Coupling of Cobalt−Carbon Bond Homolysis and Hydrogen Atom Abstraction in Adenosylcobalamin-Dependent Glutamate Mutase†

E. Neil G. Marsh*,‡ and David P. Ballou§

Department of Chemistry, University of Michigan, and Department of Biological Chemistry, University of Michigan Medical School, Ann Arbor, Michigan 48109

Received March 4, 1998; Revised Manuscript Received June 15, 1998

ABSTRACT: Adenosylcobalamin-dependent glutamate mutase catalyzes an unusual carbon skeleton rearrangement that proceeds through the formation of free radical intermediates generated by the substrate-induced cleavage of the coenzyme cobalt−carbon bond. The reaction was studied at 10 °C with various concentrations of L-glutamate and L-*threo*-3-methylaspartate and with use of stopped-flow spectroscopy to follow the formation of cob(II)alamin. Either substrate induces rapid formation of cob(II)alamin, which accumulates to account for about 25% of the total enzyme species in the steady state when substrate is saturating. Measurements of the rate constant for the formation of cob(II)alamin demonstrate that the enzyme accelerates the rate of homolysis of the cobalt−carbon bond by at least 10^{12}-fold. Very large isotope effects on cob(II)alamin formation, of 28 and 35, are observed with deuterated L-glutamate and deuterated L-*threo*-3-methylaspartate, respectively. This implies a mechanism in which Co−C bond homolysis is kinetically coupled to substrate hydrogen abstraction. Therefore, adenosyl radical can only be formed as a high-energy intermediate only at very low concentrations on the enzyme. The magnitude of the isotope effects suggests that hydrogen tunneling may play an important role catalysis.

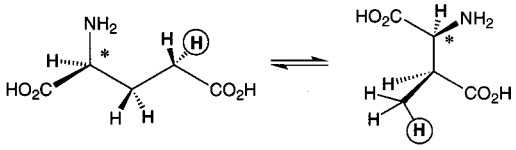
Adenosylcobalamin (coenzyme B_{12}, AdoCbl)[1] serves as the coenzyme in a number of unusual isomerizations that proceed through the intermediacy of free radical species (*1*−*3*). These isomerizations involve the 1,2-rearrangement of a group, X, on one carbon that interchanges with a hydrogen atom on an adjacent carbon atom, where X may be −OH, $-NH_2$, or, most interestingly, a carbon-containing fragment (*3*) (Scheme 1).

Scheme 1

The reversible homolysis of the bond between the central cobalt atom of the coenzyme and the 5′-carbon of adenosine generates a carbon-based radical that then proceeds to initiate the free radical-mediated 1,2-rearrangement (*1*) (Figure 1).

We are studying the mechanism of glutamate mutase, which catalyzes the carbon skeleton rearrangement of L-glutamate to L-*threo*-3-methylaspartate, as the first step in the fermentation of glutamate by *Clostridium tetanomorphum* (Scheme 2).[2]

Scheme 2



Glutamate mutase, one of several coenzyme B_{12} enzymes that catalyze similar carbon skeleton rearrangements (*4*, *5*), comprises two weakly associating subunits, MutE and MutS, which combine to form the coenzyme binding site (*6*, *7*). MutS contains a conserved cobalamin-binding domain that shares structural and sequence similarities with several AdoCbl- and methylcobalamin-dependent enzymes (*8*, *9*). The active holoenzyme assembles in a kinetically complex process in which the stoichiometry, the apparent K_d for AdoCbl, and the specific activity of the enzyme are dependent on the relative concentrations of the two subunits (*6*). To facilitate mechanistic and structural studies on the enzyme, we have recently engineered a fusion protein in which the S subunit is joined to the C-terminus of the E subunit (*10*). This enzyme, designated GlmES, exhibits only threefold lower catalytic activity than the wild-type two-subunit enzyme and binds substrate and coenzyme with similar affinities. Importantly, neither the affinity for AdoCbl nor the turnover number for GlmES depends on protein concentration. GlmES, therefore is much better suited to

†This research has been supported by NIH Research Grants GM 55163 to E.N.G.M. and GM20877 to D.P.B.

* Correspondence should be addressed to this author at the Department of Chemistry, University of Michigan, Ann Arbor, MI 48109-1055. Tel (734) 763-6096. Fax (734) 764-8815. E-mail nmarsh@umich.edu.

‡ University of Michigan.

§ University of Michigan Medical School.

[1] Abbreviations: AdoCbl, adenosylcobalamin; GlmES, glutamate mutase fusion protein; EPR, electron paramagnetic resonance; Cbl(II), cob(II)alamin.

[2] We apply the term "forward" to refer to the reaction proceeding in the direction L-glutamate to L-*threo*-3-methylaspartate and "reverse" to refer to the reaction proceeding in the direction L-*threo*-3-methylaspartate to L-glutamate.

S0006-2960(98)00512-1 CCC: $15.00
Published on Web 08/06/1998

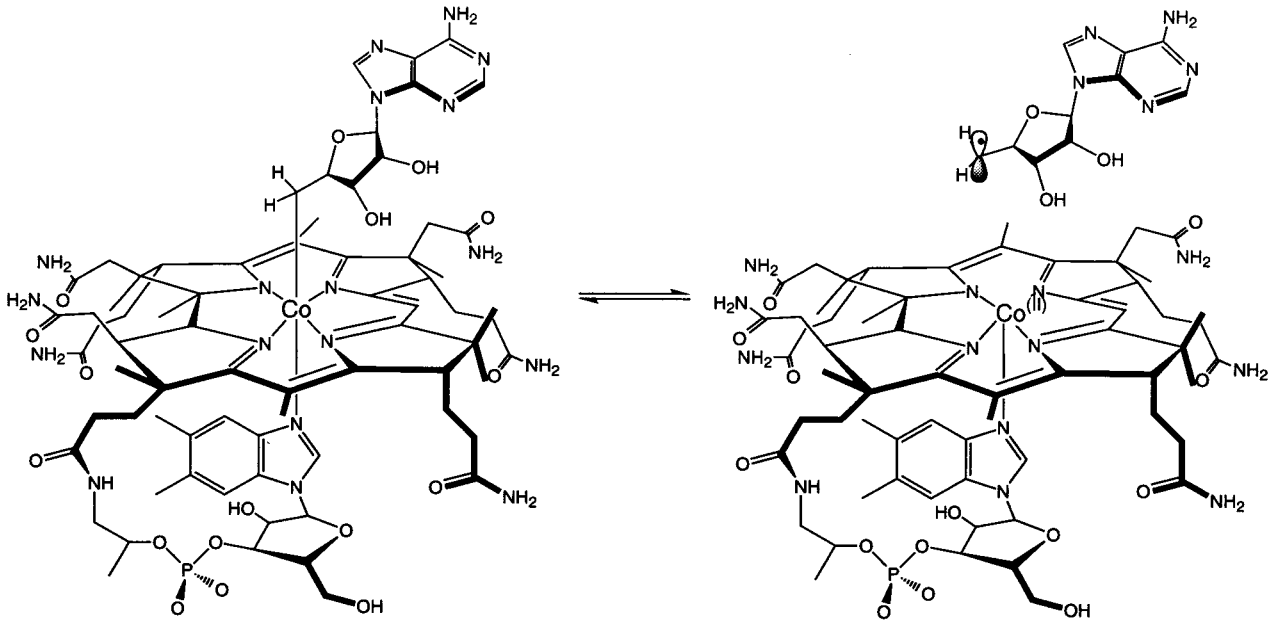


FIGURE 1: Formation of carbon-based free radicals through the reversible homolysis of the cobalt−carbon bond in adenosylcobalamin.

characterization by stopped-flow spectroscopy than is the wild-type two-subunit glumate mutase.

A proposed mechanism for the reversible rearrangement catalyzed by glutamate mutase is shown in Figure 2. In the first step, substrate-binding triggers homolysis of the coenzyme to form the adenosyl radical (species **II**), which then abstracts the migrating hydrogen atom from glutamate to form a glutamyl radical (**III**). The rearrangement of the glutamyl radical has been proposed to occur by a fragmentation−recombination mechanism with glycyl radical and acrylate as intermediates (**IV**) (*4*), although these putative intermediates have yet to be observed experimentally. After rearrangement of the substrate radical, a hydrogen atom from 5′-deoxyadenosine is transferred to the methylaspartyl radical (**V**) to yield methylaspartate and a 5′-deoxyadenosyl radical (**VI**) that, in the final step, recombines with cobalt to regenerate the coenzyme.

One poorly understood aspect of AdoCbl-dependent catalysis is how these enzymes facilitate homolysis of the coenzyme cobalt−carbon bond. This rate acceleration has been roughly estimated as $10^{12\ \pm 1}$ by comparing the rate of thermolysis of the coenzyme in free solution at 25 °C with $k_{cat} \sim 100\ s^{-1}$ for a "typical" B_{12} enzyme (*11*). However, accurate data for the rate of AdoCbl homolysis on enzymes are lacking. A related question is how homolysis is coupled to hydrogen abstraction from the substrate, since for all the enzymes examined, cleavage of the coenzyme depends on the substrate being bound (*12*−*15*). Furthermore, despite its central role in the mechanism of these enzymes, adenosyl radical itself has never been observed, for example by electron paramagnetic resonance (EPR), as an intermediate.

To address these questions, we have used stopped-flow spectroscopy to measure the rate of substrate-induced cleavage of AdoCbl by glutamate mutase. Here we report the results of experiments using various concentrations of glutamate and methylaspartate, both protiated and deuterated in the abstractable position. These experiments provide insight into the reaction mechanism and have allowed determination of several of the rates associated with the reaction.

MATERIALS AND METHODS

Materials. The purification of the glutamate mutase fusion protein, GlmES, from a recombinant *Escherichia coli* strain has been described previously (*10*). AdoCbl was purchased from Sigma Chemical Company. The sources of other materials have been described previously (*10*) or were purchased from commercial suppliers.

Substrates. L-*threo*-3-Methylaspartate and L-*threo*-[3-$^2H_3$-methyl]aspartate were prepared enantiomerically pure by enzymic synthesis (*16*). D,L-Glutamic acid was purchased from Sigma Chemical Company and D,L-[2,4,4-$^2H_3$]glutamic acid from Cambridge Isotope Laboratories Inc. Because deuterated glutamate was available only in racemic form, we used racemic protiated glutamate in these experiments for comparative purposes. However, control experiments established that D-glutamate is neither a substrate nor an inhibitor of the enzyme, and in all the experiments described here the concentrations refer only to the active L-isomer.

Pre-Steady-State Kinetic Experiments. Pre-steady-state kinetic experiments were performed at 10 °C with a Hi-Tech Scientific (U.K.) SF-61 stopped-flow apparatus controlled by KISS, a Kinetic Instruments Macintosh-based software suite. The temperature of the mixing chamber was controlled by a circulating water bath. The enzyme solution contained 125 μM GlmES in 50 mM potassium phosphate buffer containing 1 mM EDTA and 10% glycerol. Immediately before the experiment AdoCbl was added to a final concentration of 100 μM so that the effective concentration of holoenzyme was 100 μM. Solutions containing AdoCbl were handled carefully to avoid exposure to bright light. The solution was placed in a glass tonometer and made anaerobic by repeated cycles of evacuation and flushing with purified argon. Substrates, dissolved in the same buffer as the enzyme, were placed in glass syringes and made anaerobic by bubbling purified argon through them for 10 min before use. Mixing in the stopped-flow apparatus diluted both

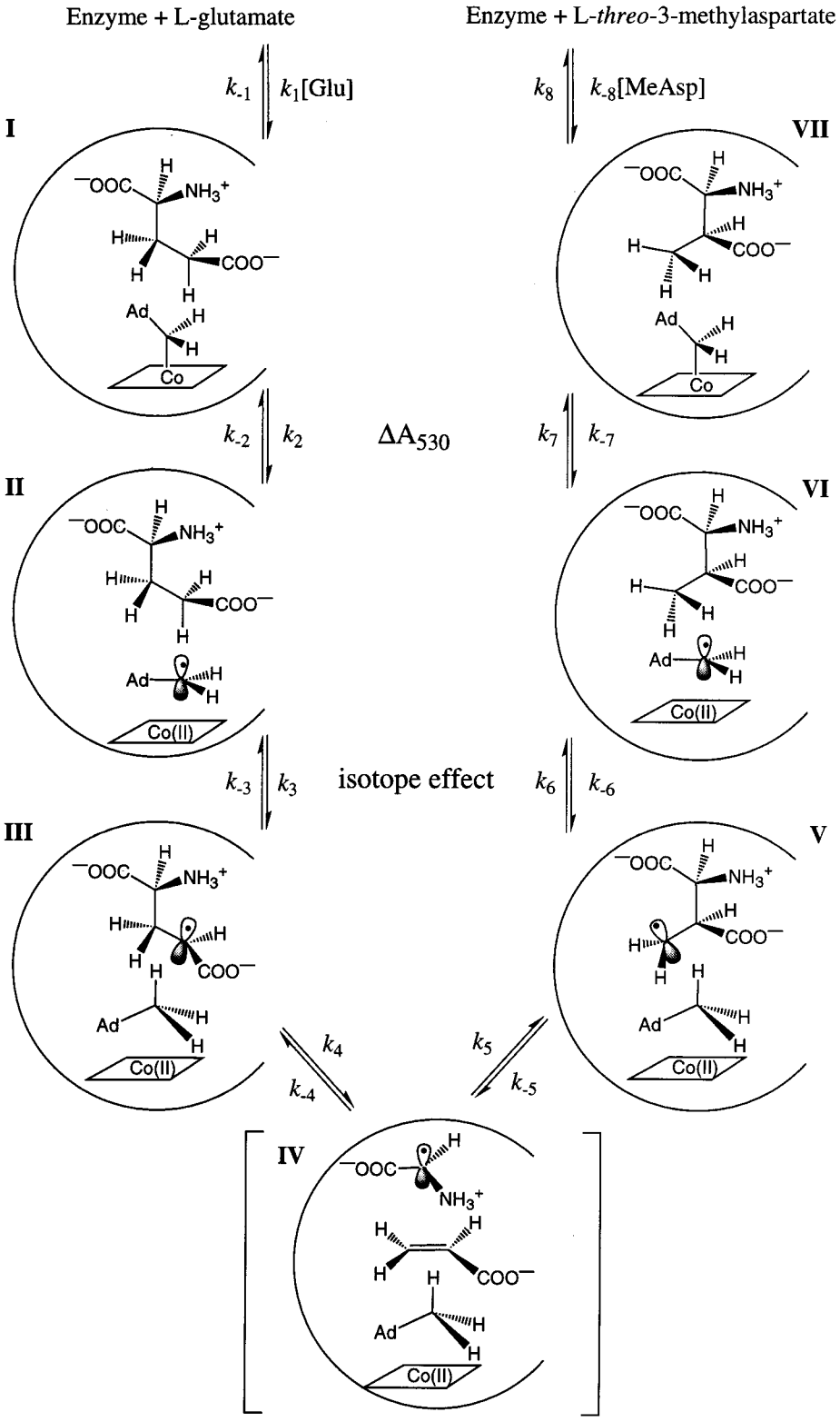


FIGURE 2: Proposed mechanism and kinetic scheme for the reversible rearrangement of L-glutamate to L-*threo*-3-methylaspartate catalyzed by glutamate mutase. Steps giving rise to a change in absorbance at 530 nm and steps giving rise to a deuterium isotope effect are indicated. The skeletal rearrangement is envisaged to occur through fragmentation and recombination, with acrylate and glycyl radical, shown in brackets as **IV** as putative intermediates.

substrate and enzyme twofold, making the concentration of holoenzyme in the measured reaction mixture 50 μM.

The reaction was monitored by following the change in absorbance at 530 nm that accompanies cobalt−carbon bond homolysis. For each concentration of substrate used, the data from at least three shots were averaged and fitted to either single or multiple parallel exponential functions to obtain rate constants through use of the KISS program. Secondary plots of data and curve-fitting were performed with the Kaleidagraph program (Abelbeck Software).

RESULTS

Homolysis of the AdoCbl Co−C bond can be monitored by changes in absorbance that occur upon the formal

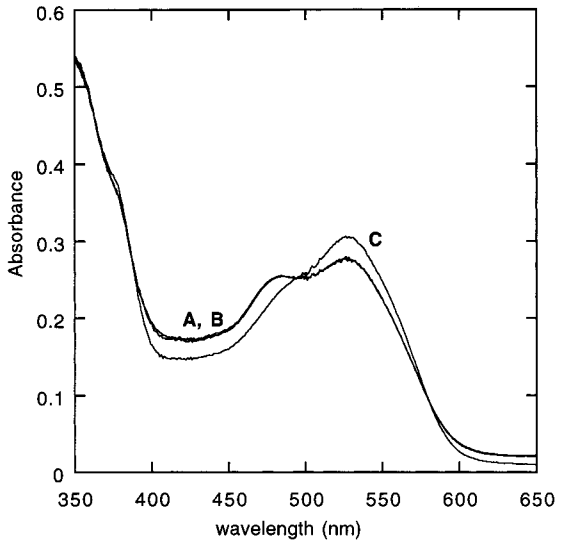


FIGURE 3: UV-visible spectra recorded in the stopped-flow spectrophotometer showing the partial formation of Cbl(II) on the enzyme after mixing with: (A) 1 mM L-*threo*-3-methylaspartate or (B) 1 mM L-glutamate; (C) control experiment showing spectrum obtained by mixing holo-enzyme with buffer. For details see the text.

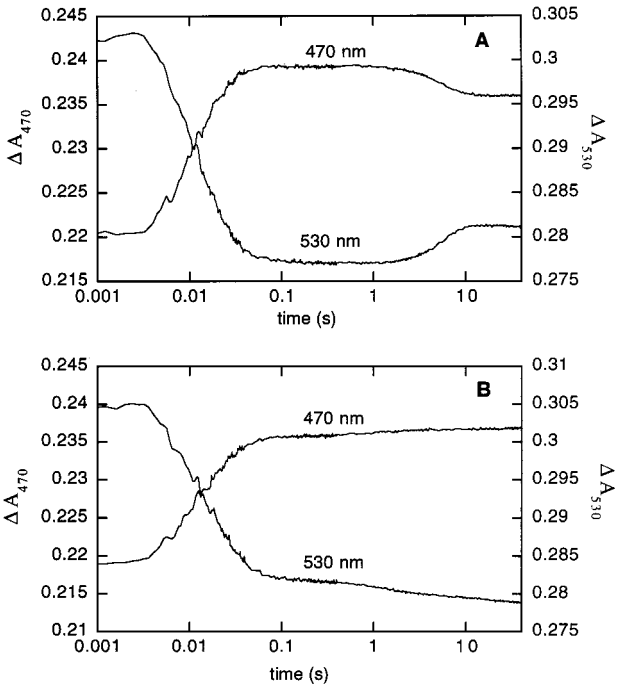


FIGURE 4: Reaction of holo-glutamate mutase (40 μM final concentration) with (**A**) 1 mM L-*threo*-3-methylaspartate or (**B**) 1 mM L-glutamate. The reactions were monitored at both 470 and 530 nm to record the formation of Cbl(II) and the disappearance of AdoCbl, respectively; the kinetics of these processes are seen to mirror each other for each substrate. The time axis is plotted on a logarithmic scale.

reduction of Co(III) to Co(II). Figure 3 shows spectra of holo-glutamate mutase recorded in the stopped-flow apparatus ~2 min after mixing with either buffer, 1 mM L-glutamate, or 1 mM L-*threo*-3-methylasparate, after which time no further spectral changes were evident. Comparison of these spectra reveals that addition of substrate led to a decrease in absorbance at between 500 and 580 nm and an increase in absorbance at between 390 and 490 nm, consistent with the formation of Cbl(II) on the enzyme.

The time-course of AdoCbl homolysis can be followed by monitoring either the disappearance of AdoCbl at 530 nm or the appearance of Cbl(II) at 470 nm. Figure 4 shows the time course of the reaction initiated by mixing either 1 mM glutamate or 1 mM methylasparate with the enzyme and monitored at both 470 and 530 nm. The stopped-flow traces mirror each other, confirming that the spectral changes arise from the formation of Cbl(II) from AdoCbl on the enzyme. With either substrate, the spectra obtained at the end of the reaction (Figure 3) superimposed almost exactly, as would be expected for a reversible reaction that had reached chemical equilibrium. Slight differences in the final absorbance (~0.002 AU) are evident from the kinetic traces in Figure 4. These most probably reflect small changes in the detector offset between shots.

The reaction catalyzed by glutamate mutase is freely reversible with an equilibrium constant of ~12 in favor of L-glutamate (*17*). Therefore, both forward and reverse reactions contribute to the pre-steady-state kinetics. The time-dependent changes in Cbl(II) concentration are therefore best described by relaxation times, τ, where $\tau_n = 1/(k_n + k_{-n})$ (*18*). The kinetic phenomena we observed are interpreted with reference to the mechanism in Figure 2. Except for the parenthetical intermediate, **IV**, our kinetic experiments are sensitive to the formation of all the proposed intermediates in mechanism. Homolysis of AdoCbl (i.e., formation of species **II** and **VI** in Figure 2) can be observed directly by the change in absorbance at 530 nm. The formation of enzyme:substrate complexes, species **I** and **VII**, can be probed by examining how the rate of AdoCbl homolysis varies with substrate concentration. Finally, hydrogen transfer from substrate to coenzyme, to form species **III** and **V**, can be probed by examining how deuteration of the substrates affects the kinetics of AdoCbl homolysis.

Kinetic Behavior with L-threo-3-Methylaspartate. The stopped-flow traces obtained when enzyme was reacted with various concentrations of methylaspartate at 10 °C are shown in Figure 5A. Three prominent phases are evident from the traces. Initially, there is a rapid decrease in absorbance at 530 nm, with a rate and amplitude that approach limiting values as substrate concentration increases. This first phase is attributable to homolysis of the Co−C bond to form Cbl(II). This is followed by a time region where very little absorbance change occurs; moreover, this region lengthens when more substrate is present. This phase can be attributed to steady-state turnover, during which the enzyme is using the excess substrate. Note that in the top trace, where enzyme and substrate are equimolar, essentially no steady-state phase is observed. Finally, there is a slower partial recovery of absorbance that is also concentration dependent; at higher substrate concentrations, recovery is slower and the amplitude of the recovery phase is diminished. Beyond 30 s, a slow decrease in absorbance occurred over several minutes (data not shown). Control experiments established that this was caused by the slow photolysis of AdoCbl in the light beam of the spectrometer.

When the measurements were repeated with L-*threo*-[3-$^2H_3$-methyl]aspartate, the initial rate of AdoCbl homolysis was substantially decreased and, interestingly, an additional kinetic intermediate was seen (Figure 5B), resulting in two downward phases of similar amplitude. The steady-state

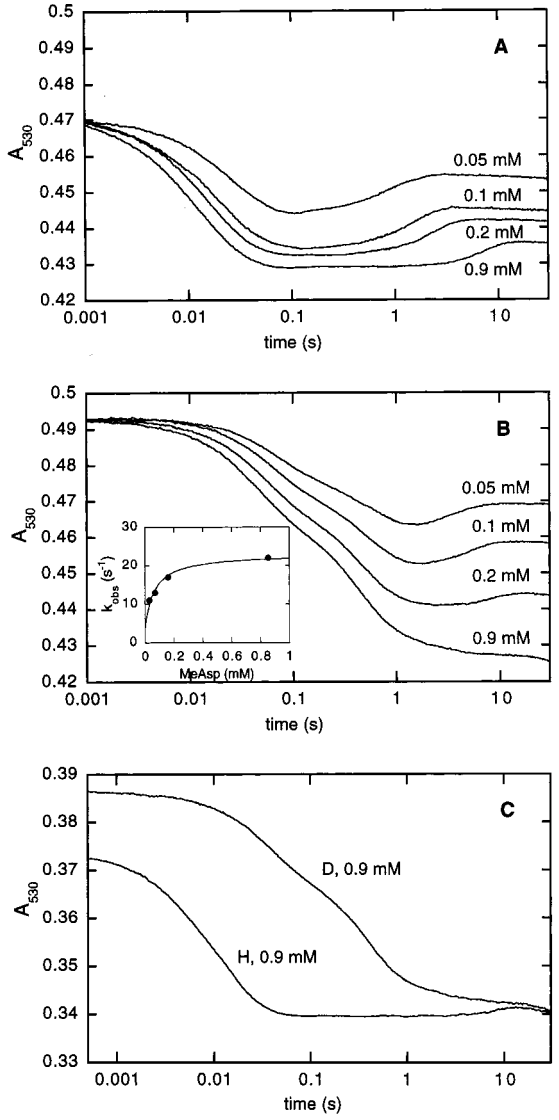


FIGURE 5: Changes in absorbance at 530 nm, indicative of homolysis of AdoCbl, following rapid mixing of holo-glutamate mutase (50 μM final concentration) with various concentrations of L-*threo*-3-methylaspartate. The concentration of substrate is indicated for each trace. The time axis is plotted on a logarithmic scale. (**A**) Traces obtained with protiated substrate; (**B**) traces obtained with substrate deuterated at the methyl group; the variation of k_{obs} measured for the first phase of the reaction with substrate concentration. (**C**) direct comparison of traces obtained with 0.9 mM deuterated and 0.9 mM protiated methylaspartate in consecutive shots (the concentration of holo-enzyme in this experiment was ~45 μM).

phase was less prominent and the final recovery phase was slower than that of the protiated substrate. One puzzling feature was that the amplitude of the traces obtained with deuterated methylaspartate appeared larger than those obtained with protiated methylaspartate. Initially, because the measurements with deuterated substrates were carried out first, we attributed this phenomenon to a loss of enzyme activity (by ~20%) that occurred over the several hours that it took to obtain the full set of traces, so that comparisons between the amplitudes of the traces in the first and last sets of data were unreliable. Therefore, in a separate experiment, the measurements were repeated first with high concentrations of protio-methylaspartate and immediately afterward with deutero-methylaspartate, so that changes in enzyme activity were negligible.

The traces obtained when first 0.9 mM L-*threo*-methylaspartate and then L-*threo*-[3-$^2H_3$-methyl]aspartate were reacted with the enzyme, one after another, are compared in Figure 5C. evidently, substantial homolysis occurred in the presence of protiated methylaspartate during the ~2 ms dead time of the experiment. The amplitude of this very fast phase is similar to that of the first phase seen when the enzyme is reacted with deuterated methylaspartate.

By fitting the first two phases of the traces obtained with various concentrations of substrates to either single or double exponential functions, the apparent rate constants, and hence the relaxation times, for these processes could be obtained. These were then extrapolated to obtain the values for τ at saturating concentrations of substrate. With protiated methylaspartate, the first homolytic phase is almost complete within the dead time of the spectrometer and therefore must be occurring with a relaxation time, $^H\tau_1$, of $\leq$2 ms. The relaxation time of the second homolytic phase of the reaction, $^H\tau_2$, is 12.5 $\pm$ 0.4 ms (k_{obs} = 80 s^{-1}). With deuterated methylaspartate, the relaxation times for the first and second phases were $^D\tau_1$ = 45 $\pm$ 0.3 ms (k_{obs} = 22 s^{-1}) and $^D\tau_2$ = 430 $\pm$ 3 ms (k_{obs} = 2.3 s^{-1}), respectively. From these data the isotope effect on the second homolytic phase, $^D\tau_2/^H\tau_2$, is 35 and that on the first homolytic phase, $^D\tau_1/^H\tau_1$, is probably at least 20.

We interpret the phase that occurs after the steady-state period of the reaction as the establishment of the chemical equilibrium between methylaspartate and glutamate. Because glutamate is bound about 10-fold less tightly than methylaspartate, and because the equilibrium favors glutamate by 12:1 (*17*), once formed, some glutamate diffuses from the enzyme and decreases the total concentration of the enzyme:substrate complex, and hence the amount of enzyme in the Cbl(II) form. The ratios of methylaspartate to enzyme used were 1:1, 2:1, 4:1, and 18:1; thus, at higher concentrations of methylaspartate, multiple turnovers are needed to establish chemical equilibrium. Also, as the initial concentration of methylaspartate and hence the final concentration of glutamate increase, the equilibrium concentrations of the enzyme:methylaspartate and enzyme:glutamate complexes, both of which have some Cbl(II) character, also increase. This is evident from the kinetic traces that show a longer steady-state region with a greater final proportion of enzyme remaining in the Cbl(II) form at equilibrium with higher concentrations of substrates. At the highest concentration of methylaspartate used, the turnover number, calculated as the number of turnovers required to achieve equilibrium divided by the time taken for the system to reach equilibrium, is ~2 s^{-1}. This is similar to the value of 1.5 s^{-1} measured for k_{cat} at 10 °C by the conventional spectroscopic assay.

With deuterated methylaspartate the slow recovery of absorbance is similar to that seen with protiated substrate and can also be attributed to the establishment of chemical equilibrium. As expected, the traces show evidence for a significant kinetic isotope effect on turnover. The exception is that with 0.9 mM deuterated methylaspartate a slight decrease in absorbance is seen after 10 s. We attribute this to photolysis of the coenzyme. The slower reaction with deutero-substrate is more susceptible to photolysis, and this masks the recovery phase. The steady-state deuterium isotope effect estimated by comparing the traces obtained with 0.2 mM methylaspartate is ~5, in reasonable agreement

with a 6.3 value obtained previously from steady-state measurements (*10*).

It is informative to examine the kinetic behavior at the lowest concentration of methylaspartate used, 0.05 mM, where the enzyme and substrate are present in stoichiometric amounts. In this case equilibrium can be reached within a single turnover and the formation of glutamate should be a first-order process. Indeed, the recovery phases seen with both deuterated and protiated substrates are reasonably well fitted by single exponentials with relaxation times ${}^{H}\tau_3 = 0.83$ s and ${}^{D}\tau_3 = 1.7$ s, respectively. This yields an observed kinetic isotope effect of 2 for the second hydrogen transfer step (step 6 in Figure 2), far smaller that the ~30-fold isotope effect seen for the first step or the ~6-fold isotope effect on steady-state turnover. This may result in part from the dilution of the 3 deuterium atoms in the substrate by equilibration with the 2 protium atoms from the coenzyme. Also, the preceding step, isomerization of the substrate, may be slower than hydrogen transfer and thus may partially suppress the isotope effect.

Kinetic Behavior with L-*Glutamate.* The stopped-flow traces obtained when enzyme was reacted with various concentrations of L-glutamate are shown in Figure 6A. Two phases are immediately evident. The initial rapid decrease in absorbance becomes greater in both amplitude and rate, tending toward a limiting value at saturating concentrations of substrate. In contrast to the case with methylaspartate, the second, much slower, phase exhibits a decrease in absorbance at 530 nm, the amplitude and rate of which appear to vary only slightly with the substrate concentration.

When the measurements were repeated with L-[$^2H_4$]-glutamate, the initial rate of AdoCbl homolysis was greatly decreased, and again an additional kinetic intermediate was seen (Figure 6B), resulting in two phases of similar amplitude, analogous to the case with L-*threo*-[3-$^2H_3$-methyl]-aspartate. Again, because some loss of enzyme activity occurred over the course of this experiment, the measurements were repeated back-to-back in a separate experiment with high concentrations of protiated and deuterated glutamate. The traces obtained when first 4.5 mM L-glutamate, and then L-[$^2H_4$]glutamate, were reacted with the enzyme are compared in Figure 6C. Again a substantial fraction of the enzyme clearly has reacted with the protiated substrate within the dead time of the experiment.

The relaxation times associated with each phase of the glutamate-initiated reaction were obtained by curve-fitting the traces and extrapolating the values to saturating concentrations of substrate. With protiated glutamate, the initial homolytic phase was almost complete within the dead time of the spectophotometer and only the tail end of the decay could be observed. The relaxation time, ${}^{H}\tau_1$, is estimated as 2 ms or less. The second homolytic phase of the reaction occurs with a relaxation time, ${}^{H}\tau_2$, of 10.3 ± 0.5 ms ($k_{obs} = 97$ s^{-1}). With deuterated glutamate, the relaxation times for the first and second phases were ${}^{D}\tau_1 = 16 \pm 0.04$ ms ($k_{obs} = 64$ s^{-1}) and ${}^{D}\tau_2 = 294 \pm 8$ ms ($k_{obs} = 3.4$ s^{-1}), respectively. From these data the isotope effect on the second homolytic phase, ${}^{D}\tau_2/{}^{H}\tau_2$, is 28 and that on the first homolytic phase, ${}^{D}\tau_1/{}^{H}\tau_1$, is probably at least 10.

In the presence of protio-glutamate, there is a much slower phase, associated with a small further decrease in absorbance that occurs with ${}^{H}\tau_3 = 0.55$ s, ($k_{obs} = 1.8$ s^{-1}). With deuterated glutamate a slow phase with a relaxation time, ${}^{D}\tau_3$, of ~3 s is also just discernible from the kinetic traces. We tentatively assign these slow reactions to the formation of methylaspartate on the enzyme. However, in contrast to the case when methylaspartate is the substrate, these relaxation times are independent of substrate concentration, which makes them harder to interpret.

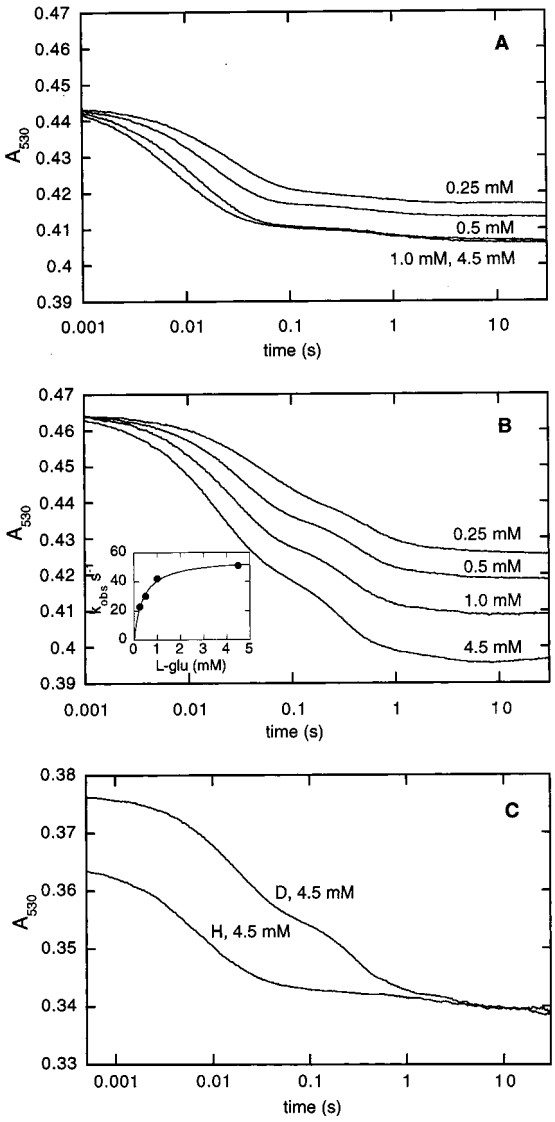


FIGURE 6: Changes in absorbance at 530 nm, indicative of homolysis of AdoCbl, following rapid mixing of holo-glutamate mutase (50 μM final concentration) with various concentrations of L-glutamate. The concentration of substrate is indicated for each trace. The time axis is plotted on a logarithmic scale. (**A**) Traces obtained with protiated substrate; (**B**) traces obtained with substrate di-deuterated at the C-4 carbon; variation of k_{obs} measured for the first phase of the reaction with substrate concentration. (**C**) direct comparison of traces obtained with 4.5 mM deuterated and 4.5 mM protiated glutamate in consecutive shots (the concentration of holoenzyme in this experiment was ~45 μM).

With L-glutamate, no recovery in absorbance is seen as the reaction approaches chemical equilibrium. We explain this by noting that, in these experiments, L-glutamate is at high concentrations relative to the enzyme concentration, because at equilibrium only 10% of the substrate is converted to methylaspartate, the glutamate concentration changes only slightly throughout the reaction. Furthermore, because the methylaspartate produced binds more tightly to the enzyme

than does the glutamate, the formation of methylaspartate will lead to a greater fraction of the enzyme being present as enzyme:substrate and enzyme:product complexes at equilibrium, and thus in the Cbl(II)-containing forms of the enzyme will be increased.

Dissociation Constants for L-Glutamate and L-threo-3-Methylaspartate. The homolysis of AdoCbl was studied at four concentrations of each substrate, chosen to be approximately 0.5 K_M, K_M, 2 K_M, and 9 K_M. By plotting the variation of the observed first-order rate constant against the substrate concentration, we obtained the apparent dissociation constant for the substrate, K_S (*18*). Because the initial phase of the reaction occurred too fast to be measured with protiated substrates, we used the data obtained with deuterated substrates to estimate apparent K_s for methylaspartate and glutamate. Plots showing the variation of the observed first-order rate constant for the formation of Cbl(II) (first phase of reaction) with increasing concentrations of deutero-methylaspartate and deutero-glutamate are shown inset in Figures 4B and 5B, respectively. The fact that the observed first-order rate constant for Cbl(II) formation tends to a limiting value at high substrate concentrations indicates that formation of the enzyme−substrate complex is rapid compared with the chemical step (*18*). In the case of L-*threo*-3-methylaspartate, wherein the substrate concentrations used were comparable with the enzyme concentration, we introduced a correction to reflect the concentration of free substrate.

The apparent K_S for L-glutamate (k_{-1}/k_1 in Figure 2) was 0.61 ± 0.07 mM, whereas the K_S for L-*threo*-3-methylaspartate (k_8/k_{-8} in Figure 2) was 0.037 ± 0.005 mM. The K_S for L-glutamate is very similar to the K_M of 0.58 mM, previously determined for this substrate. The K_S for L-*threo*-3-methylaspartate, however, is considerably lower than its K_M, 0.14 mM. These observations are in accord with predictions of K_S based on previous steady-state measurements of deuterium isotope effects (*10*). At steady state with L-glutamate as the substrate, ^DV and $^DV/K$ are similar in size, 3.9 and 4.0, respectively, which is expected when $K_s = K_m$. However, when the substrate is L-*threo*-3-methylaspartate, ^DV is noticeably larger than $^DV/K$ (6.3 and 3.4, respectively), a result that is expected when $K_s < K_m$.

DISCUSSION

Our experiments have shown that binding of either L-glutamate or L-*threo*-methylaspartate elicits rapid homolysis of AdoCbl by glutamate mutase. For both substrates this process is characterized by two relaxation times. Furthermore, when deuterated substrates are used, the kinetic isotope effects on both relaxation times are very large. Here we discuss the implications of our results for the mechanism of AdoCbl homolysis and the energetics of AdoCbl-mediated isomerizations.

Deuterium Isotope Effects and Their Significance for the Mechanism. The fact that deuterated substrates react with the enzyme to induce AdoCbl homolysis at much slower rates than protiated substrates is at first sight surprising, since homolysis does not formally involve hydrogen abstraction from the substrate. However, because the glutamate mutase−catalyzed reaction is readily reversible, one can consider the steps in isomerization as a series of coupled equilibria. In this view, addition of substrate perturbs the entire series of equilibria shown in Figure 2. The establishment of the new equilibrium involves all of the rate constants in the system, including k_3, k_{-3}, k_6, and k_{-6}, which are sensitive to isotopic substitution. Thus, the isotope effect arises because the adenosyl radical reacts more slowly with the deuterated substrate then with the protiated substrates and equilibrium is established more slowly. However, for a large isotope effect to be observed on the formation of Cbl(II), the formation of species **II** or **VI** must be both rapid and energetically unfavorable in comparison with the subsequent formation of species **III** or **V**. In other words, the adenosyl radical can exist only in very low concentration as a high-energy intermediate.

An alternative explanation for the isotope effect is that Co−C bond cleavage and hydrogen abstraction occur in a single, concerted step, so that the 5′-deoxyadenosyl radicals, species **II** and **VI** in Figure 2, are never actually formed. However, we are aware of no chemical precedent for such a reaction. Therefore, whereas our experiments do not rule out a concerted reaction, we consider it a less likely possibility.

EPR spectra showing an organic radical spin-coupled to Co(II) have been observed in the presence of substrates for many AdoCbl-dependent enzymes (*12*−*14*, *19*), including glutamate mutase (*15*). However, in cases where the nature of the organic radical has been investigated by isotopic labeling, the organic radical has been shown to reside on the substrate, e.g., ethanolamine ammonia-lyase (*12*), or, in the case of ribonucleotide reductase, on the protein (*20*) and not on 5′-deoxyadenosine. Furthermore, isotope effects on Cbl(II) formation have recently been observed for the AdoCbl-dependent enzyme methylmalonyl-CoA mutase in experiments with using deuterated methylmalonyl-CoA as the substrate (*21*). These observations all support a mechanism in which substrate binding is coupled to homolysis of the Co−C bond and the adenosyl radical is present only at very low steady-state concentrations during turnover. We envisage that substrate-binding may supply the requisite energy, perhaps through a conformational change of the protein, for a small concentration of adenosyl radical to be generated by the enzyme and subsequently react with the substrate. In this way, the reactive adenosyl radical is produced at the active site only when substrate is present, and deleterious side reactions are minimized.

The Biphasic Nature of Cbl(II) Formation. The fact that homolysis of AdoCbl appears to occur in two phases with rate constants that differ by 5- to 10-fold is puzzling. This phenomenon was apparent when the enzyme was reacted with either substrate, and both phases were subject to large isotope effects when deuterated substrates used. It seems unlikely that we could be observing coupling of two equilibrium steps in the mechanism, e.g., an unfavorable equilibrium for homolysis of AdoCbl (steps k_2/k_{-2} and k_{-7}/k_7 in Figure 2) coupled to a favorable equilibrium for the generation of substrate radicals (steps k_3/k_{-3} and k_{-6}/k_6 in Figure 2) as discussed above, because the relaxation times are sufficiently different that the isotope effect should affect only one step, not both. Thus, in the above example, when deuterated substrates were used, the initial homolytic step should still have occurred within the dead time of the experiment; only the second step, arising from redistribution

of the first equilibrium through the reaction of adenosyl radical with the substrate, should have shown an isotope effect.

One explanation is that the enzyme exists in two forms, possibly representing different conformers of the protein or coenzyme that catalyze coenzyme cleavage/hydrogen abstraction at different rates. Since the enzyme is a dimer, and the two phases are of similar amplitude, we speculate that this phenomenon may be due to a "half-of-sites" effect; i.e., binding of substrate to one site in the dimer may change the ability of the enzyme to catalyze the reaction at the other site. This hypothesis could be tested experimentally by examining the reaction in the presence of substoichiometric amounts of substrate. In this context, we note that even the slower phase of the reaction is sufficiently fast (~50-fold faster than k_{cat}) to be a kinetically competent step in the mechanism.

The deuterium isotope effects, of 28 and 35 measured with glutamate and methylaspartate, respectively, are much larger than those commonly encountered for the cleavage of C−H bonds by enzymes. However, very large deuterium isotope effects have been observed for several enzymes that catalyze hydrogen abstraction by free radical mechanisms. In a pre-steady-state study of methylmalonyl-CoA mutase, an isotope effect of >20 was estimated when deuterated methylmalonyl-CoA was used to trigger AdoCbl homolysis (*21*). For soybean lipoxygenase a deuterium isotope effect of 48 was found for hydrogen abstraction from linoleic acid (*22*), and an isotope effect of 50−100 was measured for the oxidation of CD_4 by methane mono-oxygenase (*23*). Such anomolously large isotope effects are often associated with hydrogen tunneling. Indeed, there is good evidence for extensive tunneling of both hydrogen and deuterium in the lipoxygenase-catalyzed reaction (*22*). The very large tritium isotope effects, of 125 and 150, measured for AdoCbl-dependent diol dehydratase and ethanolamine ammonia-lyase, respectively (*24*, *25*), may also arise from quantum tunneling, although alternative explanations involving partioning of tritium between the substrate and a protein-based radical have been proposed (*26*, *27*). As quantum tunneling is detected in more enzymes (*28*), this phenomenon may prove to be an important feature of AdoCbl-mediated catalysis and possibly of radical-requiring enzymes in general.

Energetics of AdoCbl Homolysis. From the preceding discussion we can see that the relaxation times associated with the formation of Cbl(II) on the enzyme do not relate to the equilibrium between species **I** and **II** (or **VII** and **VI**) in Figure 2, but rather to that between species **I** and **III** (or **VII** and **V**). The molar absorptivity change, $\Delta\epsilon_{530}$, for the homolysis of AdoCbl, estimated from the difference spectra of AdoCbl and Cbl(II) free in solution, is 4000 $M^{-1}cm^{-1}$. From the amplitude of the absorbance changes that occur when the enzyme is reacted with saturating concentrations of substrates, the equilibrium constant for the formation of Cbl(II) on the enzyme ~0.25. Thus, the forward and reverse rate constants for this equilibrium can be calculated since $K_{eq} = k_f/k_r$ and $\tau = 1/(k_f + k_r)$. With L-glutamate the formation of Cbl(II) (conversion of **I** to **III**) in the slower phase of the reaction, characterized by $^{H}\tau_2 = 10.3$ ms, proceeds with a rate constant of 19 s^{-1}, whereas the reverse reaction occurs with a rate constant of 78 s^{-1}. With L-*threo*-methylaspartate the slower phase of the homolysis reaction (conversion of **VII** to **V**) occurs with a rate constant of 16 s^{-1}, whereas the reverse reaction occurs with a rate constant of 64 s^{-1}. For comparison, the nonenzymic thermolysis of free AdoCbl is calculated to occur with rate constant of 10^{-11} s^{-1} at 10 °C, according to the thermodynamic data of Hay and Finke (*11*). Therefore, at 10 °C the enzyme must accelerate the rate of Co−C bond cleavage by at least 10^{12}-fold, with the substrate effectively acting to trap the adenosyl radical as it is formed.

Although much attention has focused on the origin of the remarkable rate acceleration for coenzyme homolysis exhibited by AdoCbl enzymes, equally noteworthy is the degree to which free radical species are stabilized by the enzyme during catalysis. When substrates are added, Cbl(II) accumulates to be a substantial proportion of the total enzyme species, a phenomenon that has been observed with several AdoCbl-dependent enzymes (*21*, *22*). This implies that the enzyme destabilizes the Co−C bond, dissipating nearly all of the dissociation energy (~30 kcal mol^{-1} in free solution) (*11*). This is a remarkable example of the destabilization of a single chemical bond by an enzyme. Some of the unfavorable bond dissociation energy may be offset if the substrate radicals, which appear to be the species that accumulate on the enzyme, are intrinsically more stable than adenosyl radical. However, simple chemical considerations make it unlikely that the methyl radical of methylaspartate, in particular, would be more stable than the 5′-deoxyadenosyl radical, and the mechanism by which the Cbl(II) form of the enzyme is stabilized remains poorly understood.

Free Energy Profile and the Nature of the Rate-Determining Step. The pre-steady-state isotope effects of 28 and 35 measured with glutamate and methylaspartate, respectively, are probably close to the intrinsic isotope effects for the transfer of deuterium between substrate and coenzyme. However, the deuterium isotope effects on V_{max} measured for glutamate mutase under steady-state conditions are much lower: 3.9 and 6.3 for glutamate and methylaspartate, respectively. This suggests that in either direction a step other than AdoCbl homolysis/hydrogen transfer, which appears to be kinetically coupled, is partially rate-limiting in the overall catalytic cycle.

The slow step(s) could be the isomerization of the substrate radical to product radical, or product dissociation that we are not able to observe directly in our experiments, on both. We suggest that product dissociation is less likely to be rate-limiting because neither substrate binds especially tightly and k_{cat} is quite slow (~1.5 s^{-1} in either direction at 10 °C). More likely, with protiated substrates the isomerization (step **III** to **V**) is partially rate determining. Under single-turnover conditions with methylaspartate, a large isotope effect is observed on hydrogen abstraction from methylaspartate and a much smaller isotope effect on the formation of glutamate. Thus, the isomerization step cannot be completely rate-determining, otherwise no isotope effect would be observed on glutamate formation. However, such findings are consistent with isomerization being partially rate-determining because the second isotope effect is significantly diminished from its intrinsic value. Further support for the rate-determining nature of this step comes from experiments set up to examine the partitioning of tritium from 5′-tritiated AdoCbl between substrate and product in the glutamate

mutase reaction (B. Hilbert and E.N.G.M., unpublished results). As discussed by Leadlay and colleagues (*29*), if the interconversion of **III** and **V** is slow relative to the steps leading to their formation, then tritium in the coenzyme will tend to partition back toward the substrate rather than appearing in the product. Those experiments indicate a small tendency for tritium to partition back toward substrate, again indicating that isomerization is partially rate-determining.

CONCLUSIONS

The kinetic experiments described here provide some insight into the mechanism of the unusual isomerization catalyzed by glutamate mutase, and have allowed us to estimate the values for several of the equilibria and elementary rate constants in Figure 2. These results have been used to construct a qualitative profile of free energy for the glutamate mutase reaction shown in Figure 6. Experiments are now in progress to determine more fully the free energy profile for this reaction in hopes of improving our understanding of how B_{12} enzymes generate and control these highly reactive free radical species.

Apparently, the 5′-deoxyadenosyl radical, a species central to mechanistic thinking regarding the role of AdoCbl, can exist on the enzyme only at very low concentrations and indeed may not even be a true intermediate in these reactions. This may explain why this species has never been observed spectroscopically. The magnitude of the isotope effects observed in these experiments raises the possibility that quantum tunneling may be important in the transfer of hydrogen between substrate and coenzyme.

ACKNOWLEDGMENT

We thank Brent Hilbert for synthesizing deutero- and protio-L-*threo*-3-methylaspartates, Hao-Ping Chen for advice on the purification of GlmES, and Rowena Matthews for reading the manuscript and helpful discussions.

REFERENCES

1. Halpern, J. (1985) *Science 227*, 869−875.
2. Ochiai, E.-I. (1994) *Metal Ions in Biological Systems 30*, 255−278.
3. Marsh, E. N. G. (1995) *Bioessays 17*, 431−441.
4. Buckel, W., & Golding, B. T. (1996) *Chem. Soc. Rev.* 329−337.
5. Banerjee, R. (1997) *Chemistry and Biology 4*, 175−186.
6. Holloway, D. E., & Marsh, E. N. G. (1994) *J. Biol. Chem. 269*, 20425−20430.
7. Switzer, R. L., & Barker, H. A. (1967) *J. Biol. Chem. 242*, 2658−2674.
8. Marsh, E. N. G., & Holloway, D. E. (1992) *FEBS Lett. 310*, 167−170.
9. Ludwig, M. L., & Matthews, R. G. (1997) *Annu. Rev. Biochem. Mol. Biol. 66*, 269−311.
10. Chen, H.-P., & Marsh, E. N. G. (1997) *Biochemistry 36*, 14393−14945.
11. Hay, B. P., & Finke, R. G. (1986). *J. Am. Chem. Soc. 108*, 4820−4829.
12. Babior, B. M., Moss, T. H., Orme-Johnson, W. H., & Beinert, H. (1974).. *J. Biol. Chem. 249*, 4537−4542.
13. Michel, C., Albracht, S. P. J., & Buckel, W. (1992). *Eur. J. Biochem. 205*, 767−773.
14. Keep, N. H., Smith, G. A., Evans, M. C. W., Daikun, G. P., & Leadlay, P. F. (1993) *Biochem. J. 295*, 387−392.
15. Zelder, O., Leutbecher, U., Beatrix, B., & Buckel, W. (1994). *Eur. J. Biochem. 226*, 577−585.
16. Eagar, R. G., Baltimore, B. G., Herbst, M. M., Barker, H. A., & Richards J. H. (1972) *Biochemistry 11*, 253−264.
17. Barker, H. A., Rooze, V., Suzuki, F., & Iodice, A. A. (1964) *J. Biol. Chem. 239*, 3260−3266.
18. Fersht, A. R. (1985) *Enzyme Structure and Mechanism*, pp 136−138, Freeman: New York.
19. Zhao, Y., Abend, A., Kunz, M., & Retey, J. (1994) *Eur. J. Biochem. 225*, 891−896.
20. Licht, S., Gerfen, G. J., & Stubbe, J. (1996) *Science 271*, 477−481.
21. Padmakumar, R., Padmakumar, R., & Banerjee, R. (1997) *Biochemistry 36*, 3713−3718.
22. Jonsson, T., Glickman, M. H., Sun, S., & Klinman, J. P. (1996) *J. Am. Chem. Soc. 118*, 10319−10320.
23. Nesheim J. C., & Lipscomb J. D. (1996) *Biochemistry 35*, 10240−10247.
24. Essenberg, M. K., Frey, P. A., & Abeles, R. H. (1971) *J. Am. Chem. Soc. 93*, 1242−1251.
25. Weisblat, D. A., & Babior, B. M. (1971) *J. Biol. Chem. 246*, 6064−6071.
26. Cleland, W. W. (1982) *C. R. C. Crit. Rev. Biochem 13*, 385−428.
27. O'Brien, R. J., Fox, J. A., Kopczynski, M. G., & Babior, B. M. (1985). *J. Biol. Chem. 260*, 16131−16136.
28. Bahnson, B. J., & Klinman, J. P. (1995) *Methods Enzymol. 249*, 374−397.
29. Meier, T. W., Thomä, N. H., & Leadlay, P. F. (1996) *Biochemistry 35*, 11791−11796.

BI980512E