Local Effects of Amino Acid Substitutions on the Active Site Region of Lysozyme: A Comparison of Physical and Immunological Results†

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ABSTRACT: Differences in the binding of the substrate analogue chitotriose to lysozymes correlate with amino acid substitutions in the binding site and not with substitutions elsewhere. This is evident from binding studies done with an immunological method as well as a conventional spectroscopic method. The immunological technique, based on the microcomplement fixation assay, required thousands of times less lysozyme than did the conventional technique. For eight bird lysozymes of known amino acid sequence, the immunologically and physically measured association constants were in approximate agreement. Five of the eight lysozymes have about the same affinity for chitotriose and have identical amino acids at the sites of contact between substrate and enzyme. In contrast, the three lysozymes that have altered affinities have amino acid substitutions in the binding site. Some of the lysozymes with similar affinities for chitotriose differ greatly in amino acid sequence outside the binding site. This suggests that evolutionary substitutions do not generally have long-range effects on the active site region of lysozyme.

How local are the effects of the amino acid substitutions that accumulate during protein evolution? The possibility that the majority of such substitutions in monomeric, globular proteins produce long-range effects has arisen as the result of immunochemical studies. Antigenic comparisons of such proteins as lysozyme, ribonuclease, and azurin from closely related species suggest that at least 80% of the evolutionary substitutions are detectable antigenically (White et al., 1978). Yet the most prominent models of antigenic structure available until recently indicate that only a small fraction of the amino acid residues in such proteins, typically about 15%, are located within antigenic sites (Crumpton, 1974; Reichlin, 1975; Atassi, 1975, 1978).

The lysozymes of birds present a good example of this contrast. On the one hand, Atassi and co-workers have delineated three antigenic sites, which are claimed to bind all of the antibodies in a typical polyclonal antiserum to lysozyme (Atassi & Lee, 1978). In these three sites, there is a total of 16 amino acid residues, which comprise only 12% of the residues in lysozyme. On the other hand, workers in our laboratory routinely detect antigenic differences between bird lysozymes of known sequences that are identical with respect to these 16 antigenic residues (Ibrahimi et al., 1979). If the three-site model is correct, our results imply that the majority of evolutionary substitutions have long-range effects on the structure and binding properties of the three sites. If, however, lysozyme has more antigenic sites, as is implied by recent monoclonal studies (Smith-Gill et al., 1982; Benjamin et al., 1984), the immunological detectability of the majority of substitutions could be due chiefly to local effects.

The active site of lysozyme provides an independent opportunity to examine whether evolutionary substitutions generally have local effects. Our approach is to measure the binding of the substrate analogue chitotriose to a series of lysozymes of known amino acid sequence, some having substitutions in the binding site and others lacking substitutions in the site but possessing substitutions elsewhere in the lysozyme molecule. Besides using the conventional spectroscopic method, we employ a sensitive immunological method to measure the binding constant of a ligand. This method is based on von Fellenberg & Levine's (1967) observation that chitotriose interferes with the binding of a fraction of rabbit antibodies raised against chicken lysozyme.

Materials and Methods

Chitotriose (i.e., tri-*N*-acetylglucosamine) was produced by partial acid hydrolysis of chitin and linear gradient elution from water to 60% ethanol on a charcoal–Celite column, 6 cm × 70 cm (Rupley, 1964). The peaks from the charcoal–Celite column that were enriched for the trimer were further separated on a Bio-Gel P-2 column. The trimeric fraction was shown to be pure with descending paper chromatography in ethyl acetate–pyridine–H_2O (5:3:2).

Antigens. Chicken lysozyme *c* (Sigma grade 1, lot L-6875) was further purified according to methods previously described (Prager & Wilson, 1971a). Bobwhite quail, California quail, turkey, Japanese quail, ring-necked pheasant, duck II, and chachalaca lysozymes were supplied by E. M. Prager and I. M. Ibrahimi of this laboratory. Their purifications and characterizations are described elsewhere (Ibrahimi et al., 1979; Jollès et al., 1979). Embden goose egg white, a source of lysozyme *g* (Grütter et al., 1983), was obtained from E. M. Prager.

Antisera. Antisera to chicken lysozyme were produced in rabbits as described by Arnheim & Wilson (1967) and Prager & Wilson (1971a). Antiserum A24 was obtained from an individual rabbit 17 weeks after the initial immunization (Arnheim & Wilson, 1967). Pool 10 is a pool of four antisera taken after immunizing four other rabbits for 45 weeks (Prager & Wilson, 1971a). The quantitative precipitin test was used to measure the content of lysozyme-specific antibodies in these sera (Prager & Wilson, 1971b). V. M. Sarich of this laboratory provided rabbit antiserum to bovine serum albumin, and N. Arnheim provided rabbit antiserum to goose lysozyme *g*.

Immunological Titration. The microcomplement fixation technique, as described by Champion et al. (1974), was modified by the addition of chitotriose, a specific and partial inhibitor of the fixation of complement by lysozyme–antibody aggregates (von Fellenberg & Levine, 1967). The antisera used were chosen because they registered large decreases in

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percent complement fixed in the presence of saturating amounts of chitotriose. The usual order of addition to the duplicate reaction tubes was (1) 0.5 mL of lysozyme serial dilutions, (2) 1.5 mL of buffer with or without increasing amounts of chitotriose, (3) 0.5 mL of a complement dilution capable of lysing about 80% of the sensitized red blood cells in 30 min, and (4) 0.5 mL of that antiserum dilution which fixes approximately 75% of the complement at the peak of the curve when no saccharide was present. The tubes were incubated for 18 h at 4 °C before the addition of sensitized sheep red blood cells. All solutions were prepared in isotris buffer, which contains 0.14 M NaCl, 0.01 M Tris-HCl, 5×10^{-4} M $MgSO_4$, and 1.5×10^{-4} M $CaCl_2$, at a final pH of 7.7 (measured at 4 °C).

Ultraviolet Difference Spectra and the Association Constant (K). Association constants of lysozyme for chitotriose were determined by ultraviolet difference spectroscopy as previously described (Dahlquist et al., 1966). Reactions were in isotris, pH 7.7, at 6.5 °C, and spectra were recorded with a Cary 118 difference spectrophotometer. The molar concentrations of bound and unbound reactants in these titrations were determined with the assumptions that $[PL]^1 = (\Delta A/\Delta A_0)[P_0]$, $[P] = [P_0] - [PL]$, and $[L] = [L_0] - [PL]$, where P is unbound protein (i.e., free lysozyme), P_0 is total protein, PL is the protein–ligand complex, L is the free ligand, and L_0 is the total ligand. The association constant $K = [PL]/([P][L])$ was solved graphically as the intercept of the linear regression of log ([PL]/[P]) on log [L].

Determination of the Apparent Association Constant (K_{app}). The immunological data from titrations with an antigen-specific ligand (chitotriose) were analyzed kinetically with three equations: $[P] = [P_0] - [PL]$, $[L] = [L_0] - [PL]$, $[PL] = [P_0](Y_0 - Y_{\%})(Y_0 - Y_\infty)^{-1}$, where Y_0 is the percent complement fixed by the protein in the absence of ligand, $Y_{\%}$ is the percent complement fixed in the presence of ligand at fractional saturation, and Y_∞ is the percent complement fixed at ligand saturation. Since $P_0 \ll L_0$ throughout the range of the immunological experiments, $[L] = [L_0]$. The value of K_{app} was determined graphically by plotting log ([PL]/[P]) vs. log [L]. This association constant is described as apparent because of the presence in the assay of an additional reactant (a constant amount of antibody), which competes with the ligand for binding to the specific protein.

Measurement and Analysis of Interatomic Distances. The inter α-carbon distances from the site of each radical substitution (see Figure 3) to the chitotriose contact residues (Table III) were from lysozyme crystallographic coordinates (Moult et al., 1976) as obtained from computer interactive color graphics (Huang et al., 1983) at the Computer Graphics Laboratory, University of California, San Francisco, CA. Distances from each substitution site to the closest two contact residues were averaged.

Results

Immunological Titrations with Chicken Lysozyme. Chicken lysozyme was allowed to react with a constant amount of antiserum in the presence of increasing concentrations of chitotriose. The amount of complement fixed decreased as the saccharide concentration increased, as described previously (von Fellenberg & Levine, 1967). The inhibition of complement fixation increased asymptotically toward a plateau value as the ligand concentration increased, as if the ligand were displacing a class of antibodies that compete with it for binding. This is shown in Figure 1. In control experiments, neither goose lysozyme, which does not specifically bind chitotriose, nor albumin was inhibited by chitotriose when reacted with their homologous antisera.

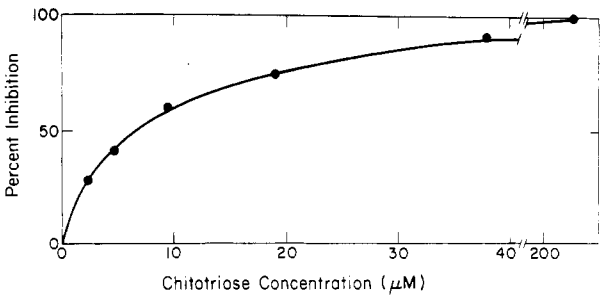

FIGURE 1: Chitotriose inhibition of complement fixation by chicken lysozyme and antiserum A24. Total antibody concentration was 4.2×10^{-10} M, lysozyme concentration was 3.6×10^{-10} M, and percent inhibition was $(Y_0 - Y_{\%})/(Y_0 - Y_\infty) \times 100$.

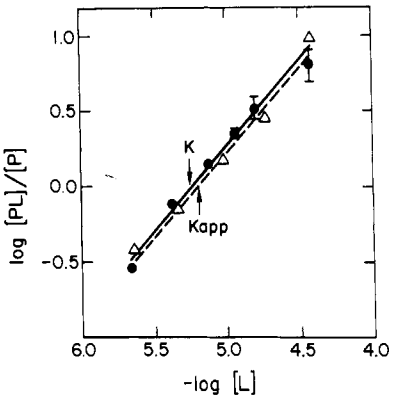

FIGURE 2: Association constant of chicken lysozyme for chitotriose determined by immunological and ultraviolet difference spectral methods. Both experiments were done in isotris buffer ($I = 0.14$ M, pH 7.7). The immunological results (Δ) were obtained with a lysozyme concentration of 3.6×10^{-10} M at 4 °C. To obtain the difference spectral results (●), the concentration of lysozyme used was 1.97×10^{-5} M at 6.5 °C.

Assuming that the percent inhibition is a measure of the degree of saturation of lysozyme with saccharide, one may determine the apparent association constant for the interaction of lysozyme with the saccharide. This was done by plotting log ([PL]/[P]) vs. log [L], as shown in Figure 2 (dashed line). The value obtained is 1.6×10^5 M^{-1}, and the slope of the line (1.1) is consistent with a protein to ligand binding ratio of approximately 1:1 over the concentration range examined.

Spectral Titrations with Chicken Lysozyme. The solid line in Figure 2 plots log ([PL]/[L]) vs. log [L] determined from spectrophotometric observations, and the intercept value gives an association constant of 1.8×10^5 M^{-1} for chicken egg white lysozyme and chitotriose at 6.5 °C and $I = 0.14$ M. This value is about 3 times higher than the association constants determined at temperatures between 20 and 25 °C (Dahlquist et al., 1966; Chipman & Sharon, 1969). Such an increase of the binding constant at lower temperatures is consistent with previous observations (Banerjee et al., 1975).

Lysozymes from Other Species. K and K_{app} were determined for seven additional avian lysozymes. This allowed us to compare immunologically derived binding constants with

[1] Abbreviations: P, unbound protein; P_0, total protein; PL, protein–ligand complex; L, free ligand; L_0, total ligand; Y_0, percent complement fixed in the absence of ligand; $Y_{\%}$, percent complement fixed in the presence of ligand when only a fraction of the protein exists in protein–ligand complexes; Y_∞, percent complement fixed in the presence of ligand where the protein is essentially all bound in protein–ligand complexes; K, association constant; K_{app}, apparent association constant obtained from immunological assay; I, inhibitor; K_i, association constant of inhibitor.

Table I: Comparison of Association Constants of Eight Purified Lysozymes for the Ligand Chitotriose Measured by Immunological and Spectroscopic Titrations[a]

lysozyme (ref)[b]	K_{app} ($\times 10^5$ M^{-1})[c]	K ($\times 10^5$ M^{-1})[d]
chicken (1)	1.6	1.8
bobwhite quail (2)	1.3	1.7
California quail (3)	1.1	1.7
turkey (4)	0.4	0.3
Japanese quail (5)	1.0	1.2
ring-necked pheasant (6)	2.1	2.0
duck II (7)[e]	0.3	0.3
chachalaca (8)	1.7	2.1

[a] Ionic strength = 0.14 M. [b] References to the amino acid sequences of these lysozymes: (1) Shrake & Rupley (1973), (2) Prager et al. (1972), (3) Ibrahimi et al. (1979), (4) LaRue & Speck (1970), (5) Kaneda et al. (1969), (6) Jollès et al. (1979), (7) Hermann & Jollès (1970), Prager & Wilson (1972), Kondo et al. (1982), and (8) Jollès et al. (1976). [c] Apparent association constants determined from microcomplement fixation experiments. Temperature = 4 °C. Lysozyme concentrations at the peak of the complement fixation curve (Y_0) ranged from 3.2×10^{-10} to 67×10^{-10} M (see Table III). [d] Association constants calculated from ultraviolet difference spectra titrations. Lysozyme concentration was $(1.92\text{–}2.09) \times 10^{-5}$ M. Temperature = 6.5 °C. [e] See footnote *b*, Table II.

affinities measured from difference spectra. The K_{app} values closely approximate the K values (Table I). This is especially evident in the turkey and duck comparisons, where a 6-fold reduction in the K values is closely paralleled by a similar reduction in the K_{app} values. The possibility that the Japanese quail lysozyme has a slightly lower affinity for chitotriose than does chicken lysozyme is suggested by multiple titrations employing both methods. We also obtained K_{app} values similar to those measured with antiserum A24 for chicken and turkey lysozyme using another antiserum [pool 10 of Prager & Wilson (1971a)].

Table II lists the amino acids at contact residues (Shrake & Rupley, 1973) and association constants for the eight lysozymes. The 6-fold decrease in affinity of turkey lysozyme corroborates previous observations (Arnheim et al., 1974; Banerjee et al., 1975) and is apparently due to the substitution of glycine for aspartic acid at position 101. The aspartyl residue forms a hydrogen bond to the first sugar residue. In contrast to the turkey lysozyme, the duck and Japanese quail lysozymes possess substitutions of residues that are occluded by, but not bonded to, the ligand. The low affinity of duck II lysozyme may be associated with the substitution of a contact residue at position 75. The slightly lower affinity of the Japanese quail lysozyme correlates with the substitutions of contact residues 102 and 103, which could sterically hinder the ligand fit or alter the local environment of aspartate-101.

Reactant Concentrations. Table III shows the concentrations of lysozyme-specific antibodies used in the above microcomplement fixation experiments. These concentrations, which were calculated from quantitative precipitin results obtained with undiluted antiserum, were generally less than 1 nM.

The low lysozyme concentrations required for immunological estimation of the chitotriose binding constants are also evident in Table III. These concentrations range from 3×10^{-10} to 67×10^{-10} M, whereas those used for the conventional spectral titrations were about 2×10^{-5} M (see Table I, footnotes *c* and *d*). The immunological method is minimally 1000 times more conservative of protein than the conventional method.

Discussion

Immunological Measurement of Ligand Binding. Our recognition and demonstration that antibodies can be used, under some conditions, to measure the affinity of a ligand for a protein antigen stems from the finding that antibodies can detect the antigenic differences caused by binding of ligands to such proteins as hemoglobin (Reichlin et al., 1964), lysozyme (von Fellenberg & Levine, 1967), aspartate transcarbamylase (von Fellenberg et al., 1968), and chymotrypsin (Bucci & Bowman, 1970; Gundlach, 1970).

The particular immunological method that we have used for monitoring ligand binding is notable because of the extremely low concentrations of protein required. Another valuable feature is that an antiserum prepared against one purified lysozyme was used to accurately measure the binding constants of every other avian lysozyme *c* examined. Additionally, this technique should be applicable to impure preparations of the protein, provided that none of the specific ligand contaminates the protein preparation. In these respects, the immunological approach has advantages over conventional physical techniques for measuring the binding of ligands to proteins.

The main practical disadvantage of the method is that ligand binding can be measured only under a restricted range of pH and ionic conditions. The main theoretical disadvantage, that the binding of antibodies may influence the binding of the ligand, is discussed below.

Under the conditions used in microcomplement fixation experiments, it is possible for K_{app} to approximate K. This

Table II: Comparison of Association Constants of Lysozymes for Chitotriose with Amino Acid Substitutions Occurring at Residues in Contact with the Bound Trisaccharide

	chitotriose contact residues[a]														K ($\times 10^5$
lysozyme	56	57	58	59	62	63	75	98	101	102	103	107	108	112	M^{-1})
chicken	Leu	Gln	Ile	Asn	Trp	Trp	Leu	Ile	Asp	Gly	Asn	Ala	Trp	Arg	1.8
bobwhite quail															1.7
California quail															1.7
turkey									Gly						0.3
Japanese quail										Val	His				1.2
ring-necked pheasant															2.0
duck II		Glu[b]					Ala								0.3
chachalaca															2.1

[a] For chicken lysozyme, the contact residues at positions 56–112 are given by three-letter abbreviations. For the other lysozymes, the residues at these positions are only given if different from that in chicken lysozyme. The information about contact residues in chicken lysozyme comes from Shrake & Rupley (1973) and is extracted from the contact data for hexa-*N*-acetylglucosamine. [b] There is uncertainty as to whether duck II lysozyme has a glutamyl or a glutaminyl residue at position 57. This uncertainty arises because this lysozyme does not differ in electrophoretic mobility from DL-1 (Prager & Wilson, 1972), a duck lysozyme that has a glutaminyl residue at this position and that is identical in amino acid sequence at other positions with duck II lysozyme (Kondo et al., 1982).

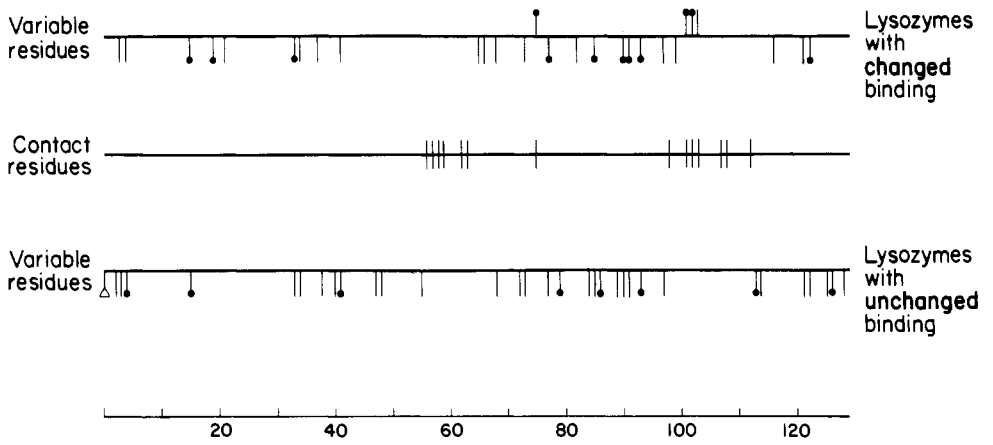

FIGURE 3: Locations of chitotriose contact residues in relation to radical and conservative substitutions in two groups of lysozymes. The scale at the bottom refers to residue position in the amino acid sequence of lysozyme, which is 129 residues long in most bird species examined. The positions of the 14 contact residues, which are listed in Table II, are indicated on the second horizontal line from the top. The 45 variable positions are indicated on the remaining two horizontal lines: the vertical lines *above* the horizontal ones represent substitutions at contact residues while those *below* represent substitutions at noncontact residues. Radical substitutions are indicated by filled circles; the triangle signifies the addition of one amino acid. The terms radical and conservative refer to the degree of chemical difference between amino acids on the basis of size, composition, and polarity of side chains; we designate as radical those substitutions for which the chemical difference is greater than 73 as reported in Table II of Grantham (1974).

Table III: Concentrations of Reactants in the Immunological Tests[a]

lysozyme	percent of antibodies precipitated	antibody concn used in MCF expt ($\times 10^{-10}$ M)[b]	lysozyme concn ($\times 10^{-10}$ M)[c]
chicken	100	4.2	3.2
bobwhite quail	(95)	4.7	4.8
California quail	(97)	4.7	5.4
turkey	89	7.3	6.3
Japanese quail	(81)	7.6	7.5
ring-necked pheasant	77	7.4	7.7
duck II	27	8.8	11.4
chachalaca	23	41.6	67.0

[a] Rabbit antibodies to chicken lysozyme were reacted with eight different lysozymes. Column 2 gives the percent of the lysozyme-specific antibodies in antiserum A24 which is precipitated by a particular lysozyme. The values in parentheses were calculated from microcomplement fixation data with the equation $y = 2.24(100 - z)$, where z is the percent cross-reactivity in the quantitative precipitin test and y is the immunological cross-reactivity measured by complement fixation and expressed in immunological distance units (Prager & Wilson, 1971b). The concentration of lysozyme-specific antibodies in the undiluted antiserum is 2.8×10^{-5} M. [b] Antibody concentration in the 3-mL microcomplement fixation reaction mixture. Obtained by multiplying the maximal amount of antibody precipitated with chicken lysozyme (2.8×10^{-5} M), the fraction of antibody precipitated by each lysozyme, and the experimental dilution factors. [c] Required to produce maximal fixation of complement.

result may be explained in terms of classical binding kinetics. In our experiments, the protein–ligand titration is carried out in the presence of a constant amount of antibody, which inhibits the ligand binding. In competitive inhibition

$$K/K_{app} = [I]K_i + 1$$

where [I] is the concentration of inhibitor, in this case antibody, which in the lysozyme experiments is displaced by chitotriose, and K_i is the affinity of the inhibiting antibody. Isolation and characterization of polyvalent rabbit antibodies that compete with chitotriose for binding lysozyme indicated binding constants on the order of 10^6 M^{-1} (Imanishi et al., 1969). The concentration of inhibiting antibody in the microcomplement fixation experiments is generally less than 10^{-10} M. If we assume that the competing antibodies from the present study possess affinities close to 10^6 M^{-1}, then the product $[I]K_i$ in the above equation has a value of less than 10^{-4}, and as a result, K/K_{app} is approximately 1.0.

We now consider the conditions under which K_{app} values will be substantially lower than K. These occur, first, when the value of $1/K_i$ approaches or is smaller than [I] and, second, when the binding of antibodies not affected by ligand binding causes an allosteric transition. For the lysozyme/chitotriose interaction, K_{app} values are about 9% lower than the K values. This small difference may be artifactual or may be the result of an allosteric effect.

Microcomplement fixation appears to be a particularly suitable immunological technique for measuring ligand binding. This technique is unusually sensitive to small differences in antigenic structure and can detect differences that are not apparent with other immunological assays (Prager & Wilson, 1971b; Champion et al., 1974; Levine et al., 1980).

Local Effects of Evolutionary Substitutions on Lysozyme. We have used the binding of chitotriose to the active site of lysozyme as a model system with which to probe the long-range effects of amino acid substitutions. When compared to chicken lysozyme, those lysozymes with substitutions of substrate-contact residues exhibited altered binding constants (Table II). In contrast, those lysozymes with identical contact residues had similar affinities in spite of extensive sequence divergence (32 substitutions and one addition) outside the substrate-contact region. This could imply a lack of long-range conformational effects of most evolutionary substitutions in bird lysozymes.

Before accepting this implication, however, it is necessary to examine the possibility that the enzymes with unchanged binding constants had fewer radical substitutions than those with changed binding properties. We, therefore, considered the location and nature of the amino acid substitutions in the two groups of lysozymes: those with changes in binding constants (top line of Figure 3) and those without such changes (third line down of Figure 3) relative to that of the chicken protein. At positions outside the binding site, there were eight radical substitutions and one addition in the lysozymes with similar binding constants and nine such substitutions in the group with altered binding constants. Thus, the number of

radical substitutions of noncontact residues is similar between the two groups; moreover, there is no significant difference ($p < 0.27$, Student's t test) in the distances between these residues and the contact residues for the two groups (11.8 ± 3.8 Å for the group with altered binding constants vs. 12.7 ± 4.6 Å for the group with unchanged binding constants). Strikingly, the three lysozymes with altered binding constants all have radical substitutions at contact residues.

Predominantly local effects are also implied by recent comparisons of the binding of a monoclonal antibody to a particular antigenic site present on lysozyme from six species of birds. Thus, antibody 5, which recognizes a site including residues 45 and 68, binds equally well to all these lysozymes, despite substitutions at a total of 18 positions elsewhere in the molecule—some being close to the antigenic site (Smith-Gill et al., 1982). When the antigen binding sites of this and other monoclonal antibodies have been defined more fully and the affinities measured more precisely, it will be possible to conduct a better test for subtle, long-range effects of amino acid substitutions on lysozyme.

Predominance of Local Effects in Other Proteins. Analogous inferences to those drawn from the chitotriose binding results are derived from comparisons of serine proteases of known structure and substrate specificity. Trypsin and chymotrypsin, for example, have accumulated more than 100 amino acid substitutions since divergence, but only one of these mutations, the replacement of a contact residue in the active site, is needed to explain the difference in substrate specificity between these two proteases (Kraut, 1977). None of the other evolutionary substitutions appear to have had significant long-range effects on their active sites.

Even in the case of hemoglobin, a classic example of an allosteric protein, the great majority of evolutionary substitutions seems to have had only local effects. We estimate from the analysis of Perutz & Imai (1980) that about 4% of the evolutionary substitutions in mammalian hemoglobins have long-range conformational effects on the binding of oxygen or diphosphoglycerate.

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Registry No. Lysozyme, 9001-63-2; chitotriose, 41708-93-4.

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