Structural Evidence for Entropic Contribution of Salt Bridge Formation to a Protein Antigen-Antibody Interaction

THE CASE OF HEN LYSOZYME-HyHEL-10 Fv COMPLEX*

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To elucidate the roles of the noncovalent forces involved in antigen-antibody complementary association, both structural and thermodynamic information are crucial (7). X-ray crystal analysis can clarify the structural aspects for the complementary interaction (2–5), and titration calorimetry can provide useful information for the quantitative assessment of the contribution of residues to the interaction (8–10). Thus, the combination of these two approaches should give more precise insight into proteinaceous antigen-antibody interactions (11).

We have focused on the interaction between hen egg white lysozyme (HEL)3 and its monoclonal antibody HyHEL-10 (12–15), whose structural features have been analyzed by x-ray crystallography (16) in the Fab-HEL complex. The bacterial expression system of the HyHEL-10 Fv fragment, which consists of the associated variable domains of an antibody, has been established (17, 18), and the interactions have been thermodynamically investigated using mutant Fv fragments (19–21). Recently, we reported the crystal structure of the HyHEL-10 wild-type (WT) Fv-HEL complex at a resolution of 2.3 Å (22). The combination of thermodynamic data with structural results should be powerful for the precise description of the mutant Fv-HEL interactions.

The ability of proteins to bind to one another in a highly specific manner is an important feature of biological phenomena, and the mechanism of antibody specificity and affinity toward target proteins has been studied as a model of protein-protein interactions (1). X-ray crystal structures of antibodies complexed with proteinaceous antigens (2–6) suggest that the strict specificity of an antibody originates from a high degree of complementarity between the antigen-combining sites and the antigen. The complementarity is composed of two factors: 1) apolar surfaces interacting by van der Waals and salt bridge formation, where the precise positioning of atoms allows charge-charge interactions (i.e. salt bridge formation and hydrogen bond formation).

A structural and thermodynamic study of the entropic contribution of salt bridge formation to the interaction between hen egg white lysozyme (HEL) and the variable domain fragment (Fv) of anti-HEL antibody, HyHEL-10, was carried out. The variable fragment (Fv) mutants (HD32A, HD96A, and HD32AD96A) were prepared, and the interactions between the mutant Fvs and HEL were investigated. Crystallography revealed that the overall structures of these mutant complexes were almost identical to that of wild-type Fv. Little structural changes were observed in the HD32AD96A mutant-HEL complex, and two water molecules were introduced into the mutation site, indicating that the two water molecules structurally compensated for the complete removal of the salt bridges. This result suggests that the entropic contribution of the salt bridge originates from dehydration. In the singly mutated complexes, one water molecule was also introduced into the mutated site, bridging the antigen-antibody interface. However, a local structural difference was observed in the HD32A Fv-HEL complex, and conformational changes occurred due to changes in the relative orientation of the heavy chain to the light chain upon complexation in HD96A Fv-HEL complexes. The reduced affinity of these single mutants for the antigen originates from the increase in entropy loss, indicating that these structural changes also introduced an increase in entropy loss. These results suggest that salt bridge formation makes an entropic contribution to the protein antigen-antibody interaction through reduction of entropy loss due to dehydration and structural changes.
The role of salt bridge formation in antigen-antibody interaction is particularly important since electrostatic complementarity is critical for high specificity and affinity of protein antigen-antibody interactions (2-5). The results of alanine scanning mutagenesis of protein-protein complexes have demonstrated that, in the majority of cases, mutations of polar and charged residues result in complex destabilization (23, 24). Two salt bridges have been observed in the Fv-HEL complex; Lys97 of HEL electrostatically interacts with both Asp32 and Asp96 of HyHEL-10 VH (Fig. 1). We recently reported the role of salt bridge formation in the HyHEL-10 Fv-HEL complex from the thermodynamic viewpoint (21). We demonstrated that, in the majority of cases, mutations of polar and charged residues result in complex destabilization (23, 24). Two salt bridges have been observed in the Fv-HEL complex; Lys97 of HEL electrostatically interacts with both Asp32 and Asp96 of HyHEL-10 VH (Fig. 1). We recently reported the role of salt bridge formation in the HyHEL-10 Fv-HEL complex from the thermodynamic viewpoint (21). We replaced Asp32 in VH with Ala, Asn, and Gln and observed increases in both negative enthalpy and entropy changes. We concluded that salt bridge formation makes an entropic contribution to the interaction. Thus, structural analyses of mutant Fv-HEL complexes should give further insights into the molecular basis for the entropic contribution of the salt bridge to the interaction.

Here, using isothermal titration calorimetry and x-ray crystallography, we report the effect of deleting antibody side chains participating in salt bridge formation on the complex formation. Moreover, we discuss, from the structural viewpoint, the molecular basis for the entropic contribution of the salt bridge to the protein antigen-antibody interaction.

### MATERIALS AND METHODS

**Site-directed Mutagenesis**—The general recombinant DNA technology was essentially that of Sambrook et al. (25). Site-directed mutagenesis was performed with phagemid pTZ18U (Bio-Rad, Tokyo, Japan) according to the method of Kunkel (26). The DNA oligonucleotides for mutation at sites 32 and 96 of VH were 5’-ATACACCTCGGCTACTG-3’ and 5’-GCCAAGCTTGGGCGCGGACTAC-3’, respectively (mutated sites are underlined). Correctness of mutations was confirmed by DNA sequencing using an Auto-Read sequencing kit and an ALF express sequencer (Amersham Pharmacia Biotech, Tokyo, Japan).

**Preparation of HyHEL-10 Mutant Fv-HEL Complex**—The mutant Fv fragment was prepared essentially as described previously (17). The purified Fv fragment was concentrated by ultrafiltration centrifugation (0.45-μm membrane) at 3000 × g (Centriprep-10). The antigen, HEL (Seikagaku Kogyo Inc., Tokyo, Japan), was purified on Superdex equilibrated with phosphate-buffered saline. Purities of the proteins were confirmed by SDS-polyacrylamide gel electrophoresis in the buffer system described by Laemmli (27). The eluate was lyophilized and dissolved in water at a concentration of 0.54 mM prior to use.

**Circular Dichroism Spectroscopy of Mutant Fv Fragment**—Circular dichroism (CD) spectra in the near- and far-UV regions were obtained with a Jasco J-720 spectropolarimeter (Tokyo, Japan) equipped with a water bath to control the temperature at 25 °C. The concentration of antibody fragments was 0.1 mg/ml for the near- and far-UV experiments. Sixteen scans were averaged to obtain the spectrum. Cells with path lengths of 1 and 0.1 mm were used for near- and far-UV data acquisition, respectively.

**Inhibition Assay of HEL Enzymatic Activity**—Experimental procedure for inhibition assay has been in principle followed by Ueda et al. (17). In brief, the various concentrations of the Fv fragment were mixed with 1.5 μl HEL and incubated at 25 °C for 1 h in 30 μl of phosphate-buffered saline. Each mixture was then added to 970 μl of 50 mM NaH2PO4 buffer (pH 6.20, adjusted with NaOH) containing 340 μg of Micrococcus luteus cells. The initial rate of the decrease in A540 nm was monitored at 25 °C.

**Isothermal Titration Calorimetry**—Thermodynamic parameters of the interactions between HEL and the wild-type or mutant Fv fragments were determined by isothermal microtitration calorimetry using an Omega titration calorimeter (MicroCal, Inc.) (28). 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 200 mM NaCl was titrated with a 125 μM solution of HEL in the same buffer at four different temperatures (25 °C, 30 °C, 35 °C, and 40 °C). The ligand solution was injected 16 times in portions of 7 μl over 15 s. Thermogram data were analyzed with the computer program Origin (MicroCal, Inc.).

The enthalpy change (ΔH) and binding constant (Kb) on antigen-antibody interaction were directly obtained from the experimental titration curve. The Gibbs free energy change (ΔG = -RT ln Kb) and the entropy change (ΔS = (ΔG + ΔH)/T) on the association were calculated from ΔH and ΔG. The heat capacity change (ΔCp) was estimated from the temperature dependence of the enthalpy change.

**Estimation of Protein Concentration**—The concentration of HEL was estimated using A280 = 26.5 (29), and the concentration of each mutant HyHEL-10 Fv fragment was estimated using A280 = 20.6 (17).

**Crystallization, Data Collection, and Structural Determination of the HyHEL-10 Mutant Fv-HEL Complex**—The HyHEL-10 mutant Fv fragment-HEL complexes were crystallized as described previously (22). The best crystals of the Fv-HEL complex were grown from 100 mM Hepes buffer (pH 7.6–7.8), 9–11% w/v polyethylene glycol 6000, and 7–9% w/v 2-methyl-2,4-pentanediol. They were elongated bipyramidal-shaped crystals.

All crystallographic data were collected on a DIP-2000 diffractometer (MAC Science, Yokohama, Japan) with a 200-mm diameter imaging plate (CuKα radiation; 50 kV, 90 mA). The diffraction images were integrated with the kkl program DENZO (30) and the intensity data were processed with SCALA (2) and AGROVATA (32) in the CCP4 suite (33). The crystallographic data and the statistics of the data collection are summarized in Table I.

The crystals of these three mutant Fv-HEL complexes were isomorphous with that of wild-type complex. The structure of HEL-Fv was determined by a molecular replacement method with the program AMoRe (34) in the CCP4 package. The model coordinates of the mutant complexes were derived from the wild-type complex structure (Protein Data Bank code 1e08). Refinements of the structure of the Fv-HEL complex were carried out with the programs XPLOR (35) and REFMAC (36) as described previously (22). The atomic parameters of the mutant Fv-HEL complexes have been deposited in the Protein Data Bank.

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2 Unpublished data.
Salt Bridge Formation and Hen Lysozyme-HyHEL-10 Fv Complex

TABLE I
Crystalllographic data of mutant Fv-HEL complexes

|                | HD32A | HD96A | HD32A96A | Wild type
|----------------|-------|-------|----------|----------
| Space group    |       |       |          |          |
| Unit cell dimensions | a = b = 57.2 Å | a = b = 57.0 Å | a = b = 57.2 Å | a = b = 57.0 Å |
| V<sub>M</sub> (Å³) | 2.5 | 2.3 | 2.1 | 2.3 |
| Wavelength (Å) | 1.5418 | 1.5418 | 1.5418 | 1.5418 |
| Resolution (Å) | 8–2.5 | 8–2.2 | 8–2.1 | 8–2.3 |
| Completeness (%) | 96.6 (96.1) | 95.6 (92.5) | 98.9 (98.9) | 98.9 (97.6) |
| Multiplicity   | 3.1 | 4.2 | 4.5 | 8.08 |
| Resolution range (Å) | 8–2.5 | 8–2.2 | 8–2.1 | 8–2.3 |
| R<sub>merge</sub><sup>b</sup> | 0.100 (0.318) | 0.071 (0.289) | 0.058 (0.342) | 0.076 (0.265) |
| R<sub>factor</sub><sup>c</sup> | 0.184 | 0.165 | 0.177 | 0.175 |
| Free R factor  | 0.279 | 0.252 | 0.230 | 0.235 |
| Root mean square deviation bond length (Å) | 0.013 | 0.019 | 0.015 | 0.020 |
| Root mean square deviation bond angle (°) | 2.7 | 2.9 | 2.1 | 2.5 |
| Water molecules | 66 | 112 | 133 | 125 |

<sup>a</sup> Kondo et al. (22).

<sup>b</sup> R<sub>merge</sub> = Σ|I<sub>obs</sub> - I<sub>calc</sub>| / ΣI<sub>obs</sub>.

<sup>c</sup> R<sub>factor</sub> = Σ|F<sub>obs</sub> - F<sub>calc</sub>| / ΣF<sub>obs</sub>.

RESULTS

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

To elucidate the structural and thermodynamic effects of deleting salt bridges in the HyHEL-10 Fv-HEL complex, we constructed two new mutants (HD96A and HD32AD96A). These new mutants and the HD32A mutant were expressed in *Escherichia coli* and the mutant Fv fragments were purified by affinity chromatography using HEL-Sepharose, followed by gel filtration on 75 pg of Superdex. Purities of greater than 95% were obtained with these steps, and the purified Fv fragments were subjected to precise analysis. The final yield of each mutant was greater than 10 mg/liter of culture.

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

Inhibition of Enzymatic Activity of Hen Lysozyme by Mutants

It has been 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, resulting from the binding of the Fv to the active site of HEL (17). Therefore, the inhibition of the enzymatic activity of HEL by three mutants (HD32A, HD96A, and HD32AD96A) was investigated (Fig. 2).

In comparison with wild-type Fv, these clones showed decreased inhibition toward HEL to different degrees. The HD32AD96A mutant Fv exhibited the largest degree of decreased inhibition of HEL enzymatic activity, suggesting that deleting both side chains resulted in a significant reduction of affinity for HEL.

![Graph of Inhibition of lysozyme enzymatic activity by HyHEL-10 Fv](image)

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

To quantitatively investigate the interactions between mutants and HEL from a thermodynamic viewpoint, we performed an isothermal titration calorimetry study of the association between mutant Fv fragments and lysozyme under the same conditions as described previously (19–21). Calorimetric titration was performed at four different temperatures. Thermodynamic parameters (30 °C, pH 7.2) calculated from the titration curves are presented in Table II, and temperature dependences of the enthalpy and entropy changes are shown in Figs. 3 and 4, respectively.

On comparison of all mutant Fv fragments with the wild-type Fv, the negative values of the enthalpy change for the interaction between each mutant and HEL were increased by 22 kJ mol⁻¹ at 30 °C. The binding constants were decreased slightly in two mutants (HD32A and HD96A) and significantly in one mutant (HD32AD96A), resulting in a smaller change in the Gibbs energy. These results indicate that the increase in the negative enthalpy change for each mutant Fv fragment was compensated for by the increase in negative enthalpy change, leading to the decrease in affinity. From the temperature dependence of the enthalpy change shown in Fig. 4, the
Experimental protocols are described under “Materials and Methods.” Each value is the average of at least three independent measurements. Errors of all values are within 5%. 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; $\Delta \Delta G$, $\Delta \Delta H$, $\Delta \Delta S$, and $\Delta \Delta C_p$, differences in each of values from those of wild-type Fv.

| Mutant  | $n$ | $K_a \times 10^6$ M$^{-1}$ | $\Delta G$ | $\Delta \Delta G$ | $\Delta H$ | $\Delta \Delta H$ | $\Delta S$ | $\Delta \Delta S$ | $\Delta C_p$ | $\Delta \Delta C_p$ |
|---------|-----|--------------------------|------------|------------------|------------|------------------|---------|-----------------|------------|------------------|
| Wild type | 1.0 | 4.2 | $-50.2$ | $-91.5$ | 0 | $-0.137$ | 0 | $-4$ | 0 | 1.4 |
| HD32A | 1.0 | 1.0 | $-46.4$ | 3.8 | $-112.9$ | $-21.3$ | $-0.219$ | $-0.082$ | $-1.8$ | $-0.4$ |
| HD96A | 1.0 | 2.1 | $-48.2$ | 2.1 | $-113.7$ | $-22.3$ | $-0.216$ | $-0.079$ | $-1.9$ | $-0.5$ |
| HD32A96A | 1.0 | 0.4 | $-44.1$ | 6.0 | $-114.2$ | $-22.7$ | $-0.231$ | $-0.094$ | $-2.1$ | $-0.7$ |

No major changes in the relative orientations of VL, VH, and HEL were observed in the HD32A mutant Fv-HEL and HD32AD96A-HEL complexes. However, superimposition of the C$\alpha$ coordinates of Fv (VL and VH) of the HyHEL-10 HD96A mutant complex onto those of the wild-type Fv complex yielded root-mean-square differences of 0.21 for VL, 0.26 for VH, and 0.58 for HEL. These root-mean-square differences originated from the different relative orientations of Fv to HEL in the HD96A mutant complex and the wild-type complex (Fig. 5).

The calculated interfacial areas of the mutant Fv-HEL complexes were 1715, 1729, and 1722 Å$^2$ for the HD32A, HD96A, and HD32AD96A mutant complexes, respectively (Table IV); these values were about 100 Å$^2$ smaller than the value for the wild-type Fv (1835 Å$^2$). The most significant decreases in the areas, >20 Å$^2$, were in Asn$^{19}$, Arg$^{23}$, and Asn$^{77}$ of HEL.

C$_p$, heat capacity change was estimated to be 1.8–2.1 kJ mol$^{-1}$ K$^{-1}$ at 30 °C for the mutant Fvs, which was 0.4–0.7 kJ mol$^{-1}$ K$^{-1}$ larger than the $\Delta C_p$ of wild-type Fv.

**Crystal Structures of Mutant Fv-HEL Complexes**

To investigate the mutant Fv-HEL complexes from the structural viewpoint, we determined the crystal structures of the three mutant-HEL complexes. Crystallographic data are summarized in Table I. The maximum resolution of the x-ray data used in the refinement ranged from 2.1 to 2.5 Å, and the R factors of the refined structures were 0.165–0.194 (37). The overall structures of these mutant complexes are almost identical to that of wild-type Fv.

The mutant Fv-HEL complexes were superimposed onto the wild-type Fv-HEL complex. The root-mean-square differences between the C$\alpha$ atoms of the mutant Fv structures and those of the wild-type Fv are summarized in Table III. The results demonstrated that the overall structures of the HyHEL-10 mutant Fv-HEL complexes are similar to that of the HyHEL-10 wild-type Fv-HEL complex (Table III).

HD32A Mutant-HEL Complex—Although little structural change occurred in the antigen HEL in the HD32A Fv-HEL complex, a structural difference was observed in the complementarity-determining region 1 in the heavy chain (CDR-H1) (Fig. 6). One water molecule (W33) was introduced into the mutation site, bridging HAsp$^{32}$ and HAla$^{96}$ with the main chain of CDR-H1 (Fig. 6A). Tyr$^{53}$ in CDR-H2 penetrated into the antigen, making more van der Waals contacts with HEL (Fig. 6B).

In addition, one water molecule (W2) formed a hydrogen bond with the hydroxy group of HTyr$^{53}$, bridging the loop of the lysozyme with the antibody. These observations suggest that deletion of the carboxyl group at site 32 in VH led to the local structural changes of the antibody in the complex.

HD96A Mutant-HEL Complex—No structural changes were observed in the Fv of the HD96A Fv-HEL complex, except at the mutated site. One water molecule was introduced into the mutation site (W15, Fig. 7), bridging HAsp$^{96}$ and HAla$^{56}$ with HAsn$^{44}$, and Lys$^{97}$ of HEL, respectively. The structural change of the CDR-H3 and the change of the relative orientation of Fv to HEL occurred, resulting from the deposition of the water molecule. These results suggest that the effect of deleting the Asp$^{96}$ side chain in VH is compensated for by introducing one water molecule and the change of relative orientation of each chain.

HD32AD96A Mutant-HEL Complex—In contrast to the HD32A Fv-HEL and HD96A Fv-HEL mutant complexes, there were few structural changes in both the antigen and antibody of the HD32AD96A mutant Fv-HEL complex. One water molecule (W4) was located in the mutated site and formed two hydrogen bonds, filling a local structurally imperfect complementarity (Fig. 8A). In addition, one water molecule (W123) was retained in the interface, participating in hydrogen-bonding networks in the antigen-antibody interface (Fig. 8B).
DISCUSSION

We recently reported the entropic contribution of salt bridge formation to the HyHEL-10 Fv-HEL interaction (21). To obtain the structural basis for the contribution to the interaction, two mutants (HD96A and HD32AD96A) were newly constructed, and the interactions of these two mutants and a third mutant (HD32A) with HEL were investigated by thermodynamic and structural analyses. As shown previously (15, 42) and in the present study, each mutation does not lead to major structural changes in the Fv fragment, and the stability of each mutant is almost identical to that of wild-type Fv. Thus, the structural and thermodynamic changes in the antigen-antibody complexes by mutation do not originate from changes in the structure of the mutants in the antigen-free state. In the following subsections, we discuss and correlate our thermodynamic and structural findings.

**Thermodynamic Analysis of Mutant Fv-HEL Interactions**

| Mutant     | VL fit | VH fit | HEL fit | Fv fit | All fit |
|------------|--------|--------|---------|--------|---------|
| HD32A      | 0.15   | 0.29   | 0.25    | 0.33   | 0.17    |
| VL         | 0.33   |        | 0.52    | 0.33   | 0.29    |
| VH         | 0.29   | 0.57   | 0.15    | 0.39   | 0.19    |
| HEL        | 0.17   | 0.49   | 0.80    | 0.21   | 0.25    |
| HD96A      | 0.36   | 0.20   | 0.60    | 0.28   | 0.29    |
| VL         | 0.70   | 0.61   | 0.18    | 0.58   | 0.34    |
| VH         | 0.11   | 0.25   | 0.18    | 0.15   | 0.14    |
| HEL        | 0.28   | 0.13   | 0.35    | 0.18   | 0.18    |
| HD32AD96A  | 0.13   | 0.18   | 0.10    | 0.18   | 0.12    |

**TABLE IV**

Interfacial areas of the mutant Fv-HEL complexes (Å²)

| Complex   | VL     | VH     | HEL   | Polar | Apolar | All   |
|-----------|--------|--------|-------|-------|--------|-------|
| Wild type | 355    | 513    | 987   | 1042  | 793    | 1835  |
| HD32A     | 337 (−18) | 495 (−18) | 883 (−84) | 943    | 772    | 1715 (−120) |
| HD96A     | 341 (−14) | 477 (−36) | 921 (−46) | 937    | 791    | 1729 (−106) |
| HD32AD96A | 334 (−21) | 486 (−27) | 902 (−65) | 917    | 804    | 1722 (−113) |

The values for both the enthalpic (−ΔH) and entropic (−TΔS) contributions to the interaction between the mutants and HEL became increasingly negative in the order HD32A < HD96A < HD32AD96A. The binding constants (Kₐ) were decreased slightly in two mutants (HD32A and HD96A) and significantly in one mutant (HD32AD96A), resulting in a smaller change in the Gibbs energy (−ΔG). These results indicate that deletion of salt bridges in the HyHEL-10 Fv-HEL complex is enthalpically unfavorable and entropically unfavorable, suggesting that the salt bridges in the HyHEL-10 Fv-HEL interaction make an entropic contribution to the interaction. Substitution of Asp32 with other residues (e.g. Asn and Glu) also led to increases in both negative enthalpy and entropy changes (21), which is almost identical to the results reported here. Thus, we conclude that the salt bridges in the HyHEL-10-HEL complex make an entropic contribution to the interaction.

Precise analyses of another protein antigen-antibody interaction, namely the HyHEL-5-HEL interaction, demonstrated that salt bridge formation made a significant contribution to the interaction (42–44). The salt bridge formed in an environment of low dielectric constant and, thus, the contribution of the salt link may be highly enthalpic (43, 44). On the other hand, an entropic contribution of the salt bridge was indicated in the HyHEL-10-HEL interaction.

To correlate the results obtained from thermodynamic analyses with structural information, the crystal structures of mutant Fv-HEL complexes were solved. We now discuss the role of salt bridge formation in the HyHEL-10-HEL interaction on the basis of structural information.

**Comparison of the Mutant Fv-HEL Structure with the Wild-type Fv-HEL Structure**—Mutations did not affect the crystal packing. Thus, the local structural changes due to the mutations, including interface solvents, can be discussed directly by comparison of the mutant structures with the wild-type structure.

The overall structures of the mutant Fv-HEL complexes are
similar to that of the wild-type Fv-HEL complex (Fig. 5). However, the area of HEL covered by mutant Fvs decreased by 46–84 Å² in comparison with the wild-type Fv-HEL complex, indicating a decrease in burial of surface areas by mutation. The changes in thermodynamic parameters can be accounted for in terms of changes in solvent-accessible polar and apolar surface area (1, 45–49). Thus, the Gibbs energy changes of the mutant Fv-HEL interactions have been estimated based on the polar and apolar buried surface areas of the crystal structures.

On comparison of all mutant Fv fragments with the wild-type Fv, the calculated negative values of the Gibbs energy change for the mutant-HEL interactions were decreased by 9.2, 8.6, and 10.0 kJ mol⁻¹ for HD32A, HD96A, and HD32AD96A, respectively, at 30 °C. These values indicate that the degree of reduction of the changes in polar and apolar surface areas does not correspond to that of the decrease in affinity for the antigen determined by experiments (Table II), suggesting the compensation by other non-covalent bond formations.

The changes in the interaction between the double mutant (HD32AD96A) and HEL reflect the complete deletion of salt bridges in the HyHEL-10-HEL complex. Double mutation led to an order of magnitude decrease in affinity (Table II), suggesting that recognition of the amino group in Lys⁹⁷ by Asp residues made a positive contribution to the interaction. Little changes, including the relative orientation of three chains and local loop structures, were observed in the double-mutant com-

**Fig. 6.** Comparison of local structures in HD32A-HEL and WT-HEL complexes. A, stereo view of region around the mutated site (32 of VH); B, stereo view of region around site 53 of VH. Hydrogen bonds made by water molecule and salt bridge are depicted as dotted lines. The positions marked W correspond to the water molecules. Sky blue, HD32A antibody VH chain; pink, HEL; red, water; gray, WT-HEL complex.

**Fig. 7.** Comparison of local structures in HD96A-HEL and WT-HEL complexes. Stereo view of region around the mutated site (96 of VH). Hydrogen bonds made by water molecule and salt bridge formation are depicted as dotted lines. Sky blue, HD32A antibody VH chain; pink, HEL; red, water; gray, WT-HEL complex.
plex. Two water molecules (Fig. 8, W4 and W123) were introduced into the mutated sites. These two water molecules bridge the antigen-antibody interface by hydrogen bonding (Fig. 8). These results indicate that structural compensation by water molecules leads to the increase in negative values of $\Delta C_p$ and $\Delta H$, suggesting that the salt bridges make an entropic contribution through complete dehydration.

The negative values of heat capacity change ($\Delta C_p$) calculated for each mutant complex increased by 0.4–0.7 kJ K$^{-1}$ mol$^{-1}$ in comparison with the wild-type value (Table II, Fig. 3). A negative $\Delta C_p$ has been proposed to be a consequence of an increase in hydrophobic interaction (50, 51), burial of interfacial surface area (51), the presence of waters buried in the interface (52), and/or local folding due to binding (53, 54). A recent report of the interaction between mutant DNA gyrase and the antibiotic novobiocin proposed that the negative values of $\Delta C_p$ increase by 200 $\pm$ 300 J K$^{-1}$ mol$^{-1}$ per interfacial water molecule (52). The increases in negative values of $\Delta C_p$, $\Delta H$, and $\Delta S$ may originate from refilling water molecules in the interface.

On the other hand, in the HD32A and HD96A mutant complexes, at least one water molecule was newly retained at the mutated site, and structural changes (e.g., changes in the relative orientation of the variable domains and local loop structure) were observed.

The side chain at site 32 of VH is almost buried (the calculated accessible surface area is 3 Å$^2$) in the complex. An Asp$^{32}$ → Ala mutation resulted in refilling one water molecule at the mutated site in the mutant Fv-HEL complex (Fig. 6). The water molecule makes two hydrogen bonds with the side chain of HEL Lys$^{97}$ and the main chain of the H chain Ala$^{32}$, bridging HEL with the Fv. In addition, the mutation led to structural changes in CDR-H1, suggesting that structural changes in the loop were introduced into CDR-H1 by substitution of Asp$^{32}$ with Ala. These results indicate that deleting the side chain at site 32 of VH is compensated for by one water molecule and local structural changes in the CDR-H1 loop. It has been suggested that the unfavorable entropy change results in part from reduction of conformational flexibility in the antibody upon complexation (55, 56).

Replacement of Asp$^{96}$ with Ala resulted in refilling one water molecule at the mutated site in the mutant Fv-HEL complex. The water molecule makes hydrogen bonds with Lys$^{97}$ of HEL and HAsp$^{32}$, HAsn$^{94}$, and HAla$^{96}$ of Fv, bridging HEL with the Fv. There were no significant changes in the main chains of the polypeptides (i.e., VH, VL, and HEL). The side chain at site 96 of VH is highly exposed (the calculated accessible surface area is 34 Å$^2$). In addition, the relative orientation of VH to VL was changed by the mutation at site 96 of VH (Table III). These results suggest that one water molecule and structural changes (i.e., changes in the relative orientations of the chains) compensate for deleting the side chain at site 96 of VH. It has been indicated that the changes in the relative orientations of the chains reflected the induced fitting of the Fv to the protein antigen (57). The results reported here might suggest that removal of the side chain of Asp$^{96}$ in VH HyHEL-10 Fv require the excess induced fitting for the recognition of the antigen.

It should be noted that the values of $\Delta H$ of the interaction between each of the single mutants (HD32A and HD96A) and HEL are almost the same as that of the HD32AD96A mutant (Table II). Moreover, the difference in affinity between each of the single mutants and the HD32AD96A mutant originates...
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from the entropy change. Structural analyses have indicated that both a refilling water molecule and structural changes may lead to an increase in negative entropy change. Therefore, an additional decrease in negative entropy change might be required for further enhancement of the antibody affinity for the antigen. Introducing two Asp residues in the antibody reduces the entropy loss upon binding of the antibody to the antigen. These results suggest that the salt bridge makes an entropic contribution to the interaction through a decrease in entropy loss due to dehydration and structural changes.

Role of Salt Bridge Formation in the HyHEL-10-HEL Interaction: Conclusion—Salt bridge formation is a major contributor to various biomolecular interactions (17, 58). The salt bridges in the HyHEL-10-HEL complex, as shown here, are buried (Asp2-Lys97) and partially buried (Asp96-Lys97); deletion of these salt bridges led to structural compensation, including refilling water molecules. We conclude that salt bridge formation makes an entropic contribution to the interaction through a decrease in entropy loss due to dehydration and structural changes.

In this study, we found that deleting salt bridges resulted in an unfavorable Gibbs energy change of 3–5 kJ mol⁻¹ per salt bridge (Table III). Water molecules, however, were introduced into the mutated sites, and the water molecules bridge the antigen-antibody interface by hydrogen bonding (Figs. 6–8). It has been suggested that burial of water molecules makes a favorable contribution to biomolecular interactions (59–62), and that one water molecule retained at the interface of proteins can make as many as four hydrogen bonds, leading to a favorable contribution of ~10 kJ mol⁻¹ per interfacial water molecule (51, 63). Supposing a favorable contribution of the water newly retained in the interface, salt bridges themselves in the interaction make a favorable contribution of 13–15 kJ mol⁻¹ to the Gibbs energy, which is indistinguishable from the values for salt bridges in completely dehydrated environments (e.g. the HyHEL-5-HEL interaction) (43). Recent analyses of mutant human lysozymes have suggested the contributions of salt bridge to the thermodynamic stability of the protein correlated with the solvent inaccessibility of the salt bridges, the salt bridge contribution was small when 100% accessible, whereas it was about 9 kJ mol⁻¹ if 100% inaccessible (64).

Computational studies of binding free energies based on the results reported here may provide further precise description of the interaction (23, 65–67).

Finally, the present study demonstrates that effects of mutation on the interaction cannot be discussed without structural information (68, 69). Changes in the thermodynamic parameters include all changes due to the mutation, including water refilling and/or conformational changes. In this study, combination of thermodynamic data with the results of structural analyses could give further insight into the role of salt bridge formation in the interaction. Structural analyses of HyHEL-10 single mutant-HEL complexes, already investigated thermodynamically, are now in progress; these analyses will be useful in correlating thermodynamic parameters with structural changes due to mutation.

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Structural Evidence for Entropic Contribution of Salt Bridge Formation to a Protein Antigen-Antibody Interaction: THE CASE OF HEN LYSOZYME-HyHEL-10 Fv COMPLEX

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