Evidence that Incorporation of Exogenous Fatty Acids into the Phospholipids of *Escherichia coli* Does Not Require Acyl Carrier Protein

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Received 5 December 1983/Accepted 8 May 1984

Cells of an *Escherichia coli* *acpS* mutant were prepared with decreased intracellular concentrations (to 10% of the normal level) of the holo form of acyl carrier protein. These cells incorporated exogenous oleic acid into phospholipid at a normal rate.

Escherichia coli, as well as many other bacteria, incorporates exogenously supplied long-chain fatty acids into its membrane phospholipids (22). The pathway whereby the fatty acids are transported into the cell and then incorporated into phospholipids is unclear (3, 18). Intracellular transport of long-chain fatty acids is known to require at least two gene products, acyl coenzyme A (CoA) synthetase, the product of the *fadD* gene (9), and a protein of unknown function encoded by the *fadL* gene (11, 12). Both genes are components of the β -oxidation (*fad*) regulon (9, 11, 12). Since the *fadD* gene product is strictly required for transport (9, 11, 12), it seems clear that the first intracellular form of the newly transported fatty acid is the acyl-CoA thioester. The acyl-CoA can then be either oxidized by the *fad* system or incorporated into phospholipid (9, 12, 13). The mechanism of this latter process is the subject of this note.

Silbert et al. (22) originally suggested that exogenously supplied fatty acids are incorporated into phospholipid by direct esterification of *sn*-glycerol 3-phosphate by acyl-CoA molecules, whereas endogenously synthesized fatty acids would be incorporated by reaction of *sn*-glycerol 3-phosphate with the acyl thioesters of acyl carrier protein (ACP) produced by fatty acid synthesis. This suggestion was consistent with the observation that both acyl-CoA and acyl-ACP are good substrates in the acylation reaction as assayed in vitro (3, 18). However, direct means of testing this notion have only recently become available. Röck and Jackowski (20) have recently reported strong evidence that endogenously synthesized fatty acids are incorporated into phospholipid via their acyl-ACP thioesters. Two pathways seem possible for the incorporation of exogenously supplied fatty acids. The more straightforward possibility is the use of acyl-CoA to directly esterify *sn*-glycerol 3-phosphate (22). However, as suggested by Greenaway and Silbert (5), it also is possible that the intracellular acyl-CoA molecules would be hydrolyzed to free fatty acids plus CoA, and the resulting acids would be esterified with ACP. The ACP thioesters thus formed would then be the acyl donors for phospholipid synthesis (5). This second possibility, although more complex, has the advantage of suggesting possible physiological functions for certain enigmatic enzyme activities of *E. coli*, the acyl-CoA thioesterases (2, 23) (two forms are known) and acyl-ACP synthetase (15, 17). The thioesterases are specific for acyl-CoA (23) and thus could produce free fatty acids for acyl-ACP synthetase (which activates the fatty acid carboxyl group with ACP) to produce acyl-ACP for acyl transfer into phospholipid. In this regard, it should be noted

that intracellularly located free fatty acids are incorporated into phospholipid by a reaction that proceeds in the absence of acyl-CoA synthetase (16). To test the role (if any) of ACP in the incorporation of exogenous fatty acids into phospholipids, I have altered the intracellular level of ACP and determined the effects of ACP concentration on the rate of incorporation of exogenous fatty acids into phospholipid.

The intracellular concentration of ACP was manipulated by using a mutant (*acpS*) defective in the transfer of the 4'-phosphopantetheine moiety from CoA to apo-ACP that is required to form holo-ACP (the holo-ACP synthase reaction) (14). The *panB acpS* mutant is a pantothenate auxotroph that requires unusually high levels of pantothenate for growth (14). The *acpS* phenotype can only be observed when the strain also carries a defect in pantothenate biosynthesis (*panB*). The increased pantothenate concentration is thought to be needed to produce a high intracellular CoA concentration, which overcomes a CoA binding defect in the mutant holo-ACP synthase (14). The *panB acpS* mutant synthesizes ACP (albeit at a two- to threefold-lower rate than does a *panB acpS*⁺ strain) at high external pantothenate concentrations (7, 14), but is severely defective in ACP synthesis at low pantothenate concentrations (14) and accumulates apo-ACP, the form lacking the prosthetic group (7). In contrast, the synthesis of CoA proceeds normally at both high and low pantothenate concentrations in both the mutant and its parent (14). The *panB acpS* mutant therefore provides a means to decrease the cellular ACP content without a concomitant decrease in CoA content. It should be noted that some decrease in ACP levels can be obtained by starvation of a *panB acpS*⁺ strain for pantothenate, but the effect on CoA levels is much more severe (1, 6, 17). Under such conditions, the acyl-CoA synthetase is expected to function poorly in the transport of fatty acid into the cell (4), and, thus, a test of the effect of decreased ACP levels on fatty acid incorporation into phospholipid would not be valid. (Indeed, a decreased rate of oleic acid incorporation [$<50\%$ of normal] was observed in cells depleted [to ca. 1 pmol/ 10^8 cells] of CoA by pantothenate starvation [data not shown].)

The key experiment in this work was to grow strain MP4 (*panB acpS*) for several generations on a medium with succinate as a carbon source and a high concentration (25 μ M) of [3 H]pantothenate (1 Ci/mmol) (14). The cells were then collected, washed with medium lacking pantothenate, and resuspended in the succinate medium containing a low level (0.25 to 0.5 μ M) of [3 H]pantothenate at the same

specific activity. This pantothenate concentration is sufficient for normal growth of strain MP3, the *acpS*⁺ *panB* strain, but the level of ACP in the *acpS* *panB* strain MP4 drops rapidly from its already abnormally low level, and growth ceases after 1.5 to 2 generations (14). Every 30 to 40 min after shift to low pantothenate, a cell sample (4 ml) was collected, the cells were lysed, and the intracellular levels of CoA and ACP were determined by the amount of [³H]pantothenate incorporation into these compounds. A second sample was exposed to [1-¹⁴C]oleic acid (0.1 mM; 5.7 mCi/mol) for 10 to 20 min. The phospholipids were extracted, and the incorporation of exogenously added oleic acid phospholipid was then determined by thin-layer chromatography (13).

The incorporation of [³H]pantothenate into CoA and ACP was determined by chromatography of cell extracts on small columns of DEAE-cellulose followed by scintillation counting of column fractions (6, 14). It should be noted that the ACP value included any acyl-ACP present (7, 15). To assay incorporation of [1-¹⁴C]oleic acid into *E. coli* phospholipids, the cellular phospholipids were extracted. The phospholipids and unincorporated fatty acids were then separated by thin-layer chromatography followed by scintillation counting of the appropriate areas of silica gel. Identical results were obtained by assaying esterified fatty acids by sodium methoxide-catalyzed transmethylation (13). Oleic acid incorporation into phospholipid was linear with time and was done at a fatty acid concentration (0.1 mM) sufficient to saturate the rate of incorporation into phospholipid (4, 9, 12).

Several aspects of the experiment require mention. First, both strains MP3 and MP4 carried a lesion (*fadE*) in β -oxidation to prevent complications due to competition between β -oxidation and incorporation into phospholipid (11). Second, the carbon source was succinate rather than the glucose previously used (14) to prevent catabolite repression of expression of the *fadL* and *fadD* genes (9, 11, 12). The level of fatty acid transport did not limit the rate of incorporation of fatty acids into phospholipid because introduction of a *fadR* lesion (to give a constitutive level of the transport proteins [9, 11, 12]) into strain MP3 gave no increase in the rate of incorporation into phospholipid (data not shown).

The results of several experiments are shown in Fig. 1, where the rate of oleic acid incorporation is plotted versus the intracellular content of CoA (Fig. 1A) or ACP (Fig. 1B). The data show that the cellular ACP level can be reduced to $\leq 10\%$ of the normal level without a significant decrease in the rate of oleic acid incorporation into phospholipid. It should be noted that Loewen (10) showed that the intracellular levels of CoA in *E. coli* depended on the carbon source used; fermentable carbon sources generally gave three to fourfold-higher levels of CoA than did nonfermentable sources, a result consistent with the present (Fig. 1) and previous (14) data on strains MP3 and MP4.

A caveat to the finding that the ACP pool in strain MP4 can be greatly decreased without affecting incorporation of exogenous fatty acids into phospholipid is that some ACP remains in these cells. Thus, a pathway with a high affinity for ACP could still proceed since the concentration present in strain MP4 would not be rate limiting. An argument against this possibility is that neither of the two enzymes involved in the putative ACP-requiring reaction sequence has a particularly high affinity for its oleic acid substrate as assayed in vitro. The Michaelis constants of acyl-ACP synthetase for oleic acid (20 to 30 μ M) (15, 17) and of *sn*-glycerol-3-phosphate acyltransferase for oleoyl-ACP (26 μ M)

FIG. 1. The rate of oleic acid incorporation into phospholipid is plotted versus the intracellular levels of either CoA (A) or ACP (B). The rates of transport are normalized to those of a parallel culture of strain MP3 growing in excess pantothenate and varied between 12 and 19 pmol incorporated per min per 10⁸ cells in different experiments. The open circles are data from strain MP4 (*panB6 acpS*⁻), and the solid circles are data from strain MP3 (*panB6 acpS*⁺). The medium was minimal medium E (24) supplemented with 0.4% sodium succinate, 0.05% vitamin-free casein hydrolysate, 1 μ g of thiamine per ml, and 1 μ g of biotin per ml. The doubling times (37°C with vigorous aeration) were about 85 and 105 min for strains MP3 and MP4, respectively. Upon shift to a low pantothenate concentration (0.25 to 0.5 μ M), cell growth continued at the pre-shift rates in strains MP3 and MP4, except that growth of strain MP4 began to slow at 1.5 to 2 h after shift and ceased at about 0.5 h thereafter. Strains MP3 and MP4 are F⁻ strains carrying the well-characterized *panB6* and *fadE62* mutations. Both strains also carry a Δ (*bio-gal*) deletion.

(19) are not particularly low; thus, if these enzymes were involved (and the in vitro constants are an accurate indication of the in vivo situation), the low levels of ACP obtained in strain MP4 (about 15 μ M) would be expected to result in a decreased rate of incorporation into phospholipid. It should also be noted that oleoyl-ACP is a poor acyltransferase substrate in vitro (19), whereas oleoyl-CoA is reported to be an excellent substrate (5, 18).

I thank S. Jackowski and C. O. Rock for a preprint of their recent work (7).

This work was supported by research grant AI15650 from the National Institute of Allergy and Infectious Diseases.

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