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Tyrosine 89 Accelerates Co−Carbon Bond Homolysis in Methylmalonyl-CoA Mutase

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Abstract: The contribution of the active-site residue, Y89, to the trillion-fold acceleration of Co−carbon bond homolysis rate in the methylmalonyl-CoA mutase-catalyzed reaction has been evaluated by site-directed mutagenesis. Conversion of Y89 to phenylalanine or alanine results in a 10^3-fold diminution of k_{cat} and suppression of the overall kinetic isotope effect. The spectrum of the enzyme under steady-state conditions reveals the presence of AdoCbl but no cob(II)alamin. Together, these results are consistent with homolysis becoming completely rate determining in the forward direction in the two mutants and points to the role of Y89 as a molecular wedge in accelerating Co−carbon bond cleavage.

The cofactor, AdoCbl or coenzyme B_{12}, functions as a radical generator in enzymes that catalyze 1,2-rearrangement reactions.[1,2] A remarkable aspect of the enzyme-catalyzed cleavage of the Co−carbon bond of the cofactor to generate radicals is the ca. trillion-fold rate acceleration compared to the uncatalyzed reaction,[3] and the strategies used by these enzymes have been a subject of ongoing debate. A member of this family of enzymes, methylmalonyl-CoA mutase, catalyzes the isomerization of methylmalonyl-CoA to succinyl-CoA (Figure 1) and is found in both bacteria and mammals. The first step common to all mutases, homolysis of the Co−carbon bond, is observed upon addition of substrate and leads to the formation of cob(II)alamin and the deoxyadenosyl radical (Figure 1, step I). This is followed by hydrogen atom (H-atom) abstraction from the substrate, methylmalonyl-CoA, by the deoxyadenosyl radical to generate a reactive primary substrate radical (step ii). Rearrangement, by a mechanism that is at present poorly understood, generates a secondary product-centered radical (step iii). Finally, a second H-atom transfer from deoxyadenosine yields product (step iv), and recombination of the cofactor-centered radical pair (step v) completes a turnover cycle. Evidence for the intermediacy of radical species in the reaction has been obtained from stopped-flow spectroscopy which reveals the presence of cob(II)alamin[4,5] and from EPR spectroscopy which demonstrates the presence of a biradical intermediate comprising cob(II)alamin and an organic radical.[6,7]

The wild-type enzyme controls the reactivity of the cofactor by kinetic coupling of the first two chemical steps: homolysis (Figure 1, step i) and abstraction of a hydrogen atom from the substrate (step ii).[4] This renders the homolysis step isotope sensitive, and large deuterium isotope effects have been measured (49.9 at 5 °C) with [CD_3]-labeled substrate, which indicates the involvement of quantum mechanical tunneling in this H-atom transfer step.[8] X-ray crystallography studies have

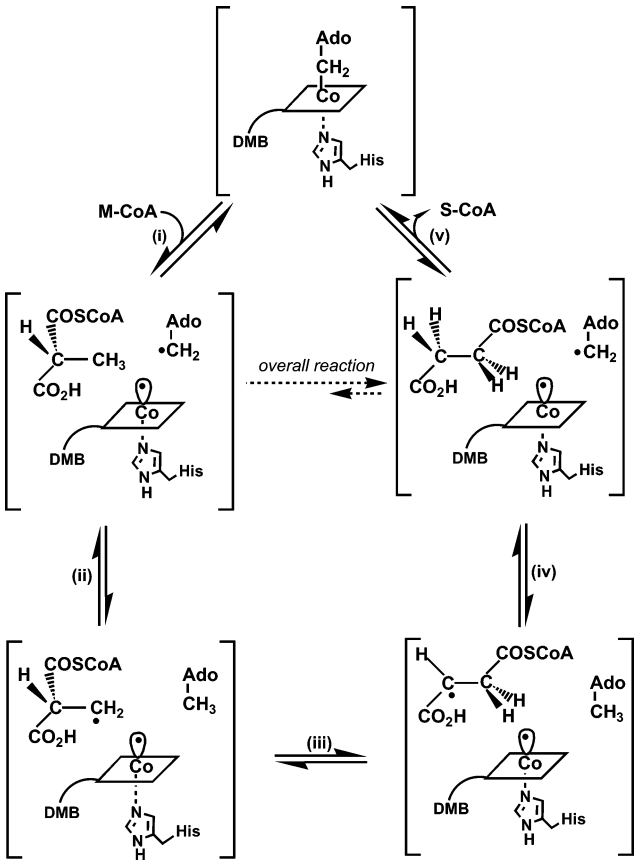

Figure 1. Postulated reaction mechanism of methylmalonyl-CoA mutase. M-CoA and S-CoA denote methylmalonyl-CoA and succinyl-CoA, respectively.

(1) Halpern, J. *Science* **1985**, *227*, 869−875.
(2) Banerjee, R. *Biochemistry* **2001**, *40*, 6191−8.
(3) Finke, R. G.; Hay, B. P. *Inorg. Chem.* **1984**, *23*, 3041−3043.
(4) Padmakumar, R.; Padmakumar, R.; Banerjee, R. *Biochemistry* **1997**, *36*, 3713−3718.
(5) Chowdhury, S.; Banerjee, R. *Biochemistry* **2000**, *39*, 7998−8006.
(6) Zhao, Y.; Such, P.; Retey, J. *Angew. Chem., Int. Ed. Engl.* **1992**, *31*, 215−216.
(7) Padmakumar, R.; Banerjee, R. *J. Biol. Chem.* **1995**, *270*, 9295−9300.

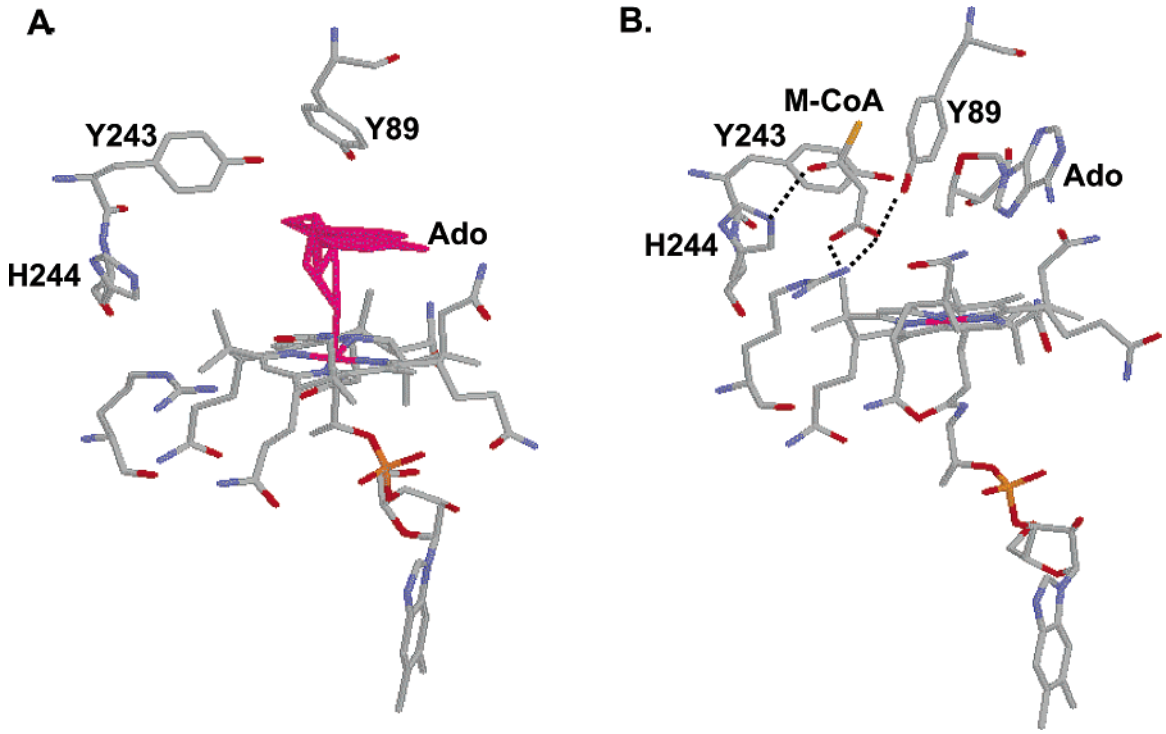

Figure 2. Movement of Y89 in the active site of methylmalonyl-CoA mutase induced by binding of substrate. (A) Structure of active-site residues in the absence of substrate taken from PDB file 3REQ with partial density for the adenosine moiety (in red) of AdoCbl. (B) Structure of active-site residues following binding of substrate (only a fragment up to the thioester bond is shown here) taken from PDB file 4REQ. Significant motion of Y89 is observed.

revealed a large conformational change that accompanies substrate binding and results in a TIM barrel snapping in around the CoA tail.[9] The active-site residue that undergoes the most significant motion in the process is Y89, which moves from its position above and roughly parallel to the adenine ring to one that destroys the adenine-binding site and establishes new hydrogen-bonding contacts with the substrate and the cofactor (Figure 2). This observation has led to the hypothesis that Y89 plays a key role in labilizing the Co−carbon bond by physically wedging into the space occupied by deoxyadenosine.[9]

The effects of the conservative substitution, Y89F, have been partially evaluated by Leadlay and co-workers who also reported the crystal structure of this mutant enzyme.[10] This structure was obtained in the presence of the nonhydrolyzable substrate, succinylcarbadethia-CoA, and revealed that it was essentially superimposable on that of the wild-type enzyme except at the locus of the alteration. However, the electron density on the aromatic ring of F89 was broadened, indicating increased mobility in the absence of the hydrogen-bonding interaction with the substrate that is observed with the wild-type Y89 residue.

The Y89F substitution was found to lower k_{cat} in the reverse direction, i.e., conversion of succinyl-CoA to methylmalonyl-CoA, by 580-fold but had little effect on the K_m value.[10] The overall deuterium isotope effect in the reverse direction was similar in the mutant ($^{H}V/^{D}V = 5.2 \pm 0.8$) and wild-type ($^{H}V/^{D}V = 3.4 \pm 0.5$) enzymes within the limits of experimental error. On the basis of the directional sensitivity of the fraction of tritium partitioning from AdoCbl to substrate versus product, which is in marked contrast to the behavior of the wild-type enzyme,[11] it was concluded that loss of the hydroxyl group resulted in an increase in the rearrangement barrier between substrate and product radicals. However, the effect of this mutation on the Co−carbon homolysis step was not examined.

In this study, we have examined the role of Y89 in accelerating the Co−carbon bond homolysis reaction by creating a conservative mutation, F89, and by creating a cavity at that position with the A89 mutation. Both mutants have small effects on the K_m for substrate (3- to 7-fold) but profound effects on the homolysis and the kinetically coupled substrate radical generation step and exhibit $\sim 10^3$-fold lower k_{cat} value in the forward direction. The absence of an overall isotope effect in conversion of methylmalonyl-CoA to succinyl-CoA is consistent with a change in the rate-determining step from product release in wild-type enzyme to Co−carbon bond homolysis preceding the first isotope-sensitive step. We estimate that $\sim 10^3$ to 10^4 of the total 10^{12}-fold acceleration of the homolysis rate may be derived from the contributions of a single residue, Y89, to this reaction.

Methods

Chemicals. AdoCbl and methylmalonlyl-CoA were purchased from Sigma. Radioactive [^{14}C]-CH_3-malonyl-CoA (56.4 Ci/mol) was purchased from New England Nuclear. All other chemicals were reagent grade commercial products and were used without further purification.

Construction of Site-Specific Mutants. Site-directed mutants were created using the Quick Change strategy (Stratagene) and the following primers for Y89F (forward: CCCTGGACGATTCGCCAG*TTC*GCCGGTTTCTCCACGGC) and Y89A (forward: CCCTGGACGATTCGCCAG*GCC*GCCGGTTT CTCCACGGC). The mutagenic codons specifying F and A are italicized. The reverse primers had the complementary sequences.

(8) Chowdhury, S.; Banerjee, R. *J. Am. Chem. Soc.* **2000**, *122*, 5417−5418.
(9) Mancia, F.; Evans, P. *Structure* **1998**, *6*, 711−720.
(10) Thomä, N. H.; Meier, T. W.; Evans, P. R.; Leadlay, P. F. *Biochemistry* **1998**, *37*, 14386−14393.
(11) Meier, T. W.; Thomä, N. H.; Leadlay, P. F. *Biochemistry* **1996**, *35*, 11791−11796.

Table 1. Comparison of Kinetic Parameters of Y89 Mutants and Wild-Type Methylmalonyl-CoA

	mutase		
	wild type[a]	Y89F	Y89A
k_{cat} (s^{-1} at 37 °C)	120	0.125 ± 0.02	0.137 ± 0.02
K_d (AdoCbl), μM	0.17 ± 0.01	0.135 ± 0.03	0.29 ± 0.05
K_m(M-CoA), μM	133 ± 37	357 ± 83	926 ± 153
$^{H}V/^{D}V$	5 ± 0.6	1.2 ± 0.09	0.96 ± 0.12

[a] These parameters are taken from ref 17. The radiolabel assay containing an (*R*,*S*) mixture of methylmalonyl-CoA was employed as described previously.[12]

Enzyme Expression and Purification. The mutant enzymes were purified through the step preceding reconstitution with cofactor using a modified procedure described previously for isolation of wild-type enzyme.[7]

Enzyme Assays. Specific activity of the mutase was determined in the radiolabeled assay at 37 °C as described previously.[12] One unit of activity catalyzes the formation of 1 μmol of succinyl-CoA min^{-1} at 37 °C. The concentration of mutant enzymes was increased 1000-fold with respect to the wild-type enzyme in the standard assay. Kinetic parameters for the two mutants were determined by increasing the duration of the fixed timed assay from 3 to 5 min in the presence of varying concentrations of [^{14}C]-methylmalonyl-CoA (100 to 5000 μM). Protein concentration was determined using the Bradford reagent (BioRad) and bovine serum albumin as a standard.

Determination of Equilibrium Binding Constants for AdoCbl by Fluorescence Spectroscopy. Addition of the cofactor to apo-methylmalonyl-CoA mutase results in a decrease in fluorescence emission at 340 nm and has been used to determine the equilibrium dissociation constant for the wild-type enzyme.[13] The enzyme concentration used in this experiment was 0.8 μM in 50 mM potassium phosphate buffer, pH 7.5. Successive aliquots (2−5 μL) of a stock 50 μM AdoCbl solution prepared in the same buffer were added followed by incubation at 4 °C for 30 min prior to measurement of the fluorescence emission.

Determination of Equilibrium Binding Constant by UV−Visible Absorption Spectroscopy. Binding of AdoCbl to methylmalonyl-CoA mutase was followed spectrophotometrically as previously described.[13] The enzyme concentration used in this experiment was 30 μM, and total AdoCbl concentration added was varied from 1.5 to 40 μM. The K_d values obtained from the equilibrium fluorescence experiments were used to calculate the free ligand concentrations. The experimentally obtained values for ΔA_{562} nm were plotted versus the concentration of free AdoCbl, *L*, and the data were fitted using eq 1.

$$L_b = E_0L/(K_d + L) + cL \quad (1)$$

where *cL* is the contribution from a low-affinity nonspecific binding site.

Results and Discussion

Steady-State Kinetic Properties of Mutant Proteins. The mutant proteins were expressed at levels comparable to the wild-type protein, and were purified using the same procedure.[7] A comparison of the steady-state kinetic parameters (Table 1) reveals significant differences. Thus, k_{cat} for the conversion of methylmalonyl-CoA to succinyl-CoA is diminished ~10^3-fold, whereas the K_m for substrate and K_{m-app} for cofactor are only modestly affected. Significantly, the overall reaction in the forward direction is insensitive to isotopic substitution, suggesting that a step preceding one of the two isotope-sensitive steps (ii and iv in Figure 1) is rate-determining. Of the two possibilities, homolysis (step i) and rearrangement (step iii), the former appears to be significantly impacted since the spectrum of the enzyme under steady-state conditions reveals only the presence of AdoCbl (Figure 3). This is in contrast to wild-type enzyme in which the ratio of AdoCbl:cob(II)alamin is ~4:1 under steady-state conditions.[4] If an increase in the rearrangement barrier were chiefly responsible for suppression of the isotope effect, the enzyme would be predicted to accumulate in the cob(II)alamin state.

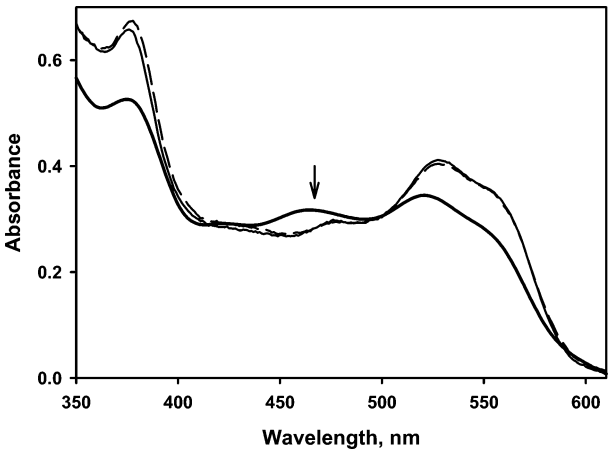

Figure 3. Comparison of wild-type and Y89F enzyme spectra under steady-state conditions. UV−visible spectra of holo-Y89F (55 μM in AdoCbl concentration, thin solid line) in 50 mM potassium phosphate buffer, pH 7.5 at 37 °C. Addition of *(R,S)*-methylmalonyl-CoA to a final concentration of 2 mM, prepared in the same buffer, did not show any significant change in the spectra of enzyme (dashed line) over a 2-h incubation. The spectrum of wild-type enzyme under steady-state turnover conditions (thick line) is from ref 4. The arrow at 470 nm indicates formation of cob(II)alamin in wild-type enzyme.

Presteady-State Kinetics. In principle, the absence of observable cob(II)alamin under steady-state turnover conditions could result from a completely rate-determining product-release step which would result in accumulation of the cofactor in the AdoCbl state. However, the presteady-state formation of cob(II)alamin would not be affected under these conditions. To distinguish between these possibilities, we attempted to measure the homolysis reaction under presteady-state conditions with the F89 mutant as described previously for the wild-type enzyme.[5] However, we were unable to measure this rate constant since the spectroscopic changes accompanying formation of cob(II)alamin were not observed in the presence of either protiated or deuterated substrate, consistent with the spectral observations under steady-state conditions. These results indicate that the barrier or equilibrium for the homolysis step have been dramatically altered by loss of the hydroxyl group in the F89 mutant and support the conclusion that homolysis rather than the rearrangement step represents the kinetic bottleneck in the forward direction. In wild-type enzyme, the rate-limiting step is believed to be product release, leading to the predominance of AdoCbl in the steady-state spectrum of the enzyme. The absence of detectable levels of cob(II)alamin under presteady-state conditions in the Y89F mutant is also consistent with an earlier step along the reaction coordinate, limiting conversion of substrate to product.

In the wild-type enzyme, k_{hom} is >10-fold faster than k_{cat}.[4] Thus, an upper limit for the net effect of the Y89F mutant on homolysis is an ~10^4-fold deceleration, corresponding to 5.4

(12) Taoka, S.; Padmakumar, R.; Lai, M.-t.; Liu, H.-w.; Banerjee, R. *J. Biol. Chem.* **1994**, *269*, 31630−31634.
(13) Chowdhury, S.; Banerjee, R. *Biochemistry* **1999**, *38*, 15287−94.

kcal mol^{-1}, which could be attributed to loss of a single hydrogen-bonding group, i.e., the OH in Y89.

Stereoselectivity of Mutant Enzymes. The bulk and position of the Y89 residue have been postulated to be important in the stereoselectivity for the (*R*)-isomer exhibited by the wild-type enzyme.[14] A plausible explanation for the higher K_m for substrate displayed by the Y89 mutants is loss of stereochemical discrimination in binding and possibly, in substrate utilization. The equilibrium concentrations of substrate, (*R,S*)-methylmalonyl-CoA, and product, succinyl-CoA, were determined following HPLC separation of the two.[15] The K_{eq} for the mutase reaction is reported to be ~20 in favor of succinyl-CoA when starting with (*R*)-methylmalonyl-CoA.[16] In both mutants and the wild-type enzyme, succinyl-CoA and the unreacted (*S*)-isomer of methylmalonyl-CoA were present at ca. equal concentrations (not shown). Thus, these results preclude utilization of (*S*)-methylmalonyl-CoA by the Y89 mutants as substrate but do not rule out its binding.

Oxygen Stability of Mutant Enzymes. Mutations in the active-site residue, H244, which is in hydrogen-bonding contact with the substrate (Figure 2), lead to acute oxygen sensitivity of the reaction due to increased interception of the cob(II)alamin intermediate.[17,18] In contrast, mutations at Y89 lead to a highly stable enzyme whose activity is insensitive to oxygen and which is very resistant to photolysis even when irradiated with a tungsten lamp in the presence or absence of substrate (not shown). Qualitatively, the latter result is consistent with decreased accumulation of the cob(II)alamin intermediate, leading to reduced susceptibility of the reaction to oxidative side reactions.

Qualitative Free Energy Profile. The consequences of mutating the active-site Y89 residue on the energetics of the reaction are discussed within the framework of a qualitative free energy profile shown in Figure 4. In wild-type enzyme, the overall deuterium isotope effect is suppressed, and AdoCbl and cob(II)alamin are observed in a 4:1 ratio under steady-state conditions. Furthermore, the homolysis rate is fast (~10-fold greater than k_{cat}). Taken together, the predominance of AdoCbl is consistent with accumulation of the enzyme in the E·P complex and with product release being rate limiting.

Tritium-partitioning experiments furnish two additional pieces of information.[10,11] First, the fraction of tritium released from AdoCbl to product versus that from substrate is independent of the direction in which the reaction is monitored, and the isotope partitions 75:25 in favor of succinyl-CoA over methylmalonyl-CoA with wild-type enzyme. This indicates that the substrate and product radicals are in rapid equilibrium and that the rearrangement barrier is low and not rate limiting. Second the tritium isotope effect, monitored by the rate of appearance of tritium in succinyl-CoA from 5′-tritiated AdoCbl when the reaction is initiated with methylmalonyl-CoA, reports on the isotope effect on the second H-atom transfer step and is influenced by the rate of product release, i.e., the subsequent

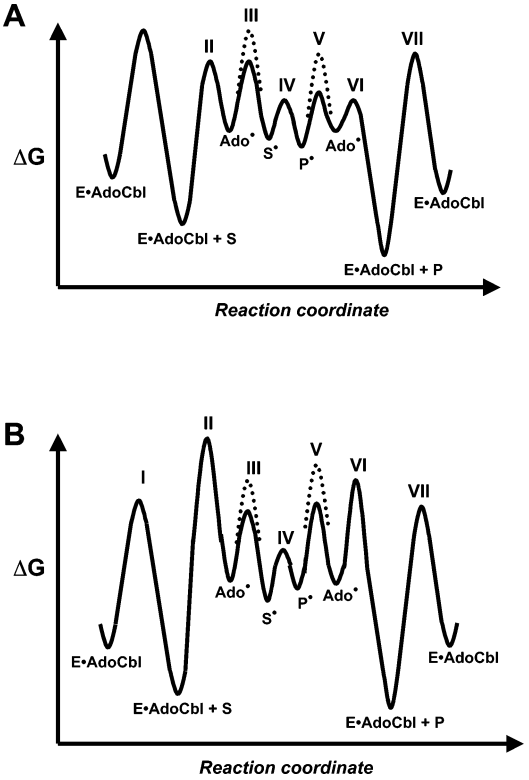

Figure 4. Qualitative free energy profiles of wild-type (A) and Y89F (B) forms of methylmalonyl-CoA mutase. The energy barriers that are isotope sensitive are depicted by the dotted lines, and the isotope effect is shown on the transition state for clarity although it actually affects the ground state. For wild-type enzyme (A), the barrier for product release is shown as being high in both forward and reverse directions. These barriers have been arbitrarily set at the same height in the mutant and wild-type enzyme and the relative increase in the other barriers in the mutant is shown. Ado•, S•, and P• refer to the deoxyadenosyl-, substrate-, and product radicals respectively, and the cob(II)alamin radical that is present along with these organic radicals is not shown for clarity.

step.[11] For wild-type enzyme, the tritium isotope effect on H-atom transfer from deoxyadenosine to product radical is suppressed ($^{H}k/^{T}k = 3.2$),[19] consistent with product release being rate limiting. The tritium isotope effect on the H-atom transfer from deoxyadenosine to the substrate radical (i.e. when the reaction is initiated with succinyl-CoA) has not been measured. However, the deuterium isotope effect on cob(II)alamin formation has been measured in the forward direction (i.e. on the first H-atom transfer step) under presteady-state conditions and found to be anomalously high, suggesting quantum mechanical tunneling.[8]

The Y89F mutant displays several kinetic features that distinguish it from wild-type enzyme and reveals that loss of the hydroxyl group in the tyrosine residue has a marked influence on the energetics of the reaction. First, while the overall deuterium isotope effect is suppressed in the forward direction, it is comparable to that exhibited by the wild-type enzyme in the reverse direction. Second, cob(II)alamin does not accumulate at detectable levels under presteady-state or steady-state conditions. Third, the partitioning of tritium from AdoCbl to substrate and product is sensitive to the direction in which the reaction is initiated.[10] When methylmalonyl-CoA is em-

(14) Mancia, F.; Smith, G. A.; Evans, P. R. *Biochemistry* **1999**, *38*, 7999−8005.
(15) Padmakumar, R.; Padmakumar, R.; Banerjee, R. V. *Methods Enzymol.* **1997**, *279*, 220−224.
(16) Kellermeyer, R. W.; Allen, S. H. G.; Stjernholm, R.; Wood, H. G. *J. Biol. Chem.* **1964**, *239*, 2562−2569.
(17) Maiti, N.; Widjaja, L.; Banerjee, R. *J. Biol. Chem.* **1999**, *274*, 32733−32737.
(18) Thoma, N. H.; Evans, P. R.; Leadlay, P. F. *Biochemistry* **2000**, *39*, 9213−21.
(19) The tritium kinetic isotope effects reported in ref 10 were overestimated since an equivalence factor of 2 rather than 3 was employed. The derivation of this correction factor is discussed in Chih, H. W.; Marsh, E. N. *Biochemistry* **2001**, *40*, 13060−13067.

ployed as substrate, the tritium partitions 60:40 in favor of succinyl-CoA whereas the ratio is 90:10 in favor of succinyl-CoA when the latter is employed to initiate the reaction. Fourth, the tritium isotope effect on the conversion of product radical to product is 19.[10,19]

These kinetic properties can be interpreted in the context of the free energy profile shown in Figure 4B. Increase in the homolysis barrier (II) results in suppression of the deuterium isotope effect in the forward direction and the failure to observe cob(II)alamin under presteady-state and steady-state conditions. The altered tritium partitioning ratios reveal an increase in the rearrangement barrier (IV) relative to the wild-type enzyme, while the sensitivity of the fractionation to the direction of the reaction indicates that the barriers connecting deoxyadenosine and substrate (III) or product (V) radicals have been differentially altered. Thus, the barrier between the product radical and deoxyadenosine (V) is increased relative to that between the substrate radical and deoxyadenosine (III), leading to less favorable partitioning toward product when the reaction is initiated with methylmalonyl-CoA. This is also consistent with the increase in the magnitude of the tritium isotope effect for the H-atom transfer between deoxyadenosine and product radical.

Suppression of the overall deuterium isotope effect on V_{max} in the forward but not reverse direction is explained by differences in the balance of energetic barriers encountered during progression of the reaction in the two directions. Thus, in the forward direction, the highest barrier, Co−carbon bond homolysis (II), suppresses the isotope effect, whereas in the reverse direction, homolysis (VI) and product release (I) contribute to its partial suppression.

Can the multiple energetic perturbations resulting from loss of a single hydrogen-bonding side chain in the active site be rationalized on chemical grounds? Structural insights into the active site of methylmalonyl-CoA mutase in the presence and absence of substrate are helpful in guiding our speculations here. The crystal structures reveal significant repositioning of Y89 accompanying substrate binding that has two discernible effects. First, the apparent destruction of the deoxyadenosine binding site in the E·S complex appears to result from motion of Y89 and could explain the contribution to the Co−carbon bond homolysis step. The deoxyadenosine binding site is not well determined in the structures of methylmalonyl-CoA mutase, and it is not known if there are direct interactions between Y89 and deoxyadenosine. In principle, Y89 may play a role in stabilizing the deoxyadenosyl radical and thereby promote the homolysis reaction. Second, the hydroxyl group of Y89 is engaged in hydrogen-bonding interactions with the substrate carboxylate and with a cofactor side chain (Figure 2). These interactions may be important in precise positioning of the substrate and in modulating the distance between the H-atom transfer sites, i.e., between substrate (or product) and deoxyadenosine, a consideration that becomes particularly important in tunneling and in promoting homolysis by kinetic coupling.

Loss of the hydrogen bond could differentially affect precise positioning of the product versus substrate radical leading to increase in barrier V versus III in Figure 4B. Furthermore, the relative stabilities of the substrate and product radicals that are β and α to the carboxylate, respectively, may be influenced by loss of this hydrogen-bonding interaction. Thus, the product radical is likely to be preferentially destabilized in the Y89F mutant as shown in Figure 4B. However, it is important to note that the substrate carboxylate is engaged in additional electrostatic interactions via a two-pronged hydrogen-bonding contact with the guanidino group of R207 in the active site. Thus, neutralization of the negative charge on the carboxylate is unlikely to be governed primarily by Y89. This view is supported by a recent computational study on the effect of the Y89F mutant on the rearrangement reaction.[20] Loss of the hydrogen-bonding interaction is predicted to increase the isomerization barrier from methylmalonyl-CoA to succinyl-CoA by 1 kcal mol^{-1}. The hydrogen-bond-accepting oxygen of the substrate carboxyl is affected primarily and displays an increased charge in the mutant.[20]

Conclusions

Movement of Y89 propelled by occupation of the substrate-binding site, plays a pivotal role in initiating radical chemistry by cleavage of the Co−carbon bond in methylmalonyl-CoA mutase. Tritium partitioning studies, in which isotope transfer from the 5′ position of AdoCbl to either substrate or product was monitored, revealed an altered and increased barrier to rearrangement in the Y89F mutant.[10] In addition, together with the large tritium kinetic isotope effect on H-atom transfer step between deoxyadenosine and the product radical in the mutant versus a highly suppressed one in wild-type enzyme,[10,11] they suggest a change in the relative barriers connecting deoxyadenosine and substrate versus product radicals.[10] In combination with the present study, which reveals that the cob(II)alamin intermediate is not visible under presteady-state or steady-state conditions and that the overall isotope effect in the forward direction is suppressed, the major role of the aromatic wedge residue, Y89, is ascribed to accelerating Co−carbon bond homolysis driven by substrate-binding energy.

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(20) Loferer, M. J.; Webb, B. M.; Grant, G. H.; Liedl, K. R. *J. Am. Chem. Soc.* **2003**, *125*, 1072−1078.