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Immunochemical Studies on the Tobacco Mosaic Virus Protein. II. The Specific Binding of a Tryptic Peptide of the Protein with Antibodies to the Whole Protein*

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ABSTRACT: Direct measurements are presented for the specific binding of a tobacco mosaic virus protein (TMVP) tryptic peptide having the amino acid sequence Ileu-Ileu-Glu-Val-Glu-AspNH_2-GluNH_2-Ala-AspNH_2-Pro-Thr-Thr-Ala-Glu-Thr-Leu-Asp-Ala-Thr-Arg to antibodies to TMVP. The specific binding of the acetyl-^{14}C tryptic peptide with anti-tobacco mosaic virus protein was shown by precipitation of the complex with ammonium sulfate, by gel filtration of the complex, and by equilibrium dialysis. No binding occurred with anti-tobacco mosaic virus protein which had been previously absorbed with tobacco mosaic virus protein.

Several protein fragments have been shown to possess immunological activity related to that of the whole protein from which they have been derived. This has been shown by methods which involve measuring the effect of the fragment on the reaction between the whole protein and the antibody or by demonstrating immunological cross reactivity between the whole protein and the fragment conjugated to an unrelated carrier.[1]

In studies on the immunological relationship of the tryptic peptides of tobacco mosaic virus protein (TMVP)[2] to the full antigen (Benjamini *et al.*, 1964), tryptic peptide 8[3] was found to specifically inhibit the fixation of complement by TMVP and anti-TMVP. This peptide has the amino acid sequence Ileu-Ileu-Glu-Val-Glu-AspNH_2-GluNH_2-Ala-AspNH_2-Pro-Thr-Thr-Ala-Glu-Thr-Leu-Asp-Ala-Thr-Arg. Preliminary experiments directed toward the elucidation of the antigenic determinant(s) of peptide 8 revealed that acetylated peptide 8 was still fully inhibitory in the complement fixation system.

The data presented in this communication deal with the binding between ^{14}C-acetylated peptide 8 and anti-TMVP. These data offer direct evidence that peptide 8 specifically binds with antibodies to TMVP.

Materials and Methods

Tobacco Mosaic Virus Protein. TMVP was obtained

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[1] Tryptic peptide 12 of TMVP was conjugated to bovine serum albumin by Anderer (1963), who studied the immunological relationship between this peptide and the tobacco mosaic virus.

[2] Abbreviations used in this work: TMVP, tobacco mosaic virus protein; ^{14}C8, [2-^{14}C]acetyl peptide 8; AChE, acetylcholinesterase.

[3] Nomenclature according to that proposed by Tsugita *et al.*, 1960.

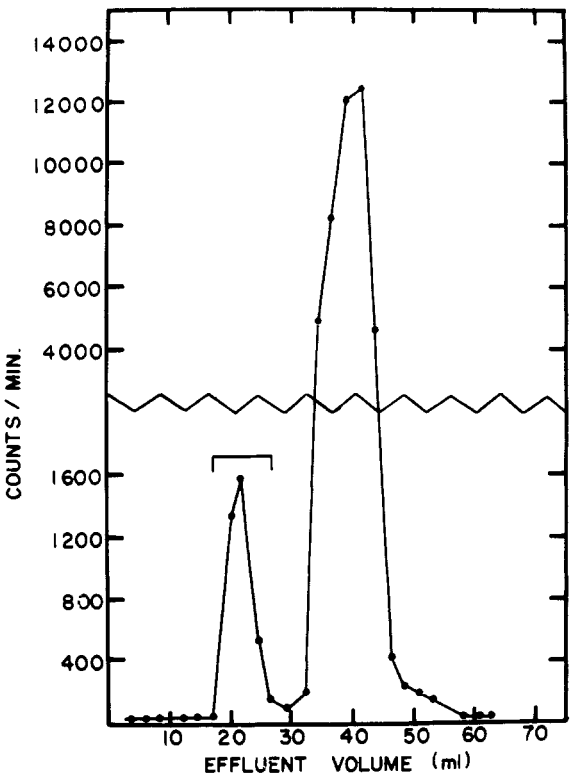

FIGURE 1: Sephadex G-25 elution pattern of tryptic peptide 8 of TMVP following acetylation with [^{14}C]-acetic anhydride. Eluted with 50% acetic acid. The smaller peak represents [^{14}C]acetyl peptide 8.

from tobacco mosaic virus[4] by treatment with 67% acetic acid according to the method of Fraenkel-Conrat (1957).

TMVP Tryptic Peptide 8. Tryptic peptide 8 used for the experiments reported in this communication was received from Dr. D. T. Gish,[5] who obtained the peptide by its precipitation at pH 2.5 followed by purification using countercurrent distribution in 1-butanol–pyridine–0.1% acetic acid (5:3.5:12) (Gish, 1961). Amino acid analysis of the peptide following a 16-hr hydrolysis in 6 N HCl was performed in our laboratory using the Spinco Model 120B amino acid analyzer. Results of the analysis showed that the molar ratios of the amino acids of the peptide were as expected, with no more than 0.04 μM of any amino acid contaminant present for each 1 μM of the peptide preparation.

Measurements of Radioactivity. Radioactivity was measured by using the Nuclear Chicago Model D-47 gas flow detector, and Model C100B Actigraph II with Model 1620B analytical count rate meter. Samples were counted after evaporation in planchets 3.2 cm in diameter and 0.13 cm deep. No corrections were made for self-absorption unless specified.

Preparation of [2-^{14}C]Acetyl Peptide 8. Tryptic peptide 8 was acetylated with [2-^{14}C]acetic anhydride, using the conditions recommended by M. D. Collams[6] (to be published). Ten μmoles of [^{14}C]acetic anhydride[7] was used for the acetylation of 1.1 μM of peptide 8 at pH 8.0 and at room temperature. The ^{14}C-acetylated peptide (henceforth designated as ^{14}C8) was separated from the reacting mixture by passage through a 1 × 50 cm G-25 Sephadex column, equilibrated, and eluted with 50% acetic acid (Crestfield *et al.*, 1963). Aliquots (10 μl) from each tube to which 0.1 ml of 1 N NaOH in 95% ethanol was added were placed in planchets for measurements of radioactivity. The separation of the acetylated peptide from the rest of the reacting mixture is shown in Figure 1. The tubes containing ^{14}C8 (17–27 ml) were pooled and lyophilized. The acetylated peptide was taken up in distilled water and the pH adjusted to 8 with 1 N NaOH. The final solution contained 0.073 μM/ml (as determined by amino acid analysis of an aliquot), with a specific activity of 69,400 cpm/ml or 9.55×10^5 cpm/μM.

Paper electrophoresis of 20 μl of ^{14}C8 on Whatman 3MM paper in pyridine–acetic acid–water (100:4:900), pH 6.4, for 3 hr at 30 v/cm revealed only one radioactive area migrating 6 cm toward the anode, which was also arginine positive as expected for tryptic peptide 8 (Benjamini *et al.*, 1964).

Antisera and Globulins. Anti-TMVP serum was obtained from rabbits following three or more weekly intramuscular injections of 10 mg of TMVP incorporated in an emulsion consisting of 1 ml of saline and 1 ml of Freund's Complete Adjuvant (Difco Laboratories, Detroit, Mich.). Globulins were obtained by repeated (three times) precipitation of the serum at 50% saturation of ammonium sulfate. The final fraction was dialyzed against sodium borate–buffered saline,[8] pH 8.0, and the volume was adjusted to the volume of serum from which the globulins were derived.

The data presented in this paper were obtained from experiments utilizing antiserum or globulins obtained from a single rabbit; however similar results were obtained from several other rabbits. Anti-acetylcholinesterase (AChE) serum or globulins were used as controls to demonstrate specificity.

Experimental Section

Ammonium Sulfate Precipitation of ^{14}C8-Antibody Complex. The method used by Farr (1958) for precipitating labeled antigen–antibody complexes at 50% saturation of ammonium sulfate was used in these experiments. Various amounts of globulins containing antibodies to TMVP or to acetylcholinesterase were

[4] The tobacco mosaic virus was generously supplied by Dr. C. A. Knight of the Virus Laboratory, University of California, Berkeley.

[5] Present address: The Upjohn Co., Kalamazoo, Mich.

[6] Dept. of Virology, University of California, Berkeley, Calif.

[7] Obtained from New England Nuclear Corp. (Lot 141-107-3) with specific activity of 0.5 mcurie/100 μmoles.

[8] Borate-buffered saline: 7.81 g of NaCl, 7.00 g of H_3BO_3, and 5.05 g of $Na_2B_4O_7 \cdot 10H_2O$ per liter.

mixed with 0.05 ml containing 3.65 mμmoles of ^{14}C8 in a total volume of 1 ml made up with borate-buffered saline, pH 8.0. To these mixtures 1 ml of a saturated solution of ammonium sulfate was added, and the tubes were centrifuged at 5000 rpm for 30 min at room temperature. Radioactivity of 0.2 ml of the resultant supernatants was counted. The precipitate was washed twice with 50% ammonium sulfate and was then taken up in 2 ml of saline. Radioactivity of 0.2 ml of the resulting solution was counted. Results shown in Figure 2 demonstrate the specific binding of ^{14}C8 to anti-TMVP.

In order to demonstrate that the specific binding of ^{14}C8 with anti-TMVP is due to antibodies which combine with TMVP, 0.8 ml of anti-TMVP serum was first completely absorbed with TMVP. The absorbed serum was adjusted with saline to a total volume of 2 ml to which 0.05 ml containing 3.65 mμmoles of ^{14}C8 was added, followed by the addition of 2 ml of saturated ammonium sulfate. After centrifugation the radioactivity of 0.4 ml of the supernatant was measured. Controls consisted of similar tests using 0.8 ml of unabsorbed anti-TMVP and 0.8 ml of anti-AChE. Results shown in Table I demonstrate that ^{14}C8 did not bind to the absorbed anti-serum.

TABLE I: Comparison of Binding of [^{14}C]Acetyl Tryptic Peptide 8 of TMVP with Absorbed and Unabsorbed Anti-TMVP.[a]

Antiserum	Activity Remaining in Supernatant[c,d] (cpm)
Anti-TMVP, nonabsorbed	6
Anti-AChE	65
Anti-TMVP, absorbed with TMVP[b]	63

[a] ^{14}C8, 3.65 mμmoles; antisera, 0.8 ml. [b] 40.5 mμmoles of TMVP needed for total absorption. [c] Radioactivity was counted in 0.4 ml of the supernatant from a total volume of 4 ml 50% saturated ammonium sulfate. [d] The supernatant was obtained by precipitation at 50% saturation of ammonium sulfate.

Globulins obtained from anti-TMVP were heated in borate saline at 90° for 15 min prior to the addition of 3.65 mμmoles of ^{14}C8. Radioactivity was determined in the supernatants resulting from precipitation at 50% saturation of ammonium sulfate. Results given in Table II show that no ^{14}C8 was bound. These data demonstrate that the component of the antiserum which binds ^{14}C8 is heat labile.

Gel Filtration of ^{14}C8–Antibody Complex. Anti-TMVP or anti-AChE globulin (1 ml) was mixed with 0.07 ml (5.11 mμmoles) of ^{14}C8. The mixture was then passed through a 1 × 50 cm column of P-60 gel (Bio-Rad Laboratories, Richmond, Calif.) equilibrated and eluted at room temperature with 0.2 M ammonium bicarbonate buffer, pH 8.2. Fractions of 20 drops were collected and diluted with 5 ml of the buffer. The optical density of each fraction was measured at 280 mμ in a Beckman Model DB spectrophotometer. Radioactivity of a 1-ml aliquot of each fraction was also measured. The elution pattern with respect to optical density and radioactivity of anti-TMVP or of anti-AChE mixed with ^{14}C8 is shown in Figure 3.

TABLE II: Loss of Binding of [^{14}C]Acetyl Tryptic Peptide 8 of TMVP with Heat-Treated Globulins to TMVP.[a]

Globulins	ml[b]	Activity Remaining in Supernatant[c] (cpm)
Anti-TMVP	0.1	124
Anti-TMVP	0.3	35
Anti-TMVP	0.5	3
Anti-TMVP	0.7	0
Anti-TMVP, heat treated	0.1	167
Anti-TMVP, heat treated	0.3	155
Anti-TMVP, heat treated	0.5	123
Anti-TMVP, heat treated	0.7	115
Anti-AChE	0.1	183
Anti-AChE	0.3	130
Anti-AChE	0.5	116
Anti-AChE	0.7	131
Anti-AChE, heat treated	0.1	167
Anti-AChE, heat treated	0.3	167
Anti-AChE, heat treated	0.5	151
Anti-AChE, heat treated	0.7	132

[a] ^{14}C8, 3.65 mμmoles/tube; the heat treatment consisted of heating at 90° for 15 min. [b] Equivalent to milliliters of serum from which globulins were obtained. [c] Following precipitation at 50% saturation ammonium sulfate the radioactivity was counted in 0.2 ml of the supernatant from a total volume of 2 ml of 50% saturated ammonium sulfate.

Equilibrium Dialysis. Since the purpose of this experiment was to show the specific binding of ^{14}C8 to anti-TMVP rather than to determine binding constants, equilibrium dialysis was performed using a single free hapten concentration.

Preliminary experiments on the dialysis through 0.6 cm in diameter cellophane dialysis tubing of ^{14}C8 in borate-buffered saline, pH 8.0, at 4° showed that the 50% escape time under these conditions was 24 ± 2 hr.

Anti-TMVP and anti-AChE sera were diluted with four volumes of borate-buffered saline; 0.5-ml aliquots

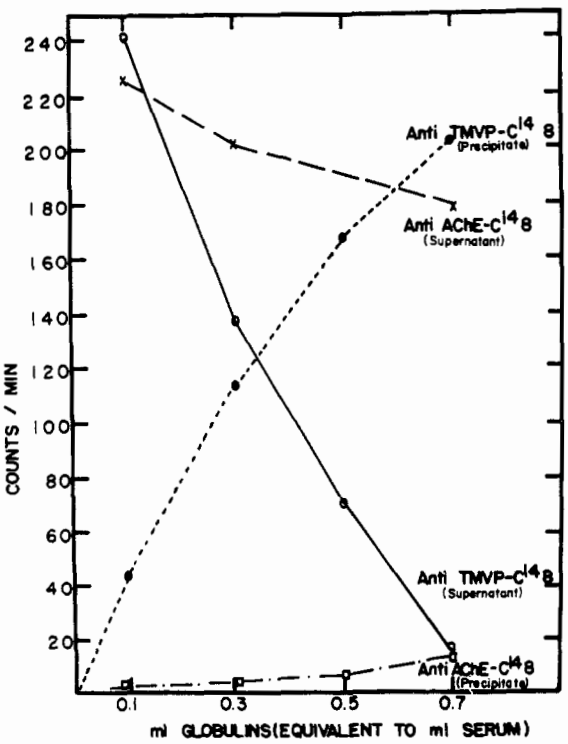

FIGURE 2: The binding between [^{14}C]acetyl tryptic peptide 8 of TMVP with immune globulins to TMVP. Anti-TMVP or anti-AChE globulins were mixed with 3.65 mμmoles of ^{14}C8 and the complex was precipitated at 50% saturation of ammonium sulfate. (○—○), radioactivity remaining in supernatant of anti-TMVP and ^{14}C8; (●—●), radioactivity in precipitate of anti-TMVP and ^{14}C8; (×—×), radioactivity remaining in supernatant of anti-AChE and ^{14}C8; (□-·-□), radioactivity in precipitate of anti-AChE and ^{14}C8.

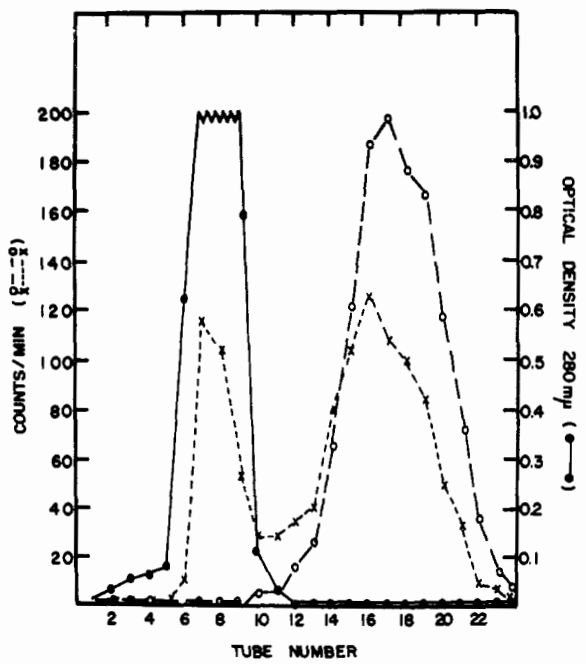

FIGURE 3: P-60 elution pattern of anti-TMVP or anti-AChE mixed with [^{14}C]acetyl tryptic peptide 8 of TMVP. Globulins derived from 1 ml of serum were combined with 5.11 mμmoles of ^{14}C8; eluting buffer: 0.2 M ammonium bicarbonate, pH 8.0. Anti-TMVP-^{14}C8: radioactivity (×- - -×), absorbance at 280 mμ (●—●). Anti-AChE-^{14}C8: radioactivity (○—○); the protein elution pattern of anti-AChE is the same as that of anti-TMVP (●—●) and was omitted for graphical clarity.

were placed in dialysis bags. Bags containing 0.5 ml of 8 mg of bovine serum albumin/ml[9] in borate saline were also prepared. All the bags were immersed in 7 ml containing 8 mg of bovine serum albumin/ml in borate saline; 2.78 mμmoles of ^{14}C8 was added to the outside solution. The entire system was slowly rotated for 10 days at 4°. At the end of this period the contents of each bag was weighed and diluted with 1 ml of 0.2 M ammonium bicarbonate. Protein concentrations of the contents of the bags were determined by their optical densities at 280 mμ. Each sample in its entirety was then counted for radioactivity. After correction for protein concentration and self-absorption (as described by Kamen, 1957) the bindings between ^{14}C8 and the antisera were calculated. Results shown in Table III demonstrate the specific binding of ^{14}C8 to anti-TMVP. Completion of equilibration was confirmed by analyses of the samples containing bovine serum albumin.

[9] Powder, fraction V from bovine plasma, obtained from The Armour Laboratories, Kankakee, Ill.

Discussion

The first implication that tryptic peptide 8 of the tobacco mosaic virus protein exhibited specific immunological activity related to that of the whole protein was based on data obtained from the inhibition by this peptide of the fixation of complement by TMVP and anti-TMVP (Benjamini *et al.*, 1964). By the use of labeled peptide 8 with radioactive carbon, data presented in this communication offer direct evidence for the specific binding of an immunologically active protein fragment of TMVP (peptide 8) with the antibody to the protein (TMVP). These data have been obtained utilizing methods currently employed to demonstrate specific binding between haptens and anti-haptens.

The data presented in Figure 2 demonstrate the capacity of anti-TMVP serum or globulins to bind with ^{14}C8. Although some ^{14}C8 was bound also to anti-AChE serum or globulins, the difference between the binding of the peptide to anti-TMVP and anti-AChE is quite marked.

The fact that ^{14}C8 did not bind with anti-TMVP serum which had been absorbed with TMVP (Table I) indicates that the binding of the peptide with anti-TMVP is through antibodies to the protein. These anti-

TABLE III: Binding of [^{14}C]Acetyl Tryptic Peptide 8 of TMVP to Anti-TMVP or Antiacetylcholinesterase Serum as Measured by Equilibrium Dialysis.

Fraction	Cpm/ml[a]	Protein Concentration (mg/ml)[b]	Bound Peptide 8 (mμmole /ml)[c]
Solution outside bags			
Bovine serum albumin	167	8.0	..
Solution inside bags			
Bovine serum albumin	156	7.9	−0.01
Antiacetylcholinesterase	186	14.6	+0.08
Anti-TMVP	1089	16.2	+0.83

[a] Counts corrected for self-absorption. [b] Determined by measuring optical densities at 280 mμ. [c] Binding values were corrected for changes in protein concentrations during dialysis.

bodies, as expected, are heat labile (Table II). Data presented in Figure 3 show that a considerable amount of radioactivity was eluted with serum proteins when ^{14}C8 was mixed with anti-TMVP serum prior to gel filtration. The lack of radioactivity in the tubes containing protein resulting from the gel filtration of a mixture of ^{14}C8 and anti-AChE serum indicates that the peptide complexed specifically with antibodies to TMVP. Although some binding between ^{14}C8 and anti-AChE was found by salt precipitation of the complex (Figure 2), this binding appears to be weak since practically no binding was observed after gel filtration of a mixture of ^{14}C8 and anti-AChE (Figure 3).

Another indication of the specific binding between ^{14}C8 and anti-TMVP is shown in the data obtained from the experiments dealing with equilibrium dialysis (Table III). Here again, although some nonspecific binding between ^{14}C8 and anti-AChE was observed, the specific binding between the peptide and anti-TMVP is apparent. Current experiments on the binding constant of ^{14}C8 to anti-TMVP indicate that this is over 10^9 l./mole.

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