Two crystal structures of the leupeptin–trypsin complex

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Abstract

Three-dimensional structures of trypsin with the reversible inhibitor leupeptin have been determined in two different crystal forms. The first structure was determined at 1.7 Å resolution with R -factor = 17.7% in the trigonal crystal space group $P3_121$, with unit cell dimensions of $a = b = 55.62$ Å, $c = 110.51$ Å. The second structure was determined at a resolution of 1.8 Å with R -factor = 17.5% in the orthorhombic space group $P2_12_12_1$, with unit cell dimensions of $a = 63.69$ Å, $b = 69.37$ Å, $c = 63.01$ Å. The overall protein structure is very similar in both crystal forms, with RMS difference for main-chain atoms of 0.27 Å. The leupeptin backbone forms four hydrogen bonds with trypsin and a fifth hydrogen bond interaction is mediated by a water molecule. The aldehyde carbonyl of leupeptin forms a covalent bond of 1.42 Å length with side-chain oxygen of Ser-195 in the active site. The reaction of trypsin with leupeptin proceeds through the formation of stable tetrahedral complex in which the hemiacetal oxygen atom is pointing out of the oxyanion hole and forming a hydrogen bond with His-57.

Keywords: enzyme–inhibitor complex; leupeptin; serine protease; X-ray crystallography

Leupeptin, *N*-acetyl-leucine-leucine-arginal, is a naturally occurring protease inhibitor of microbial origin. The arginal residue at the C-terminus of leupeptin is essential for its inhibitory activity. Leupeptin has broad specificity. It inhibits serine proteases, such as trypsin, thrombin, plasmin, as well as certain cysteine proteases, such as papain and cathepsin-B (Umezawa, 1977, 1982). Leupeptin and similar protease inhibitors, such as antipain and chymostatin, have a wide range of biological activities (Miura et al., 1986; Leto et al., 1990; Kennedy, 1994). These activities include the inhibition of growth of cancer cells caused by both chemical or radiation mutagenesis (Kennedy, 1993; Troll & Kennedy, 1993) and the competitive inhibition of steroid hormone binding (Brtko et al., 1993). The specific targets of leupeptin anticancer activity are poorly understood (Eto & Grubbs, 1992), and there are even less data on the steroid-binding inhibition activity.

There are several structures of protease inhibitor complexes that have been refined at high resolution. These include the cysteine protease papain with leupeptin (Schroder et al., 1993), the structures of aldehyde inhibitors with *Streptomyces griseus* protease A (SGPA) (James et al., 1980; Delbaere et al., 1985), and the proline–arginine methylketone complex with thrombin (Bode et al., 1992). The X-ray crystallographic structure of such a complex between leupeptin and trypsin is presented in here (Kinemage 1). It is the inhibitor complex and not trypsin that is interesting.

Aldehyde inhibitors such as leupeptin are promising for use as lead compounds in the design of anticancer protease inhibitors. They are mostly composed of amino acids and therefore have few metabolic degradation products that are toxic. However, they are unstable chemically, degrading in solution, and difficult to synthesize because the active aldehyde is oxidized readily to the inactive carboxylic acid. Although the general mechanism of inhibition of serine proteases by leupeptin has been established, no three-dimensional structures of serine proteases complexes with leupeptin have been done to date. Therefore, in order to aid in the process of developing better inhibitors with more specific activity, we have determined the structure of the leupeptin complex with trypsin. These are important basic data needed to design peptido-mimetic as opposed to peptide-derivative compounds (such as an arginine methylketone).

Trypsin was chosen because it is a well-studied representative of the family of serine proteases that possess a similar active site geometry, reaction mechanism, and substrate specificity (Bode & Schwager, 1975; Steitz & Shulman, 1982; Marquart et al., 1983; Bartunik et al., 1989), and because the crystals of inhibitor complexes with trypsin usually diffract to high resolution. Leupeptin binds reversibly to trypsin. The reaction with the active site serine 195 proceeds by a two-step mechanism beginning with the formation of a weakly bound complex followed by formation of an acetal bond to make a hemiacetal (Kuramochi et al., 1979; Ortiz et al., 1991). The canonical mechanism for trypsin-catalyzed peptide hydrolysis involves two steps (Stroud et al., 1975). First, an acyl-enzyme intermediate is formed. This step cleaves the substrate and releases one product. Then the acyl intermediate is hydrolyzed, resulting in both products and ac-

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tive enzyme. The formation of the acyl enzyme is thought to proceed by the formation of a tetrahedral intermediate, which is formally an amino ketal. Such ketals are not stable and preferentially lose the amino group (March, 1992). Leupeptin forms a hemiacetal instead of a ketal, because it lacks an amino-leaving group. The structure of this hemiacetal corresponds to the ketal tetrahedral intermediate. The crystal structure of trypsin with leupeptin confirms that the reaction mechanism proceeds via formation of a tetrahedral complex.

The complex of trypsin with leupeptin crystallized in two different space groups under identical conditions. Because it is not understood how much the crystal structure of a protein depends on the crystal lattice, comparison of the two structures will show how much variation occurs under optimal conditions. Although comparisons have been made of crystal structures of the same protein determined in different labs (Chamber & Stroud, 1979), it is unusual to find a comparison of the same protein in two space groups grown from the same conditions (Kishan et al., 1994).

Results and discussion

First, the common structural features for two crystal forms of trypsin-leupeptin complex will be discussed and then the two crystal structures will be compared in detail.

Details of the active site

Leupeptin is a very strong reversible trypsin inhibitor, and it is easily cocrystallized with trypsin. Leupeptin acts as a transition-state analogue. Transition-state analogues make good inhibitors because they are bound to the enzyme more tightly than the sub-

strates (Kuramochi et al., 1979). Leupeptin forms a covalent complex with trypsin and is a much better inhibitor than benzamidine or other small compounds (Kurinov & Harrison, 1994) that simply fill an active site pocket. Tight binding of leupeptin with trypsin is supported by close examination of the enzyme-inhibitor complex. The initial $2F_o - F_c$ electron density maps for both crystal forms showed continuous density in the active site pocket, which protruded along the protein surface. This density was easily fitted with a leupeptin molecule. The first cycles of structure refinement were done assuming no covalent interactions of leupeptin with trypsin. Both $2F_o - F_c$ and $F_o - F_c$ difference maps showed strong continuous electron density between aldehyde carbonyl of leupeptin and the hydroxyl group of serine 195, as shown in Figure 1. This is a direct confirmation for the formation of a covalent complex between leupeptin and trypsin. So, in the subsequent refinement, the structure data file was modified to create a covalent bond between the aldehyde carbonyl carbon of leupeptin and the oxygen of serine 195, with a bond length of 1.42 Å and force constant of 400 kcal/mol/Å², and the aldehyde carbonyl of leupeptin was set to tetrahedral coordination with a force constant of 50 kcal/mol/rad². The precision of protein X-ray crystallography at 1.8 Å resolution is not good enough to determine the above geometric values per se. A nearly tetrahedral complex is seen in the final model with the distance C_{leu}-O₁₉₅ of 1.42 Å and all angles around the aldehyde carbonyl fall within 8° of their ideal tetrahedral value of 109.5°. The geometry of this hemiacetal complex closely resembles a transition state of peptide hydrolysis (Stroud et al., 1975; Kuramochi et al., 1979).

In the active site pocket, the arginal residue of leupeptin forms a salt bridge with aspartate 189 of trypsin in the S1 subsite and forms several hydrogen bond interactions with trypsin residues (Table 1; Fig. 2; and Kinemage 1). There is one water molecule

Fig. 1. Omit $2F_o - F_c$ electron density map showing continuous density between Ser-195 and the aldehyde carbonyl of leupeptin. Leupeptin and Ser-195 were omitted for the map calculation. The map is contoured at 1.5σ .

Table 1. Summary of the leupeptin-trypsin interactions in two crystal forms

Leupeptin atoms	Trypsin atoms or water	Orthorhombic P ₂ ₁ 2 ₁ 2 ₁ (Å)	Trigonal P ₃ ₁ 2 ₁ (Å)
3-Arg-NH1	189-Asp-OD1	2.84	2.67
3-Arg-NH2	189-Asp-OD2	2.89	2.79
3-Arg-NH2	190-Ser-OG	3.00	2.95
3-Arg-NH2	349-Wat-O	3.32	3.37
3-Arg-NH1	219-Gly-O	2.81	2.82
3-Arg-NE	381-Wat-O	3.16	3.12
3-Arg-N	214-Ser-O	2.96	2.89
3-Arg-O	57-His-NE2	2.77	2.89
2-Leu-O	192-Gln-NE2	2.83	3.06
1-Leu-O	216-Gly-N	2.93	2.74
Acetyl-O	219-Gly-N	3.86	3.45

in the active site pocket that additionally stabilizes the arginal group through hydrogen bond interactions with carbonyl oxygens of Gly-216 and 219 and N ϵ of Gln-192. The backbone of leupeptin forms a two-residue stretch of antiparallel β -sheet with surface residues 214–216 of trypsin. Additional stabilization of the enzyme-inhibitor complex comes from the formation of three hydrogen bonds from the leupeptin backbone atoms to the nearest main-chain amide of Gly-216, the carbonyl oxygen of Ser-214, and side-chain amino nitrogen of Gln-192, and a weaker interaction between the oxygen of the acetyl group to main-chain amide of Gly-219 (details in Table 1). There are additional hydrogen bonds to two strongly bound water molecules. The leucine side chains of leupeptin point mainly toward the solvent in both crystal forms, so the definition of the S2 and S3 sites on the enzyme surface is not well defined. It is likely that the side chains of both leucines form weak hydrophobic contacts with trypsin atoms: the P3 leucine of leupeptin is near Leu-99, Trp-215, and Gly-216, the P2 leucine is near Leu-99 and His-57 (within the distance of 4.5 Å).

Early in the refinement of the two crystal forms, detailed examination of the electron density showed that the leucine side chains were disordered: the electron density for C $_{\gamma}$ and C $_{\delta}$ atoms was like a "3-cornered hat." There are no special constraints on the leucine side chains of leupeptin and the electron density shows the same disorder in both leucines in both crystal forms. This peculiarity suggests that all three rotamers are possible, but the simplest structure that reproduces the data uses two conformations for each crystal form. Experimental data were fitted using two conformations for the side chains and can be described roughly as a rotation around C $_{\beta}$ -C $_{\gamma}$ bond. The occupancy factors for both conformations are nearly the same and the *B*-factors for C $_{\delta}$ atoms were 32–34 Å² in the orthorhombic form and 20–21 Å² in the trigonal form. The electron density is shown in Figure 3.

Hemiacetal oxygen of leupeptin and "oxyanion hole"

Another interesting feature is the unusual position of the hemiacetal oxygen in the tetrahedral complex of leupeptin. There is a controversy concerning the orientation of the atoms of the tetrahedral complex. The oxygen atom can face the "oxyanion hole" with formation of two hydrogen bonds with the main-chain amides NH₁₉₅ and NH₁₉₃, or it can point toward the active site His-57. NMR data for the solution structure of leupeptin-trypsin complex (Ortiz et al., 1991) and crystallographic data for papain-leupeptin complex (Schroder et al., 1993) show the oxygen atom in the "oxyanion hole," although, in the latter case, it was mentioned that the high concentration of methanol could alter the observed orientation of hemiacetal oxygen. In the 1.8-Å structure of the complex between chymostatin and SGPA (James et al., 1980; Delbaere et al., 1985), both configurations of the hemiacetal oxygen occur in equal populations.

During structure refinement, no special dihedral or improper angles were imposed on the tetrahedral carbonyl carbon, and the final position of the hemiacetal oxygen was fitted to maximum electron density. In the refined trypsin-leupeptin complex, the hemiacetal oxygen is not in the oxyanion hole, but is in po-

Fig. 2. Details of the active site region of trypsin (thin lines) with bound leupeptin (thick lines). Hydrogen bond interactions are indicated by dashed lines.

Fig. 3. Alternative conformations for both leucine side chains of leupeptin. Omit $2F_o - F_c$ electron density map is contoured at 1.5σ . Leupeptin and Ser-195 were omitted for the map calculation.

sition to form a strong hydrogen bond to His-57 (distance from $H_{\epsilon 2}$ of histidine to hemiacetal oxygen of arginal is 1.86 Å; 1.82 Å in trigonal form; see also Table 1), thus stabilizing the whole structure. Only a small bump can be seen on the electron density map looking toward the main-chain amides NH_{195} and NH_{193} . This peculiarity was modeled as an alternative conformation for the hemiacetal oxygen, but the refinement gave the occupancy factor for this conformation around 26% (Fig. 4). At this resolution, this is close to the limit of data accuracy.

Differences between two crystal forms

Comparison of identical proteins for which the structure has been determined independently, or in different crystal forms, give RMS differences (RMSDs) of 0.16–0.79 Å for the backbone atoms of the core (Wlodawer et al., 1987; Zegers et al., 1994). The values usually reflect differences due to experimental errors and to molecular environment. In the case when the same protein (trypanosomal triosephosphate isomerase) has been crystallized in four different space groups, the RMSDs did not exceed 0.3 Å (Kishan et al., 1994). Examination of two trypsin structures in the orthorhombic and trigonal space groups indicates that the overall protein structure remains very similar (Kinemage 1). Overall RMSD between the two structures is small, despite the different intramolecular interactions, protein packing, and water content in the crystals (Fig. 5). RMSD for main-chain atoms is 0.27 Å, which is slightly higher than the estimated error of the coordinates (0.18 Å). The differences are mainly due to deviations in the autolysis loop (residues 145–153) and surface loop fragments (residues 88–93 and residues 165–173) (Kinemage 1). The last two regions are included in different intermolecular interactions, forming different contacts between symmetry-related molecules in the two space groups. The same value of 0.27 Å was reported as an RMSD for main-chain atoms for independently refined trypsin structures with different inhibitors (Chamber & Stroud, 1979). Although the active site of trypsin is accessible to solvent in the orthorhombic crystal, and the intermolecular contacts are further from it than in the trigonal crystal, the geometry of the active site region is nearly identical in both crystal forms (Kinemage 1). RMSD for main-chain atoms near the active site is only 0.13 Å, which is mainly due to errors in the experimental data and uncertainty

in structure refinement (for details, see the Materials and methods). The leupeptin backbone also appears to be rather rigid when bound to trypsin: RMSD for main-chain atoms is 0.16 Å. The active sites of two trypsin structures are compared in Figure 6.

The two structures also differ in the water content of the unit cell: 56% solvent in orthorhombic and 37% in trigonal unit cell. There is somewhat different thermal behavior of the bound waters. Both structures were refined with the same number of ordered water molecules. Many water molecules occupy the same positions in both crystal forms, but the water molecules in the orthorhombic form have a higher average thermal factor (40.5 Å^2) than the waters in the trigonal form (36.4 Å^2). When there is more room for water, diffusion and exchange with disordered bulk solvent will be higher. Solvent content and different protein packing density are probably responsible for the differences in protein average B -factor (22.2 Å^2 in orthorhombic versus 19.6 Å^2 in trigonal form). There are more disordered, long side-chain residues in the orthorhombic form than in the trigonal form. The variation of B -factor along the protein chain is highly correlated in two crystal forms, as shown in Figure 7.

Conclusions

The results described here clearly indicate that the reaction of trypsin with leupeptin in two different crystal forms proceeds through the formation of a stable tetrahedral hemiacetal complex with the catalytic serine residue of trypsin, in which the hemiacetal oxygen atom is pointing out of the oxyanion hole to form a hydrogen bond with His-57. Both leucine side chains of leupeptin are partially disordered, and do not make highly specific interactions with trypsin, thus explaining broad specificity of leupeptin toward serine and cysteine proteases. This apparent lack of specific interaction is not surprising, because trypsin is not very specific in its selection of substrate. Most of the affinity of the inhibitor is from other interactions, including the arginine salt bridge in the active site binding pocket, the hemiacetal covalent bond, and the hydrogen bonding of peptide backbone of the leupeptin.

Experiments with leupeptin analogues (I.V. Kurinov & R.W. Harrison, unpubl. data) suggest strongly that almost all of the affinity is due to the hemiacetal and the arginine binding pocket.

Fig. 4. Refined structure of covalently bound leupeptin to trypsin (from the tetragonal crystal form), showing the orientation of hemiacetal oxygen toward His-57 (A) and a minor alternative conformation toward "oxyanion hole" (B). $2F_o - F_c$ electron density map is contoured at 1.5σ . Leupeptin and Ser-195 were omitted for the map calculation.

For example, agmatine-leucine-leucine, which lacks the hemiacetal, is not an inhibitor. The leucines simply may be hydrophobic place-holders for the peptide backbone. This suggests that Leu-Leu is a useful default sequence for protease inhibitor design.

Materials and methods

Crystallization and data collection

Bovine trypsin (EC 3.4.21.4; lot 62H8090) and leupeptin (55123-66-5) were purchased from Sigma Chemical Co. and were used

without additional purification. Crystals of trypsin-leupeptin complexes were obtained by vapor diffusion either using hanging or sitting drop experiments with 24–26% PEG 8000, 0.02 M inhibitor, and 0.2 M $(\text{NH}_4)_2\text{SO}_4$ (50 mM Tris-HCl buffer, pH 8) at room temperature. Protein concentration was 30–40 mg/mL. Trypsin-leupeptin crystals were grown in two space groups: $\text{P}2_12_12_1$ and $\text{P}3_12_1$. Crystals with the space group $\text{P}2_12_12_1$ appeared mainly in the hanging drop experiments, whereas trigonal crystals appeared in the sitting drops. Otherwise, the crystallization conditions were identical. Unit cell parameters and details of data collection are presented in Table 2. Diffraction data were collected on an R-Axis II imaging plate mounted

Fig. 5. RMSDs (in Å) between two trypsin structures in orthorhombic and trigonal space groups. Values were averaged for main-chain atoms, after a best fit was performed.

on an R-AXIS Rigaku2 RU-200 rotating anode generator (50 kV, 100 mA; graphite monochromated $\text{CuK}\alpha$ radiation; 0.5-mm collimator). Data were measured to 1.8–1.7 Å resolution (completeness of the data in the last shell, 70–80%) and processed using the program package PROCESS (Higashi, 1992). The data of the trigonal crystal had a relatively high *R*-merge factor (9.9%), but the data also had high multiplicity and yielded very good electron density maps.

Structure determination and refinement

The correct orientation of trypsin molecule in both space groups was determined easily by using the molecular replacement technique from X-PLOR (Brünger, 1992). The starting model for structure refinement was our trypsin structure with a small inhibitor—phenyl-cyclopropylamine (Kurinov & Harrison, 1994; access code pdb1tnl.ent). Conventional rotational search in Patterson space yielded a single significant peak 7σ above the mean of the map for $P3_121$ space group. The next peak was

Fig. 7. Averaged isotropic temperature factors ($\text{\AA}^2$) for all protein atoms. A: Trigonal form. B: Orthorhombic form.

3.5σ above the mean of the map. The PC-refinement (Brünger, 1990) of the highest peaks of the cross-rotation function gave refined rotation angles of $\alpha = 180.24^\circ$, $\beta = 16.622^\circ$, $\gamma = 120.05^\circ$. These angles from the rotational search were then used in the translational function. A single peak in translational search was 11.7σ above the mean of the map. The same procedure was applied for $P2_12_12_1$ space group (single peak 6σ above the mean of the map). The molecular replacement solution was initially refined by rigid-body minimization followed by slow-cooling annealing with restrained refinement of individual *B*-factors using the X-PLOR package. All data with $I/\sigma > 2$ with a low-resolution limit of 8 Å were used for structure refinement. Nonpolar hydrogens were implicitly included in their associated heavy atoms;

Fig. 6. Superposition of orthorhombic (thin line) and trigonal (thick line) structures of trypsin in the active site region.

Table 2. Details of data collection and refinement

	Orthorhombic $P2_12_12_1$	Trigonal $P3_121$
Unit cell (Å)	63.686 69.373 63.013	55.615 55.615 110.510
Resolution (Å)	1.8	1.7
Total number of observed reflections ($\sigma > 1$)	78,707	64,660
Completeness of data (%)	88.8	92.5
<i>R</i> -merge (%)	4.40	9.89
Number of independent reflections	24,285	22,728
Number of reflections used for the refinement ($\sigma > 2$)	23,223	20,553
<i>R</i> -factor	0.175	0.177
<i>R</i> -free	0.207	0.218
Number of water molecules	170	169
Protein <i>B</i> -factor ($\text{\AA}^2$)	22.2	19.6

polar and hydrogen bonding hydrogen atoms were explicitly included. Molecular geometry was taken from the *param19x.pro* and *toph19x.pro* sets. A total of 170 water molecules were included in both model structures. $2F_o - F_c$ and $F_o - F_c$ difference maps were calculated and were used to guide manual fitting of leupeptin, structure rebuilding, and searching for alternative conformations, using FRODO (Jones, 1985) and CHAIN (Sack, 1988). Leucine side chains of leupeptin in both structures were refined in two conformations with nearly equal occupancies. At the end of the refinement, the *R*-factor was ~18%, with RMSDs from ideal values of 0.013 Å for bonds and 2.6° for angles. The qualities of the stereochemistry of final protein models were assessed with the PROCHECK package (Laskowski et al., 1993). In both structures, all residues fall within the most favored and allowed regions; no residues are in the generously allowed or disallowed regions. The overall error in the coordinates was estimated using σ_A plots (Read, 1986); they indicate an RMSD error of 0.18 Å. All procedures during data collection and processing and structure refinement were identical for two different crystal forms, which made it easy to compare the final structures. As a better guide to the quality of the structure, a free *R*-factor was monitored during the course of refinement. The final value of free *R*-factor was no more than 5% higher the overall *R*-factor. Atomic coordinates have been deposited with the Protein Data Bank (access code 1JRS and 1JRT).

Enzyme assay

The inhibition constant for trypsin with leupeptin was measured by a spectrophotometric assay of enzymatic cleavage of N-benzoyl-L-arginine-*p*-nitroanilide. This was done to ensure that the active inhibitor and enzyme were used. The rate of change of absorption at 410 nm from release of nitroaniline during the reaction was measured. The buffer was 0.05 M Tris-HCl at pH 8.0 with 10 mM CaCl₂ and the reaction temperature was 20 °C. The concentration of substrate was varied for a given concentration of leupeptin. The *K_i* was estimated in a standard procedure from double inverse plot to be 0.4 μM. For comparison, *K_i* for well-known trypsin inhibitor, benzamidine, in the same condition is 0.1 mM.

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