Involvement of Acyl Carrier Protein in Acylation of Glycerol 3-Phosphate in Clostridium butyricum

I. PURIFICATION OF CLOSTTRIDIUM BUTYRICUM ACYL CARRIER PROTEIN AND SYNTHESIS OF LONG CHAIN ACYL DERIVATIVES OF ACYL CARRIER PROTEIN*

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SUMMARY

Clostridium butyricum grown in medium supplemented with radioactive pantothenic acid incorporates this compound into both protein and nonprotein fractions of the cell. Acyl carrier protein (ACP) is the major protein into which pantothenic acid is incorporated. C. butyricum ACP was isolated as a homogeneous protein, as judged by column chromatography on DEAE-Sephadex, disc gel electrophoresis, and analytical ultracentrifugation. This protein contains a single sulphydryl residue, 2-mercaptoethylamine, which is a component of the prosthetic group, 4'-phosphopantetheine. The prosthetic group is cleaved from the protein when the protein is incubated at pH 12, 70°, for 1 hour. Although this protein has properties similar to those of ACP of Escherichia coli, the amino acid compositions of the two proteins are different.

The chemical synthesis of long chain fatty acyl-ACP derivatives is described. In this procedure other unidentified groups of the protein are acylated as well as the sulphydryl group. The effects of acylation or acylation and alkylation of groups other than the sulphydryl group of ACP were studied by testing ACP regenerated from such derivatives in the malonyl coenzyme A-CO₂ exchange reaction.

Investigations of fatty acid synthetase preparations from bacteria (1–5), plants (6), rat adipose tissue (7), yeast (8), and pigeon liver (9) have indicated the involvement of acyl carrier protein in fatty acid biosynthesis. The biosynthetic intermediates in this pathway as well as the primary products occur as acyl thioester derivatives of ACP. The finding that long chain fatty acyl-ACP derivatives are formed has raised the possibility that these thioesters, rather than acyl thioster of CoA, are acyl group donors to glycerol 3-phosphate in complex lipid biosynthesis. Investigation of this possibility in yeast has indicated that acyl groups are not directly transferred from the fatty acid synthetase complex (where they are presumably present as acyl thioester of ACP) to glycerol 3-phosphate; prior transfer to CoA appears to be required (10). The yeast synthetase, however, contains ACP as a fixed component of a multienzyme complex (8). Thus, in this situation a mechanism is required for transfer of the completed fatty acyl group from the synthetase complex to the site where it will be utilized in complex lipid synthesis. CoA can serve this acyl transfer function. On the other hand, ACP in bacteria and plants does not appear to be present as a component of a tightly associated synthetase complex. Acyl groups linked to ACP in these systems may therefore be available for direct acylation of glycerol 3-phosphate.

Preliminary studies with an enzyme system from Clostridium butyricum indicated that Escherichia coli ACP stimulates the conversion of glycerol 3-phosphate to lysophosphatic acid and suggested that long chain fatty acyl-ACP derivatives might be intermediates in this synthetic process (11, 12). In order to investigate this possibility further, it was desirable to ascertain that C. butyricum contains ACP, and to test the effect of ACP butyricum in the reaction.

The present papers show that C. butyricum does contain ACP and that in this organism long chain fatty acyl ACP derivatives, rather than acyl-CoA derivatives, are involved in the acylation

* The abbreviations used are: ACP and ACP-SH, acyl carrier protein with free sulphydryl group; acyl-ACP, acyl thioester derivative of acyl carrier protein; ACP butyricum, acyl carrier protein isolated from E. coli or C. butyricum, respectively.

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of glycerol 3-phosphate (13). Preliminary communications of this work have been reported (11, 12, 14). The first paper describes the purification of ACP<sub>butyricum</sub> and the chemical synthesis of long chain acyl-ACP derivatives.

**EXPERIMENTAL PROCEDURE**

**Materials**—"Chromatographically pure" CoA was purchased from P-L Laboratories. Phosphotransacetylase was obtained from CalBioChem. 1-<sup>14</sup>C-Pantothenic acid, 9,10-<sup>H</sup>-palmitic acid, and 9,10-<sup>3</sup>H-oleic acid were purchased from New England Nuclear Corporation and were utilized without further purification. Oleic and palmitic acids (99% pure) were obtained from Applied Science Laboratories.

**Analytical Procedures**—Polyacrylamide disc gel electrophoresis was carried out as described by Davis (15) with 30% gel and Tris-glycine buffer, pH 8.3. The gels were stained with 0.5% Amido Schwarz in 10% acetic acid. The gels were cut into sections with a razor blade and dissolved by adding 1 ml of 30% H<sub>2</sub>O<sub>2</sub> and heating at 80°. The radioactivity in the dissolved gels was measured in Bray's solution (10) with a Packard Tri-Carb spectrometer.

Sulfhydryl titrations were carried out as previously described (3) by means of a micromodification of the Ellman procedure (17).

4′-Phosphopantetheine, released from ACP by treatment with dilute alkali, was identified by enzymatic conversion to CoA (18), which was measured with the phosphotransacetylase assay of Stadtman (19).

Amino acid analyses were performed by Dr. P. W. Majerus on a Phoenix amino acid analyzer with spherical resin and an accelerated flow system of 80 ml per hour, and by Dr. J. Ozols with a Spinco amino acid analyzer with accelerated flow system and expanded scale accessories, both according to the method of Spackman, Stein, and Moore (20).

Sedimentation and diffusion constants were determined in a Spinco model E analytical ultracentrifuge with the use of a synthetic boundary cell with schlieren optics. The molecular weight was calculated from these data, utilizing a partial specific volume of 0.731 g per ml derived from the amino acid composition.

ACP<sub>cat</sub> and ACP<sub>butyricum</sub> were assayed enzymatically with the malonyl-CoA-CO<sub>2</sub> exchange reaction with the use of an enzyme fraction from E. coli (21). Protein was determined either by the method of Gornall, Bardawill, and David (22) or of Lowry et al. (23).

**Preparation of Acyl Carrier Protein**

_Growth of Clostridium butyricum—_C. butyricum_ was grown according to the method of Broquist and Snell (24) in Medium C supplemented with 1-<sup>14</sup>C-pantothenate at various concentrations between 1.05 and 12.6 μM. After overnight growth the cells were harvested with a Sharles centrifuge and washed once with 0.01 M potassium phosphate, pH 6.2, containing 0.01 M 2-mercaptoethanol. Approximately 50 g of cells (wet weight) were obtained from 20-liter cultures.

_Purification of ACP—_ACP<sub>cat</sub> was purified as previously described (21). ACP was also purified from extracts of _C. butyricum_ grown in medium supplemented with 10.5 μM 1-<sup>14</sup>C-pantothenate (0.66 μC per mmole). Radioactive pantothenate is incorporated into the prosthetic group of ACP, 4′-phosphopantetheine. Thus, the purification of ACP<sub>butyricum</sub> could be monitored by measuring the radioactivity of the protein fractions.

All the purification procedures were carried out at 4°. Radioactive _C. butyricum_ (25 g) was suspended in 130 ml of 0.02 M potassium phosphate, pH 6.2, containing 0.01 M 2-mercaptoethanol and ruptured in a French pressure cell at 9,000 p.s.i. The resulting homogenate was centrifuged at 48,000 × g for 30 min and the precipitate was discarded. The supernatant solution (100 ml, 33 mg of protein per ml) was poured in 33-ml batches onto a Sephadex G-50 column, 3 × 20 cm, equilibrated with the same buffer. Elution of this column yielded two radioactive peaks. The first was associated with the protein fraction. The second radioactive peak was not associated with proteins. The protein fractions from these two peaks were pooled (2.63 g of protein; 1.03 × 10<sup>6</sup> dpm) and applied directly to a 5 × 20 cm DEAE-cellulose column which had been equilibrated with 0.25 M LiCl containing, as in all subsequent operations, 0.02 M potassium phosphate, pH 6.2, and 0.01 M 2-mercaptoethanol. The column was washed with 1 liter of the same solution and then eluted with 3 liters of the same buffer containing LiCl in a linear gradient from 0.25 to 0.55 M. Approximately 440,000 dpm were washed from the column before the gradient was started. A majority of this radioactive material was ACP<sub>butyricum</sub> that was not adsorbed under these conditions. The rest of the radioactivity emerged as a single peak which was eluted at 0.37 to 0.44 M LiCl. Since the fractions in this peak contained the ACP, as measured in the malonyl CoA-CO<sub>2</sub> exchange assay, they were pooled (182 mg of protein, 500,000 dpm), diluted 2-fold with distilled water, and poured onto a 2 × 11 cm DEAE-cellulose column equilibrated with buffer containing 0.3 M LiCl. Elution of this column with 500 ml of buffer containing LiCl in a linear gradient from 0.3 to 0.55 M yielded a single radioactive peak (0.37 to 0.45 M) containing 83 mg of protein and 410,000 dpm. The fractions in this peak were pooled, diluted 2-fold with distilled water, and applied to a 2 × 28 cm DEAE-Sephadex A-50 column equilibrated with buffer containing 0.3 M LiCl. The column was eluted with 1 liter of buffer containing LiCl in a linear gradient from 0.3 to 0.6 M. A single radioactive peak was eluted at 0.48 to 0.48 M. Fractions in this peak were pooled (2.66 mg of protein; 270,000 dpm), and the radioactive ACP was precipitated by adjusting the solution to 0.1 N hydrochloric acid and 0.13 N perchloric acid. 4′-ACP was recovered quantitatively by centrifugation at 48,000 × g for 30 min. The precipitate was dissolved in 20 ml of buffer and applied to a 1 × 10 cm DEAE-Sephadex A-50 column equilibrated with buffer containing 0.3 M LiCl. This column was eluted as before with a 200-ml linear gradient of LiCl from 0.35 to 0.6 M. Fractions of the single radioactive peak were pooled and acid-precipitated as above. The precipitate was dissolved in a small volume of buffer. This preparation contained 1.6 mg of protein and 210,000 dpm and was homogeneous, as will be described below. A summary of the purification is given in Table I. This procedure resulted in a 334-fold purification with a yield of 20.4%.

For larger scale preparations of ACP<sub>butyricum</sub> the procedure was modified as follows: 900 g of _C. butyricum_ grown in medium supplemented with 4.2 μM 1-<sup>14</sup>C-pantothenate (0.35 μC per mmole) were suspended in 1800 ml of phosphate buffer containing 2-mercaptoethanol, as above, and ruptured in a Manton Gaulin submicron disperser at 9,000 p.s.i. Additional buffer was added to the homogenate to bring the volume to 4 liters, and the suspension was centrifuged at 27,500 × g for 1 hour. The super-
natant solution was adjusted by the addition of buffer to contain approximately 20 mg of protein per ml, and then brought to 50% ammonium sulfate saturation by the addition of solid ammonium sulfate. The precipitated protein was removed by centrifugation, and the supernatant solution was adjusted to pH 1.0 by the dropwise addition of 2 N HCl. The resulting precipitate was recovered by centrifugation and was extracted with approximately 1200 ml of the potassium phosphate buffer containing 2-mercaptoethanol by homogenization in a Waring Blender. This suspension was centrifuged at 27,000 x g for 1 hour to remove the remaining particulate material. The supernatant solution (900 ml containing 23.6 g of protein and 13.3 \( \mu \)C) was poured over a Sephadex G-50 column (4.5 x 40 cm) in four 225-ml batches and eluted as described above for the smaller scale preparation. The remaining steps of the procedure, starting with the first DEAE-cellulose column, were identical to those described above, except that the chromatographic columns and elution solutions were adjusted to accommodate the larger quantity of protein. This modified procedure yielded homogeneous ACP with a lower yield, 13% compared to approximately 20%, but it allowed the processing of larger quantities of ACP.

**Preparation of Acyl Carrier Protein Derivatives**

**Long Chain Acyl-ACP Synthesis**—Mixed anhydrides were prepared from \( ^3H \)-acids and ethylchloroacetate according to the method of Goldman and Vagelos (25). Specific radioactivities of the acids used varied from 3 to 63 mCi per pmole. Subsequent operations were carried out at room temperature unless otherwise specified. Both ACP\(_\text{coli} \) and ACP\(_\text{butyricum} \) were utilized in the same procedures.

ACP, 0.1 to 0.5 pmole in 0.5 ml, was initially reduced by incubation in 0.1 M Tris-HCl, pH 8.5, containing 0.02 M 2-mercaptoethanol for 10 min at 30°. To this solution was added 0.5 ml of pyridine followed by a total of 3 eq of \( ^3H \)-mixed anhydride (in freshly distilled tetrahydrofuran) per mole of sulfhydryl compound in two additions at 5-min intervals. The disappearance of the sulfhydryl groups was followed grossly by a spot test on filter paper with Ellman's reagent (17). The spot test became negative in about 10 min, at which time the reaction mixture was extracted three times with 5 ml of ethyl ether. The pH of the solution was adjusted to 1 by the addition of hydrochloric acid, and the acidified solution was extracted four times with 5 ml of ethyl ether. These extractions removed unreacted mixed anhydride, free acid, and mercaptoethanol ester. Traces of ether were evaporated and the suspension was centrifuged 15 min at 48,000 x g. The ACP was precipitated in 0.5 ml of 0.1 M imidazole HCl, pH 6.2. This solution, which was slightly cloudy at pH 6.2, was centrifuged and the particulate material was discarded. Radioactivity measurements indicated that this material contained 0.8 to 1.0 mole of \( ^3H \)-acyl per mole of ACP.

**Results**

Incorporation of Radioactive Pantothenate by Clostridium \( \text{butyricum} \)—Investigations of ACP in numerous biological sources have indicated the presence of the prosthetic group, \( ^4 \) phosphopantetheine (5). Thus, growth of \( \text{C. butyricum} \) on \( ^{14} \)C-pantothenic acid should lead to the formation of \( ^{14} \)C-ACP labeled in the prosthetic group, which facilitates isolation of this protein. Fig. 1 illustrates the concentration-dependent incorporation of \( ^{14} \)C-pantothenic acid into protein and nonprotein fractions by growing cells of this organism. Although \( \text{C. butyricum} \) does not require pantothenic acid supplementation for growth, \( ^{14} \)C-pantothenic acid in the medium is readily incorporated into cellular constituents. Maximum incorporation into both fractions was observed at 4 to 8 \( \mu \)M pantothenic acid.
FIG. 1. Incorporation of 14C-pantothenic acid into protein and nonprotein fractions of C. butyricum. C. butyricum was grown anaerobically in 10-ml cultures supplemented with various concentrations of L-L-enantiopantothenic acid, as indicated. After overnight growth, the cells were harvested by centrifugation and ruptured by sonic disruption. Homogenates were centrifuged 20 min at 48,000 X g, and the supernatant solutions were chromatographed on Sephadex G-50 columns (2 X 20 cm) to give protein and nonprotein fractions as described in "Experimental Procedure." Protein recoveries were essentially identical in all experiments. Radioactivity associated with the protein and nonprotein fractions was measured at each pantothenate concentration. Percentage of 14C-pantothenic acid incorporated into the cells was 28.5, 42.5, 12, and 6.9 at pantothenic acid concentrations of 1.05, 2.1, 8.4, and 12.6 μM, respectively.

Properties of ACP<sub>butyricum</sub>: Criteria of Purity—Chromatography on DEAE-cellulose of the protein fraction obtained after Sephadex G-50 filtration indicated a single radioactive peak that contained 48.5% of the total radioactivity associated with the protein fraction. This same peak contained ACP as measured with the E. coli enzyme fraction that catalyzes the malonyl-CoA-CO₂ exchange reaction. Further purification yielded a homogeneous protein which chromatographed as a symmetrical peak on the second DEAE-Sephadex column. (Fig. 2). Essentially superimposed upon the protein peak were the peaks of radioactivity, and ACP activity assayed in the exchange reaction. Polyacrylamide gel electrophoresis of this preparation (pooled Fractions 32 through 62) indicated a single protein band which contained all the radioactivity. Furthermore, a single symmetrical peak was obtained during sedimentation in the analytical ultracentrifuge (Fig. 3). These data are in agreement with the conclusion that the protein is essentially homogeneous.

Assay of this preparation in the malonyl-CoA-CO₂ exchange reaction indicated that it is 70% as active as ACP<sub>coli</sub> on a molar basis. The sedimentation constant, d₂₀,₅₀ = 1.25, was determined in 0.4 M KCl to minimize the charge effect of this very acidic protein. The diffusion constant, D₅₀ = 1.48 X 10⁻⁸ cm² sec⁻¹, was determined in a synthetic boundary cell (27). The molecular weight calculated from these two constants is 8100.

Amino Acid Composition—Table II gives the results of the amino acid analysis. In addition to most of the usual amino acids found in proteins, 1 mole of β-alanine and 1 mole of taurine (after performic acid oxidation of the protein) per mole of protein were found. There is no cysteine in this protein, since no cysteic acid was found in the performic acid-oxidized sample. The protein was not assayed for tryptophan. The minimum molecular weight based upon this amino acid analysis is 8600.

Sulfhydryl Content—After reduction with 2-mercaptoethanol followed by acid precipitation to remove the reducing agent, ACP was found to contain 0.97 mole of sulfhydryl group per mole of protein (based upon mol wt = 8600). This corroborated the finding of 1 mole of taurine, which is formed from the oxidation of 2-mercaptoethanol, in the amino acid analysis.

Identification of 4'-Phosphopantetheine—The finding that ACP<sub>butyricum</sub> contains 1 mole each of β-alanine and 2-mercaptoethanol per mole of protein suggested that it, like ACP<sub>coli</sub> contains 4'-phosphopantetheine as a prosthetic group. Incubation of the 14C-ACP at pH 12 and 70°C for 1 hour, conditions which cause β elimination of 4'-phosphopantetheine from ACP<sub>coli</sub> (18), released 100% of the radioactivity from the protein. This radioactive compound, which remained in the supernatant solution after the protein was acid-precipitated, was identified as 4'-phosphopantetheine by converting it enzymatically to CoA (Table III). These experiments establish that ACP<sub>butyricum</sub> contains 4'-phosphopantetheine as a prosthetic group.

Effect of Acylation or Alkylation of Groups Other Than Sulfhydryl Group of ACP—Chemical acylation of both ACP<sub>butyricum</sub> and ACP<sub>coli</sub> under the usual circumstances (see "Experimental Procedure") leads to the conversion of approximately 25% of the protein to thioester, although the protein always contains more than 0.25 mole of acyl group per mole of ACP. It was therefore evident that protein groups other than the sulfhydryl group are acylated in the procedure. The effect of acyl groups that are
FIG. 3. Ultracentrifugation pattern of ACP butyricum. ACP, 1.6 mg per ml, was dialyzed overnight against 0.4 M KCl containing 0.02 M potassium phosphate, pH 6.5, and 0.01 M 2-mercaptoethanol.

Sedimentation measurements were made in a synthetic boundary cell at a rotor speed of 59,780 rpm. This exposure was made after 4 min at bar angle of 70°. See "Experimental Procedure."

### Table II

Timed hydrolysis of ACP butyricum

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Amino acid residues per molecule of protein</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>24 hours</td>
</tr>
<tr>
<td>Lysine</td>
<td>4.15</td>
</tr>
<tr>
<td>Histidine</td>
<td>2.10</td>
</tr>
<tr>
<td>Arginine</td>
<td>0</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>13.1</td>
</tr>
<tr>
<td>Threonine</td>
<td>1.00</td>
</tr>
<tr>
<td>Serine</td>
<td>3.35</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>13.6</td>
</tr>
<tr>
<td>Proline</td>
<td>1.03</td>
</tr>
<tr>
<td>Glycine</td>
<td>1.06</td>
</tr>
<tr>
<td>Alanine</td>
<td>7.10</td>
</tr>
<tr>
<td>Valine</td>
<td>7.30</td>
</tr>
<tr>
<td>Methionine</td>
<td>7.98</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>6.18</td>
</tr>
<tr>
<td>Leucine</td>
<td>5.60</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>0.91</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>2.91</td>
</tr>
<tr>
<td>β-Alanine</td>
<td>1.95</td>
</tr>
</tbody>
</table>

**Note:**
- Amino acid composition of E. coli ACP (5).
- Based upon performic-oxidized sample of ACP.

### Table III

Identification of 4'-phosphopantetheine

<table>
<thead>
<tr>
<th>Conditions of incubation</th>
<th>ACP treated</th>
<th>CoA formed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete system</td>
<td>2.5</td>
<td>2.8</td>
</tr>
<tr>
<td>Complete system</td>
<td>5.0</td>
<td>5.7</td>
</tr>
<tr>
<td>Omit ATP in second incubation</td>
<td>2.5 + 2 mmoles of 4'-phosphopantetheine</td>
<td>4.9</td>
</tr>
<tr>
<td>Omit dephospho-CoA pyrophosphorylase-dephospho-CoA kinase in second incubation</td>
<td>5.0</td>
<td>0</td>
</tr>
</tbody>
</table>

**Note:**

- Based upon performic-oxidized sample of ACP.

Not in thioester linkage on the reactivity of ACP col in the malonyl-CoA-Co2 exchange reaction is shown in Fig. 4. H3-Palmityl-ACP (containing 0.3 μ mole of thioester and 0.2 μ mole of ACP-SH) was treated with hydroxylamine to convert the thioester to the hydroxamic acid plus free ACP-SH. Although the ACP preparation at this point contained approximately 1 mole of sulphydryl group per mole of protein, it was only 20% as active as native ACP in the malonyl-CoA-Co2 exchange reaction. Exposure of native ACP to the solvents used in the chemical acylation (pyridine and tetrahydrofuran) caused only a 15% decrease in activity. Thus it is evident that the presence of palmitoyl groups on ACP groups other than the sulphydryl group decreases the activity of this protein in the exchange reaction.

Similar studies with acylated derivatives of ACP butyricum indicated that this protein also is less active in the malonyl-CoA-Co2 exchange reaction when it is acylated at sites other than the sulphydryl group. Comparative studies with acylated preparations of ACP col and ACP butyricum indicated that ACP butyricum is
ACP<sub>butyricum</sub> is similar to ACP<sub>coli</sub> in that it is heat- and acid-stable (5). It contains a single sulfhydryl residue, 2-mercaptoethanolamine, which is a component of a prosthetic group, 4'-phosphopantetheine. The prosthetic group is released from the protein under the same conditions that caused β elimination of 4'-phosphopantetheine from ACP<sub>coli</sub>. In addition, ACP hydrolase, an E. coli enzyme which catalyzes the specific cleavage of ACP<sub>coli</sub> to yield 4'-phosphopantetheine and the apoprotein, is ¼ as active with ACP<sub>butyricum</sub> as it is with ACP<sub>coli</sub> (29). These facts suggest that in both ACP<sub>butyricum</sub> and ACP<sub>coli</sub> the prosthetic group is bound through phosphodiester linkage to the hydroxyl group of a serine residue of the protein. The difference in activity in the malonyl-CoA-CO₂ exchange reaction of ACP's from the two sources reflects differences in the apoproteins which are indicated in the amino acid compositions (5).

Chemical acylation of ACP under the conditions used in these studies leaves a great deal to be desired. The over-all yield of thioester is poor, and it is difficult to separate the products from the precursors; thus, the thioester preparations always contain ACP-SH which can complicate the interpretation of some experiments. More important, however, is the finding that sites of modification of ACP, other than the sulfhydryl group, are acylated during the reaction. The number of acyl groups per mole of ACP is always higher than that expected from the thioester concentration; thus it is assumed that amino groups of certain residues are acylated as well as the sulfhydryl group of the prosthetic group. These nonthioester long chain acyl groups decrease the activity of ACP in the malonyl-CoA-CO₂ exchange reaction, the most sensitive enzymatic assay for ACP. Decreased enzymatic reactivity of acylated ACP must be kept in mind when long chain acyl-ACP derivatives are tested in reactions in which they are presumed to be substrates (13).

In experiments where ACP-SH in preparations of acyl-ACP might complicate interpretation, alkylation of the sulfhydryl group with N-ethylmaleimide effectively removes the interference by ACP-SH. However, this procedure is not without hazard since it appears that ACP is alkylated by N-ethylmaleimide in other sites in addition to the sulfhydryl group, even under mild conditions. Thus, ACP regenerated from the thioester, which contains both extra alkyl and acyl groups, is less reactive in the malonyl-CoA-CO₂ exchange reaction than ACP that has only undergone chemical acylation.

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