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Localization of Acyl Carrier Protein in *Escherichia coli*

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Acyl carrier protein was localized by immunoelectron microscopy in the cytoplasm of *Escherichia coli*. These data are inconsistent with the previous report of an association between acyl carrier protein and the inner membrane (H. Van den Bosch, J. R. Williamson, and P. R. Vagelos, Nature [London] 228:338–341, 1970). Moreover, bacterial membranes did not bind a significant amount of acyl carrier protein or its thioesters in vitro. A thioesterase activity specific for long-chain acyl-acyl carrier protein was associated with the inner membrane.

Acyl carrier protein (ACP) functions as the carrier of acyl intermediates in fatty acid biosynthesis (22, 23). The protein has a molecular weight of 8,847 (25), and the acyl groups are bound to the active sulfhydryl site located at the terminus of the 4′-phosphopantetheine prosthetic group (25). ACP is an asymmetric acidic protein (11, 13, 25) that is highly soluble in aqueous buffers, even after the attachment of a long-chain acyl moiety (11). Likewise, all the enzymes of fatty acid biosynthesis are found in the soluble fraction of the cell (22, 23). *sn*-Glycerol-3-phosphate acyltransferase (15) and 2-acylglycerophosphoethanolamine acyltransferase (10) are the only known acyl-ACP-dependent enzymes that are localized in the cytoplasmic membrane.

ACP was previously localized in *Escherichia coli* to a site on or just inside the plasma membrane by analysis of the grain distribution in electron microscope autoradiographs of strain M99-2 (*panD*) grown on a limiting concentration of β-[^{3}H]alanine (26). This report was of particular interest since a nonrandom distribution of ACP in the cell suggested a degree of organization of the fatty acid synthesis components that was not apparent from other techniques. This result (26) along with the inability of numerous investigators to reproduce the identical properties of the fatty acid synthase system in vitro (8, 9, 18, 28) led to the conclusion that the enzymes of fatty acid biosynthesis probably exist as an organized complex localized close to or on the inner surface of the cytoplasmic membrane in vivo (22, 23). In light of the physical data showing the high water solubility of the fatty acid synthase components, we have reevaluated the intracellular localization of ACP using affinity-purified ACP-specific antibodies (6).

MATERIALS AND METHODS

The bacterial strains used in this study were derivatives of *E. coli* K-12. Strain SJ16 (3) is a *zad*::Tn*10 panD2* derivative of strain UB1005 (*metB1 relA1 gyrA216* λ λ^r F$^-$) (2). Culture conditions were the same as described previously (5). Pure ACP (12), affinity-purified ACP-specific rabbit immunoglobulin G (IgG) (6), [*pan*-^{3}H]ACP (5), and [1-^{14}C]acyl-ACPs (14) were prepared as described elsewhere.

Immunoelectron microscopy was performed essentially as described by Bendayan and Zollinger (1). Cells were fixed at 2°C overnight in 2% glutaraldehyde and postfixed for 2 h in 1% osmium tetroxide and 20 min in 2% uranyl acetate dissolved in 50% ethanol. Fixation of the majority of the samples was performed at low temperature to preserve lipid structures (20). Cells were dehydrated and embedded in Spurr resin. Sections showing a light gold interference color were deposited on nickel grids, oxidized with saturated sodium metaperiodate for 60 min, thoroughly rinsed with 0.2 M Tris-HCl (pH 7.6), and then soaked in 50 mM Tris-HCl (pH 7.6)–0.15 M NaCl containing 1% bovine serum albumin (1). The sections were treated with sodium metaperiodate to restore protein antigenicity after osmium fixation (1). Hydrogen peroxide (10%) treatment for 3 min was substituted for the sodium metaperiodate step in selected sections and did not alter the experimental results. Affinity-purified ACP-specific rabbit IgG (6) (0.3 mg/ml) in the same buffer was then applied to the sections for 1.5 h at room temperature. Controls for nonspecific binding included several dilutions of the primary antibody, nonimmune serum, and primary antibody preabsorbed with homogeneous ACP. The grids were then washed with Tris-albumin buffer and treated with 5-nm colloidal gold attached to goat anti-rabbit IgG (Janssen Life Sciences). The grids were then washed with the Tris-albumin buffer, dried, and examined with a Philips 301 transmission electron microscope operated at 60 kV. To determine if the fixation process damaged a significant number of antigenic sites or altered the distribution of gold particles, a short fixation scheme (1% glutaraldehyde for 15 min at 22°C; no postfixation) was also used in one set of experiments.

The distribution of gold particles was evaluated by dividing the cytoplasm into grids 0.11 μm on a side (0.0121 μm^2) and counting the number of particles per grid, the number of grids per cell, and the number of empty grids. A statistical approach based on the Poisson probability distribution was used to determine for each cell (n = 13) if the observed number of empty grids could be attributed to chance (3).

Strain UB1005 was lysed with a French pressure cell, and a washed total membrane fraction was prepared as described previously (4, 15). Binding assays were performed in 100 μl of 50 mM Tris-HCl (pH 7.0) containing 5 mg of total membrane protein and 25 μM [*pan*-^{3}H]ACP or [1-^{14}C]acyl-ACP. After 10 min at 25°C the incubation mixture was loaded onto a 5-ml sucrose step gradient and centrifuged at 40,000 rpm in a Beckman SW 50.1 swinging bucket rotor for 2 h at 4°C (4). Fractions were collected from the bottom of the tube and assayed for the distribution of radioactivity. The inner membrane fraction was localized at the 15%/53% sucrose interface and the outer membrane was localized at the 53%/70% sucrose interface (4, 15).

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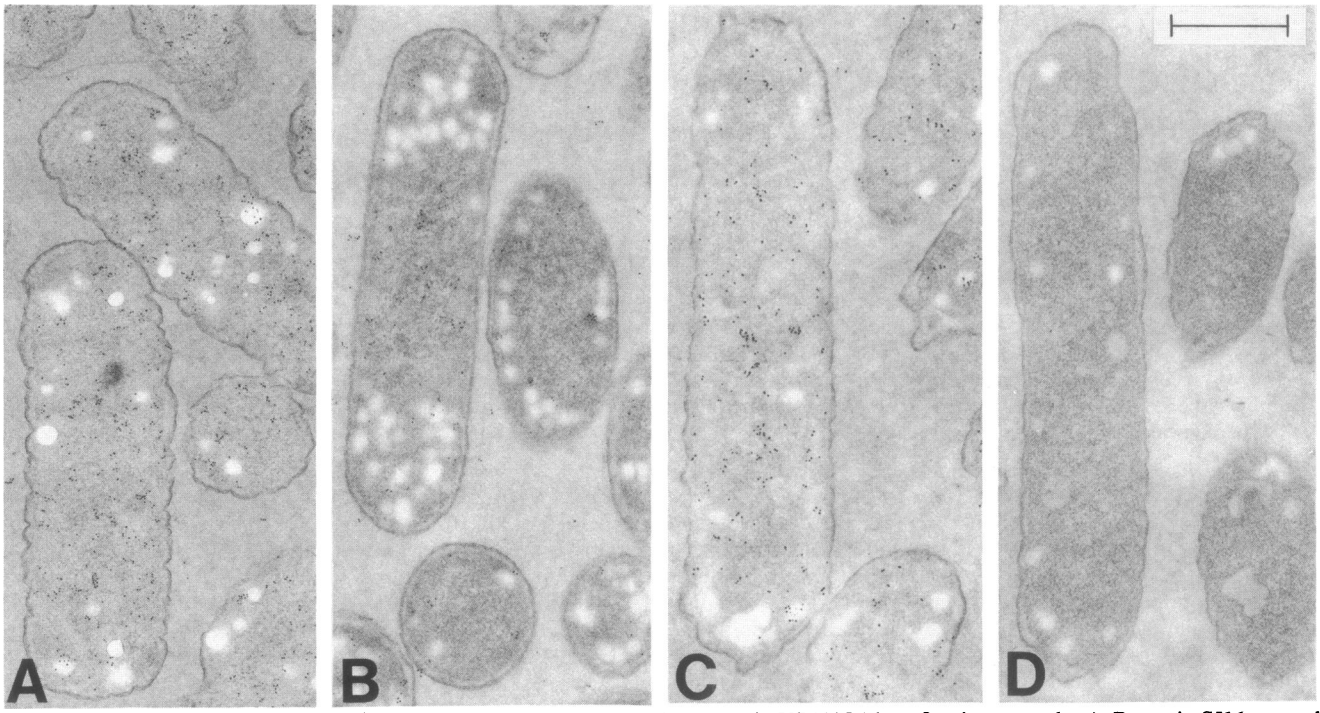

FIG. 1. Localization of ACP by immunoelectron microscopy. Panels: A, strain UB1005 (short fixation procedure); B, strain SJ16 starved for β-alanine (standard fixation procedure); C, strain UB1005 (standard fixation); D, strain UB1005 (standard fixation with the primary antibody preabsorbed with pure ACP). Magnification, ×30,000; marker, 500 nm.

Thioesterase activity was measured in assays containing 0.1 M Tris-HCl (pH 8.5), 20 μM [1-$^{14}C_{16:0}$]ACP (50,000 cpm/nmol), and 10 μg of membrane protein in a final volume of 40 μl. The reaction was stopped by the addition of 150 μl of ethanol, and the fatty acid released from the substrate was separated from unreacted acyl-ACP on Silica Gel H layers developed in butanol–acetic acid–water (5:2:4, vol/vol).

RESULTS AND DISCUSSION

Immunoelectron microscopy showed that ACP was distributed throughout the cytoplasm of the cell and was not associated with the membrane (Fig. 1). The 5-nm IgG-gold particles were consistently observed exclusively in the cytoplasm of cells isolated during exponential growth. The cells were subjected to either mild (Fig. 1A) or standard (Fig. 1C) fixation schemes, and gold particles were rarely found on the membrane in both instances. Routine fixation procedures often destroy the antigenic properties of proteins, but in the case of ACP the difference between the two fixation procedures was not very great (Fig. 1), probably because of the low amino group content of ACP (25). Preabsorption of the primary rabbit antibody with pure ACP abolished the specific reaction of IgG-gold with the cytoplasm (Fig. 1D), as did the use of nonimmune serum (data not shown). Dilutions of the primary antibody resulted in fewer gold particles and the particles were absent from sections that were not treated with primary antibody. To repeat the experiments of Van den Bosch et al. (26) in an exact fashion, we also examined the distribution of ACP in β-alanine-starved cells (Fig. 1B). As in the exponentially grown *E. coli* cultures, the IgG-gold was exclusively distributed in the cytoplasm of the coenzyme A-depleted cells (Fig. 1B). Also, fewer total gold particles were observed in β-alanine-starved strain SJ16 (99 ± 23 particles per μm^2) than in strain UB1005 (290 ± 30 particles per μm^2), which is consistent with earlier indications that the total ACP content of strain SJ16 decreased as the β-alanine supplement was reduced (5–7).

The reason for the discrepancy between our results and the earlier report that ACP was localized at a site just inside the cytoplasmic membrane (26) is not immediately obvious, but the use of IgG-gold to establish the intracellular location of antigens is clearly a better approach than autoradiography. The IgG-gold technique locates an antigen to within 10 nm of its actual site in the cell (1). In comparison, only 50% of the autoradiography grains will be within 250 nm of a tritiated point source when Ilford L4 emulsion is used, and the remaining grains are even further away (17). Since the bacterium is only 500 nm wide (Fig. 1), resolution represents a serious problem. A complicating feature was the appearance of numerous vacuolelike structures in the cytoplasm of β-alanine-starved cells that were not nearly as numerous in the cytoplasm of normal cells (Fig. 1B). The location of these vacuoles near the inner membrane caused many cross-sections to have just a thin portion of cytoplasm close to the surface, thus increasing the probability of finding grains over the membrane.

Our data do not support a homogeneous distribution of cellular ACP but instead indicate that there are regions of the cytoplasm that appear to have a locally high concentration of ACP (Fig. 1). The distinct separation between the gold particles (Fig. 1) argues that aggregation of the IgG-gold probe was not a significant factor in creating regions of high gold particle density (21). Statistical analysis of the gold particle distribution in strain UB1005 indicated that clustering was present in these cells regardless of the fixation procedure (all $P < 0.05$). On the other hand, only 8 of the 13 cells examined in β-alanine-starved strain SJ16 statistically displayed clustering. This type of clustering has also been observed in the subcellular localization of alkaline phosphatase in *Bacillus licheniformis* by using IgG-gold and was

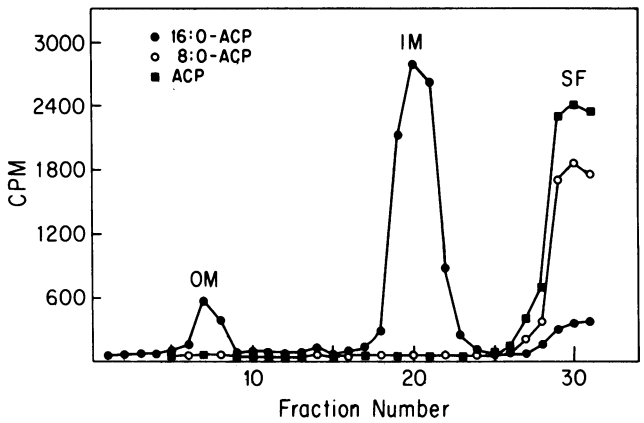

FIG. 2. Interaction of ACP and acyl-ACP with *E. coli* membranes. Incubation of the membrane fraction with labeled substrates and sucrose density centrifugation are described in the text. OM, Outer membrane location; IM, inner membrane location; SF, position of the soluble fraction.

interpreted as the association of secreted alkaline phosphatase with a cluster of specific receptors on the plasma membrane (21). On the other hand, the lack of clustering or the absence of locally high concentrations of gold particles indicates a random distribution of the LamB protein in the outer membrane of *E. coli* (27). The distribution of the cellular ACP pool among several areas of high ACP concentration suggests that the fatty acid biosynthetic activity may occur in localized regions of the cytoplasm that also contain elevated levels of other pathway enzymes.

We cannot rule out the possibility that a small fraction of the ACP pool is bound to the inner membrane. Gold particles were occasionally found over the membrane. Also, a small fraction of biosynthetically labeled [*pan*-^{3}H]ACP was always found associated with the inner membrane fraction (see below). Some association of ACP with the cytoplasmic membrane can be explained by the recent finding that the inner membrane 2-acylglycerophosphoethanolamine acyltransferase has a tightly bound ACP subunit (10).

ACP does not associate with lipid systems or biological membranes in vitro. Strain SJ16 (*panD*) was grown in the presence of 1 μM β-[3-^{3}H]alanine to uniformly label the ACP pool (5), and membrane fractions were separated from the soluble fraction by the step gradient method (4). Less than 0.5% of the [*pan*-^{3}H]ACP pool was associated with the membrane fractions. Likewise, we were unable to demonstrate binding of [*pan*-^{3}H]ACP or [1-$^{14}C_{16:0}$]ACP to phospholipid vesicles prepared by sonication of *E. coli* phospholipids. As a final test, the binding of [*pan*-^{3}H]ACP and [1-^{14}C]acyl-ACPs to purified bacterial membranes was determined (Fig. 2). [*pan*-^{3}H]ACP and short-chain [1-^{14}C]acyl-ACD did not bind to either the inner or outer membrane systems (Fig. 2). In contrast, when the membrane preparation was incubated with [1-$^{14}C_{16:0}$]ACP the majority of the label (90%) was recovered with the inner membrane fraction (Fig. 2). The radioactivity associated with the outer membrane (Fig. 2) can be accounted for by the degree of inner-membrane contamination typically encountered in this procedure (4, 15). Thin-layer chromatography on Silica Gel H layers developed in butanol–acetic acid–water (5:2:4, vol/vol) demonstrated that the membrane-bound radioactivity was [1-$^{14}C_{16:0}$] and not [1-$^{14}C_{16:0}$]ACP.

An acyl-ACP thioesterase activity was associated with the membrane fraction. The thioesterase activity was not removed by washing with 50 mM KCl–50 mM Tris-HCl (pH 7.0). A linear protein assay was developed and the membrane-associated thioesterase activity was localized to the inner membrane. Long-chain acyl-ACPs ($C_{16:0}$ and $C_{18:1}$) were the best substrates for the thioesterase (3 nmol/min per mg). Short acyl-ACP chain lengths ($C_{8:0}$ and $C_{10:0}$) were not hydrolyzed, and intermediate chain lengths ($C_{12:0}$ and $C_{14:0}$) were only marginally reactive (5 to 10% of the long-chain rate). This acyl chain specificity is similar to the specificity exhibited by the two soluble thioesterases in *E. coli* that hydrolyze acyl-ACPs (19), and the tight association of a small percentage of one or both of these enzymes with the inner membrane could account for the observed activity. Membrane-associated thioesterase activity had a pH optimum of 8.5 and was inhibited 90% by the addition of 5 mM spermidine or 5 mM Mg^{2+} to the assay system. These data illustrate that the thioesterase is most active on the expanded, denatured form of acyl-ACP (13, 24). Since biosynthetic enzymes that utilize acyl-ACP are highly specific for the compact, native acyl-ACP conformation (24), the membrane-associated thioesterase could not effectively compete with these enzymes for the low concentration of long-chain acyl-ACP normally found in vivo (16).

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LITERATURE CITED

1. **Bendayan, M., and M. Zollinger.** 1983. Ultrastructural localization of antigenic sites on osmium-fixed tissues applying the Protein A-gold technique. J. Histochem. Cytochem. **31**:101–109.
2. **Booth, B. R.** 1980. Cell surface properties of *E. coli*. Biochem. Biophys. Res. Commun. **94**:1029–1036.
3. **Feller, W.** 1968. An introduction to probability theory and its applications, 3rd ed., vol. 1, p. 101–105. John Wiley & Sons, Inc., New York.
4. **Ito, K., T. Sato, and T. Yura.** 1977. Synthesis and assembly of the membrane proteins in *E. coli*. Cell **11**:551–559.
5. **Jackowski, S., and C. O. Rock.** 1981. Regulation of coenzyme A biosynthesis. J. Bacteriol. **148**:926–932.
6. **Jackowski, S., and C. O. Rock.** 1983. Ratio of active to inactive forms of acyl carrier protein in *Escherichia coli*. J. Biol. Chem. **258**:15186–15191.
7. **Jackowski, S., and C. O. Rock.** 1984. Metabolism of 4′-phosphopantetheine in *Escherichia coli*. J. Bacteriol. **158**:115–120.
8. **Okuyama, H., S. Saitoh, and R. Hiramatsu.** 1982. Fatty acid synthetase system in the regulation of membrane lipid synthesis in *Escherichia coli* after shifts in temperature. J. Biol. Chem. **247**:4812–4817.
9. **Okuyama, H., K. Yamada, Y. Kameyama, H. Ikezawa, Y. Akamatau, and S. Nojima.** 1977. Regulation of membrane lipid synthesis in *Escherichia coli* after shifts in temperature. Biochemistry **16**:2668–2673.
10. **Rock, C. O.** 1984. Turnover of fatty acids in the 1-position of phosphatidylethanolamine in *Escherichia coli*. J. Biol. Chem. **259**:6188–6194.
11. **Rock, C. O., and J. E. Cronan, Jr.** 1979. Reevaluation of the solution structure of acyl carrier protein. J. Biol. Chem. **254**:9778–9785.
12. **Rock, C. O., and J. E. Cronan, Jr.** 1980. Improved purification of acyl carrier protein. Anal. Biochem. **102**:362–364.
13. **Rock, C. O., and J. E. Cronan, Jr.** 1982. Solution structure of acyl carrier protein, p. 333–337. *In* A. N. Martonosi (ed.), Membranes and transport, vol. 1. Plenum Publishing Corp.,

New York.
14. **Rock, C. O., and J. L. Garwin.** 1979. Preparative enzymatic synthesis and hydrophobic chromatography of acyl-acyl carrier protein. J. Biol. Chem. **254**:7123–7128.
15. **Rock, C. O., S. Goelz, and J. E. Cronan, Jr.** 1981. Phospholipid synthesis in *Escherichia coli*: characteristics of fatty acid transfer from acyl-acyl carrier protein to *sn*-glycerol-3-phosphate. J. Biol. Chem. **256**:736–742.
16. **Rock, C. O., and S. Jackowski.** 1982. Regulation of phospholipid synthesis in *Escherichia coli*: composition of the acyl-acyl carrier protein pool *in vivo*. J. Biol. Chem. **257**:10759–19765.
17. **Salpeter, M. M., L. Bachmann, and E. E. Salpeter.** 1969. Resolution in electron microscope radioautography. J. Cell. Biol. **41**:1–20.
18. **Silbert, D. F., and P. R. Vagelos.** 1967. Fatty acid mutant of *E. coli* lacking a β-hydroxydecanoyl thioester dehydrase. Proc. Natl. Acad. Sci. U.S.A. **58**:1579–1586.
19. **Spencer, A. K., A. D. Greenspan, and J. E. Cronan, Jr.** 1978. Thioesterases I and II of *Escherichia coli*: hydrolysis of native acyl-acyl carrier protein thioesters. J. Biol. Chem. **253**:5922–5926.
20. **Stein, O., and Y. Stein.** 1971. Light and electron microscopic radioautography of lipids: techniques and biological applications. Adv. Lipid Res. **9**:1–72.
21. **Tinglu, G., A. Ghosh, and B. K. Ghosh.** 1984. Subcellular localization of alkaline phosphatase in *Bacillus licheniformis* 749/C by immunoelectron microscopy with colloidal gold. J. Bacteriol. **159**:668–677.
22. **Vagelos, P. R.** 1971. Regulation of fatty acid biosynthesis. Curr. Top. Cell. Regul. **4**:119–165.
23. **Vagelos, P. R.** 1973. Acyl group transfer (acyl carrier protein), p. 155–199. *In* P. D. Boyer (ed.), The enzymes, vol. 8. Academic Press, Inc., New York.
24. **Vallari, D. S., and C. O. Rock.** 1982. Role of spermidine in the activity of *sn*-glycerol-3-phosphate acyltransferase from *Escherichia coli*. Arch. Biochem. Biophys. **218**:402–408.
25. **Vanaman, T. C., S. J. Wakil, and R. L. Hill.** 1968. The complete amino acid sequence of the acyl carrier protein of *Escherichia coli*. J. Biol. Chem. **243**:6420–6431.
26. **Van den Bosch, H., J. R. Williamson, and P. R. Vagelos.** 1970. Localization of acyl carrier protein in *Escherichia coli*. Nature (London) **228**:338–341.
27. **Vos-Scheperkeuter, G. H., E. Pas, G. J. Brakenhoff, N. Nanninga, and B. Witholt.** 1984. Topography of the insertion of lamB protein into the outer membrane of *Escherichia coli* wild-type and *lac-lamB* cells. J. Bacteriol. **159**:440–447.
28. **Weeks, G., and S. J. Wakil.** 1968. Studies on the mechanism of fatty acid synthesis. XVIII. Preparation and general properties of the enoyl acyl carrier protein reductases from *Escherichia coli*. J. Biol. Chem. **243**:1180–1189.