

MAPPING THE ANTIGENIC EPITOPE FOR A MONOCLONAL ANTIBODY AGAINST LYSOZYME¹

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A monoclonal antibody (HyHEL-5), prepared to chicken lysozyme c by the method of Köhler and Milstein, identified an antigenic site (epitope) that was shared by the lysozymes of seven different species of galliform birds. The lysozymes of two galliform species, bobwhite quail and chachalaca, shared only partial antigenic identity with the epitope defined by this antibody. Duck lysozyme did not react with the antibody at all. Amino acids that determined the epitope structure were tentatively identified by comparing the amino acid sequences of these lysozymes and assuming the antigenic changes produced by evolutionary substitutions are not due to long-range conformational changes. Arg 68 was identified as a determining amino acid. Arg 68 is hydrogen-bonded to Arg 45, and together these two amino acids form a basic cluster that may be a subsite of the epitope. The antibody inhibited lysis of *Micrococcus lysodeikticus* by chicken lysozyme. Additionally, Biebrich Scarlet, a dye that binds to the catalytic site, inhibited antibody binding to this lysozyme, which indicates that the epitope extends into the cleft region between Arg 45 and Arg 114. The epitope was hypothesized to involve a region measuring at least 13 x 6 x 15 Å including the Arg 68-Arg 45 complex that borders the enzymatic catalytic site. Four other monoclonal antibodies to lysozyme have been partially characterized; each had a distinct pattern of binding specificity for various species of bird lysozymes.

Antigenic specificity derives from an interaction between an antigenic determinant and a complementary combining site on an antibody. To understand the molecular basis of antigen-antibody interactions, it is necessary to define the molecular basis of both the antibody-combining site and the antigen. The antigen-binding sites of antibodies with specificity for a variety of non-protein antigens, in particular carbohydrates, have been studied extensively (1-3). Furthermore, studies on antibodies to a variety of haptens indicate that families of heavy and light chains arising from clusters of closely related genes are associated with particular binding specificities (see Reference 4). However, little is known about the binding specificities of antibodies for protein molecules or the genetic basis of anti-

protein specificity, although proteins are probably the most abundant source of antigenic configurations. It is not known, for example, how much of the surface of an antibody molecule or of the protein antigen is involved in binding to an antigenic site (epitope) of a protein. Further, globular proteins, in contrast to small haptens, potentially have multiple epitopes that may be recognized by antibodies, and the total immune response to even a small protein may involve multiple specificities. A study of the molecular basis of antibody recognition of a protein must therefore be approached in terms of specific epitopes. Hypothetically the interaction of antibody and a protein epitope requires compatibility of contours as well as the apposition of reactive groups. The definition of a protein epitope could therefore lead to a better understanding of the mechanisms underlying antibody diversity.

Definition of the individual epitopes on a protein antigen and of the molecular basis of antibody interaction with a protein epitope requires one or more monoclonal antibodies that can be sequenced and structurally studied. Additionally, a panel of closely related proteins whose sequences and three-dimensional structures are known is also necessary. The bird lysozymes c (E.C.3.2.1.17) are ideal for such studies; they are relatively small proteins (m.w. 14,300), many of whose primary and three-dimensional structures are well characterized (5-10). The primary structures of lysozymes c from 10 species have been determined (see Reference 10). Further, crystallographic studies of lysozymes c from such divergent species as chicken, turkey, human, and bacteriophage have shown that they all have a similar backbone structure and many of the amino acid side chains are oriented similarly (11-15). In addition, the structural and functional basis of the catalytic activity of chicken lysozyme has been well characterized (16-19). The bird lysozymes are excellent antigens, and the antigenic structure of chicken lysozyme has been studied extensively with polyclonal antibodies (20, 21). A comparative antigenic study of avian lysozymes indicates that there are cross-reactivities as well as distinctive specificities (10, 22, 23). This extensive structural, functional, and immunologic background makes bird lysozymes particularly suitable for monoclonal analysis.

We describe here an approach to mapping the epitopes defined by mouse monoclonal antibodies to chicken lysozyme. In this study the monoclonal antibodies were reacted with 10 bird lysozymes whose primary structures have been rather well characterized. Amino acids were identified that probably determined the reactivity of each monoclonal antibody for lysozyme. These amino acids were then located on a space-filled model of lysozyme constructed by computer graphics from the x-ray structure. The epitope of one monoclonal antibody was partially mapped by this method.

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MATERIALS AND METHODS

Immunizations. Female BALB/c mice 6 to 8 wk of age were immunized by a modification of the method of Tung and Nisonoff (24). On days 0, 14, and 21, 500 μ g of chicken lysozyme, mixed in complete Freund's adjuvant, was injected i.p. On day 28 another 500 μ g of lysozyme in incomplete Freund's adjuvant was given. After a rest of 6 to 8 wk, mice were given a boost of 10 μ g of purified lysozyme in saline in the retroorbital sinus. Three days after the booster injection the splenic lymphocytes were isolated for fusion. On day 27 all mice were bled or tapped for ascites fluid; all samples showed positive anti-lysozyme activity when tested for direct binding in the enzyme-labeled immunosorbent assay (ELISA)³ below.

Hybridomas. The hybridomas were obtained by fusing spleen cells of immunized BALB/c mice to azaguanine-resistant SP2/0 cells, obtained from Dr. G. Köhler of the Basel Institute, with a protocol modified slightly from that described by Köhler and Milstein (25, 26). After 10 to 15 days the supernatants of all wells containing clones were tested for anti-lysozyme secretion by using the ELISA described below. All positive clones were then recloned at least twice by using 2×10^6 /ml thymocyte feeder cells.

Hybridoma cells were grown in ascites form by injecting pristane-primed CD2F1 mice i.p. with 1 to 5×10^5 cells. The hybridoma cells are highly malignant and generally kill mice without producing much harvestable antibody. However, if the mice have been injected with pristane (0.5 ml/mouse) 4 to 6 wk before inoculation with hybridoma cells, a large volume of ascites tissue containing the monoclonal antibody can usually be obtained (27, 28). The ascites fluid is harvested by tapping the peritoneum with a 19-gauge syringe needle, and the cells and debris are removed by centrifugation at $3000 \times G$ for 20 min. The supernatant, which generally contains 1 to 10 mg of antibody per milliliter, is frozen until use for assays or for affinity purification of the antibody.

Antibodies. Ascites and tissue culture supernatants containing monoclonal antibodies for each clone were pooled and stored frozen until used for assay or for affinity purification. Aliquots from pooled samples were frozen for later use in assays; repeated freezing and thawing was then avoided. Monoclonal antibody 5 was affinity-purified on a column of chicken lysozyme coupled to AH-Sepharose 4B. Ascites fluid mixed in equal volumes with borate-buffered saline, pH 8.4, was applied to the column, the column was washed, and then was eluted with 0.2 M glycine-HCl, pH 2.0, directly into a neutralizing buffer of 2 M Tris 0.15 M NaCl, pH 7.6. The antibody was then dialyzed into 0.15 M Tris, 0.15 M NaCl, pH 8.0, concentrated by ultrafiltration, and stored frozen until used. For the experiments to test for inhibition of catalysis, buffer exchange was performed on a Sephadex G-25 column to suspend the antibody in the assay buffer.

Most of the ELISA were performed with tissue culture supernatant fluid, although inhibition curves could be replicated equally well with appropriate dilutions of culture supernatant, ascites fluid, or affinity-purified antibody. All antibody dilutions were done in 1% bovine serum albumin (BSA) in phosphate-buffered saline, pH 7.4, (PBS) with sodium azide added to a final concentration of 0.08%.

Lysozyme purification. The lysozymes of bobwhite, Japanese, California, and Gambel's quails, and the ring-necked pheasant were those prepared in Berkeley as described previously (10, 22, 29). Small White Turkey eggs were obtained from the Avian Research Unit of the Beltsville Agricultural Station, Beltsville, MD; Peking duck eggs were obtained from Truslow Farms, Chestertown, MD. The whites were separated from the yolks and frozen until used. Chachalaca and guinea fowl egg white were from the Berkeley collection (30). Turkey, duck, guinea fowl, and chachalaca lysozymes were purified in Bethesda from thawed egg white by a protocol modified slightly from Prager and Wilson (29): 0.5 M ammonium acetate buffer, pH 9.4, was used instead of the ammonium carbonate buffer in the ion exchange steps; gel filtration was performed on a BioGel P-10 column (2.5 $\times$ 60 cm); all lysozyme fractions were dialyzed overnight against distilled water before lyophilization. Two duck lysozymes were separated from pooled egg whites by using a linear gradient of 0.05 M and 0.5 M ammonium acetate buffer on a C-25 CM-Sephadex column. They probably correspond to lysozymes A and B of Prager and Wilson (31); no fraction corresponding to lysozyme C was recovered. Lysozymes A and B are both closely related to duck lysozyme II of Hermann *et al.* (32); duck A is identical to duck II electrophoretically and in amino acid composition and differs only slightly in the microcomplement fixation test (31). The final products of the purifications carried out in Bethesda were free of high m.w. contaminants by the criterion of electrophoresis in sodium dodecyl sulfate (SDS) gels at pH 8.3, and gave high specific activity when assayed for the ability to lyse *Micrococcus lysodeikticus* cells. Chicken lysozyme (Worthington, Freehold, NJ), 3 $\times$ recrystallized, was further purified by the same procedure except that the initial step with Cellex-CM was omitted. Repurified chicken lysozyme was used in all procedures unless noted otherwise.

Enzyme-labeled immunosorbent assay (ELISA). For rapid screening of culture supernatants, a plate-binding ELISA was developed. Flexible V-bottomed microtiter plates (Dynatech) were coated overnight at 4°C with

50 μ l lysozyme (50 μ g/ml) in PBS, washed with 1% BSA in PBS, and then incubated with the same solution for 60 min at room temperature. Fifty-microliter aliquots of tissue culture supernatant solution were added to the wells and incubated 60 min at 37°C. The plates were washed, and 50 μ l of a mixture of peroxidase-coupled rabbit anti-mouse IgG (heavy and light chains) and goat anti-mouse IgM (μ -chain-specific) (Chapel Laboratories, Downingtown, PA) were added and incubated for 1 hr at 37°C. After a 30-min wash with five changes of 1% BSA, 100 μ l of substrate (o-phenylenediamine, 100 μ g/ml, and 0.003% H_2O_2) were added and the plates were incubated 15 min at 37°C; positive wells could be rapidly screened visually due to the brown product. Supernatant from the parent SP2/0 culture was used as a negative control.

For competitive inhibition assays, plates were coated with chicken lysozyme (1 μ g/ml) or bobwhite quail lysozyme (25 μ g/ml). Twenty-five microliters of competitor lysozyme or of Biebrich Scarlet (Sigma Chemical, St. Louis, MO) and 25 μ l of antibody solutions were incubated overnight at 37°C, and the plates were processed as above; for competitive inhibition with the mono- and disaccharides, *N*-acetylglucosamine and chitobiose (Sigma), the plate was preincubated with 25 μ l of saccharide solution for 15 min in the cold, then 25 μ l supernatant were added and incubated for 1 hr at 37°C. Dilutions of competitors were made in 0.1% albumin, and 100% binding was determined when 0.1% albumin alone was used. Background nonspecific binding was determined by coating the wells within albumin. The amount of peroxidase product in each well was quantitated spectrophotometrically, on a Dynatech MR580 MicroELISA Autoreader, at a wavelength of 450 nm with a reference wavelength of 630 nm.

Lysozyme assays. The ability of the hybridoma antibodies to inhibit lysozyme catalytic activity was tested by using a microassay that allowed rapid processing of multiple samples. First, 5 mg/ml of chicken lysozyme were dissolved in 0.1 M potassium phosphate buffer, pH 7.0. Equal volumes of enzyme solution and an appropriate dilution of antibody were incubated in a screw cap vial for 90 min in a 37°C water bath. This mixture was then diluted 10-fold with buffer, and 25 μ l of diluted enzyme was added to each well of 96-well V-bottomed microtiter plate. The reaction was started by adding 200 μ l *Micrococcus lysodeikticus* suspension (0.33 mg/ml buffer). The reaction in each well was immediately monitored spectrophotometrically (with a Dynatech MR580 MicroELISA Autoreader) at a wavelength of 450 nm; the first reading was designated zero time, and readings were made at 1- to 2-min intervals thereafter for 15 min. Activity was measured as a decrease in absorbance per unit time. Preliminary experiments established that activity was not inhibited by ascites fluid containing a galactan-binding hybridoma antibody, or by ascites fluid from which anti-lysozyme antibodies had been removed by affinity purification.

Space-filling molecular models. Species and sources of primary sequences are outlined in Table I. The coordinates of chicken lysozyme, obtained from the Division of Computer Research and Technology, National Institutes of Health, and from the Protein Data Bank (33), were those of Phillips (6) as refined by Diamond (9). Related lysozymes were assigned three-dimensional structures with chicken lysozyme used as a model on a computer display system as described previously (34). Briefly, differing side chains of the related lysozymes were substituted for the corresponding chicken lysozyme side chains on the chicken backbone, and were rotated by the computer to positions compatible with the Van der Waals radii of surrounding groups. After adjustment, no substituted residues made contacts in any of the derivative lysozymes constructed. The skeletal model was then converted by computer to a space-filling model representing each atom as a nondeformable sphere with a characteristic Van der Waals radius (35, 36). Photographs of the space-filling model were made from the television screen of an Evans and Sutherland picture system 2-vector display.

RESULTS

Four separate fusions from the hyperimmunized mice gave eight stable clones with specificity for chicken lysozyme, one of the IgM class, one of the IgG2 class, and six of IgG1 class. The antibody clones were characterized as sufficient antibody became available, with no prior selection for fine specificity. The abilities of the various lysozymes of known amino acid sequences to inhibit binding of five antibodies to chicken lysozyme are summarized in Table II. Each antibody had a unique pattern of fine specificity of binding. In addition, when undiluted ascites fluid was preincubated with an equal volume of a 5 mg/ml solution of chicken lysozyme, and the lysozyme was then tested for its ability to lyse *M. lysodeikticus* cells, antibodies 5, 8, 9, and 12 partially inhibited catalysis (Table II).

Antibody 5 was characterized in detail, in order to map the epitope on lysozyme recognized by this antibody. The abilities of various kinds of lysozyme to inhibit binding of antibody 5 to

³ Abbreviations used in this paper: ELISA, enzyme-labeled immunoabsorbent assay.

TABLE I
Species^a and primary amino acid sequences^b of lysozymes used in this study

	-1.....5.....1.....1.....2.....2.....3.....3.....4.....4.....5.....5.....6.....6	0.....5.....0.....5.....0.....5.....0.....5.....0.....5.....0.....5.....0.....5.....0.....5.....9
Chicken	KVFGRCELAAAMKRHGLDNYRGYS LGNWVCAAKFESNFNTQATNRRTDGDSTDYGIQLNSRWWCN	
California quail ^c	-----S-----V-----	
Bobwhite quail	-----S-----V-----	
Japanese quail	-Y-----K-Q-----	
Turkey	-Y-----L-----H-----	
Ring-necked pheasant	G-Y-----M-----G-----	
Chachalaca	-IYK-----Y-----RY-Y-----SN-----	
Duck A(II)	-YE-----L-----NY-S-----E-----	
Guinea fowl	-----S-----V-----	
Chicken	DGRTPGSRNLCNIPCSALLSSDITASVNC AKKIVSDGNGMNAWVAWRNRCKGTDVQAWIRGCR L	
California quail	-----T-----H-----	
Bobwhite quail	-K-----T-----	
Japanese quail	-----VH-----N-----	
Turkey	-----K-----A-G-----H-----	
Ring-necked pheasant	-----K-H-----KH-NV-----	
Chachalaca	-----TK-H-S-MGA-AP-R-R-----KH-ST-KD-K-----	
Duck A(II)	-K-K-A-G-V-R-EA-R-R-----R-SK-----	
Guinea fowl	-----Q-TA-----KH-RV-K-----	

^a Species names, and sources of primary sequences: chicken, *Gallus gallus* (5, 10, 23); California quail, *Lophortyx californicus* (10); Bobwhite quail, *Colinus virginianus* (60); Japanese quail, *Coturnix coturnix* (61); Turkey, *Meleagris galapavo* (62); Ring-necked pheasant, *Phasianus colchicus* (22); Chachalaca, *Ortalis vetula* (30); Duck A, a fraction of Peking Duck, *Anas platyrhynchos* (31) which probably corresponds in sequence to Duck II (32), given here; guinea fowl, *Numid meleagris* (30, 63).

^b The standard one letter code for amino acids is: A-Ala; B-Asx; C-Cys; D-Asp; E-Glu; F-Phe; G-Gly; H-His; I-Ileu; K-Lys; L-Leu; M-Met; N-Asn; P-Pro; Q-Gln; R-Arg; S-Ser; T-Thr; V-Val; W-Trp; Y-Tyr; Z-Glx. If no residue is given for a position it should be assumed that the lysozyme is identical to chicken lysozyme at the position.

^c Gambel's quail, *Lophortyx gambeli* was also used; this lysozyme is identical to California quail (10).

TABLE II
Comparative characterization of antilysozyme hybridomas

	No. of Amino Acid Differences from Chicken	Hybridoma Clone Number ^a				
		5	8	9	11	12
Relative binding of lysozyme: ^b						
Chicken	0	1	1	1	1	1
California quail	4	1	1	1	1	1.5
Bobwhite quail	4	100	1	1	1	1
Japanese quail	6	1	50	1	1	14
Turkey	7	1	1	1	2	1.5
Ring-necked pheasant	10	1	2.5	400	2	<1
Guinea fowl	10			>100		
Duck ^c	20 ^d	>1000	8	500	500	>200
Chachalaca	27	170	3	150	2	<1
% Inhibition of catalysis ^e		27	44	50	NI	22

^a All 5 monoclonal antibodies were IgG1.

^b Relative lysozyme concentrations required to give 50% inhibition of antibody binding to chicken lysozyme in the ELISA plate-binding assay.

^c A pooled preparation containing both Duck A and Duck B.

^d This value assumes homology with chicken lysozyme at positions Asn 65-Asp 66. A homology was originally reported (32) when chicken was listed as Asp 65-Asn 66. Recent studies (30) have retained the Asp 65-Asn 66 assignment for duck, giving 22 amino acid differences from chicken. The recent sequencing of the chicken lysozyme gene (64) confirmed the Asn 65-Asp 66 order for the chicken.

^e Percent inhibition by ascites fluid containing antibody of lysis of *M. Lyso-deikticus* cells by chicken lysozyme.

NI, no inhibition.

chicken lysozyme are summarized in Figure 1 and Tables II and III. Five of the lysozymes tested were equivalent to chicken lysozyme but three were not. Bobwhite quail and chachalaca lysozymes gave partial inhibition, and duck lysozyme gave no inhibition at the maximum concentration tested (4 mg/ml). Those lysozymes showing nonidentity to chicken lysozyme

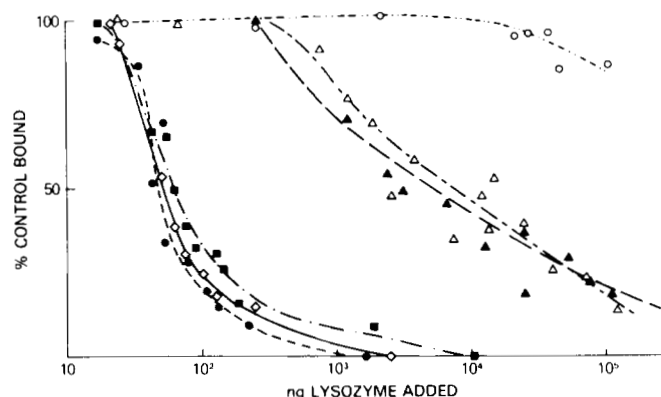


Figure 1. Competitive inhibition of the binding of antibody 5 binding to chicken lysozyme. The lysozymes used as inhibitors were chicken (◇—◇) turkey (●—●), California quail (■—■), Bobwhite quail (△—△), chachalaca (▲—▲), and duck (○—○). Gambel's quail, Japanese quail, and ring-necked pheasant gave curves similar to those of chicken, turkey, and California quail. Each point represents the means of at least 6 determinations curves were fitted by using a weighted log-logit transformation.

were tested for their ability to inhibit binding of antibody 5 to bobwhite quail lysozyme (Fig. 2, Table III). The two duck lysozymes were both equivalent to that of bobwhite quail, whereas chachalaca lysozyme inhibited binding slightly better than bobwhite quail lysozyme but still less efficiently than chicken lysozyme.

Affinity-purified antibody 5 inhibited catalysis in a concentration-dependent manner, the kinetics of which are shown in Figure 3; these results with purified antibody confirmed the results obtained with ascites fluid (Table II). Neither the monosaccharide *N*-acetylglucosamine nor the disaccharide, chito-

TABLE III

Lysozyme concentrations required to give 50% inhibition of antibody 5 binding to chicken or bobwhite quail lysozyme

Lysozyme Used as Inhibitor	Inhibitor Concentration (ng/25 λ)	
	Binding to chicken lysozyme	Binding to bobwhite lysozyme
Chicken	52	75
California quail	64	
Gambel's quail	75	
Turkey	49	
Japanese quail	70	
Ring-necked pheasant	58	
Bobwhite quail	6110	2545
Chachalaca	8974	988
Duck ^a	>10 ⁵	
Duck A		3340
Duck B		2113

^a Pooled preparation containing both duck A and duck B.

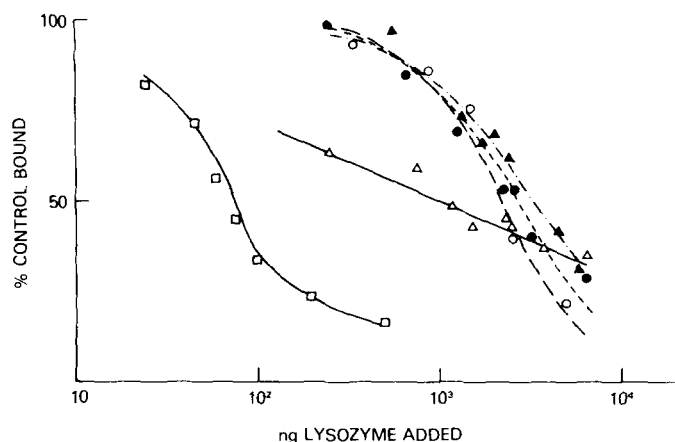


Figure 2. Competitive inhibition of the binding of antibody 5 to bobwhite quail lysozymes. The lysozymes used as inhibitors were chicken ($\square$ — $\square$), bobwhite quail ($\bullet$ — $\bullet$), chachalaca ($\triangle$ — $\triangle$), duck A ($\blacktriangle$ — $\blacktriangle$), and duck B ($\circ$ — $\circ$). Sample size and curve fitted as in Figure 1.

biose, inhibited antibody 5 binding to chicken lysozyme at final concentrations up to 5 mM (Fig. 4). Biebrich Scarlet, a dye that competitively binds the F position of the catalytic site (37), did inhibit antibody 5 binding to both chicken and pheasant lysozymes (Fig. 5), with 50% inhibition achieved at 0.47 mM and 0.22 mM, respectively. In contrast the dye failed to inhibit binding of the antibody to bobwhite quail lysozyme at the highest concentration tested, 5 mM, and in fact reproducibly enhanced binding at intermediate concentrations.

Mapping the Antigenic Epitope

The epitope recognized by antibody 5 can be localized by comparing the relative ability of avian lysozymes to inhibit the binding of this antibody to chicken lysozyme. The following assumptions are made: 1) the antigenic epitope is determined by external residues that are grouped in all the lysozymes studied; 2) the substitutions at positions 3, 15, 19, 21, 40, 41, 55, 73, 77, 91, 99, 101–103, 113, 114, 121, 122 in lysozymes showing antigenic identity which chicken lysozyme, namely the lysozymes of the Japanese, California, and Gambels quails, and of the turkey and pheasant (see Tables I and II), do not influence the binding site for antibody 5; 3) residues that differ in lysozymes with partial or no antigenic relation to chicken lysozyme are potential subsites in the epitope.

Arginine 68 as a determining amino acid for antibody 5. Amino acid comparisons of the antigenically nonidentical lysozymes of bobwhite quail and chachalaca (Table I) focus on those substitutions that are unique to these lysozymes. The bobwhite quail is particularly helpful because there are only

four substitutions with respect to chicken; three of these, Thr 40 $\rightarrow$ Ser, Ile 55 $\rightarrow$ Val, and Ser 91 $\rightarrow$ Thr are found in species (California and Gambel's quails) that react with identity to chicken in the ELISA test, and hence can be ruled out as determining the epitope. These results with bobwhite quail lysozyme definitively establish Arg 68 as a determining amino acid of the epitope recognized by antibody 5. In chicken lysozyme Arg 68 is hydrogen-bonded to Arg 45, and the two

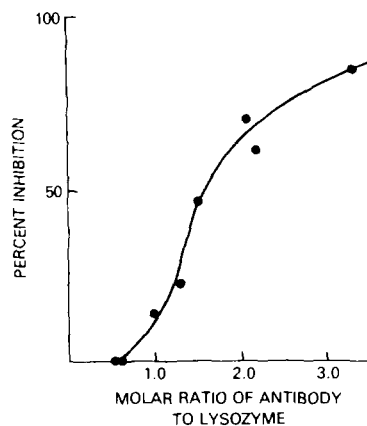


Figure 3. Inhibition of lysis of *M. lysodeikticus* by chicken lysozyme as a function of concentration of antibody 5.

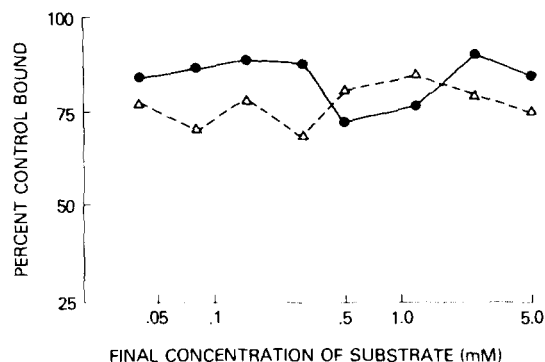


Figure 4. Binding of antibody 5 to chicken lysozyme in the presence of the saccharides *N*-acetylglucosamine ($\bullet$ — $\bullet$) and chitobiose ($\triangle$ — $\triangle$).

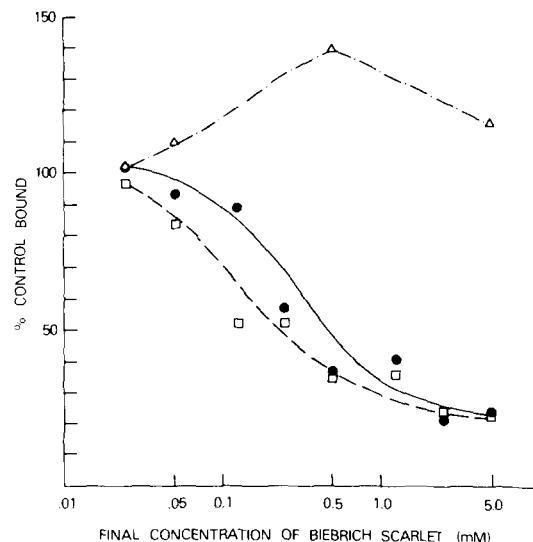


Figure 5. Biebrich Scarlet inhibition of antibody 5 binding to 3 lysozymes, chicken ($\bullet$ — $\bullet$), ring-necked pheasant ($\square$ — $\square$), and bobwhite quail ($\triangle$ — $\triangle$). Each point represents the mean of at least 4 determinations; curves for chicken and pheasant fitted as in Figure 1.

residues form a tight basic cluster (38, 39). The Arg 45-Arg 68 complex is therefore hypothesized as a subsite of the epitope. The lysine substitution in bobwhite quail lysozyme disrupts this association, thus partially destroying the epitope and reducing the binding ability of antibody 5 by two orders of magnitude (Fig. 1). Duck lysozymes also have the same substitution at 68, which can account in part for their failure to be bound by antibody 5.

Location of Arg 68 and Arg 45 on topographical models. A space-filling model of chicken lysozyme was used to locate external amino acids in the epitope defined by antibody 5. Chicken lysozyme is an oblong, globular protein approximately 42 Å x 31 Å, containing three regions of α -helix, and two parallel β -sheets (Fig. 6). The front viewing surface is arbitrarily oriented with Arg 68 and Arg 45 facing forward so the binding site cleft extends across the long axis and divides the globular protein into an upper sector (comprising about 30% of the mass) and a lower sector (Fig. 7A). The left and right views are seen when the front surface is rotated 90° to the left or right, respectively (Figs. 7B and 7C) and the top surface is seen when the molecule is rotated 90° forward (Fig. 7D). Residues 64–80, known as the 'loop' of lysozyme, are located on the top of the molecule, whereas both the amino and carboxyl termini are found in the lower sector (Fig. 7B).

Relationship of the epitope to the catalytic site of lysozyme. The inhibition of catalytic activity by antibody 5 suggests that the epitope is closely associated with the catalytic site. The catalytic site is in the form of a cleft extending from Asp 101 on the right side of the molecule to Arg 114 on the front face. The catalytic site can be occupied by six sugar residues, and the positions occupied by each residue have been designed by a letter, in order from A on the Asp 101 end to F on the Arg 114 end (16) (Fig. 8).

The mono- and disaccharides occupy positions C and B-C, respectively (16); the lack of competitive inhibition of antibody 5 binding to chicken lysozyme by either saccharide indicates that neither the B nor C catalytic sites are part of the epitope. This is consistent with the observation that Japanese quail lysozyme, with a Asn 103 → His substitution, binds antibody 5 with identity. Because sugar residues in the C position are hydrogen-bonded to Trp 62 and Trp 63 (16), we can conclude that neither of these residues are determining residues.

On the other hand the Biebrich Scarlet inhibition data (Fig. 5) implicate involvement of amino acids near the F catalytic site in the epitope. Biebrich Scarlet binds competitively in the F position in chicken lysozyme, displacing hexasaccharide bind-

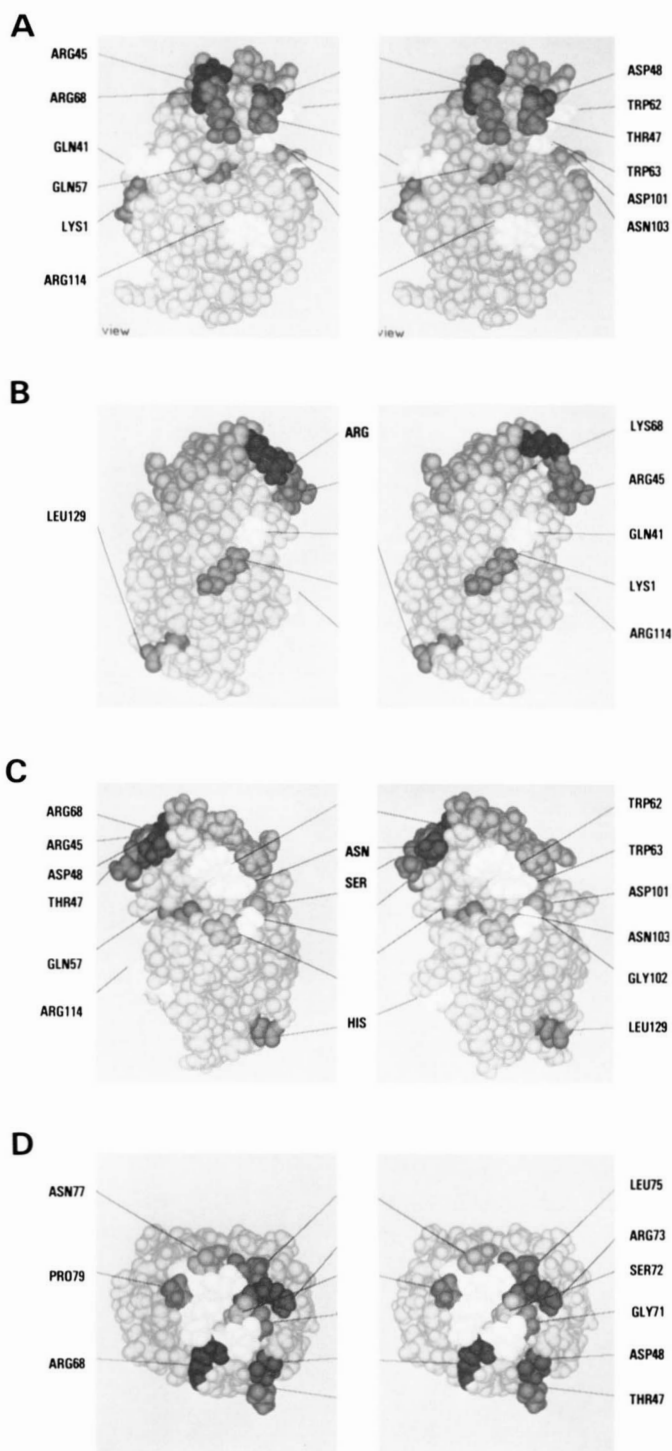


Figure 7. Stereographic pairs of spacefilling models of bird lysozymes showing position of Arg 68, Arg 45, and various landmark residues; in contrast to Figure 6, these models include the side chains. A, front view of chicken lysozyme with "loop" residues shaded slightly darker. B, left view, with chicken lysozyme in the left panel and bobwhite quail lysozyme in the right panel showing Arg 68 → Lys substitution (in black) and locations of amino (Lys 1) and carboxyl (Leu 129) termini; the 2 lysozymes may be compared by alternately winking the right and left eyes while viewing the 2 panels through a stereoscopic viewer. C, right view, with chicken lysozyme in left panel and chachalaca lysozyme in right panel, showing Thr 47 → Ser and Asp 48 → Asn substitutions. D, top view of chicken lysozyme with positions of evolutionarily variable amino acids in 'loop' region indicated; nonvariable "loop" residues shown in white. In addition to the substitutions summarized in Table I, the position of the Gly 71 → Arg substitution found in duck III (32) is also indicated; duck III was not used in the present study.

ing and thereby inhibiting catalytic activity (37). Biebrich Scarlet inhibited the binding of antibody 5 to both chicken and pheasant lysozyme but did not inhibit the binding of antibody

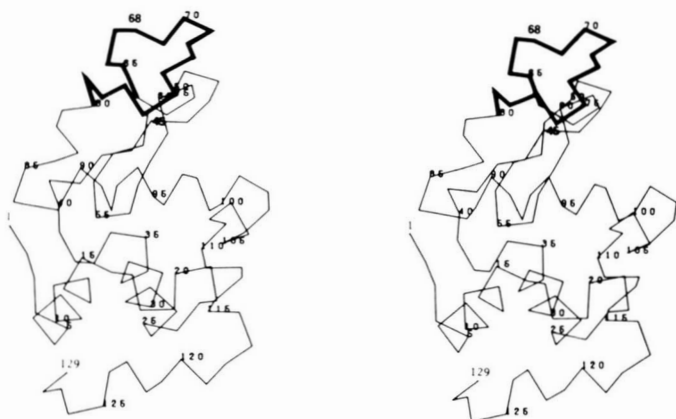


Figure 6. Stereographic pairs of Kendrew stick diagrams showing the carbon backbone of chicken lysozyme, with emphasis on the position of "loop" (residues 64 to 80) at top of the molecule.

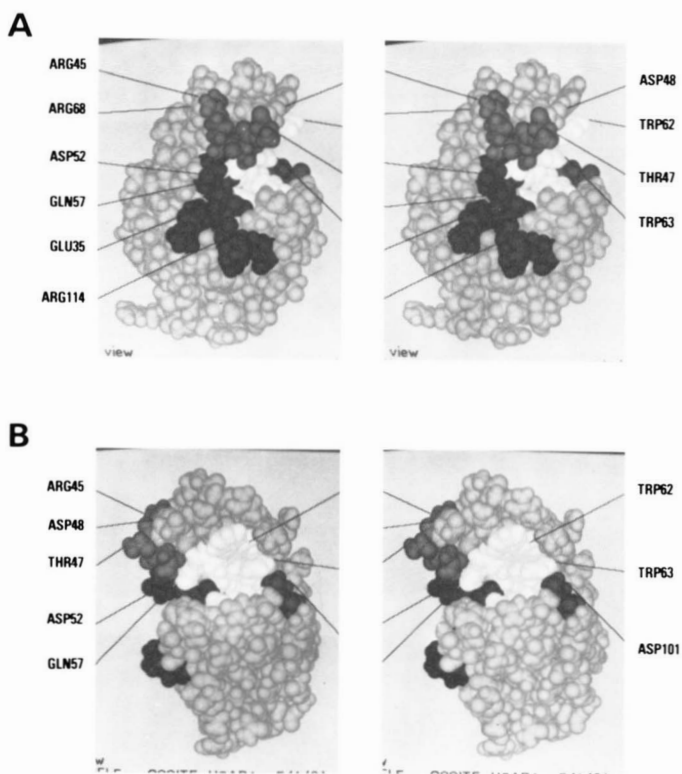


Figure 8. Stereographic pairs of front (panel A) and right (panel B) view of chicken lysozyme, showing cleft of the catalytic site which extends from Asp 101 on the right to Arg 114 on the front. The catalytic cleft can be occupied by 6 sugar residues, and the amino acids making contact with the sugar in each position have been designated by a letter in order from A to F (16): Positions A–B, black; position C–D, white; position E–F, black; position F', gray, an alternate mode of binding proposed from energy minimization studies (19). Marker residues which make contact with the sugar in each position are: A-101; B-101; C-62,63; D-57; E-57,35; F-114; F'-45.

5 to bobwhite quail lysozyme, which has a lysine at position 68 and therefore does not have the Arg 45–Arg 68 complex. Biebrich Scarlet, when bound, occupies the space between Arg 45 and Arg 114. Because the F catalytic site extends a considerable distance (i.e., 20 Å) from Arg 45 and includes the cleft space between Gln 57 and Arg 114, the ability of Biebrich Scarlet to inhibit binding of antibody 5 to chicken lysozyme suggests part of the epitope recognized by antibody 5 may extend downward from Arg 45 towards Arg 114. Experiments that will be published in detail elsewhere show that chitopenose and chitohexose competitively inhibit binding of the antibody to chicken lysozyme, implicating the E position of the catalytic site as well. The epitope does not appear to cross the cleft to include residue 114 (Fig. 7A) in the F region, however, because pheasant lysozyme, which has an Arg 114 → His substitution, binds antibody 5 with identity to chicken lysozyme.

Relationship of the epitope to the "loop". Because Arg 68 has been associated with immunogenicity of the region known as the "loop", residues 64–80 (23, 39, 40), the morphology of the "loop" will be described briefly. The "loop" amino acids can be found in the α -carbon backbone of lysozyme (in Fig. 6) that corresponds to the orientation in Figure 7A. The entirety of the loop is best seen from the top view where "loop" amino acids cover a large part of the top surface (Fig. 7D). When viewed from the top position, Arg 68 is located on the front, left part of the "loop," and the side chain projects toward the front surface. Between the "loop" and the binding site cleft (on the front and right) is a cluster of amino acids, consisting of residues 46–49 and 61, that form the top border of the catalytic site cleft.

Figure 7D depicts the position of amino acids 68, 72, 73, 75, 77, and 79 on the loop, which vary as detailed in Table I. Of these positions 73 and 77 may be eliminated because turkey and pheasant lysozymes bind antibody 5 with identity to chicken lysozyme. Therefore, at most the front half of the "loop" can be determining residues in the epitope, and the front portion of the "loop" may be considered as an outside limiting boundary for the epitope.

Possible limiting boundaries of the epitope recognized by antibody 5. When viewing chicken lysozyme from the front defined in Fig. 7A, and considering the Arg 45–68 complex as a central reference point, the potential boundaries of the epitope recognized by antibody 5 may be outlined. The boundaries are defined by positions that vary in lysozymes showing antigenic identity to chicken lysozyme. When a residue is highly accessible (41), it is assumed that it would affect binding if it were included within the epitope, and therefore the epitope boundary must lie between position 68 and that particular residue. The back boundary is defined by the front of the "loop"; it is not possible to specify whether the epitope actually includes other "loop" residues besides Arg 68, or whether the boundary lies in front of the "loop". The front and right boundaries are defined by the active site cleft; the right boundary lies between Arg 68 and residues 62, 63, 102, and 103 of the B + C positions; the front boundary is between Arg 68 and Arg 114. The left boundary is between Arg 68 and Gln 41, which varies in both turkey and pheasant lysozymes. These hypothesized boundaries define the maximum size of the epitope; residues outside these boundaries do not appear to influence antibody binding. It is not possible to establish the precise borders that delineate the actual residues in contact with the antibody.

Determining residues for antibodies 8, 9, and 12. Antibodies 8, 9, and 12 are all associated with the catalytic site. Binding of antibody 9 to lysozymes of pheasant, chachalaca, duck, and guinea fowl is significantly reduced. Of these lysozymes, pheasant, chachalaca, and guinea fowl uniquely share substitutions at 113 and 114, whereas duck has a substitution at nearby residue 116. Arg 114 is a postulated contact residue for the F position of the catalytic site (Fig. 8).

Antibodies 8 and 12 both have reduced binding to Japanese quail lysozyme compared to chicken lysozyme. Japanese quail differs from chicken at positions 3, 19, 21, 102, 103, and 121. Residue 121 may be eliminated as it is also substituted in California quail and turkey, which bind both antibodies, whereas residue 3 may be eliminated by the binding data with pheasant and turkey. Residues 102 and 103 are intimately associated with the C position of the catalytic site (Fig. 8); residues 19 and 21 are in near proximity on the same side of the molecule in the lower sector. Further experimentation will be necessary to determine which of these residues are determining residues for binding of antibodies 8 and 12.

The data do not allow specification of determining residues for antibody 11, which does not bind the catalytic site.

DISCUSSION

We have mapped the antigenic site recognized by a monoclonal antibody to chicken lysozyme (HyHEL-5), by comparing the reactivities of a panel of closely related, completely sequenced lysozymes with the antibody.⁴ In addition, we were

⁴ A similar approach has been utilized to map epitopes recognized by monoclonal antibodies to myoglobin by Berzofsky, J., G. K. Buckenmeyer, G. Hicks, F. R. N. Gurd, R. J. Feldmann, and J. Minna. 1981. Topographic antigenic determinants recognized by monoclonal antibodies to sperm whale myoglobin. *J. Biol. Chem.* In press.

able to capitalize on the extensive information that exists concerning substrate binding and catalysis by lysozyme. This combined approach has allowed us to specify potential boundaries of the epitope, which we have visualized on a computer-constructed, space-filling model.⁴

Arg 68 plays a critical role in binding of antibody 5 to chicken lysozyme, but this role may be either direct or indirect due to its hydrogen bonding to Arg 45. The importance of position 68 to antigenicity of lysozyme has been established (23, 39, 40), but in previous studies Arg 68 has been considered in association with anti-''loop'' responses (10, 40, 42-45). The conclusion that the epitope extends towards the E and F positions of the catalytic site rather than towards the front portion of the 'loop' is consistent with previous studies that established that anti-''loop'' antibodies do not inhibit catalysis (43, 44).

Three lines of evidence were interpreted as indicating that antibody 5 binds in close proximity to the E and F positions of the catalytic site. First, lysis of *M. lysodeikticus* was inhibited by the antibody. A direct correlation between efficiency of neutralization of enzymatic activity and specificity of antibodies for the active site has been demonstrated (43, 44, 46). Second, binding of the antibody to lysozyme was inhibited by Biebrich Scarlet, a dye that adsorbs to lysozyme in a 1:1 molar ratio in the F site (37), suggesting the antibody binds in close proximity to the F position of the catalytic site. Finally, the penta- and hexasaccharide, but not the mono- and disaccharide, substrates inhibited antibody binding, suggesting competition between the antibody and the substrates in the E and F positions of the catalytic site; inhibition of antibody activity by the disaccharide has been demonstrated previously to be specifically associated with ''active site-directed'' antibodies (47). An alternative interpretation is that the substrate effects are mediated by conformational changes that are induced when the sugars bind. However, structural studies have indicated significant rotations of the side chains of residues that directly interact with the mono-, di-, and trisaccharide substrates, most notably in Trp 62, but only very minor secondary changes (14, 16, 48). No data are available for the penta- and hexasaccharides, as the lysozyme crystal is not accessible to these sugars (14, 16).

An important assumption underlying our analyses is that the influences of substituted residues are local rather than long-range conformational effects by which substitutions occurring outside of an epitope indirectly influence residues that are part of the epitope. However, the data we have presented provide evidence that substituted residues do not exert long-range effects with respect to antibody binding.

First, of the other hybridomas analyzed, no influence of the Arg 68 → Lys substitution on antibody binding can be detected; antibodies 8, 9, 11, and 12 all bind bobwhite quail lysozyme with identity compared to chicken lysozyme. Hybridoma 9 is particularly interesting in this regard. Residues 113-114, which appear to be determining residues for the binding of antibody 9 to lysozyme, are located in close proximity to the hypothesized epitope for antibody 5. No effect of the Arg 68 → Lys substitution can be detected on binding of antibody 9, and, conversely, no effect of the Arg 114 → His substitution is detectable on binding of antibody 5. In fact, we were able to identify a total of 18 residues for which no influence on binding of antibody 5 to chicken lysozyme could be detected. Thus there is no evidence that interactions of Arg 68 with neighboring residues significantly influence antibody binding.

Second, all the antibodies bind turkey lysozyme with identity or near-identity compared to chicken lysozyme. Despite x-ray

analysis of turkey lysozyme, which revealed possible conformational differences in the protein backbone of residues 97-110 and 119-125 (14), even clones 8 and 12, which recognize residues close to and possibly even within the region of the conformational changes, bind turkey lysozyme. These results suggest minor conformational changes resulting from evolutionary substitutions do not exert long-range influences on antibody binding.

These lines of evidence support the hypothesis that when an external substitution significantly changes binding of a monoclonal antibody, it probably reflects a local involvement of that residue in antibody binding rather than a long-range conformational influence. Because the majority of evolutionary substitutions in a variety of proteins are antigenically detectable (49), this evidence supports the hypothesis that lysozyme contains multiple determinants (10). It is notable that each of the hybridomas we have characterized to date has a distinct pattern of binding specificity,⁵ a result consistent with a multi-determinant hypothesis.

Of the lysozymes used in this study, those of chachalaca and duck differ most from chicken in primary sequence, and we cannot rule out the possibility of conformational changes in these lysozymes. However, for the reasons outlined above, we hypothesize that the large changes in binding efficiency of these lysozymes for antibody 5 probably reflect substitutions in residues that are directly involved in antibody binding. Of the residues that are substituted in these two species, only 47-48 in chachalaca, and 68 and 57 in duck A, are within the hypothesized boundaries of the epitope.

We hypothesize that the epitope for antibody 5 includes Arg 45, Arg 68, Thr 47, and Asp 48; residues 68 and 45-49 form a wedge approximately 6 x 13 Å on the upper lip of the active site cleft. This region is part of an anti-parallel β -sheet, and has been implicated previously as an antigenic site that is associated with the catalytic site of lysozyme (46, 50). If the antibody combining site is considered a complementary cavity or groove (1, 2), the dimensions of this concavity would have to be at least 13 x 6 Å; if the edges of the antibody extend all the way into the E-F positions of the lysozyme cleft, the depth of the antibody cavity would be approximately 15 Å. These dimensions are within the range of dimensions reported both for carbohydrate and peptide haptens, as well as the combining site of several immunoglobulin proteins (2, 3).

Four of five of the antibodies we have described are associated with the catalytic site, a result that would not have been predicted from previous studies (46, 50; see Reference 53). It has been reported that active site-associated antibodies may have higher avidities for lysozyme than antibodies directed towards other regions of lysozyme (50). Our screening protocol probably selects for clones with high avidity, and thus may inadvertently select for a population associated with the catalytic site. Further characterization of the BALB/c anti-lysozyme repertoire will be necessary to clarify this point.

The determining residues recognized by antibodies 5, 8, and 12 do not correspond to any of the three antigenic sites of lysozyme defined by Atassi and Lee (21) with rabbit and goat antisera, although the residues recognized by antibody 9 may correspond to Atassi and Lee's antigenic site 3. Differences of species and methods of immunization may account for this. The epitope for antibody 5 may be related to a previously described antigenic region associated with residues 38-54

⁵ Similar results for lysozyme have been found by Metzger, D. W., L.-K. Ch'ng, A. Miller, and E. Sercarz. 1981. The lysozyme-specific repertoire: I. Specificity repertoire of anti-hen egg white lysozyme antibodies probed with hybridomas. Unpublished manuscript.

(46, 50). This latter region has been shown to be completely independent of the synthetic sites of Atassi and Lee (21, 50). The antigenic structure of lysozyme as recognized by the murine immune response apparently includes epitopes that are distinct from the three sites defined by Atassi and Lee (21), a conclusion that has been suggested previously in studies in which polyclonal antisera were used (10, 50, reviewed in References 2, 20).

Defining the protein epitope recognized by a monoclonal antibody is the first step in understanding the molecular basis of antibody interaction with a protein antigen. Utilization of a panel of closely related protein antigens has been used successfully to identify residues critical to antigenicity in lysozyme as well as in several other protein antigens^{4,5} (51-53), and also provides a powerful tool for the examination of antigenic recognition by T cells (54). Interaction of both T and B lymphocytes with antigens is mediated via receptor molecules, and to understand the total immune response we must understand the molecular basis of epitope recognition by both types of receptors. Most T lymphocytes have binding activity for carrier proteins. Although the recognition of protein epitopes by T cells differs from that of B cells in several important ways (54, 55), serologic and genetic studies suggest the T cell receptor may be at least in part structurally related to the immunoglobulin heavy chain V-region domains (55, 56). Thus, definition of antibody-epitope interactions for a well-defined protein such as lysozyme is essential to understanding the molecular basis of both types of receptor interactions.

The results of this study emphasize the importance of single amino acid substitutions on the antigenicity of a protein epitope for a monoclonal antibody, as has been observed previously in several protein-antibody systems (51-53). A complementary substitution in the complementarity-determining region of the antibody could similarly change the antibody specificity so that it could combine with an epitope region containing a substitution. While somatic mutations appear to add significantly to antibody diversity (57-59), studies to date fail to reveal a functional significance to this diversity in terms of antibody specificity or affinity (59). Avian lysozyme presents an excellent system in which to examine the potential significance of somatic mutations on antibody-binding specificity.

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