Analysis of crystal structures of aspartic proteinases: On the role of amino acid residues adjacent to the catalytic site of pepsin-like enzymes

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(RECEIVED June 27, 2001; FINAL REVISION August 22, 2001; ACCEPTED August 29, 2001)

Abstract

To elucidate the role of amino acid residues adjacent to the catalytic site of pepsin-like enzymes, we analyzed and compared the crystal structures of these enzymes, their complexes with inhibitors, and zymogens in the active site area (a total of 82 structures). In addition to the water molecule (W1) located between the active carboxyls and playing a role of the nucleophile during catalytic reaction, another water molecule (W2) at the vicinity of the active groups was found to be completely conserved. This water molecule plays an essential role in formation of a chain of hydrogen-bonded residues between the active site flap and the active carboxyls on ligand binding. These data suggest a new approach to understanding the role of residues around the catalytic site, which can assist the development of the catalytic reaction. The influence of groups adjacent to the active carboxyls is manifested by pepsin activity at pH 1.0. Some features of pepsin-like enzymes and their mutants are discussed in the framework of the approach.

Keywords: Aspartic proteases; pepsin-like enzymes; protein three-dimensional structures; comparison of protein structures; active site region of pepsin-like enzymes

Pepsin-like enzymes are aspartic proteases, which belong to the A1 family of peptidases (Rawlings and Barrett 1998). This family comprises proteins with a three-dimensional structure close to that of pepsin. The single molecular chain of these enzymes forms two domains with different amino acid sequences, but basically similar folds. In contrast, molecules of retroviral aspartic proteases, making up the A2 family, consist of two identical monomers resembling pepsin domains. The catalytic site of pepsin-like enzymes is formed at the junction of the domains and contains two aspartic acid residues, Asp 32 and Asp 215 (in pepsin num-

bering), one in each domain. An essential member of the active site is the water molecule bound between the active carboxyls, which becomes deprotonated on substrate binding to initiate the general base catalysis (Antonov et al. 1978, 1981). In accordance with the accepted mechanism of pepsin-like enzyme function (Suguna et al. 1987b; Davies 1990), Asp 215 has to be charged, whereas Asp 32 has to be protonated. A remarkable property of this catalytic center is adaptation for the action in a wide range of pH from pH 1.0 up to pH 7.0.

The three-dimensional structure of the porcine pepsin active site area is presented in Figure 1 (pdb code: 4pep; Sielecki et al. 1990). The active water molecule is labeled W1. As can be seen, the hydroxyl groups of Ser 35 and Thr 218 are near the active carboxyls of Asp 32 and Asp 215, respectively. Unlike Thr 218, whose hydroxyl can make only one hydrogen bond with the outer oxygen (O⁸²) of the Asp 215 carboxyl, the hydroxyl of Ser 35 is at a hydrogen-

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Article and publication are at <http://www.proteinscience.org/cgi/doi/10.1101/ps.25801>.

bonding distance from the outer oxygen of the Asp 32 carboxyl ($O^{\delta 1}$) and another water molecule, W2. The W2 is hydrogen-bonded itself with the carbonyl oxygen of Asn 37 and the hydroxyl of the active site flap residue Tyr 75, while the hydroxyl of Tyr 75 is fixed by a hydrogen bond with the Trp 39 $N^{\epsilon 1}$ atom. The active carboxyls have additional hydrogen bonds (not shown in Fig. 1), which connect their inner oxygens with the NH groups of glycine residues located in the Asp-Thr-Gly segments of both active loops. The conserved nature of these bonds and their role were discussed previously (Davies 1990).

The residues outlined in Figure 1 are conserved in pepsin-like enzymes, with a few exceptions. Protein engineering experiments revealed that all of them have a marked influence on enzymatic activity. A prominent drop in k_{cat} and small changes in K_M were detected for two mutants, Ser 35 Ala and Thr 218 Ala of porcine pepsin and rhizopuspepsin (pepsin numbering of residues is used for all enzymes discussed in this article), whereas the pH optimum and pK_a values of the active carboxyls changed only slightly (Lin et al. 1992). The authors of this work hypothesized that the hydrogen bonds formed by the active carboxyls with Ser 35 and Thr 218 did not control their ionization properties but provided the structural rigidity in the catalytic apparatus. However, the presence of Ala 218 instead of Thr 218 in some renins suggests that this explanation cannot be applied to all pepsin-like enzymes. The opposite point of view was used to explain the decrease in activity of the chymosin mutant Thr 218 Ala in acidic media (Mantaounis and Pitts 1990). The decrease in activity was interpreted as a consequence of a partial sacrifice of the negative charge of Asp 215 at low pH, which supposedly was stabilized by a hydrogen bond with the Thr 218 hydroxyl. This conclusion was discussed also in studies on mechanism of endothiapepsin, and the same role to stabilize the negative charge of Asp 32 was prescribed to the Ser 35 hydroxyl after formation of the tetrahedral intermediate (Veerapandian et al. 1992). The role of Ser 35 in the unbound enzyme was not considered. Attempts to change the pH optimum of the pepsin-like enzymes by point mutation experiments resulted in a small shift of this parameter (Mantaounis and Pitts 1990; Lin et al. 1992). Perutz (1992) proposed a convincing explanation of this result, suggesting that the pH optimum of pepsin-like enzymes is determined by a combined contribution of many polar residues in molecules. Special calculations in more recent studies (Yang et al. 1997) support this point of view.

Several Tyr 75 mutants were studied during protein engineering experiments with chymosin (Suzuki et al. 1989) and *Rhizomucor pusillus* protease (Park et al. 1996). The replacement of Tyr 75 with various amino acid residues (Thr, Ile, Val) in chymosin resulted in a complete loss of activity, whereas the mutant Tyr 75 Phe caused marked changes in the kinetic parameters, depending on the sub-

strates used. The replacements of Tyr 75 by 17 residues in *R. pusillus* protease resulted in negligible activity for 15 mutants, weakened activity for the Tyr 75 Phe mutant, and increased catalytic efficiency for the Tyr 75 Asn mutant. A marked drop in k_{cat} was observed for rat renin mutants Tyr 75 His, Tyr 75 Phe, and especially for the mutant Tyr 75 Ala (Suzuki et al. 1996). The results of all these experiments were explained by the special role of Tyr 75 in stabilizing the transition state of the substrate during the catalytic reaction, as proposed in studies of endothiapepsin (Blundell et al. 1987). However, the exceptional case of the increased catalytic efficiency of the Tyr 75 Asn mutant of *R. pusillus* protease was not interpreted.

The activity decreased also after the replacement of Trp 39 with a set of residues in *R. pusillus* protease (Park et al. 1997). Trp 39 was suggested to stabilize the position of the Tyr 75 phenolic ring by the hydrogen bond between the Trp 39 $N^{\epsilon 1}$ atom and the Tyr 75 hydroxyl. However, the natural replacement of Trp 39 by alanine in β -secretases (Rawlings and Barrett 1998) does not prevent these enzymes from being active.

The controversial interpretation of the role of residues surrounding the catalytic site of pepsin-like enzymes prompted us to perform a detailed structural analysis of groups in the active site area. The initial purpose of the present work was to understand the combined functional property of pepsin residues, which form a continuous chain of hydrogen bonds, with the active carboxyls being members of this chain (Fig. 1). However, the problem was more complicated than we originally had thought, and data known only for pepsin were not enough to solve it. The experimental approach involving a protein engineering introduction of the chain of hydrogen-bonded residues observed in pepsin into HIV-1 protease molecules (Dergousova et al. 1997) and a subsequent structural analysis of the mutant met many difficulties. Therefore, at this stage, all known three-dimensional structures of pepsin-like proteases, their complexes with inhibitors, and zymogens have been inspected and compared in the active site area (a total of 82 structures; Bernstein et al. 1977; Berman et al. 2000). Some observations and hypotheses ensuing from this study are described.

Results

First, we determined the degree of porcine pepsin conservation for the interactions shown in Figure 1. The threonine residue at the position 218 in pepsin numbering is present in all analyzed structures except renins, where it is replaced by alanine (human) or serine (mouse). With few exceptions, the location of the Thr 218 hydroxyl at a hydrogen bond distance from the Asp 215 carboxyl is a common feature of all active enzymes and their complexes with inhibitors. The exceptions among active enzymes include Atlantic cod pep-

Fig. 1. The catalytic site and the surrounding region of unbound porcine pepsin in monoclinic crystals (pdb code: 4pep; resolution 1.8 Å, Sielecki et al. 1990). In these crystals, the flap is involved in intermolecular contacts, and it is close to the molecular surface. The hydroxyl of Tyr 75 forms hydrogen bonds with the interior of the molecule, resulting in formation of a continuous chain of hydrogen-bonded residues Trp 39–Tyr 75–W2–Ser 35–Asp 32 extending from the flap to the active site. The same chain of hydrogen bonds is a characteristic property of all complexes of pepsin-like enzymes with inhibitors. The Thr 218 hydroxyl group, locating at a hydrogen-bond distance from the Asp 215 carboxyl, protects this carboxyl from protonation in acid media.

sin (pdb code: 1am5; Karlsen et al. 1998) and cathepsin D crystallized at alkaline pH (pdb code: 1lyw; Lee et al. 1998), where this distance is larger than that of a hydrogen bond. In *Rhizomucor miehei* unbound protease (pdb code: 2asi; Yang et al. 1997), Thr 218 is present as an unusual rotamer, and its hydroxyl group does not form any hydrogen bond. The exceptions among complexes with inhibitors comprise *Saccharomyces cerevisiae* protease with the helical IA₃ inhibitor (pdb codes: 1dpj and 1dp5; Li et al. 2000) and mouse submaxillary renin complexed with CH-66 inhibitor (pdb code: 1smr; Dealwis et al. 1994), where Thr 218 is replaced by Ser 218.

This last exception deserves special attention. The replacement of threonine by serine at position 218 in mouse submaxillary renin cannot affect the ability of the hydroxyl to form a hydrogen bond with the Asp 215 carboxyl. However, our analysis has shown the Ser 218 hydroxyl to turn around the dihedral angle χ_1 to form a hydrogen bond with the main chain carbonyl oxygen of Phe 220 at the opposite side of Asp 215 in the complex of this renin with the inhibitor (pdb code: 1smr; Dealwis et al. 1994). The distance between the inhibitor and Ser 218 is too large to induce such reorientation. The nonstandard for these enzymes' rotamer of Thr 218 is present also in the complexes of *S. cerevisiae* protease with helical inhibitor IA₃ crystallized at pH 6.6 (pdb codes: 1dpj and 1dp5; Li et al. 2000). The presence of the same nonstandard rotamer of Thr 218 is the property of zymogens (the exception is proplasmepsin that has an arrangement of residues in the region of plasmepsin active site unusual for other zymogens (pdb code 1pfz; Bernstein et al. 1999)), as shown in Figure 2 (pdb code: 2psg, Sielecki et al. 1991; pdb code: 3psg, Hartsuck et al. 1992; pdb code, 1htr; Moore et al. 1995; pdb code: 1qdm, Kervinen et al. 1999),

and the intermediate of progastricsin activation (pdb code: 1avf, Khan et al. 1997).

Ser 35 is completely conserved in all pepsin-like enzymes. The position of this residue in the N-terminal domain is symmetrical to that of Thr 218 in the C-terminal domain; however, the interaction patterns formed by Ser 35 and Thr 218 are not quite similar. The proximity of the Ser 35 hydroxyl to the Asp 32 carboxyl found in porcine pepsin was observed in all complexes with inhibitors, but not in all active enzyme structures. The most conserved interaction of the Ser 35 hydroxyl is a hydrogen bond with the water molecule W2 (Fig. 1) found in all analyzed structures. Here, we show that this internal water molecule is a characteristic feature of all pepsin-like enzymes, their complexes with inhibitors, and zymogens. This characteristic feature first was revealed in our previous studies based on limited data (Andreeva et al. 1995; Kashparov et al. 1997); however, it has not been discussed in any other publication to our knowledge. In some structures, Ser 35 is present as a non-standard rotamer, but in all of them the Ser 35 hydroxyl is located within a hydrogen-bond distance from W2.

Besides Ser 35, water molecule W2 interacts with the carbonyl oxygen of a residue at the 37th position in pepsin numbering, and this bond is conserved. In complexes of enzymes with inhibitors, W2 also forms the third hydrogen bond with the hydroxyl of the conserved residue Tyr 75, as in porcine pepsin (Fig. 1), and the Tyr 75 hydroxyl itself accepts a hydrogen bond from the Trp 39 N^ε1 atom. These bonds provide the formation of a continuous chain of hydrogen-bonded residues, Trp 39–Tyr 75–W2–Ser 35–Asp 32, connecting the flap with the catalytic site. Summarizing our observations on the structure of the active site area in complexes of pepsin-like enzymes with inhibitors, we can

Fig. 2. The conformation of Thr 218 residue in porcine pepsinogen (pdb code: 3psg; resolution 1.65 Å, Hartsuck et al. 1992). In pepsinogen, a hydrogen bond between Thr 218 and Asp 215 is not possible because Asp 215 is involved in hydrogen bonds with Lys 36 and Tyr 37 of the propeptide. The hydroxyl group of Thr 218 can form only a weak hydrogen bond with water molecule W540, although it is close to Ser 219 NH group. In this structure, Thr 218 residue is present as a rotamer unusual for active enzymes.

advocate that the formation of this chain is their essential feature (an unique exception is the enlarged distance between the Ser 35 hydroxyl and Asp 32 carboxyl in the complex of saccharopepsin with 081282 inhibitor [pdb code: 2jxr, Aguilar et al. 1997]). We have revealed that in memapsin 2 (β -secretase), where tryptophan 39 is replaced by alanine, the Trp residue at the 80th position in pepsin numbering (Trp 76 in memapsin numbering) forms a hydrogen bond with the Tyr 75 hydroxyl as is shown in Figure

3 for the complex of this enzyme with the inhibitor OM99-2 (pdb code: 1fkn, Hong et al. 2000). The temperature factors for all members of this chain, including water molecule W2, decrease markedly on ligand binding in all enzymes. The greatest changes are observed for Ser 35, W2, and the Tyr 75 hydroxyl (Table 1).

The continuous chain of hydrogen-bonded residues also was observed in several crystal structures of active enzymes, and porcine pepsin is the example. At the same time,

Fig. 3. The catalytic site and the surrounding region of inhibited memapsin 2 by OM 99-2 inhibitor (pdb code: 1fkn; resolution 1.9 Å, Hong et al. 2000). The figure shows the conserved nature of the chain of hydrogen bonds connecting the flap with the active site, which forms on inhibitor binding. Trp 80(76) plays the role of Trp 39, replaced in memapsin by Ala.

Table 1. Temperature *B* factors of atoms in Å² involved in continuous chain of hydrogen-bonded residues

Enzyme	PDB code	Ser35 O ^γ	W2	Tyr75 OH	Trp35 N ^{ε1}
PP	3app	21.25	31.68	30.56	10.46
PP-complex	1apt	6.76	5.86	12.28	6.60
PP-complex	1apu	9.56	10.12	19.40	6.89
PP-complex	1apv	3.86	8.40	9.67	7.10
PP-complex	1apw	5.29	10.24	9.70	9.27
PP-complex	1ppk	7.08	10.71	10.53	8.35
PP-complex	1ppl	5.03	10.37	7.22	4.87
PP-complex	1ppm	9.76	14.39	11.00	9.54
PR	2apr	11.32	15.38	33.16	13.71
PR-complex	3apr	9.20	9.68	14.11	10.19
PR-complex	4apr	6.67	2.00	19.00	5.84
PR-complex	5apr	9.52	8.68	22.07	9.66
PR-complex	6apr	6.24	6.46	13.68	7.29

PP, penicillopepsin; PR, rhizopuspepsin.

in penicillopepsin (pdb code: 3app, James and Sielecki 1983) and in some other active enzyme structures, the flap is not fixed to the interior of the molecule by the intramolecular interactions, and the continuous chain of hydrogen-bonded residues is not formed (Fig. 4). In penicillopepsin,

the distances from the Tyr 75 hydroxyl to W2 and Trp 39 are 4.83 and 3.61 Å, respectively. The position of the flexible flap in crystals of active enzymes is often dependent on molecular packing. However, in any case, the flap in free enzymes has at times to adopt in solution a conformation allowing a polypeptide substrate to occupy the cleft. At these moments, it cannot be fixed to the interior of molecules by the chain of intramolecular hydrogen bonds.

One more structural property of active enzymes should be mentioned. In these structures, the absence of the hydrogen bond of Tyr 75 with W2 and Trp 39 correlates every time with the enlarged distance between the Ser 35 hydroxyl and the Asp 32 carboxyl, preventing the formation of a hydrogen bond.

Discussion

The accepted mechanism of pepsin-like enzyme function assumes that one of the active aspartic acid residues, acting as a general base (Asp 215), has to be charged, whereas the other one, acting as a general acid (Asp 32), has to be protonated (Suguna et al. 1987b; Davies 1990; James et al.

Fig. 4. The catalytic site and the surrounding region of active penicillopepsin (pdb code: 3app; resolution 1.8 Å, James and Sielecki 1983) representing the arrangement of residues in the active site area, when the flap is close to the open position. The flap does not form contacts with the Trp 39 O^{ε1} atom or water molecule W2. At the same time, the distance between the Ser 35 O^γ atom and Asp 32 carboxyl is too large for the formation of a hydrogen bond.

1992; Parris et al. 1992; Veerapandian et al. 1992). The same structure of the catalytic site and the surrounding region in all pepsin-like enzymes implies a universal mechanism of their action. It means that the situation when the Asp 215 carboxyl is charged and the Asp 32 carboxyl is protonated should hold in any conditions. Therefore, the first important role of residues adjacent to the catalytic centre of pepsin-like enzymes is to preserve the charged state of Asp 215 and the protonated state of Asp 32.

Are structural data consistent with this role? In all known structures of enzymes functioning at acidic pH, the Thr 218 hydroxyl takes up a guard position near the Asp 215 carboxyl at a distance of a hydrogen bond, as in porcine pepsin (Fig. 1). This hydrogen bond utilizes the anti lone pair electrons of the outer O^{δ2} oxygen, while the syn lone pair of this oxygen is engaged in the hydrogen bond with the water molecule W1. As a result, the ability of the outer oxygen of Asp 215 to bind protons from bulky solvent becomes rather low. The analogous role of Thr 218 was proposed first in studies of chymosin mutant (Mantafounis and Pitts 1990) and discussed in the work on endotheiapepsin mechanism (Veerapandian et al. 1992). The efficiency of such a mechanism of charge protection is shown by the activity of pepsin at pH 1.0. The preservation of the charged state of several carboxyl groups at low pH is the feature of pepsin (Sielecki et al. 1990; Andreeva and James 1991), but in the case of the active Asp 215 carboxyl it should be the property of all pepsin-like enzymes acting in acidic media. Therefore, the rearrangement of the Thr 218 hydroxyl group by changing the χ_1 torsion angle and the formation of the hydrogen bond with the Asp 215 carboxyl is an important step in the activation of pepsinogen and other zymogens (cf. Figs. 1 and 2).

The absence of a hydrogen bond between the residue at the 218 position and the Asp 215 carboxyl in all renins because of the change of Thr 218 to Ala or to a nonstandard rotameric form of Ser 218 excludes the proposed mechanism of the charge protection. However, these enzymes act at neutral pH, and additional stabilization of the charged state of the Asp 215 carboxyl is not significant for them. In contrast, for pepsin, chymosin, and rhizopuspepsin, the ability of Thr 218 Ala mutants to keep the charged state of the Asp 215 carboxyl decreases substantially on lowering the pH, which results in a decrease of activity, shown in a decrease of k_{cat} . Some residual activity of the mutants can be explained by the occasional appearance of a charge at the Asp 215 carboxyl owing to fluctuation processes.

In previous studies, the role of Thr 218 and Ser 35 was considered the same, because of the symmetrical arrangement of these residues in relation to the active carboxyls. However, the absence of a hydrogen bond between the Ser 35 hydroxyl and the Asp 32 hydroxyl in free enzymes (Fig. 4) can make the role of Ser 35 opposite that of Thr 218, as it does not impede the protonation of Asp 32 in acidic media. At the same time, the functional properties of the Ser

35 residue, which, unlike Thr 218, is absolutely conserved in all pepsin-like enzymes regardless of their acting media, seem to be more complex than that of Thr 218.

Structural data suggest the existence of a mechanism assisting the proton leaving from the Asp 32 carboxyl at the initial stage of the catalysis and proton acceptance after substrate cleavage. It follows from the ability of the Ser 35 hydroxyl and the water molecule W2 to exchange their donor and acceptor roles while being hydrogen-bonded (Fig. 5a,b). If the relative orientation of Ser 35 and W2 is such as shown in Figure 5a, where W2 donates a proton to the Ser 35 hydroxyl, then this hydroxyl donates a proton to the anti lone pair electrons of the Asp 32 outer O^{δ1} carboxyl oxygen, thus enhancing its acidic properties. However, in the opposite situation (Fig. 5b), W2 accepts a proton from the Ser 35 hydroxyl. As a result, Ser 35 does not prevent the protonation of O^{δ1} atom. In the case of unbound enzymes acting at neutral pH, the captured proton can be trapped between Asp 32 and Ser 35, which stabilizes the protonated state of the active Asp 32 carboxyl. Although this property is not yet experimentally proven for unbound pepsin-like enzymes acting at elevated pH, we emphasize that such a possibility follows from the structure.

The high temperature factor of W2 in free enzymes suggests that this water molecule can have rotational freedom (Table 1). What happens when the substrate binds in the active site cleft? When the flap takes up the closed conformation, the hydroxyl of Tyr 75 approaches the Trp 39 N^{ε1} atom and W2. The hydrogen bond formed between the Tyr 75 hydroxyl and W2 fixes the orientation of this water molecule. As Trp 39 donates its proton to the Tyr 75 hydroxyl, it forces Tyr 75 to become a proton donor to W2, making this water donate protons to the Ser 35 hydroxyl and the carbonyl oxygen of the residue at the 37th position. The Ser 35 hydroxyl, approaching the Asp 32 carboxyl after substrate binding, turns to donate a proton to the O^{δ1} carboxyl oxygen of Asp 32 (Figs. 5a and 6b), enhancing its acidic properties and assisting the transfer of a proton from the Asp 32 carboxyl to the carbonyl oxygen of the scissile bond. This bond is concomitantly attacked by W1 polarized into a nucleophilic state by the charged Asp 215 carboxyl (Fig. 6). The same consideration is relevant to memapsin, in which Trp at the 80th position in pepsin numbering plays the role of Trp 39 (Fig. 3). The chain of the hydrogen bonds, Trp 39–Tyr 75–W2–Ser 35, acts as a bridge for transmitting a signal from the flap to the active site. In memapsins, this chain of hydrogen bonds is formed by Trp 80(76)–Tyr 75(71)–W2–Ser 35.

After substrate cleavage, a proton should be accepted by the Asp 32 carboxyl to return the active site to its initial state. The Ser 35 hydroxyl can assist this process if it alters a proton orientation toward W2. It is possible that W2 also changes its orientation, which may be initiated by the movement of the flap (Tyr 75) after substrate cleavage and re-

Fig. 5. (a) Scheme showing the arrangement of hydrogen bonds in the active site area of pepsin-like enzymes during formation of enzyme-substrate complex when Tyr 75 approaches Trp 39 and water molecule W2. At the same time, Ser 35 becomes close to the Asp 32 carboxyl. Trp 39 donates proton to the Tyr 75 hydroxyl and forces it to donate proton to water molecule W2. As a result, Ser 35 becomes a proton donor to the Asp 32 carboxyl, enhancing its acidic properties (Asp 32 presumes to leave its proton participating in formation of the gem-diol structure of the tetrahedral intermediate). Hydrogen-bond distances shown in both *a* and *b* are not relevant to any structure. In the scheme, they show only the presence of hydrogen bonds. (b) Scheme showing a possible reorientation of water molecule W2 after moving of Tyr 75 out of the interior of a molecule. Reorientation of the proton of the Ser 35 hydroxyl results in some increase of basic properties of the Asp 32 carboxyl, which helps to return its initial protonated state after the catalytic reaction.

lease of the C-terminal part of the product. One can suppose that these movements are concerted, and they force Ser 35 to become a proton donor to W2. Therefore, the W2 molecule, returning a rotational degree of freedom, may act as a switch, utilizing consecutively its donor and acceptor properties in a hydrogen bond with Ser 35 during the catalytic cycle.

Analogously, Thr 218 can assist proton acceptance by the Asp 215 carboxyl from the gem-diol unit of the tetrahedral intermediate if the hydrogen bond Thr 218–Asp 215 becomes weaker after substrate binding. In the unique work on the experimental localization of hydrogen positions in transition state analogs of penicillopepsin (pdb code: 1bxo, Khan et al. 1998), the hydrogen of the Thr 218 hydroxyl is turned away from the Asp 215 carboxyl. The Thr 218 hydroxyl also can form a hydrogen bond with a substrate in some complex structures (Fraser et al. 1992; Aguilar et al. 1997).

This consideration shows that the formation of a chain of hydrogen-bonded residues Trp 39–Tyr 75–W2–Ser 35–Asp 32 (or Trp 80(76)–Tyr 75(71)–W2–Ser 35–Asp 32 in memapsins) can be an important step for the reaction cata-

lyzed by pepsin-like enzymes. The breakage of this chain by a mutation of any of these residues results in a decrease of enzymatic activity (k_{cat}). An increase in the catalytic efficiency of *R. pusillus* protease after the replacement Tyr 75 Asn suggests that the chain of hydrogen bonds remains on this mutation.

Of course, the possibility of directly verifying these hypotheses is limited by only one known structure of pepsin-like enzyme, containing experimental data on hydrogen positions, except for water molecules and carboxyl groups (pdb code: 1bxo, Khan et al. 1998). Theoretically, calculated hydrogen positions included in some structures are obviously not suitable for the purpose. However, the many self-consistent data support the chosen approach to explaining the role of residues adjacent to the catalytic site of pepsin-like enzymes. They also show how the structure of the active site can be adapted for the function in a wide range of pH from 1.0 up to 7.0. A large variety of conditions in living organisms, in which pepsin-like enzymes should act, made the development of interactions helping their function very important. The physiological adaptation of the same structural motif for the action in diverse conditions

Fig. 6. Formula representation of the two steps of the catalytic reaction, showing the interaction of the catalytic groups with the surrounding residues. (a) The initial step before substrate binding. (b) Tetrahedral intermediate (Davies 1990; Parris et al. 1992; James et al. 1992; Veerapandian et al. 1992) and its interaction with groups, adjacent to the catalytic site. We suppose the hydrogen bond of the Asp 215 carboxyl with the Thr 218 carboxyl is weakened after substrate binding, while the bond of the Asp 32 carboxyl with the Ser 35 hydroxyl becomes stronger.

can be a gain of evolution. Simple homodimeric structures of retroviral aspartic proteases do not possess such a regulating system; their function in cells is limited to a narrow interval of pH common to all of them not far from the pK_a of carboxyl groups, and the asymmetry of the active site arises after substrate binding.

Materials and methods

The three-dimensional structures of pepsin-like enzymes deposited with Protein Data Bank (Bernstein et al. 1977; Berman et al. 2000) and used in this work are listed in Table 2. Data corresponding to the highest resolution are analyzed for proteins studied by various groups of authors. Medium resolution data are not considered. The internal coordinate system (ICS) approach is applied to compare three-dimensional structures (Andreeva and Pechik 1995). (The exception is the coordinate file If34 corresponding to the complex of porcine pepsin with *Ascaris* inhibitor.).

ICS is the right-handed orthogonal internal coordinate system based on PDB atomic coordinates of three reference points $p_1(x_1, y_1, z_1)$, $p_2(x_2, y_2, z_2)$, and $p_3(x_3, y_3, z_3)$ corresponding to positions of certain atoms, identical in compared structures. The conversion of PDB atomic coordinates into ICS coordinates can be performed by the standard coordinate translation and rotation procedures. In the program used, the origin of ICS is placed to the position of the first point. The first coordinate axis coincides with the vector, connecting the first and the third reference points. The

direction of the second coordinate axis corresponds to the vector perpendicular to the plane, containing all reference points. The third axis is defined as the vector product of the first two.

The relations of ICS ($X^{ics}, Y^{ics}, Z^{ics}$) and PDB (x, y, z) coordinates are determined by the following formula:

$$X^{ics} = (x - x_1)\cos\alpha_1 + (y - y_1)\cos\beta_1 + (z - z_1)\cos\gamma_1$$

$$Y^{ics} = (x - x_1)\cos\alpha_2 + (y - y_1)\cos\beta_2 + (z - z_1)\cos\gamma_2$$

$$Z^{ics} = (x - x_1)\cos\alpha_3 + (y - y_1)\cos\beta_3 + (z - z_1)\cos\gamma_3$$

where $\cos\alpha_1, \dots, \cos\gamma_3$ are direction cosines of the internal system coordinate axes in relation to the PDB axes. A good use of the ICS depends on the strategy of reference points selection and the quality of analyzed structures.

One of the advantages of the ICS approach is the convenience of the work with a large family of homologous proteins. A set of atomic coordinate files for their structures, converted to common ICS system, forms a mini ICS bank. Atomic positions in any new structure described in terms of the ICS coordinates become automatically superimposed, including water molecules, with all structures of the family presented in the ICS bank.

The conception of the internal coordinate system, its advantages and pitfalls for comparison of protein structures, and the strategy for the appropriate selection of reference points are described in the previous publication (Andreeva and Pechik 1995). The internal coordinate system used in the current work is based on the position of origin close to the active site.

Visual analysis of compared structures was performed with WebLabViewer (WebLabViewer Pro 3.0; Molecular Simulations Inc.) and Swiss-PdbViewer (Swiss-PdbViewer 3.5, Glaxo Wellcome Research and Development S.A.) programs.

Table 2. Atomic coordinate PDB files of pepsin-like proteases

Protein	PDB ID	Resolution (Å)	Reference
Zymogens			
Porcine pepsinogen	2psg	1.8	Sielecki et al. 1991
Porcine pepsinogen	3psg	1.65	Hartsuck et al. 1992
Human progastricsin (hpge)	1htr	1.62	Moore et al. 1995
Intermediated of hpge activ.	1avf	2.4	Khan et al. 1997
Prophytepsin	1qdm	2.3	Kervinen et al. 1999
Proplasmepsin	1pfz	1.85	Bernstein et al. 1999
Pepsins			
Human pepsin (hp)	1psn	2.2	Fujinaga et al. 1995
Complexes of hp with:			
Pepstatin	1pso	2.0	Fujinaga et al. 1995
Phosphonate inhibitor	1qrp	1.96	Fujinaga et al. 2000
Porcine pepsin (pp)			
Porcine pepsin monoclinic	3pep	2.3	Abad-Zapatero et al. 1990
Porcine pepsin monoclinic	4pep	1.8	Sielecki et al. 1990
Porcine pepsin hexagonal	5pep	2.3	Cooper et al. 1990
Complexes of pp with:			
Inhibitor of renin	1psa	2.9	Chen et al. 1992
<i>Ascaris</i> inhibitor-3	1f34	2.45	Ng et al. 2000
Atlantic cod pepsin	1am5	2.15	Karlsen et al. 1998
Chymosin			
Bovine chymosin	1cms	2.3	Gilliland et al. 1990
Bovine chymosin	4cms	2.2	Newman et al. 1991
Bovine chymosin mutant	3cms	2.0	Štrop et al. 1990
Complex with reduced peptide	1czi	2.3	Groves et al. 1998
Cathepsin D			
Human cathepsin D	1lya	2.5	Baldwin et al. 1993
Complex with pepstatin	1lyb	2.5	Baldwin et al. 1993
Cathepsin D at alkaline pH	1lyw	2.5	Lee et al. 1998
Renin			
Human renin tetragonal	2ren	2.5	Sielecki et al. 1989
Human renin cubic	1bbs	2.8	Dhanaraj et al. 1992
Complexes with renin			
1908 inhibitor	1bil	2.4	Tong et al. 1995a
2151 inhibitor	1bim	2.8	Tong et al. 1995a
GP 38,560 inhibitor	1rne	2.4	Rahuel et al. 1991
Polyhydroxymonoamide 980	1hrn	1.8	Tong et al. 1995b
Mouse renin with CH-66 inh.	1smr	2.0	Dealwis et al. 1994
Memapsin (β-secretase)			
Complex with OM99-2 inh	1fkn	1.9	Hong et al. 2000
Rhizopuspepsin			
Rhizopuspepsin	2apr	1.8	Suguna et al. 1987a
Complexes with:			
Reduced peptide inhibitor	3apr	1.8	Suguna et al. 1987b
Renin inhibitor	4apr	2.5	Suguna et al. 1992
Renin inhibitor	5apr	2.1	Suguna et al. 1992
Pepstatin	6apr	2.5	Suguna et al. 1992
Penicillopepsin			
Penicillopepsin	3app	1.8	James and Sielecki 1983
Complexes with:			
Iva-Val-Val-Lyssta-OEt	1apt	1.8	James et al. 1985
Iva-Val-Val-Sta-Oet	1apu	1.8	James et al. 1982
Difluorostatine inhibitor	1apv	1.8	James et al. 1992
Difluorostatone inhibitor	1apw	1.8	James et al. 1992
Iva-Val-Val-Sta(P)-Oet	1ppk	1.8	Fraser et al. 1992
Iva-Val-Val-Leu(P)-O(Phe)-OMe	1ppl	1.7	Fraser et al. 1992
Cbz-Ala-Ala-Leu(P)-OPhe-OMe	1ppm	1.7	Fraser et al. 1992
Macrocyclic inhibitor PPI4	1bxo	0.95	Khan et al. 1998
Macrocyclic inh.1 at 100°K	2wea	1.25	Ding et al. 1998
Macrocyclic inh.1 at 293°K	2wed	1.5	Ding et al. 1998
Acyclic deriv. (3) of inh.1	2web	1.5	Ding et al. 1998

(continued)

Table 2. Continued

Protein	PDB ID	Resolution (Å)	Reference
Acyclic deriv. (4) of inh.1	2wec	1.5	Ding et al. 1998
Macrocyclic inh.PPi3	1bxq	1.41	Khan et al. 1998
Endothiapepsin			
Endothiapepsin	4ape	2.1	Blundell et al. 1990
Complexes with:			
Inhibitor PD-125754	1eed	2.0	Cooper et al. 1992
Inhibitor Pd 130328	1ent	1.9	Bailey and Cooper 1994
Inhibitor PS1	1epl	2.0	Bailey and Cooper 1994
Inhibitor PS2	1epm	1.6	Bailey and Cooper 1994
Inhibitor CP-80, 794	1epn	1.6	Bailey and Cooper 1994
Inhibitor CP-81, 282	1epo	2.0	Veerapandian et al. 1992
Inhibitor PD-130693	1epp	1.91	Lunney et al. 1993
Inhibitor PD-133,450	1epq	1.9	Lunney et al. 1993
Inhibitor PD-135,040	1epr	2.3	Bailey and Cooper 1994
Inhibitor H-77	1er8	2.0	Cooper et al. 1989
Inhibitor L-364,099	2er0	3.0	Cooper et al. 1989
Inhibitor H-256	2er6	2.0	Cooper et al. 1987
Inhibitor H-261	2er7	1.6	Veerapandian et al. 1990
Inhibitor L-363,564	2er9	2.2	Cooper et al. 1989
Inhibitor CP-71,362	3er3	2.0	Bailey and Cooper 1994
Inhibitor H-189	3er5	1.8	Bailey et al. 1993
Inhibitor PD-125,967	4er1	2.0	Cooper et al. 1992
Inhibitor pepstatin A	4er2	2.0	Bailey et al. 1993
Inhibitor H-142	4er4	2.1	Foundling et al. 1987
Inhibitor BW 624	5er1	2.0	Cooper et al. 1988
Inhibitor CP-69,799	5er2	1.8	Šali et al. 1989
Rhizomucor proteases			
<i>Rhizomucor miehei</i> proteinase	2ast	2.15	Yang et al. 1997
Complex with pepstatin	2rmp	2.7	Yang and Quail 1999
<i>Mucor pusillus</i> proteinase	1mpp	2.0	Newman et al. 1993
Saccharopepsin			
Saccharopepsin complexes with			
Inhibitor 081282	2jxr	2.4	Aguilar et al. 1997
Helical inhibitor IA ₃	1dp5	2.2	Li et al. 2000
Truncated inhibitor IA ₃	1dpj	1.8	Li et al. 2000
Plant enzyme cardosin A	1b5f	1.72	Frazao et al. 1999
Plasmepepsin complexes with pepstatin:			
From <i>Plasmodium vivax</i>	1qs8	2.5	Khazanovich-Bernstein et al. (in prep.)
From <i>Plasmodium falciparum</i>	1sme	2.7	Silva et al. 1996
Candidapepsin complexes			
With inhibitor A70450	1eag	2.1	Cutfield et al. 1995
With inhibitor A70450	1zap	2.5	Abad-Zapatero et al. 1996

Acknowledgments

We are grateful to Dr. A.Yu. Borisov, L.M. Ginodman, I.V. Kashparov, and I.V. Pechik for helpful discussions. We thank Dr. I.V. Pechik for the help with the figures. The work was supported by the Grants of Russian Foundation of Fundamental Researches N 99-04-49241 and the Grant of Ministry of Science of Russian Federation N 00-15-97835.

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Note added in proof

Extraordinary structural properties of free chymosin molecule, where Tyr75 was found to occlude S1/S3 substrate binding pock-

ets, are not discussed in this paper, being a subject of special studies.

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