k_{f(T)}$, is close to unity, 1.05 ± 0.08 . The equilibrium secondary isotope effect, K_H/K_T , is calculated by allowing $t \rightarrow \infty$ in eq 2 and is therefore given by $(k_{f(H)}/k_{f(T)})(k_{H,H,D}5'-dA/k_{H,D,T}5'-dA)$. This gives a value for K_H/K_T of 0.70 ± 0.14 when deuterium is in the primary position.

Table 1: Isotope Effects Measured for the Formation of 5'-dA from AdoCbl and L-Glutamate Catalyzed by Glutamate Mutase

isotope effect	ΔV		ref
	kinetic	equilibrium	
primary	2.4 ± 0.4		21
secondary			
protium in primary position	0.76 ± 0.02	0.72 ± 0.04	15
deuterium in primary position	1.05 ± 0.08	0.70 ± 0.14	this work

DISCUSSION

The primary and secondary isotope effects measured for the formation of 5'-dA in glutamate mutase are summarized in Table 1. The secondary tritium isotope effects measured with protium in the primary position are among the larger secondary effects measured for an enzyme reaction, whereas with deuterium in the primary position the kinetic isotope effect is close to unity. These measurements were made in the pre steady state, so that the isotope effects should not be affected by the kinetics of substrate binding or product dissociation. Changing the isotope in the primary position to deuterium should not alter substrate binding or product dissociation and is thus very unlikely to be the reason that the secondary kinetic isotope effect is diminished.

We present two plausible explanations for the unusual kinetic behavior that we have encountered. First, we explore whether a change in the rate-determining step upon substitution of deuterium for protium in the primary position, in conjunction with a conformational change in the protein, might adequately account for our data. Second, we consider whether hydrogen tunneling and coupled motion in the transition state could result in the change in secondary isotope effects, an explanation that we find more appealing.

First we note that in these experiments the tritium at the 5'-carbon reports upon two bond-making/breaking steps: the first involved in breaking the Co-C bond of AdoCbl (step **I** \rightarrow **II** in Figure 1) and the second involved in hydrogen transfer from glutamate to 5'-dA (step **II** \rightarrow **III** in Figure 1). The overall equilibrium isotope effect, which is simply the product of the equilibrium isotope effect for each step, is large, inverse, and, as expected, independent of whether protium or deuterium is in the primary position. This implies that the 5'-hydrogens of 5'-dA make stiffer bonds to carbon than do the 5'-hydrogens of AdoCbl and, as discussed previously (15), suggests that the equilibrium isotope effect on the conversion of **I** to **II** is closer to unity than might be expected based simply on the changes in bonding involved.

The secondary kinetic isotope effect is determined by the differences in 5'-C-H bonding between AdoCbl and the transition state for the overall reaction. Again, this will reflect contributions from both steps **I** \rightarrow **II** and **II** \rightarrow **III**; however, a large body of experimental data from this and other AdoCbl-dependent enzymes strongly suggests that the second step in the reaction (**II** \rightarrow **III**) is the slower step (7, 9, 10, 21).

Change of Rate-Determining Step? By introducing deuterium at the primary position, we expect to slow the hydrogen transfer step (**II** \rightarrow **III**) through the action of the primary isotope effect, but this should not affect the transition state of the Co-C bond cleavage reaction (**I** \rightarrow **II**). In doing

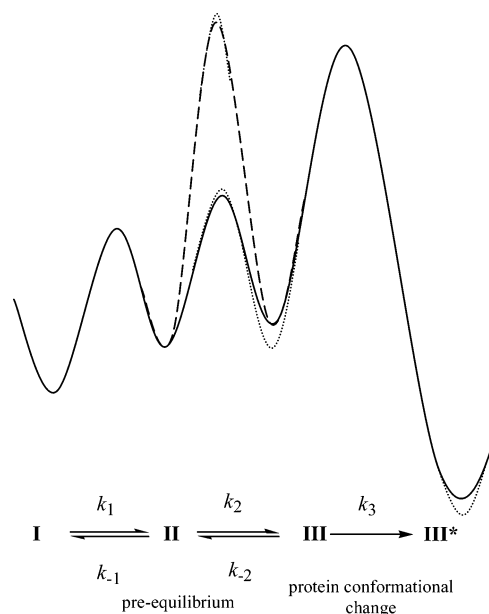


FIGURE 3: Kinetic scheme illustrating a three-step process involving a hypothetical rate-determining protein conformational change that stabilizes posthomolysis radical intermediates. The enzyme-bound species **I**, **II**, and **III** are those indicated in Figure 1; **III*** represents the conformational change in the protein. The hypothesized change in the rate-determining step when deuterated substrate is used is indicated by a dashed line. The secondary kinetic and equilibrium isotope effects are indicated by dotted lines. For discussion see the text.

so, we should make the second step more rate-determining and thus isolate the secondary kinetic isotope effect associated with the conversion of **II** \rightarrow **III** from that associated with the conversion of **I** \rightarrow **II**. This would be predicted to have either no effect on the magnitude of the secondary kinetic effect, if it is already full expressed, or actually make it more inverse, if it is partially suppressed by other steps.

However, it is possible that a more complex kinetic scheme involving a change in the rate-determining step when deuterium is in the primary position might lead to the apparent diminution in the kinetic isotope effect. This could occur if a three-step process was involved, consisting of (a) cobalt-carbon bond homolysis (**I** \rightarrow **II**), (b) hydrogen transfer between substrate and coenzyme (**II** \rightarrow **III**), and (c) a conformational change in the protein that stabilizes the resulting substrate radicals (**III** \rightarrow **III***), which are then trapped by the acid quench. If the protein conformational change was rate-determining with protiated substrate and allowed a rapid preequilibrium to be established in the hydrogen transfer step, this could explain why the values of the apparent secondary kinetic and equilibrium isotope effects are very similar with protiated substrate. In effect, the experiment would only measure the secondary *equilibrium* isotope effect. However, if step **II** \rightarrow **III** became (partially) rate-determining with deuterated substrate, then the secondary *kinetic* isotope effect would now be manifested (distinct from the equilibrium isotope effect), and if this was relatively small, it could account for why the kinetic isotope effect is very different with deuterated substrate. The conformational change would also mask the intrinsic primary kinetic isotope effect, resulting in a relatively small deuterium isotope effect being measured. This kinetic mechanism is illustrated by the free energy profile shown in Figure 3.

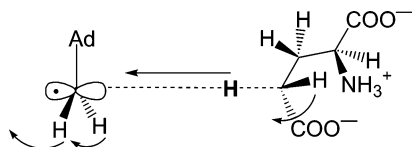


FIGURE 4: Illustration of the coupled motions involved in the transition state for hydrogen transfer between the 5'-dA radical and glutamate.

Whereas such a mechanism would explain the current isotope data, it requires an extra step be inserted at this point in the mechanism, i.e., a protein conformational change, which adds complexity and is aesthetically less pleasing. The crystal structure of glutamate mutase with substrate and coenzyme bound indicates that the entire catalytic cycle can be accomplished with minimal movement of protein side chains (22), suggesting that a rate-determining protein conformational change is unlikely. Also, for this mechanism to explain the data it requires that the isotope effect be large enough to significantly change the rate-determining step and that the hypothetical protein conformational change occurs at a rate that is just right to allow the isotope effect to change the rate-determining step. This imposes a significant constraint on this mechanism, so although it cannot be rigorously excluded, we think it less likely.

Hydrogen Tunneling and Coupled Motion? Kinetic secondary isotope effects arise mainly from differences in bending modes between the ground and transition states. The secondary kinetic effect is often interpreted with respect to the equilibrium secondary isotope effect, which reflects differences in the bending modes between the reactant and product ground states. A secondary kinetic isotope effect of 1 represents a very early transition state, resembling the structure of the reactants, whereas a secondary isotope effect equal to the equilibrium isotope effect indicates a very late transition state, resembling the structure of the products. Furthermore, substitution of a heavy isotope at the primary position should not change the secondary kinetic isotope effect. However, this interpretation is only valid in the absence of hydrogen tunneling, i.e., in the semiclassical regime; these assumptions break down if tunneling and/or coupled motion is involved in the transition state.

In general, isotope effects obey the "rule of the geometric mean", which in effect states that multiple isotopic substitutions tend to be independent; i.e., there are no isotope effects on isotope effects. As Schowen has pointed out, there are cases where this rule breaks down for isotope effects, and the failure of the rule is mechanistically significant (16). In this case the rule is broken because the secondary isotope effect is reduced by the substitution of a heavy isotope at the primary position. As discussed below, this observation is consistent with the motion of the hydrogen atoms in the secondary position (which move from planar sp^2 geometry to tetrahedral sp^3 geometry around the 5'-carbon) being coupled to that of the hydrogen atom undergoing transfer in a transition state that involves a significant degree of quantum tunneling (23, 24). This is illustrated for glutamate mutase in Figure 4.

The phenomenon of hydrogen tunneling in enzymes has been studied in a number of enzymes (25–30); in particular, it was first identified in studies of hydride transfer between NAD^+ and various alcohols catalyzed by yeast alcohol

dehydrogenase (YADH) (30–33). For this enzyme, Klinman and colleagues demonstrated tunneling and coupled motion using labeled benzyl alcohols as substrates, for which both the primary and secondary isotope effects deviated from the Swain–Schaad exponential relationship that relates the k_H/k_T to the k_D/k_T isotope effect² (33). Moreover, theoretical studies have shown that, to reproduce the large secondary isotope effects (which in YADH are larger than predicted by semiclassical models), both coupling of the secondary hydrogens to the reaction coordinate and quantum tunneling are necessary (34).

Of particular relevance to the present case is the observation that introducing deuterium at the primary position reduced the secondary tritium kinetic isotope effect measured in YADH from a value of 1.35 to 1.10 (33). A similar diminution of the secondary isotope effect in response to deuteration at the primary position has been observed with formate dehydrogenase (35). In glutamate mutase we observe a qualitatively similar phenomenon, with the secondary tritium isotope effect being deflated from 0.76 to about 1.05. The precision of our measurements is somewhat less than the those made on dehydrogenases, because our measurements have to be made under single turnover conditions, whereas most secondary isotope effects are measured under steady-state conditions.

This behavior is predicted for cases where coupled motion and hydrogen tunneling are involved, as tunneling behavior will propagate from the primary hydrogen to secondary hydrogens and inflate the value of the secondary kinetic isotope effects beyond the semiclassical limit. When a heavy isotope is present in the primary position, tunneling is reduced, or eliminated, and the secondary kinetic isotope effects are reduced to within the semiclassical limit (23, 34). Note that this behavior has the effect of reducing the primary kinetic isotope effect, which is "stolen" by the coupled motion of the secondary hydrogens; for example, in YADH $k_H/k_{D\text{ primary}}$ is 3–5, depending upon substrate, values that are well within the semiclassical range.

The secondary kinetic isotope effect measured with deuterium in the primary position is more likely to represent the "true" value of the isotope effect; i.e., the semiclassical value is unaffected by quantum tunneling. As such, this should provide a more reliable indication of the position of the transition state along the reaction coordinate. In the absence of tunneling, secondary kinetic isotope effects may be interpreted with respect to the secondary equilibrium isotope effect; the value of the kinetic isotope effect should lie somewhere between unity, reflecting an early transition state, and the equilibrium isotope effect, reflecting a late transition state (16).

The fact that the secondary isotope effect measured for glutamate mutase reacting with deuterated glutamate is close to unity suggests an early transition state for the reaction or, more specifically, that rehybridization of the 5'-carbon, from planar to tetrahedral, occurs after the transition state for hydrogen transfer. This is in accord with the position of the

² Later work on horse liver alcohol dehydrogenase mutants demonstrated that steric interactions between protein and substrate also change the exponential relationship between secondary k_H/k_T and k_D/k_T isotope effects. This suggests that the secondary isotope effect may also be sensitive to the more rigorous constraints that deuterium tunneling in the primary position imposes on the geometry of the reactants (30).

transition state predicted from Hammond's postulate, i.e., that the transition state will resemble the structure of the higher energy reactant. In this case the 5'-dA radical is of significantly higher energy than the glutamyl radical formed by hydrogen abstraction, and we would expect the transition state structure to be closer to that of the 5'-dA radical. We note that the *opposite* conclusion would be drawn from the large inverse secondary kinetic isotope effect measured with protium in the primary position, if tunneling was not suspected!

The existence of hydrogen tunneling in the glutamate mutase reaction has been suspected because of the very large primary isotope effects ($k_H/k_D > 20$) on the formation of cob(II)alamin observed with several AdoCbl-dependent enzymes. In particular, Banerjee and co-workers have conducted an investigation of the temperature dependence of the primary deuterium isotope effects in methylmalonyl-CoA mutase and showed that the isotope effects on the Arrhenius factors and activation energies are consistent with hydrogen tunneling in this enzyme (13). However, coupled motion has not been observed before for an AdoCbl-dependent enzyme, and indeed the secondary isotope effect measurements necessary to diagnose coupled motion have not been conducted for other AdoCbl-dependent enzymes.

Although we originally reported a very large primary deuterium isotope effect for the formation of cob(II)alamin and 5'-dA upon reaction of holo-glutamate mutase with deuterated substrates ($k_H/k_D = 28$ with glutamate and 35 with methyl aspartate) (7), we have recently had cause to revise our interpretation of the stopped-flow data upon which these numbers were based. As we described recently (21), rapid-quench measurements of the deuterium isotope effect, combined with mass spectral analysis of 5'-dA formed in the reaction, indicate that the intrinsic primary isotope effect is likely much smaller; see Table 1.

As Klinman has pointed out (23, 25), hydrogen tunneling does not necessarily have to occur with coupled motion of the secondary hydrogen atoms. For example, bovine serum amine oxidase (BSAO) exhibits a very large primary kinetic isotope effect for proton abstraction from the Schiff base of the amine, and the isotope effect on the Arrhenius factor is significantly less than one, consistent with tunneling. However, the secondary isotope effects for this enzyme are in the normal range, which appears to rule out coupled motion. This raises the intriguing possibility that in AdoCbl-dependent enzymes two different mechanisms of hydrogen transfer may be operating. In methylmalonyl-CoA mutase, in which the intrinsic deuterium isotope effects approach 50 at low temperatures (13), quantum tunneling of the hydrogen atom undergoing transfer may occur without coupled motion. Whereas in glutamate mutase, where the primary isotope effects appear to be much lower, quantum tunneling of the primary hydrogen may be coupled to the movement of the secondary hydrogen atoms.

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