Rotation of the *exo*-Methylene Group of 2-Methyleneglutarate Catalyzed by Coenzyme B_{12}-Dependent 2-Methyleneglutarate Mutase from *Clostridium barkeri*

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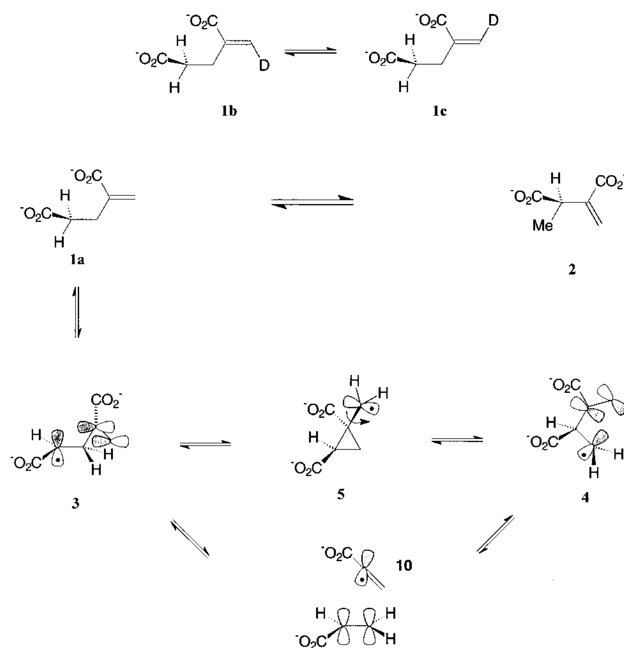
2-Methyleneglutarate mutase from *Clostridium barkeri* is a coenzyme B_{12}-dependent enzyme that catalyses a remarkable carbon skeleton rearrangement, namely the equilibration of 2-methyleneglutarate **1a** with (*R*)-3-methylitaconate [(*R*)-2-methyl-3-methylenesuccinate **2**] (see Scheme 1).[1] This process is believed to be initiated by binding of the substrate to the holoenzyme, which brings about homolysis of the Co−C σ-bond of the coenzyme B_{12} (adenosylcobalamin) yielding cob(II)alamin and a methylene (5′-deoxyadenosyl) radical.[2] The latter abstracts H_{Re} from C-4 of 2-methyleneglutarate[1c] generating the substrate-derived radical **3** shown in Scheme 1.[3] An attractive mechanism for the rearrangement of this species to a product-related radical **4** postulates the intermediacy of a cyclopropylcarbinyl radical **5** (see Scheme 1). The rapid interconversion of but-3-enyl and cyclopropylcarbinyl radicals is well known in nonenzymic chemistry,[4] and model systems have been described in support of the mechanism of Scheme 1.[3] We report a critical test of this mechanism. If the energy barrier to rotation about the C-1/methylene bond in **5** is sufficiently low then a stereospecifically deuteriated specimen of 2-methyleneglutarate (say **1b**) would equilibrate with **1c** when incubated with 2-methyleneglutarate mutase holoenzyme. We have found that this is indeed the case.

Reaction of diethyl 2-methyleneglutarate[5] **6** with 1 molar equiv of bromine in dichloromethane gave diethyl 2-bromo-2-(bromomethyl)glutarate **7**, which was dehydrobrominated using 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) in tetrahydrofuran (cf. Scheme 2 in supporting information). The stereochemistry of diethyl 2-(bromomethylene)glutarate obtained was inferred as (*E*) [see structure **8**] after saponification to diacid **9**, the configuration of which was proved by X-ray analysis.[6] Diacid **9** was reductively debrominated by sodium amalgam in D_2O.[7bc] The partially deuteriated 2-methyleneglutaric acid formed contained 79% [*E*-$^2H_1$]-isomer **1b**, 6% [Z-$^2H_1$]-isomer **1c**, and 15% unlabeled material **1a** according to analysis by ^1H (see Figure 1a) and ^2H NMR. The assignment of configuration is based on a ^1H NMR NOE experiment with 2-methyleneglutaric acid **1a** in [$^2H_6$]acetone (see supporting information), which showed that the resonance at δ 5.69 (δ 5.3 in D_2O) is the proton *syn* to the 3-methylene group, whereas the resonance at δ 6.17 (δ 5.7 in D_2O) is the proton *anti* to this group. As it is the resonance at δ 5.69 (δ 5.3 in D_2O) which is much reduced in intensity in the deuteriated 2-methyleneglutarate, the deuterium is largely in the position *syn* to the 3-methylene group as shown in structure **1b**. Hence, the reductive debromination proceeds by predominant retention of configuration in the substitution of bromine by deuterium, as expected.[7,8]

The deuterium-labeled 2-methyleneglutarate (mainly **1b**, final concentration 40 mM in 1 mL D_2O) was incubated in an NMR tube with 8 mM potassium phosphate, 20 mM imidazole (internal standard), and 0.2 mg of *apo*-2-methyleneglutarate mutase purified from *Escherichia coli*,[1e] nominal p^{2}H = 7.4 at 25 °C. After recording an NMR spectrum the reaction was initiated by addition of 10 μM coenzyme B_{12} (adenosylcobalamin). ^1H NMR spectra were run every few minutes and showed that within the first 4 min, equilibration of the 2-methyleneglutarate with (*R*)-3-methylitaconate (*ca.* 6% at equilibrium, evident from the appearence of a doublet *J* 7.1 Hz at δ 1.24 for the methyl protons) was complete. Over *ca.* 20 min the [*E*-$^2H_1$]-isomer **1b** equilibrated with the [*Z*-$^2H_1$]-isomer **1c** {see Figure 1 for spectra at 0 and 30 min [n.b. the positions

Scheme 1. Equilibration of 2-Methyleneglutarate **1a** and (*R*)-3-Methylitaconate **2** and Their Corresponding Radicals **3** and **4**[a]



[a] Equilibration occurs either *via* cyclopropylcarbinyl radical **5** (for this intermediate, the configuration at C-2 must be *R*, while at C-1, one possible stereochemistry is shown) or by fragmentation to acrylate and the 2-acrylate radical **10**.

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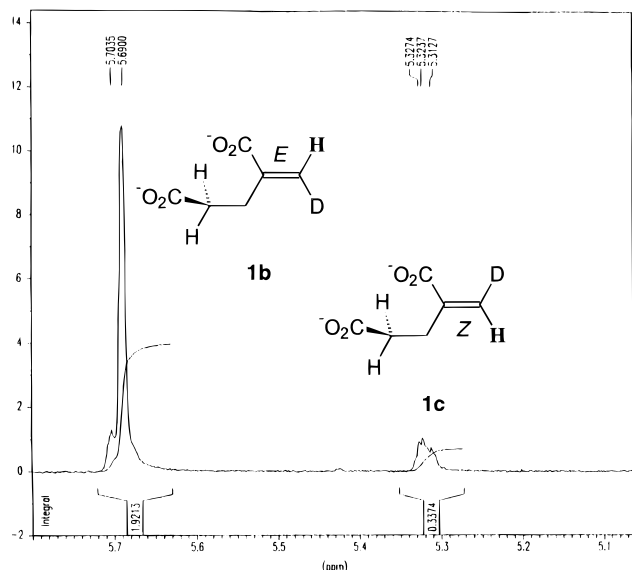
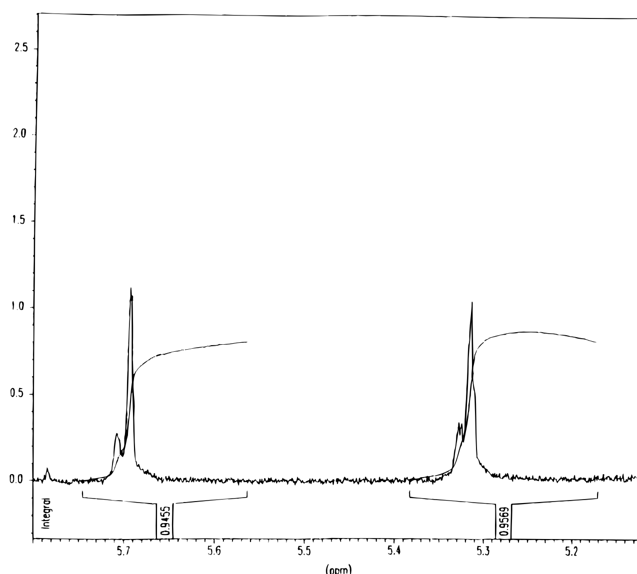


a



b

Figure 1. Equilibration of (*E*-methylene-$^2H_1$)-2-methyleneglutarate with its *Z*-isomer catalyzed by 2-methyleneglutarate mutase in D_2O. ^1H NMR spectra (400 MHz) were taken at 0 (a) and 30 (b) min. For further experimental details see text.

of the resonances from the methylene protons of 2-methyleneglutarate in D_2O are δ 5.3 (proton *syn* to methylene chain) and 5.7 (proton *anti* to methylene chain); see Figure 2 for the complete time course for equilibration]}. The results shown in Figures 1 and 2 were reproduced in five independent experiments. No incorporation of deuterium from the solvent into the substrate was observed, and the enzyme remained active for >50 min. In control experiments, no equilibration of **1b** with **1c** was observed when the enzyme was omitted or replaced by the H356Q mutant.[9] The mutant was also inactive in the standard assay because glutamine substitutes for the histidine that displaces the coenzyme's 4,5-dimethylbenzimidazole and coordinates to the cobalt (n.b. the homology of the cobalamin-

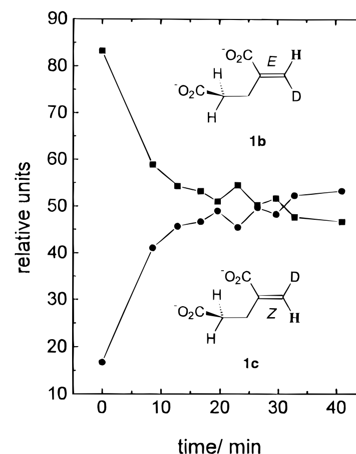


Figure 2. Time course for the equilibration of (*E*-methylene-$^2H_1$)-2-methyleneglutarate with its *Z*-isomer catalysed by 2-methyleneglutarate mutase: ■, *E*-^{2}H; ●, *Z*-^{2}H. See text for further experimental details.

binding domains in 2-methyleneglutarate mutase and methionine synthase[1d,10]).

The results described might be taken to support the mechanism of Scheme 1. But we have recently reported[11] experiments suggestive of an intriguing alternative mechanism (Scheme 1) in which the substrate-derived radical **3** *fragments* to acrylate and the 2-acrylate radical **10**. Recombination of these species either regenerates **3** or leads to **4**, hence bypassing **5**. According to EPR evidence,[12] the 2-acrylate radical prefers a linear configuration, arising from sp hybridization at C-2, as shown in Scheme 1. To explain the loss of stereochemical integrity of the substrate **1b** requires either a movement (or rotation) of the acrylate moiety or a rotation within the 2-acrylate radical. The observation that the equilibration of **1b** and **1c** lags behind the conversion of 2-methyleneglutarate to (*R*)-3-methylitaconate indicates that the postulated movement (or rotation) of acrylate or bond rotation within the radical **10** or **5** occurs in approximately every fifth turnover. Experiments are in progress to distinguish the two possible pathways of Scheme 2 (supporting information).

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Supporting Information Available: Preparative procedures and spectroscopic data for compounds **7**−**9** and **1b**, and an illustration of the crystal structure of **9** (6 pages). This material is contained in libraries on microfiche, immediately follows this article in the microfilm version of the journal, can be ordered from the ACS, and can be downloaded from the Internet; see any current masthead page for ordering information and Internet access instructions.

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