Role of Arg100 in the Active Site of Adenosylcobalamin-Dependent Glutamate Mutase†

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ABSTRACT: Arginine-100 is involved in recognizing the gamma carboxylate of the substrate in glutamate mutase. To investigate its role in substrate binding and catalysis, this residue was mutated to lysine, tyrosine, and methionine. The effect of these mutations was to reduce k_{cat} by 120−320-fold and to increase $K_{m(apparent)}$ for glutamate by 13−22-fold; $K_{m(apparent)}$ for adenosylcobalamin is little changed by these mutations. Even at saturating substrate concentrations, no cob(II)alamin could be detected in the UV-visible spectra of the Arg100Tyr and Arg100Met mutants. However, in the Arg100Lys mutant cob(II)-alamin accumulated to concentrations similar to wild-type enzyme, which allowed the pre-steady-state kinetics of adenosylcobalamin homolysis to be investigated by stopped-flow spectroscopy. It was found that homolysis of the coenzyme is slower by an order of magnitude, compared with wild-type enzyme. Furthermore, glutamate binding is significantly weakened, so much so that the reaction exhibits second-order kinetics over the range of substrate concentrations used. The Arg100Lys mutant does not exhibit the very large deuterium isotope effects that are observed for homolysis of the coenzyme when the wild-type enzyme is reacted with deuterated substrates; this suggests that homolysis is slowed relative to hydrogen abstraction by this mutation.

Glutamate mutase catalyzes the reversible isomerization of L-glutamate to L-*threo*-3-methylaspartate (*1*−*5*) and belongs to a group of adenosylcobalamin-dependent (AdoCbl, coenzyme B_{12})[1] enzymes that catalyze unusual isomerization reactions that proceed by radical mechanisms; for recent reviews see refs *6*−*10*. A mechanistic scheme for the enzyme is shown in Figure 1. The first step in the mechanism, common to all AdoCbl-dependent isomerases, is homolysis of the labile cobalt−carbon bond of AdoCbl to generate a 5′-deoxyadenosyl radical; this is followed by abstraction of hydrogen from the substrate to form 5′-deoxyadenosine (5′-dA) and a substrate radical. For glutamate mutase, the rearrangement of the C-4 radical of glutamate to form the methylaspartyl radical has been shown to proceed by a fragmentation−recombination mechanism with glycyl radical and acrylate as intermediates (*11*).

The crystal structure of glutamate mutase has identified a number of residues that appear to make important hydrogen-bonding interactions with the substrate and coenzyme (*12*, *13*). These include Arg149 and Arg66 that hydrogen bond to the α-carboxyl group of the substrate; Glu171, which makes a hydrogen bond to the amino group of the substrate, and Arg100 that forms a salt bridge with the γ-carboxylate, as illustrated in Figure 2. As part of our effort to determine how the enzyme promotes homolysis of the coenzyme and directs the reactive free radical intermediates formed toward productive catalysis, we have embarked upon a series of experiments to investigate the roles of various active site residues in the enzyme mechanism.

Recently, we constructed and characterized several sterically and functionally conservative mutations of Glu171. The properties of the mutant proteins were consistent with the hypothesis that Glu171 acts as a general base that serves to deprotonate the amino group of the substrate during catalysis (*14*). Subsequently, pre-steady-state kinetic analysis of the Glu171Gln mutant revealed that several steps in the kinetic mechanism were altered by this mutation. Thus, substrate binding was weakened, and the apparent rate constants for homolysis of AdoCbl were significantly slowed compared with wild type. Furthermore, the mutation appeared to significantly reduce the large kinetic isotope effects on AdoCbl homolysis that are observed when the enzyme is reacted with deuterated substrates (*15*, *16*).

Here we describe experiments aimed at probing the role of Arg100 in substrate binding and catalysis. We have introduced several mutations at this position and investigated their effect on the steady-state kinetic properties of the enzyme, the ability of the enzyme to stabilize radical intermediates, and the effect of the mutations on the pre-steady-state kinetics of AdoCbl homolysis.

MATERIALS AND METHODS

Materials. Oligonucleotide synthesis and DNA sequencing were performed by the Biomedical Research Core facilities

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[1] Abbreviations: AdoCbl, adenosylcobalamin; Cbl(II), cob(II)alamin; 5′-dA 5′-deoxyadenosine; GlmES, glutamate mutase fusion protein.

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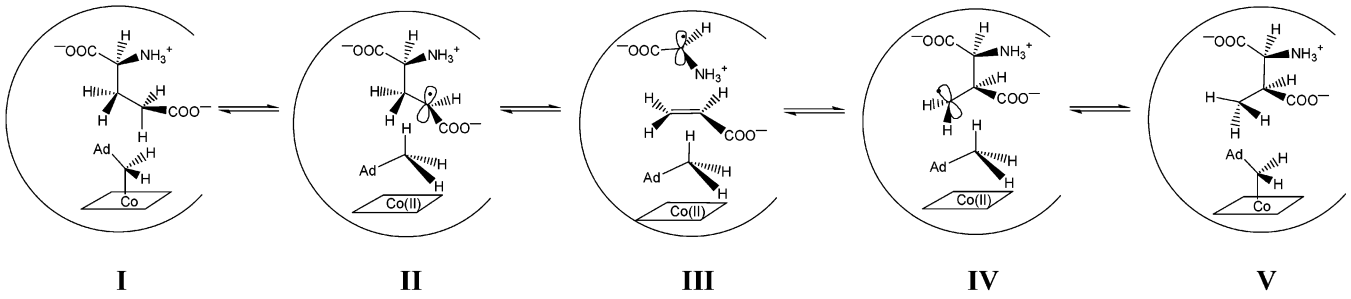

FIGURE 1: Mechanistic scheme for the rearrangement of L-glutamate to L-*threo*-3-methylaspartate catalyzed by glutamate mutase.

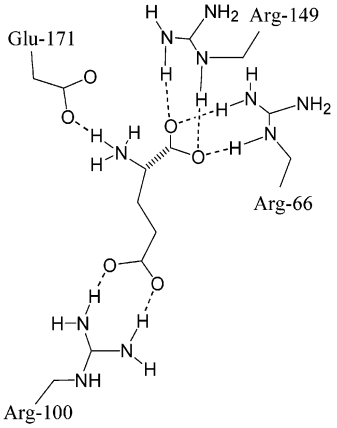

FIGURE 2: Hydrogen-bonding interactions between active site residues and the substrate in glutamate mutase; here glutamate is depicted as the substrate.

(University of Michigan). *Vent* polymerase was obtained from New England Biolabs (Beverly, MA). Restriction enzymes were obtained from New England Biolabs, Promega (Madison, WI), or Boehringer Mannheim (Indianapolis, IN). *Escherichia coli* XL Blue1 supercompetent cells were obtained from Stratagene (La Jolla, CA). D,L-[2,4,4]-Glutamic acid-d_3 was obtained from Cambridge Isotope Laboratories (Andover, MA). AdoCbl was purchased from the Sigma Chemical Company. The purification of recombinant glutamate mutase fusion protein and the sources of all other materials have been described previously (*17*).

Construction of Mutant Proteins. Mutations were introduced into the *GlmES* gene using the Quickchange Protocol (Stratagene, La Jolla, CA) according to the manufacturer's instructions, except that *Vent* polymerase was used in place of *Pfu* polymerase, as described previously (*14*). The following pairs of complementary primers were used to incorporate mutations at the site of Arg100 (the site of the mutation is underlined):

Arg100Lys, coding − ATTGATGCTTATACAAAG-CAAAATAGAATATGACG

noncoding − CGTCATATATATTTTGCTTTGTATAAG-CATCAAT;

Arg100Tyr, coding − ATTGATGCTTATACATAC-CAAAATAGAATATGACG

noncoding − CGTCATATATATTTTGGTATGTATAAG-CATCAAT;

Arg100Met, coding − ATTGATGCTTATACAATG-CAAAATAGAATATGACG

noncoding − CGTCATATATATTTTGCATTGTATAAG-CATCAAT.

The mutations were confirmed by automated DNA sequencing.

Substrates. L-*threo*-3-Methylaspartate and L-*threo*-(3-$^2H_3$-methyl)-aspartate were prepared enantiomerically pure by enzymic synthesis (*18*). D,L-Glutamic acid was purchased from Sigma Chemical Company and D,L-[2,4,4-$^2H_3$]-glutamic acid was from Cambridge Isotope Laboratories Inc. Deuterated glutamate was only available in racemic form, and therefore in these experiments racemic protiated glutamate was used 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 concentrations refer only to the active L-isomer.

Kinetic Analysis. Steady-state kinetic analyses of wild-type and mutant enzymes to determine apparent K_M's for L-glutamate and AdoCbl were performed at 25 °C using the spectrophotometric assay described by Barker (*19*), in which glutamate mutase activity is coupled to the formation of mesaconate using methylaspartase and monitored at 240 nm. Data were plotted and fitted to the appropriate equations using the KaleidaGraph graphing program (Abelbeck Software).

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 close to 100 μM. Solutions containing AdoCbl were handled so as 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 were dissolved in the same buffer as the enzyme, 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 substrate and enzyme 2-fold, so that the concentration of holoenzyme in the measured reaction mixture was 50 μM.

The reaction was monitored by following the changes in absorbance at either 525 or 470 nm that accompany 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 using the program KISS.

Table 1: Steady-State Kinetic Parameters for Wild-Type and Arg-100 Mutant Glutamate Mutase Enzymes

glutamate mutase mutant	k_{cat} (s^{-1})	$K_{m(app)}$ L-Glu (mM)	$k_{cat}/K_{m(app)}$ (M^{-1} s^{-1})	$K_{m(app)}$ AdoCbl (μM)	accumulates Cob(II)?
wild type	5.8 ± 0.3	0.58 ± 0.08	$(1.0 \pm 0.1) \times 10^3$	5.5 ± 0.7	yes
Arg100Lys	0.048 ± 0.004	10 ± 2	4.8 ± 1.0	5.3 ± 0.6	yes
Arg100Tyr	0.018 ± 0.001	7.6 ± 1.2	2.4 ± 0.4	3.9 ± 0.8	no
Arg100Met	0.021 ± 0.001	13 ± 2	1.6 ± 0.25	8.7 ± 1.2	no

Secondary plots of data and curve fitting were performed using the Kaleidagraph program (Abelbeck Software).

RESULTS

The crystal structure of glutamate mutase shows Arg100 to make a salt bridge interaction with the γ-carboxyl group of the substrate (*12*, *13*). To determine whether this interaction is important for catalysis and/or binding, three mutations were introduced into the protein in which arginine was replaced by lysine, methionine, or tyrosine. The lysine mutation represents the most conservative substitution, as it maintains the positive charge, although it is slightly shorter and does not allow the same bidentate hydrogen-bonding interactions as arginine. Methionine was chosen because its hydrophobic side chain approximates that of arginine but affords no opportunity for hydrogen bonding or electrostatic interactions. Last, tyrosine was chosen as a semi-conservative mutation that approximates the steric bulk and length of the arginine side chain and allows the possibility of hydrogen bonding to the substrate through the phenolic hydroxyl group.

These experiments used the engineered, "single-subunit" form of glutamate mutase, GlmES, in which the S subunit has been genetically fused to the E subunit (the numbering of residues in the E subunit is the same for the wild-type GlmE and engineered GlmES proteins). This protein has properties very similar to the wild-type two-subunit enzyme, and its advantages for kinetic analysis of the enzyme mechanism have been discussed previously (*17*). The mutations were introduced using standard PCR-based methods, and the mutant proteins were purified as described previously for the parent GlmES protein (*17*).

Steady-State Kinetic Properties of Mutant Enzymes. For each mutant, the apparent K_m for both glutamate and AdoCbl, together with k_{cat}, were determined using the standard spectroscopic assay (*19*). The kinetic properties of the mutants are summarized in Table 1. It is apparent that any change at this position is extremely deleterious to catalytic efficiency, with k_{cat}/K_m for glutamate being reduced by between 2000- and 7000-fold, depending upon the mutation. As might be expected, the Arg100Lys mutation is less deleterious than the change to either tyrosine or methionine, although even with lysine k_{cat} is reduced by more than 100-fold. The apparent K_m for AdoCbl is not significantly altered in these mutants, consistent with the fact that Arg100 makes no direct interaction with the coenzyme.

To determine how these mutations affect the ability of the enzyme to stabilize radical species during catalysis, we recorded the UV-visible spectra of the mutant holoenzymes in the presence and absence of saturating concentrations (100 mM) of L-glutamate. Formation of Cbl(II), indicative of the accumulation of free radical species, results in a characteristic increase in absorbance at 470 nm and a decrease at 525 nm.

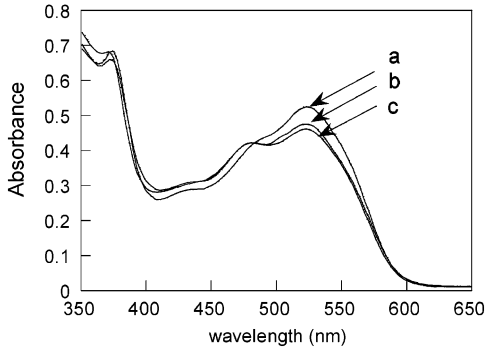

FIGURE 3: UV-spectra of glutamate mutase Arg100Lys mutant holoenzyme: (a) the resting state of the enzyme (50 μM concentration); (b) after reaction with 100 mM L-glutamate; (c) after reaction with 50 mM L-*threo*-3-methylaspartate.

No spectral changes were observed with either the Arg100Tyr or Arg100Met mutants, implying that less than 1% of the enzyme active sites accumulate Cbl(II) during steady-state turnover (based on a conservative estimate of the limit of detection). However, as shown in Figure 3, the Arg100Lys mutant does accumulate significant concentrations of Cbl(II) under steady-state turnover in the presence of either glutamate or methylaspartate. From the difference spectra, we calculate that about 35% of the enzyme active sites contain radical species, which is similar to that observed for the wild-type enzyme (*16*, *20*). This suggests that the rate-determining step in the Arg100Lys mutant is different from that in Arg100Tyr or Arg100Met mutants.

Kinetics of Cbl(II) Formation in the Arg100Lys Mutant. Because addition of substrate to the Arg100Lys mutant resulted in a significant amount of Cbl(II) being formed during catalysis, we used UV-visible stopped-flow spectroscopy to investigate the pre-steady-state kinetics of AdoCbl homolysis and Cbl(II) formation. Our previous stopped-flow studies on wild-type glutamate mutase uncovered an important aspect of the mechanism by which the enzyme catalyzes homolysis of the coenzyme (*16*). We found the rates of AdoCbl homolysis for the wild-type enzyme reacting with deuterated substrates to be dramatically slower than with protiated substrates, implying a mechanism whereby cleavage of the Co−C bond and abstraction of hydrogen from the substrate are kinetically coupled. Similar kinetic coupling has also been observed in the B_{12}-dependent enzymes methylmalonyl-CoA mutase, ethanolamine deaminase, and class II ribonucleotide reductase (*21*−*23*). Therefore, we examined how the Arg100Lys mutation alter the coupling of homolysis to hydrogen abstraction. The kinetics of AdoCbl homolysis were measured for the mutant enzyme reacting with either L-glutamate or L-*threo*-3-methylaspartate, bearing either protium or deuterium at the abstractable position. For convenience, the kinetic data obtained in these experiments are summarized in Table 2 and discussed in detail below.

Table 2: Rate Constants and Equilibrium Constants Determined from Pre-Steady-State Kinetic Measurements of the Substrate-Induced Homolysis of AdoCbl by the Arg100Lys Glutamate Mutase Mutant

substrate	$k_{forward}$ M^{-1} s^{-1}	$k_{reverse}$ s^{-1}	K_{eq} M^{-1} [a]	K_{eq} M^{-1} [b]
L-glutamate	32 ± 4	2.2 ± 0.2	14.4	14.3
[2,4,4]-L-glutamate-d_3	37 ± 6	2.0 ± 0.3	18.5	14.2
	$k_{observed}$ s^{-1}		K_{eq} M^{-1} [b]	$k_{obs(H)}/k_{obs(D)}$
methylaspartate	7.0 ± 0.6		24 ± 5	3.5
[methyl]-methylaspartate-d_3	2.0 ± 0.4		24 ± 6	

[a] Equilibrium constant for the enzyme and substrate reacting to form the enzyme/substrate-radical complex determined from the ratio of $k_{forward}$ to $k_{reverse}$. [b] Equilibrium constant for the enzyme and substrate reacting to form the enzyme/substrate-radical complex determined from variation of DA_{525} as a function of substrate concentration.

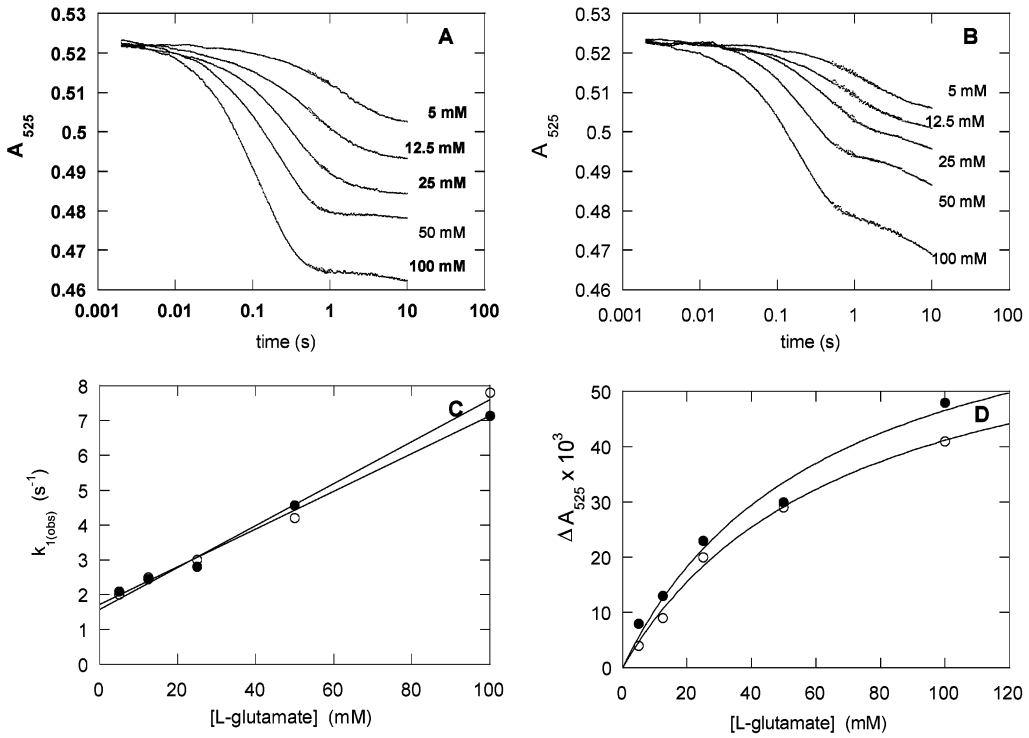

FIGURE 4: Reaction of Arg100Lys mutant with L-glutamate. (A) Changes in absorbance at 525 nm, indicative of homolysis of AdoCbl, following rapid mixing of holo-glutamate mutase (50 μM final concentration) with various concentrations of L-glutamate. The concentration of substrate is indicated by each trace. The time axis is plotted on a logarithmic scale. (B) Traces obtained in an analogous experiment where L-[2,4,4]-glutamate-d_3 was the substrate. (C) Variation of observed rate constants for homolysis as a function of substrate concentration for the enzyme reaction with protiated (●) and deuterated (○) glutamates. (D) Variation of the amplitudes associated the observed rate constants for homolysis (plotted in panel C) as a function of substrate concentration for the enzyme reaction with protiated (●) and deuterated (○) glutamates.

The stopped-flow experiments were performed at 10 °C under anaerobic conditions. Typically, reactions were monitored for times ranging from 10 to 60 s after mixing, although after about 10 s homolysis of AdoCbl in the beam of the spectrometer became significant. The reaction of substrate with the holoenzyme can be monitored either by the decrease in absorbance at 525 nm due to the disappearance of AdoCbl or by the increase in absorbance at 470 nm due to the formation of Cbl(II). Preliminary experiments demonstrated that, as expected, the formation of Cbl(II) on the mutant enzyme mirrored the decrease in AdoCbl (data not shown). In general, the reaction was monitored at 525 nm because kinetic data obtained at this wavelength were of slightly better quality than that obtained at 470 nm.

Kinetic Behavior of the Arg100Lys Mutant with L-*Glutamate.* The stopped-flow traces obtained when the Arg100Lys mutant enzyme was reacted with concentrations of L-glutamate ranging from 5 to 100 mM are shown in Figure 4A. As predicted from the elevated K_m for glutamate, much higher concentrations of glutamate were needed to observe homolysis with the mutant enzyme. Both the amplitude and the rate of the reaction increased as the substrate concentration was increased, but, consistent with low k_{cat} of the mutant, the rate of homolysis was much slower than that measured previously for the wild-type enzyme.

Although the major portion of the absorbance change is described by a single exponential function, to properly fit the kinetic traces required three exponential functions. There is evidence for an initial, relatively rapid phase of the reaction, $k_{obs} = 10-20$ s^{-1}, but the very small amplitude associated with phase, $\Delta A_{525} \sim 0.005$, meant that this phase could not be characterized quantitatively.

The major portion of the absorbance change was described by a single-first order process, for which the apparent rate constants increase in a roughly linear fashion with increasing substrate concentration, with no sign of saturation. This implies that a second-order reaction between enzyme and substrate is occurring to generate Cbl(II). A plot of the

apparent first-order rate constants for this phase of the reaction as a function of substrate concentration (Figure 4C) yielded values for the forward and reverse rate constants of $k_{f(L-glu)} = 32 \pm 4\ M^{-1}\ s^{-1}$ and $k_{r(L-glu)} = 2.2 \pm 0.2\ s^{-1}$ respectively. From these data, the equilibrium constant for glutamate reacting with the enzyme in this process may be calculated as $\sim$14.4 M^{-1}. The equilibrium constant for glutamate reacting with the enzyme to generate the enzyme/Cbl(II)/substrate radical complex may also be obtained by plotting of the change in A_{525} [which is proportional to Cbl(II) concentration] against glutamate concentration. As shown in Figure 4D, these data fit well to a simple binding isotherm, and the calculated equilibrium constant of 14.3 M^{-1} is in excellent agreement with that obtained from kinetics.

Finally, there is a slower phase to the reaction that is characterized by an apparent rate constant, $k = 0.3-0.6\ s^{-1}$. Again, because of the very small amplitude of this phase and the fact that photolysis of the coenzyme begins to interfere at longer times, it was not possible to obtain reliable fits to this portion of the data.

Kinetic Behavior of the Arg100Lys Mutant with Deuterated Glutamate. The stopped-flow traces obtained when the Arg100Lys mutant was reacted with various concentrations of [2,4,4]-glutamate-d_3 are shown in Figure 4B. It is apparent that the deuterated material reacts at a similar rate to the protiated substrate, in contrast to the large isotope effects that are observed for the wild-type enzyme (*16*). In this case, the traces are clearly biphasic, and in contrast to the reaction with protiated glutamate could be readily fitted to a double exponential function. The major absorbance change increases in both amplitude and rate as the substrate concentration is increased. A plot of apparent rate constant for the faster phase against substrate concentration was roughly linear (Figure 4C), as was observed in the reaction of protiated glutamate. From the plot, the apparent rate constants for the forward and reverse components of the reaction are $k_{f(d3L-glu)} = 37 \pm 6\ M^{-1}\ s^{-1}$ and $k_{r(d3L-glu)} = 2.0 \pm 0.3\ s^{-1}$. From these values, the equilibrium constant for this reaction may be calculated as 18.5 M^{-1}. The equilibrium constant for the formation of the enzyme/Cbl(II)/substrate radical complex, calculated from a plot of the amplitude against substrate concentration (Figure 4D), was 14.2 M^{-1}, in reasonable agreement with the kinetic data. These values are very similar to those measured for the reaction with protiated glutamate, discussed above, implying that the isotope effect on homolysis for this mutant form of glutamate mutase is very small.

The slower phase of the reaction with deuterated glutamate is marked by a smaller decrease in absorbance that occurs with an apparent rate constant of about 0.2 s^{-1} and an amplitude, $\Delta A_{525} \sim 0.010$, which is much larger than that observed with protiated glutamate. Neither the amplitude nor the rate constant for this phase appears to show a marked dependence on the substrate concentration. This part of the reaction occurs sufficiently fast for it to represent a kinetically competent step in catalysis, but because it shows no marked concentration dependence it is unclear what chemical step gives rise to this behavior, given the coupled nature of the reaction.

Kinetic Behavior of the Arg100Lys Mutant with L*-threo-3-Methylaspartate.* A set of stopped-flow traces for the enzyme reacting with concentrations of methylaspartate ranging from 2.5 to 50 mM is shown in Figure 5A. Again, it is evident that the mutant enzyme reacts much more slowly with methylaspartate than does the wild-type enzyme (*16*). Furthermore, even at the highest substrate concentrations used, the reaction does not appear to approach saturation, suggesting that the mutant enzyme binds methylaspartate considerably more weakly than wild-type glutamate mutase.

The reaction with methylaspartate comprises two phases that are well fitted by double exponential function. Interestingly, the major, faster component of the reaction displays quite different kinetic behavior to that observed with glutamate. The observed rate constant for this phase of the reaction varies little with substrate concentration (Figure 5C), with an average value, $k_{obs(MeAsp)} = 7 \pm 0.6\ s^{-1}$, whereas the amplitude of this phase increased with increasing substrate concentration and could be fitted to a simple binding isotherm. This suggests that methylaspartate binds rapidly and reversibly, reaching equilibrium on a time scale that is fast compared with homolysis. From plots of the amplitude for this phase as a function of substrate concentration (Figure 5D), the equilibrium constant for the enzyme reacting with methylaspartate to form the enzyme/Cbl(II)/substrate radical complex is $K_{eq} = 24 \pm 5\ M^{-1}$. The amplitude of this phase, extrapolated to infinite substrate concentration, was $\Delta A_{525} = 0.060 \pm 0.006$, which would correspond to about 30% of enzyme active sites containing Cbl(II), similar to what was observed when the mutant enzyme was reacted with saturating concentrations of glutamate. (It is unclear whether the small apparent decrease in $k_{obs(MeAsp)}$ at 12.5 mM methylaspartate and subsequent increase at higher concentration is simply the result of random error, possibly combined with some systematic error involved in deconvoluting the data, or represents a real effect of substrate concentration on the reaction. We suspect the former explanation is more likely.)

In contrast, for the slower phase of the reaction the amplitude appears invariant with concentration, whereas the apparent rate constant increases with increasing substrate concentration toward a limiting value, as shown in Figure 5E. Extrapolation of the data to infinite and zero substrate concentrations yielded values of 3.3 ± 0.6 and $0.3 \pm 0.07\ s^{-1}$ for the forward and reverse rate constants for homolysis, respectively, and a K_s for methylaspartate binding to the enzyme of 50 ± 20 mM. This suggests that there may be two reactive forms of the enzyme present.

Kinetic Behavior of the Arg100Lys Mutant with Deuterated Methylaspartate. A set of stopped-flow traces for the enzyme reacting with concentrations of [d_3-methyl]-methylaspartate ranging from 2.5 to 50 mM is shown in Figure 5B. It is evident that the deuterated methylaspartate reacts more slowly than the protiated material, although the reduction in rate is not nearly as dramatic as that seen previously for the wild-type enzyme. Two phases are clearly evident in the reaction, especially at higher substrate concentrations, and the data were well fitted by a double exponential function.

The faster phase appears to display a concentration dependence very similar to that seen with the protiated substrate, i.e., the apparent rate constant for Cbl(II) formation does not vary significantly with increasing substrate concentration, whereas the amplitude of the reaction increases with concentration and could be fitted to a simple binding isotherm. Fits to these data gave an observed rate constant

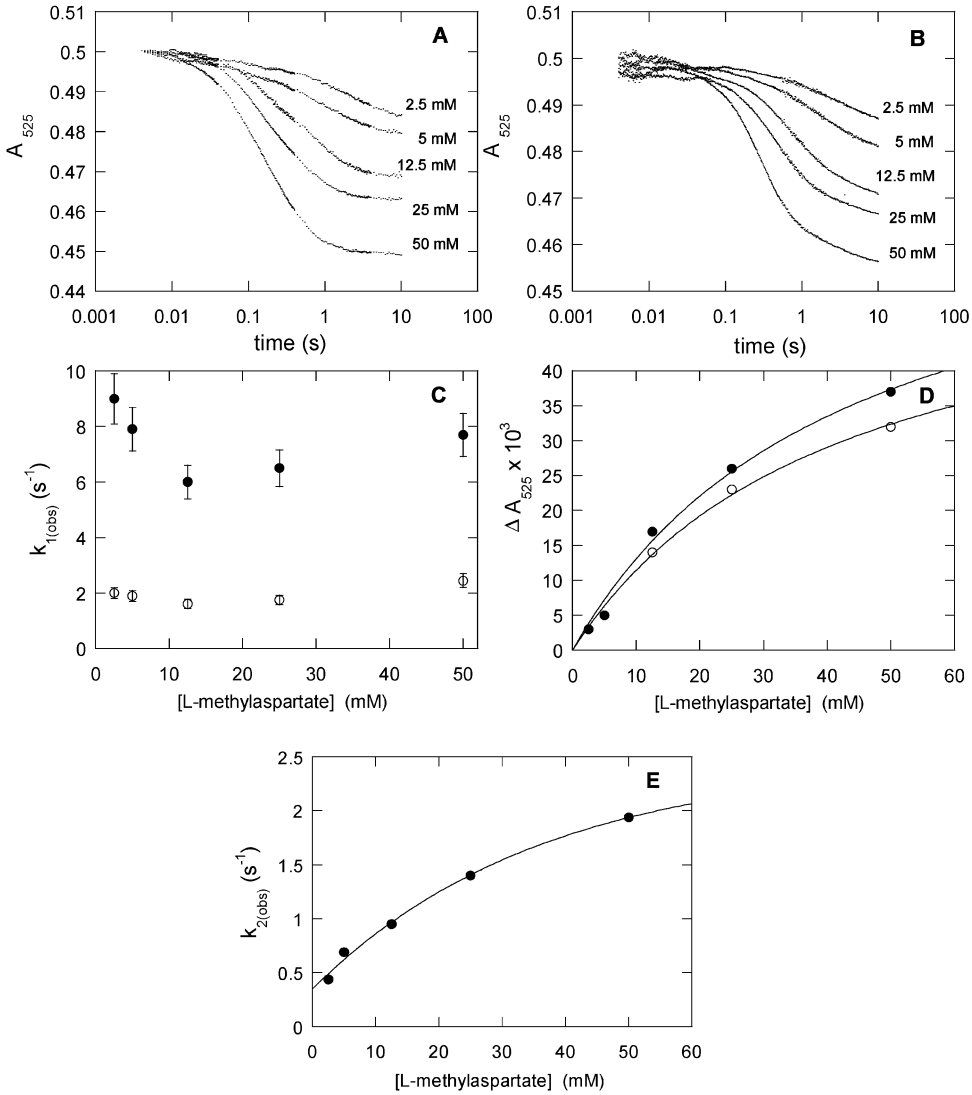

FIGURE 5: Reaction of Arg100Lys mutant with L-*threo*-3-methylaspartate. (A) Changes in absorbance at 525 nm, indicative of homolysis of AdoCbl, following rapid mixing of holo-glutamate mutase (50 μM final concentration) with various concentrations of L-*threo*-3-methylaspartate. The concentration of substrate is indicated by each trace. The time axis is plotted on a logarithmic scale. (B) Traces obtained in an analogous experiment where [d_3-methyl]-methylaspartate was the substrate. (C) Plot of observed rate constants for homolysis for the faster phase of the reaction, $k_{1(obs)}$, as a function of substrate concentration for the enzyme reacting with protiated (●) and deuterated (○) methylaspartates. (D) Variation of the amplitudes associated the observed rate constants for homolysis (plotted in panel C) as a function of substrate concentration for the enzyme reaction with protiated (●) and deuterated (○) methylaspartates. (E) Plot of observed rate constant for homolysis for the slower phase of the reaction, $k_{2(obs)}$, as a function of substrate concentration for the enzyme reacting with protiated (●) methylaspartate.

for homolysis, $k = 2 \pm 0.4$ s^{-1} and an equilibrium constant for the formation of the enzyme/Cbl(II)/substrate radical complex $K_{eq} = 24 \pm 6$ M^{-1} for [d_3-methyl]-methylaspartate binding, identical, within error, to that measured for the protiated substrate. The isotope effect on homolysis may be calculated as $^{D}V \sim 3.5$, which is much smaller than the unusually large isotope effects on homolysis, $^{D}V \sim 35$, observed previously (*16*) for the wild-type enzyme reacting with methylaspartate.

The slower phase of the reaction exhibits kinetics that are very similar to those observed with deuterated glutamate. The apparent rate constant for this phase varies very little with concentration, $k \sim 0.2$ s^{-1}, and the amplitude for this phase is also approximately constant, $\Delta A_{525} \sim -0.01$, although it appears more distinct on the traces obtained at high substrate concentration because the fast component of the reaction is better separated from the slower phase.

DISCUSSION

The crystal structure of glutamate mutase identified three arginine residues that make hydrogen bonds to the substrate. Two of these, Arg149 and Arg66, the so-called arginine claw (*12*), make bidentate interactions with the α-carboxyl group, whereas the third residue, Arg100, forms a bidentate salt-bridge with the side chain carboxylate (Figure 2). The position of Arg100 suggested that it may play an important role both in substrate recognition, and in correctly orienting the glutamate or methylaspartate side chain for promoting hydrogen abstraction. We therefore decided to investigate the effect of mutating this residue on the kinetics of the glutamate mutase-catalyzed reaction.

The properties of the Arg100 mutants described above demonstrate that this residue plays an important role, not only in substrate binding as we anticipated, but also in

catalysis. The most conservative mutation we could make, that of arginine to lysine, results in the K_m for glutamate being increased more than 10-fold, whereas k_{cat} is decreased by 2 orders of magnitude. The more drastic mutations of Arg100 to methionine or tyrosine only result in a modest further decrease in catalytic efficiency beyond that exhibited by the lysine mutant. However, these mutations do have a dramatic effect on the ability of the enzyme to stabilize radical species, as judged by our inability to detect any Cbl(II) accumulating on the enzyme during turnover.

At sufficiently high concentrations of substrate, the Arg100Lys mutant accumulates Cbl(II) to ~35% of active sites, a concentration similar to that observed for the wild-type enzyme (*20*). This indicates that even though the enzyme is significantly impaired in catalysis, this mutation does not affect the ability of the protein to stabilize substrate radicals. The fact that no Cbl(II) could be detected in the Arg100Tyr or Arg100Met mutants suggests that an electrostatic interaction between the positively charged arginine or lysine residue and the substrate carboxylate is important in stabilizing radicals. The theoretical work of Radom's group has emphasized the importance of protonation state in stabilizing radicals formed in B_{12}-dependent rearrangements (*24*). In particular, the neutral carboxyl group is predicted to stabilize the C-4 glutamyl radical much more effectively than the negatively charged carboxylate. Thus, in addition to its role in substrate binding, an important function of the arginine residue may be to neutralize the charge on the carboxylate. Whether a proton would be formally transferred to the substrate, thereby acting a general acid, or whether a "partial" protonation, as envisaged by Radom and co-workers (*24*), occurs must remain a matter of speculation. Although in free solution the pK_a's of carboxylate and guanidinium ions are sufficiently different to make proton transfer quite unfavorable, in the relatively hydrophobic active site of an enzyme the pK_a's are likely to be moved much closer toward neutral, thereby facilitating proton transfer.

Kinetics of AdoCbl Homolysis. Previously, we have examined the pre-steady-state kinetics of AdoCbl homolysis for both wild-type glutamate mutase (*16*) and for the active site Glu171Gln mutant enzyme reacting with glutamate and methylaspartate. We found the kinetics of Cbl(II) formation in the wild-type enzyme complicated by the presence of at least two exponential phases that we attribute to negative cooperativity between the active sites of the dimeric enzyme. The kinetics of homolysis in the Arg100Lys mutant are also complicated by the presence of more than one exponential phase, and we are unable to interpret all of the observed kinetic processes. However, it is clear that the mutation affects the reaction of the holoenzyme with either substrate in three significant ways.

First, the mutation weakens substrate binding significantly, indeed by a much greater degree than a simple examination of K_m would suggest. In fact, the kinetic behavior of the Arg100Lys mutant did not allow the association constants for the substrates to be calculated, although the small equilibrium constants for the formation of the enzyme/Cbl(II)/substrate radical complexes indicate much weaker binding. It is particularly interesting that the rate of reaction with glutamate is second order, implying that glutamate binding is involved in the rate-determining step for homolysis, whereas the reaction with methylaspartate is independent of substrate concentration, suggesting that a step other than methylaspartate-binding is rate-determining in this case.

We speculate that these kinetics can be explained by a model in which the resting state of the holoenzyme is able to bind glutamate productively and react with it to initiate homolysis of AdoCbl, whereas to bind methylaspartate productively a protein conformational change is first necessary before binding and/or homolysis can take place. The shapes of glutamate and methylaspartate suggest that any conformational change in the enzyme necessary to go from a glutamate-binding to a methylaspartate-binding state must be a rather subtle one. In the wild-type enzyme, this postulated conformational change would have to be fast relative to the chemical step because the observed rate constant for AdoCbl homolysis exhibits the same dependence on substrate concentration with either glutamate or methylaspartate. However, in the mutant enzyme, which binds substrates much more weakly, this putative conformational change may be slowed sufficiently to induce the kinetic behavior described above.

Second, the Arg100Lys mutation significantly slows the rate of cobalt−carbon bond homolysis. For the wild-type enzyme, the observed rate constants for AdoCbl homolysis are 97 and 80 s^{-1} with glutamate and methylaspartate, respectively (*16*). These rate constants were measured for the "slow" active site of the enzyme; the "fast" active site reacts within the dead time of the spectrometer and so cannot be accurately measured by stopped-flow techniques. By comparison, when methylaspartate is the substrate the observed rate constant for homolysis of AdoCbl is 7 s^{-1}, more than 10-fold slower than the "slow" active site of wild-type enzyme. The reaction of the Arg100Lys mutant with glutamate exhibits second-order kinetics, with $k_{f(L-glu)} = 32\ s^{-1}\ M^{-1}$, whereas the second-order rate constant for wild-type enzyme reacting with glutamate to form Cbl(II) may be calculated as $k_{(homolysis)}/K_{d(glutamate)} = 4 \times 10^4\ s^{-1}\ M^{-1}$. From this comparison, it is apparent that the mutant enzyme catalyzes the reaction 1200-fold less efficiently than wild-type. Thus, the slower rate at which the mutant is able to generate free radicals would explain a major portion of the 2000-fold decrease in k_{cat}/K_m measured for the Arg100Lys mutant for the overall reaction. It interesting that although the rate of homolysis is slowed by this mutation, during turnover the mutant still accumulates Cbl(II) to concentrations similar to wild-type enzyme. This implies that the rate of recombination of Cbl(II) with adenosyl radical must be similarly slowed by the mutation, so that the internal equilibrium constant for the formation of Cbl(II) on the enzyme remains unchanged.

The third significant effect of the mutation is to reduce the very large kinetic isotope effects associated with homolysis in the wild-type enzyme, where $^DV \sim 30$, to values that are close to 1 when the enzyme is reacted with deuterated substrates. As we have discussed previously (*16*), the glutamate mutase reaction is fully reversible and the various mechanistic steps in the reaction are best described as a series of coupled equilibria (*25*). In this view, addition of substrate perturbs the entire series of equilibria shown in Figure 1. The establishment of the new equilibrium involves all of the rate constants in the system, including those describing hydrogen transfer between either glutamate or methylaspartate, which are sensitive to isotopic substitution. Thus, the

apparent isotope effect on Cbl(II) formation arises because the adenosyl radical reacts more slowly with the deuterated substrate so that the equilibrium is established more slowly. However, for a large isotope effect to be observed on the formation of Cbl(II) (as is the case with the wild-type enzyme), the formation of adenosyl radical must be both rapid *and* energetically unfavorable compared with the subsequent formation of substrate radicals.

There are two explanations for the reduction in the apparent pre-steady-state isotope effects on Cbl(II) formation observed for the Arg100Lys mutant. The first is that the mutation slows down the rate of AdoCbl homolysis sufficiently that this step, rather than hydrogen abstraction, becomes rate determining in the mutant. The second is that the mutation actually diminishes the intrinsic isotope effect, possibly by reducing the degree of quantum tunneling associated with hydrogen transfer. Although our experimental data are consistent with either one or both possibilities occurring, we favor the former explanation as being more likely, in part because it is consistent with the slower rate of homolysis exhibited by the mutant. Also, recent kinetic studies on a model chemical system designed to mimic the hydrogen abstraction step in diol dehydrase have shown a similar high degree of quantum tunneling (*26*), suggesting that tunneling is intrinsic to the hydrogen abstraction reaction and therefore unlikely to be affected by a mutation in the enzyme.

Finally, we note that the pre-steady-state kinetic properties of the Arg100Lys mutant form of glutamate mutase are very similar to those we observed in another active site mutant, Glu171Gln (*15*). This residue hydrogen bonds to the amino-group of the substrate, and likely functions as a general base involved in adjusting the protonation state of the amino-group during catalysis. Although Glu171 recognizes a different part of the substrate, changing this residue to glutamine weakens substrate binding by 50−100-fold, slows homolysis of AdoCbl by 10-fold, and the large apparent deuterium isotope effects on homolysis are greatly reduced; however, the ability of the enzyme to stabilize radical species is unimpaired. This suggests that it is the reduction in substrate binding energy caused by these mutations that is responsible for slowing the kinetics of cobalt−carbon bond cleavage, rather than direct mechanistic linkage between these active residues and the coenzyme. Indeed, Arg100 and Glu171 are both some distance from the coenzyme binding site, and therefore the coupling of substrate binding to homolysis must presumably involve subtle and coordinated movement of protein residues.

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