Preparative Enzymatic Synthesis and Hydrophobic Chromatography of Acyl-Acyl Carrier Protein

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We have used purified preparations of acyl-acyl carrier protein synthetase to prepare pure, native acyl-acyl carrier proteins (acyl-ACP) ranging in chain lengths from C16 to C18:1. Factors affecting yield are explored and reaction conditions are presented that yield 0.8 to 0.9 mg of C16:0-ACP/ml of reaction mix. Other acyl groups, such as C18:2 and C18:1, are poorer substrates and gave correspondingly lower yields. ACP and acyl-ACP are separated by hydrophobic chromatography on octyl-Sepharose CL-4B. Mixtures of acyl-ACPs could be resolved according to acyl chain length using octyl-Sepharose CL-4B columns eluted with a 2-propanol gradient. The high resolution obtained using 2-propanol gradients to separate acyl-ACP species suggests that similar techniques would be applicable to the chromatography of protein mixtures on hydrophobic supports.

In order to circumvent these difficulties, long chain acyl-ACP has been enzymatically synthesized using plant cell extracts (2, 7). However, only saturated acyl moieties are produced and the synthesis results in a mixture of C16:0 and C18:0 ACPS (2). Recently, a new ligase activity has been found in E. coli extracts that synthesizes acyl-ACP from free fatty acid, ACP, and ATP (12). Acyl-ACP synthetase is an inner membrane enzyme (11) that has been solubilized and extensively purified (13). Acyl-ACP synthetase is capable of ligating a variety of fatty acids to ACP (12) and inner membrane preparations of this enzyme have been used to prepare several acyl-ACPs for use as substrates for E. coli thioesterases (11). However, this procedure gives a relatively low yield of acyl-ACP/ml and results in acyl-ACP preparations contaminated by Triton X-100 and a variable amount of ACP. In this manuscript we describe a general method for synthesizing milligram quantities of native acyl-ACPs using purified acyl-ACP synthetase. Methods for preparing homogeneous acyl-ACP and for fractioning acyl-ACPs according to chain length are also presented.

**EXPERIMENTAL PROCEDURES**

**Materials**—New England Nuclear provided [1-14C]C16:0 (specific activity, 55 Ci/mmol), [1-14C]C18:0 (specific activity, 55 Ci/mmol), [1-14C]C18:1 (specific activity, 55 Ci/mmol). [1-14C]C18:2 (specific activity, 28 Ci/mmol) was purchased from Amersham and [1-14C]C16:1 (specific activity, 55 Ci/mmol) was obtained from Applied Science. ATP, dithiotreitol, and octyl-agarose were purchased from P-L Biochemicals. Blue-Sepharose CL-6B and octyl-Sepharose CL-4B were from Pharmacia. The DGase-cellulose (DG59) was purchased from Whistam. All other materials were reagent grade or better.

**Protein Preparations**—Acyl-ACP synthetase was purified as described previously through the hydroxylapatite step (13). The number of enzyme units (nanomoles/min) were determined using incubation conditions exactly as described (13) and the synthesis of [1-14C]acyl-ACP was followed using the filter disc assay (11, 13). A preparation of acyl-ACP synthetase from a 250-g batch of frozen E. coli B cells (Grain-Processing) will yield a sufficient number of enzyme units (13) to prepare micromole quantities of acyl-ACP. ACP was purified, essentially as described previously (14). The protein was homogeneous as judged by electrophoresis through 15% polyacrylamide gels containing 3.5% Tris-Cl (pH 8.0), 0.1% sodium dodecyl sulfate, and 8 M urea using the discontinuous buffer system of Laemmli (15). Protein concentration was measured by determining the A280 - A260 (16), using a standard curve prepared with bovine serum albumin. Protein concentrations determined in this manner were in good agreement with the amino acid analysis.

**General Chromatographic Procedures**—Because solutions of 2-propanol are less dense, more volatile, and have stronger solvent properties than the solutions normally encountered in protein chromatography, strict precautions were followed to ensure reproducible results. All material was checked for resistance to 2-propanol. Common laboratory tubing, such as Tygon or polyethylene, are unacceptable. Most columns used in this study were fabricated from glass tubing, silicone stoppers, and Teflon tubing (1.2 mm in diameter). The top fittings for the columns contained two lengths of tubing; one running to the solvent reservoir and the other to a 3-ml syringe to
regulate the solvent volume above the gel and to assist in initiating a gradient. Linear gradients were formed using two aspirator bottles connected by Teflon tubing held in place by silicone stoppers. The tubing was looped once below the base of the bottle to prevent stratified flow and the bevels cut in the tubing were adjusted to face up. Our laboratory magnetic stirrers generated heat and the gradient-forming apparatus was elevated to avoid heating the mixing chamber and volatilizing the 2-propanol.

All 2-propanol solutions are given in weight per cent solution. Equal weights of 2-propanol solutions were placed in the two chambers of the gradient forming apparatus. All 2-propanol solutions, and other buffers, were exhaustively degassed immediately prior to their application to the column. 2-Propanol concentrations were determined using a refractive index measurement (sodium D line) and a standard curve. The refractive index is a linear function of 2-propanol concentration up to 30 weight % (1.3533 to 1.3574). Specific chromatographic conditions are given in the figure legends. All chromatographic runs, except blue-Sepharose, were carried out at room temperature (23-25°C). Since hydrophobic interactions are temperature-dependent (17, 18), octyl-Sepharose chromatography at temperatures different from those used in this study would be expected to have different 2-propanol elution requirements.

RESULTS AND DISCUSSION

Enzymatic Synthesis of Acyl-ACP—Optimum conditions for the synthesis of acyl-ACP were determined using [1-14C]C16:0 as the model substrate. Incubations contained Tris-HCl (0.1 M, pH 8.0), ATP (5 mM), MgCl2 (10 mM), dithiothreitol (2 mM), LiCl (0.4 M), fatty acid (160 μM), Triton X-100 (2%), ACP, and acyl-ACP synthetase. The last two components of the synthesis mixture may be varied to maximize either the yield of acyl-ACP (based on ACP) or the total amount of acyl-ACP synthesized. If complete acylation of ACP is desired, the concentration of ACP in the reaction mixtures should be kept below 15 μM (Fig. 1). At these ACP concentrations, acylation of ACP with [1-14C]C16:0 was nearly quantitative when incubations were carried out for 1 h in the presence of 5 units/ml of acyl-ACP synthetase. As the ACP concentration in the reaction mixture increased, the per cent conversion of ACP to acyl-ACP decreases, but the absolute amount of acyl-ACP synthesized/ml/h increases (Fig. 1). At ACP concentrations higher than 50 μM the total amount of acyl-ACP produced/ml was found to decrease under these experimental conditions.

Although reactions carried out as described above are satisfactory for small scale acyl-ACP synthesis, milligram quantities of acyl-ACP are needed for use as substrates for other lipoprotein biosynthetic enzymes. Since ACP and acyl-ACP can be resolved easily (see below), reaction conditions were sought that would maximize the yield of acyl-ACP/ml. Although we found 155 μM ACP (1.4 mg/ml) to be kinetically inhibitory in short term incubations, extension of the reaction time to 16 h results in a substantial yield of C16:0 - ACP (Table I). When reactions are performed in this manner the final yield of acyl-ACP was insensitive to the acyl-ACP synthetase concentration in the mixture (Table I). At the early time points, a concentration of 5 units/ml of acyl-ACP synthetase synthesized nearly 3 times as much acyl-ACP as 1.25 units/ml of acyl-ACP synthetase, but by the end of 16 h, incubations performed at both concentrations of enzyme gave an identical yield of 86 nmol/ml of acyl-ACP (Table I). Extension of the reaction time past 16 h did not result in a further increase in the acyl-ACP yield. It should be emphasized that pure acyl-ACP synthetase preparations (13) must be used in these experiments. Less pure preparations of acyl-ACP synthetase contain a contaminant that causes the absolute yield of acyl-ACP to decline after 3 to 6 h of incubation (11) and are, therefore, unsuitable for the preparative synthesis of acyl-ACP. Using acyl-ACP synthetase, purified as described previously (13), we have not observed degradation of acyl-ACP even at 16-h incubation times.

Separation of ACP Species from Acyl-ACP Synthetase—Acyl-ACP synthetase may be recovered from the reaction mixture for use in further reactions by binding the enzyme to blue-Sepharose. Even if it is not intended to recycle the acyl-ACP synthetase, this step is recommended because all proteins in the acyl-ACP synthetase preparation bind tenaciously to blue-Sepharose (13). The reaction mixture at 0-4°C is applied without dilution to a 3-ml column of blue-Sepharose equilibrated with 50 mM Tris-HCl, pH 8.0, containing 2% Triton X-100. Acyl-ACP and ACP do not bind to blue-Sepharose under these conditions. The gel is washed with 3 column volumes of 50 mM Tris-HCl, pH 8.0, containing 2% Triton X-100. This wash is combined with the previous flow-through. Acyl-ACP synthetase can be eluted from the blue-Sepharose column with 50 mM Tris-HCl, pH 8.0, containing 2% Triton X-100 and 0.5 mM KSCN (13). The recovery of enzyme activity was approximately 60 to 85%, and this enzyme preparation can be used in further synthetic procedures.

Removal of Triton X-100 from Acyl-ACP—The blue-Sepharose wash, containing ACP and acyl-ACP, is diluted with 3 volumes of 20 mM Tris-HCl, pH 8.0, to reduce the ionic strength to approximately that of 0.1 M LiCl and loaded onto a DEAE-cellulose column. We routinely have used a 2-ml DE52 column (Fig. 2) and have found that loading 5 mg of
ACP or less/ml of DE52 gave recoveries greater than 97%. After using with a few column volumes of buffer to remove the bulk of the Triton X-100, the column is eluted with 90% 2-propanol (Fig. 2). The 14C-labeled material in this fraction was not acyl-ACP as judged by the filter disc assay and is presumably free fatty acid. After removing 2-propanol from the column with 3 column volumes of 20 mM Tris-HCl, pH 7.4, ACP and acyl-ACP are eluted with 0.45 M LiCl in 20 mM Tris-HCl, pH 7.4 (Fig. 2).

Separation of ACP and Acyl-ACP—The proper choice of hydrophobic matrix is important for these experiments. Commercially available octyl-agaroses that were synthesized using CNBr-agarose and octylamine had a small capacity for ACP (2 μg/ml), but this capacity is high enough to bind radiochemical quantities of [14C]ACP. Radiochemical quantities of [14C]acyl-ACP were also bound to octyl-agarose. Elution of the column with a LiCl gradient resulted in the separation of ACP (0.15 μg/ml) and acyl-ACP (0.35 μg/ml); however, the peaks were broad and not well resolved. Synthesis of supports using the CNBr procedure has been shown to yield N-substituted isoureas (20), resulting in a matrix with a high degree of positive charge. Accordingly, acidic proteins have been found to be more tightly adsorbed to alkyl-agaroses than basic proteins (21). Further evidence for the charged nature of this support was provided by Wilczek and Miron (22) who showed that the isourea groups on the support could be acetylated with acetic anhydride to yield a gel that possesses no net charge. Model proteins (ovalbumin and α-lactalbumin) that bind to alkyl-agaroses were found not to be adsorbed by the acetylated, uncharged resin (22). Since ACP is a very acidic, highly charged protein (23), we feel that ACP and acyl-ACP are bound to octyl-agarose primarily by electrostatic interactions. This conclusion is supported by the fact that moderate concentrations of salt will elute both components from the matrix. The low affinity of acyl-ACP for these gels is probably due to their low substitution (10 μmol of octane/ml).

To avoid these difficulties, we have used octyl-Sepharose CL-4B. These gels have a high degree of substitution (40 μmol/ml) and do not possess detectable amounts of charged groups (17, 18). Acyl-ACP binds tenaciously to octyl-Sepharose CL-4B. Neither molar concentrations of salt (LiCl or KCl), chaotrophic agents (KSCN), 50% ethanol, or 50% ethylene glycol solutions proved satisfactory for the quantitative removal of acyl-ACP. Tetrahydrofuran and 2-propanol were found to quantitatively remove acyl-ACP from octyl-Sepharose. The solvent we chose was 2-propanol, due to the higher volatility of tetrahydrofuran. In sharp contrast to acyl-ACP, ACP was not adsorbed to octyl-Sepharose. Furthermore, we were unable to elute ACP onto octyl-Sepharose using higher concentrations of lyotropic salts (80% [NH4]2SO4 or 2 mM KCl). These data indicate ACP to be a very hydrophilic protein devoid of any hydrophobic surface available for interaction with aliphatic ligands. The amino acid composition of ACP (23) is consistent with this interpretation, being deficient in the most hydrophobic amino acids (24).

We recommend that Triton X-100 bound to acyl-ACP be removed at the DE52 step, although these two compounds are separable using octyl-Sepharose. The 25% 2-propanol used to remove acyl-ACP from octyl-Sepharose is insufficient to displace Triton X-100 from the column. Concentrations of excess of 80% are required to remove Triton X-100 from the gel. We found that octyl-Sepharose had a maximum capacity for Triton X-100 of approximately 10 μmol/ml of gel. Acyl-ACP does not bind to a Triton-saturated column, and we found that acyl-ACP could be displaced from octyl-Sepharose by elution with Triton X-100 solutions. Therefore, a larger octyl-Sepharose column will be required to adsorb acyl-ACP contaminated with Triton X-100 than would be required for the adsorption of an equivalent amount of Triton-free acyl-ACP. Also, an octyl-Sepharose column loaded with both acyl-ACP and Triton X-100 must be regenerated by elution with 80% 2-propanol before reuse or serious deterioration of column performance results.

The separation of ACP and acyl-ACP on octyl-Sepharose is shown in Fig. 3. The maximum capacity of octyl-Sepharose for C16:0-ACP was determined to be 40 nmol/ml of gel; however, we recommend using only half the available capacity. Under these conditions, quantitative absorption of all [14C]acyl-ACPs prepared in this report was achieved. We have efficiently loaded acyl-ACP onto octyl-Sepharose columns in solutions ranging in ionic composition from 0.02 to 0.45 M LiCl between pH 7.0 and 8.0. The minimum 2-propanol concentration required to elute a specific acyl-ACP depends on the structure of the acyl moiety (see below).

Acyl-ACP was bound to DE52 after elution from octyl-Sepharose. The DE52 column was rinsed with several column volumes of 20 mM Tris-HCl, pH 7.4, to remove the 2-propanol and the acyl-ACP was eluted using the same buffer containing 0.45 M LiCl. The acyl-ACP can be stored for months in the DE52 elution buffer at −20°C without perceptible hydrolysis occurring. The ACP collected in the flow-through of the octyl-Sepharose column may be concentrated using DE52 chromatography and reused.

The purified C16:0-ACP contained less than 1% free ACP as determined by the 5,5′-dithiobis(2-nitrobenzoic) acid assay for free sulphydryl groups (25). Acyl-ACP synthetase can be used as a sensitive assay for free ACP since 5 pmol of ACP can be readily detected (13). C16:0-ACP (0.5 μmol) was completely inactive in the acyl-ACP synthetase assay system, indicating a purity of 99% or better. In addition, [1-14C]C16:0-ACP was quantitatively cleaved by hydroxylamine treatment at pH 6.5 (26) and by borohydride treatment in 30% tetrahydrofuran for 20 min at 37°C (27). The latter reaction has been shown to be specific for thioesters (28). ACP and acyl-ACP can be separated by sodium dodecyl sulfate-gel electrophoresis (7). Acyl-ACP migrates faster than ACP presumably because the acyl
moiety binds additional sodium dodecyl sulfate resulting in a higher charge/mass ratio (7). As noted by these investigators, significant deacylation of acyl-ACP occurred when electrophoresis was performed at pH 7.0 or higher. Electrophoresis of C₁₀₀₀ACP using the buffer system of Laemmli (15) (pH 8.8) revealed only one protein band corresponding to the free ACP standard. At pH 7.0 two bands were observed in the C₁₀₀₀ACP preparation: one band migrating with free ACP and another faster migrating component identified as acyl-ACP. At pH 6.0 the ratio of acyl-ACP/ACP was found to be 9:1. The 10% free ACP seen in these experiments is attributed to hydrolysis of acyl-ACP during the electrophoresis run since the sample did not contain free thiol groups (see above) and was quantitatively bound to octyl-Sepharose.

Fractionation of Acyl-ACPs—ACP does not interact with octyl-Sepharose; therefore, we conclude that acyl-ACP binds to the hydrophobic gel via the acyl chain. Since the hydrophobicity of fatty acids is proportional to the chain length (24), it is not surprising that C₁₀₀₀ACP is adsorbed less strongly to octyl-Sepharose than is C₁₀₀₀ACP. An octyl-Sepharose column will resolve acyl-ACPs according to chain length when developed with a 2-propanol gradient (Fig. 4). The optimum gradient slope was determined to be 0.0312% 2-propanol/vertical cm of column, for the resolution of the chain lengths examined in this report (Fig. 4). We found a linear relationship between ln [2-propanol concentration required to elute] and acyl chain length (Fig. 5). The elution pattern (Fig. 4) closely resembles the separation of fatty acid methyl esters obtained on nonpolar gas-liquid chromatography phases, such as Apiezon L (29). The elution position of C₂₀₀₀ACP was found to correspond to the elution position of a C₁₇₅₀ACP (Figs. 4 and 5). Unsaturated acyl chains are less hydrophobic than the corresponding saturated acyl chain, and introduction of one double bond has been shown to be essentially equivalent to removing one CH₂ group from the fully saturated chain (24). Thus, finding the elution position of C₁₅₀₀ACP to correspond to the calculated elution position of a C₁₁₅₀ACP (Figs. 4 and 5) is consistent with the idea that sorption and desorption of acyl-ACPs to octyl-Sepharose is primarily mediated by hydrophobic interactions.

The attraction of nonpolar groups for each other plays only a minor role in the hydrophobic effect. The primary driving force for these interactions arises from the large entropy change associated with the local ordering of water molecules about the hydrophobic solute (24). In fact, the standard free energies for transfer of hydrocarbons from aqueous solution to organic solvent are linearly related to chain length and are probably closely proportional to the number of water molecules that must contact the hydrophobic solute (24). All the
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Fig. 5. Relationship between fatty acid chain length and elution position of acyl-ACPs. 2-Propanol concentrations were determined as weight percent 2-propanol occurring in the peak fraction. Conditions for separation were identical to those in Fig. 4.

Fig. 6. Yield of acyl-ACP as a function of fatty acid chain length. All acyl-ACPs were synthesized using the reaction conditions described in Table I (1.5 to 3.0 acyl-ACP synthetase units/ml). All acyl-ACPs were purified as described in the text. The acyl-ACP yield is the total amount of pure acyl-ACP recovered from a 10-ml incubation mixture measured by the A235-A220. Fatty acids were present at 160 μM in all incubations.

data in this report are consistent with the hydrophobic effect being the primary force involved in the adsorption of acyl-ACP to octyl-Sepharose. Therefore, it seems likely that even in the shortest homolog of acyl-ACP tested (C8:0-ACP), a significant portion of the acyl moiety is exposed to solvent.

In addition to synthesizing the radioactive acyl-ACPs used for the chromatographic experiments in this report, we have prepared a series of unlabeled acyl-ACPs ranging in chain length from C8:0 to C18:0. All syntheses were carried out as described in Table I (1.5 to 3.0 acyl-ACP synthetase units/ml) in a final volume of 10 ml. Purification of the acyl-ACP was accomplished exactly as described in the text. The values reported in Fig. 6 are the final yield of pure acyl-ACP as determined by the A235-A220. Acyl-ACP synthetase most efficiently ligates C12:0, C14:0, and C18:2 acids. As the chain length decreased the overall yield of acyl-ACP also decreased (Fig. 6). Unsaturated fatty acids were found to give lower yields than their saturated homologs (Fig. 6). This chain length dependence of acyl-ACP yield is similar to that observed previously in smaller scale experiments (11, 12).

We anticipate that these techniques will be used extensively to prepare native acyl-ACP substrates for enzymes capable of metabolizing acyl-ACP. We have found that acyl-ACPs prepared by our methods are excellent substrates for β-ketoacyl-ACP synthases I and II and for sn-glycerol-3-phosphate acyltransferase from E. coli. In addition, milligram quantities of pure acyl-ACP prepared by these methods will permit rigorous examination of the physical and chemical properties of this important intermediate. The hydrophobic chromatography procedure described in this report should be applicable to the determination of product distributions in enzymatic reactions and the acyl-ACP content and chain length distribution in vitro. Of more general interest, the high resolution obtained using aqueous gradients of increasing nonpolar content to fractionate proteins on the basis of their hydrophobicity should prove useful for the purification of hydrophobic proteins.

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