Critical Contribution of Aromatic Rings to Specific Recognition of Polyether Rings

THE CASE OF CIGUATOXIN CTX3C-ABC AND ITS SPECIFIC ANTIBODY 1C49

To address how proteins recognize polyether toxin compounds, we focused on the interaction between the ABC ring compound of ciguatoxin 3C and its specific antibody, 1C49. Surface plasmon resonance analyses indicated that Escherichia coli-expressed variable domain fragments (Fv) of 1C49 had the high affinity constants and slow dissociation constants typical of antigen-antibody interactions. Linear van’t Hoff analyses suggested that the interaction is enthalpy-driven. We resolved the crystal structure of 1C49 Fv bound to ABC ring compound of ciguatoxin 3C at a resolution of 1.7 Å. The binding pocket of the antibody had many aromatic rings and bound the antigen by shape complementarity typical of hapten-antibody interactions. Three hydrogen bonds and many van der Waals interactions were present. We mutated several residues of the antibody to Ala, and we used surface plasmon resonance to analyze the interactions between the mutated antibodies and the antigen. This analysis identified Tyr-91 and Trp-96 in the light chain as critical for the specific recognition of polyether compounds by proteins.

Ciguatera is a form of food poisoning caused by the ingestion of reef fish that have accumulated trace amounts of ciguatoxins of dinoflagellate origin via the food chain. More than 50,000 people suffer from ciguatera annually, making it one of the most common sources of food poisoning (1–4). The disease is characterized by gastrointestinal, neurological, and cardiovascular disturbances that often persist for months or years, and in severe cases, paralysis, coma, and death may occur (1). Ciguatoxins exert their effects by binding to voltage-sensitive sodium ion channels, causing persistent activation of the channels (1).

Ciguatoxin and its congener, CTX3C, are structurally classified as ladder-like polyethers (Fig. 1) (5, 6). A major obstacle to avoiding the disease is that ciguateric fish look, taste, and smell the same as uncontaminated fish. In addition, neither cooking nor freezing detoxifies the heat-stable ciguatoxins. Despite the seriousness of ciguatera, there is currently no rapid and reliable method for detecting these toxins at fisheries. The traditional method is a mouse bioassay of lipid extracts (7). Several additional methods for detecting ciguatoxins have recently been developed, including assays based on cytotoxicity (8), radioligand binding (8, 9), high performance chromatography (10), mass spectrometry (11–13), and an antibody-based immunoassay (14–16). Among these, the antibody-based immunoassay is attractive, because it is accurate, sensitive, easily performed, and portable. During the development of this immunoassay, extensive studies focused on preparing antibodies with high specificity for ciguatoxin, and recently a sandwich enzyme-linked immunosorbent assay using high affinity antibodies specific to both ends of CTX3C was established that can detect CTX3C down to the parts per billion level without cross-reactivity against other related marine toxins (14, 15), including brevetoxin A (17), brevetoxin B (18), okadaic acid (19), and maftoxin (5).

A monoclonal antibody, 1C49, was obtained from in vitro selection using a biotin-linked ABC ring fragment of CTX3C (CTX3C-ABC) Fig. 1) and an Fab library prepared from the spleens of mice.
immunized with CTX3C-ABC conjugated to keyhole limpet hemocyanin (16). Fab fragment of 1C49 binds CTX3C-ABC and CTX3C-ABCD with dissociation constants \( K_d \) of 8.6 \( \times \) 10^{-8} M and 2.4 \( \times \) 10^{-5} M, respectively (16). 1C49 also bound to CTX3C, although the affinity is reduced as compared with CTX3C-ABC (16). Elucidation of the molecular mechanism of the specific binding of the antibody to the ciguatoxin fragment would enable the antibody-based ciguatoxin immunoassay to be improved. Moreover, many marine toxins have polyether structures. These toxins include maitotoxin (5) and gambierol, which also cause ciguatera (5), brevetoxin, which causes neurotoxic shellfish poisoning (17, 18), dinophysistoxins, which cause diarrheic shellfish poisoning (19), palytoxin (20), and yessotoxin (21). Therefore, research focused on antibodies that can recognize polyether compounds may produce a more effective antibody-based immunoassay based on well understood mechanisms. Here, we investigated the binding of 1C49 to the CTX3C-ABC polypeptide from a structural viewpoint. First, we resolved the crystal structure of the 1C49 Fv fragment in complex with CTX3C-ABC at a resolution of 1.7 Å. CTX3C-ABC is deeply buried in the binding pocket and interacts with 1C49 by using three hydrogen bonds and many van der Waals interactions. Kinetic analyses of mutant proteins indicated that L-Tyr-91 and L-Trp-96 are critical residues, i.e. hot spots, for the interaction. Systematic mutation of L-Tyr-91 showed that aromatic interactions and the bulkiness by L-Tyr-91 are essential for the association and the inhibition of dissociation, respectively.

**EXPERIMENTAL PROCEDURES**

**Construction of an Expression Vector for the Fv Fragment of Monoclonal Antibody 1C49 and Its Mutant Proteins**—The gene encoding a fragment of the immunoglobulin variable regions (Fv) was amplified with KOD-Plus DNA polymerase (Toyobo) by using a vector expressing the Fab fragment of 1C49 (16) as a template. To amplify a fragment of the variable region of the immunoglobulin heavy chain ( VH), we used the following primer sets (restriction enzyme sites are underlined): 1C49-VH-NcoI-back \( (5'-'NNNCCATGG-GCCCAGGTGCAGCTGCTGAGCTGGGCTGA-3') \), 1C49-VH-SacII-forward \( (5'-NNNCCGGCGAAAAGACCGTGACTGAGG-3') \). The PCR products were digested by NcoI and SacII and then inserted into a T7 promoter-based expression vector (22) to attach a His tag at the C terminus and a pel-B signal sequence at the N terminus of VH. A fragment of the variable region of the light chain (VL) was first amplified with the primer set 1C49-VL-NcoI-back \( (5'-NNNCCGATGCCGACATTCTAGACAT-CAGTCTCTC-3') \), 1C49-VL-SacII-forward \( (5'-NNNCCCGGCGGCCCC- TTTGATTTCAGATTG-3') \) (restriction enzyme sites are underlined), and the product was also inserted into the T7 promoter-based expression vector to attach a His tag at the C terminus and a pel-B signal sequence at the N terminus. Subsequently, the His-tagged VL fragment was amplified by using the resulting product from the first amplification and the primer set SpeI-pel-B-back \( (5'-CCTAGTTTTCTCAAGAGACAGTCAATGAAATACC-3') \) and BamHI-His tag-forward \( (5'-NNNGGATCCGCTATTAATGTGTTGATGATGTTG-3') \), followed by digestion by SpeI and BamHI. By inserting the product downstream of the VH-expressing vector described above, we created an expression vector with a gene for the Fv fragment where the genes for VH and VL are connected in tandem. pel-B signal sequences and His tags were attached to the N and C termini of both chains, respectively. The expression vector is shown in supplemental Fig. S1. The expression vectors for the mutant proteins were constructed by amplifying a DNA fragment by the two-stage PCR method using synthesized primers designed for each mutation, and inserting these instead of the VH or VL fragment described above into the vectors. The accuracy of the DNA sequences was confirmed by sequencing using an ABI 310 Genetic Analyzer (Applied Biosystems, Tokyo, Japan).

**Expression and Purification of the 1C49 Fv Fragment and Its Mutant Proteins**—Transformed *Escherichia coli* strain BL21(DE3) harboring the expression vector for the desired protein was grown until the early stationary phase at 28 °C in LB medium supplemented with 100 μg ml^{-1} ampicillin. To induce expression of the desired protein, isopropyl-β-d-thiogalactopyranoside was added to a final concentration of 1 mM, and the culture was continued for 14 h.
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The culture was centrifuged at 5000 × g for 15 min at 4 °C, and the supernatant was subjected to ammonium sulfate precipitation at 60% saturation, followed by centrifugation at 7000 × g for 30 min at 4 °C. The protein pellet was solubilized in 30–40 ml of 20 mM Tris-HCl (pH 8.0), 500 mM NaCl, and then dialyzed against the same buffer. Fv fragments were purified with His Bind Resin (Novagen) previously charged with NiSO₄. The protein solution was loaded onto the column, and the column was washed with wash buffer (20 mM Tris-HCl (pH 8.0), 500 mM NaCl, 20 mM imidazole). Fv fragments were eluted with elution buffer (20 mM Tris-HCl (pH 8.0), 500 mM NaCl, 20 mM imidazole). Fv fragments were eluted by using a 0–0.8 M gradient of NaCl in 50 mM phosphate (pH 6.5). Fractions containing Fv fragments were dialyzed against 50 mM phosphate buffer (pH 6.5) and then further purified on a Resource S column (GE Healthcare Biosciences AB) equilibrated with the same buffer. Absorbed proteins were eluted by using a 0–0.8 M gradient of NaCl in 50 mM phosphate (pH 6.5). Fractions containing the Fv fragments were collected and used for further experiments. For the H-W33A, L-Y91L, and L-Y91V mutants, because of the insolubility of the expressed proteins they were prepared by refolding from the insoluble fraction of cells with a stepwise dialysis system described previously (23–25).

Crystallization of the 1C49 Fv Fragment—Purified 1C49 Fv fragment was dialyzed against 10 mM Tris-HCl (pH 8.0) and then concentrated to 10.5 mg ml⁻¹. CTX3C-ABC solution (10 mM in DMSO) was added to the protein solution to a final molar ratio of protein:CTX3C-ABC of 1:1.2, followed by a static incubation at room temperature for 1 h. The initial crystallization conditions were screened by the sparse matrix method at 20 °C, using a Crystal Screen kit and Crystal Screen2 kit (Hampton Research) and Wizard I and Wizard II (Emerald Biostructures, Bainbridge Island, WA). Crystals of the 1C49 Fv fragment in complex with CTX3C-ABC most suitable for further analyses were grown from 0.1 M MES buffer (pH 5.7) containing 25% polyethylene glycol monomethyl ether 5000 and 2 M ammonium sulfate by the hanging drop, vapor-diffusion method.

Diffraction Data Collection and Processing—X-ray diffraction data were collected on the beamline NW12 at Photon Factory (Tsukuba, Japan) under cryogenic conditions (100 K). Crystals were soaked in a crystallization buffer containing 15% glycerol before mounting. Diffraction data were collected up to a resolution of 1.7 Å. The data were indexed and integrated with the program HKL2000 (26) and were scaled and merged with the program SCALEA (27) within the CCP4 program suite (28). The data collection and processing statistics are summarized in Table 1.

Structure Solution and Refinement—The structure of the 1C49 Fv fragment in complex with CTX3C-ABC was determined by the molecular replacement method using the program CNS (29). VH and VL regions of an Fab (PDB codes 1AXS and 1FJ1) were used as search models for VH and VL, respectively. An electron density derived from CTX3C-ABC was observed at the CDR region (supplemental Fig. S2). The structure coordinates of CTX3C-ABC were prepared with the program Chem Office 6.0 (CambridgeSoft, Cambridge, MA), and the topology file and the parameter file of CTX3C-ABC were prepared at the HIC-UP web site (30). To monitor the refinement, a random 5% subset of all reflections was set aside for calculation of the Rfree factor. The positional and individual B factor refinements were carried out automatically with the program LAFIRE (31). After automatic refinement and model fitting by LAFIRE, several cycles of refinement with the program CNS and manual model fitting were carried out. Finally, the water molecules were picked automatically, and then ligand molecules were placed manually. The crystallographic R values and Rfree values converged to 19.8% and 21.0%, respectively. The stereochemical quality of the final refined models was analyzed by the program PROCHECK (32). The refinement statistics are summarized in Table 1. Residues are numbered, and framework regions and complementarity-determining regions (CDRs) are designated according to Kabat et al. (33).

The atoms that form the contact between 1C49 Fv and CTX3C-ABC were identified with the CONTACT program in the CCP4 suite, in which the length thresholds of C-C, C-N, C-O, O-O, O-N, and N-N are 4.1, 3.8, 3.7, 3.3, 3.4, and 3.4 Å, respectively. The figures were drawn with the program PyMOL (Delano Scientific LLC). The surface area was calculated with the program AREAIMOL in the CCP4 suite using a probe radius of 1.4 Å.

Analyses of the Interaction between CTX3C-ABC and the 1C49 Fv Fragment or Its Mutants—The interaction between CTX3C-ABC and the 1C49 Fv fragment or its mutant proteins were analyzed by surface plasmon resonance (SPR) spectroscopy with BIACORE 2000 (GE Healthcare Bio-Science AB, Upssala, Sweden). Bovine serum albumin-conjugated CTX3C-ABC was immobilized onto the cells in a CM5 sensor chip. Various concentrations of the 1C49 Fv fragment or its mutants in HBS-EP buffer (10 mM HEPES (pH 7.4), 150 mM NaCl, 3.4

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**TABLE 1**

| Data collection and refinement statistics |
|-----------------------------------------|
| **Data collection**                      |
| Space group                             | P2₁2₁2₁                                    |
| Cell dimensions (Å)                     | a = 47.2, b = 97.5, c = 115.7               |
| Resolution (Å)                          | 1.81–1.70                                  |
| Wavelength (Å)                          | 1.0000                                     |
| Rsym (%)                                | 5.8 (15.2)                                 |
| Completeness (%)                        | 98.8 (95.8)                                |
| Observed reflections                    | 203,645                                    |
| Unique reflections                      | 29,463                                     |
| I/σ (I)                                 | 25.7 (9.6)                                 |
| Multiplicity                             | 6.9 (6.5)                                  |

**Refinement and model quality**

| Resolution range (Å)                    | 20–1.70 (1.81–1.70)                        |
| No. of reflections                      | 29,447                                     |
| R-factor*                               | 0.198 (0.207)                              |
| Rfree-factor*                           | 0.210 (0.249)                              |
| Total protein atoms                     | 1,767                                      |
| Total ligand atoms                      | 32                                         |
| Total water atoms                       | 196                                        |
| Average B-factor (Å²)                   | 20.5                                       |
| Root mean square deviation from ideal   | 0.005                                      |
| Bond lengths (Å)                        | 1.4                                        |
| Ramachandran plot                       | 87.2                                       |
| Residues in generously allowed regions  | 12.2                                       |
| Residues in disallowed regions          | 0.0                                        |
| Residues in disallowed regions          | 0.5                                        |

* The values in parentheses refer to data in the highest resolution shell.
mm EDTA, 0.05% surfactant P20) were flowed over the CTX3C-ABC. The data were normalized by subtracting the response from a blank cell in which bovine serum albumin alone was immobilized. BLAevaluation software (GE Healthcare Bio-Science AB) was used to analyze the data. Kinetic parameters were calculated by a global fitting analysis with the assumptions of the 1:1 Langmuir binding model. The association constants of L-Y91A and L-Y91S mutants were calculated by using a Scatchard plot because of their fast dissociation rate constants.

RESULTS

Crystal Structure of the 1C49 Fv Fragment in Complex with CTX3C-ABC—We solved the crystal structure of the 1C49 Fv fragment in complex with CTX3C-ABC by the molecular replacement method at a resolution of 1.7 Å. The CDRs of the antibody formed a cavity around the ligand binding site, and the antigen, CTX3C-ABC, lay in the cavity (Fig. 2, A and B). A number of aromatic residues, H-Tyr-100a, L-Tyr-49, L-Tyr-91, H-Trp-33, L-Trp-96, and L-Phe-94, were located around the ligand binding site and contributed to ligand binding by van der Waals interactions (Fig. 2C). However, there were no polar residues around the binding site, suggesting that hydrogen bonds or ionic interactions have little effect. Fig. 2B represents an electrostatic surface of 1C49 viewed from above the ligand binding site. Although the entrance of the cavity is charged, the inside is relatively noncharged, also suggesting that van der Waals interactions have greater effects than electrostatic interactions. Ether groups in CTX3C-ABC do not act as proton acceptors for hydrogen bonding despite their abundance. CTX3C-ABC is deeply buried in the cavity, with 457 Å² of its 529 Å² surface area (86.4%) within the cavity, suggesting a well-organized shape complementarity.

The contacts between 1C49 and CTX3C-ABC observed in the crystal structure are listed in supplemental Table S1. CTX3C-ABC has 62 interactions with 15 residues, which are mainly in CDRs. Despite these extensive interactions, only three residues, H-Gln-35, H-Tyr-100a, and L-Trp-96, formed hydrogen bonds, again suggesting the predominance of van der Waals interactions. The binding of CTX3C-ABC is achieved mainly through many aromatic rings, such as those in Trp and Tyr. The three water molecules (Wat) that construct hydrogen bonds with both CTX3C-ABC and 1C49 are observed at the interface (Table 2). Wat-8 and Wat-10 are exposed to solvent above the ligand binding pocket, whereas Wat-50 is located inside the cavity and fills the interface between CTX3C-ABC and the antibody. Fig. 3 is a schematic of the residues around the ligand.

Kinetic Analysis of the Interaction between CTX3C-ABC and the 1C49 Fv Fragment—We used SPR to explore the kinetics of the interaction between CTX3C-ABC and the 1C49 Fv fragment. 1C49 Fv was injected into flow cells on which bovine...
serum albumin-conjugated CTX3C-ABC was immobilized. We applied a global curve-fitting analysis to the SPR sensorsgrams to generate response curves (Fig. 4A) using the assumptions of the Langmuir binding model and a stoichiometry of 1:1. We then used these responses to determine the association rate constant and dissociation rate constant \( k_{\text{on}} \) and \( k_{\text{off}} \) of the interaction between CTX3C-ABC and the 1C49 Fv fragment at 10 °C; \( k_{\text{on}} \) was determined to be \( 7.77 \times 10^8 \text{M}^{-1}\text{s}^{-1} \), and \( k_{\text{off}} \) was determined to be \( 3.50 \times 10^{-4} \text{s}^{-1} \), resulting in an association constant \( K_a \) of \( 2.22 \times 10^9 \text{M}^{-1} \) (Table 3). The fast association rate constant, slow dissociation rate constant, and resulting high association constant are typical of antigen-antibody interactions. To obtain the activation energy for the interaction of CTX3C-ABC with the 1C49 Fv fragment, the kinetic analyses were performed at a range of temperatures (10, 15, 20, and 25 °C). On the basis of the temperature dependence of the association constant (Fig. 4B), the van’t Hoff enthalpy \( \Delta H_{\text{van’t Hoff}} \) was calculated to be \(-31.2 \text{ kJ mol}^{-1} \), suggesting that this interaction is enthalpy-driven.

**Effects of Mutating Residues around the CTX3C-ABC Binding Site**—To investigate the contribution of residues around the CTX3C-ABC binding site, we created Ala mutants of L-Y49, L-Y91, L-F94, L-W96, H-S99, H-D100, H-W33, H-Q35, and H-Y100a, which are residues in CDR regions and interact with plural atoms in CTX3C-ABC, and used SPR to evaluate their binding affinities to CTX3C-ABC (supplemental Fig. S3). Because of the fast dissociation rate constant, the association constant of the L-Y91A mutant was determined by a Scatchard plot of the response in equilibrium. All other mutants were analyzed by global curve fitting of the SPR sensorsgrams. The SPR analyses were performed at various temperatures (10, 15, 20, and 25 °C), and the van’t Hoff enthalpies were obtained from

**TABLE 2**

| Water molecule | Interacting atom | Residue | Atom | Distance |
|----------------|------------------|---------|------|----------|
| Wat-8          | CTX3C-ABC        | O24     |      | 2.66     |
| Wat-10         | CTX3C-ABC        | O23     |      | 2.86     |
| Wat-50         | CTX3C-ABC        | O11     |      | 3.64     |
| Wat-10         | H-Lys-32         | Nɛ      |      | 2.98     |
|                | H-Lys-32         | O       |      | 2.75     |
|                | H-L-Pro-94       | Nɛ      |      | 2.96     |
|                | CTX3C-ABC        | O11     |      | 3.64     |
|                | H-Gln-35         | Ne2     |      | 2.83     |
|                | H-Ser-99         | O       |      | 2.79     |

**FIGURE 3.** A schematic of the ligand binding pocket. CTX3C-ABC is shown as a structural formula. The side chains of two hot-spot residues are also shown as structural formulas. Residues interacting with the ligand by van der Waals interactions are shown as cycle segments. Water molecules that interact with the ligand by hydrogen bonds are also shown (Wat). Hydrogen bonds are represented as dashed lines. The \( \pi-\pi \) and \( \text{CH}-\pi \) interactions by L-Tyr-91 are shown as thick dashed lines.

**FIGURE 4.** Kinetic analysis of the interaction of wild-type 1C49 Fv with CTX3C-ABC. A, SPR sensorsgrams obtained with various analyte concentrations are shown as gray lines. The results of global fitting kinetic analyses are also shown as black lines. B, temperature dependence of association constants. An approximating curve is also shown. The kinetic and thermodynamic parameters are listed in Table 4.

**TABLE 3**

| Kinetic parameters at 10 °C and van’t Hoff enthalpy of wild-type and mutant 1C49 Fv fragments |
|-----------------|-----------------|-----------------|-----------------|
|                  | \( k_{\text{on}}^a \) | \( k_{\text{off}}^b \) | \( K_a \)      | \( \Delta H_{\text{van’t Hoff}}^c \) |
| WT               | \( 7.77 \times 10^8 \) | \( 3.50 \times 10^{-4} \) | \( 2.22 \times 10^9 \) | \(-31.2 \) |
| L-Y49A           | \( 1.03 \times 10^6 \) | \( 8.06 \times 10^{-4} \) | \( 1.28 \times 10^9 \) | \(-28.4 \) |
| L-Y91A           | \( 5.53 \times 10^5 \) | \( 2.30 \times 10^{-3} \) | \( 2.41 \times 10^9 \) | \(-16.0 \) |
| L-W96A           | ND \(^d\)        | ND              | ND              | ND |
| H-S99A           | \( 5.19 \times 10^5 \) | \( 4.88 \times 10^{-3} \) | \( 1.06 \times 10^9 \) | \(-24.7 \) |
| H-D100A          | \( 1.48 \times 10^5 \) | \( 5.70 \times 10^{-3} \) | \( 2.60 \times 10^9 \) | \(-28.2 \) |
| H-W33A           | \( 7.93 \times 10^4 \) | \( 1.35 \times 10^{-2} \) | \( 5.87 \times 10^9 \) | \(-8.60 \) |
| H-Q35A           | \( 1.54 \times 10^4 \) | \( 5.58 \times 10^{-2} \) | \( 2.67 \times 10^9 \) | \(-20.7 \) |
| H-Y100aA         | \( 8.21 \times 10^3 \) | \( 3.74 \times 10^{-1} \) | \( 2.20 \times 10^9 \) | \(-26.9 \) |

\(^a\) These values were obtained with a global curve fitting analysis of the SPR sensorsgrams with the assumptions of the Langmuir binding model and a stoichiometry of 1:1.

\(^b\) van’t Hoff enthalpies were obtained from the slope of the van’t Hoff plot by the equation \( \ln K_a = \Delta H/RT - \Delta S/R \).

\(^c\) The association constant was determined by a steady-state fit of the binding data from maximal equilibrium values of the SPR sensorsgrams.

\(^d\) ND, no detectable binding.
van’t Hoff plots. The kinetic parameters at 10 °C and van’t Hoff enthalpies of the mutants are summarized in Fig. 5 and Table 3. The L-W96A mutant was completely inactivated, and its binding properties could not be determined. In the H-Y100A and L-Y49A mutants, slight increases in both the association rate constant and dissociation rate constant resulted in an association constant comparable to that of the wild type. The association constants of H-S99A and L-F94A decreased ~10- to 20-fold owing to slight decreases in the association rate constant and increases in the dissociation rate constants. In the L-W33A mutant, although the association rate constant was comparable to that of the wild type, a large increase in the dissociation rate constant caused a 40-fold reduction in the association constant. In the H-Q35A and H-D100A mutants, the association constants were reduced ~100-fold by slight decreases in the association rate constants and enormous increases in the dissociation rate constant. The association constant of the L-Y91A mutant decreased ~1000-fold (Kₐ), L-Y91A = 3.55 × 10⁶ M⁻¹, determined by Scatchard plot), and the L-W96A mutant was completely inactivated. These results suggest that L-Tyr-91 and L-Trp-96 are hot spots for the interaction of 1C49 with CTX3C-ABC.

All mutant proteins gave negative van’t Hoff enthalpies, suggesting that their interactions, like those of the wild type, are enthalpy-driven. Despite the negative enthalpy, the changes of H-W33A and L-Y91A were considerably smaller than those of the wild type.

**Effects of Substituting a Variety of Amino Acid Residues for L-Tyr-91**—The kinetic analyses described above showed an enormous reduction in the association constant and substantial changes in van’t Hoff enthalpy in the L-Y91A mutant. To investigate the role of L-Tyr-91 in more detail, we used SPR to analyze the kinetics of several additional mutants of this residue: L-Y91F, L-Y91L, L-Y91S, L-Y91V, and L-Y91W (supplemental Fig. S4). The van’t Hoff enthalpy of each mutant was also determined by the temperature dependence of the association constant at 10, 15, 20, and 25 °C. The kinetic parameters at 10 °C and the van’t Hoff enthalpy of each mutant are listed in Table 4.

The association constant of L-Y91F decreased only slightly, because the association rate constant was comparable to that of the wild type and the dissociation rate constant increased only a little. Although the association rate constant of L-Y91W increased slightly, the dissociation rate constant increased 10-fold, resulting in a decrease of ~10-fold in the association constant. These results indicate that the interactions between the aromatic ring at this site and CTX3C-ABC contribute significantly to the association rate constant. In contrast, L-Y91L and L-Y91V had association rate constants ~200-fold lower than the wild type and comparable dissociation rate constants, resulting in decreases in the association constants by two orders of magnitude. L-Y91S gave an SPR profile similar to that of L-Y91A, and the curve fitting analyses could not be applied because of the extremely high dissociation rate constant; therefore, we analyzed the association with a Scatchard plot instead. The association constant was ~250-fold lower than the wild type.

**DISCUSSION**

**Binding of CTX3C-ABC to the 1C49 Fv Fragment**—Crystal structural analysis revealed that CTX3C-ABC is deeply buried within the pocket of the 1C49 Fv fragment. It was reported previously that the association constant of the Fab fragment of 1C49 with CTX3C-ABCD (2.4 × 10⁻³ M) is much lower than that with CTX3C-ABC (Kₐ = 8.6 × 10⁻⁸ M) (16). In the structure we determined here, the end of C ring is closely surrounded by H-Trp-33 and L-Phe-94, and it is therefore possible that steric constraints at the D ring would prevent CTX3C-ABCD from entering the pocket. Interestingly, 1C49 can bind to intact CTX3C, although the affinity is much lower (16). 1C49 might have some adaptability against CTX3C fragment. CTX3C-ABC binds to the 1C49 Fv fragment through 62 interactions; of these, only 3 are hydrogen bonds, and most are mediated by aromatic rings such as those in Trp and Tyr. These binding characteristics are typical of hapten-antibody interactions (34–38). In hapten-antibody interactions, residues used for the binding are positionally conserved. For example, the residues at positions 33 and 35 in the CDR-H1 loop, the residue at position 47 in CDR-H2, and the residues at positions 91, 94, and 96 in CDR-L3 commonly participate in binding (39). The corresponding residues in 1C49 certainly contribute to the

**TABLE 4**

| Mutant | kₐ (M⁻¹ s⁻¹) | kₐ (M⁻¹ s⁻¹) | kₐ (M⁻¹ s⁻¹) | ΔHΔ (kJ mol⁻¹) |
|--------|--------------|--------------|--------------|----------------|
| WT     | 7.77 × 10⁵   | 3.50 × 10⁻⁴  | 2.22 × 10⁹   | -31.2          |
| L-Y91A | 3.55 × 10⁶   | 1.08 × 10⁹   | 1.42 × 10⁴   | -44.7          |
| L-Y91F | 7.40 × 10⁵   | 6.08 × 10⁻⁴  | 1.08 × 10⁹   | -36.3          |
| L-Y91L | 4.77 × 10³   | 3.37 × 10⁻⁴  | 1.42 × 10⁴   | -44.7          |
| L-Y91S | 9.03 × 10⁻⁹  | 9.97 × 10⁶   | 1.92 × 10⁴   | -53.9          |
| L-Y91V | 3.04 × 10³   | 3.05 × 10⁻⁴  | 1.92 × 10⁴   | -53.9          |
| L-Y91W | 9.61 × 10⁶   | 4.13 × 10⁻³  | 2.33 × 10⁷   | -24.7          |

* These values were obtained by a global curve fitting analysis of the SPR sensorgrams with the assumptions of the Langmuir binding model and a stoichiometry of 1:1.

† van’t Hoff enthalpy was obtained from the slope of the van’t Hoff plot by the equation ln Kₐ = ΔH/RT - ΔS/R.

‡ The association constant was determined by a steady-state fit of the binding data from maximal equilibrium values of the SPR sensorgrams.
interaction with CTX3C-ABC (supplemental Table S1). Also, 86.4% of the surface area of CTX3C-ABC is deeply buried in the cavity. On the basis of these structural features, we conclude that the interaction between the 1C49 Fv fragment and CTX3C-ABC is dominated by shape complementarity, as is typical of hapten-antibody interactions.

Role of Each Residue in the Interaction of CTX3C-ABC with the 1C49 Fv Fragment—CTX3C-ABC can be divided into two parts on the basis of its chemical characteristics (Fig. 1B); one is an ABC ring, and the other is a functional group that includes a methoxymethyl ether and an acid. Among the residues we mutated to Ala, H-Tyr-100a, L-Tyr-49, L-Tyr-91, H-Ser-99, and H-Asp-100 interact with the ABC ring; H-Trp-33, H-Gln-35, and L-Phe-94 interact with the functional group; and L-Trp-96 interacts with both of them. Fig. 5 is a diagrammatic representation of the kinetic parameters of the mutants. Taken together with the results from thermodynamic analyses, L-Tyr-91 and L-Trp-96 could be identified as hot spots for the interaction, and other residues made incremental contributions by conferring enthalpic advantages and reducing the dissociation rate constant. It should be noticed that critical contributions to the antigen-antibody interaction of hydrogen bond formation at H-Gln-35 and of van der Waals interactions at L-Trp-96 and H-Trp-33, which have been reported in other hapten-antibody interactions (35, 38, 40–44).

In many antibodies, Tyr residues located at the surface are critical for antigen binding (45–53), and Tyr residues often have an important role in other protein-protein interactions (45, 46, 54, 55). In the following sections, we focus on the role of an aromatic ring to the polyether-antibody interaction, which was revealed by systematic mutation of L-Tyr-91.

Effects of Substituting a Variety of Amino Acid Residues for L-Tyr-91—Our structural analysis showed that L-Tyr-91 forms many contacts with CTX3C-ABC, and an SPR kinetic analysis demonstrated that this residue contributes substantially to the interaction. These results strongly indicate that L-Tyr-91 is a hot-spot residue for binding to CTX3C-ABC; this is similar to the role of the residue at position 91 in other anti-hapten antibodies (35, 36, 39). The L-Y91A mutant had a drastically increased dissociation rate constant, and the resultant association constant decreased ~1000-fold. There is no possibility that this mutation decreased the stability of the Fv fragment, because L-Tyr-91 does not participate in the VH-VL interaction. Therefore, it is likely that L-Tyr-91 is a hot-spot residue that contributes only to antigen binding. Examination of the crystal structure shows that the aromatic ring in L-Tyr-91 is parallel to the A ring in the antigen, suggesting that π–π interactions and CH–π interactions contribute to the interaction with the A ring.

We further investigated the effects of substituting a variety of amino acid residues for L-Tyr-91 using SPR. In L-Y91F, we observed a slight decrease in association constant owing to a slight increase in dissociation rate constant. This result indicates that aromaticity and van der Waals interaction are important for the affinity, and the hydroxyl group additionally helps suppress dissociation of the antigen. However, the hydroxyl group of L-Tyr-91 does not form any interaction with antigen in the crystal structure, and therefore it is likely that the role of the hydroxyl group is distinct from hydrogen bonding with the antigen (49, 56, 57). A hydrogen atom located between the A ring and B ring of the antigen (indicated with an asterisk in Fig. 1) is thought to be a protogenic atom, because there is an oxygen atom next to the CH. Thus, when a CH–π interaction is formed between the A ring and the aromatic ring of L-Tyr-91, the proton atom is thought to act as an effective CH. Tyr residues contain a hydroxyl group that can act as a π-electron donor and therefore can form much more stable CH–π interactions than Phe (58–60).

In the L-Y91W mutant, the association rate constant increased slightly and the dissociation rate constant increased >10-fold, resulting in a reduction in the association constant of ~10-fold. The substitution of a bulky Trp residue would have affected the shape complementarity, leading to the increase in the dissociation rate constant. Replacing L-Tyr-91 with Trp increased the association rate constant despite the possible disruption of the shape complementation, suggesting that the Trp side chain is favorable for the association with the antigen. The order of the magnitude of the CH–π interaction is as follows; Trp > Tyr > Phe (58–60). Therefore, it is possible that the L-Y91W mutant is able to bind to CTX3C-ABC through a CH–π interaction, but changes in shape complementarity due to its bulkiness might not allow it.

In the case of the L-Y91L and L-Y91V mutants, although dissociation rate constant did not decrease drastically, the decrease in the association rate constant caused a remarkable decrease in the association constant. Considering that Leu and Val are bulky hydrophobic residues, it is likely that van der Waals interactions contribute to the suppression of antigen dissociation. The result that removal of the aromatic ring at this site led to a decrease in the association rate constant but did not affect the dissociation rate constant suggests that the aromatic ring in Tyr is essential for binding with CTX3C-ABC at the association stage; this agrees with the conclusion from the L-Y91F and L-Y91W substitutions described above.

On the basis of these results focusing on L-Tyr-91, we conclude that the aromatic ring of the residue at VL position 91 forms a CH–π interaction with the CH located between the A and B rings and a π–π interaction with the double bond in the A ring, and that these interactions are essential for the binding with CTX3C-ABC at the association phase. The following stable complex is achieved through van der Waals interactions with the bulky hydrophobic side chain. Aromaticity and bulkiness are critical for the specific recognition of polyether compounds by proteins.

Finally, this is the first report of the importance of aromatic interactions for the recognition of polyether compounds. These findings will provide fundamental information for the study of the specific recognition of other polyether compounds.

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