Regulation of Fatty Acid Elongation and Initiation by Acyl-Acyl Carrier Protein in Escherichia coli*

(Received for publication, November 13, 1995)

Richard J. Heath† and Charles O. Rock‡§

From the †Department of Biochemistry, St. Jude Children’s Research Hospital, Memphis, Tennessee 38101 and the ‡Department of Biochemistry, University of Tennessee, Memphis, Tennessee 38163

Long chain acyl-acyl carrier protein (acyl-ACP) has been implicated as a physiological inhibitor of fatty acid biosynthesis since acyl-ACP degradation by thioesterase overexpression leads to constitutive, unregulated fatty acid production. The biochemical targets for acyl-ACP inhibition were unknown, and this work identified two biosynthetic enzymes that were sensitive to acyl-ACP feedback inhibition. Palmitoyl-ACP inhibited the incorporation of [14C]malonyl-CoA into long chain fatty acids in cell-free extracts of Escherichia coli. A short chain acyl-ACP species with the electrophoretic properties of β-hydroxybutyryl-ACP accumulated concomitant with the overall decrease in the amount of [14C]malonyl-CoA incorporation, indicating that the first elongation cycle was targeted by acyl-ACP. All of the proteins required to catalyze the first round of fatty acid synthesis from acetyl-CoA plus malonyl-CoA in vitro were isolated, and the first fatty acid elongation cycle was reconstituted with these purified components. Analysis of the individual enzymes and the pattern of intermediate accumulation in the reconstituted system identified initiation of fatty acid synthesis by β-ketoacyl-ACP synthase III (fabB) and enoyl-ACP reductase (fabI) in the elongation cycle as two steps attenuated by long chain acyl-ACP.

Although the steps in the fatty acid biosynthetic pathway are well characterized, the mechanisms that regulate the production of fatty acids by the type II, dissociated fatty acid synthase systems typified by Escherichia coli are largely unknown (for review, see Ref. 1). Recent experiments implicate long chain acyl-ACPs as feedback inhibitors of the type II pathway. Blocking phospholipid synthesis at the first acyltransferase step by shifting either glycerol-phosphate acyltransferase (pslB) or glycerol-phosphate synthase (gapsA) mutants to the nonpermissive condition results in the concomitant inhibition of fatty acid synthesis (2) and the accumulation of long chain acyl-ACPs (2, 3). The idea that long chain acyl-ACPs are feedback regulators of fatty acid biosynthesis arose from experiments with strains engineered to overexpress thioesterases capable of degrading acyl-ACP (2, 4). Strains overexpressing either of the E. coli thioesterases (tesA or tesB) (2) or a truncated form of tesA that is not exported to the periplasm (4) fail to accumulate acyl-ACPs when phospholipid production is blocked at the glycerol-phosphate acyltransferase step and exhibit constitutive fatty acid synthesis in the absence of phospholipid synthesis. Bacteria ordinarily cease fatty acid and phospholipid synthesis in stationary phase, but when acyl-ACP-specific thioesterases cloned from plants are expressed in E. coli, such strains continue to synthesize and secrete copious amounts of fatty acids into the medium after growth ceases (5–7). The inhibition of phospholipid synthesis at the acyltransferase step following the induction of ppGpp synthesis leads to the accumulation of acyl-ACP and the concomitant cessation of fatty acid synthesis. Overexpression of the ppGpp target, pslB, restores phospholipid synthesis, eliminates the accumulation of acyl-ACP, and relieves the inhibition of fatty acid synthesis, suggesting that the ppGpp-dependent inhibition of fatty acid synthesis is a physiological response mediated by acyl-ACP (8). Taken together, these in vivo experiments provide compelling evidence that long chain acyl-ACPs are involved in a regulatory loop that controls the rate of fatty acid synthesis.

Criticality important to the development of this hypothesis is the identification of the enzymatic steps in fatty acid synthesis that are sensitive to acyl-ACP inhibition. The goal of this work was to identify candidate enzymes that are regulated by acyl-ACP.

EXPERIMENTAL PROCEDURES

Materials—Sources of supplies were: Moravek Biochemicals Inc., [2,14C]malonyl-CoA (specific activity, 57 mCi/mmol) and [1-14C]acetate-CoA (specific activity, 54 mCi/mmol); Sigma, fatty acids, ACP, cerulenin, and acyl-CoAs; Promega, molecular biology reagents; Novagen, pET vectors and expression strains; Qiagen, N2-agarose column; and Pharmacia Biotech Inc., acetyl-CoA and malonyl-CoA. Acyl-ACPs were synthesized using the acyl-ACP synthetase purified from an overproducing strain (9) as described previously (10). The acyl-ACPs were concentrated and the buffer exchanged by centrifugal filtration in a Centricon-3 concentrator (Amicon). Yields were judged by Bradford protein determination (11) and conformationally sensitive gel electrophoresis in a 2.5 mM urea, 13% acrylamide gel (12). All other chemicals were of reagent grade or better.

Assay of Fatty Acid Synthesis in Cell Extracts—The crude cell extract was prepared essentially as described previously (13, 14). Briefly, a 500-ml culture of E. coli K-12 strain UB1005 was grown to late log phase in rich medium at 37 °C, and the cells were harvested by centrifugation. Cells were resuspended in 5 ml of lysis buffer (0.1 M sodium phosphate, pH 7.0, 5 mM β-mercaptoethanol, 1 mM EDTA) and disrupted by passage through a French pressure cell at 12,000 p.s.i. Cell debris was removed by centrifugation at 20,000 rpm in a J-A-21 rotor at 4 °C for 1 h, and protein in the supernatant was fractionated by precipitation with ammonium sulfate. The 45–80% ammonium sulfate pellet was resuspended in 2 ml of lysis buffer and dialyzed at 4 °C against two changes of lysis buffer.

The fatty acid synthase assay contained 100 μM ACP, 1 mM β-mercaptoethanol, 25 μM malonyl-CoA (specific activity, 57 mCi/mmol), 100 μM acetyl-CoA, 0.1 mM sodium phosphate buffer, pH 7.0, 0.1 mM L-UCI, 1 mM NADH, and 1 mM NADPH in a final volume of 40 μl. A mixture of ACP, β-mercaptoethanol, and buffer was preincubated at 37 °C for 30 min prior to the assay to ensure complete reduction of the ACP, and then the remaining components (except protein) were added. The mixture was then aliquoted into the assay tubes and the reaction initiated by the addition of 45 μg of cell extract protein. The reaction mixture was
Inhibition of Malonyl-CoA Incorporation into Fatty Acids—The first approach to investigate the regulation of fatty acid elongation by acyl-ACP was to determine if palmitoyl-ACP affected the incorporation of \(^{14}C\)malonyl-CoA into fatty acids in a cell-free system that contained all of the enzymes necessary for fatty acid formation. Palmitoyl-ACP was an effective inhibitor of \(^{14}C\)malonyl-CoA incorporation into long chain fatty acids in the cell-free system (Fig. 1A). Since the saponification/hexane extraction method used to measure \(^{13}C\)malonyl-CoA incorporation in Fig. 1A did not provide information on whether the samples treated with palmitoyl-ACP contained short chain acyl moieties that are not efficiently extracted into hexane, the samples were also subjected to conformationally sensitive gel electrophoresis. This electrophoretic analysis showed that increasing palmitoyl-ACP concentration caused a decrease in the amount of label associated with acyl-ACP having chain lengths greater than 8 carbons and the accumulation of a new short chain acyl-ACP species (Fig. 1B). This short chain acyl-ACP was identified as \(\beta\)-hydroxybutyryl-ACP based on its comigration with a \(\beta\)-hydroxybutyryl-ACP standard (15). These data demonstrate that fatty acid elongation is inhibited by acyl-ACP in vitro and point to the first cycle of the pathway as a relevant target.

Inhibition of Individual Enzymes in Fatty Acid Elongation by Acyl-ACP—To determine which enzyme(s) in the initiation and elongation of fatty acid biosynthesis were targets for acyl-ACP inhibition, we purified to homogeneity the five His-tagged enzymes required to complete the first cycle of fatty acid elongation (15). These enzymes were malonyl-CoA:ACP transacylase, encoded by the fabD gene; \(\beta\)-ketoacyl-ACP (acetocacetyl-ACP) synthase III (fabH); the NADPH-dependent \(\beta\)-ketoacyl-ACP reductase (fabG); the \(\beta\)-hydroxyacyl-ACP dehydrase (fabA); and the NADH-dependent enoyl-ACP reductase (fabB). The enzymes required to complete one cycle of fatty acid elongation were combined sequentially in the presence and absence of palmitoyl-ACP to determine which step in the cycle was inhibited by acyl-ACP (Fig. 2). Palmitoyl-ACP did not inhibit the formation of malonyl-ACP. The first condensation step in the pathway was inhibited by palmitoyl-ACP as evidenced from the appearance of malonyl-ACP in every incubation containing palmitoyl-ACP and FabH. The recovery of \(\beta\)-ketoacyl-ACP following conformationally sensitive gel electrophoresis was poor due to the instability of acetocacetyl-ACP to the high pH and urea concentrations used to perform the separations, as noted previously (15). Therefore, we also used a specific coupled filter disc assay for FabH, which measured the incorporation of \(^{14}C\)acetoacetyl-ACP into acetocacetyl-ACP in the presence of FabH, FabD, ACP, and malonyl-CoA (13, 14). These experiments verified that acyl-ACP inhibited the FabH reaction (data not shown). The addition of FabG resulted in the efficient conversion of acetocacetyl-ACP to \(\beta\)-hydroxybutryl-ACP, and the extent of conversion was not significantly altered by the presence of palmitoyl-ACP. The slight reduction in the overall yield of \(\beta\)-hydroxybutryl-ACP was attributed to the inhibition of FabH rather than FabG. The equilibrium for the FabA reaction lies in favor of the \(\beta\)-hydroxybutryl-ACP by a ratio of 10:1 (15), which...
toward the elongation (15). Since the equilibrium for the FabA reaction lies in the cell and is required for each cycle of fatty acid elongation (15). The direction and thickness of the arrows denote the equilibrium position of each of the individual reactions and illustrate that FabI pulls the cycle to completion (15). Butyryl-ACP is converted to long chain acyl-ACP through several additional elongation cycles. Acyl-ACPs regulate fatty acid elongation by feedback inhibition of the elongation step at FabI and at the initial condensation of acetyl-CoA and malonyl-ACP by FabH.

cells (13, 14). Thus, FabH is ideally positioned in the biosynthetic pathway to regulate initiation and hence the total number of fatty acids synthesized. A tenable hypothesis for the regulation by acyl-ACP, twopotential regulatory points related to the production and degradation of malonyl-CoA could not be tested in our reconstituted system. Malonyl-CoA does not accumulate when acyl-ACP levels rise in response to the cessation of phospholipid synthesis in \( \text{pl} \text{S} \text{B} \) mutants (16). This observation suggests at first glance that acetyl-CoA carboxylase is, in fact, operational in the presence of acyl-ACP. A futile cycle consisting of the transfer of malonyl moieties from CoA to ACP followed by acyl-ACP-dependent malonyl-ACP de-carboxylation catalyzed by \( \beta \)-ketoacyl-ACP synthases I and II and transacylation of the acetyl-ACP to acetyl-CoA by FabH governs the fate of malonyl-CoA and may contribute to the down-regulation of fatty acid synthesis by recycling malonyl-ACP to the acetyl-CoA pool, thus down-regulating fatty acid elongation and initiation (16). Determining the biochemical mechanisms for the regulation of these key enzymes by acyl-ACP and understanding the relative contributions of each control point to the physiological regulation of the pathway are important goals that will be the focus of future research.

Fig. 4. Two sites for the regulation of fatty acid biosynthesis.

The first cycle of fatty acid synthesis is initiated by \( \beta \)-ketoacyl-ACP synthase III (FabH), which condenses acetyl-CoA with malonyl-ACP to form acetocacetyl-ACP. This intermediate is reduced by \( \beta \)-ketoacyl-ACP reductase (FabG), and the \( \beta \)-hydroxybutyryl-ACP is dehydrated to crotonyl-ACP by \( \beta \)-hydroxyacyl-CoA dehydrase (FabA). Butyryl-ACP is formed by the reduction of crotonyl-ACP by enoyl-ACP reductase (FabI). The direction and thickness of the arrows denote the equilibrium position of each of the individual reactions and illustrate that FabI pulls the cycle to completion (15). Butyryl-ACP is converted to long chain acyl-ACP through several additional elongation cycles. Acyl-ACPs regulate fatty acid elongation by feedback inhibition of the elongation step at FabI and at the initial condensation of acetyl-CoA and malonyl-ACP by FabH.

While our present observations illustrate that the initiation and elongation steps in fatty acid biosynthesis are subject to regulation by acyl-ACP, two potential regulatory points related to the production and degradation of malonyl-CoA could not be tested in our reconstituted system. Malonyl-CoA does not accumulate when acyl-ACP levels rise in response to the cessation of phospholipid synthesis in \( \text{pl} \text{S} \text{B} \) mutants (16). This observation suggests at first glance that acetyl-CoA carboxylase is, in fact, operational in the presence of acyl-ACP. A futile cycle consisting of the transfer of malonyl moieties from CoA to ACP followed by acyl-ACP-dependent malonyl-ACP de-carboxylation catalyzed by \( \beta \)-ketoacyl-ACP synthases I and II and transacylation of the acetyl-ACP to acetyl-CoA by FabH governs the fate of malonyl-CoA and may contribute to the down-regulation of fatty acid synthesis by recycling malonyl-ACP to the acetyl-CoA pool, thus down-regulating fatty acid elongation and initiation (16). Determining the biochemical mechanisms for the regulation of these key enzymes by acyl-ACP and understanding the relative contributions of each control point to the physiological regulation of the pathway are important goals that will be the focus of future research.

Fig. 3. Dose response for the inhibition of fatty acid synthesis by palmitoyl-ACP. The purified enzymes were incubated in the presence of increasing concentrations of palmitoyl-ACP, and the radiolabeled products were separated by conformationally sensitive gel electrophoresis as described under "Experimental Procedures."
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*J. Biol. Chem.* 1996, 271:1833-1836.  
doi: 10.1074/jbc.271.4.1833

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