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Research Paper

The role of the active site glutamate in the rearrangement of glutamate to 3-methylaspartate catalyzed by adenosylcobalamin-dependent glutamate mutase

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Abstract

Background: Adenosylcobalamin (coenzyme B_{12})-dependent enzymes catalyze a variety of chemically difficult reactions that proceed through the generation of free radical intermediates. A long-standing question is how proteins stabilize what are normally regarded as highly reactive organic radicals and direct them towards productive reactions. In glutamate mutase the carboxylate of Glu171 hydrogen bonds with the amino group of the substrate. We have investigated the role of this residue in the enzyme mechanism.

Results: Several sterically and functionally conservative mutations were introduced at position 171. In the most impaired mutant, Glu171Gln, k_{cat} is reduced 50-fold, although the K_m for glutamate is little affected. In the wild-type enzyme activity was pH-dependent and the acidic limb of the activity curve titrated with an apparent pK_a of 6.6 on V_{max}, whereas for the sluggish Glu171Gln mutant activity is independent of pH. The steady state deuterium kinetic isotope effect is reduced in the mutant enzyme, but the steady state concentration of free radical species on the enzyme (as measured by the steady state concentration of cob(II)alamin) is unaffected by the mutation.

Conclusions: The properties of the mutant proteins are consistent with the hypothesis that Glu171 acts as a general base that serves to deprotonate the amino group of the substrate during catalysis. Deprotonation is expected to facilitate the formation of the glycyl radical intermediate formed during the inter-conversion of substrate and product radicals, but to have little effect on the stability of product or substrate radicals themselves.

Keywords: Enzyme; Coenzyme B_{12}; Free radical; Mutagenesis; Isotope effect

1. Introduction

Glutamate mutase catalyzes the reversible isomerization of L-glutamate and L-*threo*-3-methylaspartate [1]. It is one of a group of enzymes that use adenosylcobalamin (AdoCbl), a biologically active form of vitamin B_{12}, to catalyze unusual 1,2-rearrangements in which an electron-withdrawing group is interchanged with a hydrogen atom on an adjacent carbon [2–5]. The migrating group may be –OH, $–NH_2$ or, as in the case of glutamate mutase, a carbon-containing fragment so that a skeletal rearrangement is effected (Fig. 1).

The role of AdoCbl as the source of carbon-based radicals, which are unmasked by homolysis of the coenzyme cobalt–carbon bond, is well established. Experiments with isotopically labeled substrates and coenzyme have demonstrated that for glutamate mutase, and all the other isomerases examined, 5′-deoxyadenosine acts as the intermediate carrier of the migrating hydrogen [6,7]. Pre-steady state kinetic studies on glutamate mutase and other B_{12}-dependent isomerases have shown that homolysis of the cobalt–carbon bond and hydrogen abstraction from the substrate are kinetically coupled processes [8–10]. This observation is consistent with a mechanism in which adenosyl radical is formed only as a transient, high energy species, with the stability of the substrate radical providing the thermodynamic driving force for homolysis.

A further intriguing and poorly understood aspect of these reactions is how the enzyme controls the rearrangement of substrate-radical to product-radical. The carbon skeleton rearrangement catalyzed by glutamate mutase is

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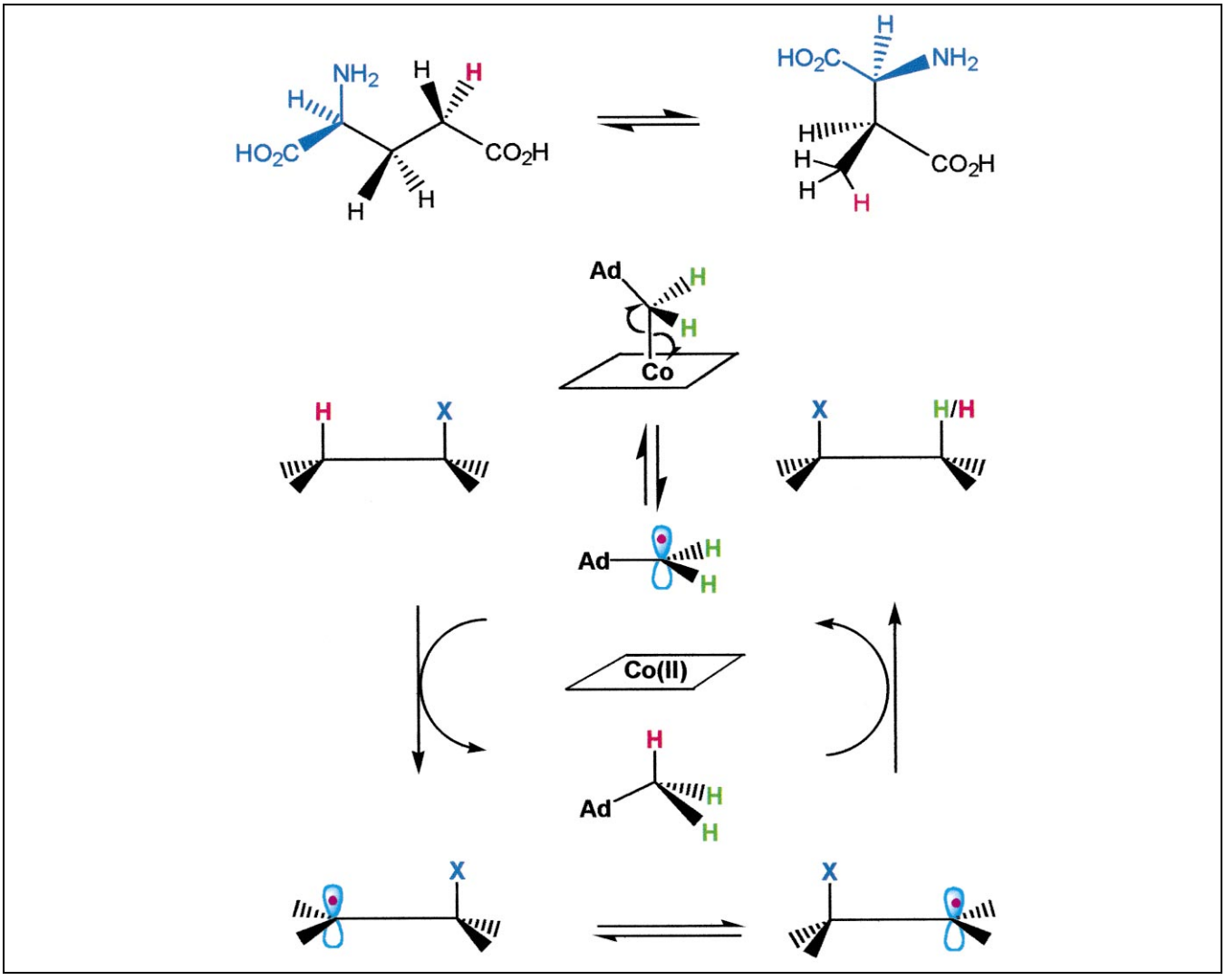

Fig. 1. The rearrangement catalyzed by glutamate mutase and a general mechanistic scheme for the rearrangements catalyzed by AdoCbl-dependent isomerases, where X may by HO–, HN_2– or a carbon-containing fragment.

the only AdoCbl-dependent rearrangement known in which an sp^3-hybridized carbon undergoes migration. Recently, experimental evidence has been obtained that the interconversion of glutamyl and methylaspartyl radicals involves fragmentation of the substrate radical to give glycyl radical and acrylate as intermediates, followed by recombination of these intermediates to yield the product radical [11].

Glutamate mutase comprises two subunits, designated E and S. The E subunit is a dimer of subunit M_r 54 000, whereas the S subunit is a monomer of M_r 14 800; AdoCbl is bound at the interface of the two subunits [12–14]. The recently solved crystal structure of the enzyme reveals that the active site is situated in the lumen of an eight-stranded β-barrel formed by the E subunit. As illustrated in Fig. 2, a number of residues from the E subunit make hydrogen bonds to the substrate: in particular, Glu171 makes a hydrogen bond with the amino group of the substrate [15].

In this study we have examined the role of Glu171 in the reaction. This residue is of interest because it may contribute both to substrate recognition and to catalysis by acting as a general base to deprotonate the amino group of the substrate. Deprotonation of the amino group would be expected to stabilize the intermediate glycyl radical thereby facilitating the rearrangement of substrate and product radicals. The results reported here are consistent with this hypothesis.

2. Results and discussion

The crystal structure of glutamate mutase shows Glu171 to make a hydrogen bond with the amino group of the substrate [15]. To determine whether this interaction is important for catalysis and/or binding, four conservative mutations were introduced into the protein in which Glu171 was replaced by aspartate, glutamine, asparagine

or alanine. These mutations introduce progressively more drastic deletions of functionality into the active site. Thus, the Glu171Gln mutation removes negative charge from the active site, while preserving steric bulk and hydrogen-bonding ability, whereas the Glu171Asp mutation preserves the carboxylate group but removes the charge ~1 Å away from the substrate. The Glu171Asn mutation combines the effects of the first two mutations and finally the Glu171Ala mutation serves to remove functionality entirely from this position.

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 [16]. The mutations were introduced using standard PCR-based methods, and the mutant proteins purified as described previously for the parent GlmES protein [16]. Each of the mutations resulted in stably folded proteins as judged by their circular dichroism spectra (data not shown) and each of the mutants retained a measurable level of activity. These properties of the mutants suggest that the mutations do not significantly alter the global fold of the protein.

2.1. *Steady state kinetic properties of mutant enzymes*

The steady state kinetic properties of the mutant enzymes, with glutamate and AdoCbl as substrate and coenzyme respectively, were measured and are summarized in Table 1. The Glu171Asp mutant is the least impaired, with k_{cat} reduced less than two-fold. This suggests either that the precise positioning of the carboxylate group is not important for catalysis, or that the active site possesses sufficient flexibility for the shorter aspartate side chain to contact the substrate. However, other mutations that remove the carboxylate group significantly decrease k_{cat} by up to 50-fold in the case of the Glu171Gln mutant. The apparent K_ms for glutamate of the mutant proteins are similar to that of the wild-type enzyme. Thus, based on this criterion, the carboxylate of Glu171 does not appear to provide significant binding interactions with the substrate. The apparent K_ms for AdoCbl of the mutant proteins are also very similar to the wild-type enzyme (Table 1). This is not surprising, given that Glu171 is relatively distant from the coenzyme and makes no contacts with it. Overall, the steady state kinetic properties of the mutant proteins are consistent with the carboxylate group of Glu171 being important, but not essential, for catalysis.

The Glu171Gln mutant enzyme was selected for further comparative studies with the wild-type enzyme. This mutant was attractive for further study because it is the most sterically conservative mutation and exhibited a large decrease in catalytic activity.

2.2. *pH dependence of catalysis*

To investigate whether the ionization of Glu171 is important in the mechanism of glutamate mutase the effect of pH on enzyme activity was investigated for both wild-type GlmES and the Glu171Gln mutant. Fig. 3A shows a plot of V_{max} as a function of pH for the wild-type enzyme. The activity titrates with an apparent pK_a of 6.6 ± 0.1. The enzyme is maximally active between pH 7.5 and 8.0. The K_m for glutamate was measured at several pHs between 6.4 and 8.5 and did not vary significantly with pH. In contrast, the activity of the Glu171Gln mutant (Fig. 3B) remains almost invariant over the same pH range. Even at the lowest pH for which the activity of the wild-type enzyme could be measured, the wild-type enzyme was about 10-fold more active than the Glu171Gln mutant.

The steady state kinetic properties of GlmES appear

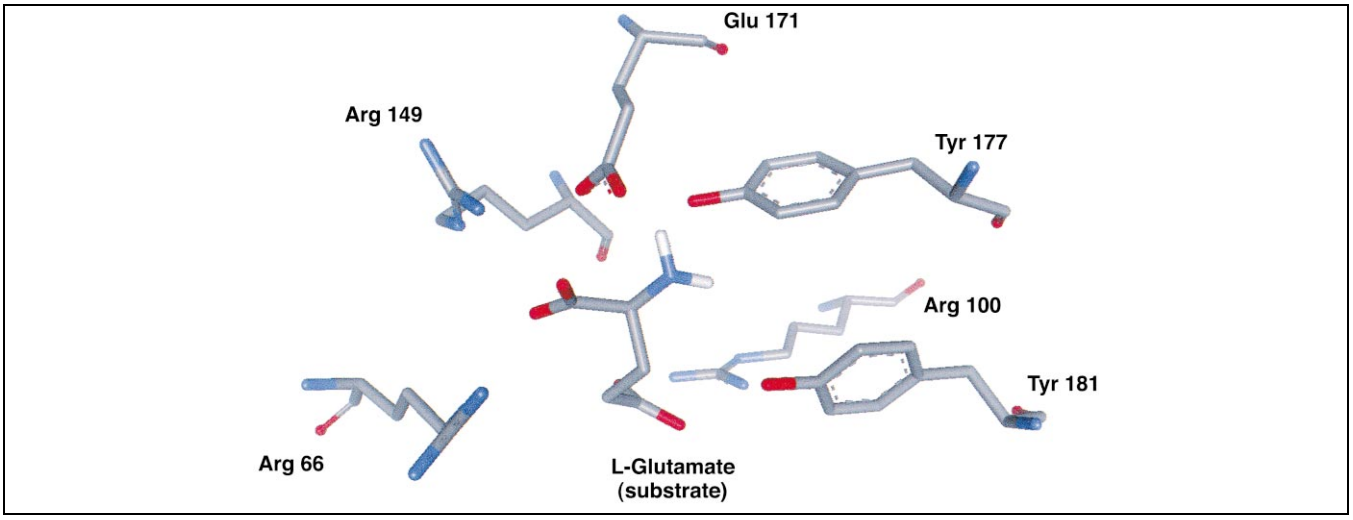

Fig. 2. Active site residues in glutamate mutase that make hydrogen bonds with L-glutamate. Coordinates taken from [15].

Table 1
Kinetic parameters for wild-type and Glu171 mutant glutamate mutase enzymes

Glutamate mutase variant	k_{cat} (s^{-1})	appK_M for L-glutamate (mM)	appK_M for AdoCbl (μM)
Wild-type[a]	5.8 ± 0.3	0.58 ± 0.08	5.5 ± 0.7
Glu171Gln	0.11 ± 0.038	0.24 ± 0.03	2.68 ± 0.35
Glu171Asp	3.19 ± 0.12	0.38 ± 0.08	2.04 ± 0.39
Glu171Ala	0.21 ± 0.09	0.65 ± 0.09	4.47 ± 0.62
Glu171Asn	0.025 ± 0.09	1.07 ± 0.35	7.63 ± 1.6

[a]Data taken from [16].

consistent with the proposed role of Glu171 as a general base involved in deprotonating the amino group of the substrate. The pH dependence of V_{max} indicates that it is the ionization state of the enzyme:substrate complex is kinetically significant. The apparent pK_a of 6.6 measured for the presumed general base is higher than that for the γ-carboxylate of glutamate in free solution. However, this increase in pK_a is consistent with the introduction of the carboxylate into the relatively low dielectric medium of the enzyme active site, and well within the range of pK_as determined for carboxylate groups in the active sites of many other enzymes. The fact that mutation of Glu171 to the non-ionizable glutamine side chain abolishes the pH dependence of enzyme activity strongly suggests that it is this residue that is being titrated on the enzyme.

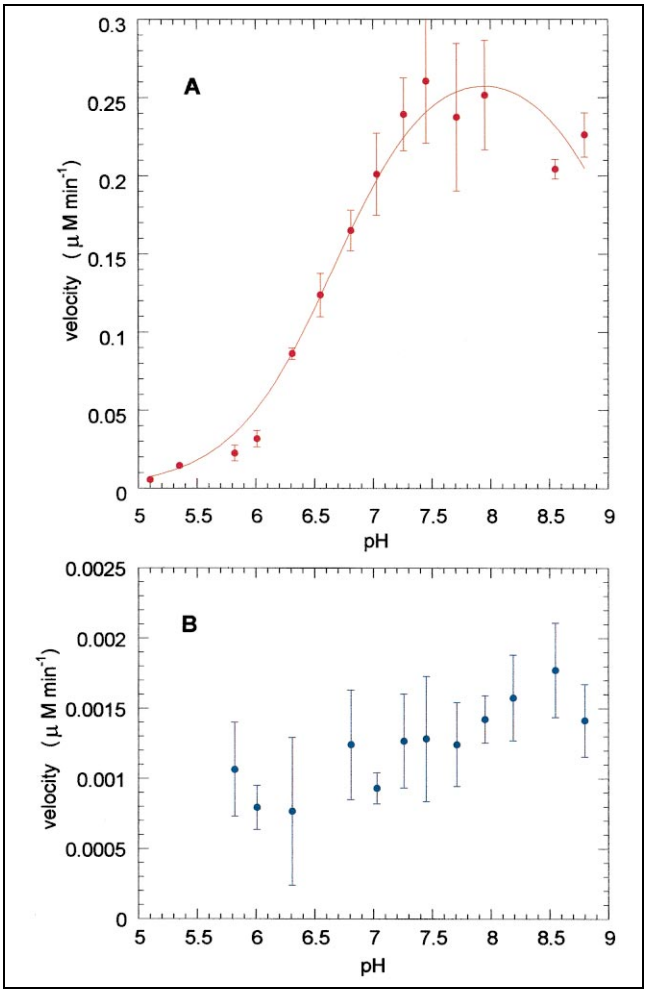

Fig. 3. Variation of glutamate mutase activity under V_{max} conditions with pH. A: Wild-type enzyme. B: Glu171Gln mutant enzyme. Error bars represent the standard deviation associated with at least three measurements for each pH.

2.3. Kinetic isotope effects

To investigate further which step or steps in the mechanism are slowed by the mutation of Glu171, the steady state deuterium kinetic isotope effects on V_{max} were measured for wild-type GlmES and the Glu171Gln mutant enzyme. Measurements were made with at pH 7.3, where the wild-type GlmES shows maximal activity, with [4-d_2]-L-glutamate as the substrate. For wild-type GlmES, $^{D}V = 4.6 \pm 0.06$, whereas for the Glu171Gln mutant $^{D}V = 2.1 \pm 0.2$. The suppression of the isotope effect in the mutant enzyme suggests that, relative to the overall reaction, the steps in the mechanism involving hydrogen transfer have become less rate-determining. This would be consistent with the rearrangement of glutamyl radical to methylaspartyl radical having been slowed by the mutation of Glu171.

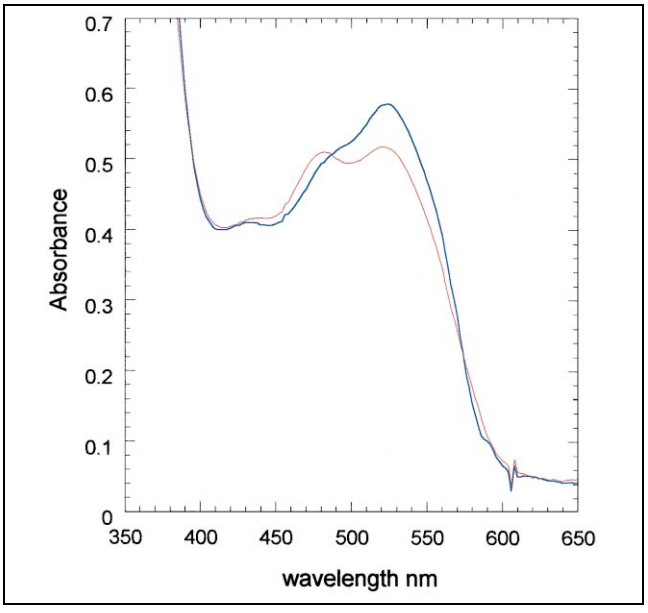

Fig. 4. UV-visible spectral changes indicative of cob(II)alamin formation observed when the glutamate mutase Glu171Gln mutant holoenzyme was incubated with 10 mM L-glutamate. Blue trace: spectrum before addition of substrate; red trace: spectrum after addition of substrate.

2.4. UV-visible spectrum of Glu171Gln mutant

The UV-visible spectrum of AdoCbl changes significantly upon homolysis of the Co–C bond to form Cbl(II). These spectral changes provide a sensitive and convenient method to assess the steady state concentration of free radical species formed on the enzyme since for each molecule of Cbl(II) formed one organic radical is generated. The changes in the UV-visible of the Glu171Gln mutant holoenzyme upon addition of a saturating concentration of L-glutamate are shown in Fig. 4. As has previously been observed with wild-type enzyme, addition of substrate results in a decrease in absorbance at 530 nm and an increase in absorbance at 470 nm characteristic of the formation of Cbl(II). From the decrease in absorbance at 530 nm, we calculate that about 30% of the enzyme active sites contain Cbl(II) during steady state turn-over. This is very similar to the steady state concentration of free radicals measured previously for the wild-type enzyme [8,17]. We conclude, therefore, that the mutation of Glu171 to glutamine does not significantly change the overall steady state concentration of free radical species within the enzyme active site.

2.5. Conclusions

The results described above are consistent with the hypothesis that Glu171 acts as a general base to aid in the deprotonation of the amino group of the substrate, as illustrated in Fig. 5. Thus, the Glu171Asp mutant, which retains the ability to act as a general base, is minimally impaired, whereas the Glu171Gln is 50-fold less active than the wild-type enzyme. It is evident that none of the mutants are completely inactive and thus the residue is not essential for catalysis. The pK_a of the ammonium group of glutamate is only about 9, so that at pH values where the enzyme is maximally active there will be low but significant concentrations of unprotonated glutamate molecules in free solution. The mutant enzymes could potentially selectively bind this sub-population of substrate molecules, but since the activity of the Glu171Gln mutant is pH-independent this seems unlikely. Alternatively, a solvent molecule or other protein residue may substitute as a general base in the active site.

Electron paramagnetic resonance measurements on glutamate mutase have established that the C-4 radical of glutamate is the major organic radical that accumulates during turn-over [17]. This is consistent with recent computational studies that indicate that this radical is significantly more stable than either 5′-deoxyadenosyl radical or methylaspartyl radical [18]. Given that Glu171 is remote from C-4 of the substrate it is reasonable that mutations at this position should have little effect on the enzyme's ability to initiate homolysis and generate the glutamyl radical. Computational studies indicate that the protonation state of both the amino group and the α-carboxyl group strongly influences the stability of the intermediate glycyl radical, and thus the feasibility of the rearrangement reaction [18]. In particular, the neutral glycyl radical (carboxylate protonated, amino group unprotonated) was found to be the most stable form and protonation of the amino group destabilized this radical by 21 kcal mol^{-1}, whereas our studies find that catalysis is only modestly impaired by deletion of the carboxylate functionality (corresponding to an increase in activation energy of only 2–3 kcal mol^{-1}). However, it should be noted that the calculations were performed for a gas phase reaction, and are likely to overestimate the energetic penalty for protonation of the glycyl radical in solution. By the same token, it is possible that other residues or solvent molecules may take over the role of general base in the mutated enzyme, thereby compensating for the loss of Glu171, thus the mutagenesis experiments probably underestimate the importance of protonation state.

Finally, we note that computational studies on both diol dehydrase and methylmalonyl-CoA mutase have also highlighted the importance of the protonation state of the substrate in facilitating the rearrangements catalyzed by these enzymes [19,20]. In methylmalonyl-CoA mutase, mutagenesis of His244, which provides a hydrogen bond to the carbonyl of the migrating acyl moiety, have demonstrated that this residue plays an

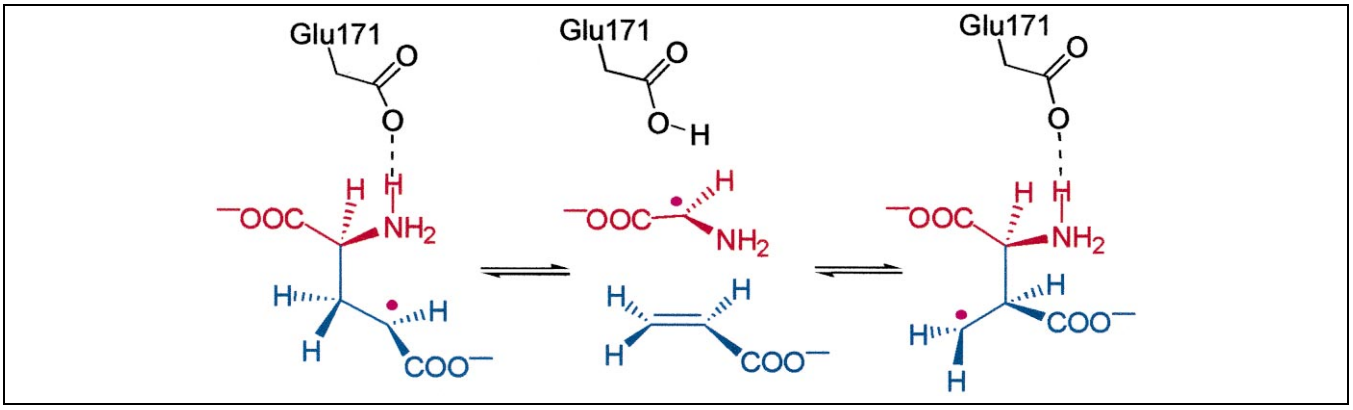

Fig. 5. Proposed mechanism for the rearrangement of glutamyl and methylaspartyl radicals catalyzed by Glu171.

important role in catalysis [21,22]. The protonation and deprotonation of substrate functional groups adjacent to the site of carbon-centered radicals appears to be emerging as an important and general mechanism by which B_{12} enzymes modulate the reactivity of radical intermediates.

3. Significance

The mechanisms by which enzymes stabilize highly reactive free radical species are poorly understood. For example, it is estimated that organic radical species formed in AdoCbl-dependent reactions are 25–30 kcal mol^{-1} more stable on the enzyme than in free solution, a remarkable feat. The solution of the crystal structure of glutamate mutase has allowed us to begin to investigate how individual active site residues contribute to catalysis. The mutagenesis and kinetic studies that we present here demonstrate how an enzyme may stabilize (or indeed destabilize) an organic radical by controlling the protonation state of a neighboring group on the substrate. The properties of the mutant enzymes are in accord with mechanistic expectations and with predictions based on chemical intuition and high-level computational studies. These studies provide further support for the idea that enzymes can significantly alter the reactivity of free radical intermediates through the protonation or deprotonation of functional groups on the substrate.

4. Materials and methods

4.1. Materials

Oligonucleotide synthesis and DNA sequencing were performed by the Biomedical Research Core facilities (University of Michigan). *Vent* polymerase was obtained from New England Biolabs (Beverly, MA, USA). *Dpn*I and other restriction enzymes were obtained from New England Biolabs, Promega (Madison, WI, USA) or Boehringer Mannheim (Indianapolis, IN, USA). *Escherichia coli* XL Blue1 supercompetent cells were obtained from Stratagene (La Jolla, CA, USA). 1,3-bis[Tris(hydroxymethyl) methylamino] propane (BTP) was obtained from Sigma (St. Louis, MO, USA). D,L-[2,4,4-d_3]-Glutamic acid was obtained from Cambridge Isotope Laboratories (Andover, MA, USA). The purification of recombinant glutamate mutase fusion protein and the sources of all other materials have been described previously [16].

4.2. Construction of mutant proteins

Mutations were introduced into the *GlmES* gene using the Quickchange Protocol (Stratagene, La Jolla, CA, USA) according to the manufacturer's instructions except that *Vent* polymerase was used in place of *Pfu* polymerase. Complementary primers were designed incorporating the desired mutation (see Table 2). Where possible, a silent mutation that introduced a new restriction site into the plasmid was also incorporated into the oligonucleotides to aid in the identification of mutant plasmids.

Table 2
Oligonucleotides used for introducing mutations in recombinant glutamate mutase

Mutation	Primers 5′→3′
Glu171Gln	GGTGGATGGACTTCAAACCAAGGAGGAGGTATCTCC
	GGAGATACCTCCTCCTTGGTTTGAAGTCCATCCACC
Glu171Ala	GGATGGACTTCAAATGCTGGAGGAGGTATCTCC
	GGAGATACCTCCTCCAGCATTTGAAGTCCATCC
Glu171Asp	GATGGACTTCAAATGACGGAGGAGGTATCTCC
	GGAGATACCTCCTCCGTCATTTGAAGTCCATC
Glu171Asn	GGTGGATGGACTTCAAACAATGGAGGAGGTATCTCCTAC
	GTAGGAGATACCTCCTCCATTGTTTGAAGTCCATCCACC

PCR reactions included the mutagenic primers (1 pmol of each), the template plasmid, pglmES [16] (62 ng), 1 U of high-fidelity *Vent* polymerase, 250 μM of each dNTP, 3 mM $MgCl_2$ in 20 μl of 'thermo pol' buffer supplied by the manufacturer. The following temperature cycle was followed: 95°C for 1 min, 45°C for 1 min, 72°C for 5 min and the cycle was performed 15 times. The PCR mixture was treated with 6 U of *Dpn*I for 1 h to digest the parental DNA strands. The reaction mixture containing PCR product and *Dpn*I was then used to transform *E. coli* XL Blue1 supercompetent cells. Plasmid DNA from the transformants was isolated and initial screening was performed by restriction analysis. Mutations were confirmed by DNA sequencing.

4.3. Kinetic analysis

Steady state kinetic analyses of wild-type and mutant enzymes to determine apparent K_Ms for L-glutamate and AdoCbl were performed at 23°C using the spectrophotometric assay described by Barker [23], 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).

4.4. pH dependence of enzyme activity

Lyophilized protein was dissolved in 100 mM BTP buffer pH 7.0. This buffer was chosen because this has two pK_as at 6.8 and 9.0 that allowed activity measurements to be made over the pH range 5.1–8.8 without the need to change buffers. Assays were performed in 50 mM BTP buffered to the appropriate pH and containing 1 mM $MgCl_2$ and 10 mM KCl. For assays under V_{max} conditions, the final concentrations of substrate and coenzyme were 10 mM L-glutamate, 25 μM AdoCbl. To insure that the assay remained linear across the pH range studied, the pH dependence of the activity of methylaspartase, used as a coupling enzyme in the assay, was investigated. At all pH values studied, the coupling enzyme was found to be at least 100-fold more active than glutamate mutase, and the assay remained linear.

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