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Malonyl-Coenzyme A Reductase in the Modified 3-Hydroxypropionate Cycle for Autotrophic Carbon Fixation in Archaeal *Metallosphaera* and *Sulfolobus* spp.[▽]

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Autotrophic members of the *Sulfolobales* (*Crenarchaeota*) contain acetyl-coenzyme A (CoA)/propionyl-CoA carboxylase as the CO_2 fixation enzyme and use a modified 3-hydroxypropionate cycle to assimilate CO_2 into cell material. In this central metabolic pathway malonyl-CoA, the product of acetyl-CoA carboxylation, is further reduced to 3-hydroxypropionate. Extracts of *Metallosphaera sedula* contained NADPH-specific malonyl-CoA reductase activity that was 10-fold up-regulated under autotrophic growth conditions. Malonyl-CoA reductase was partially purified and studied. Based on N-terminal amino acid sequencing the corresponding gene was identified in the genome of the closely related crenarchaeum *Sulfolobus tokodaii*. The *Sulfolobus* gene was cloned and heterologously expressed in *Escherichia coli*, and the recombinant protein was purified and studied. The enzyme catalyzes the following reaction: malonyl-CoA + NADPH + H^+ → malonate-semialdehyde + CoA + $NADP^+$. In its native state it is associated with small RNA. Its activity was stimulated by Mg^{2+} and thiols and inactivated by thiol-blocking agents, suggesting the existence of a cysteine adduct in the course of the catalytic cycle. The enzyme was specific for NADPH (K_m = 25 μM) and malonyl-CoA (K_m = 40 μM). Malonyl-CoA reductase has 38% amino acid sequence identity to aspartate-semialdehyde dehydrogenase, suggesting a common ancestor for both proteins. It does not exhibit any significant similarity with malonyl-CoA reductase from *Chloroflexus aurantiacus*. This shows that the autotrophic pathway in *Chloroflexus* and *Sulfolobaceae* has evolved convergently and that these taxonomic groups have recruited different genes to bring about similar metabolic processes.

Chloroflexus aurantiacus, a green nonsulfur phototrophic eubacterium, uses the 3-hydroxypropionate cycle for autotrophic CO_2 fixation (Fig. 1) (10, 15, 16, 18, 19, 30, 31). This cyclic pathway starts with acetyl-coenzyme A (CoA) carboxylation to malonyl-CoA, catalyzed by acetyl-CoA carboxylase. Malonyl-CoA not only serves as a precursor for fatty acid biosynthesis but also under autotrophic growth conditions is reduced with NADPH by malonyl-CoA reductase to 3-hydroxypropionate, a characteristic intermediate of this CO_2 fixation pathway (21). 3-Hydroxypropionate is reductively converted to succinyl-CoA in a process in which propionyl-CoA is carboxylated, thereby fixing another molecule of CO_2 (2). Succinyl-CoA, which has been thus formed from one acetyl-CoA and two CO_2 molecules, is used to regenerate the initial CO_2 acceptor molecule acetyl-CoA, and glyoxylate is released as the primary CO_2 fixation product (13, 17). Glyoxylate is converted in a second cyclic pathway to pyruvate, a central carbon precursor (16).

A similar not-yet-resolved 3-hydroxypropionate cycle was found in members of the *Sulfolobales* (*Crenarchaeota*) (6, 22, 24, 26, 27). Autotrophic members of this archaeal taxonomic group differ from the other archaebacteria by harboring a very active acetyl-CoA/propionyl-CoA carboxylase (7, 23). Because archaebacteria do not synthesize fatty acids, this activity is required for a different central metabolic process. The strict up-regulation of acetyl-CoA carboxylase activity under autotrophic growth conditions suggests that it is the primary CO_2-fixing enzyme during autotrophy in these organisms (6, 22, 23, 24). So far all enzymatic steps leading from acetyl-CoA to succinyl-CoA via 3-hydroxypropionate and propionyl-CoA have been demonstrated in extracts of autotrophic members of the *Sulfolobaceae* (22, 26); however, acetyl-CoA/propionyl-CoA carboxylase is the only enzyme studied so far (7, 23).

In this work, malonyl-CoA reductase in *Metallosphaera sedula* and *Sulfolobus tokodaii*, two members of the *Sulfolobaceae*, was investigated. In *C. aurantiacus*, malonyl-CoA is reduced by two NADPH molecules via malonate-semialdehyde to 3-hydroxypropionate. Malonyl-CoA reductase from *C. aurantiacus* is a large enzyme of 145-kDa subunits, which shows little similarity to known oxidoreductases (21). It combines both aldehyde and alcohol dehydrogenase function. A database search identified only two other malonyl-CoA reductase genes similar to that of *C. aurantiacus*, a gene from the close relative *Roseiflexus* sp. strain RS-1 (GenBank accession number EAT28741) and an uncharacterized gene from the α-proteobacterium *Erythrobacter* sp. strain NAP1 (GenBank accession number EAQ29650). However, no archaeal representatives were found to contain this gene. This fact pointed to the existence of an alternate malonyl-CoA reductase in archaebacteria that is part of the modified 3-hydroxypropionate cycle. We show that archaeal malonyl-CoA reductase has only aldehyde dehydrogenase activity and suggest that it has evolved from the duplication of the aspartate-semialdehyde dehydrogenase gene. Genes for this kind of enzyme were

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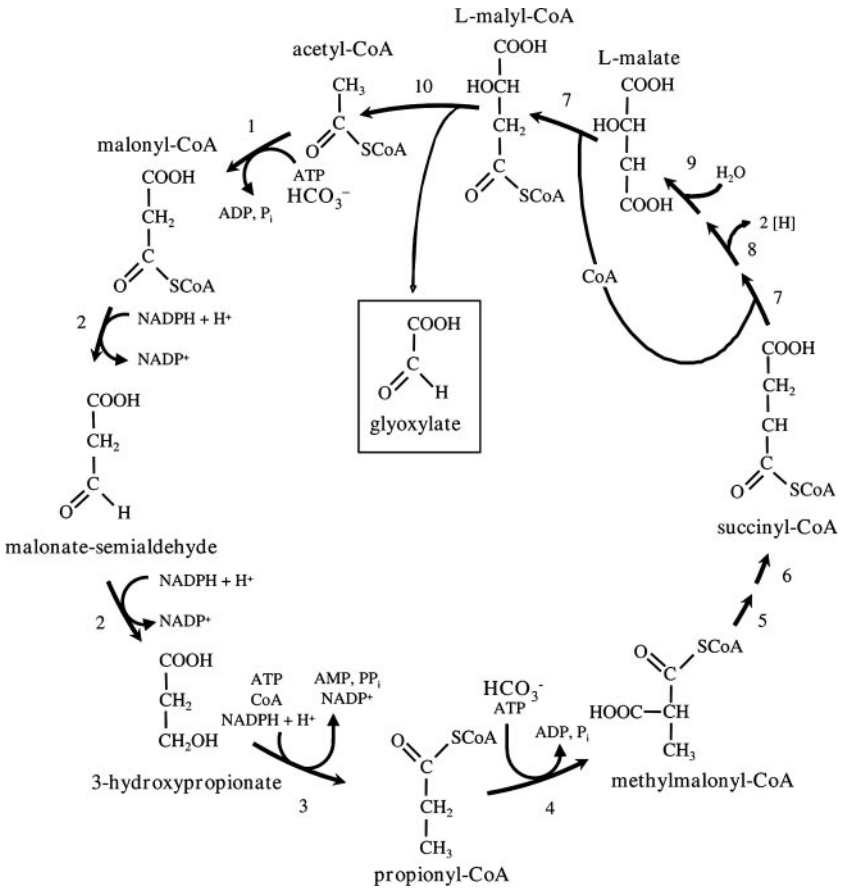

FIG. 1. 3-Hydroxypropionate cycle of *Chloroflexus aurantiacus*. Enzymes: 1, acetyl-CoA carboxylase; 2, malonyl-CoA reductase (NADPH); 3, propionyl-CoA synthase; 4, propionyl-CoA carboxylase; 5, methylmalonyl-CoA epimerase; 6, methylmalonyl-CoA mutase; 7, succinyl-CoA:L-malate-CoA transferase; 8, succinate dehydrogenase; 9, fumarate hydratase; 10, L-malyl-CoA lyase.

only found in *Sulfolobaceae*, underlining the role of this aspartate-semialdehyde dehydrogenase paralogue in the *Sulfolobaceae*-specific type of the 3-hydroxypropionate cycle.

MATERIALS AND METHODS

Materials. Chemicals and biochemicals were obtained from Roche Diagnostics (Mannheim, Germany), Fluka (Neu-Ulm, Germany), Merck (Darmstadt, Germany), Roth (Karlsruhe, Germany), Sigma-Aldrich (Deisenhofen, Germany), Bio-Rad (München, Germany), and Genaxxon (Biberach, Germany). Gases were obtained from Sauerstoffwerke Friedrichshafen (Friedrichshafen, Germany), and radioisotopes were obtained from American Radiolabeled Chemicals Inc./Biotrend Chemikalien GmbH (Köln, Germany). Enzymes and primers were obtained from MBI Fermentas (St. Leon-Rot, Germany) and Genaxxon Biosciences GmbH (Biberach, Germany). Materials and equipment for protein purification were obtained from Amersham Biosciences (Freiburg, Germany). Plasmids were obtained from Invitrogen (Karlsruhe, Germany).

Strains and culture conditions. *Metallosphaera sedula* TH2 (DSM 5348) was grown autotrophically at 75°C on a chemically defined medium (pH 2.0) under gassing with a mixture of 19% CO_2, 3% O_2, and 78% H_2 (generation time, 8 h) (20). Control cells were grown aerobically and heterotrophically with 0.05% yeast extract (generation time, 8 h) (20). *Sulfolobus tokodaii* (DSMZ 16993) was grown aerobically and heterotrophically at 75°C on a chemically defined medium (pH 3.0) with 1 g of glucose per liter (generation time, 6 h) (32). Cells were stored in liquid nitrogen until use. *Escherichia coli* strain DH5α and *E. coli* strain Rosetta 2 (Merck, Germany) were grown at 37°C in Luria-Bertani medium (28). Antibiotics were added to *E. coli* cultures up to the following final concentrations: ampicillin, 100 μg ml^{-1}; chloramphenicol, 34 μg ml^{-1}.

Preparation of cell extracts. Cells from *M. sedula*, *S. tokodaii*, and *E. coli* were resuspended in a twofold volume of 50 mM Tris/HCl (pH 7.8) containing 5 mM $MgCl_2$ and 0.1 mg ml^{-1} DNase I. The cell suspension was passed through a French pressure cell at 137 MPa and ultracentrifuged (100,000 × *g*) at 4°C for 1 h. The cell extract was used immediately or kept frozen at −70°C.

Enzyme assays. Malonyl-CoA reductase activity was monitored in a spectrophotometric assay. The malonyl-CoA-dependent oxidation of NADPH was studied spectrophotometrically at 365 nm ($\varepsilon_{365\ nm\ NADPH}$ = 3,400 M^{-1} cm^{-1}). The assay mixture contained 100 mM Tris/HCl (pH 7.8), 5 mM $MgCl_2$, 5 mM 1,4-dithioerythritol, 0.5 mM NADPH, 0.2 mM malonyl-CoA, and cell extract, partially purified enzyme, or purified enzyme. The addition of malonyl-CoA started the reaction. Buffers used to determine the pH optimum were morpholinepropanesulfonic acid (MOPS)/NaOH (pH 6.5 to 8.5), HEPES/NaOH (pH 6.2 to 7.8), and Tricin/NaOH (pH 7.8 to 8.2). To study the effect of thiol-blocking agents, 1,4-dithioerythritol was omitted, and iodoacetate was added to the reaction mixture at a concentration of 0.01 to 1 mM. After incubation of the mixture at 65°C for 5 min, the reaction was started by addition of malonyl-CoA. The apparent K_m values were determined by varying the concentration of NADPH (0.1 to 0.5 mM) or malonyl-CoA (0.02 to 0.4 mM) while keeping the cosubstrate at saturating concentration (0.2 mM malonyl-CoA or 0.5 mM NADPH). One unit of enzyme activity refers to 1 μmol of NADPH oxidized per min, corresponding to 1 μmol of malonyl-CoA reduced to malonate-semialdehyde per min. Protein was quantitated by the method of Bradford (5) using bovine serum albumin as the standard.

Purification of malonyl-CoA reductase from *M. sedula*. All purification steps were done aerobically at 4°C.

(i) DEAE-Sepharose chromatography. Cell extract (4.5 ml) was applied to a DEAE-Sepharose column (fast flow; diameter, 1.6 cm; volume, 10 ml; Pharmacia), which had been equilibrated with 20 mM triethanolamine/NaOH (pH 8.5) and 10% (vol/vol) glycerol (referred to as buffer A) at a flow rate of 1 ml min^{-1}. The column was washed with 2 bed volumes of buffer A and eluted with a step gradient of 50 mM NaCl in buffer A.

TABLE 1. Partial purification of malonyl-CoA reductase from *M. sedula* and purification of heterologously expressed malonyl-CoA reductase from *S. tokodaii*

Source of enzyme	Purification step	Amt of protein (mg)	Total activity (μmol min^{-1})	Sp act (μmol mg^{-1} min^{-1})	Purification (*n*-fold)	Yield (%)
M. sedula	Cell extract	94	40	0.42	1	100
	DEAE-Sepharose	9.4	20	2.1	5	50
	Phenyl-Sepharose	0.5	2.4	4.6	11	6
S. tokodaii	Extract after heat precipitation	65	728	11	1	100
	Gel filtration	12.4	220	18	1.6	31
	Resource-phenyl	1.6	70	44	4	10

(ii) Phenyl-Sepharose chromatography. Saturated ammonium sulfate solution was added to the combined active fractions obtained from DEAE-Sepharose chromatography to a final concentration of 1 M. The protein fraction was then centrifuged and the supernatant was directly applied to a Phenyl-Sepharose column (Pharmacia; diameter, 5.0 cm; volume, 20 ml) at a flow rate of 1 ml min^{-1}. The column had been equilibrated with 100 mM Tris/HCl (pH 7.8) and 1 M $(NH_4)_2SO_4$. After washing the column with 5 bed volumes of this buffer and carrying out a subsequent step gradient to 500 mM $(NH_4)_2SO_4$, the column was developed with a 200-ml decreasing linear gradient of 500 to 0 mM ammonium sulfate at 1 ml min^{-1}. The activity eluted between 10 mM and 0 mM salt. Active fractions were pooled and kept frozen at −20°C until use.

Molecular mass determination. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described by Laemmli (25), using 12.5% gels. Proteins were visualized by Coomassie blue staining (35). The native molecular mass of the enzyme was estimated by gel filtration chromatography. Protein from the phenyl-Sepharose chromatography step was applied to a Superdex 200 HR 16/60 gel filtration column (diameter, 1.6 cm; volume, 120 ml; Pharmacia), which had been equilibrated with 100 mM Tris/HCl (pH 7.8) containing 100 mM NaCl and 10% (vol/vol) glycerol. The flow rate was 1 ml min^{-1}. The column was calibrated with the following molecular mass standards: ferritin (450 kDa), catalase (240 kDa), alcohol dehydrogenase (150 kDa), bovine serum albumin (67 kDa), and ovalbumin (45 kDa).

N-terminal amino acid analysis. Determination of the N-terminal amino acid sequence of malonyl-CoA reductase from *M. sedula*, after blotting on a poly (vinylidene difluoride) membrane, was performed by use of TopLab (Martinsried, Germany) using an Applied Biosystems Procise 492 sequencer (Weiterstadt, Germany).

Heterologous expression of the malonyl-CoA reductase (*mcr*) gene from *S. tokodaii* and production of the protein in *E. coli*. Chromosomal DNA from *S. tokodaii* was isolated using standard techniques. Two synthetic oligonucleotides were designed to amplify the complete *mcr* gene using chromosomal DNA from *S. tokodaii* as a template: the forward primer, 5′-ATTATCCCATGGGGAGA ACATTAAAAGC-3′ introduces a NcoI site (underlined) at the initiation codon; the reverse primer, 5′-CGGGATCCTTACTTTTCAATATATCC-3′ introduces a BamHI site (underlined) after the stop codon. PCR, including denaturation at 94°C for 1 min, annealing at 45°C for 1 min, and extension at 72°C for 5 min, was performed for 30 cycles. The PCR product was isolated and cloned into pCR T7/CT Topo. The sequence of the insert was determined to ensure that no errors had been introduced. The plasmid was digested with NcoI and BamHI, and the fragment containing the *mcr* gene was ligated into pTrc99A, resulting in plasmid pTrc99A-mcr. A plasmid-derived *lac* promoter in front of *mcr* allows expression of the gene after induction of isopropyl thiogalactopyranoside (IPTG). Competent *E. coli* Rosetta 2 cells were transformed with pTrc99A-mcr, grown in a 200-liter fermentor at 37°C in Luria-Bertani broth containing ampicillin (100 μg ml^{-1}), and induced at an optical density of 0.6 with IPTG (0.5 mM). After 3 h of additional growth the cells were harvested and stored in liquid nitrogen until use.

Purification of heterologously expressed malonyl-CoA reductase from *S. tokodaii*. (i) Heat precipitation. Cell extract from 10 g (wet weight) of cells of *E. coli* (supernatant obtained by centrifugation at 100,000 × *g*) was incubated at 85°C for 15 min and cooled on ice for 10 min to precipitate unwanted protein, lipids, and pigments, followed by centrifugation (17,000 × *g*) at 4°C for 15 min. The supernatant was concentrated to a final volume of 5 ml by ultrafiltration (Amicon YM30 membrane; Millipore).

(ii) Gel filtration chromatography. The concentrated fraction was applied to a Superdex 200 HR 26/60 gel filtration column (diameter, 2.6 cm; volume, 320 ml; Pharmacia), which had been equilibrated with 20 mM Tris/HCl (pH 7.0) containing 100 mM KCl. The flow rate was 2.5 ml min^{-1}. The active protein eluted with a retention volume of 160 to 185 ml, and fractions were pooled and concentrated immediately by ultrafiltration to a final volume of 5 ml. The native molecular mass of the enzyme was estimated using this gel filtration column. The column was calibrated with thyroglobulin (660 kDa), ferritin (450 kDa), catalase (240 kDa), alcohol dehydrogenase (150 kDa), bovine serum albumin (67 kDa), and ovalbumin (45 kDa) as molecular mass standards.

(iii) Resource phenyl chromatography. Saturated ammonium sulfate solution was added to the concentrated active fractions obtained from gel filtration chromatography to a final concentration of 1 M. The protein fraction was then centrifuged, and the supernatant was directly applied to a Resource phenyl column (1 ml of Resource-PHE; Amersham Biosciences) at a flow rate of 2 ml min^{-1}. The column had been equilibrated with 100 mM Tris/HCl (pH 7.8) and 1 M $(NH_4)_2SO_4$. The activity eluted within the flowthrough of the column.

RNA analysis. Optical absorption spectra of the purified recombinant malonyl-CoA reductase from *E. coli* (16 μg in 500 μl of 50 mM Tris/HCl, pH 7.8) were collected at room temperature using a Perkin-Elmer Life Science Lambda 2S spectrometer (1-cm light path) and the same buffer as a blank. RNA was extracted from an aliquot (40 μg) of purified recombinant malonyl-CoA reductase from *Sulfolobus tokodaii* by the phenol-chloroform method (1). The concentration of RNA was determined at 260 nm (1.0 A_{260} unit corresponds to 40 μg ml^{-1}). An aliquot of isolated RNA (1.4 μg) was incubated at 37°C in 12 μl of 100 mM Tris/HCl–2.5 mM $MgCl_2$–0.1 mM $CaCl_2$ (pH 7.5) for 20 min in the presence of either 1.5 μl of 10 mg ml^{-1} bovine pancreas RNase (DNase free after boiling; Boehringer-Mannheim, Germany) or 1.5 units of DNase I (RNase free; Fermentas, St. Leon-Rot, Germany), or it was left on ice as a control. Samples were analyzed by agarose gel electrophoresis (2% agarose) as described previously (28).

Computer analysis. The BLAST program was used to search databases at the National Center for Biotechnology Information (Bethesda, Md.). GraphPad-Prism (GraphPad Software, Inc., San Diego, CA) was used to fit data obtained from kinetic measurements. The graphical codon usage analyzer (14) was used to determine the difference in codon usage between *E. coli* and *S. tokodaii*. Multiple sequence alignment and phylogenetic tree construction were performed with MultAlin (9).

RESULTS

Malonyl-CoA reductase in cell extracts of *Metallosphaera sedula*. Extracts were tested for the malonyl-CoA-dependent oxidation of NADPH or NADH. The enzyme activity was measured at 65°C (the optimal temperature for growth is 85°C) in a spectrophotometric assay by following the malonyl-CoA-dependent oxidation of NAD(P)H. The rates were calculated based on the assumption that the enzyme catalyzed only malonyl-CoA reduction to malonate-semialdehyde and no further reduction of malonate-semialdehyde to 3-hydroxypropionate. Extracts of autotrophically grown cells catalyzed the NADPH-dependent reaction at a specific activity of 420 nmol NADPH oxidized min^{-1} mg^{-1} of soluble protein (65°C); no activity was measured with NADH. This activity was down-regulated in heterotrophically grown cells to 40 nmol min^{-1} mg^{-1}.

Partial purification and characterization of the enzyme. Malonyl-CoA reductase was partially purified from autotrophically grown cells in three chromatographic steps. Because the

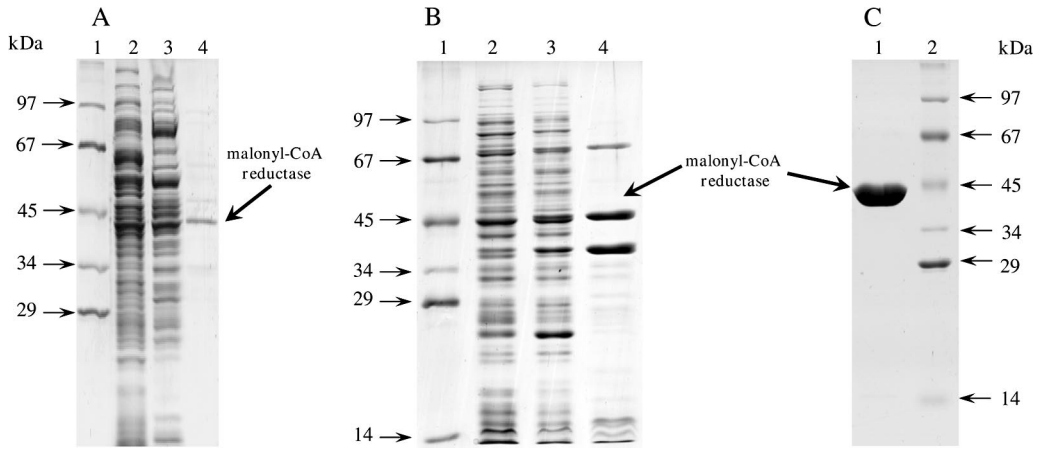

FIG. 2. SDS-PAGE (12.5%) of fractions obtained during purification of native and recombinant malonyl-CoA reductase. Proteins were stained with Coomassie blue. (A) Enzyme fractions during purification of the native enzyme from *M. sedula*. Lanes: 1, molecular mass standard proteins; 2, cell extract of autotrophically grown cells (20 μg); 3, enzyme fraction after DEAE-Sepharose chromatography (20 μg); 4, enzyme fraction after chromatography on phenyl-Sepharose (5 μg). (B) Heterologous expression of malonyl-CoA reductase gene from *S. tokodaii* in *E. coli* Rosetta 2. Lanes: 1, molecular mass standard proteins; 2, cell extract of *E. coli* before induction (20 μg); 3, cell extract of *E. coli* after 3 hours of induced growth (20 μg); 4, cell extract of *E. coli* after heat precipitation (10 μg). (C) Purified recombinant malonyl-CoA reductase from *S. tokodaii*. Lanes: 1, fraction after gel filtration chromatography and chromatography on Resource-phenyl (10 μg); 2, molecular mass standard proteins.

enzyme was rather labile, purification was performed within 24 h, and the enzyme was kept in 10% (vol/vol) glycerol. The preparation obtained with 6% yield showed a specific activity of 4.6 μmol min^{-1} mg^{-1} (Table 1). Gel filtration indicated a native molecular mass of approximately 150 kDa. SDS-PAGE of the preparation showed a strong band at 43 kDa (Fig. 2A), indicating that the enzyme may be a homotetramer. In addition, several other faint bands indicated that the protein was approximately 50% pure. The partially purified enzyme had a pH optimum of 7.8, with half-maximal activities at pH 6.5 and 8.5. The enzyme followed Michaelis-Menten kinetics, with an apparent K_m value of 0.1 mM for malonyl-CoA (measured at 0.5 mM NADPH).

Identification of a homologous gene in *Sulfolobus tokodaii* and its cloning and expression in *Escherichia coli*. The N-terminal amino acid sequence of the dominant 43-kDa band of partially purified malonyl-CoA reductase from *M. sedula* was determined to be MRRTLKAAILGATGLVGIEY. A search of the protein databases revealed a perfect match to the deduced N-terminal amino acid sequence of a gene from the closely related *Sulfolobus tokodaii*, which had been annotated as aspartate-semialdehyde dehydrogenase (accession number NP_378167). Aspartate-semialdehyde dehydrogenase catalyzes the NADPH-dependent reduction of aspartoyl-4-phosphate to aspartate-semialdehyde, and phosphate is released (Fig. 3A). We propose that this gene encodes malonyl-CoA reductase, catalyzing a similar reaction (Fig. 3B), and it was named *mcr*. No similarity with the malonyl-CoA reductase gene from *Chloroflexus aurantiacus* was found. The deduced amino acid sequence of *mcr* showed 53% amino acid sequence similarity and 38% sequence identity with a second protein encoded by another open reading frame of the *Sulfolobus tokodaii* genome (accession number NP_377174) (Fig. 4A). We determined this open reading frame to encode aspartate-semialdehyde dehydrogenase (the product of the gene *asd*), because it is located on a gene cluster together with aspartokinase and threonine synthase (Fig. 4B), which is in line with its role in the biosynthesis of amino acids of the aspartate family (34).

S. tokodaii was cultivated under heterotrophic conditions, chromosomal DNA was isolated, and the *mcr* gene was amplified and cloned into the expression vector pTrc99A. However, *mcr*, which is proposed to encode malonyl-CoA reductase, could not be expressed in *E. coli* DH5α. Comparison of the codon usage of *E. coli* and *S. tokodaii* revealed large differences. Rare codons were found to accumulate at the 5′ end of the mRNA. Therefore, *E. coli* DH5α was replaced by strain Rosetta 2, which carries a high-copy-number plasmid with genes for rare tRNA species. Expression in this system was successful. Heat (15 min at 85°C)-treated extracts revealed an induced band in SDS-PAGE analysis at 45 kDa (Fig. 2B). The activity in cell extracts could not be determined due to heat precipitation of *E. coli* proteins at 65°C. The supernatant of

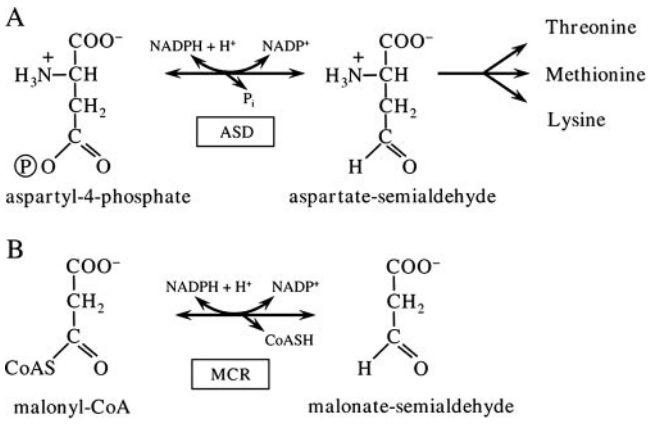

FIG. 3. Reactions catalyzed by aspartate-semialdehyde dehydrogenase (ASD) (A) and malonyl-CoA reductase (MCR) (B).

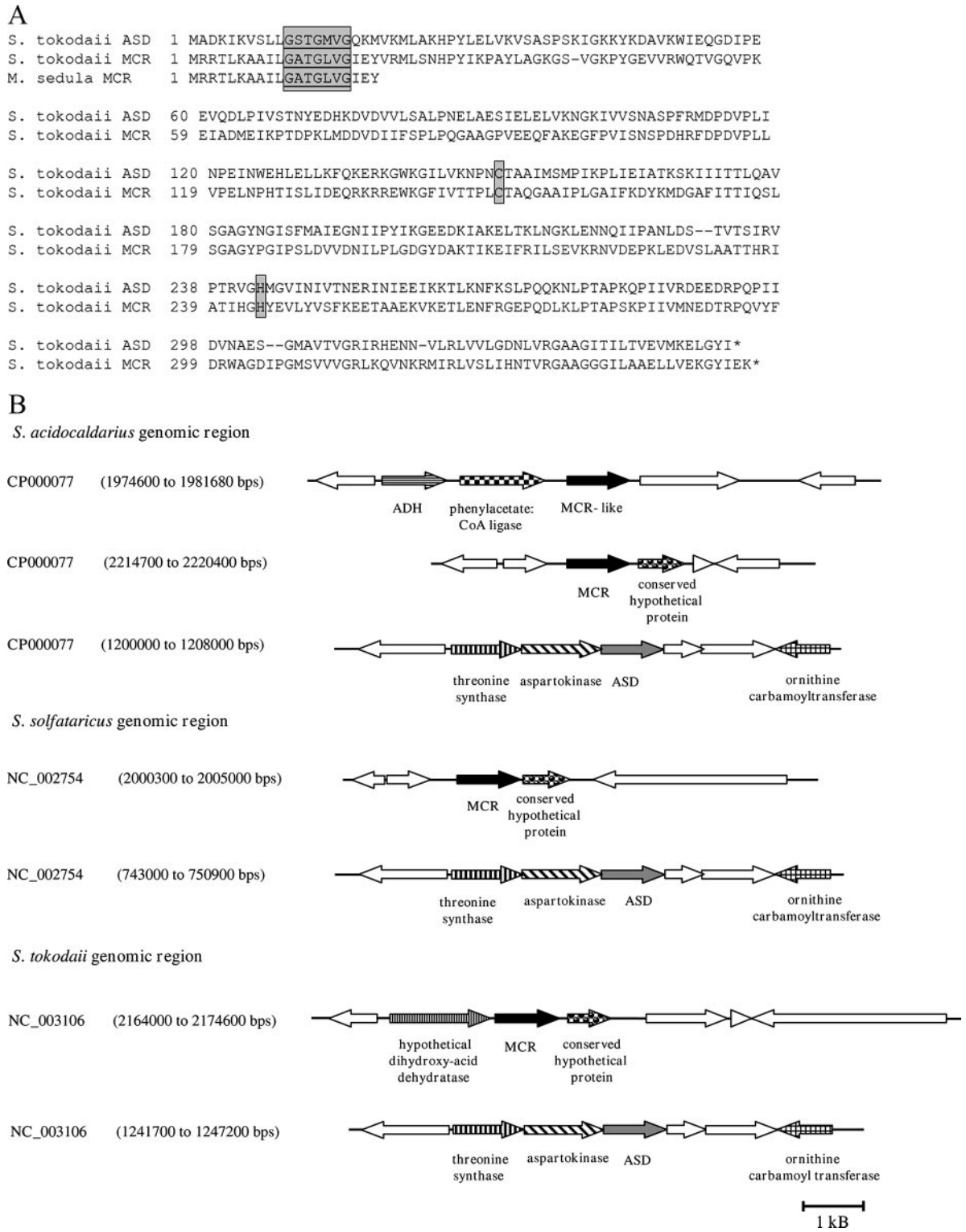

FIG. 4. (A) Alignment of the N-terminal amino acid sequence of malonyl-CoA reductase (MCR) from *M. sedula* and two hypothetical proteins from *S. tokodaii. S. tokodaii* ASD, hypothetical aspartate-semialdehyde dehydrogenase; *S. tokodaii* MCR, hypothetical malonyl-CoA reductase; *M. sedula* MCR, N-terminal amino acid sequence of purified malonyl-CoA reductase from *M. sedula*. The gray boxes indicate the NADPH binding motif (GxxGxxG) and the conserved cysteine and histidine residues. (B) Genetic environment of the hypothetical genes for aspartate-semialdehyde dehydrogenases (ASD) and malonyl-CoA reductase (MCR) from *S. acidocaldarius*, *S. solfataricus*, and *S. tokodaii*; ADH, alcohol dehydrogenase.

heat-precipitated extract, however, catalyzed the NADPH-dependent reduction of malonyl-CoA with a specific activity of 11 μmol min^{-1} mg^{-1} of protein (65°C). These results indicate that *mcr* from *S. tokodaii* (accession number NP_378167) encodes a functional malonyl-CoA reductase, which is similar to aspartate-semialdehyde dehydrogenase.

Purification of recombinant enzyme and molecular properties of *S. tokodaii* malonyl-CoA reductase. The recombinant enzyme was purified from 10 g of *E. coli* cells. Purification of the heterologously expressed enzyme required three steps: heat precipitation of most contaminating *E. coli* protein, gel filtration chromatography, and Resource phenyl chromatography. The yield was 1.6 mg of pure enzyme (recovery, 10%) (Table 1). The molecular mass calculated from the deduced amino acid sequence of the *mcr* gene is 39 kDa; SDS-PAGE analysis, however, revealed an apparent subunit molecular mass of 45 kDa (Fig. 2C). Aberrant migration in SDS-PAGE was also reported for aspartate-semialdehyde dehydrogenase from *Sulfolobus solfataricus* (11). Gel filtration chromatography showed a native molecular mass of 160 kDa, which would suggest a homotetrameric subunit composition (however, see below).

Catalytic properties of heterologously expressed and purified malonyl-CoA reductase from *S. tokodaii*. The specific activity of malonyl-CoA reductase at 65°C was 44 μmol mg^{-1} min^{-1} (Table 1), corresponding to a turnover rate of 28 s^{-1} per subunit. Assuming a similar specific activity for the *Metallosphaera* enzyme, it is estimated that malonyl-CoA reductase amounted to 1% of the soluble protein of *M. sedula* when the organism was grown autotrophically. The malonyl-CoA reductase catalyzed reaction showed a pH optimum of 7.2, with half-maximal activities at 8.0 and 6.0. The apparent K_m value for malonyl-CoA, determined at 0.5 mM NADPH, was 40 μM; the apparent K_m value for NADPH, determined at 0.2 mM malonyl-CoA, was 25 μM. NADH could not substitute for NADPH as hydride donor. Addition of the divalent metal ion Mg^{2+} or Mn^{2+} (5 mM) resulted in twofold-higher activity, and therefore Mg^{2+} was routinely included in the assay mixture. The stoichiometry of the reaction was determined. For each mole of malonyl-CoA added, 0.9 mol NADPH was oxidized, indicating that malonyl-CoA reductase from *S. tokodaii* catalyzes the reduction of malonyl-CoA to malonate-semialdehyde but not the further reduction to 3-hydroxypropionate. This was supported by the fact that trapping of the semialdehyde by addition of semicarbazide did not alter the observed stoichiometry. Succinyl-CoA was also reduced by the enzyme at a 10-fold-lower rate than malonyl-CoA. Malonyl-CoA reductase also catalyzed the oxidation of succinate semialdehyde with $NADP^+$ and CoA in the assay mixture at 25% of the rate for malonyl-CoA; no reduction of $NADP^+$ was observed when inorganic phosphate instead of CoA was added. These results indicate that malonyl-CoA reductase from *S. tokodaii* is a monofunctional enzyme, catalyzing the CoA-ester-specific reduction of malonyl-CoA with NADPH to malonate-semialdehyde. This is in contrast to the bifunctional malonyl-CoA reductase of *C. aurantiacus*, which also catalyzes the NADPH-dependent reduction of malonate-semialdehyde to 3-hydroxypropionate (21).

Inhibition of malonyl-CoA reductase of *S. tokodaii*. The reverse reaction of malonyl-CoA reductase represents a general example for the $NAD(P)^+$-dependent oxidation of an aldehyde to the corresponding CoA- or phosphate-activated carboxylic acid. Enzymes of this group (e.g., aspartate-semialdehyde dehydrogenase, glyceraldehyde-3-phosphate dehydrogenase, aldehyde dehydrogenase) generally form a thiol adduct of the aldehyde with a cysteine residue in the active site (3, 4, 29, 33). Testing was carried out to determine whether thiols affected malonyl-CoA reductase activity. In fact, dithioerythritol (5 mM) increased the rate twofold and therefore was routinely included in the assay mixture. Conversely, preincubation of the enzyme with the thiol-blocking agent iodoacetamide (0.1 mM) resulted in complete inactivation (50% inhibitory concentration = 4 μM). This suggests that the enzyme may use a cysteine residue in the catalytic mechanism.

Interaction of the malonyl-CoA reductase from *S. tokodaii* with RNA. The molar absorption coefficient at 280 nm, calculated based on the amino acid composition derived from the *mcr* gene encoding malonyl-CoA reductase from *S. tokodaii*, is 32.9 mM^{-1} cm^{-1}. Surprisingly, the UV-visible spectrum (250 to 800 nm) of the purified enzyme showed a single peak with an absorbance maximum at 260 nm rather than 280 nm (Fig. 5A) and a molar absorption coefficient at 260 nm of 550 mM^{-1} cm^{-1} per subunit of the enzyme. This indicated that the enzyme preparation, which appeared pure when tested by SDS-PAGE and protein staining (Fig. 2C), may contain nucleotides or nucleic acids. Heat precipitation (at 95°C for 1 h) or acid precipitation (with H_2SO_4 at a pH of 2 to 3) of the protein did not result in the release of nucleic acid or nucleotides from the enzyme, as judged from the lack of material in the supernatant absorbing at 260 nm (data not shown). However, nucleic acid could be extracted from the enzyme preparation by phenol-chloroform extraction. The obtained nucleic acid fraction was separated by agarose gel electrophoresis and resulted in a broad but distinct band at 120 ± 60 nucleotides (Fig. 5B). This nucleic acid material was identified as RNA because it could be digested by RNase rather than DNase (Fig. 5B). For each milligram of pure malonyl-CoA reductase, 0.5 mg RNA was isolated. A concentration of 560 μg RNA per mg of enzyme protein was determined based on the absorbance at 260 nm of the native enzyme. The contribution of the protein to the absorption at 260 nm was estimated to be <5% and was ignored. RNA that is 120 nucleotides long corresponds to a mass of approximately 36 kDa. The predicted molecular mass of the enzyme subunit was 39 kDa. Because the RNA-to-protein mass ratio was approximately 1:2, it follows that two enzyme subunits on average contained one bound RNA species. Native gel filtration of malonyl-CoA reductase indicated a molecular mass of 160 kDa. Because the enzyme contains bound RNA, this figure can only be taken to indicate that the native enzyme is at least a homodimer and possibly a homotetramer.

DISCUSSION

Properties and function of malonyl-CoA reductase, an aspartate-semialdehyde dehydrogenase paralogue in *Sulfolobaceae* (*Crenarchaeota*). We have purified and studied malonyl-CoA reductase from *M. sedula*. This enzyme activity was 10-fold up-regulated under autotrophic growth conditions, suggesting a role in the modified autotrophic 3-hydroxypropionate cycle in this and related organisms. A protein with an identical

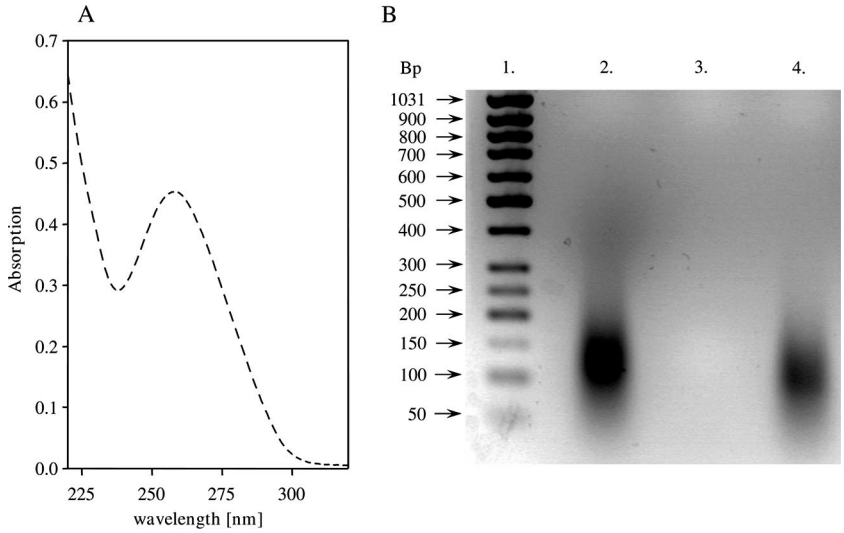

FIG. 5. (A) UV-visible spectrum of purified recombinant malonyl-CoA reductase (0.032 mg/ml). (B) Study of nucleic acid binding to malonyl-CoA reductase. Bound nucleic acid was extracted from protein by the phenol-chloroform extraction method. Samples were separated on a 1.2% agarose gel. Lanes: 1, 50-bp DNA ladder; 2, extracted nucleic acid from malonyl-CoA reductase, without treatment (1 μg); 3, extracted nucleic acid after treatment with RNase (1 μg); 4, extracted nucleic acid after treatment with DNase (1 μg).

N-terminal amino acid sequence was encoded by a gene present in *Sulfolobus tokodaii* (Fig. 4A), and closely related proteins are encoded by genes of other members of the *Sulfolobaceae* (Fig. 6). Surprisingly, malonyl-CoA reductase was homologous to aspartate-semialdehyde dehydrogenase, suggesting a common ancestor for these proteins (Fig. 6). Malonyl-CoA reductase, which consists of 39-kDa subunits, catalyzed the NADPH-specific reduction of malonyl-CoA but not the further reduction of the product malonate-semialdehyde to 3-hydroxypropionate. An NADPH-specific separate 3-hydroxypropionate dehydrogenase must exist, because cell extracts readily catalyzed the NADPH-dependent reduction of malonyl-CoA to 3-hydroxypropionate.

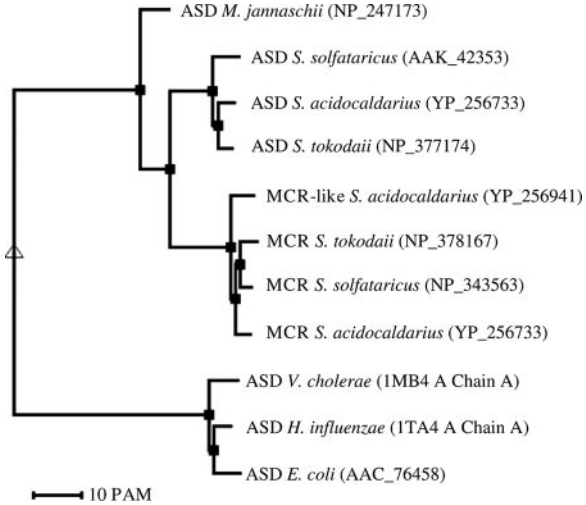

FIG. 6. Phylogenetic tree of homologs of malonyl-CoA reductase (MCR) and aspartate-semialdehyde dehydrogenases (ASD) based on amino acid sequences. The BLOSSUM 62 matrix was used. The PAM scale indicates the percentage of point accepted mutations. *V. cholerae*, *Vibrio cholerae*; *H. influenzae*, *Haemophilus influenzae*.

Comparison with *Chloroflexus* malonyl-CoA reductase. The archaeal malonyl-CoA reductase is not homologous to the bifunctional malonyl-CoA reductase from *Chloroflexus aurantiacus* (21). The *Chloroflexus* enzyme (α_2, 300 kDa) catalyzes both the reduction of the CoA-activated carboxylic acid and the reduction of the semialdehyde. Hence, the modified 3-hydroxypropionate cycle in the *Crenarchaeota* has recruited different enzymes/genes to bring about the same metabolic process. This points to a convergent evolution of a similar yet not identical autotrophic pathway. No similar malonyl-CoA reductase gene was found in *Eubacteria* or *Archaebacteria* outside of the *Sulfolobaceae*, indicating that the modified 3-hydroxypropionate cycle may be restricted to this crenarchaebacterial family.

Genetic surroundings and the problem of autotrophy in *Sulfolobaceae*. The genetic environment of the true aspartate-semialdehyde dehydrogenase gene in the three sequenced genomes of members of the *Sulfolobaceae* is very similar and supports the assigned function for the gene in amino acid metabolism (Fig. 4B): the gene clusters comprise threonine synthase, aspartokinase, and ornithine carbamoyltransferase, among other genes. The malonyl-CoA reductase gene is found next to a hypothetical gene with unknown function in all three *Sulfolobus* species (Fig. 4B). Other genes in the cluster in which the malonyl-CoA reductase gene is embedded are not conserved among different species. In *Sulfolobus acidocaldarius* a second malonyl-CoA reductase-like gene (38% amino acid identity to malonyl-CoA reductase of *S. acidocaldarius*), of which the genetic environment is completely different, is present (Fig. 4B). The malonyl-CoA reductase-like gene is found in a gene cluster which comprises, among others, CoA ligase and alcohol dehydrogenase genes. This suggests that this

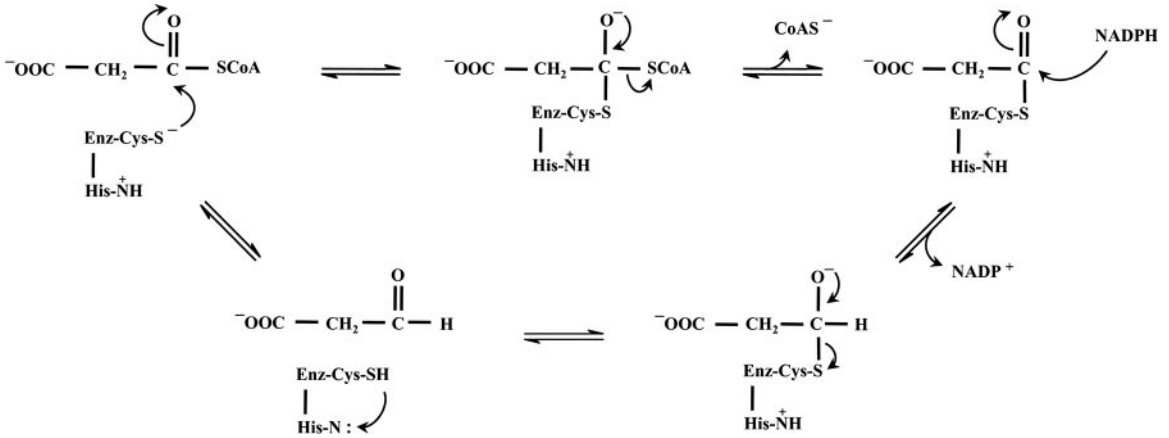

FIG. 7. Proposed reaction mechanism of malonyl-CoA reductase.

gene cluster codes for the activation and reduction of an organic acid other than malonate to its corresponding alcohol.

All of the *Sulfolobaceae* member genomes that have been sequenced contain the malonyl-CoA reductase gene as well as genes for acetyl-CoA carboxylase. However, some of these archaebacteria have not been reported to grow autotrophically; this capability remains to be demonstrated. Some of the members of the *Sulfolobaceae* were reported to have been initially enriched, isolated, and grown under autotrophic conditions. They may have lost this capability after continuous transfer in the laboratory on heterotrophic growth medium.

The reduction of malonate-semialdehyde to 3-hydroxypropionate by an NADPH-specific alcohol dehydrogenase and the further reductive conversion of 3-hydroxypropionate to propionyl-CoA, a three-step process, is at issue. We have preliminary indications that these reactions are catalyzed by individual catalytic entities rather than by multifunctional proteins, as reported for *C. aurantiacus* (2). It will be interesting to see which genes have been recruited for this purpose.

Proposed catalytic mechanism and comparison of malonyl-CoA reductase with aspartate-semialdehyde dehydrogenase. The stimulation of activity by thiols and the inactivation by thiol-blocking agents suggest an essential role for a cysteine residue in catalysis. A conserved cysteine-and-histidine residue was identified in aspartate-semialdehyde dehydrogenase and in malonyl-CoA reductase. The proposed catalytic mechanism, as shown in Fig. 7, is similar to that in other aldehyde dehydrogenases forming an activated carboxylic acid or catalyzing the reduction of an activated carboxylic acid to the aldehyde level. The role of Mg^{2+} seems not to be mandatory. EDTA only slightly inhibited enzyme activity. Whether Mg^{2+} is not accessible by EDTA or the enzyme is active in the absence of Mg^{2+} cannot be decided. Mg^{2+} may help to stabilize the C-O bond as Lewis acid, effectively making the C electropositive and thus setting it up for transfer of the hydride ion.

Malonyl-CoA reductase clustered with the archaeal branch of aspartate-semialdehyde dehydrogenases (Fig. 6). Structural features which distinguish the *Methanococcus jannaschii* enzyme from its bacterial counterpart are conserved for malonyl-CoA reductase based on sequence alignments (not shown); these features especially concern cofactor binding and include the following: an extended N-terminal surface loop resulting in an altered positioning of the cofactor $NADP^+$ and an exchange of Arg to Thr at position 3 in the $NADP^+$ consensus sequence GxTGxVG, which allows productive binding of NADH instead of NADPH for the *M. jannaschii* enzyme (12). This altered conformation for cofactor binding at the N terminus of the enzyme may allow the binding of RNA and/or CoA in the case of malonyl-CoA reductase. For aspartate-semialdehyde dehydrogenase from *S. solfataricus* it has been shown that RNA binds to the same region of the enzyme as NADPH (11). However, sequence comparison does not identify a structural feature responsible for the use of a CoA-activated acid substrate in the case of malonyl-CoA reductase versus a phosphate-activated acid substrate in the case of aspartate-semialdehyde dehydrogenase. A conserved Glu residue of aspartate-semialdehyde dehydrogenases (E210 for the *M. jannaschii* enzyme), which forms hydrogen bonds with the α-amino group of the aspartoyl moiety of the substrate, is replaced by Tyr in malonyl-CoA reductases.

RNA binding of malonyl-CoA reductase. Recombinant malonyl-CoA reductase from *S. tokodaii* binds small RNA. The RNA size varies from 60 to 180 nucleotides, which could be interpreted as a hint for nonspecific cleaving of RNA. RNA cleavage has been shown for other metabolic enzymes, e.g., glyceraldehyde-3-phosphate dehydrogenase, lactate dehydrogenase, phosphoglycerate kinase, glucose 6-phosphate dehydrogenase, and glutamate dehydrogenase; however, the physiological role is not known (8, 11) Testing to determine whether malonyl-CoA reductase cleaves RNA or RNA affects malonyl-CoA reductase activity, either positively or negatively, has not been done. Thus, an answer to the question of whether the RNA binding and possibly RNA cleavage by malonyl-CoA reductase plays a physiological role requires further detailed investigation and was out of the scope of this work.

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