Accelerated Publications

Pre-Steady-State Measurement of Intrinsic Secondary Tritium Isotope Effects Associated with the Homolysis of Adenosylcobalamin and the Formation of 5′-Deoxyadensosine in Glutamate Mutase†

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ABSTRACT: Glutamate mutase is one of a group of adenosylcobalamin-dependent enzymes that catalyze a variety of reactions that proceed through organic radical intermediates generated by homolytic fission of coenzyme's unique cobalt−carbon bond. For all the enzymes that have been examined, the homolysis step is kinetically indistinguishable from abstraction of hydrogen from the substrate (or protein), implying that deoxyadenosyl radical is formed only as a fleeting intermediate. To examine how these two steps are coupled together, we have used pre-steady-state, rapid quench techniques to measure the α-secondary tritium isotope effect associated with the formation of 5′-deoxyadenosine when the enzyme is reacted with [5′-^{3}H]-adenosylcobalamin and L-glutamate. Surprisingly, a large inverse equilibrium isotope effect of 0.72 ± 0.04 was found for the overall reaction, indicating that the 5′-C−H bonds become significantly stiffer on going from adenosylcobalamin to 5′-deoxyadenosine, even though the 5′-carbon remains formally sp^3 hybridized. The kinetic isotope effect for the formation of 5′-deoxyadenosine was 0.76 ± 0.02, which suggests a late transition state for the reaction.

Glutamate mutase is one of a group of adenosylcobalamin (AdoCbl,[1] coenzyme B_{12})-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*−*5*). 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 B_{12} enzymes ,and in each case homolysis and hydrogen abstraction are found to be kinetically coupled (*6*−*9*), 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.

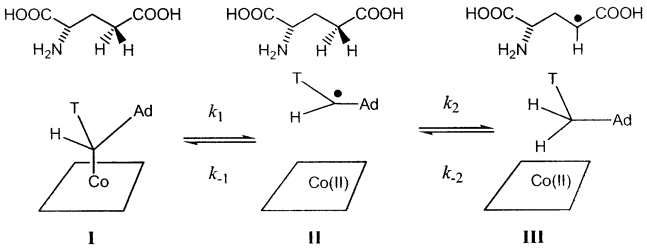

FIGURE 1: Secondary isotope effects associated with the homolysis of AdoCbl and the formation 5′-dA when hologlutamate mutase is reacted with L-glutamate.

Arguments have been advanced for a formally concerted mechanism for homolysis and hydrogen abstraction (*10*), although this is now considered to be less likely (*11*). 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 (*12*, *13*). To gain further insight into this unusual reaction, we have used rapid quench methods to measure the α-secondary tritium isotope effects associated with the formation of 5′-deoxyadensosine under single turnover conditions, as illustrated in Figure 1.

Secondary isotope effects provide a powerful tool to probe the nature of transition states in both enzyme-catalyzed and nonenzymic reactions. Notably, secondary isotope effects have provided insight into the mechanism of carbonyl reduction (*14*, *15*) and, when combined with normal-mode analysis, have allowed transition-state geometries to be

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[1] Abbreviations: AdoCbl, adenosylcobalamin; 5′-dA, 5′-deoxyadenosine; ^{H}5′-dA, 5′-deoxyadenosine specifically containing protium in the 5′-position; ^{T}5′-dA, 5′-deoxyadenosine specifically containing tritium in the 5′-position.

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calculated in several enzyme-catalyzed *N*-ribosyl and *N*-deoxyribosyl transferase reactions (*16*, *17*). In the absence of tunneling or coupled motion, the value of the secondary kinetic isotope effect should lie between unity and the value of the secondary equilibrium isotope effect (*18*). The latter reflects the change in stiffness of the isotopically substituted bond that occurs upon reaction and may be normal, i.e., greater than one, or inverse, i.e., less than one. At 25 °C, values of 0.8−1.2 and 0.65−1.35 are typical for C-^{2}H and C-^{3}H secondary equilibrium isotope effects, respectively, although at lower temperatures a larger range of values is expected. Secondary isotope effects are particularly sensitive to changes in hybridization at the carbon center undergoing reaction and are generally expected to be normal if the rate-determining step involves a change from sp^3 to sp^2 hybridization and inverse in the opposite direction.

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 (*19*). L-[G,-^{3}H]-glutamate, 250 Ci/mol, was purchased from Amersham Pharmacia Biotech, and [8-^{14}C]-adenosine, 50 Ci/mol, was purchased from American Radiochemicals. AdoCbl was purchased from Sigma Chemical Co.

Synthesis of [5′-^{3}H]-AdoCbl and [8-^{14}C]-AdoCbl. [5′-^{3}H]-AdoCbl was synthesized enzymically by exchange of tritium from L-[G,-^{3}H]-glutamate to unlabeled AdoCbl and purified by reverse-phase HPLC as described previously (*20*). [8-^{14}C]-Adenosylcobalamin was synthesized using established protocols described by Brown et al. (*21*) from [8-^{14}C]-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 HOCbl with zinc. The resulting [8-^{14}C]-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 μM glutamate mutase, 120 μM [5′-^{3}H, 8-^{14}C]-AdoCbl (specific activity of each isotope ~3500 dpm/nmol), 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 L-glutamate in the same buffer. After various times, reactions were quenched with 80 μL of 5% trifluoroacetic acid (TFA). 5′-dA was recovered by reverse-phase HPLC (*22*), and the ^{14}C:^{3}H ratio of the 5′-dA was determined by dual-label scintillation counting. Isotope effects were calculated by comparing the ratio of ^{14}C:^{3}H in the recovered 5′-dA with that of the starting AdoCbl. The progress of the reaction with time was followed by monitoring the ^{14}C content of the 5′-dA; the radioactivity of individual samples was normalized by reference to the tryptophan internal standard.

RESULTS AND DISCUSSION

The kinetics of 5′-dA formation were studied for glutamate mutase, reconstituted with ^{14}C,^{3}H-labeled AdoCbl, reacting with a saturating concentration (10 mM) of L-glutamate at 10 °C. Under these conditions, substrate binding is rapid and the rate of 5′-dA formation is independent of substrate concentration (*6*, *22*). The reaction was allowed to proceed for various times between 7 and 100 ms, after which time the concentration of 5′-dA had essentially reached internal equilibrium on the enzyme. The time courses for formation of both ^{H}5′-dA (as followed by the ^{14}C counts) and ^{T}5′-dA were well 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.

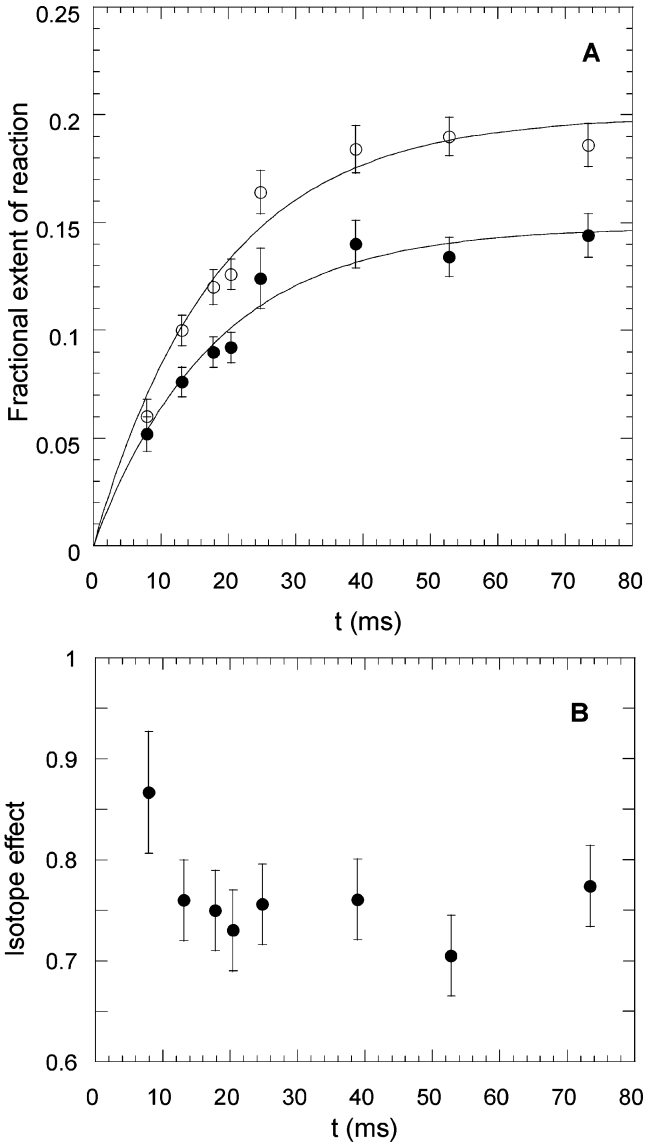

FIGURE 2: (A) Kinetics of 5′-dA formation (approach to internal equilibrium) for holo-glutamate mutase reacting with L-glutamate (20 mM). (●) Formation of [5′-H]-dA, as determined by ^{14}C counts; (○) formation of [5′-^{3}H]-dA, as determined by tritium counts. Each time point represents an average of three independent measurements. (B) Variation of the apparent α-secondary tritium kinetic isotope effect on 5′-dA formation as a function of time.

Since the reaction is reversible, the concentration of 5′-dA as a function of time, $[5'\text{-dA}]_t$, is described by eq 1, in

$$[5'\text{-dA}]_t = \frac{[\text{AdoCbl}]_0 k_f}{k_f + k_r}(1 - e^{-(k_f + k_r)t}) \qquad (1)$$

which k_f and k_r are the forward and reverse rate constants

associated with the equilibrium between the E:AdoCbl:S and E:5′-dA:Cbl(II):S$^{\bullet}$ complexes (species **I** and **III** in Figure 1) and $[AdoCbl]_0$ is the initial concentration of enzyme-bound AdoCbl. Fitting the ^{14}C data to eq 1 yielded an observed first-order rate constant for the formation of ^{H}5′-dA, $^{H}(k_f + k_r) = 53 \pm 5\ s^{-1}$, and at equilibrium the fraction of enzyme active sites containing ^{H}5′-dA, determined from the amplitude of the exponential, was 15.2 ± 0.6%. These values are in good agreement with those determined previously for formation of 5′-dA on the enzyme using unlabeled AdoCbl (*22*). The tritium counts were also fitted to eq 1 to obtain the observed first-order rate constant for the formation of ^{T}5′-dA, $^{T}(k_f + k_r) = 53 \pm 5\ s^{-1}$, a result identical within error to that obtained for the formation of ^{H}5′-dA. The presence of tritium at the secondary position, however, significantly perturbs the equilibrium concentration of 5′-dA, because, for those enzymes originally containing ^{T}5′-AdoCbl, the fraction of enzyme active sites containing ^{T}5′-dA at equilibrium was 20 ± 0.8%. A significant *inverse* equilibrium secondary tritium isotope effect therefore appears to be operating.

Calculation of Secondary Isotope Effects. Unlike most measurements of tritium isotope effects, these experiments do not rely on competition between labeled and unlabeled molecules because the measurements were made under single-turnover conditions. In this case, the tritiated and unlabeled AdoCbl molecules are reacting in parallel reactions under identical conditions. The ratio $^{H}[5′\text{-dA}]_t$:$^{T}[5′\text{-dA}]_t$ as a function of time is therefore described by eq 2 (in which the superscripts denote whether a term refers to a tritium- or protium-bearing molecule), and extrapolation of this ratio

$$\frac{^{H}[5′\text{-dA}]_t}{^{T}[5′\text{-dA}]_t} = \frac{^{H}[AdoCbl]_0\,{}^{H}k_f(^{T}k_f + {}^{T}k_r)(1 - e^{-(^{H}k_f + {}^{H}k_f)t})}{^{T}[AdoCbl]_0\,{}^{T}k_f(^{H}k_f + {}^{H}k_r)(1 - e^{-(^{T}k_f + {}^{T}k_r)t})} \quad (2)$$

to $t = 0$ gives the secondary *kinetic* isotope effect, $^{T}k_f/^{H}k_f$, on 5′-dA formation. Note that if $^{T}(k_f + k_r) = {}^{H}(k_f + k_r)$, as is the case here, then $^{H}[5′\text{-dA}]_t/^{T}[5′\text{-dA}]_t$ should be constant with time and is equal to $^{T}k_f/^{H}k_f$. As shown in Figure 2B, $^{H}[5′\text{-dA}]_t/^{T}[5′\text{-dA}]_t$ is indeed approximately constant with time. The secondary kinetic isotope effect, $^{H}k_f/^{T}k_f$, calculated from the average of all the measurements is 0.76 ± 0.02, representing a large inverse kinetic effect.

The secondary *equilibrium* isotope effect, $^{H}K/^{T}K$ is derived from eq 2 by allowing t to approach infinity, which, after rearrangement, gives eq 3, in which $^{H}[5′\text{-dA}]_{eq}$ and $^{T}[5′\text{-dA}]_{eq}$

$$\frac{^{H}[5′\text{-dA}]_{eq}}{(^{H}[AdoCbl]_0 - {}^{H}[5′\text{-dA}]_{eq})} \frac{(^{T}[AdoCbl]_0 - {}^{T}[5′\text{-dA}]_{eq})}{^{T}[5′\text{-dA}]_{eq}} = \frac{^{H}k_f\,{}^{T}k_r}{^{T}k_f\,{}^{H}k_r} = \frac{^{H}K}{^{T}K} \quad (3)$$

are the equilibrium concentrations of unlabeled and tritiated 5′-dA, respectively. The secondary equilibrium isotope effect, $^{H}K/^{T}K$, calculated from eq 3 is 0.72 ± 0.04, which is very close to the kinetic isotope effect. This implies that the kinetic isotope effect for the reverse reaction, $^{H}k_r/^{T}k_r$, is close to 1 (1.06 ± 0.05). These data also allow the individual rate constants $^{H}k_f$, $^{H}k_r$, $^{T}k_f$, and $^{T}k_r$ to be calculated, although these are determined rather less accurately than the isotope effects.

One potential source of systematic error in this experiment is that tritium can be transferred to glutamate by reversal of the reaction after formation of 5′-dA, a problem that will be most significant at longer times. However, we have previously measured the rate of tritium loss from AdoCbl (*23*), and because of the large primary isotope effect operating against this process, only about 3% of the tritium should be lost after 100 ms. This will lead to a slight underestimate of the equilibrium isotope effect but is not a major source of experimental error.

Interpretation of Isotope Effects. First we note that secondary isotope effects in enzyme-catalyzed reactions are generally measured by competition experiments where they may be suppressed by a large forward commitment factors. However, since these measurements were made in the pre-steady state, the isotope effects observed are not affected by the kinetics of substrate binding or product dissociation and, as such, should measure the intrinsic isotope effects.

In these experiments, the tritium at the 5′-carbon reports upon two bond-making/bond-breaking steps: the first is involved in breaking the Co−C bond of AdoCbl, and the second is involved in hydrogen transfer from glutamate to 5′-dA. We hypothesized that the isotope effect associated with the first step (**I** → **II**) is likely to be normal, i.e., greater than 1, whereas that for the second step (**II** → **III**) is likely to be inverse, i.e., less than 1, reflecting the change in hybridization of the 5′-carbon from sp^3 to sp^2 in the first step, and from sp^2 to sp^3 in the second step. It is therefore somewhat surprising that the equilibrium isotope effect we measured for the conversion of **I** → **III**, which might be predicted to be close to unity, is in fact large and inverse. This implies that the 5′-hydrogens of 5′-dA make stiffer bonds to carbon than do the 5′-hydrogens of AdoCbl, and it further suggests that the equilibrium isotope effect on the conversion of **I** to **II** is closer to unity than might be expected simply on the basis of the changes in bonding involved. This result might also be interpreted as implying that the hybridization state of the 5′-carbon of adenosyl moiety of AdoCbl is "sp^2-like" in character.

The kinetic isotope effect will be determined by the differences in 5′-C−H bonding between AdoCbl and the transition state for the overall reaction. The large primary isotope effects on AdoCbl homolysis/hydrogen transfer, previously measured by stopped-flow spectroscopy when the enzyme was reacted with deuterated substrates (*6*), strongly suggest that the second step in the reaction **II** → **III** is rate determining. The large inverse kinetic effect we observe is certainly consistent with this interpretation.

It is noteworthy that the kinetic isotope effect is very similar in magnitude to the equilibrium isotope effect. This result would normally be interpreted as evidence for the transition state for the reaction occurring very late in the reaction, so that the transition state closely resembles 5′-dA. More specifically, it implies that rehybridization of the 5′-carbon, from planar to tetrahedral, occurs either before or at the transition state for hydrogen transfer. One caveat to this interpretation is that hydrogen transfer between substrate and adenosyl radical probably occurs with a high degree of quantum tunneling. Under these conditions, the secondary kinetic isotope effect could, in principle, exceed the equilibrium isotope effect (*18*), and the location of the transition state cannot be conclusively determined.

For many reactions, interpretation of the isotope effects associated with enzyme catalysis is greatly facilitated by comparing them with isotope effects measured for the

corresponding nonenzymic reaction. An extensive literature on the primary and secondary isotope effects associated with hydrolysis, nucleophillic substitution, elimination, and carbonyl reduction has proved extremely useful in dissecting the mechanisms of the corresponding enzyme-catalyzed reactions. In contrast, rather less is known about radical reactions, and B_{12}-dependent rearrangements in particular represent some of the most challenging reactions to model. Recently, interesting model studies demonstrating a large primary isotope on hydrogen abstraction in a model designed to mimic the diol dehydrase-catalyzed reaction have been reported (*24*). However, we are aware of no pertinent model systems in which secondary isotope effects have been measured that we might compare with our data.

One often-discussed mechanism for the activation of the cofactor toward homolysis, the "mechanochemical hypothesis", postulates that, upon substrate binding, the enzyme induces a distortion of the corrin ring, weakening the Co−C bond and thereby promoting homolysis. We might expect that secondary isotope effects would be sensitive to changes in bonding at the 5′-carbon and could thus provide a test for this hypothesis. It would therefore be very informative to compare the equilibrium isotope effect for this reaction in the absence of enzyme. We would predict that if the enzyme distorts the adenosyl moiety of AdoCbl toward a more "radical-like" form, then the equilibrium isotope effect should be more inverse on the enzyme than in free solution.

In principle, if the vibrational frequencies of the 5′-C−H bonds were known for AdoCbl, adenosyl radical, and 5′-dA, then the equilibrium isotope effects for each step could be calculated. Similarly, these isotope effects could be determined computationally by a normal-mode analysis. Although a detailed resonance Raman study of the vibrational modes associated with the cofactor Co−C bond has been reported (*25*), and various computational studies on the homolysis of AdoCbl have been undertaken (*26*, *27*), currently the requisite data that would facilitate this comparison with our experiments are unavailable.

We hope that the results we report here may provide the impetus for further spectroscopic and computational studies and the measurements of isotope effects in pertinent model systems that, when combined with kinetic data, should provide further detailed insight into this intriguing class of enzymes.

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