Immunochemical Studies on Tobacco Mosaic Virus Protein. IV. The Automated Solid-Phase Synthesis of a Decapeptide of Tobacco Mosaic Virus Protein and Its Reaction with Antibodies to the Whole Protein*

John Morrow Stewart,† Janis Dillaha Young, E. Benjamini, M. Shimizu, and Cherry Y. Leung

ABSTRACT: It has been shown previously that tobacco mosaic virus protein (TMVP) tryptic peptide 8 having the amino acid sequence Ile-Ile-Glu-Val-Glu-Asn-Gln-Ala-Asn-Pro-Thr-Thr-Ala-Glu-Thr-Leu-Asp-Ala-Thr-Arg possesses immunological activity related to that of the whole protein (TMVP). This paper reports the synthesis and immunological activity of the C-terminal decapeptide of peptide 8. The peptide was synthesized using the automatic solid-phase synthesis method of Merrifield and Stewart [Merrifield, R. B., and Stewart, J. M. (1965), *Nature 207*, 522]. *t*-Butyloxycarbonylnitro-L-arginine was esterified to the polystyrene–divinylbenzene resin, and the remaining nine L-amino acids were coupled in a 36-hr run of the automatic instrument. The peptide was purified by ion-exchange chromatography and was characterized by amino acid analysis and paper electrophoresis. The synthetic peptide was found to possess specific immunological activity related to TMVP. This was demonstrated by its successful competition with peptide 8 for anti-TMVP and by the direct binding of the [^{14}C]acetyl decapeptide to anti-TMVP.

Tryptic peptide 8 of tobacco mosaic virus protein (TMVP), containing residues 93–112 of TMVP and having the amino acid sequence Ile-Ile-Glu-Val-Glu-Asn-Gln-Ala-Asn-Pro-Thr-Thr-Ala-Glu-Thr-Leu-Asp-Ala-Thr-Arg (Gish, 1961; Funatsu *et al.*, 1964; Wittmann, 1965), has been demonstrated by several methods to bind specifically with rabbit antibodies directed toward the whole protein, TMVP (Benjamini *et al.*, 1964, 1965). Chemical and enzymic degradation of TMVP tryptic peptide 8 revealed that certain portions of the peptide were not required for this immunological activity,—the peptide was still active after the removal of five residues from the N terminus and two residues from the C terminus (Young *et al.*, 1966). In the search for the areas responsible for the antigenic activity of peptide 8, its C-terminal decapeptide was synthesized. This paper reports the automated solid-phase synthesis of the decapeptide Thr-Thr-Ala-Glu-Thr-Leu-Asp-Ala-Thr-Agr, and studies on the reaction between this decapeptide and antibodies to the whole protein.

Experimental Section

Paper electrophoresis was performed at 1000 v on a 11.5 × 35.5 cm Whatman No. 1 paper, using a pH 6.4 pyridine–acetic acid–water buffer (10:0.4:90). The time of electrophoresis was that which was required for the migration of picric acid to 10 cm (*ca.* 1.5 hr). Radioactivity measurements were performed as previously described (Benjamini *et al.*, 1965). Quantitative amino acid analyses of peptide hydrolysates were done using the Spinco, Model 120B, amino acid analyzer (Spinco Division, Beckman Instruments, Palo Alto, Calif.). The chloromethylpolystyrene–2% divinylbenzene copolymer resin and all of the *t*-butyloxycarbonyl amino[1] acids were obtained from the Cyclo Chemical Corp., Los Angeles, Calif. Purity of the *t*-BOC-amino acids was ascertained by thin layer chromatography (Merrifield, 1964).

Automated Solid-Phase Synthesis of Thr-Thr-Ala-Glu-Thr-Leu-Asp-Ala-Thr-Arg. *t*-Butyloxycarbonylnitro-L-arginine was esterified to the chloromethylpolystyrene–2% divinylbenzene copolymer resin as described by Merrifield (1964), except that the reaction time was extended to 65 hr. An aliquot of the product was hydrolyzed by refluxing a suspension of the resin for 18 hr in a mixture of equal volumes of dioxane and concentrated hydrochloric acid (Marshall and Merrifield, 1965). The hydrolysate was analyzed on the amino acid analyzer. Summation of the ornithine, arginine, and nitroarginine peaks indicated that the resin contained 0.248 mmole of arginine/g.

A 10-g batch (2.5 mmoles) of the *t*-butyloxycarbonylnitroarginine resin was loaded into the reaction vessel

* From the Laboratory of Medical Entomology, Kaiser Foundation Research Institute, and Allergy Research Division, Allergy Department, Kaiser Foundation Hospitals, San Francisco, California 94115. *Received June 28, 1966.* These studies were supported in part by the U. S. Public Health Service Grant No. AI 06040. A preliminary report of this study has been presented (Stewart *et al.*, 1966).

† Rockefeller University, New York, N. Y. 10021.

[1] Abbreviation used: *t*-BOC, *t*-butyloxycarbonyl.

TABLE I: Amino Acid Ratios of Decapeptide Preparations.

Amino Acid	Resin Hydrolysate	Crude Peptide[a]	Peptides from Dowex 1 Column			Theoretical Ratio
			Peak I	Peak II	[^{14}C]-Acetylated Peak II	
Arg	1.20[b]	1.03	0.91	1.07	0.98	1
Asp	0.87	1.03	1.0	1.01	1.02	1
Thr	4.60	4.49	5.23	3.81	3.73	4
Glu	0.32[c]	0.97	0.94	1.04	1.05	1
Ala	2.0	2.0	2.25	2.04	2.02	2
Leu	1.05	0.97	1.0	0.99	1.02	1

[a] After hydrogenation. [b] The sum of arginine, ornithine, and nitroarginine. [c] Values for glutamic acid are characteristically low in peptide–resin hydrolysates.

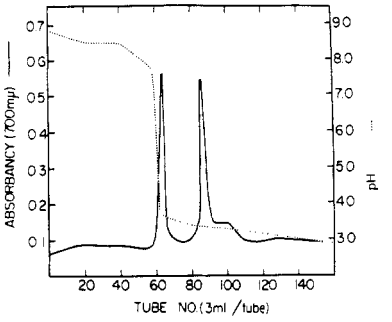

FIGURE 1: Chromatography on Dowex 1-X2 column (0.9 × 150 cm) of 26 mmoles of decapeptide which had previously been cleaved from the resin and deprotected by hydrogenation. The decapeptide was applied to the column which had been equilibrated with a pH 8.8 collidine–pyridine–acetic acid buffer and was eluted by means of an acetic acid gradient (Young *et al.*, 1966); flow rate, 42 ml/hr; temperature, 40°. The Folin–Lowry color (in a total volume of 2.2 ml) was obtained from 0.05-ml aliquots which were evaporated to dryness prior to analysis.

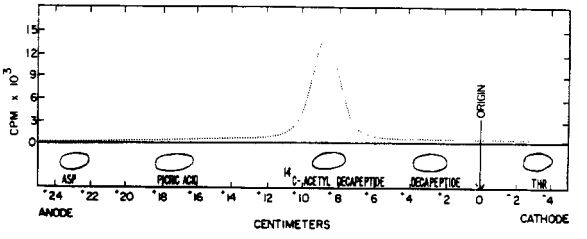

FIGURE 2: Electrophoretic patterns of [^{14}C]acetyl decapeptide, decapeptide, and markers. The electrophoresis was performed using a pH 6.4 buffer (pyridine–acetic acid–water (10.0:0.4:90)), on Whatman No. 1 paper at 22 v/cm. Radioactivity of the [^{14}C]acetyl decapeptide was scanned using a Nuclear-Chicago Model C 100B Actigraph II. The positions of [^{14}C]acetylated decapeptide, unlabeled decapeptide, threonine, aspartic acid, and picric acid which were electrophoresed under the same conditions are also indicated.

of the automatic peptide synthesis instrument (Merrifield and Stewart, 1965), which consists of the reaction vessel with components to store, select, and transfer reagents into and out of the vessel, and a programmer which automatically controls the operation of the various components. The next nine residues were coupled to the arginine in a 36-hr run of the instrument. The sequence of operations used for the coupling of each amino acid to the peptide on the resin was as described for the manual synthesis of bradykinin by the solid-phase method (Merrifield, 1964), except that chloroform was used as a solvent for the triethylamine neutralization step and for the washes immediately preceding and following this step, and except that each *t*-BOC-amino acid (5.0 mmoles) was coupled to the free amino group on the peptide–resin in 40 ml of dichloromethane in the presence of 5.5 mmoles of dicyclohexylcarbodiimide. Dichloromethane was also used for the washes preceding and following the coupling reaction. Using the above operations, the following protected amino acids were coupled in turn to the nitroarginine on the resin: *t*-BOC-L-threonine, *t*-BOC-L-alanine, *t*-BOC-L-aspartic acid β-benzyl ester, *t*-BOC-L-leucine, *t*-BOC-L-threonine, *t*-BOC-L-glutamic acid γ-benzyl ester, *t*-BOC-L-alanine, *t*-BOC-L-threonine, and *t*-BOC-L-threonine. Following the coupling of the last threonine residue the product was removed from the reaction vessel and dried (weight, 11.6 g). Hydrolysis, as described above, of a portion of the protected decapeptide–resin in dioxane–HCl followed by amino acid analysis gave the ratios shown in Table I (column 2). The yield of the peptide was 0.179 mmole/g of resin.

The peptide was cleaved from the resin with anhy-

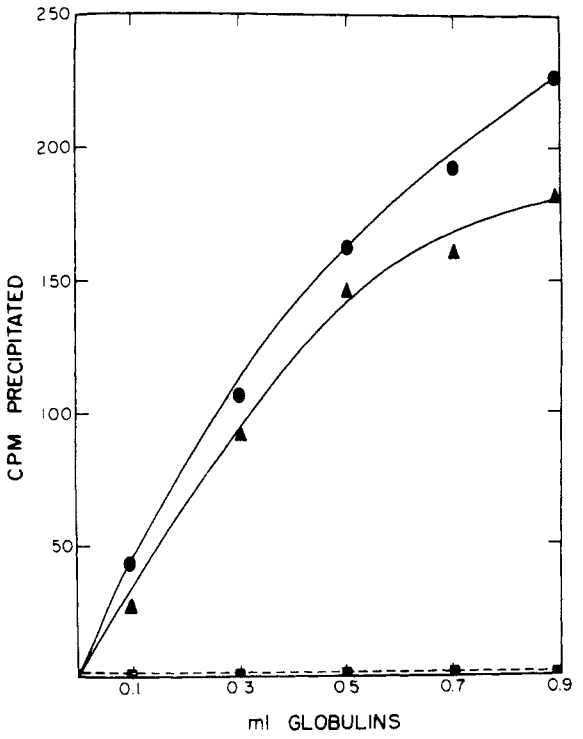

FIGURE 3: The binding of [^{14}C]acetyl peptide 8 (1.46 × 10^6 cpm/μmole) and [^{14}C]acetyl decapeptide (1.2 × 10^6 cpm/μmole) with immune globulins to TMVP and with control globulins. Globulins were mixed with 3.65 mμmoles of [^{14}C]acetylated peptides and the complex was precipitated at 50% saturation of ammonium sulfate. The term "globulins" represents the volume of serum from which they were obtained. (●—●), [^{14}C]acetyl peptide 8 with anti-TMVP; (▲—▲), [^{14}C]acetyl decapeptide with anti-TMVP; (■- - -■), [^{14}C]acetyl peptide 8 or [^{14}C]acetyl decapeptide with antiacetylcholinesterase.

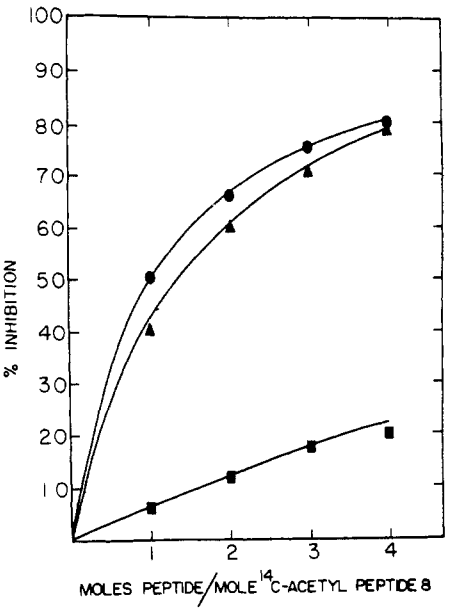

FIGURE 4: Inhibition of [^{14}C]acetyl peptide 8–anti-TMVP binding by peptide 8 (▲—▲) and by decapeptide (■—■). Globulins derived from 0.5 ml of anti-TMVP serum were mixed with unlabeled peptides and 3.65 mμmoles of [^{14}C]acetyl peptide 8; the complex was precipitated at 50% saturation of ammonium sulfate. The precipitate was washed with 50% saturated ammonium sulfate and dissolved in saline, and the radioactivity was determined on an aliquot. Expected inhibition of [^{14}C]-acetyl peptide 8–anti-TMVP by peptides having the same binding as [^{14}C]acetyl peptide 8 (●—●) (Young *et al.*, 1966). In no case did [^{14}C]acetyl peptide 8 bind to control globulins.

drous HBr in trifluoroacetic acid by the procedure of Merrifield (1964). The crude peptide (650 mg, 0.31 mmole) from 2.0 g of resin was dissolved in a mixture consisting of 40 ml of methanol, 4 ml of water, and 4 ml of acetic acid and hydrogenated for 16 hr in the presence of 1.0 g of palladium oxide–barium sulfate catalyst (Kuhn and Haas, 1955). Amino acid analysis of this crude deprotected peptide is shown in Table I (column 3). Paper electrophoresis of the crude peptide showed the presence of the expected peptide, having a net negative charge at pH 6.4, and, in addition, a large amount of an electrophoretically neutral peptide.

The crude peptide was chromatographed on a 150-cm column of Dowex 1-X2 ion-exchange resin equilibrated with collidine–pyridine buffer, pH 8.8, and eluted with a gradient of increasing acetic acid concentration (Young *et al.*, 1966). Two peptide peaks were obtained from the column effluent (Figure 1). Peak I, which emerged where the pH dropped sharply, was electrophoretically neutral at pH 6.4 and was contaminated with a small amount of negatively charged material having the same electrophoretic mobility as that of the material from peak II. Although peak I seems to be a mixture, its amino acid analysis (Table I) revealed approximately the amino acid ratios expected for the decapeptide, except for threonine, which was high. The material from peak II was electrophoretically homogeneous and had the expected negative charge. The R_{asp} of this peptide was 0.13 (Figure 2). Amino acid analysis of peak II revealed the expected ratios for the desired decapeptide (Table I). From 2.0 g of decapeptide–resin 59 μmoles of peak I peptide and 89 μmoles of peak II peptide were obtained, giving a 29% yield of pure decapeptide (peak II) from the crude peptide, or 25% yield from the decapeptide–resin.

The pure decapeptide (peak II) was acetylated with a 5 M excess of [^{14}C]acetic anhydride at pH 8 at room temperature for 1 hr (Benjamini *et al.*, 1965). The acetylated decapeptide was separated from acetate by means of a 0.9 × 100 cm Sephadex G-10 column which was equilibrated and eluted with collidine–pyridine–acetic acid buffer, pH 8 (20:20:0.75, ml/l.) (Young *et al.*, 1966). Paper electrophoresis of the [^{14}C]-

acetyl decapeptide showed a single arginine-positive spot (R_{asp} 0.37) with no ninhydrin-reacting areas (Figure 2). Scanning of the electrophoretogram for radioactivity revealed only one area of radioactivity (Figure 2). Amino acid analysis of the [^{14}C]acetylated decapeptide is given in Table I. The [^{14}C]acetyl decapeptide thus obtained had a specific activity of 1.2×10^6 cpm/μmole.

Immunological Assay. The anti-TMVP used was obtained from a single rabbit. Sensitization of the animal and preparation of anti-TMVP globulins, as well as of the antiacetylcholinesterase globulins which were used as controls, were carried out as previously described (Benjamini *et al.*, 1965). The immunological activity of the synthetic decapeptide was measured by direct binding of the [^{14}C]acetyl peptide to anti-TMVP, as described by Benjamini *et al.* (1965). The [^{14}C]acetyl peptide was mixed with globulins, which were then precipitated at 50% saturation of ammonium sulfate. The precipitates were washed twice and redissolved in saline, and aliquots were assayed for radioactivity. The activity of the peptide was also measured by its ability to inhibit the binding between [^{14}C]acetyl tryptic peptide 8 of TMVP and anti-TMVP as described by Young *et al.* (1966). Results of these experiments are shown in Figures 3 and 4.

Discussion

Synthesis. The automated solid-phase peptide synthesis method has previously been employed only for the synthesis of bradykinin (Merrifield and Stewart, 1965). The successful automated solid-phase synthesis of a TMVP decapeptide sequence demonstrates the applicability of this new technique to the synthesis of another peptide. The synthesis was carried out with ease and rapidity, and resulted in an adequate yield.

The presence in the crude peptide of a neutral component in addition to a component having the desired negative electrophoretic mobility at pH 6.4 was unexpected. However, ion-exchange chromatography using Dowex 1 effected the isolation of the negatively charged peptide (Figure 1) with an amino acid analysis corresponding to that of the desired decapeptide (Table I). It was observed that electrophoretograms of the crude peptide prior to Dowex 1 chromatography contained much more neutral material than was obtained upon chromatography. This may indicate that some of the neutral material was converted to the free peptide by the passage through the column, suggesting that one of the negative charges in the peptide was masked by a group that was labile at the relatively high pH (8.8) used at the beginning of the column, or that the ion-exchange resin itself was liberating the acidic group. The exact nature of the neutral material in the product is under investigation.

Immunological Activity. The immunological activity of the [^{14}C]acetyl decapeptide is apparent from data presented in Figure 3. The results show that as in the case of [^{14}C]acetyl peptide 8 the radioactive synthetic decapeptide remained associated with the anti-TMVP globulins following their precipitation at 50% saturation of ammonium sulfate. The binding between the radioactive peptide and anti-TMVP was found to be specific since no radioactivity was detected in precipitates obtained from mixtures of comparable amounts of the radioactive peptide and antiacetylcholinesterase globulins. A quantitative comparison of the relative affinities of the synthetic decapeptide and peptide 8 for anti-TMVP is shown in Figure 4. The data on the ability of these peptides to inhibit the binding between [^{14}C]acetyl peptide 8 and antibody indicate that the binding between anti-TMVP and the synthetic decapeptide is approximately tenfold lower than that between peptide 8 and antibody; *i.e.*, 4 moles of decapeptide or 0.4 mole of eicosapeptide (peptide 8) were able to achieve the same degree of inhibition of the binding of [^{14}C]acetyl peptide 8 to the antibody (the theoretical considerations of this inhibition technique were discussed by Young *et al.*, 1966).

Although the data of Figure 4 show that the binding between the decapeptide and anti-TMVP is lower than that between peptide 8 and anti-TMVP, the binding between the synthetic peptide and antibodies is quite substantial in view of the high binding (10^9 l./mole) between peptide 8 and anti-TMVP (Benjamini *et al.*, 1965). It would appear that the synthetic decapeptide does indeed contain those structural features necessary for specific binding to anti-TMVP. The minimal size, amino acid sequences, or functional groups responsible for this specific binding cannot at present be stated, although the high threonine content and presence of charged groups are conspicuous. Further studies to delineate exactly the minimum requirements for antibody binding are currently in progress.

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Studies on the Mechanism of Action of the Δ^5-3-Ketosteroid Isomerase from Rat Adrenal Small Particles*

Nancy L. Oleinick† and Seymour B. Koritz

ABSTRACT: Isomerization of androst-5-ene-3,17-dione has been carried out in a deuterated medium under conditions of (a) enzyme catalysis by a rat adrenal microsomal fraction activated by oxidized diphosphopyridine nucleotide (DPN^+); (b) nonenzymatic reaction at pH 7.0; (c) acid catalysis; and (d) base catalysis. In all cases, incorporation of deuterium into the 6β position of the product is found. Incorporation at C-4 of the product is also found for all conditions except acid catalysis. A similarity in mechanism between alkaline isomerization and catalysis by the rat adrenal enzyme is suggested, and the proposed intermediate is the conjugate enolate anion. A profound deuterium isotope effect is seen in the enzymatic isomerization, and this is decreased in the presence of DPN^+ or reduced DPN (DPNH).

The mechanism of action of Δ^5-3-ketosteroid isomerase was initially investigated with the enzyme from *Pseudomonas testosteroni* by Talalay and Wang (1955), Kawahara and Talalay (1960), and Wang *et al.* (1963). When the reaction was conducted in deuterium-labeled water, they found no incorporation of label into the Δ^4-3-ketone product, in contrast to the acid- and base-catalyzed reactions, from which they concluded that the enzymatic isomerization proceeds *via* direct transfer of a hydrogen atom from C-4 to C-6 of the steroid nucleus. Malhotra and Ringold (1965) have prepared various C-4- and C-6-deuterated substrates and have shown that the bacterial enzyme catalyzes a stereospecific intramolecular transfer of a proton from the axial 4β to the axial 6β position. The rate-determining step is loss of the 4β proton. Based on the lack of deuterium incorporation at C-4, the proposed intermediate is a $\Delta^{3,5}$-dienol rather than the enolate anion, in common with the acid-catalyzed reaction.

In direct contrast, Werbin and Chaikoff (1963, 1964) have indicated that the mammalian adrenal isomerase must catalyze the reaction by another mechanism which involves loss of the 4β proton to the medium. Thus, when guinea pigs were fed a mixture of cholesterol-4-^{14}C and stereospecifically labeled cholesterol-4β-^{3}H, the ^{3}H:^{14}C ratio was greatly reduced in the urinary cortisol and 6β-hydroxycortisol. Furthermore, when these two labeled cholesterols were incubated with a mitochondrial acetone powder from beef adrenals, the tritium label was retained in the pregnenolone formed but lost in progesterone. This is strong evidence that the loss of the 4β-^{3}H occurred at the isomerase step.

It seemed reasonable that if the reaction catalyzed by the mammalian isomerase were carried out in a D_2O medium, deuterium should be found in the 6 position of the product, and that additional information concerning a mechanism could be obtained by this more direct approach. The authors have now isomerized Δ^5-AND[1] under the following conditions: (1) enzyme catalysis by a rat adrenal microsomal fraction activated by DPN^+ (Oleinick and Koritz, 1966); (2) nonenzymatic isomerization at pH 7.0; (3) acid catalysis; and (4) base catalysis. Application of the infrared absorption spectra for the various possible deuterium-labeled androstenediones (Malhotra and Ringold, 1964) has allowed us to detect similarities between the enzymatic catalysis and the base-catalyzed reaction.

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† This report is from the Ph.D. dissertation of Nancy L. Oleinick, predoctorate fellow at the University of Pittsburgh. Present address: Department of Biochemistry, Western Reserve University, School of Medicine, Cleveland, Ohio.

[1] Abbreviations: Δ^5-AND, androst-5-ene-3,17-dione; Δ^4-AND, androst-4-ene-3,17-dione; DPN^+, oxidized diphosphopyridine nucleotide; DPNH, reduced diphosphopyridine nucleotide; BSA, bovine serum albumin.