Purification and characterization of acyl-acyl carrier protein synthetase from oleaginous yeast and its role in triacylglycerol biosynthesis

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Fatty acids are activated in an ATP-dependent manner before they are utilized. We describe here how the 10 S triacylglycerol biosynthetic multienzyme complex from *Rhodotorula glutinis* is capable of activating non-esterified fatty acids for the synthesis of triacylglycerol. The photolabelling of the complex with [³²P]azido-ATP showed labelling of a 35 kDa polypeptide. The labelled polypeptide was identified as acyl-acyl carrier protein (ACP) synthetase, which catalyses the ATP-dependent ligation of fatty acid with ACP to form acyl-ACP. The enzyme was purified by successive PAGE separations to apparent homogeneity from the soluble fraction of oleaginous yeast and its apparent molecular mass was 35 kDa under denaturing and reducing conditions. Acyl-ACP synthetase was specific for ATP. The K_m values for palmitic, stearic, oleic and linoleic acids were

found to be 42.9, 30.4, 25.1 and 22.7 μ M, respectively. The antibodies to acyl-ACP synthetase cross-reacted with *Escherichia coli* acyl-ACP synthetase. Anti-ACP antibodies showed no cross-reactivity with the purified acyl-ACP synthetase, indicating no bound ACP with the enzyme. Immunoprecipitations with antibodies to acyl-ACP synthetase revealed that this enzyme is a part of the 10 S triacylglycerol biosynthetic complex. These results demonstrate that the soluble acyl-ACP synthetase plays a novel role in activating fatty acids for triacylglycerol biosynthesis in oleaginous yeast.

Key words: acyltransferase, fatty acid activation, photolabelling, *Rhodotorula glutinis*, triacylglycerol biosynthetic complex.

INTRODUCTION

All metabolic processes involving fatty acids are preceded by the activation of fatty acid to form a thioester derivative. The cellular location and product of the activation process usually define the metabolic fate of the fatty acid. At least a few distinct mechanisms of fatty acid activation occur in *Escherichia coli* [1]. Endogenous fatty acids are synthesized as derivatives of acyl carrier protein (ACP), attached to the phosphopantetheine moiety of this small, acidic dissociable protein [2]. Depending on the chain lengths of acyl-ACP intermediates, they undergo further elongation or serve as acyl donors in the synthesis of phospholipids [3], lipopolysaccharides [4] and acyl proteins [5]. In contrast, exogenous fatty acids are transported across the outer membrane by a specific protein [6,7] and activated to form acyl-CoA by acyl-CoA synthetase at the inner membrane [8,9]. A limited activation of exogenous fatty acids to acyl-ACP occurs during phosphatidylethanolamine turnover in *Escherichia coli* that is catalysed by 2-acyl-glycerophosphatidylethanolamine acyltransferase/acyl-ACP synthetase [10]. Neither of these intermediates dissociates from the enzyme *in vivo* nor are they exchanged with the biosynthetic acyl-ACP pool [11]. In marine bioluminescent bacteria there is an ATP-dependent activation of myristic acid ($C_{14:0}$) for the synthesis of the myristyl aldehyde, a substrate of the light-emitting luciferase reaction [12]. In the bioluminescent bacterium *Vibrio harveyi*, acyl-ACP synthetase is capable of activating exogenous fatty acids for both β -oxidation and phospholipid synthesis [13]. Unlike in *E. coli* [14], these fatty acids can also be elongated in a cerulenin-sensitive biosynthetic pathway [13,15].

We have demonstrated recently that the triacylglycerol biosynthetic complex (TBC) can activate fatty acids, which then serve as substrates for the TBC in oleaginous yeast [16]. The objective of this study was to determine the mechanism of fatty

acid activation and the components involved in this process. In the present study, we have confirmed the presence of a soluble acyl-ACP synthetase from oleaginous yeast. This enzyme has been purified, characterized and shown to catalyse the following reaction:

The enzymic features suggest that it may play an important role in the activation of fatty acids for triacylglycerol (TAG) biosynthesis in oleaginous yeast.

EXPERIMENTAL

Materials

Rhodotorula glutinis (Microbial Type Culture Collection 1151) was obtained from the Institute of Microbial Technology, Chandigarh, India. [¹⁴C]Palmitoyl-CoA (51 mCi/mmol), [¹⁴C]fatty acids (55 mCi/mmol), [α -³²P]ATP (3000 Ci/mol) and [³⁵S]protein-labelling mix were obtained from PerkinElmer Applied Biosystems. [α -³²P]Azido-ATP (3000 Ci/mol) was obtained from ICN. Protein-assay reagents were from Pierce. TLC plates were from Merck. All other reagents were obtained from Sigma.

Growth conditions

Yeast cells were grown in malt/yeast-extract medium (pH 7.0) containing 0.3 % yeast extract, 0.5 % peptone and 0.3 % malt extract supplemented with 1 % glucose with aeration at 30 °C. Cell density was determined in terms of colony-forming units (1 attenuation unit at 600 nm = 9×10^7 cells).

Preparation of soluble and membrane fractions

Cells in the exponential growth phase (21 h) were suspended in 10 mM Tris/HCl (pH 7.5), 0.1 M NaCl, 5 mM MgCl₂, 1 mM

Abbreviations used: ACP, acyl carrier protein; LPA, lysophosphatidic acid; TAG, triacylglycerol; TBC, triacylglycerol biosynthetic complex; DTT, dithiothreitol.

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PMSF, 100 μ M leupeptin and 5 % sucrose (lysis buffer). The cells were lysed using glass beads (0.45–0.6 μ m). Equal volumes of acid-treated glass beads were added to the cell suspension and vortexed for 1 min, followed by incubation on ice for 1 min. This treatment was repeated $\approx$ 12 times. The lysate was pre-cleared by centrifugation at 300 g for 15 min. The supernatant was subjected to centrifugation at 10000 g for 15 min. The supernatant (soluble fraction) thus obtained was centrifuged again at 240000 g for 60 min to obtain the cytosol. The pellet was washed, re-suspended in lysis buffer and centrifuged at 240000 g for 60 min to obtain the membrane pellet. All the operations were carried out at 4 °C.

Purification of TBC

All operations were conducted at 4 °C. The soluble fraction from the exponentially growing cells was used for purification. Cytosol was resolved on to a 7 % native polyacrylamide gel under constant current (80 V) at 4 °C. After the run, the resolving gel was cut progressively into 0.5 cm slices and the protein was eluted by finely crushing the gel pieces in 10 mM Tris/HCl (pH 7.5) buffer containing 0.1 M NaCl, 5 mM $MgCl_2$ and 5 % sucrose, and incubated overnight at 4 °C. The gel-eluted protein was used for further studies. Protein concentration was estimated as described in [17].

Purification of acyl-ACP synthetase

The TBC was purified by native PAGE as described above. The fraction of the native gel containing the TBC was resolved by SDS/PAGE (15 % gel) under constant current (100 V) at 4 °C. After the run, the resolving gel was cut progressively into 0.5 cm slices and the protein was eluted as described above. The eluted protein was renatured by subjecting it to dialysis against lysis buffer (without protease inhibitors) containing 0.1 % SDS for 6 h, followed by dialysis against lysis buffer (without protease inhibitors) overnight at 4 °C. The acyl-ACP synthetase-containing fraction was detected by estimating the capacity of the renatured proteins to form acyl-ACP.

Purification of ACP

ACP was purified according to the method described in [2] with a few modifications. In brief, an *R. glutinis* cell pellet (35 g, wet weight) was suspended in 0.1 vol. of a stock solution containing 1 M Tris, 1 M glycine and 0.25 M EDTA, adjusted to pH 8.0 with HCl, followed by dilution to a final concentration of 1 g of cells/ml with distilled water. To the mixture, 0.5 % Triton X-100 was added and the cells were lysed using glass beads. Upon lysis, the suspension was centrifuged at 270 g for 15 min. The pellet was lysed again and centrifuged. To the supernatant, an equal volume of propan-2-ol was added slowly with stirring. The mixture was stirred for 30 min and centrifuged at 4000 g for 20 min. ACP was purified by passing the propan-2-ol supernatant through a DEAE-cellulose column. ACP was precipitated from the eluate by acid precipitation. The purified ACP had a molecular mass of 11 ± 0.5 kDa as determined by SDS/PAGE (15 % gel). The purified protein was injected into rabbits to obtain polyclonal antisera.

ACP and acyl-ACP synthetase assays

ACP and acyl-ACP synthetase assays were carried out as described in [18]. The reaction mixture consisted of 0.1 M Tris/HCl (pH 8.0), 0.4 M LiCl, 5 mM $MgCl_2$, 5 mM ATP, 1 mM dithiothreitol (DTT), 0.1 % Triton X-100, 10 mM NaF,

25 μ M ACP from either *R. glutinis* or *E. coli*, 100 μ M [^{14}C]palmitate (0.5 μ Ci) and 25–50 μ g of acyl-ACP synthetase in a final volume of 100 μ l. When TBC eluted by native PAGE was used as the enzyme source, ACP was omitted from the above reaction mixture. *E. coli* acyl-ACP synthetase was used as the positive control. Increasing concentrations of ACP (0–500 μ M) were used for calibration with *E. coli* acyl-ACP synthetase (10 m-units) under the assay conditions described. A calibration experiment was also performed with increasing concentrations of TBC (0–25 μ g/100 μ l). The reaction mixture was incubated at 30 °C for 30 min. The amount of labelled acyl-ACP formed was determined by spotting the reaction mixture on to Whatman 3MM filter discs, washing them thrice with chloroform/methanol/acetic acid (3:6:1, by vol.) and drying. The dried filter discs were subjected to liquid scintillation counting. The acyl-ACP formed was resolved by SDS/PAGE (15 % gel) using the method of Laemmli [19] in the absence of 2-mercaptoethanol. The radiolabelled acyl-ACP was visualized by fluorography.

Formation of acyl-CoA

Formation of acyl-CoA was carried out as described in [20]. The assay mixture consisted of 50 mM Tris/HCl (pH 8.0), 7.5 mM $MgCl_2$, 5 mM ATP, 0.5 mM CoA, 100 μ M [^{14}C]palmitate (0.5 μ Ci) and 25–50 μ g of enzyme (cytosolic or native-PAGE-purified TBC or commercial acyl-ACP synthetase) in a final volume of 100 μ l. The reaction mixture was incubated at 30 °C for 30 min and stopped by boiling for 2 min. The reaction mixture was centrifuged at 10000 g for 5 min at room temperature and the supernatant separated. The amount of labelled acyl-CoA formed was determined by resolving the reaction mixture on silica-TLC plates using *n*-butanol/acetic acid/water (5:2:3, by vol.) as the solvent system. The lipids were visualized by staining with iodine vapour followed by autoradiography.

TAG biosynthetic assay

The assay mixtures consisted of 0.1 M Tris/HCl (pH 8.0), 0.4 M LiCl, 5 mM $MgCl_2$, 5 mM ATP, 2 mM DTT, 0.1 % Triton X-100, 100 μ M [^{14}C]palmitate (0.5 μ Ci), 50 μ M lysophosphatidic acid (LPA) and 25–50 μ g of native-PAGE-purified TBC in a final volume of 100 μ l. The incubation was carried out at 30 °C for 30 min and stopped by extracting lipids [21]. Lipids were separated on silica-TLC plates using petroleum ether/diethyl ether/acetic acid (70:30:1, by vol.) and chloroform/methanol/acetic acid/water (170:25:25:4, by vol.) as the solvent systems for separating neutral lipids and phospholipids, respectively. The lipids were visualized by staining with iodine vapour and spots corresponding to phosphatidic acid, diacylglycerol and TAG scraped off for measurement of radioactivity.

Photoaffinity labelling

Photolabelling was carried out as described earlier [20]. The reaction mixture consisted of 0.1 M Tris/HCl (pH 8.0), 0.4 M LiCl, 5 mM $MgCl_2$, 1 mM DTT, 10 μ M LPA, 0.2 μ M [α - ^{32}P]azido-ATP (0.5 mCi) or 0.1 μ M [α - ^{32}P]ATP and 5–10 μ g of enzyme (native-PAGE-purified *R. glutinis* TBC or purified acyl-ACP synthetase) in a total volume of 100 μ l. The samples were placed in 1.5 ml Eppendorf caps on ice/water slurry and irradiated with short-wavelength UV light (5000 mW/cm², model UVG-54 UV illuminator; Ultraviolet Products) for 3 min. The distance between the lamp and the samples was 5 cm. Upon photolabelling, half of the sample was boiled in SDS sample buffer and the other half was precipitated with trichloroacetic acid and radioactivity measured by liquid scintillation counting. The

proteins were resolved by SDS/PAGE (12 % gel) [19] followed by autoradiography.

Antiserum production

Rabbits were immunized by subcutaneous injection of $\approx 350 \mu\text{g}$ of SDS/PAGE-purified *R. glutinis* acyl-ACP synthetase emulsified in Freund's complete adjuvant. Two booster doses of $\approx 125 \mu\text{g}$ of protein emulsified in Freund's incomplete adjuvant were administered at 3 week intervals. Following the last injection (10 days), blood was collected and serum separated and stored at -20°C . The antibody specificity and titre were analysed by dilution and inhibition ELISA [22].

Western blotting

Proteins were separated by either native PAGE or SDS/PAGE and transferred on to a nitrocellulose membrane for immunoblotting [23]. Acyl-ACP synthetase antibody was used at a dilution of 1:300 in 0.1 % BSA in Tris-buffered saline containing 0.05 % Tween 20.

Cross-linking of proteins

For cross-linking, preparations of the native-PAGE-purified TBC ($5 \mu\text{g}$) were mixed with disuccimidyl suberate to a final concentration of 0.5 mM in a total volume of $50 \mu\text{l}$. The cross-linking was performed for 60 min at 4°C . The reaction was stopped by the addition of $5 \mu\text{l}$ of 0.25 M Tris/HCl, pH 7.5. The cross-linked products were resolved by SDS/PAGE (6 % gel) and electroblotted on to a nitrocellulose membrane. The membrane was probed with anti-ACP antibodies for the detection of cross-linked products.

Protein labelling and immunoprecipitation

Oleaginous yeast cells (10^7 cells/ml) in the exponential growth phase were labelled with $30 \mu\text{Ci/ml}$ [^{35}S]protein-labelling mix for 3 h at 32°C . The labelled cells were lysed and centrifuged at $10000 g$ for 15 min. The lysate was incubated with primary antibodies for 1 h and the immunocomplex was precipitated with Protein A-Sepharose beads. The bound proteins were analysed by SDS/PAGE (12 % gel) followed by fluorography.

Indirect immunofluorescence

Exponential-growth-phase (21 h) *R. glutinis* cells were fixed with 4 % *p*-formaldehyde for 20 min, followed by 4 % formaldehyde for 60 min. The fixed cells were washed thrice with 0.1 M phosphate buffer (pH 5.9) and resuspended with 50 mg of crude lytic enzyme in 1.2 M sorbitol for 6 h at 30°C to obtain spheroplasts. The cells were washed, resuspended (10^7 cells/ml) and plated on to 12 mm coverslips, which were pretreated with poly-L-lysine. The coverslips smeared with *R. glutinis* spheroplasts were treated with ice-cold methanol for 6 min and acetone for 30 s. Anti-(acyl-ACP synthetase) antibodies were added at a dilution of 1:20, and anti-ACP antibody was used at a dilution of 1:200. Secondary-antibody FITC/tetramethylrhodamine β -isothiocyanate (TRITC) conjugates were used for detection. The slides were viewed using a confocal laser-scanning microscope (Leica TCS SP, Heidelberg, Germany).

RESULTS

Identification of acyl-ACP synthetase

To understand the mechanism by which fatty acids were utilized by the TBC to generate TAG, we examined the formation of

Figure 1 Identification of acyl-ACP synthetase

(a) The TBC is capable of accepting non-esterified fatty acids as substrates for TAG biosynthesis. The formation of acyl-ACP was monitored by resolving the acyl-ACP synthetase reaction product by SDS/PAGE (15 % gel), in the absence of thiol-reducing agents, followed by fluorography. The TBC was incubated in the presence of ATP with [^{14}C]linoleic acid (lane 2) or [^{14}C]palmitic acid (lane 3) or all the components except ATP (lane 1). The positions of molecular-mass markers (kDa) are indicated on the left. (b) The formation of acyl-CoA was monitored by resolving the reaction product with silica-TLC using an *n*-butanol/acetic acid/water (5:2:3, by vol.) solvent system followed by fluorography. The TBC was incubated in the presence of ATP with [^{14}C]oleate and 0.5 mM CoA (lane 2) or the reaction was carried out as for lane 2 using 20 m-units of *Pseudomonas aeruginosa* CoA synthetase as the enzyme source (lane 3). The mobilities of standard [^{14}C]oleate and [^{14}C]oleoyl-CoA are indicated in lanes 4 and 1, respectively. FFA, non-esterified fatty acid. (c) Effect of increasing concentrations of azido-ATP on the formation of acyl-ACP by the TBC in the dark. The amount of natural ATP in the reaction was 2.5 mM. Values are the means $\pm$ S.E.M. from three different experiments, each performed in duplicate. (d) Photolabelling of TBC with [^{32}P]azido-ATP was carried out for 3 min and the photolabelled samples were resolved by SDS/PAGE (12 % gel) followed by autoradiography. Lane 1, photolysed [^{32}P]azido-ATP before adding TBC; lane 2, photolabelling of the TBC with the photoprobe; lane 3, as in lane 2, but the reaction contained 5 mM excess ATP.

acyl-ACP. The formation of acyl-ACP was monitored by using [^{14}C]linoleic acid or [^{14}C]palmitic acid as a substrate for the TBC and resolving the reaction mixture by SDS/PAGE (15 % gel), in the absence of thiol-reducing agent, followed by fluorography. It is evident from Figure 1(a) that acyl-ACP was formed. In another set of experiments, the formation of acyl-CoA was monitored by resolving the acyl-CoA synthetase reaction product on silica-TLC. We found that there was no formation of acyl-CoA (Figure 1b). These results demonstrated that the TBC was

(a)

<i>E. coli</i> ACP (μ M)	+	0	400	300	200	100	-	-	-	-	-
<i>E. coli</i> acyl-ACP synthetase	-	+	+	+	+	+	-	-	-	-	-
<i>R. glutinis</i> TBC (μ g)	-	-	-	-	-	-	0	5	10	15	25

(b)

(c)

Figure 2 Validation of acyl-ACP synthetase assay

(a) Effect of increasing concentrations of ACP on the formation of acyl-ACP by *E. coli* acyl-ACP synthetase and the effect of increasing concentration of purified TBC instead of ACP in the reaction. No enzyme and no substrate were used as negative controls. Effect of ACP (b) and TBC (c) on the formation of acyl-ACP (filter-binding assay). Values are the means $\pm$ S.E.M. from five different experiments, each performed in duplicate. In (c), the amounts of TBC shown are with respect to the total assay volume of 100 μ l.

capable of acylating ACP and that this could be due to the presence of acyl-ACP synthetase. As acyl-ACP synthetase is an ATP-dependent enzyme in prokaryotes [1,13], we examined the effect of azido-ATP on the TBC. Acyl-ACP formation decreased with increasing concentration of azido-ATP in the dark, suggesting possible competition with the substrate ATP (Figure 1c). These results suggest that azido-ATP interacts with acyl-ACP synthetase. Thus we used the radiolabelled analogue of azido-ATP to identify acyl-ACP synthetase in the TBC. Azido-ATP forms an irreversible bond with the enzyme upon exposure to UV light, making it inactive. Photoaffinity labelling of the TBC with [32 P]azido-ATP was carried out and it was observed that the azido-ATP was covalently bound to a 35 kDa polypeptide under denaturing and reducing conditions (Figure 1d). These results suggest the presence of acyl-ACP synthetase in the TBC of *R. glutinis*.

The acyl-ACP synthetase assay was validated by performing a calibration experiment with TBC as well as with *E. coli* acyl-ACP synthetase. With increasing concentrations of ACP in the reaction mixture there were increasing amounts of acyl-ACP formed with acyl-ACP synthetase from both *E. coli* and *R. glutinis* (Figure 2a). Similar results were obtained with filter-binding assay with *E. coli* ACP (Figure 2b). This confirmed the authenticity of the assay for acyl-ACP synthetase. It can be seen that at low concentrations of TBC there was a faster rate of acyl-ACP

formation, whereas at higher concentrations of TBC a slower rate of acyl-ACP formation occurred in the reaction (Figure 2c). This could be due to the occurrence of two forms of the enzyme, the form present at low concentrations being more active than the form present at higher concentrations. However, further experiments are needed to confirm this possibility. Under these assay conditions saturating amounts of ATP and fatty acid were used and it is unlikely that these substrates limited the reaction.

Purification of acyl-ACP synthetase

Acyl-ACP synthetase activity was found only in the soluble fraction of *R. glutinis* and this fraction was electrophoresed by native PAGE (7% gel). Acyl-ACP synthetase, LPA acyltransferase, phosphatidic acid phosphatase, diacylglycerol acyltransferase and ACP were detected in the same region of the gel. The native PAGE step was very effective and resulted in a 358-fold purification of TBC, with a recovery of 41% (Table 1). The specific activity was determined to be 494 pmol/min per mg for palmitic acid. Acyl-ACP synthetase was purified to homogeneity from the TBC by subjecting the native-PAGE-eluted active fraction (TBC) to SDS/PAGE (Figure 3a). The SDS/PAGE-eluted proteins were renatured and used to assay for acyl-ACP synthetase activity. The fifth fraction was capable of generating acyl-ACP (Figure 3b). The specific activity of the

Table 1 Purification of acyl-ACP synthetase from oleaginous yeast

The results summarize a typical purification of acyl-ACP synthetase from *R. glutinis*. The exponential-growth-phase oleaginous yeast cells from 1 litre of culture were lysed with glass beads and the soluble fractions were obtained after ultracentrifugation. Enzyme activities were measured as described in the Experimental section.

Step	Protein (μ g)	Specific activity (pmol/min per mg)	Total activity (pmol/min)	Yield (%)	Purification (-fold)
Cytosol	9500	1.38	13.11	100	1
Native PAGE	11	494	5.43	41	358
SDS/PAGE	8	60	0.48	4	44

SDS/PAGE-eluted acyl-ACP synthetase was 60 pmol/min per mg with a yield of 4%. The fold purification of acyl-ACP synthetase decreased radically following the SDS/PAGE purification step. Also, the enzyme activity could not be restored completely upon renaturation. As a small amount of acyl-ACP synthetase activity was associated with the renatured fraction, the purified TBC was used as the enzyme source for further studies. The final purification step gave a single band with an apparent molecular mass of 35 kDa, determined by SDS/PAGE (12% gel) and silver staining (Figure 3c). TBC was used as the source of acyl-ACP synthetase, as the enzyme activity could not be restored completely upon renaturation.

The purified protein was subjected to N-terminal sequencing, and the sequence VHLAVALYGLAAVRVSRIVR shared 46% similarity with the amino acid region 276–295 of the putative acyl-ACP synthetase from *Campylobacter jejuni* [24]. The N-terminal sequence of *R. glutinis* acyl-ACP synthetase did

not share any similarity with the *E. coli* 2-acyl-glycerophosphatidylethanolamine acyltransferase/acyl-ACP synthetase.

Nucleotide requirements for acyl-ACP synthetase activity

The nucleotide dependence of acyl-ACP synthetase is shown in Figure 4. [α - 32 P]Azido-ATP cross-linking was performed in the presence of various concentrations of unlabelled ATP, GTP, CTP and UTP. Formation of the enzyme–AMP intermediate was detected by analysing the products of the photolabelling reaction by native PAGE and SDS/PAGE followed by autoradiography (Figure 4a) and filter-paper-disc assay (Figure 4b). TBC and a 35 kDa protein were labelled under native and denaturing conditions, respectively, and the intensity of labelling decreased with increasing concentrations of unlabelled ATP. Addition of 1 mM ATP to the photolabelling reaction reduced the intensity of labelled acyl-ACP synthetase to half. GTP, CTP and UTP did not compete with ATP and showed no reduction in the labelling intensity of acyl-ACP synthetase (Figure 4b). TLC analysis of the reaction product in which [α - 32 P]ATP was used as the substrate revealed that AMP was formed during the reaction (results not shown).

Kinetic parameters of acyl-ACP synthetase

The substrate specificity of acyl-ACP synthetase was studied by providing fatty acids of varying chain lengths and degrees of unsaturation as substrates for the native-PAGE-purified TBC. Acyl-ACP synthetase activity was the highest for linoleic acid ($C_{18:2}$) and lowest for palmitic acid ($C_{16:0}$). Based on the Hanes–Woelf plot [25], $V_{\max}$ and K_m values for fatty acids and ATP under the standard assay conditions were obtained, as

Figure 3 Acyl-ACP synthetase is a part of the multienzyme TBC

(a) Native-PAGE-purified TBC [16] was resolved by SDS/PAGE (12% gel). The mobility of the molecular-mass markers (kDa) is shown above. (b) TBC was electrophoresed by SDS/PAGE (12% gel) and the proteins were eluted from the gel pieces followed by renaturation, as described in the Experimental section. Acyl-ACP synthetase activity was measured from the eluted proteins. Values are the means $\pm$ S.E.M. from six different experiments. (c) The area of the SDS/polyacrylamide gel corresponding to the active acyl-ACP synthetase was reloaded on to a SDS/polyacrylamide gel (12%) and visualized by silver staining. Molecular-mass markers are indicated.

Figure 4 Nucleotide specificity of acyl-ACP synthetase

(a) UV cross-linking of [α -³²P]azido-ATP was carried out with the purified complex or the cytosol for 3 min in the dark, and the samples were resolved on native 7% polyacrylamide and SDS/12% polyacrylamide gels followed by autoradiography. The complex and a 35 kDa polypeptide were visualized under non-denaturing and denaturing conditions, respectively. Lane 1, irradiated [³²P]ATP before adding to the reaction mixture as the control. Lanes 2, 3 and 4 represent the cross-linking of the TBC with [α -³²P]azido-ATP in the presence of 0.0, 0.5 and 5.0 mM ATP, respectively. Lane 5 represents the labelling of cytosol. (b) Effect of increasing concentrations of ATP (◆), GTP (■), CTP (▲) and UTP (×) on the photolabelling of TBC with [³²P]azido-ATP. Values are the means $\pm$ S.E.M. from four different experiments, each performed in duplicate.

Table 2 Kinetic parameters of acyl-ACP synthetase

Enzyme activity was measured in the TBC as a function of fatty acid concentration with a fixed concentration of ATP (5 mM) or as a function of ATP concentration with a fixed fatty acid concentration (100 μ M). Each value is the mean of two determinations, each performed in triplicate. The Hanes–Woolf plot was used to obtain K_m and V_{max} values. The acyl-ACP synthetase was measured as described in the Experimental section.

Substrate	K_m	V_{max} (nmol/min per mg)	V_{max}/K_m
Palmitic acid (C _{16:0})	42.9 μ M	0.51	0.012
Stearic acid (C _{18:0})	30.4 μ M	1.99	0.066
Oleic acid (C _{18:1})	25.1 μ M	3.05	0.122
Linoleic acid (C _{18:2})	22.7 μ M	4.81	0.209
ATP	2.5 mM	5.02	—

summarized in Table 2. The unsaturated acids linoleic acid and oleic acid (C_{18:1}) had higher V_{max} values than the saturated fatty acids (palmitic acid and stearic acid, C_{18:0}), and lower apparent K_m values (Table 2). These results suggested that acyl-ACP

Figure 5 Properties of acyl-ACP synthetase with respect to the TBC

(a) Photolabelling with [α -³²P]ATP in the presence and absence of fatty acids (FA) and LPA as indicated. (b) The acylation of ACP with [¹⁴C]linoleic acid followed by the formation of TAG by the TBC was monitored. TBC was incubated with exogenous ACP (*E. coli* or *R. glutinis*) or acyl-ACP synthetase (*E. coli*), and the formation of TAG was measured as described in the Experimental section. Values are the means $\pm$ S.E.M. from five different experiments, each performed in duplicate.

synthetase had specificity towards C₁₈ fatty acids, with preference for linoleic acid. The acyl-ACP synthetase activity was the highest in the presence of ATP as compared with other nucleotides, and the K_m for ATP was determined to be 2.5 mM (Table 2). As ACP is a part of the TBC, it was not possible to determine the kinetic parameters of ACP alone.

Properties of acyl-ACP synthetase

Maximum enzyme activity was observed in the presence of fatty acid in the reaction mixture. Labelling of acyl-ACP synthetase with [α -³²P]ATP was maximum when either fatty acids or both LPA and fatty acids were supplied in the reaction mixture (Figure 5a). The acylation of ACP followed by the formation of TAG by the TBC in the presence of exogenous ACP from either *E. coli* or *R. glutinis* and acyl-ACP synthetase was determined. The results indicated that the exogenous supply of ACP or acyl-ACP synthetase to the TBC had no significant effect in TAG formation (Figure 5b).

Characterization of acyl-ACP synthetase

To establish the presence of acyl-ACP synthetase in the TBC, the purified 10 S complex was resolved by two-dimensional gel electrophoresis and transferred on to nitrocellulose membrane, which was probed with polyclonal antibodies raised against the

Figure 6 Western-blot analysis for acyl-ACP synthetase

(a) Displacement of the binding of *R. glutinis* ACP antibodies to immobilized acyl-ACP synthetase by the free acyl-ACP synthetase as determined by competitive inhibition ELISA. *R. glutinis* acyl-ACP synthetase (1 µg/well) was coated in a microtitre plate followed by blocking of the unoccupied sites. ELISA was then performed as described in the Experimental section. Various concentrations of *R. glutinis* ACP (0.02–10 µg/100 µl) were incubated with the *R. glutinis* acyl-ACP synthetase antibodies at dilution that gave $\approx 50\%$ of the maximum binding to 1 µg of immobilized ACP. (b) The presence of *R. glutinis* acyl-ACP synthetase in the TBC was demonstrated by probing the two-dimensional gel with SDS/PAGE-eluted anti-(acyl-ACP synthetase) antibodies in immunoblots. The diagonal arrow indicates the position of acyl-ACP synthetase in both gels. IEF, isoelectric focusing.

purified acyl-ACP synthetase. The specificity of the antibodies was determined by competitive inhibition ELISA (Figure 6a). The antibodies recognized a single polypeptide with an apparent molecular mass of 35 kDa. The pI of acyl-ACP synthetase was estimated to be ≈ 8.7 by two-dimensional gel electrophoresis (Figure 6b). The same antibody was used for probing the 10 mM CHAPS-solubilized microsomal membranes and found that there was no immunoreactive protein in this fraction, suggesting that this enzyme is absent from the membranes (results not shown). The antibodies reacted with *E. coli* acyl-ACP synthetase but did not show any reactivity towards acyl-CoA synthetase (Figure 7a). When the purified *R. glutinis* acyl-ACP synthetase was probed with anti-ACP antibodies, whose specificity was determined by competitive inhibition ELISA (Figure 7b), it was found that the antibodies did not recognize the 35 kDa protein

(Figure 7c), suggesting the absence of bound ACP in the purified enzyme.

To validate whether acyl-ACP synthetase was a part of the TBC, exponential-phase *R. glutinis* cells were metabolically labelled with [35 S]methionine, followed by immunoprecipitation with acyl-ACP synthetase antibodies. The antibodies specifically immunoprecipitated the TBC but normal rabbit serum or Protein A–Sepharose did not immunoprecipitate the complex. Resolution of the immunoprecipitate by SDS/PAGE followed by fluorography exhibited five distinct polypeptides corresponding to TBC that contained 35 kDa acyl-ACP synthetase (Figure 7d). This result demonstrated that acyl-ACP synthetase is indeed a part of the TBC. A similar immune complex pattern was also observed with LPA acyltransferase and phosphatidic acid phosphatase antibodies [16].

The acyl-ACP synthetase antibodies were capable of inhibiting only 40% of the enzyme activity. The inhibition of acyl-ACP synthetase activity with the antibodies subsequently reduced TAG formation by the TBC (Table 3). There was almost complete inhibition of acyl-ACP and TAG formation when the complex was pre-incubated with anti-ACP antibodies. This showed that one of the components of the 10 S complex was indeed acyl-ACP synthetase.

To determine the subcellular localization of acyl-ACP synthetase, *R. glutinis* spheroplasts were probed with anti-(acyl-ACP synthetase) and anti-ACP antibodies for indirect immunofluorescence. The staining pattern revealed the cytosolic nature of acyl-ACP synthetase (results not shown). The staining pattern for acyl-ACP synthetase was similar to those of LPA acyltransferase, phosphatidic acid phosphatase and ACP [16]. The latter was used as the cytosolic marker. These results confirmed the cytosolic nature of the acyl-ACP synthetase.

DISCUSSION

Acyl-ACP synthetase was purified to homogeneity from the soluble fraction of *R. glutinis*, and its activity was restored partially upon renaturation after SDS/PAGE as a purification step. Due to this limitation, TBC was used as the enzyme source for characterization of acyl-ACP synthetase. The following experiments have demonstrated the association of this enzyme with the 10 S TBC: (i) isolation of acyl-ACP synthetase from the 10 S complex, (ii) photoaffinity labelling of the TBC with [32 P]azido-ATP, (iii) immunoprecipitation of the TBC with anti-(acyl-ACP synthetase) antibodies, and (iv) immuno-inhibition of TBC activity with anti-(acyl-ACP synthetase) and anti-ACP antibodies. The enzyme activity was found to be maximum with unsaturated fatty acids and showed a preference for linoleic acid, indicating that linoleate was the preferred substrate. The fatty acid specificity of acyl-ACP synthetase was comparable with that of the *in vivo* accumulation of fatty acids in TAG. Fatty acid specificity of *R. glutinis* acyl-ACP synthetase was different from that of the *E. coli* enzyme, which prefers fatty acids ≥ 14 carbons in length [26]. The *V. harveyi* enzyme prefers medium-chain fatty acids [27].

Even though LPA does not participate directly in the activation of fatty acids, it is required for the maximum activity of the enzyme, suggesting that substrate channelling could occur in the 10 S TBC without releasing the intermediates. It is possible that the substrate channelling is perhaps important for the efficient accumulation of TAG in oleaginous yeast. Exogenous acyl-ACP did not serve as the acyl donor for the complex. The addition of 10 S TBC components such as ACP or acyl-ACP synthetase did not alter TAG biosynthetic capacity, suggesting that the alteration of stoichiometric amounts of some of the

Figure 7 Cross-reactivity with fatty acid-activating enzymes and immune-complex analysis using acyl-ACP synthetase antibodies

(a) Acyl-CoA synthetase (lane 2) and acyl-ACP synthetase (lane 3) from *E. coli* were probed with anti-(acyl-ACP synthetase) antibodies in immunoblots. Lane 1 represents the negative control with normal rabbit serum. (b) Displacement of the binding of ACP antibodies to immobilized ACP by free ACP as determined by competitive inhibition ELISA. ACP was coated in a microtitre plate ($1 \mu\text{g}/\text{well}$) followed by blocking of the unoccupied sites. Various concentrations of ACP ($0.125\text{--}4 \mu\text{g}/100 \mu\text{l}$) were incubated with the ACP antibodies at a dilution that gave $\approx 50\%$ of the maximum binding to $1 \mu\text{g}$ of immobilized ACP. (c) Immunoblot of purified acyl-ACP synthetase (lane 2) and ACP (lane 3) from *R. glutinis* probed with anti-ACP antibodies. Normal rabbit serum was used as the negative control (lane 1). (d) Immunoprecipitation of ^{35}S -labelled *R. glutinis* cell lysate with anti-(acyl-ACP synthetase) antibodies. The immunoprecipitate was resolved by SDS/PAGE (12% gel) according to the method of Laemmli [19] followed by fluorography. Lane 1, *R. glutinis* cell lysate; lane 3, cell lysate immunoprecipitated with anti-(acyl-ACP synthetase) antibody. The cytosol was first immunoprecipitated with normal rabbit serum (pre-clearing) and the supernatant thus obtained was immunoprecipitated with acyl-ACP synthetase antibodies (lane 4). Normal rabbit serum was used as the negative control (lane 2). Molecular-mass markers (kDa) are indicated on the left.

components did not have any effect. ACP was found to be a part of the TBC and it was difficult to determine the kinetic constants for ACP as we were unsuccessful in depleting the endogenous ACP.

The integral inner-membrane enzyme 2-acyl-glycero-phosphoethanolamine acyltransferase/acyl-ACP synthetase [11,26] catalyses an ATP-dependent activation and transfer of exogenous fatty acids to lysophosphatidylethanolamine [11,28]. The enzyme is involved in the reacylation of the *sn*-1 position of phosphatidylethanolamine during synthesis of outer-membrane lipoproteins in *E. coli* [10,29,30]. Acyl-ACP is a tightly bound intermediate of the *E. coli* enzyme. The purified *R. glutinis* acyl-ACP synthetase did not show any activity in the absence of exogenous ACP. In addition, the purified acyl-ACP synthetase

did not have any bound ACP, as demonstrated by testing the reactivity of anti-ACP antibody. However, the tight binding of ACP to the native enzyme preparation was not established in this study. *V. harveyi* contains a soluble 62 kDa acyl-ACP synthetase which plays a different role from the *E. coli* enzyme in fatty acid metabolism. It is proposed to be involved in the elongation of exogenous fatty acids [15]. In this study the *R. glutinis* enzyme was optimally active at low Triton X-100 concentrations compared with the *E. coli* acyl-ACP synthetase. The novel properties of this enzyme reflect a different physiological role of the acyl-ACP synthetase in this species.

The current opinion is that TAG biosynthesis occurs in the microsomal membranes and that fatty acyl-CoA esters are the substrates for acyltransferases. This study provides the first

Table 3 Inhibition of acyl-ACP and TAG synthesis by antibodies

TBC was pre-incubated with acyl-ACP synthetase or ACP antibodies for 15 min at 30 °C. The formation of acyl-ACP was measured by providing [14 C]linoleic acid and ATP as the substrates, and TAG formation was measured by providing [14 C]linoleic acid, LPA and ATP. The enzyme activities were measured as described in the Experimental section. Values are the means $\pm$ S.E.M. from four separate experiments, each performed in duplicate.

Antibody	Acyl-ACP		TAG	
	Product formed (nmol/min per mg)	Inhibition (%)	Product formed (pmol/min per mg)	Inhibition (%)
No antibody	5.21 $\pm$ 0.24	0	0.79 $\pm$ 0.05	0
Anti- (acyl-ACP synthetase)	3.13 $\pm$ 0.17	40	0.47 $\pm$ 0.03	41
Anti-ACP	0.29 $\pm$ 0.01	94	0.02 $\pm$ 0.00	98

evidence for the existence of acyl-ACP synthetase in eukaryotes. *R. glutinis* acyl-ACP synthetase has a role in the activation of fatty acids, which are channelled for the biosynthesis of TAG. Thus the identification of acyl-ACP synthetase has significant implications for the understanding of TAG biosynthesis and regulation in oleaginous yeast.

This research was supported by Department of Science and Technology, New Delhi, India. The help provided by S. Srivani and Sumana Raychaudhuri for photoaffinity labelling and kinetic experiments is acknowledged. Technical assistance provided by Dr P. Sarla for confocal studies, the Confocal Microscope Facility, Division of Biological Science and Bioinformatics Center, Indian Institute of Science is acknowledged. Protein sequencing was performed at the Protein Sequencing Facility, Rockefeller University, New York, U.S.A.

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Received 17 May 2001/3 September 2001; accepted 28 September 2001