Pre-Steady-State Kinetic Investigation of Intermediates in the Reaction Catalyzed by Adenosylcobalamin-Dependent Glutamate Mutase†

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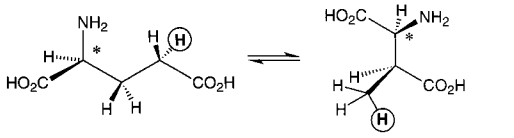
Received May 10, 1999; Revised Manuscript Received August 2, 1999

ABSTRACT: Glutamate mutase catalyzes the reversible isomerization of L-glutamate to L-*threo*-3-methylaspartate. Rapid quench experiments have been performed to measure apparent rate constants for several chemical steps in the reaction. The formation of substrate radicals when the enzyme was reacted with either glutamate or methylaspartate was examined by measuring the rate at which 5′-deoxyadenosine was formed, and shown to be sufficiently fast for this step to be kinetically competent. Furthermore, the apparent rate constant for 5′-deoxyadenosine formation was very similar to that measured previously for cleavage of the cobalt−carbon bond of adenosylcobalamin by the enzyme, providing further support for a mechanism in which homolysis of the coenzyme is coupled to hydrogen abstraction from the substrate. The pre-steady-state rates of methylaspartate and glutamate formation were also investigated. No burst phase was observed with either substrate, indicating that product release does not limit the rate of catalysis in either direction. For the conversion of glutamate to methylaspartate, a single chemical step appeared to dominate the overall rate, whereas in the reverse direction a lag phase was observed, suggesting the accumulation of an intermediate, tentatively ascribed to glycyl radical and acrylate. The rates of formation and decay of this intermediate were also sufficiently rapid for it to be kinetically competent. When combined with information from previous mechanistic studies, these results allow a qualitative free energy profile to constructed for the reaction catalyzed by glutamate mutase.

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

One important and poorly understood aspect of AdoCbl-dependent enzymes is their remarkable ability to stabilize what are normally considered highly reactive free radical species. Under conditions of steady-state turnover, most AdoCbl enzymes accumulate significant concentrations of free radicals, leading to the conclusion that the protein must destabilize the cobalt−carbon bond of the coenzyme by about

Scheme 1



L-glutamate L-*threo*-3-methylaspartate

30 kcal/mol (*11*). Recently, there have been several pre-steady-state kinetic studies that have focused on the mechanism of AdoCbl homolysis in the B_{12}-dependent ribonucleotide triphosphate reductase, in methylmalonyl-CoA mutase, and in glutamate mutase (*12*−*14*). These studies all reach the same basic conclusion: that homolysis of AdoCbl and hydrogen abstraction from the substrate (or protein in the case of ribonucleotide reductase) are either strongly kinetically coupled or concerted events, requiring that adenosyl radical is never formed at significant concentrations during the catalytic cycle. This finding is in accord with EPR experiments on these and other AdoCbl-dependent enzymes which have failed to detect adenosyl radical (*15*−*19*).

A mechanism for the glutamate mutase-catalyzed reaction, that incorporates the findings discussed above, is shown in Figure 1. The formation of the substrate radical is shown as a single step in which homolysis of AdoCbl and hydrogen abstraction from the substrate occur simultaneously. The interconversion of glutamyl and methylaspartyl radicals (species II and IV) is envisioned to occur through the intermediacy of glycyl radical and acrylate (species III) (*6, 15*).

† This research has been supported by NIH Research Grant GM 59227 to E.N.G.M.

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

10.1021/bi991064t CCC: $18.00
Published on Web 09/23/1999

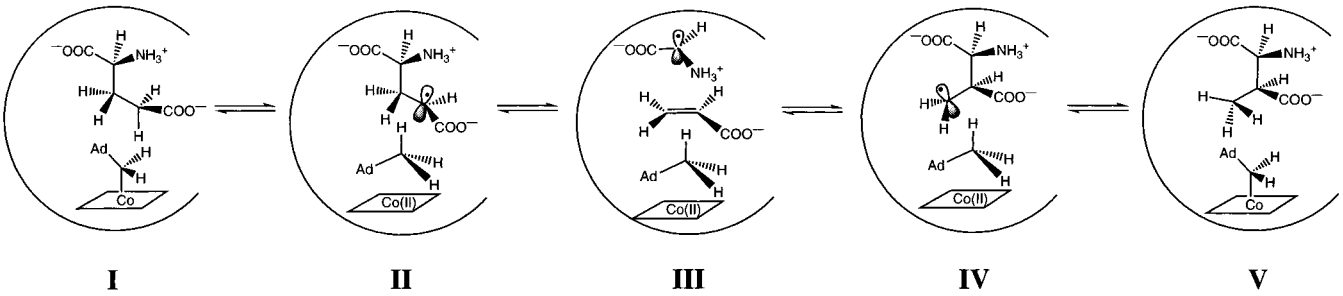


FIGURE 1: Mechanism for the reversible rearrangement of L-glutamate to L-*threo*-3-methylaspartate catalyzed by glutamate mutase. In this mechanism, homolysis of AdoCbl and hydrogen abstraction from the substrate are shown as a single step as discussed in the text.

Glutamate mutase provides one of the simplest systems with which to study the phenomenon of radical-mediated enzymatic catalysis: both the substrates are small, stable molecules; the reaction is freely reversible; and the enzyme requires no cofactors other than AdoCbl (*3*). As a prerequisite to understanding how the enzyme stabilizes radical species and directs them toward productive catalysis, we have embarked on a series of experiments to determine the free energy profile for glutamate mutase. Under steady-state conditions, the enzyme exhibited large deuterium isotope effects with both glutamate ($^{D}V = 3.9$) and methylaspartate ($^{D}V = 6.3$), initially suggesting that hydrogen transfer between substrate and coenzyme was significantly rate-limiting in both directions (*4*). However, recent pre-steady-state measurements indicate that the intrinsic deuterium isotope effects are unusually large, $^{D}V \approx 30$ for both substrates, and that with protium hydrogen transfer is unlikely to be rate-determining (*13*). These findings leave the nature of the rate-determining step open to question.

Here we report the results of rapid quench flow experiments in which the pre-steady-state kinetics of product formation have been examined to investigate whether product release is rate-determining. With neither substrate does this appear to be the case; instead, when L-*threo*-methylaspartate is the substrate, the appearance of L-glutamate is preceded by the formation of an intermediate which both forms and decays with apparent rate constants $\approx 22\ s^{-1}$. We have also measured the kinetics of 5′-dA formation when the enzyme is reacted with saturating concentrations of either L-glutamate or L-*threo*-3-methylaspartate. When these data are combined with information from previous mechanistic studies, they allow a qualitative free energy profile for the glutamate mutase reaction to be constructed.

MATERIALS AND METHODS

Materials. The purification of the glutamate mutase fusion protein, GlmES, from a recombinant *E. coli* strain has been described previously (*4*). AdoCbl, 5′-dA, L-glutamate, D,L-*threo*-3-methylasparate, glycine, and dansyl chloride were purchased from Sigma Chemical Co. The sources of other materials have been described previously (*3*, *4*, *13*) or were purchased from commercial suppliers.

Rapid Quench Kinetic Experiments. Pre-steady-state kinetic experiments were performed at 10 °C with a Hi-Tech Scientific (U.K.) RQF-63 rapid mixing apparatus. The temperature of the mixing chamber was maintained at 10 °C using a circulating water bath. The enzyme solution contained 200 μM GlmES in 50 mM potassium phosphate buffer, pH 7.0. Immediately before the experiment AdoCbl was added to a final concentration of 240 μM. The effective concentration of holoenzyme after mixing was 95 μM. The substrate solution contained either L-glutamate or L-*threo*-3-methylaspartate at twice their desired final concentration dissolved in the same buffer as the enzyme and also contained 200 μM glycine as an internal standard. In later experiments, 200 μM L-leucine was substituted for glycine as the internal standard. Reactions were started by mixing 80 μL of enzyme solution with 80 μL of substrate solution. The solution was allowed to age for various times (5−1600 ms) before being quenched with a further 80 μL of 5% trifluoroacetic acid. Samples were stored at −20 °C prior to derivatization and HPLC analysis. Solutions containing AdoCbl were handled so as to avoid exposure to bright light.

Derivatization and HPLC of Substrates. The pH of the quenched reaction mixture (240 μL) was raised above 8.5 with 200 μL of 1 M $NaHCO_3$ and the solution treated with ≈5 mg of activated charcoal to remove AdoCbl. The charcoal was removed by centrifugation and 100 μL of a 100 mg/mL solution of dansyl chloride in acetone added to the supernatant. The sample was incubated at 45 °C for 1 h to derivatize the substrate, product, and internal standard. Dansylated glutamate, methylaspartate, and glycine (or leucine) were separated by HPLC using a 25 cm C_{18} reverse phase column (Spherisorb S5 ODS2). The column was preequilibrated in 92.5% solvent A: 25 mM potassium phosphate buffer containing 7% acetonitrile and 3% methanol; and 7.5% solvent B: 70% acetonitrile, 30% methanol. Then 30−50 μL of sample was injected onto the column, and the derivatized amino acids were eluted with an ascending gradient of solvent B as follows: 0−10 min, 7.5% B; 10−22 min, 7.5−50% B; 22−26 min, 50−80% B. A typical chromatograph is shown in Figure 2. The flow rate was 1.2 mL/min, and compounds were detected by monitoring absorbance at 360 nm. The peak areas were determined by computer integration and standardized relative to the glycine or leucine internal standard. The amount of product formed was calculated from standard curves constructed by derivatization and chromatography of known amounts of either glutamate or methylaspartate. In general, three portions of the quenched, derivatized sample were chromatographed separately, and the average of the standardized peak areas was used to determine the amount of product formed.

Several different batches of enzyme were used in the experiments reported here, which require quite large amounts of protein. The experiments were reproducible from batch to batch, although small differences in the specific activity of the protein were evident. The data presented here were all obtained from one batch of protein, with the rapid quench

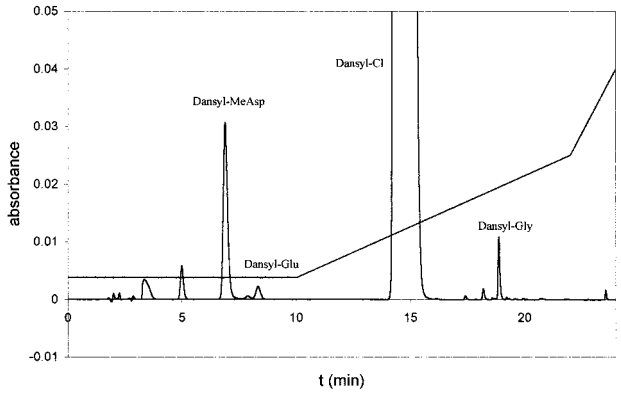


FIGURE 2: Reverse phase HPLC analysis of the products from rapid quench reactions. The chromatograph shows the separation of the dansyl derivatives of L-glutamate and L-*threo*-3-methylaspartate; dansylglycine is included in the assay as an internal standard. The solvent gradient is indicated by the ascending line.

experiments being performed as close together in time as possible (over several hours) to minimize errors due to possible losses in enzyme activity.

Analysis of 5′-dA. Rapid quenched flow experiments were conducted and analyzed as described above but with the following modifications to the reaction and chromatography conditions. The enzyme solution contained 100 μM GlmES and 120 μM AdoCbl so that the effective concentration of holoenzyme after mixing was 45 μM. The substrate solutions contained 20 μM L-tryptophan as an internal standard. 5′-dA, AdoCbl, and L-tryptophan were separated by HPLC using a 25 cm C_{18} reverse phase column (Spherisorb S5 ODS2). The column was preequilibrated in 0.1% trifluoroacetic acid, and eluted with a 20 mL linear gradient of 0−45% acetonitrile containing 0.1% trifluoroacetic acid. 5′-dA eluted at ≈10%, L-tryptophan at 40%, and AdoCbl at 45% acetonitrile. The amount of 5′-dA in the sample was calculated from standard curves obtained by chromatography of known amounts of 5′-dA.

Curve Fitting. Plots of kinetic data were generated and curve fitting performed using the KaleidaGraph program (Abelbeck Software). The data for the formation of glutamate from methylaspartate were fitted to either eq 1 or eq 2. Equation 1 describes the formation of product, $[P_t]$, as a function of time for an enzyme in which the rate constants for the formation, k_1, and decay, k_2, of a single intermediate are both kinetically significant in the overall reaction, and $k_1 \neq k_2$; $[E_0]$ is the total concentration of active enzyme (*20*). Equation 2 is the limiting form of eq 1 in which $k_1 = k_2$. The equations assume that substrate is at saturating concentration and remains saturating throughout the time course of the measurements.

$$[P_t] = k_{cat}[E_0]t\left(1 + \left(\frac{1}{k_1 - k_2}\right)(k_2 e^{-k_1 t} - k_1 e^{-k_2 t})\right) \quad (1)$$

$$[P_t] = k_{cat}[E_0]t(1 - e^{-k_1 t}(k_1 t + 1)) \quad (2)$$

Computer Simulation of Reactions. The Chemical Kinetics Simulator (v 1.0) suite of software, developed by IBM Corp., was used to perform kinetic simulations. The program uses stochastic methods to simulate reactions; typically, simulations employed 5×10^5 molecules, and the state of the system was recorded at intervals of 75 reaction events. Calculations were performed on a Macintosh 7200 power PC computer.

RESULTS

Wild-type glutamate mutase comprises two weakly associating subunits, MutE and MutS, that combine to form the coenzyme binding site. 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 upon the relative concentrations of the two subunits (*3*). 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, designated GlmES (*4*), that we have employed in previous kinetic studies (*4, 13, 21*). k_{cat} for this enzyme is only 3-fold lower than the wild-type two-subunit enzyme, and it binds coenzyme with similar affinity (*4*). Importantly, neither the affinity for AdoCbl nor the turnover number for GlmES depends on protein concentration. This enzyme is therefore much better suited to characterization by stopped-flow spectroscopy.

Glutamate mutase binds AdoCbl reversibly with $K_d = 2$ μM. In these experiments, we have assumed that association and dissociation of the coenzyme−protein complex do not affect the kinetic behavior of the enzyme. Although the holoenzyme is diluted 2-fold upon mixing, raising the possibility that some dissociation may occur, even after dilution the protein and coenzyme concentrations are still 50-fold greater than K_d, so the equilibrium will be very little perturbed. Also, previous studies on the wild-type two-subunit enzyme indicate that coenzyme exchange is very slow relative to turnover, $k_{off} = 0.01$ s^{-1}, $k_{cat} = 15$ s^{-1} (*22*), and coenzyme exchange in GlmES appears to be similarly slow. Therefore, in the rapid time scale of the present experiments, exchange of coenzyme is unlikely to occur.

Kinetics of Methylaspartate Formation. A rapid quench flow apparatus was used to mix samples of holo-glutamate mutase (95 μM final concentration) with various concentrations of L-glutamate and to quench the reaction with TFA after times ranging between 0.025 and 1.6 s. The shortest reaction times studied correspond to about one-fifth the time required for the enzyme to catalyze the formation of 1 equiv of product. The products of the reaction mixture were then derivatized with dansyl chloride, and the amount of methylaspartate formed was determined by HPLC as described under Materials and Methods; a typical chromatograph is shown in Figure 2. Glutamate and methylaspartate eluted as relatively broad peaks with quite similar retention times; nevertheless, it was possible to achieve base line separation of the two amino acids which allowed the peak areas to be integrated reliably.

Three concentrations of L-glutamate were used in these experiments: 5 mM, 10 mM, and 20 mM. The initial portions of the plots of methylaspartate formation versus time (Figure 3) were linear and intercepted the *y*-axis very close to the origin; no burst phase was evident, which indicates that product release does not contribute significantly to the overall reaction rate. There is no discernible lag phase associated with methylaspartate formation, suggesting that a single chemical step dominates the overall rate. The equilibrium between glutamate and methylaspartate favors glutamate by

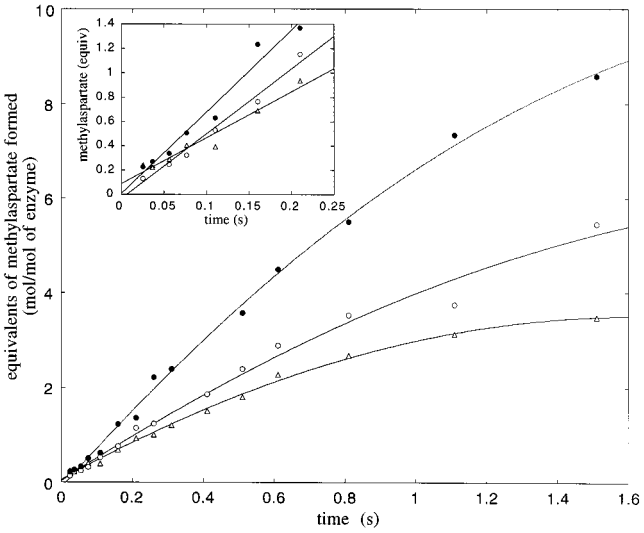


FIGURE 3: Time course for the formation of L-*threo*-3-methylaspartate by glutamate mutase obtained with three different concentrations of L-glutamate: (●) 20 mM L-glutamate; (○) 10 mM L-glutamate; (△) 5 mM L-glutamate (initial concentrations immediately after mixing). Inset: expanded view of the pre-steady-state region showing the approximately linear formation of methylaspartate during the first turnover.

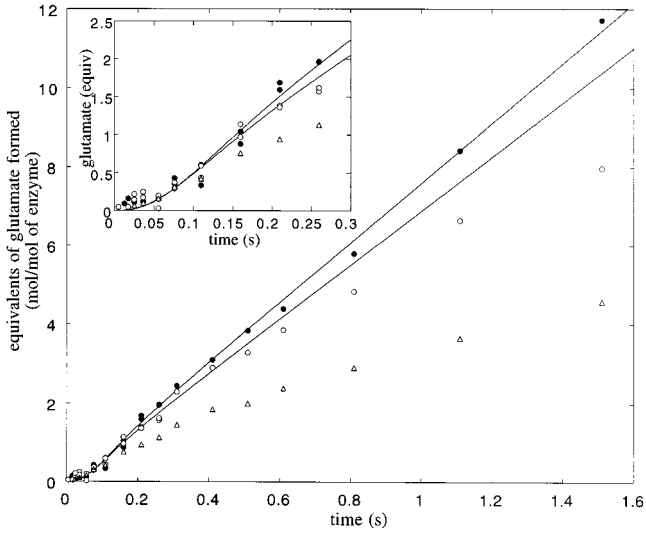


FIGURE 4: Time course for the formation of L-glutamate by glutamate mutase obtained with three different initial concentrations of L-*threo*-3-methylaspartate, showing computer fits to eq 2: (●) 2 mM L-*threo*-3-methylaspartate, with all data fitted to eq 2; (○) 1 mM L-*threo*-3-methylaspartate, with the first 0.5 s of data fitted to eq 2; (△) 0.5 mM L-*threo*-3-methylaspartate (reliable fits to eq 2 could not be obtained). Inset: expanded view of the pre-steady-state region showing the lag phase associated with the formation of glutamate during the first turnover.

about 12 to 1 (*1*); therefore, at the high enzyme concentrations necessary to observe potential burst or lag phases, relatively few turnovers are needed for the reaction to approach equilibrium. This is apparent from the plots which show significant curvature as the reaction approaches the predicted equilibrium concentration of methylaspartate.

The K_M for L-glutamate, measured by the normal spectroscopic assay, is 0.6 mM. Therefore, one might expect that even at 5 mM L-glutamate the initial rate of methylaspartate formation would be nearly maximal. Inspection of the plots reveals this is clearly not so: the most likely explanation for the much higher apparent K_M is that even after a single turnover the concentration of methylaspartate is sufficient to cause a significant degree of product inhibition. This is apparent when one considers that the K_s for methylaspartate is about 40 μM whereas the concentration of enzyme used in these experiments was 100 μM. This complicates the estimation of the 'true' k_{cat} for the enzyme because the concentration of methylaspartate varies significantly during the course of the measurement. In the presence of 20 mM L-glutamate, where product inhibition should be least important, the apparent k_{cat}, calculated from the first 300 ms of data, is 7.9 $\pm$ 0.1 s^{-1}. If one assumes the concentration of free methylaspartate to be approximately 100 μM, then about 7% of the enzyme should be product-inhibited. Therefore, the true k_{cat} is probably nearer 8.5 s^{-1}.

Kinetics of L-Glutamate Formation. The rapid quench flow technique was used to study the conversion L-*threo*-3-methylaspartate to L-glutamate for reaction times between 5 ms and 1.6 s. The initial concentrations of methylaspartate used were 0.5, 1.0, and 2.0 mM. In this direction, the equilibrium favors product (glutamate) formation, and because the enzyme binds glutamate relatively weakly, product inhibition does not become significant until the reaction is close to equilibrium. The first experiment was conducted with 0.5 mM (initial concentration) methylaspartate. Under these conditions, the reaction *does* approach equilibrium during the time course of the experiment, since only 5 turnovers are required to consume all the substrate. Consequently, the plot demonstrates curvature due to depletion of the substrate (Figure 4). No burst phase was evident within the first turnover, again indicating that product release was not rate-determining. Instead, there appeared to be a lag phase of about 50 ms associated with the formation of glutamate, suggesting that an intermediate accumulates prior to the formation of glutamate.

Subsequently, when the enzyme was reacted with higher concentrations of methylaspartate, an initial lag phase was again seen before the reaction reached steady-state (Figure 4). At 2.0 mM methylaspartate, the steady-state phase of the reaction remained linear over the full time-course of the experiment. k_{cat}, calculated from the linear region of this plot, was 7.6 $\pm$ 0.1 s^{-1}. At 1 mM methylaspartate concentration, essentially the same pre-steady-state behavior was observed; however, product formation was only linear for about the first 500 ms before the reaction slowed as the substrate was depleted.

We first attempted to fit the rate data obtained with 2.0 mM (initial concentration) methylaspartate as substrate to eq 1, which describes the behavior of the system in the general case where the rate constants for the formation (k_1) and breakdown (k_2) of the intermediate are different. Trial fits using the KaleidaGraph program established, however, that k_1 and k_2 are very similar, which leads to very poor estimation of the errors associated with the rate constants. The data were therefore fitted to eq 2, which is the limiting form of eq 1 as k_1 tends to k_2: this yields values for the apparent rate constants $k_1^{app} = k_2^{app} = 22 \pm 3$ s^{-1} and $k_{cat} = 7.6 \pm 0.2$ s^{-1}. Therefore, both steps are sufficiently rapid for the formation and breakdown of the intermediate to be kinetically competent. Equation 2 also provided a good fit to the data obtained for the first 500 ms of the reaction with 1.0 mM methylaspartate, before the steady-state rate of

glutamate formation begins to slow. In this case $k_1^{app} = k_2^{app} = 25 \pm 4\ s^{-1}$ and $k_{cat} = 6.9 \pm 0.2\ s^{-1}$. Reliable kinetic constants could not be extracted from data obtained with 0.5 mM methylaspartate because the reaction approaches equilibrium too rapidly for eqs 1 and 2 to be valid.

Most experiments described above used glycine as an internal standard, which has a convenient retention time in the HPLC assay, but during the course of this work a report appeared that indicated that small quantities of glycyl radical may be formed as an intermediate in the reaction (*15*). The aerobic conditions of the quench reaction would tend to destroy any glycyl radical, but to ensure that our results were not affected by this, one set of experiments, employing 2 mM methylaspartate as substrate, was repeated using L-leucine as the internal standard. Essentially identical kinetic behavior was observed in this experiment, and negligible amounts of glycine appeared to be formed.

Kinetics of 5′-Deoxyadenosine Formation. To investigate further the identity of the intermediate observed in the pre-steady-state formation of glutamate from methylaspartate, we examined the kinetics of 5′-deoxyadenosine formation. This stable intermediate is formed by abstraction of a hydrogen atom from the substrate (*23*), and therefore the concentration of 5′-dA will equal the total concentration of free radical species on the enzyme.

The rapid quench flow technique was used to follow the formation of 5′-dA between 5 and 300 ms, by which time the 5′-dA concentration had reached steady-state levels. The reaction was initiated by mixing samples of holo-glutamate mutase (45 μM after mixing) with either L-glutamate (20 mM after mixing) or L-*threo*-3-methylaspartate (2 mM after mixing). At various times, the reaction was quenched with 5% TFA; 5′-dA was recovered and quantified by HPLC using L-tryptophan as an internal standard, as described under Materials and Methods.

5′-dA was formed rapidly when the enzyme was reacted with either substrate, and the rate of its appearance was adequately fitted by a single exponential curve. AdoCbl was corresponding consumed in the reaction, although because the total concentration of AdoCbl decreases by a relatively small amount the data were much noisier. Because the formation of 5′-dA is reversible, the kinetic behavior of this intermediate is best described by the relaxation time, τ ($\tau = 1/k_{obs}$), that contains contributions from all of the elementary steps in the mechanism that lead to the formation and decay of 5′-dA (*20*, *24*). The relaxation time, τ_{Glu}, for the formation of 5′-dA from L-glutamate and AdoCbl was 13.7 ± 1.5 ms ($k_{obs} = 73 \pm 8\ s^{-1}$), whereas with L-*threo*-3-methylaspartate τ_{MeAsp} was 15.6 ± 2.6 ms ($k_{obs} = 64 \pm 11\ s^{-1}$). The steady-state concentration of 5′-dA obtained in the presence of 20 mM L-glutamate was $13 \pm 0.3\ \mu$M, which corresponds to 29% of the enzyme active sites being in the free radical form. In the presence of 2 mM methylaspartate, the steady-state concentration of 5′-dA was $9 \pm 0.5\ \mu$M (20% of the enzyme in the free radical form). The two experiments were performed with the same solution of enzyme, and therefore the differences in the steady-state concentration of 5′-dA most likely reflect real differences in the steady-state concentrations of enzyme-bound intermediates as opposed to differences in enzyme activity or concentration.

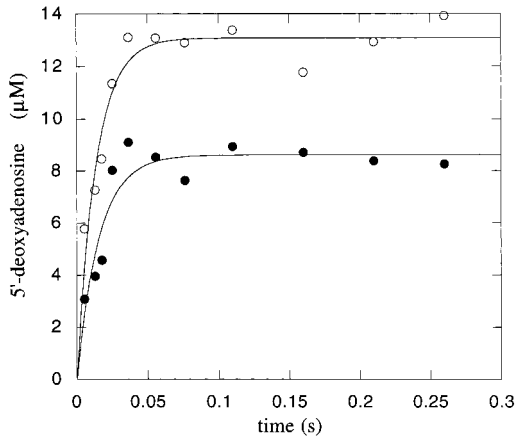


FIGURE 5: Time course for the formation of 5′-dA when glutamate mutase was reacted with either 2 mM L-*threo*-3-methylaspartate (●) or 20 mM L-glutamate (○). The data are fitted to a single-exponential function.

DISCUSSION

Nature of the Rate-Determining Step. In these experiments we have employed rapid quench flow methods to measure the first-order rate constants for the chemical steps in the isomerization of L-glutamate to L-*threo*-3-methylaspartate catalyzed by glutamate mutase. We have also measured the relaxation times for the formation of 5′-dA, both from AdoCbl and glutamate, and from AdoCbl and methylaspartate, that describe how the total concentration of radical species changes as the reaction approaches steady-state. When combined with information obtained from other mechanistic studies on the enzyme, these measurements allow some deductions about the nature of the rate-determining step to be made.

The "large" ($^DV = 4-6$) kinetic isotope effects measured previously with either deuterated glutamate or deuterated methylaspartate as the substrates, under steady-state conditions, suggested that hydrogen transfer, either from substrate to coenzyme or from coenzyme to product, would be the rate-determining step in the glutamate mutase reaction (*4*). However, subsequent measurements of the pre-steady-state kinetics of AdoCbl homolysis (*13*) revealed a mechanism in which cleavage of the coenzyme carbon−cobalt bond was kinetically coupled to hydrogen abstraction from the substrate. Furthermore, the intrinsic kinetic isotope effects for hydrogen abstraction were remarkably large ($^DV \approx 30$), indicating both that nonclassical behavior such as hydrogen tunneling might be occurring, and that in the steady-state some other step, such as product release, was significantly rate-determining and led to suppression of the isotope effects.

We have examined the glutamate mutase-catalyzed reaction in both directions on a time scale that has allowed us to measure the amount of product formed at less than one-tenth the time required for one turnover. The pre-steady-state formation of neither glutamate nor methylaspartate exhibits the characteristic burst phase that would be expected if product release were contributing to the steady-state rate of turnover. Indeed, the plots of product formation vs time (Figures 4 and 5) extrapolate very closely to the origin. This result is in accord with expectations given that k_{cat} is relatively slow and that neither substrate is bound especially tightly by the enzyme; K_s values for glutamate and methyl-

Scheme 2

$$\text{E:AdoCbl} + \text{S} \xrightarrow{fast} \text{E:AdoCbl:S} \underset{k_r}{\overset{k_f}{\rightleftharpoons}} \text{E:Cbl(II):5'-dA:S}^{\bullet} \xrightarrow{k_{p,app}} \text{E:AdoCbl:P} \xrightarrow{fast} \text{E:AdoCbl} + \text{P}$$

aspartate are 0.6 and 0.04 mM, respectively (*4*, *13*). At the lowest concentrations of substrates studied, it has been possible to follow the reaction as it approaches chemical equilibrium. The end points observed are in good agreement with those predicted by the equilibrium constant which lies 12:1 in favor of glutamate (*1*).

Most interesting is the lag phase that is associated with the formation of glutamate from methylaspartate. The lag phase is unlikely to be due to a slow substrate binding step because the experiments were conducted at near-saturating concentrations of substrate. Furthermore, the apparent rate constants associated with the lag phase are, within error, the same at both 1 and 2 mM methylaspartate concentrations. If substrate binding were contributing to the lag phase, one rate constant would be second order, and therefore doubling the concentration of substrate should increase this rate constant by a factor of 2, which is not seen.

More likely the lag phase results from a chemical intermediate (or intermediates) that is formed with an apparent rate constant $k_1^{app} \approx 22\ s^{-1}$ and that in a second slow step, $k_2^{app} \approx 22\ s^{-1}$, is either converted to glutamate directly or converted to another intermediate that is subsequently converted rapidly to glutamate. In principle, these slow steps might be associated with formation of methylaspartyl radical, isomerization of methylaspartyl radical to glutamyl radical, or quenching of glutamyl radical to form glutamate; as discussed below, we think it probable that the first two of these steps are the slow ones.

Kinetic Competence of 5′-dA. To determine to what extent formation of substrate radicals is rate-determining in the glutamate mutase reaction, we have followed the time course of 5′-dA formation when the enzyme is reacted with saturating concentrations of either substrate. The formation of 5′-dA is characterized by the relaxation times, τ_{Glu} and τ_{MeAsp}, that are associated with the equilibrium between the enzyme−substrate complex (species I or V in Figure 1) and the enzyme−radical complexes (species II, III, and IV in Figure 1). As discussed above, both product release and substrate binding appear to be much faster than the chemical steps in the glutamate mutase reaction, and under the initial conditions of the reaction product formation can be considered irreversible. In this case, the kinetics of 5′-dA formation may be described by Scheme 2, in which the relaxation time, τ, is related to the forward and reverse rate constants for 5′-dA formation, k_f and k_r, and the apparent rate constant for product formation, k_p^{app}, by eq 3. The steady-state concentration of 5′-dA is described by eq 4, where $[E_t]$ is the total enzyme concentration. From these equations, the rate constant for 5′-dA formation, k_f, can be determined.

$$\tau = \frac{1}{(k_f + k_r + k_p^{app})} \tag{3}$$

$$[5'\text{-dA}] = \frac{[E_t]k_f}{(k_f + k_r + k_p^{app})} \tag{4}$$

When methylaspartate is the substrate, the rate constant for 5′-dA formation $k_{f,MeAsp}$, is $13 \pm 3\ s^{-1}$; when glutamate is the substrate, $k_{f,Glu}$ is $22 \pm 3\ s^{-1}$. Thus, 5′-dA is formed sufficiently fast for it to be a kinetically competent intermediate in the reaction.

Previously we have measured the observed rate constants for Cbl(II) formation with both proteo- and deutero- glutamates and methylaspartates, and on the basis of the isotope effects we observed proposed that homolysis of the coenzyme and hydrogen abstraction from the substrate are coupled processes (*13*). Furthermore, the formation of Cbl(II) appeared to be biphasic, with approximately half of the enzyme reacting within the dead time (≈2 ms) of the stopped flow spectrometer. We attributed this phenomenon to negative cooperativity or 'half of sites' behavior arising from the dimeric nature of the enzyme.

The coupled mechanism predicts that 5′-dA formation should exhibit the same kinetic behavior as Cbl(II) formation, and therefore one would expect that 5′-dA would also be formed with biphasic kinetics. If this is the case, the experimental limitations of the quench flow technique do not allow us observe this. The shortest time accessible by quench flow is ≈5 ms, which is much too slow to observe the 'fast' site on the enzyme, and, indeed, about half the reaction had occurred within 5 ms. The data we obtain by quench flow are adequately fitted by a single exponential. However, we do not rule out the possibility that at very early times in the reaction the kinetic behaviors of 5′-dA and Cbl(II) may be different. The relaxation times associated with 5′-dA formation are $\tau_{Glu} = 13.7 \pm 1.5$ ms and $\tau_{MeAsp} = 15.6 \pm 2.6$ ms when the enzyme is reacted with glutamate and methylaspartate, respectively, whereas the corresponding relaxation times for Cbl(II) formation for the 'slow' site are 10.3 ± 0.5 and 12.5 ± 0.4 ms (*13*). The rate of 5′-dA formation appears to be slightly slower than the rate of Cbl(II) formation, but given the experimental error associated with these measurements and the fact that small systematic errors may be present, we consider the two sets of measurements to be in reasonably good agreement.

Relative Rates of the Chemical Steps in the Glutamate Mutase Reaction. Buckel and co-workers have recently used isotopically labeled substrates and EPR spectroscopy to investigate the identity of the organic radicals that accumulate on the enzyme under steady-state conditions (*15*). They have shown that the C-4 radical of glutamate is the major radical species present during turnover and obtained tentative evidence that the glycyl radical intermediate may be present at low concentration, but have found no evidence for methylaspartyl radical. Our previous studies on the kinetics of AdoCbl homolysis rule out 5′-deoxyadenosyl radical as a stable intermediate in glutamate mutase (*13*), and chemical reasoning would suggest methylaspartyl radical would be similarly unstable and thus also very short-lived. Furthermore, in this work we have shown that 5′-dA is both formed more rapidly and accumulates to a higher concentration with glutamate as the substrate than with methylaspartate. This is consistent with glutamyl radical being the most stable organic radical species formed on the enzyme.

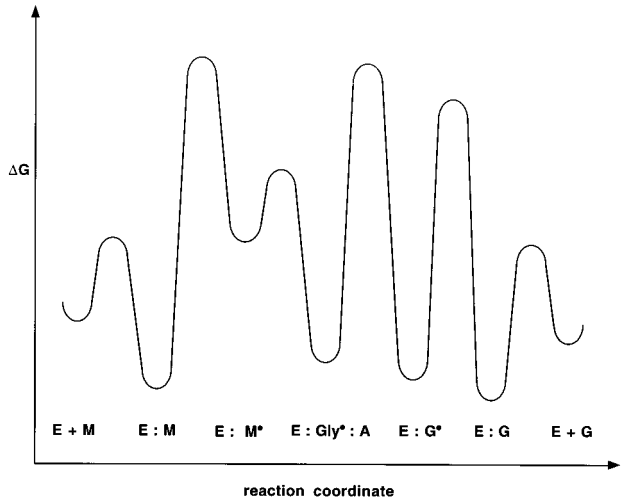


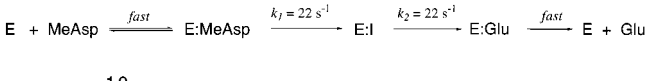
FIGURE 6: Qualitative free energy profile for the glutamate mutase catalyzed reaction.

Since product release is not rate-limiting for either substrate, the suppression of the intrinsic deuterium isotope effects during steady-state turnover (*4*, *13*) must arise from an isotopically insensitive chemical step involving the rearrangement of the substrate radical. Thus, the observed deuterium kinetic isotope effect for the re-abstraction of hydrogen from 5′-dA by glutamyl radical, determined previously from stopped flow measurements (*13*), is only 2. This value is lower than either the steady-state isotope effect or the intrinsic isotope effect for hydrogen abstraction, indicating that a slow step precedes the final hydrogen transfer step. For the enzyme-catalyzed isomerization of glutamate to methylaspartate, the pre-steady-state kinetic behavior indicates that a single chemical step dominates the rate of catalysis. In the reverse reaction, a lag phase is observed, consistent with the accumulation and decay of an intermediate, and therefore two or more chemical steps must contribute significantly to the overall rate of catalysis in this direction. (We note that a conformational change in the protein could also account for the kinetic behavior, although we consider this rather less likely.)

To account for these observations, we propose the following kinetic scheme, that is illustrated by the qualitative free energy profile in Figure 6. When the substrate is glutamate, hydrogen abstraction to form glutamyl radical is relatively rapid. The slow step is likely to be recombination of the glycyl radical and acrylate to give methylaspartyl radical, that once formed is very rapidly quenched by hydrogen transfer from 5′-dA to form methylaspartate and regenerate AdoCbl. In the reverse direction, the first slow step is likely to be formation of methylaspartyl radical, which we assume then very rapidly fragments into glycyl radical and acrylate. The second slow step would be the recombination of glycyl radical and acrylate to give glutamyl radical, with the final hydrogen transfer step, to form glutamate and AdoCbl, again occurring relatively rapidly.

The above mechanism predicts that the lag phase observed during the conversion of methylaspartate to glutamate is due to the formation of methylaspartyl radical and 5′-dA from methylaspartate and AdoCbl. At first sight, this hypothesis appears to be contradicted by the fact that the rate constant calculated for the formation of 5′-dA ($k_{f,MeAsp} = 13 \pm 3\ s^{-1}$) is significantly slower than the apparent rate constant

Scheme 3



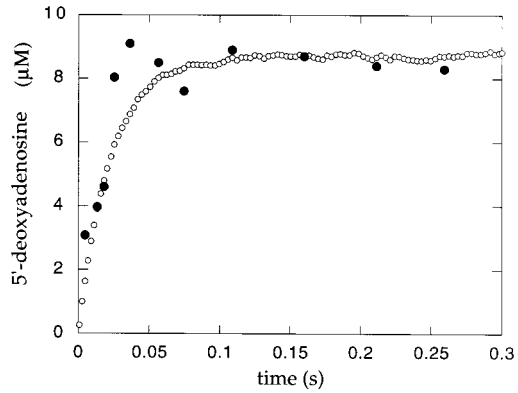


FIGURE 7: Comparison of simulated and experimental data for the formation of 5′-dA when glutamate mutase was reacted with 2 mM L-*threo*-3-methylaspartate. (○) Computer-simulated data for formation of intermediate E:I in Scheme 3; (●) experimental data from Figure 5.

measured for the lag phase ($k_1^{app} = 22 \pm 3\ s^{-1}$). However, the calculation of these rate constants involves some approximations, and these might be the cause of the discrepancy. Thus, eq 2, from which k_1^{app} is derived, and which describes the data quite well, implicitly assumes that the concentration of the intermediate decays to zero after the initial pre-steady-state phase, whereas the concentration of 5′-dA in fact tends toward a finite steady-state value.

To clarify this point, we modeled the reaction using the Chemical Kinetics Simulator software (v. 1.0, IBM) that employs stochastic methods (*25*), which do not require a deterministic mathematical expression for the rate equation, to simulate the reaction. The conversion of methylaspartate to glutamate was modeled according to Scheme 3.

The binding of methylasparate to glutamate mutase was assumed to be rapid and reversible, and the interconversion of methylaspartate to glutamate was modeled by two steps to generate first an enzyme-bound intermediate and then glutamate, each with a rate constant of 22 s^{-1}. The final step was the rapid diffusion of glutamate from the enzyme which was assumed to be irreversible. The reaction was simulated for 0.3 s, with initial concentrations of enzyme and methylaspartate of 0.045 and 2 mM, respectively, and the formation of the intermediate, E:I, monitored as a function of time. As shown in Figure 7, the formation of 5′-dA is modeled quite well by this simple reaction scheme. This result suggests that the lag phase we observe is consistent with the formation with 5′-dA and methylaspartyl radical on the enzyme.

In conclusion, we have demonstrated that chemical steps, rather than product release or conformational changes in the protein, are rate-determining in the glutamate mutase reaction. It appears that the isomerization of glutamyl and methylaspartyl radicals contributes significantly to the rate-determining step in both directions. Even so, it is evident that the energetic barriers to the interconversion of each of the chemical intermediates in Figure 1 are quite similar in height (with the possible exception of those associated with the decomposition of methylaspartyl radical), and that simply substituting the migrating hydrogen by deuterium may be enough to alter the rate-determining step.

Recently, the crystal structure of glutamate mutase has been solved (*26*). This will allow the role of various residues at the active site to be examined by mutagenesis. The qualitative free energy profile that we have determined for the wild-type enzyme should provide a useful reference point for interpreting the effects of a mutation on the relative rates of the individual steps in the reaction. This, in turn, will move us closer toward our goal of understanding how enzymes generate and control these highly reactive free radical species.

ACKNOWLEDGMENT

We thank Dr. Mark Banaszak Holl for advice on stochastic simulations of chemical reactions and the anonymous reviewers of the manuscript for their comments and suggestions.

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BI991064T