Mechanism of Glutamate Mutase: Identification and Kinetic Competence of Acrylate and Glycyl Radical as Intermediates in the Rearrangement of Glutamate to Methylaspartate

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Glutamate mutase is one of a group of adenosylcobalamin (AdoCbl, coenzyme B_{12}) dependent enzymes that catalyze unusual carbon skeleton isomerizations. These rearrangements formally involve a 1,2 hydrogen atom migration and proceed through a mechanism involving carbon-based free radical intermediates.[1−5] The initial steps of these reactions involve homolysis of the reactive cobalt−carbon bond of the coenzyme to form cob(II)-alamin and 5′-deoxyadenosyl radical. The adenosyl radical then abstracts the migrating hydrogen from the substrate to form 5-deoxyadenosine and substrate radical. Studies on several B_{12} enzymes have demonstrated the formation of Cbl(II), 5-deoxyadenosine, and substrate radicals as intermediates.[6−10] Furthermore, we have previously investigated the kinetics of B_{12} homolysis in glutamate mutase and shown that homolysis and hydrogen abstraction are kinetically coupled,[11] a phenomenon that has been observed for other AdoCbl-dependent enzymes.[12,13]

In contrast, the mechanism by which substrate radical intermediates rearrange to product radicals is far less well understood. In the rearrangements catalyzed by methylmalonyl-CoA mutase[14] and isobutyryl-CoA mutase,[15] the migrating carbon is the thioacyl carbon of the thioester with coenzyme A, whereas the rearrangement catalyzed by 2-methyleneglutarate mutase[16] involves the migration of a vinylic carbon. In these cases the migrating carbon is sp^2 hybridized, and this provides a low energy pathway for the radical rearrangement to occur through an associative mechanism involving a cyclopropyl intermediate.[17,18] However, the carbon skeleton rearrangement catalyzed by glutamate mutase is unique in that the migrating carbon is sp^3 hybridized. A cyclopropyl intermediate cannot form in this case and, significantly, model studies in free solution have failed to demonstrate 1,2-migrations of sp^3 carbon atoms under radical conditions,[19,20] although the identification of products arising through fragmentation of radical intermediates was not specifically addressed.

Various mechanisms have been proposed[3,21] to explain the glutamate mutase-catalyzed rearrangement of glutamyl radical to methylaspartyl radical, although very little experimental evidence has accumulated to substantiate any of them. To address this long-standing mechanistic problem, we have used rapid quench techniques, followed by HPLC analysis, to test the hypothesis that the rearrangement occurs through a dissociative mechanism, as shown in Figure 1. In this mechanism, glutamyl radical undergoes fragmentation to yield glycyl radical and acrylate as intermediates, followed by addition of glycyl radical to the other end of the acrylate double bond to form methylaspartyl radical.[3]

We have demonstrated that ^{14}C-labeled glutamate is converted into glycyl radical (trapped as glycine) and acrylate during the course of the glutamate mutase-catalyzed reaction in a kinetically competent manner. Furthermore, it appears that fragmentation of of the C-4 glutamyl radical occurs spontaneously once released from the enzyme active site.

The engineered single subunit glutamate mutase protein, GlmES, was used in these experiments; the purification and steady state kinetic properties of the enzyme have been described previously.[22] Rapid quench flow experiments were performed at 10 °C using a HiTech RQF-63 apparatus. Eighty microliters of a solution containing 200 μM glutamate mutase and 240 μM AdoCbl in 50 mM potassium phosphate buffer, pH 7.0, was rapidly mixed with an equal volume of 2 mM uniformly ^{14}C-labeled L-glutamate (specific activity 5800 dpm/nmol). After various times reactions were quenched with 5% trifluoroacetic acid (TFA) and either glycine (0.4 mM final concentration) or acrylate (0.08 mM final concentration) as carrier. Samples were treated with charcoal to remove AdoCbl. Acrylate was recovered by HPLC on a reverse-phase C_{18} column equilibrated in 0.1% TFA and eluted with an ascending gradient of acetonitrile. To recover glycine, amino acids were derivatized with dansyl chloride and separated by reverse-phase HPLC using protocols described previously.[23]

Radioactivity from ^{14}C-labeled L-glutamate could be detected in both carrier glycine and acrylate when the enzyme reaction was quenched with TFA after 400 ms, by which time the reaction had reached steady state.[24] 0.058 ± 0.003 mol of glycine and 0.062 ± 0.003 mol of acrylate were recovered per mol of enzyme. As expected, glycine and acrylate were formed in a 1:1 ratio; however, the total amounts of glycine and acrylate formed were surprising large, ∼6% of enzyme active sites, indicating that during steady-state turnover glycyl radical comprised about one-quarter of all of the free radical species present on the enzyme at steady state. This result appeared to contradict earlier EPR studies which demonstrated that the C-4 radical of glutamate is the major organic radical that accumulates on the enzyme[6] and which failed to find substantive evidence for the C-2 radical of the putative glycyl intermediate.

We therefore considered the possibility that after denaturation of the protein by acid, the glutamyl radical, liberated from the active site, might *spontaneously* fragment to form acrylate and glycyl radical before being quenched by solvent or abstraction

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(1) Marsh, E. N. G. *Bioorg. Chem.* **2000**, *28*, 176−189.
(2) Banerjee, R. *Chem. Biol.* **1997**, *4*, 175−186.
(3) Buckel, W.; Golding, B. T. *Chem. Soc. Rev.* **1996**, 329−337.
(4) Marsh, E. N. G. *Bioessays* **1995**, *17*, 431−441.
(5) Halpern, J. *Science* **1985**, *227*, 869−875.
(6) Bothe, H.; Darley, D. J.; Albracht, S. P.; Gerfen, G. J.; Golding, B. T.; Buckel, W. *Biochemistry* **1998**, *37*, 4105−4113.
(7) Licht, S.; Gerfen, G. J.; Stubbe, J. *Science* **1996**, *271*, 477−481.
(8) Zhao, Y.; Abend, A.; Kunz, M.; Such, P.; Retey, J. *Eur. J. Biochem.* **1994**, *225*, 891−6.
(9) Keep, N. H.; Smith, G. A.; Evans, M. C.; Diakun, G. P.; Leadlay, P. F. *Biochem. J.* **1993**, *295*, 387−392.
(10) Tan, S. L.; Kopczynski, M. G.; Bachovchin, W. W.; Orme-Johnson, W. H.; Babior, B. M. *J. Biol. Chem.* **1986**, *261*, 3483−3485.
(11) Marsh, E. N. G.; Ballou, D. P. *Biochemistry* **1998**, *37*, 11864−11872.
(12) Licht, S. S.; Booker, S.; Stubbe, J. *Biochemistry* **1999**, *38*, 1221−1233.
(13) Padmakumar, R.; Banerjee, R. *Biochemistry* **1997**, *36*, 3713−3718.
(14) Thoma, N. H.; Meier, T. W.; Evans, P. R.; Leadlay, P. F. *Biochemistry* **1998**, *37*, 14386-14393.
(15) Moore, B. S.; Eisenberg, R.; Weber, C.; Bridges, A.; Nanz, D.; Robinson, J. A. *J. Am. Chem. Soc.* **1995**, *117*, 11285−11291.
(16) Beatrix, B.; Zelder, O.; Linder, D.; Buckel, W. *Eur. J. Biochem.* **1994**, *221*, 101−109.
(17) Ashwell, S.; Davies, A. G.; Golding, B. T.; Haymotherwell, R.; Mwesigyekibende, S. *J. Chem. Soc., Chem. Commun.* **1989**, 1483−1485.
(18) Wollowitz, S.; Halpern, J. *J. Am. Chem. Soc.* **1984**, *106*, 8319−8321.
(19) Dowd, P.; Choi, S.-C.; Duah, F.; Kaufman, C. *Tetrahedron* **1988**, *44*, 2137−2148.
(20) Murakami, Y.; Hisaeda, Y.; Song, X.-M.; Ohno, T. *J. Chem. Soc., Perkin Trans. 2* **1992**, 1527−1528.
(21) Golding, B. T.; Rao, D. N. R. *Adenosylcobalamin-dependent enzymic reactions*; Page, M. L., Williams, A., Eds.; Royal Society of Chemistry: London, 1986; pp 404−428.
(22) Chen, H.-P.; Marsh, E. N. G. *Biochemistry* **1997**, *36*, 14939−14945.
(23) Chih, H.-W.; Marsh, E. N. G. *Biochemistry* **1999**, *38*, 13684−13691.
(24) Control experiments in which either the protein or coenzyme was omitted, and the reaction mixture quenched and subjected to HPLC, established that the formation of acrylate and glycine required active holo-enzyme.

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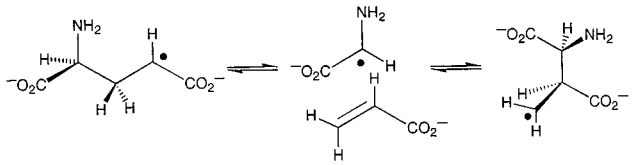

Figure 1. Proposed mechanism for the rearrangement of glutamyl radical to methylaspartyl radical catalyzed by glutamate mutase.

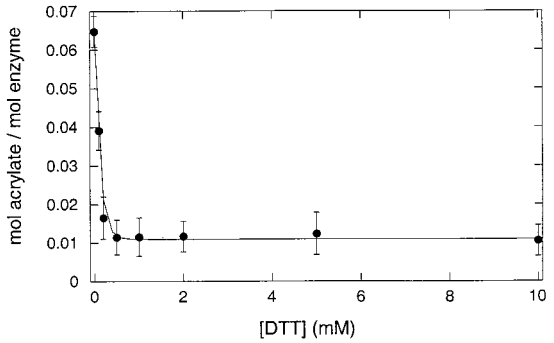

Figure 2. Effect of increasing the concentration of dithiothreitol in the quenching solution on the amount of acrylate recovered from the enzyme.

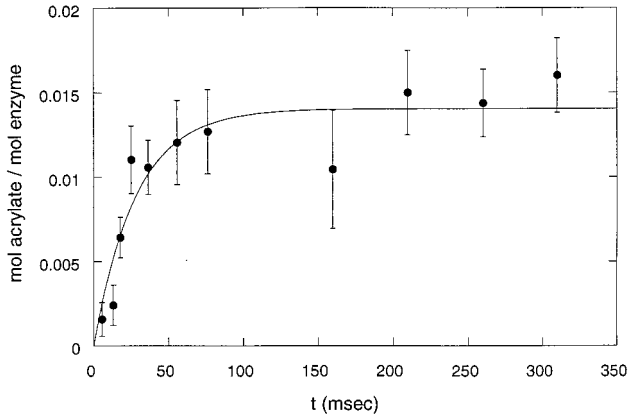

Figure 3. Kinetics of acrylate formation: 20 mM DTT was included with the quenching solution to ensure rapid reduction of radical species.

of hydrogen from the protein. To test this hypothesis we examined the effect of including increasing concentrations of dithiothreitol in the quench solution as this thiol is an efficient reducing agent for organic radicals.[25] Figure 2 shows the effect of DTT concentration on the amount of acrylate produced when the enzyme reaction was quenched with acid. Consistent with DTT reacting with an intermediate that is formed *before* acrylate, the amount of acrylate *decreases* with increasing DTT concentration. The limiting concentration of acrylate is about 1% of enzyme active sites, and this may be taken as an upper estimate of the steady state concentration of acrylate and glycyl radical during turnover. Although this represents a very small fraction of enzyme active sites it is certainly large enough to be mechanistically significant and lies well within the sensitivity range of our experiments.

Figure 3 describes the kinetics of acrylate formation in experiments in which the holo-enzyme was rapidly mixed with ^{14}C-labeled L-glutamate and the reaction subsequently quenched with 5% TFA containing 20 mM DTT. The observed rate constant for acrylate formation is 35 ± 8 s^{-1}, which is significantly faster than $k_{cat} = 5.6$ s^{-1},[22] but slower than the rate of 5′-deoxyadenosine formation, $k_{obs} = 73 \pm 8$ s^{-1}, measured previously.[23] Thus, the kinetic data are consistent with acrylate being a kinetically competent intermediate that is formed after 5′-deoxyadenosine and glutamyl radical, which is an important requirement of the fragmentation−recombination mechanism. (We were unable to investigate the kinetics of glycyl radical formation in the presence of DTT as it interfered with the derivatization and HPLC analysis of the amino acids.)

Given that fragmentation of the glutamyl radical appears to occur spontaneously, the question arises as to whether the residual glycine and acrylate that are still formed, even with high concentrations of DTT in the quench solution, might also be formed by a nonenzymatic side reaction. We cannot rigorously exclude this possibility, but we consider it unlikely. First, there is no obvious mechanism whereby a sub-population of glutamyl radical molecules would be protected from reaction with DTT and undergo fragmentation, unless they were tightly associated with the protein, which is tantamount to a protein-meditated fragmentation. Second, the requirement for the reaction to be kinetically competent, i.e., k_{obs} for acrylate formation must be greater than 5.6 s^{-1} and less than 73 s^{-1}, imposes a further quite restrictive constraint. Thus, for the observed rate of acrylate formation (35 s^{-1}) to be both kinetically competent and due to a nonenzymatic reaction is an unlikely coincidence.

It is unclear what species quenches the presumed glycyl radical in the absence of DTT, but even without added reducing agent the environment into which free radicals are released is strongly reducing. The protein is present in high concentration (100 μM) in these experiments and contains numerous side chains that could potentially reduce organic radicals, including cysteine and tyrosine residues. Cbl(II) could also act to reduce the glycyl radical, followed by protonation by the solvent to form glycine.

The observation that glutamyl radical, once liberated from the enzyme, appears to undergo spontaneous fragmentation to glycyl radical and acrylate was unexpected but is chemically quite reasonable. Fragmentation is entropically favorable and the C-2 radical of glycine is expected to be stabilized by delocalization of the radical onto the carboxyl group and the amino nitrogen; indeed several examples of enzymes that form protein-based glycyl radicals are known[26,27] (although in these cases glycine is incorporated into the pepide backbone and therefore the chemical stability of these radicals may be somewhat different). This explains why attempts to demonstrate the radical rearrangement in model systems have only been partially successful, and require the presence of lipid micelles where, presumably, diffusion of intermediates is restricted.[20]

An important function of the enzyme must therefore be to prevent diffusion of the glycyl radical and acrylate out of the active site and maintain these intermediates in the correct relative orientations so that recombination of these fragments can occur and the reaction can proceed to form the rearranged product. In fact, these results and the EPR studies of Buckel and co-workers[6] point to the equilibrium between fragmentation and recombination and favoring formation of glutamyl radical on the enzyme. The recently solved crystal structure of glutamate mutase[28] shows a substrate analogue, tartarate, to make extensive hydrogen-bonding interactions with a number of active site residues. This supports the idea that the protein controls the rearrangement of substrate radicals by binding the substrate in the correct conformation for fragmentation and recombination to occur.

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(25) Goshe, M. B.; Anderson, V. E. *Radiat. Res.* **1999**, *151*, 50−58.

(26) Becker, A.; Fritz-Wolf, K.; Kabsch, W.; Knappe, J.; Schultz, S.; Wagner, A. F. V. *Nature Struct. Biol.* **1999**, *6*, 969−975.
(27) Logan, D. T.; Andersson, J.; Sjoberg, B. M.; Nordlund, P. *Science* **1999**, *283*, 1499−1504.
(28) Reitzer, R.; Gruber, K.; Jogl, G.; Wagner, U. G.; Bothe, H.; Buckel, W.; Kratky, C. *Structure* **1999**, *7*, 891−902.