Salt Links Dominate Affinity of Antibody HyHEL-5 for Lysozyme through Enthalpic Contributions*

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The binding of murine monoclonal antibody HyHEL-5 to lysozyme has been the subject of extensive crystallographic, computational, and experimental investigations. The complex of HyHEL-5 with hen egg lysozyme (HEL) features salt bridges between Fab heavy chain residue Glu50, and Arg48 and Arg68 of HEL. This interaction has been predicted to play a dominant role in the association on the basis of molecular electrostatic calculations. The association of aspartic acid and glutamine mutants at position 50 of the cloned HyHEL-5 Fab with HEL and bobwhite quail lysozyme (BQL), an avian variant bearing an Arg68 → Lys substitution in the epitope, was characterized by isothermal titration calorimetry and sedimentation equilibrium. Affinities for HEL were reduced by 400-fold (E50HQ) and 40,000-fold (E50H) (ΔΔG° estimated at 4.0 and 6.4 kcal mol−1, respectively). The same mutations reduce affinity for HEL and BQL, an avian variant containing a arginine to lysine mutation at position 68, was characterized using isothermal titration calorimetry (ITC) and/or sedimentation equilibrium.

In this paper, we report on the energetic contributions of the salt links formed between residue E50H in the Fab and arginine residues 45 and 68 in lysozyme. By mutating a single amino acid residue, the affinity and specificity of the interaction has been dramatically reduced. Furthermore, our results demonstrate the dominantly enthalpic character of bonds formed between charged residues in this highly desolvated interface.

**EXPERIMENTAL PROCEDURES**

Site-directed Mutagenesis of Recombinant HyHEL-5 Fab—Mutagenesis was done using crossover polymerase chain reaction or the M13 method as described by Kunkel et al. (17). Polymerase chain reaction was performed in a Perkin-Elmer Gene Amp System 2400 with Taq DNA polymerase from Promega. Three polymerase chain reaction reactions were necessary for the E50H mutant. Two primers (GCTAC-CACTCCCCGAGATGAAGC (sense) and TGATTGGA-CAGATTCTACCGAGGTAGTGC (antisense); both from Biorad Biotechnologies) were designed to introduce both the mutant nucleotide sequence (shown in bold) and an additional 1-base pair change to introduce a SmaI site (shown underlined). The first set of reactions contained the heavy chain DNA template (pET MH5wt), the sense primer containing the mutation, and a T7 reverse primer (T7 AS), while the companion reaction used the antisense primer containing the complementary sequence along with a T7 forward primer (T7 S). After the polymerase chain reaction was completed (15 cycles of 94 °C/55 °C/74 °C for 1 min each with a final 6-min extension at 72 °C), an aliquot of each purified fragment was added to a final reaction containing only the T7 sense and antisense primers. This “crossover” reaction resulted in a 900-base pair band which was double-digested with BamHI and HindIII to release a 490-base pair fragment to subclone into the double-digested (BamHI/HindIII) and dephosphorylated PET MH5wt vector. Positive clones bearing the primer-encoded SmaI site were identified by the appearance of a band at 1200 base pairs when restricted by SmaI (the vector contains one constitutive SmaI restriction site) and further verified by DNA sequencing on an automated ABI fluorescence se-
quencing (University of Texas Health Science Center-Houston) prior to protein expression.

The E50D mutant was prepared by the Kunkel method after subcloning a BamH1/HindIII fragment of the heavy chain gene into M13mp19. The primer used, GGAGATCATTTACGCCGGAATGCT, included a HindIII site, and an additional BamHI site was generated by the appearance of a 1.6-kilobase band upon digestion with EcoRV. The mutated fragment was subcloned into the MH5wt vector and the sequence verified by DNA sequencing.

Purification of Mutant Proteins—The mutant proteins were purified as described previously for the wild-type Fab (16). Briefly, 2-liter cultures of induced cells expressing either the mutant heavy chain or the wild-type light chain were harvested by centrifugation. Cells containing the light chain were lysed by French Press. Inclusion bodies were washed once with buffer (100 mM Tris, 2.5 mM EDTA, 0.02% Tween 20, pH 7.9), recentrifuged, and the final pellet resuspended in denaturation buffer (100 mM Tris, 6 mM guanidine HCl, 2.5 mM EDTA, 0.4 mM 1-arginine HCl, pH 7.9) to a final protein concentration of 2 mM. Whole cells expressing the heavy chain were dissolved in the denaturation buffer (plus 100 mM dithiothreitol) to a protein concentration of 2 mg/mL. After stirring at room temperature for 1 h, the solutions were mixed in a 1:2 (light:heavy) molar ratio in the presence of 4 mM glutathione and diluted 10-fold with denaturation buffer. The solutions were exhaustively dialyzed against Tris buffer to remove, through successive changes of dialysis against 0.5 M guanidine and 1-arginine.

The refolded protein was concentrated using tangential flow filtration and purified by Q Sepharose and nickel-charged chelating Sepharose Fast Flow chromatography. The resulting protein was at least 95% pure based on SDS-polyacrylamide gel electrophoresis and sedimentation equilibrium experiments.

Isothermal Titration Calorimetry—Hen egg lysozyme (2 times crystallized) was purchased from Worthington Biologicals. BQL was prepared from eggs purchased from Stevenson’s Game Bird Farm (River- side, TX) as described by Shick et al. (9). Concentrations of all proteins were determined spectrophotometrically using extinction coefficients reported previously (16, 17).

ITC was performed in an MCS Isothermal Titration Calorimeter (Microcal) essentially as in previous work (11). Briefly, the calorimeter was equilibrated overnight at a temperature 5 °C below that intended for the experiment. HyHEL-5 Fab and HEL or BQL were allowed to co-dialyze overnight in 100 mM sodium phosphate buffer containing 150 mM NaCl (ITC buffer), pH 8.0. Experiments were also performed in Tris buffer to measure protonation effects. A typical experiment used 1.7 mL of HyHEL-5 Fab (about 20 μM) in the sample cell. The antigen (HEL or BQL, about 300 μM) was titrated into the HyHEL-5 Fab in 16 injections of 10 μL, 300 s apart. An initial injection of 2 μL was made to clear the syringe of any Fab from the sample cell which might have mixed with the lysozyme in the syringe during equilibration. All titrations were performed at least in duplicate. The reported errors are the standard deviations.

Data analysis was performed using the software program Origin, and is based on the amount of heat liberated upon association as described (19). The change in Gibbs free energy upon association, ΔG°, was calculated using the formula,

\[ ΔG^o = -RTlnK_a \] (Eq. 1)

where R is the gas constant and T is the absolute temperature. The entropy change of association, ΔS°, was calculated using,

\[ ΔG^o = ΔH^o - TΔS^o \] (Eq. 2)

where ΔH° is a measured enthalpy of association of the reaction at temperature T.

The equilibrium number, K_a, and ΔH° could be determined for all interactions except the E50Q/BQL and the E50A associations, which were too weak to be measured by ITC.

Sedimentation Equilibrium Experiments—Sedimentation equilibrium experiments were performed in a Beckman Optima XL-A analytical ultracentrifuge using an 8-hole An 50 Ti rotor, at several rotor speeds between 15,000 and 25,000 rpm at 17 °C. Two-channel epon centerpieces were filled with an 0.18-mL sample, giving column heights of approximately 5 mm, and the compartment adjoining each sample was filled with ITC buffer. Loading concentrations of Fab and lysozyme were varied in a series of experiments, covering a range of concentrations from 0.1 to 44 μM Fab and 2 to 30 μM lysozyme. The buoyant molar mass of lysozyme M°,b = M°,b(1 – ip) and of the Fab fragments M°,b = M°,b(1 – ip) were measured in independent sets of experiments. The absence of non-ideality effects or self-aggregation in the measured sedimentation profiles of the individual species at high concentrations was demonstrated by their constant apparent molar mass in the centrifugal field over the entire range of concentrations used in the experiments. Sedimentation equilibrium was established by random radial distribution of the difference of signals from successive scans.

The absorbance distributions were measured at wavelengths of 250, 250, or 305 nm, chosen so that the measured maximum absorbance was smaller than 1.5 OD. The extinction coefficients were based on a molar extinction coefficient of 83,800 OD280 nm m-1 cm-1 for the Fab fragments and 37,700 OD280 nm m-1 cm-1 for the lysozyme. The extinction coefficients at 230, 250, and 305 nm were measured in the analytical ultracentrifuge in sedimentation equilibrium experiments with the single species, by taking multiple scans at the respective wavelengths and obtaining the ratio of the absorbance at the reference radius after modeling the radial absorbance distribution (see below).

Data analysis employed the global least-squares modeling of between 5 and 13 absorbance profiles a_i(r) taken at different loading concentrations, rotor speeds, and wavelengths. The fitting function for the mixtures was the sum of the expressions for the distributions of individual components in the centrifugal field,

\[ a_i(r) = a_{i,exp}(r)e^{\frac{L}{2RT}} + a_{i,b}(r)e^{\frac{L}{2RT}} + \delta \] (Eq. 3)

where \( a_{i,exp}(r) \) and \( a_{i,b}(r) \) denote the absorbance contribution of free Fab fragment or lysozyme, respectively, at a reference radius \( r_0 \). \( \omega \) denotes the angular velocity of the rotor, \( R \) denotes the gas constant, \( T \) denotes the absolute temperature, \( \delta \) denotes a baseline offset, \( K_a \) denotes the association equilibrium constant in molar units, \( d \) denotes the optical path length, and \( e_i \) denotes a predetermined extinction coefficient,

\[ e_i = e_i,b + e_i,exp(1 + \delta) \] (Eq. 4)

which transforms the association constant from absorbance units into molar units. Fitting parameters local to each of the data sets were \( a_{i,exp}(r_0), \delta, a_{i,b}(r_0), \) and \( d \); the global fitting parameter was \( ln(K_a) \) (20). Global fits were performed using the modeling software MLAB (Civilized Software, Inc., Bethesda, MD). Confidence intervals were estimated using projections of the objective function (21) and F-statistics (22), taking into account propagated errors resulting from the uncertainty in the determination of the buoyant molar masses of the lysozyme (1.6%) and the Fab fragments (4.2%).

RESULTS

Throughout the denaturation and refolding process, each of the mutant proteins behaved identically to the wild-type HyHEL-5 Fab, eluting at the same gradient positions from the Q Sepharose and the metal-chelate columns. Sedimentation equilibrium experiments demonstrated that each of the recombinant proteins was non self-aggregating, all running with the same apparent molar mass at concentrations up to 40 μM. Preliminary x-ray crystallographic analysis indicates the E50Q/Q/HEL crystals are isomorphous with the proteolytic HyHEL-5 Fab-HEL complex (PDB 3HFL; 1).

Isothermal Titration Calorimetry—A typical isothermal titration calorimetry experiment is illustrated in Fig. 1. Table I lists the values determined at temperatures ranging from 10 to 30 °C in ITC buffer. To correct for any heat of dilution, the integrated areas of the last three injections (after saturation) were averaged and subtracted from the raw data. Heats of dilution were less than 1 kcal mol-1 at Fab concentrations around 20 μM. Experimental equivalence ratios, N, are also shown.

The affinity of the wild-type Fab/HEL interaction has been estimated to be about 4 × 1012 M-1 at 25 °C (23). The HEL
affinity at 25 °C of the E50HQ mutant was reduced by 40,000-fold, while that of the E50HQD was reduced by 400-fold, corresponding to an overall decrease in free energy of 6.4 and 4.0 kcal mol⁻¹, respectively. An E50HQD mutant showed no evidence of HEL binding in ITC experiments, indicating a decrease of at least 10⁴-fold in affinity for the single mutation based on a lower ITC detection limit of 1 × 10⁻⁶ M⁻¹.

The affinity of wild-type Fab association with BQL, the R68K avian mutant, has been measured by ITC to be 6.6 × 10⁻⁶ M⁻¹ at 25 °C (16). Only the E50HD mutant showed BQL affinity high enough to be measured through ITC; the association constant Ka of each mutant/lysozyme interaction studied is lower than that of the wild-type Fab/HEL, and decreases as the temperature increases. Both ΔH° and ΔS° become more negative throughout the temperature range, leading to a relatively constant ΔG° (Fig. 2). The ΔCp of each interaction is less negative than that of the wild-type Fab/lysozyme interactions (Table II). Negligible protonation effects (±0.1 mol⁻¹) were observed for all associations.

Sedimentation Equilibrium—Sedimentation equilibrium experiments resulted in a Kd of 1.16 × 10⁻⁶ M⁻¹ (error limits 1.3 × 10⁴ to 1.6 × 10⁵) for the E50HQ/BQL interaction at 17 °C, a 55-fold decrease in the affinity, corresponding to a ΔΔG° of 2.7 kcal mol⁻¹ from the wild-type interaction.

Double-mutant Cycle Analysis—To further understand the energetic changes observed upon mutating position 50H in the HyHEL-5 Fab, double-mutant cycles were constructed (Fig. 3). Each cycle was based on the substitution at position 50H of the Fab, and the R68K substitution (and other mutations known to exert much smaller effects) in BQL.

In each of the interactions examined, the sums of ΔΔG° of the single-mutant interactions were different from those obtained for the double-mutant interactions, indicating coupling between the mutations (Table III). Subtracting the sum of the free energy changes of the two single mutations from that of the double-mutant gave a coupling energy of +2.9 kcal mol⁻¹ for the wt/HEL → E50HQ/BQL pathway and +3.7 kcal mol⁻¹ for the wt/HEL → E50HQQ/BQL pathway (Fig. 3).

**TABLE I**

| Temperature °C | ΔH° kcal mol⁻¹ | Kd (× 10⁻⁶ M⁻¹) | ΔG° kcal mol⁻¹ | ΔS° cal mol⁻¹ K⁻¹ | Equivalence ratio |
|---------------|----------------|-----------------|---------------|-------------------|-----------------|
| E50HQD/HEL association | 9.9 | -12.6 ± 0.10 | 1460 ± 298 | -10.6 ± 0.11 | -7.36 ± 0.13 | 0.87 ± 0.01 |
| | 16.6 | -15.4 ± 0.04 | 979 ± 242 | -10.6 ± 0.11 | -16.5 ± 0.23 | 0.85 ± 0.00 |
| | 25.0 | -18.7 ± 0.56 | 562 ± 168 | -10.5 ± 0.13 | -27.2 ± 1.44 | 0.88 ± 0.00 |
| | 30.1 | -20.3 ± 0.10 | 447 ± 156 | -10.6 ± 0.16 | -31.9 ± 0.27 | 0.90 ± 0.00 |
| E50HQQ/HEL association | 11.0 | -12.5 ± 0.34 | 17.0 ± 3.0 | -8.07 ± 0.11 | -15.7 ± 0.80 | 0.93 ± 0.04 |
| | 18.1 | -13.5 ± 0.35 | 10.3 ± 6.7 | -8.01 ± 0.02 | -18.8 ± 1.14 | 0.90 ± 0.03 |
| | 25.1 | -16.4 ± 0.44 | 9.4 ± 4.3 | -8.09 ± 0.27 | -27.8 ± 0.55 | 0.89 ± 0.00 |
| | 30.2 | -17.2 ± 0.43 | 4.4 ± 0.3 | -7.83 ± 0.04 | -31.0 ± 1.26 | 0.88 ± 0.05 |
| E50HQDBQQL association | 10.4 | -15.3 ± 0.01 | 35.8 ± 5.0 | -8.50 ± 0.01 | -23.9 ± 0.01 | 0.95 ± 0.01 |
| | 16.6 | -16.9 ± 0.29 | 22.1 ± 2.0 | -8.37 ± 0.07 | -28.4 ± 0.76 | 0.85 ± 0.03 |
| | 25.1 | -17.8 ± 0.42 | 9.7 ± 8.0 | -8.22 ± 0.06 | -32.2 ± 1.29 | 0.88 ± 0.03 |
| | 30.1 | -18.1 ± 1.08 | 7.4 ± 1.0 | -8.17 ± 0.04 | -30.1 ± 0.25 | 0.86 ± 0.00 |

**DISCUSSION**

**Mutational Effects on the Affinity of the Interaction**—In the HyHEL-5/lysozyme interaction, glutamic acid residues at heavy chain positions 50 in CDR 1 and 35 in CDR 2, have long been predicted to form the core of the functional combining site. In crystal structures, E50H is juxtaposed with and believed to be far removed from solvent, in an environment of low dielectric constant. To study the role of the glutamate at position 50 in these interactions, three mutations, an alanine, aspartate, and glutamine, were introduced into the Fab and the association with HEL and BQL measured by either ITC or sedimentation equilibrium. Substitution of Ala, which both neutralizes the charge and decreases the length of the side chain, produced the greatest affinity changes. When the charge was maintained but the length of the side chain decreased, the E50HQD mutant, binding was decreased by 400-fold (HEL) and 6.6-fold (BQL). The E50HQQ maintained the side chain length, but neutralized the charge of the side chain decreasing the binding by 40,000-fold.
and wt Fab/BQL (●) results from Wibbenmeyer et al. (16); E50D/HEL (■); E50Q/HEL (△); E50D/BQL (□).

Mutational studies of interacting surfaces in other proteins have demonstrated that only a small number of residues play a dominant role in binding (24). For example, in the interaction of human growth hormone with its receptor, 11 residues contribute the functional hormone epitope; mutation of either of these residues reduces association energy by over 6 kcal mol\(^{-1}\) (HEL) and 55-fold (BQL), reducing the free energy of association by more than 6 kcal mol\(^{-1}\) in the HEL interaction and 2.7 kcal mol\(^{-1}\) in the BQL interaction.

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Thermodynamic Characterization of the Interactions—For both mutants studied, the entropic contribution to HEL association was indistinguishable from that of the wild-type Fab (−8.3 kcal mol\(^{-1}\)) at 25 °C (Table II). \(\Delta H^o\), in contrast, decreased by an average of 5.3 kcal mol\(^{-1}\) and by up to 6.5 kcal mol\(^{-1}\) from that of the wild-type Fab. Consequently, the affinity decrease for each mutant results almost entirely from loss of favorable enthalpic interactions.

For each mutant studied, the loss of enthalpic contribution to HEL association is among the highest reported, which may be due to the favorable desolvated local environment in which the electrostatic interaction occurs. Ross and Subramanian (27) have suggested that salt links may contribute to enthalpy changes when formed in an environment of low dielectric constant. Other studies have associated salt bridge formation in desolvated environment with enthalpic contributions (9, 24). Entropic contributions appear to play a greater role when electrostatic bonds are formed in solvated environments, where their contributions to binding affinity are lower (e.g. the anti-lysozyme antibody HyHEL-10) (27).

In each of the mutant Fab/lysozyme interactions examined, enthalpy-entropy compensation exists (Table I). As the temperature increases, the enthalpy change of each interaction becomes more favorable (Fig. 2), while the entropy change becomes less favorable and affinity is relatively constant. This behavior (and the observed negative values of \(\Delta C_p\)) has been suggested to be an indicator of hydrophobic interactions. The unfavorable entropy change upon association may arise from loss of protein translational, rotational, and conformational degrees of freedom on complex formation, part of which arises from the hydrophobic interactions.

The mutational \(\Delta G^o\) of 6.4 kcal mol\(^{-1}\) for E50Q/HEL and 4.0 kcal mol\(^{-1}\) for E50D/HEL are generally consistent with those predicted by Slagle et al. (14) who calculated a \(\Delta G^o_{elc}\) of +11 kcal mol\(^{-1}\) for E50Q and +2.5 kcal mol\(^{-1}\) for E50D. Most importantly, in both the model and the experimental results, each mutation yields a positive \(\Delta G^o\) and the effect of the E50Q mutation is larger.

**Fig. 3. Double-mutant cycle to test for coupling between HyHEL-5 Fab 50H mutations and the BQL R68K mutation.**

**TABLE II**

| Lysozyme | Fab | \(K_a\) (mM) | \(\Delta H^o\) (kcal mol\(^{-1}\)) | \(\Delta S^o\) (cal mol\(^{-1}\) K\(^{-1}\)) | \(\Delta C_p\) (kcal mol\(^{-1}\)) | \(\Delta G^o\) (kcal mol\(^{-1}\)) | \(\Delta T\Delta S^o\) (kcal mol\(^{-1}\)) | \(\Delta C_p\) (kcal mol\(^{-1}\)) |
|----------|-----|--------------|-----------------|-----------------|---------------|-----------------|-----------------|---------------|
| Wt Fab   | 4 \times 10^{16} | −26.9 ± 0.2 | −17.6 | −8.3 | −18.1 ± 0.2 | 2.9 | −0.4 | 119 |
| HEL E50Q/HEL | 0.4 × 10^{16} | −26.7 ± 0.1 | −17.6 | −8.3 | −18.1 ± 0.2 | 2.9 | −0.4 | 119 |
| HEL E50D/HEL | 0.1 × 10^{16} | −26.7 ± 0.1 | −17.6 | −8.3 | −18.1 ± 0.2 | 2.9 | −0.4 | 119 |

**TABLE III**

| Interaction | E50Q | E50Q |
|-------------|------|------|
| wt Fab/HEL → wt Fab/BQL | 5.2  | 5.2  |
| wt Fab/BQL → wt Fab/BQL | 5.2  | 5.2  |
| Total \(\Delta G^o_{association}\) (kcal mol\(^{-1}\)) | 9.2  | 11.6 |
| Coupling energy (kcal mol\(^{-1}\)) | 2.9  | 3.7  |
from the stabilization of the mobile loop found in the HyHEL-5 epitope. Crystal structures reveal that, upon complex formation, this epitope is immobilized (1). Amide exchange studies show that residues in HEL far removed from the interface are also perturbed upon binding, possibly altering the dynamics of the molecule and adding to an unfavorable entropy (29). The ordering of water molecules in and around the interface will decrease entropy, while likely contributing positively to the enthalpy, while burial of hydrophobic residues will increase the solvent entropy through water liberation.

The $\Delta C_p$ value calculated for each mutant complex was less negative than that obtained for the wild-type HyHEL-5 Fab (Table II). For the E50HQ/BQL interaction, $\Delta C_p$ is only approximately one-half of that of the wild-type Fab/BQL interaction.

A negative $\Delta C_p$ has been interpreted as an indication of hydrophobic interactions; burial of interfacial surface area (31) and/or the presence of waters buried in the interface (32–35) have been suggested to be contributors. While the large changes in $\Delta C_p$ produced by relatively conservative mutations in this study agree against the former (Table II), water uptake values estimated using the method of Spolar and Record (36) argue in favor of the latter. The number of water molecules taken up by each mutant complex was estimated to be less from that of the wild-type interaction (wt Fab/HEL, 17; E50HQ/D/HEL, 16; E50HQ/Q/HEL, 10; wt Fab/BQL, 10; E50HQ/D/BQL, 4). Along with the large changes in affinity and enthalpy seen in the mutant interactions, this result is consistent with a less well ordered interface, leading to a lower water capture contribution to $\Delta C_p$. Structural analysis of mutant complexes now underway may address these issues.

**Changes in the Specificity of the Interaction**—Each mutation reduces the specificity of the interactions, increasing cross-reactivity. The E50HQ mutation decreased the affinity of HyHEL-5 interaction with HEL by 400-fold; with BQL, less than one-half of that of the wild-type Fab/BQL interaction. Approximately one-half of that of the wild-type Fab/BQL interaction.

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**Thermodynamic Cycle Analysis of Mutant Interactions**—Double-mutant cycles were constructed to test the expectation that the E50HQ mutations and the R68K substitution in BQL perturb the same critical interactions (Table III; Fig. 3). In each of the interactions examined, the sum of the $\Delta G^\circ$ of the single mutations differed from $-\Delta G^\circ$ for the double mutation. The effect of each double mutation is well less than the sum of the effects of individual mutations, confirming that the same interactions are perturbed (26). The calculated coupling energies in these interactions are comparable to the 3.5 to 4.3 kcal mol$^{-1}$ attributed to buried salt links in the folding pathway of barnase (38) and in an anti-HIV Fv/peptide interaction (25).

**Conclusion**—By mutating a single residue in the HyHEL-5 Fab involved in a buried salt bridge, the affinity of the interaction with HEL was decreased up to 100,000-fold, corresponding
down to a decrease in $\Delta G^\circ$ of over 8 kcal mol$^{-1}$. Our results verify the predicted importance of $\Delta G^\circ$ in the interaction; we find this to be a “hot spot” in the functional epitope. The energy changes can be assigned almost entirely to enthalpic contributions and are among the highest reported $\Delta H^\circ$'s for a single mutation. This result is in contrast to those reported for the deletion of salt bridges in a more heavily solvated environment in the interface between another anti-lysozyme antibody, HyHEL-T10, and HEL (28). The mutations studied in this work also greatly reduce the specificity of the antibody for HEL. Crystallographic analyses presently underway should aid in further understanding the overall association process.

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