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Substrate Specificity of 2-Hydroxyglutaryl-CoA Dehydratase from *Clostridium symbiosum*: Toward a Bio-Based Production of Adipic Acid

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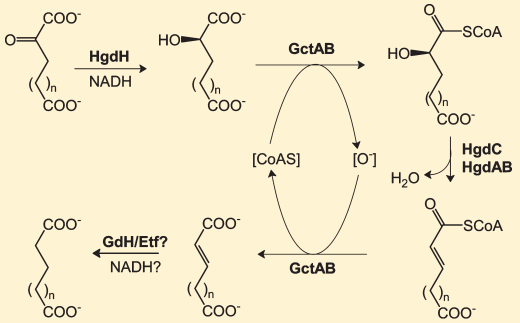
Supporting Information

ABSTRACT: Expression of six genes from two glutamate fermenting clostridia converted *Escherichia coli* into a producer of glutaconate from 2-oxoglutarate of the general metabolism (Djurdjevic, I. et al. **2010**, *Appl. Environ. Microbiol. 77*, 320−322). The present work examines whether this pathway can also be used to reduce 2-oxoadipate to (*R*)-2-hydroxyadipic acid and dehydrate its CoA thioester to 2-hexenedioic acid, an unsaturated precursor of the biotechnologically valuable adipic acid (hexanedioic acid). 2-Hydroxyglutaryl-CoA dehydratase from *Clostridium symbiosum*, the key enzyme of this pathway and a potential radical enzyme, catalyzes the reversible dehydration of (*R*)-2-hydroxyglutaryl-CoA to (*E*)-glutaconyl-CoA. Using a spectrophotometric assay and mass spectrometry, it was found that (*R*)-2-hydroxyadipoyl-CoA, oxalocrotonyl-CoA, muconyl-CoA, and butynedioyl-CoA, but not 3-methylglutaconyl-CoA, served as alternative substrates. Hydration of butynedioyl-CoA most likely led to 2-oxosuccinyl-CoA, which spontaneously hydrolyzed to oxaloacetate and CoASH. The dehydratase is not specific for the CoA-moiety because (*R*)-2-hydroxyglutaryl-thioesters of *N*-acetylcysteamine and pantetheine served as almost equal substrates. Whereas the related 2-hydroxyisocaproyl-CoA dehydratase generated the stable and inhibitory 2,4-pentadienoyl-CoA radical, the analogous allylic ketyl radical could not be detected with muconyl-CoA and 2-hydroxyglutaryl-CoA dehydratase. With the exception of (*R*)-2-hydroxyglutaryl-CoA, all mono-CoA-thioesters of dicarboxylates used in this study were synthesized with glutaconate CoA-transferase from *Acidaminococcus fermentans*. The now possible conversion of (*R*)-2-hydroxyadipate via (*R*)-2-hydroxyadipoyl-CoA and 2-hexenedioyl-CoA to 2-hexenedioate paves the road for a bio-based production of adipic acid.



Proposed production of glutaconic (n = 1) or adipic (n = 2) acids

Several anaerobic bacteria of the orders *Clostridiales* and *Fusobacteriales* ferment (*S*)-glutamate via (*R*)-2-hydroxyglutarate, (*R*)-2-hydroxyglutaryl-CoA, (*E*)-glutaconyl-CoA, and crotonyl-CoA to ammonia, CO_2, acetate, butyrate, and H_2.[1−4] Recently, we demonstrated that the expression of six genes of this pathway is able to convert *Escherichia coli* into a glutaconate producer.[5,6] Glutaconic acid [(*E*)-pentenedioic acid] and the saturated glutaric acid (pentanedioic acid) may be useful for the production of polyesters and polyamides. In *E. coli* the new pathway most likely starts by the NADH-dependent reduction of 2-oxoglutarate from the central metabolism to yield (*R*)-2-hydroxyglutarate (**2**, *n* = 1, Figure 1). Subsequent activation to the CoA thioester (**3**) and dehydration affords glutaconyl-CoA (**4**), which liberates the final product glutaconate (**5**) by a second CoA-transfer.

In this work we examine whether the three enzymes, 2-hydroxyglutarate dehydrogenase (HgdH) and glutaconate CoA-transferase (GctAB) from *Acidaminococcus fermentans* as well as 2-hydroxyglutaryl-CoA dehydratase (HgdC + HgdAB) from *Clostridium symbiosum*, can also convert 2-oxoadipate (2-oxohexanedioic acid, **1**, *n* = 2) to 2-hexenedioic acid (**5**). Reduction of this unsaturated dicarboxylic acid would lead to adipic acid (hexanedioic acid) that is chemically produced from benzene at a world scale of 2.4 million tons in the year 2008.[7] Adipic acid is a constituent of the polyamide Nylon-6,6 and several polyesters. It is already known that glutaconate CoA-transferase catalyzes the formation of adipoyl-CoA from adipate and acetyl-CoA. The substrate specificities of the two other enzymes, however, the dehydrogenase and the dehydratase, remain to be established. In a recent patent, the pathway shown in Figure 1 (*n* = 2) has been proposed for the production of adipic acid, but no experimental evidence has been presented.[8]

The key biochemical transformation in the glutamate fermentation pathway is the reversible dehydration of (*R*)-2-hydroxyglutaryl-CoA to (*E*)-glutaconyl-CoA.[3,9−12] The reaction is catalyzed by a heterodimeric dehydratase, which probably contains in each subunit

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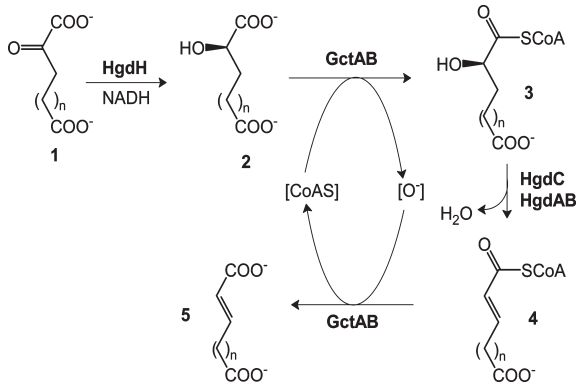

Figure 1. Pathway of the conversion of 2-oxoglutarate (**1**, $n = 1$) to (*E*)-glutaconate (**5**, $n = 1$) in a recombinant *E. coli* strain. Starting with 2-oxoadipate (**1**, $n = 2$), the product would be (*E*)-2-hexenedioic acid (**5**, $n = 2$). The intermediates are (*R*)-2-hydroxyglutarate/(*R*)-2-hydroxyadipate (**2**, $n = 1/2$), (*R*)-2-hydroxyglutaryl-CoA/(*R*)-2-hydroxyadipoyl-CoA (**3**, $n = 1/2$), and (*E*)-glutaconyl-CoA/(*E*)-2-hexenedioyl-CoA (**4**, $n = 1/2$). HgdH = (*R*)-2-hydroxyglutarate dehydrogenase; GctAB = glutaconate CoA-transferase that exchanges [CoAS] by [O$^-$] and vice versa; HgdAB = (*R*)-2-hydroxyglutaryl-CoA dehydratase; HgdC = activator of HgdAB.

one [4Fe-4S] cluster as revealed for the related (*R*)-2-hydroxyisocaproyl-CoA dehydratase from *Clostridium difficile*.[13] The dehydratase requires activation by a homodimeric protein with one $[4Fe\text{-}4S]^{1+/2+}$ cluster between the two subunits,[14] the so-called "archerase".[15] The moderately oxygen-sensitive dehydratase from *C. symbiosum* (α, 48 Da and β, 43 kDa) contains in addition to the two $[4Fe\text{-}4S]^{2+}$ clusters, 1.0 reduced riboflavin-5′-phosphate (FMN), 0.3 riboflavin, and 0.1−0.2 molybdenum.[10,16] For activation, the protein from *A. fermentans* (HgdC, 2 × 27 kDa) is used, which is produced with a C-terminal Strep-tag in *Escherichia coli* and acts with the same efficiency as the activator from *C. symbiosum*. The activation is preceded by one-electron reduction of the extremely oxygen-sensitive activator with reduced ferredoxin or flavodoxin in vivo[17] or by Ti(III)citrate or dithionite in vitro. In the presence of Mg^{2+}, the electron is transferred to the dehydratase driven by hydrolysis of ATP. Most likely one of the aconitase-like $[4Fe\text{-}4S]^{2+}$ clusters of the dehydratase stores the electron until a substrate molecule binds to the other cluster and accepts the electron to initiate the catalytic cycle.[13]

In (*R*)-2-hydroxyglutaryl-CoA, the β-hydrogen to be removed has an approximate $pK_a = 40$, which precludes simple acid−base catalysis by the dehydratase. The requisite acidification of this proton is achieved by transient addition of a high-energy electron to the thioester carbonyl to form a ketyl radical (Figure 2). This allows elimination of the α-hydroxyl group[18,19] and lowers the pK_a of the β-hydrogen in the enoxy radical intermediate by about 26 units.[20] The resulting allylic ketyl radical returns the electron to the enzyme and the product is released. Now the electron can be recycled for many turnovers until it is lost by accidental oxidation and the enzyme requires a new "shot" of the activator. In the case of (*R*)-2-hydroxyisocaproyl-CoA dehydratase, the allylic ketyl radical intermediate was structurally characterized by electron paramagnetic resonance (EPR) spectroscopy with isotopically labeled substrates.[18] In 2-hydroxyglutaryl-CoA dehydratase from *C. symbiosum*, none of the proposed substrate radicals have been detected yet.

In this work, it is shown that 2-hydroxyglutaryl-CoA dehydratase is able to catalyze the dehydration of (*R*)-2-hydroxyadipoyl-CoA as well as the hydration of muconyl-CoA, oxalocrotonyl-CoA,

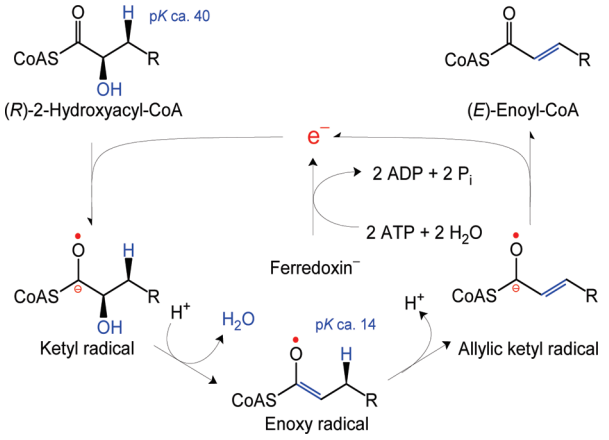

Figure 2. Proposed mechanism for the dehydration of 2-hydroxyaxcyl-CoA, with 2-hydroxyglutaryl-CoA dehydratase R = acetate, propionate, or derivatives thereof.

and butynedioyl-CoA, which are synthesized with glutaconate CoA-transferase. The results further indicate that the pathway of Figure 1 may also work with six carbon substrates (Figure 1, $n = 2$) and pave the road for the production of adipic acid. 2-Hydroxyglutaryl-CoA dehydratase also accepts substrates, in which CoA is replaced by *N*-acetylcysteamine and pantetheine.

EXPERIMENTAL PROCEDURES

Bacterial Cultures. *C. symbiosum* DSM 934 (German collection of microorganisms and cell cultures, Braunschweig, Germany, DMSZ) was grown anaerobically on a glutamate/yeast extract medium.[21] *Escherichia coli* strain XL1-blue MRF′ (Stratagene, Heidelberg, Germany) was used for the activator synthesis by expression of *hgdC* from *A. fermentans* cloned in pASK-IBA3 to yield the plasmid pMH6 (IBA-GmbH, Göttingen, Germany).[11] For production of the recombinant activator, HgdC, the cells were grown anaerobically in 10 L of Standard 1 medium (Merck, Darmstadt, Germany) containing 20 mM morpholine propanesulfonic acid (MOPS) pH 7.4 and carbenicillin (50 μg/L) at 37 °C. When the optical density at 578 nm of the culture reached 0.25, the cells were induced with 100 μM anhydrotetracyline for 3 h and harvested under anaerobic conditions. The cells were stored at −80 °C. Recombinant *E. coli* cells carrying the pJF118HE plasmid coding for glutaconate CoA-transferase (GctAB) from *A. fermentans*[22] were cultivated aerobically in Standard 1 medium containing carbenicillin (50 μg/L) at 37 °C with shaking. For production of the transferase, the cells were grown aerobically in 2 L of the same medium until A_{578} reached 2.0. The cells were induced with 300 μM β-isopropyl thiogalactoside and grown until A_{578} reached 5.5. The cells were harvested and stored at −80 °C.

Chemical and Enzymatic Syntheses. (*R,S*)-2-Hydroxyadipic acid (2-hydroxyhexanedioic acid) was prepared by adding dropwise under stirring 9.2 mmol of aqueous $NaNO_2$ to 6.2 mmol of commercial (*R,S*)-2-aminoadipate[23] in 10 mL of 6 M HCl at −5 °C. Stirring was continued overnight at room temperature. After extraction with ethyl acetate and drying with anhydrous Na_2SO_4, the solvent was evaporated and the residue was dissolved in absolute ethanol. A few drops of conc. HCl were added and the mixture was refluxed overnight. The resulting mixture was purified on a silica gel (200-mesh Sigma-Aldrich) column with a 1:1 solvent mixture of petrol 40/60 and diethyl ether. Two fractions separated and only the lesser polar first

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fraction was concentrated by rotary evaporation and saponified with 1 M NaOH. The product was then recrystallized from ether-petrol and identified as 2-hydroxyadipate by analyzing its NMR spectrum, yield 60%. ^{1}H NMR ($CDCl_3$) δ ppm: 4.3 (m, 1H), 2.3 (t, 2H), 1.8 (m, 2H), 1.6 (dt, 2H). ^{13}C NMR ($CDCl_3$) δ ppm: 180 (C-6), 178 (C-1), 73 (C-2), 34 (C-5), 32 (C-3), 22 (C-4).

Oxalocrotonic acid (2-hydroxymuconic acid, 2-oxo-4-hexenedioic acid) was synthesized by a modified procedure.[24,25] Potassium tertiary butoxide (35.5 mmol) was added under nitrogen to a reaction flask maintained at 0 °C. Ethyl crotonate (32.4 mmol) and sufficient dry ether were added and the mixture was stirred for 15 min. Then, ethyl oxalate (32.4 mmol) was introduced under anhydrous conditions and the mixture was stirred at the same temperature for 30 min. The reaction mixture was left overnight at 0−4 °C. The resulting bright yellow mixture was filtered and the solid material was dissolved in ice water. The solution was acidified with 8 M acetic acid, and the precipitate was collected and washed with cold water to obtain diethyl oxalocrotonate (85% yield). ^{1}H NMR ($CDCl_3$) δ ppm: 7.7 (1H, t), 6.3 (1H, d), 6.0 (1H, d), 4.4 (2H, q), 4.2 (2H, q), 1.5 (3H, t), 1.3 (3H, t). ^{13} C NMR ($CDCl_3$) δ ppm: 169 (C-1), 167 (C-6), 143 (C-2), 136 (C-3), 123 (C-5), 108 (C-4), 63 (CH_2), 59 (CH_2), 13 (CH_3), 10 (CH_3). Diethyl oxalocrotonate dissolved in 200 mL of cold 6 M sodium hydroxide was kept at room temperature until no more precipitate formed with acetic acid. The solution was diluted with water to 700 mL, filtered, cooled, and acidified with 8 M acetic acid. After the solution stood overnight at room temperature, 2-hydroxymuconic acid separated. It was removed by filtration, washed with a small amount of water on a Büchner funnel, and dried, yield 70%. ^{1}H NMR (CD_3OD) δ ppm: 7.16 (1 H, dd), 5.95 (1H, d), 5.84 (1H, d). ^{13}C NMR (CD_3OD) δ ppm: 109.0 (C-3), 122.2 (C-5), 139.6 (C-4), 148.2 (C-2), 166.6 (C-1), 170.5 (C-6).

Chemical Synthesis of Thioesters − General. CoASH, *N*-acetylcysteamine, or pantetheine were dissolved in aqueous 0.5 M potassium or sodium bicarbonate at ambient temperature. Then the activated acid (anhydride, acyl chloride, or acyl imidazole) dissolved in acetonitrile or tetrahydrofuran was added in slight excess. After stirring for 10 min, the SH-test with 5,5′-dithiobis(2-nitrobenzoic acid) usually was negative. Finally, the reaction mixture was acidified with 6 M HCl to pH 2 and the solvent was removed by freezing and centrifuging on a Speed-Vac concentrator (Bachofer, Burladingen, Germany). Addition of the anhydride without organic solvent to the thiol in aqueous bicarbonate as described in ref 26 often resulted in over acylation of CoASH as determined by matrix-assisted laser desorption ionization-time of flight (mass spectrometry) (MALDI-TOF) mass spectrometry (T. Selmer, Fachhochschule Aachen, Germany, personal communication) or Michael addition in the case of crotonic anhydride.[27] On the other hand, acyl chlorides added without organic solvent hydrolyzed rather than reacted with CoASH (H. Eggerer, TU München, Germany, personal communication). All thioesters were purified by solid phase extraction with Sep-Pak C_{18} cartridges (Waters, Massachusetts, USA) prewashed with methanol and equilibrated with 0.1% TFA (v/v). After loading, the columns were washed with 3 vol of 0.1% TFA. Elution was performed with 0.1% TFA containing 50% acetonitrile (v/v). Acetonitrile was removed from the eluted thioesters by freezing and centrifuging on a Speed-Vac concentrator. The thioesters were then lyophilized (Alpha 1-4, Martin Christ, Osterode am Harz, Germany). The obtained powders were stored at −80 °C.

Acetyl-CoA, crotonyl-CoA, and glutaryl-CoA were prepared by adding 35 μmol of acetic anhydride, crotonic anhydride, or glutaric anhydride in 0.5 mL of acetonitrile to 25 μmol of CoA in 5 mL of 0.1 M $KHCO_3$. (*R*)-2-Hydroxyglutaryl-CoA was synthesized starting from commercial available lactone, (*R*)-2-oxotetrahydrofuran-carboxylic acid, via the acylchloride as described earlier.[12] In the same manner, the acyl chloride was coupled to the thiol group of *N*-acetylcysteamine to yield (*R*)-*S*-2-hydroxyglutaryl-*N*-acetylcysteamine. ^{1}H NMR (D_2O) δ ppm: 5.1 (m, 1H), 3.35 (m, 2H), 3.1 (m, 2H), 2.6 (m, 3H, i.e., H_S +2H), 2.3 (m, 1H, H_R), 2.0 (s, 3H). Pantethine was reduced to pantetheine in water with a 2-fold molar excess of sodium borohydride, the pH being kept at 8. The sample was acidified to pH 2 and degassed. Pure pantetheine was obtained by a passage through a Sep-Pak C_{18} cartridge. Pantetheine was coupled to the acid chloride to yield (*R*)-2-hydroxyglutaryl-pantetheine. ^{1}H NMR (D_2O) δ ppm: 5.0 (m, 1H), 3.85 (s, 1H), 3.4 (m = overlaid dt, 4H), 3.25 (s, 2H), 2.7 (t, 2H), 2.55 (m, 2H), 2.35 (t, 2H), 2.25 (m, 2H), 0.75 (s, 6H).

Pentanoyl-CoA, 2-pentenoyl-CoA, and 2,4-pentadienoyl-CoA were synthesized according to the mixed anhydride method.[28] About 250 μmol of free acid in 2 mL of dry toluene, 300 μmol of dry triethylamine, either from a new bottle or freshly distilled, and 300 μmol of ethyl chloroformate were mixed under an N_2 atmosphere for 1 h. Then, toluene, triethylamine, and ethyl chloroformate were removed by a stream of N_2. The residue was dissolved in 5 mL of dry tetrahydrofuran. A 2−3 fold molar excess of the resulting anhydride was used for acylation of CoASH.

(*R*)-2-Hydroxybutyryl-CoA was synthesized via an imidazole intermediate.[29] The carboxylic acid (50 μmol) and carbonyl-diimidazole (70 μmol, Sigma, Deisenhofen, Germany) were mixed in 0.5 mL of dry acetonitrile. After about 2 min, the mixture was dropped into 40 μmol of CoA dissolved in 0.5 mL of 0.5 M $NaHCO_3$.

Enzymatic Synthesis of CoA Esters. Glutaconate CoA-transferase was used to synthesize glutaconyl-CoA,[12,30] 3-methylglutaconyl-CoA,[31] butynedioyl-CoA, 2-hydroxyadipoyl-CoA, oxalocrotonyl-CoA, and muconyl-CoA with acetyl-CoA or glutaryl-CoA as CoA donor. The enzyme was prepared from *E. coli* cells expressing the genes of glutaconate CoA-transferase from *A. fermentans* grown as described above. The cells (5 g) were suspended in 17 mL of 20 mM potassium phosphate, pH 7.4, and sonicated in two 15-min intervals at 0 °C with a Branson sonifier. Cell debris was removed by centrifugation at 100000*g* for 1 h. Solid ammonium sulfate was added to the cell-free extract to achieve 50% saturation. After centrifugation at 25000*g* for 30 min, the supernatant was brought to 80% ammonium sulfate saturation and centrifuged as described above. The protein pellet was dissolved in 40 mL of 20 mM potassium phosphate, pH 7.4 and passed through a Centricon cutoff membrane of 100 kDa. The sample was stored at 4 °C. Using this method, glutaconate-CoA transferase was partially purified with a specific activity of 100 U mg^{-1} using glutaryl-CoA as a substrate (1 U = 1 μmol min^{-1}). The pure enzyme with a specific activity of 130 U mg^{-1} [32] was not necessary for the synthesis of CoA esters. However, it was necessary to check the formation of free CoA from acetyl-CoA and phosphate, which was a competing reaction catalyzed by phosphate acetyltransferase. A slight phosphate acetyltransferase activity of 1.5 U/mg was found. When the buffer was changed from 100 mM potassium phosphate pH 7 to 100 mM Tris-HCl pH 7.2, the activity was suppressed. The replacement of acetyl-CoA with glutaconyl-CoA also circumvented the problem.

For the synthesis of CoA esters, acetyl-CoA (20 μmol), 200−400 μmol of the respective carboxylic acid, and 50 μg of

Table 1. Summary of Michaelis-Menten Parameters with Various Substrates of 2-Hydroxyglutaryl-CoA Dehydratase[a]

substrate	K_m μM	V_{max} U mg^{-1}	$k_{cat} \times K_m^{-1}$ M^{-1} s^{-1}
(*R*)-2-hydroxyglutaryl-CoA	52 ± 3	55 ± 5	1.6×10^6
(*R*)-2-hydroxyglutaryl-*N*-acetylcysteamine	115 ± 7	43 ± 6	5.6×10^5
(*R*)-2-hydroxyglutaryl-pantetheine	90 ± 5	48 ± 4	8.0×10^5
glutaconyl-CoA	250 ± 50	4.5 ± 0.5	2.7×10^4
(*R*)-2-hydroxyadipoyl-CoA	100 ± 10	29.0 ± 0.3	4.4×10^5
muconyl-CoA	570 ± 50	3.1 ± 0.4	8.2×10^3
oxalocrotonyl-CoA	1100 ± 100	0.6 ± 0.2	8×10^2
butynedioyl-CoA	2100 ± 100	1.9 ± 0.4	14×10^2

[a] The unit of enzymatic acitivity (U) is defined as the conversion of 1 μmol of substrate × min^{-1}. If the enzyme has a molecular mass of 60 kDa, the values of the specific activity (U mg^{-1} protein) and k_{cat} (s^{-1}) are identical.

transferase were mixed in a volume of 3−5 mL 100 mM potassium phosphate pH 7.0. After 10 min to 1 h at 37 °C, the mixture was acidified to pH 2 and filtered through a 10-kDa membrane (Amicon, Amersham Biosciences) to remove the enzyme. To obtain preferably (*R*)-2-hydroxyglutaryl-CoA or (*R*)-2-hydroxyadipoyl-CoA, the incubation time was 10 min or less, whereas 4-hydroxyglutaryl-CoA or 5-hydroxyadipoyl-CoA dominated the reaction mixture after 1 h. *trans,trans*-Muconic acid and oxalocrotonic acid were converted to their potassium salts by neutralization with 1 M KOH, and the dried salts were used instead of the acids as before. The CoA esters were purified as described above for the chemical synthesized species.

The activity of 2-hydroxyglutaryl-CoA dehydratase was measured under strict anaerobic conditions in a quartz cuvette (d = 1 cm), total volume 0.5 mL at 25 °C with 50 mM Tris/HCl (pH 8.0), 5 mM $MgCl_2$, 5 mM dithiothreitol, 0.4 mM ATP, and 0.1 mM dithionite, as well as activator from *A. fermentans* (0.5 μg) and dehydratase from *C. symbiosum* (2.0 μg). After incubation for 5 min, the reaction was started by addition of (*R*)-2-hydroxyglutaryl-CoA. The formation of (*E*)-glutaconyl-CoA was measured at 290 nm (ε_{290} = 2.2 mM^{-1} cm^{-1}). In the reverse direction and with the other substrates (Table 1) up to 100 times more enzyme and activator were used. The values for k_{cat} were calculated with m = 91 kDa and the following extinction coefficients: ε_{290} = 2.2 mM^{-1} cm^{-1} for (*E*)-glutaconyl-CoA and 2-hexenedioyl-CoA (derived from 2-hydroxyadipoyl-CoA), 6.3 mM^{-1} cm^{-1} for muconyl-CoA, 5.8 mM^{-1} cm^{-1} for oxalocrotonyl-CoA, and 9.6 mM^{-1} cm^{-1} for butynedioyl-CoA. 2-Hydroxyglutaryl-CoA dehydratase was purified from *C. symbiosum* under anaerobic conditions.[12,33] The activator from *A. fermentans* produced in *E. coli* was purified as described.[11]

Glutaconate CoA-transferase activity was measured by the 5,5′-dithiobis(2-nitrobenzoate) assay in potassium phosphate pH 7.0 with glutaryl-CoA and acetate as substrates via acetyl-CoA and citrate synthase + oxaloacetate.[30] The increase of absorbance was followed at 410 nm (ε_{410} = 14.2 mM^{-1} cm^{-1}).[34] To check for phosphate acetyltransferase activity, controls were run without citrate synthase. The concentrations of CoASH, acetyl-CoA, and glutaconyl-CoA, or any other CoA-ester substrate of the transferase were determined with 5 μg CoA-transferase in a single assay.[30] Malate dehydrogenase,[35] crotonase [36] and fumarase [37] were assayed by described procedures.

2-Hydroxyglutarate dehydrogenase activity was measured in cuvettes of 0.5 mL total volume containing 0.1 M Tris-HCl pH 8.0, 0.2 mM NADH, and 20 ng of enzyme. After addition of 1.0 mM 2-oxoglutarate, the absorbance decrease of NADH was monitored at 340 nm (ε = 6.3 mM^{-1} cm^{-1}).[35] With 2-oxoadipate and oxalocrotonate as substrates, 1 μg of enzyme was used. The enzyme from *A. fermentans* was prepared by overproduction in a recombinant *E. coli* strain.[38,39] The homogeneity of the purified proteins was checked by SDS−PAGE.[40] The protein concentrations were estimated with the Biorad-Microassay reagent (Bio-Rad-Laboratories, Munich, Germany). Bovine serum albumin (Sigma, Germany) was used as a standard.

HPLC was performed on a reverse phase C18 column (size 125/2) in 5% acetonitrile in water with UV detection at 215 nm. A linear gradient to 99% acetonitrile was applied within 30 min. The retention times of acetyl-CoA and muconyl-CoA were 22 and 29 min, respectively.

All NMR spectra were recorded at the Department of Chemistry, Philipps Universität, Marburg. ^{1}H and ^{13}C NMR spectra were routinely measured with 3−30 mg samples in standard NMR solvents (Sigma-Aldrich, Germany) on a Bruker AVANCE 300 B (300 MHz) spectrometer in automated mode. The ^{1}H spectra were measured at 300 MHz and ^{13}C spectra at 75.45 MHz. Chemical shifts were determined with respect to a 3-(trimethyl)-[$^2H_6$]propane-1-sulfonate standard.

MALDI-TOF Mass Spectrometry. The CoA samples were purified from their synthesis or enzyme reaction mixtures as described above and the lyophilized samples were dissolved in 10−40 μL of water. Close external calibration was performed with the DP mass calibration kit from BRUKER Daltonics (Bremen, Germany). Acetyl-CoA or free CoA was used as internal standards when present in the mixture. The matrix was CHCA (alpha-cyano-4-hydroxycinnamic acid from Sigma) dissolved in 70% acetonitrile/0.1% TFA. The sample (1 μL) was mixed with 1 μL of α-cyano-4-hydroxycinnamic acid as matrix and spotted onto a gold plate in a dilution series. Measurements were performed with a 355 nm laser in positive reflector mode with a delayed extraction with a positive polarity on the 4800 *Plus* MALDI TOF/TOF Analyzer (Applied Biosystems, Framingham, USA). For each spectrum, 40 subspectra with 90 shots each were acquired.

Electrospray ionization (ESI) mass spectrometry was performed on a Finnigan LTQ-FT (Thermo Fisher Scientific, Germany) mass spectrometer at Department of Chemistry, Philipps University, Marburg, in certain cases where the ^{1}H or ^{13}C NMR spectra were not enough to assess the purity of compounds. The sample (15 mg) was dissolved in 0.5 mL of methanol and subjected to ESI mass spectrometry in the negative ion mode.

EPR Spectroscopy. The EPR measurements were performed on an X-Band-EPR-spectrometer EMX-6/1 with a rectangular standard cavity from Bruker and cooled with an ER-4112HV helium-flow cryostat from Oxford Instruments. Each sample contained 3 mg of 2-hydroxyglutaryl-CoA dehydratase and 50 μg of activator, 5 mM $MgCl_2$, 5 mM dithiothreitol, 0.4 mM ATP, 0.1 mM sodium dithionite, and 100 mM Tris/HCl pH 8.0 in a total volume of 0.25 mL. The CoA ester concentration was 1 mM in all cases. The enzymes were mixed anaerobically with buffer and all components except substrate and incubated for 2 min; substrate was then added and the mixture was filled into an

EPR tube and quickly frozen. The microwave power was 20 mW, microwave frequency 9.458 GHz, modulation amplitude 1.28 mT, and temperature 9.5 K. For detection of ketyl radical signals, the same conditions were applied as were used in ref 18.

RESULTS AND DISCUSSION

Assay and Purification of 2-Hydroxyglutaryl-CoA dehydratase. The reaction of (*R*)-2-hydroxyglutarate with acetyl-CoA catalyzed by glutaconate CoA-transferase from *A. fermentans* led to a mixture of the kinetically favored (*R*)-2-hydroxyglutaryl-CoA and to the thermodynamically favored (*R*)-4-hydroxyglutaryl-CoA.[30,41] Hence, short incubations predominantly yielded the (*R*)-2-isomer, the substrate of 2-hydroxyglutaryl-CoA dehydratase, whereas the inactive 4-isomer accumulated with time. Therefore, in routine measurements of the dehydratase activity, the 2-isomer was generated in situ by incubation of the dehydratase and activator with ATP, Mg^{2+}, dithiothreitol, dithionite, (*R*)-2-hydroxyglutarate, and acetyl-CoA. Addition of glutaconate CoA-transferase started the reaction, which was monitored by the absorption of the formed (*E*)-glutaconyl-CoA at 290 nm ($\Delta\varepsilon_{290}$ = 2.2 mM^{-1} cm^{-1}); at the absorption maximum of α,β-unsaturated thioesters at 260 nm,[27] the background absorption due to the adenine moiety of CoA was too high. Accurate measurements of Michaelis−Menten kinetics with (*R*)-2-hydroxyglutaryl-CoA using the purified enzyme (Figure S1, Supporting Information), however, required the production of this substrate via a defined chemical route starting from the γ-lactone of (*R*)-2-hydroxyglutarate, (*R*)-2-oxotetrahydrofurane-5-carboxylic acid.[12] Substitution of CoASH yielded (*R*)-2-hydroxyglutaryl-thioesters of *N*-acetylcysteamine and pantetheine.

The synthesis of glutaconyl-CoA with (*E*)-glutaconate, acetyl-CoA, and glutaconate CoA-transferase gave only the "correct" isomer, glutaconyl-1-CoA, in which the double bond is conjugated with the thioester. This is surprising because the saturated glutarate is also a good substrate for the transferase and indicates that an enoate is not much favored over a saturated carboxylate. The exclusive formation of (*E*)-glutaconyl-1-CoA (see supplement for the evidence) may due to the intrinsic isomerase activity of glutaconate CoA-transferase.[42] Hence, any binding of glutaconate to the transferase in the wrong orientation is apparently corrected by isomerization. Presumably, π-π interactions of the double bond with two aromatic residues (Tyr74A and Phe26A)[43] stabilize the correct orientation of glutaconate in the enzyme. In this orientation, the carboxylate-1 can directly react with the intermediate CoA-thioester at the catalytic residue Glu54B (A and B denote the α- and β-subunits of the enzyme, respectively), which renders (*E*)-glutaconyl-1-CoA as the kinetically favored isomer. The conjugation of the thioester with the double bond favors the 1-isomer also thermodynamically, estimated as $\Delta G^{\circ\prime}$ ca. −5 kJ mol^{-1} ($K' \sim 10$). These considerations also apply to the synthesis of oxalocrotonyl-CoA from oxalocrotonate, which when bound to glutaconate CoA-transferase in the same orientation as glutaconate should be preferred and yield the 2-enoyl-CoA rather than the 2-oxoacyl-CoA. The formation of CoASH during the reaction indicated, however, that approximately 30% of the oxalocrotonate attached in the opposite orientation to the enzyme leading to the 2-oxoacyl-CoA that hydrolyzed spontaneously. Hence, in this case the correction occurred by hydrolysis of the "wrong" product.

The purifications of 2-hydroxyglutaryl-CoA dehydratase from *C. symbiosum* and its recombinant activator from *A. fermentans*, produced with a C-terminal Strep-tag in *E. coli*, have been described earlier.[16] The reported procedures led to pure activator and dehydratase with a specific activity of 54 U mg^{-1} protein (k_{cat} = 82 s^{-1}) as measured with the synthesized (*R*)-2-hydroxyglutaryl-CoA (Figure S1, Supporting Information) and in the reverse direction with glutaconyl-CoA (4.5 U mg^{-1}, Figure S2, Supporting Information). The metal content of 2-hydroxyglutaryl-CoA dehydratase was redetermined by plasma emission spectroscopy and found per heterodimer: 7.3 Fe, 1.2 Zn, 0.7 Mn, and 2.2 Ca but only traces of the transition elements Mo and Cu. As the closely related 2-hydroxyisocaproyl-CoA dehydratase was devoid of any molybdenum[44] and, besides 8 Fe per heterodimer, no other metal was found in the crystal structure,[13] the other detected metals are currently considered to be impurities. The stoichiometric content of 1.0 $FMNH_2$ and 0.3 riboflavin, which is also completely absent in 2-hydroxyisocaproyl-CoA dehydratase, is rather puzzling. 2-Hydroxyisocaproyl-CoA dehydratase contains two [4Fe-4S] clusters, each of which is coordinated by three cysteines. The noncoordinated iron of the cluster of the α-subunit binds the substrate via the thioester carbonyl, whereas that of the β-subunit comprises a sulfido (HS^-) ligand. It has been proposed that the "β-cluster", which is located within the electron transferring distance of 12 Å from the "α-cluster", acts as reservoir for the recycling electron during product release and entry of a new substrate.[13] Recent crystallographic studies with the inactive subunit of 2-hydroxyglutaryl-CoA dehydratase revealed a β-cluster and the isoalloxazine moiety of riboflavin or FMN at a distance of about 13 Å. Surprisingly, in this β-cluster the sulfido ligand was replaced by a tyrosine. Possibly, the flavin together with the β-cluster provides an alternative electron reservoir in 2-hydroxyglutaryl-CoA dehydratase (personal communication by Betra Martins and Holger Dobbek, Humboldt Universität zu Berlin, Germany).

Alternate Substrates of 2-Hydroxyglutaryl-CoA Dehydratase. The enzyme catalyzed the dehydration of (*R*)-2-hydroxyglutaryl-*N*-acetylcysteamine (Figure S3, Supporting Information) and (*R*)-2-hydroxyglutaryl-pantetheine (Figure S4, Supporting Information) almost as efficiently as the CoA-substrate. Within the error of determination, the V_{max} values were identical, whereas the K_m values were about twice as high (Table 1). Hence, the ADP and pantothenic acid moieties of CoA appear to be remarkably unimportant for thioester binding and catalysis of the dehydratase. This observation agrees very well with the crystal structure of 2-hydroxyisocaproyl-CoA bound to the related 2-hydroxyisocaproyl-CoA dehydratase, in which only the pantetheine part of the CoA molecule interacts with the enzyme.[13]

The structures of the CoA-monothioesters that act as substrates of 2-hydroxyglutaryl-CoA dehydratase are illustrated in Figure 3. Table 1 summarizes the kinetic data of the dehydrations that are described in detail below. (*E*)-Crotonyl-CoA, (*R*)-2-hydroxybutyryl-CoA, (*E*)-2-pentenoyl-CoA, and (*E,E*)-2,4-pentadienoyl-CoA did not serve as substrates of the dehydratase. Interestingly, 3-methylglutaconyl-CoA, a mixture of *E*- and *Z*-isomers,[31] was also not hydrated. This agrees with the observation that clostridia reduce leucine but not valine and isoleucine via their (*R*)-2-hydroxyacids to the corresponding branched chain fatty acids. The 2-hydroxyacyl-CoAs that could be derived from valine and isoleucine contain a methyl group at C-3, which probably impedes the interaction of the dehydration at the [4Fe-4S] cluster.[13]

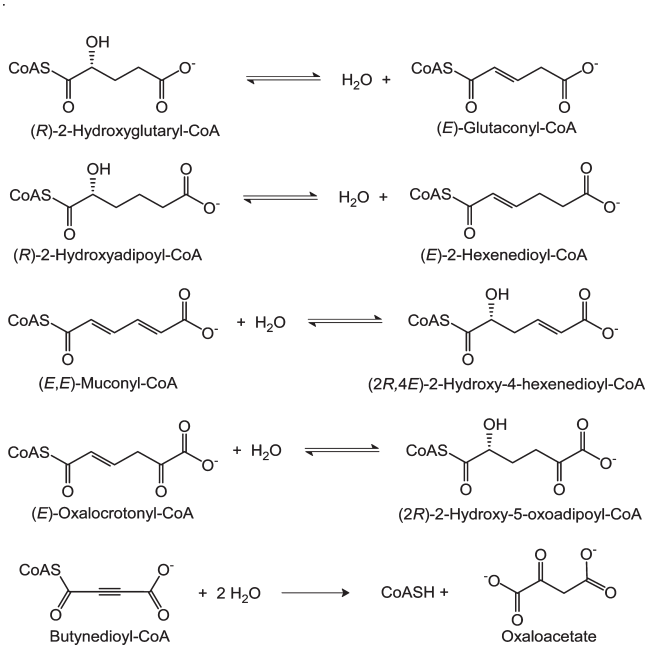

Figure 3. Summary of the most probable structures of the established substrates and products of 2-hydroxyglutaryl-CoA dehydratase.

(*R*)-2-Hydroxyadipoyl-CoA. To synthesize (*R*)-2-hydroxyadipoyl-CoA, (*R,S*)-2-hydroxyadipate and acetyl-CoA were incubated with glutaconate CoA-transferase (Figure 4). As with 2-hydroxyglutarate, the kinetically favored product was expected to be (*R*)-2-hydroxyadipoyl-CoA and the thermodynamically favored product (*R,S*)-5-hydroxyadipoyl-CoA.[30] Because the catalytic efficiency of the transferase is 17 times higher with (*R*)-2-hydroxyglutarate than with its *S*-enantiomer, only a minor amount of (*S*)-2-hydroxyadipoyl-CoA should be formed. The reaction was followed with a one-cuvette assay containing oxaloacetate, acetate, and 5,5′-dithiobis(2-nitrobenzoate). Addition of a denatured sample from the incubation gave an absorbance increase at 420 nm due to free CoA. Further addition of citrate synthase converted acetyl-CoA + oxaloacetate + H_2O to citrate + CoA. Finally, glutaconate CoA-transferase in combination with citrate synthase + oxaloacetate catalyzed the formation of CoA via acetyl-CoA from hydroxyadipoyl-CoA + acetate. The reaction with the transferase was biphasic. A rapid increase in absorbance, most likely due to (*R*)-2-hydroxyadipoyl-CoA, was followed by a slow one due to (*S*)-2-hydroxyadipoyl-CoA and (*R,S*)-5-hydroxyadipoyl-CoA.

Figure 4 shows the kinetics of the formation of two different species of hydroxyadipoyl-CoA. The concentration of the thioester substrate acetyl-CoA decreased rapidly, that of the fast forming (*R*)-2-hydroxyadipoyl-CoA reached its maximum already at 3 min, whereas the concentrations of the slow forming isomers, most likely (*R*)- and (*S*)-5-hydroxyadipoyl-CoA, increased until 20 min. At this time hydrolysis of the thioesters became the main reaction as indicated by the steadily increasing level of free CoA. For synthetic purposes, the reaction was stopped after a short time to predominantly obtain the (*R*)-2-isomer (+ acetyl-CoA). A six-times longer incubation was chosen for a preparation enriched with the 5-isomer. The UV−visible absorption spectrum of a preparation with mainly (*R*)-hydroxyadipoyl-CoA was identical to that of (*R*)-2-hydroxyglutaryl-CoA but different from that of acetyl-CoA. The maxima of the hydroxyl compounds are shifted from 258 to 263 nm,

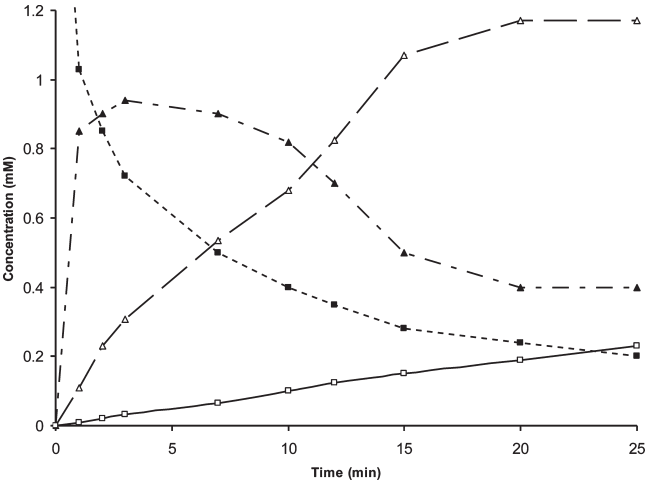

Figure 4. The kinetics of hydroxyadipoyl-CoA formation from (*R,S*)-2-hydroxyadipate and acetyl-CoA catalyzed by glutaconate CoA-transferase. The reaction mixture contained 50 mM (*R,S*)-2-hydroxyadipate, 2 mM acetyl-CoA, and 3.5 U glutaconate-CoA transferase in 100 mM potassium phosphate pH 7.0 in a total volume of 2 mL and incubated at 37 °C. Aliquots (100 μL) were withdrawn at the time-points indicated in the figure, acidified to pH 2, and neutralized after 5 min. The composition of the samples was assayed in one cuvette that contained 5,5′-dithiobis(2-nitrobenzoate), acetate, oxaloacetate, and phosphate pH 7.0. The sample was added first and caused an absorbance increase at 420 nm due to free CoASH (empty squares). The second addition of citrate synthase gave an absorbance increase due to acetyl-CoA (filled squares). The third addition of glutaconate CoA transferase initiated a rapid increase in absorbance due to (*R*)-2-hydroxyadipoyl-CoA (filled triangles) followed by a much slower increase due to a mixture of (*S*)-2-hydroxyadipoyl-CoA and (*R,S*)-5-hydroxyadipoyl-CoA (empty triangles).

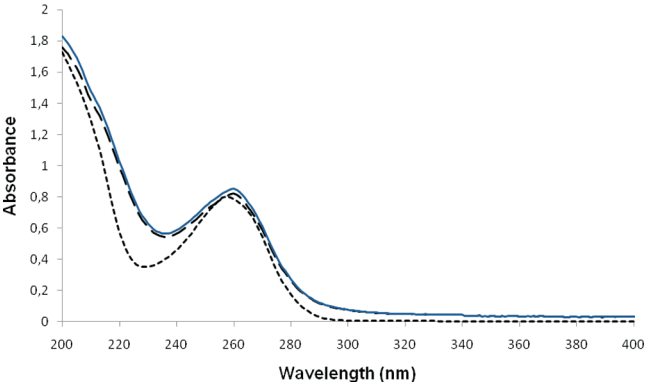

Figure 5. UV−visible spectra of 2-hydroxyadipoyl-CoA (solid line), acetyl-CoA (dotted line), and (*R*)-2-hydroxyglutaryl-CoA (dashed line), all concentrations 0.06 mM.

and the higher absorptions below 250 nm and above 270 nm are notable (Figure 5).

2-Hydroxyglutaryl-CoA dehydratase catalyzed the formation of 2-hexenedioyl-CoA from (*R*)-2-hydroxyadipoyl-CoA as judged by the increase in absorption at 290 nm (Table 1). With this substrate, the catalytic efficiency ($k_{cat} \times K_m^{-1}$) of the dehydratase was only 4-times lower than with (*R*)-2-hydroxyglutaryl-CoA (Figure 6; Table 1). With the preparation, in which the 5-isomer was enriched, the activity was much lower. MALDI-TOF mass analysis of the reaction mixture showed the presence

of hydroxyadipoyl-CoA at m/z = 912 and the dehydration product at m/z = (912 − 18) = 894, most likely the mono-CoA-ester of (*E*)-2-hexenedioic acid (Table 2 and Figure S5).

Muconyl-CoA. (*E,E*)-Muconate, 2,4-hexadienedioic acid, is a symmetrical compound that gives rise to one mono-CoA-thioester only. Muconyl-CoA was prepared with glutaconate CoA-transferase, purified by HPLC, and characterized by its UV−vis spectrum exhibiting a peak at 260 nm and a broad shoulder between 290 and 350 nm (Figure 7) with an extinction coefficient at 260 nm of 26 mM^{-1} cm^{-1}. Comparison of the spectrum with those of 2,4-pentadienoyl-CoA (peak at 260 nm and broad shoulder between 280 and 320 nm) and 2,4-hexadienoyl-CoA (peaks at 260 and 300 nm)[45] shows that the spectrum of muconyl-CoA more resembles that of the former. This demonstrates that the electron withdrawing carboxylate moiety in muconyl-CoA does not contribute significantly to the spectrum, whereas the electron donating methyl group of 2,4-hexadienoyl-CoA in combination with the electron withdrawing thioester increases the resonating system.

According to the proposed mechanism of 2-hydroxyglutaryl-CoA dehydratase, the first step of the hydration of muconyl-CoA should be its reduction to a ketyl radical stabilized by resonance with two double bonds. Hence, the reaction should stop at this stage analogous to that observed with (*R*)-2-hydroxy-4-penteneoyl-CoA and 2-hydroxyisocaproyl-CoA dehydratase.[18] However, incubation of muconyl-CoA with 2-hydroxyglutaryl-CoA dehydratase revealed a decrease of absorbance at 290 nm and thus hydration (Table 1, Figure S6, Supporting Information). The MALDI-TOF mass spectrum of the assay mixture showed two peaks, one corresponding to muconyl-CoA at m/z = 893 and the other to the hydrated product at m/z = 892 + 18 = 910, most likely (*R*)-2-hydroxy-4-hexenedioyl-CoA (Figure S7, Supporting Information). Furthermore, no substrate derived radical could be detected by EPR with muconyl-CoA and 2-hydroxyglutaryl-CoA dehydratase (see below).

Oxalocrotonyl-CoA. In the crystalline state, 4-oxalocrotonate (5-oxo-2-hexenedioic acid) exists as 2-hydroxymuconic acid (2-hydroxy-2,4-hexadienedioic acid), a dienol. A detailed study of the properties of hydroxymuconate demonstrated that in aqueous phosphate pH 7.0, the dienol rapidly tautomerized to oxalocrotonate. A specific tautomerase catalyzes the further conversion to the most stable isomer, 2-oxo-3-hexenedioate.[25] The mono-CoA-thioester prepared with glutaconate CoA-transferase was characterized by its UV−visible spectrum with a maximum at 265 nm (ε_{265} = 24 mM^{-1} cm^{-1}), a shoulder at 300 nm, and a broad minor peak between 290 and 350 nm (Figure 8). The mono-CoA-thioester is hydrated by incubation with 2-hydroxyglutaryl-CoA dehydratase as monitored by a decrease in absorption at 290 nm (Figure S8, Supporting Information) and by MALDI-TOF mass spectrometry, which exhibited the substrate and the hydrated product, m/z = 908 + 18 = 926 Da (Figure S9, Supporting Information). Hence, the structure of the thioester should be that of a 2-enoyl-CoA, most likely 5-hydroxymuconyl-CoA, because its UV-spectrum closely resembles that of muconyl-CoA (compare Figures 7 and 8). The Michaelis−Menten parameters of the hydration of oxalocrotonyl-CoA are listed in Table 1.

Butynedioyl-CoA. The mono-CoA-thioester butynedioyl-CoA obtained with acetylene dicarboxylate (butynedioate) and acetyl-CoA catalyzed by glutaconate CoA-transferase was characterized by its UV−visible spectrum showing a broad peak at 262 nm (ε = 26 mM^{-1} cm^{-1}) with a relatively high extinction coefficient at 290 nm ($\Delta\varepsilon$ = 9.6 mM^{-1} cm^{-1}) (Figure 9). 2-Hydroxyglutaryl-CoA dehydratase catalyzed the hydration of this substrate as assayed

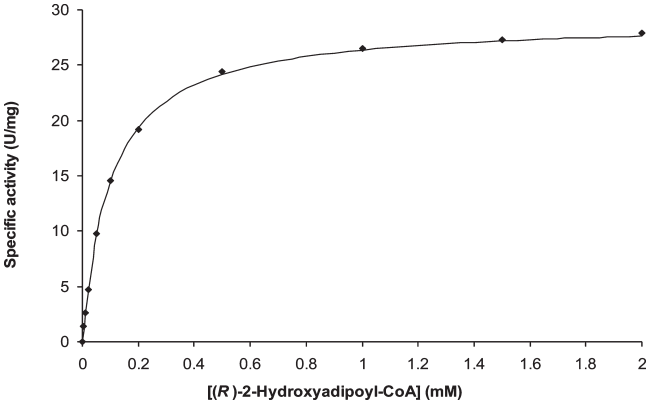

Figure 6. Kinetics of the dehydration of (*R*)-2-hydroxyadipoyl-CoA to 2-hexenedioyl-CoA catalyzed by 2-hydroxyglutaryl-CoA dehydratase. The points represent measured intial velocities. The line has been calculated using the Michaelis−Menten equation: K_m = 0.10 ± 0.01 mM, V_{max} = 29.0 ± 0.3 U mg^{-1}, k_{cat} K_m^{-1} = 4.4 × 10^5 M^{-1} s^{-1}.

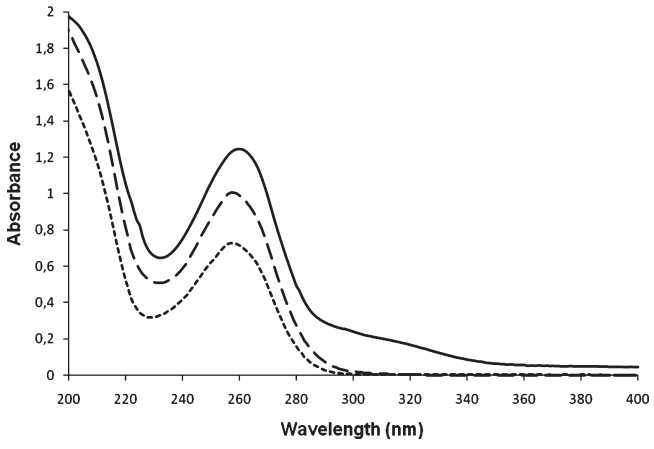

Figure 7. UV−visible spectra of HPLC purified muconyl-CoA (solid line), acetyl-CoA (dotted line), and crotonyl-CoA (dashed line) at 0.045 mM concentrations.

Table 2. Summary of the MALDI-TOF Mass Spectrometric Data of Substrates of 2-Hydroxyglutaryl-CoA Dehydratase[a]

substrate (*reaction*)	2-hydroxyacyl-CoA	enoyl-CoA	acetyl-CoA from synthesis
2-hydroxyglutaryl-CoA (*dehydration*)	2-hydroxyglutaryl-CoA, m/z = 898	glutaconyl-CoA, m/z = 880	
2-Hydroxyadipoyl-CoA (*dehydration*)	2-hydroxyadipoyl-CoA, m/z = 912.17 (100%)	2-hexenedioyl-CoA, m/z = 894.10 (45%)	m/z = 810.14 (45%)
muconyl-CoA (*hydration*)	2-hydroxy-4-hexenedioyl-CoA, m/z = 910.17 (80%)	muconyl-CoA, m/z = 892.12 (100%)	m/z = 810.12 (60%)
oxalocrotonyl-CoA (*hydration*)	2-hydroxy-5-oxohexanedioyl-CoA, m/z = 926.09 (50%)	oxalocrotonyl-CoA, m/z = 908.10 (100%)	m/z = 810.13 (100%)
butynedioyl-CoA (*hydration*)	2-hydroxyfumaryl-CoA, spontaneously hydrolyzed to CoA, m/z = 768.11 (70%)	butynedioyl-CoA, m/z = 864.04 (30%)	m/z = 810.11 (45%)

[a] The relative peak heights (highest peak = 100%) are given in brackets. The spectra are shown in the supplement.

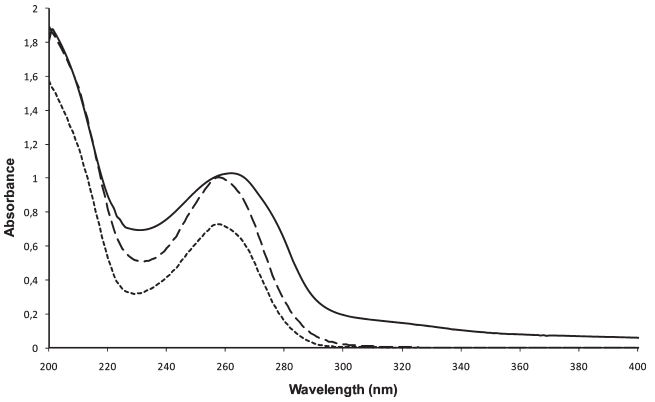

Figure 8. UV−vis spectra of oxalocrotonyl-CoA (solid line), acetyl CoA (dotted line), and crotonyl-CoA (dashed line) at 0.05 mM concentrations.

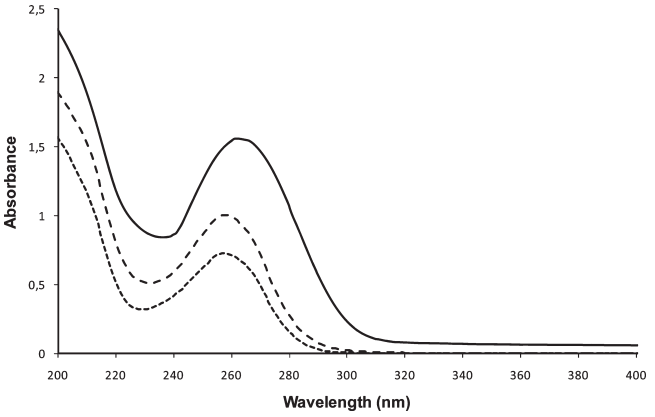

Figure 9. UV−visible spectra of butynedioyl-CoA (solid), crotonyl-CoA (dashed), and acetyl-CoA (dotted) at 0.06 mM each.

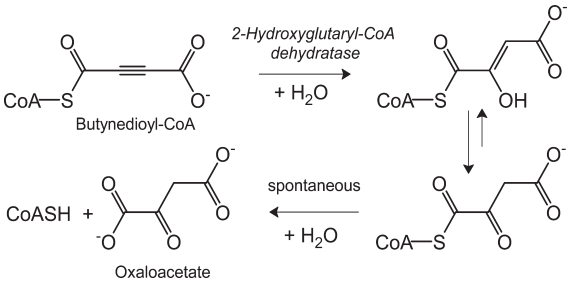

Figure 10. Proposed reaction scheme of the hydration of butynedioyl-CoA.

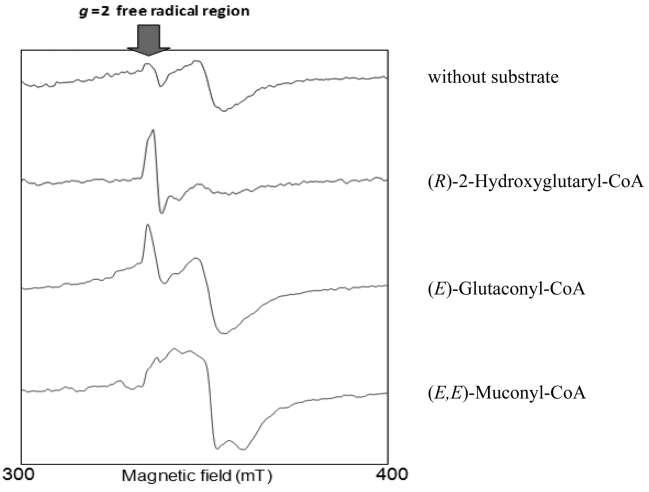

Figure 11. EPR spectra of activated 2-hydroxyglutaryl-CoA dehydratase from *C. symbiosum* with different substrates; substrate concentration 1 mM in each case (the names of the substrates are shown alongside the respective EPR traces), microwave power 20 mW, microwave frequency 9.458 GHz, modulation amplitude 1.28 mT, and temperature 9.5 K.

at 290 nm (Table 1, Figure S10, Supporting Information). The MALDI-TOF mass spectrum revealed the substrate (m/z = 864), small amounts of residual acetyl-CoA from the synthesis (810), and as the major product free CoA (768) (Table 2, Figure S11, Supporting Information). Hydration of butynedioyl-CoA in the orientation as observed with glutaconyl-CoA would lead to 2-hydroxyfumaroyl-CoA or 2-hydroxymaleyl-CoA. This would then tautomerize to the keto-form, oxaloacetyl-CoA (2-oxosuccinyl-CoA), which spontaneously hydrolyzes to oxaloacetate and free CoA (see below). The free CoA was determined with 5,5′-dithiobis(2-nitrobenzoate) from an aliquot from the assay of the dehydratase with 2.0 mM butynedioyl-CoA as substrate. A high background due to dithiotreitol in the dehydratase assay had to be subtracted. The formation of 0.19 mM CoA matched well with 0.20 mM butynedioyl-CoA consumed during the dehydration. The determination of oxaloacetate with NADH and malate dehydrogenase afforded 0.20 mM. Hence, 2-hydroxyglutaryl-CoA dehydratase catalyzed the stoichiometric hydrolysis of butynedioyl-CoA to oxaloacetate and CoA (Figure 10). Controls in the absence of dehydratase or without activation of the dehydratase by ATP neither resulted in hydrolysis of the CoA ester nor in formation of oxaloacetate. Crotonase purified from beef liver or unpurified from cell-free extracts from *C. symbiosum* did not catalyze the hydration of butynedioyl-CoA. Although acetylenedicarboxylate acts as a good substrate of fumarase,[37] this enzyme did not accept butynedioyl-CoA. Furthermore, assays with malate or fumarate did not detect any fumarase activity in cell-free extracts from *C. symbiosum*.

The hydration of butynedioyl-CoA fits well to the proposed radical mechanism (Figure 2). The initial ketyl radical guides the proton to the β-carbon and the water/hydroxide to the α-carbon. This leads to an enol, which tautomerizes to the rapidly hydrolyzing 2-oxosuccinyl-CoA (1-CoA-thioester of oxaloacetate, see below). In contrast, fumarase adds water only to the free acetylenedicarboxylate or butynedioate to yield also oxaloacetate, but certainly, in the opposite orientation, which cannot be verified experimentally with this symmetric molecule.

EPR Spectra. Incubation of 2-hydroxyglutaryl-CoA dehydratase with activator, ADP, dithiothreitol, and dithionite as in vitro reducing agent, revealed a featureless X-band EPR spectrum in the high field region (300−400 mT). In earlier measurements a sharp signal of the [4Fe-4S] cluster of the reduced activator ($S = 1/2$) at g = 2.02 was observed in this region.[16] With the current preparation only the main signal of the activator ($S = 3/2$) appeared around $g = 4-6$ (not shown). Replacement of ADP by ATP afforded broad signals in the $S = 1/2$ region (Figure 11), similar to that observed with 2-hydroxyisocaproyl-CoA dehydratase in the absence of substrate.[18] Hence, the electron was transferred to the dehydratase and reduced the iron sulfur cluster(s).

In the presence of (*R*)-2-hydroxyglutaryl-CoA, a sharp signal appeared at g = 2.008, whereas the intensity of the broad signal

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Table 3. Kinetic Constants of Other Enzymes of the 2-Hydroxyglutarate Pathway of Glutamate Fermentation[a]

enzyme/reference	substrate (second substrate)	K_m mM	V_{max} U mg^{-1}	$k_{cat} \times K_m^{-1}$ M^{-1} s^{-1}
(*R*)-2-hydroxyglutarate dehydrogenase[38]	2-oxoglutarate (NAD^+)	0.13	4.8×10^3	2.2×10^7
(*R*)-2-hydroxyglutarate dehydrogenase, this paper	2-oxoadipate (NAD^+)	0.88	8.0×10^2	5.5×10^5
(*R*)-2-hydroxyglutarate dehydrogenase, this paper	oxalocrotonate (NAD^+)	1.1	392	2.2×10^5
glutaconate CoA-transferase[30]	(*E*)-glutaconate (acetyl-CoA)	0.20	82	4.4×10^5
glutaconate CoA-transferase[30]	(*R*)-2-hydroxyglutarate (glutaconyl-CoA)	1.5	82	5.9×10^4
glutaconate CoA-transferase[30]	(*S*)-2-hydroxyglutarate (glutaconyl-CoA)	14	49	3.8×10^3
glutaconate CoA-transferase[30]	adipate (glutaconyl-CoA)	8.0	49	6.6×10^3

[a] For calculation of k_{cat} values, the molecular masses of the heterodimer (65 kDa) of the octameric glutaconate CoA-transferase and of the monomer (36.5 kDa) of the dimeric 2-hydroxyglutarate dehydrogenase were taken.

around $g = 1.93$ disappeared (Figure 11). The signal at $g = 2.008$ was recorded at various temperatures and microwave power settings (not shown). It did not exhibit resolved proton hyperfine couplings and was broader than the signal of the organic radical obtained with 2-hydroxyisocaproyl-CoA dehydratase.[18] Because of the reversibility of the dehydratase reaction, we anticipated a similar spectrum with glutaconyl-CoA. However, the $g = 2.008$ signal was weaker and the $g = 1.93$ signal stronger. Possibly, with this 60-times less efficient substrate (Table 1) the equilibrium was not reached within the 2 min incubation time. The absence of a $g = 2.008$ signal and the presence of a very broad signal with muconyl-CoA, an even slower substrate (200-time less), is in agreement with the observation with glutaconyl-CoA.

The failure to get a well-resolved EPR spectrum with 2-hydroxyglutaryl-CoA dehydratase as compared to that obtained with 2-hydroxyisocaproyl-CoA dehydratase could be due to the different substrates and/or to different mechanisms as indicated by the presence and absence of flavins, respectively. The electron donating isopropyl group of 2-hydroxyisocaproyl-CoA may stabilize the radical much better than the carboxymethyl group of 2-hydroxyglutaryl-CoA. In addition as indicated above, the sufido ligand of the β-cluster in 2-hydroxyisocaproyl-CoA dehydratase is replaced by an OH-group of a tyrosine together with a flavin at 13 Å distance in 2-hydroxyglutaryl-CoA dehydratase. Maybe these different modes of electron storage during catalysis shorten the lifetimes of the radical intermediates and thus lower the concentration at which such intermediates are present excluding detection by EPR spectroscopy.

Substrate Specificity of Glutaconate CoA-transferase and (*R*)-2-Hydroxyglutarate Dehydrogenase. In contrast to (*R*)-2-hydroxyglutaryl-CoA dehydratase, glutaconate CoA-transferase is highly specific for the complete CoA molecule; even with acetyl-3′-dephospho-CoA and glutaconate as acceptor, $k_{cat} \times K_m^{-1}$ decreased 20-fold. No reaction was observed with acetyl-4′-phosphopantetheine.[30] With succinyl-CoA:3-oxoacid CoA-transferase, it has been proposed that the binding energy of the whole CoA-molecule is used to enhance the catalytic rate by 10 orders of magnitude.[46] Apparently, binding of the CoA moiety to 2-hydroxyglutaryl-CoA dehydratase is not required for rate acceleration. This dehydratase uses the barrier-less reactivity of radicals (Figure 2) and therefore does not depend on the conversion of binding energy into catalytic efficiency. On the other hand, glutaconate CoA-transferase is less specific for the acyl part of the substrate. It accepts acetate and propionate as well as glutarate, (*E*)-glutaconate, (*R*)-2-hydroxyglutarate, and adipate as good substrates.[30] Therefore, we note that this enzyme is an extremely valuable tool for the preparation of the mono-CoA-thioesters of dicarboxylates different from 2-hydroxyglutarate, because of the lack of suitable chemical synthesis methods. Glutaconate CoA-transferase does not accept *cis* or (*Z*)-glutaconate.[30] Therefore, (*Z*)-glutaconyl-CoA could not be synthesized and tested with the dehydratase.

Interestingly, attempts to synthesize the CoA-thioesters of 2-oxoglutarate and oxaloacetate with the same enzymatic procedure using acetyl-CoA as donor resulted in hydrolysis to CoA, which was assayed continuously with 5,5′-dithiobis(2-nitrobenzoate). The kinetic parameters of the transferase were measured for 2-oxoglutarate as $V_{max} = 5.0 \pm 0.3$ U mg^{-1}, $K_m = 1.02 \pm 0.05$ mM and for oxaloacetate as $V_{max} = 3.50 \pm 0.02$ U mg^{-1}, $K_m = 1.23 \pm 0.06$ mM. This spontaneous hydrolysis of 2-oxoacyl-CoA, if formed at all, was presumably due to the electrophilic activation of the thioester carbonyl by the electron withdrawing 2-oxo group. The formation of CoA during hydration of butynedioyl-CoA was due to the proposed intermediate 2-oxosuccinyl-CoA (see above). The induction of CoA-thioester hydrolysis by oxaloacetate in the presence of glutaconate CoA-transferase explains the complex saturation behavior of the coupled 5,5′-dithiobis(2-nitrobenzoate) assay (see above) with increasing acetate concentrations. With glutaryl-CoA and acetate as substrates, the assay works well at the standard concentrations of 200 mM acetate and 1 mM oxaloacetate. At low acetate (1−10 mM), however, oxaloacetate competes with acetate and hydrolyses glutaryl-CoA without forming acetyl-CoA. Thus, the Michaelis−Menten plot becomes biphasic.

2-Hydroxyglutarate dehydrogenase mediates the NADH-dependent reduction of 2-oxoadipate (Figure S12, Supporting Information) and oxalocrotonate (Figure S13, Supporting Information), though the kinetic constants are significantly lower than with the natural substrate 2-oxoglutarate (Table 3). Thus, the substrate-binding pocket of the dehydrogenase must less flexible than those of glutaconate CoA-transferase and 2-hydroxyglutaryl-CoA dehydratase. The available structures of dehydrogenase and transferase reveal two very different binding modes. The transferase binds the terminal carboxylate anion via several hydrogen bonds to serine residues,[43] which may adjust to the different chain lengths. In contrast, the dehydrogenase applies a cluster of three arginines, which probably allows less plasticity.[38]

Toward a Biobased Production of Adipic Acid. Recently, expression of six genes converted *E. coli* into a modest glutaconate producer: The genes encoding 2-hydroxyglutarate dehydrogenase, glutaconate CoA-transferase, and 2-hydroxyglutaryl-CoA dehydratase with its activator stem from the glutamate fermenting anaerobic bacteria *C. symbiosum* and *A. fermentans*.[5] Reduction of the intermediate glutaconyl-CoA to glutaryl-CoA could lead to the biotechnological conversion of glucose to glutarate, a precursor of polyesters. As the first three enzymes have now been shown to accept six-carbon substrates, this engineered

pathway appears to be applicable also for the biological production of adipic acid, a constituent of Nylon-6,6. One has to surpass many obstacles, however, before a biological pathway for the production of adipate might work. Whereas the precursor 2-oxoadipate could be synthesized via homocitrate, a main problem is 2-hydroxyglutarate dehydrogenase, which prefers the metabolically more important 2-oxoglutarate over 2-oxoadipate. Thus, the only solution would be the construction of a 2-hydroxyadipate dehydrogenase, which does not use 2-oxoglutarate as substrate. Brain cancer cells contain a mutant of isocitrate dehydrogenase, which is inactive with $NADP^+$ and isocitrate but catalyzes the NADPH-dependent reduction of 2-oxoglutarate to (*R*)-2-hydroxyglutarate.[47] Hence, a similar mutation could transform homoisocitrate dehydrogenase to a specific (*R*)-2-hydroxyadipate dehydrogenase.

Another problem is the reduction of the unsaturated glutaconic and 2-hexenedioic acids to the saturated glutaric and adipic acids, respectively. Although this could be achieved by a chemical procedure using hydrogen and a platinum catalyst (see below), a direct reduction by the engineered organism would be more efficient. Glutaconyl-CoA could be reduced to glutaryl-CoA catalyzed by the recently discovered nondecarboxylating dehydrogenase from *Desulfococcus multivorans*.[48] Probably the enzyme may also accept 2-hexenedioyl-CoA as substrate. Like the butyryl-CoA dehydrogenase/electron transferring complex (Bcd/Etf), glutaryl-CoA dehydrogenase (Gcd) and Etf may form a similar complex (Gcd/Etf) that catalyzes the reduction of glutaconyl-CoA with 2 NADH coupled to the reduction of ferredoxin, a process called electron bifurcation.[49,50] The reduced ferredoxin could give rise to molecular hydrogen. If one considers that in the path from glucose to 2-oxoglutarate a second hydrogen molecule is produced via pyruvate formate lyase and formate hydrogen lyase, the fermentation of glucose to glutarate can be described by a simple balanced equation (1).

$$\text{glucose} = CO_2 + \text{glutarate}^{2-} + 2\,H_2 \qquad (1)$$

In the case of a balanced equation for the production of adipic acid from glucose, hydrogen would be not formed but *consumed* (2).

$$\text{glucose} + H_2 = \text{adipate}^{2-} + 2\,H^+ + 2\,H_2O \qquad (2)$$

Therefore, the fermentation requires hydrogen as second substrate; alternatively, some of the glucose could be oxidized to gluconic acid or more efficiently to CO_2 via the pentose phosphate cycle.

Already a decade ago, an efficient aerobic pathway had been developed in an *E. coli* strain that converted glucose via shikimi acid to catechol. A catechol 1,2-dioxygenase catalyzed the subsequent oxidation to *cis,cis*-muconic acid that accumulated in the medium at the high concentration of 38.6 g L^{-1} (270 mM). The reduction to adipic acid, however, had to be performed chemically by using a platinum catalyst at 3400 kPa H_2.[51] In an enzymatic reduction sequence, a yet unknown *cis-trans* isomerase, glutaconate CoA-transferase (see above), an engineered pentadienoyl-CoA reductase[45] and glutaryl-CoA dehydrogenase/electron transferring flavoprotein (see above) could be involved.

■ ASSOCIATED CONTENT

Supporting Information. This part comprises experiments that support the structure of glutaconyl-CoA as (*E*)-2-pentenedioyl-CoA. It also contains the MALDI-TOF mass spectra of the CoA thioesters, inhibitor studies with 2-hydroxyglutaryl-CoA dehydratase as well as the kinetics of the dehydrations of 2-hydroxyglutaryl-thioesters and hydrations of 2-enoyl-CoA derivatives catalyzed by 2-hydroxyglutaryl-CoA dehydratase. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ ABBREVIATIONS USED

EPR, electron paramagnetic resonance; MALDI-TOF, matrix-assisted laser desorption ionization-time-of-flight (mass spectrometry); TFA, trifluoroacetic acid

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