Structural Consequences of Target Epitope-directed Functional Alteration of an Antibody

THE CASE OF ANTI-HEN LYSOZYME ANTIBODY, HyHEL-10*

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Decreased affinity of an antibody for a mutated epitope in an antigen can be enhanced and reversed by mutations in certain antibody residues. Here we describe the crystal structures of (a) the complex between a naturally mutated proteinaceous antigen and an antibody that was mutated and selected in vitro, and (b) the complex between the normal antigen and the mutated antibody. The mutated and selected antibody recognizes essentially the same epitope as in the wild-type antibody, indicating successful target site-directed functional alteration of the antibody. In comparing the structure of the mutated antigen-mutant antibody complex with the previously established structure of the wild-type antigen-wild-type antibody complex, we found that the enhanced affinity of the mutated antibody for the mutant antigen originated not from improvements in local complementarity around the mutated sites but from subtle and critical structural changes in nonmutated sites, including an increase in variable domain interactions. Our findings indicate that only a few mutations in the antigen-binding region of an antibody can lead to some structural changes in its paratopes, emphasizing the critical roles of the plasticity of loops in the complementarity-determining region and also the importance of the plasticity of the interaction between the variable regions of immunoglobulin heavy and light chains in determining the specificity of an antibody.

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Often only a few mutations in the epitope of an antigen enable it to evade immunological attack. Major examples occur in viral coat proteins, e.g. hemagglutinin of influenza virus, gp120 of the human immunodeficiency virus, and human hepatitis B virus surface antigen (1–5). Despite only a few mutations in the antigenic epitope, the specificity and affinity of neutralizing antibodies for the mutated antigen can be completely abolished. The ability to generate specific antibodies directed against the mutated antigenic epitope thus should prove very useful. However, site-directed random mutation, chain shuffling, and DNA shuffling methods have led to only limited success in improving the affinity of antibodies for target antigens (6–9).

Extensive analyses of antigen-antibody interactions have led to the conclusion that the high specificity and affinity of antibody molecules toward target antigens (10–14) essentially originate from complementarity between their molecular shapes (10–16). Thus, structural alterations arising from changes in the antigen and/or interfacial antibody residues reduce antigen-antibody affinity. Several studies of structural changes involved in the ability of influenza virus to evade the immune system have shown that minimal structural changes in the antigen are enough to prevent antibody binding (17–20).

Recent advances in antibody engineering have made it possible to prepare tailor-made antibody fragments by in vitro selection (21–24). In particular, enhancement of the affinity of humanized mouse antibodies and of human antibodies selected from naive phage display libraries without alteration of target sites offers scope for the development of functional and useful antibody molecules (25–27). Based on hundreds of studies of antibody structures, precise procedures for modeling these proteins have been developed. Therefore, a combination of structural information and in vitro selection, e.g. by phage display technology, should be a realistic and reasonable way to achieve epitope-directed improvements in antibody-antigen recognition.

Recently, we reported the selection and functional characterization of an anti-hen egg white lysozyme antibody (HyHEL-10) from a focus library of antibody variable domains (i.e. mini-libraries containing random mutations at four identical sites in the complementarity determining region (CDR)) of the heavy chain (CDR-H2) region. Phage display was used to

1 The abbreviations used are: CDR, complementarity-determining region; HEL, hen egg white lysozyme; TEL, turkey egg white lysozyme; VH, variable region of immunoglobulin heavy chain; VL, variable region of immunoglobulin light chain; Fv, variable-region fragment of immunoglobulin; WT, wild-type; SSFSF, mutant, mutant HyHEL-10 Fv in which Tyr35–Ser36–Ser38–Tyr39 in the VH chain is replaced with Ser35–Phe36–Ser38–Phe39.
enhance its specificity toward turkey lysozyme (a naturally mutated antigen) (28). Isothermal titration calorimetric studies have revealed the following (28). 1) Mutants selected have an affinity toward turkey lysozyme with an order of 10^8 and a reduced affinity toward the original antigen, hen lysozyme, with an order of 10^6. 2) Increase in negative enthalpy change (\(\Delta H\)) has driven the enhancement of an affinity for the target antigen, whereas decrease in \(\Delta H\) led to reduction of the affinity for hen lysozyme.

Structural information of the complexes would give several insights into the molecular mechanism for target site-directed functional alteration of antibodies. Here we report structural analyses of complexes between the naturally mutated antigen and an artificially mutated and selected antibody and between the normal antigen and the same antibody. On the basis of the results obtained, we discuss the critical effects on antibody specificity of the plasticity of the CDR loops (including the roles of some antibody residues that support the loop structures) and also of the plasticity of the interaction between the variable regions of immunoglobulin heavy and light chains.

### EXPERIMENTAL PROCEDURES

**Materials**—Hen egg white lysozyme (HEL) was obtained from Seikagaku-Kogyo Inc. (Tokyo, Japan) and turkey egg white lysozyme (TEL) from Sigma. The expression vector for the HyHEL-10 variable region fragment (Fv) (wild-type and an SFSF mutant in which sites 53, 54, 56, and 58 are Ser, Phe, Ser, and Phe, respectively) was described previously (28). All other reagents were of biochemical research grade.

**Expression and Purification of Soluble Fv Fragments**—A transformed *Escherichia coli* strain, BL21 (DE3) (29), harboring an expression vector was grown at 28°C; C. The bacterial supernatant and periplasmic fractions were as follows (30). The culture was added to a final concentration of 0.1 mM, and the culture was grown overnight at 28°C. The supernatant was removed, and the cell pellet was resuspended in 10 ml of 0.1M Tris-HCl (pH 7.5), 0.5 M sucrose, and 0.1 mM EDTA and was incubated for 5 min at room temperature. Then 40 ml of water was added to give an osmotic shock, and the cells were left on ice for 30 min. The cells were collected by centrifugation at 7000 × g for 15 min at 4°C, the culture supernatant was removed, and the cell pellet was resuspended in 10 ml of 20 mM Tris-HCl (pH 7.5), 0.5 M sucrose, and 0.1 mM EDTA and was incubated for 5 min at room temperature. Then 40 ml of water was added to give an osmotic shock, and the cells were left on ice for 30 min. The cells were collected by centrifugation at 7000 × g for 60 min at 4°C, and the supernatant was saved as the periplasmic sample.
monium sulfate at 80% saturation, and the precipitates were collected by centrifugation at 7000 × g for 30 min at 4 °C. The protein precipitates were dissolved in phosphate-buffered saline and were dialyzed against the same buffer for 2 days at 4 °C. The precipitates that formed during dialysis were removed by centrifugation at 10,000 × g for 15 min. The supernatant was loaded onto a TEL-Sepharose column, in which about 10 mg of TEL per milliliter of gel was bound to CNBr-activated Sepharose 4B (Amersham Bioscience) previously equilibrated with phosphate-buffered saline. The column (inner diameter, 10 mm × 5 cm) was washed with 100 mM Tris HCl (pH 8.5) containing 500 mM NaCl; the adsorbed protein was then eluted with 100 mM glycine buffer (pH 2.0). The eluate was quickly neutralized with 1M Tris HCl (pH 7.5).

The Fv fragment obtained by affinity chromatography was further purified on a Superdex 75pg column (inner diameter, 10 mm × 100 cm), equilibrated with 50 mM Tris HCl (pH 7.5) containing 200 mM NaCl, and finally dialyzed overnight against phosphate-buffered saline at 4 °C.

Crystallization, Data Collection, and Structure Determination—Although the complex of the HyHEL-10 SFSF Fv fragment with HEL was crystallized as described previously (32), the crystal of the SFSF-HEL complex most suitable for further analyses could be grown from 100 mM HEPES buffer (pH 7.6–7.8), 10% w/v polyethylene glycol 8000, 10% ethylene glycerol. The crystal of the SFSF-TEL complex suitable for further analyses was grown from 200 mM ammonium acetate, 100 mM tri-sodium citrate dihydrate buffer (pH 6.6), 20% w/v polyethylene glycerol 4000, 10% ethylene glycerol. They were elongated, bipyramidal crystals.

All crystallographic data were collected at 100 K by using synchrotron radiation on beam line 6A of the Photon Factory (Tsukuba, Japan) with a Weissenberg camera (33). The diffraction images were integrated with the hkl program DENZO, and the intensity data were processed with SCALA and AGROVATA in the CCP4 suite (34). The crystallographic data and statistics are summarized in Table I. The crystal of SFSF Fv complex with HEL was isomorphous with crystals of the wild-type (WT) Fv complex with HEL (32), whereas the crystal of SFSF Fv complex with TEL was grown in the different crystal form. The model coordinates of the SFSF complexes were derived from those of the wild-type complex structure (Protein Data Bank ID code 1c08).

The structure of the SFSF-HEL complex was refined with the pro-
gram CNS (35). Although symmetry of diffraction intensities of SFSF/TEL showed Laue group of 4/mmm, the corresponding unit cell cannot contain one molecule in an asymmetric unit under the space group symmetry. Test for hemihedral twinning (36) has shown this crystal as a perfect twinning crystal, i.e. the twin fraction was estimated as 0.5. The structures of this crystal were determined by a molecular replacement method with the program AMoRe (37) in the CCP4 suite. Refinement was carried out by program SHELX97 (38), introducing TWIN and BASF options.

The atomic coordinates of the mutant Fv-lysozyme complexes were deposited in the Protein Data Bank (ID codes 1UA6 and 1UAC for HEL-SFSF and TEL-SFSF, respectively).

RESULTS

Overall Structure of Mutant Lysozyme-HyHEL-10 Complexes—Earlier, we reported the selection and functional characterization of an anti-hen egg white lysozyme antibody (HyHEL-10) from a focus library of antibody variable domains (i.e. mini-libraries containing random mutations at four identical sites in the CDR-H2 region). Phage display was used to enhance its specificity toward turkey egg white lysozyme (28). Several mutants were selected. Here we report structural analyses of one of them (SFSF), complexed with TEL (essentially a naturally mutated antigen) and with the wild-type antigen HEL. The contact residues in each complex are summarized in Table II.

Superposition of the Cα backbones of the TEL-SFSF, HEL-SFSF, and HEL-WT complexes indicated that, despite four mutations in the CDR-H2 loop of SFSF relative to WT and four mutations in TEL relative to HEL, the overall structures of the TEL-SFSF, HEL-SFSF, and HEL-WT complexes were almost identical (Fig. 1), except for some residues in the flexible loops.

The crystal structure of HEL-WT indicates that the following noncovalent bonds occur in the interface between antigen and antibody: 16 hydrogen bonds, 2 salt bridges, and 94 van der Waals interactions. In the SFSF complex, these numbers are 14 hydrogen bonds, 2 salt bridges, and 97 van der Waals interactions.

Differences in amino acid residues participating in the interactions were also observed (Table II). In the TEL-SFSF complex, Thr54VH, Trp62TEL, Lys79TEL, and Asn79TEL were found to be direct contact residues in the antigen-antibody interface, but Ser93VH, Tyr96VH, and Ser52VH were not. In the HEL-SFSF complex, Thr54VH, Trp62VH, and Arg73HEL were included in the direct contact residues, but Ser93VH, Tyr96VH, and Thr54VH were not.

Shape Complementarities—Shape complementarities of the molecular surfaces between antibody Fv fragment and two antigen lysozymes have been estimated using program SC (15) in the CCP4 program suit. Sc values for HEL-WT, HEL-SFSF, and TEL-SFSF were 0.702, 0.729, and 0.705, respectively, suggesting that the interface of these antigen-antibody complexes have almost identical shape complementarity to each other. On the other hand, shape complementarities of the molecular surfaces between VH and VL are calculated to be 0.700, 0.733, and 0.695, for HEL-WT, HEL-SFSF, and TEL-SFSF, respectively.

Interfacial Water Molecules—In the TEL-SFSF complex, five water molecules bridged the imperfect complementarity of the antigen-antibody chains, and in HEL-SFSF, seven interfacial water molecules were observed. The hydrogen bond networks (via interfacial water molecules) were almost identical in the TEL-SFSF and HEL-SFSF complexes, except for the two additional interfacial water molecules in HEL-SFSF.

Local Structural Changes in Mutated CDR-H2—Superposition of the three complexes indicated that the orientation of CDR-H2 changed in the mutants (Fig. 2). A major change occurred around Phe54VH. The epitope recognized by residue 54 has a flexible structure, suggesting induced conformational changes depending on whether WT or SFSF antibody binds to it.

As shown in Fig. 3a, Phe54VH of SFSF interacts with Trp62HEL, Leu75HEL, and Arg103HEL leading to clustering of the residues in the HEL-SFSF complex. In contrast, Phe54VH of SFSF interacts only with Trp62TEL in the TEL-SFSF complex. The different orientations of Phe54VH in HEL-SFSF and TEL-SFSF may lead to other changes in orientation of the CDR-H2 loop in the complex structure.

Other local structural changes were observed around Phe54VH. In the HEL-SFSF complex, the amide groups of Arg21HEL (gray) make hydrogen bonds with the hydroxyl group of Tyr50VH (red), and the aromatic ring of Tyr58VH (red) interacts with Arg21HEL (gray) via an amino-acromatic interaction (Fig. 3, b and c). In the TEL-SFSF complex, one of the amide groups in Arg21TEL (green) makes a hydrogen bond with the hydroxyl group of Tyr50VH (violet) and also interacts with the aromatic ring via an amino-acromatic interaction (Fig. 3, b and c). In addition, contacts between Tyr58VH and Phe54VH in TEL-SFSF are increased compared with the HEL-WT and HEL-SFSF complexes. Isothermal titration calorimetric studies have suggested an enthalpic contribution of Phe54VH in lysozyme-mutant antibody interactions (28). Deletion of the hydroxyl group of Tyr50VH from HyHEL-10 WT may lead to reorientation of the aromatic ring of VH at site 58 toward the loop consisting of residues 99–102, which might enhance the affinity of the antibody for lysozyme.
crystallographic studies have revealed several local differences between the TEL/H18528SFSF and HEL/H18528SFSF complexes.

**CDR-L3**—In TEL/H18528SFSF, the amide side chain of Asn92VL made hydrogen bonds with the main chain carbonyl of Asn19TEL (3.16 Å) and the side chain of Asn32VL. In HEL/SFSF, the length of the hydrogen bond between Asn92VL and Asn32VL is 3.27 Å, and one interfacial water molecule mediates the contacts with the main chain carbonyls of Asp32VH and Gly16HEL via hydrogen bonds.

**CDR-H1**—In TEL/H18528SFSF and HEL/SFSF, hydrogen bond formations between Ser31VH and Asp32VH and the main chain carbonyls of Arg73HEL (or Lys73TEL) and between the hydroxyl group of Tyr33VH and the main chain carbonyl of Lys97HEL (or TEL) were observed. In addition, the side chain carboxyl group of Asp32VH made a hydrogen bond with the side chain of Asn77TEL in TEL/H18528SFSF. Asn77TEL participated in a hydrogen bond network via an interfacial water molecule located in the interface between TEL/SFSF and CDR-H3 (Fig. 4), which was not observed in the HEL/SFSF and HEL/WT complexes.

**CDR-H3**—No major conformational changes occurred between the three complexes. The contact area of TEL-VL in the TEL/SFSF complex, however, increased. Additionally, a hydrogen bond network in TEL/SFSF via an interfacial water molecule, distinct from that in HEL/SFSF, was observed (Fig. 4), suggesting that the interfacial water molecules control the complementarity of the antigen-binding site of the antibody, as was suggested for anti-steroid hormone antibody 4155 (40) and other antigen-antibody and protein-ligand interactions (41–43).

**CDR-CDR and VH-VL Interactions**—Although the contact

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**Fig. 3.** Local structural changes in mutated CDR-H2: HEL/SFSF, VL chain, blue; VH chain, red; HEL, gray; TEL/SFSF, VL chain, sky blue; VH chain, mauve; TEL, green. Residue numbers are according to Kabat et al. (39). a, the region around residue 54. Aromatic-aromatic interaction in TEL/SFSF is depicted as a dotted line. b and c, the region around residues 50 and 58 in different orientations. Hydrogen bonds and amino-aromatic interactions are depicted as dashed lines and a hatched line, respectively.

**Fig. 4.** Local structural changes in CDR-H1 and CDR-H3. Hydrogen bonds via water molecules (large dots) are depicted as dashed red lines, salt bridges as dashed blue lines, and the hydrogen bond as a dashed black line. HEL and SFSF in HEL/SFSF complex are shown in gray and red, respectively; TEL and SFSF in TEL/SFSF are as green and mauve, respectively. Residue numbers are according to Kabat et al. (39).

**Fig. 5.** Relative ratio of each CDR loop to the total interfacial areas of the antibody covered by the target antigens.
Epitope-directed Engineering of an Antibody

Here we report the crystal structure of two antigen-antibody complexes. One is a complex between a mutant antibody fragment and its target antigen (the mutant antibody was selected from focus libraries containing random mutations at four sites via phage display using TEL as a natural mutant for the HEL antigen). The other is a complex between the same mutant antibody fragment and the original (HEL) antigen. To the best of our knowledge, this is the first report of the structures of complexes of engineered antibody fragments with their target (mutated) and wild-type antigens.

The significant contribution of VL-VH interactions to the epitope binding and its target antigen (the mutant antibody was selected from focus libraries containing random mutations at four sites via phage display using TEL as a natural mutant for the HEL antigen). The other is a complex between the same mutant antibody fragment and the original (HEL) antigen. To the best of our knowledge, this is the first report of the structures of complexes of engineered antibody fragments with their target (mutated) and wild-type antigens.

Introduction of amino acids rarely found in immune proteins into the antibody loops often causes structural disruption of other parts of the antibody molecule. The results reported here emphasize the critical role of the CDR loops, via subtle but significant structural changes in other CDR loops.

The significant contribution of VL-VH interactions to the
Epitope-Directed Engineering of an Antibody

specificity and affinity of antibody molecules was first pointed out by Colman (interface adaptor hypothesis) (44) and was experimentally demonstrated by Stanfield et al. (45) using complexed and ligand-complexed forms of anti-human immunodeficiency virus type 1 peptide antibody (Fab 50.1). The antibody fragment has a smaller VL-VH contact area, perhaps because of the shorter length of CDR-H3, which may lead to changes in VL-VH contact upon complexation, i.e. induced fitting upon complexation. Light chain shuffling of an antibody molecule resulted in drifts in epitope recognition, supporting the importance of cooperativity between variable regions to the tintering upon complexation. Light chain shuffling of an antibody changes in VL-VH contact upon complexation, because of the shorter length of CDR-H3, which may lead to nodeficiency virus type 1 peptide antibody (Fab 50.1). The complexed and ligand-complexed forms of anti-human immu-

experimentally demonstrated by Stanfield (interface adaptor hypothesis) (44) and was also highlighted by Colman (44) and was also highlighted by Colman (44). We have also demonstrated that the shortening of CDR-H3 of an antibody fragment results in lower affinity for the ligand, because of the shorter length of CDR-H3, which may lead to changes in VL-VH contact upon complexation.

Firstly, we should point out the relative insensitivity of the antibody structure to the introduction of amino acids rarely used in the immune system into an antigen. The target site-directed functional alteration of antibody molecules via focus libraries containing random mutations at several sites should be promising for promising for improving in various fields. Our results should encourage researchers, especially in the fields of evolu-
tionary engineering, antibody engineering, and rational protein design. Additional accumulation of structural data on interactions between engineered proteins, including antibody molecules, is especially important.

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