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Toward an Improved Understanding of the Glutamate Mutase System

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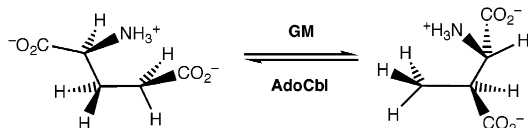
Abstract: High-level quantum chemistry calculations have been used to examine the catalytic reactions of adenosylcobalamin-dependent glutamate mutase (GM) with the natural substrate (*S*)-glutamic acid. We have also examined the rearrangement of (*S*)-2-hydroxyglutaric acid, (*S*)-2-thiolglutaric acid, and 2-keto-glutaric acid, all of which have previously been shown to react as substrates or inhibitors of the enzyme. Our calculations support the notion that the 100-fold difference in k_{cat} between glutamate and 2-hydroxy-glutarate is associated with the relatively high energy of the glycolyl radical intermediate compared with the glycyl radical. More generally, calculations of radical stabilization energies for a variety of substituted glycyl radical analogues indicate that modifications at the radical center can profoundly affect the relative stability of the resulting radical, leading to important mechanistic consequences. We find that the formation of a thioglycolyl radical, derived from (*S*)-2-thiolglutaric acid, is highly dependent on the protonation state of sulfur. The neutral radical is found to be of stability similar to that of the glycolyl radical, whereas the S^- form of the thioglycolyl radical is much more stable, thus providing a rationalization for the inhibition of the enzyme by the substrate analogue 2-thiolglutarate. Two possible rearrangement pathways have been examined for the reaction of GM with 2-ketoglutaric acid, for which previous experiments had suggested no rearrangement took place. The fragmentation−recombination pathway is associated with a fragmentation step that is very endothermic (by 102.2 kJ mol^{-1}). In contrast, the addition−elimination pathway has significantly lower energy requirements. An alternative possibility, namely, that 2-ketoglutaric acid is bound in its hydrated form, 2,2-dihydroxyglutaric acid, also leads to a pathway with relatively low energy requirements, suggesting that some rearrangement might be expected under such circumstances.

1. Introduction

Glutamate mutase (GM) is an adenosylcobalamin (AdoCbl)-dependent enzyme that catalyzes the reversible isomerization of (*S*)-glutamate to (2*S*,3*S*)-3-methylaspartate:[1]



It is used by many anaerobic bacteria in the first step in the metabolic pathway to ferment (*S*)-glutamate as a carbon and energy source.[2] More recently, GM activity has been measured in the biosynthetic pathways of various peptidyl antibiotics.[3]

The general pattern for the mechanism of GM-catalyzed reactions is similar to that for other AdoCbl-dependent enzymes; they all operate via radical intermediates whereby the AdoCbl cofactor mediates vital chemistry.[4,5] The generally accepted mechanism for these enzymes begins with an initial hydrogen abstraction from the substrate **1** by 5′-deoxyadenosyl radical (Ado•), generated via homolytic cleavage of the Co−C bond of AdoCbl, to form a substrate-derived radical **2** plus 5′-deoxyadenosine (step A, Scheme 1). The substrate-derived radical **2** then rearranges to a product-related radical **3** (step B), which then reabstracts hydrogen from 5′-deoxyadenosine (Ado−H) to regenerate Ado• and form the closed-shell product **4** (step C). In some cases, enzyme-catalyzed elimination then occurs to give the final product (not shown).[5]

While the principal features of this mechanism are common to many AdoCbl-dependent systems, each enzyme possesses

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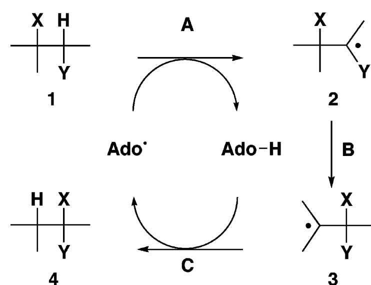
Scheme 1. Minimal Mechanism for the Reactions Catalyzed by AdoCbl-Dependent Isomerases[a]



[a] **X** = $CH(CO_2^-)NH_3^+$ and **Y** = CO_2^- for the glutamate mutase reaction.

unique characteristics[1c,d,4−6] that lead to significant mechanistic differences in how the rearrangement of the substrate radical to the product radical is catalyzed (Scheme 1, step B).[7] A common aspect is the importance of partial proton transfer (either protonation or deprotonation) through hydrogen bonding with the enzyme.[7] On the other hand, a differentiating feature among the AdoCbl-dependent enzymes is the finding that certain classes are easily susceptible to suicide inactivation by suitable substrate analogues while others are not. For example, the class II eliminases diol dehydratase and ethanolamine ammonia-lyase[5] have been shown to be fairly promiscuous in their substrate selectivity, and this has resulted in various degrees of complete and irreversible inactivation.[8] In contrast, there is a much lower susceptibility to inactivation for the class I mutases such as glutamate mutase.

We have recently examined the basis for the suicide inactivation in a number of eliminases and found that it largely results from the formation of a very stable radical intermediate that prevents the hydrogen reabstraction reaction (Scheme 1, step C) from taking place.[9] Under such circumstances, 5′-deoxyadenosyl radical is not regenerated, and the catalytic cycle cannot continue.

For the mutases, the majority of research to date on inactivation has focused on analogues relevant to the GM-catalyzed reaction,[10−13] with fewer studies directed toward methylmalonyl-CoA mutase.[14] On the whole, these experiments imply that only very conservatively modified substrate analogues can bind in the active sites of the mutase family of AdoCbl-dependent enzymes.

In this connection, it is especially interesting that reactivating factors have been identified for the class II eliminases diol dehydratase,[15] ethanolamine ammonia-lyase,[16] and glycerol dehydratase.[17] Inactivation of the holoenzymes involves irreversible cleavage of the Co−C bond of AdoCbl (as might occur, for example, following the formation of a very stable radical intermediate[9]), but rapid and continuous reactivation has been observed in the presence of adenosine triphosphate (ATP), Mg^{2+}, and free AdoCbl. The mechanisms of reactivation have been attributed to chaperone-like reactivating factors operating via a two-step ATP-dependent cobalamin exchange.[16,18] The first step is proposed to involve an ADP-dependent cob(II)alamin release from the inactivated holoenzyme to form a complex between the apoenzyme and the reactivating factor. The second step is believed to require an ATP-dependent dissociation of this complex to liberate free apoenzyme, thereby allowing it to associate with free AdoCbl for renewed catalysis.

It is not presently known whether such reactivating factors are common for all AdoCbl-dependent enzymes. Banerjee and co-workers have investigated the function of a protein (MeaB) that binds methylmalonyl-CoA mutase in a GTP-dependent manner[19] and proposed that its function is to transfer AdoCbl from an additional auxiliary protein (adenosyltransferase) to methylmalonyl-CoA mutase. Also, Zelder et al. have noted a gene in the operon for clostridial glutamate mutase (*mutL/glmL*) that has homology to an ATP-binding protein,[20] and Mori et al. have speculated that this gene may code for a reactivating factor.[16]

It is clear that further research in this area is both essential and exciting. Therefore, in an attempt to broaden our understanding of the GM system, we have carried out high-level quantum chemistry calculations on small model systems to examine catalytic and inhibitory reactions relevant to GM. In a manner similar to that of our recent work on the suicide inactivation of the coenzyme B_{12}-dependent enzymes ethanolamine ammonia-lyase and diol dehydratase,[9] we begin by comparing and contrasting the energy requirements available to GM within the context of the catalytic mechanism involving (*S*)-glutamic acid as the substrate (section 3.1). Armed with this knowledge, we then examine the energy profile for the only other known true substrate for GM, namely, (*S*)-2-hydroxyglutaric acid (section 3.2). Section 3.3 examines the use of radical stabilization energies (RSEs) to provide a measure of the relative stabilities of a selection of substituted glycyl radicals relevant to the fragmentation−recombination step in the reaction catalyzed by GM. The utility of this approach is illustrated with an examination of the recently discovered inhibitor of GM, (*S*)-

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2-thiolglutaric acid (section 3.4). The paper concludes with an analysis of the energy profiles for the substrate analogues 2-ketoglutaric acid (section 3.5.1) and its hydrate 2,2-dihydroxyglutaric acid (section 3.5.2).

2. Theoretical Methodology

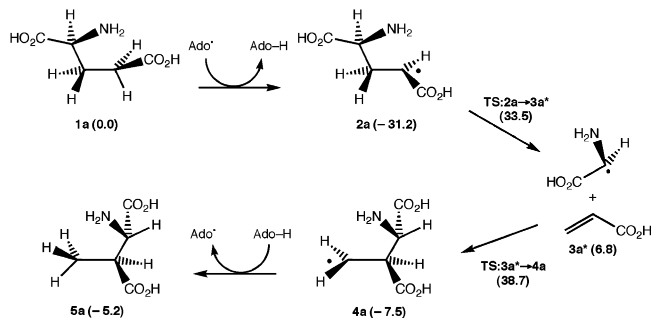
Standard ab initio[21] and density functional theory[22] calculations were performed with the MOLPRO 2002.6[23] and Gaussian 03[24] programs. Geometries and scaled (by 0.9806)[25] vibrational frequencies have been obtained at the B3-LYP/6-31G(d,p) level. Relative energies were obtained with the high-level composite method G3(MP2)-RAD.[26] This method approximates the URCCSD(T)/G3MP2Large level of theory by performing a series of single-point energy calculations, including URCCSD(T)/6-31G(d), RMP2/6-31G(d), and RMP2/G3MP2Large, on the B3-LYP/6-31G(d,p) geometries. A higher level correction, optimized to accurately reproduce experimental thermochemical data,[26b] is also included in the final G3(MP2)-RAD expression. All energies in this paper refer to isolated molecules in the gas phase at 0 K.

The neutral forms of substrates, analogues, products, and intermediates were chosen instead of their zwitterionic forms. The motivation for this choice is based on a previous computational study on the rearrangement mechanism of GM, which identified the fragmentation−recombination barrier to be highly dependent on the protonation state of the substrate.[7] Results that remained consistent with experiment involved (partially) deprotonating the NH_3^+ group and (partially) protonating the COO^- groups. Such action is compatible with the known architecture of the active site of glutamate mutase;[27] key residues include Arg149 and Arg66, which hydrogen bond to the α-carboxyl group of the substrate, Arg100, which interacts with the γ-carboxyl group of the substrate, and Glu171, which has been shown to act as a general base serving to deprotonate the NH_3^+ group during catalysis.[28] On these grounds, we have adopted neutral forms for the substrate, intermediates, and products to simulate more accurately the electrostatic environment of glutamate mutase.

The conformers chosen for the present study are based on a carbon-backbone arrangement similar to that found in the crystal structure,[27b] rather than necessarily the lowest energy conformers in the gas phase. It was found that the lowest energy conformers of the species related to the reactants and products did not always correspond to the lowest energy transition structures (TSs) for fragmentation of the substrate-derived radicals, sometimes by a significant margin (up to 25 kJ mol^{-1}). Indeed, in most cases it was the crystal-structure-like arrangements for the TS that were found to be associated with the lowest barrier. Because the energy differences between the lowest energy and crystal-structure-like conformers for the species related to the *reactants* and *products* are relatively small (up to ca. 5 kJ mol^{-1}), we chose the crystal-structure-like conformers in all our calculations in an attempt to model more accurately the kinetics that the enzyme may enforce.

Energies relative to the reactants were obtained for each reaction scheme by maintaining a constant stoichiometry throughout that scheme. Energies for specific processes within the scheme (e.g., the barrier to fragmentation) may then be straightforwardly obtained by taking the appropriate energy differences. We have chosen tetrahydro-5-methylfuran-3,4-diol as our model for Ado−H.

Scheme 2. Schematic Energy Profile for the Conversion of (*S*)-Glutamic Acid (**1a**) to (2*S*,3*S*)-3-Methylaspartic Acid (**5a**) by Glutamate Mutase[a]



[a] Energies relative to **1a** are given in parentheses, kJ mol^{-1}.

3. Results and Discussion

3.1. Natural Substrate (*S*)-Glutamic Acid. GM catalyzes the reversible conversion of (*S*)-glutamate to (2*S*,3*S*)-3-methylaspartate in a reaction that requires AdoCbl.[1] As in previous work,[9] we have characterized the catalytic mechanism of GM to provide a framework within which to understand other rearrangements that this enzyme might catalyze. Thus, Scheme 2 depicts the calculated thermochemistry for the GM-catalyzed conversion of (*S*)-glutamic acid (**1a**) to (2*S*,3*S*)-3-methylaspartic acid (**5a**).[29]

Substrate rearrangement begins with the removal of the *pro-S* hydrogen atom by Ado• at C4 of **1a** to generate the corresponding substrate-derived radical **2a** plus Ado−H.[30] This step is calculated to be exothermic by 31.2 kJ mol^{-1}.[31] Fragmentation of the substrate-derived radical **2a**, via the so-called fragmentation−recombination pathway,[32] then occurs to form glycyl radical (**3a**) and acrylic acid as discrete species[33] (we refer collectively to the combination of fragments as **3a***). The barrier for fragmentation is calculated to be 64.7 kJ mol^{-1}, and the fragmentation step is endothermic by 38.0 kJ mol^{-1}. Recombination of these fragments leads to the product-related radical **4a** in a process that is predicted to be exothermic by 14.3 kJ mol^{-1} with an associated barrier of 31.9 kJ mol^{-1}. At this point, we note that the product-related radical **4a** lies 23.7 kJ mol^{-1} higher in energy than the substrate-derived radical **2a**. This difference can be primarily understood in terms of a stabilizing effect at the radical center provided by the adjacent carboxylic acid functional group of **2a**.[34] In the last step, the transfer of hydrogen from Ado−H to the product-related radical **4a** regenerates Ado• and forms the closed-shell product **5a** in a reaction that is calculated to be slightly endothermic (by 2.3 kJ mol^{-1}).

We note that our calculations predict the overall reaction to be exothermic by 5.2 kJ mol^{-1}. Interestingly, the equilibrium

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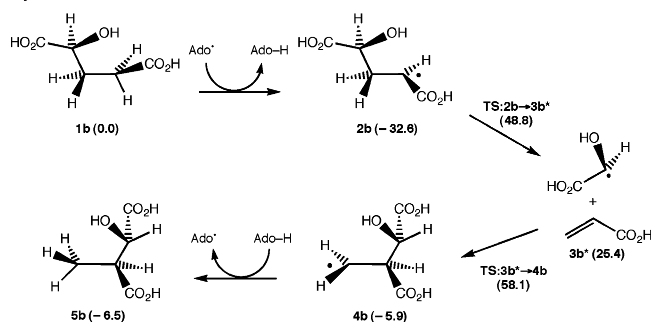
Scheme 3. Schematic Energy Profile for the Conversion of (*S*)-2-Hydroxyglutaric Acid (**1b**) to (2*S*,3*S*)-3-Methylmalic Acid (**5b**) by Glutamate Mutase[a]



[a] Energies relative to **1b** are given in parentheses, kJ mol^{-1}.

for the reaction in aqueous solution lies in the opposite direction, favoring **1a** over **5a** by about 6 kJ mol^{-1}.[35] Although such an energy difference is within the uncertainty limits of our (gas-phase) calculations, it is tempting to speculate that it may be the result of the enzyme binding the substrate in a higher energy conformation to facilitate efficient catalysis. This conjecture gains some support from our findings of significantly reduced barriers for fragmentation when using crystal-structure-like conformers compared with those obtained using the lowest energy reactant conformers (see Theoretical Methodology).

3.2. Substrate Analogue (*S*)-2-Hydroxyglutaric Acid. It has also been established that glutamate mutase can facilitate the isomerization of (*S*)-2-hydroxyglutarate to (2*S*,3*S*)-3-methylmalate, the only other substrate identified for the enzyme.[11] Compared with the k_{cat} of 5.6 s^{-1} for isomerization of (*S*)-glutamate,[36] the rate for the isomerization of (*S*)-2-hydroxyglutarate is much slower (k_{cat} = 0.05 s^{-1}). Electron paramagnetic resonance (EPR) spectroscopy identified the major organic radical produced during steady-state turnover as the C4 radical of the reactant (*S*)-2-hydroxyglutarate. Concurrent studies revealed that the low rate of activity of GM with (*S*)-2-hydroxyglutarate was unlikely to be a result of inefficient substrate binding.[11] Therefore, it was postulated that the 100-fold reduced activity resulted from the intervention of a higher energy fragmentation product, namely, the glycolyl radical.[11] Given that (*S*)-2-hydroxyglutarate is the only known true substrate for GM other than (*S*)-glutamate itself, the energy profile for this reaction is of importance, and we have examined it with the aim of providing insight into the operational framework accessible to holoenzyme GM.

The energy profile for the various steps in the proposed isomerization of (*S*)-2-hydroxyglutaric acid (**1b**) to (2*S*,3*S*)-3-methylmalic acid (**5b**) is depicted in Scheme 3. Initial hydrogen abstraction by Ado• from C4 of **1b** produces Ado−H plus the substrate-derived radical **2b** in a process that is exothermic by 32.6 kJ mol^{-1}.[37] This result is in qualitative agreement with the EPR observations of a buildup of the C4-derived radical of **1b** (i.e., **2b** in Scheme 3).[11] In addition, an exothermic initial abstraction reaction for **1b** is consistent with results obtained for **1a** (Scheme 2), where the analogous reaction is found to be exothermic by 31.2 kJ mol^{-1}. These results also indicate that the modification of the amino group in **1a** to a hydroxyl group in **1b** does not substantially influence the initial hydrogen abstraction step.

In a manner analogous to that of the isomerization mechanism of (*S*)-glutamic acid (Scheme 2), cleavage of the C2−C3 bond of the substrate-derived radical **2b**, for which we calculate a barrier to fragmentation of 81.4 kJ mol^{-1}, yields acrylic acid plus glycolyl radical (**3b**). These fragments (**3b***) lie 58.0 kJ mol^{-1} higher in energy than **2b**, significantly above the analogous value for **1b** (38.0 kJ mol^{-1}, Scheme 2). Our results thus support the original rationalization for the reduced catalytic activity of GM with (*S*)-2-hydroxyglutarate as the substrate.[11] We will address this issue more fully below. In the meantime, however, we note that the barrier for recombination of **3b*** is calculated to be 32.7 kJ mol^{-1} and leads to the product-related radical **4b** in a step that is exothermic by 31.3 kJ mol^{-1}. Having formed the product-related radical **4b**, generation of the product **5b** requires hydrogen atom reabstraction by **4b** from Ado−H, which we calculate to release 0.6 kJ mol^{-1} of energy.

A number of similarities between the energy profiles for (*S*)-glutamic acid (Scheme 2) and its analogue (*S*)-2-hydroxyglutaric acid (Scheme 3) can be highlighted. First, the initial hydrogen atom abstraction reactions for both species are moderately exothermic processes, while the reabstraction reactions are nearly thermoneutral. The difference in enthalpies between the abstraction and reabstraction reactions largely results from the formation of the CO_2H-stabilized (relative to Ado•) substrate-derived radicals **2a** and **2b** in the initial hydrogen atom abstraction reaction. On the other hand, the hydrogen atom transfer between the product-related radical **4a** or **4b** and Ado−H is nearly thermoneutral because the H atom is transferred between two quite similar environments involving unstabilized methylene carbons. Equivalently, the substrate-derived radicals **2a** and **2b** lie roughly 25 kJ mol^{-1} lower in energy than the product-related radicals **4a** and **4b**, once again largely as a result of the stabilizing effect of the carboxylic acid functional group on the radical centers. These results are consistent with the experimental observations of the substrate-derived radicals **2a**[30] and **2b**.[11]

Bearing in mind the relative rate data from experiment, it is informative to make a more quantitative comparison of the reaction profiles for (*S*)-glutamic acid and (*S*)-2-hydroxyglutaric acid. In a simplistic picture, the rate reduction from 5.6 s^{-1} (for **1a**) to 0.05 s^{-1} (for **1b**) would correspond to a relative increase in the barrier of approximately 12 kJ mol^{-1}. The calculated increase of 16.7 kJ mol^{-1} for the forward fragmentation barrier (from 64.7 kJ mol^{-1} for **2a** to 81.4 kJ mol^{-1} for **2b**) is in reasonable agreement with this value.

To the extent that the change in the kinetics of the overall rearrangement reactions parallels the relative increase in fragmentation enthalpy (from 38.0 kJ mol^{-1} for **2a** to 58.0 kJ mol^{-1} for **2b**), one might expect the difference between those values (20.0 kJ mol^{-1}) to provide a less accurate but more easily accessible indication of the overall effect. Indeed, by noting that the acrylic acid moiety could be expected to make only a small contribution to the difference in fragmentation enthalpies, it is possible to extend this line of thinking even further. More specifically, by simply determining the relative stabilities of appropriate fragment radicals (e.g., **3a** and **3b**), it may well prove

(35) Barker, H. A.; Rooze, V.; Suzuki, F.; Iodice, A. A. *J. Biol. Chem.* **1964**, *239*, 3260−3265.
(36) Chen, H.-P.; Marsh, E. N. G. *Biochemistry* **1997**, *36*, 14939−14945.
(37) We note that if hydrogen abstraction were to occur from C3 of **1b**, then the resulting radical would be 24.4 kJ mol^{-1} higher in energy than **2b**.

Table 1. Comparison of Calculated (0 K, kJ mol^{-1}) C−H BDEs and RSEs for Species Pertinent to the Fragmentation Step of the Glutamate Mutase Reaction[a]

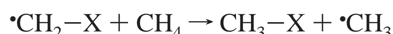
radical	BDE	RSE	radical	BDE	RSE
$H-^{\bullet}(CO_2H)-H$	407.5	21.2	$H-^{\bullet}C(CO_2H)-F$	385.8	43.0
$H-^{\bullet}C(CO_2H)-NH_2$ (**3a**)	334.4	94.3	$H_2N-^{\bullet}C(CO_2H)-NH_2$ (**3s**)	314.0	114.8
$H-^{\bullet}C(CO_2H)-SH$ (**3c**)	347.4	81.4	$HO-^{\bullet}C(CO_2H)-NH_2$	326.0	102.7
$H-^{\bullet}C(CO_2H)-S^-$ (**3d**)	302.3	126.4	$F-^{\bullet}C(CO_2H)-NH_2$	363.6	65.1
$H-^{\bullet}C(CO_2H)-OH$ (**3b**)	351.3	77.5	$HO-^{\bullet}C(CO_2H)-OH$ (**3f**)	341.6	87.1
$H-^{\bullet}C(CO_2H)-O^-$	345.4	83.4	$O{=}^{\bullet}C(CO_2H)$ (**3e**)	377.7	51.1

[a] The calculated C−H BDE for methane is 428.8 kJ mol^{-1}.

possible to arrive at a convenient means by which to estimate the effect of various substituents on the GM-catalyzed reaction.

An established manner by which to obtain a measure of the relative stabilities of radical species is to determine their RSEs. It is in this light that we now examine the RSEs for various substituted radicals relevant to the fragmentation step of the reactions catalyzed by glutamate mutase.

3.3. Radical Stabilization Energies of Substituted Glycyl Radicals. The calculation of RSEs provides a useful tool in the assessment of the effect of substituents on the stabilities of radicals relative to the effect in the corresponding closed-shell molecules.[34] The RSE for the radical $^{\bullet}CH_2-X$ is defined as the enthalpy change in the isodesmic reaction.



$$^{\bullet}CH_2-X + CH_4 \rightarrow CH_3-X + ^{\bullet}CH_3$$

It can be seen that calculated RSEs correspond to the differences in C−H bond dissociation energies (BDEs) between the reference species (in this case CH_4) and the substituted analogue (in this case CH_3-X). A positive RSE indicates that the substituent X has a stabilizing effect in $^{\bullet}CH_2-X$ relative to its effect in CH_3-X, whereas a negative RSE has the opposite meaning. Calculations of RSEs have proved useful in other biologically relevant applications.[38] In the present context, we seek to use RSEs to assess which substituents are stabilizing and which are destabilizing in relation to the radicals formed in the fragmentation step of the reactions catalyzed by glutamate mutase.[39]

Table 1 presents the C−H bond dissociation energies and radical stabilization energies of various analogues of glycine.[40] As mentioned in section 3.2, an important criterion for efficient isomerization in the reaction catalyzed by glutamate mutase is likely to be associated with the stability of the migrating radical fragment.

The first entry of Table 1 lists the BDE and RSE for acetic acid. The 21.2 kJ mol^{-1} RSE demonstrates the relative stabilizing effect of a carboxylic acid group adjacent to the radical center in the methyl radical. The CO_2H substituent stabilizes the methyl radical center by permitting delocalization of the unpaired electron into its π-system. Entry 2 is **3a**, which is a fragment formed in the isomerization of (*S*)-glutamic acid (Scheme 2). The calculated RSE of 94.3 kJ mol^{-1} for **3a** reflects a synergistic stabilizing effect from the CO_2H and NH_2 groups at the radical center.[41] This outcome is a consequence of the captodative effect[42] and arises whenever strongly π-electron-donating and strongly π-electron-accepting substituents are attached to the same radical center. In the case of the glycyl radical, the CO_2H substituent acts as the π-electron-accepting substituent, while the NH_2 moiety acts (via the nitrogen lone pair) as the π-electron donor.

This effect is further demonstrated in the third entry of Table 1 where the BDE and RSE for a thiol-substituted radical, $H-^{\bullet}C(CO_2H)-SH$ (**3c**), is shown. In this case, the RSE of 81.4 kJ mol^{-1} for **3c** is 12.9 kJ mol^{-1} lower than that determined for the glycyl radical, demonstrating the weaker π-donating ability of the thiol substituent relative to the NH_2 moiety at the radical center. On the other hand, if the thiol moiety is deprotonated, the RSE for the resultant $H-^{\bullet}C(CO_2H)-S^-$ (**3d**) radical is markedly higher at 126.4 kJ mol^{-1}, reflecting the very strong π-donating ability of S^-. We will return to the effects of thiol substitution at a radical center in section 3.4 when we examine the reaction of GM with (*S*)-2-thiolglutaric acid.

The fifth entry of Table 1 shows the BDE and RSE for **3b**, which is the fragmentation product relevant to the GM-catalyzed reaction when (*S*)-2-hydroxyglutaric acid is the substrate (Scheme 3). We see that replacement of the NH_2 group of **3a** by an OH group in **3b** leads to a reduction of 16.8 kJ mol^{-1} in the RSE. In addition, and similar to what is observed with **3d**, deprotonating the OH moiety in **3b** has the effect of increasing the RSE of the anionic species relative to its neutral counterpart. Thus, the RSE of $H-^{\bullet}C(CO_2H)-O^-$ is calculated to be 83.4 kJ mol^{-1}, which is 5.9 kJ mol^{-1} higher than that determined for **3b**. The smaller RSE with the neutral OH and anionic O^- substituents compared with NH_2 and S^- reflects the poorer π-donating ability of these species relative to NH_2 and S^-.

The above trend continues with fluoro substitution. The low RSE of 43.0 kJ mol^{-1} for $H-^{\bullet}C(CO_2H)-F$ reflects the weaker π-donor ability and stronger σ-accepting ability of the fluorine substituent. To the extent that the RSE really does parallel the effect on the GM-catalyzed reaction, it follows that turnover of 2-fluoroglutarate by GM is unlikely to occur to any great extent beyond the initial hydrogen abstraction step, since the formation of the migrating radical moiety in the fragmentation process is likely to be an energetically demanding process.

Having examined some monosubstituted analogues of acetic acid, we now explore disubstituted analogues in the eighth entry of Table 1 with the 2-aminoglycyl radical, $H_2N-^{\bullet}C(CO_2H)-NH_2$ (**3s**). Although the parent compound from which this

(38) See, for example: (a) Rauk, A.; Yu, D.; Taylor, J.; Shustov, G. V.; Block, D. A.; Armstrong, D. A. *Biochemistry* **1999**, *38*, 9089−9096. (b) Croft, A. K.; Easton, C. J.; Radom, L. *J. Am. Chem. Soc.* **2003**, *125*, 4119−4124. (c) Wood, G. P. F.; Moran, D.; Jacob, R.; Radom, L. *J. Phys. Chem. A* **2005**, *109*, 6318−6325.

(39) It should be kept in mind that the RSEs measure the effect of a substituent in the radical relative to its effect in the corresponding closed-shell molecule (which may not be negligible), but for simplicity we do not always repeat this cautionary comment.

(40) Because of numerical rounding, differences in the BDEs of Table 1 may not reproduce the tabulated RSEs to the accuracy quoted.

(41) See, for example: (a) Armstrong, D. A.; Yu, D.; Rauk, A. *Can. J. Chem.* **1996**, *74*, 1192−7207. (b) Rauk, A.; Yu, D.; Taylor, J.; Shustov, G. V.; Block, D. A.; Armstrong, D. A. *Biochemistry* **1999**, *38*, 9089−9096.

(42) Viehe, H.-G.; Janousek, Z.; Merényi, R.; Stella, L. *Acc. Chem. Res.* **1985**, *18*, 148−154.

fragmentation product would be generated, i.e., 2,2-diaminoglutaric acid, is unstable under aqueous conditions, it is nevertheless informative to consider the combined effects of two π-electron donors (via the nitrogen lone pairs) on the stability of the radical intermediates. For $H_2N-^{\bullet}C(CO_2H)-NH_2$, we calculate an RSE of 114.8 kJ mol^{-1}, implying that amino substitution in **3a** further stabilizes the radical center by 20.5 kJ mol^{-1}.[43] Similarly, OH substitution in **3a** to give $HO-^{\bullet}C(CO_2H)-NH_2$ leads to an RSE of 102.7 kJ mol^{-1}, which demonstrates that the OH moiety provides an additional 8.4 kJ mol^{-1} in stabilization energy relative to **3a**. In contrast, F substitution in **3a** leads to a destabilization relative to glycyl radical, in this instance by 29.2 kJ mol^{-1} (cf. entries 2 and 10). Again, the trend in the calculated RSEs for these disubstituted glycyl radicals can be understood in terms of the π-donating ability, which decreases across the series NH_2 > OH > F, and the σ-accepting capacity, which increases in the order NH_2 < OH < F.

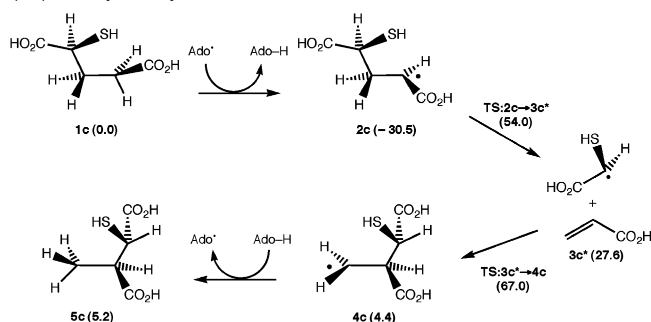
The penultimate entry of Table 1 shows a calculated RSE of 87.1 kJ mol^{-1} for the dihydroxylated analogue of acetic acid, $HO-^{\bullet}C(CO_2H)-OH$ (**3f**). This result indicates that OH substitution in **3b** leads to a radical that is further stabilized by 9.6 kJ mol^{-1}. We will look more closely at **3f** in section 3.5.2 when we examine the energy profile for the isomerization of 2,2-dihydroxyglutaric acid. The last entry of Table 1 shows the BDE and RSE associated with $O{=}^{\bullet}C(CO_2H)$ (**3e**). The relevance of this result will become clearer in section 3.5.1. For the time being, it is sufficient to note that the calculated RSE of 51.1 kJ mol^{-1} is low and implies that a fragmentation pathway involving **3e** is likely to be one of relatively high energy.

These results highlight the potential utility of calculating RSEs to obtain estimates of the stabilities of radicals with related but different substitution patterns. As pointed out in section 3.2, the 100-fold rate reduction observed for GM with (*S*)-2-hydroxyglutarate relative to (*S*)-glutamic acid[11] may be (loosely) regarded as an approximate increase in energetic demands of 12 kJ mol^{-1}. While the complete characterization of the reaction pathway is always preferable, acceptable estimates for this difference can apparently be obtained by evaluating the relative fragmentation enthalpies of **2a** and **2b** (20.0 kJ mol^{-1}) and even more conveniently from the relative RSEs of **3a** and **3b** (16.8 kJ mol^{-1}).

To the extent that this reasoning is sound, and assuming that aberrant side reactions do not occur,[44] we may expect that any radical with an RSE close to that of either **3a** or **3b** could potentially be a kinetically competent radical intermediate in the reaction catalyzed by GM. On the other hand, radicals with RSEs significantly higher or smaller than that of **3a** or **3b** may be expected to be relatively poor radical intermediates in reactions catalyzed by GM. In the next section, we investigate some of these aspects in terms of a specific example.

3.4. Substrate Analogue (*S*)-2-Thiolglutaric Acid. The reaction of the substrate analogue 2-thiolglutarate with GM has been investigated very recently.[45] This analogue was shown to elicit Co−C bond homolysis with concomitant formation of 5′-deoxyadenosine. Interestingly, the associated EPR spectrum is unlike any previously observed for this enzyme, and simulations suggest the interaction of a low-spin Co^{2+} in cob(II)alamin with a sulfur-stabilized thioglycolyl radical, presumably derived from fragmentation of the C4-centered radical produced from an initial H atom abstraction by Ado$^{\bullet}$. The putative thioglycolyl radical was found to accumulate on the enzyme, and for this reason it was proposed that it is too stable to undergo further transformation.[45] To understand this system further, we have calculated the energies of the various radical intermediates. In particular, we have examined the effect of the protonation state of the sulfur on the reaction.

Scheme 4. Schematic Energy Profile for the Conversion of (*S*)-2-Thiolglutaric Acid (**1c**) to (3*R*)-2-Thiol-3-methylsuccinic Acid (**5c**) Catalyzed by Glutamate Mutase[a]



[a] Energies relative to **1c** are given in parentheses, kJ mol^{-1}.

Scheme 4 displays an energy profile for the conversion of the neutral (at S) forms of (*S*)-2-thiolglutaric acid (**1c**) to (3*R*)-2-thiol-3-methylsuccinic acid (**5c**). Initial H atom abstraction by Ado$^{\bullet}$ from **1c** to produce the C4-centered substrate-derived radical **2c** is determined to be exothermic by 30.5 kJ mol^{-1}.[46] Fragmentation of **2c** to yield thioglycolyl radical (**3c**) plus acrylic acid is associated with a barrier of 84.5 kJ mol^{-1} and is endothermic by 58.1 kJ mol^{-1}. Recombination of the cleavage products **3c** and acrylic acid gives the product-related radical **4c** with a barrier of 39.4 kJ mol^{-1} and a reaction enthalpy of −23.2 kJ mol^{-1}. Hydrogen atom reabstraction by **4c** from Ado−H is calculated to be slightly exothermic at 0.8 kJ mol^{-1}.

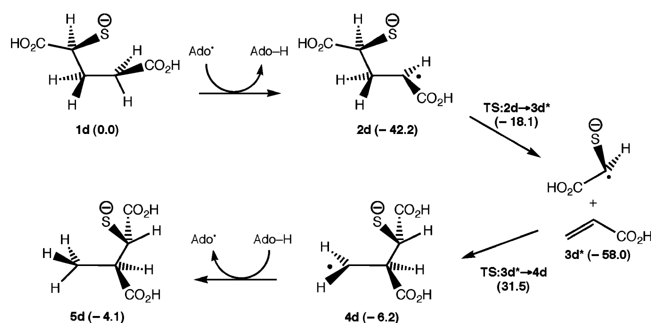
We observe that both the fragmentation barrier and reaction enthalpy for the reaction of (*S*)-2-thiolglutaric acid with GM (84.5 and 58.1 kJ mol^{-1}, respectively, Scheme 4) are very similar to those determined for (*S*)-2-hydroxyglutaric acid (81.4 and 58.0 kJ mol^{-1}, respectively, Scheme 3). In addition, the recombination barrier and reaction enthalpy are also quite similar (compare 39.4 and −23.2 kJ mol^{-1}, respectively, for **1c** in Scheme 4 with 32.7 and −31.3 kJ mol^{-1}, respectively, for **1b** in Scheme 3). Moreover, the RSE of **3c** (Table 1) is determined to be 81.4 kJ mol^{-1}, which is 3.9 kJ mol^{-1} higher than that determined for **3b** (Table 1) but 12.9 kJ mol^{-1} lower than that determined for **3a** (Table 1). Taken together, these results suggest that **3c** does not possess any significant stability that would cause the reaction to terminate. Thus, one might expect the reaction of 2-thiolglutarate with GM to proceed at a rate similar to that observed for (*S*)-2-hydroxyglutarate. However, this is not observed experimentally. Instead, an organic radical

(43) See the Supporting Information for an expanded discussion of the use of RSEs to assess the propensity of isomerization to occur within the GM-catalyzed reactions using a specific example of the hypothetical isomerization of 2,2-diaminoglutaric acid.

(44) Rétey, J. *Angew. Chem., Int. Ed. Engl.* **1990**, *29*, 355−361.
(45) Yoon, M.; Patwardhan, A.; Qiao, C.; Mansoorabadi, S. O.; Menefee, A. L.; Reed, G. H.; Marsh, E. N. G. *Biochemistry* **2006**, *45*, 11650−11657.

(46) We note that if hydrogen abstraction were to occur from C3 of **1c**, then the resulting radical would be 19.1 kJ mol^{-1} higher in energy than **2c**.

Scheme 5. Schematic Energy Profile for the Conversion of the S^- Anion of (*S*)-2-Thiolglutaric Acid (**1d**) to the S^- Anion of (3*R*)-2-Thiol-3-methylsuccinic Acid (**5d**) Catalyzed by Glutamate Mutase[a]



[a] Energies relative to **1d** are given in parentheses, kJ mol^{-1}.

intermediate is found to accumulate on the enzyme, and hydrogen abstraction from 2-thiolglutarate appears to be irreversible.[45]

One possible explanation for these events is the generation of a sulfur-centered radical subsequent to initial H atom abstraction from C4 of the substrate. In one scenario, an intramolecular H atom transfer from the thiol group of 2-thiolglutarate to the C4 position could occur. We have examined this possibility and find the reaction to be exothermic by 35.7 kJ mol^{-1} and possess a barrier of 44.9 kJ mol^{-1}. The large reverse barrier (at 80.6 kJ mol^{-1}) to re-form the reactant radical, in addition to the inability of the S-centered radical to be able to proceed in the forward direction, is consistent with the absence of tritium exchange found experimentally. However, despite being an attractive possibility on the basis of energy considerations, the involvement of an S-centered radical is at odds with the recorded EPR spectrum, which is well simulated by a C-centered radical perturbed by an adjacent sulfur moiety.

In search of an alternative explanation, we next examine how deprotonation of the thiol affects the energy profile for the isomerization of the S^- form of (*S*)-2-thiolglutaric acid (**1d**; Scheme 5). Given that the pK_a of a typical SH group is approximately 8, (partial) deprotonation of the SH moiety in 2-thiolglutarate by GM is conceivable. In this connection, Glu171 has previously been demonstrated to act as a general base in the GM-catalyzed reaction.[7,28] Assuming that 2-thiolglutarate binds in an orientation similar to that of the natural substrate glutamate, which is quite likely given that the C4 hydrogen is abstracted from 2-thiolglutarate,[45] Glu171 will be well-positioned to partially deprotonate the thiol moiety of 2-thiolglutarate.

Initial H atom abstraction by Ado$^\bullet$ from **1d** to generate the substrate-derived radical **2d** is predicted to be exothermic by 42.2 kJ mol^{-1}.[47] The barrier for fragmentation of **2d** to yield acrylic acid plus the S-centered anion of the thioglycolyl radical (**3d**) is calculated to be only 24.1 kJ mol^{-1}. The next step in the isomerization of **1d** to **5d** involves recombination of the cleavage products **3d*** to produce the product-related radical **4d**, for which we calculate a barrier of 89.5 kJ mol^{-1} and an endothermicity of 51.8 kJ mol^{-1}. The reaction concludes with a slightly endothermic hydrogen atom reabstraction by **4d** from Ado−H to form **5d** plus Ado$^\bullet$.

There are several striking features in the reaction profile depicted in Scheme 5. In the first place, the fragmentation barrier of 24.1 kJ mol^{-1} for **2d** is considerably smaller than that observed for the fragmentation barriers of the substrate-derived radicals of (*S*)-glutamic acid (64.7 kJ mol^{-1}, Scheme 2) and (*S*)-2-hydroxyglutaric acid (81.4 kJ mol^{-1}, Scheme 3). In addition, the associated fragmentation reaction of **2d** is *exothermic* by 15.8 kJ mol^{-1}, placing the cleavage products **3d*** 58.0 kJ mol^{-1} below the starting material. This is particularly intriguing since the fragmentation reactions of **2a** and **2b** are predicted to be *endothermic* by 38.0 and 58.0 kJ mol^{-1}, respectively. Finally, the energy requirements for recombination of **3d*** to form the product-related radical **4d** are relatively high when compared with the analogous requirements for the substrates **2a** and **2b**, suggesting that further reaction is likely to be much slower.

The rather low energy of **3d*** can be rationalized in terms of enhanced captodative stabilization of **3d** relative to the fragment radicals derived from alternative substrates or substrate analogues. Previously we noted that the RSEs provide a convenient measure for assessing the influence of a substituent at a radical center. The RSE of **3d** is calculated to be 126.4 kJ mol^{-1}, which is significantly higher than that shown in Table 1 for **3a** (94.3 kJ mol^{-1}), **3b** (77.5 kJ mol^{-1}), or **3c** (81.4 kJ mol^{-1}). The distinguishing factor that contributes to the enhanced stability of **3d** is the anionic sulfur moiety adjacent to the radical center. This acts as a much stronger π-electron donor than NH_2, OH, or SH. Thus, it seems as though the use of RSEs to provide an indication of the propensity for isomerization to occur is reasonable.[43]

The reaction profile depicted in Scheme 5 for the reaction of the S^- anion of 2-thiolglutaric acid with GM provides an attractive rationalization of the experimental data. In the first place, it involves a radical (**3d**) consistent with the previously reported EPR data. It is also consistent with the tritium exchange studies between AdoCbl and 2-thiolglutarate, which demonstrate that tritium exchange occurs either very slowly or not at all. With regard to this latter point, we note that the barrier for the reverse recombination of the cleavage products **3d*** to give **2d** is not large (39.9 kJ mol^{-1}) but that this reaction is endothermic by 15.8 kJ mol^{-1}. Consequently, the equilibrium concentration of **2d** (for possible tritium exchange) would be very small.[48]

The remarkable dependence of the energy demands for radical formation on the protonation state of sulfur provides yet another example where partial proton transfer resulting from hydrogen bonding is important in enzyme radical catalysis.[7,51] In the present context, (partial) deprotonation at S in the thioglycolyl radical intermediate has the profound effect of suspending the progress of the intermediate radical toward product.

3.5. Substrate Analogues 2-Ketoglutaric Acid and Its Hydrate 2,2-Dihydroxyglutaric Acid. 3.5.1. 2-Ketoglutaric Acid. 2-Ketoglutarate has previously been reported to elicit cleavage of the Co−C bond of adenosylcobalamin in glutamate mutase.[12] At the same time, rapid exchange of tritium between C5′ of the coenzyme and C4 of the substrate was observed,

(47) We note that if hydrogen abstraction were to occur from C3 of **1d**, then the resulting radical would be 20.8 kJ mol^{-1} higher in energy than **2d**.

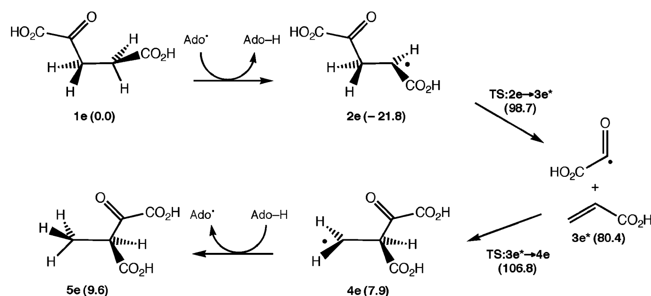
(48) On the basis of an enthalpy difference of 15.8 kJ mol^{-1} between **3d*** and **2d**, we estimate that less than 0.2% of **2d** would be present at 298 K.

(49) See section 3.5.2.

(50) Eggerer, H.; Overath, P.; Lynen, F.; Stadtman, E. R. *J. Am. Chem. Soc.* **1960**, *82*, 2643−2644.

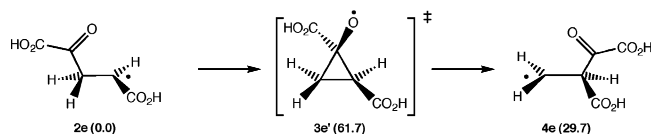
(51) (a) Smith, D. M.; Golding, B. T.; Radom, L. *J. Am. Chem. Soc.* **1999**, *121*, 1383−1384. (b) Smith, D. M.; Golding, B. T.; Radom, L. *J. Am. Chem. Soc.* **1999**, *121*, 9388−9399.

Scheme 6. Schematic Energy Profile for the Conversion of 2-Ketoglutaric Acid (**1e**) to 3-Methyloxaloacetic Acid (**5e**) by Glutamate Mutase[a]



[a] Energies relative to **1e** are given in parentheses, kJ mol^{-1}.

Scheme 7. Rearrangement Barrier for the Addition−Elimination Pathway Relevant to the Conversion of 2-Ketoglutaric Acid to 3-Methyloxaloacetic Acid by Glutamate Mutase[a]



[a] Energies relative to **2e** are given in parentheses, kJ mol^{-1}.

though rearrangement of the resulting substrate-derived radical to product could not be detected. These results suggest that initial hydrogen abstraction is a relatively facile process, but that the rearrangement step is too demanding to be made possible by GM. At the time, it was not possible to determine whether it was the keto or hydrated form of the substrate analogue that was reacting. In an attempt to provide further insight into these observations, we have calculated energy profiles for the isomerization of 2-ketoglutaric acid (**1e**) to 3-methyloxaloacetic acid (**5e**) and for that of the corresponding hydrated forms, 2,2-dihydroxyglutaric acid (**1f**) to 2-hydroxy-3-methylmalic acid (**5f**).

Scheme 6 shows that initial hydrogen atom abstraction by Ado• from C4 of **1e** is exothermic by 21.8 kJ mol^{-1} and produces Ado−H and the substrate-derived radical **2e**. The next step in a catalytic pathway is fragmentation of **2e** to yield acrylic acid plus **3e**. We calculate the barrier for fragmentation of **2e** to be 120.5 kJ mol^{-1} and the process to be endothermic by 102.2 kJ mol^{-1}. The latter value is significantly greater than the analogous endothermicities for the catalytic substrates (*S*)-glutamic acid and (*S*)-2-hydroxyglutaric acid of 38.0 and 58.0 kJ mol^{-1}, respectively. Similar trends are seen in the corresponding RSEs (Table 1), the value of 51.1 kJ mol^{-1} for **3d** being significantly lower than the RSEs of 94.3 kJ mol^{-1} for **3a** and 77.5 kJ mol^{-1} for **3b**. Therefore, if it is indeed 2-ketoglutarate that binds with GM,[49] it is possible that the large energy requirement for fragmentation, which results primarily from the high energy of radical **3e**, does not allow the reaction to proceed to product.

However, there is a possible alternative rearrangement pathway connecting the substrate-derived radical **2e** and the product-related radical **4e**, namely, an addition−elimination-type mechanism.[32] This pathway involves an intramolecular addition of the radical center to the π bond of the ketone moiety in **2e** to form a cyclic transition structure. An addition−elimination pathway is believed to be operative in the isomerization reaction catalyzed by methylmalonyl-CoA mutase, where the migrating moiety also includes an sp^2-hybridized carbon.[50] Given this precedent, it is reasonable to address the feasibility of this pathway for glutamate mutase.

As seen in Scheme 7, the barrier for rearrangement of the substrate-derived radical **2e** to the product-related radical **4e** via an addition−elimination pathway is calculated to be 61.7 kJ mol^{-1}. This barrier is substantially less than either the barrier or reaction endothermicity for the fragmentation of **2e** in the fragmentation−recombination pathway (Scheme 6).

Furthermore, and in line with previous work on the rearrangement mechanism for methylmalonyl-CoA mutase,[51] we find that protonating the carbonyl group of the migrating moiety substantially reduces the barrier for conversion. Indeed, we find that full protonation of the migrating moiety reduces the barrier from 61.7 to just 23.7 kJ mol^{-1}. We would expect that partial protonation will also lead to a reduced barrier (but to a smaller extent).[51]

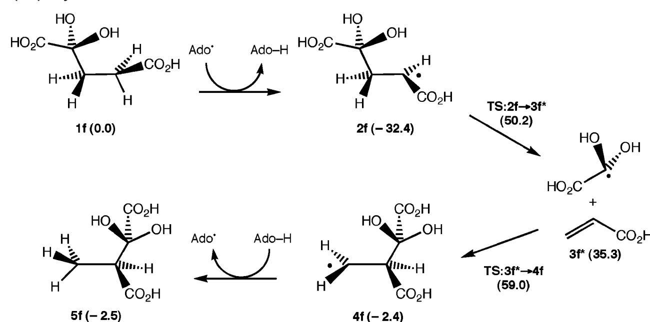
However, considering the nature of the crystal structure of GM and the likely binding mode of 2-ketoglutaric acid, it is not clear that protonation of the migrating carbonyl group (partial or otherwise) in the enzyme environment could easily take place. More specifically, if 2-ketoglutarate were to bind to GM with its carboxylate groups held by the "arginine claw" (as is the case for the natural substrate and its analogue tartaric acid),[27] the migrating carbonyl group would be positioned adjacent to Glu171 and several other electronegative entities. This environment may well be expected to have an inhibitory effect on the rearrangement, i.e., the opposite of the effect of protonation. As a first-order approximation, we have simulated such an electron-rich environment with a deprotonated migrating carboxylic acid moiety and indeed find the barrier for rearrangement to increase from 61.7 to 80.4 kJ mol^{-1}.

At this stage, it is interesting to point out that if initial H atom abstraction were to occur from C3 of **1e**, the resulting radical species is predicted to be 13.5 kJ mol^{-1} lower in energy than the C4 substrate-derived radical **2e** (Scheme 6). This energy difference reflects the differential stabilization at the radical center by the adjacent CO_2H and RC=O groups of **2e** and the C3-derived radical, respectively. In addition to being attractive on energetic grounds, an initial C3 H atom abstraction is consistent with the failure to observe product experimentally. However, it is not consistent with tritium exchange experiments,[12] which give results in line with initial H atom abstraction from the C4 position. Another possibility for the reaction of **1e** with GM is that it is the hydrate of **1e**, i.e., 2,2-dihydroxyglutaric acid, that is the actual species undergoing reaction with GM. We consider this scenario in the next section.

Before doing so, however, we note the desirability of determining the mechanism for rearrangement in the closely related AdoCbl-dependent 2-methyleneglutarate mutase (2-MGM) reaction. This has not yet been firmly established experimentally, despite extensive efforts.[32b] Theoretical calculations indicate a preference for addition−elimination over the fragmentation−recombination pathway for this system.[52] We are unaware of any experimental results reported to date with 2-MGM and 2-ketoglutarate, but this would also be of interest in helping to further our understanding of this enzyme.

(52) Smith, D. M.; Golding, B. T.; Radom, L. *J. Am. Chem. Soc.* **1999**, *121*, 1037−1044.

Scheme 8. Schematic Energy Profile for the Conversion of 2,2-Dihydroxyglutaric Acid (**1f**) to 2-Hydroxy-3-methylmalic Acid (**5f**) by Glutamate Mutase[a]



[a] Energies relative to **1f** are given in parentheses, kJ mol^{-1}.

3.5.2. 2,2-Dihydroxyglutaric Acid. As noted above, it is not known whether 2-ketoglutarate itself or its hydrate, 2,2-dihydroxyglutarate, is the actual species in the active site of GM.[12] To obtain further information in this regard, we have examined the energy profile for the isomerization of **1f** to **5f** (Scheme 8).

Initial hydrogen atom abstraction by Ado• from C4 of **1f** to generate Ado−H and the substrate-derived radical **2f** is calculated to be exothermic by 32.4 kJ mol^{-1}.[53] Fragmentation of **2f** to form acrylic acid plus the 2-hydroxyglycolyl radical (**3f**) is calculated to require 82.6 kJ mol^{-1}, and the reaction is predicted to be endothermic by 67.7 kJ mol^{-1}. The barrier for recombination of these fragments (**3f***) is calculated to be 23.7 kJ mol^{-1}, leading to the product-related radical **4f** in a reaction that is exothermic by 37.7 kJ mol^{-1}. Overall, the rearrangement step (**2f** → **4f**) is endothermic by 30.0 kJ mol^{-1}. Hydrogen atom reabstraction by the product-related radical **4f** from Ado−H is calculated to be essentially thermoneutral.

To establish the propensity for **1f** to serve as a substrate for GM, it is valuable to compare the energy profiles of Schemes 8 and 3, the latter of which displays the energy requirements for the alternative substrate for GM, i.e., **1b**. The fragmentation barriers for **2f** and **2b** are 82.6 and 81.4 kJ mol^{-1}, respectively, while their corresponding reaction enthalpies are 67.7 and 58.0 kJ mol^{-1}, respectively. The fact that the fragmentation barrier for **2f** is only marginally higher than that for **2b** implies that, should **1f** bind to GM and undergo H atom abstraction, the subsequent rearrangement could be expected to proceed at a rate similar to that of **1b**. There is a larger difference between the fragmentation reaction enthalpies, but this appears to reflect differential stabilization introduced, via intramolecular H bonds, into species along the reaction pathway of Scheme 8, which are not as dominant as the species in Scheme 3. Such artifacts are likely to be minimized in the enzyme environment, where these particular substrate−enzyme interactions are likely to be roughly constant along the reaction pathway.

Although it is not known whether GM binds the keto or hydrated form of 2-ketoglutarate, the calculations above suggest that, for either compound, once a substrate radical is generated, an energetically feasible pathway exists for its rearrangement. Furthermore, the pathways are no less favorable than for the rearrangement of (*S*)-2-hydroxyglutarate, which has been demonstrated to be a substrate for GM.[11] This has caused us to reevaluate the experimental data that led us to the conclusion that the enzyme was unable to catalyze the rearrangement of 2-ketoglutarate.[12] A combination of factors may have led to turnover not being observed, even if it was occurring.

One possibility is that the equilibrium constant for the formation of 3-methyloxaloacetate is sufficiently unfavorable that, even though the enzyme is able to catalyze the full reaction, insufficient product accumulates at equilibrium for it to be reliably detected by proton NMR, which is a relatively insensitive technique.[54] Another problem, not noted in the original study, is that 3-methyloxaloacetate is quite unstable and undergoes spontaneous decarboxylation to give 2-ketobutyrate with a half-life of about 1.5 h at neutral pH.[55] Given that the NMR experiment designed to detect turnover involved incubating the enzyme with 2-ketoglutarate over the course of 24 h (after which time the enzyme had lost all activity), it is very possible that any 3-methyloxaloacetate formed by the enzyme would have already decomposed under the conditions of the experiment. We therefore cannot confidently state that the enzyme does not fully process 2-ketoglutarate as a substrate, but only that we have not been able to detect its turnover. The computational results therefore provide the impetus to experimentally reexamine the reaction of GM with this pair of substrates. In particular, experiments to examine whether the enzyme can convert 3-methyloxaloacetate to 2-ketoglutarate, which is the thermodynamically preferred direction, may prove more definitive.

To summarize the results presented in sections 3.5.1 and 3.5.2, we first note that these data do not provide a definitive explanation of the experimental results for GM with 2-ketoglutarate but rather offer a range of possibilities to be considered. After an initial C4 abstraction of **1e**, rearrangement via fragmentation−recombination or addition−elimination pathways is in principle possible. The former pathway is calculated to be associated with very large energy requirements for fragmentation because of the high-energy fragmentation product, and this may offer a convenient, though unsubstantiated, explanation of the failure to observe product formation experimentally. The addition−elimination pathway offers a lower energy alternative. However, it is not clear whether GM possesses the necessary machinery to execute this transformation. There is the possibility in principle that initial H atom abstraction may occur in **1e** from C3 instead of C4. While such a scenario can easily account for the formation of a substrate radical that does not react further, it would be at odds with experimental evidence based on tritium exchange experiments in favor of an initial C4 abstraction. Finally, consideration of the involvement of the hydrate of **1e**, namely, 2,2-dihydroxyglutaric acid, reveals an energy profile which suggests that, if it is bound by the enzyme, this substrate might be expected to rearrange. This again would not be compatible with the original interpretation of the experimental observations. On the whole, the results in sections 3.5.1 and 3.5.2 suggest that additional experiments dealing with the reaction of 2-ketoglutarate with GM are highly desirable.

(53) We find the C3-derived substrate radical of **1f** to lie 21.5 kJ mol^{-1} higher in energy than **2f**.

(54) On the basis of our calculated enthalpy change of 9.6 kJ mol^{-1} for the isomerization of 2-ketoglutaric acid to 3-methyloxaloacetic acid depicted in Scheme 6, we estimate that less than 2.1% of the product would be present at 298 K.

(55) Kubala, K.; Martell, A. E. *J. Am. Chem. Soc.* **1981**, *103*, 7609−7615.

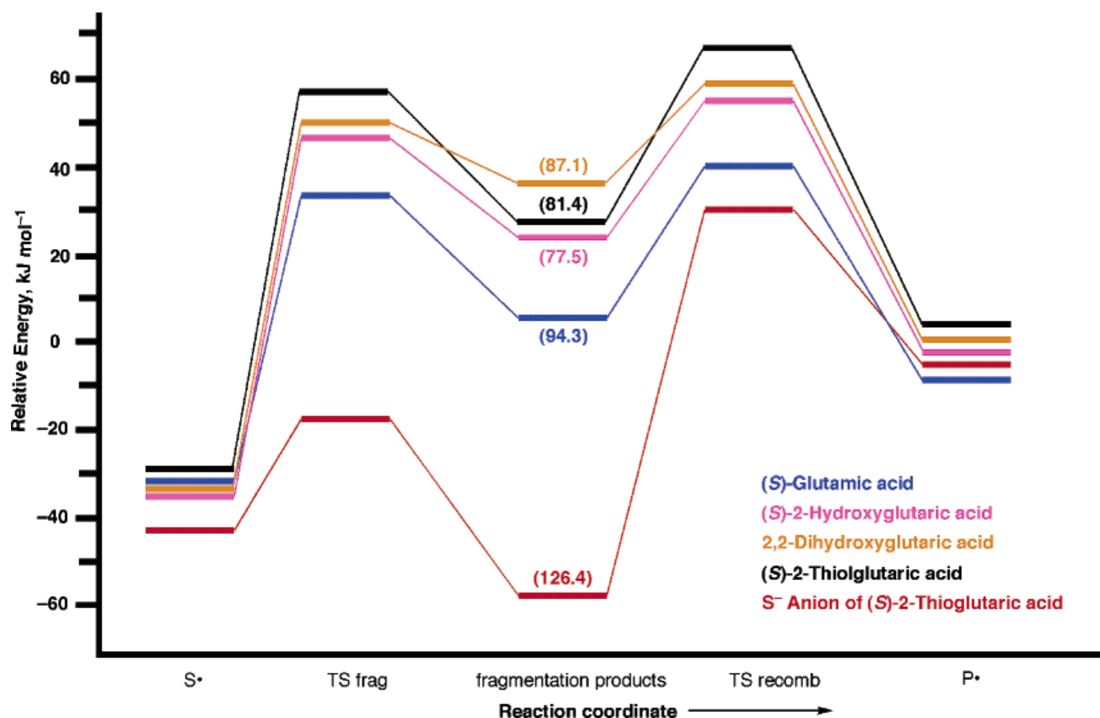


Figure 1. Schematic energy profiles for the rearrangement of various substrates and substrate analogues for glutamate mutase. The zero level is the energy of the substrate SH plus Ado•. RSEs for the intermediate fragment radicals are given in parentheses (kJ mol^{-1}).

4. Concluding Remarks

The remarkable substrate specificity of the adenosylcobalamin-dependent enzyme glutamase mutase has motivated us to examine the rearrangement mechanisms of various substrates, analogues, and inhibitors of this unique enzyme to gain insight into its catalytic threshold. Apart from the isomerization of the natural substrate (*S*)-glutamate, only one other true substrate has been previously uncovered for GM, namely, (*S*)-2-hydroxyglutarate. Comparison of the energy profiles for these two substrates reveals several similar features. However, a significant distinction is the sizable difference, amounting to 20.0 kJ mol^{-1}, calculated for the energy requirements for the fragmentation step. This difference results from the decreased stabilization of the glycolyl radical in the reaction of (*S*)-2-hydroxyglutaric acid relative to the glycyl radical in the reaction with (*S*)-glutamic acid. Calculated RSEs are consistent with this argument, as we find the RSE of the glycolyl radical to be 16.8 kJ mol^{-1} lower than that of the glycyl radical.

The notion that intrinsic radical stability can alter the kinetics of the GM-catalyzed reactions prompted us to carry out calculations of RSEs for various additional substituted analogues of glycyl radical. Generally speaking, and assuming that aberrant side reactions do not take place, we expect any substituted glycyl radical with an RSE similar to that of glycyl or glycolyl radical to be a kinetically competent reaction intermediate. In contrast, radicals with RSEs substantially higher or lower than the RSEs of these radicals are likely to be less effective reaction intermediates.

(*S*)-2-Thiolglutaric acid might be expected to rearrange to product on the basis of energy requirements that are similar to those of (*S*)-2-hydroxyglutaric acid, yet such turnover is not observed experimentally. The failure of GM to catalyze this reaction has been attributed to the intervention of a very stable radical intermediate, specifically the thioglycolyl radical. Whereas we do not find the neutral form of the thioglycolyl radical to show special stability (compare, for instance, the RSE of the thioglycolyl radical (81.4 kJ mol^{-1}) with those of the glycyl and glycolyl radicals (94.3 and 77.5 kJ mol^{-1}, respectively)), the deprotonated (S^-) form of the thioglycolyl radical *is* particularly stable, as demonstrated by its rather high RSE of 126.4 kJ mol^{-1}. The relatively high forward barrier for recombination of the cleavage products associated with the anion fragment radical and the disproportionate equilibrium in favor of these cleavage products suggest that this may be the inactivating state in the reaction of GM with 2-thiolglutarate.

The energy profiles for (*S*)-glutamic acid and (*S*)-2-hydroxyglutaric acid serve as benchmarks with which to evaluate the propensity for biological activity for substrate analogues, since these two compounds act as substrates for GM. Figure 1 summarizes the relative energy requirements for rearrangement for the variety of substrates and analogues examined in the present work, together with the RSEs calculated for the associated fragment radicals. The similar energy requirements of (*S*)-2-hydroxyglutaric acid and 2,2-dihydroxyglutaric acid suggest that the latter analogue might serve as a substrate for GM. Although it is not known whether it is 2-ketoglutarate or its hydrate 2,2-dihydroxyglutarate that is the actual species in the active site of GM, our calculations suggest substrate turnover may occur for 2,2-dihydroxyglutarate.

It is interesting to note that two rearrangement pathways are possible with 2-ketoglutaric acid as the substrate following an initial H atom abstraction at C4, i.e., fragmentation−recombination or addition−elimination. We calculate the former to be associated with a very large endothermicity of 102.2 kJ mol^{-1}, while there are significantly smaller (by more than 40 kJ mol^{-1}) energy requirements for the addition−elimination pathway, and this is further reduced if (partial) protonation of the migrating moiety takes place. On the other hand, the barrier for rearrange-

ment is predicted to increase when the migrating group is in an electron-rich environment, which may be more likely in the active site of GM. Overall, our results with 2-ketoglutaric acid and 2,2-dihydroxyglutaric acid suggest that additional experiments with GM and 2-ketoglutarate or 3-methyloxaloacetate are warranted.

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Supporting Information Available: Hypothetical isomerization of 2,2-diaminoglutaric acid, GAUSSIAN archive entries of the B3-LYP/6-31G(d,p) geometries, corresponding G3(MP2)-RAD total energies, and full citations for refs 23 and 24. This material is available free of charge via the Internet at http://pubs.acs.org.

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