ACYL CARRIER PROTEIN FROM MYCOBACTERIUM PHLEI[1]

S. Matsumura*, D. N. Brindley**, and Konrad Bloch
James Bryant Conant Laboratory, Harvard University
Cambridge, Massachusetts 02138

Received November 10, 1969

SUMMARY

A heat-stable, 4'-phosphopantetheine-containing protein has been isolated in pure form from extracts of Mycobacterium phlei. The material has a molecular weight of 10,600 and properties similar to the acyl carrier proteins (ACP)[2] isolated from other bacterial sources.

In extracts of Mycobacterium phlei the formation of long-chain fatty acids from acetyl-CoA, malonyl-CoA and TPNH is catalyzed by a fatty acid synthetase of the multienzyme type (1). The mycobacterial synthetase has a molecular weight of 1.7×10^6, and it is exceptionally unstable in solutions of low ionic strength. To facilitate enzyme isolation, M. phlei cells were grown in the presence of ^{3}H-β-alanine as a marker for 4'-phosphopantetheine. Radioactivity was incorporated into the fatty acid synthetase and was

---

[1]Supported by grants-in-aid from the United States Public Health Service, the National Science Foundation, the Life Insurance Medical Research Fund, and the Eugene P. Higgins Trust Fund of Harvard University.

[2]The abbreviations used are: ACP, acyl carrier protein; $ACP_{E.\ coli}$ and $ACP_{M.\ phlei}$ denoting origin from Escherichia coli and Mycobacterium phlei, respectively.

*Present address: Mental Retardation Unit, Neuropsychiatric Institute, UCLA School of Medicine, Los Angeles, California 90024.

**Present address: Department of Biochemistry, University of Nottingham, England.

shown to be present in the purified multienzyme complex as alkali-releasable 4'-phosphopantetheine (1). Thus, in common with the fatty acid synthetases of yeast (2, 3) and liver (4), the M. phlei synthetase appears to contain a tightly-bound ACP-like component. However, a substantial portion of the radioactivity accompanying the crude labelled synthetase separated from enzyme activity on purification. The second radioactive fraction was eventually isolated in the form of a heat-stable protein of relatively low molecular weight. The properties of this material are those of an ACP-like molecule of the type isolated in recent years from various bacterial and plant sources (5-8).

METHODS

DEAE-cellulose DE-23 was obtained from Whatman and Sephadex G-25, G-75, G-100, and G-150 from Pharmacia. Dithiothreitol and dithionitrobenzoic acid were products of Calbiochem. ^{14}C-Malonyl-CoA, ^{3}H-acetyl-CoA, and $ACP_{E.\ coli}$ were kindly provided by Mr. J. Boullon. M. phlei ATCC-356 was grown in the presence of ^{3}H-β-alanine as described (1). Protein concentrations were determined either by the biuret reaction or spectrophotometrically (9). Polyacrylamide disc gel electrophoresis was carried out as described by Davis and Ornstein with 15% gels in Tris-glycine buffer, pH 8.3 (10). Gels were stained with 0.25% Coomassie Brilliant Blue in 10% acetic acid. Radioactive gel fractions were counted in Bray's solution with a Packard Tricarb spectrometer according to the method of Ailhaud et al. (11). The sulfhydryl content of protein fractions was determined by the procedure of Ellman (12). Amino acid analyses of protein hydrolysates were performed according to Spackman, Stein and Moore (13) in a Beckman amino acid analyzer, Model 120.

Purification of M. phlei ACP ($ACP_{M.\ phlei}$). Cells (80 g) which had been

grown in the presence of ^{3}H-β-alanine were suspended in 160 ml of ice-cold 0.01 M phosphate buffer, pH 7.0, containing 0.01 M mercaptoethanol (the buffer used throughout purification) and disrupted by sonic oscillation in the presence of glass beads. The cells were homogenized for a total of 40 min in 5-min periods with 5-min intervals for cooling. The crude homogenate was centrifuged at 15,000 x g for 30 min, the sediment washed 3 times with 50 ml portions of phosphate buffer, and supernatant and washings combined. The resulting solution was centrifuged at 105,000 x g for 60 min in a Spinco Model L ultracentrifuge. After decanting from insoluble material, solid ammonium sulfate was added to the supernatant to 70% saturation. The precipitated protein[1] was collected, dissolved in 40 ml of the phosphate-mercaptoethanol buffer, and the solution dialyzed overnight against 8 ℓ of the same buffer. After diluting it to 400 ml with buffer, the solution was heated to 90° for 5 min and then cooled rapidly in ice. Denatured protein was removed by centrifugation and the supernatant applied to a 5 x 20 cm DEAE-cellulose column. The column was washed with 300 ml of buffer and protein was eluted with 800 ml of a linear gradient of 0.1 - 0.6 M buffered NaCl. Elution of the column yielded two radioactive peaks. The first contained no protein and was discarded. The radioactive fractions comprising the second peak were pooled, dialyzed against buffer for 8 hrs, and lyophilized. The residue was dissolved in 3 ml of distilled water containing 0.01 M mercaptoethanol and passed through Sephadex G-100, previously equilibrated with buffer. Fractions of 1.2 ml were collected and those which were radioactive were pooled. The protein solution was then

[1] By contrast, ACP from other bacterial sources is not precipitated by 70% $(NH_4)_2SO_4$.

applied to a second DEAE-cellulose column (1.5 x 15 cm) and the adsorbed protein eluted by a linear gradient of NaCl in buffer. The $ACP_{\underline{M.\ phlei}}$ emerged as a symmetrical radioactive peak between 0.35 and 0.45 M NaCl. All radioactive fractions were pooled, lyophilized, and the redissolved protein dialyzed against 0.01 M phosphate buffer, pH 7.0, containing 0.01 M dithiothreitol. Isolations on the scale described normally yielded 4 mg of pure $ACP_{\underline{M.\ phlei}}$.

RESULTS

Criteria of Purity

Protein purified as described was homogeneous as judged by the following criteria. During the second DEAE chromatography radioactivity and protein emerged as coincident and symmetrical peaks (Fig. 1). Analysis of the same fractions by polyacrylamide gel electrophoresis revealed a

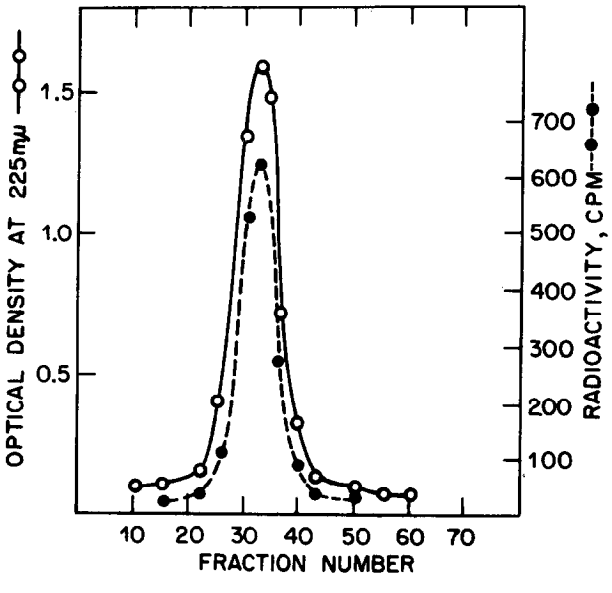

Figure 1. Elution pattern of $ACP_{\underline{M.\ phlei}}$ chromatographed on DEAE-cellulose.

single protein which contained all of the radioactivity. Finally, the protein sedimented as a single symmetrical peak in the analytical ultracentrifuge (14). Purified $ACP_{\underline{M.\ phlei}}$ did not absorb light between 240 and 300 mμ.

TABLE 1

Acid hydrolysis of $ACP_{\underline{M.\ phlei}}$

Amino acid	Amino acid residues per molecule of protein			
	24 hrs	72 hrs	Nearest integer	$ACP_{\underline{E.\ coli}}$ [a]
Lysine	5.44	5.25	5	4
Histidine	0.13	0.11	0	1
Arginine	2.93	2.82	3	1
Aspartic acid	10.27	10.73	11	9
Threonine	5.24	4.82	5	6
Serine	5.29	4.59	5	3
Glutamic acid	19.82	20.46	20	18
Proline	3.61	3.61	4	1
Glycine	6.12	5.49	6	4
Alanine	12.63	12.88	13	7
Valine	8.31	7.92	8	7
Methionine	1.02	1.07	1	1
Isoleucine	6.55	6.26	6	7
Leucine	7.36	8.29	8	5
Tyrosine	1.74	1.74	2	1
Phenylalanine	1.82	1.80	2	2
Cysteic acid [b]	0.13	0.12	0	0
Taurine [b]	0.86	0.78	1	1
β-Alanine	1.14	1.22	1	1

[a] Data from Vanaman et al., 1968 (16).

[b] Based upon performic-oxidized sample of ACP.

Amino Acid Composition

Protein hydrolyzed in 6 N HCl for 24 and 72 hrs respectively had the amino acid composition shown in the table (Table 1). The hydrolysate, after performic acid oxidation, contained β-alanine and taurine in the expected molar ratio of 1:1. Assuming one mole of 4'-phosphopantetheine per mole of protein, a molecular weight of 10,640 is calculated for $ACP_{M.\ phlei}$ in good agreement with the value of 10,450 obtained by sedimentation velocity analysis (14).

$ACP_{M.\ phlei}$ solutions were reduced with dithiothreitol and the reducing agent removed by gel filtration on Sephadex G-25. Titration of these solutions with Ellman's reagent revealed 1.10 moles of SH groups per mole of protein, in agreement with the content of 1 mole of cysteamine determined by amino acid analysis.

Identification of 4'-Phosphopantetheine

Five mμmoles of purified $ACP_{M.\ phlei}$ containing 2×10^5 dpm of ^3H were heated to 70° for 60 min at pH 12.0 (4, 15). The released radioactivity was co-chromatographed on a DEAE cellulose column with authentic 4'-phosphopantetheine (obtained by alkaline hydrolysis of $ACP_{E.\ coli}$). For further identification the radioactive DEAE fractions were incubated with pig liver dephospho-CoA pyrophosphorylase and dephospho-CoA kinase (16) and the enzymatic product identified as CoA by DEAE chromatography (4, 15). Seventy percent of the radioactivity originally associated with the $ACP_{M.\ phlei}$ samples was recovered in the form of CoA.

DISCUSSION

The heat-stable protein isolated from M. phlei extracts is conjugated with 4'-phosphopantetheine and therefore chemically related to the acyl

carrier proteins found in various bacteria and plants.

Histidine is not present in $ACP_{M.\ phlei}$. The most notable feature is the presence of 4 moles of proline per mole of protein in contrast to the single proline residue in ACP's from other bacterial sources.

In M. phlei, fatty acid synthesis is catalyzed by a multienzyme system which includes a 4'-phosphopantetheine containing component as an integral part of the complex (1). The presence of substantial amounts of free ACP in these extracts is therefore unexpected. Other cells in which fatty acid synthesis is also catalyzed by multienzyme complexes (e.g. yeast and liver), do not contain free ACP. Conversely, free ACP has heretofore been encountered only in organisms (e.g. E. coli) which employ individual enzymes rather than a multienzyme aggregate for fatty acid synthesis. To rationalize the occurrence in M. phlei of free ACP side by side with a complex that contains 4'-phosphopantetheine we record the following observations: 1) The M. phlei synthetase complex is exceptionally labile (1) and may partly dissociate into its constituent parts during isolation. In solutions of low ionic strength (< 0.1 M) it rapidly loses enzymatic activity with release of radioactive fragments of low molecular weight ($< 100,000$). Whether free ACP is liberated during this dissociation has not yet been established. 2) M. phlei contains a second, independent fatty acid synthetase (System II) with the following properties. System II, in contrast to the multienzyme complex, is retained on Sephadex G-150, synthetase activity emerging with a retention volume corresponding to an average molecular weight of less than 250,000 (Catalase marker). For chain initiation, System II can use palmitoyl-CoA or stearoyl-CoA, but not acetyl-CoA or octanoyl-CoA (Fig. 2A). This chain-lengthening activity

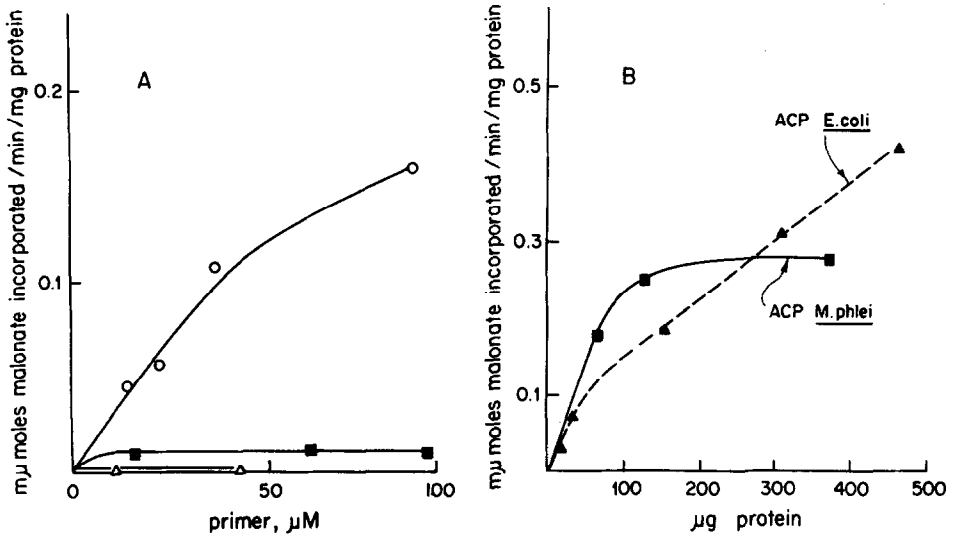

Figure 2A. Effect of primer concentration on ^{14}C-malonate incorporation into fatty acids by System II. The enzyme system was prepared by precipitating M. phlei extracts with $(NH_4)_2SO_4$, dialyzing the precipitate against 0.1 M phosphate buffer, pH 7.0, and by chromatography on Sephadex G-150. System II enzymes were eluted with 0.1 M phosphate buffer containing 1 mM EDTA and 1 mM dithiothreitol (DTT). The assay system contained, in 0.5 ml, 100 mM KPO_4 buffer, pH 7.0, 5 mM EDTA, 5 mM DTT, 250 μM TPNH, 40 μM ^{14}C-malonyl-CoA, 155 μg $ACP_{M. phlei}$ and primer as indicated. (o—o), stearoyl-CoA; (■—■), octanoyl-CoA and (Δ—Δ), acetyl-CoA.

Figure 2B. Effect of ACP on System II activity. The enzymes and the assay conditions were the same as described for 2A except that the concentration of primer was kept constant (stearoyl-CoA, 210 μM). (■—■), $ACP_{M. phlei}$ and (▲---▲), $ACP_{E. coli}$.

is strictly dependent on an external supply of ACP. Both $ACP_{M. phlei}$ and $ACP_{E. coli}$ can serve in this capacity (Fig. 2B). On all these grounds, System II is clearly distinct from the M. phlei multienzyme fatty acid synthetase complex which we have described elsewhere (1). Apart from the exceptional chain-length specificity for the primer, the properties of System II are typically those of the well-studied bacterial (E. coli) fatty acid synthetase (6, 7). 3) The existence of an additional bacterial-type fatty acid synthetase in M. phlei has been documented further by the iso-

lation of an ACP-dependent malonyl transacylase (17). This enzyme also responds to either $ACP_{\underline{M.\ phlei}}$ or $ACP_{\underline{E.\ coli}}$.

REFERENCES

1. Brindley, D. N., Matsumura, S., and Bloch, K., Nature, in press.
2. Wells, W. W., Schultz, J., and Lynen, F., Proc. Nat. Acad. Sci. U.S., 56, 633 (1966).
3. Willecke, K., Ritter, E., and Lynen, F., European J. Biochem., 8, 503 (1969).
4. Chesterton, C. J., Butterworth, P. H. W., Abramovitz, A. S., Jacob, E. J., and Porter, J. W., Arch. Biochem. Biophys., 124, 386 (1968).
5. Majerus, P. W., Alberts, A. W., and Vagelos, P. R., Proc. Nat. Acad. Sci. U.S., 51, 1231 (1964).
6. Vagelos, P. R., Majerus, P. W., Alberts, A. W., Larrabie, A. R., and Ailhaud, G. P., Fed. Proc., 25, 1485 (1966).
7. Wakil, S. J., Pugh, E. L., and Sauer, F., Proc. Nat. Acad. Sci. U.S., 52, 106 (1964).
8. Simoni, R. D., Criddle, R. S., and Stumpf, P. K., J. Biol. Chem., 242, 573 (1967).
9. Murphy, J. B., and Kies, M. W., Biochem. Biophys. Acta, 45, 382 (1960).
10. Davis, B. J., and Ornstein, L., Ann. N. Y. Acad. Sci., 121, 404 (1964).
11. Ailhaud, G. P., Vagelos, P. R., and Goldfine, H., J. Biol. Chem., 242, 4459 (1967).
12. Ellman, G. L., Arch. Biochem. Biophys., 82, 70 (1959).
13. Spackman, D. G., Stein, W. H., and Moore, S., Anal. Chem., 30, 1190 (1958).
14. Matsumura, S., Biochem. Biophys. Res. Commun., in press.
15. Majerus, P. W., Alberts, A. W., and Vagelos, P. R., J. Biol. Chem., 240, 4723 (1965).
16. Novelli, G. D., In: Methods in Enzymology, S. P. Colowick and N. O. Kaplan, Eds. (Academic Press, New York, 1955), Vol. II, p. 667.
17. Etemadi, A. H., unpublished observation.