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Isolation and Properties of Acyl Carrier Protein Phosphodiesterase of *Escherichia coli*

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The acyl carrier protein (ACP) phosphodiesterase of *Escherichia coli* catalyzes the hydrolytic cleavage of the 4′-phosphopantetheine residue from ACP, with the generation of apo-ACP (P. R. Vagelos and A. R. Larrabee, J. Biol. Chem. 242:1776–1781, 1967). Although it has been postulated to play a role in the regulation of fatty acid synthesis, presently available evidence makes this unlikely, and its physiological function requires further investigation. We have now purified the enzyme from *E. coli* more than 3,000-fold and have identified it as a protein of M_r 25,000, as judged from its migration during electrophoresis in gels containing sodium dodecyl sulfate. The enzyme has remarkable thermostability, being protected against irreversible inactivation at 90°C by the presence of sodium dodecyl sulfate. A partial sequence of the amino terminus of the enzyme is as follows: H_2N-Ser-Lys-Val-Leu-Val-Leu-Lys-Ser-?-Ile-Leu-Ala-Gly-Tyr-Ser-. Other properties of the enzyme are also described.

The acyl carrier protein (ACP) of *Escherichia coli* is a small, anionic protein that functions as an essential component of enzyme systems for the biosynthesis of fatty acids (17), membrane phospholipids (4), and lipopolysaccharides (1). Each of these functions involves an acyl residue linked to the sulfhydryl group of a phosphopantetheine residue covalently bound to serine 36 through a phosphodiester bond. A very different function for the ACP of *E. coli* in the enzymatic synthesis of membrane-derived oligosaccharides from UDP-glucose was reported by Therisod et al. (16); this function in the biosynthesis of a cell surface carbohydrate does not require the phosphopantetheine prosthetic group (15).

In cells of *E. coli*, the phosphopantetheine residue turns over more rapidly than the protein moiety of ACP (7, 12). Vagelos and Larrabee (18) discovered an enzyme (ACP phosphodiesterase) in *E. coli* that specifically cleaves the phosphopantetheine residue from holo-ACP, with the formation of free phosphopantetheine and apo-ACP. It presumably accounts for the turnover of the phosphopantetheine residue, but the physiological role of this enzyme remains poorly understood. It was originally thought that the enzyme might regulate the synthesis of fatty acids by controlling the amounts of holo-ACP needed for that process, but this hypothesis is rendered less likely by the findings of Jackowski and Rock (6), who showed that the ratio of holo-ACP to apo-ACP is always high in cells of *E. coli* and cannot be correlated to rates of lipid biosynthesis.

The possibility of enzymatic functions of apo-ACP, as well as of holo-ACP, must now also be considered, as indicated above. Furthermore, species of *Rhizobium* may contain two distinct forms of ACP, one constitutive and another the inducible product of the *nodF* gene (14). A recent report by Bibb et al. (2) offers evidence that a specific form of ACP in *Streptomyces glaucescens* performs a specific function in the biosynthesis of polyketides. These discoveries point to the need for further information about the enzymatic machinery for transfer of the phosphopantetheine residue to and from different forms of ACP which may carry out different functions within the same cell.

In this paper, we report the purification of the ACP phosphodiesterase of *E. coli* more than 3,000-fold, its identification as a protein of apparent M_r 25,000, and other properties of the enzyme. As an aid to the development of probes for later genetic studies, we also report a partial amino-terminal sequence of the enzyme.

MATERIALS AND METHODS

Materials. Reactive blue 2-Sepharose CL-6B, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), and phenylmethylsulfonyl fluoride were obtained from Sigma Chemical Co.; Sepharose 4B, Sephadex G-50, and blue dextran were from Pharmacia; leupeptin and pepstatin were from Boehringer Mannheim Biochemicals; and DEAE-cellulose (DE52) was from Whatman, Inc. Reagents for electrophoresis and protein standards were obtained from Bio-Rad Laboratories; β-[^{3}H-(*N*)]alanine was from Dupont, NEN Research Products.

Preparation of labeled ACP. ACP labeled in the phosphopantetheine residue was prepared by growth of *E. coli* SJ16 *panD* in medium containing ^{3}H-labeled β-alanine as described by Jackowski and Rock (5). The labeled ACP was purified by a procedure based on that of Therisod et al. (16).

Assay of ACP phosphodiesterase. Activity was measured by monitoring the release of phospho-[^{3}H]pantetheine, soluble in trichloroacetic acid, from labeled ACP in a procedure based on that of Vagelos and Larrabee (18). The standard assay system contained 50 mM Tris hydrochloride (pH 8.5), 0.02 mM $MnCl_2$, 25 mM $MgCl_2$, 1 mM dithiothreitol, 5 to 15 μM tritiated ACP (30,000 cpm/nmol), and enzyme in a final volume of 0.1 ml. After incubation at 35°C, bovine serum albumin (10 ml of a 5% [wt/vol] solution) was added, followed by 20 ml of 20% (wt/vol) trichloroacetic acid. The sample was mixed and centrifuged in a Beckman Microfuge for 5 min. Phospho-[^{3}H]pantetheine released from the labeled ACP was measured by counting 0.10 ml of supernatant solution. ACP phosphodiesterase activity was linear with time and with added enzyme. A unit was defined as the

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amount of enzyme that catalyzed the formation of 1 pmol of product per min.

Determination of protein. The method of Lowry et al. (9) or of Bradford (3) was used to determine protein concentration, with bovine serum albumin as the standard.

Electrophoresis. Analytical polyacrylamide gel electrophoresis in buffer containing sodium dodecyl sulfate (SDS) was performed as described by Laemmli (8), with 12% (wt/vol) acrylamide and 2.7% (wt/vol) bisacrylamide in running gels and 4% (wt/vol) acrylamide and 2.7% (wt/vol) bisacrylamide in stacking gels. Samples for electrophoresis were first treated at 100°C for 2 min in buffer containing 62.5 mM Tris hydrochloride (pH 6.8), 2% (wt/vol) SDS, 10% (wt/vol) glycerol, and 5% (wt/vol) 2-mercaptoethanol. The electrophoresis buffer (pH 8.3) contained 25 mM Tris, 0.192 M glycine, and 0.1% (wt/vol) SDS. Gels were stained with Coomassie blue. M_r values of protein standards were as follows: phosphorylase *b*, 97,400; bovine serum albumin, 66,200; ovalbumin, 42,700; carbonic anhydrase, 31,000; soybean trypsin inhibitor, 21,500; and lysozyme, 14,400.

Preparation of blue dextran-Sepharose. Blue dextran-Sepharose was prepared as described by Ryan and Vestling (13). The procedure was modified by activating Sepharose 4B with cyanogen bromide in acetonitrile as described by March et al. (10).

RESULTS

Purification of ACP phosphodiesterase. All procedures were carried out at 4°C unless otherwise stated.

(i) Step 1: preparation of cell extract. Frozen cells (Grain Processing Co.) of *E. coli* K-12 (100 g) were suspended in 500 ml of buffer A (50 mM Tris hydrochloride [pH 7.5], 2 mM $MnCl_2$, 10 mM $MgCl_2$, 5 mM 2-mercaptoethanol, 10% [wt/vol] glycerol, 1 mM pepstatin, 1 mM leupeptin, 0.2 mM phenylmethylsulfonyl fluoride) and broken by a single passage through a French pressure cell. Unbroken cells and debris were removed by centrifugation at 3,000 × *g* for 20 min. The suspension was centrifuged at 38,000 × *g* for 90 min to obtain the cell extract.

(ii) Step 2: ammonium sulfate fractionation. The cell extract was treated with streptomycin sulfate (0.2 mg/mg of protein) to remove nucleic acids as described by Vagelos and Larrabee (18) before fractionation with ammonium sulfate. After removal of the streptomycin-nucleic acid precipitate by centrifugation, ammonium sulfate (114 mg/ml of supernatant solution) was added, and the suspension was stirred for 40 min. The precipitate was removed by centrifugation and discarded. Solid ammonium sulfate was added to the supernatant (157 mg/ml), and after 40 min of stirring, the precipitate was collected by centrifugation. This fraction, containing proteins precipitated between approximately 0.25 and 0.50% saturation with ammonium sulfate, was suspended in buffer A and stored at −80°C.

(iii) Step 3: blue dextran-Sepharose chromatography. A portion of the ammonium sulfate fraction of step 2 was prepared for chromatography on blue dextran-Sepharose by dilution with four volumes of buffer B (50 mM morpholine propanesulfonic acid [MOPS; pH 7.0], 2 mM $MnCl_2$, 10 mM $MgCl_2$, 5 mM 2-mercaptoethanol, 10% [wt/vol] glycerol, 1 mM pepstatin, 1 mM leupeptin, 200 mM phenylmethylsulfonyl fluoride). The extract (520,000 U of ACP phosphodiesterase) was applied to a 50-ml column (10 by 2.5 cm) of blue dextran-Sepharose that had been equilibrated in buffer B. The column was washed successively with 100 ml of buffer B, 500 ml of buffer B containing 0.5 M NaCl, and 200 ml of buffer B containing 1 M NaCl. Little activity was contained in these wash fractions. The phosphodiesterase activity was then eluted from the column with buffer B containing 2 M NaCl, and active fractions were pooled and stored at −80°C.

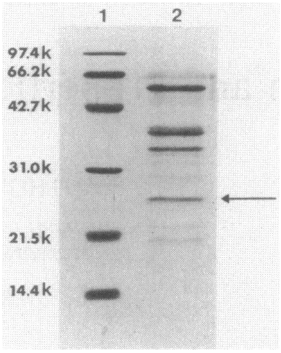

FIG. 1. Electrophoresis of ACP phosphodiesterase purified through step 5 in gels containing SDS (see Materials and Methods). Lanes: 1, standard proteins for calibration, with indicated values of M_r (in thousands [k] shown on the left); 2, partially purified ACP phosphodiesterase. Arrow marks the position of protein of M_r 25,000 identified as enzyme.

(iv) Step 4: chromatography on octyl-Sepharose. Enzyme from step 3 (121,000 U) was applied to a column of octyl-Sepharose (5 by 2.5 cm) that had been equilibrated in buffer A. The column was washed with 250 ml of buffer A. ACP phosphodiesterase activity was then eluted from the column with 200 ml of buffer A containing 1% (wt/vol) Triton X-100. Active fractions were pooled and stored at −80°C.

(v) Step 5: chromatography on reactive blue-Sepharose. Enzyme from step 4 (300,000 U) was applied to a 5-ml column of reactive blue-Sepharose that had been equilibrated in buffer A. The column was then washed successively with 100 ml of buffer A, 100 ml of buffer A containing 3 M NaCl, and again with 100 ml of buffer A. The enzyme was then eluted in a sharp peak from the column with buffer A containing 0.5 M NaCl and 10 mM CHAPS. Fractions containing ACP phosphodiesterase were pooled and stored at −80°C. SDS electrophoresis of the enzyme at this stage of the purification always revealed the presence of a protein of apparent M_r 25,000 (Fig. 1) together with other proteins whose sizes and relative amounts varied from batch to batch. To determine whether the protein of apparent M_r 25,000 was in fact the ACP phosphodiesterase, a vertical strip was cut from an SDS-gel such as that in Fig. 1 and divided into 0.5-cm horizontal slices. Each gel slice was homogenized in 0.5 ml of buffer A containing 10 mM CHAPS, held on ice for 30 min, and centrifuged to remove the acrylamide. A portion of the supernatant solution was diluted and assayed for phosphodiesterase activity. Only the slice corresponding to the band at M_r 25,000 was associated with ACP phosphodiesterase activity (Fig. 2).

(vi) Step 6: preparative SDS-polyacrylamide gel electrophoresis. A portion (0.5 ml) of the enzyme from step 5 was subjected to preparative electrophoresis in buffer containing 0.1% (wt/vol) SDS essentially as described in Materials and Methods but with a gel of 1.5-mm thickness. A thin vertical strip was cut from the gel and stained with Coomassie blue to localize the band at M_r 25,000. The phosphodiesterase was then recovered from the corresponding region of the preparative gel with an electroelution device (Elutrap; Schleicher & Schuell, Inc.). Recovery was about 45% of the units applied to the gel. Enzyme purified through step 6 was stored at 4°C.

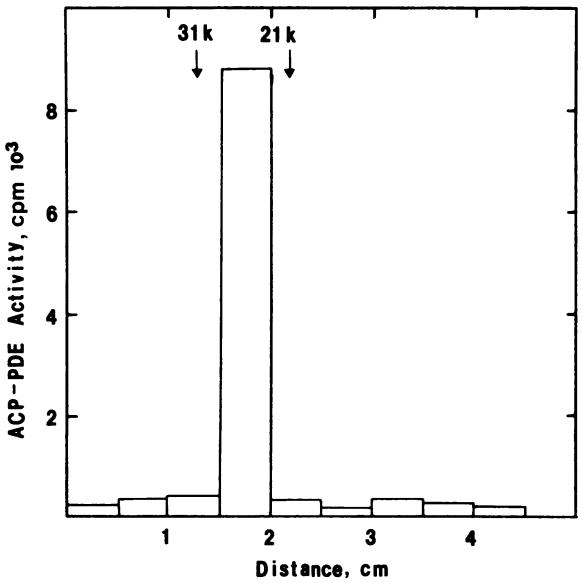

FIG. 2. Distribution of enzyme activity in SDS gel. A strip from a gel essentially like that of Fig. 1 was cut into horizontal strips, which were then assayed for their content of ACP phosphodiesterase as described in the text. M_r values are indicated in thousands (k). PDE, Phosphodiesterase.

A summary of the purification of ACP phosphodiesterase is shown in Table 1. The enzyme was purified 3,000-fold through step 5 and further purified by preparative electrophoresis, but the amount of protein recovered was too small for accurate measurement.

Amino-terminal sequence. The band of ACP phosphodiesterase localized on an SDS-gel as in the experiment of Fig. 1 was transferred to a polyvinylidene difluoride membrane in 10 mM 3-(cyclohexcylamino)-1-propanesulfonic acid buffer (pH 11) containing 10% (vol/vol) methanol as described by Matsudaira (11) and subjected to automated Edman degradation sequence analysis in the Microchemical Facility of the Harvard Biological Laboratories. The sequence of the first 15 residues from the amino terminus is given in Table 2.

Identification of phosphopantetheine released from ACP. Unlabeled 4′-phosphopantetheine was prepared by partial acid hydrolysis of coenzyme A, followed by purification by chromatography on DEAE-cellulose, essentially as described by Jackowski and Rock (5). The labeled, trichloroacetic-soluble product obtained by treatment of labeled ACP with the phosphodiesterase was mixed with unlabeled 4′-phosphopantetheine. Chromatography of the labeled product was indistinguishable from that of the carrier on DEAE-cellulose and on thin-layer chromatography on a cellulose-coated plate (Polygram CEL 300; Macherey Nagel), developed with a mixture of ethanol and 0.5 M ammonium acetate. The R_f in this system was 0.83. In confirmation of the identification of the labeled product as a phosphomonoester, it was found to be converted to a neutral product, no longer adsorbed on DEAE-cellulose, by treatment with purified alkaline phosphatase from *E. coli*.

TABLE 1. Purification of ACP phosphodiesterase

Step	Protein (mg)	U	Sp act	Purification factor	Yield (%)
1. Extract	9,905	495,240	50	1	100
2. Ammonium sulfate	1,743	520,000	298	6	105
3. Blue dextran	4.7	121,340	25,817	516	25
4. Octyl-sepharose		300,400			61
5. Reactive blue-Sepharose	1.02	153,247	150,252	3,005	31
6. Preparative SDS-polyacrylamide gel electrophoresis		68,962			14

TABLE 2. Sequence of the amino terminus of ACP phosphodiesterase

Cycle	Residue	Yield (pmol)
1	Ser	54.9
2	Lys	5.9
3	Val	5.9
4	Leu	15.0
5	Val	3.4
6	Leu	4.6
7	Lys	2.7
8	Ser	6.2
9		
10	Ile	3.1
11	Leu	4.8
12	Ala	2.3
13	Gly	2.5
14	Tyr	1.3
15	Ser	4.4

Effect of detergents on enzyme activity. The addition of certain detergents to the standard assay system caused a two- to threefold stimulation of activity (Fig. 3). The response to the addition of Triton X-100 was distinctly biphasic, with optimum stimulation at a final concentration of 62.5 mg/ml in the assay system. Optimal stimulation by CHAPS was at a final concentration of 2.9 mg/ml in the assay system.

SDS in the assay system at levels of 10 to 200 μg/ml was strongly inhibitory. At levels of less than 10 μg/ml, in the presence of CHAPS (2.9 mg/ml) SDS had little effect.

Thermal stability. The optimum temperature for assay of

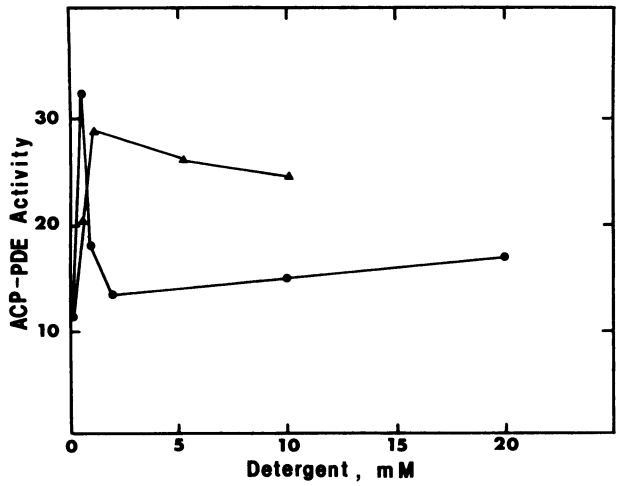

FIG. 3. Effect of detergents on enzyme activity. CHAPS or Triton X-100 was added to the standard assay system at the concentrations indicated. PDE, Phosphodiesterase.

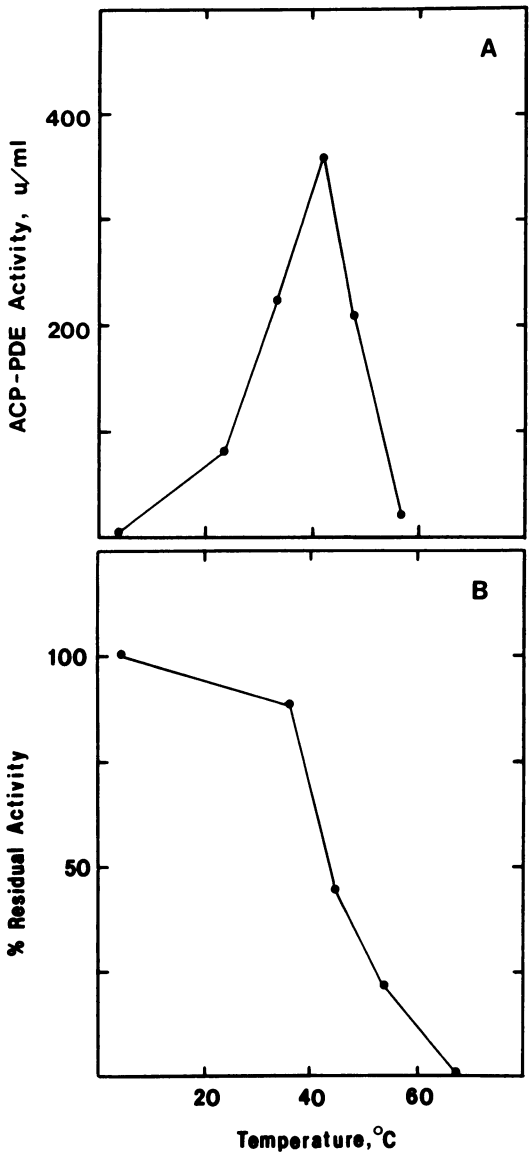

FIG. 4. (A) Effect of varying the temperature of the standard assay system; (B) effect of prior heat treatment on activity of ACP phosphodiesterase (ACP-PDE). Enzyme purified through step 5 was incubated for 15 min at the indicated temperatures in 25 mM Tris hydrochloride buffer (pH 7.5) containing 0.1 mM $MnCl_2$ and 10 mM $MgCl_2$. After treatment, the samples were held in an ice bath for 20 min before assay.

ACP phosphodiesterase was found to be 42°C (Fig. 4A). The enzyme lost all activity if heated prior to assay to 60°C in the absence of detergent (Fig. 4B).

The rapid loss of activity at 60°C was inconsistent with the high recovery of activity from SDS-gels, because preparation of the sample for electrophoresis in such gels involves heating with SDS at 100°C (see above). Surprisingly, the activity was strongly stabilized against heat inactivation by SDS. In the absence of SDS, the enzyme lost all activity in less than 1 min at 90°C. In the presence of SDS (1% [wt/vol]), 80% of the activity was retained after 10 min. (In experiments with SDS, the enzyme was diluted before assay to reduce the concentration of SDS in the assay system.) The addition of Triton X-100 or CHAPS to enzyme extracts

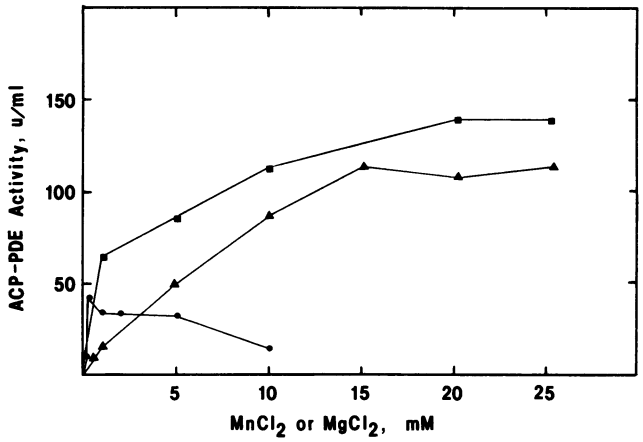

FIG. 5. Effect of added manganese and magnesium on ACP phosphodiesterase (ACP-PDE) activity. Enzyme purified through step 5 was equilibrated with 25 mM Tris hydrochloride buffer (pH 7.5) by passage over a column of Sephadex G-25. The enzyme was assayed at the indicated final concentrations in the assay system of $MnCl_2$ (●) or $MgCl_2$ (▲). The concentration of $MgCl_2$ was also varied in the presence of 0.02 mM $MnCl_2$ (■).

before heat treatment did not prevent the heat inactivation of the enzyme (data not shown).

Effects of Mn and Mg. Vagelos and Larrabee (18) noted the stimulation of activity by 25 mM $MnCl_2$. We have confirmed this observation and further found that low levels of manganese in combination with higher amounts of magnesium ion led to better activity than was seen with either alone (Fig. 5).

DISCUSSION

The effect of SDS, ordinarily considered a strong denaturing agent, in promoting recovery of activity of ACP phosphodiesterase heated to 90 to 100°C is a remarkable property of the enzyme. One plausible explanation of this phenomenon is that prevents irreversible aggregation of the enzyme which otherwise occurs upon heat denaturation, thus permitting the renaturation of the enzyme upon subsequent cooling. Indeed, a marked tendency of the enzyme to aggregate was noted during the course of its purification. During chromatography on DEAE-cellulose, the enzyme was not eluted in a clearly defined peak but was eluted from the column in many fractions. The addition of the detergent Triton X-100 to the elution buffer greatly improved the chromatography, and the enzyme was then recovered in a sharper single peak (data not shown). The stimulation of activity by the addition of detergents to the test system (Fig. 3) is also consistent with the notion that the enzyme readily aggregates or sticks to glass or other surfaces.

The physiological function of ACP phosphodiesterase remains obscure. There appear to be two principal alternatives. First, the enzyme may play a role in regulation of lipid metabolism by limiting the supply of holo-ACP. This suggestion has the merit of offering a direct explanation of the observed hydrolytic activity of the enzyme, but the studies of Jackowski and Rock (6) offer strong evidence that the ratio of holo-ACP to apo-ACP is always very high in *E. coli* and unrelated to the rates of synthesis of fatty acids. Perhaps the postulated regulatory function of the hydrolase is to provide a constant supply of apo-ACP. Therisod and Kennedy (15) have shown that apo-ACP can function as a cofactor for the membrane-bound transglucosylase needed

to catalyze the synthesis of the beta-1,2-glucan backbone of membrane-derived oligosaccharides in *E. coli*. However, holo-ACP works equally well in this enzyme system. As yet, there is no report of a function in which apo-ACP is active but not holo-ACP, as would be needed to rationalize the postulated role of ACP phosphodiesterase.

A second view of the ACP phosphodiesterase is based on the idea that the hydrolytic reaction that it catalyzes in the test tube is not its function in the living cell. Perhaps the enzyme transfers phosphopantetheine residues efficiently from one species of ACP to another, or to macromolecules other than ACP, and only slowly reacts with water in a hydrolytic side reaction. Evidence for multiple, distinct forms of ACP in *Rhizobium leguminosarum* (14) and in *S. glaucescens* (2) suggests that such transfer may offer a plausible route for the generation of multiple forms of ACP. This view implies that the "phosphodiesterase" is essentially a biosynthetic enzyme.

A definitive test of the function of ACP phosphodiesterase awaits the isolation of mutants in which it is defective. Because its function is not known, loss of the enzyme may be lethal. Search for temperature-sensitive conditional lethal mutations, however, is made less attractive by our finding that the enzyme has a remarkable thermostability under certain conditions. Genetic approaches dependent on recombinant DNA technology are therefore more attractive. For this reason, information on the amino-terminal sequence of the enzyme, although limited, may be helpful in designing probes for identifying and cloning the ACP phosphodiesterase gene as a preliminary to more comprehensive studies.

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