Properties of Succinyl-Coenzyme A: d-Citramalate Coenzyme A Transferase and Its Role in the Autotrophic 3-Hydroxypropionate Cycle of Chloroflexus aurantiacus

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The phototrophic bacterium Chloroflexus aurantiacus uses the 3-hydroxypropionate cycle for autotrophic CO₂ fixation. This cycle starts with acetyl-coenzyme A (CoA) and produces glyoxylate. Glyoxylate is an unconventional cell carbon precursor that needs special enzymes for assimilation. Glyoxylate is combined with propionyl-CoA to β-methylmalyl-CoA, which is converted to citramalate. Cell extracts catalyzed the succinyl-CoA-dependent conversion of citramalate to acetyl-CoA and pyruvate, the central cell carbon precursor. This reaction is due to the combined action of enzymes that were upregulated during autotrophic growth, a coenzyme A transferase with the use of succinyl-CoA as the CoA donor and a lyase cleaving citramalyl-CoA to acetyl-CoA and pyruvate. Genomic analysis identified a gene coding for a putative coenzyme A transferase. The gene was heterologously expressed in Escherichia coli and shown to code for succinyl-CoA: d-citramalate coenzyme A transferase. This enzyme, which catalyzes the reaction d-citramalate + succinyl-CoA → d-citramalyl-CoA + succinate, was purified and studied. It belongs to class III of the coenzyme A transferase enzyme family, with an aspartate residue in the active site. The homodimeric enzyme composed of 44-kDa subunits was specific for succinyl-CoA as a CoA donor but also accepted d-malate and itaconate instead of d-citramalate. The CoA transferase gene is part of a cluster of genes which are cotranscribed, including the gene for d-citramalyl-CoA lyase. It is proposed that the CoA transferase and the lyase catalyze the last two steps in the glyoxylate assimilation route.

The phototrophic bacterium Chloroflexus aurantiacus, a member of the green nonsulfur bacteria, grows optimally at 55°C under heterotrophic conditions but can also grow autotrophically in mineral salt medium with the use of CO₂ as the sole carbon source (23, 33, 34, 41). A novel CO₂ fixation pathway termed the 3-hydroxypropionate cycle operates under autotrophic conditions (1, 14, 15, 19–25, 44, 45). The pathway results in the fixation of three molecules of bicarbonate and forms pyruvate as the central carbon precursor molecule. The main CO₂-fixing enzyme is acetyl-coenzyme A (CoA)/propionyl-CoA carboxylase. The pathway can be divided into two metabolic cycles (Fig. 1) (20).

In the first cycle, acetyl-CoA is carboxylated to malonyl-CoA, which is subsequently reduced and converted into propionyl-CoA via 3-hydroxypropionate as a free intermediate (1, 25). Propionyl-CoA is carboxylated to methylmalonyl-CoA, which is converted to succinyl-CoA. Succinyl-CoA is used to activate t-malate by succinyl-CoA:t-malate coenzyme A transferase, which forms t-malyl-CoA and succinyl-CoA (15). Succinate is oxidized to t-malate via conventional steps. t-Malyl-CoA is cleaved by t-malyl-CoA/β-methylmalyl-CoA lyase, thus regenerating the starting acetyl-CoA molecule and releasing glyoxylate as the primary fixation product (19).

Glyoxylate is an unconventional cell carbon precursor that needs special enzymes to be used in cell carbon biosynthesis. A second cycle was proposed to serve as a glyoxylate assimilation pathway. Glyoxylate is combined with propionyl-CoA to β-methylmalyl-CoA, catalyzed by t-malyl-CoA:β-methylmalyl-CoA lyase (19). This promiscuous enzyme not only cleaves t-malyl-CoA into acetyl-CoA and glyoxylate, but also synthesizes β-methylmalyl-CoA from glyoxylate and propionyl-CoA (19). β-Methylmalyl-CoA was shown to be converted by cell extracts to mesaconyl-CoA and citramalate (20); however, details of this process are not yet known. Cell extracts also catalyzed the succinyl-CoA-dependent conversion of citramalate to acetyl-CoA and pyruvate (20, 21).

The succinyl-CoA-dependent cleavage of citramalate regenerates acetyl-CoA, which is carboxylated and reductively converted to propionyl-CoA by using the same enzymes as those in the first cycle. Biosynthesis starts with pyruvate, using pyruvate phosphate dikinase to form phosphoenolpyruvate, and phosphoenolpyruvate carboxylase functions as an anaplerotic enzyme which fixes additional bicarbonate (21). Thus, all CO₂-fixing enzymes (acetyl-CoA/propionyl-CoA carboxylase and phosphoenolpyruvate carboxylase) use bicarbonate as the actual inorganic carbon substrate (Fig. 1).

We set out to analyze the enzymes that catalyze the conversion of citramalate to acetyl-CoA and pyruvate. We supposed that this reaction is a two-step process, CoA transferase catalysis, in which the reaction succinyl-CoA + citramalate → succinate + citramalyl-CoA is followed by a reaction in which citramalyl-CoA cleavage is catalyzed by a lyase, citramalyl-CoA → pyruvate + acetyl-CoA (Fig. 1).

We have already characterized an enzyme which can activate the t-isomer of citramalate to t-citramalyl-CoA with succinyl-CoA as the CoA donor (15). Here, we describe an enzyme which catalyzes the CoA transfer from succinyl-CoA to...
**MATERIALS AND METHODS**

**Bacteria and growth conditions.** *C. aurantiacus* strain OK-70-fl (DSM 636) was grown in 2-, 5- or 12-liter glass fermentors to an optical density at 578 nm (1-cm light path) of 3.5 to 4.0 at 55°C and a pH of around 8 as described elsewhere (9, 45). Cells were stored under liquid nitrogen until use. *Escherichia coli* strains BL21(DE3) and DH5α were grown at 37°C in Luria-Bertani (LB) medium (38). Ampicillin was added to *E. coli* cultures to a final concentration of 100 μg/ml.

**Materials.** Chemicals were obtained from Fluka (Neu-Ulm, Germany), Sigma-Aldrich (Deisenhofen, Germany), Merck (Darmstadt, Germany), and Roth (Karlsruhe, Germany). Biochemicals were from Roche Diagnostics (Mannheim, Germany), Applichem (Darmstadt, Germany), and Gerbu (Gaiberg, Germany). Materials for cloning and expression were purchased from MBI Fermentas (St. Leon-Rot, Germany), New England Biolabs (Frankfurt, Germany), Genaxxon Bioscience GmbH (Ebersberg, Germany), and QIAGEN (Hilden, Germany). Materials and equipment for protein purification were obtained from Amersham Biosciences (Freiburg, Germany) and Millipore (Eschborn, Germany).

**Syntheses.** (i) Succinyl-CoA, acetyl-CoA, and propionyl-CoA. The CoA-thioesters of succinate, acetate, and propionate were synthesized from their anhydrides by a slightly modified method described previously (40, 42), and dry powders were stored at −20°C. (ii) Malonyl-CoA. Malonyl-CoA was chemically synthesized as described previously (21), and dry powders were stored at −20°C. The intermediate mono-thiophenylmalonate was chemically synthesized as described previously (26, 35) and stored under nitrogen gas at −20°C. (iii) Malyl-CoA. L-Malyl-CoA was chemically synthesized as described previously (13), with a slight modification (19). The synthesis intermediate L-malyl-caprylcysteamine ([S-(β-hydroxysuccinyl)-N-caprylcysteamine] was synthesized by Richard Krieger (Institut für Organische Chemie, Universität Freiburg, Germany) as described previously (13, 32). L-Malyl-CoA was stored as freeze-dried powder at −20°C. It contained 80% CoA-thioester and 20% CoA, as determined by high-pressure liquid chromatography (HPLC) separation and detection at 260 nm.

**Cloning and expression of a putative succinyl-CoA:D-citramalate CoA transferase (sct) gene in E. coli BL21.** Standard protocols were used for preparation, cloning, transformation, amplification, and purification of DNA (2, 38). Plasmid DNA was isolated by the method of Birnboim and Doly (4). On the basis of a protein sequence alignment of the BbsF (β-benzylsuccinate CoA-transferase [GenBank accession number AAF89841] from *Thauera aromatica*, a highly conserved region on the *C. aurantiacus* genome was found located next to the gene of a putative 3-hydroxy-3-methylglutaryl-CoA lyase. Two oligonucleotides were designed upstream and downstream of the gene coding for *C. aurantiacus sct*: (i)
FIG. 2. (A) Organization of ORFs on the gene cluster of the C. aurantiacus strain J-19-11 genome (15,400 to 27,500 bp; GenBank accession number NZ_AAHA20000057) containing the sct gene for succinyl-CoA: D-citramalyl-CoA transferase. The circle number refers to RT-PCR experiments as described in the legend to Fig. 6. orf1, putative transcriptional regulator, COG1802 (446 bp); sct, succinyl-CoA:D-citramalyl-CoA transferase, COG1804 (1,206 bp); ccl, D-citramalyl-CoA lyase, COG119 (953 bp); orf2, putative N-hydantoinase A/acetone carbonylase, beta subunit, COG0145 (2,045 bp); orf3, putative N-hydantoinase B/acetone carbonylase, alpha subunit, COG0146 (1,934 bp); orf4, putative amidases related to nicotinamidase, COG1335 (644 bp); orf5, putative ABC-type dipeptide oligopeptide/nickel transport system, ATPase component, COG444 (746 bp); orf6, putative ABC-type oligopeptide transport system, ATPase component, COG468 (1,103 bp). (B) Recombinant plasmid pAST2. The PCR product for sct and ccl was cloned into the expression vector pT7/7 by using the NdEl and BamHI restriction sites.

Preparation of cell extract. Cells were suspended in a threefold volume of 50 mM morpholinoethanesulfonic acid (MOPS)-KOH (pH 7.0) containing 4 mM MgCl₂ and 0.2 mg DNase I per ml of cell suspension and passed twice through a French pressure cell at 137 kPa. The lysate was ultracentrifuged at 100,000 × g at 4°C for 1 h.

Enzyme assays. Succinyl-CoA: D-citramalyl-CoA transferase was tested at 55°C, routinely in the forward direction.

Coupled spectrophotometric assay. The succinyl-CoA- and D-citramalyl-CoA-dependent formation of pyruvate and acetyl-CoA in the presence of excess (0.05 units) recombinant D-citramalyl-CoA lyase was monitored photometrically at 324 nm with phenylhydrazine in a continuous assay (ε₃₂₄ for pyruvate-phenylhydrazone, 10,400 M⁻¹ cm⁻¹). Succinyl-CoA: D-citramalyl-CoA transferase was limited. The assay mixture (0.5 ml) contained 200 mM MOPS-KOH buffer (pH 6.5), 5 mM MgCl₂, 3.5 mM phenylhydrazinium chloride, 1 mM succinyl-CoA, 10 mM D-citramalyl-CoA, 0.05 units D-citramalyl-CoA lyase, 2.5 units D-citramalyl-CoA transferase. Either substrate could be used to start the reaction. Buffers used to determine the pH optimum were 200 mM 2-(N-morpholino)ethanesulfonic acid (MES)-KOH buffer (pH 5.5 to 6.0) and 200 mM MOPS-KOH (pH 6.0 to 8.0). The apparent Kₘ values were determined at saturating concentrations of the substrates (1 mM succinyl-CoA and 10 mM D-citramalyl-CoA).

HPLC assay. The assay mixture (0.5 ml) contained 200 mM MOPS-KOH buffer (pH 6.5), 1 mM succinyl-CoA, 10 mM D-citramalyl-CoA, and 0.18 units recombinant succinyl-CoA:D-citramalyl-CoA transferase. D-Citramalyl-CoA was omitted in a control experiment. In a second experiment, the assay mixture also contained 5 mM MgCl₂ and 0.06 units D-citramalyl-CoA lyase. The reaction was started by the addition of D-citramalyl-CoA. Samples of 110 μl were taken after 5 min of incubation at 55°C, and the reaction was stopped by addition of 3 ml of 25% HCl. Protein was removed by centrifugation, and samples were analyzed for CoA-thioester by HPLC. A reversed-phase column (LiChrospher 100, end capped, 5 μm; Merck) was used for separation of CoA-thioesters. First, a gradient of 1 to 8% acetonitrile in 50 mM potassium phosphate buffer, pH 6.7, with a flow rate of 1 ml min⁻¹ over 30 min, was used. CoA-thioesters were detected at 260 nm. Retention times were 2 min (free organic acids), 10.8 min (D-citramalyl-CoA), 11.6 min (succinyl-CoA and free CoA), and 17.8 min (acetyl-CoA).

Second, a gradient of 2 to 10% acetonitrile in 40 mM potassium phosphate buffer, 50 mM formic acid, pH 4.0, with a flow rate of 1 ml min⁻¹ over 40 min, was used. CoA-thioesters were detected at 260 nm. Retention times were 2 min (free organic acids), 9 min (t-malyl-CoA), 10 min (free CoA and malonyl-CoA), 14 min (t-citramalyl-CoA), 15 min (l-methylmalonyl-CoA), 17 min (succinyl-CoA), 18 min (acetetyl-CoA), 20 min (itaconyl-CoA), and 26 min (propionyl-CoA).

Purification of recombinant succinyl-CoA: D-citramalyl-CoA transferase from E. coli. The purification was performed at 4°C, followed by measurement of the activity of the coupled spectrophotometric assay and gel filtration on a Sephacryl S-200 column. The column was calibrated with ferritin (440 kDa), ovalbumin (45 kDa), and aldolase (158 kDa) as molecular mass standards.

(i) Heat precipitation. Cell extract (supernatant obtained by centrifugation at 100,000 × g from 2 g of cells (wet mass) was incubated at 65°C for 20 min to precipitate unwanted protein from E. coli cells, followed by centrifugation (20,800 × g) at 4°C for 10 min. The supernatant was incubated again at 65°C for 10 min, followed by centrifugation (20,800 × g) at 4°C for 10 min.

(ii) Size exclusion chromatography. The supernatant after heat precipitation (4 ml) was reduced to 1 ml by ultrafiltration (Amicon YM 30 membranes, Millipore) and applied to a 120-ml Highload Superdex 200 16/60 column (Amersham Biosciences) equilibrated with 20 mM Tris-HCl buffer (pH 7.0), 100 mM KCl.

The column was developed at a flow rate of 1 ml min⁻¹. The peak was collected, and the active fraction was concentrated as described above, and glycerol was added to a final concentration of 10% and stored at −20°C. The native mass of the enzyme was estimated using this gel filtration column. The column was calibrated with ferritin (450 kDa), catalase (240 kDa), alcohol dehydrogenase (150 kDa), bovine serum albumin (67 kDa), and ovalbumin (45 kDa) as molecular mass standards.
zyme activity of 5 nmol min\(^{-1}\) of photoheterotrophically grown cells contained a specific enzyme activity not only on L-malyl-CoA, but also catalyzes the reaction L-citramal-CoA → pyruvate + acetyl-CoA (unpublished data). D-Citramal-CoA lyase catalyzes the analogous reaction D-citramal-CoA → pyruvate + acetyl-CoA (Fig. 1) (unpublished data). Therefore, the \textit{C. aurantiacus} genes for the two lyses, which catalyze L-citramal-CoA or D-citramal-CoA cleavage, were heterologously expressed in \textit{E. coli}, and the two proteins were partially purified and added individually in excess as coupling enzymes to the assays for L- and D-citramal-CoA transferase activity. The specific enzyme A transferase activities for L-citramalate and D-citramalate in the presence of excess lye activity were not significantly different from the activities which were measured before with cell extracts. This indicates that the CoA transferase(s) is upregulated under autotrophic conditions. We expect two different enzymes that specifically act on the L- or D-stereoisomer.

**Cloning of a putative succinyl-CoA::citramalate coenzyme A transferase gene** \( \text{(set)} \), \textit{overexpression in E. coli, and proof of function}. In a previous work, we showed that purified succinyl-CoA::malate coenzyme A transferase (Smt) from \textit{C. aurantiacus} also activates L-citramalate to its CoA thioester with the use of succinyl-CoA as the CoA donor (15). This enzyme activity was upregulated under autotrophic conditions, and its side activity therefore can account for the observed high succinyl-CoA::citramalate coenzyme A transferase activity in extracts of autotrophically grown cells. The nature of the other CoA transferase that was also upregulated and specifically act on the L- and D-citramalate in the presence of excess lye activity was not significantly different from the activities which were measured before with cell extracts. This indicates that the CoA transferase(s) is upregulated under autotrophic conditions. We expect two different enzymes that specifically act on the L- or D-stereoisomer.

**RESULTS**

Pyruvate formation from citramalate in the presence of succinyl-CoA in cell extracts. Cell extracts of photoautotrophically grown \textit{C. aurantiacus} (supernatant obtained by centrifugation at 100,000 \( \times \) g) catalyzed the citramalate- and succinyl-CoA-dependent formation of pyruvate and acetyl-CoA at 55°C, the optimal growth temperature of the organism. The coupled spectrophotometric assay followed the formation of the phenylhydrazone of pyruvate that is released from citramalyl-CoA (Fig. 1). The specific enzyme activity in extracts of autotrophically grown cells was 40 to 60 nmol min\(^{-1}\) 1 mg protein\(^{-1}\) with L-citramalate and 11 to 13 nmol min\(^{-1}\) mg protein\(^{-1}\) with D-citramalate, depending on the batch of cells. Extracts of photoheterotrophically grown cells contained a specific enzyme activity of 5 nmol min\(^{-1}\) mg protein\(^{-1}\) with L-citramalate and 2 nmol min\(^{-1}\) mg protein\(^{-1}\) with D-citramalate. This shows that the enzyme systems catalyzing D- and L-citramalate conversion to acetyl-CoA and pyruvate are upregulated under autotrophic conditions and therefore are likely to play a role in the \( \text{CO}_2 \) assimilation pathway. Yet, it could not be decided whether the presumed CoA transferase(s) or the citramalyl-CoA lyase(s) was rate limiting in the assay.

Succinyl-CoA::citramalate coenzyme A transferase activity in cell extracts. A previously characterized L-malyl-CoA lyase was visualized by Coomassie blue staining (48). Protein levels were determined (SDS-PAGE; 12.5%) was performed as described by Laemmli (29). Proteins were visualized by Coomassie blue staining (48). Protein levels were determined by the method of Bradford (5), using bovine serum albumin as the standard.

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**TABLE 1. Primers used for RT-PCR**

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<td>pol_rev</td>
<td>TGAAGGCAAGGTGACTGATCAG</td>
<td>RT-PCR, fragment 8</td>
</tr>
</tbody>
</table>

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Pyruvate formation from citramalate in the presence of succinyl-CoA in cell extracts. Cell extracts of photoautotrophically grown \textit{C. aurantiacus} (supernatant obtained by centrifugation at 100,000 \( \times \) g) catalyzed the citramalate- and succinyl-CoA-dependent formation of pyruvate and acetyl-CoA at 55°C, the optimal growth temperature of the organism. The coupled spectrophotometric assay followed the formation of the phenylhydrazone of pyruvate that is released from citramalyl-CoA (Fig. 1). The specific enzyme activity in extracts of autotrophically grown cells was 40 to 60 nmol min\(^{-1}\) 1 mg protein\(^{-1}\) with L-citramalate and 11 to 13 nmol min\(^{-1}\) mg protein\(^{-1}\) with D-citramalate, depending on the batch of cells. Extracts of photoheterotrophically grown cells contained a specific enzyme activity of 5 nmol min\(^{-1}\) mg protein\(^{-1}\) with L-citramalate and 2 nmol min\(^{-1}\) mg protein\(^{-1}\) with D-citramalate. This shows that the enzyme systems catalyzing D- and L-citramalate conversion to acetyl-CoA and pyruvate are upregulated under autotrophic conditions and therefore are likely to play a role in the \( \text{CO}_2 \) assimilation pathway. Yet, it could not be decided whether the presumed CoA transferase(s) or the citramalyl-CoA lyase(s) was rate limiting in the assay.

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class III CoA transferases. Four genes were identified, two of which were previously shown to encode the two subunits of succinyl-CoA:L-malate CoA transferase (15). A putative CoA transferase gene coding for a 44-kDa protein (402 amino acids) is located in a gene cluster (GenBank accession number NZ_AAAH02000037) next to the gene for a putative 3-hydroxy-3-methylglutaryl-CoA lyase (which turned out to code for D-citramalyl-CoA lyase and was termed ccl [unpublished results]) (Fig. 2).

A 2,169-bp DNA fragment that contained both genes, for the putative CoA transferase sct and the D-citramalyl-CoA lyase ccl, was cloned and expressed in E. coli. Cell extract was heat precipitated, because succinyl-CoA: D-citramalate coenzyme A transferase activity in cell extracts of C. aurantiacus tolerated 30 min of incubation at 65°C. The soluble supernatant was analyzed by SDS-PAGE. It contained a strongly induced protein band that migrated at approximately 40 kDa (Fig. 3); apparently, the gene coding for the lyase (expected size of the gene product, 34 kDa) was only weakly expressed. The supernatant exhibited high succinyl-CoA:D-citramalate coenzyme A transferase activity (6.8 μmol min⁻¹ mg protein⁻¹); this activity was missing in heat-treated cell extract from recombinant E. coli cells lacking the DNA insert. These results indicate that the sct gene codes for succinyl-CoA: D-citramalate CoA transferase.

**Purification of the enzyme and molecular properties.** Sct was further purified by gel filtration (Table 2) to apparent homogeneity (Fig. 3). By SDS-PAGE, only one protein of 40 kDa was observed. This molecular mass differed from the predicted one, which was 44 kDa. A discrepancy in molecular mass was also observed for succinyl-CoA:L-malate CoA transferase (15). The purified enzyme had a specific activity of 17.5 μmol min⁻¹ mg protein⁻¹. Comparison of this specific activity for the purified enzyme with the specific activity observed in cell extract of autotrophically grown C. aurantiacus (13 nmol min⁻¹ mg protein⁻¹) indicated that the CoA trans-

![Diagram](http://example.com/diagram.png)

**FIG. 4.** Formation of D-citramalyl-CoA and further of acetyl-CoA and pyruvate from D-citramalate by the combined action of recombinant succinyl-CoA:D-citramalate CoA transferase and recombinant D-citramalyl-CoA lyase at 55°C. (A) HPLC detection of CoA-thioesters after 5 min of incubation with recombinant succinyl-CoA:D-citramalate CoA transferase (I), in a control experiment as in panel I but with D-citramalate omitted (II), with recombinant succinyl-CoA:D-citramalate CoA transferase and recombinant D-citramalyl-CoA lyase (III), and in a control experiment as in panel III but with D-citramalate omitted (IV). Retention times: 10.8 min for D-citramalyl-CoA, 11.6 min for succinyl-CoA and free CoA, and 17.8 min for acetyl-CoA. The reaction mixture (0.5 ml) for experiments I and II contained 200 mM MOPS-KOH buffer (pH 6.5), 10 mM D-citramalate, 1 mM succinyl-CoA, and 10 μg of succinyl-CoA:D-citramalate CoA transferase and recombinant D-citramalyl-CoA lyase (0.18 units). The reaction mixture for experiments III and IV contained in addition 5 mM MgCl₂ and 60 μg of D-citramalyl-CoA lyase (0.06 units). (B) Spectrophotometric assays for pyruvate-phenylhydrazone formation dependent on succinyl-CoA (1 mM) and D-citramalate (10 mM) at 55°C. In experiment III, the addition of 10 mM L-citramalate could not start the reaction. The assay mixture (0.5 ml) contained 200 mM MOPS-KOH buffer (pH 6.5), 5 mM MgCl₂, 3.5 mM phenylhydrazinium chloride, (0.003 units to 0.02 units) succinyl-CoA:D-citramalate CoA transferase, and excess (0.05 units) D-citramalyl-CoA lyase. (C) Stoichiometry of pyruvate-phenylhydrazone formation in the spectrophotometric assay, with dependence on the amount of added D-citramalate, when D-citramalyl-CoA lyase was added in excess.

<table>
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<th>Purification step</th>
<th>Total enzyme activity (μmol min⁻¹)</th>
<th>Total protein (mg)</th>
<th>Sp act (μmol min⁻¹ mg protein⁻¹)</th>
<th>Recovery (%)</th>
<th>Purification (n-fold)</th>
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* Purification started from 26 mg of total protein.
ferase represents approximately 1% of the soluble protein of autotrophically grown cells.

Size exclusion chromatography resulted in a molecular mass of 90 ± 10 kDa, indicating that the native protein was a homodimer of 44-kDa subunits. The UV-visible spectrum had a protein absorption maximum at 280 nm (ε280 = 112 mM⁻¹ cm⁻¹) for the a subunit. The extinction coefficient was similar to the one calculated from the deduced amino acid sequence for the Sct gene (ε280 = 124 mM⁻¹ cm⁻¹). The enzyme could be stored for 1 week at 4°C without significant loss of activity at pH 7 in the presence of 100 mM KCl or kept frozen for months in the presence of 10% glycerol (vol/vol). It appeared not to contain any cofactor or to need additional cofactors for activity (see below).

Catalytic properties. The enzymatic conversion of succinyl-CoA at 55°C by succinyl-CoA:D-citramalate CoA transferase in the absence and presence of d-citramalate was studied (Fig. 4A). After 5 min of incubation, approximately half of the succinyl-CoA was converted to a new product, as studied by HPLC analysis, when d-citramalate was added (Fig. 4A, panel I). No such product was formed when l-citramalate was added or when d-citramalate was omitted (Fig. 4A, panel II). The protein and time dependence of the reaction were followed using the coupled spectrophotometric assay including d-citramal-CoA lyase (Fig. 4B). The reaction was linearly protein dependent in a large range, as long as lyase activity was present in excess. The reaction was linearly time dependent for minutes (Fig. 4B). This indicates that the enzyme was reasonably stable in diluted form at 55°C. The stoichiometry of the reaction was approximately 1 mol pyruvate phenylhydrazone formed per mol d-citramalate added, using an excess of succinyl-CoA (Fig. 4C), indicating that the equilibrium of the combined two reactions is on the side of pyruvate formation in the presence of phenylhydrazone.

The enzyme exhibited optimal activity at pH 6.5. Activity did not depend on Mg²⁺ and was not inhibited by EDTA, nor was it stimulated by dithioerythritol. The turnover rate per monomer was 13 s⁻¹. The apparent Km values were determined using the coupled spectrophotometric assay under the saturating concentrations for the cosubstrates (succinyl-CoA, 1 mM; d-citramalate, 10 mM) and were 0.08 mM for succinyl-CoA and 2.2 mM for d-citramalate. As indicated above, the enzyme was specific for d-citramalate. It was inactive with l-citramalate, l-malate, mesaconate, acetoacetate, adipate, β-hydroxy-β-methylglutarate, citrate, and methylmalonate, as tested by HPLC assay. However, d-citramalate could be partially substituted by D-malate, itaconate, and β-erythro-methylmalate. As a CoA donor, only succinyl-CoA could be used; the enzyme was inactive with propionyl-CoA, malonyl-CoA, acetyl-CoA, and l-malyl-CoA.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value or description for Sct</th>
</tr>
</thead>
<tbody>
<tr>
<td>Substrates</td>
<td>d-Citramalate, succinyl-CoA</td>
</tr>
<tr>
<td>Products</td>
<td>D-Citramalyl-CoA, succinate</td>
</tr>
<tr>
<td>Apparent Km values</td>
<td>0.08 mM (succinyl-CoA), 2.2 mM (d-citramalate)</td>
</tr>
<tr>
<td>Catalytic no. (per monomer)</td>
<td>13 s⁻¹</td>
</tr>
<tr>
<td>pH optimum</td>
<td>6.5 (55°C)</td>
</tr>
<tr>
<td>Native molecular mass</td>
<td>90 kDa</td>
</tr>
<tr>
<td>Subunit molecular mass</td>
<td>44 kDa</td>
</tr>
<tr>
<td>Sp act</td>
<td>17.5</td>
</tr>
<tr>
<td>Products</td>
<td>D-Citramalyl-CoA, succinate</td>
</tr>
<tr>
<td>Apparent Km values</td>
<td>80 μM ± 20 μM (succinyl-CoA), 2.2 mM (d-citramalate)</td>
</tr>
</tbody>
</table>

Mechanistic studies. Three enzyme families are known to catalyze CoA transferase reactions with the use of various CoA donors and acceptors (3, 6–8, 11, 12, 16–18, 27, 28, 30, 31, 36, 37, 39, 43). Enzymes of family I use a ping-pong mechanism and are specifically inactivated by millimolar concentrations of borohydride or hydroxylamine in the presence of the CoA donor. Enzymes of family II are subunits of complex lyases and contain a covalently bound (5′-phosphoribosyl)-3′-dephospho-CoA moiety; these features obviously do not apply to our enzyme. Enzymes of family III seem to use a different mechanism, and they are insensitive to borohydride and hydroxylamine. Therefore, we tested the effect of these compounds on succinyl-CoA:D-citramalate CoA transferase. When the enzyme was preincubated for 10 min in the presence of 1 mM succinyl-CoA and 10 mM borohydride or 10 mM hydroxylamine, more than 80% of the activity was retained. This suggests that the enzyme may belong to family III. This assignment is corroborated by sequence comparisons (15) (see Discussion).

Gene organization and cotranscription. The organization of different ORFs near the sct gene on a cluster of eight genes, which are orientated in the same direction, is shown in Fig. 2. Cotranscription of ORFs during autotrophic growth of C. aurantiacus was studied by performing RT-PCR experiments with mRNA from autotrophically grown cells, and the results were compared with the results obtained with the use of genomic DNA from C. aurantiacus as a positive control.

FIG. 5. Structures of different substrates that were tested as potential CoA acceptors by using the HPLC assay.
The product of the reversible transferase reaction is specifically utilized by a d-citramalyl-CoA lyase. This enzyme activity is also induced under autotrophic conditions (unpublished results), and the genes for CoA transferase sct and lyase ccl are cotranscribed.

There is one intriguing observation that needs explanation. Extracts also catalyzed the two-step conversion of L-citramalate to pyruvate and acetyl-CoA, and the responsible enzymes were also upregulated under autotrophic conditions. Their specific activities were even higher than those for the d-citramalate-specific metabolic steps. This apparent discrepancy may be explained as follows. Succinyl-CoA:L-malate CoA transferase (Smt) is an essential enzyme preparing L-malate for cleavage by L-malyl-CoA lyase. We have shown (15) that succinyl-CoA:L-malate CoA transferase acts not only on L-malate, but also on L-citramalate. Furthermore, L-malyl-CoA lyase acts not only on L-malyl-CoA, but also on L-citramalyl-CoA (unpublished data). The respective d-isomers are not used by the two enzymes. The upregulation of the l-specific enzymes under autotrophic conditions is explained by the promiscuity of the l-specific CoA transferase Smt and l-malyl-CoA lyase. The presence and regulation of enzymes acting specifically on the d-isomers of citramalate and citramalyl-CoA are taken as indication that the d-isomers may represent natural intermediates in the glyoxylate assimilation cycle. However, the possibility that both stereoisomers were formed in the course of β-methylmalyl-CoA conversion to citramalate, which would then require two sets of CoA transferases and lyases, cannot be excluded. A final decision requires the knockout of genes, which is a difficult task with this organism.

Succinyl-CoA:d-citramalate CoA transferase shows a strong preference for its natural CoA donor and for d-enantiomers of the acceptor molecules. The CoA acceptor could partially be replaced by d-malate, itaconate, and β-erythro-methylmalate. This suggests that the enzyme is active with C4 dicarboxylic acids that are substituted at C-2 (and C-3), provided that the d-conformation is given. It remains to be shown whether itaconate activation has a physiological function and which of the two carboxyl groups reacts with CoA.

The conversion of d-L-citramalate and succinyl-CoA to pyruvate and acetyl-CoA has been reported earlier for Pseudomonas sp. (10), but the stereospecificity of this reaction was not studied. This system is involved in itaconate utilization.
Why is a CoA transferase needed for the conversion of \(\beta\)-erythro-methylmalonyl-CoA to citramalate-CoA? As can be seen in Fig. 1, \(\beta\)-methylmalonyl-CoA carries the CoA thioester group at the carboxyl group at the \(\beta\) position to the methyl group. In contrast, citramalyl-CoA carries CoA at the carboxyl group at the \(\gamma\) position. The CoA moiety therefore needs to be transferred from the “upper” to the “lower” carboxyl group. Hydrolysis of the energy-rich thioester bond and resynthesis would require one or two ATP equivalents. We therefore anticipated that the CoA group for the precursor of \(\alpha\)-citramalate, mesaconyl-CoA or a compound derived from it, is scavenged in the same way by transfer to succinate. So far, only \(\beta\)-methylmalonyl-CoA dehydratase, which catalyzes the reaction \(\beta\)-erythro-methylmalonyl-CoA \(\rightarrow\) mesaconyl-CoA + H\(_2\)O, has been identified in this interconversion of \(\alpha\), \(\beta\), and \(\gamma\) compounds (B. Alber and G. Fuchs, unpublished results). In other words, another CoA transferase is expected to operate in this process.

Neither the presently studied CoA transferase nor succinyl-CoA: \(\alpha\)-malate coenzyme A transferase acted on mesaconate.

We found another ORF in the incomplete genome sequence of *Chloroflexus aurantiacus* encoding a putative CoA transferase of 45 kDa. The gene is located on genome segment NZ_AAAH020000019 (15). The 1,227-bp DNA fragment was also cloned and expressed in *E. coli*. After heat precipitation, the heterologously expressed protein still was in the supernatant. We checked this putative CoA transferase for activity with itaconate, mesaconate, \(\delta\)-/\(\epsilon\)-malate, and \(\delta\)-/\(\epsilon\)-citramalate as the CoA acceptor and acetyl-CoA and succinyl-CoA as the CoA donor, but the protein was inactive in all cases (S. Friedmann, B. Alber, and G. Fuchs, unpublished results). Hence, the question of how citramalate is formed from mesaconyl-CoA is unsolved.

**Succinyl-CoA: \(\alpha\)-citramalate CoA transferase, a member of the class III CoA transferases.** The inactivation experiments and sequence comparisons indicate that succinyl-CoA: \(\alpha\)-citramalate CoA transferase belongs to the class III enzymes (for details, see references 15 and 18). The identities/similarities of the amino acids to different representatives of this family are as follows: SmtA (GenBank accession number ABF14399), 41%/61%; SmtB (GenBank accession number ABF14400), 39%/60%; CaiB (GenBank accession number CA52112), 24%/43%; BbsF (GenBank accession number AAF89841), 28%/43%; and FrC (GenBank accession number AAC45298), 24%/42%. A highly conserved aspartate residue (Asp 169 in the CaiB nomenclature), which is located in the active site and binds the organic acid in an anhydride bond (17, 28, 43), is conserved in all. Other residues that are important for folding are conserved as well, namely, Arg 16, Gly 37, Ala 38, Val 40, Asp 90, Leu 184, His 185, Thr 190, and Gly 193 (43), with the exception of Thr 190.

Genes adjacent to the succinyl-CoA: \(\alpha\)-citramalate CoA transferase gene (*sct*) on the chromosome of *C. aurantiacus*. All together, a cluster of six genes, including the *sct* and *ccl* genes (15,400 to 27,500 bp; GenBank accession number NZ_AAAH02000037), forms a transcriptional unit and therefore may play a role in autotrophic carbon metabolism. *orf1* could be a transcriptional regulator gene (COG1802). The proteins encoded by *orf2* and *orf3* have similarities to subunits of hydantoinases (COG0145 and COG0146), and *orf4* belongs to a group of amidase genes (COG1335). No plausible answer can be given yet as to whether these additional genes play a role in CO\(_2\) fixation and, if so, what their role might be.

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