Pre-Steady-State Kinetic Studies on the Glu171Gln Active Site Mutant of Adenosylcobalamin-Dependent Glutamate Mutase†

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ABSTRACT: Glutamate-171 is involved in recognizing the amino group of the substrate in glutamate mutase. The effect of mutating this residue to glutamine on the ability of the enzyme to catalyze the homolysis of adenosylcobalamin has been investigated using UV−visible stopped-flow spectroscopy. Although Glu171 does not contact the coenzyme, the mutation results in the apparent rate constants for substrate-induced homolysis of the coenzyme that are slower by 7-fold and 13-fold with glutamate and methylaspartate, respectively, than those measured for the wild-type enzyme; furthermore, it weakens the binding of these substrates by ∼50-fold and ∼400-fold, respectively. These observations lend support to the idea that the enzyme may use substrate binding energy to accelerate homolysis of the coenzyme. The mutation also results in isotope effects on coenzyme homolysis that are much smaller than the very large effects observed when the wild-type enzyme is reacted with deuterated substrates. This observation is consistent with adenosylcobalamin homolysis being slowed relative to hydrogen abstraction from the substrate.

Glutamate mutase catalyzes the reversible isomerization of L-glutamate to L-*threo*-3-methylaspartate (*1*, *2*) and belongs to a group of adenosylcobalamin-dependent (AdoCbl,[1] coenzyme B_{12}) enzymes that catalyze unusual isomerization reactions that proceed by radical mechanisms (for recent reviews, see refs *3*−*7*). A detailed 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 (k_2 or k_{-7} in Figure 1); this is followed by abstraction of hydrogen from the substrate to form 5′-dA and a substrate radical (k_3 or k_{-6} in Figure 1). For glutamate mutase, the rearrangement of the C-4 radical of glutamate to the methylaspartyl radical has been shown to proceed by a fragmentation−recombination mechanism with glycyl radical and acrylate as intermediates (*8*).

Stopped-flow spectroscopic experiments on glutamate mutase to examine the substrate-induced homolysis of the AdoCbl cobalt−carbon bond have shown that there is a large kinetic isotope effect on homolysis when the enzyme is reacted with substrates deuterated in the abstractable position (*9*). This implies that hydrogen abstraction from the substrate and homolysis of AdoCbl are kinetically coupled processes, suggesting that the adenosyl radical is formed only as a transient, high-energy species. Similar kinetic behavior has been found for other AdoCbl-dependent enzymes, suggesting that this is a common mechanistic feature (*10*−*12*).

Glutamate mutase comprises two subunits, designated E and S (*13*, *14*), with the functional form of the holoenzyme being an E_2S_2 tetramer (*15*). The E subunit (M_r 54000) adopts an eight-stranded β-barrel structure whereas the S subunit (M_r 14800) possesses an $\alpha_5\beta_5$ canonical nucleotide-binding Rossmann fold. AdoCbl is bound at the interface of the E and S subunits, with the reactive face of the coenzyme and the substrate-binding site being situated in the lumen of the β-barrel formed by the E subunit (*16*).

The crystal structure of glutamate mutase identified Glu171 in the E subunit as a potentially important active site residue that makes a hydrogen bond to the amino group of the substrate (*17*). Recently, we introduced several sterically and functionally conservative mutations at this position. 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 (*18*). Thus, for the wild-type enzyme, activity is pH dependent and titrates with an apparent pK_a of 6.6 on V_{max}, whereas for the Glu171Gln mutant k_{cat} is reduced 50-fold and is independent of pH.

To investigate the role of Glu171 in more detail, we have used stopped-flow UV−visible spectroscopy to measure the rate of substrate-induced homolysis of AdoCbl by the Glu171Gln mutant enzyme. Here we report the results of experiments using glutamate and methylaspartate, both protiated and deuterated in the abstractable position. The mutation appears to change several steps of the kinetic mechanism. Substrate binding is weakened, and the apparent rate constants for homolysis of AdoCbl are slowed compared with wild type. Furthermore, the mutation appears to significantly reduce the large kinetic isotope effects on

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[1] Abbreviations: AdoCbl, adenosylcobalamin; Cbl(II), cob(II)alamin.

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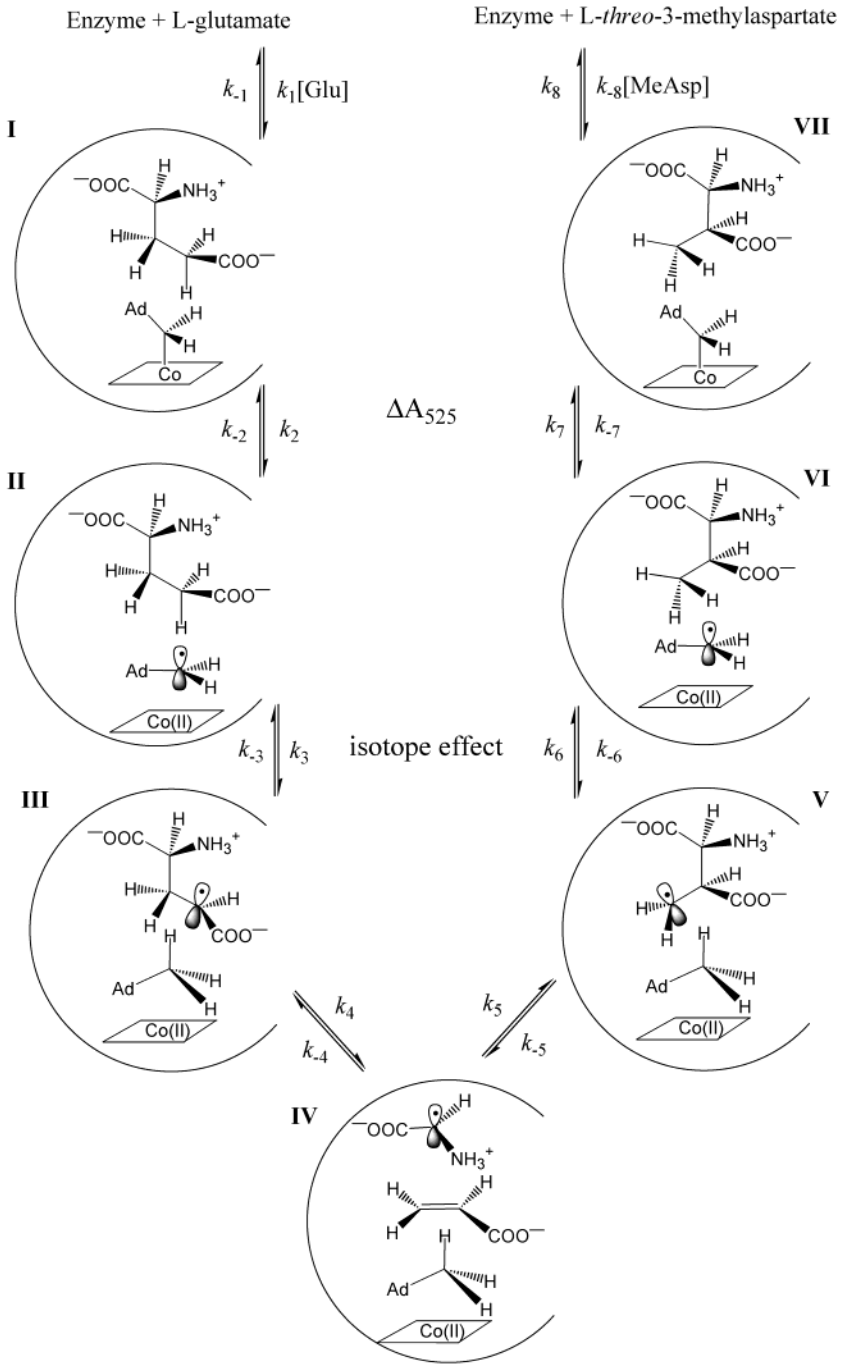

FIGURE 1: Mechanistic scheme for the rearrangement of L-glutamate to L-*threo*-3-methylaspartate catalyzed by glutamate mutase. Steps giving rise to the change in absorbance at 525 nm and steps giving rise to a deuterium isotope effect are indicated.

AdoCbl homolysis that are observed when the enzyme is reacted with deuterated substrates.

MATERIALS AND METHODS

Materials. The purification of the glutamate mutase fusion protein, GlmES, from a recombinant *Escherichia coli* strain has been described previously (*19*). AdoCbl was purchased from the Sigma Chemical Co. The sources of other materials have been described previously (*9*, *19*) or were purchased from commercial suppliers.

Substrates. L-*threo*-3-Methylaspartate and L-*threo*-3-[$^2H_3$]-methylaspartate were prepared enantiomerically pure by enzymic synthesis (*20*). D,L-Glutamic acid was purchased from Sigma Chemical Co. and D,L-[2,4,4-$^2H_3$]glutamic acid 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.

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

RESULTS

UV−visible stopped-flow spectroscopy was used to investigate the pre-steady-state kinetics of AdoCbl homolysis and Cbl(II) formation when the glutamate mutase Glu171Gln mutant was reacted with substrates, either unlabeled or deuterated at the position of hydrogen abstraction. These experiments paralleled our previous investigation of substrate-induced coenzyme homolysis in the wild-type enzyme (*9*). The reactions were performed at 10 °C under anaerobic conditions. Typically, reactions were monitored for times ranging from 10 s up to 60 s after mixing, although after about 30 s the rate of 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 proved slightly less noisy than that obtained at 470 nm. At the end of each reaction the UV−visible spectrum of sample was recorded, and from these spectra the proportions of AdoCbl and Cbl(II) could be determined.

Kinetic Behavior of the Glutamate Mutase Glu171Gln Mutant with L*-Glutamate.* The stopped-flow traces obtained when the Glu171Gln mutant was reacted with various concentrations of L-glutamate are shown in Figure 2A. Two phases are evident in the reaction. Initially there is a rapid phase that increases in both amplitude and rate as the concentration of glutamate increases. This phase is hard to discern at the lowest concentrations of substrate but is quite evident at higher substrate concentrations. This is followed by a slower phase that comprises the major absorbance change, the rate and amplitude of which tend toward limiting values at saturating concentrations of substrate.

By fitting the first two phases of the traces obtained with various concentrations of substrates to double exponential functions, apparent first-order rate constants for these phases could be obtained. The apparent rate constant for the faster phase increases in a roughly linear fashion with increasing substrate concentration (with no sign of saturation), implying that a second-order reaction between enzyme and substrate is occurring. A plot of the apparent first-order rate constants for this rapid phase of the reaction as a function of substrate concentration (Figure 2B) yielded values for the forward and reverse rate constants of 370 ± 20 M^{-1} s^{-1} and 11 ± 1.5 s^{-1}, respectively. From these data the equilibrium constant for glutamate reacting with the enzyme in this process may be calculated as ∼33 M^{-1}. The plot of rate constants for the second phase of the reaction (Figure 2B) followed a more usually observed binding isotherm leading to saturation at high substrate concentrations. Extrapolation of the data to infinite and zero substrate concentrations yielded values of 10.8 ± 0.7 s^{-1} and 1.2 ± 0.8 s^{-1} for the forward and reverse rate constants for homolysis respectively, and a K_s for glutamate binding to the enzyme of 33 ± 6 mM.

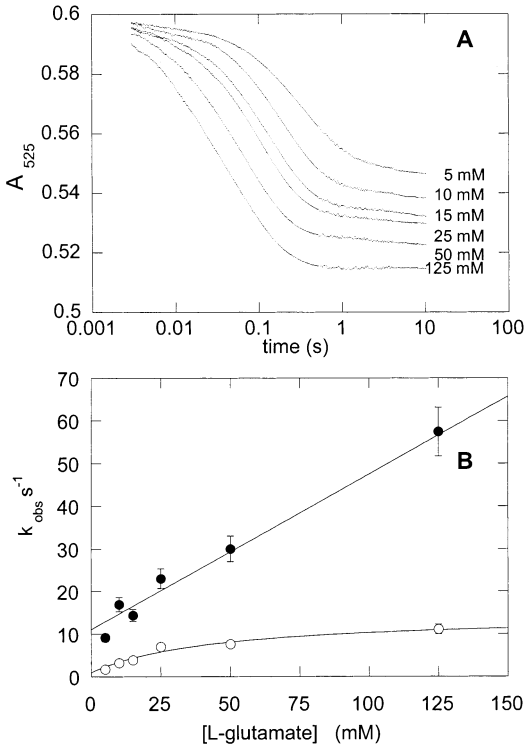

FIGURE 2: Reaction of the Glu171Gln mutant with L-glutamate. (A) Changes in absorbance at 525 nm, indicative of homolysis of AdoCbl, following rapid mixing of hologlutamate mutase (50 μM final concentration) with various concentrations of L-glutamate. The concentration of substrate is indicated by each trace. The time axis is plotted on a logarithmic scale. (B) Plots of the observed rate constants obtained by computer fitting of the traces as a function of substrate concentration: (●) rate constants obtained from fitting the faster phase; (○) rate constants obtained from fitting the slower phase of the reaction.

Kinetic Behavior of the Glutamate Mutase Glu171Gln Mutant with L*-threo-3-Methylaspartate.* The reaction of the Glu171Gln mutant enzyme with L-*threo*-3-methylaspartate was next examined. A set of stopped-flow traces for the enzyme reacting with various concentrations of methylaspartate is shown in Figure 3A. The results are qualitatively similar to those obtained with glutamate. Again, although less evident from visual inspection of the traces, the reaction comprises two phases that require a double exponential

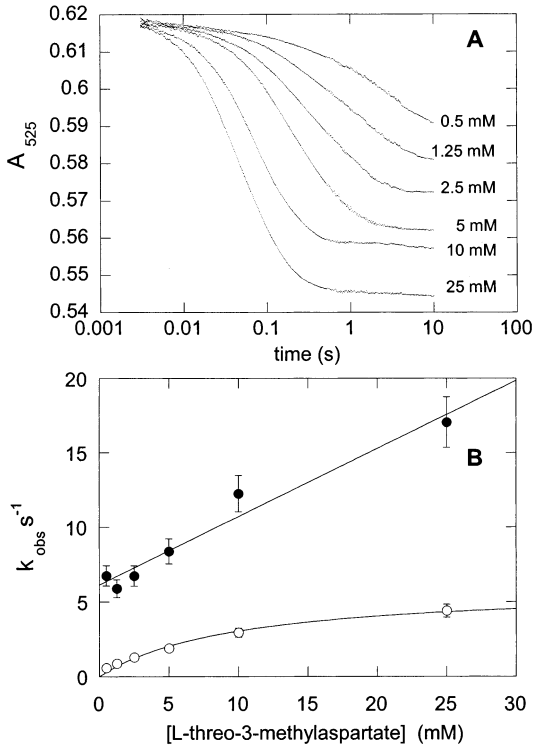

FIGURE 3: Reaction of the Glu171Gln mutant with L-*threo*-3-methylaspartate. (A) Changes in absorbance at 525 nm, indicative of homolysis of AdoCbl, following rapid mixing of hologlutamate mutase (50 μM final concentration) with various concentrations of L-*threo*-3-methylaspartate. The concentration of substrate is indicated by each trace. The time axis is plotted on a logarithmic scale. (B) Plots of the observed rate constants obtained by computer fitting of the traces as a function of substrate concentration: (●) rate constants obtained from fitting the faster phase; (○) rate constants obtained from fitting the slower phase of the reaction.

function to adequately fit the data. The mutant enzyme reacts much more slowly with methylaspartate than does the wild-type enzyme, and it also appears to bind methylaspartate more weakly.

Secondary plots of the apparent rate constants for the reaction of various concentrations of methylaspartate with the mutant enzyme are shown in Figure 3B. Again, the faster reaction appears to be a second-order process, as the apparent rate constant for homolysis appears to increase linearly with substrate concentration. Analysis of these data yielded values for the forward and reverse rate constants of 460 ± 50 M^{-1} s^{-1} and 6.0 ± 0.5 s^{-1}, respectively, and the equilibrium constant for methylaspartate reacting with the enzyme in this process may be calculated as ~ 77 M^{-1}. The slower phase of the reaction followed saturation kinetics, and extrapolation of the data to infinite and zero substrate concentrations yielded values of 6.6 ± 0.2 s^{-1} and 0.4 ± 0.04 s^{-1} for the forward and reverse rate constants for homolysis, respectively, and a K_s for methylaspartate binding to the enzyme of 16 ± 2 mM.

Kinetic Behavior of the Glu171Gln Mutant with Deuterated Substrates. Previous stopped-flow studies using deuterated substrates with wild-type glutamate mutase have uncovered an important aspect of the mechanism by which the enzyme catalyzes homolysis of the coenzyme (*9*). The rates of AdoCbl homolysis for the wild-type enzyme reacting with deuterated substrates were found to be dramatically slower than with protiated substrates, implying a mechanism

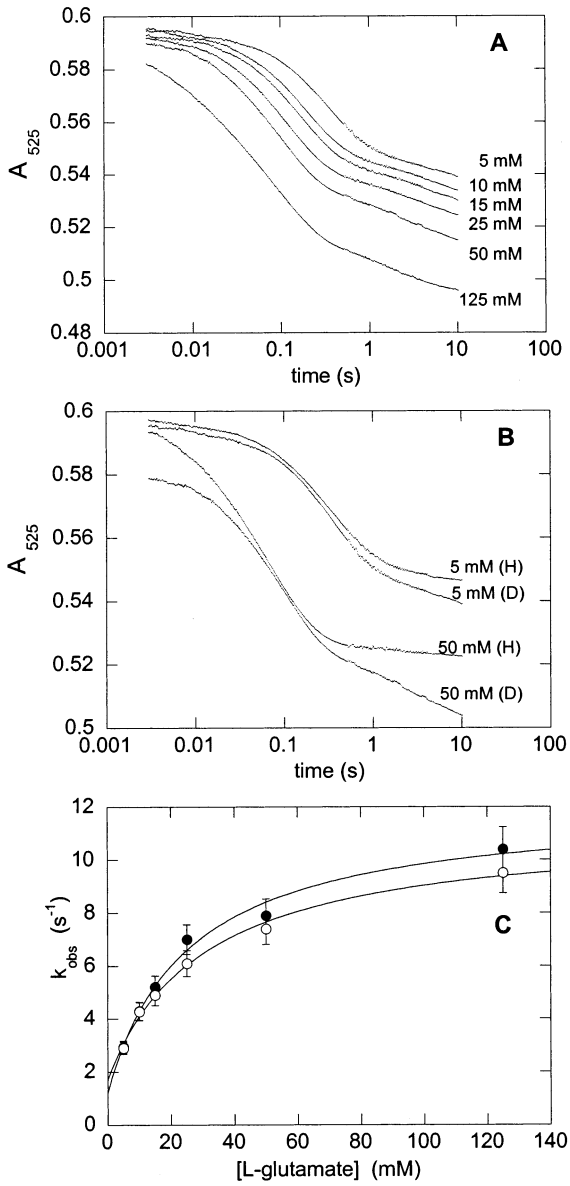

FIGURE 4: Reaction of the Glu171Gln mutant with L-[2,4,4-$^2H_3$]-glutamate. (A) Changes in absorbance at 525 nm following rapid mixing of hologlutamate mutase (50 μM final concentration) with various concentrations of deuterated L-glutamate. The concentration of substrate is indicated by each trace. The time axis is plotted on a logarithmic scale. (B) Selected traces comparing the reaction of protiated and deuterated glutamate with the mutant enzyme. To facilitate comparison, some of the traces have been offset on the *y* axis. (C) Variation of observed rate constants for homolysis as a function of substrate concentration for the enzyme reaction with protiated (●) and deuterated (○) glutamates.

where cleavage of the Co−C bond and hydrogen abstraction are kinetically coupled. Furthermore, the apparent isotope effects on Co−C bond homolysis were much larger than expected from simple zero-point energy considerations and are most likely manifestations of quantum tunneling by the hydrogen atom undergoing transfer (*21*). We were therefore interested in examining the behavior of the mutant enzyme with deuterated substrates.

The stopped-flow traces obtained when the Glu171Gln mutant was reacted with various concentrations of [2,4,4-d_3]-glutamate are shown in Figure 4A. Although less apparent from inspection, the traces again show evidence for a rapid phase that increases in both rate and amplitude as the

concentration of glutamate is increased. This is followed by a slower phase, which comprises the major absorbance change, the rate of which tends toward a limiting value as the substrate concentration increases. Finally, and rather puzzlingly, there is an additional slow phase, the rate of which appears independent of substrate concentration but whose amplitude increases as substrate concentration increases. The traces could be adequately fitted by a three exponential function. However, in contrast to the reaction with protiated glutamate, it was not possible to get robust fits for the most rapid phase of the reaction, probably because of the small amplitude of this phase. Reliable fits were obtained to the second phase of the reaction, which comprises the major amplitude change, and extrapolation of the observed rate constants to infinite and zero substrate concentrations yielded values of $9.5 \pm 0.4\ s^{-1}$ and $1.7 \pm 0.4\ s^{-1}$ for the forward and reverse rate constants for homolysis, respectively.

The slowest phase, which does not appear to be present in the reaction of the mutant with protiated glutamate, occurs with an observed rate constant of $\sim 0.3\ s^{-1}$. This part of the reaction occurs sufficiently fast for it to represent a kinetically competent step but shows no marked concentration dependence. Given the coupled nature of the reaction, it is unclear what chemical step gives rise to this behavior.

For comparison, overlays of selected traces obtained for the mutant enzyme reacting with protiated and deuterated glutamates are shown in Figure 4B. It is evident that the protiated and deuterated substrates react at very similar rates. At low substrate concentrations the traces initially superimpose but diverge at longer times as the deuterated substrate continues to react. At higher concentrations (e.g., 50 mM glutamate) the amplitude of the most rapid phase is somewhat larger with protiated glutamate, although the overall amplitudes of the two traces are similar.

A further comparison is provided by an overlay of the plots of k_{obs} extracted from the data obtained with protiated and deuterated substrates, which closely superimpose (Figure 4C). Thus, although the substitution of deuterium in the substrate does result in noticeable differences in the pre-steady-state kinetics of AdoCbl homolysis by the mutant enzyme, it appears that the very large kinetic isotope effect on Co−C bond homolysis observed for the wild-type enzyme ($^{D}V = 28$) is absent the Glu171Gln mutant ($^{D}V \sim 1$).

The reaction of the mutant enzyme with L-*threo*-3-[d_3]-methylaspartate is shown in Figure 5A. The stopped-flow traces are similar to those obtained with protiated methylaspartate and are again characterized by a biphasic reaction. There is a rapid phase that increases in both rate and amplitude as the concentration of methylaspartate is increased. This comprises a small fraction of the total absorbance change, and again it was not possible to obtain reliable fits to this portion of the reaction. The major amplitude change is characterized by a slower phase that tends toward a limiting rate at saturating concentrations of substrate. Extrapolation of k_{obs} for this portion of the reaction to infinite and zero substrate concentrations yielded values of $0.52 \pm 0.02\ s^{-1}$ and $0.1 \pm 0.02\ s^{-1}$ for the forward and reverse rate constants for homolysis, respectively.

Comparisons of traces obtained with deuterated and protiated methylaspartates (Figure 5B) reveal that the deuterated substrate reacts significantly slower, in contrast to the behavior observed with deuterated glutamate. Plots of k_{obs} for AdoCbl homolysis as a function of methylaspartate concentration also demonstrate that a large isotope effect is present (Figure 5C). Extrapolation of the plot to infinite substrate concentrations yields a value for the V_{max} isotope effect, $^{D}V = 12$, which, although large, is significantly smaller than that measured for the wild-type enzyme reacting with methylaspartate, $^{D}V = 35$ (*9*).

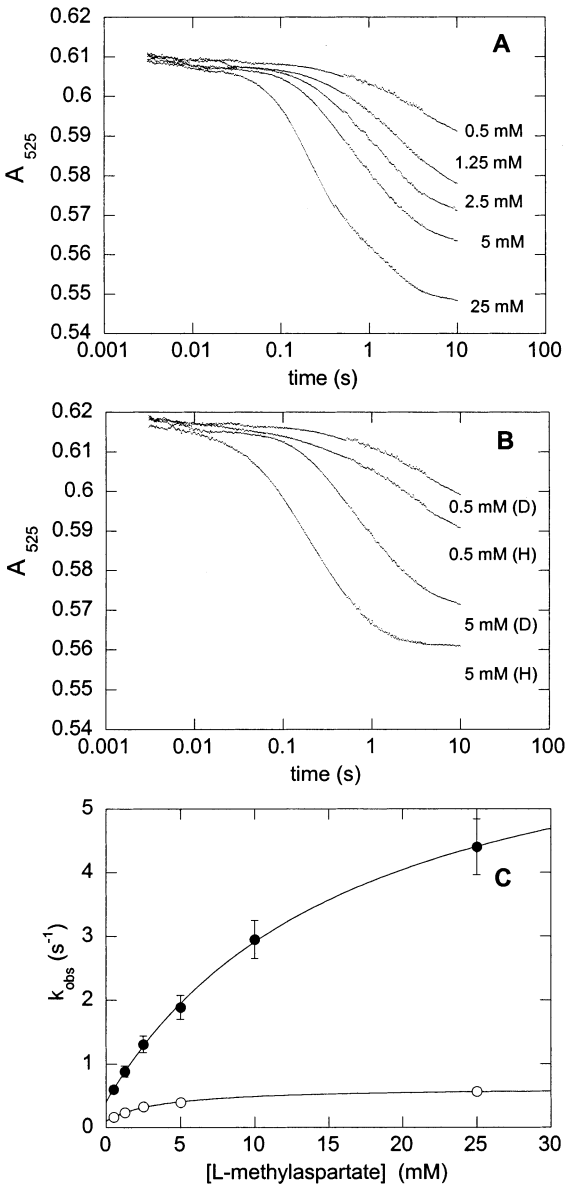

FIGURE 5: Reaction of the Glu171Gln mutant with L-*threo*-3-[$^2H_3$]-methylaspartate. (A) Changes in absorbance at 525 nm following rapid mixing of hologlutamate mutase (50 μM final concentration) with various concentrations of methylaspartate. The concentration of substrate is indicated by each trace. The time axis is plotted on a logarithmic scale. (B) Selected traces comparing the reaction of protiated and deuterated methylaspartate with the mutant enzyme. To facilitate comparison, some of the traces have been offset on the *y* axis. (C) Variation of observed rate constants for homolysis as a function substrate concentration for the enzyme reaction with protiated (●) and deuterated (○) methylaspartates.

DISCUSSION

Previously, we have examined the pre-steady-state kinetics of AdoCbl homolysis for wild-type glutamate mutase reacting with glutamate and methylaspartate (*9*). From these experi-

Table 1: Kinetic Parameters for Wild-Type and Glu171Gln Mutant Glutamate Mutase Enzymes[a]

	L-glutamate						L-*threo*-3-methylaspartate		
substrate	k_{cat} (s^{-1})	K_m (mM)	^DV (steady state)	k_{obs} (s^{-1}) (homolysis)	K_s (mM)	^DV (homolysis)	k_{obs} (s^{-1}) (homolysis)	K_s (mM)	^DV (homolysis)
wild-type enzyme	5.8[b]	0.58[b]	4.6[c]	97[d]	0.61[d]	28[d]	80[d]	0.04[d]	35[d]
Glu171Gln mutant	0.11[c]	0.24[c]	2.1[c]	12	33	1	7	16	12

[a] As discussed in the text, the pre-steady-state data refer to the "slow" active site of the enzyme. [a] Data taken from ref *19*. [b] Data taken from ref *18*. [c] Data taken from ref *9*.

ments we were able to measure the rate constants for homolysis and the dissociation constants for substrate binding. By using substrates deuterated in the abstractable position, we also established that homolysis of the coenzyme was kinetically coupled to hydrogen abstraction from the substrate. Here we compare the kinetic properties of the Glu171Gln mutant enzyme with the wild-type enzyme and discuss the implications of these results for the role of the active site glutamate residue in substrate binding and catalysis. To facilitate comparisons between the wild-type and mutant enzymes, the pertinent kinetic data are collected together in Table 1.

Effect of the Glu171Gln Mutation on Substrate Binding. In both the wild-type and mutant enzymes the kinetics of AdoCbl homolysis exhibit biphasic kinetic behavior that is characterized by two sets of observed rate constants. Similar behavior has also been observed for glutamate mutase reacting with the slow substrate, (*S*)-2-hydroxyglutarate (*22*). One postulate attributes this to negative cooperativity arising from interactions between the two active sites of the dimeric enzyme (*9*). The pre-steady-state kinetic properties of the mutant enzyme also appear to be consistent with a two-site model, although substrate binding appears to be much weaker. For the wild-type enzyme reacting with protiated substrates, homolysis at the "fast" active site is nearly complete within the dead time of the spectrometer, so that only the slower active site may be observed. The Glu171Gln mutation appears to slow the homolysis reaction sufficiently so that the fast phase of the reaction can now be observed.

The Glu171Gln mutation significantly weakens the binding of both substrates to the enzyme. With either substrate, the fast active site of the reaction appears to obey a second-order rate law, with no evidence for saturation over the range of substrate concentrations examined. This suggests that the initial Michaelis complex formed between the substrate and enzyme is extremely weak. For the "slow" active site, for which a direct comparison between the wild-type and mutant enzymes can be made, the apparent K_s for methylaspartate and glutamate binding are raised by factors of ~400- and ~50-fold, respectively. In energetic terms, this represents a $\Delta\Delta G°$ of 2−3 kcal in the free energy of substrate binding caused by the substitution of the negatively charged carboxylate of glutamate for the neutral amide function of glutamine as the hydrogen-bonding partner with the positively charged amino group of the substrate.

Interestingly, although substrate binding is weakened, the apparent K_m for the glutamate of the mutant is actually about 2-fold *lower* than that of the wild-type enzyme (*18*). This provides a dramatic illustration of how K_m can be a misleading measurement of substrate binding affinity and implies a large forward commitment to catalysis for the overall reaction.

Effect of the Glu171Gln Mutation on Catalysis. Previous steady-state measurements had shown that the Glu171Gln mutation lowers k_{cat} for the isomerization of glutamate to methylaspartate by 50-fold (*18*). We had postulated a role for Glu171 as an active site base poised to deprotonate the amino group of the substrate and thereby facilitate the carbon skeleton rearrangement by stabilizing the intermediate glycyl radical. The steady-state kinetic properties of the mutant enzyme appeared consistent with this mechanistic view. The more detailed pre-steady-state investigation presented here clearly demonstrates that the mutation has more wide-ranging effects on catalysis by the enzyme. In particular, homolysis of AdoCbl is significantly slower in the mutant enzyme. At the slow-reacting site, for which it is possible to make a direct comparison with the wild-type enzyme (see Table 1), the observed rate constants for homolysis are slower by 7-fold when glutamate is the substrate and 13-fold when methylaspartate is the substrate. However, the reduction in the rate constants for homolysis is not sufficient to explain the overall reduction in k_{cat}, and it seems reasonable to propose that Glu171 may still play an important role as an active site general base.

We did not expect that the mutation of Glu171 would have a large effect on the rate at which the enzyme catalyzes the homolysis of AdoCbl, as this residue does not make contact with the coenzyme. The mechanism by which Glu171 assists in homolysis is unclear; however, because the mutation also greatly weakens the binding of substrate to the protein, this suggests a link between the binding energy of the substrate and catalysis of coenzyme homolysis. This might be achieved through a substrate-induced conformational change of the protein, and although there is no direct evidence for this in glutamate mutase, large changes in the structure of the closely related enzyme, methylmalonyl-CoA mutase, do occur when this enzyme binds its substrate (*23*).

Effects of Deuterated Substrates on the Rate of AdoCbl Homolysis. A striking effect of the mutation is the changes that it induces in the behavior of the enzyme with deuterated substrates. Most noteworthy are the reduction in the isotope effects associated with homolysis from the very large values measured with wild-type enzyme to about 12 with methylaspartate and close to 1 with glutamate. As we have discussed previously (*9*), the glutamate mutase reaction is fully reversible, and the various mechanistic steps in the reaction are best described as a series of coupled equilibria (*24*). In this model, perturbation of one step in the reaction by, for example, isotopic substitution will affect the approach to equilibrium of all the other steps.

The reduction in the apparent pre-steady-state isotope effects on homolysis may occur in two ways: (a) the mutation stabilizes the products of homolysis, adenosyl radical and Cbl(II), so that the kinetic coupling of this step

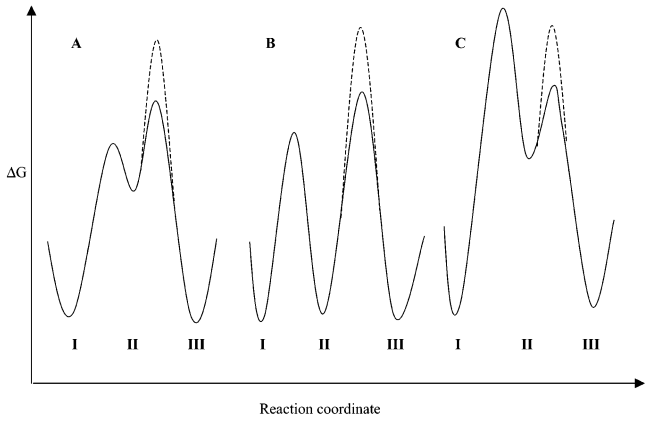

FIGURE 6: Possible effects of the Glu171Gln mutation on the free energy profile for homolysis of AdoCbl and hydrogen abstraction from glutamate. The intermediates represented by Roman numerals are those depicted in Figure 1. The deuterium isotope effect is represented by the dashed lines. (A) Free energy profile for the wild-type enzyme. (B) Free energy profile in which the mutation stabilizes the adenosyl radical, thereby uncoupling coenzyme homolysis from hydrogen abstraction. (C) Free energy profile in which the mutation slows homolysis of AdoCbl, thereby suppressing the isotope effect on hydrogen abstraction.

to hydrogen abstraction is reduced or eliminated, or (b) the mutation slows the rate of AdoCbl homolysis significantly enough that this step, rather than hydrogen abstraction, becomes rate determining so that these two steps, while still coupled, are no longer isotopically sensitive, or less so. These two possibilities are illustrated in Figure 6. We prefer the latter explanation for the reduction in isotope effects, as this is supported by the observation that homolysis is indeed slowed by the mutation and also that the steady-state isotope effect on the rearrangement of glutamate to methylaspartate is reduced in the mutant enzyme (*18*).

Stabilization of Radicals by Wild-Type and Glu171Gln Mutant Enzymes. The fraction of enzyme that accumulates in the Cbl(II) form can be readily determined from analysis of UV−visible spectra measured after the reaction has reached the steady state. Under saturating substrate concentrations, both wild-type and mutant enzymes accumulate similar amounts of Cbl(II) in the steady state, 25−30% of active sites, suggesting the mutation does not significantly affect the ability of the enzyme to stabilize free radicals. This is, perhaps, not surprising since the most stable organic radical that accumulates on the enzyme is the C-4 radical of glutamate (*25*), and therefore the hydrogen bond formed between the Glu171 carboxylate and the amino group of the substrate would not be expected to greatly influence the stability of the glutamyl radical.

In conclusion, these studies provide further insight into the mechanism by which glutamate mutase catalyzes homolysis of the AdoCbl cobalt−carbon bond, leading to the generation of radicals. Glu171 appears to play a dual role in the enzyme mechanism, serving to both deprotonate the amino group of the substrate and facilitate homolysis of the coenzyme. Most interestingly, the Glu171Gln mutation slows the rates at which radicals are generated on the enzyme but does not alter the stability of the radicals once formed. Although the details remain to be elucidated, it appears that the weakened binding of the substrates by the enzyme results in slower rates of coenzyme homolysis.

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