Kinetic and Molecular-Modelling Study of the Interaction between *Staphylococcus aureus* PC1 Enzyme and Imipenem

by **Bartolomé Vilanova**, **Miguel Coll**, **Juan Frau**, **Francisco Muñoz**, and **Josefa Donoso***

Departament de Química, Universitat de les Illes Balears, E-07071 Palma de Mallorca (fax: 34-971-173426; e-mail, dqujdp0@ps.uib.es)

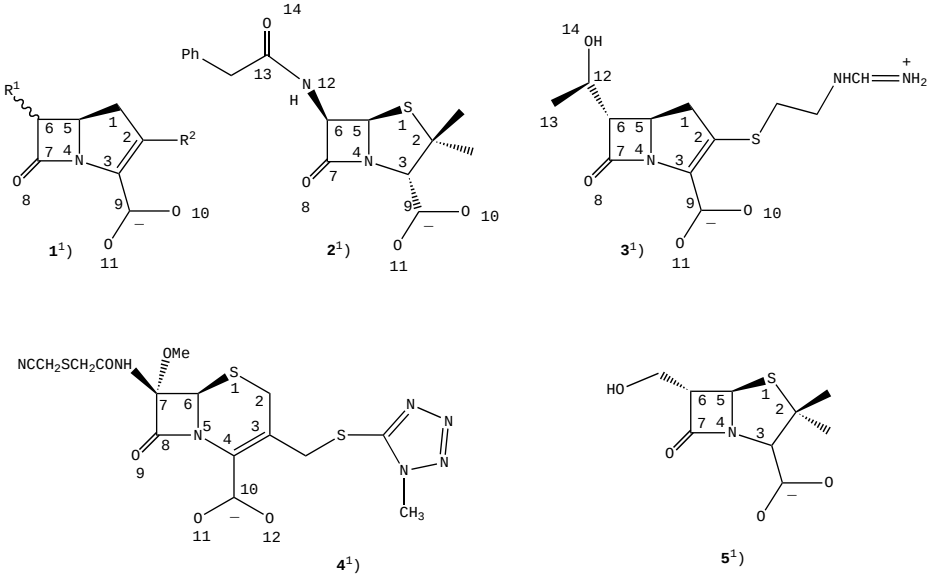
The interactions between imipenem (**3**), a clinically significant carbapenem antibiotic, and *Staphylococcus aureus* PC1 enzyme, were studied in detail. Imipenem behaves as a slow substrate that reacts by a branched pathway, which suggests the formation of a second acyl-enzyme intermediate. The individual microscopic rate constants for the process were determined. The results were analysed in the light of molecular-modelling considerations. Based on the analysis, the Ser-70(O^{γ}) group in the *Michaelis-Menten* complex formed between **3** and PC1 is very distant from the carbonyl group of the β-lactam ring of **3**, which is consistent with the decreased value of k_2 (*Model 2*, see *Scheme 2*) for imipenem relative to an appropriate substrate such as benzylpenicillin (**2**). The deacylation is the rate-determining step of the turnover process. This can be ascribed to the fact that in the deacylation of the second acyl-enzyme, the H_2O molecule lying closest to the ester group, Wat81, is in an unfavorable orientation to hydrolyse the intermediate.

Introduction. – β-Lactam antibiotics possess a β-lactam ring, the integrity of which must be preserved with a view to inactivate a series of transpeptidases that catalyse the final cross-linking reactions involved in peptidoglycan synthesis. Resistance to β-lactams in clinical isolates has been found to be primarily the result of the hydrolysis of the antibiotic by a β-lactamase. Mutational events resulting in the modification of penicillin-binding proteins (PBPs) or cellular permeability can also lead to β-lactam resistance [1].

β-Lactamases constitute a heterogeneous group of enzymes. The classification initially proposed by *Ambler* [2], which is based on the amino acid sequence, recognizes four molecular classes that are designated A to D. Classes A, C, and D gather evolutionary distinct groups of serine enzymes [3][4], whereas class B comprises metalloproteins that require the presence of bivalent transition metal ions (most often Zn^{2+}) to be active [5][6]. A more recent functional classification of β-lactamases, proposed by *Bush et al.* [7], defines four main categories according to substrate and inhibitor profiles.

Bacterial resistance has been tackled in two different ways. One uses β-lactamase-stable compounds, while the other involves the administration of a β-lactamase inactivator together with a classical β-lactam. Kinetics and molecular-modelling studies have shown that the poor activity of most class-A β-lactamases against the compounds used in the first approach is due to a low acylation rate, that, in turn, results from steric hindrance with active-site residues [8][9] or perturbation of a catalytic H_2O molecule [10], or even from the potential formation of inert acyl-enzyme intermediates leading to transient inhibition of the enzyme [11][12].

Carbapenems are among the most promising classes of β-lactam antibiotics. These compounds exhibit a broad spectrum of activity against *Gram*-positive, *Gram*-negative, aerobic, and anaerobic bacteria, including strains resistant to many drugs; unfortunately, they are hydrolyzed by class-B enzymes [13][14] and also by a few class-A β-lactamases such as Sme-1 (isolated from *Serratia marcescens* S6) [15] and NMCA (isolated from *Enterobacter cloacae* NOR-1) [16]. Carbapenems are essentially 1-carbapen-2-em-3-carboxylic acids (= 7-oxo-1-azabicyclo[3.2.0]hept-2-ene-2-carboxylic acids) **1** bearing various substituents at positions 2 and 6[1]). Instead of a C(6) acylamino substituent at the β-lactam ring, carbapenems contain a hydroxyethyl side chain or other substituent. The most salient differences between carbapenems and penicillins, *e.g.* **2**, are the absence of the ring S-atom and a more-strained dihydropyrrole-azetidinone ring system. The expansion of carbapenems has been hindered by their chemical instability, nephrotoxicity, and neurotoxicity [17]. Imipenem (=(5*R*,6*S*)-6-[(1*R*)-1-hydroxyethyl]-3-{{2-[(iminomethyl)amino]ethyl}thio}-7-oxo-1-azabicyclo[3.2.0]-hept-2-ene-2-carboxylic acid; **3**) is the prototypical carbapenem antibacterial agent and possesses a very broad spectrum of antibacterial activity. It is commercially available in combination with cilastin, which prevents its degradation by renal dehydropeptidase I [18][19].



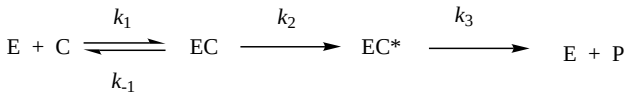
The interaction between imipenem (**3**) and class-A β-lactamases, such as I from *Bacillus cereus* [11], and TEM-1 from *Escherichia coli* [20–23], *Streptomyces albus G*, *Streptomyces cacaoi*, and *Actinomadura* R39 [24], has been examined. Imipenem behaves as a slow substrate for class-A β-lactamases with k_{cat}/K_m values in the range 1–10^3 M^{-1} s^{-1}, except for *Actinomadura* R39 (with $k_{cat}/K_m = 9000 \pm 1000$ M^{-1} s^{-1}). Class-A β-lactamases react with **3** *via* a three-step pathway (*Model 1*) that involves the

[1]) Arbitrary numbering.

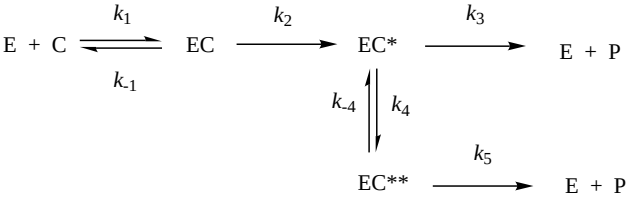
formation of a *Henri-Michaelis* complex, further reaction to an acyl-enzyme intermediate EC*, and deacylation of EC* (see *Scheme 1*). However, the results of *Monks* and *Waley* [11], and those of *Taibi* and *Mobashery* [21] suggest that the acyl-enzyme EC* formed between **3** and other class-A enzymes in the acylation step may rearrange and yield a second, more stable acyl-enzyme EC** (*Model 2*, see *Scheme 2*). *Maveyraud et al.* [22] recently reported the crystal structure of an acyl-enzyme intermediate for imipenem bound to the enzyme TEM-1. The structure of the intermediate suggests a conformational rearrangement in the first acyl-enzyme formed in the acylation step that leads to the complex observed; this is consistent with the ability of this antibiotic to resist hydrolytic deactivation by β-lactamases.

Whether the acyl-enzyme rearrangement observed with some class-A β-lactamases is the general mechanism for the interaction of this enzyme class with imipenem (**3**) remains uncertain. Should it be the case, some enzymes might react so rapidly that the steady state would be reached within a very short time. This explanation is consistent with the fact that no burst suggesting the presence of two acyl-enzymes has been detected in the interaction of **3** with *Streptomyces albus G* or *Streptomyces cacaoi* β-lactamases.

Scheme 1. *Model 1*. E = Enzyme, C = carbopenen, EC = *Henri-Michaelis* complex, EC* = acyl-enzyme **I**, and P = product



Scheme 2. *Model 2*. E = Enzyme, C = carbapenen, EC = *Henri-Michalis* complex, EC* = acyl-enzyme **I**, EC** = acyl-enzyme **II**, and P = product.



To clarify the mechanism by which imipenem (**3**) interacts with class-A enzymes, we have now examined the interactions of **3** with the *Staphylococcus aureus* PC1 enzyme, the structure of which is known to a resolution of 2.0 Å [25]. As found in this work, the turnover kinetics of PC1 with **3** involves two different steps and, thus, suggests a branched pathway and the presence of two distinct acyl-enzyme species. Molecular modelling allowed us to examine the most significant interactions in the *Henri-Michaelis* complex formed between **3** and the *Staphylococcus aureus* enzyme. In previous work, we studied the interactions between PC1 and compounds bearing a MeO group on the α side of the β-lactam ring [26]. The kinetics and molecular-modelling results for the reactions of this enzyme with various substrates led us to understand its affinity for the different substrates and the slight differences in their microscopic rate constants.

Finally, we subjected the two possible acyl-enzyme intermediates to energy minimization to obtain additional insight into the mechanism by which the enzyme PC1 is inhibited during imipenem turnover.

Experimental. – *Materials.* The PC1 β-lactamase from *Staphylococcus aureus* was obtained from *Speywood*, UK, and used as supplied. The specific activity of the enzyme against benzylpenicillin (**2**) was 300 units/mg at pH 7.0 and at 30°. Benzylpenicillin (**2**) and 6-aminopenicillanic acid (6-APA) were purchased from *Sigma*. Imipenem (**3**) was a gift from *Merck, Sharp & Dohme*. All buffer materials were reagent grade.

Analytical Methods. All kinetics experiments were performed at 25° in 0.1M potassium phosphate, pH 7.0. Absorbance measurements were performed by means of a *Uvikon 940* spectrophotometer.

Imipenem (**3**) *Hydrolysis.* The hydrolytic cleavage of **3** occurs with a substantial decrease in the absorption band at 300 nm ($\Delta\varepsilon_{300} = 5300 \pm 300$ M^{-1} cm^{-1}). The pseudo-first-order phosphate-catalyzed hydrolysis of **3** was followed by UV spectroscopy.

Inactivation Experiments. Inactivation experiments were performed by the reporter-substrate method [27]: pseudo-first-order inactivation rate constants were measured at increasing inactivator concentrations (10^{-5} to $2.5 \cdot 10^{-4}$ M). The reporter substrate was 6-aminopenicillanic acid (6-APA; 10^{-4} M). The absorbance variations were monitored at 230 nm, and the conditions were chosen so that its utilization remained below 10%.

Partial-Inactivation Experiments. The enzyme was incubated at different C_0/E_0 ratios such that partial inactivation was observed. After 5 min, the samples were diluted in buffer, and residual activity was assayed on benzylpenicillin (**2**; $9 \cdot 10^{-4}$ M) at 230 nm, 25°.

Reactivation Experiments. The inactive acyl-enzyme of the PC1 β-lactamase was generated by reaction of the enzyme (10^{-6} M) with **3** ($3.5 \cdot 10^{-5}$ M). After 30 min of incubation, the enzyme activity was less than 10% of the experiment control. A mixture containing enzyme and **3** was dialysed at least 4 h at 5° against 0.5 l of 0.1M phosphate (one buffer change) to remove excess inhibitor. The incubation mixture was then diluted 50-fold in phosphate buffer and maintained at 25°. Recovery of enzymatic activity was monitored periodically by adding the incubation mixture to benzylpenicillin (**2**; 0.9 mM) and following initial rates as a function of time. Note that prolonged incubation of uninhibited staphylococcal β-lactamase resulted in some loss of activity at 25°.

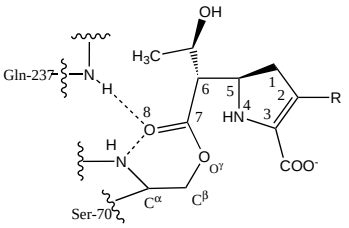
Pre-Steady-State Kinetics. The pre-steady-state phases of the reaction between the PC1 β-lactamase (0.99 and 1.37 μM) and **3** ($2.89 \cdot 10^{-5}$, $3.85 \cdot 10^{-5}$, and $4.82 \cdot 10^{-5}$ M) were studied spectrophotometrically at 300 nm. The resulting absorption changes were fitted to reaction schemes by the program OPKINE [28][29].

Molecular Modelling. The three-dimensional structure of *Staphylococcus aureus* PC1 enzyme [25] was obtained from the *Brookhaven Protein Data Bank* [30] (refcode: 3BLM). We determined the pre-acylation complex by the minimization protocol of *Juteau et al.* [31] with slight modifications [26]. The acyl-enzyme structure was generated by cleaving the β-lactam amide bond and subsequently connecting it to the Ser-70(O^γ) residue (the consensus numbering scheme proposed by *Ambler et al.* [32] is used throughout this paper), which was followed by global optimization. This intermediate (acyl-enzyme **I**) was used to generate the second, which was equivalent to that crystallographically determined for TEM-1 [22] by rotating the $C(7)-O^\gamma$ bond (acyl-enzyme **II**). To avoid a global displacement in the enzymatic structure, the $C^\beta-O^\gamma-C(6)-C(7)$ improper dihedral and the $N-C^\alpha-C^\beta-O^\gamma$ dihedral were restricted to the crystallographic values, and complete minimization was performed. The acyl-enzyme structures were generated on the assumption of a deprotonated Glu-166 group.

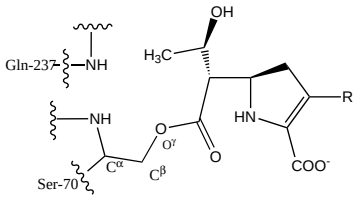
The energy-minimized acyl-enzymes were used as the starting conformations for the molecular-dynamics simulations. These minimized complexes were heated at 300 K for 25 ps, by means of the shake algorithm. A dynamic simulation of the two acyl-enzymes for 165 ps was performed with a time step of 1 fs. Conformations were sampled at 0.2 ps intervals and then minimized until the largest energy change was less than 0.01 kcal mol^{-1}.

Calculations were performed on a *Silicon-Graphics-Origin-200-R10000* computer, with the AMBER* force field [33][34] as implemented in the Macromodel 6.0 software package [35].

Results and Discussion. – Imipenem (**3**) is slowly hydrolysed by the enzyme PC1, such that the time course of the process can be monitored by UV/VIS spectroscopy. With the initial carbopenem-substrate (C) concentration $[C]_0$ *ca.* 20 times higher than that of the enzyme ($[E]_0$), the curve obtained by monitoring at 300 nm suggests the occurrence of two steps (*Fig. 1*), namely, rapid, but partial hydrolysis of the β-lactam,



Acyl-enzyme I (EC*)



Acyl-enzyme II (EC)**

followed by much slower turnover. The burst size ($4 \cdot 10^{-6}$ M) is greater than the enzyme concentration ($1.37 \cdot 10^{-6}$ M). A molar ratio between the burst size and the enzyme concentration that exceeds 1.0 cannot be accommodated by the kinetics pathway of *Model 1*. The observation of a rapid burst reaction in which more than 2 mol-equiv. of substrate is consumed can be accommodated by a branched pathway such as that of *Model 2*, where EC denotes the *Henri-Michaelis* complex, EC* the acyl-enzyme initially formed, and EC** an altered form of the original acyl-enzyme intermediate (*Scheme 2*). Previous results [11][21][22] are consistent with a branched model for the interactions of imipenem (**3**) with other class-A enzymes.

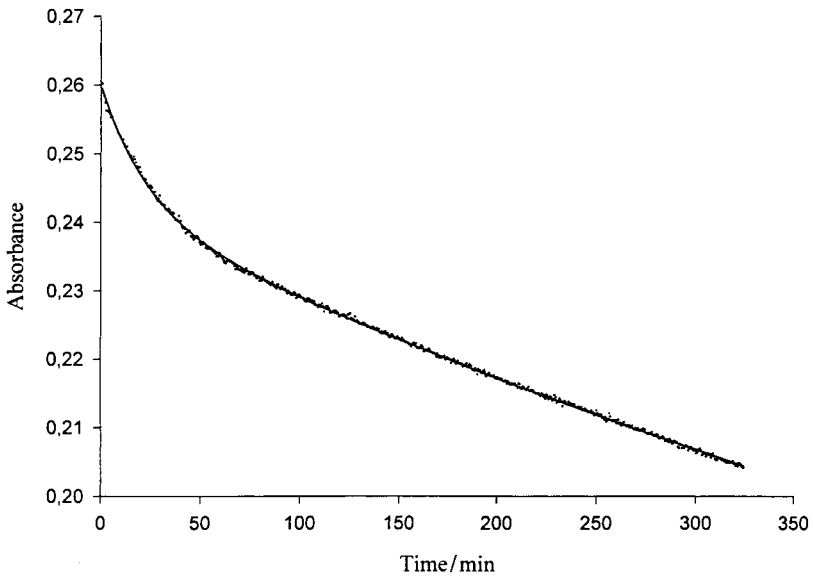


Fig. 1. *Absorbance changes at 300 nm on mixing PC1 β-lactamase* (1.37 μM) *with imipenem* (**3**; 28.9 μM) *at pH 7.0 and at 25°*. The points are experimental and the line calculated as described in the text.

The general model admits two borderline cases, *i.e.* $k_{-4} = 0$ (*Model 2a*) or $k_5 = 0$ (*Model 2b*), which are kinetically indistinguishable. The data reported in this work do not allow one to choose among the different possibilities (*viz.* the general model or one

of the borderline cases). For simplicity, the situation will be further discussed by assuming $k_{-4}=0$; this, however, does not mean that this borderline case is more likely than either the other or the general model.

Constants k_2 and K_S were calculated by means of the method of *Faraci* and *Pratt* [36] for substrates conforming to the kinetics *Model 2a*. On incubation, the amount of residual β-lactamase activity (A) at any time t_c after mixing will be given, according to *Model 2a*, by *Eqn. 1*, where A_0 is the initial enzyme activity and $[C]_0$ (the initial substrate concentration) is greater than $[E]_0$. The plot of $t_c/\ln(A/A_0)$ against $1/[C]_0$ is a straight line ($r^2=0.989$) that provided the k_2 and K_S values shown in *Table 1*.

Table 1. *Kinetics Parameters for the Interaction between PC1 β-Lactamase and Different Substrates*

	k_2 [s^{-1}]	K_S [μM]	k_2/K_S [M^{-1} s^{-1}]	k_{OH} [M^{-1} s^{-1}]
Imipenem (**3**)	$(1.5\pm0.2)\cdot10^{-2}$	280 ± 30	54 ± 10	2.5[a])
Benzylpenicillin (**2**)	173 ± 10[b])	9 ± 2[b])	$(20\pm6)\cdot10^{6}$[b])	$15\cdot10^{-2}$[c])
Cefmetazole (**4**)[d])	$(4.0\pm0.6)\cdot10^{-2}$	50–100	530 ± 100	$2\cdot10^{-2}$

[a]) Taken from [37]. [b]) Taken from [38]. Reaction conditions: 20°, pH 7.0. [c]) Taken from [39]. Reaction conditions 30°, $I=0.5$M. [d]) Taken from [26]. Reaction conditions: 25°, pH 7.0.

$$A = A_0 \cdot \exp\{-k_2[C]_0 t_c/(K_S+[C]_0)\} \tag{1}$$

The dissociation constant for the enzyme-substrate complex, K_S, is inversely proportional to the enzyme's affinity for the substrate. The K_S value obtained for imipenem (**3**) is *ca.* 30 times greater than that for benzylpenicillin (**2**) and *ca.* 3–4 times greater than that for cefmetazole (**4**) (*Table 1*). K_S for **3** and some class-A enzymes is strongly dependent upon the particular class-A enzyme (*e.g.* 3.5 μM for TEM-1 [21], 9–30 mM for β-lactamase I [11] and, as found in this work, 280 μM for PC1). Imipenem possesses a k_2/K_S value of 54 M^{-1} s^{-1}, so it is not a good substrate for the PC1 β-lactamase.

The reporter-substrate method [27] was used to monitor enzyme inactivation at high $[C]_0/[E]_0$ ratios. The dependence of the inactivation rate, k_i, on the imipenem concentration (with $k_{-4}=0$), is given by *Eqn. 2*, the factor $\alpha=1+([S]/K_{m,S})$ ($K_{m,S}$ = *Michaelis* constant for the reporter substrate) being a correction factor accounting for the protection of the enzyme by the substrate (S).

$$k_i = k_5 + \frac{A[C]}{(B\alpha + [C])} \tag{2}$$

$$\text{where } A=\frac{k_2k_4}{k_2+k_3+k_4} \text{ and } B=\frac{(k_3+k_4)K'}{(k_2+k_3+k_4)}, \text{ with } K'=\frac{(k_{-1}+k_2)}{k_{+1}}$$

With 100 μM 6-APA as reporter substrate, the *pseudo*-first-order rate constant for inactivation was found to remain proportional to the imipenem concentration up to $2.5\cdot10^{-4}$ M. Based on these results, and taking into account the correction factor ($\alpha=1.7\pm0.1$), an A/B ratio of 55 ± 15 M^{-1} s^{-1} was obtained. The rate constant of

reactivation (k_5) of the enzyme transiently inactivated by **3** was determined in reactivation experiments as described in the *Exper. Part*. Deacylation occurred over a considerably long period (several days), which allowed for recovery of no more than 25% of activity in 3 days ($k_5 \approx 2 \cdot 10^{-6}$ s^{-1}).

The software OPKINE [28][29] performs a numerical integration of the differential equations defined for a specific kinetics scheme and allows the above-described results for the pre-steady state to be mathematically fitted to the kinetics scheme of *Model 2a*. OPKINE not only allows calculation of kinetics constants, but also optimizes molar-absorption coefficients. The experimental coefficients obtained for the substrate and enzyme were $\varepsilon_C = 8700 \pm 200$ M^{-1} cm^{-1} and $\varepsilon_E = 18000 \pm 1000$ M^{-1} cm^{-1}, respectively. To decrease the number of variables to be optimized, the following approximations were used: $\varepsilon_{EC} = \varepsilon_E + \varepsilon_C$ and $\varepsilon_{EC^*} = \varepsilon_{EC^{**}} = \varepsilon_E + \varepsilon_P$. *Table 2* shows the results for the different microscopic constants as calculated for the proposed model. The theoretical curve obtained by fitting the experimental results to the kinetics equations of *Model 2a* and by using the kinetics constants of *Table 2* is shown in *Fig. 1*.

Table 2. *Kinetics Parameters for the Interaction between Imipenem* (**3**) *and Different Class-A β-Lactamases*

	k_2 [s^{-1}]	K_S [μM]	k_3 [s^{-1}]	k_4 [s^{-1}]	k_5 [s^{-1}]
β-Lactamase I[a])	1.5–5.0	$(9–30) \cdot 10^3$	0.27–0.4	$(1.6–3.3) \cdot 10^{-3}$	–
TEM-1[b])	$5 \cdot 10^{-3}$	3.5 ± 0.1	–	–	$3.3 \cdot 10^{-3}$
PC1	$(1.6 \pm 0.2) \cdot 10^{-2}$	215 ± 35	$(6.4 \pm 2) \cdot 10^{-3}$	$(4.3 \pm 0.6) \cdot 10^{-3}$	$(4.8 \pm 0.4) \cdot 10^{-6}$

[a]) Taken from [11]. Reaction conditions: 30°, pH 7.0. [b]) Taken from [21]. Reaction conditions: pH 7.0, room temperature.

The A/B ratio $[k_2k_4/K'(k_3 + k_4)]$ obtained from the microscopic constants, 25 ± 15, was similar to that determined by the reporter-substrate method.

As shown above, the experimental results are consistent with the branched model, which assumes the presence of two acyl-enzyme complexes with a different hydrolysis constant. By MM and MD calculations, the structures of EC, EC*, and EC** were modelled to study the inhibitory process at the molecular level by examining the amino acids involved in each of the processes of *Model 2a*.

Henri-Michaelis *Complex*. The structure of the active site of β-lactamase from *S. aureus* was minimized, and docking of imipenem (**3**) into it was analysed in detail. *Fig. 2* illustrates potential interactions in the minimized structure of the *Henri-Michaelis* complex by **3** docked into the *S. aureus* active site. *Table 3* gives the distances between heteroatoms of **3** and the major residues in the minimized *S. aureus* PC1-β-lactam complex, as well as the corresponding values for a very good substrate (benzylpenicillin (**2**)) and a β-lactamase-stable substrate (cefmetazole (**4**)).

As previously found with other β-lactamase substrates [26][31][40], the carboxylate function of imipenem (**3**) forms H-bonds with the side-chain functions of Ser-130, Ser-235, and Arg-244 upon active-site anchoring of the substrate. Lys-234 is one other important active-site residue involved in both ground-state and transition-state binding [40]. As can be seen from *Table 3*, the carboxy group is strongly bound to Arg-244($N^{\eta 1}$) and to the OH group of the Ser-235 side-chain in the three β-lactams compounds compared. The essential difference between **3** and the other substrates lies in the

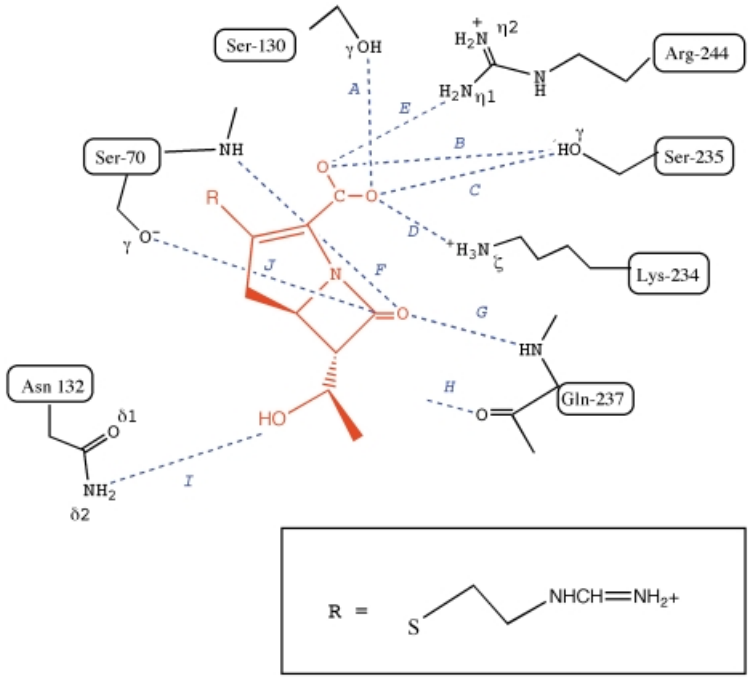


Fig. 2. *Diagram defining the distances* (see *Table 3*) *in the* Henri-Michaelis *complex between imipenem* (**3**) *and* Staphylococcus aureus *PC1 enzyme*. For clarity, H_2O molecules are omitted.

Table 3. *Calculated Intermolecular Distances* [Å] *between Heteroatoms in the Minimized* Staphylococcus aureus *PC1 β-Lactam* Henri-Michaelis *Complexes*. Arbitrary numbering.

Distance	Imipenem (**3**)[a])	Benzylpenicillin (**2**)[a,b])	Cefmetazole (**4**)[b])
A: Ser-130(O^{γ})···O(10)	2.66 (2.67)	3.29 (2.69)	3.64
B: Ser-235(O^{γ})···O(11)	2.69 (3.27)	2.66 (2.86)	2.74
C: Ser-235(O^{γ})···O(10)	3.43	3.34	3.10
D: Lys-234(N^{ζ})···O(10)	3.35	3.23	2.74
E: Arg-244($N^{\eta 1}$)···O(11)	2.67 (2.61)	2.67 (2.70)	2.68
F: Ser-70(NH)···O(8)	3.42 (3.64)	3.02 (3.18)	4.33
G: Gln-237(NH)···O(8)	2.81 (2.81)	2.73 (2.83)	2.80
H: Gln-237(O)···N(12)	–	2.89	3.03
I: Asn-132($N^{\delta 2}$)···O(14)	2.78	3.80	4.94
J: Ser-70(O^{γ})···C(7)	3.57	3.02	3.24

[a]) The values in parentheses refer to the intermolecular distances determined by *Raquet et al.* [23] in the minimized TEM-1 β-lactam complexes. Note that the TEM-1 β-lactamase has an Ala at the position 237. [b]) Taken from [26].

interaction between the carboxy group and the OH group of the Ser-130 sidechain, which is very strong in **3**, and also in distance *D*, according to which **3** is the substrate establishing the weakest interaction with Lys-234(N^{ζ}).

Other major interactions in the *Henri-Michaelis* complex worth noting are those related to the carbonyl group in the β-lactam ring, *i.e.*, distances *F*, *G*, and *J* in *Fig. 2*.

While distance *G* is virtually identical in the three substrates, distance *F* of imipenem (**3**) suggests that the interaction between Ser-70(NH) and O(8) is weaker than in benzylpenicillin (**2**), but stronger than in cefmetazole (**4**).

The results of the modelling analysis of **3** suggest a strong interaction of the atom O(4) of the OH group at the side chain at C(6), with both Asn-132($N^{\delta 2}$) and a H_2O molecule (Wat81). These two H-bonds were previously found by other authors in the complex formed by **3** and the TEM-1 β-lactamase [23]. For these two interactions to be feasible, the side chain at C(6) of **3** must previously rotate to allow for H-bonding. A strong interaction (2.81 Å) was also observed between the imino group of the side chain at C(2) of **3** and Asp-276($O^{\delta 1}$).

Table 3 shows some of the interaction distances for the minimum-energy structure calculated by *Raquet et al.* [23] for the *Henri-Michaelis* complexes of benzylpenicillin (**2**) and imipenem (**3**) with the enzyme TEM-1. From those distances, it follows that the interaction between O(8) of **2** or **3** and the Ser-70(NH) and Ala-237(NH) residues of TEM-1 is virtually identical with that of the two substrates with the enzyme PC1 (in PC1, the 237 residue is Gln); on the other hand, the interactions involving the carboxylate group in the β-lactam differ between the two enzymes. Distance *E* is identical in the different complexes and suggests the formation of a strong H-bond; on the other hand, distances *A* and *B* depend on the particular substrate and enzyme. Thus, in the complex formed by **3** and PC1, the *A* and *B* values obtained indicate the formation of two strong H-bonds, whereas those for the structure formed with TEM-1 suggest the presence of a strong bond and a weak one. Penicillin **2** exhibits the opposite results: it forms two strong H-bonds with TEM-1, but a strong bond and a weak one with PC1.

Mechanism of Active-Site Acylation. The mechanism for the acylation step in class-A β-lactamases is somewhat controversial since such residues as Lys-73 [41], Ser-130 [40], and Glu-166 [42–46] have been assigned a general acid-base role for activation of the Ser-70 residue. Glu-166 appears to be the most likely residue of the three proposed by different authors [44–46]. Based on the proposed mechanism, k_2 might be related to H-bonding parameters required for Ser-70(OH) to be activated by the Glu-166 residue (as suggested by *Matagne et al.* [47]) and to the distance between the C-atom of the carbonyl group of the β-lactam ring and the Ser-70 group (*i.e.* with distance *J*).

The experimental k_2 value for **3** was four orders of magnitude smaller than the calculated value for benzylpenicillin (**2**) and roughly of the same order as those for cefmetazole (**4**) and other cephamycins [26]. *Fig. 3* illustrates the parameters involved in the enzyme acylation mechanism and *Table 4* gives their values. As can be seen, the H-bonding distances for **3** are similar to those for **2**. However, the Ser-70 residue is rather distant from the C-atom of the carbonyl group (distance *J* for **3** is 0.6 Å greater than for **2**), consistent with the decreased k_2 value for **3** relative to a good substrate.

Table 4 also shows the parameter values for cefmetazole (**4**). In this case, the H_2O molecule (Wat81) forms no H-bond with the Ser-70(O^{γ}) residue, so the activation process must be less favorable than in benzylpenicillin (**2**). As shown elsewhere [26][36], k_2 is smaller for cephamycins than for benzylpenicillin (**2**).

Deacylation of the Acyl-Enzyme Intermediate. The step following the formation of the acyl-enzyme structure in the enzymatic mechanism is its deacylation (*Model 1*, see *Scheme 1*), which regenerates the enzyme. The crystal structure of the acyl-enzyme

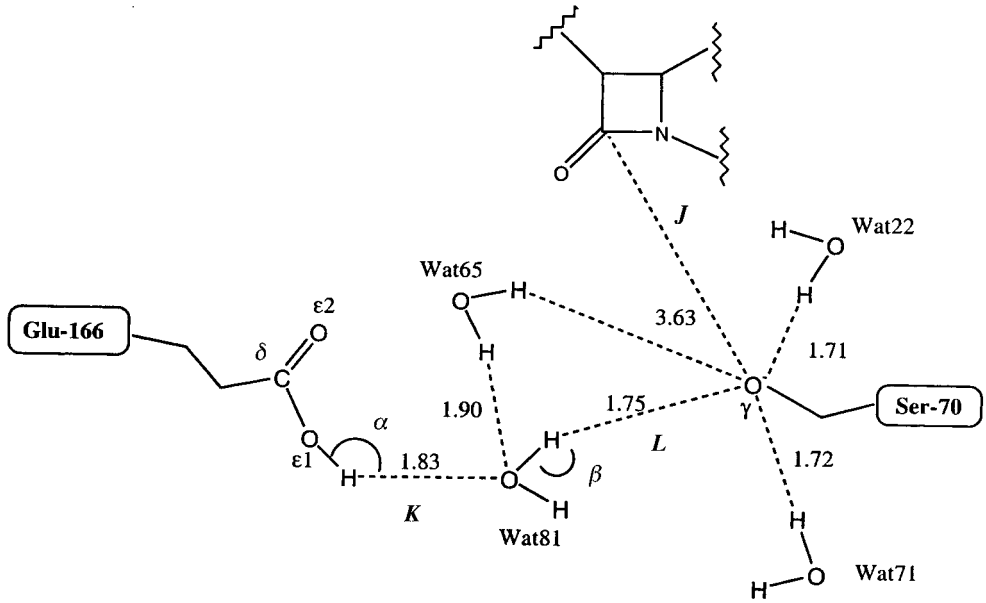


Fig. 3. *Diagram showing the possible H-bonds in the active site of PC1 during the activation of Ser-70(O$^{\gamma}$) by the carboxylate group of Glu-166*. Some of the most important H-bonds are shown as dashed lines (– –). Values in the free enzyme are included. H-Bonding parameters described in *Table 4* (distances *K* and *L*, and angles α and β) for the *Henri-Michaelis* complex are represented in the diagram.

Table 4. *Hydrogen-Bonding Parameters between Ser-70(O$^{\gamma}$), Wat81, and Glu-166 (O$^{\varepsilon 1}$H)*

	Hydrogen-bonding parameters			
	K [Å]	*L* [Å]	α [°]	β [°]
Imipenem (**3**)	1.94	1.68	165.7	168.3
Benzylpenicillin (**2**)[a])	1.85	1.71	161.0	159.0
Cefmetazole (**4**)[a])	1.78	3.61	167.2	96.3
Free enzyme	1.83	1.75	173.3	174.9

[a]) Taken from [26].

formed between TEM-1 and 6α-(hydroxymethyl)penicillanic acid (**5**) was recently reported [48].

The kinetics mechanism for some carbapenems has been found to involve the formation of a second acyl-enzyme intermediate and the transient inhibition of the enzyme. *Knowles* and co-workers [49][50] suggested that the second acyl-enzyme arises from tautomerization of the double bond in the five-membered ring of the β-lactam. However, the crystal structure obtained by *Maveyraud et al.* [22] for the acyl-enzyme intermediate formed between imipenem (**3**) and TEM-1 suggests that the five-membered ring has the double bond between C(2) and C(3) and that the formation of the second acyl-enzyme is due to a conformational change. The ester group formed in the first acyl-enzyme, which lies in the oxy-anion hole, exhibits two strong interactions

with the Ser-70(NH) and Ala-237(NH) residues (acyl-enzyme **I**, Ala-237 instead of Gln-237). These H-bonds can be broken and allow the ester group to rotate and leave the oxy-anion hole to form acyl-enzyme **II**, which is more resistant to deacylation. Molecular-dynamics computations suggest that this process takes place on the nanosecond scale, whereas the catalytic process occurs on the millisecond scale [22].

To derive information about the process by which imipenem (**3**) inhibits the enzyme PC1, the minimum-energy structures for the two acyl-enzymes were examined, by means of the experimentally determined structures for the complexes between **3** and TEM-1 [22] on the one hand, and between 6α-(hydroxymethyl)penicillanic acid (**5**) and TEM-1 [48] on the other, as models. To this end, the substrate was positioned at the active site, the two acyl-enzymes were manually constructed, and the two were subjected to molecular-dynamics simulation. The most stable among the calculated minimum-energy structures were chosen.

Table 5 shows the distances between heteroatoms in the two acyl-enzyme intermediates **I** and **II**. The values for acyl-enzyme **I** suggest that this intermediate possesses roughly the same interaction distances as those experimentally determined for crystal structure *1* of the complex of 6α-(hydroxymethyl)penicillanic acid (**5**) with TEM-1. The *F* and *G* distances obtained suggest the formation of two strong H-bonds between the ester group and the Ser-70(NH) and Gln-237(NH) residues.

On the other hand, the ester group in the acyl-enzyme **II** forms no H-bonds with the Ser-70(NH) and Gln-237(NH) residues, which is consistent with the experimental results for the crystal structure *2* of the complex between imipenem (**3**) and TEM-1 (see *Table 5*). Of the other distances shown in *Table 5*, *I* is the only one that differs appreciably from its crystallographic counterpart; the remaining ones are consistent. However, one must bear in mind that the two enzymes compared differ in some structural features even though they belong to the same class.

Table 5. *Calculated Intermolecular Distances* [Å] *between Heteroatoms in the Minimized* Staphylococcus aureus *PC1 Imipenem* (**3**) *Acyl-Enzymes.* Arbitrary numbering.

Distance	Acyl-enzyme **I**	Crystal structure *1*[a])[c])	Acyl-enzyme **II**	Crystal structure *2*[b])[c])	Benzylpenicillin (**2**) acyl-enzyme
A: Ser-130(O^{γ})···O(10)	4.62	4.86	4.91	5.21	3.32
B: Ser-235(O^{γ})···O(11)	2.66	2.67	2.65	3.12	2.65
C: Ser-235(O^{γ})···O(10)	3.59	4.16	4.00	4.71	3.42
D: Lys-234(N^{ζ})···O(10)	4.66	4.64	4.59	6.44	3.03
E: Arg-244($N^{\eta 1}$)···O(11)	2.66	2.72	2.72	2.61	2.71
F: Ser-70(NH)···O(8)	2.71	2.82	3.71	4.71	2.72
G: Gln-237(NH)···O(8)	2.71	2.80	5.10	5.36	2.70
H: Gln-237(O)···N(12)	–	–	–		2.04
I: Asn-132($N^{\delta 2}$)···O(14)	3.05	3.09	4.65	2.70	3.79

[a]) Complex of **5** and TEM-1; values taken from [48]. [b]) Complex of **3** and TEM-1; values taken from [22]. [c]) In the crystal structures *1* and *2*, the residue at 237 of the enzyme TEM-1 is Ala. The mean MD H(N-Ser-70)··· O(8) and H(N-Gln-237)···O(8) distances obtained were 1.88 and 1.91 Å, respectively, for acyl-enzyme **I**, and 3.97 and 4.17 Å for acyl-enzyme **II**. These values should be compared to 1.75 and 1.79 (*F* and *G* distances between the H-atom and the O-atom for acyl-enzyme **I**) and to 3.45 and 4.13 (for acyl-enzyme **II**). Comparison of these values ensure that the conclusions drawn by examination of the most stable conformation are also valid when the whole trajectories are taken into account.

Table 5 also shows the distances between the more significant groups in the minimum-energy structure of the acyl-enzyme intermediately formed by benzylpenicillin (**2**) and PC1. The *F* and *G* values obtained for benzylpenicillin (**2**) suggest that this structure is similar to that obtained for acyl-enzyme **I**.

There is wide consensus that the carboxylate group in Glu-166 is the general-base catalyst for the deacylation step in the catalytic mechanism for class-A β-lactamases, where an occluded H_2O molecule next to the carboxylate group in the crystal structure is believed to act as the nucleophile [25][40][51][52]. Based on this mechanism, where a H_2O molecule would act as the nucleophile, the deacylation constant should be related to H-bonding parameters (see *Fig. 4*, where the two H_2O molecules lying closest to the carbonyl group in the acyl-enzyme intermediate, Wat65 and Wat81, are considered). *Table 6* shows the corresponding parameter values, according to which both Wat81 and Wat65 are activated by H-bonding with Glu-166 (distance *N* and *R*,

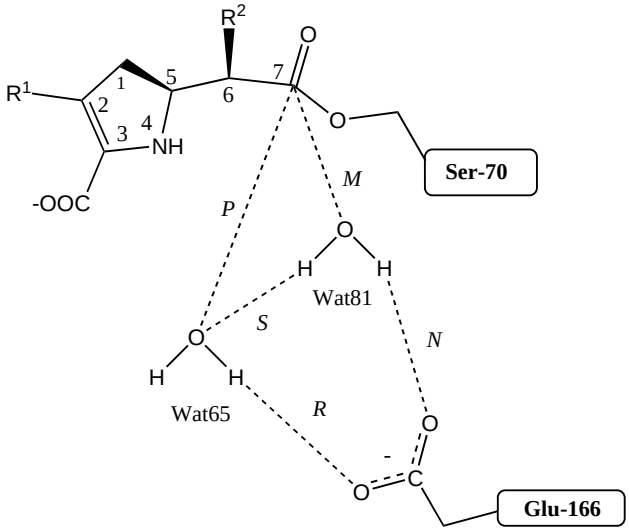


Fig. 4. *Diagram showing the possible H-bonds in the active site of PC1 during the activation of Wat65 and Wat81 by the carboxylate group of Glu-166 in the deacylation of the acyl-enzyme intermediate.* Some of the most important H-bonds are shown as dashed lines (– –). H-Bonding parameters described in *Table 6* (distances *M*, *N*, *P*, *R*, and *S*, and dihedral angles) for the acyl-enzyme intermediate are represented in the diagram.

Table 6. *Hydrogen-Bonding Parameters between C(7), Wat81, Wat65, and Glu-166, and Dihedral Angles in the Acyl-enzymes*

	Distances [Å]					Dihedral angle [°]	
	M	*N*	*P*	*R*	*S*	C(6)–C(7)–Ser-70 (O^γ)–O(Wat81)	C(6)–C(7)–[Ser-70 (O^γ)]–O(Wat65)
Acyl-enzyme **I**	3.52	3.31	5.80	2.56	1.88	– 94.6	– 75.2
Acyl-enzyme **II**	3.70	2.67	3.82	2.78	1.82	146.7	138.3
Acyl-enzyme with Benzylpenicillin (**2**)	3.60	2.75	4.06	2.49	1.82	– 88.6	– 48.3

resp.). However, distance *M* is much shorter than distance *P* in acyl-enzyme **I**, which suggests that Wat81 is the molecule acting as nucleophile in the last step of the enzymatic deacylation process.

The deacylation rate of the first acyl-enzyme intermediate has been experimentally found to be three orders of magnitude greater than that of the second. Such a large difference cannot be solely explained by differences in H-bond distances, as these differ little between the acyl-enzymes. However, careful examination of the orientation of the H_2O molecule, *i.e.*, of the dihedral angle formed by C(6)–C(7)–Ser-70(O^{γ})–O(Wat), reveals that the orientation of the Wat81 molecule in the complex between benzylpenicillin (**2**) and the acyl-enzyme – the most favorable – is *ca.* 90 degrees; whilst this orientation is unfavorable in acyl-enzyme **II**, so k_3 must be smaller that k_5, as indeed shown by the experimental results.

In conclusion, the results obtained in this work suggest that the enzyme PC1 reacts with imipenem (**3**) *via* a branched pathway that involves the formation of a second acyl-enzyme intermediate **II** (EC**). Such an intermediate is more resistant to deacylation than the first (acyl-enzyme **I** (EC*)), which results in the transient deactivation of the enzyme. The stabilizing effect arises from an unfavorable orientation of the H_2O molecule that effects the hydrolysis of the acyl-enzyme intermediate. The results obtained for PC1 and those reported for two other class-A β-lactamases [11][21][22] are consistent with a rearrangement in the acyl-enzyme EC* to structure EC** being the origin of the inhibitory action of imipenem (**3**) on these enzymes (see *Scheme 2*).

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