Published in final edited form as:

J Mol Biol. 2007 February 9; 366(1): 230–243.

Rigidification of a Flexible Protease Inhibitor Variant Upon Binding to Trypsin

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Abstract

The Tyr35→Gly replacement in bovine pancreatic trypsin inhibitor (BPTI) has previously been shown to dramatically enhance the flexibility of the trypsin-binding region of the free inhibitor and to destabilize the interaction with the protease by about 3 kcal/mol. The effects of this replacement on the enzyme-inhibitor interaction were further studied here by x-ray crystallography and isothermal titration calorimetry. The co-crystal structure of Y35G BPTI bound to trypsin was determined using 1.65 Å resolution x-ray diffraction data collected from cryopreserved crystals, and a new structure of the complex with wild-type BPTI under the same conditions was determined using 1.62 Å data. These structures reveal that, in contrast to the free protein, Y35G BPTI adopts a conformation nearly identical to that of the wild-type protein, with a water-filled cavity in place of the missing Tyr side chain. The crystallographic temperature factors for the two complexes indicate that the mutant inhibitor is nearly as rigid as the wild-type protein when bound to trypsin. Calorimetric measurements show that the change in enthalpy upon dissociation of the complex is 2.5 kcal/mol less favorable for the complex containing Y35G BPTI than for the complex with the wild-type inhibitor. Thus, the destabilization of the complex resulting from the Y35G replacement is due to a more favorable change in entropy upon dissociation. The heat capacity changes for dissociation of the mutant and wild-type complexes were very similar, suggesting that the entropic effects probably do not arise from solvation effects, but are more likely due to an increase in protein conformational entropy upon dissociation of the mutant inhibitor. These results define the biophysical role of a highly conserved core residue located outside of a protein-binding interface, demonstrating that Tyr35 has little impact on the trypsin-bound BPTI structure and acts primarily to define the structure of the free protein so as to maximize binding affinity.

Keywords

bovine pancreatic trypsin inhibitor; x-ray crystallography; isothermal titration calorimetry; protein flexibility; binding entropy

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Abbreviations used: BPTI, bovine pancreatic trypsin inhibitor; Amino acid replacements are indicated by the wild-type residue type (using the one letter code for the 20 standard amino acid residues), followed by the residue number and the mutant residue type; Elsewhere, amino acid residue types are indicated by the standard 3-letter code. IAS, iso-aspartate; HEPES, *N*-2-Hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; Tris, tris(hydroxymethyl)-aminomethane; NMR, nuclear magnetic resonance.

1 Introduction

“Standard mechanism” serine protease inhibitors and their targets have been valuable model systems for understanding the principles governing protein-protein interactions. Comprised of at least 18 different families with little or no similarity in global architecture, standard mechanism inhibitors all bind their respective proteases with structurally similar loop segments (1;2;3;4). Often described as “lock-and-key” interactions, the backbones of the inhibitor binding loops are preorganized into rigid, extended conformations. Side chains presented by this scaffold are evolved to be highly complementary to the structure of the enzyme active sites, particularly the P1 residue, which inserts directly into the S1 specificity pocket of the enzyme. Proteins within this class of inhibitors interact with cognate proteases in an apparently substrate-like manner, yet they are cleaved very slowly. The dissociation constants for the enzyme inhibitor complexes lie in the range of 10^{-13} M to 10^{-7} M and are generally substantially lower than K_m values measured for peptide substrates. Thus, these proteins function as very effective competitive inhibitors.

The extreme stability of inhibitor-protease interactions and the resistance of the inhibitors to proteolytic cleavage are widely attributed to the preorganization of the protease-binding loop conformations (5;6;7;8;9;10;11;12). Restricted access to alternate conformations presumably limits the conformational entropy lost on the part of the inhibitor's active site loops upon protease binding, and thus enhances the stability of the resulting complex. Rigidity of the inhibitor active site loops within the inhibitor-protease complex may also restrict the atomic motions necessary for catalysis to proceed, further distinguishing inhibitor loops from substrates. The preorganized structure of the inhibitor active site loops is presumably maintained via intramolecular interactions among atoms of the loops themselves and atoms within the remaining protein core.

Bovine pancreatic trypsin inhibitor (BPTI) is one of the best characterized standard mechanism inhibitors and is the defining member of the BPTI-Kunitz inhibitor family (13). The BPTI structure consists of a C-terminal α -helix packed against a three-stranded β -sheet and is stabilized in part by three disulfide bonds (Figure 1). The disulfide between Cys14 and Cys38, connects two loop segments responsible for interactions with trypsin. The structure of the outer loop segment from residues 13–16 (drawn in magenta in Figure 1) is held in an extended conformation with β -strand backbone dihedral angles. This loop region forms backbone-backbone hydrogen bonds with main-chain atoms of trypsin resulting, in a small intermolecular β -sheet, orienting the Lys15 side chain for insertion into the S1 specificity pocket of trypsin. The resulting complex has a dissociation constant of 6×10^{-14} M, the lowest known for an inhibitor-protease interaction, and the hydrolysis reaction proceeds with an estimated half-time of approximately 20 years (14;15).

Previous work has shown that the Tyr35 side chain, a central component of the network of intramolecular interactions linking the active site loops to the protein core, plays a critical role in maintaining the preorganized, functional conformation of BPTI(6;16;17). Figures 1a and 1b summarize previous crystallographic and solution NMR characterization of the effects of the Y35G substitution on the structure of free BPTI (6;18). The blue spheres identify backbone sites known from NMR relaxation studies to undergo conformational exchange on the ms– μ s time scale. A crystal structure of Y35G BPTI reveals large rearrangements within the active site loop region, including residues 10–20 and 33–44. Notably, the outer loop segment from residues 13–16 no longer displays β -strand backbone dihedral angles. In solution, the replacement also induces ms– μ s backbone motions at nearly every position throughout the same region. The altered structure and extensive conformational fluctuations in Y35G BPTI are accompanied by a 250-fold reduction in trypsin binding affinity(6), and the rate of cleavage

for this variant is approximately 1000-fold greater than for the wild-type protein, with a half-time of approximately 100 h (E. Zakharova and D.P. Goldenberg, unpublished results).

Here we describe a 1.65 Å resolution limit crystal structure of Y35G BPTI in complex with trypsin. The co-crystal structure shows Y35G BPTI adopting a conformation nearly identical to the wild-type protein, with a water-filled cavity in place of the missing side chain. In contrast to the NMR results with the mutant inhibitor, crystallographic *B*-values show that Y35G BPTI in complex with trypsin displays very little conformational heterogeneity. Calorimetric experiments indicate that the reduced affinity of the mutant inhibitor for trypsin is due to entropic factors, most likely associated with a loss of conformational entropy upon binding to the protease. The present results suggest that pre-organization of the BPTI active site loops is not essential to achieving substantial resistance to hydrolysis, so long as rigidity within the inhibitor-protease complex is maintained.

2 Results

2.1 Structure of trypsin-bound Y35G BPTI

To determine whether the structure of Y35G BPTI is altered upon binding its target protease, we have determined the co-crystal structure of this variant in complex with bovine trypsin using data from cryopreserved crystals frozen at 100 K. For comparison, a wild-type BPTI-trypsin co-crystal structure was also determined under similar conditions. Both crystals belonged to the same space group, *I*222, as those used in previously described crystallographic studies of the wild-type complex (19;20). Figure 2 shows portions of the

$|F_o^{Y35G}| - |F_o^{wt}|$ electron density difference map calculated with structure factor amplitudes measured for the Y35G and wild-type crystals and phases calculated from the refined 100 K wild-type structure. Negative density differences are shown with red mesh (panel a), and positive differences are shown in blue (panel b). The maps are superimposed onto the structure of the 100 K wild-type complex, and the atoms shown are from the regions in and around the replacement site as well as the catalytic triad of trypsin. Intense negative density surrounds the ring of Tyr35, as expected from the Tyr to Gly replacement. Near the replacement site are additional sections of negative density around the side chain of Arg20 and a surface bound sulfate ion. Complementary positive density was also observed in the immediate vicinity of the replacement site. A sphere of positive density, likely corresponding to a water molecule, was found in the region normally occupied by the center of the Y35 ring, with another similar sphere in an adjacent position. Next to the negative density surrounding the Arg20 side chain is a patch of positive density suggesting this side chain adopts a new position. Spheres of positive density were also observed near the sulfate ion, which is clothed in negative density, suggesting that the sulfate is displaced by two new solvent molecules.

The prominent features of the electron density difference map are limited to the vicinity of Tyr35. Except for the replacement site and the neighboring region, the trypsin-bound wild-type and Y35G BPTI proteins thus appear to adopt very similar conformations. Of special note, no electron density differences were observed in the region of the Y35G BPTI scissile peptide bond or within the catalytic triad of trypsin, consistent with the idea that the complex is trapped in an inhibited state similar to that of the complex with wild-type BPTI.

Model refinement of the present BPTI-trypsin complexes was performed in the *I*222 space group, and the resulting wild-type structure can be compared to two previously determined crystal structures, 2PTC and 3BTK, also determined in *I*222, but at room temperature (Table 1) (19;20). Root mean square deviations between C_α atoms of the 100 K structure and 2PTC and 3BTK were 0.2 and 0.1 Å, respectively. The relatively small atomic shifts are within the 0.2 Å Luzatti coordinate error and likely reflect the somewhat contracted size of the unit cell

incurred upon freezing. By collecting data with cryopre-served crystals at 100 K we were able to extend the resolution limit from 1.85 to 1.62 Å with an improved data to parameter ratio of 4.8. Though the 100 K wild-type crystal structure is nearly identical to those determined previously, the quality of the structure is improved, leading to greater confidence in the comparison with the Y35G structure discussed below.

Figure 3 shows average backbone and side-chain RMS deviations between the BPTI portions of the wild-type and Y35G co-crystal structures. Backbone atoms of the Y35G protein, including the active site region, generally superimpose onto the wild-type structure within experimental error, with an average RMS deviation of 0.14 Å. For the majority of residues, the average side-chain RMS deviations are comparable to the differences observed for backbone atoms, with the exceptions of the Arg20, Leu29, Arg39 and Lys46. In the Y35G structure, the electron density indicates that Arg20 occupies two positions, one that is similar to that seen in the wild-type protein and one in which the side chain has moved into part of the cavity generated by removal of the Tyr35 side chain. Two of the other side chains that display significant differences in the two structures, Arg39 and Lys46 are largely surface exposed and in regions of relatively weak electron density, suggesting that the differences in the final refined structures may not be significant. The final residue that displayed differences in the final structures, Leu29, is quite distant from the replacement, and, in each structure, the electron density was consistent with two alternate structures, but with different relative occupancies. Again, this difference is unlikely to be significant.

Given that the unbound form of Y35G BPTI is highly flexible in solution and that increased flexibility has often been correlated with enhanced susceptibility to proteolysis (5;6;7;8;9; 10), we were particularly interested in the structure of the active site regions of the inhibitors and protease. Figure 4 shows the geometry of the active site region in the refined structure of Y35G BPTI, with the corresponding distances and bond angles for the wild-type structure shown in parentheses. In both structures, the peptide bond between Lys15 and Ala16 is planar, with no indication of a shift toward the tetrahedral geometry expected of the transition state. The γ -oxygen atom of the catalytic Ser195 residue of the protease approaches the carbonyl carbon of the Lys15-Ala16 bond of Y35G and wild-type BPTI at distances of 2.8 and 2.7 Å, respectively. The angle of approach formed between the plane of the peptide bond and the vector linking the carbonyl carbon atom and the approaching nucleophilic oxygen atom are also very similar in the two structures. All of these geometric parameters are similar to those previously observed for other inhibitor-protease complexes (10) and belie any enhanced tendency of the Y35G structure to form the tetrahedral transition state.

The major structural perturbation associated with the Y35G replacement is the formation of a large cavity in the space normally occupied by the aromatic ring of Tyr35. Lining this large cavity are side-chain atoms from residues 18, 20, 33, 40, and 44 and backbone atoms from residues 10, 11 and 35–40. Two water molecules partially occupy the space vacated by the Tyr ring, and the cavity appears to be open to the bulk solvent. Similar cavities have been observed in structures of other mutant proteins with aromatic residues replaced with Ala or Gly, including the F22A, Y23A, N43G, and F45A replacements in free BPTI(21).

Unrelated to the effects of the replacement in BPTI, the crystallographic studies also revealed that trypsin residue Asn115 was converted to isoaspartate (IAS) in a substantial fraction of the molecules in both crystals. Spontaneous isomerization of Asn residues has been observed in many proteins and is known to take place via a succinimide intermediate that can also give rise to either L- or D-Asp residues (22). For the complex with wild-type BPTI, the electron density calculated for this region of the structure was clearly more compatible with L-IAS than with either L- or D-Asn (or Asp), indicating that at least 80% of the molecules had undergone isomerization. In the complex with Y35G BPTI, the Asn (or Asp) and IAS forms appeared to

be present at roughly equal levels. Using neutron crystallography, Kossiakoff previously observed deamidation of Asn115, as well as Asn48 and Asn95, in trypsin (23). However, the deamidated side chains had the normal Asp/Asn configuration. Evidence for IAS in trypsin appears to have been observed in only one other crystal structure, a complex of porcine trypsin and the inhibitor bdellestasin, where isomerization of Asn115 likely played a role in the formation of an alternate crystal form (24). In the crystals described here, the region of residue 115 is exposed to a solvent filled portion of the lattice, so that IAS, Asn, and Asp, or a mixture of these isomers, are accommodated without perturbing the crystal packing contacts essential for construction of the *I*222 space group.

2.2 Buried solvent within the trypsin-bound Y35G BPTI structure

Figure 5 illustrates the patterns of bound solvent molecules observed in the crystal structures of trypsin-bound wild-type BPTI, free Y35G BPTI, and trypsin-bound Y35G BPTI. In the wild-type protein (Figure 5a), solvent molecule X106 resides in a small cavity near the 14-38 disulfide bridge, while another set of water molecules, X103–X105, form a hydrogen-bonded network and bridge the polypeptide segments composed of residues 8–10 and 41–44. For wild-type BPTI, the pattern of buried solvent molecules is identical in the crystal structures of the free and trypsin-bound proteins. However, the crystal structure of free Y35G BPTI displays a radically different pattern of buried water molecules, reflecting the different positions of the polypeptide segments (Figure 5b). Two water molecules are located near the replacement site, and a third lies in a position analogous to X103 in the wild-type protein.

In contrast, the trypsin-bound Y35G BPTI structure (Figure 5c) revealed a pattern of bound solvent similar to that of the free and trypsin-bound wild-type protein, augmented by additional water molecules occupying the space created by the missing Tyr35 side chain. These additional solvent molecules provide a bridge between the four molecules seen in the wild-type structure, resulting in a continuous hydrogen bonding network. Water molecules X101 and X102 hydrogen bond with the main-chain atoms of Tyr10 and Thr11, and Gly37 respectively. In the wild-type protein, the ring of Tyr35 forms π -hydrogen bonds with the backbone amide of Gly37 and the side chain of Asn44. The π -hydrogen bonds are partially replaced by a hydrogen-bond between X102 and Gly37 in trypsin-bound Y35G BPTI. X102 in trypsin-bound Y35G BPTI is exposed to the bulk solvent, forming one mouth of a nearly continuous aqueous channel with another opening at the position of X103.

2.3 Temperature factors for trypsin-bound wild-type and Y35G BPTI

Solution NMR relaxation measurements(6;16;17) have shown that the backbone of free Y35G BPTI undergoes extensive motions on the μ s-ms time scale, involving segments 11–19 and 34–41. While similar motions also occur in the wild-type protein, they are limited to the immediate vicinity of the 14-38 disulfide bond(25;6;26;27). As a measure of conformational dynamics in the complexes with trypsin, temperature factors (*B*-values) for the bound forms of Y35G and wild-type BPTI are compared in Figure 6. The shaded portions in the figure correspond to the segments previously shown by NMR spectroscopy to have enhanced mobility in the free Y35G protein. To a first approximation, temperature factors reflect positional variation of atoms over time and over the molecular ensemble in the crystal, though they are also influenced by crystal order, diffraction resolution and atom occupancy(28). Because the wild-type and mutant crystals are isomorphous and diffract x-rays to similar resolution limits, differences in temperature factor values for particular atoms are expected to be well-correlated with differences in atomic positional variation.

The overall patterns of temperature factors were quite similar for the two complexes, though the values for the Y35G complex were systematically larger than those for the complex with the wild-type protein. These differences most likely arise from a difference in the overall order

of the two crystals, as reflected by the resolution limits of 1.60 and 1.65 Å for the wild-type and mutant complexes, respectively. For backbone atoms, relatively large temperature factors were observed for atoms close to the N- and C-termini as well as for the atoms in residues 25–28, which form an exposed β -turn. Similar patterns have been reported in previous crystal structures of wild-type BPTI and variants bound to trypsin (20). In spite of the systematic shift towards larger *B*-values for the mutant inhibitor, the residue-by-residue pattern of backbone *B*-values was remarkably similar for the two proteins, suggesting that there is very little difference in main-chain flexibility once bound to trypsin.

The most notable difference in the temperature factors for the two proteins were for the side-chain atoms of Arg 20. This side chain normally forms van der Waals interactions with the ring of Tyr35, and the electron density in this region of the mutant complex indicated the presence of two side-chain conformations with roughly equal occupancy, suggesting considerable conformational freedom. Aside from this quite localized effect, however, it appears that nearly all of the flexibility observed for the free mutant protein in solution is lost upon binding to the enzyme. Although some of the apparent rigidification could be associated with formation of the crystal, the regions that are flexible in solution are well isolated from direct crystal contacts.

2.4 Thermodynamic effects of the Y35G replacement on trypsin binding

Using an enzymatic assay to distinguish free from inhibitor-bound trypsin, we previously determined the dissociation constant for the Y35G BPTI-trypsin complex to be 1.6×10^{-11} M, as compared to 6×10^{-14} M for the wild-type BPTI complex (6;14). To further compare the binding thermodynamics of the wild-type and mutant inhibitors, isothermal titration calorimetry (ITC) was used to measure directly the enthalpy and heat capacity changes of dissociation. At the concentrations used for these experiments (1–10 μ M), the binding reactions with both wild-type and Y35G BPTI were essentially irreversible, and the heat change observed for each injection corresponds to the enthalpy change for the reaction. The enthalpy change was measured as a function of temperature at pH 8 in the presence of two buffers, Tris-HCl and HEPES.

The observed molar enthalpy changes for dissociation (ΔH_d) are plotted as a function of temperature in Figures 7a (Tris) and 7b (HEPES). The enthalpy changes observed in the presence of Tris buffer were consistently larger than in HEPES, by about 3 kcal/mol for both proteins. The heats of protonation of Tris and HEPES have been reported to be –5.02 and –11.34 kcal/mol, respectively, and the differences in the observed values of ΔH_d in the two buffers could reflect the release of an average of ≈ 0.5 protons, which then bind to the buffer, upon the dissociation of the complex. At lower pH values (4–6), studies of BPTI and other standard mechanism inhibitors have indicated that approximately one proton is released upon *formation* of the complex, and it is generally believed that this reflects a decrease in the pK_a of the imidazole of trypsin residue His57 upon binding the inhibitor. At pH 8, however, His57 is expected to be fully deprotonated in both the free and inhibited enzyme and this residue is unlikely to contribute to the net release of protons under these conditions. The observed difference in ΔH_d in the two buffers could be due to a change in ionization of another site, but it is also possible that one or both of the buffers could interact directly with one of the free proteins or the complex. Regardless of its origin, the magnitude of the buffer effect was very similar for both proteins and over the range of temperatures examined, indicating that it is unlikely to influence the interpretation of the mutational effects.

For both proteins, and in each of the buffers, the observed values of ΔH_d were positive, *i.e.* favorable for binding. Under all of the conditions examined, ΔH_d was larger for the Y35G variant than for wild-type BPTI, by approximately 2–3 kcal/mol, indicating that the reduced affinity of the mutant inhibitor was due to entropic, rather than enthalpic, factors. Values of

ΔG_d and ΔS_d for the two complexes at 25 °C were calculated from the published dissociation constants and the values of ΔH_d at 25 °C (in Tris), and are summarized in Table 2. For the wild-type complex, ΔS_d was -12 cal/deg-mol. Though the dissociation of the two proteins would be expected to increase their total entropy, the negative ΔS_d can be accounted for by a decrease in solvent entropy, as discussed further below. For the Y35G complex, $\Delta S_d = 7$ cal/deg-mol, with the more positive value likely reflecting the increased flexibility of the free mutant inhibitor.

Further insights into the energetic factors distinguishing the wild-type and mutant complexes can be obtained from the changes in heat capacity for dissociation, $\Delta C_{p,d}$, *i.e.* the derivative of ΔH_d with respect to temperature. For each of the four data sets, the temperature dependence of ΔH_d was approximately linear over the range of 20 to 45 °C, and the data were analyzed assuming that $\Delta C_{p,d}$ is constant with respect to temperature. From the slopes of the lines fit to the plots in Figure 7, $\Delta C_{p,d}$ was estimated to be 410 and 350 cal/deg-mol for the wild-type BPTI complex in the Tris and HEPES buffers, respectively, and 360 and 330 cal/deg-mol for the Y35G complex in the two buffers.

On the basis of a large body of experimental data, including both thermodynamic studies of the transfer of small molecules from non-polar solvents to water and protein unfolding experiments(29;30;31;32;33), it is widely believed that the major contribution to the heat capacity change for protein structural transitions arises from presumed, but poorly understood, changes in solvation associated with an increase or decrease in accessible surface area(34). Though there is some uncertainty about the relative contributions of polar and non-polar groups, the heat capacity change for the transfer of protein fragments from non-polar environments to water is positively correlated with accessible surface area, and parameters derived from these correlations have proven moderately reliable in predicting $\Delta C_{p,d}$ for unfolding transitions and other structural changes (33;35;36). From the crystal structure of the wild-type BPTI-trypsin complex, the change in total accessible surface area for dissociation was calculated to be 1400 Å², with 870 Å² arising from non-polar groups and 530 Å² from polar groups. Using parameters derived by Murphy and Friere from transfer experiments (32), the heat capacity change predicted from the calculated change in accessible surface area is 250 cal/deg mol, while the parameters of Spolar et al.(31) predict $\Delta C_{p,d} = 200$ cal/deg-mol. These calculations suggest that a large fraction, but perhaps not all, of the observed heat capacity changes are due to solvation effects. The relatively small differences between the values of $\Delta C_{p,d}$ for the complexes with the wild-type and Y35G BPTI suggest that the overall changes in solvation are similar for the two complexes.

If the heat capacity change is assumed to arise primarily from solvation effects, the data from model compound studies can also be used to estimate the entropy change associated with the change in solvation, according to the relationship:(29)

$$\Delta S_{solv} = \Delta C_{p,d} \ln(T / T_0) \quad (1)$$

where T is the temperature of interest, and T_0 is the temperature at which ΔS_{solv} is assumed to be zero, usually taken to be 385 K. For both the wild-type and mutant complexes, the calculated values of ΔS_{solv} at 25 °C are approximately -100 cal/deg-mol, corresponding to a favorable contribution to binding of approximately 30 kcal/mol. This contribution is presumably offset by losses in translational, rotational and conformational entropy to yield the total entropy changes of -12 and 7 cal/deg-mol for the wild-type and mutant complexes. Although magnitudes of the translational and rotational entropy changes are difficult to assess (37;38; 39;40), they are unlikely to be greatly affected by the amino acid replacement. Since the replacement also appears to have minimal effects on the contributions from solvation, it is most likely that the substantial difference in ΔS_d calculated for the dissociation of the two complexes

$(\Delta S_d^{wt} - \Delta S_d^{Y35G} = -19 \text{ cal / deg} \cdot \text{mol})$ arises primarily from differences in protein conformational entropy.

3 Discussion

3.1 Structural and thermodynamic aspects of the mutant inhibitor and its interaction with trypsin

The crystallographic results presented here reveal that the structure of trypsin-bound Y35G BPTI is almost identical to that of the complex with the wild-type inhibitor, aside from the formation of a water-filled cavity in the space normally occupied by the Tyr35 side chain. The structure of the inhibitors in these complexes are also virtually identical to that of the free wild-type protein, as previously determined in multiple crystal forms(41;42) and by solution NMR (43). On the other hand, these structures differ dramatically from a crystal structure of free Y35G BPTI(18), and NMR relaxation studies indicate that in solution the trypsin binding region of the free mutant inhibitor undergoes extensive internal motions on the ms- μ s time scale(6;16;17). It thus appears that binding induces a rigidification of the mutant inhibitor.

This conclusion is also supported by the thermodynamic measurements summarized in Table 2. The total entropy change for binding is 19 cal/deg-mol less favorable for the Y35G mutant inhibitor than for the wild-type, corresponding to a free energy difference of 5.7 kcal/mol at 25 °C. Although the observed entropy changes for each of the proteins almost certainly include contributions from solvation effects, the very similar values of $\Delta C_{p,d}$ observed for the two proteins suggest that the contributions of solvation, as defined by transfer experiments with small molecules, to ΔS_d are also similar. On the other hand, it could well be that the binding of specific molecules to sites in the Y35G structure contributes to the observed differences in ΔS_d .

If the difference in ΔS_d for the two proteins is assumed to be due solely to the greater flexibility of the free mutant protein, the observed entropic penalty would correspond to a reduction in the number of accessible isoenergetic conformations by a factor of about 10^4 . An effect of this magnitude is most readily accounted for by a modest number of substructures that can independently take on multiple conformations in the free mutant protein but have fixed conformations in the complex with trypsin. It should be noted, however, that the NMR relaxation experiments indicate that the time constants for the motions of the trypsin binding regions in the free mutant protein are very similar for all of the mobile residues, an observation that we have previously interpreted as an indication that the motions are concerted. Thus, it is presently unclear exactly how the motions observed by NMR are related to the changes in conformational entropy implied by the thermodynamic measurements. One possibility is that the conformational freedom in the free mutant inhibitor that allows for extensive, but concerted, motions of the backbone also allows for additional and more independent side-chain motions.

The rigidification upon binding of the mutant protein could involve the selective binding of very rare inhibitor molecules already possessing the wild-type structure, or the final structure may form following an initial weak association of the enzyme with an inhibitor molecule in an altered conformation. These alternative models are often described as “preexisting equilibrium” and “induced fit” mechanisms, respectively, and, in some cases, can be distinguished kinetically (44;45;46). Formation of the trypsin complex with the wild-type inhibitor is known to involve at least one loosely-bound intermediate, as represented by the following scheme:

where E and I are the free enzyme and inhibitor, and (E · I) and EI are the loose and stable complexes, respectively (1). Except under conditions of very high protein concentration, the apparent kinetics of forming the stable complex are second order, with an apparent rate constant, k_{on} , that is equal to the product of the equilibrium constant, K_i , for forming (E · I) and the first-order rate constant, k_2 , for the conversion of (E · I) to EI(47;48). At neutral pH, K_i and k_2 have been measured to be approximately 10^5 M^{-1} and 10 s^{-1} , respectively, to give rise to an apparent rate constant, k_{on} , of about $10^6 \text{ s}^{-1}\text{M}^{-1}$ (48). Preliminary experiments (S.A. Beeser, personal communication) indicate that the rate of forming the stable complex with the Y35G inhibitor is approximately the same as for wild-type BPTI, indicating that the values of K_i and k_2 are either unchanged by the substitution or that changes in the two parameters compensate one another. These observations suggest that a relatively large fraction of the free mutant inhibitor molecules are able to form the initial complex with the enzyme, as described by the induced fit model, but more complete kinetic studies are necessary to define the mechanism.

Although the Y35G substitution makes the free energy change for trypsin binding approximately 3 kcal/mol less favorable, the calorimetric measurements indicate that the enthalpy change for binding is actually more favorable for the mutant inhibitor, by 2.5 kcal/mol. This effect could be due to an ability of the more flexible mutant inhibitor to form slightly more favorable attractive interactions with the enzyme, or the binding to the enzyme may be coupled to the binding of water molecules to the cavity in the mutant inhibitor, which could contribute to the more favorable enthalpy change.

3.2 Functional consequences of the Y35G replacement

Perhaps the most remarkable feature of the standard mechanism protease inhibitors is the very low rate at which they are hydrolyzed after binding to their target proteases, even though the structures of the complexes appear to position the carbonyl carbon of the scissile bond ideally for nucleophilic attack by the serine hydroxyl oxygen (Fig. 4). In this regard, BPTI represents the most extreme known example, with an estimated half time for hydrolysis of approximately 20 years(15). In addition to reducing the affinity of BPTI for trypsin, the Y35G replacement also increases the rate of hydrolysis by approximately 2,000-fold, reducing the half time to about 4 days at pH 8 (E. Zakharova and D.P. Goldenberg, unpublished results). The increased rate of cleavage observed for Y35G BPTI suggests that the restriction of particular motions within the enzyme-inhibitor complexes may be an important factor in the function of standard-mechanism inhibitors, as has been suggested by several authors previously (49;5;50;7;9).

Each step in the serine protease mechanism requires atomic motions of the substrate or the enzyme (51), and the extensive interactions formed between the two molecules could, in principle, impede any of these steps. Most of the available evidence, however, has focussed attention on the initial formation of a tetrahedral transition state and the acyl-enzyme intermediate. More than 50 crystal structures of standard-mechanism inhibitor-protease complexes have been determined, and in each the scissile bond is intact and has a nearly planar geometry (49;2;3;10). Furthermore, the chemical shifts of the carbonyl carbon in ^{13}C labeled complexes are consistent with a planar configuration (52;53). From these observations, it has generally been concluded that the normal protease mechanism is blocked before the formation of the acyl intermediate.

An alternative model, recently described as a “clogged gutter mechanism” by Radisky and Koshland (10), is that the acyl intermediate can be formed at a significant rate, but is converted back to the EI complex much more rapidly than it reacts with water to generate the hydrolyzed product. During the cleavage of a normal substrate, the polypeptide fragment with the new free amino terminus is released from the enzyme as the acyl intermediate is formed. In the protease-inhibitor complexes, however, the α -amino group is retained in the active site, thus favoring

the reverse reaction. In addition, the presence of the inhibitor likely limits entry of the water molecule necessary for the forward reaction. Both of these factors would disfavor product formation (5;9;7). This model is supported by gel electrophoresis experiments showing that a small fraction (5-10%) of chymotrypsin inhibitor 2 (CI2) molecules in complex with subtilisin form an acyl intermediate, even though the intermediate cannot be detected in crystal structures of the complex(10). Using similar electrophoresis methods, we have been unable to detect an acyl intermediate in BPTI-trypsin complexes, but it may be present at levels too low to detect in this way. A quantum mechanics/molecular mechanics simulation of the BTPI-trypsin complex indicates that the formation of the acyl intermediate is disfavored both kinetically and thermodynamically (9).

Increased rates of hydrolysis have previously been associated with enhanced flexibility in other modified forms of BPTI and other standard-mechanism inhibitors (54;7;55;8;56;16;11). While the results with the Y35G mutant are consistent with this pattern, the data presented here indicate that much of the flexibility of this mutant inhibitor is lost upon forming the complex. Thus, any remaining flexibility that facilitates hydrolysis is likely to involve quite subtle motions or, perhaps, affects states representing later steps in the catalytic reaction that are not observable in the crystal structure.

Though the crystal of the Y35G complex used for structure determination was allowed to grow for nearly a month at room temperature, compared to a half-time of cleavage of about 4 days in solution, the cleavage site appeared to be fully intact in the crystal structure. The apparent inhibition of hydrolysis in the crystal could reflect reversible resynthesis of the peptide bond, since the cleaved product presumably cannot diffuse readily. Indeed, resynthesis of the cleaved forms by incubation with the protease under appropriate conditions is a characteristic feature of the standard-mechanism inhibitors (57;58;1;59). It is also possible, however, that the crystal restricts a structural rearrangement during one of the catalytic steps. Though the motions necessary for the chemical steps are highly localized to the active site (51), displacement of the new N-terminus from the acyl intermediate may require more substantial motions.

Finally, it is important to note that the Y35G mutant inhibitor retains a level of activity that is still comparable to that of many other natural inhibitors. For example, the complexes of trypsin with soy bean trypsin inhibitor(47) and with the squash inhibitors(59) from *Cucurbita maxima*, as well as the complex of subtilisin with CI2 (60;5) all have dissociation constants in the range of 10^{-12} to 10^{-11} M and half times for hydrolysis on the order of one to ten days. The functionality of Y35G BPTI persists despite the extensive motions of the trypsin-binding region in the free inhibitor, and likely depends on rigidity imparted to the loops as a result of the intermolecular interactions formed upon binding. Prior to this study, preorganization of standard mechanism inhibitor active sites was thought to be a distinguishing feature differentiating inhibitors from substrates. The crystallographic results presented here show that protease binding can result in the formation of the stereotypical inhibitor active site loop conformation even when this structure is not stably formed in the free inhibitor. So long as enough rigidity is maintained in the complex, it appears that substantial inhibition can be achieved despite the large loss in flexibility incurred upon forming the inhibitor-protease complex.

4 Materials and Methods

4.1 Crystallography

Bovine trypsin and wild-type BPTI (aprotinin) were obtained from Sigma Chemical Co. and Roche Applied Sciences, respectively. Trypsin was further purified by affinity chromatography using immobilized soybean trypsin inhibitor and then exchanged into a buffer containing 5 mM HEPES, 5 mM CaCl_2 at pH 7.5. Aprotinin was dissolved in the same buffer solution. The

Y35G BPTI protein was produced by heterologous expression in *Escherichiacoli* and purified as described previously (61). To form complexes, wild-type or Y35G BPTI was mixed with trypsin in a 1.25:1 molar ratio and a total protein concentration of 30 mg/mL. Crystals were grown at 36 °C or room temperature using the hanging dropvapor diffusion technique. Drops contained 1.5 μ L of the protein mixture and 1.5 μ L of well solution composed of 0.2 M HEPES, 10 mM CaCl_2 , 0.02% NaN_3 , and 1.5 - 2.5 M $(\text{NH}_4)_2\text{SO}_4$.

Crystals were soaked briefly in reservoir solution plus 20% (v/v) ethylene glycol as cryoprotectant prior to freezing in liquid propane at 100 K. During data collection, crystals were maintained at 100 K in a stream of evaporated nitrogen (Oxford Cryosystems). Data were collected with use of a CCD detector and a rotating anode FR591 generator (Nonius Delft, Netherlands). Reflections were indexed, scaled and merged using the HKL suite of programs (62). Molecular replacement solutions were obtained using the cross-rotation search functions implemented with CNS (63). For the wild-type BPTI-trypsin complex, the structure was determined and refined in an *I*222 space group, which is nearly but not exactly isomorphous with the *I*222 lattice observed for this complex at room-temperature (PDB entry 2PTC; see Table 1). For the Y35G BPTI-trypsin complex, the structure was first determined and partially refined in a *C*2 space group before moving the structure into a higher-symmetry *I*222 space group.

Refinement of both trypsin-complex structures proceeded by iterative cycles of torsion angle simulated annealing, geometry and atom positional minimization, and restrained individual *B*-factor refinement using maximum likelihood target functions implemented with CNS. Each cycle of refinement was interleaved with manual adjustment and rebuilding using the program O (ref. (64)) while inspecting σ_A -weighted $2|F_o| - |F_c|$, $3|F_o| - |F_c|$ and $|F_o| - |F_c|$ difference maps. As phases improved, peaks with intensities greater than 5σ and with appropriate geometry and disposition of H-bonding donors or acceptors were assigned to solvent molecules including water molecules, sulfate ions and, in a few instances, ethylene glycol molecules. Where indicated by residual positive and negative electron density peaks, certain residues were modeled with two or three alternate conformations.

In the initial electron density maps for both complexes, positive and negative peaks surrounding trypsin residue 115 suggested that the expected L-Asn residue had been replaced with a non-standard amino acid. With D-Asp introduced at this site in the model, the side chain pointed in the direction of observed positive density but also extended into a region of negative density. However, a model of an L-isoaspartate (L-IAS) residue at this site matched the envelope of positive density quite well. In the case of the wild- type BPTI-trypsin complex, the best fit with the experimental data was obtained using a model in which residue 115 was fully occupied by an L-IAS residue, while for the Y35G BPTI-trypsin structure, this position was best fit as a 50%-50% mixture of L-IAS and L-Asp. The relative occupancies of each isomer were obtained by first modeling this residue as a glycine to remove the side chain, followed by multiple rounds of refinement including torsion angle simulated annealing. The resulting omit maps were inspected to judge whether Asn, L-IAS, or a mixture of L-IAS and L-Asp best fit the residual density. Stereochemical restraints appropriate for L-IAS were obtained from the Hetero-Compound Information Center - Uppsala (<http://xray.bmc.uu.se/hicup/>).

The atomic coordinates for both complexes have been deposited in the Protein Data Bank, with accession codes 2FTL (wild type) and 2FTM (Y35G).

4.2 Isothermal titration calorimetry

Protein samples for ITC experiments were prepared as described above and were extensively dialyzed against buffer solutions containing 10 mM CaCl_2 and either 50 mM Tris-HCl or 50 mM HEPES at 4 °C. The buffer solutions were adjusted to pH 8 at the temperature to be used

for the calorimetric measurements (20–45 °C). Trypsin and BPTI concentrations were typically about 0.3 mg/mL (0.013 mM) and 1.5 mg/mL (0.23 mM), respectively. Actual concentrations after dialysis were determined by absorbance at 280 nm using extinction coefficients of $3.6 \times 10^4 \text{ cm}^{-1} \text{ M}^{-1}$ (trypsin), $5.4 \times 10^3 \text{ cm}^{-1} \text{ M}^{-1}$ (wild-type BPTI) and $4.0 \times 10^3 \text{ cm}^{-1} \text{ M}^{-1}$ (Y35G BPTI). Samples were degassed under vacuum immediately before use.

Heats of binding were measured using a MicroCal VP-ITC titration calorimeter. For each measurement, 1.7 mL of trypsin solution was placed in the calorimeter sample cell, and 20 aliquots of 5 μL each of BPTI were injected at 200 second intervals. The total heat observed for each injection was determined by integrating the titration peaks using the Origin software provided with the instrument. Because the experiments were carried out at protein concentrations much greater than the dissociation constant, the injection heats remained approximately constant until all of the trypsin was titrated. Subsequent injections generated a much smaller heat change that was attributed to mixing. The first injection in an experiment typically gave rise to a slightly lower than average heat change, presumably because of diffusion of titrant from the needle during the pre-equilibration period, and the data from these injections were discarded. Data for the final injection for which a heat of binding was clearly detected were also discarded. The average value of the post-titration peak area was subtracted from the areas of the titration peaks to correct for the small heats of mixing, and the corrected values for each experiment were averaged. For each temperature and buffer combination, at least two independent experiments were performed.

5 Acknowledgments

We thank Dr. Bruce Yu for helpful discussions and the use of the VP-ITC microcalorimeter. This research was supported by grants GM42494 (to DPG) and GM067994 (to MPH) from the U.S. National Institutes of Health.

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Figure 1.

Backbone representations of wild-type BPTI (a and c) and the Y35G variant from crystal structures of the free proteins (a and b) and the complex of wild-type BPTI with trypsin (c). (a) Free wild-type BPTI drawn using the atomic coordinates of entry 4PTI in the Protein Data Bank. (b) Free Y35G BPTI drawn from PDB entry 8PTI. (c) Wild-type BPTI in complex with bovine trypsin (white) drawn from PDB entry 2PTC. The C_{α} and side-chain atoms of BPTI residue 35 are drawn in ball-and-stick form, as are the disulfide-bonded Cys residues of BPTI. The BPTI loop segment containing the scissile peptide bond is colored magenta. The blue spheres in panels A and B identify backbone nitrogen atoms shown by solution NMR to undergo conformational exchange on the ms-μs time scale. The images were generated using the computer programs MOLSCRIPT(65) and Raster3D(66).

Figure 2.

$|F_o^{Y35G}| - |F_o^{wt}|$ difference electron density maps illustrating the localized differences between the structures of the two complexes. (a) Negative density differences shown as red cages contoured at 5σ . (b) Positive density differences shown as blue cages. The maps were calculated from diffraction data with a resolution limit of 1.65 Å and phases calculated from the refined structure of the wild-type complex. The density difference maps are superimposed onto the wild-type structure showing inhibitor and enzyme atoms in the active site region. These images and those in Figures 4, 5 and 6 were created using the program PyMOL (<http://www.pymol.org>).

Figure 3. Average RMS deviations of backbone (solid curve) and side-chain (filled circles) atoms between the inhibitor portions of the wild-type and Y35G co-crystal structures plotted as a function of residue number.

Figure 4.

Geometry of the approach of the O_{γ} atom of trypsin residue Ser195 toward the scissile bond of Y35G BPTI. The dashed line represents the interatomic vector between the Ser195 O_{γ} and the Lys15 carbonyl carbon atoms. The angles formed between this vector and those connecting the carbonyl carbon with either the carbonyl oxygen of Lys15 or the amide nitrogen of Ala16 are also indicated, showing that the attack is perpendicular to the plane of the peptide bond. For comparison, the corresponding parameters from the crystal structure of the complex with wild-type BPTI are shown in parenthesis.

Figure 5. Internal water-binding sites in free wild-type BPTI (a), free Y35G BPTI (b) and trypsin-bound Y35G BPTI (c). The dashed lines represent hydrogen bonds involving water molecules.

Figure 6.

Average backbone and side-chain temperature factors for the inhibitor portions of the wild-type and Y35G BPTI-trypsin co-crystal structures, plotted as a function of residue number. Values for the wild-type protein are represented by solid lines and those for the Y35G variant by broken lines. The shaded regions correspond to the region of the free Y35G protein known to have enhanced flexibility, relative to the wild-type protein, in solution.

Figure 7.

Heats of dissociation (ΔH_d) for the complexes of trypsin with wild-type (filled symbols) and Y35G (open symbols) BPTI. Enthalpy changes were measured by isothermal titration calorimetry at pH 8 in the presence of 20 mM CaCl_2 and 50 mM Tris-HCl (a) or 50 mM HEPES (b). At least two measurements were made for each combination of BPTI variant, temperature and buffer. The lines represent least-squares fits to the experimental data, from which estimates of the heat capacity change, $\Delta C_{p,d}$, and ΔH_d at 25 °C were derived. The parameters for the data obtained with Tris-HCl are listed in Table 2. For the wild-type complex in HEPES, the parameters were: $\Delta H_d = 9.6 \pm 0.3$ kcal/mol, $\Delta C_{p,d} = 350 \pm 20$ cal/deg·mol. For the Y35G complex in HEPES: $\Delta H_d = 11.9 \pm 0.2$ kcal/mol, $\Delta C_{p,d} = 330 \pm 20$ cal/deg·mol.

Table 1

Data collection and refinement statistics

	2FTL (WT)	2FTM (Y35G)	2PTC	3BTK
A. Diffraction				
Temperature (K)	100	100	293	293
Space group	<i>I</i> 222	<i>I</i> 222	<i>I</i> 222	<i>I</i> 222
Unit cell (Å) <i>a</i>	74.73	74.59	75.5	75.37
<i>b</i>	81.91	81.74	84.4	84.57
<i>c</i>	124.26	123.59	122.9	122.81
Resolution (Å)	20 - 1.60	20 - 1.65	6.8 - 1.9	15 - 1.85
<i>R</i> _{crystal} (%)	5.0	4.6	5.0	6.9
Completeness (%)	99.9	99.4	70.8	99.1
B. Refinement				
Resolution (Å)	20 - 1.62	20 - 1.65	6.8 - 1.9	8 - 1.85
Bulk solvent ¹	Yes	Yes	No	No
Average <i>B</i> -value (Å ²)	17.3	18.7	24.4	25.4
<i>R</i> (%) ²	21.4	20.7	19.6	22.8
<i>R</i> _{free} (%) ²	22.7	21.9	N.A.	22.3
RMSD bonds (Å)	0.0049	0.0050	0.0215	0.012
RMSD angles (°)	1.30	1.30	2.36	1.55
C. Data/Parameter				
Unique reflections	48,719	45,459	21,630	33,142
Number of atoms	2,572	2,550	2,241	2,166
Data-to-parameter ratio ³	4.7	4.5	2.4	3.8

N.A., not available

¹ A bulk solvent model adds two parameters to the refinement, average electron density and *B*-value of regions not occupied by defined atoms, and allows inclusion of low-resolution reflections.

² *R* and *R*_{free} were calculated using structure factors and atomic coordinates deposited with the PDB and differ slightly (± 1%) from literature-reported values.

³ Data-to-parameter ratio calculated as (Unique reflections) / 4(Number of atoms).

Table 2
Dissociation Thermodynamics for BPTI-trypsin complexes at 25° C, pH 8

	wild-type	Y35G	wt – mutant
ΔG_d (kcal/mol) ^a	18±0.4	14.7±0.4	3.3
ΔH_d (kcal/mol) ^b	14.4±0.2	16.9±0.1	-2.5
ΔS_d (cal/deg·mol) ^c	-12±2	7±2	-19
$\Delta C_{p,d}$ (cal/deg·mol)	410±20	360±20	50

^a ΔG_d values were calculated from the published dissociation constants for trypsin complexes with wild-type and Y35G BPTI [?, ?]. Uncertainties were calculated assuming a 2-fold uncertainty in the measurement of K_d .

^b ΔH_d and $\Delta C_{p,d}$ and the uncertainties in these parameters were calculated from the intercept and slope, respectively, of a linear fit to the plots of ΔH_d (measured in the presence of 50 mM Tris pH, 20 mM CaCl₂) versus temperature shown in Figure 7a.

^c ΔS_d was calculated as $(\Delta H_d - \Delta G_d)/T$, and the uncertainties in this parameter were calculated from propagation of the indicated uncertainties in ΔG_d and ΔH_d .