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Immunochemical Studies on the Poly-γ-D-glutamyl Capsule of *Bacillus anthracis*. III. The Activity with Rabbit Antisera of Peptides Derived from the Homologous Polypeptide*

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ABSTRACT: The poly-γ-D-glutamic acid capsular polypeptide from a strain of *Bacillus anthracis* was partially hydrolyzed with 3 N HCl at room temperature for 6 days. Peptides from the tripeptide to the hexapeptide were resolved by high-voltage electrophoresis of the hydrolysate on sheets of DEAE-cellulose paper at pH 3.5. The peptides were recovered by elution from the papers and characterized for molecular size and optical activity. The peptides were assayed for reactivity with three rabbit antisera specific for the parent polypeptide, prepared by immunization with either intact bacilli or purified polypeptide–methylated albumin complexes, by inhibition of the quantitative precipitin reaction. With two of the sera, increments in inhibiting efficiency were obtained up to the pentamer, which was equal in inhibiting capacity to the hexamer. The third antiserum could distinguish between the pentapeptide and the hexapeptide. These results indicate a maximum size for the region on the polypeptide which combines with the antibody site of six residues of D-glutamic acid, and also demonstrate heterogeneity of the combining sites of the antibodies in the sera tested. However, an unresolved mixture of larger peptides, with an average polymer size of nine amino acids, gave almost fourfold better inhibition than the hexapeptide on a molar basis with all three sera.

The capsular polypeptide of *Bacillus anthracis*, a polymer composed exclusively of residues of D-glutamic acid (Hanby and Rydon, 1946; Goodman and Nitecki, 1966) linked by γ-peptide bonds (Bruckner and Kovács, 1957), and its homologous antibody comprise an ideal system for investigating the extent of the region on a protein antigen which combines with the antibody molecule. In the initial stages of this study, the eight possible dipeptides and four branched tripeptides of glutamic acid were synthesized (Nitecki and Goodman, 1966) and used to inhibit the precipitin reaction of rabbit antiserum and the homologous anthrax polypeptide (Goodman and Nitecki, 1966). The best inhibitor of these proved to be a branched tripeptide consisting of a residue of L-glutamic acid substituted at both carboxyl groups with residues of D-glutamic acid.

In the present communication, the study has been extended using a series of peptides of increasing chain length up to the hexapeptide, obtained from a partial acid hydrolysate of the native capsular polypeptide of *B. anthracis*, to delineate the extent of the combining region on the polypeptide with three different rabbit antisera.

Materials and Methods

Polypeptide and Antisera. The preparation of purified polypeptide from strain M36 of *B. anthracis* and the pool of rabbit antisera prepared by immunization with heated suspensions of the organism have been previously described (Goodman and Nitecki, 1966). Rabbit antisera from individual animals prepared by immunization with purified polypeptide–methylated bovine serum albumin electrostatic complexes have also been detailed (Goodman and Nitecki, 1967). These are designated antisera 7 and 10. The former had a precipitating antipolypeptide antibody content of 400 μg of protein/ml, while the content of the latter was about 165 μg of protein/ml.

Degradation of Peptides of Glutamic Acid by Serum Enzymes. It has been shown that serum enzymes degrade peptides of L-amino acids and influence their apparent interaction with antibody (Schechter *et al.*, 1966). Accordingly, the eight synthetic dipeptides of glutamic acid (Nitecki and Goodman, 1966) were assayed for susceptibility to proteolysis by serum. A 1% solution of each peptide was mixed with an

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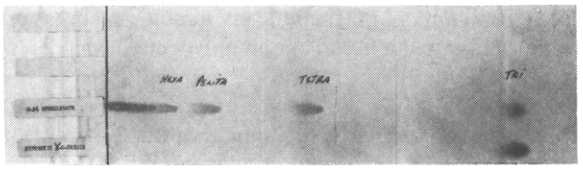

FIGURE 1: Electropherogram of partial acid hydrolysate of *B. anthracis* strain M36 polypeptide (upper) and synthetic γ-D-Glu-γ-D-Glu-D-Glu (lower). The anode is to the right.

equal volume of fresh rabbit serum which contained merthiolate at a concentration of 0.1% (w/v). The mixtures were incubated at 37°. Aliquots were withdrawn at intervals to test for degradation by electrophoresis on sheets of Whatman No. 1 paper at pH 3.5, 2000 v, for 1 hr. The papers were developed with ninhydrin. Under these conditions, glutamic acid was clearly resolved from each of the dipeptides. A mixture of glutamic acid and serum served as a control. α-L-Glu-L-Glu was noticeably degraded within 1 hr and the dipeptide spot disappeared completely within several days. Free glutamic acid appeared in the tube containing γ-L-Glu-L-Glu after 24 hr; this peptide was degraded much more slowly than α-L-Glu-L-Glu. None of the six peptides containing either one or two residues of D-glutamic acid were detectably hydrolyzed even after 21 days of incubation at 37°. Thus, in inhibition assays involving only peptides of the D isomer, unfractionated antiserum was used. This precluded the elimination of a portion of the total antibody, which usually accompanies fractionation of serum.

Partial Hydrolysis of Poly-γ-D-glutamyl Polypeptide. Polypeptide (900 mg) was dissolved in 100 ml of 3 N HCl and incubated at room temperature for 6 days. The solution was evaporated *in vacuo* and the residue was dissolved in water and lyophilized. The hydrolysate (20–25 mg) was applied across sheets of Whatman DEAE-cellulose paper and electrophoresed at 2000 v for 3.5 hr in a pyridine–acetate buffer at pH 3.5 (Katz *et al.*, 1959). Guide strips were developed with ninhydrin and the areas on the paper sheets which contained peptides larger than the dipeptide were cut out and eluted, first with water and then with 9.5 M acetic acid. The peptides were removed with the acetic acid eluent and were subsequently lyophilized. They were redissolved in a volatile buffer consisting of 0.4% pyridine and 0.8% acetic acid (pH 5.0) and each was passed through a carboxymethylcellulose column equilibrated with the same buffer. The eluted peptides were again lyophilized, redissolved in water, dried *in vacuo*, and stored in a desiccator until use. The preparation and characterization of synthetic γ-D-Glu-D-Glu and α,γ-L-Glu-(D-Glu)$_2$ have been described (Nitecki and Goodman, 1966).

Determination of Optical Purity of Peptides. The optical purity of each peptide derived from poly-γ-D-Glu was ascertained by gas–liquid partition chromatography (Westley, 1967). The hydrolyzed peptides were converted to *N*-trifluoroacetyl-L-prolylglutamic acid dimethyl esters and analyzed on a Varian Aerograph 1200, using a column with dimensions of 60 × 1/8 in. (0.5% HI-EFF-2AP on Gas Chrom ø, 60–80 mesh). During the analyses, the temperature was maintained at 200° and the nitrogen flow rate was 30 cc/min. Under these conditions, the retention times for the D and L isomers of *N*-trifluoroacetyl-L-prolylglutamic acid dimethyl ester were 10.7 and 12.0 min, respectively. The *N*-trifluoroacetyl-L-prolyl chloride reagent contained at least 3.5% of the D-prolyl form as determined by assay with sterically pure (−)-ephedrine. Hence, peptide analyses had to be corrected for this quantity of impurity.

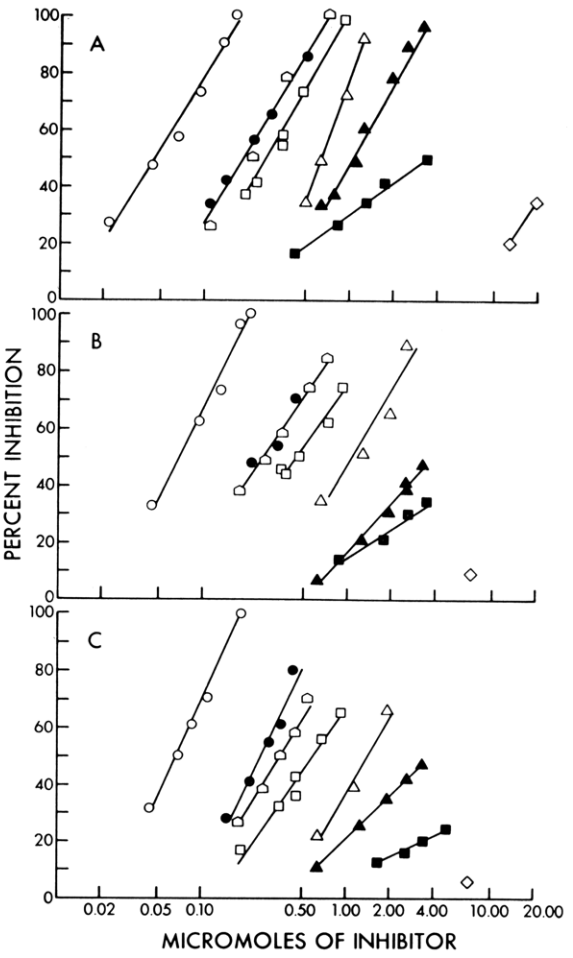

FIGURE 2: Inhibition by peptides of the quantitative precipitin reaction between M36 glutamyl polypeptide and three rabbit antisera. (A) Serum pool from rabbits immunized with killed bacilli; (B) serum 7, and (C) serum 10, both from animals immunized with polypeptide–methylated albumin complexes. (◇) D-Glutamic acid; (■) γ-D-Glu-D-Glu; (▲) α,γ-L-Glu-(D-Glu)$_2$; (△) M36 tripeptide; (□) M36 tetrapeptide; (○) M36 pentapeptide; (●) M36 hexapeptide; (○) M36 > hexapeptide.

Quantitative Assay of Inhibition by Peptides. The ability of synthetic peptides and the peptides derived

TABLE I: Activities of Peptides as Inhibitors of γ-D-Glutamyl Polypeptide–Antipolypeptide Precipitin Reactions.

	Ninhydrin Color Hydrolyzed/ Unhydrolyzed	Rabbit Antiserum								
		Quantity of Peptide for 50% Inhibition								
		Pool			7			10		
		μmoles	Peptide/ Hexapeptide	Peptide/ >Hexapeptide	μmoles	Peptide/ Hexapeptide	Peptide/ >Hexapeptide	μmoles	Peptide/ Hexapeptide	Peptide/ >Hexapeptide
Synthetic γ-D-Glu-D-Glu		3.60	19	80	11.5[a]	43	164	62[a]	238	886
Synthetic α,γ-L-Glu-(D-Glu)$_2$		1.10	5.8	24	3.9[a]	14	56	3.8[a]	14	54
M36 tripeptide	3.1	0.65	3.4	14	1.10	4.1	16	1.40	5.4	20
M36 tetrapeptide	3.9	0.27	1.4	6.0	0.46	1.7	6.6	0.60	2.3	8.6
M36 pentapeptide	4.5	0.19	1.0	4.2	0.27	1.0	3.9	0.36	1.4	5.1
M36 hexapeptide	5.9	0.19		4.2	0.27		3.9	0.26		3.7
M36 >hexapeptide	9.1	0.045[b]			0.070[b]			0.070[b]		

[a] Estimated by extrapolation of lines of Figure 2. [b] Calculated for a nonapeptide.

from *B. anthracis* polypeptide to inhibit precipitation of antibody by the homologous polypeptide was determined. Solutions of the peptides were adjusted to pH 7.3–7.7 prior to use. Quantities of peptide were added to a volume of serum containing about 20 μg (0.14×10^{-3} μmole, assuming a molecular weight of 140,000) of precipitable antipolypeptide antibody in 3.0-ml conical centrifuge tubes. After incubation at room temperature for 30–60 min, a suitable quantity of antigen was added to each tube and to control tubes which contained antiserum but no inhibitor. Final volumes were 0.7 ml. The tubes were refrigerated for 5 days and then centrifuged and washed three times with 0.5 ml of saline. The washed precipitates were dried *in vacuo* over phosphorus pentoxide and analyzed for protein colorimetrically using a modification of the Folin–Ciocalteu method (Heidelberger and MacPherson, 1943a,b) in which the analyses were done directly in the centrifuge tubes in a total volume of 1.0 ml. The volume of each reagent employed in the procedure was scaled down correspondingly using calibrated microliter pipets. This modified procedure permitted the use of smaller quantities of materials in short supply and was found to give highly reproducible results. The glutamyl polypeptide does not react with the Folin reagent and no correction is necessary to obtain antibody protein values.

Results

An electropherogram of the partially hydrolyzed glutamyl polypeptide is shown in Figure 1. Under the conditions employed, glutamic acid and γ-D-Glu-D-Glu migrated off the anodal end of the paper, but good resolution of the hexapeptide from the unresolved mixture of larger peptides was achieved. A sample of synthetic γ-D-Glu-γ-D-Glu-D-Glu (Fox Chemical Co.) is also shown on the paper. The major component of the synthetic tripeptide has a mobility identical with that of the tripeptide from the hydrolyzed bacterial polypeptide; a rapidly migrating impurity is not seen in the illustration. The final yields of the eluted and chromatographed peptides, obtained from 450 mg of the polypeptide, were: tripeptide, 31 mg; tetrapeptide, 28 mg; pentapeptide, 32 mg; hexapeptide, 36 mg; and larger peptides, 155 mg. The polymer size of each peptide was determined by quantitative ninhydrin analysis before and after hydrolysis with 6 N HCl. The values are given in Table I and are consistent with a stepwise sequence of peptides, from the trimer to the hexamer, which decrease in electrophoretic mobility as molecular size increases. The mixture of peptides larger than the hexapeptide had a hydrolyzed: unhydrolyzed ninhydrin ratio of 9:1. The gas–liquid partition chromatographic analyses showed that each peptide, as well as the mixture of larger peptides, was composed of at least 96% D-glutamic acid. The trace of L form found in each preparation had to be corrected for the racemic reagent, which introduced considerable uncertainty in the small values found. The 4% of L form is a maximum estimate, and since the analytical

method has a precision of about ±3%, it is questionable whether L-glutamic acid was at all present in the peptides.

The results of assays of the peptides as inhibitors of the precipitin reaction between the polypeptide and three rabbit antisera are given in Figure 2 and Table I. With all three antisera, the tripeptide was appreciably more effective than the synthetic branched tripeptide, α,γ-L-Glu-(D-Glu)$_2$, which, in turn, was a better inhibitor on a molar basis than γ-D-Glu-D-Glu. However, there were pronounced differences between the three sera with respect to the relative efficiencies of these peptides. Thus, with the pool of rabbit sera prepared by immunization with heat-killed bacilli, 3.6 μmoles of γ-D-Glu-D-Glu, 1.10 μmoles of α,γ-L-Glu-(D-Glu)$_2$, and 0.65 μmole of M36 tripeptide gave 50% inhibition of the precipitin reaction. For sera 7 and 10 the corresponding values were 11.5, 3.9, and 1.10, and 62, 3.8, and 1.40, respectively. The branched tripeptide was, therefore, a relatively more effective ligand with the antibody sites in the serum pool than with those in either of the two antisera prepared against the purified polypeptide complexed with methylated albumin. On the other hand, the dipeptide was much poorer with antiserum 10 than with either of the other two antisera. These relationships are also shown by the ratios of the molar quantities of peptide:hexapeptide required for 50% inhibition of the precipitin reactions (Table I).

With each serum, increments in inhibiting efficiency were found with successive members of the series of peptides up to the pentamer. The pool and serum 7 did not distinguish between the pentapeptide and the hexapeptide within the limits of the experimental method. With the pool, 0.19 μmole of either peptide gave 50% inhibition, while 0.27 μmole of either peptide gave the equivalent result with serum 7. However, serum 10 did significantly discriminate between these two peptides (Figure 2), since 0.36 μmole of the pentapeptide gave 50% inhibition while only 0.26 μmole of the hexapeptide was required. Although larger quantities of the dipeptide through the pentapeptide were needed to give equivalent results with serum 10 than with serum 7, the hexapeptide behaved indistinguishably with the two sera. On the other hand, smaller quantities of all the peptides were needed for equivalent activity with the pool, suggesting a lower average binding affinity for the population of antibody molecules in this serum.

The mixture of peptides larger than the hexapeptide gave markedly enhanced inhibition, relative to the hexapeptide, with all three antisera. On the basis of an average nonapeptide, only 0.045 and 0.070 μmole were needed for 50% inhibition of precipitation with the serum pool and the two individual sera, respectively. In each case, about four times as much hexapeptide on a molar basis was required for the same degree of inhibition. Even if the calculations were made on the basis of a heptapeptide for the mixture, it would still be three times more effective than the hexapeptide on a molar basis.

Each of the peptides was assayed for specificity of inhibition using an azohapten–rabbit antiazohapten system and quantities of peptide which gave more than 50% inhibition of the polypeptide–rabbit antipolypeptide system. None of them perceptibly influenced precipitation of the unrelated reaction.

Discussion

The experimental data presented here indicate that the maximum size of the region on poly-γ-D-glutamic acid which is involved in combination with homologous rabbit antibody is equivalent to a hexapeptide. Comparative data with the three antisera used show that there is a heterogeneity of the combining sites in the antisera, and that antiserum 10 contains a higher proportion of antibody molecules with larger combining sites than do the pool or antiserum 7. These conclusions are based on the relative efficiencies of the peptides in the inhibition assays and the demonstration that only serum 10 could distinguish between the penta- and hexapeptides (Table I). The finding that the ratio of the quantities of hexapeptide:>hexapeptide required for 50% inhibition was about four for each antiserum (Table I) also supports the conclusion that the hexapeptide represents the maximum size of the antigen determinant. Since serum 10 contained a higher proportion of antibody molecules with larger combining sites, the ratio should have been greater for this serum than for the other two if the maximum size had not been reached. It can be seen that such was the case for the smaller peptides (Table I).

Delineation of the combining region on a linear antigen by inhibition of the quantitative precipitin reaction was first applied by Kabat to the dextran–human antidextran system (Kabat, 1960). Using a series of isomaltose oligosaccharides as inhibitors, that study showed that the maximum extent of the combining region on the dextran molecule was represented by isomaltohexaose, the dimensions of which are 34 × 12 × 7 Å in its most extended form. By comparison, the corresponding dimensions of hexaglutamic acid are 36 × 10 × 6 Å. Thus, the results are in very close coincidence for the two different antigens in different species of animals.

Heterogeneity in the extent of antibody combining sites has been demonstrated previously for human antidextran (Kabat, 1960) and rabbit antipneumococcal type III antibodies (Mage and Kabat, 1963). The present findings represent the first such demonstration for antibodies other than antipolysaccharides.

A number of investigations employing synthetic polypeptide antigens have yielded determinant group sizes in reasonable consonance with those of dextran and polyglutamic acid (Sage *et al.*, 1964; Schechter *et al.*, 1966; Arnon *et al.*, 1965). These have involved multichain polymer–protein conjugates as antigens, in which the average polymer chain length was five to eight residues. Two recent studies using polypeptides of longer chain length are of particular interest. Schlossman and Levine (1967) immunized guinea pigs with

a mixture of α-*N*-DNP-lysyl peptides with an average chain length of eleven amino acids. In inhibition tests, maximum efficiency was reached with the heptamer, which was indistinguishable from the octa- and nonapeptides. The provocative finding was made that α-*N*-DNP-heptalysine, which defined the maximum determinant group size, was also the smallest immunogenic peptide in the series.

Antibodies produced against a large synthetic polymer of D-lysine complexed with phosphorylated bovine serum albumin had combining sites complementary to a pentapeptide when assayed by complement fixation (Van Vunakis *et al.*, 1966). Oligomers of up to 14 lysine residues did not give appreciably better activity than penta-D-lysine.

The observation in the present study that a mixture of larger peptides produced markedly better inhibition than did hexa-D-glutamic acid contrasts somewhat with those described above. Clarification of this point must await studies with purified peptides larger than the hexapeptide, since the more active component or components in the mixture is presently unknown. However, several possible explanations might be considered. Based on an average molecular weight of about 33,500 for the polymer produced by strain M36 (Goodman and Nitecki, 1966), the molecule contains over 200 residues of D-glutamic acid. It has been shown that the glutamyl polypeptides formed by organisms of the genus *Bacillus* are composed of essentially linear, unbranched peptide chains (Chibnall *et al.*, 1958). Thus, it seems reasonable that the antigenic determinants of such a polymer might be removed from the N- and C-terminal positions. Perhaps an internal sequence of six D-glutamic acid residues in a larger peptide would better satisfy the configurational requirements of the antibody site. Another possibility is that the larger peptides possess a three-dimensional configuration which is more complementary than the free hexapeptide to the antibody site. Finally, larger peptides would present more combinations of six residues to the antibody molecule, although data of others (*e.g.*, Van Vunakis *et al.*, 1966) suggest that this exerts no palpable effect on the results of inhibition assays, and when large enough would possess more than a single combining region. A multivalent hapten would be expected to produce markedly better inhibition than one which is univalent (Kabat, 1966).

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