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A Substrate Variant as a High-Affinity, Reversible Inhibitor: Insight from the X-ray Structure of Cilastatin Bound to Membrane Dipeptidase

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Abstract—An analysis of the X-ray structure of cilastatin bound to membrane dipeptidase, together with docking studies, is presented here to reveal how a simple amide may act as a high-affinity, reversible, amidase inhibitor. Cilastatin binds as a normal substrate and is orientated in a perfect near-attack conformer for formation of a tetrahedral intermediate with the zinc-bound water/hydroxide. This intermediate is fated, however, only to revert to its starting components as scission of the amide bond is prevented by the precise fit of cilastatin within the active site. The cilastatin alkyl end groups that are tightly buttressed against amino acid residues on opposite sides of the active site, are aligned along the C–N reaction coordinate axis thereby preventing collapse of the intermediate via rupture of the C–N bond. Such a feature could have more general applicability in the explicit design of substrate variants as selective, tight-binding, and reversible inhibitors.

Introduction

Membrane dipeptidase (MDP) is an important mammalian enzyme that occurs in the kidneys[1] and lungs.[1,2] It is a zinc-based metalloenzyme, with a unique zinc-binding motif, that is involved in the conversion of leukotriene-D_4 (LTD_4) to leukotriene-E_4 (LTE_4), amongst other metabolic processes. A key attribute of MDP is its ability to catalyse the hydrolysis of the β-lactam ring of imipenem and of a number of other carbapenem antibiotics (Scheme 1).[3] This latter feature led to the (empirical) development of cilastatin as a selective, high-affinity ($K_i = 0.1\ \mu M$) reversible inhibitor of MDP that was then co-administered with imipenem.[4] The basis of the inhibitory action of cilastatin is not apparent—it is neither a transition state analogue nor a metal ion chelator; in essence, it is a simple substrate-type structure with a scissile amide bond. Indeed, at the developmental stage no Hansch-type structure activity correlation with inhibitory potency was found for a set of (16) cilastatin-related structures spanning a 2000 fold variation in K_i.[4b] Understanding how cilastatin behaves as an amidase inhibitor is important per se but also in terms of defining a potentially novel principle of inhibitor design.

The successful refinement, by Nitanai and co-workers,[5] of the X-ray structure of MDP with cilastatin bound at the active site has provided a detailed picture of its mode of binding. Herein, an analysis of this X-ray structure is presented, together with docking studies of three distinct MDP substrates, to provide a rationale for the mode of action of cilastatin as an inhibitor, and from this analysis a novel paradigm for inhibitor design has been extracted. Cilastatin binds in a catalytically-competent, substrate-like manner and is ideally positioned for formation of a tetrahedral intermediate by addition of the zinc-bound water/hydroxide. Furthermore, the imidazole of His152 is ideally positioned to stabilize the oxyanion of this intermediate via a hydrogen bond. The tetrahedral intermediate so formed is constrained, however, to revert back to its starting components as collapse via scission along the C–N axis is prevented due to the position of the dimethylcyclopropyl and the alkyl endgroup of cilastatin. These are

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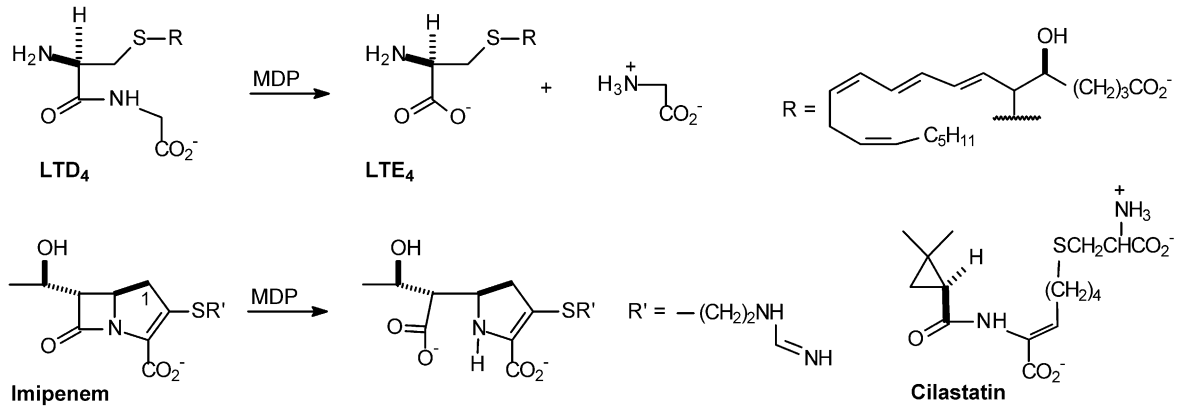


Scheme 1.

tightly buttressed against active site residues and, critically, are aligned along the C–N reaction coordinate axis. The substrates, glycyldehydrophenylalanine (Gly-D-Phe) and LTD_4, bind in the same catalycally-competent manner as cilastatin, but, neither contains a pair of substituents to buttress against the opposite sides of the active site. Thus, Gly-D-Phe has an alkyl group at its carboxyl terminus but lacks a group at its amino terminus, whereas, LTD_4 has an alkyl group at its amino terminus but lacks a group at its carboxyl terminus.

Discussion

The X-ray structure shows that the active site of MDP is a small pocket on the protein surface;[5b] this is in contrast to the extended groove of some zinc-based enzymes such as the β-lactamases from *B. cereus* (1BVT) and from *B. fragilis* (1ZnB).[6] The key internuclear separations of cilastatin (truncated sidechain)[7] bound to MDP are shown in Figures 1 and 2 (polar hydrogens only have been added). Significant hydrogen bonding interactions occur between the carboxylate group of cilastatin and the polar hydrogens of Arg230 and also with that of Tyr255. A hydrogen bond is also established between the cilastatin carbonyl oxygen and the imidazole ring of His152; this ring is shown here as being positively charged (imidazole $pK_a = 6.80$). The cyclopropyl group and the alkyl endgroup of cilastatin are tightly buttressed by Tyr68 and Tyr252 respectively (Figs 1 and 3). At a separation of 3.52 Å the interaction energy of a cyclopropyl carbon with that of Tyr68 is only very marginally stabilizing, whereas, at a separation of 3.90 Å the interaction energy of the alkyl-sidechain carbon with a carbon of Tyr252 is just about optimal (see Fig. 4).

The separation of the zinc-bound water/hydroxide to the amide carbonyl (2.82 Å) and its angle of approach, $\alpha = 102.9°$, indicate that it is ideally positioned for addition to this group; also note that < HO–C–N = 83.2° and < HO–C–C = 83.1° (Fig. 2; see NAC-1 in

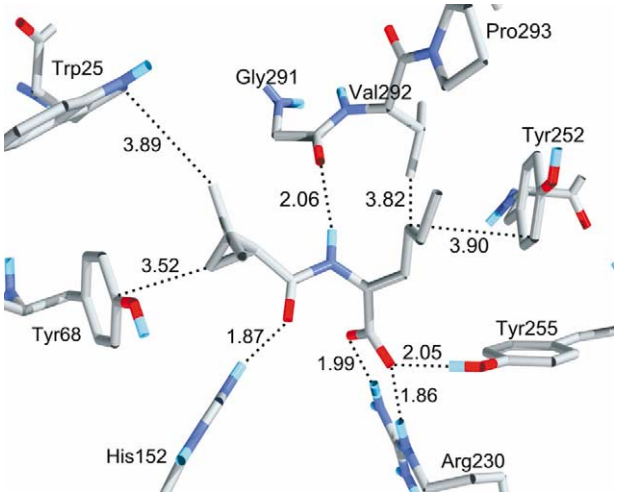


Figure 1. Internuclear separations (Å) between MDP active site residues and cilastatin.

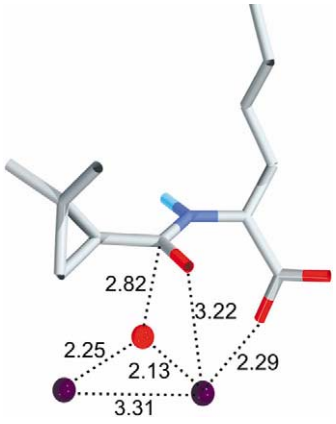


Figure 2. Heavy-atom separations (Å) in cilastatin-bound MDP: Zn^{2+} (black) and water/hydroxide (red). < HO–C–O = 102.9°, < HO–C–N = 83.2° and < HO–C–C = 83.1°.

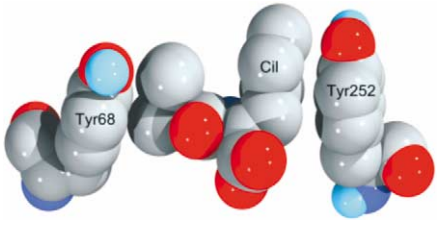


Figure 3. Spacefilling view of cilastatin between active-site residues of MDP.

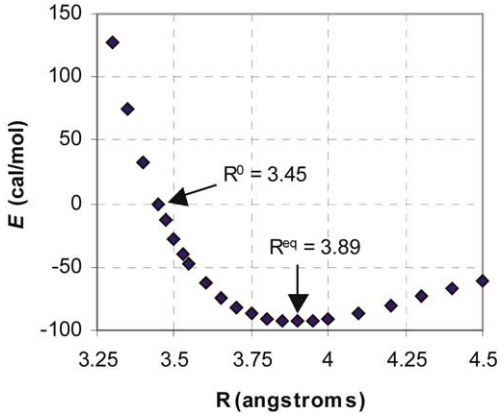
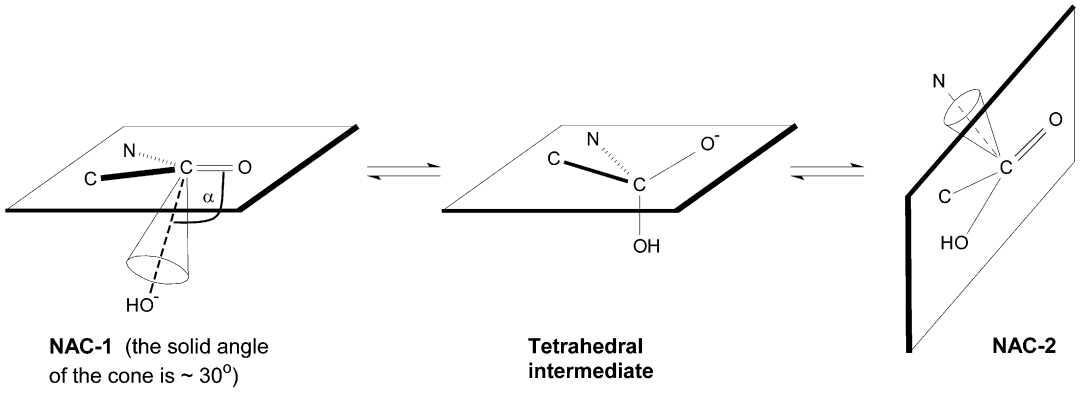


Figure 4. Potential energy (E) versus internuclear separation (R) for C/C non-bonded interaction.[8]

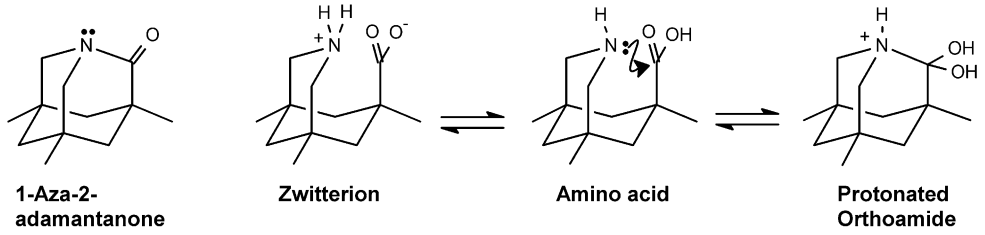
Scheme 2).[9] There is no deformation from planarity of the carbonyl carbon indicating that a ground-state structure is observed here. The cilastatin–water/hydroxide ensemble is, therefore, a perfect near-attack conformer (NAC) in the terminology of nucleophile addition to a carbonyl group (Scheme 2).[9] Furthermore, the hydrogen bonding interaction between the cilastatin carbonyl and the imidazole of His152 (Fig. 1) is well suited for stabilization of the tetrahedral intermediate resulting from hydroxide ion addition. A zinc ion is also appropriately positioned to stabilise the oxyanion in this intermediate. These characteristics parallel those identified as being important in the stabilization of the (analogous) tetrahedral intermediate of a typical β-lactam in the active sites of zinc-based β-lactamases.[6,10] Overall, these features indicate that a normal tetrahedral intermediate can occur with cilastatin as with a typical amide substrate. So the question that must be posed is 'why is cilastatin an inhibitor and not a substrate?'

The tetrahedral intermediate can, potentially, fragment along two reaction coordinates (Scheme 2). The hydroxide ion can be expelled by displacement along the C–OH coordinate to reform the starting components, or the amine moiety can be expelled by displacement along the C–N coordinate to form the amide-hydrolysis products. As a first approximation, the maximum displacement along the C–N coordinate is set by the closest approach of a cyclopropyl carbon to a carbon of Tyr68, combined with the closest approach of an alkyl-side-chain carbon of cilastatin to a carbon of Tyr252. The closest approach in each case is taken as 3.45 Å, that is the separation distance at which the interaction energy is zero for two non-bonded carbon atoms (see Fig. 4). This would allow for a total displacement of 0.52 Å along the C–N reaction coordinate (without significant further energy input) which would result in a separation of 1.90 Å between the amine leaving group and the carbonyl moiety of cilastatin [i.e., 0.52 + 1.38 Å (the amide bond length)]. At this distance the amine-like nitrogen and carboxyl-like carbonyl remain substantially inside the sum of their van der Waals radii and therefore strongly interacting (bonding). Crystallographic studies by Dunitz and co-workers have shown that a bonding interaction between an amine nucleophile and keto-carbonyl group is clearly initiated at a separation of 2.60 Å and is well established at 2.46 Å.[11] Kirby and co-workers showed that the enforced proximity of an amine and a carboxylic acid carbonyl can drive the reaction rapidly toward amide bond formation where this reaction is thermodynamically favoured.[12] Thus, the protonated orthoamide, related to 1-aza-2-adamantanone, was readily formed from the neutral amino acid in aqueous solution as shown in Scheme 3 (an AMPAC/AM1 gas-phase model (this work) of the neutral amino acid gives an internuclear separation of 2.69 Å between the nitrogen and carboxyl carbon). Basically, the corollary applies here, namely, enforced proximity (due to an intermolecular constraint) prevents displacement of a leaving group from the tetrahedral intermediate. This leaves the reaction ensemble in the only available energy minimum, namely, the amide ground state with the zinc-bound water/hydroxide juxtaposed in an NAC position.

The inherent flexibility of the protein structure may well accommodate a separation somewhat larger that 1.90 Å. A minimum separation of 2.7 Å may be required for facile release of the amino moiety as indicated by the data above from the neutral amino acid structure related to 1-aza-2-adamantanone (Scheme 3). Comparison of the crystal structure of native and cilastatin-bound MDP provides some data on the flexibility of the active-site dimensions; Tyr68-$O\cdots$Tyr255-O 10.74 (complex)



Scheme 2.



Scheme 3.

versus 10.87 Å (native); Gly291-*O*···His152-NE2 7.40 (complex) versus 7.84Å (native); Gly291-*O*···Arg230-NH1 8.45 (complex) versus 9.50 Å (native). The largest difference is along the Gly291-to-Arg230 axis (see Fig. 1) and results from the hydrogen bonding interaction of Gly291-*O* with the amide hydrogen of cilastatin. Some other observations point to limited flexibility of the active site to readily expand much beyond the dimensions occurring in the native protein. Thus, 1α- and 1β-methyl carbapenem structures are not substrates for MDP[13]—this points to a limited flexibility to expand along the Gly291-to-Arg230 axis. Cephalosporins are not accommodated in the active site—they are neither substrates nor inhibitors. The bicyclic structure of a cephalosporin is somewhat flatter than that of a carbapenem, due to the six-membered dihydrothiazine ring. Hence, the 3′-side chain of cephalosporins, which protrudes outward more than the corresponding side group of a carbapenem (its side chain protrudes more upwards), will generate a conflict with Tyr252. The scope to expand along the Tyr68-to-Tyr252 axis thus appears to be restricted also; the active site is flanked by a disulfide bond at each end of this axis.

A number of naturally occurring peptides, which can be classed as substrate variants, are potent inhibitors of serine-based proteases.[14] These structures bind in a substrate-like, catalytically-competent manner at the active site. The scissile amide bond of (some of) these inhibitors has been shown to cleave and readily reform at the active site—diffusion of the cleaved amino moiety from the active site is restricted.[14c,d] These inhibitor structures are complex (considerably more so than cilastatin) and the basis of their exceptional inhibitory potency is multifaceted due to the extent of their interaction with the protease enzyme. A detailed analysis indicated that restricted diffusion of the cleaved leaving group is an important contributing factor.[15]

A significant difference occurs in the position of the zinc-bound water/hydroxide in the native and cilastatin-bound MDP (Fig. 5). In native MDP the Zn···O separations of 2.77 and 2.79 Å indicate that a water molecule is coordinated. The corresponding values (2.25 and 2.13 Å) in the cilastatin-bound MDP indicate that on binding a substrate (here cilastatin) the water molecule is displaced closer to the zinc ions setting the scene for incipient hydroxide formation and subsequent nucleophilic addition. The carboxylate of Asp288 could act as the basic component to accept the proton from the displaced water molecule[16] and, ultimately, relay it to the nucleofugic nitrogen. Asp288-OD1 is at 3.21 Å from the oxygen of the zinc-bound water in native MDP whereas this separation is 2.55 Å in cilastatin-bound MDP. Thus, the cohesive application of binding forces (van der Waals, polar, hydrogen bonding) stabilizes the substrate (here cilastatin) within the active site with respect to bulk solution and, in so doing, drives the active-site components forward along the reaction coordinate for nucleophilic addition.

Docking of substrates

AutoDock 3.0[17] was used to assess binding to MDP of the following structures: a truncated LTD_4 ($R = CH_3$, see Scheme 1), Gly-D-Phe ($K_m = 1$ mM),[18] and imipenem (with $R' = CH_3$, see Scheme 1). Initially, cilastatin was docked to the active site of MDP (cilastatin-bound structure) for validation (see Computational Methods for details). A typical result is shown in Figure 6; here the top-ranked conformer (docked energy = −11.51 kcal/mol)[19] had an RMSD of 0.15 Å from the crystal structure. Overall, this supports our application of the docking process to the metalloenzyme active site.

In the case of LTD_4, the L configuration of the cysteinyl carbon requires the amino group to point downwards to allow the extended thioalkyl moiety to protrude above the active site. This implies that the (neutral) amino group of LTD_4 must coordinate with a zinc ion.[20] The truncated LTD_4 structure was constructed in this orientation and its binding to the active site evaluated

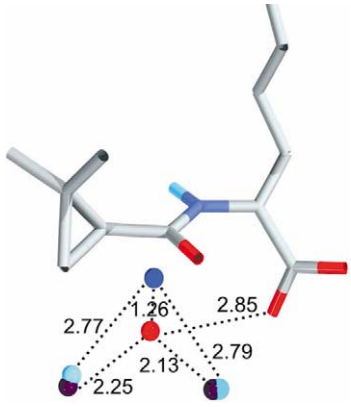


Figure 5. Heavy-atom separations (Å) in native MDP: Zn^{2+} (light blue) and water/hydroxide (dark blue), and in cilastatin-bound MDP: Zn^{2+} (black) and water/hydroxide (red); the separation of the water/hydroxide and the carboxylate oxygen is also shown. The enzyme structures were superimposed using the alpha carbons (RMSD = 0.69 Å).

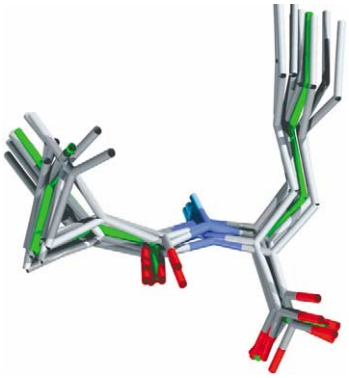


Figure 6. Cilastatin conformers docked to MDP (cilastatin-bound protein structure) superimposed on cilastatin (green) from the crystal structure. The top-ranked conformer here had an RMSD of 0.15 Å from the crystal structure and a docked energy of −11.51 kcal/mol.

with AutoDock. A typical result, showing a single,[21] low-energy conformer (docked energy = −8.87 kcal/mol), is given in Figures 7 and 8. The data indicate that coordination of the amino group to a zinc ion is compatible with the binding of LTD_4 in a catalytically-competent, substrate-like manner. The separation of Tyr252 and the end alkyl carbon of LTD_4 of 4.98 Å (Fig. 8) is 1 Å more than the corresponding value for cilastatin (Fig. 1). This should be sufficient to allow for scission of the tetrahedral intermediate (derived from LTD_4) by a relatively unhindered displacement of the amine group along the C–N axis; this is consistent with the behaviour of LTD_4 as a substrate.

A similar picture emerges from the docking of Gly-D-Phe. A single, low-energy conformer docked to MDP (cilastatin-bound protein structure), with the amino group coordinated to a zinc ion, is shown in Figures 9 and 10 (docked energy = −7.17 kcal/mol).[22] The separation of 4.41 Å between Tyr68 and the glycyl carbon (Fig. 10) is almost 1 Å more than the corresponding value for cilastatin (3.52 Å; see Fig. 1). There is also ample separation between Tyr252 and the phenyl ring of Gly-D-Phe (4.54 Å compared to 3.90 Å in the case of bound cilastatin), although, there is a closer contact (3.35 Å) with Pro293-CD. The other parameters shown in Figs 9 and 10 are typical of a substrate bound in a catalytically-competent manner: α = 109° at a distance of 3.01 Å and a good hydrogen bond is established with His152. Thus, the tetrahedral intermediate derived from Gly-D-Phe should be able to fragment, without undue constraint, by scission of its C–N bond.

The carbapenem structure was docked to the active site of native MDP, as this is more open than the cilastatin-bound protein structure. The zinc-bound water was positioned, however, at the same coordinates as in the cilastatin-bound MDP as this is an appropriate location when a substrate is bound to the active site (see Fig. 5). Inspection of Figure 1 shows that placing a bicyclic β-lactam structure, in a planar manner, in the same orientation as the cilastatin amide bond will generate a conflict between Gly291-*O* and the β-lactam bridgehead carbon. Relief of this conflict requires that the β-lactam structure be tilted about its C–N amide bond; this is borne out by the data from docking of the carbapenem structure. In addition, the docking results show that tilting of the β-lactam structure about the C–N bridgehead axis serves to relieve conflict of the sidechain sulfur atom with Tyr252, Tyr255 and Pro293. The greater the tilt about both axes the better the clearance between the carbapenem and Gly291-*O* and also with Tyr252, but, the lower (poorer) is the angle, α, for attack of the water/hydroxide to the β-lactam carbonyl. The data in Figures 11–14, showing two low-energy docked conformers of the carbapenem structure, provide snapshots of the dynamics of this scenario. In conformer **A** (Figs 11 and 12) α = 84°, which is rather low, but, the clearance to Gly291-*O* is adequate and to Tyr252 is more than adequate (the separation of 4.71 Å is larger than the sum of the van der Waals radii of sulfur and carbon by 1.2 Å). In conformer **B** (Figs 13 and 14) α = 92°, which is better than for conformer **A**, but here the

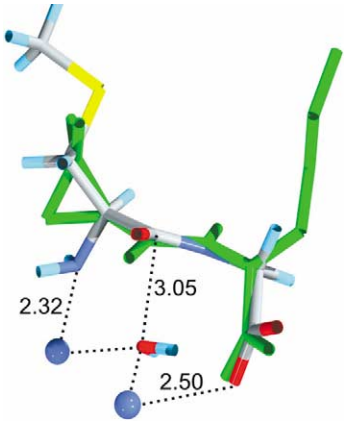


Figure 7. Heavy-atom separations (Å) of the water molecule and Zn^{2+} (light blue) to a conformer of truncated LTD_4 (all hydrogens shown) docked to MDP (cilastatin-bound protein structure). Docked energy = −8.87 kcal/mol, < HO–C–O = 121°, < HO–C–N = 71°, < HO–C–C = 79°. Cilastatin (green; only the polar hydrogen is shown) from the crystal structure has been superimposed for reference.

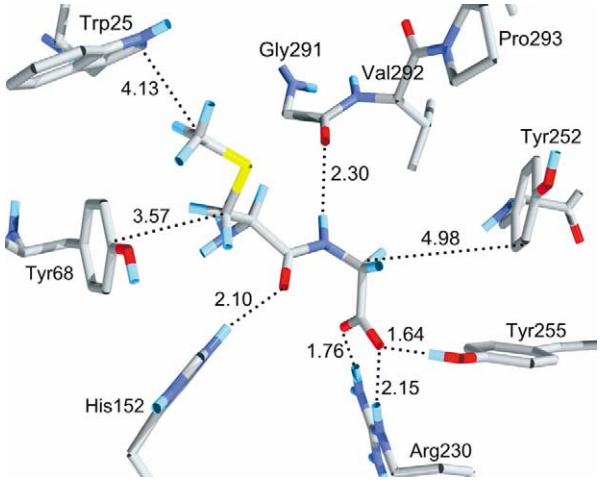


Figure 8. Internuclear separations (Å) between MDP (cilastatin-bound protein structure) active-site residues and a docked conformer of LTD_4 (R = $-CH_3$ in Scheme 1); (docked energy = −8.87 kcal/mol).

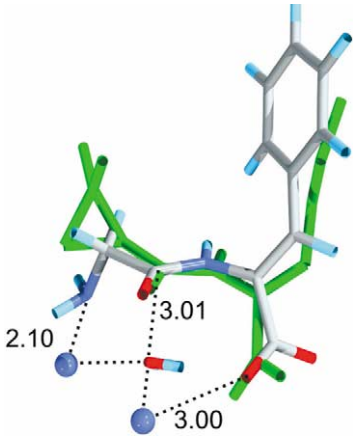


Figure 9. Heavy-atom separations (Å) of the water molecule and Zn^{2+} (light blue) to a conformer of Gly-D-Phe (all hydrogens shown) docked to MDP (cilastatin-bound protein structure). Docked energy = −7.17 kcal/mol, < HO–C–O = 109°, < HO–C–N = 84°, < HO–C–C = 79°. Cilastatin (green; only the polar hydrogen is shown) from the crystal structure has been superimposed for reference.

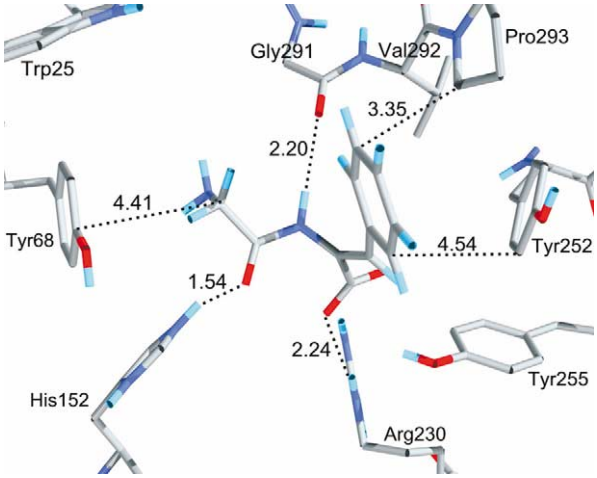


Figure 10. Internuclear separations (Å) between MDP (cilastatin-bound protein structure) active-site residues and a docked conformer of Gly-D-Phe (docked energy = −7.17 kcal/mol).

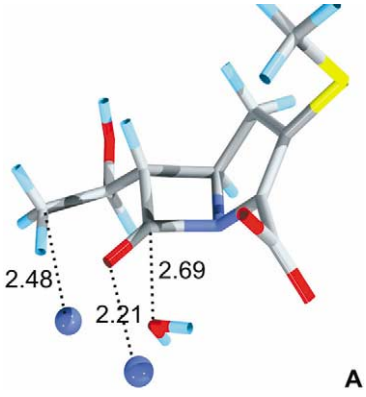


Figure 11. Heavy-atom separations (Å) of the water molecule and Zn^{2+} (light blue) to a conformer of imipenem (with R′ = CH_3; all hydrogens shown) docked to MDP (native protein structure). Docked energy = −8.47 kcal/mol, < HO–C–O = 84°, < HO–C–N = 87°, < HO–C–C = 96°).

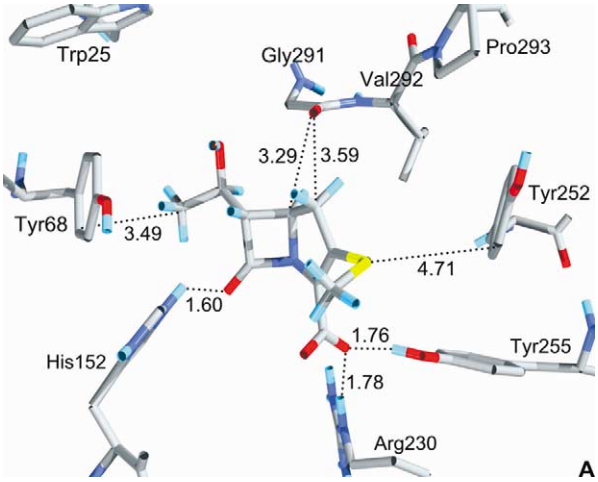


Figure 12. Internuclear separations (Å) between MDP (native protein structure) active-site residues and a docked conformer of imipenem (with R′ = CH_3); (docked energy = −8.47 kcal/mol).

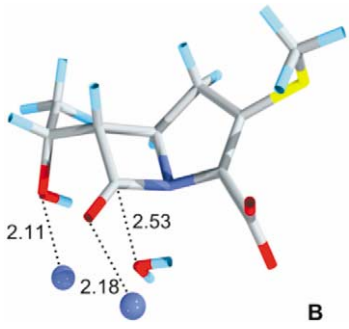


Figure 13. Heavy-atom separations (Å) of the water molecule and Zn^{2+} (light blue) to a conformer of imipenem (with R′ = CH_3; all hydrogens shown) docked to MDP (native protein structure). Docked energy = −9.26 kcal/mol, < HO–C–O = 92°, < HO–C–N = 62°, < HO–C–C = 112°).

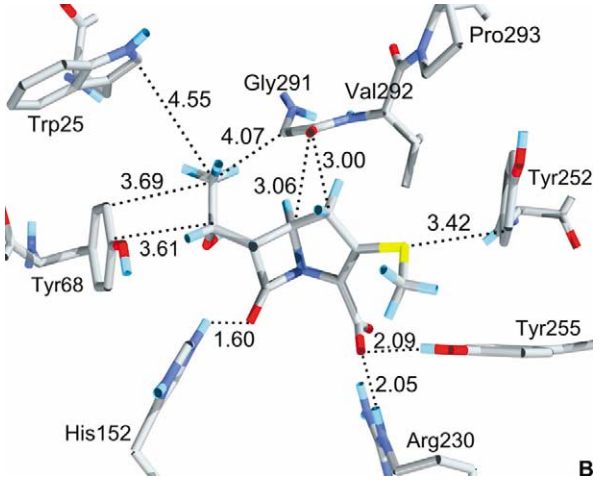


Figure 14. Internuclear separations (Å) between MDP (native protein structure) active-site residues and a docked conformer of imipenem (with R′ = CH_3); (docked energy = −9.26 kcal/mol).

clearance to Gly291-*O* and to Tyr252 is low. For each conformer the tilting of the β-lactam structure places the lactam carbonyl oxygen in close proximity to a zinc ion: such an interaction should facilitate nucleophilic addition of the water/hydroxide where the angle, α, is somewhat less than optimal. [Rotation of the hydroxyethyl sidechain was allowed during the docking process; the tighter binding conformer (B) has the side-chain oxygen coordinated to a zinc ion.] The data from these two snapshots indicate that: (a) this carbapenem structure can bind in a substrate-like manner (see conformer B), and (b) fragmentation of the tetrahedral intermediate, by scission of its C–N bond, could occur without undue constraint (see conformer A). It is apparent from the docking data of the carbapenem that its substituent array does not buttress it against Tyr68 and Tyr252 to the same degree as does the substituent array of cilastatin (see Figs 3 and 6); this is consistent with the experimental data which show imipenem to be a substrate and cilastatin to be an inhibitor.

In summary, the data obtained on docking of these distinct structures provides a valid picture of their binding as substrates to the MDP active site, in particular, for LTD_4 and Gly-D-Phe. This is fully consistent with our interpretation of the action of cilastatin—a substrate variant—as an inhibitor.

Conclusions

Cilastatin is a simple amide that acts as a high-affinity, reversible and highly selective amidase inhibitor. An analysis of the crystal structure of this amide bound to MDP has been presented here that provides a rationale for its inhibitory action. The crystal structure is a snapshot of this substrate variant locked in a perfect near-attack conformer (NAC) for hydroxide ion addition. It is argued that this NAC can readily generate a normal tetrahedral intermediate, but, that collapse of this intermediate through loss of the amino group to form the products of amide hydrolysis, is prevented by its tight fit along a specific reaction coordinate axis relating to heavy-atom displacement.

This feature may have general applicability in the design of substrate variants to act as selective, tight-binding, and reversible[23] inhibitors—in particular for pocket-like active sites. Such structures would: retain the primary functional group of the substrate itself, have a substituent pattern designed to fit tightly against active site residues, this tight binding would be orientated such that heavy-atom displacement is impeded along a key reaction coordinate axis. Measured exploitation of such an approach depends on accurate methodologies of docking/binding organic structures to proteins and on knowledge of reaction path determinants within an enzyme active site. Crystallographic studies are, increasingly, providing this type of information and have highlighted the importance of heavy-atom displacements as reaction path determinants.[24,25]

Computational Methods

Docking studies

The simulated annealing algorithm in AutoDock 3.0, together with the associated cluster analysis routine, was used for all docking experiments. Default parameters were used: 10 runs; 50 cycles; accepted steps, 25,000; rejected steps, 25,000; starting temperature, 616 K, temperature reduction factor per cycle, 0.95. Several repetitions of each docking experiment were carried out. Docking experiments were run with the A monomer unit of the homodimeric MDP (cilastatin-bound protein PDB[26] code 1ITU, 1.9 Å resolution; native protein PDB code 1ITQ, 2.3 Å resolution). Only the zinc-bound water molecule was incorporated (see below). A grid size of 40 × 40 × 40 $Å^3$ with a spacing of 0.375 Å, which fully encompasses the active site when centered on the cilastatin position, was used. Polar hydrogens only were added to the protein structure. This renders imidazole rings as positively charged units; this was the case with His152. In the case of His20, His198 and His219 only, which are complexed via an imidazole-ring nitrogen to the zinc ions, the relevant polar hydrogens were deleted prior to the addition of Kollman charges, leaving these groups neutral. The standard metal (M) parameters were used without modification for the zinc ions, which were given formal charges: this is consistent with the essentially formal charges (Kollman) applied to the protein atoms by AutoDock. The zinc-bound water was generated by placing the oxygen atom of an AMPAC/AM1 water molecule in the same coordinates as those found in the crystal structure of cilastatin-bound MDP, with the hydrogens orientated toward the carboxylate oxygens of Asp288. The atomic charges used were −0.66 for O and +0.33 for each H. The charges on hydrogen here are larger than the AM1 values but were used to reflect the effect of coordination to the zinc ions. For other docked structures the AMPAC/AM1 atomic charges were used directly. Gly-D-Phe, truncated-LTD_4 and the imipenem structure were constructed using AMPAC/AM1. The output of the docking runs was initially evaluated with ADT1.5 (the AutoDock 3.0 GUI) and, subsequently, Swiss-PdbViewer,[27] POV-Ray[28] and Picture It Express (Microsoft) were used to generate the graphics shown here.

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References and Notes

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