Acyl Carrier Protein

XVIII. CHEMICAL SYNTHESIS AND CHARACTERIZATION OF A PROTEIN WITH ACYL CARRIER PROTEIN ACTIVITY*

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SUMMARY

A protected linear polypeptide of 74 amino acids with the sequence of Escherichia coli 1 to 74 apo-acyl carrier protein (ACP) was synthesized by the automated solid phase method. The polypeptide was removed from the solid support and partially deprotected by treatment of the peptide-resin with hydrobromide and trifluoroacetic acid, and the product was purified by gel filtration. The removal of protecting groups was completed by hydrogenation, and the prosthetic group, 4'-phosphopantetheine, was introduced enzymatically with holo-ACP synthetase. Ion exchange chromatography of the product yielded a preparation in which 55% of the protein in the purified fraction contained the prosthetic group. This synthetic 1 to 74 holo-ACP was as active as native holo-ACP in the malonyl pantetheine-CO₂ exchange reaction which is dependent upon malonyl-coenzyme A-ACP transacylase and β-ketoacyl-ACP synthetase.

The purified synthetic 1 to 74 holo-ACP preparation was found to be homogeneous and similar to native 1 to 74 holo-ACP as judged by co-chromatography on DEAE-cellulose and Sephadex G-50 and by sodium dodecyl sulfate disc gel electrophoresis. In addition the synthetic and native proteins were similar with respect to their ultraviolet spectra, amino acid compositions, and their immunological activity with antisera prepared against native ACP.

* For the preceding report of this series see Reference 1. This investigation was supported in part by Grants 5-RO1-HL 10406 and AM-5142X from the National Science Foundation, and the George M. and AM-13025 from the United States Public Health Service. Grant age to a serine residue (13, 14). The sulfhydryl group of the 4'-phosphopantetheine is the substrate binding site of ACP,1 and the intermediates of fatty acid biosynthesis are bound as thioesters.

The enzymes that catalyze reactions involving ACP have a high degree of specificity for the protein component of this coenzyme, and little perturbation of its structure is tolerated (15-20). It has been shown, for example, that replacement of the ACP of a plant fatty acid-synthesizing system with Escherichia coli ACP causes a change in the spectrum of fatty acids produced (19). Structure-function studies have been initiated through investigations of ACP peptides (15-18) and investigations of the effects of chemical modifications of specific amino acid residues (16, 20). Both of these procedures, however, are limited in their ability to define the role of specific residues. It is only by chemical synthesis that specific amino acid substitutions can be systematically prepared, and for this reason the synthesis of ACP was undertaken as a necessary prelude to such a study. The synthesis of even a small protein, such as ACP containing 77 residues, presents a challenge since at the present time the syntheses of only a very limited number of proteins have been attempted, e.g. bovine insulin (21, 22), ferredoxin (23), ribonuclease A (24, 25), fragment Ps of Staphylococcus aureus nuclease T (26), cytochrome c (27), a protein with growth hormone activity (28), and soybean trypsin inhibitor (29). It is only by chemical synthesis that specific amino acid substitutions can be systematically prepared, and for this reason the synthesis of ACP was undertaken as a necessary prelude to such a study. The synthesis of even a small protein, such as ACP containing 77 residues, presents a challenge since at the present time the syntheses of only a very limited number of proteins have been attempted, e.g. bovine insulin (21, 22), ferredoxin (23), ribonuclease A (24, 25), fragment Ps of Staphylococcus aureus nuclease T (26), cytochrome c (27), a protein with growth hormone activity (28), and soybean trypsin inhibitor (29). It was decided to use the solid phase synthetic procedure of Merrifield because this method has been found to be much faster and to give a higher yield than the classical approach (30).

Several important prerequisites for the synthesis of ACP have been accomplished. The complete amino acid sequence of E. coli ACP was determined by Vanaman et al. (31), while Tagaki and Tanford (32) showed that denatured ACP can be readily renatured. Holo-ACP synthetase, which catalyzes the synthesis of holo-ACP from CoA and apo-ACP, as shown in Reaction 1 (see "Experimental Procedures") was purified from extracts of E. coli and characterized (18, 33). Thus if apo-ACP could be synthesized by the solid phase method, the enzyme could be utilized to add the prosthetic group to the deprotected product. E. coli 1 to 74 ACP, formed by digestion of native 1 to 74 ACP, was purified by gel titration. The removal of protecting groups was completed by treatment of the peptide-resin with hydrobromide and trifluoroacetic acid, and the product was purified by gel filtration. The removal of protecting groups was completed by hydrogenation, and the prosthetic group, 4'-phosphopantetheine, was introduced enzymatically with holo-ACP synthetase. Ion exchange chromatography of the product yielded a preparation in which 55% of the protein in the purified fraction contained the prosthetic group. This synthetic 1 to 74 holo-ACP was as active as native holo-ACP in the malonyl pantetheine-CO₂ exchange reaction which is dependent upon malonyl-coenzyme A-ACP transacylase and β-ketoacyl-ACP synthetase.

The purified synthetic 1 to 74 holo-ACP preparation was found to be homogeneous and similar to native 1 to 74 holo-ACP as judged by co-chromatography on DEAE-cellulose and Sephadex G-50 and by sodium dodecyl sulfate disc gel electrophoresis. In addition the synthetic and native proteins were similar with respect to their ultraviolet spectra, amino acid compositions, and their immunological activity with antisera prepared against native ACP.

* The abbreviations used are: ACP, acyl carrier protein; holo-ACP, acyl carrier holoprotein; apo-ACP, acyl carrier protein lacking the 4'-phosphopantetheine prosthetic group. Nomenclature and abbreviations, where possible, follow the tentative rules of the IUPAC-IUB Commission on Biological Nomenclature (1966) J. Biol. Chem. 241, 2401; (1967) 242, 555.
to 77 ACP with carboxypeptidase A, was shown to possess full biological activity with ACP synthetase (18) and in fatty acid biosynthesis (19). The synthesis of 1 to 74 apo-ACP would have an important advantage over that of 1 to 77 apo-ACP since ACP 1 to 74 does not contain histidine; histidine residues tend to complicate peptide syntheses (34).

The pentapeptide 33 to 37, around the active site of E. coli ACP, has been synthesized previously by Miura and Sato (35), while the manual synthesis of 1 to 74 ACP has been the subject of a previous communication (36). In this paper the preparation and purification of a protein with ACP activity is described, together with a comparison of the synthetic product with native ACP.

MATERIALS AND METHODS

Materials—""""-Butoxycarbonyl-amino acids and t-butoxycarbonylb-glutamic acid esterified to a styrene-1%-divinylbenzene resin were purchased from Schwartz BioResearch. The following side-chain blocking groups were used: lysine, benzoylcarbonyl; arginine, N'-nitro; aspartic acid, benzyl ester; threonine, serine and tyrosine, benzyl ethers; glutamic acid, benzyl ester, while the t-butoxycarbonyl group was used for α-amino protection. Methionine was purchased at its 2-mercaptoethanesulfonic acid salt. Palladium (5%) on barium sulfate was purchased from Engelhard.

Sephadex G-25 and G-50 were purchased from Pharmacia; DEAE-cellulose was obtained from Whatman. CoA—SH (Chromatopure) was purchased from P-L Biochemicals. Sodium [3H]bicarbonate, 20 μCi per mmole, was obtained from New England Nuclear. Dithiothreitol was obtained from Calbiochem. E. coli B, harvested in early exponential growth, was purchased from the Grain Processing Corporation, Muscatine, Iowa. All other chemicals of reagent grade or better were purchased from the Grain Processing Corporation, Muscatine, Iowa. All other chemicals of reagent grade or better were purchased from common sources.

Unlabeled E. coli ACP and E. coli ACP and CoA labeled with 1H- or 14C-labeled β-alanine in the prosthetic group were isolated as described previously (37, 38). Purified Fraction A and holo-ACP synthetase were prepared according to the method of Elvolson and Vagelos (33) and desalted immediately before use.

Experimental Procedures—The two stage assay for apo-ACP utilized holo-ACP synthetase and measured the holo-ACP formed in a coupled system utilizing the malonyl pantetheine/CO2 reaction as described previously (33). The reactions involved are as follows:

CoA + apo-ACP + Mg++ → holo-ACP + adenosine 3',5'-bisphosphate
(1)

Malonyl-pantetheine + holo-ACP → malonyl holo ACP + pantetheine
(2)

Caproyl-pantetheine + holo-ACP → caproyl-holo-ACP + pantetheine
(3)

Malonyl-holo-ACP + caproyl-holo-ACP
→ β-ketoacetyl-holo-ACP + holo-ACP + CO2
(4)

Reaction 1 is catalyzed by holo-ACP synthetase. The holo-ACP thus formed was measured by the holo-ACP-dependent incorporation of 14CO2 into malonyl pantetheine that involves the reversibility of Reactions 2 to 4. Reaction 2 is catalyzed by malonyl-CoA-ACP transacylase, and Reactions 3 and 4 are catalyzed by β-ketoacyl-ACP synthetase (39). Malonyl-CoA-ACP transacylase and β-ketoacyl-ACP synthetase are present in Fraction A, a crude enzyme preparation from E. coli (33). In the first stage of the assay the standard reaction mixtures contained 2 μmoles of Tris-HCl, pH 8.0, 0.2 μmole of dithiothreitol, 1 to 30 pmole of apo-ACP, 24 μmoles of reduced CoA, 0.8 μmole of MgCl2, and 0.001 unit of holo-ACP synthetase in a total volume of 0.04 ml. After 10 min at 33° the holo-ACP that was synthesized was assayed in the second stage of the assay by addition of 0.05 ml of a mixture containing 85 nmole of malonyl pantetheine, 17 nmole of caproyl pantetheine, 8.5 μmoles of imidazole-HCl, pH 6.2, 2 μmoles of EDTA, 4.5 μmoles of K23CO3 (200 μCi per mmole), and 0.5 mg of Fraction A. The addition of EDTA immediately terminated the holo-ACP synthetase reaction, which requires Mg++. After 15 min at 33° the exchange reaction was stopped by the addition of 0.01 ml of 4 N HCl, the reaction mixtures were transferred to liquid scintillation counting vials and dried at 100° for 15 min. The samples were counted after the addition of 1 ml of water and 10 ml of Bray's solution (40). Native apo-ACP residues 1 to 77 and 1 to 74 gave identical values when assayed in this system.

Antibody against pure E. coli native ACP was prepared by Dr. D. A. K. Roncari from rabbits receiving subcutaneous injections of ACP mixed with Freund's complete adjuvant as described previously (41) and characterized by Ouchterlony double diffusion patterns (42) and quantitative precipitin tests (43).

Protein concentrations were determined by the method of Lowry et al. (44) and by amino acid analysis. Radioactive [3H-alanine-14C]ACP, 3.4 μCi per mmole, was used as a protein standard when the concentration of ACP was measured. All radioactive measurements were carried out in a Packard 3280 scintillation counter.

Yields for coupling reactions in the synthesis were based on the limiting reactant, the peptide, and were calculated from the amount of the first amino acid, glycine, attached to the resin. Peptide-resin (2 mg) was hydrolyzed with 2 ml of 12 N HCl and propionic acid (1:1) for 2 hours at 130° according to the method of Scatches et al. (45). Free peptides were hydrolyzed with 6 N HCl in sealed, evacuated tubes for 24 hours at 110°. Amino acid compositions of peptides were determined on a Beckman Spinco amino acid analyzer.

Solid Phase Synthetic Procedure—A Schwarz automated synthesizer was used for the automated syntheses. The synthesis was based on the stepwise addition of protected amino acids to the carboxyl-terminal amino acid, glycine, which was esterified via a benzyl ester to a polystyrene resin. Most of the procedures common to the solid phase method (46) were used, although some significant modifications were introduced. The rationale for these changes will be described in a separate publication (3).

After the desired sequence of 74 amino acids had been assembled (Fig. 1), the peptide was cleaved from the resin, deprotected, and isolated as described below.

In a typical synthesis, 0.66 mmole of t-butoxycarbonyl-glutamic acid esterified to 2 g of a 1% cross-linked polystyrene resin support was used. The sequence of washes utilized in the addition of a single amino acid residue is outlined in Table I. Since the number of washes was increased greatly in our modified procedure, a single manual synthesis of ACP required 3 months, while automation of the procedure decreased this time to 3 weeks. The results of the automated synthesis of ACP are described in this paper, while the manual synthesis was the subject of a previous communication (30). The coupling steps were carried out with...
Washes used during automated synthesis of acyl carrier protein

The cycle of washes constitutes one double addition of a residue and involves 84 washes of the resin. If 2 g of peptide-resin were used, then each wash was of 25 ml, except for trifluoroacetic acid washes (10 ml) and coupling reactions (14 ml). All amino acids were added by the dicyclohexylcarbodiimide method except glutamine and asparagine, which were added as the p-nitrophenyl esters. Coupling reactions with these two esters were carried out twice in dimethylformamide (1:1) and involved 84 washes of the resin. If 2 g of peptide-resin were used, then each wash was of 25 ml, except for trifluoroacetic acid washes (10 ml) and coupling reactions (14 ml). All amino acids were added by the dicyclohexylcarbodiimide method except glutamine and asparagine, which were added as the p-nitrophenyl esters. Coupling reactions with these two esters were carried out twice in dimethylformamide (10 ml) for 6 hours each time, and three washes of dimethylformamide were included before each coupling reaction.

| Reagent                        | number of applications | time (each application) |
|--------------------------------|------------------------|-------------------------|
| CHCl₃                          | 3                      | 1/2                     |
| Trifluoroacetic acid-CHCl₃ (1:1) | 2                      | 1/2, 10                 |
| CH₂Cl₂                         | 3                      | 1/2                     |
| 3-Butanol                      | 3                      | 1/2                     |
| CH₂Cl₃                         | 3                      | 1/2                     |
| Trifluoroacetic acid-CH₂Cl₃ (1:1) | 2                      | 1/2, 10                 |
| CH₂Cl₃                         | 3                      | 1/2                     |
| 3-Butanol                      | 3                      | 1/2                     |
| CH₂Cl₃                         | 3                      | 1/2                     |
| Et₂N·CH₂Cl₂                    | 3                      | 1/2                     |
| CH₂Cl₃                         | 3                      | 1/2                     |
| 3-Butanol                      | 3                      | 1/2                     |
| CH₂Cl₃                         | 3                      | 1/2                     |
| t-Butoxycarbonyl-amino acid, δ diceloyloxyhexylcarbodiimide·CH₂Cl₃ | 1 | 120 |
| CH₂Cl₃                         | 3                      | 1/2                     |
| 3-Butanol                      | 3                      | 1/2                     |
| CH₂Cl₃                         | 3                      | 1/2                     |
| Et₂N·CH₂Cl₂                    | 3                      | 1/2                     |
| CH₂Cl₃                         | 3                      | 1/2                     |
| 3-Butanol                      | 3                      | 1/2                     |
| CH₂Cl₃                         | 3                      | 1/2                     |

* Before the addition of diceloyloxyhexylcarbodiimide, the resin was shaken with the amino acid for 5 min.

RESULTS

Synthesis of Protected 74 Amino Acid Residue Polypeptide Chain of 1 to 74 Acyl Carrier Protein—The course of the synthesis was followed by the removal of samples at steps in the synthesis where the first of a given amino acid was added. The amino acid ratios were determined on the acid hydrolysate of the peptide-resin. Even with our modifications to the synthetic procedure, the yield of the growing peptide dropped considerably from Gly to Val (Table II). However, from that point, the yield of the synthesis remained constant at 40 to 50%, which indicated that the peptide chains that had become unavailable for coupling early in the synthesis did not reinitiate growth at a later stage.

The final weight of the fully protected 1 to 74 ACP-resin was...
A sample of N-nitro-t-butoxycarbonyl-arginine was hydrogenated for the minimum time necessary for complete removal of the nitro group. Although N-nitro-arginine residues in some proteins have been found to be resistant to hydrogenation (49), presumably due to steric hindrance, this study would at least give the minimum time necessary for complete removal of the nitro group. The efficiency of the palladium on barium sulfate catalyst was determined by amino acid analysis.

| Amino acid | Residue number | Amount incorporated |
|------------|----------------|---------------------|
| Gly        | 74             | 1.0                 |
| Asp        | 73             | 0.85                |
| His        | 72             | 0.80                |
| Tyr        | 71             | 1.00                |
| Ala        | 68             | 0.60                |
| Glu        | 66             | 0.60                |
| Val        | 65             | 0.40                |
| Thr        | 64             | 0.50                |
| Lys        | 61             | 0.50                |
| Pro        | 55             | 0.50                |
| Phe        | 50             | 0.50                |
| Leu        | 46             | 0.40                |
| Met        | 44             | 0.45                |
| Ser        | 36             | 0.40                |
| Phe*       | 28             | 0.40                |
| Lys*       | 18             | 0.40                |
| Arg        | 6              | 0.35                |

* Second residue of this amino acid.

3.6 g, while the product contained 1 NO₂, 31 benzyl, and 4 benzoyl groups and had a calculated molecular weight of 11,685. The product contained 0.065 mmole of protein per g of protected ACP-resin as estimated by amino acid analysis and by the amount of peptide obtained from the HBr-trifluoroacetic acid cleavage reaction. This corresponds to 20% of the initial value for the amount of glycine esterified to the resin.

**Stability of Apo-ACP in Conditions Necessary for Cleavage of Peptide-Resin**—Before the removal of the protected peptide from the resin could be attempted, it was necessary to determine the stability of native apo-ACP in the conditions necessary for the cleavage of the benzyl ester. Treatment of apo-ACP with HF as described in “Experimental Procedures” led to complete inactivation of the protein (Fig. 2). When equal volumes of HF and trifluoroacetic acid were utilized under similar conditions, the recovered apo-ACP was 40% active. The best retention of biological activity (72%) was obtained when apo-ACP was treated with HBr and trifluoroacetic acid. Therefore, this procedure appeared to be suitable for use with the synthetic product.

As HF could not be used to cleave the synthetic apo-ACP from the resin, it was necessary to investigate the use of a second deprotection step, hydrogenation, which would remove the nitro group protecting Arg. Apo-ACP was hydrogenated using the conditions described in “Experimental Procedures.” Samples (10%) were removed at different time intervals during the hydrogenation, and the activity of the peptide was measured in the two stage assay for apo-ACP (Fig. 3). In a parallel experiment, the efficiency of the palladium on barium sulfate catalyst was tested in the reduction of N-nitro-t-butoxycarbonyl-arginine. Although N-nitro-arginine residues in some proteins have been found to be resistant to hydrogenation (49), presumably due to steric hindrance, this study would at least give the minimum time necessary for complete removal of the nitro group. A sample of N-nitro-t-butoxycarbonyl-arginine was hydrogenated under conditions identical with those used for apo-ACP deprotection; the reduction was rapid (Fig. 3) as no further release of ammonia was observed after 10 min. The preceding experiments indicated that native apo-ACP could not be quantitatively recovered with any of the cleavage or deprotection procedures.
The reaction conditions that were used are described in “Experimental Procedures” and text. The amount of protein cleaved was measured by Lowry protein determination on an aliquot of the isolated protein or by amino acid analysis on the acid hydrolysate of a weighed sample of the residual resin. The yield of cleavage was based on the amount of peptide, as measured by amino acid analysis, present in the peptide-resin before the cleavage reaction.

| Method of cleavage | Conditions | Weight of peptide cleaved from 50 mg of peptide resin | Yield% |
|--------------------|------------|------------------------------------------------------|--------|
| HF                 | 1 hr at 0°C| 12.5 mg                                              | 92%    |
| HF-trifluoroacetic acid (1:1) | 30 min at 10-15°C | 9.2 mg | 88% |
| HBr-trifluoroacetic acid | 30 min and 1 hr at 25°C | 10 mg | 74% |
| Methanol-Et$_3$N | 40 hrs at 25°C | 3.0 mg | 22% |
| Benzyl alcohol-Et$_3$N | 40 hrs at 60°C | 1.0 mg | 7% |

* Yield of cleavage.
* Measured by Lowry protein determination on an aliquot of weighed sample of residual resin.
* Measured by amino acid analysis on an aliquot of isolated protein.
* Material from both cleavages was combined.

It appeared that HBr-trifluoroacetic acid treatment for cleavage of the peptide from the resin, followed by hydrogenation to remove the N-nitro protecting group, might yield the best results.

**Isolation and Purification of Synthetic Product**—Samples of the peptide-resin were subjected to the HF, HF-trifluoroacetic acid and HBr-trifluoroacetic acid cleavage procedures in an attempt to verify the optimal procedure for cleavage of the peptide from the resin. The yield and conditions of cleavage are described in Table III. The general procedure used for the trans-esterification studies was based on the work of Bayerman et al. (51), but even with the most vigorous conditions only a small portion of the peptide was cleaved from the resin with methanol-Et$_3$N or benzyl alcohol-Et$_3$N (Table III).

From studies of the stability of apo-ACP (Figs. 2 and 3) and the extent of cleavage of the peptide-resin (Table III), it was decided to use HBr-trifluoroacetic acid as the method of cleavage and deprotection. The amino acid composition of the cleaved product was found to be very similar to that of the peptide-resin (Table IV). The ratio of the values for tyrosine (residue 71) to phenylalanine (residues 28 and 50), which should be 0.5, was 1.3 in the crude cleaved peptide, indicating the presence of small incomplete peptides in the product. For comparison, the amino acid analysis of the peptide-resin obtained in the manual synthesis (36) is also shown in Table IV.

The crude, partially deprotected material was purified by gel filtration on Sephadex G-25. The elution profile of the column is shown in Fig. 4. Peak 1, which had the same elution volume as native ACP, contained 3/4% of the protein (Table V). Only material from Peak I had any biological activity in the assay with ACP synthetase (see below). The rest of the material presumably consisted of ACP fragments formed by incomplete coupling reactions. The amino acid analysis for Peak I is shown in Table IV, and the improvement in the composition, relative to the crude peptide mixture, can be attributed to the removal of short peptides, e.g. the ratio of tyrosine to phenylalanine decreased to 0.7. For comparison, the elution profile obtained with peptide from the manual synthesis (Fig. 4) and the peptide yields in the different peaks of the chromatogram (Table V) are shown. The deprotection of the synthetic product was then completed.
by removal of the N-nitro group protecting arginine as described under "Experimental Procedures." The recovery of protein from this reaction was 88% and the amino acid analysis of the product was not significantly different from that obtained with material of Peak I (under the acidic conditions required to hydrolyze the sample for amino acid analysis most of the nitro-arginine was converted to arginine).

An attempt was then made to convert the synthetic 1 to 74 apoprotein to 1 to 74 holo-ACP by reacting it with holo-ACP synthetase and CoA. To facilitate identification of the holo-ACP, [panetheine-3H]CoA was used so that the product would be radioactive, and the reaction mixture was chromatographed on DEAE-cellulose-52 (Fig. 5). Peak I was readily identified as the synthetic ACP, as the holo-ACP activity in the malonyl pantetheine-CO2 exchange reaction was catalyzed by fractions in this peak. The fractions of Peak I were pooled, concentrated, and desalted on a Sephadex G-25 column. The desalted material contained 6 x 10^4 dpm of 3H radioactivity, which corresponded to 1.5 pmoles of synthetic 1 to 74 holo-ACP. Lowry protein determination and amino acid analysis indicated that the sample contained 22 mg of protein (2.75 μmoles). Thus 55% of the protein in this sample contained the radioactive 4'-phosphopantetheine. This material was used without further purification in the following studies which attempted to establish the identity of the synthetic product.

The amino acid analysis of the material from Peak I gave a composition which agreed closely with the expected values for 1 to 74 ACP (Table IV). In addition, β-alanine, a constituent of the prosthetic group, could be identified in the analysis, and the value of 0.7 residue per molecule was in close agreement with the above finding that 55% of the protein contained 4'-phosphopantetheine.

Because of the small difference in properties of 1 to 74 ACP and CoA on DEAE-cellulose, a sample of Peak I (4H radioactivity), unlabeled CoA, and native 1 to 77 ACP (4C radioactivity) were chromatographed on DEAE-cellulose-52, and the peak of 4H radioactivity was shown to be well resolved from the peak of CoA (Fig. 6). It is of interest that 1 to 77 ACP, which contains three additional residues including a histidine residue, was eluted at a much higher salt concentration than 1 to 74 ACP.

Comparison of Properties of Synthetic Product and Native Acp Carrier Protein—Synthetic 1 to 74 [panetheine-3H]holo-ACP and native 1 to 74 [panetheine-14C]holo-ACP were shown to co-

![Fig. 5. Chromatography of products formed by holo-ACP synthetase from synthetic 1 to 74 apo-ACP and CoA. The incubation mixture contained Tris-HCl, pH 8.5, 0.2 mM dithiothreitol, 0.1 mM [panetheine-3H]CoA, 5 μmoles (2.0 x 10^6 dpm); synthetic apoprotein, 3.3 μmoles; and ACP synthetase, 30 μg (1.0 enzyme unit) in a total volume of 20 ml. The reaction mixture was incubated at 37° for 20 hours. The mixture was then diluted with water to a conductivity of 0.1 mmdsi, and applied to a DEAE-cellulose-52 column (2 x 10 cm), which had been equilibrated with 0.01 M Tris-HCl, pH 7.3, 0.001 M dithiothreitol. The column was eluted with a linear gradient composed of 250 ml of 0.01 M Tris-HCl, pH 7.3, 0.001 M dithiothreitol, and 250 ml of the same buffer containing 0.5 M LiCl (15.2 mmmhos). Fractions (5 ml) were collected and were assayed for 3H radioactivity (—•—•), absorbance at 290 nm (O—O—O), conductivity (A—A—A), and holo-ACP activity, 14C radioactivity (©). Holo-ACP activity was measured in the malonyl pantetheine CO2 exchange reaction, described in "Experimental Procedures" as the second stage of the two-stage assay for apo-ACP. OD, Optical density.]

![Fig. 6. Chromatography of synthetic 1 to 74 ACP, native 1 to 77 ACP, and CoA on DEAE-cellulose. A solution containing synthetic 1 to 74 ACP (4H radioactivity, 2 x 10^4 dpm), unlabeled CoA (2 mg), and native 1 to 77 ACP (4C radioactivity, 3 x 10^4 dpm) in 1 ml of 0.01 M Tris-HCl, pH 7.3, and 0.001 M dithiothreitol was applied to a column (2 x 10 cm) of DEAE-cellulose-52 which had been equilibrated with 0.01 M Tris-HCl, pH 7.3, 0.001 M dithiothreitol. The column was eluted with a linear gradient composed of 100 ml of 0.01 M Tris-HCl, pH 7.3, 0.001 M dithiothreitol, and 100 ml of the same buffer containing 0.7 M LiCl (conductivity 29 mmmhos). Fractions (1.8 ml) were collected and assayed for 3H radioactivity (O—•—•), 14C radioactivity (©—©—©), CoA (A—A—A), and conductivity (A—A—A). The CoA was measured by the phosphotransacetylase assay (52). OD, optical density. Chromatograph on a DEAE-cellulose column that was developed with a shallow lithium chloride gradient (36), indicating that the synthetic and native products had similar charge properties. Synthetic 1 to 74 [panetheine-3H]ACP and native 1 to 74 [panetheine-14C]ACP were shown to co-

| Table V Protein content of different peaks obtained from Sephadex G-25 chromatography of crude synthetic protein |
| --- |
| Manual synthesis | Automated synthesis |
| Protein | Vg | Total | Protein | Vg | Total |
| Peak I (Fractions 30 to 50) | 1.2 | 74 | 25 | 11.4 | 7.5 | 57 |
| Peak II (Fractions 51 to 80) | 0.7 | 112 | 23 | 7.4 | 110 | 57 |
| Peak III (Fractions 81 to 120) | 1.9 | 185 | 48 | 1.3 | 180 | 10 |

a A total of 4 mg was chromatographed on the column, but the optical densities were increased proportionally in Fig. 4 to match the other sample.

b A total of 20 mg was chromatographed on the column.

c The elution volume for native ACP was found to be 72 ml.
Fig. 7 (left). Cochromatography of synthetic and native 1 to 74 ACP on Sephadex G-50. A mixture of synthetic 1 to 74 [pantetheine-3H]ACP (1.25 nmoles, 1 x 10⁶ dpm) and native 1 to 74 [pantetheine-14C]ACP (20 nmoles, 1 x 10⁶ dpm) in 0.1 ml of 0.01 M Tris-HCl, pH 7.3, 0.001 M dithiothreitol was applied to a column (1 x 24 cm) of Sephadex G-50. The column was eluted with the same buffer and fractions (0.4 ml) were assayed for 3H radioactivity (O---O) and 14C radioactivity (O--O).

Fig. 8 (center). Sodium dodecyl sulfate polyacrylamide gel electrophoresis of synthetic 1 to 74 [pantetheine-3H]holo-ACP and native 1 to 77 [pantetheine-3H]holo-ACP. The samples were subjected to electrophoresis on 15% gels containing 0.1% sodium dodecyl sulfate at pH 8.9 according to the method of Laemmli (53). A, 80 µg of synthetic 1 to 74 ACP (0.6 x 10⁶ dpm of 3H radioactivity). B, 50 µg of native 1 to 77 ACP (19 x 10⁶ dpm of ³H radioactivity). The gels were scanned for absorbance at 280 nm (O--O) and then sliced. The slices were digested in 0.5 ml of 33% hydrogen peroxide by heating at 100° for 10 min. Bray's solution (10 ml) was added and the ³H radioactivity (bar graphs) was measured. OD, optical density.

Fig. 9 (right). Ultraviolet absorption spectra of ACP preparations. Absorption spectrum of native 1 to 74 ACP (--), synthetic 1 to 74 ACP (---) and product from HF-trifluoroacetic acid cleavage (O---O) in 0.01 M Tris-HCl, pH 7.3. The concentration of protein was 0.45 mg per ml. A Spectronic 600 recording spectrophotometer was used for the measurements of the spectra.

As 1 to 74 ACP contains only 1 tyrosine, 2 phenylalanine, and no histidine or tryptophan residues, there is little absorption in the ultraviolet spectrum of native ACP, and one could expect that the ultraviolet spectrum of synthetic ACP would be significantly different in the presence of protecting groups such as benzylxoycarbonyl and benzyl derivatives. It was found that the ultraviolet spectra of synthetic and native 1 to 74 ACP (Fig. 9) were completely superimposable at all wave lengths, which indicated that all of the protecting groups had been removed by the procedures used for deprotection. This was in distinct contrast to the crude material from the HF and trifluoroacetic acid cleavage which had a much greater ultraviolet absorption than the native protein.

Synthetic 1 to 74 holo-ACP was examined for activity in the malonyl pantetheine-CO₂ exchange reaction, a coupled assay based upon the activities of malonyl-CoA-ACP transacylase and β-ketoacyl ACP synthetase (39). As seen in Fig. 10, synthetic 1 to 74 holo-ACP was as active as native 1 to 77 holo-ACP in this assay. Furthermore the activity of synthetic 1 to 74 holo-ACP in the exchange reaction (Table VI) was dependent upon
the presence of capryl pantetheine, malonyl pantetheine, and Fraction A, which contained both malonyl-CoA-ACP transacylase and β-ketoacyl-ACP synthetase, and these requirements are characteristic of this exchange reaction with native ACP.

Antiserum specific for ACP was prepared from rabbits immunized against native ACP (41). This antiserum was found to react equally well with native apo-ACP and holo-ACP. A mixture of synthetic 1 to 74 ACP (2H radioactivity) and native 1 to 74 ACP (14C radioactivity) was incubated with increasing amounts of antiserum. As is shown in Fig. 11, synthetic 1 to 74 and native 1 to 74 ACP were precipitated equally well by the antibodies to ACP independent of antibody concentration. This indicated that the synthetic protein and the native protein contained antigentic sites that had similar affinity for the antibodies.

**DISCUSSION**

The synthesis of ACP was not monitored, except by amino acid analysis, because none of the available analytical methods was found to be satisfactory for use with a rapid automated synthesis. Fortunately, the first residue of each amino acid is scattered throughout the ACP sequence (Table II), and the yield of the addition of these residues gave an estimate of the amount of peptide that was growing at any stage of the synthesis. The analyses indicated that the amino acid additions occurred in good yield even toward the end of the synthesis, so that an increase in size of the peptide chain did not cause additional steric hindrance for the coupling reactions. To ensure an adequate yield, however, it was found necessary to repeat all coupling and deprotection reactions and to include a much larger number of washes into the synthetic scheme; for example, extensive use was made of a 3-butanol wash to shrink the resin. Thus the resin was subjected to a swell-shrink-swell cycle before a deprotection or coupling reaction was repeated (Table I). Such a wash scheme has been shown to expose buried functional groups so that repetition of a given step will lead to further reaction with newly exposed terminal residues. The extra steps, however, greatly increased the time that was required for a single synthesis, which now involved 6216 washes of the resin. If the synthesis of analogues was to be attempted, it was obvious that the process had to be automated so that the protein could be prepared with a reasonable expenditure of effort. The automation of the synthesis has the further advantage of much greater reproducibility as human error becomes a significant factor in long syntheses. A commercially available peptide synthesizer was adapted so that the synthesis of ACP could be carried out continuously for 24 hours a day. The automated synthesis gave a product, in a similar yield, indistinguishable from that prepared by the manual method (see "Appendix"). As both coupling and deprotection reactions were repeated, the time of each reaction was decreased to 2 hours and 10 min, respectively, except for active esters which were still coupled for 6 hours. There is considerable evidence that extended dicyclohexylcarbodiimide-mediated coupling reactions are not useful because of a rapid side reaction, the formation of N-acyl ureas, which consumes the reactants. One could expect, therefore, that two 2-hour couplings would be much more effective than one 4-hour coupling. As is common with other syntheses, the molar excess of the reagents increased during the synthesis, since the same quantity of reagents was used even though the number of available peptide chains decreased.

The side chain protecting groups were chosen so that cleavage of the benzyl ester linkage of the peptide chain to the resin would remove all of the protecting groups. This goal could not be achieved because of the instability of ACP to anhydrous HF, and this necessitated the use of HBr and trifluoroacetic acid as the cleavage procedure, with the consequent retention of N-nitro protecting groups. Although benzoyloxycarbonyl groups were used to block the e-amino groups of lysine, careful sizing of the cleaved product on Sephadex G-50 indicated that little growth had occurred on side chains because of premature removal of this derivative during the synthesis. Methionine was used without protection of the thioether, and amino acid analysis of the cleaved peptides (Table IV) indicated that little destruction of this amino acid had occurred during the synthesis or the cleavage step.

**Table VI**

Activity of synthetic 1 to 74 holo-ACP in malonyl pantetheine-CO₂ exchange reaction

| System                                      | Amount of 14CO₂ fixed |
|---------------------------------------------|-----------------------|
| Complete                                    | 2.6                   |
| Complete, minus synthetic ACP               | 0.75                  |
| Complete, minus caproyl pantetheine         | 0.44                  |
| Complete, minus malonyl pantetheine         | 0.04                  |
| Complete, minus caproyl pantetheine and malonyl pantetheine | 0.41                  |
| Complete, minus Fraction A                  | 0.01                  |

**Fig. 11.** Activity of synthetic 1 to 4 ACP with antibodies specific for native ACP. A mixture of synthetic 1 to 74 (0.13 mg, 2H radioactivity, 2 × 10⁶ dpm) and native 1 to 74 ACP (0.13 mg, 14C radioactivity, 2 × 10⁶ dpm) was incubated with increasing amounts of antiserum. The incubation mixtures contained the ACP mixture and 1 to 50 μl of serum in 0.2 ml of 0.02 M potassium phosphate buffer, pH 7.4, 0.15 M sodium chloride, and 0.001 M dithiothreitol.

After incubation at room temperature for 2 hours and at 0° for 16 hours, the ACP-antibody complex was precipitated by the addition of 0.2 ml saturated ammonium sulfate solution (pH 7.0), and the precipitate was removed by centrifugation at 10,000 × g for 10 min. A sample (5%) of the supernatant was assayed for 2H radioactivity (○○○) and 14C radioactivity (○○○). Ratio of 2H:14C radioactivity (▲▲▲) in the supernatant.
The use of HBr-trifluoroacetic acid was by far the best method for cleavage of the peptide from the resin, as apo-ACP had reasonable stability under the reaction conditions, a good yield of peptide was released (74%), and the product, after hydrolygregation, was biologically active. It was found necessary to repeat the HBr-trifluoroacetic acid treatment as the first cleavage only released 50 to 70% of the available material. Amino acid analysis of the material left on the resin after two HBr-trifluoroacetic acid cleavages was in close agreement with the expected values for ACP, but repetition of the cleavage reaction did not release any more material.

The amino acid analysis of the crude mixture of cleaved peptides indicated that the product contained significant quantities of short peptides, which were readily removed by gel filtration on Sephadex G-25. The short peptides contained a preponderance of tyrosine, and as the only tyrosine in ACP occurs at residue 71, this amino acid was present in incomplete peptides in much higher molar quantities than amino acids such as phenylalanine, leucine, or serine, which were added much later in the synthesis when the percentage of chains growing was decreased. The presence of these short peptides, together with the data obtained from amino acid analysis of samples taken at different stages of the synthesis (Table II), indicated that residues which became unavailable at a given stage of synthesis did not reinitiate growth to a significant extent. The unavailability of truncated sequences for further reaction was consistent with the suggestion2 that the solvation of the peptide-resin changes as the synthesis proceeds, because of the increasing protein character of the solid phase matrix. A decrease in the solvation could cause a loss of certain reaction sites that are situated in the less accessible regions of the resin beads. This problem could be expected to become intensified as the synthesis proceeded, so that, with minor exceptions, one would not expect buried sites to reinitiate growth at a later stage of the synthesis.

When native apo-ACP was subjected to the conditions of deprotection, i.e. HBr-trifluoroacetic acid treatment followed by hydrolygregation, the protein retained only 40% of its biological activity. It is interesting to note that 55% of the pure synthetic product was active with holo-ACP synthetase. In addition, the synthetic product which accepted the prosthetic group in the reaction with holo-ACP synthetase was as active as native holo-ACP in the malonyl pantetheine-CO₂ exchange reaction. Although a direct comparison between the activities may not be valid, as the stability of a denatured, partially deprotected peptide may be different from the native protein, it is clear that a very significant proportion of the product has biological activity very similar to that of native ACP. It has been shown that both holo-ACP synthetase (18) and β-ketoacyl-ACP synthetase (15) possess an unusually high degree of specificity for the intact protein chain. One can, therefore, assume that the synthetic product which is biologically active has a close resemblance to the native protein.

The unique nature of the prosthetic group, which contains the only sulfhydryl in the molecule, could serve as the basis for a further purification step to remove the remaining inactive protein. This approach, as well as the preparation of analogues for carrying out the polyacrylamide disc gel electrophoreses, and Dr. R. A. Bradshaw for numerous amino acid analyses.

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**TABLE VII**

Comparison of yields obtained with manual and automated syntheses

|                        | Manual synthesis | Automated synthesis |
|------------------------|------------------|--------------------|
| Yield of this step     | Overall yield    | Yield of this step | Overall yield |
| Peptide resin          | 15               | 20                 | 74             |
| Cleaved peptide       | 12               | 1.8                | 20             |
| Peptide in Peak I of Sephadex G-25 | 25 | 0.45             | 57              |
| Peptide after hydroregeneration | 94 | 0.42             | 88              |
| Peptide active with holoACP synthetase | 25 | 0.11             | 46              |

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**TABLE VIII**

Extraction of cleaved peptides by various solvents

| Solvent used in extraction | Total extracted protein (%) |
|----------------------------|-----------------------------|
|                            | Manual synthesis | Automated synthesis |
| 1st trifluoroacetic acid wash | 33             | 70             |
| 2nd trifluoroacetic acid wash | 10             | 25             |
| Dioxane                     | 67             | 5              |
| Acetic acid-water (1:1)      | 12             | 74             |

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**APPENDIX**

The manual solid phase synthesis of ACP, which was reported previously (30), utilized several modifications which were not used in the subsequent automated syntheses. As is shown in Table VII, the yield of the automated synthesis was significantly better than that of the manual synthesis, although it has been shown that automation of the procedure is not responsible for this increase in the yield.3 It was of interest, therefore, to examine these modifications in the manual synthesis procedure and to determine the reasons for this lower yield. In the manual synthesis all coupling reactions were repeated, but the second coupling reaction was done with 1.5 M urea included in the solvent. The use of this reagent has been reported to increase the yield of difficult coupling reactions (54). It can be noted in Table IV that the amino acid analysis of the peptide-resin at the end of the manual synthesis was closer to the expected values for ACP than in the automated synthesis, which would suggest that the urea treatment increased the yield of the synthesis. Unfortunately, the properties of the resin after the urea treatment were quite different from the normal polystyrene resin; it had a "shriveled" appearance when dry, as if the urea treatment caused cross-linking of the resin. This was reflected in the difficulty experienced in the cleavage and extraction of the product (Table VIII); in fact, only 12% of the product could be

3 W. S. Hancock, unpublished results.
extracted from the resin with a wide variety of solvents. A disadvantage of the use of urea is the presence of isocyanate impurities, which can cause undesirable side reactions such as chain termination by reaction with free amino groups to form thioureas (55).

Acetylation was also used in the first manual synthesis to block partially complete sequences after the addition of residues 2, 10, 20, 47, 62, and 70 (36). Subsequent studies, however, have shown that acetylation is often not effective in blocking unreactive amino groups2 (56, 57) and, in fact, the product from material (Tables V and VII and Fig. 4). The use of acetic anhydride has been found to cause side reactions (58), and if such reactions occurred in the manual synthesis, they would explain the greater amount of short peptides in the product.

It was clear that these modifications were harmful in the synthesis of ACT, and, therefore, the use of urea and acetic anhydride were abandoned.

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