9688 *Biochemistry* **2010,** *49,* 9688–9697
DOI: 10.1021/bi1012222

BIOCHEMISTRY
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Current Topic

Structural Relationship between the Active Sites of β-Lactam-Recognizing and Amidase Signature Enzymes: Convergent Evolution?†

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Received August 2, 2010; Revised Manuscript Received October 17, 2010

ABSTRACT: The β-lactam-recognizing enzymes (BLRE) make up a superfamily of largely bacterial proteins that include, principally, the DD-peptidases and β-lactamases. The former enzymes catalyze the final step in bacterial cell wall biosynthesis and are inhibited by β-lactam antibiotics, while the latter enzymes catalyze the hydrolytic destruction of β-lactams and represent a major source of bacterial resistance to these antibiotics. The active site of this superfamily of enzymes includes a Ser1/Ser2(Tyr)/Lys1(His)/Lys2 tetrad in which Ser1 is a nucleophilic catalyst that becomes acylated in the formation of an acyl−enzyme intermediate. An oxyanion hole is also present. The amidase signature (AS) enzymes represent another serine amidohydrolase superfamily with no overall structural resemblance to the BLRE. The active site is characterized by a Ser1/Ser2/Lys1/NH tetrad and an oxyanion hole. We point out that there is a close spatial overlap between the two tetrads and speculate that this has arisen from a process of convergent evolution driven by a mechanistic imperative. Conversion of the backbone NH group of the AS tetrad into Lys2 of the BLRE is rationalized and leads to another mechanistic possibility that may dominate BLRE catalysis. The active site triads of other serine amidohydrolases are also briefly and comparatively discussed.

Structural studies of proteins reveal a large but finite number of backbone folds (*1*−*3*). Enzymes of common function often arise by processes of divergent evolution from a common ancestral structure. Common folds and function usually correlate with common active site structure, i.e., the common architecture of the catalytically active residues. A much-cited example is the catalytic triad of serine proteases. It is also found that enzymes with a common function have similar active site residues but a different overall fold. These proteins are often thought to arise through a process of convergent evolution under the influence of a mechanistic imperative (*4*); i.e., for catalysis of a particular reaction, there is only a small number of very effective ways of arranging active site functional groups, e.g., into a catalytic triad [as, again, in serine proteases (*5*−*7*)]. Examples of convergent evolution may not be unusual (*8*), although some at least may represent the products of divergent evolution from very distant sources (*9*). In this paper, we point out the close active site similarities between two enzyme superfamilies, the β-lactam-recognizing enzymes (BLRE)[1] and the amidase signature (AS) enzymes.

The serine amidohydrolases, located in EC classification groups 3.4 and 3.5, represent a large and important group of enzymes that have the common function of catalyzing the hydrolysis of amides and, in particular, although not exclusively, peptides and proteins. The proteases/peptidases of EC 3.4 have been classified into a series of clans, each of which represents a separate protein fold (*10*), and further folds are found in EC 3.5. The active site of each of these enzymes contains a nucleophilic serine (or, occasionally, threonine) residue, surrounded by one to three auxiliary residues. The latter are generally believed to have roles in acid−base catalysis or participate in hydrogen bonds that stabilize the transition state of the catalyzed reaction. The auxiliary residues are commonly His, Asp, Glu, Lys, Ser, and Tyr, and quite a wide variety of combinations of these have been identified (*7*). An additional common theme of the active sites of these enzymes is the "oxyanion hole," comprised of one or more hydrogen bond donors situated in space to stabilize the anionic tetrahedral transition states of the acyl transfer reaction being catalyzed (*11*, *12*).

It has been found that the combination of active site residues (nucleophile and auxiliaries) is not always the same within a particular clan (fold). Certain functional groups apparently may be gained or lost during divergent evolution, presumably under the pressure of a newly encountered substrate. Conversely, the same combination of functional groups may be found in different clans (folds), achieved, presumably, by convergent evolution, driven by the mechanistic imperative mentioned above.

DD-PEPTIDASES

One important serine peptidase clan [SE (EC 3.4.16.4)] contains the bacterial DD-peptidases [penicillin binding proteins (PBPs)]. These enzymes catalyze the final transpeptidation and carboxypeptidation steps of bacterial cell wall biosynthesis. Their substrates, therefore, are D-alanyl-D-alanine-terminating peptides (Scheme 1). Catalysis proceeds by a double-displacement acyl transfer mechanism involving a covalent acyl−enzyme

†This research was supported by National Institutes of Health Grant AI-17986 (R.F.P.) and National Science Foundation Grant EF-0425719 (M.J.M.).

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[1]Abbreviations: AS, amide signature; BLRE, β-lactam-recognizing enzyme; FAAH, fatty acid amide hydrolase; HMM, high-molecular mass; LMM, low-molecular mass; PDB, Protein Data Bank.

 Published on Web 10/26/2010

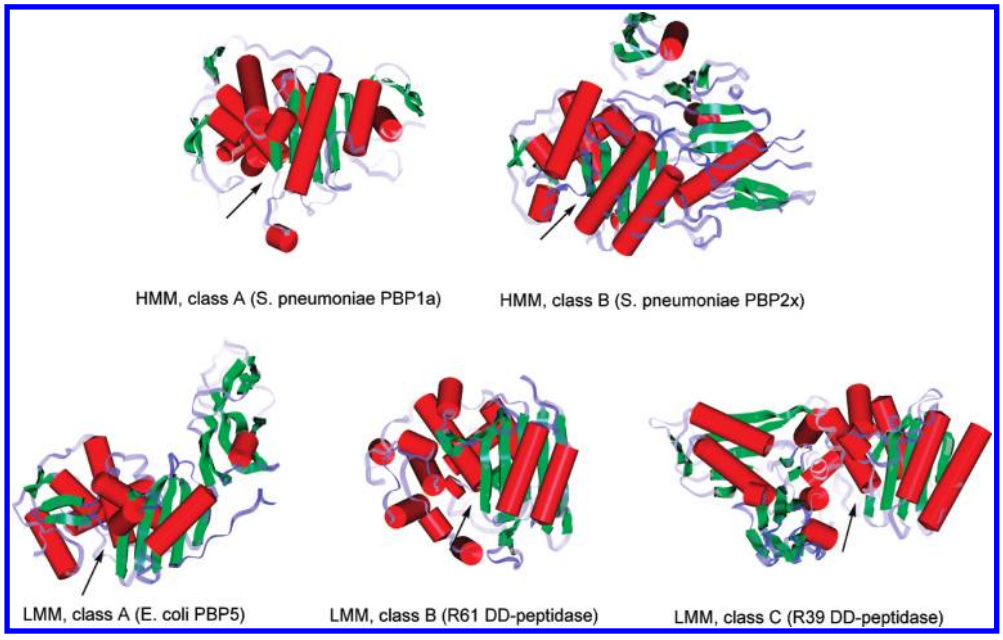
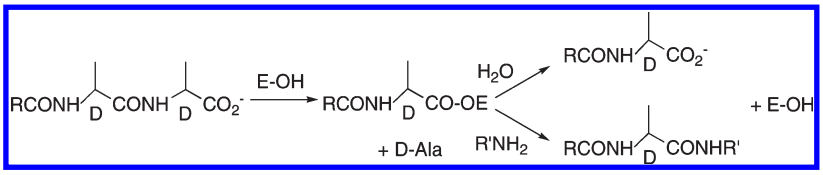


FIGURE 1: Common fold of the DD-peptidase module in the major classes of β-lactam-recognizing enzymes. The entrance to the active site lies between the helix/sheet and helix domains of the peptidase module and is indicated by an arrow in each case.

Scheme 1



intermediate, where an active site serine residue becomes transiently acylated.

The active sites of bacterial DD-peptidases are characterized by a combination of three distinctive amino acid sequence motifs, SXXK (including the nucleophilic serine of Scheme 1), S(Y)XN, and K(H)T(S)G (*13*−*15*). On the basis of more detailed sequence comparisons, they can be subdivided into two groups, high-molecular mass (HMM) and low-molecular mass (LMM) groups, where the cutoff between the groups is around 50000 kDa. The latter group is further subdivided into three subgroups, A−C, and the former into two, A and B. The LMM enzymes are not essential to bacterial survival and are thought to adjust the degree of cross-linking in mature peptidoglycan, whereas the HMM group is essential, catalyzing the synthetic transpeptidation reaction. The latter enzymes are the targets of β-lactams as antibiotics (*16*, *17*).

Crystal structures of representative examples of all subgroups of DD-peptidases are now available (*18*, *19*). They demonstrate a unique protein fold that is common to all members of what is today described as a superfamily (*15*) (Figure 1). This fold can be seen as a combination of two domains, one α and the other α/β; between them lies the active site (Figure 1). The HMM enzymes have at least one additional module, which, in the HMMA group, catalyzes the transglycosylation reaction of peptidoglycan biosynthesis (*20*). These enzymes will here be termed members of the β-lactam-recognizing enzyme (BLRE) superfamily that contains, principally, the DD-peptidases and β-lactamases.

Inspection of the active site region of DD-peptidases shows the presence of the three distinctive conserved motifs mentioned above (Figure 2). The example presented in Figure 2 shows the

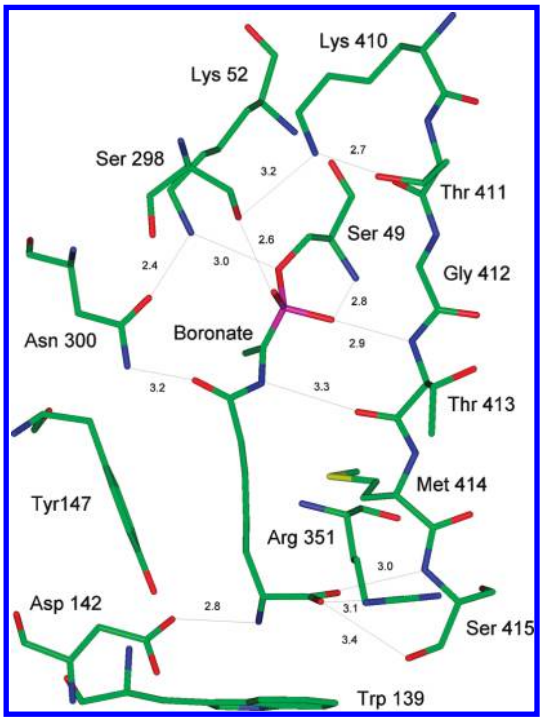


FIGURE 2: Active site structure of a typical β-lactam-recognizing enzyme, the *Actinomadura* R39 DD-peptidase, with a boronate transition state analogue bound to the nucleophilic serine residue (PDB entry 2XDM). In this structure, Ser1 is Ser49, Lys1 is Lys410, Ser2 is Ser298, and Lys2 is Lys52.

active site of a LMM class C enzyme, the DD-peptidase of *Actinomadura* R39 (*21*), which also contains a boronate transition

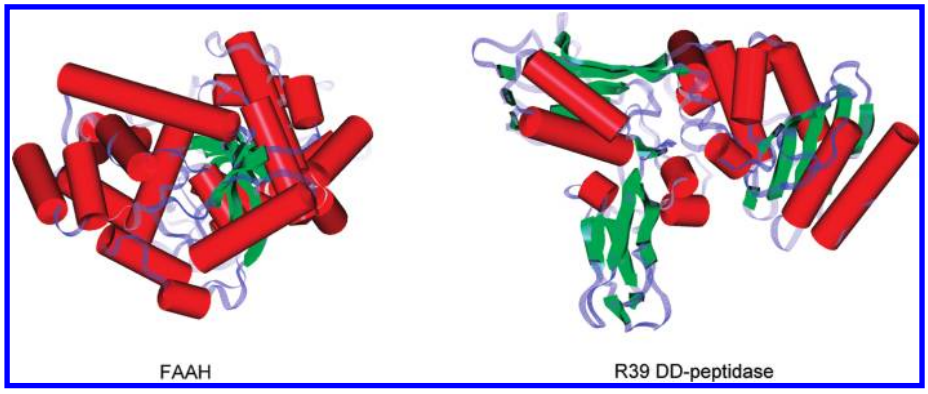


FIGURE 3: Protein folds of a typical amidase signature enzyme (fatty acid amide hydrolase, FAAH, PDB entry 1MT5) and a typical β-lactam-recognizing enzyme (*Actinomadura* R39 DD-peptidase, PDB entry 1W79).

state analogue inhibitor modeled after a specific peptidoglycan-mimetic substrate, D-α-aminopimelyl-D-alanyl-D-alanine (*22*). The tetrahedral boronate, mimicking the tetrahedral intermediates of catalysis, is covalently bound to the active site nucleophile (Ser49, of the SXXK motif), as expected. The main feature of this diagram for our purposes is the cluster of functional groups surrounding the boronate presumably in a conformation relevant to catalysis, and including Lys52 (of the SXXK motif), Ser298 (of the SXN motif), and Lys410 [of the K(H)T(S)G motif]. These four residues will be referred to below as Ser1, Lys2, Ser2, and Lys1, respectively. These four are found at the active site of all known DD-peptidases, with the exception of the LMMB group in which Tyr replaces Ser2 and His replaces Lys1. The latter three are often found at hydrogen bonding distances from each other in crystal structures (*18*, *19*) and are believed to represent the auxiliary catalytic residues of this superfamily of serine amidohydrolases. There has been considerable discussion of their specific roles in catalysis (*21*−*23*), although a generally accepted consensus has not yet been achieved. This combination of functional groups seems as unique to DD-peptidases as their protein fold.

There has apparently been little expansion of function of the BLRE superfamily. The major one has been in the evolution of β-lactamases, although this represents only a short aside in which a more facile β-lactam deacylation process has been included (*24*). Both combinations, SXN and YSN, have been incorporated into β-lactamases, the former in class A and the latter in class C. Some other bacterial enzymes have also inherited the BRLE fold and active site features. For example, a D-aminopeptidase (*25*) and an amino acid amidase (*26*) have been isolated and characterized from *Ochrobactrum anthropi*, and both have the classical LMMB active site seen in the *Streptomyces* R61 DD-peptidase (*27*). However, while the D-aminopeptidase retains the HT(S)G motif of the LMMB R61 DD-peptidase, the amino acid amidase exhibits the KT(S)G motif of the other DD-peptidase classes. Several bacterial esterases with the BRLE fold have also been identified. These include EstB from *Burkholderia gladioli* (*28*), EstA from *Arthrobacter nitroguajacolicus* (*29*), and a 6-aminohexanoate linear dimer hydrolase from *Arthrobacter* sp. (*30*). These enzymes have the BLRE fold and the SXXK and YXN motifs, but only glycine of the K(H)T(S)G motif. Finally, a group of bacterial glutaminases has been found (*31*, *32*) that, like the amino acid amidase described above, has the general active site structure of a LMMB DD-peptidase, but with the KT(S)G motif rather than the HT(S)G motif as the third structural motif.

Beyond bacteria, there appear to be very few examples of the BLRE superfamily. As deduced from phylogenetic analysis, the esterases mentioned above may exist in lower metazoans (*33*). Similar analysis has discovered one BLRE-derived protein, LACTB, in higher metazoans, including humans (*33*, *34*). Its functional role in these organisms, however, is not completely clear at present, although there is no evidence that it is an enzyme (*35*). In summary, the BLRE fold and active site appear to be unique and are characteristic of a superfamily that has not yet been related to any other major protein group.

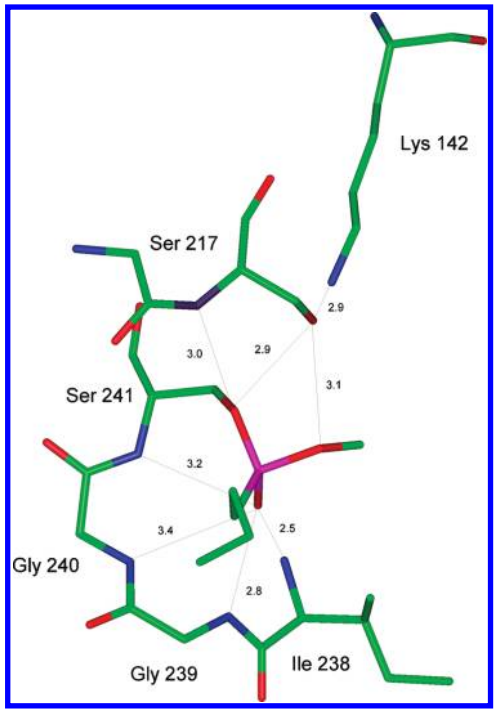
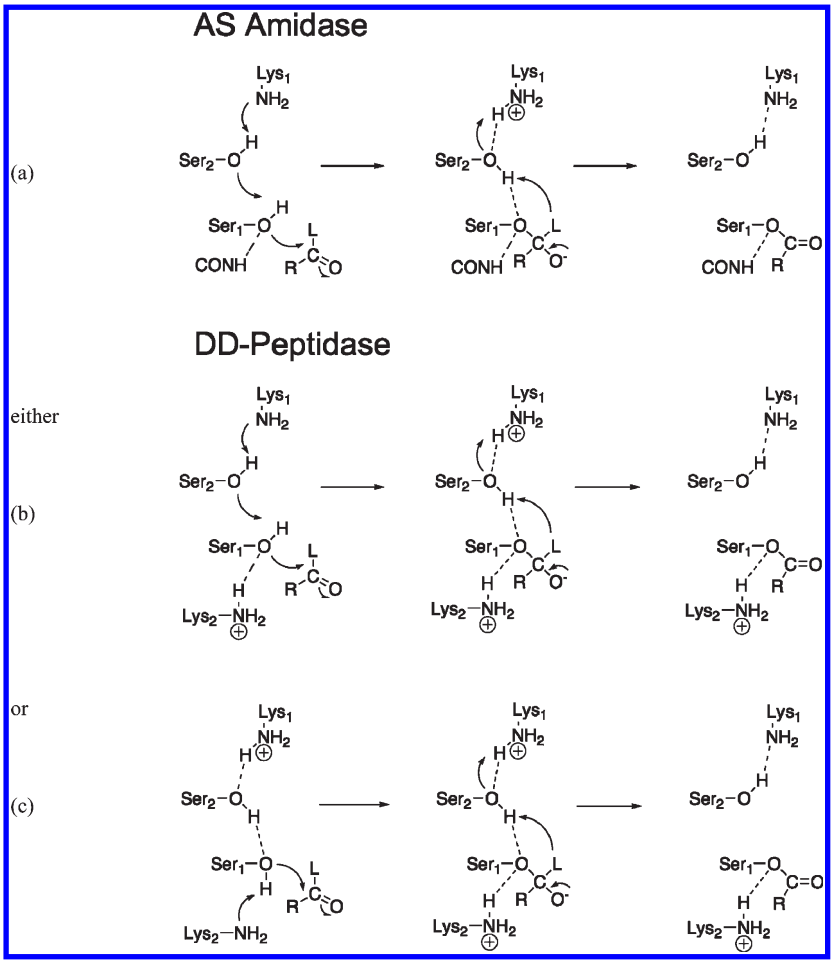


FIGURE 4: Active site structure of a typical amidase signature enzyme, fatty acid amide hydrolase, with a phosphonate transition state analogue bound to the nucleophile serine residue (PDB entry 1MT5). In this structure, Ser1 is Ser241, Lys1 is Lys142, and Ser2 is Ser217.

AMIDASE SIGNATURE (AS) ENZYMES

Another large group of serine hydrolases classified under EC 3.5 is the amidase signature (AS) family of enzymes (*36*, *37*). This family is characterized by the presence of a highly conserved serine- and glycine-rich stretch of approximately 50 amino acids, and members of this family have been identified in more than 90 different organisms, including archaea, eubacteria, fungi, nematodes, plants, insects, birds, and mammals (*38*, *39*). Given that the function of these enzymes is simply to hydrolyze an amide bond,

Scheme 2



the amidases play a surprisingly wide variety of biological roles. These include carbon−nitrogen metabolism in prokaryotes and eukaryotes (*40*, *41*), formation of indole-3-acetic acid in plant bacterial pathogens (*42*), degradation of neuromodulatory fatty acid amides in mammals (*38*), metabolism of atrazine in soil bacteria (*43*), and formation of Gln-tRNAGln by transfer of ammonia from glutamine (*44*, *45*).

Crystal structures of AS enzymes from a variety of organisms are available, including those of fatty acid amide hydrolase (FAAH) from rat (*46*), malonamidase E2 (MAE2) from *Bradyrhizobium japonicum* (*39*), a peptide amidase (PAM) from *Stenotrophomonas maltophilia* (*47*), glutamyl-tRNAGln amidotransferases from *Staphylococcus aureus* (*48*) and *Thermotoga maritima* (*49*), and a 6-aminohexanoate cyclic dimer hydrolase from *Arthrobacter* sp. (*50*). Structurally, they present a unique mixed α/β-fold comprising a core of 11 β-strands covered by layers of α-helices. As can be seen in Figure 3, their backbone fold is distinctly different from that of a typical BLRE such as the R39 DD-peptidase. This structural dichotomy suggests that the two families are unlikely to be evolutionarily related.

The active site of the AS enzymes is distinguished by a Ser-*cis*-Ser-Lys triad (*39*, *46*−*51*). By analogy to the corresponding elements of the BLRE active site, as discussed below, this triad is subsequently termed the Ser1-Ser2-Lys1 triad. Figure 4 shows details of the active site from the crystal structure of the covalent complex of FAAH with a phosphonate transition state analogue inhibitor (*46*). A very similar picture is provided by the covalent pyrophosphate complex of malonamidase E2 (*39*). On the basis of the former structure and other biochemical evidence (*52*), a double-displacement mechanism with an acyl−serine intermediate was proposed for the AS enzymes (Scheme 2a). In this mechanism, Lys1 acts as a general base to remove a proton from Ser2 (the *cis*-Ser) and then, via a hydrogen bond chain, probably in a concerted fashion, a proton is removed from Ser1 as it attacks the substrate carbonyl group to form the first tetrahedral intermediate. This collapses to the acyl−enzyme intermediate with protonation of the leaving group, forming ammonia, by the protonated Lys1, again indirectly via Ser2. Under the simplest assumption of a symmetrical mechanism, deacylation would proceed by general base (Lys1 via Ser2)-protonated attack of water on the acyl−enzyme carbonyl, yielding the second tetrahedral intermediate. Collapse of this species, involving departure of the original nucleophile, Ser1, would be aided by the transfer of a proton from the protonated Lys1 via Ser2. Subsequently, computational studies have provided support for this mechanism (*53*−*55*).

It is notable that other components of the active site appear to assist catalysis by forming hydrogen bonds to the substrate at critical times. To promote nucleophilic attack on the carbonyl and stabilize the anionic tetrahedral intermediate, an oxyanion hole is provided via the donation of a hydrogen bond from the NH groups of Ile238 and Gly239 [FAAH numbering (Figure 4)]. Another significant hydrogen bond appears to be formed between the backbone NH group of Ser217 and the nucleophilic Ser241 O_γ. It is clearly important to the mechanism (see below). This interaction was noted by Shin et al. (*39*) and is made possible by the unusual *cis* peptide bond between Gly216 and Ser217. It is interesting to note that two other backbone NH groups, those of Gly240 and Ser241, are also directed toward the phosphonate

oxygen, although apparently not as closely as the above-mentioned Ile238 and Gly239. It is possible that these may be more effective contributors to the oxyanion hole with other substrates or inhibitors.

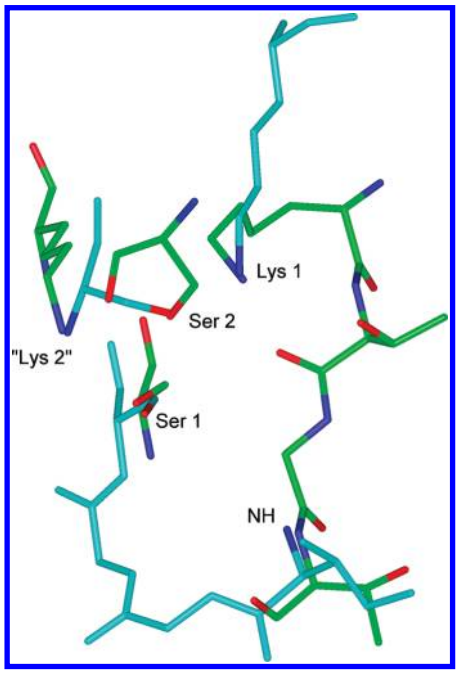


FIGURE 5: Five-point overlap of critical active site elements of the typical β-lactam-recognizing (elemental colors) and amidase signature (turquoise) enzymes shown in Figures 2 and 4. The overlapped atoms (shown in elemental colors in both cases) are the Lys1 side chain nitrogens (Lys410 and Lys142, respectively), the hydroxyl oxygens of Ser2 (Ser298 and Ser217, respectively), the nitrogen atoms of "Lys2" (Lys52 and backbone N of Ser217, respectively), the hydroxyl oxygens of Ser1 (Ser49 and Ser241, respectively), and the N of the oxyanion hole NH group (backbone N of Thr413 and Ile238, respectively).

IS THERE A LINK BETWEEN THE DD-PEPTIDASE AND THE AS ENZYMES?

Leaving aside, for the moment, the details of the mechanism, we find the similarity in the distribution of active site functional components in Figures 2 and 4 very striking. For example, the root-mean-square (rms) difference in position between Ser1 O_γ, Ser2 O_γ, and Lys1 N_ζ in the FAAH phosphonate complex and Ser1 O_γ, Ser2 O_γ, and Lys1 N_ζ in the R39 DD-peptidase boronate complex is 0.189 Å. It should be noted that the rms difference between the analogous atoms of Ser1, Ser2, and Lys1 of FAAH and Ser1, Ser2, and Lys2 of the R39 DD-peptidase is 0.770 Å. Thus, the overlap including Lys1 of R39 with FAAH is much closer than the overlap including Lys2 of R39; the latter combination will not be considered further. If a five-atom overlap is considered, where the oxyanion hole NH group of Ile238 in FAAH and Thr413 in R39 and the NH group of Ser217 paired with N_ζ of Lys 52 are included (Figure 5), the rms difference is 0.404 Å. If the Ser241 N of FAAH, which is obviously close to the Ser49 N of R39, is included, the six-atom rms difference is 0.539 Å. However, because the interaction between the Ser241 NH group and the phosphonate oxygen is not optimal in the FAAH structure, as mentioned above, only the five-atom overlap will be further considered. The analogous comparisons between the FAAH phosphonate and the malonamidase E2 phosphate (PDB entry 1OCM) are 0.211 and 0.225 Å for the three-atom and five-atom overlaps, respectively, and between the free enzymes malonamidase E2 (PDB entry 1OCK) and R39 (PDB entry 1W79) 0.233 and 0.380 Å, respectively.

Figure 6 shows that when the structures are overlapped as described above, the ligands in the FAAH phosphonate and the R39 boronate are linearly extended in approximately the same direction. This shows that substrates approach the active site of each enzyme from the same direction, i.e., onto the same face of the active site residues. This point is also seen in Figure 7, which shows that the active site crevices of the two enzymes and the

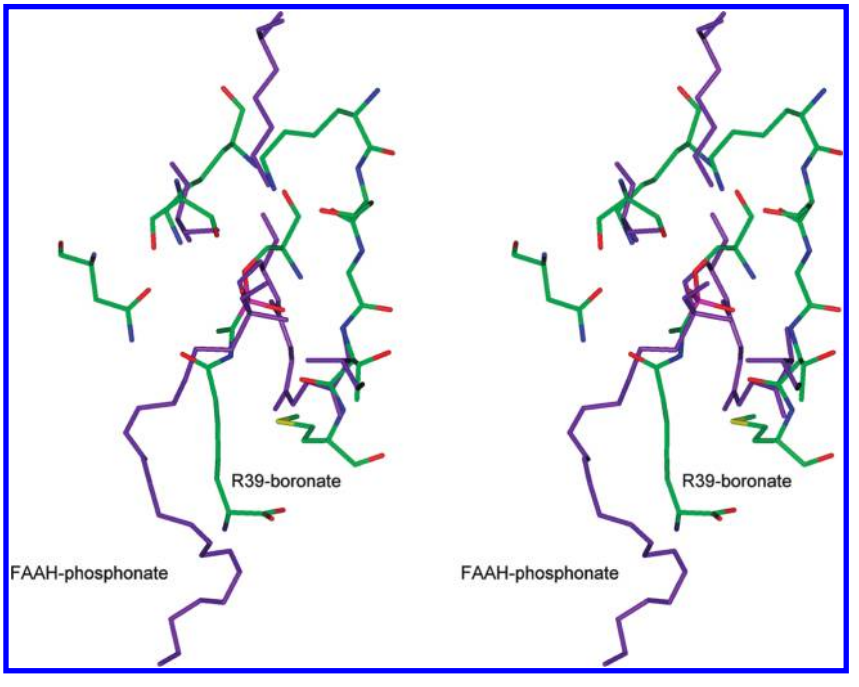


FIGURE 6: Active site overlap of the *Actinomadura* R39 DD-peptidase boronate adduct of Figure 2 (elemental colors) and the fatty acid amide hydrolase of Figure 4 (purple). This diagram, in stereoview, highlights the overlap depicted in Figure 5 but also includes the side chains of the inhibitors. The latter extend out in the same direction from the nucleophilic center, suggesting that substrates are oriented in a similar manner.

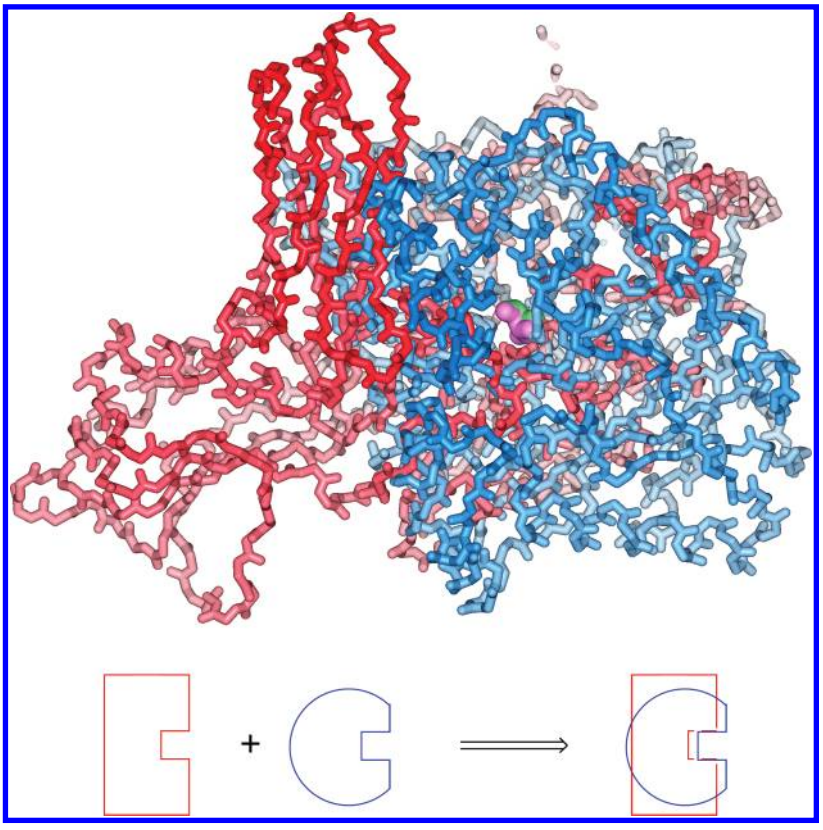


FIGURE 7: Same overlap as in Figure 6 but showing the backbone of each of the enzymes, with the R39 DD-peptidase colored red and the fatty acid amide hydrolase colored blue. In spite of the very different overall architectures, a common opening from the outside into the active site (pink and green for the two enzymes, respectively) is visible. The schematic below makes the major point.

openings to free solution overlap when the active site residues are superimposed as described above.

The three-dimensional arrangements of the active-site functional groups of all DD-peptidases are known to be very similar from the available crystal structures. Not surprisingly, therefore, the active site overlap of FAAH with a variety of BLRE enzymes shows a marked numerical similarity (Table 1). The three-atom and five-atom means are 0.27 ± 0.14 and 0.57 ± 0.30, respectively. Generally, the higher extremes involve structures in which the relevant side chains are displaced from hydrogen bonding position by rotation around the terminal C−C bond, e.g., the Cα−Cβ bond in serine. In some cases, e.g., *Escherichia coli* PBP5 (*56*), *S. aureus* PBP4 (*57*), and *S. aureus* PBP2a (*58*), more elaborate active site distortions have been previously noticed and conformational changes proposed to accompany substrate binding and catalysis (the data for these enzymes are not included in the averages quoted above). With respect to *S. aureus* PBP2a, the β-lactam complex probably gives a more realistic picture, but this improvement is not general; of course, the β-lactam complex is nonproductive. In principle, transition state analogue complexes would be preferable for comparison, but these are generally not available.

As described above, a likely mechanism of catalysis for AS enzymes is shown in Scheme 2a (*52*). The spectator-like role of the hydrogen bond from the Ser2 NH group (CONH in the scheme) to Ser1 O_γ can be seen as orienting Ser1 and perhaps increasing its acidity. By analogy, one can write the mechanism of Scheme 2b for a BLRE, where Lys2 has replaced the CONH group. The advantage of the second lysine to a BLRE is that it increases the positive electrostatic potential of the active site, which is probably necessary for stabilization of the anionic tetrahedral intermediate when the substrate is already negatively charged. It also affords the possibility, depending on the lysine pK_a values, of a transition to the mechanism of Scheme 2c where the primary general base became Lys2 rather than Lys1. It is noticeable that the tetrahedral intermediates generated from parts b and c of Scheme 2 are identical (although the transition states are not). The mechanism shown in Scheme 2c is, in fact, a popular mechanism for DD-peptidase catalysis (*21*, *23*).

It may be that the arrangement of catalytic groups in the AS enzymes represents an optimal set for amide hydrolysis (*52*). It is not clear, however, what the particular need is for the intermediate Ser2 between the reaction center and Lys1. Serine proteases, which also hydrolyze amides, manage with only an adjacent base, usually histidine (although its position and basicity are controlled by the third member of the triad). With respect to the AS enzymes, it has been suggested that the particular active site arrangement is important for the optimization of amide versus ester selectivity (*52*, *54*).

RELATIONSHIP TO OTHER SERINE PROTEASES

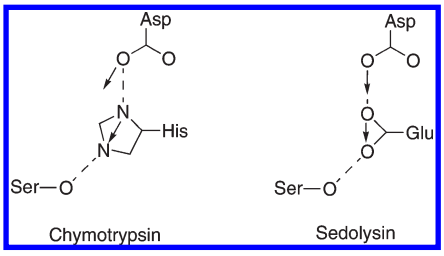
With respect to serine proteases, it is interesting to note the similarities between the classical Asp-His-Ser triad and the Lys-Ser-Ser triad of a BLRE. To explore this issue, the R39 DD-peptidase−boronate structure was superimposed onto a chymotrypsin boronate structure (PDB entry 6CHA) (*59*). Direct superimposition is not possible, however, because chymotrypsin has two nitrogen atoms of the His57 imidazole, hydrogen bonded to Ser195 and Asp102, respectively, and a "spacer", the imidazole ring, between them. If, however, the Cartesian coordinates of His57 ND1 and Asp102 OD2 are adjusted so that they superimpose His57 ND1 on His57 NE2 (Scheme 3), a more direct

Table 1: Root-Mean-Square Differences (angstroms) between BLRE and the FAAH Phosphonate (PDB entry 1MT5)

enzyme	class	ligand	PDB entry	three-atom	five-atom
Streptococcus pneumoniae PBP1a	HMMA	−	2C6W	0.289	0.784
St. pneumoniae PBP1a	HMMA	cefotaxime	2C5W	0.342	0.627
St. pneumoniae PBP1b	HMMA	−	2FFF	0.083	0.305
S. aureus PBP2	HMMA	−	2OLU	0.288	0.419
S. aureus PBP2a	HMMB	−	1MWR	0.781	1.871
S. aureus PBP2a	HMMB	benzylpenicillin	1MWT	0.222	0.530
St. pneumoniae PBP2x	HMMB	−	1QME	0.183	0.545
St. pneumoniae PBP2x	HMMB	cefuroxime	1QMF	0.544	0.828
Neisseria gonorrheae PBP2	HMMB	−	3EQU	0.479	1.329
St. pneumoniae PBP3	LMMA	−	1XP4	0.256	0.314
E. coli PBP5	LMMA	−	1NZO	0.811	1.370
E. coli PBP5	LMMA	boronate	1Z6F	0.704	1.217
Haemophilus influenzae PBP5	LMMA	−	3A3J	0.585	1.260
S. aureus PBP4	LMMA	−	3HUM	0.320	0.683
Streptomyces R61	LMMB	−	3PTE		0.725[a]
Streptomyces R61	LMMB	benzylpenicillin	1PWC		0.322[a]
Actinomadura R39	LMMC	−	1W79	0.132	0.317
Actinomadura R39	LMMC	cephalosporin	2VGJ	0.225	0.436
Actinomadura R39[b]	LMMC	−	1W79	0.234	0.380
Actinomadura R39[c]	LMMC	−	1W79	0.341	0.395
E. coli PBP4	LMMC	−	2EX2	0.999	0.988
E. coli PBP4	LMMC	benzylpenicillin	2EX8	0.128	0.286
TEM-1 β-lactamase	A	−	1BTL	0.263	0.609
SHV-1 β-lactamase	A	−	1SHV	0.181	0.591
P99 β-lactamase	C	−	1XX2	0.039	0.235

[a]Four-atom overlap; the His replacing Lys1 is omitted. [b]Malonamidase E2 (PDB entry 1OCK) replaces FAAH. [c]The pyrophosphate complex of malonamidase E2 (PDB entry 1OCM) replaces FAAH.

Scheme 3



comparison can be made. Under these conditions, chymotrypsin (Ser O, His N, and Asp OD1) overlaps with Ser1-Ser2-Lys1 triad of the R39 DD-peptidase with a rms difference of 0.153 Å. Inclusion of either or both of the oxyanion hole nitrogens gives a much poorer overlap (rms difference of > 0.6 Å). This superimposition does, however, lead to a similar overall orientation of the ligand as observed in the comparison of FAAH versus R39 (Figures 6 and 7). The same superposition of histidine nitrogens in the clan SH (*7*) His-His-Ser cytomegalovirus protease (PDB entry 1CMV) yields a three-point overlap with an rms difference of 0.286 Å. A similar result was obtained with the Ser-Glu-Asp serine protease, sedolysin (clan SB, PDB entry 1GA4), in which OE1 and OE2 of the intermediate Glu80 were superimposed to remove the spacer inherent in the carboxylate (Scheme 3); the rms difference was 0.258 Å in this case.

The evidence discussed above suggests that in all three superfamilies, serine protease, AS, and BLRE, there is a common disposition of a hydrogen-bonded triad that may well be optimal for catalysis. This may arise simply because of preferred angles in hydrogen bond formation, although this preference is usually quite weak (*60*, *61*). Attempted superimposition of the additional elements found in the five-atom AS versus BLRE overlap is less precise with the serine proteases. It is not unreasonable to speculate that this could be advantageous as it would provide some flexibility in the positioning of the substrate.

Another interesting comparison is with certain Ser-Lys dyad peptidases that are found in distinct clans of EC 3.4. In many cases, it seems, however, that they are really Ser-Lys-Ser(Thr) triad enzymes with the Lys hydrogen bonded centrally to both serine residues (*7*, *62*). In the case of *E. coli* signal peptidase I (clan SF, PDB entry 1KN9), the Ser-Ser-Lys triad (side chain OON) overlap with the R39 DD-peptidase Ser1-Ser2-Lys1 triad is poor, but the overlap of the Ser-Lys-Ser triad (side chain ONO) with the Ser-Ser-Lys triad of R39 is very good (rms difference of 0.158 Å). It thus appears that this triad superimposes very closely with that of the R39 DD-peptidase. The mechanism of reaction might, however, be different because it is unlikely that the unaided serine hydroxyl is a proton donor or acceptor, and more similar, perhaps, to a classical serine protease mechanism, where by analogy to the role of Asp102, the non-nucleophilic serine may be used to position the immediate general base (lysine) during catalysis. Of more interest, perhaps, is the fact that another peptidase of the SF clan, the LexA protease (PDB entry 1JHF), has a Ser-Lys-Thr triad that does not superimpose well on the signal peptidase I triad (rms difference of 0.668 Å) (*7*, *62*). The threonine hydroxyl, although hydrogen-bonded to the lysine residue, is very differently placed with respect to the apparently analogous serine of signal peptidase I. This suggests that although the specific placement of the functional atoms of a triad may perform the same function, they need not be identically distributed in space. With this perspective, the superimposition of the functional atoms of the AS enzymes with those of BLRE becomes more striking.

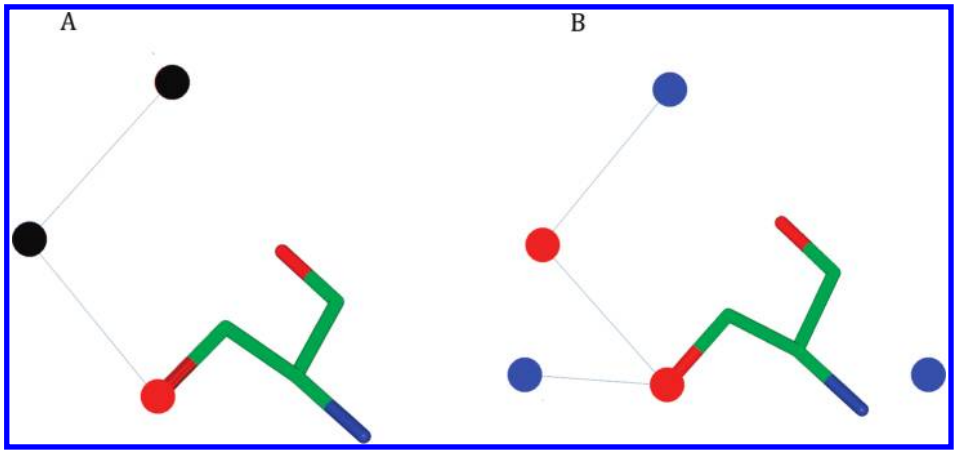


FIGURE 8: (A) Conserved (three-point) positions of the nitrogen or oxygen atoms of the classic serine protease catalytic triad and (B) extended five-point overlap ("catalytic pentad") between the β-lactam-recognizing and amidase signature enzyme active sites. The red and blue circles represent O and N, respectively. The black circles represent either O or N [depending on the serine amidohydrolase (see the text)]. The lines represent hydrogen bonds.

SUMMARY

Some time ago, Kobayashi et al. suggested that there may be an evolutionary relationship between AS amidases and peptide bond-cleaving enzymes, in particular the aspartic proteases (*63*). In that paper, it was shown that the amidases were not sulfhydryl enzymes as previously thought. Instead, evidence that the catalytic residues were an aspartate and a serine (D237 and S241, respectively, in FAAH numbering) was presented. This proposed evolutionary relationship between the amidases and the peptidases was essentially based on sequence alignments involving the "active site" aspartate (*63*). More recent structural studies have made it clear that D237 is not in the active site, but the question of a relationship between amide bond- and peptide bond-cleaving enzymes still lingers. Here we have brought to light the striking five-point superimposition of active site functional group atoms of the BLRE and AS enzymes. Because the backbone folds of the two groups of enzyme are so very different, it seems likely that this situation has resulted from independent convergent evolution. The reactions catalyzed by members of the two superfamilies, i.e., amide hydrolyses, are chemically very similar, and there is even some direct overlap of function, as in the glutaminases cited above. It is likely that this common placement of active site residues is particularly effective for amide hydrolysis and thus the convergent evolution has been driven by a mechanistic imperative.

The evolution of an enzyme employing a covalent mechanism of amide hydrolysis (and, thus, an acyl−enzyme intermediate) presumably would proceed from selection of the nucleophile to selection of a general acid−base catalyst in cases where it was necessary to activate the nucleophile (e.g., the serine hydroxyl) or protonate the leaving group (e.g., amide nitrogen). Recruitment of a third member to form a catalytic triad is apparently advantageous, where the latter is useful for modulation of the general acid−base pK_a and positioning of the catalytic apparatus during the reaction. Further, judicious placement of electrophiles, such as the oxyanion hole and the Ser217 NH group of FAAH (Figure 4 and Scheme 2a), would also facilitate the reaction. Finally, conversion of the latter electrophilic NH group to a lysine ammonium ion (Figure 2 and Scheme 2b) would likely promote reaction of a negatively charged substrate, while placement where it may act as an alternative general acid and/or base may provide, under particular circumstances, a more effective mechanism. The latter conversion may have occurred for the BLRE (Scheme 2c). Indeed, this mechanism appeared most likely from consideration of the R39/boronate structure (*21*).

The hydrolysis of amides, including peptides, is a reaction that is central to biochemistry as we know it today and whose mechanism must have been explored in enzyme evolution from the time of emergence of the earliest living organisms. From our present vantage point of this process, it is evident that one successful strategy for catalyzing amide hydrolysis has been employment of a double-displacement mechanism with a covalent acyl−serine intermediate. Activation of the serine hydroxyl group for attack on the amide carbonyl group to form this intermediate has been accomplished by various combinations of base catalysts. It is striking that the most common auxiliary catalytic apparatus consists of a functional dyad containing a base or a proton transfer catalyst (a bifunctional hydrogen bond proton donor and/or acceptor) directly hydrogen bonded to the serine hydroxyl, with another base or hydrogen bond donor and/or acceptor hydrogen bonded to it. Overall, a catalytic triad is formed. We see that it is further striking that the position of the nitrogen or oxygen atoms central to these hydrogen bonds and proton transfers is quite strictly conserved (Figure 8A) and arrived at, presumably, by convergent evolution. It seems likely that this arrangement of atoms is mechanistically driven and may be optimal for controlled proton transfer to the amine leaving group, a challenging aspect of the reaction. We show here that the β-lactam-recognizing enzymes and amidase signature enzymes are members of this broad group, but with even more in common (Figure 8B and Scheme 2).

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