oxygen (Figure 1B). However, the existence during catalysis of a reduced form of the enzyme which in the absence of tryptophan reacts at significant rates with CO or O_2 is incompatible with the present evidence and may no longer be considered in proposing reaction mechanisms. Thus, the dithionite-treated enzyme, which has been shown to bind CO in the absence of tryptophan, does not seem to be a normal participant in catalysis. As no form of the divalent enzyme which cannot bind CO has yet been described, we therefore conclude, on the basis of existent evidence, that the second possibility is improbable (Figure 1B) and that oscillation in valence of the heme of tryptophan oxygenase probably occurs during the catalytic process (Figure 1A).

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Chemical and Immunological Characterization of a Unique Antigenic Region in Lysozyme*

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ABSTRACT: A peptide composed of the amino acid sequence 60–83, containing one intrachain disulfide bond (between half-cystine residues 64 and 80), and denoted "loop"-peptide, was obtained from hen egg-white lysozyme. The peptide was identified by amino acid analysis, determination of its amino- and carboxy-terminal residues, and the identification of the half-cystine residues which constitute the disulfide bond. Antibodies specific toward this region were prepared either by their isolation from the totality of anti-lysozyme antibodies with an immunoadsorbent containing the loop-peptide, or by immunization with a synthetic conjugate composed of multichain poly-DL-alanine to which the loop-peptide was attached (denoted loop-A—L). In the last case the anti-loop antibodies, namely, those recognizing the natural loop, were isolated on a lysozyme immunoadsorbent. The antibodies to the loop region, prepared according to these two approaches, showed restricted heterogeneity as compared to the totality of anti-lysozyme antibody population. This was manifested both in the acrylamide electrophoresis of their respective light chains and in the isoelectric focusing of the intact antibodies. The reactivity and specificity of the anti-loop antibodies, as compared to that of anti-lysozyme antibodies, was assessed both by their capacity to bind the labeled free loop-peptide as well as labeled intact lysozyme, and by their ability to inactivate modified bacteriophage, to which either the loop-peptide or lysozyme were chemically attached. In both these methods the anti-loop antibodies were indeed shown to react with the loop region of lysozyme exclusively and not with other parts of the molecule. Unfolding of the peptide chain of the loop-peptide, achieved by either reduction and alkylation or by performic acid oxidation, resulted in a drastic decrease in the reactivity with the specific antibodies.

These findings are indicative of the decisive role played by the spatial conformation in the antigenic specificity of this unique region in the lysozyme molecule.

Studies on the antigenic structure of hen egg-white lysozyme have been reported in recent years from several laboratories (*e.g.*, Shinka *et al.*, 1967; Imanishi *et al.*, 1969; Atassi and Habeeb, 1969; Habeeb and Atassi, 1969; Arnon, 1968; Bonavida *et al.*, 1969; Strosberg and Kanarek, 1970). In a previous communication from this laboratory (Arnon and Sela, 1969) we reported the preparation of antibodies with specificity directed toward a unique region, denoted loop-peptide,[1] in the lysozyme molecule. This peptide included the loop between half-cysteines-64 and -80. We describe now

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[1] Abbreviations used are: TPCK, L-(1-tosylamido-2-phenyl)ethyl chloromethyl ketone; dansyl, 1-dimethylaminonaphthalene-5-sulfonyl; PFU, plaque-forming units; loop, amino acid sequence 60–83 of lysozyme; A—L, multichain poly-DL-alanyl—poly-L-lysine; loop-A—L, the loop conjugate of A—L; 1-CM-loop, loop-peptide carboxymethylated at cysteine residue 76. RCM-peptide, completely reduced and carboxymethylated peptide of the sequence 60–83 of lysozyme.

an improved method for the preparation of the loop peptide, its chemical and immunological characterization, as well as the limited heterogeneity of the anti-loop antibodies.

The loop-peptide used in our previous study was prepared from a peptic fragment of lysozyme (Canfield and Liu, 1965) by reduction of the disulfide bonds in order to disconnect an adjoining peptide, followed by reoxidation of the intrachain disulfide bridge and re-formation of the loop. One of the difficulties encountered in this procedure was the formation of aggregates which led to unpredictable yields of the loop-peptide. In the present study we describe a reproducible method for preparing the loop-peptide, and show that the re-formed disulfide bridge is identical with the one present in intact lysozyme.

Loop-specific antibodies were prepared, as described before, either by selective absorption with a loop immunoadsorbent from an anti-lysozyme serum, or by immunization with a synthetic conjugate of the loop-peptide and subsequent isolation of the specific antibodies on a lysozyme immunoadsorbent. Thus, two populations of antibodies, directed most probably against the same unique region within the lysozyme molecule, were available. It was of interest, therefore, to compare these antibodies to each other and to the total anti-lysozyme antibody population, both as regards their specificity and their heterogeneity. Since the sensitivity of the assay method used is a decisive factor in studies of specificity, we employed here an extremely sensitive method for the comparison of the antibodies, namely, the inactivation of bacteriophages (Haimovich *et al.*, 1970a) on to which proteins are chemically attached. Using this technique, very small amounts of anti-protein antibodies can be specifically detected by their capacity to inactivate the protein–bacteriophage conjugate. If the bacteriophage conjugate contains only a fragment of the protein antigen, the inactivation studies are focused solely on that specific region of the antigen molecule. Moreover, the capacity of the protein, its fragments or analogs, to inhibit the inactivation of the modified phage by the antibodies (Haimovich *et al.*, 1970b) may serve for further elucidation of the specificity of the antibodies and for comparison of different antibody populations.

Heterogeneity of antibodies is assumed to be due, to a large extent, to the presence of multiple specificity determinants on the antigen (Haber, 1968). Hence, antibodies with specificity directed toward limited regions of the antigen would be expected to be less heterogeneous. Indeed, it has been reported that antibodies with restricted heterogeneity had been elicited in several cases when antigens with very regular repeating structure were used for immunization (*e.g.*, Mamet-Bratley, 1966; Richards and Haber, 1967; Richards *et al.*, 1969; Krause, 1970; Haber, 1970; Roholt *et al.*, 1970; Brenneman and Singer, 1970). Furthermore, in one case (Givas *et al.*, 1968), selective fractionation of homogeneous antibodies was achieved. Using an immunologically active peptide of sperm-whale myoglobin for the elution of antibodies from an immunoabsorbent, a fraction of antibodies was obtained which was restricted to a narrow range of electrophoretic mobility in polyacrylamide gel, indicative of a pure protein. There is also experimental evidence that a purified immunologically active peptide of oxidized ribonuclease (Isagholian and Brown, 1970) binds to the antibodies with a heterogeneity index of 0.98, indicating binding to a rather homogeneous fraction of the antibodies. We wish to report here the restricted heterogeneity of the two types of loop-specific antibodies.

Experimental Section

Materials

Lysozyme (two-times-crystallized, lot nos. LY 8AA, LY 8HB, and LY 9FA), pepsin (lot no. PM 8JC), TPCK-treated trypsin, (lot TRTPCK 8DA), and α-chymotrypsin (three-times crystallized, lot no. CDI 6JG) were purchased from Worthington Biochemical Co. Subtilisin (crystallized, lot no. 60420) was obtained from Novo Co., Copenhagen. Pronase (lot no. 62282) was purchased from Calbiochem, Los Angeles.

Loop-peptide was prepared by reduction of a peptic fragment of lysozyme as described in detail in the Results. Loop-A—L was prepared by conjugating the loop-peptide to multichain poly-DL-alanine (A—L), as described elsewhere (Arnon and Sela, 1969). All other reagents were analytical grade or the best grade available.

Antisera were prepared by immunizing rabbits and goats with lysozyme or loop-A—L. The immunization was carried out by multisite intradermal injections of 10–15 mg of the antigen in complete Freund's adjuvant (Difco). The resultant anti-lysozyme sera contained 1–2 mg of antibody/ml of serum, whereas anti-loop-A—L sera contained about 200 μg/ml of loop-specific antibodies.

Methods

Separation of Peptides. Peptides from peptic digest of lysozyme were fractionated by gel filtration on Sephadex G-25 fine (according to Canfield and Liu, 1965), followed by a pH gradient column chromatography on CM-cellulose in 0.3 M ammonium acetate. The fraction containing peptide 1 of Canfield and Liu was reduced, reoxidized in a dilute solution of NH_4OH, and separated on Sephadex G-25 column, as described under the Results, to yield the loop peptide.

Carboxymethylation of sulfhydryl groups was carried out either on the loop-peptide directly upon its release from the column, or on its reduced derivative obtained by previous reduction in 0.1 M 2-mercaptoethanol. The alkylation was performed with iodoacetic acid in 0.05 M Tris-HCl buffer (pH 8.2). In several preparations ^{14}C-labeled iodoacetic acid (Amersham) was used for the reaction. The carboxymethylated derivatives were purified by gel filtration on Sephadex G-25 coarse, in 0.1 M acetic acid. The totally reduced and carboxymethylated loop-peptide was denoted RCM-peptide.

Performic Acid Oxidation. Oxidation of the disulfide bond in the loop-peptide was carried out with performic acid, according to Hirs (1967).

Amino Acid Analyses. The amino acid composition of lysozyme fragments was analyzed in a Beckman-Spinco automatic amino acid analyzer, Model 120B, after hydrolysis under reduced pressure in constant-boiling hydrochloric acid (6 N) for 22 hr (Spackman *et al.*, 1958).

The molecular weight of the loop-peptide was determined in the ultracentrifuge by low-speed sedimentation equilibrium using interference optics (Richards and Schachman, 1959).

Determination of the Amino-Terminal Residues. The amino-terminal residues were determined according to the procedure of Gray and Hartley (1963). The peptide fragments (dissolved in 0.1% triethylamine–CO_2 buffer, pH 8.0) were allowed to react overnight with dansyl-chloride (equal volume of an 0.1% solution in acetone) at room temperature. Following gel filtration on Sephadex G-10 in 0.1% triethylamine-CO_2 buffer (pH 8.0), containing 50% alcohol, the dansylated peptides were hydrolyzed either by pronase or in 6 N HCl

at 90° for 18 hr. Identification of the dansylamino acids was carried out by silica gel thin-layer chromatography in a system consisting of methyl acetate–2-propanol–NH_4OH (9:7:4, v/v) or in a system of benzene–pyridine–acetic acid (16:4:1, v/v).

Determination of the Carboxy-Terminal Residues. The hydrazinolysis method (Hirs *et al.*, 1960) was used. To 0.2–0.4 μmole of peptide was added 65 mg of hydrazine sulfate and the mixture was dried in a vacuum desiccator for 2 days. Anhydrous hydrazine (0.3 ml) was added and allowed to react in a sealed tube at 96° for 7 hr. The hydrazinolysate was dried in a desiccator over concentrated H_2SO_4, and then dissolved in 1.5 ml of water and mixed with 0.3 ml of benzaldehyde. Following stirring for 2 hr the aqueous layer was evaporated and analyzed in the amino acid analyzer.

Identification of Disulfide Bonds. The diagonal paper electrophoresis technique (Brown and Hartley, 1963, 1966) was used for this purpose. The loop-peptide was digested either with chymotrypsin and trypsin or with subtilisin. The first procedure was as follows: 20 mg of loop-peptide was dissolved in 2 ml of a 1% triethylamine–CO_2 buffer (pH 7.0). Chymotrypsin (0.2 mg) in 0.2 ml of the same buffer was added and the digestion proceeded for 18 hr at 37°. Trypsin (0.2 mg) in 0.2 ml of buffer was then added and the reaction mixture kept for additional 18 hr at 37°. The subtilisin digestion was carried out with 20 mg of loop-peptide and 0.2 ml of subtilisin in 1% triethylamine–CO_2 buffer (pH 7.0) at 37° for 18 hr. The digests were applied onto a large sheet of Whatman (No. 3MM) paper and the procedure was continued under the conditions described by Neuman *et al.* (1967), except that the electrophoresis in both dimensions was carried out at 55 V/cm for 45 min at pH 3.5.

Immunospecific Isolation and Fractionation of Antibodies. Antibodies were isolated by means of specific immunoadsorbents prepared by binding of the antigen to Sepharose 4B (Pharmacia) (Porath *et al.*, 1967) according to Cuatrecasas *et al.* (1968). The totality of antibodies against lysozyme was adsorbed from anti-lysozyme serum on lysozyme–Sepharose. In a typical preparation lysozyme–Sepharose immunoadsorbent (in an amount containing 2 g of dry Sepharose and 400 mg of lysozyme) was dispersed in 1 l. of antiserum. The adsorbed antibodies were eluted with 1 N acetic acid. From the above purified antibodies the fraction of *antibodies specific for the loop alone* was isolated using an immunoadsorbent in which the loop-peptide was bound to Sepharose (20–40 mg of loop-peptide bound to 1 g of dry Sepharose). In this manner 8–10 mg of specific anti-loop antibodies were obtained from 100 mg of pure anti-lysozyme antibodies. *Specific anti-loop antibodies* were also isolated from antiserum against the synthetic conjugate loop-A—L. In this case lysozyme–Sepharose immunoadsorbent was utilized for the adsorption of the antibodies. Antibodies (200 mg) were isolated in that manner from 1 l. of serum.

Disc Electrophoresis. Analytical electrophoresis of light chains on polyacrylamide gel was performed by the disc electrophoretic method of Davis (1964). Electrophoresis was carried out at pH 8.3 in 7.5% polyacrylamide gel. Protein components were stained with 1% Amido Schwarz in 7% acetic acid.

Isoelectric Focusing. Gel electrofocusing was performed according to Wrigley (1968), using pH 5–8 Ampholytes (LKB). The protein solution in 10% sucrose was applied above the gel layer and not polymerized in it. The proteins were stained with 0.025% coumassie blue in methanol–water–acetic acid (5:5:1, v/v).

Preparation of Modified Bacteriophages. Lysozyme–bacteriophage T4 conjugate, prepared by making use of tolylene 2,4-diisocyanate as cross-linking agent, was a gift from Dr. J. Haimovich. The conjugate of loop-peptide and bacteriophage T4 was prepared according to the general procedure described by Haimovich *et al.* (1970a), using glutaraldehyde as the bifunctional reagent.

Inactivation of Modified Bacteriophage by Antibodies (according to Haimovich *et al.*, 1970a). Antibody preparations at several dilutions were mixed with the modified bacteriophage solution (containing *ca.* 500 PFU) and kept at 37° for 2 hr. At the end of the inactivation reaction, 2.5 ml of soft agar containing 3×10^9 bacteria (*Escherichia coli* B) were added to the test tubes and the whole mixture poured onto plates of bottom layer agar. Plaques were counted after 10–18 hr at 37°.

Inhibition of Bacteriophage Inactivation. Various concentrations of the inhibitors were allowed to react at 4° overnight with the amount of antibodies that would have caused approximately 90% inactivation of the bacteriophage conjugate. The modified bacteriophage (containing 500 PFU) was then added to the mixture and the number of surviving bacteriophage was determined as described above. The inhibitory capacity of the various inhibitors was calculated as the concentration of inhibitor required for 50% inhibition of the bacteriophage inactivation.

Antigen Binding Capacity. These experiments were carried out with purified rabbit antibodies and with labeled antigens—^{125}I-labeled lysozyme (prepared according to McFarlane (1958)) and ^{14}C-labeled loop-peptide (alkylated with [^{14}C]-iodoacetic acid), or its performic acid oxidized derivative. Increasing amounts of anti-lysozyme antibodies (200–900 μg) were incubated for 1 hr at 37° with 10 μg of labeled lysozyme or 1.6 μg of labeled loop-peptide. Goat antiserum (1 ml) against rabbit IgG (containing 8 mg of antibodies/ml) was then added, and the final mixture was kept at 37° for 1 hr, followed by 24 hr at 4°. In some experiments a lower amount of labeled loop-peptide (0.08 μg) was used. The degree of binding was calculated by measuring the radioactivity of the washed precipitate.

Results

The fraction denoted peptide 1 (Canfield and Liu, 1965) was obtained upon gel filtration of a peptic digest of lysozyme. This peptide consists of the loop peptide and a linear peptide, linked between them through the disulfide bridge between half-cystine-76 and -94.

Preparation and Characterization of the Loop-peptide. Peptide 1 was further fractionated on CM-cellulose in 0.3 M ammonium acetate, using a pH gradient in the pH range 4–6. (The pH of the buffers was adjusted with NH_4OH.) The elution profile of such a column is given in Figure 1 (fractions under peaks 2, 3, 4, and 6 were obtained in small amounts only and were, therefore, discarded). The main fractions, under peaks 1 and 5, were pooled, respectively, as illustrated in Figure 1 (shaded areas), and were analyzed for their amino acid composition as well as the amino- and carboxy-terminal residues. The results showed relatively minor differences in the amino acid composition, and in the carboxy-terminal residues. These results suggest that the primary structures of both fractions are almost the same, except that the carboxy terminus of fraction 1 has several amino acid residues, additional to those present in fraction 5, in the "linear" peptide part.

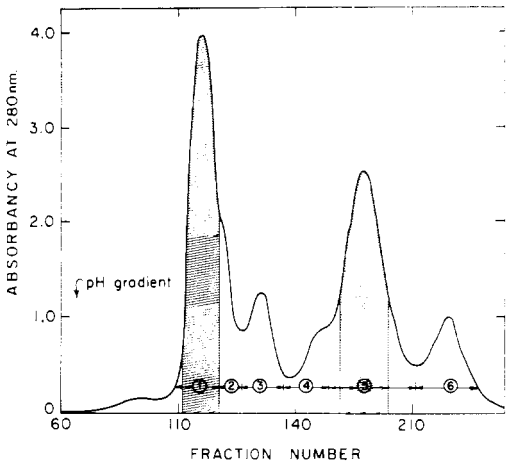

FIGURE 1: Elution pattern of peptide 1 isolated from a peptic digest of lysozyme. A 1.5 × 66 cm CM-cellulose column equilibrated with 0.1 M of ammonium acetate buffer (pH 3.5) was used. Peptide 1 (400 mg) was applied with the starting buffer. Elution took place in 0.3 M ammonium acetate buffer with a linear pH gradient between pH 4.0 and pH 6.0; 5-ml fractions were collected.

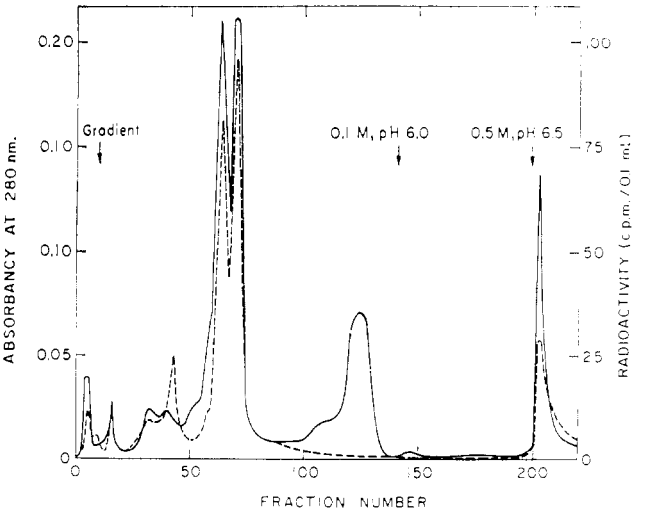

FIGURE 3: Elution pattern of ^{14}C-labeled 1-CM-loop-peptide on CM-cellulose column (1.5 × 15 cm) in 0.05 M acetic acid with a linear gradient of 0.1 M ammonium acetate (pH 4.0), followed by stepwise gradients as indicated by the arrows. The specific radioactivity of the 1-CM-loop preparation was 5300 cpm/mmole. Absorbancy at 280 nm (——); radioactivity (---).

The two fractions differed in their capacity to yield the loop-peptide. Only fraction 5 gave rise to loop-peptide upon reduction and reoxidation in dilute solution, as described previously (Arnon and Sela, 1969), whereas no such peptide was derived from fraction 1, using the same conditions. This is apparently due to side reactions leading to peptic cleavages within the loop itself. The loop-peptide was, therefore, prepared from fraction 5 as follows. Fraction 5 was reduced using dithiothreitol (0.025 mM), reoxidized in 0.05 M NH_4OH containing 5×10^{-5} M copper sulfate, and gel filtered through Sephadex G-25 (Figure 2). (The copper ions were added, as it was observed that presence of EDTA in the mixture caused extensive aggregation. Similarly, aggregates were obtained in excess of copper ions.) In order to prevent possible aggregation due to the presence of the free sulfhydryl group, this function was carboxymethylated immediately upon release from the column, and the loop-peptide obtained was denoted 1-CM-loop. Further purification of this derivative and removal of small amounts of contamination could be achieved by CM-cellulose column chromatography (Figure 3).

The 1-CM-loop had mostly serine, but also some arginine at its amino-terminal residue, and leucine at its carboxyl terminus (Table I). It contained two tryptophan residues, as estimated from the absorbency in the ultraviolet region at 280 nm ($E^{1\%}_{1cm}$ 40.2). According to these data and the results of the amino acid analysis (Table I), the loop-peptide consists mainly of the amino acid sequence 60–83 of lysozyme, although in part of it the amino-terminal serine residue is missing. The calculated weight of the loop-peptide, assuming the above sequence, is 2713. The actual value for the molecular weight as obtained by ultracentrifugal analysis is 2750.

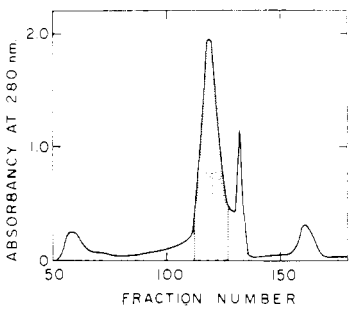

FIGURE 2: Chromatographic separation on a Sephadex G-25 (fine) column (3 × 270 cm) equilibrated with 0.05 M NH_4OH, of the reduced and reoxidized fraction 5 (150 mg). The shaded zone contains the loop-peptide.

TABLE I: Amino Acid Composition of 1-CM-loop-peptide.

Amino Acid Residue	For Residues 60–83		Carboxy-Terminal Residue[b]	Amino-Terminal Residue[c]
	Calcd	Found[a]		
Arginine	3	2.89		±
Aspartic acid	4	3.85		
Threonine	1	1.02		
Serine	3	2.44		+
Proline	2	1.87		
Glycine	2	2.00		
Alanine	1	1.05		
Half-cystine	2	1.72		
CM-cysteine	1	0.76		
Isoleucine	1	1.08		
Leucine	2	1.93	+	
Tryptophan[d]	2	2.02		

[a] The samples were hydrolyzed for 22 hr in 6 N HCl. The results were calculated from the average recoveries of four different preparations, assuming that the loop-peptide contains two residues of glycine. [b] The carboxy-terminal residue was determined in the amino acid analyzer after hydrazinolysis as described in the text. [c] The amino-terminal residues were identified after dansylation as described in the text. [d] Tryptophan content was calculated from the absorbancy at 280 nm, assuming a molecular weight of 2713 (the calculated value for sequence 60–83).

TABLE II: Identification of the Disulfide Bond in the Loop-peptide. Amino Acid Composition of the Off-Diagonal Peptides.[a]

Amino Acid Residue	Chymotryptic + Tryptic Digest		Subtilisin Digest	
	Peptide a	Peptide b	Peptide c	Peptide d
Arginine	1.01 (1)			
Cysteic acid	0.87 (1)	1.48 (1)	0.85 (1)	1.09 (1)
Aspartic acid	1.84 (2)	1.00 (1)	1.00 (1)	1.00 (1)
Serine	0.14	0.88 (1)	0.28	0.66 (1)
Glutamic acid	0.07	0.24	0.16	0.07
Proline		1.08 (1)		0.99 (1)
Glycine	1.00 (1)	0.29	0.28	0.10
Alanine	0.08	0.92 (1)	0.11	0.04
Isoleucine	0.03	0.99 (1)	0.09	1.00 (1)
Leucine	0.07	1.06 (1)	0.19	0.12

[a] The peptides containing the disulfide bond were identified by the diagonal paper electrophoresis technique as described in the text. The numbers in parentheses indicate the residues assumed to be present in the respective peptides.

The identification of the disulfide bond present in the loop-peptide was established by the diagonal paper electrophoresis technique (Brown and Hartley, 1963, 1966). Chymotryptic, followed by tryptic, digestion of the loop-peptide yielded only one pair of off-diagonal peptides (Figure 4A). These peptides had the amino acid composition listed in Table II, indicating that the fragment containing the disulfide bond which was obtained by this enzymatic digestion was

$$\begin{array}{ccccccccccc} & & & & 64 & & & & & & 68 \\ & & & & \text{Cys} & \text{-} & \text{Asn} & \text{-} & \text{Asp} & \text{-} & \text{Gly} \text{-} \text{Arg} \\ & & & & | & & & & & & \\ \text{Asn} & \text{-} & \text{Ile} & \text{-} & \text{Pro} & \text{-} & \text{Cys} & \text{-} & \text{Ser} & \text{-} & \text{Ala} \text{-} \text{Leu} \\ 77 & & & & & & 80 & & & & 83 \end{array}$$

Similarly, a subtilisin digest of the loop-peptide yielded in the diagonal technique (Figure 4B) mainly one pair of off-diagonal peptides (Table II), which implies that the fragment containing the disulfide bond in this case was

$$\begin{array}{ccccccc} & & & & 64 & & \\ & & & & \text{Cys} & \text{-} & \text{Asn} \\ & & & & | & & \\ \text{Asn} & \text{-} & \text{Ile} & \text{-} & \text{Pro} & \text{-} & \text{Cys} \text{-} \text{Ser} \\ 77 & & & & & & 80 \end{array}$$

It appears, therefore, that most of the loop-peptide molecules contain the disulfide bond in a position identical with that present in the native lysozyme molecule.

Binding of Loop-peptide to Antibodies. The loop-peptide, prepared according to the above procedure, using radioactive iodoacetic acid, was capable of binding to anti-lysozyme antibodies and to anti-loop antibodies, as shown in Figure 5. Within the antibody concentration range described in Figure 5, up to 30% of the peptide added could bind to both antibody preparations, whereas none of it bound to normal rabbit IgG. Oxidation of the disulfide bridge of the loop-peptide resulted in a drastic decrease in its capacity to bind both to the anti-lysozyme and to the anti-loop antibodies. With the same amount of antibodies, labeled lysozyme exhibited a marked difference in its binding of the two types of antibodies. Whereas in experiments with anti-lysozyme antibodies up to 90% of the labeled protein was bound, under the same conditions only 25–30% of it could bind to the anti-loop antibodies.

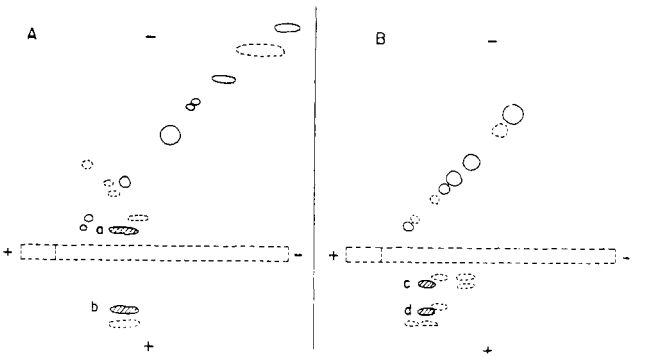

FIGURE 4: Schematic "diagonal map" of the disulfide bridges of the loop-peptide. A digest by chymotrypsin followed by trypsin (A), and a digest by subtilisin (B), of the loop-peptide were fractionated by electrophoresis at pH 3.5. A marker strip cut out along each paper was oxidized with performic acid vapor. The strips were then sewn on new sheets of paper and run again at pH 3.5 at right angle to the original direction. The peptides drawn in broken lines appeared in trace amounts only. The peptides a, b, c, and d were eluted from the main part of the electrophoresis sheets and analyzed for their amino acid contents (Table II).

When relatively higher antibody concentrations were used (experiments performed with lower amounts of labeled antigens) both lysozyme and the loop-peptide were bound to the two types of antibodies to an extent of 90%. On the other hand, the open-chain loop-peptide showed only limited capacity to bind to the antibodies even in this concentration range (Figure 6).

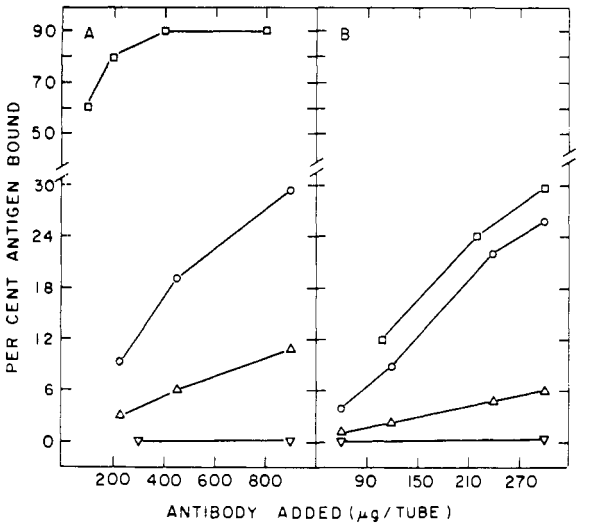

FIGURE 5: Antigen binding capacity by different antibody species. Binding of lysozyme (□); 1-CM-loop-peptide (○); performic acid oxidized loop-peptide (△), by purified rabbit anti-lysozyme antibodies (A), and by purified anti-loop antibodies obtained from rabbit anti-loop-A—L serum (B). Binding of these various substances by normal rabbit IgG (▽). Lysozyme, labeled by iodination with ^{125}I, was added in an amount of 10 μg/sample; the loop-peptide labeled by [^{14}C]carboxymethyl group and the performic acid oxidized peptide prepared from it, were added in an amount of 1.6 μg/sample.

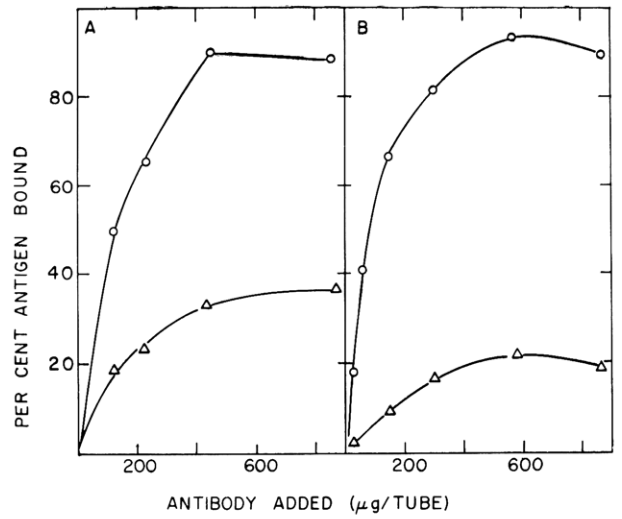

FIGURE 6: Antigen binding capacity by different antibody species. Binding of 1-CM-loop-peptide (○) and performic acid oxidized loop-peptide (△), by purified rabbit anti-lysozyme antibodies (A), and by purified anti-loop antibodies obtained from rabbit anti-loop-A—L serum (B). ^{14}C-Labeled peptides were added at the amount of 0.08 μg/sample.

Heterogeneity of Anti-loop Antibodies. Antibodies specific toward the loop-peptide, prepared both from goat anti-lysozyme serum by isolation on loop-peptide–immunoadsorbent and from goat anti-loop-A—L serum by isolation on lysozyme–immunoadsorbent, showed restricted heterogeneity as compared to the total antibody preparation isolated from the same antisera. The extent of heterogeneity was tested by isoelectric focusing of the intact antibody molecules, and by the acrylamide gel electrophoresis of the light chains prepared from these antibodies.

Figure 7 depicts the patterns obtained by gel electrofocusing. Whereas the purified anti-lysozyme antibodies (column b) yielded at least 24 bands, the fractionated loop-specific antibodies from the same sample of antiserum (column a) yielded only 9 bands, of which only two appear as major bands. In the amount applied on the gel these two major bands are of the same intensity as the corresponding bands in the total antibody preparation, whereas the other bands present in the total anti-lysozyme antibodies are either much less intense or completely missing in the loop-specific antibodies. The antibodies with the same specificity obtained from a serum of a goat immunized with the synthetic conjugate (column c) yielded a very similar pattern, showing approximately ten bands, in positions almost equivalent to the bands in column a.

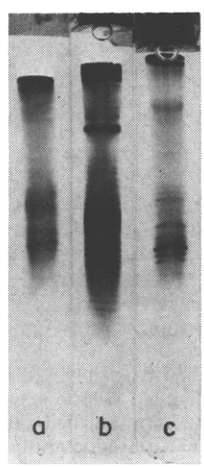

FIGURE 7: Isoelectric focusing of purified goat antibodies, at pH range 5.0–8.0. (a) Loop-specific antibodies purified from anti-lysozyme serum (30 μg), (b) purified anti-lysozyme antibodies (140 μg), (c) anti-loop antibodies obtained from anti-loop-A—L serum (40 μg).

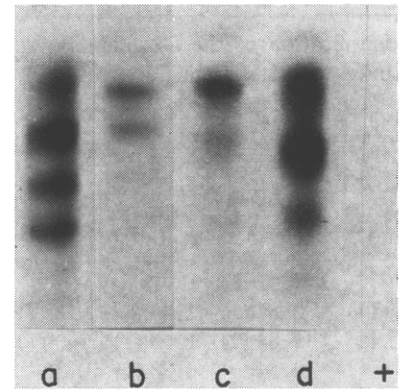

FIGURE 8: Disc electrophoretic patterns of light chains of (a) total purified anti-lysozyme antibodies. (b) Loop-specific antibodies fraction from anti-lysozyme serum. (c) Loop-specific antibodies from anti-loop-A—L serum. (d) Total IgG fraction from anti-loop-A—L serum. In each sample 0.1 ml of a 3-mg/ml solution was applied on the gel.

In acrylamide electrophoresis of light chains (Figure 8), a similar phenomenon was observed—the two types of anti-loop antibodies showed fewer bands than the total antibody preparation. The light chains obtained from anti-lysozyme antibodies separated into at least six bands, as compared to the two bands obtained in the case of anti-loop antibodies prepared from the same serum sample. Similarly, antibodies purified from anti-loop-A—L serum also yielded only two bands as compared to at least five bands derived from the IgG fraction of the same serum sample.

Specificity of Isolated and Fractionated Antibodies. The specificity of the different antibody preparations was established by the use of the sensitive technique of inactivation of the chemically modified bacteriophage. For this purpose, two different modified bacteriophage conjugates were employed, consisting of either the intact lysozyme molecule or the loop-peptide attached to bacteriophage T4. Figure 9 illustrates the capacities of purified anti-lysozyme antibodies, and of the fractions originating from it, to inactivate both bacteriophage preparations. It is thus shown that lysozyme–T4 conjugate is inactivated by the fractionated loop-specific antibodies to the same extent as by the total anti-lysozyme antibodies (Figure 9A). The extent of inactivation brought about by the antibodies remaining in solution following exhaustive adsorption with loop-peptide immunoadsorbent, was only slightly lower. In contradistinction, the loop–bacteriophage conjugate was differently affected by each of the three antibody fractions derived from the anti-lysozyme serum (Figure 9B). Whereas the loop-specific antibodies served as a very efficient inactivating agent, the total anti-lysozyme antibodies exerted a less pronounced effect. The fraction of antibodies, which had not been adsorbed on the loop-peptide immunoadsorbent, was not inhibitory at all, implying that it contains antibodies to other parts of the lysozyme molecule but not to the loop region.

TABLE III: Inhibition of the Inactivation of Modified Bacteriophages by Specific Antibodies.[a]

Modified Bacteriophage: Antibody Preparation:	Lysozyme-T4		Loop-T4	
	Anti-lysozyme[b] (1.9×10^{-5} mg/Sample)	Anti-loop[c] (4.3×10^{-3} mg/Sample)	Anti-lysozyme[b] (2.9×10^{-4} mg/Sample)	Anti-loop[c] (1.3×10^{-3} mg/Sample)
Lysozyme	1.6×10^{-6}	1.7×10^{-4}	1.8×10^{-5}	7.4×10^{-4}
1-CM-loop	*d*	1.8×10^{-2}	4.3×10^{-6}	8.6×10^{-5}
R-CM-peptide	*d*	0.9	6.1×10^{-5}	2.0×10^{-4}

[a] The number in the table denotes the concentration of the inhibitor (milligram per sample) required for 50% inhibition of the inactivation of the modified bacteriophage preparation. Details of inactivation procedure are described in the text. [b] Purified total goat anti-lysozyme antibodies. [c] Anti-loop-peptide antibodies prepared from goat antiserum against the synthetic conjugate loop-A—L. [d] No inhibition was observed with an inhibitor concentration as high as 1 mg/sample.

In Figure 10 anti-lysozyme antibodies are compared to the loop-specific antibodies derived from antiserum against the synthetic loop-A—L conjugate. In this experiment, as in the previous one, it is demonstrated that anti-lysozyme antibodies inactivate more efficiently the lysozyme–bacteriophage conjugate than the loop-bacteriophage conjugate (Figure 10A). Actually, the amount of antibodies causing 50% inactivation of the loop-bacteriophage is around 10 times higher than that required for 50% inactivation of the lysozyme–T4 preparation. This is in agreement with the finding that approximately 10% of the anti-lysozyme antibodies are specific toward the loop region.

On the other hand, in the inactivation by the loop-specific antibodies, the loop-modified phage is affected to a higher extent than the lysozyme–bacteriophage conjugate (Figure 10B), indicating that the sepcificity of this antibody preparation is indeed directed toward the loop region exclusively.

The specificity of these two types of antibodies, namely, anti-lysozyme and anti-loop, was further investigated by experiments of inhibition of the bacteriophage inactivation, as shown in Table III. Lysozyme was an efficient inhibitor in the inactivation of both lysozyme–phage and loop–phage with either anti-lysozyme or anti-loop antibodies. The loop-peptide, on the other hand, was a good inhibitor in all loop-specific reactions, but not in the inactivation of lysozyme–phage by anti-lysozyme antibodies.

The open-chain peptide obtained by reduction and carboxymethylation of the loop (RCM peptide) was a much weaker inhibitor. The decrease in the inhibitory capacity resulting from the unfolding of the chain was more pronounced in the inactivation by anti-lysozyme antibodies, where a 15-fold difference was observed in comparison to the effect of the intact loop, than in the inactivation of the loop–phage, where only a threefold decrease in inhibitory capacity was observed.

Discussion

The first part of this study describes the modified procedure used in the preparation of the loop-peptide from lysozyme. As was mentioned in our previous communication (Arnon and Sela, 1969), large amounts of aggregates were formed during the preparation of the loop-peptide, mainly during the stage of reoxidation of the intrachain disulfide bond.

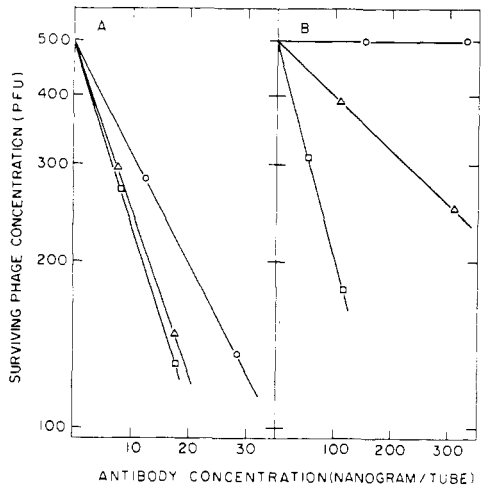

FIGURE 9: Inactivation of modified bacteriophage preparations by different purified goat antibody species. Inactivation of lysozyme-bacteriophage T4 conjugate (A) and loop-peptide-bacteriophage T4 conjugate (B) by total purified anti-lysozyme antibodies (△), by loop-specific antibodies selected from anti-lysozyme serum by loop immunoadsorbent (□), and by the fraction of antibodies remaining in the solution following adsorption with the loop immunoadsorbent (○).

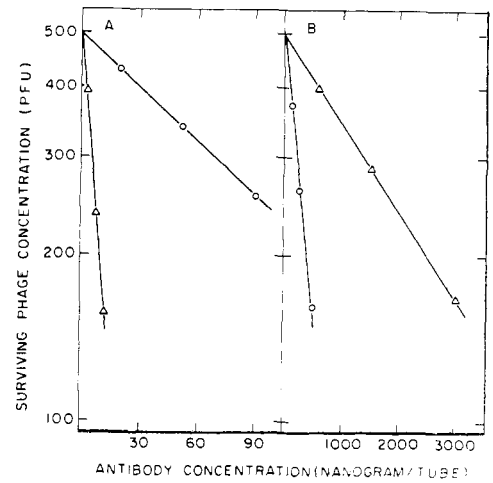

FIGURE 10: Inactivation of modified bacteriophage preparations by antibodies of different specificities. Inactivation of lysozyme-bacteriophage T4 conjugate (△), and loop-peptide bacteriophage T4 conjugate (○), by purified goat anti-lysozyme antibodies (A) and by purified goat anti-loop antibodies obtained from anti-loop-A—L serum (B).

This phenomenon could either stem from the nonhomogeneity of peptide 1 preparation from which the loop-peptide was prepared, or result from the formation of interchain disulfide bonds leading to the aggregation. In order to overcome these difficulties, peptide 1 was first fractionated (Figure 1), and only the fraction which led to the formation of the loop-peptide in a high yield was further employed. Furthermore, immediately upon release from the column, the free sulfhydryl group (presumably Cys-76) was blocked by carboxymethylation to prevent aggregation. An interesting observation was the possible role played by trace elements such as Cu in the formation of aggregates. Presence of a large amount of metal resulted in a high extent of aggregation. Similarly, complete removal of metal by EDTA also caused aggregation. The optimal amount of copper salt in the mixture appears to be 5×10^{-5} M. The mechanism by which the copper affects the loop formation is not understood.

The detailed characterization of the loop-peptide was achieved by analysis of the terminal residues (Table I) and by the identification of the disulfide bond it contains (Table II). The determination of terminal residues indicated that the loop-peptide consists mainly of the amino acid sequence 60–83 of lysozyme, and not 64–83 as stated in our previous report. Thus the presence of tryptophan and higher contents of arginine are inherent in the loop-peptide and not due to contamination with larger fragments. The molecular weight found for the loop-peptide by ultracentrifugal analysis (2750) was in very good agreement with the calculated value for the above sequence (2713). The molecular weight calculated from the absorbancy at 280 nm, assuming the presence of two tryptophan residues is 2690, in agreement with these two values.

The last point in the characterization of the loop-peptide was the establishment that the position of the disulfide bond is identical with the bond present in the intact lysozyme. About 70–80% of the re-formed loop-peptide contained this particular disulfide bond. Thus, even though the peptide which led to the loop upon reoxidation contained three half-cystine residues, one of the three possible disulfide bridges was preferentially formed, namely, the same disulfide bridge which was present in the native lysozyme.

The purified loop-peptide is bound by the specific antibodies (Figures 5 and 6). However, to achieve complete binding of the loop, the amounts of antibodies required are higher than the amount that binds completely an equal molar amount of intact lysozyme. This may imply that even though the loop-peptide retains the structure it originally occupies in the lysozyme molecule, it may still have in its isolated state lower affinity toward the antibodies. Alternatively it is possible that in the intact lysozyme molecule the loop comprises the main part but not a complete antigenic determinant.

Antibodies specific to the loop-peptide, whether originating from anti-lysozyme serum or derived from anti-loop-A—L serum, were less heterogeneous than the total anti-lysozyme antibody population. This was manifested both in acrylamide electrophoresis of their respective light chains (Figure 8), and in the isoelectric focusing of the intact antibodies (Figure 7). It should be noted that the isoelectric focusing technique is a more sensitive method, leading to a much more discriminating resolution of different species of antibodies.

The specificity of the various antibody preparations was established by experiments on inactivation of modified bacteriophages. The advantage of this technique, as applied in the present study, was the possible attachment of intact lysozyme, on the one hand, and the loop-peptide, on the other hand, to phage preparations. In this manner the relative reactivity of the various antibody species with the loop-peptide as opposed to other antigenic determinants of lysozyme could be compared. It was thus shown that in the reaction with the lysozyme molecule, antibodies with specificity directed toward either the loop-peptide or other regions of lysozyme exhibited exactly the same efficiency, whereas in the reaction with the loop peptide, only loop-specific antibodies can participate and hence the reactivity of the total anti-lysozyme antibody preparation is a reflection of the level of anti-loop antibodies it contains (Figure 9).

Inhibition of the bacteriophage inactivation provides means for comparison of the extent of relatedness of various derivatives to the moiety which is attached to the bacteriophage conjugate. The homologous antigen in each system is expected to serve as the most efficient inhibitor, whereas in the reaction with the loop-peptide, only loop-specific its analogs will exert gradual inhibition with respect to their resemblance to the homologous antigen. In the inactivation of lysozyme–bacteriophage the intact lysozyme was indeed a much better inhibitor than the loop-peptide. However, in the reactions with the loop–phage, the loop-peptide was a more efficient inhibitor than lysozyme. In fact, to reach the inhibition level of the loop-peptide, comparable molar amounts of lysozyme have to be used, indicating that indeed the loop moiety is the only active component in lysozyme in the inhibition reaction (Table III).

Unfolding of the peptide chain of the loop-peptide, achieved by either complete reduction and alkylation or by performic acid oxidation, resulted in a drastic decrease in the reactivity with the antibodies as manifested both in the capacity to bind to the antibodies (Figures 5 and 6) and in the efficiency of inhibiting the bacteriophage inactivation (Table III). These findings are indicative of the role of conformation (Sela *et al.*, 1967) in the antigenic specificity of the loop-peptide.

The main point demonstrated in this paper is the possibility to fractionate selectively anti-protein antibodies and to obtain a fraction with specificity directed toward a unique region in the molecule. These antibodies are far less heterogeneous than the total anti-protein antibody population, and indeed interact specifically with that region exclusively and not with any other antigenic determinants of the protein molecule. Moreover, if the same region of the protein is attached to a different carrier, the resultant antigen elicits antibodies very similar, both in their homogeneity and in their antigenic specificity, to the selectively fractionated antibodies elicited by the protein. In both cases the spatial structure of the immunopotent region serves as a decisive factor in its antigenic properties. Thus, in the last case, we were able to "graft" a conformation-dependent antigenic determinant from a native protein onto a synthetic polymer, and to use the resulting conjugate for provoking antibodies reacting with the native molecule.

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Varying Luminescence Behavior of the Different Tryptophan Residues of Papain*

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ABSTRACT: The fluorescence of papain at 25° is dominated by a tryptophan residue with unusually high quantum yield and excited lifetime. This residue is largely shielded from the action of external perturbants or quenchers but can be oxidized by *N*-bromosuccinimide. The dominant tryptophan may be quenched by protonation of a histidine or carboxyl group in its vicinity, or by the Hg atom of mercuripapain. The residual tryptophans have quite different properties, including a lower average quantum yield and lifetime and a different spectral distribution of fluorescence.

In an earlier publication the luminescence of papain was examined as a function of conditions (Weinryb and Steiner, 1970). Preparations of unactivated commercial crystalline papain were used in this study. (The term "unactivated papain" refers to the enzymically inert material lacking a free sulfhydryl group; treatment with a sulfhydryl reagent produces the activated form which contains a sulfhydryl group and possesses enzymic activity.) The principal conclusions may be briefly summarized as follows.

It was found that, at 25° in aqueous solution, the fluorescence emission spectrum was essentially characteristic of tryptophan, with little or no indication of any significant contribution from the tyrosine residues of papain. The implication was that the tyrosine emission is largely or entirely abolished either by quenching or as a consequence of radiationless energy transfer to tryptophan. Under conditions where the organized three-dimensional structure of papain is eliminated, as in 6 M guanidine hydrochloride, a tyrosine emission band appears.

The fluorescence of unactivated papain was partially quenched at pH values acid to 8, the midpoint of the quenching process being about pH 6.6 in agreement with Barel and Glazer (1969). In the pH range 3–5, where the fluorescence intensity was constant, direct evidence for energy transfer from tyrosine to tryptophan was obtained, the quantum yield based upon tryptophan absorption increasing for excitation wavelengths within the absorption band of tyrosine.

Recently, the picture has been complicated by the finding of Sluyterman and Wijdenes (1970) that crystalline papain may be resolved into two components, one of which (PI) cannot

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