Unbound Form of Tomato Inhibitor-II Reveals Interdomain Flexibility and Conformational Variability in the Reactive Site Loops*

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The Potato II (Pot II) family of proteinase inhibitors plays important roles in the constitutive and inducible defense of plants against predation by a wide range of pests. The structural basis of inhibition by a multidomain Pot II family inhibitor was revealed recently by the structure of the ternary complex between the two-headed tomato inhibitor-II (TI-II) and two molecules of subtilisin Carlsberg. Here we report the 2.15-A resolution crystal structure of the unbound form of TI-II that reveals significant conformational flexibility in the absence of bound proteinase molecules. The four independent copies of unbound TI-II in the asymmetric unit of the unit cell display a range of different conformations when compared with the bound form of the inhibitor, most strikingly in the orientations of the inhibitory domains and in the conformations of the reactive site loops. One of the two linker segments (residues 74 to 79) between the two domains as well as the adjacent beta-strand in Domain I (residues 80–85) is well ordered in all four copies of the unbound inhibitor, even though this region appeared to be disordered in the structure of the ternary complex. Conformational flexibility seen in the reactive site loops of unbound TI-II suggests a mechanism by which the inhibitor can balance the need for tight binding with the need for broad inhibitory function.

Tomato inhibitor-II (TI-II)† is a member of the Potato II (Pot II) proteinase inhibitor family of serine proteinase inhibitors (PIs). This 13.5-kDa protein is a potent inhibitor of subtilisin (\(K_i = 9\) nM) that also possesses a remarkable dual specificity toward chymotrypsin (\(K_i = 30\) nM) and trypsin (\(K_i = 80\) nM) (1). Pot II inhibitors accumulate systemically in plant tissue as a result of wounding or pest attack and have been isolated from wounded tomato and tobacco leaves (1, 2), green tomatoes (3), potato tubers (4, 5), eggplant fruits (6), paprika seeds (7), and ornamental tobacco flower stigma (8). The systemic response to wounding or pest attacks originates from complex signaling cascades initiated by the binding of systemin (9) to a cell-surface receptor (10). This results in the release of jasmonic acid and the activation of signaling pathways that drive the synthesis of PIs (11, 12). After insect attack or wounding, PIs can accumulate to levels of 2% or more of the total soluble protein in leaves (13, 14) and exert their pesticidal properties by interfering with the digestive physiology of the attacking insects (15). The wide distribution and inducible expression of Pot II PIs underline the fundamental importance of these proteins to pest defense in commercially important crops. To this effect, the inhibitory properties of PIs have already been exploited in the production of transgenic plants overexpressing PIs in an attempt to control destruction by pests (16–20). However, greater understanding of the molecular mechanisms of inhibition in PIs is required to fully harness the potential benefits of these proteins to crop protection.

TI-II and other Pot II PIs belong to the class of “standard mechanism, canonical” proteinaceous inhibitors of serine proteinases (21), which consists of at least 18 nonhomologous families. The PIs from this large and diverse class bind to the active sites of proteinases in a substrate-like manner (22) through “reactive site loops” (23). According to the nomenclature of Schechter and Berger (24), where P1 is the residue N-terminal to the scissile bond and P1′ is the residue C-terminal to the scissile bond, residues P4 to P2′ of each inhibitor typically interact with binding pockets S4 to S2′ on the proteinase. The reactive site loop adopts a relatively extended conformation and forms a distorted antiparallel beta-strand pair with residues near the active site of the proteinase (25).

Similar to many other families in the standard, canonical mechanism class of PIs, the Pot II family has many members that consist of multiple inhibitory domains, each of which contains a reactive site loop. The crystal structure of the TI-II:

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The abbreviations used are: TI-II, tomato proteinase inhibitor-II; Pot II, Potato II inhibitor; PI, proteinase inhibitor; PI-II, potato proteinase inhibitor; PCI-I, polypeptide chymotrypsin inhibitor-I from potatoes; PEG, polyethylene glycol; r.m.s.d., root mean squared difference; SGPB, streptomyces griseus proteinase B.

1 The abbreviations used are: TI-II, tomato proteinase inhibitor-II; Pot II, Potato II inhibitor; PI, proteinase inhibitor; PI-II, potato proteinase inhibitor; PCI-I, polypeptide chymotrypsin inhibitor-I from potatoes; PEG, polyethylene glycol; r.m.s.d., root mean squared difference; SGPB, streptomyces griseus proteinase B.
Conformational Variability in Unbound TI-II

A central paradox in understanding the mechanism of proteinase inhibitors is how these proteins can bind tightly to proteinases in a substrate-like manner without being rapidly hydrolyzed like a substrate. Many structural and biochemical studies (21, 31) on a wide range of structurally diverse PIs and their cognate proteinases have revealed a variety of mechanisms allowing PIs to achieve their biologically important functions. These mechanisms include various strategies for stabilizing the reactive site loop using disulfide bridges, extensive hydrogen bonding networks, salt bridges, and van der Waals interactions between the reactive site loop and the proteinase. Crystallographic studies of multidomain Pot II inhibitors, starting with the structure of the TI-II:(subtilisin)2 complex, are just beginning to suggest the importance of multiple inhibitory domains within a single PI.

To further extend the earlier x-ray crystallographic structure determination of a ternary complex consisting of TI-II bound to two molecules of subtilisin Carlsberg (26), the structure of unbound TI-II has now been determined to 2.15-Å resolution. Four copies of the unbound inhibitor are contained within the asymmetric unit of the crystalline unit cell, thereby providing a unique opportunity to examine the significant range of conformational flexibility present in the global structure of the inhibitor, as well as in the local structure of the reactive site loops. An analysis of the range of conformations sampled by the four independent molecules in the asymmetric unit provides substantial insight into the importance of conformational flexibility between domains as well as within reactive site loops.

EXPERIMENTAL PROCEDURES

Purification and Crystallization—TI-II was prepared from transgenic tomato plants that overexpressed a prosystemin transgene, resulting in the synthesis and accumulation of high levels of TI-II in the leaves (~1 mg/ml leaf juice). Leaves from 1000 young tomato plants were collected (~800 g of leaf tissue) and blended with 1600 ml of a buffer containing 0.01 M sodium citrate, 0.5 M sodium chloride, and 0.7 M sodium hydrosulfite, pH 4.3. The homogenate was expressed through eight layers of cheesecloth by hand squeezing. The debris retained in the cloth was resuspended in 400 ml of buffer, and the mixture was further expressed. The combined, expressed liquid was clarified by centrifugation at 12,000 × g for 20 min. Protein in the filtered liquid were precipitated by adding solid ammonium sulfate to 80% saturation and stirring at 4 °C for 2 h. The precipitate was recovered by centrifugation as above. The pellet was solubilized in 600 ml of water, and the remaining debris was removed by centrifugation. The resulting solution was placed in a flask and immersed in a boiling water bath with stirring until the temperature of the liquid was 70 °C. The contents of the flask were then cooled rapidly in an ice bath to room temperature, and the precipitated proteins were repelletted by centrifugation. The solution was dialyzed overnight at 4 °C against 0.01 M Tris, 0.1 M KC1 buffer, pH 8.1. The retentate was passed through a 3 × 12-cm column containing chymotrypsin-Sepharose CL4B affinity resin (26) that had been equilibrated with the buffer. The column was then washed with three bed volumes of buffer and then with 5 M urea. The column was washed with three bed volumes of buffer and then with 5 M urea, to elute the inhibitor proteins that were bound to chymotrypsin. The eluate was dialyzed against several changes of 50 mM ammonium bicarbonate and lyophilized and stored. The preparation yielded about 80 mg of dry material containing about 24 mg of TI-II and 14 mg of inhibitor I. TI-II protein was purified to homogeneity using reverse phase high pressure liquid chromatography. A Vydac (Hesperia, CA) 218TP510 C18 semi-preparative column (10 × 250 mm, 5 μm, 300 Å) was used with a 90-min gradient of 0–60% acetonitrile in 0.1% trifluoroacetic acid. TI-II eluted as a single peak at 53 min. The yields of inhibitor from this step were nearly quantitative. TI-II protein was obtained as homogenous as judged by SDS-PAGE (data not shown).

Crystals were grown at room temperature by the hanging-drop, vapor-diffusion method by mixing 2 μl of a 1 mM solution of TI-II and 2 μl of 7% (w/v) PEG 10000 and 50 mM ammonium sulfate. Small crystals grew within 1–2 weeks. Macroseeding was used to produce crystals with typical dimensions of 0.2 × 0.1 × 0.05 mm. The crystals belonged to space group C2 with cell dimensions a = 58.31 Å, b = 81.35 Å, a = 90°, β = 104.14°, and γ = 90°. The Vm for four TI-II molecules (13,500 Da) per asymmetric unit is 2.26 Å3/Da.

Data Collection, Structure Solution, and Refinement—Crystals used for data collection were prepared by harvesting into a solution identical to the crystallization solution with the addition of 5% (w/v) PEG 400 and equilibrating overnight against a well solution containing the crystallization solution with the addition of 20% (w/v) PEG 400. Immediately before flash freezing in a nitrogen gas stream at 100 K, the crystals were briefly transferred into a cryoprotectant solution identical to the crystallization solution, with the addition of 25% (w/v) PEG 400. Data were measured from an ADSC Quantum-315 CCD detector using 0.5° oscillation frames with wavelength radiation (9-2 mm) (Stanford Synchrotron Radiation Laboratory). Data were recorded as 180° oscillations at a crystal-to-detector distance of 250 mm and processed using the HKL suite of programs (37). Intensities were converted to amplitudes using TRUNCATE (38).

The solvent content of the unbound inhibitor crystals was calculated to be 59, 45, or 32% if three, four, or five copies of TI-II were present in the asymmetric unit, respectively. Molecular replacement searches were performed using BEAST (39) and CNS (40). The structure of TI-II from the TI-II:(subtilisin)2 complex (PDB code 1OYV), with all the side chains and temperature factors refined, was used as the search model for molecular replacement calculations. Cross-rotation functions calculated from the maps data to a maximum resolution of 3.5 Å, each yielding one clear solution (log-likelihood-gain scores of 23.0 and 21.1). Each rotation function solution was used to calculate a translation function using data to a maximum resolution of 3.5 Å, each yielding one clear solution (log-likelihood-gain scores of 22.5 and 14.9). Using CNS (40), the two solutions were subjected to Patterson correlation refinement, and the relative orientation of the two solutions was determined using a translation search, followed by rigid body refinement (R-factor of 0.51). A third solution was identified using CNS (40) by carrying out Patterson correlation refinement and translation searches on other high-scoring solutions from the cross-rotation search. The three solutions were subjected to rigid body refinement, yielding an R-factor of 0.46. The fourth solution should not be identified with Patterson correlation refinement and translation searches. Instead, a non-crystallographic, 2-fold rotational symmetry element relating two of the three positioned molecules was identified and was applied to the third molecule to generate the fourth molecule in the asymmetric unit. After rigid body refinement of the four molecules, the R-factor was 0.42.

The electron density map calculated using the molecular replacement solution revealed a good fit of the model to the core regions of the inhibitor. In addition, there was clear electron density for the interdomain regions 74–85 in all four copies of TI-II in the asymmetric unit. Because these residues were not modeled in the structure of TI-II in complex with subtilisin, they were placed by manual inspection using the program XFIT (41). The N and C termini, and the loop encompassing residues 101–105, showed the greatest variation among the four copies. The model was subjected to iterative rounds of manual model building using XFIT (41) and refinement against a maximum-likelihood target using CNS (40). Further refinement of the structure was performed using REFMAC v. 5.0.36 (42) with individual B-factors refined for Domain B. The solvent content of the unbound TI-II was 70%, and the loop encompassing residues 101–105, showed the greatest variation among the four copies. The model was subjected to iterative rounds of manual model building using XFIT (41) and refinement against a maximum-likelihood target using CNS (40). Further refinement of the structure was performed using REFMAC v. 5.0.36 (42) with individual B-factors refined for Domain B. The solvent content of the unbound TI-II was 70%.
Global Structure of the Unbound Form of TI-II—The structure of unbound TI-II (Fig. 1) was determined by the molecular replacement technique to 2.15 Å resolution, using the coordinates of TI-II in complex with two molecules of subtilisin Carlsberg (26) (PDB code 10YV) as the search model (refer to Table I for crystallographic parameters). There are four copies of TI-II (designated as A, B, C, and D) in the asymmetric unit, and the overall conformation of each copy is similar in most regions to that of TI-II from the complex (26).

TI-II adopts an elongated shape with approximate overall dimensions of 50 \times 25 \times 15 Å; it consists of two structurally similar inhibitory domains (Domains I and II). Each inhibitory domain adopts the fold determined previously for the single-domain Pot II inhibitors (48–51) and consisting of only a small amount of regular secondary structure in the form of a small antiparallel β-sheet, as well as a series of polypeptide chain segments interconnected by four disulfide bonds. Both domains also contain one turn of a 310 helix, comprising residues 30–32 in Domain II and residues 90–92 in Domain I. An inhibitory reactive site loop is found in each domain, and these loops are positioned at opposite ends of the elongated molecule (Fig. 1), thereby allowing a single inhibitor to bind to two protease molecules simultaneously.

As first deduced from the structure of PCI-I in complex with SGPB and proven by the structure of TI-II in complex with subtilisin Carlsberg, the sequence repeats present in TI-II do not correspond to individual structural domains (26, 48). Instead, Domain I consists of the first 15 residues and of residues 80–116, whereas Domain II consists of residues 18–73. Although both domains adopt the same fold, the structures of each domain differ in detail. The two most important structural differences between Domain I and Domain II are: (1) the N terminus of the reactive site loop in Domain I lies at the N terminus of the protein, but in Domain II, this segment is connected by a loop to the last β-strand of the small β-sheet (corresponding to the C terminus of Domain I); and (2) the loop composed of residues 20–26 in Domain II is shortened by four residues in Domain I and includes the only cis-peptide bond in the structure (Tyr-83 to Pro-84).

Domains I and II are linked together by two polypeptide segments consisting of residues 16–17 and 74–79. The linker consisting of residues 74–79, as well as several adjacent residues (residues 80–85), appeared to be disordered in the structure of TI-II bound to subtilisin, because electron density corresponding to this region could not be detected. In contrast, in all four copies of unbound TI-II, the entire region was well defined by unbiased electron density maps calculated before modeling the structure of this region. The linker region consisting of residues 74–79 is highly solvent exposed and appears to be stabilized primarily by three hydrogen bonds. It is not clear why there is such a dramatic change in the conformational flexibility of residues 74–85 between the unbound and bound forms of TI-II. In the TI-II:(subtilisin)2 complex, the loop of subtilisin comprising residues from 158 to 164 is located adjacent to where residues 81–84 of the inhibitor would be expected. It is puzzling why this region of the inhibitor appears to be disordered only in the presence of bound protease, because it might be expected that the protease would interact with this region and possibly stabilize it.

A possible explanation for the conformational flexibility seen in the linker region may lie in the different orientations of Domain I relative to Domain II that are observed in the bound and unbound forms of TI-II. DYNDOM (52) reveals a rotation (residues 17 and 75 as the hinge residues) of Domain II relative to Domain I of 7.4° and 8.8° when comparing copies A to C and A to D, respectively. The two inhibitory domains can clearly adopt different orientations relative to each other, which may allow the multidomain inhibitor molecule to accommodate the binding of multiple protease molecules simultaneously. In the complex of TI-II with subtilisin Carlsberg, the two protease molecules are well separated from each other, but complexes of TI-II with other proteases may require slight changes in the orientation of the inhibitory domains to avoid steric clashes between bound protease molecules.

A feature of the structure of TI-II that allows for interdomain flexibility is the relatively small interdomain interface. An analysis of this interface reveals that the interdomain buried surface area found in the unbound form of TI-II ranges from 559 to 627 Å², which is 15–34% larger than that found for the bound form (487 Å²). Similar to the bound form, the interdomain interface in unbound TI-II consists of a small cluster of highly conserved hydrophobic residues (Ile-14, Pro-16, Tyr-98, Phe-100, and Phe-106 from Domain I; and Tyr-34, Pro-54, and Pro-57 from Domain II), as well as Arg-17 and Lys-55. When comparing the different copies of bound and unbound TI-II, there are a number of changes in the positions and conformations of residues at the interdomain interface, most notably Ile-14, Arg-17, Pro-54, Pro-57, and Phe-100. Perhaps the most striking difference in conformation among the interface residues arises in Arg-17. In copies A and D, the side chain points toward Domain I, forming a salt bridge with Glu-114, whereas in copies B and C, as well as in the TI-II:(subtilisin)2 complex,
the side chain extends out into solution (Fig. 2). Because these two conformations place the side chains on opposite sides of the linker segment consisting of residues 77–78, it appears that a concerted conformational change involving the linker and Arg-17 would be required to interconvert between the two side chain conformations.

In solution, it is likely that both bound and unbound forms of TI-II exhibit conformational variability in the orientation of one inhibitory domain relative to the other, and that this conformational flexibility is mediated by slight changes in packing at the interdomain interface. The range of conformations as seen in different copies of both the bound and unbound forms of TI-II likely arises from the trapping of different solution conformations of the inhibitor by the crystal lattice. Table II reveals the overall range of conformational variability seen among the four copies of unbound TI-II in the asymmetric unit and in comparison with the bound form. Globally, copies A and B (r.m.s.d. of 0.62 Å) are similar, as are copies C and D (r.m.s.d. of 0.44 Å). This is also reflected in comparisons of Domain II by itself with r.m.s.d.’s of 0.19 and 0.24 Å when comparing copies A and B, and copies C and D, respectively. In contrast, Domain I differs strikingly between copies A and B (r.m.s.d. of 0.75 Å), although it is quite similar when comparing copies C and D (r.m.s.d. of 0.38 Å).

Comparison of Unbound TI-II with That of Bound TI-II—The overall conformation of the core of the unbound TI-II reveals no major structural rearrangements between the bound and unbound forms (Fig. 3), but there are clear differences in the orientation of the two domains relative to one another as well as localized variations in the structures of several loops. Superposition of all main-chain atoms reveals large r.m.s.d.’s among all four copies of the unbound inhibitor in comparison with the bound form of the inhibitor (refer to Table II). A hinge motion consisting of a rotation of 7.5° between Domains I and

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**Fig. 1.** Crystallographic structure of unbound TI-II. A, ribbon diagram of unbound TI-II. Domain I is shown in red, whereas Domain II is shown in blue. Reactive site loops 1 and 2 are shown in black and green, respectively. The side chain of the P1 residue in each reactive site loop is drawn in black. Disulfide bonds are drawn in yellow and are assigned residue numbers in black. B, stereoscopic view of the four copies in the asymmetric unit of unbound TI-II. Domain I is shown in red, whereas Domain II is shown in blue. Domain II of each copy has been superimposed using main-chain atoms.
II is revealed when comparing copies C and D with the bound form of TI-II. A similar hinge motion of $5^\circ$ can be detected when comparing copies A and B with the bound form of TI-II. The structure of the proteinase-inhibitor ternary complex suggests that proteinase binding restricts the orientation of the two inhibitory domains, because the proteinase molecule bound to Domain II can form interactions with residues in Domain I (26). In the only other structure of a proteinase inhibitor bound to multiple proteinase molecules, individual trypsin molecules bind to single domains of two-headed Bowman-Birk inhibitors and do not appear to affect the orientation of the two inhibitory domains (53–55). In contrast, the flexible orientation of the two domains of TI-II may allow the inhibitor to fine tune its interactions with different proteinase molecules bound to Domain II.

In addition to differences in orientations of domains, the bound and unbound forms of TI-II reveal significant localized changes in the conformations of several loops. In particular, the loop consisting of residues 100–106 in Domain I, as well as the reactive site loops and adjacent regions in both domains, show large conformational differences. The loop consisting of residues 100–106 is situated at the interdomain interface and is near residues 130–132 of the subtilisin molecule bound to Domain II in the TI-II:(subtilisin)$_2$ complex. The different conformations of this loop appear to reflect differences in the orientation of the two inhibitory domains as well as the presence of a proteinase molecule bound to Domain II.

Although there have been many previous studies (32–35, 56–58) examining the conformations of bound and unbound forms of reactive site loops in canonical, standard mechanism proteinase inhibitors, the structures of four independent copies of unbound TI-II in the crystalline asymmetric unit provide a unique opportunity to examine the range of conformations adopted by reactive site loops of two different sequences in both unbound and bound forms. As in other members of the Pot II family, the reactive site loops are flanked by disulfide bonds formed by cysteine residues at the P3 and P2$'$ positions (Cys-3 to Cys-91 and Cys-7 to Cys-87 in Domain I and Cys-60 to Cys-64 to Cys-27 in Domain II). These disulfide bridges are thought to hold the reactive site loop in a relatively rigid conformation that may help to prevent proteolytic cleavage of the inhibitor upon interaction with proteinases. Reduced conformational flexibility in the loop is also thought to enhance proteinase binding by reducing entropic loss to achieve tighter binding (32, 59).

**Conformational Flexibility**—As analyzed previously (26), main-chain torsional angles of the reactive site loops in the TI-II:(subtilisin)$_2$ complex are quite similar to those seen in other PIs (Table III). Although the overall conformation of reactive site loops from different inhibitors bound to a variety of serine proteinases is generally conserved, each proteinase substrate binding cleft is unique. Broad-specificity inhibitors that have evolved to inhibit a wide range of proteinases must balance the need for flexibility in recognizing proteinases with different substrate recognition sites against the need to maintain conformational rigidity (34).

The range of conformational flexibility in the reactive site loops of TI-II is revealed by variations in main-chain torsional angles, side-chain rotamers, and hydrogen bonding networks in the four copies of unbound TI-II. Some of these features underlying the conformational flexibility of reactive site loops have been analyzed previously (33–35,58). Significant changes in

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**Fig. 2. Conformational change in linker region of unbound TI-II.** A stereoscopic view of linker residues 15–18 (shown in magenta) and residues 76–80 (shown in black) of copy A (A) and copy B (B) is shown. Omit map electron density corresponding to Arg-17 has been contoured at 3.4$\sigma$. 

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**TABLE III.**

| Reaction Enzyme Type | Reactive Site Loop | Residues | Main-Chain Torsional Angles | Side-Chain Rotamers | Hydrogen Bonding Networks |
|----------------------|-------------------|----------|---------------------------|--------------------|--------------------------|
| Serine proteinase    | Bright yellow     | 100–106  | $\phi$, $\psi$            | $\chi_1$, $\chi_2$ | $\cdots$                |
| Cystine proteinase   | Dark blue         | 130–132  | $\phi$, $\psi$            | $\chi_1$, $\chi_2$ | $\cdots$                |
| Aspartate proteinase | Light green       | 60–64    | $\phi$, $\psi$            | $\chi_1$, $\chi_2$ | $\cdots$                |
main-chain torsional angles are found in the reactive site loops from Domains I and II (refer to Table III and Fig. 4). Most of these changes reflect the higher flexibility of the unbound reactive site loop. The overall path of the polypeptide chain is quite similar for residues P3 to P5, but numerous changes in main-chain torsional angles cause carbonyl oxygen and amide nitrogen atoms to occupy positions differing significantly from those seen in the bound form of TI-II.

Particularly dramatic changes are seen in the ϕ angle of the P1 residue and the ψ angle of the P1′ residue in the reactive site loop of Domain II (refer to Table III and Fig. 5). The reactive site loop in Domain II appears to adopt two distinct conformations in the unbound state, with copies A and B adopting one conformation and copies C and D adopting the other. Both of these conformations appear to differ significantly from the bound conformation. The change in the ϕ and ψ angles flanking the scissile bond causes the carbonyl carbon-to-carbonyl oxygen vector of the P1 residue to point either 45° toward (copies A and B) or 70° away from (copies C and D) Asn-29. The P1 side chain in copies A and B (x1 = −78° and x2 = −21°) adopts a similar conformation to that seen in the bound form of the inhibitor (x1 = −88° and x2 = −4°), but in copies C and D, the P1 side chain adopts a distinctly different rotamer (x1 = −173° and x2 = 76°).

Conformations and Interactions of Reactive Site Residues—Side chains of other residues in the reactive site loop also adopt distinct rotamers in the unbound form of the inhibitor. In the reactive site loop of Domain I, the most dramatic changes can be seen in the side chains of the P2, P1, and P1′ residues. The P1 arginine residue adopts a somewhat unusual rotamer to fit into the S1 pocket when bound to subtilisin Carlsberg (26). In the absence of constraints imposed by a bound peptidase molecule, this highly solvent exposed side chain adopts an extended conformation. The P2 and P1′ side chains in both domains also change significantly in conformation in the unbound form of TI-II, resulting in the loss of a commonly found hydrogen bond formed between the side chains of these two residues; this hydrogen bond is thought to confer stability in the reactive site loop (35). In Domain I of the unbound form of TI-II, the conformation of Thr-4 at the P2 position is quite similar to that seen in the bound form, but the conformation of Glu-6 at the P1′ position is distinctly different. In copies A and B, the rotamer adopted by Glu-6 places the carboxylate oxygen atoms too far from Thr-4 to form a direct hydrogen bond, whereas in copies C and D, a hydrogen bond can still be formed between the P2 and P1′ residues. In Domain II, the rotamers adopted by Thr-61 (P2) and Asn-63 (P1′) all differ markedly from the rotamers adopted in the bound form of TI-II, which disrupts the hydrogen bond between the P2 and P1′ residues in all four copies of the unbound inhibitor.

Interactions of Reactive Site (P2, P1, and P1′) with the Core of the Inhibitor—A second dramatic alteration in the hydrogen bonding network stabilizing the reactive site loop in Domain II is also seen in the unbound form of the inhibitor. In the bound form of the inhibitor, a key set of interactions stabilizing the reactive site loop in Domain II is contributed by the hydrogen bonds between the Nε2 atom of the side chain of Asn-29 and the main-chain carbonyl oxygen atoms of Thr-61 and Asn-63 at the P2 and P1′ positions, as well as the hydrogen bond between the main-chain carbonyl oxygen atom of Ile-28 and the main-chain amide nitrogen atom of Asp-65. The same set of interactions are observed in copies A and B of the unbound inhibitor, but in copies C and D, the hydrogen bond between Asn-29 and Thr-61 is missing because the reactive site loop appears to have moved slightly farther away from the core of Domain II. The corresponding interaction in Domain I consists of a van der Waals contact between the side chains of Thr-89 and Glu-6, replacing the hydrogen bonds donated by Asn-29 in Domain II. This van der Waals contact is missing in all four copies of the unbound inhibitor, because the side chain of Glu-6 adopts different rotamers that place it farther from Thr-89.

In addition to the many differences seen in the conformations

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**Table II**

r.m.s.d. of superimposed coordinates for bound and unbound forms of TI-II

| Copy No. | Unbound TI-II | Bound TI-II |
|----------|---------------|-------------|
| A        | 0.62          | 0.88        |
| B        | 0.77          | 0.87        |
| C        | 0.44          | 1.01        |
| D        | 0.88          | 0.86        |

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*Only main chain atoms are included in the superpositions. All superpositions were performed using the program LSQMAN (62). The "Copy No." entry refers to the individual copies in the asymmetric unit of the crystal.

452 atom pairs.

**A. Global superpositions**

| Unbound TI-II | Bound TI-II |
|---------------|-------------|
| A             | 0.75        | 0.70        |
| B             | 0.33        | 0.79        |
| C             | 0.42        | 0.71        |
| D             | 0.71        | 0.71        |

**B. Superpositions of Single Domains**

| Unbound TI-II | Bound TI-II |
|---------------|-------------|
| A             | 0.19        | 0.77        |
| B             | 0.61        | 0.79        |
| C             | 0.61        | 0.81        |
| D             | 0.57        | 0.84        |

**C. Superpositions of Reactive Site Loops**

| Unbound TI-II | Bound TI-II |
|---------------|-------------|
| A             | 0.29        | 0.61        |
| B             | 0.20        | 0.72        |
| C             | 0.18        | 0.67        |
| D             | 0.40        | 0.59        |
of the reactive site loops of the unbound inhibitor, conformational differences are also seen in a region immediately N-terminal to the reactive site loop in Domain II consisting of residues 53–59. This loop adopts a significantly different conformation in all four copies of unbound TI-II compared with the conformation of the bound form. This region is close to the terminal to the reactive site loop in Domain II consisting of

\[ \text{Table III} \]

Main-chain torsion angles of reactive site loops

Angles for PCI-I, OMTKY3, CI-2 (chymotrypsin inhibitor-2 from barley), eglin-C, and BPTI (bovine pancreatic trypsin inhibitor) were obtained from Greenblatt et al. (48).

|       | P4  | P3  | P2  | P1  | P1' | P2' | P3' |
|-------|-----|-----|-----|-----|-----|-----|-----|
| ubTI-II (1A)* | 117 | 155 | 72  | 167 | -79 | -2  | 85  |
| ubTI-II (1B)  | -27 | 171 | -87 | 6   | -69 | 131 | -84 |
| ubTI-II (1C)  | 139 | 129 | 76  | 95  | 72  | 152 | 92  |
| ubTI-II (1D)  | -78 | 168 | -58 | 165 | -84 | 116 | -75 |
| ubTI-II (2A)  | -80 | 165 | -124| 166 | 132 | 135 | 84  |
| ubTI-II (2B)  | -84 | 166 | -158| 166 | 115 | 115 | 87  |
| ubTI-II (2C)  | -88 | 169 | -120| 166 | 82  | 132 | 132 |
| ubTI-II (2D)  | -84 | 166 | -158| 166 | 115 | 115 | 87  |
| OCI-I         | -27 | 171 | -87 | 6   | -69 | 131 | -84 |
| OCI-KY3       | -80 | 165 | -124| 166 | 132 | 135 | 84  |
| OCI-KY3       | -84 | 166 | -158| 166 | 115 | 115 | 87  |
| OCI-2         | -88 | 169 | -120| 166 | 82  | 132 | 132 |
| OCI-CY3       | -84 | 166 | -158| 166 | 115 | 115 | 87  |
| BPTI          | -88 | 169 | -120| 166 | 82  | 132 | 132 |
| sBBI          | -84 | 166 | -158| 166 | 115 | 115 | 87  |

\[ *\text{ub, unbound.} \]

\[ **\text{Ovmucoid third domain bound to Streptomyces griseus proteinase B (61).} \]

\[ ***\text{Ovmucoid third domain bound to } \alpha \text{-chymotrypsin (33).} \]

\[ ***\text{Soybean Bowman-Birk inhibitor bound to bovine trypsin (54).} \]
interdomain interface, and small changes in conformation in this loop may stabilize different orientations of the two inhibitory domains.

Comparison with Inhibitors from the Potato II Family—Apart from the structure of TI-II in complex with subtilisin Carlsberg (26), the only other crystallographic structure of a Pot II family inhibitor is the structure of PCI-I in complex with SGPB (48). The structure of PCI-I in complex with SGPB is very similar to that of Domain II in TI-II, because the two domains share 86% sequence identity (refer to Table III). Most of the conformational changes observed between the structure of Domain II of TI-II bound to subtilisin Carlsberg and the unbound inhibitor structures can also be seen when comparing the bound form of PCI-I and the unbound forms of TI-II. One interesting difference between PCI-I and TI-II is the identity of the P2 residue in the reactive site loop. In PCI-I and most other Pot II family PIs, proline occupies this position, whereas in TI-II it is occupied by threonine in both reactive site loops. In the bound form of TI-II, this residue forms a hydrogen bond with P1’, but this hydrogen bond is not present in six of the eight independent reactive site loops of the unbound form. It has been proposed that proline at the P2 position helps to rigidify the reactive site loop, and that threonine in the P2 position can also help to rigidify the reactive site loop by forming a hydrogen bond with the P1’ residue (35). These two different mechanisms for stabilizing reactive site loop conformation may be of functional significance because the hydrogen bond between the threonine at P2 and the P1’ residue can be broken in the unbound form of the inhibitor to provide additional flexibility. On the other hand, proline at P2 presumably rigidifies both the bound and unbound forms of the reactive site loops.

The structures of several single domain Pot II family inhibitors from Nicotiana alata have been determined by NMR (49–51). These studies reveal that the solution structures of Pot II family inhibitors are similar to the crystallographically determined structures. The regions of ϕ-ψ space occupied by residues in the reactive site loop appear to be similar to those

**Fig. 4. Conformations of the reactive site loops.** The reactive site loops from Domain I of unbound TI-II copies A, B, C, and D are shown superimposed onto that of bound TI-II in panels A, B, C, and D, respectively. The reactive site loops from Domain II of unbound TI-II copies A, B, C, and D are shown superimposed onto that of bound TI-II in panels E, F, G, and H, respectively. The bound form of TI-II is shown in magenta, and the unbound form is shown in cyan.

**Fig. 5. Variations in main-chain torsional angles between the bound and unbound forms of TI-II in the reactive site loop from Domain I (A) and Domain II (B).** Angles from copies A and B of unbound TI-II are shown in blue-filled circles. Angles from copies C and D of unbound TI-II are shown in red-filled circles. Angles from bound TI-II are shown in open black circles.
occupied in both the bound and unbound structures of TI-II and PCI-I. The NMR structures reveal that the atomic r.m.s.d.'s over the reactive site loop are significantly larger than the other regions of the inhibitor, which is consistent with the greater conformational variability seen in the reactive site loops of the unbound form of TI-II.

A highly conserved feature of the bound form of reactive site loops in many Pot II family inhibitors is a hydrogen bond between the side chain of the P1' residue and its amide nitrogen. It has been proposed that this hydrogen bond stabilizes the reactive site loop from proteolytic cleavage at the scissile bond immediately adjacent to the amide nitrogen (48, 60, 61). Although this hydrogen bond is seen in the bound forms of PCh-I and TI-II, it is missing in six of the eight reactive site loops of unbound TI-II. The loss of this hydrogen bond is consistent with NMR studies of unbound T1 to T4 (50), where this hydrogen bond also appears to be absent. The absence of this hydrogen bond in unbound forms of proteinase inhibitors may provide additional flexibility to the unbound form of the reactive site loop that may be of importance in proteinase recognition and binding. However, once bound, the P1' NH to P1' side-chain oxygen (Glu, Asp, or Asn) is important to the inhibitory nature of the domain.

Conformational Changes in Reactive Site Loops upon Complexation: Comparison with Inhibitors from Other Families—The increased conformational variability of the reactive site loops in unbound TI-II is similar to that seen in other families of proteinase inhibitors (34, 35). Main-chain torsional angles, particularly at the P2 to P1' positions, show greatly increased variability in the unbound form of a wide range of PIs, and this is also seen in TI-II (refer to Table III and Figs. 4 and 5). A similar increase in variability in side-chain torsional angles is also seen, particularly at positions P1, P1', and P2', which is also the case in TI-II. Surprisingly, variation at the P2 position in other inhibitors appears to be quite limited, but in TI-II, Thr-61 which occupies the P2 position in the reactive site loop in Domain II can adopt three different rotamers ranging over 250° in χ1 torsion angle space in the unbound form. Variation in side-chain conformation at this position as well as at the P1' position in unbound inhibitors appears to be coupled to the loss of the stabilizing hydrogen bond between the P2 and P1' residues as discussed above. Dramatic changes in the conformations of the P2 threonine residue and the P1' glutamate residue in the unbound form of chymotrypsin inhibitor-2 from barley (58) also accompany the loss of a stabilizing hydrogen bond between the P2 and P1' residues in the bound form of the inhibitor. In contrast, smaller changes in conformation between bound and unbound forms are seen in other PIs, such as the Kazal family of PIs, where the network of hydrogen bonds and van der Waals contacts stabilizing the reactive site loop are preserved in the unbound form (33). Specifically, the hydrogen bonding network between the side chain of Asn-33 and the main-chain carbonyl groups of Thr-17 and Glu-19 (P2 and P1' residues of the reactive site loop) are seen in both the bound and unbound forms of the ovomucoid third domain inhibitors.

In conclusion, the structures of four independent copies of the unbound form of the two-headed TI-II reveal significant conformational flexibility that may be of functional importance in the inhibition of a wide range of proteinases. TI-II is an integral part of the constitutive and inducible defensive mechanisms that protect plants from attacking pests (bacteria, fungi, and insects). To this effect, the structure must have sufficient flexibility to be able to accommodate to the substrate binding clefts of a wide range of proteinases. High-resolution structures of TI-II in bound and unbound forms reveal that flexibility in the orientation of the two inhibitory domains as well as in the reactive site loops allow the inhibitor to achieve a balance between tight binding and broad specificity.

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