Role of Salt Bridge Formation in Antigen-Antibody Interaction

ENTROPIC CONTRIBUTION TO THE COMPLEX BETWEEN HEN EGG WHITE LYSOZYME AND ITS MONOCLONAL ANTIBODY HyHEL10*

(Received for publication, May 29, 1996, and in revised form, September 9, 1996)

Kouhei Tsumoto‡, Kyoko Ogasahara§, Yoshitaka Ueda¶, Kimitsuna Watanabe‖, Katsuhide Yutani¶, and Izumi Kumagai¶¶

From the ‡Department of Biochemistry and Engineering, Graduate School of Engineering, Tohoku University, Aoba-ku, Sendai 980-77, the §Institute for Protein Research, Osaka University, Suita, Osaka 565, and the ¶Department of Chemistry and Biotechnology, Graduate School of Engineering, The University of Tokyo, Bunkyo-ku, Tokyo 113, Japan

For elucidation of the role of salt bridge formation in the antigen-antibody complex, the interaction between hen egg white lysozyme (HEL) and its monoclonal antibody HyHEL10, the structure of which has been well characterized and forms one salt bridge (Lys97 of HEL and Asp32 of HyHEL10 heavy chain variable region (VH)), was investigated. Asp32 of VH was substituted with Ala, Asn, or Glu by site-directed mutagenesis, and the interaction between HEL and the mutant fragments of the variable region of light chain was investigated by inhibition of the enzymatic activity of HEL and isothermal titration calorimetry. Inhibition assay indicated that these mutations lowered the inhibition only slightly. Thermodynamic study indicated that the negative enthalpic change in the interaction between each of the mutant variable regions of light chain and HEL was significantly increased, although the association constant was slightly decreased, suggesting that these mutations increased the entropy change upon antigen-antibody binding. These results indicate that the role of salt bridge formation in the HyHEL10-HEL interaction is to lower the entropic loss due to binding. In the mutant proteins, the numbers of residues that were perturbed structurally on binding increased, suggesting that the salt bridge suppresses excess structural movement of the antibody upon binding.

A major goal of molecular biology is to describe biological phenomena in terms of interaction between molecules. Recently, thanks to progress in structural biology, several quaternary structures of biomolecular complexes have been clarified, and now a number of biological events can be described precisely at the atomic level. Among them, DNA-repressor (1), enzyme-inhibitor (2, 3) and antigen-antibody interactions (4, 5) have been most extensively investigated at the atomic level.

Antigen-antibody interactions have been studied in detail not only from an immunological but also a biochemical viewpoint as a model of protein-ligand interaction. The most striking feature of antigen-antibody interactions is the creation of strict specificity and high affinity of antibodies for their antigens. It is known that unique conformations and special locations on the surfaces of proteinaceous antigens are recognized by antibodies (6). Among the amino acids located on protein surfaces, it is believed that antibodies recognize charged residues by forming (1) hydrogen bonds and (2) salt bridges and that salt bridge plays a significant role in the interaction (7–9).

For detailed elucidation of antigen-antibody interactions, a number of approaches have been attempted, including structural analysis (10–13) and calculation and modeling (14–16). However, thermodynamic analysis of antigen-antibody interaction for analyzing precisely the contribution of noncovalent forces to the interaction is most suitable (17–22), especially when combined with site-directed mutagenesis of structurally well defined ones (23–25). We have focused on the interaction of hen egg white lysozyme (HEL) with the anti-HEL monoclonal antibody HyHEL10 interaction as a model of proteinaceous antigen-antibody interaction (26, 27). This system is advantageous for studying the mechanism of antigen-antibody interaction because the structure of the antigen-antibody complex has already been clarified by x-ray crystallographic analysis (10), and also a secretory expression system for the Fv fragment, which is composed of variable domains of immunoglobulin heavy and light chains, has been established in Escherichia coli (28, 29).

The side chain of Lys97 in HEL interacts with the contact region of HyHEL10 and is recognized by HASp32, HTyr33, and HTryp95. HASp32 contributes to the interaction by formation of a salt bridge with Lys97 of HEL (10). In this study, for elucidation of the role of the salt bridge in the antigen-antibody interaction, HASp32 of the Fv fragment was replaced with Ala (HD32A), which deleted a carboxyl group; Glu (HD32E), which added a methylene group; or Asn (HD32N), which added an amino group. The interactions among antigen, HEL, and the engineered HyHEL10 Fvs were examined using the inhibition of HEL enzymatic activity and isothermal titration calorimetry. The contribution of the salt bridge to the interaction will be discussed thermodynamically here.

EXPERIMENTAL PROCEDURES

Materials—All enzymes used for genetic engineering were purchased from Toyobo, Takara Shuzo, or Boehringer Mannheim. Hen egg white lysozyme was from Seikagaku Kogyo, Inc. (Tokyo, Japan). Micrococcus lysodeikticus for measuring the enzymatic activity of HEL was obtained from Sigma. HEL-Sepharose was prepared from CNBr-activated Sepharose (Pharmacia). Oligonucleotide DNA primers were synthesized by a 381-Å DNA synthesizer (Applied Biosystems). A mutagenesis kit was obtained from Bio-Rad (Tokyo, Japan). All other reagents were of biochemical research grade.

Site-directed Mutagenesis of HASp32—The method used for site-
directed mutagenesis was followed as described previously (27). Correctness of the mutation was confirmed by DNA sequencing using a Bca-BEST sequencing kit (Takara).

Expression and Purification of HAsp32-mutated Fv Fragments—Secretory expression in E. coli and the affinity chromatography purification procedures using HEL-Sepharose were performed as described previously (26).

Measurements of CD Spectra of Wild-type and Mutant HyHEL10 Fv Fragments—Measurements of CD spectra of the wild-type and mutant HyHEL10 Fv fragments (25 μM) were carried out using a Jasco J-720 spectropolarimeter. Far- and near-UV CD spectra were measured using a cell with 1- and 10-mm optical path lengths, respectively, at pH 7.2 and 25°C.

Measurement of Inhibitory Activity toward HEL—Measurement of inhibition of HEL enzymatic activity was done as described previously (28). The experimental condition was described in the legend to Fig. 1.

Isothermal Titration Calorimetry—Thermodynamic parameters of the interactions between HEL and the mutant Fv fragments were determined by microtitration calorimetry using an OMEGA titration microcalorimeter (30) from MicroCal, Inc. (Northampton, MA). The experimental conditions were the same as those reported previously (27). The wild-type Fv HEL at a concentration of 5 μM in 50 mM phosphate buffer (pH 7.2) containing 100 mM NaCl in a calorimeter cell was titrated with a 125 μM solution of the mutant Fv fragments in the same buffer at five different temperatures (20, 25, 30, 35, and 40°C). The ligand solution was injected 16 times in portions of 7 μl during 15 s. Thermogram data were analyzed using a computer program (Origin) supplied by MicroCal, Inc. (30).

Estimation of Protein Concentration—The concentration of HEL was estimated using AEmax = 26.5 (31) and that of mutant HyHEL10 Fv fragments using AEmax = 20.6 (26).

RESULTS

Expression, Purification, Overall Structure, and Antigen-binding Activities of Mutated HyHEL10 Fv Fragments—All of the mutants were obtainable in the E. coli secretory expression system and were purified by affinity chromatography using HEL-Sepharose. The yield was about 10 mg/liter of culture.

No difference in far- and near-UV CD spectra between wild-type and mutant Fv fragments was observed (data not shown), indicating that the overall structure of mutant Fv fragments is identical with that of the wild type.

As described previously (28), the HyHEL10 Fv fragment has complete inhibitory activity toward its antigen, HEL, in a Fv: HEL molar ratio of 1:1. Although the association constant of the interaction is high, a sigmoidal curve was observed (Fig. 1). This might be due to the dilution of Fv-HEL solution and the competition with the substrate in the measurements of HEL enzymatic activity. The inhibitory activities of the HAsp32-mutated Fv fragments were measured (Fig. 1). The inhibition profile indicated that the inhibitory activities for the D32N mutant and D32E mutant Fv fragments were slightly decreased and that for the D32A mutant Fv was significantly decreased, indicating that all mutations lowered the association.

Thermodynamic Analyses of the Interactions between Asp32 mutant Fv and HEL—To estimate the thermodynamic parameters of the interactions between the Asp32 mutant HyHEL10 Fv fragments and HEL, we performed titration calorimetry of the association between the HAsp32 mutant Fv fragments and HEL (Fig. 2). The enthalpy change (ΔH) and binding constant (K) on antigen-antibody interaction were directly obtainable from the experimental titration curve shown in Fig. 2 (30), and the heat capacity change (ΔCp) of the interaction was estimated from the temperature dependence of the enthalpy change. The Gibbs energy change (ΔG = -RT ln K) of binding was calculated from the binding constant, and the entropy change (ΔS = ΔG + ΔH) on the association could also be estimated.

Thermodynamic parameters at 30°C calculated from the titration curves are presented in Table I, and the temperature dependence of the enthalpy and entropy change is shown in Figs. 3 and 4, respectively.

On comparison of all of the mutant Fv fragments with the wild-type Fv fragment, the negative values of the enthalpy change of the interaction between each mutant Fv fragment and HEL were increased by 17–24 kJ mol⁻¹ at 30°C. The binding constants were decreased only slightly in all mutants, resulting in a small change in Gibbs energy. The increases in the negative enthalpy change (−ΔH) for the mutant Fv fragments were completely compensated by the increase in negative entropy change (−TΔS), which was superior to −ΔH, suggesting that Asp32 contributed to the interaction by sup-
pressing the decrease in entropy change (i.e. the entropy loss due to binding). From the temperature dependence of the enthalpy change shown in Fig. 3, the heat capacity change was estimated to be \(-2.2\) kJ mol\(^{-1}\) K\(^{-1}\) for all the mutant Fvs, which was \(-0.8\) kJ mol\(^{-1}\) K\(^{-1}\) larger in negative value than that of the wild type (Table I).

**DISCUSSION**

**Thermodynamic Features for Deletion of the Salt Bridge in the HyHEL10-HEL Interaction**—We discuss the result reported here on the supposition that the effect of any change of protonation upon complex formation by mutation is completely compensated by the deprotonation of buffer. Although the structure of free Fv fragment has not been determined, far- and near-UV CD spectra of free mutant Fv fragments are identical with that of wild-type Fv (data not shown), indicating that no difference in overall structure between wild-type Fv and mutants exists. This suggests that the difference in thermodynamic parameters of the interaction between mutant Fv and HEL from those of wild-type Fv does not originate from the reconstruction of the partly unfolded structure of the mutant protein in the free state.

The data reported here indicated that deletion of the salt bridge in the HyHEL10-HEL interaction by mutation led to increased changes in both negative enthalpy and negative entropy. The negative Gibbs energy was slightly decreased in the same direction as the previously reported mutants (27). These results suggest that although the interaction between HAsp32 mutant Fv and HEL is enthalpically more favorable than that for the wild-type Fv fragment, the entropic loss due to binding is also increased, indicating that deletion of the salt bridge is enthalpically favorable and entropically unfavorable. This
means that the thermodynamic role of salt bridge formation is
to decrease the entropic loss due to binding.

Increased changes in both negative enthalpy and negative
entropy by mutagenesis of the antigen-binding site of an anti-
body have also been observed in the mutant antibody-antigen
Salmonella serogroup B O-polysaccharide interaction (20).
Substitution of His101, which is exposed to solvent and forms
a hydrogen bond with O-4 of mannose, with Gly or Asp pro-
duces a dramatic increase of negative enthalpy change in the
mutant antibody-antigen interaction. However, the unfavor-
able entropy change outweighs the enthalpic advantage, result-
ing in a 10-fold lower binding constant. In the case of the
interaction between Y82F mutant FK506-binding protein
(FKBP-12) and its ligand (FK506), large increases in negative
enthalpy (− ΔH) and negative entropy (− T ΔS) change have
been observed in comparison with the wild-type FKBP-12 li-
gand interaction (32). The binding constant of the interaction
between Y82F mutant FKBP-12 and its ligand is slightly de-
creased in comparison with the wild type. 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 Tyr82 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 due to removal of the
water molecules (32).

A structural study of a free HEL has suggested that Lys97 is
exposed and hydrated (33). Although the structure of a free
HyHEL10 Fv fragment has not yet been determined, HAasp32 is
thought from calculation to be exposed and hydrated (34, 35).
Because no water molecule is observed in the HyHEL10-HEL
complex (10), the solvent molecules must be removed on bind-
ing of HEL. The increase in entropy change might originate
mainly from the removal of solvent molecules on formation of
the HyHEL10-HEL complex, because desolvation is accompa-
nied by a considerable entropy advantage (36).

The present data indicate that a significant increase in neg-
ative enthalpy change is observed in the interactions between
the three mutants and HEL. Substitution of the Asp at site 32
of VH with Glu resulted in a large increase in negative en-
thalpy and entropy, although both the enthalpy and entropy
changes produced by dehydra tion of Asp or Asn are almost the
same (37). Furthermore, although the entropic change of dehy-
dration of the side chain of Ala is quite different from that of
Asn (37), the difference in the negative entropy changes of the
interactions between HD32A Fv and HD32N Fv was only sub-
tle (1 kJ mol−1 K−1 at 30 °C; Table I). These results suggest
that the changes in thermodynamic parameters produced by
mutation cannot be understood merely by the changes in de-
hydration occurring upon binding. Why, then, is negative en-
tropy change increased by mutation?

### Entropy Changes Resulting from Conformational Change on
### Binding of HyHEL10 Fv with HEL—

According to the considera-
tions of Murphy et al. (38), the total ΔS of binding is given as

$$\Delta S = \Delta S_{\text{olv}} + \Delta S_{\text{conf}} + \Delta S_{\text{crat}}$$

(Eq. 1)

where ΔSolv is the change in entropy derived from solvent
release upon binding, ΔSconf is the change in entropy resulting
from conformational changes due to formation of the antigen-
antibody complex, and ΔScrat is the cratic entropy change.

ΔSolv is given by

$$\Delta S_{\text{olv}} = \Delta C_p \ln (T_2/T_1)$$

(Eq. 2)

(\(T_2 = 386 \text{ K} \); \(T_1\) is the temperature at which the desolvation
entropy change is considered to be zero), and ΔScrat can be
considered a constant value (− 0.033 kJ mol−1 K−1) (39, 40).
From their experimental data, they have suggested that the
overall conformational entropy loss is, to a large extent, com-
penated by the desolvation entropy. On the basis of this view-
point, the present data can be analyzed as follows. In the
interaction between HEL and the wild-type HyHEL10 Fv frag-
ment, ∆Cp was estimated to be − 1.42 kJ mol−1 K−1 (Table I);
and from Equation 2, ΔSolv is calculated to be 0.36 kJ mol−1
K−1. From the present experimental data, the total entropy
change is − 0.14 kJ mol−1 K−1, and then ΔSconf is estimated to be
− 0.45 kJ mol−1 K−1 from Equation 1. On the other hand, as
shown in Table II, in the interaction between HEL and each
mutant HyHEL10 Fv fragment, ΔSolv is estimated to be
0.54− 0.57 kJ mol−1 K−1, which is 0.18− 0.21 kJ mol−1 K−1
larger than that of wild-type Fv fragment. This indicates that
the entropic advantage gained by desolvation is enhanced by
the mutations. However, the total entropy change is about
− 0.22 kJ mol−1 K−1; thus, ΔSconf is calculated to be − 0.72−
0.75 kJ mol−1 K−1, which is about 0.27− 0.29 kJ mol−1 K−1
smaller than that of wild-type Fv fragment, indicating that the
negative entropy change produced by the conformational
change is significantly increased. This suggests that confor-
matonal changes are increased by the mutation and that the
entropy loss due to the enhanced conformational changes is not
completely compensated by the increased desolvation entropy
change. Thus, the extra unfavorable ΔSconf (negative value)
produces a decrease in affinity.

### Table II

| Fv | Total ΔS | ΔS | ΔSolv | ΔSconf | ΔScrat |
|----|----------|----|--------|--------|--------|
| Wild type | −137 | 0 | 355 | 0 | −459 | 0 |
| HD32A | −219 | −82 | 538 | 183 | −724 | −265 |
| HD32N | −222 | −85 | 563 | 208 | −752 | −293 |
| HD32E | −201 | −64 | 210 | −733 | −274 |

* Calculations were done according to the method of Murphy et al. (38), and procedures are summarized briefly in the text. ΔS, ΔSolv, and ΔSconf are the differences in each of the values from those of wild-type Fv.
* Data from Table I.
* Calculated from the equation
Salt Bridge Formation in Antigen-Antibody Interaction

TABLE III

| Mutants       | \( T^* \) | \( \Delta S_{HE} (T^*) \) | \( R \) |
|---------------|----------|------------------------|------|
| Wild type     | 245      | 203.9                  | 27.5 |
| HD32A         | 270      | 248.2                  | 35.4 |
| HD32N         | 265      | 273.3                  | 39.9 |
| HD32E         | 275      | 247.5                  | 35.3 |

\( ^* \) Temperature where entropic contribution to the interaction is zero.

Values were estimated from the plot in Fig. 4.

Calculated from the equation

\[
\Delta S = \Delta S_{HE} + \Delta S_{RT} + \Delta S_{other} \tag{3}
\]

where \( \Delta S_{HE} \) is the entropy change from hydrophobic interaction, and \( \Delta S_{RT} \) is that from rotational and translational change. Where \( \Delta S = 0 \) at a particular temperature \( (T^*) \),

\[
0 = \Delta S_{HE}(T^*) + \Delta S_{RT}(T^*) + \Delta S_{other} \tag{4}
\]

is obtained. \( \Delta S_{HE}(T^*) \) is related to the heat capacity change, and \( \Delta S_{RT} \) is considered to be constant \((50 \text{ cal mol}^{-1} \text{ K}^{-1}) \) (41). If the entropy change due to conformational changes is considered to be equal for each residue, the entropy change is calculated to be 5.6 cal mol\(^{-1} \) K\(^{-1} \), and thus division of \( \Delta S_{other} \) by \(-5.6 \) yields the number of residues involved in the conformational changes:

\[
R = \frac{\Delta S_{other}}{-5.6} \tag{5}
\]

We have found that in the HyHEL10-HEL interaction, the folding in the antibody HyHEL10 is induced by binding with HEL (26). In this study, the numbers of residues \((R) \) involved in the folding induced by the association between the mutant Fv fragments and HEL were estimated according to the above proposal (Table III). The results indicated that \( R \) values were increased by substitution with Ala, Asn, or Glu at position 32 of VH, suggesting that local conformational changes for the interaction between mutant Fv and HEL are increased by the mutation if the mutual structures of VH are not affected by the substitutions. Thus, it can be concluded that a large proportion of the increased negative entropy change induced by mutation arises from increased structural change, suggesting that the salt bridge suppresses excess local conformational change upon binding.

Conclusion—We previously analyzed thermodynamically the HyHEL10-HEL interaction using mutant Fv fragments (Tyr residues were substituted with Phe) (27). The hydroxyl groups of the Tyr residues at sites 33, 50, and 55 form hydrogen bonds with the antigen, HEL. The changes in the thermodynamic parameters induced by removal of a hydroxyl group from each of the Tyr residues at positions 33 and 50 in HyHEL10 VH are as follows: 1) the negative values of both the enthalpy and entropy change are decreased by substitution; 2) the \( R \) number is decreased. These data indicate that hydrogen bonds created by complementary association of the antibody with the antigen are one of the major contributors to the gain in binding enthalpy. However, the data reported here indicate that the salt bridge makes a different thermodynamic contribution to the interaction.

In conclusion, the removal of the salt bridge produced an increase in the negative entropy \((-\Delta T\Delta S)\) of the interaction, and \(-\Delta T\Delta S\) was almost compensated by an increase in negative enthalpy \((-\Delta H)\), resulting in a small decrease in negative binding Gibbs energy \((-\Delta G)\). The entropic contribution of salt bridge removal arose not only from the desolvation effect also but also from the structural effect (i.e. excess conformational change). The salt bridge may be formed to decrease this entropic loss and to suppress the extra complementary association in the HyHEL10-HEL interaction.