Immunochemical Studies on the Tobacco Mosaic Virus Protein. VII. The Binding of Octanoylated Peptides of the Tobacco Mosaic Virus Protein with Antibodies to the Whole Protein*

E. Benjamini, M. Shimizu, Janis D. Young, and Cherry Y. Leung

ABSTRACT: It has been previously shown that a pentapeptide having the sequence Leu-Asp-Ala-Thr-Arg, representing residues 108–112 of the tobacco mosaic virus protein (TMVP), exhibited specific immunological binding with antibodies to the whole protein, whereas shorter C-terminal portions of this peptide did not. Subsequent experiments performed in this laboratory indicated that hydrophobicity of the N-terminal portion of the peptide may enhance binding with antibodies. The present communication reports on the synthesis of *N*-[^{14}C]octanoyl derivatives of the C-terminal tetra-, tri-, and dipeptides of the above pentapeptide and on their capacity to bind with anti-TMVP and with antiacetylcholinesterase globulins. Results showed that the octanoyl-Asp-Ala-Thr-Arg and octanoyl-Ala-Thr-Arg exhibited specific binding with anti-TMVP whereas octanoyl-Thr-Arg did not.

It has been reported from this laboratory that a tryptic eicosapeptide of the tobacco mosaic virus protein, representing residues 93–112 of the protein, exhibited antigenic activity with antibodies produced in rabbits following sensitization with the whole protein (Benjamini *et al.*, 1964, 1965). It was subsequently shown that antigenic activity related to that of the whole protein was exhibited by the synthetic C-terminal decapeptide portion of the eicosapeptide (Stewart *et al.*, 1966). More recent communications from this laboratory reported that the synthetic C-terminal pentapeptide portion of the eicosapeptide, having the sequence Leu-Asp-Ala-Thr-Arg, exhibited binding with anti-TMVP produced by some rabbits, whereas shorter peptides did not (Young *et al.*, 1967b; Benjamini *et al.*, 1968).

In the search for the factors which determine the binding of the peptide with antibodies several analogs of the pentapeptide were synthesized and tested for their ability to bind with those globulins which exhibit binding with the native pentapeptide. One of the findings was that the substitution of the N-terminal leucine with alanine resulted in an immunologically inactive peptide (Young *et al.*, 1967a). Another finding was that the binding of the *N*-acetyl pentapeptide with anti-TMVP was higher than that of the nonacetylated peptide (Benjamini *et al.*, 1968). It was suspected that the hydrophobicity of the N terminus was a factor in the binding of the pentapeptide with anti-TMVP. Consequently, the *N*-octanoyl derivatives of the C-terminal tetra-, tri-, and dipeptides of the antigenic pentapeptide were synthesized and tested for binding with anti-TMVP. The results of these studies are reported in the present communication.

Experimental Section

Synthesis of Peptides[1] *and of Octanoylated Peptides.* TMVP peptide-resins were synthesized by the Merrifield solid-phase peptide synthesis method (Merrifield, 1964) as described by Stewart *et al.* (1966) and Young *et al.* (1967b). The decapeptide having the sequence Thr-Thr-Ala-Glu-Thr-Leu-Asp-Ala-Thr-Arg, the pentapeptide having the sequence Leu-Asp-Ala-Thr-Arg, and the $(Ala)_5$-pentapeptide conjugate having the sequence Ala-Ala-Ala-Ala-Ala-Leu-Asp-Ala-Thr-Arg were cleaved from the resin, purified, and acetylated with [^{14}C]acetic anhydride as previously described (Stewart *et al.*, 1966). Approximately 10-mg portions of each of the N-terminal protected (*t*-butyloxycarbonyl, Boc) peptide-resins Asp-Ala-Thr-Arg-resin, Ala-Thr-Arg-resin, and Thr-Arg-resin were used for the octanoylation with [^{14}C]octanoic acid. Octanoic acid was prepared from sodium [^{14}C]octanoate (specific activity 8 mCi/mmole, obtained from Nuclear-Chicago Corp., Des Plaines, Ill.) by acidifying with 0.1 N HCl and extracting the octanoic acid with chloroform. The chloroform was removed under vacuum using a water aspirator and the octanoic acid was dissolved in methylene chloride. The peptide-resin was swollen in methylene chloride and washed three times with glacial acetic acid. The peptide-resin was then depro-

* From the Laboratory of Medical Entomology, Kaiser Foundation Research Institute, and the Allergy Research Division, Allergy Department, Kaiser Foundation Hospitals, San Francisco, California. *Received November 14, 1967.* These studies were supported in part by a U. S. Public Health Service grant (AI 06040).

[1] The following designations are used throughout the manuscript: decapeptide for Thr-Thr-Ala-Glu-Thr-Leu-Asp-Ala-Thr-Arg; pentapeptide for Leu-Asp-Ala-Thr-Arg; $(Ala)_5$-pentapeptide conjugate for Ala-Ala-Ala-Ala-Ala-Leu-Asp-Ala-Thr-Arg; tetrapeptide for Asp-Ala-Thr-Arg; tripeptide for Ala-Thr-Arg; dipeptide for Thr-Arg.

TABLE I: Amino Acid Analyses of Peptides.

Sequence	Molar Ratio of Amino Acids					
	Thr	Ala	Glu	Leu	Asp	Arg
[^{14}C]Acetyl-Thr-Thr-Ala-Glu-Thr-Leu-Asp-Ala-Thr-Arg	3.82	2.00	1.05	1.00	1.03	1.00
[^{14}C]Acetyl-Ala-Ala-Ala-Ala-Ala-Leu-Asp-Ala-Thr-Arg	1.01	5.91		0.99	1.04	1.04
[^{14}C]Acetyl-Leu-Asp-Ala-Thr-Arg	1.00	1.06		1.00	1.03	1.00
[^{14}C]Octanoyl-Asp-Ala-Thr-Arg	0.96	1.04			1.02	1.05
[^{14}C]Octanoyl-Ala-Thr-Arg	0.97	1.02				1.02
[^{14}C]Octanoyl-Thr-Arg	1.01					0.99

tected, washed, treated with triethylamine, and washed as previously described (Stewart *et al.*, 1966). Octanoic acid in methylene chloride was then introduced into the reaction vessel (at a ratio of 2 moles of acid/mole of peptide) followed by the addition of 2.1 moles of *N,N'*-dicyclohexylcarbodiimide/mole of peptide. The reaction was allowed to proceed at room temperature for at least 2 hr, at the end of which time the peptide-resin was washed with methylene chloride, acetic acid, and absolute alcohol. The peptide was then cleaved from the resin with anhydrous HBr in trifluoroacetic acid and was reduced with hydrogen using 5% palladium on barium sulfate (Matheson Co. Inc., Cincinnati, Ohio) as the catalyst. The [^{14}C]octanoyl tetrapeptide ([^{14}C]octanoyl-Asp-Ala-Thr-Arg) was purified by paper electrophoresis performed at pH 6.4 under the conditions described by Stewart *et al.* (1966). The [^{14}C]-octanoyl tri- and dipeptides ([^{14}C]octanoyl-Ala-Thr-Arg and [^{14}C]octanoyl-Thr-Arg, respectively) were purified by passage through a G-10 Sephadex column (0.8 × 110 cm) equilibrated and eluted with collidine–pyridine–acetic acid buffer (pH 8.0) (20:20:0.75 ml made up to 1 l. with water). Each peptide was electrophoresed at pH 6.4, sprayed with ninhydrin, stained for arginine with the Sakaguchi reagent, and scanned for radioactivity as previously described (Benjamini *et al.*, 1965). A single radioactive spot coinciding with a single Sakaguchi-positive material indicated the purity of each peptide. Each peptide was taken up in water, and aliquots were hydrolyzed under open reflux in 6 N HCl at 110° for 24 hr and analyzed for amino acids using the Spinco Model 120B amino acid analyzer. Results of the analyses shown in Table I agree closely with the theoretical values for these peptides. The specific radioactivity of the [^{14}C]acetyl peptides was approximately 2×10^6 cpm/μmole and that of the [^{14}C]octanoyl peptides was approximately 4×10^6 cpm/μmole.

Immunological Assay of Peptides. Anti-TMVP globulins used throughout the reported experiments were derived from pooled sera obtained from a single rabbit. Antiacetylcholinesterase globulins were used as control. The sensitization and preparation of globulins were performed as previously described (Benjamini *et al.*, 1965). Since the optical density at 280 mμ of anti-TMVP globulins was slightly higher than that of antiacetylcholinesterase, the latter were concentrated and adjusted with borate-buffered saline to a concentration having the same optical density as that of anti-TMVP globulins. The assay for immunological activity of the radioactive peptides (as previously described for the TMVP peptides by Benjamini *et al.*, 1965) was performed using 10 mμmoles of peptide (or sodium octanoate) and 0.5 ml of globulins. Following precipitation at 50% saturation of ammonium sulfate and two washings, the precipitates were dissolved in 1 ml of saline and the radioactivity of three aliquots was measured. The entire experiment was performed three times, and the number of counts obtained was corrected for the specific radioactivity of each peptide, using the [^{14}C]acetyl pentapeptide as the standard so that the number of counts reported is directly proportional to the molar concentration of the peptides. Results presented in Table II represent an average of three separate runs. In one experiment TMVP was used to inhibit the reaction between [^{14}C]octanoyl-Ala-Thr-Arg and anti-TMVP. For this purpose, 100 mμmoles of TMVP was introduced to 0.25 ml of globulins prior to the introduction of 10 mμmoles of the radioactive peptide, and the total volume of the reaction mixture was adjusted with saline to 0.5 ml. Following precipitation and washings the precipitates were dissolved in 1 ml of saline and aliquots were counted as described above. Results are shown in Table III.

Discussion

Previous communications from our laboratory (Young *et al.*, 1967b; Benjamini *et al.*, 1968) reported that binding with anti-TMVP, obtained from some rabbits which had been immunized with TMVP, started with the C-terminal pentapeptide Leu-Asp-Ala-Thr-Arg of the previously described antigenic tryptic peptide of TMVP representing residues 93–112 of the protein. Whereas shorter C-terminal peptides did not exhibit binding with anti-TMVP (as demonstrated by the lack of binding between anti-TMVP and [^{14}C]-acetyl peptides and by the inability of the native peptides to inhibit the reaction between anti-TMVP and [^{14}C]acetyl pentapeptide), the average association constant (reflected by radioactivity bound to globulins) increased with the addition of N-terminal native resi-

TABLE II: Immunological Activity of TMVP Peptides and Their Derivatives.

Peptide (0.1 ml of 100 mμmoles/ml)	Cpm Bound to 0.5 ml of Globulins		Net cpm Bound to 0.5 ml of Anti-TMVP	Act. Related to [^{14}C]Acetyl Pentapeptide (as 1.00)	Binding Specificity $\left(1 - \frac{\text{CPM anti-ChE}}{\text{CPM anti-TMVP}}\right)$[a]
	Anti-TMVP	AntiChE[a]			
[^{14}C]Acetyl-Thr-Thr-Ala-Glu-Thr-Leu-Asp-Ala-Thr-Arg	390	56	334	1.21	0.86
[^{14}C]Acetyl-Ala-Ala-Ala-Ala-Ala-Leu-Asp-Ala-Thr-Arg	502	79	423	1.53	0.84
[^{14}C]Acetyl-Leu-Asp-Ala-Thr-Arg	350	73	277	1.00	0.79
[^{14}C]Octanoyl-Asp-Ala-Thr-Arg	785	195	590	2.13	0.75
[^{14}C]Octanoyl-Ala-Thr-Arg	1129	343	786	2.84	0.70
[^{14}C]Octanoyl-Thr-Arg	455	409	46	0.17	0.10
[^{14}C]Sodium octanoate ($CH_3(CH_2)_6COONa$)	56	50	6	0.02	0.10

[a] Anti-ChE, antiacetylcholinesterase.

TABLE III: The Reaction between 10 mμmoles of [^{14}C]Octanoyl-Ala-Thr-Arg and 0.25 ml of Globulins, and Its Inhibition by 100 mμmoles of TMVP.

Cpm Bound to 0.25 ml of Globulins						% Inhibition of Net Anti-TMVP
Without Inhibitor			With Inhibitor			
Anti-TMVP	Anti-ChE	Net Anti-TMVP	Anti-TMVP	Anti-ChE	Net Anti-TMVP	
610	83	527	212	148	64	87.8

dues. It has also been recently ascertained (Benjamini *et al.*, 1968) that the average association constant of the decapeptide Thr-Thr-Ala-Glu-Thr-Leu-Asp-Ala-Thr-Arg with anti-TMVP obtained from the same rabbit as that used in the present experiments (rabbit 31500; Benjamini *et al.*, 1968) was approximately one order of magnitude higher than that of the pentapeptide, and similar in magnitude to that of $(Ala)_5$-pentapeptide conjugate. Results presented in Table II also show that the average association constant (which is reflected by counts bound to globulins) of $(Ala)_5$-pentapeptide conjugate and that of the native decapeptide with anti-TMVP is higher than that of the pentapeptide. Since not only the decapeptide but also $(Ala)_5$-pentapeptide conjugate exhibited increased binding over that of the pentapeptide, the increase may be attributed to the increase in peptide size by five N-terminal residues; *i.e.*, the increase in size may contribute in some nonspecific manner to the higher binding of the antigenic pentapeptide with anti-TMVP. It is doubtful that the five alanine residues have enough similarity to the Thr-Thr-Ala-Glu-Thr N-terminal portion of the native decapeptide to account for the increased binding of the $(Ala)_5$-pentapeptide conjugate, in view of the finding that even very minor changes in the antigenic C-terminal pentapeptide markedly decreased its binding with anti-TMVP.

As mentioned earlier, it was suspected that hydrophobicity of the N-terminal portion of the peptide may play a role in the binding with anti-TMVP. It was therefore decided to substitute the leucine with [^{14}C]octanoic acid and to investigate the binding of the resulting octanoylated tetrapeptide with anti-TMVP. The results in Table II clearly demonstrate not only that the octanoyl tetrapeptide exhibits specific binding with anti-TMVP but also that its average association constant is higher than that of [^{14}C]acetyl pentapeptide, decapeptide, or $(Ala)_5$-pentapeptide conjugate. Similarly high specific activity with anti-TMVP was also exhibited by the [^{14}C]octanoyl tripeptide.

The high specific antigenic activity of the octanoylated tetra- and tripeptides suggests that the increased ac-

tivity of these peptides over that of [^{14}C]acetyl pentapeptide, decapeptide, or $(Ala)_5$-pentapeptide conjugate is not due to increase in the number of peptide bonds, but rather due to the increase in hydrophobicity of the N-terminal portion of the determinant Ala-Thr-Arg. Just how this increase in hydrophobicity enhances the binding with anti-TMVP is still not clear. It may be brought about through the orientation of the peptide away from water and into the antibody site, or it may be due to the stabilizing effect of the fatty acid on a given conformation which exhibits a good fit to the site of the antibody. These alternatives are under investigation. The finding that hydrophobicity enhances the binding perhaps points out to a hydrophobic interaction between an antigenic area and antibodies similar to that suggested by Metzger *et al.* (1963) for a hapten–antibody interaction.

Irrespective of the mode by which the fatty acid enhances the binding of the peptides, it is clear that while the N-terminal leucine of the N-terminal dipeptide leucylaspartate is essential for binding of the remaining C-terminal portion of the pentapeptide, neither the leucine nor the aspartic acid residues are essential for binding with anti-TMVP when the C-terminal tripeptide is assayed as the *N*-octanoyl derivative.

Data presented in Table II on the binding of [^{14}C]-octanoyl dipeptide ([^{14}C]octanoyl-Thr-Arg) show that while the binding with anti-TMVP and with anti-acetylcholinesterase is substantial, the net binding of the octanoyl dipeptide with anti-TMVP (Table II) is insignificant.

The specificity of binding of each of the assayed peptides with anti-TMVP is presented in Table II. The figures have been derived by computing the ratio of the number of counts bound with antiacetylcholinesterase over the number of counts bound with anti-TMVP and subtracting the value from unity. Thus a value of 1 represents binding with anti-TMVP only, whereas a value of zero represents equal binding to anti-TMVP and antiacetylcholinesterase. The data indicate that except for the octanoyl dipeptide, the binding of the other peptides with anti-TMVP is specific. Moreover, data presented in Table III which show that the reaction between [^{14}C]octanoyl-Ala-Thr-Arg and anti-TMVP can be almost completely inhibited by TMVP provide further support for the specificity of the binding.

From the foregoing, a speculation on the role of some of the amino acid residues of the antigenic pentapeptide Leu-Asp-Ala-Thr-Arg of TMVP in the binding with anti-TMVP can be made. It appears that the N-terminal leucine and/or the N-terminal leucylaspartyl residues contribute greatly to the binding with antibodies to TMVP but that their effect can be substituted by the hydrophobic octanoyl group. The tripeptide Ala-Thr-Arg, however, seems to contribute to the specific binding with anti-TMVP. Whether the octanoyl-Ala-Thr-Arg will exhibit binding with anti-TMVP globulins derived from other rabbits, including those which do not exhibit binding with the nonoctanoylated C-terminal pentapeptide portion of the antigenic eicosapeptide of TMVP (Benjamini *et al.*, 1968), is currently under investigation. We are also presently investigating the factors affecting the specificity of the Ala-Thr-Arg region by testing the binding of octanoylated analogs of this tripeptide.

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