A 2.3 Å resolution structure of chymosin complexed with a reduced bond inhibitor shows that the active site β-hairpin flap is rearranged when compared with the native crystal structure

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Introduction

Calf chymosin, an aspartic proteinase used for many centuries in the manufacture of cheese, is a bilobal neonatal gastric proteinase of molecular weight ~36 kDa consisting of 323 residues in its active form (Foltmann, 1970). The two lobes have a similar fold and are related by a pseudo twofold axis that runs perpendicular to the 25 Å substrate binding cleft (Tang, 1977). A catalytic aspartate in an Asp–Thr–Gly–Thr sequence is situated in a topologically equivalent position on each lobe of the enzyme.

Chymosin is secreted as an inactive precursor (prochymosin) and is activated at acidic pH by the proteolytic cleavage of the 42 residues of the N-terminal propeptide (Pedersen et al., 1975). The main physiological function of chymosin is to cleave milk protein κ-casein, which acts as a stabilizer of the micelle (Jollès et al., 1968), at an exposed Phe–Met bond. At the pH of milk (pH 6.6) chymosin cleaves the Phe105–Met106 peptide bond releasing the C-terminal peptide residues 106–169 known as glyco-macropeptide (GMP) which, in the presence of Ca2+ ions, results in the coagulation of whole milk micelles, leading to clotting (Raap et al., 1983). Using circular dichroism and computer modelling techniques, it has been postulated that the κ-casein adopts an extended conformation in the region of the 105–106 bond and so could be easily accommodated within the substrate binding cleft of chymosin (Pedersen, 1977).

Chymosin is moderately specific to small substrates, as shown by its action on the B-chain of oxidized insulin (Pedersen and Foltmann, 1975); this implies that there exist interactions with the κ-casein substrate other than those within the active site cleft, which would account for the increased specificity. It is possible that a helical region of chymosin (residues 248–255, pepsin numbering) may form a recognition surface for the micelle system, although this has yet to be demonstrated.

The specificity of chymosin must be a consequence of the structure of the substrate binding pockets $S_4 \rightarrow S_3$ (Shechter and Berger, 1967; Lunney et al., 1993), which have been identified by analogy with other high resolution mammalian, fungal and retroviral aspartic proteinase inhibitor complexes, such as renin (Dhanaraj et al., 1992), pepsin (Chen et al., 1992), penicillopepsin (James et al., 1982), rhizopusspepsin (Parris et al., 1992; Suguna et al., 1992), endothiapsin (Veerapandian et al., 1990; Lunney et al., 1993) and HIV-1 protease (Miller et al., 1989; Priestle et al., 1995). The $S_1$ and $S_2$ subsites form well defined but continuous pockets, accommodating hydrophobic residues; in chymosin the $S_1$ pocket is specific for large hydrophobic residues such as Phe. Residue Val111 in chymosin (pepsin numbering) was hypothesised to play a role in substrate recognition, lying as it does on the junction of the $S_1$ and $S_2$ pockets (Strop et al., 1990). The cloning and expression of the enzyme, together with knowledge of its three-dimensional structure, now provide the opportunity of protein engineering new specificities for other peptide substrates that may have commercial applications in protein processing in the food or feed processing industries.

In designing such protein engineering experiments we need a crystal structure of the native and the inhibitor complexed enzyme. Surprisingly the native crystal structure (Gilliland et al., 1990; Newman et al., 1991) showed that the active site differed radically from that of other closely related mammalian aspartic proteinases, such as pepsin and renin. Indeed the extended β-hairpin known as ‘the flap’ has a similar extended conformation in all other aspartic proteinases for which there are crystal structures available. In the crystal structure of native chymosin the mainchain of the flap differs from this conformation and the conserved tyrosine (Tyr75) (pepsin numbering) is flipped by 180° around the CB-Cy bond so that
it occludes the space that would be expected to be occupied by the inhibitor or substrate at P1; this movement and the consequences for self-inhibitory behaviour of chymosin were discussed by Andreeva et al. (1992). Furthermore all previous efforts to crystallize chymosin with inhibitors led to orthorhombic, inhibitor-free crystals identical to the native. These results posed two questions. Is the conformation observed in the native crystals characteristic of the native enzyme? Is the conformation of the enzyme in its complex with an inhibitor or substrate similar to that of other aspartic proteinases?

Recently, as a result of a systematic attempt to crystallize a series of chymosin inhibitor complexes, we obtained a novel rhombohedral crystal form of triangular prisms with the iodine-containing, reduced bond renin inhibitor (CP-113972). This inhibitor-containing inhibitor was the only synthetic inhibitor that produced inhibitor complex crystals. In this paper we describe the X-ray analysis at 2.3 Å resolution of these crystals. We describe the conformation of the inhibitor which lies in pockets S4-S1. We compare the structure of the chymosin inhibitor complex with complexes of renin, pepisin and various fungal aspartic proteinases and show that the chymosin inhibitor complex is very similar. We compare the conformations of chymosin in the uncomplexed and complexed forms, and we discuss the nature and possible cause of the conformational change.

Materials and methods

Synthesis of CP-113972
Proton NMR spectra were obtained on a Varian Unity 400 spectrometer at 23°C. Liquid secondary ion mass spectra (LSIMS) were obtained on a VG70-250-S spectrometer using a liquid matrix consisting of 3:1 dithiothreitol/dithioerythritol. Microanalyses were performed by Schwarzkopf Microanalytical Laboratory (Woodsind, NY). Chromatographic purification was performed using silica gel (30 µM) eluted with ethyl acetate-hexanes. Each substance was homogenous by thin layer chromatography and 1H NMR.

Preparation of (2R,3S)-isopropyl 3-[N-(t-butoxycarbonyl)-L-phenylalanyl-S-methyl-L-cysteiny1]-4-cyclohexyl-2-hydroxybutanoate (3)

Boc derivative 3 (0.95 g, 1.1 mM) was deprotected by the procedure described for the preparation of compound 2, except that the crude product was titrated with hot acetonitrile and dried, giving a colourless solid (685 mg, 81%); HPLC (Rainin Microsorb C-18, 250×4 mm, 1.0 ml/min, 60/40 acetonitrile/pH 2.1 0.1M KH2PO4 buffer) 5.1 min (95%); 1H NMR (400 MHz, DMSO-δ6) δ 8.68 (d, 1H, J = 8.5 Hz), 8.39 (d, 1H, J = 8.3 Hz), 7.70 (d, 1H, J = 7.4 Hz), 7.57 (d, 2H, J = 8.3 Hz), 7.06 (d, 2H, J = 8.3 Hz), 5.33 (d, 1H, J = 6.2 Hz), 4.58 (M, 1H), 4.43 (M, 1H), 4.17 (M, 1H), 4.03 (M, 1H), 3.96 (dd, 1H, J = 5, 14 Hz), 2.68 (dd, 1H, J = 4, 10 Hz), 2.50 (dd, 1H, J = 9, 14 Hz), 2.23 (m, 2H), 2.04 (s, 3H), 1.84-1.70 (m, 4H), 1.62-1.50 (m, 4H), 1.35-1.28 (m, 2H), 1.23-1.00 (m, 4H). Anal. (C31H47N4O8SI.HCl) C, H, N.

Data collection and reduction
Data were collected at the SRS Daresbury (station 9.5) using a wavelength of 0.88 Å on a graphite monochromator to a resolution of 2.3 Å. 74% of data were collected on image plates. The data were indexed in spacegroup R32 with unit cell dimensions of a = b = 132.8 Å, c = 82.0 Å and processed using the MOSFLM (Leslie, 1993) suite of programs. The Rmerge value for fully recorded observations was 0.095. The 62 777 observations of I > σ(I) were reduced to 12 125 independent reflections (11 348 acentric and 777 centric).
The crystal structure of a chymosin inhibitor complex

The inhibitor was clearly identifiable, especially for the sulphur atom in P$_2$ and the iodine atom in P$_4$, which appeared as 4σ and 5σ peaks in the map. The inhibitor was built into the density in an extended conformation, although density for the P$_4$ Pro residue was initially ambiguous. Two further rounds of restrained refinement and model building were performed, after which the final R-factor for 11 988 reflections between 2.3 and 10 Å with I ≥ σ(I) is 0.19.

Results and discussion

The chymosin inhibitor complex crystallized in a new space-group R32, cell dimensions a = b = 132.8 Å, c = 82.0 Å, with one molecule in the asymmetric unit and a solvent content of approximately 34%. This contrasts with the native enzyme, which crystallized in the spacegroup I222, cell dimensions a = 79.7 Å, b = 113.8 Å, c = 72.8 Å, with one molecule in the asymmetric unit and approximately 50% solvent content (Newman et al., 1991). Molecular replacement and least-squares refinement led to a structure with good geometry and a final crystallographic R-factor of 0.19. The final electron density is illustrated in Figure 1a. The flap region 70–83 has reasonable electron density for all residues of the loop, while other surface loops such as 156–163, 239–245 and 289–291 still show high temperature factors in the complex, with breaks in density for the loop 289–291. In hexagonal porcine pepsin (Cooper et al., 1990) and native chymosin (Newman et al., 1991) these residues are also disordered, although they have crystallized in a different spacegroup. This suggests that the disorder is due to conformational variability rather than a consequence of the crystal packing. The disorder in the region 239–245 may be a consequence of sequence differences in this region resulting from a mixture of chymosin A, B and C (Foltmann, 1970).

The final model was aligned with the uncomplexed structure and rigid body shifts identified using the program X5S (SåI, unpublished program); as shown in Table II. The tertiary structure is characteristic of previously solved structures of aspartic proteinases; r.m.s. deviation comparisons with other inhibitor complexes are given in Table III.

Aspartic proteinases show a rigid body movement of residues 190–302 (pepsin numbering) when complexed with inhibitors and even in differing crystallographic environments (SåI et al., 1989, 1992). When compared with the pepsin inhibitor complex (pdb:4pep; Chen et al., 1992) where the domain movement is very large it is noticeable that there is a smaller domain movement upon complexation for the chymosin complex, which accounts for the poor degree of similarity between chymosin and pepsin inhibitor complexes. Although both complexes show the inhibitor bound in an extended conformation, the inhibitor in the chymosin complex occupies a greater volume of space within the active site cleft than the inhibitor in the pepsin complex; hence there is no requirement of the protein to undergo large domain movements to minimize the volume of unoccupied space. Most of the differences in the positions of residues in the active site cleft result from local conformational changes due to the presence of the inhibitor, with the largest being that of the β-hairpin over the active site (Figure 1b).

Inhibitor–protein contacts that define the specificity pockets $S_1$→$S_4$

This is the first chymosin inhibitor complex to be reported, although many attempts have been made to co-crystallize
various inhibitors with chymosin. The inhibitor lies in an extended conformation within the binding cleft making numerous electrostatic and hydrophobic interactions as in other aspartic proteinase complexes (Figure 1c). The catalytic aspartates are found in the expected coplanar conformation with the OH of the inhibitor lying in the plane of Asp32 and Asp215. A network of hydrogen bonds is formed around these essential residues, known as the 'fireman's grip' (Pearl and Blundell, 1984), which holds the aspartates in this rigid conformation. In inhibitor complexes of other aspartic proteinases (Dhanaraj et al., 1992) the Asp215 is usually found closer to the OH of the inhibitor (3.4 Å, 2.9 Å respectively). The extra hydrogen bond available to Asp215 is made to the carbonyl oxygen of Gly34 and the Oδ1 of Asp215 are within hydrogen bonding distance of the oxygen of the O=C=H moiety of the inhibitor (3.4 Å, 2.9 Å respectively). The extra hydrogen bond available to Asp215 in the chymosin inhibitor complex may account for this difference (Figure 1d).

The flap region 71–81 is rearranged when compared with that of the native, accommodating the large cyclohexyl ring of P1. Gly76 makes a hydrogen bond between its nitrogen to the OG oxygen in the inhibitor (3.5 Å) which, along with the hydrogen bond from Trp39 to Tyr75, appears to stabilize the flap in this closed conformation.

The S1′ pocket lies between the tip of the flap (residues 70–76) and the active site (residues 30–34 and 215–218), with contributions from residues 112–120; it is predominantly non-polar and accommodates the isopropyl ester moiety (O=C=H_3). The cyclohexyl ring at P1 occupies the S1′ pocket and is surrounded by the aromatic rings of Trp39, Tyr75, Phe112, Phe117 and the aliphatic sidechains of Ile120 and Leu30. The cyclohexyl ring also contacts the para-iodo-phenylalanine (pIPhe) at P3, and thus is totally enclosed within a hydrophobic pocket, suggesting that this pocket is specific for a large, hydrophobic residue at P1.

The specificity pocket S2 is again comprised mainly by non-polar residues, although Thr77 and Thr218 make stabilizing contributions to the Sγ and N of P2. This pocket is much more open around the S-methyl cysteine (SMC) of P2 and could easily accommodate a larger residue, such as His, Phe or Met. P2 contributes to some of the hydrophobic packing around the O=C=H moieties at the C-terminus of the peptide inhibitor; which partially occupies S1′.

S3 is a large pocket, which accommodates the pIPhe P3 residue. The main chain of P1 makes contact with Ser219 (O–219 N:2.8 Å, N–219 O:2.8 Å). The pocket is not very tight around P3 allowing for a degree of movement of this residue; this may account for the incomplete pIPhe ring density in the final maps.

The S4 specificity pocket lies towards the end of the binding cleft, so that P4 Pro makes few interactions with protein atoms, although there are several well-ordered water molecules in this region. The P4 Pro appears to have a large degree of freedom for rotation around the peptide bond with P3, leading

### Table II. Rigid body shifts of the inhibitor complex with respect to the native enzyme

<table>
<thead>
<tr>
<th>Structure</th>
<th>Rotation (°)</th>
<th>Translation (Å)</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-terminal domain (~2 to 189) (excluding the active site flap)</td>
<td>0.95</td>
<td>0.06</td>
</tr>
<tr>
<td>Active site flap (71–81) (excluding helix 248–255)</td>
<td>27.13</td>
<td>0.11</td>
</tr>
<tr>
<td>C-terminal domain (190–322) (excluding helix 248–255)</td>
<td>2.75</td>
<td>0.02</td>
</tr>
<tr>
<td>N-terminal lobe (303–327)</td>
<td>0.96</td>
<td>0.25</td>
</tr>
<tr>
<td>Helix 248–255</td>
<td>1.67</td>
<td>0.39</td>
</tr>
</tbody>
</table>

### Table III. Structural alignment of chymosin–inhibitor complex with other aspartic proteinases

<table>
<thead>
<tr>
<th>Protein</th>
<th>R.m.s. (Å)</th>
<th>Average difference (Å)</th>
<th>No. of structural equivalences</th>
<th>Poorly aligned regions (pepsin numbering)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Recombinant human renin complex 1</td>
<td>1.06</td>
<td>0.96</td>
<td>242</td>
<td>144–147,156–162,233–254,278–282</td>
</tr>
<tr>
<td>Mouse submaxillary renin complex 2</td>
<td>1.08</td>
<td>0.98</td>
<td>264</td>
<td>142–147,187–204,233–244,280,289–299</td>
</tr>
<tr>
<td>Porcine pepsin complex 3</td>
<td>0.90</td>
<td>0.79</td>
<td>292</td>
<td>56–159,236–244,275–280,289–297</td>
</tr>
<tr>
<td>Endothia parasitica complex 4</td>
<td>0.90</td>
<td>0.79</td>
<td>198</td>
<td>7–10,46–52,156–161,183–188,194–211,222–244,249–282,286–297,316–319</td>
</tr>
<tr>
<td>Bovine chymosin 6</td>
<td>0.69</td>
<td>0.61</td>
<td>303</td>
<td>74–77,156–161,289–297</td>
</tr>
<tr>
<td>Human recombinant renin 7</td>
<td>0.97</td>
<td>0.86</td>
<td>272</td>
<td>144–147,156–162,199–204</td>
</tr>
<tr>
<td>Porcine Pepsin 8</td>
<td>0.91</td>
<td>0.81</td>
<td>302</td>
<td>40–24,241–276–282,289–297</td>
</tr>
<tr>
<td>Endothia parasitica 9</td>
<td>0.98</td>
<td>0.82</td>
<td>195</td>
<td>156–160,290–297</td>
</tr>
<tr>
<td>Rhizopus chinensis 10</td>
<td>1.45</td>
<td>1.16</td>
<td>261</td>
<td>109–112,156–161,185–188,200–203,238–244,249–254,274–281</td>
</tr>
</tbody>
</table>

1pdb:1rme (Raheul et al., 1991); 2pdb:1smr (Dealwis et al., 1994); 3pdb:1psa (Chen et al., 1992); 4pdb:2er7 (Veerapandian et al., 1990); 5pdb:4apr (Suguna et al., 1992); 6pdb:4cms (Newman et al., 1991); 7pdb:1smr (Sielecki et al., 1992); 8pdb:5pep (Cooper et al., 1990); 9pdb:4ape (Pearl and Blundell, 1984); 10pdb:2apr (Suguna et al., 1987).

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Fig 1. (a) A figure showing the electron density for the bound inhibitor CP-113972 and the flap (residues 71–80) in the closed conformation (contoured at 1.2σ). (b) A figure comparing the conformation of the flap (residues 71–80) in chymosin/CP-113972 complex and native chymosin (Newman et al., 1991) (light blue). Tyr75 rotates by approximately 180° around the Cα–Cβ bond. (c) A figure showing the superposition of CP-113972 with previously determined inhibitors of renin (Tong et al., 1995; Dhanaraj et al., 1992). CH-66 is shown in blue, CGP 38’960 is shown in green and BILA 980 is shown in red. (d) A figure indicating the hydrogen bonds made between CP-113972 and residues of the flap and the active site of chymosin.
to relatively poor density. Several residues make contributions to two pockets simultaneously; for example, Val111 makes contributions to specificity pockets S1 and S3 and has already been the subject of site-directed mutagenesis studies, showing it to have an effect on the $k_{cat}$ of this enzyme (Strop et al., 1990). Ile300 lies between the sulphur of the S-methyl cysteine (SMC) $P_2$ and the isopropyl ester moiety of the $P_1$ norstatine (Nor), which partially fills the $S_1'$ pocket, and may play a part in keeping these two regions apart, producing the extended conformation in the $P_1'$–$P_2$ region.

Conformational changes within the binding cleft

The co-ordinates of the uncomplexed enzyme (Newman et al., 1991) were aligned with the complexed enzyme using MNYFIT (Sutcliffe et al., 1987), omitting the loop region in both enzymes, to a r.m.s. deviation of 0.7 Å. The same algorithm was then used to align only the loop regions of both the uncomplexed and complexed enzymes.

In the region of the specificity pocket $S_1$, the flap (residues 71–81) is rotated by approximately 27° with respect to the uncomplexed enzyme co-ordinates and displaced by a maximum of 4 Å at the tip of the loop, closing down onto the bound inhibitor. There is an associated difference in the neighbouring strand 110–115. The main chain conformational changes in the flap (residues 71–81) are essentially localized to the region 73–78 with Gly76 and Gly78 undergoing the largest changes in $(\Phi, \Psi)$ of (157°,126°) and (179°,22°) respectively. There are few significant differences within the pocket, although the ring of Phe112 moves in towards the cyclohexyl ring of $P_1$ by 1 Å to give closer packing. The water molecule, which in the native structure sits in the plane of the two catalytic aspartates, is displaced by the hydroxyl group of $P_1$, which makes an almost identical network of hydrogen bonds with surrounding main chain and side chain atoms.

The flap movement of 71–81 allows more favourable contacts between Gly76, Thr77 and $P_2$. Ser219 undergoes a change in its sidechain orientation such that the Oy is brought 0.5 Å closer to the $S_1'$ of $P_2$, although the distance is too great for a hydrogen bond; this change in orientation may be due to either a long-range electrostatic interaction or local changes in side chain orientation due to the presence of the bound inhibitor. The hydrogen bond from Ser219 Oy to the $P_1$ peptide nitrogen, seen in other aspartic proteinase inhibitor complexes (Dhanaraj et al., 1992), is retained.

The terminal methyl group of $P_2$ occupies the same position in space as that of a well-ordered water molecule in the uncomplexed enzyme. The displacement of this water molecule allows the C$\gamma$ of Ile30 to move in towards the CH$_2$ moiety of $P_2$ by 0.5 Å, providing a more favourable van der Waals interaction. A movement of 1 Å for Gln287 may be a consequence of inhibitor–protein interactions in the region around $P_2$, but, alternatively, it may be a result of a conformational change in the region 289–297 due to crystal contacts in this region.

The flap provides good van der Waals contacts for the $P_2$/$P_1$ peptide bond, although only Thr77 is close enough to form hydrogen bonds with the inhibitor. The most noticeable difference is the reorientation of the side chain of Ser12, which brings the Oy 2 Å closer to the iodine of $P_1$. Val111, which makes a large contribution both to this pocket and to $P_1$, is displaced by 1.5 Å to bring the carboxylate oxygen closer to the iodine and Gln13 shifts by about 0.5 Å to bring the Nε closer to the iodine. These two differences contribute to the main electrostatic interactions around the iodine of $P_3$, with the remainder provided by the N of Tyr114. Ala115 and Phe117 are displaced by 1 and 0.5 Å respectively, away from $P_2$, providing space for the large $P_1$ residue. The shift of residues Ser12, as mentioned above, of Gln287 towards $P_2$ and Lys220 provides more space for the $P_4$ residue in this spacious pocket.

Clearly the most important conformational differences between native and complexed chymosin come from the repositioning of the flap region over the active site. In the uncomplexed crystal form the flap region is involved in intermolecular contacts, possibly stabilizing the observed conformation. In the uncomplexed crystal form the tip of the flap, Thr77, packs onto Ala147 (Thr77 Cβ–Val1 Cγ1: 3.1 Å) and Val1 (Thr77 Cγ1–Ala147 Cβ: 3.0 Å) of symmetry related molecules. The contacts between Thr77 and hydrophobic residues of crystallographically related molecules provide stabilization to Thr77 in this position that is not available in solution. Ser79 Cβ is within 3.6 Å of Pro172 Cγ and also provides good van der Waals contacts between the flap and symmetry related molecules. In the crystals of the inhibitor complex the flap region is far from any intermolecular interactions.

Comparison with a renin–inhibitor complex

The inhibitor in the chymosin–inhibitor complex adopts an extended conformation similar to that found in other aspartic proteinase inhibitor complexes. An alignment of CP-113972 is given with a variety of other aspartic proteinase inhibitors in Figure 1d.

There are two renin–inhibitor complexes available in the Protein Data Bank: 1smr (Dhanaraj et al., 1992; Dealwis et al., 1994), a complex of mouse submaxillary renin with a decapeptide portion of angiotensin (CH-66) and Irre (Raheul et al., 1991; Dhanaraj et al., 1992), a complex of human recombinant renin with CGP 38’560, an artificial substrate analogue. Although renin and chymosin share only 38% sequence identity, both 1smr and 1rne superpose well with the chymosin–inhibitor complex. With a cut-off of 3.5 Å for structurally equivalent residues, the program MNYFIT (Sutcliffe et al., 1987) found 261 and 264 equivalent residues with chymosin from 1smr and 1rne, respectively, displaying a r.m.s. Cα difference of 1.0 Å in each case. However the two renin structures differ with respect to chymosin in the loop regions 246–252 and 156–161, which could be attributed to differing crystal environments, and an insertion in the loop region at 288–292. Because of the similarity between the two renin structures, we have only made detailed comparisons between the chymosin–inhibitor complex and the human recombinant renin–inhibitor complex.

The loop regions 71–81 are found in the same conformation, closed down upon the bound inhibitor, with Tyr75 making a hydrogen bond to Trp39, enclosing the cyclohexyl ring of $P_1$. In both complexes a hydrogen bond from the main chain nitrogen of 76 (Ser in renin, Gly in chymosin) to an oxygen atom in the inhibitor further stabilize the loop. The remaining differences in the $S_1$ pocket are the replacement of Ile30 with Val and Val120 with Ile; the residues differ only by a $\text{CH}_2$ group and are both hydrophobic. In the chymosin inhibitor complex, Asp215(Oβ1) and Gly34(O) pick up additional hydrogen bonds (2.9 Å, 3.4 Å) with the inhibitor $P_1$ main chain oxygen (part of the blocking group); this oxygen is not present in the renin inhibitor CGP 38’560. In both models the
The crystal structure of a chymosin inhibitor complex

rest of this pocket is made up of mainchain contributions from 32→35, 217→219 and hydrophobic contributions from Tyr75, Phe112 and Phe117.

The S2 pockets are again similar, although some sequence differences give rise to a slightly larger pocket in renin, with alanines replacing Ile300 and Thr218, maintaining hydrophobic contacts, and Ser replacing Val222. In the human renin inhibitor complex the P2 histidine Ne1 makes a weak hydrogen bond to the Oγ of Ser222 (3.2 Å). The mutation Ser to Val makes this pocket more suitable for the Met-like P2 SMC of the chymosin inhibitor. The remainder of the pocket is made from mainchain contributions from 213→218. The 288→292 loop in chymosin is a shorter version of the equivalent loop in renin, in which it folds over the active site along with the flap.

S3 is almost identical in the two enzyme species, the only differences being that renin has a Leu for Tyr114 and a Pro for Val111; both substitutions conserving the hydrophobic nature of the pocket. As with the chymosin complex the renin complex S4 pocket is very open and the changes Tyr220, Lys and His287Gln (renin/chymosin) can make little difference to this pocket, even allowing for the changes in charge and hydrophobicity. The inhibitor in the chymosin complex does not extend far onto the prime side of the scissile bond, where there are significant differences in the specificity pockets.

Some of the reasons for the primary specificity of chymosin for the exposed Phe105→Met106 bond of κ-casein are revealed in the crystal structure of the inhibitor complex. The norstatine residue at P1 is a good substitute for the phenylalanine of the substrate. The residues comprising S2 provide tight packing around the cyclohexyl ring at P1. The same arrangement of side chains in S2 can be expected around the phenylalanine of the substrate. On the other side of the scissile bond the O=C,H moiety of the inhibitor, which partially occupies S1′ makes electrostatic contacts with the enzyme that cannot mimic interactions made by the substrate, which is a methionine at P1′. On the non-prime side of the scissile bond the inhibitor residues of SMC-pIPhe-Pro can have few sidechain interactions in common with the substrate sequence Ser-Leu-His. On either end of the substrate binding cleft there are groups of acidic sidechains which may help to align the substrate within the active site cleft, by interacting with the basic residues more than four positions from the scissile bond (N:His-Pro-His-Pro-His-Leu-Ser-Ph-Pro-Ale-Ile-Pro-Pro-Lys-Lys-Asn: C).

Conclusion

The crystal structure of the complex of chymosin with a reduced bond inhibitor strongly supports the view that the transition state complex of chymosin with its substrate closely resembles that of other aspartic proteinases such as human renin. This is important for the design of engineered chymosins with differing specificities (for example Strop et al., 1990; Nugent et al., 1996; Williams et al., 1997). In these experiments either single residue substitutions or loop replacements were engineered on the basis of the crystal structure of uncomplexed chymosin and modelled inhibitors. The crystal structure of the complex, described here, provides a firmer basis for predicting the effects of these mutations and making more informed designs.

The question of the conformation of the uncomplexed state remains open, although the fact that the flap conformation found in the orthorhombic crystals is also found as a major component in solution (Andreeva,N., personal communication). As a consequence the specificity of chymosin to κ-casein may be linked to a conformational change of the flap, triggered by interactions between chymosin and κ-casein far from the catalytic residues as suggested by Gustchina et al. (1996).

References


M.R. Groves et al.


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