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Changes in the Free Energy Profile of Glutamate Mutase Imparted by the Mutation of an Active Site Arginine Residue to Lysine

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Abstract

Arginine 100 plays an important role in substrate recognition in adenosylcobalamin-dependent glutamate mutase. We have examined how the mutation of this residue to lysine affects the partitioning of tritium, incorporated at the exchangeable position of the coenzyme, between substrate and product. We find that partitioning of tritium back to the substrate predominates in the mutant enzyme, regardless of whether the reaction is run in the forward or reverse direction. This contrasts with the behavior of the wild-type enzyme in which tritium partitions equally between substrate and product, independent of the direction of the reaction. From this we conclude that the mutation significantly impairs the ability of the enzyme catalyze the rearrangement of substrate radical to product radical. The results illustrate the importance of electrostatic interactions in stabilizing free radical intermediates in this class of enzymes.

Keywords

enzyme; coenzyme-B₁₂; protein-radical; isomerization; free energy profile; isotope effect; isotope partitioning

Glutamate mutase (EC 5.4.99.1) catalyses 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 [1-4]. It is one of a group of Adenosylcobalamin- (AdoCbl, coenzyme B₁₂) 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 [5-10]. These rearrangements proceed through a mechanism involving free radical intermediates that are generated by homolysis of AdoCbl.

Glutamate mutase provides an attractive system 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]. The crystal structures of the enzyme complexed with substrate and coenzyme analogs have been determined at high resolution [11,12]. The enzyme has been the subject of extensive mechanistic studies, both from our laboratory and other groups [13-22]. From these studies a fairly detailed mechanistic description of the wild-type enzyme has emerged and is summarized in Fig. 1. Most of intermediates postulated in this mechanism have been experimentally verified and the rates with which they are formed and decay have been measured, allowing a qualitative free energy profile for the enzyme to be constructed.

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To understand better how the enzyme stabilizes these highly reactive radical intermediates and directs them towards productive catalysis, we have mutated a number of active residues in the enzyme and undertaken detailed kinetic analyses of the effects of these mutations on catalysis [23-26]. In particular, we recently described the effects of mutating Arg-100, which forms a salt bridge with the γ -carboxylate of the substrate (Fig. 2), on the steady state kinetic properties of glutamate mutase [26]. Three mutations were introduced, Arg100Lys, Arg100Tyr and Arg100Met, that reduced k_{cat} by 120 – 320 fold and increased $K_{\text{m(appeant)}}$ for glutamate by 13 – 22 fold. No cob(II)alamin could be detected in the U.V.-visible spectra of the Arg100Tyr and Arg100Met mutants. However, the Arg100Lys mutant does accumulate cob(II)alamin during turn-over, which allowed us to investigate the pre-steady state kinetics of adenosylcobalamin homolysis by stopped-flow spectroscopy. Compared with wild-type enzyme, homolysis of the coenzyme is slower by an order of magnitude. Furthermore, substrate binding is significantly weakened, so much so that with glutamate the homolysis reaction exhibits second order kinetics and does not saturate over the range of experimentally accessible substrate concentrations.

To examine how the Arg100Lys mutation affects the overall free energy profile of the glutamate mutase-catalyzed reaction, we have undertaken tritium partitioning experiments on this mutant, which are reported here. The partitioning of hydrogen isotopes from an enzyme-bound intermediate between products and substrates provides an elegant way of investigating the relative heights of energetic barriers in an enzyme-catalyzed reaction. We have previously applied the same technique to investigate the partitioning of tritium at the 5'-carbon of AdoCbl between substrate and product in the wild-type enzyme [27]; tritium partition experiments have also been used to investigate the free energy profile of AdoCbl-dependent methylmalonyl-CoA mutase [28-30]. We compare the results of our present studies with the previous data, which affords some insights into the role of active site residues in promoting the rearrangement of substrate radicals in these enzymes.

Materials and Methods

Materials

These experiments used the engineered single-subunit form of glutamate mutase, GlmES [4]. The construction of the glutamate mutase fusion protein mutant, GlmES-Arg100Lys and its purification from a recombinant *E. coli* strain has been described previously [26]. AdoCbl, L-glutamate, D,L-*threo*-3-methylaspartate, leucine and dansyl chloride were purchased from the Sigma Chemical Company. The sources of other materials have been described previously [3,4,22,27] or were purchased from commercial suppliers. [5'- ^3H]-AdoCbl was prepared enzymatically by using GlmES protein to exchange tritium into the coenzyme from ^3H -labelled glutamate in a slight modification of the procedure described previously [31]. Racemic *threo*-3-methylaspartate was used in these experiments as the D-isomer has previously been shown to be neither a substrate nor an inhibitor of the enzyme [4].

Acid Quench Experiments

Tritium partitioning experiments were performed at 10 °C. Solutions were prepared containing 100 μM GlmES-Arg100Lys in 50 mM potassium phosphate buffer, pH 7.0 and 120 μM 5'-[^3H]-AdoCbl, specific activity of 40,000 dpm/nmol. (Radiolabeled material was added immediately before the experiment.) The substrate solution contained either L-glutamate or L-*threo*-3-methylaspartate at twice the desired final concentrations dissolved in the same buffer as the enzyme and also contained 500 μM Leucine as an internal standard. The reactions were initiated by syringing an equal volume of substrate solution into a tube containing the enzyme solution (typically 50 μL) and rapidly mixing by drawing the liquid in and out of the syringe several times. The effective concentration of holoenzyme after mixing was 45 μM . The solution

was allowed to age for various times 10 – 100 s before being quenched with a further volume of 0.2 M HCl. The sample was centrifuged for 20 minutes in order to remove precipitated protein. The supernatant was removed and stored at -20°C prior to derivatization with dansyl chloride and HPLC analysis for glutamate and methylaspartate content.

Derivatization and HPLC analysis of glutamate and methylaspartate

The volume of quenched reaction mixture was reduced to 50 μL using a Speedvac apparatus and then 100 μL of 0.5 M NaHCO_3 was added to raise the pH above 8.5. 50 μL of a 20 mg/mL solution of dansyl chloride in acetone was added and the sample was incubated at 45°C for 1 h to derivatize the substrate, product, and internal standard. Dansylated glutamate, methylaspartate and leucine were separated by HPLC using a 25 cm C_{18} reverse phase column (Alltech Alltima C18 5 μ). The column was pre-equilibrated 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. 200 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 - 8.5 min, 7.5-50 % B; 8.5 - 18.5 min, 50 - 80 % B; 18.5 - 20 min, 80 % B; 20 - 20.5 min, 80 - 100 % B; 20.5 - 25.5 min, 100% B; 25.5 - 26 min, 100 - 7.5 % B; 26 - 36 min, 7.5 % B. The flow rate was 1.4 mL/min and compounds were detected by monitoring absorbance at 330 nm. Dansyl-glutamate and dansyl-methylaspartate peaks were collected and tritium content determined by scintillation counting. A blank run was performed prior to each sample injection to remove any contamination from the compounds left from previous sample analysis.

Results

Steady state kinetic properties of the glutamate mutase Arg100Lys mutant

We initially sought to measure the steady state kinetics for the Arg100Lys mutant catalyzing the “reverse” reaction, i.e., the conversion of methylaspartate converted to glutamate, (the enzyme is conventionally assayed in the direction of glutamate to methylaspartate) to establish conditions for partitioning experiments. This was achieved through a discontinuous assay in which the enzyme was allowed to react with various concentrations of methylaspartate for times between 30 s and 5 min before the reaction was quenched with HCl. The amino acids were then derivatized with dansyl chloride and separated and quantified by HPLC as described in the materials and methods section. Using this assay k_{cat} and K_{m} for methylaspartate were determined to be $0.073 \pm 0.007 \text{ s}^{-1}$ and $9.5 \pm 2.3 \text{ mM}$ respectively. When compared with $k_{\text{cat}} = 5.8 \text{ s}^{-1}$ and $K_{\text{m}} = 0.14 \text{ mM}$ for the wild type enzyme reacting with methylaspartate as substrate [4] it is evident that this mutation significantly impairs the catalytic efficiency of the enzyme.

We previously measured a similarly large decrease in k_{cat} and increase in K_{m} when the steady state kinetic parameters for the Arg100Lys mutant enzyme were determined with glutamate as the substrate. In the forward direction k_{cat} is 0.048 s^{-1} and K_{m} for glutamate is 10 mM [26]. Given the readily reversible nature of the reaction it is not surprising that this mutation causes a similar reduction in k_{cat} and increase in K_{M} for both substrates.

Partitioning of tritium with glutamate as substrate

We next investigated how tritium in the exchangeable 5'-position of AdoCbl partitioned between substrate and product when glutamate was the substrate. As we have discussed previously [27], to avoid the complication of multiple passages of tritium between coenzyme and substrates, these measurements need to be made on a time-scale that is sufficiently rapid that the reaction does not approach equilibrium. Under such conditions the probability of product molecules, once released from the enzyme, partitioning back to form glutamate is negligible. Turn-over of the Arg100Lys mutant enzyme is much slower than wild-type enzyme, and therefore experiments could be performed by manually mixing and quenching reactions.

In a typical experiment 45 μ M holoenzyme was reacted with 12.5 mM L-glutamate (effective concentrations after mixing) at 10 °C for various times between 0 and 80 s. After quenching with HCl, portions of the reaction mixture were analyzed to determine the amount of tritium in glutamate, methylaspartate and 5'-dA. As shown in Fig. 3, formation of methylaspartate was linear over the time scale of the experiment. After 80 s ~ 5 nmol of methylaspartate were formed, corresponding to about 4 turn-overs. Less than 1 % of the glutamate was consumed, insuring that the reaction remained far from equilibrium.

The tritium content of the glutamate and methylaspartate recovered from the reaction both increased in an approximately linear manner over the period of the experiment (Fig. 3). After 80 s the tritium counts in substrate accounted for about 4 % of the radioactivity originally present in AdoCbl. It is evident that there is strong tendency for tritium to partition back to glutamate rather than forward into methylaspartate. The partitioning ratio is 5 : 1 in favor of glutamate, indicating that there is a large kinetic barrier to conversion of the glutamyl radical to methylaspartyl radical. In contrast for the wild-type enzyme we found that, with glutamate as substrate, tritium partitions in an almost 1 : 1 ratio between glutamate and methylaspartate.

Partitioning of tritium with methylaspartate as substrate

We employed similar protocols to investigate the partitioning behavior of the Arg100Lys mutant for the reaction proceeding in the reverse direction with methylaspartate as substrate. The Arg100Lys mutant enzyme, 45 μ M, was reacted with 12.5 mM L-threo-3-methylaspartate (final concentrations after mixing) at 10 °C for various times between 0 and 80 s. The rate of glutamate formation was linear over the course of the experiment (Fig.4). Under the conditions of the experiment about 4 nmol of glutamate were formed, which corresponds to about 5 turnovers. Again, less than 1 % of the substrate was consumed, so the reverse reaction is negligible.

The transfer of tritium from AdoCbl to methylaspartate and glutamate was roughly linear over the time course of the experiment, as shown in Fig. 4. However, there was some evidence for a burst phase for the transfer of tritium to methylaspartate, as the counts do not extrapolate to zero at $t = 0$. After 80 s about 10 % of the radioactivity originally present in AdoCbl was transferred to the substrate and product. The partitioning of tritium back into substrate was even more pronounced, with the partitioning ratio calculated as 11 : 1 in favor of methylaspartate. Furthermore, tritium appeared to be transferred to methylaspartate at about twice the rate of at which it was transferred to glutamate when it was the substrate. Although far fewer counts were found in the product, glutamate, there again appeared to be a burst phase followed by a linear increase in tritium counts. Again, the pronounced partitioning of tritium back to methylaspartate in the Arg100Lys mutant contrasts with the behavior of the wild-type enzyme in which tritium partitions almost equally between substrate and product when methylaspartate is the substrate.

Discussion

We have previously investigated the partitioning of tritium at the 5'-position of AdoCbl between substrate and product catalyzed by wild-type glutamate mutase [27]. In the wild-type enzyme the isotope partitions between glutamate and methylaspartate in nearly 1:1 ratio, regardless of the direction in which the overall reaction is proceeding. This is consistent with a free energy profile in which the inter-conversion of the intermediate glutamyl and methylaspartyl radicals is rapid relative to the transfer of tritium from 5'-deoxyadenosine to either substrate or product.

The introduction of the Arg100Lys mutation has a pronounced effect on not only the steady state kinetic properties of the enzyme but also on tritium partitioning. With glutamate as

substrate this mutation reduces k_{cat} by 120 fold and increases $K_{\text{m(appeant)}}$ 17 fold; with methylaspartate as substrate k_{cat} is reduced by 80 fold and $K_{\text{m(appeant)}}$ increased by ~ 70 fold. The side-chain of lysine is slightly shorter than that of the arginine it replaces and cannot form the bidentate salt bridge with the γ -carboxylate of the substrate that is observed in the crystal structure of the enzyme substrate complex. This would explain the weaker binding of the substrates as evidenced by the increased K_{m} s and the higher K_{d} s for glutamate and methylaspartate that we previously measured by stopped flow [26]. The apparent K_{m} for AdoCbl is little affected by the mutation, which is not surprising because Arg100 does not make contact with the coenzyme.

Two effects may explain the overall decrease in k_{cat} caused by the Arg100Lys mutation. Firstly, it appears from our earlier studies that the ability of the enzyme to catalyze AdoCbl homolysis is impaired. For the wild-type enzyme the observed rate constant for AdoCbl homolysis was 80 s^{-1} with methylaspartate as substrate [22]. However, for the Arg100Lys mutant the observed rate constant for homolysis of AdoCbl was 7 s^{-1} with methylaspartate as substrate, more than ten-fold slower than the wild-type enzyme [26]. The Arg100Lys mutant exhibited second-order kinetics in the reaction of AdoCbl with glutamate, implying that glutamate binding was greatly weakened; the efficiency of the reaction was calculated to be 1200-fold lower than wild-type.

Secondly, the tritium partitioning studies indicate that the free energy profile of the reaction is further altered by the Arg100Lys mutation such that the inter-conversion of the glutamyl and methylaspartyl radicals becomes slow relative to hydrogen transfer between substrate and coenzyme. This implies that once the substrate radical is formed through hydrogen abstraction by the coenzyme there is a significantly greater probability that the substrate radical will re-abstract hydrogen from 5'-dA to generate tritiated substrate than that the substrate radical will rearrange and go on to form product. This is consistent with the observation that regardless of the direction the reaction is run in, tritium is found to preferentially partition back to whichever molecule is the substrate in the reaction. The changes imparted by the Arg100Lys mutation to the free energy profile of glutamate mutase are illustrated in Fig. 5.

An obvious question that arises is why this mutation should impair the inter-conversion of the glutamyl and methylaspartyl radicals on the enzyme? Clearly, as noted above, the Arg100Lys mutation weakens substrate binding by disrupting the bidentate salt bridge formed with the γ -carboxylate of the substrate. In general terms this may be expected to reduce the amount of binding energy available for catalysis. More specifically, however, it will alter the protonation state of the γ -carboxylate group, and this is likely to have a significant effect on the stability of the substrate radical intermediates. Indeed, in a series of computational studies Radom and coworkers have highlighted the importance of the protonation state of acidic groups in stabilizing adjacent organic radicals in a number of AdoCbl-dependent rearrangements [32-35] including the glutamate mutase-catalyzed reaction [36].

Leadlay and colleagues have used the partitioning of tritium between AdoCbl and substrates [28] to examine the free energy profile of the related AdoCbl-dependent enzyme methylmalonyl-CoA mutase (MMCM), which catalyzes the rearrangement of methylmalonyl-CoA to succinyl-CoA. For wild-type MMCM, tritium partitioning is independent of whether methylmalonyl-CoA or succinyl-CoA is the substrate, indicating that rearrangement of the substrate radicals is rapid compared with hydrogen transfer. However, two active site mutations, His244Gln and Tyr248Phe have been identified which cause tritium in AdoCbl to preferentially partition back to the substrate [29,30]. These mutations appear to exert effects very similar to what we observe in the glutamate mutase Arg100Lys mutant.

Most interesting is the MMCM His244Gln mutation as this histidine residue forms electrostatic interactions with both the terminal carboxylate and thioester carbonyl group of the substrates.

In this respect it may play a similar role to Arg100 in glutamate mutase in adjusting the protonation state of the substrate. Although it was not remarked upon by the authors, previous theoretical studies had predicted that protonation of the thioester carbonyl group would be expected to facilitate the 1,2-migration of the acyl-CoA moiety necessary for the rearrangement of the intermediate methylmalonyl-CoA radical to the succinyl-CoA radical [32].

In conclusion, our experiments point to the importance of positively charged residues in promoting the rearrangements of the substrate- and product-radical intermediates in glutamate mutase. Taken together with similar observations for the MMCM-catalyzed rearrangement, they suggest that adjustment of the protonation state is likely to be a general strategy employed by AdoCbl-dependent enzymes to facilitate the formation and rearrangement of radical intermediates. The experimental data on these enzymes are in qualitative agreement with computational studies on MMCM, glutamate mutase, and other AdoCbl-dependent enzymes, that have examined the effect of “partial” protonation on the stabilities of radical intermediates and the activation energies for their inter-conversion.

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Fig 1.
Mechanistic scheme for the rearrangement of L-glutamate to L-threo-3-methylaspartate catalyzed by glutamate mutase.

Fig 2. Hydrogen-bonding interactions between active site residues and the substrate in glutamate mutase. The substrate (depicted here as glutamate) and the side-chain of Arg100 are shown in bold.

Fig 3. Partitioning of tritium from AdoCbl with glutamate as substrate *Top*: formation of methylaspartate during the first 80 s of reaction. *Bottom*: tritium counts incorporated into substrate (glutamate, ●), and product (methylaspartate, ○) during the first 80 s of reaction.

Fig 4. Partitioning of tritium from AdoCbl with methylaspartate as substrate *Top*: formation of glutamate during the first 80 s of reaction. *Bottom*: tritium counts incorporated into substrate (methylaspartate, ○), and product (glutamate, ●) during the first 80 s of reaction.

Fig 5.

Changes to the free energy profile of the glutamate mutase catalyzed reaction imparted by the mutation of Arg100 to lysine. *Top*: the free energy profile of the wild-type enzyme, adapted from reference 27; *Bottom*: the free energy profile of the Arg100Lys mutant. Tritium isotope effects are indicated by dashed lines. The intermediates represented by Roman numerals correspond to the chemical species illustrated in Fig. 1.