BIOORGANIC CHEMISTRY 10, 277–282 (1981)

An Approach to the Semisynthesis of Acyl Carrier Protein

R. L. PRESTIDGE, D. R. K. HARDING, C. H. MOORE, AND W. S. HANCOCK

Department of Chemistry, Biochemistry and Biophysics, Massey University, Palmerston North, New Zealand

Received July 7, 1980

A scheme has been devised for the preparation of semisynthetic derivatives of acyl carrier protein (ACP). Acetylated synthetic ACP_{1-6} is coupled via its activated pentachlorophenol ester to native ACP (7-77), which had previously been acetylated and converted to the *S*-5′-dithiobis(2-nitrobenzoate)(DTNB) derivative. Removal of the DTNB moiety after the coupling yielded active ACP in good yield.

INTRODUCTION

Semisynthesis has been proposed as a useful alternative to total synthesis for the study of structure–function relationships of proteins (*1*). To date only a few examples of this approach exist where a synthetic peptide has been covalently joined to the corresponding native structure (*2–8*). It was decided to develop a semisynthesis of acyl carrier protein which would allow the efficient preparation of analogs of the amino terminal region. Previous studies (*9, 10*) have shown that the amino terminal hexapeptide is required for maintenance of the correct 3D structure of the protein, and hence its biological activity.

The semisynthetic scheme used is shown in Fig. 1. Since acetylated native ACP has full biological activity (*9*), the synthesis was designed to prepare this derivative.

EXPERIMENTAL

The materials used for the isolation of *E. coli* ACP and preparation of the 7-77 fragments were the same as used previously (*11*). The solid phase synthesis techniques have been described elsewhere (*12*). All reagents or solvents used were AR grade except when purified for synthetic work.

Isolation of the native fragment. Native ACP was isolated, acetylated, and cleaved with trypsin at the single arginine residue (position 6) using well-established procedures (*11*). The acetylation was carried out with [^{3}H]acetic anhydride, to facilitate location of the product during later chromatographic procedures. The active site thiol group was converted to the 5′-dithiobis(2-nitrobenzoic acid (DTNB) derivative (*13*) to prevent its oxidation during the coupling

0045-2068/81/030277-06$02.00/0

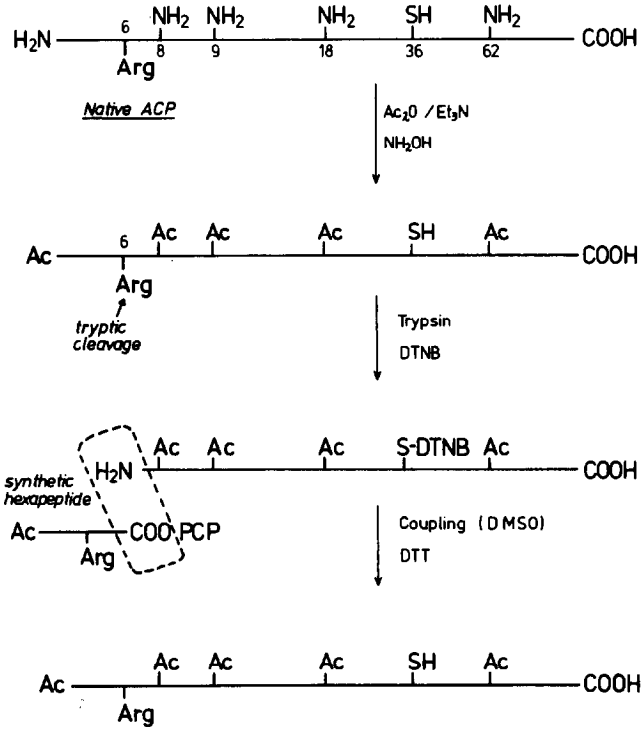

FIG. 1. The semisynthesis of ACP.

reaction. The 7-77 fragment of acetylated ACP was isolated by chromatography on Sephadex G-25 and Whatman DE52 DEAE-cellulose.

Preparation of the synthetic hexapeptide. The protected hexapeptide corresponding to ACP_{1-6} (Fig. 2) was prepared by the solid phase method (see Fig. 2). The synthesis of the acetylated protected hexapeptide has been described (*12*). The crude hexapeptide was then purified by gel filtration on Sephadex G-25 in 50% acetic acid and partition chromatography on Sephadex LH-20 with chloroform : hexane : methanol (15 : 4 : 1) as the eluant. The pentachlorophenyl active ester of the protected peptide was formed by the method of Kovacs *et al.* (*13*). The peptide was purified on a preparative hplc system which consisted of two columns (60 cm long and 7 mm i.d.) connected in series. The columns were packed with silanized Bondapak phenyl-Porasil B (37 to 50 μm, Waters Associates) and the mobile phase consisted of 40% water and 60% methanol, with 1% acetic acid. A flow rate of 5 ml/min was used. After this chromatographic step, the protected peptide was shown to be homogeneous by analysis on a μBondapak alkylphenyl column with the same mobile phase.

The *p*-nitrobenzyl ester and N^G-nitro protecting groups were removed by catalytic hydrogenation using the conditions shown (Fig. 2) and the product was isolated by gel filtration on Sephadex G-25 in 50% acetic acid and freeze-drying.

Semisynthetic coupling. A typical coupling procedure is described in the legend

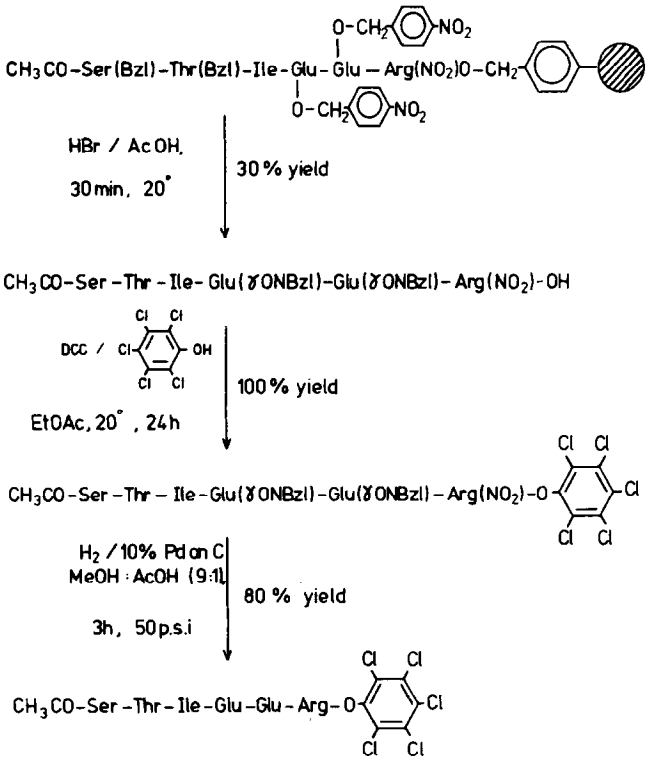

FIG. 2. The synthesis of the activated hexapeptide corresponding to ACP_{1-6}.

to Table 1. After removal of the DTNB protecting group, the product was purified by chromatography on Sephadex G-50 and DEAE-cellulose (*11*). The product was finally isolated by freeze-drying.

RESULTS AND DISCUSSION

The preparation and isolation of the 7-77 fragment of acetylated ACP was carried out using established procedures (*9, 11*). The purified material was shown to be homogeneous by chromatographic techniques, amino acid analysis, and the presence of valine as the sole amino terminal residue. The purified 7-77 peptide was found to have less than 2% of the activity of native ACP in the $^{14}CO_2$ exchange assay (*9*).

The synthesis of the amino terminal hexapeptide, in a protected form suitable for semisynthesis (Fig. 2), was achieved with acid stable side chain protecting groups and the solid phase method (*12*).

The protected peptide was removed from the resin by HBr and acetic acid. Although a single 20-min cleavage was used, a considerable amount of *p*-nitrobenzyl alcohol was observed in the crude product. A possible explanation for this side product was that the nitrobenzyl ester was partially hydrolyzed during the acidolytic cleavage.

TABLE 1

SEMISYNTHETIC COUPLING YIELDS[a] USING DIFFERENT SOLVENTS

Solvent	Yield[b]
0.1 *M* $NaHCO_3$, pH 8.15	0.20
DMSO + 1 mol Et_3N	0.34
N-Methylpyrrolidone + 1 mol Et_3N	0.53
N-Methylpyrrolidone/25 m*M* potassium phosphate buffer, pH 7.0 (1 : 1)	0.60

[a] In a typical reaction the DTNB derivative of [^{3}H] acetylated ACP 7-77 peptide (1 mg, 0.1 μM, 0.03 μCi) and the ACP 1-6 hexapeptide pentachlorophenyl ester (0.3 mg, 0.3 μM) were suspended under nitrogen in *N*-methylpyrrolidone (0.5 ml) and 25 m*M* potassium phosphate buffer, pH 7, was added. After 16 hr at room temperature the DTNB group was removed with dithiothreitol. The product was then purified by chromatography on Sephadex G-25 and on DEAE-cellulose.

[b] Moles of arginine incorporated into the final product as purified by gel filtration and ion-exchange chromatography.

The protected peptide was purified by gel filtration and by chromatography on Sephadex LH-20. Amino acid analysis of the purified peptide was in agreement with the theoretical values, and high-voltage paper electrophoresis at pH 6.5 indicated that the peptide had a single free carboxyl group. As is shown in Fig. 2 the peptide was then activated by conversion of the C terminal carboxyl group to the pentachlorophenyl ester, and this product was purified by preparative hplc. The side chain protecting groups were removed by catalytic hydrogenation (see Fig. 2) under conditions which did not cleave the active ester. More vigorous reduction conditions resulted in the formation of tetra- and trichlorophenyl esters, the existence of which could be demonstrated by mass spectrometric examination of the crude product. Analysis of the product by uv absorbance at 275 nm and the Sakaguchi assay (*15*) indicated that the N^G-nitro group was completely removed by these conditions, while the hydroxylamine assay (*16*) revealed that the active ester was still intact. Electrophoresis at pH 6.5 indicated that the peptide contained two free carboxyl groups, presumably formed by hydrogenolysis of the *p*-nitrobenzyl ester groups. The product was shown to be homogeneous by tlc, by analytical hplc (μBondapak alkylphenyl column, 50 : 50, methanol : water, 1% acetic acid, t_R 3.0 min), and by amino acid analysis.

The pentachlorophenyl ester was chosen because of its resistance to racemization under normal coupling conditions (*14*). A feature of the semisynthetic scheme was that the synthetic peptide was fully deprotected after introduction of the C terminal active ester. In this manner, the semisynthetic protein was not exposed to deprotection reactions which could damage the protein.

TABLE 2

AMINO ACID ANALYSIS DATA[a,b]

Amino acid	Native ACP[c]	7-77 Fragment	Semisynthetic ACP	Theoretical
Lys	3.8	3.4	3.8	4
His	0.73	0.76	0.55	1
Arg	1.0	0.01	0.6	1 (+1)[e]
Asp	9.4	8.7	9.7	9
Thr	6.1[d]	4.5[d]	5.5[d]	6 (+1)[e]
Ser	2.9[d]	2.2[d]	3.0[d]	3 (+1)[e]
Glu	17.7	15.5	17.0	18 (+2)[e]
Pro	1.1	1.2	1.1	1
Gly	4.3	4.2	4.7	4
Ala	7.4	6.4	6.7	7
Val	6.8	6.4	7.3	7
Met	1.0	1.2	1.4	1
Ile	6.5	5.5	6.4	7 (+1)[e]
Leu	5.3	4.3	4.3	5
Tyr	1.0	0.81	0.53	1
Phe	2.2	3.6[f]	4.0[f]	2
β-Ala	0.97	ND[g]	ND[g]	1

[a] Values are expressed as moles per mole of ACP (calculated from the average of the number of moles of all amino acids).

[b] Data from a 24-hr hydrolysis with 6 *M* HCl.

[c] Values taken from Ref. (*17*).

[d] Calculated from 24-, 48-, and 64-hr hydrolyses and extrapolation to zero time of hydrolysis.

[e] Expected increase on addition of hexapeptide.

[f] The high value of Phe is attributed to unresolved components of the 4′-phosphopantetheine prosthetic group, i.e., β-alanine and 2-mercaptoethylamine. A similar observation was made by F. Sauer *et al.*, *Proc. Nat. Acad. Sci. USA*, **52**, 1360 (1964). This assignment was further confirmed by treatment of a sample of ACP with 0.1 *M* NaOH at 70°C for 1 hr to eliminate the prosthetic group (*17*). Isolation of the protein by gel filtration on Sephadex G-10 and amino acid analysis of a 6 *M* HCl hydrolysate gave a value of Phe as 1.9 mol/mol of ACP.

[g] Not determined.

The coupling reaction between the synthetic and native fragments was found to be slow due to steric hindrance caused by valine as the amino terminal residue of the 7-77 fragment. As is shown in Table 1, the most effective solvent mixture used in the coupling was 50% *N*-methylpyrrolidone in 20 m*M* potassium phosphate, pH 7. Even with these carefully optimized conditions, the highest coupling yield obtained was 60%, which was further indication of a slow coupling reaction.

After purification the semisynthetic protein was isolated as a white powder (0.6 mg, 60%). The semisynthetic product was found to exhibit, with Sephadex G-75 and DEAE-cellulose, chromatographic properties identical to those of the native protein. As is shown in Table 2, the amino acid compositional data for the product was consistent with theoretical values (*17*). Peptide mapping by reversed phase hplc has been shown to be an extremely sensitive technique for detecting slight differences between closely related proteins (*18*). A thermolytic digest of the semisynthetic product was found to be identical to a corresponding digest of

native ACP on reversed phase hplc (*19*). This result indicates that the hexapeptide had been successfully rejoined in the semisynthesis. The CO_2 exchange activity of ACP has been shown to be a highly specific test of the ability of the carrier protein to function in fatty acid biosynthesis (*9–11*). The observed activity of the semisynthetic protein in this assay was equivalent to 71% of the activity of native ACP, and was again consistent with the success of the semisynthesis. The lower than theoretical activity could be attributed to some damage to the protein during the prolonged coupling reaction. Amino acid analysis and free –SH measurement suggested that there was a slow loss of tyrosine and the sulfhydryl group, due to irreversible oxidation reactions during the slow coupling of the synthetic and native fragments. The CO_2 exchange assay is particularly sensitive to inhibition to derivatives of ACP, and thus damage to the protein in the coupling reaction could be expected to decrease the activity of the semisynthetic product.

In conclusion, the semisynthetic scheme outlined in Fig. 1 has been successfully applied to acyl carrier protein. The application of the technique to other proteins, such as apolipoprotein A-I, is currently under investigation.

ACKNOWLEDGMENTS

We thank Mr. J. E. Battersby for expert technical assistance, Dr. G. Midwinter for amino acid analyses, and Professor R. Hodges for mass spectrometry studies. Supported by University Grants Committee (New Zealand) Grant 73/94, Medical Research Council (New Zealand) Grant 77/30, National Heart Foundation of New Zealand Award 153, and Lottery Distribution Committee, Grant 20/12508.

REFERENCES

1. R. E. Offord, *Nature (London)* **221,** 37 (1969).
2. G. Corradin and H. A. Harbury, *Biochem. Biophys. Res. Commun.* **61,** 1400 (1974).
3. D. F. Dyckes, T. Creighton, and R. C. Shepperd, *Nature (London)* **247,** 202 (1974).
4. H. Jering and H. Tschesche, *Angew. Chem. Int. Ed. Engl.* **13,** 660 (1974).
5. D. Kowlaski and M. Laskowski, *Biochemistry* **15,** 1300 (1976).
6. E. T. Lode, C. L. Murray, W. V. Sweeny, and J. C. Rabinowitz, *Proc. Nat. Acad. Sci. USA* **71,** 1361 (1974).
7. R. Obermeier and R. Geiger, *Z. Phys. Chem.* **357,** 759 (1976).
8. H. Zahn, D. Brandenberg, and H. G. Gattner, *Diabetes* **21,** 468 (1972).
9. P. W. Majerus, *Science* **159,** 428 (1968).
10. W. S. Hancock, G. R. Marshall, and P. R. Vagelos, *J. Biol. Chem.* **248,** 2424 (1973).
11. P. W. Majerus, A. W. Alberts, and P. R. Vagelos, *Biochem. Prep.* **12,** 56 (1968).
12. R. L. Prestidge, D. R. K. Harding, and W. S. Hancock, *J. Org. Chem.* **41,** 2579 (1976).
13. G. L. Ellman, *Arch. Biochem. Biophys.* **82,** 70 (1959).
14. J. Kovacks, L. Kisfaludy, and M. Q. Ceprini, *J. Amer. Chem. Soc.* **89,** 183 (1967).
15. J. M. Stewart and J. D. Young, "Solid Phase Peptide Synthesis," p. 63. Freeman, San Francisco, 1969.
16. F. Lipman and L. C. Tuttle, *J. Biol. Chem.* **159,** 21 (1945).
17. T. C. Vanaman, S. J. Wakil, and R. L. Hill, *J. Biol. Chem.* **243,** 6420 (1968).
18. W. S. Hancock, C. A. Bishop, R. L. Prestidge, D. R. K. Harding, and M. T. W. Hearn, *Science* **20,** 1168 (1978).
19. W. S. Hancock, C. A. Bishop, R. L. Prestidge, and M. T. W. Hearn, *Anal. Biochem.* **89,** 203 (1978).