Activation of long chain fatty acids with acyl carrier protein: Demonstration of a new enzyme, acyl-acyl carrier protein synthetase, in Escherichia coli

(fatty acid thioesters/phospholipid synthesis/fatty acid incorporation)

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ABSTRACT A soluble enzyme activity which catalyzes the synthesis of acyl-acyl carrier protein from acyl carrier proteins, a long chain fatty acid, and ATP has been demonstrated in E. coli. The reaction requires high concentrations of both Ca$^{++}$ and Mg$^{++}$ for activity, and cleaves ATP to AMP and PP.$^1$. The fatty acyl product has been identified as acyl-acyl carrier protein by its solubility, thioester linkage, molecular weight, charge, and biological activity. Several criteria indicate the enzyme is distinct from acyl-CoA synthetase. The fatty acid specificity of the enzyme suggests a role of acyl-acyl carrier protein synthetase in the incorporation of fatty acids into phospholipid.

A major step in the synthesis of the membrane phospholipids of Escherichia coli may involve the transfer of fatty acids from acyl carrier protein (ACP) thioesters to sn-glycerol 3-phosphate to form phosphatidic acid, a key intermediate in phospholipid synthesis (1, 2). It has been proposed that long chain fatty thioesters of ACP arise as the end product of de novo fatty acid synthesis, whereas long chain fatty acids that are exogenously supplied become incorporated into phospholipid via transfer from CoA thioesters (3-5). Recent results from our laboratory (6) challenge this proposal, and have led us to seek an enzyme which catalyzes the acylation of ACP with a long chain free fatty acid. In this paper, we shall provide evidence of the presence of such an enzyme which we will call acyl-ACP synthetase. This enzyme catalyzes the following reaction:

$$RCOH + ACP - SH + ATP \rightarrow RC\_\_ACPP + AMP + PP\_I$$

The enzyme commission name for this enzyme is fatty acid: ACP ligase (AMP). No enzyme capable of the direct activation of long chain fatty acids with ACP-SH has been reported. Indeed, Samuel and Ailhaud (7) reported the absence of acyl-ACP synthetase in E. coli.

EXPERIMENTAL PROCEDURES

Bacterial Strains. Various E. coli K12 strains were used. These were the wild-type strain, Ymel, and the fadD$^-$ (acyl-CoA synthetase deficient) mutant strains, TR3 and K27. These strains are described in previous papers (4, 6). Cultures were grown to late logarithmic phase in R broth (8) (unless otherwise indicated), then harvested, and washed by centrifugation.

Enzyme Preparations. Cells of strain TR3 (unless otherwise indicated) were suspended in buffer A (0.1 M Tris-HCl buffer at pH 7.5, 0.01 M 2-mercaptoethanol) and ruptured in a French pressure cell (18,000 psi, 1.24 $\times$ 10$^6$ Pa). The resulting crude homogenate was then centrifuged at 48,000 $\times$ g for 30 min and the supernatant retained. The crude supernatant was adjusted to 45% saturation with (NH$_4$)$_2$SO$_4$ (Mann Enzyme Grade) and was then centrifuged at 10,000 $\times$ g for 15 min. The pellet was resuspended in buffer A and dialyzed overnight against 200 volumes of buffer A.

Enzyme Assay. The standard reaction mixture contained 0.1 M Tris-HCl at pH 8.0; 10 mM ATP; 35 $\mu$M ACP-SH; 50 mM CaCl$_2$; 40 mM MgCl$_2$; 0.5 mM dithiothreitol; 30 $\mu$M [1-14C] palmitic acid (10,000 cpm/nmol); 2 mg/ml Triton X-100 and crude enzyme (0-0.1 mg) in a total volume of 0.1 ml. After incubation at 37$^\circ$C for 10 min, incorporation of fatty acid into acyl-ACP was assayed by either of two methods. The first method was solvent extraction to remove free fatty acid as described by Mancha et al. (9). A second and less cumbersome method was to pipet the reaction mixture onto a filter paper disc (Whatman 3MM, 2.4 cm diameter). The discs were then washed twice in a beaker containing chloroform–methanol–acetic acid (1:2:0.3, vol/vol) in order to remove free fatty acid (fatty acid-CoA is also removed). Under these conditions, the enzyme assay gave a linear response with both protein and time. Blank values (minus enzyme, ACP, or ATP) showed <5% of the incorporation given by complete reaction mixtures. A unit of enzyme activity is a nmol of acyl-ACP formed per minute.

Separation of ACP-SH and Acyl-ACP. Standard incubation mixtures, in which about 30% of the [3H]palmitoyl-ACP-SH (4100 cpm/nmol) was converted to [14C]palmitoyl-[3H] palmitoyl-ACP by enzyme purified through the ammonium sulfate step, were extracted to remove fatty acid and then acid precipitated at pH 3.5. The precipitate was resuspended in 1 ml of 0.1 M Tris-HCl buffer at pH 8.0 which contained 1 M KCl (to prevent nonspecific binding), 1 mM NaEDTA, and a 10-fold excess of dithiothreitol. After reduction, the ACP-SH in the mixture was then converted to its thionitrobenzoic acid derivative by addition of a 10-fold excess (over dithiothreitol) of 5,5'-dithiobis(2-nitrobenzoic) acid (DTNB). Excess DTNB and the other reaction products were removed from the protein fraction via a Sephadex G-25 column. The ACP derivatives from the void of the G-25 column were then applied to a 5 ml column of DTNB-agarose (10). The DTNB-agarose column was slowly (0.1 ml/min) eluted with the Tris-KCl-EDTA buffer. This treatment removed >95% of the ACP-SH in a parallel sample incubated without ATP. The ratio of palmitate to ACP in the eluate was then determined by dual label scintillation counting, by using the appropriate standards.

Electrophoresis. Gels of 12% acrylamide were run in 0.1% sodium dodecyl sulfate (NaDodSO$_4$) as previously described (8, 11) except that sample preparation and quantitation were done as described by Ferro-Luzzi Ames (12).

Chromatography. The Sephadex G-75 column (1 × 36 cm,
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Table 1. Requirements for acyl-ACP synthesis

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Complete</th>
<th>-ACP-SH</th>
<th>-ACP-SH + ACP-NEM</th>
<th>-ATP</th>
<th>-Ca++</th>
<th>-Ca++ + EGTA</th>
<th>-Mg++</th>
<th>-Mg++ + EDTA</th>
<th>-Ca++ and Mg++</th>
<th>-Ca++ and Mg++ + EDTA and EGTA</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.00</td>
<td>0.02</td>
<td>0.27</td>
<td>0.02</td>
<td>0.15</td>
<td>0.10</td>
<td>0.25</td>
<td>0.05</td>
<td>0.02</td>
<td>0.02</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

Assay of acyl-ACP synthesis by ammonium sulfate-purified enzyme was measured by either incorporation of [1-14C]palmitate in the filter disc assay (Exp. 1) or by conversion of [14C]ATP to [14C]AMP (Exp. 2). ACP-NEM was a sample of ACP-SH in which about 70% of the sulphydryl groups had been blocked with treatment with N-ethylmaleimide (21). The chelators, EDTA (ethylenedinitrilo)tetraacetic acid) and EGTA (ethylene glycol-bis[β-aminopropyl]ether)-N,N'-tetraacetic acid), were added at 10 mM and 1 mM, respectively.

The void volume of 16.5 ml was run in 2 mM potassium phosphate buffer at pH 6.1 containing 10 mM 2-mercaptoethanol. The column was run at 0.7 ml/min and 1.5 ml samples were collected.

The DEAE-Sephadex A-25 column (1.5 x 21 cm) was equilibrated in 0.01 M potassium phosphate buffer at pH 6.1 containing 10 mM 2-mercaptoethanol. The column was eluted with 10 column volumes of a linear gradient of 0.2–0.6 M KCl in the equilibration buffer.

Hydroxylamine treatment at pH 6.5 was carried out as described by Rosenfeld et al. (13). The borohydride treatment was carried out in 30% tetrahydrofuran for 15 min at 37°C as described by Barron and Mooney (14). Under these conditions, the reaction is quite specific for thiosterols (15). The acetyl derivatives (13) as well as the free hydroxamates and alcohols were chromatographed with the appropriate standards, as described by Rosenfeld et al. (13), except the developing solvent system was petroleum ether-ether-acetic acid (70:30:2, vol/vol).

Materials. ACP and [3H pantothenate]-ACP (5 x 10⁶ cpm/μmol) were prepared essentially as described by Major et al. (16). These preparations were shown to be >95% pure when run on 12% acrylamide gels by the method of Laemmli (17) and had the expected high activity in the malonyl-CoA-CoA exchange reaction (16). Radioactive compounds were from New England Nuclear and Amersham-Searle. Palmitoleic and cis-vaccenic acids, both 14C-labeled in the odd numbered carbons, were made biosynthetically (18) and purified by argentation chromatography (18, 20). Nucleotides were from PL Biochemicals.

RESULTS

Properties of the Reaction. The synthesis of acyl-ACP was determined by measuring the conversion of [14C]palmitic acid to a form insoluble in organic solvents. By using this assay, we found the formation of acyl-ACP requires ATP, ACP-SH, Mg²⁺, and Ca²⁺ (Table 1). The requirement for both divalent cations is unusual. Quite high [though physiological (22)] concentrations of both cations are required for optimal activity. If ATP hydrolysis is used as an assay, then a requirement for fatty acid can also be demonstrated (Table 1, experiment 2). The assay is stimulated 2- to 4-fold by the addition of the detergent Triton X-100, and has a broad pH optimum between 7.5 and 8.5. If CoA-SH (100 μM) is substituted for ACP-SH in the assay, acyl-CoA is formed at <5% of the rate of acyl-ACP formation.

Acyl-ACP synthetase is found in the soluble fraction of cells disrupted in a French pressure cell. The membrane fraction has <1% of the total activity. The enzyme is precipitated by ammonium sulfate at 45% of saturation which provides a simple method to remove endogenous ACP-SH (16).

Identification of the 14C-Labeled Product as Palmitoyl-ACP. The 14C-labeled product is bound to ACP by a thioester linkage to the single sulphydryl (that of the 4′-phosphopantotheine prosthetic group) of the protein. This conclusion is supported by the following data: (i) greater than 97% of the radioactivity is converted to an ether soluble form by treatment either with hydroxylamine at pH 6.5 (13) or with sodium borohydride (14). The ether soluble products of these reactions cochromatograph with the appropriate authentic standards (palmitylhydroxamate and palmityl alcohol, respectively). (ii) The 14C-labeled product is not retained by the DTNB-agarose column which quantitatively retains ACP-SH and other sulphydryl-containing proteins (10). (iii) Experiments using [3H]pantothenate]-ACP-SH and [14C]palmitic acid as substrates, and then purification on the DTNB-agarose column gives a product with a molar ratio of fatty acid to ACP of 0.92.

The 14C-labeled product of the reaction is not palmitoyl-CoA as shown by the selective extraction procedures of Mancha et al. (9). The product, like acyl-ACP, was precipitated by ammonium sulfate in the presence of chloroform/methanol whereas acyl-CoA is soluble under these conditions (9). When the ammonium sulfate precipitate was resuspended and chromatographed on Sephadex G-75, the radioactivity eluted as a single peak in the included volume coincident with an internal standard of ACP-SH (Fig. 1a). The product also was eluted from DEAE-Sephadex in a manner very similar to the elution profile of ACP-SH (Fig. 1b). From these experiments, we concluded that the 14C-labeled product had a molecular weight and charge distribution very similar to that of ACP-SH. Because ACP-SH is one of the smallest (8900 daltons) and most acidic proteins found in E. coli (23), this behavior strongly suggested that the product was palmitoyl-ACP.

We further characterized the product by NaDodSO₄ acrylamide gel electrophoresis. Jaworski and Stumpf (11) reported that stearoyl-ACP, synthesized by plant tissue extracts, migrated ahead of ACP-SH on NaDodSO₄ gels. In earlier work, we confirmed this observation with palmitoyl ACP (8), and it seems clear [as proposed by Jaworski and Stumpf (11)] that the anomalous behavior of acyl-ACP can be attributed to an increased binding of NaDodSO₄ by the hydrophobic fatty acid chain. The 14C-labeled product was purified by ammonium sulfate precipitation in the presence of chloroform/methanol, then by chromatography on Sephadex G-75, and finally by chromatography on DEAE-Sephadex (overall yield of about 60%). Upon electrophoresis in 0.1% NaDodSO₄ on 12% acrylamide gels run at pH 6.0, these preparations gave a single Coomassie brilliant blue staining band which coincided with ACP-SH. However, all the 14C-labeled radioactivity detected by autoradiography migrated slightly ahead of ACP-SH. Quantitation of one of these gels (Fig. 1c) showed that >95% of the radioactivity on the gel migrated in a band slightly ahead...
demonstrate (i) the transfer of the acyl moiety by the fatty acid synthetase of *E. coli*. In the first experiments, we incubated a sample of \[^{14}C\]palmitoyl-ACP [purified by the procedure of Mancha et al. (9)] followed by acid precipitation at pH 3 with sn-glycerol 3-phosphate, and a washed membrane fraction (24) from *E. coli* in our standard sn-glycerol 3-phosphate acyltransferase reaction mixture (8). After incubation, >90% of the lipid products were recovered as a mixture of lysophosphatidic acids (8, 24) and thus the acyl moiety was efficiently transferred from ACP to sn-glycerol 3-phosphate.

We also tested the elongation of acyl-ACP by the *E. coli* fatty acid synthetase. Elongation was tested in incubation mixtures containing a \[^{14}C\]-labeled fatty acyl-ACP (see Table 3) plus nonradioactive malonyl-CoA, ACP-SH, an NADPH generating system, and the other components of the fatty acid synthetase assay (20) with the exception of acetyl-CoA. After incubation and treatment with base, the fatty acids were extracted from the reaction mix, methylated, and analyzed by argentation and reverse phase thin-layer chromatography (19). The ACP thioesters of decanoyl, lauryl, and myristyl acids were efficiently elongated to a mixture of palmitic, stearic, and arachidic (C20) acids, whereas the elongation products of octanoyl-ACP were a mixture of long chain unsaturated and saturated fatty acids, the major product being cis-vaccenic acid. Decarboxylation of the elongated saturated acids yielded \[^{14}C\]CO₂ under conditions that gave a quantitative release of carboxyl carbon (25).

In conclusion, the \[^{14}C\]-labeled fatty acid product is a molecule which contains 1 mol of fatty acid bound to 1 mol of ACP-SH through a thioester linkage. The molecule has the size, charge distribution, and NaDodSO₄ binding properties expected of acyl-ACP. In addition, the acyl moiety can be transferred to sn-glycerol 3-phosphate by the membrane bound sn-glycerol 3-phosphate acyl-transferase and elongated by the fatty acid synthetase. These data, therefore, fully support our identification of the product as acyl-ACP.

### Table 2. Nucleotide specificity of acyl-ACP synthetase

<table>
<thead>
<tr>
<th>Nucleotide</th>
<th>Acyl-ACP synthesis (units/mg of protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0.01</td>
</tr>
<tr>
<td>ATP</td>
<td>1.91</td>
</tr>
<tr>
<td>dATP</td>
<td>1.08</td>
</tr>
<tr>
<td>GTP</td>
<td>0.01</td>
</tr>
<tr>
<td>CTP</td>
<td>0.28</td>
</tr>
<tr>
<td>UTP</td>
<td>0.02</td>
</tr>
<tr>
<td>TTP</td>
<td>0.01</td>
</tr>
<tr>
<td>5'-AMP-PNP</td>
<td>0.23</td>
</tr>
</tbody>
</table>

The incorporation of \[^{14}C\]palmitic acid into acyl-ACP by ammonium sulfate purified enzyme was assayed by the filter disc assay. ATP and dATP were added at 10 mM. The other nucleotides were tested at 15 mM. GTP was also inactive at 5 and 20 mM.

of ACP-SH. The single radioactive band found when incubation mixtures were run on NaDodSO₄ gels after only the purification procedure of Mancha and coworkers (9) also ran slightly ahead of ACP-SH.

The final identification of the product as acyl-ACP was to demonstrate its biological activity in two enzyme systems. These were (i) the transfer of the acyl moiety to sn-glycerol 3-phosphate, and (ii) the elongation of the acyl moiety by the fatty acid synthetase of *E. coli*. In the first experiments, we incubated a sample of \[^{14}C\]palmitoyl-ACP [purified by the procedure of
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Table 3. Fatty acid specificity of acyl-ACP synthetase

<table>
<thead>
<tr>
<th>Fatty acids</th>
<th>Structure</th>
<th>$K_m$ (µM)</th>
<th>$V_{max}$ (units/mg of protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saturated</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Octanoic</td>
<td>C8</td>
<td>166</td>
<td>1.20</td>
</tr>
<tr>
<td>Decanoic</td>
<td>C10</td>
<td>100</td>
<td>0.93</td>
</tr>
<tr>
<td>Lauric</td>
<td>C12</td>
<td>25</td>
<td>0.66</td>
</tr>
<tr>
<td>Myristic</td>
<td>C14</td>
<td>15</td>
<td>0.99</td>
</tr>
<tr>
<td>Palmitic</td>
<td>C16</td>
<td>15</td>
<td>0.90</td>
</tr>
<tr>
<td>Stearic</td>
<td>C18</td>
<td>13</td>
<td>0.33</td>
</tr>
<tr>
<td>Unsaturated</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Palmitoleic</td>
<td>C16A&lt;sup&gt;+&lt;/sup&gt;</td>
<td>20</td>
<td>1.20</td>
</tr>
<tr>
<td>cis-Vaccenic</td>
<td>C18A&lt;sup&gt;+&lt;/sup&gt;</td>
<td>20</td>
<td>1.35</td>
</tr>
<tr>
<td>Oleic</td>
<td>C18A&lt;sup&gt;-&lt;/sup&gt;</td>
<td>20</td>
<td>1.05</td>
</tr>
<tr>
<td>Linoleic</td>
<td>C18A&lt;sup&gt;-&lt;/sup&gt;</td>
<td>20</td>
<td>1.02</td>
</tr>
</tbody>
</table>

Incorporation of $^{14}$C-labeled fatty acids into acyl-ACP by crude supernatant enzyme was assayed by the filter disc assay. The approximate kinetic constants were derived by the method of Lineweaver and Burk (29).

Table 4. Nonidentity of acyl-ACP and acyl-CoA synthetases

<table>
<thead>
<tr>
<th>Strain</th>
<th>fadD</th>
<th>Carbon source</th>
<th>Acyl-ACP synthetase (units/mg of protein)</th>
<th>Acyl-CoA synthetase (units/mg of protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ymel</td>
<td>+</td>
<td>Acetate</td>
<td>0.50</td>
<td>1.00</td>
</tr>
<tr>
<td>Ymel</td>
<td>+</td>
<td>Oleate</td>
<td>0.49</td>
<td>4.70</td>
</tr>
<tr>
<td>K27</td>
<td>–</td>
<td>Acetate</td>
<td>0.38</td>
<td>0.20</td>
</tr>
<tr>
<td>TR3</td>
<td>–</td>
<td>Succinate</td>
<td>1.05</td>
<td>0.15</td>
</tr>
</tbody>
</table>

Supernatant protein from a low-speed centrifugation (to remove unbroken cells) of a French press homogenate was assayed for both synthetase activities. Acyl-CoA synthetase was assayed as described previously (6). The mutations in the particular fadD strains used are known to be somewhat leaky (6). Cultures were grown on medium E (31) plus the carbon source indicated (0.1-0.4%).

carbon source (Table 4). Growth on oleate results in a large increase in acyl-CoA synthetase activity (4, 7). However, the most direct proof of the nonidentity of the two synthetases is that mutants in the gene (fadD) that codes for acyl-CoA synthetase (4, 32, 33) have normal levels of acyl-ACP synthetase, although these strains are deficient in acyl-CoA synthetase (Table 4).

Purification of acyl-ACP synthetase is currently in progress in this laboratory and will be the subject of a future publication. The enzyme seems of quite high molecular weight in that it is excluded from Sephadex G-150. This property and its behavior on DEAE-cellulose clearly show that the acyl-ACP synthetase molecule is distinct from $\beta$-ket酰基-ACP synthetase (34), and from the two E. coli thioesters which have been purified (35, 36).

DISCUSSION

We previously showed that E. coli can incorporate endogenous long chain fatty acids into phospholipid in the absence of acyl-CoA synthetase activity (6). This finding suggested the presence of another enzyme capable of activating free fatty acid for transfer into phospholipid. Furthermore, our data suggested that acyl-CoA was not an intermediate in this transfer, and hence mechanisms involving transfer of the acyl group from CoA to ACP [such as that catalyzed by $\beta$-ket酰基-ACP synthetase I for short chain acids (34)] seemed unlikely. We therefore sought an enzyme which would directly activate fatty acids with ACP-SH. In contrast to the results of Samuel and Ailhaud (7), we found an enzyme activity which catalyzes acyl-ACP synthesis. The discrepancy between our results and the previous report (7) can be attributed to the unusual metal ion requirements of acyl-ACP synthetase.

Acyl-ACP synthetase could function to activate free fatty acids into a form that can be transferred into phospholipid. The free fatty acids might arise from two sources, either by hydrolytic turnover of phospholipid fatty acids or by thioesterase mediated cleavage of acyl-CoA molecules formed during transport of extracellular fatty acids. In the latter situation, the exogenous fatty acid would enter the cell as an acyl-CoA molecule [Overath and coworkers have shown that acyl-CoA synthetase is required for fatty acid transport (4, 33)]. The acyl-CoA would then be either degraded via $\beta$-oxidation or cleaved to free fatty acid by the thioesterase (35, 36) known to be in E. coli. The resulting free acid would then be activated by acyl-ACP.

<sup>†</sup> A. Spencer, T. Ray, M. Rao, and J. Cronan, unpublished experiments.
synthetase and transferred into phospholipid. If this proposed pathway is true, the selection of mutants lacking acyl-ACP synthetase should be straightforward.

The physiological function of acyl-ACP synthetase is unknown. The occurrence of this enzyme presents an apparent paradox which arises because E. coli is unable to elongate exogenously supplied fatty acids (5, 30, 37, 38), although such acids are readily incorporated into phospholipid. It could be expected that since long chain fatty acids can be converted to acyl-ACP thioesters, elongation by the fatty acid synthetase should also occur. The resolution of this apparent contradiction may lie in the specificity of acyl-ACP synthetase (Table 3). The only fatty acids which are effective substrates for this enzyme are those that are readily incorporated into phospholipid by E. coli. Thus, after conversion to an ACP thioester, an entering long chain fatty acid might be transferred into phospholipid rather than becoming elongated. Recent data from this laboratory indicate that the chain length of the fatty acids synthesized by E. coli is determined by just such a competition between elongation and transfer to phospholipid (19).

Acyl-ACP synthetase seems ideal for the synthesis of long chain acyl-ACP derivatives. Difficulties in the chemical synthesis of these compounds has hindered study of various enzymes in the latter stages of fatty acid synthesis and the early steps of phospholipid synthesis (8, 11). Jaworski and Stumpf (11) recently reported acyl-ACP synthetase by fatty acid synthetase preparations from various plant tissues. However, the acyl-ACP synthetase activity of E. coli should become the method of choice for production of acyl-ACP thioesters, because the enzyme permits the synthesis of a much wider variety of acyl-ACP derivatives than does the plant system and is also much less laborious.

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