Multiple Acyl-Coenzyme A Carboxylases in *Pseudomonas citronellolis*†

Mina L. Hector‡ and R. Ray Fall*

ABSTRACT: *Pseudomonas citronellolis* was shown to contain four different acyl-coenzyme A carboxylases, including acetyl-, propionyl-, 3-methylcrotonyl-, and geranyl-CoA carboxylases, when grown on the appropriate carbon sources. Acetyl-CoA carboxylase activity in crude extracts was stimulated approximately 40-fold by inclusion of 0.4–0.5 M ammonium sulfate in the assay. Unexpectedly high levels of propionyl-CoA carboxylase activity, also stimulated by ammonium sulfate, were found in acetate-grown cells. That these acetyl- and propionyl-CoA carboxylase activities were due to different enzymes was shown by their resolution during purification by a procedure that stabilized acetyl-CoA carboxylase as a complex and separated propionyl-CoA carboxylase into two required protein fractions. Propionate- or valine-grown cells contained a propionyl-CoA carboxylase activity that was strongly inhibited by ammonium sulfate in the assay, and which may represent an inducible form of the enzyme. Geranyl- and 3-methylcrotonyl-CoA carboxylases that catalyze the carboxylation of the 3-methyl groups of homologous acyl-CoA acceptors, were induced by growth on the monoterpenes, citronellic or geranoic acid; only 3-methylcrotonyl-CoA carboxylase was induced by growth on leucine or isovaleric acid. Induction of either carboxylase was associated with the appearance of similar high-molecular-weight, biotin-containing proteins as measured by gel filtration. These two carboxylases are probably distinct enzymes since 3-methylcrotonyl-CoA carboxylase from isovalerate-grown cells does not carboxylate geranyl-CoA, while geranyl-CoA carboxylase will carboxylate both acyl-CoA homologues. *P. citronellolis* appears to be a useful system for studying the structural aspects of pairs of homologous acyl-CoA carboxylases.

Many different biotin-dependent carboxylases have been isolated from a variety of biological sources, and it is now clear that such enzymes, including acetyl-coenzyme A carboxylase (ACase),[1] propionyl-coenzyme A carboxylase (PCase), 3-methylcrotonyl-coenzyme A carboxylase (MCase), urea carboxylase, and pyruvate carboxylase, contain at least three functional elements: a biotin carboxylation site, a transcarboxylation site, and a covalently bound biotin prosthetic group that acts as a "CO_2" carrier between these two catalytic sites (for a review see Moss and Lane, 1971; Alberts and Vagelos, 1972). The interaction of these three subsites is shown schematically in Figure 1. All of these enzymes catalyze an analogous ATP and divalent metal ion dependent carboxylation of the biotin moiety at the biotin carboxylation subsite, and a "CO_2" transfer from carboxybiotin to the appropriate carboxyl acceptor at the transcarboxylation subsite. The distinct nature of the subsites has been confirmed by the findings that these three subsites are localized on three separate subunits, termed biotin carboxylase, biotin carboxyl carrier protein, and transcarboxylase, in *Escherichia coli* (Alberts and Vagelos, 1968; Alberts et al., 1969) and spinach chloroplast (Kannangara and Stumpf, 1972) ACases. Several other biotin-dependent carboxylases apparently contain two or more of these subsites fused into one or two polypeptide chains (Lynen, 1974). For example, *Pseudomonas citronellolis* and *Azotobacter vinelandii* pyruvate carboxylases (Barden et al., 1975), and *Achromobacter* MCase (Schiele et al., 1975) each contain two distinct subunits, the larger of which contains the biotin moiety; in the case of the *Achromobacter* MCase the larger subunit also contains the biotin carboxylase subsite, and the smaller subunit is presumed to be the transcarboxylase component. On the other hand, recent evidence suggests that yeast and liver pyruvate carboxylases (Utter et al., 1975), and rat liver ACase (Tanabe et al., 1975) are composed of only a single type of subunit resulting from fusion of the three subsites into a single polypeptide chain.

Because of the similarity in reaction mechanism it seems plausible that different biotin-dependent carboxylases in the same organism might share common structural features in the biotin carboxylase and biotin subsites, and differ primarily at the transcarboxylase subsite that confers carboxyl acceptor specificity (Alberts et al., 1969). Two studies aimed at testing this possibility focused on structural comparisons of ACase and pyruvate carboxylase isolated from either yeast (Sumper and Riepertinger, 1972) or *P. citronellolis* (Fall et al., 1975), and concluded that there are major structural differences between these two enzymes in both organisms. However, in order to test this possibility with biotin-dependent carboxylases that contain subsites fused into one or two polypeptide chains it will be necessary to determine amino acid sequence homology between different enzymes. In addition, the studies cited above have involved comparisons of only two enzymes, ACase and pyruvate carboxylase, that represent two different classes of biotin enzymes, acyl-CoA carboxylases and α-keto acid carboxylases, respectively (Moss and Lane, 1971). A more relevant comparison might be between biotin enzymes that carboxylate homologous substrates (e.g., ACase vs. PCase). We decided to search for a single microorganism that would be a useful source of several different biotin-dependent, acyl-CoA carboxylases. An organism with this potential is *P. citronellolis*,

† From the Department of Chemistry, University of Colorado, Boulder, Colorado 80309. *Received March 9, 1976.* This work was supported by a grant from the National Institutes of Health (HL 16628).

‡ Present address: Department of Chemistry, California State University, Chico, Calif. 95929.

[1] Abbreviations used are: ACase, acetyl-coenzyme A carboxylase; PCase, propionyl-coenzyme A carboxylase; MCase, 3-methylcrotonyl-coenzyme A carboxylase; GCase, geranyl-coenzyme A carboxylase; ATP, adenosine 5′-triphosphate; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; NADH, reduced nicotinamide adenine dinucleotide; BSA, bovine serum albumin; DEAE, diethylaminoethyl.

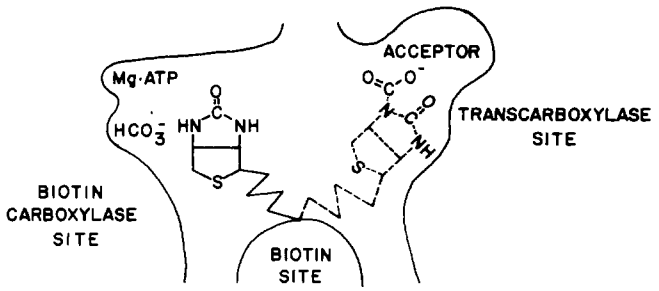

FIGURE 1: Schematic representation of the three functional sites of biotin-dependent carboxylases. The acceptor is the carboxyl accepting substrate.

since it contains an *E. coli*-type ACase (Fall et al., 1975), and an inducible geranyl-CoA carboxylase (GCase). GCase is a biotin-dependent carboxylase involved in the degradation of acyclic terpenes, such as citronellol, geraniol, and farnesol (Seubert, 1960; Seubert and Remberger, 1963; Seubert et al., 1963). In addition, it seemed possible that two other biotin-dependent carboxylases, PCase and MCase, might be obtained from *P. citronellolis,* since these two enzymes have been described in related fluorescent pseudomonad species. MCase is induced in *P. putida* as part of the leucine degradation pathway (Massey et al., 1974), and PCase has been detected in *P. aeruginosa* when grown on valine as carbon source (Sokatch et al., 1968). The studies described below demonstrate that, indeed, *P. citronellolis* is a potentially useful source of four different acyl-CoA carboxylases, including ACase, PCase, GCase, and MCase.

Materials and Methods

Organisms and Growth Conditions. P. citronellolis 13674 was obtained from the American Type Culture Collection. Wild-type strains of *P. citronellolis* and *P. aeruginosa* were kindly provided by M. F. Utter and J. R. Sokatch, respectively. Cultures were routinely grown on the following basal medium (medium T): 50 mM Tris-Cl, pH 7.4; 2 mM potassium phosphate, pH 7.4; and the following in g/l: $(NH_4)_2SO_4$, 2; KCl, 0.74; $MgSO_4$, 0.2; $CaCl_2 \cdot 2H_2O$, 0.015; $FeSO_4 \cdot 7H_2O$, 0.015; Na_2MoO_4, 0.002; $MnSO_4 \cdot H_2O$, 0.002; yeast extract (Difco), 0.5. The concentrations of the various carbon sources were: glucose, 0.2%; sodium acetate, 1%; propionic acid, 0.5%; DL-leucine, 0.3%; DL-valine, 0.3%; citronellic acid, 0.1%; geranic acid, 0.1%; and isovaleric acid, 0.3%. Where necessary the pH of the medium was adjusted to pH 7.4 with NaOH. In some experiments the media was supplemented with 50 μCi of [^{3}H]biotin (3.2 Ci/mmol)/l.; the [^{3}H]biotin was prepared (New England Nuclear) and purified as previously described (Fall and Vagelos, 1973). Cells were grown aerobically with shaking at 33 °C to late-logarithmic stage, and were harvested by centrifugation at 4 °C. Cells were washed twice with 0.02 M potassium phosphate, pH 7.0, and the cell pellets were stored frozen.

Enzyme Preparation. All operations were carried out at 4 °C, and where the protein content was determined a microbiuret procedure was used (Koch and Putnam, 1971). Cells were suspended in 2–3 volumes of 0.02 M imidazole-Cl, pH 7.0, 0.05 M ammonium sulfate (buffer IA), containing 1 mM dithiothreitol, and ruptured by passage through a French pressure cell, and then centrifuged at 48 000*g* for 40 min. The resulting supernatant was used as the crude extract.

In some cases crude extracts from cells that had been grown in the presence of [^{3}H]biotin were adjusted to 60% saturation with ammonium sulfate (361 g/l.), and the precipitated protein was collected by centrifugation at 10 000*g* for 20 min. This procedure results in quantitative precipitation of all the biotin-containing carboxylases described herein with good recovery of activity. The resulting precipitate could be stored frozen for several weeks without loss in activity. The precipitates were dissolved in buffer IA, containing 0.1 mM dithiothreitol, adjusted to a protein concentration of 50 mg/ml, and then 3 ml of these preparations were applied to a column of Sepharose-4B (2.5 × 77 cm) and eluted with the same buffer. Fractions of approximately 3 ml were collected, and [^{3}H]biotin protein peaks were detected by liquid scintillation counting in 3a70 fluid (Research Products International). Carboxylase assays were conducted as described below.

The purification of ACase will be described in detail elsewhere (R. Fall, manuscript in preparation); the following general procedure was used. Crude extracts were prepared as described above, diluted to approximately 20 mg of protein/ml, and then adjusted to 45% saturation with ammonium sulfate (258 g/l.). The precipitated protein was collected by centrifugation. The precipitate was dissolved in buffer IA, containing 1 mM dithiothreitol, to a protein concentration of 50 mg/ml and fractionated with alumina gel (Sigma) as previously described (Fall and Vagelos, 1972). The 0.4 M potassium phosphate eluate from the gel contained >50% of the initial ACase activity. The ACase was concentrated by adjusting the solution to 50% saturation with ammonium sulfate (291 g/l.) and collecting the precipitate by centrifugation. The precipitate was dissolved in 0.02 M imidazole-Cl, pH 7.0, 0.5 M ammonium sulfate, 1 mM dithiothreitol, and stored at 4 °C.

For the preparation of PCase components crude extracts were treated as above, except that ammonium sulfate fractionations between 0–40% (226 g/l.), 40–50% (58 g/l.), and 50–65% (92 g/l.) were carried out. The 0–40% and 50–65% precipitates were saved and dissolved in a minimal volume of buffer IA containing 1 mM dithiothreitol just before assay.

Enzyme Assays. The assay for ACase, PCase, MCase, or GCase in crude extracts is based upon the formation of acid-stable ^{14}C-carboxylated products derived from [^{14}C]$NaHCO_3$ (Flavin et al., 1957). The reaction mixtures contained 0.1 M Hepes, pH 8, 1.67 mM $MgCl_2$, 2.2 mM ATP, 13.9 mM [^{14}C]$NaHCO_3$ (2 mCi/mmol), 0.33 mM appropriate acyl-CoA, 25 μg of bovine serum albumin, and enzyme extract in a total volume of 0.09 ml. The reactions were initiated with the ATP component, and were routinely carried out in 6 × 50 mm tubes for 5 min at 30 °C. Control reactions with the acyl-CoA omitted were always run to determine background fixation. The reactions were terminated by the addition of 0.01 ml of 3 N HCl, and each sample tube was cut in half and the reaction mixture plus tube was inverted into a scintillation vial, and then heated to dryness in an oven at 80–90 °C for 15 min. Water (0.5 ml) and scintillation fluid (5 ml of 3a70) were added and radioactivity was counted in either a Packard 3310 or Beckman LS 3145 scintillation counter. A unit of enzyme activity is defined as that amount of enzyme that causes the formation of 1 μmol of carboxylated product/min.

Spectral assays for MCase or GCase were carried out essentially as described by Seubert et al. (1963). The reaction mixtures contained 100 mM Tris-HCl, pH 8, 10 mM $MgCl_2$, 0.5 mM ATP, 10 mM $KHCO_3$, 0.2 mM phosphoenolpyruvate, 6.3 units/ml of pyruvate kinase, 13 units/ml of lactic dehydrogenase, 0.10 mg/ml of NADH, 0.5 mg/ml of BSA, and 0.3 mM 3-methylcrotonyl-CoA or geranyl-CoA (*cis*-geranyl-CoA was routinely used in these assays); and the appropriate enzyme (0–5 milliunits) in a total volume of 0.2 ml. Reactions were carried out at 30 °C. Spectral assays of pyruvate car-

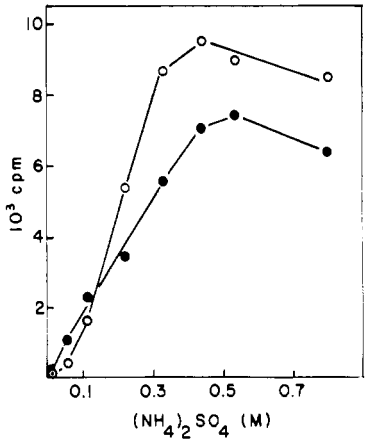

FIGURE 2: Ammonium sulfate stimulation of ACase and PCase in an extract of acetate-grown cells. A crude extract of acetate-grown *P. citronellolis* was prepared and assayed for ACase and PCase by the $^{14}CO_2$ fixation assay as described under Materials and Methods. Each assay mixture contained 128 μg of crude extract protein, the amount of $(NH_4)_2SO_4$ indicated, and the appropriate acyl-CoA. ^{14}C-fixation in the absence of acyl-CoA was determined for each $(NH_4)_2SO_4$ level and subtracted from the acyl-CoA-dependent $^{14}CO_2$-fixation values. Assays were carried out for 5 min. (O-O) ACase activity; (●-●) PCase activity.

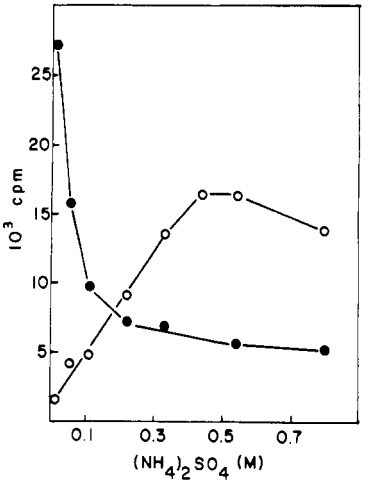

FIGURE 3: Effects of ammonium sulfate on ACase and PCase activities in an extract of propionate-grown cells. $^{14}CO_2$-fixation assays were carried out on an extract of propionate-grown cells (250 μg of protein/assay) as described in the legend of Figure 1. (O-O) ACase; (●-●) PCase.

boxylase were conducted as described by Taylor et al. (1972).

Substrates and Other Reagents. Acetyl-CoA was synthesized by the method of Simon and Shemin (1953). 3-Methylcrotonyl-CoA was synthesized from 3-methylcrotonic acid (J. T. Baker Chemical Co.) by the mixed anhydride method described by Stadtman (1956); geranyl-CoA was also synthesized by this procedure from geranoic acid except that the $KHCO_3$ solution was prepared in 50% tetrahydrofuran to solubilize the geranoic acid. *cis*-Geranoic acid was prepared by oxidation of citral (Pfaltz and Bauer) essentially as previously described (Seubert et al., 1963). The coenzyme A esters were assayed by the hydroxamate method of Shapiro (1953). Coenzyme A was obtained from P-L Biochemicals. Avidin and [^{14}C]-$NaHCO_3$ were obtained from Worthington and Research Products International, respectively.

Results

Assay of ACase and PCase in Crude Extracts. We initially experienced great difficulty in obtaining linear, reproducible ACase and PCase assays using a standard $^{14}CO_2$-fixation assay (see Materials and Methods). As part of a concurrent study to isolate an intact ACase complex from *P. citronellolis*, we determined that the complex is stabilized by the addition of ammonium sulfate to extraction and purification buffers (R. Fall, manuscript in preparation). Addition of this salt to the assay mixture markedly affected the apparent ACase and PCase activities in crude extracts. Figure 2 illustrates these effects. The ACase activity in a crude extract of acetate-grown *P. citronellolis* was stimulated by increasing levels of ammonium sulfate up to a maximal stimulation at approximately 0.45 M; the ACase activity decreased at ammonium sulfate levels higher than 0.5 M (Figure 2). In addition to providing an approximately 40-fold stimulation of ACase, the presence of 0.4–0.5 M ammonium sulfate in the assay decreased the ^{14}C-background fixation (i.e., in the absence of acetyl-CoA), and resulted in linear, reproducible assays. Similar ammonium sulfate stimulation of ACase was observed in all extracts of *P. citronellolis*, *P. aeruginosa*, and *P. putida* examined, although the concentration of ammonium sulfate required for maximal stimulation varied between 0.3–0.5 M. Figure 3 shows the ammonium sulfate stimulation of ACase activity in a crude extract from propionate-grown *P. citronellolis*.

The effect of ammonium sulfate in the assay was not extensively investigated, but it may be related to stabilization of the ACase complex or salt inhibition of interfering enzymes, such as malonyl-CoA decarboxylase (Hayaishi, 1955), that are present in *Pseudomonas* extracts. For unknown reasons, substitution of other salts, such as ammonium chloride or sodium sulfate, for ammonium sulfate in the assays resulted in much lower stimulations.

When crude extracts were assayed for PCase in the presence of ammonium sulfate two different patterns emerged. Extracts of acetate-grown *P. citronellolis* contained a lower level of PCase compared to ACase, but the PCase activity followed a stimulation curve (Figure 2) that paralleled the ACase curve. In contrast, PCase activity in crude extracts of propionate-grown *P. citronellolis* was inhibited by the addition of ammonium sulfate (Figure 3); similar results were obtained with valine-grown cells. These two contrasting patterns may explain in part the results of Sokatch et al. (1968) who detected PCase activity in extracts of *P. aeruginosa* grown on valine, but not in extracts of cells grown on acetate. We obtained their strain of *P. aeruginosa* and assayed PCase in extracts of cells grown on either acetate or valine as carbon source. The results are shown in Table I along with data for PCase in extracts of *P. citronellolis*. When assayed in the absence of added ammonium sulfate PCase activity in crude extracts of either *P. aeruginosa* or *P. citronellolis* is very low in acetate-grown cells, and is apparently induced 80- to 120-fold by growth on carbon sources that lead to the production of propionyl-CoA (i.e., valine or propionate). However, when the same extracts were assayed in the presence of 0.44 M ammonium sulfate there was clearly a significant amount of salt-stimulated PCase activity in acetate-grown cells. These results suggest the possibility that two different PCase activities are present in *Pseudomonas*, a constitutive salt-stimulated form in acetate-grown cells (also noted in malate, glucose, isovalerate, and citronellate-grown cells), and an inducible salt-inhibited form in propionate and valine-grown cells. However, since PCase activity in extracts of cells grown on any of these carbon sources readily dissociates as described below, it hasn't yet been possible to isolate two different forms of PCase. It is also possible that these salt ef-

TABLE I: Apparent Induction of PCase Activity in *P. aeruginosa* and *P. citronellolis*.

Strain	Carbon Source	PCase Act.[a] (milliunits/mg of protein) $-(NH_4)_2SO_4$	PCase Act.[a] (milliunits/mg of protein) $+(NH_4)_2SO_4$
P. aeruginosa[b]	Acetate	0.05	0.8
	DL-Valine[c]	4.1	0.7
P. citronellolis	Acetate	0.1	3.5
	Propionate	12.1	2.2
	DL-Valine[c]	7.8	1.4

[a] The values shown represent the averages from two separate cultures grown on the indicated carbon source. Where ammonium sulfate was added to the assay, the final concentration was 0.44 M. [b] This strain was the same as that used in a previous study (Sokatch et al., 1968). [c] The medium also contained 0.005% L-leucine.

TABLE II: Demonstration of ATP, Acyl-CoA, and Biotin Dependencies of ACase and PCase in *P. citronellolis* Extracts.

Reaction Mixture	Carboxylase Act.[a] (cpm of $^{14}CO_2$ fixed/5 min) Acetate Grown ACase[b]	Acetate Grown PCase[b]	Propionate Grown ACase[b]	Propionate Grown PCase[c]
Complete	9 770	6 820	15 010	33 870
−Acyl-CoA	380	380	1 730	3 440
−ATP	420	350	950	2 140
+Avidin	800	970	2 110	3 220
+Biotin-saturated avidin	9 960	6 880	13 440	29 580

[a] Preparation of cell extracts and assays were carried out as described under Materials and Methods. Assay mixtures contained crude extract from either acetate-grown cells (128 μg of protein) or propionate-grown cells (148 μg of protein). When avidin was added, crude extract, assay buffer and avidin (20 μg) were preincubated for 5 min at 4 °C before adding the rest of the components; biotin-saturated avidin was prepared by mixing 6 μg of D-biotin with 20 μg of avidin before the addition of the crude extract. [b] Assayed in the presence of 0.44 M ammonium sulfate. [c] Assayed in the absence of added ammonium sulfate.

TABLE III: Loss of PCase Activity during ACase Purification.[a]

Enzyme Preparation	Enzyme Units ACase	Enzyme Units PCase	Ratio ACase/PCase
Acetate-grown cells			
Crude extract	5.33	3.97	1.3
0–45% $(NH_4)_2SO_4$ fractionation	6.68	0.38	17.6
Alumina gel eluate	4.93	0.09	54.8
Propionate-grown cells			
Alumina gel eluate	1.05	0.02	52.5

[a] ACase was partially purified from acetate-grown cells (30 g wet wt) or propionate-grown cells (10 g wet wt) as described under Materials and Methods. Assays included optimal levels of ammonium sulfate.

TABLE IV: Resolution of PCase Activity into Two Required Fractions.[a]

Preparation	PCase Act. (units)	Yield (%)
Crude extract	10.3	100
0–40% $(NH_4)_2SO_4$ fraction	0.9	9
50–65% $(NH_4)_2SO_4$ fraction	0	0
0–40% fraction plus 50–65% fraction (excess)[b]	6.0	58
0–40% fraction (excess) plus 50–65% fraction[c]	4.2	41

[a] Ten grams (wet wt) of propionate-grown *P. citronellolis* was disrupted and fractionated as described under Materials and Methods. [b] The assays included 105 μg of protein of the 0–40% fraction and 377 μg of protein of the 50–65% fraction. [c] The assays included 85 μg of protein of the 50–65% fraction and 240 μg of protein of the 0–40% fraction.

fects on PCase activity in crude extracts are the result of differential inhibition or activation of interfering enzymes.

To confirm that the $^{14}CO_2$-labeled products of the ACase and PCase reactions were due to ATP and biotin-dependent carboxylation reactions, samples of the crude extracts were assayed either in the absence of ATP or in the presence of avidin, a specific inhibitor of biotin enzymes (Green, 1975). These results are shown in Table II. Assays were routinely carried out in the presence and absence of the acyl-CoA substrates in order to determine acyl-CoA dependent carboxylation rates (see Materials and Methods), and typical data are also in Table II. For these experiments the ACase and PCase sources were crude extracts of acetate- and propionate-grown *P. citronellolis*. In each case the optimal level of ammonium sulfate was added to the assays, except those for the PCase of propionate-grown cells that is inhibited by this salt (Figure 3). The data clearly demonstrate that the CO_2-fixation assays in crude extracts are measuring ATP and acyl-CoA-dependent reactions. In addition, both ACase and PCase reactions are inhibited greater than 85% by inclusion of avidin in the assays, but not by biotin-saturated avidin, results consistent with biotin dependencies typical of this class of enzyme (Moss and Lane, 1971).

Acyl-CoA Specificity of Partially Purified ACase. The ACases that have been isolated from several sources (see Alberts and Vagelos, 1972; Erfle, 1973), except for *E. coli* ACase (Alberts and Vagelos, 1968), are capable of carboxylating propionyl-CoA at substantial rates. For this reason it was important to establish if *P. citronellolis* ACase is capable of propionyl-CoA carboxylation, and if ACase was contributing to the apparent PCase activity detected in crude extracts. When ACase was partially purified by a procedure designed to stabilize it as a complex (R. Fall, manuscript in preparation; see Materials and Methods) the PCase activity in the preparation did not copurify, as shown in Table III. During the purification of ACase from acetate-grown cells the ratio of ACase to PCase increased from 1.3 to 55; this change is the result of removal of PCase activity during ammonium sulfate fractionation (as described below) and the fact that propionyl-CoA is a poor substrate for the ACase. ACase purified from propionate-grown cells shows a similar low rate of carboxylation of propionyl-CoA (Table III). These results indicate that ACase is not contributing significantly to the PCase activity seen in crude extracts, and that distinct ACase and PCase enzymes must exist in *P. citronellolis*.

Resolution of PCase Activity into Two Fractions. Initial

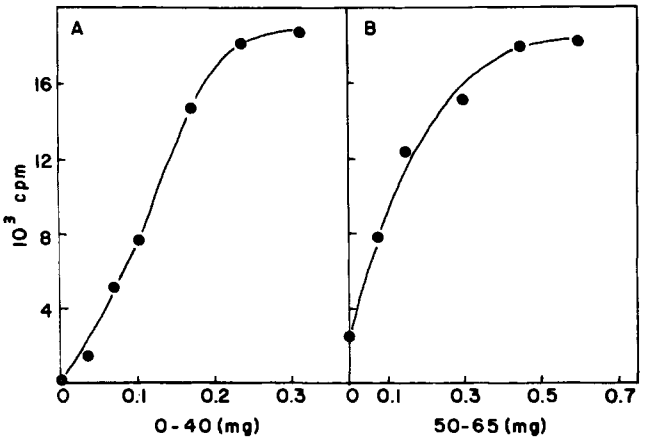

FIGURE 4: Effect of varying levels of two protein fractions required for the carboxylation of propionyl-CoA. The preparation of the fractions precipitating between 0–40% saturation and 50–65% saturation with $(NH_4)_2SO_4$ is described under Materials and Methods. An extract from propionate-grown cells was used. In (A) the effect of different levels of the 0–40% fraction in the presence of excess 50–65% fraction (377 μg of protein) was measured by the $^{14}CO_2$-fixation assay. In B the effect of different levels of the 50–65% fraction in the presence of excess 0–40% fraction (240 μg of protein) was measured. All assays were carried out for 5 min.

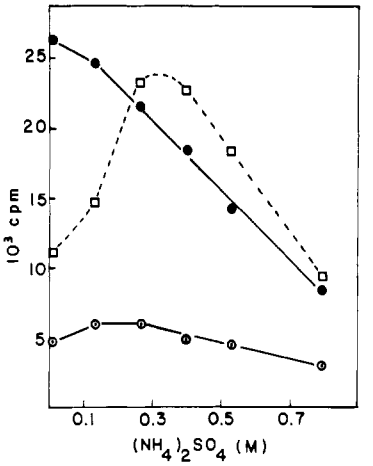

FIGURE 5: Effects of ammonium sulfate on GCase and MCase activities in cell extracts. $^{14}CO_2$-fixation assays were carried out as described under Materials and Methods and Figure 1 on an extract of citronellate-grown cells (66 μg of protein/assay), (●-●) GCase, (⊙-⊙) MCase, or on an extract of isovalerate-grown cells (75 μg of protein), (□- -□) MCase.

attempts to purify PCase via ammonium sulfate fractionation of crude extracts resulted in large losses of the enzyme. We considered that this loss of PCase activity could be due to the separation of functional subunits of PCase if the enzyme is an unstable complex in vitro analogous to the ACase of *E. coli* (Alberts and Vagelos, 1972; Guchhait et al., 1974). When a crude extract from propionate-grown cells was fractionated with ammonium sulfate, and each fraction was mixed with combinations of the others, addition of the 0–40% and 50–65% fractions resulted in restoration of PCase activity. Typical data are shown in Table IV. The 0–40% and 50–65% fractions alone had almost no PCase activity, but when recombined there was a 41–58% yield of PCase, depending on which fraction was in excess. PCase activity was proportional to the concentration of each fraction over a limited range as shown in Figure 4. Boiling either fraction for 1 min at 100 °C abolished the stimulatory effect. Similar results were obtained with the PCase from acetate-grown cells. These results suggest that the two fractions contain protein components necessary for the reconstitution of PCase activity. These components have been partially purified and are under investigation (L. Fall and R. Fall, unpublished observations).

Induction of GCase. The original demonstration of GCase in *P. citronellolis* by Seubert et al. (1963) utilized cells grown with the monoterpene, citronellol, as the sole carbon source. Using a strain of *P. citronellolis* obtained from the American Type Culture Collection (ATCC 13674), poor growth yields were obtained with citronellol or citronellal, geraniol or geranial as carbon sources in the medium of Seubert (1960) or in medium T (Materials and Methods). Similar results were obtained with a strain of *P. citronellolis* that was obtained from and has been maintained for several years by Dr. M. F. Utter. Apparently, since the original isolation (Seubert, 1960) the strain has mutated and no longer effectively oxidizes these acyclic terpene alcohols and aldehydes (W. E. Seubert, personal communication), or is now especially sensitive to the toxic surface effects of these compounds. To circumvent this problem, we prepared citronellic acid and geranoic acid by oxidation of the corresponding aldehydes, and used these as carbon sources. Excellent growth rates and yields were obtained with either acid with both strains of *P. citronellolis*.

Assays of GCase in cell extracts were conducted with a $^{14}CO_2$ fixation assay as described by Seubert et al. (1963) with the modifications described under the Materials and Methods. In addition, the same extracts were assayed for MCase activity, because we were interested in the possibility that growth on geranoic or citronellic acid would induce GCase plus a unique MCase. The latter enzyme might be induced because the postulated pathway for geranyl-CoA degradation in this organism involves the production of 3-methylcrotonyl-CoA as an intermediate (Seubert and Remberger, 1963). Alternatively, GCase itself may be responsible for carboxylation of 3-methylcrotonyl-CoA; indeed, Seubert et al. (1963) reported that a partially purified GCase preparation from *P. citronellolis* also carboxylates 3-methylcrotonyl-CoA, although at a low rate. Figure 5 shows the levels of GCase and MCase activities in a crude extract of citronellate-grown *P. citronellolis*, and the effects of addition of ammonium sulfate. For comparison, Figure 5 also shows the MCase activity of isovalerate-grown cells; these results are described below. In cells grown on citronellate, high levels of GCase were found and the enzyme was strongly inhibited by the addition of ammonium sulfate in the assay. A much lower level of MCase activity was present, and was slightly stimulated by addition of 0.1–0.3 M ammonium sulfate and inhibited at higher levels.

The data shown in Table V show that GCase is an inducible enzyme. In cells grown on either acetate, glucose, leucine, or isovalerate as carbon source, only traces of GCase were detected. However, high levels of the enzyme were detected in cells grown on either citronellate or geranoate. The induction of GCase could be correlated with the appearance of a new [^{3}H]biotin-labeled protein peak when extracts from cells grown in the presence of [^{3}H]biotin were chromatographed on Sepharose-4B columns. Typical results are shown in Figure 6. In each case extracts from [^{3}H]biotin-labeled cells were concentrated before chromatography by ammonium sulfate precipitation as described under Materials and Methods. Figure 6A shows the profile of [^{3}H]biotin proteins present in extracts of acetate-grown cells. Two major [^{3}H]biotin protein peaks were detected, corresponding to approximate molecular weights of 250 000 and 150 000, respectively, when compared to the elution volumes of proteins of known molecular weight. The former peak contains the pyruvate carboxylase present

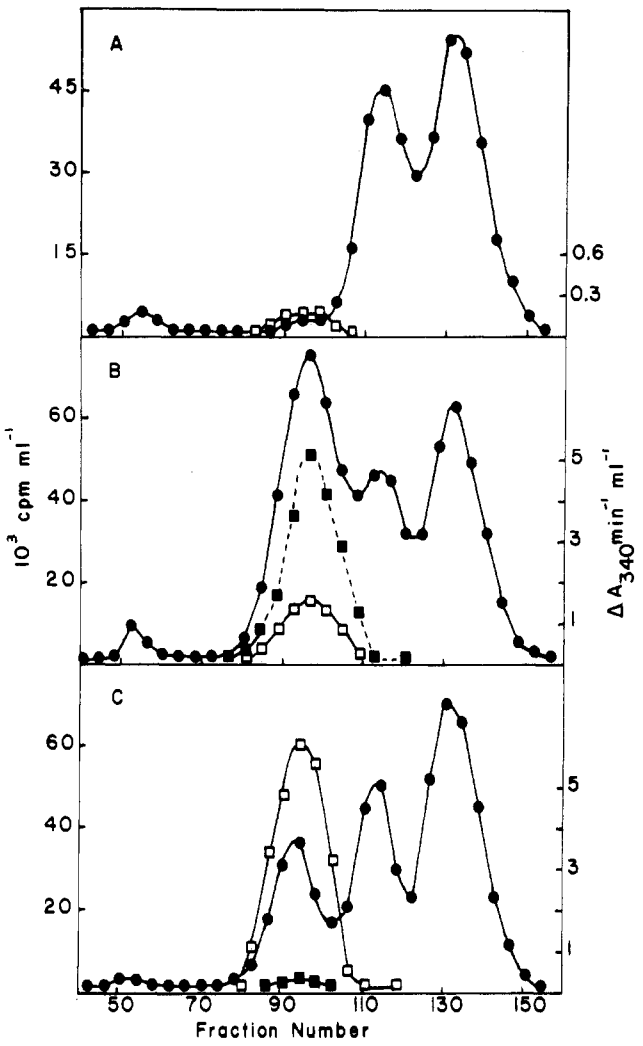

FIGURE 6: Gel filtration of [^{3}H]biotin-labeled extracts of *P. citronellolis* grown on different carbon sources. Chromatograph of [^{3}H]biotin-labeled extracts on Sepharose-4B was performed as described under Materials and Methods. Aliquots were assayed for [^{3}H]biotin (left ordinate, ●–●) and MCase (□–□) or GCase (■- - -■) by spectral assays (right ordinate). In A the profile of an extract from acetate-grown cells is shown. B shows the profile of an extract of citronellate-grown cells. C shows the profile of an extract of isovalerate-grown cells.

in these extracts. The lower molecular weight [^{3}H]biotin protein peak contained no detectable pyruvate carboxylase, ACase, PCase, GCase, or MCase. Studies in progress have revealed that this peak represents the biotin-containing substructure of ACase and PCase that results from the dissociation of these two enzymes under the conditions of the chromatography. Figure 6B shows the profile of [^{3}H]biotin proteins present in extracts of citronellate-grown cells. In this case, three major [^{3}H]biotin protein peaks were detected. As in the case of acetate-grown cells, [^{3}H]biotin peaks corresponding to pyruvate carboxylase and dissociated ACase/PCase were found, plus a new higher molecular weight peak of GCase. This peak of GCase has an approximate molecular weight of 550 000 and contains a lower level of MCase activity as indicated on the figure (Figure 6B).

Induction of MCase. MCase has been reported as an inducible enzyme in *P. putida* when grown on leucine, isovaleric acid, or 3-methylcrotonic acid, all of which lead to the production of 3-methylcrotonyl-CoA (Massey et al., 1974). The enzyme has also been reported in extracts of *P. oleovorans* grown on hexane (Rilling and Coon, 1960). We determined

TABLE V: Induction of GCase and MCase in *P. citronellolis*[a]

Carbon Source	Sp. Act. (milliunits/mg of protein)	
	GCase	MCase
Acetate	0.4	1.9
Glucose	0.4	0.8
Citronellate	34.1	6.6
Geranoate	40.3	8.8
Isovalerate	0.5	29.6
DL-Leucine	2.1	33.1

[a] Cells were grown and cell extracts were prepared and assayed as described under Materials and Methods. MCase assays included optimal levels of ammonium sulfate as described in the text.

that *P. citronellolis* was capable of growth on leucine and isovaleric acid but not 3-methylcrotonic acid as sole carbon sources. The best growth yields were obtained with isovaleric acid. MCase could be assayed in crude extracts by the CO_2-fixation assay, and was stimulated 2- to 3-fold by inclusion of ammonium sulfate in the assay as shown in Figure 5. That MCase was induced by growth on isovalerate or leucine is shown in Table V. Extracts of cells grown on acetate or glucose contained only traces of MCase, while growth on leucine or isovalerate led to an approximately 25-fold induction of the enzyme. The intermediate levels of MCase induced by growth on citronellate and geranoate are probably due in part to carboxylation of 3-methylcrotonyl-CoA by GCase as noted above.

The induction of MCase could be correlated with the appearance of a new [^{3}H]biotin-labeled protein peak when extracts were chromatographed on Sepharose-4B as described above for GCase. The results are shown in Figure 6C. As with GCase, the ^{3}H-labeled MCase peak had a molecular weight of approximately 550 000. This partially purified MCase fraction showed only a trace of GCase activity, suggesting that MCase doesn't readily bind the larger acyl-CoA substrate (i.e., geranyl-CoA) at its transcarboxylase site. These results are suggestive of the possibility that MCase and GCase are distinct inducible enzymes with very similar molecular weights. Whether growth on citronellate or geranoate induces distinct GCase and MCase enzymes has been difficult to establish, since they exhibit virtually identical chromatographic behavior upon gel filtration, ion-exchange, or hydroxylapatite chromatography. Thus, it hasn't yet been possible to resolve MCase activity from GCase activity in preparations from citronellate-grown cells.

Discussion

The results described here strongly suggest that depending on growth conditions *P. citronellolis* can serve as a source of four different acyl-CoA carboxylases, including ACase, PCase, MCase, and GCase. This means that *P. citronellolis* is a potentially useful system for studying the structural aspects of pairs of homologous acyl-CoA carboxylases: ACase and PCase that both carboxylate the α-carbon of their acceptor substrates, acetyl-CoA and propionyl-CoA, respectively, and MCase and GCase that carboxylate the 3-methyl group of their acceptor substrates, 3-methylcrotonyl-CoA and geranyl-CoA, respectively. Structural comparisons of this type have not been made before. Since each of these enzymes contain the three functional elements (or subsites), including biotin carboxylase, biotin protein, and transcarboxylase, it will be of great interest to determine the arrangement of the elements in each enzyme's

substructure, and to eventually determine the degree of structural homology between each of these elements.

Based on the findings that the biotin carboxylation half-reaction is mechanistically similar if not identical in all biotin-dependent carboxylases (Moss and Lane, 1971), it is reasonable to expect that there will be common structural features in the biotin carboxylase and biotin protein regions of *P. citronellolis* ACase, PCase, MCase, and GCase, and that their transcarboxylase sites that specify acceptor binding will be different. That the latter sites are distinct is clear from the studies shown here on the substrate specificity of ACase and MCase. Both of these enzymes are quite specific for their acyl-CoA substrates, not effectively carboxylating larger substrate homologues, propionyl-CoA and geranyl-CoA, respectively, presumably because of steric restrictions at their transcarboxylase sites.

In order to rigorously test these structural considerations, it will be necessary to isolate each enzyme and analyze its substructure. In cases where subsites are fused into a single polypeptide chain structural studies may be technically difficult. Thus, as in the case of *Achromobacter* MCase that contains the biotin carboxylase and biotin protein subsites fused (Schiele et al., 1975), a separation of these subsites will necessarily involve hydrolytic procedures that may destroy activity. It may be necessary to compare such fused enzymes by peptide mapping and amino acid sequence techniques. Since it is relatively easy to label the biotin enzymes of *P. citronellolis* with radioactive biotin by supplying [^{3}H]biotin in the growth medium, we are planning to compare the biotin peptides isolated from each of the purified enzymes. Hopefully, this approach will provide information about the degree of sequence homology around the biotin site in several different biotin enzymes from the same cell.

Some aspects of the purification of the biotin enzymes from *P. citronellolis* were suggested by the work described here, and the purification of ACase, PCase, MCase, and GCase is in progress. ACase has been stabilized and purified to near homogeneity (R. Fall, manuscript in preparation) by a modification of the procedure outlined in Table III. The stabilization is effected by inclusion of ammonium sulfate in purification buffers. The stimulation of ACase activity by inclusion of this salt in the assays may be a related phenomenon. The enzyme appears to be structurally similar to *E. coli* ACase, containing four distinct subunits including biotin carboxylase, biotin carboxyl carrier protein, and two different transcarboxylases. The major distinction between the ACases of *P. citronellolis* and *E. coli* is that the latter has never been isolated as an intact complex and its subunits are isolated separately, while the former can be readily isolated as an intact complex under stabilizing conditions. In contrast, stabilizing conditions for *P. citronellolis* PCase have not yet been found, and even in the presence of ammonium sulfate the enzyme dissociates into at least two protein fractions as shown in Table IV. The purification of these components is under investigation.

While the purification of MCase free from GCase and other biotin enzymes has been easy, the purification of GCase has presented problems. MCase is induced in *P. citronellolis* grown on isovalerate as carbon source, and extracts from these cells contain almost no GCase activity (Table V). The MCase in such extracts is a high-molecular-weight complex that is readily resolved from the other biotin enzymes present by gel filtration (Figure 6). GCase is induced by growth of *P. citronellolis* on citronellate or geranoate as carbon sources, but this also results in the apparent induction of MCase (Table V). The MCase activity in these extracts may be due to (a) carboxylation of 3-methylcrotonyl-CoA by GCase or (b) the induction of a unique MCase to metabolize the 3-methylcrotonyl-CoA fragment produced in the catabolism of geranyl-CoA (Seubert and Remberger, 1963). All preliminary attempts to separate GCase and MCase activities in these extracts have failed. The two activities cochromatograph on DEAE-cellulose, hydroxylapatite, and Sepharose-4B; MCase from isovalerate cells exhibits identical chromatographic behavior. Thus, MCase and GCase are very similar protein complexes, lending support to the idea that they may share common structural features. The properties of these two enzymes are under investigation.

In addition to the four biotin-dependent acyl-CoA carboxylases mentioned, *P. citronellolis* also contains pyruvate carboxylase, another biotin enzyme, that has been extensively characterized by Utter and co-workers (Utter et al., 1975). Thus, *P. citronellolis* may be a useful system for comparing structural (sequence) homology between different categories of biotin-dependent enzymes. Its pyruvate carboxylase is composed of two different polypeptide chains, one containing biotin, but the distribution of the biotin carboxylase and transcarboxylase functions has not been determined. Fall et al. (1975) reported that the biotin-containing subunit has a distinctly larger molecular weight than the biotin protein subunit from the ACase of this organism. It seems likely that the biotin site in pyruvate carboxylase is fused with one of the other subsites as in *Achromobacter* MCase. Whether the biotin site of pyruvate carboxylase shares structural features with the biotin sites of the acyl-CoA carboxylases from the same organism is also under investigation.

Acknowledgment

We thank Lana Fall for excellent technical assistance.

References

Alberts, A. W., Nervi, A. M., and Vagelos, P. R. (1969), *Proc. Natl. Acad. Sci. U.S.A. 63,* 1319–1326.

Alberts, A. W., Vagelos, P. R. (1968), *Proc. Natl. Acad. Sci. U.S.A. 59,* 561–568.

Alberts, A. W., and Vagelos, P. R. (1972), *Enzymes 6,* 37–82.

Barden, R. E., Taylor, B. L., Isohashi, F., Frey, II, W. H., Zander, G., Lee, J. C., and Utter, M. F. (1975), *Proc. Natl. Acad. Sci. U.S.A. 72,* 4308–4312.

Erfle, J. D. (1973), *Biochim. Biophys. Acta 316,* 143–155.

Fall, R. R., Alberts, A. W., and Vagelos, P. R. (1975), *Biochim. Biophys. Acta 379,* 496–503.

Fall, R. R., and Vagelos, P. R. (1972), *J. Biol. Chem. 247,* 8005–8015.

Fall, R. R., and Vagelos, P. R. (1973), *J. Biol. Chem. 248,* 2078–2088.

Flavin, M., Castro-Mendoza, H., and Ochoa, S. (1957), *J. Biol. Chem. 229,* 981–996.

Gerwin, B. I., Jacobson, B. E., and Wood, H. G. (1969), *Proc. Natl. Acad. Sci. U.S.A. 64,* 1315–1322.

Green, N. M. (1975), *Adv. Protein Chem. 29,* 85–135.

Guchhait, R. B., Polakis, S. E., Dimroth, P., Stoll, E., Moss, J., and Lane, M. D. (1974), *J. Biol. Chem. 249,* 6633–6645.

Hayaishi, O. (1955), *J. Biol. Chem. 215,* 125–136.

Kannangara, C. G., and Stumpf, P. K. (1972), *Arch. Biochem. Biophys. 152,* 83–91.

Koch, A. L., and Putnam, S. L. (1971), *Anal. Biochem. 44,* 239–245.

Lynen, F. (1974), *Lipmann Symp. Energy, Regul. Biosynth. Mol. Biol.* 671–698.

Massey, L. K., Conrad, R. S., and Sokatch, J. R. (1974), *J. Bacteriol. 118,* 112-120.
Moss, J., and Lane, M. D. (1971), *Adv. Enzymol. 35,* 321-442.
Rilling, H. C., and Coon, M. J. (1960), *J. Biol. Chem. 235,* 3087-3092.
Schiele, U., Niedermeier, R., Stürzer, M., and Lynen, F. (1975), *Eur. J. Biochem. 60,* 259-266.
Seubert, W. (1960), *J. Bacteriol. 79,* 426-434.
Seubert, W., Fass, E., and Remberger, U. (1963), *Biochem. Z. 338,* 265-275.
Seubert, W., and Remberger, U. (1963), *Biochem. Z. 338,* 245-264.
Shapiro, B. (1953), *Biochem. J. 53,* 663-666.
Simon, E. J., and Shemin, D. (1953), *J. Am. Chem. Soc. 75,* 2520.
Sokatch, J. R., Sanders, L. E., and Marshall, V. P. (1968), *J. Biol. Chem. 253,* 2500-2506.
Stadtman, E. R. (1956), *Methods Enzymol. 3,* 931-941.
Sumper, M., and Riepertinger, C. (1972), *Eur. J. Biochem. 29,* 237-248.
Tanabe, T., Wada, K., Okazaki, T., and Numa, S. (1975), *Eur. J. Biochem. 57,* 15-24.
Taylor, B. L., Barden, R. E., and Utter, M. F. (1972), *J. Biol. Chem. 247,* 7383-7390.
Utter, M. F., Barden, R. E., and Taylor, B. L. (1975), *Adv. Enzymol. 42,* 1-72.

Evidence against an Acyl-Enzyme Intermediate in the Reaction Catalyzed by Clostridial Phosphotransacetylase†

Jack Henkin‡ and Robert H. Abeles*

ABSTRACT: Clostridial phosphotransacetylase catalyzes acyl group transfer between coenzyme A (CoA) and inorganic phosphate and also the arsenolysis of acetyl-coenzyme A (AcCoA) to yield acetate and CoA-SH. The enzyme mobility on sodium dodecyl sulfate electrophoresis corresponds to a molecular weight of 70 000. Kinetics of both forward and reverse reactions are of the ternary type as previously reported and product inhibition data are consistent with a random binding scheme. One essential sulfhydryl group per 70 000 daltons was inactivated in a pseudo-first-order process by either *N*-ethylmaleimide or 5,5′-dithiobis(nitrobenzoic acid). Reduction of the rate of this inactivation by 50% in the presence of AcCoA or acetyl phosphate concentrations near their kinetic $\bar{K}$ values demonstrates binding of these acyl donors in simple enzyme-substrate complexes. Moreover, pulse-chase experiments show these binary complexes to be functional and also show that they do not dissociate rapidly compared with their rates of catalytic turnover. Incubation of the enzyme with ^{14}C-labeled acyl donors failed to produce labeled protein after passage through Sephadex. This was true despite efforts to mimic "substrate synergism" with desulfo-CoA or to compensate for unfavorable equilibria by means of CoA traps. Very slow isotope exchange reactions of $^{32}P_i$ into acetyl phosphate and [^{3}H]CoA into AcCoA were at first observed. As in the cases of several other enzymes recently reexamined, these were shown on careful inspection to be artifacts of contamination by second substrates. Attempts to detect exchange reactions between acetyl phosphate and P_i, even in the presence of the CoA analogue, desulfo-CoA, were also unsuccessful. Therefore, no evidence for an acyl-enzyme could be detected. Furthermore, our data allow us to develop arguments which, we believe, indicate that an acyl-enzyme intermediate is extremely improbable in the reaction catalyzed by phosphotransacetylase.

It is now well established that the reactions catalyzed by many group transfer enzymes involve covalent intermediates (Spector, 1973). The existence of such covalent intermediates is not surprising, and, as a matter of fact, is expected, for enzymes which show ping-pong kinetics. For enzymes where the kinetic pattern gives intersecting lines, it had been assumed that covalent intermediates are unlikely. This view was seriously altered when it was reported that hexokinase, an enzyme which does not display ping-pong kinetics, catalyzed ATP-ADP[1] exchange and glucose-glucose-6-P exchange (Walsh and Spector, 1971a). The existence of these exchange reactions led to suggestions that this reaction involves a phospho-enzyme intermediate. Subsequently, the occurrence of exchange reactions was reported for phosphoribosyl pyrophosphate synthetase (Switzer, 1970) and glycerate kinase (Walsh and Spector, 1971b). Neither of these enzymes shows ping-pong kinetics. These discoveries led to suggestions that all transfer reactions may involve covalent intermediates, and that proof of their nonexistence is the task of the researcher (Spector, 1973).

In this context, it was decided to reexamine some properties of phosphotransacetylase (EC 2.3.1.8) from *Clostridium*

† This is publication No. 1092 from the Graduate Department of Biochemistry, Brandeis University, Waltham, Massachusetts 02154. *Received March 16, 1976.* This research was supported in part by grants from the National Science Foundation (GB 31952) to R.H.A. and the National Institutes of Health Training Grant GM 212 (J.H.).

‡ Present address: Chemistry Department, Harvard University, Cambridge, Massachusetts 02138.

[1] Abbreviations used: CoA, coenzyme A; AcCoA, acetyl-coenzyme A; E, enzyme; S, substrate; ATP, adenosine 5′-triphosphate; ADP, adenosine 5′-diphosphate; glucose-6-P, glucose 6-phosphate; P_i, inorganic phosphate; NAC, *N*-acetylcysteamine; Tris, tris(hydroxymethyl)aminomethane; NADH, reduced nicotinamide adenine dinucleotide; NAD, nicotinamide adeninedinucleotide; NEM, *N*-ethylmaleimide; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid).