# Aspartic proteinases: From the first X-ray photos of pepsin crystals to hundreds of 3-D structures

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## Historical background

ALTHOUGH the existence of pepsin in the digestive juices had been noted in the early part of the 18th century and Darwin had identified a similar activity in pitcher plants in 1875, the pepsin family first attracted the attention of biochemists in the 1920s. They were characterized as a group of proteinases, active in the pH range 1–5 and subsequently crystallized in Svedberg's laboratory, leading to the demonstration in the 1930s by Dorothy Crowfoot (Hodgkin) and her supervisor J. D. Bernal that the crystals diffracted X-rays. To quote from Bernal and Crowfoot[1]: 'Four weeks ago, Dr G. Millikan brought us some crystals of pepsin prepared by Dr Philpot in the laboratory of Prof. The Svedberg, Uppsala. They are in the form of perfect hexagonal bipyramids up to 2 mm in length of axial ratio $c/a = 2.3 \pm 0.1$. When examined in their mother liquor, they appear moderately birefringent and positively uniaxial, showing a good interference figure. On exposure to air, however, the birefringence rapidly diminishes. X-ray photographs taken of the crystals in the usual way showed nothing but a vague blackening. This indicates complete alteration of the crystal and explains why previous workers have obtained negative results with proteins, so far as crystalline pattern is concerned.' They go on to comment that, 'At this stage, such ideas are merely speculative, but now that a crystalline protein has been made to give X-ray photographs, it is clear that we have the means of checking them and, by examining the structure of all crystalline proteins, arriving at far more detailed conclusions about protein structure than previous physical or chemical methods have been able to give.' These observations set the scene for modern protein crystallography and enzymology.

Unfortunately, the hexagonal crystals (Figure 1) diffracted weakly and the *c*-axis of the unit cell was more than 300 Å in length, making X-ray data collection difficult in the early days of protein crystallography. But the influence of Dorothy Hodgkin was felt in other ways. The X-ray crystal structures of three fungal aspartic proteinases were each solved by a former member of the Hodgkin/Powell Laboratory of Chemical Crystallography, Oxford University. Thus, Mike James, a student of Dorothy Hodgkin in the 1960s, solved the penicillopepsin structure in Edmonton, Canada[2]; David Davies, who was a student in Oxford with H. M. Powell, but sharing a laboratory with Dorothy Hodgkin in the 1950s, solved rhizopuspepsin in Washington, USA[3–5], and Tom Blundell, who did his post-doctoral work with Dorothy Hodgkin in the late 1960s, solved the structure of endothiapepsin in Sussex, UK[3,6], the latter two structures being first published together.

Dorothy Hodgkin made other indirect contributions to the work on pepsins. When the method of isomorphous replacement proved to be difficult for chymosin and endothiapepsin, Dorothy provided much encouragement. Charles Bunn and his team at the Royal Institution were frequent visitors to Oxford in the 1960s, and on his retirement he passed these projects to the laboratory of Tom Blundell, where they were solved in the following years. The hexagonal pepsin (Figure 2 *b*) itself proved quite a difficult analysis and was not solved until many years later in the Blundell laboratory at Birkbeck[7], sometime after the structure of monoclinic pepsin (Figure

**Figure 1.** Hexagonal crystals of pepsin.

2 *a*) had been solved in the Andreeva laboratory in Moscow[8].

Many laboratories are now involved in structure analyses of aspartic proteinases; three-dimensional structures for about fifteen different enzymes and hundreds of inhibitor complexes, for example, Figure 2 *c* and *d* and Figure 3, and site-directed mutants have allowed us to make good progress in defining binding subsites and suggesting transition states and intermediates of the catalytic mechanism.

## Three-dimensional structures

Pepsin itself and the closely related calf chymosin[9,10] are bilobal enzymes with rather open active site clefts, consistent with binding a wide range of amino acid sequences. More recently the three-dimensional structures of several other enzymes, some of which have more specific roles, have been reported. These include human renin[11–14], the most specific enzyme of the group, which is involved in the formation of angiotensin II, a potent pressor and aldosterogenic substance. Crystal structures have been reported for cathepsin D (refs 15, 16), an enzyme implicated in the invasiveness of breast cancer, and proteinase A (ref. 17), a lysosomal proteinase which is found in the vacuoles of *Saccharomyces cerevisiae* and involved in proteolysis during nitrogen starvation and proteolytic maturation of other vacuolar enzymes. These structures have a similar general topology to pepsin, but in addition a further flap partially covers the active site in a way that might contribute to specific interactions with the substrate.

## Inhibitor complexes

The first inhibitor complexes to be studied by X-ray analysis were pepstatin with rhizopuspepsin[4,18] and a pepstatin fragment with penicillopepsin[2]; these confirmed that the hydroxyl group of the statine mimicks a tetrahedral intermediate, being hydrogen bonded to both catalytic aspartates and replacing the water bound at this position in the uncomplexed enzymes.

These and later studies[5,7,13–16,19–27] showed that the active site cleft (Figure 2 *c* and *d*) is formed between

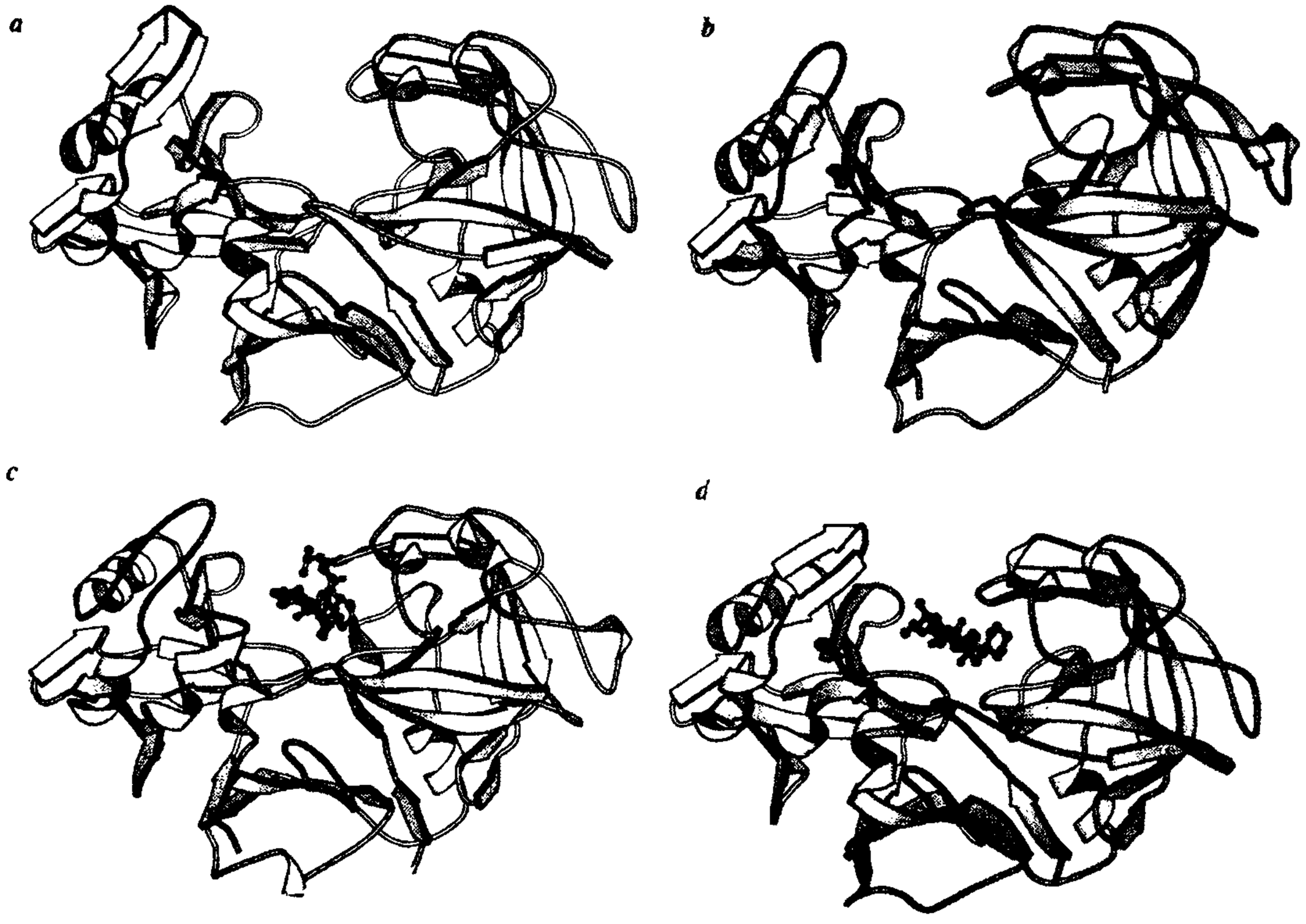


**Figure 2.** Crystal structures of pepsin: (*a*) 4PEP; monoclinic, (*b*) 5PEP; hexagonal, and pepsin inhibitor complexes: (*c*) 1PSO and (*d*) 1PSA.

the two lobes, each of which provides one of the essential aspartates. The inhibitors have an extended $\beta$-strand conformation and make hydrogen bonds through their main chains to each lobe of the enzyme (Figure 3). The side chains alternate on either side of the main chain and make hydrogen bonds and van der Waals interactions with both domains in the molecule, so defining subsites, S6 to S3′. An antiparallel $\beta$-sheet, comprising residues 71 to 82 and known as the 'flap', interacts with the central region of the inhibitor and shields the active site from the solvent region. The carboxylates of the active site aspartates form hydrogen bonds to the hydroxyl oxygen of the transition-state isostere (Figure 3). Comparisons of the many inhibitor complexes show that the general mode of binding is conserved over very great evolutionary distances.

In modelling exercises, it was often assumed that specificities derive from differences in the sizes of the residues in the specificity pockets, which complement the corresponding side chains in the substrate/inhibitor[28,29]. This was correct for the S1′ subsites for the human and mouse renins where differences in the enzymes are complementary to the valine and leucine at P1′ in human and mouse angiotensinogens; residue 213 is leucine in human renin and valine in mouse renin. However, the three-dimensional structures of human renin, complexed with a P4 to P1′ norstatine-containing inhibitor CP-85,339 and of mouse renin, complexed with a decapeptide hydroxyethylene isostere inhibitor CH-66 (refs 13, 14), show that the situation can be more complex. For example, the S1′ pockets of chymosin, pepsin and endothiapepsin have an aromatic side-chain at residue 189, while the renins have amino acids with smaller side-chains (valine in human renin and serine in mouse renin). This would be expected to make the pocket larger in renins. However, the structure of the mouse renin complex shows that the substrate moves closer to the enzyme in renins as a result of the smaller residue at 189 and the pocket is made even more compact due to changes in the position and composition of the poly-proline loop (residues 290–297). Thus, the specificity difference at this site arises not only from the movement of a secondary structure, in this case a loop region, but also from the substitution of a residue that allows the substrate to come closer to the body of the enzyme.

The specificity pockets are also influenced by the elaboration of loops on the periphery of the binding cleft in renins. This is most marked at S3′ and S4′. For instance, in endothiapepsin, which has been the

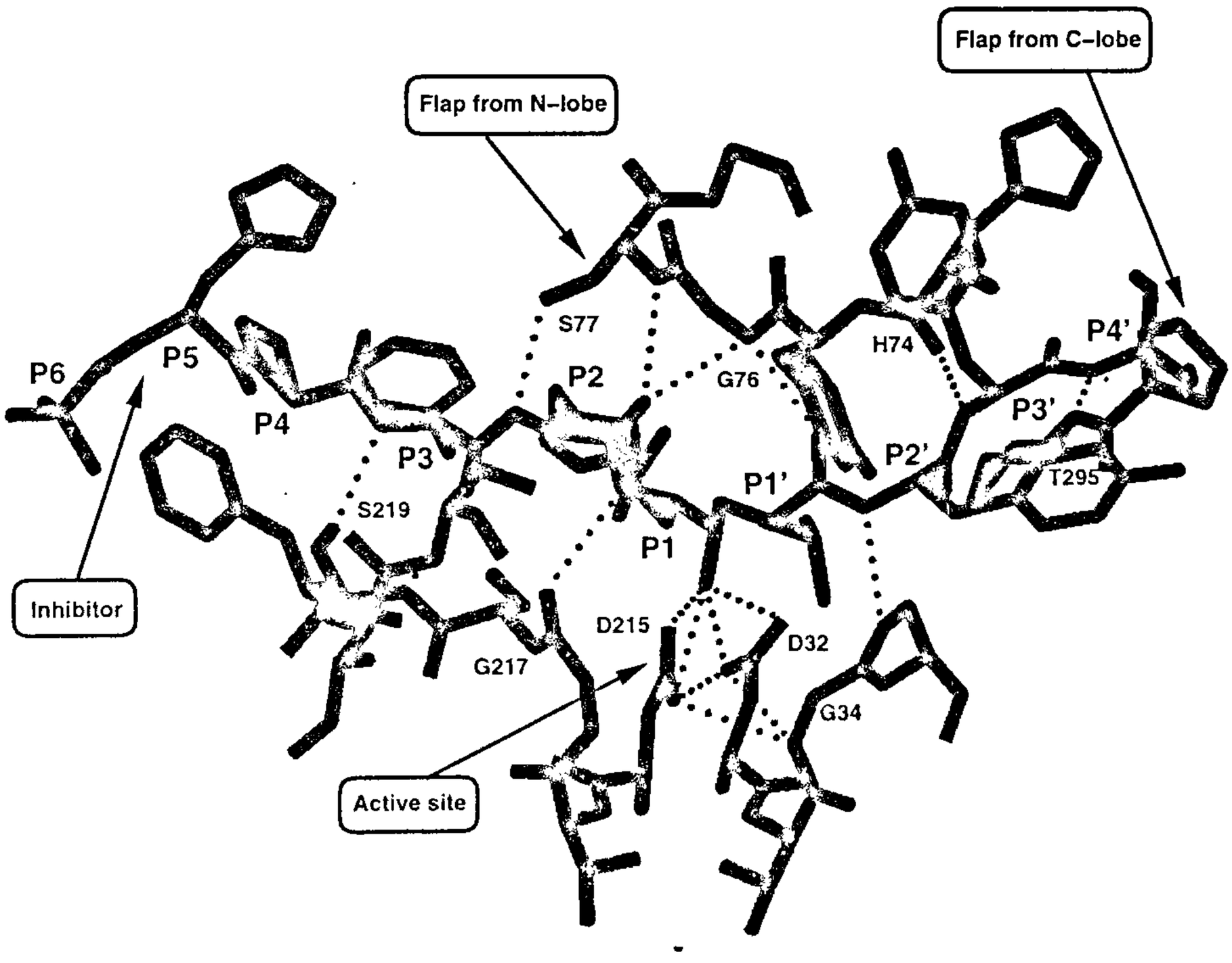


**Figure 3.** Active site of the mouse renin–inhibitor complex.

subject of the greatest number of studies, different conformations are adopted at P3′, and the residue at P4′ is generally disordered. In contrast these residues are clearly defined in mouse renin. This is mainly a consequence of the poly-proline loop, involving residues 294 to 297. The X-ray analysis of the mouse renin complex shows that the S3′ & S4′ subsites are formed by the poly-proline loop together with residues of the 'flap' and a similar situation is likely to occur in human renin. The well-defined interactions of P3′ described in the mouse renin complex explain the significant affinity when inhibitors have phenylalanine or tyrosine at P3′ as well as the importance of a P3′ residue for catalytic cleavage of a substrate by renin. In human and bovine cathepsin D and in yeast proteinase, this proline-rich loop has a similar conformation but the fact that it occupies a position further away from the inhibitors probably accounts for their decreased specificity for substrates at P3′ & P4′.

## Transition-state isosteres and the catalytic mechanism

Although there is no general agreement about the details of the catalytic mechanism, it is clearly established that there is no covalent intermediate involving the catalytic aspartates. It is generally accepted that the nucleophilic attack by a water molecule leads to tetrahedral intermediates and/or transition states involving groups such as $-C(OH)O^{-}-NH_2^{+}-$. There have been several attempts to simulate such tetrahedral intermediates. These have included phosphostatine ($-PO(OH)-CH_2-$), crystal structures of complexes of which demonstrate that the second oxygen binds close to the outer oxygen of Asp-32 (ref. 30). However, this is probably not a very good model as there is likely to be one less proton in the complex than in the real intermediates/transition states. A better model is the hydrated difluorostatone. Thus in the complex of endothiapepsin with the potent difluorostatone-containing tripeptide renin inhibitor (CP-81,282), the scissile bond surrogate is fully hydrated with the pro-(R) hydroxyl oxygen of the inhibitor within 3.4 Å of all four carboxyl oxygens of the two active site aspartates[31].

Two possible complexes with differing locations of the negative charge can be envisaged. In the first, a negatively charged Asp-32 is stabilized by four hydrogen bonds, two from the hydrate hydroxyls, one from Ser-35 $\gamma$-OH and one from Gly-34 NH. The outer oxygen of protonated Asp-215 donates a hydrogen bond to the statine-like hydroxyl. In the second, a negatively charged Asp-215 is stabilized by three hydrogen bonds, one from the statine-like hydroxyl of the hydrate, one from Thr-218 $\gamma$-OH and one from Gly-217 NH, while the inner oxygen of protonated Asp-32 donates a hydrogen to the statine-like hydroxyl and the outer hydroxyl of the hydrate donates a further hydrogen bond to the outer oxygen of Asp-32. Thus, the high resolution structures of the enzyme inhibitor complexes suggest a mechanism that involves the stabilization of a negatively charged aspartic acid carboxylate first at Asp-215 and then at Asp-32. In each case the stabilization involves complete inaccessibility of the carboxylate from bulk solvent and the formation of three or four hydrogen bonds to the two carboxylate oxygens. This is similar to the environment of several other carboxylates that are conserved in pepsins and which contribute to the negative overall charge at low pH. The absence of any strong stabilization of a developing oxyanion at the peptide carbonyl argues against a possible analogy with serine proteinases, in which main chain nitrogen atoms are thought to stabilize the negative charge of an oxyanion. In the case of the aspartic proteinases the anion stabilized by the enzyme is the negatively charged carboxylate of an aspartic acid, and the intermediate is a gem diol.

## Retroviral proteinases

The discovery of pseudo two-fold symmetry in the 3-D structures of the pepsin-like aspartic proteinases by Tang *et al.*[32] has led to a productive series of hypotheses in subsequent years. These hypotheses concern a dimeric ancestor of the aspartic proteinases[32], the close equivalence of the two active site aspartates[33], the similarity of the specificity sites on either side of the scissile bond[28], and the structure of the retroviral proteinases as dimeric homologues of the pepsins[34,35]. The crystal structures of the RSV[36] and HIV proteinases[37,38] have generally confirmed these ideas.

Figure 4 shows representative 3-D structures of monomeric pepsin-like and dimeric retroviral proteinases viewed from equivalent directions perpendicular to the 2-fold axis. The similarity in general shape can be seen although the monomeric pepsin is roughly 60% larger than the retroviral dimer. The close relationship between their folds is best seen by considering the arrangements of the $\beta$-strands and $\alpha$-helices. Each subunit of the retroviral proteinase and each lobe of a pepsin-like proteinase comprises two similar motifs formed from anti-parallel strands: *a*, *b*, *c* and *d* for the first and *a*′, *b*′, *c*′ and *d*′ for the second[6,36,38,39], organized together in a distorted sheet (sheet 1). Strands *c* and *d*′ and strands *c*′ and *d* form two pairs of parallel strands. Strands *b* and *c* and strands *b*′ and *c*′ form anti-parallel $\beta$-hairpins that are folded over sheet 1 and hydrogen-bonded together around intra-domain two-fold axis to give a second sheet (sheet 2), which is orthogonal to the first. In the retroviral proteinases the motifs are more symmetrical and less distorted than in the pepsin-like enzymes, where both sheets are fragmented.

Much of strand *a* of the first motif is displaced from the main sheet (1) and forms an anti-parallel $\beta$-sheet with the carboxy-terminal strands of the subunit or lobe,

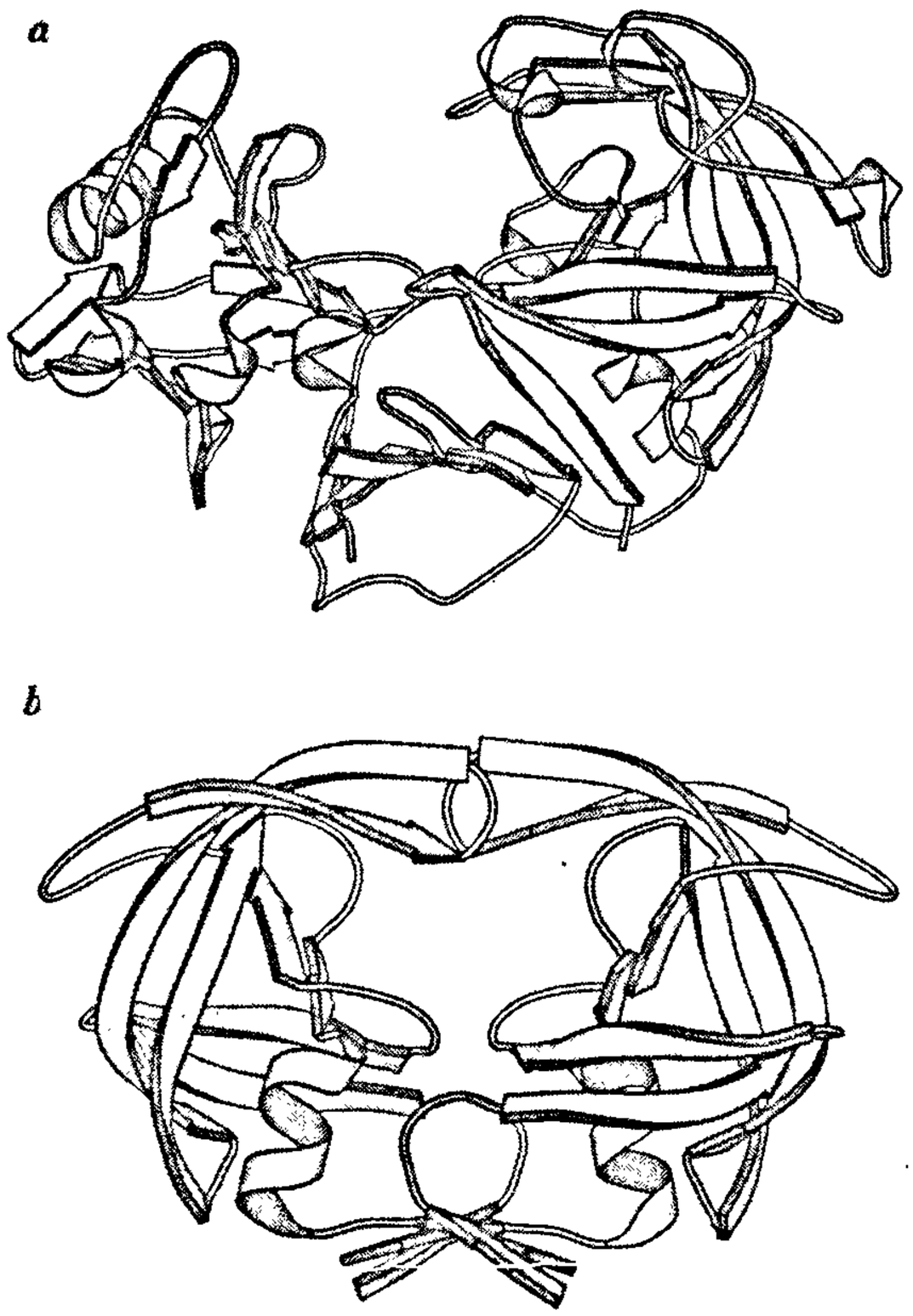


**Figure 4.** A schematic view perpendicular to the two-fold axis and along the active site cleft of a pepsin-like (*a*) aspartic proteinase[6] and (*b*) the HIV-1 proteinase[37,39].

and their equivalents in the second subunit or lobe (sheet 3). In the retroviral proteinases there is only one carboxy-terminal strand and so the inter-subunit $\beta$-sheet contains four antiparallel strands. In the pepsin-like proteinases two carboxy-terminal strands of each lobe contribute to a six stranded antiparallel $\beta$-sheet. Figure 4 shows that the strands of $\beta$-sheet 3 form the base of the enzymes below the well-defined cleft. In the retroviral and pepsin-like enzymes the equivalent $\beta$-sheets occupy the same volume but have different orientations.

The conserved active site residues on the loop between strands *c* and *d*, for example, Asp-25–Thr-26–Gly-27 of the HIV proteinase, form a symmetrical and highly hydrogen-bonded arrangement, virtually identical to that in pepsin-like aspartic proteinases (residues Asp-32–Thr-33–Gly-34 and Asp-215–Thr-216–Gly-217 of pepsin). This includes the two threonines, which are inaccessible to solvent and hydrogen-bonded so that the $\gamma$-O forms hydrogen bonds with the mainchain NH and CO functions of the other subunit or lobe in a fireman's grip[33]. They provide a good example of high conservation of buried, hydrogen-bonded polar residues that are important for maintenance of 3-D structure. The two aspartates lie approximately planar with their inner carboxylate oxygens hydrogen bonded to the NH functions of Gly-27 and within hydrogen-bonding distance of each other. The conservation of the glycines appears to be a consequence of the fact that the existence of sidechains at this position would disrupt the structure of the aspartic acid sidechains.

A further significant conservation in the retroviral and pepsin-like aspartic proteinases is the sequence hydrophobic–hydrophobic–glycine found on strand *d'*, for example Ile-84–Ile-85–Gly-86 found in HIV-1 proteinase, which crosses a loop containing an active site aspartate and forms a $\psi$ structure. The equivalents of Ile-85 in other retroviral proteinases and in pepsin-like proteinases are conserved as hydrophobic because they contribute to the core. Gly-86 is packed close to the active site residues in a way that does not allow a sidechain. Ile-84 is conserved for purposes of binding substrate. This pattern is characteristic of both monomeric

and dimeric aspartic proteinases. In all retroviral proteinases the following residue is an arginine. Such a basic residue is never found in the pepsins, where it is either a hydrophobic residue or an aspartic acid. In both HIV-1 proteinase and RSV-proteinase[36] this conserved arginine participates in an intersubunit cluster of ionic and hydrogen bonding interactions, and is therefore probably important in dimer formation.

## Pepsins in biotechnology and drug discovery

Aspartic proteinases have turned out to have fascinating applications. They have been traditionally used in cheese, soya and cocoa processing and recombinant enzymes are in commercial use in the cheese industry.

Renin is the most specific aspartic proteinase yet discovered and is involved in the formation of angiotensin II, a potent pressor and aldosterogenic substance controlling vascular tone, fluid volume and sodium excretion. Because of its unique specificity, its inhibition was widely expected to provide selective therapy for hypertension, congestive heart failure and associated degenerative disorders linked to angiotensin II. Useful renin inhibitors have been developed by several companies in the 1980s. In the absence of crystal structures most of the early work was based on models of renin[28,29]. Crystal structures of human renin were much later reported at medium resolutions both for the uncomplexed enzyme[11,12] and for various complexed enzymes with inhibitors[12,13]. A higher resolution structure of a mouse renin complex with a decapeptide inhibitor is also reported[13,14]. The detailed analyses of inhibitor complexes indicate the general structural features that may contribute to specificity, especially of renin but demonstrate the need for careful, high resolution X-ray analyses for more confidence in drug design. In particular, they show that even minor alterations in the positions of secondary structural elements can lead to major changes in the disposition of the subsites (see above). Considerable progress was made exploiting structure-based approaches in developing highly specific and orally active molecules. However, most activity in the synthesis of renin inhibitors was stopped when it became apparent that the sophisticated chemistry would require a high price and that they would consequently not be competitive with that of generic antihypertensives, based on the inhibition of angiotensin converting enzyme, as patent protection of these falls. The same technology offers possibilites for anticancer (cathepsin D and E), antimalarial and antifungal agents against candidapepsins, the latter of which can be used to treat infections associated with AIDS.

Most exciting of all has been the production of antivirals targeted at HIV proteinase, which are now proving their value in the clinic. This has been one of the first cases where structure-based approaches have convincingly played a role in the discovery of drugs in the clinic[40]. Structures of several hundred inhibitor complexes have been experimentally defined, providing a previously unparalleled structural database for design. These have exploited a range of different structural features (see Whittle and Blundell[41] for a review) including two-fold symmetry in the ligand, replacement of a bound water molecule, cyclization and replacement of scissile peptide bonds; see for example the cyclic, symmetric inhibitor of the Dupont-Merck team[42]. Several studies have used programs like DOCK or fragment searching to identify non-peptidic structures. Useful molecules are now exploited as cocktails in the treatment of AIDS, with encouraging results, although it is evident that mutation in HIV allows the virus to quickly escape if challenged with a single antiviral.

## Conclusions

The structural studies of the pepsins, begun by J. D. Bernal and Dorothy Crowfoot in the 1930s, have had an enormous impact on structural biology, enzymology, biotechnology and drug discovery. Bernal and Crowfoot immediately saw the potential importance for X-ray crystallography in their early studies of pepsin crystals and this has been more than justified. They were also aware of the implications for enzymology; enzymes must be discrete, well-ordered, globular proteins. They could not have foreseen the fascinating story of protein evolution that has since been unravelled, starting from an ancestral dimer to give bilobal enzymes with broad specificites such as pepsin and the fungal aspartic proteinases or highly specific enzymes with higher optimal pHs such as renins. But they would not have been surprised! Neither would they have been surprised by the great value that the knowledge of their structures would have in drug discovery and biotechnology. Dorothy Hodgkin always kept in close collaboration with the pharmaceutical and biotechnology industries, whether she was working on penicillin, vitamin $B_{12}$ or insulin. She was a firm believer that beautiful science will very often be useful. The studies of the aspartic proteinases give further support to her vision.


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