Biochemistry

Volume 9, Number 14 July 7, 1970

Immunochemical Studies on Lysozyme and Carboxymethylated Lysozyme*

Janis Dillaha Young and Cherry Y. Leung

ABSTRACT: Antibodies from rabbits sensitized with crystalline lysozyme were found to react with reduced and *S*-carboxymethylated lysozyme (CM-lysozyme) as well as with lysozyme.

The titer to CM-lysozyme was about 0.1 of that to lysozyme and decreased with time and repeated lysozyme injections while the titer to lysozyme remained relatively constant. The anti-CM-lysozyme antibodies found in the antilysozyme serum were not simply antibodies to lysozyme which cross-reacted with CM-lysozyme. This was evidenced by two observations: (1) the reaction between CM-lysozyme and antilysozyme could be inhibited only with very high concentrations of lysozyme (2000-fold excess); (2) following absorption with lysozyme, the antilysozyme serum contained all of the CM-lysozyme binding activity. Complement fixation by the lysozyme–antilysozyme complex could not be inhibited by chymotryptic or tryptic peptides of CM-lysozyme or by two cyanogen bromide peptides of CM-lysozyme representing residues 1–12 and 106–129. The CM-lysozyme–antilysozyme reaction, however, could be inhibited by the tryptic digest of CM-lysozyme, the C-terminal cyanogen bromide peptide (residues 106–129), and, to a lesser extent, by the N-terminal cyanogen bromide peptide (residues 1–12). The chymotryptic peptides did not inhibit this reaction. The complement fixation reaction by CM-lysozyme–anti-CM-lysozyme could be inhibited by the same cyanogen bromide peptides obtained from CM-lysozyme, the C-terminal peptide being more inhibitory than the N-terminal one.

Hen egg white lysozyme is valuable for use as a model protein antigen since it has a well-known amino acid sequence and three-dimensional configuration (Phillips, 1966; Chipman and Sharon, 1969). In addition, it is a good immunogen, is enzymatically active, and is one of many structurally related bird egg white lysozymes.

The antigenic determinants of hen egg white lysozyme have been studied comprehensively by Shinka *et al.* (1967), Fujio *et al.* (1968a, 1968b), and Imanishi *et al.* (1969) of Osaka University. In addition, Gerwing and Thompson (1968) have studied antigenic regions of the reduced and alkylated molecule, CM-lysozyme.[1] Bonavida (1969) and Bonavida *et al.* (1969) have compared the immune response of cyanogen bromide treated lysozyme with native lysozyme. Habeeb and Atassi (1969) and Atassi and Habeeb (1969) have studied the immunological changes obtained by chemical modification of tryptophan and tyrosine residues in lysozyme.

The present paper presents the results of our investigation of the lysozyme–antilysozyme system and the reaction of CM-lysozyme with antilysozyme and with its homologous antibody. The inhibition of these antigen–antibody reactions by tryptic, chymotryptic, and cyanogen bromide peptides of CM-lysozyme utilizing complement fixation and radioimmunoassay techniques is presented.

Experimental Section

Hen egg lysozyme (Lots 19 and 2X, Pentex Inc., Kankakee, Ill.) was used without further purification. Reduced and *S*-carboxymethylated lysozyme (CM-lysozyme) was prepared by the method of Canfield and Anfinsen (1963) where only the cysteine residues obtained by reduction were found to be alkylated. Tobacco mosaic virus (TMV) protein, which was used

* From the Laboratory of Medical Entomology, Kaiser Foundation Research Institute and Allergy Research Division, Allergy Department, Kaiser Foundation Hospitals, San Francisco, California 94115. *Received September 18, 1969.* This work was supported in part by a grant from the United States Public Health Service (AI 06040). A preliminary report of this study has been published (Young and Leung, 1969).

[1] Abbreviation used, but not listed in *Biochemistry 5*, 1445 (1966), is: CM-lysozyme, *S*-carboxymethylated lysozyme, obtained by carboxymethylation of lysozyme after reduction of the disulfide bonds to cysteine.

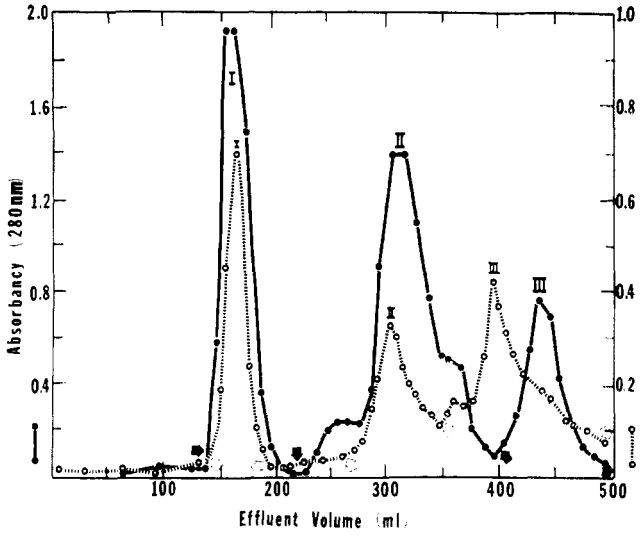

FIGURE 1: G-50 Sephadex elution pattern of a chymotryptic digest of CM-lysozyme (31 mg) (O- - -O) and a tryptic digest of CM-lysozyme (172 mg) (●——●); eluting solvent, 5% acetic acid, 0.15 M ammonium acetate; 6 ml/tube; 36 ml/hr; column size 2.5 × 70 cm.

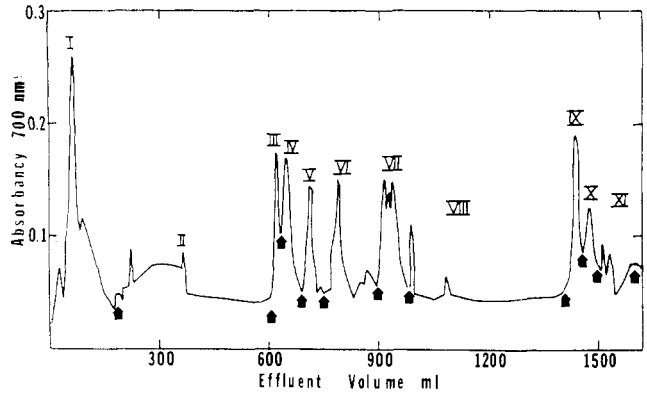

FIGURE 2: Dowex 1-X2 elution pattern of G-50 peak II of a tryptic digest of 43 mg of CM-lysozyme (see Figure 1) using the gradient of Funatsu (1964); 3 ml/tube; 45 ml/hr; Folin color in 1.2 ml using 0.1 ml from alternate tubes. Fractions were pooled as indicated by the arrows (⬇).

as a control antigen, was obtained from TMV[2] by 66% acetic acid treatment (Fraenkel-Conrat, 1957).

CM-lysozyme was digested with chymotrypsin and trypsin as described by Jollès *et al.* (1963). The CM-lysozyme, 5 mg/ml, was solubilized at pH 10.8, then brought to pH 7.8 and digested with either chymotrypsin (Worthington, chromatographically homogeneous, Lot CDC51) or trypsin (Sigma Chemical Co., Lot 105B-2280) at 37° for 4 hr at an enzyme to CM-lysozyme weight ratio of 1:100. The digest was brought to pH 4 using 30% acetic acid and then lyophilized. The digests were fractionated on a G-50 Sephadex column (2.3 × 70 cm) using 5% acetic acid in 0.15 M ammonium acetate as the eluting solvent (see Figure 1).

The low molecular weight material from the G-50 separation of the tryptic digest was lyophilized, dissolved in a pyridine–collidine–acetic acid buffer, pH 8.8, and fractionated on a Dowex 1-X2 column using the eluting gradient described by Funatsu (1964) (see Figure 2).

CM-lysozyme, 5 mg/ml, was cleaved with 2.5 mg/ml of cyanogen bromide in 70% formic acid overnight at room temperature as described by Steers *et al.* (1965). Ten volumes of water were added and the contents of the flask dried by rotary evaporation. The cleaved product was dissolved in 16% formic acid and fractionated on a G-50 Sephadex column using 16% formic acid in 0.15 M ammonium acetate (see Figure 3).[3] The low molecular weight material was dissolved in 0.2 N acetic acid and brought to pH 8.8 just prior to application to a Dowex 1-X2 (1 × 150 cm) column. The column was equilibrated with a pH 8.8 collidine–pyridine–acetic acid buffer (40:40:0.25 ml, in 4 l.) and eluted with a Varigrad (Technicon) containing 135 ml of the following solvents in chambers: 1–3, pH 7.3, collidine–pyridine–acetic acid buffer (40:40:8 ml, in 4 l.); 4 and 5, 0.02 N acetic acid; 6, 0.2 N acetic acid; 7, 0.35 N acetic acid; 8, 0.5 N acetic acid; 9, 0.65 N acetic acid (see Figure 4).

Amino acid analyses were performed on carefully evacuated 6 M HCl hydrolysates using a Beckman/Spinco 120B amino acid analyzer.

Proteins used in the radioimmunoassays were iodinated according to the method of Greenwood *et al.* (1963). Iodination was performed using 0.1–2 mg of protein and 0.04 ml of [^{125}I]NaI (5 mCi/0.1 ml, New England Nuclear Corp., Boston, Mass.) in 0.1 N NaOH. The [^{125}I]protein was separated from [^{125}I]iodide on a G-25 Sephadex column (1.5 × 75 cm) eluted with 0.1 M $NaHCO_3$. The [^{125}I]proteins were frozen in 0.1 M $NaHCO_3$ containing 1% bovine serum albumin and aliquots were diluted with cold protein to approximately 1000 cpm/0.1 μg of protein before use.

Antisera. Rabbits were sensitized by three intramuscular injections at weekly intervals using 10 mg of lysozyme in 1 ml of saline with 1 ml of Freund's complete adjuvant per injection. Rabbits were sensitized to CM-lysozyme by one intramuscular injection of 200 μg in 1 ml of saline and 1 ml of Freund's complete adjuvant followed 1 month later by three intravenous injections of 100 μg in 1 ml of saline on alternate days. The CM-lysozyme was solubilized in 0.04 N HCl and adjusted to pH 7, and NaCl added to give a final concentration of 0.15 M. In all cases, antisera were obtained from the ear vein at 2 week intervals. Occasional booster shots were administered 10 days before bleeding. The control anti-TMV protein serum was obtained as previously described by Benjamini *et al.* (1964).

The antilysozyme globulin preparation noted in Table II was prepared by three 50% ammonium sulfate precipitations of an antiserum obtained 3 months after the first injection (Benjamini *et al.*, 1965). The equivalence point of the globulin preparation was determined by mixing various amounts of lysozyme with 0.1-ml portions of the globulin preparation and allowing the mixtures to stand overnight at 4° prior to centrifugation. The supernatant obtained at the equivalence point (0.3 μg of lysozyme/0.1 ml of globulin) was used as the lysozyme absorbed globulin preparation.

[2] The TMV was generously supplied by Dr. C. A. Knight of the Department of Molecular Biology, University of California, Berkeley, California.

[3] We have found that the cyanogen bromide cleaved CM-lysozyme peptides are easier to fractionate on Sephadex using 0.2 N acetic acid instead of 16% formic acid as the solvent.

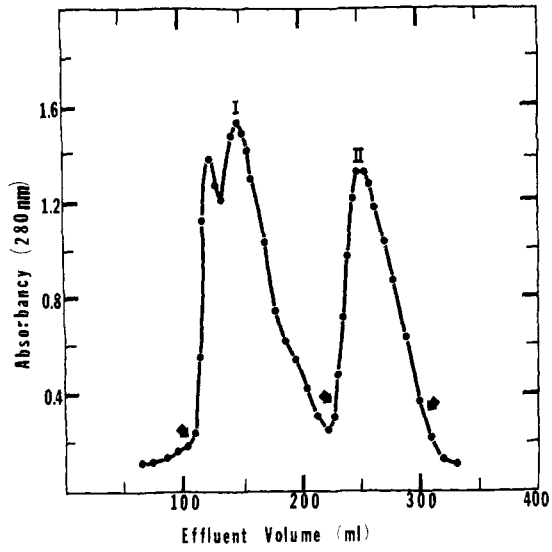

FIGURE 3: G-50 Sephadex elution pattern of cyanogen bromide cleaved CM-lysozyme (84 mg); eluting solvent, 16% formic acid in 0.15 M ammonium acetate; column size 2.5 × 70 cm; 6 ml/tube; 12 ml/hr (see footnote 3).

Immunoassays. Complement fixation inhibition assays were performed as described by Benjamini *et al.* (1964) using titrated complement and hemolysin. The diluent was a barbital buffer containing NaCl, Mg^{2+}, and Ca^{2+} (Campbell *et al.*, 1963). Decomplemented diluted antisera, 1 ml, and inhibitors, 1 ml, were incubated 15 min at 37° before adding the antigen, 0.5 ml, and complement, 1 ml, and the mixture was incubated 30 min at 37°. Red blood cells, 2%, 0.5 ml, and hemolysin, 0.5 ml, were then added and the mixture was incubated until the controls lacking antigen or antibody showed 100% lysis (usually about 15 min). The tubes were centrifuged and the optical density of the supernatants was read at 542 nm.

Radioimmunoassays were performed by mixing diluted antisera, 0.1 ml, inhibitors, 0.1 ml, and 1% bovine serum albumin in 0.1 M $NaHCO_3$ to a total volume of 0.4 ml in 1-ml conical centrifuge tubes. After incubation at 37° for 30 min [^{125}I]antigen, 0.1 ml, and goat antirabbit globulins, 0.5 ml, were added and the mixtures left overnight at 4°. The precipitates were packed by centrifugation at 3500 rpm in a 7-in. radius swinging bucket head for 1 hr. The precipitates were washed twice with 1.0 ml of 1% bovine serum albumin in 0.1 M $NaHCO_3$ and transferred to test tubes with 1 ml of 0.1 N NaOH for the radioactivity measurement. Using this method of assay, 80–100% of the [^{125}I]-lysozyme could be precipitated by antilysozyme. The amount of [^{125}I]CM-lysozyme precipitated by antilysozyme varied with the particular antisera and the particular preparation of [^{125}I]CM-lysozyme. In Figure 9, some of the antisera shown could precipitate as much as 80% of the [^{125}I]CM-lysozyme, whereas in Figure 8, only 46% of the [^{125}I]CM-lysozyme could be precipitated. Neither [^{125}I]lysozyme nor [^{125}I]CM-lysozyme precipitated with the control anti-TMV protein serum.

Results

Fractionation and Analysis of Peptides. The separation patterns obtained from chymotrypsin, trypsin, and cyanogen bromide cleavage of CM-lysozyme on Sephadex G-50 columns are given in Figures 1 and 3. The low molecular weight components (peaks II of Figures 1 and 3) from the trypsin and cyanogen bromide cleavage were chromatographed on Dowex 1 as shown in Figures 2 and 4. Amino acid analyses of some of the peptide fractions are given in Table I. Analysis of fraction 1, Table I, indicated it was tryptic peptide T-9 comprising residues 62–68, Trp-Trp-CM-Cys-Asp-Asn-Gly-Arg as designated by Canfield (1963). Cleavage of CM-lysozyme at the two methionines at positions 12 and 105 by cyanogen bromide treatment are expected to yield three peptides (Gross, 1967): an N-terminal peptide containing residues 1–12, Lys-Val-Phe-Gly-Arg-CM-Cys-Glu-Leu-Ala-Ala-Ala-Homoser; a C-terminal peptide containing residues 106–129, Asn-Ala-Trp-Val-Ala-Trp-Arg-Asn-Arg-CM-Cys-Lys-Gly-Thr-Asp-Val-Gln-Ala-Trp-Ile-Arg-Gly-CM-Cys-Arg-Leu; and a large peptide containing residues 13–105 with 105 converted into homoserine. The amino acid analysis of fraction 2 shown in Table I agreed with the expected analysis for the large cyanogen bromide peptide representing residues 13–105. The analysis of fraction 3 indicated a mixture of the expected N- and C-terminal cyanogen bromide peptides. Dowex 1 chromatography of fraction 3 resulted in two major peaks, fractions 4 and 5, which gave amino acid analyses corresponding to the expected C-terminal and N-terminal cyanogen bromide peptides, respectively. Peak IB (Figure 4) was not characterized. The yield was a mixture of 2.6 μmoles of the N-terminal and 2.6 μmoles of the C-terminal peptides, fraction 3, from 4 μmoles of CM-lysozyme. Fraction 3 then yielded 1.2 μmoles or 46% of the C-terminal peptide, *i.e.*, fraction 4, and 2.6 μmoles or 100% of the N-terminal peptide, *i.e.*, fraction 5.

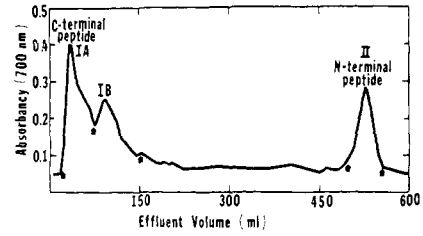

FIGURE 4: Dowex 1-X2 elution pattern of G-50 peak II from cyanogen bromide cleavage of 60 mg of CM-lysozyme (see Figure 3). The eluting gradient is given in the text with other conditions the same as in Figure 3.

Bonavida *et al.* (1969) used disulfide exchange to open the disulfide bonds both before and after cyanogen bromide cleavage of lysozyme. The expected peptides were then obtained using a G-25 Sephadex column.

Antilysozyme Serum Reactions. EFFECT OF PEPTIDES ON COMPLEMENT FIXATION BY LYSOZYME–ANTILYSOZYME. Complement fixation by the lysozyme–antilysozyme complex was performed by incubating 2 μl and 1.33 μl of antisera (obtained 4.5 months after initial sensitization) with 0.1–1 μg of lysozyme (Figure 5). In all cases, a 30 μg of CM-lysozyme equivalent[4] of each peptide was used. With this amount of

[4] A CM-lysozyme equivalent is here defined as the weight of CM-lysozyme starting material from which the digested peptide fraction was obtained. If the particular peptide was analyzed, the CM-lysozyme equivalent is defined more precisely as the molecular weight of CM-lysozyme containing this molecular weight of peptide.

TABLE I: Amino Acid Ratios of Peptide Fractions.

	Peptide Fractions[a]										
	1		2		3		4			5	
Amino Acid	Found	Expected (62–68)[b]	Found	Expected (13–105)	Found	Expected (1–12) + (106–129)	Found	Found[c]	Expected (106–129)	Found	Expected (1–12)
Lys			3.85	4	1.8	2	1.03	1.04	1	0.91	1
His			0.85	1	0.2	0					
Arg	0.90	1	6.20	6	4.2	5	4.02	3.98	4	1.00	1
S-CM-Cys	1.04[d]	1		5		3		1.67	2		1
Asp	1.99	2	18.2	18	3.7	3	2.91	3.17	3		
Thr			5.96	6	1.1	1	1.03	1.11	1		
Ser			9.81	10	0.5	0					
Glu			3.57[e]	3	2.2	2	1.05	1.06	1	1.44	1
Pro			1.94	2							
Gly	1.00	1	9.19	9	3.7	3	2.12	2.21	2	1.04	1
Ala			7.29	6	5.4	6	2.86	3.24	3	3.00	3
Val			3.03	3	2.5	3	1.90	1.95	2	0.64	1
Ile			4.66	5	0.8	1	0.93	0.62	1		
Leu			5.99	6	2.1	2	1.07	10.7	1	1.04	1
Tyr			2.06	3							
Phe			2.09	2	0.9	1				0.90	1
Trp	2.29[f]	2		3		3		2.92[f]	3		
Homoser			0.71[e]	1	0.25	1				0.55	1

[a] Peptide fractions obtained from CM-lysozyme were as follows: (1) tryptic G-50 peak III material, Figure 1; (2) cyanogen bromide cleaved high molecular weight material, G-50 peak I, Figure 3; (3) cyanogen bromide cleaved low molecular weight material, G-50 peak II, Figure 3; (4) Dowex 1 -X 2 fractionation of fraction 3 of this table, fraction IA, Figure 4; (5) fractionation on Dowex 1 -X 2 of fraction 3 of this table, fraction II, Figure 4. [b] The expected peptide of CM-lysozyme is designated in parentheses by residue numbers. [c] This analysis was generously performed by Dr. Hirohsi Matsubara of the Space Science Laboratories, University of California, Richmond, Calif. [d] *S*-CM-cysteine was not found unless the hydrolysis was performed in carefully evacuated tubes. [e] Homoserine eluted with glutamic acid which accounts for the high value for glutamic acid. The homoserine was estimated as the amount of glutamic acid found over that expected times 1.25. The 1.25 corrects for the difference in constants of the two amino acids. [f] The tryptophan analyses were performed on separate hydrolysates containing thioglycolic acid as described by Matsubara and Sasaki (1969).

each peptide, and a range of lysozyme from 0.1 to 1 μg, a 300- to 30-fold molar excess of peptide was achieved. None of the low molecular weight fractions from chymotrypsin, trypsin, or cyanogen bromide digests (Figures 1 and 3, peaks II and III) inhibited the antigen–antibody interaction.

EFFECT OF PEPTIDES ON COMPLEMENT FIXATION BY CM-LYSOZYME–ANTILYSOZYME. Complement fixation by the CM-lysozyme–antilysozyme complex is shown in Figures 6 and 7. In these experiments, the same serum and the same range of concentration, 0.1–1 μg, of the CM-lysozyme antigen were used as in Figure 5. Approximately ten times more antibody, 20 and 13.3 μl, was required to obtain the same degree of complement fixation. The peptides in peaks II and III of Figures 1 and 3 were tested with a 4 μg of CM-lysozyme equivalent for their ability to inhibit this reaction (figure not shown). The cyanogen bromide fraction (peak II, Figure 3) and the tryptic fraction (peak II, Figure 1) were both inhibitory, whereas the chymotryptic fractions (peaks II and III) and the tryptic fraction (peak III) were not inhibitory. Tryptic peptide fractions from the Dowex-1 column (Figure 2) were tested for their ability to inhibit complement fixation by this system. A 19-μg equivalent of each of the CM-lysozyme peptides was used in the tests. Some of the results of the complement fixation experiments using these fractions are shown in Figure 6. The fractions having little or no inhibitory activity were I, III, and X (Figure 2); those having some inhibitory activity were II, V, VI, IX, and XI; and those having the greatest inhibitory effect were IV, VII, and VIII. A mixture of the most inhibitory fractions, IV and VII, as well as a pool of all the fractions, was tested. More inhibition was obtained with the mixtures than any single fraction. The N-terminal cyanogen bromide peptide (fraction 5, Table I) and the C-terminal cyanogen bromide peptide (fraction 4, Table I) inhibited the CM-lysozyme–antilysozyme complement fixation reaction as shown in Figure 7. A 40-μg CM-lysozyme equivalent of these cyanogen bromide peptides was used in this experiment. These experiments were repeated using lesser amounts

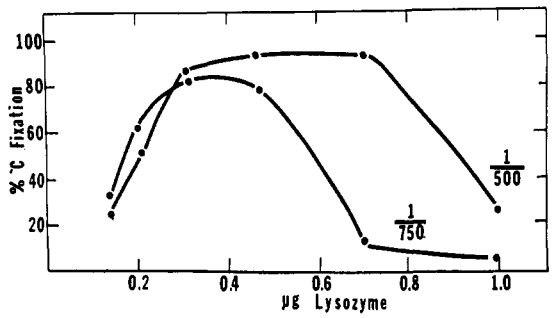

FIGURE 5: Complement fixation of lysozyme–antilysozyme. The antilysozyme was obtained from a bleeding 4.5 months after the initial lysozyme injection, and was assayed at dilutions of $^1/_{500}$ and $^1/_{750}$ which represent 2 and 1.33 μl of antiserum in each assay tube, respectively. See text for method of assay.

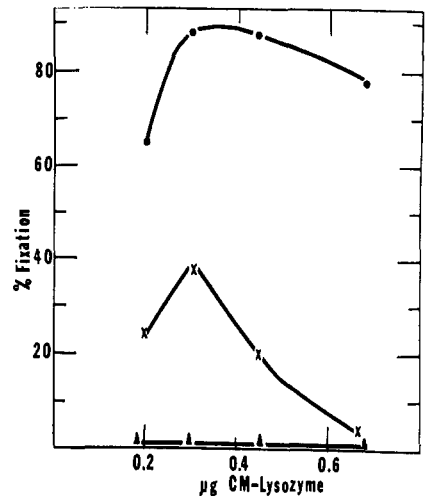

FIGURE 7: Complement fixation of CM-lysozyme–antilysozyme (20 μl) (●——●) and its inhibition with a 40-μg CM-lysozyme equivalent[4] of each CNBr peptide; N-terminal peptide (×——×); C-terminal peptide (▲——▲). The antilysozyme was from the same bleeding as in Figure 5.

of the peptides. The smallest amount of C-terminal cyanogen bromide peptide exhibiting maximum inhibition was a 5-μg equivalent of this peptide. The N-terminal cyanogen bromide peptide exhibited significant inhibition using a 5-μg equivalent with little demonstrable below this concentration. The specificity of the inhibitory peptides was ascertained from the fact that the peptides did not inhibit the complement fixation reaction of either the lysozyme–antilysozyme system or the TMV protein–anti-TMV protein system.

RADIOIMMUNOASSAY WITH LYSOZYME AND CM-LYSOZYME. The reaction of [^{125}I]lysozyme (0.1 μg) with antilysozyme (0.25–2 μl) is shown in Figure 8. The antilysozyme serum used in these experiments was the same as that used in the experiments illustrated in Figures 5, 6, and 7. This reaction is inhibited by 10 μg of unlabeled lysozyme but not by 10 μg of unlabeled CM-lysozyme. The reaction of 0.05 μg of [^{125}I]CM-lysozyme and 0.5–4 μl of antilysozyme is also shown. This reaction is completely inhibited by unlabeled CM-lysozyme but only slightly by 10 μg of unlabeled lysozyme. TMV protein (10 μg) had no effect on the binding of either [^{125}I]antigen with antilysozyme. In another experiment (not illustrated) [^{125}I]CM-lysozyme (0.05 μg) was completely inhibited from binding with antilysozyme (1–5 μl) by 100 μg of lysozyme.

BINDING OF [^{125}I]CM-LYSOZYME WITH ABSORBED SERUM. An antilysozyme globulin preparation (serum obtained 3 months after sensitization) was assayed for its ability to bind with [^{125}I]CM-lysozyme both with and without prior absorption with lysozyme. The data in Table II show that the binding of antilysozyme globulins following absorption with lysozyme was the same as in the unabsorbed antibody preparation.

CHANGE OF TITER TO CM-LYSOZYME WITH TIME. Figure 9 compares the reaction of antilysozyme sera, obtained from

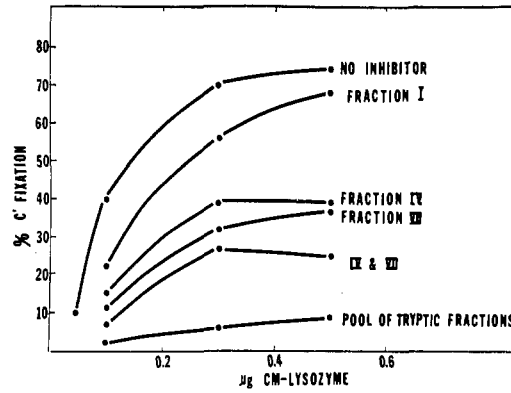

FIGURE 6: Complement fixation of CM-lysozyme–antilysozyme (20 μl) and its inhibition by tryptic peptide fractions obtained by Dowex 1-X2 chromatography (see Figure 2). Each tryptic peptide fraction was assayed at 19 μg equivalent of CM-lysozyme. The pool was prepared by combining a portion of the 11 fractions obtained by Dowex chromatography. The antilysozyme was from the same bleeding as used in Figure 5.

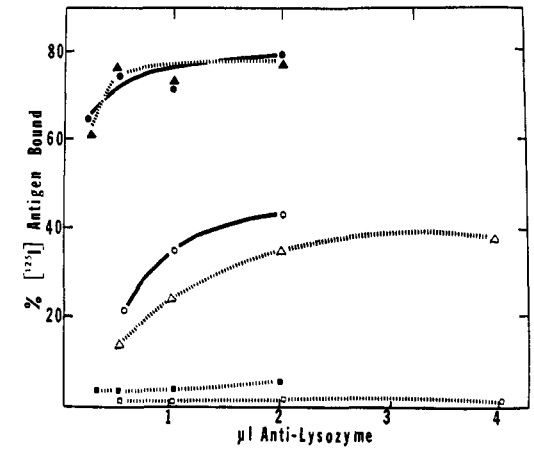

FIGURE 8: The binding of 0.1 μg of [^{125}I]lysozyme with antilysozyme (●——●) and its binding in the presence of 10 μg of unlabeled lysozyme (■- - -■) or 10 μg of unlabeled CM-lysozyme (▲- - -▲). The binding of 0.05 μg [^{125}I]CM-lysozyme with antilysozyme (○——○) and its binding in the presence of 10 μg of unlabeled CM-lysozyme (□- - -□) or 10 μg of unlabeled lysozyme (△- - -△). TMVP (10 μg) had no effect on the binding on either the [^{125}I]lysozyme with antilysozyme or the [^{125}I]CM-lysozyme with antilysozyme at the concentrations used above. The antilysozyme was from the same bleeding as that used in Figure 5.

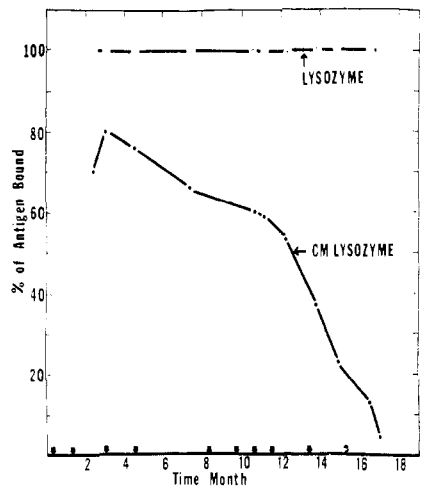

FIGURE 9: Per cent of [^{125}I]CM-lysozyme (0.05 μg) or [^{125}I]lysozyme (0.1 μg) precipitated by 2 μl of antilysozyme serum at various times of bleeding. Sensitizing doses are noted with arrows (⬇) on the abscissa.

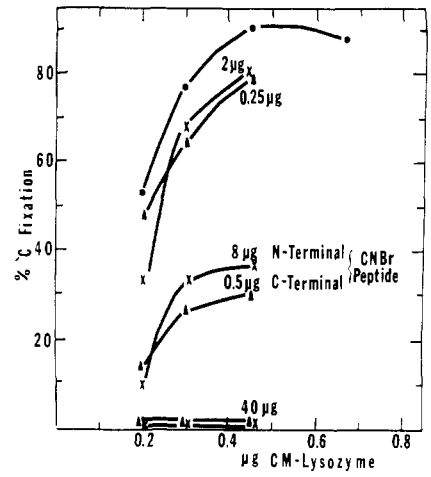

FIGURE 10: Complement fixation on CM-lysozyme–anti-CM-lysozyme (10 μl) and its inhibition by various amounts of the N-terminal (×——×) and C-terminal (▲——▲) CNBr peptides of CM-lysozyme expressed in μg-equiv of CM-lysozyme.[4] The anti-CM-lysozyme was from a bleeding taken 2 months after the first sensitizing injection.

various bleedings, with CM-lysozyme using the radioimmunoassay method. Booster injections are indicated on the figure. The titer to lysozyme did not appreciably change since 1–2 μl of each antiserum was required to precipitate 0.1 μg of [^{125}I]lysozyme. However, the amount of serum (2 μl) that would totally precipitate this amount of lysozyme bound 80% of the [^{125}I]CM-lysozyme (0.05 μg) at the third month after the initial injection. By the 17th month, no more than 5% of the [^{125}I]CM-lysozyme was bound by 2 μl of antilysozyme.

Effect of Cyanogen Bromide Peptides on Complement Fixation by CM-lysozyme–Anti-CM-lysozyme. Complement fixation by 0.2–1 μg of CM-lysozyme and 10 μl of anti-CM-lysozyme is shown in Figure 10. Also shown in Figure 10 is the complete inhibition of this reaction by a 40-μg equivalent of either the C-terminal or N-terminal cyanogen bromide peptide. On further testing, the C-terminal peptide completely inhibited the reaction with a 1-μg equivalent and exhibited significant inhibition using a 0.5-μg equivalent of the peptide with 0.2–0.45 μg of CM-lysozyme. Similarly, the N-terminal cyanogen bromide peptide significantly, though not completely, inhibited the reaction at the 8-μg equivalent level. As mentioned before, the peptides specifically inhibited the CM-lysozyme–anti-CM-lysozyme reaction since they had no inhibitory effect on either the TMV protein–anti-TMV protein or the lysozyme–antilysozyme complement fixation reaction.

TABLE II: Binding of [^{125}I]CM-Lysozyme to Antilysozyme Globulins.[a]

	% [^{125}I]CM-Lysozyme (0.05 μg) Bound	
μl of Antibody	Unabsorbed Antibody	Antibody Absorbed with Lysozyme
5	29	24
2.5	20	20
1.7	16	16
1.2	14	13

[a] Control globulins, anti-TMVP, did not bind any [^{125}I]CM-lysozyme.

Discussion

The data presented in this paper show that tryptic, chymotryptic, and cyanogen bromide cleavage products of CM-lysozyme do not inhibit the reaction of lysozyme with rabbit antilysozyme. This confirms the findings of Gerwing and Thompson (1968) and Shinka *et al.* (1967). There were, however, in rabbits injected with lysozyme, antibodies which bind CM-lysozyme. These antibodies could be inhibited with trypsin and cyanogen bromide cleaved peptides of CM-lysozyme and were found to bind preferentially to CM-lysozyme rather than lysozyme.

It is possible that the antibodies reacting with CM-lysozyme were produced by the presence in the immunogen of small amounts of denatured lysozyme more similar to CM-lysozyme than to native lysozyme. Lysozyme preparations are known to chromatograph heterogeneously on IRC-50 resin columns (Tallan and Stein, 1953). Alternatively, the lysozyme could be altered either by Freund's adjuvant or by *in vivo* degradation. The altered lysozyme molecules could evoke antibodies to determinants more similar to those exposed on the CM-lysozyme molecule than on the lysozyme molecule.

The presence of CM-lysozyme specific antibodies in rabbits injected with lysozyme illustrates that absorption of an antiserum with the immunizing antigen does not necessarily remove all antibodies evoked by the antigen.

Since the CM-lysozyme binding antibodies disappear with time and with continued lysozyme booster injections in one animal (Figure 9), other animals are now under study.

The observation that several components from Dowex 1 fractionation (Figure 2) of the tryptic peptides were active and that combinations of these were more inhibitory than the individual fractions indicates the presence of antibodies which recognize several areas of the CM-lysozyme primary sequence.

The cyanogen bromide peptides of CM-lysozyme, representing residues 1–12 and 106–129, respectively, were found to inhibit the complement fixation reaction of CM-lysozyme–antilysozyme. As seen in Figure 7, the C-terminal cyanogen bromide peptide gave more inhibition with less material than the N-terminal peptide. Although not reported here, the [^{125}I]CM-lysozyme–antilysozyme reaction was also inhibited by those cyanogen bromide peptides.

There is a similarity in specificity between the anti-CM-lysozyme antibodies and the antibodies in antilysozyme sera which react with CM-lysozyme. Both antibodies are inhibited in their reaction with CM-lysozyme by the N- and C-terminal cyanogen bromide peptides. As noted, the C-terminal peptide (Figures 7 and 10) was more effective.

Gerwing and Thompson (1968) found that anti-CM-lysozyme reacted with only one tryptic peptide of CM-lysozyme, namely tryptic peptide T-11, representing residues 74–96. We have not tested the inhibition of individual tryptic peptides with our anti-CM-lysozyme sera. In preliminary experiments, the C-terminal cyanogen bromide peptide inhibited CM-lysozyme–anti-CM-lysozyme more than the unfractionated tryptic peptides (peak II, Figure 1).

The antigenic determinate areas of CM-lysozyme and lysozyme are depicted in Bragg's drawing of lysozyme in Figure 11. The CM-lysozyme determinate region, residues 74–96, falls within one of the two determinate regions of lysozyme, residues 57–107. The cyanogen bromide peptides which are also determinants of CM-lysozyme are in the region of the other lysozyme determinant, *i.e.*, residues 1–27, 122–129. These correlations between the determinate regions of CM-lysozyme and lysozyme may be due to the fact that the combined determinate sequences of lysozyme involve 67% of the amino acids in the molecule. We are presently engaged in the synthesis of peptides in the N- and C-terminal areas, which when combined through the appropriate disulfide bridges will provide a more precise definition of the lysozyme and CM-lysozyme determinants in this region of the two molecules.

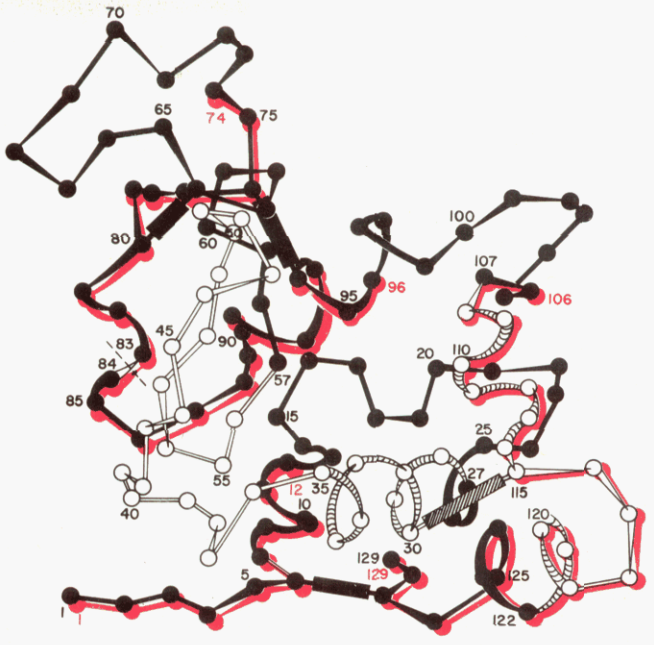

FIGURE 11: Schematic drawing of lysozyme molecule based on Bragg's drawing in Blake *et al.* (1965). The numerals refer to the residue number in the amino acid sequence. The blacked sequences are determinant areas found for lysozyme (Fujio *et al.*, 1968a,b). The dotted line between residues 83 and 84 represents a break in the peptide chain in the lysozyme determinate area contained in residues 57–107. The red lines depict the determinate areas for CM-lysozyme reported by Gerwing and Thompson (1968) (residues 74–96) and reported in this paper (residues 1–12 and 106–129).

Acknowledgments

We wish to thank Dr. Ben F. Feingold, the Director of the Laboratory of Medical Entomology, Kaiser Foundation Research Institute, for his interest and encouragement in this work. Drs. E. Benjamini, J. R. Kettman, D. Michaeli, W. J. Peterson, Allan C. Wilson, Norman Arnheim, and Mr. M. Shimizu are thanked for their valuable advice and discussions. Drs. Eugene Gardner and Karen Thompson are especially thanked for their help in editing the manuscript. Miss Carol Carpenter and Mr. Alan Singer are acknowledged for their technical help and Mr. Daniel Leung is thanked for performing many of the animal sensitizations and bleedings. We also appreciate the work of Miss Diane Karasik and Mr. M. Shimizu in drawing and photographing the figures.

References

Atassi, M. Z., and Habeeb, A. F. S. A. (1969), *Biochemistry 8*, 1385.

Benjamini, E., Young, J. D., Peterson, W. J., Leung, C. Y., and Shimizu, M. (1965), *Biochemistry 4*, 2081.

Benjamini, E., Young, J. D., Shimizu, M., and Leung, C. Y. (1964), *Biochemistry 3*, 1115.

Blake, C. C. F., Koenig, D. F., Mair, G. A., North, A. C. I., Phillips, D. C., and Sarma, V. R. (1965), *Nature 206*, 757.

Bonavida, B. (1969), *Fed. Proc. 28*, 326.

Bonavida, B., Miller, A., and Sercarz, E. E. (1969), *Biochemistry 8*, 968.

Campbell, D. H., Garvey, J. S., Cremer, N. E. and Sussdorf, D. H. (1963), Methods in Immunology, New York, N. Y., W. A. Benjamin, p 246.

Canfield, R. E. (1963), *J. Biol. Chem. 238*, 2698.

Canfield, R. E., and Anfinsen, C. B. (1963), *J. Biol. Chem. 238*, 2684.

Chipman, D. M., and Sharon, N. (1969), *Science 165*, 454.

Fraenkel-Conrat, H. (1957), *Virology 4*, 1.

Fujio, H., Imanishi, M., Nishioka, K., and Amano, T. (1968a), *Biken J. 11*, 207.

Fujio, H., Imanishi, M., Nishioka, K., and Amano, T. (1968b), *Biken J. 11*, 219.

Funatsu, G. (1964), *Biochemistry 3*, 1251.

Gerwing, J., and Thompson, K. (1968), *Biochemistry 7*, 3888.

Greenwood, F. C., Hunter, W. M., and Glover, J. S. (1963), *Biochem. J. 89*, 114.

Gross, E. (1967), *Methods Enzymol. 11*, 238.

Habeeb, A. F. S. A., and Atassi, M. Z. (1969), *Immunochemistry 6*, 555.
Imanishi, M., Miyagawa, N., Fujio, H., and Amano, T. (1969), *Biken J. 12*, 85.
Jollès, J., Jauregui-Adell, J., Bernier, I., and Jollès, P. (1963), *Biochim. Biophys. Acta 78*, 668.
Matsubara, H., and Sasaki, R. M. (1969), *Biochem. Biophys. Res. Commun. 35*, 175.
Phillips, D. C. (1966), *Sci. Amer. 215*, 78.
Shinka, S., Imanishi, M., Miyagawa, N., Amano, T., Inouye, M., and Tsugita, A. (1967), *Biken J. 10*, 89.
Steers, E., Craven, G. R., and Anfinsen, C. B. (1965), *J. Biol. Chem. 240*, 2478.
Tallan, H. H., and Stein, W. H. (1953), *J. Biol. Chem. 200*, 507.
Young, J. D., and Leung, C. Y. (1969), *Fed. Proc. 28*, 326.

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

Barbara Mitchell and Julia Gerwing Levy

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

* From the Department of Microbiology, The University of British Columbia, Vancouver 8, British Columbia, Canada. *Received March 20, 1970.* This work was supported by a grant from the Medical Research Council of Canada.