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Immunological Studies on Peptides from the Haptenic C-Terminal Octapeptide of *Clostridium pasteurianum* Ferredoxin*

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ABSTRACT: Various sequences of a haptenic octapeptide of *Clostridium pasteurianum* ferredoxin were synthesized and tested for their haptenic activity with purified rabbit antiserum to performic acid oxidized ferredoxin. Sequences between the C-terminal tripeptide and the whole octapeptide were N-^{14}C-acetylated and tested for their ability to bind directly to antisera from two rabbits. In one instance, the tri- and tetrapeptide showed limited capacity to bind, and the penta- through octapeptide bound to an equivalent extent. The second antiserum tested showed no binding with the tripeptide, but showed appreciable binding with the tetra-, penta-, and octapeptide.

The various peptides were also tested for their ability to inhibit complement fixation between purified antiserum and oxidized ferredoxin. These tests indicated that the tripeptide had no haptenic activity, the tetrapeptide had limited capacity, whereas the penta- and octapeptide had close to equivalent haptenic activity.

It has generally been accepted that the minimum size at which antigenic determinants consisting of oligopeptides from protein antigens will combine appreciably with antibody is in the region of five to six amino acid residues. This was demonstrated graphically by Benjamini and his coworkers with a pentapeptide which formed the C-terminal portion of a haptenic eicosapeptide from tobacco mosaic virus protein (Young *et al.*, 1967). They also showed that some rabbits produced antibody which could bind the pentapeptide, whereas others produced antibody which could only bind the hexapeptide (Benjamini *et al.*, 1968a). These workers (Benjamini *et al.*, 1968b) postulated that the hydrophobicity of the amino acid residues at the N-terminal region of this pentapeptide played a more important role in antibody binding than did their actual configuration. To test this, they octanoylated the C-terminal di-, tri-, and tetrapeptides of their pentapeptide and showed that while the octanoylated dipeptide did not bind significantly to antibody, the octanoyl derivatives of both the tri- and tetrapeptide did. They concluded that the N-terminal residues of the pentapeptide contributed stability to the hapten–antibody complex by their hydrophobic nature, and that the specificity was designated by the C-terminal residues.

Using peptides isolated from silk fibroin, Cebra (1961) showed that a tetrapeptide was capable of inhibiting immune precipitation between silk fibroin and specific antisera from several rabbits. In one serum tested, a dipeptide comprising the probable C-terminal of the haptenic peptide was shown to produce marginal inhibition.

In this laboratory, it was shown that the C-terminal octapeptide of the ferredoxin of *Clostridium pasteurianum* constituted an antigenic determinant of this molecule (Mitchell *et al.*, 1970). The work reported here involves examination of the tri-, tetra-, penta-, hexa-, and heptapeptide of this octapeptide for haptenic properties. Each peptide was tested, after N-^{14}C-acetylation, for its ability to bind directly to purified rabbit antiserum against performic acid oxidized ferredoxin (O-Fd) and for its ability to inhibit complement fixation between this antiserum and its homologous antigen.

Materials and Methods

The ferredoxin was prepared and purified as described by Tanaka *et al.* (1964) and Mortenson (1964). Performic acid oxidized ferredoxin was prepared according to previously described methods (Mitchell *et al.*, 1970). Antiserum to O-Fd was raised in two rabbits as described previously (Nitz *et al.*, 1969). These animals had been immunized over a period of 18 months and had received booster shots intramuscularly of 1.0 mg of O-Fd in a 50% saline suspension of Freund's adjuvant (total volume was 0.2 ml) at 6-month intervals. In

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these experiments, only antiserum purified by means of an immunoabsorbent was used. The antigen (O-Fd) was conjugated to bromoacetylcellulose and used as an immunoadsorbent according to the methods of Robbins *et al.* (1967). The purified antibodies were titrated by complement fixation as previously described (Gerwing and Thompson, 1968) and titered at 1:1200.

Solid Phase Synthesis and Purification of Peptides. Peptides were synthesized basically according to the methods of Merrifield (1964) with the modifications suggested by Stewart and Young (1969). The *t*-Boc amino acids were purchased from Sigma Chemical Co. The C-terminal octapeptide shown originally to have haptenic activity contained the sequence NH_2-Pro-Val-Gly-Ala-Pro-Val-Gln-Glu-COOH. The C-terminal Glu was attached to 2.0 g of the chloromethylated copolystyrene–divinylbenzene resin (Bio-Rad) and after two further additions and the removal of the last blocking group, an aliquot of the resin was removed and the tripeptide (NH_2-Val-Gln-Glu-COOH) was detached from it. After each subsequent amino acid was added and the blocking group removed, an aliquot of resin was removed from the reaction vessel, so that crude preparations of the tri-, tetra-, penta-, hexa-, hepta-, and octapeptide were obtained.

Each peptide thus synthesized was cleaved from the resin, lyophilized, and chromatographed according to the procedure of Konigsberg and Hill (1962) with the previously described modifications (Mitchell *et al.*, 1970).

Quantitative and Qualitative Analysis. Peptide samples were checked for purity by high-voltage paper electrophoresis in pyridine–acetate buffer at pH 6.5 (100 ml of pyridine and 4.0 ml of acetic acid per l.) on a Gilson high voltage electrophoretor at 3000 V for 45 min. Peptides were also examined by chromatography on silica gel thin layer plates in a solvent of butanol, pyridine, acetic acid, and water (15:10:3:12). Plates were run for 3 hr at 37°. Electrophoretograms and chromatograms were developed with ninhydrin (5% in acetone) at room temperature. Quantitation of both antigen and peptides was performed by amino acid analysis on a Spinco Model 120 amino acid analyzer according to the method of Spackman *et al.* (1958).

Acetylation of Peptides. Samples of each of the synthesized peptides, containing between 2.0 and 4.0 μmoles, were N-acetylated with [^{14}C]acetic anhydride and purified according to the methods of Benjamini *et al.* (1965), with the modifications described previously (Mitchell *et al.*, 1970).

Radioactive Measurements. All measurements of ^{14}C-labeled materials were made on a Nuclear-Chicago scintillation counter (Model 725). Samples for counting were mixed with 8.0 ml of scintillation fluid and cooled to 4° before counts were made. The scintillation fluid contained 60% toluene, 40% methanol, and 42.0 ml of Liquifluor (New England Nuclear) in a final volume of 1.0 l.

Hapten Studies. 1. Complement fixation. The haptenic activity of the synthesized peptides was measured by testing their relative abilities to inhibit complement fixation between O-Fd and its homologous purified antiserum. The procedure has been described previously (Gerwing and Thompson, 1968). Negative controls using lysozyme and its homologous antiserum were done simultaneously.

2. Direct binding studies. The ability of these peptides to bind directly and specifically with antiserum to O-Fd was measured by a modification of the method originally described by Farr (1958). Purified antiserum (0.5 ml) and the acetylated peptide (0.01 ml containing 0.004 μmole of peptide) were mixed. Similarly, negative controls were set up using 0.5 ml of nonspecific immunoglobulin (in an equivalent concentration as that of the specific antiserum) and 0.5 ml of NaCl–Tris buffer (0.1 M NaCl in 0.01 M Tris at pH 7.0), each with 0.01 ml of peptide. All tubes were set up in triplicate and incubated for 1 hr at 37° and then 6 hr at 4°. Ethanol (95%) which had been cooled to −15° was added in 1.0-ml amounts to each tube and the tubes were left for 18 hr at −15°. The precipitates thus formed were centrifuged, the supernatants removed, and 0.1 ml of each was taken for counting. The precipitates were washed three times with 95% ethanol and then dissolved in 0.1 ml of the NaCl-Tris buffer at pH 7.0. The redissolved precipitates in 0.1 ml were transferred to scintillation vials to which a total volume of 8.0 ml of scintillation fluid was added (some of this was used to wash out the tubes containing the samples). Each sample was counted for 10 min. Ten minute counts were also done on the 0.1-ml samples of the supernatants. This was done mainly as a means of confirming that no errors had been made when the labeled peptides were added to each tube.

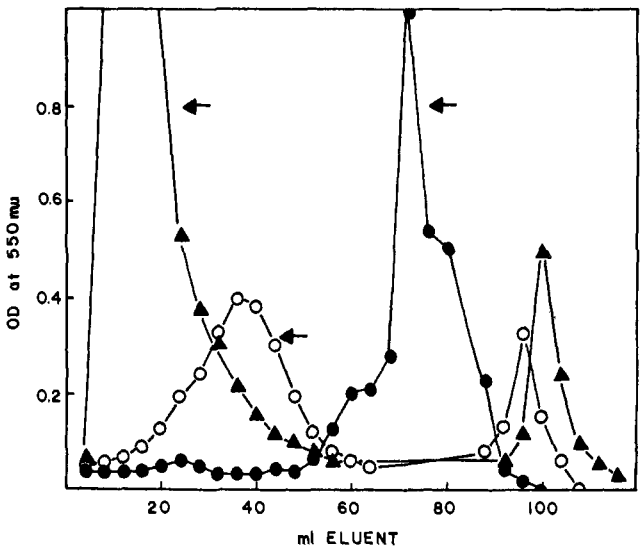

FIGURE 1: Elution profiles of the tri-, tetra-, and pentapeptides from Dowex 1-X8. The arrows designate the ninhydrin positive peaks containing peptide material with the most favorable amino acid ratios: ●——●, tripeptide (Val-Gln-Glu); ○——○, tetrapeptide (Pro-Val-Glu-Gln); ▲——▲, peptapeptide (Ala-Pro-Val-Gln-Glu).

Results

After chromatography of the synthesized peptides on Dowex 1-X8, all ninhydrin positive peak fractions eluting from these columns were pooled, and aliquots were analyzed by amino acid analyses. In each case, those containing the most favorable amino acid ratios were selected for further study. The elution profiles of the tri-, tetra-, and pentapeptide are shown in Figure 1. The hexa- through octapeptides chromatographed in the same position as the pentapeptide. The selected peptide fractions were examined by both high-voltage paper electrophoresis and thin-layer chromatography. In each instance, only one major ninhydrin positive spot was

TABLE I: The Amino Acid Compositions, Molar Ratios, and Sequences of Peptides from the C-Terminal of *C. pasteurianum* Ferredoxin, Prepared by the Solid Phase Synthetic Method.

Peptide	Amino Acids Added									
	Glutamic Acid		Valine		Proline		Alanine		Glycine	
	μmole	Molar Ratio	μmole	Molar Ratio	μmole	Molar Ratio	μmole	Molar Ratio	μmole	Molar Ratio
Tripeptide Val-Gln-Glu	0.061	2.34	0.026	1.00						
Tetrapeptide Pro-Val-Gln-Glu	0.027	2.25	0.012	1.00	0.013	1.08				
Pentapeptide Ala-Pro-Val-Gln-Glu	0.033	2.06	0.017	1.06	0.017	1.06	0.015	0.93		
Hexapeptide Gly-Ala-Pro-Val-Gln-Glu	0.059	1.84	0.029	0.91	0.027	0.85	0.035	1.09	0.035	1.09
Heptapeptide Val-Gly-Ala-Pro-Val-Gln-Glu	0.031	1.82	0.029	1.71	0.018	1.05	0.017	0.94	0.018	1.05
Octapeptide Pro-Val-Gly-Ala-Pro-Val-Gln-Glu	0.037	1.85	0.034	1.70	0.034	1.70	0.020	1.00	0.020	1.00

TABLE II: The Specific Activity of the Various N-Acetylated Peptides and Their Relative Binding Properties with Purified Antiserum to O-Fd from Two Rabbits. Counts Shown Are Those Obtained from Precipitated Immunoglobulins (both Specific and Nonspecific) after Incubation with 0.004 μmole of Each Peptide.

Peptide	Specific Activity (cpm/μmole of peptide)	Antiserum O-Fd A1 (1.01 mg/ml), cpm in Averaged Samples			Antiserum O-Fd A2 (0.946 mg/ml), cpm in Averaged Samples		
		Specific Antiserum	Nonspecific Antiserum	μmoles of Peptide Bound/μmole of Antibody	Specific Antiserum	Nonspecific Antiserum	μmoles of Peptide Bound/μmole of Antibody
Tripeptide Val-Gln-Glu	1.0×10^6	22 ± 4.7	2 ± 1.7	2.9×10^{-3}	10 ± 3.6	2 ± 1.5	
Tetrapeptide Pro-Val-Gln-Glu	2.2×10^6	80 ± 2.0	18 ± 1.3	4.0×10^{-3}	85 ± 5.8	44 ± 7.6	2.2×10^{-3}
Pentapeptide Ala-Pro-Val-Gln-Glu	7.0×10^6	311 ± 15.1	89 ± 5.0	4.6×10^{-3}	90 ± 9.7	32 ± 4.7	2.2×10^{-3}
Hexapeptide Gly-Ala-Pro-Val-Gln-Glu	4.0×10^6	170 ± 10.8	44 ± 4.7	4.5×10^{-3}			
Heptapeptide Val-Gly-Ala-Pro-Val-Gln-Glu	3.1×10^6	114 ± 4.7	18 ± 0.3	4.5×10^{-3}			
Octapeptide Pro-Val-Gly-Ala-Pro-Val-Gln-Glu	3.1×10^6	99 ± 11.0	11 ± 1.0	4.2×10^{-3}	73 ± 5.6	28 ± 3.0	2.6×10^{-3}

observed when either technique was used. The presumed sequence and the amino acid ratios of each peptide are shown in Table I.

The N-^{14}C-acetylated derivatives of each of the peptides were tested for their ability to bind specifically with purified rabbit antiserum to O-Fd. Serum from two individuals was used and the results are correlated in Table II, which shows the specific activity of each peptide tested and the actual counts obtained in precipitates of both specific antiserum and nonspecific immunoglobulin when they were incubated with 0.004 μmole of each peptide. The counts represent the average of tests run in triplicate. The experiments on the two sera (A1

and A2) were done at separate times. This accounts for the differences noted in background counts for the nonspecific controls. Because the specific activities of the peptides differed somewhat from each other, the counts in the precipitated material were not directly related to the binding capacity of each peptide. Consequently, the data were equalized by calculating the binding at this level in micromoles of peptide bound per micromole of antibody. This is also shown in Table II and gives a better indication of the progressive degree of binding. However, since the specific activities of the peptides differed and no attempts were made to isolate acetylated from nonacetylated materials, low binding, particularly in the case of the tripeptide, could be accounted for on this basis (the specific activity in this instance was much lower than that found in the other peptide preparations). Counts on the Tris–NaCl controls are not shown since these were lower than those in the immunoglobulin controls and therefore were not considered significant. It can be seen that in the case of both antisera, the binding of the tetrapeptide and pentapeptide is virtually equivalent to that found with the octapeptide. It was also noted that antiserum A2, although at the same protein concentration and complement fixation titer as serum A1, demonstrated about 50% of the capacity to bind these peptides as does antiserum A1.

The ability of the tri-, tetra-, penta-, and octapeptide to inhibit complement fixation between antiserum A1 and O-Fd was tested. The hexa- and heptapeptides were not tested since it appeared that no significant difference existed between the binding of peptides between 5 and 8 amino acid residues. The positive results are shown in Figure 2. The tripeptide exhibited no haptenic activity under these test conditions whereas the tetrapeptide, pentapeptide, and octapeptide all exerted considerable inhibitory activity. Only at the lower levels of hapten concentration (0.005 and 0.0025 μmole) did the tetrapeptide exhibit significantly less haptenic activity than the penta- and octapeptides. Negative controls using lysozyme and its homologous antiserum were not inhibited by any of the test peptides.

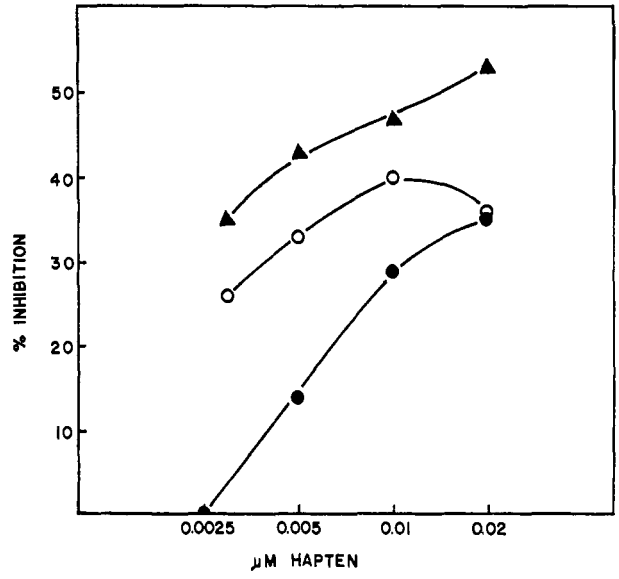

FIGURE 2: The ability of the octa-, penta-, and tetrapeptide, at various concentrations, to inhibit complement fixation between O-Fd and its specific antiserum: ▲——▲, (Pro-Val-Gly-Ala-Pro-Val-Gln-Glu) octapeptide; ○——○ (Ala-Pro-Val-Gln-Glu) pentapeptide; ●——● (Pro-Val-Gln-Glu) tetrapeptide. Complement dilution, 1:125; antiserum dilution, 1:1200.

Discussion

The work reported here is basically in agreement with the findings reported by other workers studying different antigenic determinants. Our initial binding studies showed that one test antiserum, O-Fd A1, possessed a limited capacity to bind the C-terminal tripeptide of the haptenic octapeptide. However, when this antiserum was used to test the capacity of these peptides to inhibit complement fixation, only the tetrapeptide showed limited inhibitory behavior. This capacity to bind the tripeptide was not seen in the second test antiserum, O-Fd A2. It has been recognized that different animals produce antibody with different degrees of affinity for small haptenic groups (Benjamini *et al.*, 1969) and that stability is enhanced by the addition of hydrophobic groups to the hapten (Benjamini *et al.*, 1968b). Antiserum A1 exhibited a higher affinity for binding with all the peptides tested, so that the inability of antiserum A2 to bind the tripeptide may reflect an animal difference in the affinity of the specific antibody for this haptenic structure. It is worth mentioning that the complement fixation titer of both antisera with O-Fd was the same (1:1200) so that these affinity differences do not appear to be directly related to the complement fixation properties. We realize that the binding shown by antiserum A1 with the tripeptide is marginal. However, this is probably a real effect since it was easily reproducible.

There seems no question regarding the capacity of the tetrapeptide either to bind directly to either test antiserum or to act as a hapten in complement fixation reactions with O-Fd and its homologous antiserum. A tetrapeptide certainly represents a lower limit for the size of antigenic determinants, although both Cebra (1961) and Benjamini *et al.* (1968b) have observed that, in the former case, tetrapeptides of silk fibroin bind to specific antiserum and, in the latter case, tetrapeptides may designate antigenic specificity but require stabilization with hydrophobic groupings. It is worth noting here that the sequence of the tetrapeptide under test is Pro-Val-Gln-Glu, in which three out of four of the amino acid residues are quite hydrophobic; this peptide thus presents a likely sequence for reasonably stable binding with antiserum.

The pentapeptide (sequence Ala-Pro-Val-Gln-Glu) under the test conditions used here bound with antiserum and exhibited haptenic activity equal to that observed with the octapeptide, and it was concluded that the optimal size for the antigenic determinant in this instance was in the range of 5 amino acid residues, which is in keeping with most observations on both oligopeptides from protein antigens and on synthetic oligopeptides which constitute antigenic determinants.

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An Effect of Calcium Ions on the Activity, Heat Stability, and Structure of Trypsin*

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ABSTRACT: A temperature-dependent activation of trypsin by calcium was observed. The activation effect of calcium always occurred at or above the temperature optimum for the calcium-free enzyme system and was influenced by pH and the calcium concentration. In the presence of high concentrations of calcium an altered form of trypsin was stabilized which possessed greater enzyme activity than calcium-free trypsin. Addition of calcium to trypsin at pH 7.8 and 20–40° caused the development of a positive ultraviolet differential spectrum. The positive differential spectrum indicated a conformational change to a more compact structure and the formation of a calcium–enzyme complex.

Optical rotatory dispersion of trypsin in the presence of calcium showed a major conformational transition between 40 and 45° which nearly coincided with the ultraviolet differential thermal transitions and the enzyme velocity–temperature transition points. SE-Sephadex chromatography, Sephadex gel filtration, and sedimentation studies were used to show the nature of the calcium–trypsin complex and its relationship to the other components in the heterogeneous trypsin system.

It has long been known that calcium promotes the formation of active trypsin from trypsinogen (McDonald and Kunitz, 1941) and stabilizes trypsin against autolysis (Gorini, 1951; Bier and Nord, 1951). Reports in the literature (Delaage and Lazdunski, 1967) attribute this stabilization effect of calcium to the existence of a specific calcium binding site on trypsinogen which is also preserved in the active trypsin molecule. Accordingly, the binding of calcium to trypsinogen induces a conformational change which protects the molecule against the formation of inert proteins. The recent results of Abita *et al.* (1969) indicate that calcium at high concentration binds to the four N-terminal aspartyl residues on trypsinogen without inducing a structural change, and increases the affinity of the Lys^6–Ile^7 bond to trypsin hydrolysis.

Radhakrishnan *et al.* (1969) attribute the relatively slow hydrolysis rate of the Lys^6–Ile^7 bond during zymogen activation to the chemical character of the amino acid sequence in the N-terminal end of the zymogen, since the calcium effect could be abolished by partial substitution of the N-terminal aspartyl residues with glycinamide groups.

Earlier Bier and Nord (1951) showed that calcium not only protects trypsin against self-digestion, but it also slightly increased its proteolytic activity. Gorini (1951) concluded that calcium promotes the formation of a calcium–trypsin complex from a reversible inactive form.[1] Gorini also sug-

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[1] "Reversible inactive" trypsin was the designation of Kunitz and Northrop (1934) for the form of trypsin obtained by raising the temperature or by making the solution strongly alkaline. This form was in equilibrium with active trypsin ($T_a \rightleftarrows T_i$) but if allowed to stand, unless at very low pH (2), trypsin soon irreversibly lost its activity (irreversible inactive). Evidently, if considerably ionized by either acid or base,