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Hypothesis

The catalytic mechanism of aspartic proteinases

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The highly symmetric active site of an aspartic proteinase, endothiapepsin, binds a water molecule ideally situated for nucleophilic attack on a substrate peptide bond whose distortion from planarity is stabilised by interactions of the substrate with the extended binding cleft. The *apparent* electrophilicity of the catalysis results from this distortion. The scissile peptide bond is orientated with the carbonyl oxygen hydrogen bonding to the tip of the β-hairpin 'flap' which lies over the cleft. Nucleophilic attack by the bound water leads to a tetrahedral intermediate similar to observed complexes with hydroxyl inhibitors and stabilised by hydrogen bonds with the flap.

Active site; Aspartic proteinase; Catalytic mechanism; Enzyme conformation; Peptide hydrolysis; Protein crystallography

1. INTRODUCTION

The aspartic proteinases are the fourth major class of proteolytic enzymes, distinct from the serine, thiol and metallo proteinases. With the exception of the renins, all the known examples are optimally active at acidic pH, which led to the now abandoned name of acid proteinases. Crystal structures have been determined for the archetypal enzyme, porcine pepsin [1], microbial enzymes from *Penicillium janthinellum* (penicillopepsin) [2,3], *Rhizopus chinensis* (rhizopuspepsin) [4,5] and *Endothia parasitica* (endothiapepsin) [6–8], and the inactive zymogen of pepsin, pepsinogen [9]. Structures have also been determined for several complexes of aspartic proteinases with natural and synthetic inhibitors containing modified peptide bonds [5,10–13].

Any mechanistic model must account for several biochemical phenomena observed in catalysis by these enzymes, particularly the marked variation in catalytic rate (k_{cat}) observed for substrates of differing length and composition, compared with the lack of variation in the corresponding K_m values shown for the same substrates [14,15]. Here, we present a comprehensive catalytic mechanism for the aspartic proteinases, based upon the crystallographic structure of endothiapepsin, which can explain the majority of the biochemical data.

2. EXPERIMENTAL

The crystal structure of endothiapepsin was refined by restrained least-squares and difference Fourier techniques, to an R factor of 0.16 at 2.1 Å resolution [7,8]. The full amino acid sequence has been chemically determined and aligned with the electron density. In addition, 345 solvent molecules with reasonable temperature factors

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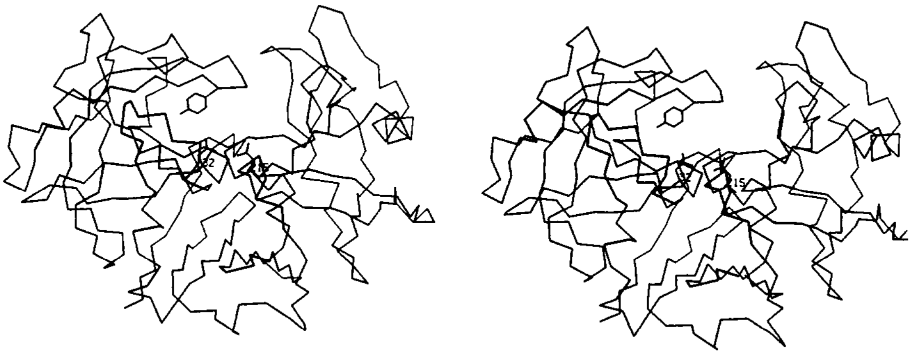

Fig.1. Stereo pair showing overall structure of endothiapepsin, viewed along the substrate-binding cleft. The catalytic aspartates 32 and 215 lie in the base of the cleft, with the flap containing Tyr 75 lying over the cleft.

have been located. Details of the structure solution and refinement, will be presented elsewhere.

3. ROLE OF THE ACTIVE-SITE BOUND WATER

The highly symmetric structure of the active site of endothiapepsin has already been described [16]. A clear solvent peak, seen in the refined electron density maps of both endothiapepsin (fig.2) and penicillopepsin [3], has been interpreted as a water molecule, polarised by hydrogen bonding interactions with the carboxyl side chains of aspartates 32 and 215. A water molecule in this situation would be a powerful nucleophile.

Earlier mechanisms [17–19] have assumed an electrophilic rate-limiting step, based on the observation that aspartic proteinases, in common with known electrophilic systems such as H^+ and carboxypeptidase A, catalyse hydrolysis of esters and peptides at comparable rates, whereas nucleophilic systems, such as chymotrypsin and OH^-, hydrolyse esters much more readily than peptides. Paradoxically, no significant deuterium isotope effect is observed [20]. Distortion of the scissile peptide bond has been suggested as the means by which the rate-limiting step is effected by binding of substrate residues distant from that bond [21]. This distortion would reduce the double bond character in the C–N bond and polarise the car-

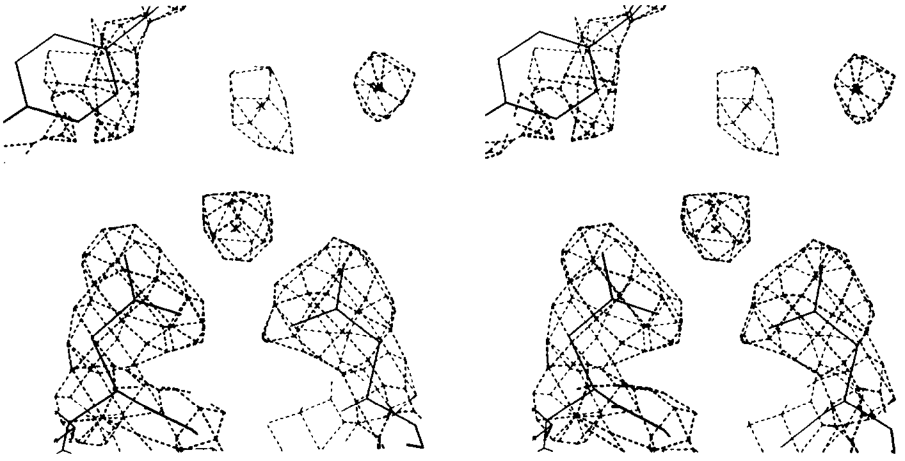

Fig.2. Crystallographic electron density for the active-site aspartate residues 32 and 215, and the bound water molecule.

bonyl, making it electronically similar to an ester, and consequently similar in susceptibility to a rate-limiting nucleophilic attack by the bound water. Such a process would appear electrophilic by the criterion described above, but involves no transfer of electrophilic protons and would display no deuterium isotope effects.

4. ORIENTATION OF THE SCISSILE PEPTIDE

Structures of enzyme-inhibitor complexes [5,10–13] show the N-terminal side of the peptide inhibitor binding in an extended conformation in the substrate-binding cleft, with its main chain

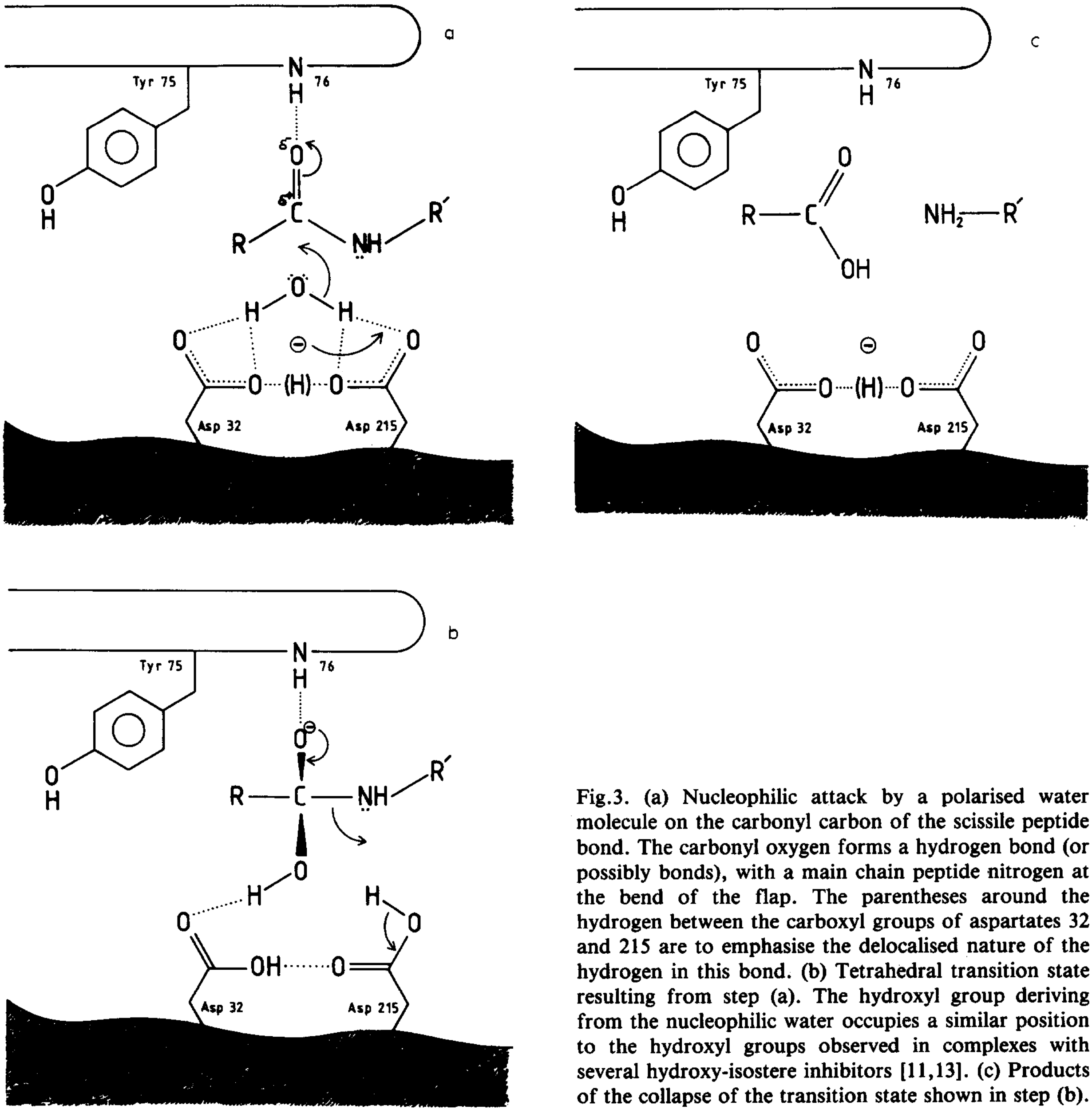

Fig.3. (a) Nucleophilic attack by a polarised water molecule on the carbonyl carbon of the scissile peptide bond. The carbonyl oxygen forms a hydrogen bond (or possibly bonds), with a main chain peptide nitrogen at the bend of the flap. The parentheses around the hydrogen between the carboxyl groups of aspartates 32 and 215 are to emphasise the delocalised nature of the hydrogen in this bond. (b) Tetrahedral transition state resulting from step (a). The hydroxyl group deriving from the nucleophilic water occupies a similar position to the hydroxyl groups observed in complexes with several hydroxy-isostere inhibitors [11,13]. (c) Products of the collapse of the transition state shown in step (b).

forming several β-sheet hydrogen bonds with residues in the enzyme. In particular, the NH groups of the peptide bonds on either side of the scissile peptide hydrogen bond to the carbonyl oxygens of Gly 34 and Gly 217 in the base of the cleft, while the carbonyl oxygens of these inhibitor residues form hydrogen bonds with the NH groups of Gly 76 and Asp 77 which form the flexible tip of the β-hairpin 'flap' overlying the binding cleft. If the scissile carbonyl (absent in the inhibitors) were orientated with its oxygen towards the flap, it would also receive favourable hydrogen bonding interactions from the peptide NH groups of flap residues 76 and possibly 77. In this orientation, the electrostatic dipole of the scissile peptide would be aligned favourably, with its negative end directed towards the δ^+ peptide NH groups at the tip of the flap, and its δ^+ carbonyl carbon directed towards the nucleophilic water bound by the aspartate pair.

5. CATALYTIC MECHANISM

Initial rate-limiting attack by the bound water (fig.3a) leads to an oxyanion tetrahedral intermediate (fig.3b) with the original water retaining its interaction with the aspartates. The analogous arrangement is seen in several inhibitor complexes where a secondary alcohol function replaces the scissile peptide [11,13]. Protonation of the scissile peptide NH occurs after the rate-limiting step, and may be concomitant with collapse of the intermediate. The oxyanion could be stabilised by hydrogen bonds with the peptide NH of residue 76 and possibly of residue 77, in a manner analogous to that proposed for serine proteinases [22], with minor contributions from the dipole of the helix formed by residues 304–310, whose δ^+ end is directed towards the active site. Hydrogen bonds from the flap to the charged oxygen may serve to orientate the orbitals so that there is a lone pair antiperiplanar to the C–N bond but not to the hydroxyl C–O bond, thereby ensuring that on collapse of the tetrahedral intermediate, the leaving group is the free amine rather than the original nucleophile [23]. Pyrimidalisation of the peptide nitrogen may be favoured by the binding-induced peptide rotation, which would direct the free lone pair generally towards the catalytic groups, and consequently no inversion of the lone pair and proton directions would be required for protonation of the NH leaving group, in contrast to that suggested for the serine proteinases [24]. Release of the neutral products, and binding of a new water molecule, restores the enzyme.

6. CONCLUSION

The mechanism described above accounts for much of the hitherto unexplained phenomena surrounding these enzymes, and utilises the available structural data without invoking any as yet unobserved phenomena such as the charge relays and large conformational changes required by other mechanistic models current in the literature. The critical assertions of peptide distortion and the orientation of the carbonyl bond place theoretical restrictions on the conformation of the substrate in enzyme-substrate complexes, that may be testable by structural studies of enzyme-inhibitor complexes. Detailed analysis of the conformational parameters required by this mechanism will be presented elsewhere. A precise identification of the nature of the solvent peak must await a neutron diffraction study.

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