α-Keto Acid Chain Elongation Reactions Involved in the Biosynthesis of Coenzyme B (7-Mercaptoheptanoyl Threonine Phosphate) in Methanogenic Archaea[†]

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ABSTRACT: The biochemistry of the 13 steps involved in the conversion of α-ketoglutarate and acetylCoA to α-ketosuberate, a precursor to the coenzymes coenzyme B (7-mercapto heptanoylthreonine phosphate) and biotin, has been established in *Methanosarcina thermophila*. These series of reactions begin with the condensation of α-ketoglutarate and acetylCoA to form *trans*-homoaconitate. The *trans*-homoaconitate is then hydrated and dehydrated to *cis*-homoaconitate with (*S*)-homocitrate serving as an intermediate. Rehydration of the *cis*-homoaconitate produces (−)-*threo*-isohomocitrate [(2*R*,3*S*)-1-hydroxy-1,2,4-butanetricarboxylic acid], which undergoes a $NADP^+$-dependent oxidative decarboxylation to produce α-ketoadipate. The resulting α-ketoadipate then undergoes two consecutive sets of α-ketoacid chain elongation reactions to produce α-ketosuberate. In each of these sets of reactions, it has been shown that the homologues of *cis*-homoaconitate, homocitrate, and (−)-*threo*-isohomocitrate serve as intermediates. The protein product of the *Methanococcus jannaschii* MJ0503 gene *aksA* (AksA) was found to catalyze the condensation of α-ketoglutarate and acetylCoA to form *trans*-homoaconitate. This gene product also catalyzed the condensation of α-ketoadipate or α-ketopimelate with acetylCoA to form, respectively, the (*R*)-homocitrate homologues of (*R*)-2-hydroxy-1,2,5-pentanetricarboxylic acid and (*R*)-2-hydroxy-1,2,6-hexanetricarboxylic acid. The α-ketosuberate resulting from this series of reactions then undergoes a nonoxidative decarboxylation to form 7-oxoheptanoic acid, a precursor to coenzyme B, and an oxidative decarboxylation to form pimelate, the precursor to biotin. Of the 13 intermediates in this pathway, eight have not previously been reported as occurring in biological systems.

The biosynthesis of the alkyl portions of the coenzymes 7-mercaptoheptanoylthreonine phosphate (coenzyme B)[1] and biotin in the Archaea is an interesting dilemma considering that these cells contain no fatty acids (*1*) and have no genes homologous to the fatty acid synthases (*2*). Several years ago, a pathway for the formation of the 7-mercaptoheptanoic acid moiety of coenzyme B was proposed based on ^{13}C-labeling studies (*3*) and the identification of α-ketoglutarate, α-ketoadipate, α-ketopimelate, and α-ketosuberate in a series of methanogenic bacteria (*4*). In the proposed pathway, the repeated application of the α-ketoacid chain elongation series of reactions was used to increase the number of methylenes from two, as found in α-ketoglutaric acid, to five, as found in the product α-ketosuberic acid. The α-ketosuberic acid then served as a precursor to the biosynthesis of the 7-mercaptoheptanoic acid moiety of coenzyme B (*5*). We would now like to report on the identification of each of the reactions leading to α-ketosuberic acid in *Methanosarcina thermophila* by demonstrating their occurrence in cell extracts and establishing the stereochemistry of the intermediates involved. The gene for the enzyme catalyzing three of the reactions in the overall biosynthetic scheme was also established. This was accomplished by the overexpression of the corresponding gene products from *Methanococcus jannaschii* in *Escherichia coli* and demonstrating that it catalyzed the expected reactions.

MATERIALS AND METHODS

(±)-Homocitric acid lactone, α-ketoglutaric acid, and α-ketoadipic acid were all obtained from Sigma. Dimethyl α-ketoglutarate, (*R*)-(−)-citramalic acid, (*S*)-(+)-citramalic acid, methyl(triphenylphosphoranylidene)acetate, methyl 3-bromopropionate, and methyl 5-bromovalerate were obtained from Aldrich. Factor F_{420} was isolated from *Methanobacterium formicicum* as previously described (*6*). α-Ketopimelic acid and α-ketosuberic acid were prepared as previously described (*7*). (*S*)-Homocitrate was prepared by the brucine resolution of the synthetic (±)-homocitrate

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[1] Abbreviations: coenzyme B, 7-mercaptoheptanoylthreonine phosphate; GC, gas chromatography; GC-MS, gas chromatography−mass spectrometry; α-ketoglutarate, 2-oxopentanedionic acid; α-ketoadipate, 2-oxohexanedionic acid; α-ketopimelate, 2-oxoheptanedionic acid; α-ketosuberate, 2-oxooctanedionic acid; *trans*-homoaconitate, (*E*)-1,2,4-but-1-enetricarboxylic acid; *cis*-homoaconitate, (*Z*)-1,2,4-but-1-enetricarboxylic acid; homocitrate, 2-hydroxy-1,2,4-butanetricarboxylic acid; isohomocitrate, 1-hydroxy-1,2,4-butanetricarboxylic acid; *trans*-$(homo)_2$-aconitate, (*E*)-1,2,5-pent-1-enetricarboxylic acid, *cis*-$(homo)_2$aconitate, (*Z*)-1,2,5-pent-1-enetricarboxylic acid; $(homo)_2$citrate, 2-hydroxy-1,2,5-pentanetricarboxylic acid; iso$(homo)_2$citrate, 1-hydroxy-1,2,5-pentanetricarboxylic acid; *trans*-$(homo)_3$aconitate, (*E*)-1,2,6-hex-1-enetricarboxylic acid; *cis*-$(homo)_3$aconitate, (*Z*)-1,2,6-hex-1-enetricarboxylic acid; $(homo)_3$citrate, 2-hydroxy-1,2,6-hexanetricarboxylic acid; iso-$(homo)_3$citrate, 1-hydroxy-1,2,6-hexanetricarboxylic acid; (+)-*threo*-isocitrate, (2*R*,3*S*)-1-hydroxy-1,2,3-propanetricarboxylic acid; (−)-*threo*-isohomocitrate, (2*R*, 3*S*)-1-hydroxy-1,2,4-butanetricarboxylic acid; (+)-*threo*-isohomocitrate, (2*S*, 3*R*)-1-hydroxy-1,2,4-butanetricarboxylic acid.

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lactone as described by Maragoudakis and Strassman (*8*). A sample of homocitrate enriched in (*R*)-homocitrate was obtained from the mother liquor of this resolution. (*R*)-Homocitrate was also prepared by the condensation of acetylCoA with α-ketoglutaric acid using the cloned *Azotobacter vinelandii* homocitrate synthase (NifV) (*9*). Carboxylic acids (0.01−1 mg) were converted into their methyl esters by heating at 50 °C for 12 h with ∼1 mL of 1 M HCl in anhydrous methanol and processed as described below under the incubation of cell extracts with precursors heading.

The cis and trans homologues of the trimethyl esters of aconitate were prepared by the reaction of the appropriate dimethyl α-ketodiacid with methyl(triphenylphosphoranylidene)acetate as described by Massoudi (*10*), but using the dimethyl esters of the α-ketoacids in place of the di-*tert*-butyl esters. By using the dimethyl esters in this synthesis, a mixture of both the cis and trans isomers were produced, which were then completely separated by preparative TLC using the solvent system methylene chloride/methyl acetate (90:10, v/v). In this solvent, the trans isomers always have R_f values higher than the cis isomers. The separated isomers were readily distinguished by the chemical shifts of their C-2 vinyl hydrogen which was at ∼5.8 ppm for the cis isomers and ∼6.7 ppm for the trans isomers. Each isomer was converted into its sodium salt by saponification with 6 equiv of 0.5 M NaOH/mol of compound in 50% aqueous methanol for 1 h at 50 °C. The samples were then titrated to neutral pH with 1.0 M HCl, the methanol was removed by evaporation, and the samples dissolved in a known volume of water to give 0.1 M solutions. After sparging with argon to prepare anaerobic solutions, portions of the solutions were used as substrates in the incubations.

The homologues of citrate were prepared by the Reformatsky condensation of methyl bromoacetate with the dimethyl esters of α-ketoadipate, α-ketopimelate, and α-ketosuberate (*11*). The derived products were purified by preparative TLC and showed only one peak upon gas chromatography. The citrate homologues were converted into their sodium salts by saponification with 6 equiv of 0.5 M NaOH/mol of compound in 50% aqueous methanol for 15 min at 100 °C. The samples were then titrated to neutral pH with 1.0 M HCl, the methanol and water removed by evaporation, and the sample dissolved under argon in a known volume of anaerobic water to give 0.1 M standard solutions.

The racemic homologues of isocitrate were prepared by the catalytic hydrogenation of the corresponding oxalo acid esters (*12*) followed by base saponification as described above. The Yamashita hydrogenation led to a mixture of the (±)-threo and (±)-erythro isomers with the biologically important threo isomers being the major isomers produced in each synthesis. The (−)-threo isomer was the isomer identified as being involved in the biosynthesis of lysine (*13*). The (±)-threo and (±)-erythro isomer pairs could be easily distinguished by the chemical shifts and coupling constants of their C-2 hydrogen doublets when dissolved in ^2H_2O, or as the methyl esters dissolved in $CDCl_3$. Thus, the chemical shifts of the (±)-threo isomers of isohomocitric acid, iso(homo)$_2$citric acid, and iso(homo)$_3$citric acid in ^2H_2O occurred at 4.23, 4.23, and 4.25 ppm, respectively, and the chemical shifts of the (±)-erythro isomers occurred at 4.38, 4.35, and 4.37 ppm, respectively. The splitting of the C-2 hydrogen doublet could also be used to assign the isomers since the coupling constants ($J_{2\rightarrow3}$) for the threo isomers of isohomocitric acid, iso(homo)$_2$citric acid, and iso(homo)$_3$citric acid of 4.27, 4.57, and 4.89 Hz, respectively, were always smaller than the erythro isomers of 4.89, 5.15, and 5.34 Hz, respectively. The percentage of erythro isomers prepared by the Yamshita reduction in the preparations of isohomocitric acid, iso(homo)$_2$citric acid, and iso(homo)$_3$citric acid was about 11, 17, and 17%, respectively.

Pure (>98%) samples of (−)-*threo*-isohomocitrate and (+)-*threo*-isohomocitrate were prepared by the condensation of dimethyl D-malate or dimethyl L-malate with methyl 3-bromopropionate in a reaction patterned after that described by Seebach and Wasmuth (*14*). (−)-*threo*-Iso(homo)$_3$ citrate was prepared by the condensation of dimethyl D-malate with methyl 5-bromovalerate in the same type of reaction. In each case, the pure products were isolated by preparative TLC of the reaction mixtures. In the solvent system, diethyl ether/hexane (3:1, v/v), the trimethyl ester of *threo*-isohomocitrate and *threo*-iso(homo)$_3$citrate both had an R_f of 0.17. The trimethyl ester of the *threo*-isohomocitrate enantiomers gave the same ^{1}H NMR and mass spectra (see below) as the major isomer produced by the Yamshita reduction. ^{1}H NMR ($CDCl_3$): δ 2.02 (1H, m, H-4), 2.19 (1H, m, H′-4), 2.47 (2H, m, H-5), 3.01 (1H, dq, H-3), 3.35 (1H, d, $J_{1\rightarrow OH} = 6.86$ Hz, OH), 3.69, 3.71, 3.81 (3H each, s, methyl ester groups), 4.31 (1H, q, $J_{2\rightarrow3} = 3.43$, $J_{2\rightarrow OH} = 6.86$ Hz, H-2). The (−)-*threo*-isohomocitrate sample gave the same specific rotation as previously reported (*15*).

The purity and structures of all of the synthetic compounds used were confirmed by gas chromatography−mass spectrometry of their methyl esters and by ^{1}H NMR and ^{13}CMR data. The NMR data was obtained on a Varian Unity 400 spectrometer.

Incubation of Cell Extracts with Precursors. Cell extracts of *M. thermophila* (50−100 μL), prepared as previously described (*16*), were mixed with the indicated amounts of 0.1 M anaerobic solutions of the desired compounds and/or cofactors and incubated at 50 °C for 2−3 h under either argon or air as indicated. To the resulting incubation mixtures were added, with mixing, 400 μL of methanol and 50 μL of 6 M HCl, and the resulting precipitate was removed by centrifugation (16000*g* for 2 min). The clear methanol/water layer was removed and evaporated to dryness with a stream of nitrogen gas. The carboxylic acids in the resulting residue were converted into their methyl esters by reaction with 1 mL of 1 M HCl in anhydrous methanol at 50 °C for 12 h. The methyl esters contained in the sample were recovered by evaporation of the samples to dryness with a stream of nitrogen gas, dissolving the residue in dichloromethane (0.5 mL), and washing with half saturated $NaHCO_3$ solution (0.5 mL). The methylene chloride layer was then dried with anhydrous Na_2SO_4 (∼20 mg), the solvent concentrated by evaporation to 20 μL, and the sample analyzed by GC-MS. When required, the sample was converted into its trifluoroacetyl derivative by reaction overnight at room temperature with 100 μL of a mixture of methylene chloride/trifluoroacetic anhydride (1:1, v/v). After evaporation of the solvent with a stream of dry nitrogen gas, the resulting sample was dissolved in 10 μL of methylene chloride for GC-MS analysis.

Analysis of Carboxylic Acid Methyl Esters. The identification and quantification of the carboxylic acid methyl esters were obtained by GC-MS using a VG-70-70EHF gas chromatrography-mass spectrometer operating at 70 eV and equipped with a HP-5 column (0.32 mm by 30 m) programmed from 95 to 280 °C at 10 °C/min. Under these conditions, the following compounds as their methyl esters had the following retention times (s) and mass spectral data [molecular weight, base peak (in italics), the two five most abundant ions with masses over 100 *m*/*z* listed in order of decreasing intensities]: dimethyl ester of α-ketoglutaric acid, 236 (174, *55*, 115, 113, 143); dimethyl ester of α-ketoadipic acid, 312 (188, *59*, 101, 129, 157); dimethylketal dimethyl ester of α-ketoglutaric acid, 360 (220, *101*, 161, 129, 115, 133); dimethyl ester of α-ketopimelic acid, 398 (202, *55*, 111, 143, 115); dimethylketal dimethyl ester of α-ketoadipic acid, 439 (234, *101*, 175, 129, 133, 202); trimethyl citrate, 439 (234, *143*, 101, 175, 111); trimethyl *trans*-homoaconitate, 471 (198, *139*, 166, 111, 198); trimethyl *cis*-homoaconitate, 480 (198, *139*, 166, 111, 198); dimethyl homocitrate lactone, 488 (216, *129*, 157, 115); trimethyl homocitrate, 514 (248, *129*, 157, 115, 125, 189); dimethylketal dimethyl ester of α-ketopimelic acid, 516 (202, *55*, 111, 143, 115); trimethyl *threo*-isohomocitrate, 534 (250, *157*, 125 116, 189); trimethyl *erythro*-isohomocitrate, 537 (250, *157*, 125 116, 189); trimethyl *trans*-(homo)$_{2-}$aconitate, 545 (198, *139*, 166, 111, 198); trimethyl *cis*-(homo)$_2$aconitate, 563 (198, *139*, 166, 111, 198); trimethyl (homo)$_2$citrate, 589 (262, *171*, 129, 101, 139, 203); trimethyl iso(homo)$_2$citrate, 615 (262, *55*, 111, 139, 171, 153, 203); trimethyl *trans*-(homo)$_3$aconitate, 616 (244, *152*, 180, 213, 124, 111); trimethyl *cis*-(homo)$_3$aconitate, 635 (244, *152*, 180, 214, 124, 111); trimethyl (homo)$_3$citrate, 660 (276, *185*, 111, 153, 139, 143, 217); and trimethyl iso-(homo)$_3$citrate, 682 (276, *185*, 125, 167, 153, 103, 217). Quantitation was accomplished by comparison to known amounts of samples of known samples processed in the identical manner.

Analysis of the Absolute Stereochemistry of the Intermediates. The absolute stereochemistry of specific intermediates was established by GC-MS of volatile derivatives using a type G-TA Chiraldex column (0.25 mm by 40 m; Advanced Separation Technologies Inc., Whippany, NJ) programmed from 95 to 180 °C at 3 °C/min. GC-MS was used for all of the analyses so that positive identification of the GC peaks could be established even in the complex mixtures. The general procedure used to establish the absolute stereochemistry of a metabolic intermediate was to first determine a suitable derivative of the compound of interest that would separate the racemic mixture of the isomers into two peaks. Then the synthetic or resolved isomer of known stereochemistry was co-injected with this racemic mixture to determine which of the peaks corresponded to which isomer. After the same procedure was performed with the isomers produced in an incubation mixture, the absolute stereochemistry of the biosynthetic product was deduced.

The R and S isomers of the methyl esters of homocitrate were prepared as described above from a racemic sample of homocitrate and eluted from the Chiraldex GC column as two pairs of peaks. The first pair to elute consisted of the S and R isomers of dimethyl homocitrate lactone and the second contained the R and S steroisomers of the trimethyl-ester of homocitrate. The first peak of the dimethyl homocitrate lactone was identified as the S stereoisomer, whereas the first peak of the trimethyl ester of homocitrate was identified as the R stereoisomer.

Gas chromatography of the trimethyl ester of (*R*,*S*)-(homo)$_2$citrate or (*R*,*S*)-(homo)$_3$citrate on the same Chiraldex GC column each produced one peak with the same mass spectra as the trimethyl esters of each, showing that the stereoisomers of these compounds were not resolvable on this column. Treatment of the samples with a equal mixture of trifluoroacetic anhydride and methylene chloride for 24 h at 30−40 °C produced two new, earlier eluting peaks for the (*R*,*S*)-(homo)$_2$citrate sample, but did not change the elution pattern for the (*R*,*S*)-(homo)$_3$citrate sample. On the basis of the mass spectrum of these two peaks for the (*R*,*S*)-(homo)$_2$citrate sample ($M^+ = m/z$ 230), they corresponded to the two γ-lactone enantiomers of the (homo)$_2$citrates. On the basis of analogy to the elution positions of the δ-lactones of the homocitrate, the first peak was assigned to the S isomer and the second to the R isomer. The (homo)$_2$citrate produced in the incubation and derivativitized in the same manner coeluted with the R peak, establishing that this metabolic intermediate was the R isomer.

Many attempts to resolve the (homo)$_3$citrate stereoisomers were undertaken by forming other types of derivatives but none of these allowed for the separation of the enantiomers. It should be noted that this separation of the R and S isomers of (homo)$_2$citrate was done at the limits of resolution of this chiral column and could only be accomplished after the column was freshly regenerated by treatment with trifluoroacetic anhydride as described by the manufacturer.

The same Chiraldex GC column was also used for the stereochemical analysis of the stereoisomers of the homologues of the trimethyl esters of the isohomocitrates. GC-MS of the trimethyl esters of the synthetic isohomocitrate produced by hydrogenation, and consisting of a mixture of all four of the isohomocitrate isomers, produced three peaks in the ratio of 90:5:5, each with the same mass spectra. On the basis of the intensities of these peaks, the first peak corresponded to a mixture of the (+)- and (−)-*threo*-isohomocitrate isomers and the two minor peaks corresponded to the (+)- and (−)-*erythro*-isohomocitrate isomers. This same mixture of isomers as their trifluoroacetyl derivatives also produced three peaks, but this time, the ratios of the peaks were 5:45:50. From these observed peak intensities, it was clear that the first peak was one of the resolved *erythro*-isohomocitrate peaks, the second peak was one of the resolved *threo*-isohomocitrate isomer, and the third peak was a mixture of the other *erythro*- and *threo*-isohomocitrate isomers. Co-chromatography with the trifluoroacetyl derivative of the trimethyl ester of synthetic (−)-*threo*-isohomocitrate confirmed that the middle of the three peaks corresponded to the (−)-threo isomer. Analyses of the iso(homo)$_2$citrate and iso(homo)$_3$citrate homologues gave similar results.

On the Chiraldex GC column, dimethyl (*S*)-citramalate was found to elute before dimethyl (*R*)-citramalate. The opposite elution pattern was observed for the same compounds as their trifluoroacetyl derivatives.

Identification, Cloning and High-Level Expression of Enzymes. The approach to establishing the genes involved in these reactions is to survey the genome of organisms that produce coenzyme B for genes homologous to those known

Table 1: Analytical Data for the First Set of α-Ketoacid Chain Elongation Reactions in *Methanosarcina thermophila*

	concn of intermediates (mM)				
experiment	*trans*-homoaconitate	(*S*)-homocitrate[a]	*cis*-homoaconitate	isohomocitrate[b]	α-ketoadipate[c]
1. blank	0.04	0.06	0.01	nd[d]	nd
2. α-ketoglutarate (3.5 mM), acetylCoA (5.2 mM)	0.07	0.7	0.02	nd	nd
3. *cis*-homoaconitate (4.8 mM)	0.1	4.4	0.3	0.03	nd
4. *trans*-homoaconitate (4.8 mM)	3	0.5	0.1	0.03[e]	nd
5. isohomocitrate (4.3 mM)					
a. anaerobic, plus, NAD^+, $NADP^+$ (4.3 mM each)	0.1	1	0.04	3.1[f]	0.02
b. aerobic[g]	0.14	2	0.04	2.3[f]	0.04
6. (−)-*threo*-isohomocitrate (4.2 mM)					
a. anaerobic NAD^+, $NADP^+$ (4.2 mM each), acetylCoA (8.4 mM)	0.14	1	0.1	0.6	0.04[h]
b. anaerobic, $NADP^+$ (4.1 mM), acetylCoA (4.2 mM)	0.03	0.9	0.1	0.8	0.06[i]

[a] Sum of the amounts of trimethyl ester of (*S*)-homocitrate and the dimethylester of (*S*)-homocitrate lactone. The (*S*) isomer was confirmed in each case by GC-MS analysis using the chiral column. [b] The sum of all of the diasteresomers. [c] Sum of the amount of the dimethyl ester and the dimethyl dimethylketal ester derivative. [d] Not detected because of the low concentrations of the product present. [e] An equal mixture of the *erythro* and *threo* isomers were detected. The *erythro* isomer is involved in the biosynthesis of 1,3,4,6-hexanetetracarboxylic acid. [f] GC-MS analysis using the chiral column showed only the (−)-*threo*-isohomocitrate was consumed during the incubation. [g] Also included in this incubation was 0.17 mM F_{420}. [h] Also detected in this experiment was 0.1 mM α-ketopimelate; 0.06 mM *cis*-(homo)$_2$aconitate, 2.0 mM (homo)$_2$citrate, and 0.03 mM (homo)$_3$citrate. [i] As in Experiment 6a, α-ketopimelate, *cis*-(homo)$_2$aconitate, (homo)$_2$citrate, and (homo)$_3$citrate were also detected.

to encode enzymes that carry out similar reactions. The searches were performed using the blast_to_rpraze method. At present, the genomes of two methanogens, *M. jannaschii* and *Methanobacterium thermoautotrophicum* Δ*H*, both of which produce methane and thus coenzyme B, have been completely sequenced. The genome of *M. jannaschii* contains three genes homologous to homocitrate synthase (*nifV*) (MJ0503, MJ1392, and MJ1543), four genes homologous to aconitase (MJ0499, MJ1003, MJ1271, and MJ1277), and two genes homologous to isocitrate dehydrogenase (MJ1596 and MJ0720) (*2*). *M. thermoautotrophicum* Δ*H* has three genes homologous to homocitrate synthase (*nifV*) (MTH1630, MTH723, and MTH1481), four genes homologous to aconitase (MTH1631, MTH1386, MTH1387, and MTH829), one gene homologous to isocitrate dehydrogenase (MTH184), and two genes homologous to citrate synthase (MTH962 and MTH1726) (*17*). The protein products from one or more of these genes were targets as possible genes for the enzymes involved in the biosynthesis of α-ketosuberate and thus coenzyme B.

Plasmid constructs AMJGS60, AMJHK30, and AMJHH70, each containing the desired *M. jannaschii* gene MJ1392, MJ0503, and MJ1195, respectively, and the PUC18 vector, were obtained from TIGR/ATCC microbial genome special collection. The oligonucleotide primers used to direct the polymerase chain reaction formation of a specific gene cartridge had the following sequences. For MJ1392, 5′ CATGCATATGATGGTAAGGATATTTGAT3′ and 5′ GATCGGATCCTTAATTCAATAACATATTGAT 3′, for MJ0503, 5′CATGCATATGACAAAAGTGCTGGTGATG3′ and 5′-GATCGGATCCTTAATTTTTATTTCTCTTCCT3′, and for MJ1195, 5′CATGCATATGATAATTTATAGGGAAGAG3′ and 5′ATCGGATCCAAATCATTTTTCTCTTTTT3′.

The high-level expression of the MJ1392, MJ0503, and MJ1195 gene products in the *E. coli* host strain BL21(DE3) was accomplished by constructing a gene cartridge in vitro and cloning this cartridge into the pT7-7 plasmid (*18*) such that the gene expression is controlled by the T7 phage transcriptional and translational regulatory elements, which in turn are regulated by the lac control elements. Each recombinant plasmid was transformed into the host strain BL21(DE3) *E. coli* cells, and the BL21(DE3) *E. coli* cells containing the pT7-7 plasmid with insert were grown in the LB medium supplemented with 100 mg of ampicillin/mL at 37 °C to an absorbance at 600 nm of 1.0. Protein production was then induced by the addition of isopropylthio-β-D-galactoside (IPTG) to a final concentration of 1 mM. After the addition of IPTG, the cells were cultured for another 2 h, then harvested by centrifugation (4000*g*, 5 min), and frozen at −20 °C until used. High expression of the desired protein was confirmed by SDS−PAGE (12% polyacrylamide) of the SDS soluble cellular proteins, removing the overexpressed protein band, and sequencing its amino-terminus. The presence of a particular enzyme was also confirmed by the measurement of its corresponding activity.

Enzymatic activities of the overexpressed *M. jannaschii* enzymes were measured in *E. coli* cell extracts prepared by sonication of the *E. coli* cell pellets, using the same methods reported above for the preparation and assay of *M. thermophila*.

RESULTS

Identification of the Intermediates Involved in the First Set of α*-Ketoacid Chain Elongation Reactions.* Incubation of a cell extract of *M. thermophila* with α-ketoglutarate and acetylCoA led to the production of (*S*)-homocitrate, *cis*-homoaconitate, and *trans*-homoaconitate (Table 1, experiment 2). For each of these compounds, the amount produced was higher than that found in an unincubated blank (Table 1, experiment 1). The amount of (*S*)-homocitrate produced corresponded to a 20% conversion of the added α-ketoglutarate to (*S*)-homocitrate. Incubation with *cis*-homoaconitate (Table 1, experiment 3) resulted in its almost quantitative conversion into (*S*)-homocitrate. Also detected in this experiment was an increase in the concentration of *trans*-homoaconitate and isohomocitrate. Likewise, the results of experiment 4 in Table 1 showed that incubation of a cell extract with *trans*-homoaconitate gave rise to *cis*-homoaconitate, (*S*)-homocitrate, and isohomocitrate, although not as efficiently as the cis isomer was converted into its products. Together, these results indicated that (*S*)-homocitrate can be derived from either *trans*- or *cis*-homoaconitate and establishes a link between these two molecules through

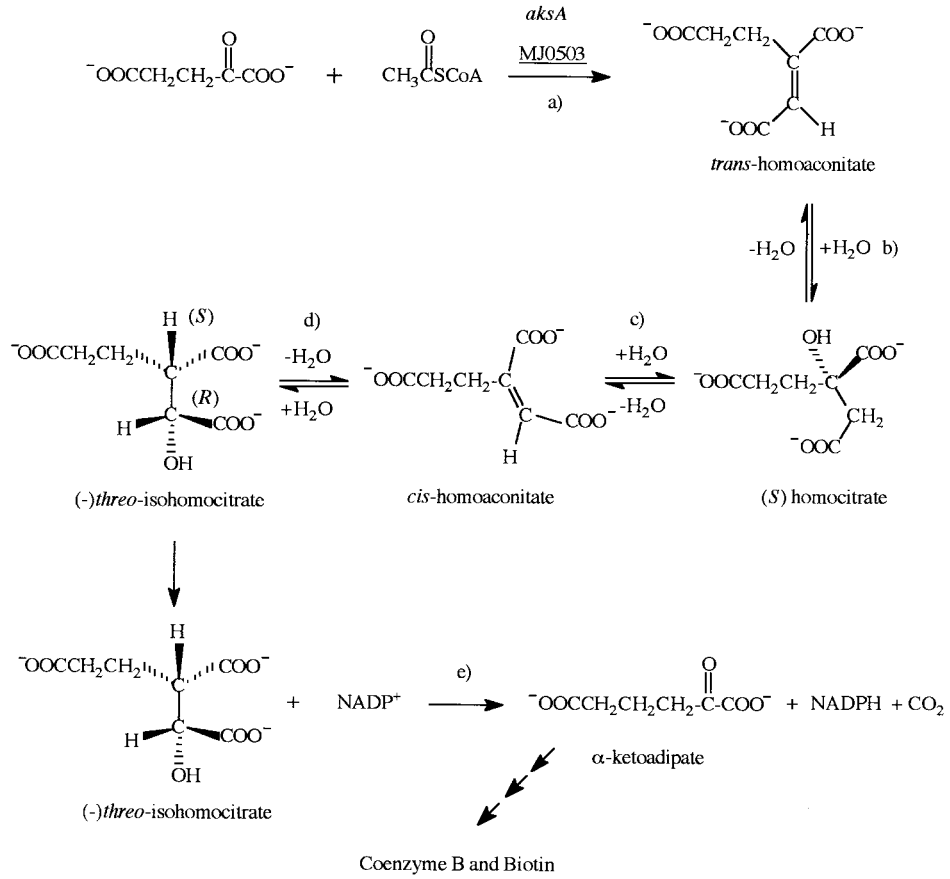
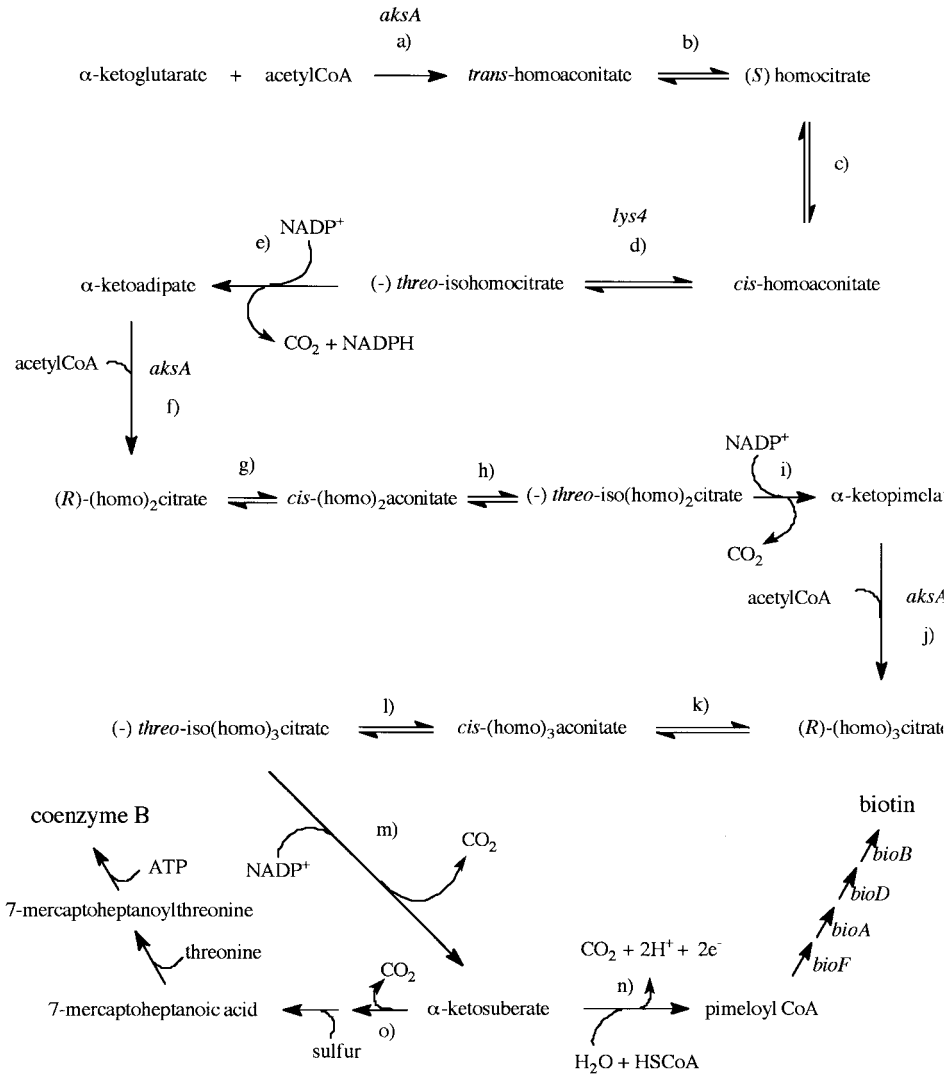


FIGURE 1: Stereochemistry of the intermediates in the first four steps of α-ketosuberate biosynthesis.

(*S*)-homocitrate. Incubation of the cell extract with the mixture of the isomers of isohomocitrate produced by hydrogenation, under anaerobic conditions in the presence of NAD^+ and $NADP^+$, resulted in the conversion of 23% of one of the isohomocitrate isomers into (*S*)-homocitrate (Table 1, experiments 5a), whereas incubation in the presence of air resulted in a 46% conversion of this substrate into (*S*)-homocitrate (Table 1, experiment 5b). In each of these isohomocitrate incubations, increased amounts of *trans*-homoaconitate, *cis*-homoaconitate, and α-ketoadipic acid over the background levels were also detected. These results indicated that these products can be produced from one or more of the isohomocitrate isomers present in the sample. The results also demonstrate that reactions d, c, and b in Figure 1 can proceed in the reverse direction and that reaction e can provide α-ketoadipic acid from one or more of the isohomocitrate isomers. Analysis of the abundance of the isomers of the different isohomocitrates remaining in the incubation mixture after the incubation showed that, of the four isomers added, only the (−)-*threo*-isohomocitrate enantiomer was utilized. Its decrease corresponded closely to the amount of (*S*)-homocitrate detected after the incubation. To confirm the specific ulitization of the (−)-*threo*-isohomocitrate isomer, the incubation was repeated with only this isomer in the presence of NAD^+ and $NADP^+$ or $NADP^+$ alone, and the same group of products was generated (Table 1, experiments 6a and 6b). In these incubations, 80−85% of the (−)-*threo*-isohomocitrate was metabolized to the other intermediates in the biosynthetic pathway. The corresponding (+)-*threo*-isohomocitrate isomer was not metabolized.

Identification of the Intermediates Involved in the Second Set of α*-Ketoacid Chain Elongation Reactions.* The second set of reactions are those involved in the conversion of α-ketoadipate to α-ketopimelate (reactions f, g, h, and i in Figure 2). Incubation of a cell extract with α-ketoadipic acid and acetylCoA readily transformed 44% of the α-ketoadipic acid to (*R*)-(homo)$_2$citrate (Table 2, experiment 1). Also indentified in this incubation was *cis*-(homo)$_2$aconitate and iso(homo)$_2$citrate. Noteworthy among these products is the absence of *trans*-(homo)$_2$aconitate. This finding indicates that the second set of reactions uses only the *cis*-(homo)$_2$-aconitate in the interconversion of (homo)$_2$citrate to iso-(homo)$_2$citrate. GC analysis using the chiral column showed only the (−)-*threo*-iso(homo)$_2$citrate was produced during the incubation. Incubation of the cell extract with (±)-*threo*-iso(homo)$_2$citrate resulted in the production of (homo)$_2$citrate, *cis*-(homo)$_2$aconitate, and α-ketopimelate (Table 2, experiment 2). At the end of the incubation, only the concentration of the (−)-*threo*-iso(homo)$_2$citrate isomer was reduced from its initial starting concentration, indicating that only this isomer was metabolized. The incubations reported in Table 4, to be discussed later, confirm the $NADP^+$ dependency of reaction i. In total, these results demonstrate the occurrence of the next four of the reactions leading to α-ketopimelate (reactions f, g, h, and i in Figure 2).

Identification of the Intermediates Involved in the Third Set of α*-Ketoacid Chain Elongation Reactions.* In an analogous manner, the incubation results shown in Table 3 demonstrate the occurrence of the last four required reactions (reactions j, k, l, and m in Figure 2). Reaction m was found



Biosynthesis of coenzyme B and biotin

FIGURE 2: Pathway for the biosynthesis of α-ketosuberate, coenzyme B, and biotin.

Table 2: Analytical Data for the Second Set of α-Ketoacid Chain Elongation Reactions in *Methanosarcina thermophila*

	concn of intermediates (mM)[a]					
experiment	α-keto-adiptic[b]	(*R*)-(homo)$_2$-citrate	*cis*-(homo)$_3$-aconitate	iso(homo)$_2$-citrate	α-keto-pimelate[b]	*trans*-(homo)$_2$-aconitate
1. α-ketoadipate (3.5 mM), acetylCoA (5.2 mM)	2.3	2.0	0.2	0.09[c]	nd[d]	nd
2. (±)-*threo*-iso(homo)$_2$citrate, (4.7 mM), aerobic[e]	nd	0.2	0.5	4.0[f]	0.06	nd

[a] None of the indicated products were detected in a nonincubated cell extract. [b] Sum of the dimethyl and dimethyl dimethylketal derivatives. [c] GC analysis showed that only the (−)-*threo*-iso(homo)$_2$citrate isomer was produced. [d] Not detected. [e] Also included in the incubation was 0.2 mM F_{420}. [f] GC analysis using the chiral column showed only the (−)-*threo*-iso(homo)$_2$citrate was consumed during the incubation.

Table 3: Analytical Data for the Third Set of α-Ketoacid Chain Elongation Reactions in *Methanosarcina thermophila*

	concn of intermediates (mM)[a]					
experiment	α-keto-pimelate	(homo)$_3$-citrate	*cis*-(homo)$_3$-aconitate	iso(homo)$_3$-citrate	α-keto-suberate[b]	*trans*-(homo)$_3$-aconitate
1. α-ketopimelate (3.5 mM), acetylCoA (5.2 mM)	0.1	4	0.07	0.03	0.3	nd[c]
2. iso(homo)$_3$citrate (4.8 mM)[d], aerobic	nd	0.1	0.09	4.8	0.2	nd

[a] None of the indicated products were detected in a nonincubated cell extract. [b] Sum of the amount of the dimethyl ester and the dimethyl dimethylketal derivative. [c] Not detected. [d] Also included in the incubation was 0.17 mM F_{420}.

to be an $NADP^+$-dependent oxidative decarboxylation of (−)-*threo*-iso(homo)$_3$citrate. This was confirmed by demonstrating that only this isomer underwent an $NADP^+$-dependent oxidative decarboxylation to α-ketosuberate (data not shown). One noteable difference in these data from that of the preceding homologues is the much more efficient

Table 4: Metabolism of (−)-*threo*-Isohomocitrate and AcetylCoA to Homologs of Citrate in *Methanosarcina thermophila*

experiment	concn of intermediates (mM)		
	(−)-*threo*-isohomocitrate	(*R*)-(homo)$_2$citrate	(*R*)-(homo)$_3$citrate
(−)-*threo*-isohomocitrate (4.2 mM), acetylCoA (8.2 mM)[b]			
a. NAD^+, $NADP^+$ (4.1 mM each)	0.6	2.0	0.03
b. $NADP^+$ (4.2 mM)	0.8	2.2	0.05
c. NAD^+ (4.2 mM)	2.4	0.05	0.006
d. control	2.8	0.1	0.002

[a] None of the indicated intermediates were detected in the cell extracts unless they were incubated with substrates. [b] In addition to the products indicated (*S*)-homocitrate, *cis*- and *trans*-homoaconitate, and α-ketoadipic acid were also detected in each experiment and accounts for most of the remainder of the substrate metabolized.

condensation of the α-ketopimelate with acetylCoA to form (homo)$_3$citrate (Table 3, experiment 1).

Characterization of the Cloned Enzymes. The overexpressed protein for MJ0503 (calculated molecular mass of 44.6 kDa) showed a SDS−polyacrylamide gel electrophoresis band at 45 kDa with the expected amino sequence of MTKVL. The overexpressed protein for MJ1392 (calculated molecular mass of 51 kDa) showed a band at 51 kDa and the overexpressed protein for MJ1195 (calculated molecular mass of 56.5 kDa) showed a band at 56 kDa. SDS−polyacrylamide gel electrophoresis analysis of the crude *E. coli* cell extracts and the insoluble pellets resulting from the preparation of these extracts from the overexpressed cells showed that most of the overexpressed enzyme was in the pellet. This result indicated that most of the overexpressed enzyme was present as inclusion bodies.

The crude *E. coli* cell extracts containing overexpressed *M. jannaschii* gene MJ0503 product produced 120 nmol of *trans*-homoaconitate/mg of protein/h from 2.2 mM α-ketoglutarate and 2.2 mM acetylCoA at 50 °C. This crude cell extract also produced 12 nmol of (homo)$_2$citrate and (homo)$_3$citrate/mg protein/h from α-ketoadipate and α-ketopimelate under the same conditions. Considering that MJ0503 is homologous to homocitrate synthase (NifV), which produces only (*R*)-homocitrate, these results strongly suggest that the homologues of homocitrate produced by this enzyme are homologues of (*R*)-homocitrate. We are calling the protein product of the MJ0503 gene AksA, and it represents the first gene (*aksA*) in the biosynthesis of α-ketosuberate.

The cloned product of the MJ1392 gene was shown to catalyze the formation of (*R*)-citramalate from pyruvate and acetylCoA and is thus citramalate synthase (CtmA). This is a previously unknown enzyme, which is now designated as the product of the *ctmA* gene. This cloned gene product did not produce homocitrate, (homo)$_2$citrate, (homo)$_3$citrate, or *trans*-homoaconitate when incubated with acetylCoA and either α-ketoglutarate, α-ketoadipate, or α-ketopimelate.

The cloned product of the MJ1195 gene was shown to catalyze the formation of 2-isopropylmalate from 2-ketoisovalerate and acetylCoA and is thus a 2-isopropylmalate synthase (LeuA), the product of the *leuA* gene. This enzyme is thus involved in the biosynthesis of leucine.

Coenzyme Specificity of the Citrate Dehydrogenase Steps. The question as to the specificity of the oxidant in the above isohomocitrate dehydrogenase-like reactions could not be established from the above data reported, since each pertinent experiment was found to produce some α-ketoadipic acid. To overcome this problem, the incubations were repeated with (−)-*threo*-isohomocitrate and acetylCoA in the presence and absence of the different pyridine nucleotides. As discussed below, the acetylCoA would react with the α-ketoadipic acid to product (homo)$_2$citrate which could then be measured. As can be seen from the data reported in Table 4, $NADP^+$ was by far the most efficient oxidant for the production of (homo)$_2$citrate, indicating that reaction e is analogous to an $NADP^+$-dependent isohomocitrate dehydrogenase. The detection of (homo)$_3$citrate in these groups of experiments indicate that Reaction i is also analogous to an $NADP^+$-dependent isohomocitrate dehydrogenase.

DISCUSSION

On the basis of the detection of α-ketoglutarate, α-ketoadipate, α-ketopimelate, and α-ketosuberate in several different methanogenic Archaea and the labeling pattern of these compounds produced by *M. thermophila* grown with labeled acetates, a pathway for their biosynthesis was proposed (*3*, *4*). This pathway consisted of the repeated application of the α-ketoacid chain elongation series of reactions to extend α-ketoglutarate to α-ketosuberate. The α-ketosuberate then serves as a precursor to 7-mercaptoheptanoic acid (*5*), a component of the methanogenic cofactor coenzyme B. On the basis of analogy with known biochemical reactions and on the chemistry required to perform these series of reactions, one would expect that at least three different enzyme activities would be required for each group of α-ketoacid chain elongation reactions. These would consist of an enzyme catalyzing the condensation of an α-ketodiacid with acetylCoA to form a homologue of citrate, an enzyme catalyzing the isomerization of this citrate homologue to the isocitrate homolog, and finally an enzyme catalyzing an oxidative decarboxylation of the isocitrate homologue to the α-ketodiacid containing one additional methylene group. These reactions would be analogous to the reactions catalyzed by citrate synthase, aconitase, and isocitrate dehydrogenase in the citric acid cycle and the reactions catalyzed by (*R*)-homocitrate synthase, homoaconitase, and (−)-*threo*-isohomocitrate dehydrogenase in the biosynthesis of lysine (*19*). Each time one sequence of reactions is completed, one additional methylene group is added to the growing molecule.

The approach that has been used to establish the biosynthetic pathway to α-ketosuberate involves first establishing the presence of the types of compounds that could be involved in the biosynthetic pathway in *M. thermophila* and then determinating as to whether a cell extract of *M. thermophila* will catalyze the desired interconversions of these or other possible compounds. Finally, when the reaction is confirmed in the cell extract, the gene encoding the enzyme for the reaction is established by demonstrating that the cloned gene product catalyzes the desired reaction.

Of the five possible compounds that could be involved in the first set of α-ketoacid chain elongation reactions, *trans*-homoaconitate, homocitrate, *cis*-homoaconitate, isohomocitrate, and α-ketoadipate, three were identified in the cell extracts of *M. thermophila*, *trans*-homoaconitate, homocitrate, and *cis*-homoaconitate (Table 1). The homocitrate present in the cell extract was identified as the S stereoisomer based on its mass spectra and GC retention time by GC-MS on the chiral column. α-Ketoadipate, which has been previously reported to be present in these cells (*4*), was not detected since its concentration in the extracts was too low be detected considering the small amounts of the samples assayed here. The discovery of the presence of both (*S*)-homocitrate and *trans*-homoaconitate in the cell extracts, as well as their production in the incubation mixture, was quite unexpected since neither of these compounds have ever been previously reported as natural products. Their discovery complicates the expected simplicity for the formation of isohomocitrate from α-ketoglutarate and acetylCoA, which was expected to be formed via *cis*-homoaconitate and (*R*)-homocitrate as occurs in the biosynthesis of lysine via the α-ketoadipate pathway (*19*).

Knowing that the precursor to the α-ketoadipate is (−)-*threo*-isohomocitrate, the same isohomocitrate isomer that is involved in lysine biosynthesis, and the expected occurrence that only trans additions and eliminations of water are involved in the interconversions of the intermediates (*20*), the reaction sequence shown in Figure 1 is proposed to account for the first set of reactions. This pathway accounts for the formation of all of the intermediates observed. Reactions b and c could *not* be carried out by an enzyme homologous to aconitase or homoaconitase, since these enzymes use only the *cis*-homoaconitate and produce (*R*)-homocitrate as substrates (*7, 21*). Reaction d, however, is that expected for an aconitase or a homoaconitate-like enzyme. It is possible that reaction c in Figure 1 is catalyzed by the aconitate hydrase described by Neilson (*22, 23*). Unfortunately, this enzyme appears to have never been purified and isolated in a pure form, so no information is currently available as to the stereospecificity of its reaction. Reaction b in Figure 2 could be catalyzed by an enzyme related to mesaconase, an enzyme which catalyzes the reversible addition of water to mesaconate (*trans*-1,2-prop-1-enedicarboxylic acid) to form (*S*)-citramalate (*24, 25*). The direct conversion of *trans*- to *cis*-homoaconitate with an enzyme analogous to aconitate isomerase is not possible on mechanistic grounds (*26*).

At present, three homologous enzymes are known to catalyze the condensation of acetylCoA with α-ketoacids to produce products with the R stereochemistry. The reactions catalyzed by these enzymes as well as the accompanying isomerization reactions are outlined in Figure 3 and include the *re*-citrate synthases found in some bacteria (*27*−*29*), (*R*)-homocitrate synthase (*8, 9, 12*), and 2-isopropylmalate synthase (*30*). Only the *si*-citrate synthase produces a product with the same stereochemistry (*31*) as the (*S*)-homocitrate reported here. Although *si*-citrate synthases have been found in members of the Archaea (*32*) and *si*-citrate synthases with homologies to other bacterial and eukarya citrate synthases and both have been shown to be present in *Sulfolobus* (*33*), *Thermoplasma* (*34*), and *Pyrococcus* (*35*), it is unlikely that this enzyme is involved in the biosynthesis of the (*S*)-homocitrate. The strongest support for the lack of involvement of this enzyme comes from the absence of a citrate synthase in *M. thermophila* (*2*), discussed below, the observation that *trans*-homoaconitate is the product of the condensation of α-ketoglutaric acid with acetylCoA, and that this compound is infact the first biosynthetic intermediate in the pathway.

On the basis of the stereochemistry of the aconitase-type reactions d, g, h, k, and l in Figure 2, they could each be expected to be catalyzed by an aconitase type of enzyme (*35*). At least three different biochemical reactions are known to be catalyzed by enzymes homologous to that of aconitase and homoaconitase. These include the conversion of citrate to (+)-*threo*-isocitrate catalyzed by aconitase (*20*), the conversion of (*R*)-homocitrate to (−)-*threo*-isohomocitrate catalyzed by homoaconitate (*36*), and the conversion of 2-isopropylmalate to 3-isopropylmalate catalyzed by 3-isopropylmalate dehydratase (*37, 38*). The stereochemical courses of these reactions are shown in Figure 3. Viewed from the stereochemical perspective of the homocitrate reaction, each of these reactions begins with a substrate that has the R stereochemistry, proceeds through a cis-alkene intermediate, and produces a product with the same relative stereochemistry as (−)-*threo*-isohomocitrate and (+)-*threo*-isocitrate. This same series of reactions can account for most of the desired conversions described here.

The one unprecedented step in this first set of reactions is the generation of *trans*-homoaconitate from the condensation of α-ketoglutarate and acetylCoA. This reaction was shown to be catalyzed by the protein product of the *M. jannaschii* MJ0503, a gene homologous to homocitrate synthase. Since *M. thermophila* is also a methanogenic Archaea, requiring coenzyme B for methanogenesis, it is most likely that the protein product of this gene is also the one used to catalyze the first step in the biosynthesis of α-ketoadipate. The unusual aspect of this reaction is that the dehydrated product homoaconitate is the product of the reaction. Considering the homology between this enzyme and homocitrate synthase, we would expect the enzyme to catalyze not only the condensation reaction to form homocitrate but also a second dehydration reaction to generate the observed product. Oddly, this same enzyme was observed to also catalyze the formation of $(homo)_2$citrate and $(homo)_3$citrate from their respective reactants without the dehydration step occurring. The possibility that the dehydration reaction is carriered out by an *E. coli* enzyme present in the crude extract is unlikely since the incubations are done at 50 °C, which is too high a temperature for most *E. coli* enzymes to operate.

The data for the next two sets of α-keto acid chain elongation reactions from α-ketoadipate to α-ketosuberate support the conclusion that each proceeds with homologues with the same stereochemistry as the intermediates involved in the conversion of α-ketoglutarate to α-ketoadipate during the biosynthesis of lysine. These include homologues of (*R*)-homocitrate, *cis*-homocitrate, and (−)-*threo*-isohomocitrate.

Finally, we must consider the possible genes for the $NADP^+$-dependent isocitrate dehydrogenase-type enzyme-catalyzing reactions e, i, and m. *M. jannashii* has two genes which could produce proteins homologous to isocitrate dehydrogenase, the NAD^+-dependent isocitrate dehydrogenase or the 3-isopropylmalate dehydrogenase gene MJ0720 and the $NADP^+$-dependent isocitrate dehydrogenase gene

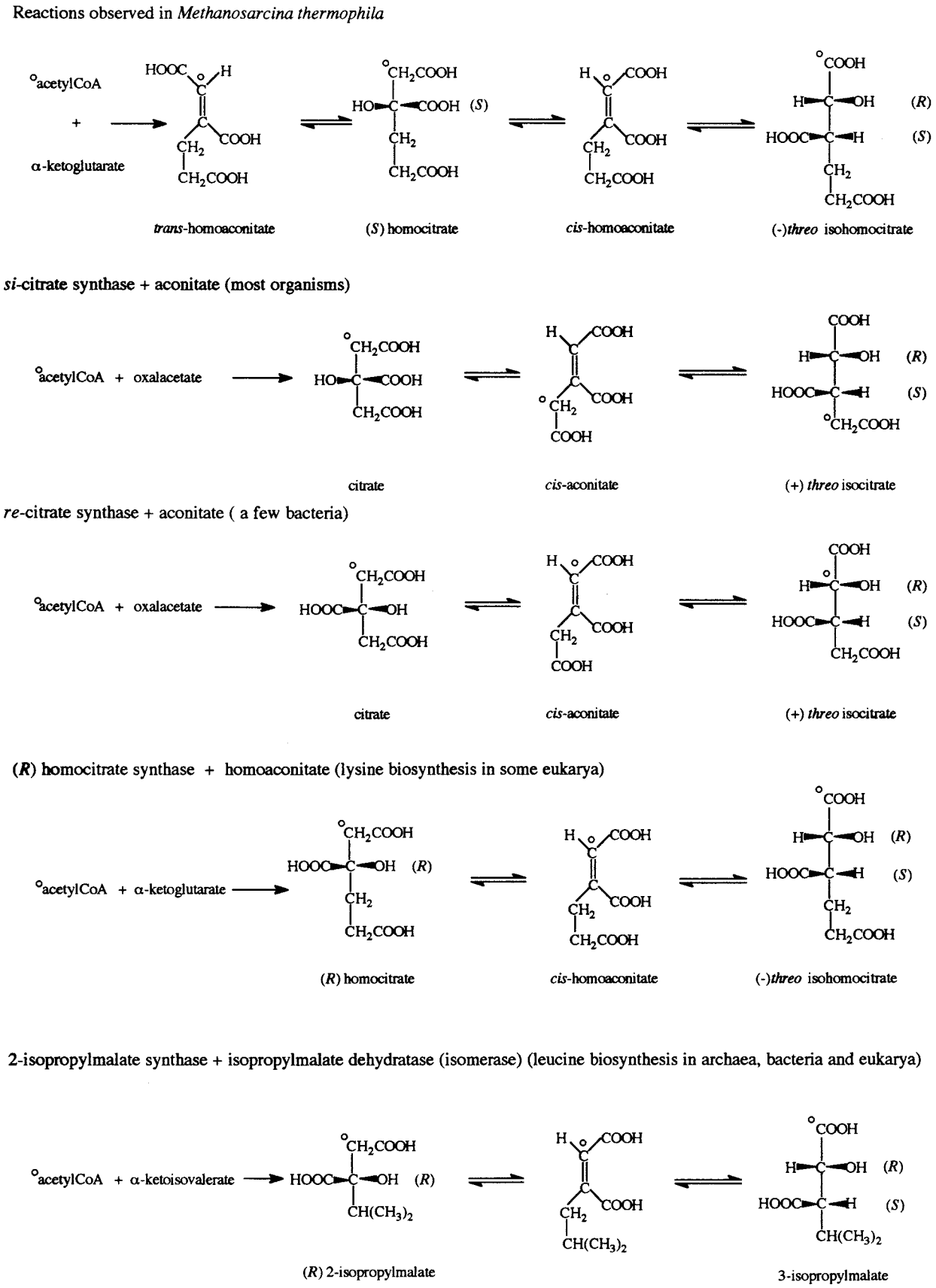


FIGURE 3: Stereochemistry of the known α-ketoacid chain elongation reactions.

MJ1596. These correspond to the MTH184 and MTH1388 genes, respectively, in *M. thermoautrophicium*. The data presented here indicate that the latter enzyme is the one likely to be involved in the reactions reported here. It is also likely that only one enzyme is able to catalyze each of the reactions with the three different homologous substrates.

A precedent for a single enzyme being involved for each different type of analogous reactions comes from a consid-

eration of the enzymes which are involved in the biosynthesis of fatty acids which are part of the fatty acid synthase. This multienzyme complex is able to extend a growing fatty acid alkyl chain two methylenes at a time using the same set of enzymes for each different type of analogous reactions. Thus, each enzymatic step proceeds using the same enzymes but with the different homologues of the fatty acids. Evidence that this could be occurring in the methanogens comes from the observations reported above, where the cloned MJ0503 gene product is able to catalyze the formation of both $(homo)_2$citrate and $(homo)_3$citrate. It is equally likely that only one of the isohomocitrate and one of the homoaconitase gene products would be required to catalyze their respective group of reactions.

The above results strongly suggest that the enzymes used in biosynthesis of 7-mercaptoheptanoic acid have evolved in the Archaea from enzymes that are already known to exist in Bacteria and Eukarya. This same situation is in contrast to what is being discovered for the biosynthesis of the modified folate methanopterin. Here it appears that, despite the fact that many of the same reactions are occurring as occur in folate biosynthesis, the overall biosynthesis proceeds with enzymes which have little or no primary sequence homologies to the folate biosynthetic enzymes (White, unpublished results).

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