

Evidence for interactions of acyl carrier protein with glycerol-3-phosphate acyltransferase, an inner membrane protein of *Escherichia coli*

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We [(1989) FEBS Lett., in press] have previously shown that membrane vesicles from *Escherichia coli* contain protein-binding sites for the acyl carrier protein (ACP). We report now that membrane vesicles prepared from a strain amplified for glycerol-3-phosphate acyltransferase (GPAT) contain a higher number of ACP-binding sites than the membrane vesicles prepared from a wild type strain. In addition, we show that GPAT is retained specifically on an ACP-Sepharose affinity column and that [³H]ACP binds to the enzyme solubilized by detergent. We conclude that GPAT, an inner membrane protein which catalyses the transesterification of a fatty acyl group from acyl coenzyme A or acyl ACP to glycerol-3-phosphate, possesses a binding site for ACP.

Acyl carrier protein; Plasma membrane; Glycerol-3-phosphate acyltransferase; (*E. coli*)

1. INTRODUCTION

Acyl carrier protein (ACP) is a small acidic protein which plays a central role in the synthesis and subsequent metabolism of fatty acids and phospholipids in plants and bacteria [1]. We have previously shown that ACP is also implicated in the biosynthesis of the polyglucose chains of membrane derived oligosaccharides (MDO) [2,3]. Although ACP is described as a soluble protein, we have recently shown that the inner membrane of *E. coli* contains binding sites for ACP [4] and we have proposed that the 2-acylglycerolphosphoethanolamine acyltransferase/acyl ACP synthetase (implicated in the reacylation of lysophospholipids) [5], the glycerol-3-phosphate acyltransferase (implicated in the biosynthesis of phospholipids) [6] and the glucosyl transferase (implicated in the biosynthesis of MDO) [7] might constitute the

ACP-binding sites on the inner membrane of *E. coli* [4].

Recently, Cooper et al. [5] have shown that acylglycerolphosphoethanolamine acyltransferase/acyl ACP synthetase has a tight binding site for ACP. In this paper, we present evidence that ACP binds to the glycerol-3-phosphate acyltransferase (GPAT) protein located on the inner membrane of *E. coli*, which catalyses the transesterification of a fatty acyl group from acyl coenzyme A or acyl ACP to the *sn*-1 position of *sn*-glycerol-3-phosphate [6].

2. MATERIALS AND METHODS

2.1. Materials

β -[³H]Alanine was obtained from New England Nuclear. ACP was obtained from Calbiochem or prepared according to Rock and Cronan [8] from *E. coli* B. Purity of these two preparations was 99%, as judged by non-denaturing gel electrophoresis [2]. Labelled ACP was prepared according to Rock and Cronan [8]. HPLC experiments were conducted using a TSK Gel 3000SW column (300 × 7.5 mm). The hydrogenated form of Triton X-100 was obtained from Aldrich. Activated thiol-Sepharose 4B was obtained from Pharmacia.

ORV30/pWW20 strain was a generous gift from R. M. Bell. This strain was amplified for GPAT and was grown according

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to [9]. The wild type strain FB8 is a derivative of *E. coli* K12 and was grown as described previously [4].

2.2. Methods

Binding experiments on ORV30/pWW20 and FB8 membrane vesicles were done as described in [4].

HPLC experiments were conducted in 30 mM Hepes, 10 mM imidazol buffer, pH 7.0, containing 0.1 mM CaCl_2 , 0.2 M sodium acetate and 0.5% of hydrogenated Triton. A membrane ORV30/pWW20 Triton extract (around 50 μg of protein) was incubated with labelled ACP (5 nM, 250 000 cpm/pmol) 30 min at 20°C before injection on a TSK Gel 3000SW column. The column was eluted at a flow rate of 0.5 ml/min, the eluate monitored at 280 nm, fractions of 0.5 ml were collected and the radioactivity determined.

Glycerol-3-phosphate acyltransferase was solubilized with Triton and assayed for activity according to Green et al. [10].

ACP-Sepharose 4 B column was prepared as described by Jakowski and Rock [11] except for the size of the column (200 μl instead of 4 ml). 50 μl of Triton extract obtained from ORV30/pWW20 membrane vesicles (around 200 μg of protein) was incubated (1 h, 20°C with gentle stirring) with either 100 μl of ACP-Sepharose or 100 μl of thiol-Sepharose, equilibrated in 50 mM Tris-HCl, pH 7.0, containing 0.5% of Triton. Both gels were allowed to sediment for 15 min and a 20 μl aliquot of each supernatant was analyzed by SDS electrophoresis. Then gels were packed into columns and washed with the equilibrating buffer ($4 \times 300 \mu\text{l}$), with buffer containing 0.2 M NaCl ($4 \times 300 \mu\text{l}$) and finally with buffer containing 0.5 M NaCl ($4 \times 300 \mu\text{l}$). Aliquots (20 μl) of the fractions were analyzed by SDS electrophoresis as described [9]. Protein bands were visualized by silver staining [12]. Microbiuret [13] and the method of Lowry et al. [14] were used to measure protein content.

3. RESULTS

3.1. Binding of ACP to ORV30/pWW20 membranes

Recently, we showed that ACP binds to the inner membrane of *E. coli* (FB8 strain) with a dissociation constant around 0.4 μM and a maximal number of protein-binding sites around 50 pmol per mg of membrane protein [4]. In order to determine if the glycerol-3-phosphate acyltransferase is an ACP receptor on the membrane, we examined the binding of ACP on an *E. coli* strain that overproduces GPAT. The gene encoding GPAT, *plsB*, has been cloned and its DNA sequence determined [15,16]. Wilkison et al. [9] constructed plasmids bearing the *plsB* gene [9]. These plasmids increase expression of GPAT. pWW20 plasmids are under the control of the *tac* promoter. A 30-fold increase in GPAT activity was obtained after induction with IPTG. When the cells are not induced an 8-fold increase was obtained relative to a wild type strain which contains no plasmid [9]. It was shown that

with induced cells overproducing GPAT, intracellular tubular structures composed of ordered arrays of the enzyme are formed [9]. In order to avoid the formation of these structures and to have an homogeneous membrane material, we did not induce ORV30 cells with IPTG.

The binding of ACP to membrane vesicles of this strain was performed as described [4]. When [^3H]ACP (300 cpm/pmol) was incubated with the membrane, a fraction of ACP remained associated with the membrane after centrifugation. For a constant membrane protein concentration, the binding of ACP to the membrane saturated as the ACP concentration increased (fig.1A). The binding was reversible as it was completely displaced by the addition of unlabelled ACP (data not shown). Non-specific binding and water contamination (approx. 10%, fig.1A) were estimated as described previously [4] by performing parallel incubations to which a 50-fold excess of unlabelled ACP was added. Scatchard analysis of the specific binding showed that there was only one apparent class of binding sites with a dissociation constant (K_d) equal to 0.9 μM ($\pm 0.1 \mu\text{M}$) and a maximal number of binding sites of 300 pmol/mg membrane protein (± 10 pmol) (fig.1B). This should be compared to the 50 pmol obtained in the wild type cells. This difference most probably reflects binding of ACP to GPAT.

3.2. Interaction of ACP with GPAT solubilized in detergent

3.2.1. High-performance liquid chromatography

GPAT was solubilized by the extraction of membrane vesicles from the ORV30 strain with Triton X-100 according to [9] except that a reduced form of Triton was used. The Triton extract was incubated with tritiated ACP and chromatographed on an HPLC size-exclusion column (fig.2). Two radioactive peaks were obtained. The first one corresponded to the radioactivity associated with ACP, while the second one corresponded to a molecular mass of approx. 120 kDa. SDS-polyacrylamide gel electrophoresis showed that this latter fraction contained only one protein with a molecular mass of 83 kDa (inset of fig.2) corresponding to GPAT [9,10]. This fraction was also active in the acyl transfer assay. In the case of a wild type strain, we could not detect the higher molecular weight peak. The binding of phospho-

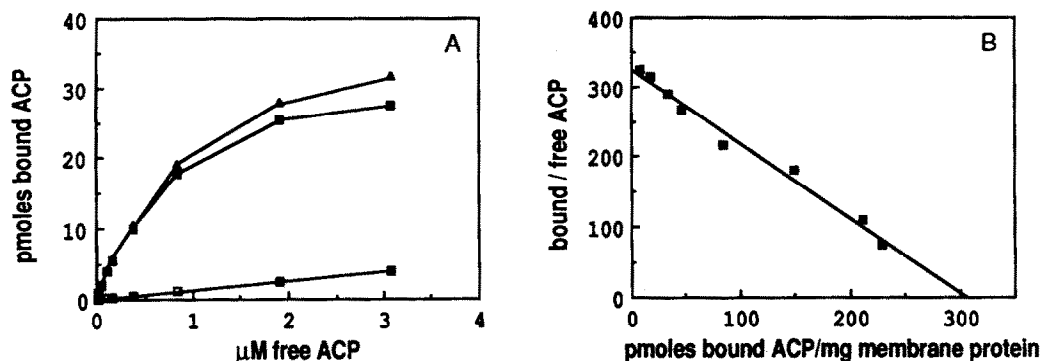


Fig.1. Binding of ACP to membrane vesicles of *E. coli* ORV30/pWW20. 120 μg of ORV30 membrane vesicles (an overproducing strain for GPAT) were incubated with different [3 H]ACP concentration and binding was performed as described previously [4]. (A) Total binding (▲), non-specific binding (□) and specific binding (■). (B) The Scatchard representation of the specific binding.

lipids [9] and Triton on GPAT may explain the difference that we found in the molecular weight of GPAT determined in HPLC and SDS electrophoresis experiments.

3.2.2. ACP-Sepharose chromatography

Triton X-100 extract obtained from ORV30 membrane vesicles was incubated with either an ACP-Sepharose or a thiol-Sepharose gel as described in section 2. The proteins which were not adsorbed on the gels were analyzed by SDS electrophoresis. Clearly, almost all GPAT was retained on the ACP-Sepharose column as indicated by the disappearance of the band corresponding to the molecular weight of GPAT [9]. On the other hand, GPAT was not at all retained on the thiol-Sepharose column (fig.3, lanes 2 and 3). The gels were then packed into columns and washed with the equilibrating buffer until no more material was eluted from the column (as ascertained by polyacrylamide gel electrophoresis). At this step no significant amount of GPAT was eluted from the ACP-Sepharose column. The columns were then washed with $4 \times 300 \mu\text{l}$ of the same buffer containing 0.2 M NaCl. No material was eluted (data not shown). Next the column was eluted with $4 \times 300 \mu\text{l}$ of buffer containing 0.5 M NaCl. Analysis of these fractions by polyacrylamide gel electrophoresis (fig.3, lanes 4 and 5) revealed that a single protein band corresponding to the molecular weight of GPAT was eluted from the ACP-Sepharose column while no material was eluted from the thiol-Sepharose column. This fraction

was also active in the acyl transfer assay. Bands observed in the area of 60 kDa (fig.3) are not proteins as described by Tasheva and Dessev [17] and are an artefact due to the presence of mercaptoethanol in the sample buffer during SDS-polyacrylamide gel electrophoresis.

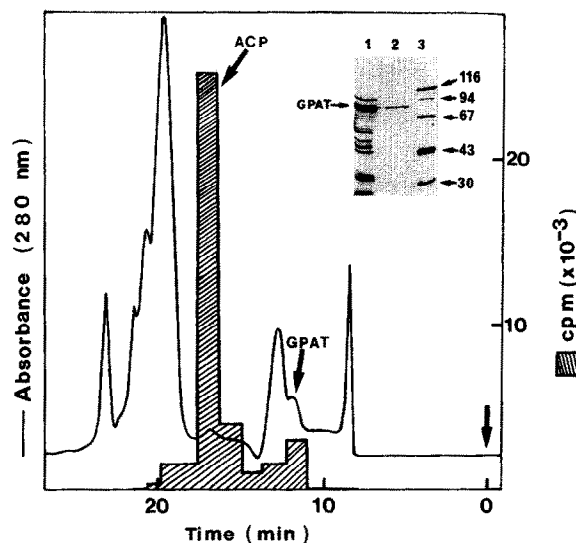


Fig.2. Association of [3 H]ACP to GPAT solubilized in Triton. 20 μl of a Triton extract obtained from ORV30 membrane vesicles (50 μg of protein) were incubated with [3 H]ACP (5 nM, 250 000 cpm/pmol) then chromatographed on a TSK Gel 3000SW column. Elution of the column was performed as described in section 2. The inset shows the SDS-polyacrylamide gel electrophoresis of the labelled fraction eluted at 12 min. Lanes: 1, ORV 30 Triton extract; 2, fraction eluted at 12 min.; 3, molecular mass standards (kDa).

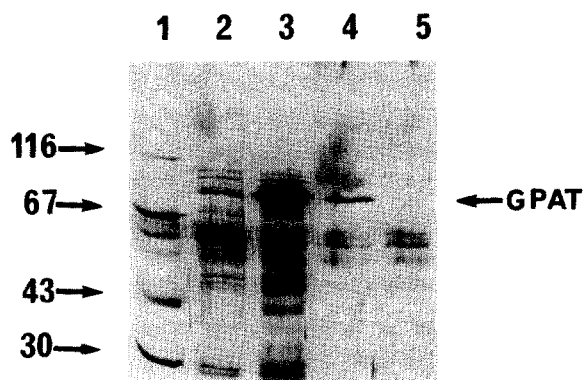


Fig.3. Binding of GPAT to an ACP-Sepharose affinity column analyzed by SDS gel electrophoresis. Approximately 200 μ g of protein Triton extract were chromatographed on an ACP-Sepharose affinity column or on a thiol-Sepharose column. The two columns were eluted and fractions analyzed by SDS-polyacrylamide gel electrophoresis as described in section 2. Lanes: 1, molecular mass standards (kDa); 2, Triton extract after incubation with ACP-Sepharose; 3, Triton extract after incubation with thiol-Sepharose; 4, aliquot of the fraction eluted from the ACP-Sepharose column with Tris-HCl buffer containing 0.5 M NaCl; 5, aliquot of the fraction eluted from the thiol-Sepharose column with Tris-HCl buffer containing 0.5 M NaCl.

4. DISCUSSION

We show in this paper that membrane vesicles of *E. coli* prepared from a strain amplified GPAT contain significantly more binding sites for ACP than a wild type strain. 300 pmol of ACP bound (instead of 50) per mg of membrane protein were found with an apparent dissociation constant around 0.9 μ M. This 6-fold increase in the number of ACP-binding sites correlates well with the increase of GPAT activity observed in this strain (8-fold) [9].

An interaction between GPAT, localized in the inner membrane of *E. coli* and ACP is supported by the result obtained with GPAT solubilized in detergent. First, GPAT extracted with Triton binds [3 H]ACP, second GPAT was entirely retained on an ACP-Sepharose affinity column.

In vivo, GPAT catalyses the transfer of fatty acid chains from acyl ACP to the 1 position of glycerol-3-phosphate. This is one of the first steps in the phospholipid biosynthesis [18]. Kinetic experiments have shown that ACP is a competitive inhibitor of acyl ACP in the acyl transfer reaction [19]. This result suggests that acyl ACP and ACP

have the same binding site on GPAT. The acyl transfer catalysed by GPAT is apparently very efficient since the acyl ACP pool in growing cells was found to be small and particularly devoid of acyl ACP with chain lengths that could serve as acyltransferase substrates [20]. On the other hand ACP is one of the most abundant proteins. Its concentration in *E. coli* cells ranges between 100 and 200 μ M [18]. Consequently, and since the dissociation constant that we found [4] is in the micromolar range, all the membrane-binding sites for ACP are occupied. Under these conditions it is difficult to understand how acyl transfer takes place in vivo. One explanation is that acyl ACP could have a very high affinity for GPAT (at least 10-fold higher than ACP). Another possibility is that fatty acid synthetase (proposed to be cytoplasmic) and GPAT (an inner membrane protein) exhibit, in vivo, some kind of structural organization in which ACP might constitute a common subunit. This possibility was also suggested by Van der Bosch et al. [21] on the basis of ACP localization studies.

Other proteins susceptible to bind ACP are probably contained in the inner membrane; in particular, the glucosyl transferase implicated in MDO biosynthesis [2,3,7]. We are currently investigating this possibility.

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