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Acyl Coenzyme A Carboxylase of *Propionibacterium shermanii*: Detection and Properties

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An acyl coenzyme A (CoA) carboxylase, which catalyzes the adenosine triphosphate-dependent fixation of CO_2 into acetyl-, propionyl-, and butyryl-CoA, was detected in fractionated cell extracts of *Propionibacterium shermanii*. Catalytic activity was inhibited by avidin but was unaffected by avidin pretreated with excess biotin. The carboxylase levels detected were relatively small and were related to cellular growth. Maximal carboxylase activity was detected in cells grown for about 96 h. Thereafter, the activity declined rapidly. Optimal CO_2 fixation occurred at pH 7.5. Other parameters of the assay system were optimized, and the apparent K_m values for substrates were determined. The end product of the reaction (with acetyl-CoA as the substrate) was identified as malonyl-CoA. The stoichiometry of the reaction was such that, for every mole of acetyl-CoA and adenosine triphosphate consumed, 1 mol each of malonyl-CoA, adenosine diphosphate, and orthophosphate was formed. These data provide the first evidence for the presence of another biotin-containing enzyme, an acyl-CoA carboxylase, in these bacteria in addition to the well-characterized methylmalonyl-CoA carboxyltransferase.

In a wide variety of living systems, acetyl coenzyme A (CoA) carboxylase (EC 6.4.1.2), a biotin-containing enzyme, catalyzes the first committed reaction in the de novo synthesis of fatty acids (3, 20). The overall reaction occurs via the two partial reactions:

$$\text{biotinyl enzyme} + \text{ATP} + HCO_3^- \rightleftharpoons \text{biotinyl enzyme-}CO_2 + \text{ADP} + P_i \quad (1)$$

$$\text{biotinyl enzyme-}CO_2 + \text{acetyl-CoA} \rightleftharpoons \text{biotinyl enzyme} + \text{malonyl-CoA} \quad (2)$$

Combining reactions 1 and 2 yields:

$$\text{acetyl-CoA} + \text{ATP} + HCO_3^- \rightleftharpoons \text{malonyl-CoA} + \text{ADP} + P_i \quad (3)$$

Until now, the only biotinyl enzyme documented in *Propionibacterium shermanii* has been methylmalonyl-CoA carboxyltransferase (EC 2.1.3.1), which plays a central role in the formation of propionate when the organism ferments glucose, glycerol, etc. (27, 29). This enzyme catalyzes the following reaction:

$$\text{methylmalonyl-CoA} + \text{pyruvate} \rightleftharpoons \text{oxaloacetate} + \text{propionyl-CoA} \quad (4)$$

Transcarboxylase can also catalyze the reversible transfer of a carboxyl group from oxaloacetate to acetyl-CoA:

$$\text{oxaloacetate} + \text{acetyl-CoA} \rightleftharpoons \text{malonyl-CoA} + \text{pyruvate} \quad (5)$$

The rate of acetyl-CoA carboxylation by this reaction is about 50% that of propionyl-CoA (27). Previous attempts to demonstrate acetyl-CoA carboxylase or propionyl-CoA carboxylase in *P. shermanii* were unsuccessful (25, 27), and in addition Fall et al. (12) noted the lack of a biotinyl polypeptide in these organisms in the molecular weight range of 20,000 to 25,000 considered to be characteristic of the biotin carboxyl carrier protein components of microbial acetyl-CoA carboxylases. Thus, it was considered that the malonyl-CoA required for fatty acid synthesis in *P. shermanii* most likely arose from reaction 5 catalyzed by transcarboxylase. If this were indeed the case, then transcarboxylase would function in both anabolic (malonyl-CoA synthesis) and catabolic (propionate formation) processes, and such bifunctionality seemed unlikely.

Besides transcarboxylase, propionic acid bacteria also contain pyruvate orthophosphate dikinase (EC 2.7.9.1) and phosphoenolpyruvate (PEP) carboxykinase (pyrophosphate) (EC 4.1.1.38). The combined actions of these enzymes can (10, 25) result in the formation of malonyl-CoA via the following sequence of reactions:

$$\text{ATP} + \text{pyruvate} + P_i \rightleftharpoons \text{PEP} + \text{AMP} + PP_i \quad (6)$$

$$\text{PEP} + CO_2 + P_i \rightleftharpoons \text{oxaloacetate} + PP_i \quad (7)$$

$$\text{oxaloacetate} + \text{acetyl-CoA} \rightleftharpoons \text{malonyl-CoA} + \text{pyruvate} \quad (5)$$

$$\text{acetyl-CoA} + \text{ATP} + CO_2 + 2P_i \rightleftharpoons \text{malonyl-CoA} + \text{AMP} + 2PP_i \quad (8)$$

Thus, if small diffusible metabolites, e.g., pyruvate, phosphoenolpyruvate, P_i, and oxaloacetate are not removed before assay and if the stoichiometry of the reaction is not determined, some confusion might arise as to whether fixation of CO_2 was occurring by the sum of reactions given in reaction 8 or whether malonyl-CoA formation was catalyzed by acetyl-CoA carboxylase as shown in reaction 3. This investigation provides reliable evidence that *P. shermanii* contains an acyl-CoA carboxylase activity capable of carboxylating acetyl-CoA, propionyl-CoA, and butyryl-CoA. Optimal conditions for assay, the stoichiometry of the reaction with acetyl-CoA as the substrate, and some properties of the *P. shermanii* acyl-CoA carboxylase are described.

MATERIALS AND METHODS

Acetyl-CoA, propionyl-CoA, butyryl-CoA, and malonyl-CoA were obtained from P-L Biochemicals, Inc. [2-^{14}C]malonyl-CoA (15 mCi/mmol), [γ-^{32}P]ATP (2 Ci/μmol), [2-^{14}C]malonic acid (50 mCi/mmol), and Aquasol 2 were purchased from New England Nuclear Corp. D-[*carbonyl*-^{14}C]biotin (40 to 60 mCi/mmol) was purchased from Amersham Corp. Avidin, ATP, dATP, ITP, GTP, UTP, NADH, lactate dehydrogenase, malate dehydrogenase (EC 1.1.1.37), pyruvate kinase (EC 2.7.1.40), oxaloacetate, streptomycin sulfate, chymotrypsinogen, cytochrome *c*, lysozyme, ovalbumin, bovine serum albumin, and bromcresol green were obtained from Sigma Chemical Co. Bio-Gel P4 and all reagents for sodium dodecyl sulfate-polyacrylamide gel electrophoresis, including diallyltartardiamide, were purchased from Bio-Rad Laboratories. Polyethyleneimine-cellulose F plates were from Merck & Co., Inc. All other reagents were of the highest purity commercially available.

Organism, media, and growth conditions. The stock cultures of *P. shermanii* were maintained in "stabs" containing 1.5% (wt/vol) agar, 1% (wt/vol) glucose, 2% (wt/vol) peptone, and 1% (wt/vol) yeast extract; they were subcultured periodically and stored at 4°C. Cells were routinely grown in 4-liter conical flasks containing 3.5 liters of a complex medium described elsewhere (P. M. Ahmad, L. A. Stirling, and F. Ahmad, J. Gen. Microbiol., in press) or were grown in a semidefined medium (8). Growth flasks were inoculated with approximately 80 ml of a vigorously fermenting culture and were incubated at 30°C. The flasks were shaken manually for a few minutes once daily to ensure adequate suspension of cells. Bacteria were harvested during late logarithmic growth by centrifugation at 10,000 × *g* for 10 min, and cell suspensions were washed twice with 0.1 M Tris-hydrochloride buffer (pH 7.5) containing 1 mM β-mercaptoethanol. Cell pastes were stored at −20°C until use.

For studies involving growth of the organism on D-[*carbonyl*-^{14}C]biotin, cells were grown in a semidefined medium (8) supplemented with radioactive biotin (50 μCi/liter).

The results of experiments given in Fig. 1 and 2 and Table 2 were obtained on the cellular protein derived from cells grown on the complex medium, whereas all other experiments were conducted on *P. shermanii* grown on a semidefined medium.

Preparation of cell extracts. Frozen cell pastes (20 to 40 g) were thawed and suspended in 0.1 M Tris-hydrochloride buffer (pH 7.5) containing 1 mM β-mercaptoethanol. The cell suspensions were disrupted by sonication (10 2-min bursts) in a Heat Systems model W375A sonicator operating at maximum power output while the temperature was maintained below 5°C.

Fractionation of cell extract with ammonium sulfate. All fractionation procedures were carried out at 0 to 4°C. The crude cell extract was diluted to 15 mg of protein per ml with buffer. To this solution, we added 0.3 volume of 5% (wt/vol) streptomycin sulfate while stirring. The solution was then centrifuged at 37,000 × *g* for 15 min. The resultant pellet was discarded. Solid ammonium sulfate was added to the supernatant solution, and the proteins precipitating at 0 to 45%, 45 to 55%, 55 to 65%, and 65 to 100% saturation levels were collected by centrifugation. All precipitated protein fractions were retained, were dissolved in a minimal amount of 0.1 M Tris-hydrochloride buffer (pH 7.5) containing 1 mM β-mercaptoethanol, and were quick-frozen in an alcohol-dry ice bath before storage at −70°C. Before use, samples were dialyzed for 3 h at 4°C against the above mentioned buffer, with one change after 1.5 h, to remove ammonium sulfate.

Acyl-CoA carboxylase assay. The acyl-CoA carboxylase assay method used, based on that of Alberts and Vagelos (2), measured the rate of incorporation of $^{14}CO_2$ into an acid-stable product. Optimal concentrations of reactants were determined. The reaction mixtures contained (total volume, 0.125 ml): 15.0 μmol of *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) buffer (pH 7.5), 0.1 mg of bovine serum albumin, 0.5 μmol of glutathione; 0.5 μmol of $MnCl_2$, 0.5 μmol of ATP, 1.25 μmol of $NaH^{14}CO_3$ (1,000 cpm/nmol), 0.6 μmol of acetyl-CoA, propionyl-CoA, or butyryl-CoA; and 250 to 500 μg of protein contained in the fraction precipitating between 45 and 55% ammonium sulfate saturation. The reaction was started by the addition of ATP, and mixtures were incubated at 30°C for 5 min. Reactions were terminated by the addition of 0.025 ml of 4 M HCl, 0.075-ml portions were removed into scintillation vials, and the contents were dried at 90°C in a forced-air oven before the addition of 0.5 ml of water. After the addition of scintillant (5.0 ml of Tritosol [15]), radioactivity was

determined in a Mark III (Searle Analytic) scintillation spectrometer with 85 to 90% counting efficiency. Assay conditions were selected to ensure that the reaction rate was proportional to the enzyme protein concentration and to the time of incubation. Control reactions lacking enzyme, ATP, and acyl-CoA were carried out routinely, and the low-background radioactivity was subtracted. Specific activity is expressed as nanomoles of $^{14}CO_2$ incorporated per minute per milligram of protein.

Protein was estimated by the biuret method (21), using bovine serum albumin as a standard, or by the method of Bradford (5), using ovalbumin as a standard. All assays were done in duplicate with ±10% variation. Variation in activity from batch to batch of cells grown for the same duration was negligible.

End product identification. A reaction mixture, scaled up 10-fold, was incubated at 30°C for 2 min. After the reaction was halted by acidification, the mixture was centrifuged to remove protein and the supernatant fraction was neutralized. This material was then applied to a Bio-Gel P4 column (1.5 by 30 cm), which was then eluted with water. Fractions were collected, and those fractions possessing radioactivity were pooled and lyophilized. The lyophilized material was dissolved in a minimal amount of water, and portions were applied to polyethyleneimine-cellulose F thin-layer chromatography sheets. The developing solvent was 0.75 M NaCl; with this solvent, good separation of malonyl-CoA, acetyl-CoA, and ATP could be achieved. Authentic malonyl-CoA was similarly chromatographed, and its location was visualized under UV light. The plates were then cut into small sections, and the radioactivity in each was estimated by the method already described.

A second reaction mixture was treated as described above to the neutralization step. The mixture was subjected to overnight alkaline hydrolysis (1 M KOH, at room temperature) to split the thioester bond. The sample was then acidified with HCl to pH 4.0 and extracted three times with ethyl ether. The extract was taken to dryness under a stream of N_2, and the residue was dissolved in a minimal amount of methanol. Carrier malonic acid was added, and portions of the sample were subjected to thin-layer chromatography on silica gel plates along with authentic radiolabeled malonic acid. The plates were developed in absolute ethanol-concentrated NH_4OH (70:30 [vol/vol]). Chromatograms were dried, and malonic acid was located on one-half of the plate by spraying with 0.04% bromcresol green in ethanol. The nonsprayed half of the plate was cut up and counted as described above.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Sodium dodecyl sulfate-gel electrophoresis was performed by an established procedure (26), except that to provide gels that could later be solubilized methylenebisacrylamide was replaced by diallyltartardiamide as described by Anker (4). Protein samples (~2 mg/ml) in 0.01 M sodium phosphate buffer (pH 7.0)–1.0% (wt/vol) sodium dodecyl sulfate–1.0% (vol/vol) β-mercaptoethanol were denatured by heating at 100°C for 10 min. Samples containing 20 to 40 μg of protein were then applied to 15% gels, and electrophoresis was performed at a constant current of 3 mA per gel in the presence of sodium dodecyl sulfate until the bromophenol blue tracking dye had migrated to the opposite ends of the gels. Gels were stained overnight in 0.025% Coomassie blue R 250–10% (vol/vol) glacial acetic acid–25% (vol/vol) isopropyl alcohol and were destained in a Bio-Rad 255 destainer containing 30% (vol/vol) methanol–10% (vol/vol) glacial acetic acid. After destaining, the gels were manually cut into small sections, which were placed in scintillation vials containing 0.5 ml of 2% (wt/vol) periodic acid. After the gel slices were allowed to solubilize at room temperature for 30 min, 5 ml of Aquasol 2 was added to each vial and the radioactivity was determined. Proteins of known molecular weights (ovalbumin, chymotrypsinogen, lysozyme, cytochrome *c*, and bovine trypsin inhibitor) were used to construct a standard curve.

Determination of reaction stoichiometry. To determine the amount of P_i formed in reaction 3, [γ-^{32}P]ATP was used instead of unlabeled ATP. The reaction mixture was scaled up 10-fold and incubated at 30°C for 2 min, and the reaction was halted by the addition of 4 M HCl. Inactivated protein was removed by centrifugation, and the pH of the supernatant solution was adjusted to 4.5. $^{32}P_i$ was separated from labeled ATP by thin-layer chromatography on polyethyleneimine-cellulose F sheets, which were developed with 0.75 M NaCl. P_i was also estimated by the colorimetric method of Hess and Derr (19). The concentration of ADP was quantitated enzymatically with pyruvate kinase and lactate dehydrogenase (6).

The concentrations of acetyl-CoA (combination of reactions 5 and 9) and malonyl-CoA (combination of reactions 5 and 10) were determined with transcarboxylase (30) by the reactions:

$$\text{acetyl-CoA} + \text{oxaloacetate} \rightleftharpoons \text{malonyl-CoA} + \text{pyruvate} \quad (5)$$

$$\text{pyruvate} + \text{NADH} \rightarrow \text{lactate} + \text{NAD} \quad (9)$$

$$\text{malonyl-CoA} + \text{pyruvate} \rightleftharpoons \text{acetyl-CoA} + \text{oxaloacetate} \quad (5)$$

$$\text{oxaloacetate} + \text{NADH} \rightarrow \text{malate} + \text{NAD} \quad (10)$$

RESULTS

Fractionation of cell extracts of *P. shermanii* and determination of acyl-CoA carboxylase activity. When the crude cell-free protein from *P. shermanii* was tested for acyl-CoA carboxylase activity, only extremely low levels of activity (<0.1 nmol of CO_2 fixed per min per mg of protein) could be detected. Experiments were therefore conducted to determine whether fractionation of *P. shermanii* cell extracts with ammonium sulfate would facilitate detection of acyl-CoA carboxylase activity. This procedure will also eliminate small interfering metabolites. The results obtained showed that acyl-CoA carboxylase activity could be readily detected in these fractions and that the majority

of activity was present in protein precipitating between 45 and 55% ammonium sulfate saturation. Fractions precipitating between 0 and 45%, 55 and 75%, and 75 and 100% saturation levels of ammonium sulfate had negligible activity. There was no stimulation of carboxylase activity when various fractions were combined and assayed.

Acyl-CoA carboxylase activity as a function of cellular growth. Cells were harvested and fractionated after the incubation periods ranging from 24 to 168 h (Fig. 1). The carboxylase activity increased as the cells entered the logarithmic phase of growth and reached a maximum toward the late-logarithmic, early-stationary phase. Thereafter, the carboxylase levels decreased rapidly, suggesting that the activity was closely related to cellular growth processes. Doubling times for *P. shermanii* under the conditions used were approximately 10 h. Similar results were obtained when these experiments were repeated. Most of the results reported below were obtained with cells grown for 96 h.

Optimal assay conditions for catalysis. (i) pH optimum. The optimal pH for catalysis was found to be 7.5 (Fig. 2). Carboxylase activity was greatest in the presence of HEPES, and this buffer was used in all subsequent assays. Independent experiments showed that its optimal concentration in the assay system was 120 mM.

(ii) Divalent metal ion requirements. Carboxylase assays were carried out in the presence

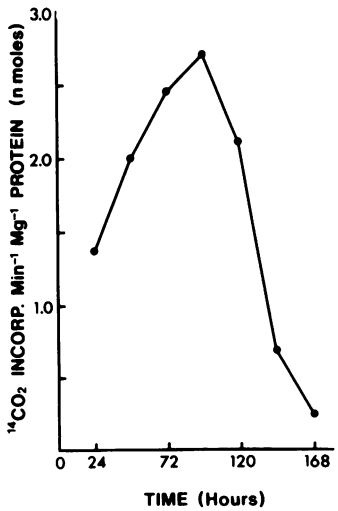

FIG. 1. *Acyl-CoA carboxylase activity during the growth cycle of P. shermanii. Cultures of P. shermanii were harvested, disrupted, and fractionated at the time periods indicated. The acyl-CoA carboxylase activity in the 45 to 55% ammonium sulfate fraction was assayed with acetyl-CoA as substrate as described in the text.*

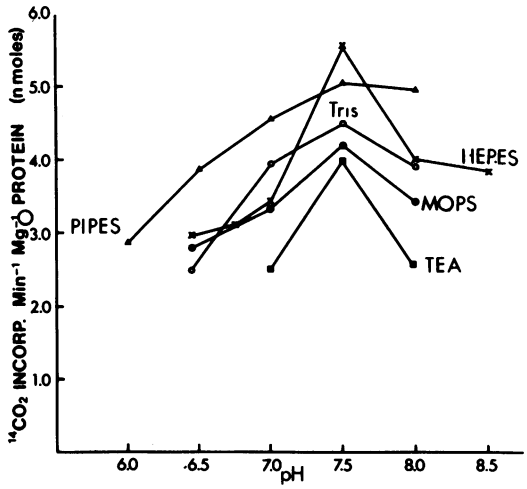

FIG. 2. *Optimal pH and buffer system for acyl-CoA carboxylase activity. Assays were performed as described in the text with acetyl-CoA as substrate. All buffers were added to a final concentration of 120 mM. PIPES, piperazine-N,N'-bis(2-ethanesulfonic acid); MOPS, morpholinopropanesulfonic acid; and TEA, triethanolamine.*

of different cations. The reaction proceeded optimally in the presence of Mn^{2+} (100% relative activity), with no difference in activity when the chloride or the sulfate salt was used. Optimal fixation of $^{14}CO_2$ occurred in the presence of Mn^{2+} at a final concentration of 4 mM, and this concentration was used in all subsequent assays. Instead of Mn^{2+}, when assays were performed in the presence of 4 mM Co^{2+}, Mg^{2+}, Fe^{2+}, Ca^{2+}, or Zn^{2+}, the activity decreased to 73, 52, 25, 3, and 2.3%, respectively.

Substrate specificity. Acyl-CoA. Incorporation of $^{14}CO_2$ into an acid-stable product occurred in the presence of acetyl-CoA, propionyl-CoA, and butyryl-CoA. Hexanoyl-CoA was not carboxylated.

Nucleotide. The incorporation of $^{14}CO_2$ into products is dependent upon a source of energy, usually in the form of ATP. We examined the carboxylase activity of *P. shermanii* in the presence of various nucleotides and also pyrophosphate, because of its implication as an energy source for some reactions in propionic acid bacteria (28). Both ATP and dATP were active in the carboxylase reaction. At identical concentrations (4 mM), the specific activity of the carboxylase in the presence of dATP was 16.9, whereas in the presence of ATP it was 11.2. ITP, GTP, UTP, and pyrophosphate were essentially inactive.

Stability of acyl-CoA carboxylase. It was observed that the carboxylase was relatively un-

stable, especially upon storage at 4°C or higher. This perhaps explains why similar enzyme preparations stored for different time intervals possess different specific activities. Activity losses incurred at 4°C were comparable for acetyl-CoA, propionyl-CoA, and butyryl-CoA.

When the carboxylase was subjected to heat treatment (35°C for 10 min), 50 to 60% of the activity was lost regardless of acyl-CoA substrate used. Higher temperatures (e.g., 45°C for 1 min) resulted in more than 90% loss of carboxylase activity.

K_m values for the substrates of the carboxylase reaction. The apparent K_m values for all the substrates of the carboxylase reaction, obtained from double-reciprocal plots, summarized in Table 1 and show that acetyl-CoA had the lowest K_m value. ATP and dATP had similar kinetic constants.

In addition to those factors already described, bovine serum albumin and glutathione were required for optimal catalysis. The optimal HCO_3^- concentration was determined to be 10 mM.

Effect of *p*-chloromercuribenzoate on enzyme activity. The enzyme preparation was incubated at 30°C for 2 min with various concentrations of *p*-chloromercuribenzoate and then the carboxylase activity was assayed in the usual fashion to determine the effect of the added *p*-chloromercuribenzoate. Carboxylase activity decreased as the concentration of *p*-chloromercuribenzoate increased (Fig. 3). The losses in activity with acetyl-CoA and propionyl-CoA as substrates were seen to follow an almost identical pattern. This sulfhydryl reagent had a somewhat more profound inhibitory effect when butyryl-CoA was used as the substrate.

Analysis of biotin-containing polypeptides from *P. shermanii*. Cell extracts from *P. shermanii* grown in the presence of radiolabeled biotin were fractionated as described in Materials and Methods. The fraction precipitating between 45 and 55% ammonium sulfate saturation was treated with sodium dodecyl sulfate in the presence of β-mercaptoethanol and then electrophoresed in the presence of the denaturant. Most of the recovered radioactivity was located in a gel segment where protein(s) of molecular weight(s) of 11,500 was found to migrate (data not shown). The molecular weight of the biotin carboxyl carrier protein of transcarboxylase is approximately 12,000 (16).

TABLE 1. *Apparent K_m values for the substrates of the carboxylase-catalyzed reaction*[a]

Substrate	Apparent K_m (mM)
Acetyl-CoA	0.09
Propionyl-CoA	0.17
Butyryl-CoA	0.83
$H^{14}CO_3$	3.00
ATP	1.43
dATP	1.14

[a] Assays were run for 1-min periods so that initial rates could be measured. The concentrations of the nonvaried substrates are given in the text. K_m values of HCO_3^-, ATP, and dATP were determined in the presence of acetyl-CoA.

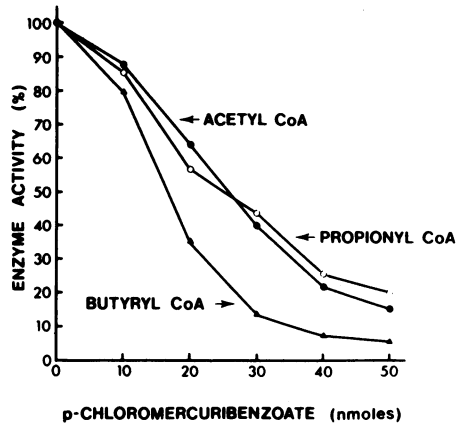

FIG. 3. *Effect of p-chloromercuribenzoate on acyl-CoA carboxylase activity. Portions of the 45 to 55% ammonium sulfate fraction (50 μl) were incubated for 2 min at 30°C with the amounts of p-chloromercuribenzoate shown. After this period 20-μl samples were added to a mixture containing all the other requisite components for the carboxylase reaction, and assays were done as described in the text. Control samples lacking p-chloromercuribenzoate, but preincubated for the same 2-min periods at 30°C, were also run.*

Additional evidence for the presence of acyl-CoA carboxylase activity in *P. shermanii*. (i) Effect of avidin on $^{14}CO_2$ incorporation. Avidin is a very potent inhibitor of biotin-containing enzymes. Incorporation of $^{14}CO_2$ was completely inhibited when avidin was present in the assay system but the activity was unaffected in the presence of avidin pretreated with excess biotin. This result provided reliable evidence for a biotin-dependent carboxylase-catalyzed $^{14}CO_2$ incorporation. However, transcarboxylase is also a biotin-containing enzyme which would be similarly inhibited in the presence of avidin. Therefore, the sequence of reactions culminating in reaction 8 will be likewise inhibited.

(ii) Removal of small metabolites before assay. As stated above, the presence of small metabolites can lead to some confusion concerning ATP-dependent CO_2 fixation in *P. shermanii*. When care was taken to remove pyruvate, oxaloacetate, or PEP, thus preventing re-

actions 5, 6, 7, and consequently 8 from occurring, the rate of $^{14}CO_2$ incorporation was essentially unchanged (Table 2). This provided firm evidence that malonyl-CoA formation was not occurring via the sum of reactions shown in reaction 8, but via reaction 3 catalyzed by an acyl-CoA carboxylase. In addition it is known that EDTA is an inhibitor of phosphoenolpyruvate carboxykinase (7). When the enzyme was treated with EDTA before assay the incorporation of $^{14}CO_2$ was unaltered, lending further support for an acyl-CoA carboxylase-catalyzed reaction.

(iii) End product identification in the absence of small metabolites. The radiolabeled end product of the reaction, with acetyl-CoA as the substrate, cochromatographed with authentic malonyl-CoA after thin-layer chromatography on polyethyleneimine-cellulose F plates (Fig. 4). In addition, after alkaline hydrolysis and ether extraction of a second reaction mixture, the radiolabeled product was found to have the same R_f value as authentic malonic acid when both were subjected to thin-layer chromatography on silica gel plates.

(iv) Reaction stoichiometry. The overall stoichiometry of carboxylase-catalyzed reaction 3 dictates that, for every mole of acetyl-CoA and ATP utilized, 1 mol of malonyl-CoA, ADP, and P_i should be formed. An average of three determinations (with percent variation ±5%) yielded 0.41 μmol of malonyl-CoA, 0.49 μmol of ADP, and 0.47 μmol of P_i per ml when 0.46 μmol of acetyl-CoA per ml was utilized. Our results were consistent with the mechanism of reaction 3 and provided firm support for the presence of an acyl-CoA carboxylase in *P. shermanii*. Under the experimental conditions used, the other plausible route for CO_2 fixation (reaction 8) into acetyl-CoA in *P. shermanii* can be ruled out because neither PP_i nor AMP was detected during catalysis.

TABLE 2. *Effect of the removal of small metabolites before assay of acyl-CoA carboxylase*

Addition	$^{14}CO_2$ incorporated per minute per milligram of protein (nmol)
None	5.7
NADH + LDH[a]	5.8
None	5.0
HCO_3^- + MDH + P_i + NADH[b]	4.6

[a] For the removal of pyruvate (reaction 9), samples of the 45 to 55% ammonium sulfate fraction were incubated for 2 min at 30°C with 0.1 μmol of NADH and 1 U of lactate dehydrogenase (LDH). After this period, the other components of the assay were added, and reactions were carried out as described in the text.

[b] For the removal of phosphoenolpyruvate (reactions 7 and 10), oxaloacetate (reaction 10), or both, samples of the 45 to 55% ammonium sulfate fraction were incubated for 2 min at 30°C with 2 μmol of NADH, 1 U of malic dehydrogenase (MDH), and 10 μmol of phosphate. After this treatment, the other components of the assay were added, and reactions were carried out as described in the text.

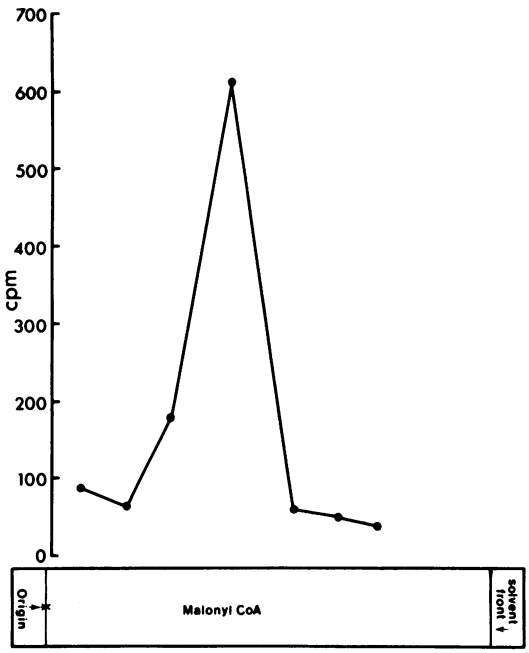

FIG. 4. *Identification of malonyl-CoA as reaction end product. Thin-layer chromatography was carried out on polyethyleneimine cellulose F plates as described in the text with 0.75 M NaCl as the developing solvent. Malonyl-CoA was visualized under UV light.*

DISCUSSION

An acyl-CoA carboxylase system from *P. shermanii* has been studied in detail for the first time. We refrain from using the term acetyl-CoA carboxylase because the system also has the ability to carboxylate propionyl-CoA and butyryl-CoA. At present, it is unknown whether more than one carboxylase activity was responsible for the observed results or whether there existed one enzyme with a relatively broad substrate specificity. Assuming that one enzyme was responsible, the apparent K_m values indicated that acetyl-CoA was the preferred substrate. That only one enzyme was involved was suggested by the fact that when activity was lost, either by storage of enzyme preparations at 4°C or by heat treatment, these losses were of a similar magnitude irrespective of the acyl-CoA substrate used. In addition, when enzyme prep-

arations were treated with various amounts of *p*-chloromercuribenzoate, the activity losses followed similar patterns for all three acyl-CoA substrates. Further studies are necessary, however, before the presence of a single enzyme capable of carboxylating acetyl-CoA, propionyl-CoA, and butyryl-CoA can be unequivocally demonstrated.

Acyl-CoA carboxylases have been isolated from several microbial sources. The acetyl-CoA carboxylase from *Escherichia coli* has been studied most extensively and has been shown to consist of three readily separable subunits (biotin carboxylase, biotin carboxyl carrier protein, and carboxyltransferase) and in addition displays an absolute specificity for acetyl-CoA (1, 17, 20). The acetyl-CoA carboxylase from *Achromobacter* sp. strain 1VS is structurally similar to that from *E. coli* (22). The carboxylase system from *Mycobacterium phlei* has been isolated as an aggregated complex and is active in the presence of propionyl-CoA and acetyl-CoA, but only one enzyme is implicated here (9). Multiple acyl-CoA carboxylases, including acetyl-CoA and propionyl-CoA carboxylase, have been isolated as separate entities from *Pseudomonas citronellolis* (18). The latter activity has been shown to contain at least two subunits, whereas the former has been stabilized and isolated as an intact complex (11).

It is of the utmost importance to exercise caution when measuring apparent carboxylase activity in relatively crude fractions derived from propionic acid bacteria because these organisms contain enzymes that, in the presence of low-molecular-weight, naturally occurring metabolites, could effect an ATP-dependent incorporation of CO_2 without involving acyl-CoA carboxylase activity. The evidence presented strongly favors a carboxylase-catalyzed reaction, and the stoichiometry data substantiate its unequivocal presence in *P. shermanii*. The requirements and optimal conditions for assaying the carboxylase activity have been established, and the end product has been identified by both the chemical and the enzymatic methods.

The carboxylase can use either ATP or dATP as its energy source. The kinetic constants for each are very similar, and one merely substitutes for the other. Pyruvate carboxylase from chicken liver is equally active in the presence of ATP or dATP (24) as is the acetyl-CoA carboxylase from rat mammary gland (23).

Attempts to identify the biotin-containing peptides by using radiolabeled biotin in the growth medium followed by an electrophoretic analysis of ammonium sulfate fractions revealed the presence of a single biotin peptide with a molecular weight of 11,500. A biotin peptide of similar size is part of methylmalonyl-CoA transcarboxylase (16). Thus, it is possible that the radiolabeled peptide found in fraction catalyzing acyl-CoA carboxylation is part of the transcarboxylase complex. However, this peptide could also represent the native or a modified form of the biotin carboxyl carrier protein from an acyl-CoA carboxylase described in this report. In the *E. coli* system, several species of biotin carboxyl carrier protein have been isolated. Biotin peptides with molecular weights of 9,000 and 10,400 were initially found which were later shown to represent degradation products, via proteolysis, of a larger biotin carboxyl carrier protein with a molecular weight of 22,000 (13). The larger form was found to be more active biologically than the smaller fragments derived from its cleavage. Subsequent studies have shown that the native form of biotin carboxyl carrier protein in *E. coli* is in fact best represented by a dimeric structure with a molecular weight of 45,000 consisting of two subunits with a molecular weight of 22,500, each containing a biotinyl group (14).

It is possible that a similar situation occurs in *P. shermanii* and that the peptide with a molecular weight of 11,500 could be the degradation product of a larger biotin carboxyl carrier protein. However, before this question is resolved, it will be necessary to remove the relatively large amounts of methylmalonyl-CoA transcarboxylase from acyl-CoA carboxylase preparations. It may also be necessary to find means to inhibit protease activity in cell-free preparations.

Attempts at further purifying the carboxylase have been hampered because of its instability, but preliminary work indicates that it does not dissociate to any appreciable extent during ammonium sulfate fractionation and by passage through ion exchange and gel filtration matrices. This indicates that the carboxylase system from *P. shermanii* is unlike that of *E. coli*, which readily dissociates into its three components during purification procedures. In contrast, the fatty acid synthase of *P. shermanii* has been found to be similar to the *E. coli* synthase system (Ahmad et al., in press).

The *P. shermanii* carboxylase system may be more closely related to that of *P. citronellolis* or *M. phlei*. It is apparent that, although they are similar in function, there is a great deal of structural variability among biotin enzymes of different origins, making them good candidates for the study of their evolutionary development.

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