Interaction between class B β-lactamases and suicide substrates of active-site serine β-lactamases

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Received 12 December 1998

Abstract The most widely used inactivators of active-site serine β-lactamases behave as substrates of four class B metallo-β-lactamases, but the efficiency of the catalytic process can vary by several orders of magnitude. A comparison of the kinetic parameters for the α and β isomers of 6-iodopenicillanic acid shows that there is no general preference for the α isomer and that the efficient hydrolysis of imipenem by these enzymes must rest on other factors.

Key words: Metallo-β-lactamase; Clavulanic acid; Sulbactam; Tazobactam; 6-β-Iodopenicillanic acid

1. Introduction

Since class B metallo-β-lactamases rapidly hydrolyse carbapenem antibiotics and, with the exception of the *Aeromonas hydrophila* enzyme, exhibit a broad activity spectrum against most β-lactam compounds, production of these enzymes by pathogenic strains results in clinical problems [1,2]. Only monobactams appear to escape their activity. Moreover and not unexpectedly, mechanism-based inactivators of the active-site serine β-lactamases are not only useless against the Zn^{2+}-dependent metalloenzymes, but can also behave as substrates of these enzymes [2].

Thus, in mixed infections, metallo-β-lactamase-producing strains might protect their serine-β-lactamase-producing counterparts by destroying the inactivators and the acquisition of the genes encoding the former proteins by numerous strains is a distinct and frightening possibility, as exemplified by the isolation of strains which can synthesise up to three different β-lactamases [3,4].

In this paper, the interactions between a representative set of four metallo-β-lactamases and four β-lactam molecules which usually act as potent inactivators of active-site serine β-lactamase have been investigated; three of them (clavulanic acid, tazobactam and sulbactam) are in current clinical use.

The stereospecificity of the enzymatic process was analysed by comparing the kinetic parameters for 6-β and 6-α-iodopenicillanic acids (6-β-IP and 6-α-IP).

2. Materials and methods

The β-lactamases from *Bacillus cereus* 5/B/6, *Bacteroides fragilis* CfiA (CfiA), *Aeromonas hydrophila* AE036 (CphA) and *Pseudomonas aeruginosa* 101/1477 were produced and purified as described before [1,2,5,6]. Clavulanic acid and imipenem were kindly given by SmithKline Beecham Pharmaceuticals (Harlow, Essex, UK) and Merck Sharp and Dohme Research Laboratories (Rahway, NJ, USA) respectively. Tazobactam and sulbactam were gifts of Lederle Laboratories (Pearl River, NY, USA), α- and β-iodopenicillanic acid were synthesised as described [7] and nitrocefin was purchased from Unipath (Oxford, UK). The chemical structures of the tested β-lactam compounds are detailed in Fig. 1.

The hydrolysis of all antibiotics except clavulanic acid was monitored by following the absorbance variations resulting from the opening of the β-lactam ring, using a Uvikon 860 spectrophotometer equipped with thermostatically controlled cells and connected to a Copam PC 88C microcomputer via an RS232C serial interface. Cells with 0.2–1.0 cm pathlengths were used, depending on the substrate concentrations. The absorbance variations were as follows: α-IP and β-IP: $\Delta\varepsilon^{305} = 8500\ M^{-1}\ cm^{-1}$, tazobactam: $\Delta\varepsilon^{233} = 3600\ M^{-1}\ cm^{-1}$, sulbactam: $\Delta\varepsilon^{235} = 1780\ M^{-1}\ cm^{-1}$.

The k_{cat} and K_m values were derived as described by De Meester et al. [8] by analysing complete hydrolysis time courses. When the K_m was high, initial rates of hydrolysis were determined and the kinetic parameters derived on the basis of Hanes-Woolf plots. In some cases, K_m was so high that only the k_{cat}/K_m ratio could be determined. K_m values lower than 10 μM were measured as K_is in competition experiments with 8 μM imipenem (*Aeromonas* enzyme) or 50 μM nitrocefin (all other enzymes).

The total reaction volume was 0.5 ml in all cases. All assays were performed at 25°C in 0.1 M Na phosphate buffer (pH = 7) containing 0.1 mM $ZnSO_4$ with the exception of the *A. hydrophila* enzyme to which no $ZnSO_4$ was added since the enzyme retains one zinc ion/molecule after dialysis against ordinary bidistilled water.

Although the initial product of clavulanic acid hydrolysis can be assayed on the basis of its UV spectrum [9], it decays with a half-life of about 50 s. Since the activities of the various enzymes were rather low with this substrate, it was more convenient to directly monitor its disappearance by NMR spectroscopy. ^1H spectra were recorded with a Bruker AM 400 MHz spectrometer. All programs were from the Bruker library. Proton NMR spectra with water presaturation were obtained with a spectral width of 4 kHz for 16 000 frequency and time domain data points. All assays were performed at 25°C in 0.5 ml of the same buffer as above but prepared in 99% D_2O. A typical spectrum was the average of 80 scans over 8 min. For each metallo-β-lactamase, the reaction was followed at two different substrate concentrations (10 mM and 20 mM), with 10 μM enzyme. During and after hydrolysis, several products appeared, some of them transiently, phenomena which precluded their identification from the resulting complex spectra.

Chemical shifts for ^1H (expressed in ppm relative to an internal dimethylsilapentanesulfonate reference) and coupling constants were determined by using an iteration program (WIN-DAISY, Bruker, based on the spiral algorithm of A. Jones [10]) on the basis of the experimental spectrum. The protons in the NMR spectrum of clavulanic acid have been assigned by Howarth et al. [11].

3. Results

Table 1 gives an overview of the catalytic properties of the four enzymes studied.

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PII: S0014-5793(98)01689-5

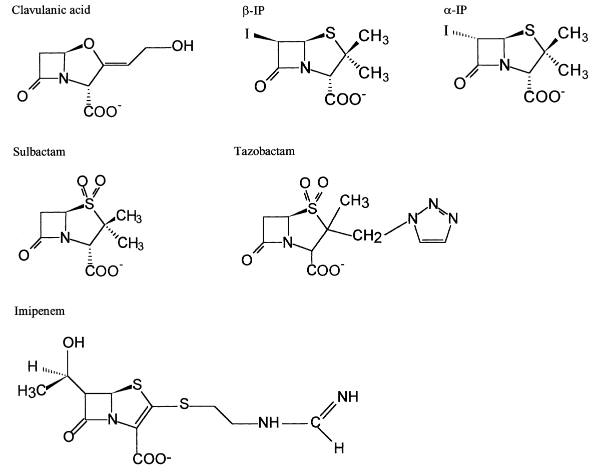

Fig. 1. Structures of the β-lactam compounds used in the study.

3.1. Clavulanic acid

Fig. 2 shows the disappearance of clavulanic acid in the presence of the *B. cereus* β-lactamase. With all the enzymes the clavulanic acid concentration was monitored by measuring the relative intensities of the peaks corresponding to protons H5 (δ5.79), H6A (δ3.20), H6B (δ3.63) and H9A (δ4.24) and the derived rate constants were the means of the four measured values.

With the *B. cereus* enzyme these rate constants were proportional to the substrate concentrations and only the k_{cat}/K_m value could be deduced. Conversely with the *A. hydrophila* and *B. fragilis* enzymes, zero-order kinetics were obtained at both substrate concentrations, thus directly yielding the k_{cat} values. The K_m values were thus significantly lower than 10 mM. With the *P. aeruginosa* enzyme, substrate inhibition was observed above 5 mM, a concentration at which a v_0/E_0 value of 0.4 s^{-1} was obtained. A 5 mM solution of clavulanic acid (1 ml) was then hydrolysed by incubation with the *B. fragilis* enzyme. After various periods of time (8–16 h), the enzyme was eliminated with the help of a Centricon device and the product solution added to a nitrocefin solution. The final concentrations were 100 µM for nitrocefin and 1–4.5 mM for the clavulanic acid degradation product. No inhibition was observed, suggesting that the inhibition recorded above was indeed due to an excess of substrate.

3.2. Sulbactam

Sulbactam was hydrolysed by all the class B β-lactamases with K_m values above 1 mM. The activity of the *A. hydrophila* enzyme was much lower than reported by other authors [1,2].

3.3. Tazobactam

B. cereus was the only enzyme to exhibit a significant activity on tazobactam. With *A. hydrophila* enzyme, no significant hydrolysis was observed at a 250 µM substrate concentration, indicating v_0/E_0 values lower than 10^{-3} s^{-1}. The *B. fragilis* enzyme slowly hydrolysed the compound but the K_m value was too high to be determined. When the enzyme was added to a mixture of 1 mM tazobactam and 100 µM nitrocefin, a 20% inhibition of the hydrolysis of the latter was recorded, which indicated a poor binding of the compound to the enzyme. This was confirmed by the low k_{cat}/K_m value at the steady state.

Table 1
Kinetic parameter values of metallo-β-lactamases with different substrates

Substrate	*A. hydrophila*			*P. aeruginosa*			*B. cereus* 5B6			*B. fragilis*		
	K_m (µM)	k_{cat} (s^{-1})	k_{cat}/K_m (M^{-1} s^{-1})	K_m (µM)	k_{cat} (s^{-1})	k_{cat}/K_m (M^{-1} s^{-1})	K_m (µM)	k_{cat} (s^{-1})	k_{cat}/K_m (M^{-1} s^{-1})	K_m (µM)	k_{cat} (s^{-1})	k_{cat}/K_m (M^{-1} s^{-1})
Clavulanic acid[a]	< 5000	0.14	> 28	ND	> 0.4 (substrate inhibition)	ND	> 10 000	> 0.13	13	96 ± 4[c]	0.1	1 042
α-IP	157 ± 5[c]	~0.005	3	< 500	~0.01 (lag)	> 4	< 500	~0.0016 (lag)	> 3.2	NH	NH	NH
β-IP	530 ± 50	0.64	1 200	> 1000	> 3.98	3 980	7 500 ± 300[b]	450[b]	60 000[b]	1.3 ± 0.2[c]	0.32	242 000
Sulbactam	> 1000	> 0.01	10	> 1000	> 13.7	13 700	5 200 ± 300[b]	10[b]	1 900[b]	> 1 000	> 5.9	5 900
Tazobactam	NH	NH	NH	> 1000	ND	400 (lag)	420 ± 10[b]	6.5[b]	15 500[b]	> 500	> 0.3	700
Imipenem	80 ± 8[b]	168[b]	2 100 000[b]	39 ± 4[d]	46 ± 3[d]	1 200 000[d]	> 1 000[b]	> 100[b]	120 000[b]	140[d]	210[d]	1.5[d]

[a]In 99% D_2O.
[b]Data from [1,2].
[c]Determined as K_i in a competition experiment.
[d]Data from [14,15].
NH: no significant hydrolysis, see text. ND: not determined. When not stated, S.D. values were ± 10%.

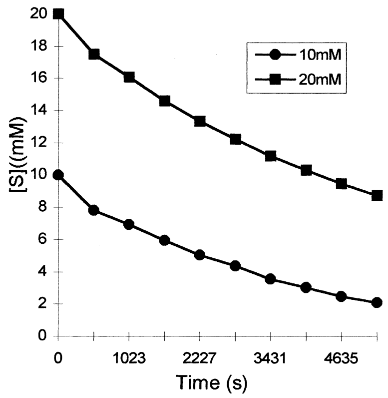

Fig. 2. Disappearance of clavulanic acid in the presence of the *B. cereus* β-lactamase. The conditions were as described in Section 2. S.D. values were below 10% ($n = 8$).

The activity of the *P. aeruginosa* enzyme was low but not negligible. A lag was observed and the k_{cat}/K_m value shown in Table 1 was that obtained at the steady state.

3.4. α-IP and β-IP

β-IP was a substrate for all the enzymes studied and was especially well hydrolysed by the *B. cereus* and *B. fragilis* enzymes. By contrast, the α isomer was a very poor substrate with k_{cat} values never exceeding 10^{-2} s^{-1}. Moreover, with *P. aeruginosa* and the *B. cereus* enzymes, a lag was observed and the values shown in Table 1 are those obtained at the steady state which was reached after 1–2 min, but even under these conditions the rate of the reaction remained very low. With the *B. fragilis* enzyme, no significant hydrolysis of α-IP was recorded at a 500 μM substrate concentration ($v_0/E_0 < 10^{-3}$ s^{-1}).

4. Discussion

The four mechanism-based inactivators of active-site serine β-lactamases are recognised and hydrolysed by most of the metalloenzymes, although the k_{cat}/K_m values are usually not very high. The highest k_{cat}/K_m values were observed with β-IP and the *B. cereus* and *B. fragilis* enzymes, but this is of little clinical relevance since β-IP is not used in combination therapies. In this respect, the non-negligible hydrolysis of sulbactam by the *P. aeruginosa* and *B. fragilis* enzymes appears more worrying. In the other cases, the enzymes produced by pathogenic strains do not represent a threat to combination therapies, at least in the absence of very strong overproduction phenomena. It should be noted, however, that our results with the *P. aeruginosa* enzyme are at variance with those of Bush et al. [12] who observed IC_{50} values in the micromolar range for sulbactam and tazobactam with the identical enzyme produced by *Serratia marcescens* S6.

The comparison between α- and β-iodopenicillanate shows that the exceptional activity of class B β-lactamases versus imipenem cannot be solely explained by the α position of the side chain. Indeed, even with the *A. hydrophila* enzyme, which can be considered a strict carbapenemase [13], the β isomer was a much better substrate than its α counterpart and all the other enzymes exhibited an overwhelming preference for the former compound. The k_{cat}/K_m values were even significantly larger than the k_2/K values determined for the interaction between β-IP and the class C enzymes [8]. Finally, in several cases, the reaction time courses indicated complex kinetic pathways accompanied by lags which remain unexplained at the present time. However, these phenomena occurred at very high concentrations of the studied compounds (0.5 mM and higher) and are unlikely to be of physiological interest.

Acknowledgements: This work was supported in part by a grant from the European Union (No. ERBFMRCX-CT98-0232) in the framework of the 'Training and Mobility of Researchers' Programme and by the 'Poles d'Attraction Interuniversitaire' initiated by the Belgian state, Prime Minister's Office, Services Fédéraux des Affaires Scientifiques, Techniques et Culturelles (PAI No. P4/03). C. Prosperi-Meys is a Fellow of the Fonds pour la Formation à la Recherche dans l'Industrie et dans l'Agriculture (FRIA, Brussels, Belgium).

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