The capability to use carbon dioxide as the sole source of cell carbon (autotrophy) is found in almost all major groups of prokaryotes. The CO₂ fixation pathways differ between groups, and there is no clear distribution pattern of the four presently known autotrophic pathways (8).

The reductive pentose phosphate cycle (Calvin-Bassham-Benson cycle) represents the CO₂ fixation pathway in almost all aerobic autotrophic bacteria, for example, the cyanobacteria. This cycle became the autotrophic pathway of plants, and all aerobic autotrophic bacteria, for example, the cyanobacteria (anaerobic bacteria, such as the phototrophic Chlorobiaceae [1, 6, 9, 21]) and the sulfate-reducing Desulfovibrio desulfuricans (8, 45), as well as in the microaerobic thermophilic hydrogen bacteria Hydrogenobacter thermophilus (36) and Aquifex pyrophilus (early branch-off of bacteria [2]), and in the sulfur-reducing crenarchaeon Thermoproteus neutrophilus (2). This pathway is characterized by the enzymes ATP citrate lyase, 2-oxoglutarate:acceptor oxidoreductase (2-oxoglutarate synthase), and pyruvate synthase.

The reductive citric acid cycle was found in several strictly anaerobic bacteria, such as the phototrophic Chlorobium limicola (Chlorobiaceae [1, 6, 9, 21]) and the sulfate-reducing Desulfobacter hydrogenophilus (delta subgroup of Proteobacteria [33]), as well as in the microaerobic thermophilic hydrogen bacteria Hydrogenobacter thermophilus (36) and Aquifex pyrophilus (early branch-off of bacteria [2]), and in the sulfur-reducing crenarchaeon Thermoproteus neutrophilus (2). This pathway is characterized by the enzymes ATP citrate lyase, 2-oxoglutarate:acceptor oxidoreductase (2-oxoglutarate synthase), and pyruvate synthase.

The reductive acetyl coenzyme A (acetyl-CoA) pathway is confined to the strict anaerobic bacteria, such as gram-positive acetogenic bacteria (8, 45), the sulfate-reducing Desulfbacterium autotrophicum and relatives (delta subgroup group of Proteobacteria [32]), and the methanogenic bacteria (8). It is also found in euryarchaeota, e.g., in the denitrifying Ferroglobus placidus (40), and the sulfate-reducing Archaeoglobus lithotrophicus (41). This pathway is characterized by the CO₂-fixing enzyme carbon monoxide dehydrogenase.

A new autotrophic pathway, the 3-hydroxypropionate cycle, has been discovered in Chloroflexus aurantiacus OK-70, a facultatively aerobic, phototrophic bacterium (5, 12, 13, 37, 38). The postulated outline of this pathway and the enzymes involved are shown in Fig. 1. Glyoxylate is formed from acetyl-CoA after the fixation of two molecules of CO₂ by acetyl-CoA and propionyl-CoA carboxylases, while acetyl-CoA is regenerated. Phosphoenolpyruvate (PEP) carboxylase may have an anaplerotic function. The assimilation of the CO₂ fixation product glyoxylate into cell material is at issue (20).
been studied in only a limited number of anaerobic species (see above), and the CO₂ fixation pathways of aerobic autotrophic species are unknown. In the aerobic *Haloferax mediterranei*, ribulose-1,5-bisphosphate carboxylase was detected and purified from heterotrophically grown cells. Although the catalytic number of this key enzyme of the Calvin cycle was 100-fold lower than that in enzymes from other sources (30), this euryarchaeon may use the Calvin cycle for CO₂ fixation under certain conditions, e.g., microaerobic conditions. Interestingly, the *Methanococcus jannaschii*, *Archaeoglobus fulgidus*, and *Pyrococcus horikoshii* genomes contain ribulose-1,5-bisphosphate carboxylase-like sequences (23, 24, 43). The situation is equally complex in the crenarchaeota. A reductive citric acid cycle has been reported for the strictly anaerobic sulfur-reducing *T. neu-
trophilus (2, 37). Autotrophic members of the order Sulfolobales include Sulfolobus metallicus, Metallophaga sedula, Metallophilus prunae, Acididium brierleyi, Acididium infernus, Acididium ambivalens, and Stigmatulus azoricus (34). Early studies, performed with aerobic or microaerophilic representatives of the Sulfolobales, indicated the presence of a nondefined carboxylic acid cycle in these organisms (22). Sulfolobus species grown aerobically under CO2 starvation showed an induced acetyl-CoA carboxylase activity (28). More recently, some enzymes of the proposed 3-hydroxypropionate cycle were detected in both autotrophs and heterotrophs, including a CoA carboxylase, propionyl-CoA carboxylyase, and malonate semialdehyde dehydrogenase (18). Furthermore, labelled products formed in vitro from 14CO2 in the presence of acetyl-CoA or propionyl-CoA included malate, fumarate, and succinate. 3-Hydroxypropionate was not found, and maloyl-CoA lyase activity was undetectable. The authors concluded that a modified 3-hydroxypropionate cycle operates in A. brierleyi (18).

The aims of the present work were to study the pathway of autotrophic CO2 fixation in C. aurantiacus and in a representative of the aerobic crenarchaeota, M. sedula, and to screen other archaea for characteristic enzymes of the 3-hydroxypropionate cycle.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The following strains were used: T. neutralis V2481ta (DSM 2338), Thermoproteus tenax Kral (DSM 2078), M. sedula TH2 (DSM 5348), S. metallicus Kr25 (DSM 6482), A. infernus Sofia (DSM 1391), Methanobacterium thermoautotrophicum YTB (DSM 1850), Methanothermus fervidus UB5 (DSM 1311), Methanobrevibacter smithii (DSM 3959), Methanococcus voltae (DSM 1537), Methanopyrus kandleri MP-K1 (DSM 6534), C. aurantiacus OK-701 (DSM 636), A. pyrophilus KO15a (DSM 6838), D. autotrophicum HRM2 (DSM 3382), D. hydrogenophilus AcKD1 (DSM 3380), Alkaligenes eutrophus H16 (DSM 426), and Escherichia coli K-12 (DSM 423).

The Sulfolobales members were grown on Allen mineral medium as previously described (34). M. sedula was grown microaerobically with a gas phase of H2-CO2 (24:76) at 5°C and pH 2.0 (generation time, 10 h [15, 16]). As a control, cells grown aerobically with 0.05% yeast extract were used (generation time, 10 h [15]). A. infernus was grown at pH 2.5, aerobically with sulfur and anaerobically with sulfur and H2, both in the presence of 0.02% yeast extract at 85°C (29). M. sedula was grown anaerobically either on metal ore or sulfur at 65°C (14). T. neutralis was grown anaerobically on mineral medium at 85°C under a gas phase of H2-CO2 (80:20) and elemental sulfur (37). T. tenax was also grown with CO2, H2, and thiosulfate but under anaerobic conditions at 85°C (38). C. aurantiacus strain A. was anaerobically with H2 and CO2 at 87°C and 80°C, respectively (26, 42). M. barkeri was grown anaerobically on salt medium with acetate at 37°C, and M. organophilus was grown on salt medium with ethanol and CO2 at 30°C as reported in reference 44. A. pyrophilus was grown microaerobically on MME medium at 85°C with a gas phase of H2-CO2-O2 (78:19:3; 250 kPa). ATP citrate lyase was measured by the citrate-, Mg2+-, and CoA-dependent oxidation of NADH. The enzyme was assayed as described in reference 38. Typically, the carboxylase assay (1 ml; 2 ml headspace) contained 100 mM Tris-HCl (pH 7.8), 0.4 mM substrate, 2 mM ATP, 4 mM MgCl2, 5 mM DTE, 10 mM NaHCO3, and 17 kBg of [14C]Na2CO3. The reaction mixture was preincubated at the reaction temperature, and the reaction was started by the addition of different volumes of cell extract (50 to 100 μl). When both carboxylating activities were measured simultaneously, 0.4 mM acetyl-CoA and 0.4 mM propionyl-CoA were added. The malonyl-CoA reduction to 3-hydroxypropionate was monitored spectrophotometrically by the oxidation of NADPH. The assay mixture (0.5 ml) contained 100 mM Tris-HCl (pH 7.8), 5 mM MgCl2, 5 mM DTE, 0.5 mM NADPH, 0.5 mM malonyl-CoA, and 0.2 mg of protein. The reaction was started by the addition of the substrate. The redox conversion of 3-hydroxypropionate to propionyl-CoA was measured as the Mg2+-, ATP-, and CoA-dependent oxidation of NADPH. The assay mixture contained 5 mM MgCl2, 3 mM ATP, 10 mM KCl, 0.5 mM CoASH, 0.5 mM NADPH, and different amounts of cell extract, and the reaction was started by adding the substrate 3-hydroxypropionate to the assay mixture. PEP carboxylase and pyruvate carboxylase activities were assayed as described in references 3 and 35 with minor modifications. The tests were carried out in a system in which the oxaloacetate formed was reduced to malate in the presence of endogenous malate dehydrogenase. Both tests were monitored spectrophotometrically at 365 nm. The PEP carboxylase assay mixture contained 100 mM Tris-HCl (pH 7.8), 5 mM MgCl2, 0.5 mM NADH, 10 mM NaHCO3, and different amounts of cell extract, and the reaction was started by adding PEP at a 2 mM concentration. The pyruvate carboxylase assay mixture contained a similar composition but with 3 mM ATP, and the reaction was started with 3 mM pyruvate. CO2 dehydrogenase and pyruvate:acceptor and 2-oxoglutarate: acceptor oxidoreductases were oxygen sensitive and therefore were assayed anaerobically. All other enzyme assays were oxygen independent.

Synthesis of [14C]Acetyl-CoA. Radioactively labelled acetyl-CoA was enzymatically synthesized. The 1 ml reaction mixture contained 0.3 mM CoA, 0.2 mM [U-14C]acetate (54 μCi/μmol) (Amersham, Braunschweig, Germany), 1 mM ATP, 0.4 U of acetyl-CoA synthetase (Boehringer Mannheim, Mannheim, Germany), and 1 mM MgCl2 in 50 mM Tris-HCl (pH 8.4). In addition, an ATP-generating system, consisting of 1 mM PEP, 0.4 mM NADH, and the enzymes myokinase (1 U), pyruvate kinase (1.5 U), and lactate dehydrogenase (2.5 U), was added. The reaction was started at 37°C, and the radioactivity was monitored photometrically at 365 nm. The radioactive acetyl-CoA was purified by using an RP-18 extraction minicolumn (ICT, Bad Homburg, Germany) following the protocols of the supplier.

Detection of [14C]acetate-CoA. Radioactive products generated during the 14CO2 assimilation tests were separated and identified by both high-pressure liquid chromatography (HPLC) and thin-layer chromatography (TLC). The reactions were stopped by adding 30 μl of 6 M H2SO4, and the reaction mixtures were incubated on ice for 10 min and centrifuged. The supernatant was retained and divided into two fractions. For scintillation counting, the free 14CO2 was removed from the sample by adding 100 μl of 6 M formic acid and gassing the samples for 15 min with a CO2 stream; later, 150 μl of 1 M KOH was added and the gassing step was repeated for another 15 min. For HPLC, 60 μl of the centrifuged sample was injected onto a C18 RP-HPLC column (LicroCART; Merck, Darmstadt, Germany) and chromatographed with a 20-min gradient of 1 to 80% acetoniitrite in 50 mM phosphate buffer, pH 6.7. Simultaneous detection of standard compounds and reaction products was possible by using two detectors (UV and radioactivity) in series. The acyl-CoA esters present in 100 μl of the same nonvolatile sample were hydrolyzed at pH 12 for 30 min at 60°C. After cooling and neutralizing of the sample, 4 ml of cell extract. Carbon monoxide dehydrogenase was tested as the reductive conversion of 3-hydroxypropionate to propionyl-CoA thioesters of acetate, propionate, malonate, and succinate were synthesized according to the method described in reference 31, and the thioester of 3-hydroxypropionate was synthesized according to the method described in reference 11. 3-Hydroxypropionate was obtained by hydrolyzing 3-hydroxypropionitrile.

Detection of cytochrome oxidations. Radioactive products generated during the 14CO2 assimilation tests were separated and identified by both high-pressure liquid chromatography (HPLC) and thin-layer chromatography (TLC). The reactions were stopped by adding 30 μl of 6 M H2SO4, and the reaction mixtures were incubated on ice for 10 min and centrifuged. The supernatant was retained and divided into two fractions. For scintillation counting, the free 14CO2 was removed from the sample by adding 100 μl of 6 M formic acid and gassing the samples for 15 min with a CO2 stream; later, 150 μl of 1 M KOH was added and the gassing step was repeated for another 15 min. For HPLC, 60 μl of the centrifuged sample was injected onto a C18 RP-HPLC column (LicroCART; Merck, Darmstadt, Germany) and chromatographed with a 20-min gradient of 1 to 80% acetoniitrite in 50 mM phosphate buffer, pH 6.7. Simultaneous detection of standard compounds and reaction products was possible by using two detectors (UV and radioactivity) in series. The acyl-CoA esters present in 100 μl of the same nonvolatile sample were hydrolyzed at pH 12 for 30 min at 60°C. After cooling and neutralizing of the sample, 4 ml of cell extract. Carbon monoxide dehydrogenase was tested as the reductive conversion of 3-hydroxypropionate to propionyl-CoA thioesters of acetate, propionate, malonate, and succinate were synthesized according to the method described in reference 31, and the thioester of 3-hydroxypropionate was synthesized according to the method described in reference 11. 3-Hydroxypropionate was obtained by hydrolyzing 3-hydroxypropionitrile.
sodium dodecyl sulfate-polyacrylamide gel electrophoresis on 15% gels, the proteins were electrotransferred to nitrocellulose membranes. The membranes were first blocked by shaking them for 2 h in a solution containing 5% skim milk powder in Tris-buffered saline (TBS) (20 mM Tris-HCl [pH 7.5], 500 mM NaCl). They were then washed three times with TBS and subsequently incubated for 1.5 h with 8.5 μg of avidin-peroxidase conjugate (Sigma, Deisenhofen, Germany) per ml in TBS. The membranes were washed again, and bound peroxidase was detected by developing the sheets with 4-chloronaphthol and hydrogen peroxide as described in reference 10.

RESULTS

Search for key enzymes of established autotrophic pathways in M. sedula. M. sedula grows autotrophically at 65°C with CO₂, S, and O₂ as carbon, electron, and energy sources, respectively, with a generation time of 20 h. This corresponds to a specific carbon assimilation rate of 48 nmol of CO₂ fixed min⁻¹ mg⁻¹ of total cell protein⁻¹. The calculation is based on an assumed carbon and protein content of 50% of cell dry matter each. Extracts of M. sedula, grown autotrophically under these conditions, were tested at 55 or 65°C for CO₂ fixing and other key enzymes of known autotrophic pathways. As a control, cell extracts of A. pyrophilus, which efficiently catalyzed both reactions at 75°C, were used. Pyruvate synthase activity was detectable (>180 nmol min⁻¹ mg⁻¹ of cell protein) in M. sedula. This corresponds to a specific carbon assimilation rate of 48 nmol of CO₂ fixed min⁻¹ mg⁻¹ of total cell protein⁻¹. Similarly to M. sedula, C. aurantiacus crude extracts showed a low activity of pyruvate:acceptor oxidoreductase at 55°C. With methyl viologen, the specific activity reached 15 nmol min⁻¹ mg⁻¹ of cell protein⁻¹, and in the isotope exchange reaction 10 nmol min⁻¹ mg⁻¹ of cell protein⁻¹ was measured. 2-Oxoglutarate:acceptor oxidoreductase activity was below the detection limit (<0.1 nmol min⁻¹ mg⁻¹ of cell protein⁻¹ measured with both methyl viologen and the isotope exchange reaction). No NAD⁺-dependent pyruvate dehydrogenase or 2-oxoglutarate dehydrogenase activities were detectable.

C. aurantiacus grows autotrophically at 55°C under anerobic conditions with light, H₂, and CO₂ as energy, electron, and carbon sources, respectively, with a generation time of approximately 20 h. This corresponds to a specific carbon assimilation rate of 48 nmol of CO₂ fixed min⁻¹ mg⁻¹ of total cell protein⁻¹. Serch for key enzymes of established autotrophic pathways in M. sedula. M. sedula grows autotrophically at 65°C with CO₂, S, and O₂ as carbon, electron, and energy sources, respectively, with a generation time of 20 h. This corresponds to a specific carbon assimilation rate of 48 nmol of CO₂ fixed min⁻¹ mg⁻¹ of total cell protein⁻¹. The calculation is based on an assumed carbon and protein content of 50% of cell dry matter each. Extracts of M. sedula, grown autotrophically under these conditions, were tested at 55 or 65°C for CO₂ fixing and other key enzymes of known autotrophic pathways. As a control, cell extracts of A. pyrophilus, which efficiently catalyzed both reactions at 75°C, were used. Pyruvate synthase activity was detectable (>180 nmol min⁻¹ mg⁻¹ of cell protein) in M. sedula. This corresponds to a specific carbon assimilation rate of 48 nmol of CO₂ fixed min⁻¹ mg⁻¹ of total cell protein⁻¹. Similarly to M. sedula, C. aurantiacus crude extracts showed a low activity of pyruvate:acceptor oxidoreductase at 55°C. With methyl viologen, the specific activity reached 15 nmol min⁻¹ mg⁻¹ of cell protein⁻¹, and in the isotope exchange reaction 10 nmol min⁻¹ mg⁻¹ of cell protein⁻¹ was measured. 2-Oxoglutarate:acceptor oxidoreductase activity was below the detection limit (<0.1 nmol min⁻¹ mg⁻¹ of cell protein⁻¹ measured with both methyl viologen and the isotope exchange reaction). No NAD⁺-dependent pyruvate dehydrogenase or 2-oxoglutarate dehydrogenase was detectable (<1 nmol min⁻¹ mg⁻¹ of cell protein⁻¹).

These results suggested that the Calvin cycle, the reductive acetyl-CoA pathway, and the reductive citric acid cycle do not function in M. sedula when grown with S and O₂. Rather, the presence of a fourth autotrophic pathway has to be postulated.

Detection of CO₂ fixation enzymes of the postulated 3-hydroxypropionate cycle in M. sedula. It was tested whether M. sedula uses the postulated 3-hydroxypropionate cycle as an autotrophic pathway. This cycle uses acetyl-CoA carboxylase (reaction a) and propionyl-CoA carboxylase (reaction b) for the fixation of two molecules of CO₂ per cycle. Since intermediates of the 3-hydroxypropionate cycle participate in the citric acid cycle, an active anaplerotic reaction is also required. Candidates for possible anaplerotic enzymes are pyruvate carboxytransphosphorylase (reaction c), PEP carboxylase (reaction d), and PEP carboxytransphosphorylase (reaction e).

\[
\text{Acetyl-CoA + MgATP + HCO}_3^- \rightarrow \text{malonyl-CoA + MgADP + Pi}
\] (a)

\[
\text{Propionyl-CoA + MgATP + HCO}_3^- \rightarrow \text{methylmalonyl-CoA + MgADP + Pi}
\] (b)
When NADPH was included in this assay, 3-[14C]hydroxypropionate was completely abolished by 1 nM avidin. When both substrates, acetyl-CoA (0.4 mM) and propionyl-CoA (0.4 mM), were simultaneously added to the assay, specific enzyme activities were similar to those found in autotrophically grown M. sedula at the same temperature. These results showed that both C. aurantiacus and M. sedula convert acetyl-CoA to 3-hydroxypropionate via malonyl-CoA, as postulated for the 3-hydroxypropionate cycle.

Demonstration of enzyme activities converting acetyl-CoA via malonyl-CoA to 3-hydroxypropionate. The acetyl-CoA carboxylase assay depends on the fixation of the radioactivity from [14C]CO2 into acid-stable products in the presence of MgATP and acetyl-CoA. Under these conditions, cell extracts of M. sedula catalyzed the MgATP- and CO2-dependent conversion of [14C]acetyl-CoA to [14C]malonyl-CoA (Fig. 3A). Formation of this intermediate was completely abolished by 1 nM avidin. When NADPH was included in this assay, 3-[14C]hydroxypropionate was formed in addition to [14C]malonyl-CoA (Fig. 3B). In this experiment, [14C]malonate was released from labelled malonyl-CoA by alkali treatment. Similar results were obtained when [14CO2 and acetyl-CoA were used (Fig. 4). This shows that acetyl-CoA is first carboxylated to malonyl-CoA by the avidin-sensitive acetyl-CoA carboxylase and then converted, by reduction, to 3-hydroxypropionate. The intermediate semialdehyde could not be identified, partly because it was not commercially available as a reference. The reduction of malonyl-CoA to 3-hydroxypropionate requires the oxidation of two molecules of NADPH per molecule of malonyl-CoA. This reaction was monitored in an aerobic spectrophotometric assay catalyzed by M. sedula cell extracts (Fig. 3C). The absorption decrease followed a biphasic curve, which may be due to different specific activities of the two sequential oxidoreductases, the first one catalyzing a fast reduction of malonyl-CoA to malonate semialdehyde, followed by a slower reduction of malonate semialdehyde to 3-hydroxypropionate (see also Fig. 1). The initial high rate corresponded to a specific NADPH oxidation rate of 156 nmol min⁻¹ mg of cell protein⁻¹, while the final lower rate was 78 nmol min⁻¹ mg of cell protein⁻¹. Both rates were linearly protein dependent in the range 0 to 0.15 mg of cell protein ml of assay mixture⁻¹. Noreaction occurred when NADH was used instead of NADPH in the assay (data not shown).

Control experiments were performed with C. aurantiacus cell extracts with acetyl-CoA and 14CO2 (Fig. 5) or with 14Cmalonyl-CoA and CO2 (data not shown), in the presence of MgATP and NADPH. It is to be expected that malonyl-CoA is formed only transiently because this intermediate is rapidly reduced by NADPH to 3-hydroxypropionate. As expected, in both cases the early formation of 3-[14C]hydroxypropionate via [14C]malonate-CoA was observed. According to the working hypothesis, 3-hydroxypropionate should subsequently be converted to succinyl-CoA via propionyl-CoA. As expected, after alkali treatment [14C]succinate and an unidentified labelled product (X) accumulated in time and were observed in two different TLC solvent systems (Fig. 5, lane 4).

These results showed that both C. aurantiacus and M. sedula convert acetyl-CoA to 3-hydroxypropionate via malonyl-CoA, as postulated for the 3-hydroxypropionate cycle.

**Pyruvate + MgATP + HCO₃⁻ → oxaloacetate + MgADP + P_i (c) PEP + HCO₃⁻ → oxaloacetate + P_i (d) PEP + CO₂ + P_i → oxaloacetate + P_i (e)**

Extracts of autotrophically grown M. sedula were tested at 55°C for the presence of these enzymes. Control extracts of autotrophically grown C. aurantiacus were tested in parallel at the same temperature. M. sedula contained both acetyl-CoA and propionyl-CoA carboxylase activities (Table 1). The specific enzyme activities were similar to those found in C. aurantiacus. When both substrates, acetyl-CoA (0.4 mM) and propionyl-CoA (0.4 mM), were simultaneously added to the assay, the initial CO2 fixation rates measured with M. sedula extracts were roughly additive (Table 1). This result suggests the presence of two different carboxylases, since both activities were measured near substrate saturation (Fig. 2). Both activities were completely abolished by low concentrations (1 nM) of avidin. The rate of 14CO2 fixation in these assays rapidly decreased for unknown reasons. The rates given refer to the amount of 14CO2 fixed after 2 min of incubation.

In addition to the two CO2-fixing enzyme activities, extracts of both organisms, M. sedula and C. aurantiacus, contained PEP carboxylase (Table 1), while pyruvate carboxylase and PEP carboxytransferase activities were not detectable (<1 nmol min⁻¹ mg of cell protein⁻¹).

Demonstration of enzyme activities converting acetyl-CoA to 3-hydroxypropionate. The 3-hydroxypropionate cycle postulates the MgATP- and CoA-dependent activation, by a CoA ligase enzyme, of the characteristic intermediate of the cycle, 3-hydroxypropionate, to 3-hydroxypropionyl-CoA. This is followed by K⁺-dependent beta-elimination of water, generating acryl-CoA as the next intermediate. This intermediate is subsequently reduced to propionyl-CoA by NAD(P)H (Fig. 1). After sufficient formation of CoA thioesters, this sequence of reactions results in the oxidation of NADPH. This oxidation was the basis for an aerobic spectrophotometric assay of the reactions (Fig. 6A). NADPH oxidation required approximately 2 min to reach its maximal rate after the coupled spectrophotometric assay was started by addition of 3-hydroxypropionate. This is to be expected, because NADPH oxidation is initiated only after substantial amounts of the intermediates 3-hydroxypropionyl-CoA and acryl-CoA are formed. The final rate of NADPH oxidation with M. sedula cell extracts was even higher than that found in C. aurantiacus (Table 1). The final rate of NADPH oxidation observed after 3-hydroxypropionate addition was linearly dependent on the amount of cell protein in the range 0 to 0.15 mg ml of assay mixture⁻¹. It has been shown before with C. aurantiacus that NADPH oxidation could also be started with acrylate instead of 3-hydroxypropionate (38).

HPLC analysis of the samples taken at different reaction times (Fig. 6B) showed that cell extracts of M. sedula produced 3-hydroxypropionyl-CoA and subsequently reduced it to propionyl-CoA in the presence of the corresponding cofactors and
cosubstrates. In control experiments, without 3-hydroxypropionate, none of these products was detected. Under the chromatographic conditions used, the substrate, 3-hydroxypropionic acid, does not separate and elutes together with other polar substances during the first 2 min. The chromatograms also show the consumption of the corresponding cofactor CoASH and cosubstrate NADPH and the production of NADP.

Evidently, 3-hydroxypropionate is not a metabolic dead-end product but can be reduced, in vitro, to propionyl-CoA, the substrate of the second postulated carboxylating enzyme.

Demonstration of enzyme activities converting propionyl-CoA to succinate via methylmalonyl-CoA.

The second CO₂ fixation step proposed in the 3-hydroxypropionate cycle involves the carboxylation of propionyl-CoA to methylmalonyl-CoA. This reaction is catalyzed by the biotin-dependent enzyme propionyl-CoA carboxylase and requires ATP and Mg²⁺.

The product of this reaction, methylmalonyl-CoA, is further isomerized to succinyl-CoA and later de- or transesterified to succinate (Fig. 1).

Cell extracts of *M. sedula* catalyzed the MgATP-dependent carboxylation of propionyl-CoA, measured as the incorporation of CO₂ into acid-stable products. HPLC analysis of the reaction time course showed the formation of [¹⁴C]methylmalonyl-CoA, in initial stages of the reaction (Fig. 7A). Later, [¹⁴C]methylmalonyl-CoA was consumed in favor of [¹⁴C]succinate production. The identity of these intermediates was confirmed by performing a second HPLC analysis after alkaline hydrolysis of the samples. The radioactive peaks coeluted with the free acids [¹⁴C]methylmalonate and [¹⁴C]succinate (Fig. 7B). The [¹⁴C]methylmalonate and [¹⁴C]succinate formed during the reaction were also identified by TLC with two different solvent systems (not shown).

The presence of the two enzymes methylmalonyl-CoA epimerase and methylmalonyl-CoA mutase can be inferred from the detection of the reaction product [¹⁴C]succinate. These
enzymes catalyze reactions 8 and 9 of the 3-hydroxypropionate cycle (Fig. 1).

Detection of carboxylase activities and biotin-containing peptides in M. sedula, S. metallicus, and A. infernus. Since archaea do not contain substantial amounts of fatty acids—if any—neither acetyl-CoA carboxylase nor propionyl-CoA carboxylase plays a significant role in their central carbon metabolism. Therefore, it was not expected that these carboxylases would be found in archaea. All bacterial acetyl-CoA carboxylases studied so far contain a small and abundantly synthesized biotin-carboxy-carrier protein subunit (BCCP; 16 to 24 kDa) (reviewed in reference 39).

The 3-hydroxypropionate cycle involves the interconversion of acetyl-CoA and propionyl-CoA to succinate. In vitro the essential partial reactions of this cycle from acetyl-CoA via 3-hydroxypropionate and propionyl-CoA to succinate were investigated in several archaea with known autotrophy pathways. T. tenax, a strictly anaerobic autotrophic member of the Crenarchaeota, which probably uses the reductive citric acid cycle for CO₂ fixation (as shown for T. neutrophilus), did not contain detectable amounts of biotin-carrying peptides (Fig. 8A, lanes 9 and 10). A small BCCP-like protein was also absent in autotrophic Euryarchaeota with a reductive acetyl-CoA pathway for CO₂ fixation. These bacteria either contained virtually no biotin enzymes, as in the case of M. organophillum (Fig. 8B, lanes 5 and 6), M. kandleri (Fig. 7B, lanes 7 and 8), and M. thermoaerotrophicum (Fig. 8B, lanes 9 and 10), or contained various biotinylated proteins of higher molecular mass, as found in M. voltae (Fig. 8B, lanes 1 and 2) and M. Barkeri (Fig. 8B, lanes 3 and 4).

Acetyl-CoA and propionyl-CoA carboxylase activities were tested with extracts of various archaea. Acetyl-CoA plus propionyl-CoA-dependent fixation of CO₂ was detected, albeit at a low rate, in S. metallicus (2.5 mmol min⁻¹ mg of total cell protein⁻¹ at 60°C) and A. infernus (0.18 mmol min⁻¹ mg of total cell protein⁻¹ at 60°C). Note, however, that A. infernus was grown at 80°C in the presence of 0.02% yeast extract. In T. tenax (grown at 80°C, measured at 60°C) and M. voltae (grown and measured at 37°C), no activity was observed (<0.01 mmol min⁻¹ mg of total cell protein⁻¹).

These results indicate that acetyl-CoA and propionyl-CoA carboxylase are actively synthesized not only in M. sedula but also in S. metallicus and A. infernus, whereas these enzymes appear to be lacking in T. tenax and in the methanogens tested. The optimal conditions for autotrophic growth and enzyme assay in these bacteria are yet to be established.

**DISCUSSION**

In vitro evidence for the 3-hydroxypropionate cycle of CO₂ fixation in Chloroflexus and in archaea. The 3-hydroxypropionate cycle of CO₂ fixation (5, 13, 38) has been proposed as the fourth, alternative pathway for autotrophic growth in nature. It was first described in the early-branching phototrophic thermophilic bacterium C. aurantiacus. We have demonstrated in vitro the essential partial reactions of this cycle from acetyl-CoA via 3-hydroxypropionate and propionyl-CoA to succinate.
The radioactive intermediates were formed irrespective of which carbon precursor, acetyl-CoA or bicarbonate, was 14C labelled. At a generation time of 20 h, the specific CO2 fixation rate reaches 48 nmol min$^{-1}$ mg of protein$^{-1}$. Since two molecules of CO2 are fixed per turn of the postulated CO2 fixation cycle, a minimal specific activity of the enzymes of the cycle of 24 nmol min$^{-1}$ mg of protein$^{-1}$ is required to explain the rate of autotrophic growth. The reactions rates measured approximated these postulated in vivo rates of autotrophically growing cells.

*C. aurantiacus* remains, so far, the only representative of the bacteria where strong evidence for this cycle has been found. K$^+$, MgATP, CoA, and NADPH were required for reaction. We could not confirm the operation of an alternative CO2 fixation cycle as proposed elsewhere (19, 25), since pyruvate:acceptor oxidoreductase activity was low and the labelling pattern of alanine precluded synthesis of pyruvate from acetyl-CoA and CO2 (5). We assume that the low levels of pyruvate:acceptor oxidoreductase function in acetyl-CoA synthesis. PEP carboxylase activity is required as an anaplerotic enzyme of the 3-hydroxypropionic acid cycle.

Recent evidence has indicated the presence of a similar pathway operating in *A. brierleyi* (18). This organism belongs to the *Sulfolobales*, a crenarchaeal order consisting of lithoautotrophic aerobes, facultative anaerobes, and obligate anaerobes; characteristically, these organisms thrive by aerobic sulfur oxidation or anaerobic sulfur reduction (34). We have extended these studies and have examined *M. sedula*, another autotrophic member of the *Sulfolobales* (15), for the presence of the 3-hydroxypropionate cycle. This organism does not express key enzymes of the reductive pentosephosphate cycle (ribulose-1,5-bisphosphate carboxylase), the reductive acetyl-CoA pathway (carbon monoxide dehydrogenase), or the reductive citric acid cycle (ATP citrate lyase). In contrast, the two carboxylating enzymes of the 3-hydroxypropionate cycle, acetyl-CoA carboxylase and propionyl-CoA carboxylase, were found. Additionally, the enzyme activities and intermediates involved in the CO2 fixation following the 3-hydroxypropionate cycle up to succinate have been verified. The specific activities of the enzymes of this cycle measured in cells grown on O2, S, and CO2 (generation time, 20 h) were close to the calculated minimal enzyme activities required for growth. It should be noted that the actual specific activity of the carboxylating enzymes at growth temperature (65°C) should be twice as high as that measured with the assay at 55°C.

**Acetyl-CoA and propionyl-CoA carboxylase activities and small biotin-carrying proteins in archaea.** A fast, specific, and sensitive test for the presence of biotin-containing proteins in cell extracts, with particular focus on the BCCP fragment of the acetyl-CoA and propionyl-CoA carboxylases, was used to screen autotrophic archaea. The rationale behind the test was that lipids of archaea do not contain fatty acids, since their membranes are formed by isoprenol glycerol ether lipids. It is therefore assumed that archaea normally do not require, and therefore do not synthesize, acetyl-CoA and propionyl-CoA carboxylases. The presence of significant amounts of a small BCCP-like protein and acetyl-CoA plus propionyl-CoA carboxylase activities in cell extracts of archaea would indicate that these carboxylases are being synthesized and involved in a process other than lipid biosynthesis.

A clear correlation was found between the presence of a
small biotin-containing protein and the detection of acetyl-CoA- and propionyl-CoA-dependent carboxylation activity in cell extracts. This positive correlation was found only in members of the *Sulfolobales* (*M. sedula*, *S. metallicus*, and *A. infernus*). In all other autotrophic archaea tested, which use one of the other pathways for CO₂ fixation, neither an abundant BCCP-like fragment nor acetyl-CoA and propionyl-CoA carboxylase activities were detected.

FIG. 7. Time course of the carboxylation of propionyl-CoA to methylmalonyl-CoA with ¹⁴CO₂ and subsequent conversion to succinate catalyzed by cell extracts of *M. sedula* at 55°C. (A) HPLC chromatograms showing the incorporation of ¹⁴CO₂ into propionyl-CoA, generating ¹⁴Cmethylmalonyl-CoA as transient intermediate. This intermediate is subsequently converted to ¹⁴Csuccinate. (B) HPLC chromatograms after alkaline hydrolysis of the CoA esters present in the samples. The labelled compounds in both panels were preliminarily identified by cochromatography with authentic compounds. Assay conditions (1-ml assay mixture, 2-ml headspace): 100 mM Tris-HCl (pH 7.8), 1 mM ATP, 5 mM MgCl₂, 3 mM DTE, 0.5 mM propionyl-CoA, 5 μmol of ¹⁴CNa₂CO₃ (10 kBq), 0.4 mg of protein.

Occurrence of acetyl-CoA carboxylase in archaea. The acetyl-CoA carboxylase protein is well conserved in nature; it has been purified and characterized not only in bacteria but also in yeasts, plants, and animals. Two basic types of this enzyme are found. The “bacterial type” contains four different subunits organized in three functional domains. Four different genes (accABCD) encode the peptides (AccABCD) of approximately 35, 17, 49, and 33 kDa, respectively. The “eukaryotic type” is composed of a single large peptide of approximately 280 kDa (39). The subunit composition of the acetyl-CoA carboxylase was found in the genomes available to date.

Further reactions of the 3-hydroxypropionate cycle and possible role in acetyl-CoA assimilation. At the present stage of our research with M. sedula, the fate of the CO₂ fixed after the formation of succinate remains an open question. However, it can be assumed that the rest of the reactions postulated in the 3-hydroxypropionate cycle involving the regeneration of the initial substrate, acetyl-CoA, are in operation. Tests aimed at clarifying this issue and the fate of glyoxylate are under way.

Furthermore, the assimilation of acetyl-CoA into C₃ compounds via the initial reactions of the 3-hydroxypropionate cycle should be considered as a possible pathway in those microorganisms that lack one, or both, of the characteristic enzymes of the glyoxylate cycle (isocitrate lyase and malate synthase). The new pathway of succinate synthesis from acetyl-CoA and 2CO₂ would allow growth on ethanol, acetate, or fatty acids and may be responsible for the assimilation of these substrates in the microorganisms studied here.

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