Propionyl-Coenzyme A Synthase from Chloroflexus aurantiacus, a Key Enzyme of the 3-Hydroxypropionate Cycle for Autotrophic CO₂ Fixation

Birgit E. Alber‡ and Georg Fuchs

From the Institut Biologie II, Mikrobiologie, Universität Freiburg, Schänzlestrasse 1, Freiburg D-79104, Germany

The 3-hydroxypropionate cycle has been proposed as a new autotrophic CO₂ fixation pathway for the phototrophic green non-sulfur eubacterium Chloroflexus aurantiacus and for some chemotrophic archaeabacteria. The cycle requires the reductive conversion of the characteristic intermediate 3-hydroxypropionate to propionyl-CoA. The specific activity of the 3-hydroxypropionate-, CoA-, K⁺-, and MgATP-dependent oxidation of NADPH in autotrophically grown cells was 0.09 μmol min⁻¹ mg⁻¹ protein, which was 2-fold down-regulated in heterotrophically grown cells. Unexpectedly, a single enzyme catalyzes the entire reaction sequence: 3-hydroxypropionate + MgATP + CoA + NADPH + H⁺ → propionyl-CoA + MgAMP + PPi + NADP⁺ + H₂O. The enzyme was purified 30-fold to near homogeneity and has a very large native molecular mass between 500 and 800 kDa, with subunits of about 185 kDa as judged by SDS-PAGE, suggesting a homotetrameric or homometrameric structure. Upon incubation of this new enzyme, termed propionyl-CoA synthase, with the proteinase trypsin, the NADPH oxidation function of the enzyme was lost, whereas the enzyme still activated propionyl-CoA to its CoA-thioester and dehydrated it to acrylyl-CoA. SDS-PAGE revealed that the subunits of propionyl-CoA synthase had been cleaved once and the N-terminal amino acid sequences of the two trypsin digestion products were determined. Two parts of the gene encoding propionyl-CoA synthase (pcs) were identified on two contigs of an incomplete genome data base of C. aurantiacus, and the sequence of the pcs gene was completed. Propionyl-CoA synthase is a natural fusion protein of 201 kDa consisting of a CoA ligase, an enoyl-CoA hydratase, and an enoyl-CoA reductase, the reductase domain containing the trypsin cleavage site. Similar polyfunctional large enzymes are common in secondary metabolism (e.g. polyketide synthases) but rare in primary metabolism (e.g. eukaryotic type I fatty acid synthase). These results lend strong support to the operation of the proposed pathway in autotrophic CO₂ fixation.

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‡ To whom correspondence should be addressed. Tel.: 49-761-203-2685; Fax: 49-761-203-2626; E-mail: alber@uni-freiburg.de.

The proposed 3-hydroxypropionate cycle is shown below in Fig. 1. Each turn of the cycle results in the net fixation of two molecules of bicarbonate into one molecule of glyoxylate. Acetyl-CoA is carboxylated to malonyl-CoA by conventional ATP-dependent acetyl-CoA carboxylase. In vitro, a straightforward reductive conversion of malonyl-CoA to propionyl-CoA was observed when NADPH, K⁺, CoA, and MgATP were added to cell extract (6). This finding was surprising, because this conversion formally requires five enzymatic reactions and 3-hydroxypropionate as a free intermediate (see Fig. 1). 3-Hydroxypropionate formation is characteristic for the cycle; this metabolite is even excreted when cell growth becomes limited (3). Recently, we have purified the enzyme reducing malonyl-CoA and have shown that it is a bifunctional enzyme that requires two NADPH and forms 3-hydroxypropionate (15); 3-hydroxypropionate semialdehyde is an intermediate in this process. This new enzyme, malonyl-CoA reductase, has both alcohol and aldehyde dehydrogenase (CoA acylating) activity; the two partial reactions catalyzed are shown below in Fig. 1.

The subsequent reductive conversion of 3-hydroxypropionate to propionyl-CoA formally requires three enzymatic reactions. The first step, activation to 3-hydroxypropionyl-CoA, requires MgATP; the product of ATP hydrolysis, ADP or AMP, has not been identified. This knowledge is important for estimating the energy need for autotrophic CO₂ fixation. So far, a CoA ligase catalyzing this reaction was assumed. The second step is the dehydration of 3-hydroxypropionyl-CoA to acrylyl-CoA by an enoyl-CoA hydratase. The third step is the NADPH-specific reduction of acrylyl-CoA to propionyl-CoA by an enoyl-CoA reductase.

This study originally aimed at purifying the first of these enzymes, the presumptive CoA ligase, converting 3-hydroxypropionate to its CoA-thioester. Much to our surprise, an enzyme is present in cell extracts of C. aurantiacus, which catalyzes the overall reductive conversion of 3-hydroxypropionate to propionyl-CoA. The enzyme was purified and characterized, and the gene encoding this new enzyme was identified.
was incubated for 30 min at 63 °C and centrifuged at 20,000 × g for 15 min. Saturated ammonium sulfate solution was added to the supernatant to a final concentration of 10% (NH₄)₂SO₄ and centrifuged at 100,000 × g for 1 h.

(iii) Phenyl-Sepharose Chromatography—The supernatant from step ii was loaded onto a 25-ml Phenyl-Sepharose column (Amersham Biosciences, Inc.) equilibrated with buffer A containing 200 mM ammonium sulfate. After an 80-ml wash, the column was developed with a 250-ml decreasing linear gradient of 200–0 mM ammonium sulfate at 1 ml min⁻¹. The peak of activity was eluted between 120 and 80 mM salt, and the pooled fractions were concentrated using an ultrafiltration unit fitted with a 10-ml membrane cell (cutoff, 10 kDa; Filtron, Karlsruhe, Germany).

(iv) MonoQ Chromatography—The concentrated enzyme solution from step iii was applied onto an 8-ml MonoQ HR 10/10 anion-exchange column (Amersham Biosciences, Inc.) equilibrated with buffer B (25 mM MOPS/NaOH (pH 7.2), 5 mM MgCl₂). The column was washed with 15 ml of buffer B and developed with a linear gradient of 0–1 mM NaCl at 1 ml min⁻¹. Propionyl-CoA synthase activity eluted between 300 and 340 mM NaCl. The column was developed at a flow rate of 1 ml min⁻¹. The combined active fractions were concentrated as described above, and glycerol and DTE were added to a final concentration of 20% and 1 mM, respectively, and stored at −20°C.

Characterization of the Enzyme

The Kₘ values of CoA, ATP, 3-hydroxypropionate or acrylate, and NADPH of the ATP-, CoA-, and NADPH-dependent reduction of 3-hydroxypropionate or acrylate were determined using propionyl-CoA synthase, obtained after the MonoQ chromatography step, and the standard reaction assay (a). The concentration of one substrate was varied, while keeping the concentration of the other substrates constant; the concentrations given were saturating. The stoichiometry of the reaction was determined by changing the concentration of either ATP, CoA, or 3-hydroxypropionate of the standard reaction assay to concentrations between 10 μM and 0.1 mM. After the reaction had come to completion, the amount of NADPH consumed was determined by measuring the overall absorption change at 365 nm. The stoichiometry of the 3-hydroxypropionate-dependent formation of AMP was determined using assay (b) in the presence of NADH and NADPH. The pH optimum at 55 °C of the ATP-, CoA-, and NADPH-dependent reduction of 3-hydroxypropionate was determined by using the standard reaction assay (a) with 100 mM instead of 10 mM KCl and, instead of 100 mM Tris/HCl, the following buffers at 100 mM concentrations (pH at 55 °C): MOPS/NaOH pH 6.8–7.3, HEPES/NaOH pH 7.2–8.1, TAPS/NaOH pH 7.7–8.9. The thermostability of the enzyme was determined by incubating the enzyme at 30, 40, 50, 60, 65, 72, 77, and 90 °C for 15 min. The enzyme solution was cooled to 4 °C before determining the activities relative to those of samples kept at 4 °C throughout the experiment. K⁺ and Mg⁴⁺ dependencies were measured by omitting KCl or MgCl₂ from the standard reaction assay and adding them back at defined concentrations. The specificity of the ATP-, CoA-, and NADPH-dependent reduction of 3-hydroxypropionate toward its substrate was determined by substituting 3-hydroxypropionate in the standard reaction assay by 10 mM neutralized 3-hydroxybutyrate, crotonate, acrylate, β-alanine, or by 1 mM glycolate and malonate; ATP by 3 mM GTP and UTP, NADPH by 0.4 mM NADH. An optical absorption spectrum of the purified enzyme (1.3 mg ml⁻¹ in 20 mM MOPS/NaOH, pH 7.5, 5 mM MgCl₂, 300 mM KCl, 10% (v/v) glycerol) was collected at 25 °C using a PerkinElmer Life Sciences Lambda 25 spectrometer and the same buffer as a blank.

Trypsin Digestion of the Native Enzyme

To an aliquot of 200 μl of purified propionyl-CoA synthase (1.0 mg ml⁻¹) 320 μl of 10 mM ammonium bicarbonate buffer (pH 8.0), containing 20 μl of trypsin (Sigma), was added. To a second aliquot of 320 μl of 10 mM ammonium bicarbonate buffer (pH 8.0), and no trypsin, was added and used as a control ("undigested"). After incubation at 37 °C for 30 min, 20 μl of 1 mg ml⁻¹ trypsin inhibitor was added. The activity of ATP-, CoA-, and 3-hydroxypropionate-dependent oxidation of NADH and/or NADPH and the stoichiometry of 3-hydroxypropionate used per NADPH supplied, was measured using assay (b), containing either 0.5 mM NADPH or 0.5 mM NADH or 0.25 mM NADPH plus 0.25 mM NADH.
Propionyl-CoA Synthase

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TABLE I

| Step          | Total activity | Protein | Specific activity | Recovery | Purification |
|---------------|----------------|---------|------------------|----------|--------------|
|               | µmol min⁻¹      | mg      | µmol min⁻¹ mg⁻¹  | %        | fold         |
| Cell extract  | 37.4            | 410     | 0.091            | (100)    | (1)          |
| 30 min at 63 °C, 10% (NH₄)₂SO₄ | 29.2          | 280     | 0.10             | 78       | 1.1          |
| Phenyl-Sepharose | 21.3          | 17      | 1.3              | 57       | 14           |
| MonoQ         | 15.3            | 7.4     | 2.1              | 41       | 23           |
| Gel filtration| 6.1             | 2.4     | 2.5              | 16       | 28           |

HPLC Analysis

An optimized reaction mixture (0.6 ml), based on the standard reaction assay (a), containing 100 mM Tris/HCl (pH 8.5), 2 mM DTE, 2 mM MgCl₂, 100 mM KCl, 2 mM ATP, 0.5 mM CoA, 0.7 mM NADPH, and 25 µg of purified propionyl-CoA synthase was used. The enzymatic reaction was started by the addition of 1 mM 3-hydroxypropionate and stopped at different time points by transferring 100 µl of the reaction mixture to 3 µl of 25% HCl. CoA-thioesters were analyzed by reversed-phase HPLC using an RP-C₁₈ column (LiChrospher 100, endcapped, 5 µm, 150 × 4 mm; Merck, Darmstadt, Germany). A 35-min gradient from 2% to 10% (v/v) acetonitrile, in 50 mM potassium phosphate-buffer, pH 6.7, at a flow rate of 1 ml min⁻¹, was used. All CoA-esters were detected at 280 nm. Propionyl-CoA and acrylyl-CoA were synthesized enzymatically at 37 °C (100 mM Tris/HCl, pH 8.5, 2 mM MgCl₂, 20 mM KCl, 3 mM phosphoenolpyruvate, 0.5 mM NADH, 2 mM ATP, 0.5 mM CoA, 0.2 unit of myokinase, 0.1 unit of pyruvate kinase, 0.1 unit of lactate dehydrogenase, 0.1 unit of acetyl-CoA synthetase (Sigma), and 20 mM neutralized propionate or acrylate, reaction time: 3 min for propionate and 5 min for acrylate) and used as standards.

Molecular Mass Determination

SDS-PAGE (7%) was performed as described previously (17). The native molecular mass was determined on a 125-ml Sephacryl S-300 (Amersham Biosciences, Inc.) gel filtration column calibrated with ovalbumin (45 kDa), catalase (250 kDa), ferritin (450 kDa), and thyroglobulin (669 kDa).

N-terminal Amino Acid Analysis

To an aliquot of 200 µl of purified propionyl-CoA synthase (0.6 mg ml⁻¹) 320 µl of 10 mM ammonium bicarbonate buffer (pH 8.0), containing 20 µg of trypsin (Sigma), was added. After incubation at 37 °C for 30 min, trichloroacetic acid was added to a final concentration of 6% (v/v). The sample was centrifuged at 10,000 × g for 10 min, and the pellet was resuspended in 50 µl of 0.1 M NaOH. The trypsin-digested protein fragments were separated by 8% SDS-PAGE and transferred to an Immobilon-P™ transfer membrane (Millipore, Bedford, MA). N-terminal sequencing was performed by TopLab (Martinsried, Germany) using Applied Biosystems Precise 492 sequencer (Weiterstadt, Germany). The phenylthiohydantoin derivatives were identified with an on-line Applied Biosystems Analyzer 140 C.

Identification of the pcs Gene, Encoding Propionyl-CoA Synthase

Small scale chromosomal DNA from C. aurantiacus was isolated using a standard technique (18). Two 23-mer oligonucleotides (primer echB: 5'-TTCCATCATATTGGCGAAGCCG-3', partially corresponding to nucleotides 11841–11855 of contig 1024 of the genome data base of C. aurantiacus (www.jgi.doe.gov/JGI_microbial/html/chloroflexus/chloro_homepage.html) and primer echEcoRI: 5'-ACACGCGAATTCGGGTCAACCACT-3', partially corresponding to nucleotides 445–459 of contig 799 of the same genome data base), 1 µg of chromosomal C. aurantiacus DNA, 2.5 units of Pfu polymerase (Promega, Erlangen, Germany), and the Pfuql DNA amplification kit were used to amplify a 974-bp genomic region, which is part of the pcs gene. The PCR product was purified (19) and sequenced at the sequencing facility to amplify a 974-bp genomic region, which is part of the pcs gene. The PCR product was purified (19) and sequenced at the sequencing facility.

MATERIALS

Chemicals were obtained from Fluka (Neu-Ulm, Germany), Merck (Darmstadt, Germany), Sigma-Aldrich (Deisenhofen, Germany), or Roth (Karlsruhe, Germany); biochemicals were from Roche Diagnostics (Mannheim, Germany), Applichem (Darmstadt, Germany), or Gerbu (Craiberg, Germany).

RESULTS

Reductive Conversion of 3-Hydroxypropionate by Cell Extracts—Extracts of autotrophically grown cells of C. aurantiacus catalyzed the 3-hydroxypropionate-dependent oxidation of NADPH, provided that Mg²⁺, ATP, K⁺, and CoA were added. The specific activity at 55 °C was 0.09 µmol min⁻¹ mg⁻¹ protein; this activity was 2-fold down-regulated in heterotrophically grown cells.

Purification of the Enzyme Transforming 3-Hydroxypropionate in the Presence of Mg²⁺, ATP, K⁺, CoA, and NADPH—The enzyme catalyzing the reductive transformation of 3-hydroxypropionate was purified from 10 g (wet weight) of autotrophically grown cells (Table I). An initial heat precipitation step was essential to remove large amounts of pigments. Three chromatographic steps yielded a nearly homogeneous protein, as judged by SDS-PAGE (Fig. 2). The enzyme eluted as a symmetrical peak from the gel filtration column. The overall recovery was 16% and the enrichment about 30-fold, which means that this protein amounts to at least 3% of the soluble cell protein. Comparison of the soluble protein profile of cells grown hetero- versus autotrophically (Fig. 2) shows that the amount of enzyme is elevated under autotrophic growth condition, consistent with the higher specific activity measured under these conditions.

Molecular Properties of the Enzyme—SDS-PAGE of the purified enzyme showed a single protein band of ~185 kDa (Fig. 2). Gel filtration chromatography of the native protein gave a molecular mass of 500–800 kDa. This indicates that the enzyme is a homotrimer or homotetramer. The UV-visible spectrum (250–800 nm) of the purified enzyme showed a single peak at 280 nm, with no indication for the presence of a chromophoric cofactor (data not shown). The enzyme was stable when incubated for 15 min at temperatures up to 65 °C and during storage in the presence of 20% glycerol and 1 mM DTE at −20 °C for a few weeks.

Stoichiometry and Products of the Reaction—The stoichiometry of the reaction was determined. Per mole of 3-hydroxypropionate added, 1 mol of NADPH was oxidized. The same stoichiometry was observed for ATP and for CoA. The products of the reaction were determined in different ways. HPLC analysis showed that propionyl-CoA was formed. To determine whether AMP plus pyrophosphate or ADP plus phosphate was formed from ATP, a coupled spectrophotometric assay was used. In the presence of NADH and NADPH the rate of NADP(P)H oxidation was increased by 190% over the rate of NADPH oxidation alone; this effect was strictly dependent on the addition of myokinase. Furthermore, the stoichiometry of NAD(P)H oxidized per mole of 3-hydroxypropionate added was 2.9:1. This shows ATP was hydrolyzed to AMP and pyrophosphate. The overall reaction catalyzed by the enzyme is as follows,

\[ \text{3-hydroxypropionate} + \text{MgATP} + \text{CoA} + \text{NADPH} \rightarrow \text{propionyl-CoA} + \text{MgAMP} + \text{PP} + \text{H}_2\text{O} + \text{NADP}^+ \]
The enzyme is referred to as propionyl-CoA synthase.

**Catalytic Properties of Propionyl-CoA Synthase**—The enzyme showed high affinities for its substrates. The reaction followed Michaelis-Menten kinetics with apparent $K_m$ values of 15 $\mu$M for 3-hydroxypropionate, 10 $\mu$M for NADPH, 50 $\mu$M for ATP, and 10 $\mu$M for CoA. The enzyme is also very specific; it does not act on glycolate, malonate, $\beta$-alanine, 3-hydroxybutyrate, or crotonate. Acrylate was transformed at the same rate as 3-hydroxypropionate, albeit with a much higher apparent $K_m$ value (0.5 $\mu$M). NADH could not substitute for NADPH, whereas ATP (100%) could be substituted by GTP (24%) and UTP (20%). Activity was dependent on $K^+$, and half-maximal activity was observed at 4 mM KC1. The acrylate-dependent oxidation of NADPH in the presence of ATP and CoA was also dependent on $K^+$, indicating that the CoA ligase step is $K^+$-dependent. Monovalent cations with similar ionic radii (Rb$^+$, NH$_4^+$, and Cs$^+$) can partially substitute for $K^+$ (95%, 85%, and 20%). There is a clear correlation of decrease in activity with the increase in ionic radius, relative to that of $K^+$, indicating that this $K^+$ dependence is not a general ionic strength effect.

The pH dependence at 55 °C of the propionyl-CoA synthase catalyzed reaction shows a rather narrow pH optimum around pH 8 (Fig. 3). Between pH 6.8 and 7.9 the specific activity of the propionyl-CoA synthase increased 30-fold, indicating that a functional group in the enzyme with an estimated $pK_a$ of 7.3 has to be deprotonated for maximum turnover. The $v_{max}$ value was 4 $\mu$mol min$^{-1}$, corresponding to a turnover of 12 s$^{-1}$ per subunit of 185 kDa.

**Possible Intermediates**—Product formation from 3-hydroxypropionate in the presence or absence of NADPH was studied to detect possible intermediates. In the absence of NADPH an unknown CoA-thioester (compound X) and acrylyl-CoA were formed (Fig. 4B). After addition of 1 mM NADPH, propionyl-CoA was formed (Fig. 4C), and the amount of compound X and acrylyl-CoA decreased. The consumption of compound X and acrylyl-CoA upon addition of NADPH suggests that both are intermediates of the reaction, and it is, therefore, likely that compound X represents 3-hydroxypropionyl-CoA. The assignment of compound X as 3-hydroxypropionyl-CoA is also consistent with its elution time after CoA and before propionyl-CoA.

**Domain Character of Propionyl-CoA Synthase**—Native (undenatured) propionyl-CoA synthase was digested with the proteinase trypsin. SDS-PAGE analysis indicated that the subunits of the enzyme were principally cleaved once, each subunit yielding two polypeptides, with estimated molecular masses of 150 and 40 kDa (Fig. 5). The trypsin-digested propionyl-CoA synthase was no longer able to catalyze the ATP-, CoA-, and 3-hydroxypropionate-dependent oxidation of NADPH (Table II). By coupling the formation of 1 mol of AMP to the oxidation of 2 mol of NADH using myokinase, phosphoenolpyruvate, pyruvate kinase, and lactate dehydrogenase, it was shown that the trypsin-digested enzyme, however, still catalyzed the 3-hydroxypropionate- and CoA-dependent hydrolysis of ATP (Table II). The rate of 3-hydroxypropionate-, ATP-, and CoA-dependent formation of AMP for the trypsin-digested enzyme (0.5 $\mu$mol min$^{-1}$, mg$^{-1}$) was even increased over the rate of the same reaction catalyzed by undigested propionyl-CoA synthase (0.34 $\mu$mol min$^{-1}$, mg$^{-1}$).

![Figure 2](image1.png) **Fig. 2.** Denaturing PAGE (7%) of cell extract of phototrophic and photoautotrophically grown *C. aurantiacus* and propionyl-CoA synthase at various steps of the purification. Lanes: A, 20 $\mu$g of cell extract protein of phototrophically grown cells; B, 20 $\mu$g of cell extract protein of photoautotrophically grown cells; C, 20 $\mu$g of protein after the heat/ammonium sulfate precipitation step; D, 5 $\mu$g of protein from the Phenyl-Sepharose column step; E, 3 $\mu$g of protein from the MonoQ column step; F, 1 $\mu$g of protein from the gel filtration column step; G, molecular mass marker (rabbit myosin, 205 kDa; Escherichia coli $\beta$-galactosidase, 116 kDa; rabbit phosphorylase b, 97 kDa; rabbit fructose-6-phosphate kinase, 84 kDa; bovine albumin, 66 kDa; bovine glutamic dehydrogenase, 55 kDa; egg ovalbumin, 45 kDa; rabbit glyceraldehyde-3-phosphate dehydrogenase, 36 kDa); H, 2 $\mu$g of protein from the gel filtration column step. The gel was stained with Coomassie Brilliant Blue R-250.

![Figure 3](image2.png) **Fig. 3.** pH dependence of the propionyl-CoA synthase at 55 °C. Propionyl-CoA synthase activity was measured in 100 mM MOPS/NaOH (●), HEPES/NaOH (▲), or TAPS/NaOH (■), 100 mM KCl, 2 mM DTE, 5 mM MgCl$_2$, 3 mM ATP, 0.5 mM CoA, 0.4 mM NADPH, and 9.8 $\mu$g of protein.

![Figure 4](image3.png) **Fig. 4.** HPLC analysis of CoA-thioesters formed during the reductive conversion of 3-hydroxypropionate by propionyl-CoA synthase. The reaction mixture contained 100 mM Tris/HCl (pH 8.5), 100 mM KCl, 2 mM MgATP, 0.5 mM CoA, and 25 $\mu$g of propionyl-CoA synthase at 55 °C. Samples were withdrawn immediately after addition of 1 mM 3-hydroxypropionate (A), after 5 min incubation in the absence of NADPH (B), and after 5 additional minutes of incubation following the addition of 1 mM NADPH (C). D and E, the reaction mixture contained 0.7 mM NADPH at the start of the reaction and 25 $\mu$g of propionyl-CoA synthase incubated at 37 °C for 30 min in the absence (D) and presence (E) of the proteinase trypsin. Samples were withdrawn 5 min after the addition of 1 mM 3-hydroxypropionate. The elution times of known standards were: CoA, 7.3 min; propionyl-CoA, 18.8 min; acrylyl-CoA, 23.6 min. Compound X elutes at 10.6 min and is likely 3-hydroxypropionyl-CoA.
μmol min⁻¹ mg⁻¹). In the presence of NADPH and trypsin-digested enzyme, only traces of propionyl-CoA were formed, whereas compound X was the major CoA-thioester formed (Fig. 4E). Acrylyl-CoA was also formed (Fig. 4E), indicating that the trypsin-digested propionyl-CoA synthase retained 3-hydroxypropionate-CoA ligase and acrylyl-CoA hydratase activity, whereas the acryl-CoA reductase activity was almost completely lost.

Primary Sequence Analysis of the Propionyl-CoA Synthase—The N-terminal sequences of both polypeptides of the trypsin-digested propionyl-CoA synthase were determined as MIDTA for the 150-kDa polypeptide and ASTLLAAGAH for the 40 kDa polypeptide. A search of the data base of the almost completed genome of C. aurantiacus at the DOE Joint Genome Institute revealed a perfect match of the N-terminal sequence of the second (40 kDa) polypeptide. A search of the data base of the almost completed genome of C. aurantiacus at the DOE Joint Genome Institute revealed a perfect match of the N-terminal sequence of the second (40 kDa) polypeptide. The N-terminal sequence of the second (40 kDa) polypeptide revealed a perfect match of the N-terminal sequence of the second (40 kDa) polypeptide. The N-terminal sequence of the second (40 kDa) polypeptide revealed a perfect match of the N-terminal sequence of the second (40 kDa) polypeptide. It is, therefore, concluded, that the MIDTA sequence represents the true N terminus of the propionyl-CoA synthase. The N-terminal sequence of the second (40 kDa) polypeptide revealed a perfect match to an internal sequence of a putative protein encoded by an incomplete open reading frame (ORF) on contig 799. Based on the deduced amino acid sequence of this ORF, the N-terminal alanine of the 40-kDa polypeptide is preceded by an arginine residue in the native protein, which itself is preceded by four consecutive serine residues. This indicates a likely cleavage site for the proteinase trypsin. The missing DNA sequence between the two contigs was completed and deposited in the GenBank™ data base (accession number AF445079, Fig. 6). The gene encoding propionyl-CoA synthase from C. aurantiacus is referred to as pcs. The pcs gene encodes a protein with 1822 amino acids and a calculated molecular weight of 201,404. This value is within the estimated subunit molecular mass of 185 kDa for the purified protein (Fig. 2). The calculated molecular masses of the two polypeptides, after cleavage with trypsin, are 159 and 42 kDa, respectively.

**DISCUSSION**

We have described a new enzyme, propionyl-CoA synthase, which functions in autotrophic CO₂ fixation in C. aurantiacus and possibly other prokaryotes. Propionyl-CoA synthase catalyzes the irreversible reductive conversion of 3-hydroxypropionate to propionyl-CoA. The reaction consists of three partial reactions as shown in Fig. 1. The properties of the enzyme are summarized in Table III.

New Enzyme—Propionyl-CoA synthase is a trifunctional enzyme. It belongs to the following enzyme classes: EC 6.2.1., ligases forming carbon–sulfur bonds (acid-thiol ligases); EC 4.2.1., carbon–oxygen lyases (hydro-lyases); and EC 1.3.1., oxidoreductases acting on the CH–CH groups of donors with NAD(P)⁺ as acceptor. The first partial reaction corresponds to organic-CoA ligase (AMP-forming), the second one to enoyl-CoA hydratase, and the third partial reaction to NADPH-dependent enoyl-CoA reductase.

Domain Structure of Propionyl-CoA Synthase—A search of protein data bases indicated that propionyl-CoA synthase consists of three protein domains (Fig. 6). Residues 18–850 of the deduced amino acid sequence of pcs showed significant sequence similarities to several acetyl-CoA and short fatty acid acyl-CoA synthetases from various organisms and was, there-

![Fig. 5. Denaturing PAGE (8%) of propionyl-CoA synthase after treatment with the proteinase trypsin. Both lanes contain 3.5 μg of propionyl-CoA synthase from C. aurantiacus after incubation at 37 °C for 30 min in the absence (left lane) or presence of 0.35 μg trypsin (right lane). After this incubation 0.35 μg of trypsin inhibitor was added. The gel was stained with Coomassie Brilliant Blue R-250.](image1)

**Fig. 6. Schematic representation of the propionyl-CoA synthase (1822 amino acids) indicating the three functional domains.** The location of the N-terminal sequences of two trypsin digestion products (150 and 40 kDa) and the connecting sequence for contigs 1024 and 799 are shown (GenBank™ accession number AF445079). For details see text.

**Table II**

* Rates of the ATP-, CoA-, 3-hydroxypropionate-dependent oxidation of NADPH and/or NADH by propionyl-CoA synthase and stoichiometry of NAD(P)/H used per 3-hydroxypropionate supplied

| Propionyl-CoA synthase | NADPH (μmol) | NADH (μmol) | Specific activity (μmol NAD(P)/H oxidized) | Stoichiometry ( equivalents) |
|------------------------|--------------|-------------|------------------------------------------|-----------------------------|
| Undigested             | 0.5          | 0           | 1.5                                      | 1:1.0                       |
|                        | 0            | 0.5         | 0.67                                     | 1:2.2                       |
|                        | 0.25         | 0.25        | 4.3                                      | 1:2.8                       |
| Trypsin digest         | 0.5          | 0           | <0.1                                     | NA⁺                        |
|                        | 0            | 0.5         | 1.0                                      | 1:2.2                       |
|                        | 0.25         | 0.25        | 1.1                                      | 1:2.0                       |

* Ratio is 3-hydroxypropionate used:NAD(P)/H oxidized.

⁺ Not applicable, because no activity was detected under these conditions.
fore, named acyl-CoA synthetase (ACS) domain. It showed highest percent sequence identity (35%) to an acetyl-CoA synthetase from Pyrobaculum aerophilum (GenBank accession number AAD09253). A multiple alignment of several homologous sequences indicated an untypical insertion of about 110 amino acids in the ACS domain of propionyl-CoA synthase, located within a consensus AMP binding domain (Pfam 00501) identified in all aligned sequences. The acyl-CoA synthetase (3-hydroxypropionate-CoA ligase) activity of propionyl-CoA synthase is K⁺-dependent, as has been shown for the acetyl-CoA synthetase from animal tissue (21). The enoyl-CoA hydratase (ECH) domain (Pcs amino acid residues 858–1051) showed 43% sequence identity to the 3-hydroxybutyryl-CoA dehydratase (crotonase) of Clostridium acetobutylicum (GenBank accession number AAK80658) and significant sequence similarity to other members of the enoyl-CoA hydratase family (Pfam 00378). Both glutamate residues shown to be involved in the enoyl-CoA hydratase-catalyzed reaction (22) are conserved in propionyl-CoA synthase (E975 and E995). The enoyl-CoA reductase (ECR) domain (Pcs amino acid residues 1201–1816) is placed within the zinc-binding dehydrogenase family of proteins (Pfam 00107), however, the zinc-binding site is not conserved. An untypical insertion of about 150 amino acids is present in propionyl-CoA synthase compared with other members of this family, where this insertion is absent. Conserved residues of a NAD(P)H consensus binding motif (GXGXCA_X_A) correspond to Glys-1415 to Ala-1424 of propionyl-CoA synthase. The highest sequence similarity (overall 28% sequence identity) was found with an alcohol dehydrogenase of CoA synthase. The highest sequence similarity (overall 28% sequence identity) was found with an alcohol dehydrogenase of CoA synthase. The highest sequence similarity (overall 28% sequence identity) was found with an alcohol dehydrogenase of CoA synthase. The highest sequence similarity (overall 28% sequence identity) was found with an alcohol dehydrogenase of CoA synthase. The highest sequence similarity (overall 28% sequence identity) was found with an alcohol dehydrogenase of CoA synthase.
due to its double bond a reactive intermediate, is rapidly converted by the enzyme and, therefore, does not accumulate in the cell. These results show that propionyl-CoA synthase is a key enzyme in the 3-hydroxypropionate cycle of autotrophic CO₂ fixation. It remains to be shown whether, using the 3-hydroxypropionate cycle, a similar trifunctional enzyme exists in other autotrophs, or whether a multifunctional enzyme complex or even separately functioning enzymes are operating.

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