Structural Consequences of Mutations in Interfacial Tyr Residues of a Protein Antigen-Antibody Complex

THE CASE OF HyHEL-10-HEL

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Tyrosine is an important amino acid in protein-protein interaction hot spots. In particular, many Tyr residues are located in the antigen-binding sites of antibodies and endow high affinity and high specificity to these antibodies. To investigate the role of interfacial Tyr residues in protein-protein interactions, we performed crystallographic studies and thermodynamic analyses of the interaction between hen egg lysozyme (HEL) and the anti-HEL antibody HyHEL-10 Fv fragment. HyHEL-10 has six Tyr residues in its antigen-binding site, which were systematically mutated to Phe and Ala using site-directed mutagenesis. The crystal structures revealed several critical roles for these Tyr residues in the interaction between HEL and HyHEL-10 as follows: 1) the aromatic ring of Tyr-50 in the light chain (LTyr-50) was important for the correct ternary structure of variable regions of the immunoglobulin light chain and heavy chain and of HEL; 2) deletion of the hydroxyl group of Tyr-50 in the heavy chain (HTyr-50) resulted in structural changes in the antigen-antibody interface; and 3) the side chains of HTyr-33 and HTyr-53 may help induce fitting of the antibody to the antigen. Hot spot Tyr residues may contribute to the high affinity and high specificity of the antigen-antibody interaction through a diverse set of structural and thermodynamic interactions.

Protein-ligand interactions via noncovalent bonds play a central role in many biological processes. To exhibit their functions, most proteins specifically recognize their partners or targets with certain affinities. Recently, analyses of the three-dimensional structures of many protein-protein complexes, mutational analyses, and analyses of the kinetics and thermodynamics of protein-protein interactions have revealed the roles of noncovalent bonds (i.e. hydrogen bonds, salt bridges, and van der Waals contacts). In general, the factors that determine the affinity and specificity of an interaction are the surface area and structure of the binding interface, including the electrostatic, hydrophilic, and hydrophobic complementarities. It has been reported that aromatic residues, especially Tyr and Trp, are more frequently found at the core of interaction sites and make more energetically favorable contributions to binding than do other amino acids.

The concept of a protein-binding hot spot, defined as the critical residues for a protein interaction, was first proposed by Clackson and Wells (4). Hot spot residues make energetically significant contributions to binding, typically contributing more than 2 kcal mol⁻¹ in Gibbs energy change (ΔG) to the interaction. The frequency of Tyr residues in hot spots is far greater than that of Trp, suggesting that Tyr residues are a preferred amino acid for protein interaction hot spots (2). Tyr has both a hydrophobic aromatic ring and a hydrophilic hydroxyl group in one side chain. Thus, from a structural point of view, Tyr residues can potentially contribute to binding via the following: 1) hydrogen bonding via the side-chain hydroxyl group; 2) hydrophobic interactions; 3) van der Waals interactions; and 4) amino-aromatic (cation-π) interactions via the aromatic ring (5).

Antibodies change the amino acid composition in their complementarity-determining regions (CDRs) to bind specifically to various kinds of target antigens with high affinity. Tyr residues are commonly found in the CDRs of many antibodies (6, 7). Among the three-dimensional structures of 26 proteinaceous antigen-antibody and 14 peptide-antibody complexes, we observed that there were, on average, nearly 17 contacting residues in the antigen-binding site, of which 24% were Tyr.
Thus, it appears that Tyr is used with the highest frequency during natural selection of antibodies. Tyr residues contribute to ~17% of the overall interfacial area of antigen-antibody complexes (1), and buried Tyr residues tend to be interaction hot spots (2). These observations suggest that Tyr residues are critical during the development of antibodies with high specificity and high affinity for their targets. Although Phe also has an aromatic ring, the frequency of Phe at antigen-binding sites was ~4%, much lower than that of Tyr.

In a recent report (8), the ankyrin repeat protein off7, which was selected from a ribosome display library against maltose-binding protein, has a binding site, including seven aromatic residues, four of which are Tyr residues. In addition, the structure of artificially selected antibody against human vascular endothelial growth factor shows that antigen recognition is mediated mostly by Tyr residues, which cover 71% of the antigen-binding site (9). These results suggest that Tyr residues might play an important role in generating high affinity, high specificity binding sites during artificial selection processes as well.

We have focused on the interaction between hen egg lysozyme (HEL) and the variable domain fragment (Fv) of the anti-HEL antibody HyHEL-10, which is one of best studied proteinaceous antigen-antibody interactions (10–18). Of the 19 residues in HyHEL-10 involved in antigen binding, six are Tyr residues, two in the variable regions of the immunoglobulin light chain (VL) and four in the heavy chain (VH) (Fig. 1, a and b) (13, 19). These Tyr residues contact 12 residues of HEL through van der Waals contacts, hydrogen bonding, or amino-aromatic (cation–π) interactions, or via interfacial water molecules. Together, these contacts encompass almost the entire HEL epitope recognized by HyHEL-10 (Fig. 1c). Because of the abundance of Tyr residues in the binding site, the HyHEL-10-HEL system may be a good model for investigating the role of Tyr residues in protein-protein interactions.

In a previous study, we investigated the role of the Tyr residues in the VH region of HyHEL-10 (HyHEL-10 VH) using site-directed mutagenesis and isothermal titration calorimetry (ITC) (11). We found that Tyr residues located in the CDR of HyHEL-10 VH had a variety of significant roles in binding as follows: formation of hydrogen bonds via hydroxyl groups; hydrophobic interactions; van der Waals interactions (due to their large volume); and stabilization of local structure (due to their aromatic rings). Structural analyses of the complex between HEL and antibody mutants with one or two Tyr residues in the interfacial region of HyHEL-10 would provide more precise insights into the role of these residues in the antigen-antibody interaction.

To elucidate the contribution of Tyr residues to high affinity and high specificity antigen binding, in combination with thermodynamic analyses we performed crystallographic studies of HyHEL-10 Fv mutants in complex with HEL, in which the six interfacial Tyr residues of HyHEL-10 were mutated to Phe and Ala. On the bases of the results obtained, we discuss how Tyr residues contribute to the specificity and affinity of proteinaceous antigen-antibody interactions.

**EXPERIMENTAL PROCEDURES**

**Preparation of HyHEL-10 Mutant Fvs**—The gene structure of the expression vector of the HyHEL-10 Fv fragment was described in our previous paper (20). LY50A, LY96F, and HY53A mutants were produced by the method of Kunkel as described in our previous paper (16, 21). The double Ala substitution mutant of the HyHEL-10 Fv fragment, HY33A/Y53A, was generated by insertion of an Xhol/EcoRI fragment of the gene encoding the HY53A mutant into the identical site of the gene encoding the HY33A mutant. We obtained wild-type and mutant Fv fragments as secreted proteins using the Escherichia coli BL21(DE3) expression system. BL21(DE3) cells harboring the appropriate expression plasmid were precultured in 3 ml of LB medium, then inoculated into 2×YT medium with 100

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6 The abbreviation used for mutants is, for example, LY50A, the mutant of HyHEL-10 Fv in which Ala is substituted for Tyr-50 of the VL chain.
mg/liter ampicillin, and shaken overnight at 28 °C. The culture was centrifuged, and the bacteria were resuspended in 2× YT medium containing 100 mg/liter ampicillin and isopropyl 1-thio-β-D-galactopyranoside at a final concentration of 1 mM and then shaken overnight at 28 °C again. The culture was centrifuged at 3000 × g for 20 min, and the supernatant was subjected to ammonium sulfate precipitation at 80% saturation, followed by centrifugation. The protein pellet was solubilized in 30–40 ml of PBS buffer and then dialyzed against PBS buffer. Fv fragments were purified using affinity chromatography. The protein solution was loaded onto an HEL-Sepharose column (20), and the column was washed with PBS buffer and then wash buffer (50 mM Tris-HCl (pH 8.5) containing 0.5 mM NaCl). Fv fragments were eluted using elution buffer (0.1 M Gly-HCl (pH 2.0) containing 0.2 mM NaCl) and buffered rapidly with 1 M Tris-HCl (pH 7.5). Fv-containing fractions were centrifuged, and minor impurities were removed by gel filtration with a Sephacryl S-200 column (GE Healthcare) pre-equilibrated with 50 mM Tris-HCl (pH 7.5) containing 0.2 mM NaCl. Purified Fv fragments were concentrated using a Centriprep-10 column (Millipore, Bilerica, MA). The antigen, HEL, was purchased from Seikagaku-Kogyo Inc. (Tokyo, Japan).

**Isothermal Titration Calorimetry**—Thermodynamic parameters of the interaction between HEL and wild-type or mutant HyHEL-10 Fv fragments were determined by ITC using a VP-ITC (MicroCal, Inc., Northampton, MA). The experimental conditions were as follows: in a calorimeter cell, the Fv fragment, at a concentration of 5 μM in 50 mM phosphate buffer (pH 7.2) containing 0.2 mM NaCl, was titrated with a 50 μM solution of HEL in the same buffer at four different temperatures (25, 30, 35, or 40 °C). The HEL solution was injected 20 times in portions of 10 μl over 15 s. Thermograms were analyzed with the program Origin7 (MicroCal, Inc.) after subtraction of the thermogram against the buffer only. The enthalpy change (ΔH) and binding constant (K_b) for the antigen-antibody interaction were obtained directly from the experimental titration curve. The Gibbs free energy change (ΔG = −RT ln K_b) and the entropy change (ΔS = (−ΔG + ΔH)/T) for the association were calculated from ΔH and K_b. The heat capacity change (ΔC_p) was estimated from the temperature dependence of the enthalpy change.

**Crystallization, Data Collection, and Structural Determination of the HyHEL-10 Mutant Fv-HEL Complex**—Except for the Hy33A/Y53A-HEL complex, all mutant Fv-HEL complexes were crystallized under conditions similar to those of the wild-type Fv-HEL complex (13). The best crystals of Fv-HEL complexes were grown in 0.1 M Hepes buffer (pH 7.6–7.8), 9–11% w/v polyethylene glycol 6000, and 7–9% (w/v) 2-methyl-2,4-pentanediol. The resultant crystals were elongated bipyramidal shapes. Crystals of the Hy33A/Y53A-HEL complex were obtained in 0.1–0.2 M ammonium sulfate, 15–20% w/v polyethylene glycol 4000, and 0.1 M sodium acetate trihydrate (pH 4.6); hexagonal pillar-like crystals were obtained. All crystallization conditions included glycerol at a final concentration of 15% as a cryoprotectant.

Data sets for the wild-type Fv-HEL complex and all mutant Fv-HEL complexes were obtained using the synchrotron x-ray source at beamline BL6A at the Photon Factory (Tsukuba, Japan). The diffraction images were processed by the interactive data processing package DPS/MOSFLM/CCP4. Integration was carried out using the MOSFLM software (22); scaling was carried out using SCALA software (23), and the final file of structural factors was obtained using TRUNCATE (24) and MTZ2VARIous of the CCP4 suite (25). The structure of the Fv-HEL complexes was refined by using the CNS program (26). The graphic program O (27) was used for making adjustments to the molecular model. Crystallographic and refinement data for each Fv mutant-HEL complex are summarized in the supplemental Table 1.

Calculation of root-mean-square deviations (r.m.s.d.) for structural comparison was done using the programs LSQKAB (28) and COMPAR in the CCP4 software suite. Interfacial areas were calculated with NACCESS (29). Shape complementarity scores were calculated with SC (30) in the CCP4 suite. Determination of contacting atoms between Fv and HEL was performed with the CONTACT program in the CCP4 suite. Figures were drawn with the program WebLab Viewer Lite (Accelrys Inc., San Diego, CA).

Atomic coordinates and structural factors for each mutant Fv-HEL complex have been deposited in the Protein Data Bank. The Protein Data Bank accession codes are as follows: wild type (2DQJ), LY50A (2DQI), HY33F (2DQC), HY33A/Y53A (2DQF), HY50F (2DQD), HY53A (2DQE), HY53F (2DQG), and HY58A (2DQH).

**RESULTS**

Mutant Fv fragments were expressed using VL-VH coexpression vector and an E. coli expression system. They were purified to more than 95% purity by HEL-Sepharose affinity chromatography following size exclusion chromatography. As shown in our previous study, single Ala substitution mutants at positions H33 (HY33A mutant) and H50 (HY50A mutant) could not be purified by affinity chromatography, despite their high expression levels, because of their extremely low affinity for HEL (11). Ala substitutions at HTyr-33 and HTyr-50 cause large decreases in binding affinity using the HyHEL-10 Fab-HEL or HyHEL-63 Fab-HEL system; the latter antibody is related to HyHEL-10 and recognizes a similar epitope (17, 31). However, the double Ala substitution mutant of HTyr-33 and HTyr-53, HY33A/Y53A, could be purified using affinity chromatography.

**Thermodynamic Analyses**—The interactions between mutant Fv fragments, constructed additionally in this study (LY50A, LY96F, HY33A/Y53A, and HY53A), and HEL were analyzed thermodynamically using ITC. The thermogram of each experiment was obtained by titrating the HEL solution into the Fv solution, from which the base line obtained by titrating HEL solution into the buffer was subtracted (Fig. 2, a–c). Together with the previous data on the other mutants, thermodynamic parameters are summarized in Table 1. In the Ala substitution mutant of LTyr-50 (LY50A), the thermodynamic analysis showed that the decrease in enthalpy change (ΔΔH, 10.4 kJ mol⁻¹) was smaller than that of the Phe substitution mutant (ΔΔH, 16.7 kJ mol⁻¹) (Fig. 2a and Table 1). In contrast, the

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7 The abbreviation used for residues is, for example, H33, residue number 33 of the VH chain.
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entropic change was similar to that of the wild type (TΔΔS, 3.2 kJ mol⁻¹). Ala substitution at HTyr-53 did not lead to the large Gibbs energy change compared with the wild-type-HEL interaction (ΔG; 0.9 kJ mol⁻¹) (Fig. 2b and Table 1). However, large decreases in binding enthalpy (ΔH; 15.9 kJ mol⁻¹) and entropic loss (TΔΔS; 15.0 kJ mol⁻¹) were observed in the HY53A-HEL interaction. The binding constant of the HY33A/Y53A-HEL interaction (Kₜ = 1.9 × 10⁶ M⁻¹) was higher than that of the HY33A mutant but much lower than that of the wild type (Fig. 2c and Table 1). The large decrease in Gibbs energy (ΔG; 31.3 kJ mol⁻¹) resulted from the large loss of binding enthalpy in HY33A/Y53A mutant.

Crystal Structure of Fv Mutant-HEL Complexes—The crystal structures of mutated HyHEL-10 Fv-HEL complexes were solved at resolutions sufficient for determining local structural differences (1.8–2.5 Å) (supplemental Table 1). Most of the interfacial water molecules, which mediate the Fv-HEL interaction, were conserved between each mutant Fv-HEL complex and the wild-type complex (supplemental Table 2).

Although LTyr-50 does not directly contact the residue of the VH chain, the relative orientation of VL, VH, and HEL is altered in the LY50A-HEL complex. In particular, the difference in the orientation of Fv and HEL between the wild-type and mutant complexes is large (Table 2). The large space generated by the Ala substitution at site I50 is occupied by water molecules that form a hydrogen-bonding network.

The structure of the HY33F-HEL complex shows that the overall structure, the interfacial water molecules, and the local structure around the mutation site of the HY33F-HEL complex are almost the same as those of the wild-type complex (Table 2 and supplemental Table 2 and Fig. 3b). The structure also showed that the decrease in polar area (–26 Å²) and the increase in nonpolar area (+32 Å²) that occurred at the site of H33 compared with the wild type were the result of the substitution of HTyr-33 with Phe (supplemental Table 3).

In the HY50F-HEL complex structure, there are no overall structural differences compared with wild type (Table 2). Two hydrogen bonds are absent because of deletion of the hydroxyl group of HTyr-50, leading to large structural changes in the side chain of Arg-21 of HEL. The amino- aromatic (cation-π) interaction between HTyr-58 and Arg-21 of HEL is also absent, because of structural perturbation of the side chain of Arg-21 (Fig. 3c). In addition, there is a significant rearrangement of the interfacial water molecules. In the wild-type complex, a water molecule (Wat-10) is completely buried in the Fv-HEL interface and forms hydrogen bonds bridging the Fv-HEL interface. In the HY50F-HEL complex, although newly arranged water molecules (Wat-46, Wat-74, Wat-100, and Wat-250) form a network of hydrogen bonds at the Fv-HEL interface, some of

![Figure 2. Thermodynamic analyses by isothermal titration calorimetry of interactions of HyHEL-10 Fv mutants with HEL.](image)

**TABLE 1**

Thermodynamic parameters of mutant Fv-HEL interactions at 30 °C and pH 7.2

Experimental protocols are described under the “Experimental Procedures.” Data represent the averages of at least three independent measurements. Errors of all values are within 5% for several experiments. The abbreviations used are as follows: n, stoichiometry; Kₜ, binding constant; ND, not determined; ΔG, ΔH, ΔS, and ΔCₚ, changes in Gibbs energy, binding enthalpy, entropy, and heat capacity, respectively.

| Mutant    | n  | Kₜ × 10⁶ M⁻¹ | ΔG kJ mol⁻¹ | ΔH kJ mol⁻¹ | ΔS  | TΔΔS kJ mol⁻¹ K⁻¹ | ΔCₚ kJ mol⁻¹ K⁻¹ |
|-----------|----|--------------|-------------|-------------|-----|-----------------|-----------------|
| Wild type | 0.99 | 42           | –50.2       | –91.5       | –41.3 | –1.38           |
| LY50A     | 0.99 | 2.6          | –43.0       | –81.1       | 10.4 | –38.1           | 3.2             |
| LY50F     | 1.05 | 11           | –46.7       | –74.8       | 16.7 | –28.1           | 13.2            |
| LY96F     | 1.0  | 12           | –46.8       | –94.6       | –3.1  | –47.8           | –6.5            |
| HY33A     | 1.12 | 7.1          | –45.6       | –73.2       | 18.3 | –27.6           | 13.7            |
| HY33AY53A | 1.07 | 0.19         | –36.4       | –60.2       | 31.3  | –23.8           | 17.5            |
| HY33F     | 1.0  | 2.9          | –43.1       | –59.8       | 31.7  | –16.7           | 24.6            |
| HY50A     | 0.96 | 30           | –49.3       | –75.6       | 15.9  | –26.3           | 15.0            |
| HY50F     | 1.03 | 15           | –47.2       | –84.4       | 7.1   | –37.2           | 4.1             |
| HY58A     | 0.97 | 2.6          | –43.1       | –72.3       | 19.2  | –29.2           | 12.1            |
| HY58F     | 1.01 | 23           | –48.5       | –85.3       | 6.2   | –36.8           | 4.5             |

*Data are from Ref. 11.
*Data are from Ref. 16. ΔΔH and TΔΔS are the differences in binding enthalpy and entropy between mutant and wild type.
*HY33A and HY50A could not be purified by HEL-Sepharose affinity chromatography.
these water molecules are exposed to solvent, suggesting that the network of hydrogen bonds contributes less energetically than that seen in the wild-type complex. Some hydrogen bonds, an amino-aromatic interaction, and many van der Waals interactions between Arg-21 of HEL and HTyr-58 are lost because of these structural changes (supplemental Table 4).

In the HY58A-HEL complex structure, there are no large changes in overall structure or in the local structure of the mutation site (Table 2 and Fig. 3d). Two water molecules newly introduced at the mutated site make no hydrogen bonds bridging Fv and HEL. The substitution of Ala for HTyr-58 results in the loss of an amino-aromatic interaction, hydrogen bonding via a water molecule, and some van der Waals contacts.

The structure of the HY53A-HEL complex shows that both the overall and local structures of the HY53A-HEL complex were similar to those of the wild-type complex (Table 2 and Fig. 3e). Notably, the B factor in the loop around Asp-101 of HEL in the HY53A-HEL complex is higher than that in the wild-type complex by 4.7 Å² compared with the overall B factor difference between the mutant and wild type (+0.7 Å²).

We solved the structure of the HY33A/Y53A-HEL complex from the trigonal crystals (space group P3_121) obtained under conditions different from those for the wild-type and the other mutant complexes. Two complexes (HY33A/Y53A_1 and HY33A/Y53A_2) are present in an asymmetric unit (Fig. 4a). These two complexes have almost identical structures. The structure of each main chain of the mutant complex is similar to the corresponding main chain of the wild-type complex (average r.m.s. d. of each chain in the mutant complexes compared with that of the wild type was 0.30 Å for VL, 0.55 Å for VH, and 0.50 Å for HEL; Table 2). However, large differences in the orientation of HEL to VL and/or VH were observed compared with the wild type. Furthermore, the r.m.s.d. of the main chain of HEL was 2.72 Å when the Fv fragment of the mutant complex was superposed on that of the wild-type complex (Table 2 and Fig. 4, a and b). One salt bridge, some hydrogen bonds, and many van der Waals contacts were lost as a result of the removal of the side chains of HTyr-33 and HTyr-53 and the large change in the relative orientation between Fv and HEL (supplemental Table 4). Thus the large decrease in binding enthalpy in the HY33A/Y53A-HEL interaction may result from the loss of these bonds and contacts. Furthermore, these structural changes led to a decrease in the interfacial area and shape complementarity between Fv and HEL (supplemental Tables 3 and 5). When each chain of the mutant complex is compared with the corresponding chain of the wild-type complex, two large local structural differences are observed in the mutant complex. One is the large structural difference observed in the CDR-H2 loop by maximal 1.7 Å (shift of the C-α atom at H53) (Fig. 4c). The other is the structure of the loop around Asp-101 of HEL. In the HY33A/Y53A complex, the loop structure is similar to that seen in unbound lysozyme, and the peptide flip in Asp-101 seen in the wild-type complex is not observed (Fig. 4d).

**DISCUSSION**

In the following sections, we discuss the role of each Tyr residue on the basis of the crystal structure of the mutants, in combination with previous and/or current thermodynamic data.

**LTyr-50, the Aromatic Ring Is Important for Maintaining the Correct Orientation of the Ternary Complex**—Our previous report showed that the hydroxyl group of LTyr-50 formed a hydrogen bond via interfacial water molecules with a large binding enthalpy and a large entropic loss, indicating that this “stiffened” the antigen-antibody complex by hydrogen bonding via the interfacial water molecules (16). The relative orientation between Fv and HEL is largely different between the wild-type and mutant complexes, suggesting that the aromatic ring of LTyr-50 is important not only for making interfacial contacts but also for maintaining the correct orientation of the antigen-antibody complex. The large space generated by the Ala substitution at site L50 is occupied by water molecules that form a hydrogen-bonding network. The presence of water molecules in this space could induce structural changes in the side chain of Lys-49, change the direction of the side chain of HAsp-99, and ultimately change the relative orientations of VL-HEL and VL-VH (Fig. 3a and Table 2). A rearrangement of the relative orientation of VL and VH was also observed in the Ala substitution mutant of HAsp-99, which forms a salt bridge to Lys-97 of HEL, by the single new water molecule introduced to the mutation site (15). Such rearrangements of interfacial water molecules have been observed in other antigen-antibody complexes when large residues (such as Tyr and Trp) are replaced by smaller residues (32–34). The smaller decrease in enthalpy change in the Ala substitution mutant (LY50A) than that of the Phe substitution mutant (LY50F), despite the large decrease in interfacial area (~128 Å²) (supplemental Table 3) and the loss of many van der Waals contacts (supplemental Table 4), is because of the formation of a hydrogen-bonding network by the water molecules introduced at the mutation site of the LY50A mutant complex. No new water molecules were introduced in the LY50F complex (16). These newly formed hydrogen bonds presumably led to larger negative enthalpy changes compared with the LY50F mutant.

### Table 2

**r.m.s.d. in the C-α atoms of each chain (Å)**

|             | VL fit | VH fit | HEL fit | Fv fit | All fit |
|-------------|--------|--------|---------|--------|---------|
| LY50A vs. WT | 0.28   | 0.67   | 1.62    | 0.41   | 0.56    |
| VH          | 0.81   | 0.20   | 0.98    | 0.31   | 0.37    |
| HEL         | 1.68   | 1.04   | 0.33    | 1.11   | 0.65    |
| HY33F vs. WT | 0.07   | 0.09   | 0.16    | 0.08   | 0.09    |
| VH          | 0.12   | 0.07   | 0.17    | 0.07   | 0.09    |
| HEL         | 0.18   | 0.17   | 0.09    | 0.15   | 0.10    |
| HY33AY53A vs. WT | 0.30  | 1.15   | 1.98    | 0.44   | 0.76    |
| VH          | 0.88   | 0.55   | 3.38    | 0.68   | 1.09    |
| HEL         | 2.21   | 2.73   | 0.50    | 2.72   | 1.10    |
| HY50F vs. WT | 0.09   | 0.20   | 0.71    | 0.10   | 0.14    |
| VH          | 0.18   | 0.12   | 0.82    | 0.13   | 0.17    |
| HEL         | 0.46   | 0.45   | 0.12    | 0.42   | 0.24    |
| HY33F vs. WT | 0.13   | 0.14   | 0.22    | 0.13   | 0.14    |
| VH          | 0.15   | 0.13   | 0.21    | 0.13   | 0.14    |
| HEL         | 0.17   | 0.19   | 0.14    | 0.19   | 0.15    |
| HY33A vs. WT | 0.07   | 0.08   | 0.13    | 0.07   | 0.07    |
| VH          | 0.09   | 0.07   | 0.16    | 0.07   | 0.08    |
| HEL         | 0.09   | 0.09   | 0.07    | 0.09   | 0.08    |
| HY58A vs. WT | 0.14   | 0.20   | 0.29    | 0.15   | 0.15    |
| VH          | 0.21   | 0.18   | 0.33    | 0.18   | 0.20    |
| HEL         | 0.19   | 0.28   | 0.13    | 0.18   | 0.15    |

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**LTyr-50, the Aromatic Ring Is Important for Maintaining the Correct Orientation of the Ternary Complex**—Our previous report showed that the hydroxyl group of LTyr-50 formed a hydrogen bond via interfacial water molecules with a large binding enthalpy and a large entropic loss, indicating that this “stiffened” the antigen-antibody complex by hydrogen bonding via the interfacial water molecules (16). The relative orientation between Fv and HEL is largely different between the wild-type and mutant complexes, suggesting that the aromatic ring of LTyr-50 is important not only for making interfacial contacts but also for maintaining the correct orientation of the antigen-antibody complex. The large space generated by the Ala substitution at site L50 is occupied by water molecules that form a hydrogen-bonding network. The presence of water molecules in this space could induce structural changes in the side chain of Lys-49, change the direction of the side chain of HAsp-99, and ultimately change the relative orientations of VL-HEL and VL-VH (Fig. 3a and Table 2). A rearrangement of the relative orientation of VL and VH was also observed in the Ala substitution mutant of HAsp-99, which forms a salt bridge to Lys-97 of HEL, by the single new water molecule introduced to the mutation site (15). Such rearrangements of interfacial water molecules have been observed in other antigen-antibody complexes when large residues (such as Tyr and Trp) are replaced by smaller residues (32–34). The smaller decrease in enthalpy change in the Ala substitution mutant (LY50A) than that of the Phe substitution mutant (LY50F), despite the large decrease in interfacial area (~128 Å²) (supplemental Table 3) and the loss of many van der Waals contacts (supplemental Table 4), is because of the formation of a hydrogen-bonding network by the water molecules introduced at the mutation site of the LY50A mutant complex. No new water molecules were introduced in the LY50F complex (16). These newly formed hydrogen bonds presumably led to larger negative enthalpy changes compared with the LY50F mutant.
Hydroxyl Group of HTyr-33, Single Hydrogen Bond with Large Enthalpic Advantage and Entropic Loss—HTyr-33 is located at the center of the antigen-antibody interface and is one of the hot spots in the HyHEL-10-HEL interaction. The structure of HY33F-HEL complex is almost identical to that of the wild-type complex. This suggests that the decrease in binding enthalpy ($\Delta H$; $-18.3$ kJ mol$^{-1}$) could be attributed simply to the loss of a hydrogen bond between the hydroxyl group of HTyr-33 and the carbonyl oxygen of Lys-97 in HEL, and the contribution toward the Gibbs energy of this hydrogen bond could be estimated to be $4.6$ kJ mol$^{-1}$ by simply subtracting the $\Delta G$ of HY33F from wild type (Table 1) (11). The hydroxyl groups of HTyr-33 are energetically significant. In general, the energetic contribution of the hydrogen bond completely buried at the interface is quite large (34). Such strong hydrogen bonds have also been observed in other protein-protein interactions, such as the thermolysin-inhibitor interaction (35) and the influenza hemagglutinin-antibody interaction (36). The structure also showed that the decrease in polar area ($-26$ Å$^2$) and the increase in nonpolar area ($+32$ Å$^2$). Therefore, the reduction in entropic loss ($T\Delta S$; $13.7$ kJ mol$^{-1}$) compared with wild type is most likely due to the increase in hydrophobic area, which is in agreement with the consensus that the more hydrophobic the interface, the less entropic loss the interaction tends to have (Table 1) (37).

HTyr-50, Hydroxyl Group That Makes High Energy Hydrogen Bonds with Antigen Residues—HTyr-50 is also one of the hot spots in the HyHEL-10-HEL interaction. According to a previous study (11), the hydrophobic and bulky side chain at H50 is necessary for this interaction. The hydroxyl group of HTyr-50 forms three hydrogen bonds to nitrogens N$_1$ and N$_2$ of Arg-21 and to the carbonyl oxygen (O) of Ser-100 in the wild-type Fv-HEL interaction. In the Phe mutant (HY50F)-HEL interaction, loss of the hydroxyl group led to a large reduction in binding affinity. Thermodynamic analysis showed a large decrease in binding enthalpy ($\Delta H$; $-31.7$ kJ mol$^{-1}$), which was compensated for by the large decrease in entropic loss ($T\Delta S$; $24.6$ kJ mol$^{-1}$), resulting in some loss of Gibbs energy ($\Delta G$; $7.1$ kJ mol$^{-1}$) (11). It is conceivable that the large decrease in the negative enthalpy change originates mainly from the loss of hydrogen bonds and the amino-aromatic interaction. On the other hand, the large decrease in entropic loss might be due to a lack of stability or “rigidity” around the mutated site. It has been reported that HEL Arg-21 contributes strongly to the affinity of the HyHEL-10-HEL and HyHEL-63-HEL interactions ($\Delta G$; 6 to 8 kJ mol$^{-1}$) (17, 18, 38, 39) The large structural change around Arg-21 may be a result of the loss of hydrogen bonds with HTyr-50, suggesting that the hydroxyl group of HTyr-50 forms important hydrogen bonds that tether the

![FIGURE 3. Comparison of local structures (at the mutation site) between mutant and wild-type Fv-HEL complexes. a, LY50A-HEL; b, HY33F-HEL; c, HY50F-HEL; d, HY58A-HEL; and e, HY53A-HEL. C-α atoms of all polypeptide chains of each mutant complex are superimposed on those of the wild-type complex. Wild-type complex is shown in gray. Residues of VL, VH, and HEL of the mutant complexes are shown in green, cyan, and pink, respectively. Water molecules are shown as red balls. Hydrogen bonds in the mutant complexes and wild-type complexes are depicted as red dotted lines and gray dotted lines, respectively. The amino-aromatic (cation-π) interaction is represented as a gray thick dashed line.](image-url)
side chain of HEL Arg-21 and stabilize the structure in the vicinity of HTyr-50 and Arg-21.

HTyr-58, Substitution of Ala for Tyr Shows Enthalpic Contribution of the Amino-Aromatic (Cation-π) Interaction—In the HyHEL-10 Fv-HEL complex, the aromatic ring of HTyr-58 participates in an amino-aromatic interaction with Arg-21 of HEL, and the hydroxyl group participates in hydrogen bonding via a water molecule in the wild-type complex. The energetic contribution of the hydroxyl group of HTyr-58 is very small (11). Structural analysis of the SFSF-HEL complex, in which HTyr-58 is mutated to Phe, has shown that there are no structural changes associated with the mutation (40). Although the affinity constant of the Ala or Leu mutant of HTyr-58 was almost the same as that of the wild type, suggesting that the aromatic ring of HTyr-58 is important for binding (11). The Ala mutant (HY58A)-HEL interaction showed a large decrease in binding enthalpy ($\Delta H; 19.2$ kJ mol$^{-1}$) and an entropic loss ($T\Delta S; 12.1$ kJ mol$^{-1}$) (Table 1).

Although HTyr-58 has much fewer van der Waals contacts than HTyr-53, the decrease in binding enthalpy of the HY58A-HEL interaction was larger than that of HY53A, indicating that the energetic contribution of the amino-aromatic interaction is quite high. Levitt and Perutz (41) proposed that the binding energy of an amino-aromatic interaction is worth $12$ kJ mol$^{-1}$, with a nitrogen-benzene distance between $2.9$ and $3.6$ Å. The distance between the N-atom of the guanidinium group of Arg-21 and the aromatic ring of HTyr-58 is about $3.4$ Å. Furthermore, the orientation between the guanidinium group of Arg-21 of HEL and the aromatic ring of HTyr-58 has a hydrogen bond-like geometry. Therefore, the enthalpic contribution of the amino-aromatic interaction is estimated to be nearly $13$ kJ mol$^{-1}$ by subtracting the contributions of the hydrogen bond and van der Waals contacts. This might be the first report of a structural and thermodynamic analysis of the contributions of amino-aromatic interactions to a protein-protein interaction.
**Structural Consequences of Mutations in Interfacial Tyrosine**

HTyr-33 and HTyr-53, Double Ala Mutation into Sites H33 and H53 Suggests the Mechanism of Fit Induced by the HTyr-33 Residue—Ala substitution at HTyr-53 did not lead to the large Gibbs energy change; however, large decreases in binding enthalpy and entropic loss were observed in the HY33A-HEL interaction. Although the structure of the HY53A-HEL complex is almost identical to the wild-type-HEL complex, the $B$ factor in the loop around Asp-101 of HEL increased by interacting with the aromatic ring of HTyr-53, suggesting that the aromatic ring of HTyr-53 compacts and stiffens this region and might cause the entropic loss.

Hydropathy, in particular, because of the aromatic ring is important for binding at the site of H33 (11, 42). In our previous report, we were unable to purify the Ala substitution mutant of HTyr-33 (HY33A) by HEL-Sepharose affinity chromatography (11). Recently, Li et al. (42) performed a thermodynamic analysis of an HY33A mutant of the anti-HEL antibody HyHEL-63 and showed that the affinity of HY33A for HEL was significantly decreased ($K_a = 2.0 \times 10^5 \text{m}^{-1}$) compared with that of the wild type. Thus, the aromatic ring of H33 is crucial for binding of the antibody to HEL.

Ala substitution at HTyr-33 is likely to cause the large shift in the CDR-H2 loop in the direction of the mutated site, because substitutions at HTyr-53 do not lead to such a structural change. This suggests that HTyr-33 plays an important role in stabilizing the CDR-H2 loop upon antigen-antibody binding. On the other hand, the local structure around Asp-101 of HEL in the HY33A/Y53A-HEL complex is different from that of the wild type but similar to that of the free HEL. The local structure around these same positions in the HY33A mutant complex is similar to that in the wild type, indicating that HTyr-33 contributes to the induced fitting. Structural perturbation as a result of induced fitting around Trp-62 and Asp-101 of HEL has been investigated by thermodynamic analyses of the Gly substitution mutants of Trp-62 and Asp-101 of HEL in our previous report (10).

Interestingly, the double Ala mutant (HY33A/Y53A) had higher affinity than the single HY33A mutant. HTyr-53 seems to have a negative effect on binding without the aid of HTyr-33. In light of our results, we propose the following two mechanisms. One is that HTyr-33 plays a central role in induced fitting. As a result of induced fitting by HTyr-33, HTyr-53 can be at the proper position to form hydrogen bonds and van der Waals interactions, compacting and stabilizing the local structure around Asp-101 of HEL. Several examples of induced fitting in antigen-antibody interactions have been reported (43, 44), including the interaction of the anti-HEL antibody D1.3 with HEL. In these cases, induced fit appears to have involved conformational rearrangements between the VL and VH chains, rather than local structural changes (43). However, in our case, Tyr residues appears to involve local structural changes in the antigenic structure. The other possible role of HTyr-33 is that it stabilizes the CDR-H2 loop. HTyr-53 in the CDR-H2 loop protrudes from the binding surface of HyHEL-10. It should be noted that multiple conformations have been observed in the CDR-H2 loop of unbound HyHEL-63 (42, 45), indicating that the CDR-H2 loop has some flexibility. Loss of stabilization of the CDR-H2 loop by the aromatic ring of HTyr-33 might result in a fluctuation in HTyr-53, which hinders the binding of HyHEL-10 to HEL. The protruding HTyr-53 on the flexible loop of CDR-H2 obstructs binding to HEL in the absence of stabilization by the hydrophobic side chain of HTyr-33.

As observed in the HyHEL-10 Fv-HEL complex, the structure around Asp-101 of HEL in the Fab-HEL complex also changed in the same way upon complexation (13, 19). In addition, there are no large structural differences between Fab and Fv complexes in CDR-H2 loop and the surrounding residues, suggesting that this binding mode might occur in the Fab-HEL interaction.

**Roles of Tyr Residues in Protein-Protein Interactions; Conclusion**—In general, Tyr residues can contribute to binding via the following: 1) hydrogen bonding via the side-chain hydroxyl group; 2) hydrophobic interactions; 3) van der Waals interactions; and 4) amino-acromatic (cation-π) interactions via the aromatic ring. In addition to these general roles, our combinational study of crystallographic and thermodynamic analyses of HyHEL-10 Fv mutant-HEL complexes revealed several critical roles for these Tyr residues as follows: a contribution to stabilize the neighboring residues and/or a loop region, and a contribution to the induced fitting of HEL upon antibody binding.

The distribution of Tyr residues is biased toward the antigen-binding sites of antibodies. T-cell receptors, whose structure and mechanism of production are similar to those of antibodies, specifically recognize peptide antigen displayed by the major histocompatibility complex. The affinity of T-cell receptors for major histocompatibility complex molecules is much lower than the affinity of antigen-antibody interactions. The number of Tyr residues participating in the T-cell receptor interaction is less than in the case of antibodies (data not shown). Therefore, the utilization of many Tyr residues might be one of the characteristics of antibodies, which have high affinity for their antigens. In a series of elegant studies of antibody design, Fellouse et al. (9, 46) showed that high specificity, high affinity antibody can be created using only four amino acids, Tyr, Ser, Ala, and Asp, and that Tyr contributes over 70% of the interfacial area of the antibody. Presumably, this versatile nature of the Tyr residue is used preferentially by antibodies to create high affinity. This property might also be useful in creating artificial proteins with increased affinity for a variety of targets, such as proteins, DNA, and chemical compounds.

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