The Role of Hydrogen Bonding via Interfacial Water Molecules in Antigen-Antibody Complexation

THE HyHEL-10-HEL INTERACTION*  

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To study the role of hydrogen bonding via interfacial water molecules in protein-protein interactions, we examined the interaction between hen egg white lysozyme (HEL) and its HyHEL-10 variable domain fragment (Fv) antibody. We constructed three antibody mutants (L-Y50F, L-S91A, and L-S93A) and investigated the interactions between the mutant Fvs and HEL. Isothermal titration calorimetry indicated that the mutations significantly decreased the negative enthalpy change (8–25 kJ mol⁻¹), despite some offset by a favorable entropy change. X-ray crystallography demonstrated that the complexes had nearly identical structures, including the positions of the interfacial water molecules. Taken together, the isothermal titration calorimetric and x-ray crystallographic results indicate that hydrogen bonding via interfacial water enthalpically contributes to the Fv-HEL interaction despite the partial offset because of entropy loss, suggesting that hydrogen bonding stiffens the antigen-antibody complex.

Specific recognition of ligands by proteins is fundamentally important in biological phenomena. Recent high resolution x-ray crystallographic determinations of the structures of various protein-protein complexes have shown that protein-protein interfaces are generally characterized by a high degree of both shape and charge complementarity (1–3). The specificity and affinity of protein-protein interactions are in principle created by the complementarity of the interface surfaces, which allows the formation of various noncovalent bonds (hydrogen bonds, salt bridges, and van der Waals interactions) (1–3). Solvent water molecules, however, have been observed at protein-protein interfaces, mediating imperfect surface complementarity via hydrogen bond formation (1, 4–7).

Solvent plays a significant role in biologically important protein association systems (8), e.g. the adhesion interface of the cell adhesion protein cadherin (9), the barnase-barstar complex (5), the cytochrome c-cytochrome c oxidase interaction (10, 11), antigen-antibody interactions (12–16), the bacterial periplasmic protein (OppA-peptide interaction (17, 18), the DNA-repressor interaction (19, 20), the interaction between T-cell receptor and self-peptide major histocompatibility complex antigen (21), and the natural killer cell receptor-major histocompatibility complex antigen interaction (22). Ladbury (5) proposed that water at the interface of a complex can participate in various types of interaction, yet can also lead to increased specificity and affinity. However, the energetic contribution of hydrogen-bonded interfacial water molecules to protein-protein interactions has been determined for only a few complexes (18, 23).

Elucidation of the roles of the hydrogen bonds involved in antigen-antibody complementary association requires both structural and thermodynamic information (24). X-ray crystal analysis can clarify the structural aspects of the complementarity of the interactions (25–28), and titration calorimetry can provide useful information for the quantitative assessment of the energetic contribution of residues to the interaction (29–31). Thus, the combination of these two approaches should be especially valuable in providing insight into hydrogen bonding via interfacial water molecules.

We have focused on the interaction between hen egg white lysozyme (HEL) and its monoclonal antibody HyHEL-10 (32–34), whose structural features have been analyzed by x-ray crystallography in the Fab-HEL (35) and Fv-HEL (36) complexes. The bacterial expression system of the HyHEL-10 Fv fragment, which consists of the associated variable domains of an antibody, has been established (37–39); and the Fv-HEL interactions have been investigated by using mutant Fv fragments (40–45). The combination of thermodynamic data with structural results should be a powerful tool for the precise description of the mutant Fv-HEL interactions.

In the wild-type Fv-HEL complex, Kondo et al. (36) observed 12 water molecules bridging the imperfect antigen-antibody interface (Fig. 1). Kondo’s x-ray crystallographic study indicated that the temperature factor (B factor) of these water

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The atomic coordinates and structure factors (code 1J1O, 1J1P, and 1J1X for L-Y50F, L-S91A, and L-S93A, respectively) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

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† The abbreviations used are: HEL, hen egg white lysozyme; VH, variable region of immunoglobulin heavy chain; VL, variable region of immunoglobulin light chain; Fv, fragment of immunoglobulin variable regions; Fab, antigen binding fragment of immunoglobulin; WT, wild-type; L-Y50F, mutant; mutant HyHEL-10 Fv in which Tyr50 of the VL chain is substituted with Phe; ITI, isothermal titration calorimetry; CDR, complementarity determining region.
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molecules is small, and thus, the contribution of these solvent water molecules to the Fv-HEL interaction is assumed to be significant. Therefore, thermodynamic analyses of mutant Fv-HEL interactions combined with structural analyses of the Fv-HEL complexes should give further insight into the molecular basis for the role of hydrogen bond formation via interfacial water molecules in the interaction. In this report, we have focused on three residues (Tyr\(^{50}\), Ser\(^{91}\), and Ser\(^{98}\)) in the light chain. These residues participate in the formation of the hydrogen bond network (Fig. 1B), and thus, the mutational analyses should give further insight into the role of the hydrogen bonds.

Here, using isothermal titration calorimetry and x-ray crystallography, we report the effect on complex formation of truncating atoms of antibody side chains participating in hydrogen bond formation via water molecules. On the basis of our thermodynamic and structural results, we discuss the role of the hydrogen bonds in the protein antigen-antibody interaction.

EXPERIMENTAL PROCEDURES

Materials—All enzymes for genetic engineering were obtained from Takara Shuzo (Kyoto, Japan), Toyobo (Osaka, Japan), and New England Biolabs (Beverly, MA). Isopropyl-\(\beta\)-D-thiogalactopyranoside was obtained from Wako Fine Chemicals Inc. (Osaka, Japan). All other reagents were of biochemical research grade. The antigen, HEL (Seikagaku-Kogyo, Tokyo, Japan), was purified by ion exchange chromatography on SP-Sephadex FF (Amersham Biosciences, Tokyo, Japan), followed by gel filtration on Superdex 75 pg equilibrated with phosphate-buffered saline. The eluate was lyophilized and dissolved in water at a concentration of 0.54 mg prior to use.

Site-directed Mutagenesis—Site-directed mutagenesis was performed with phagemid pTZ18U (Bio-Rad) according to the method of Kunkel et al. (46). The DNA oligonucleotide primers for mutation at sites 50, 91, and 93 of VL were 5'-CTGATCAAGTTCCGAGGCAAGCAG-3', 5'-CGCCACAGCGGCAAACGCTCG-3', and 5'-GCTGCTGAAGCCTGCCCTAC-3', respectively (mutated sites are underlined). The correctness of the mutants was confirmed by DNA sequencing (ABI 310 Genetic Analyzer, Applied Biosystems, Tokyo, Japan).

Preparation of HyHEL-10 Mutant Fv—The mutant Fv fragment was prepared essentially as described previously (35). The purified Fv fragment was concentrated by ultrafiltration centrifugation (0.45-\(\mu\)m membrane) at 3000 \(\times\) g (Centrigrad-10, Japan-Millipore, Tokyo). The purified Fv-HEL complexes were confirmed by SDS-PAGE in the buffer system described by Laemmli (47).

Inhibition Assay of HEL Enzymatic Activity—The experimental procedure for the inhibition assay was essentially that of Ueda et al. (37). In brief, the various concentrations of the Fv fragment were mixed with 1.5 \(\mu\)M HEL and incubated at 25 °C for 1 h in 30 \(\mu\)l of phosphate-buffered saline. Each mixture was then added to 970 \(\mu\)l of 50 mm NaHPO\(_4\) buffer (pH 6.20, adjusted with NaOH) containing 340 \(\mu\)g of Micrococcus luteus cells. The initial rate of the decrease in A\(_{540}\) nm was monitored at 25 °C.

Isothermal Titration Calorimetry—The thermodynamic parameters of the interactions between HEL and the wild-type or mutant Fv fragments were determined by isothermal microcalorimetry (VP-ITC, MicroCal, Northampton, MA) (48) under conventional conditions as follows. In a calorimeter cell, a 5 \(\mu\)M solution of HEL in 50 mm phosphate buffer (pH 7.2) containing 200 mm NaCl was titrated with a 50 \(\mu\)M solution of Fv in the same buffer at five temperatures (20, 25, 30, 35, and 40 °C). The ligand solution was injected 25 times in portions of 10 \(\mu\)l over 20 s. Thermogram data were analyzed with the computer program Origin version 5 (MicroCal) after subtraction of the heats of dilution of Fv fragment into buffer and buffer into HEL (obtained in separate experiments).

To correlate thermodynamic data with structural data, the thermodynamic parameters of the interactions were determined under crystallization conditions as follows. In a calorimeter cell, a 5 \(\mu\)M solution of HEL in 100 mm Hepes buffer (pH 7.6), 1% (w/v) polyethylene glycol 6000, 15% glycerol, and 1% (w/v) 2-methyl-2,4-pentanediol was titrated with a 50 \(\mu\)M solution of Fv in the same buffer at five temperatures (20, 25, 30, 35, and 40 °C). The ligand solution was injected 25 times in portions of 10 \(\mu\)l over 20 s. Thermogram data were analyzed as described above after subtraction of the heats of dilution of the Fv fragment into buffer and buffer into HEL (obtained in separate experiments).

The enthalpy change (\(\Delta H\)) and binding constant (\(K_b\)) for the antigen-antibody interaction were directly obtained from the experimental titration curve. The Gibbs free energy change (\(\Delta G = -RT\ln K_b\)) and the entropy change (\(\Delta S = (-\Delta H + \Delta G)/T\)) for the association were calculated from \(\Delta H\) and \(K_b\). The heat capacity change (\(\Delta C_p\)) was estimated from the temperature dependence of the enthalpy change.

Estimation of Protein Concentration—The concentration of HEL was estimated by using \(A_{540}^{11000}\) = 26.5 (49). The concentrations of the HyHEL-10 Fv fragments were estimated by using \(A_{540}^{150}\) = 20.6 (37) for the WT and l-s91A and l-s93A mutants, and \(A_{540}^{150}\) = 20.2 (38) for the l-y50F mutant.

Crystallization, Data Collection, and Structural Determination of the HyHEL-10 Mutant Fv-HEL Complexes—The HyHEL-10 mutant Fv fragment-HEL complexes were crystallized as described previously (36). The best crystals of the Fv-HEL complexes were grown from 100 mm Hepes buffer (pH 7.8), 9–11% (w/v) polyethylene glycol 6000, 15% glycerol, and 7–9% (w/v) 2-methyl-2,4-pentanediol. They were elongated bipyramid-shaped crystals.

All crystallographic data were collected at 100 K using synchrotron radiation on beam line 6A of the Photon Factory (Tsukuba, Japan) and a Weissenberg camera (50). The diffraction images were integrated and scaled with the crystal of the wild-type complex. The structures of the mutant complexes were determined by molecular replacement with the program AMoRe (52) in the CCP4 suite. The model coordinates of the mutant complexes were derived from the structure of the wild-type complex (Protein Data Bank ID code 1e08). Refinement of the structures of the Fv-HEL complexes were carried out with the programs XPLOR (53) and REFMAC (54) as described previously (36).
Inhibition of Enzymatic Activity of Hen Lysozyme by Mutants

Tsumoto, Kumagai, and co-workers (37, 38, 41) have shown that the HyHEL-10 Fv fragment inhibits the enzymatic activity of its antigen, HEL, in the presence of a slight molar excess of the Fv fragment. Thus, we investigated the inhibition of the enzymatic activity of HEL by three mutants (L-Y50F, L-S91A, and L-S93A) (Fig. 2).

The L-S93A mutant Fv, L-S91A mutant Fv, and wild-type Fv fragments showed identical inhibition activities, whereas the L-Y50F mutant Fv fragment showed a slight decrease in inhibition activity toward HEL, suggesting that the L-Y50F, L-S91A, and L-S93A mutations do not lead to a significant reduction of the affinity for the target.

Thermodynamic Analysis of the Interactions between Hen Lysozyme and Mutant Fv Fragments

To investigate the interactions between the mutants and HEL from a thermodynamic viewpoint, we carried out an iso-
thermal titration calorimetry (ITC) study of the association between the mutant Fv fragments and lysozyme (41–45) (Fig. 3). We initially performed ITC at five temperatures under conventional conditions, i.e. phosphate buffer. Thermodynamic parameters (30 °C, pH 7.2) calculated from the titration curves are presented in Table II, and the temperature dependence of the enthalpy changes are shown in Fig. 4A.

The binding enthalpies for each of the mutant Fv-HEL interactions relative to the wild-type Fv-HEL interaction were decreased by 24.9, 11.0, and 8.5 kJ mol⁻¹ at 30 °C for L-Y50F, L-S91A, and L-S93A, respectively (Table II). The binding constants for the mutant interactions were smaller than the binding constant for the wild-type interaction, resulting in a smaller change in the Gibbs energy. These results indicate that the decreases in the negative enthalpy change for each mutant Fv fragment were compensated for by the decrease in the negative entropy change, leading to the slight decrease in affinity. From the temperature dependence of the enthalpy change shown in Fig. 4A, the heat capacity change was estimated to be −1.62 to −1.86 kJ mol⁻¹ K⁻¹ at 30 °C for the mutant Fvs.

To correlate the results obtained from ITC to the crystallo-
graphic study, we next performed an ITC study of the association at four temperatures under the crystallization buffer conditions. Thermodynamic parameters (30 °C, pH 7.6) calcu-

TABLE I
Crystallographic data of mutant Fv-HEL complexes

|                | L-Y50F | L-S91A | L-S93A | Wild type* |
|----------------|--------|--------|--------|------------|
| Space group, P4,2,2 |        |        |        |            |
| Unit cell dimensions | a = b = 56.2 Å | a = b = 56.2 Å | a = b = 56.2 Å | a = b = 57.0 Å |
| c = 234.5 Å | c = 234.8 Å | c = 234.6 Å | c = 236.1 Å |
| Vₐ (Å³) | 2.4 (Z = 8) | 2.4 (Z = 8) | 2.4 (Z = 8) | 2.4 (Z = 8) |
| Wavelength (Å), 1.0000 |        |        |        |            |
| Resolution (Å) | 1.8 | 1.8 | 1.8 | 1.8 |
| Rₚ⁸₉₉⁺, (%) | 0.065 (0.252) | 0.060 (0.156) | 0.067 (0.156) | 0.076 (0.265) |
| Compl. (%) | 99.5 | 100 | 99.9 | 99.4 |
| Multiplicity | 5.8 | 14.0 | 14.1 | 7.0 |
| Resolution range (Å) | 8–1.8 | 8–1.8 | 8–1.8 | 8–1.8 |
| R factor | 0.190 | 0.191 | 0.189 | 0.194 |
| Free R factor | 0.215 | 0.212 | 0.217 | 0.220 |
| Root mean square deviation bond length (Å) | 0.004399 | 0.004437 | 0.004468 | 0.004464 |
| Root mean square deviation bond angle (°) | 1.29 | 1.28 | 1.28 | 2.5 |
| No. of water molecules | 343 | 364 | 357 | 330 |

* Kondo et al. (36).

Rₚ⁸₉₉⁺ = (Σobs (I) – (I))/[Σcalc (I)].
R factor = (Σ|Fobs| – |Fcalc|)/Σ|Fobs|.

The atomic parameters of the mutant Fv-HEL complexes have been deposited in the Protein Data Bank (ID codes 1J1O, 1J1P, and 1J1X for L-Y50F, L-S91A, and L-S93A, respectively).

RESULTS

Secretory Expression, Preparation, and Spectral Properties of Mutant Fv Fragments

To determine the energetic contribution of hydrogen bonds bridging the imperfect interface of the HyHEL-10 Fv-HEL complex to the antigen-antibody interaction, three mutants (L-Y50F, L-S91A, and L-S93A) were constructed and expressed in Escherichia coli. The mutant Fv fragments were purified by affinity chromatography using HEL-Sepharose, followed by gel filtration on Superdex 75 pg. Purities of greater than 95% were obtained by this procedure. The final yield of each mutant was greater than 10 mg/liter of culture.

The peptide and aromatic circular dichroism (CD) spectra of the mutants under the native condition (pH 7.2, 25 °C) were measured to examine the effect of mutation on the conformational stability. Comparison of these CD spectra with those of the wild-type (WT) revealed no differences in the far- and near-UV CD spectra of the wild-type Fv and mutant Fv fragments (data not shown), indicating that the secondary and tertiary structures of the mutants are quite similar to the structure of WT. Thus, the mutations have no significant influence on the overall structure.

Inhibition of Enzymatic Activity of Hen Lysozyme by Mutants

The peptide and aromatic circular dichroism (CD) spectra of the mutants under the native condition (pH 7.2, 25 °C) were measured to examine the effect of mutation on the conformational stability. Comparison of these CD spectra with those of the wild-type (WT) revealed no differences in the far- and near-UV CD spectra of the wild-type Fv and mutant Fv fragments (data not shown), indicating that the secondary and tertiary structures of the mutants are quite similar to the structure of WT. Thus, the mutations have no significant influence on the overall structure.
The heat capacity change was estimated to be slight decrease in, or almost identical, affinity. From the temperatures indicated that the decreases in the negative enthalpy changes are obtained with the OMEGA calorimeter (MicroCal). In several experiments, the values obtained with the VP-ITC calorimeter (MicroCal) were found to be slightly different from the previous values.

Experimental protocols are described under "Experimental Procedures." Each value is the average of at least three independent measurements. Abbreviations used: n, stoichiometry; \( K_a \), binding constant; \( \Delta G \), \( \Delta H \), \( \Delta S \), and \( \Delta C_p \), changes in Gibbs energy, binding enthalpy, entropy, and heat capacity, respectively. \( \Delta G_e \), \( \Delta H_e \), \( \Delta S_e \), and \( \Delta C_p \) are the differences in each of these values from those of the wild-type Fv.

| Mutant         | n   | \( K_a \) \( \times 10^4 \mu \text{M}^{-1} \) | \( \Delta G \) kcal mol\(^{-1} \) | \( \Delta G_e \) kcal mol\(^{-1} \) | \( \Delta H \) kcal mol\(^{-1} \) | \( \Delta H_e \) kcal mol\(^{-1} \) | \( \Delta S \) | \( \Delta S_e \) | \( \Delta C_p \) | \( \Delta C_p \) |
|----------------|-----|--------------------------------|----------------|----------------|----------------|----------------|-----------|-----------|-------------|-------------|
| Wild type*     | 1.05 ± 0.05 | 8.21 ± 1.64 | -51.7 ± 2.6 | 0 | -99.7 ± 5.0 | -0.158 ± 0.010 | 0 | -1.53 ± 0.10 | 0 |
| L-Y50F         | 1.05 ± 0.05 | 1.12 ± 0.20 | -46.7 ± 2.2 | 5.0 | -74.8 ± 3.5 | 24.9 | -0.093 ± 0.008 | 0.065 | -1.77 ± 0.12 | -0.24 |
| L-S91A         | 1.04 ± 0.05 | 3.63 ± 0.72 | -49.7 ± 2.4 | 2.0 | -88.7 ± 4.4 | 11.0 | -0.129 ± 0.009 | 0.029 | -1.62 ± 0.12 | -0.09 |
| L-S93A         | 1.07 ± 0.06 | 7.41 ± 1.52 | -51.4 ± 2.5 | 0.3 | -91.2 ± 4.6 | 8.50 | -0.131 ± 0.009 | 0.027 | -1.86 ± 0.13 | -0.33 |

* In several experiments, the values obtained with the VP-ITC calorimeter (MicroCal) were found to be slightly different from the previous values obtained with the OMEGA calorimeter (MicroCal).

The binding enthalpies for each of the mutant Fv-HEL interactions relative to the wild-type Fv-HEL interaction were decreased by 27.5, 18.4, and 5.1 kcal mol\(^{-1} \) at 30 °C for L-Y50F, L-S91A, and L-S93A, respectively (Table III). The values of the binding constants (\( K_a \)) for the L-Y50F and L-S91A mutant interactions were smaller than the \( K_a \) for the wild-type interaction, whereas the \( K_a \) for the L-S93A mutant interaction was almost identical to the \( K_a \) for the wild-type interaction. These results indicate that the decreases in the negative enthalpy change for each mutant Fv fragment were compensated for by the decrease in the negative entropy change, leading to the slight decrease in, or almost identical, affinity. From the temperature dependence of the enthalpy change shown in Fig. 4, the heat capacity change was estimated to be -1.28 to -2.05 kcal mol\(^{-1} \) K\(^{-1} \) at 30 °C for the mutant Fvs.

Crystal Structures of Mutant Fv-HEL Complexes

The crystallographic data are summarized in Table I. The maximum resolution of the x-ray data used in the refinements was 1.8 Å, and the \( R \) factors of the refined structures were 0.189–0.194. The free \( R \) factors were within 3% of the \( R \) factors.

The mutant Fv-HEL complexes were superimposed onto the wild-type Fv-HEL complex. The root mean square differences between the CO atoms of the mutant Fv structures and those of the wild-type Fv structure are summarized in Table IV. The results demonstrated that the overall structures of the HyHEL-10 mutant Fv-HEL complexes are similar to the structure of the HyHEL-10 wild-type Fv-HEL complex.

No major changes in the relative orientations of VL, VH, and HEL were observed in the mutant Fv-HEL complexes (data not shown). The calculated interfacial areas of the mutant Fv-HEL complexes were 1853, 1879, and 1851 Å\(^2\) for the L-Y50F, L-S91A, and L-S93A mutant complexes, respectively (data not shown). Among 330 water molecules observed in the wild-type complex, 296, 307, and 296 water molecules in L-Y50F, L-S91A, and L-S93A complexes, respectively, have been completely on the surface of the structure. The locations of the interfacial water molecules of the mutant Fv-HEL complexes are almost the same as the overall structure of the wild-type Fv complex.

Local Structural Changes Observed in Mutant Fv-HEL Complexes

L-Y50F Mutant-HEL Complex—Although significant structural changes occurred around sites 17–19 of the antigen HEL in the L-Y50F Fv-HEL complex, no structural differences were observed around other sites, including the mutated site (Fig. 6). In addition, the locations of the interfacial water molecules...
were unchanged. Thus, two hydrogen bonds involving the hydroxyl group of Tyr\(^{50}\) in CDR-L2 were removed because of the mutation, and no structural changes involving interfacial water molecules and the same number of hydrogen bonds as wild-type were observed.

**L-S91A Mutant-HEL Complex**—No structural changes were observed in the Fv fragment of the L-S91A Fv-HEL complex, except at the mutated site. One water molecule was newly introduced into the mutation site (W3; the \(2F_o - F_i\) electron density map of the WT-HEL complex around L-S91A is shown in Fig. 7A, and the corresponding region of the L-S91A-HEL complex is shown in Fig. 7B), bridging i-Ase\(^{32}\), i-His\(^{34}\), and a water molecule (W13, corresponding to W2 in the wild-type complex), and this new water molecule contributed to the formation of the hydrogen-bonding network at the interface. The B factor of the newly introduced water molecule (W3) is estimated to be 12.5, which is smaller than the average value (16.8) of the Ca chains of the complex. Thus, two hydrogen bonds have been increased, and no other structural changes have been induced because of the mutation.

**L-S93A Mutant-HEL Complex**—In contrast to the structures of the L-Y50F Fv-HEL and L-S91A Fv-HEL mutant complexes, the structures of the antigen and antibody of the L-S93A mutant Fv-HEL complex were unchanged except for deletion of one hydrogen bond because of the mutation (Fig. 8). Thus, one hydrogen bond has been removed because of the mutation, and no other structural changes have been induced.

**DISCUSSION**

Many researchers have proposed that interfacial water molecules make important contributions to the specificity and affinity of antigen-antibody interactions (6–16). To investigate the energetic contribution of hydrogen bonding via interfacial water molecules, we constructed three mutants (L-Y50F, L-S91A, and L-S93A) and investigated the interactions of these mutants with HEL by thermodynamic and structural analyses. As reported in previous work (8, 55) and in this study, the mutations do not lead to major structural changes in the Fv fragment, and the stabilities of the mutant Fv fragments are almost identical to the stability of the wild-type Fv fragment. Thus, the structural and thermodynamic changes in the antigen-antibody mutant complexes do not originate from changes in the structure of the mutants in the antigen-free state. In the following sections, we discuss and correlate our thermodynamic and structural findings.

**Thermodynamic Analysis of Mutant Fv-HEL Interactions**—The values for both the enthalpic (\(-\Delta H\)) and the entropic (\(-T\Delta S\)) contributions to the interaction between the mutants and HEL became increasingly positive in the order L-S93A < L-S91A < L-Y50F. The values of the binding constants (\(K_b\)) for the L-S91A mutant-HEL and L-S93A mutant-HEL interactions were slightly smaller than the value of \(K_b\) for the wild type-HEL interaction, whereas \(K_b\) for the L-Y50F mutant-HEL interaction was significantly smaller than \(K_b\) for the wild type-HEL interaction, resulting in a smaller change in the Gibbs energy (\(-\Delta G\)). These results indicate that deletion of hydrogen bonds via interfacial water molecules in the HyHEL-10 Fv-HEL complex is enthalpically unfavorable and entropically favorable, suggesting that the hydrogen bonds mediated by interfacial water molecules in the HyHEL-10 Fv-HEL interaction make an enthalpic contribution to the interaction. Substitution of Tyr\(^{53}\) with Phe also led to decreases in both enthalpy and entropy changes (42). Thus, we conclude that hydrogen bonding via interfacial water molecules in the HyHEL-10 Fv-HEL complex makes an enthalpic contribution to the interaction.

To correlate the results obtained from the thermodynamic analyses with structural information, we next performed ITC experiments under crystallization conditions (100 mM HEPES buffer (pH 7.6), 15% glycerol, 1% (w/v) polyethylene glycol 6000, and 1% (w/v) 2-methyl-2,4-pentanediol). The values for both the enthalpic (\(-\Delta H\)) and entropic (\(-T\Delta S\)) contributions to the interaction between the mutants and HEL became increasingly positive in the same order as in the case of phosphate buffer. The values of the binding constants (\(K_b\)) for the L-S91A mutant-HEL and L-S93A mutant-HEL interactions were slightly smaller than the value of \(K_b\) for the wild type-HEL interaction, whereas \(K_b\) for the L-Y50F mutant-HEL interaction was significantly smaller than \(K_b\) for the wild type-HEL interaction, resulting in a smaller change in the Gibbs energy (\(-\Delta G\)). These results correlate qualitatively well with the data obtained with the phosphate buffer; deletion of hydrogen bonds via interfacial water molecules in the HyHEL-10 Fv-HEL complex is enthalpically unfavorable and entropically favorable.

The affinity of the antibody, including mutants, for the target was significantly reduced in the crystallization buffer, which included polyethylene glycol 6000, glycerol, and 2-methyl-2,4-pentanediol (cf. Tables II and III). For WT, L-Y50F, and L-S93A, the enthalpy changes under the crystallization buffer conditions were almost the same as those under the phosphate buffer conditions, indicating that an increase in entropy loss led to a significant reduction of affinity. Additives such as polyethylene glycol and glycerol are known to reduce water activity and thus stabilize proteins by stabilizing the exposed hydrophobic regions of proteins (56, 57). These results highlight, therefore, the entropic contribution of dehydration upon complexation to the affinity of an antibody for the antigen.
The abbreviations used are: $n$, stoichiometry; $K_a$, binding constant; $\Delta G$, $\Delta H$, $\Delta S$, and $\Delta C_p$ changes in Gibbs energy, binding enthalpy, entropy, and heat capacity, respectively. $\Delta G$, $\Delta H$, $\Delta S$, and $\Delta C_p$ are the differences in each of these values from those of the wild-type Fv.

| Mutant    | $n$ | $K_a \times 10^4 M^{-1}$ | $kd$ mol$^{-1}$ | $kd$ mol$^{-1}$ | $kd$ mol$^{-1}$ K$^{-1}$ | $kd$ mol$^{-1}$ K$^{-1}$ |
|-----------|-----|--------------------------|------------------|------------------|--------------------------|--------------------------|
| Wild type | 1.01±0.10 | 2.66±0.50 | 43.1±2.60 | 0 | -100±5.00 | 0 | -188±0.010 | 0 | -203±0.10 | 0 |
| L-Y50F    | 1.05±0.08 | 0.62±0.05 | 39.4±2.00 | 3.7 | -72.5±3.5 | 27.5 | -109±0.006 | 0.079 | -1.28±0.05 | 0.75 |
| L-S91A    | 0.89±0.10 | 1.90±0.10 | 42.2±2.20 | 0.9 | -81.6±4.0 | 18.4 | -130±0.008 | 0.058 | -2.05±0.15 | -0.02 |
| L-S93A    | 0.98±0.08 | 3.07±0.12 | 43.4±2.50 | -0.3 | -94.9±4.5 | 5.1 | -170±0.010 | 0.018 | -1.89±0.14 | 0.14 |

Experimental protocols are described under “Experimental Procedures.” Each value is the average of at least three independent measurements.

The positions marked W correspond to the water molecules (parentheses indicate wild-type water molecules). L-Y50F antibody VL chain, green; VH chain, sky blue; HEL, pink. Generated with WebLab Viewer.

Fig. 5. Overall structure of the mutant HyHEL-10 Fv-HEL complex. The structure of the L-Y50F Fv-HEL complex, whose Ca coordinates of VL are superimposed on the Ca coordinates of VL complexed with the wild-type Fv, is superposed on the structure of Fv (gray). VL chain, green; VH chain, sky blue; HEL, pink. Generated with WebLab Viewer.

Fig. 6. Comparison of local structures in the L-Y50F-HEL and WT-HEL complexes. Region around the mutated site (50 of VL). Hydrogen bonds made by water molecules are depicted as dotted lines. The positions marked W correspond to the water molecules (parentheses indicate wild-type water molecules). L-Y50F antibody VL chain, green; VH chain, sky blue; HEL, pink; water, red; WT-HEL complex, gray.

Comparison of the Mutant Fv-HEL Structure with the Wild-type Fv-HEL Structure—Mutations did not affect the crystal packing. The overall structures of the mutant Fv-HEL complexes are similar to the structure of the wild-type Fv-HEL complex (Fig. 5). The area of HEL covered by the mutant Fvs was slightly decreased by 1–29 Å$^2$ in comparison with the wild-type Fv-HEL complex (Table IV), indicating that almost the same areas are buried upon complexation. In addition, ~90% of the water molecules in the structures of the mutant Fv-HEL complexes are identical to those in the wild-type complex. Therefore, the local structural changes because of the mutations, including interface solvents, can be discussed directly by comparing the mutant structures with the wild-type structure.

The overall structures of the L-Y50F and L-S93A mutant Fv-HEL complexes are almost identical to the structure of the wild-type Fv-HEL complex, including the interfacial areas, the number of noncovalent interactions, the location of interfacial solvent molecules, and the local structure around the mutated site. These results indicate that changes in the thermodynamic parameters (e.g., the Gibbs energy and the enthalpy changes) reflect the effect of truncating the hydrogen bonds on the interactions. Removal of the hydroxyl group from Tyr50 in the
light chain resulted in a significant reduction of the negative enthalpy change, and the overall and local structures are almost identical to the wild-type structure except for truncation of hydrogen bonds, suggesting that the decrease in the negative enthalpy change (about 25 kJ mol\(^{-1}\)) originated from deletion of two hydrogen bonds. The side chain at site 50 of VL is almost buried (the calculated accessible surface area is 3 Å\(^2\)) in the complex, which might lead to a significant reduction of the negative enthalpy change because of the mutation. However, a smaller reduction in the Gibbs energy change, originating from a smaller negative entropy change, was observed. Wilcox \textit{et al.}(58) and Murphy \textit{et al.}(59) suggested that the unfavorable entropy change results, in part, from reduction of the conformational flexibility in the antibody upon complexation. In the case of the interaction between Y82F mutant FK506-binding protein (FKBP-12) and its ligand (FK506), large increases in negative enthalpy and negative entropy change have been observed in comparison with the wild-type FKBP-12 ligand interaction (60). A crystallographic study of the complex formed between the wild-type FKBP-12 and its ligands has indicated that two water molecules are located in the hydroxyl group of Tyr\(^{82}\) in unliganded FKBP-12 and that on ligand binding these water molecules are lost and the hydroxyl group forms a hydrogen bond with the ligand. From these experimental data, it has been suggested that the formation of a hydrogen bond with the ligands results in a large decrease of negative entropy change because of removal of the water molecules (60).

In contrast, the values of the thermodynamic parameters of the interaction between the L-S93A mutant Fv and HEL are smaller than those of the other mutants. The side chain of L-Ser\(^{93}\) is partially exposed, and thus, the effect of the mutation on the interaction might be smaller than in the case of the other two mutations.

Substitution of Ser\(^{91}\) with Ala resulted in the appearance of a new water molecule at the mutated site in the mutant Fv-HEL complex. The electron density map confirmed that this water molecule, not found in the wild-type complex, was located near the mutated site, suggesting that the solvent compensated for removal of the hydroxyl group at site 91 of VL.

### Table V

| Antibody | Buried ΔASA [Å\(^2\)] | ΔC\(_p\)^b | ΔC\(_v\)^c | ΔC\(_d\)^d |
|----------|------------------------|------------|------------|------------|
|          | Total                  | Apolar     | Polar      | J K\(^{-1}\) mol\(^{-1}\) |
| Wild type| −1801                  | −865       | −936       | −607       | −1530 | −2026 |
| l-Y50F   | −1791                  | −889       | −902       | −658       | −1766 | −1278 |
| l-S91A   | −1813                  | −870       | −943       | −610       | −1628 | −2054 |
| l-S93A   | −1809                  | −882       | −927       | −635       | −1856 | −1866 |

\(^a\) Calculation was performed according to Kondo \textit{et al.} (36).

\(^b\) Calculated with the equation, ΔC\(_p\) = 1.34 ΔASA\(_{apolar}\) − 0.59 ΔASA\(_{polar}\) (62).

\(^c\) Experimentally determined in phosphate buffer. From Table II.

\(^d\) Experimentally determined in crystallization buffer. From Table III.
Two water molecules in the wild type-HEL complex were retained in the mutant complex, and these water molecules formed hydrogen bonds with the hydroxyl group of Tyr^{30} of HEL and with L-Asn^{102} and L-His^{93} of Fv, bridging HEL with the Fv (Fig. 7C). One hydrogen bond was lost because of the mutation, and three new hydrogen bonds were formed. Thus, the number of hydrogen bonds in the mutant complex increased. However, the mutation resulted in a reduction of the negative enthalpy change. These changes in the thermodynamic parameters have been described for other antigen-antibody interactions (e.g., the D1.3-HEL interaction (4, 61)) and for OppA-peptide interactions (17, 18). Recently, we reported the effect of deleting side chains participating in salt bridge formation on the HyHEL-10-HEL interaction (38). The newly introduced water molecules that were observed around the mutated sites led to the formation of several hydrogen bonds. Thermodynamic analyses of the salt bridge-deleted mutant Fv-HEL interactions showed a significant increase in the negative enthalpy change because of the formation of the hydrogen bonds (38). Taken together, these results suggest that the new water molecule in the L-Ser^{91}-HEL complex might not be newly introduced upon complexation (i.e., from bulk solvent water); rather the ligand-free antibody or antibody-free antigen might be involved in the formation of hydrogen bonds. As described above, the number of hydrogen bonds via water molecules is increased; however, less favorable ΔH and more favorable ΔS have been observed when an additional water molecule is found to bind in the interface. One possible explanation for this would be that the presence of the water molecule in the unliganded protein will reduce the entropic cost of ligand binding, as adequately discussed in OppA system (18).

The most useful tie between the thermodynamic and structural data is based on the relationship between the change in heat capacity and the change in the surface area that is exposed to solvent on going from one equilibrium state to another (62). For example, Spolar and Record (63) successfully related the thermodynamic and structural changes on formation of a protein-DNA complex. Thus, from the temperature dependence of the enthalpy changes (Figs. 4 and 5), we estimated the heat capacity changes (ΔC_p) of the interactions between the antibodies and the antigen (Tables II and III). We calculated the ΔC_p values on the basis of structural data and the equation, ΔC_p = 1.34ΔASA_{spolar} – 0.59ΔASA_{apolar}, reported by Spolar and Record (63) (Table V). The data clearly indicate a large discrepancy between the calculated values and the experimentally determined values. The discrepancy was first pointed out by Ladbury et al. (64). Possible explanations for this discrepancy are restriction of water molecules in the interface (62–65) and recognition-coupled structural changes because of induced fitting (63, 66). Precisely estimating the contribution of water molecules and structural changes is difficult; nonetheless, significant contributions of larger negative ΔC_p values may originate from interfacial water molecules, because no major conformational changes have been observed in the antigens and/or in the antibodies (6, 25–28, 67).

Role of Hydrogen Bond Formation via Interfacial Water Molecules in the HyHEL-10-HEL Interaction: Conclusion—Hydrogen bond formation via interfacial water molecules has been observed in various biomolecular interactions, and significant contributions of the hydrogen bonds to the interaction has been proposed (6, 7). Here, we have shown that the hydrogen bonds in the HyHEL-10-HEL complex are either buried (L-Tyr^{90} and L-Ser^{23}) or partially exposed (L-Ser^{50}); deletion of these hydrogen bonds spoiled to thermodynamic compensation because little structural change was introduced by the mutations. We conclude that hydrogen bond formation via interfacial water molecules makes an enthalpic contribution to the interaction despite a partial offset because of an unfavorable entropy loss. As shown previously in several biomolecular interactions, the favorable enthalpy changes are compensated for by an unfavorable entropy change (68–70), suggesting that the antigen-antibody interaction tolerates truncation of these hydrogen bonds. It has been suggested that the unfavorable entropy change results, in part, from reduction of the conformational flexibility in the antibody upon complexation (71, 72), suggesting that the hydrogen bonds stiffen the antigen-antibody complex.

Finally, we note that almost all of the interfacial water molecules were observed in the same positions as in the wild-type complex (72), despite truncation of some of the residues participating in hydrogen bond formation via interfacial water molecules. These results suggest that interfacial water molecules might make a significant contribution to the interaction through other factors, e.g., structural stabilization of the antigen, the antibody, or its complex with a smaller energetic contribution. Constant positioning of the water molecules with different bound ligands has also been described for the OppA-peptide system because of the fact that these sites are at positions of lowest energy for occupancy of water (17). Computational studies of the binding free energies based on the results reported here may provide a more precise description of the role of hydrogen bond formation via interfacial water molecules (73, 74).

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