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Research Article

Substrate binding and catalytic mechanism of class B β-lactamases: a molecular modelling study

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Abstract. Increased resistance to β-lactam antibiotics is mainly due to β-lactamases whose production by pathogenic bacteria makes their broad activity spectrum especially frightening. X-ray structures of several zinc β-lactamases have revealed the coordination of the two metal ions, but their mode of action remains unclear. Geometry optimisation of stable complexes along the reaction pathway of benzylpenicillin hydrolysis highlighted a proton shuttle occurring from D120 of the *Bacillus cereus* β-lactamase to the β-lactam nitrogen via Zn2 which is central to the network. First, the Zn1 ion has a structural role maintaining Zn-bound waters, WAT1 and WAT2, either directly or through the Zn1 tetrahedrally coordinated histidine ligands. The Zn2 ion has a more catalytic role, stabilising the tetrahedral intermediate, accepting the β-lactam nitrogen atom as a ligand. The role of Zn2 and the flexibility in the coordination geometry of both Zn ions is of crucial importance for catalysis.

Key words. Metallo-β-lactamase; catalytic mechanism; molecular modelling; penicillin binding; molecular mechanics; zinc enzyme.

The main mechanism by which bacteria develop resistance to β-lactam antibiotics involves the production of β-lactamases, enzymes that inactivate the antibiotics by hydrolysing the C-N bond of the β-lactam ring [1]. Of the four structural classes of β-lactamases, classes A, C and D use a serine-dependent mechanism, while in class B β-lactamases, zinc ions participate in β-lactam cleavage [2]. Zinc β-lactamases do not share any sequence or structure similarity with active-site serine β-lactamases.

The increase in resistance to β-lactam antibiotics and the production of metallo-β-lactamases in pathogenic bacteria make the broad activity spectrum that most of them exhibit especially frightening. They are unselective and hydrolyse almost all so far designed β-lactam drugs including carbapenems. They comprise three different structural classes exhibiting considerable sequence diversity despite the conservation of some motifs. The most populated is class B1, containing enzymes that share relatively high sequence similarity to the *Bacillus cereus* and *Bacteroides fragilis* enzymes and the plasmid-borne IMP-1 enzyme, whose three-dimensional structures have been elucidated [3–8]. The members of class B2, e.g. the *Aeromonas hydrophila* and *A. veronii* enzymes, have almost identical amino acid sequences but their sequence isology with the class B1 enzymes is low. The *Stenotrophomonas maltophilia* L1 and the plasmid-encoded enzymes share the lowest sequence identity with the other known metallo-β-lactamases and belong to class B3.

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In the *B. cereus* enzyme, the two zinc ions are liganded by active-site residues that are generally conserved in all known B1 metallo- β-lactamase sequences. One zinc ion is coordinated by three histidines and a water molecule (WAT1), in a tetrahedral arrangement. The second zinc-binding site contains an aspartate, a cysteine, a histidine, WAT1 and a second water molecule (WAT2). The resulting coordination environment for Zn2 is a distorted trigonal bipyramidal arrangement.

Despite the relatively large number of known X-ray structures, the mode of action of class B β-lactamases remains unclear. The different mechanisms proposed consider D120, WAT1 and the Zn1 ion itself as playing a role in catalysis, but the protonation state of the aspartic residue and WAT1 (H_2O or hydroxide) is still a subject of controversy [9, 10]. The reaction mechanism proposed below is based on the sequential changes that occur in the optimised complexes, obtained after docking three entities representing discrete possible intermediates along the benzylpenicillin hydrolysis reaction pathway (fig. 1). This method was successfully applied to explain the mechanism of acyl transfer by class A serine β-lactamases [11]. Comparison of the energy-minimised complexes highlighted a proton shuttle that may explain the catalytic mechanism of the di-zinc metallo-β-lactamases.

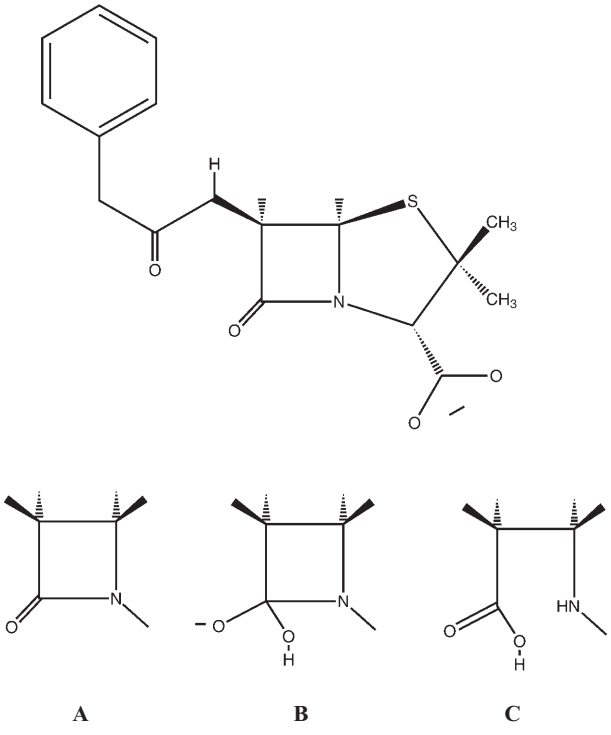

Figure 1. Benzylpenicillin and the corresponding stable entities (*A*)–(*C*) used for geometry optimisation and modelling of the *B. cereus* metallo-β-lactamase-catalysed reaction.

Material and methods

Modelling of protein structures

All calculations were based on the high-resolution X-ray structure of the *B. cereus* 569/H/9 enzyme (BcII) from crystals obtained at pH 7.5, as described in Paul-Soto et al. [12]. Histidines present in the active site were taken as neutral. For the other three histidines, the tautomeric form of the imidazole ring was chosen according to the X-ray structure, after geometrical analysis of the potential hydrogen bonds. The Zn2-coordinated cysteine was in the thiolate form. Other titratable sites were assigned their standard protonation states at pH 7. Hydrogen atoms were added to the crystal structure using the PROTONATE module of AMBER 4.1 [13]. BBL numbering [14] has been used throughout this paper. The molecular structures were manipulated with InsightII (Molecular Simulations, San Diego, Calif.).

Molecular mechanics calculations

Molecular mechanics programs treat metal-ligand interactions either by a pure 'bonded' approach [15] (i.e. defining all metal-ligand bonds as covalent bonds and using appropriate parameters for bond stretching and angle bending) or by a pure 'non-bonded' approach [16] (i.e. by treating the interactions by means of electrostatic and van der Waals forces). The disadvantage of the 'bonded' method is that the coordination of the metal ion cannot be easily changed during the refinement. On the other hand, 'non-bonded' models generally require constraints in order to avoid strong repulsion between the metal ions. To overcome these difficulties, a 'bonded' approach was used here, with the zinc involved in only one covalent bond, allowing free motion of the other ligands. The force-field potential parameters for the zinc ion were those developed by Hoops et al. [17]. The equilibrium values for the bond and angle potential were set according to X-ray structures. Atomic charges of the AMBER 4.1 all-atom library were generally used in the calculations, except for the residues of the active site. For the zinc ions, the zinc-coordinating residues and the active-site water molecules, atomic point charges were those proposed by Bianci et al. [18]. The structure was optimised with AMBER running on an SGI Indy workstation, first by steepest-descent energy minimisation of atoms with forces above 500 kcal mol^{-1} $Å^{-1}$, and then by conjugate gradient energy minimisation until the r.m.s gradient was less than 0.1 kcal mol^{-1}.

Penicillin models along the reaction pathway

The entities **(a)–(c)** (fig. 1) represent different intermediates that could be adopted by the β-lactam moiety during the β-lactamase-catalysed reaction. The geometry of the benzylpenicillin structures containing each of these moieties was optimised by an AM1 semi-empirical

method [19]. Each optimised structure was then docked into the enzyme active site and the energy of the corresponding complex minimised as described for the native enzyme, using CNDO charges to compute the coulombic term. In these entities, the bond lengths, bond and dihedral angles were constrained to the AM1 values while allowing rotation around the free bonds.

Results

Native enzyme

Three different protonation states of the D120-WAT1 pair have been proposed in the mechanisms considered carboxylic/hydroxide, carboxylate/hydroxide or carboxylate/water. The geometry of the β-lactamase structure containing each of the three models was optimised by molecular mechanics and compared to that derived from the X-ray structure of BcII containing two zinc ions and the two catalytically important water molecules in the active site [6].

Table 1 summarises the results of these comparisons giving the r.m.s. distances between pairs of Cα atoms, and the distances between pairs of the same atoms of the active site when the X-ray structure and the considered models are superimposed.

From this table, the Asp^-/OH^- model is apparently the less realistic one, as these distances exceed 0.3 Å, a difference that could not be explained by experimental errors and molecular flexibility of the X-ray structure. In the two other models, the differences are smaller, and both models are probably good reflections of reality. Indeed, the proton should be shared by the two oxygen atoms (D120Oδ1 and WAT1) between which it is located. However, the positions of both zinc ions and of WAT2 are better reproduced in the Asp^-/HOH model. This model will thus be used in the following calculations, as molecular mechanics methods need hydrogen atoms to be attached to a heavy atom.

Table 1. Comparison between the X-ray structure of the *B. cereus* β-lactamase and each of the models differing by the protonation state of D120 (Asp or Asp^-) and WAT1 (HOH or OH^-): r.m.s. distances between pairs of Cα atoms and distances between pairs of the same atoms of the active site when the X-ray structure and the considered models are superimposed.

	Asp^-/HOH	Asp/OH^-	Asp^-/OH^-
r.m.s. Cα	0.43	0.49	0.63
H116(Nε)	0.1	0.59	0.77
H118(Nδ)	0.09	0.52	0.88
H196(Nε)	0.36	0.44	0.3
H263(Nε)	0.15	0.38	0.54
D120(Oδ2)	0.27	0.28	0.45
C221(Sγ)	0.31	0.41	0.45
Zn1	0.09	0.74	0.58
Zn2	0.29	0.77	1.25
WAT1(O)	0.45	0.25	0.32
WAT2(O)	0.69	0.91	2.58

Figure 2 shows the hydrogen-bonding network found between the zinc ions and their direct and indirect ligands in the native enzyme model. These interactions were conserved in the energy-minimised structure of the enzyme-substrate complexes described above. As found in the X-ray structures, the line between D120Oδ and WAT2 is perpendicular to a plane containing the three other Zn2 ligands (H263Nε, C221Sγ and WAT1), forming a scalene triangle in which WAT1-Sγ is the largest side (fig. 3 A).

Enzyme-benzylpenicillin complexes

The starting geometry for the complexes formed by docking entities **(a)–(c)** in the enzyme active site (fig. 4) was such that the β-lactam carbonyl oxygen was located between Zn1 and the side chain NH_2 group of N233. The position of the partners is such that the nucleophilic attack takes place from the less hindered alpha side of the β-lactam, as generally proposed [20].

The antibiotic carboxylic group is oriented towards the ammonium group of K224. The carbonyl oxygen of the penicillin acetamido side chain interacts with the backbone NH groups of residues 119 and 120, while the phenyl group of penicillin fits into a hydrophobic pocket formed by the side chains of F61 and W87. In turn, the thiazolidine methyl groups interact with V67. The reaction steps modelled in the calculations are illustrated in figure 5 A–C. The bond lengths, valence and dihedral angles around the bonds involved in the interactions are given in table 2.

Entity (a)

Binding of the β-lactam ligand hardly modifies the active-site geometry. A slight reorientation of the F61 and W87

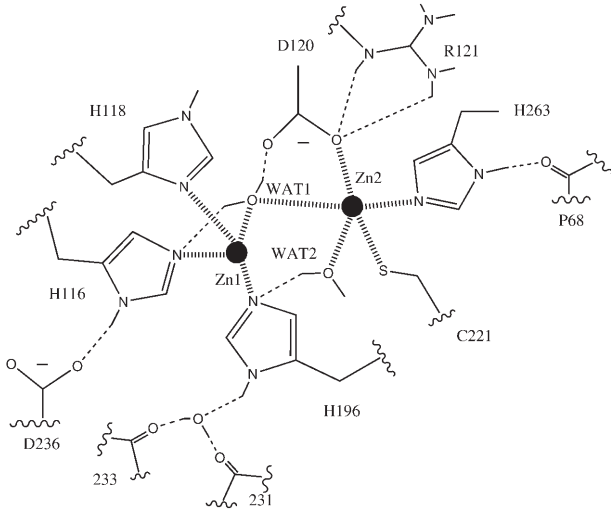

Figure 2. Schematic representation of the hydrogen-bonding network between the zinc ions and their direct and indirect ligands in the native enzyme model.

Table 2. Evolution of different distances (Å) and dihedral angles (°) in the complexes occurring along the reaction pathway.

	Free enzyme	Henri Michaelis complex with benzylpenicillin (a)	Tetrahedral intermediate (b)	Product (c)
D120 (χ1)[a]	−58.5	−86.5	−23.9	−93.2
Zn1-Zn2	3.35	4.18	3.83	4.19
Zn1-WAT1 (O)	1.95	1.92	2.45[b]	2.09[b]
Zn2-D120(Oδ2)	1.93	2	2.11	2.03
Zn2-C221(Sγ)	2.78	2.62	3.22	2.74
Zn2-WAT1 (O)	2.11	2.5	2.51[b]	2.99
Zn2-WAT2 (O)	2	2.1	1.95	1.96
Zn1-Olact	_	2.64	2.01	3.23
N233(NH2)-Olact	_	2.96	2.98	2.8
Zn2-Nlact	_	3.7	2.2	4.8

[a] D120 (χ1): N–Cα–Cβ–Cγ dihedral angle of D120 measuring the rotation of the Asp side chain.
[b] OH on the β-lactam.

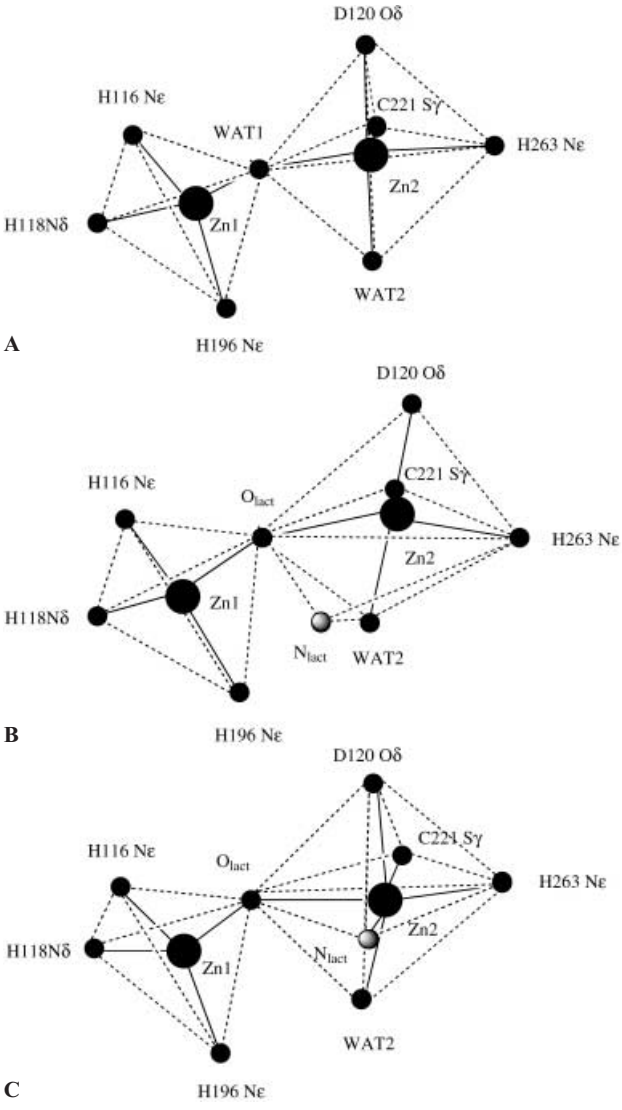

Figure 3. Coordination polyhedron around the two zinc ions as found in the models representing the different steps along the reaction pathway. (*A*) in the free enzyme. (*B*) in the Henri-Michaelis complex with benzylpenicillin. (*C*) in the complex with the tetrahedral intermediate.

side chain is observed, increasing the aromatic interaction with the phenyl ring of penicillin. The distance between the antibiotic carboxylate and the Nε of K224 is too long (4.62 Å) to allow formation of a salt bridge, but a hydrogen bond occurs between one of the carboxylate oxygens and WAT2 (2.74 Å between oxygen atoms). In turn, WAT1 forms a hydrogen bond with WAT2 and reorients with one of its hydrogen atoms directed towards the lactam carbonyl carbon (fig. 5A). The lactam carbonyl oxygen atom is located in an oxyanion hole formed by the side chain NH2 group of N233 (Nδ-Olact 2.96 Å) and the Zn1 ion (Zn1-Olact 2.64 Å). In this model, the distance between the two zinc atoms increases slightly. The D120 side chain rotates around Cβ-Cγ to remain hydrogen bonded to WAT1, with one WAT1 hydrogen atom close to the plane of the carboxylic head. The lactam nitrogen is in the plane of WAT1, H263Nε and C221Sγ, the four atoms disposed as a distorted square (fig. 3B). The distance between Zn2 and the lactam nitrogen is long (3.7 Å).

Entity (b)

The Zn1-carbonyl interaction polarises the C–O bond. WAT1 can readily attack the carbonyl carbon while the Zn2-bound Asp carboxylate can accept a proton from the WAT1 molecule, thereby increasing its nucleophilicity. In a concerted manner, the hydroxyl ion attacks the carbonyl carbon of the scissile lactam bond. A negatively charged adduct is formed, in which the lactam carbonyl carbon adopts a tetrahedral geometry (fig. 5B). Generation of the tetrahedral adduct has the following consequences (table 2, fig. 3C). (i) Most interatomic distances (including Zn-His) and interactions are conserved. (ii) The D120 side chain rotates around the Cβ-Cγ bond. (iii) The protonated Asp retains an interaction (via Oδ1) with the former WAT1 (now an OH group attached to the β-lactam carbonyl carbon) while remaining a Zn2 ligand (via Oδ2), although the distance slightly increases. The Zn2-Cys221 distance increases due to a displacement of Zn2 towards Zn1 and the lactam nitrogen atom. (iv) The lactam carbonyl oxygen now binds strongly to the metal ion (Zn1-Olact 2.01 Å), (v) Due to shortening of the Zn2-N-lactam distance (2.2 Å), the latter becomes a fourth ligand of Zn2 in the plane perpendicular to D120Oδ2-WAT2 (fig. 3C), so that Zn2 adopts an octahedral conformation (mean side length of the octahedron 3.15 Å).

Entity (c)

Different β-lactam intermediates have been proposed to occur along the reaction pathway of antibiotic hydrolysis

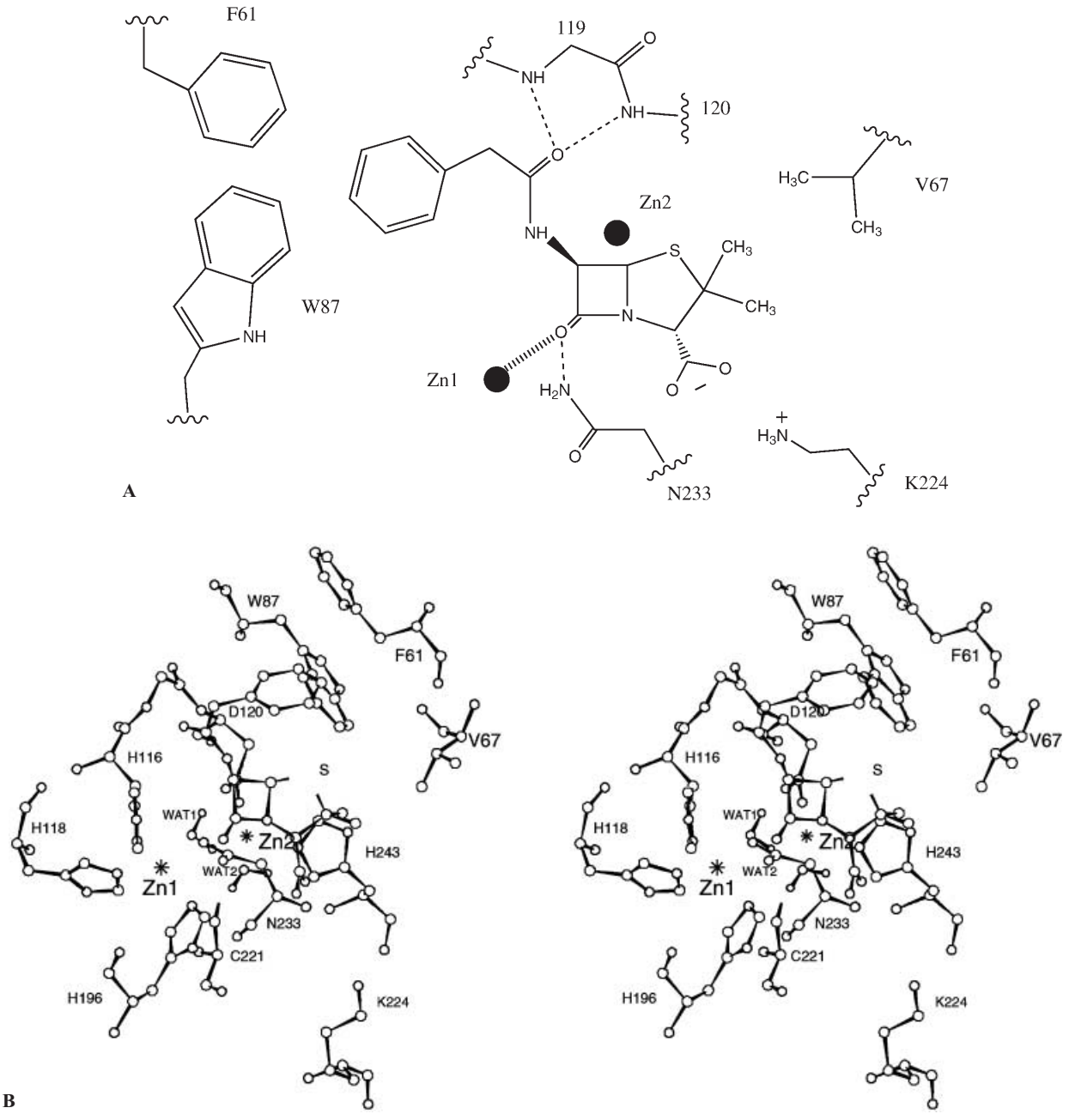

Figure 4. Starting geometry for the docking of benzylpenicillin in the enzyme active site, as used for energy minimisation of the complexes. (*A*) Schematic representation. (*B*) Stereo-view.

[21]. Among them, the tetrahedral intermediate in which two negatively charged oxygens are bonded to the lactam carbonyl carbon appears to be highly improbable, as the AM1 optimisation of this molecule induces the opening of the C-N bond. In turn, collapse of the tetrahedral adduct occurs by opening of the lactam C-N bond with restoration of an sp^2 carbonyl carbon. The Zn1-Olact distance now increases to 3.23 Å.

Proton transfer to the leaving amino group gives rise to the product, with a return to the enzyme geometry found in the complex with benzylpenicillin. A water molecule, WAT1′, can now re-enter the enzyme cavity, replacing WAT1, which has been utilised in the catalytic process (fig. 5C).

Discussion

In its resting state, the active site of a class B β-lactamase shows intricate electrostatic interactions between the zinc

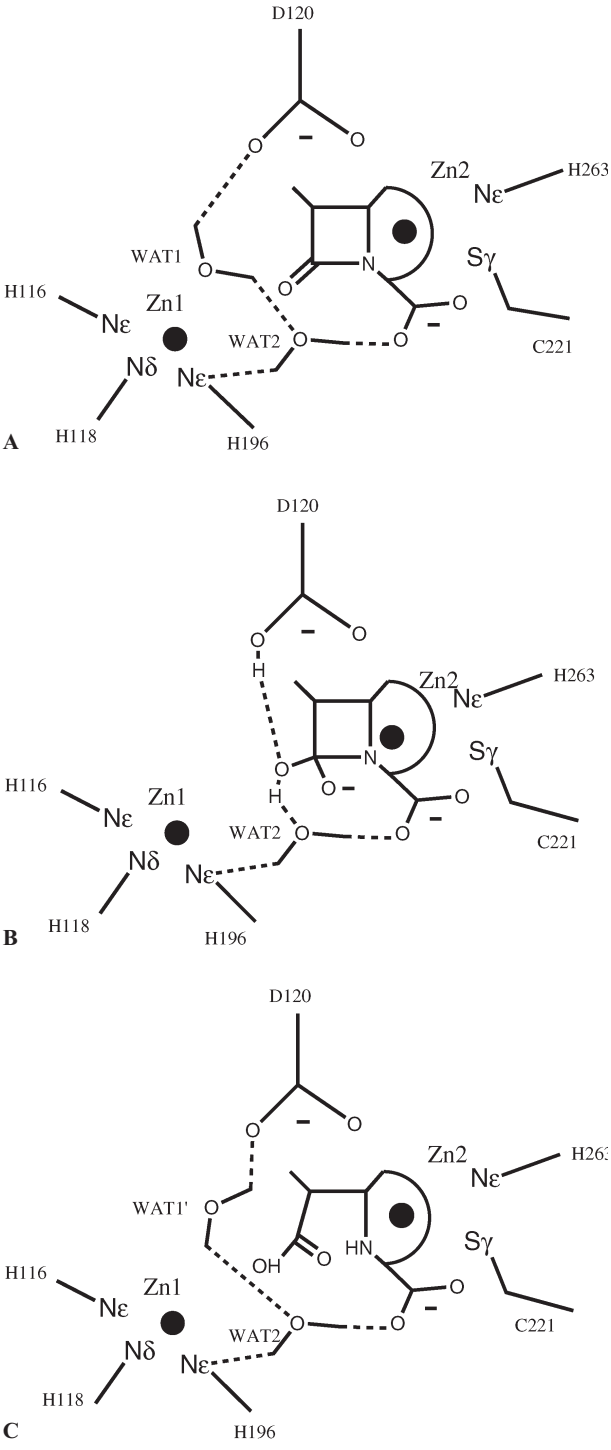

Figure 5. Schematic representation of the active-site residues in the different steps of the reaction. (*A*) Henri-Michaelis complex with benzylpenicillin. (*B*) Tetrahedral intermediate. (*C*) Product.

ions, the zinc direct and indirect ligands and two solvent molecules.

The calculations propose the simultaneous presence of WAT1 as HOH and the Asp residue in its deprotonated form.

Binding of benzylpenicillin gives rise to an integrated functional complex. The backbone NH groups of residue A119 and D120 interact with the carbonyl group of the antibiotic exocyclic amide bond. In the hydrophobic pocket, two aromatic side chains (the phenyl groups of F61 and W87) are in interaction with the phenyl group of benzylpenicillin. The ring centre-to-centre separation (6.1 and 6.6 Å) and dihedral angle (49° and 69° for Phe and Trp, respectively) are in the range of distances (between 4.5–7 Å) and dihedral angles (between 50–90°) observed in the most potentially stabilising hydrophobic interactions [22].

On the other side of the antibiotic, the carboxylate helps to orient the molecule in the active site by electrostatic interaction with the Nε of K224. However, this interaction remains loose, without formation of a hydrogen bond, in contrast to the situation observed in the X-ray structure of the IMP1-mercaptocarboxylate inhibitor complex [8]. Indeed, the amide nitrogen of the inhibitor is not involved in a four-membered ring, so the distance between the carbonyl carbon and the carboxylate group is longer than in benzylpenicillin. The orientation of the carboxylate with respect to the scissile bond is different (in the inhibitor, this group rather takes the place of the methyl groups in benzylpenicillin). By contrast, the carboxylate-Nε distance would be too long for a hydrogen bond to be formed with cephalosporins, though the carboxylate group is oriented differently in these antibiotics.

The Zn1 ion and the NH2 group of N233 interact with the carbonyl of the scissile amide bond, at the central position of the ligand. The relative spatial disposition adopted by the two interacting partners results in the polarisation of this carbonyl oxygen (by Zn1 and N233), while the carbonyl carbon is close to the OH group of the water molecule WAT1. The Zn1-Olact distance is short (table 2), thus confirming that the metal centre plays a direct catalytic role by binding the substrate. The Zn1 coordination is not affected by penicillin binding.

In the complex with entity (b), the reorientation of D120 is observed as a result of the transfer of one of the WAT1 protons to the Asp carboxylate group. The Zn1-Olact distance strongly decreases on formation of the tetrahedral adduct, showing that the zinc ion plays a crucial role in stabilising this intermediate and polarising the C–O bond. Zn–O distances of the same order of magnitude have also been found in a zinc enzyme-substrate complex studied by ab initio methods [23].

In a concerted motion, the Zn2–C211Sγ distance increases while the distance between Zn2 and the β-lactam nitrogen decreases. Such a 'valence buffer' in zinc enzyme catalysis has been observed previously: in cytidine deaminase complexes, the zinc-sulphur bond changes as the substrate approaches the tetrahedral transition state [24]. Similar structural features in metallo-enzymes sug-

gest that analogous mechanisms may be a general feature of catalysis by zinc enzymes. The resulting modification of the coordination around Zn2 is facilitated by the fact that zinc ions lack any preference for a specific coordination geometry because of their closed shell of electrons ($3d^{10}$).

Opening of the β-lactam ring and back delivery of the proton on the β-lactam nitrogen atom depends on the relative orientation of D120 and WAT2 with respect to the lactam ring. The proton could travel from D120 to nitrogen through an H-bond network that includes WAT2. A more detailed description of the different steps should be supported by quantum mechanical calculations.

The protonation step leading to entity (**c**) could occur after cleavage of the C-N bond through a mechanism that does not require protonation of the leaving lactam nitrogen before the bond is broken [25].

The catalytic pathway described here for penicillins should also be valid for cephalosporins, though in this case, hydrolysis is followed by elimination of the C3-leaving group [26]. This side chain would extend between loops L1 (residues 61–68) and L3 (residues 227–231) [14], with enzyme substrate interactions, that depend on the cephalosporin side-chain and on the amino-acid in the loops, which are specific to each enzyme.

In the metallo-β-lactamases, as in other metallo-enzymes, the Zn ions have essentially two roles: a structural role in stabilising the local conformation and/or an active role in catalysis. First, the Zn1 ion has a structural role in maintaining WAT1 and WAT2, either directly or through the Zn1 tetrahedrally coordinated histidine ligands. Together with the NH2 group of N233, it also polarises the β-lactam carbonyl bond and thus also plays a catalytic role.

The Zn2 ion has a more catalytic role, by stabilising the tetrahedral intermediate and accepting the β-lactam nitrogen atom as a ligand. This flexibility in the coordination geometry for Zn^{2+}, of crucial importance for catalysis, may be enhanced by the fact that zinc is generally less tightly bound in the second site [2]. Finally, the catalytic mechanism proposed here for a dizinc enzyme might also explain the mechanism of a protein containing only one equivalent of zinc. In the mononuclear species, the metal ion, whether Zn or Cd, has been shown to be shared between the two binding sites [12] and, moreover, preliminary nuclear magnetic resonance results (unpublished observations) suggest that the zinc ion moves between the two sites rather rapidly. Atomic absorption measurements also showed that the BcII enzyme can bind two equivalents of zinc per molecule of enzyme. The efficiency of the catalytic mechanism would then depend on the population of the Zn2 site at any given time, which would vary depending on the zinc concentration and the pH.

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