3-Hydroxypropionyl-coenzyme A synthetase from *Metallosphaera sedula*, an enzyme involved in autotrophic CO$_2$ fixation

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A modified 3-hydroxypropionate cycle has been proposed as the autotrophic CO₂ fixation pathway for the thermoacidophilic crenarchaeon *Metallosphaera sedula*. The cycle requires the reductive conversion of 3-hydroxypropionate to propionyl-coenzyme A (CoA). The specific activity of the 3-hydroxypropionate-, CoA-, and MgATP-dependent oxidation of NADPH in autotrophically grown cells was 0.023 μmol min⁻¹ mg protein⁻¹. The reaction sequence is catalyzed by at least two enzymes. The first enzyme, 3-hydroxypropionyl-CoA synthetase, catalyzes the reaction: 3-hydroxypropionate + ATP + CoA → 3-hydroxypropionyl-CoA + AMP + PP. The enzyme was purified 95-fold to a specific activity of 18 μmol min⁻¹ mg protein⁻¹ from autotrophically grown *M. sedula* cells. An internal peptide sequence was determined and a gene encoding a homologous protein identified in the genome of *Sulfolobus tokodaii*; similar genes were found in *S. solfataricus* and *S. acidocaldarius*. The gene was heterologously expressed in *Escherichia coli* and the His-tagged protein purified. Both, the native enzyme from *M. sedula* and the recombinant enzyme from *S. tokodaii*, not only activated 3-hydroxypropionate to its CoA ester but also activated propionate, acrylate, acetate, and butyrate; however, with the exception of propionate, the affinities for these substrates were reduced. 3-Hydroxypropionyl-CoA synthetase is up-regulated 8-fold in autotrophically versus heterotrophically grown *M. sedula*, supporting its proposed role during CO₂ fixation in this archaeon and possibly other members of *Sulfolobaceae.*

**key words:** 3-hydroxypropionate cycle, autotrophic CO₂ fixation, 3-hydroxypropionate ligase, *Crenarchaeota, Metallosphaera*
INTRODUCTION

A new autotrophic pathway, the 3-hydroxypropionate cycle, has been described for the phototrophic green non sulfur bacterium *Chloroflexus aurantiacus* (9 – 12, 22, 23, Fig. 1). It represents the fourth known CO$_2$ fixation pathway; the others being the reductive pentose phosphate cycle (Calvin-Bassham-Benson cycle), the reductive citric acid cycle and the reductive acetyl-coenzyme A (acetyl-CoA) pathway. However, additional pathways for the assimilation of CO$_2$ may exist (14).

Autotrophic members of the *Sulfolobaceae* appear to use a modified 3-hydroxypropionate cycle for CO$_2$ fixation (6, 14, 17, 18, 21). The primary carboxylation steps are catalyzed by a bifunctional acetyl-CoA/propionyl-CoA carboxylase (7, 15).

Malonyl-CoA, the carboxylation product of acetyl-CoA, is reduced to malonate semialdehyde by malonyl-CoA reductase (2) and further to the characteristic intermediate of the pathway, 3-hydroxypropionate (21). For *C. aurantiacus* a single enzyme catalyzes both reduction steps (16, Fig. 1). However the bifunctional enzyme from *C. aurantiacus* does not exhibit any significant sequence similarity with malonyl-CoA reductase from *M. sedula*, indicating that the autotrophic pathway in *Chloroflexus* and *Sulfolobaceae* have evolved convergently. The further reductive conversion of 3-hydroxypropionate to propionyl-CoA requires three enzymatic steps: the activation of 3-hydroxypropionate to its CoA-ester, dehydration to acrylyl-CoA and reduction of acrylyl-CoA to propionyl-CoA. For *C. aurantiacus* a single trifunctional enzyme, called propionyl-CoA synthase, catalyzes these steps (1, Fig. 1). This reaction sequence has not been studied for *Sulfolobaceae* in detail, however, reductive conversion of 3-hydroxypropionate to propionyl-CoA was catalyzed by cell extracts of *M. sedula* (21). Propionyl-CoA is carboxylated to methylmalonyl-CoA by acetyl-CoA/propionyl-CoA carboxylase and the carbon skeleton is rearranged to form succinyl-CoA (14, 21). From here on out the autotrophic pathway for *Chloroflexus* and *Sulfolobaceae*
appear to differ. In the case of *C. aurantiacus*, succinyl-CoA is oxidatively converted to L-
malyl-CoA (Fig 1). L-Malyl-CoA lyase regenerates acetyl-CoA and releases glyoxylate as the
primary CO$_2$ fixation product (9). L-Malyl-CoA lyase activity was not detected in cell extract
of *M. sedula* (14). Therefore, the subsequent conversion of succinyl-CoA to acetyl-CoA and
the formation of the initial CO$_2$ fixation product in *Sulfolobaceae* are at issue.

In this work, the reductive conversion of 3-hydroxypropionate to propionyl-CoA for
*M. sedula* was studied and shown to involve at least two enzymes. This is in contrast to *C.
aurantiacus* where all three reaction steps are catalyzed by propionyl-CoA synthase (1). The
first enzyme of the reaction sequence, the 3-hydroxypropionyl-CoA synthetase, was purified
and studied. A homologous protein is encoded by the genomes of *Sulfolobus tokodaii*. The
corresponding gene was heterologously expressed in *E. coli* and the recombinant protein was
also catalyzed the CoA-, and MgATP-dependent activation of 3-hydroxypropionate.

**MATERIALS AND METHODS**

**Cell material.** *Metallosphaera sedula* TH2 (DSM 5348) was grown microaerobically
and autotrophically on minimal salt medium with a gas phase of CO$_2$-O$_2$-H$_2$ (19:3:78) at 75°C
and pH 2.0 (13). As a control, cells grown microaerobically and heterotrophically with 0.05 %
yeast extract were used. The cells were harvested by centrifugation at an OD$_{578}$ of 0.2 to 0.3
and stored in liquid nitrogen until used. *Sulfolobus tokodaii* (DSMZ 16993) was grown
aerobically under heterotrophic conditions and chromosomal DNA was isolated as described
previously (2).

**Enzyme Assay.** *Reduction of 3-hydroxypropionate to propionyl-CoA.* ATP-, CoA-,
and NADPH-dependent reduction of 3-hydroxypropionate was measured
spectrophotometrically at 65°C using cell extracts. The standard reaction mixture (0.5 ml)
contained 100 mM Tris/HCl (pH 7.8), 2 mM MgCl$_2$, 6 mM ATP, 0.5 mM CoA, 0.5 mM NADPH, 2 mM dithioerythritol (DTE), and 0.5 to 1.7 mg cell extract protein. The reaction was started by the addition of 2 mM 3-hydroxypropionate and followed at 365 nm ($\varepsilon_{\text{NADPH}} = 3,400 \text{ M}^{-1} \text{ cm}^{-1}$).

3-Hydroxypropionyl-CoA synthetase. Two spectrophotometric assays were used to measure 3-hydroxypropionyl-CoA synthetase activity. (a) A discontinuous assay was used to measure the ATP-, 3-hydroxypropionate-dependent disappearance of free CoA at 65 °C. The standard reaction mixture (0.9 ml) contained 100 mM Tris/HCl (pH 8.4), 2 mM MgCl$_2$, 3 mM ATP, 0.15 mM CoA, and protein. After 0 and 1 minute at 65 °C, 200 µl of the test mixture was removed and diluted in 200 µl 200 mM N-(2-hydroxyethyl)-piperazine-N’-2-ethanesulfonic acid (HEPES)/NaOH (pH 7.2), 0.5 mM 5,5’-dithiobis(2-nitrobenzoic acid) (DTNB, from 10 mM stock solution in 0.5 M potassium phosphate (pH 7.2), 1 mM EDTA) at 0°C. To the remaining test mixture 2 mM 3-hydroxypropionate was added to start the reaction. Samples (200 µl each) were taken after an additional 1 and 2 minutes and diluted as described above. Absorbance at 412 nm was measured to determine the amount of free CoA used during the reaction ($\varepsilon_{412\text{nm}} = 13,600 \text{ M}^{-1} \text{ cm}^{-1}$). (b) For the purified enzyme the formation of AMP was measured using a coupled assay at 45°C. The reaction mixture (0.5 ml) contained 100 mM Tris/HCl (pH 8.5), 2 mM DTE, 2 mM MgCl$_2$, 3 mM ATP, 0.1 to 0.5 mM CoA, 3 mM potassium phosphoenolpyruvate (PEP), 0.3 units of myokinase, 0.1 units pyruvate kinase, 0.3 units of lactate dehydrogenase, 0.5 mM NADH, and to 4 to 11 µg purified 3-hydroxypropionate synthetase. The reaction was started by the addition of 3 mM 3-hydroxypropionate and followed at 365 nm ($\varepsilon_{\text{NADH}} = 3,400 \text{ M}^{-1} \text{ cm}^{-1}$). For the purified recombinant enzyme the coupled assay was modified. The reaction mixture (0.5 ml) contained 200 mM morpholinepropanesulfonic acid (MOPS)/NaOH (pH 7.2), 2 mM DTE, 10 mM MgCl$_2$, 9 mM ATP, 0.1 mM CoA, 9 mM PEP, 3.7 unit of myokinase, 3.2 unit pyruvate...
kinase, 1.8 unit of lactate dehydrogenase, 0.5 mM NADH, and protein. The reaction was
started by the addition of 2 mM 3-hydroxypropionate and followed at 365 nm.

*Malate dehydrogenase.* The oxaloacetate-dependent oxidation of NADH was measured
spectrophotometrically at 65°C. The reaction mixture (0.5 ml) contained 100 mM Tris/HCl
(pH 7.8), 2 mM MgCl₂, 0.5 mM NADH, and protein. The reaction was started by the addition
of 5 mM oxaloacetate and followed at 365 nm (ε₅₄₀ = 3,400 M⁻¹ cm⁻¹).

Protein concentrations were determined by the method of Bradford using bovine serum
albumin as standard (4).

**Purification of 3-hydroxypropionyl-CoA synthetase from M. sedula.** All
procedures were done aerobically at 4°C. *(i) Preparation of cell extract* – Thawed cell paste
(10 g wet weight) of autotrophically grown cells was resuspended in 10 – 15 ml of 100 mM
Tris/HCl (pH 8.5), 4 mM MgCl₂ containing 0.2 mg DNase I and passed twice through a
chilled French pressure cell at 138 kPa. The cell lysate was centrifuged at 20,000 × g for 10
min, and the supernatant was recentrifuged at 100,000 × g for 1 h. *(ii) Ammonium Sulfate
Precipitation* – To the cell extract saturated ammonium sulfate solution was added to a final
saturation of 30% (NH₄)₂SO₄ and centrifuged at 100,000 × g for 1 h. *(iii) Phenyl-Sepharose
Chromatography* – The supernatant from step ii was loaded onto a 20-ml Phenyl-Sepharose
column (Amersham Bioscience, Inc.) equilibrated with 50 mM Tris/HCl (pH 7.9), 1 mM
DTE, 5 mM MgCl₂, 1 M (NH₄)₂SO₄. After a 20 ml wash with equilibration buffer, the
column was developed with a 250-ml linear gradient at 1 ml min⁻¹ from 100 % equilibration
buffer to 100 % 2 mM Tris/HCl (pH 7.9). The peak of activity eluted between 20 and 0 mM
ammonium sulfate, and to the pooled active fractions DTE was added to a final concentration
of 2 mM. *(iv) MonoQ Chromatography* – The enzyme solution from step iii was applied onto
an 8-ml MonoQ HR 10/10 anion-exchange column (Amersham Bioscience, Inc.) equilibrated
with buffer A (20 mM potassium morpholinopropanesulfonic acid (MOPS/KOH), pH 7.2).
The column was washed with 15-ml buffer A and developed with a 100-ml linear gradient of
0 – 1 M KCl in buffer A at 0.8 ml min\(^{-1}\). Activity eluted between 50 and 120 mM KCl. (v)

**Reactive Green Chromatography** – Active pooled fractions from step iv were applied onto a 10-ml Reactive Green 19 column (Sigma-Aldrich) equilibrated with buffer A. The column was washed with 25 ml buffer A containing 250 mM KCl and developed with a 100-ml increasing linear gradient of 0.25 – 1 M KCl in buffer A at 0.5 ml min\(^{-1}\). The enzyme eluted between 300 and 500 mM KCl. The combined active fractions were concentrated in dialysis tubing (cutoff, 3.5 kDa) embedded in dry polyethylene glycol 20,000 and dialyzed against 2 liters of 20 mM Tris/HCL (pH 8.1), 5 mM MgCl\(_2\). (vi) **Gel Filtration Chromatography** – Aliquots of 1 ml from step v were applied onto a 120-ml Superdex 200 HiLoad 16/60 column (Amersham Biosciences) equilibrated with buffer B (20 mM MOPS/KOH (pH 7.2), 150 mM KCl). The column was developed at a flow rate of 0.4 ml min\(^{-1}\). Active fractions were pooled, concentrated as in step v, and dialyzed against 1 liter of buffer B. DTE was added to a final concentration of 1 mM and the enzyme was stored at -20°C.

**Heterologous expression of the lig gene from S. tokodaii and production of the enzyme in E. coli.** The gene encoding 3-hydroxypropionyl-CoA synthetase was amplified by PCR from S. tokodaii chromosomal DNA by using the primer combinations ligNdeI_for 5’-CACGAGTCATATGACTGAAAAACTTCT-3’ / ligKpnI1_rev 5’-A\(_{\text{TAGGTACC}}\)CATCTTCATCAATCATTG-3’ and lig2KpnI_for 5’-A\(_{\text{TAGGTACC}}\)TATGGGTAATGGGTAGA-3’ / lig2BamHI_rev 5’-GG\(_{\text{AGGATCC}}\)TTAGAAAATCATTGG-3’ introducing a NdeI site (underlined) at the initiation codon, a KpnI site (underlined) in the middle of the gene (resulting in an amino acid change I518L in the protein), and a BamHI site (underlined) after the stop codon. The PCR products were isolated and cloned separately into pUC19 or pCRT7/CT TOPO resulting in plasmids pAR1 and pAR2. Ligation of the KpnI/BamHI fragment of pAR2 into the likewise restricted pAR1 resulted in plasmid pAR3. The sequence of the insert was determined to ensure that no errors (beside the purposely introduced changes) had occurred. pAR3 was
digested with NdeI and BamHI, and the fragment containing the lig gene was ligated into pET16b, resulting in plasmid pAR5. Introduction of the NdeI/BamHI fragment into the expression vector pET16b results in the expression of an extended 5’ coding region, resulting in a N-terminal deca-His-tag for the recombinant protein. Competent E. coli Rosetta 2 (DE3) cells were transformed with pAR5, grown in a 12-liter fermenter at 37 °C in Luria-Bertani broth containing 100 µg ml⁻¹ of ampicillin and 34 µg ml⁻¹ chloramphenicol, and induced at an optical density of 0.5 with 0.4 mM isopropyl thiogalactopyranoside (IPTG). After additional growth for 6.5 h at 30 °C, the cells (22 g wet weight) were harvested and stored in liquid nitrogen until use.

**Purification of heterologously produced 3-hydroxypropionyl-CoA synthetase from S. tokodaii.**

1. **Preparation of cell extract** – Thawed cell paste (11 g wet weight) was resuspended in 11 ml of 100 mM Tris/HCl (pH 8.0), 5 mM MgCl₂ containing 0.2 mg DNase I and passed twice through a chilled French pressure cell at 138 kPa. The cell lysate was centrifuged at 20,000 x g for 15 min.
2. **Heat precipitation** – Cell extract of E. coli was incubated at 85°C for 15 min, cooled on ice for 15 min, and centrifuged at 100,000 x g at 4°C for 60 min.
3. **Nickel-affinity chromatography** – 12 ml of heat precipitated cell extract was applied onto a Ni²⁺-chelating sepharose affinity column (Amersham Biosciences, Freiburg, Germany) equilibrated with 20 mM Tris/HCl (pH 7.9), 250 mM KCl (buffer C). The column was washed with buffer C containing 200 mM imidazole and developed with a 75-ml increasing linear gradient of 0.2 – 0.5 M imidazole in buffer A at 1.0 ml min⁻¹. Active fractions were pooled, washed with 20 mM Tris/HCl (pH 8.5), and concentrated to a final volume of 7 ml by ultrafiltration (Amicon YM 10 membrane; Millipore, Bedford, MA). The protein (18 mg) was stored at -20°C with 30 % glycerol.

**Characterization of the enzymes.** The apparent $K_m$ values of ATP, 3-hydroxypropionate, propionate, and acrylate for 3-hydroxypropionyl-CoA synthetase were determined using the coupled assay (b). The concentration of one substrate was varied, while keeping the
concentration of the other substrates constant. The apparent $K_m$ value for ATP was also determined using the discontinuous assay (a). Both methods agreed well. The thermostability of the enzyme was determined by incubating the enzyme at 70, 80, 90, and 100°C for 15 minutes. The enzyme solution was cooled to 4°C before determining the activities relative to those of samples kept on ice throughout the experiment. The specificity of the ATP- and CoA-dependent activation of 3-hydroxypropionate was determined by substituting 3-hydroxypropionate in the standard coupled assay (b) by propionate, acrylate, acetate, butyrate, $(\pm)3$-hydroxybutyrate, crotonate, glycolate, malonate, mesaconate, methylsuccinate, glycine, glycolate, succinate, $\beta$-alanine, 3-mercaptopropionate or 3-chloropropionate. The specificity for ATP was determined by substituting ATP in the standard discontinuous assay (a) by 3 mM ADP, GTP, ITP, UTP or CTP. Pyrophosphate was determined by a colorimetric method. Inorganic pyrophosphate is hydrolyzed by inorganic pyrophosphatase (Sigma-Aldrich) to orthophosphate, which was quantified by the ammonium molybdate reagent (3). A sample blank without pyrophosphatase served as control.

**Molecular mass determination.** SDS-PAGE (10 %) was performed as described (20). The native molecular mass was determined on a 300-ml Superdex 200 column (Amersham Biosciences) gel filtration column calibrated with ovalbumin (43 kDa), bovine serum albumin (67 and 134 kDa), aldolase (158 kDa), and ferritin (440 kDa).

**Peptide sequence.** An aliquot (20 $\mu$g) of the protein solution of the last purification step was precipitated with 8 % trichloroacetic acid, separated by 9 % SDS-PAGE and transferred to an Immobilon-P$^{\text{TM}}$ transfer membrane (Millipore, Bedford, MA). Endoproteinase Lys-C digestion, HPLC separation of peptides and N-terminal sequencing of one peptide was performed by TopLab (Martinsried, Germany).

**Materials.** Chemicals were obtained from Fluka (Neu-Ulm, Germany), Sigma-Aldrich (Deisenhofen, Germany), VWR (Darmstadt, Germany), Genaxxon Biosciences (Biberach, Germany), MBI Fermentas (St. Leon-Rot, Germany), Novagen (Schwalbach, Germany),
MWG Biotech (Ebersberg, Germany) or Roth (Karlsruhe, Germany). 3-Hydroxypropionate was obtained by alkaline hydrolysis of 3-hydroxypropionitrile as previously described (10).

RESULTS AND DISCUSSION

Reductive conversion of 3-hydroxypropionate by cell extracts. Extracts of autotrophically grown cells of M. sedula catalyzed the 3-hydroxypropionate-dependent oxidation of NADPH, provided that Mg$^{2+}$, ATP, and CoA were added. The specific activity at 65 °C was 23 nmol min$^{-1}$ mg$^{-1}$ protein; this activity was 3-fold down-regulated in heterotrophically grown cells (Table 1). Malate dehydrogenase activity was used as a control and its activity was about twice as high in heterotrophically as compared to autotrophically grown cells. These results indicate that the reductive conversion of 3-hydroxypropionate is involved in autotrophic CO$_2$ fixation by M. sedula.

Search for enzyme activating 3-hydroxypropionate. The reaction catalyzed by cell extracts forms propionyl-CoA (21). This requires first the activation of 3-hydroxypropionate to its CoA thioester. A discontinuous assay was developed, which measured the 3-hydroxypropionate-, ATP-, and Mg$^{2+}$- dependent consumption of CoA in the presence of cell extract. The specific activity at 65 °C in cell extracts was 91 nmol min mg$^{-1}$ protein; this activity was 8-fold down-regulated in heterotrophically grown cells (Table 1). In a first chromatographic step, protein was separated on a Phenyl-Sepharose column. 3-Hydroxypropionate- and Mg$^{2+}$/ATP-dependent CoA consuming activity eluted between 0 to 20 mM ammonium sulfate. These pooled active fractions, however, were no longer able to catalyze the (entire) reductive conversion of 3-hydroxypropionate to propionyl-CoA, but required the addition of protein fractions eluting from the same column at 400 mM ammonium sulfate. These results indicate that in contrast to C. aurantiacus (1), the reductive
conversion of 3-hydroxypropionate to propionyl-CoA for *M. sedula* requires at least two enzymes, which could be separated by hydrophobic interaction chromatography.

**Purification of the 3-hydroxypropionate-activating enzyme.** The enzyme catalyzing the activation of 3-hydroxypropionate to its CoA ester was up-regulated in autotrophically *versus* heterotrophically grown cells (Table 1), indicating a function of the enzyme in autotrophic CO$_2$ fixation. The enzyme was purified 95-fold from 10 g (wet weight) of autotrophically grown cells (Table 2) to apparent homogeneity as indicated by a single protein band after electrophoresis in a denaturing gel (Fig. 2). The enzyme is tentatively referred to as 3-hydroxypropionyl-CoA synthetase.

**Molecular properties of 3-hydroxypropionate-CoA synthetase.** Gel filtration chromatography of the native enzyme gave an estimated molecular mass of 340 kDa. SDS/PAGE produced one protein band of 78 kDa (Fig. 2). 3-Hydroxypropionyl-CoA synthetase, therefore, exists as a homotetramer. The enzyme was stable when incubated for 15 min at temperature up to 80°C and during storage at -20°C for several weeks. After incubation for 15 min at 100°C, 50 % of the activity remained relative to that of a sample kept at 4°C throughout the experiment. Acyl-CoA synthetases are usually described as monomers or homodimers composed of 70 to 75 kDa subunits. A homotetrameric structure therefore is unusual and may relate to the thermostability of the enzyme (5).

**Stoichiometry and products of the reaction.** HPLC analysis showed that 3-hydroxypropionyl-CoA was formed (data not shown). To determine whether AMP or ADP was formed, a coupled spectrophotometric assay was used. The stoichiometry of NADH oxidation per mole of 3-hydroxypropionate added was 1:1.8. This shows that ATP was hydrolyzed to AMP. Pyrophosphate was the second product of the reaction: the amount of pyrophosphate formed was determined in an assay containing 0.4 mM coenzyme A (80 % pure) and 2 mM MgATP; 0.3 mM pyrophosphate (0.6 mM phosphate was determined after addition of pyrophosphatase) was formed after equilibrium of the reaction was reached. This
indicates that pyrophosphate and AMP were the products of the ligase reaction. Hence, 3-
hydroxypropionyl-CoA synthetase catalyzes the following reaction:

\[
3\text{-hydroxypropionate} + \text{ATP} + \text{CoA} \rightarrow 3\text{-hydroxypropionyl-CoA} + \text{AMP} + \text{PP}_i
\]

**Catalytic properties of 3-hydroxypropionyl-CoA synthetase.** The dependency of
the 3-hydroxypropionyl-CoA synthetase towards CoA did not follow Michaelis-Menten
kinetics (Fig. 3). Maximal activity was obtained with 100 µM CoA, which was chosen to
determine the apparent \( K_m \) values for other substrates. The enzyme had a high affinity for
ATP and 3-hydroxypropionate with apparent \( K_m \) values of 45 µM for ATP and 180 µM for 3-
hydroxypropionate. ATP (100 %) could be substituted by UTP (32 %) and CTP (13 %). 3-
Hydroxypropionyl-CoA synthetase can also use propionate, acrylate, acetate, and butyrate as
substrates (Table 3). At saturating substrate concentrations the turnover of propionate and
acrylate was similar to the turnover for 3-hydroxypropionate (Table 4). The specificity
\( (k_{cat}/K_m) \) for the enzyme towards 3-hydroxypropionate and propionate were similar, however,
was decreased by about 10-fold for acrylate (Table 4). We assume that the lower activities
determined for the other substrates at 4 mM (non-saturating) concentrations (Table 3) is due
to decreased affinities of 3-hydroxypropionyl-CoA synthetase towards these substrates rather
than a lower turnover number. The enzyme, therefore, is specific for 3-hydroxypropionate
and propionate.

**Identification of a homologous protein for Sulfolobus tokodaii.** An internal peptide
sequence of the purified enzyme from *M. sedula* was determined as VVITADAYPRRGK. A
search of protein databases revealed a match, with only one amino acid residue exchange (Y
for P), to an internal sequence of a 659 amino acid long (74 kDa) hypothetical acetyl-CoA
synthetase from the closely related crenarchaeum *S. tokodaii* strain 7 (accession number
NP_376686, ref. 19). The gene encoding this protein was amplified, cloned into the
expression vector pET16b, and heterologously expressed in *E. coli* Rosetta (DE3) strain,
which carries a plasmid with genes for rare tRNA species. Cell extract of this *E. coli* strain
catalyzed the 3-hydroxypropionate-dependent oxidation of NADPH in the presence of Mg-
ATP and CoA with a specific activity of 0.2 μmol min⁻¹ mg⁻¹ of protein (65 °C). His-tagged
3-hydroxypropionyl-CoA synthetase was readily purified 34-fold by heat precipitation of cell
extract at 85 °C and Ni²⁺-affinity chromatography with almost 60% recovery. The catalytic
properties of the purified recombinant enzyme from *S. tokodaii* are summarized in Table 3
and compared to the properties of native enzyme from *M. sedula*. With the exception of the
determined subunit composition, both enzymes are almost indistinguishable. It is possible
that the presence of the deca-His-tag may prevent the recombinant enzyme from *S. tokodaii* to
form a homotetramer and might also explain the somewhat lower turnover number compared
to the enzyme from *M. sedula*.

**3-Hydroxypropionyl-CoA synthetases from Sulfolobaceae.** Recently the genome
sequence for *M. sedula* has been completed by the Joint Genome Institute and deposited at the
NCBI databank (accession number CP000682). Based on the internal peptide sequence
determined for purified 3-hydroxypropionyl-CoA synthetase, a gene was identified in the
genome of *M. sedula*, which encodes for a protein with an internal sequence matching the
determined peptide sequence by 100%. The protein (accession number YP_001191537) has
a calculated molecular mass of 74 kDa and has 76% sequence identity to the enzyme from *S.
tokodaii*, which was heterologously produced in *E. coli* in this work. A similar protein is also
encoded by the genome of *S. solfataricus* (NP_344510, 73% sequence identity) and *S.
acidocaldarius* (YP_255824, 69% sequence identity). It is assumed that all four proteins are
3-hydroxypropionyl-CoA synthetases. The genomic contexts of the genes encoding these 3-
hydroxypropionyl-CoA synthetases, however, are different in each organism and none appear
to be cotranscribed with other genes. A putative acetyl-CoA synthetase from *Thermofilum
pendens* (YP_920297) represents the next best hit to the *M. sedula* enzyme with an overall
sequence identity of 51%. The 3-hydroxypropionyl-CoA synthetase domain of the
propionyl-CoA synthase of *C. aurantiacus* constitutes only the forth best hit among possible
acyl-CoA synthetases in *C. aurantiacus* when compared to the enzyme from *M. sedula*. The broad substrate specificity of 3-hydroxypropionyl-CoA synthetase (and acyl-CoA synthetases in general) supports the idea, that only small changes in the active site of these enzymes are necessary to increase catalytic efficiency of one over another substrate. Therefore, it cannot be ruled out that other 3-hydroxypropionate-specific acyl-CoA synthetases are present in the database, which may play a role in a (modified) 3-hydroxypropionate cycle for other organisms.

**Modified 3-hydroxypropionate cycle in *Cenarchaeum symbiosum***? Yet another possibility may be realized for the marine chemolithoautotrophic sponge symbiont *C. symbiosum*. Hallam *et al.* recently proposed that the organism uses the modified 3-hydroxypropionate cycle for autotrophic CO$_2$ fixation based on genomic analyses (8). The genome, however, does not appear to encode for any homologs of the here described 3-hydroxypropionyl-CoA synthetase. Instead genes for ADP-forming acyl-CoA synthetases (COG1042) are found, which show no significant sequence similarity to the AMP-forming enzymes (COG0318). In *C. symbiosum* 3-hydroxypropionate may be activated to its CoA-ester by means of an ADP-forming enzyme. The here described 3-hydroxypropioyl-CoA synthetase is the third enzyme of the modified 3-hydroxypropionate cycle for which the corresponding genes have been identified in *M. sedula*; the other two being acetyl-CoA/propionyl-CoA carboxylase and malonyl-CoA reductase (2, 15). Genes encoding the subunits of a biotin-dependent acyl-CoA carboxylase are present in the genome of *C. symbiosum* and are likely to encode for acetyl-CoA/propionyl-CoA carboxylase. However, the homolog of malonyl-CoA reductase encoded by the *C. symbiosum* genome is probably an aspartate semialdehyde dehydrogenase involved in threonine biosynthesis. Although the modified 3-hydroxypropionate cycle for autotrophic CO$_2$ fixation by *C. symbiosum* cannot be ruled out, different enzymes may have been recruited to catalyze individual steps during the process or parts of the pathway may be quite different.
In summary, the rate of reductive conversion of 3-hydroxypropionate to propionyl-CoA was up-regulated in autotrophically- versus heterotrophically-grown cells of *M. sedula* and the reaction sequence is catalyzed by at least two enzymes. In a first step, 3-hydroxypropionate is activated to its CoA-ester. The gene encoding the 3-hydroxypropionyl-CoA synthetase has been identified in *M. sedula* and *S. tokodaii*. The gene(s) encoding the enzyme(s) responsible for dehydration of 3-hydroxypropionyl-CoA (enoyl-CoA hydratase) and further reduction of acrylyl-CoA to propionyl-CoA (enoyl-CoA reductase) could not be identified based on genomic analyses: (i) there are several candidates present in the genome of *M. sedula*, which could encode proteins able to catalyze either reactions; a gene encoding a possible fusion protein, however, was not found; (ii) genes encoding the enzymes catalyzing the consecutive steps in the conversion of 3-hydroxypropionate to propionyl-CoA appear not to be cotranscribed. In the genomic surrounding of the identified 3-hydroxypropionyl-CoA synthetase, catalyzing the first step of the reaction sequence, genes possibly encoding enzymes catalyzing the second or third steps are absent. Therefore, in order to identify the enzyme(s) responsible for the reductive conversion of 3-hydroxypropionyl-CoA to propionyl-CoA during autotrophic growth of *M. sedula*, it will be necessary to purify the protein(s). The recombinant 3-hydroxypropionyl-CoA synthetase from *S. tokodaii* not only activates 3-hydroxypropionate but also acrylate to its CoA ester and, therefore, can be used to assay the reduction of acrylyl-CoA to propionyl-CoA independently from the dehydration reaction using a coupled assay.

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plasmid constructs, R. Teufel for determination of pyrophosphate, M. Kies of TopLab, München, for peptide sequence analysis, and G. Igloi, Freiburg, for DNA sequencing. We especially like to thank N. Gad’on, Freiburg, for his expertise in growing cells. We also thank I. A. Berg for very helpful discussions and critical review of the manuscript.
REFERENCES


Table 1. Regulation of some enzymes possibly relevant for autotrophic CO₂ fixation by *Metallosphaera sedula*, as determined by activity measurements in cell extracts at 65 °C.

<table>
<thead>
<tr>
<th>Growth conditions</th>
<th>3-Hydroxypropionate reduction</th>
<th>3-Hydroxypropionyl-CoA synthetase</th>
<th>Malate dehydrogenase (control)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>nmol min⁻¹ mg⁻¹</td>
<td></td>
</tr>
<tr>
<td>autotrophic</td>
<td>23</td>
<td>91</td>
<td>1400</td>
</tr>
<tr>
<td>heterotrophic</td>
<td>8.5</td>
<td>12</td>
<td>3200</td>
</tr>
</tbody>
</table>
Table 2. Purification of 3-hydroxypropionyl-CoA synthetase from 10 g (wet cell mass) of autotrophically grown *Metallosphaera sedula*.

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Volume $ml$</th>
<th>Total Activity $\mu mol min^{-1}$</th>
<th>Total Protein $mg$</th>
<th>Specific Activity $\mu mol min^{-1}mg^{-1}$</th>
<th>Recovery %</th>
<th>Purification $-fold$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell Extract</td>
<td>31</td>
<td>150</td>
<td>810</td>
<td>0.19</td>
<td>(100)</td>
<td>(1)</td>
</tr>
<tr>
<td>30% (NH$_4$)$_2$SO$_4$</td>
<td>25</td>
<td>108</td>
<td>550</td>
<td>0.20</td>
<td>72</td>
<td>1.1</td>
</tr>
<tr>
<td>Phenyl-Sepharose</td>
<td>35</td>
<td>60</td>
<td>160</td>
<td>0.38</td>
<td>40</td>
<td>2.0</td>
</tr>
<tr>
<td>MonoQ</td>
<td>12</td>
<td>42</td>
<td>37</td>
<td>1.1</td>
<td>28</td>
<td>5.8</td>
</tr>
<tr>
<td>Reactive Green</td>
<td>1.5</td>
<td>31</td>
<td>4.7</td>
<td>6.6</td>
<td>21</td>
<td>35</td>
</tr>
<tr>
<td>Gel Filtration</td>
<td>1.1</td>
<td>16</td>
<td>0.9</td>
<td>18</td>
<td>11</td>
<td>95</td>
</tr>
</tbody>
</table>

*Activity was determined at 65 °C using the discontinuous assay.*
Table 3. Molecular and catalytic properties of 3-hydroxypropionyl-CoA synthetase from *M. sedula* and the recombinant enzyme from *S. tokodaii*.

<table>
<thead>
<tr>
<th>Property</th>
<th><em>M. sedula</em></th>
<th><em>S. tokodaii</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Substrates</td>
<td>3-hydroxypropionate, CoA, ATP</td>
<td></td>
</tr>
<tr>
<td>Products</td>
<td>3-hydroxypropionyl-CoA, AMP, PPi</td>
<td></td>
</tr>
<tr>
<td>Specific activity</td>
<td>18 μmol min⁻¹ mg⁻¹&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6.7 μmol min⁻¹ mg⁻¹&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Apparent $K_m$ values</td>
<td>3-hydroxypropionate: 180 μM</td>
<td>190 μM</td>
</tr>
<tr>
<td></td>
<td>ATP: 45 μM</td>
<td>110 μM</td>
</tr>
<tr>
<td></td>
<td>CoA: n.a&lt;sup&gt;b&lt;/sup&gt;</td>
<td>CoA: n.a&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Native molecular mass</td>
<td>340 kDa</td>
<td>140 kDa</td>
</tr>
<tr>
<td>Subunit molecular mass</td>
<td>78 kDa</td>
<td>74 kDa</td>
</tr>
<tr>
<td>Suggested composition</td>
<td>$\alpha_4$</td>
<td>$\alpha_2$</td>
</tr>
<tr>
<td>Turnover number</td>
<td>23 s⁻¹ (per subunit)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>8 s⁻¹ (per subunit)&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Specificity&lt;sup&gt;c&lt;/sup&gt;</td>
<td>ATP 100%, UTP 32%, CTP 13%, ADP 5%, ITP 3%, GTP &lt; 3%</td>
<td>not determined</td>
</tr>
<tr>
<td></td>
<td>3-hydroxypropionate 100%</td>
<td>3-hydroxypropionate 100%</td>
</tr>
<tr>
<td></td>
<td>propionate 103%, acrylate 77%</td>
<td>propionate 98%, acrylate 96%</td>
</tr>
<tr>
<td></td>
<td>acetate 42%, butyrate 20%</td>
<td>acetate 65%, butyrate 27%</td>
</tr>
<tr>
<td></td>
<td>3-hydroxybutyrate, crotonate, glycerate, malonate &lt; 1%</td>
<td>3-hydroxybutyrate, crotonate, glycerate, malonate, β-alanin, glycine, succinate, mesaconate, methylsuccinate &lt; 1%</td>
</tr>
</tbody>
</table>

<sup>a</sup> activities were determined using the discontinuous enzyme assay at 65 °C.

<sup>b</sup> n.a. non applicable; CoA dependency does not follow Michaelis-Menten kinetics

<sup>c</sup> the final concentration of nucleotides was 3 mM each, the final concentration of other substrates was 4 mM each
Table 4. Substrate specificity of 3-hydroxypropionyl-CoA synthetase from *Metallosphaera sedula*

<table>
<thead>
<tr>
<th>Substrate</th>
<th>apparent <em>K_m</em> (mM)</th>
<th><em>k_cat</em> (s⁻¹)</th>
<th>apparent <em>k_cat</em>K_m* (× 10⁻⁴ M⁻¹ s⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3-hydroxypropionate</td>
<td>0.18 ± 0.03</td>
<td>5.7 ± 0.3</td>
<td>3.2 ± 0.2</td>
</tr>
<tr>
<td>propionate</td>
<td>0.12 ± 0.02</td>
<td>5.6 ± 0.2</td>
<td>4.7 ± 0.2</td>
</tr>
<tr>
<td>acrylate</td>
<td>1.42 ± 0.20</td>
<td>5.4 ± 0.2</td>
<td>0.4 ± 0.04</td>
</tr>
</tbody>
</table>

Activities were determined using the coupled spectrophotometric assay at 45 °C.
FIGURE LEGENDS

Figure 1. 3-Hydroxypropionate cycle of autotrophic CO₂ fixation in the phototrophic green non-sulfur eubacterium *Chloroflexus aurantiacus*. 1, acetyl-CoA carboxylase; 2, malonyl-CoA reductase (bifunctional, 16); 3, propionyl-CoA synthase (trifunctional, 1); 4, propionyl-CoA carboxylase; 5, methylmalonyl-CoA epimerase; 6, methylmalonyl-CoA mutase; 7, succinyl-CoA:L-malate CoA transferase; 8, succinate dehydrogenase, electron acceptor unknown; 9, fumarase, 10, L-malyl-CoA/β-methylmalyl-CoA lyase (bifunctional, 9).

Figure 2. SDS-PAGE (10 %) of 3-hydroxypropionyl-CoA synthetase of *Metallosphaera sedula* at various steps during purification. Lane A, 20 μg of cell extract protein from autotrophically grown *M. sedula*; lane B, 10 μg of protein from the Phenyl-Sepharose column step; lane C, 7 μg of protein from the Mono Q column step; lane D, 3 μg of protein from the Reactive Green column step; lane E, 1 μg of protein from the Gelfiltration column step. The gel was stained with Coomassie brilliant blue.

Figure 3. CoA dependency of 3-hydroxypropionyl-CoA synthetase of *Metallosphaera sedula*. The coupled enzyme assay at 45 °C and 3.7 μg of purified 3-hydroxypropionyl-CoA synthetase were used.
Figure 1.
Figure 2.
Figure 3.

[Graph showing specific activity (µmol min$^{-1}$ mg$^{-1}$) against CoA concentration (mM).]