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Catalytic triads and their relatives

Guy Dodson and Alexander Wlodawer

Interactions among the residues in the serine protease Asp-His–Ser catalytic triad, in the special environment of the enzyme–substrate complex, activate the nucleophilic potential of the seryl Oγ. In the subtilisin and trypsin families, the composition and arrangement of the catalytic triad do not vary significantly. However, the mechanisms of action of many other hydrolytic enzymes, which target a wide range of substrates, involve nucleophilic attack by a serine (or threonine) residue. Review of these enzymes shows that the acid–base–ser/thr pattern of catalytic residues is generally conserved, although the individual acids and bases can vary. The variations in sequence and organization illustrate the adaptability shown by proteins in generating catalytic stereochemistry on different main-chain frameworks.

CHEMISTRY IS THE engine that drives biology, and many enzyme families are responsible for making this chemistry possible. The catalytic ability of enzymes rests on the spatial organization of the active atoms, through which the chemical and structural steps of the reaction are orchestrated. Structural and sequence comparisons of a variety of proteins have been carried out by a number of groups (see Ref. 1, for example). Such comparisons of enzyme families show that catalytic groups have distinctive patterns of variation[2] and that the proteins themselves sometimes have unexpected, even surprising, evolutionary and structural relationships with other protein families that have utterly different functions.

In this article, we examine how the catalytic groups of enzymes that cleave amide or ester bonds by nucleophilic attack, a particularly important reaction in biology, can vary among different enzyme families. This kind of analysis has been made possible by the remarkable activity in the field of X-ray crystallography – activity that is producing an avalanche of new protein structures and accurate details of their functional surfaces[3].

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Figure 1 shows the well-known series of chemical events that occurs during catalysis by a serine protease. It also shows the different chemical branches that can exist on each side of the amide or ester bond, although we discuss here only reactions involving the amide bond. In the scheme shown, the unifying chemistry of nucleophilic attack at the carbonyl carbon can be appreciated. In reviewing the variations in the catalytic structures among different enzyme families, we consider the acid, the base and the nucleophile separately.

The architecture of the classical triad in serine proteases

The first protease catalytic site, revealed by David Blow and colleagues[4] about 30 years ago using X-ray crystallography, was that of α-chymotrypsin. Their analysis showed initially that two residues were directly involved in catalysis: Ser195 and His57. Chemical evidence for the involvement of Ser195 and His57 in the catalytic reaction already existed, and the crystal structure revealed that the catalytic serine residue was indeed near enough to His57 to form a hydrogen bond. However, the nearby residue 102 was not an asparagine residue, as originally thought, but rather an aspartate residue that was hydrogen-bonded to His57 and could potentially form a salt bridge with this histidine residue. The invariance of this aspartate residue in all the related sequences showed that the residue was an important component of the catalytic structure, which the authors identified as a Ser–His–Asp triad; because of the triad's polarization, they referred to it as a charge-relay system. An account of the

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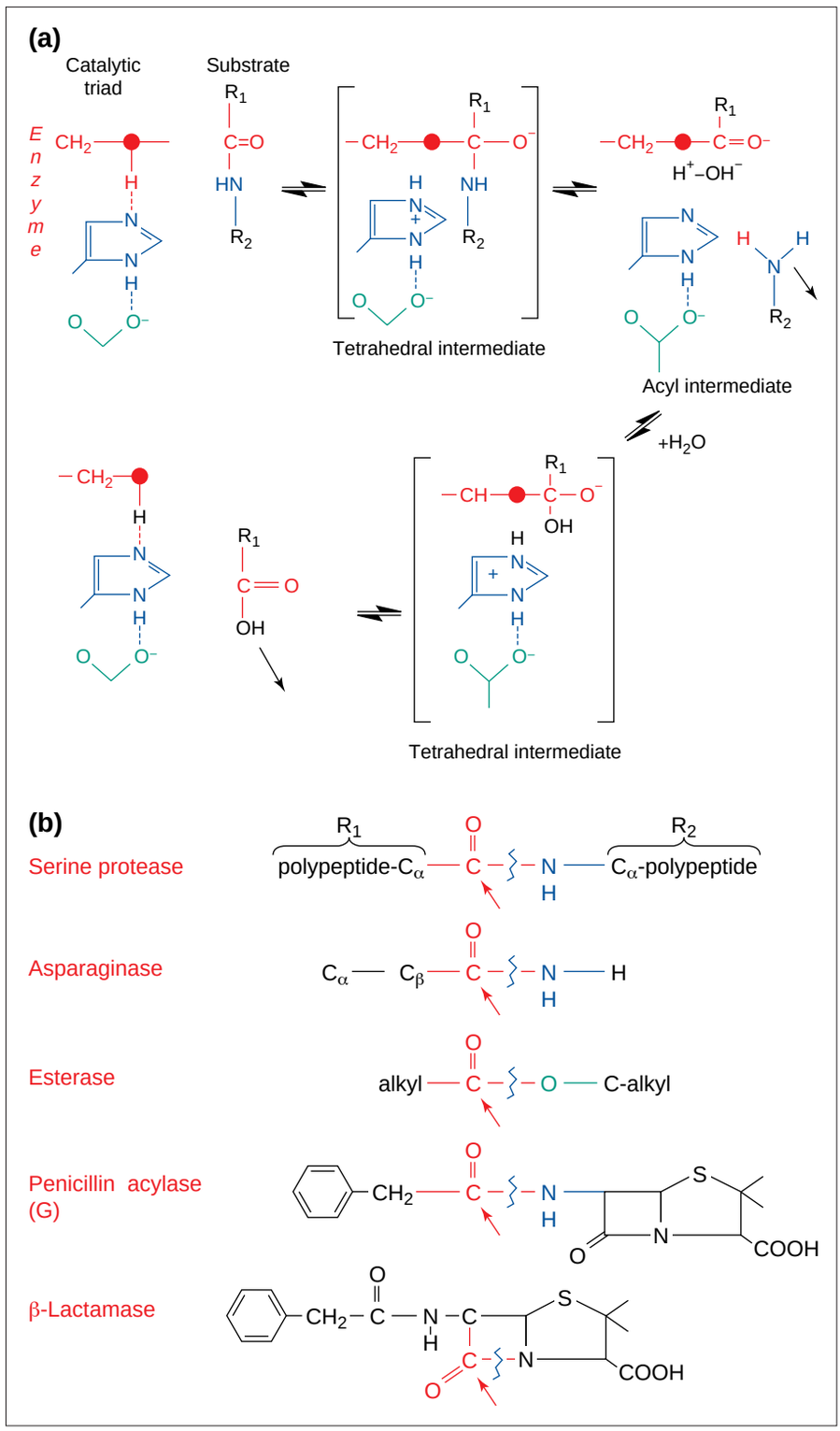

Figure 1

(a) Nucleophilic attack on the peptide bond by the catalytic triad. The nucleophile-bearing residue is shown in red; the red dot indicates the nucleophilic atom. The histidine and acidic residues are shown in blue and green, respectively. **(b)** The bonds cleaved by various classes of enzymes discussed in the text. Arrows indicate the sites of nucleophilic attack.

structural determination of α-chymotrypsin and the first ideas about the active site was published recently in *TiBS*[5].

An identical triad was later found in the trypsin structure (see Fig. 2), which was determined in the laboratories of Huber[6] and Stroud[7], as well as in the elastase structure, which was determined by Watson *et al.*[8] These two enzymes are closely related to α-chymotrypsin, and the existence of the same triad was expected. Even more significant was the discovery by Kraut *et al.*[9] and by Drenth *et al.*[10], independently, that the same triad is present in the protease subtilisin (see Fig. 2b). Subtilisin's main-chain structure is completely different from that of α-chymotrypsin; thus, the presence of an essentially identical stereochemistry in the subtilisin active residues demonstrated that there was an independent evolutionary path to the triad.

Several investigators have analysed in detail the roles of the side chains in serine-protease catalysis; these studies have revealed the synergistic nature of the side chains' contribution to the enhancement of the reaction rate[11,12]. There are, nonetheless, still many outstanding questions about the nucleophilic mechanism and its evolution – in particular, that of just how the arrival of the substrate favours the abstraction of the proton from the attacking oxygen. The recent review in *TiBS*[13], which addresses the chemical interactions in differently constituted catalytic structures and the existence of a catalytic dyad, discusses some of these issues.

Generally speaking, in serine proteases, the three residues of the classic catalytic triad each have a specific role in generating the nucleophilic potential at the seryl Oγ, although many problems in defining these in detail remain. The aspartate residue forms a hydrogen bond with the adjacent imidazole group. This interaction orients the imidazole group in the triad and increases the group's pK_a and basicity somewhat[12]. This is an advantage because a histidine residue, when buried by the substrate during the reaction, would otherwise have a reduced pK_a and be more reluctant to accept a proton. In the catalytic reaction, the imidazole group (acting as a base) accepts the seryl Oγ proton, becomes positively charged and forms a transient salt bridge with Asp102. Significantly, the well-defined interaction between the aspartate and histidine residues is not repeated between the histidine and serine residues. There is usually unfavourable geometry at the Oγ and/or the Nε in both the free and inhibited enzyme[14,15]. This strained geometry reduces the energy of the hydrogen bond, which might be important in the relaying of the proton from the seryl Oγ to the imidazole and, subsquently, on to the departing peptide.

The hydrogen-bonding interaction between the Oγ and the Nε is important, even if energetically weak, in that it allows a closer than van der Waals approach, which might favour the transfer of the

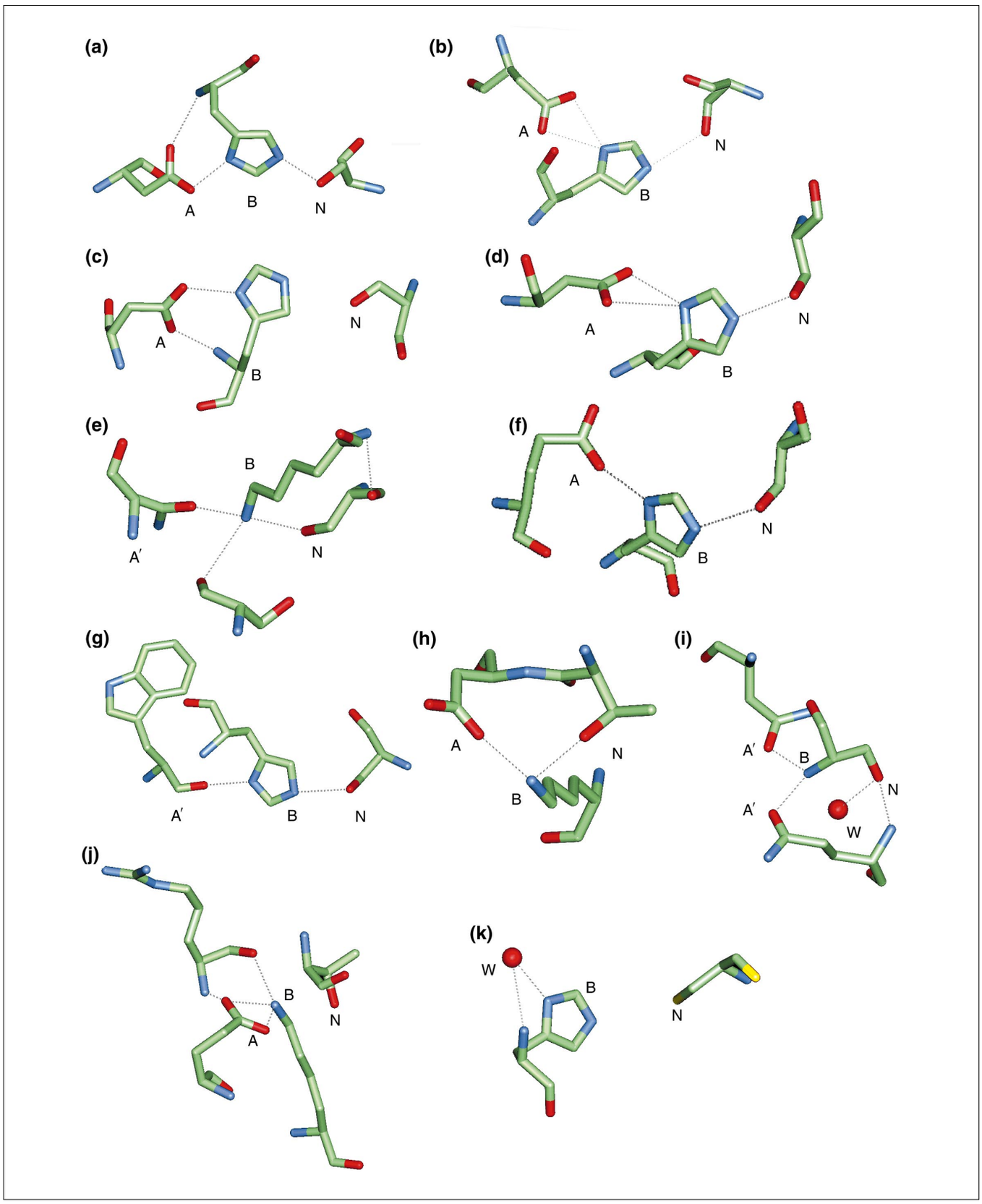

Figure 2

The catalytic triads of enzymes discussed in the text. Nitrogen atoms are shown in blue; oxygen atoms are shown in red; sulphur atoms are shown in yellow. H-bonds are indicated by dotted lines. The base (B) is shown in the centre; the acid (A) and/or H-bond acceptor (A′) are shown on the left; the residue that contains the nucleophilic atom (N) is shown on the right. Brookhaven accession numbers, where available, are given in parentheses. **(a)** Trypsin (1sgt). **(b)** Subtilisin (1svn). **(c)** Brain acetyl hydrolase (coordinates provided by Zygmunt Derewenda). **(d)** Lipase (3gtl). **(e)** β-Lactamase (coordinates provided by Michael James). **(f)** Acetyl cholinesterase (2ace). **(g)** *Streptomyces scabies* esterase (coordinates provided by Zygmunt Derewenda). **(h)** Asparaginase (coordinates provided by Alex Wlodawer). **(i)** Penicillin acylase (1pnk). **(j)** The prokaryotic proteasome catalylic subunit (1pma). **(k)** The trypsin-like enzyme from picornavirus, in which cysteine provides the nucleophile (coordinates provided by Michael James). W, water.

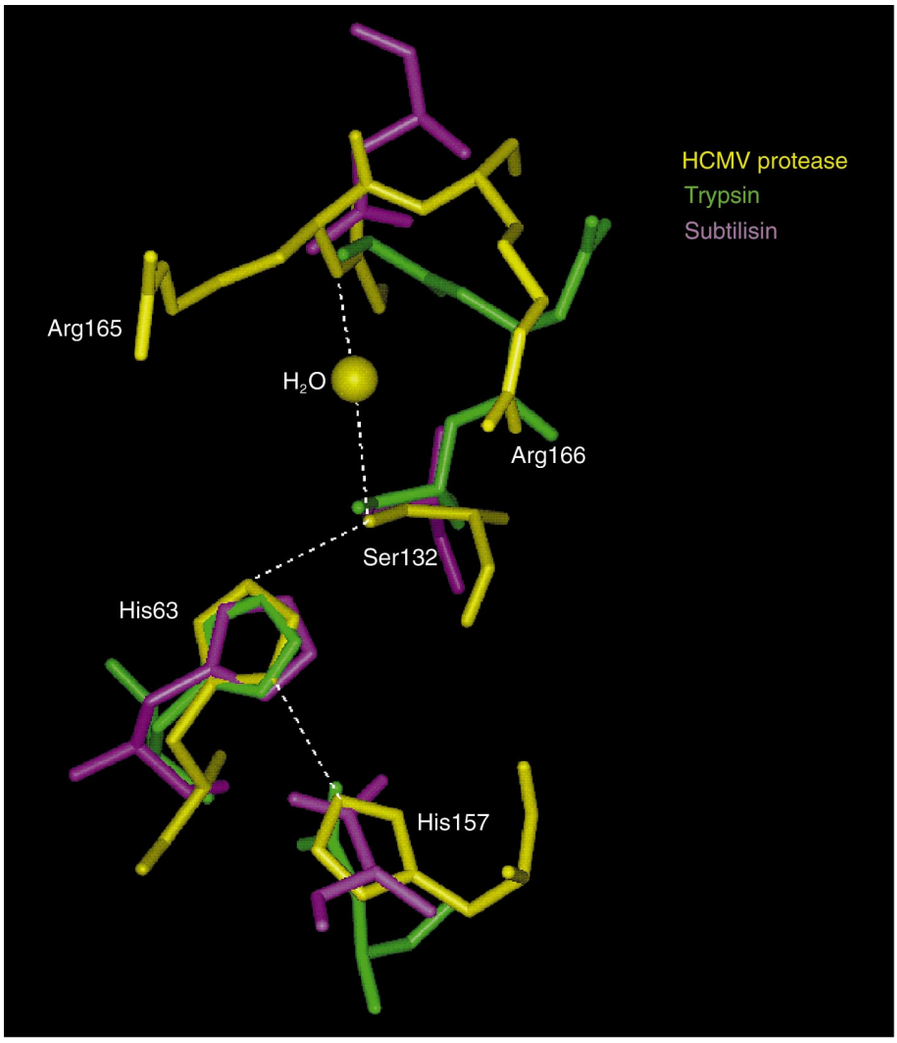

Figure 3

Superposition of the catalytic triads of human cytomegalovirus, trypsin and subtilisin. Adapted, with permission, from Ref. 26.

proton. The small energetic advantage might also be important: a strong bond could interfere with proton transfer[6]. The ability of the catalytic histidine residue to accept the proton from the serine Oγ obviously arises from a great deal more than the difference in the relative pK_as of the two residues. Other important factors in the mechanism include the effects of substrate binding, which will bury the active centres and cause transient structural adjustments that favour the proton's transfer from the seryl Oγ and make the latter a powerful nucleophile that can attack the substrate carbonyl carbon.

Variations in the 'serine' catalytic triad

No real variation (but see below) in the chemical make-up of the catalytic sites of the serine-protease families has been observed; the three active residues all sit at the same location in their respective structures. An equivalent alignment of the active atoms in the aspartate, histidine and serine side chains occurs in the α/β-hydrolase family (e.g. the lipases[16,17]; see Fig. 2d) and in a recently discovered esterase family[18]. The catalytic triad of brain acetyl hydrolase (a member of another recently discovered esterase family), which has a fold similar to that of a small G protein, is illustrated in Fig. 2c. The triad is located near to what would normally be the nucleotide-binding site. Whether other triad-containing folds will be discovered remains to be seen, but given the wide occurrence of the catalytic chemistry, this seems likely.

The methylesterase CheB catalytic domain is a member of yet another new serine-hydrolase family and contains a catalytic triad that appears to hydrolyse the methyl-ester bond. The positioning of the active serine residue and the enzyme's secondary structure is reminiscent of the α/β hydrolases; however, the connectivity in these enzymes is different, as is the placing of the catalytic histidine and aspartate residues[19].

One very interesting change in the chemistry and internal structure of the catalytic triad is evident in an α-chymotrypsin-like protease, the so-called HAV-3C gene product of the hepatitis A picornavirus[20,21]. Here, the catalytic nucleophile is a cysteine residue not a serine residue (Fig. 2k). There is also a structural change in the side-chain geometry of His44 and the adjacent Asp84 (equivalent to His57 and Asp102 in chymotrypsin, respectively): in the HAV-3C gene product, the side-chains of these residues are not in contact. The aspartate residue is replaced by a water molecule (Fig. 2). At first sight this arrangement is surprising; however, it is reminiscent of the substitution of an asparagine residue for an aspartate residue in the cysteine proteases and reflects the greater nucleophilic character of sulphur compared to that of oxygen.

The preservation of the spatial relationships of the active atoms in chymotrypsin, subtilisin, lipase and brain-acetyl-hydrolase families is not in itself surprising: the stereochemical factors that govern the nucleophilic cleavage of the peptide (and ester) bond are specific and require precisely defined, and identical, structural and chemical events. As emphasized before, however, the chemical reaction depends on much more than just the stereochemistry of the catalytic residues. The substrate makes transient interactions as it approaches, binds to and adapts to the active-site surface of the enzyme. The surfaces involved extend from the catalytic serine residue to the structure that stabilizes the transition state – the so-called oxyanion hole. These complex structural contacts are essential for the catalytic process. For reasons of space, we exclude consideration of the oxyanion hole from the following discussion. Suffice it to say that the oxyanion hole often contains main-chain atoms and varies greatly in structure, although the atoms that interacting are presented with the same general stereochemistry.

Variations at the hydrogen-bond-acceptor/acid component

Some years ago, lipases were also shown to contain the Asp–His–Ser catalytic triad[22,23]; this provided another example of convergent evolution of this catalytic triad. The chirality of the catalytic clusters in lipases and other α/β hydrolases, however, differs from that of the serine proteases: the positions of the attacking and leaving atoms are reversed. Analysis of the many other

lipase structures and some related enzymes has revealed other interesting variations in the catalytic triad[17,18]. For example, the positioning of the catalytic aspartate in the secondary structure of human lipases is different from that of other lipases. These enymes have an invariant structural platform composed of five β-strands. In the human lipase, the catalytic aspartate is on strand III; in all other lipases that have been studied, this residue is on strand IV. In addition, in both acetylcholinesterase from *Torpedo californica* and the lipase from *Geotrichum candidum*, the active-site aspartate residue is substituted by a glutamate residue[24]. Chemically, these residues are equivalent and can form a very similar local structure (see Fig. 2); however, a glutamate residue at this position has not yet been observed in naturally occurring serine proteases. Protein-engineering experiments on subtilisin have shown that replacement of the catalytic aspartate residue by glutamate is detrimental to catalysis[11].

An esterase from *Streptomyces scabies* (Fig. 2g) reveals a further variation in nucleophilic activation[25]. The enzyme is related structurally to lipases and to the other α/β hydrolases but displays very unusual chain folding. Furthermore, two main-chain carbonyl oxygens (instead of an acidic residue) contact the catalytic histidine residue. Although these main-chain oxygens will still make the stereochemically important hydrogen bonds, they will increase the pK_a of the catalytic histidine residue to a lesser extent than a carboxylate group would, and a transient salt-bridge cannot form during the catalytic reaction. Thus, the carbonyl-oxygen–histidine hydrogen bond should reduce the system's capacity to promote the nucleophilic activity of the catalytic serine residue, although this activity would probably be less important for an esterase than for a protease or peptidase.

Four recent, simultaneous reports of the structure of a serine protease from human cytomegalovirus illustrate the extraordinary activity in the field of protein crystallography and the intense interest in viral proteases[26–29]. The enzyme has a novel fold but cleaves substrates by the usual mechanism of nucleophilic attack by an activated serine residue. This serine protease, however, possesses a novel His–His–Ser catalytic triad (Fig. 3). The substitution of the aspartate residue by a histidine residue, a much weaker acid, is, unsurprisingly, associated with considerably reduced activity.

Variations at the base

The enzymes that break open the β-lactam ring in penicillin, and the enzyme penicillin acylase, which removes the side group, also exploit serine residues for nucleophilic attack. The other components of the catalytic structure, however, vary. The structural chemistry of these enzymes is discussed below because there are changes in the base and, in the case of the penicillin acylase, other important changes as well.

In β-lactamase enzymes, a lysine residue replaces the histidine base (Fig. 2e). This enzyme attacks the β-lactam ring by splitting the C–N bond (which is analogous to the peptide bond cleaved by serine proteases)[30]. Three crystallographic studies have revealed that a lysine residue is adjacent to the active-site serine residue; this lysine residue presumably plays a role analogous to that of the histidine base in serine proteases. The pK_a of the free lysine ϵ-amino group is ~11. Thus, if this group is to act as a base, the environment must be able to stabilize the energetically unfavourable deprotonated form[31]. Interactions between the active serine (Ser 70) and lysine (Lys 73) residues, and the surrounding residues differ in the different crystals. Interestingly, a nearby glutamate residue (Glu166) can contact Lys73[32–34]. The study by Strynadka *et al.*[35] shows that the serine residue forms one contact to the nitrogen atom of this lysine residue which in turn contacts two carbonyl oxygen atoms. The variation observed among the catalytic residues in the different β-lactamase crystal structures suggests that these residues are flexible and have weakly defined interactions – in contrast to the enzymes discussed above. The catalytic requirements might be generated by more than one arrangement; alternatively, the catalytic stereochemistry might only be assumed when the correct productive complex is established.

Enzymes that belong to the asparaginase/glutaminase family convert side-chain amide groups to carboxylate groups. These enzymes also possess a putative catalytic triad. Although the mechanistic implications are not yet clear, the spatial arrangement of the lysine and aspartate residues allows delineation of a triad in which lysine could serve as a catalytic base that is linked to an aspartate residue (Fig. 2h)[36]. The generation of a deprotonated lysine residue (in order to accept a proton) in this triad demands considerable energy and steric organization, even in the presence of the bound substrate. This apparently inappropriate chemistry might be explained by the presence of a second nucleophile side chain in the active site; indeed, this second nucleophile might be even more important. This enzyme differs from serine proteases in another respect: the catalytic serine residue is replaced by a threonine residue (discussed below)[37].

Variations at the nucleophile

The use of a threonine residue as the nucleophile in asparaginases (Fig. 2h) is not surprising[37]. There are no significant chemical differences between the Oγ atoms of threonine and serine residues, but these residues do different significantly in conformation and bulk. The relatively simple structure of the substrate (the side-chain amide group of asparagine or glutamine residues) might permit a threonine residue to present the catalytic Oγ correctly. The threonine side chain might have an additional advantage: the Cγ could help to exclude water. Recently, catalytic threonine residues have been identified in other hydrolytic enzymes (e.g. the proteasome catalytic subunit[36] and aspartyl glucosaminidase[38]); proteases that utilize the theonine side chain as a nucleophile are now an accepted phenomenon.

In asparaginase, an interesting complication is the presence of a second threonine residue that is appropriately positioned for nucleophilic attack and lies on the opposite side of the substrate but is not part of a catalytic triad. Mutations at either threonine residue inactivate the enzyme. These experiments suggest that the side chains of both threonine residues participate in the reaction[39] and that, in this case, a single triad might not be sufficient for enzymatic activity.

Catalytic economy in Ntn-hydrolases

Another class of hydrolytic enzymes, the so-called Ntn-hydrolases, has been recognized recently[40]; two such enzymes are penicillin acylase and the prokaryotic proteasome catalytic subunit. In these two enzymes (Fig. 2i,j), the side chain that provides the nucleophile is an N-terminal residue whose α-amino nitrogen atom acts as a base. Thus, nucleophile and base exist in the same amino acid. There is no acid component – a property that is shared by β-lactamase and some esterases. Although only about six members of the Ntn hydrolase family have been described, variation in the form of serine, threonine

or cysteine nucleophiles has been identified[36,41]. This kind of variation at the nucleophile-containing residue is not seen in other serine-hydrolase families, which suggests that the Ntn-hydrolases are more adaptable and perhaps more primitive. The Ntn-hydrolases share a further biologically important property: autocatalytic processing of a precursor peptide, through which the N-terminal catalytic residue is created[40]. It will be interesting to see what structural arrangements are responsible for this autocatalytic chemistry.

Summary

The catalytic triad first observed as Ser–His–Asp in serine proteases exists in a variety of forms and evolved a number of times independently. The comparisons between the different catalytic systems that led to the recognition of these chemical and structural relationships were made possible by the availability of results from many crystallographic analyses. There is little doubt in our minds that other discoveries of relationships in catalytic chemistry and in protein function will be made in the future, and that the understanding of enzyme mechanisms will be amplified and deepened by such comparisons.

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