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Membrane-binding sites for acyl carrier protein in *Escherichia coli*

Nicolas Bayan and Helene Therisod

Laboratoire des Biomembranes, UA 1116, Université Paris Sud, Bât 433, 91405 Orsay Cedex, France

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We report that membrane vesicles of *Escherichia coli* contain protein-binding sites for acyl carrier protein. Scatchard analysis of the binding indicates a dissociation constant around 0.35 μM and a maximum number of protein-binding sites around 50 pmol per mg of membrane protein. Binding is on the inner membrane while the outer membrane is devoid of binding sites. These results are consistent with the fact that some acyl carrier protein-dependent enzymes implicated in phospholipid- and membrane-derived oligosaccharide biosynthesis are localized in the cytoplasmic membrane.

Acyl carrier protein; Plasma membrane; Scatchard analysis; (*E. coli*)

1. INTRODUCTION

Acyl carrier protein (ACP) is a small acidic protein that plays a central role in the synthesis and subsequent metabolism of fatty acids and phospholipids in plants and bacteria [1]. Recently, we have shown that ACP is also implicated in the biosynthesis of the polyglucose chains of the membrane derived oligosaccharides (MDO) of *E. coli* [2,3]. ACP is one of the most abundant proteins in *E. coli* [4], but its localisation in the cell is still not well established. In 1970 Van der Bosch et al. [5], on the basis of autoradiography and electron microscopy of intact cells, concluded that ACP was localised to a site on or just inside the plasma membrane. In 1985 Jackowsky et al. [6], using a colloidal gold antibody technique, reported that ACP was exclusively localized in the cytoplasm. These authors also reported that ACP did not, in vitro, associate with lipids or membranes. This last result is not consistent with the function of ACP in the phospholipid metabolism as well as in the biosynthesis of MDO. In fact, phospholipid biosynthesis requires the cooperation between several inner membrane proteins and ACP (or acyl-ACP), suggesting an interaction between these proteins [7,8]. The synthesis, in vitro, of the polyglucose chains of MDO involves ACP and a proteinaceous membrane fraction [2,9]. The role of ACP in this system is not known but an interaction between ACP and the membrane component could be necessary for this biosynthesis. For this reason we have reexamined the binding of ACP on *E. coli* membrane vesicles.

We show that ACP binds to the inner membrane of *E. coli*. This binding is protein dependent. Scatchard analysis reveals a single class of binding sites with an apparent dissociation constant (K_d) around 0.35 μM and a maximum number of binding sites around 50 pmol/mg of membrane protein.

We conclude that in vivo ACP is not entirely distributed throughout the cytoplasm of *E. coli* but that a small fraction remains associated with the inner membrane.

2. MATERIALS AND METHODS

2.1. *Materials*

β-[^{3}H]Alanine was obtained from New England Nuclear. ACP was obtained from Calbiochem or prepared according to Rock and Cronan [10] from *E. coli* B. Purity of these two preparations was 99%, as checked by non-denaturing gel electrophoresis [2].

Correspondence address: H. Therisod, Laboratoire des Biomembranes, Bât 433, Université Paris Sud, 91405 Orsay Cedex, France

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2.2. *Bacterial strains*

Strain FB8 (F^-, prototroph) used in this study is a derivative of *E. coli* K-12. This strain was grown at 37°C on medium M63, supplemented with 0.2% glucose and 0.1% casamino acids.

Strain SJ16 (a β-alanine auxotroph) was grown on minimal medium E [10] containing thiamine (0.001%), methionine (0.01%), glucose (0.4%) and β-[^{3}H]alanine (0.25 μM; 1.11 TBq/mmol).

2.3. *Preparation of labelled ACP*

Labelled ACP was prepared according to Rock and Cronan [10]. The purity of [^{3}H]ACP was assessed by autoradiography after native electrophoresis.

2.4. *Preparation of membrane vesicles*

Membrane vesicles were prepared according to Rock [11] except that FB8 cells were broken in a French pressure cell at 8000 psi. The membranes were stored on ice and used within two days of their preparation.

2.5. *Binding of ACP on membrane vesicles of* E. coli

Membrane vesicles were washed 4 times with the appropriate buffer before each experiment and then resuspended in the same buffer at a protein concentration of 10 mg/ml. Protein was determined using the microbiuret procedure [12] with bovine serum albumin as a standard. The binding assays were conducted in 80 μl of 50 mM Tris-HCl buffer, pH 7.0, with 5 mM dithiothreitol (DTT) and 50 to 200 μg of membrane protein. The concentration of [^{3}H]ACP varied from 0.1 μM to 4 μM (300 cpm/pmol). The reaction was initiated by the addition of [^{3}H]ACP. Incubations were performed at 20°C for 10 min (longer times gave the same result) and terminated by a centrifugation at 100000 × g for 15 min at 20°C in a TL 100 Beckmann centrifuge. The pellets were dried and radioactivity was counted. Non-specific binding and pellet water contamination were estimated by performing parallel incubations to which a 50-fold excess of unlabelled ACP was added. All experiments were run in duplicate.

2.6. *Trypsin digestion of membrane fraction*

The membrane fraction (1500 μg of membrane protein) was digested with trypsin (90 μg) for 30 min at 37°C before the addition of soybean trypsin inhibitor (630μg) to stop the reaction.

3. RESULTS

3.1. *Binding of ACP on FB8 membrane vesicles*

When [^{3}H]ACP was incubated with membranes of FB8 strain, a fraction of ACP remained associated with the membranes after centrifugation. The amount was proportional to the quantity of added membranes: for a fixed ACP concentration of 0.30 μM, the amount of bound ACP increased to 5 pmol, as the concentration of membrane protein increased to 220 μg (fig.1). Binding equilibrium was reached within a few minutes and was stable for over an hour.

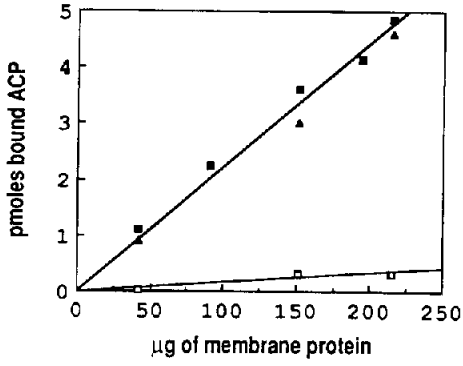

Fig.1. Specific binding of [^{3}H]ACP to membrane vesicles of *E. coli* FB8 as a function of membrane protein amount. Different amounts of membrane vesicles were incubated with 0.30 μM of labelled ACP in 50 mM Tris-HCl, pH 7, containing 5 mM DTT. Binding was conducted as described in section 2. (■) Intact membrane vesicles; (▲) membrane vesicles incubated with soybean trypsin inhibitor prior to trypsin digestion; (□) membrane vesicles digested with trypsin before addition of soybean trypsin inhibitor.

Treatment of membranes with trypsin led to the loss of 95% of the binding sites. That this loss of binding was indeed caused by proteolysis was demonstrated by the fact that addition of trypsin inhibitor prior to the addition of trypsin completely prevented the disappearance of the binding sites (fig.1). The binding was reversible as it was completely displaced by the addition of unlabelled ACP (fig.2).

For a constant membrane protein concentration, the binding of ACP to the membrane saturated as the ACP concentration increased (fig.3A). Non-specific binding and water contamination (equal approximately to 10 to 35% (fig.3A) of the binding) were estimated 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 0.35 μM ($\pm$ 0.1 μM within a single experiment) and a maximal number of binding sites of 50 pmol/mg membrane protein ($\pm$ 5 pmol within a single experiment) (fig.3B).

Different membrane preparations gave different ACP binding values: the results of six independent experiments indicated that the apparent dissociation constant (K_d) is between 0.2 and 0.6 μM and the maximal number of sites (n) is in the range

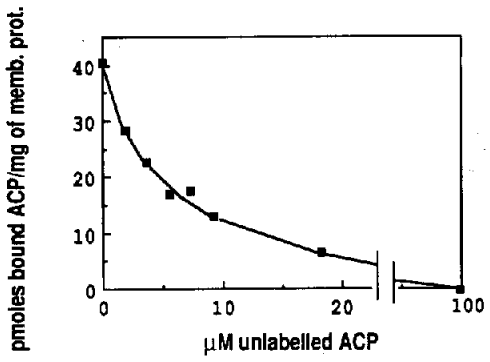

Fig.2. Displacement of [^{3}H]ACP binding by unlabelled ACP. 200 μg of FB8 membrane vesicles were incubated 10 min with 2 μM [^{3}H]ACP in 50 mM Tris-HCl, pH 7.0, 5 mM DTT. Parallel incubations were performed with increasing amounts of unlabelled ACP.

Table 1

Effect of divalent cations on ACP binding

Experimental conditions	pmol bound ACP/mg of membrane protein[a]
Standard[b]	20
+ Mg^{2+} 1.25 mM	20
6.25 mM	18
12.5 mM	18
+ Ca^{2+} 1.25 mM	15
6.25 mM	9
12.5 mM	9
+ EDTA 2 mM	20

[a] Standard deviation is ± 5%

[b] Standard conditions are described in section 2. In this experiment ACP was 0.3 μM

40–60 pmol/mg of membrane protein. Consequently, absolute binding values should be compared only within a series of experiments in which a single membrane preparation was used.

3.2. *pH dependency of ACP binding*

The number of specific binding sites was measured between pH 6 and pH 8 in 50 mM Tris-HCl buffer or phosphate buffer. The number of binding sites was relatively constant with a maximum around pH 7.0. The calculated dissociation constant followed a similar profile (data not shown).

3.3. *Effect of bivalent cations on ACP binding*

ACP is not commonly regarded as a metalloprotein, although divalent ions sites were recently located on ACP [13]. Stimulation of fatty acid synthesis, upon addition of divalent cations, that correlated with ion induced changes in the structure of ACP was observed [14]. Mg^{2+} was also found necessary for the in vitro biosynthesis of MDO [9]. For this reason we have studied the ACP binding in the presence of different divalent cations. Table 1 shows that, if Mg^{2+} does not influence the binding, part of the fixation is inhibited by Ca^{2+}. EDTA has no influence on the binding.

3.4. *Binding of ACP on the inner membrane*

E. coli and other Gram negative bacteria are enclosed by an envelope consisting of two membranes. The biosynthesis and turnover of lipids are catalyzed by membrane proteins which are localized in the inner membrane [7,8]. Consequently, the

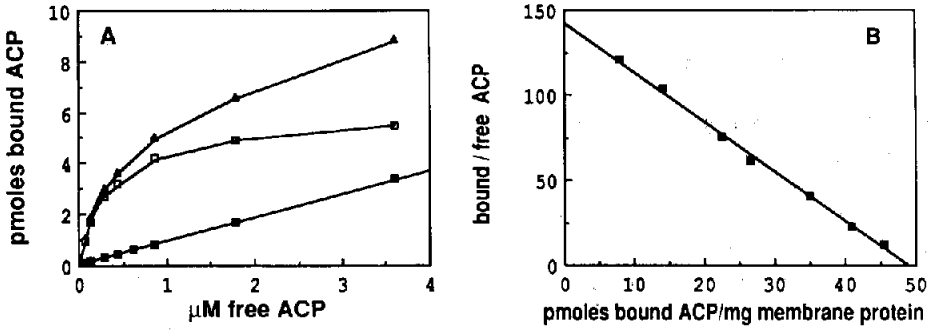

Fig.3. Binding of ACP to membrane vesicles of *E. coli* FB8. 120 μg of FB8 membrane vesicles were incubated with different [^{3}H]ACP and binding was performed as described in section 2. (A) Represents total binding (▲), non-specific binding (■) and specific binding (□) plotted as a function of free ACP concentration. (B) The Scatchard representation of the specific binding.

Table 2

Binding of ACP on inner and outer membrane of *E. coli*

Membrane fraction	pmol bound ACP/mg of membrane protein[a]
Inner membrane	21 ± 1
Outer membrane	1.5 ± 1

[a] In this experiment [^{3}H]ACP was 0.3 μM

binding of ACP was assumed to take place on this cytoplasmic membrane. Inner and outer membranes were prepared according to Ishidate et al. [15]. Binding on these two membranes was examined. The results show clearly that 95% of the binding was specific to the inner membrane (table 2).

4. DISCUSSION

Two contradictory reports have been published regarding the localisation of ACP in *E. coli* cells. The first one concluded that ACP was associated with the plasma membrane [5]; the second stated that ACP was localized in the cytoplasm and did not associate with biological membranes nor lipids vesicles [6].

In the present study we show that ACP binds to *E. coli* membrane vesicles. Scatchard analysis of the binding revealed one class of sites with an apparent dissociation constant around 0.35 μM. These binding sites are proteins, located in the inner membrane, and represent 50 pmol per mg of membrane protein. Consequently, since membrane proteins represent approx. 15% of the total protein content of *E. coli* cell [16], we can estimate that there are around 1000 ACP-binding sites per cell. This is a value commonly found for membrane proteins [17].

ACP is one of the most abundant proteins in *E. coli* [4]. Its internal concentration is between 100 and 200 μM [4]. Consequently, and since the dissociation constant (K_d) is around 0.35 μM, in vivo all the available sites are occupied. Yet the membrane bound ACP would represent only 1.5% to 3% of the total ACP pool. This could explain why Jackowsky et al. [6] did not detect any membrane-bound ACP by immunoelectron microscopy. The reason for such a large pool of ACP in the cytoplasm is not clear inasmuch as a ten-fold decrease in its concentration does not affect lipid biosynthesis [8]. In any event, in phospholipid biosynthesis the functional ACP could be a membrane bound form which, in view of the apparent K_d (0.35 μM), is not dissociated even when the cells are severely depleted in ACP.

The functions of ACP require an interaction with most of the enzymes implicated in the biosynthesis of phospholipids, fatty acids and MDO. While the enzymes involved in fatty acid biosynthesis are cytosolic, those of phospholipid and MDO biosynthesis are located in the inner membrane [7–9]. In fact, the glycerol-3-phosphate acyltransferases [18,19], the 2-acyl glycerophosphoethanolamine transferase [11] and the acyl-ACPsynthase [20], which are implicated in the biosynthesis of phospholipids and the reacylation of lysophospholipids, as well as the glucosyltransferase complex [2,9], which is implicated in MDO biosynthesis, might constitute the ACP-binding sites on the inner membrane of *E. coli*. Although we find only one class of binding sites in the present study, this does not preclude the existence of different classes of sites with similar dissociation constants. The identity of these ACP-binding sites is now being investigated.

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