Communication

A Cerulenin Insensitive Short Chain 3-Ketoacyl-Acyl Carrier Protein Synthase in *Spinacia oleracea* Leaves

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ABSTRACT

A cerulenin insensitive 3-ketoacyl-acyl carrier protein synthase has been assayed in extracts of spinach (*Spinacia oleracea*) leaf. The enzyme was active in the 40 to 80% ammonium sulfate precipitate of whole leaf homogenates and catalyzed the synthesis of acetoacetyl-acyl carrier protein. This condensation reaction was five-fold faster than acetyl-CoA:acyl carrier protein transacylase, and the initial rates of acyl-acyl carrier protein synthesis were independent of the presence of cerulenin. In the presence of fatty acid synthase cofactors and 100 micromolar cerulenin, the principal fatty acid product of *de novo* synthesis was butyric and hexanoic acids. Using conformationally sensitive native polyacrylamide gel electrophoresis for separation, malonyl-, acetyl-, butyryl-, hexanoyl-, and long chain acyl-acyl carrier proteins could be detected by immunoblotting and autoradiography. In the presence of 100 micromolar cerulenin, the accumulation of butyryl- and hexanoyl-acyl carrier protein was observed, with no detectable long chain acyl-acyl carrier proteins or fatty acids being produced. In the absence of cerulenin, the long chain acyl-acyl carrier proteins also accumulated.

It has been known for decades that fatty acid biosynthesis involves extending an acyl-ACP[1] stepwise by two carbons. In plants and bacteria, this synthesis is catalyzed by a type II fatty acid synthase, a readily dissociable group of enzymes which can be individually isolated and studied (1). One of the initial reactions leading to chain elongation is catalyzed by the enzyme 3-ketoacyl-ACP synthase, frequently referred to simply as the 'condensing enzyme.' The spinach fatty acid synthase was initially separated into its individual components by Shimakata and Stumpf (13, 14). In that study, they found two 3-ketoacyl-ACP synthases, designated I and II. 3-Ketoacyl-ACP synthase I was responsible for the majority of the condensations, using acyl-ACPs ranging from 2:0-ACP to 14:0-ACP. 3-Ketoacyl-ACP synthase II had its primary function in stearic acid synthesis, and thus was most active with 14:0-ACP and 16:0-ACP. A diagnostic difference between the two synthases was their sensitivity to the antibiotic cerulenin. 3-Ketoacyl-ACP synthase I was very sensitive and was inhibited 100% by as little as 10 μM cerulenin. In contrast, 3-ketoacyl-ACP synthase II was inhibited only 50% by 50 μM cerulenin. Thus, the characteristic sensitivity of fatty acid

biosynthesis to cerulenin can be attributed entirely to the inhibition of 3-ketoacyl-ACP synthase I.

Similarly, the 3-ketoacyl-ACP synthases found in *Escherichia coli*, are characteristically cerulenin sensitive (5). Recently, a third condensing enzyme has been reported in *E. coli*. which has been named acetoacetyl-ACP synthase (6). This 3-ketoacyl-ACP synthase is distinctly different from the other 3-ketoacyl-ACP synthases in several important respects. It is cerulenin insensitive, is specific for very short chain acyl-ACPs, and it prefers acetyl-CoA over acetyl-ACP (7). As a consequence of this last property, it would appear that this enzyme can bypass the much slower acetyl transacylase step and as a result, the acetyl transacylase would not be rate limiting in fatty acid biosynthesis.

In light of these findings in the *E. coli* system, we have reexamined the spinach system and report here the presence of a third 3-ketoacyl-ACP synthase which is completely insensitive to cerulenin, active only with short chain acyl thioesters, and appears to prefer acetyl-CoA to acetyl-ACP.

MATERIALS AND METHODS

Materials

Spinach (*Spinacia oleracea*) was purchased locally. Spinach ACP was the generous gift of J. Ohlrogge, Michigan State University. Malonyl-CoA was prepared by the method of Rutkoski and Jaworski (12). [1-^{14}C]AcetylCoA was prepared from [^{14}C]sodium acetate by preparing an acetyl-imidazole intermediate (3). Acyl-ACPs were prepared by the method of Cronan and Klages (4). All other chemicals were reagent grade or best available.

Enzyme Preparation

A crude spinach leaf extract was prepared by the method of Shimakata and Stumpf (14). The 40 to 80% ammonium sulfate precipitate was dissolved and dialyzed against 50 mM potassium phosphate (pH 8.0), 20% (v/v) glycerol, and 2 mM dithiothreitol. The protein concentration of this crude spinach leaf extract was 19 mg/mL as determined by the method of Bradford (2).

Enzyme Assays

The *in vitro* synthesis of acyl-ACPs was carried out under assay conditions for acetyl-CoA:ACP transacylase and fatty

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[1] Abbreviation: ACP, acyl carrier protein.

acid synthase. For acetyl-CoA:ACP transacylase, the reaction mixture contained 100 mM Tris (pH 8.0), 0.5 mM 2-mercaptoethanol, 60 μg of spinach ACP, 10 μM [1-^{14}C]acetyl-CoA (56 mCi/mmol), and 15 μL of crude spinach leaf extract (0.29 mg protein) in a total volume of 150 μL. Malonyl-CoA:ACP transacylase was assayed the same as the acetyl transacylase, but 100 μM [2-^{14}C]malonyl-CoA (10 mCi/mmol) replaced the acetyl-CoA. For fatty acid synthase assays, the reaction mixture contained in addition to the components of the acetyl transacylase reaction, 100 μM malonyl-CoA, 1 mM NADH, 2 mM NADPH, and 100 μM cerulenin when indicated. Cerulenin was maintained as a 1.0 mM stock solution (pH 4.0) at 4°C. Reactions were stopped by transferring an aliquot of the reaction mix at the designated interval to an equal volume of 10% (w/v) TCA, mixing, and storing on ice for 5 to 15 min. All samples were diluted with 1 mL of 5% TCA, centrifuged, and the supernatant discarded. Pellets were washed with an additional 1 mL of 5% TCA and then redissolved in 25 μL of 50 mM Mes, approximate pH of 6.3. An aliquot of this solution was analyzed by liquid scintillation spectrometry or used immediately for subsequent analysis.

Electrophoretic Separation and Analysis

Native PAGE (11), with 13% acrylamide and 1 mm thickness were run at 15 mA and at 15°C. Samples in 50 mM Mes were diluted with an equal volume of 20% (v/v) glycerol, 0.002% (w/v) bromophenol blue, and loaded onto gels directly. Proteins were transferred to nitrocellulose (8). For autoradiography, nitrocellulose was dried, and exposed to film for 48 h.

Immunoblots were carried out after proteins were transferred to nitrocellulose. Nitrocellulose was blocked with 5% (w/v) nonfat dry milk (Carnation Co., Los Angeles, CA) in TBST (10 mM Tris [pH 8.0], 150 mM NaCl, 0.05% [v/v] Tween 20) for 1 h. Spinach ACP I antibody was added to the blocking solution and incubated an additional 1 h. The antibody solution was poured off and the nitrocellulose was washed three times with TBST for 10 min each. Immunostaining was carried out using the anti-rabbit IgG alkaline phosphatase conjugate.

HPLC

Acyl groups esterified to the ACP in the electrophoresis samples were analyzed on HPLC following mild alkaline hydrolysis. These samples were hydrolyzed by adjusting to approximately pH 9.5 and treating with 100 mM 2-mercaptoethanol for 1 h at 37°C (9). Samples were directly injected into an HPLC system equipped with ODS column, eluted by the solvent system indicated in Table I and the radioactive fractions collected. Retention time of each fatty acid was compared to the available standard.

RESULTS AND DISCUSSION

Initial Velocity Measurements

In *E. coli*, the acetoacetyl-ACP synthase was distinguished from the 3-ketoacyl-ACP synthases by its insensitivity to

cerulenin (6). In addition, it preferred acetyl-CoA as a substrate and could be distinguished from acetyl transacylase by a 5- to 10-fold higher activity (7). Since Shimakata and Stumpf (15) had demonstrated that the acetyl transacylase was the slowest reaction step in fatty acid biosynthesis, we reasoned that if the acetyl transacylase step was bypassed by a more active 3-ketoacyl-ACP synthase, the overall rate of fatty acid biosynthesis should exceed the rate limiting acetyl transacylase step. The reactions used [^{14}C]acetyl-CoA as the only labeled substrate, in the absence of ATP, to ensure that any fatty acids synthesized would be labeled only once and thus have the same specific activity as the product of the acetyl transacylase reaction. Furthermore, the commonly employed methods for assaying fatty acid synthase were not used, in that these methods are dependent on measuring longer chain (10-18 carbons) fatty acids only. Since we were interested in measuring true initial velocities, we expected that a significant amount of the fatty acid synthase products would still be shorter chain fatty acids, that they would still be esterified to ACP, and they could be measured by acid precipitation. Acid precipitation was also the method used for the measurement of the acetyl transacylase, and thus would allow a direct comparison of the rates of the two reactions. The assay conditions for acetyl transacylase and fatty acid synthase differed only by supplementing the transacylase assay mixture with NADH, NADPH, and malonyl-CoA to allow for the 3-ketoacyl-ACP synthase and reductase reactions to occur. As illustrated in Figure 1, the initial velocity of the fatty acid synthase was 5-fold higher than the acetyl transacylase (fatty acid synthase: 11.7 pmol/min/mg protein *versus* acetyl transacylase 2.2 pmol/min/mg protein). These initial velocities were calculated from the linear portion of the progress curves, *i.e* 1 to 4 min. The initial velocity data indicate that since the fatty acid synthase was much faster than the acetyl transacylase, the transacylase reaction was not rate-limiting and was bypassed by fatty acid synthase. Furthermore, it would indicate that acetyl-CoA rather than acetyl-ACP was the primer in these reactions.

To further examine if acetyl-CoA was preferred over acetyl-ACP as the primer for fatty acid synthesis, incubations containing 10 μM [^{14}C]acetyl-CoA were supplemented with 10 and 20 μM unlabeled acetyl-ACP. If there was equal or greater preference for acetyl-ACP over acetyl-CoA, the incorporation of label should have been decreased by 50% or more with equimolar acetyl-ACP. However, the incorporation of acetyl-CoA into fatty acids was diminished by only 25%, even with a twofold excess of acetyl-ACP. This decrease may even be attributed to dilution of label in the acetyl-CoA by the acetyl-ACP as a result of the reversible acetyl transacylase activity. Thus, this data was consistent with a strong preference for acetyl-CoA as the primer for fatty acid synthesis.

The effect of cerulenin on the rate of fatty acid synthase under these assay conditions was also determined (Fig. 1). The initial rate of fatty acid synthase was essentially unchanged by the addition of 100 μM cerulenin. This concentration was 10-fold higher than the concentration needed to completely inhibit 3-ketoacyl-ACP synthase I (14), and indicated that a cerulenin insensitive 3-ketoacyl-ACP synthase was functioning in these reactions. Enzyme extracts were

Table I. *HPLC Data that Confirm the Identity of the Acyl Groups Attached to the ACPs*

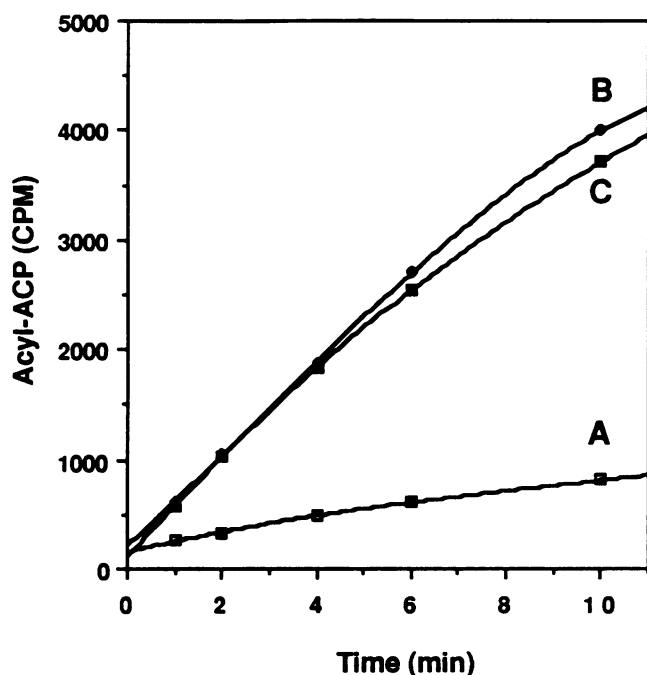| Acyl Group	Retention Time	Elution Solvent
	min	
Acetate	5.3	5 mM triethylamine (pH 5.0) adjusted with H_3PO_4
Malonate	6.1	5 mM triethylamine (pH 5.0) adjusted with H_3PO_4
Acetoacetate	6.5	5 mM triethylamine (pH 5.0) adjusted with H_3PO_4
Butyrate	6.3	50% MeOH in 10 mM H_3PO_4
Hexanoate	17.1	50% MeOH in 10 mM H_3PO_4

Figure 1. *In vitro* synthesis of acyl-ACP was measured under conditions for acetyl-CoA:ACP transacylase (curve A), fatty acid synthase activity (curve B), and fatty acid synthase activity with 100 μM cerulenin (curve C) as described in "Materials and Methods." Reactions were stopped by transferring 25 μL of the reaction mix at the designated interval to an equal volume of 10% (w/v) trichloroacetic acid (TCA), mixing, and storing on ice until the end of the experiment. Samples were prepared for liquid scintillation spectrometry as described in "Materials and Methods."

Figure 2. Autoradiogram of a native 13% PAGE of [¹⁴C]acyl-ACPs produced *in vitro* from spinach under the indicated reaction conditions. Enzyme assays were carried out and acyl-ACPs were prepared as described in "Materials and Methods." Each reaction was run for 2, 5, or 15 min, as indicated at the top of each lane. A, Acetyl-CoA:ACP transacylase—[¹⁴C] precursor was [1-¹⁴C]acetyl-CoA; B, fatty acid synthase with 100 μM cerulenin, but omitting both NADPH and NADH; C, fatty acid synthase with 100 μM cerulenin; D, fatty acid synthase without cerulenin present; E, malonyl-CoA:ACP transacylase (same as acetyl-CoA:ACP transacylase, but with [2-¹⁴C]malonyl-CoA as the substrate. Native gel was run at 15 mA and 15°C. Proteins were transferred to nitrocellulose, dried, and exposed to film for 48 h.

treated with cerulenin in several ways to avoid any artifacts due to stability or solubility of the antibiotic. Typically, a 1 mM stock at pH 4 stored 4°C was used (10). Freshly prepared solutions were also used as well as a freshly prepared ethanol solution (6), with no inhibition observed. Preincubation of the enzyme with cerulenin for as long as 30 min had no effect, as was the case with using lower cerulenin concentrations (10 and 50 μM).

Acrylamide Gel Analysis of Products

As the kinetic data were based on measuring acid precipitable radiolabeled products, it was necessary to confirm that these products were acyl-ACPs as well as to determine the identity of the acyl groups in the fatty acid synthase products. The native acrylamide gel system employed by Rock and Cronan (11) was used to successfully separate the acyl-ACPs
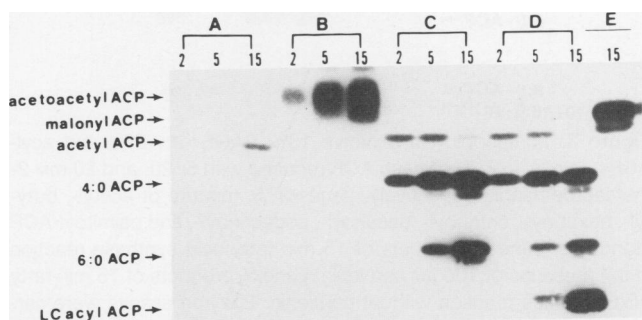
(Fig. 2). The acid precipitable product of the acetyl transacylase assay was exclusively [¹⁴C]acetyl-ACP, present in the expected low quantity. When the assay mixture was supplemented with only malonyl-CoA, a single product, acetoacetyl-ACP, was expected. There was only one radioactive band, tentatively identified as acetoacetyl-ACP, detected in these incubation products, migrating slightly behind the malonyl-ACP (compare lanes B and E, Fig. 2). These bands always lacked sharpness and we attribute this to instability of acetoacetate at pH 9.0. Supplementing this assay with NADH and NADPH resulted in production of saturated acyl-ACPs. When cerulenin was present, the major products were butyryl- and hexanoyl-ACP, with the precursor-product relationship apparent between them. In the absence of cerulenin, longer chain acyl-ACPs were also produced, as expected. Thus, these results were consistent with the presence of a cerulenin insensitive short chain 3-ketoacyl-ACP synthase, analogous to the acetoacetyl-ACP synthase found in *E. coli*.

The identities of the radiolabeled proteins as acyl-ACPs were determined by immunoblotting with spinach ACP antibody (Fig. 3) and HPLC analysis of the acyl groups (Table I). The products of 15 min reactions (Fig. 3, lanes 4 and 5) were compared with standards and the bands observed on the autoradiogram. The radiolabeled bands corresponding to malonyl-, butyryl-, hexanoyl-, and long chain acyl-ACPs were
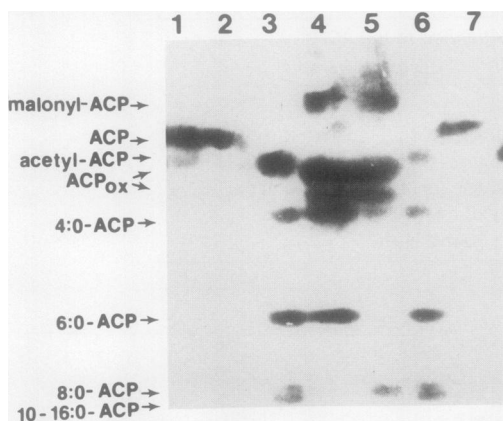
Figure 3. Immunoblot of a native 13% PAGE of ACPs and acyl-ACPs. Lanes 1, 2, 7, spinach ACP reduced with 5, 20, and 20 mM 2-mercaptoethanol, respectively. Lanes 3, 6, mixture of acetyl-, butyryl-, hexanoyl-, octanoyl-, decanoyl-, dodecanoyl-, and palmitoyl-ACP standards. Lane 4, products of 15 min fatty acid synthase reaction in the presence of 100 μM cerulenin. Lane 5, products of 15 min fatty acid synthase reaction without cerulenin. Enzyme assays were carried out and acyl-ACPs were prepared as described in "Materials and Methods." Native gel was run at 15 mA and 15°C. Proteins were transferred to nitrocellulose, dried, and immunoblotted with an antibody to spinach ACP I.

immunoreactive with the ACP antibody. Several unlabeled immunoreactive bands were detected, including two uncharacterized forms of ACP. These two bands, which migrated just ahead of reduced ACP, disappeared upon reduction of the samples with 2-mercaptoethanol, and thus appeared to be oxidized forms of ACP. Because these bands were present in our incubation products, they obscured any acetyl-ACP band that might have been present. Acetyl-ACP (upper band in lanes 3 and 6) migrated at almost the same rate as one of these ACP bands. Since 2-mercaptoethanol catalyzed the rapid hydrolysis of the acyl-ACPs (data not shown), we could not add 2-mercaptoethanol to our samples (lanes 4 and 5) to examine for the presence of acetyl-ACP. Note in Figure 2, lanes C and D, that there was a minor radiolabeled protein, migrating between malonyl- and acetyl-ACP, which was consistently observed when NADH and NADPH was present during the incubation. This band is about where we expected to see reduced ACP (Fig. 3, lanes 1, 2, and 7), but there was no corresponding band visible in our samples on the immunoblots (Fig. 3, lanes 4 and 5). Thus, the labeled band observed on the autoradiogram presumably was not an acyl-ACP and was not further characterized. Finally, acetoacetyl-ACP could not be clearly identified on the immunoblots. The incubations which produced [^{14}C]acetoacetyl-ACP also contained high levels of unlabeled malonyl-ACP, which obscured the diffuse acetoacetyl-ACP band.

In addition to comparison with standard acyl-ACPs on PAGE, the identification of the acyl group esterified to ACP was also determined by HPLC. Each of the acyl-ACP samples was hydrolyzed, and the free acids analyzed directly by HPLC. Because of the wide range of hydrophobicity of the acyl groups, several different isocratic solvent systems (see Table

I) were used to analyze free acids. Samples containing only one or two radioactive acyl-ACPs were used in this analysis to eliminate ambiguity in the identification of the acyl-ACP. For each acyl-ACP, the retention of the corresponding free acid was compared with an authentic standard.

In summary, we have reported here that a third 3-ketoacyl-ACP synthase was identified in spinach. It had the distinctive characteristics of being cerulenin insensitive, specific for the synthesis of short chain fatty acids, *i.e.* C4 and C6, and apparently could use acetyl-CoA as a substrate. This enzyme appeared to be completely analogous to the *E. coli* acetoacetyl-ACP synthase reported by Jackowski and Rock (6) with an important distinction that *in vitro* products included C6 fatty acids. Thus, it would more appropriately be referred to as a short chain 3-ketoacyl-ACP synthase rather than a acetoacetyl-ACP synthase.

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