ENZYMATIC SYNTHESIS OF ACYL–ACYL CARRIER PROTEIN AND ASSAY OF ACYL CARRIER PROTEIN

Allen K. SPENCER+, A. D. GREENSPAN and John E. CRONAN, jr*
Departments of Molecular Biophysics and Biochemistry and +*Chemistry Yale University, New Haven, CT 06520* and
**Department of Microbiology, University of Illinois, Urbana, IL 61801, USA*

Received 28 February 1979
Revised version received 20 March 1979

1. Introduction

Acyl carrier protein (ACP) of *Escherichia coli* is a small protein of 77 residues to which is attached a prosthetic group, 4-phosphopantetheine (reviewed in [1]). The amino acid sequence of ACP is known [2] and its central role in fatty acid biosynthesis has been well documented [1]. Most of the fatty acid biosynthetic enzymes are active only on acyl-ACP substrates [1] and thus such substrates are a requirement for the study of these enzymes. Chemical methods have been developed for the synthesis of acyl-ACP derivatives and the use of these substrates have given considerable information [1]. However, the interpretation of these data is complicated because the amino groups of the ACP protein moiety as well as the prosthetic group sulfhydryl have been acylated. Furthermore, these modifications have been shown to have rather profound effects on the structure of the protein (reviewed in [3,4]). Recently, an alternative to chemical synthesis became available, the acyl-ACP synthetase activity of *E. coli*. Using this enzyme, acyl-ACP derivatives have been synthesized [3–5]. However, the yields based on ACP were rather low and we, therefore, examined the factors that limit the yield of acyl-ACP. Here we report an improved preparative method for the synthesis of acyl-ACP derivatives and also demonstrate that acyl-ACP synthetase can be used for the assay of ACP.

2. Materials and methods

2.1. *Preparation of acyl-ACP synthetase*

Cells of the *E. coli* K12 strains, Ymel or TR3, were grown and harvested as in [3,4]. The cells were disrupted by passage through a French pressure cell and centrifuged at 30 000 × *g* for 30 min. Solid sucrose (0.8 g/ml) was added to the supernatant either directly or after a 4-fold concentration by ultrafiltration. This solution (8 ml/tube) was overlaid first with 5 ml 40% (w/w) sucrose (1.18 g/ml) in 10 mM Tris–HCl (pH 8.0) 10 mM 2-mercaptoethanol from the top, then with a 0.2–0.4 ml layer of 30% (w/w) sucrose (1.13 g/ml) in the same buffer. The tubes were centrifuged for 60 h at 200 000 × *g* in an SW40 Ti rotor. After centrifugation, a third of the original acyl-ACP synthethase activity, functionally free of contaminating outer membranes, was recovered by removing the top 1.5 ml solution with a pipet. The acyl-ACP synthetase assay is that in [3]. One unit of activity is 1 nmol fatty acyl-ACP formed per minute; a 5 l culture yields 100–150 units. ACP was purified essentially as in [6].

2.2. *Assay of acyl-carrier protein*

The reaction mixture is prepared by mixing (in order) 5 μl 0.1 M ATP solution (titrated to pH 7 with NaOH), 10 μl 48 μg/ml [U-^{14}C]-palmitic acid (55 mCi/mmol) in 10 mM Tris–Cl (pH 8.0, containing 2% Triton X-100), 5 μl 0.5 M $MgCl_2$ and 10 μL 0.8 M $CaCl_2$. Just before use, 0.50 μl of an acyl-ACP synthetase preparation (1.0 unit/ml to give 0.5 unit/ml final conc.) is added to the reaction mix.

The samples to be assayed for ACP are reduced by addition of an equal volume a freshly prepared solution of 5 mM dithiothreitol in 1.0 M Tris–Cl (pH 8.0), followed by incubation at 37°C for 10 min. A 20 μl

portion of the reduced sample is then added to 80 μl of the complete reaction mix and the mixture is incubated at 37°C for 1 h. After incubation 80 μl of each assay mixture is pipetted onto a filter paper disc (Whatman 3 MM, 2.4 cm diam.). The disc is air-dried then washed twice in methanol–chloroform–acetic acid (6:3:1, v/v/v) to remove free palmitic acid and counted in a scintillation counter [3].

3. Results

In attempting to synthesize acyl-ACP using acyl-ACP synthetase we noticed a perplexing characteristic of the acyl-ACP synthetase reaction (fig.1). Although each of the various enzyme preparations had the same amount of synthetase activity as judged by assay at low enzyme concentrations (0.01–1 U/ml), at the high enzyme concentrations used in the preparative reactions there was a wide variation in the time course of the reaction and in the yield of acyl-ACP. For example, a crude lysate converted only ~10% of the ACP to acyl-ACP and most of the acyl-ACP formed was subsequently degraded whereas a 30 000 × *g* supernatant gave a yield of > 30% with less subsequent degradation of acyl-ACP (fig.1). We found that addition of benzamidine–HCl (0.1 M), a protease inhibitor, to reaction mixtures increased the yield of acyl-ACP (data not shown). Since several *E. coli* proteases are outer membrane enzymes, we prepared purified inner membranes. Inner membranes purified by isopycnic sucrose gradient centrifugation gave much higher yields of acyl-ACP than more crude preparations, but appreciable degradation of acyl-ACP still occurred (fig.1). We therefore designed the flotation technique described here to prepare extremely pure inner membranes. These inner membrane preparations give yields of acyl-ACP of > 90% with no detectable loss of acyl-ACP (fig.1). If these inner membrane preparations were intentionally contaminated with purified outer membrane, the yields of acyl-ACP were decreased.

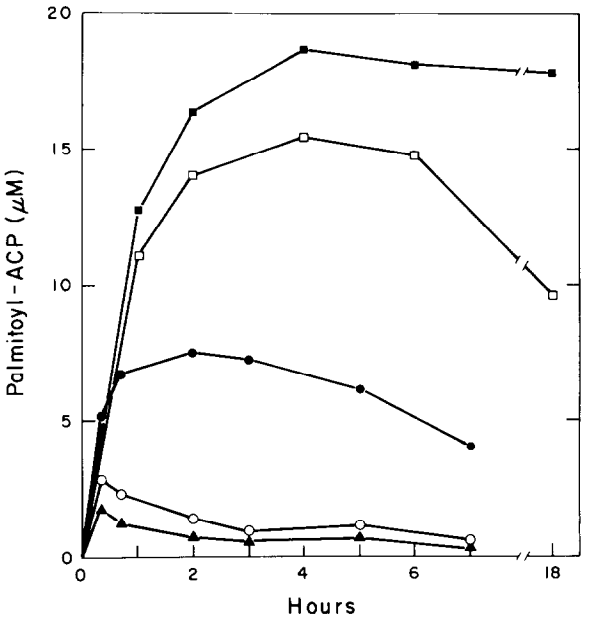

Fig.1. Time course at 37°C of acyl-ACP accumulation by various cellular fractions. All fractions contained the same final concentration of acyl-ACP synthetase activity (0.5 U/ml), ACP (20 μM), [^{14}C]-palmitic acid (20 μM), $MgCl_2$ (40 mM), $CaCl_2$ (50 mM), Triton X-100 (0.2%), dithiothreitol (0.5 mM), ATP (10 mM) and Tris–Cl (100 mM, pH 8.0). The fractions tested were: (▲) crude extract; (○) low speed supernatant of crude extract; (●) high speed supernatant of crude extract; (□) inner membranes from isopycnic sucrose gradient; (■) inner membranes purified by flotation. The last 12 h of the 19 h incubation were done at 22°C. The low and high speed supernatants result from centrifugation at 12 500 × *g* for 10 min or 30 000 × *g* for 30 min, respectively.

3.1. *Assay of ACP-SH*

The virtually quantitative conversion of ACP to acyl-ACP, the simplicity of the acyl-ACP synthetase assay, and the stability of the enzyme suggested that acyl-ACP synthetase could be a useful system for the assay of ACP. As shown in fig.2, the amount of acyl-ACP synthesized is a linear function of the amount of ACP over a wide concentration range. The background (minus ACP) of the reaction is reproducibly low (70 ± 10 cpm, $n = 8$), thus allowing measurement of as little as 2 ng ACP-SH. These results were obtained using inner membranes purified by isopycnic sucrose gradient centrifugation. However, ACP can also be assayed using a crude (30 000 × *g*) supernatant as the source of acyl-ACP synthetase (fig.3). Due to the increased background (resulting from endogenous ACP) the reaction is less sensitive, and since the extent of acylation is not stoichiometric, the reaction gives only relative values unless standardized with pure ACP. We have monitored the purification of

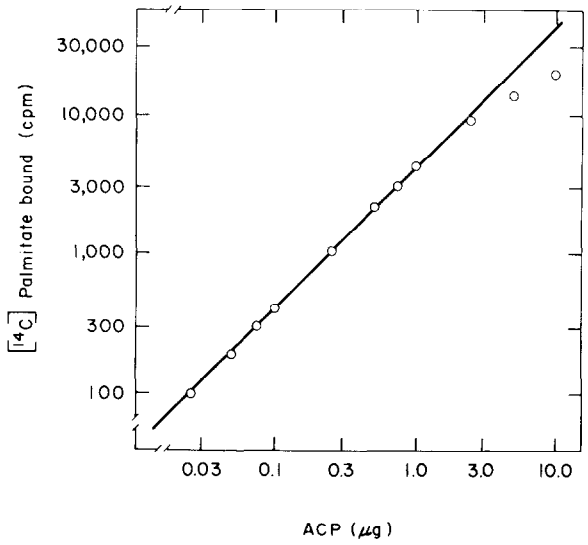

Fig.2. Assay of ACP using inner membrane acyl-ACP synthetase. ACP was assayed using inner membrane purified on isopycnic sucrose gradients as in section 2. The values plotted are the log of cpm above background (70 ± 10 cpm) versus the log of the ACP concentration and represent > 90% conversion of ACP to acyl-ACP.

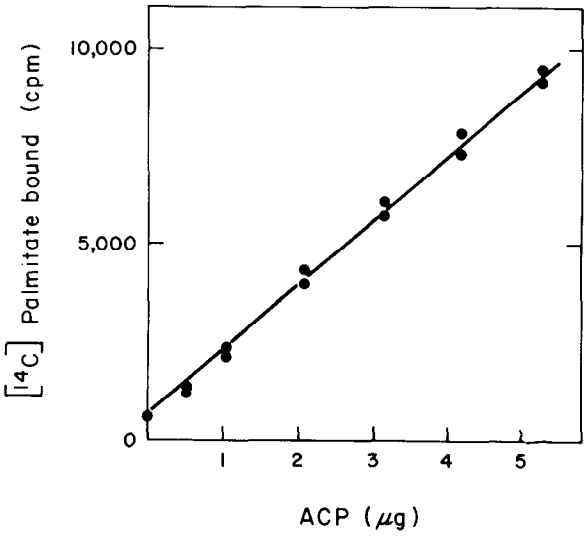

Fig.3. Assay of ACP using crude acyl-ACP synthetase. ACP was assayed using a high speed supernatant (fig.1) as in section 2.

Table 1
Purification of ACP via the acyl-ACP synthetase assay

Step	μg ACP/mg protein	mg ACP
Crude extract	2.1	322
$(NH_4)_2SO_4$ supernatant	6.9	138
Redissolved acid precipitate	21.5	112
DEAE-cellulose	120.0	72
DEAE–Sephadex A25	1000.0	80

The method used for ACP purification is that in [6]. Each sample was diluted 10-fold in 10 mM Tris–HCl (pH 8.0) containing 10 mM 2-mercaptoethanol (freshly prepared) and assayed as in section 2. *E. coli* B cells (1 kg frozen paste) was the source of ACP. The portion of the crude extract to be assayed was centrifuged at 160 000 × *g* for 5 h to remove inhibitory material (presumably outer membrane). Inhibition was monitored by addition of a known amount of pure ACP. The expected increment in ACP concentration was obtained at all steps. However, inhibition was observed in the crude extract that had not been cleared of outer membranes or if the $(NH_4)_2SO_4$ supernatant was not diluted before assay. Protein was determined by the Lowry method [9]

ACP using the acyl-ACP synthetase assay (table 1). Under the proper conditions the assay functioned well at each step of the purification as determined by internal standardization. The protein was purified ~500-fold to homogenity using only this assay.

4. Discussion

Acyl-ACP substrates can be synthesized with very good efficiency using purified inner membranes as a source of acyl-ACP synthetase. The inefficiency of more crude preparations can be attributed to contamination with outer membranes. Outer membrane contamination probably also explains the failure to detect acyl-ACP synthetase activity in 50 000 × *g* pellets of *E. coli* [3]. Acyl-ACP substrates can also be synthesized using the purified acyl-ACP synthetase [7] and can be separated from ACP via hydrophobic chromatography [8].

Acyl-ACP synthetase also provides an alternative to the malonyl-CoA–CO_2 exchange reaction [6] as an assay for ACP. The exchange reaction requires frequent purification of short-lived enzyme fractions (with the resultant problems of reproducibility) and the chemical synthesis of several substrates. The

specific activity of radioactive substrate used in the exchange reaction ($^{14}CO_2$) is also difficult to control and thus frequent standardization is necessary for quantitative results. In contrast, the acyl-ACP synthetase activity of purified inner membranes is stable to storage for > 1 year, all the components of the reaction are commercially available, and if a pure inner membrane preparation is used, the assay does not require standardization.

Acknowledgements

The work was supported by NIH grants AI 10186 and AI 15650 from the National Institute of Allergy and Infectious Diseases.

References

[1] Prescott, D. J. and Vagelos, P. R. (1972) Adv. Enzymol. 36, 269–309.
[2] Vanaman, T. C., Wakil, S. J. and Hill, R. L. (1968) J. Biol. Chem. 243, 6420–6431.
[3] Ray, T. K. and Cronan, J. E., jr (1978) Proc. Natl. Acad. Sci. USA 73, 4374–4378.
[4] Spencer, A. K., Greenspan, A. D. and Cronan, J. E., jr (1978) J. Biol. Chem. 253, 5922–5926.
[5] Gally, H.-U., Spencer, A. K., Armitage, I., Prestegard, J. H. and Cronan, J. E., jr (1978) Biochemistry 17, 5377–5382.
[6] Majerus, P. W., Alberts, A. W., and Vagelos, P. R. (1969) Methods Enzymol. 14, 43–50.
[7] Rock, C. O. and Cronan, J. E. jr (1979) J. Biol. Chem. in press.
[8] Rock, C. O. and Garwin, J. L. (1979) J. Biol. Chem. in press.
[9] Lowry, O. H., Rosebrough, N. J., Fan, A. L., and Randall, P. J. (1951) J. Biol. Chem. 193, 263–275.