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REVIEW

X-ray, neutron and NMR studies of the catalytic mechanism of aspartic proteinases

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Abstract Current proposals for the catalytic mechanism of aspartic proteinases are largely based on X-ray structures of bound oligopeptide inhibitors possessing non-hydrolysable analogues of the scissile peptide bond. Until recent years, the positions of protons on the catalytic aspartates and the ligand in these complexes had not been determined with certainty due to the inadequate resolution of these analyses. There has been much interest in locating the catalytic protons at the active site of aspartic proteinases since this has major implications for detailed understanding of the mechanism of action and the design of improved transition state mimics for therapeutic applications. In this review we discuss the results of studies which have shed light on the locations of protons at the catalytic centre. The first direct determination of the proton positions stemmed from neutron diffraction data collected from crystals of the fungal aspartic proteinase endothiapepsin bound to a transition state analogue (H261). The neutron structure of the complex at a resolution of 2.1 Å provided evidence that Asp 215 is protonated and that Asp 32 is the negatively charged residue in the transition state complex. Atomic resolution X-ray studies of inhibitor complexes have corroborated this finding. A similar study of the native enzyme established that it, unexpectedly, has a dipeptide bound at the catalytic site which is consistent with classical reports of inhibition by short peptides and the ability of pepsins to catalyse transpeptidation reactions. Studies by NMR have confirmed the findings of low-barrier and single-well hydrogen bonds in the complexes with transition state analogues.

Keywords Aspartic proteinase · Neutron diffraction · Atomic resolution X-ray · Catalytic mechanism · Low-barrier hydrogen bond

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Introduction

The aspartic proteinases are a family of enzymes involved in a number of important biological processes (Cooper 2002; Dunn 2002). In animals the enzyme renin has a hypertensive action through its role in the rennin–angiotensin system. The retroviral aspartic proteinases, such as the HIV proteinase, are essential for maturation of the virus particle and inhibitors have a proven therapeutic record in the treatment of AIDS. The enzyme β-secretase has been implicated in amyloidosis and the stomach enzyme pepsin is known to be involved in various gastric disorders. All enzymes in this class are characteristically inhibited by the microbial peptide pepstatin A which contains the unusual amino acid statine. Statine is an analogue of L-leucine, differing from this amino acid by the

insertion of a –CHOH–CH_2– group between the Cα and the main chain carbonyl group.

Most eukaryotic aspartic proteinases are monomeric and consist of a single chain of around 330 amino acids which forms two similar domains with the active site located in between (Davies 1990). In contrast, retroviral aspartic proteinases are dimeric, consisting of two identical subunits, each roughly equivalent to one domain of a eukaryotic aspartic proteinase. Accordingly, the amino acid sequences of the eukaryotic enzymes have signs of an internal repeat relating the two halves of the molecule, their identity being greatest in the vicinity of the active site which involves two conserved Asp-Thr-Gly sequences (Tang et al. 1978; Pearl and Blundell 1984). Hence the eukaryotic aspartic proteinases may have evolved divergently from a primitive dimeric enzyme (resembling the retroviral proteinase) by gene duplication and fusion. In all aspartic proteinases, the base of the active site cleft is made of β-strands which contain the catalytic aspartate residues (32 and 215). The side chains of the aspartates are held coplanar and within hydrogen bonding distance by an intricate arrangement of H-bonds involving surrounding main chain and conserved side chain groups. A solvent molecule is found between both aspartate carboxyls in all native aspartic proteinase crystal structures and is presumed to be a water molecule. In numerous chemical studies, the failure to trap covalently bound substrate indicated that the reaction involves an intermediate which binds non-covalently to the enzyme (Hofmann et al. 1984). NMR studies using an inhibitor with a ketone analogue of the scissile bond suggested that it binds to the enzyme in a hydrated *gem*-diol form (–$C(OH)_2$–) (Rich et al. 1982). Thus current proposals for the catalytic mechanism (such as that shown in Fig. 1) invoke nucleophilic attack of the active site water molecule on the scissile bond carbonyl generating a tetrahedral *gem*-diol intermediate (Veerapandian et al. 1992).

The best synthetic inhibitors of aspartic proteinases are those in which one or both of the hydroxyl groups of the putative transition state are mimicked (intermediate 2 in Fig. 1). One hydroxyl binds by hydrogen bonds to both of the catalytic aspartates in the same position as the solvent molecule in the native enzyme and most of the transition state analogues (e.g. statine) mimic this group alone. In contrast, fluoroketone analogues (–CO–CF_2–) mimic both hydroxyls of the putative intermediate since they readily hydrate to the *gem*-diol form (–$C(OH)_2$–CF_2–). In current mechanisms, the active site water molecule becomes partly displaced upon substrate

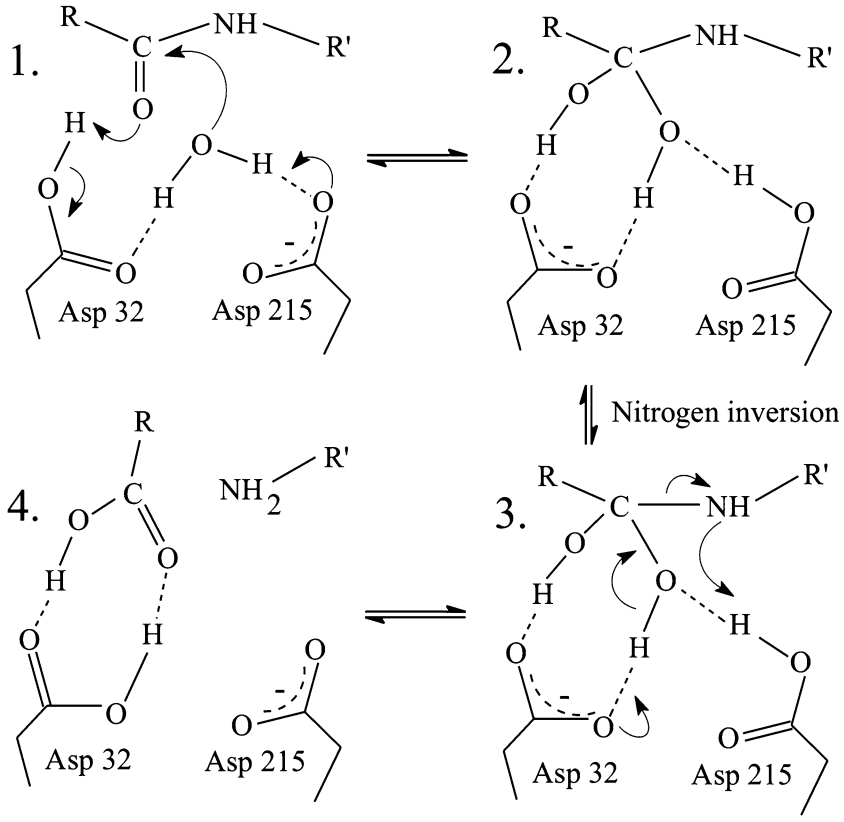

Fig. 1 The catalytic mechanism for aspartic proteinases proposed by Veerapandian et al. (1992). This mechanism is based on the X-ray structure of a difluoroketone (*gem*-diol) inhibitor bound to endothiapepsin. A water molecule tightly bound to the aspartates in the native enzyme is proposed to nucleophilically attack the scissile bond carbonyl. The resulting tetrahedral intermediate (2) is stabilised by hydrogen bonds to the negatively charged carboxyl of aspartate 32. Fission of the scissile C–N bond is accompanied by transfer of a proton to the leaving amino group either from Asp 215 (with nitrogen inversion) or from bulk solvent. *Dashed lines* indicate hydrogen bonds

binding and polarised by one of the aspartate carboxyls, as suggested by Suguna et al. (1987). The water may then nucleophilically attack the scissile bond carbonyl group to form the tetrahedral intermediate. Structural comparison of numerous inhibitor complexes (e.g. Bailey and Cooper 1994) largely confirmed earlier predictions by Pearl (1987) that the mechanism involved strain imposed by tight binding of the substrate at sub-sites adjacent to the catalytic centre.

Early proposals for the mechanism based on crystal structures generally invoked proton transfer between the two inner carboxyl oxygen atoms of the catalytic diad (Polgar 1987; Pearl 1987). However inspection of the molecular geometry of the catalytic carboxyls in numerous high resolution structures suggests that a direct hydrogen bond interaction between these two groups would have poor geometry. Furthermore there are shorter hydrogen bond contacts between the aspartate carboxyls and the active site water molecule suggesting that the active site protons are involved in these interactions rather than in the interaction between the two aspartate carboxyls. In the ground state of the enzyme-substrate complex (shown as intermediate 1 in Fig. 1) it is assumed that the protons on the active site water molecule are oriented towards the two aspartates so that both of the water oxygen lone pairs are pointed towards the substrate for nucleophilic attack. Thus in more recent proposals, which are based on the bound structures of difluoroketone inhibitors (e.g. Veerapandian et al. 1992; James et al. 1992; Silva et al. 1996), there are no direct proton transfers between the two catalytic carboxyl groups and instead all proton transfers are mediated by the nucleophilic water molecule. These mechanistic proposals were consistent in general terms but lacked direct experimental evidence for the protonation states of the active site groups. Since, the active site hydrogen atoms cannot be located by X-ray analysis of proteins even at high resolution, their putative positions have to be inferred from the local geometry of surrounding polar atoms. One of the key features of the mechanism proposed by Veerapandian et al. (1992) (shown in Fig. 1) is the stabilisation of the transition state by a negative charge localised on Asp 32. The assignment of a negative charge to this residue was made solely on the basis that its hydrogen bonding capacity is satisfied to a greater extent than that of Asp 215 in complex with a *gem*-diol inhibitor. However, the protonation states of the catalytic aspartates had not been determined with any certainty at that stage.

Neutron diffraction studies

Crystals of endothiapepsin bound to the hydroxyethylene inhibitor (H261) of sufficient size for neutron data collection were obtained and were partially deuterated by vapour diffusion (Cooper and Myles 2000). Neutron Laue data were collected using the LADI detector at ILL (Grenoble) on large crystals of the H261 inhibitor complex allowing the structure to be refined at a resolution of 2.1 Å (Coates et al. 2001). This represents one of the largest protein structures to be refined in molecular detail using neutron data. The final neutron dataset had a merging R-factor of 7.5% and a completeness of 84.5%. The final neutron refinement R-factor was 23.5% and the R-free was 27.4%, which are slightly high in comparison with the values expected for a refined X-ray structure at comparable resolution. However, they are consistent with *R*-values obtained in other analyses which have used neutron Laue data. The neutron structure superimposes very well with the previously solved X-ray structure of this complex (Veerapandian et al. 1990); the rms Cα deviation is only 0.2 Å.

Whilst partial deuteration of the crystal was undertaken primarily to reduce incoherent scatter from bulk solvent, the neutron data show that the endothiapepsin molecule itself has become extensively deuterated by the vapour diffusion protocol. The parts of the molecule most protected from H-D exchange are the buried β-strand regions. The majority of polar side chains have exchanged and, in general, the loops and helical regions have exchanged to a greater extent than the β-sheet regions. Many aspartic proteinases have pI values which are unusually low, e.g. pepsin has a pI value close to 2.0 (Fruton 1976). It has been suggested that these low pI values are due to buried carboxylate groups which remain deprotonated even at very low pH by interacting with conserved neutral polar residues. Evidence for this was provided by the neutron data since there are a number of buried carboxylates in endothiapepsin which the neutron data show conclusively to be deprotonated (Coates et al. 2001).

The bound inhibitor (H261) possesses a hydroxyethylene analogue (–CHOH–CH_2–) in place of the scissile peptide bond. The analogue –OH group mimics one hydroxyl of the putative tetrahedral intermediate and replaces the water molecule found at the catalytic centre of the native enzyme. A neutron difference Fourier for the structure, without hydrogens or deuteriums modelled at the catalytic centre, shows positive difference density for deuterium atoms on the inhibitor hydroxyl and the outer carboxyl oxygen of Asp 215 (Fig. 2). To test possible interpretations, the structure

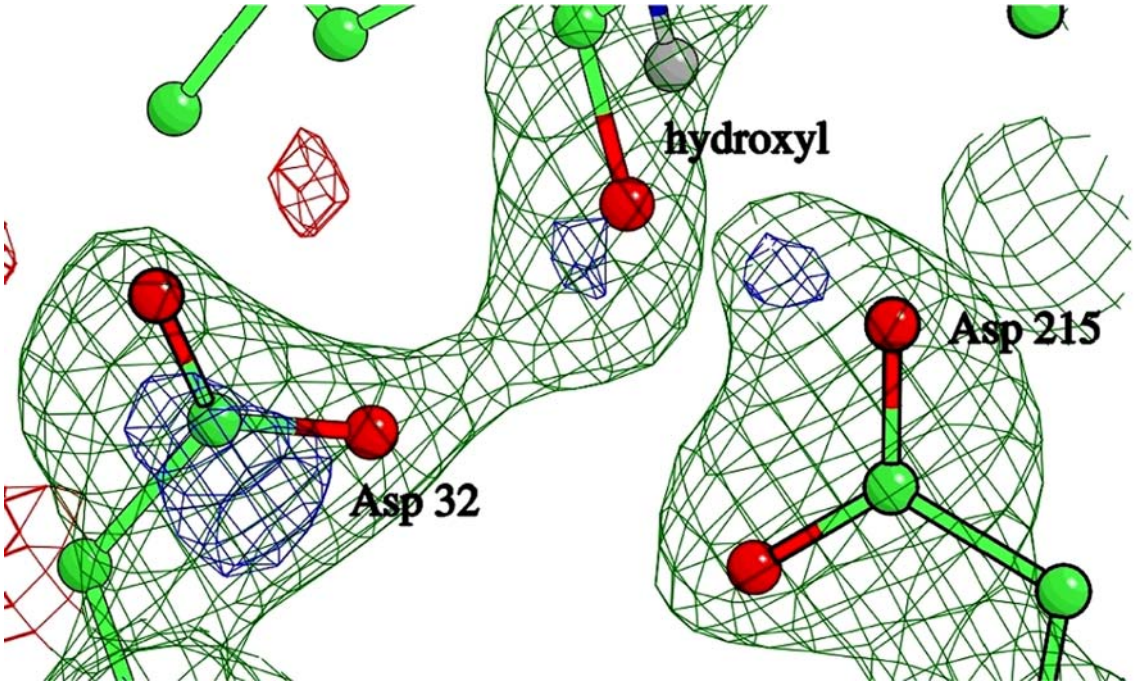

Fig. 2 The neutron map at the catalytic centre of the aspartic proteinase endothiapepsin formed by aspartates 32 and 215. *Green lines* indicate the $2F_o$–F_c neutron density at 2.1 Å resolution contoured at 1.2 rms. Positive difference density (contoured at 2.5 rms is shown with *dark blue lines*) indicates that the outer oxygen of Asp 215 is deuterated and that the inhibitor hydroxyl has a D oriented towards Asp 32 (Coates et al. 2001) (*red* indicates negative difference density at a contour level of –2.5 rms). This provided the first experimental evidence for the mechanism shown in Fig. 1

was refined using different tautomeric models of the deuterium positions and the resulting maps and deuterium occupancies inspected. These results again strongly suggested that Asp 215 is deuterated (or protonated in vivo) in the transition state complex and provided the first direct experimental evidence in favour of the mechanism proposed by Veerapandian et al. (1992).

Atomic resolution X-ray analysis

Atomic resolution X-ray data have been collected from crystals of native endothiapepsin allowing the structure to be refined at 0.9 Å resolution giving a final R-factor of 12.1% and free *R*-value of 14.7% (Erskine et al. 2003). The quality of the map showed conclusively that one region of the protein involving an -Asp-Gly- dipeptide had cyclised forming a succinimide. Biochemical studies have shown that -Asp-Gly- sequences undergo slow succinimide formation when the glycine's main chain nitrogen nucleophilically attacks the side chain carboxyl of the aspartate. Succinimide and subsequently β-aspartic acid formation in proteins is a characteristic of ageing and it has been suggested that the accumulation of these unusual residues may be a triggering factor in amyloidogenesis (Lindner and Helliger 2001).

The electron density map of the native enzyme at atomic resolution also demonstrated that a short peptide (probably a Ser-Thr dipeptide) was bound non-covalently in the active site cleft (Fig. 3) (Erskine et al. 2003). The N-terminal nitrogen of the dipeptide interacts with the aspartate diad of the enzyme by hydrogen bonds involving the carboxyl of Asp 215 and the catalytic water molecule. The two amino acids of the dipeptide lie in the same positions as the P_1' and P_2' residues of bound inhibitors. In refinement, the occupancy of the Ser-Thr dipeptide converged to a value of 39%, i.e. unlike the tight-binding transition state analogue inhibitors, the dipeptide is not present in all enzyme molecules in the crystal. This is consistent with classical findings that the aspartic proteinases can be inhibited weakly by short peptides and that these enzymes can catalyse transpeptidation reactions (Fruton 1976). In this process the ability of the active site cleft to retain one of the products of the hydrolytic reaction is clearly important for the subsequent condensation step involving a second peptide that binds at the active site. Previously, transpeptidation has been cited as evidence for a covalently bound intermediate. However, these results demonstrated that a covalent

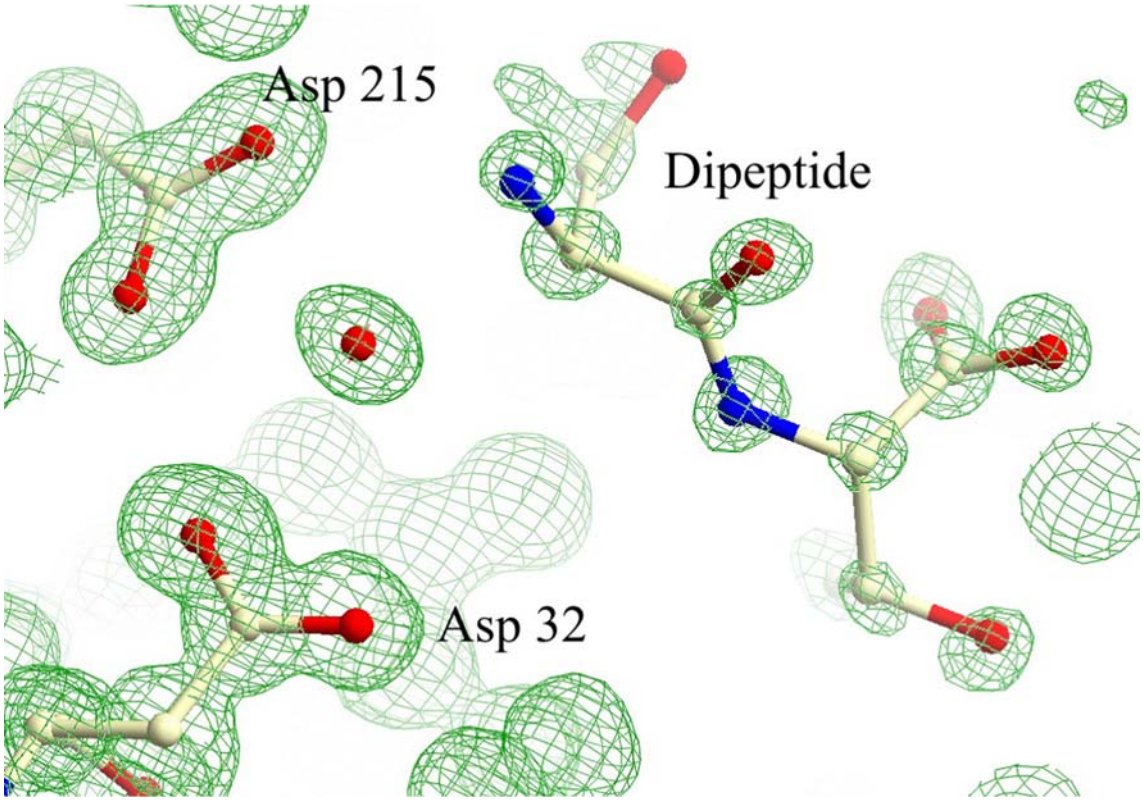

Fig. 3 The 0.91 Å resolution X-ray map of endothiapepsin showing the dipeptide bound to the 'native' enzyme at the catalytic centre (Erskine et al. 2003). The $2F_o$–F_c density (shown in *green*) is contoured at 1.0 rms. The occupancy of this dipeptide refined to 39%. The dipeptide probably originates from autolysis of the enzyme's N-terminal Ser-Thr-dipeptide during crystallisation

intermediate may not be necessary for transpeptidation to occur.

It is conceivable that the dipeptide could have originated from autolysis of the enzyme's N-terminal Ser-Thr- sequence during crystallisation. The exact sequence of the dipeptide, as deduced from the electron density, is not certain but several efforts at refinement with different amino acids built in to the density lent faith to the conclusion that it is a Ser-Thr dipeptide. In addition, the following 'structurally similar' dipeptides were synthesised to test whether any possessed inhibitory activity against endothiapepsin: Ser-Thr, Pro-Thr and Ser-Val (Erskine et al. 2003). These dipeptides were chosen since they could fit the electron density for the ligand almost equally well. The finding that only the Ser-Thr dipeptide was able to inhibit the enzyme lends further credence to the idea that this is the ligand observed in the active site although it is conceivable that a mixture of short peptides are bound. A similar finding of a short peptide bound in the 'prime' side of the active site was reported by Symersky et al. (1997) for the aspartic proteinase from *Candida tropicalis*.

As mentioned above, the protonation state of the catalytic residues has been a long-standing controversy in the aspartic proteinase field. In the optimal pH range it is likely that the aspartate diad possesses a single negative charge. The putative salt-bridge interaction made by the active site dipeptide's amino group with Asp 215 indicates that this aspartate could be negatively charged in the ground state of the enzyme. In general, unrestrained refinement using atomic resolution X-ray data provides a powerful means of defining the protonation states of carboxyl groups in well-ordered parts of the structure. Neutral carboxyl groups have a significant difference between their C–OH and C = O bond lengths (1.21 Å for the C = O bond and 1.32 Å for the C–OH bond) whereas ionised carboxylates have identical C–O bond lengths (typically 1.27 Å) due to resonance. Unrestrained refinement of the carboxyl groups of native endothiapepsin at 0.9 Å resolution showed that both catalytic aspartates have C–O bond lengths which are almost identical within the errors of measurement (ESDs = 0.01 Å). Whilst this suggests that both are ionised, this is unlikely due to the proximity of both groups. Instead it is

likely that both are equally likely to be charged in the ground state i.e. Asp 32 and 215 will each be negatively charged (and able to make a salt-bridge) in only 50% of the active sites in the crystal at any one time due to proton transfer. This correlates with the finding that the occupancy of the dipeptide is less than 50%. Thus, atomic resolution studies of the native endothiapepsin enzyme suggest that the distribution of charge at the catalytic centre in the absence of substrate is symmetric—an effect which was first predicted earlier (Pearl and Blundell 1984) but has not been substantiated until now. However, an asymmetric charge distribution may arise in the presence of substrate.

To analyse this, X-ray data have been collected to atomic resolution on a total of seven peptide-mimetic inhibitors bound to endothiapepsin (Coates et al. 2002a, b; Erskine et al. 2003). These represent a wide range of transition state analogues including the hydroxyethylene analogue (–CHOH–CH_2–) as well as the statine (–CHOH–CH_2–CO–), reduced bond (–CH_2–NH–), norstatine (–CHOH–CO–), phosphinate (–PO(OH)–CH_2–) and the difluoroketone (–$C(OH)_2$–CF_2–) analogues. In one structure there is excellent electron density for a hydrogen atom on the inhibitor hydroxyl orientated toward the inner oxygen of Asp 32, as shown in Fig. 4. This X-ray structure represents the most ordered of the inhibitor complexes analysed in terms of average B-factor—something which may stem from favourable freezing conditions for this particular crystal. Significantly, the occurrence of a proton at this position is consistent with the neutron data (Coates et al. 2001) and unrestrained refinement of the carboxyl bond lengths (Coates et al. 2002a).

The atomic resolution X-ray data showed that the transition state analogues of these inhibitors bind by making several hydrogen bonds with the catalytic aspartates, some of which have donor–acceptor distances as short as 2.5 Å and, in the case of the phosphinate analogue, as short as 2.4 Å. Whilst distances as short as these had been observed in earlier analyses at around 2.0 Å resolution, the errors in the bond lengths of atomic resolution structures are considerably lower (of the order of 0.01 Å). This confirms that these short hydrogen bonds are a real feature of

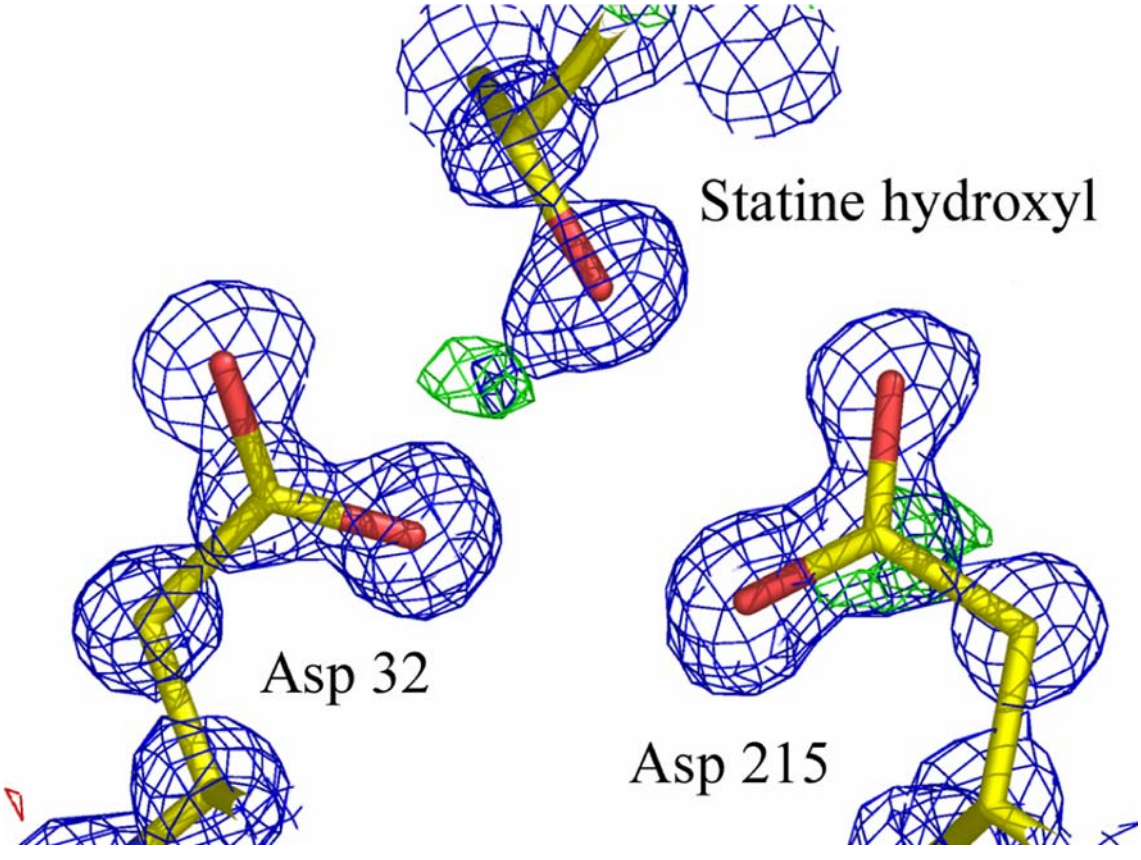

Fig. 4 The electron density at 0.98 Å resolution for a hydrogen atom on a transition state analogue (*paler contours* indicate positive difference density) (Coates et al. 2002a). The putative hydrogen atom on the inhibitor hydroxyl is oriented towards Asp 32. This is consistent with the mechanism shown in Fig. 1

the enzyme-inhibitor complex and not errors in the structure analysis. Hydrogen bonds as short as this are referred to as low barrier hydrogen bonds (LBHB) since the proximity of the donor and acceptor atoms reduces the energy barrier which normally prevents transfer of the hydrogen atom from the donor to the acceptor group (Cleland et al. 1998). The locations of the LBHB's observed in the endothiapepsin complexes are shown in Fig. 5 where it can be seen that the inhibitor hydroxyl forms an LBHB with the inner oxygen of Asp 32 and another with the outer oxygen of Asp 215. This is consistent with the deuterium positions found in the neutron analysis (Coates et al. 2001) and other atomic resolution X-ray structures of aspartic proteinases (e.g. Fujimoto et al. 2004).

Analysis by NMR

A distinguishing property of LBHBs is a large downfield shift detected by ^{1}H-NMR typically between 16 and 21 ppm. Thus, to confirm the existence of the short interactions suggested by the atomic resolution X-ray data, 1D ^{1}H-NMR solution spectra of native endothiapepsin and a number of complexes were recorded (Coates et al. 2002a). Whilst the NMR spectrum of free endothiapepsin (or the free inhibitor) showed no peaks outside the normal region for protein signals 0–11 ppm, the complexes gave several peaks between 15.5 and 18.5 ppm (Fig. 6). In the spectra of endothiapepsin complexed with the phosphinic acid analogue inhibitor, there is a peak with an even larger

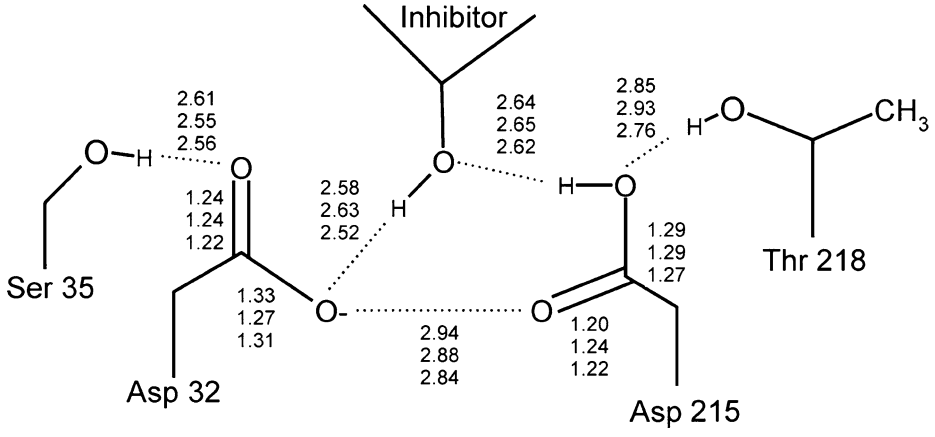

Fig. 5 The hydrogen bond distances for three atomic resolution structures of endothiapepsin bound to hydroxyl-containing transition state analogues (Coates et al. 2002a). The presence of hydrogen bonds as short as 2.5–2.6 Å suggests that low barrier hydrogen bonds are involved

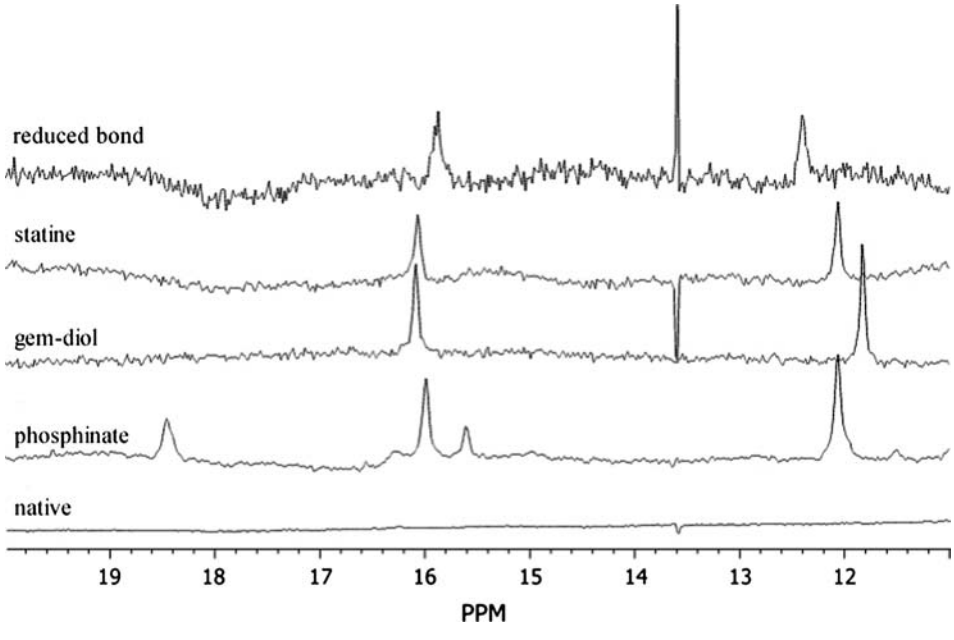

Fig. 6 The proton NMR spectra of native endothiapepsin and that of complexes with four different transition state analogues (Coates et al. 2002a). The peaks around 16 and 18 ppm observed in the complexes are indicative of low-barrier and single-well hydrogen bonds, respectively. There are no peaks in this region in the native enzyme spectrum or in the spectra of the inhibitors themselves (data not shown). The sharp peak and dip at around 13.6 ppm in some spectra are an artifact

downfield shift of 18.5 ppm. Most likely, this corresponds to a very short hydrogen bond (2.41 Å) observed in the X-ray structure between the outer oxygen of Asp 32 and an oxygen of the phosphinate group. Such extremely short hydrogen bonds are referred to as single-well hydrogen bonds.

Concluding remarks

Since LBHB formation facilitates rapid proton transfer, the role of these interactions in enzyme catalysis is the subject of much discussion in the enzymology field (Cleland et al. 1998). An early proposal for the involvement of LBHBs in aspartic proteinase catalysis based on deuterium isotope effects (Northrop 2001) invoked a somewhat different pattern of LBHB formation at the catalytic centre involving a proton shared equally between the two inner aspartate oxygens. Overall, recent work using a range of techniques (described above) has provided the first structural evidence that low-barrier hydrogen bonds may be significant for aspartic proteinase catalysis and has helped to pin-point where the LBHB interactions are at the catalytic centre, thus giving a sounder footing for evaluating their role in the mechanism.

The neutron and atomic resolution work described in this review strongly suggest that Asp 32 is negatively charged in the transition state complex of aspartic proteinase catalysis. The negatively charged carboxyl of this aspartate residue forms hydrogen bonds with both of the hydroxyls of the neutral *gem*-diol intermediate in a symmetric manner (Fig. 1, intermediate 2). In contrast with serine proteinases where the enzyme provides an oxyanion binding hole or pocket, the oxyanion of the aspartic proteinase mechanism appears to be part of enzyme itself, rather than part of the transition state intermediate.

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