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Conformation of the Primary Binding Loop Folded through an Intramolecular Interaction Contributes to the Strong Chymotrypsin Inhibitory Activity of the Chymotrypsin Inhibitor from *Erythrina variegata* Seeds

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We previously demonstrated that amino acid residues Gln62 (P3), Phe63 (P2), Leu64 (P1), and Phe67 (P3′) in the primary binding loop of *Erythrina variegata* chymotrypsin inhibitor (ECI), a member of the Kunitz inhibitor family, are involved in its strong inhibitory activity toward chymotrypsin [Iwanaga *et al.* (1998) *J. Biochem.* 124, 663-669]. To determine whether or not these four amino acid residues predominantly contribute to the strong inhibitory activity of ECI, they were simultaneously replaced by Ala. The results showed that a quadruple mutant, Q62A/F63A/L64A/F67A, retained considerable inhibitory activity (K_i, 5.6×10^{-7} M), indicating that in addition to the side chains of these four amino acid residues, the backbone structure of the primary binding loop in ECI is essential for the inhibitory activity toward chymotrypsin. Two chimeric proteins, in which the primary binding loops of ECI and ETIa were exchanged: an isoinhibitor from *E. variegata* with lower chymotrypsin inhibitory activity, were constructed to determine whether the backbone structure of the primary binding loop of ECI was formed by the amino acid residues therein, or through an interaction between the primary binding loop and the residual structure designated as the "scaffold." A chimeric protein, ECI/ETIa, composed of the primary binding loop of ECI and the scaffold of ETIa showed weaker inhibitory activity (K_i, 1.3×10^{-6} M) than ECI (K_i, 9.8×10^{-8} M). In contrast, a chimera, ETIa/ECI, comprising the primary binding loop of ETIa and the scaffold of ECI inhibited chymotrypsin more strongly (K_i, 5.7×10^{-7} M) than ETIa (K_i, 1.3×10^{-6} M). These results indicate that the intramolecular interaction between the primary binding loop and the scaffold of ECI plays an important role in the strong inhibitory activity toward chymotrypsin. Furthermore, surface plasmon resonance analysis revealed that the side chains on the primary binding loop of ECI contribute to both an increase in the association rate constant (k_{on}) and a decrease in the dissociation rate constant (k_{off}) for the ECI-chymotrypsin interaction, whereas the backbone structure of the primary binding loop mainly contributes to a decrease in the dissociation rate constant.

Key words: chymotrypsin inhibitor, *Erythrina variegata*, Kunitz family proteinase inhibitor, primary binding loop, scaffold.

Genus *Erythrina* comprises a family of deciduous leguminous trees widely spread throughout the subtropics and tropics. Several Kunitz family proteinase inhibitors have been isolated from various *Erythrina* species and characterized in terms of proteinase specificity (*1, 2*). We previously isolated three Kunitz family proteinase inhibitors: one chymotrypsin inhibitor, ECI, and two trypsin inhibitors, ETIa and ETIb, from *Erythrina variegata* seeds obtained in Okinawa (*3-5*). All three inhibitors exhibit inhibitory activity toward chymotrypsin, ECI inhibiting chymotrypsin more strongly than the other two isoinhibitors, ETIa and ETIb (*3, 4*). ECI consists of 179 amino acid residues with two disulfide bonds, and more than 60% of the amino acid residues are identical with those of ETIa and ETIb. It was estimated by titration of chymotrypsin that the stoichiometry of ECI and chymotrypsin was 1:2 (*3*). However, limited digestion of ECI with chymotrypsin under acidic conditions unambiguously identified only Leu64 as the P1 residue for chymotrypsin.

In foregoing work, the stoichiometry of the interaction between ECI and chymotrypsin was reinvestigated by estimation of the molecular mass of the ECI-chymotrypsin

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Abbreviations: Ac-Ala-Aphe-ONp, *N*-acetyl-L-alanyl-α-azaphenylalanine-*p*-nitrophenyl ester; ECI, *Erythrina variegata* chymotrypsin inhibitor; ETIa, *E. variegata* trypsin inhibitor a; ETIb, *E. variegata* trypsin inhibitor b; HBS, HEPES buffer saline; RU, resonance units; SDS-PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis; SPR, surface plasmon resonance.

complex by ultracentrifugation as well as determination of the amounts of ECI and chymotrypsin in the complex. From the results of these analyses, we concluded that the stoichiometry of the interaction of ECI and chymotrypsin is 1:1 (*6*), despite that ECI shows an apparent inhibitory stoichiometry of 1:2. To further investigate the inhibitory mechanism of ECI, the contribution of amino acid residues (from P3 to P3′) surrounding the P1 residue in the primary binding loop to the inhibitory activity was investigated by alanine-scanning mutagenesis. As a result, it was indicated that in addition to P1 residue Leu64, Gln62 (P3), Phe63 (P2), and Phe67 (P3′) also play important roles in the inhibitory activity of ECI toward chymotrypsin (*6*). In the present study, we examined whether or not these four amino acid residues are predominantly involved in the strong inhibitory activity of ECI toward chymotrypsin by means of simultaneous substitution of these four residues by alanine. Furthermore, the contribution of the intramolecular interaction between the primary binding loop and the residual structure, designated as the "scaffold," was proved by constructing two chimeric proteins, in which the primary binding loops of ECI and ETIa were mutually exchanged.

MATERIALS AND METHODS

Materials—α-Chymotrypsin was obtained from Sigma Chemical, USA. The oligonucleotides used in this study were purchased from Pharmacia Biotech. A Chameleon™ double stranded site-directed mutagenesis kit was obtained from Stratagene. Restriction enzymes and T4 polynucleotide kinase were purchased from MBI Fermentas and Toyobo, respectively, and used as recommended by the suppliers. A sensor chip, CM5, HEPES buffer saline (HBS), and an amine-coupling kit for a BIAcore™ instrument (Pharmacia Biosensor) were obtained from Pharmacia Biotech. All other chemicals were of reagent grade and obtained from Nacalai Tesque or Wako Pure Chemicals.

Preparation of Alanine-Substituted Mutants—The amino acid residues from Gln62 (P3) to Phe67 (P3′) were replaced with Ala by the unique site elimination method developed by Deng and Nickoloff (*7*), as described previously (*6*). Two chimeric proteins, in which the primary binding loops of ECI and ETIa were exchanged, were generated by means of cassette mutagenesis. That is, novel recognition sites for *Kpn*2I (TCCGGA) were created by the unique site elimination method at the positions corresponding to the P7-P6 and P10′-P11′ sites in plasmids pmECI (*8*) and pmETIa (*9*) which include cDNA fragments encoding ECI and ETIa, respectively. After digestion of mutated pmECI and pmETIa with *Kpn*2I, the gene fragments encoding the primary binding loops of ECI and ETIa were then mutually exchanged. The oligonucleotide primers used in this study are listed in Table I. The mutation in each mutant was confirmed by DNA sequencing using a thermo sequenase fluorescent labeled primer cycle sequencing kit containing 7-deaza-dGTP (Amersham) and a DNA sequencer DSQ-1000 (Shimadzu) to ensure that no alteration other than that expected had occurred. All mutated genes were overexpressed in the *Escherichia coli* BL21(DE3) strain using the expression vector, pET-22b (Novagen) (*10*), and purification of the expressed proteins was performed by the same procedures as those described for recombinant ECI (*6*).

Assay for Chymotrypsin Inhibitory Activity—Chymotrypsin inhibitory activity was assayed at 37°C as described previously (*3*) with casein (Merck) as the substrate. The concentration of active chymotrypsin molecules was determined using an azapeptide, Ac-Ala-Aphe-ONp, by the method of Frank *et al.* (*11*). The concentrations of ECI and the mutant were determined with proteins bicinconinic acid assay reagent (Pierce). From the inhibitory profile obtained, the inhibitory constant (K_i) was estimated by the method of Henderson (*12*).

Surface Plasmon Resonance (SPR) Analysis—Real time analysis of the interaction of ECI or its mutants with chymotrypsin was performed using a BIAcore™. The principle and application of the system, with SPR detection, were described by Karlsson *et al.* (*13*), and detailed kinetic analysis of the interaction of ECI with chymotrypsin was previously described (*6*).

Proteolysis with Chymotrypsin—Chymotrypsin and a tenfold molar amount of ECI or one of its mutants were mixed and incubated at 37°C for 24 h in 10 mM Na-phosphate buffer, pH 7.4. Proteolytic degradation was evaluated by SDS-PAGE under reduced or non-reduced conditions (*14*). The scissile peptide bonds were determined by sequencing the resulting peptide fragments with a gas-phase sequencer, PSQ-1 (Shimadzu).

RESULTS

Preparation of Alanine-Substituted Mutant Proteins—In our previous study, it was demonstrated that the ECI-chymotrypsin interaction involves not only Leu64 (P1) but also the flanking residues, Gln62 (P3), Phe63 (P2), and Phe67 (P3′), in the primary binding loop (*6*). Thus, in order to determine whether or not amino acid residues Gln62 (P3), Phe63 (P2), Leu64 (P1), and Phe67 (P3′) are predominantly involved in the strong inhibitory activity toward chymotrypsin, the amino acid residues between Gln62 and Phe67 were simultaneously replaced by Ala. We chose Ala

TABLE I. **Oligonucleotide primers used in this study.** Underlining and boxes indicate nucleotide sequences designed for mutagenesis and the introduced restriction site for *Kpn*2I, respectively.

Mutant	Oligonucleotide primer
L64A/F67A	5′-TTCGCTTCAACGGCTATCCCTGAT-3′
Q62A/F63A/L64A/F67A	5′-AGGATTGCATCCGCTGCTGCTTCAACGGCT-3′
Q62A/F63A/L64A/S65A/T66A/F67A	5′-GCTGCTGCTGCTGCTGCTATCCCTGATGGC-3′
N-*Kpn*2I(ECI)	5′-GGAGAACCCATCCGGATTGCATCCCAATTC-3′
C-*Kpn*2I(ECI)	5′-TCCCTGATGGCTCTCCAGTCCGGATTGGCTTTGC-3′
N-*Kpn*2I(ETIa)	5′-CTAATGGAAAACCCATCCGGATTGAATCACGAC-3′
C-*Kpn*2I(ETIa)	5′-CCCGATGACGACAAGGTCCGGATTGGGTTCGC-3′

because it has the smallest hydrocarbon side chain and thus the effects of Ala mutation could be expected to not disrupt the global protein structure. It could be further expected that the interaction of the side chains of Ala residues with chymotrypsin could be negligible because of their small side chains. All multiple mutants were expressed in *E. coli* BL21(DE3) cells using the expression vector, pET-22b, renatured from inclusion bodies, and purified by gel filtration chromatography on Sephadex G-75, as described for the wild type ECI (*6*). Throughout the purification, all mutants behaved like the wild type ECI and exhibited the same elution pattern as ECI. The amounts of all multiple substituted mutants expressed were similar to that of the wild type ECI; the yields of protein from 1 liter of culture broth ranged from 10 to 15 mg.

Inhibitory Activities of the Mutant Proteins—The inhibitory activities of the multiple mutants were assayed using casein as a substrate, as described under "MATERIALS AND METHODS." Figure 1 shows the inhibitory profiles of the multiple mutants. Double mutant L64A/F67A, in which residues Leu64 (P1) and Phe67 (P3′) were simultaneously replaced by Ala, exhibited slightly lower inhibitory activity than mutants L64A and F67A, in which Leu64 and Phe67 were individually substituted by Ala, respectively. The K_i value (5.4×10^{-7} M) of mutant L64A/F67A was about 5.5-fold higher than that of ECI (9.8×10^{-8} M), and about 1.5-fold higher than those of L64A and F67A (L64A, 3.7×10^{-7} M; F67A, 3.6×10^{-7} M) (Table II). Further replacement of residues Gln62 (P3) and Phe63 (P2) in L64A/F67A, however, did not decrease the inhibitory activity of the resulting quadruple mutant, Q62A/F63A/L64A/F67A, as shown in Fig. 1. Furthermore, additional replacement of Ser65 (P1′) and Thr66 (P2′) by Ala did not cause a further decrease in the inhibitory activity toward chymotrypsin (Fig. 1). As a result, it was found that a multiple mutant, Q62A/F63A/L64A/S65A/T66A/F67A, retained considerable inhibitory activity toward chymotrypsin (K_i, 5.5×10^{-7} M). The pattern of the interaction between Kunitz inhibitor and its cognate proteinase was investigated in detail for a complex composed of soybean trypsin inhibitor and trypsin (*15, 16*). Tertiary structure analyses of the complex revealed that most of the contacts involve amino acids located in the primary binding loop of the inhibitor. It is further likely that because of their small size, the side chains of introduced Ala residues in the primary binding loop in multiple mutants hardly interact with the amino acid residues at subsites of chymotrypsin. It is thus suggested that the inherent backbone structure of the primary binding loop may be responsible for the remaining inhibitory activity of the multiple mutants toward chymotrypsin. In other words, the strong inhibitory activity of ECI toward chymotrypsin can be attributed not only to the side chains of the amino acid residues in the primary binding loop but also to the backbone structure of the primary binding loop of ECI.

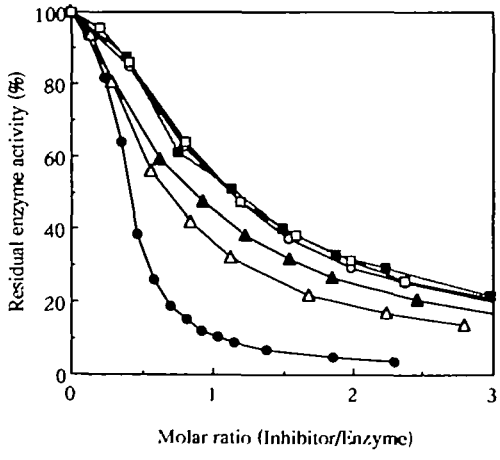

Fig. 1. **Inhibitory activity of alanine-substituted mutants toward chymotrypsin.** Various amounts of ECI or alanine-substituted mutants were examined as to their potential to inhibit chymotrypsin. Inhibition is expressed as the remaining activity of chymotrypsin with casein as the substrate. ECI (●), L64A (▲), F67A (△), L64A/F67A (□), Q62A/F63A/L64A/F67A (■), Q62A/F63A/L64A/S65A/T66A/F67A (⁻).

As shown in Table II, the decrease in affinity of mutant protein Q62A/F63A/L64A/F67A to chymotrypsin corresponds to a decrease in binding energy of 4.50 kJ/mol. On the assumption that positive interaction between the side chains of Ala residues and subsites in chymotrypsin would be negligible, it is possible to estimate from this value that the side chains on the primary binding loop account for about 11% of the total binding energy for the complex formation between ECI and chymotrypsin.

Preparation and Characterization of Chimeric Proteins—The results described above raised the question of whether the primary binding loop of ECI can adopt a favorable backbone structure that exhibits strong inhibitory activity toward chymotrypsin or interaction between the primary binding loop and the residual structure, designated as the "scaffold," of ECI is involved in formation of the backbone structure of the primary binding loop. We addressed this question by generating two chimeric proteins, ECI/ETIa and ETIa/ECI, in which the primary binding loops of ECI and ETIa were exchanged. As described in the Introduction, ETIa is an isoinhibitor in the seeds of *E. variegata* and exhibits weaker inhibitory activity toward chymotrypsin than ECI does.

The genes encoding the two chimeric proteins were constructed by means of cassette mutagenesis as described under "MATERIALS AND METHODS," and overexpressed and purified by the same procedures as those described for the multiple mutants. The inhibitory activities of the

TABLE II. **Inhibitory constants and different binding energies of ECI mutants.** The inhibitory constants (K_i) were obtained by analysis of the data for the inhibitory potencies toward chymotrypsin by the method of Henderson (*12*). ΔG was derived from the equation, $\Delta G = -RT\ln(1/K_i)$. $\Delta\Delta G$ was calculated according to the relationship, $\Delta\Delta G = \Delta G_{mt} - \Delta G_{wt}$. (a) This value was calculated using the relationship, $\Delta\Delta G = \Delta G_{ETIa} - \Delta G_{ETIa\ ECI}$. ΔG values (kJ/mol) of ECI and ETIa were calculated to be −41.60 and −34.89, respectively.

Name	K_i ($\times10^{-8}$ M)	$\Delta\Delta G$ (kJ/mol)
ECI	9.8±0.9	
L64A	36.9±1.5	3.42
F67A	36.2±1.9	3.37
L64A/F67A	54.0±6.0	4.40
Q62A/F63A/L64A/F67A	56.0±1.0	4.50
Q62A/F63A/L64A/S65A/T66A/F67A	54.9±4.0	4.44
ECI/ETIa	133.0±27.0	6.72
ETIa/ECI	57.2±2.2	2.17[a]
ETIa	132.81±13.1	

purified chimeric proteins were measured (Fig. 2). Chimeric protein ECI/ETIa comprising the primary binding loop of ECI and the scaffold of ETIa inhibited chymotrypsin (K_i, 1.3×10^{-6} M) more weakly than ECI (K_i, 9.8×10^{-8} M); it showed approximately the same inhibitory activity as ETIa (K_i, 1.3×10^{-6} M) (Table II). This result suggests that the primary binding loop of ECI/ETIa does not strongly interact with chymotrypsin, although ECI/ETIa and ECI share the primary binding loop. The decrease in affinity of ECI/ETIa as compared with ECI corresponds to a decrease in binding energy of 6.73 kJ/mol, that could be due to differences in the intramolecular interactions between the primary binding loop and the scaffold within the two molecules. In contrast to ECI/ETIa, chimeric protein ETIa/ECI composed of the primary binding loop of ETIa and the scaffold of ECI exhibited stronger inhibitory activity toward chymotrypsin than ETIa: the K_i value for ETIa/ECI (5.7×10^{-7} M) was approximately 2-fold lower than that of ETIa (Fig. 2 and Table II). The enhancement of the inhibitory activity of ETIa/ECI, as compared with that of ETIa, is assumed to be caused by the intramolecular interaction between the primary binding loop and the scaffold of ETIa/ECI to make the backbone structure of the primary binding loop favorable for chymotrypsin. It was estimated from the K_i values of ETIa and ETIa/ECI that the difference in binding energy between ETIa and ETIa/ECI was 2.16 kJ/mol. The present results indicate that the primary binding loop of ECI is not sufficient for the strong inhibitory activity toward chymotrypsin, and that the intramolecular interaction between the primary binding loop and the scaffold of ECI is required to enhance the affinity of the primary binding loop to chymotrypsin.

It is generally known that the P1 residue of a serine proteinase inhibitor interacts with a target enzyme in a substrate-dependent manner, and the proteinase inhibitor is therefore partially hydrolyzed at a peptide bond between P1 and P1′; the resulting proteinase inhibitor is referred to as the modified inhibitor. Hence, conformational differences in the reactive sites on the primary binding loops of ECI and ECI/ETIa were examined by determined their susceptibilities to chymotrypsin upon complex formation. Figure 3 shows the results of SDS-PAGE analyses of the peptides derived on chymotryptic digestion of ECI and ECI/ETIa. As expected, ECI produced two peptide fragments, being digested at the peptide bond between Leu64 (P1) and Ser65 (P1′). In contrast, chimeric protein ECI/ETIa, which showed weaker inhibitory activity than ECI, was digested in a different manner from ECI, despite that its primary binding loop was identical to that of ECI. Sequence analysis of the peptide fragments revealed that the primary binding loop derived from ECI was not digested by chymotrypsin, but rather that the peptide bond (Phe77-Ala78) within the scaffold derived from ETIa was accessible to chymotrypsin. The results demonstrate that the conformation of the reactive site (P1-P1′) of ECI/ETIa is somehow different from that of ECI.

Surface Plasmon Resonance (SPR) Analysis—The interaction of ECI or one of its mutants with chymotrypsin was further quantitatively investigated by SPR with use of the

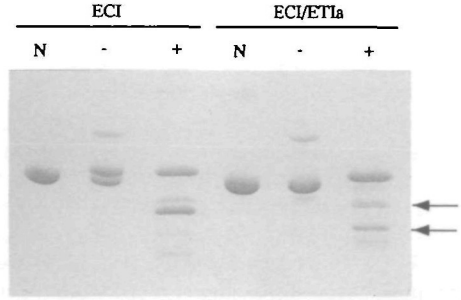

Fig. 3. **Susceptibilities of ECI and a chimeric protein toward chymotrypsin.** Fixed amounts of ECI and ECI/ETIa were mixed with a 1/10-fold molar amount of chymotrypsin, followed by incubation at 37°C for 24 h in 10 mM Na-phosphate buffer, pH 7.4. The aliquot of each mixture was subjected to SDS-PAGE. N indicates undigested protein. − and + indicate the results under non-reduced and reduced conditions, respectively. Arrows indicate peptide fragments derived from ECI/ETIa on digestion with chymotrypsin and analyzed with a gas-phase sequencer.

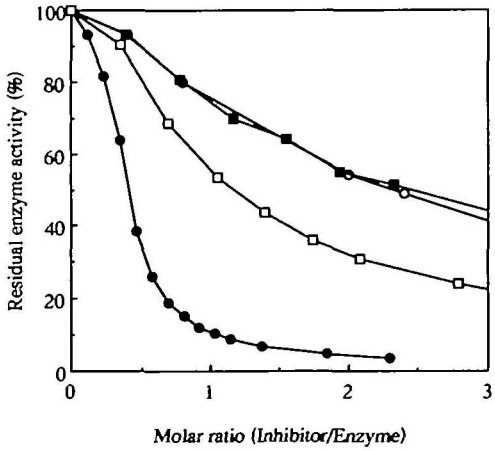

Fig. 2. **Inhibitory activity of chimeric proteins ECI/ETIa and ETIa/ECI toward chymotrypsin.** Various amounts of ECI (●), ETIa (○), ECI/ETIa (■), and ETIa/ECI ( ) were incubated with chymotrypsin, and then the remaining activity of chymotrypsin was measured using casein as the substrate, as described in Fig. 1.

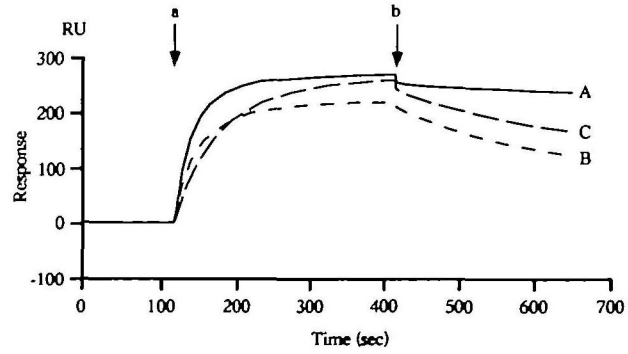

Fig. 4. **Sensorgrams obtained on real time analyses with a BIAcore™ of the interactions of ECI and ECI mutants with chymotrypsin.** The immobilization of chymotrypsin on a sensor chip, CM5, and binding analyses were performed as described under "MATERIALS AND METHODS." A, B, and C indicate sensorgrams of ECI, Q62A/F63A/L64A/S65A/T66A/F67A, and ECI/ETIa, respectively. Arrows a and b indicate the times of injection of either ECI or a mutant proteins, and the end of association by replacement with HBS, respectively.

TABLE III. **Kinetic constants of ECI mutants.** The apparent dissociation constants (K_d) were calculated according to the relationship, $K_d = k_{off}/k_{on}$. The association constants rate constants (k_{on}) and dissociation rate constants (k_{off}) were determined from sensorgram curves using the kinetic evaluation software installed in the BIAcore™.

Name	K_d ($\times 10^{-9}$ M)	k_{on} ($\times 10^5$ $M^{-1}\cdot s^{-1}$)	k_{off} ($\times 10^{-4}\cdot s^{-1}$)
ECI	3.0±0.0	2.95	8.89
Q62A/F63A/L64A/S65A/T66A/F67A	10.8±1.6	1.57	17.0
ECI/ETIa	14.1±0.5	2.14	30.1
ETIa/ECI	11.7±1.2	1.59	18.6
ETIa	15.2±1.0	1.78	27.0

BIAcore™ system. Real time analysis of the interaction of each protein with chymotrypsin was performed three times with a sensor chip of 731.1 resonance units (RU). Figure 4 shows typical sensorgrams of the association and dissociation of the interaction of ECI or one of its mutants with chymotrypsin. The association rate constant (k_{on}) and dissociation rate constant (k_{off}) values were calculated from the curves of sensorgrams using the kinetic evaluation software installed in the BIAcore™, and the apparent dissociation constants (K_d) were derived from the relationship between k_{on} and k_{off} ($K_d = k_{off}/k_{on}$). The kinetic constants obtained are summarized in Table III. This analysis showed that the association rate constant of the multiple mutant was about half (k_{on}, 1.5×10^5 $M^{-1}\cdot s^{-1}$), while that of chimeric protein ECI/ETIa (k_{on}, 2.0×10^5 $M^{-1}\cdot s^{-1}$) was comparable with that of ECI (k_{on}, 3.0×10^5 $M^{-1}\cdot s^{-1}$). On the other hand, the dissociation rate constants of the multiple mutant and ECI/ETIa were about 2- and 3-fold, respectively, higher than that of ECI. As for the kinetic constants for ETIa and ETIa/ECI, it was shown that their association rate constants were comparable with each other, while the dissociation rate constant of ETIa/ECI was decreased to about 69% as compared with that of ETIa.

DISCUSSION

Herein, we presented direct evidence that the strong inhibitory activity of ECI toward chymotrypsin is attributable not only to the side chains, but also to the backbone structure of the primary binding loop folded through an intramolecular interaction. From the inhibitory constants (K_i) of Q62A/F63A/L64A/F67A and ECI, the binding energy due to the side chains on the primary binding loop was estimated to be 4.50 kJ/mol, which accounts for about 11% of the total binding energy for the complex formation between ECI and chymotrypsin. On the other hand, inhibitory analysis of the chimeric proteins showed that the differences in binding energy between ECI/ETIa and ECI, and ETIa and ETIa/ECI are 6.73 and 2.16 kJ/mol, respectively. It is thus supposed that the difference (4.57 kJ/mol) between 6.73 and 2.16 kJ/mol might correspond to the difference in the contribution of the intrinsic side chains on the primary binding loops between ETIa and ECI to the inhibitory activity toward chymotrypsin. Interestingly, this value (4.57 kJ/mol) is in good accordance with the value (4.50 kJ/mol) obtained from the binding energies of ECI and Q62A/F63A/L64A/F67A, as described above. This finding suggests that the side chains on the primary binding loop of ETIa contribute little to the inhibitory activity of ETIa toward chymotrypsin.

The SPR analyses with the aid of the BIAcore instrument provided the kinetic parameters for the interaction of ECI, one of its mutants, or ETIa with chymotrypsin (Table III). The results revealed that the decreased affinity of the multiple mutant was due to both a decreased association rate constant and an increased dissociation rate constant, whereas that of chimera ECI/ETIa was mainly due to an increased dissociation rate constant. Furthermore, it was found that the increased affinity of chimera ETIa/ECI, as compared with that of ETIa, was due to a decreased dissociation rate constant. These results indicate that the side chains on the primary binding loop of ECI contribute to both the increase in the association rate constant and the decrease in the dissociation rate constant, whereas the backbone structure of the primary binding loop of ECI mainly contributes to the decrease in the dissociation rate constant.

It has been pointed out that an intramolecular interaction within a serine proteinase inhibitor molecule is involved in stabilization of the conformation of its primary binding loop containing the reactive site for a target enzyme. As for Kunitz family proteins, it was previously suggested that two disulfide bonds (Cys40 to Cys84 and Cys134 to Cys143, ECI numbering) conserved in all Kunitz family proteins play a role in stabilization of the structure of the primary binding loop. However, the chemical modification of ETI indicated that the reduced form retained full trypsin inhibitory activity toward trypsin (*17*), and furthermore site-directed mutagenesis of cysteine residues Cys39, Cys83, Cys132, and Cys139 in ETI did not cause any decrease in the inhibitory activity (*18*), indicating that the two disulfide bonds are not directly involved in stabilization of the primary binding loop.

Meanwhile, X-ray crystallographic studies have revealed the detailed three-dimensional structures of a large number of serine proteinase inhibitors, including four Kunitz family proteins, *i.e. E. caffra* trypsin inhibitor (ETI) (*19*), soybean trypsin inhibitor (*16*), winged bean chymotrypsin inhibitor (*20*), and proteinase K/α-amylase inhibitor from wheat germ (*21*). These studies revealed that most serine proteinase inhibitors possess a common exposed primary binding loop with a characteristic canonical conformation, but that the scaffolds have quite different folding motifs (*22*). It was shown that the crystal structures of Kunitz family proteins are highly conserved, consisting of 12 antiparallel β-strands joined by long loops, and the reactive site residue was found to be located in the loop structure between β-strands A4 and B1. It has been suggested from these studies that the primary binding loop of a Kunitz family protein is stabilized by hydrogen bonds networks derived from the N-terminal segment and also by intramolecular interactions between β-strands. Our preliminary experiments shows that the N-terminal amino acid truncation of ECI drastically decreased its inhibitory activity toward chymotrypsin, demonstrating that the N-terminal segment is involved in the inhibitory activity, probably by stabilizing the primary binding loop of ECI (S. Iwanaga *et al.* unpublished results). Furthermore, our previous study on ECI showed that it consists of two distinct structural domains: a hydrophobic N-terminal domain (positions 1-107) and a hydrophilic C-terminal

domain (positions 108–179), and that the N-terminal domain retained slight inhibitory activity, while the C-terminal domain exhibited no inhibitory activity at all. It was further shown that the inhibitory potency of the N-terminal domain was enhanced by the presence of the C-terminal domain in the reconstituted mixture (*23*). Overall, it can be assumed that the higher hydrophobicity of the N-terminal domain of ECI, as compared with those of other known Kunitz family proteins, may enhance the intramolecular interaction within the ECI molecule, and this characteristic may be involved in the strong inhibitory activity toward chymotrypsin. The crystallization and preliminary X-ray structural analysis of a chymotrypsin inhibitor: ECI homologue, from *E. caffra* seeds was reported some years ago (*24*). The inhibitory mechanism of ECI will doubtless be better understood when its three-dimensional structure is known.

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