Noninvolvement of Acyl Carrier Protein with Citrate Synthase and Malate Synthase

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Acyl carrier protein (ACP coli) was isolated from commercially grown Escherichia coli B and was acetylated by chemical methods. Biological activity of the synthesized acetyl-ACP coli was checked in an in vitro fatty acid-synthesizing system isolated from E. coli B. Since acetyl-ACP is preferred over acetyl-coenzyme A (CoA) as a substrate in these reactions, the possibility that it may substitute for acetyl-CoA in biosynthetically and oxidatively important cellular pathways (glyoxylate and Krebs cycles, respectively) was examined. Acetyl-ACP was tested for substrate activity with the enzyme of each cycle which has been found to utilize acetyl-CoA. Crystalline citrate synthase (EC 4.1.3.7) of porcine origin (Calbiochem) was found to be inactive with acetyl-ACP coli, which acted neither as a substrate nor as an inhibitor in the presence of acetyl-CoA. Malate synthase (EC 4.1.3.2) of the acetate type was isolated from acetate-grown cells of a mutant of E. coli K-12 (VGD3H3) and was also found to be inactive with acetyl-ACP coli. The significance of these results and of the recent discovery of another phosphopantetheine-containing protein are discussed.

Evidence for the general biological significance of acyl carrier protein (ACP) has been steadily accumulating since Majerus et al. (8) reported that all of the intermediate reactions of fatty acid biosynthesis in Escherichia coli occur with the substrates bound as thioesters to ACP. Overath and Stumpf (Federation Proc., p. 166, 1964) showed that E. coli ACP (ACP coli) can function in the fatty acid synthesizing system of the avocado mesocarp. In spite of probable species specificity, Alberts et al. (2) demonstrated that acetoacetyl-ACP coli undergoes further metabolism with the fatty acid synthetase isolated from rat adipose tissue than does acetoacetyl-coenzyme A (CoA). Acetoacetyl-CoA was metabolized to β-hydroxybutyryl-CoA, whereas acetoacetyl-ACP coli underwent a second reduction to form a product which, the authors suggest, “is probably” butyryl-ACP.

Attempts to utilize ACP as an acyl carrier in systems not related directly to fatty acid biosynthesis, e.g., in the biosynthesis of acetylhomoserine (11), have not been successful. In systems more closely related to fatty acid metabolism, however, some success has been achieved. Rudney et al. (12), in studies with the β-hydroxy-β-methyl-glutaric acid (HMG) CoA-condensing enzyme of yeast, observed that E. coli acetoacetyl-ACP and acetyl-CoA can react in the presence of HMG-CoA-condensing enzyme to form β-hydroxy-β-methylglutaric acid at about one-sixth the rate observed with acetoacetyl-CoA. Furthermore, HMG-ACP coli can undergo a nonenzymatic transacylation with CoA. It was suggested that the above reactions may serve to unify the malonate and acetate pathways of HMG biosynthesis (12). Significantly, ACP has been found to be essential for the conversion of glycerophosphate to lysophosphatidic acid by a particulate fraction from Clostridium butyricum (1). Palmityl-ACP butyricum was found to be an efficient donor for lysophosphatidic acid synthesis (1), whereas acyl-CoA derivatives were used inefficiently. These results contrast with those of Althaud and Vagelos (1), who found that E. coli membrane and particulate fractions can utilize either acyl-ACP coli or acyl-CoA for the acylation of glycerophosphate. The aforementioned studies indicated that ACP may be utilized in other cellular processes related to fatty acid metabolism.

Acetyl-CoA is a principal intermediate in the cellular biosynthetic and oxidative pathways of the tricarboxylic acid and the glyoxylate cycles. Therefore, it would appear absolutely essential that the acetyl carrier be present for maximal growth under aerobic conditions. Alberts and Vagelos (3) studied the incorporation of pantothen-
ate into CoA and ACP in pantothenate-requiring mutants of *E. coli*. They discovered that, whereas the concentration of CoA varied with the concentration of pantothenate in the growth medium, the amount of ACP present did not vary significantly. The concentrations of ACP, the authors stated (3), "are maintained at the expense of CoA." It appeared that CoA could also transfer its 4' phosphopantetheine moiety to be used in ACP biosynthesis. Although the study was done on a specially selected auxotroph, it suggested that CoA is sacrificed to maintain ACP levels because of the greater functional importance of ACP. ACP might, therefore, be expected to function as an acyl carrier in energy-producing and biosynthetic reactions other than those involving fatty acids. We have attempted to clarify this hypothesis by testing the effects of acetyl-ACP on the two enzymes in the tricarboxylic acid and glyoxylate cycles, citrate-condensing enzyme (EC 4.1.3.7) and malate synthase (EC 4.1.3.2), respectively, which have been found to catalyze the condensation of acetyl-CoA with another substrate.

**MATERIALS AND METHODS**

ACP was isolated from *E. coli* B (Grain Processing Co., Muscatine, Iowa) by the method of Majerus et al. (9) and was assayed by the malonyl-pantetheine-14CO2 exchange method (9). 1-14C-acetyl-ACP was prepared according to the method of Alhaud et al. (1).

Fatty acid synthesis was assayed by a modification of the method of Kass and Bloch (7). The fatty acid synthesizing system was obtained from *E. coli* B. A 12.5-g amount of cells (wet-frozen) was suspended in a final volume of 50 ml of friethanolamine-HCl buffer (pH 7.5; 0.01 M) and was disrupted in a Raytheon sonic oscillator (10 kHz at full power) for 12 min. Cell debris was removed by centrifugation at 37,000 × g for 30 min. 14C-acetyl-CoA was synthesized by the method of Schweizer (Ph.D. Thesis, Univ. of Munich, Germany, 1963), and malonyl-CoA was synthesized according to the method of Trams and Brady (13). The reaction mixture contained, in a total volume of 0.5 ml: friethanolamine-HCl (pH 7.2), 50 μmole; ACP coil, 8 μmole; acetyl-1-14C-CoA, 50 nmole containing 125,000 counts/min, or acetyl-1-14C-ACP coil, 15 μmole containing 62,000 counts/min; malonyl-CoA, 0.16 μmole; and a reduced nicotinamide adenine dinucleotide phosphate-generating system consisting of glucose-6-phosphate (2.8 μmole), glucose-6-phosphate dehydrogenase (10 units; Calbiochem, Los Angeles, Calif.), and nicotinamide adenine dinucleotide phosphate (1.0 μmole).

Fatty acids were determined after incubation at 37°C for 30 min by adding 0.2 ml of 50% aqueous KOH to each tube and placing the tubes in a boiling-water bath for 20 min. They were allowed to cool and then were acidified with concentrated HCl to pH 1. The fatty acids were extracted twice with 5 ml of hexane, the extract was collected, and the hexane was evaporated off with nitrogen. After the hexane had completely evaporated, 10 ml of scintillation fluid, consisting of 0.4% 2,5-bis(2-tert-butybenoxazolyl-thiophene) in toluene-ethyl alcohol (2:1, v/v), was added to each tube; the contents of the tubes were mixed thoroughly and were poured into plastic scintillation vials. Radioactivity was determined with a Packard model 3003 scintillation spectrometer set at maximal efficiency.

**Citrate-condensing enzyme assay.** The assay was carried out according to the method of Buckel et al. (4). An increase in absorbancy at 340 nm was measured with a Cary model 14 recording spectrophotometer. The reaction mixture contained tris(hydroxymethyl)aminomethane (Tris)-chloride buffer (pH 8.0), 55 μmole; DL-malic acid, 7.5 μmole; nicotinamide adenine dinucleotide (NAD), 0.23 μmole; malic dehydrogenase (EC 1.1.1.37), 5 aliters (Calbiochem); and acetyl-ACP coil or dil. Malic dehydrogenase and thioster were omitted from the control cuvette. The volume was adjusted to 1.0 ml with distilled water, and the reaction was started by the addition of 5 μliters of citrate-condensing enzyme (Calbiochem, of porcine origin) to the experimental cuvette.

**Malate synthase.** This enzyme was isolated from a mutant (VGD₃H₂) of *E. coli* K-12 which is constitutive for malate synthase. The organisms were grown to late log phase at 37°C on mineral salts medium containing 1% sodium acetate plus L-histidine (100 mg/liter) and thiamine (200 μg/liter) to satisfy the auxotrophic requirements of the mutant. After harvesting the cells, malate synthase was obtained by a method based on that of Falmagne et al. (5), with the exception that diethylaminomethyl(DEAE)-Sephadex A-25 was substituted for the DEAE cellulose used by Falmagne. We obtained 150-fold purification of the acetate-type malate synthase as a DEAE-Sephadex column fraction, and this fraction was used for all studies with acetyl-ACP. The enzyme assay method was the microassay reported by Furmansi et al. (6).

To observe any inhibition by acetyl-ACP, activity was also measured by observing the thioester cleavage of acetyl-CoA at 232 nm (Dixon and Kornberg, 1959).

**RESULTS**

[1-14C]Acetyl-ACP was checked for biological activity in the fatty acid synthesizing system of *E. coli* B; the results are shown in Table 1. These results demonstrate the biological activity of the [1-14C]acetyl-ACP. Maximal incorporation of [1-14C]acetyl-CoA into hexane-extractable material was dependent on the presence of added free ACP, and omission of malonyl-CoA from the reaction mixture resulted in a significant decrease in the quantity of fatty acid formed.

To determine whether acetyl-ACP might be involved with the biosynthetic and oxidative
In the level of citrate formation (as is acetyl-CoA), citrate-condensing enzyme assays were performed with commercially prepared, crystalline enzyme. Figure 1 shows the results of assays performed with acetyl-CoA and acetyl-ACP.

In cuvette A, containing 30 nmoles of acetyl-CoA, the total change in absorbancy at 340 nm after 4 min was 0.15, indicating the reduction of 25 nmoles of NAD. Cuvette B, which contained 30 nmoles of acetyl-ACP, showed no difference in absorbancy at 340 nm. Only after the addition of 30 nmoles of acetyl-CoA, to cuvette B did we observe an increase in absorbance at 340 nm equivalent to that seen in cuvette A. Thus, acetyl-ACP did not react with the citrate-condensing enzyme in the presence of enzymatically formed oxalacetate to produce citrate. Furthermore, the data in Fig. 1 indicate that acetyl-ACP coli does not prevent the reaction of acetyl-CoA with the enzyme from being carried to completion.

The glyoxylate pathway provides additional amounts of C₄ intermediates for use in cellular biosynthesis during growth on acetate and under conditions in which the tricarboxylic acid cycle functions only as an oxidative, energy-producing pathway. Malate synthase isolated from E. coli VGD₃H₃ was assayed for activity by measuring the disappearance of ¹⁴C-glyoxylate in the presence of enzyme and acetyl-CoA over a 15-min period. Acetyl-ACP coli was also tested for substrate activity and for any possible reaction with glyoxylate (Table 2).

Acetyl-ACP did not serve as a substrate for malate synthase nor was it found to react with glyoxylate. Spectrophotometric assays were performed with both acetyl-CoA and acetyl-ACP present in the same cuvette. No inhibition of enzyme activity by acetyl-ACP was observed.

### Table 1. Formation of fatty acids from [¹⁴C] acetyl-CoA and [¹⁴C] acetyl-ACP

<table>
<thead>
<tr>
<th>Test system</th>
<th>Radioactivity in hexane extract (counts/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>I-¹⁴C-Acetyl-CoA</td>
</tr>
<tr>
<td>Complete⁵</td>
<td>2,646</td>
</tr>
<tr>
<td>Without ACP</td>
<td>907</td>
</tr>
<tr>
<td>Without malonyl-CoA</td>
<td>127</td>
</tr>
</tbody>
</table>

* A 50-nmole amount containing 125,000 counts/min.
* A 15-nmole amount containing 62,000 counts/min. Free ACP was omitted from the reaction mixture.

### Table 2. Activity of malate synthase with acetyl-CoA or acetyl-ACP

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Enzyme</th>
<th>Free [¹⁴C]glyoxy late remaining</th>
<th>Amt of glyoxy late utilized</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>-</td>
<td>3,300</td>
<td>0</td>
</tr>
<tr>
<td>None</td>
<td>+</td>
<td>3,335</td>
<td>0</td>
</tr>
<tr>
<td>Acetyl-CoA (10 nmoles)</td>
<td>Heated</td>
<td>3,462</td>
<td>0</td>
</tr>
<tr>
<td>Acetyl-CoA (10 nmoles)</td>
<td></td>
<td>1,140</td>
<td>7.9</td>
</tr>
<tr>
<td>Acetyl-ACP (15 nmoles)</td>
<td>-</td>
<td>3,274</td>
<td>0</td>
</tr>
<tr>
<td>Acetyl-ACP (15 nmoles)</td>
<td>+</td>
<td>3,310</td>
<td>0</td>
</tr>
</tbody>
</table>

* Activity was measured by isolating glyoxylate from the reaction mixture by thin-layer chromatography, removing the glyoxylate spot, and measuring radioactivity (counts/min) with a Packard model 3003 scintillation counter set at maximal efficiency. Before starting reaction, 12 nmoles of I-¹⁴C-glyoxylate was present. Reaction time was 15 min.

### Discussion

The finding by Alberts and Vagelos (3) that pantothenate auxotrophs of E. coli sacrifice CoA in order to maintain the cellular ACP concentration at optimal levels suggests that ACP is of
primary importance to the cell, perhaps of greater importance than CoA. Thus, ACP may be able to substitute for CoA in numerous pathways which the cell depends upon for survival. These pathways would most likely be those involved in energy production or in the production of bio-synthetic intermediates. Since the tricarboxylic acid and glyoxylate cycles represent such pathways, we investigated the possibility of replacing acetyl-CoA with acetyl-ACP in these enzymatic reactions which have been found to utilize acetyl-CoA. The negative findings reported here may be significant in view of the recent discovery by Matsumura (10) of a protein, isolated from E. coli K-12, containing 4'-phosphopantetheine. The protein, termed "protein c," is quite similar to ACP in its amino acid composition and its prosthetic group. Protein c, however, is unable to replace ACP in the malonyl-CoA CO2 exchange reaction. Matsumura, in the same communication, suggests that "protein c" may function as an acyl carrier for a series of reactions other than those involved in fatty acid synthesis. These additional reactions may well include some which are presently thought to occur only with CoA as the requisite cofactor. Our negative findings do not rule out the possibility of another acyl protein functioning in the pathways mentioned at times when acetyl-CoA concentration is limiting.

It should be noted that, whereas the ACP used in these studies was isolated from E. coli B and the crystalline citrate condensing enzyme was of porcine origin, earlier work (P. Overath and P. K. Stumpf, Federation Proc., p. 166, 1964; 2) has shown that species specificity for reactions involving ACP does not appear significant.

The noninvolvement of acetyl-ACP col with crystalline condensing enzyme and malate synthase may indicate that ACP only serves as an acyl carrier in the fatty acid biosynthetic pathway and in those pathways more directly associated with it (i.e., steroid biosynthesis).

ACKNOWLEDGMENTS

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LITERATURE CITED