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Catalytic Pathways of Aspartic Peptidases

Introduction

The history of the carboxyl peptidases, initially encompassing only the enzymes containing catalytic aspartates, is a long one with many fascinating turns of event. This history has been told beautifully in a review by Fruton [1], whereas a review of the historical aspects of the structural results on aspartic peptidases has been presented by Blundell et al. [2].

The early mechanistic history of aspartic peptidases was fraught with problems revolving around the question of whether or not the intermediates on the hydrolytic pathway were covalently attached to the enzyme [3,4]. It was Fruton who first suggested that the intermediate in the hydrolytic reaction may not be covalently attached to pepsin as an acyl nor as an amino enzyme [5]. Subsequent work on the structural aspects of several aspartic peptidases confirmed that access to the two active
site aspartate residues from which this family of peptidases derives its name (Asp32 and Asp215 in pepsin numbering that will be used consistently in this chapter, rather than the numbers from the original publications), was very restricted due to the limited solvent accessibility of these residues [6,7].

Aspartic peptidases are either cellular, pepsin-like enzymes (clan AA, family A1), or homodimeric enzymes originally identified in retroviruses (retropepsins, clan AA, family A2), but later discovered also in higher organisms. Enzymatic properties of these peptidases will be discussed here in greatest detail. Some other enzymes were also considered at one time to be aspartic peptidases and were historically listed together with pepsins, but were later shown to have unique catalytic mechanisms that utilize the side chains of glutamic acid and glutamine. Enzymatic mechanisms of the latter family of enzymes will also be discussed in this chapter, albeit briefly.

**Enzymatic Mechanism of Aspartic Peptidases**

The substrate binding cleft of the cellular aspartic peptidases extends for 7 or 8 amino acid residues (approximately from P5 to P3′ — Figure 2.1) and is formed where the N-terminal domain and the C-terminal domain meet. Centrally located in this cleft are the two aspartic acid residues, each surrounded by virtually identical supporting motifs that are related by an approximate 2-fold axis running between the carboxyl groups of the two aspartates. The two aspartates (32 and 215) are commonly found in the Asp-Thr/Ser-Gly-Thr/Ser motif that is important for establishing and maintaining the proper environment for the carboxyl groups. The clefts in retropepsins are slightly shorter, usually accommodating only residues from P3 to P3′ [8].

A schematic diagram of the most important active site residues in pepsins is shown in Figure 2.2A, whereas the corresponding hydrogen bonding interactions in selected uninhibited forms of pepsins and retropepsins are summarized in Table 2.1. The carboxyl groups of the two aspartate residues are approximately co-planar and the water molecule that is bound between them lies in the common plane. In spite of the different resolution of the several determinations and the differences in the pH of the crystals, the distances are sufficiently similar for common bonds to be averaged. The distance between the two inner oxygen atoms of the aspartates (A) is greater than or equal to the sum of their van der Waals radii (2 × 1.4 Å). This does not necessarily imply the presence of a hydrogen bond between the two aspartates. Distances B and C are approximately equal suggesting that one of the protons on the water molecule sits midway between the two oxygen atoms in a bifurcated hydrogen bond. Distances D and E differ by 0.45 Å and are consistent with a proton on the outer oxygen of Asp32 and a hydrogen bond from the second water proton to the inner oxygen of Asp32 (O...O distance = 2.76 Å).

The simplest interpretation of these hydrogen-bonding distances is shown in Figure 2.2B. In this interpretation of the active site of the enzyme in the ground state, Asp215 would be negatively charged and Asp32 would be a neutral carboxylic acid. The active center of aspartic peptidases has two pKa values, one of ~1.5 and the other ~4.5. The proton distribution shown in Figure 2.2B is consistent with established, experimental observations [4,9,10]. However, the requirement that the aspartates and the catalytic water be co-planar was recently questioned on the basis of a re-refined structure of plasmepsin II [11] in which considerable deviation from planarity was noticed. A suggestion was made that the planarity is

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**FIGURE 2.1** A view of the substrate binding cleft in endothiapepsin based on the atomic resolution crystal structure of a complex with the hydroxyethylene-containing inhibitor H261 (PDB code 1OEX — [38]). The surface of the enzyme is rendered and colored according to charge, whereas H261 (Boc-His-Pro-Phe-Ala-Lov-Ile-His) bound at the active site is shown as a ball-and-stick model. The residues from the flap make intimate contact with the P1 residue of the substrate (or the inhibitor). Hydrogen bonding and van der Waals interactions position the scissile peptide appropriately for nucleophilic attack by the general-base activated water molecule. Atomic color coding for the inhibitor: green, carbon; blue, nitrogen; red, oxygen.
enforced by the cryogenic temperature of data collection but not necessarily present under biologically relevant conditions. That argument, however, is counteracted by the many structures of aspartic peptidases solved at room temperature, including some at very high resolution and/or solved using neutrons rather than X-rays [12,13].

The enzymatic mechanism is most fully characterized for serine peptidases, thus providing a point of comparison with the mechanism of pepsins. A large number of structures of the serine peptidases show the detailed interactions of enzyme-substrate complexes [14]. These structures are protein inhibitors (e.g. turkey ovomucoid third domain [OMTKY3] [15] complexed to a variety of serine peptidases, e.g. α-chymotrypsin [16]. Streptomyces griseus peptidase B (SGPB) [17], etc. Although the protein inhibitors are excellent inhibitors of the serine peptidases, they are also poor substrates. The enzyme-protein inhibitor complexes are therefore reasonable mimics of enzyme-substrate complexes expected for peptide hydrolysis catalyzed by serine peptidases.

Comparisons of the serine peptidase-protein inhibitor complexes (α-chymotrypsin -OMTKY3) with the tetrahedral intermediate mimics of the aspartic peptidase hydrolytic reaction are very revealing (Figure 2.3). Figure 2.3A shows the structure of the Iva-Val-Val-difluorostatone methylester inhibitor bound in the active site of penicillopepsin alone [18]; Figure 2.3B is a superposition of this structure and OMTKY3 bound in the active site of α-chymotrypsin. A comparison of these views shows that the formation of the tetrahedral intermediate in both the serine peptidases and the

TABLE 2.1 Interatomic distances in the active sites of selected pepsin-like aspartic proteases and retropepsins. Only apoenzyme structures were used in the comparisons. Since the two catalytic aspartates should be symmetric in the structures of uninhibited retropepsins, the orientation in which distance E was longer than B was arbitrarily selected

<table>
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<tr>
<th>Pepsins</th>
<th>PDB</th>
<th>Resol.Å</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
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<tr>
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<td>2.89</td>
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<td>3.11</td>
<td>2.92</td>
<td>2.83</td>
<td>2.81</td>
<td>3.37</td>
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<td>2.90</td>
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</tr>
<tr>
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<td>2.3</td>
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<td>2.77</td>
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<td>3APP</td>
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<td>2.86</td>
<td>2.83</td>
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<tr>
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<td>2.96</td>
<td>3.15</td>
<td>2.97</td>
<td>3.38</td>
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<td>3.02</td>
<td>2.91</td>
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<td></td>
<td>2.95</td>
<td>2.89</td>
<td>2.90</td>
<td>2.76</td>
<td>3.21</td>
</tr>
</tbody>
</table>

| Retropepsins          |      |         | 2.98| 2.78| 2.92| 2.98| 3.33|
| RSV PR                | 2RSP | 2.0     | 2.81| 2.56| 2.69| 2.58| 3.18|
| XMRV PR               | 3NR6 | 2.0     | 3.07| 3.11| 2.73| 2.62| 3.23|
| HIV PR (subtype A)    | 3IXO | 1.7     | 3.07| 2.67| 3.33| 3.73| 3.59|
| Average               |      |         | 2.98| 2.78| 2.92| 2.98| 3.33|

ΔE-D=0.45 Å for pepsins, 0.35 Å for retropepsins
two carboxyl groups (Figure 2.2) and activated to an hydroxide ion by the general base carboxylate of Asp215. In the serine peptidases the nucleophile is the Oγ of Ser195 and the general base is the imidazole of His57.

The electrophilic component assisting in the nucleophilic attack of Ser195 Oγ is the oxyanion hole. This feature in the serine peptidases is made up of two backbone NH groups (of Ser195 and Gly193) that form strong hydrogen bonds to the carbonyl oxygen atom of the P1 residue in the scissile bond. This interaction enhances the polarization of the carbonyl group and increases the electropositive character of the carbonyl-carbon atom thereby assisting in the proton transfer from Ser195 Oγ to His57 Nε2 by reducing the pKα of Ser195. The analogous feature in the aspartic peptidases is comprised of two components: (1) the protonated carboxyl group of Asp32 that forms a hydrogen-bonded interaction with the carbonyl oxygen atom of the substrate in the E-S complex; and (2) an electrostatic interaction of the edge of the phenol ring from Tyr75 with the carbonyl oxygen of the substrate [18,20]. This latter interaction is speculative, but support for a catalytic function for Tyr75 comes from mutagenesis experiments with Rhizomucor pusillus pepsin in which it was replaced by 17 other amino acids and the catalytic activity of these variants assessed [21]. The only active tyrosine replacements were Phe75 (weak activity) and Asn75 (enhanced activity — kcat/Km 5.6 times higher than that of Tyr75). Presumably the side chain amide of Asn75 forms a hydrogen bond with the scissile peptide carbonyl oxygen, thereby enhancing the polarization of the carbonyl bond and the electrophilicity of the carbonyl carbon atom of the scissile peptide.

Following the nucleophilic attack on the re face of the scissile peptide by the general-base activated water molecule (OH−), the resulting tetrahedral intermediate adopts a gem-diol hydrogen-bonded interaction with the now negatively-charged carboxylate of Asp32 (Figure 2.4B). Independently determined crystal structures of penicillopepsin [18] and of endothiapepsin [22] complexed with hydrated difluoroketones confirm the possibility of such a gem-diol association for the tetrahedral intermediate.

At this stage in the mechanism cleavage of the peptide bond requires protonation of the leaving group nitrogen atom. Reference to the chemical hydrolytic pathway in Figure 2.4B shows that application of the stereoelectronic effect results in the lone pair electrons on the nitrogen of the scissile bond pointing away from Asp215. In order for proton transfer to take place the lone pair on the nitrogen should point towards the protonated carboxyl group of Asp215. This can take place by inversion of the absolute configuration of the nitrogen atom, a process that is extremely rapid [23].

aspartic peptidases results from nucleophilic attack on the carbonyl carbon of the scissile peptide from the re face of the peptide [19]. The nucleophile of the aspartic peptidases is the water molecule bound between the
2. Catalytic Pathways of Aspartic Peptidases

Transfer of the proton of the leaving group nitrogen (Figure 2.4C) is coupled to the regeneration of the protonated state of the carboxyl of Asp32. It is also coupled to cleavage of the peptide bond in a concerted fashion. Thus, the protonated carbonyl oxygen transfers the proton to the carboxylate of Asp32. The carbonyl of the newly formed carboxyl results in addition to the peptide NH-C=O cleavage that would result on protonation of the nitrogen.

Several crystal structures of transition state intermediates (i.e., phosphonates) bound to penicillopepsin and endothiapepsin contain low barrier hydrogen bonds (LBHB) between the outer carboxyl oxygen of Asp32 and the phosphonate oxygen atom [13,24–26]. This bond is short (2.42–2.54 Å) and would qualify for a high energy LBHB. Release of the energy of this LBHB would assist in the peptide bond cleavage by lowering the activation energy barrier of the reaction and would return the enzymes active site to the native state. It is likely that an equivalent low barrier hydrogen bond between the analogous oxygen atoms is present in the transition state on the hydrolytic pathway of a good substrate. Such a bond would have a strong effect on the two proton transfers required during the bond cleavage reaction (Figure 2.3C, D). As the proton of the LBHB becomes associated more with Asp32, the energy would be used to facilitate the proton transfer from Asp215 to the leaving group nitrogen as well as lowering the activation energy barrier for peptide bond cleavage. It is imperative that the LBHB occurs in the transition state as it does in this mechanism and as suggested by Cleland [27].

The LBHB mentioned above is different from the putative LBHB between the carboxyl groups of Asp32 and Asp215, previously invoked to explain the kinetic mechanism of aspartic peptidases [28]. That argument hinged on a ten atom hydrogen-bonded cyclic structure for the water molecule and the two carboxyl groups of the active site (see Figure 2.5) and was principally based on ab initio molecular dynamic simulations on HIV-1 protease [29]. An LBHB between the two catalytic aspartates would require the distance between their inner oxygens to be shorter than 2.5 Å [27]. As shown in Table 2.1, there is no case in which such distance (A) is shorter than 2.76 Å (average 2.95 Å), thus it represents a normal oxygen-oxygen contact. In addition, for the LBHB to be effective in lowering the activation

![Figure 2.4](image1)

**FIGURE 2.4** (A) Stylized representation of the enzyme-substrate complex. The substrate approaches the active site with the P1 side chain in the S1 binding pocket. Such models were determined from a large number of crystal structures of aspartic peptidases complexed to transition-state peptidomimetics. For the present discussion, an accurate model of an E-S complex of an aspartatic peptidase has been deduced on the basis of superimposing the tetrahedral transition state mimics with the complexes of the serine peptidases with protein inhibitors such as OMTKY3 (Figure 2.3B). The nucleophilic water of the aspartic peptidases is positioned appropriately for attack on the carbonyl-carbon atom of the scissile peptide. General base assistance is achieved via the ionized carboxylate of Asp215; electrophilic assistance comes from a short hydrogen bond (LBHB) from the protonated carboxyl of Asp32. In this mechanism the proton is transferred from Asp32 to the carbonyl-oxygen atom of the scissile peptide. It could, however, be an LBHB and reside midway between the two oxygen atoms. (B) Tetrahedral intermediate. The hydroxyl attack on the carbonyl-carbon atom is assisted by the stereoelectronic effect [39] in that lone pair electrons on the carbonyl oxygen atom and on the nitrogen atom of the scissile peptide are arranged antiperiplanar to the direction of hydroxide ion attack (black lone-pair orbitals). The carboxylate of Asp32 receives hydrogen bonds from the gem diol of the tetrahedral intermediate. The tetrahedral intermediate is also stabilized by the proximity of the positive edge of the phenol ring of Tyr75 (not shown here). (C) Model for protonation of leaving group. In order for the peptide bond to break, the nitrogen of the scissile bond (now a tetrahedral sp3 nitrogen atom) must be protonated to make a good leaving group (—NH2). In order to facilitate the protonation from Asp215 the nitrogen atom must invert its configuration from that in (B) to place the lone-pair orbital appropriately to receive the proton. Cleavage of the peptide is also enabled by the antiperiplanar lone pair electrons (black orbitals) on each of the oxygens in the gem diol. (D) The bond cleavage. The proton transfer from the outer oxygen atom of the gem diol to Asp32 carboxylate (general base in this second case) results in the peptide bond cleavage to release a new free amino group (R'-NH3) and a new free carboxyl group (R-COOH). Dissociation of these groups and reassociation of the active site carboxyl groups with a water molecule regenerates the active form of the enzyme (Figure 2.1B).

![Figure 2.5](image2)

**FIGURE 2.5** A proposed configuration of the two aspartic acid residues and the bound water in the HIV peptidase structure as deduced from *ab initio* molecular dynamics simulations [29]. There is no evidence for the low barrier hydrogen bond between the carboxyl groups from any of the X-ray or neutron crystallographic studies done on aspartic peptidases or their inhibitor complexes (see Table 2.1).
energy barrier of the reaction being catalyzed, it should not occur in the ground state but rather should be formed in a transition state or in a transient intermediate. However, none of the structures of transition-state mimics, i.e. the hydrated difluorostatone [18] discussed here or the phosphonate inhibitors of penicillopepsin [24–26] have a short distance between the inner carboxylate oxygens of the catalytic aspartates.

Finally, atomic resolution X-ray and medium resolution neutron diffraction studies of endothiapepsin complexed with a transition state analog inhibitor, PD-135,040, has determined with reasonable certainty the protonation states of the active site groups [13]. Asp32 is best interpreted as ionized, i.e. negatively charged, and Asp215 is protonated. This charge distribution corresponds exactly with that of the chemical mechanism deduced from the many crystallographic structures and depicted in Figure 2.4B,C. The neutron structure determination has no evidence of a proton between the inner oxygen atoms of Asp32 and Asp215. The distance between these atoms is 2.93 Å, again confirming that there is no LBHB between the active site aspartate residues.

The mechanism of retropepsins follows most likely the mechanism described above for the pepsin-like enzymes. The main difference is that there cannot be any distinction between the two aspartates (Asp25 in the enzyme from the human immunodeficiency virus) in the native enzyme, since it is a symmetric homodimer. However, the symmetry is broken as soon as a substrate is bound, allowing one of the aspartates to assume the function of Asp32, and the other of Asp215. Structural data for the complexes of retropepsins with inhibitors support this assumption, and indeed the mechanism employed by this family of peptidases was discussed in some detail in the past [30].

Histo-aspartic protease from Plasmodium falciparum presents an unusual variant of aspartic peptidases. Despite the fold being similar to other pepsin-like enzymes [31], Asp32 is replaced in this enzyme by histidine, and several other active site residues also differ from the consensus. Whereas the detailed mechanism of enzymatic activity of histo-aspartic protease is still not fully established, modeling and computational studies suggested that Asp215 might act as both a nucleophile and an electrophile, whereas the role of His32 would be only to assist in the reaction and increase the rate by up to four orders of magnitude [32].

On the other hand, the mechanism of peptide bond hydrolysis in the aspartic peptidase family of enzymes seems to be reasonably well established [18,33,34]. The majority of the details have been gleaned from structural studies of these enzymes with transition state mimics bound to the active sites. Although proton locations cannot be inferred with confidence from the X-ray crystal structures, the donor–acceptor distances are consistent with the mechanism depicted in Figure 2.4. In this mechanism, the hydroxide ion is the nucleophile and its formation is assisted by the general-base function of Asp215. The tetrahedral intermediate resulting from the nucleophilic attack on the carbonyl carbon atom is stabilized by gem diol hydrogen bonding to Asp32. One of these hydrogen bonds is a LBHB whose energy can be used to lower the activation energy of the peptide bond cleavage reaction. The leaving group nitrogen is protonated by the general acid function of protonated Asp215.

**Enzymatic Mechanism of Glutamic Peptidases**

The three-dimensional structures of several enzymes known to utilize carboxyl-bearing residues in their catalytic mechanism, yet not being inhibited by pepstatin A, have shown that their overall structures and the catalytic centers were not related to those of pepsins. This new family of carboxyl peptidases, now designated clan GA, family G1, was identified in 2004, with the founding members being scylalidocarboxyl peptidase B (SCP-B) from Scyltidium lignicolum ATCC24568 [35] and aspergillo glutamic peptidase from Aspergillus niger [36]. On the basis of the conserved catalytic residues in the active site (a glutamic acid and glutamine), the name given to the enzyme family was eqolisin.

Unlike the pepsins, eqolisin molecules consist of a β-sandwich, with the catalytic residues residing on two non-adjacent strands (Figure 2.6A). A catalytic mechanism
for this family was first proposed by Fujinaga et al. [35] on the basis of the structures of native SCP-B and its complex with hydrolytic products of angiotensin II (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe) (Figure 2.6B). A water molecule, analogous to the catalytic water in aspartic peptidases, is located within hydrogen bonded distances from the side chains of Glu136 and Gln53. In the structure of the product complex, the ligands included a tripeptide, Ala-Ile-His, occupying subsites P1-P3, and a single tyrosine. The tripeptide most likely corresponds to the sequence Tyr-Ile-His, with the side chain of Tyr disordered and thus represented as Ala. The ligand binds in an extended conformation, with the OXT atom of the P1 His carboxyl group at its C terminus forming a hydrogen bond with the side-chain carboxyl group of Glu136. A van der Waals contact is made between O=1 of Gln53 and O of P1 His. The interpretation of this observation is that the side-chain carboxyl group of Glu136 is protonated and a second hydrogen bond to the P1 His OXT is donated by the Nε2 atom of Gln53. In addition, the Nε2 atom of the P1 His imidazole group forms a hydrogen bond to the carboxylate of Asp57.

The structures of SCP-B can thus be used to postulate the catalytic mechanism of the eqolins. The water molecule bound between Glu136 and Gln53 (Figure 2.6B) acts as the nucleophile that is activated to a hydroxide ion by the general base functionality of the carboxylate of Glu136. Electrophilic assistance by polarizing the carbonyl bond of the scissile peptide is provided by the side-chain amide of Gln53. The hydrogen-bonded interaction from Gln53 Nε2 to the substrate carbonyl oxygen is bifunctional, by assisting in the formation of the tetrahedral intermediate as well as providing stabilization of the resulting oxyanion. Gln53 provides for stabilization of the tetrahedral intermediate by donating a hydrogen bond from its Nε2 to the oxyanion of the tetrahedral intermediate and by receiving a hydrogen bond to O=1 from the OH of the attacking water molecule. The nucleophilic attack takes place on the si-face of the scissile peptide. This direction of attack is seen only in the papain-like cysteine peptidase family; it is the re-face attack that takes place in the serine, aspartic, and metallopeptidases.

Protonation of the leaving-group nitrogen is an obligatory step in any hydrolytic mechanism for an amide. By analogy with the aspartic and metallopeptidases, it is likely that the proton donor will be the protonated Glu136. The tetrahedral character of the nitrogen atom in the intermediate will place the lone-pair electrons on the nitrogen in an appropriate position to abstract the proton from the carboxyl group of Glu136.

Another family of glutamic peptidases, G2, is represented by the C-terminal domain of gene product 12 of the tailed bacteriophage φ29 [37]. It has been postulated that self-processing of this protein is catalyzed by Glu695 which attacks the carbonyl carbon of the substrate residue Ser691, possibly assisted by the side chain of Asp692. Mutation of either Glu695 or Asp692 prevented autoprocessing, but structural data are not detailed enough to enable full analysis of the catalytic mechanism.

It is thus clear that although the mechanisms of enzymatic activity of the aspartic and glutamic peptidases share some similarities, there are also very important differences between these enzymes which are now separated into different catalytic types.

References

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