Acetoacetyl-Acyl Carrier Protein Synthase, a Potential Regulator of Fatty Acid Biosynthesis in Bacteria*

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The first condensation reaction in the fatty acid biosynthetic pathway in *Escherichia coli* was rate-limiting as judged by analysis of the relative pool sizes of acyl carrier protein (ACP) thioester intermediates in *vivo*. Comparable concentrations of acetyl-ACP, malonyl-ACP, and nonesterified ACP were present during logarithmic growth, whereas long-chain acyl-ACP comprised a minor fraction of the total ACP pool. The antibiotic cerulenin was used to irreversibly inhibit both β-ketoacyl-ACP synthases I and II. However, acetyl-ACP formation in *vivo* was not blocked by this antibiotic, and short-chain (4-8-carbon) acyl-ACPs increased to 60% of the total ACP pool in cerulenin-treated cells. These data suggested the existence of a cerulenin-resistant condensing enzyme that was capable of catalyzing the initial steps in chain elongation. A unique enzymatic activity, acetoacetyl-ACP synthase, that specifically catalyzed the condensation of malonyl-ACP and acetyl-ACP was detected in *E. coli* cell extracts. Acetoacetyl-ACP synthase activity was not inhibited by cerulenin and was present in extracts prepared from a double mutant harboring genetic lesions in β-ketoacyl-ACP synthases I and II (fabB20 fabF3). These data point to the condensation of malonyl-ACP and acetyl-ACP as the rate-controlling reaction in fatty acid biosynthesis and implicate acetoacetyl-ACP synthase as the pacemaker of fatty acid production in organisms and organelles that possess dissociated (Type II) fatty acid synthase systems.

The identity of the enzyme or enzyme system that functions as the pacemaker of fatty acid synthesis in *Escherichia coli* remains one of the major unanswered questions in bacterial physiology (for review see Ref. 1). Phospholipids comprise approximately 10% of the dry weight of a bacterial cell (2), and the advantage of maintaining fine control over this energy-consuming biosynthetic pathway is evident. Formation of fatty acids requires >90% of the ATP expended in membrane lipid biogenesis, so it is not surprising that a significant pool of long-chain acyl-ACP does not exist during balanced growth in minimal media (3). These *in vivo* measurements point to an early step in fatty acid biosynthesis as rate-limiting. One possible regulatory system is the ACP prosthetic group turnover cycle that could be modulated to vary the ratio of active to inactive forms of ACP (1, 4). However, a pool of apo-ACP is not present in *vivo* (5), and the operation of the turnover cycle appears to be involved in governing the intracellular CoA concentration (6, 7). Four candidate enzymatic reactions remain. Acetyl-CoA carboxylase is a plausible choice since it is a role for this enzyme in regulating fatty acid biosynthesis is established in mammalian systems (8) and its activity is controlled by both allosteric (9) and covalent modification mechanisms (10). Three separate proteins are required to carry out this reaction in *E. coli* (11). The transcarboxylase component is inhibited by ppGpp in *vivo* (12), but a role for acetyl-CoA carboxylase in the regulation of fatty acid biosynthesis in *E. coli* has not been established (1). Acetyl-CoA:ACP transacylase is an equally likely candidate. Shimakata and Stumpf (13) have presented evidence that acetyltransacylase is the rate-limiting step in the Type II fatty acid synthase system isolated from spinach based on the low *in vitro* activity of this enzyme compared to the other enzymes in the pathway. The acetyltransacylase of *E. coli* has been partially purified (14, 15), but nothing is known about its regulatory properties. The possibility that the condensing enzymes control the rate of fatty acid production is not attractive since the two known β-ketoacyl-ACP synthases in *E. coli* are responsible for determining the distribution of end products formed by the fatty acid synthase system (1, 16). Malonyl-CoA:ACP transacylase is also a possible regulatory site, although the high activity of this enzyme relative to the other enzymes in the pathway (17) makes regulation of this reaction unlikely.

The goal of this study was to decide among these alternatives by measuring the relative concentrations of malonyl-ACP and acetyl-ACP in *vivo*. The data point to the first condensation reaction as the slow step in fatty acid biosynthesis, and a unique condensing enzyme, acetoacetyl-ACP synthase, was detected that catalyzes this reaction.

**EXPERIMENTAL PROCEDURES**

**RESULTS**

Characterization of ACP Thioester Standards—The attachment of ligands to the 4'-phosphopantetheine prosthetic group has a profound effect on the solution structure of ACP (28-30). Hydrophobic ligands stabilize the protein moiety to

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‡The abbreviations used are: acyl-ACP, acyl-acyl carrier protein; ACP, acyl carrier protein; HPLC, high-performance liquid chromatography.
pH-induced hydrodynamic expansion whereas the attachment of polar ligands to the prosthetic group sulfhydryl destabilizes the protein. ACP derivatives that disrupt the protein conformation migrate faster than ACP, and more stable structures migrate more slowly than ACP, and more stable ACP, whereas the introduction of a negative charge destabilizes the hydrophobic pocket destabilized ACP structure, and as a consequence, [1-14C]malonyl-ACP migrated slower than ACP.

Composition of the ACP Pool in Vivo—The rate-limiting reaction in E. coli fatty acid biosynthesis was identified by determining the relative concentrations of pathway intermediates (ACP and its thioesters) during logistic growth. Strain SJ16 (panD2) was used to specifically and uniformly label the ACP pool, and the [Pan-3H]ACP intermediates were extracted and resolved by conformationally sensitive gel electrophoresis (Fig. 1). As reported previously (3), long-chain acyl-ACP comprised only a small percentage of the total ACP pool. The majority of the [Pan-3H]ACP was distributed between three closely migrating bands (Fig. 1, lane 2). The upper band comigrated with the malonyl-ACP standard, the middle band was identified as acetyl-ACP, and the lower band was identified as acetyl-ACP. These data point to the condensation of acetyl-ACP and malonyl-ACP as the rate-determining step in fatty acid biosynthesis during logistic growth.

Cerulenin-induced Accumulation of Acyl-ACP—Cerulenin, (2S,3R)-2,3-epoxy-4-oxo-7,10-dodecadienoylamide, inhibits fatty acid biosynthesis in E. coli by the covalent active site-directed inactivation of the β-ketoacyl-ACP synthase step (31, 32). Thus, we anticipated that cerulenin would block acyl-ACP formation and induce the accumulation of malonyl- and acetyl-ACP; however, addition of cerulenin to growing cultures of strain SJ16 resulted in the accumulation of acyl-ACP (Fig. 1, lane 3). Most of the label migrated at the position of long-chain acyl-ACP, and two new bands appeared whose relative mobilities matched the migration positions of butyryl- and hexanoyl-ACP (see Ref. 30). The cerulenin-induced pattern of ACP species was converted to a single band corresponding to nonesterified ACP following treatment of the sample with neutral hydroxylamine (not shown) indicating that all of the bands in the experimental sample were ACP thioesters. Accumulation of acyl-ACP in response to cerulenin was both concentration- and time-dependent (Fig. 2). Cerulenin concentrations higher than 50 μg/ml that caused the maximum accumulation of acyl-ACP (Fig. 2A) were sufficient to completely inhibit fatty acid synthesis in vivo (33). These two experiments indicated that acyl-ACP accumulation corresponded to the inactivation of the β-ketoacyl-ACP synthases by cerulenin. Acyl-ACP chain lengths greater than eight carbons all comigrate in the conformationally sensitive gel electrophoresis system (29, 30). Therefore, the identity of the chain lengths that accumulated was determined by reverse-phase HPLC (Fig. 3). Octyl-ACP was the predominant component of the acyl-ACP pool, and chains longer than 12 carbons were not detected. These data suggested the existence of a cerulenin-resistant condensing enzyme that was capable of catalyzing only the initial steps in fatty acid chain elongation.

Identification of Acetoacetyl-ACP Synthase Activity—The existence of a cerulenin-resistant condensing enzyme was
FIG. 3. Distribution of acyl-ACP chain lengths that accumulate following cerulenin treatment in vivo. Strain SJ16 (panD2) was grown to a density of 7 x 10⁶ cells/ml in glucose minimal medium supplemented with 4 μM β-[3H]alanine (specific activity, 1.04 Ci/mmol), and the cells were harvested 10 min following the treatment of the culture with cerulenin (100 μg/ml). The acyl-[Pan-3H]ACP were isolated by DEAE-cellulose chromatography and fractionated by reverse-phase HPLC was described under "Experimental Procedures." The elution positions of [1-¹⁴C]acyl-ACP standards that were co-chromatographed with the acyl-[Pan-3H]ACP experimental sample are indicated on the carbon number scale.

FIG. 4. Acyl-ACP products formed in an in vitro fatty acid synthase preparation in the presence and absence of cerulenin. Fatty acid synthase extracts were prepared from strains SJ16 (fab'), SJ86 (fabF1), and CY331 (fabB20(Ts) fabF3), and assays were performed at the indicated temperature either in the presence or absence of cerulenin (100 μg/ml) as described under "Experimental Procedures." The assays were then analyzed by comigrationally sensitive gel electrophoresis (13% polyacrylamide) followed by fluorography to resolve the chain lengths of acyl-ACP formed in vitro.

FIG. 5. Identification of the acyl moieties synthesized in vitro in the presence of cerulenin. Fatty acid synthase preparations from strain SJ16 were assayed as described under "Experimental Procedures" except that nonradioactive malonyl-CoA was omitted to increase the labeling of acyl-ACP in the presence of cerulenin (100 μg/ml). The assay volumes were scaled up 5-fold, and the [1⁴C]acyl-ACP formed during the incubation was purified by DEAE-cellulose chromatography. The acyl groups were hydrolyzed from the protein and analyzed by reverse-phase HPLC as described under "Experimental Procedures." Panel A, standard assay conditions; Panel B, NADP and NADPH were not added to the assay in this experiment.
Acetoacetyl-ACP synthase is a unique enzyme that is ideally positioned in the biosynthetic pathway to function as the pacemaker of fatty acid biosynthesis. Control points are commonly found at the first step in a biochemical pathway, and the existence of comparable concentrations of acetyl-ACP and malonyl-ACP in vivo (Fig. 1) point to the condensation of these two species as the rate-limiting step in fatty acid biosynthesis. Acetoacetyl-ACP synthase specifically catalyzes the formation of four-carbon intermediates in vitro (Figs. 4 and 5), and in the presence of β-ketoacyl-ACP synthases I and II, the first step in the pathway is likely to be the only significant reaction catalyzed by this enzyme in vivo. However, in the absence of other condensing enzyme activities in cerulenin-treated cells, specificity is not absolute and acetyl-ACP synthase can elongate acyl-ACPs up to six carbons (Fig. 3). The perturbation of normal cell physiology also results in synthases I and II catalyzing reactions that normally do not occur in vivo. Abnormally long-chain fatty acids accumulate when acyl-ACP utilization is blocked at the glycerol phosphate acyltransferase step (34), and the overproduction of synthase I leads to the formation of cis-vaccenate in the absence of synthase II activity (37). Butyryl-ACP elongation is slightly reduced in extracts from synthase II (fabB) mutants but is severely depressed in extracts from synthase I (fabB) mutants (Fig. 4) implicating synthase I as the enzyme primarily responsible for the elongation of butyryl-ACP. Whether synthases I and II can catalyze acetoacetyl-ACP formation in vivo and thereby circumvent the requirement for acetoacetyl-ACP synthase activity awaits the isolation of mutants defective in this third condensing enzyme.

The β-ketoacyl-ACP synthases are emerging as the key regulators of fatty acid biosynthesis. Our data suggest acetoacetyl-ACP synthase is involved in determining the total amount of fatty acids produced by regulating chain initiation. Physiological experiments indicate that the production of phospholipid is governed by the supply of fatty acids (1, 3), suggesting that acetoacetyl-ACP synthase may control the rate of membrane lipid biosynthesis in E. coli. The two other synthases are responsible for modulating the product distribution of the pathway. β-Ketoacyl-ACP synthase I is responsible for the elongation of palmitoleic to cis-vaccenic acid and is responsible for the temperature-dependent regulation of fatty acid composition (for review see Ref. 16). Other enzymes may also be involved in regulation of the pathway, but there is too little known about these enzymes or their interactions with the β-ketoacyl-ACP synthases to speculate on their roles.

β-Ketoacyl-ACP synthases catalyze two reactions that may be relevant to generating acetyl-ACP for fatty acid biosynthesis. It is generally accepted that a distinct enzyme, acetyl-CoA:ACP transacylase, is responsible for the formation of acetyl-ACP (for review see Ref. 40). Numerous acyl-CoA chain lengths serve as substrates for the malonyl-CoA:ACP transacylase reactions catalyzed by β-ketoacyl-ACP synthases I and II, with the notable exception of acetyl-CoA (35, 41). By analogy, acetoacetyl-ACP synthase should promote acetyl-CoA:ACP transacylation, and it is possible that acetyltransacylase is not a distinct enzyme but a partial reaction of acetoacetyl-ACP synthase. In this case, there would be no difference between our proposal and the proposal of Shimakata and Stumpf (13) who conclude that acetyl-CoA:ACP transacylase is the rate-controlling factor. Acetyl-ACP is also generated by the malonyl-ACP decarboxylase activity of condensing enzymes accounting for the observation that a requirement for acetyl-ACP is not apparent in the in vitro fatty acid synthase system (35, 42). Cerulenin inhibits the malonyl-ACP decarboxylase of β-ketoacyl-ACP synthases I and II (32, 36); therefore, the cerulenin-resistant malonyl-ACP decarboxylase observed in our experiments (Fig. 5) is likely to be catalyzed by acetoacetyl-ACP synthase. Further work is needed to determine the importance of these two partial reactions of acetoacetyl-ACP synthase to the overall regulation of the fatty acid biosynthetic pathway.

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REFERENCES

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Supplemental Material to

**Acetoacetyl-ACP Synthase in E. coli**

**Materials**

- Source of ACP (strains B. subtilis and E. coli): New England Nuclear
- [1-14C]acetate (specific activity 11 Ci/m mole)
- [1-6H]glycine (specific activity 6 Ci/m mole)
- Separation media: Lye 2 (10250) and 3 (11250) mmol Hepes, pH 7.5
- Tris-HCl (pH 7.0), 0.025 M so as to give 1 M
- 0.05 M NaOH (pH 13)
- 0.05 M HCl (pH 2.0)
- 0.01 M EDTA, 5 M KCl
- [1-14C]acetyl-CoA, 100 nCi/mg, acetyl-CoA (7 M, 25 Ci/mole)
- Malonyl-CoA

**Methods**

- ACP was purified by chromatography on a glass column packed with DEAE-cellulose (200 ml) equilibrated with 0.1 M Tris-HCl, pH 7.0
- The column was eluted with a linear gradient of 0.01 M to 0.3 M Tris-HCl, pH 7.0
- The purity of the ACP was confirmed by analytical PAGE and SDS-PAGE

**Experimental Procedures**

**Preparation of [1-14C]Acetyl-ACP.**

- ACP was synthesized using [1-14C]acetate and acetyl-CoA in an in vitro system
- The reaction mixture contained [1-14C]acetate, acetyl-CoA, ATP, SAM, and protein extract
- The reaction mixture was incubated at 37°C for 60 min
- The product was isolated by chromatography on a DEAE-cellulose column

**Determination of Specific Activity.**

- The specific activity of the ACP was determined using a liquid scintillation counter
- The radioactivity was measured in a Beckman LS-5000 apparatus

**Results**

- The specific activity of the ACP was determined to be 1.04 Ci/mole
- The radioactivity was found to be linear with time of incubation}

**References**

- deMeo, A. W., Balch, W. E., and Vogel, P. R. (1972) J. Biol. Chem. 247, 3190–3198

**Proline biosynthesis in bacteria**

- The intracellular concentration of ACP was determined by monitoring the effluent at 210 nm
- The specific activity of the ACP was determined to be 1.04 Ci/mole
- The radioactivity was found to be linear with time of incubation

**References**

- deMeo, A. W., Balch, W. E., and Vogel, P. R. (1972) J. Biol. Chem. 247, 3190–3198