Kinetic and Molecular-Modelling Studies of Reactions of a Class-A β-Lactamase with Compounds Bearing a Methoxy Group on the β-Lactam Ring

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

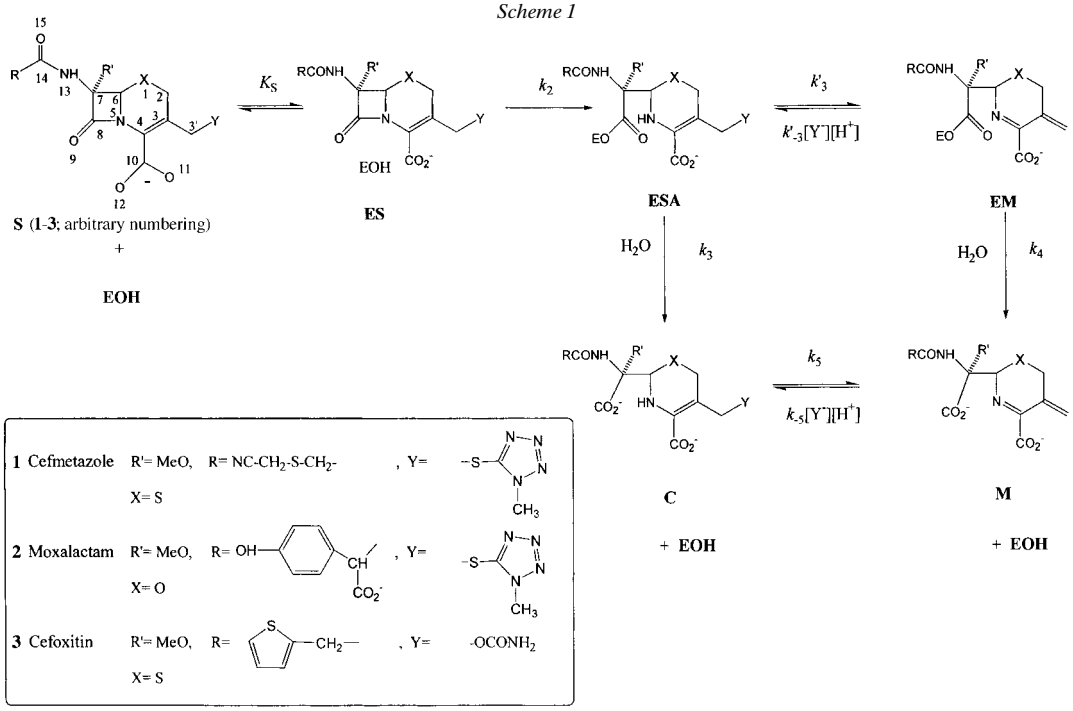
Departament de Química. Universitat de les Illes Balears, E-07071 Palma de Mallorca, Spain
(Phone: + 34-971-173254, Fax: + 34-971-173426, e-mail: dqubvc4@ps.uib.es)

The interactions between *Staphylococcus aureus* PC1 enzyme and compounds bearing a methoxy group on the α-face of the β-lactam ring (cefmetazole (**1**), moxalactam (**2**) and cefoxitin (**3**)) were studied. With these compounds, a partitioning of the acyl-enzyme between deacylation and a transiently inactivated form of the enzyme were observed. The individual microscopic rate constants were determined, indicating for **1** and **3** that $k'_3 > k_3$ (*Scheme 1*), whereas for **2** $k'_3 \approx k_3$. This different behavior could be attributed to the presence of the α-carboxy group at the C(7) side chain of **2**, which is able to act as a general-base catalyst in the deacylation step. Molecular-modelling studies allowed correlation of K_S and k_2 with the structures of the *Henri-Michaelis* complexes that these compounds formed with the *S. aureus* enzyme. The acylation rate constant (k_2) for these 'β-lactamase-stable' compounds was lower than that observed with substrates lacking the methoxy group. Molecular-modelling studies indicated that the methoxy group increases the displacement of the crystallographically observed water molecule (Wat81), which is involved in the acylation mechanism. On the other hand, an average of the most important interactions in the *Henri-Michaelis* complexes was related to K_S. An increase of 0.2–0.5 Å in this average value was found to result in an increase in K_S by about one order of magnitude.

Introduction. – β-Lactamases (EC 3.5.2.6) are a family of bacterial enzymes that inactivate β-lactam antibiotics by hydrolyzing the β-lactam amide bond typical of this group of compounds, giving rise to biologically inactive products [1][2]. The main division of β-lactamases is based on the chemistry of their catalytic mechanisms, and distinguishes serine and zinc enzymes; the former have an active-site serine residue and function by a covalent acyl-enzyme mechanism, whereas the latter are metalloenzymes and appear to involve only noncovalent intermediates. On the basis of their amino-acid sequences, the active-site serine β-lactamases are divided into three molecular classes A, C, and D [3][4]. All zinc β-lactamases are often lumped together as class-B enzymes. Of the serine β-lactamases, class-A enzymes are most common among pathogens. Detailed mechanistic understanding of these enzymes can be expected to guide the design of new inhibitors and antibacterial compounds resistant to their action. Several mechanisms have already been proposed for this family of enzymes on the basis of crystallographic, molecular-modelling, and mutagenesis studies [5][6].

An interesting aspect of catalysis by class-A β-lactamases is that they exhibit a phenomenon of reversible inactivation with certain substrates [7–9]. It is well established that serine β-lactamases and transpeptidases form inert acyl-enzymes (*EM* in *Scheme 1*, X = S) with cephems that possess good leaving groups at position 3′, and that its formation leads to transient inhibition of the enzyme. These inert species arise by elimination of the leaving group Y from the initially formed and more rapidly hydrolysing acyl-enzyme (**ESA**, X = S), which has the normal cephalosporate structure [10–13].

Scheme 1



Cephamycins (**S** in *Scheme 1*, X = S and R′ = MeO; *e.g.* cefmetazole (**1**) and cefoxitin (**3**)) and other β-lactam antibiotics with a MeO group in the α-face of the β-lactam ring (*i.e.* moxalactam (**2**)) behave as very poor substrates of the active-site serine β-lactamases [14]. This resistance to hydrolysis by various bacterial β-lactamases has been correlated with the presence of the MeO group [11][15][16]. Hydrolysis of cefoxitin (**3**) proceeds through an acyl-enzyme intermediate with very low acylation and deacylation rate constants [17]. The enzyme is thus transiently inactivated as a rather stable acyl-enzyme. Direct observation by NMR spectroscopy of the stable acyl-enzyme indicated clear modifications of the enzyme structure [18]. *Faraci* and *Pratt* [19] showed that a strong nucleophile, thiophenoxide, causes the reactivation of this inert acyl-enzyme. This reactivation is proposed to arise through nucleophilic addition to the exocyclic methylene C-atom of the inert acyl-enzyme to regenerate a more rapidly hydrolysing cephalosporoate.

Cefmetazole (**1**) shows poor activity against *Pseudomonas aeruginosa*, *Streptococcus faecalis*, and the methicillin-resistant *Staphylococcus aureus*; however, it is two times more active than cefoxitin (**3**) [20]. Moxalactam (**2**) does not interact very efficiently with the TEM-1/2 and *Klebsiella pneumoniae* K1 enzymes [21][22] (class A) and is a potent inhibitor of several class-C β-lactamases from *E. coli*, *E. cloacae*, and *P. vulgaris*. Its inhibitory properties have been related to the carboxylate moiety at the C(7) side chain [14]. With class-C β-lactamases, biphasic kinetics were observed, indicating a possible rearrangement of the acyl-enzyme [23].

In the last five years, a number of theoretical studies have appeared that attempt to address some of the more specific details of the microscopic mechanism for the PC1 β-lactamase. *Vijayakumar et al.* [24] have confirmed by molecular dynamics that the switch of the H-bond network is important for the catalysis, and this switch is mainly due to the mobile conformation of the Lys-73 residue (throughout this paper, the consensus numbering scheme proposed by *Ambler et al.* [25] will be used). *Ishiguro* and *Imajo* [26] have reported the model building of the complex structures of the β-lactamase with penicillin G and discussed a hydrolytic mechanism of class-A β-lactamases through the modelling study. *Frau* and *Price* [27] have reported a study of the electrostatic forces within the active site of the β-lactamase PC1 to predict structures for the precatalytic complex with ampicillin, methicillin, clavulanate, and imipenem. The authors found significant differences in the orientation of those β-lactams within the binding site, which explains the differences in their resistance to the lactamase. *Wladkowski et al.* [28] have studied the initial acylation step in the β-lactamase-catalysed hydrolysis of β-lactams using an *ab initio* quantum-mechanical approach. However, none of these works have investigated the model building of the complex structures of the PC1 β-lactamase with cephamycins and/or moxalactam.

In the present study, we analyze the interactions of cefmetazole (**1**), moxalactam (**2**), and cefoxitin (**3**) with the *Staphylococcus aureus* PC1 enzyme (class-A β-lactamase). With these compounds, a partitioning of the acyl-enzyme between deacylation and the transiently inhibited form of the enzyme was observed. On the basis of the kinetic parameters, we propose a plausible explanation for the branched pathway, and with molecular-modelling studies we attempt to correlate the kinetic data (K_S and k_2) with the structures of the *Henri-Michaelis* complexes that these compounds form with the *S. aureus* enzyme, the structure of which is known at 2.0-Å resolution [29].

Experimental. *Materials.* The PC1 β-lactamase from *Staphylococcus aureus* was obtained from *Speywood*, U.K., and used as supplied. The specific activity of the enzyme against benzylpenicillin was 300 units/mg at pH 7.0 and at 30°. Cefmetazole (**1**), moxalactam (**2**), cefoxitin (**3**), and benzylpenicillin were purchased from *Sigma*. 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.

Reactivation of the PC1 β-Lactamase. The inactive acyl-enzyme of the PC1 β-lactamase was generated by reaction of the enzyme (6.4 μM) with **1** (80 μM). A portion of this soln. was then immediately diluted (1000-fold) into an assay mixture in a cuvette, containing the good substrate benzylpenicillin (2.3 mM). The initially measured activity was small but it increased with time, corresponding to hydrolysis of the inactive acyl-enzyme, and was monitored by the absorption changes at 240 nm. The rate constant for reactivation could then be calculated from progress curves by using *Eqn. 1* derived by *Monks* and *Waley* [30] where Ab_0 and Ab are the initial and measured absorbances, respectively, $\Delta\varepsilon$ is the increment in the absorption coefficient, V is the maximum velocity attained after all of the transiently inhibited enzyme has reactivated, B is V minus the activity at zero time, and k is the first-order rate constant for the conversion of the inhibited enzyme to active enzyme. Data were fitted to *Eqn. 1* by using the ENZFITTER program [31].

$$Ab = Ab_0 - \frac{\Delta\varepsilon}{\left(1 + \frac{K_m}{[S]_0}\right)}\{Vt - [B(1 - e^{-kt})/k]\} \quad (1)$$

Similarly, the acyl-enzyme was generated by reaction of the PC1 β-lactamase (4 μM) with **2** (2.6 mM) and **3** (1.4 mM). Again, the return of activity was monitored by measuring benzylpenicillin hydrolysis at 240 nm.

Steady-State Kinetics. Michaelis-Menten parameters were obtained from initial-velocity measurements of the hydrolysis of the various β-lactam compounds. With the substrates studied in this work, these velocities are, of course, those measured for the steady state subsequent to the burst described below. The reactions were followed spectrophotometrically at 270 nm for **1** ($\Delta\varepsilon = 8000\ \text{M}^{-1}\ \text{cm}^{-1}$) and for **2** ($\Delta\varepsilon = 9700\ \text{M}^{-1}\ \text{cm}^{-1}$), and at 265 nm for **3** ($\Delta\varepsilon = 6000\ \text{M}^{-1}\ \text{cm}^{-1}$). The concentration of PC1, β-lactamase was between 1 and 3 μM, while substrate concentrations were between 3 and 40 μM for **1**, between 60 and 200 μM for **2**, and between 9 and 200 μM for **3**.

Pre-steady-State Kinetics. The pre-steady-state phases of the reactions between the PC1 β-lactamase (2 μM) and **1** (8.1 and 28 μM), and between PC1 (18 μM) and **2** (0.42 and 0.83 mM) were studied spectrophotometrically at 270 nm. The resulting absorption changes were fitted to reaction schemes by non-linear least-squares procedures (see *Appendix*).

Measurement of the Extent of Inhibition of the Enzyme. The kinetics of formation of the PC1 β-lactamase transiently inhibited by cefmetazole (**1**) could be determined from the decrease in immediately active enzyme as a function of time. Thus, portions of a mixture containing the enzyme (1–2 μM) and various concentrations of **1** (45–600 μM), 90 s after mixing, were added to a standard benzylpenicillin assay mixture. The initial velocity of benzylpenicillin hydrolysis in each case was taken as a measure of the amount of immediately active enzyme remaining. Similar experiments were performed with PC1 and the substrates moxalactam (**2**; 0.4–3.0 mM) and cefoxitin (**3**; 0.1–0.8 mM).

Hydroxide-Catalyzed Hydrolysis. The pseudo-first-order hydroxide-ion-catalyzed hydrolysis of β-lactam compounds were followed spectrophotometrically in KOH soln. (pH 11–13, $I = 0.5$M with KCl) at 25°. Second-order rate constants were obtained from the pseudo-first-order rate constants.

Molecular Modelling. The three-dimensional structure of the *Staphylococcus aureus* PC1 enzyme [29] was obtained from the *Brookhaven Protein Data Bank* (refcode: 3BLM [32]). To calculate the pre-acylation complex, we followed the minimization protocol developed by *Juteau et al.* [33]. The polypeptide backbone and the O-atoms of the crystallographically observed H_2O molecules were initially strongly constrained (100 kcal/mol per Å^2) during the addition of H-atoms (step 1). In the second step, the H_2O molecules were then allowed to move, while the backbone was constrained (100 kcal/mol per Å^2) to preserve the tertiary structure. The protein backbone was then slightly constrained (20 kcal/mol per Å^2) to allow gradual relaxation of the crystallographic structure (step 3), and a continuum dielectric was used to simulate the solvation effects.

In the fourth step, the β-lactam structures, which had been previously optimized by the AM1 semiempirical method [34], were manually fitted into the active site to position the β-lactam carbonyl O-atom in the oxy-anion hole. The N(5) (N(4)) atom of the six-membered (five-membered) ring of the β-lactam occupied a nearby site for a H_2O molecule (Wat71). Thus, four H_2O molecules (Wat22, Wat64, Wat71, and Wat111) observed in the crystal structure were displaced by the β-lactam. This solvated enzyme-substrate complex was completely minimized with no constraints. The enzyme-substrate complexes were minimized to convergence using for the β-lactams the partial atomic charges determined from AM1 calculations [34]. These charges were fitted to the *e*lectrostatic *s*urface *p*otencial (ESP) obtained from an AM1 wavefunction. Several mechanisms for the acylation step have been proposed (see *Discussion*), but in all of them, the Ser-70 must be activated. For that reason, and in accord to the results obtained by *Juteau et al.* [33], the reactive Ser-70 was made anionic, as it would be just prior to nucleophilic attack on the β-lactam carbonyl C-atom, and the carboxylic-acid group of Glu-166 was protonated. The same authors [33] indicated that MOPAC charges did not include the induced charge polarization in the carbonyl bond of the β-lactam when it was positioned to accept the two H-bonds of the enzyme's oxy-anion hole. Therefore, to better model the pre-acylation complex, it was necessary to make some modifications to the atomic charges. Following this suggestion, the AM1 charges were arbitrarily changed to $+1.26$ and -1.00 for C- and O-atoms of the β-lactam carbonyl group. Obviously, these charges are not realistic, but their inclusion is necessary for the initial formation of the complex. For that reason, it has been necessary to include a final step (step 5), where the previous charges ($+1.26$ and -1.00) were modified to the AM1 values, and the complex was fully minimized again.

Calculations were performed on a *Silicon Graphics Origin 200 R10000* using the AMBER* force field [35][36] implemented in the Macromodel 6.0 package [37].

Results and Discussion. – *Table 1* gives the values of the steady-state parameters for the hydrolysis of the three substrates studied, in addition to those for benzylpenicillin and cephaloridine for comparison. Cefmetazole (**1**) and cefoxitin (**3**) gradually inactivate the PC1 enzyme, as does moxalactam (**2**) at higher concentrations

Table 1. *Kinetic Parameters for the Interactions between PC1 β-Lactamase and Different Substrates*

	k_{cat} [s^{-1}]	K_m [μM]	k_{cat}/K_m [$M^{-1}s^{-1}$]	k_2 [s^{-1}]	K_S [μM]	k_2/K_S [$M^{-1}s^{-1}$]	k_4 [s^{-1}]	k_{OH} [$M^{-1}s^{-1}$]
Cefmetazole (**1**)	$(4.6\pm1.0)\cdot10^{-4}$	3 ± 2	155 ± 100	$(3.5-4.6)\cdot10^{-2}$	50–100	530 ± 100	$(3.6\pm0.4)\cdot10^{-4}$	$2\cdot10^{-2}$
Moxalactam (**2**)	$(1.4\pm0.2)\cdot10^{-3}$	325 ± 70	4.6 ± 1.2	$(4.0-5.0)\cdot10^{-2}$	7000–11000	5 ± 1	$(4.8\pm0.7)\cdot10^{-4}$	$12\cdot10^{-2}$
Cefoxitin (**3**)	$(4.0\pm0.7)\cdot10^{-4}$	10 ± 2	40 ± 15	$(6.0-8.0)\cdot10^{-2}$	1000–1400	55 ± 10	$(2.8\pm0.7)\cdot10^{-4}$	$4\cdot10^{-2}$
Benzylpenicillin	62 ± 3 [a])	6 ± 1 [a])	$(10\pm1)\cdot10^{6}$ [a])	173 ± 10 [a])	9 ± 2 [a])	$(20\pm6)\cdot10^{6}$ [a])	–	$15\cdot10^{-2}$ [b])
Cephaloridine	$(8\pm1)\cdot10^{-3}$ [c])	<1 [c])	>8000 [c])	–	–	–	–	$53\cdot10^{-2}$ [b])

[a]) Taken from [38]. Reaction conditions: pH 7.0, 20°. [b]) Taken from [39]. Reaction conditions: 30°, $I=0.5$M. [c]) Taken from [10]. Reaction conditions: pH 7.5, 20°.

(*ca.* 1 mM). The residual activity decrease for the enzyme conforms to a first-order kinetics.

Scheme 1 illustrates the general model of *Faraci* and *Pratt* [19] as applied to the interactions between active-site serine β-lactamases and cephems with a leaving group at position 3′. On incubation, the amount of residual β-lactamase activity (A) at any time t_c after mixing will be given, according to *Scheme 1*, by *Eqn. 2* where A_0 is the initial enzyme activity, and $[S]_0$ (the initial substrate concentration) is greater than $[E]_0$. As can be seen in *Fig. 1*, a plot of $t_c/\ln(A/A_0)$ against $1/[S]_0$ was a straight line and provided the k_2 and K_S values given in *Table 1*.

$$A = A_0 \cdot \exp\{-k_2[S]_0 t_c/(K_S + [S]_0)\} \quad (2)$$

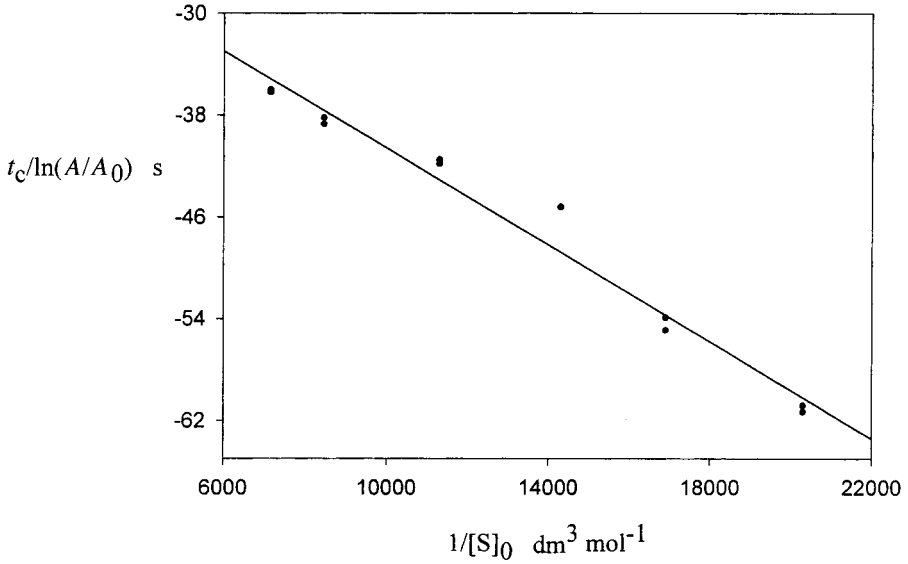


Fig. 1. *Determination of* k_2 *and* K_S *for cefmetazole* (**1**) *and the* S. aureus *PC1 enzyme.* For exper. conditions, see text.

Cefmetazole (**1**), moxalactam (**2**), and cefoxitin (**3**) are poor substrates for PC1 β-lactamase; in fact, their k_{cat} values are extremely low ($<2\cdot10^{-3}$ s^{-1}) relative to those for a good substrate such as benzylpenicillin. The K_m values for these compounds are

rather different from each other – that for **2** is the largest; also, the k_{cat}/K_m ratios are much lower than those for benzylpenicillin and cephaloridine. We also determined the dissociation constant for the enzyme-substrate complex, K_S, which is inversely proportional to the enzyme's affinity for the substrate. Penicillinase exhibits a high affinity for **1** but a low affinity (*viz*, a large K_S value) for the other two substrates. It is worth noting that the K_S values for **1** and **2** are roughly 17 times lower and 7 times higher, respectively, than that for **3**. We examined these differences with the aid of modelling techniques.

The structure of the active site of β-lactamase from *S. aureus* was minimized and docking of the substrates into it was analyzed in detail. *Fig. 2* shows potential

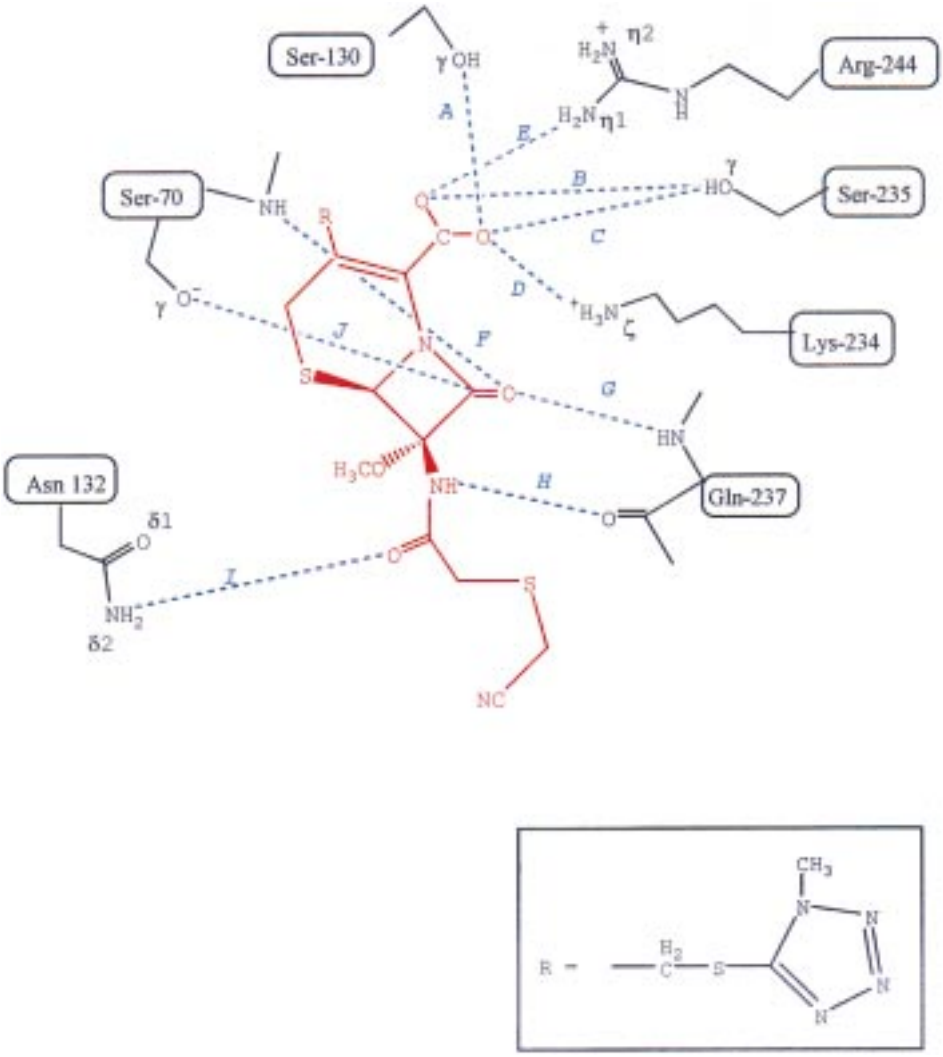


Fig. 2. *Diagram defining the distances* (see *Table 2*) *in the* Henri-Michaelis *complex between cefmetazole* (**1**) *and* Staphylococcus aureus *PC1 enzyme*. For clarity, H_2O molecules are omitted.

Table 2. *Calculated Intermolecular Distances* [Å] *between Heteroatoms in the Minimized* Staphylococcus aureus *PC1 β-Lactam Complexes.* Arbitrary numbering for β-lactams.

Distance	Benzylpenicillin[a])	Cephalothin	Cefmetazole (**1**)	Moxalactam (**2**)	Cefoxitin (**3**)
A: Ser-130 (O^{γ})···O(11)	3.29 (2.76)	3.52	3.64	3.77	4.58
B: Ser-235(O^{γ})···O(12)	2.66 (2.74)	2.71	2.74	5.00	4.45
C: Ser-235(O^{γ})···O(11)	3.34 (3.72)	3.21	3.10	4.87	3.22
D: Lys-234(N^{ζ})···O(11)	3.23 (3.36)	2.80	2.74	4.24	4.43
E: Arg-244($N^{\eta 1}$)···O(12)	2.67 (2.63)	2.70	2.68	3.54	2.69
F: Ser-70(NH)···O(9)	3.02 (2.80)	4.23	4.33	4.64	4.39
G: Gln-237(NH)···O(9)	2.73 (2.80)	3.09	2.80	2.79	2.82
H: Gln-237(O)···N(13)	2.89	3.80	3.03	3.12	4.13
I: Asn-132($N^{\delta 2}$)···O(15)	3.80	4.94	4.94	4.94	4.20
J: Ser-70(O^{γ})···C(8)	3.02 (3.09)	3.29	3.24	3.65	3.48
$\bar{d}_{A-GJ}$	3.00	3.19	3.16	4.06	3.76

[a]) The values in parentheses refer to the intermolecular distances determined by *Ishiguro* and *Imajo* [26].

interactions in the minimized structure of the *Henri*[1])-*Michaelis* complex by cefmetazole (**1**) docked into the *S. aureus* enzyme active site. *Table 2* gives the distances between heteroatoms in the substrates **1**–**3** and the major residues in the minimized *S. aureus* PC1 β-lactam complexes, as well as the values for a very good substrate (benzylpenicillin) and a fair one (cephalothin).

Binding of the substrate to the active site has been hypothesized to involve H-bonding interactions between carboxylate O-atoms in the substrate and the side chains in Ser-130, Ser-235, and Arg-244 [33][40]. Lys-234 is one other important active-site residue involved in both ground-state and transition-state binding; in fact, it forms an ion-pair with the carboxylate moiety on the ring to avoid buried unbalanced charges [40]. As can be seen from *Table 2*, the OH group on the Ser-130 side chain interacts closely with the carboxy group in each β-lactam (*A* distance); the interaction, however, is less strong in cefoxitin (**3**). Except in **3** and moxalactam (**2**), the carboxy group binds strongly to Ser-235 (O^{γ}); also, all substrates except **2** bind strongly to Arg-244 ($N^{\eta 1-}$). *Zafarralla et al.* [41] reported a moderate interaction (3.2 Å) between the Arg-244 side chain in a class-A β-lactamase (RTEM-1) and the carboxy group in penicillin G, in both the ground and in the transition states of the enzyme-substrate complex. Such a distance is slightly shorter in the *Henri-Michaelis* complexes formed with PC1 β-lactamase (*E* distance 2.7 Å) and is consistent with the value reported by *Ishiguro* and *Imajo* [26]. All these values are quite consistent with each other; the slight differences among them can be ascribed to the differential orientation of the carboxy group at the active sites of the two enzymes (PC1 and RTEM-1).

Additional major interactions in the *Henri-Michaelis* complexes involve the O-atom of the β-lactam carbonyl group and Ser-70 (O^{γ}) (*J* distance) and between the O-atom of the β-lactam carbonyl group and the amino group of Ser-70 and Gln-237 (*F* and

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[1]) *Victor Henri*, the scientist for whom this complex is named, was a pioneer in the field of enzymology. *Henri*'s 1902 article in the *Comptes Rendus de l'Académie des Sciences*, entitled '*Théorie générale de l'action de quelques diastases*' applied the equilibrium approximation to the *Michaelis-Menten* mechanism for the first time. The more famous paper of *Michaelis* and *Menten* was published eleven years later.

G distances, resp.). The *F* distance for benzylpenicillin (a good substrate) is shorter than that obtained for cephalothin (a cephalosporin) or cephamycins; this suggests that the four-membered ring in the *Henri-Michaelis* complex of the penicillin is close to the Ser-70 residue and thus well accommodated. The same conclusion can be drawn for the *J* distance, except that the differences between substrates are smaller. This is quite logical as it involves the same residue. On the other hand, the *G* distance is virtually the same in all complexes, so the residue Gln-237 must be flexible enough to interact strongly with the O(9) atom in all.

Table 2 also gives the distances of the β-lactam acylamide side chain to the backbone carbonyl moiety of residue Gln-237 and to Asn-132 ($N^{\delta 2}$) (*H* and *I* distances). None of the β-lactams binds strongly to the Asn-132 residue (the *I* distance exceeds 3.8 Å in every case, except for benzylpenicillin); on the other hand, all bind moderately strongly to Gln-237(CO) (*H* distance, except **3**). The *H* and *I* distances in the cephamycins are possibly mutually dependent – a large value in one distance suggests a small value in the other. This dependence might be the result of rotation about the side chain at C(7).

The free enzyme exhibits a strong interaction between Ser-130(O^{γ}) and Lys-234(N^{ζ}), which plays a structural role in the enzyme. It should be noted that this distance remained constant at 2.8 Å in every *Henri-Michaelis* complex studied.

The modelling analysis of cefmetazole (**1**) suggests an interaction (3.83 Å) between Asn-170($N^{\delta 2}$) and the CN group at the C(7) side chain. This interaction allows the substrate to accommodate itself readily in the enzyme. The end of the C(7) side chain in cefoxitin (**3**) exhibits no interaction at the active site, which cannot accommodate it so easily. As a result, **3** exhibits a high K_S value. As shown below, moxalactam (**2**) interacts strongly with some amino acids at the active site, which prevents it from adopting an appropriate orientation at the active site and raises its K_S.

According to *Murakami* and *Yoshida* [42], compounds with an α-carboxy group at the C(7) side chain exhibit poor affinity for penicillinases and the *P. vulgaris* enzyme (cephalosporinase); this suggests that the negatively charged α-carboxy substituent cannot interact with an amino group or positive charge at the active site of the enzyme, and hence that the carboxy group cannot stabilize the substrate against these enzymes. The results of our molecular-modelling computations for moxalactam (**2**) suggest that the α-carboxy group causes rotation and displacement of the Ile-239 chain (Ile-239 is shifted by 1.7 Å relative to its initial position in the free enzyme, thereby also shifting the neighboring Gln-237 and Ser-235 residues by *ca.* 1 Å). The phenol group in Tyr-171 also exhibits a strong interaction with that of the C(7) side chain in **2** (2.84 Å). Such an interaction results in a slight displacement of the Tyr-171 residue and hence of the neighboring Glu-166 residue (see *Table 3* and *Fig. 3*). It should be noted that the Ser-70 (*Table 3*), Lys-73, and Lys-234 residues (the latter two in *Fig. 3*) are scarcely shifted from their initial positions by effect of the presence of the substrate. These results suggest that the bulky side chain at C(7) can fit in the binding site only provided the enzyme undergoes significant conformational changes. The large K_S values obtained can thus be ascribed to a grossly distorted binding site.

Recently, *Ishiguro* and *Imajo* [26] reported the structure of the complex formed between the PC1 enzyme and penicillin G (*Table 2*). The slight differences between their results and ours for penicillin G may be a consequence of differences in the

Table 3. *Disposition of the Water Molecule, Ser-70, and Glu-166 in the Complexes; Hydrogen-Bonding Parameters between Ser-70*(O^{γ}), Wat81, and Glu-166 ($O^{\varepsilon 1}H$)

	Relative displacement [Å] of					Hydrogen-bonding parameters			
	Wat81	Ser-70(O^{γ})	Glu-166(C^{δ})	Glu-166($O^{\varepsilon 1}$)	Glu-166($O^{\varepsilon 2}$)	*K* [Å]	*L* [Å]	α [°]	β [°]
Benzylpenicillin	1.58	0.33	1.01	1.06	1.38	1.85	1.71	161.0	159.0
Cephalotin	1.75	0.52	1.22	1.22	1.39	1.87	1.74	167.2	161.8
Cefmetazole (**1**)	2.01	0.51	1.45	1.39	1.62	1.78	3.61	167.2	96.3
Moxalactam (**2**)	2.23	0.44	1.62	1.67	1.71	1.78	3.48	167.6	97.3
Cefoxitin (**3**)	2.04	0.66	1.30	1.51	1.53	1.82	1.68	173.4	176.8
Free enzyme	0.0	0.0	0.0	0.0	0.0	1.83	1.75	173.3	174.9

conditions used to build the *Henri-Michaelis*-complex model. In fact, these authors used version 3 of AMBER and a 'Monte-Carlo' water bath in the minimization protocol.

The distances for the *Henri-Michaelis* complexes formed between the studied compounds and the *S. aureus* enzyme (*Table 2*) can be classified into four different groups in terms of interactions according to whether they involve the carboxy group (*A* – *E*), the O-atom of the carbonyl group (*F* and *G*), the Ser-70(O^{γ}) group and C(8) (*J*), and the side chain at C(7) (*H* and *I*). The average theoretical distances for the *Henri-Michaelis* complex may be good indicators for the affinity of a substrate for the enzyme (K_S). However, one should bear in mind that the side chains in β-lactams exhibit not only *H* and *I* interactions, but also others that are specific to each substrate and hence difficult to assign an accurate weight. Consequently, the specific interactions of the C(7) side chain were excluded in computing the above-mentioned average distances. This does not mean that the C(7) side chain was ignored since, as shown above for moxalactam (**2**), its orientation at the active site directly influences that of

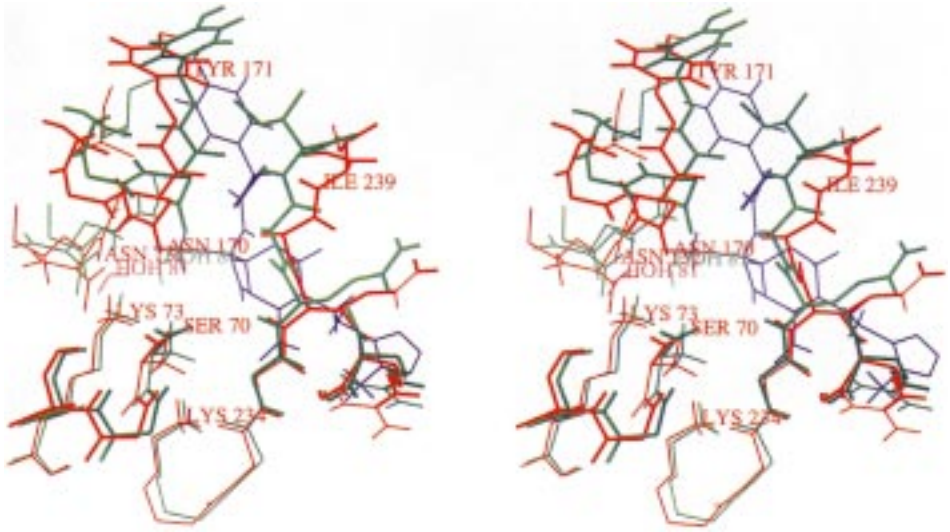

Fig. 3. *Stereoplot of the superposition of the active site of the free enzyme* (green) *and the* Henri-Michaelis *complex formed between moxalactam* (**2**; blue) *and the enzyme* (red). The crystallographic water molecule Wat81 is included.

the substrate and hence every enzyme-substrate interaction (*Fig. 3*). For this reason, we only used an average of the $A-G$ and J distances, ($\bar{d}_{A-G,J}$), which was related to K_S. An increase of 0.2–0.5 Å in $\bar{d}_{A-G,J}$ was found to result in an increase in K_S by about one order of magnitude. We intend to extend the modelling analysis to other β-lactam complexes in the future to confirm these results.

We conducted several pre-steady-state experiments to obtain individual microscopic constants. The burst observed in the reactions of 7α-methoxy substrates with PC1 β-lactamase reflect a branched pathway. *Fig. 4* shows typical data for cefmetazole (**1**) at a given concentration. The graph shows the time course of events preceding the steady state. Based on these results and on available knowledge of β-lactamase mechanisms [19][43][44], these data were analyzed in quantitative terms in the light of *Scheme 1*. The reversible rate constants k_{-5} and k'_{-3} were excluded because the leaving group Y at C(3′) of **1** and **2** is not a nucleophile at a neutral pH. In fact, 1-substituted 1*H*-tetrazole-5-thiols are reportedly in equilibrium with the tautomer dihydro-1*H*-tetrazolthione, the prevailing form [45][46]; also, the 1-methyl-1*H*-tetrazole-5-thiol has been shown not to react with *Ellman*'s reagent at a neutral pH. The value of k_5 for the spontaneous elimination from **C** in solution, (25.8 ± 5.0) s^{-1}, was obtained from a paper by *Faraci* and *Pratt* [12]. As can be seen in *Fig. 4*, the model of *Scheme 1* fitted data quite well. The mean rate constants are given in *Table 4*, which includes the values for PADAC for comparison.

Combined steady-state and pre-steady-state kinetic data for the reaction of PC1 β-lactamase with the substrates were analyzed in the light of the detailed kinetic scheme for branched paths reported by *Waley* [47]. The expressions for the steady-state parameters for the branched path of *Scheme 1* are given in *Table 5*. The k_{cat} and K_m

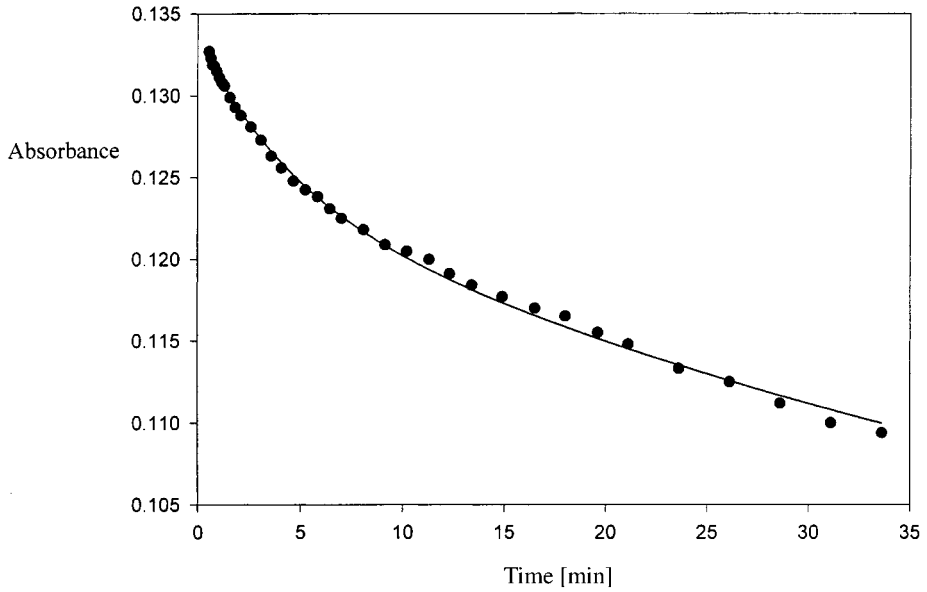

Fig. 4. *Absorbance changes at 270 nm on mixing β-lactamase* (2 μM) *with cefmetazole* (**1**; 8.1 μM). The points are experimental and the line calculated as described in the text.

Table 4. *Best Kinetic-Constant Values Obtained from Fitting the Experimental Results to Kinetic Equations from* Scheme 1

	K_S [mM]	k_2 [s^{-1}]	k_3 [s^{-1}]	k'_3 [s^{-1}]	k_4 [s^{-1}]	k_5 [s^{-1}]	k_{cat}^{calc} [s^{-1}]	K_m^{calc} [μM]
Cefmetazole (**1**)	$(6.5\pm0.4)\cdot10^{-2}$	$(3.8\pm0.4)\cdot10^{-2}$	$(1.1\pm0.1)\cdot10^{-3}$	$(3.3\pm0.3)\cdot10^{-3}$	$(3.5\pm0.2)\cdot10^{-4}$	25.8 ± 3	$(4.2\pm0.3)\cdot10^{-4}$	0.8 ± 0.2
Moxalactam (**2**)	8.6 ± 0.8	$(4.0\pm0.4)\cdot10^{-2}$	$(2.4\pm0.2)\cdot10^{-3}$	$(2.4\pm0.2)\cdot10^{-3}$	$(5.0\pm0.3)\cdot10^{-4}$	25.0 ± 3	$(9\pm1)\cdot10^{-4}$	180 ± 20
Cefoxitin (**3**)	1.3 ± 0.1	$7.3\cdot10^{-2}$	$1.5\cdot10^{-3}$ [a])	$4.4\cdot10^{-3}$ [a])	$2.8\cdot10^{-4}$	[b])	$(3.5\pm0.3)\cdot10^{-4}$	6.3 ± 0.6
PADAC [c])	0.01	16.5 ± 2.5	1.8 ± 0.3	1.8 ± 0.3	$1.1\cdot10^{-3}$	11	–	–

[a]) Taken from [19]. Reaction conditions: pH 7.0, 30°. [b]) Not determined. [c]) Taken from [10]. Reaction conditions: pH 7.5, 20°.

values derived from the microscopic rate constants according to the expression of *Table 5* (k_{cat}^{calc} and (K_m^{calc}) are given in *Table 4*, and are consistent with the experimental values (*Table 1*). The rate constants for reactivation (k_4) of the enzyme transiently inactivated by **3** and cefmetazole (**1**) were similar to k_{cat}, thus suggesting that deacylation of the inert acyl-enzyme intermediate is rate-determining in the steady-state turnover at saturation. From the expression for k_{cat} in *Table 5* it follows that $k'_3 > k_3$. By contrast, k_4 for moxalactam (**2**) is smaller than k_{cat}, so $k'_3 \leq k_3$. The microscopic rate constants k'_3 and k_3 obtained in the pre-steady-state computations confirm these assumptions. *Jamin et al.* [18] reported the steady-state kinetic parameters for the reactions of **2** and **3** with β-lactamase from *B. licheniformis*. Their k_{cat} values were $(0.9\pm0.1)\cdot10^{-3}$ s^{-1} for **2** and $(0.13\pm0.01)\cdot10^{-3}$ s^{-1} for **3**. These compounds were found to transiently inactivate the enzyme; also, the rate constants for reactivation were found to be similar to k_{cat}, which suggests that **2** is deacylated at a higher rate (k_4) than is **3**.

Table 5. Michaelis-Menten *Parameters for the Steady-State Phase*

$$k_{cat} = \frac{k_2\,k_4\,(k_3+k'_3)}{k_2\,k'_3 + k_4\,(k_2+k_3+k'_3)}\,,\quad K_m = \frac{K_S\,k_4\,(k_3+k'_3)}{k_2\,k'_3 + k_4\,(k_2+k_3+k'_3)}\,,\quad \frac{k_{cat}}{K_m} = \frac{k_2}{K_S}$$

A comparison of the k_2 values in *Tables 1* and *4* exposes the well-documented behavior of class-A β-lactamases and compounds containing a MeO group on the α-face of the β-lactam ring: acylation, if it occurs, is extremely slow (several orders of magnitude slower than in good substrates).

The catalytic mechanism for class-A β-lactamases involves three steps, namely, formation of a *Henri-Michaelis* complex, nucleophilic attack of the Ser-70(O^γ) residue on the β-lactam amide bond leading to the formation of an acyl-enzyme intermediate, and deacylation of the acyl-enzyme. There is wide consensus that the Glu-166 carboxylate group is the general-base catalyst for the deacylation step, where an occluded H_2O molecule next to the carboxylate in crystal structures is believed to act as the nucleophile [40][48]. On the other hand, the mechanism for the acylation step is somewhat controversial since such residues as Lys-73, Ser-130, and Glu-166 [5][6] have been assigned a general acid-base role. One mechanism includes the hypothesis that, in solution, the carboxylate group of Glu-166 can readily contact the nucleophile at the active site and establish a stable H-bond with it, at least in the absence of substrate. Another mechanism is based on the suggestion that the intervening H_2O molecule (Wat81) between the carboxylate group of Glu-166 and the OH group of Ser-70 acts, together with the carboxylate, as an extended general-base catalyst in the acylation. Still another mechanism rests on the assumption that the unprotonated NH_2 group of

Lys-73 serves as the general-base catalyst. This last mechanism calls for an anomalously low pK_a for the ammonium ion in Lys-73. Recently, *Raquet et al.* [49] used a continuum electrostatic model to calculate the pK_a for Lys-73, which they found to be 10 (consistent with experimental data [50] but inconsistent with Lys-73 acting as the general base in the acylation reaction).

Matagne et al. [6] reported a molecular-modelling structural study of the *Henri-Michaelis* complexes of the acyl-enzymes formed between three class-A β-lactamases and substrates bearing a MeO group on the α-face of the β-lactam ring. They suggested a major displacement of the crystallographically observed H_2O molecule between the Glu-166 and Ser-70 side chains, which made acylation very unlikely. Based on this suggestion, we examined the position of the crystallographically observed H_2O molecule (Wat81) in both the free enzyme and the *Henri-Michaelis* complexes. We found the H_2O molecule in substrates bearing a MeO group to be displaced by 2.0–2.3 Å from their initial position in the free enzyme and by only 1.5–1.8 Å in the substrates containing no such substituent (Fig. 5). As can be seen from *Table 3*, the incorporation of the substrate into the active site alters the position of the carboxylate group of Glu-166. On the other hand, Wat81 in the free enzyme is bonded to Wat65, and Ser-70(O^γ) interacts strongly with Wat22, Wat65, Wat71, and Wat81 (*Fig. 6*). To accommodate itself in the active site, the β-lactam displaces 4 H_2O molecules, two of which are Wat22 and Wat71. As a result, Ser-70 is less markedly hindered and can be slightly shifted to allow Wat81 to reach the Glu-166 residue (*Table 3*). For these reasons, the Wat81 molecule in compounds lacking the MeO group is displaced, the magnitude of the displacement being increased by the presence of the bulky MeO group.

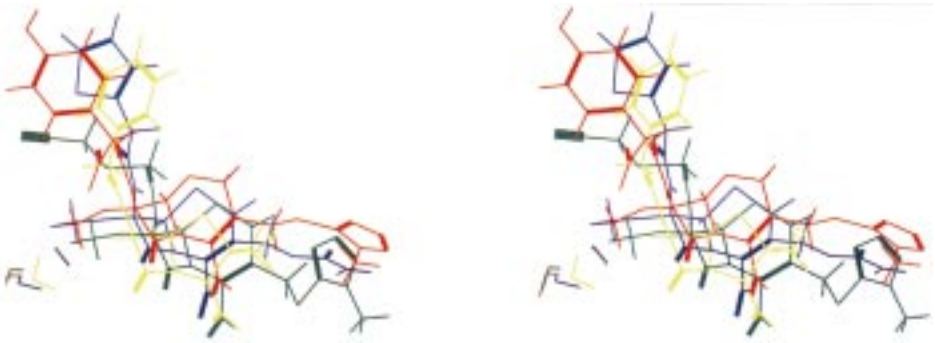

Fig. 5. *Stereoplot of the relative orientations cefmetazole* (**1**; green), *moxalactam* (**2**; red), *and cefoxitin* (**3**; blue) *with respect to benzylpenicillin* (yellow) *in the minimized* Henri-Michaelis *complexes.* Relative orientations of Wat81 are included for every complex with respect to Wat81 in the free enzyme (black).

The rate of acylation (k_2) can be correlated to the chemical structure of the substrate *via* two different factors, namely, H-bonding parameters for the potential activation of Ser-70(O^γ) by the Glu-166 residue (as suggested by *Matagne et al.* [6]) and the distance between the C-atom of the β-lactam carbonyl group and the Ser-70(O^γ) residue (*J* distance). From the data of *Table 3* for Ser-70(O^γ), Wat81, and Glu-166 (COO^-), it follows that H-bonding in the *Henri-Michaelis* complexes of cefmetazole

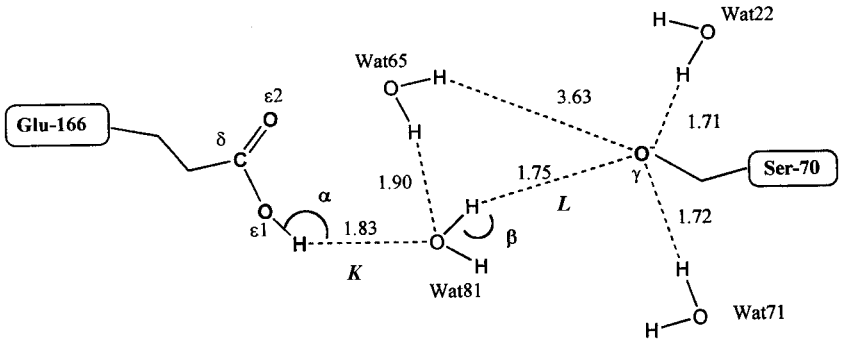

Fig. 6. *Diagram showing the possible hydrogen bonds in the active site of PC1 during the activation of Ser-70(Oγ) by the carboxylate group of Glu-166.* Some of the most important H-bonds are shown as dashed lines (– – –). Values in the free enzyme are included. H-Bonding parameters described in *Table 3* (distances *K* and *L*, and angles α and β) for the *Henri-Michaelis* complex are represented in the diagram.

(**1**) and moxalactam (**2**) is not very strong (H-bond distances are quite long and/or angles are anomalous); this suggests small k_2 values. The H-bonding parameters for cefoxitin (**3**) are similar to those for the free enzyme; however, the distance from Ser-70(O^{γ}) to the C-atom in the β-lactam carbonyl group is too long for an appreciable k_2 value to be obtained. On the other hand, based on the second-order rate constants for the hydroxide-ion-catalyzed hydrolysis (*Table 1*), k_2 should be higher for **2** than for **1**. However, the *J* distance in the *Henri-Michaelis* complex formed by **2** is longer than in that formed by **1**. As shown above, the strong H-bonds formed between the C(7) side chain and the amino-acid moieties Ile-239 and Tyr-171 account for the increased distance between Ser-70[O^{γ}) and C(8) in the case of **2** relative to **1**.

Finally, it is interesting to note that the k_3 and k_4 values for the acyl-enzymes formed by moxalactam (**2**) are slightly greater than those for the acyl-enzymes formed by cefmetazole (**1**) and cefoxitin (**3**). Moxalactam (**2**) differs from **1** in the side chain at C(7). Taking into account that the Glu-166 carboxylate group acts as a general-base catalyst for the deacylation step by interacting with a H_2O molecule that acts as the nucleophile when the acyl-enzyme is formed, the α-carboxy group at the C(7) side chain of **2** can approach the H_2O molecule and activate it.

APPENDIX

Numerical Analysis of Enzyme Progress Curves. Most interesting progress curves describing enzyme activity cannot be directly integrated, so they require numerical computation. Absorption changes can be fitted to reaction schemes by a nonlinear least-squares procedure [51].

The absorbance *Ab* at any time after mixing the enzyme and substrate can be calculated from *Eqn. 3* (see *Scheme 1*), where ε denotes extinction coefficients for the differenct species. On the assumption that $\varepsilon_{\mathbf{ES}} \approx \varepsilon_{\mathbf{EOH}} + \varepsilon_{\mathbf{S}}$, $\varepsilon_{\mathbf{ESA}} \approx \varepsilon_{\mathbf{EOH}} + \varepsilon_{\mathbf{C}}$, and $\varepsilon_{\mathbf{EM}} \approx \varepsilon_{\mathbf{EOH}} + \varepsilon_{\mathbf{M}}$, and taking into account that $[\mathbf{Y}^-] = [\mathbf{M}] + [\mathbf{EM}]$, one can write *Eqn. 4*.

$$Ab = \varepsilon_{\mathbf{EOH}}[\mathbf{EOH}] + \varepsilon_{\mathbf{S}}[\mathbf{S}] + \varepsilon_{\mathbf{ES}}[\mathbf{ES}] + \varepsilon_{\mathbf{ESA}}[\mathbf{ESA}] + \varepsilon_{\mathbf{EM}}[\mathbf{EM}] + \varepsilon_{\mathbf{Y}^-}[\mathbf{Y}^-] + \varepsilon_{\mathbf{C}}[\mathbf{C}] + \varepsilon_{\mathbf{M}}[\mathbf{M}] \quad (3)$$

$$Ab = \varepsilon_{\mathbf{EOH}}([\mathbf{EOH}] + [\mathbf{ES}] + [\mathbf{ESA}] + [\mathbf{EM}]) + \varepsilon_{\mathbf{S}}([\mathbf{S}] + [\mathbf{ES}]) + \varepsilon_{\mathbf{C}}([\mathbf{ESA}] + [\mathbf{C}]) + (\varepsilon_{\mathbf{M}} + \varepsilon_{\mathbf{Y}^-})([\mathbf{EM}] + [\mathbf{M}]) \quad (4)$$

If reasonable initial values for all rate constants are assumed – notice that those of K_S, k_2, k_4, and k_5 are experimentally determined – the concentrations of all the species as a function of time can be determined with the kinetic-data-processing software GIT [52], which performs numerical integrations of the differential equations for a given kinetic scheme. We used a multivariate nonlinear regression programme based on the *Marquardt* algorithm [53] to optimize fits to *Scheme 1:* by assuming the initial values of the rate constants, and from the calculated concentrations of all the species, we fitted *Eqn. 4* to the exper. data. This process was carried out iteratively. The intensity parameters $\varepsilon_{\mathbf{EOH}}$, $\varepsilon_{\mathbf{S}}$, and $\varepsilon_{\mathbf{M}}+\varepsilon_{\mathbf{Y}^-}$ in *Eqn. 4* were experimentally determined; in each run, three parameters (k_3, k'_3, and $\varepsilon_{\mathbf{C}}$) were optimized. The ruggedness of each fit was checked by systematically changing the initial estimates.

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