A Bicyclic Autotrophic CO₂ Fixation Pathway in Chloroflexus aurantiacus*

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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]methylmalalate and [1,2,2-13C₃]citramalate. Similar experiments using a partially purified protein fraction afforded erythro-β-[1,2,2-13C₃]methylmalalyl-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-methylmalalyl-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 this bicyclic autotrophic CO₂ fixation pathway is pyruvate serving as an universal building block for anabolic reactions.

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 hydrolyzes malyl-CoA to malate and CoA (7, 13–15).

Previous studies have shown that C. aurantiacus can use pyruvate for anaplerotic reactions (3, 7, 11, 16). Pyruvate is converted to phosphoenolpyruvate (PEP)¹ 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 C. aurantiacus is still unknown. 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: [2-14C]propionate (1.98 MBq μmol⁻¹) from Hartmann Analytic (Braunschweig, Germany), [1,2,3-13C₃]sodium propionate (99.9% ¹³C enrichment) from Cambridge Isotope Laboratories (Andover, MA), acetyl-CoA synthetase, L-lactate dehydrogenase, pyruvate kinase, and myokinase from Roche Diagnostics (Mannheim, Germany). Malonyl-CoA was prepared as described in Ref. 7. Succinyl-CoA, acetyl-CoA, and propionyl-CoA were synthesized according to published procedures (17, 18).

Preparation of erythro-β-Methylmalate—Ethyl-3-methyl-2-oxobutyrate-1,4-dioate was reduced with sodium borohydride. The product was hydrolyzed affording erythro-β-methylmalate (19–21). NMR δH (500 MHz, D₂O): 4.25 (1H, d, J = 4.2 Hz, H-2), 2.95 (1H, m, H-3), 1.05 (H, d, J = 7.2 Hz, methyl).

Preparation of [2-14C]Propionyl-CoA—[2-14C]Propionyl-CoA was synthesized according to the protocol described previously for synthesis of [1,2-14C]acetil-CoA (7).

Preparation of [1,2,3-13C₃]Propionyl-CoA—A reaction mixture (20 ml) containing 100 mM Tris/HCl buffer, pH 8.4, 2 mM MgCl₂, 3 mM CoA, 3 mM [1,2,3-13C₃]sodium propionate, 7.5 mM ATP, 4 mM NADH, 10 units of acetyl-CoA synthetase, 10 units of myokinase, 5 mM PEP, 30 units of pyruvate kinase, and 28 units of 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

1 The abbreviations used are: PEP, phosphoenolpyruvate; erythro-methylmalate, [2R,3S]- and [2S,3R]-2-hydroxy-3-methylbutan-2-one; citramalate, 2-hydroxy-2-methylsuccinate; mesaconate, trans-2-methylene fumarate; HPLC, high performance liquid chromatography; MOPS, 4-morpholinospiroproanesulfonic acid; DTNB, 5,5'-dithiobis(2-nitrobenzoate); HMQC, heteronuclear multiple quantum coherence; TOCSY, total correlation spectroscopy; HMBC, heteronuclear multiple bond connectivity.

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was added to be 2% by addition of 6.6 ml HCl. The mixture was centrifuged, and the supernatant was extracted twice with 30 ml of diethyl ether. [2-13C]Propionyl-CoA (18.3 kBq) was added as tracer, and the aqueous phase was subjected to reverse phase HPLC (Grom-Sil 120 ODS-4 HE, 250 × 20 mm, 10 μM) (Grom, Herrenberg, Germany). Propionyl-CoA was eluted in a stepwise gradient (4, 6, and 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 13C- and 14C-labeled propionyl-CoA was adjusted to pH 2 by adding 6 ml HCl, and acetonitrile was evaporated by flash evaporation at 30 °C (30 mbar). The sample was lyophilized and stored at −20 °C, yield, 50%. The protein content of the cell extracts was determined by the Bradford method (23) and ranged from 15 to 50 mg 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 (20,000 × g, 4 °C, 20 min). The supernatant (3.7 ml) was added to a DEAE-Sepharose Fast Flow column (10 ml, Amersham Biosciences, Freiburg, Germany, flow rate 5 ml min⁻¹), which had been equilibrated with 20 mM MOPS/KCl buffer, pH 7.2 (buffer A). The column was washed with 30 ml of 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 4-fold with 20 mM MOPS/KCl buffer, pH 7.6 (buffer B), and applied in two runs each to a Resource Q column (Amersham Biosciences; 1 ml, flow rate 5 ml min⁻¹), which had been equilibrated with buffer B. The column was washed with 4 ml of 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 the Presence of Glyoxylate—The formation of free CoA from propionyl-CoA after addition of glyoxylate was followed spectrophotometrically (412 nm) with 5,5'-diethylthiobis(2-nitrobenzoate) (DTNB; ε₁₀₀₀ = 13,600 M⁻¹ cm⁻¹). The assay mixture (0.5 ml) contained 200 mM MOPS/KCl buffer, pH 7.5, 0.25 mM DTNB, 2 mM MgCl₂, 1 mM propionyl-CoA, 5 mM glycylglycine, and 5–50 μl of cell extract (0.1–1.0 mg of protein) or 100 μl of partially purified protein fraction (0.1 mg of protein).

Conversion of [1,2,3-13C₃]Propionyl-CoA by Cell Extract—A reaction mixture (40 ml) containing 100 mM potassium phosphate buffer, pH 7.3, 1 mM [1,2,3,13C₄]propionyl-CoA, 1 mM glycylglycine, 2 mM MgCl₂, 36.6 kBq of [2-13C]propionyl-CoA, and 2 ml of cell extract (48 μg of 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 (30 mbar), and the pH was adjusted to pH 10 by addition of NaOH. The solution was extracted with diethyl ether (100 ml), and the aqueous phase was applied onto a column of Dowex WX 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% NH₄H₂O₂ solution (v/v) and concentrated to 2 ml by flash evaporation at 30 °C (30 mbar). Aliquots (50 μl) were spotted onto a Polyphase OA HY column (300 × 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, Baytest, 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: glyoxylyte, 5.7 ml; citramalate, 6.2 ml; erythro-β-methylmalonate, 6.7 ml; propionate, 9.0 ml; mesaconate, 12.7 ml.

Conversion of [1,2,3-13C₃]Propionyl-CoA by a Partially Purified Protein Fraction—A reaction mixture (20 ml) containing 100 mM potassium phosphate buffer, pH 7.3, 2 mM glycylglycine, 2 mM MgCl₂, 1.2 mM [1,2,3,13C₄]propionyl-CoA, 54.7 kBq of [2-13C]propionyl-CoA, and 5 ml of partially purified protein fraction (8 mg of protein) was incubated at 55 °C. After 10 min, the mixture was adjusted to pH 2 by the addition of 6 M HCl. Protein was removed by centrifugation. The supernatant was lyophilized, dissolved in 5 ml of water, and applied onto a reverse phase column (Grom-Sil 120 ODS-4 HE, 250 × 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 and 320 ml, respectively, were adjusted to pH 2 by addition of 6 M HCl and lyophilized.

Citrullate Conversion to Pyruvate and Acetyl-CoA in the Presence of Succinate—Conversion of 200 mM MOPS/KCl 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 of cell extract (0.1–0.5 mg of protein) were monitored photometrically (324 nm) at 55 °C. Pyruvate phenylhydrazide formation was followed (ε₂₄₈ = 11,520 M⁻¹ cm⁻¹, experimentally determined). 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 of cell extract (1.0 mg of protein) were incubated at 55 °C. Aliquots (0.1 ml) were retrieved at intervals and were mixed with 10 μl of concentrated HCl. Protein was removed by centrifugation, and the supernatant was analyzed by reverse phase HPLC (LiChrospher 100, endcapped, 125 × 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 16 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 HMJC, HMQC–TOCSY, and HMBC experiments were performed according to standard Bruker software (XWINNMR). The duration of the ¹H spin-lock was 60 ms in the HMJC–TOCSY experiment.

RESULTS

Condensation of Glyoxylate with Propionyl-CoA—The condensation of glyoxylate with propionyl-CoA with formation of methylmalonate has been reported in Rhodospirillum rubrum and Bacillus sp. (24–26), but had not been observed in C. aurantisus. Our preliminary experiments showed that cell extracts of C. aurantisus 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...
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 I).

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 hydrogen 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 I 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 “Experimental Procedures”), it appears safe to conclude that the elusive residues in 11 and 10 are carboxylic acids. 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-thiosteres, 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-$^{13}$C$_3$]propionyl-CoA containing trace amounts of [2-$^{14}$C]propionyl-CoA. HPLC analysis showed two fractions containing 40% of the proffered radioactive elution. The elution conditions were typical for CoA derivatives. The fraction eluted at a retention time of 36 min (12) showed three $^{13}$C multiplets (Table I: experiment B). A doublet centered at 204.5 ppm was suggestive of a thioester carbonyl atom. The $^1$H NMR spectrum confirmed the presence of a CoA residue (data not shown). Two-dimensional $^1$H$^{13}$C experiments (HMQC and HMQC-TOCSY, Fig. 3) identified a $\beta$-methylmalyl spin system comprising H-2, H-2 and H-3 protons (Fig. 3B) as well as to H-2 and H-3 protons (Fig. 3B). The HMQC spectrum (Fig. 3A) showed correlations of $^{13}$C-2 and $^{13}$C-2 with their directly attached protons. In the HMQC-TOCSY spectrum (Fig. 3B) of the same sample, extended $^1$H spin systems connected by $^1$H TOCSY transfer are correlated to individual carbon atoms. Thus, C-2 ($^{13}$C NMR signal at 51.6 ppm) showed correlation to the directly attached H-2 proton ($^1$H NMR signal at 3.0 ppm) as well as to H-2 ($^1$H NMR signal at 1.0 ppm) and to H-3 ($^1$H NMR signal at 4.1 ppm). Confirmation of the $^1$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 (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 $\beta$-[1,2,2-$^{13}$C$_3$]methylmalyl-CoA. In the experiment with cell extracts of C. aurantiacus erythro-$\beta$-[1,2,2-$^{13}$C$_3$]methylmalate (11) was identified as a reaction product (see above), and therefore, it appears plausible that 12 is the erythro form of $\beta$-[1,2,2-$^{13}$C$_3$]methylmalyl-CoA. The fraction eluted at 40 min (13) displayed three $^{13}$C multiplets (Table I: 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 I) identified the molecular fragment...
boxed in Fig. 4. The \(^1\)H NMR spectrum indicated the presence of a CoA residue. On this basis, 13 was assigned as \([1,2,2/-\text{H}^{13}\text{C}3]\)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 extracts of \(C. \text{aurantiacus}\) 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 (not shown). 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

\begin{table}
\centering
\caption{NMR data of products from \([1,2,3-\text{H}^{13}\text{C}3]\)propionyl CoA in reaction mixtures with cell extracts (experiment A) and with a partially purified protein fraction of \(C. \text{aurantiacus}\) (experiment B)}
\begin{tabular}{|c|c|c|c|c|c|}
\hline
\textbf{Position} & \textbf{Chemical shifts, ppm} & \textbf{Coupling constants, }\textit{J} & \textbf{Correlation pattern} \\
\textbf{1} & \textbf{\text{C}} & \textbf{\text{H}} & \text{HMQC} & \text{HMQC-TOCSY} & \text{HMBC} \\
\hline
\begin{enumerate}
\item \([1,2,3-\text{H}^{13}\text{C}3]\)Propionyl-CoA (4)
\item \([1,2,2-\text{H}^{13}\text{C}3]\)Methylmalate (11)
\item \([1,2,2-\text{H}^{13}\text{C}3]\)Citramalate (10)
\item \([1,2,2-\text{H}^{13}\text{C}3]\)Mesoconyl-CoA (13)
\end{enumerate}
\end{tabular}
\end{table}

\begin{figure}
\centering
\includegraphics[width=\textwidth]{fig3}
\caption{Two-dimensional HMOC (A) and HMOC-TOCSY (B) spectra of compound 12 (\(\beta\)-methylmalyl-CoA) formed by incubation of a mixture containing \([1,2,3-\text{H}^{13}\text{C}3]\)propionyl-CoA, glyoxylate, and a partially purified protein fraction of \(C. \text{aurantiacus}\). A part of the one-dimensional \(^{13}\text{C}\) NMR spectrum of \(\beta\)-methylmalyl-CoA (12) is shown as a projection. For reasons of intensity, only signals of \(^{13}\text{C}\)-enriched carbon atoms are displayed in the one-dimensional \(^{13}\text{C}\) NMR spectrum and the two-dimensional HMOC spectrum.}
\end{figure}
suggests that the reaction catalyzed is due to two enzymes, a CoA transferase and a citramalyl-CoA lyase, catalyzing Reactions 1 and 2, respectively.

\[
\begin{align*}
\text{Succinyl-CoA} & \rightarrow \text{succinate} + \text{citramalyl-CoA} \\
\text{REACTION 1} \\
\text{Citramalyl-CoA} & \rightarrow \text{acetyl-CoA} + \text{pyruvate} \\
\text{REACTION 2}
\end{align*}
\]

**DISCUSSION**

The present study aimed at elucidating the fate of glyoxylate in autotrophic CO₂ 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 to minimize the risk of decomposition or structural modification. For this purpose, we used [1,2,3-¹³C₃]propionyl-CoA as the substrate to enhance the sensitivity and selectivity of ¹³C NMR analysis in crude reaction mixtures. Any products formed from the uniformly ¹³C-labeled propionate without breakage of ¹³C⁻¹³C-bonds must contain a group of three contiguous ¹³C atoms, which form a spin system that is easily assigned via ¹³C⁻¹³C coupling. Moreover, the ¹³C spin system can be extended to identify ¹H atoms bound directly to one of the respective ¹³C atoms. Using two-dimensional ¹H⁻¹³C correlation techniques, the spin system can be extended still further to include ¹H atoms bound to one of the ¹³C-labeled positions via two or three bonds. This approach enabled us to assign the structures of enzyme products resulting from condensation of the ¹³C-labeled propionate moiety with glyoxylate (Fig. 5). We could show that [1,2,3-¹³C₃]propionyl-CoA (4) can be condensed with glyoxylate (9) to form *erythro*-β-[1,2,2-¹³C₃]methylmalyl-CoA (12), which can be transformed further to [1,2,2-¹³C₃]mesaconyl-CoA (13) (Fig. 5) and [1,2,2,13C]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 with heterotrophically grown cells. This regulatory pattern suggests that these reactions are part of the autotrophic carbon metabolism of *C. aurantiacus*. The forma-
tion of erythro-β-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 CO₂ fixation cycle of *C. aurantiacus* (4–7) (Fig. 1 and 6A). 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 in acetate-grown *R. 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 (methylmalonyl-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) (Figs. 1 and 6, A and 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 [1-¹³C]acetate or [2-¹³C]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.

**Fig. 6.** Hypothetical bicyclic pathway for autotrophic CO₂ fixation in *C. aurantiacus*. A, schematic representation of the inner and outer cycle. B, carbon atoms highly ¹³C-enriched in feeding experiments (2, 6) of *C. aurantiacus* proferred with [1-¹³C]acetate (5), [2-¹³C]acetate (6), and ¹³CO₂ (*) are indicated. 1, Acetyl-CoA; 4, propionyl-CoA; 8, malyl-CoA; 9, glyoxylate; 12, erythro-β-methylmalyl-CoA; 13, mesaconyl-CoA; 14, citramalyl-CoA; 15, pyruvate; 16, hexose. Note that the actual substrate in the carboxylation reactions is HCO₃⁻ rather than CO₂.
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-CoA transferases. The other two putative CoA transferase genes are located nearby and may be involved in such a CoA transfer shuttle. There might be another, fourth CoA transferase gene on another contig.

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 and reduced to butyryl-CoA followed by conversion to succinyl-CoA and glyoxylate. We thank Prof. Ruslan N. Ivanovsky, Moscow State University, Russia, for fruitful suggestions. Special thanks to Dr. Richard Krieger, Institut für Organische Chemie, Universität Freiburg, Germany, for the synthesis of erythro-β-methylmalate. We thank Fritz Wendling and Angelika Werner for expert help with the preparation of the manuscript.

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