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High-resolution mapping of the HyHEL-10 epitope of chicken lysozyme by site-directed mutagenesis

(epitope mapping/protein–protein interaction)

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ABSTRACT **The complex formed between hen egg white lysozyme (HEL) and the monoclonal antibody HyHEL-10 Fab fragment has an interface composed of van der Waals interactions, hydrogen bonds, and a single ion pair. The antibody overlaps part of the active site cleft. Putative critical residues within the epitope region of HEL, identified from the x-ray crystallographic structure of the complex, were replaced by site-directed mutagenesis to probe their relative importance in determining affinity of the antibody for HEL. Twenty single mutations of HEL at three contact residues (Arg-21$_{HEL}$, Asp-101$_{HEL}$, and Gly-102$_{HEL}$) and at a partially buried residue (Asn-19$_{HEL}$) in the epitope were made, and the effects on the free energies of dissociation were measured. A correlation between increased amino acid side-chain volume and reduced affinity for HELs with mutations at position 101 was observed. The D101G$_{HEL}$ mutant is bound to HyHEL-10 as tightly as wild-type enzyme, but the $\Delta\Delta G_{dissoc}$ is increased by about 2.2 kcal (9.2 kJ)/mol for the larger residues in this position. HEL variants with lysine or histidine replacements for arginine at position 21 are bound 1.4–2.7 times more tightly than those with neutral or negatively charged amino acids in this position. These exhibit 1/40 the affinity for HyHEL-10 Fab compared with wild type. There is no side-chain volume correlation with $\Delta\Delta G_{dissoc}$ at position 21. Although Gly-102$_{HEL}$ and Asn-19$_{HEL}$ are in the epitope, replacements at these positions have no effect on the affinity of HEL for the antibody.**

A major goal of immunochemistry has been to provide a quantitative understanding of the specificity of immune receptors for antigens. Such knowledge requires the elucidation of both the structural and the functional bases of complementarity. We have focused on monoclonal antibodies (mAbs) specific for hen egg-white lysozyme (HEL) as a model for understanding protein–protein interactions in general. The detailed topography of the interaction between a mAb and a macromolecular antigen became clear when high-resolution x-ray structures for three HEL·mAb complexes were published (1–3). The three antibodies, D1.3, HyHEL-5, and HyHEL-10, recognize different sites (epitopes) on the HEL molecule. There is a small overlap of the D1.3 and HyHEL-10 epitopes. All three sites are discontinuous, comprising between 14 and 17 lysozyme amino acids in contact with 17–19 antibody residues. There are numerous van der Waals interactions (up to 111), 12–14 hydrogen bonds, and 0–3 salt bridges. The contact areas of the three epitopes range from 690 to 774 Å^2 (4). The recently refined x-ray structure of D1.3 complexed with its anti-idiotype revealed a similar picture (5). Although x-ray structures have given us detailed information about the structural contacts in the interface, the quantitative contributions of each residue to specificity and affinity have not yet been defined.

Before the advent of site-directed mutagenesis technology, epitopes were broadly defined by examining the cross-reactivity of mAbs raised against defined proteins with naturally occurring variants of those proteins (reviewed in ref. 6), but the residues which could be evaluated were limited by availability of sequenced naturally occurring variants. Previous investigations with chicken and Japanese quail lysozymes identified residues 19, 21, 102, and/or 103 as critical residues for HyHEL-10 binding but failed to implicate Asp-101$_{HEL}$ (7). Other experimental approaches to epitope mapping have included chemical modification of amino acids, protein footprinting, studies with peptide binding, and rates of solvent exchange of individual protons within the epitope determined by two-dimensional NMR (reviewed in ref. 8). These techniques allow identification of potential contact residues, but they do not evaluate individual contributions to the free energy of association. Replacement of specific amino acids in proteins by site-directed mutagenesis allows for a rationally designed, systematic, and quantitative analysis of the interaction between antibodies and protein antigens. Several mAb·protein complexes have been partially studied by this technique (9–14), but high-resolution x-ray structures are not yet available for these complexes to allow structural interpretation.

HEL has been heterologously expressed in the laboratory of J.F.K. (15). We report here an investigation of the thermodynamic contributions of specific antigen residues within the crystallographically defined epitope by site-directed mutagenesis. The results on the HEL·HyHEL-10 complex together with those discussed on the HEL·HyHEL-5 complex (16) allow us to estimate the quantitative contribution of specific antigenic residues to antibody–protein recognition.

MATERIALS AND METHODS

Materials. HEL was purchased from either Sigma or Worthington. *Micrococcus lysodeikticus* was purchased from Sigma. Japanese quail lysozyme (JQEL), Montezuma quail lysozyme (MQEL), and turkey lysozyme (TEL) were a generous gift from Ellen Prager (University of California, Berkeley). The preparation of mAb HyHEL-10 has been described (7).

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Abbreviations: HEL, chicken (hen) egg-white lysozyme; JQEL, Japanese quail egg-white lysozyme; MQEL, Montezuma quail egg-white lysozyme; TEL, turkey egg-white lysozyme; mAb, monoclonal antibody; PCFIA, particle concentration fluorescence immunoassay; V_H, heavy-chain variable region; V_L, light-chain variable region.

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Mutant Lysozyme Preparation. Site-directed mutagenesis and expression of chicken lysozyme mutants in yeast were performed as described by Malcolm *et al.* (15) with the following modifications: A single transformed yeast colony was inoculated into 5 ml of minimal medium and shaken at 30°C for 36–48 hr. This seed culture was used to inoculate 50 ml of minimal medium in a 125-ml Erlenmeyer flask, which was shaken for 48 hr. The flask was used to inoculate 500 ml of 1% yeast extract/2% bactopeptone/8% glucose medium in a 2.8-liter Fernbach flask. The cells were grown for 7–9 days. They were harvested in a Beckman model J2-21 centrifuge (JA-10 rotor at 5000 rpm, 15 min), washed twice with 60 ml of 0.5 M NaCl, and collected by centrifugation. The supernatants were pooled, diluted 5-fold with deionized H_2O, and loaded onto a 20-ml column of CM-Sepharose Fast Flow (Pharmacia) equilibrated with 0.1 M potassium phosphate, pH 6.24. The column was washed with 200 ml of 0.1 M potassium phosphate, pH 6.24, and lysozyme was eluted with 50 ml of 0.5 M NaCl/0.1 M potassium phosphate, pH 6.24. Fractions were assayed by activity (decrease in OD_{450} of *M. lysodeikticus* cell wall suspensions). Those containing lysozyme were concentrated in Centricon-10 (Amicon) filter units, washed with 0.1 M potassium phosphate buffer, pH 6.24, and stored at 4°C. Protein concentration was determined from $A_{280}^{1\%} = 26.4$ (17).

Competitive Inhibition Assays. All natural variants and site-directed mutants of lysozyme used in this study exhibited at least 50% of wild-type activity. The values of the lysozyme–HyHEL-10 dissociation constants were determined by taking advantage of the fact that the antibody (Ab) occludes the enzyme (E) active site (3). As a result, the HyHEL-10·HEL complex is essentially catalytically inactive (6) (see the scheme below, in which S = substrate and P = product).

$$\begin{array}{c} E + S \rightleftharpoons E{\cdot}S \rightarrow E + P \\ Ab \downarrow\uparrow \\ E{\cdot}Ab \text{ (inactive)} \end{array}$$

This was demonstrated in all cases tested by the nearly total loss of enzyme activity upon addition of excess antibody. The level of residual catalytic activity can therefore be used to estimate the concentration of free (uncomplexed) enzyme. The lysozyme species were preequilibrated with various concentrations of antibody in the presence of added bovine serum albumin as described in ref. 18 (1 hr in 66 mM potassium phosphate, pH 6.24, at 25°C). Catalytic activity was measured by following a modified procedure of Shugar (19). Dissociation constants, K_d, were calculated by nonlinear regression to the model described in Eq. 1, using the SAS program (20):

$$V = V_0 - (V_0 - V_1) \times \frac{(K_d + 2[Ab] + [Lz]) - \sqrt{(K_d + 2[Ab] + [Lz])^2 - 8[Ab][Lz]}}{2[Lz]}, \quad [1]$$

where [Ab] = concentration of total antibody, [Lz] = concentration of total lysozyme, V is the remaining activity in the solution, and V_0 and V_1 are the activities of lysozyme in the absence of added antibody and the presence of excess antibody, respectively. The dissociation constants listed in Tables 1 and 3 were calculated as weighted averages of several determinations for each lysozyme mutant.

The free energies of dissociation of the lysozyme–antibody complexes were calculated from Eq. 2:

$$\Delta\Delta G = \Delta G_{WT} - \Delta G_{Mut} = -RT\ln(K_{WT}/K_{Mut}), \quad [2]$$

where WT = wild type, Mut = mutant, R is the gas constant, and T is absolute temperature. The errors in $\Delta\Delta G$ (WT − Mut) were calculated from the errors in the measured K_d values from Eqs. 3 and 4:

$$\sigma(K_{Mut}/K_{WT}) = \sqrt{(\sigma K_{Mut}/K_{WT})^2 + [K_{Mut}/(K_{WT})^2]^2(\sigma K_{WT})^2} \quad [3]$$

$$\sigma(\Delta\Delta G) = RT\sigma(K_{Mut}/K_{WT})/(K_{Mut}/K_{WT}). \quad [4]$$

Particle Concentration Fluorescence Immunoassays (PCFIAs). Antigen inhibition PCFIAs were performed with an automated immunoassay system (Screen Machine; Idexx Laboratories, Westbrook, ME) as described in detail elsewhere (18). The percent bound was calculated relative to a control containing trace amounts of lysozyme and a background reading with diluent in the place of antibody. Binding data were corrected for monovalent/bivalent binding to the solid phase according to Stevens (21). The concentration of antigen (B_{50}) required to give a bound-to-free ratio = 0.5 maximum was calculated for each antigen by regression analysis on log-logit transformed data. K_{Mut} was approximated from the PCFIA B_{50} values by the relationship of Eq. 5 (22):

$$\log\frac{B_{50WT}}{B_{50Mut}} = \log\frac{K_{WT}}{K_{Mut}}, \quad [5]$$

where B_{50WT} and B_{50Mut} represent the B_{50} values of wild type lysozyme and mutant lysozyme, respectively. Wild-type (naturally derived) HEL was used as a standard with $K_{WT} = 2.22 \times 10^{-11}$ M as determined previously by PCFIA according to the method of Friguet *et al.* (23) under conditions described by Lavoie *et al.* (24). Calculated values of K_{WT} and K_{Mut} were used to determine the $\Delta\Delta G_{dissoc}$ as described above.

RESULTS

Contributions of the Asp-101$_{HEL}$ Side Chain to the Free Energy of Association. Asp-101$_{HEL}$ is of particular interest because it contributes to the free energy of association of substrate ligands with the enzyme (25, 26), and both side-chain and backbone atoms of this residue form contacts with HyHEL-10 in the HEL·HyHEL-10 complex (3). Nine single mutations were made at position 101 of HEL to test the importance of both the size and charge of the side chain. Generally, similar results were found whether measured by catalytic activity or by antigen inhibition (PCFIA) (Table 1). Although the quantitative effects of some replacements differed slightly between the two assays (Table 1), all mutants with the exception of D101G$_{HEL}$ have a K_d significantly greater than that of the wild-type enzyme as determined by both methods.

Role of Arg-21$_{HEL}$ in Determining the $\Delta\Delta G_{dissoc}$. There are three hydrogen bonds between Arg-21$_{HEL}$ and three antibody residues, Asn-92$_{VL}$, Tyr-50$_{VH}$, and Tyr-96$_{VL}$ (main-chain N hydrogen bonds to Asn-92$_{VL}$ O and side-chain NH1 hydrogen bonds to the OH groups of Tyr-96$_{VL}$ and Tyr-50$_{VH}$; VL and VH indicate variable regions of the light and heavy chains, respectively) (3). The seven mutations at position 21 were also designed to investigate the importance of side-chain charge and volume. Mutations R21Q$_{HEL}$ and R21W$_{HEL}$ were made because two natural variants, JQEL and MQEL, have glutamine and tryptophan, respectively, at position 21 (Table 2). These quail homologues each show an affinity approximately 1/30 to 1/45 of that for the antibody compared with HEL (Table 3). All mutations of Arg-21$_{HEL}$ to neutral side chain amino acids result in an increase of ≈2.2 kcal/mol in

Table 1. Dissociation constants and $\Delta\Delta G$ values for the complexes formed with mAb HyHEL-10 and lysozymes varying at position 101

Lysozyme position 101*	Activity assay K_{Mut}/K_{WT}† (SD)	Activity assay $\Delta\Delta G_{[WT\ HEL\text{-}Mut]}$ (SE), kcal/mol	PCFIA K_{Mut}/K_{WT}‡ (SD)	PCFIA $\Delta\Delta G_{[WT\ HEL\text{-}Mut]}$ (SE), kcal/mol
WT HEL	1.0	0.00	1.00	0.00
Gly	1.4 (0.8)	0.21 (0.31)	2.4 (0.9)	0.52 (0.21)
Ala	7.7 (2.9)	1.21 (0.23)		
Asn	9.6 (4.2)	1.34 (0.26)	5.2 (1.6)	0.98 (0.18)
Ser	18 (6)	1.71 (0.20)	3.3 (1.0)	0.71 (0.18)
Gln	26 (9)	1.92 (0.22)		
Arg	36 (17)	2.13 (0.28)	410 (120)	3.55 (0.17)
Glu	28 (10)	1.97 (0.21)	7.4 (2.2)	1.13 (0.18)
Phe	39 (16)	2.17 (0.25)		
Lys	28 (10)	1.97 (0.21)	88 (26)	2.65 (0.17)
TEL (Gly)	9.5 (4.4)	1.33 (0.27)	1.9 (0.8)	0.38 (0.24)

1 kcal = 4.18 kJ.

*Wild-type HEL was produced in yeast, as were the mutant HELs. The position 101 mutants shown were made in the chicken sequence. TEL has a glycine at position 101 in addition to six other substitutions compared with the chicken enzyme, two of which are in the epitope (Table 2).

†Relative dissociation constants were measured by a competitive inhibition activity assay at pH 6.24. Experimentally determined K_{WT} is 0.13 ± 0.04 nM.

‡PCFIA assays were performed in 150 mM Tris·HCl/150 mM NaCl at pH 7.4 with a final mAb concentration of 2.3 nM. Experimentally determined K_{WT} is 0.031 ± 0.019 nM.

$\Delta\Delta G_{dissoc}$, independent of side-chain volume. There may be a small positive charge effect, since mutant lysozymes containing lysine or histidine replacements bind 0.6 and 0.2 kcal/mol, respectively, more tightly; but a glutamic acid replacement yields a protein which binds about as well as lysozymes with neutral side chains at position 21. Thus, the $R21W_{HEL}$ replacement alone accounts for the lowered affinity of MQEL for HyHEL-10, since position 21 is the only difference from HEL in the epitope. It also appears that the lower affinity of JQEL to the antibody can be attributed to position 21, although additional replacements at positions 19 and 102 are present in its epitope (Table 2) (see below).

Asn-19_{HEL} and Gly-102_{HEL}. Table 2 shows the amino acids within the HyHEL-10 epitope of four natural variant lysozymes. Asn-19_{HEL} is of particular interest because the crystal structure shows that it is a partially buried residue in the epitope (3), and JQEL has a lysine at this position (28), which may possibly bury a positive charge. To test the importance of Asn-19_{HEL} in the association reaction, three single mutations were constructed. The data show that all three mutant proteins bind nearly as tightly as wild-type HEL to HyHEL-10 (Table 3), indicating that the amino acid side chain at this position is not a significant immunodeterminant.

Gly-102_{HEL}, which forms a main chain hydrogen bond with the antibody in the complex, is replaced by valine in JQEL. This mutation made directly on HEL has no discernible effect on $\Delta\Delta G_{dissoc}$ (Table 3), indicating the absence of any positive or negative contributions from the increased bulk of the valine side chain.

Table 2. Naturally occurring lysozyme amino acid replacements within the HyHEL-10 epitope

Lysozyme*	Amino acid residue at position 15	19†	21	73	101	102	103†
HEL	His	Asn	Arg	Arg	Asp	Gly	Asn
JQEL	—	Lys	Gln	—	—	Val	His
MQEL	—	—	Trp	—	—	—	—
TEL	Leu	—	—	Lys	Gly	—	—

Dashes indicate the amino acid residue is the same as in HEL.

*Amino acid sequence sources: HEL (27), JQEL (28), MQEL (33), TEL (29).

†Partially buried residue.

The conclusion from these experiments is that the total free energy differences distinguishing the association of HyHEL-10 with HEL, MQEL, and JQEL are accounted for by the amino acid at position 21.

DISCUSSION

Asp-101_{HEL}. The effect of each replacement on ΔG_{dissoc} was examined by calculating the $\Delta\Delta G_{dissoc}$ for each mutant lysozyme in comparison with wild-type HEL (Tables 1 and 3). An estimate of the specific electrostatic and hydrogen bond contributions to the ΔG_{dissoc} of the Asp-101_{HEL} side chain might be obtained from a comparison of the near isosteres aspartic acid and asparagine. The 9.6-fold increase in K_d occasioned by the mutation yields a $\Delta\Delta G_{dissoc}$ value of 1.34 kcal/mol (Table 1). If charge is the dominant contributor to the stabilization energy effected by Asp-101_{HEL}, which makes a hydrogen bond to Tyr-53_{VH}, the placement is

Table 3. Dissociation constants for the complexes formedwith mAb HyHEL-10 and lysozymes varying at positions 21, 19, and 102

Lysozyme*	Activity assay K_{Mut}/K_{WT}† (SD)	Activity assay $\Delta\Delta G_{[WT\ HEL\text{-}Mut]}$ (SE), kcal/mol
WT HEL	1.0	0.00 (0.24)
Arg-21 → Trp	28 (10)	1.97 (0.28)
Gln	44 (11)	2.24 (0.23)
Glu	48 (12)	2.30 (0.22)
Gly	46 (19)	2.26 (0.30)
Asn	39 (6)	2.17 (0.20)
His	30 (13)	2.01 (0.31)
Lys	15 (5)	1.61 (0.26)
Asn-19 → Lys	1.5 (0.5)	0.23 (0.25)
Gln	0.93 (0.73)	−0.04 (0.49)
Asp	2.0 (1.1)	0.41 (0.37)
Gly-102 → Val	1.5 (0.3)	0.24 (0.21)
MQEL (Trp-21)	32 (16)	2.06 (0.34)
JQEL (Gln-21)	45 (23)	2.25 (0.34)

*Wild-type and mutant HELs were produced in yeast. The position 21, 19, and 102 mutants shown were made in the HEL sequence.

†Dissociation constants were measured by a competitive inhibition assay. Experimentally determined K_{WT} is 0.13 ± 0.04 nM.

critical, as seen by the experimentally nearly identical $\Delta\Delta G_{dissoc}$ values introduced by the neutral D101Q$_{HEL}$ and anionic D101E$_{HEL}$ replacements (Table 1).

A plot of $\Delta\Delta G_{dissoc}$ versus the side-chain volumes of the amino acids replacing aspartate at position 101 demonstrates that this latter parameter is the overwhelming determinant of complex stability (Fig. 1). An unexpected finding is that D101G$_{HEL}$ binds the antibody about as well as wild-type HEL (Table 1). It appears that the deletion of the aspartic acid side chain allows for the substitution of compensating favorable protein–protein contacts. Novotny (32) has recently calculated the total contribution of Asp-101$_{HEL}$ to the free energy of formation of the HyHEL-10·HEL complex as −1.8 kcal/mol. The figure is the sum of hydrophobic (−2.3 kcal/mol), entropic (+1.3 kcal/mol), and electrostatic and hydrogen bonding (−0.8 kcal/mol) terms. The observation that the D101G$_{HEL}$ mutant binds as tightly to HyHEL-10 as does wild type might seem to be at variance with the calculations. However, most of the free energy contributed by Asp-101$_{HEL}$ to the HEL·HyHEL-10 complex is contributed by the backbone atoms, and the Asp-101$_{HEL}$ side chain actually has a calculated entropic penalty of 1.3 kcal/mol. Replacement of the Asp-101$_{HEL}$ side chain with a glycine would give a predicted $\Delta\Delta G_{dissoc}$ of significantly less than 1 kcal/mol (J. Novotny, personal communication).

TEL, which has a glycine at position 101 and has 1/5 to 1/6 the affinity for chitotriose compared with HEL between pH 4.5 and pH 6.0 (26), exhibits a similarly reduced (1/9.4) affinity for HyHEL-10 (Table 1). Of the six additional amino acid replacements between HEL and TEL, Leu-15$_{TEL}$ and Lys-73$_{TEL}$ are contact residues in the HyHEL-10 epitope (Table 2), and they presumably account for the difference in affinities for the antibody observed between TEL (Gly-101$_{TEL}$) and mutant D101G$_{HEL}$. This hypothesis has not yet been tested experimentally.

There is a correlation between side-chain volume and $\Delta\Delta G_{dissoc}$ of all of the position 101 mutants of lysozyme (Fig. 1). This correlation could reflect steric interference of bulky side chains with favorable contacts made by other residues. Nearby hydrogen bonds between HyHEL-10 and the backbone of lysozyme residues flanking position 101 include Ser-100$_{HEL}$ main-chain O···Tyr-50$_{VH}$ OH, Gly-102$_{HEL}$ main-chain N···Tyr-58$_{VH}$ OH, and Gly-102$_{HEL}$ main-chain N···Tyr-58$_{VH}$ OH (3).

The observed effects of substitutions at position 101$_{HEL}$ on binding of lysozyme to HyHEL-10 likely reflect a combination of direct effects related to the contribution of the Asp-101$_{HEL}$ side chain to ΔG_{dissoc}, as well as the indirect ones of steric interference with backbone contacts at this position and possible interactions of adjacent residues. The combination of direct and indirect effects, such as those associated with Asp-101$_{HEL}$, can lead to apparent ambiguities in assignment of a given amino acid as a contact residue in epitope mapping experiments. For example, in the original characterization of the HyHEL-10 epitope (6), Asp-101$_{HEL}$ was neither assigned nor excluded as a contact residue. In binding experiments, one is limited to investigating the role of side-chain atoms (13), and a combination of effects may produce seemingly contradictory results even with an x-ray structure available for interpretation. Site-directed mutagenesis of cloned antibody genes has allowed evaluation of the contribution of individual antibody residues to complementarity (24, 33, 34). Thus, mutagenesis of complementary contact residues in HyHEL-10 might clarify the precise contribution of Asp-101$_{HEL}$ to the complex. A caveat which must be appreciated, however, is the fact that the observed $\Delta\Delta G_{dissoc}$ is composed of two effects, the decrease in the stability of the mutant lysozyme·HyHEL-10 complex and any change in free energy of interaction of the uncomplexed mutant relative to uncomplexed wild-type enzyme with solvent.

Arg-21$_{HEL}$. Contrasting with the near linear correlation of $\Delta\Delta G_{dissoc}$ values with side-chain volume observed for position 101$_{HEL}$ mutations is the nearly uniform increase of ≈2.2 kcal/mol obtained by mutation of Arg-21$_{HEL}$ to any neutral amino acid (Fig. 2). Novotny (32) has calculated that the free energy contribution of Arg-21$_{HEL}$ to the stability of the complex is −4.0 kcal/mol, but he does not address the expected effects of site-directed replacements. The guanidino moiety of Arg-21$_{HEL}$ makes two hydrogen bonds with the OH groups of Tyr-96$_{VL}$ and Tyr-50$_{VH}$ (3). The 2.2 kcal/mol decrease in affinity in this instance can be unambiguously attributed to the hydrogen bonds. Although the hydrogen bond acceptor on the antibody is neutral, the positive charge of Arg-21$_{HEL}$ may have some influence on the $\Delta\Delta G_{dissoc}$, as the R21K$_{HEL}$ mutant is bound only 1.6 kcal/mol worse than wild-type HEL to HyHEL-10 (Fig. 2).

Other Variants of the HyHEL-10 Epitope. The mutations at residues 19, 21, and 102 were designed to probe the contribution of these residues to the reduced affinity of JQEL and MQEL to HyHEL-10. The data suggest that the R21W$_{HEL}$ replacement can by itself account for the lowered affinity of MQEL for HyHEL-10, since the ΔG_{dissoc} of the R21W$_{HEL}$ mutant is equivalent to that of the MQEL, which has no other

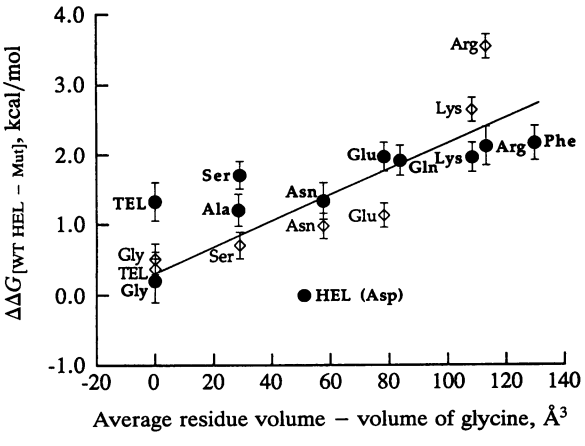

FIG. 1. Dependence of the free energies of dissociation of the mAb HyHEL-10·lysozyme complexes on the volume of the amino acid side chains (taken from refs. 30 and 31) at HEL position 101. Filled circles and boldface print = enzyme activity assay; open diamonds = PCFIA.

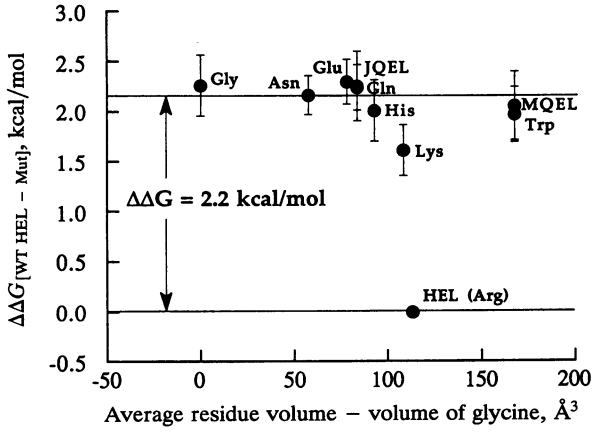

FIG. 2. Importance of HEL position 21 in determining the free energies of dissociation of mAb HyHEL-10·lysozyme complexes. Wild-type HEL has Arg at position 21. The single amino acid replacements were made at this position. JQEL has Gln in position 21 as well as the other changes in the HyHEL-10 epitope shown in Table 2. The HyHEL-10 epitope for MQEL differs from that of the chicken enzyme in having Trp at position 21.

substitutions in the HyHEL-10 epitope other than Trp-21_{MQEL} (Table 2). Similarly, the observed ΔG_{dissoc} of the $R21Q_{HEL}$ mutant is equivalent to that of JQEL, suggesting that the amino acid change at position 21 is sufficient to account for the lowered reactivity of JQEL for HyHEL-10. Preliminary results with the antigen inhibition assay, however, show that the B_{50} values of the $R21Q_{HEL}$ and $R21N_{HEL}$ mutants were significantly lower than those of JQEL and the $R21W_{HEL}$ mutant (data not shown). The apparent discrepancy between catalytic activity and antigen inhibition results could be due either to an effect of pH or to the kinetics of the assays themselves. As noted above, the affinity measurements in the present experiments were done under conditions which were optimal for enzymatic activity, at pH 6.24, and in conditions of constant total antigen with varying concentration of antibody, while the antigen inhibition assays were done under conditions optimal for antibody binding, at neutral pH, with constant total antibody and varied antigen concentration. Under the latter conditions, we have obtained a slightly higher affinity of HyHEL-10 for HEL, and somewhat lower affinity for JQEL than in the present experiments (24). Since Arg-21_{HEL} forms hydrogen bonds with tyrosine residues from HyHEL-10, and our results clearly indicate a contribution of charge to the position 21_{HEL} interactions, it is likely that the apparent discrepancies between the affinity and antigen inhibition data are at least in part attributable to pH effects.

Conclusions. This report demonstrates that site-directed mutagenesis can be employed to probe the detailed chemistry of immunocomplex formation with greater precision than was afforded by the natural variant epitope mapping method (6). The well-characterized HEL has proven ideal for this study. The specific amino acid replacements of the present investigation were guided by data obtained from these earlier studies and by the high-resolution x-ray structures of the HyHEL-10:HEL complex (3). We have shown that mutations in two sites provoke qualitatively different responses in $\Delta\Delta G_{dissoc}$. The size of the introduced side chain at position 101 is directly related to the increase in the difference in free energy of the interaction, while steric bulk is not a factor in the position 21 mutants. Side-chain charge reversal at position 101 (e.g., aspartate to arginine or lysine) does not contribute additionally, while a slight preference for a positive charge is exhibited at position 21. The present results emphasize the importance of introducing a variety of amino acids at the probed position to resolve the complexities due to charge, polarity, and size in determining the overall effect on $\Delta\Delta G_{dissoc}$.

The surviving authors dedicate this work to the memory of our friend and colleague, Allan C. Wilson. We thank Dr. Jiri Novotny for help with interpretation of the energetics calculations. This work was supported by National Service Research Award AI07989 from the National Institute of Allergy and Infectious Diseases (to L.N.W.K.-M.) and National Institute of General Medical Sciences Award GM14514-01 (to M.G.T.), by a National Institutes of Health grant to A.C.W., and by the Director, Office of Energy Research, Office of Basic Energy Science, Divisions of Material Sciences and Energy Biosciences of the U.S. Department of Energy under Contract DE-AC03-76SF00098.

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