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Rotation of the *exo*-Methylene Group of (*R*)-3-Methylitaconate Catalyzed by Coenzyme B_{12}-Dependent 2-Methyleneglutarate Mutase from *Eubacterium barkeri*

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Abstract: 2-Methyleneglutarate mutase from the anaerobe *Eubacterium (Clostridium) barkeri* is an adenosylcobalamin (coenzyme B_{12})-dependent enzyme that catalyzes the equilibration of 2-methyleneglutarate with (*R*)-3-methylitaconate. Two possibilities for the mechanism of the carbon skeleton rearrangement of the substrate-derived radical to the product-related radical are considered. In both mechanisms an acrylate group migrates from C-3 of 2-methyleneglutarate to C-4. In the "addition−elimination" mechanism this 1,2-shift occurs via an intermediate, a 1-methylenecyclopropane-1,2-dicarboxylate radical, in which the migrating acrylate is simultaneously attached to both C-3 and C-4. In the "fragmentation−recombination" mechanism the migrating group, a 2-acrylyl radical, becomes detached from C-3 before it starts bonding to C-4. In an attempt to distinguish between these two possibilities we have investigated the action of 2-methyleneglutarate mutase on the stereospecifically deuterated substrates (*Z*)-3-methyl[2′-$^2H_1$]itaconate and (*Z*)-3-[2′-$^2H_1$,*methyl*-$^2H_3$]methylitaconate. The enzyme catalyzes the equilibration of both compounds with their corresponding *E*-isomers and with a 1:1 mixture of the corresponding (*E*)- and (*Z*)-2-methylene-[2′-$^2H_1$]glutarates, as shown by monitoring of the reactions with ^1H and ^2H NMR. In the initial phase of the enzyme-catalyzed equilibration a significant excess (8−11%) of (*E*)-3-methyl[2′-$^2H_1$]itaconate over its equilibrium value was observed ("*E*-overshoot"). The *E*-overshoot was only 3−4% with (*Z*)-3-[2′-$^2H_1$,*methyl*-$^2H_3$]methylitaconate because the presence of the deuterated methyl group raises the energy barrier from 3-methylitaconate to the corresponding radical. The overshoot is explained by postulating that the migrating acrylate group has to overcome an additional energy barrier from the state leading back to the substrate-derived radical to the state leading forward to the product-related radical. It is concluded that the fragmentation−recombination mechanism can provide an explanation for the results in terms of an additional energy barrier, despite the higher calculated activation energy for this pathway.

Introduction

2-Methyleneglutarate mutase from the anaerobe *Eubacterium (Clostridium) barkeri*[1,2] is a coenzyme B_{12}-dependent enzyme that catalyzes an unusual reaction, the equilibration of 2-methyleneglutarate **1a** with (*R*)-3-methylitaconate **2a** (Scheme 1; reviews[3,4]). In this rearrangement the 4-H_{re} of 2-methyleneglutarate migrates intermolecularly via the coenzyme to the 3-methylene group to yield a completely racemized methyl group, whereas the (*R*)-methine group of (*R*)-3-methylitaconate is formed with inversion of configuration from the 4-methylene group of 2-methyleneglutarate.[5,6] 2-Methyleneglutarate mutase has been isolated as a homotetramer (4 × 67 kDa) containing various cobalamin species, including coenzyme B_{12} (adenosylcobalamin) and cob(II)alamin.[7−9] The gene coding for the enzyme was cloned and overexpressed in *Escherichia coli*[10] to give apoenzyme that was reconstituted to active holoenzyme by addition of coenzyme B_{12}.[11]

The carbon skeleton rearrangement catalyzed by the mutase belongs to a family of more than 10 similar reactions mediated by coenzyme B_{12}-dependent enzymes.[12,13] These reactions are

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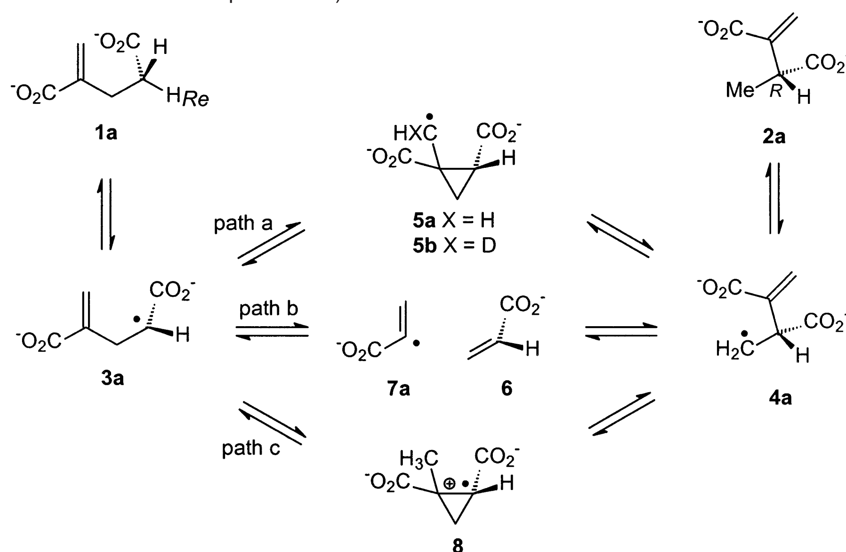
Scheme 1. Possible Pathways for 2-Methyleneglutarate Mutase (Path a, Addition−Elimination; Path B, Fragmentation−Recombination; Path C, Addition−Elimination with Protonation/Deprotonation)



believed to be initiated by the substrate-induced homolysis of the carbon−cobalt bond of the coenzyme. The resulting 5′-deoxyadenosyl radical abstracts a hydrogen atom from the substrate (e.g., 2-methyleneglutarate **1a** in Scheme 1) to generate a protein-bound substrate-derived radical S• (2-methyleneglutar-4-yl radical **3a**), which rearranges to the product related radical P• [(*R*)-3-methyleneitaconate radical **4a**]. Redonation of the hydrogen atom to P• yields the product [(*R*)-3-methylitaconate **2a** from **1a**] and the 5′-deoxyadenosyl radical, which upon reaction with the cob(II)alamin spectator reforms the carbon−cobalt bond. A critical issue to resolve is the nature of the rearrangement of S• into P•, in which a group [e.g. an acrylate moiety C(=CH$_2$)CO$_2$H, Scheme 1] migrates from one carbon atom (C-3 of 2-methyleneglutarate) to an adjacent carbon atom (C-4 of 2-methyleneglutarate). For 2-methyleneglutarate mutase and the other carbon skeleton mutases containing a suitably placed π-bond in the migrating group [e.g. C(=O)SCoA for methylmalonyl-CoA], two possibilities for the migration are a 1,2-shift without the migrating group X ever becoming detached from its carbon termini (addition−elimination mechanism, shown for 2-methyleneglutarate mutase in Scheme 1, path a via the cyclopropylmethylene radical **5a**) or a fragmentation−recombination mechanism, in which the migrating group is detached from one carbon before it starts bonding to the other (Scheme 1, path b). The fragmentation is shown in Scheme 1 to give an alkene (i.e., acrylate **6**) + a radical (the 2-acrylyl species **7a**), although in principle charge separation could occur, for example, giving an alkene radical cation plus an anion.[14]

Possible pathways for the carbon skeleton mutases were explored using ab initio molecular orbital calculations.[15−17] For methylmalonyl-CoA mutase, substantial acceleration of the addition−elimination pathway through "partial protonation" of the carbonyl oxygen of the COSCoA group was demonstrated.[15] There is experimental evidence to support this proposal.[18,19] Similar calculations on the 2-methyleneglutarate mutase reaction showed that an addition−elimination pathway via intermediates **3a**, **4a**, and **5a** (Scheme 1, path a) was energetically more favorable than fragmentation−recombination via acrylate **6** and the 2-acrylyl radical **7a** (Scheme 1, path b).[16] Furthermore, an even lower energy pathway was addition−elimination with partial protonation at the *exo*-methylene group (Scheme 1, path c). The extreme form of this mechanism with a full protonation of the *exo*-methylene group would generate an intermediate methylcyclopropane radical cation (**8**).

Despite the theoretical results, the available experimental evidence for 2-methyleneglutarate mutase has provided support for the fragmentation−recombination pathway (Scheme 1, path b). Thus, the enzyme is inhibited by acrylate with a square dependence on acrylate concentration.[11] Furthermore, when added to 2-methyleneglutarate mutase, acrylate induced an EPR spectrum that resembles the spectra produced by the corresponding substrates.[11] The addition−elimination route for 2-methyleneglutarate mutase (Scheme 1, path a) proceeds via cyclopropylmethylene radical **5a**. However, none of the stereoisomers of 1-methylcyclopropane-1,2-dicarboxylate, one of which must have the same chirality as radicals **5a** and **8** (1*R*,2*R* or 1*S*,2*R*), inhibited the enzyme.[11] For glutamate mutase, fragmentation−recombination via acrylate and the 2-glycinyl radical seems to be the only possibility and is in accord with EPR data[20] and the crystal structure analysis of the enzyme.[21] Furthermore, glutamate mutase is inhibited by an equimolar mixture of glycine and acrylate, but by neither glycine nor acrylate alone.[11] Glycine and acrylate in a 1:1 ratio have been trapped after incubating radiolabeled glutamate with glutamate mutase and quenching the reaction with trifluoroacetic acid. This experiment demonstrated that under steady-state conditions 6% of the enzyme-bound substrate fragments to the 2-glycinyl radical and acrylate,[22] whereas ca. 50% of the enzyme bound

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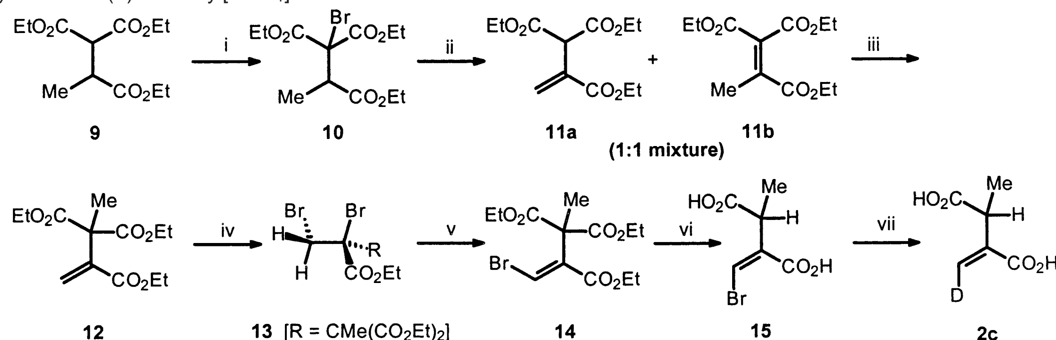
Scheme 2. Synthesis of (*Z*)-3-Methyl[2′-$^2H_1$]itaconate **2c**[a]



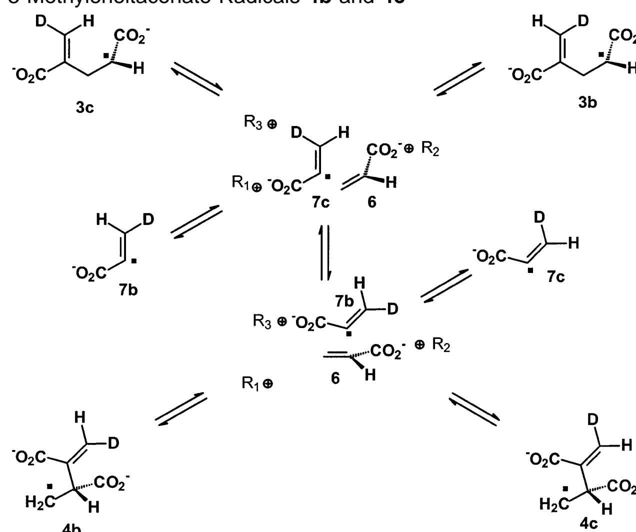
[a] [*Reagents*: i, KO^{t}Bu/*N*-bromosuccinimide (77%); ii, DBU (86%); iii, NaH/THF, MeI (80%); iv, Br_2 in CH_2Cl_2 (94%); v, Bu_4N^+ F^- in HMPA + molecular sieves (21−38%); vi, 20% HBr (57%); vii, Na−Hg/D_2O (97%)]; (*Z*)-3-methyl[2′-$^2H_1$,methyl-$^2H_3$]itaconate **2e** was prepared in a similar manner from triethyl propane-1,1,2-tricarboxylate **9**, but using trideuteriomethyl iodide in step iii. N.b. all chiral compounds are a racemate. For compounds **2a−2f** only the biologically active *R*-enantiomer is shown.

substrate occurs as the 4-glutamyl radical.[23] The relative amounts of 6 vs 50% are consistent with the EPR data.[20]

We have previously reported the enzyme-catalyzed rotation of the *exo*-methylene group of 2-methyleneglutarate.[24] To detect this rotation, (*E*)-2-[2′-$^2H_1$]methyleneglutarate **1b** was incubated with 2-methyleneglutarate mutase and coenzyme B_{12}. Equilibration of the *E*- and *Z*-isomers (**1b** and **1c**, respectively) of 2-[2′-$^2H_1$]methyleneglutarate was indeed observed, concomitant with the formation of (*R*)-3-methylitaconate (presumably **2b** and **2c**). The rate of rotation was found to be comparable to substrate turnover.[25] Interpretation of this result requires an intermediate that enables a low barrier rotation of this methylene group. The addition−elimination mechanism (Scheme 1, path a) provides a seemingly plausible candidate—cyclopropylmethylene radical **5a**. In this radical, the *exo*-methylene group has been calculated to rotate with a relatively low barrier (15.2 kJ mol^{-1},[16] cf. 14.5 kJ mol^{-1} for the parent cyclopropylmethylene radical[26]). Similarly, if the radical cation **8** were an intermediate, the rotation barrier around the bond between the methyl group and cyclopropane ring should be extremely low.[16] The fragmentation−recombination mechanism (Scheme 1, path b) can also provide an explanation for the observed rotation. According to ab initio molecular orbital (m.o.) calculations the nonlinear 2-acrylyl radical **7b** can invert to **7c** (see Scheme 3) with a barrier of only 5.2 kJ mol^{-1}.[16]

We now report results from incubating (*Z*)-3-methyl[2′-$^2H_1$]-itaconate **2c** and (*Z*)-3-[2′-$^2H_1$,*methyl*-$^2H_3$]methylitaconate **2e** with 2-methyleneglutarate mutase. The advantage of using these labeled substrates is that the deuterium content of all of the products can be more accurately measured than in the case of (*E*)-2-[2′-$^2H_1$]methyleneglutarate **1b**. This is because the equilibrium constant for the reaction greatly favors 2-methyleneglutarate over 3-methylitaconate (K_{eq} = 0.06).[24] During these studies we have discovered an unexpected distribution ("*E*-overshoot") of (*E*)-3-methyl[2′-$^2H_1$]itaconate **2b** and (*E*)-3-[2′-$^2H_1$,*methyl*-$^2H_3$]methylitaconate **2d**, respectively, arising during the initial phase of the enzyme-catalyzed equilibration. In addition to the NMR studies described, we have determined isotope effects under steady-state conditions for 2-methylene-[4-$^2H_2$]glutarate **1d** and 3-[*methyl*-$^2H_3$]methylitaconate **2f**. The data obtained from NMR experiments and measurements of isotope effects are discussed in the context of the mechanistic options described above.

Scheme 3. Rearrangement of the 2-Methyleneglutar-4-yl Radicals **3b** and **3c** via Fragmentation−Recombination to the 3-Methyleneitaconate Radicals **4b** and **4c**[a]



[a] R_1, R_2, and R_3 symbolize the three arginine residues, which form the "arginine claw" in glutamate mutase.[4] The interconversion of the 2-acrylyl radicals **7b** and **7c** may occur when bound to the same arginine residue or during the "hand-over" from R_1 and R_3 or vice versa.

Results and Discussion

Synthesis of (*Z*)-3-Methyl[2′-$^2H_1$]itaconate 2c and (*Z*)-3-[2′-$^2H_1$,*methyl*-$^2H_3$]Methylitaconate 2e. These compounds were both prepared from triethyl propane-1,1,2-tricarboxylate **9** as shown in Scheme 2. The carbanion derived from the triester **9**, in the presence of a catalytic quantity of potassium *tert*-butoxide, was treated with *N*-bromosuccinimide to give bromotriester **10**. The succinimide anion released during the bromination allowed the generation of more anion from the triester. Treatment of the bromotriester **10** with 1,8-diazabicyclo[5.4.0]undec-7-en (DBU) gave a 1:1 mixture of triethyl prop-1-ene-2,3,3-tricarboxylate **11a** and triethyl prop-1-ene-1,1,2-tricarboxylate **11b**, which was deprotonated to a common carbanion with sodium hydride. Trapping of this carbanion with iodomethane gave **12**,

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Table 1. Kinetic Constants for 2-Methyleneglutarate Mutase under Steady-State Conditions[a]

substrate	kinetic constants			
	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m (s^{-1} M^{-1})	$^D(k_{cat}/K_m)$
2-methyleneglutarate **1a**	30 ± 3^b	3.1 ± 0.8	9700	
2-methylene[4-$^2H_2$]glutarate **1g**	2.3 ± 0.3^b	2.3 ± 0.6	1000	10 ± 2
(*R*)-3-methylitaconate **2**a	18 ± 3^c	0.4 ± 0.1	45000	
(*R*)-3-[*methyl*-$^2H_3$]methylitaconate **2f**	1.1 ± 0.1^c	0.38 ± 0.05	2900	16 ± 4
(*R,Z*)-3-methyl[2′-$^2H_1$]itaconate **2c**	17 ± 3^d	nd	43000[e]	
(*R,Z*)-3-[*methyl*-$^2H_3$]methyl[2′-$^2H_1$]itaconate **2e**	1.4 ± 0.3^d	nd	3500[e]	12 ± 4^e

[a] N.b. The data for the 3-methylitaconates were determined with the racemic compounds. [b] 3-Methylitaconate formation was measured with the coupled spectroscopic assay. [c] 2-Methyleneglutarate formation was measured discontinuously by HPLC. [d] 2-Methyleneglutarate formation was measured by NMR. [e] K_m's for **2c** and **2e** were assumed to be equal to those for **2a** and **2f** (0.4 mM); nd = not determined.

which was converted into the corresponding dibromide **13** by treatment with bromine. Successful dehydrobromination of **13** required considerable experimentation. Eventually it was found that tetra-*n*-butylammonium fluoride in dry hexamethylphosphorus triamide produced a sufficient yield of triethyl 1-bromobut-1-ene-2,3,3-tricarboxylate, which was almost exclusively the *E*-isomer **14**. This stereochemical outcome can be rationalized as kinetic control arising from the minimization of steric and polar interactions in the transition state for an E2 elimination (cf. the conformer shown for **13** in Scheme 2). Remarkably, during heating of **14** with 20% hydrobromic acid, conversion of the *E*- to the *Z*-isomer concomitant with ester hydrolysis and decarboxylation afforded **15**. Presumably, this isomerization is caused by reversible addition of hydrogen bromide to the bromomethylene group of **14**/**15**, leading to an intermediate dibromomethyl compound, which eliminates HBr. The conversion of (*Z*)-2′-bromo-3-methylitaconic acid **15** into (*Z*)-3-methyl-[2′-$^2H_1$]itaconic acid (**2c**) was accomplished stereospecifically using sodium amalgam in deuterium oxide.[24] The sequence described from the mixture **11a**/**11b** was repeated using iodo-[$^2H_3$]methane in place of unlabeled iodomethane for the methylation step, to give (*Z*)-3-[2′-$^2H_1$,*methyl*-$^2H_3$]methylitaconate **2e**. Finally, 3-[*methyl*-$^2H_3$]methylitaconate **2f** was obtained using [$^2H_3$]iodomethane, but omitting the steps which introduced deuterium into the *exo*-methylene group. ^{1}H NMR studies of **2c** and **2e** showed that each compound contained 96−97% of the *Z*-isomer and 3−4% of the *E*-isomer. Since each chiral compound synthesized was racemic and 2-methyleneglutarate mutase only utilizes the *R*-isomer of 3-methylitaconate,[5] 50% of **2c**, **2e**, and **2f** did not participate in the enzymatic conversions. In the NMR experiments described below, the integrals of the resonances determined for the 3-methylitaconate in a reaction mixture were therefore corrected by subtracting half of the value of the integral of the initial signal. The K_m values derived for 3-methylitaconates **2a** and **2f** refer to the *R*-isomer.

Isotope Effects under Steady-State Conditions. Initial NMR experiments showed that (*Z*)-3-[2′-$^2H_1$,*methyl*-$^2H_3$]methylitaconate **2e** reacted much more slowly to 2-methyleneglutarate than (*Z*)-3-methyl[2′-$^2H_1$]itaconate **2c**, indicative of a relatively large primary kinetic isotope effect for the initial abstraction of a hydrogen (deuterium) atom from the substrate methyl group. The isotope effect on the initial rate of enzymatic turnover was determined by an HPLC-based discontinuous assay as $^D(k_{cat}/K_m) = 16 \pm 4$ with 3-[*methyl*-$^2H_3$]methylitaconate **2f** as compared to unlabeled 2-methylitaconate (Table 1). The k_{cat} values are in accord with the NMR data showing the need for 20 times more enzyme to achieve comparable initial rates of formation of 2-methyleneglutarate from **2e** versus **2c**. For 2-methyleneglutarate, a similar isotope effect of $^D(k_{cat}/K_m) = 10 \pm 2$ was determined using a coupled UV spectrophotometric assay[27] with 2-methylene[4-$^2H_2$]glutarate **1g** as compared to unlabeled 2-methyleneglutarate (Table 1). On the basis of the data given for isotope effects, the rate-limiting steps of the 2-methyleneglutarate mutase reaction can be taken to be hydrogen atom abstraction from 2-methyleneglutarate (**1b**/**1c** → **3b**/**c**) or 3-methylitaconate (**2b**/**c** → **4b**/**c**), analogous to glutamate mutase[28,29] and methylmalonyl-CoA mutase.[30,31] In this work, it is proposed that a conformational change during the rearrangement step occurs at a rate comparable to hydrogen atom abstraction.

Detection of the Rotation of the *exo*-Methylene Group. To observe the expected rotation of the *exo*-methylene group of the methylitaconates **2b** and **2c** during conversion to the corresponding 2-methyleneglutarates **1b** and **1c**, several series of independent ^{1}H NMR experiments were performed using different preparations of 2-methyleneglutarate mutase, which ensured the quality and reproducibility of the data (cf. Figures 1 and 2). Since signals at concentrations of less than 0.2 mM proton could not be reproducibly quantified, the reaction was performed with the high concentration, for an enzymatic conversion, of 50 mM *rac*-methylitaconate **2c**. Owing to inhibition of 2-methyleneglutarate mutase at high ionic strength, higher concentrations of the dicarboxylate could not be used. A particular problem was the sensitivity of the enzyme−coenzyme complex to light and dioxygen, and the time-dependent inactivation of the enzyme during turnover. On account of these problems not all experiments proceeded to equilibrium (Figure 1, B and D). However, for every experiment the same trends were evident. It was found that for each NMR experiment quantities ≥0.08 mg of 2-methyleneglutarate mutase in a total volume of 0.75 mL were sufficient to reach equilibrium *and* observe the “overshoot” phenomenon (Figure 1A).

The ^{1}H NMR spectrum shown in Figure 3, derived from a typical experiment, exhibits resonances in the spectral region δ 5.25−5.85 for the protons connected to the *exo*-methylene group of the reaction species (**1b**, **1c**, **2b**) derived from (*Z*)-3-methyl-[2′-$^2H_1$]itaconic acid (**2c**). Resonances were observed at δ 5.29 (2′-H of 2-methyleneglutarate **1c**), 5.33 (2′-H of 3-methylitaconate **2c**), 5.67 (2′-H of 2-methyleneglutarate **1b**), and 5.79 (2′-H of 3-methylitaconate **2b**). The assignment of the reso-

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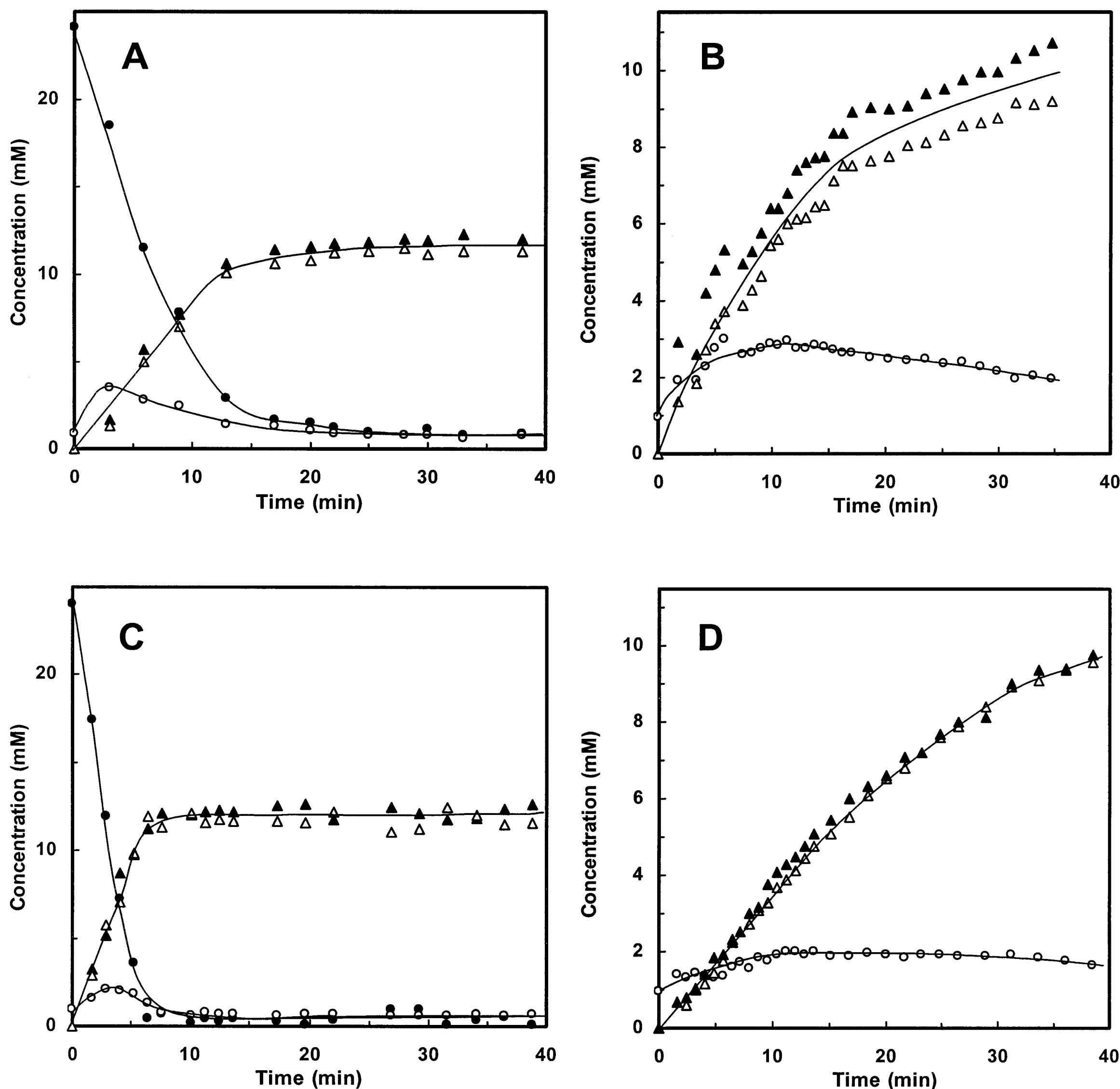


Figure 1. Kinetics of the rotation of the *exo*-methylene groups of 3-methylitaconate and 2-methyleneglutarate catalyzed by 2-methyleneglutarate mutase. Each incubation contained in a total volume of 0.75 mL of 50 mM potassium phosphate, pD = 7.4 (uncorrected), 50 mM racemic 3-methyl[2′-$^{2}H_{1}$]itaconate comprising 24 mM *Z*-isomer **2c** and 0.8 mM *E*-isomer **2b** (tubes A and B), 50 mM racemic 3-[2′-$^{2}H_{1}$,*methyl*-$^{2}H_{3}$]methylitaconate comprising 24 mM *Z*-isomer **2e** and 1.0 mM *E*-isomer **2d** (tubes C and D), and *apo*-2-methyleneglutarate mutase, 0.08 mg in tube A, 0.05 mg in tube B, 1.5 mg in tube C, and 0.6 mg in tube D. The isomerizations were initiated by addition of adenosylcobalamin, 10 μM in tubes A and B, 50 μM in tubes C and D. At times indicated, ^{1}H NMR spectra were taken, and the intensities of the signals were obtained by integration (see Experimental Section). Tubes A and B: (●) (*Z,R*)-3-methyl[2′-$^{2}H_{1}$]itaconate **2c**; (○) (*E,R*)-3-methyl[2′-$^{2}H_{1}$]itaconate **2b**; (▲ (*Z*)-2-methylene[2′-$^{2}H_{1}$]glutarate **1c**; (△) (*E*)-2-methylene[2′-$^{2}H_{1}$]glutarate **1b**. Tubes **C** and **D**: (●) (*Z,R*)-3-methyl[2′-$^{2}H_{1}$,*methyl*-$^{2}H_{3}$]itaconate **2e**; (○) (*E,R*)-3-methyl[2′-$^{2}H_{1}$,*methyl*-$^{2}H_{3}$]itaconate **2d**; (▲ (*Z*)-2-methylene[2′-$^{2}H_{1}$, 3-$^{2}H_{2}$, 4-$^{2}H_{1}$]-glutarate **1e**; (△) (*E*)-2-methylene[2′-$^{2}H_{1}$, 3-$^{2}H_{2}$, 4-$^{2}H_{1}$]glutarate **1d**. The slightly lower values for the *E*-isomers of the 2-methyleneglutarates as compared to those of the *Z*-isomers are probably due to a small systematic error in the integrations. The relatively high errors of the values for the *Z*-isomers of the 3-methylitaconates are due to subtraction of 24 mM enzymatically inactive *S*-enantiomer.

nances for **1b** and **1c**, and by inference for **2b** and **2c**, was confirmed by a ^{1}H NMR NOE experiment performed with unlabeled 2-methyleneglutaric acid, which showed that the resonance at higher field (i.e., lower δ value) is the *exo*-methylene proton *syn* to the 3-methylene group.[24] The coupling constants observed at higher dispersion in this work (500 vs 400 MHz) are consistent with this assignment. The *exo*-methylene proton of **1c** exhibits a triplet by interaction with the 3-methylene protons in the *syn* position ($J = 4$ Hz), whereas the corresponding signal of **2c** consists of a doublet through coupling with the methine proton ($J = 4$ Hz). The couplings of the protons in the *anti* positions of **1b** and **2b** are much weaker ($J = 1$ Hz, not resolved in Figure 3). The integrated areas of resonances from the *exo*-methylene protons were plotted against time (Figure 1). The equilibration finally led to 0.75 mM (3%) each of **2b** and **2c**, concurrent with the generation of about 12 mM (47%) each of the *E*-isomer (**1b**) of 2-[2′-$^{2}H_{1}$]methyleneglutarate and the corresponding *Z*-isomer (**1c**) (Figure 1A). These results are in agreement with the known equilibrium constant ($K_{eq} = 0.06$).[24]

In the early stages of the reaction, the amount of **2b** substantially exceeded its equilibrium concentration of 0.75 mM (Figure 1A). From several experiments ($n = 7$) with a good signal-to-noise ratio in the first few minutes, a maximum amount of the *E*-isomer **2b** in the range 2.8−3.5 mM was observed, corresponding to an *E*-overshoot of 1.9−2.6 mM (8−10%). Changing the temperature from 22 to 4 °C did not significantly affect the magnitude of the overshoot. The data of Figure 2

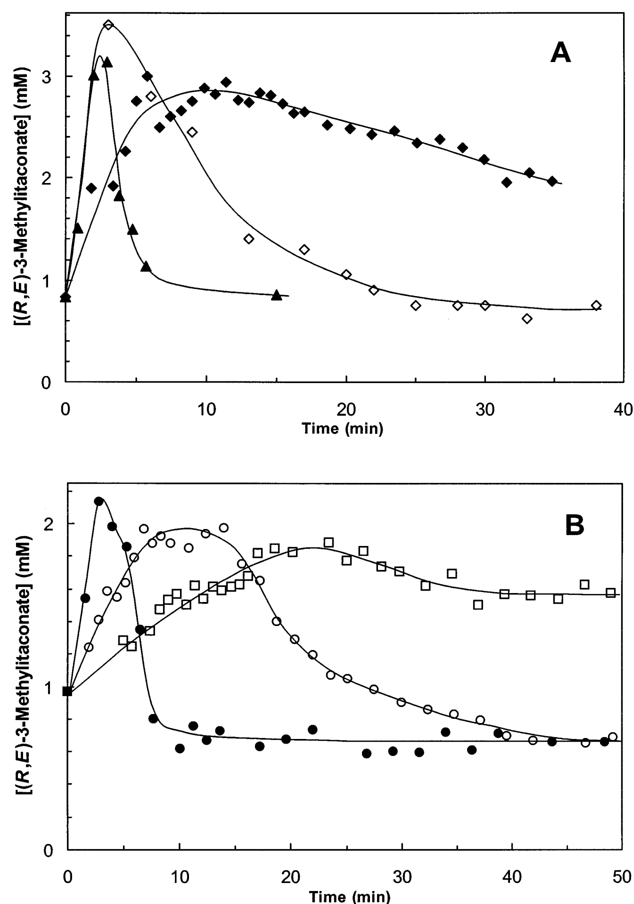


Figure 2. Time course of the formation of the *E*-isomers of the (*R*)-3-methyl[2′-$^{2}H_1$]itaconates (*E*-overshoot) at various concentrations of 2-methyleneglutarate mutase. (A) with (*Z,R*)-3-methyl[2′-$^{2}H_1$]itaconate and (B) with racemic (*Z,R*)-3-methyl[2′-$^{2}H_1$,*methyl*-$^{2}H_3$]itaconate as substrates. The incubations were performed as described in the legend of Figure 1. The amounts of enzyme used per NMR-tube were (A): (◆) 0.05 mg, (◆) 0.08 mg, (▲) 0.28 mg; (B): (□) 0.3 mg, (○) 0.9 mg, (●) 1.5 mg. The reactions were initiated in A with 10 μM and in B with 50 μM adenosylcobalamin. After about 20−30 min gradual losses of 2-methyleneglutarate mutase activity account for the slow or absent approach to equilibrium.

show that the amount of the *E*-overshoot apparently was independent of the concentration of the enzyme, whereas the higher the enzyme concentration, the shorter was the time to reach the maximum. Experiments with less enzyme (Figure 1B) indicated that the initial rate of the formation of the *E*-isomer of 3-methylitaconate **2b** was about that of either the *E*- or the *Z*-isomer of 2-methyleneglutarate, **1b** or **1c**.

To explore whether the *E*-overshoot occurs before or after hydrogen abstraction from the methyl group, the experiments were repeated with (*Z*)-3-[2′-$^{2}H_1$,*methyl*-$^{2}H_3$]methylitaconate **2e**. Since hydrogen abstraction from the methyl group is associated with the very high primary kinetic isotope effect $^{D}(k_{cat}/K_m) = 16 \pm 4$ (Table 1), the deuterated compound should enhance an *E*-overshoot if the rotation occurs prior to hydrogen abstraction, since the reaction toward 2-methyleneglutarate formation is slowed. On the other hand, rotation after hydrogen abstraction would decrease the *E*-overshoot, since the rate of the back reaction to the *E*- and *Z*-isomers should be similarly diminished by the isotope effect of the deuterium transfer from the intermediate [5′-$^{2}H_3$]deoxyadenosine to the 3-methyleneitaconate radical. With (*Z*)-3-[2′-$^{2}H_1$,*methyl*-$^{2}H_3$]methylitaconate **2e**, it was necessary to use ca. 20-fold more enzyme to achieve a similar time course for the equilibrium (Figure 1C). Since the rates were lower, the initial velocities could be determined more accurately. In these experiments ($n = 8$), the maximum amount of (*E*)-3-[2′-$^{2}H_1$,*methyl*-$^{2}H_3$]methylitaconate **2d** was in the range 1.8−2.2 mM corresponding to an overshoot of only 0.9−1.1 mM (3.5−4.5%). Furthermore, experiments with less enzyme indicated that the initial rate of formation of the *E*-isomer **2d** was slowed by a factor of ca. 2 compared to that of either the *E* or the *Z*-isomer of 2-methyleneglutarate, **1d** or **1e** (Figure 1D). The rates, however, were not directly comparable, since the concentration of 2-methyleneglutarate started at zero, whereas that of the *E*-isomer of 3-methylitaconate commenced already at 0.9 mM, which is 45% of the overshoot.

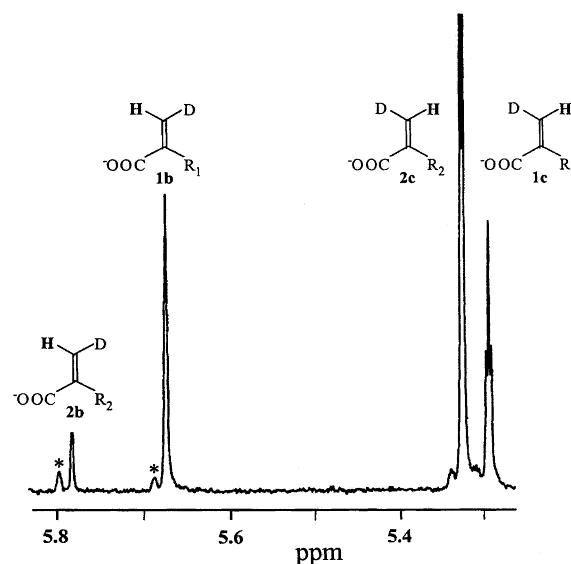


Figure 3. ^{1}H NMR spectrum (500 MHz) in D_2O, pD 7.4 (uncorrected), of the *exo*-methylene protons of a mixture of (*E*)- and (*Z*)-2-[2′-^{2}H]methyleneglutarate (**1b** and **1c**, respectively; $R_1 = -CH_2-CH_2-COO^-$) as well as of (*E*)- and (*Z*)-3-methyl[2′-^{2}H]itaconate (**2b** and **2c**, respectively; $R_2 = -CH(CH_3)-COO^-$). The incubation mixture contained in a total volume of 0.75 mL of 10 mM potassium phosphate, 50 mM racemic 3-methyl[2′-^{2}H]-itaconate, comprising 48 mM *Z*-isomer **2c** and 1.7 mM *E*-isomer **2b**, and 0.1 mg of *apo*-2-methyleneglutarate mutase. The isomerization was initiated by addition of 10 μM adenosylcobalamin. The spectrum was taken after 30 min at 22 °C, when the reaction had reached equilibrium. The equilibrium mixture consisted of about 1.6 mM **2b**, 25 mM **2c**, 12 mM **1b**, and 12 mM **1c**. Whereas **2b** represents a nearly equal mixture of the (*R*)- and (*S*)-isomers of (*E*)-3-methyl[2′-^{2}H]itaconate, **2c** mainly consists of the enzymatically inactive (*S*)-enantiomer (97%). The asterisks indicate unlabeled material.

Results were obtained using ^{2}H NMR that complemented those from the ^{1}H NMR experiments. The ^{2}H NMR spectrum of (*Z*)-3-[2′-$^{2}H_1$,*methyl*-$^{2}H_3$]methylitaconate **2d** in H_2O showed broad resonances at δ 1.1 (CD_3) and 5.6 (=CH*D*). After addition of enzyme and coenzyme to initiate the enzymatic reaction and attainment of equilibrium, new resonances were observed at δ 2.1 (CD_2CHD of 2-methyleneglutarate **1d** and **1e**), 2.2 (CD_2-CHD of 2-methyleneglutarate **1d** and **1e**), 5.1 (2′-D of 2-methyleneglutarate **1d**), and 5.5 (2′-D of 2-methyleneglutarate **1e**) (Figure 4). Owing to the inherent lower sensitivity of ^{2}H NMR it was not possible to follow the time dependence of exchange or to detect quantitatively a change in intensity of the resonance at δ 5.6 [masked by unreacted (*S*)-**2e**] or to observe a resonance for **2d**. The data showed, as expected, that a deuterium is transferred from the CD_3 group of (*Z*)-3-[2′-$^{2}H_1$,*methyl*-$^{2}H_3$]methylitaconate **2e** to take up the 4-*re*[5] position of the 2-methyleneglutarates **1d** and **1e**. The carbon atom and

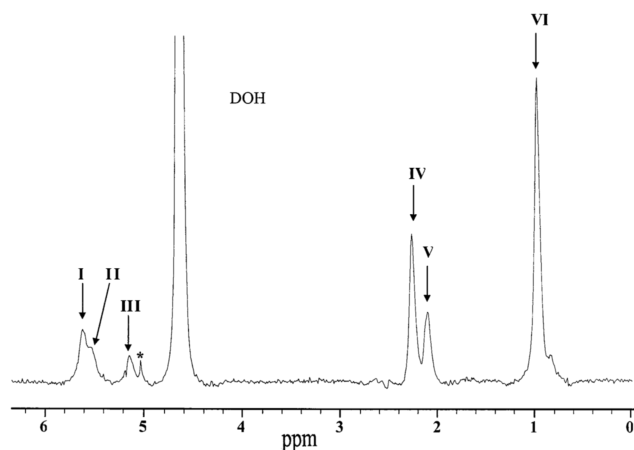


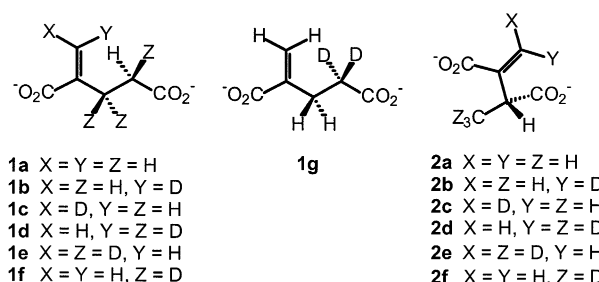
Figure 4. ^{2}H NMR spectrum (76.7 MHz) in H_2O, pH 7.4, of the mixture obtained by incubation of racemic (*Z*)-3-methyl[2′-$^2H_1$,*methyl*-$^2H_3$]itaconate **2e** with 2-methyleneglutarate mutase until equilibrium was reached. The reaction mixture was contained in a total volume of 0.75 mL of 10 mM potassium phosphate, with 48 mM racemic (*Z*)-3-methyl[2′-$^2H_1$,*methyl*-$^2H_3$]-itaconate **2e** and 2 mM racemic (*E*)-isomer **2d**, and 3 mg of *apo*-2-methyleneglutarate mutase. The isomerization was initiated by addition of 50 μM adenosylcobalamin. The spectrum was taken after 30 min at 22 °C, when the reaction had reached equilibrium. The equilibrium mixture consisted of about 1.6 mM **2d**, 25 mM **2e**, 12 mM **1d**, and 12 mM **1e**. Whereas **2d** represented a nearly equal mixture of the (*R*)- and (*S*)-isomers of (*E*)-3-methyl[2′-^{2}H, *methyl*-$^2H_3$]itaconate, **2e** mainly consisted of the enzymatically inactive (*S*)-enantiomer (97%). Signal I, *exo*-methylene group of **2e**; signal II, *exo*-methylene group of **1e**; signal III, *exo*-methylene group of **1d**; the signal of the *exo*-methylene group of **2d** is too small to be visible; the large signal at 4.65 ppm is DOH; signal IV, 3-CD_2 of **1d** and **1e**; signal V, 4-CDH of **1d** and **1e**; signal VI, methyl groups (CD_3) of **2d** and **2e**. The asterisk indicates an artifact signal.

the remaining two deuterons of the CD_3 group became the 3-methylene group (CD_2) of the 2-methyleneglutarates **1d** and **1e**. Although the deuterium transfer occurred via the adenosyl 5′-methylene group of adenosylcobalamin, the two hydrogen atoms did not significantly dilute the migrating deuterium due to the low concentration of the coenzyme (10−50 μM) compared to the active substrate (25 mM).

For ^{1}H NMR spectra, careful comparison of the integral of the *exo*-methylene proton of **1b**, **1c**, **2b**, or **2c** with the integrals from other C−H protons did not reveal any loss of the alkene hydrogen to D_2O solvent during the reaction. Analogous observations were made for tetradeuterated 3-methylitaconate **2e** and its associated species. Similarly, ^{2}H NMR analysis did not show any gain of deuterium in the 2-methyleneglutarate **1d** and **1e** produced from **2e** for an enzymatic experiment performed in D_2O. In this case the reaction was worked up, and the sample was redissolved in H_2O for analysis. These observations are in agreement with an earlier result, in which no incorporation of tritium into 2-methyleneglutarate or 3-methylitaconate occurred from tritiated water.[32]

Mechanistic Implications. As described in the Introduction, there are opposing hypotheses concerning the mechanism of action of 2-methyleneglutarate mutase [addition−elimination, addition−elimination with (partial) protonation, fragmentation−recombination: see Scheme 1, paths a−c]. Although ab initio m.o. calculations show that fragmentation−recombination is energetically less favorable than either of the addition−elimination pathways,[16] the enzyme could, in principle, use fragmentation−recombination to achieve rearrangement pro-

(32) Kung, H.; Tsai, L. *J. Biol. Chem.* **1971**, *246*, 6436.

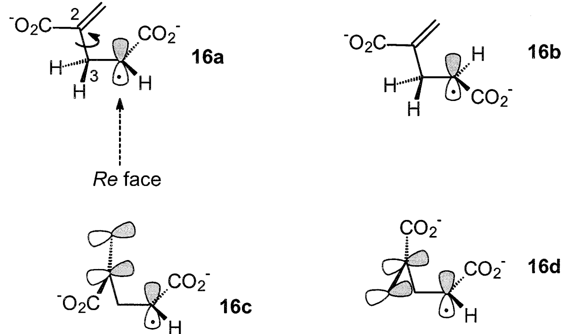


vided that this pathway is *not* energetically more difficult than the substrate/product C−H abstraction steps. Starting from 2-methyleneglutarate, stereoelectronic considerations indicate that both the fragmentation−recombination and addition−elimination mechanisms require the C-2/C-3 bond of the 2-methyleneglutar-4-yl radical **3a** to be near parallel to the axis of the p orbital at C-4. Conformations exemplified by **16a** but not **16b** (Scheme 4) fulfill this requirement and account for the stereochemical course of the reaction,[5] if hydrogen addition to the radical center approaches from the less hindered *re* face. Rotating the acrylate moiety of **16a** gives an infinite number of conformers with a maximum (ca. 4.6 Å) and minimum (ca. 3.0 Å) carboxylate separation (i.e., carbon-to-carbon distance). All of these allow fragmentation, but only two [carboxylate separation = ca. 4.1 Å (**16c**) and ca. 3.5 Å (**16d**)] maximize orbital overlap between the p orbital of the radical and the p orbital at C-2, as required for addition−elimination. Hence, the enzyme could bind 2-methyleneglutarate or 3-methylitaconate and the derived radicals in such a way that fragmentation−recombination is allowed but that the addition−elimination pathway is precluded.

In principle, any of the mechanisms for the rearrangement of the substrate-derived radical to product-related radical under consideration can explain the observed rotation of the *exo*-methylene group because they all provide a sufficiently low barrier pathway (see Introduction). An addition−elimination mechanism in which full protonation of the *exo*-methylene group occurs generating an intermediate methylcyclopropane radical cation (**8**)[16] can be excluded because of the lack of exchange of *exo*-methylene deuterons or protons with solvent. Furthermore, the addition−elimination mechanism (Scheme 1, path a) cannot readily explain the observed *E*-overshoot. In this mechanism the 2-methyleneglutar-4-yl radicals **3b** and **3c**, as well as the 2-methyleneitaconate radicals **4b** and **4c**, are directly obtained from the same transient cyclopropylmethylene radical **5b**. The ring-opening of **5b** is expected to occur preferentially toward the 2-methyleneglutar-4-yl radicals **3b**/**3c**, because of the stabilization of the radical center provided by the adjacent carboxylate group. This expectation is based on experimental[33] and theoretical data,[16] although in principle the enzyme could modulate the relative stabilities of the radicals and the activation barriers linking these species. In the absence of a significant effect of the enzyme on intrinsic radical stabilities, the formation of (*E*)-3-methyl[2′-$^2H_1$]itaconate **2b** should lag behind the formation of (*E*)- and (*Z*)-2-methylene[2′-$^2H_1$]glutarate **1b**/**1c** according to the addition−elimination mechanism. In reality, the rates of formation of **2b** and **1b**/**1c** are similar. A comparable argument can be used against addition−elimination assisted by

(33) Ashwell, S.; Davies, A. G.; Golding, B. T.; Hay-Motherwell, R.; Mwesigye-Kibende, S. *J. Chem. Soc., Chem. Commun.* **1989**, 1483.

Scheme 4. Conformational Analysis of the Addition−Elimination and Fragmentation−Recombination Pathways for 2-Methyleneglutarate Mutase



partial protonation, because ring-opening of the intermediate cyclopropylmethylene radical **5b** should occur preferentially to the 2-methyleneglutar-4-yl radicals **3b**/**3c**.

A possible explanation of the *E*-overshoot and working hypothesis for the design of further experiments is that there is an energy barrier associated with a conformational change within the sequence of steps from substrate radical to product radical. The free energy profile for the 2-methyleneglutarate mutase reaction may be analogous to that proposed for glutamate mutase,[29b,34] with barriers corresponding to substrate/product binding to the enzyme, and to the hydrogen abstraction and rearrangement steps. The additional barrier must be comparable in height to either of the C−H abstraction steps, and it must occur after the formation of a species that allows *EZ*-isomerization but before the formation of a 2-methyleneglutar-4-yl radical when starting from 3-methylitaconate. The putative energy barrier may be associated with a structural reorganization that occurs, say, between fragmentation and recombination (Scheme 3), thus allowing the 2-acrylyl radical to move to and from C-2 and C-3 of acrylate. The effect of this barrier could be to make recombination of acrylate and the 2-acrylyl radical leading to a 3-methyleneitaconate radical (**4b** or **4c**), and hence to (*E*)- and (*Z*)-3-methyl[2′-$^{2}H_{1}$]itaconate **2b**/**2c**, similar in rate to that of the recombination leading to a 2-methyleneglutar-4-yl radical (**3b** or **3c**) and hence to (*E*)- and (*Z*)-2-[2′-$^{2}H_{1}$]-methyleneglutarate **1b**/**1c**. The observed decrease of the *E*-overshoot using (*Z*)-3-[2′-$^{2}H_{1}$,*methyl*-$^{2}H_{3}$]methylitaconate **2d** can be ascribed to the increased barrier between tetradeuterated 3-methylitaconates and their corresponding radicals, while the reorganization step connecting (**6** + **7b**) and (**6** + **7c**) should be hardly affected by deuterium substitution in the methyl group.

The crystal structure of glutamate mutase[21] shows three arginine residues at the catalytic site. The 5-carboxylate of glutamate is bound to one arginine, whereas the α-carboxylate, which is part of the migrating glycine moiety, is interacting with two arginines. This arrangement has been termed the "arginine claw"[4] and could enable a "hand-over" of the carboxylate of the intermediate 2-glycinyl radical from one arginine to the other, thus permitting the structural change from substrate to product. Comparison of the primary sequence of component E of glutamate mutase from *Clostridium cochlearium*[10] with the corresponding domain of 2-methyleneglutarate mutase shows no significant identity. However, this does not exclude the possibility of the presence of an arginine claw in 2-methyleneglutarate mutase. Thus, the proposed additional energy barrier for 2-methyleneglutarate mutase could pertain to a hand-over step, that is, the transfer of the 2-acrylyl radical between C-2 and C-3 of acrylate.

(34) Chih, H. W.; Marsh, E. N. *Biochemistry* **1999**, *38*, 13684.

Conclusions

The experiments described in this work provide further insights into the rearrangement step in a coenzyme B_{12}-dependent mutase. The deuterated substrates used provided delicate probes to assess energy barriers on the catalytic pathway of the mutase. The data presented can be accommodated by the fragmentation−recombination mechanism for 2-methyleneglutarate mutase. This conclusion corroborates earlier inhibition and EPR studies using acrylate and all four stereoisomers of 1-methylcyclopropane-1,2-dicarboxylate.[21]

Experimental Section

Materials. Commercial reagents were utilized without further purification. Dichloromethane and *tert*-butyl alcohol were distilled from calcium hydride. Ethanol was distilled from magnesium/iodine. Tetrahydrofuran (THF) was distilled from sodium benzophenone ketyl. Melting points were determined using a Gallenkamp apparatus and are uncorrected. Proton and carbon nuclear magnetic resonances were recorded at the frequency stated. Chemical shifts are reported as δ in units of part per million (ppm) using residual protons in the deuterated solvents as an internal standard. Thin-layer chromatography was carried out using TLC aluminum sheets precoated with silica gel (Kieselgel 60 F_{254}, 0.2 mm). Silica gel ("flash", Kieselgel 60) was used for column chromatography procedures. All biochemicals were purchased from Merck (Darmstadt, Germany) apart from coenzyme B_{12} that was from Sigma (Deisenhofen, Germany).

Triethyl Propane-1,1,2-tricarboxylate (9). Freshly distilled diethyl malonate (16.1 g, 100 mmol) was added to ethanolic sodium ethoxide [prepared by reaction of sodium (2.6 g, 113 mmol) with 40 mL of dry ethanol], followed by ethyl 2-bromopropionate (18.0 g, 100 mmol), which was added dropwise with ice cooling. The resulting mixture was stirred and boiled under reflux for 3 h. After cooling, sodium bromide was filtered off, and the solvent was removed from the filtrate in vacuo. The resulting oily residue was fractionally distilled to give the title compound (22.8 g, 88%), bp 96−98 °C/0.5 mmHg; ^{1}H NMR (200 MHz, $CDCl_3$) δ 1.18−1.29 (m, 12H), 3.13 (dq, *J* = 7.2, 9.7 Hz, 1H), 3.67 (d, *J* = 9.7 Hz, 1H), 4.16 (m, 6H); ^{13}C NMR (50 MHz, $CDCl_3$) δ 174.1, 168.0, 61.6, 60.9, 54.7, 39.1, 15.1, 14.1, 14.0.

Triethyl 1-Bromopropane-1,1,2-tricarboxylate (10). *N*-bromosuccinimide (4.11 g, 23.1 mmol) was added to a stirred solution of triethyl propane-1,1,2-tricarboxylate **9** (6.0 g, 23.1 mmol) in dry *tert*-butyl alcohol (110 mL). Potassium *tert*-butoxide (130 mg, 1.16 mmol) was added, and the reaction mixture was stirred for 40 h at 35 °C. The solvent was removed in vacuo to give a residue that was partitioned between water and ether. The aqueous phase was extracted three times× with ether. The combined ethereal phases were washed with brine and dried with magnesium sulfate. The solvent was removed in vacuo, and the residue was purified by column chromatography (silica, elution with dichloromethane) to afford the title compound as a colorless oil (6.08 g, 77% yield); R_f 0.45 (dichloromethane); ^{1}H NMR (200 MHz, $CDCl_3$) δ 1.23 (t, *J* = 7.1 Hz, 3H), 1.27 (t, *J* = 7.1 Hz, 3H), 1.70 (s, 3H), 1.29 (t, *J* = 7.1 Hz, 3H), 1.45 (d, *J* = 7.1 Hz, 3H), 3.50 (q, *J* = 7.1 Hz, 1H), 4.14 (q, *J* = 7.1 Hz, 2H), 4.26 (q, *J* = 7.1 Hz, 2H), 4.27 (q, *J* = 7.1 Hz, 2H); ^{13}C NMR (50 MHz, $CDCl_3$): δ 174.1, 168.2, 61.6, 60.9, 54.7, 39.1, 15.1, 14.1, 14.0.

Triethyl Prop-1-ene-2,3,3-tricarboxylate[x] (11a) and Triethyl Prop-1-ene-1,1,2-tricarboxylate[y] (11b). 1,8-Diazabicyclo[5.4.0]undec-7-ene (DBU; 1.84 g, 12.3 mmol) was added to an ice-cold solution of triethyl 1-bromopropane-1,1,2-tricarboxylate 10 (4.0 g, 11.8 mmol) in anhydrous THF (100 mL). The reaction mixture was stirred with ice-

cooling for 3 h. Acetic acid was added, the hydrobromide salt of DBU was filtered off, and the solvent was removed in vacuo. The residue was purified by column chromatography (silica, elution with 10% ethyl acetate in petrol) to afford the title compounds (2.63 g, 86%) as an inseparable 1:1 mixture; R_f 0.32 (petrol/ethyl acetate, 9:1); ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.15−1.26 (m, 18H)^{x,y}, 2.13 (s, 3H)^y, 4.07−4.53 (m, 12H)^{x,y}, 4.53 (s, 1H)^x, 5.78 (s, 1H)^x, 6.43 (s, 1H)^x; HRMS: found 258.1100, $C_{12}H_{18}O_6$ requires 258.1097.

Triethyl But-1-ene-2,3,3-tricarboxylate (12). To a suspension of sodium hydride (60% w/w NaH in mineral oil, 640 mg, 16 mmol) in anhydrous THF (40 mL) was added **11a**/**11b** (4.0 g, 15.5 mmol) with ice-cooling. The mixture was stirred for 1 h, after which it was cooled in an ice bath. Methyl iodide (2.3 g, 16 mmol) was added dropwise within 5 min, and the mixture was stirred at 60 °C for 2.5 h. The solvent was removed, and the residue was partitioned between water and ether. The ethereal phases were washed with brine and dried over magnesium sulfate. The solvent was removed, and the oily residue was purified by chromatography (silica, elution with a mixture of 20% ethyl acetate in petrol) to afford 3.41 g (12.5 mmol, 80%) of a colorless oil; R_f 0.30 (15% ethyl acetate in petrol); ^{1}H NMR (200 MHz, $CDCl_3$) δ 1.23 (t, $J = 7.1$ Hz, 6H), 1.27 (t, $J = 7.1$ Hz, 3H), 1.70 (s, 3H), 4.18 (q, $J = 7.1$ Hz, 6H), 5.67 (s, 1H), 6.32 (s, 1H); ^{13}C NMR (50 MHz, $CDCl_3$) δ 170.4, 165.6, 140.1, 125.9, 61.8, 61.8, 61.1, 57.4, 21.6, 14.1, 13.1.

Triethyl 1,2-Dibromobutane-2,3,3-tricarboxylate (13). To a solution of triethyl but-1-ene-2,3,3-tricarboxylate **12** (830 mg, 3.01 mmol) in dry dichloromethane (15 mL) was added bromine (2.9 mL, 50 mmol). The mixture was stirred for 3 days in the dark. After cooling at 0 °C, a saturated solution of sodium hydrogen sulfite was carefully added, and the sulfur formed was removed by filtration. The aqueous phase was extracted with dichloromethane, and the combined organic phases were dried over magnesium sulfate and concentrated in vacuo. The yellowish residue was purified by chromatography (silica, elution with dichloromethane) to afford the title compound as a colorless oil (1.24 g, 2.84 mmol, 94%); R_f 0.48 (dichloromethane); ^{1}H NMR (200 MHz, $CDCl_3$) δ 1.23−1.40 (m, 9H), 1.74 (s, 3H), 3.88 (d, $J = 11.3$ Hz, 1H), 4.15−4.31 (m, 6H), 4.73 (d, $J = 11.3$ Hz, 1H); ^{13}C NMR (50 MHz, $CDCl_3$) δ 168.8, 168, 167.2, 71.5, 63.5, 63.2, 62.5, 62.3, 38.7, 20.0, 14.0, 13.8.

(*E*)-Triethyl 1-Bromo-but-1-ene-2,3,3-tricarboxylate (14). To a solution of tetra-*n*-butylammonium fluoride trihydrate (4.9 g, 15.5 mmol) in hexamethylphosphorus triamide (24 mL) was added molecular sieve beads (3 Å, ca. the volume of the solvent). The mixture was stirred for 30 min. Triethyl 1,2-dibromobutane-2,3,3-tricarboxylate **13** (9.2 mmol) was added under nitrogen, and the reaction mixture was stirred overnight. The molecular sieves were filtered off and washed with ether. After addition of water to the filtrate, the solution was acidified to pH 1 with 1 M sulfuric acid. The aqueous layers were extracted three times with ether, and the combined organic phases were washed with brine and dried over magnesium sulfate. The solvent was removed, and the residue was purified by chromatography (silica, elution with dichloromethane) to afford the title compound as a colorless oil (690 mg, 1.94 mmol, 21%); R_f 0.22 (dichloromethane); ^{1}H NMR (200 MHz, $CDCl_3$) δ 1.18−1.33 (m, 9H), 1.68 (s, 3H), 4.13−4.30 (m, 6H), 6.76 (s, 1H); ^{13}C NMR (50 MHz, $CDCl_3$) δ 171.5, 167.8, 143.1, 129.8, 61.8, 61.9, 61.3, 54.4, 21.9, 14.2, 13.3.

(*Z*)-2′-Bromo-3-methylitaconic acid (15). A mixture of (*E*)-triethyl 1-bromo-but-1-ene-2,3,3-tricarboxylate **14** (105 mg, 0.29 mmol) and 20% hydrobromic acid (4 mL) was heated for 4 days at 80 °C. The solvent was removed in vacuo, and water (2 mL) was added to the residue. The solution was extracted with ethyl acetate (5×), and the combined organic extracts were dried over magnesium sulfate. The solvent was removed, and the residual solid was purified by chromatography [silica, elution with petrol−ethyl acetate−acetic acid (7:2:1)] to afford the title compound as a white crystalline solid (37 mg, 0.166 mmol, 57% yield); mp 108 °C; R_f 0.26 (petrol−ethyl acetate−acetic acid, 7:2:1); ^{1}H NMR (200 MHz, d_6-acetone) δ 1.52 (d, J 7.2 Hz, 3H), 3.78 (q, J 7.2, 0.9 Hz, 1H), 7.12 (d, J 0.9 Hz, 1H), 11.15 (br, 1H); ^{13}C NMR (125 MHz, d_6-acetone) δ 173.8, 166.5, 139.1, 113.5, 45.0, 16.4.

(*Z*)-3-Methyl[2′-^{2}H$_1$]itaconic acid (racemic 2c). (*Z*)-2′-Bromo-3-methylitaconic acid **15** (290 mg, 1.29 mmol) was dissolved in D_2O, lyophilized, and redissolved in D_2O (11 mL). To the rapidly stirred solution was added 5% sodium amalgam (9.1 g, British Drug Houses), and the mixture was stirred for 50 min. The aqueous phase was decanted from the mercury, which was washed with water. The combined aqueous solutions were acidified with 2 M HCl to pH 1 and extracted with ethyl acetate (5×). The organic phases were washed with brine and dried with sodium sulfate. The solvent was removed in vacuo to afford the title compound as a white crystalline solid (181 mg, 97%). An analytical sample was obtained by recrystallization with acetonitrile; mp 148−149 °C (lit. value for unlabeled 3-methylitaconic acid: 152−154 °C[5]); ^{1}H NMR (200 MHz, d_6-acetone): δ 1.50 (d, J 7.3 Hz, 3H), 3.78 (q, J 7.3 Hz, 1.1 Hz, 1H), 5.75 (s, 1H); ^{13}C NMR (125 MHz, d_6-acetone): δ 174.9, 167.5, 142.6, 125.4 (t, $^1J_{D-C}$ 24.6 Hz), 41.4, 16.5.

(*Z*)-3-[2′-^{2}H$_1$,*methyl*-^{2}H$_3$]Methylitaconate (racemic 2e). This was prepared in a manner similar to that described for (*Z*)-3-methyl[2′-^{2}H$_1$]-itaconate **2c**, except that trideuteriomethyl iodide was used in the methylation step: mp 147−148 °C; ^{1}H NMR (200 MHz, d_6-acetone): 3.55 (s, 1H), 5.83 (s, 1H); ^{13}C NMR (125 MHz, d_6-acetone): δ 174.9, 167.6, 141.6, 125.4 (t, $^1J_{D-C} = 23.6$ Hz), 41.4.

2-Methylene[4-^{2}H$_2$]glutaric acid (1g). Sodium metal (144 mg, 6.25 mmol) was reacted with dry ethanol (60 mL). Diethyl malonate (1.0 g, 6.25 mmol) was added, and the solution was cooled in an ice bath. Ethyl α-bromoacrylate (1.21 g, 6.25 mmol) was added and the reaction stirred at room temperature for 1 h. The solvent was removed, and the resulting oil was dissolved in ether. The solution was filtered, and the ether was removed. The crude product was purified by flash column chromatography on silica, eluting with 15% ethyl acetate/petrol to afford triethyl but-3-ene-1,1,3-tricarboxylate as a colorless oil (912 mg, 53%);^{1}H NMR (200 MHz, $CDCl_3$): δ 1.20−1.32 (m, 9H), 2.87 (d, $J = 7.8$ Hz, 2H), 3.70 (t, $J = 7.8$ Hz, 1H), 4.11−4.25 (m, 6H), 5.62 (s, 1H), 6.20 (s, 1H); ^{13}C NMR (50 MHz, $CDCl_3$): δ 168.6, 166.3, 136.8, 127.6, 61.5, 60.9, 50.9, 31.4, 14.2, 14.1.

A mixture of triethyl but-3-ene-1,1,3-tricarboxylate (1.0 g, 3.67 mmol) and 20% DCl in D_2O (8 mL) was refluxed for 7 days. The solvent was removed to yield a yellow solid. The crude product was recrystallized from acetonitrile to afford the title compound as a white crystalline solid (335 mg, 62%): mp 128−129°C; ^{1}H NMR (200 MHz, d_6-acetone): δ 2.63 (s, 2H), 5.75 (s, 1H), 6.23 (s, 1H); ^{13}C NMR (50 MHz, d_6-acetone): δ 174.7, 168.6, 140.7, 126.4, 32.9 (septet, $^1J_{CD} = 19.6$ Hz), 28.1.

Enzymology. Apo-2-methyleneglutarate mutase and the 3-methylitaconate isomerase were overproduced and purified from *E. coli* as described recently.[6] Holo-2-methyleneglutarate mutase was reconstituted just by addition of an excess of coenzyme B_{12}. For the determination of the primary deuterium isotope effect exhibited by 2-methylene[4-^{2}H$_2$]glutarate in the reaction catalyzed by 2-methyleneglutarate mutase, the standard assay was used. It is based on the subsequent isomerization of the product 3-methylitaconate to 2,3-dimethylmaleate, the absorbance of which is measured at $\lambda = 256$ nm, using 3-methylitaconate isomerase as an auxiliary enzyme.[27] The assay mixture contained in a total volume of 500 μl: 100 mM potassium phosphate, pH 7.4, 10−20 units of 3-methylitaconate isomerase, 0.05−0.2 units of *apo*-2-methyleneglutarate mutase, and either unlabeled **1a** or 2-methylene[4-^{2}H$_2$]glutarate **1g**. The reaction was started by addition of 10 μM adenosylcobalamin. The initial velocity was plotted against the substrate concentration. The data were fitted to the Michaelis−Menten equation using the method of nonlinear least-squares.

The activity of 2-methyleneglutarate mutase with (*R*)-2-methylitaconate as substrate was measured in an incubation containing in 50 mM potassium phosphate, pH 7.4, 0.05−0.2 units of *apo*-2-methyleneglutarate mutase, and either unlabeled **2a** or 3-[*methyl*-^{2}H$_3$]-

methylitaconate **2f**. The reaction was started by addition of 10 μM adenosylcobalamin. Samples (0.1 mL) were withdrawn at times and acidified with 0.1 mL of 1 M HCl. After at least 5 min the samples were neutralized with 1 mL of 1 M NaOH and centrifuged, and the concentration of 2-methyleneglutarate in the supernatant was measured at 206 nm by reversed phase HPLC in 0.086% trifluoroacetic acid and 7% methanol in water at a flow rate of 2 mL/min at 40°C. A LiChroCART cartridge (100 mm × 4.6 mm) filled with LiChrosphere 100 RP-18/5 μm (Merck, Darmstadt, Germany) was used.

NMR Studies. Proton NMR spectra were recorded at 500.16 MHz on a JEOL Lambda 500 spectrometer on samples in 5-mm o.d. spinning tubes using the signal from the 99% D_2O solvent as a lock and that from residual HDO as a reference at 4.65 ppm. Typically, 8−32 free induction decays were acquired with a 45° pulse angle (5 μs) and repetition interval of 4 s into 32k data points over a spectral range of 10 kHz. The data were exponentially weighted to produce an additional broadening of 0.15 Hz and zero-filled to 128k data points prior to Fourier transformation and subsequent integration. Deuterium spectra were obtained on the same instrument at 76.7 MHz without any lock and were referenced to the HOD signal at 4.65 ppm. Normally 64 free induction decays were acquired into 8k data points with a 45° pulse angle (10.5 μs) and repetition interval of 4 s. It was found unnecessary to suppress the solvent resonance to observe weak peaks as close to it as 0.1 ppm (7.6 Hz). Kinetic runs were done at 22 ± 1 °C, the temperature stability during any run being better than ± 0.1°. Sets of 8−32 spectra were acquired over periods of about 50 min, the first spectrum being obtained within 1−2 min of mixing. Therefore, limited data were available for calculation of the initial rates.

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