Cry1Aa binding to the cadherin receptor does not require conserved amino acid sequences in the domain II loops

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Synopsis
Characterizing the binding mechanism of Bt (Bacillus thuringiensis) Cry toxin to the cadherin receptor is indispensable to understanding the specific insecticidal activity of this toxin. To this end, we constructed 30 loop mutants by randomly inserting four serial amino acids covering all four receptor binding loops (loops α8, 1, 2 and 3) and analysed their binding affinities for Bombyx mori cadherin receptors via Biacore. High binding affinities were confirmed for all 30 mutants containing loop sequences that differed from those of wild-type. Insecticidal activities were confirmed in at least one mutant from loops 1, 2 and 3, suggesting that there is no critical amino acid sequence for the binding of the four loops to BtR175. When two mutations at different loops were integrated into one molecule, no reduction in binding affinity was observed compared with wild-type sequences. Based on these results, we discussed the binding mechanism of Cry toxin to cadherin protein.

Key words: Bacillus thuringiensis, cadherin receptor, Cry toxin, insecticide, ligand binding

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INTRODUCTION
Bt (Bacillus thuringiensis) is a Gram-positive soil bacterium that produces insecticidal proteins called Cry toxins during sporulation. Cry toxins are specific to insects and do not affect humans and domestic animals. In addition, these toxins are environmentally friendly because they are proteinaceous and easily degrade in the soil and on plant surfaces. Therefore Bt formulations represent pesticides that possess a low impact on human health and the environment. For this reason, Cry toxin genes have been used in the development of GMOs (genetically modified organisms) [1,2]. However, acquisition of Cry toxins with high insecticidal activity against coleopteran, hemipteran, dipteran and hymenopteran insects from the soil is difficult. The development of a method for engineering Cry toxins with improved insecticidal activity against target pests and possessing a broader target pest spectrum is desired. To establish protein-engineering methods for the Cry toxin, the mode of action by which Cry toxin binds to the cadherin receptor must be characterized.

One hypothesis for the insecticidal mechanism of Cry toxins is the pore-forming model [3]. A recent version of the pore-formation model proposed the following sequence of events: protease-activated Cry toxin monomers bind to GPI (glycosylphosphatidylinositol)-anchored proteins, such as APN (aminopeptidase N) and ALP (alkaline phosphatase). This interaction promotes the binding of toxin monomers to the cadherin BtR (Bt receptor), which facilitates protease cleavage of the toxin’s N-terminus, including helix α1 of domain I, and induces oligomerization of the toxin. Then toxin oligomers bind with increased affinity to GPI-anchored receptors and create pores in the midgut membrane, leading to osmotic shock and...
cell death. In contrast, the signalling model hypothesises that an adenylate cyclase/PKA (protein kinase A) signalling pathway is activated by BrR-bound monomer toxins, inducing programmed cell death [4]. Thus, BrR is an important receptor, although the correct hypothesis remains unknown. The generation of various Cry toxin-resistant insect strains has been attributed to a mutation or deletion in BrR [5–8]. In addition, there have been reports that cultured BrR-expressing insect or human cells are susceptible to Cry1A toxins, thus validating this hypothesis [9–12].

Seven 3D (three-dimensional) Cry toxin structures have been determined by X-ray crystallography [13–19]. Similar 3D structures consisting of three domains (domains I, II and III of the N-terminus) were confirmed to be associated with these toxins. Domain I is composed of a seven-α-helix bundle with a central helix and six surrounding helices, domain II is a β-prism consisting of three antiparallel β-sheets and loops and domain III is a β-sandwich consisting of two antiparallel β-sheets [13, 14, 18]. Loops α8, 1, 2 and 3 of domain II are thought to be BrR-binding regions [20–26]. However, it is unclear whether all regions are required for binding to BrRs.

A high-efficacy medicine is defined as a compound that can bind to and act on receptors at low concentrations. Accordingly, a major goal for the improvement of a lead medicinal compound is receptor-affinity maturation. Based on this concept, we anticipate that mutant toxins with higher BrR-binding affinities will likely also exhibit higher insecticidal activity. The insecticidal activity of Cry1A toxins is correlated with the binding affinity of Cry1A toxins for BBMV (brush-border membrane vesicles) prepared from insects [27]. Furthermore, binding to BrR has been determined to be indispensable to the insecticidal activity of Cry toxins [28]. Thus, we have developed a genetic-engineering method for improving the binding affinity of Cry toxin for BrR. Indeed, when a Cry1Aa loop suspected to be a BrR-binding site was replaced with that of Cry4Ba, the engineered Cry1Aa lost activity against Manduca sexta and obtained activity against Culex pipiens [29]. Similarly, when Cry4Ba loop 3 was replaced with a Cry4Aa-loop 3-mimic peptide, the engineered Cry4Ba gained activity against C. pipiens, which was originally resistant to Cry4Ba [30].

Cry1Aa, whose 3D structure has been determined, is active against the easy-bleeding insect Bombyx mori [14]. We have attempted to develop an affinity maturation process for Cry1Aa activity against B. mori BrR (BrR175). This involved applying a directed evolution-based method for the improvement of insecticidal activity using phage display and bio-panning, as described elsewhere [31]. However, as with other toxins, the only information known about this interaction is that the BrR175 binding site of Cry1Aa is near loops 1, 2 and 3 [25, 26]. To identify the critical binding sites, we performed a comprehensive binding assay that utilized mutants containing four random amino acid mutations spanning nearly all of the loop regions. Specifically, four serial amino acid residues were replaced with random amino acids at sites 275–278, 278–281 and 280–283 of loop α8; 310–313 of loop 1; 365–370, 367–370, 371–374 and 375–378 of loop 2; and 435–438, 439–442 and 443–446 of loop 3. This resulted in the generation of a total of 30 mutant proteins whose binding affinities for BrR175 were assessed by Biacore. Surprisingly, no critical binding site on Cry1Aa was found in the loop regions, leading us to propose a flexible, multi-binding model for BrR175 binding by Cry1Aa.

**EXPERIMENTAL**

**Bacterial and insect strains**

Wild-type T7 phage and Cry toxin displaying phages were propagated and titrated using Escherichia coli BLT-gene10 as described previously [31]. E. coli BL21 was used for the production of the wild-type or mutant Cry1Aa protoxin. Kinshu × Showa, a hybrid race of the silkworm, B. mori was reared as described previously [31].

**Construction of phage libraries**

To create Cry1Aa mutants-displaying-phage libraries the regions encoding regions spanning domains II loops to domain III were amplified by PCR using 5′-agaagtattaggtggggatccaatagaaactggttacaccccaa-3′, 5′-agaatatattgat-NNNNNNNNNNNNNNNNNngaaatggctcacg-3′, 5′-tttgatggtagtNNNNNNNNNNNNNNGctcagagaataagagg5′-tttatctagttgtgNNNNNNNNNNNNNntatttgagttcaggc-3′, 5′-gaagaattata-NNNNNNNNNNNNNeccaaattaccg-3′, 5′-ccttggttgctcggcNBBMV(NNNNNNNNNNNNNNNNNNNNacagtttgctgcggc-3′, 5′-cagttgctcggcNBBMV(NNNNNNNNNNNNNNNNNNNNacagtttgctgcggc-3′, 5′-cagttgctcggcNBBMV(NNNNNNNNNNNNNNNNNNNNacagtttgctgcggc-3′, 5′-cagttgctcggcNBBMV(NNNNNNNNNNNNNNNNNNNNacagtttgctgcggc-3′ as sense primers, respectively, for libraries with respect to mutations at 275–278, 278–281, 280–283 of loop α8; 310–313 of loop 1; 365–370, 367–370, 371–374 and 375–378 of loop 2; and 435–438, 439–442 and 443–446 of loop 3 and 5′-agctcatttcgtagctgcggcctctctaatctatctatcggcctacca-3′ as an antisense primer. Next, the regions encoding domains I–III of Cry1Aa toxin were amplified by PCR using above-described DNA fragments as sense mega-primers and 5′-agaagtattaggtggggatccaatagaaactggttacaccccaa-3′ as an antisense primer, digested by restriction enzymes and inserted between BamHI and XhoI sites of T7Select10-3b DNA (Novagen) as described previously [31].

**Preparation of wild-type and mutant toxins**

Wild-type and mutant toxins were prepared as described previously. Briefly, DNAs from toxins displayed on the phage were inserted between SpeI and SacI sites of GST (glutathione transferase)-Cry1Aa protoxin fusion-protein expressing vector, pB9 and E. coli BL21 was transformed with each resulting vector [31]. Fusion proteins were harvested as inclusion bodies and solubilized, and then solution of each fusion protein was applied to a DEAE column (Shodex IEC DEAE-825) connected to an HPLC.
system (Waters 600), and protoxins were activated by 0.5 mg/ml trypsin for 2 h at 37 °C in the column [25]. Activated toxins were eluted using a linear gradient of Tris/HCl buffer and protein concentrations were determined by densitometry using BSA as a standard.

**Binding kinetics analysis with SPR (surface plasmon resonance)**

Cry1Aa toxin-binding region of BtR175 (BtR175-TBR) was prepared as described previously [32] and immobilized on a CM5 Biacore sensor chip using the amine-coupling method. Eight different concentrations (3.125–200 nM) of mutant Cry toxins diluted in PBST (PBS with 0.005 % Tween 20, pH 7.4) were applied to the surface of the BtR175-TBR-immobilized CM5 sensor chip for 120 s. For dissociation, toxin flow was replaced by PBST, and the response was recorded for at least 240 s. The response curves were fit to a 1:1 Langmuir binding model and analysed using global fitting. Rate constants for association ($k_a$) and dissociation ($k_d$) were determined. The sensor chip was regenerated using 30 μl of 10 mM NaOH.

**Bioassay using insect larvae**

Solubilized GST–protoxin fusion proteins were mixed with artificial diet, Silk Mate PM, resulting in a final concentration of 1.3 μg/g diet. The diets (2.5 g) were placed in 9 cm dishes and then 25 third-instar larvae were reared on each diet at 25 °C. Dead larvae were confirmed after 48 and 72 h.

**Activation of toxins with the midgut juice**

Activation profile of toxins by the silkworm midgut juice was assessed as described previously [31]. Briefly, midgut juice was prepared from fifth-instar larvae and diluted to be 25 % (v/v) with deionized water. The inclusions of GST-protoxin fusion proteins were mixed with the diluted midgut juice to a final concentration of 1.3 μg/μl. Samples were incubated at 25 °C for 0.5, 1, 2 and 4 h, and subjected to SDS/PAGE.

**Preparation of two-site mutants**

The region encoding loop 1 to mutated loop 2 was amplified using R$^{375}$GPD$^{378}$ mutant DNA as a template and 5′-ttcggtgatggctccggt-3′ and 5′-gaaaaactccccatcacaaggaca-3′, respectively, as sense and anti-sense primers. The region encoding loop 3 was amplified using Q$^{439}$PRG$^{442}$, H$^{439}$MPR$^{442}$ or R$^{443}$LGR$^{446}$ mutant DNA as templates and 5′-ctgttgcctgtaggaacg-3′ and 5′-aagaaacgtgagctcata-3′, respectively, as sense and anti-sense primers. Both regions were connected by overlap PCR and resulting DNA fragments were digested by restriction enzymes and then inserted between SpeI and SacI sites of GST-Cry1Aa protoxin fusion-protein-expressing vector, pB9, and E. coli BL21 was transformed with each resulting vector.

**RESULTS**

**Assessment of BtR175 binding affinity of Cry1Aa loop mutants**

We have attempted to establish a model system for directed evolution to acquire mutant toxins with higher BtR-binding affinity and higher insecticidal activity using a combination of B. mori and Cry1Aa (Y. Fujii, S. Tanaka, M. Otsuki, Y. Hoshino, H. Endo and R. Sato, unpublished work). As part of this trial, a mutant library was constructed by replacing four serial amino-acid residues with random amino acids at various sites, as described above (Figure 1). To analyse the role of each of the four loops in the BtR175-binding mechanism of Cry1Aa, more than four phage-displayed Cry1Aa loop-mutants were randomly selected from each library (from each replacement site) and produced in E. coli as inclusions using the fusion protein connected to GST. The mutants were activated by the removal of GST in an anion-exchange column and purified, which resulted in successful acquisition of 30 trypsin-tolerant loop-mutants. BtR175-binding affinities were assessed by Biacore (Figure 2). Global fitting using a 1:1 Langmuir binding model was used to calculate the association rate constant [$k_a(M^{-1}s^{-1})$] and dissociation rate constant [$k_d(s^{-1})$] (Table 1). Then the dissociation constant [$K_D(M)$], a primary parameter for binding affinity, was calculated according to the formula $K_D = k_d/k_a$. All loop-mutants indicated high binding affinities for BtR175. The highest $K_D$ was that of T$^{390}$LGP$^{392}$ and the lowest $K_D$ was that of R$^{375}$GPD$^{378}$, resulting in an affinity range that spanned from 1.6 times lower to 4.2 times higher than that of Cry1Aa for all 30 mutants (Table 1, Figure 2). All mutants contained different amino acid sequences with respect to the corresponding loop region and no general trend was observed in the amino acid sequence composition compared with the wild-type toxin (Figures 1 and 2).

**Insecticidal activities of the Cry1Aa loop mutants**

Insecticidal activities toward B. mori larvae were assessed using 23 Cry1Aa loop mutants. Although no activity was observed in seven mutants (H$^{365}$AGG$^{368}$, F$^{365}$EPK$^{368}$, A$^{367}$PAP$^{370}$, S$^{367}$PSA$^{370}$, S$^{371}$APN$^{374}$, R$^{375}$GPD$^{378}$ and T$^{439}$LRT$^{442}$), insecticidal activities were confirmed for the other 16 mutants (Figure 3). At least one mutant indicated that insecticidal activity was associated with replacement of the eight sites covering all areas of loops 1, 2 and 3.

**Defects in the activation process of non-active loop-mutants in larval midgut juice**

To determine whether non-active mutants contain defects in the activation process in the midgut fluid, two non-active mutants (R$^{375}$GPD$^{378}$ and T$^{439}$LRT$^{442}$), five active mutants (K$^{310}$ASR$^{313}$, H$^{367}$NAG$^{370}$, P$^{387}$RRP$^{370}$, V$^{375}$GCA$^{378}$ and V$^{443}$ELL$^{446}$) and wild-type toxins were incubated in midgut fluid and analysed by SDS/PAGE (Figure 4). The non-active loop-mutant R$^{375}$GPD$^{378}$ underwent complete degradation, leaving no observable fluid-tolerant fragments (Figure 4B). Another non-active loop-mutant...
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Figure 1  Mutation sites introduced in loops $\alpha_8$, 1, 2 and 3 of Cry1Aa and amino acid sequences of the mutants used in this experiment

Cry1Aa mutant toxins containing four serial amino acid residue replacements in three regions of loop $\alpha_8$, one region of loop 1, four regions of loop 2 and three regions of loop 3 were expressed on the phage surface to construct 11 classes of phage libraries. Thirty mutants were prepared as activated toxins. The mutated sequence of each mutant is shown compared with wild-type sequences. Sequences in bold typeface are those that were integrated into one molecule. Sequences written in italic are high-affinity-generating mutants derived from our unpublished work. Molecular models were generated using CCP4MG.

(T$^{439}$LRT$^{442}$) contained only a faint band of activated toxin at 60 kDa (Figure 4D). The remaining mutants, as well as the wild-type toxin, had larvicidal activities with stable, activated forms present at 60 kDa. These results indicate the non-activity of R$^{375}$GPD$^{378}$ and T$^{439}$LRT$^{442}$ owing to a defect in the activation process, resulting in the suggestion that only mutants with defects in the activation process might have lost insecticidal activity.

Assessment of BtR175 binding affinity of mutants containing double mutations in different loops

Phage-displayed Q$^{439}$PRG$^{442}$, H$^{439}$MPR$^{442}$ and R$^{441}$LGR$^{446}$ mutants were selected by bio-panning during a trial of directed evolution directing Cry1Aa affinity against BtR175 (Y. Fujii, S. Tanaka, M. Otsuki, Y. Hoshino, H. Endo and R. Sato, unpublished work). Simultaneous two-site replacement was performed in different loops to assess the effects of multiple mutation sites on BtR175-binding affinity. Specifically, we were interested in determining whether binding affinity would decrease when two mutations were combined. The loop 3 mutants Q$^{439}$PRG$^{442}$, H$^{439}$MPR$^{442}$ and R$^{441}$LGR$^{446}$ had higher affinities (13, 42 and 15 times, respectively) than that of wild-type (Table 2) (Y. Fujii, S. Tanaka, M. Otsuki, Y. Hoshino, H. Endo and R. Sato, unpublished work) and were integrated with the R$^{375}$GPD$^{378}$ loop 2 mutation (four times higher affinity) into one molecule. The binding affinities of R$^{375}$GPD$^{378}$/Q$^{439}$PRG$^{442}$, R$^{375}$GPD$^{378}$/H$^{439}$MPR$^{442}$ and R$^{375}$GPD$^{378}$/R$^{441}$LGR$^{446}$ double mutants were assessed by Biacore and resulted in $K_D$ values of 0.34, 0.056 and $0.27 \times 10^{-18}$ M, respectively (Table 2), indicating affinity values that were 8, 49 and 10 times higher than the wild-type toxin. In case of R$^{375}$GPD$^{378}$/H$^{439}$MPR$^{442}$ double mutant, an increase in binding affinity was observed compared with the original mutants (Table 2).

DISCUSSION

Replacement of any four sequential amino acids within any of the four loops did not reduce BtR175-binding affinity (Table 1, Figure 2). In addition, insecticidal activities remained high in
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Figure 2 Binding and dissociation kinetics obtained by BtR175-immobilized CM5 Biacore sensor chips using concentrated phage clone-derived mutant toxins

Mutant toxins (12.5, 25, 50, 100 and 200 nM) were prepared using PBST (0.005% Tween 20) and injected over the CM5 Biacore sensor chip immobilized with BtR175 for 120 s. For dissociation measurements, the toxin flow was replaced by PBST buffer and recorded for at least 240 s. RU, response units.

many of these mutants (Figure 3). No relationship was observed between the identity of the four replaced amino acid sequences of the mutants and those of the wild-type toxin (Figure 1). Pigott et al. [33] reported that replacement of loop 1, 2 and 3 regions with CDR3 fragments from human IgG heavy chain did not affect the binding affinity between Cry1Aa and M. sexta BtR (Bt-R1). These results suggest that the amino-acid sequences of the four loops of Cry1Aa are not critical to BtR175 binding.

Many proteins do require conserved amino acid sequences for ligand binding. For example, the RGD motif is necessary for the binding of several mammalian extracellular matrix proteins, such as fibronectin, to integrin [34]. However, our results suggest that Cry1Aa activity does not depend on conserved amino-acid sequences in loops, indicating an unusual binding character compared with other proteins.

One binding mechanism that does not require specific amino-acid loop sequences for BtR binding is multipoint attachment. Indeed, loop 2 and 3 of Cry1Aa and loops 2 and α8 of Cry1Ab are reported to have a binding affinity for Bt-R1 [21,22], and loops 2 and 3 of Cry1Ab are reported to have a binding affinity for Heliothis virescens BtR [24]. Furthermore, all four loop regions are candidate Cry1A-binding regions for BtRs [25,35–38]. Thus, it is possible that these loops bind to BtR by multipoint attachment because replacing a restricted region (four amino acids in one loop) with random amino acids would not be expected to significantly affect BtR-binding affinity, which we observed in the present study.

Even if the multipoint-attachment mechanism applies to Cry toxins, it is reasonable to assume that reduction of the binding site should reduce the binding affinity. However, none of the mutants had a BtR175-binding affinity two times lower than that of wild-type toxins. Indeed, Gomez et al. [21] reported that one of the BtR1-binding sites of Cry1Aa and Cry1Ab includes 367–373 of loop 2, although only two of the seven amino acids overlap between the toxins [21]. It was postulated that amino-acid sequences displaying inverted hydropathic profiles against...
Figure 3  Insecticidal activities of wild-type and mutant toxins
Mutant toxins with a high binding affinity for BtR175 and wild-type (wt) toxin were added to the diet in the protoxin form, resulting in a final concentration of 1.3 $\mu$g/g diet. Twenty-five third-instar larvae were reared on each diet at 25 °C. Dead larvae were confirmed after 48 (A) and 72 h (B).

Figure 4  Activation profile of wild-type and mutant toxins containing high binding affinities for BtR175 using the midgut fluid of B. mori larvae
Wild-type and mutant toxins were prepared as inclusions using GST-protoxin fusion proteins. The inclusions were mixed with a 1:4 dilution of midgut fluid from fifth-instar B. mori larvae, incubated at 25 °C for 0.5, 1, 2 and 4 h, and subjected to SDS/PAGE. Asterisks indicate 160 kDa GST–protoxin fusion proteins and arrowheads indicate 60 kDa midgut fluid-tolerant toxins. (A) wild-type and K$^{310}$ASR$^{313}$; (B) H$^{367}$NAG$^{370}$ and R$^{375}$GPD$^{378}$; (C) P$^{367}$RRP$^{370}$ and V$^{375}$GCA$^{378}$; (D) T$^{439}$LRT$^{442}$ and V$^{443}$ELL$^{446}$. Lane 1: before digestion; Lane 2: 0.5 h after digestion; Lane 3: 1 h after digestion; Lane 4: 2 h after digestion; Lane 5: 4 h after digestion.
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Table 1 Binding of 30 mutant and wild-type toxins for BtR175 obtained by fitting the curves indicated in Figures 2 and 5 (wild-type) to a 1:1 Langmuir binding models

| Clone names | $K_d$ ($10^{5}$) M$^{-1}$ s$^{-1}$ | $K_a$ ($10^{-3} \cdot s^{-1}$) | $K_b$ ($10^{-8} \cdot M$) |
|-------------|----------------------------------|-----------------------------|--------------------------|
| Wild-type   | 0.96                             | 2.66                        | 2.77                     |
| G275EGG278  | 1.15                             | 1.66                        | 1.45                     |
| D278DIV281  | 1.12                             | 2.40                        | 2.14                     |
| F280RWE283  | 0.85                             | 1.92                        | 2.24                     |
| A310QMA313  | 1.12                             | 1.80                        | 1.6                      |
| K310ASR313  | 2.51                             | 6.75                        | 2.69                     |
| L365EAQ368  | 2.57                             | 4.28                        | 1.66                     |
| H365AGG368  | 2.38                             | 4.39                        | 1.84                     |
| F365EPK368  | 0.84                             | 3.1                         | 3.68                     |
| F367SQA370  | 1.26                             | 4.14                        | 3.29                     |
| A367PGP370  | 0.94                             | 3.82                        | 4.06                     |
| S367TRS370  | 1.27                             | 2.26                        | 1.77                     |
| T367LGR370  | 0.88                             | 4.00                        | 4.54                     |
| L367PPR370  | 1.34                             | 1.78                        | 1.33                     |
| L367PAP370  | 0.85                             | 2.55                        | 2.98                     |
| P367RBB370  | 1.26                             | 1.53                        | 1.21                     |
| S367QA367   | 0.71                             | 2.6                         | 3.64                     |
| H367NAQ370  | 0.71                             | 1.82                        | 5.57                     |
| A367PAP370  | 0.81                             | 2.7                         | 3.31                     |
| S367PSA370  | 0.79                             | 2.93                        | 3.68                     |
| S371APN374  | 1.25                             | 1.67                        | 1.33                     |
| E371PRH374  | 2.90                             | 3.28                        | 1.13                     |
| R375GPD278  | 1.85                             | 1.22                        | 0.65                     |
| V375GCA378  | 1.06                             | 3.01                        | 2.84                     |
| G435LVG438  | 4.14                             | 5.59                        | 1.35                     |
| S435VVG438  | 1.90                             | 3.79                        | 1.99                     |
| E439PPA442  | 1.21                             | 1.40                        | 1.16                     |
| T439LRT442  | 1.51                             | 1.58                        | 1.05                     |
| D439TGN442  | 1.20                             | 1.62                        | 1.35                     |
| I443VPV446  | 1.58                             | 3.16                        | 1.99                     |
| V443ELL446  | 1.33                             | 2.72                        | 2.04                     |

*To calculate parameters, the 1:1 Langmuir binding fit model was applied to the curves indicated in Figure 5.† Y. Fujii, S. Tanaka, M. Otsuki, Y. Hoshino, H. Endo and R. Sato, unpublished work.

Table 2 BtR175 binding by mutant toxins containing two mutated loops in one molecule

| Clone names | $K_a$ ($10^{5}$) M$^{-1}$ s$^{-1}$ | $K_d$ ($10^{-3} \cdot s^{-1}$) | $K_b$ ($10^{-8} \cdot M$) |
|-------------|----------------------------------|-----------------------------|--------------------------|
| Wild-type   | 0.96                             | 2.66                        | 2.77                     |
| G275PRG278† | 7.96†                            | 1.62†                       | 0.211†                   |
| H365MPR442† | 15.9†                            | 1.05†                       | 0.066†                   |
| R439LGR446† | 7.12†                            | 1.32†                       | 0.185†                   |
| R375GPD378/PQ439RG442 | 5.13 | 1.75 | 0.340 |
| R375GPD378/H365MPR442 | 21.6 | 1.22 | 0.056 |
| R375GPD378/R439LGR446 | 6.46 | 1.75 | 0.270 |

ligand proteins are important for binding. If binding in the Cry loop regions were governed by this rule, hydrophatic profiles would be conserved in all BtR-binding loops. To test this hypothesis, we compared the replaced sequences of the 30 mutants with the wild-type sequence. In only a few cases did the hydrophatic profile of the replaced sequences coincide with the original sequences.

Short peptides and small linear molecules can adopt different conformations, resulting in different molecules binding to the same protein. For example, various peptide fragments can bind to human HLA-A2 Class I MHC molecules [39]. Further, many kinds of volatile compounds with disparate structures can bind to the same odorant binding protein [40]. These phenomena are possible due to conformation changes or the generation of new binding sites.

It has also been reported that loops are flexible in nature, which affects the activities of loop-harbouring molecules. Indeed, the flexible nature of a catalytic loop plays an important role in the activity of a nucleotide hydrolase from Trypanosoma vivax [41]. The flexibility of a loop in E. coli class II fructose-1,6-bisphosphate aldolase was found to be important to its catalytic activity [42]. Cry1Aa loops were reported to be highly mobile and their flexible nature was postulated to play an important role in receptor recognition [14]. It is possible that mutated loops may change conformation to present suitable hydrophatic profiles to BtR175, preventing a reduction in BtR175-binding affinity. The sequences of the four loop regions of domain II of Cry1Aa, BtR1 [21]. Thus, our experimental results suggest that the high BtR175-binding affinities found for all 30 mutants containing four serially replaced amino acids depend on the flexible nature of the loops. This flexibility may facilitate multipoint attachment by exposing suitable hydrophatic profiles for the receptors without the requirement for specially conserved amino acid sequences in the loops. It is conceivable that faster association rates seen in those mutants (Tables 1 and 2) could be a consequence of longer conformational flexibility of loops involved in binding, allowing for a larger available fraction of loop conformations compatible with toxin–receptor complex formation.

The Q439PRG442, H365MPR442 and R439LGR446 loop 3 mutants had 13, 42 and 15 times higher affinity for BtR, respectively, than the wild-type toxin (Y. Fujii, S. Tanaka, M. Otsuki, Y. Hoshino, H. Endo and R. Sato, unpublished work). Each of these loop 3 mutants was independently integrated with a R375GPD378 loop 2 mutant having an affinity four times higher than that of the wild-type. Initially, it was expected that the integration of these separate sequences into the same mutant toxin would reduce the binding affinity of the mutant for BtR. However, all three mutants containing the two simultaneous replacements maintained higher binding affinity for BtR175 at levels comparable to that of the wild-type toxin (Table 2, Figure 5). Moreover, the R375GPD378/H365MPR442 double mutant further increased the binding affinity for BtR175, which suggests that loops do not require a specific amino acid sequence for...
BtR binding. Moreover, these results indicate that the mutated version containing mutations at both loops 2 and 3 possessed new binding sites for BtR175, which probably resulted from a conformational change in the double mutant. Indeed, Pigott et al. [33] reported that replacing loops 1 and 2, or 2 and 3 simultaneously with CDR3 peptide mutant toxins maintained the binding affinity for Bt-R1 [33]. This is also consistent with the idea that loops do not require specific amino acid sequences for BtR-binding and that newly created loops can form new binding sites.

In the case of scFv (single-chain fragment variable), a mutation generating 133 times higher binding affinity and a mutation derived from a different loop region generating 59 times higher affinity to an antigen were integrated into one molecule, resulting in generation of a 1200 times higher affinity mutant in contrast with the wild-type version [43]. We found that integration of the R375GPD378 mutation (four times higher affinity) with the H439MPR442 mutation (42 times higher affinity) into a R375GPD378/H439MPR442 double mutant had a resulting binding affinity 49 times higher than that of wild type. These results suggest that the binding affinity of the Cry toxin to the receptor can be increased by integrating two different high-affinity-generating mutations simultaneously into one molecule. Affinity maturation of the Cry1A toxin can be enhanced by introducing several different loop-derived high-affinity-generating mutations into one molecule. In fact, in addition to mutations on loop 3, such as H439MPR442, we succeeded in finding several mutations on loop 2 containing affinities 20–50 times higher than that of the wild-type toxin. We anticipate the generation of mutants with more than 100 greater binding affinities for BtR than the wild-type toxin by integrating multiple mutations into one molecule.

**AUTHOR CONTRIBUTION**

Ryoichi Sato conceived and designed the projects and wrote the paper. Yuki Fujii performed the experiments, analysed the data and wrote the paper. Manami Otsuki, Yasushi Hoshino, Chinatsu Morimoto, Takuya Kotani, Yoko Harashima, Haruka Endo, Shihoo Tanaka and Yasutaka Yoshizawa performed the experiments and analysed the data.

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