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An unusual orientation for Tyr75 in the active site of the aspartic proteinase from *Saccharomyces cerevisiae*

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Abstract

The structures of the native *Saccharomyces cerevisiae* proteinase A have been solved by molecular replacement in the monoclinic and trigonal crystal forms and refined at 2.6–2.7 Å resolution. These structures agree overall with those of other uninhibited aspartic proteinases. However, an unusual orientation for the side chain of Tyr75, a conserved residue on the flexible "flap" that covers the active site and is important for the activity of these enzymes, was found in the trigonal crystals. A similar conformation of Tyr75 occupying the S_1 substrate-binding pocket was previously reported only for chymosin (where it was interpreted as representing a "self-inhibited" state of the enzyme), but for no other aspartic proteinases. Since this orientation of Tyr75 has now been seen in the structures of two members of the family of aspartic proteinases, it might indicate that the placement of that residue in the S_1 substrate-binding pocket might have some functional significance, analogous to what was seen for self-inhibited structures of serine proteinases.

Keywords: Aspartic proteinases; Active site; Protein structure; Structure comparison; Crystal packing

Aspartic proteinases are characterized by the presence of two adjacent and coplanar aspartic acid side chains in their active site. Interest in the structure and function of members of this group of proteolytic enzymes is due, in part, to their importance in a number of pathological processes, including gastric ulcers, Alzheimer's disease, hypertension, malaria, and AIDS [1–3]. These enzymes can generally be categorized into one of the two sub-families: pepsin-like proteinases consist of two lobes that are similar, but not identical, whereas retroviral proteinases (retropepsins) are dimers consisting of two identical subunits [4]. Crystal structures have been determined for many members of both sub-families (see reviews [2,5]), in the free enzyme form, as complexes with a wide range of chemically synthesized and naturally occurring inhibitors, and for the zymogen forms of several pepsin-like enzymes.

The free enzymes have largely preformed active sites [2,5,6] and, upon inhibitor binding, structural changes are generally minor [7]. The exception is the single β hairpin loop known as the "flap" in pepsin-like enzymes, which can move by as much as 8–9 Å between the free and inhibited forms of an enzyme [8], or the two equivalent flaps in retropepsins [9]. In all pepsin-like aspartic proteinases, one absolutely conserved residue, Tyr75 (pepsin numbering), is present near the tip of the flap and has been postulated to be involved in the capture and cleavage of substrates [10]. This residue was found in a similar orientation in almost all structures, with the sole exception of chymosin. In the latter uninhibited enzyme, the side chain of Tyr75 occupied the S_1-binding pocket, blocking its accessibility [11,12]. It was subsequently postulated that native chymosin exists in a "self-inhibited" form [13]. This term was later invoked to establish the structural basis for the loss of activity in

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several serine proteases and phosphatases observed under certain conditions or upon enzyme maturation. Self-inhibition may be a result of the changes in the conformation of one or more residues in the active site of the enzyme as in phosphatase Cdc25A [14], or of the blockage of the substrate-binding pockets by the enzyme residues as in Sindbis virus core protein [15], or of the modification of the binding site architecture due to conformational changes of flexible loops upon enzyme activation as in factor D [16,17]. Such self-inhibited state of the enzyme could be permanent (as in Sindbis viral core protein) or transient. In the case of factor D, for example, the self-inhibited enzyme is activated only upon binding of its sole specific substrate [17].

Yeast vacuolar proteinase A (saccharopepsin) is a pepsin-like aspartic proteinase. Structures have been previously reported for its free enzyme form (at 3.5 Å resolution), for a complex with a small-molecule inhibitor CP81,282 (Mor–Phe–Nle–Chf–Nme) [18], and for further five complexes with renin inhibitors [19]. However, the structure of the native enzyme was not refined, released, or discussed in any detail. Recently, we have also solved the crystal structure of proteinase A complexed with an 8 kDa polypeptide inhibitor called IA_3, demonstrating that while residues 2–32 of IA_3 have little intrinsic structure in the free inhibitor, they become ordered upon binding to proteinase A and adopt a near perfect α-helical conformation occupying the active site cleft [8,20]. The structure of proteinase A itself in its complex with IA_3 is similar to that in the CP81,282 complex, except for the large differences in the flap conformations [8]. In our ongoing studies on the structural determination of the complexes of proteinase A with mutant forms of IA_3, we obtained by serendipity two crystal forms, monoclinic and trigonal, of the free enzyme. We report here that the structures determined for free proteinase A in either crystal form, although similar overall, have some important differences in the conformation of the flap and in the orientation of the Tyr75 side chain within the active site cleft of the enzyme.

Materials and methods

Complexes were prepared for crystallization by mixing proteinase A and a mutant of the IA_3 inhibitor in molar ratios of 1:5 in 20 mM Mes buffer, pH 6.6. The samples were concentrated at 4 °C to approximately 5 mg ml^{-1} using Centriprep (Amicon) devices with a 3-kDa cut-off. Retrospectively, however, it was established that the mutant form of the IA_3 polypeptide used for crystallization acted not only as an inhibitor, but also as a slow substrate, and thus was degraded by the final, highly concentrated preparation of proteinase A (unpublished observations). Crystals were obtained by vapor diffusion. The well solution contained 28% PEG 1500, 0.2 M ammonium sulfate in 0.1 M Mes buffer at pH 6.0, while the sample was in 20 mM Mes buffer, pH 6.6. The hanging drop was a 1:1 mixture of the sample and well solution. Two crystal forms appeared after a few days and grew to $0.15 \times 0.4 \times 0.3$ mm after 2 weeks. One of the crystal forms belongs to space group $P3_222$ with unit cell dimensions of $a = b = 84.6$ Å and $c = 108.7$ Å, containing one molecule per asymmetric unit. The other crystal form belongs to space group $P2_1$ with unit cell dimensions of $a = 82.97$ Å, $b = 49.08$ Å, and $c = 94.69$ Å, $\beta = 96.5°$, and contains two molecules per asymmetric unit.

Table 1
X-ray data collection and refinement statistics[a]

Crystal form	Trigonal	Monoclinic
Space group	$P3_221$	$P2_1$
Cell dimensions (Å)		
a	84.6	82.97
b	84.6	49.08
c	108.7	94.69
β(°)	–	96.5
Resolution (Å)	2.7	2.6
Measured reflections	78,120	75,834
Unique reflections	12,565	22,963
R_{merge} (%)	6.4 (38.6)	8.6 (56.2)
$I/\sigma(I)$	25.7 (3.9)	14.3 (2.1)
Completeness (%)	97.9 (96.5)	98.8 (97.6)
R (%)	20.71	20.44
R_{free} (%)	27.08	28.05
PDB Accession Code	1FMU	1FMX

[a] Numbers in parentheses correspond to the last shell of measured data.

Diffraction data were collected at 100 K with a MAR345 detector mounted on a Rigaku RU200 generator, operated at 50 kV and 100 mA, to a resolution of 2.6 Å for the monoclinic crystals and 2.7 Å for the trigonal crystals. The structure in space group $P2_1$ was solved by molecular replacement with *AmoRe* [21]. Proteinase A coordinates from the complex with IA_3 [8] were used as the search model. A single solution consisting of two molecules in the asymmetric unit was obtained with a correlation factor of 0.503 and *R*-factor of 0.385. The resulting model was rebuilt with the program *O* [22] and refined with *CNS* [23]. The results of the refinements are summarized in Table 1. The structure in space group $P3_221$ was isomorphous with the previously reported complex with CP81,282 [18] and was thus refined directly, using procedures outlined above. Coordinates and structure factors for the trigonal and monoclinic crystal forms of proteinase A (Table 1) have been deposited with the Protein Data Bank (Accession Codes 1FMU and 1FMX, respectively).

Results and discussion

Structures were determined for free proteinase A in both monoclinic and trigonal crystal forms (Fig. 1A), at the resolution of 2.6 and 2.7 Å, respectively. The two crystallographically independent molecules observed in the monoclinic form can be superimposed with a root-mean-square (r.m.s.) deviation of 0.52 Å for 319 Cα pairs. Each of these molecules, in turn, can be superimposed on the single molecule present in the trigonal cell with an r.m.s. deviation of 0.48 or 0.50 Å for 298 or 284 pairs, respectively.

There are some disordered regions in each molecule, where the electron density is either poor or not visible at all, such as for the residues 162–165 and 243–245 in surface loops in the trigonal crystal form. In the monoclinic crystals, the flap is partially disordered in each of the molecules in the asymmetric unit so that

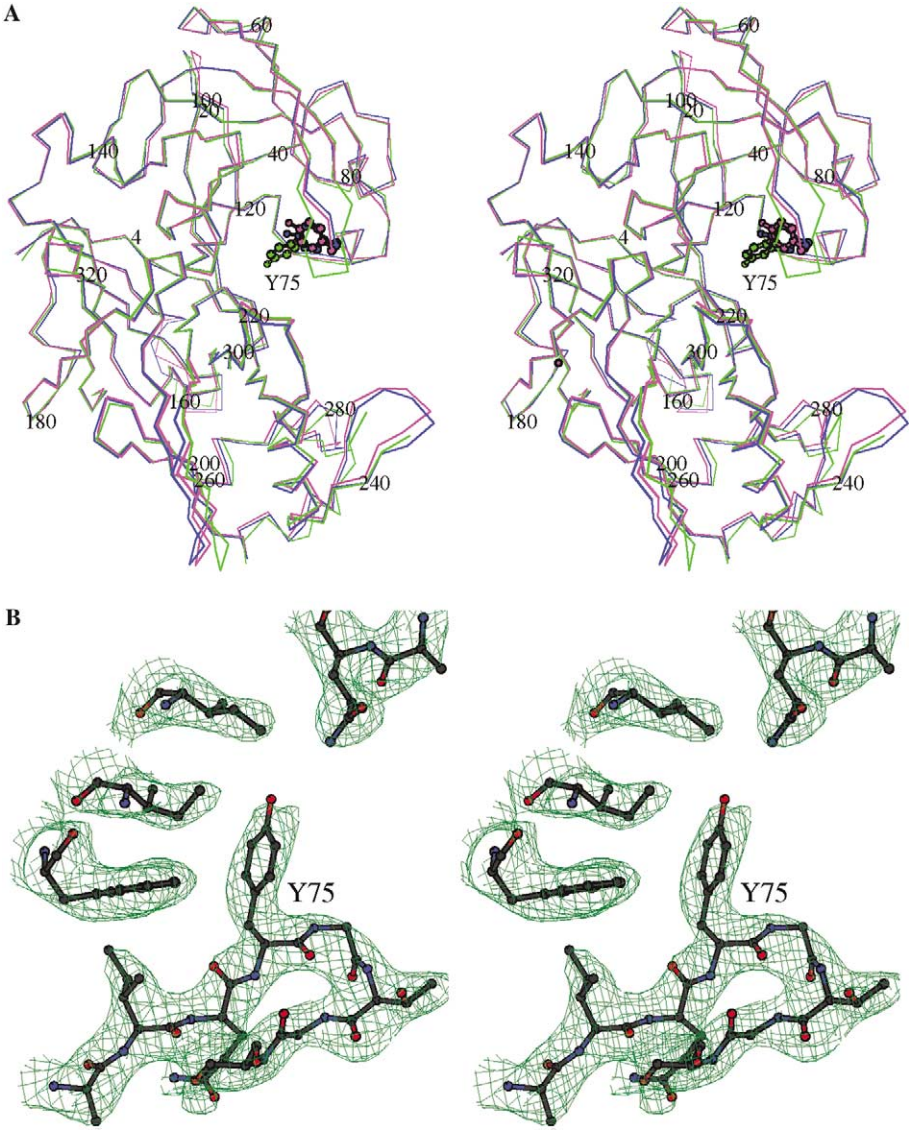

Fig. 1. Structure of proteinase A and the electron density for Tyr75. (A) Tracings of the Cα coordinates of the trigonal form of proteinase A (green) superimposed on the two crystallographically independent molecules from the monoclinic crystal form (blue and red). The side chain of Tyr75 is shown in ball-and-stick representation. (B) Electron density in the vicinity of Tyr75 in the trigonal crystals of proteinase A, contoured at 1σ level.

residues 76–79 could not be traced (Fig. 1A). However, the position of the main chain atoms and the side chain of Tyr75 could be readily discerned. In the trigonal crystal form, the electron density for the main chain atoms in the flap was contiguous, despite the relatively high temperature factors in this region, and was particularly clear for the side chain of Tyr75 (Fig. 1B). The orientation of the side chain of this residue does not correspond to that found in proteinase A complexed with IA_3 [8,20] or with a low molecular weight inhibitor, CP81,282, which contains a Phe residue as its P_1 substituent (Fig. 2A) [18]. The side chain of Tyr75 in the trigonal crystal form of free proteinase A occupies almost exactly the position of the benzene ring of the P_1 Phe in the CP81,282 inhibitor (Fig. 2A), i.e., Tyr75 in the trigonal crystal form adopts the unusual orientation of pointing unequivocally into the S_1 pocket of the enzyme's active site.

The main chain of the visible part of the flap is shifted in the two molecules in the monoclinic crystals compared to their trigonal counterpart and the conformation of the flap and the orientation of the Tyr75 side chain are similar to those in protease A complexed with IA_3, but different from those found in the trigonal crystals of free enzyme and its complexes with small-molecule inhibitor (Fig. 2B). It needs to be stressed that the native crystals were grown from a solution in which a mutant of IA_3 was initially present. We thus may be able to reconcile these observations by suggesting that some products of hydrolysis might still be present in the monoclinic crystals at low occupancy, but are too disordered to be seen in the electron density maps at this resolution.

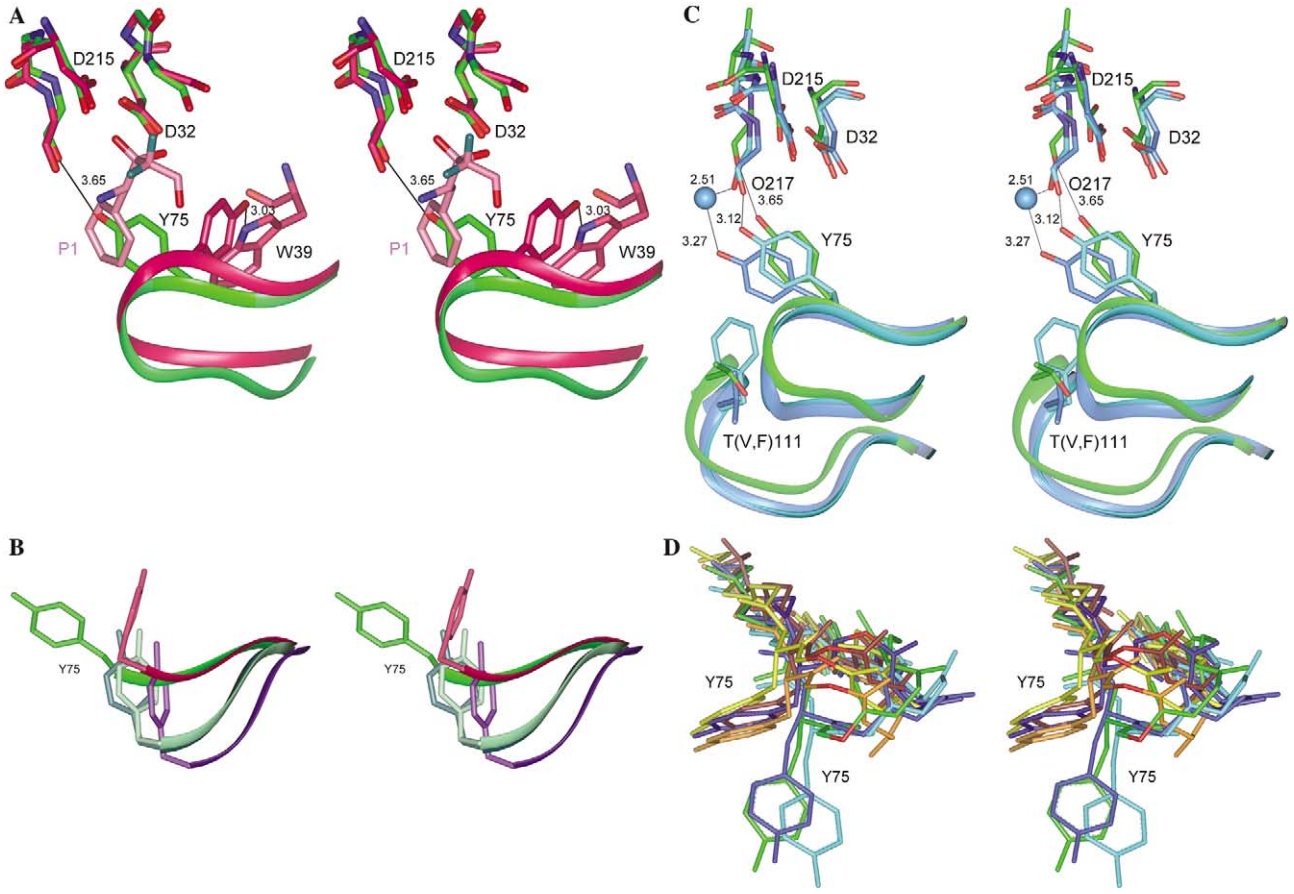

Fig. 2. Comparison of the flap conformation and the interactions made by Tyr75 in the different orientations observed in various aspartic proteinases. (A) Superposition of the flap and parts of the active site of the unliganded proteinase A in the trigonal crystal form (green) and in its complex with CP81,282 (red). In the latter, the position of the P1 Phe substituent of the inhibitor is depicted, while the side chain OH group of Tyr75 is hydrogen bonded to the ring N atom of Trp39. In the trigonal crystal form of the native proteinase A, the OH lies adjacent to the carbonyl oxygen of Gly217. (B) Three distinct orientations of Tyr75 in the crystal structures of proteinase A. Tyr75 and a fragment of the flap are shown in green in the trigonal crystals of proteinase A and in light and dark cyan in the two molecules in the asymmetric unit of the monoclinic crystals. These structures are overlaid with their counterparts reported for proteinase A complexed with the protein inhibitor IA3 (1DPJ—violet) and with the small-molecule inhibitor CP81,282 (2JXR—red). (C) Hydrogen bond arrangement between Tyr75 and the carbonyl oxygen of Gly217 in the trigonal crystal form of proteinase A (green), and in the Val111Phe mutant (3CMS, light blue) and wild-type (1CMS, dark blue) forms of chymosin. Residue 111 in these three enzymes is Thr, Phe, and Val, respectively. (D) The two different orientations adopted by Tyr75 correlate strictly with the main chain conformation around Gly76 (marked in red) in the flaps of several aspartic proteinases. Proteinase A is shown in green, wild-type chymosin (1CMS) in light blue, Val111Phe mutant of chymosin (3CMS) in dark blue for one observed conformation and in violet for the other, human pepsin (1PSN) in orange, porcine pepsin (4PEP) in yellow, and *Rhizomucor pusillus* pepsin (1MPP) in brown.

The conformation of Tyr75 found in the trigonal crystals of free proteinase A is reminiscent of that reported previously for two structures of free bovine chymosin [11,12] and to one of the two orientations reported for this residue in the Val111Phe mutant of free chymosin [24] (Fig. 2C). The OH group of the tyrosine side chain in proteinase A lies within 3.65 Å of the carbonyl oxygen of Gly217 (Fig. 2C), in a hydrogen-bonding arrangement similar to that seen in the Val111Phe mutant of free chymosin [24], where, however, this hydrogen bond is stronger (3.12 Å. In the active site of wild-type chymosin [11], the hydrogen bond between the carbonyl oxygen of Gly217 and the hydroxyl of Tyr75 is maintained via a water molecule (Fig. 2C).

To exclude the possibility that crystal packing effects might have had an influence on the orientation of Tyr75, the contacts in the flap region of free proteinase A within the trigonal crystals were examined. The distance between Tyr75 and the nearest part of another molecule related by crystallographic symmetry exceeds 15 Å, so that the conformation of the side chain of Tyr75 could not have been influenced by crystal packing forces. In free chymosin, on the other hand, although Tyr75 itself is not involved in direct crystal contacts, residues Ser81 and Glu83 on the opposite strand of the flap do make such contacts. Even in chymosin, however, crystal contacts cannot be the sole determinants for the orientation of Tyr75, since the two positions of that side chain have been seen in the Val111Phe mutant [24], despite its

isomorphism with the native enzyme. The latter structure was refined independently in two laboratories with virtually identical results [11,12], so that the location of Tyr75 in wild-type chymosin is beyond doubt. Thus, it is unlikely that the placement of this residue in the S_1 pocket of proteinase A in the trigonal form and in free chymosin is the result of crystal contacts.

In addition to bovine chymosin (PDB codes 1CMS, 3CMS), the trigonal structure solved for free proteinase A was also compared with the structures of other non-liganded aspartic proteinases with coordinates available in the Protein Data Bank. These enzymes include human pepsin (1PSN), cod pepsin (1AM5), porcine pepsin (4PEP, 5PEP), *Rhizomucor pusillus* pepsin (1MPP), rhizopuspepsin (2APR), human renin (2REN), and penicillopepsin (3APP). By contrast to the unusual orientation observed for Tyr75 in the trigonal crystal structure of proteinase A and in the wild-type and Val111Phe mutant forms of free chymosin, the side chain of this residue in the majority of the other enzymes forms a hydrogen bond with the ring nitrogen of Trp39, comparable to that depicted in Fig. 2A for the complex of CP81,282 with proteinase A. The conformation of the main chain of residues 75–77 located at the tip of the flap is also different in the enzymes with such conventional orientation of the Tyr75 side chain, compared to those in the trigonal proteinase A/chymosin structures with Tyr75 in the unusual orientation. In the latter two enzymes, the torsion angle φ of Gly76 is positive, whereas in the other enzymes it is negative. As a result, the peptide bonds involving residues 76 and 77 at the very tip of the flap are oriented at a significant angle to each other in the two groups (for example, $\sim 82°$ between the two conformations of the flap in the chymosin (3CMS) structure, Fig. 2D). Since only a Gly residue is capable of adopting a conformation with a positive φ torsion angle and any value of ψ, the presence of this residue at position 76 at the tip of the flap appears to be a crucial feature in enabling the side chain of Tyr75 to enter the S_1 binding pocket and thus lead to the "self-inhibited" [13] enzyme. For those aspartic proteinases that do not have a Gly at position 76, such as human renin where the equivalent residue is a Ser, such a conformation would not be expected to form and none has been observed to date.

In further attempts to rationalize why the unusual orientation can be adopted for proteinase A and chymosin but not for the other enzymes, the nature of the residues contributing to the S_1-binding site occupied by the side chain of Tyr75 was examined. In the trigonal form of proteinase A and in chymosin, the side chain of Tyr75 is accommodated in a generally hydrophobic pocket consisting of the side chains of Phe112 (3.7 Å), Ile120 (4.3 Å), and Phe117 (3.5 Å). The equivalent residue to Phe117 is an Asn in rhizopuspepsin and penicillopepsin and an Ile in endothiapepsin [11]. Although Phe117 is capable of making excellent aromatic–aromatic contacts with Tyr75 in the unusual orientation, this residue alone cannot account for the observed conformation of Tyr75, since Phe is also present in this position in the sequences of pepsin and renin, and no similar structures have been reported for the free enzyme forms of either of these two aspartic proteinases. In this regard, however, it may be noteworthy that renin does not have the important contribution of Gly76 at the tip of the flap, as discussed above. Residues Phe112 and Ile120 enhance the hydrophobic nature of the S_1 pocket in proteinase A. Only hydrophobic residues are present at the position corresponding to Ile120 in all aspartic proteinases, while Phe112 in the various pepsins is substituted by a variety of other residues. However, in these enzymes where such substitutions are found at position 112, the immediately upstream residue at position 111 is frequently observed to be Phe and the side chain of this phenylalanine residue is also able to form compensatory hydrophobic interactions with the residue occupying the S_1 pocket. The residue at position 30 can also potentially make contact with a large P_1 substituent and in proteinase A and chymosin, residue 30 is Ile and Leu, respectively. Vast majority of aspartic proteinases with known crystal structures have Leu, Ile or Val in this position, with the exceptions of Asp in rhizopuspepsin and endothiapepsin, and Asn in penicillopepsin. Thus, while the more hydrophobic nature of the S_1 subsite in proteinase A and chymosin relative to those of rhizopus-, endothia-, and penicillopepsins might explain the unusual orientation of Tyr75 observed in the former enzymes, the lack of such a phenomenon in non-fungal aspartic proteinases such as pepsin and cathepsin D remains somewhat enigmatic. The hydrophobic nature of the S_1 pocket in these mammalian enzymes is not substantially different from their counterparts in proteinase A and chymosin, yet structures with Tyr75 in the unusual orientation have not been reported for these mammalian enzymes.

A similar arrangement of water molecules in the structures of free proteinase A and chymosin is found in the vicinity of Ser35 and Trp39. Water W2 in the trigonal crystal form of free proteinase A (shown in blue in Fig. 3) is located close to the position occupied by the hydroxyl of Tyr75 in proteinase A complexed with a low molecular weight inhibitor [18]. This water forms a long hydrogen bond (3.38 Å) with the NE1 atom of Trp39 and another hydrogen bond with the conserved water molecule W1, interacting with Ser35 and the carbonyl oxygen of Asn37 (Fig. 3). The same network of interactions was previously described for chymosin [13] (Fig. 3). In the monoclinic crystals of proteinase A, water molecules W1 and W2 are also present at equivalent positions (not shown). However, the side chain of Tyr75 assumes a different orientation due to the larger shift of the flap (Fig. 2B) and therefore its hydroxyl is located at the hydrogen bond distance to water W2. The other interactions described above are present in this crystal form as well.

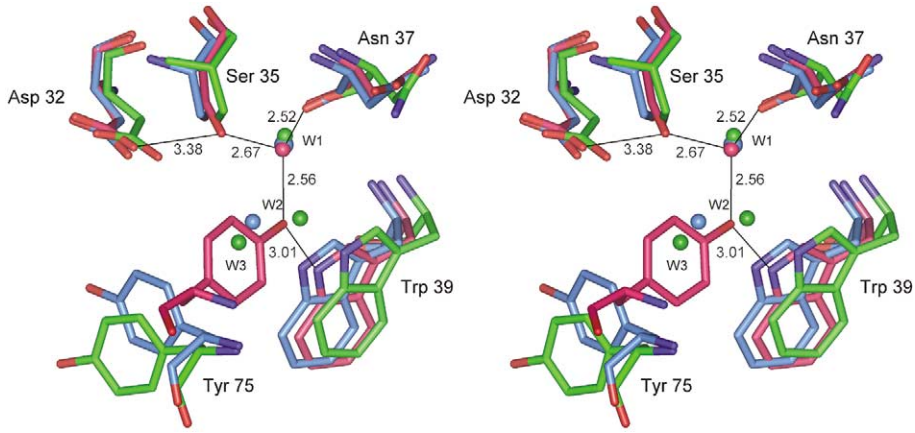

Fig. 3. Comparison of the important interactions which involve conserved water molecules between Tyr75 and the active site residues in the structures of free proteinase A (blue), its complex with a small molecule inhibitor CP81,282 (red), and chymosin (green). Hydrogen bonds are explicitly shown for the liganded proteinase A.

Functional significance of the interactions mediated via conserved water molecules in the structures of numerous aspartic proteinases has been recently discussed in detail [7,25]. It was suggested that the changes in the orientation of Tyr75 upon ligand binding lead to the formation of a network of strong hydrogen bonds (Fig. 3), assisting in the catalytic mechanism [7]. It could be proposed that the alternative conformation of Tyr75, with its side chain occupying the S_1 substrate-binding pocket as observed in free chymosin and proteinase A, might have negative impact on the activity of these two enzymes.

The mode of "self-inhibition" of proteinase A and chymosin described here may represent only a transitional structural feature rather than true inhibition and is different from the mechanisms that aspartic proteinases have evolved for modulating or stabilizing their own intrinsic activity. For example, cathepsin D at neutral pH exists in a self-inhibited form that results from a major structural rearrangement in which residues 1–10 at the N-terminus of the free enzyme are relocated into the active site cleft, enabling specific interaction of the side chain of Lys7 with the active site aspartate residues [26]. This self-inhibited form uses some of the apparatus seen in the precursor forms of aspartic proteinases, such as pepsinogen [27], progastricsin [28], or prophytepsin [29]. Similar large movements of the N terminus of the mature enzyme facilitated the formation of a self-inhibited state of factor D during its activation [17]. If such major rearrangements are readily accomplished, then it is perhaps not surprising to observe a more limited transition between the different orientations for Tyr75 in various aspartic proteinases. Nevertheless, since the unusual position of Tyr75 within the S_1 pocket has now been observed in four structures determined for two different enzymes, it would appear that this feature might have some operational significance. Whether this represents only a transitional structural feature as in factor D, or whether it reflects some deeper significance as a mechanism for stabilizing intrinsic activity, perhaps linked to substrate capture [10], poses a fascinating challenge for future investigation.

Acknowledgments

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References

[1] H. Krausslich, S. Oroszlan, E. Wimmer, Viral Proteinases as Targets for Chemotherapy, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989.
[2] D.R. Davies, The structure and function of the aspartic proteinases, Annu. Rev. Biophys. Biophys. Chem. 19 (1990) 189–215.
[3] L. Hong, G. Koelsch, X. Lin, S. Wu, S. Terzyan, A. Ghosh, X.C. Zhang, J. Tang, Structure of memapsin 2 (β-secretase) complexed with inhibitor: a template to design drugs for Alzheimer's disease, Science 290 (2000) 150–153.
[4] A.J. Barrett, N.D. Rawlings, J.F. Woessner, Handbook of Proteolytic Enzymes, Academic Press, London, 1998.
[5] A. Wlodawer, A. Gustchina, Structural and biochemical studies of retroviral proteases, Biochim. Biophys. Acta 1477 (2000) 16–34.
[6] N.K. Bernstein, M.M. Cherney, H. Loetscher, R.G. Ridley, M.N.G. James, Crystal structure of the novel aspartic proteinase zymogen proplasmepsin II from *Plasmodium falciparum*, Nature Struct. Biol. 6 (1999) 32–37.
[7] N.S. Andreeva, L.D. Rumsh, Analysis of crystal structures of aspartic proteinases: on the role of amino acid residues adjacent to the catalytic site of pepsin-like enzymes, Protein Sci. 10 (2001) 2439–2450.

[8] M. Li, L.H. Phylip, W.E. Lees, J.R. Winther, B.M. Dunn, A. Wlodawer, J. Kay, A. Gustchina, The aspartic proteinase from *S. cerevisiae* folds its own inhibitor into a helix, Nature Struct. Biol. 7 (2000) 113–117.

[9] A. Wlodawer, M. Miller, M. Jaskólski, B.K. Sathyanarayana, E. Baldwin, I.T. Weber, L.M. Selk, L. Clawson, J. Schneider, S.B.H. Kent, Conserved folding in retroviral proteases: crystal structure of a synthetic HIV-1 protease, Science 245 (1989) 616–621.

[10] J. Tang, G. Koelsch, A possible function of the flaps of aspartic proteases: the capture of substrate side chains determines the specificity of cleavage positions, Protein Peptide Lett. 2 (1995) 257–266.

[11] G.L. Gilliland, E.L. Winborne, J. Nachman, A. Wlodawer, The three-dimensional structure of recombinant bovine chymosin at 2.3 Å resolution, Proteins 8 (1990) 82–101.

[12] M. Newman, M. Safro, C. Frazao, G. Khan, A. Zdanov, I.J. Tickle, T.L. Blundell, N. Andreeva, X-ray analyses of aspartic proteinases. IV. Structure and refinement at 2.2 Å resolution of bovine chymosin, J. Mol. Biol. 221 (1991) 1295–1309.

[13] N. Andreeva, J. Dill, G.L. Gilliland, Can enzymes adopt a self-inhibited form results of X-ray crystallographic studies of chymosin, Biochem. Biophys. Res. Commun. 184 (1992) 1074–1081.

[14] E.B. Fauman, J.P. Cogswell, B. Lovejoy, W.J. Rocque, W. Holmes, V.G. Montana, H. Piwnica-Worms, M.J. Rink, M.A. Saper, Crystal structure of the catalytic domain of the human cell cycle control phosphatase, Cdc25A, Cell 93 (1998) 617–625.

[15] H.K. Choi, L. Tong, W. Minor, P. Dumas, U. Boege, M.G. Rossmann, G. Wengler, Structure of Sindbis virus core protein reveals a chymotrypsin-like serine proteinase and the organization of the virion, Nature 354 (1991) 37–43.

[16] H. Jing, Y.S. Babu, D. Moore, J.M. Kilpatrick, X.Y. Liu, J.E. Volanakis, S.V. Narayana, Structures of native and complexed complement factor D: implications of the atypical His57 conformation and self-inhibitory loop in the regulation of specific serine protease activity, J. Mol. Biol. 282 (1998) 1061–1081.

[17] H. Jing, K.J. Macon, D. Moore, L.J. DeLucas, J.E. Volanakis, S.V. Narayana, Structural basis of profactor D activation: from a highly flexible zymogen to a novel self-inhibited serine protease, complement factor D, EMBO J. 18 (1999) 804–814.

[18] C.F. Aguilar, N.B. Cronin, M. Badasso, T. Dreyer, M.P. Newman, J.B. Cooper, D.J. Hoover, S.P. Wood, M.S. Johnson, T.L. Blundell, The three-dimensional structure at 2.4 Å resolution of glycosylated proteinase A from the lysosome-like vacuole of *Saccharomyces cerevisiae*, J. Mol. Biol. 267 (1997) 899–915.

[19] N.B. Cronin, M.O. Badasso, J. Tickle, T. Dreyer, D.J. Hoover, R.L. Rosati, C.C. Humblet, E.A. Lunney, J.B. Cooper, X-ray structures of five renin inhibitors bound to saccharopepsin: exploration of active-site specificity, J. Mol. Biol. 303 (2000) 745–760.

[20] L.H. Phylip, W.E. Lees, B.G. Brownsey, D. Bur, B.M. Dunn, J.R. Winther, A. Gustchina, M. Li, T. Copeland, A. Wlodawer, J. Kay, The potency and specificity of the interaction between the IA3 inhibitor and its target aspartic proteinase from *Saccharomyces cerevisiae*, J. Biol. Chem. 276 (2001) 2023–2030.

[21] J. Navaza, An automated package for molecular replacement, Acta Crystallogr. A 50 (1994) 157–163.

[22] T.A. Jones, M. Kieldgaard, Electron-density map interpretation, Methods Enzymol. 277 (1997) 173–208.

[23] A.T. Brünger, P.D. Adams, G.M. Clore, W.L. DeLano, P. Gros, R.W. Grosse-Kunstleve, J.S. Jiang, J. Kuszewski, M. Nilges, N.S. Pannu, R.J. Read, L.M. Rice, T. Simonson, G.L. Warren, Crystallography and NMR system: a new software suite for macromolecular structure determination, Acta Crystallogr. D 54 (1998) 905–921.

[24] P. Strop, J. Sedlacek, J. Stys, Z. Kaderabkova, I. Blaha, L. Pavlickova, J. Pohl, M. Fabry, V. Kostka, M. Newman, Engineering enzyme subsite specificity: preparation, kinetic characterization, and X-ray analysis at 2.0-Å resolution of Val111Phe site-mutated calf chymosin, Biochemistry 29 (1990) 9863–9871.

[25] B.V. Prasad, K. Suguna, Role of water molecules in the structure and function of aspartic proteinases, Acta Crystallogr. D 58 (2002) 250–259.

[26] A.Y. Lee, S.V. Gulnik, J.W. Erickson, Conformational switching in an aspartic proteinase, Nature Struct. Biol. 5 (1998) 866–871.

[27] M.N.G. James, A.R. Sielecki, Molecular structure of an aspartic proteinase zymogen, porcine pepsinogen, at 1.8 Å resolution, Nature 319 (1986) 33–38.

[28] S.A. Moore, A.R. Sielecki, M.M. Chernaia, N.I. Tarasova, M.N.G. James, Crystal and molecular structures of human progastricsin at 1.62 Å resolution, J. Mol. Biol. 247 (1995) 466–485.

[29] J. Kervinen, G.J. Tobin, J. Costa, D.S. Waugh, A. Wlodawer, A. Zdanov, Crystal structure of plant aspartic proteinase prophytepsin: inactivation and vacuolar targeting, EMBO J. 18 (1999) 3947–3955.