Catalysis by Mutants of Methylmalonyl-CoA Mutase: A Theoretical Rationalization for a Change in the Rate-Determining Step

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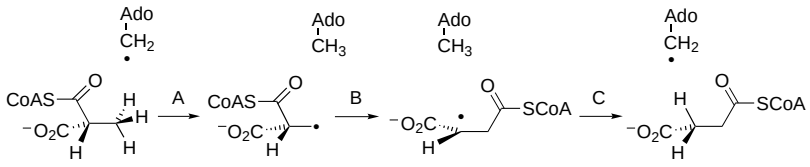
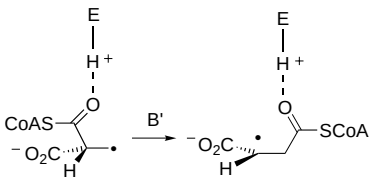
ab initio calculations · coenzyme B_{12} · enzyme catalysis · hydrogen abstraction · methylmalonyl-CoA mutase

Methylmalonyl-CoA mutase, in the presence of adenosylcobalamin (coenzyme B_{12}), catalyses the transformation of methylmalonyl-CoA to succinyl-CoA. This is an intriguing rearrangement in which the thioformyl-CoA group and a hydrogen atom on adjacent carbon atoms change places (Scheme 1).[1]



Scheme 1. *Transformation of methylmalonyl-CoA to succinyl-CoA.*

The first step in this process (A) is generally accepted to involve hydrogen abstraction from methylmalonyl-CoA by the 5′-deoxyadenosyl radical (•CH_2—Ado).[1] This step is followed by a 1,2-migration of the thioformyl-CoA group (B). The final step (C) involves re-abstraction from 5′-deoxyadenosine (CH_3Ado) to give the product. Since 1,2-shifts in radicals are normally difficult processes, the rearrangement step has been widely studied. The consensus of opinion has been that either a fragmentation/recombination mechanism or an intramolecular addition/elimination process is followed.[1, 2] However, in recent computational studies[3] we proposed a third pathway involving partial proton transfer from the enzyme to the carbonyl oxygen atom of the migrating group (Scheme 2).



Scheme 2. *Proposed pathway for the rearrangement involving partial proton transfer from the enzyme to the carbonyl oxygen atom of the migrating group.*

By using high-level ab initio calculations on small model systems we found that partial protonation reduces the energy barrier to rearrangement (B′). Furthermore, we found that energy barriers ranging from that corresponding to no protonation to that corresponding to full protonation can be obtained by altering the acidity of the protonating group (E—H^+). The partial proton transfer concept is consistent with the crystal structure of methylmalonyl-CoA mutase reported by Evans, Leadlay, and co-workers[4] which shows a histidine moiety (His 244) ideally positioned to donate a hydrogen bond to the carbonyl oxygen atom of the ester (Figure 1).[5]

More rigorous tests of the partial proton transfer proposal have subsequently been carried out in the laboratories of both Banerjee[6] and Leadlay.[7] Their elegant experiments have examined mutant enzymes in which the potentially important His 244 moiety was replaced with glycine,[6] alanine[7] or glutamine.[7] They found the catalytic efficiencies of the mutant enzymes to be 100 – 1000 times less than that of the wild-type enzyme, thus providing strong support for the assistance of His 244 in the rearrangement of the carbon skeleton.[6, 7] The experiments also suggest[6, 7] that

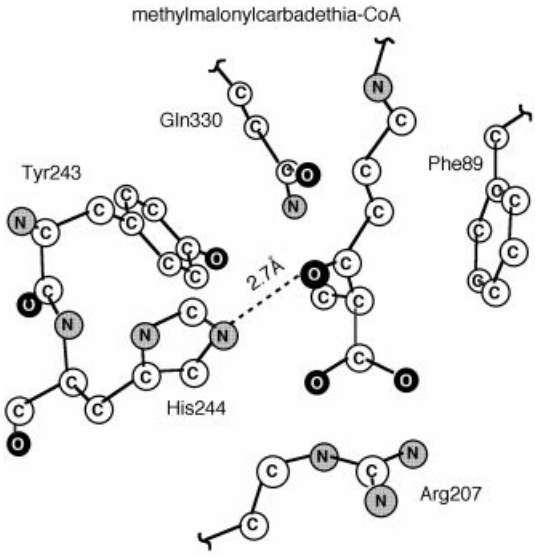


Figure 1. *The active site of a mutant of methylmalonyl-CoA mutase.[5]*

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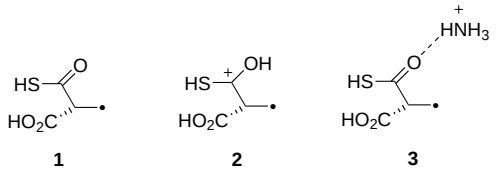
His 244 plays an important role in protecting the radical site from attack by oxygen (negative catalysis).[8]

Banerjee and co-workers[6] concluded that "these data provide the first experimental support for the ab initio molecular orbital theory-based calculations that partial proton transfer may facilitate the rearrangement reaction in this AdoCbl-dependent enzyme." However, they also stated that the magnitude of the rate change was significantly less than expected from theoretical results.[3, 6] One aim of this present study is to resolve this apparent conflict.

A clue to the resolution is provided by the tritium-labeling experiments of Leadlay and co-workers.[7] They found that the rate-limiting step for the reaction catalysed by methylmalonyl-CoA mutase is different for the mutant enzymes[7] than for the wild-type enzyme.[9] A second aim of the present article is to qualitatively determine reasons for this difference.

We have, therefore, carried out high-level ab initio molecular orbital calculations on appropriate model systems. We report results for the hydrogen-transfer steps (steps A and C, Scheme 1) for the first time and, in addition, extend our previous work on the radical rearrangement (step B).

We initially replaced the thioformyl-CoA group of the substrate by a thioacid group (−C(=O)SH (**1**)) to study the rearrangement step.[3] In addition, we used a neutral carboxylic



acid substituent in place of a carboxylate anion to reflect interactions between Arg 207 and the carboxylate group of the substrate (Figure 1) which are likely to lead to only a small net charge in this region. Finally, we used a protonated base (initially the ammonium system, **3**) to model histidine at the active site, since the results of Banerjee and co-workers suggest that His 244 is likely to be protonated.[6] We note that system **3** models the environment of the migrating group at the active site in the wild-type enzyme with His 244 present, while system **1** models that in a mutant enzyme where histidine has been replaced with an amino acid moiety (specifically Gly, Ala or Gln) unlikely to provide analogous hydrogen-bonding interactions.

The potential-energy surfaces for intramolecular rearrangements involving no protonation (**1**), full protonation (**2**) and partial proton transfer (**3**), were fully characterized at the G3(MP2)-RAD(p) level (Table 1).[10, 11] The calculated energy barrier for rearrangement (298 K) decreases from 65.0 kJ mol^{-1} with no protonation to 25.1 kJ mol^{-1} with full protonation, while partial protonation by NH_4^+ leads to an intermediate energy barrier of 47.3 kJ mol^{-1}.

In addition to using an improved level of theory, we have extended our previous study of the rearrangement mechanism by further testing the adequacy of our model systems. We first consider the description of the CoA chain (Table 2). We find that

Table 1. *Calculated energy barriers (298 K) for rearrangement (step B, Scheme 1) and hydrogen transfers (steps A and C) associated with the unprotonated (**1**), protonated (**2**), and partially protonated (**3**) model systems.[a]*

Model	Step A [kJ mol^{-1}]	Step B [kJ mol^{-1}]	Step C [kJ mol^{-1}]
1 (unprotonated)	42.7	65.0	62.9
2 (protonated)	41.8	25.1	50.7
3 (partially protonated)	41.5	47.3	54.3

[a] Calculated with G3(MP2)-RAD(p) theory. See refs. [10, 13 – 15].

Table 2. *Calculated energy barriers (298 K) for rearrangement in a sequence of models for the methylmalonyl-CoA radical.*

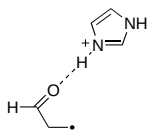
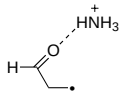
Model	B3-LYP/6-31G(d,p) [kJ mol^{-1}]	G3(MP2)-RAD(p) [kJ mol^{-1}]
thioacid (**1**)	61.6	65.0
methyl thioester[a]	64.8	65.8
aminoethyl thioester[b]	63.5	

[a] The SH group of **1** is replaced by SCH_3. [b] The SH group of **1** is replaced by $SCH_2CH_2NH_2$.

replacing the thioacid group in **1** by a methyl thioester increases the barrier to rearrangement by only 0.8 kJ mol^{-1}. B3-LYP calculations on a model which includes a more significant portion of the CoA chain (namely, replacing −SH in **1** with $-SCH_2CH_2NH_2$) also suggest only a small overall increase in the energy barrier to reaction over that for the thioacid **1**. We thus conclude that incorporation of the complete CoA chain is unlikely to significantly change the energy barriers of our basic models (Table 1).

Secondly, we consider the adequacy of the ammonium group to model His 244 by examining the degenerate rearrangement of the 3-propanal radical model[3] assisted by protonated imidazole (Table 3). We find that the effectiveness of protonated imidazole to catalyse the rearrangement lies roughly halfway between the extremes of full protonation and no protonation, and is some-

Table 3. *Calculated energy barriers (298 K) for the rearrangement of the 3-propanal radical, and partially protonated and fully protonated variants.[a]*

Model	Energy barrier [kJ mol^{-1}]
	50.0
	32.5
	19.4
	12.2

[a] Calculated with G3(MP2)-RAD(p) theory.

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what less than that of the ammonium group. We conclude that the ammonium group in **3** provides a useful representation, at least for the present purposes, of histidine at the active site.[12] With confidence in our reported energy barriers for the rearrangement step, we turn our attention to the energy barriers for hydrogen transfer.

We initially calculated energy barriers for hydrogen transfers (steps A and C, Scheme 1) in our model substrates at the G3(MP2)-RAD(p) level by using a methyl radical to represent the adenosyl radical ($^{\bullet}CH_2$—Ado) in step A (and methane to represent 5′-deoxyadenosine (CH_3—Ado) in step C).[13, 14] Our calculated energy barriers (Table 1) include temperature (298 K) and tunneling corrections.[15] Our approach leads to calculated energy barriers for abstraction leading to the formation of the substrate radical (step A) of 42.7 (no protonation), 41.5 (partial protonation by NH_4^+) and 41.8 kJ mol^{-1} (full protonation; Table 1). The energy barriers for hydrogen re-abstraction leading to product formation (step C) are 62.9 (no protonation), 54.3 (partial protonation by NH_4^+) and 50.7 kJ mol^{-1} (full protonation; Table 1).

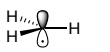
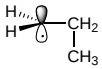
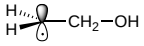
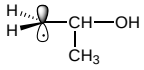
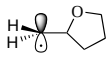
The adequacy of our approximate treatment of $^{\bullet}CH_2$—Ado (CH_3—Ado) was tested by systematically improving our model adenosyl radical, starting with the methyl radical and progressing to the (tetrahydro-2-furanyl)methyl radical (Table 4). The substrate used for this analysis was 3-propanal. From these calculations we estimate that improving our model for the adenosyl radical may lower the reported energy barriers for hydrogen transfer (Table 1) by up to 4 – 5 kJ mol^{-1}.

Our calculations of the energy barriers for hydrogen transfer, presented here for the first time, and our improved estimates for the energy barriers to rearrangement provide insight into the mechanism of action of methylmalonyl-CoA mutase. Our calculations suggest that the rate-limiting step in the wild-type enzyme (model **3**), among those investigated in the present study, is the hydrogen re-abstraction to form the (closed-shell) product (step C). Although it has been concluded that steps in addition to this re-abstraction (for example, product release) contribute to the overall rate limitation,[7, 9] our results are in qualitative agreement with the suggestion that the radical rearrangement step (B) is *not* itself rate limiting in the wild-type enzyme.[9] Removal of the histidine (ammonium) residue (model **1**), as in mutant enzymes in which this group is replaced by another amino acid, leads to an increase in the energy barriers for all the steps investigated in the present study. However, the energy barrier to rearrangement is increased to the greatest extent, with the result that the rearrangement step becomes rate limiting. These results are in accordance with experimental observations for the His 244 Gln mutant which suggest that the rearrangement of the substrate radical to the product radical becomes slow and hydrogen abstraction is even less rate determining in the mutant enzyme (model **1**) than in the wild-type enzyme (model **3**).[7]

Our calculated difference between the energy barriers for the rate-limiting steps, among those investigated in the present work, in the reactions catalysed by a mutant enzyme (model **1**) and the wild-type enzyme (model **3**) is 10.7 kJ mol^{-1}. When possible improvements to our computational models are accounted for, as discussed above, our best estimate for this difference is 10 ± 5 kJ mol^{-1}, which is consistent with the rate reductions of 100 – 1000 reported experimentally.[6, 7] If steps other than hydrogen transfer (step C) contribute to the overall rate limitation in the wild-type enzyme (model **3**),[9] the difference in the energy barriers for the rate-determining steps in the mutant and wild-type enzymes would be reduced. Our results suggest that in order to be consistent with the measured rate reduction, the barrier for the true rate-limiting step in the wild-type enzyme must be close to the energy barrier calculated for step C (model **3**). If a change in the rate-determining step is not taken into account, the rate reduction in mutase activity that accompanies removal of His 244 appears smaller than expected from the calculations, that is, smaller than predicted from the calculated increase in the energy barrier to rearrangement (step B) when the ammonium group is removed from our model (**3** → **1**).[6]

Our results should primarily be used to draw qualitative conclusions regarding the effects of mutations on the catalytic rate of methylmalonyl-CoA mutase. It is possible that there may be effects other than those considered here that could also lead to reductions in the measured rate. For example, the catalytic rate could be affected by changes in the conformation of the enzyme or the binding ability of the substrate as a consequence of mutations at the active site. Additionally, the rate could be affected by the water molecule that is found to occupy the cavity created when His 244 is replaced by alanine, which may lead directly to complete inactivation in some mutants.[7] We also note that our calculations on small models only account for a selection of active-site residues, and implicitly assume that other residues have a relatively small effect on the energy barriers investigated. For example, we have not attempted to address the question of how removal of Tyr 89 might affect the rate of rearrangement.[4c] However, we believe that our results provide important qualitative information about the relative magnitudes of the barriers of key steps associated with the reaction catalysed by methylmalonyl-CoA mutase. Most importantly, our results re-

Table 4. *Calculated energy barriers (298 K) for hydrogen abstraction from 3-propanal from a series of models for the adenosyl radical.*[a]

Model	Energy barrier [kJ mol^{-1}]
	42.1
	41.2
	39.0
	38.6
	38.0

[a] Calculated with G3(MP2)-RAD(p) theory. Energy barriers include a correction for tunneling according to Bell's formulation. See refs. [13 – 15].

emphasize the important role of the His 244 moiety in reducing the energy barrier to radical rearrangement.

The unraveling of the mechanism of action of methylmalonyl-CoA mutase demonstrates an effective synergistic interplay between theory and experiment. The present study complements previous experimental[4, 6, 7, 9] and theoretical[3] investigations in this respect. It provides a qualitative explanation for the magnitude of the measured rate reduction associated with removal of an important active-site residue (His 244), which may be ascribed to a change in the rate-determining step. Our results also re-emphasize the importance of His 244 in catalysing the radical rearrangement step.

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[1] For recent reviews, see, for example: a) B. T. Golding, W. Buckel in *Comprehensive Biological Catalysis*, *Vol. 3* (Ed.: M. L. Sinnott), Academic Press, London, **1997**, pp. 239 – 259; b) P. R. Evans, F. Mancia in *Vitamin B_{12} and B_{12}-Proteins* (Ed.: B. Kräutler, D. Arigoni, B. T. Golding), Wiley-VCH, Weinheim, **1998**, pp. 217 – 226; c) R. Banerjee, S. Chowdhury in *Chemistry and Biochemistry of B_{12}* (Ed.: R. Banerjee), Wiley, New York, **1999**, pp. 707 – 730.

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[3] a) D. M. Smith, B. T. Golding, L. Radom, *J. Am. Chem. Soc.* **1999**, *121*, 1383 – 1384; b) D. M. Smith, B. T. Golding, L. Radom, *J. Am. Chem. Soc.* **1999**, *121*, 9388 – 9399.

[4] a) F. Mancia, N. H. Keep, A. Nakagawa, P. F. Leadlay, S. McSweeney, B. Rasmussen, P. Bosecke, O. Diat, P. R. Evans, *Structure* **1996**, *4*, 339 – 350; b) F. Mancia, P. R. Evans, *Structure* **1998**, *6*, 711 – 720; c) N. H. Thomä, T. W. Meier, P. R. Evans, P. F. Leadlay, *Biochemistry* **1998**, *37*, 14368 – 14393; d) F. Mancia, G. A. Smith, P. R. Evans, *Biochemistry* **1999**, *38*, 7999 – 8005.

[5] The Protein Data Bank (PDB) code for the structure shown in Figure 1 is 5req. For additional information, see ref. [4c].

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[8] a) J. Rétey, *Angew. Chem.* **1990**, *102*, 373 – 379; *Angew. Chem. Int. Ed. Engl.* **1990**, *29*, 355 – 361; b) J. Rétey in *Chemistry and Biochemistry of B_{12}* (Ed.: R. Banerjee), Wiley, New York, **1999**, pp. 271 – 287.

[9] T. W. Meier, N. H. Thomä, P. F. Leadlay, *Biochemistry* **1996**, *35*, 11791 – 11796.

[10] G3(MP2)-RAD(p) calculations have been successfully employed to study related B_{12}-mediated rearrangements: a) S. D. Wetmore, D. M. Smith, L. Radom, *J. Am. Chem. Soc.* **2000**, *122*, 10208 – 10209; b) S. D. Wetmore, D. M. Smith, B. T. Golding, L. Radom, *J. Am. Chem. Soc.* **2001**, *123*, 7963 – 7972.

[11] These data represent a slight improvement over results from G2(MP2,SVP)-RAD(p) studies and a significant improvement over energetics reported previously that were calculated at the B3-LYP level.[3] We also note that re-investigation of the potential-energy surfaces for **1**, **2** and **3** revealed additional conformers which more closely resemble the substrate conformation in the crystal structure. The new structure for **1** is also lower in energy than that previously reported.[3] For consistency, we report all energy barriers with respect to these new conformers. For full details of the geometries, see the Supporting Information.

[12] Support for the appropriateness of our model is also provided by the good agreement between the calculated separation of the carbonyl oxygen atom of the 3-propanal radical and the nitrogen atom in the (protonated) imidazole ring (2.68 Å) or NH_4^+ group (2.59 Å) and the corresponding O ··· N distance in the crystal structure (2.7 Å, Figure 1). The O ··· N distance in the more elaborate NH_4^+-bonded model **3** is 2.59 Å.

[13] We note good agreement between energy barriers for hydrogen abstraction from methane by a methyl radical calculated at 298 K and including a tunneling correction at G3(MP2)-RAD(p) (52.7 kJ mol^{-1}) and W1 (49.9 kJ mol^{-1}) levels of theory (see refs. [14] and [15]). W1 is a high-level theoretical procedure that has been designed with the goal of reproducing experimental results to within 1 kJ mol^{-1} (see: J. M. L. Martin, G. de Oliveira, *J. Chem. Phys.* **1999**, *111*, 1843 – 1856).

[14] The energy barriers to hydrogen abstraction are reported with respect to the infinitely separated reactants.

[15] We have approximated the contribution of tunneling to the energy barriers to hydrogen transfer using Bell's tunneling correction (see: R. P. Bell, *The Tunnel Effect in Chemistry*, Chapman and Hall, London, **1980**, pp. 60 – 63). We use B3-LYP/6-31G(d,p) frequencies and G3(MP2)-RAD(p) energy barrier heights to calculate the tunnelling factor. The appropriateness of this analysis is currently being investigated in more detail.

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Kinetic Monitoring of Self-Replicating Systems through Measurement of Fluorescence Resonance Energy Transfer

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autocatalysis · self-replication · fluorescence spectroscopy · kinetics · oligonucleotides

Chemical self-replicating systems have been the subject of various investigations over the last 15 years.[1] In the simplest implementation, two starting materials A and B react with each other to give a self-complementary template molecule C through an autocatalytic reaction cycle.[2] The latter cycle involves the termolecular complex ABC between the template and its precursors as well as the template duplex C_2 and can be described by the gross reaction equation $A + B + C \rightleftharpoons ABC \rightarrow C_2 \rightleftharpoons 2C$. Three basic classes of template molecules have been utilized so far, namely oligonucleotide analogues,[3] peptides,[4] and artificial templates.[5] Despite the structural diversity of these templates, the kinetics of their autocatalytic synthesis were always found to be limited by product inhibition caused by the dimerization of template C.[6] Consequently, these systems typically exhibited the so-called square-root law and autocata-

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