A bicyclic autotrophic CO₂ fixation pathway in _Chloroflexus aurantiacus_

Sylvia Herter*, Georg Fuchs*,‡, Adelbert Bacher†, and Wolfgang Eisenreich†

*Lehrstuhl für Mikrobiologie, Institut Biologie II, Universität Freiburg, Schänzlestr. 1, D-79104 Freiburg, Germany; and †Lehrstuhl für Organische Chemie und Biochemie, Technische Universität München, Lichtenbergstr. 4, D-85747 Garching, Germany

‡To whom correspondence should be addressed. Tel.: +49-761-203-2649; Fax: +49-761-203-2626; Email: fuchsgeo@uni-freiburg.de

Running title: New CO₂ fixation pathway
SUMMARY

Phototrophic CO₂ assimilation by the primitive, green eubacterium *Chloroflexus aurantiacus* has been shown earlier to proceed in a cyclic mode via 3-hydroxypropionate, propionyl-CoA, succinyl-CoA, and malyl-CoA. The metabolic cycle could be closed by cleavage of malyl-CoA affording glyoxylate (the primary CO₂ fixation product) with regeneration of acetyl-CoA serving as the starter unit of the cycle. The pathway of glyoxylate assimilation to form gluconeogenic precursors has not been elucidated to date. We could now show that the incubation of cell extract with a mixture of glyoxylate and [1,2,3-¹³C₃]propionyl-CoA afforded *erythro* β-[1,2,2'-¹³C₃]-methylmalate and [1,2,2'-¹³C₃]citramalate. Similar experiments using a partially purified protein fraction afforded *erythro* β-[1,2,2'-¹³C₃]methylmalyl-CoA and [1,2,2'-¹³C₃]mesaconyl-CoA. Cell extracts of *C. aurantiacus* were also shown to catalyze the conversion of citramalate into pyruvate and acetyl-CoA in a succinyl-CoA-dependent reaction. The data suggest that glyoxylate obtained by the cleavage of malyl-CoA can be utilized by condensation with propionyl-CoA affording *erythro* β-methylmalyl-CoA, which is converted to acetyl-CoA and pyruvate. This reaction sequence regenerates acetyl-CoA, which serves as the precursor of propionyl-CoA in the 3-hydroxypropionate cycle. Autotrophic CO₂ fixation proceeds by combination of the 3-hydroxypropionate cycle with the methylmalyl-CoA cycle. The net product of that bicyclic autotrophic CO₂ fixation pathway is pyruvate serving as an universal building block for anabolic reactions.
FOOTNOTES

*erythro* β-methylmalate ([2R,3S and 2S,3R] 2-hydroxy-3-methylsuccinate or 2-hydroxy-3-methylbutane-1,4-dioate), citramalate (2-hydroxy-2-methylsuccinate), mesaconate (*trans* 2-methylfumarate).

INTRODUCTION

Autotrophic CO₂ fixation in the phototrophic bacterium *Chloroflexus aurantiacus* has been proposed to proceed via a novel pathway, the 3-hydroxypropionate cycle (Fig. 1) [1-7]. Briefly, acetyl-CoA (1) serves as starting unit, and biotin-dependent carboxylation of acetyl-CoA and propionyl-CoA (4) are the main CO₂ fixation reactions. One turn of the proposed cycle results in conversion of acetyl-CoA into malyl-CoA (8) with consumption of 2 HCO₃⁻ and 3 NADPH. Malyl-CoA is cleaved by malyl-CoA lyase with regeneration of the starting molecule acetyl-CoA. Glyoxylate (9) is believed to be the initial CO₂ fixation product [7].

The pathway of glyoxylate assimilation into cell material is incompletely understood [5-12]. Glycine has been ruled out as an intermediate [7]. So far, *in vitro* transformation of glyoxylate has not been observed, except for pyridine nucleotide-dependent reduction to glycolate [7]. An acetyl-CoA-dependent conversion of glyoxylate to malyl-CoA and malate was ascribed to the reverse reaction of malyl-CoA lyase forming malyl-CoA, combined with a side reaction of citrate synthase or acyl-CoA thioesterase, which hydrolyze malyl-CoA to malate and CoA [7, 13-15].
Previous studies have shown that \textit{C. aurantiacus} can use pyruvate for anaplerotic reactions [3, 7, 11, 16]. Pyruvate is converted to phosphoenolpyruvate (PEP) by pyruvate phosphate dikinase, followed by PEP carboxylation to oxaloacetate by PEP carboxylase. However, pyruvate synthase activity was hardly detectable [12], and the origin of pyruvate in \textit{C. aurantiacus} is still unknown. In order to serve as a central intermediate for anaplerotic reactions, it should be formed ultimately from one of the intermediates of the 3-hydroxypropionate cycle and/or from glyoxylate.

The aim of this work was to elucidate reactions for glyoxylate assimilation. We show that a reaction sequence starting with glyoxylate and propionyl-CoA affords acetyl-CoA and pyruvate.

**EXPERIMENTAL PROCEDURES**

**Materials.** Materials were obtained from the commercial sources indicated: \([2-^{14}\text{C}]\)propionate (1.98 MBq \(\mu\text{mol}^{-1}\)) from Hartmann Analytic (Braunschweig, Germany), \([1,2,3-^{13}\text{C}_3]\)sodium propionate (99.9 % \(^{13}\text{C}\) enrichment) from Cambridge Isotope Laboratories (Andover, MA, USA), acetyl-CoA synthetase, L-lactate dehydrogenase, pyruvate kinase, and myokinase from Roche (Basel, Switzerland). Malonyl-CoA was prepared as described in [7]. Succinyl-CoA, acetyl-CoA and propionyl-CoA were synthesized according to published procedures [17, 18].

**Preparation of \textit{erythro} \(\beta\)-methylmalate.** Ethyl-3-methyl-2-oxobutane-1,4-dioate was reduced with sodium borohydride. The product was hydrolyzed affording \textit{erythro} \(\beta\)-
methylmalate [19, 20, 21]. NMR $\delta_H$ (500 MHz, D$_2$O); 4.25 (1H, d, $J = 4.2$ Hz, H-2), 2.95 (1H, m, H-3), 1.05 (3H, d, $J = 7.2$ Hz, methyl).

**Preparation of [2-$^{14}$C]propionyl-CoA.** [2-$^{14}$C]Propionyl-CoA was synthesized according to the protocol described previously for synthesis of [1,2-$^{14}$C]acetyl-CoA [7].

**Preparation of [1,2,3-$^{13}$C$_3$]propionyl-CoA.** A reaction mixture (20 ml) containing 100 mM Tris/HCl-buffer, pH 8.4, 2 mM MgCl$_2$, 3 mM CoA, 3 mM [1,2,3-$^{13}$C$_3$]sodium propionate, 7.5 mM ATP, 4 mM NADH, 10 U acetyl-CoA synthetase, 10 U myokinase, 5 mM PEP, 30 U pyruvate kinase, and 28 U L-lactate dehydrogenase was adjusted to pH 8.4 by addition of KOH. The mixture was incubated at 30 °C. The reaction was monitored photometrically (380 nm). After 80 min, the pH was adjusted to 2 by addition of 6 M HCl. The mixture was centrifuged, and the supernatant was extracted twice with 30 ml diethylether. [2-$^{14}$C$_1$]Propionyl-CoA (18.3 kBq) was added as tracer and the aqueous phase was subjected to reversed phase HPLC (Grom-Sil 120 ODS-4 HE, 250 x 20 mm, 10 µm) (Grom, Herrenberg, Germany). Propionyl-CoA was eluted in a stepwise gradient (4 %, 6 %, 8 %) with 8 % acetonitrile (v/v) in 50 mM potassium phosphate-buffer, pH 6.7, flow rate 8 ml min$^{-1}$, at a retention volume of 180 ml. The fraction containing $^{13}$C- and $^{14}$C-labeled propionyl-CoA was adjusted to pH 2 by adding 6 M HCl, and acetonitrile was evaporated by flash evaporation at 30 °C (30 mbar). The sample was lyophilized and stored at −20 °C. Yield, 50 %.

**Bacterial culture.** *C. aurantiacus* strain OK-70-fl (DSM 636) was grown anaerobically at 55 °C and pH 8 in 12 liter glass fermenters under autotrophic or heterotrophic conditions as described earlier [5, 7, 22].
Preparation of cell extract. Cell extracts were prepared as described previously [7]. The protein content of the cell extracts was determined by the Bradford method [23] and ranged from 15-50 mg protein ml\(^{-1}\).

Partial protein purification. Buffers contained 10% glycerol (v/v). Cell extract prepared from 2 g of autotrophically grown cells was incubated at 65 °C for 10 min and was then centrifuged (20,000x g, 4 °C, 20 min). The supernatant (3.7 ml) was applied to a DEAE Sepharose Fast Flow column (10 ml, Pharmacia, Freiburg, Germany, flow rate 4 ml min\(^{-1}\)), which had been equilibrated with 20 mM MOPS/K\(^+\)-buffer, pH 7.2 (buffer A). The column was washed with 30 ml buffer A followed by 50 ml of buffer A plus 100 mM KCl, 100 ml of buffer A plus 160 mM KCl, 100 ml of buffer A plus 220 mM of KCl, and 100 ml of buffer A plus 500 mM KCl. Most of activity eluted with 100-180 mM KCl in buffer A; these fractions were pooled (25 ml), diluted fourfold with 20 mM MOPS/K\(^+\)-buffer, pH 7.6 (buffer B), and applied in 2 runs each to a Resource Q column (Pharmacia; 1 ml, flow rate 5 ml min\(^{-1}\)), which had been equilibrated with buffer B. The column was washed with 4 ml buffer B, and developed with a gradient from buffer B alone to buffer B plus 300 mM KCl over 20 ml. Active fractions (130-250 mM KCl) were pooled (8 ml) and stored at –20°C.

CoA release from propionyl-CoA in presence of glyoxylate. The formation of free CoA from propionyl-CoA after addition of glyoxylate was followed spectrophotometrically (412 nm) with 5,5'-dithiobis(2-nitrobenzoate) (DTNB; \(\epsilon_{412} = 13,600\ M^{-1}\ cm^{-1}\)). The assay mixture (0.5 ml) contained 200 mM MOPS/K\(^+\)-buffer, pH 7.5, 0.25 mM DTNB, 2 mM MgCl\(_2\), 1 mM propionyl-CoA, 5 mM glyoxylate, and 5-50 µl cell extract (0.1-1.0 mg protein) or 100 µl partially purified protein fraction (0.1 mg protein).
Conversion of \([1,2,3-^{13}C_3]\)propionyl-CoA by cell extract. A reaction mixture (40 ml) containing 100 mM potassium phosphate-buffer, pH 7.3, 1 mM \([1,2,3-^{13}C_3]\)propionyl-CoA, 1 mM glyoxylate, 2 mM MgCl_2, 36.6 kBq \([2-^{14}C]\)propionyl-CoA, and 2 ml cell extract (48 mg protein) was incubated at 55 °C. After 50 min, 200 ml of ethanol were added and protein was removed by centrifugation. The supernatant was concentrated by flash evaporation at 30 °C (20 mbar) and the pH was adjusted to pH 10 by addition of NaOH. The solution was extracted with diethylether (100 ml) and the aqueous phase was applied onto a column of DOWEX WX8 50 (H⁺-form, 10 g) (Serva, Heidelberg, Germany). The column was developed with 50 ml of water. The eluate was adjusted to pH 4.0 by addition of a 10 % NH₃ solution (v/v) and concentrated to 2 ml by flash evaporation at 30 °C (20 mbar). Aliquots (50 µl) were applied onto a Polyspher OA HY column (300 x 6.5 mm; Merck, Darmstadt, Germany), which was developed with 1 mM H₂SO₄, flow rate 0.8 ml min⁻¹. The effluent was monitored by a radiomonitor (Ramona, Raytest, Straubenhardt, Germany) and photometrically (210 nm). A radioactive fraction eluting at a retention volume of 6.4 ml was adjusted to pH 6 by addition of 2 M NaOH, lyophilized and stored at –20 °C. Retention volumes of reference samples: glyoxylate, 5.7 ml; citramalate, 6.2 ml; \(\text{erythro} \ \beta\)-methylmalate, 6.7 ml; propionate, 9.0 ml; mesaconate, 12.7 ml.

Conversion of \([1,2,3-^{13}C_3]\)propionyl-CoA by a partially purified protein fraction. A reaction mixture (20 ml) containing 100 mM potassium phosphate-buffer, pH 7.3, 2 mM glyoxylate, 2 mM MgCl_2, 1.2 mM \([1,2,3-^{13}C_3]\)propionyl-CoA, 54.7 kBq \([2-^{14}C]\)propionyl-CoA and 5 ml partially purified protein fraction (8 mg protein) was incubated at 55 °C. After 10 min, the mixture was adjusted to pH 2 by addition of 6 M HCl. Protein was removed by centrifugation. The supernatant was lyophilized, dissolved in 5 ml of water and applied onto a reversed phase column (Grom-Sil 120 ODS-4 HE, 250 x 20 mm, 10 µm), which was
developed by a step gradient (64 ml each) of 1 %, 2.9 %, 4.8 %, 5.7 %, 6.7 %, and 20 % acetonitrile (v/v) in 50 mM potassium phosphate-buffer, pH 6.7, flow rate 8 ml min⁻¹. The effluent was monitored by a radiomonitor and photometrically (210 nm). Radioactive fractions eluted at retention volumes of 288 ml and 320 ml, respectively, were adjusted to pH 2 by addition of 6 M HCl and lyophilized.

**Citramalate conversion to pyruvate and acetyl-CoA in presence of succinyl-CoA.** Assay mixtures (0.5 ml) containing 200 mM MOPS/K⁺-buffer, pH 7.0, 5 mM MgCl₂, 3.5 mM phenylhydrazine hydrochloride, 1 mM succinyl-CoA, 5 mM L- or D-citramalate, and 5-25 µl cell extract (0.1-0.5 mg protein) were monitored photometrically (324 nm) at 55 °C. Pyruvate-phenylhydrazone formation was followed (ε₃₂₄ = 11,520 M⁻¹ cm⁻¹). In control experiments, succinyl-CoA was omitted or replaced by 1 mM acetyl-CoA, propionyl-CoA or malonyl-CoA. Alternatively, reaction mixtures (0.5 ml) containing 200 mM ammonium bicarbonate, pH 7.8, 5 mM MgCl₂, 1 mM succinyl-CoA, 10 mM L-citramalate, and 60 µl cell extract (1.0 mg protein) were incubated at 55 °C. Aliquots (0.1 ml) were retrieved at intervals and were mixed with 10 µl conc. HCl. Protein was removed by centrifugation and the supernatant was analyzed by reversed phase HPLC (LiChrospher 100, endcapped, 125 x 4 mm, 5 µm, Merck). The column was developed by a gradient of 1 %-8 % acetonitrile over 30 min in 50 mM potassium phosphate-buffer, pH 6.7, flow rate 1 ml min⁻¹. The effluent was monitored photometrically (260 nm) and acetyl-CoA eluted at a retention volume of 19 ml.

**NMR spectroscopy.** Samples were dissolved in D₂O at pH 6 (uncorrected glass electrode reading). ¹H and ¹³C NMR spectra were measured at 20 °C using a four-channel Bruker DRX 500 spectrometer (Bruker, Karlsruhe, Germany). One-dimensional experiments and two-dimensional HMQC, HMQC-TOCSY and HMBC experiments were performed according to
standard Bruker software (XWINNMR). The duration of the $^1$H spin-lock was 60 ms in the HMQC-TOCSY experiment.

RESULTS

Condensation of glyoxylate with propionyl-CoA. The condensation of glyoxylate with propionyl-CoA with formation of methylmalate has been reported in Rhodospirillum rubrum and Bacillus sp. [24-26], but had not been observed in C. aurantiacus. Our preliminary experiments showed that cell extracts of C. aurantiacus could form free CoA in reaction mixtures containing propionyl-CoA and glyoxylate. The glyoxylate-dependent release of CoA from propionyl-CoA was catalyzed by cell extracts of autotrophically grown cells at a specific rate of 36 nmol min$^{-1}$ mg$^{-1}$ cell protein, by cell extracts of heterotrophically grown cells at 4 nmol min$^{-1}$ mg$^{-1}$ protein. The release of CoA set in after a lag phase of 1-2 min (cell extract) and after up to 5 min (partially purified protein fraction). In order to study that reaction in more detail, we incubated cell extract of C. aurantiacus with a mixture of 1 mM glyoxylate, 1 mM [1,2,3-$^{13}$C$_3$]propionyl-CoA and traces of [2-$^{14}$C]propionyl-CoA. Radioactive reaction products were isolated by HPLC and were analyzed by one- and two-dimensional NMR spectroscopy.

The $^{13}$C NMR spectrum displayed nine multiplets due to $^{13}$C-$^{13}$C-coupling, which could be assigned to three multiply $^{13}$C-labeled compounds (Fig. 2). A set of three signals (205.7, 37.3, 9.4 ppm, Fig. 2A, Table 1: Experiment A) was attributed to [1,2,3-$^{13}$C$_3$]propionyl-CoA (4) by comparison with the NMR signals of authentic material. The remaining six multiplets were assigned to erythro β-[1,2,2'-$^{13}$C$_3$]methylmalate (11) and [1,2,2'-$^{13}$C$_3$]citramalate (10) as
described below. Both metabolites were characterized by doublets at chemical shift ranges typical for carboxylic atoms (181.4 and 179.4 ppm). The signal at 181.4 showed $^{13}$C-coupling with the double-doublet at 73.6 ppm (coupling constant, 57 Hz), whereas the signal at 179.4 ppm was $^{13}$C-coupled with the double-doublet at 44.4 ppm (coupling constant, 54 Hz). Both double-doublets showed additional couplings to doublets resonating at chemical shifts typical for methyl atoms (25.9 and 12.9 ppm, Table 1).

Information about the respective $^1$H and $^{13}$C spin networks was gleaned from two-dimensional $^1$H$^{13}$C-correlation experiments. Specifically, HMQC experiments revealed information about H atoms directly connected to $^{13}$C atoms, HMQC-TOCSY experiments showed couplings between H atoms when at least one observed $^1$H atom was directly connected to a $^{13}$C atom, and HMBC experiments highlighted $^1$H$^{13}$C long-range couplings via two or three bonds. In conjunction with the chemical shifts, the correlation patterns summarized in Table 1 establish the structural fragments boxed in Fig. 2. Since the HPLC retention times of 11 and 10 and authentic samples of erythro $\beta$-methylmalate and citramalate, respectively, were almost identical (see Materials and Methods), it appears safe to conclude that the elusive residues in 11 and 10 are carboxylic atoms. Further confirmation was achieved by addition of authentic erythro $\beta$-methylmalate to the NMR sample. Signals assigned to erythro $\beta$-[1,2,2'-$^{13}$C$_3$]methylmalate were selectively enhanced in HMQC and HMQC-TOCSY experiments.

Since cell extracts of C. aurantiacus were expected to contain substantial amounts of thioesterases, we supposed that the immediate products of glyoxylate assimilation were CoA-thioesters, which were subsequently cleaved into the free acids detected in the experiments described above. We therefore incubated a partially purified protein fraction of C. aurantiacus, putatively containing less thioesterases, with a mixture of 2 mM glyoxylate and
1.2 mM [1,2,3-\textsuperscript{13}C_3]propionyl-CoA containing trace amounts of [2-\textsuperscript{14}C]propionyl-CoA. HPLC analysis showed two fractions containing 40% of the proffered radioactivity. The elution conditions were typical for CoA derivatives. The fraction eluted at a retention time of 36 min (12) showed three \textsuperscript{13}C multiplets (Table 1: Experiment B). A doublet centered at 204.5 ppm was suggestive of a thioester carbonyl atom. The \textsuperscript{1}H NMR spectrum confirmed the presence of a CoA residue (data not shown). Two-dimensional \textsuperscript{1}H\textsuperscript{13}C experiments (HMQC and HMQC-TOCSY, Fig. 3) identified a β-methylmalyl spin system carrying \textsuperscript{13}C in position 2 and 2′ but not 3. The HMQC spectrum (Fig. 3A) showed correlations of \textsuperscript{13}C-2 and \textsuperscript{13}C-2′ with their directly attached protons. In the HMQC-TOCSY spectrum (Fig. 3B) of the same sample, extended \textsuperscript{1}H spin systems connected by \textsuperscript{1}H TOCSY transfer are correlated to individual carbon atoms. Thus, C-2 (\textsuperscript{13}C NMR signal at 51.6 ppm) showed correlation to the directly attached H-2 proton (\textsuperscript{1}H NMR signal at 3.0 ppm) as well as to H-2′ (\textsuperscript{1}H NMR signal at 1.0 ppm) and to H-3 (\textsuperscript{1}H NMR signal at 4.1 ppm). Confirmation of the \textsuperscript{1}H spin system comprising H-2, H-2′, and H-3 was obtained from HMQC-TOCSY correlations of C-2′ to the directly attached H-2′ protons as well as to H-2 and H-3 protons (Fig. 3B). Due to sensitivity reasons additional correlations of carbon atoms belonging to the CoA moiety were observed in the HMQC-TOCSY spectrum (Fig. 3B). On this basis, 12 was assigned as β-[1,2,2′-\textsuperscript{13}C_3]methylmalyl-CoA. In the experiment with cell extracts of \textit{C. aurantiacus erythro} β-[1,2,2′-\textsuperscript{13}C_3]methylmalate (11) was identified as a reaction product (see above) and, therefore, it appears plausible that 12 is the \textit{erythro} form of β-[1,2,2′-\textsuperscript{13}C_3]methylmalyl-CoA. The fraction eluted at 40 min (13) displayed three \textsuperscript{13}C multiplets (Table 1: Experiment B, Fig. 4) at chemical shifts suggesting a thioester carbonyl atom (197.7 ppm), an olefinic carbon (149.6 ppm) and a methyl atom (14.5 ppm). Correlations observed in HMQC and HMQC-TOCSY experiments (Table 1) identified the molecular fragment boxed in Fig. 4. The \textsuperscript{1}H NMR
spectrum indicated the presence of a CoA residue. On this basis, 13 was assigned as $[1,2,2'-\text{C}_3]\text{mesaconyl-CoA}$.

**Conversion of L-citramalate to pyruvate and acetyl-CoA.** Buckel, Dimroth and their associates reported the cleavage of citramalate or citramalyl-CoA into pyruvate and acetate or acetyl-CoA [27, 28], respectively. We found that cell extract of *C. aurantiacus* could cleave L-citramalate in the presence of succinyl-CoA with formation of acetyl-CoA and pyruvate. The rate of pyruvate formation was 63 nmol min$^{-1}$ mg$^{-1}$ with cell extracts of autotrophically grown cells and 3 nmol min$^{-1}$ mg$^{-1}$ with cell extracts of heterotrophically grown cells. D-Citramalate was transformed at a rate of 24 nmol min$^{-1}$ mg$^{-1}$ by extracts of autotrophically grown cells. Acetyl-CoA formation was followed by HPLC analysis. Succinyl-CoA could not be replaced by acetyl-CoA, propionyl-CoA, or malonyl-CoA as CoA donor.

The results suggest that succinyl-CoA functions as CoA donor giving rise to citramalyl-CoA. This is in line with a short lag phase when the assay was performed with cell extract. This suggests that the reaction catalyzed is due to two enzymes, a CoA transferase and a citramalyl-CoA lyase, catalyzing reaction (1) and (2), respectively.

(1) Succinyl-CoA + citramalate $\rightleftharpoons$ succinate + citramalyl-CoA
(2) citramalyl-CoA $\rightleftharpoons$ acetyl-CoA + pyruvate.

**DISCUSSION**

The present study aimed at elucidating the fate of glyoxylate in autotrophic CO$_2$ fixation in *C. aurantiacus*. We obtained evidence for glyoxylate condensation with propionyl-CoA. The following experiments were designed to detect and identify enzyme products formed from
glyoxylate and propionyl-CoA under in situ conditions without prior isolation in order to minimize the risk of decomposition or structural modification. For this purpose, we used [1,2,3-\textsuperscript{13}C\textsubscript{3}]propionyl-CoA as the substrate in order to enhance the sensitivity and selectivity of \textsuperscript{13}C NMR analysis in crude reaction mixtures. Any products formed from the uniformly \textsuperscript{13}C-labeled propionate without breakage of \textsuperscript{13}C-\textsuperscript{13}C bonds must contain a group of three contiguous \textsuperscript{13}C atoms, which form a spin system that is easily assigned via \textsuperscript{13}C-\textsuperscript{13}C coupling. Moreover, the \textsuperscript{13}C spin system can be extended to identify \textsuperscript{1}H atoms bound directly to one of the respective \textsuperscript{13}C atoms. Using two-dimensional \textsuperscript{1}H-\textsuperscript{13}C correlation techniques, the spin system can be extended still further to include \textsuperscript{1}H atoms bound to one of the \textsuperscript{13}C-labeled positions via two or three bonds. This approach enabled us to assign the structures of enzyme products resulting from condensation of the \textsuperscript{13}C-labeled propionate moiety with glyoxylate (Fig. 5). We could show that [1,2,3-\textsuperscript{13}C\textsubscript{3}]propionyl-CoA (4) can be condensed with glyoxylate (9) to form \textit{erythro} \textbeta-[1,2,2'-\textsuperscript{13}C\textsubscript{3}]methylmalyl-CoA (12), which can be transformed further to [1,2,2'-\textsuperscript{13}C\textsubscript{3}]mesaconyl-CoA (13) (Fig. 5) and [1,2,2-\textsuperscript{13}C\textsubscript{3}]citramalate (10) or citramalyl-CoA (14). We could also show that L-citramalate (10) can be cleaved to acetyl-CoA (1) and pyruvate (15) when succinyl-CoA is present as a CoA donor. This suggests that L-citramalyl-CoA (14) is an intermediate. The enzyme activities are substantially higher in autotrophically grown cells as compared to heterotrophically grown cells. This regulatory pattern suggests that these reactions are part of the autotrophic carbon metabolism of \textit{C. aurantiacus}. The formation of \textit{erythro} \textbeta-methylmalate (11) by cell extract is believed to be due to the action of ubiquitous thioesterases.

In summary, this sequence of reactions results in the conversion of glyoxylate (9) and propionyl-CoA (4) into acetyl-CoA (1) and pyruvate (15) via mesaconyl-CoA (13) (Fig. 5). Propionyl-CoA and glyoxylate are both believed to be formed in the CO\textsubscript{2} fixation cycle of \textit{C}.
The cyclic reactions described in the present study enable the net formation of pyruvate from three carbon dioxide molecules with regeneration of acetyl-CoA serving as starter molecule (Fig. 6A). Hence, a bicyclic autotrophic pathway is operating. More specifically, passage of an acetate moiety through the inner cycle in Fig. 6A in the counterclockwise direction affords glyoxylate with regeneration of acetyl-CoA, which had served as starter unit; in other words, the inner cycle is closed. The passage of acetyl-CoA through the initial part of the inner cycle in Fig. 6A affords propionyl-CoA, which can be converted to erythro β-methylmalyl-CoA by condensation with glyoxylate by passage through the outer cycle in Fig. 6A in clockwise direction. Cleavage of citramalyl-CoA in the outer cycle affords pyruvate, again with regeneration of acetyl-CoA, which had served as starter unit; hence, the outer cycle is also closed. A similar but reverse reaction sequence (citramalate cycle) was proposed for the generation of glyoxylate from acetate grown *Rhodospirillum rubrum* [29].

The inner cycle in Fig. 6A (3-hydroxypropionate cycle) requires acetyl-CoA carboxylase [4, 5, 7], malonyl-CoA reductase [30], and propionyl-CoA synthase [31], which have all been shown to be present in *C. aurantiacus*. Some enzymes of the outer cycle (methylmalyl-CoA cycle) have been demonstrated in the present study but need to be studied in the future. One of the propionyl-CoA molecules (4) is carboxylated to methylmalonyl-CoA (5) by propionyl-CoA carboxylase and further converted to malyl-CoA (8) (Fig. 1 and 6A, B). Malyl-CoA in turn is cleaved by malyl-CoA lyase, regenerating acetyl-CoA (1) and releasing glyoxylate (9). Glyoxylate condenses with the second molecule of propionyl-CoA (4) and finally yields back the second molecule of acetyl-CoA (1) and forms pyruvate (15) as net CO₂ assimilation product. The pyruvate extruded by the joint operation of the two reaction cycles in Fig. 6A can serve as precursor for PEP, which can be carboxylated to form C₄ compounds in an
anaplerotic reaction. PEP also serves as precursor for other C₃ compounds and derived hexoses (16) and pentoses.

This proposed glyoxylate assimilation pathway also explains the unique labeling patterns of building blocks observed in previous labeling studies (Fig. 6B) [2, 6]. When autotrophically growing cells were fed with [1-¹³C₁]acetate or [2-¹³C₁]acetate, the cellular building blocks showed a unique ¹³C-labeling pattern that could not be explained by any known pathway of carbon metabolism [6]. Specifically, carbon from C₁ of acetate was preferentially incorporated into C₃ of pyruvate (15) (alanine) and into C₁ and C₆ of hexoses (16), whereas C₂ of acetate was preferentially incorporated into C₂ of pyruvate and into C₂ and C₅ of glucose. C₁ of pyruvate was predominantly derived from CO₂. These findings are all easily explained by the proposed bicyclic pathway. Fig. 6B shows one turn of this cycle. Further cycles result in some randomization of label, which was also observed in the labeling experiment [6]. The proposed pathway of glyoxylate assimilation represents a new mechanism for incorporation of C₂ units into central precursors and like the well known glyoxylate cycle [32] explains how acetate could be assimilated.

In principle, the proposed conversion of glyoxylate plus propionyl-CoA to acetyl-CoA and pyruvate should be reversible. The role of CoA-thioester intermediates is intriguing. If all intermediates were CoA-thioesters, the problem arises that for cleavage of citramalate or citramalyl-CoA, the CoA has to move from one carboxyl group in mesaconyl-CoA to the other in citramalyl-CoA (Fig. 5). Alternatively, mesaconyl-CoA is hydrolyzed and then transformed to citramalate, followed by succinyl-CoA-dependent activation to citramalyl-CoA. A third possibility is that succinate acts as CoA shuttle between mesaconyl-CoA and
citramalate. Interestingly, the *C. aurantiacus* genome contains at least three genes, which are assumed to code for succinyl-CoA-dependent CoA transferases. One gene adjacent to a putative malyl-CoA lyase gene is likely coding for the postulated succinyl-CoA:L-malate CoA transferase [7]. The other two putative CoA transferase genes are located nearby and may be involved in such a CoA transfer shuttle.

A related problem of acceptor molecule regeneration exists in *Methylobacterium extorquens* AM1 [33] and *Streptomyces* species [34]. In these bacteria the regeneration of glyoxylate from acetyl-CoA was unknown. A complete reaction sequence was proposed by which two molecules of acetyl-CoA are condensed and reduced to butyryl-CoA followed by conversion to succinyl-CoA and malyl-CoA. Malyl-CoA is cleaved by malyl-CoA lyase to glyoxylate and acetyl-CoA. In summary, one molecule of acetyl-CoA is oxidized to glyoxylate [33].

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**REFERENCES**


FIGURE LEGENDS

Fig. 1: Proposed 3-hydroxypropionate cycle of autotrophic CO₂ fixation in *C. aurantiacus* [3-7, 16]. Acetyl-CoA, 1; malonyl-CoA, 2; 3-hydroxypropionate, 3; propionyl-CoA, 4; methylmalonyl-CoA, 5; succinyl-CoA, 6; malate, 7; malyl-CoA, 8; glyoxylate, 9.

Fig. 2: ¹³C NMR signals of compounds isolated after incubation of cell extracts of *C. aurantiacus* with [1,2,3-¹³C₃]propionyl-CoA and glyoxylate. Signals of non-enriched carbon atoms are not observed due to low intensity. ¹³C-¹³C-coupling patterns are indicated. Structural fragments derived from NMR correlation patterns are boxed. Adjacent ¹³C atoms are connected by bonds in bold type.

Fig. 3: Two-dimensional HMQC (A) and HMQC-TOCSY (B) spectra of compound 12 (β-methylmalyl-CoA) formed by incubation of a mixture containing [1,2,3-¹³C₃]propionyl-CoA, glyoxylate and a partially purified protein fraction of *C. aurantiacus*. A part of the one-dimensional ¹³C NMR spectrum of β-methylmalyl-CoA (12) is shown as a projection. For reasons of intensity, only signals of ¹³C-enriched carbon atoms are displayed in the one-dimensional ¹³C NMR spectrum and the two-dimensional HMQC spectrum.

Fig. 4: ¹³C NMR signals of compound 13 (mesaconyl-CoA) formed in a mixture containing [1,2,3-¹³C₃]propionyl-CoA, glyoxylate and a partially purified protein fraction of *C. aurantiacus*. For reasons of intensity, only the signals of ¹³C-enriched carbon atoms are observed. ¹³C-¹³C-coupling patterns are indicated. The structural fragments derived from NMR correlation patterns are boxed.
Fig. 5: Hypothetical pathway for transformation of glyoxylate (9) and [1,2,3-\textsuperscript{13}C\textsubscript{3}]propionyl-CoA (4) into acetyl-CoA (1) and [1,2,3-\textsuperscript{13}C\textsubscript{3}]pyruvate (15). \textit{erythro} β-methylmalyl-CoA (12), mesaconyl-CoA (13), citramalate (10) were identified by \textsuperscript{13}C NMR. Citramalate could be activated by succinyl-CoA to citramalyl-CoA (14). \textit{erythro} β-Methylmalate (11) is the product in a CoA releasing reaction. Adjacent \textsuperscript{13}C atoms are connected by bonds in bold type.

Fig. 6: Hypothetical bicyclic pathway for autotrophic CO\textsubscript{2} fixation in \textit{C. aurantiacus}. A: Schematic representation of the inner and outer cycle. B: Carbon atoms highly \textsuperscript{13}C-enriched in feeding experiments [2, 6] of \textit{C. aurantiacus} proffered with \textsuperscript{[1-13}C\textsuperscript{]}acetate (○), \textsuperscript{[2-13}C\textsuperscript{]}acetate (●), and \textsuperscript{13}CO\textsubscript{2} (*) are indicated. Acetyl-CoA, 1; propionyl-CoA, 4; malyl-CoA, 8; glyoxylate, 9; \textit{erythro} β-methylmalyl-CoA, 12; mesaconyl-CoA, 13; citramalyl-CoA, 14; pyruvate, 15; hexose, 16. Note that the actual substrate in the carboxylation reactions is HCO\textsubscript{3}\textsuperscript{−} rather than CO\textsubscript{2}. 
Table 1: NMR data of products from \([1,2,3-^{13}C_3]\)propionyl CoA in reaction mixtures with cell extracts (experiment A) and with a partially purified protein fraction of *C. aurantiacus* (experiment B).

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<th>Coupling constants, Hz</th>
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<td>(^{13}C)</td>
<td>(^1H)</td>
<td>(J_{cc})</td>
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Fig. 2
Fig. 3
Fig. 4
Fig. 5
A bicyclic autotrophic CO2 fixation pathway in Chloroflexus aurantiacus
Sylvia Herter, Georg Fuchs, Adelbert Bacher and Wolfgang Eisenreich

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