Searching for Intermediates in the Carbon Skeleton Rearrangement of 2-Methyleneglutarate to (*R*)-3-Methylitaconate Catalyzed by Coenzyme B_{12}-Dependent 2-Methyleneglutarate Mutase from *Eubacterium barkeri*†

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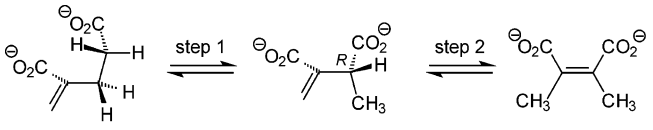
ABSTRACT: Coenzyme B_{12}-dependent 2-methyleneglutarate mutase from the strict anaerobe *Eubacterium barkeri* catalyzes the equilibration of 2-methyleneglutarate with (*R*)-3-methylitaconate. Proteins with mutations in the highly conserved coenzyme binding-motif **D**X**H**$(X)_2G(X)_{41}GG$ (D483N and H485Q) exhibited decreased substrate turnover by 2000-fold and >4000-fold, respectively. These findings are consistent with the notion of H485 hydrogen-bonded to D483 being the lower axial ligand of adenosylcobalamin in 2-methyleneglutarate mutase. (*E*)- and (*Z*)-2-methylpent-2-enedioate and all four stereoisomers of 1-methylcyclopropane-1,2-dicarboxylate were synthesized and tested, along with acrylate, with respect to their inhibitory potential. Acrylate and the 2-methylpent-2-enedioates were noninhibitory. Among the 1-methylcyclopropane-1,2-dicarboxylates only the (1*R*,2*R*)-isomer displayed weak inhibition (noncompetitive, K_i = 13 mM). Short incubation (5 min) of 2-methyleneglutarate mutase with 2-methyleneglutarate under anaerobic conditions generated an electron paramagnetic resonance (EPR) signal ($g_{xy} \approx 2.1$; $g_z \approx 2.0$), which by analogy with the findings on glutamate mutase from *Clostridium cochlearium* [*Biochemistry*, **1998**, *37*, 4105−4113] was assigned to cob(II)alamin coupled to a carbon-centered radical. At longer incubation times (>1 h), inactivation of the mutase occurred concomitant with the formation of oxygen-insensitive cob(II)alamin ($g_{xy} \approx 2.25$; $g_z \approx 2.0$). In order to identify the carbon-centered radical, various ^{13}C- and one ^{2}H-labeled substrate/product molecules were synthesized. Broadening (0.5 mT) of the EPR signal around g = 2.1 was observed only when C2 and/or C4 of 2-methyleneglutarate was labeled. No effect on the EPR signals was seen when [5′-^{13}C]adenosylcobalamin was used as coenzyme. The inhibition and EPR data are discussed in the context of the addition−elimination and fragmentation−recombination mechanisms proposed for 2-methyleneglutarate mutase.

Within adenosylcobalamin (coenzyme B_{12})-dependent isomerases is a group of carbon skeleton mutases named methylaspartate (glutamate) mutase (EC 5.4.99.1), methylmalonyl-CoA (EC 5.4.99.2), 2-methyleneglutarate (EC 5.4.99.4), and isobutyryl-CoA mutase (EC 5.4.99.13)[1,2] (*1*, *2*). These enzymes have vital functions in catabolic processes in human liver and bacterial fermentations.

Scheme 1: Isomerization of 2-Methyleneglutarate to (*R*)-3-Methylitaconate and Dimethylmaleate Catalyzed by 2-Methyleneglutarate Mutase (Step 1) and 3-Methylitaconate Isomerase (Step 2)



Methylmalonyl-CoA mutase is involved in the formation, as well as the degradation, of propionate in many organisms. Glutamate mutase initiates the fermentation of glutamate to ammonia, CO_2, acetate, butyrate, and H_2 by *Clostridium cochlearium* and *Clostridium tetanomorphum*. In the fermentation of nicotinate to ammonia, CO_2, acetate, and propionate by the strictly anaerobic bacterium *Eubacterium barkeri* (order *Clostridiales*), 2-methyleneglutarate mutase interconverts 2-methyleneglutarate, which still has the nicotinate carbon skeleton, with the more highly branched (*R*)-3-methylitaconate (*3*−*5*) (Scheme 1, step 1). The equilibrium for this reaction disfavors 3-methylitaconate (K_{eq} = 0.06) (*6*). However, the subsequent reaction in the

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[1] Abbreviations: CoA, CoASH, coenzyme A; coenzyme B_{12}, adenosylcobalamin; EPR, electron paramagnetic resonance.

[2] Enzymes: 2-methyleneglutarate mutase, EC 5.4.99.4; methylaspartate (glutamate) mutase, EC 5.4.99.1; methylmalonyl-CoA mutase, EC 5.4.99.2; isobutyryl-CoA mutase, EC 5.4.99.13; 3-methylitaconate isomerase, EC 5.3.3.6; methionine synthase, N^5-methyltetrahydrofolate-L-homocysteine *S*-methyltransferase, EC 2.1.1.13.

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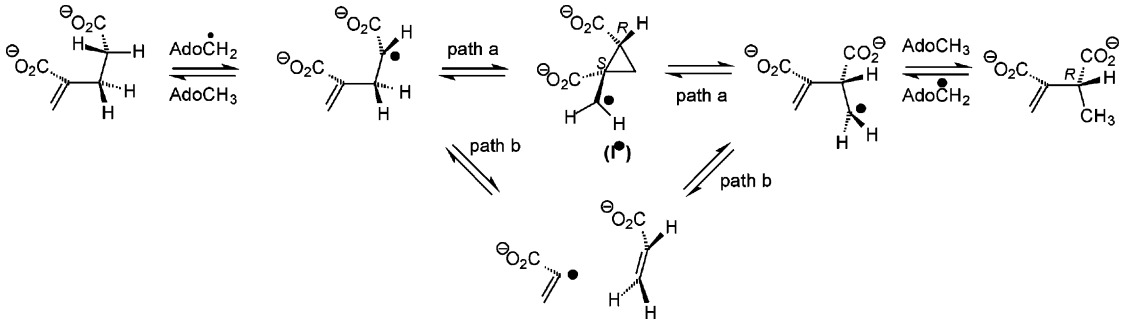
Scheme 2: Addition−Elimination and Fragmentation−Recombination Mechanisms[a]



[a] Path a: Addition−elimination mechanism with the participation of an intermediate radical I• [1-methylene-1,2-cyclopropanedicarboxylate, for which the (1*S*,2*R*)-isomer is shown] {$AdoCH_2$ = 5-deoxyadenosyl; $AdoCH_3$ = 5-deoxyadenosine}. Path b: Fragmentation−recombination mechanism for 2-methyleneglutarate mutase involving an acrylate molecule and a 2-acrylate radical ("fragment radical F•").

fermentation pathway (Scheme 1, step 2), the isomerization of 3-methylitaconate to dimethylmaleate catalyzed by 3-methylitaconate isomerase (K_{eq} = 7), overcomes this unfavorable step (*5*).

EPR spectroscopy (*7*, *8*) and X-ray crystallographic analysis have shown that the resting forms of methylmalonyl-CoA mutase (*9*) and glutamate mutase (*10*, *11*) bind adenosylcobalamin by replacing the 5,6-dimethylbenzimidazole ligand with an imidazolyl moiety from a histidine of the protein ("base off, his on"). For 2-methyleneglutarate and isobutyryl-CoA mutases, replacement of dimethylbenzimidazole in a manner similar to that for the aforementioned mutases was inferred from the primary sequence (*12*, *13*).

Concerning the reaction pathways for the mutases, the first step is the generation of the 5′-deoxyadenosyl radical and cob(II)alamin from enzyme-bound adenosylcobalamin by homolysis of the coenzyme's cobalt−carbon σ-bond in the presence of a substrate molecule SH (*14*). A 10^{12}-fold enhancement of the rate of this step compared to homolysis of the free coenzyme at room temperature has been estimated (*15*). For glutamate mutase, homolysis of the cobalt−carbon bond of the enzyme-bound adenosylcobalamin has been documented experimentally by EPR spectroscopy, rapid-quench, UV−visible, and stopped-flow techniques (*16*−*18*). Stereospecific hydrogen abstraction from a substrate molecule (e.g. 4-H_{Re} of 2-methyleneglutarate) (*19*) by the 5′-deoxyadenosyl radical gives 5′-deoxyadenosine and a substrate radical S• (e.g. 2-methylene-4-glutaryl). As observed with glutamate mutase this step generates the predominant form of the enzyme in the steady state (*20*) with a pre-steady-state rate fast enough for the observed overall reaction (*16*). The intermediacy of a substrate-derived radical (4-glutamyl) was demonstrated by an EPR study of glutamate mutase (*21*). The substrate radical S• rearranges to a product radical P•, again under strict stereochemical control (*14*). For glutamate and 2-methyleneglutarate mutases a "fragmentation−recombination" mechanism has been proposed by Buckel, Golding, and co-workers (*22*) in which a common acrylate molecule partners a fragment radical F• and enables the handover of the acrylate moiety from C-2 to C-3 (substrate numbering) (see Scheme 2, path b). In the case of glutamate mutase there is direct evidence for the kinetically competent participation of acrylate and a glycine radical in the overall reaction (*23*). With 2-methyleneglutarate mutase, however, an alternative pathway ("addition−elimination" mechanism: see Scheme 2, path a) (*24*) from S• to P• is possible, which requires the participation of an intermediate radical I• (1-methylene-1,2-cyclopropanedicarboxylate) that is isomeric with S• and P•. Transfer of hydrogen between the methyl group of 5′-deoxyadenosine and the product radical P• gives product PH and regenerates the 5′-deoxyadenosyl radical, which combines with cob(II)alamin. With reaction rates of 10^2 s^{-1} and inactivation occurring in less than 1 in 10^6 rearrangements, the mutases offer a high degree of protection against unwanted side reactions of the intermediate radicals, thus illustrating the principle of negative catalysis (*25*).

In this paper, we describe the purification of apo-2-methyleneglutarate mutase, which was converted into the active holoenzyme by addition of adenosylcobalamin. We report the effects of D483N and H485Q mutations on 2-methyleneglutarate mutase activity using a nondeleterious mutation (H464Q) as a control for expression and purification. Selected compounds that were structurally similar to the substrate or potential intermediates were synthesized and tested as inhibitors. In an attempt to detect substrate, product, intermediate, or fragment radicals (S•, P•, I•, or F•), EPR studies were performed using 2-methyleneglutarate or (*R*)-3-methylitaconate specifically labeled with one or more ^{13}C or ^{2}H atoms. The data from inhibitor studies and the EPR measurements are discussed in the context of the mechanistic alternatives presented above.

MATERIALS AND METHODS

Bacterial Strains, Phages, and Plasmids. *Eubacterium barkeri* (strain DSM 1223, formerly *Clostridium barkeri*, see ref *26* for revision of nomenclature) was obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany). Maintenance and growth were done according to ref *12*. The liquid and solid media for *Escherichia coli* DH5α were standard nutrient broth (Merck, Darmstadt, Germany); for *E. coli* XL1-Blue, the double concentrated yeast extract medium (Merck, Darmstadt, Germany) was used. *E. coli* was grown as described in refs *27*, *28*.

Overproduction in E. coli and Purification of Wild-Type 2-Methyleneglutarate Mutase. Growth of *E. coli* transformed with the plasmid pBB2 was performed essentially as described (*12*). Since *E. coli* lacks the ability to synthesize cobalamin cofactor, apo-2-methyleneglutarate mutase was

purified aerobically without exclusion of light. However, after reconstitution of the holoenzyme with adenosylcobalamin all operations were carried out under dim red light. The holoenzyme was normally handled aerobically although mutants were assayed under anaerobic conditions. All manipulations involving enzyme isolation and purification were carried out at 4 °C. Cells (typically 20 g wet mass) were harvested by centrifugation (6000*g*, 15 min), suspended in buffer A (50 mM potassium phosphate, 1 mM EDTA, pH 7.4) containing 1 mM phenylmethylsulfonyl fluoride, and disrupted by sonication (Branson sonifier B15). Cell debris and membranes were removed by ultracentrifugation (100000*g*, 60 min). Apo-2-methyleneglutarate mutase was obtained by chromatography of the soluble fraction on a Pharmacia FPLC system equipped with Pharmacia columns (Freiburg, Germany). The supernatant of the ultracentrifugation step was loaded onto DEAE Sepharose (column type HiLoad 26/10) equilibrated with buffer A (3 mL/min). After washing with 100 mL of buffer A, apo-2-methyleneglutarate mutase was eluted with a linear gradient of 0−1 M NaCl in buffer A. The fractions, which eluted at around 300 mM NaCl, contained 2-methyleneglutarate mutase activity upon reconstitution with adenosylcobalamin. Ammonium sulfate was added to a final concentration of 1 M to the pooled fractions. The clear solution was loaded onto a phenyl-Sepharose HP column (HiLoad 26/10) equilibrated with buffer B (buffer A supplemented with 1 M ammonium sulfate) operated at a flow rate of 3 mL/min. A gradient of 100−50% buffer B in buffer A (100 mL), 50−0% buffer B in buffer A (200 mL), and 200 mL of buffer A eluted apo-2-methyleneglutarate mutase after about 400 mL. Fractions which exhibited activity upon adenosylcobalamin reconstitution were concentrated in an Amicon chamber with PM30 membrane (Amicon, Witten, Germany). For EPR spectroscopic investigation requiring large amounts of protein, the apo-2-methyleneglutarate mutase was of sufficient purity at this stage (i.e. >90% as revealed by Coomassie staining of SDS−PAGE gels). For all kinetics experiments and the initial characterization an additional size-exclusion chromatography step was added. Apo-2-methyleneglutarate mutase fractions with a purity of 95−99% eluted at a volume of about 250 mL from Superdex 200 prep grade (column type 35/600) equilibrated with buffer A supplemented with 100 mM NaCl (3.0 mL/min). Analytical gel filtration was performed on Superose 12 (HR 10/30) calibrated with ferritin, catalase, aldolase, bovine serum albumin, and cytochrome C to determine the native molecular mass. Elution was performed with buffer A supplemented with 100 mM NaCl at a flow rate of 0.5 mL/min and indicated a mass of 280 kDa. Purified apo-2-methyleneglutarate mutase was stable for several months when stored at protein concentrations of 1−40 mg/mL at −80 °C. The protein content was determined by the Bradford method (*29*). Within experimental error identical protein contents were observed in control experiments using (1) the bicinchoninic acid method (Pierce kit, Merck, Darmstadt, Germany) (*30*) and (2) the UV absorbance at λ_{max} 280 nm with the extinction coefficient, $E_{1\ mg/mL} = 1.23$, calculated (*31*) from the gene-derived amino acid sequence (*12*). N-terminal sequencing of the purified apo-2-methyleneglutarate mutase was performed as described (*32*).

Site-Directed Mutagenesis and Purification of Mutants. Plasmid pBB2 carrying a PCR-generated fragment of the *mgm* gene encoding 2-methyleneglutarate mutase was originally cloned into the vector pJF119HE (*12*). Mutagenesis was carried out essentially according to the protocol of the manufacturer (Stratagene, Amsterdam, The Netherlands). For mutagenesis, the *Hin*dIII fragment of the pBB2 plasmid containing the *mgm* gene was subcloned into pBluescript SK(+) and transformed into *E. coli* XL1-Blue. A growing culture of the resulting clone was infected with the helper phage VCSM 13. The single-stranded DNA was recovered and site-directed mutagenesis was performed according to the method of Taylor et al. (*33*) with the following oligonucleotide primers (mutated nucleotides underlined):

5′-GTGGAAGTTCA<u>A</u>GTGGAAAAAGCCCCC-3′ for H464Q

5′-CCGTTGGTGCG<u>A</u>A<u>C</u>GCCCATGTCAATGGG-3′ for D483N

5′-CGGATGCCCA<u>A</u>GTCAATGGG-3′ for H485Q

For overexpression the mutated genes were cloned back into the pJF119HE expression vector. The mutated region of the *mgm* gene was sequenced using infrared dye labeled primers (synthesized by MWG-Biotech, Ebersberg, Germany) and the thermo sequenase fluorescent-labeled primer cycle kit from Amersham (Freiburg, Germany). After mixing DNA, primer, dideoxynucleotides, and solutions from the sequenase kit, a PCR amplification was performed. DNA fragments were separated on a polyacrylamide gel in a Li-Cor DNA Model 400 automated sequencer (MWG Biotech, Ebersberg, Germany). The analysis of the sequence data was performed with the supplied software.

SDS−PAGE of the crude extracts showed that the mutant genes were expressed in *E. coli* at levels similar to that of the wild-type gene. The obtained mutant proteins were purified by the method used for wild-type 2-methyleneglutarate mutase. SDS−PAGE followed by Coomassie staining or Western blotting (*12*) showed that the mutations did not significantly change the elution behavior. By size-exclusion chromatography (Sephadex G25, 20 mM potassium phosphate, pH 7.4) of a mixture of the H485Q mutant enzyme with adenosylcobalamin no coelution was observed.

Enzyme Assays. In the standard continuous assay of 2-methyleneglutarate mutase, activity (0.05−0.1 unit) was measured with 10 mM 2-methyleneglutarate as substrate in 100 mM potassium phosphate (pH 7.4, 25 °C) using 3-methylitaconate isomerase (10−30 units/mL) as auxiliary enzyme. The 2,3-dimethylmaleate formed was measured by its UV absorbance ($\Delta\epsilon_{256} = 0.66\ mM^{-1}\ cm^{-1}$). The same assay was performed in a discontinuous fashion for 2-methyleneglutarate mutase mutants in an anaerobic chamber under red light with an upper time limit of 30 min because of the instability of the coenzyme in the mutase. The reaction was stopped by acidification with 1 M HCl, which converted dimethylmaleate to its cyclic anhydride ($\Delta\epsilon_{256} = 4.3\ mM^{-1}\ cm^{-1}$) (*5*).

Effects of Potential Inhibitors on 3-Methylitaconate Isomerase. No absorbance change (<0.001 absorbance unit/min at 256 nm) was observed when inhibitor [e.g. 4 mM (*E*)- or (*Z*)-2-methylpent-2-enedioate] was incubated in the presence of 3-methylitaconate isomerase (10 units/mL), but

in the absence of the mutase. Standard isomerase assays were performed using isomerase (0.05−0.1 unit/mL) and 5 mM *rac*-3-methylitaconate. With (*Z*)-2-methylpent-2-enedioate (0.8, 2, and 4 mM) the activity of the isomerase was reduced to 96%, 83%, and 59%, respectively, but there was no significant inhibition with up to 4 mM (*E*)-2-methylpent-2-enedioate.

Effects of Inhibitors on 2-Methyleneglutarate Mutase. In a typical experiment the candidate inhibitor (up to 15 mM for sodium acrylate, 4 mM for (*E*)- and (*Z*)-2-methylpent-2-enedioate, 70 mM for 1-methylcyclopropane-1,2-dicarboxylates) was incubated with 10 μM adenosylcobalamin, 30 units of methylitaconate isomerase and 2-methyleneglutarate until a stable absorbance (<0.001 absorbance unit/min at 256 nm) was observed. 2-Methyleneglutarate mutase (0.05−0.1 unit) was added and mutase activity was recorded by the continuous assay. The data for (1*R*,2*S*)-, (1*S*,2*R*)-, and (1*S*,2*S*)-1-methylcyclopropane-1,2-dicarboxylate were corrected for the presence of 10−20% of enantiomeric impurity.

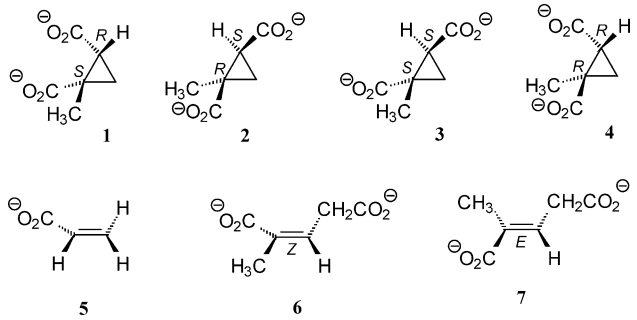
Preincubation of 2-methyleneglutarate mutase with 100 μM adenosylcobalamin and 10 mM (*E*)- or (*Z*)-2-methylpent-2-enedioate in the absence of 2-methyleneglutarate for 3 min at 0 °C in the dark showed no loss of activity (under similar conditions 10 mM 2-methyleneglutarate caused a 10% decrease in activity).

Synthesis of Unlabeled and Isotopically Labeled 2-Methyleneglutarates and 3-Methylitaconates, (E)- and (Z)-2-methylpent-2-enedioic Acid and Isomeric 1-Methylcyclopropane-1,2-dicarboxylic Acids. These compounds were prepared either by literature procedures or by adapting literature procedures (e.g., *34*−*37*) (see Supporting Information for detailed procedures).

Other Materials. Crystalline adenosylcobalamin (purity >98%) was from Sigma (Deisenhofen, Germany). 2,3-Dimethylmaleic anhydride (purity >97%) was from Fluka (Deisenhofen, Germany). [5′-^{13}C]Adenosylcobalamin was a gift from Professor H. P. C. Hogenkamp, University of Minnesota, Minneapolis−St. Paul, MN. All other chemicals were of the highest purity available from Sigma-Aldrich, Merck, or Fluka.

The cobalamin content of apo-2-methyleneglutarate mutase before and after reconstitution was determined by UV/visible spectroscopy (*38*, *39*). Alternatively the cobalt content was determined with a Zeeman/3030 atomic absorption spectrometer (Perkin-Elmer, Überlingen, Germany) using a standard solution of cobalt (990 mg/L) in 1% HNO_3 (Sigma, Deisenhofen, Germany). The formation of cob(II)alamin during the reaction was determined by UV/visible spectroscopy on the basis of the characteristic absorbance maxima of the different cobalamins (*40*). Purified apo-2-methyleneglutarate mutase (9 mg) was incubated with 1 mM adenosylcobalamin (total volume 1 mL) for 20 min in the dark, followed by separation of excess of coenzyme by size exclusion chromatography (Sephadex G25, 20 mM potassium phosphate, pH 7.4). The eluted protein was concentrated to a volume of 2 mL with an Amicon chamber, and a spectrum was taken. The probe was divided into two samples, to each of which was added 2-methyleneglutarate (final concentration 5 mM). The samples were incubated in the dark (14 h, 37 °C), one under aerobic and the other under anaerobic conditions.

Chart 1: Structures of Compounds Tested as Inhibitors of 2-Methyleneglutarate Mutase[a]



[a] **1**−**4**: 1-Methylcyclopropane-1,2-dicarboxylates. **5**: Acrylate. **6** and **7**: 2-Methylglutaconates.

EPR Spectroscopy. Samples for EPR spectroscopy (200 μL) were prepared aerobically in the dark or under dim, red light at room temperature. 2-Methyleneglutarate mutase (100−200 nmol of monomer in 160 μL of 50 mM potassium phosphate, pH 7.4) was mixed with saturated aqueous ca. 16 mM adenosylcobalamin (20 μL), incubated for 5 min, and transferred into calibrated quartz tubes (4.7 ± 0.2 mm outer diameter, 0.45 ± 0.05 mm wall thickness, and 13 cm length, Ilmasil-PN high purity quartz obtained from Quarzschmelze Ilmenau GmbH, Langewiesen, Germany). A neutralized aqueous solution (20 μL) containing 1 M 2-methyleneglutarate or 3-methylitaconate in 50 mM potassium phosphate, pH 7.4, was added, mixed, incubated for 45 s, and frozen in liquid nitrogen. For experiments with in situ generation of (*R*)-3-methylitaconate a neutralized solution of 1 M dimethylmaleate in 50 mM potassium phosphate, pH 7.4, was preincubated for 10 min with 100 units of 3-methylitaconate isomerase. The EPR samples were stored in the dark immersed in liquid nitrogen. Changes in the coupled spectra of 2-methyleneglutarate mutase were not observed during storage over >2 years.

The EPR-spectra were recorded on a Bruker EMX-6/1 X band EPR spectrometer composed of an ER-041 XG X-band microwave bridge and built-in ER-041-1161 microwave frequency counter, EMX-1101 power supply, ER-070 6-in. magnet, EMX-032T Hall field probe, an ER-4102 Universal TE102 rectangular cavity, and an ESR-900 Oxford Instruments Helium flow cryostat (3.8−300 K). Data acquisition was performed with the software supplied by Bruker (WINEPR Acquisition program, version 2.3.1.); data manipulation (determination of *g*-values, subtraction, base lining, integration, and conversion to ASCII files for use with Microsoft EXCEL) was done with the WINEPR program version 2.11.

RESULTS AND DISCUSSION

Chemical Syntheses. The compounds synthesized for EPR and inhibition studies are collected in Chart 1. 2-[2-*exo*-^{13}C]-Methyleneglutaric acid was synthesized following the procedure reported for the corresponding ^{14}C-labeled compound (*41*), but replacing ^{14}C-formaldehyde with ^{13}C-formaldehyde. Disodium dimethyl[1,4-$^{13}C_2$]maleate and disodium [2,2′,3,3′-$^{13}C_4$]dimethylmaleate were obtained by hydrolysis of the corresponding anhydrides. These anhydrides were prepared from [1,4-$^{13}C_2$]- and [2,3-$^{13}C_2$]maleic anhydride, respectively, following the procedure reported for the unlabeled compound

(*35*, *42*). 2-Methylene[2-^{13}C]glutaric acid was obtained after hydrolysis of ethyl 5-*tert*-butyl-4-*tert*-butoxycarbonyl-2-methylene[2-^{13}C]glutarate, which was made by condensation of di-*tert*-butyl malonate with ethyl 2-(bromomethyl)-[2-^{13}C]-acrylate. 2-Methylene[4-^{13}C]glutaric acid was prepared by hydrolysis of triethyl [1-^{13}C]but-3-ene-1,1,2-tricarboxylate, derived by condensation of diethyl [2-^{13}C]malonate with ethyl 2-(bromomethyl)acrylate. (*RS*)-3-Methylitaconate and (*RS*)-3-[^{13}C-*methyl*]methylitaconate were made by acidic hydrolysis of triethyl but-1-ene-2,3,3-tricarboxylate and triethyl [4-^{13}C]but-1-ene-2,3,3-tricarboxylate, respectively, which were obtained by condensation of "Malachowski's mixture" (triethyl prop-1-ene-1,1,2-tricarboxylate + triethyl prop-2-ene-1,1,2-tricarboxylate) (*43*) under basic conditions with either iodomethane or iodo[^{13}C]methane.

(*E*)- and (*Z*)-2-methylpent-2-enedioic acid were prepared from 1,1,3,3-tetracarboethoxypropenyl sodium by improving the literature procedures (*34*, *44*, *45*). Sodium methoxide induced condensation of methyl acrylate with methyl 2-chloropropionate is known to afford a mixture of the stereoisomers of methyl 1-methylcyclopropane-1,2-dicarboxylate (*36*). We found that heating the reaction mixture at reflux with sodium methoxide for 3 h gave, under thermodynamic control, a ca. 5:1 ratio of the (1*R*,2*R*)/(1*S*,2*S*) (*trans*) diesters versus the (1*R*,2*S*)/(1*S*,2*R*) (*cis*) diesters, whereas treatment with sodium methoxide at 20 °C overnight gave a ca. 1:5 ratio of *trans*/*cis* (kinetic control). From the mixture obtained under thermodynamic control, the racemic *trans*-diester was separated chromatographically and saponified to give pure (1*R*,2*R*)/(1*S*,2*S*)-1-methylcyclopropane-1,2-dicarboxylic acid. This was resolved with brucine to give (1*R*,2*R*)-1-methylcyclopropane-1,2-dicarboxylic acid. The mixture of diesters obtained under kinetic control was saponified, and the resulting diacids were heated with acetyl chloride to give the anhydride of the racemic *cis*-diacid. Hydrolysis of the anhydride gave (1*R*,2*S*)/(1*S*,2*R*)-1-methylcyclopropane-1,2-dicarboxylic acid. The racemic diacid was resolved with brucine to give (1*S*,2*R*)-1-methylcyclopropane-1,2-dicarboxylic acid. Resolution with quinine gave (1*R*,2*S*)-1-methylcyclopropane-1,2-dicarboxylic acid. Base-catalyzed equilibration of the dimethyl ester from (1*S*,2*R*)-1-methylcyclopropane-1,2-dicarboxylic acid led to (1*S*,2*S*)-1-methylcyclopropane-1,2-dicarboxylic acid. (1*R*,2*R*)-1-Methylcyclopropane-1,2-dicarboxylic acid was determined to be ca. 100% enantiopure, whereas the other diacids contained 80−90% of the desired isomer (*37*, *46*).

Enzyme Purification. 2-Methyleneglutarate mutase purified from *E. barkeri* was shown to be a homotetramer (α_4; 4 × 67 kDa) containing 2−4 cobalamin molecules (*38*, *39*). By UV/visible and EPR spectroscopy the cobalamins were identified as a mixture of adenosylcobalamin and oxygen-stable cob(II)alamin. Only 50% of cobalamin could be removed by treatment with 8 M urea, with concomitant loss of activity. Dialysis in the presence of dithiothreitol and addition of adenosylcobalamin resulted in complete activation. Thus, the tightly bound cob(II)alamin was presumed to be an artifact, not involved in catalysis (*38*, *39*), arising from endogenous cobalamin(s) and the lengthy purification of the light sensitive mutase (*32*, *47*).

Subsequently, the gene encoding 2-methyleneglutarate mutase was cloned and overexpressed in *E. coli* (*12*). A cell-free extract of the transformed strain showed no 2-methyleneglutarate mutase activity, whereas addition of adenosylcobalamin resulted in immediate activation (*12*). In the present study, the 2-methyleneglutarate mutase activity in a cell-free extract corresponded to a 20-fold overproduction of the mutase as compared to *E. barkeri*. Purification typically yielded 30 mg of homogeneous, colorless apo-2-methyleneglutarate mutase. Gel filtration showed that wild-type apo-2-methyleneglutarate mutase and the mutants prepared (see below) were already assembled into a homotetramer. After addition of adenosylcobalamin the specific activity (28 units/mg; k_{cat} 31 s^{-1}) was ca. twice the activity of enzyme prepared from *E. barkeri* (*39*). The first 10 N-terminal amino acids of the apo-mutase were identical to those of the enzyme from the eubacterial source.

Table 1: Effects on Activity of Mutagenesis of the Cobalamin-Binding Domain in Cobalamin-Dependent Enzymes

enzyme	mutation	activity (s^{-1})	rel act. (%) compared to wild type	ref
2-methylene-glutarate mutase	none	20 ± 0.5[a]	100	*b*
	H464Q	6.9 ± 0.7[a]	35	*b*
	D483N	0.012 ± 0.002[a]	0.06	*b*
	H485Q	<0.005[a]	<0.03	*b*
glutamate mutase	none	18	100	*63*
	D14N	0.021	0.12	*63*
	D14A	0.043	0.24	*63*
	D14E	0.014	0.08	*63*
	H16G	0.016	0.09	*63*
	H16Q	0.023	0.13	*63*
methylmalonyl-CoA mutase	none	120	100	*64*
	H610N	0.003	0.003	*64*
	H610A	0.024	0.02	*64*
methionine synthase	none	27.1	100	*65*
	D757N	1.5	6	*65*
	D757E	0.99	4	*65*
	H759G	0	0	*65*

[a] Measured with 10 mM 2-methyleneglutarate. [b] This work.

Site-Directed Mutagenesis. From amino acid sequence alignment of cobalamin-containing enzymes (*48*) as well as structural data on glutamate mutases (*10*, *49*) and methylmalonyl-CoA mutase (*9*), it was inferred that His485 of 2-methyleneglutarate mutase acts as the lower axial ligand to the cofactor (*12*). This His485 belongs to the fully conserved motif $DXH(X)_2G(X)_{41}GG$, which forms the N-terminal part of the cobalamin-binding domain comprising about 120 amino acids. As shown on a structural basis for other mutases and methionine synthase (*50*) it was anticipated that an aspartate residue (Asp483) forms a hydrogen bond to the His485 of 2-methyleneglutarate mutase (*12*). To confirm the postulated roles for His485 and Asp483, site-directed mutagenesis (H485Q and D483N) was performed along with mutation of the nonconserved His464 (H464Q), which should be nonessential for catalytic function and therefore acted as a control for the mutagenesis procedure and expression in *E. coli*. As expected, the H485Q mutation gave an inactive enzyme, while the D483N mutation decreased the activity by a factor of ca. 2000. Table 1 summarizes the data for mutagenesis experiments and presents comparative data for other carbon skeleton mutases and methionine synthase. Rate measurements with a sufficient accuracy for Michaelis−Menten kinetics were not possible due to the low activity and the lack of highly

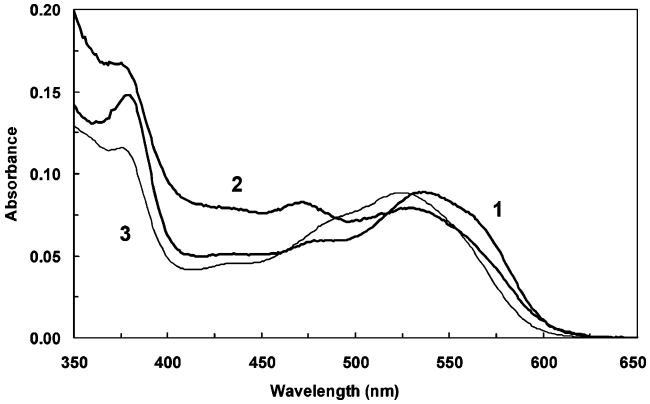


FIGURE 1: UV-visible spectrum of reconstituted 2-methyleneglutarate mutase in the absence and presence of 2-methyleneglutarate in comparison with free adenosylcobalamin. Trace 1, 20 μM holo-2-methyleneglutarate mutase (subunit concentration); trace 2, as in trace 1 after incubation with 10 mM 2-methyleneglutarate for 5 min at ambient temperature; trace 3, 11 μM adenosylcobalamin.

radiolabeled substrates. The slight activity of the D483N mutant was shown to be dependent on the added substrate concentration, thus reflecting a "real" enzymatic activity. In contrast, the H464Q mutant retained substantial activity. The conclusions regarding mutase activities were confirmed by measurements with cell-free extracts of each mutant. That loss of activity of the H485Q mutant is due to its inability to bind the B_{12} cofactor was shown by size-exclusion chromatography of a mixture of the apoprotein and cobalamin. The eluted protein contained no detectable amounts of cobalamin. Neither the H485Q nor the D483N mutant gave detectable EPR signals when incubated with adenosylcobalamin and substrate (vide infra).

Cobalamin Content of 2-Methyleneglutarate Mutase and Stability of the Enzyme. Apo-2-methyleneglutarate mutase was shown to be devoid of cobalamins and cobalt (<0.01 mol of Co/67 kDa subunit) by UV/visible and atomic absorption spectroscopy. After incubation of the apoprotein with an excess of adenosylcobalamin the maximum specific activity of 28 units/mg was immediately obtained. Removal of excess of coenzyme by size-exclusion chromatography did not change the specific activity; upon supplementation with adenosylcobalamin no significant increase was observed. Hence the coenzyme remained tightly bound to the enzyme. UV/visible spectroscopy of the holoenzyme showed λ_{max} 378 and 570 nm (Figure 1) typical for adenosylcobalamin bound to a protein (*51*). Neither cob(II)alamin (λ_{max} 470 nm) nor its oxidation product aquocobalamin (λ_{max} 352 nm) were detected. According to UV/visible spectroscopy and atomic absorption spectroscopy the adenosylcobalamin content of five independent preparations was determined as 0.5 ± 0.1 mol/67 kDa subunit. Probably the tetrameric enzyme shows half-of-the-sites reactivity (*52*); only two of the four sites are occupied by cobalamin, whereas the other two sites are empty, an arrangement different from glutamate mutase (*10*). In contrast, a 2-methyleneglutarate mutase preparation from *E. barkeri* contained 0.8 ± 0.2 mol of cobalamin/subunit, with the cobalamin being a mixture of adenosylcobalamin, aquocobalamin, and cob(II)alamin (*38*).

A mixture of apo-2-methyleneglutarate mutase with an excess of adenosylcobalamin in the absence of substrate was

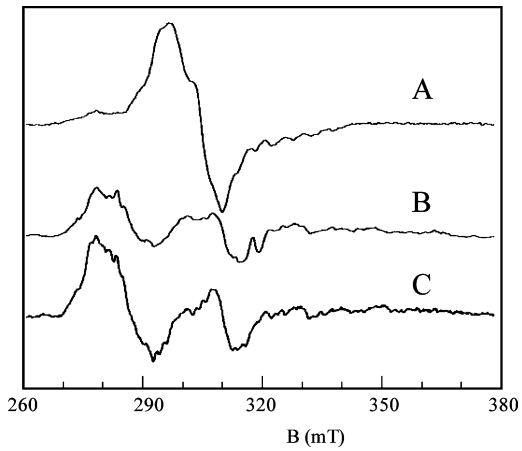


FIGURE 2: EPR spectra indicative of the substrate-induced inactivation ("suicide") of 2-methyleneglutarate mutase. (A) Apo-2-methyleneglutarate mutase (273 μM monomer) incubated under anaerobic conditions (5 min, 30 °C) in the presence of 3 mM coenzyme B_{12}, 33 mM potassium phosphate pH 7.4, 3 mM β-mercaptoethanol, and 33 mM 2-methyleneglutarate. (B) Apo-2-methyleneglutarate mutase (final concentration 130 μM) incubated under anaerobic conditions (14 h, 37 °C) in the presence of 1 mM coenzyme B_{12}, 100 mM potassium phosphate pH 7.4, and 10 mM 2-methyleneglutarate. (C) Sample as in B incubated in the presence of air (EPR conditions: temperature 77 K, microwave power 25 mW, modulation frequency 100 kHz, modulation amplitude 0.4 mT, microwave frequency 9100 ± 5 MHz).

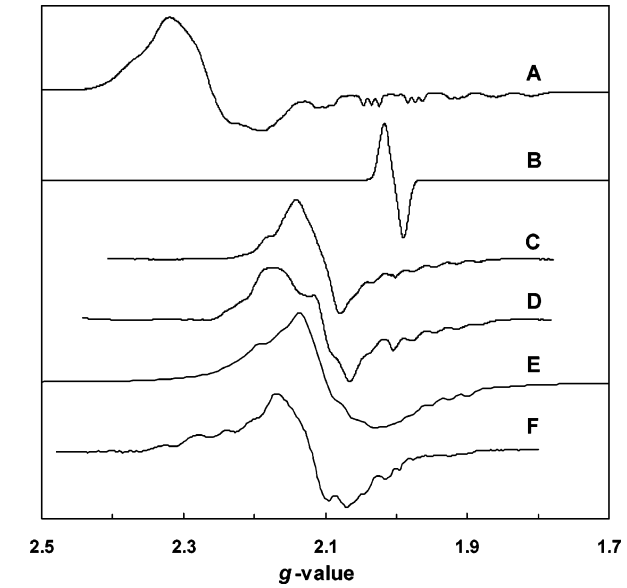


FIGURE 3: EPR spectra of enzyme bound cob(II)alamins and radicals. (A) Uncoupled cob(II)alamin in methanol:coenzyme M methyltransferase MtaC subunit, adapted from ref *66*. (B) A simulated malonyl radical, parameters see ref *21*. (C) Glutamate mutase and (*S*)-glutamate, adapted from ref *21*. (D) 2-Methyleneglutarate mutase and 2-methyleneglutarate. (E) Methylmalonyl-CoA mutase and succinyl-CoA, adapted from ref *54*. (F) Ribonucleotide triphosphate reductase, reductant and dGTP, adapted from ref *67*. The spectra have been converted to a *g*-value *x*-axis to allow better comparison.

EPR-silent, indicating that no cob(II)alamin was formed during reconstitution of the active enzyme (data not shown). After short incubations (up to 5 min) with the substrate, EPR-signals were obtained (Figures 2A, 3D, and 4A) that were similar but not identical to the substrate-induced signals of coenzyme B_{12}-dependent glutamate mutase from *C. cochlearium* (Figure 3C) (*21*), methylmalonyl-CoA mutase from

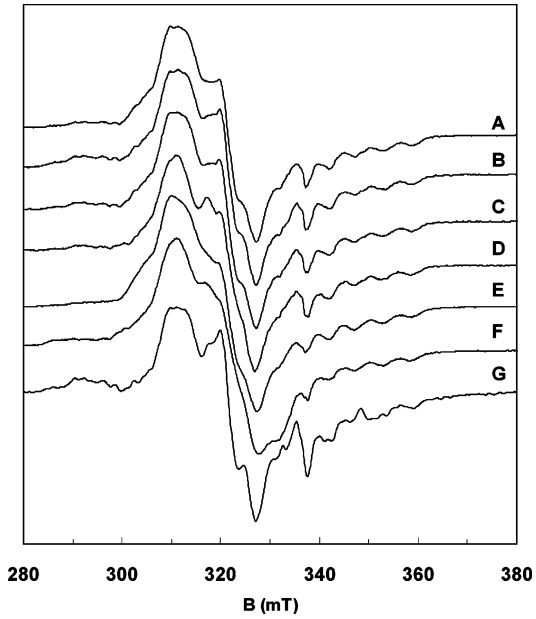


FIGURE 4: EPR spectra of 2-methyleneglutarate mutase and ^{13}C-labeled 2-methyleneglutarates. (A) Incubated with unlabeled 2-methyleneglutarate (natural abundance ^{13}C; for structures see Figure 5). (B) 2-[2′-^{13}C]Methyleneglutarate. (C) 3-[3′-^{13}C]methylitaconate. (D) 2-Methylene[4-^{13}C]glutarate. (E) 2-Methylene[2-^{13}C]-glutarate. (F) [2,2′,3,3′-$^{13}C_4$]Dimethylmaleate with (*R*)-3-methylitaconate isomerase. (G) 2-[4-$^{2}H_2$]Methyleneglutarate, for structures see Figure 5. EPR conditions: temperature 40 K, microwave power 2 mW, modulation frequency 100 kHz, modulation amplitude 0.5 mT, microwave frequency 9463 ± 1 MHz. Spectra similar to spectrum A were obtained from both (*RS*)-3-methylitaconate and (*R*)-3-methyl[1,4-$^{13}C_2$]itaconate, generated in situ by the action of 3-methylitaconate isomerase on dimethyl[1,4-$^{13}C_2$]maleate.

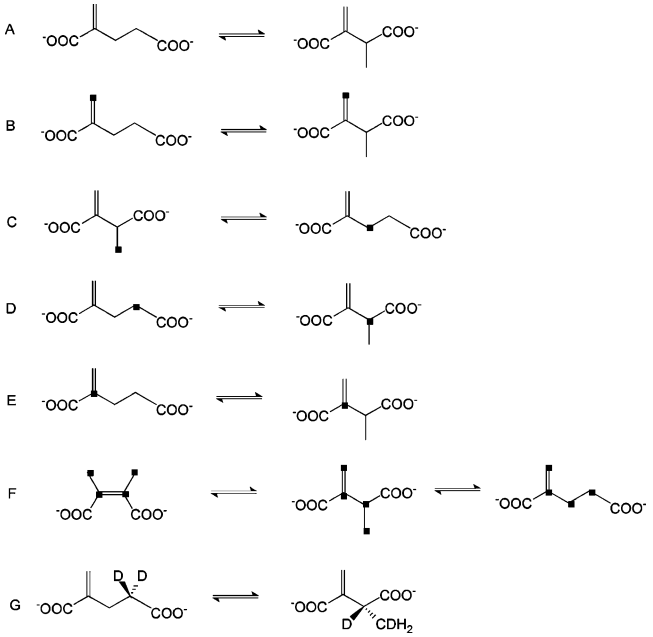


FIGURE 5: Structures of the labeled and unlabeled compounds used for the EPR spectra (Figure 4). The compound on each left side was used to start the reaction with 2-methyleneglutarate mutase. Incubation F contained in addition 3-methylitaconate isomerase. The ^{13}C atoms are indicated by black squares.

Propionibacterium shermanii (Figure 3E) (*53*, *54*), and ribonucleoside triphosphate reductase (Figure 3F) (*55*). The spectra are typical for cob(II)alamin coupled to an organic radical: the g_{xy} region (zero-crossing at $g \approx 2.10$, 310 mT) showed no clear resolution of ^{59}Co hyperfine and ^{14}N superhyperfine coupling, while the g_z line ($g = 2.00$, 320 mT) was split into eight hyperfine lines due to coupling with ^{59}Co ($I = 7/2$) without resolution of the superhyperfine coupling with the axial ^{14}N ligand ($I = 1$). For comparison the EPR spectrum of cob(II)alamin in the absence of a radical (Figure 3A) and that of a carbon centered radical alone (Figure 3B) are included in Figure 3.

Prolonged aerobic or anaerobic incubation of apo-2-methyleneglutarate mutase with cobalamin and 2-methyleneglutarate (14 h at 37 °C) elicited dramatic changes in the UV/visible (peak at 470 nm; see Figure 1) and EPR spectra (shift of the positive lobe of the spectrum from 300 to 280 mT in Figure 2C, $g_{av} = 2.10$ to 2.18 with a clear appearance of $g_{xy} = 2.3$), which demonstrates the formation of cob(II)alamin. Anaerobic incubation in the dark led to the formation of only cob(II)alamin (Figure 2B), whereas after aerobic incubation aquocobalamin was formed in addition to cob(II)alamin (Figure 2C). In both cases the enzyme was inactivated. Subsequent aeration of the anaerobic sample gave incomplete conversion of the cob(II)alamin to aquocobalamin. The results indicate that aerobic and anaerobic incubation with the substrate caused the formation of both oxygen-stable and oxygen-sensitive cob(II)alamin. Free cob(II)alamin and loosely bound cob(II)alamin were oxidized to aquocobalamin, whereas the tightly bound cobalamin is probably concealed in a pocket of the enzyme and hence is not accessible to oxygen. The 2-methyleneglutarate mutase isolated from *E. barkeri* also contained cob(II)alamin inaccessible to oxygen (*38*, *39*). In the paper by Michel et al. (*39*) it was assumed that cob(II)alamin might act as prosthetic group in addition to adenosylcobalamin. The results reported here rule out this possibility.

EPR-Spectroscopic Characterization. The EPR spectrum of 2-methyleneglutarate mutase in the presence of 2-methyleneglutarate (Figures 2A, 3D, and 4A) exhibited an average g value of 2.102 that arises from coupling between a carbon-centered radical ($g \approx 2.0023$) with cob(II)alamin ($g_{av} = 2.18$). The data are in good agreement with values for glutamate and methylmalonyl-CoA mutase (2.100 ± 0.001) (*21*, *56*). The observed spectrum was not changed by replacing adenosylcobalamin with [5′-^{13}C]adenosylcobalamin, as was also observed with glutamate mutase (Bothe, Pierik, and Buckel, unpublished results). To try to pinpoint the site(s) of the carbon-centered radical(s), a series of ^{13}C-labeled 2-methyleneglutarates and 3-methylitaconates (Figure 5) was studied. None of these showed any significant effect on the $g_{parallel}$ cobalt(II) hyperfine region of the spectrum (Figure 4B−F), unlike the dramatic effect observed when [4-^{13}C]glutamate was employed with glutamate mutase. This was true irrespective of whether the starting material was 2-methyleneglutarate or 3-methylitaconate (or dimethylmaleate + 3-methylitaconate isomerase). However, with 2-methylene[4-$^{2}H_2$]glutarate a significant change of the spectrum was observed (Figure 4G), possibly indicative of a contribution from the substrate radical (S•, 2-methylene-4-glutaryl).

Inhibition Experiments. The effects of acrylate, 1-methylcyclopropane-1,2-dicarboxylates, and (*E*)- and (*Z*)-2-methylpent-2-enedioate on 2-methyleneglutarate mutase were determined using standard assay procedures as described in

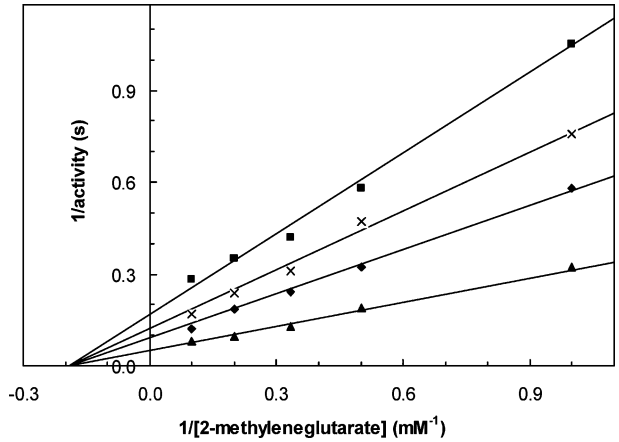


FIGURE 6: Noncompetitive inhibition of 2-methyleneglutarate by (1*R*,2*R*)-1-methylcyclopropane-1,2-dicarboxylate at 0 mM (triangles), at 10 mM (diamonds), at 20 mM (crosses), and at 30 mM (squares).

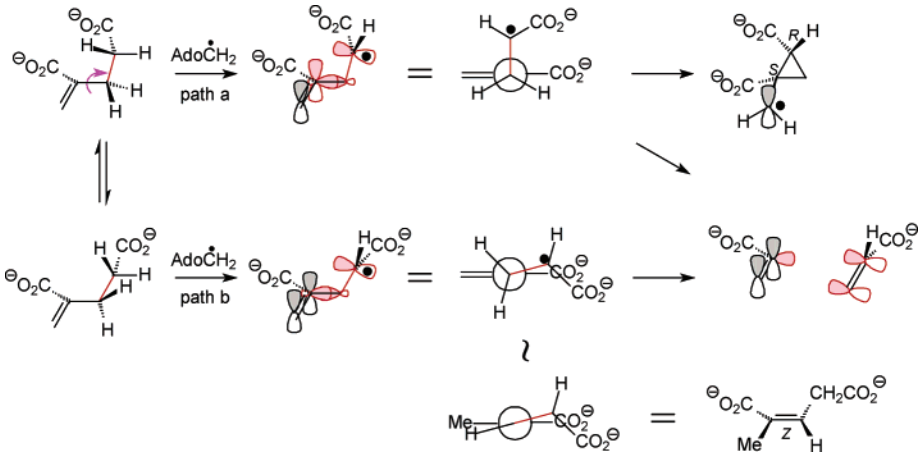
Materials and Methods. In contrast to earlier results we found that acrylate (up to 15 mM) was not an inhibitor of the enzyme. Currently we have no explanation why in earlier experiments we observed an inhibition, which, furthermore, was claimed to be linearly dependent on the square of the acrylate concentration (*22*, *57*). None of the 1-methylcyclopropane-1,2-dicarboxylates exhibited competitive inhibition, although the (1*R*,2*R*)-isomer was a weak noncompetitive inhibitor, $K_i = 13$ mM (Figure 6). (*E*)- and (*Z*)-2-methylpent-2-enedioate failed to show any competitive or noncompetitive inhibitory behavior and did not show mechanism-based inactivation in the absence of 2-methyleneglutarate.

Mechanistic Implications. The major aim of the present study was to acquire experimental evidence that would enable a distinction to be made between the addition−elimination (*24*) and fragmentation−recombination (*22*) mechanisms (Scheme 2) proposed for 2-methyleneglutarate mutase. For the related enzyme glutamate mutase, for which an analogous addition−elimination mechanism is impossible, the accumulated evidence (*11*, *21*−*23*, *58*) supports the fragmentation−recombination mechanism (*22*). For 2-methyleneglutarate mutase, ab initio molecular orbital calculations have shown that the addition−elimination pathway is energetically more favorable than fragmentation−recombination, with the latter having an intrinsic energy barrier of >100 kJ mol^{-1} (*59*). The possibility of the addition−elimination pathway was demonstrated by early model studies (*24*). Recent model studies concluded, however, that the rate of formation of the 1-methylenecyclopropane-1,2-dicarboxylate radical is "nearly 5 orders of magnitude too slow" for it to be a kinetically competent intermediate in the enzymatic pathway (*60*). Likewise, it was claimed that results from the equilibration of (*Z*)-3-methyl[2′-$^2H_1$]itaconate and (*Z*)-3-[2′-$^2H_1$,*methyl*-$^2H_3$]methylitaconate with their *E*-isomers and the corresponding (*E*)- and (*Z*)-2-methylene[2′-$^2H_1$]glutarates catalyzed by 2-methyleneglutarate mutase could not be satisfactorily explained by the addition−elimination pathway (*61*). The data were rationalized by a fragmentation−recombination pathway, in which there were two states for the fragment radical separated by an energy barrier. However, the possibility that the enzyme might destabilize the substrate radical was not taken into account. The effect of this could be to enhance the rate of formation of the 1-methylenecyclopropane-1,2-dicarboxylate radical and make the relative stabilities of substrate and product radicals closer than predicted from considerations of model radicals (i.e. a nonstabilized methylene radical versus a carboxy-stabilized methine radical).

In the present work, the possible intermediacy of the 1-methylenecyclopropane-1,2-dicarboxylate radical was explored by the synthesis and assay of all four isomers of 1-methylcyclopropane-1,2-dicarboxylate (Chart 1). The classical studies of Kung and Stadtman had claimed that the *trans*-pair (1*R*/2*R* and 1*S*/2*S*) was a competitive inhibitor of 2-methyleneglutarate mutase, while the *cis*-pair (1*R*/2*S* and 1*S*/2*R*) exhibited weak noncompetitive inhibition (*4*). Given that the absolute stereochemistry of product 3-methylitaconate is *R* (*19*), the expected stereochemistry of the intermediate 1-methylenecyclopropane-1,2-dicarboxylate radical is either (1*R*/2*R*) or (1*S*/2*R*). We found that *none* of the isomers acted as a competitive inhibitor although the (1*R*/2*R*)-isomer was a weak noncompetitive inhibitor (Figure 6). This result does not exclude the intermediacy of a 1-methylenecyclopropane-1,2-dicarboxylate radical because the bound state of this radical may not allow exchange with external 1-methylcyclopropane-1,2-dicarboxylate. Alternatively, there could be a conformational change of the enzyme during the reaction such that the free enzyme is unable to bind this compound.

In the fragmentation−recombination mechanism there is participation of a fragment radical, 2-acrylate, and an acrylate molecule. We therefore investigated whether acrylate inhibits 2-methyleneglutarate mutase and if so there was a square dependence on acrylate concentration. We have previously claimed such an effect (*57*), but careful repetition of the earlier experiments failed to demonstrate any acrylate inhibition.(*E*)- and (*Z*)-2-methylpent-2-enedioate were selected because they potentially provided interesting alternative modes of inhibition. These molecules are conformationally restricted analogues, with respect to the C-2/C-3 bond, of 2-methyleneglutarate or the derived 2-methylene-4-glutaryl radical, and one or the other could act as a competitive inhibitor. If one of the isomers binds at the active site, abstraction of a C-4 hydrogen atom would generate an allyl radical, which could be a thermodynamic sink. The conformational requirements of the addition−elimination pathway clearly differ from that of the fragmentation−recombination pathway. For addition−elimination there are only two conformers for the 2-methylene-4-glutaryl radical that permit optimal stereoelectronics for the formation of the 1-methylenecyclopropane-1,2-dicarboxylate radical (cf. Scheme 3, path a). With fragmentation−recombination there are an infinite number of conformers of the 2-methylene-4-glutaryl radical about the C-2/C-3 bond that permit fragmentation to the 2-acrylate radical (cf. Scheme 3, path b). In the event, neither (*E*)- nor (*Z*)-2-methylpent-2-enedioate showed any inhibitory properties up to the maximum concentration of 4 mM possible for the steady-state kinetics. It is notable that these isomers cannot adopt conformations that correspond to the ideal conformers for addition−elimination. Indeed, the *Z*-isomer approximates to the conformer of the 2-methylene-4-glutaryl radical, in which the dihedral angle (C-1/C-2/C-3/C-4) is 0° (cf. Scheme 3). The *E*-isomer approximates to the conformer of the 2-methylene-4-glutaryl radical in which the dihedral angle (C-2′/C-2/C-3/C-4) is 0°. These

Scheme 3: Formation of a 2-Methylene-4-glutaryl Radical and an Alternative Conformer of 2-Methyleneglutarate[a]



[a] Path a: Formation of a 2-methylene-4-glutaryl radical from a specific conformer of 2-methyleneglutarate that can undergo both addition−elimination to the (1*S*,2*R*)-1-methylenecyclopropane-1,2-dicarboxylate radical and fragmentation−recombination to acrylate and the 2-acrylate radical. Path b: An alternative conformer of 2-methyleneglutarate achieved by rotating by 90° about the C-2/C-3 bond of the conformer shown for path a; this conformer can be approximately mimicked by (*Z*)-2-methylpent-2-enedioate. It can only undergo fragmentation−recombination. Rotation by 180° about the C-2/C-3 bond of the conformer shown for path a affords the only other conformer ideal for addition−elimination [leading to the (1*R*,2*R*)-1-methylenecyclopropane-1,2-dicarboxylate radical]. Rotation by 180° about the C-2/C-3 bond of the conformer shown for path b affords a conformer that can be approximately mimicked by (*E*)-2-methylpent-2-enedioate and can only undergo fragmentation−recombination. The addition−elimination pathway requires that the C-3/C-4 bond (highlighted in red) is perpendicular to the C=C.

conformers are the least ideal for addition−elimination but can permit fragmentation−recombination (Scheme 3). The failure of the 2-methylpent-2-enedioates to inhibit 2-methyleneglutarate mutase may be taken to indicate that the bound conformation of 2-methyleneglutarate and the derived 2-methylene-4-glutaryl radical has a dihedral angle (C-1/C-2/C-3/C-4 or C-2′/C-2/C-3/C-4) that significantly deviates from 0°, but is not necessarily the 90° required by the addition−elimination pathway.

EPR data for glutamate mutase obtained in the presence of isotopically labeled substrate molecules was clearly indicative of the presence of a dominant 4-glutamyl radical (*21*). Similar measurements for 2-methyleneglutarate mutase reported in this paper cannot be so decisively interpreted. In particular, disappearance of the cobalt(II) hyperfine coupling of the octuplet between 330 and 360 mT was not observed with any of the ^{13}C-labeled substrate or product molecules. There were subtle broadening effects in the central part of the spectra (around 320 mT), but these were insufficient to draw any firm conclusions. It was however shown that 2-methylene[4-$^2H_2$]glutarate induced significant sharpening of the EPR signal around $g = 2.10$ implying a contribution from a C-4 centered radical. The degree of interaction between the coupling partners (cob(II)alamin and organic radical) depends on distance and angular factors, as well as the nature of the organic radical. The width and shape of the overall EPR signal is similar to that observed with glutamate mutase and indicates a distance of 6−7 Å between cobalt(II) and the organic radical. Owing to angular factors, it is possible that the effect of ^{13}C hyperfine splitting at g_z of the coupled system might not manifest itself as in glutamate mutase. Finally, the hybridization state of the organic radical will influence the ^{13}C-hyperfine coupling. The substrate, product, and intermediate radicals are expected to occupy *2*p-type orbitals, whereas there is a potential ambiguity for the 2-acrylate radical because this can in principle be linear (with the unpaired electron occupying a *2*p-type orbital) (*62*) or bent (unpaired electron occupying an sp^2-type orbital) with the latter able to interact more strongly with the adjacent carbon nucleus (*59*).

In summary, the EPR data presented in this paper indicates that 2-methyleneglutarate mutase involves a cob(II)alamin−organic radical partnership similar to that in glutamate mutase, although firm conclusions could not be derived on the basis of ^{13}C-labeling. To aid the interpretation of the EPR data, additional selectively deuterated 2-methyleneglutarates would be useful in conjunction with high frequency and pulsed EPR techniques. Further progress requires the crystal structure of 2-methyleneglutarate mutase. The purification of substantial quantities of enzyme as reported herein makes this now a realistic prospect.

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SUPPORTING INFORMATION AVAILABLE

Supporting Information containing synthesis procedures of unlabeled and isotopically labeled 2-methyleneglutarates, 3-methylitaconates, dimethylmaleates, (*E*)- and (*Z*)-2-methylpent-2-enedioic acid, and 1-methylcyclopropane-1,2-dicarboxylic acids. This material is available free of charge via the Internet at http://pubs.acs.org.

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